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Disease Resistance in Fruit

Proceedings of an International Workshop
held at Chiang Mai, Thailand,
18–21 May 1997

Editors: **G.I. Johnson, E. Highley, and D.C. Joyce**

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Address of Welcome

Distinguished ladies and gentlemen, it is a great pleasure for me to welcome you to Chiang Mai and to this International Workshop on Disease Resistance in Fruit. I hope that all of you had a pleasant journey and will enjoy your stay here.

The meeting that is about to begin today will review research on the management of diseases of tropical fruit. Tropical fruits are important commodities worldwide, but because they are grown in hot, wet climates they are prone to diseases caused by fungi and other organisms that affect their commercial value and eating quality. Diseases that develop after harvest are particularly important. Fungicides and pesticides are routinely used to control postharvest diseases of fruit in many parts of the world, but chemically treated fruit is becoming increasingly unacceptable to consumers, who view chemical residues on fruit as a health hazard.

In order to minimise the use of chemicals and reduce postharvest losses of fruit, researchers are now seeking means of enhancing the in-built defence mechanisms of fruit, be these constitutive or inducible, to disease. This is the topic for discussion at this workshop which, I believe, will provide a very beneficial opportunity for scientists to establish or strengthen mutual relationships and cooperation in fruit resistance research and development. The meeting will also provide a forum for experts from various countries to report on their recent work and to discuss possibilities for future collaborative research activities in Thailand.

I am looking forward to learning of your researches and suggestions contributed throughout the workshop. Your contribution in this meeting will surely benefit all attendees, especially Thai researchers. I wish the meeting every success and thank you all again for coming. I would like to say welcome to Chiang Mai and to open this workshop on fruit disease resistance.

Thank you.

Associate Professor Dr Chawalit Putthawong
Vice President, Chiang Mai University

Considerations and Future Directions for 'Exploitation' of Natural Disease Resistance in Fruit—Workshop Summary and Conclusions

D.C. Joyce* and G.I. Johnson†

THE objective of this international workshop on disease resistance in fruit was to review recent research on non-chemical control of fruit diseases, with particular emphasis on utilisation and enhancement of constitutive or inducible host defence mechanisms.

Participants in the workshop included scientists from various countries who are active in research on defence mechanisms in fruit, and researchers from the region who are involved or interested in this field.

Most of the invited review papers and contributed poster papers presented at the workshop are published in these proceedings.

The following summaries present considerations, recommendations, and strategies for the implementation of future research, as formulated during the workshop by a number of working groups of participants. The information offered represents a collation of the informed views of participants, as recorded by group reporters. Each pair of reporters was responsible for chairing one of four simultaneous round-table discussions centred on different sets of commodities. The reports were presented in the final session of the workshop. The agenda for this session, as described in the workshop handbook, was as follows:

> This session will commence with reports from groups about their analyses of priorities emerging from the two days of presentations. The reporters, and their commodity groups, are: Dr Dov Prusky/Dr David Guest—avocado and durian; Dr Nimal Adikaram/Dr Daryl Joyce—mango and papaya; Dr Connie Lizada/Dr Robert Holmes—banana, grapes and Sapindaceae; and Dr Ahmed El Ghaouth/Dr Brian Wild—citrus and melon. This session will focus on how we can make practical use of the findings of antifungal research, and discuss issues such as toxicity. A panel discussion and formulation of group resolutions will follow presentation of the group reports.

Because there were four distinct groups of participants involved, the formats of summary considerations, recommendations, and strategies for future research on the various sets of commodities inevitably vary.

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* Department of Plant Production, The University of Queensland, Gatton College, Queensland 4345, Australia.

† ACIAR, GPO Box 1571, Canberra, ACT 2601, Australia.

Avocado and Durian

Group participants: Lindy Coates, Melinda Gosbee, David Guest, Anjani Karunaratne, Lise Korsten, Le Thi Yhu Hong, T.K. Lim, Mai Van Tri, Ratiya Pongsiputa, Sawat Promin, Dov Prusky, Somsiri Sangchote, Kobkiat Sangnil, Casiana Vera Cruz.

General priorities

1. Accurate diagnosis of the disease and the pathogen(s): (i) Koch's postulates; (ii) host range; (iii) identification of virulence/pathogenicity factors; and (iv) race structure and population biology.
2. Understand the disease cycle: (i) sources of inoculum and vector relationships; (ii) infection processes—How does the pathogen overcome physical and chemical barriers presented by the host?...What are the roles of individual and combined virulence factors such as appressorial adhesion and cutinase?...How might these factors be manipulated to suppress disease development?; and (iii) develop rapid, reproducible, and meaningful screens for disease resistance to assist in selection and breeding of cultivars with reduced susceptibility....Identify genetic markers, if possible....However, resistance in fruits is a complex response resulting from the additive or synergistic interaction of many individual responses that may not, on their own, be significant....Is it possible to use QTL analysis to select combinations of quantitative traits?
3. Understand resistance mechanisms: (i) identify individual responses and determine their roles in isolation and in combinations; (ii) development of model systems may assist this analysis, and its reproducibility in annuals and perennials; (iii) use these model interactions to study induced responses to infection, elicitor treatment of exposure to signalling compounds like INA, BTH, etc.; and (iv) can tools and methods developed in the study of other systems (*Arabidopsis*?) be used in the study of fruit disease?...Specific genes thought to be involved in defence (hydrolytic enzymes, PGIPs, etc.) may be incorporated into and expressed in fruits if transformations systems are available.
4. How do orchard management factors affect susceptibility to postharvest disease? (i) effects on the pathogen and vectors; (ii) effects on susceptibility and resistance—drainage, canopy management, ventilation, light and UV exposure, etc.; and (iii) how can management practices be manipulated in integrated disease management programs?
5. Effects of ripening and storage on disease: postharvest disease is unusual because it usually involves interactions between necrotrophs and senescing plant tissue.
6. Biological interactions: (i) interactions between pathogens and rhizosphere, phylloplane, flower, fruit-surface-colonising microorganisms; (ii) interactions between pathogens and endophytes; (iii) interactions in multiple infections—induced resistance and induced susceptibility; and (iv) how can these interactions be manipulated or exploited in integrated disease management programs?
7. Methodology: (i) in all studies it is essential to develop and follow standard techniques for inoculation, incubation, and analysis of defence responses; (ii) this facilitates reproducibility and comparisons between labs; (iii) resistance is complex, so comparisons are essential for interpretation of the roles of—preformed physical and chemical barriers,...rapid localised responses such as oxidative burst, phytoalexins, papilla and lignin deposition,...delayed systemic responses, including systemic acquired resistance and wound

repair/containment mechanisms; and (iv) must use model interactions that are appropriate for the responses to be studied.

8. What are the consequences of induced resistance?: (i) fruit quality, palatability, digestibility; (ii) social risks (toxicity, carcinogenicity, environmental) and perceptions of risks; and (iii) benefit–cost analysis. The aim is to develop safer methods to produce quality fruit at an acceptable cost
9. Avocado: role of dienes well established.
10. Durian: (i) we know nothing of disease resistance mechanisms; and (ii) perhaps we can use tools that induce resistance or susceptibility to study differential responses—phosphonate, BTH, and cycloheximide.

Points for general discussion

1. We have to remember that, in considering fruit resistance to disease, we been discussing different types of general dynamic incompatibility where genes are expressed only under particular physiological conditions....Under these conditions, many genes might be involved, and battles (reactions) at different host–pathogen levels are continuously occurring during pathogen penetration and host reaction.
2. We have to remember also that resistance is composed of a series of barriers (chemical and physical), expressed at different levels and, at each particular level, activation of specific genes occurs....That is, resistance is composed of a suite of mechanisms (both physical and chemical) expressed in a cascade of reactions starting with those induced by the host's receipt of physical or chemical signals from pathogen propagules in the vicinity of or in surface contact with the host....The best for us would be to restrict the pathogens at early levels of infection....Wild et al. (these proceedings) described that failure to inhibit *Penicillium digitatum* attacking apples during the early stages of infection resulted in the subsequent development of the pathogen in normally resistant tissue.
3. For avocado, based on early developments: (i) the use of antioxidants for the prevention of the diene decrease has become a commercial treatment; and (ii) the selection of cultivars based on the presence of high concentrations of epicatechin has become a common marker for avocado cultivar selection.
4. Possible future studies based in early stages of pathogen development: (i) the use of signal transduction processes and studies for the possible elicitation of the antifungal diene; and (ii) the study of the biosynthesis of the antifungal diene and its possible involvement in resistance will further elucidate its importance.
5. We have to understand that we are observing resistance processes that are very complicated and that more than one factor is almost certainly involved in resistance.

Mango and Papaya

Group participants: Nimal Adikaram, Somboon Anantalapochai, L. Artes, Ray Carman, S. Chancumni, Parinya Chantrasri, Nuanwan Farungsang, Daryl Joyce, Ilana Koblier, John Labavitch, N. Ratanapanont, Vicha Sardsud, Damrat Supyen, Zainuri.

Outcome of SWOT (strengths, weaknesses, opportunities, and threats) analysis

1. *Mangoes*

   (i) Strengths—relatively good knowledge of preformed and inducible resorcinol compounds; and have a good physical treatment to suppress decay (55°C, 15 sec.).

   (ii) Weaknesses—relatively poor knowledge of the relationship between fruit maturity and disease susceptibility; limited understanding of the 'battery' of host defences against pathogens; need for a 'sensitivity analysis'; incomplete information on how effective disease suppression treatments work (e.g. heat-induced spreading of surface waxes); and need to improve understanding of the nature of host defence differences between varieties.

   (iii) Opportunities—potential to compare host defence mechanisms in a wide range of varieties; could utilise a wide range of growing environments in Australia to help understand preharvest effects on defence mechanisms; control measures such as heat treatment can also contribute to fruit 'quality' by promoting ripening; mango latex, like papaya latex, should be tested for antifungal activity; 'other' host-defence induction treatments (e.g. UV light) could be evaluated; solvents (e.g. petroleum-based) may have application for altering the structure of the waxy cuticle, as a first line of defence; and opportunity to test 'citrus type' control techniques (e.g. 2,4-D on the pedicel).

   (iv) Threats—any control strategy utilising natural resistance and effective against any one pathogen may not give control of other pathogens; and potential mammalian toxicity of natural antifungals occurring in mangoes warrants testing.

2. *Papayas*

   (i) Strengths—strong pre-formed defence system against insects/fungi (viz. preformed chitinase, benzyl isothiocyanate(?), pectinase inhibitor (PGIP?), capability of phytoalexin accumulation).

   (ii) Weaknesses—some pathogens can tolerate latex; no attempts have been made to use latex on (a) endogenous or (b) external agents to protect the ripe fruit; using latex as a control agent may allow those that tolerate latex to dominate; and knowledge regarding phytoalexins is incomplete.

   (iii) Opportunities—develop strategies to utilise sap; exploit the possibility of using papaya latex as a general postharvest fungicide(?); investigate phytoalexins and other defence mechanisms triggered in papaya; investigate other fruit/skin/pulp characteristics; and find ways to trigger defence mechanisms simultaneously.

   (iv) Threats—if one defence mechanism is used/overused, the pathogens might overcome it (risk = waste of money by industry); some fungi can overcome latex, will they dominate?, will the balance be upset?, toxicity to mammals?

3. *Important generic issues*

   (i) Strengths—no special strengths identified.

   (ii) Weaknesses—need for 'better methods' (e.g. ELISA tests for antifungals); and physiology of postharvest fungi is not well understood.

   (iii) Opportunities—should 'tease out' the relationship between ripening and decay (e.g. study antisense PG system in more detail; compare climacteric and non-climacteric systems); investigate why 'other pathogens' of, say, crop A are not pathogens on crop B; tighter networking between laboratories; and knowledge gained from non-climacteric fruit may be usefully applied to climacteric fruits.

   (iv) Threats—potential mammalian toxicity of natural antifungals.

Banana, Grape, and Sapindaceae

Group participants: C. Abayasekara, T. Cooke, U. Farungsang, R. Holmes, G. Johnson, Wilawan Kumpoun, S. Kooriakul, C. Lizada, T. Phatchaiyo, B. Ratanachinakorn, U. Sardsud, A. Suwanigul, S. Tongdee, J. Uthaibutra, Y. Yaguchi.

The banana, grape, and Sapindaceae group discussed generic issues relating to: pre- and postharvest factors contributing to postharvest disease; the infection process; methods for the detection and identification of constitutive and induced antifungals; describing fruit maturity; and the inconsistent use of terminology.

1. Pre- and postharvest factors: Historically, the quality of horticultural produce was controlled by emphasising end-point inspection. The ability to trace back to identify the causal factors contributing to postharvest disease was therefore limited. HACCP (hazard analysis and critical control points) offers a framework for describing the critical variables which influence postharvest disease (hazards) and the appropriate courses of action to minimise hazards at each stage of production (controls). The group recommends that HACCP plans for the pre- and postharvest production of specific crops would be a most useful tool for: (i) the development of quality assurance (QA) specifications and the implementation of QA programs; (ii) teaching the principles of postharvest pathology; and (iii) identifying gaps in the knowledge base, enabling the prioritising of research.
2. Infection a stress in itself?: (i) hypersensitive versus compatible interactions; and (ii) infection = stress stimulus signal???...tissue response (stress)...C_2H_4...increased PAL?...phenolics (anti-fungals).
3. Techniques: (i) the TLC overlay technique (bioautography) is useful for separating antifungal compounds in plant extracts and enabling the isolation of compounds for identification....It has been the convention to use *Cladosporium* as the indicator of antifungal activity....However, recent research (Holmes, pers. comm.) has shown that several antifungals isolated were inactive against *Cladosporium,* but highly active against specific fungi. It is therefore recommended that, where possible, specific pathogens are used as indicators; and (ii) use of standard internal markers (at least 2), e.g. substituted resorcinols.
4. Maturity parameters: should be related to physiological stages; (i) apples: internal C_2H_4(?), induced climacteric, days from full bloom + heat units; mangoes (cv. specific): days from nitrate-induced flowering + heat units, verify with specific gravity, internal

C_2H_4 + response to treatments, develop forecasting model for breaking of quiescence... Strict requirement, good handle on physiological maturity.

5. Terminology—the group discussed the various definitions of the term 'latency' and the definition of 'quiescence'. It was agreed that latency had become an imprecise term, and that it should be reserved for the period between infection and infectiousness, i.e. in the epidemiological context (Vanderplank). Quiescence describes arrested development of the pathogen due to adverse physiological conditions temporarily imposed by the host (T.R. Swinburne). Dr Yaguchi commented that the term quiescence was not well known in Japan, and suggested that a translation of the definition is needed. The term 'endophyte' is also imprecise because it has been defined in various ways. The group agreed to accept a definition of endophyte to include all organisms inhabiting plant organs that, at some time in their life, can colonise internal plant tissue without causing apparent harm to their host (Petrini). There was debate over whether some diseases of mango and avocado, for example, were best described as quiescent or endophytic. The group also discussed maturation and ripening, which are often confused. The agreed definitions follow: 'maturation' is characterised by anabolic processes. When a fruit has received sufficient quantities of manufactured compounds to enable it to ripen to a satisfactory quality it is said to be 'mature'. Mature fruit needs to undergo compositional and physical changes before it reaches optimum eating quality. These catabolic processes are what characterise 'ripening'. Ripening is most pronounced in climacteric fruits.

Citrus and Melon

Group participants: S. Ben-Yehoshua, Chen Nianlai, A. El Ghaouth, E. Esguerra, B. Hawthorne, P. Hofman, Y. Homma, Y. Huang, S. Nanthachai, C. Sittigul, Tang Wenhua, J. Sornsrivichai, N. Visarathanonth, B. Wild, J. Wright.

The members of the table working on either melon or citrus crops first reviewed the major pathogens which were encountered during marketing of this produce. Generally it was considered that 90% of the problems were brought about by the pathogens that cause sour rot (*Geotrichum candidum*), stem end rots (*Diplodia* and *Diaporthe* spp.), blue and green moulds (*Penicillium* spp.), transit rots (*Rhizopus* spp.), and melon rots (caused by *Fusarium* spp.).

The group resolved that, in order to implement control procedures based on host defence reactions, it was necessary to develop a better knowledge of the aetiology of these decay organisms. Factors to be considered under this heading were:

1. Synergisms that can occur between different pathogens in the infection process. This may occur when there is more than one type of organism involved in establishing a decay lesion, and that a simple Koch's postulates may not reveal the true aetiology of the disease.
2. Potential resistance by the organisms to a host-defence reaction. Just because the method of decay control was based on using natural defence reactions of the fruit, it did not mean the organisms being controlled could not develop resistance to the induced 'natural' fungicides. If anything, it was considered that, because of co-evolution between the organisms and the host, it was possibly more likely to occur than with the synthetic compounds.
3. Multi-pronged approach needed. Because of these risks of resistance development and possible synergisms in the infection process, it was considered that, if any recommenda-

tions were made for using a defence reaction for decay control, it should also include an additional treatment just to provide an increased level of security.

The group also considered the commercial potential of the host-defence reactions, but realised that if maximum use was to be made of them then it was essential to know: (i) the nature of the reaction and the processes that regulated it; (ii) the types and toxicology of the compounds formed; and (iii) what elicitors were needed and were there any others that could be found by additional research.

It was also considered that before commercial recommendations could be made for these procedures the following factors should be considered: (i) do we have enough knowledge? (ii) what was the cost to the quality of the product? (iii) what was the cost to the yield of the product? and (iv) what was the cost to consumer safety (some of the antifungal compounds formed had the potential to be toxic to consumers)?

In reviewing methodology, the group considered that there was a need to develop better bioassay techniques which would assay antifungals against the organisms causing the trouble. Also, there was a need for the use of statistics in the sampling used for chemical analysis.

In summary, the group agreed that using host-defence reactions would be feasible in the marketing chain as long as it fits in with existing practices, and that its limitations were recognised and it was not oversold as a panacea to solve all postharvest problems.

Conclusion

The considerations, recommendations, and strategies presented here tend to be somewhat disparate with regard to individual commodities and in terms of emphasis on specific issues. Nonetheless, collectively, the views represent valuable general advice for future work involving 'exploitation' of natural host-resistance mechanisms. The consensus view of the workshop participants was that natural antifungal compounds occurring in fruit should be thoroughly investigated with a view to enhancing resistance to postharvest decay.

INVITED PAPERS

The Pathogens of Fruit

Fruit Resistance to Pathogens

G.I. Johnson*

THE damage and losses caused by fruit pathogens are unacceptable to orchardists, marketers and consumers. But what is the evolutionary significance of the magnitude of the losses? In nature, and in terms of tree survival, the losses are **not** of major significance.

This is because fruit are primarily reproductive structures. They nurture and protect developing seed and can help its dispersal and germination. As a result of their central role in reproduction, natural selection has favoured the retention of structural, phenological and constitutive traits that optimise survival and reproduction of plants in the environments and regions in which they have evolved. In any ecological association, plants respond to climatic and environmental parameters to produce fruit/seed in the quantity appropriate to the conditions. One set of conditions may favour regular, abundant flowering and fruiting. However, if conditions deteriorate, fruit may be aborted, and only a portion of the crop may be retained till maturity. In another season, flowering and fruit-set may be sparse, and only a few seed may germinate. Plants have evolved constitutive traits (and natural microbial associations) which enable them to reproduce despite the presence of pathogens and pests, and in variable climates and growth substrates.

Microbial associations have also evolved. The pathogens which we try to control may actually aid host survival by preventing colonisation by mycotoxigenic fungi which tend to be avoided by vertebrate seed dispersers such as birds. Fruit restrict decay until seeds mature. When the seeds are mature, the mechanisms which limit disease and pest invasion and consumption by vertebrates deteriorate. Attachment to the tree is weakened. The fruit fall, or are forcibly detached by vertebrate feeders. The vertebrate feeders may consume entire fruit and, frequently, the seeds remain viable while passing through their digestive tracts. Alternatively (with larger fruit) the flesh may be eaten and the horny or fibrous tissues enclosing the seed discarded—in both processes, the seed are dispersed. In addition to the vertebrate pests, insect larvae and decay microorganisms compete for fruit flesh as a growth substrate. But some seed are spared—'natural selection' in action!

Cultivation/domestication upsets these natural balances. Maximum production of blemish-free fruit is required every season. Microbial associations are disrupted. Climate and growing conditions are manipulated. Fruit are harvested, treated, and stored. Consumers are demanding 'chemical-free' food. It is in the context of this 'unnatural selection' that we need to reconsider: *How do fruit defend themselves?*

This workshop will review one facet of the evolutionary development of plants—the biochemical and physiological traits used by fruits to minimise the ingress of pathogens during development and dispersal.

We will:

- review pathogen containment and modulation and breakdown of the fruit defences;
- survey what is known about specific mechanisms in several fruit;
- consider physical, chemical, and microbial treatments and strategies which elicit and enhance fruit defences; and
- discuss the research methodologies involved?

Finally we will consider the questions:

What facets of host genome, environment, and micro and macroflora and fauna could be manipulated, improved or reassembled to adjust the balance, so that disease losses are minimised and product quality is maintained at the level dictated by commerce? And how are we going to do it?

* ACIAR, GPO Box 1571, Canberra ACT 2601, Australia.

Postharvest Diseases of Tropical Fruits

S. Sangchote*

Abstract

Postharvest diseases of tropical fruits caused by different kinds of pathogens. Infection of these pathogens occurred both in the field and after harvest. Infection and disease development concerned to pathogens biology, pre- and postharvest factors. Reduction of these diseases through non-chemical practices is discussed.

Various kinds of fungi cause spoilage of tropical fruits. These fungi infect the fruit both in the field and after harvest. Disease development is influenced by many factors including pathogen biology, developmental stage of fruit, cultivar, handling, and environmental factors. This paper discusses non-chemical methods of managing fruit diseases.

MANY tropical fruit crops are grown in Thailand, including mango (*Mangifera indica* L.), durian (*Durio zibethinus* Murr), rambutan (*Nephelium lappaceum* L.), mangosteen (*Garcinia mangostana* L.), and guava (*Psidium guajava* L.). Durian, an important fruit crop, is grown mainly in the eastern and southern parts of the country. The producing areas in the east are Rayong, Chanthaburi, Trad, and Prachin Buri, and in the south, Surat Thani, Chumporn, Nakorn Sri Thammarat, and Yala. In 1993–94, the total production area was 123260 ha with total yield was 746642 t. In 1995, 48761 t were exported, valued at 1004.1 million baht (Sertapukdee 1996) (during May 1997, 25 Thailand baht (THB) = ca US$1). The other main export fruit crops are longan, lychee, mangosteen, and rambutan, with Hong Kong, Taiwan, Malaysia, and Japan the major importing countries (Udomsin 1994).

These fruit crops grow mainly during the rainy season. The warm weather and high humidity make the fruit prone to attack by microorganisms, most importantly various types of fungi (Table 1).

* Department of Plant Pathology, Faculty of Agriculture, Kasetsart University, 50 Jatuchuk, Bangkok-10900, Thailand.

The extent of losses due to these diseases has been recorded for various cultivars of crops such as durian, mango, and mangosteen, and in different producing areas (Table 2). To reduce these losses, pre- and postharvest factors affecting disease incidence and severity need further study.

Infection of Postharvest Pathogens

Infection of postharvest pathogens can occur before or after harvest, as discussed by Johnson and Sangchote (1994). *Colletotrichum gloeosporioides* is able to infect mango fruits at any time from flowering onwards. However, preharvest infection usually produces few or no symptoms, the disease developing as the fruit starts to ripen. Coates and Gowanlock (1994) reviewed infection of tropical fruits by *C. gloeosporioides* and discussed the formation of a symptomless quiescent structure and restricted lesions under conditions of high inoculum pressure. Disease development and severity were related to the stage of fruit ripening (Sangchote and Chayasombat 1986). *C. gloeosporioides*, *Phomopsis* sp., and *Botrytis cinerea* were pathogens in this category.

Table 1. Some postharvest diseases of tropical fruits in Thailand.

Fruit	Disease	Causal organism(s)	Reference
Mango	Anthracnose	*Colletotrichum gloeosporioides*	Sangchote 1987
	Stem-end rot	*Lasiodiplodia theobromae*	
		Dothiorella dominicana	
		D. mangiferae	
		Phomopsis mangiferae	
Rambutan	Fruit rot	*C. gloeosporioides*	Farungsang et al. 1994
		L theobromae	
		Gliocephalotrichum bulbilium	
		Phomopsis sp.	
Durian	Fruit rot	*L. theobromae*	Chana et al. 1991
		Phytophthora palmivora	
		Phomopsis sp.	
Guava	Fruit rot	*L. theobromae*	Sangchote et al. 1985
Banana	Anthracnose	*C. musae*	Thongthieing 1992
Mangosteen	Fruit rot	*L. theobromae*	Sangchote and Pongpisutta 1995
		Pestalotiopsis sp.	
		C. gloeosporioides	
		Phomopsis sp.	
Papaya	Anthracnose	*C. gloeosporioides*	Sangchote et al. 1985

Table 2. Percentage losses due to postharvest diseases of mango fruits cv Nam Dorkmai, Okrong, Tongdum, and Kaew from different producing areas (Sangchote 1987).

Location	Variety	Losses due to diseases				
		Anthracnose	*Lasiodiplodia* fruit rot	*Dothiorella* fruit rot	*Phomopsis* fruit rot	*Aspergillus* rot
Nonburi	Okrong	0	20	34	7	21
	Kaew	48	0	0	0	0
	Nam Dorkmai	12	23	25	10	23
	Tongdum	30	41	25	0	21
Chachoengsao	Okrong	8	37	27	4	1
	Nam Dorkmai	30	34	26	2	11
	Tongdum	10	16	23	0	2
Nan	NamDorkmai	49	14	1	0	2
Nakhonratchasima	Kaew	54	0	0	0	0
	Nam Dorkmai	96	4	1	0	0

The other groups of pathogens infect fruit during and after harvest through the stem end and parts wounded by cutting or rough handling. The pathogens in this category include *L. theobromae*, *Dothiorella* sp., and *Phomopsis* sp. (Johnson and Sangchote 1994). The symptoms of these pathogens also develop as the fruit ripens. However, the two groups of pathogen are not completely distinct. Some of these pathogens, such as *D. dominicana* and *L. theobromae* are also found infecting different parts

of sound fruit, but without symptoms. Johnson et al. (1991) showed that, during flowering and fruit set, colonisation by *Dothiorella* sp. increased as the flowers senesced and young fruit formed. An early infection caused fruitlet abortion. The infections that initiated postharvest stem-end rot occurred later. Colonisation of vascular tissue by *Phomopsis citri* was also shown by Homma et al. (1989).

Sources of Inoculum of Postharvest Pathogens

Sources of inoculum of these postharvest pathogens play an important role in the initiation of infection. *L. theobromae* is an organism with a wide host range. Isolates from different hosts and sources can cause disease on mango fruit, but those from diseased mango fruit are the most virulent isolate and those from soil the least so (Sangchote 1991). Johnson et al. (1993) note that mango fruit can be infected by *Lasiodiplodia theobromae* when the fruits are inverted on the soil after harvest to drain sap from the fruit. *L. theobromae* was also found infecting the fruit pedicel of mango, the pericarp of rambutan, the fruit stylar of mangosteen, and the spines of durian, with no symptoms. After fruit had been stored for some time or ripened, the fungal infection progressed and the fruit started to develop symptoms. Soil-borne inoculum was also an important source of infection of durian fruit caused by *Phytophthora palmivora*. Durian fruit dropped on the ground were found to develop symptoms as fruit ripened (Pongpisutta and Sangchote 1994)

Locations and Environmental Factors

Inoculum pressure in the producing area is one of the key factors of determining level of infection. Peacock (1988) reported that the source of mangoes significantly affected stem-end rot losses. Mangoes obtained from an area in which mangoes had been grown for a long time and had high inoculum pressure showed rather high levels of infection (Sangchote 1987). *C. gloeosporioides* infected mango leaves, and conidia of this fungus were spread from infected leaves by rain. Dodd et al. (1991) found a correlation between number of conidia and rain intensity. Fitzell et al. (1984) found that temperatures of 20–30°C and relative humidities above 95% were favourable for germination of conidia of *C. gloeosporioides* and subsequent infection. Symptoms become obvious at the ripening stage of fruit (Chayasombat 1987). Prusky and Gat (1992) reported that *Alternaria alternata* needed at least 350 hours of relative humidity above 80% to establish a quiescent infection on developed mango fruit.

Non-chemical Control of Postharvest Diseases

Prevention or delay of infection

The amount of inoculum available for infection can be reduced by not planting other host plants of postharvest pathogens near fruit crops. Also, other sources of inoculum such as diseased twigs or fruit should be eliminated (Fittzell and Peak 1986; Sangchote 1991), and windbreaks can be used to reduce spore dispersal (Pruvost et al. 1990)

Sangchote (1995a) found that bagging of mango fruit starting 1 month after fruit set reduced incidence of anthracnose disease from 97 to 32%. It also reduced an infestation of fruit flies. The length of pedicel also has an influence on stem-end rot infection and disease development. In fruit with no pedicel, stem-end rot severity was the highest (41%), whereas it was lowest (12%) in the fruit with the longest pedicels (4 cm) (Sangchote 1991). Usually, this fungus infects through the stem-end scar and exposed surface of pedicel of fruit.

Eradication of infection and delay of disease development

Different postharvest practices can be used to delay disease development. These include low-temperature storage and modified or controlled atmosphere storage. Also, disease-resistant cultivars can be planted. At 15°C, the growth rate of *L. theobromae* is about 30% that at 25°C. Temperatures above 35°C also affect the growth of this organism (Chana et al. 1991). Cool storage at 10–13°C delays the development of stem-end rot and anthracnose in mango for several days (Schiffmann-Nadel et al. 1985; Johnson 1992). Teerapawa et al. (1980) treated fruit using two kinds of wax (wax 1: morpholene oleate in syntletic wax; wax 2: sucrose fatty acid ester). Treatment reduced the disease rate from 23.8 % (control) to 15.5 % and 11.1 %, respectively. Moreover, a combined treatment of wax and hot water (55°C, 5 min) also increased the level of disease control. Wax delayed ripening of the fruit, so its resistance to disease was prolonged. Holding fruit in a reduced oxygen controlled atmosphere

(8% O_2, 6 % O_2, or 4 % O_2) reduced anthracnose disease incidence from 38.6 to 28, 6.3, and 3.8%, respectively, but this is a costly practice (Sangchote 1989).

Infection of postharvest pathogens can be delayed by the use of resistant cultivars.

Six cultivars of mango fruit inoculated with *L. theobromae* showed different levels of disease severity. Mangoes cv. Kaew were rather resistance (Sangchote 1991). In Thailand, this cultivar is used mainly for processing. Many durian cultivars, including Kadoom, Laung, Kob, Kanyao, Chanee, and Monthong, have different levels of susceptibility to *Phytophthora palmivora*, which causes fruit rot. Chanee is a relatively resistant cultivar (Pongpisutta and Sangchote 1994). This cultivar is exported to many countries such as Hong Kong and China (Keeratipataragul 1980). Physical treatments such as heat, high CO_2, and UV-C are used to induce resistance in fruit. Treatment of mangoes with high CO_2 concentrations for short periods after harvest enhanced levels of the antifungal resorcinols and delayed decay development (Prusky and Gat 1992).

Other measures which have been successful in delaying disease development and eradicating infection are hot water treatment, irradiation, and biological control. Irradiation delays the ripening of fruit but, to avoid unwanted effects on quality, care is needed with the dosage. Fruit irradiated at 600Gy showed reduced incidence of anthracnose and stem-end rot, but the treatment affected colour change in the fruit. Combinations of irradiation and other treatments had an additive effect (Johnson et al. 1990).

Heat treatment of fruit at 50–60°C for 510 minutes controlled many postharvest pathogens (Smith et al. 1964). Treating mango fruit with hot water at 55°C for 5 minutes reduced anthracnose severity (Sangchote 1989). Heat treatment causes protein denaturation, lipid release, destruction of hormones, asphyxiation of tissue, depletion of food reserves, or metabolic injury with or without accumulation of toxic intermediates (Baker 1962).

Biological control is at an early stage of development as compared with other treatments. It had been used to control postharvest pathogens in the field and after harvest. De Villiers and Korsten (1994) reported that postharvest dip applications of *Bacillus licheniformis* (isolates B250 and B251) controlled anthracnose and stem-end rot of mango. *Bacillus licheniformis* at 10^8 cell/mL also reduced decay of lychees (Korsten et al. 1993). Sangchote (1995b) investigated the biological control potential against stem-end rot of mango of 11 isolates of food yeast. *Candida tropicalis* (IFRPD 6010) reduced spore germination of *L. theobromae* by 43% and mycelial growth by 18%. *Candida tropicalis* competed for nutrients with the conidia of *L. theobromae*. The lesion size of stem-end rot on wounded mango fruit treated with yeast, then challenged with the pathogen conidia was reduced by 79%. Development of biological control agents has thus made good progress, but it will take some time yet for these approaches to gain full acceptance by regulatory authorities and users.

Conclusion

Postharvest diseases of tropical fruits involve various kinds of pathogens. Previously, these organisms were successfully controlled by chemical treatments. However, produce so treated has become unacceptable to consumers and importing countries because of the risk of chemical residues. Non-chemical treatments can minimise postharvest diseases. Their successful adoption will require integration and manipulation of all detailed knowledge of the postharvest pathosystem.

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The Role of the Stem End of Citrus in Infection by *Phomopsis*

Y. Homma*

Abstract

The present study demonstrates why *Phomopsis citri* does not rot fruit until the end of the storage period even though it is able to penetrate the fruit pedicel. We have found that one of three inhibitory substances is crystallised in chestnut-burr-like colonies in the tissues around the fruit stem buttons of *Citrus unshiu* when treated with formalin vapour. The others can be extracted as resinous materials from the methanol-soluble fraction of fruit stem buttons. They were purified by repeated preparative thin-layer chromatography on silica gel and HPLC to yield pale yellow crystals or resinous materials. These compounds were identified as a flavanone, hesperidin (the former), and derivatives of cinnamic acid, m-hydroxy cinnamic acid methyl, ferulic acid methyl (the latter), from the results of measurement by GC-MS, ^{1}H-NMR and ^{13}C-NMR.

A stem button has on average 0.324, 0.041, and 0.001 mg of hesperidin, ferulic acid methyl, and trans m-hydroxy cinnamic acid methyl, respectively. In a stem button 25 μg or more of these compounds could completely inhibit conidial germination of *Phomopsis citri* or other pathogens. Therefore, the level of hesperidin or ferulic acid methyl and trans m-hydroxy cinnamic acid methyl in a stem button seemed to restrict the entry of *P. citri* or other pathogens into the fruit. In addition, we have confirmed that hesperidin disappears from around the stem button towards the end of the storage period.

CITRUS melanose, scab, and canker are common diseases of citrus plants in Japan. Citrus fruits, however, do not begin to rot while they are still on the tree. Decay begins after they are picked or at the end of the storage period about 2 months after harvest, when they seem to lose their resistance (Homma and Yamada 1969). The study reported here shows why *Phomopsis* (*Diaporthe*) *citri* does not rot fruit until the end of the storage period, even though it is able to penetrate the fruit pedicel.

Relation between Citrus Melanose, Stem-end Rot Occurrence and Seasonal Change

As shown in Figure 1, symptoms of citrus melanose can be observed from early June to early October, the amount of infection governed by period of wetness from rainfall. The fungus that entered the pedicel appeared to grow into the disk through vascular bundles, but initially failed to pass over the disk. The pathogen required an extended incubation period of 8–9 months (February or March) to reach the apex of the fruit through strands of vascular bundles (Homma and Arimoto 1981, 1988).

Why Does Citrus Stem-end Rot Occur at the End of Storage Period?

As shown in Table 1, fruit peel of satsuma mandarin has an inhibitory effect. An extract of the peel completely inhibited pycnidiospore germination even at 1000 times dilution. It was not clear whether the pathogen was unable to penetrate into the oil glands. For clarification, oil gland juice from the peel of mature fruit was tested for its effect on pycnidiospore germination at concentrations of 25, 50, 75, 85, 90, and

* Department of Agricultural Chemistry, Tamagawa University, 6–1–1, Tamagawa–Gakuen, Machida–shi, Tokyo, 194, Japan.

100%. The 90% of oil gland juice completely inhibited pycnidiospore germination and germ tube elongation (Table 2). The stem buttons, the tissues under the disk at the citrus stem end, and the subsequent vascular bundles inside satsuma mandarin fruit allow the passage of the pathogen hyphae that cause stem-end rot. It was determined whether extracts from those vascular bundle systems had an inhibitory effect. As shown in Table 3, a 10-time dilution of extract from the stem button and the tissues under disk completely inhibited pycnidiospore germination, and the same dilution of the extract from subsequent vascular bundles strongly inhibited germination. A 100-times dilution of the extract from the tissues under the disk had an inhibitory effect on germ tube elongation. Therefore, fruit peel and tissues under the disk might contain factors which inhibit this pathogen.

Existence of Antifungal Substances in Stem End

A stem button of tissue under disk (7 mm in diameter and 8 mm in depth) was removed from the stem end of healthy fruit. The tissue was cut longitudinally (Fig. 2). It was kept on a glass slide in a petri dish and fixed with formalin-soaked filter paper. The formalin vapour sterilised the tissue, which was dried for 30 minutes under high vacuum. Scanning electron microscopy (SEM) revealed numerous chestnut-burr-like colonies of needle crystals around the central part of the underside of the abscission zone of the disk. Figure 3 shows the SEM image of the central part under a disk.

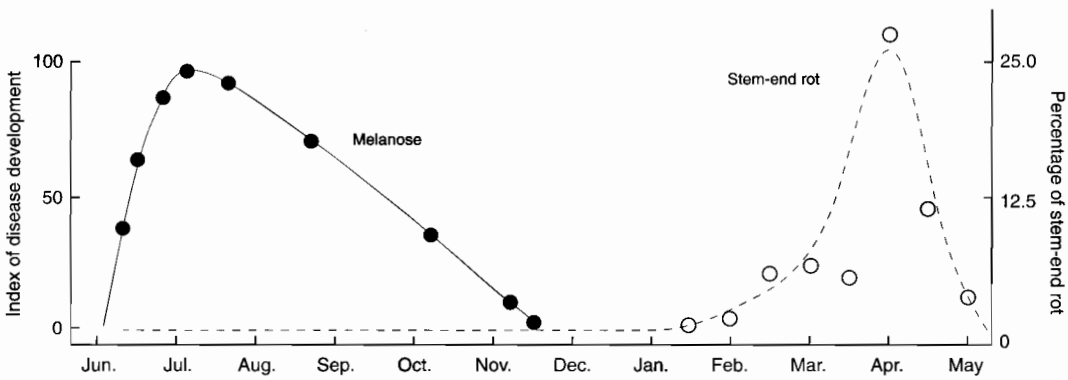

Figure 1. Relation between citrus melanose, stem-end rot occurrence, and seasonal change.

Table 1. Effect of the extracts from various parts of satsuma mandarin on pycnidiospore germination and germ tube elongation of *P. citri*.

Extract	Treatment	Pycnidiospore germination (%)	Inhibition (%)	Index of germ tube length[a]	Inhibition (%)
Fruit peel	FC[b] × 10^4+F[c]	100	0	13	78
	FC × 10^3+F	0	100	0	100
	FC × 10^2+F	0	100	0	100
Fruit vesicle	FC × 10^2+F	39	61	16	73
Distilled water	−− +F	100	—	60	—

[a] Index of germ tube length (X) was calculated by the following equation

$$X = \frac{1n_1 + 2n_2 + 3n_3 + 4n_4 + 5n_5}{5N} \times 100$$

Where $n_1 - n_5$ indicate the number of pycnidiospores with the germ tube length equal to, less than 4 times, less than 10 times, less than 15 times and more than 15 times of major axis of the pycnidiospore, respectively. Observations were made after 24 hours incubation.

[b] FC: formulated concentrate (sampling date: November 16)

[c] F means containing 5% fructose.

Table 2. Effect of concentration of oil gland juice on pycnidiospore germination of *D. citri*.

Concentration of oil gland juice[a]	Conidial germination (%)	Index of germ tube length[b]
25	100	100
50	100	100
75	11.5	16.5
85	3.0	14.5
90	0	0
100	0	0

[a, b]See Table 1

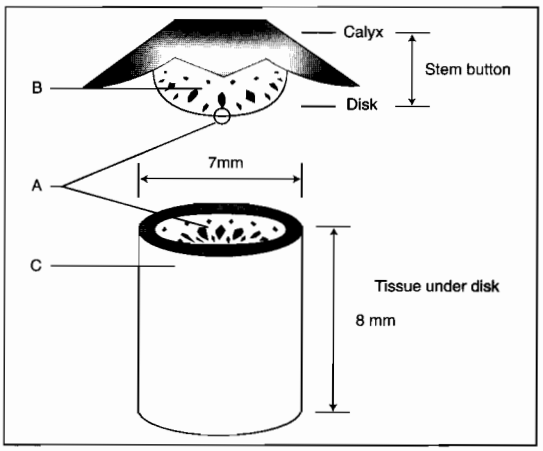

Figure 2. The diagram of the stem button and the tissue under disk for extraction of inhibitory substances. A, B, and C show the position of citrus stem end cut longitudinally and photographed under scanning electron microscopy in Figure 3.

One of the colonies was taken off with a pin, pressed on a specimen stub to make a thin film, and analysed by Fourier–transform infrared spectrophotometer (FT–IR). Figure 4 shows the FT–IR spectrum of a chestnut-burr-like colony of tissue preinhibitin. The FT–IR spectrum of the preinhibitin on tissue is almost superimposable on that of the authentic hesperidin. It can be concluded that needle crystals observed by SEM are those of hesperidin (Homma et al. 1992). Furthermore, the citrus stem buttons of 605 samples were extracted in methanol, and the filtrate also extracted with ethyl acetate. Germination inhibitory activity was in the ethyl acetate phase, not the aqueous phase. Thin-layer chromatography (TLC) was then carried out using ethyl acetate:n-hexane 1:1 as the solvent system. This experiment confirmed that the germination inhibitory activity was in a blue fluorescent band at Rf 0.7. As a 2000-times dilution of this fraction completely inhibited pycnidiospore germination of this fungus, an attempt was made to purify the substances inhibiting germination. Using the scheme shown in Figure 5, two substances, Cu-5 and Cu-6, were isolated by TLC and high-performance liquid chromatography (HPLC). Measurement by GC–MS, ^{1}H–NMR, and ^{13}C–NMR (Hosoda et al. 1997) indicated that these compounds were derivatives of cinnamic acid, namely ferulic acid methyl and trans (or cis) m-hydroxy cinnamic acid methyl. A stem button contains, on average, 0.324 mg hesperidin, 0.041 mg ferulic acid methyl, and 0.001 mg trans (or cis) m-hydroxy cinnamic acid methyl. The presence of 25 μg (0.025 mg) or more of these compounds in a stem button will completely inhibit conidial germination of *Phomopsis citri* or other pathogens.

Table 3. Effect of the extracts of disks, tissues under disks, and vascular bundles of satsuma mandarin fruit on pycnidiospore germination and germ tube elongation of *P. citri*.

Extract	Treatment	Pycnidiospore germination (%)	Inhibition (%)	Index of germ tube length[a]	Inhibition (%)
Stem button	FC[b] × 10^3+F[c]	100	0	67	1
	FC × 10^2+F	100	0	87	–28
	FC × 10+F	0	100	0	100
Tissue under disk	FC × 10^3+F	100	0	56	18
	FC × 10^2+F	95	5	38	44
	FC × 10+F	0	100	0	100
Vascular bundle	FC × 10^3+F	100	0	80	–18
	FC × 10^2+F	100	0	100	–47
	FC × 10+F	3	97	11	24
Distilled water	— +F	100	—	68	—

[a, b and c]See Table 1 (Sampling date: October 7)

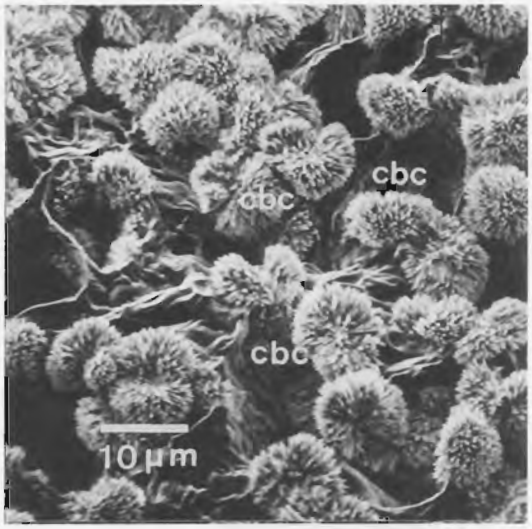

Figure 3. Longitudinally cut tissue under a disk of satsuma mandarin fruit at mature stage. CbC: chestnut-burr-like colonies consisting of needle crystals.

Disappearance of Antifungal Substance in Storage and Stem-end Rot

The tissues of satsuma fruit were extracted at increasing intervals after harvest. The results are shown in Figure 6. The extract from citrus fruit vesicles in October suppressed pycnidiospore germination of *P. citri*. An extract collected from vesicles at the end of February was less effective, and that collected in the middle of April had no effect on pycnidiospore germination and germ tube elongation. The inner part of the stem button is covered with many small crystals after treatment with formalin vapour (Fig. 7). On the other hand, at the end of the storage period (late in April), there were no crystals around the inner part of stem button (Fig. 8). The preinhibitin, hesperidin, seemed to have disappeared through self-digestion of citrus fruit. In the storage test, stem-end rot began to occur in the middle of February and increased with the length of storage. This result seemed to parallel percentage pycnidiospore germination and the index of germ tube length following treatment with the extract from citrus fruit vesicle (Homma et al. 1989).

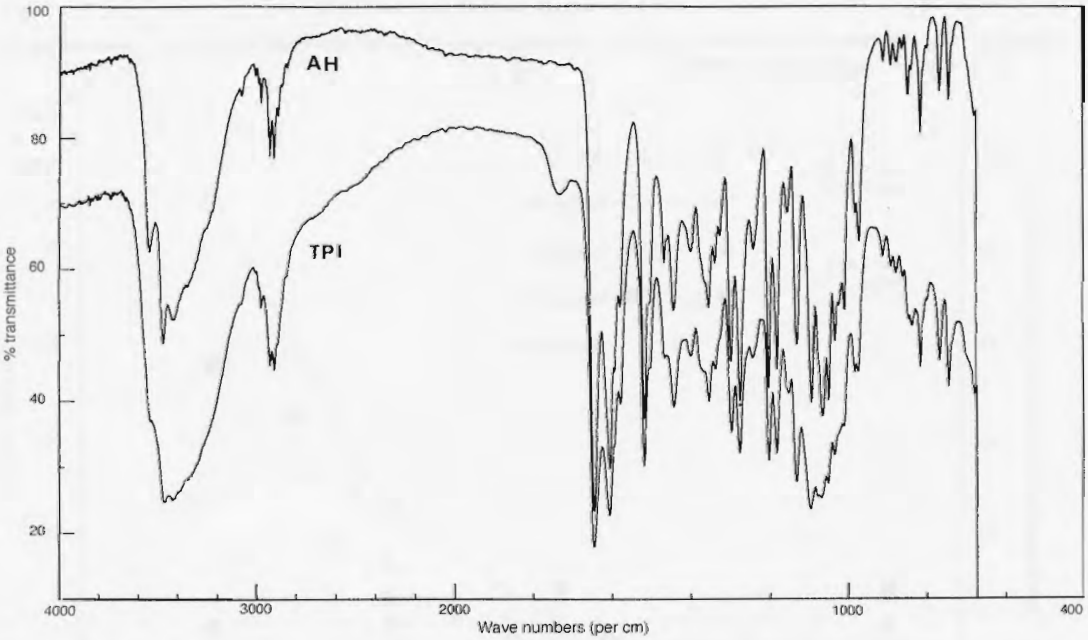

Figure 4. The Fourier–transform infrared spectra of tissue preinhibitin and authentic hesperidin. TPI: tissue preinhibitin; AH: authentic hesperidin.

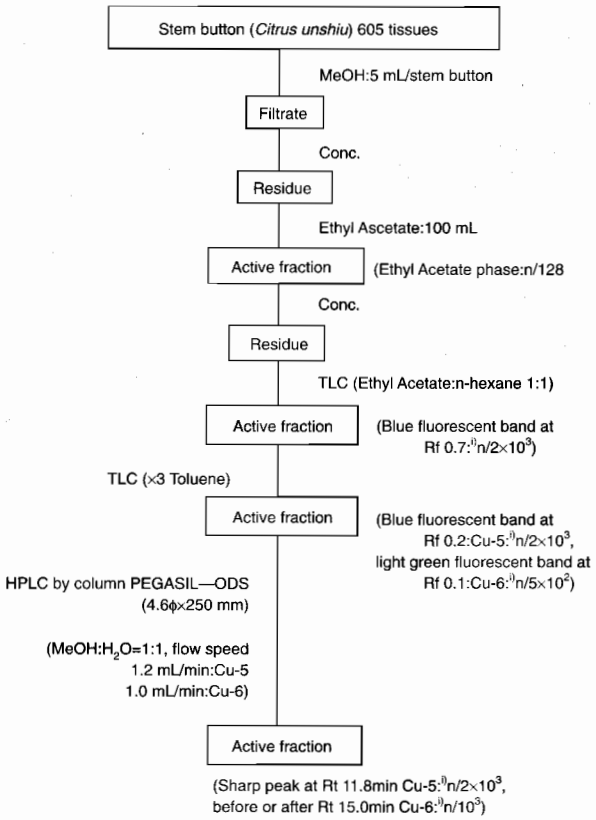

Figure 5. The procedure for isolation of inhibitory substances against pycnidiospore germination of *P. citri*. Substances marked i) gave complete inhibition.

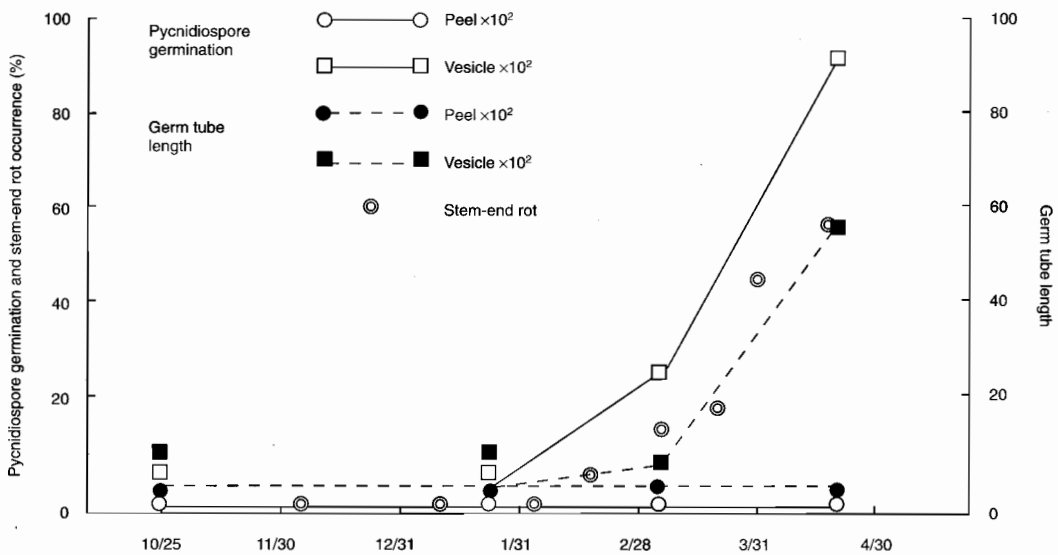

Figure 6. The relationship between inhibitory effect of stored fruit extracts on pycnidiospore germination and germ tube elongation of *P. citri* and stem-end rot occurrence.

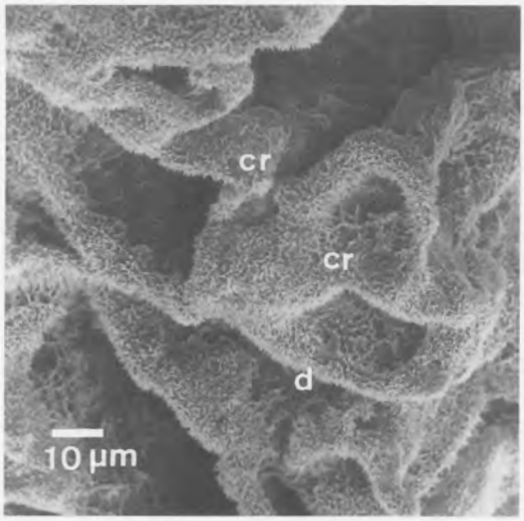

Figure 7. Inner disk of maturing fruit in late October. Many crystals cover the inner disk. cr: crystal, d: disk end.

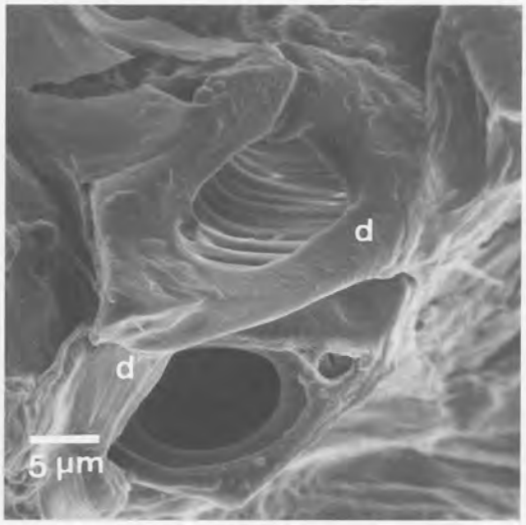

Figure 8. Inner disk of fruit stored until late April. Crystals have disappeared from the inner disk. d: disk end.

The Role of Stem End of Citrus Fruit

The citrus stem end, namely the stem button and the tissue under the disk, does not only inhibit *P. citri* and other pathogens by these preinhibitins, but also controls the number of fruit, through so called "physiological drop" when the tree is over bearing fruit.

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Containment of Infection

Mechanisms of Resistance of Fruits and Vegetables to Postharvest Diseases

D. Prusky*

Abstract

To successfully colonise a particular host, a microorganism must develop the ability to overcome defensive barriers elaborated by the plant. Dynamic and ongoing evolutionary battles have resulted in the utilisation of highly specific and sophisticated attack strategies by the pathogen and equally elaborate defence responses by the host. What are the mechanisms that control the lack or degree of virulence before and after pathogenicity has been established in postharvest host–pathogen interaction? The first barrier that the pathogen encounters is the plant cuticle. Cuticular waxes can induce the production of infection structures, and specific enzymes are needed for breaching the cuticle. Host inhibition of the production of pathogenicity factors at this early stage can affect fungal development. A fruit host also can restrict fungal colonisation by (i) preformed barriers where deposition of phenolic polymers occurs, or (ii) by preformed antifungal compounds. Preformed antifungal compounds tend to concentrate in the outer layers of the fruit hosts. Differential decrease in the level of antifungal compounds during periods of increased susceptibility was used as an initial basis to determine their involvement in host resistance. Study of the host mechanism controlling the decline of preformed antifungal compound has resulted in a better understanding of the importance of these compounds in host resistance. However, in the absence of host mutants differing in the amount of preformed compounds, the possibility that pathogen virulence could be determined by detoxification of preformed compounds has raised another option regarding their importance. Interestingly, though these compounds are considered preformed and therefore, by definition, not inducible, higher levels of these compounds were induced by biotic and abiotic factors and a recently described signal transduction process regulating this process. Inhibition of pathogenicity factors is another possibility for restriction of fungal colonisation of host fruits. Several polyphenols, tannin, and polygalacturonases inhibiting proteins were found to inactivate pectic enzymes needed for fungal colonisation of host fruits. A general understanding of the plant biosynthetic pathway which led to the formation of preformed barriers is of primary importance for the manipulation of resistance and could be the basis for novel crop protection strategies.

FUNGAL pathogens of fruit and vegetables must follow several stages during their life cycle, from spores landing on the host surface to the production of decay symptoms. Spores have to attach to the surface, germinate, produce penetration structures and breach the cuticle, or penetrate directly through wounds, and activate pathogenicity factors, in order to achieve decay development. A successful pathogen must also have the capability to overcome host defences and initiate attack under the physiological conditions prevalent.

The appearance of disease symptoms in a compatible interaction is the outcome, in most cases, of the ability of the pathogen to overcome the complexities of the host defence responses. Incompatibility, a resistance reaction that prevents or retards pathogen growth, may be conditioned by a single interaction gene pair: a host resistance (*R*) gene and a pathogen avirulence (*Avr*) gene (Flor 1971). Although gene-for-gene interactions are extremely important and specific, they are not the usual or only case of interactions occurring during pathogenesis of postharvest fruits.

* Department of Postharvest Science of Fresh Produce, The Volcani Center, Agricultural Research Organization, Bet Dagan 50250, Israel.

In fruits and vegetables the mechanism of resistance is generally not dictated only by a single gene interaction of the host and pathogen. However, there have been cases of basic incompatibility. For example, *Penicillium digitatum,* a pathogen of citrus fruit, does not attack apple fruits but does attack other deciduous fruits. This specificity defines a relationship between pathogens and host plants, that results in the inhibition of pathogen infection or growth. The genetic and physiological bases for this kind of specificity are not known.

Most of the fruit and vegetable interactions maintain a type of dynamic incompatibility. This is a situation triggered by the response of the hosts resistance genes to the pathogens avirulent genes that prevents or retards pathogen growth, under specific host physiological conditions. When the pathogen cannot trigger the hosts defence responses due to physiological changes in the harvested host, the interaction becomes compatible. This is the most common situation in postharvest interactions. Pathogens such as *Botrytis* and *Colletotrichum* attack a broad range of hosts, remaining quiescent during fruit growth and becoming virulent during senescence of the harvested host. For this type of interaction, quantitative rather than qualitative reactions are the general rule in the postharvest host–pathogen interaction. When this interaction takes place, the following questions become more significant: (i) What is the nature of preformed barriers to pathogen attack? (ii) How do *R* genes trigger defence responses and what are the physiological conditions needed for triggering defence responses? (iii) What are the roles of the various induced compounds in resistance? (iv) How are secondary defence responses such as systemic acquired resistance (SAR) elicited and maintained? Fruit–pathogen interactions are then a dynamic interaction in which the equilibrium is dependent on expression of resistant factors by the host and pathogenicity factors by the pathogen during different periods of fruit life.

Fungal Infection in Fruits and Vegetables

The evolution of fungal phytopathogens toward a high degree of specialisation for individual plant species may be reflected in the different ways of pathogen attack (Jackson and Taylor 1996). Some pathogens are opportunistic parasites, which enter the plant through wounds or natural openings because they lack enzymatic capabilities to penetrate directly, or require a weakened plant for colonisation. Other pathogens rely on the initial recognition by a living host to induce and produce, under specific circumstances, infection structures to penetrate and colonise the host. There are two key questions about the process of direct or wound penetration: (i) What are the mechanism(s) that enable the transition from of saprophytic to pathogenic conditions? (ii) What are the mechanism(s) that control the lack or degree of virulence on the host once pathogenicity has been established?. These questions are related to the process of fungal penetration and colonisation that will be the main subject of this paper.

Active Penetration of Plants

To colonise plants, fungal pathogens have evolved strategies to invade plant tissue to optimise their growth in the plant, and to propagate. The cuticle covers the outer parts of fruits and vegetables, and several pathogen events occur during the initial contact and penetration of this part of the plant (Kolatukkudy 1987). To gain entrance through the cuticle, through wounds, and then through the epidermal cell wall, fungi generally secrete a cocktail of hydrolytic enzymes, including cutinases, cellulases, pectinases, and proteases.

Constitutive Mechanisms of Resistance

Inhibitors of appressoria formation

Spores of *Colletotrichum* that landed on very hydrophobic surfaces, such as fruit wax, are induced to produce appressoria (Prusky and Plumbley 1992; Podilia et al. 1993). This suggests that surface waxes are prime candidates as signals for the fruit–pathogen interaction. Avocado wax was shown to enhance appressoria formation. This kind of signalling, according to Kolattukudy et al. (1995), was demonstrated to be specific, since other plant waxes could not induce differentiation in *Colletotrichum gloeosporioides*. The fatty alcohol fraction, which constituted only 5% of the whole wax, was the most active fraction, with C_{30} and C_{32} as the major components. Interestingly, very-long-chain alcohols are present in many plant waxes, but they do not induce appressorium formation, probably because they also contain inhibitors of this process. Addition of plant waxes from other plants, such as broccoli, jade, and

Senecio odoris leaves to avocado wax inhibited the ability of the latter to induce appressorium formation by *C. gloeosporioides*. These findings suggest that plant-surface lipids contain both inducers and inhibitors of spore germination and appressorium formation, and that the balance between them might be responsible for the selective activation of the pathogen for initiation of parasitisation.

Inhibitors of appressoria germination

Development of germinated appressoria can be affected by constitutive factors that influence appressorium germination (Muirhead 1981). Appressorium germination inhibitors may be volatile or non-volatile. The lack of appressorium germination may result from the lack of a germination-triggering factor; i.e., an inducer that appears only during specific periods of fruit life. Flaishman and Kolattukudy (1994) suggested that ethylene produced by the host specifically at ripening could be a signal for the termination of appressorial dormancy on the fruit surface. Ethylene at concentrations of less than 1 μL/L, much lower than the concentration produced during fruit ripening, induced both germination and appressorium formation in *C. gloeosporioides* and this can dictate decay initiation. However, experiments reported by Prusky et al. (1996) did not support this theory. Treatment with ethylene of unripe avocado fruits inoculated with *C. gloeosporioides* did not induce decay symptoms. This indicates that, even if ethylene induced multiple appressoria formation, several other changes must occur in the fruit peel for the germinated appressoria to be able to develop further and cause disease symptoms.

Inhibitors of germinated appressoria penetration

Several key postharvest pathogens, such as *C. gloeosporioides* and *B. cinerea,* which directly penetrate the host, secrete cutinase during their penetration. Several lines of evidence demonstrated the importance of cutinase in the pathogenicity of fruits. Dickman et al. (1983) showed that inhibition of cutinase by chemical inhibitors or cutinase-specific antibodies prevented penetration of *C. gloeosporioides* in intact, but not in breached, cuticle. Transformation of the wound pathogen *Mycosphaerella,* with a cutinase gene obtained from *Fusarium* from pea, resulted in the penetration of the intact papaya fruit (Dickman et al. 1989). However, the importance of cutinase has been questioned and may vary between fungi and physical mechanisms compensating for the lack of the enzyme.

Inhibition of fungal penetration by cuticle thickness

The resistance of sweet cherry to infection by *Monilinia fructicola* was correlated with cuticle and cell-wall thickness (Adaskaveg et al. 1989,1991; Michallidis and Johnson 1992). Also, in peaches, the length of the period until symptom expression increased with the cuticle and cell-wall thickness. Peach cultivars that were significantly more resistant had a thicker and denser epidermis than those of susceptible cultivars, and their resistance was correlated with penetration delay and longer incubation periods for infection (Adaskaveg et al. 1989, 1991). Similar findings were reported by Michaillidis and Johnson (1992) in nectarines, where the latent period of *M. fructicola* decreased with the decrease in cuticle thickness of the fruit.

Inhibitors of fungal penetration in host cuticle

The development of germinated appressoria may, at early stages, need a combination of penetration force with cutinolytic and hydrolytic enzymes.

Bostock et al. (1996) suggested that the development of *M. fructicola* in unripe fruit is inhibited by the presence on the fruit surface of phenolic acids. Chlorigenic acid and caffeic acid are the major phenols in the epidermis and subepidermal cell layers of peach fruit. Their concentrations are especially high in peach genotypes with high resistance to the brown rot fungus, and decline as fruit matures. There is a corresponding increase in disease susceptibility. It was proposed that high concentrations of chlorigenic acid and caffeic acid present in immature fruit may contribute to brown rot resistance through the interference with the production of cutinase rather than by directly affecting the pathogen.

The results reported by van Kan et al. (1997), where gene disruption of cutinase genes of *Botrytis cinerea* did not affect fungal penetration to gerbera flowers, suggested that germination of appressorium and its initial development is dependent on more than a single enzyme, cutinase. Wattad et al. (1997) tested that possibility. They purified pectate lyase from *C. gloeosporioides* and obtained antipectate lyase antibodies which did not affect germination or germ tube growth of *C. gloeosporioides* spores. However, when antipectate lyase antibodies were supplied to germinating conidia, symptoms of *C. gloeosporioides* in avocado, mango, and banana were inhibited. This suggests that the presence of pectate lyase inhibitors might become a constitutive barrier during very early stages of fruit infection.

Inhibition of fungal colonisation constitutive barriers

Fungal restriction by host barriers

A highly anionic peroxidase isolated from tomato fruits was found in the exocarp of unripe fruit (Sherf and Kolattukudy 1993); it is encoded by a single gene (*tap*). The constitutive expression of *tap* in green fruits undergoes a twofold increase during the development from immature to mature green fruits. Once fruits reach their climacteric stage, however, *tap* transcriptions diminish and remain undetectable in the ripe fruit, which is susceptible to decay development. Detaching green fruits from the vine, however, leads to complete loss of the *tap* transcripts. The rapid decline in peroxidase mRNA levels observed in detached green fruits may possibly result from the loss of a positive regulatory molecule which originates in the parent plant and functions to sustain the constitutive expression of the gene in maturing green tomatoes. The anionic peroxidase is a key enzyme in polymerisation of the phenolic polymers which act as barriers in cell walls, rendering the cell walls highly resistant to mechanical and enzymatic disruption (Kolattukudy 1987; Pearce and Rutherford 1981). Lignification and suberisation of the plant cell walls have been suggested as being part of the elaborate defence strategies of the plant. It is therefore possible that the constitutive expression of such an anionic peroxidase in green tomato fruits represents a barrier to fungal development.

Fungal restriction by preformed compounds

Plants produce a diverse constitutive array of secondary metabolites with antifungal properties (Osbourn 1996b). These types of compounds will affect either the germinated spores that have entered through wounds, or pathogens that have penetrated the host directly and have already overcome the first set of barriers. Some of these compounds are present in the plants in their biologically active form. Some other compounds occur as inactive precursors and are activated in response to pathogen attack (Osbourn 1996a). These preformed compounds or phytoanticipins differ from the inducible phytoalexins that are synthesised from remote precursors in response to pathogen attack.

The distribution of preformed inhibitors within plants is often not uniform (Bennet and Wallsgrove 1994). These compounds tend to be concentrated in the outer layers of plant organs, which suggests that they may indeed act as inhibitors to pathogens and pests. In some cases, diffusible preformed compounds like catechol and proteocatechuic acid (which are found in onion scales) can influence fungal growth at the plant surface (Osbourn 1996b). In general, preformed compounds might be compartmentalised in vacuoles or organelles in healthy plants, such as gossypol in cotton and the antifungal compounds in avocado that are compartmentalised in oil bodies in the mesocarp (Prusky and Keen 1995). Therefore, the biological activity depends on the extent of fungal damage and the amount of compound released. It might be that specific signals from the pathogen could enhance and release the preformed compounds. The release of the compounds could depend on the genotype, plant age, and environmental conditions (Davis 1991).

There have been numerous attempts to associate natural variation in levels of preformed inhibitors in plants with resistance to particular pathogens, but only a few critical tests have been described (Prusky 1997). Difficulties in determining the role of preformed compounds in fruit resistance exist primarily because of problems in (a) accurately assessing the quantities of inhibitory compounds that may contact the invading pathogen, (b) determining in vivo biological activity of the compound, and (c) relating changes in their concentration to fruit resistance.

The isolation of a plant mutant defective in the biosynthesis of preformed inhibitors could allow a direct genetic test of the importance of these compounds in host defence, but this has not been done. In its absence, correlative data were used to sustain the importance of reduction of preformed compounds in host resistance.

Changes in the level of antifungal compounds were used as the basis for determining the involvement in host resistance. The decrease from fungitoxic concentrations in unripe resistance fruits to subfungitoxic concentrations of the preformed antifungal compounds in ripening or senescing fruits, was demonstrated initially for avocado, mango, and citrus fruits, and for celery stalks. In avocado, the resistance of unripe fruits to the quiescent germinated appressoria of *C. gloeosporioides* is correlated with the presence of high concentrations of preformed antifungal compounds. The major antifungal compound has been shown to be 1-acetoxy-2-hydroxy-4-oxo-heneicosa-12,15-diene (Prusky et al. 1991); the amount of this compound decreased tenfold, to subfungitoxic concentrations during fruit ripening when susceptibility increases.

In mango fruit, a mixture of antifungal compounds consisting of 5-12-*cis*-heptadecenyl resorcinol and 5-pentadecenyl resorcinol was found at fungitoxic concentration in the peel of unripe mango fruit that were resistant to *Alternaria alternata* (Droby et al. 1986, 1987). Also in this case, 5-substituted resorcinols occurred at fungitoxic levels in the peel of unripe fruit and decreased to nontoxic levels in ripening fruit at the same time as symptoms of decay appeared in inoculated fruit.

Citrus fruit are resistant to wound pathogens during fruit growth, even though a considerable amount of *Penicillium* inoculum is present. It has been suggested by Ben Yehoshua and co-workers (Ben-Yehoshua et al. 1992), that the resistance of young mature green lemons is related to the presence of a preformed monoterpene aldehyde, citral, which decreased in older yellow fruit, enabling decay to develop rapidly (Rodov et al. 1995).

Resistance of celery stalks to *Botrytis* could result from the presence of the preformed antifungal psoralen precursor, mermesin, since this is a very active preformed compound, almost ten times as fungicidal as the inducible psoralens that were considered in the past to effect celery resistance. Decreased concentrations of mermesin during storage, and not the induction of psoralens, are the main factor affecting the resistance of celery to *B. cinerea* (Afek et al. 1994). Decay occurred earlier when mermesin concentration decreased faster (Afek et al. 1995).

Another piece of evidence suggesting the importance of preformed compounds is the differential decrease of preformed compounds during the period of increased susceptibility. Fruit of mango cv. Tommy Atkins are more susceptible to decay than those of cv. Hayden and show a faster decrease in antifungal compounds during ripening (Droby et al. 1986). Also, avocado fruit susceptible to fungal development showed a faster decrease of preformed compounds to nontoxic levels in ripening fruit, at the same time as symptoms of decay appeared earlier in inoculated fruit (Prusky et al. 1988).

The activity of preformed compounds in different tissues, depending on the distribution or compartmentalisation of compounds, can also be consistent with the importance of preformed antifungal compounds in resistance. The finding that 85% of the antifungal diene in avocado mesocarp (flesh) is compartmentalised in specific oil cells, compared with the apparent free distribution in the peel, can explain the susceptibility of flesh tissue to fungal attack (Kobiler et al. 1994).

Since disease susceptibility in avocado fruit is correlated with decreased concentrations of the preformed antifungal compound, the host mechanism controlling this reduction may support the importance of the antifungal compounds in fruit resistance against *C. gloeosporioides* infection. The antifungal diene was found to serve as a substrate for oxidation by lipoxygenase that is activated during fruit ripening (Prusky et al. 1983; Prusky 1988). Lipoxygenase activity in ripening avocado fruit is affected by an endogenous inhibitor, epicatechin, present in the avocado peel (Prusky et al. 1988; Prusky and Keen 1993). This flavan-3-ol competitively inhibited lipoxygenase activity and decreased in concentration during fruit ripening, thus permitting lipoxygenase activity. Resistance to infections was therefore related to the period required for complete reduction of epicatechin in softening fruit. This suggests that epicatechin may be acting as an inhibitor of lipoxygenase activity, thereby delaying degradation of the antifungal diene (Prusky and Keen 1993).

Induction of preformed antifungal compounds

In spite of the fact that preformed antifungal compounds are considered non-inducible compounds (Van Etten et al. 1994), significant changes in the level of preformed compounds have been observed as a result of different biotic and abiotic elicitors (Prusky and Keen 1993). Challenge inoculation in unripe fruit also induces preformed antifungal compounds (Prusky et al. 1990). Inoculation of unripe avocado fruit with spores of a nonpathogenic mutant of *Colletotrichum magna* induced a significant increase in levels of the antifungal diene, with longer persistence of the elicitors, without any expression of symptoms (Prusky et al. 1994). This effect of a nonpathogenic strain was used to delay the activation period of wild type *C. gloeosporioides*, by co-inoculation of fruit with both strains.

All these observations reinforce the view that activation of fungus development depends on significant changes that are induced in the host during fruit ripening. These observations suggest, but do not always conclusively prove, that the resistance of unripe fruit results from fungitoxic concentrations of the antifungal compounds.

Modulation of preformed resistance by pathogens

The possibility that pathogen virulence could be determined by fungal detoxification of preformed antifungal compounds, and not by biochemical changes in the host, was raised by Osbourn (1996b).

Preformed saponins exhibit potent antifungal activity and are often present at relatively high concentrations in healthy plants (Osbourn 1996a). The major saponin in tomato is the steroidal glycoalkaloid α-tomatin. Its levels are particularly high in the leaves, flowers, and green fruits (Roddick 1974). During fruit ripening, however, there is a significant reduction of α-tomatin in the fruit. These results suggested that saponins may contribute to fruit resistance. Osbourn and Van Ettens group has cloned a number of saponin-detoxifying glycosyl hydrolase genes produced by tomato-infecting fungi (Osbourn et al. 1994a,b, 1995; Sandrock et al. 1995). The cloning of the gene encoding for tomatinase was used to prove the role of preformed antifungal compounds. Gene disruption of the *Septoria lycopersici* β-tomatinase gene resulted in a loss of tolerance to α-tomatin. Similar experiments were done with *Colletotrichum coccodes,* a pathogen of tomato fruit. This is the first genetic proof of a plant–fungus interaction to determine the importance of saponin detoxification for fungal attack.

Inhibition of pathogenicity factors by constitutive barriers

The inactivation of pectic enzymes by inhibitors has been hypothesised to be a mechanism of host resistance for modulating fungal pathogenicity (Schlosser 1994).

Preformed phenols inhibiting pathogenicity factors

Phenols have been reported as possible inhibitors of *B. cinera* in strawberries. *Botrytis* infects strawberry fruit via the floral parts, but the fungus remains, in a quiescent form, at the bottom of the receptacle until the fruit matures. Infection by direct penetration through the epidermis is relatively rare (Jersch et al. 1989). The inhibition of fungal development could be entirely attributed to the presence of proanthocyanidins, which are present in a solid layer beneath the epidermis and surrounding the receptacle. These compounds are oligomers, composed of pelargonidin, cyanidin, or delphinidin subunits, cyanidin being the most common. Since Huth and Schlosser (1982) consider that extracellular hydrolases are essential for pathogenesis of *B. cinerea,* their binding to proanthocyanidins and consequent inactivation would explain the plant resistance to this pathogen in unripe fruit. When fruit mature, the proanthocyanidins are still present but have lost their inhibitory power (Jersch et al. 1989), because of a high degree of polymerisation. Pathogen enzymes produced at this stage will permit rapid decay development.

Similarly, proanthocyanidins have also been found in the skin of grapes. Their concentration in young berries is sufficient to inhibit completely the extracellular hydrolases of *B. cinerea,* but with berry development the inhibitory power declines steadily (Hill et al. 1981).

In avocado peel, a flavan-3-ol was isolated and identified as epicatechin, which inhibited pectolytic enzymes produced by *C. gloeosporioides.* Purified polygalacturonase and pectate lyase produced by *C. gloeosporioides* were inhibited in vitro by epicatechin (Prusky et al. 1989; Wattad et al. 1994). At 20 μg/mL, epicatechin inhibited the enzyme maceration capability of avocado wedges by 64%. Since the flavan is present in unripe fruit at much higher concentrations (ca 350 μg/g fresh weight, ca 270 μg/mL) than the inhibitory concentrations, epicatechin may contribute to the resistance of avocado fruit by inhibiting the activity of pectate lyase of *C. gloeosporioides* and *C. musae.*

Preformed proteins affecting host resistance

Plant cell walls contain proteins that can specifically and effectively inhibit polygalacturonases (PG) of fungal origin. These PG-inhibiting proteins (PGIPs) have been reported in numerous plant species, including pear fruit (Abu-Goukh et al. 1983a), apple fruit (Yao et al. 1995), pepper fruit (Brown and Adikaram 1983), bean leaves (Cervone et al. 1987), alfalfa leaves (Degra et al. 1988), soybean (Favaron et al. 1994), raspberry fruit (Johnson et al. 1993), and tomato fruit (Stotz et al. 1994). PGIPs are relatively heat stable glycoproteins, that may inhibit fungal PG by both competitive and non-competitive mechanisms. Purified pear PGIPs inhibited various fungal PGs, including that of *B. cinerea,* but did not affect endogenous pear fruit PG activity (Abu-Goukh et al. 1983b). In pears, PGIP activity was observed throughout development (4–14 weeks after anthesis) and changes in susceptibility to decay development after ripening of cv. Bartlett were accompanied by a decrease in the PG inhibitor content of the fruit. PGIPs from different plant species are likely to differ in their inhibition kinetics and target specificity (Abu-Goukh et al. 1983a,b). The PGIP inhibitor was approximately 100 times more abundant in fruit than in flowers and was not detectable in pear leaves. PGIP specific activity in fruit was 200 times higher than in flowers and 1400 times higher than in leaves. Stotz et

al. (1993) have suggested that the pear PGIP promoter may effect a fruit-specific expression of the gene, resulting in the inhibition of *B. cinerea* (Stotz et al. 1993). Powell et al. (1994) reported that transgenic tomato fruit expressing the pear PGIP gene were more resistant to *B. cinerea* than control fruit. However, recent experiments indicated that, under field conditions, the transgenic cultivar did not show the same degree of resistance (J. Labavitch, pers. comm.).

Adikaram et al. (1997) described the presence of constitutive chitinases and protease in papaya latex, reporting them to be involved in the inhibition of *C. gloeosporioides* development in papaya. Only after the reduction in latex secretion from the peel or stem of papaya, did fungal attack occur.

Inducible Mechanisms of Resistance

There is a considerable amount of information on the role of inducible antimicrobial compounds in disease resistance in plants (Kuc 1987). Most of this information has limited relevance to postharvest issues, since it has been obtained from plants or organs which are not normally stored. The evidence for the involvement of inducible antifungal compounds in stored produce becomes stronger when a correlation exists between a compounds accumulation and the resistance of inoculated tissue. However, with the exception of highly specific fungal *Avr* and host *R* interactions, there is no absolute resistance/susceptibility in harvested organs. Accumulation of phytoalexins in infected tissues to levels found inhibitory in vitro to a pathogen, at early stages of infection is a possible indication of their role as a resistance factor. These inducible antifungal compounds could restrict and arrest lesions, but they could not limit absolutely fungal development. In all these cases, the resistance accompanying phytoalexin accumulation does not prevent disease but does prevent or delay extensive rotting (Kuc 1987). The rate of its accumulation is particularly important, as it can determine the outcome of an interaction. Susceptible hosts usually accumulate phytoalexins at a slower rate than do resistant hosts (Kuc 1994). In either case, the amount of phytoalexins that can be accumulated by any elicitor usually diminishes during ripening or storage. Such a drop in phytoalexins potential was noted after treatment of maturing grapes and stored carrots with abiotic elicitors, and was accompanied by a concomitant increase in host susceptibility to *B. cinerea* (Creasy and Coffee 1988, Mercier et al. 1993a). Other evidence indicating a role of phytoalexins in disease resistance is the reduced pathogen development accompanying the induction of phytoalexins. Cell wall hydrolysates of *Glomerella cingulata* (Adikaram et al. 1982) enhanced phytoalexin accumulation in pepper. No rot developed on wounded fruits treated with cell wall hydrolysates, 24 or 48 hours before inoculation with *B. cinerea*. Carrots, kumquats and lemons exposed to a low dosage of short-wave ultraviolet light before storage accumulated phytoalexins and became more resistant to storage, pathogens (Ben-Yehoshua et al. 1992; Mercier et al. 1993a,b). There was a significant correlation between the levels of 6-methoxymellein in the peel of ultraviolet-treated carrots and their resistance to *B. cinerea*.

Phytoalexins involvement in host–pathogen interaction of fruits and vegetables

Several cases have been reported in which phytoalexins accumulation could affect the host–pathogen interaction.

Apples

The resistance of Bramleys seedling apples to *Nectria galligena* is the result of induced benzoic formation. *N. galligena* invades wounds and lenticels of apple fruits before harvest (Swinburne 1971), but fruit rotting does not become severe until after harvest. Limited colonisation takes place following the initial invasion, and the synthesis of benzoic acid in the necrotic tissue was readily observed (Swinburne 1975). The elicitor of benzoic acid synthesis was found to be a protease produced by the pathogen (Swinburne 1975). Two other apple pathogens inducing quiescent infections, *Diaporthe perniciosa* and *Gloeosporium perennans*, also secreted proteases in vivo. In the case of *Penicillium expansum*, *B. cinerea*, *Phytophthora cactorum*, *Sclerotinia fructigena* and *Aspergillus niger*, no protease was produced in infected tissue and, consequently, these organisms could rot immature fruit without inducing benzoic acid accumulation. Benzoic acid is toxic only as an undissociated molecule, but its fungitoxic activity decreased significantly as a result of the increase in pH during fruit ripening (Brown and Swinburne 1973). This, in conjunction with increasing sugar levels, enables the pathogen to degrade benzoic acid and resume active growth. Based in those results it is suggested that the secretion of proteases followed by the induction of benzoic acid may be the basis for the induction of the mechanism of resistance in this cultivar.

Bananas

A series of compounds accumulated in arrested lesions in banana. Resistance of green banana to *C. musae* was associated with a growing necrotic reaction within the peel (Brown and Swinburne 1981), and five antifungal compounds not present in healthy tissue were isolated. These unidentified compounds diminished as the fruit ripened. Hirai et al. (1994) identified one compound as 2-(4'-hydroxyphenyl)-naphthalic anhydride. Another six phenalenone-type phytoalexins, viz., irenolone, emenolone, and musanolones C-F, the antifungal properties of which have not been clearly stated, have been described (N.K.B. Adikaram, pers. comm.). Two of these compounds of the phenalenone group were found to be induced also when *Phyllosticta musarum* infected the host fruit. This fungus causes a pin-head-sized spot described as freckles on the banana peel (Abayasekera et al., these proceedings).

Citrus

In citrus fruit, Stange et al. (1993) reported that a wound gum accumulated in injured lemon exocarp and contained a predominantly antifungal compound, identified as 3-[4-hydroxy-3-(methyl-2-butenyl) phenyl]-2-(E)-propenal. This compound increased in concentration with time, resulting in the complete limitation of the invading pathogen. In other citrus fruit, including kumquat, accumulation of scoparone (6,7-dimethoxycoumarin) was observed when the fruit were held at 36°C after inoculation with *P. digitatum* (Ben-Yehoshua et al. 1992). In lemons, only low amounts of scoparone were detected in wound-inoculated fruits at 17°C, presumably because of the rapid decay period (Kim et al. 1991). Incubation of lemons at 36°C for 3 days, starting 24 hours after inoculation, enhanced scoparone accumulation and prevented decay. Under these conditions, inhibitory levels of scoparone were present in the inoculated flavedo 24 hours after the beginning of the heat treatment and accumulation continued for 6 days after fruit were returned to 17°C. This heat treatment, which does not induce scoparone by itself, could help restrict growth of the pathogen until the phytoalexin elicitation is completed, or accelerate the defence response in the presence of the pathogen.

Vitis

In response to *B. cinerea* infection, the leaves of *Vitis* spp. produce the stilbene, resveratrol, which is converted into the antifungal trimer ε-viniferin (Langcake and McCarthy 1979; Pryce and Langcake 1977). The viniferins are constitutive compounds in the lignified tissue but are inducible in leaves. Young leaves are highly susceptible to *B. cinerea* and synthesise only a small amount of resveratrol; as they develop, their resistance and their ability to synthesise resveratrol increase concomitantly. With the fruit of grapes, the situation is reversed: immature grapes can synthesise large amounts of resveratrol but, as they mature, the ability to synthesise it decreases steadily and the fruit become more susceptible.

In grapevine leaves, an efficient elicitation factor may alter the activity of the rate-limiting enzyme, leading to stilbene formation. Stilbene synthase activity is the rate-limiting step, and various biotic and UV-light treatments were able to activate the stilbene synthase gene to different extents (Schroder et al. 1988. Lanz et al. 1990). In grapes, more than seven genes encode for stilbene synthase, but only two of them are expressed at higher rates upon elicitation (Melchior and Kindl 1991; Weise et al. 1994). A simple way to modulate resistance is by the use of ozone (Sarig et al. 1996), which was found to induce resistance to *Rhizopus stolonifer* in treated grapes. Several *Vitis vinifera* cultivars exposed to ozone for 10 minutes showed induction of resveratrol, similar to the induction by UV-c irradiation (Sarig et al. 1996). Ozone induction of plant defence has been attributed to the induction of the phenylpropanoid pathway and other systems (Eckney-Kaltenbach et al. 1994). Experiments with transgenic tobacco (Hahn et al. 1993) and tomato (Kindl 1994) proved that the transfer of the stilbene synthase gene to a plant not capable of forming stilbene results in disease resistance.

Peppers

Phytoalexins are induced at the initial stage of infection in peppers. The capsicannol phytoalexins, 1-deoxycapsidion and endesmadienol, were associated with the induction of resistance in *Capsicum annuum* fruits infected with *Glomerella cingulata* (Adikaram et al. 1988). When harvested unripe fruits were inoculated with *G. cingulata,* capsicannol accumulated readily. In ripening fruits, both capsicannol and capsidiol accumulated but, at the stage when lesion expansion occurred, both compounds were absent. It has been suggested that these compounds also accumulate at the sites of arrested *B. cinerea* lesions, but not in progressive lesions.

