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Working with Mycorrhizas in Forestry and Agriculture

Mark Brundrett,
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MEASUREMENTS AND STANDARDS

ECM = ectomycorrhiza, ectomycorrhizal, ectomycorrhizas

VAM = Vesicular-arbuscular mycorrhiza (-s, -l), arbuscular mycorrhizas, Glomalean mycorrhizal

SI units are used throughout this book.

Unless otherwise stated, water is the solvent used to make solutions.

v/v = volume/volume; measure volume of solute and adjust final volume of solvent (i.e. 10% v/v of A in B would be 10 mL of solution A in sufficient B to make 100 mL of solution).

w/v = weight/volume; measure weight of solute and adjust final volume of solvent (i.e. 10% w/v of A in B would be 10 g of A dissolved in enough of B to make 100 mL of solution).

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SYMBOLS


Camera


Fixation


Microscope


Clearing


Forceps


Notes


Cultures


Glasshouse


Roots


ECM fungi


Growth


Sectioning


ECM roots


Lab


Seedling


ECM spores


Leaf


Spore print


Important point



Measuring


Transfer


Field trials


Dissecting microscope


VAM spores

PREFACE

This manual was initially prepared for an ACIAR-sponsored International Mycorrhizal Workshop in Kaiping, China in 1994. The main focus of this workshop was to promote the use of mycorrhizal fungi for eucalypt plantation forestry in China, but other mycorrhizal research topics were presented. This document was created to provide explanations of the main procedures employed by mycorrhizal scientists to work with mycorrhizal fungi and roots in the laboratory, nursery or natural and managed ecosystems. The targeted audience of this manual includes managers of forest nurseries and plantations, horticulturalist and agronomists, as well as students and scientific researchers. Practical applications of mycorrhizal research in forestry and agricultural situations, especially using Australian trees and fungi, in the Asian Region have been emphasised. Diagrams and recipes were designed to contain sufficient information to allow these procedures to be used without additional information or prior experience. In this manual, emphasis has been placed on techniques which require only readily obtainable and relatively inexpensive equipment and chemicals. We expect that this manual will be especially helpful to scientists who have limited access to scientific literature. We have also tried to restrict methods presented to those we have successfully used ourselves and have endeavoured to reproduce instructions and formulas as clearly an concisely as possible.



Chapter 1

INTRODUCTION

1.1. MYCORRHIZAL ASSOCIATIONS

Mycorrhizas are highly evolved, mutualistic associations between soil fungi and plant roots. The partners in this association are members of the fungus kingdom (Basidiomycetes, Ascomycetes and Zygomycetes) and most vascular plants (Harley & Smith 1983, Kendrick 1992, Brundrett 1991). In the mycorrhizal literature, the term symbiosis is often used to describe these highly interdependent mutualistic relationships *where the host plant receives mineral nutrients while the fungus obtains photosynthetically derived carbon compounds* (Harley & Smith 1983, Harley 1989).

A. Association types

At least seven different types of mycorrhizal associations have been recognised, involving different groups of fungi and host plants and distinct morphology patterns (Table 1.1). The most common associations are:

1. vesicular-arbuscular mycorrhizas (VAM) — in which Zygomycete fungi produce arbuscules, hyphae, and vesicles within root cortex cells,
2. ectomycorrhizas (ECM) — where Basidiomycetes and other fungi form a mantle around roots and a Hartig net between root cells,
3. orchid mycorrhizas — where fungi produce coils of hyphae within roots (or stems) of orchidaceous plants,
4. ericoid mycorrhizas — involving hyphal coils in outer cells of the narrow 'hair roots' of plants in the plant order Ericales, and
5. ectendo-, arbutoid and monotropoid associations which are similar to ectomycorrhizal associations, but have specialised anatomical features (Table 1.1).

Only ECM and VAM associations will be considered in this manual.

Table 1.1. Types of mycorrhizal associations (after Harley & Smith 1983).

	VAM	*ECM*	*Ectendo-*	*Arbutoid*	*Monotropoid*	*Ericoid*	*Orchid*
Root structures							
Septate hyphae	– (+)	+ –	+ –	+	+	+	+
Hyphae in cells	+	– (+)	+	+	+	+	+
Hyphal coils	+ –	–	+	+	–	+	+
Arbuscules	+	–	–	–	–	–	–
Fungal sheath	–	+ (–)	+ (–)	+	+	–	–
Hartig net	–	+	+	+	+	–	–
Vesicles	+ –	–	–	–	–	–	–
Host plants	Vascular plants	Gymnosperms & Angiosperms		Ericales	Mono-tropaceae	Ericales	Orchidaceae
Plant has chlorophyll	+	+	+	+ –	–	+	+ –
Fungi	Zygo-Glomales	Most Basid-, but some Asco- and Zygo-				Asco- (Basid-)	Basid-

Notes: – = absent, + = present, (+) = sometimes present, (–) = sometimes absent, +– = present or absent, Basid- = Basidiomycetes, Asco- = Ascomycetes, Zygo = Zygomycetes.

B. Host plants

Field surveys have found that plants with mycorrhizal associations predominate in most natural ecosystems in Australia and throughout the world (Brundrett 1991, Brundrett & Abbott 1991, Brundrett et al. 1994). VAM are the most prevalent of these associations in Australian plant communities, but trees and plants with ECM associations are important in most habitats, and non-mycorrhizal species such as members of the Proteaceae are also often common (Table 1.2). However, there is still much to learn about the fungal associations of Australian plants, including species which are economically important.

Table 1.2. Mycorrhizal associations of some economically important families and genera of woody Australian plants (Turnbull 1986). Data from Brundrett & Abbott (1991), Brundrett et al. (1995) and the additional references which are given.

Family *Genus*	*Mycorrhizal association*	*Notes and additional references:*
Anacardiaceae		
Buchanania	VAM	
Caesalpiniaceae		N-fixing nodules
Cassia, Erythrophleum, Senna, Labichea	VAM	
Casuarinaceae		N-fixing nodules
Allocasuarina,	ECM/VAM	Theodorou & Reddell (1991), Thoen
Casuarina	VAM (ECM)	et al. (1990), Dell et al. (1994)
Combretaceae		
Terminalia	VAM	
Euphorbiaceae		
Petalostigma	VAM	
Fabaceae		N-fixing nodules
Most genera	VAM	VAM most common, but many also have
Gompholobium, Jacksonia, Mirbelia,	ECM/VAM	dual ECM/VAM associations and a few
Oxylobium, etc.		are nonmycorrhizal
		Kope & Warcup (1986)
*Daviesia**	NM*	*Cluster roots
Lecythidaceae		
Planchonia	VAM	
Meliaceae		
Owenia	VAM/ECM	
Mimosaceae		N-fixing nodules
Acacia	ECM/VAM	Warcup (1980), Reddell &
	VAM	Warren (1987), Bellgard (1991)
Moraceae		
Ficus	VAM	
Myrtaceae		
Agonis	VAM/ECM	Smaller shrubs often have VAM only,
Eucalyptus, Leptospermum, Melaleuca	ECM/VAM	while larger shrubs and trees have ECM or dual ECM/VAM associations
		Chilvers & Pryor (1965), Lamont (1979),
Syzygium	VAM	Bellgard (1991)
Proteaceae		
Banksia, Grevillea, Persoonia, etc.	NM	Cluster roots
Rhamnaceae		
Trymalium	ECM/VAM	
Sapotaceae		
Planchonella	VAM	
Sterculiaceae		
Brachychiton	VAM	

Notes: NM = non-mycorrhizal, ECM/VAM or VAM/ECM = dual associations with the most important listed first.

C. Mycorrhizal fungi

Members of the fungus kingdom obtain nutrition from many sources, including decomposition of organic substrates, predation and parasitism, and involvement in mutualistic associations (Christensen 1989, Kendrick 1992). Many soil fungi are saprobes with the enzymatic ability to digest organic substrates of varying degrees of complexity, but some subsist on very low levels of organic or inorganic substrates (Wainwright 1988). Mycorrhizal fungi are a major component of the soil microflora in many ecosystems, but usually have limited saprophytic abilities (Harley & Smith 1983). Mycorrhizal fungi have many important roles in natural and managed ecosystems (Table 1.3). More information about fungi forming ECM and VAM associations is provided in Chapters 2 and 4, respectively.

In a recent review of mycorrhizal research trends, Kilronomos & Kendrick (1993) state that 'we may know less than we think about mycorrhizas, since we have consistently based broad hypotheses and conclusions on studies of a small number of taxa'. Populations of mycorrhizal fungi are thought to have occupied the same soil habitats for millions of years, slowly adapting to changes in site conditions (Trappe & Molina 1986). Some mycorrhizal fungi appear to have worldwide distribution patterns and have apparently adapted to a wide range of habitats, but it is known that soil factors such as pH restrict the distribution of many taxa (Abbott & Robson 1991, Brundrett 1991). Unfortunately, information about the soil and climatic conditions where isolates used in experiments were originally obtained is usually not provided. These data are urgently needed to allow isolates to be selected for use in inoculation programs and to establish correlations between their taxonomy and physiology. The fact that most mycorrhizal research has been concerned with plant growth responses with little consideration of the fungi involved has also helped to create the false impression that most of these fungi are functionally equivalent (Abbott & Robson 1991, Brundrett 1991). Table 1.4 lists properties of individual mycorrhizal fungi, resulting from their adaptations to soil/environmental/host factors, which can be used to select isolates for specific purposes. Fungal properties which would help determine the effectiveness of mycorrhizal associations include the amount of soil hyphae produced relative to root colonisation, the rate of hyphal growth and root colony initiation and physiological characteristics which regulate nutrient absorption or nutrient translocation by hyphae and exchange with the host (Kottke & Oberwinkler 1986, Smith & Gianinazzi-Pearson 1988, Abbott et al. 1992).

The potential for manipulating mycorrhizal associations to increase plant productivity in plantation forestry, or plant establishment during ecosystem recovery after severe disturbance, is the focus of major research initiatives. There is also much interest in their potential utilisation in agriculture and horticulture. However, we know very little about the taxonomic

or functional diversity of mycorrhizal fungi in Australia (Bougher 1995). Indeed, there is a paucity of knowledge about all Australian fungi, with only about 10% of these organisms having been named, while an estimated 80% of them remain undiscovered (Pascoe 1991). Knowledge of mycorrhizal fungus diversity is important because of their important roles in natural and managed ecosystems (Table 1.3). Since different fungal taxa would vary in their capacity to utilise resources, withstand adverse conditions, etc. (Table 1.4), mycorrhizal fungus diversity must contribute to the resilience of ecosystems. The functional diversity of mycorrhizal fungi provides opportunities to select fungi adapted to specific combinations of host/environment/soil conditions (Table 1.4) to optimise tree growth in plantations. The high diversity of mycorrhizal fungi associated with eucalypts in Australia provides a very large and valuable biological resource for plantation forestry.

Table 1.3. Important roles of mycorrhizal fungi in natural and managed ecosystems.

A. Benefits to plants
- Plant nutrient supply through mycorrhizal roots
- Antagonism of parasitic organisms
- Non-nutritional benefits due to water relations, etc.

B. Other roles in ecosystems
- Nutrient cycling and conservation by soil mycelia
- Fungi are important food sources for many animals
- Improving soil structure
- Carbon transport from plant roots to other soil organisms

C. Values to people
- Valuable food resources
- Medicinal uses
- Aesthetic values
- Fungal diversity is a bio-indicator of environmental quality

Table 1.4. The functional diversity of mycorrhizal fungi includes variations between individual species and isolates of fungi in the following capacities.

A. Obtaining limiting soil nutrients:
inorganic forms of phosphorus, nitrogen, etc.
organic forms of nitrogen, etc.
trace elements

B. Amelioration of adverse soil conditions due to:
toxic concentrations of metal ions
extremes in pH
high conductivity (salinity)
nutrient imbalances such as high Mg:Ca ratios

C. Responses to severe climatic conditions:
limited or excessive water supply
temperature extremes

D. Compatibility with different hosts

E. Tolerance of adverse soil conditions:
disturbance, microbial competition, etc.

F. Survival and spread in soil by spores, mycelia, etc.

G. Capacity for inoculum production:
spore production
growth in sterile culture (ECM)
growth in pot cultures (VAM)

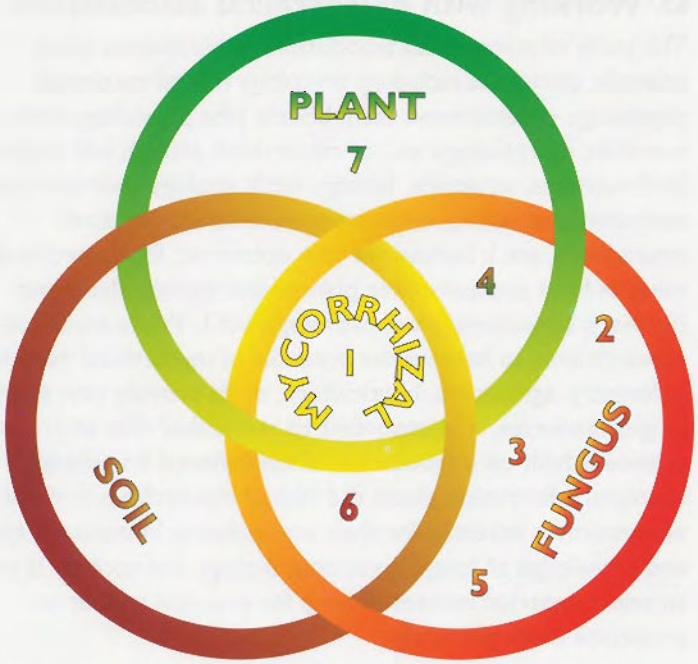


Figure 1.1 *Mycorrhizal associations involve three-way interactions between the fungus, host plant and soil factors. Numbers in this figure refer to the mycorrhizal research topics listed below and correspond to chapters in this book.*

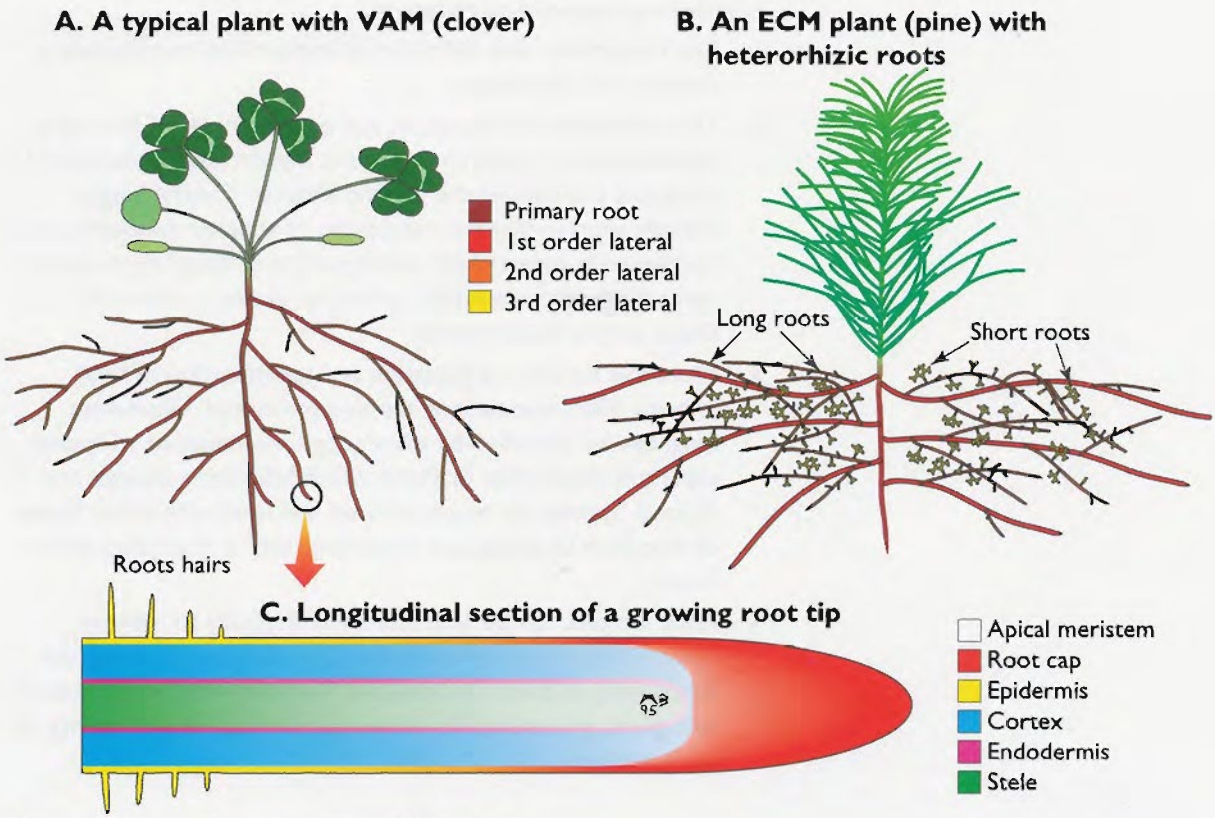


Figure 1.2. *Structural and developmental features of roots.*

A. *Components of a clover plant root system showing orders of lateral roots.*

B. *Cross-section of a growing root tip (much enlarged) with the development of major tissue types diagrammatically represented by colour gradients.*

D. Working with mycorrhizal associations

The study of mycorrhizal associations encompasses many scientific disciplines including: mycology (fungal taxonomy, physiology, development, etc.); botany (the physiology, mineral nutrition, morphology, etc. of mycorrhizal plants); soil science (soil nutrients, structure, biology, etc.); ecology (nutrient cycling, environmental quality, ecosystem reconstruction, biotic interactions, etc.); humanities (the economic, food, medicinal, etc. value of fungi and associated plants); and applied disciplines (forestry, agriculture, plant pathology, etc.). While much current research aims to harness the potential of mycorrhizal associations in forestry, agriculture, horticulture, or to provide new edible fungus resources, it is important to remember that all practical science is built on a foundation of fundamental knowledge. For example, information about the role of mycorrhizas in natural ecosystems is essential for their manipulation in managed systems and knowledge of fungal taxonomy, biology and ecology is needed to select superior isolates of fungi for practical use, or to propagate them efficiently.

Mycorrhizas are three-way associations involving plants, fungi and soils (Fig. 1.1) and all these aspects must be considered when working with mycorrhizas. Different components of mycorrhizal associations can be examined or manipulated using procedures outlined in different chapters of this manual as listed below (numbers correspond to chapters).

1. The recognition and definition of mycorrhizal associations is discussed in this chapter.
2. The collection, identification, and processing of ECM fungi is explained and a guide to important mycorrhizal associates of eucalypts provided in the second chapter. Careful fungus identification and/or the deposition of voucher specimens in a herbarium is essential for investigations of fungal diversity, to name fungi associated with particular plants, or to verify those used in experiments.
3. Protocols for the manipulation and identification of fungi forming VAM associations are also presented. Glomalean fungi can be identified by microscopic examination of spores separated from soils, or roots which have been cleared and stained. Spores can be germinated and used with other forms of inoculum to propagate these fungi with a host plant grown in soil.
4. Root samples can be processed histologically to identify mycorrhizas, and to provide valuable information about the functioning of these associations. The examination of roots of bait plants grown in soils allows mycorrhizal fungus activity to be quantified.

