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ASSESSMENT OF GENETIC AND CHEMICAL CONTROL OF BACTERIAL SPOT IN BARBADOS

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ABSTRACT: Research conducted on the bacterial spot disease caused by *Xanthomonas campestris* pv. *vesicatoria* on tomato, bell pepper and hot pepper in Barbados assessed prospects for genetic and chemical control of the disease on the island. Race and chemical resistance profiles of the pathogen, effectiveness of deployment of bacterial spot resistance genes in the field and the genetic basis for pathogen population changes with respect to pathogenicity and sensitivity to copper bactericides were examined.

Twenty-four races of *X. campestris* pv. *vesicatoria*, 20 of which were previously unreported on bell pepper and tomato in Barbados, were identified. Race specific host selection, mutation and exchange of genetic material are postulated to explain the evolution of the pathogen population in Barbados. The research also reported the disease on hot pepper for the first time, with the most abundant bacterial races isolated being capable of overcoming resistance conferred by most available bacterial spot resistance genes in bell pepper.

Chemical resistance profiles of isolates of *X. campestris* pv.*vesicatoria* indicated that 18.3 and 78.1 percent of the population were resistant to copper and zinc, respectively.

Differentially resistant bell pepper and tomato were shown to succumb to the disease at significantly lower frequencies than susceptible cultivars grown alongside them.

Copper resistance of *X. campestris* pv. *vesicatoria* was generally associated with the presence of a 0.6-0.9 kb plasmid which was transferable between strains. Reversion from copper resistance to sensitivity occurred at high frequencies in the absence of copper.

The successful management of bacterial spot will require a multi-faceted approach including deployment of mixtures of resistance genes and exploitation of the bactericide resistance status of individual pepper and tomato fields.

INTRODUCTION

Bacterial spot of pepper and tomato is caused by *X. campestris* pv. *vesicatoria* and is of economic importance worldwide. Prolonged use of the bactericidal chemicals for bacterial spot control has resulted in increased incidence of resistance in the pathogen population to such bactericides. The situation is further complicated by the frequent appearance and subsequent spread in various locations of new and/or exotic races of the pathogen, a situation which continuously poses a challenge for genetic control of the disease.

The bacterial spot pathogen has been differentiated into three pathogenic groups, namely the pepper, tomato and pepper-tomato groups. Pepper group strains affect pepper but not tomato whereas tomato group strains affect tomato but not pepper; strains of the pepper-tomato group

affect both pepper and tomato (Reifschneider et al. 1985; Minsavage et al. 1990; Ward and O'Garro 1992; O'Garro and Tudor 1994; Jones et al. 1995; Kousik and Ritchie 1995; Sahin and Miller 1995, 1996, 1998).

Within each group specific physiologic bacterial races are designated on the basis of virulence on differential tomato and pepper lines. Three common tomato races, namely T1, T2 and T3, have been differentiated on tomato cultivars carrying various or no resistance genes (Scott and Jones 1986; Yu et *al.* 1992; Jones et *al.* 1995). Nine common pepper group races (P0 to P8) have thus far been identified based on interaction with pepper cultivars carrying genes *Bs1*, *Bs2* and *Bs3* (Minsavage et al. 1990; Sahin and Miller 1995, 1996). Gene *Bs1* restricts growth of races P0, P2 and P5 whereas gene *Bs2* confers resistance to races P0, P1, P2 and P3. Gene *Bs3* restricts races P0, P1 and P4 (Minsavage et al. 1990; Kousik and Ritchie 1995, 1996). Race P6 overcomes resistance conferred by *Bs1*, *Bs2* and *Bs3* (Sahin and Miller 1995, 1996). Numerous pepper-tomato group races, each designated by the prefix PT, have been reported. These races are differentiated on the basis of the combination of compatible and incompatible responses on the sources of bacterial spot pepper and tomato resistance resistance.