Carrot

The isocoumarin 6-methoxymellein is the major phytoalexin accumulated in carrot root tissues in response to infection by the storage pathogens *B. cinerea, Mycocentrospora acerina,* and *Sclerotinia sclerotiorum* (Coxon et al. 1973; Davies and Lewis 1981; Harding and Heale 1980; Kurosaki and Nishi 1983). In carrots held at low temperature and inoculated with *B. cinerea* or *M. acerina*, 6-methoxymellein accumulated to inhibitory concentrations at the site of resistant lesions. Roots which had lost 10 or 15% of their fresh weight were more susceptible to *B. cinerea* and produced less 6-methoxymellein (Garrod et al. 1978).

Lettuce

Lettuce leaves inoculated with *B. cinerea* accumulated the terpenoid, lettucenin A (Bennet et al. 1994). In response to conidial inoculum of this fungus, the phytoalexin accumulated within 3 days at the inoculation site to levels inhibitory to spore germination and germ-tube growth. Such inoculation with conidia caused only a low incidence of spreading lesions, in contrast to *Botrytis* mycelium, which apparently overwhelmed the phytoalexin response.

Potato

In potato, numerous phytoalexins and stress metabolites were found. The presence and amount of the various phytoalexins detected in inoculated tissues vary with the pathogen, cultivar, and conditions of incubation (Ghanekar et al. 1984; Lyon 1984. The sesquiterpene rishitin may be associated with the resistance to bacterial soft rot caused by *Erwinia carotovora*, which it inhibits in vitro (Lyon and Bayliss 1975). Amounts of rishitin detected in tissues with restricted rot were twofold higher than in tissues showing extensive rot (Lyon et al. 1975). Higher incubation temperatures, which make tubers more susceptible to soft rot, also did not affect the accumulation of rishitin (Ghanekar et al. 1984; Lyon 1984; Lyon and Bayliss 1975). Low oxygen, which suppresses rishitin accumulation, enhanced the development of soft rot (Ghanekar et al. 1984). Although the levels of phytoalexins increased in more resistant potato tubers, it is not clear whether resistance is dependent on the presence of their phytoalexins.

Sweet potato

In root slices of sweet potato, several phytoalexins accumulate. Inoculation of sweet potato with *Ceratocystis fimbriata* led to accumulation of the furanoterpene ipomeamarone, and the coumarins umbelliferone and scopoletin (Akazawa and Wada 1961; Minamikawa et al. 1963). The accumulation of these three compounds was rapid in root slices of a resistant cultivar, especially between 24 and 72 hours after inoculation. Ipomeamarone levels in resistant cultivars 72 hours after inoculation were double those in susceptible cultivars. At the same time, umbelliferone levels in the resistant cultivars were more than three times higher than in susceptible cultivar (Minamikawa et al. 1963).

Phytoalexins were detected also in other vegetables. Eggplant was found to produce lubumin and other sesquiterpenes in response to *B. cinerea, Fusarium oxysporum,* and several non-pathogens. Two phytoalexins have been detected in onions inoculated with *B. cinerea:* 1,3-dion-5-octyl-cyclopentane and 1,3-dion-5-hexyl-cyclopentane.

Fungal Pathogenicity and Its Induction by the Host's Physiological Maturity

The hypothesis presented by Simmonds (1941) is that fruit and vegetable resistance could be attributable to failure of the pathogen to produce adequate levels of pathogenicity factors, such as cutinolytic or pectolytic enzymes, until the ripening process leads to suitable changes in the cell-wall structure. Theoretically, an inadequate pectolytic enzyme potential could be the result of several factors (Prusky 1996): (i) pathogen enzymes are not all constitutive and may require induction by proper substrates; (ii) enzymes of the pathogen might be produced but are blocked by cation cross-linking; and (iii) enzymes of the pathogen might be inhibited or inactivated by substances present in higher quantities in immature fruit, as described before.

The first hypothesis, supported by Simmonds (1941) and Verhoeff (1974), fails to explain the results of a simple experiment. *C. gloeosporioides* and *A. alternata* readily macerated (within 1 day) the mesocarp of peeled fruit of unharvested or freshly harvested unripe avocado and mango fruit, before the occurrence of any physiological changes which take place during ripening. Infection of the peel (pericarp) of the same unripe fruit resulted in a quiescent infection that developed 1420 days later (Kobiler et al. 1994; Prusky 1996). This simple experiment suggests that there is a difference in tissue susceptibility between the peel (pericarp) and the flesh (mesocarp) of unripe avocado and mango fruit,

and that the biochemical changes in the flesh during fruit ripening are not related directly to activation of quiescent infections in the peel.

Enzymes that degrade the cells walls in fruit might also behave as specific activators of fungal pathogenicity factors and thereby activate susceptibility. Expression of the tomato PG during the fruit climacteric is temporally correlated with the susceptibility of the fruit to fungal infection. Severity of postharvest diseases in transgenic tomato fruit lines is a function of the level of polygalacturonase expressed by the plant, suggesting that developmentally regulated plant genes can act as biochemical determinants of susceptibility. Furthermore, PG-solubilised pectic polysaccharides from tomato may act as signal molecules that specifically induce pathogenicity factors. This indicates that a highly evolved and complementary signalling process might be invoked by the plant and the pathogens in order to activate infection by the pathogens (J. Labavitch, unpublished data).

The presence of blocked cross-linked bonds in the cell-wall pectin may affect enzymatic degradation. The configuration of the polygalacturonic chain allows spaces for the binding of a series of cations between carboxyl groups. The formation of cation bridges between pectic acid molecules may make the cell wall less accessible to enzymes produced by fungal pathogens that cause decay (Tepfer and Taylor 1981). Increasing the Ca^{2+} content of apples by means of preharvest sprays and postharvest dips reduces postharvest decay. Such treatments were found to increase significantly the number of salt bridges, and consequently the structural integrity of the cell wall, thus reducing the vulnerability of the cell walls to maceration (Conway et al. 1988).

The Relevance of Preformed and Inducible Barriers in Plant Disease Control

A logical application of the information concerning defence compounds would be the possible modulation of these compounds to protect plants against disease. The plausible scenarios would involve prevention of reduction of the compounds, enhancement of their concentration, or selection of cultivars rich in such antifungal compounds. The assumptions underlying such action are that the compounds are safe because they occur naturally, and that they would be effective since they are the compounds which have evolved in nature for specific plant protection and which have enabled existing plants to survive the selection pressure of evolution. However, naturally occurring compounds are not necessarily safe (Osbourn 1996a); many of the world's most potent poisons are derived from plants, and some plant tissues are extremely toxic to animals because they contain protective compounds, e.g. potato, tomato, and tobacco foliage. Furthermore, the levels of preformed compounds can change during the hosts lifetime: usually they decrease (Prusky and Keen 1993), thus necessitating induction during specific periods in their lifetime. The exogenic application of naturally occurring defence compounds to protect plants would be uneconomical; their synthesis would be difficult and the cost of their isolation probably high.

In spite these possible disadvantages, the protection of the host during specific periods of susceptibility, by modulation of the levels of the natural compounds seems to be a safe way to preserve host resistance. The possibility, for example, of maintaining a high level of the antifungal diene in avocado fruit during fruit storage, but allowing a reduction of the compound before consumption of the fruit, could be a safe way to use this natural compound.

An alternative to the external application of defence compounds is their elicitation, specifically or not specifically within the plant, by regulation of gene expression, and hence of the gene product, leading to the synthesis and further accumulation of the desired compounds. In spite of the fact that phytoanticipins do not consider the presence of inducible preformed compounds, several preformed antifungal compounds have been shown to be enhanced to higher levels by biotic and abiotic factors (Ingham 1973; Prusky and Keen 1993; Van Etten et al. 1994). The possibility of induction of preformed compounds by nonpathogenic strains of *C. magna* suggests that this could be done by biological means during fungal colonisation. However, the use of such a biological elicitor or other physical elicitors (CO_2, γ-radiation) has not always proven effective, because the host, e.g. avocado, is receptive to the signals only during very specific periods after harvest (Prusky and Keen 1995).

Triggering early phytoalexin accumulation with elicitors allows the treated tissues to develop resistance early in the infection process. However, since the stored fruit are held at low temperature, the process of elicitation may be slowed down, thereby affecting the development of resistance. However, at the same time, the degradation of the elicited antifungal compounds may also be considerably slowed down,

allowing for an extended effect on host resistance. There have been several cases where induced resistance occurred as a result of an increase in phytoalexin levels, although such treatments have not been put into practice (Adikaram et al. 1992; Ben Yehoshua et al. 1992, Mercier et al. 1993a).

Suggestions have been made to enhance the resistance barriers of plants by incorporation of specific inhibitors of fungal enzymes that detoxify preformed antifungal compounds. If ongoing studies of the preformed α-tomatin suggest that saponin-degrading enzymes may have a more general role in pathogenicity, then inhibitors of these enzymes could become attractive as a basis for disease control strategies (Osbourn et al. 1995; Sandrock and Van Etten 1997). The extracellular location of the detoxifying enzymes should facilitate approaches involving the use of chemicals or the expression of saponinase inhibitors in genetically engineered plants, since there should be no requirements for the inhibitors to penetrate the fungal hyphae (Osbourn et al. 1995; Osbourn 1996a).

The possibility of over-expressing compounds that have preformed activity in a susceptible host which does not express those compounds, has also been tested. The transfer of stilbene synthase from grape to a resveratrol-non-producing plant such as tobacco, leading to the synthesis and accumulation of resveratrol in tobacco, is one exciting example (Hahn et al. 1993): the transgenic tobacco synthesising and rapidly accumulating resveratrol, had enhanced resistance to disease caused by *B. cinerea*. However, since there are several preformed antifungal barriers, and since secondary metabolites and their biosynthetic pathways are complex and not always known, the transgenic approach might be difficult. Another possible way to modulate the basic constitutive barriers may be to engineer hosts with such barriers. This could be done by over-expressing host constitutive inhibitors such as PGIP, or through inducible host inhibitors that had not shown any significant active preformed activity. Transgenic tomato plants expressing PGIP were found more resistant to fungal attack in the laboratory (Powell et al. 1994). However, more recent results suggested that this resistance is not matched in field experiments (J. Labavitch, pers. comm.). Also, transgenic tobacco and rape plants containing an inducible bean chitinase gene with a constitutive promoter, have been shown to exhibit higher basal levels of chitinase and concomitant increased resistance to *Rhizoctonia solani*, compared with control plants (Broglie et al. 1991). In these cases, resistance to fungal attack was obtained by creating a host with a preformed barrier as a result of engineering the plants by continuously over-expressing an inducible chitinase gene. In another study (Neuhaus et al. 1991), tobacco plants which had been transformed with a basic chitinase from tobacco, under the regulation of a constitutive promoter, accumulated up to 120 times more active chitinase than non-transformed plants. However, the transformed plants were found to be no more resistant to the fungus *Cercospora nicotiana* than were the controls. It is possible that the additional chitinase—located intracellularly in the transgenic plant—is compartmentalised in such a way that it does not come into contact with the penetrating fungus. This is a very common problem in preformed barriers. It may be also that combination with other genes encoding antifungal products is necessary for providing resistance.

The general understanding of the plant biosynthetic pathways which led to the formation of preformed and inducible barriers, and of their contribution to disease resistance, is of primary importance for the manipulation of these compounds to enhance resistance. Such an understanding offers the possibility that the potential level of preformed barriers might be modulated by genetic, biological, physical, and chemical means, to provide the basis for novel crop protection strategies that could lead to the reduction of pesticide use in the future.

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Induced Barriers to Infection

D. Guest*

Abstract

Plants grow and bear fruit under constant threats from potential pathogens. To cause disease, plant pathogens must overcome complex and tightly-coordinated defence responses, based on constitutive barriers, localised containment, and systemic protection. The aim of this review is to describe and evaluate plant disease resistance mechanisms induced by pathogen challenge.

Although pathogen recognition is often highly specific, individual responses contributing to containment and protection are nonselective, and their expression is quantitative. The effectiveness of these responses in arresting pathogen development depends on their location, speed and magnitude. The success of this strategy is demonstrated by the health of most plants most of the time, and its failure results from highly-evolved and diverse pathogenic counter-strategies, usually coinciding with adverse environmental conditions.

We do not yet have a 'grand unified theory' of disease resistance in plants, but some common patterns emerge from studies on a range of plant–pathogen interactions. Initial contact is often followed by a rapid 'oxidative burst', associated with changes in host cell membrane function, hypersensitive cell death, accumulation of phytoalexins, and antimicrobial proteins, and the repair and strengthening of plant cell walls. These responses are localised and transitory, and inhibit or contain infection, colonisation, and pathogen reproduction.

Plants that survive localised infections are often more resistant to subsequent infections by the same, or by different, pathogens. The activation of systemically acquired resistance in plants involves three phases—induction of a signal, translocation of that signal to distant parts of the plant where it conditions a state of alert and, upon subsequent challenge, expression of an enhanced defence response.

The possible exploitation of localised defence responses and systemic acquired resistance in plant protection will be discussed.

If one looks around at natural plant communities, it becomes apparent that most plants are healthy most of the time, despite the septic environments they grow in, full of potentially pathogenic microorganisms. Pathogens are distinguished from non-pathogens by their ability to penetrate the outer defensive barriers of the host cuticle, cell wall, and preformed antibiotics. The virulence of a pathogen, or its ability to colonise and reproduce, while causing disease on a host plant, depends on two interrelated factors: (i) the substrate requirements of the pathogen and; (ii) the response of the plant.

Two broad groups of pathogens can be distinguished on the basis of their substrate requirements:, necrotrophs and biotrophs. Necrotrophs are thugs in the sense that they kill plant cells by secreting toxins before invading them. Necrotrophs must establish an incompatible relationship with host cells. Many necrotrophs secrete non-specific toxins that aggravate symptom development, but do not determine host range. Other necrotrophs secrete host-specific toxins that are responsible for symptom development and host range.

* School of Botany, University of Melbourne, Parkville 3052, Australia.

Biotrophs are furtive intruders that elude or escape recognition by the host, and must establish a compatible relationship with infected cells of its host to allow colonisation. Biotrophs are highly specialised pathogens, usually with a restricted host range.

The second factor that determines whether the pathogen succeeds in colonising its host is the nature of the defence response it induces. All plants possess the potential to activate these defences; the difference between a susceptible and a resistant plant is due to the timing, magnitude, and location of complex and tightly coordinated individual and total responses (Table 1).

Table 1. Sequence of plant responses associated with disease resistance.

Time	Event
Minutes	• Membrane depolarisation & electrolyte leakage
	• Active oxygen generation
	• Expression of genes involved in phytoalexin biosynthesis
Hours	• Oxidative burst
	• Membrane lipid peroxidation
	• Rise in salicylic acid levels
	• Cytoplasmic aggregation, cell collapse, and the hypersensitive response
	• Phytoalexin accumulation
Days	• Accumulation of lignin and pathogenesis-related proteins
	• Systemic acquired resistance

Rapid Cellular Responses

Recent advances in physiological and molecular studies have improved our understanding of the initial events that follow contact between host and pathogen cells. Most models propose a role for the host membrane in recognition, because of the rapid physiological changes observed in permeability, polarity, and the oxidative burst. This burst releases the superoxide anion and hydrogen peroxide around the infection court, initiating a chain peroxidation of host and pathogen membrane lipids (Sutherland 1991). Irreversible membrane damage results, at the same time inhibiting pathogen growth, triggering hypersensitive cell death, and releasing local and systemic signals that result in acquired resistance to subsequent pathogen challenge.

Induced Mechanical Barriers to Infection

Soon after these changes in membrane function, streaming of the underlying cytoplasm intensifies, and the host nucleus and cytoplasm aggregates under the penetration site. These aggregates are thought to contain the cellular apparatus for the synthesis of cell wall fortifications. **Papillae** and lignitubers, reinforced by silicon, lignin, and proteins, and by the peroxide-induced cross-linking of hydroxyproline-rich glycoproteins, inhibit pathogen development.

Rapid **lignification** is associated with resistance to non-pathogens and to avirulent pathogens in wheat. Lignin and **suberin** deposition are also important in Solanaceae, crucifers, melons, and carrots (Vance et al. 1980). Lignification functions in several ways:

1. Lignified walls are more resistant to degradation and penetration
2. Lignified walls restrict the diffusion of pathogen enzymes and toxins, and block the uptake of water and nutrients by the fungus
3. Lignin precursors are toxic
4. Hyphal tips and bacterial cells may be lignified.

Cork layers (necrophylactic periderm) may seal off infected areas, containing the pathogen and protecting against saprophyte invasion. They are often produced in fleshy tissues, roots, fruits, and bark. **Tyloses** are ingrowths of xylem parenchyma extruded through xylem vessel pits into the lumen. If they form rapidly enough ahead of the advancing pathogen they may restrict colonisation or the spread of propagules in the xylem. The formation of tyloses has a cost to the plant, as they not only block the spread of the pathogen, but also reduce the translocation of water, inducing wilt symptoms.

Hypersensitive Cell Death

Ward (1902 and) Stakman (1915) noticed that cytoplasmic streaming and aggregation in cells of resistant wheat varieties was followed by granulation, membrane disruption, cytoplasmic browning and, after about 24 hours, cell death. The browning associated with hypersensitive cell death results from the activation of polyphenol oxidase activity, generating an even more hostile environment for invading pathogens. Stakman named this type of cell death the hypersensitive response, and it has since been described in many, but not all, incompatible

responses. There are striking similarities between hypersensitive cell death in plants and programmed cell death, or apoptosis, in mammals, suggesting that rapid cell death is an ancient response.

Induced Chemical Barriers

Bernard (1909), while studying germination of orchid seedlings, found that some fungi destroyed the seed, others penetrated several layers of cells before stopping and disintegrating, while others established a successful mycorrhizal association. He found that the fungi that penetrated, but were then destroyed, induced resistance to subsequent infections by normally pathogenic fungi.

Nobécourt (1923) found that heat, freezing, or chloroform prevented this induced resistance, presumably by preventing biosynthesis of a new inhibitor. Gäumann and Jaag (1945) and Gäumann and Kern (1959a,b) showed that inhibition was due to the synthesis of orchinol and hircinol by the challenged orchid seed.

At the same time K.O. Müller found that slices of potatoes reacting hypersensitively to *Phytophthora infestans* produced antibiotics. Working at the CSIRO in Canberra, he found in 1958 that the inside of bean pods exposed to spores of the non-host pathogen, *Monilinia fructicola*, became necrotic and produced an inhibitory chemical 24 hours after inoculation. Uninoculated droplets were stimulatory. The inhibitor was extracted in organic solvents and, although not identified, was termed a phytoalexin (plant defender). This inhibitor was purified by his successors, a team led by I.A.M. Cruickshank in the 1960s, and found to be a pterocarpan named phaseollin. They also found the related compound, pisatin, in pea pods.

Since then phytoalexins have been found in hundreds of species from 30 families of higher plants (Kuc 1995). The chemical nature of phytoalexins varies, and is taxonomically linked. For example, legumes produce isoflavanoid phytoalexins, while solanaceous plants produce terpenoids. Most species produce several variations based on a common molecular skeleton. French bean (*Phaseolus vulgaris*) produces at least five isoflavanoids; potato (*Solanum tuberosum*) at least four terpenoids. Elemental sulfur accumulates in and around xylem vessels of cocoa infected with *Verticillium dahliae*. As a result of this variety, pathogens usually confront a toxic cocktail of phytoalexins. Phytoalexins are defined as low molecular weight antibiotics produced *de novo* by plants in response to infection, that accumulate to inhibitory levels at the infection court. Their toxicity is non-selective; most are apparently membrane toxins.

Phytoalexins are synthesised in cells adjacent to the infection site. They are synthesised in cytoplasmic vesicles and exported to the infected cell where they accumulate in toxic concentrations, inhibiting or killing the pathogen. The experimental correlation between resistance and the rapid, localised accumulation of phytoalexins in many host–parasite interactions is strong. In resistant plants, gene transcription begins within one hour of recognition, phytoalexins appear within four hours and concentrations peak to fungitoxic levels about 18–24 hours after challenge. These events are delayed and diffuse in susceptible plants.

The virulence of some necrotrophic pathogens is linked to their ability to detoxify phytoalexins. Virulent biotrophs avoid or suppress phytoalexin elicitation. Resistance is lost if phytoalexin biosynthesis is blocked by inhibitors of phytoalexin biosynthesis enzymes, and is reduced in mutants that are slow to accumulate phytoalexins. Conversely, resistance is increased in plants transformed to express novel phytoalexins, or if exogenous phytoalexins are applied to the infection court.

Phytoalexin synthesis is not universal, and the dynamics of each plant–parasite interaction is unique, making generalisations pointless. Nevertheless, it is clear that in many interactions, the rapid, localised accumulation of toxic concentrations of phytoalexins plays a decisive role in the expression of resistance.

Pathogenesis-related Proteins

'Pathogenesis-related (PR) proteins' appear several days after infection, both immediately surrounding the infection site, and systemically. Some induced proteins have β-glucanase and chitinase activity, others have lysozyme activity, while others include the inhibitory proteins thaumatin and osmotin.

Sixteen small (15–30 kD) PR proteins appear in TMV-infected tobacco, constituting up to 5–10% of the total leaf protein. These include four chitinases and four glucanases, while *P. infestans*-infected potato leaves accumulate two β-1,3-glucanases and six chitinases.

Hydrolytic enzymes accumulate in vacuoles, although some glucanase is secreted to the intercellular space (Mauch and Staehelin 1989). These enzymes attack fungal cell walls, and the fragments released are able to elicit hypersensitive cell death and phytoalexin biosynthesis. They have antiviral, antibacterial, and antifungal activity.

Proteinase, polygalacturonase, and ribosome inhibitors constitute up to 10% of total proteins in cereal, legume and solanaceous seeds, and their synthesis may be induced by wounding. They provide another defence against insects and pathogens, including insect-transmitted viruses.

Systemic acquired resistance

It has been known since Bernard and Nobécourt's work in the early 20th century that plants surviving an attack by a pathogen that causes localised necrosis becomes resistant to subsequent infections. Acquired (also called induced) resistance is non-specific, and it reduces disease severity rather than providing immunity.

Three phases are involved in acquired resistance (Ryals et al. 1996). Induction usually requires a slow, progressive necrosis, associated with the release of a systemic signal, or signals. The signal is phloem translocated, but not cultivar, species, or genus specific. All of the signal originates from the induction site. There are several natural molecules with signalling activity, and a few synthetic agents are near commercialisation, including dichloroisonicotinic acid. Research interest has focused on salicylic acid, because of its ability to induce systemic acquired resistance, and its presence in protected tissue. One salicylic acid-binding protein recently described in plant cell membranes is a catalase, and binding inhibits its activity. The resulting hydrogen peroxide signals other local defence responses. A second high-affinity binding protein appears to be involved in gene activation pathways.

Several other naturally-occurring molecules have signal activity, including β-ionone, ethylene, and jasmonic acid, although evidence currently suggests the responses of induced plants result from several signals.

The expression of systemic acquired resistance is triggered following a 'challenge', and involves hypersensitive cell death, enhanced phytoalexin accumulation, pathogenesis-related proteins, peroxidase, and oxidative burst.

The Dynamics of Disease Resistance

Passive mechanisms, such as the barriers imposed by the cuticle, cell wall, and phytoanticipins, exclude saprophytic and epiphytic microbes. Wound repair mechanisms, such as cork layers, papillae, and lignitubers, exclude secondary invaders and opportunists, and may contribute to retarding colonisation and the spread of pathogens. The rapid accumulation of antimicrobial substances like phytoalexins, lignin precursors, and PR proteins in association with hypersensitive cell death, provides a formidable defence against invading pathogens. Virulent pathogens either evade or suppress these responses.

These defence responses are most likely to be effective when they are expressed in combination—the rapid deposition of papillae, lignin, and hydroxyproline-rich glycoproteins at the point of penetration of the cell wall, combined with rapid hypersensitive cell death and phytoalexin accumulation, backed up by the presence of lytic enzymes in the intercellular spaces and vacuoles, results in a very potent defence against invading pathogens (Table 1). The failure of these responses invariably leads to susceptibility.

How are these responses mediated, and what determines the discriminatory responses in resistant and susceptible hosts? What triggers the diversity of localised and systemic defence responses including hypersensitivity, phytoalexins, lignification, and lytic enzymes? These are the exciting questions of the study of host-parasite physiology.

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Further reading

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The October 1996 issue (Vol. 8, No 10) of The Plant Cell contains an excellent compilation of up-to-date reviews of plant–microbe interactions, including comprehensive descriptions of induced barriers to infection.

Does Preharvest Stress of Plants Affect Postharvest Decay of Their Fruit?

D.C. Joyce*, G.I. Johnson†, and M.J. Gosbee§

Abstract

In discussion related to the planning of the present workshop on 'disease resistance in fruit', the question arose as to whether fruit harvested from stressed plants have increased, decreased, or unchanged susceptibility to pathogens whose disease symptoms are expressed after harvest. For example, does preharvest water deficit stress affect the incidence and severity of stem-end rot (endophytic infection) or anthracnose (latent infection) on harvested ripening mango fruit? A literature survey failed to yield any research papers relating preharvest plant stress to the host–pathogen biology of the harvested fruit. Thus, it was decided to offer a largely speculative account of how preharvest stress might affect postharvest decay. The practical intent of this presentation is to stimulate informed debate and directed investigation of the proposition that 'preharvest stress may alter the ability of host fruit tissue to suppress the development of quiescent infections'. For instance, in terms of constitutive and induced defence mechanisms, might fruit from plants subjected to abiotic stress have enhanced levels of constitutive defence compounds (e.g. phenolics) but reduced capacity to synthesise new defence compounds (e.g. proteins related to pathogenesis)?

ALL fresh horticultural products succumb to pathogens at some time after harvest. This increase in disease susceptibility occurs in association with a shift in the balance of physicochemical processes of the plant tissue away from those favouring the host and towards those favouring the pathogen. That is, host metabolism shifts from a net anabolic to a net catabolic state in response to 'harvest stress'. Harvest stress associated with detachment involves permanent disruption of source/sink relationships characterised by water, nutrient (mineral, carbohydrate), and growth regulator fluxes. Harvested host tissue becomes progressively less able to mount defence reactions against pathogens and other stresses and, in fact, encourages decay in association with physiological deterioration (Sommer 1989). Defence reactions against pathogens of plant tissue include the maintenance of constitutive antifungal chemicals (e.g. phenolics), the synthesis of inducible antifungals (e.g. pathogenesis related proteins), and the laying down of physical barriers to pathogen invasion (e.g. callose) (Issac 1992). From an evolutionary perspective, physiological deterioration manifest during fruit ripening and plant organ senescence is functionally important as it favours seed dissemination in the first instance and nutrient recycling in the second.

Stress and Strain

The present review briefly considers, in the context of horticulture, the contention that exposure to stress before harvest will predispose harvested plant tissue to disease. Generically, 'stress' can be defined as 'an overpowering pressure of some adverse force of influence' (Jones and Jones 1989). Stress factors induce strains, which may be classified as elastic or

* Department of Plant Production, The University of Queensland, Gatton College, Queensland 4345, Australia.

† ACIAR, GPO Box 1571, Canberra, ACT 2601, Australia.

§ Cooperative Research Centre for Tropical Plant Pathology, The University of Queensland, St Lucia, Queensland 4072, Australia.

plastic conditions according to whether the effects are reversible or irreversible, respectively (Levitt, cited by Schoneneweiss 1975). 'Predisposition' is taken to mean 'an internal degree of susceptibility resulting from external causes' (Yarwood, cited by Schoneneweiss 1975).

Review Articles

A number of comprehensive reviews which address to varying degrees the interaction between host, disease, and environment, including stress factors, have been published over the years in the Annual Review of Phytopathology series (e.g. Cook and Papendick 1972; Colhoun 1973; Schoeneweiss 1975; Ayres 1984; Boyer 1995; Kuc 1995;). Of these reviews, and in the specific context of the present article, that entitled 'Predisposition, stress, and plant disease' (Schoeneweiss 1975) is particularly relevant. The interaction mentioned above can be represented by the classical 'disease triangle', but can be expanded to consider abiotic and biotic stress and the third dimension of time (Fig. 1; Ayres 1991). Time is of special importance in terms of postharvest horticulture, where it represents the irreversible loss of host tissue 'vitality'.

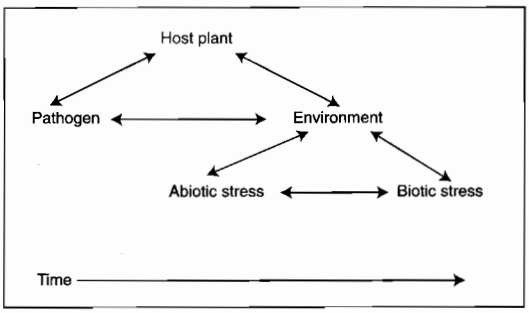

Figure 1. 'Disease triangle' relationship expanded to encompass time and biotic and abiotic stress factors (modified after Ayres 1991).

Discrete and Complex Stresses

Abiotic stresses are physical (e.g. water deficit stress, chilling injury stress) or chemical (e.g. salt stress, ozone stress), as opposed to biotic stresses (e.g. pathogen stress, insect stress) which are biological. Table 1 presents a summary of the abiotic and biotic stresses that Schoeneweiss (1975) considered in his review article on stress-induced predisposition to disease. Apart from 'discrete' stress factors, such as water deficit stress, Table 1 lists relatively 'complex' stress factors such as transplantation. In apparent contrast to distinct stresses, multiple stresses comprising complex stress factors can be mechanistically difficult to define. Transplantation stress, for example, is likely to involve, at the very least, physical wounding, water deficit, and carbohydrate deficit stresses. It follows that it is equally difficult to define the resultant strains, and therefore to develop an understanding of the physicochemical processes that determine the outcome in terms of predisposition to disease (i.e. host–pathogen biology).

Table 1. Some biotic and abiotic stresses to which plants and plant organs may be subjected during growth and development (after Schoeneweiss 1975).

Water based:	Water deficit
	Water logging
Temperature based:	Freezing injury
	Chilling injury
	High temperature injury
Defoliation	
Transplantation	
Nutrient based:	Nutrient deficiency
	Nutrient toxicity
Light based:	Low light
	Poor quality light
	High light
Toxic substance based:	Pesticides
	Pollutants
Wounding based:	Mechanical
	Biological

Cross Protection

Although stresses themselves may be discrete, they can nonetheless have degrees of commonality in terms of the strains imposed on the tissue. For example, freezing, water deficit, and salt stresses can each involve a fall in osmotic potential (increase in the osmotic concentration) of the cytoplasm of cells comprising the stressed tissue. Thus, certain responses to stress, such as accumulation of osmoprotectants, might confer a degree of cross-protection (Jones and Jones 1989). This theme is considered further below (see 'Stress-induced Disease Resistance').

Host versus Pathogen Responses

The effect of stress on host–pathogen interactions is likely to depend on the relative response of the host versus the pathogen. 'Response' may be considered as a composite of processes, which can include stress perception, degree of injury, and capacity to defend. In plant protection there are practical examples where, under certain severe stress treatments, the host is less adversely affected than the pathogen: hot water dipping of plant tissue to control pathogens is an example. However, the outcome in response to other stress regimes may be completely different. Simplistically, a 'see saw with a variable fulcrum position' model can be used to illustrate the three possible outcomes of a host-pathogen system exposed to stress (Fig. 2); viz. a shift in balance to favour the host, a shift in balance to favour the pathogen, or no shift in balance (i.e. no differential effect).

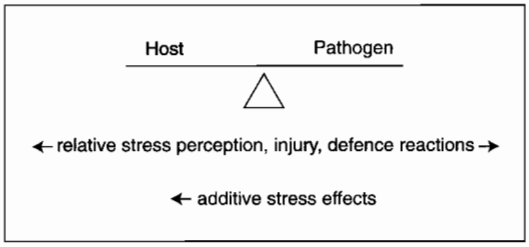

Figure 2. Simple 'see saw' model to illustrate potential shifts in host–pathogen balance as determined by the position of the fulcrum which varies according to the relative response of the two organisms to an imposed stress regime.

Stress-induced Disease Susceptibility

There are numerous reports in the literature of increased disease in response to all manner of stresses (Table 1; Schoeneweiss 1975, 1986). Instances discussed by Schoeneweiss (1975) and relating to discrete water deficit (11 hosts) and temperature stresses (low temperature, 6 hosts; high temperature, 7 hosts), and complex defoliation (8 hosts) and transplantation (3 hosts) stresses are summarised in Tables 2, 3, 4, and 5. In the case of coconut palms infected with *Lasiodiplodia theobromae*, drought induced by withholding water resulted in a significant increase in the length of disease lesions on inoculated leaves (Table 6; Warwick et al. 1993). However, it should be recognised that not all studies show an increase in disease in association with water deficit stress (Schoeneweiss 1975). Biggs (1993) reported that significant correlations between reduced plant water status and increased susceptibility of peach trees to *Leucostoma* canker were obtained only occasionally.

Table 2. Host and pathogen combinations for which water deficit stress has been reported to increase predisposition to disease (after Schoeneweiss 1975).

Host	Pathogen
Aspen	*Hypoxylon pruinatum*
Cotton	*Macrophomina phaseoli* (root rot)
Crab apple	*Physolospora obtusa*
European white birch	*Botryosphaeria dothidea*
Grain sorghum	*Macrophomina phaseoli* (stem rot)
Highland bentgrass	*Pythium ultimum*
Kent blue grass	*Sclerotinia homeocarpa*
Loblolly pine	*Fomes annosus*
Paper mulberry	*Fusarium solani*
Red-osier dogwood	*Botryosphaeria dothidea*
Sweetgum	*Botryosphaeria dothidea*

Table 3. Host and pathogen combinations for which temperature stress has been reported to increase predisposition to disease (after Schoeneweiss 1975).

Host	Pathogen (disease)
A. Freezing (winter chilling) stress:	
European white birch	*Botryosphaeria dothidea*
Juniper	*Sclerophoma pythiophila* (tip dieback)
Plum	*Cytospora* sp. (canker)
Rhamnus frangula 'Tallhedge'	*Tubercularia ulmea*
Sweet cherry	*Cytospora* sp. (canker)
Sweetgum	*Botryosphaeria dothidea*
B. High temperature (including sunburn) stress:	
Apple	Black rot canker
Beans	(Rust)
Corn	(Rust)
Peanuts	*Diplodia gossypina*
Soybeans	Miscellaneous pathogens
Tobacco	*Phytophthora parasitica* var. *nicotiana*
Walnut	*Hendersonula toruloidea*

Table 4. Host and pathogen combinations for which defoliation stress has been reported to increase predisposition to disease (after Schoeneweiss 1975).

Host	Pathogen
Cottonwood	Several genera (blackstem)
European mountain ash	*Botryosphaeria dothidea*
European white birch	*Botryosphaeria dothidea*
Oak (insect attack)	? (mortality), (anthracnose)
Peach	*Cytospora leucostoma*
Red clover (clipping stress)	*Fusarium* sp.
Sugar maple	*Armillaria mellea*, (anthracnose)
Sweetgum	*Botryosphaeria dothidea*

Table 5. Host and pathogen combinations for which transplantation stress has been reported to increase predisposition to disease (after Schoeneweiss 1975).

Host	Pathogen
European mountain ash	*Botryosphaeria dothidea*
European white birch	*Botryosphaeria dothidea*
Thornless honey locust	*Botryosphaeria dothidea*

Table 6. Effect of withholding water on the severity of coconut leaf blight disease (caused by *Lasiodiplodia theobromae*) on coconut palm leaves (data from Warwick et al. 1993).

Treatments	Average lesion length over 13 weeks
	(cm)
Unstressed (control)	5.91 a[a]
Water withheld for 30 days	7.86 b
Water withheld for 45 days	7.77 b

[a] Numbers with different letters are significantly different

Additive Stress Effects

The host–pathogen balance in plants exposed to multiple discrete stresses is likely to be shifted towards favouring the pathogen (Fig. 2). For instance, Zhang and Sutton (1994) reported that black spruce seedlings exposed to a combination of high temperature, dark, and drought stresses were predisposed to grey mould disease.

Plant Nutrition and Disease Susceptibility

Plants are subject to nutrient deficiencies, toxicities, and imbalances which may affect their susceptibility to disease. For example, increased supplies of silicon, potassium, or calcium have been associated with decreased disease severity, and elevated supply of nitrogen with increased susceptibility (Marshner 1986). High calcium levels, in particular, have been associated with reduction in disease levels in harvested horticultural produce (Marshner 1986; Conway 1989; Sugar et al. 1992; Elad et al. 1993). A reduction in the nitrogen:calcium ratio has been strongly correlated with increased resistance of apple fruit to postharvest disease (Sugar et al. 1992).

Stress-induced Disease Resistance

Just as predisposition to disease may be increased by exposure to stress, it is also conceivable that adaptation to one type of stress may confer protection against disease (viz. biological stress). An example of cross protection against biological stress is the use of one organism (e.g. a non-pathogenic mutant) to confer resistance against another (e.g. pathogenic mutant) (Jones and Jones 1989; El-Ghaouth and Wilson 1995). Similarly, exposure to abiotic stresses (e.g. ultraviolet light, heat) has been shown to confer disease resistance (Ho and Sachs 1989; El-Ghaouth and Wilson 1995). Such cross protection has been observed in both pre- (e.g. Ferraris et al. 1987; Yalpani et al. 1994) and postharvest (e.g. Spotts and Chen 1987; Ben-Yehoshua et al. 1992; Fallik et al. 1995) plant studies.

Mechanisms of Increased Susceptibility or Resistance

From the preceding discussion it remains evident that the three possible outcomes of plant exposure to stress are increased, decreased, or unchanged predisposition to disease.

With respect to increased predisposition, the biological mechanism(s) involved are generally unclear (Schoeneweiss 1975). However, it is likely that increased predisposition is related to alterations in plant metabolism (e.g. reduced carbon fixation; Table 7) which lead to a general decline in the vigour of the host. In turn, pathogen development will be favoured by the onset of senescence processes and by the

reduced ability of the host to mount defence reactions. In the case of aspen tissue, water stress resulted in lowered concentrations of three constitutive antifungal compounds; catechol, salicortin, and salicin (Kruger and Manion 1994). Under certain circumstances, the stress regime may also specifically favour the pathogen (Schnoeneweiss 1975). For example, a pathogen may have a higher temperature optimum than the host, and therefore gain the 'upper hand' in response to rising ambient temperature regimes.

In contrast to mechanisms underlying stress-induced increases in susceptibility to decay, those underlying stress-induced increases in resistance to decay are more clearly understood. They include laying down of physical barriers to invasion, and induction of the synthesis of antifungal compounds (Table 7). Treatment of tobacco with ultraviolet C radiation, for instance, resulted in an increase in salicylic acid levels, with associated increased synthesis of two pathogenesis-related proteins (PR1a and PR1b; Yalpini et al. 1994). Similarly, illumination of lemon fruit with ultraviolet radiation resulted in increased endogenous levels of the antifungal compound, scoparone.

Plant Growth Rate

While it was suggested in the preceding section that stress-induced reduction in plant vigour will increase the predisposition of host plants to disease, it does not necessarily follow that a stress-induced reduction in growth rate will have the same result. That is, it may be important to delineate between vigour and growth rate.

In a study conducted with a range of radish cultivars (host) and *Fusarium oxysporum* (pathogen), Hoffland et al. (1996) demonstrated that relative growth rate was negatively correlated (r^2 = 0.68, n = 15 cvs.) with pathogen resistance. They explained this result by invoking the 'cost of defence theory', whereby plants that are growing rapidly (viz. rapid cell division and expansion; primary metabolites) have relatively less free resources to partition into defence (viz. secondary metabolites) and vice versa. To date, the cost of defence theory has not been generally considered in relation to plant pathogen defence. However, the theory has been discussed widely with respect to plant anti-herbivore defence (Glumon and Mooney 1983; Coley et al. 1985; Bazzaz et al. 1987; Jones and Coleman 1991; Herms and Mattson 1992). For example, in a study with the *Cecropia* plant, Coley (1986) determined that individual plants with relatively low leaf production rates suffered less herbivory and had comparatively high tannin concentrations. The range in tannin concentrations was in the order of 13 to 58 mg/g dry weight.. Hoffland et al. (1996) found that the slow-growing, relatively disease resistant radish cultivars had more leaf cell wall material, greater root biomass density, more cytoplasmic elements (proteins), and higher concentrations of phenols.

Thus, it seems likely that plants growing under moderate stress regimes may also have higher concentrations of constitutive pathogen defence compounds.

Postharvest Implications

In the limited time available to review the literature before preparation of this paper, no data were found that demonstrated a direct relationship between preharvest stress of horticultural fruit (or other) crops and predisposition to postharvest disease. However, information in the general literature relating stress and predisposition to disease strongly suggests that severe preharvest stress might predispose harvested commodities to disease (see Stress-induced Susceptibility). On the other hand, moderate preharvest stress may indeed decrease their predisposition (see 'Plant Growth Rate').

Table 7. Some general biological mechanisms involved in stress-associated increased or decreased predisposition of plant tissue to disease (Schnoeneweiss 1975; Issac 1992; El-Ghaouth and Wilson 1995).

A. Increased predisposition:	
	increased production of metabolites toxic to plant cells (e.g. ethanol)
	decreased starch and increased soluble sugar levels
	decreased ability to synthesise antifungal compounds (e.g. phenolics)
B. Decreased predisposition:	
	production of physical barriers (e.g. callous deposition)
	increased production of antifungal chemicals (e.g. phytoalexins)
	increase production of pathogensis-related proteins (e.g. peroxidase)

One of us (M.J.G.) recently conducted an experiment in which the severity of stem-end rot disease on 'Kensington' mango fruit harvested from irrigated and non-irrigated trees and inoculated with *Dothiorella dominicana* was measured. The results of that experiment were negative, as the development of disease symptoms was similar for both treatments.

None-the-less, further investigation of the question as to whether fruit harvested from stressed plants have increased, decreased, or unchanged susceptibility to pathogens whose disease symptoms are expressed after harvest is warranted. Although there are few or no data relating preharvest stress levels to postharvest decay, there is clear evidence that other preharvest variables (e.g. fruit position on the plant, growing season, nitrogen and calcium supply, water deficit) have marked effects on the postharvest characteristics of fruit and other horticultural products (e.g. Ketsa et al. 1992; Sugar et al. 1992; Marguery and Sangwan 1993; Srikul and Turner 1995; Pyke et al. 1996). Since detachment of product from the host plant is itself a form of complex stress, the question posed above might be rephrased as: Can discrete, complex or additive stress regimes experienced before harvest have sufficient impact on postharvest host–pathogen relationships to override the 'harvest stress' effect?

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Botryosphaeria (Anamorphs *Fusicoccum* and *Dothiorella*), *Diaporthe* (Anamorphs *Phomopsis* spp.) and *Lasiodiplodia*: Infection and Defence

G.I. Johnson,* D.C. Joyce,† and M.J. Gosbee§

Abstract

Along with anthracnose, stem end rots (SER) caused by *Lasiodiplodia theobromae* and the anamorphs of *Botryosphaeria* and *Diaporthe*, are major causes of postharvest loss in many fruit. Infection of fruit by SER pathogens occurs by endophytic colonisation of the pedicel or via wounds and other receptive tissues.

- Water stress and defoliation of the host, microbial competition, and the production of phytotoxins and insect attractants by the pathogens could all influence their inoculum potential.
- Following infection, abscission zone barriers and antifungal compounds may restrict invasion until the fruit ripen or senesce.
- The regulation, manipulation or enhancement of host, pathogen and ecological factors all offer scope for preventing or delaying infection and reducing the incidence, onset, and severity of stem end rot.

The role of these factors and the potential for manipulating them to control stem end rot diseases is reviewed.

In another paper in these proceedings, Dr Yasuo Homma discusses the role of the stem end in infection of citrus by *Phomopsis citri* (anamorphs of *Diaporthe*) (Homma, these proceedings). He discussed the delay between penetration of the pedicel by *P. citri* and the development of decay associated with inhibitory compounds in tissues at the stem end. In many countries, the plant hormone 2,4-D is applied to prevent or delay abscission of the button end of citrus fruit and this treatment controls stem-end rot caused by *P. citri*. We have found that some control of stem-end rot of mango caused by *Fusicoccum aesculi* (syn. *Dothiorella dominicana* = the anamorph of *Botryosphaeria dothidea*) could also be obtained by treatment of mangoes with 2,4-D, while stem-end anthracnose caused by *Colletotrichum gloeosporioides* was not controlled (Table 1) (Johnson 1997). The abscission zone barrier appears to be specific for stem-end rot pathogens.

The stem-end rot pathogens, *Botryosphaeria* spp., *Diaporthe* spp., and *Lasiodiplodia theobromae* can also cause flower blight, dieback and canker diseases on many woody plants. They also occur as endophytes (symptomless inhabitants) in stem and leaf tissues (Table 2) (Johnson 1997; Wright et al. 1996). Joyce et al. (these proceedings) have summarised evidence indicating that the fungi become more destructive when the hosts are stressed. We suggest they are opportunists whose association with their hosts is well developed. The challenge in horticulture is to extend the 'control' beyond that required in nature.

* Australian Centre for International Agricultural Research, GPO Box 1571, Canberra ACT 2601, Australia.

† Department of Plant Production, University of Queensland Gatton College, Queensland 4345, Australia.

§ Department of Primary Industries and Fisheries, GPO Box 990, Darwin NT 0801, Australia.

In addition to their roles as pathogens, a variety of insect–plant associations with *Botryosphaeria, Diaporthe* and *Lasiodiplodia* have been reported.

In this paper, we review information concerning:

- infection, colonisation, and dispersal of *Botryosphaeria, Diaporthe* and *Lasiodiplodia*; and
- the implications of antifungal compounds, phytotoxins, and insect associations for understanding or manipulating host–pathogen relations.

Quiescence of SER Fungi

In the first paper in these proceedings, Johnson discusses seed production as the primary function of fruit and speculated that fruit–pathogen associations may serve to protect seed from other (mycotoxigenic) invaders that would be less attractive to disperser vertebrates (Cipollini and Stiles 1992). We suggest that constitutive antifungal compounds in plants play an important part in regulating this balance.

When *Botryosphaeria* spp. were inoculated, with or without wounding, into the sides of unharvested mango fruits (cv. Kensington) several weeks before harvest, a variety of limited lesions developed (Figure 1a–d) (Johnson and Sangchote 1994). Lesions were more extensive on wound inoculated fruit, but remained discrete until after harvest or until the fruit split due to fruit growth pressure on the damaged tissue (Figure 1e). After harvest, some lesions remained discrete during ripening, while in other cases, spreading lesions developed (Figure 1f) (Johnson 1992). When fruit were inoculated several weeks before harvest, by placing a mycelial plug at the fruit–pedicel interface or on the peduncle 10 cm from the fruit, endophytic colonisation was detected 7 weeks later and stem-end rot did not develop until after harvest (Table 3). However, the development of postharvest stem-end rot in inoculated but not uninoculated fruit indicated that quiescent infections could be initiated on the peduncle several centimetres from the fruit or at the fruit–pedicel interface several weeks before harvest and that postharvest stem end rot developed from the quiescent infections.

When other stem end rot fungi (*Lasiodiplodia theobromae, Phomopsis mangiferae*) and *Colletotrichum gloeosporioides* were inoculated onto the peduncle 10 cm away from developing fruit several weeks before harvest, local colonisation also occurred (G. Johnson and A. Cooke, unpublished data). More extensive colonisation appeared to be constrained by prior colonisation by other endophytes —*Colletotrichum gloeosporioides, Pestalotiopsis mangiferae, Alternaria alternata,* and *Eppicoccum* sp. were recorded. Endophytic colonisation by the inoculated fungi appeared to be most extensive when inoculum was introduced early in fruit development before the peduncle had become extensively colonised by other fungi.

Table 1. Effect of 2, 4-D treatment on the incidence of stem-end rot caused by *Fusicoccum* spp. and stem-end anthracnose on mangoes during storage at 23°C for 3 weeks.

Postharvest treatment	Incidence (%) of	
	Stem-end rot	Stem-end anthracnose (SEA)*
Untreated	48.3b	41.7
Hot benomyl (w.p.)	0.0a	0.0
Hot benomyl (d.f.)	3.3a	0.0
2, 4-D amine	18.3a	48.3

Means within columns not followed by the same letter differ significantly (P = 0.05) *Anthracnose at the stem end.

This work has demonstrated the ability of mango fruit and stem tissue to contain infections of stem-end rot pathogens when they are intitiated before harvest. Containment is probably due to a combination of antifungal compounds and structural barriers. The work illustrates that fruit have well developed mechanisms to limit the ingress of stem-end rot fungi on developing fruit.

Other Associations

There are other plant associations with *Botryosphaeria, Diaporthe,* and *Lasiodiplodia* which depend upon limited colonisation of the host. Presumably host antifungals play a role in limiting colonisation and the initiation of the associations may trigger or stimulate production of the antifungals. These include the following:

Gall midge larvae and *Botryosphaeria*

Bissett and Borkent (1988) have reviewed the role of fungi inhabiting the ambrosia galls produced in host plants by gall midge larvae. Conidia of *Botryosphaeria* (resembling those of *Botryosphaeria parva/Fusicoccum mangiferum* (Johnson 1994; Johnson and Cooke 1998) are deposited alongside the eggs of the gall midge inside the host tissues using a specialised conidia-carrying structure (a mycangia).

Table 2. Causes of stem-end rot or kernel decay in fruit and nut crops and the fungi recorded as endophytes from fruiting stems (Johnson 1997). * Isolated most frequently

Host	Stem-end rot	Stem endophyte
Apple	*Botryosphaeria obtusa* *Botryosphaeria* spp.	*Alternaria alternata,* *Botryosphaeria obtusa,* *Penicillium* spp.
Avocado	**Colletotrichum* spp **Botryosphaeria* spp.; *Fusicoccum* spp. **Phomopsis perseae* *Thyronectria pseudotricha*	**Colletotrichum gloeosporioides,* *Botryosphaeria* spp., *Pestalotiopsis* sp. *Phomopsis* sp.
Carambola	*Collectotrichum* sp., *Botryosphaeria* spp. *L. theobromae, *Phomopsis* sp.	*Colletotrichum gloeosporioides,* *Dothiorella dominicana,* **Phomopsis* sp.
Chestnut	*Phomopsis castanae*	
Citrus	*Botryosphaeria spp., L. theobromae* * *Phomopsis citri*	**Colletotrichum* sp., *Botryosphaeria* spp. *L. theobromae, *Phomopsis citri,* *Pestalotiopsis versicolor*
Custard Apple	*L. theobromae*	*Alternaria alternata,* *Colletotrichum* sp., **Phomopsis* sp.
Durian	*L. theobromae* *Phytophthora palmivora*	**L. theobromae* **Phomopsis* sp., *Pestalotiopsis* sp.
Kiwifruit	*Botrytis cinerea, Fusicoccum* spp. **Phomopsis* sp.	*Alternaria alternata* *Colletotrichum acutatum* *Colletotrichum gloeosporioides* *Fusicoccum* spp., **Phomopsis* sp.
Longan	*Phomopsis* sp.	*Colletotrichum* sp., *Botryosphaeria* spp. *Pestalotiopsis versicolor* **Phomopsis* sp.
Lychee	*Colletotrichum* sp., *Botryosphaeria* spp. *L. theobromae* *Phomopsis* sp.	*Colletotrichum* sp., *Botryosphaeria* spp. *Pestalotiopsis versicolor* **Phomopsis* sp.
Mango	*Colletotrichum* spp. *Cytosphaera mangiferae* **Botryosphaeria spp., L. theobromae* *Pestalotiopsis mangferae* *Phomopsis mangiferae*	*Colletotrichum* sp. *Cytosphaera mangiferae* **Botryosphaeria* spp., *L. theobromae* **Phomopsis mangiferae* *Pestalotiopsis mangiferae*
Mangosteen	*Colletotrichum* sp., *Fusicoccum aesculi* **L. theobromae* *Pestalotiopsis* sp., *Phomopsis* sp.	*Colletotrichum* sp., *Fusicoccum cajani* *L. theobromae* **Pestalotiopsis* sp., **Phomopsis* sp.
Pecan		*Colletotrichum* sp., *Botryosphaeria* spp. *Phomopsis* sp.
Rambutan	*Phomopsis* sp.	*Colletotrichum* sp., *Botryosphaeria* spp. *Pestalotiopsis versicolor* **Phomopsis* sp.
Sapodilla	*Colletotrichum* sp., *Fusicoccum cajani* *Pestalotiopsis* sp., *Phomopsis* sp.	*Colletotrichum* sp., *Fusicoccum cajani* *L. theobromae* *Pestalotiopsis* sp., **Phomopsis* sp.
Tamarillo	*Phomopsis* sp.	**Colletotrichum* sp., *Botryosphaeria* spp. *Phomopsis* sp.

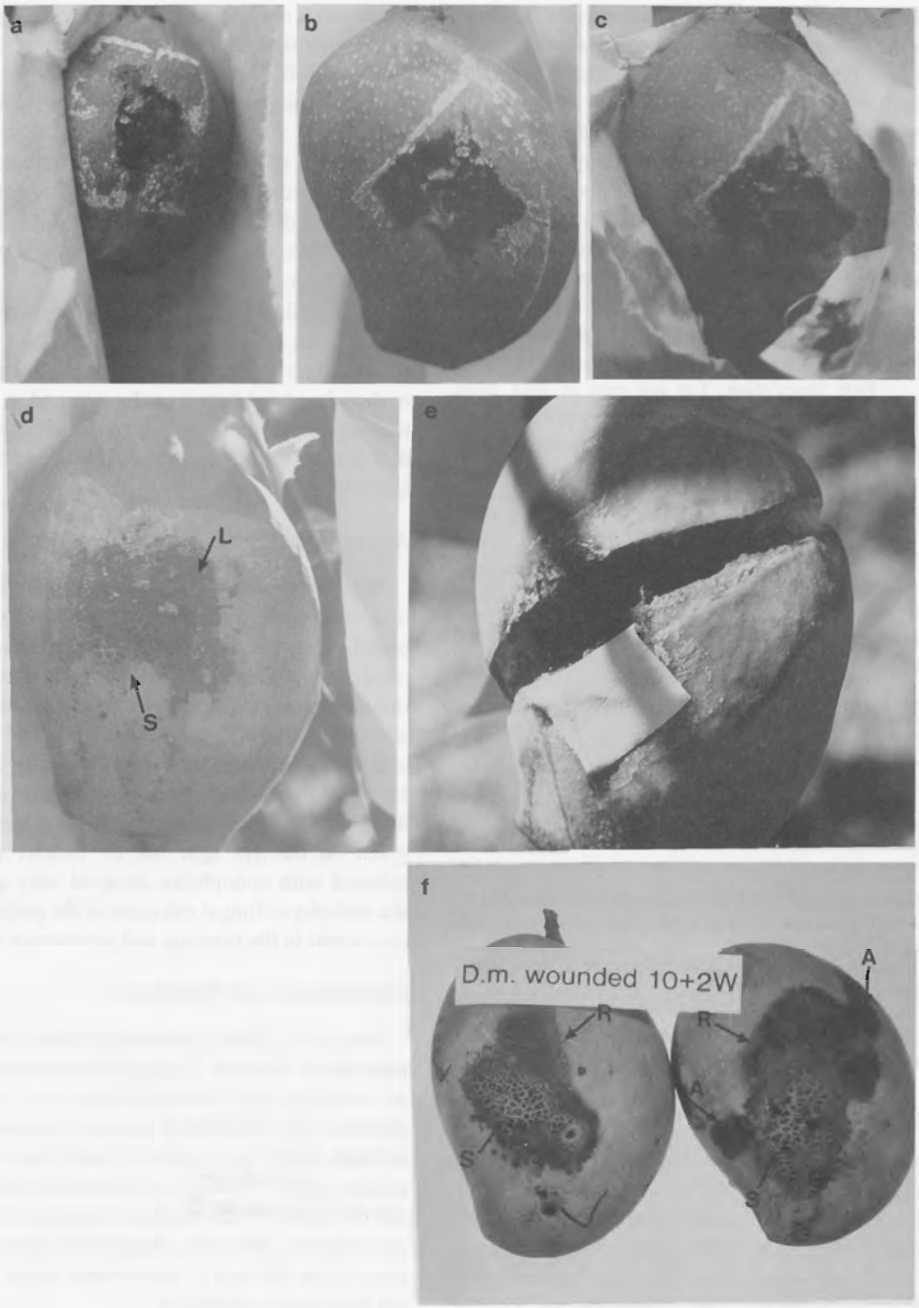

Figure 1. Unharvested fruit wound inoculated with *Botryosphaeria parva* (anamorph *F. mangiferum* = *Dothiorella mangiferae*) (a–c). A limited lesion was evident at 7 days after inoculation (a) and remained similar at 2 weeks (b) and 3 weeks. In (c) the adhesive tape has been removed to expose the lesion. Seven weeks after inoculation (d), a limited lesion (L) and scar tissue (S) are evident around the point of inoculation. In (e), 7 weeks after inoculation, latitudinal fruit splitting has occurred around the point of inoculation, and a spreading necrotic lesion has developed while the fruit still hangs on the tree. Most fruit affected in this way, subsequently fell from the tree. Ten weeks after inoculation (f), the remaining fruit were harvested and ripened at 23°C for 2 weeks. During ripening a spreading lesion (R) developed around the limited lesion and scar tissue (S) that were present at harvest. Natural infections of *C. gloeosporioides* (A) have developed on one fruit.

In the gall associations, the mycelium of the *Botryosphaeria* is intracellular and nutrition appears to be biotrophic. The larvae appear to be dependent on the mycelium of the fungus as their only food source. Bissett and Borkent (1988) speculate that the association may have evolved from evolutionary links between the insects and gall-forming species of *Botryosphaeria* and that the association, in addition to providing larval food, may also help protect the larvae from other fungal colonists and plant chemicals.

Table 3. Frequency of detection of *Botryosphaeria dothidea* from mango stem tissue 7 weeks after field inoculation. Ten per cent of fruit from stems inoculated at B or C developed stem end rot after harvest. No fruit from uninoculated stems developed stem end rot (Johnson 1992).

Inoculation point[a]	Incidence[b] (%) of detection in segments cut at points A[c], B, or C from 10 stems per treatment		
	A[c]	B	C
Stem segment (B)[a], 10 cm distal from fruit	50	40[a]	20
Fruit–pedicel connection zone (C)[a]	20	30	30[a]
Uninoculated stem at point B	0	0	0
Uninoculated fruit at point C	0	0	0

[a] Indicates the point at which the samples had been inoculated 7 weeks before harvest. Uninoculated stems had adhesive tape applied at the point indicated.
[b] The percentage of stem segments yielding *B. dothidea* from the sample nominated.
[c] A – An uninoculated stem segment, 10 cm distal from point B at which some stems were inoculated, and 20 cm distal from then fruit.

Insect repellence

'The presence of fungi and their toxins may deter colonisation, inhibit growth of, or kill phytophagous insects...infections by endophytic fungi add to the repertoire of plant constitutive and induced "defences", nutritional and physical barriers, and phenological variability that colonising and feeding stages of insect species must overcome in order to use a plant species as a host.' (Hammon and Faeth 1992). Endophytic fungi may have a similar role in supplementing and or eliciting the production of host-produced antifungal compounds. They may also serve to deter insect vectors of disease. *Phomopsis oblonga* is a common endophyte in elm trees. It produces a repellent which discourages colonisation of elm bark by the bark-boring insects which carry the Dutch elm disease pathogen (*Ceratocystis ulmi*) (Claydon et al. 1985).

The latex of mangoes contains high levels of antifungal resorcinols which delay the development of postharvest diseases. Joel (1978, 1980) has suggested that the latex also deters oviposition and colonisation by fruit flies and their larvae.

It is significant that abscission zone barriers restrict colonisation of fruit during development. The benefits that accrue from endophytic associations in the bark and peduncle must not be required in developing fruit—probably because the host's pathogen and pest deterrent structures and chemicals are present at adequate levels in vigorously growing fruit.

Are endophytes the gatekeepers of fruit ripening?

Wilson (1993, 1995) considered the various roles that endophytic fungi might play in plants. He proposed that some attributes of plant defence were for protection of fungal endophytes and were an evolutionary consequence of endophyte infection. Wilson and Carroll (1994) also proposed that leaf senescence and nutrient resorption were affected by endophytes. He noted that excised leaf discs of *Quercus garryana* that were not infected with endophytes remained green on nutrient agar for 18 months while those infected with endophytes decayed very quickly. Do the endophytic fungal colonists of the pedicel and skin play a part in the ripening and senescence of fruit?

Lasiodiplodia and butterflies

Nago et al. (these proceedings) have reviewed the association between *Lasiodiplodia theobromae* and the butterfly *Idea leuconoe* Ericson. *L. theobromae* produces (*R*)-2-octeno-d-lactone (lasiolactone) and mellein, which have a strong 'fruity' odour and could attract insects. Mellein is concentrated in the hairpencils of male butterflies and is thought to be a butterfly sex hormone. However, Nago et al. conclude that the association between *L. theobromae* and *I. leuconoe* is not necessarily mutualistic.

Attraction of butterflies to sporulating lesions caused by *L. theobromae* would facilitate spread of the fungus to other hosts, particularly nectar producing flowers. Early research on stem-end rot of citrus suggested that stem-end rot infections arose from quiescent infections in floral remnants. Sangchote et al.

(1997) found that *L. theobromae* was isolated more frequently from the stigma disk remnants than from the sepals on developing mangosteens. Transmission by nectivorous insects would favour such a distribution (as would adhesion of airborne spores to the sticky stigmatic surface).

However, Nago and Masumoto (1994) note that the mellein may also have plant regulatory effects. Mellein has also been reported as the most abundant phytotoxin produced by *Botryosphaeria obtusa* (Schwein.) Shoemaker, the cause of black rot of apple fruit. Other phytotoxins made by *B. obtusa* include tyrosol, which is also produced by *Phomopsis oblongata*, and implicated in the resistance to bark beetles of elms infected with *Endothia parasitica* (*C. ulmi*) (Venkatasubbaiah et al. 1991; Claydon et al. 1985). Venkatasubbiaah et al. (1991) found that the phytotoxins produced by *B. obtusa* could damage apple leaf tissues but there was no correlation between apple cultivar susceptibility to *B. obtusa* and the severity of damage caused by the phytotoxins. The toxins were not present in healthy apple fruit extract. In regulating ingress of *Botryosphaeria* and *Phomopsis* the host must be able to limit or contain the risk from any phytotoxic metabolites produced *in planta.*

Fruit/vertebrate dispersers/fruit rot fungi: selection pressures for secondary chemical defence

Cipollini and Stiles (1992) reviewed the relative risks of decay for fleshy fruits and the significance of the associations with respect to dispersal and selection for secondary defence. They noted the categories of Herrera (1982) for the defence of ripe fruit:

'(i) To ripen when pest pressure is lowest.
(ii) To reduce the exposure time of ripe fruits to damaging agents.
(iii) To reduce the nutritive quality of tissues for pests and pathogens by providing unbalanced or poor quality fruits.
(iv) To retain some degree of structural or chemical defence.'

and suggested that the retention of toxic compounds in fruit during ripening has been a compromise between optimal defence and optimal attraction. Because defence chemicals are toxic to dispersers, selection pressure has favoured the non-retention of these constituents during ripening. However, microbial invaders can also reduce palatability and attractiveness to frugivores, so retention of microbe-specific defence would be an evolutionary advantage. In considering these mechanisms, Cippollini and Stiles (1992) did not consider the additional important associations in tropical fruit: infestation of fruit by fruit fly larvae and abscission-zone defences.

Johnson (1997) noted that fruit fly associated Enterobacteriaceae such as *Klebsiella oxytoca* and *Enterobacter cloacae* may have fungistatic effects in infected fruit, enhancing the availability of fruit to fly larvae. Fruit are usually infested as they start to ripen and before they fall—the larval association may provide protection from fungal invaders that might kill the seed. In addition, abscission of the fruit frees it from the threat of the stem-end rot fungi in the pedicel, although the fruit will remain prone to invasion from soilborne inoculum of the same fungi.

The fruit fly associations and abscission at ripening may also provide fruit with additional mechanisms for avoiding undesirable (seed-destroying) associations.

Conclusions

We have reviewed a variety of information concerning the biology of fruit and fruit pathogens. Fruit resistance factors have evolved against this background. A greater understanding of the interplay involved will undoubtedly improve our ability to exploit fruit resistance factors and reduce reliance on chemicals to control disease.

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Fruit Ripening and Defence against Pathogens—Loss of Resistance or Gain of Susceptibility?

J.M. Labavitch*

Abstract

Developing fruits exhibit general resistance to many pathogenic fungi. The natural defences of fruits fall into the same categories (preformed and induced, physical and biochemical) as the defences expressed by other plant organs. For many years plant pathologists have worked to understand the specific mechanisms of disease resistance and how their expression is controlled. These research efforts have gained importance in recent years as restrictions have been placed on the chemicals that have been used to control postharvest diseases because of consumer concerns about the effects of these chemicals on human health. Whether these concerns are warranted or not, they have put increasing pressure on efforts to develop more 'natural' means of control for local and export markets.

When ripening begins, natural defences are no longer expressed (or are no longer effective) and the incidence of disease is high. When viewed from the perspective of angiosperm evolution, this increase in a fruit's susceptibility to pathogens can be seen as beneficial. The pathogen helps to free the mature seeds from the fruit so that their dispersal is enhanced. This kind of thinking leads us to consider whether the ripening-related decrease in fruit defences should be viewed as a loss of resistance or a gain of susceptibility. This is an important distinction. Postharvest pathologists must try to sustain the fruit's defences while millions of years of evolution of the ripening process (with its associated alterations in gene expression, cell wall polymer integrity, membrane function, metabolic pathways, etc.) have brought a loss of defence capability. The idea that susceptibility is gained during ripening may imply that there are ripening-associated signals that trigger an active dismantling of defences. If this is so, then it might be possible to specifically block the gain of susceptibility without disrupting other aspects of ripening.

MOST plants are resistant to most pathogens in spite of the fact that pathogen propagules are generally present in the environment. We conclude, therefore, that plant resistance is the general situation and that special conditions must exist in order to permit pathogen development on plant hosts. Typically, defence mechanisms are described as either preformed or pathogen-induced and as confronting the pathogen with either a physical barrier or an antagonist to its biochemical functions (Agrios 1997). Specific examples of these classes of resistance mechanisms are many and have been the object of several reviews (e.g. Prusky 1996).

* Pomology Department, University of California, Davis, CA 95616, USA.

Fruits exhibit resistance to pathogens throughout most of their development. This resistance can be defined in terms of specific mechanisms that can be categorised as described above. When fruits begin to ripen, however, they appear to lose their defences. An important consequence of this developmental transition is that infections that have remained latent throughout much of fruit development become active (Prusky 1996) and, when control is not adequate, postharvest losses to disease can be substantial The problem is made more acute because fruit have been removed from the parent plant which had served as a source of assimilate and water, and the harvest procedure often damages surface barriers to penetration. Postharvest handling may include washing which, though it is designed to clean the fruit, too often provides an opportunity for spread of spores to

previously uninfected fruit. Handling protocols for local or distant markets often emphasise high humidity and close packing; conditions which facilitate spore germination and infection spread.

Fruit ripening is a terminal developmental process. Plant biologists agree that ripening is one form of plant senescence—a programmed development that ultimately leads to cell death. However, this senescence-related death serves important functions in terms of the overall life cycle of the species. The result of ripening in most fruits is a total loss of metabolic and structural integrity. Eventually the extent of this tissue disintegration represents what horticulturists refer to as the overripe state; a state which, in many respects, resembles that of a well-rotted fruit. The ideal, from the postharvest biologist's point-of-view, is to manage the ripening process so that consumers receive the greatest number of harvested fruits with maximum organoleptic and nutritional quality. The increased susceptibility to pathogens that ripening fruits display represents an additional challenge to the postharvest horticulturist.

Control of Postharvest Pathogens

A great deal of postharvest pathogen control in the United States has been accomplished through the use of chemical fungicides. In spite of this it has been estimated that 24% of harvested fruits and vegetables is lost to spoilage (USDA 1965). Continued use of these chemicals is contributing to the development of resistant strains of microorganisms and consumer concerns are influencing regulatory agencies to limit chemical use on foods. These pressures are driving increasing research into means of controlling postharvest pathogens. The approaches taken have generally focused either on alternatives to managing fruit in the storage environment or on identifying the fruit's natural means of resistance and modifying these so that they will be more effectively expressed in the stored fruit.

'Traditional' refrigerated storage remains an effective means of pathogen control on many fruits of non-tropical origin; its use being limited by the level of chilling tolerance of the fruit. Combining refrigeration with controlled atmosphere (CA) regimes improves the efficacy. The effects of these treatments are on both of the participants in the disease interaction. The CA and cold treatments delay the onset of ripening and slow its progression (and the associated decrease in resistance), and also have fungistatic effects.

Calcium treatments (preharvest applications and postharvest dips and infiltrations (Conway et al. 1994)) have proven to be effective in enhancing resistance. These have been known to increase fruit firmness through effects on cell wall pectin cross-bridging and membrane function (Ferguson 1984; Poovaiah 1988; Mignani et al. 1995). Calcium also slows fruit ripening. It is likely that the altered pectin solubility influences pathogen development because the host cell wall becomes less subject to digestion by microbial enzymes such as polygalacturonase [PG] and pectin lyase [PL] (Conway et al. 1988).

Postharvest heat treatments (hot water dips lasting a few minutes or hot air exposures lasting for several hours) that are sufficient to elevate the temperature of the outermost fruit tissues have been shown to reduce decay incidence (Klein and Lurie 1991; Mitcham and Cantwell 1995). While the most important impact of these treatments appears to be directly on the pathogen, in some fruits the effect is partially indirect in that aspects of fruit development may be altered. For instance, ripening of climacteric fruits may be slowed because of heat effects on ethylene biosynthesis. Hot water dips of citrus may contribute to resistance because they 'melt' cuticular wax so filling in gaps that could be penetrated by the pathogen (Schirra and D'hallewin 1997).

Exposure of fruits to low doses of UV-C irradiation also reduces decay incidence (Wilson et al. 1994). The impact is not solely due to the fungicidal impact of the irradiation because beneficial effects may not be seen for some time after irradiation has ended. UV-C induces the synthesis of phytoalexins in carrots and citrus (Mercier et al. 1993). Other mechanisms of natural resistance may also be involved, particularly if the exposure is sufficient to elicit fruit wound responses.

Considerable research time has been spent studying the impact of antagonistic microorganisms (bacteria, fungi and yeasts) on pathogen development (Roberts 1994). Biocontrol agents may outcompete pathogens for important niches on the fruit surface, or they may directly oppose the pathogen by parasitising it or producing antifungal metabolites. In some cases, the biocontrol agents have been shown to elicit fruit phytoalexin production (e.g. Rodov et al. 1992). The production of phytoalexins in response to treatments with biocontrol organisms may be an indication that the fruit also perceives the biocontrol agent to be a threat.

More recently (Wilson and El Ghaouth 1993), research has shown that treatments of fruits with natural products can provide enhanced resistance to postharvest pathogens. Of particular interest is the observation that the coating of fruits with films of chitosan (the polymer produced when the N-acetylgucosamine residues of chitin are deacetylated) can reduce the tissue damage caused by pathogen (see El Ghaouth, these proceedings). The beneficial effects may be due to direct effects on the pathogen or because the fruit's ripening and/or response to the pathogen are altered.

Genetic Engineering Approaches

Molecular biologists have focused considerable research attention on studies of the interactions of pathogens and their hosts, and many putative defence genes have been identified. Genes that are involved in controlling hypersensitive death of host cells in specific gene-for-gene host–pathogen interactions have been identified (Hammond-Kosack et al. 1994; Hammond-Koscak and Jones 1995). Several families of pathogenesis-related (PR) genes are induced in infected plant tissues, and the in vitro functions of some of the protein products of these genes have been identified. For instance, it has been proposed that PR gene-encoded chitinase and β-1,3-glucanase enzymes contribute to defence by digesting structural components of pathogen cell walls (directly interfering with pathogen development), and that this hydrolysis of fungal-wall polysaccharides produces glucan and chitin oligosaccharides which have been shown to be elicitors of host defence responses in several plant tissues (Darvill and Albersheim 1984; Ryan and Farmer 1991). 'Genetic engineers' have already begun to test the effects of transgenic expression of some of these genes on the defences of transformed crop plants (Cornelisson and Melchers 1993). For instance, collaborative work in my laboratory has identified a pear fruit protein (PGIP) that inhibits the pectin-degrading polygalacturonase (PG) enzymes that are produced by many fungi. We have cloned the gene for this protein and have transformed tomato plants with it so that they express the pear PGIP at a high level. We are now testing the impact of this genetic engineering 'strategy' on fruit defence against the grey mould pathogen, *Botrytis cinerea* (Powell et al. 1994; Labavitch et al., these proceedings). Other strategies for enhancing postharvest resistance have been proposed (Mount and Berman 1994).

Another approach to enhancing fruit defences against postharvest pathogens ignores specific mechanisms of the 'typical' fruit defences and aims for effective control of fruit ripening. The logic behind this strategy is that if a fruit's generalised resistance to infection is high before the onset of ripening then effective control of ripening should maintain that natural resistance and give effective control of pathogens. The work of Flaishman and Kolattakudty (1994) may support this idea. They found that spores of *Colletotrichum gloeosporioides* would not develop on tomato fruits whose ethylene synthesis was inhibited by expression of an antisense 1-aminocylopropane-1-carboxylic acid synthase (ACC) gene unless the fruits were given an ethylene treatment. In this case, the authors suggest that the ethylene has a direct effect on fungal development (spore germination and appressorium formation) as well as on fruit ripening.

Evolution and the Connection between Fruit Ripening and Increased Susceptibility to Pathogens

The function of fruit is two-fold. Initially, it accords protection to the developing seeds it contains and provides the continuous vascular supply that allows transfer, into seeds, of assimilate that is used for the development of seed structures. It is likely that hormones produced in the seeds influence this aspect of fruit development. The attainment of maturity by fruits generally corresponds to the time at which seeds reach full development. At this point the fruit's function changes. In most species, fruits now contribute to seed dispersal. The different components of fruit ripening are listed in Table 1. Fruit ripening can be viewed as an example of plant organ senescence (i.e., a developmental sequence that ultimately leads to organ death). Nevertheless, it should be clear that the death that comes at the end of fruit ripening represents death with a purpose. Ripening-related changes in fruits require a substantial investment of cellular energy and a dramatic redirection of metabolic pathways. Presumably, natural selection has been important in determining the direction that fruit development takes after seeds are fully developed. Thus, different aspects of ripening should be interpreted as factors which contribute to the attraction of seed dispersal agents or improved chances of seed survival.

Table 1. Different aspects of ripening in the 'typical' fruit and where they are localised.

1. Change in colour (plastids and vacuoles)
2. Change in energy metabolism (mitochondria)
3. Changes in storage carbohydrates (plastids, vacuoles, cytoplasm and apoplast)
4. Changes in aroma and flavour (vacuoles and cytoplasm)
5. Changes in texture (cell walls and membranes)
6. Changes in ethylene production (vacuoles and cytoplasm — unclear)
7. Changes in 2° products (phenol oxidation — vacuoles and cytoplasm — unclear)
8. Changes in gene expression and protein synthesis (nucleus and cytoplasm — some proteins are then exported to the vacuole or apoplast)
9. Loss of resistance/gain of susceptibility (?)

Early humans were probably among the animals involved in the mutualism between fruiting plants and seed dispersal agents. Pathogens of ripe fruits may also have been part of this (presumed) co-evolving mutualistic interaction. If this is so, then the increase in fruit susceptibility to pathogens that accompanies ripening may have become as tightly associated (in terms of natural selection) with the ripening process as are changes in fruit colour and flavour. Of course, we humans have also had a substantial impact on the recent course of fruit evolution—imposing artificial selection pressures to drive 'evolution' toward extremes of fruit size, ripening rate, colour, etc.—but this selection has had little to do with enhancing the contribution of the fruit to seed dispersal. In the context of the fruit's role in dispersal, we may now be more legitimately considered to be parasites than are the postharvest pathogens we wish to control.

If an increase in fruit pathogen susceptibility can be considered to be one facet of the ripening process, then the most effective means to control postharvest pathogens may be to learn the basis of the increase in susceptibility and how it is controlled as part of the overall regulation of ripening. There is a clear correlation between resistance and the levels of antifungal chemicals in unripe avocados, mangoes, and peaches (Droby et al. 1997; Prusky and Keen 1993; Bostock and Gradziel, pers. comm.), and the loss in resistance that accompanies ripening can be explained, at least in part, by decreases in the concentrations of these molecules. One strategy for increasing resistance, therefore, might be to increase the concentrations of these compounds or eliminate the factors that cause their reduction. It is not clear, however, that such a strategy would be consistent with providing ripe fruit of maximal organoleptic or nutritional quality to consumers.

How Might the Hypothetical Ripening-related Increase in Fruit Susceptibility to Pathogens Be Controlled?

Several laboratories have examined the relationship of fruit PG production to the tissue softening that accompanies ripening in tomatoes and other fruits: see Fischer and Bennett (1991) for a review. Studies that have used molecular genetic approaches to alter PG production in tomatoes have made clear that if fruit PG is important for fruit softening it is not the only factor involved. This work also provided a surprising insight into the relationship of ripening and disease resistance. Giovannoni et al. (1989) tested PG's contribution to tomato softening by incorporating a chimeric tomato PG gene whose expression was regulated by the ethylene and propylene-inducible E8 promoter into non-ripening *rin* tomato mutants. These mutants have a PG gene in their genome but it is normally not expressed because of the pleiotropic effects of the *rin* mutation. However, when the transformed *rin* fruits were treated with propylene, the chimeric PG transgene was expressed, PG protein accumulated, and cell wall pectin digestion occurred as in normally-ripening fruits; but the transgenic *rin* fruits did not soften (DellaPenna et al. 1990). Normally, *rin* fruits that have reached maturity do not demonstrate the increase in disease susceptibility that is shown by ripening fruits. However, while the transgenic *rin* fruits that expressed PG in response to propylene treatment did not soften they did show a pronounced increase in susceptibility to pathogens like *Alternaria alternata* and *Rhizopus stolonifer*. Transformed *rin* fruits that were not treated with ethylene or propylene (and, thus, did not produce PG) retained their normal resistance to these pathogens (Bennett et al. 1992; Martin 1994).

The only change in the susceptible *rin* fruits was in their ability to produce PG and digest their cell wall pectins. Therefore, the connection between cell wall changes and susceptibility was examined (Martin 1994). Mature green (MG) and red ripe (RR) fruits were homogenised and two, roughly defined classes of cell wall pectins were collected. The first fraction was freely water soluble (WS). Pectins in this class are presumed to have been part of the insoluble cell wall fabric at some point in fruit development but have been made soluble as development of the fruit proceeded (because of cell wall hydrolysis or other kinds of wall metabolism) and so are readily separated from the major portion of the cell wall (which is relatively insoluble in water) when the fruit is homogenised. The second class of pectins (wall-bound; WB) represents all of those pectins which have retained their association with the insoluble wall and so are precipitated by the low speed centrifugation which follows the extraction in water that leaves the WS pectins in the supernatant. Most of the pectin in MG tomato fruits remains associated with the WB fraction and the pectin of MG fruits that is collected in the WS fraction tends to be of fairly high molecular weight (Brummell and Labavitch 1998). The amount of pectin that is in the WS fraction of red ripe tomatoes increases substantially (and includes low molecular weight pectin breakdown products; Melotto et al. (1994)) and there is a concomitant decrease in the amount and nature of the pectins that are WB.

The WS and WB pectins were then used as the carbon source in liquid cultures of *A. alternata*. After a few days in culture, the medium was assayed for two factors that may be indicators of the virulence of the cultured pathogen. If cultured on pectins of MG tomato fruit the fungus produced the AAL phytotoxin (Gilchrist and Grogan 1976) only if the medium had been supplemented with the WB material. In contrast, if pectins from RR tomatoes were used, the AAL toxin was produced only if the WS pectins were used as a medium supplement. PG was also produced by the cultured *A. alternata* and once again the primary elicitors of this pectin hydrolase were the WB pectins from MG fruit and the WS fraction from ripe fruit. These observations led to the suggestion that the cell wall pectin metabolism that accompanied fruit ripening produced soluble signals that enhanced pathogen virulence. Presumably, the ripening-related changes in cell wall pectins were caused by the action of fruit PG which might hydrolyse WB pectins to produce 'oligosaccharins' that could elicit changes in fungal metabolism (Martin 1994). The fungus's increased production of PG in response to the WS pectin of RR fruit would further enhance the degradation of WB pectins and, thus, cause production of more oligomeric elicitors. While several points in this scenario require more detailed study, the model developed leads directly to the conclusion that the failure of *A. alternata* to infect *rin* fruit which do not produce PG and the fungus's success in infecting the transgenic *rin* fruits which expressed PG can be explained by the absence or presence, respectively, of pectin oligomers in the fruit flesh. Thus, the cell wall pectin digestion that is a normal part of ripening can activate potential pathogens (i.e., enhance fruit susceptibility).

Furthermore, the normal control of tomato fruit ripening may depend on the breakdown of wall-bound pectins, with the concomitant production of pectin oligomers. We have shown that oligosaccharides produced when citrus pectin is hydrolysed are capable of promoting tomato ripening (Campbell and Labavitch 1991) and that wall breakdown products, similar in structure and activity to these citrus pectin oligomers, accumulate in ripening tomato pericarp (Melotto et al. 1994). When purified tomato fruit PG is incubated with extracted tomato pectins, ripening-promoting oligomeric digestion products are generated. We do not know if the initiation of tomato fruit ripening depends on the presence of these (potential) oligosaccharide signal molecules and, if so, whether oligomer production in vivo is due to the action of PG or some other pectin-degrading enzymes. Nevertheless, the suggestions that pectin-derived oligomers can both enhance fruit susceptibility to pathogens (Martin 1994) and promote fruit ripening (Melotto et al. 1994) appear to support the conclusion that these processes are mechanistically linked.

Are there other observations that suggest a linkage between the control of ripening and resistance to pathogens? It has recently been reported (Kausch and Handa 1997) that tomato fruit contain an isozyme of lipoxygenase that is specifically expressed during ripening. They have used a co-suppression approach (Handa, pers. comm.) to reduce (but not eliminate) expression of this lipoxygenase and fruit ripening is delayed. In avocado fruit, lipoxygenase is a pathogenicity factor in that its expression during fruit ripening contributes to the destruction of antifungal dienes and enhanced fruit disease susceptibility (Prusky 1988, and these proceedings; Prusky et al. 1983; Prusky and Keen 1993). It is not known if tomato lipoxygenase is also a pathogenicity factor. However, the dependence of fruit ripening on lipoxygenase expression suggested by Handa's observa-

tions and the potential contribution of the enzyme to pathogen success may be another indication of the linkage of ripening and increasing fruit susceptibility.

Ripening Control—the Key to Control of Postharvest Pathogens?

It is important to examine this potential relationship closely. Postharvest plant pathologists and physiologists hope to develop effective, biologically-based measures for controlling diseases in ripening fruits. An understanding of the developmental cues and metabolic processes involved in connecting ripening with increased susceptibility may lead the way to approaches which can disconnect the two and allow ripening to proceed while resistance remains high.

Speculations about the Evolution of a Developmental Linkage of Fruit Ripening and Increased Susceptibility to Disease

Earlier in this paper I suggested that ripening and loss of pathogen resistance are tied to one another because both are important for the dispersal of seeds, and hence for the maintenance of fruiting plant species. There is also a clear advantage to the pathogen if a given stage of its host plant's development is well-suited to support fungal growth and reproduction. Did the co-evolution of the host and its fungal pathogens establish a tight developmental connection between ripening and disease susceptibility? When unripe fruits are infected by pathogens they often show localised ripening which includes the synthesis of ethylene of plant or fungal origin. Is this a developmental accident, ethylene being produced by host tissues that are 'biologically' wounded? If the ethylene is of pathogen origin, is it produced because the pathogen has 'learned' over evolutionary time that fruit tissues that have been exposed to ethylene are easier to invade and provide metabolic substrate that is more accessible (as in what we now think of as a ripened fruit)? Evolutionary biologists suggest that the chloroplasts and mitochondria of modern cells are the descendants of prokaryotic endosymbionts that once shared a mutualistic existence with early cells. Could the highly coordinated ripening program of climacteric fruits have been 'acquired' from early fungal pathogens which promoted localised tissue senescence as they invaded, because the fruit 'learned' that the fungal program was an efficient way to carry out seed dispersal?

The linkage between fruit ripening and increasing disease susceptibility may now be too strong to disjoin. If so, we may have to accept that the best way to improve disease control in the postharvest environment is to control ripening itself.

Acknowledgments

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Disease Resistance in Fruit

Mechanism of Resistance of Avocado Fruits to *Colletotrichum gloeosporioides* Attack

D. Prusky, R. Ardi, I. Kobiler, D. Beno-Moalem and A. Leikin*

Abstract

Colletotrichum gloeosporioides is the most destructive postharvest pathogen of avocado and other tropical fruits. The pathogen infects tropical fruits throughout the period of fruit growth, but remains quiescent for weeks or months while the fruit is immature. Conidia of the fungus germinate on the peel of avocado fruit, producing appressoria. The appressoria produce infection pegs that breach the wax layer and come to rest on the underlying epidermal cells. Upon harvest and fruit ripening, quiescent infections are activated, causing extensive damage to the fruit.