5. A guide to working with ectomycorrhizal fungi in the laboratory is provided. Sterile cultures and other inoculum forms allow ECM fungi to be applied in experiments or when young trees are grown for plantation forestry.
6. Practical information pertaining to the growth of mycorrhizal plants in the glasshouse or nursery is provided. The careful management of soil fertility and other factors is necessary to optimise the yield of plants and to encourage mycorrhizal colonisation of roots.
7. The design, maintenance, measurement and analysis of data from field experiments is discussed in the final chapter. Examples of field experiments, which allow host plant responses to mycorrhizal inoculation and soil nutrient levels to be determined, are presented.

There are greater opportunities for the management of ECM associations in plantation forestry than for the manipulation of VAM associations in other cropping systems (Grove & Malajczuk 1994). This results from (1) the ubiquitous presence of VAM fungi in most soils, (2) the capacity for many ECM fungi, but not VAM fungi, to be grown in sterile culture, (3) the compatibility of conventional management practices with fungal inoculation in forest nurseries (Chapter 6), and (4) specificity between host trees and many ECM fungi (Malajczuk et al. 1982, 1984, Dighton & Mason 1985, Gardner & Malajczuk 1988, Molina et al. 1992). Even where soils already contain ECM fungi, it may still be advantageous to introduce new fungi, especially when tree species are first grown in new habitats (Grove et al. 1991). Although sufficient quantities of inoculum of VAM fungi are normally present in agricultural soils, there are opportunities to adjust management practices (fertilizer and pesticide use, crop rotations, plant genotypes, tillage practices, etc.) to obtain greater benefits from these associations (Miller et al. 1994, Thompson 1994). There is a great potential to utilise both ECM and VAM fungi (by topsoil management or inoculation) during the revegetation of disturbed habitats (Jasper 1994, Malajczuk et al. 1994, Pfleger et al. 1994). Inoculation with VAM fungi should also be of value in horticulture, especially when fumigated or pasteurised soils, soil-less potting mixes, or tissue-cultured plants are used (Chang 1994).

1.2. THE STRUCTURE AND DEVELOPMENT OF MYCORRHIZAL ROOTS

It is necessary to be familiar with the structure of non-mycorrhizal roots before examining any changes in root structure due to mycorrhizal associations. Some of the anatomical features described below are thought to provide structural strength to roots, or have defensive roles since they are most highly developed in long-lived roots (Brundrett & Kendrick 1988). Suberin in the endodermis and exodermis is considered to function as a permeability barrier, blocking apoplastic (cell wall) transport of substances (Clarkson & Robards 1975, Peterson 1988). Root anatomy features can also have the potential to regulate mycorrhizal development (Section 1.5). An introduction to important terms used to describe root structures is provided below, with an emphasis on tree roots. More information can be found in plant anatomy texts such as Esau (1977). Many of the structures described below are illustrated in photographs presented in this Chapter (Figs 1.3, 1.6, 1.10, 1.11).

A. Root systems

Root system features are illustrated in Figure 1.2. The recognition of primary and secondary roots, and different orders of lateral roots, is important because these can have different functions. Different orders of roots vary in their growth rates, life spans, structural features, etc. as well as their capacities to obtain water and nutrients, or support mycorrhizal associations. Higher order lateral roots are generally thinner, shorter, have shorter life spans and grow less rapidly than those of lower orders.

Terminology: *(underlined terms are explained in the next section)*

Seminal root — root formed initially by a germinating seed.

Lateral roots — roots which grow from another root.

Adventitious roots — arise from a stem.

First order lateral roots — arise from the seminal root or adventitious roots.

Second and third order laterals, etc. — roots which arise from first order laterals which in turn may produce third order laterals, and so on. Higher order laterals may be categorised as feeder roots or fine roots (see below).

Primary growth — initial growth of a plant organ caused by cell division in its apical meristem and cell enlargement in subapical regions.

Secondary growth — new growth activity in a mature plant organ. This normally results from radial enlargement of an organ by a new lateral meristem.

Secondary roots, woody roots — roots, which develop a periderm and additional vascular tissue as a result of secondary growth. These would normally have a much longer lifespan than feeder roots and will not contain mycorrhizas if secondary growth has resulted in cortex loss.

Coarse roots — the 'distributive' root system comprised of lower order roots, which is responsible for mechanical support and the transport of substances between fine roots and the shoot. Coarse roots of Monocotyledons do not have secondary growth, but may not form mycorrhizas if their primary cortex is protected by heavily suberized or lignified cells.

Feeder roots, fine roots — fine, higher order lateral roots that are thought to be responsible for most nutrient and water uptake, as well as mycorrhiza formation.

Brown roots, suberized roots, etc. — Additional terms that are sometimes use to designate old roots, woody roots, or roots with a suberized exodermis. These general terms are misleading and should not be used.

B. Tissues

Root tissues are produced by cell division in the root apex and cell expansion in subapical regions (Fig. 1.2). These tissues progressively mature at greater distances from the root tip and can be identified microscopically by specialised features of their cell walls or cytoplasm (see Fig. 1.3).

Terminology: *(underlined terms are explained in the next section)*

Apex — the root tip which is covered by a *root cap* (covering sheath) and secreted *mucilage* (water soluble polysaccharides which adhere to the root).

Root hair — thin hair-like cell extension of an epidermal cell on the root surface. These may be long or short and provide a dense or sparse root covering. Root hairs increase root contact with the soil and are thought to be important for water and nutrient uptake.

Apical meristem — the zone of dividing cells at the root apex which gives rise to new cells in a growing root. Actively growing roots have gradients of maturing tissues away from the apical meristem (Fig. 1.2).

Epidermis — the outermost layer of cells of the root, in direct contact with the soil. As the soil–root interface, the epidermis is an important site for nutrient uptake and the initiation of mycorrhizal associations.

Hypodermis — the layer of cells below the epidermis is called a hypodermis if it is not suberized (Peterson 1988).

Exodermis — the hypodermis is called an exodermis if its cell walls contain a Casparian band, and these cells often also have suberin lamellae (Peterson 1988). The exodermis is thought to reduce root permeability (to apoplastic flow) and increase resistance to pathogenic organisms, water loss, etc.

Passage cells, short cells — small exodermal cells that remain unsuberized, surrounded by longer suberized cells (long cells). In many plants, long and short cells alternate in a uniform pattern (called a dimorphic exodermis).

Cortex — the cell layers occurring between the epidermis and stele. Cortex cells typically have a large central **vacuole** used to store solutes and are the site of arbuscule formation in VAM associations.

Endodermis — a cortical cell layer found in all roots, next to the vascular cylinder. The cell walls contain a Casparian band and may develop suberin lamellae (Esau 1977, Clarkson & Robards 1975).

Intercellular space — spaces outside the root cells, often in the cortex at the junction of cells. These form longitudinal air channels in many roots, which can be seen by observing whole-living roots mounted in water. Air channels provide conduits for gas transport in waterlogged soils (Armstrong 1979) and influence VAM formation.

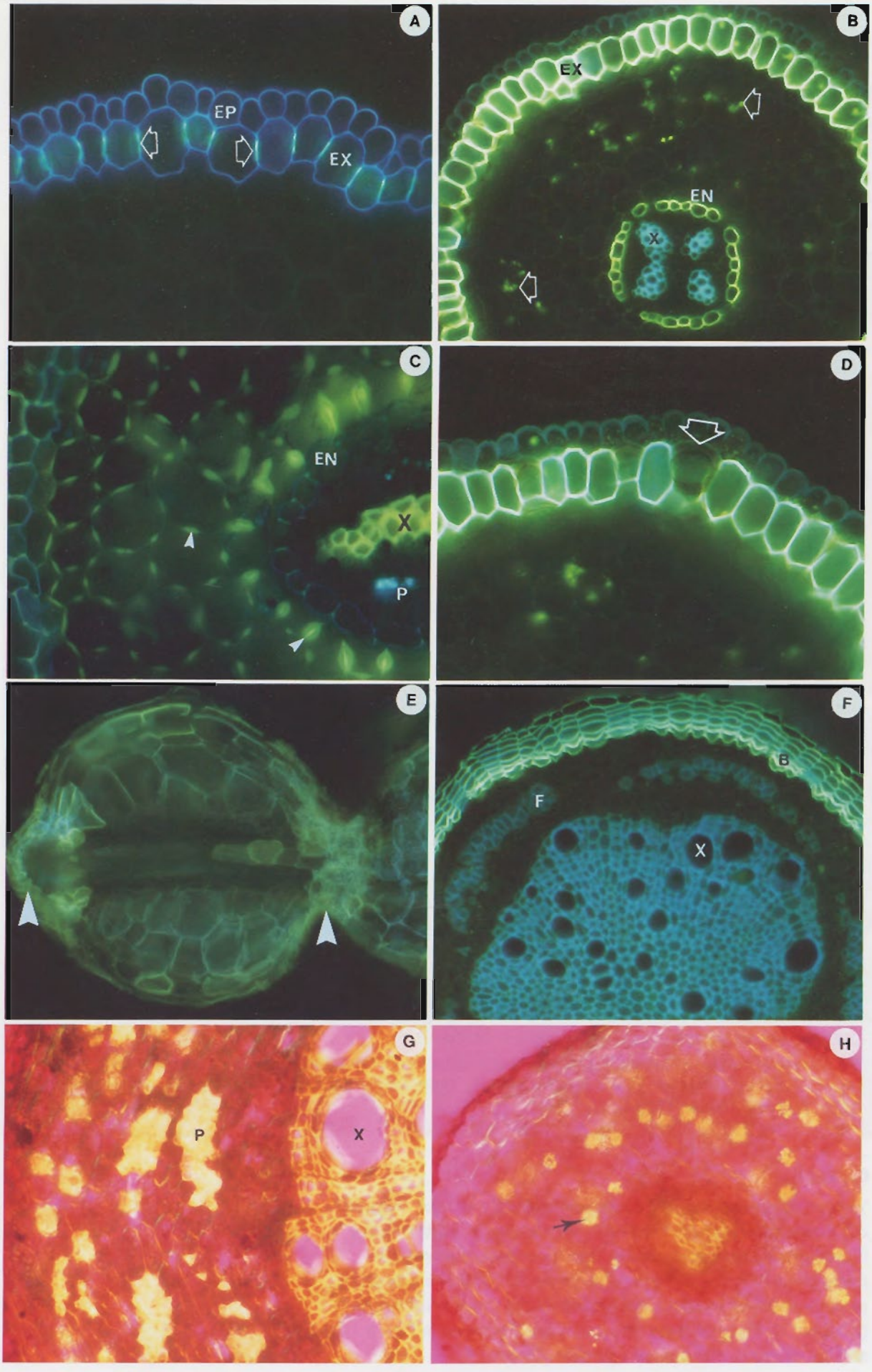
Stele or vascular cylinder — the zone internal to the endodermis which contains specialised tissue responsible for the transport of water and minerals to the shoot (xylem) or organic nutrients, such as photosynthetically fixed carbon (phloem). Additional layers of xylem and phloem form radially during secondary growth and lateral root initiation also occurs in this zone. Xylem cells develop lignified walls and are dead when mature.

Periderm — the bark layer formed on the surface of roots or branches by secondary growth. Walls of periderm cells are strengthened by suberin and lignin deposits, which reduce their permeability and susceptibility to microbial activity and adverse soil conditions.

Metacutinization — This is the protection of dormant root tips by suberization of one or more root cap cell layers (Romberger 1963). Inactive roots of many temperate forest trees develop a metacutinized apex (Brundrett et al. 1990).

C. Cells

Various components of the cytoplasm and walls of root cells, that can be identified using microscopic procedures (Chapter 4), are listed below. These components may have important roles in metabolism or transport processes in mycorrhizal roots, and can provide information about the functioning of these associations (Chapter 4). Many of these structures are illustrated in Figure 1.3, and the electron microscope figures in Chapter 4.


A
EP
EX
B
EX
EN
X
C
EN
X
P
D
E
F
B
F
X
G
P
X
H

Terminology:

A. Cell components

Plasma membrane, Plasmalemma — the outer membrane of plant cells.

Cytoplasm — the zone inside the plasmalemma of cells where most metabolic activity occurs.

Plastid — a cytoplasmic organelle with a double membrane, involved in photosynthesis (chloroplast), starch storage (amyloplast), or pigment synthesis (chromoplast) (Esau 1977).

Organelle — distinct bodies in the cytoplasm such as mitochondria and ribosomes.

Plasmodesmata — microscopic channels which cross the walls of adjacent plant cells to form a cytoplasmic continuum known as the *symplast*.

Vacuole — one or more spaces inside a plant cell, which contains water and stored substances that are bounded by a membrane called the *tonoplast*.

Crystals, secondary metabolites — Specialised root cells may contain crystals, mucilages, or other substances in their vacuoles that help to protect the root from predation.

B. Cell wall components

Cell wall — located outside the plasmalemma and primarily made of structural carbohydrates such as cellulose. The cell wall space inside plants is collectively known as the apoplast. Cell walls provide mechanical support and apoplastic transport. They often contain secondary metabolites such as tannins (phenolic pigments), suberin or lignin.

Middle lamella — a wall zone rich in the carbohydrate pectin connecting adjacent cells.

Suberin — a hydrophobic material, containing lipids and phenolics, which impregnates the cell wall of specialised cells and is thought to prevent the passage of water, and other materials, in the apoplast.

Suberin lamellae — concentric layers of suberin deposited on the inner surface of cell walls and considered to function as barriers to microbial and solute penetration.

Casparian band — suberin deposited in a radial band in the walls of epidermal and exodermal cells and thought to provide a barrier to apoplastic solute transport (Esau 1977, Clarkson & Robards 1975, Peterson 1988).

Lignin — phenolic compounds used to impregnate cell walls, which are often considerably thickened to strengthen plant organs. Xylem cells and fibres are typically lignified, but other cells in the stele or cortex can have lignified walls.

Phi thickenings — localised deposits of lignified wall material which form a thickened ring in cortical or hypodermal cell walls (von Guttenberg 1968).

Figure 1.3. *Microscope photographs of root hand sections, using different staining and microscopy procedures (see Chapter 4), to illustrate structural and developmental features.*

A, C, E. *Cross-sections with a stain for suberin and lignin (berberine/aniline blue) and viewed with fluorescence microscopy.*

B, D, F. *Cross-sections with a stain for lipids and suberin lamellae (fluorol) and viewed with fluorescence microscopy.*

G, H. *Polarised light microscopy of root cross-sections.*

A. *Cross-section of an* Allium cepa *root showing exodermal (Ex) and epidermal (Ep) cells. Exodermal cells have Casparian bands (arrows) and suberin lamellae (blue).*

B. Fraxinus *root showing intense yellow fluorescence of suberin lamellae in the endodermis (EN) and exodermis (EX) and lipids in VAM fungus hyphae in the cortex (arrows). Xylem cells (X) are also revealed by blue autofluorescence.*

C. *Cross-section of a cedar (*Thuja occidentalis*) root with many phi thickenings (arrows) in cortex cell walls. Endodermal (EN), xylem (X) and phloem (P) cells are also visible*

D. *Close-up of a* Fraxinus *root (see B) showing an unsuberized passage cell in the exodermis (arrow).*

E. *Longitudinal section of a sugar maple (*Acer saccharum*) root showing constrictions (beads) caused by root cap metacutinization (arrows) when roots resume growth.*

F. *Fluorescent staining of suberized periderm (bark) cells (B) encasing a* Quercus *root after secondary growth. Blue autofluorescence of lignified xylem (X) and phloem fibre (F) cells is also visible.*

G. *Strong refringence of thick cell walls of xylem (X) and phloem fibre (F) cells in a* Juglans nigra *secondary root.*

H. *Refringence of crystals (probably calcium oxalate) in a* Juglans nigra *primary root (arrow).*

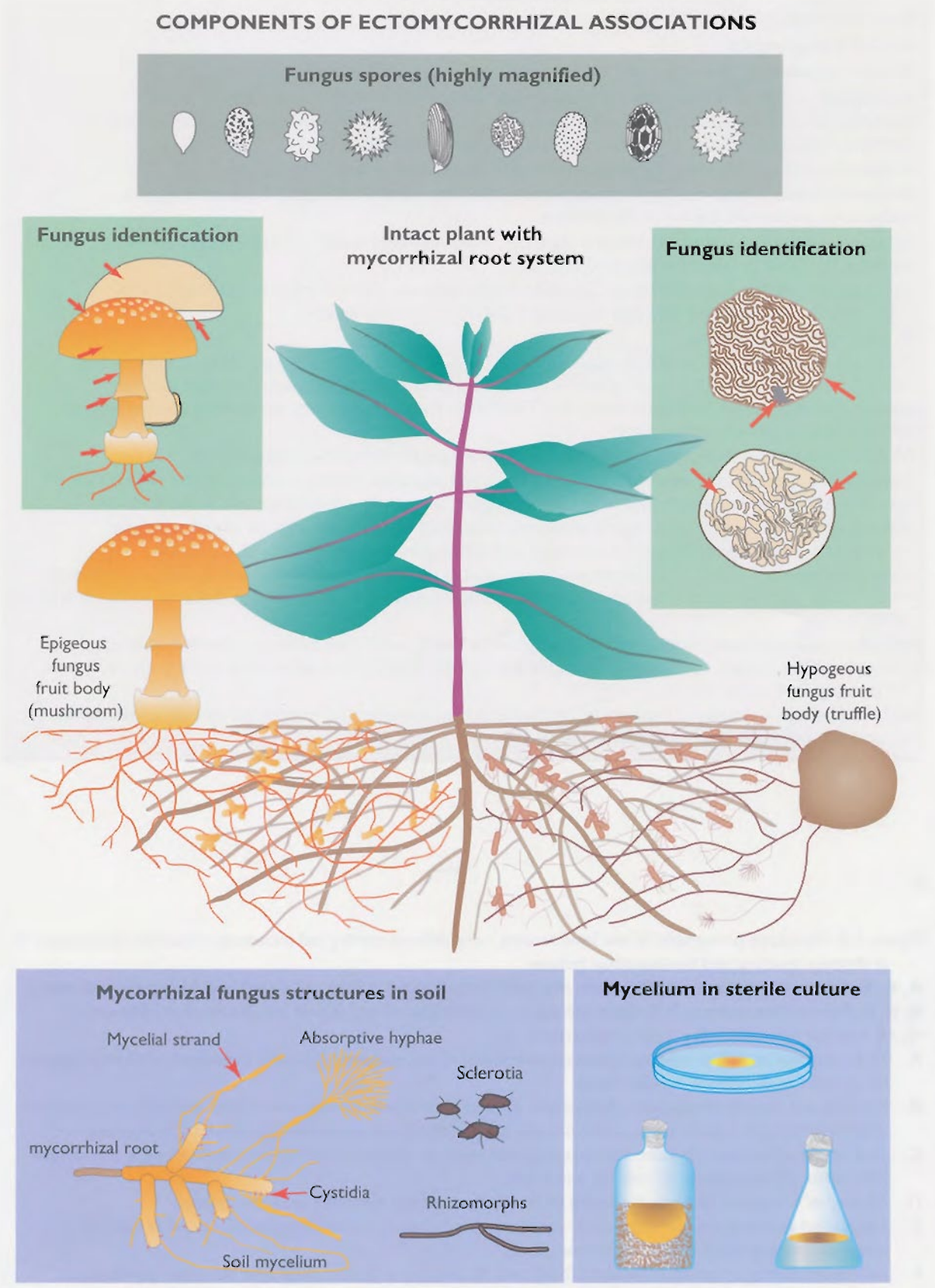


Figure 1.4. *Principal components of ectomycorrhizal associations. A diagrammatic representation showing an ectomycorrhizal plant and associated fungal structures including propagules which can be used as inoculum forms.*

1.3. ECTOMYCORRHIZAL ASSOCIATIONS

Ectomycorrhizal associations (often abbreviated as ECM in this book) are mutualistic associations between higher fungi (Chapter 2) and Gymnosperm or Angiosperm plants belonging to certain families (Table 1.5). Ectomycorrhizal associations consist of a soil mycelium system, linking mycorrhizal roots and storage or reproductive structures (Fig. 1.4). Ectomycorrhizal roots (which have also been called ectotrophic associations or sheathing mycorrhizas) are characterised by the presence of a mantle and Hartig net (Figs 1.5, 1.6), but both these structures may not be well developed (see Section 1.6). Detailed descriptions of ECM root morphology have been published elsewhere (e.g. Harley & Smith 1983, Kottke & Oberwinkler 1986, Massicotte et al. 1987).