Pathogen strains may have multiple avirulence genes and it is the inheritance of these genes which determines physiologic race type. Avirulence genes may be located on chromosomes or plasmids (Vivian and Gibbon 1997; Vivian et al. 2001). Plasmid-borne avirulence loci may be lost or acquired by bacteria due to changes in plasmid profile which may influence pathogen ability to cause disease on particular host plants (Chaterjee and Vidaver 1986; Minsavage et al. 1990a; Vivian et al. 2001)

Much of the work done on the bacterial spot disease of pepper and tomato in the Caribbean has been carried out in Barbados. The first study, conducted during the period 1985-1987, reported a prevalence of races P1 and T1 (O'Garro and Ward 1989). Two years later, three additional races, namely T2, P2T1 and P3T1 became common (Ward and O'Garro 1992). Another study conducted in the period 1990-1991 reported, for the first time, unspecified race(s) of *X. campestris* pv. *vesicatoria* capable of overcoming gene *Bs2* (O'Garro and Tudor 1994). Race classification of 24 Barbadian strains of the pathogen by Jones et *al.* (1994) indicates the presence of races P0T1, P1T1 and P4T1.

It has been proposed that durability of disease resistance genes can be improved by reducing genetic homogeneity of crops exposed to pathogen populations (Kousik and Ritchie 1996a; O'Garro 1998). Cultivating a mixture of host cultivars differing in disease resistance creates a heterogenous crop. Such a crop is expected to reduce disease resistance development mainly by barrier effects through which compatible pairs of host cultivars and pathogen races and effective against otherwise compatible races (Kousik and Ritchie 1996).

Until the current study, no bacterial spot resistance genes have been deployed and investigated for durability in the field in Barbados. The current study assesses the effect of deploying susceptible and differentially disease resistant pepper and tomato varieties on bacterial spot, including pathogen race structure in the field.

Chemical control of bacterial spot of pepper and tomato has generally relied on sprays of streptomycin and copper and zinc compounds (Sahin and Miller 1996; Ward and O'Garro 1992;; O'Garro and Ward 1989; Marco and Stall 1983). From inception, standard methods of bacterial spot control in Barbados utilized sprays of copper and/or zinc formulations (O'Garro and Ward 1989; Ward and O'Garro 1992). Most copper-resistant strains of the pathogen carry plasmids encoding copper-resistance.

METHODOLOGY

Selection of bacterial spot infested fields

Bacterial spot infested bell pepper (*C. annuum*), hot pepper (*C. chinense*) and tomato farms located at National Hatcheries, Rices, Heddings, Wiltshire, Wilcox, Graeme Hall and Sargeant's Village in Barbados were selected for study during the annual rainy season from 1993 to 1996.

Assessment of bacterial spot severity

Leaves and fruits were collected at harvest from a random sample comprising 5 percent of plants of each crop and used to assess bacterial spot severity by determination of the surface area of foliage and fruit covered by typical bacterial spot lesions as previously described (O'Garro and Tudor 1994). Analysis of variance was performed on the mean lesion sizes obtained for all races or groups of races isolated from each lesion and the significance of any difference between means determined by Tukey's significant difference test. Data were collected from 1997 to 2001 during the annual rainy season.

Isolation of Pathogen

Bacterial spot-infested pepper and tomato fruit and leaves were first washed in tap water to remove soil debris and then air-dried. Individual lesions were then excised, surface-disinfected by first dipping in ethanol (95 percent) for 2 seconds followed by sodium hypochlorite (1.25 percent) for 10 seconds, rinsed twice in sterile distilled water (SDW), and then homogenised in SDW (200ml) in an ethanol-sterilised mortar and pestle to form a suspension as previously described (Ward and O'Garro 1992). Loopsful of the homogenate were streaked onto Tween A agar (McGuire et *al.* 1986) for isolation of the bacterial spot pathogen. Three or five presumptive colonies of *X. campestris* pv. *vesicatoria* were randomly selected from each lesion and subcultured onto NA or NYGA at 25-28 ^{o}C for three to five days.

Identification of pathogen

The presumptive pathogen was identified by testing for xanthomonad-determinative characteristics including cell morphology and Gram-reaction. All isolates were tested also for activity of catalase, oxidase, urease and nitrate reductase, acid production from carbohydrates including D-glucose, D-mannose, L-arabinose and utilisation of citrate (Schaad and Stall 1988). In addition, 109 randomly-selected isolates were tested for the presence of xanthomonadin pigments (Irey and Stall 1982). Identity of the pathogen was confirmed by testing all strains for pathogenicity on the bacterial spot susceptible pepper and tomato cultivars, Early Calwonder (ECW) and Walter, respectively, as previously described (O'Garro and Tudor 1994).