Quiescent infection appears to be a fungal response to adverse physiological conditions temporarily imposed by the host. The lack of available nutrients, the presence of preformed antifungal compounds, and the lack of enzymatic potential have been tested as possible causes of quiescent fungal infections on unripe fruits. The quiescence of *C. gloeosporioides* in unripe avocado fruit has been attributed to the presence of high concentrations of several preformed antifungal compounds, the most active of them 1-acetoxy-2-hydroxy-4-oxoheneicosa-12,15-diene. Concentrations of the antifungal diene and epicatechin (a non-specific inhibitor present in the pericarp of unripe fruits but not ripe fruits) decrease during fruit ripening and were accordingly suggested as major factors in permitting activation of quiescent infections. The catabolism of the diene after harvest is attributed to the oxidation by lipoxygenase whose activity is in turn regulated by epicatechin. The level of epicatechin is a critical point regulating the mechanism of resistance. Induction of epicatechin level enhances the level of the antifungal compound by preventing its breakdown. Induced levels of epicatechin occur by activation of the phenylpropanoid pathway with enhanced activity of phenylalanine ammonia lyase, chalcone synthase, and flavoanone 3 hydroxylase.

C. gloeosporioides also decays the mesocarp of peeled unripe fruits despite fungitoxic concentrations of the antifungal compounds. This may be explained by the compartmentalisation of the antifungal diene in idioblast oil cells. These idioblasts are metabolically active and can incorporate labelled precursors and synthesise and export diene. Modulation of the level of preformed antifungal compounds could become a basis for enhancing resistance and reducing pesticide use.

PATHOGENS must perform several specific functions to overcome plant barriers if they are to initiate disease. With fungal pathogens, it is frequently necessary for them to adhere to the plant surface and produce structures which allow penetration. Spores of *Colletotrichum gloeosporioides,* the causal agent of anthracnose disease, germinate and produce appressoria for initial penetration. The germinated appressoria breach the fruit cuticle at the base of the appressoria and penetrate through the fruit wax, fruit cuticle, and become quiescent close to the host cell wall. This sequence of events for pathogen infection occurs throughout fruit growth but after the initial penetration phase the pathogen becomes quiescent until the fruit ripens after harvest. Quiescence is the result of the fungus meeting a set of barriers that prevent its development until fruit ripening. Only after ripening are symptoms of decay observed.

* Department of Postharvest Science of Fresh Produce, The Volcani Center, Agricultural Research Organization, Bet Dagan 50250, Israel.

Reports by Verhoeff (1974), and Prusky (1996) suggested that significant physiological changes must occur in the host and pathogen during fruit ripening to activate quiescent infections and enable fungal colonisation. The mechanisms affecting fungal colonisation in unripe fruits were summarised in four hypotheses (Prusky 1996): (1) the lack of nutritional requirements of the pathogen; (2) the presence of preformed antifungal compounds that decrease in ripening fruits; (3) the presence of inducible antifungal compounds whose concentration falls after harvest; and (4) activation of fungal pathogenicity factors occurring in ripening fruits. In the avocado–*Colletotrichum* interaction all four of the above aspects have been tested. Nutrients do not seem to be involved in fruit resistance. Neither have phytoalexin-like compounds been found in avocado fruit. However, strong evidence for the presence of preformed compounds has been reported. This paper will describe the role of preformed antifungal compounds in the mechanism of resistance of avocado fruit to attack by *C. gloeosporioides.*

Preformed Antifungal Compounds in Avocado Fruit

The resistance of unripe avocado fruit to attack by *C. gloeosporioides* was shown to be dependent on the presence of fungitoxic concentrations of preformed compounds. Five major compounds were identified in unripe avocado fruit (Prusky et al. 1982; Prusky and Keen 1993, 1995; Adikaram et al. 1992). The most-active antifungal compound was identified as 1-acetoxy-2-hydroxy-4-oxo-heneicosa-12,15 diene, which has an ED_{50} for germ-tube elongation of about 450 µg/mL (Fig. 1). The concentration of the diene in unripe fruits can reach 1600 µg/g fresh weight and decreases tenfold during ripening to subfungitoxic concentrations, both in the peel and in the flesh. A second antifungal compound was identified as 1-acetoxy-2,4 dihydroxy-n-heptadeca-16-ene, that is half as active with a ED_{50} of about 850 µg/mL (Fig. 1). Its initial concentration is lower than the diene and its concentration also decreases during fruit ripening. Biological activity of fruit peel extracts decreases in parallel with the fall in concentration of the antifungal diene (Fig. 2). Based on these results, where the diene is present in higher concentration and is more active than other antifungal compounds present, it was concluded that it is the main antifungal compound in the fruit peel. It was concluded accordingly that, for a quiescent infection to become active, a fall in the concentration of the diene is needed.

Disease susceptibility in avocado fruit is closely related to decreased concentrations of the preformed antifungal compounds. Consequently, the mechanism controlling this reduction may predispose fruit to disease. The antifungal diene contains a cis–cis pentadiene system easily oxidised by the enzyme lipoxygenase (Prusky et al. 1983). The experimental results listed below suggested the involvement of lipoxygenase in the breakdown of the antifungal compound and induced resistance.

1. The apparent specific activity of the enzyme increased by 80% during fruit ripening before symptom development (Prusky et al. 1983).
2. Partially purified avocado lipoxygenase oxidised the antifungal diene in vitro (Prusky et al. 1983).
3. Treatment with α-tocopherol acetate, and other nonspecific inhibitors of lipoxygenase, inhibited avocado activity in vitro and delayed the decrease of the antifungal diene and disease symptoms (Prusky et al. 1983).
4. A specific inhibitor of lipoxygenase—5,8,11,14-eicosatetraynoic acid—inhibited decay development by *C. gloeosporioides* (Prusky et al. 1983).

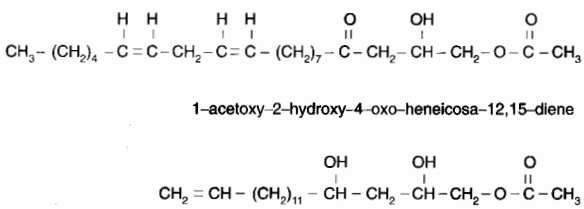

Figure 1. Chemical structures of the antifungal diene and monoene compounds isolated from avocado fruit.

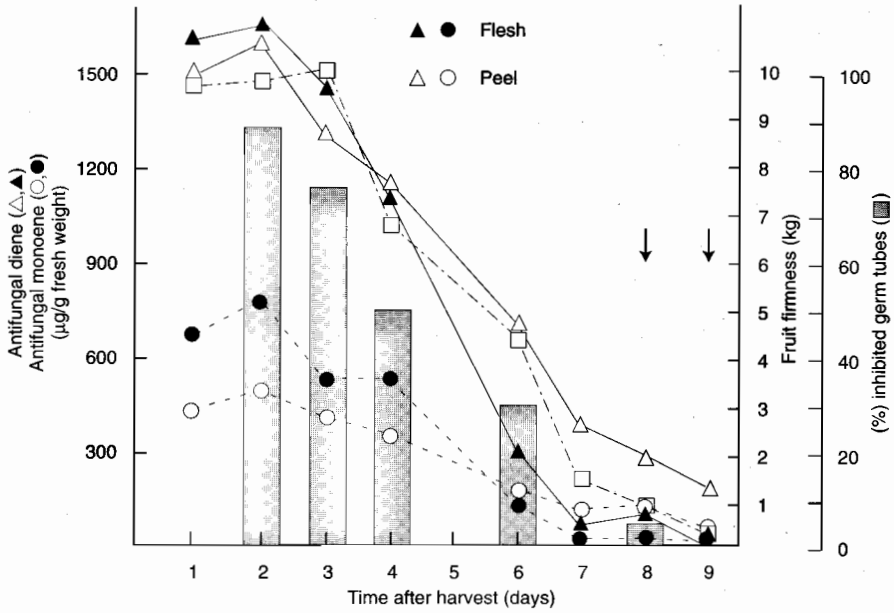

Figure 2. Antifungal diene and monoene levels in the flesh and peel, effect of germ-tube elongation (bars), and fruit firmness (open squares) in freshly harvested avocado cv. Fuerte fruit maintained at 20°C. Arrows indicate the appearance of decay symptoms.

5. Enhanced lipoxygenase activity by supplying methyl jasmonate resulted in an early decline of diene and symptom development (R. Ardi, I. Kobiler, and D. Prusky, unpublished data).

Treatments with methyl jasmonate at concentrations of 200 µM enhanced the accumulation of responsive LOX in the peel of freshly harvested and treated fruits (Fig. 3). One and two dips with methyl jasmonate enhanced the level of detectable lipoxygenase compared with untreated fruit. The treatment resulted in a fall in the antifungal diene and enhancement of decay development caused by *C. gloeosporioides* (Fig. 3). It is suggested that the increase in lipoxygenase is part of a responsive signal cascade that can be elicited by methyl jasmonate, which is considered to be an intracellular second messenger.

Lacking the possibility of avocado transformation, which could give a direct approach to demonstrate the involvement in lipoxygenase in fruit susceptibility, a indirect approach was checked, in which regulation of LOX can be correlated with changes in the presence of inhibitors (Prusky et al. 1985). It was found that LOX of the avocado peel is affected by an endogenous inhibitor, present in the pericarp of avocado fruit. The inhibitor was identified as epicatechin. This flavan 3 ol competitively inhibited LOX activity and its concentration could fall from 600 µg/g fresh weight of fruit to 20–30 µg/g in ripening fruit. These results suggest that lipoxygenase activity and degradation of the antifungal diene might be regulated in unripe fruit and ripening fruit by the level of epicatechin acting as a trap for free radicals (Fig. 4).

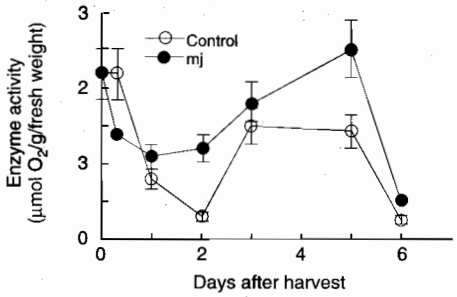

Figure 3. Effect of a methyl jasmonate dip on lipoxygenase activity on peel of freshly harvested cv. Fuerte avocado fruit.
A suspension of 100 mM MJ was used for the treatment, single dip at harvest (mj) (●). The control fruit were dipped in double-distilled water (○). Bars denote standard errors for three replications. Enzyme activity was expressed as µL O_2 consumption/hour/g fresh weight.

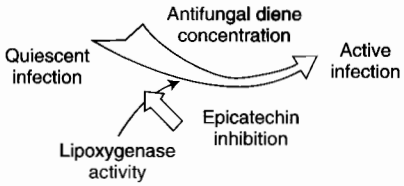

Figure 4. Apparent relationship between diene concentration (as thickness of arrow), lipoxygenase activity, and epicatechin levels during avocado fruit ripening and susceptibility to anthracnose decay.

To further elucidate the role of epicatechin in regulation of this system, two cultivars with differential susceptibility were compared. The concentration of epicatechin in fruit of a highly susceptible cultivar decreased in parallel with an increase in fruit softening during ripening, and symptom expression occurred when the epicatechin concentration was at its lowest level. In a cultivar in which the quiescent infection was activated and expressed symptoms later, the initial concentration of epicatechin was much higher, and considerable epicatechin was still present in the peel of soft, ripe fruit (Fig. 5). Resistance to infection was therefore related to the period required for complete reduction of epicatechin in softening fruit. This suggests that epicatechin may be acting as an inhibitor of lipoxygenase activity and, consequently, is delaying degradation of the antifungal diene.

Regulation of the Level of Preformed Compounds in Avocado Fruit

It is possible to modulate the level of the preformed antifungal compounds by either inducing their increase and/or preventing their decrease. Most of our work has sought to regulate the decrease of the antifungal compounds. Antioxidants and cultivars with differential epicatechin content can influence the decrease of the antifungal diene.

Modulation of the level of antifungal diene is also possible by inducing the diene levels over several days or transiently. Several abiotic treatments, such as wounding, γ-radiation, CO_2 treatment and ethylene, could activate the mechanism. Here we will describe the effect of the exposure of fruits to a stream of 30% CO_2 for 24 hours and to 30 ppm ethylene for 3 hours. These two elicitors differ significantly in their effect on the system.

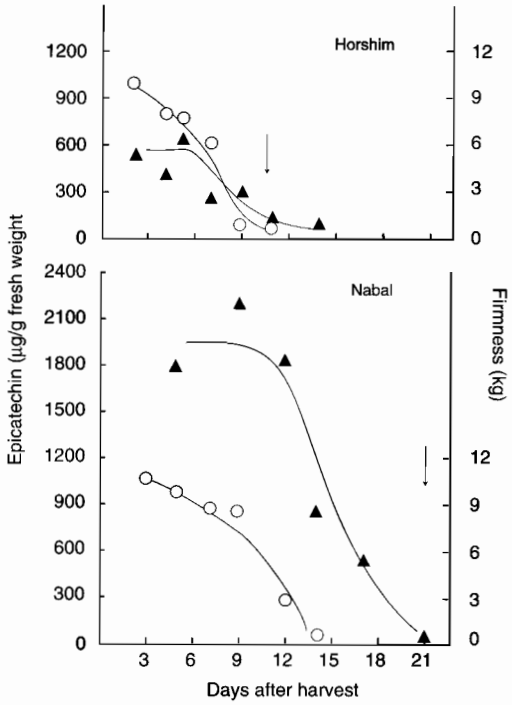

Figure 5. Epicatechin concentration (○) and firmness (▲) of fruit of two avocado cultivars at various days after harvest. Arrows indicate the appearance of decay symptoms caused by *Colletotrichum gloeosporioides*.

In studying the mechanism of elicitation the following questions were asked:

Are CO_2 and ethylene affecting fruit ripening and fruit resistance? When fruits were exposed to 30% CO_2, fruit ripening was not affected but decay development was significantly delayed (Fig. 6). If freshly harvested fruits were exposed to 35 ppm ethylene for 3 hours, no effect on fruit ripening or decay development was observed (Fig. 7). When fruits were exposed to 30% CO_2 for 24 hours a double peak behaviour was observed (Fig. 8). Untreated fruits showed a decrease in concentration of the diene during ripening, while fruits exposed to 30% CO_2 for 24 hours showed a two-peak behaviour. Exposure of avocado fruit to ethylene, showed a single transient peak increase only, followed by behaviour similar to that of untreated fruit for the rest of the ripening

period (Fig. 9). Since the CO_2 treatment has induced fruit resistance and it also induced a second increase of the antifungal diene, it is suggested that this effect is involved in the resistance of the fruit. Quantification of epicatechin levels after CO_2 treatment showed that the level of epicatechin was induced by both treatments (Figs 8–9). CO_2 induced a double peak increase also in the level of epicatechin compared with the decreasing concentration in the control, while in ethylene-treated fruit, only an initial transient increase was observed.

What are the changes affecting lipoxygenase activity? Lipoxygenase can be affected either by the level of its inhibitor or by a direct effect of this treatment at a transcriptional level. Northern blot analysis of RNA obtained at different days after CO_2 treatment showed no significant change in the expression of LOX, suggesting that the different treatments are not affecting the diene by a direct regulation of lipoxyge-

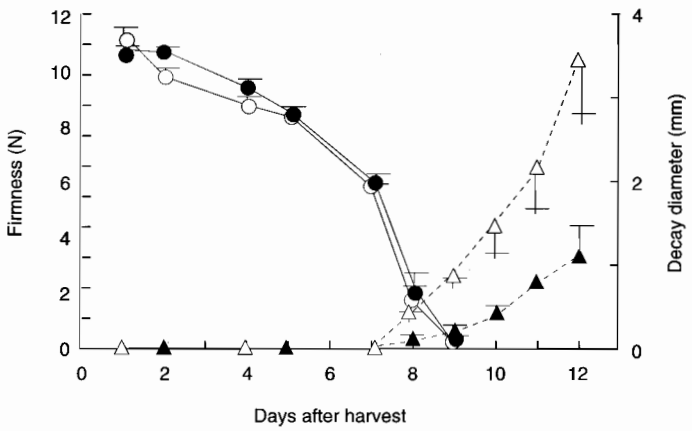

Figure 6. Effect of CO_2 treatment on decay development by *C. gloeosporioides* and fruit ripening. Decay development by *C. gloeosporioides* (△) and the rate of ripening (○) cv. Fuerte avocado fruit. Freshly harvested fruit were exposed to 30% CO_2 for 24 hours (●▲) and compared to untreated fruit (○△). Vertical lines on the graph denote standard errors of the means of six replications for fruit firmness and 60 replicates for decay.

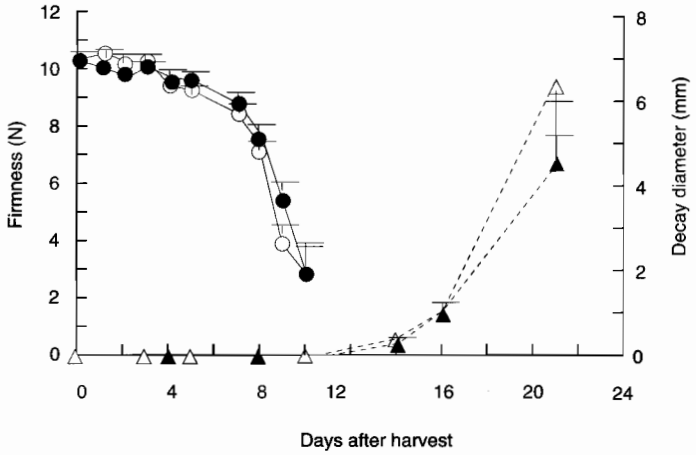

Figure 7. Effect of ethylene on decay development by *C. gloeosporioides* and fruit ripening. Decay development by *C. gloeosporioides* (△) and the rate of ripening (○) in cv. Fuerte avocado fruit. Freshly harvested fruit were exposed to 30 µL/L ethylene for 3 hours (●▲) and compared with untreated fruit (○△). Vertical lines on the graph denote standard errors of the means of six replications for fruit firmness and 60 replicates for decay.

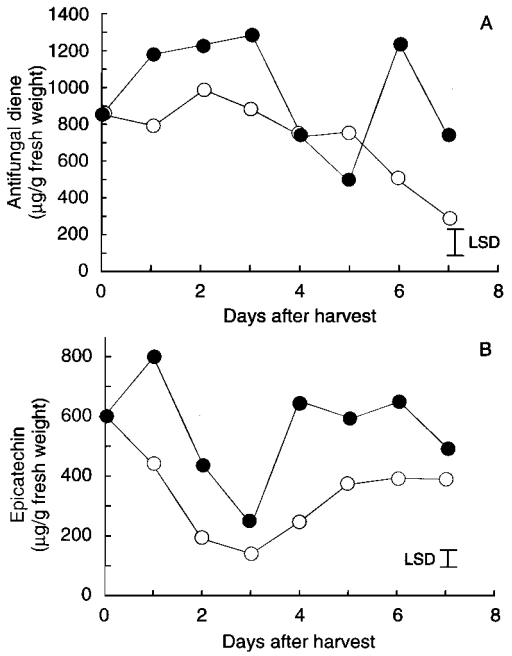

Figure 8. Effect of CO_2 on the levels of the antifungal diene and epicatechin. Levels of the antifungal diene (A) and epicatechin (B) in the peel of cv. Fuerte avocado fruit. Freshly harvested fruit were exposed to a stream of 30% CO_2 for 24 hours (●) and compared with freshly harvested fruit exposed to air (○). The 5% LSD = 150 (A) or 50 (B).

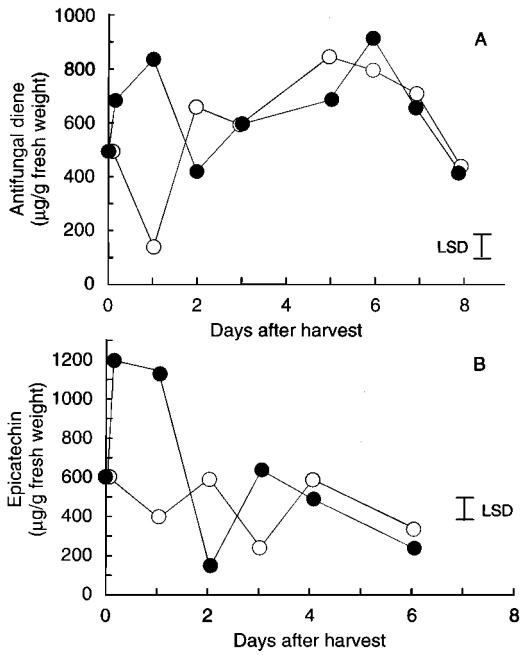

Figure 9. Effect of ethylene on the levels of the antifungal diene and epicatechin. Levels of the antifungal diene (A) and epicatechin (B) in the peel of cv. Fuerte avocado fruit. Freshly harvested cv. Fuerte fruit were exposed to 30 µL/L ethylene for 3 hours (●) and compared with untreated fruit (○). The 5% LSD=100 (A) or 90 (B).

nase but probably by regulating the level of epicatechin.

The level of epicatechin can be affected through its synthesis. The synthesis of epicatechin occurs through the phenylpropanoic pathway. One of the last steps of the biosynthesis of epicatechin is catalysed by the enzyme flavanone-3-hydroxylase (F-3-H). As a result of exposure of avocado fruit to CO_2 treatment, activity of phenylalanine ammonia lyase (PAL), chalcone synthase (CHS) and F-3-H showed a significant increase (Fig. 10). Interestingly, F-3-H the last step for epicatechin synthesis showed a double peak pattern very similar to that shown by the level of epicatechin. When fruit were exposed to ethylene, an initial, usually transient increase was observed for all the enzymes (Fig. 11).

To determine if enzyme activation is regulated at a transcriptional level, several genes were cloned from a cDNA library of cell suspension of avocado fruit. Slot blots showed a significant expression of PAL, CHS, and F-3-H as a result of CO_2, and a significant induction of PAL and F-3-H transcripts as a result of ethylene treatment. These results indicate that both treatments can affect the mechanism of resistance by affecting the level of epicatechin through the activation of genes encoding the biosynthetic pathway at a transcriptional level. The ethylene effect seems to be transient while the effect of the CO_2 seems to be long-lasting enough to affect decay development.

All these results clearly suggest the importance of F-3-H in regulation the mechanism of resistance of avocado fruit to postharvest pathogens.

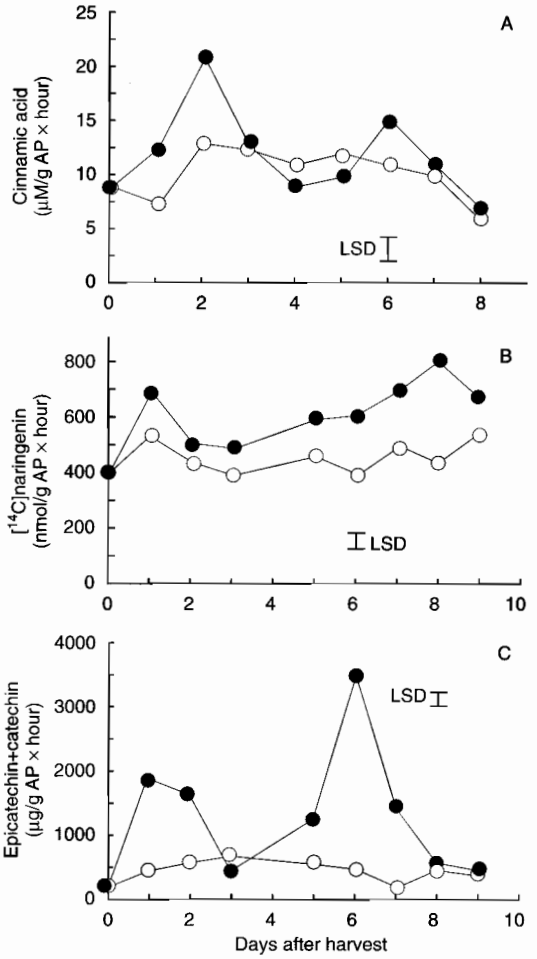

Figure 10. Effect of treatment with CO_2 on the activities of the epicatechin biosynthesis enzymes. Activities of PAL (A), CHS (B), and F-3-H (C) in peel of cv. Fuerte avocado fruit. Freshly harvested fruit were exposed to a stream of 30% CO_2 for 24 hours (●) and compared with control fruit exposed to air (○). The 5% LSD=2 (A), 60 (B) and 150 (C).

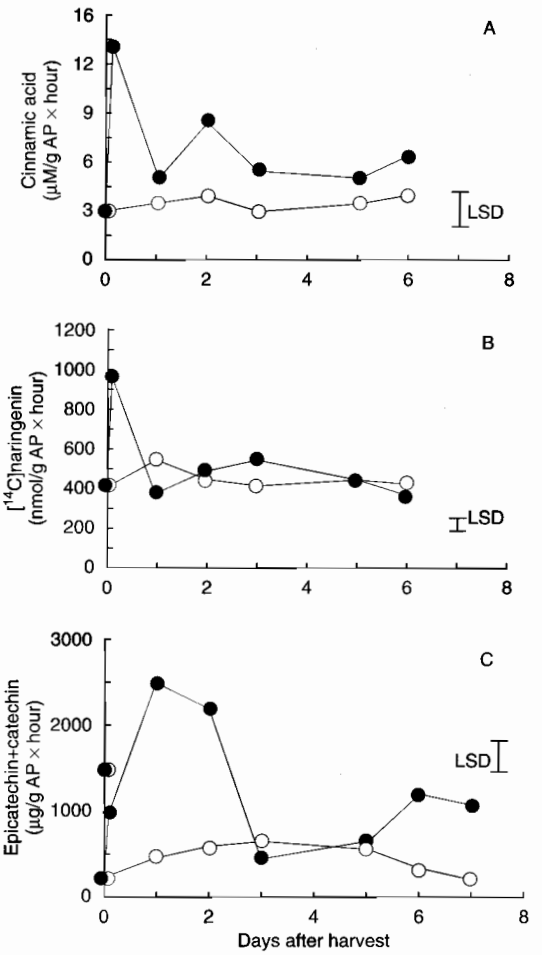

Figure 11. Effect of ethylene on the activities of the epicatechin biosynthesis enzymes. Activities of PAL (A), CHS (B), and F-3-H (C) in peel of cv. Fuerte avocado fruit. Freshly harvested fruit were exposed to 30 µL/L ethylene for 3 hours (●) and compared with untreated fruit (○) The 5% LSD=2 (A), 50 (B), and 300 (C).

Signal Transduction During the Activation of Preformed Antifungal Compounds in Avocado

One of the intriguing aspects of elicitation is to understand how signals such as CO_2 or ethylene are transduced in order to induce a resistant reaction. When membranes isolated from either cell suspension or avocado fruit peel are incubated with cell wall elicitors of *Colletotrichum gloeosporioides*, they show a significant increase in ATPase activity, suggesting an activation of the membrane by the elicitor. Membranes of avocado fruit also showed a significant increase in the activity of NADPH oxidase as a result of elicitor treatment. NADPH oxidase is a membrane enzyme involved in the production of

hydrogen peroxide. Addition of H_2O_2 to avocado peel slices induced PAL activity and a 50% increase in epicatechin level. This clearly suggests that signals are also transduced during the induction process of preformed compound in avocado.

Localisation of Antifungal Compounds in the Mesocarp of Avocado Fruit

A careful localisation study found that the antifungal diene is not evenly distributed in the tissue but is sequestered in specific oil bodies (Kobiler et al. 1994). These oil bodies, named idioblasts, contain about 85% of all the antifungal compound of the flesh and consequently, even if the compound can be extracted, it is not active for fungal attack.

Because of the high amount of antifungal compounds localised inside those cells, they were used as a model system for the study of the biosynthetic pathway of the preformed compounds. Purified cells exposed to 35 ppm ethylene for 3 hours induced a significant increase in diene concentration inside the cells (Fig. 12). Similar effects could be seen in idioblasts isolated from avocado cv. Fuerte and Reed (Fig. 13). The increase in the level of the diene was dependent on the time that the cells were exposed to ethylene.

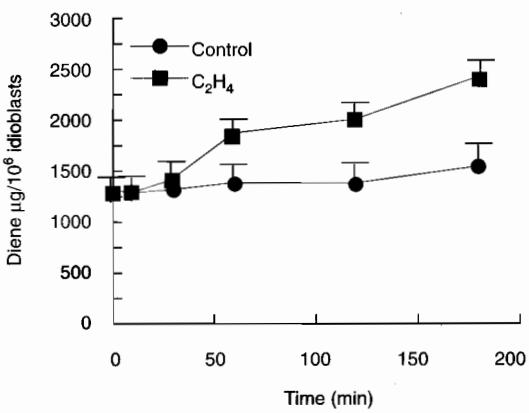

Figure 12. Antifungal diene content in idioblast expossed to ethylene. Idioblast isolated from mesocarp of avocado fruits cv. Fuerte were exposed for different periods to 40 µL/L ethylene at 20°C.

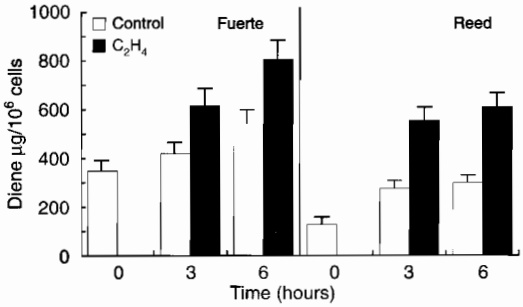

Figure 13. Antifungal diene content in idioblast exposed to ethylene. Idioblast isolated from mesocarp of avocado fruit cv. Fuerte and Reed were exposed for 3 and 6 hours to 40 µL/L ethylene at 20°C.

We believe that idioblast cells might be more important than was thought previously. It is clear that these cells are metabolically sound, they can synthesise the diene and possibly export secondary products one of which is the antifungal diene. It might be that these cells are the main source of antifungal compound and that the compound is transported to the peel where it builds up to fungitoxic concentrations and becomes biologically active.

Summary

It was found that:

- different elicitors can induce higher preformed antifungal diene levels;
- antifungal diene level is modulated by LOX activity;
- LOX activity is regulated by epicatechin level;
- epicatechin level is regulated at the transcriptional level of the phenylpropanoic pathway enzymes;
- F-3-H can serve as a criterion for fruit resistance; and idioblast cells can synthesise the antifungal diene.

These findings suggest that the antifungal diene might be a significant factor in fruit resistance and could be modulated to enhance resistance.

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Suppression of Avocado Anthracnose and Stem-end Rot Pathogens by Endogenous Antifungal Substances and a Surface Inhabiting *Pestalotiopsis* sp.

N.K.B. Adikaram and A. Karunaratne*

Abstract

Anthracnose (*Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc.) and stem-end rot (*Phoma* spp., *Botryodiplodia theobromae* and *Phomopsis* spp.) are recognised as major postharvest diseases in avocado fruit. The former originates from quiescent infections in the immature fruit long before harvest. When isolation of *C. gloeosporioides* from immature fruit skins was attempted, a surface inhabiting fungus, *Pestalotiopsis neglecta*, which apparently suppressed the growth of *C. gloeosporioides* , was often encountered.

The resistance of immature avocados to anthracnose is attributed to endogenous antifungal substances in the fruit peel. Two preformed antifungal compounds were previously reported by a group in Israel. We have characterised three more antifungal compounds from the immature avocado peel: 1,2,4-trihydroxyheptadec-16-yne (1); 1,2,4-trihydroxyheptadec-16-ene (2); and 1-acetoxy-2,4-dihydroxyheptadec-16-ene (3). These five compounds appear to constitute the total antifungal activity of the fruit peel. The most active compound against *C. gloeosporioides* was the diene which was twice as active as the monoene and compound (3). The additive effect of all five compounds was determined to be equivalent to about three times the antifungal activity of diene alone, particularly at the time of harvest. There was a gradual increase in concentration of the five compounds in the peel with increasing fruit maturity, and was highest when the fruit were ready to harvest. In the harvested and stored fruits the concentration declined during ripening, coinciding with the onset of anthracnose lesions.

Pestalotiopsis neglecta, a non-pathogenic, surface inhabitant most commonly encountered on the avocado fruit surface, exhibited strong antagonistic properties in vitro against the major stem-end rot pathogens, *Phoma* spp. Application of conidia of *P. neglecta* on to the stem-end region of harvested avocados preinoculated with *Phoma* sp. delayed stem-end symptom development by 4 days when compared with the control. A similar effect was not observed with *B. theobromae*. *In vitro* testing confirmed that *P. neglecta* was antagonistic to *Phoma* sp. The antagonism appears to be due to a heat-labile extracellular factor. Dipping avocado fruit in a suspension of conidia of *P. neglecta* delayed anthracnose and stem-end rot development.

ANTHRACNOSE disease caused by *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. and the stem-end rot caused by *Phoma* spp., *Botryodiplodia theobromae,* and *Phomopsis* spp. are recognised as major postharvest diseases in avocado fruit. Young fruit are usually free from visible symptoms and characteristic decay lesions in both diseases develop during fruit ripening. The anthracnose disease originates from quiescent infections in the immature fruit long before harvest (Binyamini and Schiffmann-Nadel 1972). In unripe fruit the fungus produced an appressorium and an infection peg which ceased the growth in the cuticle (Coates et al. 1993) before becoming quiescent.

The quiescence of *C. gloeosporioides* was attributed to the presence of substantial preformed antifungal activity in the immature fruit peel (Prusky et al. 1982; Sivanathan and Adikaram 1989a). Prusky et al.

* Department of Botany, University of Peradeniya, Peradeniya, Sri Lanka.

(1982) isolated the major antifungal principle and identified it as 1-acetoxy-2-hydroxy-4-oxo-heneicosa-12, 15-diene (diene). Another compound 1-acetoxy-2,4-dihydroxy-n-heptadec-16-ene (monoene), with lesser antifungal activity, was later reported by the same group (Prusky et al. 1991).

The other hypotheses that have been proposed to explain the quiescence in fruits based on host nutrients and the enzyme potential of the pathogen (Swinburne 1983) are not relevant to the quiescence of *C. gloeosporioides* in avocados (Sivanathan and Adikaram 1989b). Prusky et al. (1989), however, suggested that epicatechin found in the peel of unripe avocados might regulate the activity of endopolygalacturonase of *C. gloeosporioides*, affecting the enzyme potential of the fungus.

We have purified and characterised three more preformed antifungal compounds from the peel tissue of unripe avocados which together with the two previously identified compounds constitute the total antifungal activity of the immature fruit (Adikaram et al. 1992). A non-pathogenic, surface inhabiting fungus, *P. neglecta* (Steyaert 1961), was frequently isolated from immature avocado fruit skin and was found to strongly inhibit pathogens causing anthracnose and stem-end rotting (Weerasinghe and Adikaram 1995). This paper discusses the influence of endogenous antifungal compounds and the surface inhabitant, *P. neglecta*, on anthracnose and stem-end rot development in avocados.

Materials and Methods

Extraction and purification of antifungal compounds from the fruit peel

Immature avocado (*Persea americana* var. Green) fruit were obtained from the trees grown around the campus of the University of Peradeniya. For extraction of antifungal compounds, the outer skins (1–2 mm thick) were peeled, bulked, freeze-dried, and powdered in bulk. The powdered material (479 g) was extracted in hexane, dichloromethane (CH_2Cl_2), and methanol under reflux conditions. Both the hexane (23.1 g) and CH_2Cl_2 (25.2 g) extracts yielded dark green solids and displayed antifungal activity. The dichloromethane extract (25.2 g), which displayed the greatest antifungal activity, was fractionated by column chromatography (silica gel 250 g, Merck art 7734 ASTM). Sequential elution with hexane, ethyl acetate, and methanol gave 80 fractions which were reduced to 12 by pooling on the basis of TLC behaviour (Table 1). The fractions labelled AV(1)–AV(4), corresponding to four different zones of antifungal activity, were distinguished by TLC bioassay. Part of the AV(4) material (fraction 9, R_f 0.42) was further purified by flash chromatography (silica gel, 230–400 mesh, 30 g) with $CHCl_3$–MeOH (97:3) as eluent. Two fractions were obtained and the less polar, more active one was passed through a Sephadex LH20 column ($CHCl_3$–MeOH, 3:7) to remove pigments. A solid was collected and subjected to preparative HPLC on a uBondopack C_{18} column eluted with MeOH–H_2O (7:3) at a flow rate 4 mL/ min (using refractive index detection). Four species were detected: AV(4a)(14% capacity factor, k'3.5), AV(4b)(1%, k'9.3); AV(4c)(68%, k'11.7); and AV(4d)(17%, k'24). Only AV(4a) and AV(4c) showed antifungal activity. Part of the AV(3) material (faction 7, active spot Rf 0.54) was further purified by flash chromatography (column as above, $CHCl_3$ as eluent). Four fractions were obtained all showing some antifungal activity. The most polar fraction was separated by preparative HPLC (as above) into two components AV(3a)(62%, k'9.6) and AV(3b)(38%, k'23.1) of which only the former had antifungal activity (Adikaram et al. 1992). The antifungal compounds were obtained in 95% pure form. Antifungal activity was monitored throughout the purification process by a TLC bioassay (Klarman and Stanford 1968).

Table 1. Distribution of antifungal activity in chromatographic fractions.

Fraction number	Antifungal activity	Rf value
1	–	–
2	–	–
3	+	0.78 AV(1)
4	+	0.60 AV(2)
5	–	–
6	–	0.54 AV(3)
7	+	0.54 AV(3)
8	+	0.42 AV(4)
9	+	0.42 AV(4)
10	+	0.42 AV(4)
11	+	0.42 AV(4)
12	–	–

Source: Adikaram et al. 1992.

Bioassay for antifungal activity

The aliquots of the extracts were spotted on to TLC plates (Kieselgel 254 PF60 Merck, 0.5 mm thick), the plates developed with ethyl acetate and air-dried overnight. The plates were then sprayed with a suspension of conidia of *Cladosporium cladosporioides* or *Colletotrichum gloeosporioides* prepared in Czapek Dox nutrient medium and incubated in a moist chamber for 2–3 days at 26°C. The presence of compounds toxic to the fungus was indicated by a zone characterised by a lack of aerial mycelium (Klarman and Stanford 1968). The antifungal activity was also tested by spore germination assay conducted on material eluted from areas corresponding to inhibition zones (Sivanathan and Adikaram 1989a).

Quantification of antifungal compounds in peel extracts

Quantification of antifungal compounds in the avocado peel at different stages of development was done by TLC bioassay using a dose-response curve obtained from pure compounds. Series of solutions containing different concentrations of individual antifungal compound were prepared and aliquots were spotted on TLC plates. The plates were sprayed with a suspension of conidia of *C. gloeosporioides* or *C. cladosporioides*. The areas of resultant zones of inhibition were measured and dose-response curves were obtained separately for each compound by plotting the concentration against the area of inhibition. The sizes of inhibition areas produced by extracts similarly obtained on TLC plates were measured and the approximate concentrations of the compound were determined by using the calibration curve.

Antagonistic properties of *P. neglecta* isolated from avocado surface

Phoma spp. and *B. theobromae* were isolated from a stem-end rot of ripe avocado and *P. neglecta* from the peel of immature avocado on Cook's No. 2 agar following surface sterilisation with sodium hypochlorite. The fungi were maintained on the same medium. To study the antagonistic effect of *P. neglecta* in vitro on *C. gloeosporioides* and the two stem-end rot pathogens, the fungi were grown individually on agar plates together with *P. neglecta*.

The effect of *P. neglecta* on stem-end rot development was tested by inoculating five sets of immature avocados with: a) *P. neglecta* only; b) *B. theobromae*; c) *Phoma* spp.; d) *B. theobromae* + *P. neglecta*; e) *Phoma* spp. + *P. neglecta*; and another set f) by treatment with distilled water (control). Inoculated fruit were incubated in moist chambers for the first 24 hours and thereafter stored in boxes. In another experiment a set of immature avocado fruit was dipped in a suspension of *P. neglecta* for 10 minutes. Two control sets, one dipped in distilled water and another without any treatment, were maintained (Weerasinghe and Adikaram 1995).

The effect of *P. neglecta* on stem-end rot pathogens was examined by mixing the conidia and allowing them to grow on agar medium. Aliquots (25 µL) from suspensions of conidia of *P. neglecta* and the stem-end rot pathogens were mixed as follows: a) *B. theobromae* + *P. versicolor*; b) *Phoma* spp. + *P. versicolor*; c) *P. versicolor* + water; d) *B. theobromae* + water; and e) *Phoma* spp. + water. These were placed in sterile petri dishes to which 20 mL portions of Cook's No. 2 medium (after cooling to about 45°C) were added. After incubation at 28°C the plates were examined daily.

P. neglecta was grown in Czapek Dox liquid medium (without agar) for 4 days, the culture filtrates obtained were incorporated into the Cook's No. 2 agar medium in petri dishes. Mycelial discs (5 mm diameter) cut from the colonies of *Phoma* spp. or *B. theobromae* were placed on the centre of the medium. Control plates were prepared by adding sterile distilled water instead of culture filtrate. The plates were incubated at 28°C and the colony growth was measured daily. The culture filtrate was also tested for germination of conidia of the two pathogens separately on glass slides. Following a 5-hour incubation period in a moist chamber, the percentage germination was determined.

Results and Discussion

Antifungal activity of the fruit peel

The presence of preformed antifungal substances in the immature avocado peel was demonstrated by first extracting the peel tissue in ethyl acetate and then bioassaying on TLC plates. The extracts produced four distinct areas at Rf 0.30, 0.32, 0.70, and 0.75 (chloroform, methanol, 9:1). The activity was also checked by carrying out spore germination (*C. gloeosporioides*) assays on substances eluted from silica gel scraped from areas corresponding to inhibition zones in another TLC plate (Sivanathan and Adikaram 1989a). The substance eluted from Rf 0.60–0.70 caused 90% inhibition. Inhibition at a

lower region (around Rf 0.30) could not be detected by this method. Inoculation of immature avocado fruit with live or heat-killed conidia of *C. gloeosporioides* did not result in an increase of antifungal activity.

For chemical studies the outer fruit skin was peeled, bulked, and freeze-dried. This material (479 g) was extracted with dichloromethane and chromatographed on silica gel to afford four fractions with antifungal activity (Table 1). These four fractions had R_f values (ethyl acetate) of 0.78, 0.60, 0.54, and 0.42 which were labelled AV(1), AV(2), AV(3), and AV(4), respectively. The fraction AV(2) was separated and purified and the compound was found to be similar to cis-1-acetoxy-2-hydroxy-4-oxo-heneicosa-12,15-diene (1) that had previously been described by Prusky et al. (1982). Also the inhibitory compound in the fraction AV(1) at Rf. 0.78 appeared to be 1-acetoxy-2, 4-dihydroxy-n-heptadec-16-ene (2) later reported by the same group (Prusky et al. 1991).

$CH_3(CH_2)_4CH{=}CHCH_2CH{=}CH(CH_2)_7$
$COCH_2CHOHCH_2OCOCH_3$ (1)

$CH_2{=}CH(CH_2)_{11}CHOHCH_2CHOCH_2OCOCH_3$ (2)

Fraction AV(4), which was further purified by flash chromatography on silica gel, gel permeation chromatography, and finally HPLC on a reverse phase column yielded two antifungal compounds, AV(4a) and AV(4c) in the ratio 1:4. A similar purification of fraction AV(3) afforded only one active compound AV(3a). These three compounds, which were white/off-white crystals, were examined by ^1H NMR spectroscopy, ^1H–^1H correlation spectroscopy and ^{13}C, DEPT spectra, and EI mass spectra and the following structures assigned:

$CH_2{=}CH(CH_2)_{11}CHOHCH_2CHOHCH_2OH$
AV(4a) 1,2,4-trihydroxyheptadec-16-yne (3)

$CH{\equiv}C(CH_2)_{11}CHOHCH_2CHOHCH_2OH$
AV(4c) 1,2,4-trihydroxyheptadex-16-ene (4)

$CH{\equiv}C(CH_2)_{11}CHOHCH_2CHOHCH_2OCOMe$
AV(3a) a-acetoxy-2,4-dihydroxyheptadec-16-yne (5)

At this point a literature survey revealed that compounds of this type had been discovered by Kashman et al. (1969) over 25 years ago in extracts of avocado seed or dried avocado fruit. Their antifungal activity was not recognised but the structures appeared to be firmly established.

We have previously established the presence of the dihydroxyketone (1) in avocado peel and demonstrated that it is the most potent of the fungitoxic fractions which were isolated. The most striking structural feature among the five antifungal compounds is the presence of a trihydroxy fragment which could be present as a precursor to these compounds. If this structural feature is taken to be a five carbon fragment then the whole series of compounds can be represented as (6) or derived species.

$$R{-}CH_2{-}\underset{\underset{OH}{|}}{CH}{-}CH_2{-}\underset{\underset{OH}{|}}{CH}{-}\underset{\underset{OH}{|}}{CH_2} \qquad (6)$$

Toxicity of antifungal compounds

The areas of inhibition of fungal growth caused on TLC plates by different amounts of these compounds were measured and dose-response curves prepared. To compare their toxicities the amount of each compound required to bring about inhibition in a 1 cm^2 area was calculated. These values indicated that the diene (1) which is the most active against *C. cladosporioides* and *C. gloeosporioides* is twice as active as the compounds (2) and (3), the next most active compounds. The compounds (4) and (5) which were similar in level of antifungal activity were least active against the above fungi. Thus, the order of activity of the five compounds is 1>3=2>4=5.

Levels of antifungal compounds at different fruit development stages

The concentrations of these compounds in the peel tissue of fruit at different stages of maturity before harvest and during ripening following harvest were determined. All five compounds were detected in an extract taken from fruit harvested 15 days after anthesis. There was a gradual increase in their concentration with increasing maturity reaching the highest when the fruit were ready to harvest. The compounds had a parallel behaviour in their distribution with respect to depth in the fruit, with a maximum concentration in a layer 5–7 mm below the surface. These strong similarities in general behaviour of the fungitoxic fractions suggested a similar chemical structure and this has now been confirmed by our present results.

Prusky et al. (1982) found that the diene in the peel tissue could, by itself, prevent the growth of *C. gloeosporioides* in the immature avocado fruit. Our studies revealed a significant contribution by the other four antifungal compounds towards fruit resistance. Prusky et al. (1991) have obtained evidence for synergistic activity of the diene and monoene. This is

likely to be the case also for the other three antifungal compounds. The additive effect of all five antifungal compounds would be equivalent to about three times the toxicity of diene alone at the time of harvest. The concentration of the five antifungal compounds declines sharply as the harvested and stored fruit ripens, and at the time of symptom expression the antifungal activity was negligible. These studies further strengthen the hypothesis that preformed antifungal activity of the peel of immature avocados is responsible for quiescent infection of *C. gloeosporioides* and the decline of antifungal activity during ripening enables the pathogen to produce anthracnose lesions.

C. gloeosporioides exhibits diverse cultural and morphological characteristics. About 100 isolates obtained from various parts of the avocado tree were placed in four groups based on their morphological and cultural characteristics. Our preliminary experiments suggested that these isolates vary in their sensitivity to antifungal compounds, some requiring small quantities for inhibition and others requiring up to 60 times the minimal amount. This could be significant as certain isolates could tolerate the antifungal levels existing in fruit peel.

The antagonistic effect of *P. neglecta*

P. neglecta was frequently isolated from immature fruits, flower parts, leaves, and stems of avocado trees grown in the Kandy area of the Central Province of Sri Lanka. The fungus appears to be a natural inhabitant on the fruit surface and no invasion or damage to the unripe or ripe fruit tissue could be detected. Subsequent experiments revealed that *P. neglecta* can suppress the growth of *C. gloeosporioides*, and of *Phoma* sp., the major pathogen causing stem-end rot in Sri Lanka. Another species belonging to the same genus, *P. versicolor*, has been identified as a minor postharvest pathogen in avocado, occasionally encountered in South Africa (Darvas and Kotze 1987).

Application of conidia of *Phoma* sp. alone, or a mixture of conidia of *Phoma* sp. and *B. theobromae*, to the stem-end region of avocado resulted in stem-end rot symptoms equally well within 2–3 days. In the fruit that were inoculated with a mixture of *Phoma* sp. and *P. neglecta*, however, the symptoms appeared about 6–7 days later. This indicates that the presence of *P. neglecta* delays stem-end rotting. The stem-end rot development in fruit inoculated with a mixture of *P. neglecta* and *B. theobromae* was similar to those controls that were inoculated with *B. theobromae* alone, showing that *P. neglecta* can suppress only *Phoma* sp. and not *B. theobromae*. The latter fungus is less important than *Phoma* sp. as a stem-end pathogen, in Sri Lanka.

The effect of *P. neglecta* on the growth of the two stem-end rot pathogens was tested in vitro. When the conidia of both *P. neglecta* and *Phoma* sp. were mixed together and seeded on agar medium only *P. neglecta* grew. However, when *P. neglecta* and *B. theobromae* were similarly seeded together, both the fungi grew on the medium. Further, the culture filtrates of *P. neglecta* could completely inhibit the growth of *Phoma* sp. on Cook's No. 2 medium. This inhibitory effect was not observed in the autoclaved culture filtrate. The inhibitory effect was also observed when the conidia of *Phoma* sp. were allowed to germinate in culture filtrate. These experiments suggest that *P. neglecta* is suppressing the growth of *Phoma* sp. and this antagonistic effect appears to be due to a heat labile factor produced extracellularly.

Immature fruit dipped in a suspension of conidia of *P. neglecta* did not develop stem-end rott or anthracnose lesions for over 9 days when the fruit was fully ripened. The control fruit showed symptoms within a period of six days.

The fact that *P. neglecta* could be isolated frequently from various parts of avocado tree, especially from the fruit surface, indicated that the fungus is a natural inhabitant. The fungus, while not causing any damage, protects the fruit from the two most important postharvest diseases in avocado, a highly desirable situation. The antagonistic effect may be utilised in the reduction of anthracnose and stem-end rot diseases of avocado perhaps by enhancing the natural population of *P. neglecta* on the avocado fruit surface. This may be achieved by creating conditions that favour *P. neglecta* over other surface microflora.

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Constitutive and Induced Resistance of Citrus Fruit against Pathogens

S. Ben-Yehoshua, V. Rodov, and J. Peretz*

Abstract

The mechanisms of resistance of citrus fruit against pathogens were investigated. Oil cavities of citrus flavedo (exocarp) constitutively contain a range of materials, many of which (citral, perillaldehyde, perillalcohol, carveol, citronellal, hexanal etc.) possess antifungal activity. Mechanical wounding releases the contents of the cavities, which may come into contact with penetrating wound pathogen, such as *Penicillium digitatum*. In young mature-green citrus fruit, the complex of constitutive antifungal compounds acts as a first line of defence against pathogens. Depending on the physiological age of the fruit, the materials released from the oil cavity may either inhibit or encourage pathogen development. The effect of several postharvest treatments on citrus decay (growth regulators, ethylene, heat) might be related to modulation of the ageing-associated decline of constitutive antifungal materials.

In addition to the direct effect on the pathogen, the release of the contents of the destroyed oil cavities was shown to act as a primary signal triggering the induced defensive reactions in the flavedo and albedo (mesocarp) of mature-green citrus fruit. The induced resistance mechanisms (phytoalexins accumulation, cell wall reinforcement) could be elicited also by pathogen challenge followed by heat treatment, by biocontrol yeast agents, and by ultraviolet illumination. The accumulation of phytoalexins, scoparone and scopoletin, is a convenient biochemical marker of induced defensive reactions in citrus. Although PR-proteins (chitinases) were detected as constituents of the orange peel, their level was markedly affected by pathogen inoculation and subsequent heat treatment. The evolutionary advantages and practical implications of these mechanisms will be discussed.

THE biological role of the fruit is related, among others, to protection of developing seeds from various abiotic stresses and biotic hazards. Accordingly, the young and mature fruit have a highly active system of natural resistance against pathogens, composed of several preformed and induced defensive mechanisms. With completion of seed development and fruit maturation, disease resistance usually declines, especially during the postharvest period when the fruit is detached from the mother plant (Ben-Yehoshua et al. 1988, 1990). As a result, methods to control postharvest decay of fresh agricultural produce such as synthetic fungicides, cooling, etc. must be employed. These methods are not always environmentally friendly or effective. A better understanding of the endogenous mechanisms of disease resistance may help develop suitable new biological and biotechnological approaches to reducing fruit decay.

In citrus fruit, the green mould disease caused by *Penicillium digitatum* Sacc. is a main factor of postharvest decay. The presence of a peel wound is usually a prerequisite for *Penicillium* invasion (Nadel-Schiffmann and Littauer 1956). Lemons picked green have longer storage life than fruit harvested fully coloured.

* Agricultural Research Organization, The Volcani Center, P.O. Box 6, Bet Dagan 50250, Israel.

Constitutive Mechanisms

One of the constitutive antifungal materials of lemon peel exerting high inhibitory activity against *P. digitatum* is the monoterpene aldehyde citral (3,7-dimethyl-2,6-octadienal), a natural mixture of geranial and neral geometric isomers (Ben-Yehoshua et al. 1992). However, the physiological role of citral in fruit disease resistance is not completely clear, since this material, like all the compounds of citrus essential oils, is not distributed uniformly throughout the fruit surface, but is located inside oil glands (cavities) in the flavedo layer of the peel (Shaw 1977). According to the early findings of Nadel-Schiffmann and Littauer (1956) and by Homma and Arimoto (1988), a fully mature citrus fruit, including lemon, is easily infected by *Penicillium* when oil glands are artificially inoculated.

Although the antifungal activity of citral is well documented in many works (Tripathi et al. 1984; Asthana et al. 1988; Onawunmi 1989), other publications (French et al. 1978) report that it can stimulate, under certain conditions, spore germination of *P. digitatum* and *P. italicum*. Additional evidence is necessary to determine whether citral is capable of inhibiting the pathogen in situ.

Effect of fruit age on decay susceptibility

During storage of non-inoculated fruit, the young mature-green lemon (approx. 6–7 months after anthesis) exhibited significantly lower decay incidence than the older yellow fruit (approx. 10–11 months after anthesis) (Fig. 1). The decay of an old fruit started during the first month of storage while the young lemon at that period had practically no rot. At later stages, the decay percentage of yellow fruit was approximately twice as high as the green (Rodov et al. 1995).

Preformed antifungal compounds of lemon fruit

TLC bioassay revealed presence of several antifungal materials in lemon flavedo (Fig. 2). The largest inhibitory spot on Figure 2 was attributed to citral (3,7-dimethyl-2,6-octadienal) on the basis of GC-MS and ^{1}HNMR analysis (Fig. 3). Other inhibitory spots belonged to preformed antifungal materials of coumarin or furanocoumarin nature, such as 5-geranoxy-7-methoxycoumarin, limettin (5,7-dimethoxycoumarin), and isopimpinellin (5,8-dimethoxy psoralen) (Ben-Yehoshua et al. 1992).

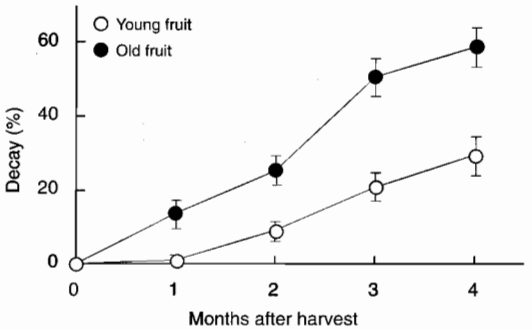


Figure 1. Percentage of fruit infected during storage of young and old noninoculated lemons. Young (mature-green, approximately 6 months after anthesis) and old (yellow, approximately 11 months after anthesis) fruit were harvested at the same time and stored at 17°C and 85% RH. Bars indicate standard errors.

The inhibitory activity of identified compounds towards *P. digitatum* was checked by their effect on spore germination and germ-tube elongation of the pathogen. Based on germ-tube elongation, the median effective dose (ED_{50}) of citral was determined to be 170 ppm, varying in different trials from 100 to 215 ppm. The activity of preformed antifungal materials of coumarin nature was markedly lower: ED_{50} = 1578 and 886 ppm for 5-geranoxy, 7-methoxycoumarin and limettin, respectively. The integral antifungal activity of the flavedo extract from yellow fruit was almost twice as low as that of green fruit (25–38 and 48–57%, respectively) (Kim et al. 1991).

Role of the oil glands in the decay susceptibility of lemon fruit

To determine the involvement of oil-gland content in the fruit–pathogen interaction, young and old lemons were inoculated by injecting spore suspensions of *P. digitatum* either into the oil glands, or into the tissues between the glands (Fig. 4). With yellow fruit, more than 80% of the inoculation sites in the oil glands rotted. However, inoculation of oil glands of young green lemons caused no decay development. No significant difference in disease susceptibility between young and old fruit was observed when inoculation was performed between oil glands. Low decay incidence in these cases could not be attributed to the lack of germination-stimulating materials since 1% orange juice was added to the spore suspension. These findings indicate that the difference in decay susceptibility between young and old fruit may be related to the changes in the composition of oil gland content (Rodov, et al. 1995).

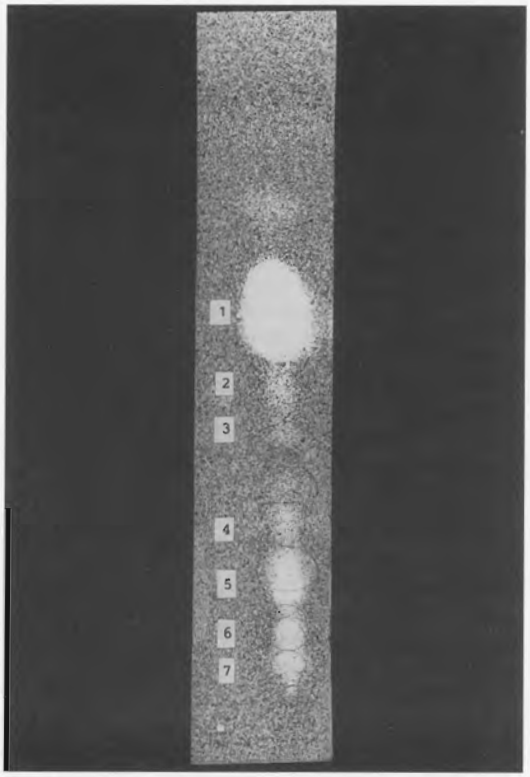


Figure 2. Detection of antifungal compounds in lemon essential oil. The essential oil was separated by thin-layer chromatography (TLC) using n-hexane-ethyl acetate (85:15) as the developing agent. The developed plate was sprayed with a spore suspension of test organism (*Cladosporidium cladosporioides*) and incubated at 24°C and saturated RH for 3 days. The zone of fungal growth inhibition indicates the presence of antifungal compounds: 1, citral; 3,5-geranoxy-7-methoxycoumarin; 4, isopimpinellin; 5, limettin; 6, unidentified psoralen derivative; 2 and 7, unidentified.

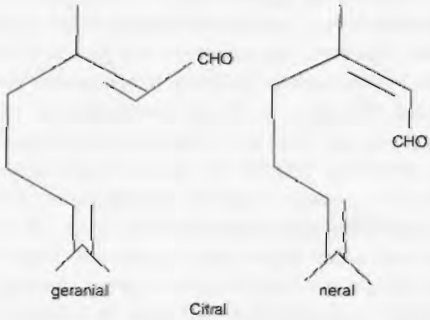


Figure 3. Citral,-3,7-dimethyl-2,6-octadienal

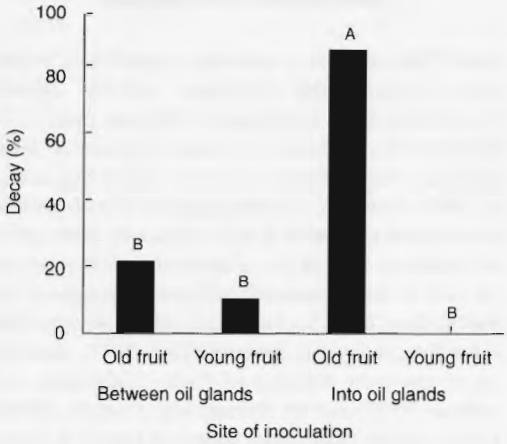


Figure 4. Role of oil glands in the susceptibility of lemon fruit to *Penicillium digitatum*. Ten microlitres of spore suspension (10^6 spores/mL) containing filtered fresh orange juice (1% v/v) were injected in the flavedo either into or between oil glands. The decay percentage was examined 4 days after inoculation. Different letters indicate significantly different values as determined by Duncan's multiple-range test ($p = 0.05$).

Effect of prolonged storage on the constitutive defensive system of lemon

During prolonged storage of lemons, the content of citral declined in parallel with the reduction of antifungal activity in flavedo extracts (Fig. 5) and with the increase in decay incidence. Citral decline was faster in young fruit so that after 3 months in storage the concentration of citral in young fruit was only slightly higher (by about 20%) than in old fruit (Fig. 5). Of two citral isomers, geranial demonstrated faster decrease during fruit storage, as compared to neral (data not shown) (Rodov et al. 1995).

These results suggest that a high level of citral in the oil glands of young lemon is related to its relative resistance to postharvest decay. In inoculation experiments, the compounds located in the oil glands of young lemon were capable of inhibiting the pathogen in situ, while the gland content of old fruit was not active and possibly even stimulated disease development (Nadel-Schiffmann and Littauer 1956).

In nature, penetration of *P. digitatum* is confined, at its early stages, to rind wounds in citrus fruit. Mechanical wounding was shown to cause rupture of citrus oil glands and consequent spillage of essential oil into the intercellular space of sub-epidermal tissue. Considering the high density of oil glands in citrus rind, even small mechanical wounds could injure and rupture

several oil glands. Consequently, the content of essential oil glands may come into contact with a penetrating wound pathogen, such as *P. digitatum*. This suggestion supports Mansfield's (1983) statement that most preformed antifungal materials are effective following pathogen penetration rather than by inhibiting fungal development on the plant surface.

Changes in citral concentration in lemon flavedo corresponded to the level of fruit disease susceptibility. It is accordingly proposed that citral may be one of the oil-gland-localised factors which determines pathogen resistance in lemon. However, the contrast between young and old lemon cannot be attributed only to different citral levels.

Dosage of monoterpenes significantly affected pathogen spore viability. This observation may partly explain the conflicting reports on citral, both inhibiting (Asthana et al. 1988; Onawunmi 1989; Ben-Yehoshua et al. 1992) and stimulating (French et al. 1978) fungal development. A similar concentration-dependent shift from stimulation to inhibition of phytopathogenic fungi has been described for other natural materials such as chlorogenic acid (see Lattanzio et al. 1994). In addition, the stimulative influence of citral and some aliphatic aldehydes on germination of *Penicillium* spores was observed on water agar in the absence of any other organic materials (French et al. 1978) but addition of nutrients caused these aldehydes to be inhibitory. We believe that the latter conditions more closely simulate the situation inside the rind wound of citrus fruit.

The effect of fruit storage on the level of constitutive antifungal compound in Satsuma mandarin was studied by Homma et al. (1989). In the calyx of just-harvested mature fruit, the authors found an antifungal substance, hesperidin (hesperitin-7-rutinoside) which disappeared by the end of the 3-month storage period (Fig. 6). The increase of stem-end rot during prolonged mandarin storage was attributed to a decline in antifungal activity in the fruit extract.

In our experiments, decline in citral concentration was also correlated with an increase in decay of stored lemons.

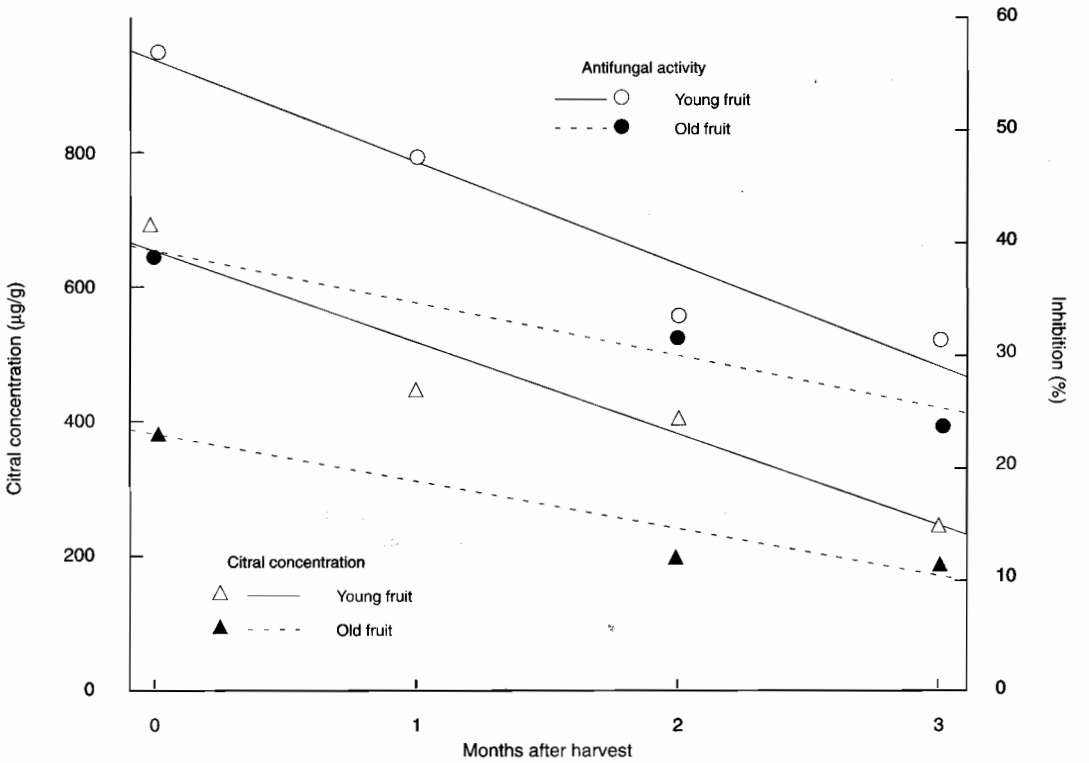


Figure 5. Effect of prolonged fruit storage on citral content (solid line) and antifungal activity (dotted line) in lemon flavedo. Young and old lemon fruit were stored at 17°C and 85% RH. Citral content was measured after different storage periods. The antifungal activity of flavedo extracts was evaluated by inhibition of germ-tube elongation of *Penicillium digitatum*.

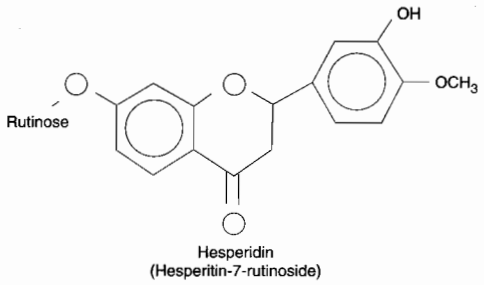


Figure 6. Hesperidin/(Hesperitin-7-rutinoside)

Thus, the shift from relative resistance to high disease susceptibility of citrus fruit after completion of its protective biological function can be related to the changes in the amount and ratio of citral in the oil glands. These changes may be part of the mechanism determining the natural life span of citrus fruit.

Effects of Various Horticultural Practices on Citral and Decay Level

Effect of heat treatment

Heat treatment was shown to drastically reduce decay of citrus fruits (Ben-Yehoshua et al. 1987a,b, 1989a) and to inhibit markedly the decline of the preformed antifungal activity of citrus fruits (Kim et al. 1991). Heat treatment of individually sealed Eureka lemon markedly reduced their decay. After 180 days at 17°C, sealed and heat-treated fruit had only 4.5% decay as compared with 46.5% in non-treated fruit (Fig. 7). The non-treated sealed fruit had 21.0% and non-sealed, heat-treated fruit had 13.0% decay.

The effects of heat treatment in reducing decay were correlated to its effect on inhibiting the decline of the antifungal activity of the crude extract of flavedo tissues of the lemon fruit (Fig. 8). Decline of antifungal activity in non-treated fruit was significantly faster than in sealed and heat-treated fruit. It is interesting that sealing and heating each had an independent effect on delaying the decline.

The decline in resistance against decay of lemon fruit during storage correlated with the reduction of both antifungal activity (Fig. 8) and the citral level (Fig. 7) of the crude extract of the flavedo tissue. Figure 9 shows that heat treatment (three days at 36°C) delayed the decline of the levels of geranial and neral, the two isomers of citral.

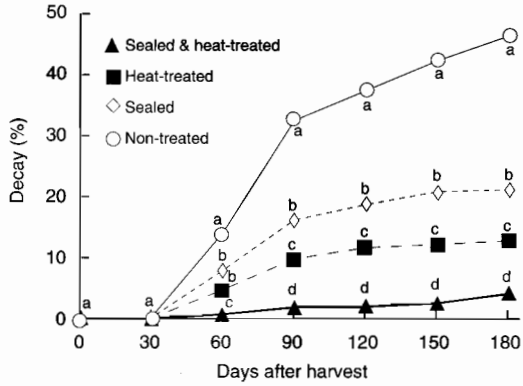


Figure 7. Effect of heat treatment on the percentage of *Penicillium*-infected fruit during storage of Eureka lemon. Fruit were kept at 36°C for 72 hours and afterwards stored at 17°C and 85% RH. The control fruit were kept at 17°C and 85% RH throughout the experiment. A different letter indicates the statistically significant difference between treatments as determined by Duncan's multiple-range test (p=0.05).

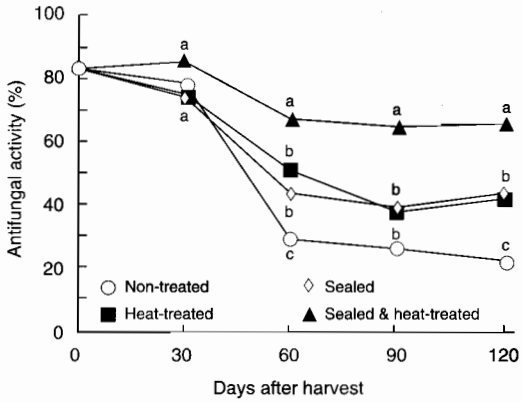


Figure 8. Effect of heat treatment on the antifungal activity on the crude extracts from lemon fruit. This antifungal activity was expressed as percent inhibition of germ tube elongation of *Penicillium digitatum* spores as compared with that of the control treatment containing 0.5% sucrose, 0.5% orange juice, and 5% ethanol. The assay was done in triplicate.

How heat affects citral level is still uninvestigated. Heat shock is known to induce various changes in cell ultrastructural and biochemical functions which lead in certain cases to enhancement of disease resistance (see Nover 1990). The inhibited decline of preformed antifungal materials may contribute to the decay reduction in lemon together with other heat-shock effects such as induced lignification

(Ben-Yehoshua et al. 1988), synthesis of stress proteins (Nover 1990), and direct thermal inhibition of the pathogen. The relative contribution of each of these mechanisms cannot yet be evaluated.

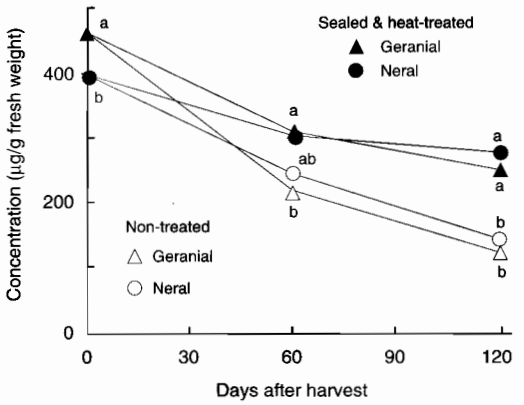


Figure 9. Effect of heat treatment on the changes of content of the two isomers of citral in Eureka lemon fruit.

Effect of various growth regulators: GA and 2,4-D

Postharvest application of gibberellic acid (GA) (in two doses: 50 ppm and 100 ppm) was very effective in delaying the decline of antifungal materials in lemon fruit. In non-treated fruit, the decline of citral concentration (Fig. 10) and antifungal activity of flavedo extract (Fig. 11) began within a month of storage and continued progressively during the entire 3-month storage period. This significant decline of antifungal materials occurred together with a rise in decay development during storage (Fig. 12).

Both GA doses largely prevented the decrease of antifungal activity in the flavedo for 90 days (Fig. 11) and markedly inhibited the decline of citral concentration (Fig. 10). Moreover, during the first 30 days of storage, citral level remained constant in fruit treated with 50 ppm GA and even demonstrated a slight but statistically significant increase in fruit treated with 100 ppm GA. Accordingly, GA applications markedly inhibited lemon decay during storage (Fig. 12).

Fruit dipped in 200 ppm 2,4-D exhibited similar results to fruit treated with 50 ppm GA. Citral content was very similar to that of GA-treated fruit and significantly higher than that of the control (Fig. 10). The antifungal activity of the 2,4-D dipped fruit was again similar to that of the two GA treatments and significantly greater than that of non-treated fruit (Fig. 11). Only at 90 days were both antifungal activity and citral level lower than those of the 100 ppm GA but similar statistically ($p = 0.05$) to the 50 ppm GA treatment. The incidence of decay in 2,4-D treated fruit was similar to the two doses of GA and much lower than those of the control fruit (Fig. 12).

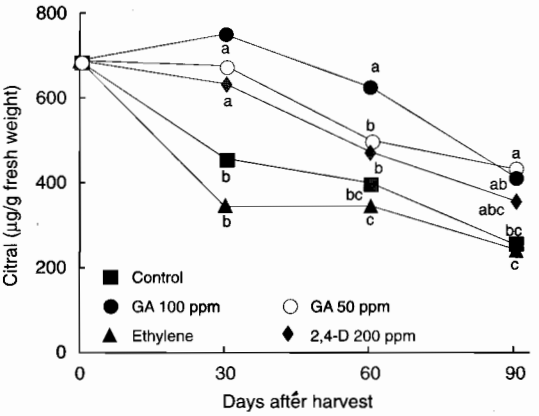


Figure 10. Effect of treatment with GA, 2,4-D, and ethylene on citral content of lemon fruit.

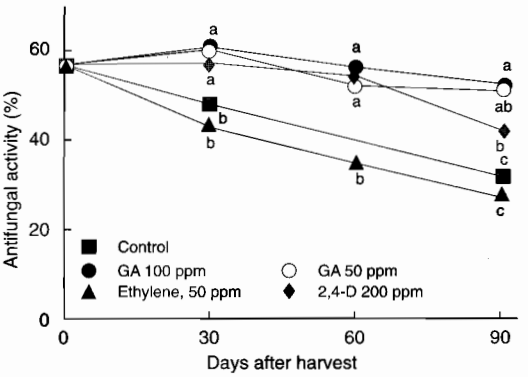


Figure 11. Effect of treatments with GA, 2,4-D and ethylene on antifungal activity of the flavedo of lemon fruit.

Reducing decay of lemon and other citrus fruit by pre- and postharvest applications of GA and 2,4-D is usually attributed to the known effects of these phytohormones on maintaining juvenility and delaying senescence of plant tissues. The inhibitory effect of GA and 2,4-D on the degradation of antifungal materials such as citral provides additional more specific explanation for the mode of action of these phytohormones in decay reduction.

Recent reports have shown that GA application reduced decay in persimmon (Perez et al. 1993) and celery (Afek et al. 1994). Similar to our observations, postharvest GA application on celery delayed the

endogenous decline of the antifungal material marmesin (Afek et al. 1994). However, gibberellic acid, indole-acetic acid or kinetin had either no significant effect or decreased the level of defensive terpenoid aldehydes such as gossypol and hemigossypolone in root-knot nematode-infected cotton plants (Khoshkhoo et al. 1993). Content of terpenoid aldehydes in cotton roots was increased by a foliar spray of salicylic acid or of a mixture of cytokinins (commercial name Burst) showing that different plant species vary in their major antifungal compounds and in their response to phytohormones.

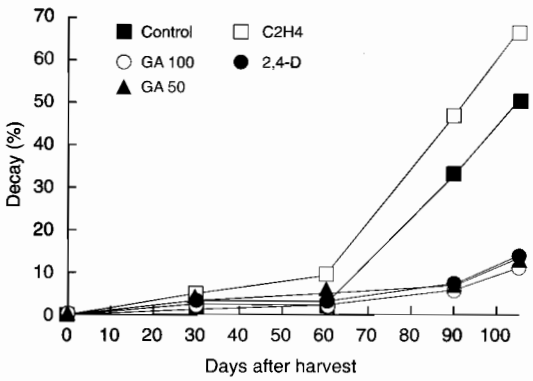


Figure 12. Effect of treatments with GA, 2,4-D, and ethylene on the percentage of infected fruit during storage of Eureka lemon.

Effect of ethylene

Interestingly, the effect of ethylene is just the opposite from that of GA: that is, ethylene treatment (25 ppm for three days) enhanced the decline of citral level in lemon fruit (Fig. 10) and of antifungal activity of crude extract in both lemons (Fig. 11) and grapefruit (Fig. 13). Ethylene application accelerated decay development (Fig. 12) in a significant way. An alternate explanation for decay stimulation by ethylene is its known effect in enhancing senescence of plant tissues. This work suggests a new role for ethylene in accelerating the degradation of the antifungal materials such as citral. Ben-Yehoshua et al. (1990) found that ethylene at temperatures beyond 30°C caused destruction of oil glands in pomelo fruit.

The important findings in this work are the marked effects that various postharvest treatments have on the level of the preformed antifungal materials and decay incidence of lemon fruit. Ethylene degreening accelerates the decline of antifungal materials in the flavedo during fruit storage, while heat treatment, 2,4-D or gibberellin applications markedly inhibit this decline. Accordingly, ethylene enhances while heat, 2,4-D, or gibberellin reduce decay incidence of the fruit during storage. The effect of these postharvest treatments on citrus decay may be related to the modulation of endogenous disease resistance of fruit by influencing the changes of preformed antifungal materials.

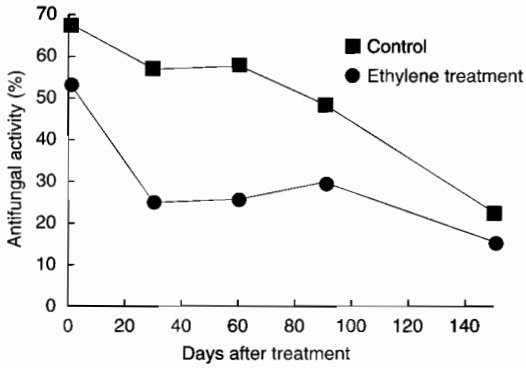


Figure 13. Effect of ethylene degreening treatment (3 days, 25 ppm) on the antifungal activity of the flavedo of grapefruit.

Brown and Lee (1993) reported that ethylene degreening treatment stimulated the stem-end rot of Valencia orange fruit caused by the pathogen *Diplodia natalensis* Pole-Evans. These authors could not explain their observations by the direct ethylene influence on the pathogen. Our data regarding the effect of ethylene on the level of constitutive antifungal materials in citrus fruit provide an alternative explanation.

Although our data consistently show that ethylene treatment enhances decay of citrus fruit, the possibility exists that applying ethylene in certain cases may reduce disease by inducing the synthesis of various enzymes related to plant defence from pathogens (Ecker and Davis 1987; Yoshikawa et al. 1990).

Recently, Flaishman and Kolattukudy (1994) proposed that fungal pathogens have a mechanism to use ethylene, the host's ripening hormone, as a signal to initiate germination, multiple appressorium formation, and infection.

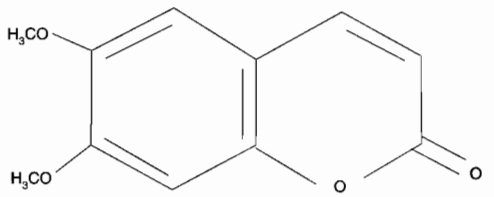
In many postharvest fruit diseases, fungi remain latent until the fruit ripens. How the fungus times its infection at ripening of the host is not known. Exposure of the spores of *Colletotrichum* fungi to ethylene, the host's ripening hormone, at ≤1 μL/L, caused germination, branching of the germ tube, and formation of up to six appressoria from a single spore. The ethylene effect on the fungi was inhibited by silver

ion and 2,5-norbornadiene; the inhibition by the latter could be reversed by higher ethylene concentrations. These results strongly suggest that these fungi must have co-evolved to develop a mechanism to use the host's ripening hormone as a signal to differentiate into multiple infection process. However, Prusky et al. (1996) contend that ethylene does not activate lesion development by *C. gloeosporioides*. They claim that the fungal invasion is inhibited by the constitutive antifungal diene compound even when high concentrations of ethylene are present.

Induced Mechanisms

Scoparone, the phytoalexin of citrus fruit

Scoparone (Fig. 14) is the most widely-studied phytoalexin of citrus fruit (Afek et al. 1986; Arimoto et al. 1986; Ben-Yehoshua, et al. 1992; Kim et al. 1991). Other inducible antifungal stress metabolites were also observed in citrus fruit. Rodov et al. (1994) showed that scopoletin was induced by the biological control antagonist *Pichia guilliermondii* yeast even before scorapone was induced. Dubery et al. (1988) reported the presence of 4-(3 methyl-2 butenoxy) isonitroacetofenone in the damaged peel of α-irradiated orange and lemon. Recently, Stange et al. (1991) found two materials with antifungal activity in extracts of healed tissues of citrus fruit. They identified one as a prenylated coumaral, 3-[4-hydroxy-3-(3-methyl-2-butenyl)phenyl]-2-(*E*)-propanal.



Scoparone

Figure 14. The structure of scoparone, 6,7-dimethoxycoumarin.

According to Kim et al. (1991), the median effective dose of scoparone against germ-tube elongation of *P. digitatum* is 29 μg/mL. Comparing the values of median effective doses one could see the prevalence in the antifungal activity of the induced compound over the preformed ones. That is, the 'weapons' developed during an enemy attack are much more effective than those available for defence in 'peace time'. However, since the flavedo has significant amounts of these preformed antifungal materials, their importance as first line defence cannot be ignored. In fact, the endogenous decline in the level of these antifungal materials is the most reasonable explanation for the increase in the sensitivity of fruit to pathogen attack during its senescence on or off the tree (Ben-Yehoshua 1989b).

Scoparone level in various citrus species

Since scoparone was demonstrated as the phytoalexin of citrus plant, a survey was made of the level of its accumulation in various citrus species (Table 1).

Table 1. Effect of the combined inoculation and heat treatment or UV-illumination on scoparone level (mg/g of fresh weight of flavedo) in citrus fruits.[a]

Species	Control	Inoculation and Heat Treatment[b]	UV Illumination[c]
Lemon, 'Eureka'	0	178 ± 7.3	247 ± 19.3
Grapefruit, 'Marsh'	0	114 ± 7.3	10.3 ± 2.3
Orange, 'Shamouti'	4.6 ± 0.2	1676 ± 97.9	130 ± 15.6
Orange, 'Valencia'	3.8 ± 0.4	848 ± 56.7	162 ± 38.9
Lime, 'Tahiti'	Trace	545 ± 65.1	23 ± 5.2
Kumquat, 'Nagami'	2.1 ± 0.8	101 ± 5.3	418 ± 50
Pomelo, 'Goliath'	Not detected	Not detected	Not detected

[a] Result was obtained 10 days after inoculation or UV illumination. Means ± S.E.
[b] Fruits were heat-treated at 36°C for 3 days 24 hours after inoculation with *Penicillium digitatum*.
[c] UV dose: 4.5×10^3 J/m^2.

Different citrus fruits varied greatly in their capacity to produce scoparone. The occurrence of scoparone in just-harvested oranges or its appearance after several days of storage has been reported by Tatum and Berry (1977). However, quantities of this substance observed in such cases were insufficient to bring about the significant antifungal activity of

scoparone, considering its median effective dose. Grapefruit was distinguished by the relatively low level of scoparone production. The fact that pomelo, which is one of the three parents of citrus fruit in general and specifically of grapefruit, was found to lack scoparone is very interesting. Possibly, pomelo evolution did not reach the stage of scoparone biogenesis. However, the many antifungal compounds present in pomelo as constitutive materials, give this fruit adequate protection.

The reaction of different fruits to two kinds of stresses—combined inoculation and heat treatment, and UV-illumination—is shown in Table 1. Thus, lemon produced similar amounts of scoparone responding to either of the two stresses. With lime, orange, and grapefruit, however, the former was much more effective in eliciting scoparone on the stressed site. Only with kumquat was the effectiveness of these two treatments inversely related, thus stressing the genealogical difference between kumquat (genus *Fortunella*) and genuine *Citrus* species.

UV-induced scoparone, lignification and decay reduction

Accumulation of scoparone in UV-illuminated lemon, kumquat, and orange was accompanied by a decrease in susceptibility to decay (Ben-Yehoshua et al. 1992; Rodov et al. 1992). Figure 15 demonstrates the relation between decay susceptibility of UV-treated lemons and the level of scoparone in their flavedo on the day of inoculation. Fruit inoculated two days after illumination displayed the lowest decay percentage, although scoparone content at that period was far below its maximal level. Nevertheless, the content was comparable to the median effective dose of scoparone and, furthermore, increased rapidly during the period of pathogen development. Although an effective inhibitory level of scoparone was established in the tissues, the lack of a greater effect in reducing decay may be related to the gradual development of UV-damage described below. This damage, overt and possibly also covert, weakened the lemon so that the fruit succumbed to the pathogen.