Trees with ECM associations are dominant in coniferous forests, in cold boreal or alpine regions, and many of the broad-leaved forests in temperate or mediterranean regions, but they also occur in some tropical or subtropical savanna or rain forests habitats (Brundrett 1991). The majority of ECM hosts are trees, or shrubs (Table 1.5), but associations are formed by a few herbaceous plants, including *Kobresia* (Cyperaceae) and *Polygonum* (Polygalaceae) species found in alpine/arctic regions (Kohn & Stasovski 1990). Some Australian herbaceous plants in the families Goodeniaceae, Asteraceae, Euphorbiaceae, and Stylidiaceae have also been reported to form ECM, at least in certain circumstances (Kope & Warcup 1986).

Ectomycorrhizal associations are formed predominantly on the fine root tips of the host, which are unevenly distributed throughout the soil profile, being more abundant in topsoil layers containing humus than in underlying layers of mineral soil (Meyer 1973, Harvey et al. 1976). There is little information on the biomass of mycorrhizas throughout the soil profile. However, Marks et al. (1968), Vogt et al. (1981), Hunt & Fogel (1983) and Malajczuk & Grove (unpublished) suggest that ECM fungi can make a significant contribution to the biomass of forest ecosystems. The hyphae of mycorrhizal fungi are widely distributed through the soil, and must make a large contribution to nutrient uptake and cycling in many forest ecosystems.

A. Development

Ectomycorrhizal structures develop as a function of genetic interactions between the particular fungus and host plant, and result from synchronised root growth and fungal differentiation. Most plants with ECM have roots with a modified lateral root branching pattern. This pattern, which is called heterorhizy, consists of short mycorrhizal lateral roots (called short roots) supported by a network of thicker, long roots (Fig. 1.4). The long and short roots in heterorhizic root systems are fundamentally similar in structure (Wilcox 1964), but short roots are normally narrower and grow much more slowly than long roots

Table 1.5. Important ectomycorrhizal woody plant families and genera. Only plants that have been carefully examined microscopically are included. Data are from Table 1.2, Alexander & Hogberg 1986, Harley & Harley 1987, and other sources.

Family	*Genera*
Betulaceae	*Alnus*, Betula*, Carpinus*, Ostrya*, Ostryopsis*
Caesalpiniaceae[2]	*Anthonotha, Afzelia, Berlinia, Brachystegia, Eperua, Gilbertiodendron, Intsia, Isoberlinia, Julbernardia, Microberlinia, Monopetalanthus, Tetraberlinia, Uapaca, etc.*
Casuarinaceae[2]	*Allocasuarina**
Corylaceae	*Corylus**
Cistaceae	*Helianthemum, Cistus, Tuberaria*
Dipterocarpaceae	*Anisoptera, Dipterocarpus*, Hopea, Marquesia, Monotes, Shorea*, Vateria, etc.*
Euphorbiaceae[2]	*Marquesia, Uapaca*
Fabaceae[2]	*Gastrolobium*, Gompholobium*, Jacksonia*, Mirbelia*, Oxylobium, Pericopsis, etc.*
Fagaceae	*Castanea, Castanopsis, Fagus*, Nothofagus*, Quercus*, etc.*
Gnetaceae[1]	*Gnetum**
Meliaceae	*Owenia**
Mimosaceae[2]	*Acacia**
Myrtaceae[2]	*Allosyncarpia*, Agonis*, Angophora, Baeckea, Eucalyptus*, Leptospermum, Melaleuca*, Tristania, etc.*
Nyctaginaceae[2]	*Neea, Pisonia*
Pinaceae[1]	*Abies*, Cathaya, Cedrus, Larix*, Picea*, Pinus*, Pseudotsuga, Tsuga*, etc.*
Rhamnaceae[2]	*Pomaderris, Trymalium*, etc.*
Rosaceae[2]	*Dryas*
Salicaceae	*Populus*, Salix**
Tiliaceae	*Tilia**

Notes: * = genera examined by the authors, 1 = Gymnosperms, 2 = families which also have many VAM plants.

(Wilcox 1964, Kubiková 1967). The restricted growth of short roots may be necessary to allow ECM fungi time to form an association, since these fungi have difficulty colonising more rapidly growing roots (Chilvers & Gust 1982). Thus trees with ECM would require slow growth of some of their lateral roots, and in time this process would result in the evolution of separate, genetically distinct long and short roots.

Ectomycorrhizas form where host roots and compatible fungi are growing in close proximity and environmental conditions are favourable. The sequence of events that results in typical ECM formation has been described in many studies (Chilvers & Gust 1982, Kottke & Oberwinkler 1986, Massicotte et al. 1987) and can be summarised as follows:

1. Hyphae contact, recognise and adhere to root epidermal cells near the apex of a young actively-growing, high-order, lateral root.
2. Mycelia proliferate on the root surface and differentiate to form the mantle.
3. Hyphae penetrate between epidermal cells (in most angiosperms) or into the cortex (in gymnosperms) to form a

labyrinthine Hartig net. Host responses may include polyphenol production in cells and the deposition of secondary metabolites in walls.

4. The active mycorrhizal zone occurs several mm behind the root tip (as a result of the time required for mycorrhizal formation), but Hartig net hyphae senesce (as indicated by ultrastructural changes—Massicotte et al. 1987) in older regions, which are further from the root tip. Consequently, Hartig net activity depends on root age and root growth.
5. The mantle in older roots generally persists long after associations become inactive. Older ECM roots probably function as storage structures and propagules.

A generalised life cycle for mycorrhizal associations is presented in Table 1.6 and shows many additional stages in fungus activity and mycorrhizal formation that can be recognised. These stages include key events that determine host–fungus compatibility, which can be examined using techniques presented in Chapter 4.

Table 1.6. The life cycle of a mycorrhizal association showing stages in the development and senescence of soil mycelia and mycorrhizas.

1. Fungal propagules (spores, hyphae, old roots, etc.)
- survival:
  - — disturbance, predation
  - — adverse conditions
- dispersal to new locations
- dormancy, quiescence
- activation — fungi may respond to:
  - — environmental conditions
  - — time intervals
  - — presence of roots or other organisms

3. Root growth *(young roots required to form association)*
- regulated by phenology and environmental factors
- production of soluble or volatile signals by roots

2. Active soil hyphae
- mineral nutrient acquisition by fungus
- microhabitat preferences ?
- limited saprobic potential ?
- spread through soil
- attraction to roots, trophic responses, recognition of host?
- contact with young host roots

4. Hyphae on root surface
- proliferation on root surface
- further recognition events?
- fungal morphology changes to form:
  - — appressoria (VAM)
  - — mantle (ECM)

5. Hyphal penetration into or between root cells
- avoidance or tolerance of host defences
- recognition by host ? (minimal response)
- further fungus morphology changes:
  - — hyphal branching patterns (VAM)
  - — inner mantle structure (ECM)

6. Formation of exchange sites
- most pronounced fungal morphology changes
- highly branched hyphal structures:
  - — arbuscules (VAM)
  - — Hartig net (ECM)
- major host cytoplasmic responses (protoplasm synthesis, etc.)

7. Active exchange processes
- limited in duration, occurs in young roots

8. Senescence of hyphal structures within roots
- disorganisation of cytoplasm in exchange site hyphae
- fungal resources withdrawn for:
  - — storage by hyphae, vesicles within root (VAM)
  - — storage in mantle (ECM)
  - — export into external mycelium

9. Propagule formation →1. (the cycle recommences)
- resting spores in soil or root (VAM)
- sexual spores from mushrooms, truffles (ECM)
- mycelial strands and sclerotia (ECM)
- mycelial networks in soil
- fungal structures associated with long-lived roots

10. Root senescence and decomposition
- root cortex lost due to:
  - — root death or secondary growth
  - — parasitism or consumption
- nutrients in root and fungus structures recycled
  - — decomposition food web

B. Root systems

Structural features of typical ECM roots are illustrated in Figure 1.6 and discussed further in Chapter 4. Terminology used to describe ECM associations is outlined below. ECM roots can often be recognised by external and internal hyphal features which are characteristic of different fungi (Chapter 4) and by changes to root growth and branching patterns (heterorhizy) which occur (Figs 1.4–1.6). However, microscopic observations should be used to confirm diagnoses (Chapter 4).

Terminology:

Mantle — layers of fungal hyphae covering the root surface. These hyphae form thin or thick, loose or compact layers with a variety of colours and surface textures depending on the fungus present (Chapter 4). The mantle may contain recognisable hyphae in an interwoven structure *(plectenchyma)* or hyphae may be modified to resemble plant cells *(pseudoparenchyma)*. Hyphal projections *(cystidia)* may occur on the mantle surface.

Hartig net — labyrinthine branching of fungal cells in a layer which grows between epidermal or cortical cells of a root. Multiple branches and wall infoldings of hyphae greatly increase the contact area between fungus and host. This is considered to be the major site of nutrient exchange between the fungus and host plant.

Heterorhizy — root system with distinct long and short elements, resulting from a reduced capacity for longitudinal growth by some of the finest lateral roots. Heterorhizic roots are a typical feature of ECM plants, but plants with VAM may also have them.

Short roots — roots with ECM often have reduced apical growth and more frequent branching, resulting in a *heterorhizic* root system with distinctive short roots supported by a network of long roots.

Long roots — the lateral roots which bear short roots and elongate more rapidly. Long roots and may undergo early secondary growth.

Dichotomous branching — equal branching of the ECM short roots of some Gymnosperm trees including *Pinus* species.

Pinnate branching — or sympodial branching, is unequal branching of mycorrhizal lateral roots where side branches grow perpendicular to the main axis of a mycorrhizal root.

C. Fungal structures

Hyphae or mycelium are fine thread-like cells which occur separately or are arranged to form the organised structures described below (see Fig. 1.4).

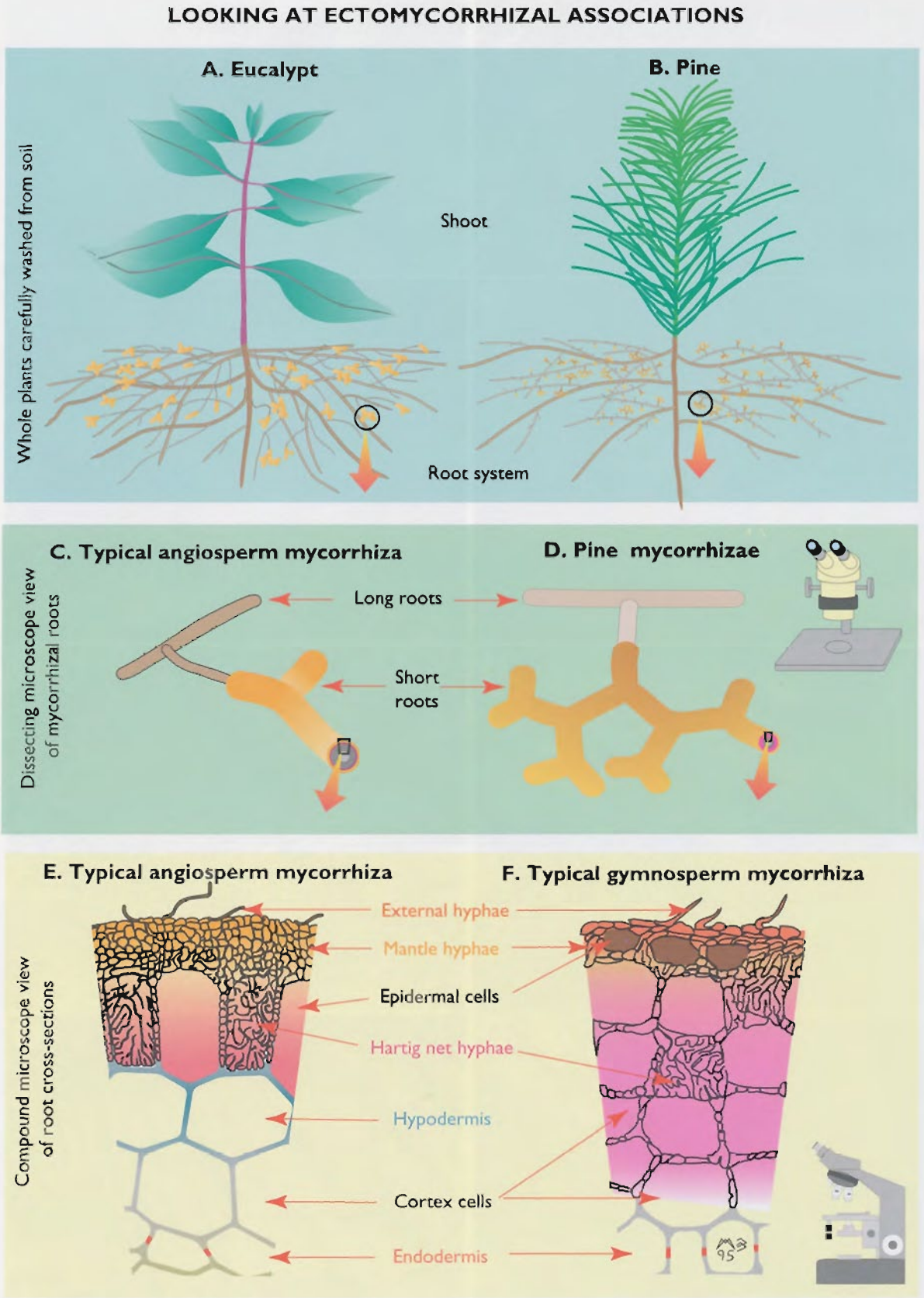
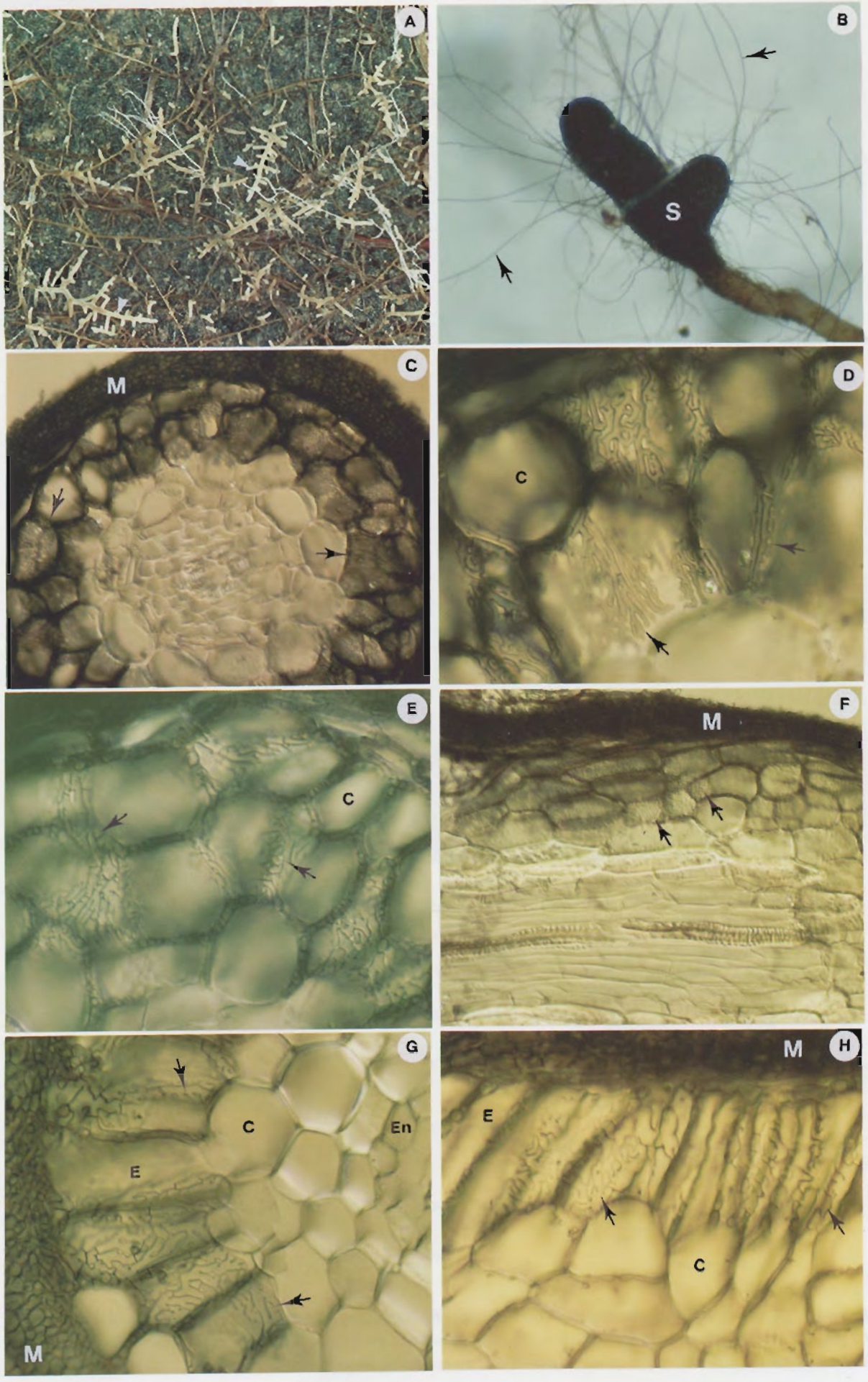


*Figure 1.5. Looking at ectomycorrhizal associations. Eucalypt (**A**) and pine (**B**) seedling root systems. Typical Angiosperm (**C**) and pine (**D**) mycorrhizal root systems with characteristically branched short roots. Details of typical Angiosperm (**E**) and Gymnosperm (**F**) ectomycorrhizal roots with a mantle and an epidermal (**E**) or cortex (**F**) Hartig net .*


A
B
S
C
M
D
C
E
C
F
M
G
C
En
E
M
H
M
E
C

Terminology:

A. Hyphal components

Septae — septae are cross-walls which divide fungal cells. In Basidiomycetes, these may be arranged as clamp connections — a semi-circular bridge between two cells used to regulate the transfer of nuclei during mycelial growth (Chapter 2).

Melanin — fungal cell walls are often modified by the inclusion of brown, black, or coloured compounds, that apparently have a protective role. These are produced by the secondary metabolic pathways of fungi and are analogous to the phenolic compounds produced by plant cells.

B. Organised structures in soil

Fruit bodies — called sporocarps, basidiocarps, ascocarps, mushrooms, truffles, etc., are relatively large reproductive structures formed by Basidiomycetes or Ascomycetes which produce sexual basidiospores or ascospores respectively. These develop from primordia produced by the mycelial system and are often associated with strands or rhizomorphs. Fruit bodies are used to identify fungi (Chapter 2) and provide a source of inoculum (Chapter 6).