Preparation of inoculum

Suspensions for inoculations were prepared by growing the bacterium overnight in NYGB or NB at 25-28^{o}C on a rotary shaker at 160 rpm. Cells were harvested by centrifugation at 7000g for ten minutes at 4^{o}C, washed twice in SDW and then resuspended in SDW to give inocula of concentration 5×10^{8} cfu ml^{-1}.

Determination of pathogen races

Tomato cultivars Walter, Hawaii 7998 and Hawaii 7981 and the near-isogenic pepper cultivars ECW, ECW10R, ECW20R and ECW30R were used to differentiate *X. campestris* pv. *vesicatoria* into races. ECW10R, ECW20R and ECW30R contain genes *Bs1*, *Bs2* and *Bs3*, respectively, for bacterial spot pepper control (Hibberd et *al.* 1987; Minsavage et *al.* 1990; Jones et al. 1995). ECW and Walter contain no genes for resistance to bacterial spot of pepper and tomato, respectively (Hibberd et *al.* 1987; Minsavage et *al.* 1990; Jones et *al.* 1995).

Fully expanded leaves of four-week-old plants were infiltrated with a bacterial suspension (10^8 - 10^9 cfu ml^{-1}) and observed for appearance of the compatible response or hypersensitivity as previously described (Hibberd et *al.* 1987; Minsavage et *al.* 1990; O'Garro and Tudor 1994). The responses were used to classify the bacterium into races (Minsavage et al. 1990; Bouzar et *al.* 1994; Kousik and Ritchie 1995; Kousik et al. 1996, 1998). Three or five strains of the bacterium from each lesion were tested in duplicate. The experiment was conducted twice.

Assessment of pathogen sensitivity to copper and zinc

A total of 2091 isolates of *X. campestris* pv. *vesicatoria* was assessed for sensitivity to zinc and copper. Bacterial suspensions (10^8 - 10^9 cells ml^{-1}) prepared in filter-sterilised aqueous solutions of $CuSO_4.5H_2O$ or $ZnSO_4.7H_2O$ were spotted in 5-10□l amounts in duplicate onto NA or NYGA after 2 - 4 hours' incubation in the presence of the corresponding chemical at 25-28°C. Bacterial isolates producing confluent growth on NA or NYGA after three to five days' in the presence of bactericides at concentrations of at least 200 □g ml^{-1} were considered resistant to the respective chemicals (Marco and Stall. 1983; Adaskaveg and Hine 1985). Each test on strains was replicated twice and the experiment was repeated twice.

Assessment of effect of deploying susceptible and differentially disease resistant pepper and tomato varieties on bacterial spot

In 2000, farms located at National Hatcheries and Wilcox were selected to test the effect of deploying susceptible and differentially resistant pepper and/or tomato on bacterial spot severity and race structure of *X. campestris* pv. *vesicatoria*. One site each at National Hatcheries and Wilcox was used for evaluating bacterial spot of pepper. The sites were previously cultivated with Calwonder 300TMR (ECW) which is susceptible to bacterial spot of pepper. Each site was divided into two plots, one of size 0.10 hectares (ha) and the other 0.30 ha. The smaller plot was cultivated with ECW only and the other with a mixture of equal amounts of ECW, ECW10R and ECW30R in random distribution.

Bacterial spot of tomato was also assessed at sites of reported infestation of Calypso with *X. campestris* pv. *vesicatoria* (Gore and O'Garro 1999). One site each at National Hatcheries and Wilcox was similarly sub-divided as described above for bacterial spot pepper assessment. The 0.10 ha plot was planted with Calypso only and the other cultivated by intercropping randomly with equal amounts of Calypso, H7998, Campbell 28 and 913214SBK representing differentially resistant cultivars.

Bacterial spot assessments utilized pepper and tomato crops established by planting 4-week-old seedlings in soil beds prepared as rows. Assessment in 2001 was repeated using different

bacterial spot infested sites of the corresponding farms at National Hatcheries and Wilcox. All experiments were conducted in the annual rainy season.

The yield of marketable fruit produced by ECW30R, Campbell-28, Hawaii 7998 and 913214SBK was assessed also at harvest by weighing fruits from each crop.