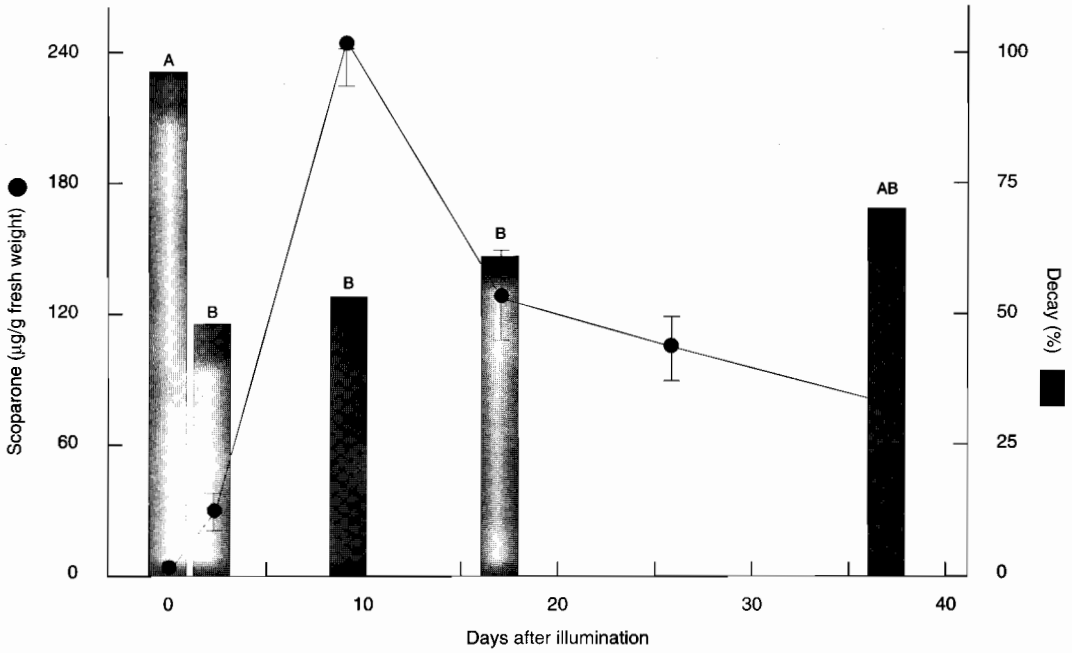


Figure 15. Effect of UV-induced scoparone accumulation on decay percentage of penicillium-inoculated lemon fruit. Fruit was illuminated with UV dose of 5×10^{-3}J/m^2 , stored and inoculated with *Penicillium digitatum* spore suspension (10^{-6} spores/mL) 2 days after UV-treatment. Scoparone was measured fluorimetrically. The position of histograms indicates the day of inoculation. Decay percentage determined 6 days after inoculation. Values separated by Duncan's multiple range test (p = 0.01).

Abiotic stress like UV-illumination may act as non-specific elicitor simulating in tissue the effect of pathogen challenge. Heat treatment by itself does not induce scoparone production, but markedly enhances the elicitation induced by the pathogen, and inhibits the degradation of the preformed antifungal materials (Ben-Yehoshua et al. 1995). Wounding and sealing induces the activity of the enzyme phenylalanine ammonia-lyase (PAL, E.C.4.3.1.5.) in the peel (Fig. 16). Furthermore, the extent of this enzyme activity was much greater at 30°C than at 20°C, and almost no activity was recorded at 10°C. PAL is known as a key enzyme in formation of lignin precursors and of several phenolic compounds that may be related to endogenous antifungal substances (Ben-Yehoshua et al. 1988; Brown and Barmore1983). Stange et al. (1993) suggest that wound gum, not lignin, is deposited in infection resistant injuries of citrus peel. Other above-mentioned defensive mechanisms: synthesis of lignin-like materials and PR proteins (Hahlbrock and Scheel 1987), as well as direct thermal inhibition of the pathogen (Ben-Yehoshua et al. 1987a,b), may also participate in decay-reducing effect of abiotic stresses.

Our results indicate that the postharvest treatments compared in this work have different relations to the natural disease resistance of the fruit. Such factors as UV-illumination or yeast biocontrol agent demonstrated high eliciting capacities even in the absence of the pathogen. Fosetyl-Al (Aliette), known to induce resistance of fruit against pathogens was demonstrated also to induce scoparone production in citrus (Ben-Yehoshua et al. 1989a; Ali et al. 1991).

Heat treatment and UV-illumination in decay prevention

The inhibition of decay development by both heat treatment and UV illumination are important developments encouraging the possibility of reducing exogenous fungicide residues by inducing the fruit to build its own defence against pathogens.

Heat treatment (combined with seal-packaging) was already demonstrated to enable export of pomelo free of toxic residues to any market in the world (Ben-Yehoshua et al. 1987a,b). Accordingly, we recently found that a similar result to the 72 hour curing at 36°C could be obtained by 2 to 3 minutes at 53°C (Rodov et al. 1995).

Disease reduction by UV treatment was evidently related to the induced internal defence mechanisms rather than to the germicidal effect of ultraviolet illumination. Decay was lowered by treating the fruit before inoculation, i.e. without direct contact of pathogen with UV light. Moreover, illumination of previously inoculated fruit failed to prevent their decay. The final result of fruit–pathogen interaction depended on the relative rates of fungal growth and resistance development. The inoculation of fruit before UV treatment gave adequate advantage to the pathogen, while fruit illuminated two days before inoculation, was capable of resisting infection.

Stevens et al. (1991) and Droby et al. (1991) described certain reduction of green mould decay in UV-treated citrus fruits accompanied with PAL enzyme activation.

UV illumination, beyond a certain threshold dose, exerted visible damage to the peel of citrus fruit. Only the flavedo tissues showed damage that appeared as brown or bronze-tan coloration (Fig. 17). Additionally, UV induced a more shiny appearance on the surface of fruit and the peel seemed to become firmer. These changes may relate to a possible induction of a lignification-like processes.

Illumination with UV appears to be an easily-applied technology. UV damage, however, is a factor which seriously effects the possible commercial utilisation of UV illumination as a new decay control. Further research is needed to arrive at optimisation of fruit treatment and storage conditions to enhance positive UV effects and diminish undesirable ones.

PR proteins and fruit resistance against pathogens

Proteins induced in the plant by pathogen attack, the pathogenesis-related (PR) proteins have been grouped into 11 families by Van Loon et al. (1994). Current PR protein families include chitinases, β -1,3-glucanases, peroxidases, proteinase inhibitors, ribonuclease-like proteins, and thaumatin-like proteins.

The chitinases and β-1,3-glucanases exhibit antifungal activity by attacking the chitin and β -1,3-glucan components in fungal cell walls. Proteins are more amenable to genetic manipulation and study because they are direct products of gene expression.

Mauch et al. (1988) isolated a chitinase and β -1,3-glucanase which separately inhibited only *Fusarium solani* f.sp. *pisi*. However, when they were combined, the growth of eight different fungi was inhibited. These experiments demonstrated that chitinases and β -1,3-glucanases act synergistically and are more effective fungal inhibitors together than either enzyme individually.

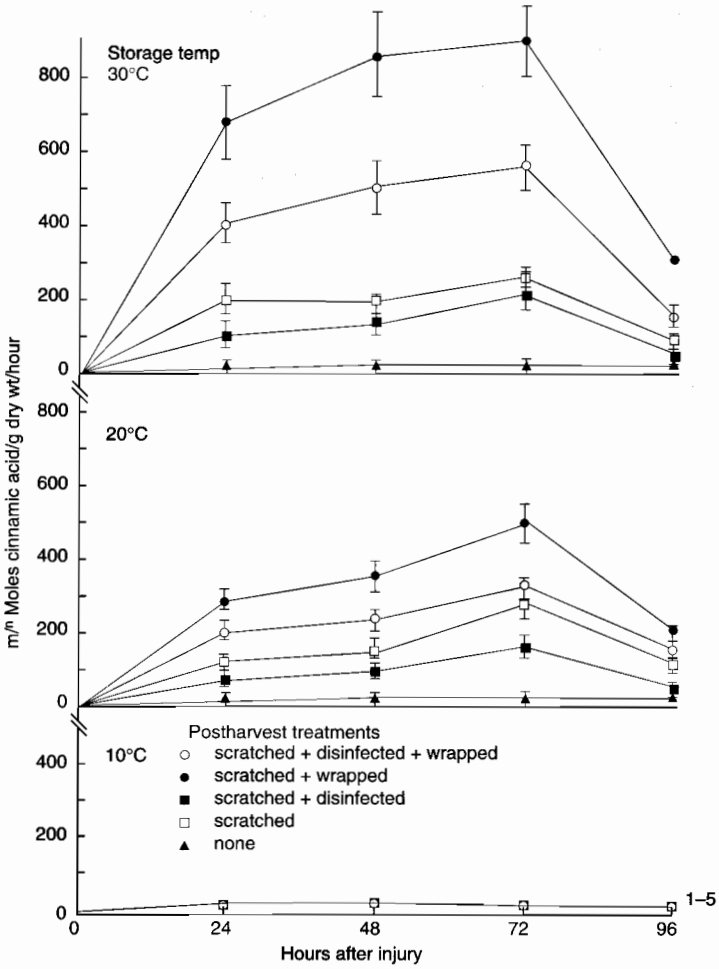


Figure 16. Effects of postharvest treatment and storage temperature on phenylalanine ammonia-lyase enzyme activity in intact or scratched flavedo of 'Marsh Seedless' grapefruit.

Chitinases, chitosanases, and β-1,3-glucanases have been isolated and partially characterised in citrus calls cultures (Gentile et al. 1992; Niedz et al. 1994; Osswald et al. 1994). Multiple forms of each enzyme were identified. It was known which forms, if any, were induced by pathogen activities. These enzymes have also been studied at the USDA citrus research laboratory in Orlando, Florida. Tissue cultures of sweet orange embryogenic callus and suspension cells can be induced to produce high levels of chitinase and β -1,3-glucanase by altering the carbon source in the culture medium (Niedz et al. 1994). To determine if these enzymes inhibit disease-causing fungi, *Penicillium digitatum* was inoculated onto media containing either sucrose or glycerol as the carbon source. Medium that contained sucrose-induced high levels of chitinase and β -1,3-glucanase in the callus, which remained undifferentiated. Medium that contained glycerol repressed the production of these enzymes in the callus, which differentiated into embryos. Fungal growth was significantly inhibited by the callus producing high levels of enzyme for up to 6 weeks. Chitinases and β-1,3-glucanases have been identified in the roots, leaves, and flavedo of 'Marsh' grapefruit, and in the leaves and blossoms of navel orange. These enzymes might be useful in developing new economical and environmentally friendly disease control measures (Niedz et al. 1994).

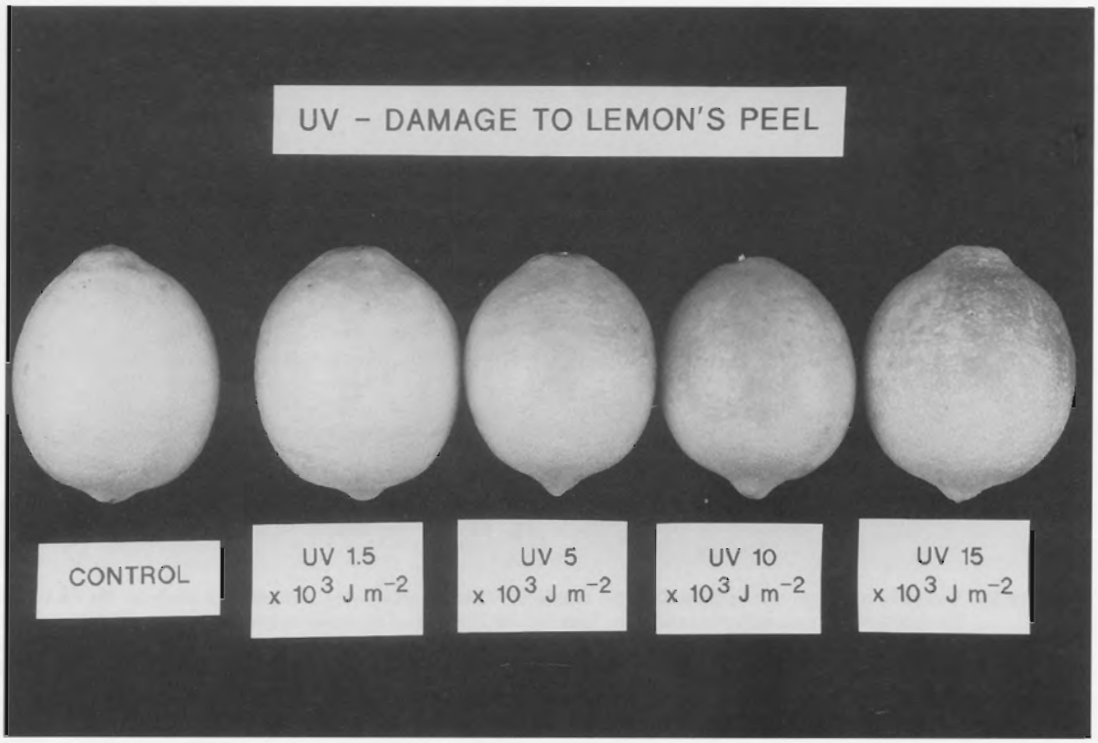


Figure 17. UV-induced damage to lemon peel. Light-green fruit were illuminated in the vertical position, stem end facing the UV source, at a distance of 25 cm. Control: nonilluminated fruit.

Through immunoblot analysis of the albedo of non-inoculated and non-treated Valencia oranges, we found (Fig. 18) two polypeptides of apparent molecular masses of 25 and 27 kDa, reacting positively to antibodies specific for the PR-protein chitinase from citrus callus cultures (Rodov et al. 1997). Earlier, Gentile et al. (1992) described four chitinase isozymes in the callus cultures of Tarocco orange, with apparent molecular masses of 23, 27, 31 and 33 kDa. No positive reaction was found in the orange albedo with antibodies specific for chitinase and for β-1,3-glucanase from tobacco.

During storage of the non-treated oranges, the amount of the two tentative chitinase polypeptides tended to decline, especially of that with lower molecular weight (Fig. 18). The heat treatment, not combined with inoculation, did not prevent this decline and even seemed to enhance this trend.

The inoculation caused rapid disappearance of the chitinase polypeptides, so that they had disappeared from the albedo within one day of inoculation (Fig. 18). However, the accumulation of the polypeptide with apparent molecular mass of 27 kDa was restored by heat treatment of the inoculated fruit which caused the appearance of the pathogen-resistant zones in the albedo. Moreover, the level of this polypeptide in the resistant zones seemed to increase with time and clearly exceeded that in the non-elicited fruit (Fig. 18)

Injection of a preparation of chitin fragments (2 to 15 glucosamine units) into the albedo of Valencia orange also elicited the accumulation of scoparone in the tissue although in lower amounts than the combined inoculation with viable pathogen and subsequent heat treatment. The level of scoparone accumulation was linearly related to the concentration of chitin fragments (Fig. 19).

The elicitation of scoparone by exogenously administered chitin fragments, observed in our work, may hint at a relationship between the two defence

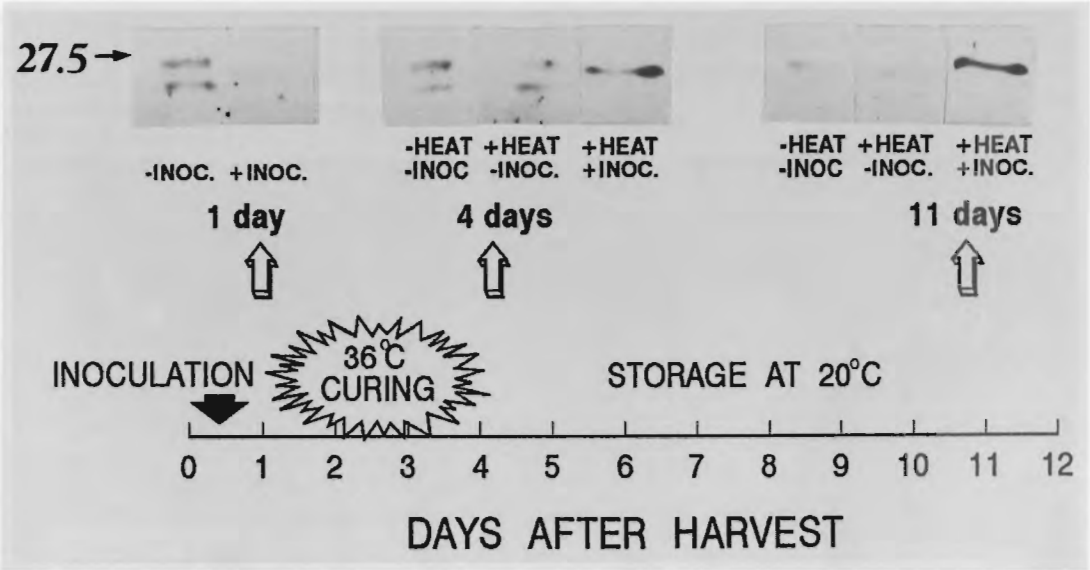


Figure 18. Detection of PR-protein (chitinase) in the albedo of Valencia oranges by Western blot analysis. On the day of harvest, the fruit were either inoculated by injecting the *Penicillium digitatum* spores into albedo (+INOC.), or left noninoculated (-INOC.). One day later, half of the fruit from each group were subjected to curing at 36°C for 72 hours (+HEAT), while another half were left untreated (-HEAT). The fruit were not sampled because of the rapid decay development.

mechanisms—chitinase and phytoalexins—similar to that reported by Yoshikawa et al. (1990). According to these authors, the activity of PR-protein (β -1,3-endoglucanase in their case) releases elicitor molecules from fungal cell walls and these, in turn, induce the production of phytoalexin exerting an inhibitory effect on the pathogen (Yoshikawa et al. 1990). On the other hand, citrus PR-proteins were shown to exert, in vitro, a direct antifungal effect on the pathogens, *P. digitatum* (Niedz et al. 1994), and *Phoma tracheiphila* (Gentile et al. 1992).

These results show that defensive mechanisms such as production of phytoalexins, reinforcement of cell walls, and PR-proteins can be elicited in citrus and are sufficient to inhibit the pathogen development in the inoculated fruit. Our findings confirm the thesis that the potential for induction of defensive mechanisms is present even in highly disease-susceptible objects, and that resistance is determined by the speed, magnitude and timing of defence response, as influenced by the environment (Kuc 1991).

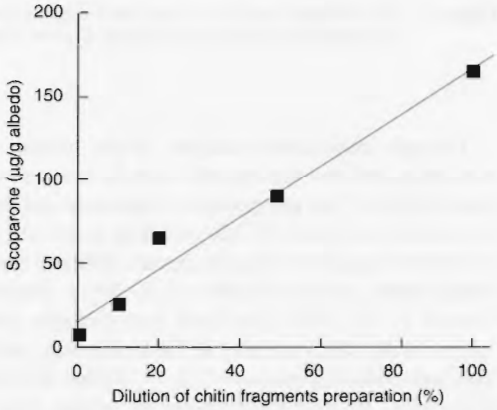


Figure 19. Accumulation of scoparone in the albedo of Shamouti oranges treated with chitin fragments. The preparation of chitin fragments (2 to 15 glucosamine units) was diluted in different rations with sterile water before injection into the albedo. Abscissa: 0 – sterile water, 100 – nondiluted preparation. The fruit was heat-treated at 36°C for 72 hours (curing). Samples for scoparone analysis were taken 10 days after injection.

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Resistance of Banana Fruit to Fungal Disease: an Overview

C. Abayasekara, S. Ratnayake, and N.K.B. Adikaram*

Abstract

Resistance of banana fruit to fungal infection appears to be accompanied by a multicomponent defence response. Preformed fungistatic substances have been reported from green Cavendish banana peel but their presence or the toxicity has not been related to the fruit resistance. At least five phytoalexins accumulate in the necrotic spots beneath the inoculum droplets of *Colletotrichum musae.* These compounds diminished as the fruit ripened. Two of these compounds have been identified as 2-(4'-hydroxyphenyl)-naphthalic anhydride and 2-benzimidazole carbamate. Another recent report described six phenalenone-type 'phytoalexins', named irenolone, emenolone, and musanolones C-F, the antifungal properties of which have not, however, been clearly stated.Since understanding of the biology of banana phytoalexins, particularly their significance in fruit resistance, appears to be incomplete, a detailed investigation was made of phytoalexin accumulation and other defence responses associated with freckle disease in banana.

Immature banana fruit var. 'Embul' (Mysore, group AAB) accumulate at least five phytoalexins in response to infection by *Phyllosticta musarum*, the cause of pin-head sized, restricted, and isolated spots—referred to as freckles—in the fruit peel. Two of these were purified and found to be new phytoalexins, one apparently belonging to the phenalenone group. Irenolone was also isolated but did not show any antifungal activity. Freckle-infected fruits continued to show antifungal activity during ripening. The incidence of anthracnose disease was observed to be significantly less in freckle-infected fruits than those without freckles. A negative correlation was observed between the density of freckle infections and the severity of anthracnose lesions that develop during fruit ripening.

Tissue extraction followed by polyacrylamide gel electrophoresis showed the presence of at least four pathogenesis-related (PR) proteins in the freckled tissue. In addition, freckling increased the PAL activity and resulted in additional peroxidase isozymes. Freckle infections were also associated with lignification and suberisation of the host cell walls at the infection sites. The possibility of development of a strain of the freckle fungus or an elicitor capable of inducing similar defence response without cellular damage is being investigated with the aim of protecting bananas from anthracnose disease.

BANANA, one of the most extensively marketed fruits in the world, comes second only to apples in consumer demand. The world trade in bananas is centred on fresh fruit of the Cavendish group. Bananas are attacked by over 20 fungal pathogens. Except for a few, all the others are capable of infecting only the ripe bananas. Anthracnose (*Colletotrichum musae*) is by far the most important disease in almost every banana-growing region and accounts for most of the postharvest losses in ripe bananas. The fungus infects the fruit at a very early stage of maturity and remains quiescent until ripening, when black lesions progressively develop. Crown and stalk rot caused by *Fusarium pallidoroseum* (Cooke) Sacc., *C. musae* (Berk. & Curt) v. Arx, *Verticillium theobromae* (Turc.) Mason & Hughes, *Botryodiplodia theobromae* Pat., *C. gloeosporioides* (Penz.) Sacc., *Ceratocystis paradoxa* (Dade) Moreau, is another destructive disease that originates as fungal colonisation at the exposed crown tissue of dehanded boxed bananas. Finger rot (*B. theobromae* Pat.), Dothiorella rot (*Dothiorella*

* Department of Botany, University of Peradeniya, Peradeniya, Sri Lanka.

gregaria), pitting disease [*Magnaporthe grisea* (Herbert) Barr], and cigar-end rot [*V. theobromae* (Turc.) Mason & Hughes] are considered less important. The freckle disease (*Phyllosticta musarum*) and speckle (*Deightoniella torulosa*) affect mainly the market quality of the fruit. Sigatoka disease (*Mycosporella musicola, M. fijiensis*) is a potentially dangerous disease that has not been reported yet in certain major banana growing regions (Snowdon 1990).

Matern and Kneusel (1988) proposed that plant defence occurred in two stages. The first involved rapid accumulation of phenols at the infection site to slow (or stop) invasion and allow activation of secondary strategies that would more thoroughly restrict the pathogen. Secondary responses included the *de novo* synthesis of phytoalexins or other stress-related substances. The defence events could include, in order, the host cell death and necrosis, accumulation of toxic phenols, modification of cell walls by phenolic substituents or physical barriers such as appositions of papillae and, finally, the synthesis of specific antibiotics such as phytoalexins (Nicholson and Hammerschmidt 1992). Signals for activation of various defences are thought to be initiated in response to recognition of pathogen avirulence determinants (elicitors) by plant receptors (Dixon and Harrison 1994). In addition, plants defend themselves from pathogen infection through a wide variety of mechanisms (Dixon 1986), which induce the synthesis of pathogenesis-related proteins, structural defence barriers such as lignin and suberin, and the presence of tannins in high concentrations. In most cases, the extent to which these various systems affect disease progression is poorly understood, and is an area of extensive biochemical and genetic research.

Resistance of Immature Bananas to *Colletotrichum musae*

Anthracnose of bananas, caused by *C. musae*, develops as quiescent infections in uninjured green fruit. The fungus penetrates under the cuticle and remains quiescent there until the fruit ripens (Simmonds 1941). Chakravarty (1957) described the development of appressoria by *C. musae* which initiate quiescent infections of green bananas. On green banana fruit, hyaline thin-walled appressoria, termed proto-appressoria are formed on short germ tubes. Some of these appressoria immediately produce hyphae which grow beneath the cuticle for a short distance and induce a hypersensitive response in the green peel. However, most hyaline appressoria become dark and thick walled, and induce no visible response in the peel. During ripening, the sub-cuticular hyphae formed previously from the hyaline appressoria remain inactive but the dark appressoria produce hyphae which invade the peel and cause anthracnose lesions. Dark appressoria were therefore regarded as the quiescent structures (Muirhead 1979). Following extensive assessment of four hypotheses based on nutrients, respiration and fungitoxic substances in unripe fruit, and enzyme potential of the fungus, Simmonds (1963) concluded that quiescence results from a combination of some or all of these factors.

Evidence for preformed antifungal substances in the banana skin

Preformed antifungal substances provide a passive chemical barrier to fungal infection and such substances have been reported from other fruits. Germination experiments conducted by Chakravarty (1957) on conidia of *C. musae* in the juice expressed from green and yellow banana skin showed that the green skin has an inhibitory effect. This effect was thought to be due to the presence of tannins, which disappear to some extent as the fruit ripens. Previous researchers (Wardlaw and McGuire 1931; Simmonds 1941; Barnell and Barnell 1945) have also mentioned the presence of tannins in the tissues of green banana and which disappear as the fruit ripens. Barnell and Barnell (1945) have demonstrated the presence of tannin in the latex vessels of the skin and the pulp of banana. They found that as ripening progresses the contents of the latex vessels undergo changes and eventually become caked and brittle. Tannins gradually disappear from these dried latex contents, in the pulp first and subsequently in the skin. With ripening, the pulp loses its astringency, and this might be associated with the observed changes in the tannins. They also found that the growth of *C. musae* is sometimes stimulated by low percentages of tannin; sporulation, for example, is frequently best on media containing a low percentage of tannin. This may explain the occurrence of anthracnose lesions on the skin of ripe banana fruit when the tannin content is low but has not completely disappeared. Evidence obtained elsewhere does not, however, support these findings.

There are contradictory findings regarding the presence of fungitoxic phenols in the banana skin. Mulvana et al. (1969) showed that ethanolic extracts from healthy, green Cavendish bananas were antifungal but the activity falls to low levels as the fruit ripens and this

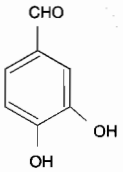
he believed is reflected by the development of *C. musae* on the fruit peel. The fungistatic substance was isolated and identified as 3,4-dihydroxybenzaldehyde (1, Fig. 1). This compound was shown to inhibit the growth of *C. musae* causing anthracnose in bananas. In contrast, a related compound, 2,3-dihydroxybenzoic acid (2, Fig. 1)was shown to stimulate germination and appressoria formation of *C. musae* (Harper and Swinburne 1979). This compound, together with catechol and a trace of pyrogallol due to degradation of anthranilic acid present in the banana fruit leachates, is formed by mycelium and conidia of *C. musae*. Muirhead (1979) found no evidence of a preformed antifungal compound that could restrict the growth of *C. musae*. He attributed the antifungal activity to oxidation products formed during extraction from the phenolic compound, dopamine. Dopamine was isolated from the peel of unripe bananas in concentrations that inhibited *C. musae* in vitro. It was therefore presumed to be a possible preformed compound. However, changes in its concentration were not synchronised with changes in decay development (Muirhead and Deverall 1984).

Detection of phytoalexins in bananas

Brown and Swinburne (1980) were the first to demonstrate the accumulation of phytoalexins in the necrotic lesions that formed beneath drops of conidia of *C. musae* applied to the surface of green bananas. Thin-layer chromatograms of ethyl acetate extracts of the necrotic tissue from green fruit, when bioassayed with *C. musae,* demonstrated the presence of five zones of inhibition, two of which were particularly prominent. Necrotic lesions developed in the peel within 48 hours of inoculation but antifungal activity continued to increase for several days and then remained at a maximum in the unripe fruit. The development of typical progressive anthracnose lesions occurred only after ripening had been initiated with ethylene, when the antifungal compounds could no longer be detected chromatographically. Following extensive investigations, Simmonds (1963) was unable to demonstrate such compounds in inoculated bananas, but his experiments relied upon the diffusion of potentially fungitoxic compounds into water droplets placed on the cuticle at the infection site. Similar experiments repeated by Brown and Swinburne (1980) apparently proved negative. A glucan-containing polysaccharide present in culture filtrates and the mycelial walls of *C. musae* provoked similar lesions on green bananas, and the resulting necrotic tissue, when bioassayed with conidia of *C. musae*, inhibited germination (Brown and Swinburne 1980). Treatment of green bananas with the glucan resulted in the accumulation of two antifungal compounds which corresponded chromatographically to the major inhibition zones on chromatograms of extracts of *C. musae* infected tissue. The antifungal compounds did not diminish with ripening in lesions caused by the glucan. Swinburne and Brown (1983) subsequently showed that the hyaline appressoria (Muirhead and Deverall 1981) initiate phytoalexin response in green bananas, making dark appressoria quiescent. The fact that iron-deficient *C. musae*, which is more aggressive, produces lesser hyaline appressoria on banana fruit and also lesser phytoalexin response, further confirms this.

Identification of phytoalexins

Using a TLC bioassay, Hirai et al. (1994) detected four antifungal compounds in the peel extracts of unripe green fruit var. Giant Cavendish (group AAA) wound-inoculated with *C. musae*. Comparison of Rf values (Rf 0.04, 0.14, 0.43, and 0.54) of the inhibition areas and their colours with those antifungal substances detected by Brown and Swinburne (1980) suggested that these four corresponded to their compounds E, C, B, and A, respectively. Two major compounds (Rf 0.04 and 0.14) were identified as 2-(4'-hydroxyphenyl)-naphthalic anhydride (5, Fig. 1) and methyl 2-benzimidazole carbamate (6, Fig. 1). The former is a new phytoalexin belonging to the phenalenone type. Two phenalenones, irenolone (3, Fig. 1) and emenolone (4, Fig. 1), have also been isolated recently from banana leaves infected with *Mycosphaerella fijiensis* (Luis et al. 1993) but their antifungal activity has not been reported (see below). Hirai et al. (1994) suggest that 2-(4'-hydroxyphenyl)-naphthalic anhydride (5, Fig. 1) could be a metabolite of irenolone (3, Fig. 1) formed by decarboxylation of the enol carbon. The latter is known as an active form of benomyl, a postharvest fungicide. Benomyl is unstable and easily decomposed to methyl 2-benzimidazole carbamate (6, Fig. 1) in aqueous solutions. This suggests that the decomposition occurs in the banana fruit soon after treatment. However, methyl 2-benzimidazole carbamate was not detected in the extract of healthy immature fruit, and was apparently induced by injury or infection. Methyl 2-benzimidazole carbamate seems to have phytoalexin-like characteristics (Hirai et al. 1994).



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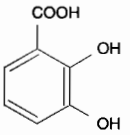

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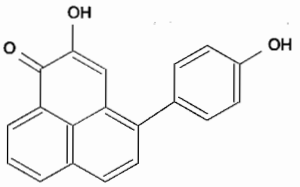

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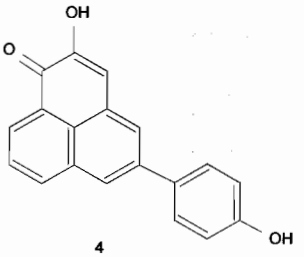

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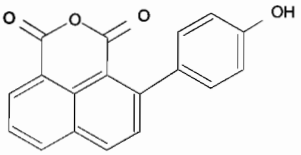

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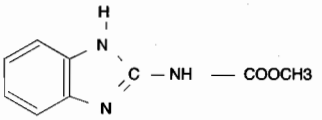

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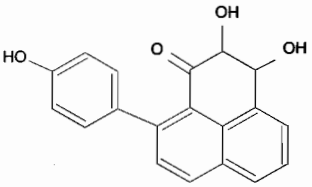

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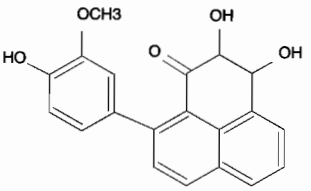

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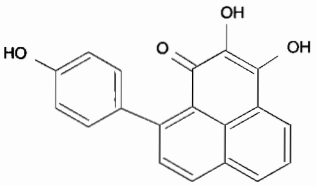

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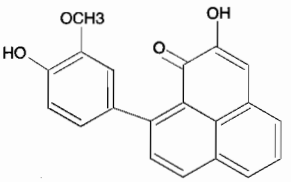

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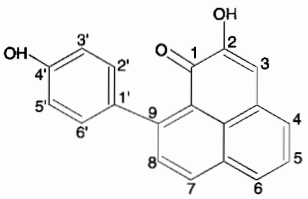

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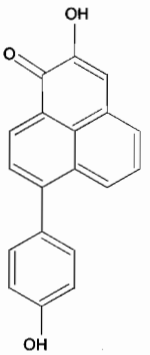

12

Figure 1. Structures of compounds 1–12.

The inoculation procedure employed by Hirai et al. (1994) had, however, been rather unconventional in that the unripe bananas which were first lightly bruised by rubbing with sandpaper were soaked with a spore suspension of *C. musae* for 10 sec. The inoculated fruit were incubated for 6 days before removing for extraction. The method of inoculation no doubt created conditions unlike those which *C. musae* would normally encounter during its natural infection process.

Luis et al. (1993) identified two phenalenone-type compounds, named irenolone (3, Fig. 1) and emenolone (4, Fig. 1), from the *Mycosphaerella fijiensis*-infected banana leaves. Although the induction of these two compounds has been shown also by treatment of the fruit with kanamycin, the antifungal activity has not been reported. The aminoglycoside, kanamycin, which has a carbohydrate nucleus and several amino groups, was used as a phytoalexin inducer in banana leaves and fruit. The tissues were extracted in ethyl acetate and the samples chromatographed on silica gel plates using diethyl ether/hexane. Several compounds were detected as red spots. Irenolone, the major constituent isolated, was found mostly in infected leaves and in lesser amounts in peel of fruit from which the pulp was removed and filled with a solution of kanamycin, and in the extract of kanamycin-treated leaves. This in fact was claimed to be the first of a new type of 'phytoalexin' structurally based on a phenalenone skeleton. In an attempt to re-examine the phytoalexins produced in banana fruit (described below) we also isolated irenolone from the extracts of freckle-infected banana peel but repeated tests proved this compound to be nonfungitoxic. Luis et al. (1993) also found a minor metabolite emenolone, most abundantly in the infected leaves and in trace amounts in fruit peel and kanamycin-treated leaf extract. A third compound with an unknown structure was isolated from the kanamycin-induced extract and trace amounts in infected leaves and fruit peel. These compounds were not detected in the extracts of leaves of fruit peel treated with water-Tween only.

Some years later, Luis et al. (1996) reported four new phenalenone-type 'phytoalexins', named musanolones C–F from rhizomes of banana var. Grand Nain (group AAA) infected by *Fusarium oxysporium* f. sp. *cubense* race 4 causing Panama disease. Combined chloroform and ethanol extracts of rhizomes of infected or healthy plants were subjected to column chromatography. The *n*-hexane-EtOAc fractions when compared by thin-layer chromatography analysis on silica gel plates showed the presence of eight coloured spots in the infected extract. The major constituent of the infected rhizomes was identified as irenolone, which was also present in trace amounts in the healthy tissue extract, probably as a result of mechanical stress (cutting). Of the other five spots purified, four of them were identified and named as musanolone C (7), D (8), E (9), and F (10) (see Fig 1.). Although the induction of irenolone (3) and emenolone (4) in relation to *M. fijiensis* and musanolones C–F in relation to *F. oxysporium* has been confirmed (Luis et al. 1993, 1996), the antifungal activity of these compounds has not been properly tested.

From the studies carried out by Hirai et al. (1994) valuable information on 'phytoalexins' in banana fruit, especially their chemical nature, has been made available. However, the technique that has been employed to elicit phytoalexin response in particular was crude and far from plant pathological significance. The information published by Luis et al. (1993, 1996) is mainly of chemical importance. It is possible that most of the compounds described by them could be stress metabolites which do not have a role in plant resistance. Phytoalexins must, by definition, be fungitoxic and this property of some of the compounds described has not been fully established. Our studies have shown that irenolone is a stress metabolite showing no fungal toxicity. Further, none of these studies seemed to address the other characteristics that a metabolite must have before it can be implicated as playing a role in host resistance. These include toxicity, concentration, time of formation, etc. in relation to pathogenesis. For these reasons the phytoalexin accumulation by banana fruit was re-examined using freckle disease as an example.

Defence Responses Associated with Freckle-infected Banana Peel

Freckle disease

Freckle disease (*Phylosticta musarum*) was first reported in Hawaii (Carpenter 1918, 1919), and subsequently spread to most parts of the world, including Sri Lanka (Abayasekara et al. 1993). On fruit, the freckles appear soon after the bunch is shot, initially as minute, grey-brown, circular spots of about 0.25 mm diameter. As the fruit matures, the individual freckles increase in size up to 1 mm and turn dark-brown or black. A typical freckle is rough to the touch, and heavy infections give a reddish-brown colour to the fruit. The disease affects the appearance and reduces the market quality of fruit. Unlike many other fungal diseases the freckle infections do not

grow and develop into lesions as the fruit ripens. The disease does not lead to fruit spoilage or quantitative crop loss (Abayasekara et al. 1993). These features may reflect the less-destructive and specialised nature of *P. musarum*.

Extraction and purification of phytoalexins from freckle-infected banana peel

Ethyl acetate extracts of the peel tissue of freely harvested, unripe bananas var. '*Embul*' (Mysore, group AAB) infected heavily with *P. musarum*, when chromatographed on TLC plates (chloroform: methanol, 95:5, v/v) and oversprayed with *C. cladosporioides* showed a large inhibitory zone (FR2) and two other smaller, but still prominent inhibitory zones (FR3 and FR4). In addition, two more less prominent inhibitory zones were also observed (FR1 and FR5) (Fig. 2). Similar extracts from non-freckled banana peel showed no antifungal areas. The freckle fungus can also infect the banana leaf, producing symptoms similar to those in the fruit. The freckle-infected leaves when tested by the above extraction and bioassay procedure displayed antifungal activity similar to that of the infected fruit. The ether phase of the peel extracts (ethyl acetate) of the freckled peel delayed germination of conidia of *C. musae*, and inhibited appressoria formation.

In order to isolate and identify the phytoalexins produced, a large-scale extraction of antifungal compounds was carried out using 200 g of banana fruit peel heavily infected with *P. musarum*. Peel tissue was extracted by vacuum infiltration in ethyl acetate with continuous magnetic stirring, and the extract was evaporated to dryness (Abayasekara et al. 1995).

The crude extract (2 g) was fractionated into nine fractions by medium pressure liquid chromatography (MPLC) with increasing solvent polarity. The fractions 3, 4, 7, and 8 showed antifungal activity when bioassayed on a TLC plate. Fraction 6 gave red needle shaped crystals (25 mg), and was identified as irenolone (3) by comparison with previous spectral data (Luis et al. 1993). Fraction 3 was further purified by a normal-phase HPLC column, coupled with a refractive index detector. A colourless oily compound (C2) (10 mg) was isolated.

Fraction 7 was purified by flash column chromatography, eluted with 2% MeOH in dichloromethane, and further purified by preparative TLC. A red coloured, active compound (C4) (15 mg) was obtained. ^{1}H and ^{13}C NMR spectra of C4 showed a similar pattern to phenyl phenalenones (Luis et al. 1993, 1996). ^{1}H NMR data of C4 were compared with those of 2-hydroxyanigorufone (11, Fig. 1), found as a constitutive natural product from *Anigozanthos rufus* (Cook and Dagley 1978) (Table 1). ^{1}H and ^{1}H-^{1}H COSY spectra indicated three aromatic spin systems. AMX at d 7.80, 7.62, and 8.01; AB at d 7.57 and 8.34; and AA'BB' at d 7.20 and 6.81, respectively. The AA'BB' system indicated the presence of para substituted phenyl group. In addition there was a signal at d 7.10 and two broad signals with low intensity at d 9.25 and 9.55, indicating the presence of two OH groups. A signal at d 179.9 in ^{13}C NMR spectrum was attributed to the carbonyl carbon, and the proton attached to the adjacent C atom gave a deshielded signal at d 8.34 in the proton NMR. The signal at d 8.34 showed coupling with proton at d 7.57 in the ^{1}H-^{1}H COSY spectrum. Due to the comparatively low chemical shift of this proton, we suggested that the adjacent C was substituted with the para hydroxy phenyl group. From these spectral data we propose the structure shown as 12 in Figure 1 for the compound C4. Further structural analysis of compounds C4 and C2 is in progress.

Table 1. ^{1}H NMR data of compounds 11 and 12.

Proton	Compound 11	Compound 12
2-OH	No signal	9.55 (br s)
3	7.15 (s)	7.10 (s)
4	7.85 (dd, 1.3, 8.3)[a]	7.80 (d, 7.2)
5	7.61 (tbr)	7.62 (dd, 8.2, 7.2)
6	7.93 (dd, 1.3, 8.3)	8.01 (dd, 8.2)
7	8.23 (d, 8.3)	–
8	7.62 (d, 8.3)	7.57 (d, 8.4)
9	–	8.32 (d, 8.4)
2', 6'	7.31 (dd, 2.0, 8.5)	7.20 (d, 8.6)
3', 5'	6.97 (dd, 2.0, 8.5)	6.81 (d, 8.6)
4'-OH	No signal	9.25 (br s)

[a] Coupling constants (in parentheses, in Hz): spectra recorded in acetone-d^6 for compound 11 and DMSO-d_6 for compound 12.

The antifungal profile in the banana fruit at different developmental stages was followed. For this purpose, bananas of following ages were harvested at 2, 4, 6, 10, and 14 weeks after bunch emergence. All fruit samples had similar freckle infections, an average of 40 freckles per cm^2. The peel tissues following extraction were bioassayed on TLC plates as above using *C. cladosporioides* or *C. musae*. All these maturity stages showed substantial antifungal activity with a prominent antifungal area corresponding to FR2.

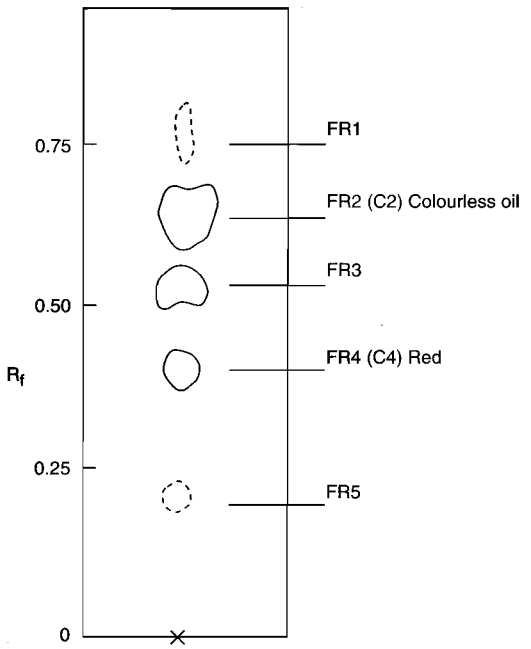

Figure 2. Antifungal activity in freckle-infected banana peel.

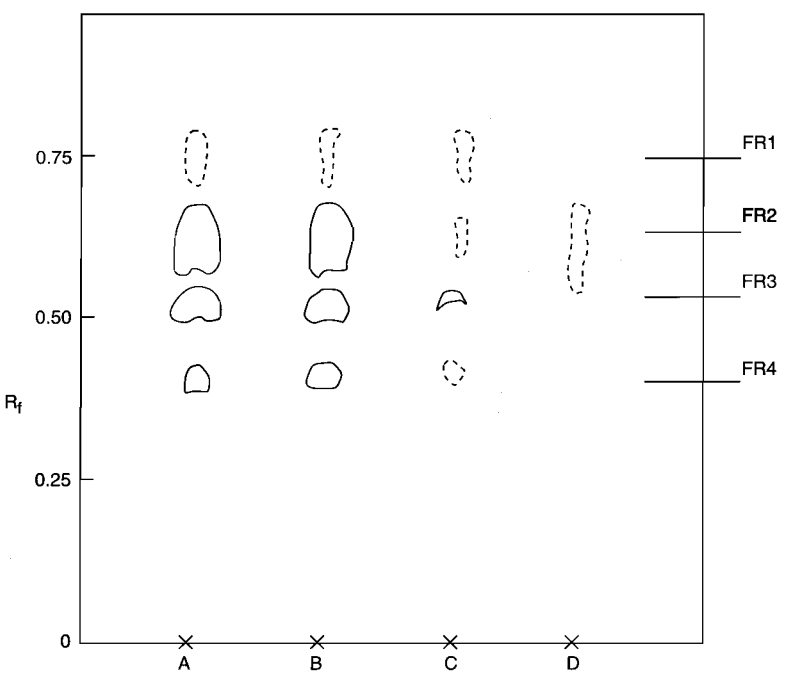

Figure 3. Comparison of antifungal profile of *C. musae*-infected banana peel extract and freckle-infected peel extract (A: freckled banana tissue; B: healthy bananas inoculated with *C. musae* after bruising; C: healthy bananas inoculated with *C. musae* without bruising; D: healthy bananas treated with H_2O after bruising).

To examine the fate of antifungal substances of the fruit peel of freckled bananas during ripening, peel tissue taken from fruit at seven consecutive days after harvest was extracted and bioassayed. This showed that the size of the inhibition area of FR4 decreased progressively during fruit ripening, but most of the activity of FR3 remained. The peel taken from fully ripened fruit still showed a considerable antifungal activity. Antifungal activity was observed in several other local varieties of bananas. Puwalu (group AAB) and Golden bananas (group AAAA) exhibiting a relatively high degree of freckling showed antifungal activity, corresponding to the previous observations, when subjected to the above method of extraction and antifungal bioassay.

Comparison of antifungal compounds associated with *C. musae*-infected banana peel with those of freckle-infected peel

In order to determine if freckle infection accumulates the same phytoalexins as *C. musae*-infected bananas (Brown and Swinburne 1980; Hirai et al. 1994), an extract of freckled tissue was compared by TLC bioassay with an extract of *C. musae*-infected tissue prepared using the same procedure as Hirai et al. (1994). A set of immature healthy bananas was inoculated with *C. musae* after bruising with sandpaper and another set was inoculated without bruising. In another set, the skin was bruised and treated with sterile distilled water. A fourth set consisted of fruit naturally infected with freckle fungus. The fruit was incubated in a moist chamber for 6 days. Following this, the tissue samples obtained were extracted in ethyl acetate by vacuum infiltration (Abayasekara and Adikaram 1995). When bioassayed, the extracts of peel from both freckled and the bruised *C. musae*-inoculated fruit, gave three prominent antifungal zones, corresponding to FR2, FR3, and FR4 and another additional, faint antifungal zone, corresponding to FR1. A similar extract from unbruised *C. musae*-inoculated fruit gave three faint antifungal zones, corresponding to FR1, FR2 and FR4 and another prominent, but smaller zone corresponding to FR3. The extract of fruit which were bruised and treated with H_2O, when bioassayed, produced a very faint inhibition zone. The above observations indicated that both freckle-infected and *C. musae* -inoculated tissue (after bruising) induce a strong and a similar profile of antifungal activity. Inoculation of intact banana skin (without bruising) with *C. musae* induces very much less antifungal activity.

Pathogenesis-related proteins associated with the freckle disease

Van Loon and van Kammen (1970) and Gianinazzi et al. (1970) independently reported the *do novo* synthesis of several proteins in tobacco plants reacting hypersensitively to infection with tobacco mosaic virus (TMV). These proteins were not detected in healthy leaves but were induced in the inoculated leaves by a localised TMV infection. Furthermore, it was reported that they were plant-coded proteins and their synthesis was induced. These proteins were subsequently called pathogenesis-related or PR proteins. Since then, PR proteins have been detected in at least 20 plant species (Nassar et al. 1988). PR proteins are characterised by their acidic nature, their resistance to proteases, and their extracellular location. More recently, basic homologues to a number of acidic PR proteins have been identified. A relationship has been suggested between the accumulation of PR proteins and disease resistance.

PR proteins extracted from freckled and non-freckled peel samples using an extraction buffer of pH 2.8 (Tuzan et al. 1989) were separated by polyacrylamide gel electrophoresis (PAGE) using a 12.5% acrylamide gel. The extracts of freckle-infected tissues contained four to five additional protein bands in the low molecular weight region, when compared to the non-freckled tissue, indicating the presence of PR proteins in the infected peel.

In another experiment, tissues were excised from regions adjacent to freckled points (ranging from 0.5 cm away from freckle infection to completely non-freckled fruit) of banana peel and extracted for PR proteins. When PAGE was performed on four such extracts, it showed that non-freckled peel samples from different locations had no additional low molecular PR protein bands such as had been observed in the freckled peel extract.

Lignified, suberised and tannin cells associated with the freckle disease

Lignification has been reported to be associated with systemic resistance (Vance et al. 1980; Touze and Rossignol 1977). Induced systemic resistance of cucumber against *C. lagenarium* appears to involve inhibition of penetration by the fungus and is associated with rapid lignification of the epidermis localised around the appressoria. Thin transverse sections of freckled and non-freckled peels were stained for lignin using phloroglucinol-HC1 (Johanson 1940). The freckled tissue consisted of lignified cells around

the pycnidia, while lignified cells were not observed in the non-freckled tissue, indicating that freckling is associated with lignification.

Suberisation is yet another relatively frequent response to infection or wounding, and has been associated with resistance in some cases. Thin transverse sections of diseased and healthy tissue were treated with chlorozinc-iodide (Johansen 1940). The tissues surrounding the pycnidia, and in some instances the tissues in between two pycnidia, and the epidermal cells adjacent to pycnidia, stained violet, indicating the presence of suberin in the diseased tissue, while in the healthy tissue no suberin could be found. The production of suberin may also be a contributing factor in preventing the expansion of progressive freckle lesions during fruit ripening, thereby limiting the development of lesions even in an advanced state of the disease.

The freckled unripe peel tissue contained significantly more tannin cells than did the healthy fruit peel. This was examined by treating tissue in a 10% solution of $FeCl_3$ with Na_2CO_3 (Johansen 1940), which turns the tannin-containing cells blue-green.

Phenylalanine ammonia lyase (PAL) and peroxidase activity associated with the freckle-infected tissue

The probable pathway for the synthesis of lignin from phenylalanine in wheat would be essentially the same in most higher plants (Gross 1980). The initial conversion of phenylalanine to trans-cinnamic acid is an irreversible step catalysed by the enzyme phenylalanine ammonia-lyase (PAL) with a small amount of carbon in the Gramineae also entering from tyrosine via tyrosine ammonia-lyase (TAL). The conversion of phenylalanine to cinnamic acid may lead to the biosynthesis of many phenolic compounds apart from lignin and hence problems arise in interpreting the importance of increased PAL (and TAL) activity in infected tissue. The possibility that infection-induced lignification might involve the utilisation of existing pools of phenolic precursors further complicates the issue. Nevertheless, the fact that increases in PAL activity have been spatially and temporarily associated with lignification in infected tissues (Maule and Ride 1976; Vance and Sherwood 1976), together with the observed incorporation of radiolabelled phenylalanine into induced lignin (Touze and Rossignol 1977; Maule and Ride 1982), suggest that PAL activity is important, and that a major portion of the carbon for lignification is derived directly from phenylalanine on infection. In addition to increases in ammonia-lyase, lignifying tissues have also shown increases in cinnamate-4-hydroxylase, hydroxycinnamate:Co A ligase *0*-methyltransferase, and peroxidase activities (Maule and Ride 1976; Maule 1977; Vance and Sherwood 1976; Vance et al. 1976).

PAL activity was estimated by measuring the rate of conversion of phenylalanine into cinnamic acid using the method described by Riov et al. (1968) and Martinez-Tellez and Lafuente (1993). The results indicated that the PAL activity of the freckled peel was 2–3 times greater than that in the non-freckled peel. PAGE was performed to estimate the soluble peroxidases (Wendel and Weeden 1990) in the freckle-infected and non-infected peel tissue. Four additional bands were observed in the freckle-infected peel when compared to the healthy peel. Increased activity of these enzymes may be significant, as the defence responses by the banana fruit tissue to freckle included the accumulation of phytoalexins and lignification.

Resistance of freckle-infected bananas to anthracnose disease

We have previously noticed that heavily-freckled bananas generally develop lesser anthracnose lesions during ripening than non-freckled or less-freckled ones. This phenomenon was tested in the laboratory using freckled bananas a) that had been naturally infected with *C. musae* at the time of collection, or b) artificially inoculating with *C. musae* after collection. Anthracnose disease originates from quiescent infections by *C. musae* that take place in the field. In Sri Lanka bananas are almost invariably infected with this fungus at the time of harvest.

Two sets of bananas, one with freckle infections and another with no freckles, were bought and allowed to ripen. From the skin tissue of both *C. musae* could be isolated which indicated the presence of quiescent infections in the skin. The non-freckled fruit developed anthracnose lesions much earlier than the freckled fruit. The lesions in the non-freckled bananas were larger than those of freckled ones (Table 2). The heavily freckled (category 5) bananas had smaller anthracnose lesions than the moderately freckled (category 3) fruit.

A similar trend was observed when the freckled and non-freckled fruit were artificially inoculated with *C. musae* and allowed to ripen (Table 3). The artificial inoculations produced anthracnose lesions several days earlier than fruit that had natural

C. musae infections. The non-freckled fruit showed the first signs of anthracnose 5 days after inoculation. These expanded into larger lesions within another two days. The heavily freckled fruit showed no symptoms on the 7th day. The moderately freckled fruit had slightly brownish lesions on the 7th day which were much smaller than those in the non-freckled fruit.

Table 2. Anthracnose lesion development in non-freckled and freckled bananas naturally infected with *C. musae* during ripening.

Days after harvest	Lesion area (cm^2)		
	Category 0[a]	Category 3	Category 5
9	0.93a[b]	0.44b	0.21b
10	2.73a	0.64b	0.51b
11	4.51a	2.36b	0.84b

[a] Category 0 = non-freckled; category 3 = 40 freckles per cm^2; ategory 5 = 200 freckles per cm^2
[b] Values followed by the same letter within each line do not differ significantly (p = 0.05, Duncan's multiple range test).

Table 3. The anthracnose lesion development in non-freckled and freckled bananas artifically inoculated with *C. musae*, during ripening (7 days after inoculation).

Category 0[a]	Category 3	Category 5
	Lesion area (cm^2)	
4.94a[b]	0.79b	0.03b

[a] category 0 = non-freckled; category 3 = 40 freckles per cm^2; ategory 5 = 200 freckles per cm^2
[b] Values followed by the same letter within each line do not differ significantly (p = 0.05, Duncan's multiple range test)

To compare the natural quiescent propagules of *C. musae* in the freckled peel with those of non-freckled fruit, peel segments were cut from both freckled and non-freckled fruit, surface sterilised, and placed separately on agar medium. The percentage peel segments that produced *C. musae* colonies was determined and this was taken as an indication of the numbers of quiescent infections of *C. musae* in the fruit peel. The peel segments removed from banana skins that had more freckles showed a significantly greater number of *C. musae* colonies (13.66) than those of non-freckled ones (4.33), indicating higher numbers of quiescent appressoria (dark) in the freckled peel.

To examine if greater amounts of phytoalexins accumulate in heavily freckled fruit peel than in peel that is less freckled, equal weights of the fruit peel with various densities of freckles (Table 4) were extracted separately and bioassayed on TLC plates. Even peel with the lowest freckle density showed significant antifungal activity. The sizes of the areas of inhibition, particularly FR2, produced by the extracts taken from the more heavily freckled peel samples were larger than those from extracts from light and moderately freckled peel, showing that greater antifungal activity is associated with heavier freckling. The inhibition zones FR3 and FR4 were observed in tissue with more than 40 freckles per cm^2, their size increasing with increase in freckling (Table 4).

Table 4. Antifungal zones in extracts taken from banana peel with different freckle densities.

Average freckles per cm^2	Area of antifungal zones (cm^2)		
	FR2	FR3	FR4
Category 1 (2.5)	1.35	–	–
Category 2 (15)	1.86	–	–
Category 3 (40)	1.97	0.71	0.09
Category 4 (125)	2.35	1.04	0.33
Category 5 (200)	4.74	2.62	0.53

In order to establish whether the resistance evoked by the freckle infections spreads beyond the freckled sites into neighbouring tissue, suspensions of conidia of *C. musae* were inoculated into the following locations on bananas obtained from a single bunch:

1. Completely freckled peel;
2. Non-freckled sites in a freckled fruit, 0.5 cm away from the freckles;
3. The non-freckled surface of bananas which had freckle infections on the other side;
4. Surface of completely non-freckled bananas taken from a hand with heavily freckled bananas;
5. Non-freckled fruit from a hand with no freckled fruit.

The development of anthracnose was significantly reduced (DMRT, p = 0.05) in the heavily freckled fruit (1) compared with all the non-freckled fruit used in the experiment. There was also a significantly lower anthracnose development in the non-freckled sites inoculated 0.5 cm away from the freckle infections (2) than in the other non-freckled sites. However, there was no reduction of anthracnose development in either the non-freckled surface of the fruit which had freckles on the other surface (3), in the non-freckled fruit taken from a freckled hand (4), or in the non-freckled fruit from a completely

non-freckled hand (5). These results may indicate that the resistance induced due to freckle infections is restricted to the freckled sites and their immediate vicinity (to about 0.5 cm away) but does not spread in a systemic manner.

These studies have clearly shown that unripe banana fruit responds defensively to freckle infections by a number of cellular, chemical, and biochemical mechanisms. The most significant response was the accumulation of substantial amounts of phytoalexins. The amount of phytoalexin that could be detected in the freckle-infected fruit is much greater than the amount accumulated in fruit in response to *C. musae* infection. These observations may indicate that the freckle fungus is a more effective inducer of host defence than *C. musae*, and that the defence elicitation property of the freckle fungus might be utilised in the protection of banana fruit from anthracnose.

An understanding of the defence mechanisms and their elicitation in fruit could lead to the development of new biological/biotechnological approaches to reduce fungal decay. These could serve as environmentally friendly alternatives for toxic fungicides, which are currently used for the control of postharvest diseases. In this study, a number of cellular, chemical, and biochemical defence responses in banana fruit to freckle infections have been revealed. The development of an elicitor substance, or a mild strain of the freckle fungus capable of inducing resistance without cellular damage, could have practical value in protecting bananas from destructive diseases such as anthracnose.

Conclusions

1. Fungitoxic tannins and phenolic substances appear to be present in the unripe banana fruit, but regarding the significance of preformed fungitoxic substances in the resistance of unripe banana peel evidence is rather conflicting.
2. Several 'phytoalexins' have been purified and identified from banana but only a few of them seem to be involved in responding to disease incursion. The fungitoxicity of irenolone is questionable. In these studies, the phytoalexin accumulation has been induced by conditions which the pathogens do not normally encounter during disease development. Further, there has been no attempt to correlate the toxicity, concentration, or the time of formation of phytoalexins with disease progression. Phytoalexins accumulate in response to *C. musae* infection, but whether they are responsible for quiescent infections has not been addressed fully.
3. Freckle infections are associated with strong antifungal activity consisting of at least five phytoalexins. Two phytoalexins were purified, and found to be new compounds. Another compound isolated was identified as irenolone. Freckle infections are also accompanied by several PR proteins, increased PAL and peroxidase activity, and certain cellular changes such as lignification and suberisation. The restricted nature of freckle infections may be due to a combination of strong defence responses by the host tissue. Some of these, e.g. phytoalexins, are retained in the ripe fruit, preventing the further development of freckles.
4. Freckle-infected bananas are resistant to anthracnose development. The resistance is enhanced with increased freckling. *P. musarum* appears to be a more effective elicitor of phytoalexins in banana than *C. musae*. The results of these studies raise the possibility that phytoalexin elicitation by *P. musarum* might be utilised in the protection of bananas from anthracnose disease.

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Antifungal Defences in Cucurbit Fruit

B.T. Hawthorne*

Abstract

This review provides an overview of evidence for both inducible and constitutive defence mechanisms proposed as components of disease resistance responses for control of fungal rots of cucurbit fruit.

THE Cucurbitaceae is a tropical or semitropical family comprising approximately 130 genera and 900 species (Jeffrey 1980). Three genera are cultivated extensively for their edible fruits: *Citrullus* (watermelon); *Cucumis* (cucumber and melons); and *Cucurbita* (pumpkins, squashes, and gourds). Within *Cucurbita* three species are widely grown, *C. pepo* L., *C. moschata* Duchesne, and *C. maxima* Duchesne. A wide range of fungi is known to cause disease in cucurbit fruit and the rots they produce are particularly damaging after harvest and during storage (Guba 1950; Sherf and McNabb 1986; Hawthorne 1988; Reddy and Reddy 1988; Rath et al. 1990; Keinath 1995). As a group these fungi can be characterised as rather weak, opportunistic pathogens, or wound parasites, that gain entry to the fruit tissue primarily through wounds and produce invasive rots mainly on fully mature fruit (Van Steekelenburg 1982). The rot fungi can become established on fruit in several ways: as quiescent, or very slow growing, infections during development of the fruit; through colonising stems, wounds, and callus tissue over the growing period and especially during harvest operations; and by lodging around the base of stems (Hawthorne and Sutherland 1991). Suggested control measures for these pathogens include avoiding damage to fruit at harvest (Van Steekelenburg 1982); Hawthorne 1989), harvesting at optimum maturity (Hawthorne 1990), postharvest fungicide treatment and/or curing (Wade and Morris 1983; Nagao et al. 1991), short duration hot water treatment (Francis and Thomson 1965; Mayberry and Hartz 1992) reducing ethylene levels (Miyazaki and Ookubo 1989; Aharoni et al. 1993) and cool temperature 10–12°C) storage (Francis and Thomson 1965; Lutz and Hardenberg 1968; Nagao et al. 1991).

This paper provides an overview of the potential for natural defence mechanisms, both constitutive and inducible, to function as components of disease-resistance responses against fruit rotting fungal pathogens in the major commercial *Cucurbita* species.

Defence Mechanisms Operating at the Fruit Surface

Cuticle and epidermis as barriers to infection

It is perhaps self-evident that an intact cuticle and epidermis could function as physical, and/or physiological, barriers to fungal infection. Indeed, the capacity for direct penetration through the skin is uncommon for most of the fruit rot fungi. It appears plausible, therefore, to think that there would be a positive correlation between skin thickness and resistance to fruit rots. Hence, breeding fruit with thicker skins may seem an appropriate strategy for reducing the incidence of storage rots. However, in work on storage rots of *C. maxima* breeding lines,

* The Horticulture and Food Research Institute of New Zealand Ltd, Private Bag 92, 169, Auckland, New Zealand.

produced from a 5 × 5 diallel reciprocal crossing experiment, Hawthorne and Rees–George (unpublished data) found there was no relationship between amounts of surface wax (Fig. 1) and incidence of rots during storage. Further, rot incidence appeared greater for fruit with greater amounts of cuticle (Fig. 1). These results suggest that the thickness of the external layers is not the crucial element for external protection against infection. Properties of the wax-cuticle layer which lead to a reduced tendency for cracking are likely more important than the actual thickness of the layer.

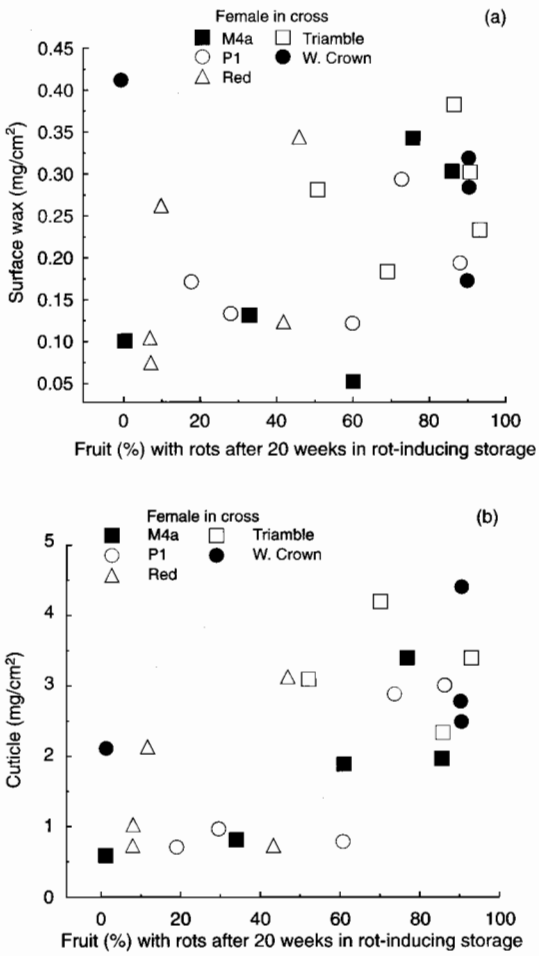

Figure 1. Relationship of amounts of (a) surface wax and (b) cuticle with incidence of storage rots for fruit from 25 crosses produced from a 5 × 5 complete diallel involving cultivars of *Cucurbita maxima* with varying keeping qualities.

Wound healing

Wound exudate

As an initial response to wounding, cucurbit fruit produce often copious amounts of a phloem exudate which rapidly hardens on contact with air to form an effective protective barrier (MacGibbon and Mann 1986; Sharrock and Parkes 1990; Walter et al. 1990; Hawthorne and Sutherland 1991; Mann and MacGibbon 1991). Phloem exudate has a high protein content and a high pH (Richardson et al. 1982) and in addition to the gelling properties there is a strong protease inhibitor activity which may be antifungal in vivo (MacGibbon and Mann 1986; Sharrock and Parkes 1990). There is evidence from work on buttercup squash that higher concentrations of protease inhibitor activity in the exudate were associated with reduced incidence of storage rots (Sharrock and Parkes 1990). In addition, in a study of eight inbred and nine hybrid lines of *C. maxima* negative correlations were found between the degree of susceptibility to wound infection by *Fusarium solani* and levels of protease inhibitor (Hawthorne et al. 1993).

Wound periderm and callus formation

Cucurbit fruit have an active wound repair mechanism. First, the exudate seals off the wound and prevents water loss; then, within a few hours, a wound periderm forms. This periderm divides rapidly and produces layers of corky callus 'scar' tissue that erupt above the fruit surface (Walter et al. 1990; Hawthorne and Sutherland 1991). This wound healing response is an effective 'first aid' but it also increases the potential during prolonged storage for subsequent invasion of the fruit by fungi capable of colonising the wound scar tissue micro-environment (Hawthorne and Sutherland 1991).

Defence Mechanisms Operating within the Fruit Tissues

Induced antifungal compounds

Chitinases

In response to wounding, pathogen invasion, and abiotic stress, plants exhibit an altered pattern of protein synthesis. The group of proteins produced, collectively known as pathogenesis-related proteins, include a number of chitinases which have the capacity to degrade fungal cell walls (Kombrink et al.

1988; Roberts and Selitrennikoff 1988; Verburg and Huynh 1991). It is possible, as suggested by Verburg and Hyunh (1991) that chitinases may play an indirect role in plant defence against fungi by releasing products from fungal cell walls which in turn stimulate the biosynthesis of phenolic compounds and lignification in plant cells. Chitinase activity has been found in tissue extracts and tissue slices from cucurbit fruits (Esaka et al. 1993; Sharrock and Parkes 1990). The role of chitinases *in vivo* in cucurbit fruit is uncertain. Sharrock and Parkes (1990) found there was no relationship between levels of chitinase activity and incidence of storage rots in buttercup squash. Elevated storage temperatures were found to increase the incidence of storage rots and the levels of chitinase activity in buttercup squash fruit (Hawthorne et al. 1993). If chitinases do play a part in protecting cucurbit fruit from fungal invasion then it appears it will be a minor part.

Systemic acquired resistance

Cucurbit plants, especially cucumber, have been used extensively in studies of the phenomenon known as systemic acquired, or induced, resistance (Kuc et al. 1975; Kuc 1983; Hammerschmidt and Becker 1997). In systemic induced resistance, localised invasions of roots, stems or leaves, by necrotic lesion-inducing pathogens or by nonpathogens induces an enhanced state of resistance throughout the plant. This type of resistance is nonspecific and can be induced by a wide range of compounds from pathogens and plants, and by plant growth-promoting rhizobacteria (Wei et al. 1996; Hammerschmidt and Becker 1997). Most studies of induced resistance have involved leaf pathogens and its occurrence in fruit is largely a matter of speculation. However, there is a report of reduced fruit rot in muskmelons treated with growth-promoting rhizobacteria (Eayre 1994).

Conclusions

Although there is much speculation about the possible involvement of a range of antifungal mechanisms, particularly induced compounds, in protecting fruit against fungal invasion there is very little direct and unequivocal evidence to support these assertions in cucurbits. It is clear, however, that within a species such as *C. maxima* susceptibility to fungal rots can vary substantially between cultivars and that this variation has a genetic basis (Grant et al. 1997). Reduced susceptibility to fungal invasion is likely to occur through the interaction of several mechanisms, each of small effect, acting collectively to condition a 'resistant phenotype'. The challenge is to determine the combination of factors that leads to this resistant phenotype.

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Antifungal Compounds Regulating Quiescent Diseases in Mango

I. Kobiler, R. Reved, L. Artez, and D. Prusky*

Abstract

The most important postharvest diseases developing in mango fruit are anthracnose caused by *Colletotrichum gloeosporioides* and black spot disease caused by *Alternaria alternata.* These pathogens infect mango fruit in the field and remain quiescent until harvest. To study the regulation of these quiescent fungal infections in mango fruit, we searched for endogenous antifungal compounds, assessed their effect on fungal symptom development, and investigated factors affecting their activity and amount.

We have described the presence of a mixture of antifungal compounds in the mango peel, consisting of substituted resorcinols: the major one was 5, 12-cis heptadecenyl resorcinol, with 5-pentadecyl resorcinol secondary. This mixture of 5-substituted resorcinols is present at fungitoxic concentrations in the peel of the mango during the period of fruit growth, and decreases after the harvest and ripening of the fruit. The concentration becomes non-toxic to fungi at the time of initiation of the decay development.

A new antifungal compound was identified recently as 5(7,12-heptadecadienyl) resorcinol. The concentration of this compound, present in the peel and the flesh of the fruit, is not reduced significantly with the fruit harvest and ripening. In order for this compound to play a functional role in delaying disease development it should be inducible to fungitoxic concentrations. Several treatments have been shown to elicit this compound. CO_2 treatment applied to the harvested mango increased the concentration of the antifungal 5-(7,12-heptadecadienyl) resorcinol, concurrently with a delay of decay development. Furthermore, challenge inoculation of mango fruit with *C. magna*, a non-pathogenic strain on mango, increased the level of the antifungal 5-(7,12-heptadecadienyl) resorcinol in the peel of the inoculated fruits, and prevented the development of decay caused by mango pathogens.

Also recently, the activity of additional antifungal compounds, still unidentified, was detected. These antifungal compounds seem to vary between different cultivars of mango, showing differential activity toward the pathogens tested. It is concluded that a family of substituted resorcinols is present in mango fruit. Some of those compounds seem to have preformed fungitoxic activity and others are present at subtoxic concentrations and can be induced to fungitoxic levels.

ONE of the limitations of long-term storage of fresh mangoes for local consumption or international trade is the fruit's susceptibility to postharvest diseases. The most important postharvest diseases developing worldwide in mango fruit are anthracnose caused by *Colletotrichum gloeosporioides* and stem-end rot caused by *Dothiorella dominicana, Lasiodiplodia theobromae*, and *Phomopsis mangiferae*. The main postharvest disease in Israel is the black spot disease caused by *Alternaria alternata.* In parts of the country with high relative humidity, 100% of fruit will decay from this pathogen if they are not treated after harvest (Prusky et al. 1986). Also, in recent years, stem-end rots have become a significant problem in Israel, and about 10–15% of the fruit develop these symptoms during long-term storage.

* Department of Postharvest Science of Fresh Produce, The Volcani Center, Agricultural Research Organization, Bet Dagan 50250, Israel.

Alternaria alternata and *Colletotrichum gloeosporioides* infect mangoes as they grow in the orchard. Fungal spores germinate on the peel and penetrate through lenticels, as in *A. alternata*, or after appressoria formation by breaching the fruit cuticle, as in *C. gloeosporioide* After penetration the hyphae remain latent intercellularly, until the fruit is harvested and ripens. During fruit ripening the fungus renews its development, which results in browning of the intercellular space and the appearance of the characteristic black spot disease symptoms.

After harvesting, significant physiological and chemical changes occur in the fruits which enable the resumption of fungal growth, resulting in active decay lesions. Four possible resistance mechanisms of unripe fruits to fungus development were described by Prusky (1996):

1. Lack of nutrients required by the pathogen in unripe fruit but which become available in ripening fruit.
2. The presence of preformed antifungal compounds in unripe fruit that decline during ripening.
3. The presence of inducible antifungal compounds in unripe fruit, but in subfungitoxic levels, that decline during ripening, and which might be induced by elicitors.
4. Lack of activation of the fungal pathogenicity factors in unripe fruits as a result of the presence of inhibitors that decline during ripening.

Hypotheses 1 and 2 are dependent mostly on the fruit itself, while the other two may depend on the interaction between the host and the pathogen. The activation of quiescent infection may result not only from the effect of a single mechanism, but also by several mechanisms acting jointly.

Our work to understand the mechanism of resistance of unripe fruit is mainly related to the presence of endogenous preformed antifungal compounds termed phytoanticipins (Van Hetten et al. 1994). The modulation of the preformed antifungal compound levels by biological, physical, and chemical means may provide new options in the control of postharvest decay and reduction of pesticide use.

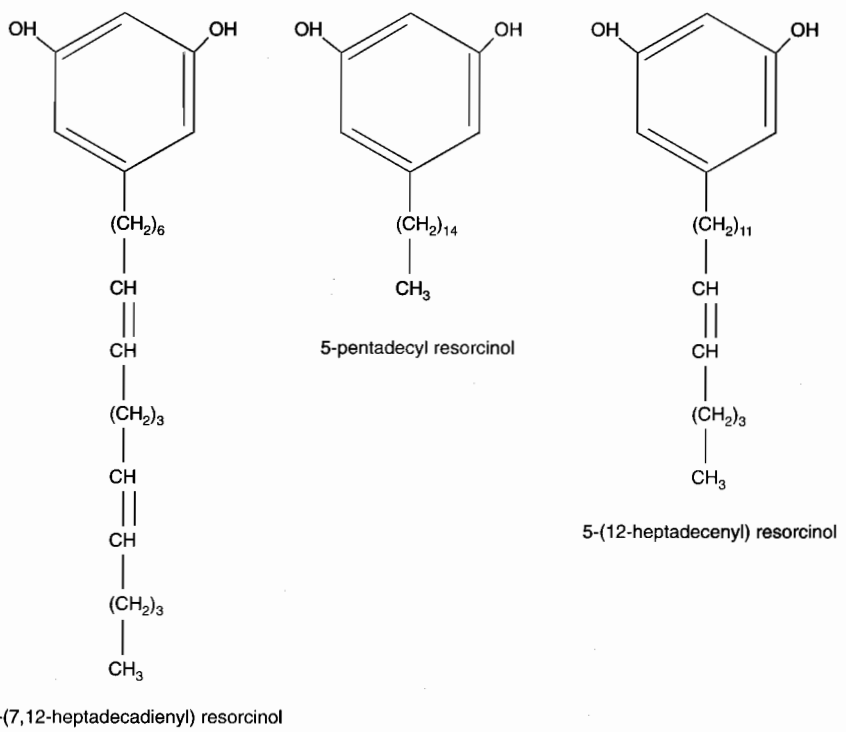

Figure 1. Structure of three resorcinols from mango peel: 5-(7,12-heptadecadienyl) resorcinol; 5-pentadecyl resorcinol; and 5-(12-heptadecenyl) resorcinol.

Preformed Antifungal Compounds

In the mid 1980s, Droby and co-workers (Cojocaru et al. 1986; Droby et al. 1986) described the presence of a mixture of antifungal compounds in mango peel, consisting of substituted resorcinols. The dominant one (about 65%) was 5,12-cis heptadecenyl resorcinol, followed by (about 15%) 5-pentadecyl resorcinol (Fig. 1). The mixture of 5-substituted resorcinols was found by Droby and co-workers in cultivars Haden, Tommy Atkins, Maya, Palmer, and Keitt.

Several experiments suggested the involvement of these two compounds in mango fruit resistance. The mixture is present at fungitoxic concentrations in the peel of unripe mango during the period of fruit growth, and decreases after the harvest and ripening of the fruits (Fig. 2). The concentration becomes subfungitoxic in parallel with the initiation of the decay development.

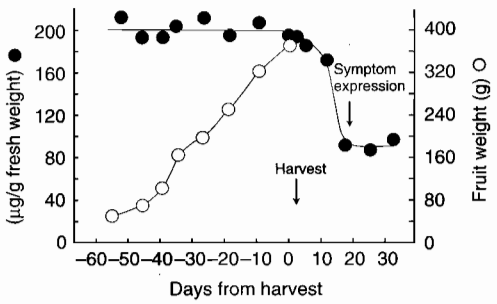

Figure 2. The concentration of antifungal recorcinols during the growing and ripening stages of mango fruit.

In addition, two cultivars, Tommy Atkins and Haden, which differ in the lengths of their latent periods before symptoms develop, were shown to have similar differences in the delays in reduction of their antifungal resorcinols (Fig. 3) (Droby et al. 1987).

From the results of those experiments it can be inferred that there is a relationship between the decrease in the concentration of the antifungal mixture and the initiation of disease.

The search for other putative antifungal compounds has been continued by R. Reved et al. (unpublished data) who purified a new antifungal compound that was identified as 5(7,12-heptadecadienyl) resorcinol (Fig. 1). This compound was extracted from the peel of Tommy Atkins and Keitt mangoes (Prusky et al. 1996) and its concentration did not change significantly after fruit harvest and ripening. Since the 5(7,12-heptadecadienyl) resorcinol was detected in the flesh of mango fruit in similar concentrations, and this tissue was found to be susceptible to disease in unripe fruit (Droby et al. 1987), it was concluded that the 5(7,12-heptadecadienyl) resorcinol is not involved in preformed resistance.

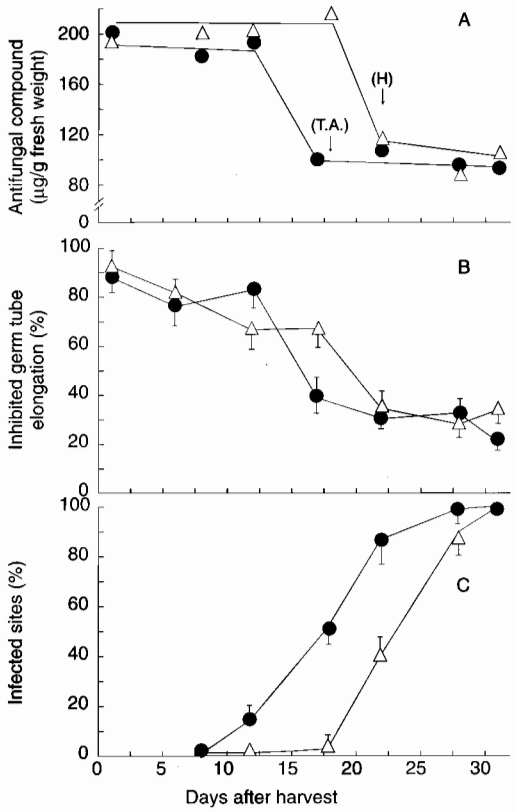

Figure 3. The concentration of antifungal compound in (A) Tommy Atkins (△) and Haden (●) mango fruit, and (B) its effect on germ-tube elongation of *Alternartia alternata* and (C) disease development.

Induction of the Antifungal Activity

Since the concentration of the 5(7,12-heptadecadienyl) resorcinol did not change during ripening, and is present in similar concentrations in the fruit flesh (Prusky et al. 1996), which is susceptible to disease, it may be that the actual concentration of 5(7,12-heptadecadienyl) resorcinol is subfungitoxic in unripe fruit. In order to determine if this new antifungal compound can be inducible and can subsequently delay disease development, several experiments were

carried out to demonstrate its phytoalexinlike activity. When fruits were inoculated at different periods after peeling it was observed that flesh inoculated up to 2 hours after peeling developed symptoms of decay within 48 hours. However, inoculations carried out 7 to 48 hours after peeling were not infected. The results of this experiment suggest that antifungal compounds might be induced in peeled flesh, and might account for fungal inhibitors in similar way to the induction shown by Droby et al. (1987).

Effect of CO_2 on Antifungal Activity

Based on the results of previous experiments (Prusky et al. 1991), in which resistance to *Colletotrichum* in avocado fruit was enhanced by exposing fruit to 30% CO_2 for 24 hours, a similar treatment was tested on mango. Mango fruit cvs Tommy Atkins and Keitt were placed in 30 L jars at 20°C, and exposed to a stream of 100 mL of CO_2/minute for 24 hours. At the end of the treatment, the fruits were inoculated and the development of disease monitored. Fruit cv. Keitt were exposed to 20, 50, and 65% CO_2 for 24 hours, but the disease was delayed only by the 20% treatment (Fig. 4). When fruit of cv. Tommy Atkins, were exposed to 20, 40, and 60% CO_2 (Fig. 5) the highest CO_2 concentration showed the greatest inhibition of the decay symptoms. When the level of 5-(7,12-heptadecadienyl) resorcinol was tested in the peel of Keitt fruits in response to 30% CO_2 treatment, a significant induction was found.

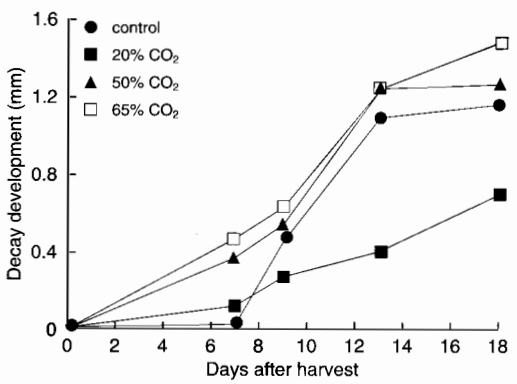

Figure 4. The effect of CO_2 treatments on the development of symptoms of *Colletrichum gloeosporioides* in mango cv. Keitt.

The results of a similar treatment applied to mango fruit in Thailand suggest that CO_2 can prevent anthracnose development also in cv. Nam Dok Mai, but in this case higher concentrations of CO_2 had to be applied and for longer periods.

One of the problems encountered in our experiments in the use of CO_2 as a postharvest treatment is the differential response of mango cultivars, this suggests that the optimal effective concentration for each cultivar has to be checked.

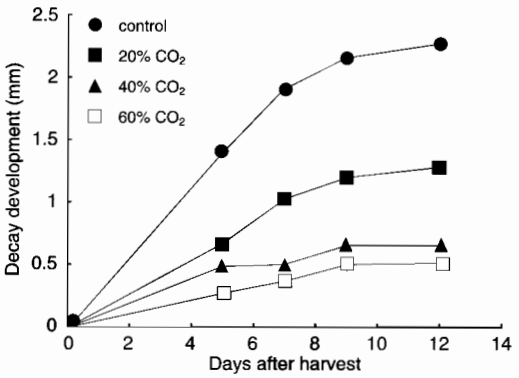

Figure 5. The effect of CO_2 treatments on symptoms development of *C. gloeosporioides* in mango cv. Tommy Atkins.

Effect of Hot Water Dipping on Decay Development

Dipping mango fruit in hot water is a common postharvest treatment for the control of anthracnose in several countries. Five-minute dipping of mango fruit at 55°C delayed decay development, but no delay in decay development was observed if the fruit were exposed to shorter periods of 2, 3, and 4 minutes. Exposure of *Colletotrichum* on PDA to a temperature of 55°C for 5 minutes did not delay its development, which may indicate that the heat treatment is not affecting the pathogen directly. In preliminary results, we were able to show a threefold increase in the concentration of the 5(7,12-heptadecadienyl) resorcinol immediately after the 55°C dip treatment, followed by a second increase several days later. These results suggest that heat treatment may induce an increase in the level of resorcinol compounds.

Control of Postharvest Diseases in Mango by Biotic Elicitation of the Antifungal Compounds

Control of diseases resulting from quiescent infections such as *Colletotrichum* and *Alternaria* could be achieved by inducing resistance in freshly harvested fruit. For this purpose, *Colletotrichum magna*, a pathogen of cucurbits, which is nonpathogenic on mango, was tested for its ability to elicit resistance to postharvest pathogens.

It was found that spot inoculation of mango fruit with *C. magna* inhibited the development of *C. gloeosporioides* inoculated either 2 or 24 hours later.

Dipping the fruit in a spore suspension of *C. magna* before inoculation with *C. gloeosporioides* significantly delayed the development of decay. Parallel extraction of the antifungal compounds from the treated fruit showed a significant increase in the level of the antifungal 5-(7,12-heptadecadienyl) resorcinol in the peel of mango fruis inoculated with *C. magna* (Fig. 6).This suggests that the nonpathogenic strain increased the levels of the 5-(7,12-heptadecadienyl) resorcinol to fungitoxic levels, and thus retarded the development of anthracnose.