Sclerotia — storage structures produced in soil by some fungi, comprised of compact fungal tissue, which is often highly melanized.

Other spores — small asexual spores (conidia) which function as propagules, may be produced by some mycorrhizal fungi.

Mycelial strands and rhizomorphs — strands of interwoven hyphae. Rhizomorphs are made of compact, specialised hyphae. These are considered to function as transport conduits and to spread the association.

Extraradical hyphae — individual hyphae extending outwards from the fungal mantle or strands into the soil. These initiate mycorrhizal associations, acquire soil nutrients, etc.

Figure 1.6. Morphology of ectomycorrhizal (ECM) associations.

A,B. *Whole roots viewed with a dissecting microscope.*

C–H. *Hand sections of field-collected root material, cleared and stained with Chlorazol black E and viewed with interference contrast microscopy (Chapter 4). Abbreviations: C = cortex cell, E = epidermal cell, M = mantle, En = endodermis.*

A. Eucalyptus *roots with ECM (arrows) after growth in chambers containing forest soil.*

B. *ECM short root (S) of* Eucalyptus globulus *with a* Cenococcum*-like mycorrhiza with radiating external hyphae (arrows).*

C, D. Pinus strobus *ECM short root with thick mantle and Hartig net hyphae (arrows) around cortex cells.*

E. *Cross-section of a* Picea glauca *ECM with labyrinthine Hartig net hyphae (arrows)*

F. *Longitudinal section of* Abies balsamea *root showing Hartig net (arrows).*

G. Populus tremuloides *ECM root cross-section showing labyrinthine Hartig net hyphae (arrows) around elongated epidermal cells.*

H. *Longitudinal section of* Betula papyrifera *ECM roots showing Hartig net hyphae (arrows) and radially elongated epidermal cells.*

1.4. VESICULAR-ARBUSCULAR MYCORRHIZAL ASSOCIATIONS PRODUCED BY GLOMALEAN FUNGI

Glomalean fungi form vesicular-arbuscular mycorrhizal associations (abbreviated as VAM in this book) within roots (Chapter 3). These associations are also known as arbuscular mycorrhizas, VA mycorrhizas, endomycorrhizas, or endotrophic mycorrhizas. There is some disagreement about whether arbuscular mycorrhizas or vesicular-arbuscular mycorrhizas is the most appropriate name for these associations, because some fungi do not produce vesicles and arbuscules are not always present in mycorrhizal roots, and the role of arbuscules in nutrient exchange has not been unequivocally demonstrated (Smith 1995, Walker 1995). VAM associations consist of soil hyphae, spores and auxiliary bodies produced in the soil, as well as hyphae, arbuscules and vesicles in roots (Figs 1.7–1.10). These structures can be used to identify fungi, quantify them and propagate these associations using methods described in Chapters 3 and 4 (Fig. 1.9).

A. Development

The stages in root colonisation by VAM fungi are listed in Table 1.6 and shown in Figure 1.7. Stages in this process are outlined below and illustrated in Figure 1.9.

1. Associations start when soil hyphae respond to the presence of a root by growing towards it, establishing contact and growing along its surface.
2. Root penetration occurs when one or more hyphae form appressoria between adjacent epidermal cells and penetrate these cells.
3. One or more hyphae manage to penetrate the hypodermis (through passage cells if present in an exodermis) and branch in the outer cortex.
4. Aseptate hyphae spread along the cortex in both directions from the entry point to form a colony (infection unit). A relatively rapid parallel spread of intercellular hyphae may occur along air channels, or intracellular hyphae may follow a convoluted path through cortex cells.
5. Arbuscules grow (see terminology below) within cortex cells from subapical branches on internal hyphae. Arbuscule formation follows hyphal growth, progressing outwards from the entry point.
6. Vesicles develop to accumulate storage products in many Glomalean associations. Vesicles are initiated soon after the first arbuscules, but continue to develop when the arbuscules senesce.
7. In later stages, arbuscules collapse (progressively, starting with the finest branch hyphae). Hyphae in the root cortex develop cross-walls in old associations.

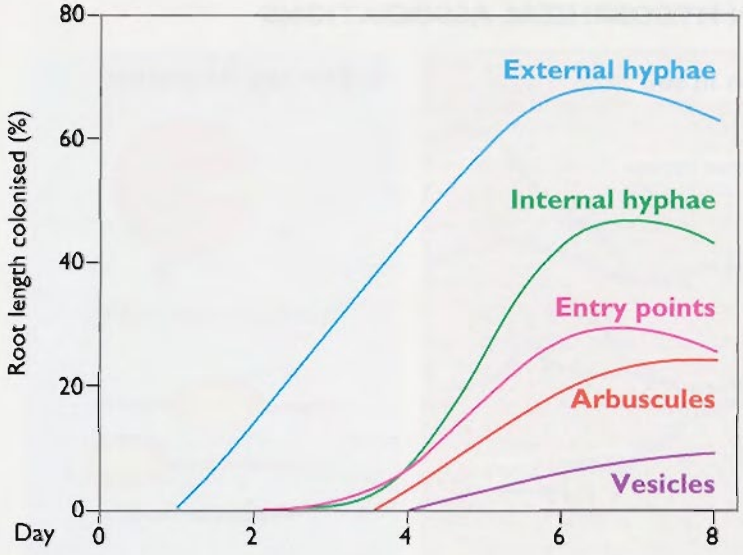


Figure 1.7. *The time course of the formation of various mycorrhizal structures within leek (Allium porrum) roots cleared and stained with Chlorazol black E (Chapter 4). These data were obtained by transplanting leek seedlings into pots containing an established association with active mycorrhizal hyphae, for different time intervals (after Brundrett et al. 1985).*

B. Mycorrhizal roots

Roots containing VAM associations look superficially similar to non-mycorrhizal roots, unless they have been processed (Chapter 4) to reveal the internal features defined below (see Figs 1.8, 1.10). The morphology of mycorrhizal associations varies with different Glomalean fungi (Chapter 3) and is influenced by root anatomy (Section 1.5).

Terminology:

Colony — hyphal colonisation of a root resulting from one external hypha (there may be several adjacent entry points). These are often called infection units.

Appressoria — hyphal swellings which occur where hyphae first penetrate root epidermal cells.

Internal hyphae, intraradical hyphae — aseptate hyphae which grow within the cortex of a root to form a colony and later develop arbuscules and vesicles.

Intracellular hyphae — hyphae which grow within the wall of root cells.

Intercellular hyphae — hyphae which grow between the walls of adjacent root cells.

Aseptate hyphae — hyphae that are normally without cross-walls. Cross-walls may form as hyphae age.

Arbuscules — named by Gallaud (1905) because they look like little trees, these are intricately branched 'haustoria' formed within root cortex cells. They remain separated from the cytoplasm by infoldings of the cell plasma membrane. Arbuscules are formed by repeated dichotomous branchings and reductions in hyphal width, starting from an initial *trunk hypha* (5–10 µm in diameter) and ending in a proliferation of fine *branch hyphae* (< 1 µm diameter). Arbuscules are considered to be the major site of symbiotic exchange with the host plant. Old arbuscules collapse progressively until only the trunk remains.

Dichotomous branching — a symmetrical branching patten which occurs when two branches arise simultaneously from the tip of a hyphae, plant or fungus organ, and grow at the same rate.

Vesicles — intercalary (-o-) or terminal (-o) hyphal swellings formed on internal hyphae within the root cortex. These may be inter- or intracellular. Vesicles accumulate lipids and may develop thick wall layers in older roots. The production and structure of vesicles vary between different genera of Glomalean fungi. They function as spores for some VAM fungi, but not for others.

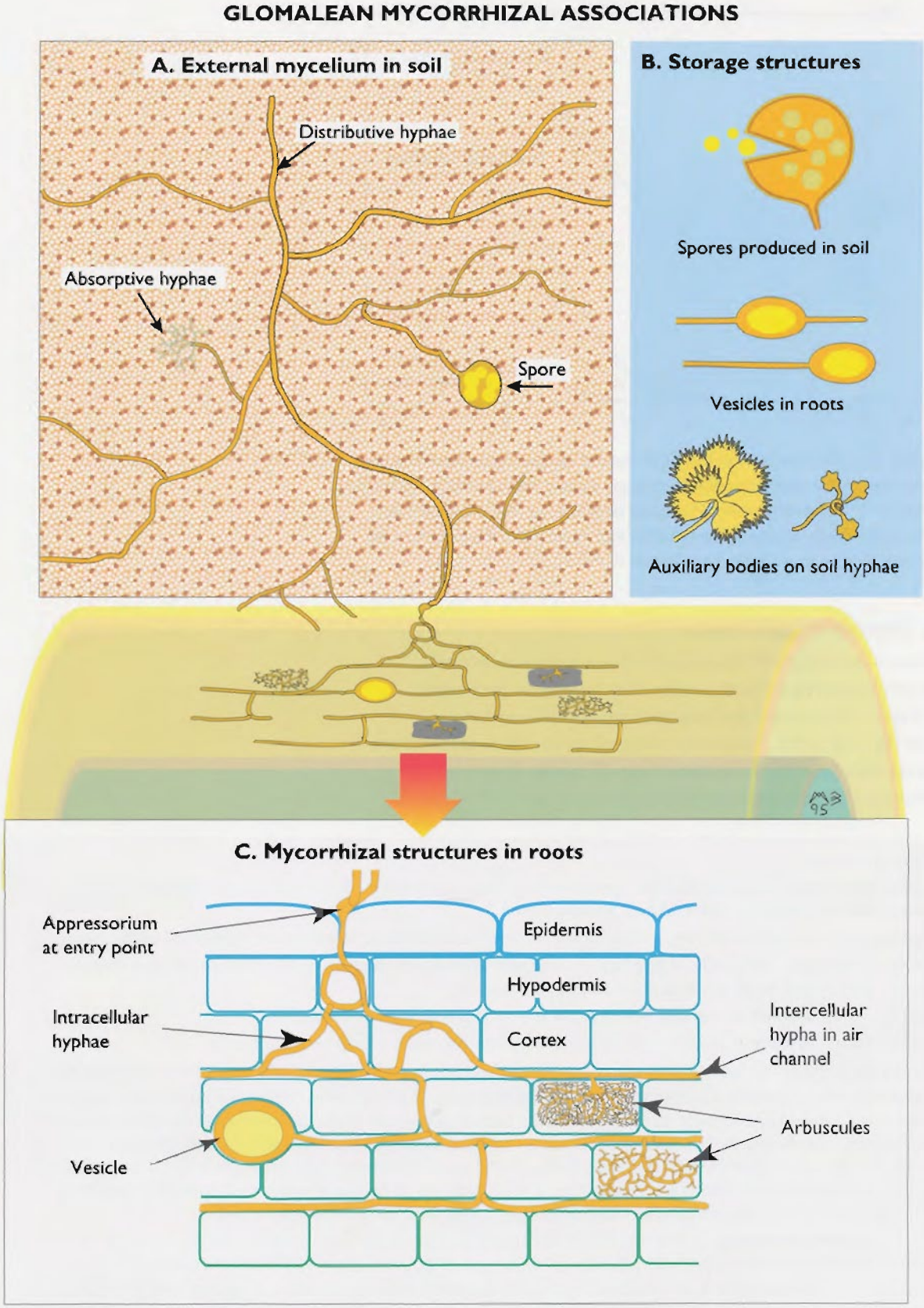


Figure 1.8. *Principal components of VAM associations:*

A. *Hyphae and other structures produced by Glomalean fungi in soil.*

B. *Storage structures (spores, vesicles and auxiliary bodies) produced by fungi in roots or soil.*

C. *Structures formed by Glomalean fungi in mycorrhizal roots.*

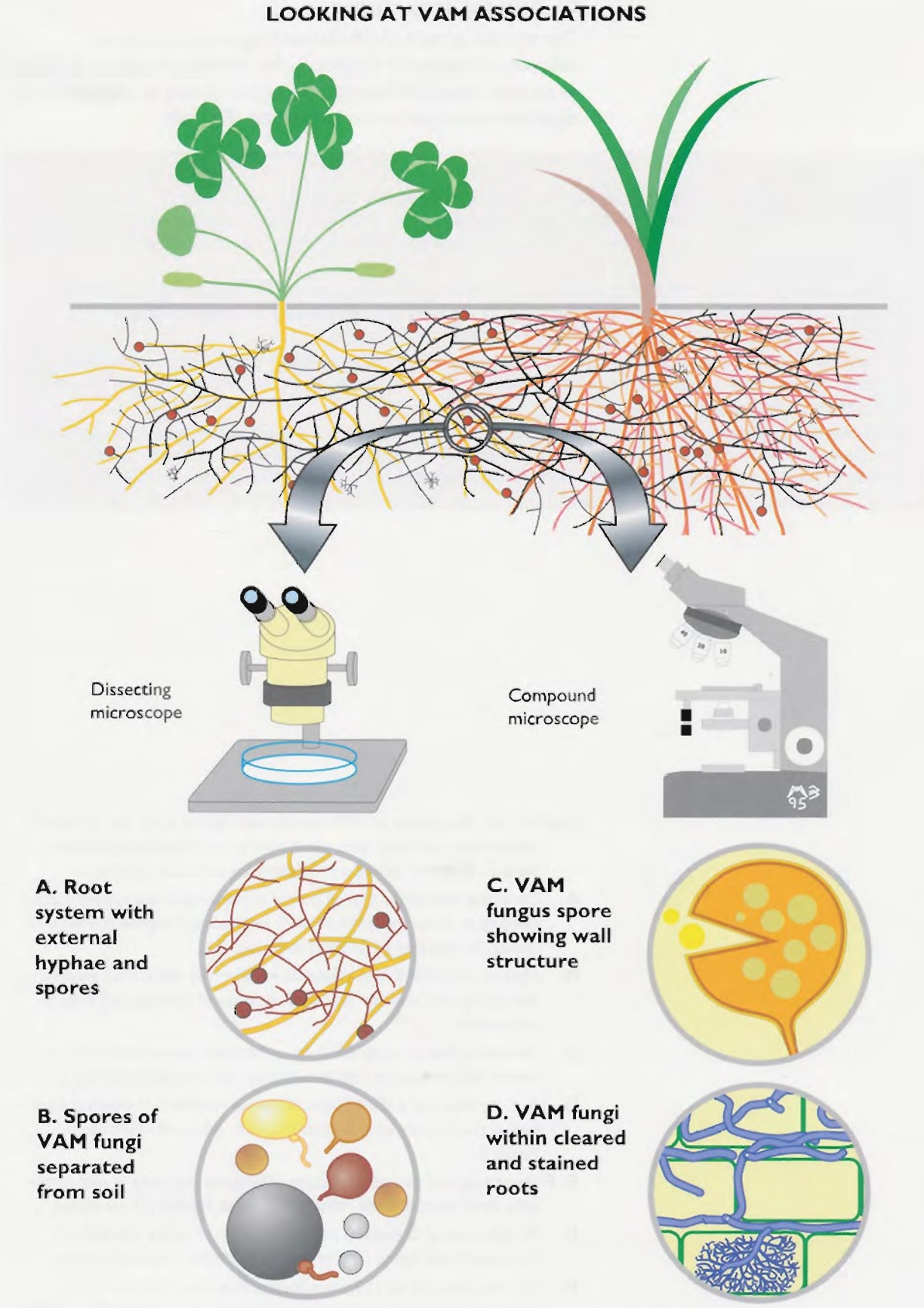


Figure 1.9. An introduction to methods used to study VAM associations.

C. Fungal structures

The mycelial system of Glomalean fungi may produce the following structures in the soil. Some of these structures function as storage organs and are capable of functioning as propagules for dispersal or survival of the fungi in soil (Fig. 1.8).

Terminology:

Soil hyphae — also known as extraradical or external hyphae, are the system responsible for nutrient acquisition, propagation of the association, spore formation, etc. VAM fungi produce different types of soil hyphae including thick 'runner' or 'distributive' hyphae as well as thin, highly branched 'absorptive' hyphae (Friese & Allen 1991).

Auxiliary bodies — also called external vesicles or accessory bodies, are clustered swellings on external hyphae. These are often ornamented by spines or knobs and are characteristic of *Scutellospora* and *Gigaspora*. These apparently do not function as propagules.

Spores — sometimes called chlamydospores or azygospores, form as swellings on one or more subtending hyphae in the soil or in roots. Spores usually develop thick walls, which often have more than one layer and can function as propagules (Chapter 3).

Sporocarps — aggregations of spores into larger structures, which may contain specialised hyphae and can be encased in an outer layer (peridium). This term can be misleading, as the sporocarps produced by most Glomalean fungi are small and relatively unorganised structures compared to those produced by ECM fungi.

Figure 1.10. *Morphology of VAM associations. Figures* **A–G** *are of cleared leek (*Allium porrum*) roots which have been stained with Chlorazol black E.* **B–G** *are viewed with interference contrast microscopy.*

A. *Dissecting microscope view of part of a mycorrhizal root system washed carefully to reveal the intact network with external hyphae (arrow) with spores (S) produced by* Glomus mosseae.