Assessment of bacterial spot on hot pepper

Until recently, bacterial spot of *C. chinense* was not observed in Barbados. Over the period September 1995 to March 1996, fruits from a commercial hot pepper field at National Hatcheries were harvested from a random sample of 0.5 percent of plants and examined for bacterial spot lesions. The sizes of typical lesions and *X. campestris* pv. *vesicatoria* races associated with them were determined and used to assess the contribution of each race to bacterial spot on hot pepper.

Susceptibility of *C. chinense* cultivar West Indian Red to several races of *X. campestris* pv. *vesicatoria* was assessed by determination of electrolyte leakage from leaf discs. The extent of electrolyte leakage elicited was determined at 4-hourly intervals for 24 hours as the conductivity produced by two leaf discs, each of area 20 mm^2, in deionised water (3 ml), as previously described by Walkes and O'Garro (1996).

Determination of genetic basis of pathogen race change and variation in response to copper

Plasmid profiles of copper resistant and copper sensitive strains were compared in order to detect plasmids which may be uniquely associated with resistant strains. Plasmid DNA from copper resistant and sensitive strains of *X. campestris* pv. *vesicatoria* was extracted using a modified version of the salt extraction method described by Kado and Liu (1981). Pulse field gel electrophoresis was carried out in 0.5X TBE buffer (0.045M Tris-borate, 0.001M EDTA) at 14°C using a CHEF-DR II electrophoresis apparatus (Biorad) set to provide constant current of 75 -100 mA and switching times varying from, for example, 60 or 75 seconds for 15 hours followed by 75 or 90 seconds for 8 hours. Separation was achieved on 0.8 to 1.5 percent agarose gels. A 50 base-pair □ step ladder, a □EcoR1 digest and/or pulsed field electrophoresis markers in the size ranges of 0.1-200 kb and 50-1000 kb [Sigma Chemical Co.] were used as size standards. Fractionated DNA was stained with ethidium bromide and visualised with ultraviolet illumination. The plasmid profiles of copper-resistant and copper-sensitive strains of *X. campestris* pv. *vesicatoria* were compared.

Assessment of stability of copper-resistance and tests for race change

The basis of the loss of copper resistance was investigated also by comparing profiles of copper resistant strains and copper sensitive variants derived from them. The possibility that change in response to copper may be associated with race change was investigated also. The assessment was conducted during prolonged bacterial culture in NYGB at 37°C, and *in planta,* and during storage in tap water and soil.

Conjugation experiments

The association between changes in response to copper and race type was investigated further by conjugation experiments. Donor strains were resistant to copper (200□g ml^{-1} $CuSO_4.5H_2O$) and

sensitive to nalidixic acid (30□g ml^{-1}) whereas recipient strains were sensitive to copper but resistant to nalidixic acid at the appropriate chemical concentrations mentioned.

RESULTS AND DISCUSSION

A total of 2186 isolates of *Xanthomonas campestris* pv. *vesicatoria* in Barbados were isolated from susceptible pepper and tomato and hot pepper and differentiated into 24 physiological races and three pathogenic groups. Of these races, 24 were detected on tomato, 22 on bell pepper and 9 on *Capsicum chinense* (Table 1). Important to note is the appearance of previously unreported races capable of overcoming resistance conferred by gene *Bs2* which offered resistance to the majority of pathogen races previously isolated from the population. A significant finding also is the isolation for the first time of the bacterial spot pathogen from hot pepper and the fact that the majority of races selected by this host, usually grown in rotation with tomato and bell pepper, defeated most of the available bacterial spot resistance genes. Electrolyte leakage patterns (not shown) obtained from inoculation of hot pepper variety, *C. chinense* cv. West Indian Red, confirmed this finding in that this host plant was able to resist disease caused by races of *X. campestris* pv. *vesicatoria* which did not possess the *Bs2* avirulence gene but was unable to resist the disease when infested with strains that did possess this gene.

With respect to the severity of the disease on the solanaceous host plants being assessed, the largest lesions were found on tomato and the smallest on hot pepper (Table 2). Severe enough damage to the fruit could render them unmarketable. Generally, the most abundant pathogenic races incited the largest lesions (data not shown).