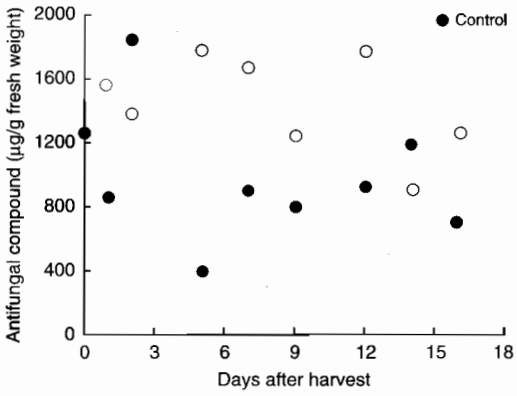

Figure 6. The effect of dipping mango fruits cv. Keitt in a spore suspension of *C. magna* on the concentration of the antifungal 5-(7,12-heptadecadienyl) resorcinol.

The Search for New Antifungal Compounds

A comparison of extracts from the peel of mango cvs Tommy Atkins and Keitt run on TLC chromatography using a chloroform:ethylacetate (70:30) system showed different patterns of inhibition. At least five different inhibition zones could be seen when the plates were sprayed with *Cladosporium* as an assay fungus. This suggests that, in addition to the five substituted resorcinols present in most of the mango cultivars, there are other unidentified compounds specific to the cultivar. Those compounds differ also in their inhibitory activity on fungi, since when the inhibitory zones were checked using *Colletotrichum* and *Alternaria* spores as assays differences in inhibition were observed. All the inhibition zones from Tommy Atkins and Keitt affected spore elongation of *Alternaria*. The most effective band, which caused total inhibition at the concentration checked, was band III from Tommy Atkins with Rf 0.89.

The same extracts tested on *Colletotrichum* were less effective than on *Alternaria*. Furthermore, band I with an Rf of 0.22 from Tommy Atkins and Band II from Keitt with Rf of 0.53 had no effect on *Colletotrichum* at the tested concentration. The compounds in those bands have yet to be identified.

Conclusion

We have demonstrated the presence of a family of preformed 5-substituted resorcinols with antifungal activities in unripe mango fruit.

The concentration of 5,12 heptadecadecenyl and 5 pentadecyl resorcinols decreases to subfungitoxic levels in the peel of ripening fruits, while the concentration of 5(7,12 heptadecadienyl) resorcinol is subfungitoxic in the peel and flesh of unripe fruit. We demonstrated that the antifungal compounds present at subfungitoxic levels can be induced to fungitoxic levels by biotic (a nonpathogenic strain of fungus) and abiotic (CO_2 and heat) elicitors.

Additional, cultivar-specific antifungal compounds are present in the mango fruit, and have yet to be identified.

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Non-resorcinol Antifungal Compounds in Mango Peel (*Mangifera indica* L.)

D. Supyen*, N. Chairangai†, and V. Sardsud§

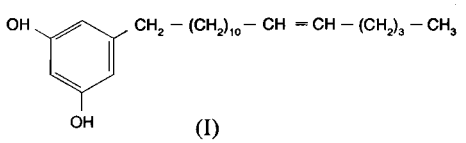
Abstract

An antifungal compound was isolated from the peel of unripe mango ('Chok-anan'). Concentrated ethanol–dichloromethane extract of the peel when bioassayed on thin-layer chromatographic plates (TLC) with conidia of *Cladosporium cladosporioides* produced an inhibition area at $R_f \approx 0.15$. Concentrations of the antifungal compound above 20 μg/μL inhibited growth of the fungal colonies. The crude extract was partitioned on a silica gel column and the antifungal compound was separated by hexane: ethyl-acetate (50:50 v/v) elution and purified again by preparative TLC technique.

Spectroscopic data indicate that these antifungal compounds consisted of two parts: the major component was di-2-ethylhexyl phthalate, the other was a mixture of various alkyl phthalate compounds. However, the same compounds bought from the BDH company could not inhibit the growth of the fungus.

The same experiments were conducted with other parts of mangoes such as leaf, inner layer (mesocarp), and seed. The substances obtained from all these parts were not found to inhibit the fungal colony expansion.

COJOCARU et al. (1986) found two resorcinol derivatives (I) and (II) as performed compounds against *Alternaria alternata* in the peel of unripe Israeli mangoes.



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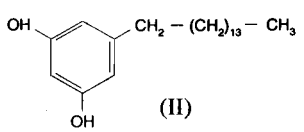

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* Chemistry Department, Faculty of Science, Chiang Mai University, Chiang Mai 50002, Thailand.

† Faculty of Graduate School, Chiang Mai University, Chiang Mai 50002, Thailand.

§ Plant Pathology Department, Faculty of Agriculture, Chiang Mai University, Chiang Mai 50200, Thailand.

In Thai mango peel we found the resorcinol (I) and non-resorcinol compounds as preformed antifungal compounds.

Materials and Methods

Extraction of the crude extract

The mango fruits (cv. 'Choke-anan') at harvest stage (ca 235 g) were taken from Prapat's plantation, Sansai Chiang Mai, Thailand during March to June.

After washing and drying in air, the mangoes were peeled to about 2 mm depth. The peel was blended in cold 95% ethanol and, after immersing in cold ethanol for 30 minutes, the mixture was filtered.

Reduction of the volume to half by evaporation under reduced pressure, and extraction with dichloromethane, gave the required solution of the active compounds. After drying with $MgSO_4$, filtering, and evaporation, the dichloromethane solution gave a crude extract of 15.9 g from 8.9 kg of peel (0.18% recovery).

Purification and identification of the non-resorcinol active compounds

Ten milligrams of the crude extract were subjected to preparative thin-layer chromatography. After developing with a mixture of hexane, ethyl acetate, and methanol (60:40:1) and drying, there were many coloured bands. The spores of the fungus *Cladosporium cladosporioides* were used because this fungus grows very easily and is black. The suspension of the spores (1×10^6 spores/mL) in PDB was sprayed on the developed chromatographic plate and it was incubated in a sterile moist chamber for 24 hours at 22–20°C. Three white zones of inhibition could be seen in the black background (Fig. 1) at $R_f = 0.66–0.70$, 0.40–0.46, and 0.10–0.23. The zone at R_f 0.40–0.46 contained recorcinols (Fig. 2). The compounds in the zone $R_f = 0.10–0.23$ were scraped off and extracted with a mixture of dichloromethane and methanol (1:1). After filtering and concentration the solution was subjected to GC–MS analysis (see Figs 3–6). Peak 3 (Fig. 3) was a major component (80%).

Figure 1. Chromatogram of the crude extract of unripe mangoes, showing the pronounced inhibition zone at R_f 0.10–0.23 (nonresorcinol) and the less pronounced zones at 0.40–0.46 (resorcinols) and 0.66–0.70 (unknown).
$R_f = 0.66–0.70$
$R_f = 0.40–0.46$
$R_f = 0.10–0.23$

Obtaining further compounds for ^{1}H–NMR

Five grams of the crude extract were purified by column chromatography using silica gel (60 mesh) as an absorbent and eluting with a mixture of ethyl acetate in hexane, increasing the concentration of ethyl acetate. The active compounds (tested by TLC–bioassay) were extracted at a concentration of 50% ethyl acetate in hexane.

Purification by column chromatography gave 2 g (40% yield) of the active compounds. From 2 g, preparative thin-layer chromatography (silica gel and the same developing solvent as above) twice gave 0.24 g of the active purified mixture.

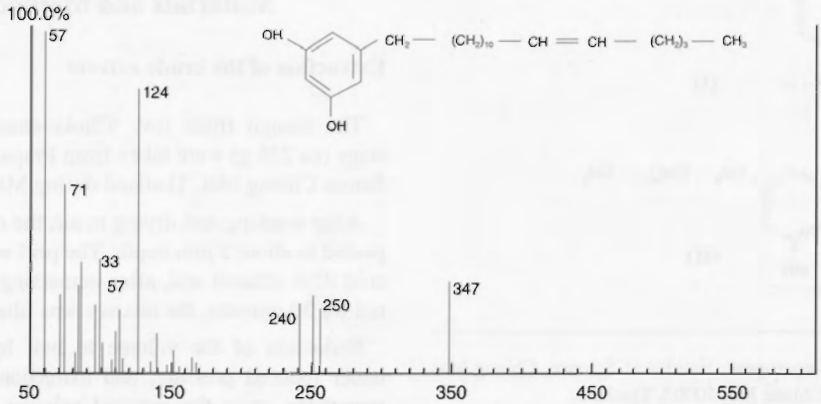


Figure 2. Mass spectrum of antifungal compound band at R_f 0.40–0.46.

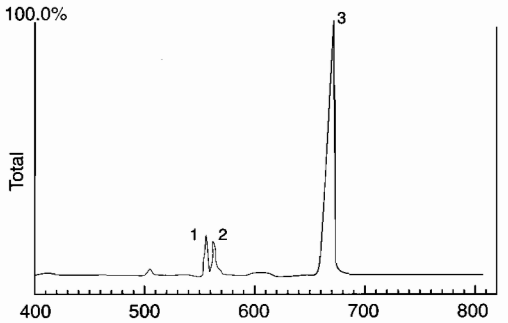


Figure 3. Total ion chromatogram of the compounds in the inhibition zone 0.10–0.23.

The ^{1}H–NMR spectrum of the purified mixture (20 mg) was obtained in $CdC1_3$ and using TMS as internal reference (Fig. 7). The GC–MS spectra were similar to those shown in Figures 3–6. They were recorded using an ionisation method (EI), with an ionisation voltage of 70 eV and ion source temperature of 250°C. Analyser rods were of the hyperbolic quadrupole type. Sample inlet had split time 0.50 min. with He (0.8 kg/cm^2) as the carrier gas. The conditions for gas chromatography in the instrument were as follows:

- column: type DB–1 (100% dimethylpolysiloxane), J&W 122–1032, length 30 m, ID 0.25 mm, film thickness 0.25 mm.
- temperatures: injection 240°C, detector 280°C, initial temperature 120°C, initial time 3 min., rate increase in temperature from 80 to 180°C = 20°C/min. and from 180° to 240°C = 15°C/min.
- Final temperature 240°C
- Final time 10 min.

The infrared spectrum was obtained by neat oil applied to the NaCl cell disc (Fig. 8). The UV spectrum was recorded in the 95% ethanol solution compared with ethanol 95% (Fig. 9). The minimum inhibitory concentration (MIC) of the active fraction was accomplished according to the standard method using *C. cladosporioides* as an assay organism. It was found that MIC was 60 µg or 20 µg/µL. Other parts of mango such as leaves, inner layer (mesocarp), and seeds were studied by the same method and found not to inhibit the fungus.

Analytical instruments

^{1}H–NMR spectrum was recorded on a Bruker AM–300 Fourier transform instrument using TMS as internal reference in $CdC1_3$ solution. GC–MS spectra were recorded on a Shimadzu QP 2000a instrument. The infrared spectrometer was a Jusco, USA model IR 810, and the UV spectrometer was a Shimadzu, Japan UV 265 instrument. The haemacytometer was from Clay–Adams, New York, USA. Dichloromethane, 95% ethanol, and methanol were technical grade and were used without distillation.

Results and Discussion

The crude extract was obtained, purified, and separated as noted above. A suspension of spores of the fungus *Cladosporium cladosporioides* (using PDB as a carbon source) was sprayed on the separated TLC-plate. After incubation for 24 hours in a sterile box (80% RH) at 22–25°C, the zones of inhibition were found (Fig. 1). The zone at R_f = 0.40–0.46 contained resorcinols (Fig. 2). The zone at R_f = 0.10–0.23 contained non-resorcinol compounds. The silica gel of this band was scraped out and extracted with a 1:1 mixture of dichloromethane and methanol. After drying, filtering, and evaporation a partially purified mixture was obtained. Examination by gas chromatography and mass spectrometry (GC–MS) indicated that it contained at least three compounds (Fig. 3), the most abundant (80% of mixture) had a highest mass of 279 (Fig. 4). The minor peaks (1 and 2 in Fig. 3) had similar mass spectra as that of peak 3 (Figs 5 and 6).

Five grams of the crude extract were purified first by column chromatography then twice by preparative thin layer chromatography to give 0.24 g of the active compounds. Subjected to GC–MS analysis the active fraction contained three compounds whose spectra were similar to those in Figures 3–6. The ^{1}H–NMR (Fig. 7) shows four aromatic protons at 7.4–7.8 ppm, four methylene protons in the ester group at 4.2 ppm, and many aliphatic protons at 0.8–1.8 ppm. The infrared spectrum showed the ester carbonyl stretching frequency at 1740 /cm. The ultraviolet spectrum of the active fraction (Fig. 8) shows the aromatic ring conjugated with the carbonyl group at λ_{max} 274 and 225 nm. The outcome of fragmentation of the major component (peak 3) is shown in Figure 9 (Safe and Hutzinger 1973). All of the peaks in Figure 3 show in their mass spectra the fragment ion m/e 149 which is a characteristic of a dialkyl phthalate (Figs 5 and 6).

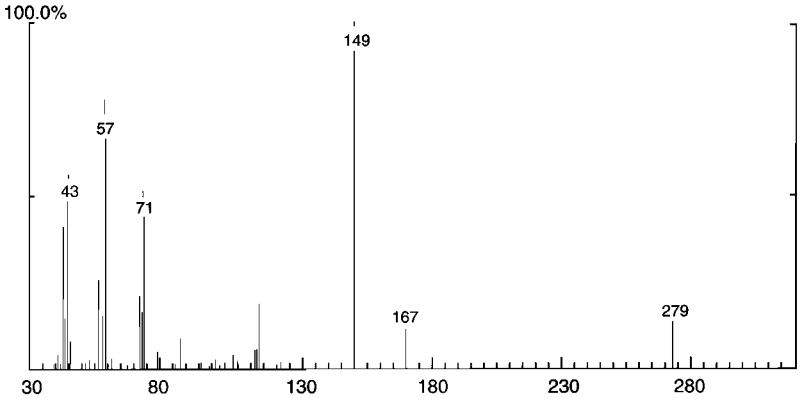


Figure 4. Mass spectrum of peak 3 in Figure 3.

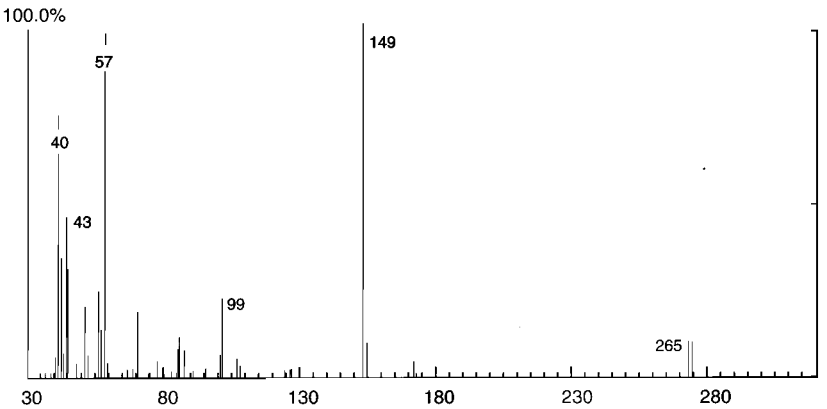


Figure 5. Mass spectrum of peak 1 in Figure 3.

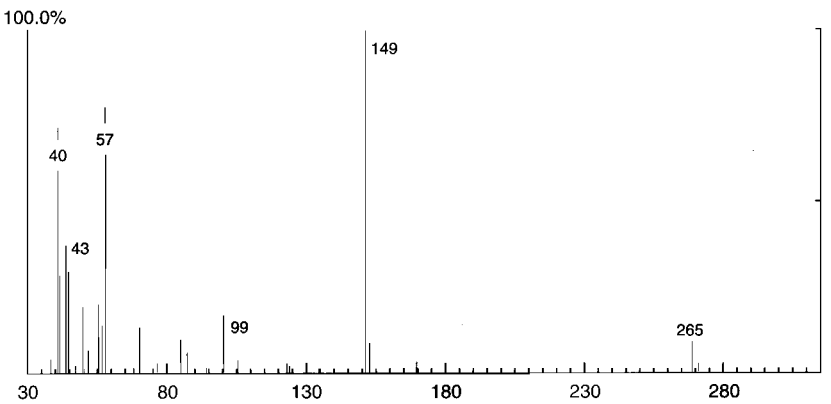


Figure 6. The mass spectrum of peak 2 in Figure 3.

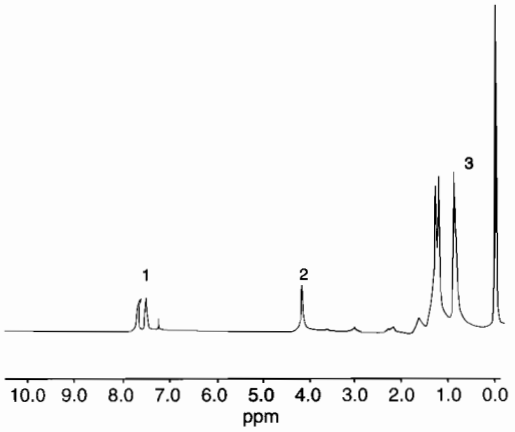


Figure 7. ^{1}H–NMR spectrum of the active fraction which contained mainly of peak 3 in Figure 3.

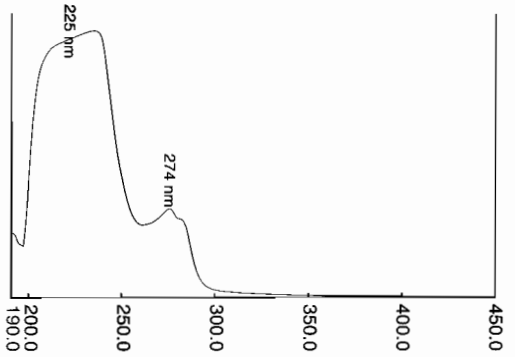


Figure 8. UV spectrum of the active fraction in 95% ethanol.

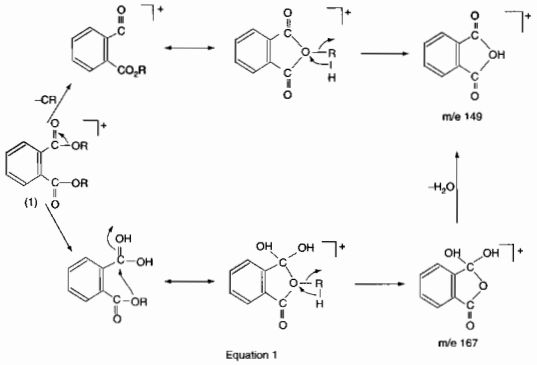
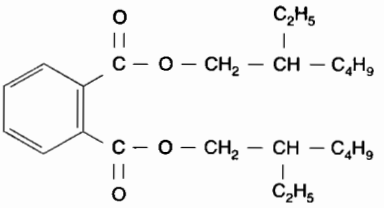


Figure 9. Fragmentation pattern of the major peak 3 in Figure 3.

From the comparison of the mass–spectrum of the major component (Fig. 4) with that of the di–2–ethyl-hexyl phthalate (Fig 10; Safe and Hutzinger 1973), and other spectroscopic evidence, it was concluded that the major component was di–2–ethylhexyl phthalate (I) and the minor components were dialkyl phthalate derivatives.



di-2-ethyl-hexyl phthalate

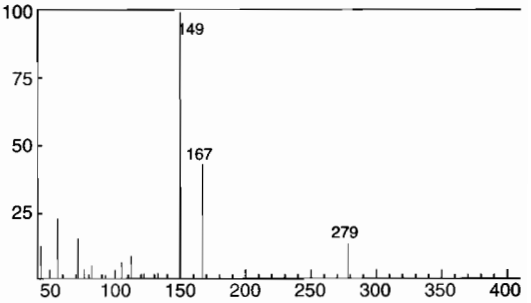


Figure 10. Mass spectrum of di–2–ethylhexyl phthalate (from Safe and Hutzinger 1973).

However, a formulation of this compound from the BDH company failed to inhibit fungal growth.

We concluded that either the asymmetric carbon atoms in the compound may be inhibiting the fungus, or the naturally active compounds may not be the phthalate but other alkyl phthalates appearing as smaller peaks in the chromatogram, or other minor peaks whose mass spectra were not recorded.

By TLC, the compound could not be detected as a contaminant in the solvents used. The active fraction was employed to determine the minimum inhibitory concentration to the fungal colonies. This was 20 μg/μL.

The same experiments were run with other parts of mangoes such as leaves, inner layer (mesocarp), and seed. None of these parts was found to inhibit the fungus.

Acknowledgments

Part of the work was supported by the Australian Centre for International Agricultural Research. The work was a part of Neeranuch Chaiwansri's MSc thesis (1996) project at Chiang Mai University.

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Safe, S. and Hutzinger, O. 1973. Mass spectrometry of pesticides and pollutants. Boca Raton, CRC Press, 87–88.

Editors' Note

There was some discussion during the workshop of the possibility of the primary compound identified in these studies being a contaminant from the experimental apparatus.

Resistance of Immature Papaya (*Carica papaya* L.) Fruit to Fungal Infection: an Overview

N.K.B. Adikaram*, A. Karunaratne*, S.R.P. Indrakeerthi*† and P. R. Menike*§

Abstract

Fungistatic tannins and phenolics have often been reported from papaya fruits. Benzyl isothiocyanate, a preformed fungal inhibitor, was detected in the latex, fruit pulp, and seed, but its significance in fruit resistance is not clear. Another protein that inhibits pectinase enzyme produced by *Colletotrichum gloeosporioides* has also been detected in papaya cell walls, the activity of which was higher in the unripe fruit. No phytoalexins have, however, been reported from papaya. Immature papaya fruit possess a milky latex, a complex mixture of enzymes—notably proteases, glycosidases, and lipase—and simple sugars. The water soluble fraction of papaya latex can completely digest the spores of many pathogenic fungi (e.g. *C. gloeosporioides*) upon brief exposure in vitro. Spores of certain other fungal species when exposed to latex loose their viability. A few fungi, for example *Rhizopus* spp., however, could grow profusely in the presence of latex. The fungicidal effect of latex is due to the degradation of polysaccharide constituents in the fungal cell wall. The differential sensitivity of fungi to latex was related to their hyphal wall composition, particularly to the presence and amount of chitin. The conidia of *C. gloeosporioides*, which undergo complete digestion in the presence of latex, contain chitin-glucan in the wall and the chitin fraction is broken down by the enzyme N-acetyl-beta-D-glucosaminidase in the papaya latex. On the other hand *Rhizopus* spp., which contain more chitosan, a substance which is not degraded by this enzyme, thrive well in latex. Immature papaya fruit contain latex in the laticifers but during ripening it disappears. Those fungi that are sensitive to latex are unable to rot papaya fruit until ripening and *C. gloeosporioides* is an example of this, whereas fungi such as *Rhizopus arrhizus* which can grow profusely in the latex develop large rots in both unripe and ripe fruit.

MORE than 15 fungal diseases have been reported from papaya (*Carica papaya*) fruit, most of them affecting the harvested and ripe fruit. The most important diseases that affect ripe papayas are: anthracnose (*Colletotrichum gloeosporioides*, *C. capsici*); stem-end rot (*Botryodiplodia theobromae*, *Phomopsis caricae-papayae*), black rot (*Mycosphaerella caricae* H. and P. Sydow); phytophthora rot [*Phytophthora palmivora* (Butler) Butler]; Rhizopus rot [*Rhizopus stolonifer* (Ehrenb. ex Fr.) Lind]; Alternaria rot (*Alternaria alternata*); Cladosporium rot (*Cladosporium herbarum*), and Fusarium rot [*Fusarium equesiti* (Corda) Sacc.]. Other papaya diseases of minor importance include greasy rot (*Corynospora cassicola*), pink mould rot (*Trichothecium roseum*), stemphylium rot (*Stemphylium botryosum*), and cercospora rot (*Cercospora pappaea*) (Snowdon 1990). Anthracnose originates from quiescent infections in uninjured immature fruit, but most of the other fungi infect injured ripe fruit.

During their early stages of development, papaya fruit are resistant to fungal pathogens. The incidence of disease increases with ripening and a concomitant decrease in latex content (Dharmasiri 1988; Hine et al. 1965). In this paper, the factors underlying the resistance of immature fruit to fungal disease are discussed, with special reference to latex.

* Department of Botany, University of Peradeniya, Peradeniya, Sri Lanka.

† Present address: National Packaging Center, Transworks House, Lower Chatham Street, Colombo 1, Sri Lanka.

§ Present address: Wall's Ceylon Ltd, Banduragoda, Sri Lanka.

Antifungal Substances in Papaya

Papaya fruit contain benzyl isothiocyanate in latex, pulp, and the seed (Tang 1973). In vitro studies have demonstrated that isothiocyanates have antifungal activity, raising the possibility that they might be involved in fruit resistance to fungal diseases (Patil et al. 1973). Leachates collected from healthy papaya fruit, both unripe and ripe stimulated germination of conidia of *Colletotrichum gloeosporioides*, the cause of anthracnose disease. Leachates collected from the unripe fruit following inoculation with conidia of *C. gloeosporioides* were slightly inhibitory (Dharmasiri 1988). This was thought to be because of the accumulation of postinfectional antifungal activity. Further examination of *C. gloeosporioides*-infected tissue following solvent extraction, however, failed to establish its presence (Dharmasiri 1988). Very recently, a phytoalexin, danielone, was isolated from papaya fruit slices following treatment with copper salts (Echeverri et al. 1997). The structure was established as 3',5'-dimethoxy-4'-hydroxy-(2-hydroxy)acetophenone. The compound showed high antifungal activity against *C. gloeosporioides* but its significance in fruit resistance to fungal infection has not been investigated.

A cell-wallbound protein separated from unripe papaya fruit was found to inhibit the pectinase enzymes of *C. gloeosporioides* and *C. capsici*, both of which cause anthracnose disease. There was a correlation between the decline of fruit resistance and the inhibitor content during ripening, suggesting a possible role of the proteinaceous inhibitor in the resistance of immature fruit to these two pathogens (Dharmasiri 1988).

Properties of Papaya Latex

Different parts of the *C. papaya* plant—the stem, leaf, petiole, and fruit—contain a milky substance referred to as latex. The dried form of latex obtained by tapping the immature fruit, commonly known as papain, is available commercially. Papaya latex possesses antifungal (Dharmasiri 1988; Giordani et al. 1996), antibacterial (Thomas 1989), nematicidal (Siddiqui et al. 1987), and anthelmintic activities (Satrija et al. 1994). Papaya latex and papain are extensively used in the pharmaceutical, food, textile, and tanning industries (Raman and Seemanthani 1968).

Proteolytic Activity

Latex from unripe papaya fruit contains several proteinase enzymes, but only one, papain, has been extensively characterised (Goodenough and Owen 1987). Papain was originally thought to be a mixture of two proteolytic enzymes, mainly papain and chymopapain (Balls and Lineweaver 1939). Subsequent reports indicated that papaya latex contains four major proteases. Goodenough and Owen (1987) classified the proteinase enzymes in papaya latex into papain (pI 8.75, MW 23 kdaltons), chymopapains A and B (pI 10.3–10.4 and 10.6–10.7, respectively, MW 24), papaya proteinase A (pI 11.0, MW24), and papaya proteinase beta (pI 11.0, MW 28). Two isozymes were purified from papaya latex by polyacrylamide gel electrophoresis: the isozyme-3X contains five components, while the isozyme-E appears to be homogeneous (Howard and Glazer 1967). Three cysteine proteinases, i.e. chymopapain, papaya proteinase IV, proteinase III, were purified to homogeneity from papaya latex using a combination of ion exchange chromatography and hydrophobic interaction chromatography (Dekeyser et al. 1994). Papaya latex can digest fungal structures and plant cell walls. Its lytic action has been attributed to the presence of proteolytic enzymes (Hine et al. 1965; Dharmasiri 1988).

Glycosidase Activity

Another striking finding was the very high chitinase activity of papaya latex (Howard and Glazer 1967). Recent studies have shown that the water soluble component of *C. papaya* latex exhibits glycosidase activity on nine different glycosides: '*p*NP-N-acetyl-b-D-glucosaminide, *p*NP-α-L-arabinoside, *p*NP-β-fucoside, *p*NP-α-D-galactoside, *p*NP-α-D-glucoside, *p*NP-α-D-mannoside, and *p*NP-β-xyloside (Giordani and Lafon 1993; Roger and Laurence 1993). Among these, N-acetyl-β-D-glucosiminidase (chitinase) activity was found to be very high. It was also suggested that the papaya latex might be classified as a chitinase rather than lysozyme.

The chitinolytic enzyme system is composed of chitinases (EC 3.2.1.14), chitobiosidases (Tronsmo and Harman 1993), and N-acetyl-β-glucosaminidase (EC 3.2.1.52). Chitinases cleave glycosidic linkages randomly along the chitin chain, eventually giving N,N-diacetyl- β-D-chitobiose $[GlcNAc)_2]_n$ as the end product (Monreal and Reese 1969), while

chitobiosidases cleave $(GlcNAc)_2$ units from the non-reducing end of the polysaccharide chain (Tronsmo and Harman 1993). The dimer $(GlcNAc)_2$ is further hydrolysed to GlcNAc by N-acetyl-β-glucosaminidase (Monreal and Reese 1969).

Though chitinase activity has been found in a number of plants (Abeles et al.1970; Pegg 1977; Pegg and Young 1982; Benhamau et al. 1994) the physiological role of the enzyme in general metabolism of plant cells has not been documented. It is difficult to envisage a general role for chitinase because its substrate, chitin, does not occur in higher plants. Chitinase in papaya latex may be important in the plant's defence against invasion of fungi, as a vast majority of fungal pathogens have a chitin–glucal cell wall, both in their mycelium and the spores (Bartnicki-Garcia 1968; Abeles et al. 1970).

Tributyroylglycerol Hydrolase Activity

From a commercial homogenate of spray-dried latex obtained from *C. papaya*, tributyroylglycerol activity has been detected. The activity was found in the particulate fraction obtained following centrifugation, and not in the soluble supernatant (Giordani et al. 1991).

Fungicidal Property of Papaya Latex

Papaya latex displays a high degree of fungitoxicity (Saxena and Saksena 1981; Indrakeerthi and Adikaram 1995). The nature and extent of fungitoxicity, however, vary with the fungal species. The fungal species tested in this study responded to latex in four different ways. The latex was highly destructive to certain species, the conidia being completely digested within a short time. These included several *Colletotrichum* species causing postharvest diseases in important fruit crops, viz. *C. capsici*, *C. gloeosporioides*, and *C. musae*. When the conidia of *C. gloeosporioides*, *C. musae*, *C. capsici*, and *Fusarium semitectum* were exposed to diluted latex (10 times), the walls of conidia began to dissolve gradually during the first 60 seconds. Within about 10 minutes the walls of most conidia completely disintegrated. By this time the conidia had lost their shape and integrity and appeared as irregular, fragmented masses. Digestion continued and after 30 minutes only smaller particles remained. The digestion of conidia of *F. semitectum* was observed to be somewhat faster than the conidia of three *Colletotrichum* spp. The digestion of conidia of *C. gloeosporioides* took place in papaya latex diluted up to 100 times and was most efficient at pH 5–7.

Conidia/sporangiospores of several other species of fungi were neither digested nor did they germinate on prolonged incubation with diluted latex. To examine if these species retained viability following exposure to latex, the conidia were washed with sterile distilled water several times after latex treatment and allowed to germinate. Of these fungi, *V. theobromae*, *B. theobromae*, *Nectria* sp., *Sodaria musae*, *Phytophthora palmivora*, *Trichoderma* sp., and *Phyllosticta musarum* failed to germinate, showing that the effect of latex was permanent. Other species—*Penicillium* sp., *Phoma* sp., *Pestalotiopsis* sp. and two *Aspergillus* spp.—showed a germination rate of about 50% of that of their respective controls.

The spores of *Rhizopus arrhizus* and *Alternaria* sp. could germinate rapidly and produce mycelium in the presence of papaya latex. The mycelial growth of these two fungi increased with an increase in latex percentage.

Role of Papaya Latex in the Differential Rot Development by Three Fungal Species in Papaya Fruit

Three fungal pathogens of papaya which behave differently with respect to their modes of infection and the stage of fruit maturity they prefer were selected for the study. *C. gloeosporioides* establishes quiescent infections in the immature fruit by direct cuticular penetration. These develop into progressive anthracnose lesions only after fruit ripening. *Phytophthora palmivora* can infect fruit at any stage of maturity, but it invariably requires a wound in order to cause infection of immature fruit. *Rhizopus arrhizus* can cause rotting in detached papaya fruit at any stage of maturity, provided there is substantial wounding on the surface.

The effect of exposure of propagules of the three pathogens to papaya latex at different concentrations was compared in vitro. Suspensions of conidia, appressoria, and hyphae of *C. gloeosporioides* were separately mixed with equal volumes of the water-soluble fraction of papaya latex at different dilutions. Aliquots were examined under the microscope at regular intervals. Suspensions of conidia and mycelium were prepared by suspending mycelium scraped from colonies in sterile distilled water and filtering through glass wool. The filtrate contained conidia whereas the

residue after repeated washing gave hyphae. Young and mature appressoria were obtained by allowing the conidia to germinate on glass slides for 6 and 24 hours, respectively. Latex was collected from immature papaya fruit attached to the tree by making incisions using a razor blade. The water soluble fraction was separated by centrifugation (1000 g for 5 min.) followed by filtration (Whatman No. 1). Different dilutions were prepared by mixing with sterile distilled water. In latex the conidia of *C. gloeosporioides* were digested rapidly (as described earlier). Conidia were digested in latex diluted up to 100 times, but the time taken for digestion increased with dilution. The digestion of hyphae of *C. gloeosporioides* also took place in the presence of latex at a similar time frame. First the contents of hyphae were clumped together, following the dissolution of hyphal wall. The diluted papaya latex could digest the young appressoria of *C. gloeosporioides*, but somewhat more slowly than they could conidia. The mature appressoria could, however, resist latex, remaining intact in latex for up to 6 hours. Subsequent tests showed that they could also germinate.

Zoospores of *P. palmivora* ceased mobility within one minute of exposure to diluted latex (25%) and thereafter became totally disorganised. When the sporangia were exposed, they became swollen within 5 minutes, and then their contents began to clump, but no change was observed in the sporangial wall. The clumped sporangial contents were subsequently released through the papillae, and empty sporangia with intact walls could be seen. The papillae appeared to have been dissolved. Papaya latex had no visible effect on the hyphae of *P. palmivora*. Hine et al. (1965) found that low concentrations of papaya latex and papain not only stopped the motility of zoospores of *P. parasitica* but also could burst them. According to them, papain, the main substance in latex reduced in concentration from 1.28 units/g to 0.27 units/g during ripening, while the fruit susceptibility increased.

Papaya latex did not damage the hyphae, sporangia or sporangiospores of *R. arrhizus*. Rather, the sporangiospores could germinate in all the dilutions of latex tested and produce extensive mycelial growth. In fact, a higher percentage germination of spores and a greater mycelial growth were observed with an increase of latex concentration.

The Relationship between the Susceptibility to Latex and Hyphal Wall Composition

The polymers present in the hyphal walls of fungi vary not only between species but also at different stages in the life cycles of individual species (Bartnicki-Garcia 1968). In most fungi, the hyphal wall has a distinct microfibrillar texture on the inner face and appears amorphous on the outer face. The microfibrillar components are made up of chitin in most fungi but in Oomaycetes they are made up of cellulose and beta-glucan. Proteins and polysachharides such as glucans, mannans, and galactans cement the structural components together (Hunsley and Burnett 1970). Chitin is constituted entirely of β-1,4 linked N acetylglucosamine, and the long chains of units may achieve a molecular weight similar to that of cellulose. Chitin, which consists of β-1,3 and α-1,4 linked monomers, makes up from 3 to 60% of the dry mass of the fungal cell wall (Hudson 1986). In certain fungi, chitosan, which is a deacetylated form of chitin, is found. The acetyl content of chitosan varies from a relatively high to nil (Foster and Webber 1960). Chitosan occurs naturally in the order Mucorales (Aronson 1965).

The fungicidal effect of latex was thought to be due to the degradation of polysaccharide constituents in the fungal cell wall. The differential sensitivity of fungi to latex may perhaps be related to the hyphal wall composition, particularly to the presence and amount of chitin. In order to clarify this, mycelial mats of three fungi grown in liquid cultures were analysed. Cell wall materials were extracted as described by Anderson and Albersheim (1975) and freeze dried. The initial tests were conducted for the presence of chitin/chitosan in the extracts by the following procedure: extracts were separately placed in boiling KOH (saturated) for 30 min. (to acetylate chitin to chitosan), removed and washed with 90% alcohol and stained with I_2/KI. A red-violet colour was taken to indicate chitosan. These tests indicated that chitin/chitosan was present in the extract of *C. gloeosporioides* and *R. arrhizus* but not in *P. palmivora*.

Gas liquid chromatography (GLC) was used to determine chitin in the cell walls, following hydrolosis with 4N HCl for 4 hours at 100°C in a heating

chamber to yield N-acetyl glucosamine. After neutralisation with $AgCO_3$, the solution was centrifuged, and the supernatant containing the monomer was dried. This was reduced with $NaBH_4$ and acetylated. Concentrated samples (5 μL) were injected into a GLC apparatus (Varian-3000 with flame ionisation detector) with DB–1 column (final column temperature 275°C; injector and detector temperatures were 250°C). The retention time for N-acetyl glucosamine in the test samples was compared with a standard sample. Acetylated test samples combined with standard N-acetyl glucosamine were injected for confirmation.

The retention time observed for standard N-acetyl glucosamine was 14.752 minutes. The chromatographs showed peaks corresponding to retention times of 14.837 and 14.803 minutes, respectively, for extracts of *C. gloeosporioides* and *R. arrhizus,* indicating the presence of the monomer. Chromatographs for combined (test samples plus standard) injections showed aggregate peaks at retention times 14.820 (*C. gloeosporioides*) and 14.798 (*R. arrhizus*), confirming the presence of N-acetyl glucosamine in the test samples. Further, the major monomer by hydrolysed samples of cell walls of both fungi was found to be this. The extract from *P. palmivora* did not show peaks at this retention time.

Determination of Chitinase and N-acetyl β-D-glucosaminadase Activity in the Papaya Latex

Chitinase activity of papaya latex was determined by a method described by Boller and Mauch (1988) using chitin (poly-1,4-β-D-N-acetyl glucosamine; from crab shell; Sigma) as the substrate. The water-soluble fraction of papaya latex was prepared in 1M sodium acetate buffer (pH 5). The reaction was carried out at 37°C for 2 hours. The control tubes had boiled papaya latex in the buffer. The hydrolysed reaction products were analysed by GLC (Varian-33000 with flame ionisation detector) with DB–1 column (final column temperature 275°C; injector and detector temperatures were 250°C). The chromatogram of the reaction mixture had a prominent peak at a retention time 14.789 min. which is comparable with a standard sample of N-acetyl glucosamine (14.755 min.). A combined injection of the reaction mixture and the standard had a retention time of 14.801.

N-acetylglucosaminidase activity of papaya latex was determined as described (Giordani and Lafon 1993) using *para* nitrophenyl glucosaminide as the substrate. The amount of *para* nitrophenol released by the enzyme action was measured by spectrophotometer at 400 nm. To determine the Michaelis constant (K_m) of N-acetylglucosaminidase, five concentrations of PNP-glucosaminide were used. The results indicated that the papaya latex exhibits N-acetyl- β -glucosaminadase activity equivalent to 0.22 U/mg protein [one unit (U) was defined as the amount of enzyme that utilises 1 micromole of substrate per minute at 30°C]. The K_m value for the enzyme was determined to be 8.3 mM/mL. Giordane and Lafon (1993) found N'-acetyl β-D-glucosaminadase activity in dried papaya latex equivalent to 28 U/mg protein.

These studies have shown that the conidia and mycelium of *C. gloeosporioides* contain chitin in the hyphal wall, and that the papaya latex has chitinase and N-acetyl b-glucosaminadase activity. The digestion of conidia when exposed to the latex of the fungus could be attributed to these enzymes. Young appressoria which are lighter in colour and thinner walled than the mature ones were also digested in a similar way, but at a slower rate. The mature appressoria were not digested. As the appressoria mature, wall components become thick and multilayered; the chitin-like components are overlayed by melanin or extracellular mucilage (Kleinschuster and Baker 1974). Bourett and Harward (1990) have suggested that chitin is masked by melanin in the appressorial wall of *Magnaporthe grisea.* The results presented here have also shown that the walls of sporangia and hyphae of *R. arrhizus* contain chitin. According to Bartnicki-Garcia (1968), Mucorales members contain higher percentages of chitosan than chitin. The tolerance of *R. arrhizus* to latex might be because there is more chitosan than chitin in the cell wall, or because to chitins are buried in chitosan thus not readily accessible to chitinase. Nevertheless, the analytical methods used in our studies did not determine chitin and chitosan separately, as both chitin and chitosan following hydrolysis were finally acetylated to the same alditol structure. Another possible explanation is the dark brown coloration of the mycelial wall of *R. arrhizus*, a result perhaps of the deposition of a melanin-like substance making chitin inaccessible to enzyme action. Melanin is universally associated with dark colour of skin, insect cuticle, fungi, and actinomycetes (Burnett 1971). The sporangial and hyphal walls of *P. palmivora* also showed resistance to papaya latex. The latex could dissolve the papillae present at the tip of the sporangia, resulting extrusion of protoplasm. Zoospores, which lack cell walls, how-

ever, were destroyed within a brief exposure. The sporangia and hyphae of this fungus did not have chitin in the walls, showing the lack of sites for enzyme action. Normally, *Phytophthora* spp. contain glucans and cellulose to about 90% of the dry mass (Hudson 1986) and chitin is absent in appreciable amounts at any stage of life cycle (Bartnicki-Garcia 1968).

From these experiments we have been able to establish a correlation between latex and rot development in three important pathogens of papaya. *C. gloeosporioides* which is sensitive to latex and unable to infect unripe fruit containing latex, could cause progressive rot in ripe fruit which are devoid of latex. *P. palmivora*, which is tolerant to latex, can initiate rots at any stage of fruit development. *R. arrhizus* is also tolerant, in spite of the presence of chitin/chitosan which is apparently masked by other cell wall constituents, and could therefore initiate rot in fruit at any maturity stage. *Rhizopus* species are generally weak pathogens showing a strong tendency for saprophytic growth and may be able to use latex and its constituents as an energy source for growth much more rapidly than any possible counteraction by the latex.

The study has also revealed that those fungi which have a higher chitin–glucan ratio and in which chitin is in accessible form are readily digested by the papaya latex. This was supported by our subsequent studies, in which *Fusarium* sp., *Phomopsis* sp. and *Cladosporium* sp. which contain chitin were also found to be easily digested by papaya latex. It would be interesting to examine this trend in other fungal pathogens that cause infections in papaya fruit.

Papaya Latex as a Fungicide

In spite of the high fungicidal properties, papaya latex has not been used in plant disease control. The investigations carried out in our laboratory have revealed that it has good potential for use as a post-harvest fungicide.

Effect of treatment with papaya latex on the development of crown rot in boxed bananas

In Sri Lanka, *Fusarium semitectum*, *C. musae*, *Verticillium theobromae*, *B. theobromae*, and *C. gloeosporioides* are the main causes of banana crown rot. A mixed suspension of conidia of these pathogens, when applied to crown tissue in the laboratory, generally causes development of initial crown rot symptoms within 2–3 days. The infection progresses rapidly, and within another 4–5 days most of the crown tissue and the finger-stalks become affected. By this time the fruits have ripened fully.

Two sets of bananas, each containing four hands (dehanded about 1 hour before the experiment) of unripe fruits (var. 'Anamalu', AAA group), were obtained. The crown tissue was inoculated with a mixed suspension of conidia of *Fusarium* sp., *C. musae*, *C. gloeosporioides*, *V. theobromae*, and *B. theobromae* (equal numbers of conidia from each species and a total of 4×10^3 conidia/ mL) by application of the suspension over the exposed crown tissue using a spatula. The water-soluble fraction of the papaya latex (diluted 10 times) was later applied by paint brush to the crowns of one set of inoculated banana hands. The second set of inoculated banana hands was treated with sterile distilled water as a control. The treated and control hands were packed in standard boxes and incubated at room temperature for one week. The extent of crown rot development was recorded daily as a percentage crown tissue affected (0–100%).

In the crowns that were treated with papaya latex the initial signs of crown rot were noticeable only after 9 days. The treated crowns showed a dark, grey-black coloration within the first 5 days, and the crown tissues remained hard during this period. Hardening and blackening of the latex-treated crown tissue may have resulted from the action of the latex, and may also have provided a mechanical or perhaps chemical barrier to pathogen invasion. In certain trials, the fruit of latex-treated crowns took longer to ripen than the untreated controls but this was not consistently observed. The latex applied to the crowns remains active for several days.

The effect of delay between dehanding and latex treatment was tested by applying latex at different times after dehanding. Seven hands of bananas with freshly exposed crown tissue were inoculated immediately after dehanding, with a mixed suspension of conidia of the five fungi as above. One inoculated hand was treated with latex soon after inoculation. The others were similarly treated with latex at 5, 15, 25, 35, and 45 minutes after dehanding. One hand treated with sterile distilled water served as a control. The treated and control hands were incubated and the development of crown rot recorded. The results showed that the crowns treated with latex within the first 35 minutes of dehanding developed crown rot, but to a lesser degree than the untreated controls. Latex applied 45 minutes after dehanding gave effective control of the disease.

We have since found that the watery exudate (banana latex) present in the exposed crown tissue of banana reacts with papaya latex, reducing the fungicidal activity of the latter. Papaya latex should thus be applied only after all banana latex has been removed.

Treatment of unripe wood apples (*Limonia acidissima*), papaya, and avocado fruit

Unripe wood apples are generally free of fungal growth at the time of harvest but a succession of fungi appears on the fruit shell, and sometimes in the pulp, during ripening (Adikaram et al. 1989). Freshly harvested, unripe fruit (20) of wood apple were dipped in a solution of diluted (twice) water-soluble fraction of papaya latex for 10 minutes. Control fruit (20) were dipped in sterile distilled water. Both the treated and control fruit were stored in aluminium trays covered with glass plates for 7 days at room temperature. The development of fungi on the fruit shell was recorded daily. In a separate trial, freshly harvested unripe avocado fruit (var. 'Green') were dipped in the water-soluble fraction of papaya latex for 10 minutes as above and the development of anthracnose and stem-end rotting was recorded daily for 8 days, by which time the fruit were fully ripe.

No fungi developed during ripening of unripe wood apples dipped in the water-soluble fraction of papaya latex for 10 minutes. All the controls treated with sterile distilled water showed fungal growth within 4 days. Most of the treated fruit could be held without fungal growth for 10 days. The immature avocado fruit similarly treated with papaya latex, however, were no different from the controls, contracting stem-end rot and anthracnose.

It was previously shown in this laboratory that dipping unripe papaya fruit in diluted papaya latex reduced anthracnose by 35% and stem-end rot by 46% during ripening (Karunaratne et al. 1996). In addition to its toxicity to fungi, the papaya latex forms a shiny layer on the fruit surface, perhaps affecting gas exchange and evaporation. Papaya latex appears to have excellent potential for development as a fungicide for postharvest treatment of fruits, though creation of a commercial product may require improvement of the substance's form, activity, and storage capability. As a natural product this substance may also offer several advantages over synthetic chemicals, the use of which is now being restricted because of perceived health and environmental problems.

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Elicitation of Defence

Use of Elicitors to Control Postharvest Diseases in Fruits and Vegetables

A. El Ghaouth*

Abstract

Public concern over food safety has placed under scrutiny the use of synthetic fungicides to control postharvest diseases of fruits and vegetables. It is clear that effective alternatives to synthetic fungicides are needed for the control of postharvest diseases of fruits and vegetables. Recently, induced resistance has been advanced as an alternative to synthetic fungicides, and there has been considerable success in using biotic and abiotic elicitors to control postharvest diseases. This paper gives an overview of the use of elicitors to enhance disease resistance of harvested commodities.

POSTHARVEST diseases represent a major limiting factor in the long-term storage of fruits and vegetables. In developing nations, losses due to fungal and bacterial diseases have been roughly estimated at 40–50%. While in developed nations losses may be lower, they are often just as serious for producers and consumers. Currently, synthetic fungicides are a primary means of controlling postharvest diseases (Eckert and Ogawa 1988). However, growing concerns over the presence of chemical residues in the food chain, the development of fungicide-resistant strains of postharvest pathogens, and the deregistration of some of the effective fungicides have generated an interest in the development of safer, alternative methods for the control of storage diseases. Among the proposed alternatives, the induction of defence reactions in postharvest tissue by pre-storage treatment with innocuous elicitors has received little attention. Recent attempts to exploit induced resistance through the pre-storage treatment with various bioelicitors show that induction of defence responses in harvested crops is feasible and may offer a new strategy for disease control. This paper presents an overview of the use of elicitors to enhance disease resistance of harvested commodities. The fundamental basis, the potential, and limitations of this strategy will be presented, with special references to biologically active elicitors such as UV-C, chitosan, and antagonistic microorganisms.

Host Defence Responses: an Overview

It is well established that plants can be rendered resistant to disease organisms by artificially activating their natural defence mechanisms (Kuc 1990; Sequeira 1990). Upon treatment with a potential pathogen or an elicitor (biotic or abiotic), plant tissue usually reacts by producing an array of defensive responses that can help ward off infection. The activated defence reactions can be restricted to tissue close to the site of the stimulus or can be expressed systemically through the tissue. These biochemical and structural responses include: reinforcement of the cell wall by deposition of lignin, callose, and hydroxyproline-rich glycoproteins; and accumulation of

* USDA Appalachian Fruit Research Station, 45 Wiltshire Road, Kearneysville, WV 25430, USA.

phytoalexins, antimicrobial secondary metabolites [for reviews, see Bailey and Mansfield (1982) and Stoessel (1983)], and glucanohydrolases such as chitinase, chitosanase, and ß-1,3-glucanase that hydrolyse the main components of fungal cell walls [for reviews, see Collinge and Slusarenko (1987), Bowes (1990), and Dixon and Harrison (1990)].

In recent years, considerable attention has been placed on induced resistance in vegetative crops as an important form of plant protection (Kuc 1990; Sequeira 1990). Although harvested produce also possesses constitutive and inducible defensive mechanisms, their potential has not received the attention they deserve. The deliberate induction of defence reactions in advance of infection, if they are large enough, can be expected to give postharvest tissue a head start in deterring disease development. The control of postharvest decay by pre-storage treatment with fungal wall components (Adikaram et al. 1988; El Ghaouth et al. 1992a,b) and UV light (Stevens et al. 1991; Mercier et al. 1993) suggests that intensification of defence mechanisms has potential in reducing postharvest decay.

Control of Decay by Ultraviolet Light

Non-ionising UV-C (190–280 nm) radiation is known to activate several biological processes in higher plants, including damage to the photosynthesis apparatus, stimulation of respiratory activity, the induction of flavonoids and phytoalexins biosynthesis, and elicitation of pathogenesis-related proteins (Hahlbrock and Scheel 1989; Haram 1980). The ability of UV-C to stimulate defence responses has been demonstrated in a variety of plant tissues (Hadwiger and Schwochau 1971; Bridge and Klarman 1972). Recently, UV-C radiation has been shown to reduce decay in a variety of commodities. The optimum doses of UV-C appear to occur in a rather narrow range ($2–10 \times 10^4$ erg/mm$^{2)}$ depending on the commodity, the cultivar, and the physiological status of tissue (Stevens et al. 1991; Chalutz et al. 1992; Mercier et al. 1993; Droby et al. 1993). Exposure of fruit above a certain threshold dose often results in severe tissue damage and increases the susceptibility of the tissue to decay (Ben-Yehoshua et al. 1992; Lui et al. 1993). Furthermore, responsiveness of harvested tissue appears to UV light diminish with an increase in tissue ripeness (Lui et al. 1993; Mercier et al. 1993). This was probably associated with the decreased ability of tissue to synthesise inhibitory compounds in response to UV treatment.

Control of postharvest decay by UV-C treatment has been attributed to induction of antifungal secondary metabolites rather than to a germicidal effect. In several commodities, UV-C treatment triggered a gradual development of tissue resistance that coincided with the induction and accumulation of PAL activity and phytoalexins (Chalutz et al. 1992; Ben-Yehoshua et al. 1992; Droby et al. 1993; Mercier et al. 1993). Implication of phytoalexins in UV-induced resistance was also reported in UV-treated lemon fruit and carrots, where the induction of scoparone and 6-metoxymellein was shown to enhance the resistance of UV-treated tissue (Ben-Yehoshua et al. 1992; Mercier et al. 1993).

Wilson et al. (1997) assessed the potential of UV-C treatment as an alternative to synthetic fungicides under semi-commercial conditions. Results from a series of semi-commercial packing-line tests on apple fruit showed that the UV-C treatment was effective in controlling natural decay of apple fruit caused by *Botrytis cinerea* and *Penicillium expansum*.. With 'Golden Delicious' and 'Empire' apples, the level of disease control obtained with UV-C treatment was comparable with that obtained with the recommended fungicide thiabendazole (Figs 1 and 2). The results obtained from semi-commercial tests demonstrate the potential of UV-C treatment as a feasible alternative to synthetic fungicides.

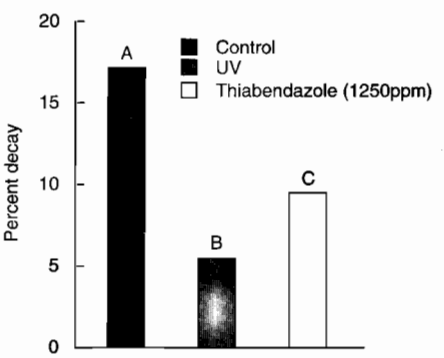

Figure 1. Effect of UV-C treatment on decay of apple fruit (cv 'Golden Delicious') stored at 18°C for 28 days. The experiment was carried out under semi-commercial conditions. Apples were irradiated using a UV-C chamber built on the processing line. Percentage of decay was based on five replicates of 56 fruit each. Columns with the same letter within the same time interval are not significantly different according to Duncan's multiple range test, P = 0.05.

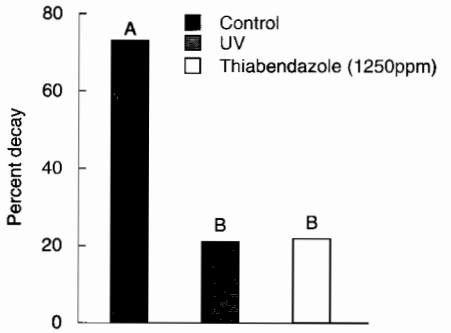

Figure 2. Effect of UV-C treatment on decay of apple fruit (cv 'Empire') stored at 18°C for 28 days. The experiment was carried out under semi-commercial conditions. Apples were irradiated using a UV-C chamber built on the processing line. Percentage of decay was based on five replicates of 56 fruit each. Columns with the same letter within the same time interval are not significantly different according to Duncan's multiple range test, P = 0.05.

Control of Decay by Chitosan

Chitosan, a ß-1,4-glucosamine polymer found as a natural constituent in the cell walls of many fungi, has shown great potential as an antifungal preservative for fresh fruits and vegetables. It is produced from arthropod exoskeleton chitin deacetylated to provide sufficient free amino groups to render the polymer readily soluble in dilute organic acids. Chitosan is known to form a semi-permeable film, to be inhibitory to a number of pathogenic fungi (Allan and Hadwiger 1979), and to activate a range of defence reactions in plant tissues (Mauch et al. 1988). That chitosan is polycationic is believed to provide the basis for its physicochemical and biological functionality. Attempts were made recently with several postharvest commodities to exploit the filmogenic and biological properties of chitosan (El Ghaouth et al. 1992b). Chitosan treatment controlled postharvest decay, stimulated several defence responses in plant tissue, and delayed ripening of strawberry, bell pepper (capsicum), tomato, and cucumber fruit by acting as a selective barrier to gas diffusion (El Ghaouth et al. 1992b). The control of decay by chitosan was attributed to a combination of antifungal and eliciting properties (El Ghaouth et al. 1992a). In vitro, chitosan not only inhibited the radial growth of major postharvest pathogens, but also induced severe morphological alterations in *Rhizopus stolonifer* and *B. cinerea*. In addition, there was increased cellular leakage in both fungi, presumably because of interference with fungal plasma membranes (El Ghaouth et al. 1992a). The antifungal activity of chitosan was also observed *in planta*. In bell pepper tissue, chitosan treatment reduced the potential of *B. cinerea* to initiate infection and caused severe cellular alteration to invading hyphae (El Ghaouth et al. 1994). The preservation of pectic and cellulosic binding sites in the host wall in contact with affected hyphae strongly suggests that chitosan might have reduced the ability of *B. cinerea* to produce macerating enzymes (El Ghaouth et al. 1997).

In addition to interfering directly with fungal growth, chitosan stimulated ß-1,3-glucanase and chitinase activities and various structural defence barriers in several postharvest commodities. In chitosan-treated tissues, ß-1,3-glucanase and chitinase activities remained elevated for up to 14 days after treatment. In bell pepper tissue, the expression of inducible defensive reactions by chitosan seems to have helped restrict fungal infection (El Ghaouth et al. 1992a, 1994, 1997). This is indirectly supported by: (1) the fact that invading hyphae were restricted mainly to the epidermal cells ruptured during wounding; and (2) the observation that fungal chitin was substantially reduced over fungal walls in contact with chitosan-treated tissue. The activation by chitosan of systemic defensive reactions that persist in the tissue could also affect the resumption of quiescent infection. If this is the case, such treatment could be important in controlling latent infections that become active when the biosynthetic potential of the tissue to produce antimicrobial compounds declines.

In laboratory tests, chitosan treatment controlled postharvest decay caused by *Botrytis cinerea*, *Penicillium expansum*, *Penicillium. digitatum*, and *Penicillium. italicum* on a variety of fruit (El Ghaouth, unpublished data). In a series of semi-commercial tests conducted in West Virginia, Florida, and California, chitosan was very effective in controlling natural decay of major apple and citrus varieties. The level of disease control obtained with chitosan on 'Red Delicious' apples was significantly higher than that obtained with thiabendazole (Fig. 3). On 'Golden Delicious' apples, the level of disease control obtained with chitosan was comparable with that obtained using thiabendazole (Fig. 4). The results obtained from semi-commercial tests demonstrate the potential of chitosan as an alternative to synthetic fungicides. The biocontrol activity of chitosan appeared to rest on synergistic interactions between its antifungal and eliciting properties.

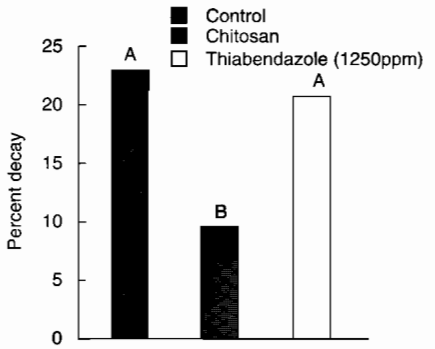

Figure 3. Effect of chitosan on decay of apple fruit (cv 'Golden Delicious') stored at 18°C for 28 days. Apples were treated under semi-commercial conditions using a processing line. Percentage of decay was based on five replicates of 80 fruit each. Columns with the same letter within the same time interval are not significantly different according to Duncan's multiple range test, P = 0.05.

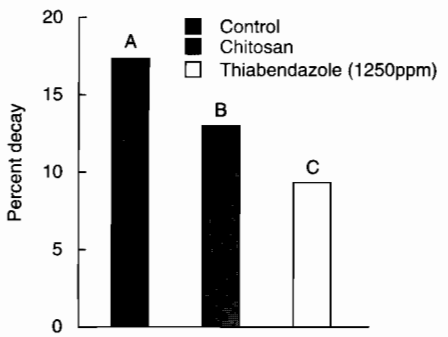

Figure 4. Effect of chitosan on decay of apple fruit (cv 'Red Delicious') stored at 18°C for 28 days. Apples were treated under semi-commercial conditions using a processing line. Percentage of decay was based on five replicates of 80 fruit each. Columns with the same letter within the same time interval are not significantly different according to Duncan's multiple range test, P = 0.05.

Control of Decay by Antagonistic Yeasts

Disease resistance resulting from the induction of plant defensive systems by treatment with certain microorganisms has been shown to offer a manageable form of plant protection and has been documented in different host–pathogen systems (Kuc 1990; Sequeira 1990). Recently, some promising antagonistic yeasts have been shown to induce defence responses in harvested tissue. *Pichia guilliermondii* (US–7) was shown to induce PAL activity in grapefruit (Wisniewski and Wilson 1992) and the accumulation of scoparone in lemon fruit (Ben-Yehoshua et al., these proceedings). Also, the induction of defence enzymes and structural barriers was reported in apples treated with the antagonistic yeast *Candida saitoana* (Wilson and El Ghaouth 1993). While it is not possible to determine exactly the extent of the role played by host-mediated responses in the biocontrol activity of antagonistic yeasts, the expression of inducible defensive reactions by antagonists is more likely to play a supporting role in restricting fungal spread. The ability of the antagonistic yeast to out-compete the pathogen for nutrient and space is likely to render the nutrient-deprived pathogen more susceptible to the host antifungal enzymes, namely chitinase and ß-1,3-glucanase.

Concluding Remarks

The use of biotic and abiotic elicitors to enhance disease resistance of harvested commodities offers great potential as an approach for the control of postharvest decay in fresh fruits and vegetables. In most cases, the biocontrol potential of the bioelicitor is attributable to the interplay of its biological and eliciting properties. Data accumulated so far from laboratory and semi-commercial studies show that induced resistance is feasible and may offer a new strategy for disease control. Although the use of elicitor as a disease control method has been demonstrated with several postharvest commodities, the elicitor-mediated protection tends to last a short period and to decline rapidly with ripening, a period where the fruit is more susceptible to infection. This may prove to be disadvantageous since in commercial packing houses fruit often come from different locations with variable inoculum load, type of infection, physiological maturity, and level of mechanical injury. More effective methods of controlling diseases through pre-storage treatment with elicitors should ultimately emerge as we learn more about: (1) the biological activity of elicitors; (2) the signal transduction pathways that link the host perception with the expression of defence genes required to ward off infection; (3) the regulation of defence genes associated with the induced resistance in harvested tissue and their role in resistance; and (4) the effect of ripening on the disease resistance potential of the harvested crop.

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Practical Approaches for Controlling Plant Diseases through Systemic Induced Resistance

Y. Huang and B.J. Deverall*

Abstract

Systemic induced resistance (SIR) for control of plant diseases is attracting great interest following the release by Ciba–Geigy of two resistance-inducing chemicals named as 'activators'. A dicholoroisonicotinic acid (INA) was the first artificial activator used widely in research from 1990. Many papers are being published on the effectiveness of INA in inducing resistance in legumes, tobacco, and cucurbits. The second activator (BTH) was released for practical application and registered in Germany in 1996 as a tonic named 'Bion' for control of powdery mildew in wheat.

The activators are chemicals that are not pesticides themselves but are very effective in inducing resistance throughout plants, in an overall process known as systemic induced (or acquired) resistance. According to Ciba–Geigy, the activators are rapidly taken up from foliar sprays or soil application and transported up and down the plant through the phloem. They can bind to receptor proteins throughout the plant and subsequently cause the release of latent defensive systems, including pathogenesis-related proteins.

EARLY work on the 'acquisition of immunity to disease in plants' was reviewed cautiously by Chester (1933). Evidence for induction of systemic resistance in plants was first unequivocally provided for some viral infections by Ross (1961), and then for fungal infections by Kuc et al.(1975). Considerable further research implied that a signal was released from initial local lesions and that it passed upwards through the phloem causing changes to the physiology of later-developing cells, so that they resisted pathogenesis by a wide range of microorganisms for a period of some weeks. This was reviewed in the first book on induced resistance to disease in plants by Hammerschmidt and Kuc (1995).

For plant protection, chemical substances have been sought for application to plants so that resistance can be induced systemically. The leading plant protection company to date in seeking such activators is 'Ciba–Geigy' (now re-named 'Novartis' following a merger).

* Department of Crop Sciences, University of Sydney, New South Wales 2006, Australia.

Systemic Induced Resistance in Control of Plant Diseases by Synthetic Chemicals

The first activator described was 2,6-dichloro-isonicotinic acid (INA). INA induced systemic resistance against a broad range of pathogens in several plant species (Table 1). INA was made available widely for research. It was effective in decreasing foliar diseases in green beans *Phaseolus vulgaris* (Dann and Deverall 1995, 1996) in growth rooms and the field. It decreased powdery mildew in cucumber (Hijwegen and Verhaar 1995) and barley (Kogel et al. 1994), and infections by *Cercospora beticola* in sugar beet (Nielsen et al. 1994). INA also decreased powdery mildew in roses (Hijwegen et al. 1996). INA has not been registered for commercial use.

Table 1. Control of plant diseases by INA.

Crops	Disease/pathogen	Reference
Barley	Powdery mildew/*Erysiphe graminis*	Kogel et al. (1994)
Cucumber	Powdery mildew/*Sphaerotheca fuliginea*	Hijwegen and Verhaar (1995)
Cucumber	Anthracnose/*Colletotrichum lagenarium*	Metraux et al. (1991)
Green bean	Anthracnose/*Uromyces appendiculatus*	Dann and Deverall (1995,1996)
Pepper	Bacterial spot/*Xanthomonas campestris*	Staub et al. (n.d.)
Rice	Rice blast/*Pyricularia oryzae*	Staub et al. (n.d.)
Tobacco	Blue mould/*Peronaspora tobacina*	Staub et al. (n.d.)

The second activator was benzothiadiazole (BTH), which was particularly useful for pathosystems in wheat, rice, tobacco, and some vegetable crops (Table 2). BTH applied early in the growth of wheat gave effective protection against powdery mildew for the season, and some protection against leaf rust and Septoria leaf spot (Gorlach et al. 1996). BTH decreased infection by fungi, bacteria, and viruses in tobacco (Friedrich et al. 1996) and in *Arabidopsis* (Lawton et al. 1996). BTH was registered for commercial use in Europe in 1996 against powdery mildew in wheat.

BTH (50 ppm) significantly reduced rust severity in experiments in which it was sprayed onto faba bean leaves 4 days before the challenge inoculation with *Uromyces viciae-fabae* spores (Fig. 1).

Neither INA for BTH shows antimicrobial activity in vitro. Neither are they converted into fungitoxic or bactericidal activity inside plants. A requirement for activity of a lag time between the treatment and the challenge inoculation indicates that plant reaction resulting from the treatments is responsible for the successful disease control. Studies in tobacco plants have led to the discovery that treatment with INA resulted in accumulation of chitinase and β–1,3–glucanase in plants that become resistant to disease, while treatment with an inactive analogue of INA (CGA 180777) did not cause accumulation of these proteins the plants so treated were not resistant to disease (Table 3).

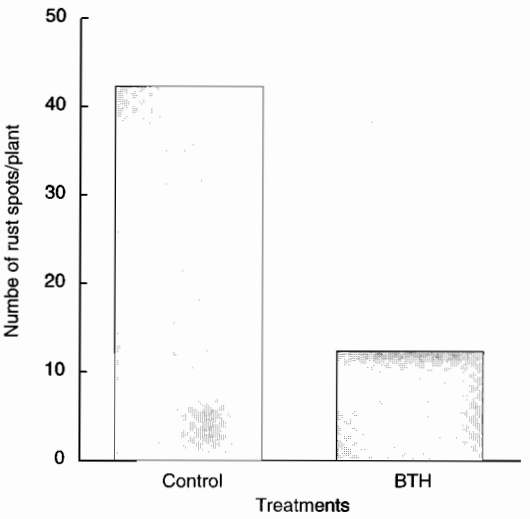

Figure 1. Effect of BTH on control of faba bean rust.

Table 2. Control of plant diseases by BTH.

Crops	Disease/pathogen	Reference
Cucumber	Downy mildew/*Pseudoperonospora cubensis*	Ciba data
Rice	Rice blast/*Pyricularia oryzae*	Ciba data
Tobacco	Blue mould/*Peronaspora tobacina*	Friedrich et al. 1996
Tomato	Bacterial spot/ *Xanthomonas* spp.	Ciba data
Wheat	Powdery mildew/*Erysiphe graminis*	Gorlach et al. 1996

Table 3. Changes of enzyme activities in tobacco plants after treatment with INA or its inactive analogue CGA 180777, comparing with the control.

Enzyme	INA	CGA 180777
Chitinase	+28	–8
β-1,3-glucanase	+120	–7
peroxidase	+10	–4
polyphenoloxidase	+5	+8
phenylalanine ammonia-lyase	0	0
acidic protease	0	+7
6-phosphogluconate dehydrogenase	+53	+11
lipoxygenase	+107	+214

Future Prospects

The continued use of traditional chemical fungicides has led to environmental and economical problems. As a consequence, integrated pest management (IPM) is favoured for plant protection. Systemic induced resistance is considered to be one of the important strategies to achieve IPM through manipulation of natural defence mechanisms in plants. Synthetic chemicals for systemic induced resistance have provided practical possibilities for induction of resistance in control of plant disease. They have shown promising effect on control of a wide range of plant diseases. Their potential for control of postharvest melon diseases is being investigated.

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Polygalacturonase Inhibitor Proteins—Do They Contribute to Fruit Defence against Fungal Pathogens?

J.M. Labavitch*, L.C. Greve*, A.L.T. Powell†, A.B. Bennett† and K.R. Sharrock§

Abstract

Polygalacturonase inhibitor proteins (PGIPs) are proteins which inhibit fungal PGs in vitro. PGIPs have been identified in extracts of many plant tissues, including a large assortment of fruits. Because PG is thought to be an important component of the fungal pathogen's infection system, PGIPs have been considered an important part of the plant's defences. The fact that all PGIPs contain a leucine-rich repeat motif similar to that found in many of the recently-identified plant defence proteins supports the proposition that PGIPs are defence molecules. PGIPs could contribute to fruit defence by blocking pathogen PG action and, hence, ingress, or by altering the population of oligomeric pectin digestion products at the infection site, thus changing the signals that influence the host's response to the pathogen.

We have cloned the PGIP genes from pear and tomato fruit, and have attempted to test the idea that PGIPs contribute to pathogen defence by expressing the pear PGIP in transgenic tomatoes. Some, but not all, of our tests of the resistance of transgenic tomato fruit to infection by *Botrytis cinerea* have indicated that the pear PGIP contributes to resistance. In order to learn why our trials have given variable results, we have carried out studies of the ability of PGIPs to inhibit PGs from different pathogens and different *B. cinerea* PG isoforms. We have also examined the population of PGs produced by *B. cinerea* when it infects control and transformed tomato tissues. These studies have told us a great deal about the interactions of PGs and PGIPs, but have not yet made clear whether and how PGIP contributes to a fruit's resistance to fungal pathogens.

CONSUMERS of horticultural products are expressing increasing apprehension about the use of chemical treatments to control insect pests and diseases on fruits and vegetables. These concerns, fuelled by the fear that pesticides may have adverse effects on human health, have led to increased efforts to identify safer control measures. To address this concern, pathologists have made efforts to identify the natural mechanisms of disease resistance in plants and then to enhance the expression of this resistance in potential host tissues.

* Department of Pomology, University of California, Davis, CA 95616, USA.

† Department of Vegetable Crops, University of California, Davis, CA 95616, USA.

§ The Horticulture and Food Research Institute of New Zealand, Ruakura Research Centre, Hamilton, New Zealand.

About 25 years ago a glycoprotein that inhibited fungal polygalacturonases (PGs) in vitro was extracted from bean hypocotyl tissues (Anderson and Albersheim 1972). PGs had been observed early in the infection of plants by soft-rotting fungal pathogens and PGs and other pectolytic enzymes degrade a significant portion of the cell wall architecture. Therefore, these and other researchers concluded that PG was an enzyme that enabled pathogens to infect their hosts (Bateman and Millar 1966). They proposed that the PG inhibitor proteins (PGIPs) limited pathogen degradation of tissues and, thus, were part of the plant defence system. We subsequently identified a PGIP in pear fruits. The observation showing more PGIP associated with unripe fruit than with ripe fruit (Egli 1987), provided correlative data that suggested that the pear PGIP contributed to fruit resistance against postharvest

pathogens. Furthermore, when pear PGIP was purified from tissue homogenates it was found to have differential activity against the PGs of several fungi. The PG of *Penicillium expansum* was only weakly inhibited by the pear PGIP, while PGs from *Botrytis cinerea* and *Dothiorella gregaria* were strongly inhibited (Abu-Goukh et al. 1983b). Inoculation studies indicated that *P. expansum* was a more aggressive pathogen of pears than were *B. cinerea* and *D. gregaria* (Abu-Goukh et al. 1983a). Thus, the 'ability' of a fungus to produce PG that could 'avoid' PGIP inhibition correlated with its ability to be an effective pathogen. These observations led us to propose: (1) that PGIP constituted a preformed biochemical barrier to pathogens; and (2) that PGIP changes during ripening might help to explain why resistance decreased with fruit maturation (Abu-Goukh and Labavitch 1983). Subsequent work with the bean PGIP (Bergmann et al. 1994) has shown that it is induced upon pathogen infection, although our work with tomato and pear PGIPs has not indicated that they are inducible.

PGIPs could contribute to host resistance in at least two ways. The first is the most direct. If the pathogen relies on its PGs to digest pectin polymers in the cell walls of its host and thus eliminate the barrier to infection that the cell wall constitutes, then PGIP would be a biochemical limitation to infection. The work of Cervone et al. (1989) suggests another way that PGIP could contribute to defence. Research over the last 25 years has shown that many plant tissues or cell systems express an assortment of plant defence responses following treatment with oligosaccharides produced during enzymic digestion of plant cell walls (Darvill and Albersheim 1984; Ryan and Farmer 1991). Presumably, this signal-response system has evolved as plants 'learned' to respond to the presence of oligosaccharide elicitors which indicate that the plant's cell walls are being digested by an 'invader'. Cervone et al. (1989) showed that when purified fungal PG is incubated in vitro with a pectin substrate it will digest the substrate into small oligomers which will not elicit phytoalexin production when applied to soybean cotyledons. However, if PGIP is added to the incubation mixture, larger oligomers accumulate, and these can elicit soybean phytoalexin production. Thus, PGIP action could contribute to the activation of defence responses when pathogens secrete PG into the host's cell wall space during infection. In addition, it is likely that PGIPs participate in protein–protein interactions, predicted by their leucine-rich repeat motifs (Stotz et al. 1994; Powell et al. 1994). These interactions could be part of a signal transduction pathway involved in pathogen recognition (DeLorenzo et al. 1994).

Does PGIP Contribute to Host Resistance to Pectolytic Fungal Pathogens?

PGIPs have been identified in many fruits (e.g. Johnston et al. 1993; Yao et al. 1995) and the idea that they are components of host defences has received increasing support. While the logic that PGIP is a defence protein is strong, it is based on correlative data and in vitro assays. No natural mutants lacking PGIP have been identified in any plant. Brown and Adikaram (1983) reported that tomato fruit contained a PGIP that inhibited the PG of *Glomerella cingulata*, but was ineffective against that of *B. cinerea*. In 1989, we extracted tomato fruits and confirmed the report of Brown and Adikaram (Labavitch, unpublished data). This suggested a genetic engineering strategy to enhance the amount of PGIP in tomatoes and thereby determine if PGIPs contribute to disease resistance. We reasoned that, since the pear PGIP was effective against *B. cinerea* PG while the tomato PGIP was not, a transgenic tomato that produced a large amount of pear PGIP might be more resistant to *B. cinerea* than unaltered tomatoes.

The pear PGIP was purified and its gene was cloned (Stotz et al. 1993). Transformation vectors including the constitutive promoter element from the cauliflower mosaic virus were constructed, and transgenic plants were produced using an *Agrobacterium*-based transformation system (Fillati et al. 1987). Proteins were extracted from leaves of these primary transformants (the T_0 generation) and PG inhibition assays indicated that several T_0 plants had elevated levels of PGIP activity. Western blot analysis using a rabbit polyclonal antiserum raised against the chemically-deglycosylated pear PGIP (Stotz 1994) indicated that there was a substantial presence of pear PGIP in the leaf extracts. When the T_0 plants set fruit, these were analysed and shown to contain pear PGIP. Most importantly, when we had obtained enough red fruit from each of several pear PGIP-expressing T_0 lines, wound inoculations with *B. cinerea* spore suspensions were made. Infection was scored after 3 days and was found to be much less than for inoculated control tomatoes (Table 1).

Table 1. Percentage of wound sites on ripe fruit that were infected 3 days after inoculation with 10^3 *B. cinerea* spores. There were four inoculation sites per fruit. These data are for one transgenic T5 tomato line that expressed abundant pear PGIP in all tissues. Another 14 transgenic lines were analysed.

Genotype	Transgenic generation		
	T_0[a]	T_1[a]	T_2[b]
Transgenic (216)	0	45	85
Control	80[c]	66	78

[a] Plants were grown in the greenhouse.
[b] Plants were grown in the field in Davis, CA.
c The control for the T_0 generation was untransformed T5 tomatoes. For the T_1 and T_2 controls, we used segregating progeny lacking the introduced pear PGIP gene.

Leaves of the T_1 populations derived from seeds of the self-pollinating T_0 plants were assayed for pear PGIP levels in order to identify segregants which no longer carried the pear PGIP gene and those which expressed it. Ripe fruit of the T_1 plants were assayed for resistance to *B. cinerea*, as described above (Table 1). Again, elevated PGIP level correlated with an increase in resistance. Seed was collected from fruits of the pear PGIP-expressing plants in the various T_1 populations, and T_2 plants homozygous for the pear PGIP gene were identified by Southern hybridisation analysis of the genomic DNA.

Seedlings from the various T_2 populations were grown in the greenhouse and then transplanted to the field. Mature green and red ripe fruits were harvested from these plants and brought into controlled conditions for a repeat of the *B. cinerea* resistance tests. In this case no differences in resistance between pear-PGIP expressing and non-expressing T_2 lines were observed (Table 1). This trial was repeated in the subsequent year (1995), and again no differences were noted.

Resistance to Pathogens is the Consequence of Several Interacting Defence Systems

In the gene-for-gene resistance systems that have received a great deal of research attention, a single pair of alleles (one in the pathogen and the other in the host) determines resistance or susceptibility. These alleles encode recognition factors, however, not specific mechanisms of defence. Resistance or susceptibility is determined by whether or not the pathogen is recognised so that a combination of defence genes is activated (Jones et al. 1994; Hammond-Kosack and Jones 1995). Our view is that the more generalised defences against pathogens that we are examining when we inoculate tomato fruit with spores of *B. cinerea* are also the result of the combined expression of genes responsible for an assortment of defence mechanisms. Thus, it is not surprising that elevated expression of the pear PGIP gene might enhance fruit resistance under one set of circumstances (e.g. in greenhouse-grown fruits) and not in another (e.g. in field-grown fruits). Undoubtedly, the stresses that a host tissue has experienced during development have an impact on the success of its resistance when challenged by a pathogen, and fruits grown in the greenhouse and field have substantially different histories of stress.

We are working to develop new approaches to the resistance testing of these transgenic tomatoes in order to make sure that the pathogen challenge we are providing is not so great that it could overwhelm any PGIP-related defence that might be expressed. Certainly, if PGIP over-expressing transgenic plants are to be used in agriculture we will need to know under what conditions they might be of value. The mixed results of our greenhouse and field trials also indicate that further testing of the idea that PGIP contributes to resistance is required. To that end, we plan to use antisense mRNA technology to generate tomato plants that no longer express their own PGIP. These PGIP-deficient plants should provide useful material for testing of the PGIP role in defence.

Does PGIP Inhibit All Fungal PGs to the Same Extent?

Our ongoing studies of the biochemistry of PGIP inhibition of fungal PGs have indicated that our initial genetic engineering approach for the use of pear PGIP to enhance tomato resistance may have been biochemically naive. Fungi generally produce a variety of cell-wall-degrading enzymes when living on plant tissues (Cooper 1984). They include several kinds of pectolytic enzymes and it is not unusual for many different PG isoforms to be produced (Collmer and Keen 1986). Studies of fungus grown in liquid culture have revealed some of the factors that determine the assortment of PG isoforms produced under different culture conditions (Cooper and Wood 1973). It is reasonable to presume that the conditions under which a given fungus grows on plant tissues

will also influence the spectrum of cell-wall-degrading enzymes, including PGs, it produces. When the PGs produced by *B. cinerea* grown in liquid culture or on apple fruit are compared, their isoform complements are found to be different (Tobias et al. 1993 and 1995).

B. cinerea produces a substantial assortment of PG isoforms when grown in modified Pratt's medium (Abu-Goukh and Labavitch 1983) supplemented with pear cell walls as a carbon source. This PG isoform heterogeneity was revealed (Sharrock and Labavitch 1994) by collecting the proteins in the medium of 5-day cultures and subjecting them to isoelectric focusing. The developed focusing gel was then overlain with a thin acrylamide gel containing polygalacturonic acid (PGA), the substrate typically used for assay of PGs. After maintaining contact between the two gels for a period to allow transfer of the focused proteins (including PGs) into the PGA-containing gel, the gel overlay was incubated. The incubation period allows the PGs that have been transferred to the substrate overlay gel to digest the PGA substrate. The location of the various PGs separated during the isoelectric focusing step is then revealed by staining the overlay with the pectin stain ruthenium red. PGA in the overlay stains red, but if PG has been transferred into the overlay and digested the substrate, an unstained zone is identified (Fig. 1). Our data indicate that several PG isoforms with isoelectric points ranging from pH 3.5 to 5.5 are produced by this strain of *B. cinerea* after 5 days growth in the above medium. The data indicate another very important fact. If purified pear fruit PGIP is included with the PGA in the substrate overlay gel it should block the digestion of substrate that is catalysed by the transferred PG isoforms. While this inhibition of clearing was seen for some of the *B. cinerea* isoforms, it was not seen for all. Pear PGIP showed a degree of selectivity, in that some of the *B. cinerea* isoforms were inhibited while others were not. We are working to determine the biochemical basis for the selectivity shown by the pear PGIP.

How Does *B. cinerea* Respond to Elevated Host Expression of PGIP?

The recognition that PGIP can be ineffective against some of the PGs produced by a given pathogen raised concerns about the validity of our 'PGIP strategy' for enhancing tomato resistance. Could the fungus respond to a plant environment that was inhibitory to its complement of PGs (because of the PGIPs present), by producing PGs that can avoid inhibition? Perhaps *B. cinerea* can. Whether or not this concern was justified, was tested (K.R. Sharrock, pers. comm.) by challenging stem tissues of transgenic, pear PGIP-expressing and control, greenhouse-grown tomato plants with *B. cinerea* spores. The stem tissues were chemically killed by subjecting them to vapours of the tissue fixative propylene oxide before exposure to the spores. This treatment allowed Sharrock to focus on direct contributions of PGIP to defence because no induced defences could be expressed. An extensive growth of mycelium developed on both control and transgenic stem explants. However, when the infected tissues were tested for PG activity, the pear PGIP-expressing stems contained only a few percent of the activity that could be isolated from the controls. Although the fungus grew on both sets of dead tissue, PGIP influenced fungal production of PG. When the PG-containing tissue extracts were subjected to isoelectric focusing followed by the PGA overlay assay (Fig. 1), the PG isoforms detected were strikingly different. The small amount of PG extracted from the transgenic stem tissues represented a set of three or four acidic isoforms that were only minor components of the ca. 14 isoforms in the extracts of infected control tissues. Although this study must be repeated, it appeared as if the *B. cinerea* had responded to the high level of pear PGIP in the transgenic tissue by reducing the range of PG isoforms produced.

Our concern about the impact of inhibition selectivity when PGIP interacts with different PGs was also revealed when the PGs of three different fungi (*B. cinerea*, *Fusarium moniliforme*, and *Aspergillus niger*) were tested with PGIPs purified from pear and tomato fruit and stems of bean (Powell et al. 1994). The bean PGIP strongly inhibited all three PGs, the tomato and pear PGIPs inhibited the *B. cinerea* PG but not the *F. moniliforme* PG, and the *A. niger* PG was weakly inhibited by the tomato PGIP and not at all affected by the pear PGIP. These results make clear that transgenic overexpression of the pear and tomato PGIPs in crop plants would likely have limited utility in enhancing pathogen defence even if the strategy of providing protection by blocking fungal PG has validity. These observations further underscore the fact that PGIPs can be effective against some of the PGs of a given fungus and not others. Brown and Adikaram (1983) had originally reported that tomato PGIP did not inhibit *B. cinerea* PG and we confirmed that observation. Nevertheless, the pure tomato PGIP

inhibits at least some of the PGs of the strain of *B. cinerea* (Del-11, from grape) which we are using. (This represents a complicating factor when we try to determine if over-expressing pear PGIP in transgenic tomatoes enhances their resistance to *B. cinerea* infection.) When we originally purified the pear PGIP (Abu-Goukh et al. 1983b) it strongly inhibited the commercial *A. niger* PG we used as a reference. Nevertheless, the pure pear PGIP does not inhibit the pure *A. niger* PG used by Cervone et al. (1989) in their characterization of the bean PGIP (H. Stotz and C. Bergmann, unpublished data).