B. *Hyphae of a VAM fungus (*Glomus versiforme*) which have grown on the surface of a root and produced appressoria (arrows), just prior to penetration.*

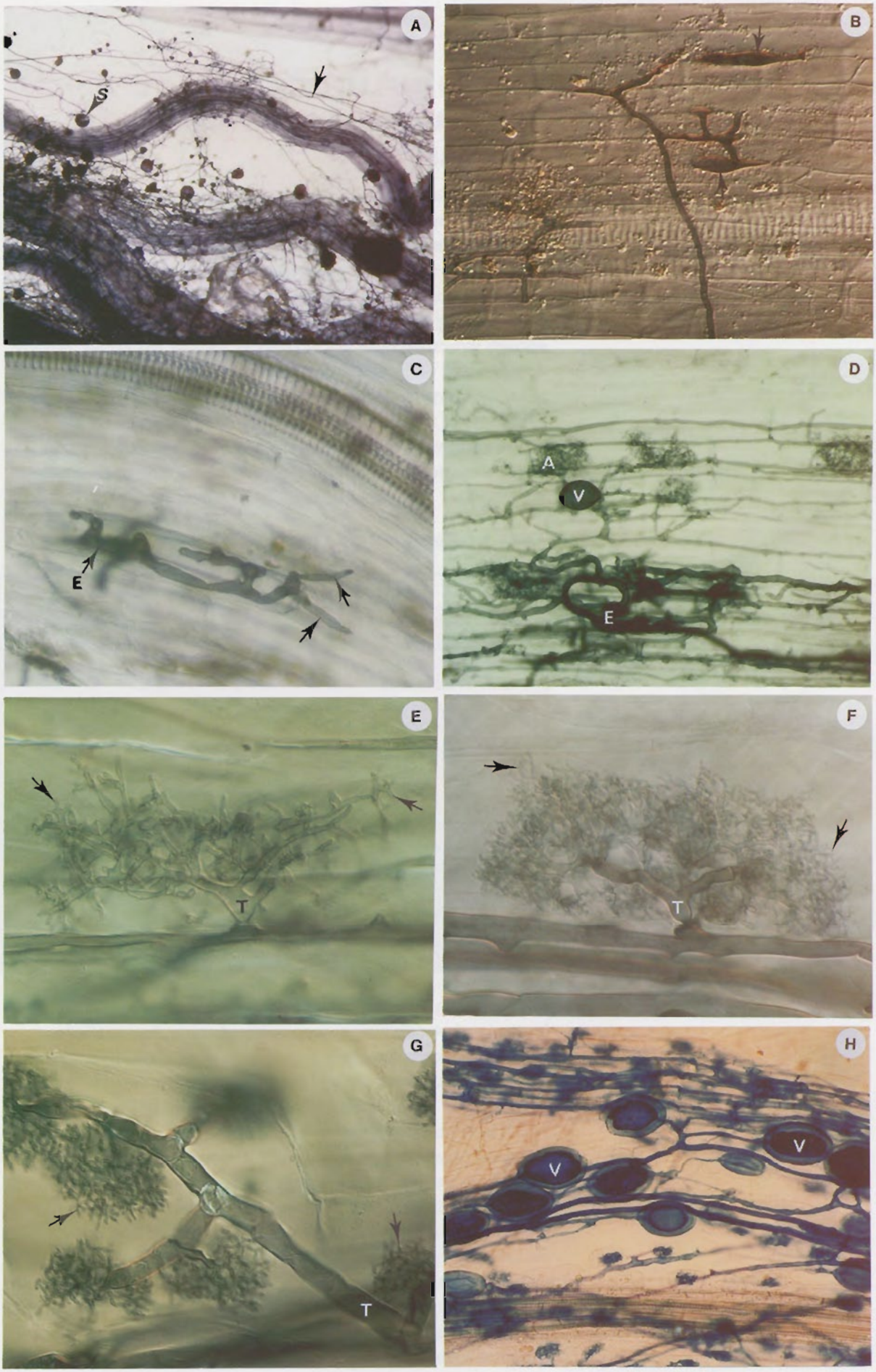
C. *Internal hyphae (arrows) which have colonised an exodermal cell and several adjacent cortex cells immediately after root penetration (E).*

D. *A young colony of a VAM fungus (*Glomus versiforme*) resulting from hyphal growth from an entry point (E) with arbuscules (A) and a vesicle (V).*

E, F. *Developing and mature arbuscules of* Glomus mosseae *in root cortex cells. Fine branch hyphae (arrows) and trunk hyphae (T) are shown.*

G. *An arbuscule of* Gigaspora margarita *in a root cortex cell with an elongated trunk hypha (T) and tufts of fine branch hyphae (arrows).*

H. *Vesicles produced by a* Glomus *species in a clover (*Trifolium subterraneum*) root (routine light microscopy of a cleared root stained with trypan blue).*


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1.5. THE INFLUENCE OF ROOT STRUCTURE ON MYCORRHIZAL FORMATION

A study of the roots and mycorrhizal associations of 20 species of Canadian temperate forest trees demonstrated substantial anatomical diversity of their roots (Brundrett et al. 1990). The ultimate lateral roots of most tree species were consistently mycorrhizal, and many species had heterorhizic root systems with separate long and short lateral roots. These tree roots displayed enough structural diversity in features such as thickened, lignified, or suberized walls, and secondary metabolite-containing cells to allow randomly collected root samples to be assigned to genera. The roots of the Canadian trees examined belonged to four major anatomical categories, resulting from major differences between angiosperm and gymnosperm roots, and between those with ECM or VAM associations (Table 1.7).

Table 1.7. Mycorrhizal root morphology of trees from eastern Canada.

	Angiosperms	*Gymnosperms*
VAM	*Juglans* (Juglandaceae), *Ulmus* (Ulmaceae), *Prunus* (Rosaceae), *Acer* (Aceraceae), *Fraxinus* (Fraxinaceae) • non-heterorhizic or partially heterorhizic roots • Arbuscules, vesicles and hyphae in cortex	*Thuja* (Cupressaceae) • non-heterorhizic roots with phi thickenings • Arbuscules, vesicles and hyphae in cortex
ECM	*Salix* (Salicaceae), *Tilia* (Tiliaceae), *Betula, Carpinus, Ostyra* (Betulaceae), *Fagus, Quercus* (Fagaceae) • heterorhizic roots • Hartig net hyphae restricted to the epidermis, cortex thin with modified walls	*Pinus, Larix, Picea, Abies, Tsuga* (Pinaceae) • heterorhizic roots • Hartig net hyphae extend deep into the thick cortex

Root anatomy features can provide valuable clues about the life history and mycorrhizal susceptibility of roots. For example, mycorrhizal formation only occurs in actively growing or recently formed roots, which can be recognised by observing the distance of xylem and endodermis cell maturation from the root tip. Roots which have stopped growing have mature xylem vessels at their apex and may also have a metacutinized root cap (Fig. 1.11A). These features are readily apparent after roots have been cleared and stained (Chapter 4). Massive aerenchyma formation in roots growing in waterlogged soil would greatly reduce the habitat available for mycorrhizal fungi in roots (Fig. 1.11H). Root anatomy features can also provide clues to the ecological function of roots. Suberin and lignin deposits in root cell walls are correlated with the life span of roots and probably also their capacity to tolerate extreme environmental conditions (Brundrett & Kendrick 1988).

Structural features of roots can influence the formation of VAM associations (Table 1.8). Gallaud (1905) observed that VAM associations in different species formed two distinctive morphology types — which he named the *Arum* and *Paris* series after two host plants. In roots with *Arum* series, VAM hyphae proliferate in the cortex by growing longitudinally between host cells (Fig. 1.11), while in *Paris* series VAM hyphae spread by forming coils within cells (Fig. 1.11). This distinction arises because hyphae grow through longitudinal intercellular air spaces (Fig. 1.11) when these are present (Brundrett et al. 1985, Brundrett & Kendrick 1988). Suberin in the cell walls of exodermal cells appears to regulate the pathway VAM fungus hyphae follow during root penetration (Fig. 1.11). *Paris* type VAM associations have been described less often than *Arum* type associations in the literature and are sometimes considered to be unusual (Malloch & Malloch 1981, Jacquelinet-Jeanmougin & Gianinazzi-Pearson 1983). However, we have observed that associations with coils are often as common as associations with linear hyphae in natural ecosystems. Experiments have demonstrated that the same VAM fungus can form either type of association in different hosts and substantial growth responses have been measured for both (Gerdemann 1965, Nemec 1987).

Ectomycorrhizal root morphology is also influenced by root anatomy features (Tables 1.7, 1.8, Fig. 1.12). Angiosperms with ECM such as *Eucalyptus, Betula, Populus, Fagus* and *Shorea* usually have a shallow Hartig net which is confined to the epidermis (Alexander & Hogberg 1986, Massicotte et al. 1987), while in Gymnosperms such as *Pinus*, Hartig net hyphae generally extend deep into the cortex (Harley & Smith 1983, Kottke & Oberwinkler 1986). Examples of typical Angiosperm and Gymnosperm ECM roots are shown in Figures 1.6 and 1.12. Structural characteristics of host roots, particularly of cells in the hypodermal layer, are thought to restrict ECM fungus hyphae to the epidermis in most Angiosperms (Ling-Lee et al. 1977, Brundrett et al. 1990). Hyphal penetration in Gymnosperms may also be stopped by inner-cortex wall features (Fig. 1.12, see Brundrett et al. 1990).

The root systems of ECM plants are typically heterorhizic and there may be major differences in root anatomy between short roots and long roots (Fig. 1.12). The long roots of many species rapidly develop a periderm, which may further limit their susceptibility to mycorrhizal formation (Fig. 1.12H).

Root structure–mycorrhizal morphology interactions require further investigation. In particular, comparisons of the time-course of mycorrhizal and root development and integrated studies using histological and ultrastructural investigations are required (Chapter 4). It is important to understand which features of ECM root morphology are caused by the host, and which are caused by fungi (Table 1.8), as the latter may be related to host growth responses.

Table 1.8. Root morphology characteristics which influence mycorrhiza formation.

Association	*Anatomical feature*	*Influence on mycorrhizas*
VAM	cortex air channels	hyphal distribution and growth rates, arbuscule distribution
	epidermis and hypodermis structure	appressorium position and path of root penetration
ECM	hypodermis structure	Hartig net position (epidermis or cortex)
	root growth rate	mycorrhiza formation

Figure 1.11. *Root anatomy and VAM formation.*

A–E, I. *Cleared roots stained with Chlorazol black E and viewed with interference contrast microscopy (Chapter 4).*

A, B. *Surface view of cleared and stained* Smilacina racemosa *roots.*

A. *Low-magnification view of a metacutinized root cap (M) and long and short cells in the dimorphic exodermis. These suberized cells protect the root during periods of inactivity. VAM fungus entry points are also shown (arrows).*

B. *Higher-magnification view of alternating long (L) and short (S) cells in the dimorphic exodermis. Hyphae of VAM fungi have penetrated the relatively unsuberized short cells (arrows).*

C, D. *Arbuscules (A) and convoluted hyphae (arrows) in the inner cortex of* Erythronium americanum *and* Asarum canadense *roots respectively. This hyphal growth pattern is typical of roots without cortical air channels.*

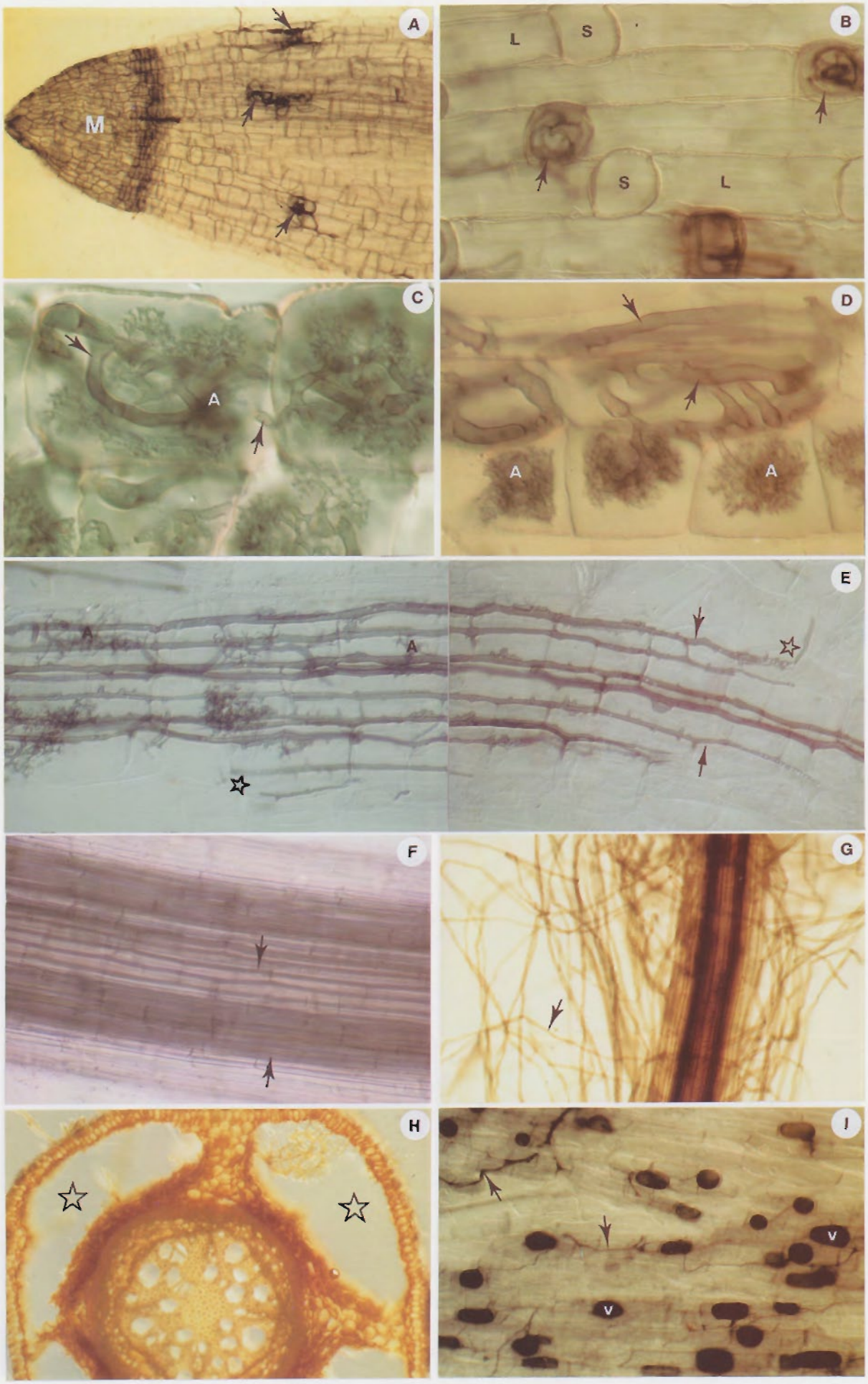
E. *Longitudinal growth of hyphae of a VAM fungus (*Glomus versiforme*) along cortex air channels in an* Allium porrum *root. Note progressive development of arbuscules (A). Some hyphae were damaged during preparation (stars).*

F. *Intercellular air channels (arrows) in a whole mount of a living leek (*Allium porrum*) root, shown for comparison with mycorrhizal development (see E). These channels run continuously from the apex to the base of roots.*

G. *Fern (*Dryopteris intermedia*) root showing extensive root hairs (arrows). This species supports only limited colonisation by VAM fungi.*

H. *Cross-section of a willow (*Salix nigra*) root from wet soil showing cortex disruption by large air channels (stars). Much of cortex below the epidermis and exodermis is gone. The stele of this root is in an early stage of secondary growth (arrows).*

I. *Saprophytic growth of a putative VAM fungus, which has produced hyphae and vesicles (arrows), within a rhizome scale of* Hydrophyllum virginianum, *a non-mycorrhizal plant.*


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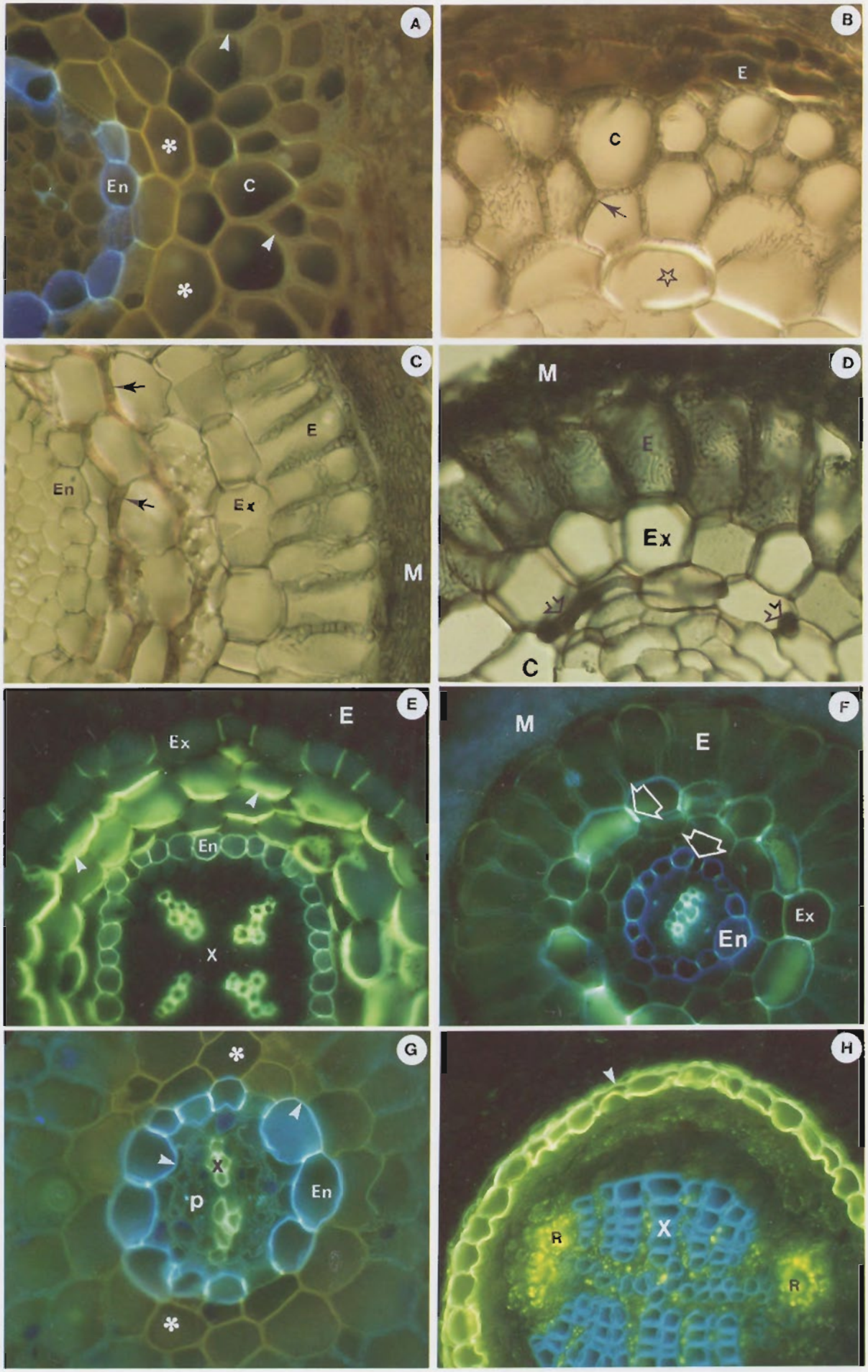

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Figure 1.12. Root anatomy and ECM formation — histochemical investigations of mycorrhizal tree roots (see Chapter 4).

E–H. *Hand sections of field-collected root material stained with the fluorochromes berberine-aniline blue (E–G) and fluorol (H).*

A, E–H. *Photographed using UV-light induced fluorescence.*

B–D. *Cleared hand sections stained with Chlorazol black E and viewed with interference contrast microscopy.*

A. *Unstained cross section of* Larix laricina *ECM root showing UV-induced autofluorescence of Hartig net hyphae (arrows), endodermal cell walls (En) and tannins in inner cortex cell walls (asterisks) — which may limit hyphal penetration.*

B. *Cross-section* Tsuga canadensis *ECM root with cortex (C) Hartig net hyphae (arrows), tannin-filled epidermal cells (E). Inner-cortex cells (star) with thickened walls apparently limit hyphal penetration.*

C. Quercus rubra *ECM root cross-section showing the mantle (M), Hartig net hyphae around elongated epidermal cells (E), massively thickened cortex cell walls (arrows), and endodermal cells (En). (Compare with E.)*

D. *Cross-section of a* Betula papyrifera *(white birch) root with mantle hyphae (M), an epidermal Hartig net (E), Exodermis (EX) and Phi thickenings (arrows) in cortex cell walls (C). (Compare with F.)*

E. *Cross-section of a* Quercus alba *(white oak) root with fluorescent staining of exodermal Casparian bands (Ex) below the epidermal Hartig net (E), as well as endodermal (En), xylem (X) and lignified cortex cell walls (arrows).*

F. *Cross-section of a* Betula papyrifera *(white birch) root with fluorescent staining of exodermal (Ex) and endodermal (En) Casparian bands (arrows). The mantle (M) and epidermal Hartig net (E) are also visible.*

G. *Similar section to A, with fluorescent staining of endodermal (En) Casparian bands (arrows), suberin lamellae (arrows) and phloem (P). Note autofluorescence of xylem cells (X) and cortex-wall tannins (asterisks).*

H. Picea glauca *long root in early secondary growth, with fluorol-induced fluorescence of suberin lamellae in periderm cells (arrow) and resin duct materials (R).*

1.6 IDENTIFYING AND DEFINING MYCORRHIZAL ASSOCIATIONS

Different criteria have been used to designate plants with mycorrhizal associations and this has sometimes led to confusion in the mycorrhizal literature (Harley and Smith 1983, Brundrett 1991). *It is vital that consistent definitions are used to distinguish mycorrhizal association types and that these definitions are stated in published work to avoid future misunderstandings.* Problems of terminology and definition used to describe VAM and ECM associations are discussed below.