The effect on disease severity and pathogen race structure of the deployment into the field of pepper and tomato cultivars carrying varied and no bacterial spot resistance genes when compared to the effect of the disease when susceptible plants were grown as monocultures was a significant reduction in the effect on susceptible pepper and tomato cultivars in heterogenous crops (Table 3).

Table 1. Race structure of *Xanthomonas campestris* pv. *vesicatoria* population on susceptible pepper and tomato in Barbados

Host	No. isolates tested	No. physiological races	Most abundant races	Frequency (%)
Tomato	1476	24	T1	16.7
			P1T1	15.9
			T2	12.0
			P1T2	9.8
Pepper	561	22	P1T1	17.3
			P1T2	12.5
			P3T1	9.1
			P6T1	7.3
Hot pepper	149	9	P4T2	44.2
			P4	19.0
			P6T2	13.6
			P1T2	6.8

Table 2. Bacterial spot severity on susceptible pepper and tomato in Barbados

Host	No. lesions assessed	Lesion size range (mm^2)	Mean lesion size (mm^2)
Tomato	580	0.9 – 38.0	10.3
Pepper	132	0.9 – 15.1	4.4
Hot pepper	56	0.1 – 5.4	1.4

Table 3. Effect of *Xanthomonas campestris* pv. *vesicatoria* on susceptible pepper and tomato grown as monocultures or as multiline crops measured as disease severity and number of races associated with the crops

Farm		Pepper crops				Tomato crops				
		monoculture	Multiline crop			monoculture	Multiline crop			
		ECW (susceptible)	ECW (susceptible)	ECW10R (*Bs1*)	ECW30R (*Bs3*)	Calypso (susceptible)	Calypso	Campbell 28	913214 SBK	Hawaii 7998
NH	*No. races*	18	11	9	14	22	19	10	3	0
	Disease severity[x,y]	0.57a	0.21b	0.26b	0.12c	0.52a	0.16a	0.14b	0.13b	0
WX	*No. races*	21	7	4	2	20	15	5	3	0
	Disease severity	0.73a	0.35b	0.41b	0.10c	0.99a	0.30b	0.22b	0.41b	0

x – disease severity measured as mean lesion size in mm^2

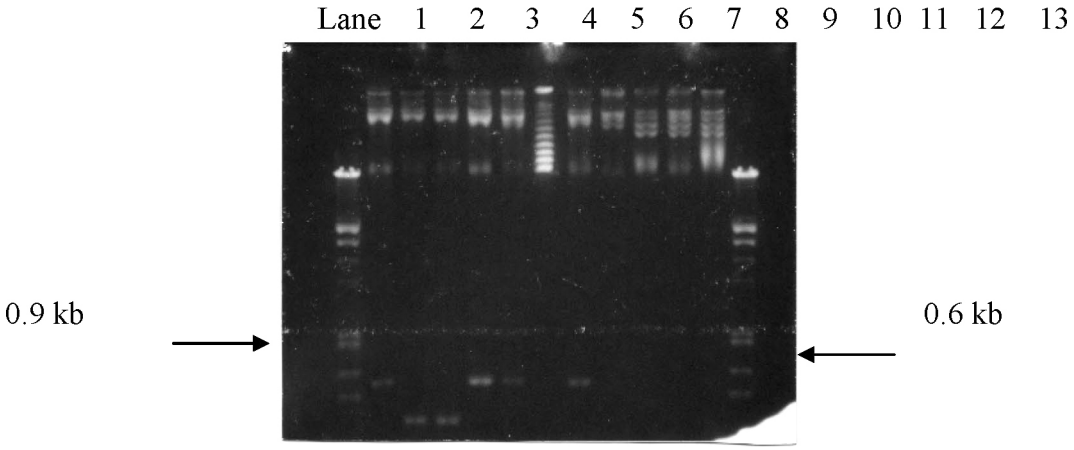
y – lesion sizes followed by the same letter within each host/cultivar combination are not significantly different ($P>0.05$) based on Tukey's significant difference test

NH: National Hatcheries; WX: Wilcox

In addition to being less susceptible than conventional varieties to bacterial spot, differentially resistant pepper and tomato cultivars assessed for yield of marketable fruit compared well with conventional varieties. Though the fruit of tomato cultivar Hawaii 7998 was small, the variety was extremely prolific, yielding 1.31 kg of marketable fruit per plant, and was not found to succumb to the disease in the field. Tomato cultivars Campbell-28 and 913214SBK yielded 0.81 kg and 0.95 kg of marketable fruit per plant, with the fruit of the former being compact with an attractive deep red colour. Neither fruit was found to crack on ripening as occurred with conventional varieties grown alongside them. Pepper cultivar ECW30R yielded 0.13 kg, comparable to that of susceptible varieties. In general, the resistant tomato and pepper varieties performed better than susceptible ones in the rainy season when the incidence of bacterial spot is highest on varieties with no resistance to the disease.