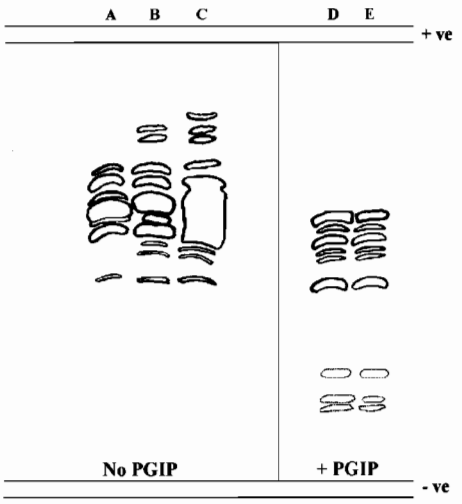

Figure 1. The differential effects of pear PGIP on the PG isoforms in liquid cultures of *Botrytis cinerea* are illustrated in this drawing. As described in the text, proteins in the culture fluid were separated by isoelectric focusing in a pH 6.0 to 3.0 gradient and the developed focusing gel was then overlain with a PGA- or PGA plus pear PGIP-containing thin gel. The lines depict the zones that were cleared of ruthenium red-stained PGA by the action of different PG isoforms. Differing amounts of culture fluid proteins were applied in the different lanes: lane A received a (relative)1× amount of protein, lanes B and E received 5×, and lanes C and D received 10×. Before pectin staining, the overlay on the right (containing PG plus PGIP) was incubated longer than the overlay on the left because the presence of PGIP slowed the digestion of PGA in the overlay. These data are from Sharrock and Labavitch (1994). [Reprinted with the permission of *Physiological and Molecular Plant Pathology*.]

Biochemical Analysis of PGIP Expression in Transgenic Plants

Our initial impressions of the level of pear PGIP in the T_0 transformants was based on analysis of PGIP protein (Western analysis) in leaf extracts. We measured leaf rather than fruit PGIP protein because we wanted to know if the transgene was functioning before fruit set and because we did not want to waste fruit, recognising that we would need several from each plant if data from inoculation tests were to have validity. When we finally obtained transgenic tomatoes that were homozygous for the pear PGIP gene we extracted proteins from both leaves and fruit and did Western analysis. We saw that the transgenic PGIP protein in leaf extracts migrated as a tight band with a molecular weight of about 41 kD. The PGIP in fruit extracts produced a diffuse band that seemed to have two parts. One of these had a molecular weight similar to that of the leaf PGIP and the other was somewhat smaller. Our analysis (Stotz et al. 1993, 1994) had indicated to us that the pear and tomato fruit PGIPs were glycoproteins with up to 20% of their molecular weight as carbohydrate. We presumed, therefore, that the differences in PGIP migration in the SDS-PAGE gel separations of leaf and fruit extracts were due to differing amounts of carbohydrate substitution on the pear PGIP polypeptide. We tested this by treating the PGIP-containing leaf and fruit extracts with trifluoromethane sulfonic acid to hydrolyse the sugars away (Karp et al. 1982). Western analysis of the deglycosylated samples indicated single bands of material that were bound by the anti-pear PGIP serum. Each of these bands appeared to have the same molecular weight (about 34 kD). Presumably, therefore, the pear PGIP transgene codes for the same pear PGIP polypeptide in the cells of fruits and leaves, but the post-translational processing (glycosylation) differs in the two organs.

We do not know what effect this differential glycosylation has on inhibitor specificity. However, Bergmann et al. (1995) have reported that the inhibitory activity of bean PGIP is influenced by the extent to which the polypeptide is glycosylated. We therefore wondered if the differential pear PGIP glycosylation observed when the pear gene is expressed in tomato leaves and fruit might be having an effect on its inhibitory activity. Several dilutions of the leaf and fruit PGIPs were subjected to SDS-PAGE separation followed by Western blotting with the anti-pear PGIP serum. The gel was then scanned with a densitometer

to determine which dilutions of the two extracts contained the same amount of cross-reactive material (i.e. PGIP protein). These dilutions were then tested for their ability to inhibit *B. cinerea* PG (Taylor and Secor 1988). The PGIP in fruit extracts was only 40% as inhibitory as the PGIP in leaf extracts. This observation provides information that is very important for our study of the role of PGIP in resistance to pectolytic pathogens. Because transgenic fruit PGIP is less active than the PGIP in transgenic leaves, our genetic engineering strategy is likely to be, at best, less effective at improving fruit resistance than it is at improving leaf resistance if the pathogen in question produces important PGs that are inhibited by the transgenic PGIP.

The observation may be very important for genetic engineering approaches for altering crop plants in general. If the genetic manipulation used involves a gene that encodes a glycoprotein, it is possible that the expression of that glycoprotein and, more importantly, its activity, will be different in different organs of the transgenic plant. It is important that the basis of this differential post-translational modification be determined so that it can be controlled.

Does PGIP Play a Role in Disease Resistance?

The logic that suggests a role for PGIP in disease resistance is persuasive and the results from our early resistance tests of greenhouse-grown transgenic tomatoes support it. However, the results from our tests of field-grown plants make any conclusions about PGIP's role in generalised defences uncertain. Our view is that the contribution that PGIP might make to host protection must be integrated with the expression of a plant's other defence mechanisms. The outcome of the confrontation with a fungal pathogen will depend also on the flexibility of the pathogen's mechanisms of infection and, of course, the impact of the plant environment on both the plant and the pathogen.

The data we have reported about differences in *B. cinerea* PG isoforms produced when the fungus infected killed control and transgenic tomato stems were collected several days after inoculation when fungal growth was well established on both types of tomato. Because the tissues tested were dead there was no integration of PGIP's hypothetical defence contribution with potential 'active' protection mechanisms. The important effect of PGIP that might contribute to a successful defence could be very early in the interaction of fungus and plant. They could involve contributions to the host's recognition of the invader's presence as well as a direct slowing of its digestion of the cell wall barrier and tissue penetration. For instance, *B. cinerea* conidia carry a constitutive PG that is likely to be important in its early development on plants (Verhoef and Liem 1978; van Kan et al. 1991). Pear PGIP inhibits the PG that can be eluted with water from the conidia of the Del. 11 strain of *B. cinerea* (Labavitch et al., unpublished data). Could this be an interaction that determines whether PGIP can, in certain situations, help with defence? We cannot know this until we have a clearer understanding of the biochemical factors that influence the interaction of the inhibitor with its PG targets and how (or if) that interaction contributes to the activation of other host defences. Then we may understand how to use the increasing number of cloned PGIP genes (Stotz et al. 1993, 1994; Toubart et al. 1992) in genetic engineering strategies aimed at improving crop defences.

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Significance of Time Lapse between Harvest and Postharvest Hot Water Treatment on a Local Avocado Variety

A. M. Karunaratne and N.K.B. Adikaram*

Abstract

Anthracnose (*Colletotrichum gloeosporioides*) contributes to about 80% of total disease in avocado. Mature avocado fruit were treated with hot water at three different temperature–time regimes: 55°C for 5 minutes, 50°C for 10 minutes, or 45°C for 15 minutes. Five separate batches of fruits were treated at different times after harvest: i.e., soon after harvest, and 12, 24, 36, or 48 hours after harvest. It was found that a treatment at 55°C for 5 minutes caused browning of the skin and enhanced disease development. The other two temperature–time regimes did not cause any discoloration of the skin.

Treatment at 50°C for 10 minutes did not appreciably reduce anthracnose development. When fruit were treated at 45°C for 15 minutes on the day of harvest, or on 1–5 days after harvest, a considerable reduction of anthracnose development was seen in fruit treated 1 day after harvest. It was found that the fruit have to be fully mature for this treatment to be effective.

The effect of the above treatments on the pathogen *Colletotrichum gloeosporioides*, and on the fruit, were determined by the following methods, but neither gave a satisfactory explanation for the above observations.

Conidial germination assays were carried out with *Colletotrichum gloeosporioides* isolates obtained from the surface of avocado fruit by exposing them separately to the three temperature–time regimes given above. It was observed that although the three temperature–time regimes were capable of reducing the percent germination progressively, this did not explain the reduced anthracnose development in fruit treated at 45°C.

The preformed antifungal compounds in the peel of avocado fruit were assayed daily for four consecutive days in fruit treated at 45°C for 15 minutes on the day of harvest, or 1 or 2 days after harvest. A TLC-bioassay showed that the daily fluctuations of the total antifungal activity present in the peels of fruit were similar for all treatments. Therefore, no convincing explanation could be found for the reduction in anthracnose development when fruit were treated a day after harvest at 45°C for 15 minutes. Formation of heat shock proteins reported by Woolf et al. (1995) may offer an explanation to this.

HEAT treatment of intact avocado fruit has been attempted to control fruit flies (Kerbel et al. 1987; Jang 1996; Jessup 1991), decrease chilling injury (Woolf et al. 1995; Sanxter et al. 1994), and reduce polyphenoloxidase activity (Trejo-Gonzalez et al. 1992). Disease control by postharvest hot water treatment of 'Pollock' avocados is reported by Flores (1992). Rupasinghe and Peiris (1992) reported that hot water and a separate benomyl treatment controlled postharvest diseases in 'Pollock' avocados. Jessup (1991) reported a reduction in anthracnose development when fruit were subject to a hot benomyl dip. Skin browning in avocados has been reported by Rupasinghe and Peiris (1992) when they are treated at 55°C. Although Jessup (1991) did not observe skin browning at the time of publishing, he observed skin browning in fruit treated at 50°C (A.J. Jessup, unpublished data) some years later, and reduced the dipping temperature to 46°C. Prusky et al. (1991) observed that lesion development in 'Fuerte' avocados was dependent on treatment time (55°C, 5 and 10 min) and occurred earlier on treated fruit.

* Department of Botany, University of Peradeniya, Peradeniya, Sri Lanka.

The resistance of unripe avocado fruit to anthracnose is attributed to preformed antifungal activity (Prusky et al. 1982, 1991; Prusky 1996; Sivanathan and Adikaram 1989; Adikaram et al. 1992). Prusky et al. (1982) observed earlier symptom expression on hot water-treated fruit, and attributed this to changes in the concentration of an antifungal compound in the fruit.

The information given above shows that there are contradictory reports on the effect of hot water on the postharvest quality of avocados, and the suitable temperature–time regime for treatment. In the study reported here, three different temperature–time regimes were tested to determine the effect of hot water treatment on anthracnose development in avocado. In view of the changes in the antifungal activity, the time delay after harvest to dipping time was considered as a variable. Antifungal compounds were also assayed to seek an explanation for the observations made. The heat sensitiveness of conidia of *Colletotrichum gloeosporioides* isolates was assessed by exposing them to the three temperature–time regimes used for dipping fruit, which was thought to reflect the influence of the treatment on the pathogen.

Materials and Methods

Fruit

All avocado (*Persea americana* cv 'Green') fruit used in the experiments were plucked from trees in the University of Peradeniya premises. Fully mature unripe avocados were used in these experiments unless otherwise stated.

Hot water treating

Five fruit at a time were placed in a net basket, and this basket was immersed into a water bath made by filling a 50 L plastic basin with hot water. Temperature of the bath was monitored by an immersed thermometer and adjusted every 3 minutes by adding and mixing excess hot water. Immediately after the dip, the fruit were exposed to flowing tap water at room temperature for ≈1 min. They were allowed to drip dry at room temperature (≈28°C), and were kept on trays and left at ambient conditions. Lesion development was scored daily on each fruit in each batch on a scale of 0 to 5, 0 indicating no disease and 5 indicating more than 50% of the fruit being diseased.

To determine the effect of three different temperature–time regimes, equal numbers of fruit were divided into three groups. Each group was hot water treated separately at the following regimes: 45°C,15 min.; 50°C, 10 min.; and 55°C, 5 min. A dip treatment was given once to separate batches of fruit at different time intervals: soon after harvesting, and 12, 24, 36 and 48 hours after harvesting. A total of 75 fruit was used in this experiment.

In a separate experiment, 10 fruit at a time were hot water treated at different time intervals after harvest at 45°C for 15 min: on the day of harvest, and 1, 2, 3, 4, and 5 days after harvest. Ten fruit were maintained as untreated controls. This experiment was repeated three times with fully mature unripe fruit, and once with somewhat less mature (approximately one month before ideal harvesting maturity) fruit. A separate batch of fruit was treated at the same time intervals after harvest as above, at 50°C for 10 min. A total of 350 fruit was used in these experiments.

Extraction of antifungal compounds from peel of hot water-treated fruit

Fourteen fruit were hot water treated separately at 45°C for 15 min, at different time intervals after harvest—on the day of harvest, and 1, 2, and 3 days after—and approx. 1 g of the outer skin (about 1–2 mm thick) was cut from the treated and control fruit daily, until 4 days after harvest. Peeled fruit were discarded. These samples were freeze-dried and stored airtight in the deep freezer (–20°C). The samples were extracted as described earlier (Adikaram et al. 1992). This experiment was repeated three times with three separate batches of fruit. A total of 42 fruit was used in this study, and the average of three replications is presented.

A TLC-bioassay was done using *Cladosporium cladosporioides* as the test fungus, as described by Sivanathan and Adikaram (1989) with the following modifications. The plates after spotting the extracts were not developed in a solvent system, but allowed to air dry overnight before spraying with the test fungus. The diameter of each clear area on the TLC plate indicated by the lack of aerial mycelia was measured in two directions at 90° angles to each other, and the average of the two values was taken as the average diameter of the inhibition area. This diameter was assumed to be proportional to the amount of total antifungal compounds present in 1 g of fresh peel.

Assay for germination of conidia

Suspensions of conidia of five isolates of *C. gloeosporioides* were made separately, by scraping the mycelium from well sporulated cultures less than 6 days old, grown on Cook's No. 2 medium and suspending it in Czapek Dox nutrient medium. These were filtered through a layer of glass wool to remove the hyphae. The filtrate that contained the conidia was collected and the concentration of the suspension was adjusted so that 15 to 20 conidia were visible when viewed through the high power (×400) of the microscope.

Approximately 10 mL portions of each suspension of conidia in glass tubes were exposed to 55°C for 5 min, 50°C for 10 min, and 45°C for 15 min separately, by placing them in hot water baths calibrated to each of the above temperatures. The temperature of the suspension was monitored by inserting a thermometer into it directly. Another set of tubes containing suspensions of conidia was placed in water baths maintained at room temperature as controls. At the end of the exposure time all tubes were transferred into a water bath at room temperature and 10 mL portions of each suspension were pipetted out separately on to clean glass slides. The slides were incubated in moist chambers for 7 hours. At least three replicate slides were prepared for each fungal isolate. At the end of the incubation period a drop of lactophenol was added to each drop on the slides to arrest germination. Percent germination in each fungal isolate was determined by counting at least 100 conidia from each isolate under the microscope and determining the proportion that had germinated.

Statistical analyses

The seven treatments (6 sets treated at 45°C for 15 min at different time lapses and the untreated control) carried out with mature fruit were compared in a randomised complete block design with three replicates. The data consisted of disease scores. The method of aligned ranks (Lehmann and D'abrera 1975) was used to eliminate the block effect and the resulting data were analysed using the Kruskal-Wallis test. A Mann-Whitney test was performed with the aligned ranks of anthracnose scores on the fruit treated one day after harvest against the other anthracnose scores obtained in this study.

The data obtained on the germination of conidia were analysed using the log linear models approach since they are count data. The best fitting model was investigated for three factors, namely the hot water treatment regimes, fungal isolates, and germination. The statistical analysis system (SAS 1987) was used in the analysis.

Results

Of the diseases observed, anthracnose (*Colletotrichum gloeosporioides*) contributed to about 80% of total disease. Of the three hot water treatment regimes tested the disease score was highest in mature green fruit treated at 55°C for 5 min. This treatment also caused browning of the skin. Disease reduction was observed in fruit treated at 50 and 45°C, the reduction being pronounced when treated at approximately 12 hours after harvest (Fig. 1). Disease reduction was considerable when fruit were treated within the time span of 12 to 24 hours of harvest at 45°C. A disease reduction which was not as pronounced was also observed in fruit treated at 50°C 48 hours after harvest (Figs 1 and 5). When mature green fruit were treated (45°C for 15 min), at 6 different time lapses after harvest, disease appearance was variable in the three replicates, but the fruit treated a day after harvest showed a reduction of disease compared with the other treatments (Fig. 2). A Kruskal-Wallis test on the aligned ranks showed a treatment effect significant at $P<0.05$. A Mann-Whitney test to compare disease scores of fruit treated at 45°C a day after harvest with the disease scores of the rest of the treatments showed that the null hypothesis could be rejected at $P = 0.056$. A consistent and significant disease reduction was not observed when fruits were hot water treated at 50°C for 10 min (Fig. 3) and when comparatively immature fruit were treated at 45°C for 15 min (Fig. 4). The earliest disease initiation was visible at 4 days after harvest.

Antifungal activity assayed by TLC-bioassay showed a high variation (Fig. 5). The activity remained stable until a day after harvest, but dropped sharply on the second day. The activity increased slightly by the 3rd day and dropped 4 days after harvest.

A progressive reduction of viability of conidial spores was observed with increasing treatment temperatures. Table 1 gives the break down of the ANOVA. The likelihood ratio test showed that even after all the 2-way interactions are fitted that the model was not adequate ($P<0.0001$). Figure 6 illustrates the interpretation of these data for each fungal isolate separately. While isolate 2 shows poor germination even without subjecting to the treatment, the other 4 isolates showed an average reduction of 93% in spore germination when treated at 45°C.

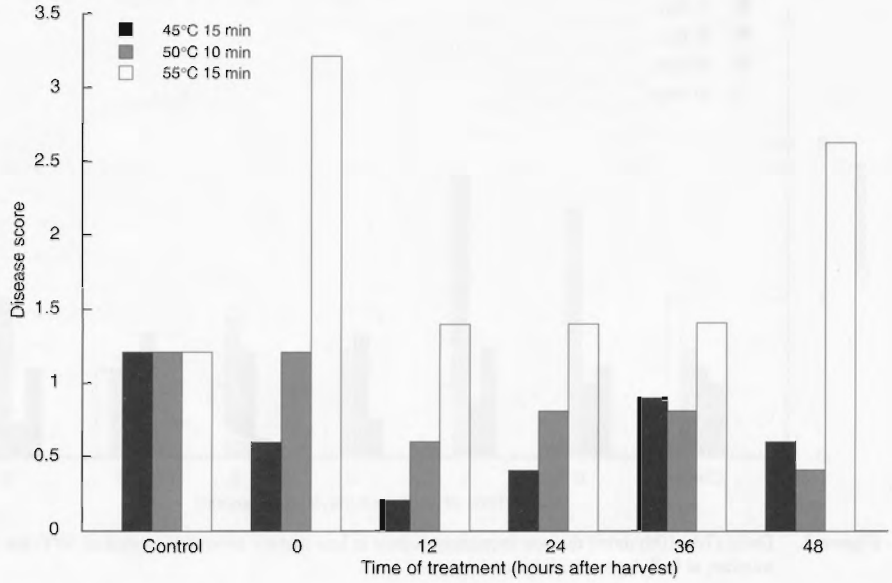

Figure 1. Effect of three hot-water treatment regimes on anthracnose score of avocados 10 days after harvest.

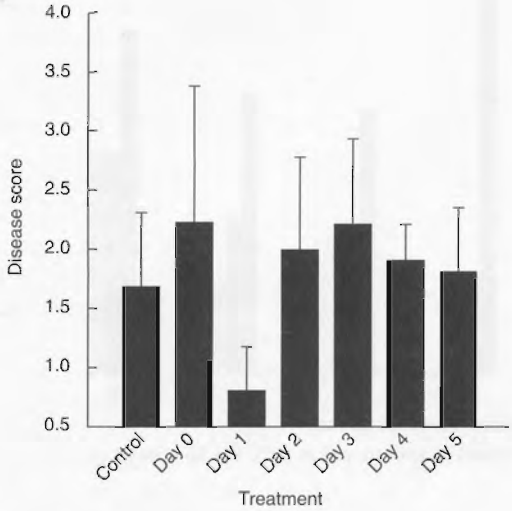

Figure 2. Anthracnose score 10 days after harvest in fruit treated at 45°C for 15 minutes at 0–5 days after harvest.

Table 1. Maximum likelihood analysis of variance table for the data on percent conidial germination of four *Colletotrichum gloeosporioides* isolates treated at the three temperature–time regimes and control.

Source	DF	Chi-square	Prob
Treatment	3	173.76	0.0000
Fungus	4	223.85	0.0000
Treatment × fungus	12	273.84	0.0000
Germination	1	190.96	0.0000
Treatment × germination	3	305.60	0.0000
Fungus × germination	4	288.90	0.0000
Likelihood ratio	5	42.42	0.0000

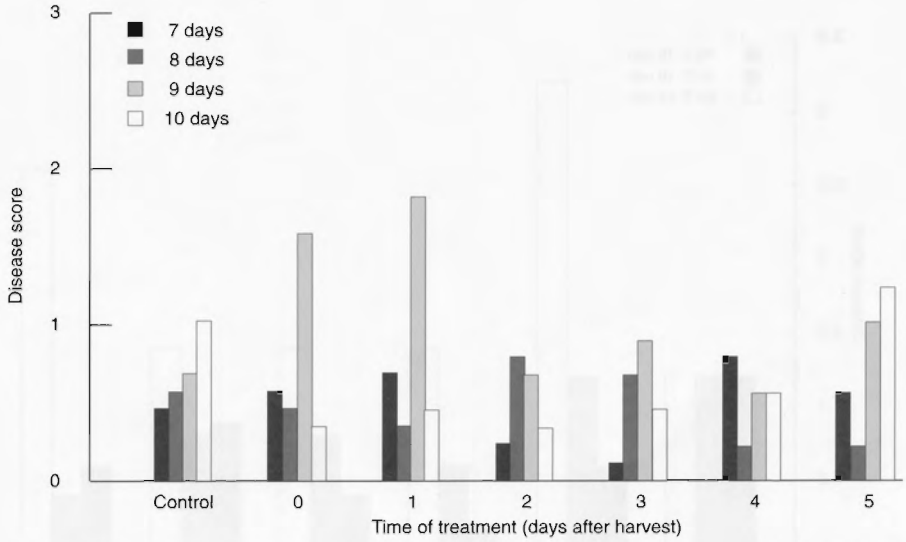

Figure 3. Daily (7th–10th days) disease increment scores in less mature avocados treated at 50°C for 10 minutes at 0–5 days after harvest.

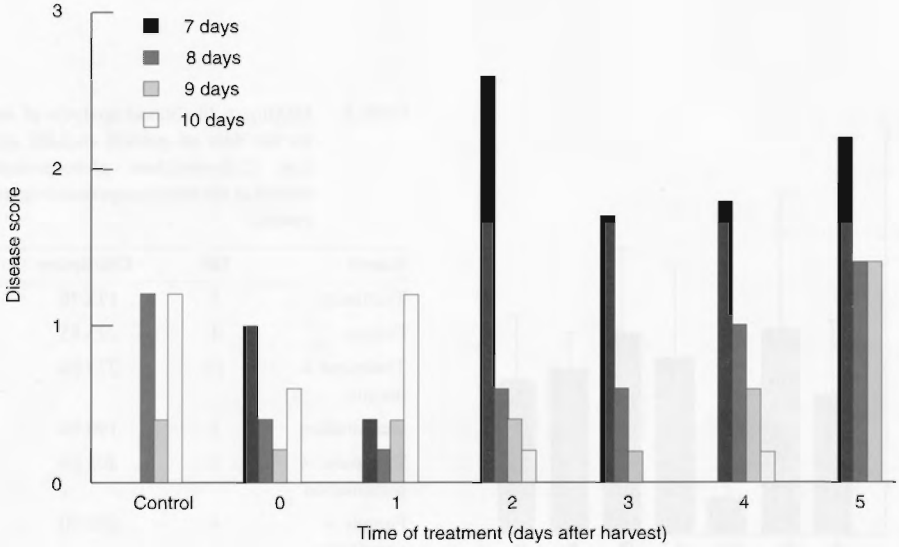

Figure 4. Daily (7th–10th days) disease increment scores of fruit treated at 50°C for 10 minutes at 0–5 days after harvest.

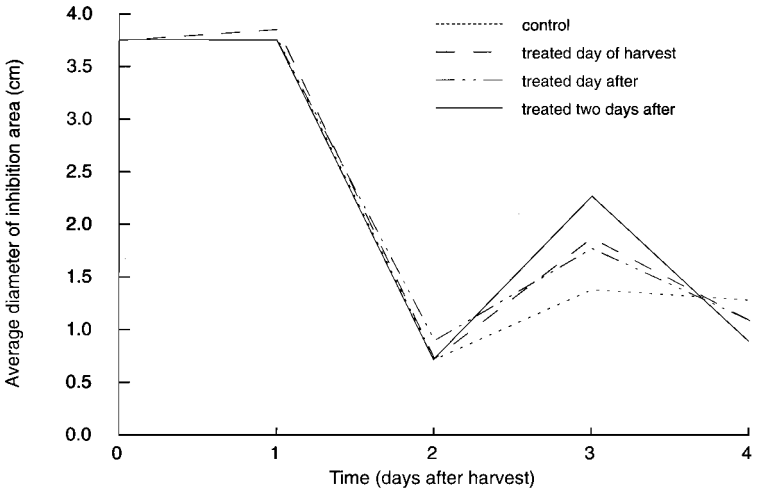

Figure 5. Average diameter of inhibition areas on TLC-bioassay plates from peel extracts of avocados obtained on four consecutive days treated at 0–2 days after harvest .

Discussion

A treatment temperature of 55°C was found to cause browning of the skin in this study as reported in an earlier study by Rupasinghe and Peiris (1992) in which 'Pollock' avocados were treated. Prusky et al. (1991) and Plumbley et al. (1993) used the same temperature for dipping 'Fuerte' avocados (for 5 and 10 min) and do not report skin browning. Although we did not observe browning in the fruit subject to the other two temperature–time regimes of treatment, Jessup (1991) who tried a 0.5% benomyl dip at 50°C for 3 min on 'Hass' avocados, observed phytotoxic effects (skin scalding) in some subsequent trials. According to him, this was not attributable to regions or maturities, and therefore subsequent treatments were done at 46°C, which did not cause any skin scalding (A.J. Jessup, unpublished data). In contrast, Trejo-Gonzalez et al., (1992) reported that hot water treatments from 30 to 45°C for 30 min of intact 'Hass' avocados, reduced extractable polyphenoloxidase activity without having detrimental effects on peel colour, pulp texture and flavour. Woolf and Laing (1996) reported that skin browning observed in 'Hass' avocados treated at 50°C (1–10 min) could be eliminated by a pretreatment at 3°C for 1 hour. This disparity in observations made by different authors on heat treatment of avocados suggests a high variability in the response of avocados (even within the same cultivar) to heat treatment, unlike many other fruit.

Disease incidence of avocados treated at 55°C increased more than twofold (Fig. 1) and it may possibly be attributable to skin scalding. Peels of skin tissues treated with a vital stain (neutral red) showed that fruit treated at 55°C did not take up the stain as readily as the control fruits, implying cell death. Therefore, more disease could probably be expected originating from damaged skin areas. However, there are reports of disease reduction in avocados treated at this temperature. For instance, Flores (1992) recorded that *Phytophthora* sp. was the only pathogen on cv. 'Pollock' which was not controlled by water dipping at 55 and 30°C for 10 min at each temperature. Some authors have reported skin browning and a disease reduction when hot water treatment was combined with a benomyl treatment (Jessup 1991; Rupasinghe and Peiris 1992). From our study, it appears that the major, if not the sole contributing factor to disease reduction in these studies would have been the fungicide treatment rather than the hot water treatment.

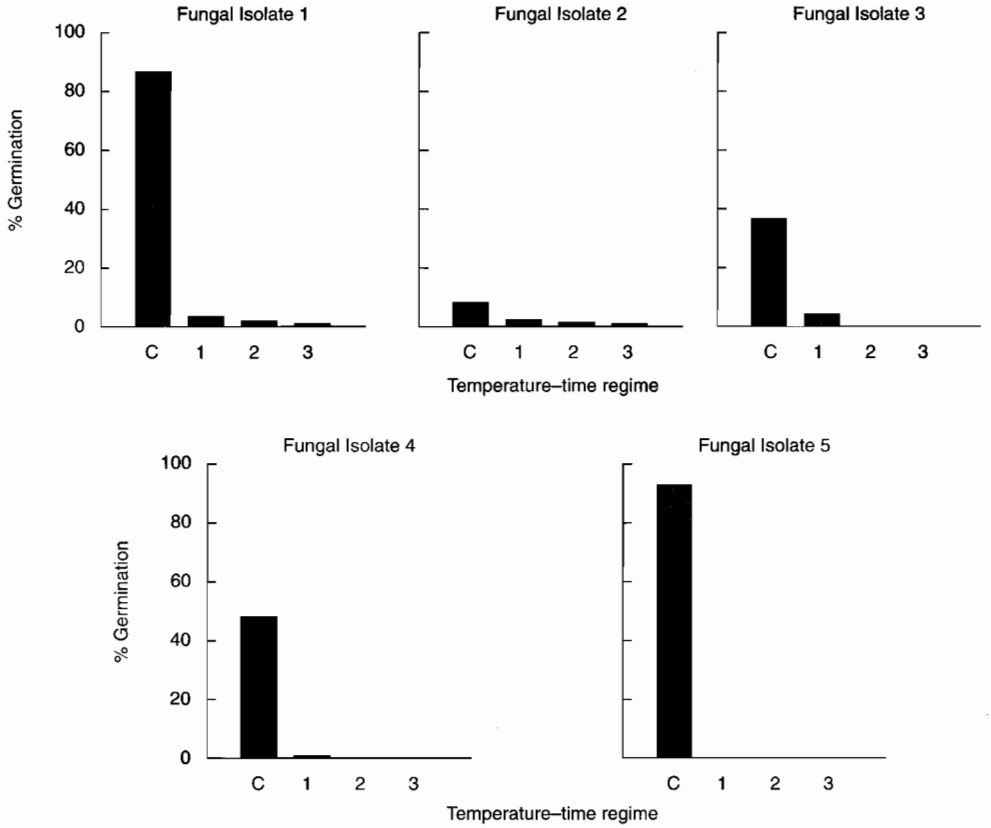

Figure 6. Effect of different temperature–time regimes (C = control; 1 = 45°C for 15min; 2 = 50°C for 10 min; 3 = 55°C for 15 min.) on percent conidial germination of five isolates of *Colletotrichum gloeosporioides*.

When the effect of the three temperature–time regimes on the viability of *C. gloeosporioides* spores was investigated, a progressive reduction of viability of conidia was observed with increasing temperature. It should be noted here that on the skin of intact avocado fruit there are quiescent infections and free-living conidia, and therefore this observation offers an explanation to the fate of the latter only. Of the five *C. gloeosporioides* isolates tested, the germination of isolate number 2 was low (8%) even at room temperature. In the other three isolates, there was a considerable reduction in spore germination at all three temperature–time regimes. It is apparent that the effect of 50 and 55°C treatments on disease development is due to a response by the fruit, rather than by the fungus.

The choice of time delay after harvest for treatment was found to be an important parameter. Contrary to the findings of Prusky et al. (1991), we observed a decrease in total antifungal activity only 2 days after harvest (Fig. 3). We found no effect of the treatments on total antifungal activity, but Plumbley et al. (1993) report that hot water treatment affects the concentration of the principal diene compound. The differences in the methods employed in the two studies should be noted; while these estimates of the total antifungal activity were by TLC-bioassays, Plumbley et al. (1993) used an HPLC method to determine concentrations of the antifungal diene only.

As there is no difference in the level of antifungal activity a day after harvest and on harvesting day, one would expect the effects of hot water treatment to be similar when fruit are treated on any of these two days, if antifungal activity was the major contributing factor for this effect. However, as a series of events takes place from the time conidia alight on the fruit surface before lesion initiation, as described by Coates et al. (1993), and as propagules on the surface might alight at different stages of fruit development, the answer to this problem may not be simple.

Plumbley et al. (1993) attribute a lag in resuming the initial concentration of the diene after its initial drop in concentration to the higher and earlier disease incidence observed when hot water-treated fruit are inoculated with *C. gloeosporioides*, 2 hours after harvest. However, it has to be established whether the concentrations of the diene during this lag phase are actually below the minimum fungitoxic concentrations. Prusky et al. (1988) reported that in susceptible 'Fuerte' avocados the level of the antifungal diene during fruit ripening is 120 µg/g fresh weight and in the resistant 'Hass' avocados it is 238 µg/g fresh weight. There is no indication that levels equal to, or lower than 120 µg/g fresh weight have been observed in the study of Plumbley et al., to justify earlier disease initiation in hot water treated fruits.

There are reports on physiological changes that avocado fruit undergo due to short-term heat treatments (Smillie 1992; Florissen et al. 1996). It is possible that heat-shock proteins (HSP), reported by Woolf et al. (1995) may be a contributing factor for these observations. Woolf et al. report that in flesh tissue (of avocado) sampled directly after heat treatments, the levels of mRNA homologous to cDNA probes for two plant heat-shock protein genes increased to a maximum at 40°C and declined at higher temperatures. Perhaps this event is species specific, and HSP are formed at a higher temperature of 45°C in the present cultivar. If HSP are formed here, it has to be established that they have the ability to delay disease occurrence, in the same way they are able to prevent chilling injury. The presence of HSP in the peel tissues has to be established.

The explanation for pronounced disease reduction seen on fruit treated at 45°C, a day after harvest, may lie in several contributing factors. Since less mature fruit did not respond by showing a reduction in disease development it may be logical to conclude that the 'factor/s' responsible for the disease reduction in the fruit is present only in fully mature fruits. It is not justifiable to seek a simple answer for a complex situation where there is an interaction among several factors in the fruit as well as the pathogen.

Acknowledgment

The authors wish to record their gratitude to Professor R.O. Thattil, for suggesting suitable statistical analyses.

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Apple Host Defence Reactions as Affected by Cycloheximide, Phosphonate, and Citrus Green Mould, *Penicillium digitatum*

B.L. Wild*, C.L. Wilson†, and E.L. Winley*

Abstract

A host defence reaction was detected in apples which reduced decay development in fruit that had been challenged by the apple blue mould organism *Penicillium expansum*. It was found that this reaction could be negated by the application of the protein synthesis inhibitor cycloheximide, which when applied to inoculated fruit resulted in more than a 700% increase in decay. The defence response was temperature dependent and was optimised by holding damaged fruit at 22°C for 48 hours before inoculation. Decay development under these conditions was reduced from 96% to 37%. Application of potassium phosphonate after fruit injury reduced decay initially by over 60%, although this compound had no *in vitro* activity against the fungus.

Inoculation of apples with the citrus green mould pathogen, *Penicillium digitatum*, normally does not result in decay in fresh apples. However, when the defence reaction was prevented by the application of cycloheximide, mould development occurred. It was also found that if mould resulting from *P. digitatum* infection progressed through an apple and came in contact with a previously damaged section, it progressed around that damaged site, leaving a halo of uninfected tissue of up to 1 cm in diameter. It is proposed that this area is where the host defence reaction is occurring and it has been visualised by *P. digitatum* development. Gel electrophoresis of protein production in these areas showed that phosphonate increased production of proteins particularly in the 25 k Dalton range, whereas cycloheximide inhibited it.

THE concept of reducing postharvest fruit decay by manipulating the host's defence mechanism has been used for many years (Adikaram 1990). In crops like potatoes (Forbes-Smith 1988), sweet potatoes (Snowdon 1992), onions (Shipway and Parkin 1985), and citrus (Hopkins and Loucks 1948) it is a technique that provides a method of decay control without the application of fungicides. It has been shown that by favouring the defence reaction by holding fruit at high temperatures for several days before cool storage, decay development can be reduced. To date a defence reaction in apple fruit has been reported only in immature fruit (Rey and Noble 1984; Swinburne 1975) and it has not been manipulated for reducing breakdown and decay control in mature fruit. Traditionally, decay control during storage and marketing has principally relied on fungicide application (Hardenburg and Spalding 1972) and manipulation of storage conditions (Koffman et al. 1989). There is, however, consumer demand for reduced fungicide application and the use of alternative 'non-chemical' means of controlling fruit rots (Wisniewski and Wilson 1992). One of the possible alternatives to fungicide application is the utilisation of induced resistance

* NSW Agriculture, Postharvest Group, Horticultural Research and Advisory Station, P.O. Box 581, Gosford NSW 2250, Australia.

† United States Department of Agriculture–Agricultural Research Service, 45 Wiltshire Road, Kearneysville, West Virginia 25430–9802, USA.

responses that occur under suitable conditions in some commodities (Wilson et al. 1994). This paper reports on investigations into a host defence reaction in mature apple fruit, and factors which govern its effectiveness in reducing decay by *Penicillium expansum* during storage. The involvement of this defence reaction in preventing infection by the citrus mould, *Penicillium digitatum*, and its potential involvement in decay development are also discussed.

Materials and Methods

Testing for a host defence reaction in apples

Golden Delicious apples were randomised into 8 experimental units each containing 20 fruit. Half of these fruit were wound inoculated four times around the equator of each fruit with a nail protruding 4 mm from a cork, and the other half were inoculated in a similar way except a smaller nail was used which only produced skin punctures 2 mm deep. To each of four of these treatment units the following dips were applied: (i) water dip only; (ii) 30 second dip in cycloheximide, 2 mg/L; (iii) dip in *P. expansum* spores at 10^6 spores/mL; and (iv) dip in cycloheximide at 2 mg/L plus *P. expansum* spores at 10^6 spores/mL. Fruit were held for 7 days at 20°C and examined for decay and per cent mould calculated.

Temperature effect on defence reaction

Experiment 1

Experimental treatment units each of 20 Red Delicious apples were held for 48 hours at 1, 18, 22, and 30°C and then punctured with a large nail as previously described. All fruit were then held for an additional 72 hours at those temperatures and then dipped in a spore suspension of *P. expansum* at 10^5 spores/mL. One additional experimental unit of fruit which was held at 22°C was dipped immediately in spores of *P. expansum* after the fruit were punctured. This enabled a comparison in the effect of the defence over the 72 hour storage period. All fruit were then brought back to 20°C for 7 days and then examined for mould development and percentage mould determined. The experiment was replicated three times.

Experiment 2

Granny Smith apples were divided into six treatment units each of 20 fruit and stored as previously described in experiment 1, except that the storage temperatures tested were 1, 14, 20, 25, 30, and 35°C. The rest of the experiment was conducted in the same manner as before.

Establishing citrus green mould infection in apples

Cycloheximide experiment

Early season Red Delicious apples were surface-sterilised by dipping them in a 70% ethanol solution, allowed to dry, then divided into four treatment units each of 20 fruit. All fruit were then punctured with a large nail as described previously. Half of these treatment units were dipped in water and the other half in 2 mg/L cycloheximide. Approximately 24 hours later, control and cycloheximide treatments were dipped in a suspension of 10^5 *P. digitatum* spores per mL and the remaining two treatment units dipped in a suspension of 10^5 *P. expansum* spores per mL. Fruit were held at 22°C for 10 days, then examined for decay. Fruit lesion diameter was measured and averaged as a measure of infection establishment.

Mature fruit experiment and induced 'halo' response

Mid-season Red Delicious apples were punctured four times around the equator with a large nail protruding 4 mm through a rubber stopper and then immediately dipped in a spore suspension of 10^5 spores/mL *P. digitatum*. The aim of this approach was to determine whether or not infections of this organism could be established before the apple had a chance to respond to the pathogen. Fruit were then held at 22°C for 10 days, then examined for decay.

Synergism in mould infection between *P. expansum* and *P. digitatum*

Eighty Red Delicious apples were surface sterilised by dipping them in 70% ethanol and, after drying, all fruit were punctured with a large nail as described previously. The following treatments were then applied to 20 fruit: (i) control—water dip only after fruit puncturing; (ii) dipped immediately in suspension of *P. digitatum* spores, 10^6 sp/mL; (iii) same as (ii) except that 24 hours after the dip in *P. digitatum* spores fruit were dipped in a *P. expansum* spore suspension, 10^5 sp/mL; and (iv) fruit dipped only in *P. expansum* spores (10^5 sp/mL) 24 hours after they were punctured. All fruit were then stored at 22°C and examined for mould 7 days later. The experiment was replicated three times.

Gel electrophoresis of protein synthesis in damaged tissue treated with phosphonate and cycloheximide

Fruit were wounded on four sides with a 5 mm diameter nail penetrating the apple tissue to a depth of 3 mm. Each wound was then inoculated with approximately 1 mL of phosphonate (10 mL/L) or cycloheximide (200 mg/mL) or distilled water. A control with no wound or treatment was used. The apples were kept at 20°C for 5 days. Tissue samples from around the wound sites were taken and frozen before extraction with a phosphate extraction buffer at a pH of 7.5 with PVP and mercaptoethanol added to reduce the occurrence of phenols. Gel electrophoresis was performed on the extracted tissue samples. A tris-glycine 10% gel was used with a native running buffer at pH8. The gel ran for 3 hours at 75 volts. A silver stain kit was used to stain the gel.

Results

Testing for a host defence reaction in apples

Levels of mould development in apples which had been punctured with the nail only were low, but fruit punctured and dipped in cycloheximide had significantly greater levels of decay (Fig. 1). With punctured fruit which had been dipped in a suspension of *P. expansum* spores, 24 hours after puncturing there was only a slight increase in decay. However, in fruit dipped in cycloheximide and then blue mould spores, decay levels increased by up to 700% in fruit punctured with both the large and small nails.

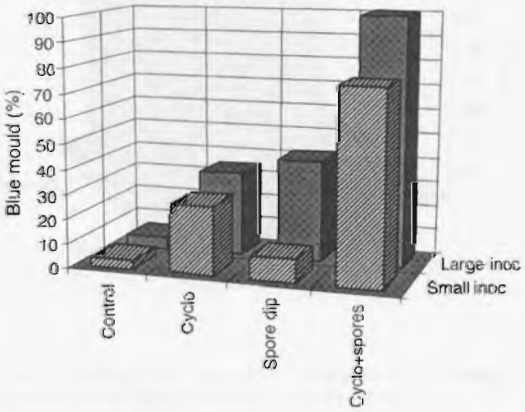

Figure 1. Influence of spore dip, skin puncture size, and cycloheximide on blue mould development in apples stored for 1 week at 22°C.

Temperature effect on defence reaction

Experiment 1

Storing Red Delicious apples at 22°C resulted in a reduction in decay of 63% when compared with the treatment where the spores were applied immediately after the fruit had been punctured (Fig. 2). Storage at lower temperatures reduced the effectiveness of the defence reaction so that at 1°C there was only a reduction of 20% in decay levels when compared with immediate inoculation after skin puncture.

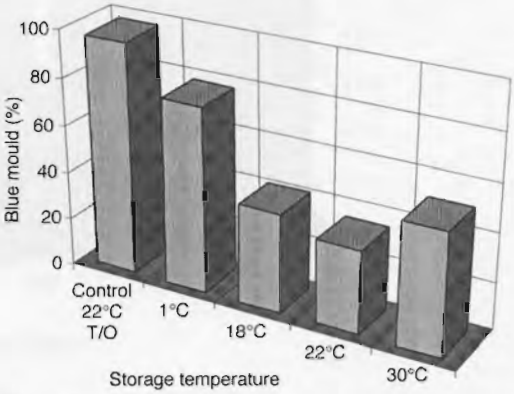

Figure 2. Effect of fruit temperature on decay development in Red Delicious apples which were inoculated with *P. expansum* spores 72 hours after the fruit had been punctured. In the control treatment, the apples were dipped in spores immediately after the puncturing of the

Experiment 2

Holding fruit at 35°C after puncturing the rind resulted in the greatest reduction in decay compared with storage at 1°C, with an initial reduction in decay of 73%. This decreased to a 45% reduction after the second examination 2 weeks after inoculation (Fig. 3).

Establishing citrus green mould infection in apples

Cycloheximide experiment

Apples treated with cycloheximide after being inoculated became 10 times more susceptible to infection by the citrus mould, *P. digitatum*, than the control (Fig. 4.) Once infection was established, lesion development with *P. digitatum* was found to be approximately twice as fast as apple blue mould, *P. expansum* (Fig. 5). Development of *P. digitatum* continued within the apple, and green mould spores were formed on the surface. Culturing of tissue samples from around the margin of the decay lesions confirmed that *P. digitatum* was the causal organism.

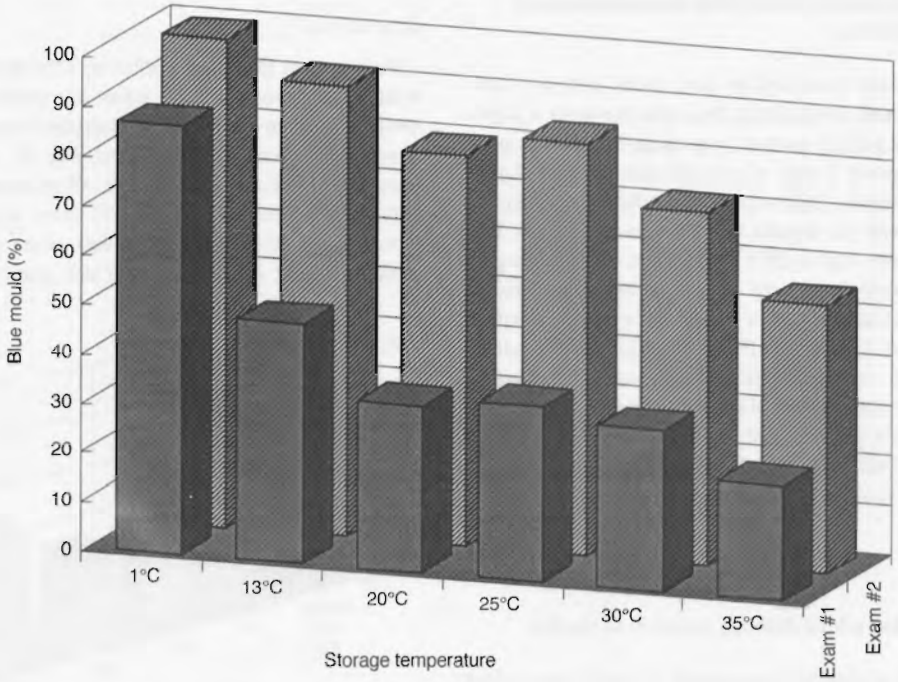

Figure 3. Effect of a range of fruit temperatures on the development of *P. expansum*, after fruit were dipped in the spores 72 hours after skin puncture.

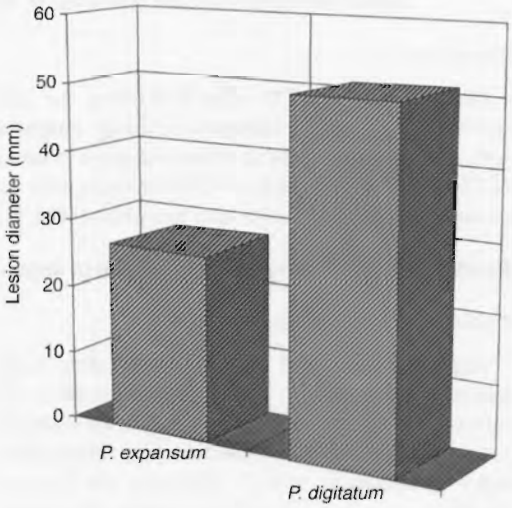

Figure 4. The effect of inhibiting protein synthesis, by using cycloheximide, on the development of decay by both apple and citrus moulds.

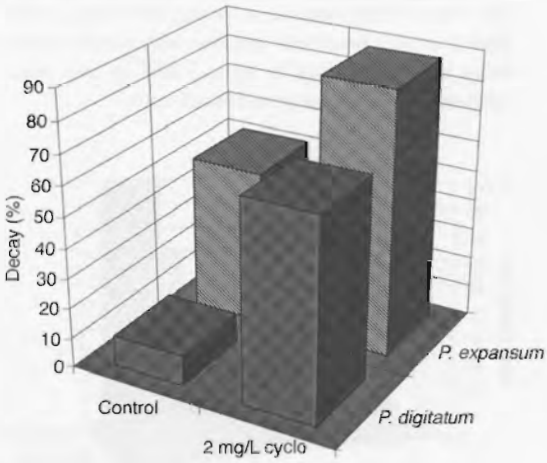

Figure 5. Comparison of the size in apples of decay lesions produced by inoculation by either citrus green mould, *P. digitatum*, or apple blue mould, *P. expansum*.

Mature fruit experiment and induced 'halo' response

Decay lesions of *P. digitatum* developed in approximately 30% of skin punctures when spores were applied at the time of skin puncturing. Where a lesion developed in one injury site on the apple and expanded so it came in contact with another damaged site which had not decayed, it was unable to invade an area of up to 10 mm in diameter around that damaged site. This left a 'halo' of uninfected tissue around the inoculation site (Fig. 6).

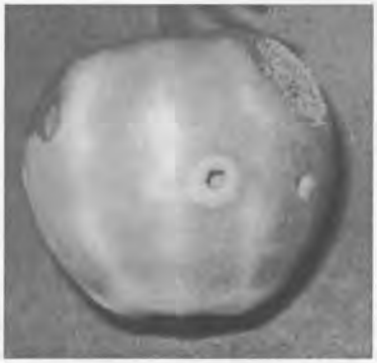

Figure 6. The development of the 'halo' response in apples as a result of invasion by the citrus green mould fungus, *P. digitatum*.

Synergism in mould infection between *P. expansum* and *P. digitatum*

When *P. digitatum* spores were applied first, followed 24 hours later by *P. expansum* spores, the levels of decay increased also three-fold from 28% for *P. expansum* alone to 79%. The level of decay with *P. digitatum* by itself was only 4% (Fig. 7).

Gel electrophoresis of protein synthesis in damaged tissue treated with phosphonate and cycloheximide

The results of gel electrophoresis of apple rind tissue which had been damaged and treated with UV light, cycloheximide, and potassium phosphonate are shown in Figure 8. Wounded tissue treated with phosphonate was found to produce denser protein bands around the 25 kDa region than unwounded fruit. The cycloheximide treatment, on similarly damaged fruit inhibited this synthesis. Protein synthesis in undamaged tissue was very slight with only one band showing up in the 70 kDa range.

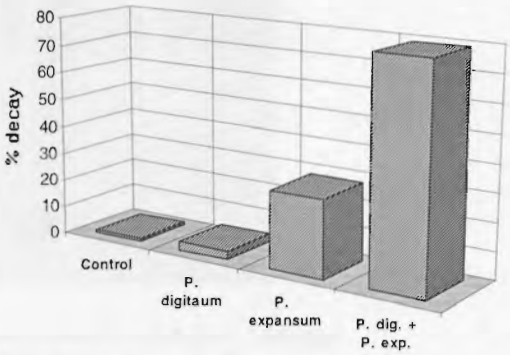

Figure 7. Synergistic effect on decay of the combination of citrus mould spores with apple blue mould, *P. expansum*.

Discussion

The dramatic increase in decay levels in apples treated with the protein synthesis inhibitor, cycloheximide, indicates that the formation of proteins in damaged apple tissue influences the infection process by apple blue mould, *P. expansum*. It also shows that the lack of protein synthesis in damaged tissue may make the apple susceptible to infection by organisms which would normally not be a problem. This was shown in the establishment of infection by citrus green mould, *P. digitatum*, and its rapid invasion of apple tissue once it had the defence reaction blocked. The presence of a defence reaction in apples was reported initially by Brown and Swinburne (1971) who analysed the formation of benzoic acid within the damaged tissue in immature apples. This paper reports for the first time that the defence reaction occurs also in mature fruit, and that it is temperature dependent with an optimum between 22 and 35°C. It is also possibly the main reason citrus green mould does not develop in apples despite its ability to produce pectolytic enzyme that are similar to those produced by *P. expansum* (Conway et al. 1988; Barmore and Brown 1979).

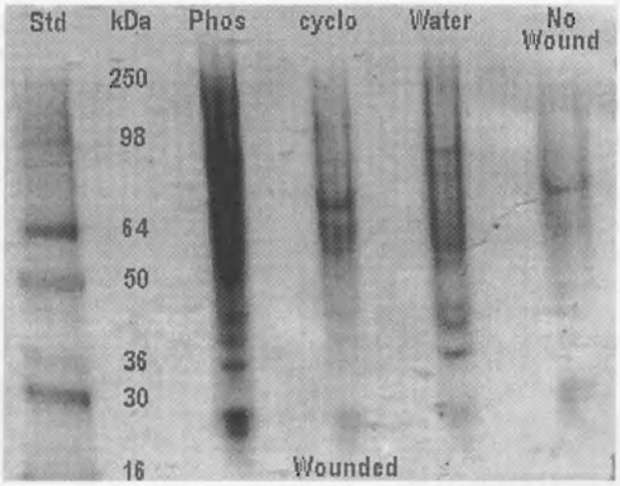

Figure 8. Stimulation of protein synthesis by phosphonate treatment in damaged tissue, compared with inhibitory effects of cycloheximide.

The synergistic association between *P. expansum* and *P. digitatum* in the infection process highlights the potential problem caused when these two pathogens are mixed. The *P. expansum* is possibly more capable of overcoming the apple defence mechanism than *P. digitatum*, but *P. digitatum* produces more rapidly developing decay lesions once it is assisted in the initial infection process by *P. expansum*. The sensitivity of *P. digitatum* to the host defence reaction is demonstrated by the 'halo' reaction that was observed when an expanding lesion came in contact with a previously damaged site. Presumably this fungus is more sensitive to the compounds formed during the defence reaction, which appears to occur within a radius of approximately 5 mm of the skin puncture.

More research is currently under way into what proteins are formed during the defence reaction and what compounds or barriers they are forming. The use of elicitors of defence reactions like potassium phosphonate (Wild 1993), ultraviolet light (Wilson et al. 1994), and salicylic acid (Delaney et al. 1994).

Acknowledgments

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Enhanced Biocontrol of Postharvest Diseases of Apples and Pears

R.J. Holmes*, S. de Alwis*, N. Shanmuganathan*†, S. Widyastuti§, and P.J. Keane§

Abstract

Major impediments to the commercial exploitation of yeasts for postharvest treatments have been their mediocre efficacy and high cost compared with synthetic fungicides. With the aim of improving the cost-efficiency of antagonistic yeasts, three treatments (phosphonate dipping, curing, and calcium dipping) with the potential to enhance fruit disease resistance were evaluated. Phosphonate (1000 µg/mL) when applied to wound-inoculated apples at the time of inoculation reduced the incidence of rot caused by *Botrytis cinerea* and *Penicillium expansum* by 50% and *Mucor piriformis* by 30%. Curing pears at 17°C for 24 hours was as effective as dipping in iprodione + benomyl or H10 *(Rhodotorula glutinis)* + $CaCl_2$ promptly after harvest. Curing Granny Smith apples at 15°C for 24 hours was as effective as H10 (1×10^7 cfu/mL) + $CaCl_2$ but less effective than vinclozolin + carbendazim. On Golden Delicious apples H10 (1×10^7 cfu/mL) + $CaCl_2$ gave complete control of blue mould and was as effective as iprodione + benomyl. The addition of $CaCl_2$ enabled highly effective biocontrol on apples with one-tenth the concentration of antagonist.

Phosphonate severely inhibited growth and pigmentation of antagonistic yeasts and is therefore not a suitable amendment. Phosphonate was also incompatible with diphenylamine (DPA) used after harvest for scald control and with $CaCl_2$ used to control bitter pit, and therefore cannot be integrated into current postharvest systems. No attempt was made to optimise curing treatments and further work to better define conditions giving rot control without compromising fruit quality could lead to practical nonchemical disease control.

SEVERAL species of yeasts have demonstrated antagonism toward postharvest diseases of apples (Wilson and Pusey 1985; Roberts 1990; Kampp 1994). However, major impediments to the commercial exploitation of yeasts for postharvest treatments have been the mediocre efficacy and the high cost compared with synthetic fungicides.

To improve the effectiveness of *Pichia guilliermondii* strain US-7, Hofstein et al. (1990) and Droby et al. (1990) amended yeast suspensions with 200 µg/mL thiabendazole. This approach, however, is counter to the philosophy of non-chemical disease control. Holmes et al. (1994) have selected and evaluated three yeast isolates H10 (*Rhodotorula glutinis*), D9 (*Rh. mucilaginosa*), and D20 (*Pichia guilliermondii*) antagonistic to the main postharvest diseases of apples and pears (caused by *Mucor piriformis, Penicillium* spp. and *Botrytis cinerea*). These isolates were consistently as efficacious as the synthetic fungicides, but only at concentrations of 1×10^9 cfu/mL or higher. It is estimated that at this concentration, the cost of fruit treatment would be over 10 times the cost of drenching in synthetic fungicides. With the aim of reducing the effective concentrations of antagonists, three treatments (phosphonate dipping, curing, and

* Agriculture Victoria, Institute for Horticultural Development, Private Bag 15, South Eastern Mail Centre, Victoria 3176, Australia.

† Retired.

§ Department of Botany, La Trobe University, Bundoora, Victoria 3083, Australia.

calcium dipping) with the potential to enhance disease resistance were evaluated.

Materials and Methods

Phosphonate dipping

Freshly harvested fruit (120 each of the cultivars Granny Smith, Lady Williams, and Packham's Triumph) were wounded in 10 places, dipped in phosphonate (1000 µg/mL; m-dKP fos-ject 200) or water and held at room temperature. Subsamples (20 fruit) were taken and inoculated with *Penicillium expansum* (10^4 cfu/mL) by dipping 2 hours after wounding then daily for up to 5 days after applying phosphonate.

The following season, fruit (Golden Delicious and Granny Smith) were wounded and dipped in phosphonate as described above and inoculated with *P. expansum, B. cinerea* and *M. piriformis* (10^4 cfu/mL) 0, 24, and 48 hours after treatment. In both seasons fruit were incubated for 7 days at 23°C and examined for rot incidence.

Diphenylamine (DPA), a powerful antioxidant, is applied to apples and pears to prevent superficial scald during storage. To establish the compatibility of DPA and phosphonate treatments, Granny Smith fruit were wounded and dipped in a suspension of *P. expansum* (10^4 cfu/mL) containing phosphonate (1000 µg/mL) with and without DPA (2100 µg/mL), 0 and 24 hours after wounding. Controls were *P. expansum* only and *P. expansum* + DPA. Rot incidence was assessed after incubation at 23°C for 7 days.

To determine if phosphonate directly inhibited the three yeasts, yeast suspensions (100 mL; 1×10^9 cfu/mL) were spread onto PDA plates containing phosphonate (1000 µg/mL; m-dKP fos-ject 200) incorporated into the medium. Yeast growth and pigmentation were examined after 4 days at 23°C.

To check if phosphonate and $CaCl_2$ when mixed were injurious to fruit, wounded and unwounded Granny Smith apples were dipped in phosphonate (1000 µg/mL; m-dKP fos-ject 200) and $CaCl_2$ (2% flake grade) for 30 seconds, dried and then incubated at 23°C for 7 days.

Calcium dipping

To establish the benefits of formulating antagonists with $CaCl_2$, freshly harvested apples cv Golden Delicious were wounded in 10 places and immersed in suspensions containing a mixture of two strains of *P. expansum* (1×10^4 cfu/mL) and one of the three antagonists with or without $CaCl_2$ (2% flake). Wounded fruit were also immersed in *P. expansum* inoculum suspended in iprodione (500 µg/mL; Rovral®) + benomyl (250 µg/mL; Benlate®), $CaCl_2$ or water. Treatments were applied randomly to 20 replicates, and fruit were incubated at 21°C for 7 days before assessment for rot incidence.

Efficacy of antagonists with and without calcium chloride, phosphonate and delayed dipping (curing)

Freshly harvested Packham's Triumph pears (6 replicates of 100 fruit per treatment) were immersed in a suspension containing pathogen inoculum (2.5×10^3 cfu/mL of benomyl-resistant *P. expansum*; 2.5×10^3 cfu/mL of benomyl-sensitive *P. expansum*; 2.5×10^3 cfu/mL of *Botrytis cinerea*; 2.5×10^3 cfu/mL of *M. piriformis*) and DPA (1050 µg/mL), and either antagonist (with or without $CaCl_2$), or fungicide (iprodione 500 µg/mL; benomyl 250 µg/mL), or phosphonate or water. A 'curing' treatment (24 hours at 17 °C and ambient humidity) before dipping was superimposed on the phosphonate and water treatments. Fruit were stored at 0°C for 140 days then incubated at 21°C for 5 days before assessment.

Freshly harvested Granny Smith apples (9 replicates of 100 fruit per treatment) were immersed in a suspension containing pathogen inoculum (5×10^3 cfu/mL dicarboximide-resistant *P. expansum*; 5×10^3 cfu/mL *Botrytis cinerea*) and DPA (2100 µg/mL), and either antagonist (H10 at 2 concentrations with $CaCl_2$), or fungicide (500 µg/mL vinclozolin; 250 µg/mL carbendazim), or phosphonate, or water. A 'curing' treatment (24 hours at 15°C and ambient humidity) before dipping was superimposed on the phosphonate and water treatments. Fruit were stored in a controlled atmosphere (–1.0°C, 1.0 % CO_2 : 1.5 O_2) for 119 days then incubated for 5 days at 21°C before assessment.

Results

Effectiveness of phosphonate

Phosphonate reduced the *incidence* of rot by 40 to 60% when applied to apples and pears 2 hours before the pathogen. In treatments inoculated 1 day or more after wounding, rots failed to develop in the fruit

dipped in water or phosphonate. This is an indication of curing. Cyclohexamide, known to inhibit disease resistance in plants, reversed the effects of phosphonate and curing, and made fruit susceptible to rot up to the 5th day in these experiments.

Phosphonate when applied with the inoculum 0, 1, and 2 days after wounding, reduced the *severity* of rot caused by *B. cinerea* and *P. expansum* by about 50%. Phosphonate reduced the *incidence* of *Mucor* rot by about 30% when applied with the pathogen inoculum.

Phosphonate severely inhibited the growth and pigmentation of the three yeast isolates and is therefore considered an incompatible treatment.

DPA greatly reduced (by between 60 and 80%) the efficacy of phosphonate applied 0 and 24 hours after wounding. DPA also increased the incidence of rot over the level in the *P. expansum* control.

The mixture of phosphonate and $CaCl_2$ caused small necrotic lesions to develop on fruit, centred on lenticels. While the pH of m-dKP fos-ject 200 (1000 µg/mL phosphonate) and of $CaCl_2$ were 6.0 and 9.9, respectively, the pH of the mixture was 4.4.

Comparative efficacy of antagonists with and without calcium chloride, phosphonate, and delayed dipping (curing)

Pears

Rot incidence was lowest where fruit were left undipped and therefore not inoculated. Delaying dipping for 24 hours after harvest (curing) gave fruit increased resistance to infection and this treatment was as effective as Rovral® + Benlate® or H10 + $CaCl_2$ applied within 6 hours of harvest (Table 1). Phosphonate, however, was no better than water. Leaving fruit at 17°C for 24 hours before dipping appeared to have no adverse effects on fruit quality after 140 days cold storage and 5 days at 20°C. Fruit that received calcium treatments ripened less rapidly and less evenly than fruit from other treatments.

Apples

This experiment was conducted at an orchard where neither *Mucor* nor benomyl-resistant *Penicillium* was present. Under these conditions, Ronilan® + Bavistan® gave the best level of control. Yeast isolate H10 + $CaCl_2$ at both concentrations and curing were also efficacious, although phosphonate was of no benefit (Table 2).

Table 1. Mean rot incidences in Packham's Triumph pears treated with DPA and either antagonistic yeasts (isolates H10, D9, or D20) with and without calcium chloride, fungicides, or phosphonate. All fruit except 'undipped' were inoculated with a mixture of benomyl-resistant and benomyl-sensitive *P. expansum*, *Botrytis cinerea*, and *M. piriformis* at the time of treatment. Numbers followed by the same letter are not significantly different at $p < 0.05$. Statistical significance on angular transformed data.

Rank	Treatment details (concentrations in cfu/mL)	% rot	
1	Undipped	5.8	a
2	Rovral® + Benlate®	6.7	ab
3	Phosphonate 24 hours postharvest	8.7	abc
4	Water 24 hours postharvest	9.3	abc
5	H10 (8×10^7) + $CaCl_2$	9.6	abc
6	H10 (8×10^7)	11.3	cd
7	D9 (1×10^8) + $CaCl_2$	12.8	cd
8	Phosphonate 0 hours postharvest	13.7	cd
9	D20 (1×10^8) + $CaCl_2$	13.9	cd
10	D20 (1×10^8)	14.0	d
11	D9 (1×10^8)	14.0	d
12	Water 0 hours postharvest	17.9	d

Table 2. Mean rot incidences in Granny Smith apples treated with DPA and either antagonist H10 plus calcium chloride, fungicides, phosphonate, or water. Fruit were inoculated with a mixture of dicarboximide-resistant *P. expansum* and fungicide-sensitive *Botrytis cinerea* at the time of treatment. Numbers followed by the same letter are not significantly different at $p < 0.05$. Statistical significance on angular transformed data.

Rank	Treatment details (concentrations in cfu/mL)	% rot	
1	Ronilan® + Bavistin®	0.5	a
2	H10 (1×10^8) + $CaCl_2$	2.6	b
3	Undipped	2.9	b
4	Water 24 hours	3.9	bc
5	H10 (1×10^7) + $CaCl_2$	4.4	bc
6	Phosphonate 24 hours postharvest	5.0	cd
7	Water 0 hours postharvest	5.6	cd
8	Phosphonate 0 hours postharvest	6.8	d

Calcium dipping

In a laboratory-scale trial yeast antagonists H10, D9, and D20 at 1×10^8 cfu/mL in combination with $CaCl_2$, and H10 and D20 at 1×10^8 without $CaCl_2$, were as efficacious as the standard fungicide treatment (Rovral® + Benlate®). $CaCl_2$ (2 %) tended to enhance the efficacy of the antagonists but it did not affect rot incidence on its own (Table 3).

Table 3. Mean rot incidences (% of wounds infected) in Golden Delicious apples treated with antagonistic yeasts and calcium chloride. Fruit were inoculated at the time of treatment with a mixture of dicarboximide-resistant *P. expansum* and fungicide-sensitive *P. expansum*. Numbers followed by the same letter are not significantly different at $p < 0.05$. Statistical significance on angular transformed data.

Rank	Treatment details (concentrations in cfu/mL)	% rot	
1	Rovral® + Benlate®	0	a
1	H10 (1×10^8) + $CaCl_2$	0	a
2	D20 (1×10^8) + $CaCl_2$	0.5	a
3	D9 (1×10^8) + $CaCl_2$	1.0	a
4	H10 (1×10^8)	2.5	a
5	D20 (1×10^8)	3.5	a
6	D9 (1×10^8)	10.5	b
7	$CaCl_2$	22.5	c
8	Water control	23.5	c

Discussion

Phosphonate

Phosphonate reduced the incidences of rots caused by the wound parasites *Mucor piriformis, Penicillium* spp. and *Botrytis cinerea*. However, it was incompatible with DPA used to control scald and $CaCl_2$ used to control bitter pit and therefore cannot be integrated into current postharvest systems. Phosphonate was also incompatible with the antagonists and it is therefore not a suitable amendment. Alternative techniques for scald control which avoid wetting fruit are under development, and it has been demonstrated in this study that when fruit are not wetted, the incidence of rot is lower than the incidence in phosphonate treatments.

Calcium dipping

Apples become susceptible to fungal attack due to the action of pectolytic enzymes, produced by the fruit, on the middle lamellae of the fruit cell walls (Knee et al. 1975). It is speculated that the calcium content of cell walls determines the rate of self-degradation, and increasing the calcium content of fruit reduces rot development in storage (Johnson 1979; Conway and Sams 1985).

$CaCl_2$ is a readily absorbed source of calcium used on apples to reduce physiological disorders. It is inexpensive, non-injurious to fruit at 2%, and an approved food additive. In this study $CaCl_2$ (2%) did not directly reduce rot incidence in long-term storage, but it did assist biological control of postharvest rots. The mode of this enhancement is not known and it may not be general. Calcium increased the efficacy of some antagonistic yeasts (Gullino et al. 1991; McLaughlin and Wilson 1992), but not *Cryptococcus* spp. (Roberts 1990).

Delayed dipping and cooling

Holding freshly harvested pears at 17°C and apples at 15°C at ambient humidity for 24 hours before postharvest treatment effectively reduced rot incidence. No attempt was made to optimise these treatments and further work may better define conditions giving higher levels of control without compromising the storage performance and quality of the fruit.

Pre-storage heat treatments have been shown to reduce rots and to have other benefits. Spotts and Chen (1987) demonstrated that heating pears to 27°C for 1–3 days at 98–100 % RH reduced decay caused by *M. piriformis* and *Phialophora malorum* but not *B. cinerea* and *P. expansum*. Klein and Lurie (1992) reported improved quality and less scald in Granny Smith apples heated to 46°C for 24 hours before storage. The contemporary practice of rapidly cooling fruit promptly after harvest should be reconsidered.

While cyclohexamide reversed the benefit of delayed dipping and cooling, this is not necessarily evidence for an induced defence mechanism. By wounding and inoculating pears before and after heat treatment, Spotts and Chen (1987) demonstrated that the effect of heating is on the wound site and speculated that the disease resistance achieved is due to a healing response.

While immature apples contain several phenolic compounds with fungicidal activity e.g. *p*-coumarylquinic and chlorogenic acids (Ndubizu 1976) and can synthesise the phytoalexin benzoic acid in response

to infections by *Nectria galligena* and *Cryptosporiopsis malicorticis,* these properties are absent in mature fruit (Brown and Swinburne 1971). The proposed resistance mechanism in mature apples is desiccation and the formation of anti-fungal compounds by the oxidation of phenolic compounds at the injury site (Kahl 1978). Further work to confirm and enhance this mechanism could enable nonchemical disease control.

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Studies on the Endophytic Mycota of *Citrus* spp.

J.G. Wright,* G.I. Johnson,† and K.D. Hyde*

Abstract

An investigation has been undertaken to determine the pattern of colonisation of citrus fruit and branches by stem-end rot pathogens (*Phomopsis citri*, *Lasiodiplodia theobromae*, and *Botryosphaeria* sp). The healthy (symptomless) flowers, fruits, pedicels, and branches (five most recent growth flushes) of *Citrus reticulata* var 'Nin Kat' that had been regularly sprayed or not sprayed with copper oxychloride were assayed monthly for endophytic fungi over two consecutive seasons. All three stem-end rot pathogens were confirmed to be endophytes. Members of other genera (including known pathogens of *Citrus*) were also recovered as endophytes including *Colletotrichum* (anthracnose), *Guignardia/Phyllostictina* (black spot), *Mycosphaerella* (greasy spot), *Cladosporium*, and *Alternaria*. Recovery levels of all fungi varied in time, reflecting the complex and dynamic nature of the host/colonist interactions.

In addition, other plantings in China and elsewhere were sampled as opportunity permitted. A greater diversity of fungi was recovered from plant material collected from Chinese sites and unsprayed sites, suggesting that domestication (outside the native range of citrus) and the application of fungicides may reduce the prevalence and diversity of the endophytic mycota of fruit trees.

The endophytic fungi present in healthy citrus tissues may represent a reservoir of:

- quiescent pathogens;
- potential pathogens;
- pathogen antagonists;
- insect repellents;
- harmless saprophytes; and
- host defence elicitors.

It is in the last-named role that they merited consideration by this workshop. Endophytic colonists are present throughout tree growth and development. In any crop centre of origin, evolution may have favoured those endophytes that provided some natural selection advantage to the host. The advantages may have included the production of anti-microbials by the endophytic colonist or the elicitation of similar compounds by the host. Further studies are needed in the centres of origin of our major fruit species to seek and evaluate fungal colonists that might confer such advantages.

In recent years there has been intensive research into endophytic fungal assemblages of grasses and woody plants. Although the concept of fungi living symptomlessly in the host-plant tissue has been around since the 1800s, it is only in recent times that the term 'endophyte' has been defined and used. The currently accepted definition is given by Wilson (1995): 'endophytes are fungi or bacteria which, for all or part of their life cycle, invade the tissues of living plants and cause unapparent and asymptomatic infections entirely within plant tissues but cause no symptoms of disease'. The great bulk of research on endophytes of dicotyledonous plants has been directed towards sampling the biodiversity of the endophytic assemblages, but the roles of most of these fungi have yet to be elucidated.

* Department of Ecology and Biodiversity, The University of Hong Kong, Pokfulam Road, Hong Kong, China.

† Australian Centre for International Agricultural Research, GPO Box 1571, Canberra, ACT 2601, Australia.

The citrus stem-end rot (SER) pathogens *Phomopsis citri* (teleomorph, *Diaporthe citri*), *Lasiodiplodia theobromae*, and *Fusicoccum* sp. (teleomorph, *Botryosphaeria* sp), have been investigated over many years (Pole Evans 1910; Fawcett 1911; Fawcett 1931; Homma et al. 1989). They had economic significance until the discovery that the application of 2, 4–dichlorophenoxyacetic acid to the fruit after harvest could control stem-end rot (Erickson 1964). In citrus, deterioration of the calyx allows the pathogens to enter the fruit (Nadel 1944), so keeping this tissue healthy is paramount, and may be achieved with 2,4-dichlorophenoxyacetic acid and gibberellic acid. The calyx is therefore the critical site for fruit infection. Current understanding of the infection process for *P. citri* and *L. theobromae* is that conidia arrive at the stem end of attached citrus fruit and colonise the necrotic tissue of the calyx surface, where they remain quiescent until harvest (Nadel et al. 1947; Brown and Eckert 1988). At harvest, hyphae grow through cracks in the abscission zone and colonise first the underlying albedo layer, then the central vascular tissue of the fruit. Symptoms usually appear from 10–20 days after harvest. Kuntz (1932) and Minz (1946) suggest that infections can occur anytime throughout the season for both *P. citri* and *L. theobromae*.

Although these results are well established, evidence of alternative infection processes can be found in the literature. A definitive experiment in which the fruit is covered to prevent infection by spores and the subsequent development of stem-end rot development in fruit is monitored could indicate the relative importance of endophytic infection. Homma and Yamada (1969) reported that not only could *Diaporthe citri* infect through wounds and scars, but also that it could penetrate into inner tissues easily through 'bearing shoots and mother branches with bearing shoots'. All three SER pathogens are capable of causing other disease symptoms in the citrus tree: melanose in the case of *Phomopsis citri*; root rot, dieback, and trunk gummosis by *Lasiodiplodia theobromae*; and gummosis by *Fusicoccum* (Whiteside, 1988a,b; Menge 1988). Colonisation leading to these symptoms is usually reported as occurring under environmental stress. and hence they are classified as secondary pathogens. The source of inoculum for these diseases may be endophytic mycelia present in host tissue, but contained, presumably, by host resistance factors. Although the link has yet to be made between endophytes and pathogenicity, these findings warrant further investigation.

Materials and Methods

Citrus reticulata variety 'Nin Kat' trees growing at Kadoorie Farm (Tai Po, New Territories, Hong Kong) were chosen as sample trees. The trees were

Table 1. Fungi isolated as endophytes from *Citrus* species.

Hyphomycetes	Coelomycetes	Ascomycetes	Unknown
Alternaria citri[a]	*Colletotrichum*	*Biscogniauxia* sp.	Cercospora- like
Alternaria tenuissima[a]	*gloeosporioides*[a,c]	*Chaetomium globosum*[b]	Various xylariaceous mycelia
Alternaria sp.	*Colletotrichum*	*Daldinia* sp.	sterilia
Aspergillus sp..	Type 2	*Diaporthe citri*[a]	Various non-xylariaceous
Aureobasidium pullulans[b,c]	*Diplodina* sp.	*Glomerella cingulata*[a]	mycelia sterilia
Cladosporium sp.[a,b,c]	*Dothiorella* sp.[a]	*Guignardia citricarpa*[a]	
Cuvularia lunata	*Lasiodiplodia theobromae*[a]	*Hypoxylon* sp.	
Fusarium spp.[a,c]	*Pestalotiopsis* sp.	*Leptosphaeria* sp.	
Microdochium sp.	*Phoma multirostrata*	*Mycosphaerella citri*[a]	
Nigrospora oryzae[c]	*Phoma tenuis*	*Ophioceras* sp.	
Nodulisporium sp.[c]	*Phoma* sp.	*Sporomiella* sp.	
Penicillium digitatum[a]	*Phomopsis citri*[a,c]	*Xylaria* sp.[c]	
Ramichloridium sp.	*Phyllostictina citricarpa*[a,c]		
Spiropes sp.			
*Trichoderma*sp.[b]			
Ulocladium sp..			
Wardomyces sp.			

[a] Pathogens of Citrus species.
[b] Fungi already exploited as biological control agents.
[c] Fungi isolated from Citrus spp. regardless of location.

about 20 years old and were hard-pruned four years before the study began. Five trees were unsprayed and five were treated with copper oxychloride (50% wettable powder; 120 g/100 L). The sprays were applied once a month from April to September in both years. Samples of five branches from each of the ten trees were collected monthly for one year (April 1995–February 1996), and taken to the laboratory for isolation, where the flowers or fruits, and the five most-recent vegetative growth flushes were assayed. The parts from which isolates were to be taken were treated with a three-step sterilisation process as follows: 1 minute in 70% ethanol; 2 minutes in a 1:5 sodium hypochlorite solution; and 30 seconds in a 90% ethanol solution. The pieces were then allowed to dry on sterilised filter paper and plated out onto half-strength potato dextrose agar. The plates were incubated at 25°C in continuous light (12 hours daylight, 12 hours near ultraviolet light). Fungi growing out were either immediately identified by their spores or sub-cultured onto media to induce sporulation, then identified.

Results

Fungi were consistently isolated from every sampling point except the albedo, which yielded fungi much less frequently (Figs 1–3). There tended to be a basic suite of fungi (see Table 1) isolated regardless of location. Rarer fungi may have been more site or host specific.

Figures 1 to 3 show the mean recovery of *Phomopsis citri, Colletotrichum gloeosporioides,* and *Nodulisporium* sp. from each assay point sampled monthly over one season.

For both unsprayed and copper-treated trees, *P. citri* was isolated significantly less frequently from the albedo than other assay points. For each assay point, copper treatment had no significant effect on recovery of *C. gloeosporioides* and *Nodulisporium* sp. when compared with assays of untreated tree samples. Similarly, there was no significant difference in the recovery level of *P. citri* from various assay points on untreated trees. However, for copper-sprayed trees, recovery of *P. citri* from the pedicel was significantly lower than from the button and older growth flushes (GF1 to 3). Copper treatment significantly reduced recovery of *P. citri* from pedicel tissue and the youngest growth flush (GF5) when compared with the same assay points of untreated trees (Figs 1 to 3).

Discussion

Stem-end rot pathogens as endophytes

This investigation has shown that the three SER pathogens occur as endophytes in company with

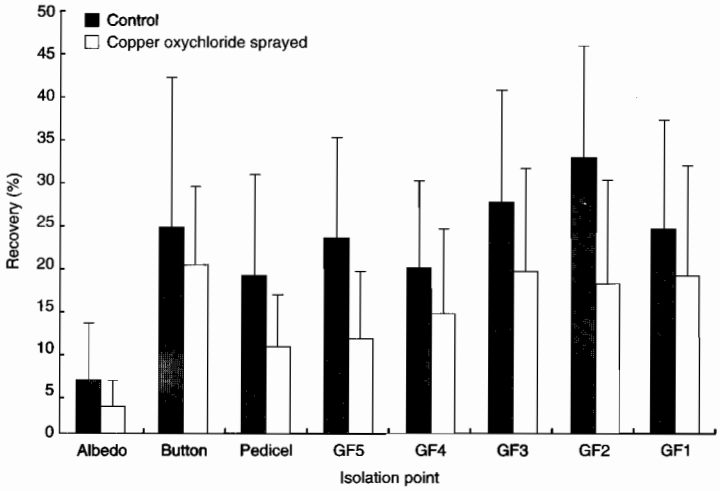

Figure 1. Mean recovery of *Phomopsis citri* as an endophyte from *Citrus reticulata* var 'Nin Kat' in Hong Kong from assay points as indicated. GF5 is the youngest growth flush closest to pedicel. Error bars show least significant difference $P \leq 0.05$.

many other fungi. The endophyte environment has proven to be dynamic, with levels of recovery of the fungi varying with each sampling over time. What causes these changes is as yet not understood. Factors such as temperature, humidity, free water, nutrient availability, cyclic microbial competition, and host anatomy and maturity must all play a part in the assemblage of fungi present at any one time, but the significance of each factor has yet to be determined. Johnson (1997) suggested that the concept of 'climax microbiota' could be applied to microbial colonists in the same manner in which 'climax vegetation' is used for describing temporal vegetation changes in an ecosystem. We suggest here that *Phomopsis citri*, *Colletotrichum gloeosporioides*, xylariaceous fungi, and their anamorphs are the

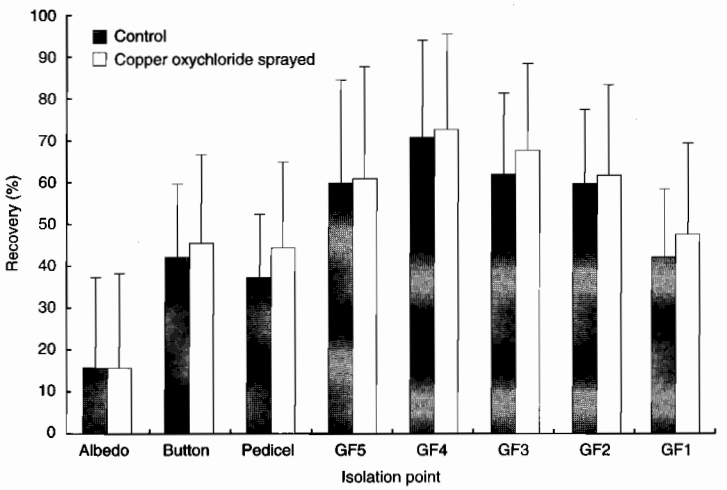

Figure 2. Mean recovery of *Colletotrichum gloeosporioides* as an endophyte of *Citrus reticulata* var 'Nin Kat' in Hong Kong from assay points as indicated. GF5 is the youngest growth flush closest to pedicel. Error bars show least significant difference $P \leq 0.05$.

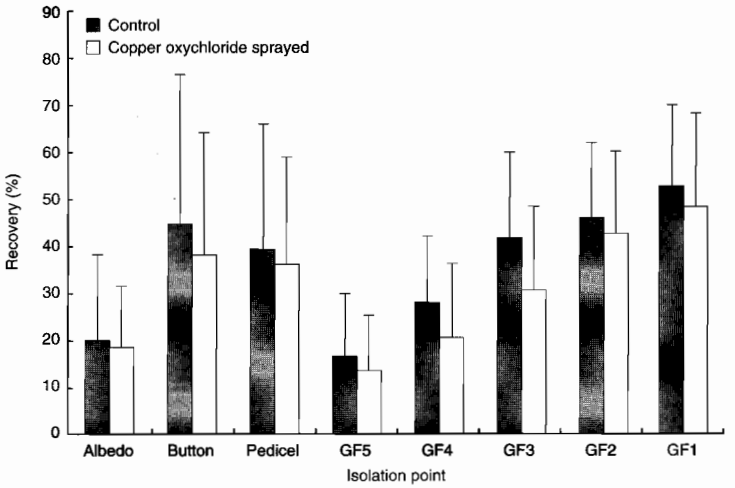

Figure 3. Mean recovery of *Nodulisporium* sp. as an endophyte of *Citrus reticulata* var 'Nin Kat' in Hong Kong from assay points as indicated. GF5 is the youngest growth flush closest to pedicel. Error bars show least significant difference $P \leq 0.05$.

major climax microfungi of citrus stem and fruit tissue. A sampling in Brunei from old citrus trees untouched by any commercial practices showed high recoveries of these fungi, particularly *P. citri* (J.G. Wright, unpublished data).

One question raised by this study is: Are there any interactions between the fungi, either direct or indirect, that affect the levels of recovery? The great challenge when studying the endophytic environment is that the investigator has little control over conditions. However, in the *C. reticulata* system, the fungi isolated as endophytes are being tested for antagonistic properties against the SER pathogens and each other (J.G. Wright, unpublished data). Copper pollution was found to change the balance of fungal species occurring as endophytes in Scots pine shoots, where those fungi shown to withstand elevated levels of copper in vitro were the fungi positively correlated with increasing concentration of copper in the shoots (Ranta et al. 1994).

This investigation was undertaken to study the effects of copper pollution on both pathogenic and non-pathogenic fungi, to see if tolerant fungi gained a competitive advantage against sensitive endophytes. Our study may have been too short for major differences to appear. Ranta et al. (1994) found that the pathogen (*G. abietina*) was as sensitive as the fungi that could possibly be antagonistic. Barklund and Unestam (1988) found that pollutants had a negative effect on the endophytic fungi of Norway spruce, suggesting that this led to susceptibility to *G. abietina*. In the case of citrus, *Phomopsis citri* appeared more affected than the other two fungi (Fig. 1). Recovery of *Colletotrichum gloeosporioides* did not appear to be affected by copper sprays at all (Fig. 2). This study encompassed only one year of copper sprays in the tropics, where such sprays may not be durable (due to the wet season), and an effect may be observed only after a number of years. To understand the effects of copper on the internal environment of the tree is vital, as the endophyte habitat may be a reservoir for both pathogens and potentially antagonistic fungi and bacteria.

Biological control relies on the understanding that resources are limited and that there will be many types of competition as organisms vie for niches, and this may be said to be true for the endophytic environment also. As such, it has potential for manipulation to exclude or destroy pathogenic organisms. Advances have been made in control of citrus pathogens after harvest (Korsten et al. 1993; Shachnai et al. 1996) and in the rhizoplane (Tsao et al. 1996). The concept of competition for nutrients has been exploited in controlling blue and green mould and sour rot of citrus (Chalutz and Wilson 1990).