A. VAM associations

The presence of arbuscules in a root is generally used to designate plants with VAM. However, these structures are ephemeral and may be absent from field-collected roots. Consequently, hyphal colonisation alone is often used to identify VAM associations, but hyphae and vesicles of VAM fungi will also occupy non-host roots (see below). Hyphal coils or longitudinal hyphae, but not arbuscules, may be seen in roots collected from the field, for the following reasons.



1. Roots of many species persist in the soil for months or years without secondary growth, but arbuscules are ephemeral structures that last for only a few weeks (Brundrett & Kendrick 1990b). Consequently, the probability of finding roots with arbuscules is quite low for many species. However, it should always be possible to find arbuscules if samples contain young roots (with growing tips). There will be certain times of the year when soil conditions (temperature and moisture) favour root growth and other times when roots are inactive. Sampling at times when young roots (with growing tips) are present is the best way to obtain roots with arbuscules.
2. Roots from the field are often heavily pigmented with phenolics and other secondary metabolites and may require a post-clearing bleaching step. However, bleaching roots with chlorine bleaches or hydrogen peroxide will reduce subsequent staining of fungal structures (see 4.2) and the delicate branch hyphae of arbuscules will be the first structures to be affected. (The stains used for mycorrhizas apparently bind to melanin in fungal walls – which is chemically similar to the phenolic pigments in roots you are trying to remove.)
3. Root anatomy features of many plants (see 1.5) results in VAM fungi producing coils of hyphae in many cortex cells, while arbuscules may be restricted to a single cortex layer (Brundrett & Kendrick 1988).
4. In ecosystem studies, saprophytic colonisation of non-host roots, rhizome scales, etc. by hyphae and vesicles of Glomalean fungi is relatively common (Brundrett & Kendrick 1988). This saprophytic activity differs from normal mycorrhizal associations, because arbuscules were never

present, the roots involved usually are senescent (they often also contain saprophytic fungi) and the plant does not benefit from fungal colonisation.

Hyphae of VAM fungi without arbuscules are often found in older roots collected in the field and care must be taken before calling them mycorrhizal. Mycorrhizal fungus hyphae can usually be identified by characteristic branching patterns (Chapter 3), but these structures also form in non-host roots. Confirming that an association was present will require a judgment to be made based on prior knowledge of that plant species. This knowledge includes root phenology information and prior observations of the same or closely related species. More information will result in safer conclusions.

B. ECM associations

The presence of a Hartig net, consisting of labyrinthine hyphae between root epidermal or cortex cells, is normally used to identify ECM roots (Harley & Smith 1983). Eucalypt ectomycorrhizas can have a typical morphology, consisting of a well-developed epidermal Hartig net and thick mantle, as first described by Chilvers & Pryor (1965). However, we have observed that other association types are common in Australian plant communities. For example, eucalypt roots with superficial sheathing ECM with a thin mantle (formed by *Cortinarius* and *Hysterangium* species, etc.) can dominate in mature eucalypt forests (Malajczuk et al. 1987). Australian plants, such as *Acacia* species, often have associations with putative ectomycorrhizal fungi, that have a mantle with little or no Hartig net, but are still considered to have ECM (Warcup 1980, Kope & Warcup 1986, Brundrett et al. 1995). The identification of atypical associations as ectomycorrhizal remains controversial and the following points should be considered before proceeding.



1. Most mycorrhizal scientists are hesitant to recognise ECM associations if (1) there is no Hartig net, (2) fungal associates are not known to form ECM with other hosts, and (3) host growth responses due to nutrient uptake have not been demonstrated.
2. Morphological definitions have always been used to identify mycorrhizal associations and should not be discarded too quickly. In the past it has been the normal practice to avoid calling unusual associations ectomycorrhizas, at least until more is known about them. Ectendomycorrhizas, arbutoid and monotropoid associations are all considered to be distinct types of mycorrhizas, but are anatomically similar to ECM associations (Table 1.1).
3. Mycorrhizal experiments have shown that Hartig net formation is a good indicator of host–fungus compatibility and is correlated with host growth responses (Tonkin et al. 1989, Burgess et al. 1994, Dell et al. 1994).
4. Ectomycorrhizal associations must be distinguished from saprophytic fungi, which grow on the surface of roots

collected in the field, and ECM fungi may colonise the surface roots of incompatible species. Thus, fungi colonising the root surface may be beneficial, harmful or neutral to plants.

5. A functional definition of ECM associations may have to be used for plants which do not form typical associations. Such a definition could be based on measurements of a substantial host growth response resulting from the presence of an ECM fungus at realistic soil nutrient levels (representative of the soils in which plants normally grow).

In conclusion, there are many strange things happening in the soil in Australia and no doubt some of the unusual associations are evolving towards or away from ECM associations, but others likely represent saprobic activity on the root surface.

It should be noted that observations of the fruiting of putative ECM fungi near a potential host plant do not provide sufficient evidence to confirm the presence of an association (Harley & Smith 1983). Problems arise if fungi fruit a considerable distance from their host tree or are wrongly assumed to form ECM associations. A number of older literature citations of this type are wrong and involve trees such as *Acer, Fraxinus and Ulmus*, which are now known not to have ECM (see Harley & Harley 1987 for examples). One such example involves the fungus *Boletinellus merulioides* which was thought for many years to be an ECM associate of *Fraxinus* – a tree which only has VAM. It is now known this fungus forms an association with aphids which are parasitic on *Fraxinus* roots (Brundrett & Kendrick 1987).

C. Dual associations

Surveys of the mycorrhizal literature have established that plants within a genus usually have the same type of mycorrhizas (ECM, VAM, etc., or else they remain non-mycorrhizal) and these relationships are generally also consistent within a family (Harley & Harley 1987, Newman & Reddell 1987, Brundrett & Abbott 1991). This high correlation between plant phylogeny and mycorrhizal relationships has been observed for families with ECM, as well as those containing species that are usually non-mycorrhizal, but there are also many exceptions (Harley & Smith 1983, Testier et al. 1987, Brundrett 1991).

In the northern hemisphere, there are some examples of trees which often form both ECM and VAM, such as *Populus* spp. (Brundrett et al. 1990). Other reports of VAM in roots of species which normally form ECM concern hyphae and vesicles, but not arbuscules (Vozzo & Hacskaylo 1974, Malloch & Malloch 1981, Harley & Harley 1987, Cázares & Trappe 1993). It is unlikely that these reports result from misidentification of the fungus, since VAM-fungus hyphae and vesicles have a characteristic appearance (Chapter 3). However, VAM fungi also colonise a variety of substrates, including soil organic material and senescing roots of non-mycorrhizal species (St John et al. 1983, Brundrett & Kendrick 1988, Cázares & Trappe 1993). It is probable that many

reports of these structures in roots which normally form ECM associations represent such saprophytic activity. Roots of non-host plants may not be able to completely exclude VAM hyphae, given the nature of the activity of these fungi and their nearly universal presence in soils (Harley & Smith 1983).

Many Australian trees and shrubs such as *Eucalyptus* and *Acacia* species have been reported to have both ECM and VAM associations (Warcup 1980, Malajczuk et al. 1981, McGee 1986, Chilvers et al. 1987, Reddell & Warren 1987, Brundrett & Abbott 1991). However, there is some controversy about the relative importance of ECM and VAM associations in plants which have both. These include economically important Australian trees and shrubs in the families Mimosaceae, Casuarinaceae, Fabaceae and Myrtaceae (Table 1.2). For example, in northern and eastern Australia, *Acacia* species were reported to have dual VAM and ECM associations or VAM only (Warcup 1980, Reddell & Warren 1987, Bellgard 1991, Brundrett et al. 1994), while acacias from south-western Australia had VAM, but not ECM (Jasper et al. 1989, Brundrett & Abbott 1991). There have also been reports that eucalypt seedlings may initially have VAM associations, which are replaced by ectomycorrhizal associations as they mature (Gardner & Malajczuk 1988). In the family Casuarinaceae, the genus *Casuarina* apparently has VAM while *Allocasuarina* has ECM and VAM (Table 1.2). From the above discussion, it can be seen that there is at present often insufficient knowledge to safely predict what type or types of mycorrhizal associations a given plant species will have, or what the functional significance of different associations is. The versatility of roots of many important Australian trees and shrubs may be an adaptation to survival in response to harsh or fluctuating environmental conditions, and provides increased options for harnessing mycorrhizal associations in plantation forestry.

D. Facultative associations and non-mycorrhizal plants

The concept that plants have varying degrees of dependence on mycorrhizal associations is gaining acceptance (see Janos 1980, Marschner 1986, Brundrett 1991). Detailed examinations of plants in natural ecosystems often show consistent differences between host plants occurring in a particular habitat in both the intensity and consistency of mycorrhiza formation (proportion of root system involved). These observations have shown that species generally either have consistently high levels of mycorrhizas, intermediate or variable levels of mycorrhizas, or are not mycorrhizal (Janos 1980, Brundrett & Kendrick 1988). Plants belonging to these categories can be called obligatorily mycorrhizal, facultatively mycorrhizal, or non-mycorrhizal, as is explained below. Characteristics of plant roots systems are usually highly correlated with these mycorrhizal formation categories, as is summarised in Table 1.9.

Obligatorily mycorrhizal plants have been defined as those which will not survive to reproductive maturity without being associated with mycorrhizal fungi in the soils (or at the fertility levels) of their natural habitats (Janos 1980). These species consistently support mycorrhizal colonisation throughout most of their young roots.

Facultatively mycorrhizal plants are those that benefit from mycorrhizal associations only in some of the least fertile soils in which they naturally occur (Janos 1980). In ecosystem surveys, inconsistent mycorrhization (Trappe 1987) or low levels of mycorrhizal colonisation (less than 25% – Brundrett & Kendrick 1988) have been used to designate facultatively mycorrhizal species when soil fertility levels could not be manipulated.

Non-mycorrhizal plants have roots that consistently resist colonisation by mycorrhizal fungi, at least when they are young and healthy. These observations provide evidence that intrinsic properties of roots can restrict mycorrhizal formation. Nutrient levels and other soil properties and mycorrhizal propagule dynamics can also reduce mycorrhizal formation, but usually do not prevent it completely.

Table 1.9. Typical features of host root systems and mycorrhizal formation that are associated with categories of mycorrhizal formation.

Designation:	*Obligate*	*Facultative*	*Non-mycorrhizal*
Colonisation:			
Arbuscules	young roots	sparse or variable	none
hyphae or vesicles	older roots	sparse or variable	may occur in old roots
Roots:			
diameter	often coarse	usually fine	usually fine
root hairs	few/short	many/long	many/long

In natural ecosystems, plants with facultatively mycorrhizal associations or non-mycorrhizal roots are more common in very dry, wet or cold habitats where plant productivity is limited by soil/environmental conditions, or in disturbed habitats where mycorrhizal fungus inoculum is limited (Brundrett 1991). Non-mycorrhizal trees are rare (members of the Australian family Proteaceae are one exception). There are a number of non-mycorrhizal genera which are important in agriculture and horticulture including members of the families Chenopodiaceae, Amaranthaceae, Caryophyllaceae, Polygonaceae, Brassicaceae, Scrophulariaceae, Commelinaceae, Juncaceae and Cyperaceae (Newman & Reddell 1987, Testier et al. 1987, Brundrett 1991).

E. Recommendations

The presence of arbuscules should be used to identify VAM associations and the presence of a Hartig net should be used to define ECM associations. However, these definitions cannot always be used and careful judgment may be required when examining mycorrhizal associations in roots collected from the field, particularly if roots are old, or associations are atypical. Some older reports of the mycorrhizal status of hosts should be disregarded if they do not include evidence that mycorrhizal definitions were rigorously applied or anatomical investigations were carefully conducted.

There is disagreement about whether arbuscular mycorrhizas or vesicular-arbuscular mycorrhizas is the most appropriate name for these associations (Section 1.4). It is important that scientists use consistent terminology, because this influences the capacity of computer searches to find papers, abstracting services to categorise them, and the accessibility of mycorrhizal literature to other scientists and students. It seems that the name arbuscular mycorrhizas is gradually replacing vesicular-arbuscular mycorrhizas in the scientific literature. It is best to use both the words arbuscule and Glomalean in the title or keywords of papers to ensure they will be retrieved by computerised search programs in the future.

The terms *ectomycorrhiza, ectomycorrhizas, ectomycorrhizal* should be used for ECM associations.

The degree of mycorrhizal formation of a host plant can be expressed as the proportion (%) of root length colonised by mycorrhizal fungi (see Chapter 4). However, roots with a periderm (bark) layer resulting from secondary growth should be excluded, since they have no cortex. In ecosystem surveys, it is best to express the degree of mycorrhizal colonisation as the proportion of roots available for colonisation that were mycorrhizal. This requires an understanding of the dynamics of root growth and mycorrhizal formation.

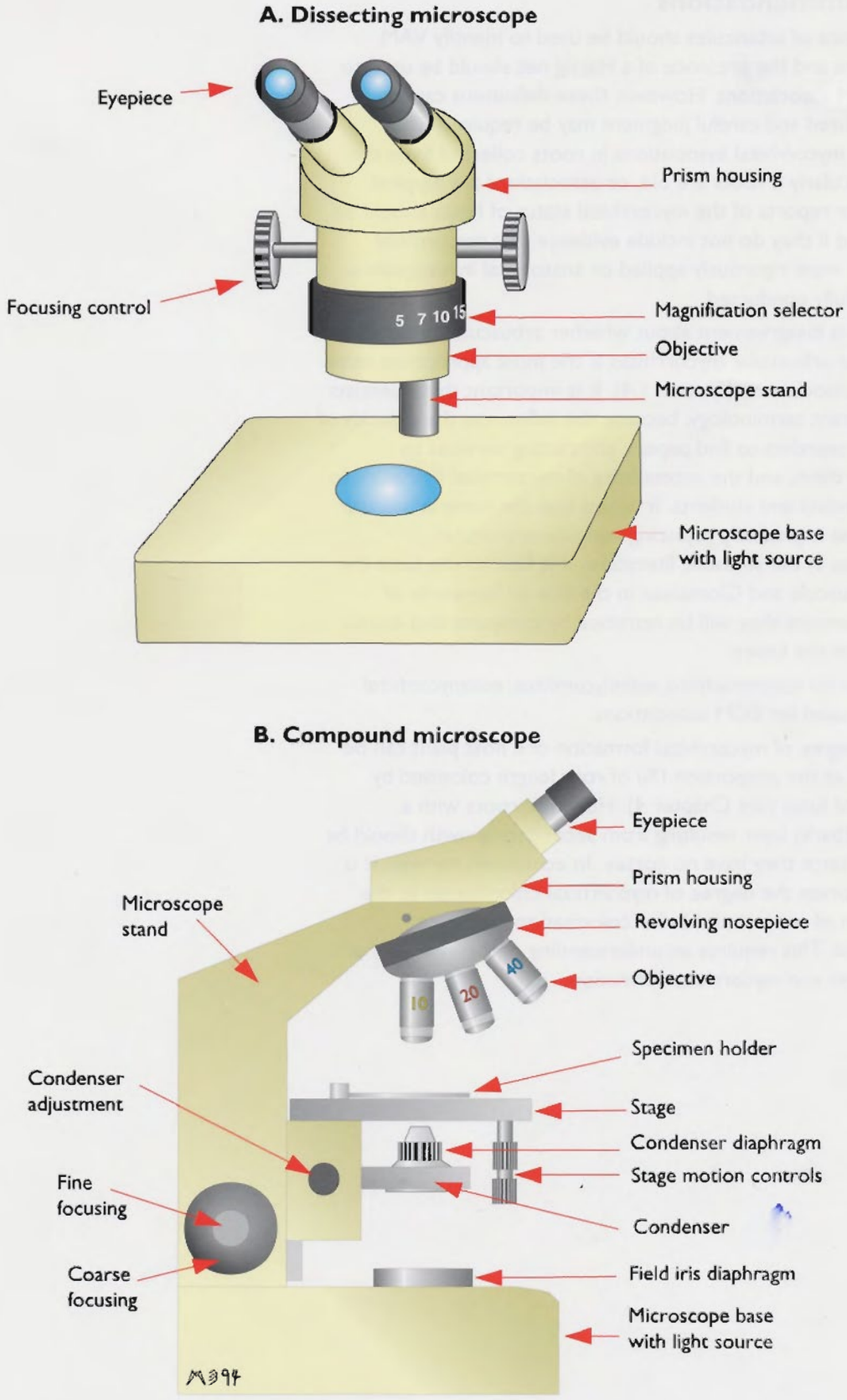


Figure 1.13. *Major components of dissecting (**A**) and compound (**B**) microscopes.*

1.7. WORKING WITH MICROSCOPES

Many of the procedures introduced in this manual require the use of a microscope to examine details of fungal or root structure that would not otherwise be visible. Mycorrhizal research activities that require access to a good-quality dissecting and compound microscopes include fungal identification, confirmation of association types, quantification of mycorrhizal roots, and identification of roots within samples. Publication of the results of mycorrhizal experiments often requires microscope photographs to illustrate details of mycorrhizal or fungal anatomy. More information on specialised microscope techniques is presented in Chapter 4.

Parts of basic compound and dissecting microscopes are shown in Figure 1.13, and some information about their use is provided here. However, it is recommended that you also refer to books on microscopy, especially if you intend to use histochemistry, fluorescence microscopy, interference contrast microscopy, or electron microscopy methods (Chapter 4).

A. Dissecting microscopes

These microscopes normally have two eyepieces to provide stereoscopic vision and are most often used to view whole structures at relatively low magnifications. In mycorrhizal studies they are used to quantify mycorrhizal roots (Chapter 4), to help identify fungi (Chapters 2, 3), check fungal cultures (Chapter 5), and to assess the results of mycorrhizal inoculation (Chapter 6). Some notes on dissecting microscope use are provided here.



1. *It is advisable to keep all microscopes separate from areas where samples are prepared to minimise exposure to dust and liquids.* If samples contained in liquids (cleared and stained roots, etc.) are used routinely, it is advisable to have a clear plastic platform made to protect the base of the microscope.
2. Several types of illumination are required for mycorrhizal studies.

(i) Incident-light illumination is required to required to count spores of VAM fungi and select them to start pot cultures (Chapter 3) and to view unstained roots, fungal fruit bodies, etc. It is best to use a cool light source when observing living material such as VAM fungus spores (halogen lamps produce less heat than incandescent light bulbs of similar intensity). Fibre-optic adjustable arms or ring lights mounted on the objective lens are very useful (obtained from microscope manufacturers).

(ii) A transmitted light source, which is built into the microscope base, is required to assess mycorrhizal colonisation in cleared and stained root samples.

(iii) Darkfield illumination is not essential, but allows unstained hyphae and roots to be observed more easily.

B. Compound microscopes

A good quality compound microscope is an essential tool for mycorrhizal research, to allow fungus identification by examining fungal structures (Chapters 2, 3), and to examine sections of mycorrhizal roots or mounted segments of cleared and stained roots (Chapter 4). Microscopes should have powerful built-in illumination, interchangeable objectives including a 100X oil-immersion objective and a fully adjustable condenser and dual eyepieces. Some notes about microscope use are provided below.