Assessment of the response to copper and zinc-based bactericides of the bacterial spot pathogen population in Barbados showed 18.3 and 78.1%, respectively, of strains tested to be resistant. A total of 16.9% of strains were resistant to both copper and zinc. Plasmid profiles of copper-resistant and sensitive *X. campestris* pv. *vesicatoria* isolates showed all strains to contain at least 3 – 4 large plasmids of sizes ranging from 50 – 400 kb in size (Figure 1). In the majority of cases, copper-resistant strains carried a 0.6 or 0.9 kb plasmid which was not present in copper-sensitive strains. Conjugation experiments confirmed that transfer frequencies of these miniplasmids occurred at frequencies varying from 2.4×10^{-7} to 4.7×10^{-6}.

Figure 1. Plasmid DNA profile of both copper-resistant and copper-sensitive strains of *Xanthomonas campestris* pv. *vesicatoria* obtained using pulsed field gel electrophoresis. Lanes 1 and 13, lambda EcoRIHindIII DNA marker; lanes 2 to 6, copper-resistant strains; lane 7, CHEF pulse marker (50-1000 kb); lane 8, copper-resistant strain; lanes 9 to 12, copper-sensitive strains.



Copper-based bactericides appear to have utility for bacterial spot control on account of the instability in the copper-resistant trait observed in *X. campestris pv. vesicatoria* (O'Garro and Durant 1993). Isolates of the bacterial spot pathogen lost resistance to copper at high frequencies, particularly when maintained in soil (Table 4). Loss of copper resistance was also shown to cause race change in isolates losing resistance and has implications for management of the disease by the selective deployment of disease resistance genes. However, the possibility exists that this genetic and chemical instability could be exploited for disease control. In the ideal situation, pepper and tomato fields infested predominantly with copper-resistant strains of the bacterium would be removed from treatment with copper bactericides, thus allowing strains to revert to sensitivity. Copper bactericides would then be used for control of bacterial spot caused by copper-sensitive strains of the pathogen. This approach to chemical control of the bacterial spot disease would enhance the effectiveness of chemical control and reduce the cost of chemical control and the release of toxic compounds into the environment.

Table 4. Effect of copper resistance status of *Xanthomonas campestris* pv. *vesicatoria* under varied conditions

Effect	Growth condition			
	In planta	Tap water	High temperature (37°C)	Soil
Reversion frequency (%) [loss of copper resistance]	9.5	15.0	0.8	40.0
Reversion frequency (%) [gain of copper resistance]	0	0	0	0
Race change(s) observed	none	P4 to P6 (93% of isolates)	none	P5T2 to P4T1, P6T1 or P6T2

As a viable alternative to high input cultivation of pepper and tomato crops, farmers should strive to adopt an integrated pest management approach. Solanaceous crops should only be rotated with other solanaceous crops at a low frequency if this practice cannot be totally avoided due to land availability. All seeds should be treated to remove infesting pathogens prior to sowing and,

where possible, soils should be solarized to reduce inoculum surviving between crops prior to planting. In place of sole reliance on the traditional copper or zinc sprays, bactericides with a variety of modes of action should be considered and rotated. More attention should be paid to the nutrition of the crops. Presently, farmers in Barbados need to adopt techniques in the application of stage-specific fertilizers and other agrochemicals according to the manufacturer's recommendations. The more robust the plant, the more likely it is to resist attack by pathogens in the environment.

With the present negative effects of excessive chemical use on the environment, cultural methods should be adopted by the farmer. Any practice which results in a decreased dependence on chemical control methods should be considered. Such methods would invariably aid the farmer in reducing inputs and maximizing output.

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