Biological control agents may be used to elicit a host response. In citrus, for example, the phytoalexin scoparone (6,7-dimethoxycoumarin) is induced by *Phytophthora citrophthora*, *Verticillium dahliae*, *Penicillium digitatum*, *P. italicum*, *Colletotrichum gloeosporioides*, *Hendersonula toruloidea*, and *Diplodia natalanesis* (*Lasiodiplodia theobromae*). Scoparone is known to be a defence against both *Phytophthora citrophthora* (Afek et al. 1986) and *Phomopsis citri* (Arimoto et al. 1986). All the fungi tested were pathogens of citrus, but it may be that other non-pathogenic fungi could elicit the same response, and that endophytic fungi (with pathogenic potential or not) could be eliciting the production of scoparone.

Afek et al. (1986) reported that scoparone was also found in susceptible control plants, but in much lower concentrations. We suggest that low levels of scoparone may be produced in response to endophytic colonisation of healthy plant tissue. Induced resistance is a phenomenon that is currently exploited by using biological control agents applied to the surface of the host. It is not inconceivable that endophytic fungi could, in the future, be used to colonise young host tissue or specific tissues and play a significant role in plant protection practices.

In addition to eliciting host responses, endophytes may provide other advantages to the host. These may include spatial colonisation that excludes invasion by a pathogen (Freeman and Rodriguez 1993) or production of compounds that play various roles in the life of their host, including: attraction of insects (Nago and Matsumotot 1994); eradication of plant parasitic nematodes by secondary metabolites and resistance to soil-borne pathogens (Hallmann and Sikora 1996); resistance to herbivores (Diamandis 1981; Webber 1981; Clay 1988); resistance to plant pathogens (White and Cole 1985); and stimulation of germination (Luginbuhl and Muller 1980). The roles of endophytic fungi in citrus have yet to be resolved.

Conclusions

The three fungi causing stem-end rot of citrus were all recovered as endophytes, and proved pathogenic in artificial inoculation studies. The importance or role of the endophytic phase in a fungus's life cycle is not well understood, except in a few cases, and a more thorough understanding of this phase may identify a

'weak link' in the life cycle of a pathogen to target in disease control. It is vital that we understand how the host 'limits and manages' endophytic colonisation, so that we might use the same methods to manage losses from disease. Exploitation of 'engineered' endophytes to deliver other benefits to the host may be possible in the future, e.g. species from genera used as biocontrol agents on other crops have also been isolated as endophytes from *Citrus reticulata* and could be employed in the control of diseases of *Citrus* species. Knowledge of the effects of fungicides (such as copper oxychloride) on endophytic fungi will expedite integrated pest management systems.

The fungal genera recovered as endophytes of *Citrus reticulata* var. 'Nin Kat' have also been recovered from a variety of hosts including eucalypts (*Eucalyptus nitens*) (Fisher et al. 1993), grape vines (*Vitis vinifera*) (Cardinali et al. 1994), and Australian fan palms (*Licuala ramsayi*) (Rodrigues and Samuels 1990). Comparisons of numbers of genera isolated indicate that the *Citrus reticulata* sampled may have a depauperate endophytic assemblage, possibly attributable to a number of factors including agricultural practices and that the plants are vegetatively propagated (genetically identical) in monoculture orchards (ten Houten 1974). Agricultural practices have long been documented as causing 'unnatural' conditions leading to the prevalence of pathogenic fungi such as *Colletotrichum gloeosporioides* (Fig. 2). They may also lead to a loss in fungal diversity, as can be observed in the copper-treated trees. Other fungi isolated also belong to genera pathogenic to citrus including: *Phyllostictina/Guignardia*, *Cladosporium*, *Phoma*, *Fusarium* and *Alternaria*. How significant is this phase of their life cycles has yet to be determined.

The distinct differences in rates of recovery of fungi from the control and copper-treated trees are noteworthy. Although the copper fungicide used was not systemic, it appeared to reduce rates of recovery of *P. citri* (Figs 1–3). Whether this is due to its effect on the entry of endophytes into the host or whether a direct effect is occurring is unknown. Stirling et al. (1994) noted that copper fungicides reduced phylloplane microbial antagonists of *Colletotrichum gloeosporioides* on avocado fruit. In other systems, heavy metal pollution has been connected with depletion of nutrients (Raitio 1992). Use of copper fungicides in citrus led to phosphorus deficiency, a result of its effect on the vesicular arbuscular mycorrhizal fungi (Graham et al. 1986). This could effect, in turn, the endophytic population, as suggested by Ranta et al. (1994).

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Research Methodologies

Methodology for Studying Defence Mechanisms against Fungal Pathogens: an Overview

N.K.B. Adikaram* and B.M. Ratnayake Bandara†

Abstract

Apart from preformed chemical and cellular barriers, several post-infection mechanisms regulate pathogen infection simultaneously in fruits. These events could include host cell death and necrosis, accumulation of toxic phenols, chemical modification of cell walls, papillae formation, and *de novo* synthesis of phytoalexins and stress-related substances. Specific analytical and assay methods are required to follow each of these events. Fresh or freeze-dried fruit tissues collected following or without inoculation may be extracted using a convenient solvent. Vacuum infiltration of tissue with organic solvents makes subsequent analysis less complicated. Antifungal activity could be tested by germination assays or thin layer chromatography-bioassay, but the use of more than one assay method is often beneficial. Purification of antifungal compounds could be done by bioactivity monitored solvent fractionation by column chromatography, HPLC etc. in which the TLC-bioassay is the best choice. Cellular alterations such as lignification and papillae formation could be studied by histochemical tests. Procedures have been developed for extraction and electrophoresis of pathogenesis-related proteins. The paper relates how our study team has tackled research into antifungal substances in fruits in relation to fungal infection at Peradeniya and illustrates key steps and pitfalls.

PLANTS have evolved complex and diverse mechanisms of defence against invading pathogens. The protection of fruits until seed development, on which the existence of a species often depends, may have been particularly subject to selection pressure. Senescence generally reduces the effectiveness of defence responses in harvested fruits. Detailed understanding of the endogenous mechanisms of disease resistance in fruit may help in the development of new biological and biotechnological approaches to extend resistance to the ripening phase and protect the ripe fruits from disease. The antifungal activity of unripe avocado, which usually declines with ripening, has been retained in the ripe fruit by external treatment (Prusky et al. 1991) protecting fruit from postharvest rot. Induced resistance also holds promise as a new technology for the control of fruit diseases (Wilson et al. 1994). *Phyllosticta musarum*, a somewhat mild pathogen in banana, is capable of accumulating phytoalexins and inducing resistance to anthracnose disease (Abayasekara and Adikaram 1995).

Defence mechanisms in plants can be systemic or localised, constitutive or inducible. Constitutive structural (e.g. cuticle and other cellular barriers) and chemical (e.g. antimicrobial substances, pathogen-produced enzyme inhibitors) barriers provide passive defence. On the other hand, a range of inducible defence mechanisms at structural (cell-wall modification) and chemical level has been reported from plants. Accumulation of phytoalexins in fruits is well documented. The induction of enzymes in the phenylpropanoid pathway, oxidative enzymes, and hydroxyproline-rich glycoproteins (HRGP, extensin) is partially related to cell-wall modifications that hold back pathogen development (Bol et al. 1990). Peroxidases are involved in the polymerisation of alcohol derivatives of aromatic compounds such as coumaric,

* Department of Botany, University of Peradeniya, Peradeniya, Sri Lanka.

† Department of Chemistry, University of Peradeniya, Peradeniya, Sri Lanka.

ferulic, and sinapic acids into lignin and suberin, and the cross-linking of these polyphenols to extensin molecules from the cell wall matrix. 'Pathogenesis-related' (PR) proteins are induced in plants after infection with fungi, bacteria, and viruses. Van Loon and Van Kammen (1970) and Gianinazzi et al. (1970) independently reported *do novo* synthesis of several proteins in tobacco plants. These proteins were characterised by their acidic nature (Van Loon 1976), resistance to proteases, and extracellular location. PR proteins in plants have been classified into groups, some having unknown function, and others such as β-1,3-glucanase, chitinase, etc. exhibiting specific enzyme activities. Chitinases and β-1,3-glucanases are thought to play a role in plant defences against fungal infection (Schumbaum et al. 1986). It is also likely that both preformed and induced antifungal substances occur simultaneously in certain plants e.g. citrus (Ben-Yehoshua et al. 1992).

This paper outlines methodology employed for the investigation of defence mechanisms, with special reference to antifungal substances.

Constitutive or Preformed Antifungal Compounds

The term 'constitutive' includes compounds which are released from inactive precursors following tissue damage (Mansfield 1983). The first compounds found to have a role in disease resistance were two constitutive inhibitors, protocatechuic acid and catechol, water soluble phenolics that occur in the outer pigmented scales of onion bulbs resistant to smudge disease caused by *Colletotrichum circinans*. The phenolic compounds diffuse from the dead cells into overlying inoculum droplets and inhibit spore germination at the bulb surface (Link et al. 1929; Link and Walker 1933). Antifungal phenols occurring constitutively have been found in all plants investigated to date and are thought to function as preformed inhibitors associated with non-host resistance. Numerous plant-derived constitutive antimicrobial compounds belonging to diverse chemical classes have now been isolated and identified. The preformed antifungal activity has been implicated in the resistance of unripe fruits to fungal invasion, e.g. avocados (Prusky et al. 1982; Sivanathan and Adikaram 1989), wood apple (Adikaram et al. 1989), citrus (Ben-Yehoshua et al. 1992).

Inducible Antifungal Compounds

The concept of induced antifungal compounds originated from Bernard's (1909) observations and was further developed by Muller and Borger (1940) in a classic paper describing the resistance response of potato tubers to *Phytophthora infestans*. The antifungal compounds that accumulated in tubers inoculated with the incompatible race of *P. infestans* were termed phytoalexins. Over the past 50 years or so the production of phytoalexins has become the most extensively studied process of disease resistance in plants. Certain phenols are also formed in response to the ingress of pathogens, and their appearance is considered as a part of an active defence response (Nicholson and Hammerschmidt 1992). Phytoalexin accumulation and its role in endogenous fruit resistance has been examined in banana (Brown and Swinburne 1980; Abayasekara and Adikaram 1995), capsicum (Adikaram et al. 1982), citrus (Ben-Yehoshua et al. 1992).

Study of Defence Systems

To understand the diversity of defence mechanisms operating in plants against pathogens requires a multidisciplinary approach. Such approaches may involve exhaustive investigations using elaborate biological, chemical, biochemical, and molecular biological procedures.

Broadly the studies can be directed in two lines, i.e. studies involving (a) tissue or cell fractionation, and (b) tissue or cell localisation. The former involves: collection of plant material (for phytoalexins and antifungal hydrolases, etc. the collection or production of diseased tissues); preparation of tissue for extraction; extraction; assay for antifungal activity; bioassay monitored fractionation of the extract leading to isolation of active fraction; chemical analysis and structure elucidation. The cell and tissue localisation studies require electron microscopy coupled with histochemical, immunofluorescent, immunolabelling and radioactive labelling, etc.

Study of Antifungal Systems

Preparation of tissue

Tissues for extraction of constitutional antifungal systems are collected from areas free of any damage

or infections. The selection of the plant part and the type of tissue must be based on type of plant–pathogen interaction under investigation. When the stem, root, or fruit is tested for activity, the tissue from the outer layer through which the pathogens normally enter and develop infections would be best. In diseases that cause vascular damage the vascular tissue may be cut and examined.

For phytoalexin assay, infected tissues are required. These could be obtained from either naturally infected or artificially inoculated plant material. Again, the selection of the appropriate stage of infection could be vital in the final interpretation of results, as the concentration, composition, etc. of the antifungal substances may vary with the disease progression. Certain compounds may be metabolised into non- or less toxic substances by the host tissue, pathogen, or both. This is particularly important when dealing with fruits as they undergo ripening when most defence mechanisms become less effective. For example, the preformed antifungal compound, diene, present in the unripe fruits is oxidised into a less-active derivative by lipoxygenase enzyme during ripening (Prusky et al. 1991).

The method of inoculation employed to produce infected tissue should, as far as possible, mimic the natural mode of infection of the pathogen. Application of drops of suspensions of spores onto the undamaged plant surface approximates this requirement. With wound pathogens, a tiny wound could be made with a fine, sterile needle. Severe wounding of plant tissue before inoculation would create artificial situations, subjecting the plant tissues to stress and leakage of nutrients. The composition of phytoalexins induced by inoculation following severe tissue damage and using unconventional inoculation methods can be quite different from that accumulating in naturally infected tissue. The phytoalexin, capsidiol, isolated from diffusates collected from the pepper (*Capsicum annuum*) fruit cavity, following injection of a spore suspension of a non-pathogen into the fruit cavity, though it had substantial antifungal activity (Stoessl et al. 1972) was of minor importance in the fruit resistance. The more important capsicannol phytoalexins (Adikaram et al. 1982) were produced by the unripe fruit when the fruit surface was inoculated with spore drops of *Glomerella cingulata*. Capsidiol could be detected only in the ripe fruit. The method used by Stoessl et al. (1972) resulted in substantial damage to the inner mesocarp tissue which had no protective barrier equivalent to that in the outer fruit surface. Several stress metabolites, some apparently having phytoalexin properties, were isolated from bananas by Hirai et al. (1994), who first bruised the entire fruit surface with sandpaper, then inoculated the fruit by dipping them in a suspension of conidia of *Colletotrichum musae*. This procedure was repeated in our laboratory. It revealed that the inoculation procedure causes extensive damage to the fruit, the skin rapidly becoming dark brown. A series of stress metabolites is produced, some of them behaving like phytoalexins. Most of these compounds could not be seen in naturally infected fruit or in fruit that were inoculated with spore drops without wounding (Abayasekara et al. 1997).

Extraction of tissue for antifungal substances

The tissues are normally cut into small pieces, weighed, and stored in polythene bags or containers. In order to avoid enzymic browning, the cut tissues may be immediately stored in a deep-freezer (at –20°C) preferably under liquid N_2. Tissues are often freeze-dried before extraction which, while preventing the formation of emulsions during extraction in organic solvents, facilitates extraction process.

Extraction of tissue may be done either by vacuum infiltration or by macerating tissue in a solvent. Vacuum infiltration is carried out by allowing the solvent to infiltrate tissues immersed in the solvent under vacuum. Tissues are first immersed in a suitable solvent, e.g. methanol or ethanol, in a conical flask with a side arm, and the flask is then connected to vacuum. The infiltration is carried out under continuous stirring for several hours. Several flasks will often be connected in series to the same vacuum source. The main advantage in vacuum infiltration is a less complex final extract that is often devoid of unwanted pigments, cellular debris, etc. Methanol or ethanol can be used as a convenient solvent for routine extraction of fresh or freeze-dried plant material. Sequential extraction with solvents with increasing polarity, e.g. *n*-hexane, dichloromethane, ethyl acetate, and methanol may be used. This method, though time consuming, furnishes preliminary fractionation in solvents of varying polarity. The ethanol extract obtained from papaya fruit treated with copper salts has, for example, been re-extracted with *n*-hexane, chloroform, and ethyl acetate, and the phytoalexin danielone has been detected only in the ethyl acetate extract (Echeverri et al. 1997).

Isolation

A plant extract is a complex mixture of a large number of compounds. Isolation of active compounds from a plant extract requires a systematic approach, often involving chromatographic techniques. A number of chromatographic techniques are available, including: thin-layer chromatography (TLC), column chromatography (CC), flash chromatography (FC), high performance liquid chromatography (HPLC), droplet counter-current chromatography (DCCC), and rotation locular counter-current chromatography (RLCC).

In CC, the extract, along with a solvent, is placed in a column packed with a polar adsorbent such as silica gel or alumina. The compounds eluted from the column are collected as fractions. The distribution of compounds in a fraction can be assayed by analytical TLC, and fractions having similar TLC patterns are combined to obtain fractions for subsequent bioassay. Conventional CC is time-consuming and sometimes gives poor resolution. FC, in which air or nitrogen is applied to the top of the column, requires much shorter times (10–15 minutes) for the entire operation and affords moderate resolution. The particle size of the adsorbent used for FC is smaller than that used for CC and is about the same as that used for TLC (Still 1978).

The active column fraction often contains more than one compound and requires further fractionation. This is achieved by preparative TLC or repetitive CC or FC using varying solvent systems. A chromatographic technique that provides very high resolution is HPLC which may be used to separate compounds in a sample that cannot be resolved by other methods. In HPLC, the solvent is pumped through the column at high pressure (6000 psi). Unlike CC and FC, HPLC thus requires special instrumentation.

By chemically attaching alkylsilane groups (e.g. C_8 and C_{18}) to silica, the stationary phase can be made very much less polar than the mobile phase. The application of such reverse-phase adsorbents in TLC, CC, FC, and HPLC has enhanced the potential of these techniques in the separation of complex mixtures (Cooke and Olsen 1980). For example, the bioassay-directed fractionation of an extract of the bacterium *Pseudomonas cepacia*, employing reverse-phase (C_{18}) CC and HPLC, has furnished 2-(2-heptenyl)-3-methyl-4-quinolinone as the antifungal principle. *P. cepacia* is antagonistic to *P. capcisi*, the plant pathogen responsible for the phytophthora blight of red pepper (Moon 1996). Reverse-phase HPLC is one of the most widely used forms of chromatography because of its flexibility and high resolution.

Gel-filtration and ion exchange chromatography are special forms of liquid–solid chromatography. In gel-filtration the adsorbent is a molecular sieve gel (e.g. agarose, dextran, and polyacrylamide) which selectively traps the smaller molecules and allows larger molecules to pass down the column in the liquid phase. Ion-exchange separations are performed in columns packed with an ion-exchanger. Ion-exchangers consist of organic macromolecules (e.g. agarose, cellulose, and polystyrene) containing ionisable groups (e.g. carboxy, sulfomethyl, and trimethylaminomethyl) capable of exchanging with the ions of a solution of an electrolyte. When choosing an ion-exchanger, the stability of the sample components, their relative molecular mass, and the specific requirements of the separation should be considered. Gel-filtration and ion-exchange chromatography can be used to separate and purify proteins associated with antifungal systems.

DCCC is a form of liquid–liquid partition chromatography where the stationary phase is retained by gravitational force in a narrow (about 2 mm) vertical tube through which droplets of immiscible phase are passed. Here, unlike in HPLC, crude extracts can also be fractionated without having to go through a clean-up or preliminary separation beforehand. As no solid adsorbent or porous support is used in DCCC, the total sample can be recovered. This is particularly useful when the active compound is present in only trace quantities in the extract and is intractable with conventional chromatography. DCCC may, however, take 1–3 days for separation of mixtures containing several compounds. In RLCC the separation time is cut down to a few hours by employing centrifugally operated units to effect a much faster flow rate. With appropriate choice of solvents, a range of structurally different compounds can be separated with good resolution using counter-current techniques (Conway 1990).

Complex biological molecules can be separated using affinity chromatography which relies upon specific biological interactions of the molecules with a specific ligand that is attached to an insoluble matrix. When the molecule to be isolated is an enzyme, the immobilised ligand may be the substrate, a reversible inhibitor, or an allosteric activator. Only the enzyme binds to the immobilised ligand and all other compounds can be washed away. The enzyme is recovered by displacement from the ligand.

Electrophoresis offers separation of molecules such as amino acids, peptides, proteins, and nucleotides. Isolation of active compounds from an extract often requires the use of a combination of chromatographic methods. The chloroform extract of freeze-dried avocado peel has, for example, been sequentially subjected to CC, FC, gel filtration, and reverse-phase HPLC to obtain the following antifungal compounds: 1,2,4-trihydroxyheptadec-16-yne, 1,2,4-trihydroxyheptadec-16-ene, and 1-acetoxy-2-4-dihydroxyheptadec-16-yne (Adikaram et al. 1992).

Structure elucidation

Spectroscopic techniques are widely used for structure elucidation of antifungal compounds. These techniques include ultraviolet spectroscopy (UV), infrared spectroscopy (IR), proton (^{1}H NMR) and carbon (^{13}C NMR) nuclear magnetic resonance spectroscopy, and mass spectroscopy (MS). The UV absorption data provide information about the presence of conjugated systems such as polyenes, α, β-unsaturated ketones and benzenoid compounds and a few larger atoms such as sulfur, bromine, and iodine having lone pairs of electrons. The IR absorption bands are correlated with the vibration of various functional groups such as hydroxy, carbonyl, and olefinic. The NMR peaks have been correlated with the molecular environment of the atom (proton or carbon) concerned and thus structural assignments can be made. A number of modern NMR techniques such as COSY, HOHAHA, HMBC, HMQC, HOESY, HETCOR, INEPT, DEPT, and INADEQUATE enable the unambiguous structural assignment of complex molecules (Friebolin 1991).

The molecular formula of a compound is extremely helpful in the elucidation of its structure, and can be deduced from its molecular mass measured accurately using high resolution mass spectrometry (HRMS). Recognisable groupings within a molecule can also be deduced from the mass spectrum. Various mass spectrometric techniques are available, including electron impact ionisation (EIMS), chemical ionisation (CIMS), field desorption (FDMS), and fast atom bombardment (FABMS). The choice of a technique depends on the nature of the compound. Relatively volatile molecules are, for example, analysed by EIMS or CIMS, whereas nonvolatile compounds may require FDMS or FABMS (Baldwin 1995).

X-ray diffraction analysis provides the structure and stereochemistry of a molecule unequivocally. X-ray studies can sometimes be used to reveal structural features which may escape detection by spectroscopic methods. Although advanced NMR techniques such as COSY, DEPT, HMQC and HMBC have been applied in the structure elucidation of danielone, a phytoalexin from papaya fruit, the relative positions of the two methoxy groups and the hydroxy group on the aromatic ring have been established by X-ray diffraction analysis of its 2,4'-diacetate; danielone has the structure, 3'5'-dimethoxy-4'-hydroxy-(2-hydroxy) acetophenone (Echeverri 1997).

Bioassay for antifungal compounds

Testing of antifungals is often done in vitro, the outstanding feature of in vitro tests being their efficiency and economy. Large numbers of samples can be handled on a routine basis in one experiment, and results are obtained within a relatively short period of time. Only minute quantities of test compound/extract are needed.

Different methods are available for assaying antifungal activity, including: thin layer chromatography bioassay; spore/slide germination assay; and agar cup plate methods. The proper conduct of bioassay demands careful selection of methods, as well as an almost constant re-examination of the methods in use. The selection and re-examination of methods should be based on four criteria: suitability, reliability, efficiency, and economy. The activity of a sample must be confirmed by examination using more than one method.

Thin-layer chromatography bioassay (TLC bioassay)

This is a very useful assay method, especially when a large number of samples is available for testing and when chemical analyses are done concurrently. The method used by Klarman and Stanford (1968) is now used widely for evaluating extracts/substances for antifungal activity. Several modifications to the original method have been developed to extend its utility. The assay technique also provides information over and above antifungal properties, e.g. the number of active constituents in the sample, their Rf value, and the relative toxicity/concentration.

Crude or partially purified extracts, or pure samples dissolved in a suitable organic solvent, are carefully spotted on a thin-layer chromatography plate coated with silica gel and the plate is developed in a suitable solvent system. The plate is air-dried over-

night to remove remaining solvents and carefully sprayed with a suspension of conidia of the test fungus using a fine air jet sprayer. The plate is then incubated in an airtight chamber lined with moist filter paper for 2–3 days at ambient temperature (25°C). The regions in which the growth is inhibited lack aerial mycelial growth and appear light coloured against dark-coloured mycelium. These are taken as inhibition areas. Spraying should be sufficient to fully wet the plate, while ensuring that the liquid does not drain away. Care must be taken not to disturb the silica gel. The suspension of conidia is prepared in a nutrient solution containing a carbon source (sugar) and basic minerals, e.g. Czapek Dox nutrient solution. Spores scraped from cultures are suspended to make a dense suspension which is filtered through glass wool. It is possible to use any fungus capable of growing on silica gel as a test fungus, but best results could be obtained with fungi that are fast growing and produce ample conidia in culture. To obtain best results the fungus should give a flat, uniform, and brightly coloured mycelium on silica gel. A *Cladosporium* species that has a characteristic flat, dirty-green-coloured mycelia is often chosen for this purpose. With this fungus the inhibition areas contrast with those on which mycelium grows. Temperatures between 15–25°C are the best if *Cladosporium* is used, as the fungus does not perform well at temperatures above 25°C. In instances where fungi that produce colourless mycelia are used for spraying, the inhibition areas may not be readily visible due to lack of contrast. In such instances, the suspected inhibition areas may be examined more closely using a low power microscope or a hand lens. Alternatively, the plates can be exposed to iodine vapour (saturated) in a container. This procedure stains the mycelium brown. We have found that this method is not very satisfactory, because the iodine tends to be absorbed by the wet silica gel. Thus, the whole plate is stained light brown, reducing the contrast between the mycelium and inhibition zones.

A slight modification of this method allows the extract/material to be bioassayed directly on silica gel plates without developing the plate in a solvent system. Known aliquots are spotted on a defined area of the plate and after allowing the small amount of solvent to evaporate, the plates can be immediately sprayed with the spore suspension. The test provides information on the total antifungal activity of the extract/material. This procedure can save time, as the plate does not have to be left overnight for solvent evaporation. The presence of inducible antifungal substances (phytoalexins) can be demonstrated by carrying out bioassay for extracts obtained separately from both healthy and infected tissue. If phytoalexins are present, the inhibition areas will be seen in extracts obtained from the infected but not the healthy tissues. The occurrence of inhibition zones in both indicates the presence of preformed antifungal substances. After the bioassay the active substance can be eluted from the silica gel scraped from the inhibition region. This may furnish a partially purified material for further analysis.

The technique has proven useful in the solvent fractionation of extracts for isolation of antifungal compounds, as the activity can be monitored throughout the course of fractionation. The Rf value of the inhibition zone is a useful guide for subsequent separation of the active principle. An advantage of this method over others, e.g. slide germination assay (see below), is that the water insolubility of the compound is not a limiting factor.

The TLC bioassay has also been used with some success to make rough estimates of the amounts the antifungal compounds in samples (Sivanathan and Adikaram 1989). The assumption here is that the inhibition area produced is related to the amount of the antifungal compound present, provided the other conditions, e.g. amount spotted, area of the spot, developing solvent, and the thickness, material, etc. used for preparation of the TLC plate, are the same. Substances with low solubility in water can be better quantified by this method than the water-soluble ones which tend to diffuse on the plate when the spores are sprayed in an aqueous medium.

As with any other method, this approach has some disadvantages. A TLC bioassay trial may take up to 2–3 days and is therefore time consuming. This might allow the potential antifungal compounds to decompose, particularly if they are unstable at the conditions used for the assay. The chance of photodecomposition may be prevented by keeping the plates in the dark. When oily substances are present in the test sample, the spore suspension sprayed on the plate may be repelled by them, giving ambiguous results if there is no fungal growth at these locations.

A TLC method has also been developed to assay antibacterial activity (Hamburger and Cordell 1987). A suspension of a bacterium (e.g. *Bacillus subtilis*, *Escherichia coli*) in a suitable nutrient broth (BBL, Lockeysville, Maryland) is dispersed evenly over a developed chromatogram using a roller device covered with chromatography paper. The plates are incubated

overnight in humid chambers and then sprayed with an aqueous solution of TTC (20 mg/mL), INT (5 mg/mL), or MTT (2.5 mg/mL). The plates are again incubated at 37°C for 4 hours. The TLC plates are treated with EtOH (70%) before examining them for inhibition areas.

Spore germination tests

A slide germination procedure developed for evaluation of protective fungicides is extensively used today (American Phytopathological Society 1943). The original procedure was to test spore germination on fungicide-sprayed slides. The principle of this technique has been incorporated into many other slide germination assays. Basically, these procedures make use of glass slides on which drops of fungal spores are allowed to germinate and grow for a time in the presence of the test sample. A suspension of spores of the test fungus is prepared by scraping the mycelium from a young colony and suspending in sterile distilled water. The suspension is filtered through glass wool, and the filtrate, which contains spores, is collected and washed by repeated centrifugation (1000 for 3–5 min.). The concentration of the final suspension is adjusted to the required level using a haemocytometer. Dense spore suspension may lead to self-inhibition (Allen 1965).

Aqueous solutions of the test compound are prepared by dissolving known amounts of the test compound. These are mixed with the spore suspension. If equal amounts are mixed, the original concentration of the test solution and the density of the spore suspension should be double the final concentration/density. Controls are prepared without the test compound. Drops (20 mL) are applied to clean glass slides (two drops per slide) and the slides are incubated in moist chambers for a period sufficient for germination of spores. To compare different test samples, replicate slides may be used. The incubation period usually ranges from 4–12 hours depending on the fungus. At the end of the incubation period the slides are removed and germination is stopped by adding a drop of lactophenol. A coverslip is carefully placed over the drop and the slide can now be left for some time before examination under the microscope. Counts are made on at least 100 randomly selected spores for germination, and average percentage germination is determined. To obtain a good cross-section of germination, the counts could be made by moving the object lens from one corner to another across the slide. The results, expressed as counts compared to untreated controls, are a measure of activity, and can be expressed graphically as dose–response curves. The germ-tube elongation of germinated spores has also been taken as a measure of toxicity of antifungal compounds. Here the length of the germ-tube is measured using a micrometer eye piece and percentage inhibition of germ-tube elongation is determined against controls.

A limitation of this method is its applicability mainly to totally or partially water-soluble substances. This may be overcome in some cases by using 5–20% methanol or ethanol as germinating medium instead of water as these would not be too harmful to most fungi. The assay could also be done on a film of the extract/substance layered on the slide surface. Here a drop of the test solution prepared in a volatile solvent is added to a defined area on the slide and, after allowing the solvent to evaporate, the spore drop is placed over the film.

Any structural damage to the spores caused by the test sample can be observed by the slide germination technique as the spores are examined under the microscope following treatment. Further, the method can be used to establish whether the effect of the test sample on spores is permanent (fungicidal) or temporary (fungistatic). For this purpose, the spores that are exposed to the test sample for set times are separated by repeated centrifugation (1000*g* for 3 min.), after which the supernatant is replaced with fresh distilled water. Following this the spores are suspended in water and allowed to germinate once again on slides. If they still germinate the effect of the test chemical is temporary (fungistatic).

Prusky et al. (1991) have used Millipore filter (0.45 mm pore size, Millipore), instead of glass, as the surface for testing germination of spores. The test sample/extract dissolved in a solvent was spotted on a Millipore filter (13 mm diameter) and small amounts of the same solvent alone were placed on a control filter. After removal of solvent by drying, a drop of a suspension of spores was placed on the sample and the control filters. The filters were incubated in a moist chamber and at various periods after the start of incubation the germination was tested after adding cotton blue in lactophenol.

In another modification by Kim et al. (1991), crude extracts dissolved in 10% ethanol were placed in wells (11 mm diameter) of 35 mm petri dishes, each well being previously supplied with a spore suspension of the test fungus. The spore suspension contained 1% fresh orange juice and 1% sucrose (pH 3.7). The dishes were placed in a humid chamber at 17°C for 24 hours. The percentage germination and

germ-tube elongation were determined by examination under the microscope after mounting on glass slides.

Agar plate tests

Agar plate tests were borne out of the need to detect activity against fungi whose spore production, germination, or size characteristics do not lend themselves to spore germination assay. The test measures the effect of a test chemical on fungal spores and/or mycelial growth on an agar medium. The basic procedure involves seeding spores or mycelial fragments in an agar medium containing the test substance. Alternatively, the test solution may be added directly to wells cut in agar or first absorbed into filter paper discs (the latter is mostly practiced for antibacterial assay) which are subsequently placed on agar medium. The substances are free to migrate though the adjacent agar medium, resulting in a gradually falling concentration in the surrounding agar. No growth will appear in the areas where the substance is present at inhibitory concentrations. The seeded plates incorporated with test substances are incubated to allow a measurable mycelial growth. Lack of growth compared with untreated controls is the measure of activity. The size of the inhibition zone is affected by the sensitivity of the test organism and the rate of diffusion through the agar (Berry and Thornsberry 1980).

PR proteins and antifungal hydrolases

In certain plants, e.g. tobacco, acidic and basic proteins appear to be strictly compartmentalised. The former accumulate predominantly in the extracellular space of the leaf which has been analysed in most detail by cell fractionation techniques for PR–1 proteins and β-1,3-glucanases (Van den Bulke et al. 1989). Location has been verified by immunofluorescence microscopy and immunogold labelling techniques (Hosokawa and Ohashi 1988). Tissue extraction and electrophoresis procedures have been developed for PR protein studies (Tuzun et al. 1989).

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Chemistry Around the Avocado Antifungal Diene

R.M. Carman, A.R. Duffield, P.N. Handley, and T. Karoli*

Abstract

The leaves (both new and old) of 17 avocado varieties have been analysed monthly throughout the year to measure the concentration and seasonal variation of the antifungal diene compound. Aspects of the chemistry of this compound, which is also active in anti-cancer screens, have been explored and analogues have been synthesised for structure/activity studies.

THE leaves of the avocado are known to be toxic to a range of animals (Kingsbury 1964). In the recent work of Seawright and colleagues (Oelrichs et al. 1995), the diene antifungal compound (1a) (see Figure 1 for the structure of this and other compounds mentioned) (named persin by Seawright) has been shown to be responsible for anti-lactation and toxic effects in goats and mice. The compound (1a) is also highly active in various anti-cancer screens (Seawright et al. 1995), and is a growth inhibitor against silk-worms (Chang et al. 1975). We have previously obtained supplies of the compound (1a) and its tetrahydro derivative (1b) (Carman and Duffield 1995), which is equally active in the anti-cancer and anti-lactation screens (Oelrichs et al. 1995), from Reed variety avocados without knowing whether this is the optimum source, and we now report a survey of the concentration of the compound (1a) from a range of avocado varieties.

We previously commented (Bull and Carman 1994) upon the considerable similarity between structures (1a) and (2a); the latter is a simple monoglyceride of linoleic acid. We speculated that structure (1) might be biologically-active because it can mimic compounds (2), perhaps irreversibly, in regular glyceride synthesis. Compound (1), lacking the ester oxygen of compound (2), will not be hydrolysed at a critical stage in the normal glyceride synthesis. Compound (1) might then be expected to interfere with fundamental lipid development within any cell; and the wide range of biological results available for the compound (1) is not inconsistent with this premise. We now report a range of synthetic analogues of compound (1).

Compound (1) is active in the mouse anti-lactation screen when administered orally, but not by other means. Is exposure in the stomach converting compounds (1) into active compounds? Compound (1) has been chemically modified in a search for more active derivatives.

Results and Discussion

Leaves of avocado varieties listed in Table 1 have been analysed monthly for the concentration of compound (1a). Both new, when they can be distinguished, and old leaves have been examined and the concentration of compound (1a) is found to alter very little with age of leaf. The monthly values are, within experimental error, the same throughout the year, and only values averaged over the whole year are listed in Table 1. Generally, Guatemalan-sourced varieties contain more compound (1a) than do other sources,

* Department of Chemistry, The University of Queensland, Brisbane, Queensland 4072, Australia.

although the number of the other varieties surveyed is not great. Reed is not necessarily the richest source, although this variety has been used by other workers (Oelrichs et al. 1955; Carman and Duffield 1995) as a source of the compound (1). The values in Table 1 can be compared with the literature; 2 mg/g isolated yield from fresh Reed leaf (Carman and Duffield 1995), 910 mg/g isolated yield from freeze-dried Reed leaf (Oelrichs et al. 1955), 0.6 mg/g isolated yield from fresh leaves of an unspecified variety (Chang et al. 1975), 1.4 mg/g (Fuerte), and 0.6 mg/g (Hass) from freshly harvested fruit peel (Prusky et al. 1988).

Table 1. Concentration of lipid diene (1a) (mg/g of fresh leaf) in various varieties of avocado.

Variety	Type	Average
Hass	Guatemalan	3.8
Wurtz	Guatemalan	3.2
Sharwil	Guatemalan (Australian-bred)	3.1
Fuerte	Mexican/Guatemalan	2.8
Pinkerton	Mexican/Guatemalan	2.7
Reed (2)	Guatemalan	2.6
Reed (1)	Guatemalan	2.3
Plowman	Guatemalan	2.1
Gwen (2)	Guatemalan	2.0
Rincon	Mexican	1.7
Gwen (1)	Guatemalan	1.6
Shepard	West Indian/Mexican	1.4
Hazzard	Guatemalan	1.2
Zutano	West Indian/Mexican	0.9
Duke-7	Mexican	0.9
Velvick	West Indian	0.6
Edranol	Mexican/Guatemalan	0.4
G775b	Guatemalan cross	0.0
G775c	Guatemalan cross	0.0
Hass (Sus)	Anthracnose susceptible	3.8
Hass (Res)	Anthracnose resistant	3.8

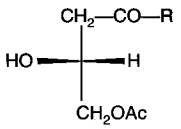
We have also analysed the concentration of diene (1a) in the leaves of two samples of Hass; one from a tree reputed to consistently give anthracnose-infected fruit (Hass-Sus), the other from a tree reputed to provide anthracnose-resistant fruit (Hass-Res). Only four samples of each tree have been examined thus far (April/May leaves), but the results (Table 1; last two entries) show little variation between the two samples. This work is being continued.

The compounds (1a) and (1b) have already been chemically synthesised by us (Bull and Carman 1994) and others (MacLeod and Schffeler 1995). We have now explored and achieved further routes to compound (1b) to make it, and analogues, available for biological screening.

Compounds (1) have an acetate ester group attached to the lipid molecule. In order to screen other esters, we have attempted to remove the acetate group to provide compound (3b), but with only limited success. Normal alkaline hydrolysis affords the cyclopentenone (4) by a mechanism which we can rationalise. Also formed are the compounds (5) and (6) through a reversed aldol condensation. Compound (3b) can be formed, together with another unknown product, from compound (1b) by cyanide hydrolysis. Compound (3b), unexpectedly, screens negatively in the mouse lactation screen; we had expected that acetate groups would be readily added or removed by normal esterases available within the cell. We note that the acetate group, however, which might appear to be an extraneous complication in these compounds, actually provides useful stability. Without the acetate group, compound (3b) converts rapidly, through an acid-catalysed reaction, into the furan (7).

Only the (**R**)-enantiomers of the compounds (1) are biologically active in the mouse lactation screen (Oelrichs et al. 1955; J. Ng and P. Oelrichs, pers. comm.). There are reports (Oelrichs et al. 1955; J. Ng and P. Oelrichs, pers. comm.) that compounds (1) are active only when they are administered orally. Is the active drug therefore an acid-catalysed derivative of (1), with the acid-catalysis occurring in the stomach? To date, acid treatment of compound (1b) has only provided the furan (7). This is not active in mouse screens, and is not predicted to be active, as it is a non-chiral molecule. We have thus far been unable to isolate any compound on the acid-catalysed pathway (1b) to (7) which still retains chirality.

The compounds (1) look superficially like monoglycerides of type (2), but lack the hydrolysable ester group, and we have synthesised the compounds (8) to (10) which also have a non-hydrolysable monoglyceride-like appearance in the hope that they might have biological activity similar to the compounds (1). However, all these compounds screen negatively in the mouse lactation test. The experimental details of these chemical syntheses and manipulations, all of which involved standard synthetic chemistry, will be reported elsewhere.



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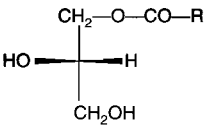

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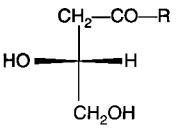

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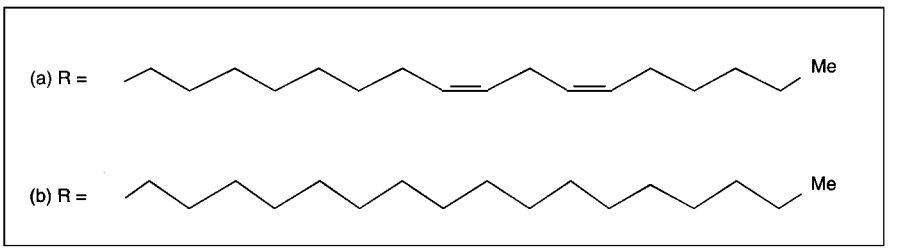

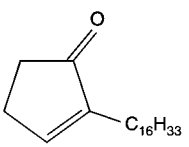

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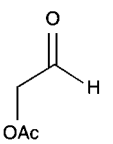

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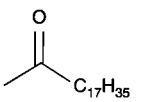

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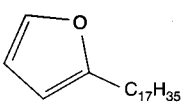

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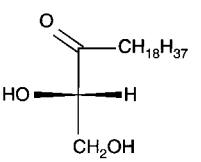

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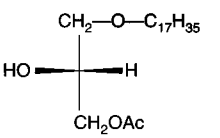

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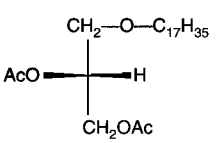

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Figure 1. Antifungal diene from avocado, and analogues synthesised for structure/function analysis.

Experimental

Leaf analyses

Avocado leaves were collected monthly from labelled mature trees growing at the Birdwood Nursery, Nambour, Queensland. Reed (1) and Reed (2), and Gwen (1) and Gwen(2), were duplicate trees from the same area. Hass (Sus) and Hass (Res) are trees considered to have consistently provided fruit susceptible to, and resistant to, anthracnose, respectively. Both old and young (when available) leaves were stored in airtight bags at 20°C. The leaf (0.9–1.1 g) was weighed and chopped finely with scissors. The leaf material was ground in a mortar with ethyl acetate (10 mL). The green liquid was decanted and filtered. Two further extractions were usually necessary to ensure that all the green colour was extracted, leaving brown fibrous residue. The combined organic filtrate was treated with an aliquot of internal standard [octadecanol (1.00 mg) in ethyl acetate]. The mixture was then taken to dryness (<40° under vacuum). Trimethylsilylimidazole (0.3 mL) was added with swirling to ensure complete dissolution, and the stoppered flask was allowed to stand (>15 min). Excess reagent was quenched with methanol (6 drops; violent reaction) followed by distilled water (5 mL) and sodium chloride (3–4 mg). The resultant mixture was shaken with hexane (distilled, 1–2 mL) and the organic layer was pipetted off and filtered through a plug of cotton wool into a GC vial. Samples were stored (–20°C) until analysis using a Varian 3300 gas chromatograph (GC) [30 m non-polar (5%-phenyl)-methylpolysiloxane column (BP-5 or DB-5); helium carrier gas; 100°C for 2 min rising to 250°C at 16°C/min, then 250°C for 17 min; flame ionisation detection]. Peak areas were measured relative to the derivatised octadecanol peak. Detector response factors were determined frequently by separate injection of a derivatised solution containing octadecanol (1.00 mg) and compound (1b) (1.00 mg). Frequent checks of the derivatised leaf sample by GC/MS analysis (Hewlett Packard MSD 5970 spectrometer using a GC inlet with a column identical or similar to those reported above) confirmed the identity of the peaks for both compound (1a) and the octadecanol standard. For most leaf samples the peak for compound (1a) was the major GC peak.

Results for both new and old leaves are collected into the Table 1 as an average value. New leaves, when available, showed only marginally higher values than did old leaves. Results over a 10-month period (analyses will continue for at least a year) show no apparent seasonal variation.

Acknowledgments

We thank Peter Young, Birdwood Nursery, Nambour, Queensland for the identification of varieties and for regular access to his nursery. The Hass resistant and susceptible samples were collected by Dr Lindy Coates, Department of Primary Industries, Queensland. Preliminary leaf analyses were performed by Peter Wynne. Mouse lactation screens were performed by Jack Ng.

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POSTER PAPERS

Control of Stem-end Rot of Papaya by Heat Treatments

Y. Yaguchi and S. Nakamura*

Abstract

Papaya fruit from Hawaii reached the market in Japan approximately 4 days after harvest, free of any symptoms of decay. However, a high incidence of decay developed as ripening progressed. The rate of development of stem-end rot due to *Lasiodiplodia theobromae* depended on the degree of ripeness, as the decay in ripe fruit advanced faster than in green fruit. It was found that the fungus had penetrated all fruit to a depth of 25 mm 96 hours after inoculation. Combined vapour heat and hot water treatment had an inhibitory effect on the disease. When fruit were immersed in hot water at 50°C for 20 minutes, 12 hours after inoculation at the stem end, or at a temperature above 55°C for 20 minutes, 24 and 48 hours after inoculation, no stem-end rot was observed. For all treatments, the pathogen was eradicated from the stem end to a depth of 5 mm, but not at a depth of more than 10 mm. Fruit immersed in hot water at 48.9°C for not more than 20 minutes developed fewer cases of heat damage than fruit immersed at 51.7°C for 10 minutes. In addition, the ripening of all fruit was inhibited when they were immersed at 51.7°C for 20 minutes. Although hot water treatment can damage fruit and affect ripening, it can reduce disease.

PAPAYAS (cv. Solo) imported into Japan come mostly from Hawaii. The import of these fruit into Japan was first permitted in March 1963 on condition that they were vapour heat treated. In addition, hot water treatment to control the anthracnose fungus *Colletotrichum gloeosporioides*, requires the dipping of fruit in hot water before vapour heat treatment. Akamine (1953) reported that the double dipping method for killing eggs of fruit flies on papaya to be exported from Hawaii to the U.S. mainland inhibited the occurrence of anthracnose. This has been put to practical use since 1964 to prevent postharvest diseases in papaya to be exported to the U.S. mainland. However, although the hot-water spray method was effective for anthracnose, it did not inhibit decay due to *Lasiodiplodia theobromae.*

* Laboratory of Electron Microscope, Faculty of Agriculture, Tokyo University of Agriculture, Setagaya-ku, Tokyo 156, Japan.

This paper reports investigations on the types of damage found in imported papaya and on the control by heat treatment of stem-end rot of papaya caused by *L. theobromae.*

Diseases and Disorders of Imported Papaya

The physiological disorders and diseases of, and mechanical injuries to, fruit were investigated when they arrived in Japan, over a period of 10 months starting in September 1986.

The overall average damage was 3.6%, made up of 0.9% physiological disorders, 1.8% mechanical injuries, and 0.9% diseases. Damage was highest in December (6.4%) and lowest in April and May (1.2%), while fruit tended to become rotten in October, because of higher rainfall (Table 1).

Table 1. Occurrence of losses of imported papaya in Japan from September 1986 to May 1987.

Month	Number of inspected fruit	Number of damaged fruit (%)		
		Physiological disorders	Mechanical injury	Diseases
Sep.	14121	115 (0.8)	231 (1.6)	113 (0.8)
Oct.	20707	174 (0.8)	350 (1.7)	451 (2.2)
Nov.	21308	144 (0.7)	728 (3.4)	202 (0.9)
Dec.	24903	480 (1.9)	899 (3.6)	212 (0.9)
Jan.	11098	153 (1.4)	167 (1.5)	113 (1.0)
Feb.	14234	64 (0.4)	102 (0.7)	73 (0.5)
Mar.	18307	50 (0.3)	155 (0.8)	177 (1.0)
Apr.	21864	102 (0.5)	115 (0.5)	56 (0.2)
May	11241	79 (0.7)	21 (0.2)	39 (0.3)
Total	157783	1361	2768	1436
Average		(0.9)	(1.8)	(0.9)

Table 2. Occurrence of rotted papaya fruit imported to Japan from September 1986 to May 1987.

Month	No. of inspected fruit	No. of rotten fruit (%)	
		Stem end	Surface
Sep.	14121	108 (0.8)	5 (0.04)
Oct.	20707	434 (2.1)	17 (0.08)
Nov.	21308	195 (0.9)	7 (0.03)
Dec.	24903	202 (0.8)	10 (0.04)
Jan.	11098	106 (1.0)	7 (0.06)
Feb.	14234	70 (0.5)	3 (0.02)
Mar.	18307	53 (0.3)	124 (0.7)
Apr.	21864	6 (0.03)	50 (0.1)
May	11241	31 (0.3)	8 (0.07)
Total	157783	1205	231
Average		(0.76)	(0.15)

Decay of fruits

Table 2 shows the results of the inspection of imported fruits immediately after customs clearance. Sites of diseases were divided into the stem end and other surfaces of the fruit.

The overall average occurrence of stem-end rot (0.76%) was significantly higher than that of disease on other parts of the fruit surface (0.15%). Stem-end rot was most prevalent during September–January, with the highest incidence (2.1%) in October. The highest incidence of diseases on other surfaces (0.7%) occurred in March. However, no tip decay was observed.

Stem-end rot

Stem-end rots encountered could be classified into five types on the basis of appearance.

Type I: A central portion of stem end begins to soften, decays, and turns black within several days. Decay spreads rapidly to a peripheral region where abundant black spherical pycnidia are formed. The pycnidia then successively exude white and blackish-green conidiospores from the tip as they mature; grey-coloured velvety aerial hyphae grow thickly when moist.

Type II: Black, spherical pycnidia are formed when the disease has advanced. These successively exude conidiospores from their tip. Although decay is similar to that of Type I, the site of decay can be distinguished because it becomes pale ivory-white in colour.

Type III: The stem end that initially softens, spreading water-soaked spots to a peripheral region, which becomes dark brown and grows thick, orange-coloured conidial layers.

Type IV: Although white-coloured hyphae grow thickly on the stem end and water-soaked spots are recognisable in a peripheral region, decay progresses more slowly and is dry.

Type V: The stem end initially exhibits a water-soaked condition, and decay gradually spreads in the form of a black colour when subepidermal pycnidia are formed. Decay due to this fungus is similar to that of Type II, but can be distinguished by its black colour as the disease advances.

When the decays investigated were classified by types.

- Surface rot was Type I: 0.03% and Type II: 0.12% throughout the investigation period. Type II did not occur during September–February, but suddenly appeared in 124 fruit in March.
- Stem-end rot Type I showed the highest incidence at 0.39%, followed by Type II: 0.16%, Type III: 0.13%, Type IV: 0.06%, and Type V: 0.02%. The results of this study indicated that October–February was the period of highest incidence of stem-end rot in imported papaya (Table 3).

Pathogens of stem-end rot

Isolates were obtained from the sites of the different types of diseases and grown on a PDA medium. The colours and conidial morphology of the colonies were as follows:

Type I isolates were white with few aerial mycelia, but became blackish-brown with more aerial mycelia 3 days after incubation, forming black pycnidia under fluorescent light 5 days after incubation.

Type II isolates were white to yellow with few mycelia. Pycnidia were formed, however, under fluorescent light.

Type III isolates were greyish-white, forming an orange-coloured conidial pile.

Type IV isolates were white, cotton-wool-like mycelial tufts, forming conidia 5 days after incubation.

Type V isolates were greyish white, but turned blackish-brown 3 days after incubation, forming pycnidia under fluorescent light.

Morphological characteristics of pathogens and their identification

When isolates from the sites of stem-end rot were inoculated into the stem end of papaya, colonies with the following characteristics were obtained.

Type I: Pycnidia were black, ostiolate, globose, 140–260 μm diameter. Conidia were initially 1-celled, hyaline, 20–30 × 14–16.4 μm (av. 25.6 × 14.4 μm). Mature conidia were 1-septate, cinnamon to fawn, ellipsoid-oblong, 20–27.2 × 12–15.9 μm (av. 24.9 × 13.3 μm). On the basis of these morphological features, this particular fungus was identified as *Lasiodiplodia theobromae* (Patouillard) Griffon & Maublanc (Syn. *Botryodiplodia theobromae* Pat.).

Type II: Pycnidia were dark, ostiolate, nearly globose, 150–400 μm. Conidia were hyaline, 1-celled, of two types, α-conidia ovoid to fusiform, 5.4–7.7 × 1.8–2.7 μm (av. 6.2 × 2.2 μm). β-conidia filiform, curved or bent, 12.8–20.5 × 0.7–1.2 μm (av. 17.4 × 0.9 μm). This fungus was considered to belong to a yet to be determined species of the genus *Phomopsis*.

Table 3. Occurrence of five types of stem-end rots on imported papaya fruit from 1986 to 1987

Month	Total no. of fruits	Rotted types of stem end					
		Total	Type I[a]	Type II[b]	Type III	Type IV	Type V
Sep.	14121	108	81	6	11	10	0
Oct.	20707	434	237	84	77	23	13
Nov.	21308	195	97	36	24	33	5
Dec.	24903	202	88	43	46	19	6
Jan.	11098	106	55	36	10	4	1
Feb.	14234	70	22	23	17	4	4
Mar.	18307	53	21	17	10	4	1
Apr.	21864	6	5	1	0	0	0
May	11241	31	14	10	7	0	0
Total	157783	1205 (0.76)	620 (0.39)	256 (0.16)	202 (0.13)	97 (0.06)	30 (0.02)

a Type I, *Lasiodiplodia*
b Type II, *Phomopsis* etc.

Type III: Acervuli were disc-shaped or cushion-shaped, waxy, subepidermal, typically with dark spines or setae at the edge or among the conidiophores. Conidiophores were simple, elongate. Conidia were hyaline, 1-celled, ovoid or oblong, to falcate parasitic, 14.1–19.7 × 3.6–5.4 μm (av. 16.7 × 4.9 μm). No perfect stage was recognised, however, in either case. From these morphological features, this fungus was identified as *Collectotrichum gloeosporioides* (Penzig) Penzig et Saccardo.

Type IV: Microconidia were 1-celled, ovoid or oblong, 7.5–16.4 × 3×5.9 μm (av. 13.8 × 4.3 μm). Macroconidia were 3–5 celled, slightly curved or bent at the pointed end. This fungus was identified as *Fusarium solani* (Martius) Saccardo.

Type V: Pycnidia were dark, globose, septate, immersed in host tissue, ostiolate, 104-208 μm. Conidia were hyaline, 2-celled, ovoid to oblong, 6–10 × 2–4 μm in size (av. 7.7 × 2.7 μm). This fungus was identified as *Ascochyta caricae* Patouillard.

Measures to Control Stem-end Rot of Papaya Caused by *L. theobromae*

Since the use of postharvest agricultural chemicals is strictly restricted in Japan, no agent has so far been approved for application to papaya.

vapour heat treatment is compulsory for killing eggs of fruit flies in papaya imported into Japan. In addition, hot water treatment for anthracnose has been introduced in recent years.

Akamine (1953) reported that hot water treatment is effective for inhibiting anthracnose and this technique was first put into practice in Hawaii in 1964, for papaya bound for the U.S. mainland. However, the efficacy of this treatment for pathogens other than anthracnose has yet to be confirmed. Thus, the occurrence of stem-end rot of papaya after hot water and vapour heat treatment were studied. Also investigated was the possibility of damage to papaya from the high temperatures required by these treatments.

Effect of hot water and vapour heat treatment

The effect of hot water and vapour heat treatments on stem-end rot caused by *L. theobromae* was studied.

Materials and methods

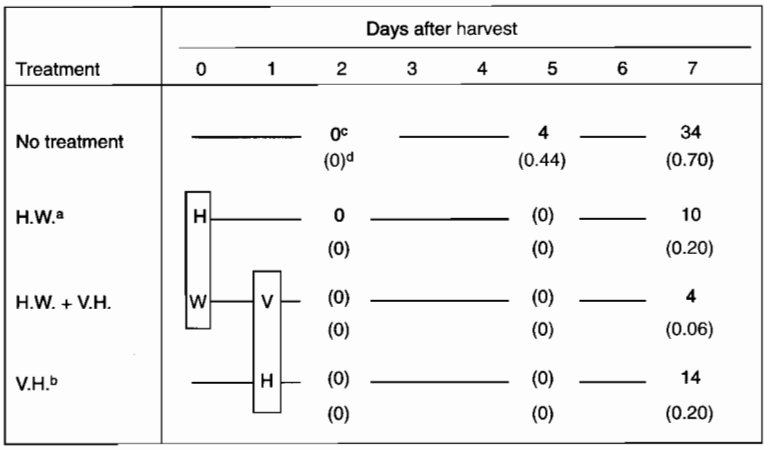
Experiment I: 50 papaya fruit were subjected to (a) hot water alone, (b) vapour heat treatment, or (c) hot water + vapour heat. The stem ends of these fruit were inspected at room temperature (26°±1°C) during storage over several days. Immediately after harvest, the fruit were immersed in hot water at 48.9°C for 20 minutes, and then cooled with cold tap water. Vapour heat treatment (b) continued until the centre of the fruit reached 47.2°C. Vapour heat treatment (b) was performed after hot water treatment (a), followed by the combined hot water and vapour heat treatment (c).

Experiment II: In an attempt to clarify the effect of hot water and vapour heat on *L. theobromae* during the initial stage of infection, a conidial suspension was inoculated on the stem end and fruit were treated with hot water and vapour heat on day 1 and day 2 following inoculation. A conidial suspension (4×10^3 pcs/mL) of *L. theobromae* was inoculated on the stem ends of papayas, which were then allowed to stand at room temperature (26° ± 1°C) for 1–2 days before being treated with hot water, hot water + vapour heat, or vapour heat treatment. The development of stem-end rot was tracked over time, with the mean degree of decay determined in accordance with established indices (0–4) of decay: 0 = no decay; 1 = initial hyphal growth and beginning of flesh softening; 2 = hyphae rapidly growing, with established and expanding decayed fresh; 3 = decayed area expands, surface tissue darkens as a result of initiation and development of pycnidia and acervuli; 4 = one third or more of fruit is decayed sporulation has begun.

Results

Experiment I: Figure 1 shows the results of (a) hot water, (b) vapour heat, and (c) hot water + vapour heat treatments of fruit immediately after harvest. Although 4% of non-treated fruit were beginning to decay 5 days after harvest, no disease was recognised at all in the fruit treated with hot water, vapour heat alone, or combined hot water and vapour heat. However, by day 7, 10–14% of fruit treated with hot water or vapour heat alone had begun to decay, indicating that the progress of decay was faster than in the fruit given the combined treatment with hot water and vapour heat (Fig. 1).

When the type of stem-end disease was investigated, *L. theobromae* was the most abundant fungus detected, followed by *C. gloeosporioides*, and *Fusarium* sp. However, *L. theobromae* and *C. gloeosporioides* were less abundant in the fruit treated with hot water and vapour heat than in the non-treated fruit, while *C. gloeosporioides* in particular was absent in the fruit treated with hot water (Table 4).



[a] Hot-water treatment (48.9°C, 20 min).
[b] Vapor-heat treatment (47.2°C).
[c] Rate of diseased fruits (%).
[d] Average of rot index.

Figure 1. Effect of hot water and vapour heat treatments on the control of stem-end rot of papaya.

Table 4. Recovery of pathogenic fungi from stem-end rot of papaya by treatments

Treatment	*L. theobromae*	*C. gloeosporioides*	*Fusarium* spp.	Unknown
No treatment	20[c]	8	2	4
H.W.[a]	6	0	2	2
H.W. + V.H.	2	0	0	2
V.H.[b]	6	2	2	4

[a] Hot water treatment (48.9°C, 20 min).
[b] Vapor heat treatment (47.2°C).
[c] Incidence of diseased fruit 7 days after harvest (%).

Experiment II: For fruit treated at day 1 after inoculation and stored for an additional 5 days, there was much less decay in fruit given the combined treatment than in those treated with hot water or vapour heat alone (Fig. 2). Further, the decay occurred less frequently in the hot water treatment than in the group treated with vapour heat. Both the incidence of decay and the severity of disease were reduced when fruit were treated 1 day after inoculation rather than 2 days after inoculation. These results indicate that respective treatments were markedly more effective when given at an early stage of infection by *L. theobromae*.

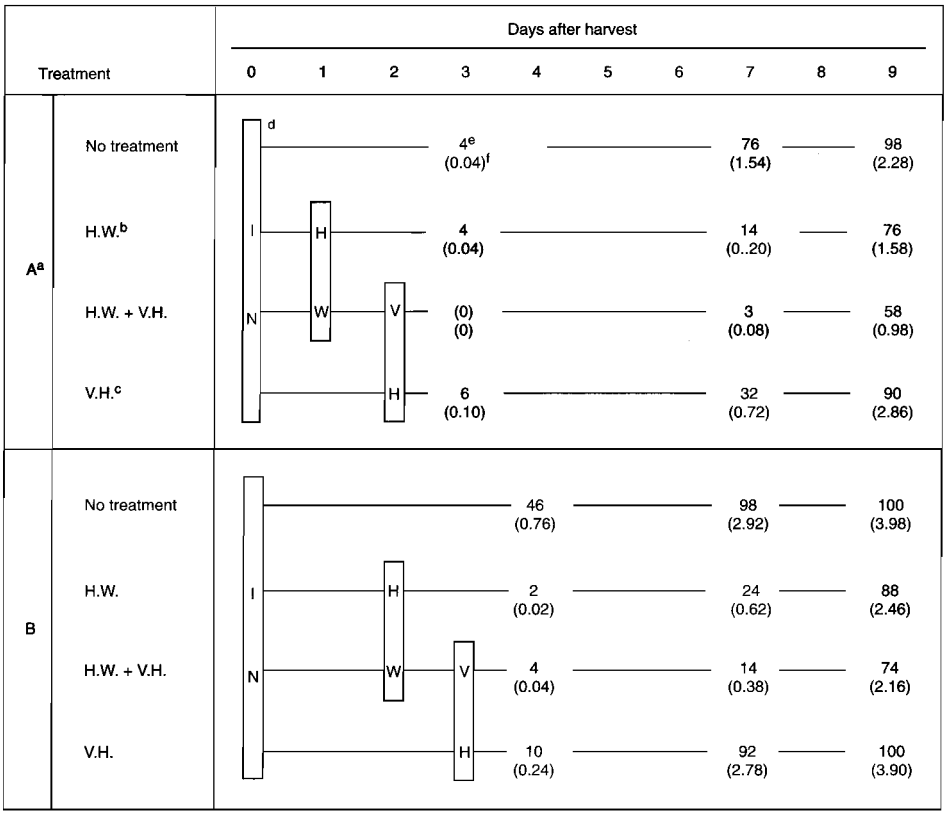
Temperature increases in fruit due to hot water treatment

Fruit at different stages of maturity and the rise in temperature in different parts of fruit were compared to improve the temperature and time required for hot water treatment.

Materials and methods

Five pieces each of green and half-ripe fruit were allotted to each experimental group immediately after arrival in Japan and were allowed to stand for 12 hours at 25°C. A temperature sensor, BS-9, and a temperature data collector (AM-7002, Anritsu K.K. Japan) which could take readings at intervals of 0.1 seconds were used to monitor temperature. A sensor part-inserted in the fruit was sealed with a filler of silicon material (Cemedain K.K. Japan) to prevent it coming into direct contact with hot water.

Temperature measurement of fruit. A sensor was hypodermally inserted in the stem end, fruit tip, and centre of green fruit to a depth of 5 mm and sealed as described above. The fruit were then immersed in hot water of 48.9°C for 30 minutes. The temperature at the various points within the fruit was measured at 1 minute intervals.



[a] Treatment A : 24 hours after inoculation. B : 48 hours after inoculation.
[b] Hot water treatment (48.9°C, 20 min).
[c] Vapor heat treatment (47.2°C).
[d] Inoculation.
[e] Rate of diseased fruits (%).
[f] Average value of rot index.

Figure 2. Effect of hot water and vapour heat treatments on the control of stem-end rot of papaya caused by *Lasiodiplodia theobromae*.

Measurement of temperature at the stem end of fruit in different stage of maturity. Green and half-ripe fruit were used. A temperature sensor was inserted parallel to the stem end to a depth of 5 mm. The fruit were immersed in hot water at 40, 45, 50 and 55°C for 30 minutes. Sensor temperature was recorded at 1 minute intervals.

Measurement of temperature at the stem end of green fruit. Temperature sensors were inserted in the stem end of green fruit at depths of 5, 10, and 15 mm. The fruit were immersed in hot water at 45, 50, and 55°C for 30 minutes. Sensor temperature was recorded at 1 minute intervals.

Results

Rise of fruit temperature. When green papaya were immersed in hot water at 48.9°C, temperature at 5 mm depth in the fruit rose most rapidly in the stem end where it was 46.1°C 10 minutes after immersion. The fruit tip temperature was 44.3°C and in the central hypodermal region it was 43.4°C. When the stem-end temperature reached 48.6°C, there was still a difference in temperature of 2.1°C between it and the central region (46.5°C). The fruit tip temperature was 47.6°C.

Rise of stem-end temperature of fruit in different stages of maturity. Ten minutes after immersion, the temperature of green fruit was found to be higher than

that of half ripe fruit by 1.7°C in hot water at 40°C, 3.8°C at 45°C, 3.2°C at 50°C, and 3.5°C at 55°C.

Rise of stem-end temperature of green fruit. When green fruit were immersed in hot water at different temperatures, the temperatures at depths of 5, 10, and 15 mm in the stem end were found to be almost equal at approximately 30 minutes after immersion. However, they were lower than the hot water temperature by 0.1–1.7°C and 1.1–2.9°C at the 10 and 15 mm depths, respectively (Fig. 3).

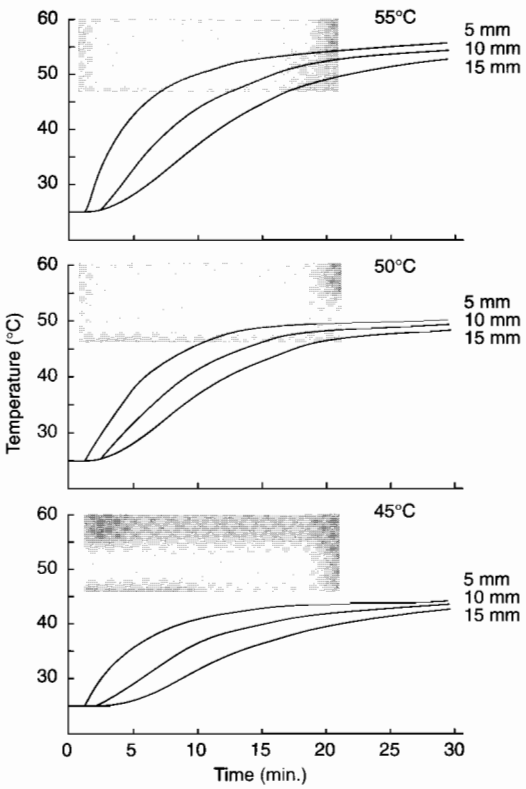


Figure 3. Temperature–time profiles at depths of 5, 10, and 15 mm in green papaya on dipping in hot water at 45, 50, and 55°C. The shaded areas denote thermal death of mycelia of *Lasiodiplodia theobromae* by treatment for 20 minutes.

Effect of hot water treatment on the progress of stem-end rot

The effect of hot water treatment on the progress of stem-end rot due to *L. theobromae* was also studied.

Materials and methods

PB-1, an isolate of *L. theobromae* obtained from papaya fruit imported from Hawaii was incubated on a PDA medium at 25°C for 7 days under artificial illumination with fluorescent light of 1500 Lux (FL20SD, K.K. Japan). A conidial suspension diluted with sterilised water (4×10^3 pc/mL) was used as inoculum.

A conidial suspension was used to inoculate green fruit on stem ends that had been freshly cut with a razor blade. At 12, 24, and 48 hours after inoculation, five fruit at a temperature of 25°C were then immersed in hot water at 45, 50, and 55°C for 20 minutes and cooled with tap water. After this treatment fruit tissue containing vascular bundles was removed from the stem end using a cork borer. Discs were excised at intervals of 5 mm (1: 0–5 mm, 2: 5–10 mm, 3:10–15 mm, 4: 15–20 mm and 5: 20–25 mm) from the inoculated stem ends for re-isolation using a PDA medium. Recovery of *L. theobromae* was assessed by the presence or absence of mycelial tufts 2 days after the tissue discs had been plated onto PDA, when the culture was observed under the above-mentioned artificial illumination.

Results

L. theobromae was recovered from tissue discs taken from the inoculated, treated fruit. Pathogens were re-isolated from the fixed depth of the stem ends when conidia of *L. theobromae* were inoculated and treated with hot water after the designated time. The results obtained are shown in Table 5. Twelve hours after inoculation, the pathogen was re-isolated from 40% of discs at 0–5 mm from the stem end of fruit not treated with hot water and from 20% of 0–5 mm discs of fruit treated with hot water at 45°C, but not at all from fruit treated with hot water at 50 or 55°C.

For fruit treated 48 hours after inoculation, *L. theobromae* was recovered from 60% of the 0–5 mm discs of the fruit not treated with hot water, but from only 40% of fruit treated at 45°C, 20% of fruit treated at 50°C, and from none of the fruit treated at 55°C.

For fruit treated with hot water 48 hours after inoculation, *L. theobromae* was recovered from 60% of the 0–5 mm discs of non-treated fruit, from 80% of 5–10 mm discs, and 60% of 10–15 mm discs. Recovery of *L. theobromae* was lower from fruit treated with hot water at 45°C. *L. theobromae* was recovered from 80% of 0–5 mm discs and 40% of 5–10 mm discs. In fruit treated at 50°C, *L. theobromae* was not recovered from 0–5 mm discs, but it was re-isolated from 80% of 5–10 mm discs and from 40% of 10–15 mm discs.

Table 5. Recovery of *Lasiodiplodia theobromae* from tissue discs cut from the stem end of papaya fruit immediately after hot water treatment for 12, 24 and 48 hours after inoculation.

Time after inoculation (hours)	Disc of stem end		No treatment	Hot water treatment (°C)		
	No.	Depth (mm)		45	50	55
12	1	0–5	40[a]	20	0	0
	2	5–10	0	0	0	0
	3	10–15	0	0	0	0
	4	15–20	0	0	0	0
	5	20–25	0	0	0	0
24	1	0–5	60	40	40	0
	2	5–10	0	0	0	0
	3	10–15	0	0	0	0
	4	15–20	0	0	0	0
	5	20–25	0	0	0	0
48	1	0–5	100	80	0	0
	2	5–10	80	40	80	0
	3	10–15	60	0	40	0
	4	15–20	0	0	0	0
	5	20–25	0	0	0	0

[a] Rate of detection of *L. theobromae* (%)

Fruit injury due to high temperatures

The effect of hot-water temperature and length of treatment on the occurrence of fruit injuries has also been examined.

Materials and methods

Papaya fruit imported immediately after harvest were treated with hot water followed by conventional vapour heat treatment at different temperatures. The occurrence of disorders due to heat and the incidence of decay at room temperature (23±2°C) were investigated 7 days after arrival in Japan. Hot water treatment was conducted on groups of 56 fruit at 4 different temperatures—46.1°C (115°F), 48.9°C (120°F), 51.7°C (125°F) and 54.4°C (130°F)—for 10 and 20 minutes.

Disorders due to high temperature were scored according to the following criteria:

– : No disorder and fruit ripened to entirely yellow

± : Fruit remained partially green, and did not ripen to all yellow

+ : Did not ripen to yellow colour, and half of the fruit surface was greyish green in colour

++: Did not ripen to yellow colour but remained entirely greyish green with light brown spots on surface of fruit.

Results

No disorders due to high temperature were observed in fruit treated with hot water at 46.1°C for 10 minutes. However, at higher temperatures, disorders became progressively more pronounced. Half the fruit treated with hot water at 51.7°C for 10 minutes did not ripen to yellow colour and 16% had further disorders. Furthermore, high temperature disorders and immature peripheral regions were observed in all fruit immersed at 51.7° for 20 minutes (Table 6).

General Discussion

The use of postharvest agricultural chemicals has been prohibited on papaya imported into Japan. However, the application of TBZ is approved for Hawaiian papaya bound to the U.S. mainland and its effect has been presented by Quimio et al.(1975) and Couey and Farias (1979).

Since the use of postharvest agricultural chemicals for imported fruits and vegetables has been taken up as a social problem by the mass media in recent years, the application of vapour heat, hot water, radiant rays, etc. not using agricultural chemicals is now being considered. Above all, heat has long been used for sterilising seeds.

Table 6. Thermal injury to papaya fruits by hot water treatment

Temperature (°C)		46.1		48.9		51.7		54.4	
Time (min)	No[a]	10	20	10	20	10	20	10	20
–[b]	98[c]	100	82	98	68	32	0	0	0
±	2	0	18	2	32	52	0	0	0
+	0	0	0	0	0	16	34	0	0
++	0	0	0	0	0	0	66	100	100

[a] No treatment.
[b] Thermal injured fruit (–:none, ±:fruits remained partially green, +:grey-green colour on half fruit surface, ++:grey fruits remained green with no sign of ripeness).
[c] Rate of injured fruit (%).

The so-called single dipping method of immersing fruits in hot water at 48.9° for 20 minutes combined with vapour heat treatment mainly designed to kill fruit flies is now being used for Hawaiian papaya to be imported into Japan. Although no effective means of eliminating stem-end rot in papaya has been developed, our studies have shown that hot water treatment is much more effective than vapour heat treatment in reducing stem-end rot.

The development of the main pathogens of stem-end rot of papaya, such as *L. theobromae, C. gloeosporioides,* etc., was reduced following the combined use of hot water and vapour heat treatment. In particular, it was found that decay due to *C. gloeosporioides* no longer occurred after hot water treatment, confirming a report of Akamine (1953).

When conidia of *L. theobromae* were inoculated at the stem end of fruit and treated with hot water for 1 and 2 days, the occurrence of stem-end rot was markedly reduced in fruit that were treated 1 day after inoculation. Since stem-end rot decreased in papaya treated with hot water and vapour heat immediately after harvest of fruit, it was considered that the treatment should remain effective until the fruit reach the market.

Further detailed investigations on the temperature and time required for treatment revealed that the pathogen was killed when fruit were treated with hot water at 50°C for 20 minutes 12 hours after conidia of *L. theobromae* had been inoculated onto the stem end. It took about 10 minutes for the temperature to reach 47°C or higher at a depth of 5 mm inside the stem end. Although *L. theobromae* in the stem end at a depth of 0–5 mm was killed when fruit were treated at 50°C for 20 minutes 48 hours after inoculation, it was not eradicated at depths of 5–15 mm. It took about 10 minutes for the temperature to exceed 47°C at a depth of 5 mm in fruit immersed in hot water at 50°C, but the temperature did not exceed 47°C at depths greater than 10 mm. The results clearly showed that more than 10 minutes treatment would be required for the stem-end temperature at 10 mm to become higher than 47°C to eradicate *L. theobromae* using hot water treatment.

However, papaya can be damaged by excessive temperatures during hot water treatment, causing a major problem in the market. Although less injury developed when fruit were immersed in hot water at 48.9°C for no more than 20 minutes, the incidence and severity of injury became worse when treated at 51.7°C for 10 minutes. Diseases were detected in 16% of the fruit, which did not ripen to yellow colour. Furthermore, not all the fruit turned yellow when immersed in hot water at 51.7°C for 20 minutes.

The above results indicated that fruit often develop injury due to hot water, necessitating precise management of temperature and the time required for treatment. Rapid treatment of fruit; at 48.9°C for 20 minutes immediately after harvest will cause less injury, and should prevent stem-end rot due to *L. theobromae*.

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Effect on Stem-end Rot and Anthracnose Levels of Dipping Mango Fruit in Host-defence-promoting Compounds

Zainuri*, D.C. Joyce*, A.H. Wearing*, L.M. Coates† and C.M. Crossley*

Abstract

Anthracnose and stem-end rot are the major postharvest diseases of mango. The causal pathogens may infect fruit in the field and remain latent during subsequent fruit development. Both diseases are usually controlled by pre- and postharvest fungicide treatments. However, in view of public concern about health risks and the possibility of pathogens developing resistance to fungicides, it is important to explore alternative control measures. To this end, an experiment was conducted to determine if treatment with the host-defence-promoting compounds, salicylic acid and potassium phosphonate, would induce fruit resistance to stem-end rot and anthracnose. Mango cv. Kensington Pride fruit were treated with 0, 10, 100, and 1000 mg/L salicylic acid or potassium phosphonate as a series of three preharvest dips and one postharvest dip. Ripening (colour, firmness) and disease (severity, incidence) parameters were assessed at various intervals during shelf life at 23°C. Neither salicylic acid nor potassium phosphonate treatments reduced disease incidence or severity under the conditions of this experiment. For both pathogens, disease symptoms (lesions) appeared when the fruit started to ripen, as indicated by colour and firmness changes. There were no significant treatment effects on colour or firmness changes. These results suggest that salicylic acid and potassium phosphonate did not induce host resistance mechanisms. However, to fully test this proposition, further investigation of the effect of these chemicals on levels of antifungal compounds in

POSTHARVEST diseases of fruits and vegetables caused by pathogen infection are a serious concern to fresh produce industries. Diseases result in major losses of total production. Anthracnose and stem-end rot, which are caused by *Colletotrichum gloeosporioides* and *Dothiorella dominicana*, respectively, are the main postharvest diseases of mango in ASEAN countries (Pordesimo et al. 1984) and Australia (Coutts et al. 1987; Johnson et al. 1993). The causal fungi can infect the fruit during the growing period but remain quiescent. Symptoms become apparent during ripening and storage (Snowdon 1990; Johnson et al. 1991). Pre- and postharvest fungicide treatments are usually applied to control these diseases. However, in view of public concern about health risks associated with fungicide treatments and the possibility of pathogens developing resistance to fungicides, research effort has been directed towards finding alternative control measures (Johnson and Sangchote 1994; Tuzun and Kloepper 1995).

Manipulating host resistance is one of the strategies that hold promise for control of postharvest diseases (Adikaram 1990). Host resistance can be induced by applying physical, chemical, or biological inducers (Wilson et al. 1994). Chemicals such as salicylic acid have been shown to induce resistance in some horticultural crops, including orange and potato (Gaur and Chenulu 1982), banana (Ram and Vir 1986), kiwifruit (Poole and McLeod 1994), and cucumber (Marry et al. 1995). Salicylic acid plays an important role in

* Department of Plant Production, The University of Queensland, Gatton College, Queensland 4345, Australia.

† Plant Protection Unit, Department of Primary Industries, 80 Meiers Road, Indooroopilly, Queensland 4068, Australia.

signal transduction leading to resistance (Gaffney et al. 1993). Phosphonate is another chemical inductant that has been reported to have antifungal effects against both oomycete (Coffey and Ouimette 1989) and non-oomycete pathogens (Heaton and Dullahide 1990). Both studies suggested that this compound acts by direct inhibition of the pathogen and also by eliciting the defence response of the host plant.

The aim of the experiment reported in this paper was to determine if treatment with salicylic acid or potassium phosphonate would elicit a host defence response in mango fruit and suppress the development of anthracnose and stem-end rot diseases.

Materials and Methods

Mango (*Mangifera indica* cv. Kensington Pride) fruit from a commercial orchard near Gatton, Queensland were used in this experiment. Fruit were treated with 0, 10, 100, and 1000 mg/L salicylic acid or potassium phosphonate as a series of three preharvest dips and one postharvest dip. Replication (individual fruit) was 20-fold. Preharvest treatments were applied by dipping individual fruit on the tree every second week over a two-month period up to harvest. Fruit were harvested at the mature green stage, surface sterilised (70% alcohol), and air dried. The postharvest dip treatment was then applied and the fruit were left for 24 hours. The fruit were then divided into two groups. One group was inoculated with *D. dominicana* by placing an inverted agar plug on which *D. dominicana* was growing onto the cut stem end of the fruit, and the other with *C. gloeosporioides* by placing a 25 µL droplet of *C. gloeosporioides* spore suspension (5×10^6 spores/mL) onto the fruit surface. Inoculated fruit were next incubated at 25°C for 24 hours, either in boxes covered with plastic bags (for *D. dominicana*, or in sealed high humidity containers (for *C. gloeosporioides*). Thereafter, the fruit were allowed to ripen at 23°C and 80 % relative humidity for approximately 2 weeks. During shelf life, fruit were assessed daily for colour and firmness changes. Colour ratings were: 1 = 100 % green, 2 = 25 % yellow, 3 = 50 % yellow, 4 = 75 % yellow, and 5 = 100 % yellow. Hand firmness ratings were: 1 = hard, 2 = firm, 3 = slightly soft, 4 = soft, and 5 = very soft. Disease incidence and severity were also assessed. Disease severity was recorded for stem-end rot as the proportion (%) of the fruit surface affected and for anthracnose as lesion diameter (mm). Disease incidence was recorded as the proportion (%) of mango fruit showing stem-end rot lesions on each day of assessment. Data were analysed by analysis of variance (ANOVA) using the Minitab statistical package.

Results

Salicylic acid and potassium phosphonate treatments apparently did not induce host resistance. Neither stem-end rot nor anthracnose disease severity or incidence were reduced (Fig. 1). With the potassium phosphonate treatments, stem-end rot disease symptoms became evident on day 3. With the salicylic acid treatments, stem-end rot symptoms appeared 4 days after inoculation. Stem-end rot symptoms were severe by day 7. Stem-end rot incidence was high for each treatment. Nonetheless, there was a suggestion in the data that higher concentrations of 100 and 1000 mg/L salicylic acid tended to reduce disease incidence (Table 1). Anthracnose lesions appeared on days 3 and 5 for potassium phosphonate-and salicylic acid-treated fruit, respectively (Fig. 1). Compared with stem-end rot, anthracnose symptoms developed slowly.

There were no significant effects of the chemical treatments on either colour or firmness changes of fruit inoculated with *D. dominicana* (Fig. 2). Changes in colour and firmness, which indicated that the fruit ripening process had started, became evident 3 days after inoculation. Similarly, salicylic acid and potassium phosponate did not have significant effect on colour and firmness changes in fruit inoculated with the anthracnose pathogen (Fig. 3).

Discussion

Assessment of colour and firmness changes for fruit inoculated with either *D. dominicana* or *C. gloeosporioides* showed that there was no effect of either salicylic acid or potassium phosphonate treatments on the ripening characteristics of mango fruit. Similarly, neither salicylic acid nor potassium phosphonate treatments reduced disease levels. This result contradicts the notion that pre-infection application of potassium phosphonate can result in protection of plant tissue against pathogens through activation of host defence mechanisms (Dercks and Creasy 1989). Failure to achieve an induced defence mechanism in this study may have been due to an overriding wound stress effect resulting from artificial inoculation. Such an effect might predispose the fruit to disease development (Hislop et al. 1971; Isaac 1992).

Table 1. Proportion (%) of mango fruit showing stem-end rot lesions on each assessment day (n=20).

Treatments	Day							
(mg/L)	0	1	2	3	4	5	6	7
(a) Phosphonate								
0	0	0	0	5	15	25	60	65
10	0	0	0	5	10	35	40	65
100	0	0	0	0	5	35	45	70
1000	0	0	0	0	5	30	50	60
(b) Salicylic acid								
0	0	0	0	0	15	25	75	90
10	0	0	0	0	25	40	60	80
100	0	0	0	0	10	25	30	40
1000	0	0	0	0	10	35	45	45

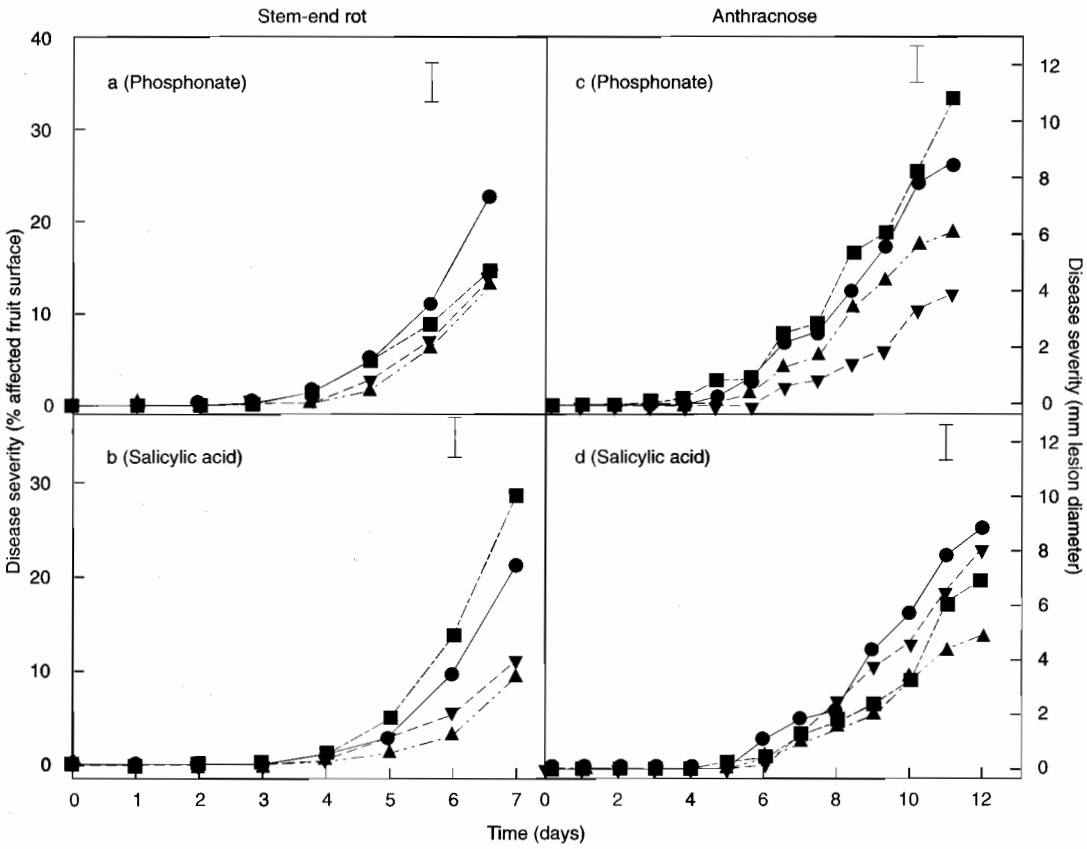

Figure 1. Effect of dipping mango fruit inoculated with either *D. dominicana* (a,b) or *C. gloeosporioides* (c,d) in 0 (●), 10 (■), 100 (▲) and 1000 mg/L (▼) potassium phosphonate (a,c) and salicylic acid (b,d) on stem-end rot [(% affected fruit surface (a,b)) and anthracnose (lesion diameter (mm) (c,d)] disease severity. Vertical bars show the averaged standard error (n = 1120; 2 chemicals, 4 concentrations, 20 replications, 7 days for stem-end rot; and, n = 1920; 2 chemicals, 4 concentrations, 20 replications, 12 days for anthracnose).