1. All parts of the microscope must be kept free of dust and cleaned carefully when they become dirty. Dust and dirt on microscope components are the most common causes of poor image quality.
2. Great care must be taken when examining liquid preparations such as unsealed slides, fungi growing on culture media, etc. to avoid contact with objectives. Immersion oil must be used very carefully to avoid damage to other objectives. Objective lenses are also easily damaged by abrasion.
3. The microscope condenser must be carefully adjusted so that illumination of the specimen is uniform and sufficiently bright. Condenser adjustments are often required when objective lens magnification is changed. Kohler illumination is required to obtain uniform lighting of the specimen with a compound microscope. This can be achieved by raising the condenser until the image of its iris diaphragm is in focus, centring that image and then opening the iris beyond the field of view of the objective that is being used.
4. Light microscopes may have additional components to allow polarised light, interference contrast, phase contrast, or fluorescence microscopy (see Chapter 4). These may be of great value in mycorrhizal studies, but require different accessories to be installed and these parts are often very expensive.

C. Photography

Microscope photographs are commonly used to document taxonomic features of fungi and the results of experiments. A relatively high quality microscope with a dedicated camera and photomicroscopy controller is normally required to obtain satisfactory results. Some information on microscope photography is provided here, but more specific instructions are provided in the manuals provided with photomicroscopes.



1. Photographic exposures are usually controlled by a dedicated camera control unit. It is often wise to bracket exposures (± 1 or 2 F stops).
2. The cleanliness of microscope parts, and proper adjustment of the condenser (see above) is most important when photographs are taken, as microscopy errors are often magnified when slides or negatives are printed.
3. Colour photographs can be taken with a microscope by using a film which is colour-balanced for the light source used (usually tungsten). The light source voltage of the microscope also needs to be adjusted to obtain the correct colour temperature (typically 3200° K). It is normally best to use a relatively 'slow' slide film (100–200 ASA) such as Ektachrome EPY for microscope photography to obtain sufficient detail of the subject material, but faster film is used for some purposes. Fine-grained black-and-white negative film is also commonly used for photography.
4. It is essential that records of information pertaining to photographs be recorded, on a record sheet or log book, when they are taken. Essential information includes the exposure number, magnification, details of the subject material, microscope procedures, or stains used, the date and photographer's initials. This will allow photographs to be to be identified and correctly labelled after processing.

Chapter 2

WORKING WITH ECTOMYCORRHIZAL FUNGI

2.1. INTRODUCTION

This chapter concerns methods used for the collection, description, management and identification of larger fungi (Fig. 2.1). Most emphasis is on mycorrhizal fungi, especially those which associate with eucalypts in Australian forests. However, these methods are also applicable to non-mycorrhizal fungi and fungi which fruit on substrates other than soil. Terms used to categorise fungi throughout this chapter are explained below. Refer to Section 1.3 for an introduction to ECM associations.

Major terms for fungi

Larger fungi — fungi which produce macroscopic fruit bodies, large enough to be readily seen with the naked eye.

Fruit bodies — reproductive structures of larger fungi containing sexual spores. These are also called sporocarps, sporophores, ascocarps, basidiocarps, etc.

Epigeous — fruit bodies produced above ground, on the soil, or on other substrates such as wood. These include fungi called mushrooms, club fungi, coral fungi, puffballs, etc.

Hypogeous — fruit bodies produced below ground, e.g. truffles. Hypogeous fungi include some Basidiomycetes and Ascomycetes. They often rely on animal vectors for spore dispersal.

Mushroom-like fungi — fruit bodies with a clearly defined stalk and cap (e.g. like the field mushroom *Agaricus*). These fungi are commonly called mushrooms, or toadstools.

Truffles — hypogeous members of the Ascomycete genus *Tuber* are the true truffles, but other hypogeous fungi (Ascomycetes and Basidiomycetes) are sometimes also called truffles.

Truffle-like fungi — hypogeous Basidiomycetes (sequestrate Hymenomycetes and Gasteromycetes) and Ascomycetes.

Sequestrate — fungi which have spores that are not forcibly discharged from their basidia and remain enclosed by the fruit body at maturity. Spores of these fungi are mostly dispersed by animals. These fungi are often referred to as secotioid or gastroid basidiomycetes and many have close ties with groups of mushroom-like fungi.

Gasteromycetes — an artificial group containing a diverse assemblage of basidiomycetes with spores that are not forcibly discharged from their basidia, but are dispersed by wind, rain, or animals after they have matured. These fungi, which are commonly called puffballs, stinkhorns, earthstars, etc. are mostly not known to be closely related to any of the mushroom-like fungus groups.

Hyphomycetes — known as conidial fungi, or anamorphic fungi, which reproduce by producing asexual spores.

Saprophytic fungi — live on dead organic matter (e.g. litter, wood).

Pathogenic fungi — invade living organisms causing disease.

Symbiotic fungi — involved in mutually beneficial relationships, especially with plants, e.g. mycorrhizal associations.

A. Fungal taxonomy

Taxonomy provides a framework on which to base biological work by providing names of organisms and by giving data about the biology of the organism and its relationship to other organisms. Communication between scientists relies largely upon

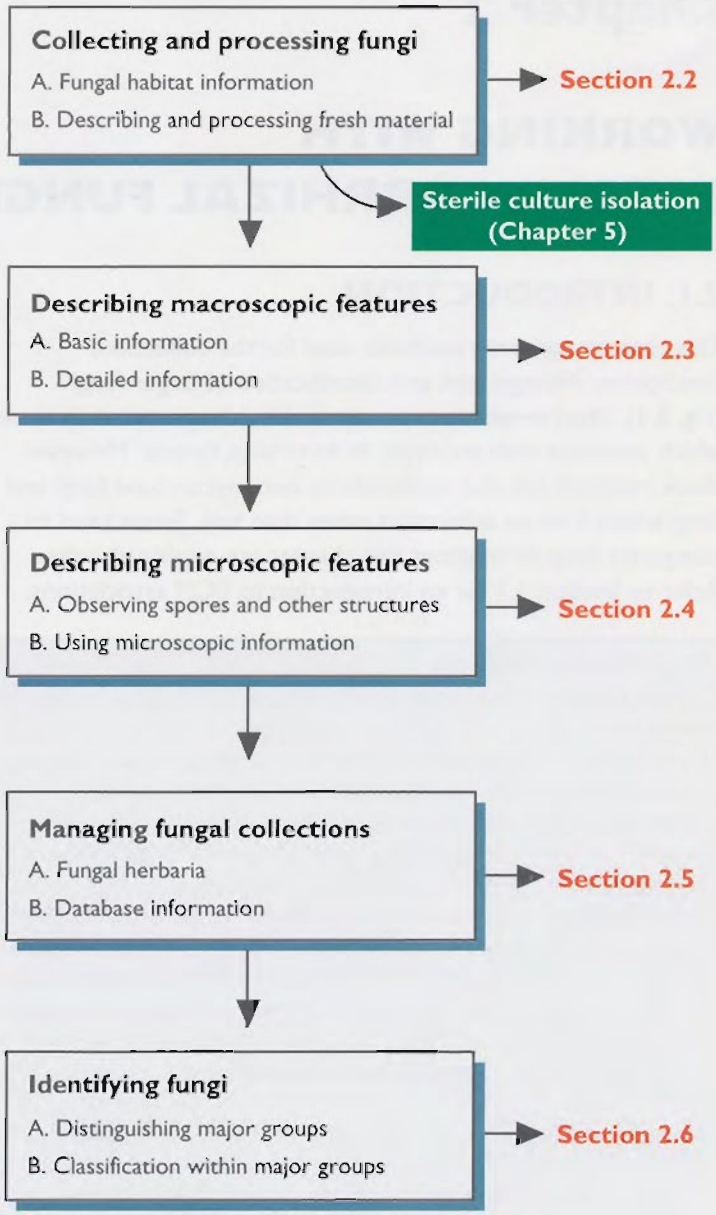


Figure 2.1. *Stages in the collection and identification of larger fungi, corresponding to sections of this chapter.*

the accurate application of names of taxa. The name of a fungus is of prime importance as it opens up access to all of the information that is known about the species and its close relatives. For example, knowledge about which host plants a particular fungus is known to form mycorrhizas with would be significant for ecological studies, especially in habitats where many ectomycorrhizal plant species coexist in close proximity. The accuracy of names applied to fungi is of great importance in enabling communication of data. Incorrect identification of organisms and application of incorrect names may lead to over-generalisations or incorrect conclusions (Trappe & Molina 1986). It is important to realise that not all fungi included in Section 2.6 in the Ascomycetes and Basidiomycetes are ectomycorrhizal, as many are saprophytic or parasitic. The best way to determine if a

specimen is likely to be mycorrhizal is to accurately identify in which taxon it belongs as the mode of life is known and published for many genera. If the fungus is not identified, determination of ectomycorrhizal status may require time-consuming processes such as isolation and testing of pure cultures (Chapter 5).

Identification of fungi relies upon accurate recognition and description of fungal characteristics. Identification may be carried out at any of the three levels described below, depending on the accuracy that is sought. To aid this task, the reader is referred to the outline and illustrations of major fungal groups presented in Section 2.6.

Levels of fungal identification

Level	*Process*	*Determination*
1	• Basic field observations	• Ascomycete vs. Basidiomycete • Family name • Possibly also genus name
2	• Superficial examination of macroscopic details • Comparison with illustrations and descriptions in field guides	• Confirm genus name • Tentative species name
3	• Detailed macroscopic and microscopic examination • Consult taxonomic literature	• Confirm species name (if described)

1. At the first level, the broad group of fungi to which the specimen belongs must be determined, for example, whether the specimen is an Ascomycete or Basidiomycete, and the family to which it belongs. This level of identification will often be insufficient to determine whether or not a specimen is ectomycorrhizal.
2. At the second level of identification, fresh specimens collected in the field can be compared to colour illustrations in commonly available regional field guides and simple keys and descriptions can be used to recognise genera of fungi. Some examples of such books include Phillips 1981, Arora 1986, and Breitenbach & Kränzlin 1991. There are many good reference books on fungi from northern temperate regions, but it is much harder to obtain information on fungi from southern and tropical regions. Identification to genus level will usually be sufficient to determine whether or not a specimen is mycorrhizal. In some cases the fungus may be so distinctive that there is no doubt as to its species name from the information and photographs in field guides. To help identify fungi to genus level, the reader is again referred to the outline and illustrations of fungal taxa presented in Section 2.6.
3. A third level of investigation is often required to accurately identify species of ectomycorrhizal fungi. This usually involves careful examination of macroscopic and microscopic characters (as discussed in Sections 2.3 and 2.4), and the



consulting of specialised monographic works. It may also be possible to obtain advice from a taxonomist, especially one who specialises in the group a particular fungus belongs to. Submitting descriptive data and specimens to a herbarium should eventually result in its identification, but this is a slow process.

No matter what level of identification is sought, three points should be remembered.



1. Macroscopic characters such as fruit body colour and size can vary according to environmental conditions and are not always reliable for determining differences between species.
2. Fungal morphology rarely falls into discrete categories, but rather is often expressed as a continuum that transgresses the formal boundaries of low level taxa such as genera and species (Bougher et al. 1993).
3. Australian fungi have co-evolved with eucalypts and other Australian plants. Consequently many of these fungi are unique and may not conform to taxonomic schemes constructed for northern hemisphere fungi (Bougher & Castellano 1993, Bougher 1995).

B. The biodiversity of Australian fungi

The taxonomy of ECM fungi in many parts of the world is poorly known, particularly in tropical regions and the southern hemisphere including Australia. It is estimated that less than 5% of Australia fungi are named (Pascoe 1991). There may be 250 000 species of fungi in Australia, including perhaps 5000 mushrooms (Pascoe 1991). About 650 species of ECM fungi have been named so far in Australia (Bougher 1995). Australian ECM fungi have co-evolved with Australian plants and are therefore quite unique and extremely diverse (Bougher & Tommerup 1996, Castellano & Bougher 1994, Bougher et al. 1994). Many do not naturally occur outside Australia. Asian fungi associated with trees such as oaks, pines and dipterocarps are mostly different to Australian fungi and are generally not compatible with Australian trees such as eucalypts.

The ECM fungi are predominantly Ascomycetes and Basidiomycetes (see Table 2.1). A few Zygomycetes are also form ECM associations (e.g. Warcup 1990) but these are infrequent and their details are not considered in this chapter. A greater proportion of Zygomycetetes produce VAM and these are dealt with in Chapter 3. Also there are some hyphomycete fungi (for which the sexual stage is lacking or not yet recognised) such as *Cenococcum* which form ECM. One estimate suggests that there are 5000–6000 species of mycorrhizal fungi with the majority being ectomycorrhizal (Molina et al. 1992). Most ECM fungi produce sexual fruiting structures commonly referred to as mushrooms, toadstools, coral fungi, puffballs, and truffles. These are the larger fungi, i.e. they produce structures visible to the naked eye. The taxonomy of ECM fungi is based almost exclusively on characteristics of their sexual fruiting structures (see Section 2.6).

Table 2.1. Ectomycorrhizal and ectendomycorrhizal fungal taxa. List compiled from data in Miller 1982, Kendrick 1992, Molina et al. 1992 and Bougher 1995. (H = hypogeous truffle-like fungi, E = epigeous fungi, P = puffballs, genera with an asterisk have many non-mycorrhizal species, genera in bold are known to occur in Australia.)

Family	*Genera*	*Type*
HYPHOMYCETES		
	***Cenococcum**, etc.*	Conidial
ZYGOMYCETES		
Endogonaceae	***Endogone, Sclerogone***	H
ASCOMYCETES		
Ascobolaceae	***Sphaerosoma***	H
Balsamiaceae	***Balsamia**, Picoa*	H
Elaphomycetaceae	***Elaphomyces***	H
Geneaceae	***Genea, Geneaba***	H
Geoglossaceae	***Geoglossum*, Leotia*, Trichoglossum***	E
Helvellaceae	***Gyromitra, Helvella***	E
	Barssia, Fischerula, ***Gymnohydnotrya, Hydnotrya, Mycoclelandia***	H
Pezizaceae	***Aleuria*, Peziza*, Phillipsia*, Pulvinia,** Sarcosphaera*	E
	Amylascus, Hydnotryopsis, Muciturbo, Pachyphloeus, Peziza, Ruhlandiella	H
Pyronemataceae	*Geopora, Humaria,* ***Jafneadelphus*, Lamprospora* Sphaerosporella,*** *Trichophaea, Wilcoxina*	E
	Choiromyces, ***Dingleya, Elderia,*** *Geopora, Hydnobolites,* ***Hydnocystis, Labyrinthomyces, Paurocotyis Reddellomyces, Sphaerozone,*** *Stephensia*	H
Sarcoscyphaceae	***Plectania*, Pseudoplectania*, Sarcocypha****	E
Terfeziaceae	***Choiromyces,** Terfezia*	H
Tuberaceae	***Mukagomyces,** Paradoxa,* ***Tuber***	H
BASIDIOMYCETES		
Amanitaceae	***Amanita, Limacella***	E
	Torrendia	H
Astraeaceae	*Astraeus*	E
	Pyrenogaster, ***Radiigera***	H
Boletaceae	***Austroboletus, Boletellus*, Boletochaete, Boletus, Buchwaldoboletus*, Chalciporus, Fistulinella,** Gyrodon,* ***Gyroporus, Heimiella, Leccinum, Phlebopus, Phylloporus, Pulveroboletus, Rubinoboletus, Suillus, Tylopilus, Xanthoconium***	E
	Alpova, Boughera, Chamonixia, Gastroboletus, Rhizopogon, Royoungia	H
	Truncocolumella	H
Cantharellaceae	***Cantharellus, Craterellus***	E
Chondrogastraceae	***Chondrogaster***	H
Clavariaceae	***Aphelaria, Clavaria, Clavariadelphus, Clavicorona, Clavulina, Clavulinopsis, Ramaria, Ramariopsis***	E
Corticiaceae	*Amphinema, Byssocorticium, Byssosporia, Piloderma, etc.*	E
Cortinariaceae	***Astrosporina, Cortinarius, Cuphocybe, Dermocybe*, Descolea, Hebeloma, Inocybe,** Leucocortinarius,* ***Rozites, Stephanopus, etc.***	E
	Cortinarius, Cortinomyces, Descomyces,** Destuntzia,* ***Hymenogaster, Quadrispora, Setchelliogaster, Thaxterogaster, Timgrovea	H
Cribbiaceae	***Cribbea,** Mycolevis*	H
Entolomataceae	***Clitopilus, Entoloma, Leptonia, Rhodocybe****	E
	Rhodogaster, Richoniella	H
Elasmomycetaceae	***Elasmomyces, Gymnomyces, Martellia, Zelleromyces***	H
Gelopellidaceae	***Gelopellis***	H
Gomphaceae	***Gomphus***	E
Gomphidiaceae	*Chroogomphus, Cystogomphus, Gomphidius*	E
Hydnaceae	*Bankera, Dentinum,* ***Hydnellum, Hydnum, Phellodon***	E
Hygrophoraceae	***Bertrandia* Camarophyllus, Gliophorus, Humidicutis*, Hygrocybe, Hygrophorus**, *etc.***	E
Hysterangiaceae	***Hysterangium, Pseudohysterangium, Trappea***	H
Leucogastraceae	***Leucogaster, Leucophleps***	H
Lycoperdaceae	***Lycoperdon****	P

Table 2.1. *(continued)*

Family	*Genera*	*Type*
Melanogastraceae	***Melanogaster***	H
Mesophelliaceae	***Castoreum, Diploderma, Gummiglobus, Malajczukia, Mesophellia, Nothocastoreum***	
Octavianinaceae	***Octavianina, Sclerogaster***	H
Paxillaceae	***Paxillus***	E
Pisolithaceae	***Pisolithus***	P
Polyporaceae	*Albatrellus*	E
Russulaceae	***Lactarius, Russula***	E
	Archangeliella, Cystangium, Macowanites	H
Sclerodermataceae	***Scleroderma***	P
	Horakiella, Scleroderma	H
Sedeculaceae	*Sedecula*	H
Stephanosporaceae	*Stephanospora*	H
Strobilomycetaceae	***Strobilomyces***	E
	Austrogautieria, Chamonixia, Gautieria, *Wakefieldia*	H
Thelephoraceae	***Boletopsis, Thelephora***	E
Tricholomataceae	***Clitocybe*, Cystoderma*, Cantharellula,*** *Catathelasma,* ***Laccaria, Lepista, Leucopaxillus, Tricholoma, Tricholomopsis****	E
	Gigasperma, ***Hydnangium, Podohydnangium***	H

2.2. COLLECTING, PROCESSING AND DESCRIBING ECTOMYCORRHIZAL FUNGI

Collecting fungi should be undertaken in a well-planned and ordered fashion, as outlined below, to achieve a high standard of retrievable information (see Fig. 2.2). This protocol for collecting fungi is described in detail in the corresponding parts (A–F) of this Section. Data on fungi and their environments, particularly associated plants and soil, need to be recorded at the time of collection as it is often impossible to retrieve such data at a later date. Morphological characteristics such as shapes, textures and colours are required for identifying fungi and these must be recorded with fungi in their fresh condition. If recorded in a systematic fashion, fungal and environmental information may be synthesised into a valuable database about particular mycorrhizal associations (Section 2.5). The value of a database will be greatly enhanced by preservation of fungal fruit bodies in a herbarium, to provide a perpetual, physical source of confirmatory data.