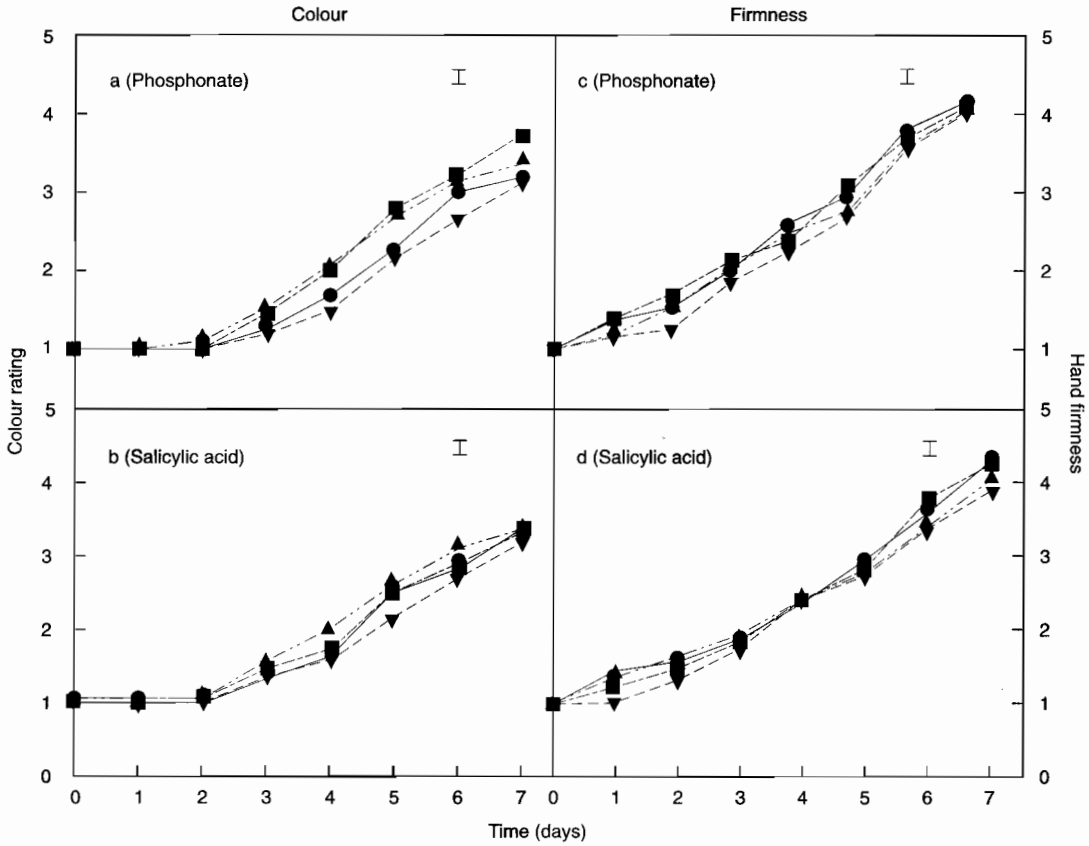

Figure 2. Effect of dipping mango fruit inoculated with *D. dominicana* in (●), 10 (■), 100 (▲) and 1000 mg/L (▼) potassium phosphonate (a,c) and salicylic acid (b,d) on colour (a,b) and hand firmness rating (c,d). Vertical bars show the averaged standard error (n=1120; 2 chemicals, 4 concentrations, 20 replications, 7 days).

Results of ethylene physiology studies suggest that salicylic acid can inhibit ethylene biosynthesis (Leslie and Romani 1986; Romani et al. 1989). However, our results seem not to support this contention. Ripening of control (untreated) and salicylic acid-treated climacteric mango fruit began around day 34 after harvest, at about the time when disease symptoms started to develop.

Loss of resistance of ripening mango fruit to disease can be attributed, in part at least, to a decrease in the concentration of endogenous antifungals; such as 5-(12-cis-heptadecenyl)-resorcinol and 5-pentadecyl resorcinol (Droby and Prusky 1987). Fungitoxic concentrations of these compounds are found in the peel of unripe mango and levels decrease during ripening. The latent period of fungi in unripe fruit might also be related to insufficient host attacking enzyme production by the fungi, such as polysaccharide degrading enzymes (Batman 1976; Droby and Prusky 1986).

Conclusion

Under the conditions of this experiment, salicylic acid and potassium phosphonate did not appear to induce host resistance mechanisms in Kensington mango fruit. However, in order to fully test this interim conclusion, we propose to determine the effects of these chemicals on levels of endogenous antifungal compounds in mango leaf and fruit skin tissue.

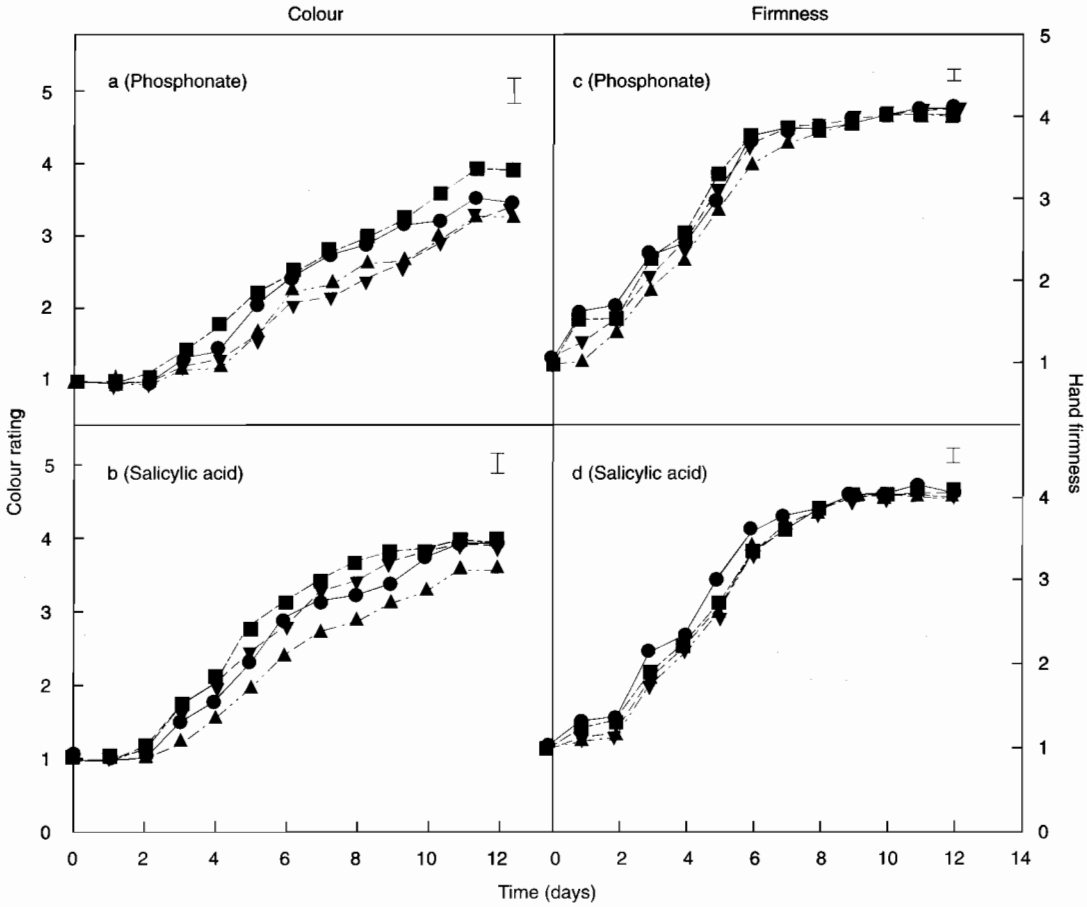

Figure 3. Effect of dipping mango fruit inoculated with *C. gloeosporioides* in 0 (●), 10 (■), 100 (▲) and 1000 mg/L (▼) potassium phosphonate (a, c) and salicylic acid (b, d) on colour (a, b) and hand firmness rating (c, d). Vertical bars show the averaged standard error (n=1920; 2 chemicals, 4 concentrations, 20 replications, 12 days).

Acknowledgments

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Lasiodiplodia theobromae and the Roles of Insects in Dispersal of the Fungi

H. Nago and M. Matsumoto*

Abstract

Lasiodiplodia theobromae GK-1, a strain isolated from the surface of fruit of the coconut palm, produced a coconut-like aroma that was identified as 2-octeno-δ-lactone, β-phenylethanol, and a compound known as mellein. Considering the general nature of the secondary metabolite and its potential role for the micro-organisms producing it, this aromatic compound might have ecological significance as an attractant for insects, which could then be vectors of the fungal spores. This hypothesis led to isolation of *L. theobromae* from the outer surface of a danaid butterfly, *Idea leuconoe*, which was caught in an area in which there were many plants infected by *L. theobromae*. Mellein uptake was investigated by studying the ingesting behaviour of danaid butterflies (male only) alighting on fungal-infected plant tissues in the field and in an insectarium. These observations indicated some kind of mutualistic relationship between the butterfly and *L. theobromae*, associated with the mellein. Mellein was not major component in the hairpencil (a kind of sex organ), and the butterfly could breed without the mellein in an insectarium, suggesting the insect could be independent of *L. theobromae*. This also suggested the role of the insect in dispersing the fungi may be rather weak. On the other hand, the fact that the plant tissues infected with *L. theobromae* outdoors were rather sound in appearance might indicate a symbiotic relationship between plant and fungi which could produce a plant hormone of some kind. This also suggests a useful application of the fungus for postharvest disease control.

SOME strains of *Fusarium solani* isolated from coconut were described previously (Nago et al. 1993). These strains accumulated a compound, (R)-(-)-2-deceno-δ-lactone (massoialactone; Fig. 1), with a coconut-like aroma in the cells as the major compound of the volatile secondary metabolites. We expected that other types of δ-lactone-producing fungi could be found in the vicinity of coconut palms, so we tried to isolate some directly from the surface of green fruits of the tree. The isolated fungus, *Lasiodiplodia theobromae*, produced (R)-(-)-2-octeno-δ-lactone and mellein, which were described in detail in an earlier paper (Matsumoto and Nago 1994). This paper deals with *L. theobromae* and its volatile compounds mainly from the aspect of chemical ecology.

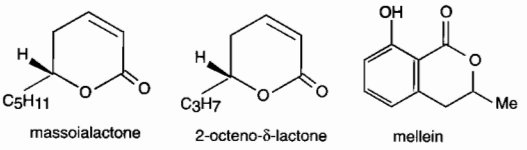

Figure 1. Massoialactone: 2-octeno- δ -lactone mellein

In general, the secondary products can have useful functions for the organisms producing them. The δ-lactones, including mellein, which have a slightly

* Department of Food and Nutrition, Setouchi Junior College, 2379, Shimokatsuma Takase-cho, Kagawa 767, Japan.

fruity odour, are supposed to attract insects (or mites), and may bring the insects into contact with the spores of the fungus. The spores, attached to the mobile insects, would be transported to new places that also attracted these insects, and these new places could be, in many cases, flowers, fruits, or some edible substances containing compounds with the same or similar aromas. Consequently, we can probably obtain the fungi that have ability to produce these aromatic substances from insects as well as from fruits or flowers containing compounds with sweet aromas like the δ-lactones. This was the reason we selected the coconut as the isolation source when we attempted to isolate the δ-lactone-producing fungi, *F. solani* and *L. theobromae*.

Regarding the relationship between the isolated fungus *L. theobromae* and insects, Filer (1969) suggested that the fungi causing sycamore canker were probably spread by crawling insects, primarily ants, because insecticide treatment of the trees prevented infection by the fungi, and large numbers of ants were observed on untreated trees.

We confirmed that the strain of *L. theobromae* IFO 30028, which had been isolated from *Pandanus boninensis* produced 2-octeno-δ-lactone and mellein. Therefore, *L. theobromae* was expected to be also isolated from the places *Pandanus odoratissmus* (screw-pine; adan in Japanese) grows where we could have a chance to investigate for the genus *Pandanus*.

In the meantime, Nishida et al. (1996) discovered that many males of a giant danaid butterfly, *Idea leuconoe* Ericson, grown outdoors, accumulated mellein in the hairpencils as a suspected sex pheromone, and the mellein was characterised as the specific attractant and exhibited potent phagostimulatory activity for the male butterflies. The male accumulated mellein in its hairpencil (a few mg/male), when the butterfly was fed with the compound. The full significance of the mellein for the life cycle of the butterfly is still unknown, but Nishida et al. (1990). suggested that the origin of the mellein might be microorganisms We presumed that one of these microorganisms would be *L. theobromae*, because the giant danaid butterfly inhabits tropical and subtropical coastal regions, in which are also found the genus *Pandanus* and coconut palm etc., the possible host plants for the fungi *L. theobromae*.

In this study, we selected ants and butterflies as possible sources of the δ-lactone-producing fungi to test this assumption.

Materials and Methods

L. theobromae isolation from coconut palm, and the volatile compounds produced by the fungi

Fresh coconuts imported from the Philippines were purchased at a local market, as a source of microorganisms. They were kept at room temperature for a month after wiping the surface with an 80% ethanol solution. The conidia emerging from the pycnidia that covered almost the entire surface of the fruit were transferred to melted potato-dextrose-agar (PDA) medium. *L. theobromae* GK-1 was the isolate used in most of the culture experiments.

Ten petri dishes containing 20 mL of PDA medium were inoculated with the hyphae of the strain GK-1, and incubated at 25°C for 15 days. The volatiles from the cultures were isolated and identified using the method of Nago et al. (1993).

Isolation of δ-lactone-producing fungi from ants

Hundreds of ants (*Anoplolepis longipes* Jerdon) which were crawling on the young fruit of the screw-pine, were caught on the coast at Kinwan (Okinawa island) in October using a sterile milk bottle and some sugar as an attractant. Six of them were placed on a PDA plate, and incubated for 5 days at 25°C. All the colonies broken out from outer surface of these ants were isolated.

Isolation of δ-lactone-producing fungi from outer surface of giant danaid butterflies

A butterfly net was washed with 80% ethanol solution before each butterfly was caught. One male giant danaid butterfly was caught at Okanzaki (Ishigaki island) and one on Maedamisaki (Okinawa island) in October 1991. One male and one female of the butterfly were caught at Haemidanohama (Iriomote island) in February 1993. The entire body of each dead butterfly was placed on the PDA medium in a large petri dish (18 cm diam.), before being incubated at 25°C for 10–20 days. Dominant colonies of the fungi emerging from the outer surface of each butterfly were isolated and identified.

Collection of the suspected host plants of *L. theobromae* involved in the danaid butterfly, and isolation of the fungi from these plants

Five kinds of plants suspected of being host of *L. theobromae* were selected in the habitats of the giant

danaid butterfly on Iriomote, Ishigaki, and Okinawa islands. Parts of each plant collected— leaves, flowers, involucres, and seed pods—, were placed on the PDA medium, and incubated at 25°C for 20 days. The *L. theobromae* growing on the PDA plate was isolated and identified by its characteristic hyphae and conidia, and also by the volatile compounds produced by the culture.

Qualitative analyses of δ-lactone compounds and mellein contained in fungal cultures and plant tissues

Culture conditions for all fungi isolated from ants, butterflies, and plant tissue, and methods for identification of the volatiles produced by these fungi, were as described in a previous paper (Matsumoto and Nago 1994). All mellein detected from various sources was identified by gas chromatography (GC) and GC-MS compared to the authentic sample obtained from the culture of *L. theobromae* GK-1 (Matsumoto and Nago 1994).

Measurement of mellein in plant tissues that were naturally infected with *L. theobromae*

Green fruit of the coconut palm, as described in a previous paper (Matsumoto and Nago 1994), was again purchased from a local market and, without artificial inoculation, was kept for 3 weeks until hyphae of *L. theobromae* covered about one fifth of the surface. After being tested for insect attraction in an insectarium, a 1 cm thick circle of the fruit flesh weighing about 40 g was cut from the outer part of the fungal colony of *L. theobromae*. In the case of screw-pine and bottle palm, part of a blackened leaf or leaf sheath which was obtained in Maedamisaki (Okinawa island) was cut into pieces of 2–4 cm^2, before being steam-distilled and analysed for mellein by GC. The GC was operated using an OT-FS column (CBP-1). The mellein was measured by comparison with the peak area of undecano-g-lactone. A sound part of each plant tissue which had stayed green was also analysed in the same way.

Mellein content in the hairpencil of giant danaid butterfly

Males of the giant danaid butterfly were caught in February 1992 in Chinen (Okinawa island) and Haemidanohama (Iriomote island), one and two individuals, respectively. Each hairpencil cut from the butterfly body was steam-distilled. Mellein in the distillate was measured in the same way as described above for plant tissues.

The butterfly-attraction test

The butterfly-attraction test was done in the Minoo-Koen Insectarium (Osaka). More than 200 giant danaid butterflies were released in a space of 2600 m^3 (200 $m^2 \times 13$ m) together with some other kinds of butterflies. (1) Purified mellein—the mellein which was obtained from the culture of *L. theobromae*—was further purified by silica-gel thin-layer chromatography. A mini-vial (2 mL) containing about 5 mg of the mellein was opened inside the insectarium for about 5 minutes. (2) Plant tissues—blackened tissues of plants which were infected with *L. theobromae*—were placed on the centre of the floor of the insectarium, together with sound parts of each kind of plant tissue as a control.

Results and Discussion

Filamentous fungi broken out on the surface of fruits of coconut palm

The surfaces of three fruits of coconut palm were almost entirely covered by filamentous fungi with stromatic and setose pycnidia in which many conidia were formed. Observing the characteristic uniseptate conidia, the dominant fungal species was identified as *Lasiodiplodia theobromae* (Punithalingam 1976) which has been known mainly as a postharvest parasite on some tropical crops, such as papaya, banana, mango, sugarcane, and others (Punithalingam 1976; Alvarez and Nishijima 1987).

Identification of aroma compounds isolated from the culture of *L. theobromae*

The strain isolated (GK-1) was cultured on PDA medium in petri dishes at 25°C for 15 days. No pleasant aroma came from the headspace of the culture at 15 days, but heating the culture mats with steam led to emission of a strong coconut-like odour. The volatiles were steam-distilled and extracted with methylene chloride. Qualitatively analysed gas chromatograms of the extracts from the cultures of 3, 8, and 15 days are shown in Figure 2. The major peak—compound 2—was identified as 2-octeno-δ-lactone. It showed negative cotton effect, suggesting an R-configuration like naturally occurring massoialactone.

Some published studies of the secondary metabolites of *L. theobromae* have reported mellein, 7-iso-jasmonic acid, lasiodiplodin, and related compounds, which are all considered to be fatty acid derivatives (Aldridge et al. 1971; Miersch et al. 1987, 1989), but

none had reported 2-octeno-δ-lactone. The lack of the 2-octeno-δ-lactone in these studies is probably due to differences in culture conditions.

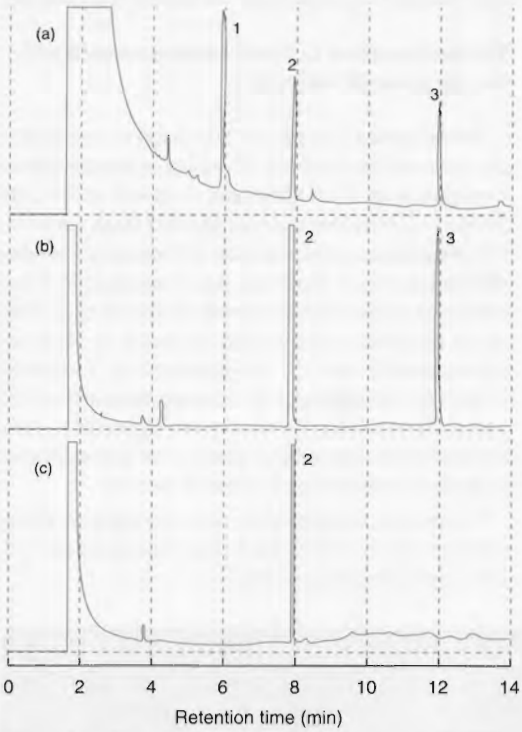

Figure 2. Gas chromatograms of the steam-distillates from cultures of a strain of *Lasiodiplodia theobromae* GK-1: (a) 3 days, (b) 8 days, and (c) 15 days old. 1 = b-phenylethanol; 2 = 2-octeno-δ-lactone; 3 = mellein.

In GC-MS fragmentation, the peak for compound 3 which appears in Figure 2(a) and (b) showed m/z (%), 178(83), 160(43), 149(23), 135(28), 134 (100), 132(28), 106(38), 105(30), 104(32), 78(57), 77(51), 65(20), 63(26), 52(36), 52(53), 43(25), 39(53), these being identical to that reported for mellein (Nishida et al. 1982). The mellein was observed in the cultures of 3 and 8 days, but it disappeared in the final culture as shown in Figure 2. The peak compound 1 was identified to be β-phenylethanol by GC-MS and GC with an authentic sample.

Isolation of δ-lactone-producing fungi from ants

Six ants (*Anoplolepis longipes* Jerdon) (ashinagaki-ari in Japanese) that were crawling on a young fruit of the screw-pine were caught on Okinawa island, and placed on PDA medium. Only a few kinds of filamentous fungi grew on the medium, one strain each of the genera *Fusarium*, *Trichoderma*, and *Aspergillus*. These strains were cultured and the volatiles produced were investigated in the same manner as described already. Massoialactone and 6-pentyl-α-pyrone (2,4-decadieno-δ-lactone) were identified from the cultures of *Fusarium* and *Trichoderma*, respectively.

The expected strain of *L. theobromae* was not isolated from ant bodies; possibly the isolation of δ-lactone-producing fungi suggests the presence of a significant ecological relation between ants and δ-lactone-producing fungi.

L. theobromae isolated from outer surface of the body of a danaid butterfly

A male giant danaid butterfly was caught in October on Ishigaki island, and placed on the PDA medium in a large petri dish (diameter, 18 cm). 20 days after inoculation, about half of the medium was blackened as shown in Figure 3. On these black stains, characteristic grey hyphae were observed, and the isolated fungal strain, named GD-1, was identified as *L. theobromae* with its characteristic uniseptate conidia, as described above. Other mellein-producing fungi could not be isolated from the same source. *L. theobromae* was also obtained from the male giant danaid butterfly that was caught at Maedamisaki (Okinawa island) in the same month.

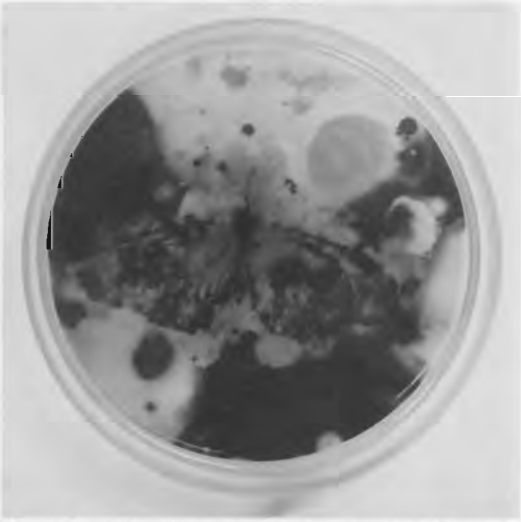

Figure 3. Microorganisms growing on potato-dextrose-agar medium inoculated with a wild male giant danaid butterfly, *Idea leuconoe*. The photograph was taken from the underside of the petri dish. Black stains were confirmed to be caused mainly by *L. theobromae*.

Three other individuals of *Idea leuconoe* (two males and one female) were caught in February on Iriomote and Okinawa islands. These butterflies did not carry any spores of *L. theobromae*, but many colonies of the genus *Aureobasidium* that were unwittingly discovered to be massoialactone-producing fungi, emerged from almost every part of the butterflies on the PDA medium. This is probably because of the cold season when *L. theobromae* and *Idea leuconoe* are inactive, although the two males had mellein in their hairpencils as described later. These suggest the need of further study on the effect of seasonal changes on the fungal flora.

Butterfly-attractivity of the mellein obtained from *Lasiodiplodia theobromae* GD-1

L. theobromae GD-1 was cultured on PDA medium for 7 days, and volatiles were obtained as described above. Using GC and GC-MS, 2-octeno-δ-lactone and mellein were identified in the volatiles, as obtained from the strain GK-1. About 5 mg of mellein, which was purified by thin-layer silica-gel chromatography, was obtained from the culture mats (300 g) of 15 petri dishes, and used in the butterfly attraction test. As shown in Figure 4, males of *Idea leuconoe* were attracted and ingested the mellein that was obtained from the fungi attached to the butterfly.

The host plants of *L. theobromae* associated with the giant danaid butterfly

The suspected host plants of the fungi were sought in the habitat of the butterfly. The parts of selected plants were placed on PDA plates and incubated at 25°C for 20 days. *L. theobromae* was isolated from some the following plants: (1) a seed pod of *Parsonsia laevigata* (Hooraikagami, in Japanese; Apocynaceae), on which the larvae of the butterflies feed exclusively; (2) flowers of *Anodendron affine* (Sakakikazura, in Japanese; Apocynaceae); and (3) an involucre of *Hernandia ovigera* L.(Hasunohagiri, in Japanese; Hernandiaceae), which the butterflies fly around. All strains of *L. theobromae* isolated from these plants were also confirmed to produce mellein, and 2-octeno-δ-lactone.

These facts suggest that many varieties of plants could be involved with the *L. theobromae* as the mellein source for the butterflies.

Figure 4. Feeding behaviour of male giant danaid butterflies at purified mellein produced by *L. theobromae*. The butterflies were attracted to the vial and its cap (right), which held purified mellein (5 mg), and attempted to ingest the chemical. An empty vial and its cap (left) were ignored

Ingesting behaviour of male giant danaid butterfly on plant tissues in the field and also in an insectarium.

We made efforts to observe the mellein-ingesting behaviour of *Idea leuconoe*, outdoors on Okinawa islands, and in the subtropical insectarium.

Ingestion was observed on a leaf of the wild-orangetree, *Taddalia asiatica* (sarukakemikan, in Japanese; Rutaceae) at a habitat of the butterfly in the Maedamisaki district. The butterfly extended the proboscis, and reciprocally spat saliva and ingested it for something on the leaf as shown in Figure 5. The same behaviour was also observed on the leaf of screw-pine, *Pandanus odoratissmus* (Adan, in Japanese; Pandanaceae) (Figure 6). This behaviour is probably associated with mellein ingestion, because it is quite similar to behaviour shown to a filter paper containing synthesised chemicals of methyl o-hydroxybenzoate and other compounds similar to mellein, as observed by Nishida et al. (1990).

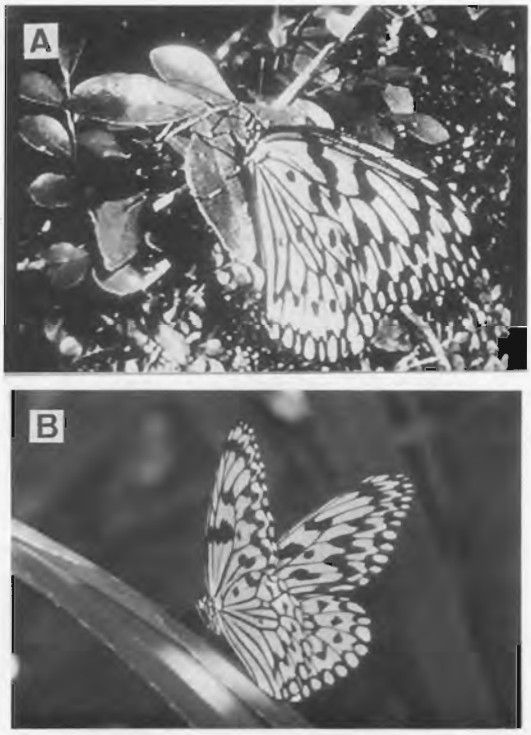

Figure 5. Feeding behaviour of male giant danaid butterflies in the field on leaves of the wild-orangetree (A), and the screw-pine (B). The butterfly extended the proboscis, released saliva, then ingested the result. The leaf was infected mostly with the fungus *Lasiodiplodia theobromae*.

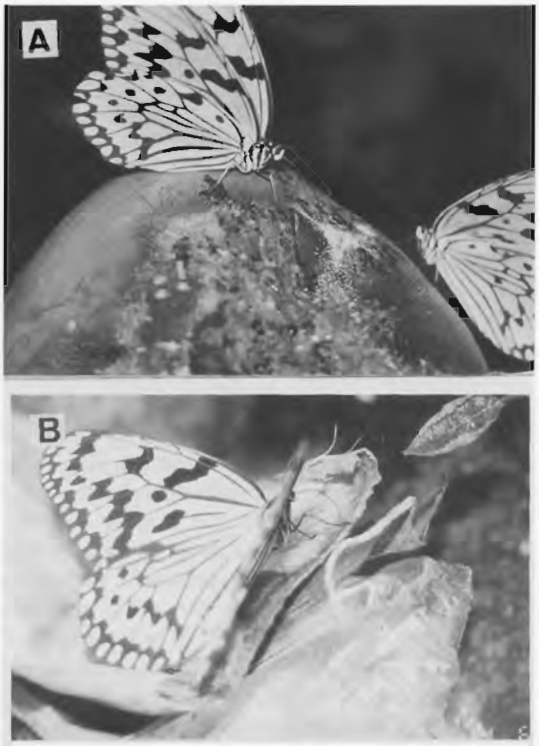

Figure 6. Feeding behaviour of a male giant danaid butterfly in an insectarium on a coconut (A), and on a leaf sheath of bottle palm (B). Both the fruit and the leaf sheath (about a week after harvest) were naturally infected with *L. theobromae*.

The leaf of each plant to which the butterfly reacted was brought into the laboratory and a part of it placed on the plate of PDA medium. More than 90% of the surface of each PDA medium plate was covered within 3 days with the hyphae of *L. theobromae*, and both strains that were isolated from the wild-orange tree and the screw-pine were also confirmed to produce both 2-octeno-δ-lactone and mellein. *L. theobromae* was also isolated from a part of the leaf of the wild-orange tree which the butterfly reacted to, even after being washed with 80% ethanol solution for about 10 seconds, this suggesting that *L. theobromae* was deep within the leaf.

L. theobromae was also obtained by transfer to a sterile tissue paper from the leaf of the screw-pine to which the butterfly reacted. The paper was first pressed onto the surface of the leaf using finger pressure, then the paper with adhering fungal spores pressed on a plate. Many colonies with characteristic hyphae of *L. theobromae* spread out from the point of

contact. This suggests that fungal spores can be easily transferred to a butterfly's body when the insect comes into contact with the surface of an infected plant leaf. In these experiments on the leaves of wild-orange tree and screw-pine, we could not isolate *L. theobromae* from sound leaves which the butterflies showed no interest in. Nevertheless, it is important to point out that the leaves that the butterflies did show interest in also looked rather sound.

In a further experiment, another green coconut was purchased from a local market and, without artificial inoculation, kept for 3 weeks until its surface was about one-fifth covered by hyphae of *L. theobromae.* The mouldy fruit was then brought into the insectarium. A few minutes after the sample had been put down, males of the giant danaid butterfly flew to the mouldy coconut in turn, and each butterfly displaying ingestion behaviour for 0.5–2 minutes as shown in Figure 5. Within 10 minutes, about 20 males of *Idea leuconoe* came had shown this behaviour. No females or any other kinds of butterfly showed any interest in the mouldy coconut. As seen in Figure 6, the males of *Idea leuconoe* were interested especially in the peripheral part of the colony of the *L. theobromae* which was growing from the stem end of the fruit. Male *Idea leuconoe* displayed the same behaviour in the insectarium to both blackened leaf of the screw-pine and leaf sheath of bottle palm, these leaves being tested about a week after harvest. It was confirmed that sound leaves and fresh fruit which had no fungi did not attract butterflies in the insectarium.

Mellein contents in the hairpencils of the butterflies and in the plant tissues infected with the *L. theobromae*, and the attractiveness of these plant tissues to butterflies

Mellein has been found in many male *Idea leuconoe* butterflies grown outdoors, but indoor grown males originally lacked mellein (Nishida et al. 1996). The author also analysed quantitatively the mellein in the hair pencils of *Idea leuconoe* caught on Okinawa and Ishigaki islands in February. The results, obtained from three individuals collected were: by mellein content per single hairpencil 110 ng (Haemidanohama, Iriomote island), 110 ng (Haemidanohama),and 210 ng (Chinen, Okinawa island).

Mellein contents of the plant tissues which were positive in the insect-attraction test in the insectarium were measured. These tissue samples were cut into pieces of 2–10 cm^2, and then steam-distilled without homogenisation. The results of analyses made in the same way as for the fungal culture are shown in Figure 7 and the Table 1, in which the results of insect-attraction test are also shown.

In Figure 7(a), which is the chromatogram of volatiles obtained from the infected fruit of coconut palm, peaks other than mellein do not appear. Thus, strong insect-attraction to the infected fruit shown in the Figure 6A and Table 1 confirms that the male giant danaid butterfly was attracted only to mellein. The normal parts of these plants, fruit flesh of coconut palm and leaf of screw-pine, were confirmed to contain no mellein. It was therefore concluded that the compound in these plant tissues infected with *L. theobromae* was produced by the fungus. As shown in the gas chromatogram (Figure 7(b)), the mellein content of the infected leaf of screw-pine was low, and the remaining miscellaneous peaks did not exclude the possibility that that there were other compounds present attractive to the butterfly in the leaf of screw-pine. Nevertheless, the weak attractiveness of the leaf to insects makes this doubtful. The mellein content of 26 ng/cm^2 in the infected leaf of screw-pine makes it not unreasonable for a male giant danaid butterfly to accumulate 110 to 210 ng of the compound in his hairpencil.

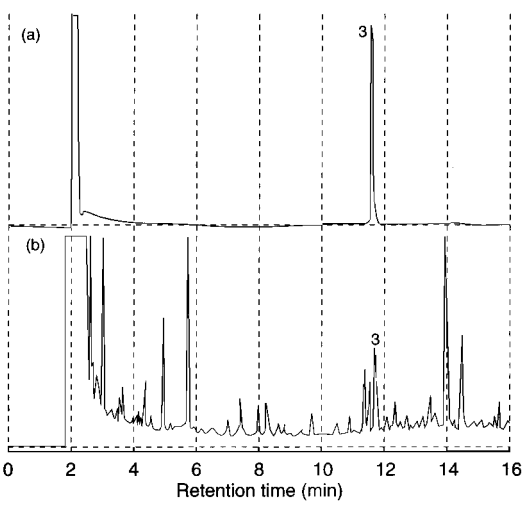

Figure 7. Gas chromatograph traces of volatiles obtained from plant tissues invaded mostly by *L. theobromae*. (a), from fruit flesh of coconut palm infected with fungi; (b), from a leaf of screw-pine infected with the fungus. Extraction conditions are described in the text. Peak 3 is mellein

Table 1. Mellein content and butterfly-attractivity of infected plant tissues with *L. theobromae*.

Sample[a]	Part of the plant	Weight of sample (mg/cm^2)	Mellein content ng/cm^2 (ppm)	Butterfly- attractivity[c]
Screw-pine	Leaf and leaf sheath	96[b]	26, (0.3)	+
Coconut palm	Surface part of the fruit	650	7000, (11)	+++
Bottle palm	Leaf sheath	70[b]	136, (2)	++
Papaya	Whole fruit		(12)	–

[a] Samples were naturally infected with *L. theobromae*, except for papaya which was inoculated with *L. theobromae* GK–1.
[b] With full thickness of sample.
[c] +; poor butterfly attraction
++; good butterfly attraction
+++;excellent butterfly attraction
–; no butterfly attraction

There is thus strong evidence that the mellein in the hairpencils of male giant danaid butterflies comes from the mellein produced by *L. theobromae* in the infected plants.

As described previously (Matsumoto and Nago 1994), a papaya fruit that was artificially inoculated with *L. theobromae* contained 12 ppm of mellein, the same level as in the fruit of coconut (Table 1). The mouldy papaya was also brought into the insectarium, but the butterflies did not fly to it. This negative result may be due to the strong aroma compounds, such as benzyl isothiocyanate and monoterpene derivatives, which are found in papaya (Flath and Forrey 1977).

Mutualistic relation of microorganisms with insect

Many examples of fungus–insect mutualism have been reported and reviewed, including the case of the genus *Ceratocystis* associated with bark beetles as the vectors of fungal pathogens (Graham 1967; Basham 1970; Beaver 1987; Webber and Gibbs 1989). Fungi, mostly *Ceratocystis*, were isolated from adult bodies of the bark beetles (Kobayashi et al. 1974). There are very few reports of fungi being found on outer bodies of insects.

Some cases of insect attractance of microorganisms have been reviewed (Ishikawa et al. 1984; Grove and Blight 1983). However, cases involving the relationship between fungus and butterfly have not reported as far as we are aware. Regarding the relationship between *L. theobromae* and insects, besides the case of ants on sycamore trees described already, Thapa (1971) observed that *L. theobromae* had been introduced by ambrosia beetles into majau trees, and developed within the tunnels, spreading to all parts of the trees.

Our observation described above suggested the presence of a kind of mutualistic relationship between the fungus *L. theobromae* and the giant danaid butterfly, centring on the compound mellein which. it was suggested might be a sex pheromone of the butterfly. However, the mutualistic relation might be rather weak, because fungal spores can also be disseminated by wind and water (Punithalingam 1976).

Kunesch et al. (1987) reported a similar case of a mutualistic relationship between a bumblebee wax moth and a mellein-producing fungus. They discovered mellein as the major component of the male wing gland pheromone of the bumblebee wax moth, *Aphomia sociella*, and a mellein-producing fungus, *Aspergillus ochraceus*, was detected in the intestines of the last-instar larvae. They argued that this might indicate a biosynthesis of mellein by the fungi present in the gut of the larva. In the case of the danaid butterflies described here, however, most of the mellein would not be extrinsic, because the butterflies were attracted to and showed ingesting behaviour to extrinsic mellein (Figure 6).

The male of *Grapholita molesta*, an insect pest of fruit trees, also has mellein in its hairpencil together with methyl 7-iso-jasmonate and other compounds (Nishida et al. 1982). This insect might be also associated with *L. theobromae* or some other mellein-producing fungus.

In the fatty-acid metabolites from the culture of *L. theobromae*, mellein (carbon number, 10; C10), (+)-7-iso-jasmonic acid and related compounds (C12), and lasiodiplodin and its derivatives (C16), except for 2-octeno-δ-lactone (C8), have been shown to have plant growth regulatory effects (Miersch et al. 1987, 1989. This suggests an association between the *L. theobromae* and the host plant. This also suggests

a useful application of the fungus in postharvest disease control.

Some papers have been published on the chemical–ecological relationship of α, β-unsaturated lactones to insects. Cavill et al. (1968), for example, obtained massoialactone from one of the formicine ants of the genus *Camponotus* collected in Western Australia. This may also indicate an ecological relationship between ants and δ-lactone-producing fungi, but we have no further data to support this contention.

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Durian—Sources of Resistance to *Phytophthora palmivora*

T.K. Lim*

Abstract

Phytophthora palmivora is the most devastating pathogen of durian. Chemicals are usually employed in attempts to control the disease, but the outcome is often erratic and unsatisfactory. The use of disease resistance, an invaluable facet of biological control, forms an important component of an integrated disease management (IDM) strategy. Resistant durian varieties in Malaysia and Indonesia have been field selected instinctively by growers down the generations. Recently in Malaysia, three field-tolerant durian hybrids were released by MARDI. They were produced by crossing a tolerant clone (D10) with a popular but susceptible clone (D24). Potential sources of *Phytophthora* resistance can also be found in other *Durio* species and allied genera—*Coelostegia*, *Kostermansia*, and *Neesia*—especially those that thrive in damp, low-lying areas, marshes, and peat swamps. Evidence is provided that the durian–*Phytophthora* interaction exemplifies a horizontal pathosystem and that host resistance is horizontal and governed by many genes. Likewise, resistance in other *Durio* species and related genera is polygenic. Resistant genes can be exploited by conventional or molecular plant breeding. The use of gene transformation in the latter approach is still a distance away in perennial tree crops such as durian. Deployment of resistant rootstocks in various combinations provides an alternative approach to exploiting resistance in durian. Besides imparting disease resistance, appropriate rootstock can increase field survival of young plants, increase habitat adaptability, and enhance precocity of bearing.

DURIAN with its high economic value has been deservedly deemed as the 'golden fruit' of Southeast Asia. The fruit is also being grown on a small commercial scale in northern Australian (Lim 1995). Besieged by besotted humans, the durian is also beset by the ravages of pests and diseases. The most destructive and economically significant diseases of durian are caused by the soil-borne fungus, *Phytophthora palmivora,* which thrives in damp soil. This oomycetous fungus causes root rot, patch canker, seedling dieback, leaf rot, and pre- and postharvest fruit rot on durian (Lim and Chan 1986; Lim 1990). It can be devastating in all the major durian-growing areas in Southeast Asia and in northern Australia.

* Horticulture Division, Department of Primary Industry and Fisheries, GPO Box 990, Darwin, Northern Territory 0801, Australia.

The *Phytophthora palmivora* group is cosmopolitan and polyphagous, attacking a wide host range of economic crops. The strain attacking durian belongs to the A1 mating type and MF1 morphological form (Lim and Chan 1986). Several fungicides—metalaxyl–mancozeb mixture, cyprofuram, milfuram and fosetyl-Al—have given effective control of seedling dieback (Chan and Lim 1987) but control of the disease on adult field trees has often been erratic and unsatisfactory. Moreover, the continual reliance on pesticides can produce many inimical side-effects such as the appearance of pesticide-resistant pathogens, contamination of soil, water, and other parts of the environment, and health hazards to humans.

With the rise in environmentally consciousness, we have seen a shift to more environmental friendly and socioeconomically acceptable approaches to plant disease control. Such a strategy is conceptualised in the systems approach of integrated disease manage-

ment (IDM), a term borrowed from entomology. IDM takes into account the environment, plant, and pathogen population dynamics. It utilises all suitable methods and techniques in a unified manner to manage the pathogen to reduce crop losses. IDM does not mean that chemicals are not used. Rather, it means judicious use of chemicals in combination with other biological, physical, and cultural methods. IDM involves consideration of the growers' preferences and productivity, and is especially suitable for tree crops as they allow for continuity of the IDM approach. The use of resistant varieties is one facet of biological approaches to disease management.

Resistance in Durian

Durian (*Durio zibethinus* Murr.) varieties

The common and most popular varieties of durian in Southeast Asia have been field selected over the years from open-pollinated seedlings resulting from frequent out-crossing for fruit quality rather than productivity or disease resistance. Reports of resistance in durian to *Phytophthora palmivora* have been based in the main on field observations, except for the inoculation studies by Tai (1971) in Malaysia and, more recently, by Pongpisutta and Sangchote in Thailand (1993). Tai (1971), using seedling inoculations, found that the cultivars D2, D10, D30, and D63 were tolerant to *Phytophthora palmivora*, whereas D4, D24, and D66 were susceptible. Pongpisutta and Sangchote, using wound inoculation of detached fruits, reported that the cultivars Chanee, Kob, Gaan-Yaow, and Monthong were susceptible. Keeratipataragul (1980) reported that two other Thai cultivars, Luang and Gradumtong, were also susceptible to *Phytophthora palmivora*.

Recently, following some 20 years research and observation, the Malaysian Agricultural Research and Development Institute (MARDI), released some new hybrids developed by crossing and selection based on fruit quality, yield, and resistance to *Phytophthora*. Using cultivar D10 as a tolerant parent, and D24 as a highly susceptible one, three hybrid clones were released with higher tolerance than D24. MDUR 79 [D24 (female parent) × D10 (male parent)] and MDUR 88 [D24 (female parent) × D10 (male parent)] had higher field resistance than the reciprocal cross MDUR 78 (D10 (female parent) × D24 (male parent) (Lee 1994; Zainal Abidin 1994). Higher field resistance was obtained when cultivar D10 was used as the male parent, with a canker rating of less than 0.5 compared with a disease severity rating of 2.2 for cultivar D24 (Table 1). Zainal Abidin (1994) also found that resistant cultivars had greater canopy area than the susceptible D24.

Also, the popular clone D145 is susceptible, while D99 is tolerant to *Phytophthora palmivora*. Field tolerance or susceptibility of the remaining 200 or so registered Malaysian clones has not been documented (Lim 1996). The same is true of the immense Indonesian varietal gene pool where only a small number had been reported to show field tolerance to *Phytophthora palmivora* (Widyastuti and Piamin 1993). Those with reported field resistance are: Bokor, Lalong, Mansau, Petruk, Sawarigading, Siriwig, Si Dodol, Si Hijau, Si Japang, Si Mas, Sukun, Sunan, Tamalatea, and Tembaga, while Kani (Chanee) was reported to be susceptible.

Table 1. Field tolerance of MARDI hybrids to patch canker.

Hybrid	Canker rating[a]
MDUR 79 (D24 × D10)	0.43
23–6 (D10 × D24)	0.45
22–5 (D24 × D 10)	0.63
D9 (D7 × D24)	1.38
15A–6 (D24 × D7)	1.58
MDUR 78 (D10 × D24)	1.78
E33 (D8 × D10)	2.00
F6 (D8 × D24)	2.12
D24	2.29

[a] <1 = 0–25% canker, 1.1 to 2 = 26–50%, 2.1 to 3 = 51–75%
Source: Lee (1994)

Other *Durio* species and allied genera

Malaysia and Indonesia are the centre of diversity for *Durio* species and possess 28 of the 29 recognised species (Kostermans 1958, 1992), while Burma has one species, *Durio mansoni* (Gamble) Bakh. Wild and semi-wild species of *Durio* and closely allied genera in the family Bombacaceae provide good sources of interesting traits like resistance to diseases, soil type tolerance, stress tolerance (waterlogging), fruit colour, and precocity of bearing. Their fitness for survival is high, as they have evolved in a harsh natural environment that would have been catastrophic for the domesticated durian.

Many *Durio* species that grow in low-lying, damp places such as marshes, peat swamps, and river banks which are periodically flooded have not been exploited as sources of resistance (Table 2). *D. carinatus* Mast. thrives gregariously in the peat swamps in Peninsular Malaysia and Sarawak; *D. lissocarpus* Mast in marshy places in Borneo; *D. acutifolius* (Mast.) Kosterm. thrives on sandy soils with high water tables; and *D. excelsus* (Korth.) Bakh. is found on sandy loam soil that is periodically inundated (Kostermans 1958). Other good sources of resistance include *D. graveolens* Becc. (Durian kuning or Durian burung) which thrives on coastal alluvial soils that are periodically flooded and also grows in peat swamps, *D. oxyleyanus* Griff. (Isu) found in damp clay-rich alluvium which is frequently flooded, and *D. testudinarum* Becc. (Durian kura) which thrives on low-lying river banks which are periodically inundated (Kostermans 1958; Voon 1994). *D. testudinarum* also has a dwarfing effect on the common durian which show smaller girth and shorter height increase (Voon 1994). Unfortunately, the highly regarded Lai (*Durio kutejensis* (Hassk.) Becc.) which is semi-wild, with delectable edible fruits like durian, cannot withstand waterlogging for even two days (Voon 1994) and is susceptible to *Phytophthora*.

There is every likelihood that other species of *Durio* (Table 2) that cannot withstand waterlogging are also tolerant to *Phytophthora palmivora*. *D. lowianus* Scort. ex King and *D. mansoni* had been reported to be resistant to *Phytophthora*, but no details on procedures and results were provided (Subhadrabandhu et al. 1992). They also reported that *D. mansoni* might have a dwarfing effect as rootstock, since it did not thicken at the same rate as the scion.

Another potential source of disease resistance can be found in the closely allied genera of *Neesia*, *Coelostegia*, and *Kostermansia* within the same family. Kostermans (1958) reported that *Coelostegia*, *Durio*, and *Neesia* are closely related and that the last two might well be united, while *Kostermansia* appears to be intermediate between *Coelostegia* and *Durio* (Kochummen 1972). *Kostermansia malayana* Soegeng, known as 'krepal', has close resemblance to the common durian and is endemic in Peninsular Malaysia in swampy places; *Coelostegia griffithii* Benth. is found in lowland forest and sometimes in poorly drained or semi-swampy soil (Kochummen 1972). Among the *Neesia*, 8 species are distributed among Thailand, Sumatra, Java, and Borneo, while 5 are found in Malaysia, mainly in the lowland swamp forest, e.g. *Neesia kostermansiana* Soepadmo, *Neesia malayana* Bakh., and *Neesia synandra* Mast. (Kochummen 1972).

Table 2. Species of *Durio* and allied genera with potential field tolerance/resistance to *Phytophthora*.

Durio acutifolius (Mast.) Kosterm.
Durio carinatus Mast.
Durio excelsus (Korth.) Bakh.
Durio graveolens Becc.
Durio lowianus Scort. ex King
Durio mansoni (Gamble) Bakh.
Durio oxyleyanus Griff.
Durio testudinarum Becc.
Coelostegia griffithii Benth.
Kostermansia malayana Soegeng
Neesia kostermansia Soepadmo
Neesia malayana Bakh.
Neesia synandra Mast.

Resistance Mechanism

Disease resistance or susceptibility is a manifestation of the constitutive host defence mechanism. The susceptibility of D24 to *Phytophthora palmivora* is uniform wherever in Peninsular Malaysia or Sarawak the clone is grown, and the tolerance of D2 is also uniformly expressed whether it is grown in Malaysia, Sarawak, or Cairns (Lim, unpublished data). This aligns with the work of Tai (1971) and Lee (1994).

These findings indicate that all individuals of D2 or D24 varieties have an identical level of horizontal resistance or susceptibility to *Phytophthora palmivora*. Thus, on a population basis, the durian–*Phytophthora palmivora* interaction represents a horizontal pathosytem (Robinson 1976) where there is no differential interaction between the horizontal pathodeme (crop host) and pathotype (physiologic races of the pathogen). In other words, on a one-to-one or host–pathogen relationship, the resistance in durian is horizontal and controlled by a few to many genes, i.e. is polygenic, in contrast to monogenic resistance which is vertical and controlled by a single gene (Van der Plank 1984).

That resistance to *Phytophthora palmivora* in durian is polygenic is further evidenced by the immense diversity and heterozygosity of durian

varieties that are produced through natural outcrossing which ensures a continuous gene-flow and shuffling of many genes. Likewise, it is also conceivable that resistance in other *Durio* species and in the allied genera previously mentioned is polygenic, horizontal resistance. The effect of horizontal resistance is that the rate of disease spread and severity are reduced, in contrast to vertical resistance that delays the onset of an epidemic by reducing the initial inoculum (Van der Plank 1984). The cultivation of mixed cultivars in a durian plantation also ensures the expression of a durable horizontal pathosystem.

Exploitation of Resistance Genes

By classical plant breeding

Resistance in durian can be exploited by classical plant breeding, or by molecular plant breeding using genetic transformation techniques. In classical plant breeding, new combinations of genes can be generated by crossing different varieties and selecting the progenies for some desirable traits, or transferring a trait by crossing a cultivar with the desirable characteristic to a recipient plant. This is exemplified by the development of new hybrids by MARDI in Malaysia (Lee 1994; Zainal Abidin 1994). Another way is to select for field resistance by exposing progenies to disease in the field and selecting for resistant ones, as has been done instinctively by growers down the generations. Both involve the uncontrolled, sexual transfer of large pieces of chromosomes containing many genes (desirable as well as undesirable or detrimental).

By molecular plant breeding–genetic transformation

With the rapid achievements in the field of genetic engineering, or more precisely genetic transformation, it is now possible to develop new, transgenic crops by inserting into the genomes of crop plants desirable genes that have been identified, isolated, and manipulated (Chrispeels and Sadava 1994). A gene is a sequence of DNA which encodes for a particular protein or functional RNA, which confers a particular trait to a cell expressing that gene. Gene transformation allows for the transfer of specific genes from unrelated species, genera or plants or other living organisms without unwanted traits, thereby not affecting other production attributes of the recipient plant. Gene introgression into crops can be achieved via two methods—biological and mechanical. Biological transfer is mediated through the use of the bacterium, *Agrobacterium tumefaciens*, involving t-DNA which carries a gene encoded for resistance. Mechanical transfer is achieved by coating beads of gold or tungsten with DNA and shooting these into an explant using high pressure gas or an electric discharge.

In perennial tree crops, it is conceivable that other traits besides disease resistance are controlled by polygenes. It is easier to insert disease resistance controlled by a single gene rather than resistance controlled by many genes. Thus, the development of a transgenic perennial tree crop may be further away than the advancement of transgenic herbaceous annual and biennial crops. Nevertheless, some early progress in the development of transformation systems for tree crops, especially in areas of fruit quality and disease resistance, has been reported (Stomp 1987; Hanover 1987). DNA finger-printing technology such as the restriction fragment length polymorphism (RFLP) technique, polymerase chain reaction (PCR) analysis, random amplified polymorphic DNA technique, and minisatellite DNA probes, has developed rapidly. It is now possible to accurately identify genes encoding for particular traits and to use these molecular markers to develop genetic maps of crops for cultivar identification, registration, and authentication; for determination of phylogenetic relationships; and for marker-aided selection of important traits such as disease resistance at the seedling stage. However, progress on the use of such technology and micropropagation techniques such as tissue culture on tropical perennial fruit trees like durian is lagging. Hitherto, only one brief report on durian has been published, on the phylogenetic relationship of the new MARDI hybrids MDUR 78 and MDUR 79 to their progenitors using RAPD (Hassan et al. 1993), and success in micropropagation of durian by tissue culture has yet to be achieved.

By rootstock manipulation

Rootstocks are important determinants for tree vigour control, graft incompatibility, habitat adaptability, and for imparting pest or disease resistance. Species of *Durio* or allied genera as described above would be excellent candidates to impart disease resistance and tolerance of damp soils or waterlogged conditions. A rapid way to harness resistance in durian is to use these species, cultivars, and related genera as rootstocks, with the desired clone as scion. Rootstocks can

be used in various combinations. Multiple rootstocks can be used as an important component in an integrated disease management strategy if one or all of the rootstocks is tolerant or resistant to the soil-borne *Phytophthora*. Even if none of the rootstocks is tolerant, the double root systems will increase the chances of tree survival during its years of field establishment.

Preliminary observations (Zappala and Lim, unpublished data) have shown that the use of double rootstocks increased the survival of grafted seedlings in the field in 90% of cases after 1 year of field establishment. They produced healthier, more vigorous trees even though ordinary seedlings were used as the second rootstock. The height of the union between the two rootstocks is important. Multiple rootstocks can produce better growth and advance maturity by their more extensive, enhanced root system which facilitates greater uptake of nutrients and water. The stronger root system can result in better anchorage and support, making the tree less prone to windthrow by strong winds. In many fruit tree species it is well documented that rootstock also influences the precocity of bearing and dwarfing for easier implementation of cultural practices. Small statured *Durio* species provide good sources for precocity (Lim 1996) and could be used directly as dwarfing rootstocks or used for rootstock breeding. *D. testudinarum* Becc. (identified as synonymous with *D. macrophyllus*), which thrives on low-lying river banks that are periodically inundated, also had a dwarfing effect on the common durian, yielding shorter plants of smaller girth (Voon 1994). Subhadrabandhu et al. (1992) reported that *D. mansoni*, besides imparting resistance to *Phytophthora,* might have a dwarfing effect as rootstock since it did not thicken at the same rate as the scion. Unfortunately, trials with these rootstocks in Thailand were discontinued because of the low percentage take of grafts. Also, scion overgrowth was considered as sign of incompatibility.

Conclusion

There is a dearth of published information on the use of resistant durian varieties, *Durio* species, and allied genera to control *Phytophthora* diseases in durian. The use of resistant planting material for breeding, or in single or multiple rootstock combinations, is an important component of IDM in durian and deserves more research and greater emphasis. More studies are needed on their graft compatibility with the common durian and on their use in the breeding of disease-resistant rootstock. Besides imparting disease resistance and tolerance, rootstock also influences survival in field establishment, increases habitat adaptability, and enhances precocity of bearing.

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Detection and Isolation of Antifungal Compounds from the Peel of Pomelo (*Citrus grandis* (L.) Osb.)

W. Kumpoun*, D. Supyen†, P. Kitsawatpaiboon†, V. Sardsud§, P. Chansri§, S. Promin§, and J. Sronsrivichai¶

Abstract

Pomelos are quite resistant to diseases after harvest. They can be stored for up to 2 months without disease, indicating that antifungal compounds may exist in the fruit. It was reported there were many kinds of preformed antifungal compounds in the flavedo (oily part) of its peel, some of them coumarin derivatives. No study has been conducted on the antifungal compound in Thai pomelos. The flavedo of three cultivars of Thai pomelo i.e. 'Thongdee', 'Kaopan', and 'Kaoyai' was extracted using dichloromethane and the compounds in the extract separated by PTLC plate using n-hexane: ethylacetate: methanal at a 60:40:10 ratio as the developing solvent. Detection of the antifungal compound in the isolates on the PTLC plate was done by bioassay with 1×10^6 spores/mL of *Cladosporium cladosporioides*. Four inhibition bands with similar Rf from 10 isolation bands were detected from each cultivar. Two of the inhibition bands could be further separated into 3 and 4 new inhibition bands by the first and second developing solvent (dichloromethane: toluene: ethylacetate 60:30:10), respectively. The effective band from the second partition was tested for spectral absorption using a UV spectrophotometer. The maximum absorbance was found to be similar to that of scopoletin, a derivative of coumarin. Molecular weight and structure determination using the GC-MS revealed that all bands were impure. However, a comparison of the mass spectra showed that none of the bands was similar to that of citral. The molecular weight was also much higher. For further research, the compound will need to be purified and identified.

POMELOS are quite resistant to disease after harvest. They can be stored for up to 2 months without disease, suggesting that they may contain antifungal compounds. Antifungal compounds have been reported in many citrus fruits. For example, citral, limettin, 5-geranoxy, 7-methoxycoumarin, and isopimpinellin have been found in the peel of limes (Ben-Yehoshua et al. 1992). In pomelo, Ben-Yehoshua et al. (1988) reported many antifungal compounds which were derivatives of coumarin i.e., osthol (7-methoxy-8-prenylcoumarin), auratene (2,3-epoxy-7-methoxy-8-prenylcoumarin), 7-[(6,7-epoxy-3,7-dimethyl-2-octyl) oxy]-coumarin, and 7-geranoxycoumarin. Other coumarin derivatives were also found in the leaf and stem of citrus. Ben-Yehoshua et al. (1988) reported that unidentified derivatives were 50 times more effective than coumarin. No study has been conducted on the antifungal compound in Thai pomelos. This paper reports the occurrence of antifungal compounds in three pomelo cultivars in Thailand, at various stages of maturity.

* Institute for Science and Technology Research and Development, Chiang Mai University, Chiang Mai 50200, Thailand.

† Department of Chemistry, Chiang Mai University, Chiang Mai 50200, Thailand.

§ Department of Plant Pathology, Chiang Mai University, Chiang Mai 50200, Thailand.

¶ Department of Biology, Chiang Mai University, Chiang Mai 50200, Thailand.

Materials and Methods

The experiment was divided into two parts:

***Experiment I.* Extraction of antifungal compound from the peel of three pomelo cultivars.**

Khao-Yai pomelos were harvested from an orchard in Chiang Mai at 7 and 8 months after anthesis. The 8-month pomelos were divided into two groups: one was used for the experiment immediately after harvest; the other was stored at room temperature for 2 weeks before being used for the experiment. Thongdee and Khao-Panne pomelos were harvested from an orchard in Nakhon-Pathom, at 8 months after anthesis (pomelo are fully mature 8 months after anthesis)

Extraction was done by submerging the peel of the pomelos overnight in dichloromethane. The extraction was repeated and the extract combined. The combined extract was evaporated and applied to a PTLC plate (silica gel 60 GF245 on 1 mm thick layer). N-hexane:ethylacetate:methanol at a 60:40:10 ratio was used as the developing solvent. The plate was sprayed with 1×10^6 spore/mL of *Cladosporium cladosporioides* and the inhibition zone recorded.

***Experiment II.* Second partitioning and identification of antifungal compounds**

A crude extract from the peel of Thongdee pomelos was developed on a PTLC plate as above. The 3 bands which had antifungal properties, i.e. band 4 (Rf = 0.36), band 5 (Rf 0.45), and band 6 and 7 (Rf = 0.48–0.65) were scraped from the plate. Methanol was used to remove the compound from the silica gel. The solution was filtered to remove the silica gel and the methanol evaporated. The remaining material was developed on the PTLC plate again, but with the developing solvent changed to N-hexane:ethyacetate:methanol at a ratio of 60:40:10, **or** dichloromethane toluene:ethylacetate at a ratio of 60:30:10, **or** dichloromethane:methanol at a ratio of 90:10. The PTLC plate with the best resolution was tested for antifungal properties as above.

Antifungal bands were extracted with methanol and approximate molecular weight and structural formula determined by gas chromatography–mass spectrometry (GC–MS). Spectral absorption properties were determined using a UV spectrophotometer. Results were compared with those of citral and scopolatin.

Result and Discussion

***Experiment I.* Extraction of antifungal compound**

All groups of 'Khao-Yai' pomelo produced 10 bands on the PTLC plate. Inhibition zones were found at band 4 (Rf = 0.36), band 5 (Rf = 0.45), and bands 6 and 7 (Rf = 0.48–0.65) in all groups (Fig. 1). Similar extraction bands and inhibition zones were found at both maturity stages of Khao-Yai pomelo, and in Thongdee and Khao-Panne pomelo (Fig. 2). The same bands and inhibition zone also appeared on the PTLC plate for Khao-Yai pomelos that were kept for 2 weeks at room temperature.

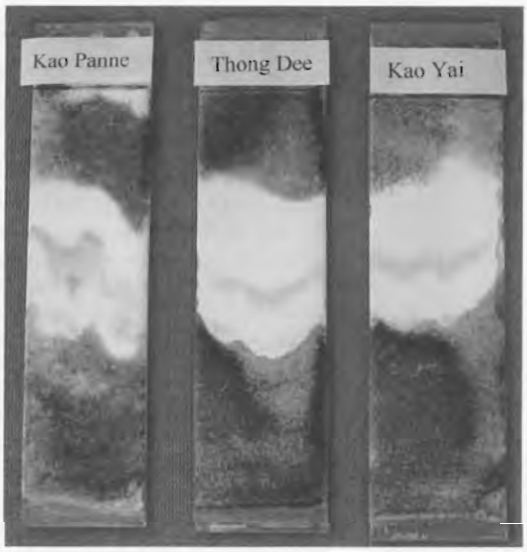

Figure 1. TLC bioassay of antifungal compounds extracted from the peel of three cultivars of Thai pomelo.

***Experiment II.* Second partitioning and identification of antifungal compound materials**

The first developing solvent (N-hexane:ethyacetate:methanol at a ratio of 60:40:10) could separate band 4 into 8 bands (Fig. 3a), three of which had effective antifungal properties (Rf = 0.17, 0.37, and 0.67).

The second developing solvent (dichloromethane:toluene:ethylacetate at a ratio of 60:30:10) separated band 5 into 6 bands (Fig. 3b), four of which had effective antifungal properties (Rf = 0.23, 0.32, 0.42, and 0.51). Bands 6 and 7 were separated into 6 bands, all of which had antifungal properties.

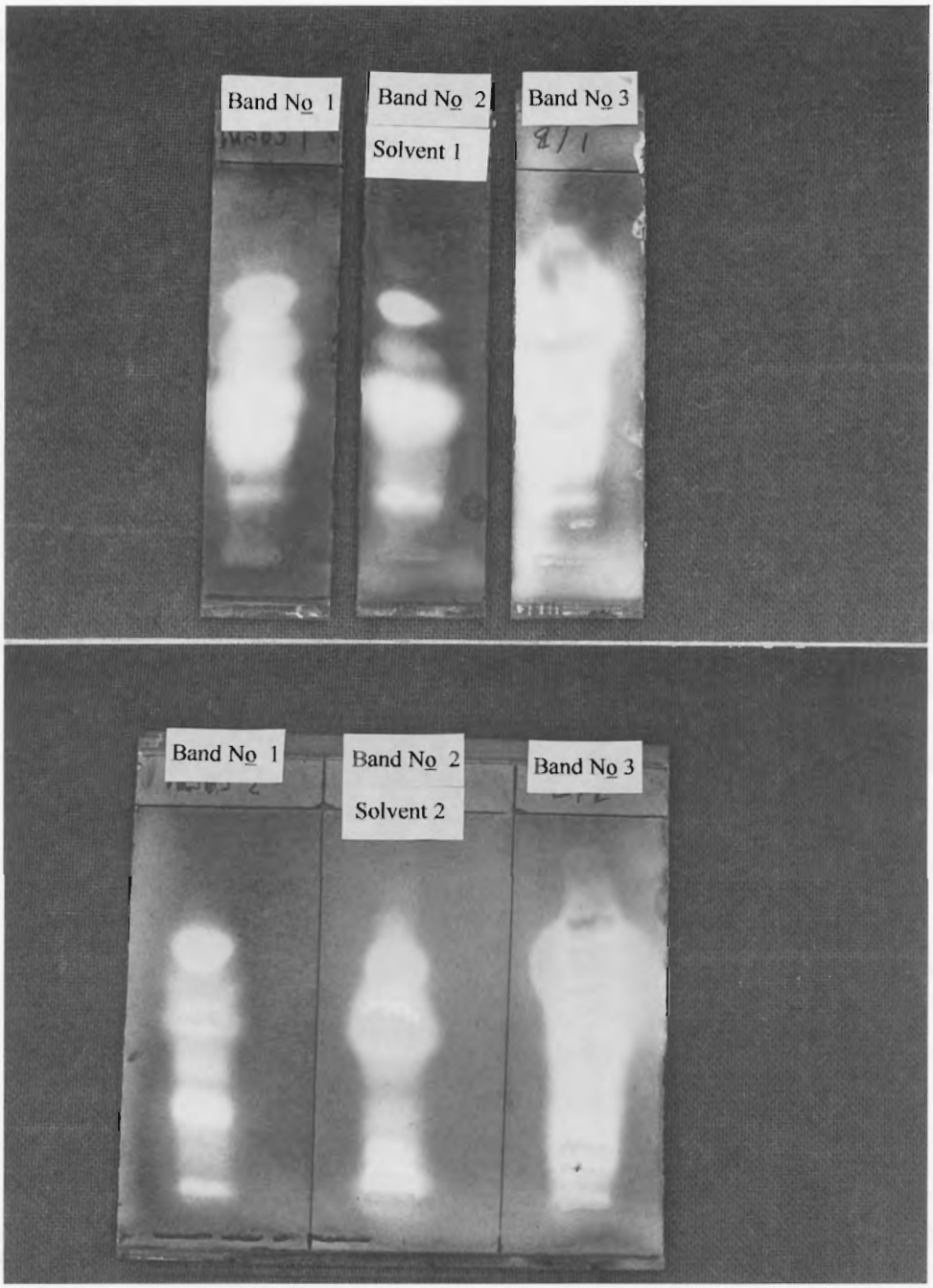

Figure 2. Second partitioning of three active antifungal bands extracted from the peel of Thongdee cultivar pomelos using two development solvents: 1. n-hexane:ethylacetate:methanol (60:40:10); 2. dichloromethane:toluene:ethylacetate (60:30:10).

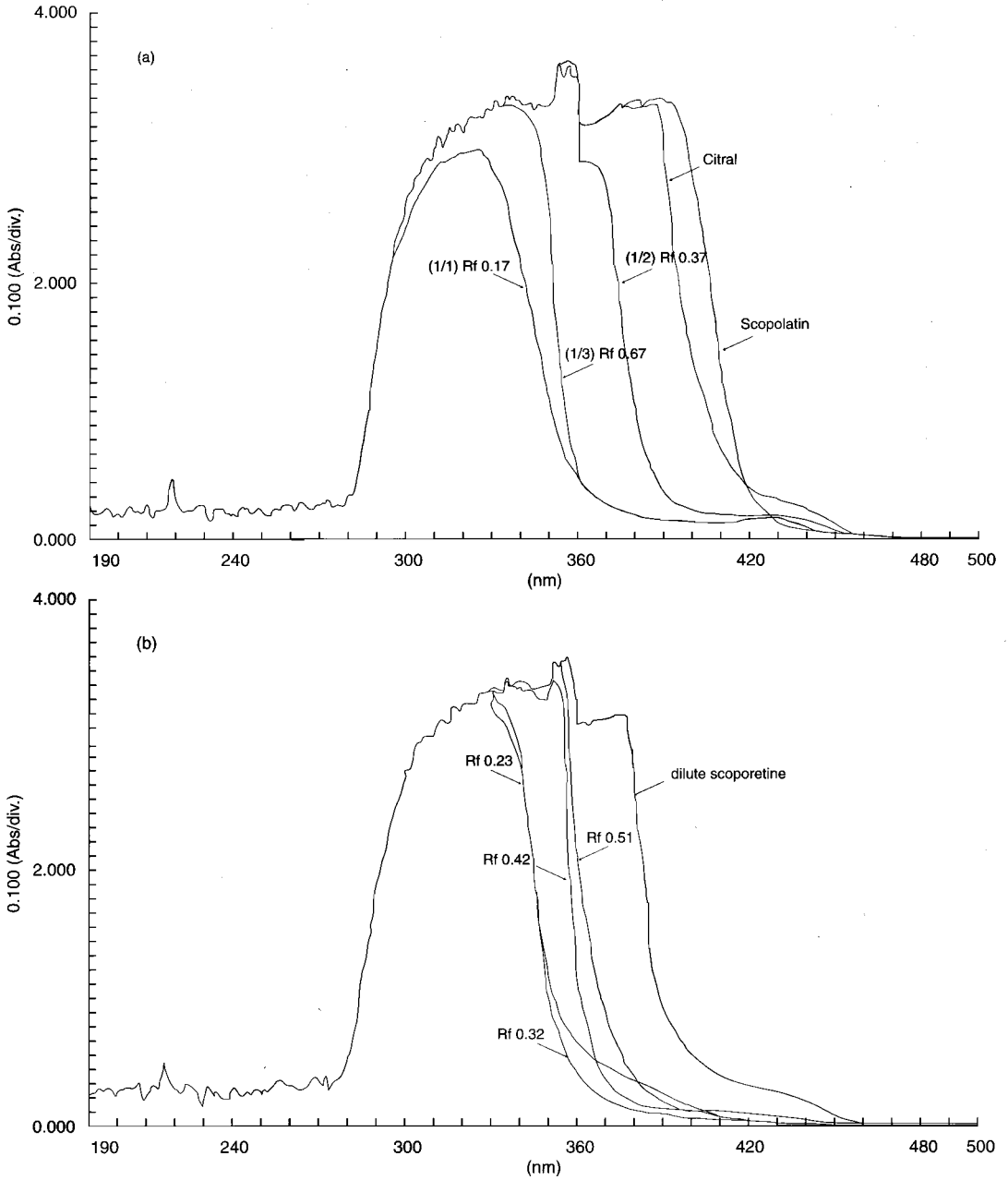

Figure 3. Absorption spectrum of the compound separated from the second partitioning of (a) band 4, Rf = 0.36, and (b) band 5, Rf = 0.45 of an extract from pomelo peel.

The third developing solvent (dichloromethane:methanol at a ratio of 90:10) was not effective in separating the extract.

When the material from the effective bands in the second partitioning was tested for spectral absorption using a UV spectrophotometer, the maximum absorbances found were similar to those for scopolatin, a derivative of coumarin. Molecular weight and structure determination using the GC–MS revealed that all bands from the second partitioning were impure, indicating a need for further purification. However, a comparison of the mass spectra showed that none of the bands was similar to that of citral. The molecular weight was also much higher.

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Participants

Australia

Dr Ray Carman
Department of Chemistry
University of Queensland
Brisbane Qld 4072
Fax: 61 7 3365 4299
Email: r.carman@chemistry.uq.edu.au

Dr Lindy Coates
Plant Protection Unit
Department of Primary Industries
80 Meiers Rd
Indooroopilly Qld 4068
Fax: 61 7 3896 9533
Email: coateslm@dpi.qld.gov.au

Mr Tony Cooke
Plant Protection Unit
Department of Primary Industries
80 Meiers Rd
Indooroopilly Qld 4068
Fax: 61 7 3896 9533
Email: cookea@dpi.qld.gov.au

Ms Melinda Gosbee
18 Meryll Ave
Baulkham Hills NSW 2153
Ph/Fax: 61 2 9639 2340
Email: emangos@ozemail.com.au

Dr David Guest
School of Botany
University of Melbourne
Parkville Vic 3052
Fax: 61 3 9347 5460
Email: d.guest@botany.unimelb.edu.au

Mr Ed Highley
Arawang Communication Group
6 Lonsdale Street
Braddon ACT 2612
Fax: 61 2 6257 7808
Email: ed@arawang.com.au

Dr Peter Hofman
Horticulture Postharvest Group
Department of Primary Industries
19 Hercules St
Hamilton Qld 4007
Fax: 61 7 3406 8663
Email: hofmanp@dpi.qld.gov.au

Dr Robert Holmes
Institute for Horticultural Development
Agriculture Victoria
Private Bag 15
South Eastern Mail Centre Vic 3176
Fax: 61 3 9800 3521
Email: holmesr@knoxy.agvic.gov.au

Dr Yong Huang
Dept of Crop Sciences
University of Sydney
NSW 2006
Fax: 61 2 9351 4172
Email: y.huang@agec.usyd.edu.au

Dr Greg Johnson
ACIAR
GPO Box 1571
Canberra ACT 2601
Fax: 61 2 6217 0501
Email: johnson@aciar.gov.au

Dr Daryl Joyce
Dept of Plant Production
University of Queensland
Gatton College
Gatton Qld 4343
Fax: 61 7 5460 1455
Email: D.joyce@mailbox.uq.edu.au

Kok Peng Yeo
40 Livermore Street
Redcliffe
Qld 4020
Tel/Fax: 07 3283 4401

Dr T.K. Lim
Dept Primary Industries and Fisheries
GPO Box 990
Darwin NT 0801
Fax:61 8 8999 2049
Email: tk.lim@dpif.nt.gov.au

Dr Brian Wild
NSW Agriculture
Horticultural Postharvest Laboratory
PO Box 581
Gosford NSW 2250
Fax: 61 43 481910
Email: wildb@agric.nsw.gov.au

Dr Chris Yuen
Department of Food Technology
University of Newcastle
Central Coast Campus
PO Box 127 (Brush Rd)
Ourimbah NSW 2258
Fax: 61 43 484155
Email: ftcmcy@cc.newcastle.edu.au

Ms Zainuri
Dept of Plant Production
University of Queensland
Gatton College Qld 4345
Fax: 61 7 5460 1455
Email: D.joyce@mailbox.uq.oz.au

China

Assoc. Prof. Chen Nianlai
Melon Research Institute
Ganshu Agricultural University
Lanzhou, Gansu 730070
Fax: 86 931 766 8010

Prof. Jun-Yan Guo
South China Institute of Botany
Academia Sinica
Guangzhou 510650
Fax: 86 20 8770 1031
Email: gzscib@public1.guangzhou.gd.cn

Prof. Ma Keqi
Melon Research Institute
Ganshu Agricultural University
Lanzhou, Gansu 730070
Fax: 86 931 766 8010

Prof. Tang Wenhua
Department of Plant Pathology
China Agricultural University
Beijing 100091
Fax: 86 10 6258 5305

Ms Jacquie Wright
Department Ecology and Biodiversity
University of Hong Kong
Pockfulam Road, Hong Kong
Fax: 852 2559 5984
Email: h9492010@hkusua,hku.hk

Prof. Xiao-Song Hu
Department of Food Science
China Agriculture University
Beijing 100091
Fax: 86 10 6258 2332

Ms Xiao-Xia Wang
Department of Food Science
China Agriculture University
Beijing 100091
Fax: 86 10 6258 2332

Israel

Ms Ilana Kobiler
Volcani Centre
Department of Fruit and Vegetable Storage
Box 6
Bet Dagan 50250
Fax: 972 3 968 3622

Dr Dov Prusky
Volcani Centre
Department of Fruit and Vegetable Storage
Box 6
Bet Dagan 50250
Fax: 972 3 968 3622
Email: vtdov@volcani.agri.gov.il

Dr Shimshon Ben-Yehoshua
Volcani Centre
Department of Postharvest Science of Fresh Produce
PO Box 6
Bet Dagan 50250
Fax: 972 3 968 3622
Email: vtsbypac@volcani.agri.gov.il

Japan

Dr Y. Homma
Department of Agricultural Chemistry
6-1-1 Tamagawa University
Tamagawa-gakuen 6-1-1
Machida-City, Tokyo 194
Fax: 81 427 39 8854

Dr Yukio Yaguchi
Electron Microscope Laboratory
Tokyo University of Agriculture
1, 1-chome, Sakuragaoka
Setagaya-ku
Tokyo 156
Fax: 81 3 5477 2636
Email: yaguchi@nodai.ac.jp

Laos

Mr Kham Sanatem
Deputy Director
Hatokkeo Agricultural Station
Department Agriculture and Extension
Vientiane

Mr Houmchitsavath Sodarak
Agriculture and Forestry Office
Luang Prabang

Malaysia

Ms Koh Siew Hua
Global Food Management Services
No 2, Jalan SS 19/6K
47500 Subang Jaya, Selangor
Fax: 60 3 254 7831
Email: siew_asean@compuserve.com

New Zealand

Dr Brian Hawthorne
HortResearch
Private Bag 92 169
Auckland
New Zealand
Fax: 64 9 815 4201
Email: BHawthorne@hort.cri.nz

Philippines

Ms Leonisa A. Artes
Postharvest Horticulture Training and Research Center
University of the Philippines
Los Baños, Laguna 4031
Fax: 63 49 536 3259

Dr Elda B. Esguerra
Postharvest Horticulture Training and Research Center
University of the Philippines
Los Baños, Laguna 4031
Fax: 63 49 536 3259

Dr M.C.C. Lizada
Postharvest Horticulture Training and Research Center
University of Philippines
Los Baños, Laguna 4031
Fax: 63 49 536 3259

Dr Casiana M. Vera Cruz
Department of Plant Pathology,
University of the Philippines
Los Baños, Laguna 4031
Fax: 63 49 536 3259

South Africa

Dr Lise Korsten
Department Microbiology and Plant Pathology
University of Pretoria
Pretoria 0002
Fax: 27 12 342 2713

Sri Lanka

Ms Charmalie Abayasekara
Department of Botany
University of Peradeniya
Peradeniya
Fax: 94 8 388018

Prof. Nimal Adikaram
Department of Botany
University of Peradeniya
Peradeniya
Fax: 94 8 388018 or 94 8 232343

Ms Anjani Karunaratne
Department of Botany
University of Peradeniya
Peradeniya
Fax: 94 8 388018 or 94 8 232343

Thailand

Kasetsart University

Mrs Nuanwan Farungsang
Central Laboratory and Greenhouse Complex
Kamphangsean
Nakhon Pathom 73140
Fax: 66 34 351 392
Email: rdinwf@nontri.ku.ac.th

Mr Udom Farungsang
Department of Plant Pathology
Faculty of Agriculture
Kamphangsean
Nakhon Pathom 73140
Fax: 66 34 351 392
Email: agrudf@nontri.ku.ac.th

Miss Ratiya Pongpisutta
Department of Plant Pathology
Faculty of Agriculture
Kasetsart University
Kamphaengsaen Campus
Nakhon Pathom 73140
Fax: 66 34 351890
Email: agrryp@nontri.ku.ac.th

Assoc. Prof. Dr Somsiri Sangchote
Department of Plant Pathology
Faculty of Agriculture
50 Jatuchuk
Bangkok-10900
Fax: 66 2 579 6152
Email: agrs@nontri.ku.ac.th

Assoc. Prof. Niphon Visarathanonth
Department of Plant Pathology
Faculty of Agriculture
50 Jatuchuk
Bangkok-10900
Fax: 66 2 579 6152
Email: agrnpv@nontri.ku.ac.th

Chiang Mai University

Dr Somboon Anantalapochai
Biology Department
Faculty of Science
Chiang Mai University
Chiang Mai 50200
Fax: 66 53 222268

Dr Danai Boonyakiat
Horticulture Department
Faculty of Agriculture
Chiang Mai University
Chiang Mai 50200
Fax: 66 53 223127

Mr Surasak Chanchumni
The Royal Project
Faculty of Agriculture
Chiang Mai University
Chiang Mai 50200
Fax: 66 53 223127

Mr Parinya Chantrasri
Institute for Science and Technology Research and Development
Chiang Mai University
Chiang Mai 50200
Fax: 66 53 892188

Ms Wilawan Kumpoun
Institute for Science and Technology Research and Development
Chiang Mai University
Chiang Mai 50200
Fax: 66 53 892188

Ms Thongmai Phatchaiyo
The Royal Project
Faculty of Agriculture
Chiang Mai University
Chiang Mai 50200
Fax: 66 53 223127

Mr Sawat Promin
Plant Pathology Department
Faculty of Agriculture
Chiang Mai University
Chiang Mai 50200
Fax: 66 53 225221

Dr Nithiya Ratanapanont
Food Science and Technology Department
Faculty of Agroindustry
Chiang Mai University
Chiang Mai 50200

Dr Uraporn Sardsud
Biology Department
Faculty of Science
Chiang Mai University
Chiang Mai 50200
Fax: 66 53 222268

Dr Vicha Sardsud
Plant Pathology Department
Faculty of Agriculture
Chiang Mai University
Chiang Mai 50200
Fax: 66 53 225221
Email: agppi002@chiangmai.ac.th

Dr Chatree Sittigul
Plant Pathology Department
Faculty of Agriculture
Chiang Mai University
Chiang Mai 50200
Fax: 66 53 225221

Dr Jinda Sornsrivichai
Biology Department
Faculty of Science
Chiang Mai University
Chiang Mai 50200
Fax: 66 53 222268

Dr Damrat Supyen
Chemistry Department
Faculty of Science
Chiang Mai University
Chiang Mai 50200

Dr Jamnong Uthaibutra
Biology Department
Faculty of Science
Chiang Mai University
Chiang Mai 50200
Fax: 66 53 892259
Email: scboi015@chiangmai.ac.th

Maejo University

Sompoch Gomolmanee
Department of Postharvest Technology
Faculty of Engineering and Agricultural Industries
Maejo University
Chiang Mai 50290
Tel: 66 53 878117
Email: sompoch@maejo.mju.ac.th

Yongyut Khamsea
Department of Postharvest Technology
Faculty of Engineering and Agricultural Industries
Maejo University
Chiang Mai 50290
Tel: 66 53 878117
Email: sompoch@maejo.mju.ac.th

Other Thailand

Ms Pakinee Akkaravessapong
Sisaket Horticultural Research Centre
Muang
Sisaket 33000
Fax: 66 45 611156

Dr Piyawat Boon-Long
Thailand Research Fund
18th Floor, Gypsum Metro Tower
539/2 Sri-Ayudhya Rd Rajthevi
Bangkok 10400
Fax: 61 2 642 5190

Rachit Chuthakorn

Mr Alastair Hicks
Regional Office for Asia and the Pacific
Food and Agriculture Organization of the United Nations
Maliwan Mansion
Phra Atit Road
Bangkok 10200

Mr Surachart Kooariyakul
Chiang Rai Horticultural Research Centre
Department of Agriculture
Muang District
Chiang Rai Province 57000
Fax: 66 53 7141023

Mr Clive Murray
Thai Agrotech
754/80 Mooban Rangsiya
Sukhumvit Soi 101
Phrakanong Bangkok
Fax: 66 2 741 3518

Sombat Srichuwong

Dr Anawat Suwanigul
Postharvest Technology Laboratory
Food Industry Department
Thailand Institute of Scientific and Technological Research
196 Phahonyothin Rd
Chatuchak
Bangkok 10900
Fax: 66 2 561 4771
Email: somsakc@mozart.inet.co.th

Mrs Sing Ching Tongdee
Postharvest Technology Laboratory
Food Industry Department
Thailand Institute of Scientific and Technological Research
196 Phahonyothin Rd
Chatuchak
Bangkok 10900
Fax: 66 2 561 4771

USA

Dr Ahmed El Ghaouth
USDA Appalachian Fruit Research. Station
45 Wiltshire Road
Kearneysville, WV 25430
Fax: 1 304 728 2340
Email: ghaouth@asrr.arsusda.gov

Dr John Labavitch
Pomology Department
University of California
Davis CA95616
Fax: 1 916 752 8502
Email: jmlabavitch@ucdavis.edu

Vietnam

Mr Mai Van Tri
Plant Protection Department
Long Dinh Fruit Research Center
Box 203
My Tho
Tien Giang

Ms Le Thi Yhu Hong
Division of Research Management and International Cooperation
Long Dinh Fruit Research Center
Box 203
My Tho
Tien Giang