A. Finding and collecting fungi

Fungal fruit bodies are produced intermittently in response to seasonal changes in environmental stimuli such as rainfall and temperature. Fungi may fruit at any time of the year, particularly in tropical and subtropical regions. In temperate regions fungi are most abundant in autumn, but another spring flush may occur. Each fungal species responds differently, and the fruiting of any particular species cannot always be predicted. Some fungi fruit every year, either once or in several different months, while others do not. Hence, the absence of fruit bodies of a particular species in a specific area at any point in time does not necessarily confirm that its mycorrhizas are absent in the area. Also, the abundance of fruit bodies does not necessarily reflect the extent of mycorrhizal formation by a particular fungus compared with other fungi. However, it is possible to infer broad changes in populations of mycorrhizal fungi in a site by regular observations of fruit bodies at that site over a period of years.

The fruit bodies of ectomycorrhizal fungi are ephemeral structures. Fruit bodies may occur for many weeks or only days. Some species of fungi have large fruit bodies that may take weeks to mature and then decay, while others have small, fragile fruit bodies that may appear and disappear within a day.

A great deal of planning and equipment (listed below) is needed to ensure a successful expedition for collecting ectomycorrhizal fungi. It is obvious that locations of interest, e.g. where particular ectomycorrhizal tree species occur, need to be targeted. If many locations are to be targeted, the most efficient travel arrangements need to be worked out. It is also obvious that collecting expeditions must be timed to coincide with the appearance of fruit bodies in the target area. Therefore the precise timing of expeditions must be as flexible as logistics

Protocol for processing fungal specimens

A. Locate and collect fungal specimens.

B. Assign a code number to each collection. Record the site and date of collection, the collector's name, and details of the habit, soil type and associated vegetation.

C. Examine, draw, and record basic macroscopic details (Section 2.4), with particular attention given to variations with age. Accurately describe the colour of the fresh specimens using daylight and a reference colour chart.

D. Clean off adhering soil and debris. Take colour photographs showing young, mature, and old specimens, some in longitudinal section, on a standard grey background with a scale (mm). (Examples of photographs are provided in this manual from Figure 2.20 onwards.)

E. Set up a spore print on white paper (epigeous fungi).

F. Cut large epigeous fruit bodies longitudinally before drying the specimens. Cut truffle-like fungi in half for drying. After drying is completed, package the specimens in a labelled paper bag or envelope. Store specimens in a dry environment.

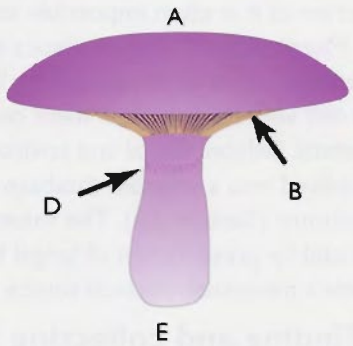
1. Select representative specimens showing all developmental stages



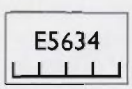
2. Record habitat, location, substrate and plant associate information

3. Keep fresh fungi in waxed paper or paper bags





4. Assign number to specimen



5. Describe macroscopic features

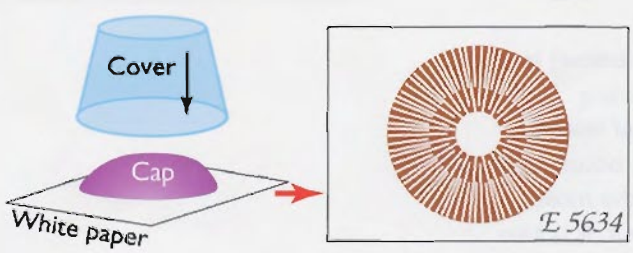
6. Describe colours by matching with colour guide



7. Photograph specimens



8. Make a spore print



9. Preserve specimens by drying

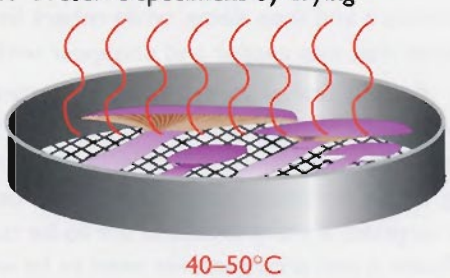


Figure 2.2. *Collecting and processing fruit bodies of ectomycorrhizal fungi.*

will allow. When planning an expedition, the broad time of the year may firstly be planned, then rainfall patterns of the target area monitored to narrow down to the best time. The most accurate way to ensure that fungi will be present in the area is to have a network of local people to monitor fruiting patterns and to provide notification when fungi are beginning to appear.

During expeditions, there are two broad strategies for collecting ectomycorrhizal fungi.

1. The first strategy involves collecting as many different fungi as possible for most of each day before returning to the laboratory at or near the end of each day. This strategy may enable a rapid assessment of biodiversity at a particular site at that particular time. This is particularly useful in cases where it may not be possible to revisit the area due to time or cost restraints, e.g. if the area is remote. A rapid assessment may also be appropriate if the area is under threat of clearing. Disadvantages of this strategy include that there will be no daylight left for assessment of colours and specimens collected early in the day may be in poor or semi-dried condition by the time one returns to the laboratory. Also there are likely to be too many collections to enable processing each one properly with attention to all details. This strategy is particularly suitable for truffle-like fungi as these are less likely to dry out than epigeous fungi, spore prints are not made with them, and they can be stored overnight or longer before being examined.
2. The first strategy is not very suitable for collecting mushroom-like fungi as these require more immediate and more detailed attention. The best strategy for collecting mushroom-like fungi is to collect fungi around a fixed base in one area for several days or more. Collecting is undertaken each morning before returning to the laboratory each afternoon. Only a limited number of fungal collections is obtained during one morning and each collection is processed with full attention to all details. In this way the biodiversity of an area can be assessed by accumulating collections over a period of several days, and the data obtained for each collection are comprehensive and therefore of high value.

Searching for fungi

1. Fruit bodies always occur near ectomycorrhizal plant species and are rarely found beyond the root zone of compatible plants. However, roots can extend considerable distances from the base of a tree (50 m or more), so it is important to assess the vegetation surrounding a collected fungus carefully before assuming what the host tree is. Epigeous fungi can occur out in the open, or may be well hidden in litter, under a shrub, or log, etc. Fruit bodies of ectomycorrhizal fungi mostly occur in the soil surrounding ectomycorrhizal plants but occasionally are found on logs or tree trunks several feet or more above the ground, reflecting the presence of their mycelium in these substrates.

Field collecting equipment list

Field gear

- maps, compass, collecting permits, etc.
- small notebook for recording data in the field
- pocket knife or trowel for unearthing entire specimens
- truffle fork for finding hypogeous fungi
- warm and waterproof clothing, sturdy shoes
- food, water, first aid kit, etc.

Transporting specimens

- collecting basket or bag
- storage containers with lids, such as margarine or ice cream tubs
- wax paper roll
- small and large paper bags to keep collections from the same location

Photography

- camera with macro (close-up) lens
- colour film (transparency)
- grey colour board as background for photographs

Care of specimens

- fungus dryer
- envelopes for storing spore prints and dried specimens



2. Fruit bodies may be conspicuously coloured or drab. They may be quite large or very small. Therefore, collecting epigeous fungi can be carried out at different levels of thoroughness, e.g. simply strolling around without stooping will reveal some, but less fungi than slowly crawling around on hands and knees.

Collecting fungi

1. The base of the stalk is an important structure for identifying fungi. Extract the whole fruit body including the base of the stalk by using a small knife or other instrument to dig well under and into the soil. Brush any excessive soil off the fruit body (being careful not to remove any part of the fungus, including any attached mycelium at the base) and immediately wrap in wax paper, or place in a sealed storage container to prevent desiccation (see below).
2. Hypogeous fungi may be found by using a three-pronged fork or similar gardening implement. In general, most of their fruit bodies occur within 10 cm of the soil surface, especially in a zone immediately below the humus layer, but they can sometimes also be much deeper. Fruit bodies are also more likely to be concentrated around the base of ectomycorrhizal trees, but otherwise finding truffle-like fungi is largely by chance. Clues to the presence of truffle-like fungi may be provided by surface scratchings made by small animals attracted to them by fungal scents. Sometimes the animal begins to unearth the fungi but leaves some behind. Hypogeous (and epigeous) ectomycorrhizal fungi may have conspicuous white or brightly coloured and abundant mycelia which can help locate them.
3. A suitable *collection of fungi* should consist of at least several fruit bodies of the same species, preferably young and mature specimens at different stages of development. All of the fruit bodies constituting a collection should ideally be produced by the same individual mycelium, and therefore be collected from a small patch, e.g. around a single tree, or in a discrete patch. Rotting or maggot-infested specimens should be discarded.
4. Avoid bruising, breaking and squashing specimens. Fresh specimens collected in the field should be immediately wrapped in wax paper or placed in wax paper bags (Fig. 2.2). Plastic bags are not suitable for storing fungi as excess humidity causes bacteria and mould development. Each different fungal collection should be placed in separate bags or wrapped separately to prevent mixture of spores and later confusion. Baskets are suitable for carrying collections of fungi in the field. Hypogeous fungi are usually less delicate and may be kept in a cloth carry bag.
5. Specimens need to be kept out of direct sunlight and heat as much as possible after collection to avoid desiccation before returning to the lab. In hot climates, a cool-box is useful for storing collections. Do not freeze specimens. Refrigeration can be used to preserve specimens for a few

days, but is not recommended as it may prevent fungi from producing a spore print.

B. Recording habitat data



1. It is essential to assign an unique number to each collection to allow field notes, descriptions, photographs and specimens to be cross-referenced in the future. Many number formats are possible, e.g. NLB-2234, H4007, 24/95 (collection 24 in 1995).
2. For each collection the identity of the most likely plant associate(s) must be noted. In a mixed stand (e.g. where eucalypts are planted, but there are a few remnant pines) all potential host plants and other dominant species should be noted. If plant species are not recognised in the field, it may be necessary to collect samples of leaves, flowers and fruits, for eventual identification back at the laboratory.
3. Site details such as the precise location should be recorded. The location can refer to a distance and direction from the nearest landmark such as a road or town, which can later be translated into a map grid reference or a latitude/ longitude value.
4. Soil information (texture, colour, organic matter content, etc.) should be recorded, especially for mycorrhizal fungi. In the case of truffle-like fruit bodies, the depth in the soil at which they were found should be noted. Identification of other substrates is very important for saprophytic or parasitic fungi.
5. Morphological details of the fresh specimens need to be recorded, as these details are important in allowing immediate identification of ectomycorrhizal fungi in the field during collection, or at any time in the future. However, for most fungi the only morphological characters that need to be recorded on site in the field are any distinctive fresh colours that appear to fade rapidly, or are obscured by bruising soon after picking. Other distinctive characters, which may quickly disappear, such as latex production, or a strong odour, should also be noted. For all but the smallest of fungi, which will dry out quickly or rapidly change colour, full details can be completed later after the specimens are transported back to the laboratory, as long as it is no more than a few hours away.
6. For recording field collection data in a systematic fashion, a well-designed standard format is recommended (see Table 2.2 — a two-page example below). Data sheets may prompt the user to record all categories of data and they can be efficiently translated into electronic databases. A more advanced design for data sheets has tear-off tags on the bottom of the page, each tag with pre-printed code numbers and a mm scale. These tags can be used for photographs, for spore prints, and for labelling specimens during drying and packaging. They minimise the chance of confusion or mixing of fungi when many collections are being processed at the same time.

Table 2.2. Data sheet for recording data about collections of mushroom-like ectomycorrhizal fungi. (For details of the characters see Section 2.3.)

FIELD DATA FOR MUSHROOM-LIKE FUNGI

COLLECTION No. ..... DATE COLLECTED .....

GENUS ..... SPECIES .....

SITE .....

PLANT ASSOCIATION AND HABIT .....

.....

CAP: size, shape, colour, surface texture and moisture.

GILLS: colour, attachment, spacing, lamellules.

STEM: size, shape, colour, surface texture and moisture.

PARTIAL VEIL: (ring, or covering over gills): presence or absence, form.

UNIVERSAL VEIL: presence or absence, form.

FLESH: colour, texture, bruising.

Continued over page

Table 2.2. *(continued)*

FURTHER NOTES AND DIAGRAMS

COLLECTION No. .....

CHEMICAL REACTIONS: 15% KOH or ammonia, $FeSO_4$, Melzer's reagent

ODOUR AND TASTE:

SPORE PRINT COLOUR:

NOTES AND DIAGRAMS

MAIN FEATURES:

1. .....
2. .....
3. .....
4. .....

IDENTIFICATION/ RELATIONSHIPS AND NOTES

.....

.....

.....

.....

.....

When collecting fungi, it is very important to make careful observations and record detailed descriptive notes of macroscopic characters, such as colour, shape, size, as soon as possible after collecting specimens. This information is required for proper taxonomic studies, but is lost once fungi are dried. Even when the utmost care is taken during the collection, packaging and transport of fungi, rapid deterioration of ephemeral structures is inevitable. Specimens should be described on the same day that they are collected. Observation of microscopic features is less urgent, as these can be described from dried specimens.



C. Describing fresh specimens

After returning to the laboratory, or place where you are staying, detailed descriptions of macroscopic fungal morphology should be made (see box). A standardised data sheet (Table 2.2) can be used, and protocols for describing macroscopic characters are provided in the next Section. It is not necessary to investigate any microscopic characters at this stage as these can be obtained from dried specimens (see Section 2.4).

1. The first step is to check collections to ensure that they contain representative specimens of only one fungus, as similar species may have been confused in the field. A representative collection should include larger, smaller and intermediate examples of young and old fungi to provide important developmental information. It may be necessary to gently clean specimens with a soft brush to remove loose soil, taking care not to remove delicate structures. Keep specimens wrapped in wax paper as they will desiccate rapidly.
2. It is best to take photographs of specimens as soon as possible after collection to minimise changes in fungal appearance. Freshly-collected mushroom caps should also be used to obtain spore prints (see E below).
3. Another immediate task may be to attempt to obtain pure cultures of the fungi by using fresh fruit body tissue (see Chapter 5).
4. It is useful to list 3 or 4 main distinguishing features of a fungus (Table 2.2). This can be written in simple language or using mycological terminology. These features can be used later to quickly construct a mental image of the fungus without reading the whole description. Other macroscopic features are then described as outlined in Section 2.4.

D. Photographing specimens

The aim is to provide a clear visual record of the main characters of specimens, e.g. shapes, textures, colours. A photographic system which enables good lighting and close focusing must be adopted. Some experimentation may be needed to establish a suitable system. Once a protocol is established, it should always be used as a standard in the future so that all photographs will be directly comparable, i.e. the same type of film, background colour, lighting system, etc. This is especially important if the colours of fungi are to be compared, or photographs are to be published.



1. *Camera type* — a single lens reflex camera for 35mm film is suitable. The camera should have a close-up, or macro, lens, e.g. 50–100 mm macro lens capable of focusing at a 1:1 or 1:2 reproduction ratio (1X or 0.5X magnification at closest focus). A less expensive option is to use supplementary, close-up lenses on a standard lens, or a bellows, but these are less effective. A minimum lens aperture of at least F 16, preferably F 22, or F 32, is required for an acceptable depth of focus. No filters should be used on the lens.

2. *Film type* — colour photographs are needed. Slide film is recommended over print film, as colour reproduction is more consistent. Prints can be produced from the slides later. ISO 100 films, such as Kodak Ektachrome, or Fujichrome, are most suitable (E6 process slide films). Standardisation of film type and ASA is advisable as different film brands have different colour balances and respond differently to lighting situations. Store film in a cool place.
3. *Lighting* — use a system that avoids harsh shadows, because otherwise the film will not be able to cope with the contrast. Flash lighting is recommended as it enables photographs to be taken at any time and provides better colour and exposure standardisation. Flash lighting may be diffused or reflected onto the specimen to soften shadows. Ring flashes are not suitable if they produce a central hot spot or strong reflections. Lighting strength should be sufficient to allow lens apertures of F 11 or smaller to be used.
4. *Specimens* — should be arranged to show a representative selection of young, mature, and old individuals of one species. Some should be sectioned longitudinally. A neutral grey background is preferable. It is essential to include a unique collection number for the specimen and a scale (mm) should also be included (see Figs 2.20 onwards in this chapter).
5. *Habitat photos* — the above applies mainly to photographs taken in the laboratory after returning from the field. Sometimes it is also useful to photograph specimens in situ, particularly to show its habitat (e.g. type of leaf litter) or if the specimen is likely to dry out or change colour rapidly after being collected. Daylight or flash lighting may be used in the field.
6. *Slide storage* — pictures of fungi contain valuable scientific information. It is important that slides are kept in a cool, dark, dry environment to avoid deterioration. Slides can be conveniently stored in indexed folders containing clear plastic A4 size sheets with pockets to slot the slides (usually 20 per page). An index system, usually based on specimen numbers, is required to find slides in a large collection (Section 2.5). Electronic storage of photographic images and attachment of these to databases is another increasingly viable option.

E. Making spore prints

Spore prints of most fungi can be made after returning to the laboratory. However, for very small fruit bodies which may dry out rapidly it is useful to take into the field a set of small containers and papers for making a spore print at the site of collection. Do not refrigerate specimens before making a spore print as they may not produce a print after refrigeration.

1. A spore print is made by placing a mushroom cap (which is mature but not too old) with the stem removed and the spore-bearing gills facing down onto white paper. It can be covered with a box or glass, etc. for protection against air



movements and desiccation. A few drops of water placed on a fungus may help to prevent desiccation.

2. After a period of one hour to overnight (depending on the size and condition of the specimen), the spores should have formed a white or coloured deposit on the paper. The spores will adhere to the paper by static but can be easily brushed off, and therefore require protection in an envelope.
3. The colour of the deposit itself is useful for broad identification purposes, and the spores can be used for later microscopic examination. Spore print colour should be noted soon after they are produced as they may change colour upon drying out, and may also change further after lengthy periods of storage.
4. Label the spore print paper with the code number of the fungal collection. Allow the paper to dry out, then fold it in half with the spores facing inwards. Store spore prints with the corresponding specimen, or in an envelope labelled with the code number.

F. Preserving fungal specimens by air drying

Air drying is the preferred method of preserving fungi. It is possible to preserve them in a pickling liquid or by freeze drying but these methods result in greater loss of the fine structures than with air drying. *Most mycologists and herbaria prefer air-dried specimens over those preserved in other ways.*



1. It is advisable to cut some specimens of each collection in half longitudinally before drying. This enables faster drying and also provides a ready-made edge for sectioning later when examining microscopic characters. Indeed some of the larger specimens, e.g. the big fleshy boletes, may need to be sliced into several pieces (±2 cm wide slices) to allow adequate drying. Hypogeous truffle-like specimens should be cut in half (sectioned through the columella or basal point of attachment), otherwise the interior may not dry adequately and can be difficult to handle after drying.
2. Air drying can best be achieved with commercially available electrical units designed for drying fruit. These have a heating element at the base and blow warm air up through a series of wire or plastic grid trays. Heating overnight at 45–50° C is sufficient to dry most fungi. *It is important not to cook specimens by overheating them as this may destroy key microscopic structures needed later for identification.* Good air circulation is another requirement for optimum preservation. If a suitable dryer is not available it may be possible to air-dry small to medium-sized fungi by:
   (a) the heat from an electric, gas or oil lamp;
   (b) silica desiccating gel;
   (c) the sun (in warm, low humidity climates only).

3. After returning to the laboratory it is advisable to re-dry specimens, before placing them in storage (see Section 2.5).
4. Dried fruit bodies should be retained for detailed examination of microscopic characters leading to identification, and to provide a permanent reference source to which a name has been attached and which may be checked at any time in the future. It is always a good idea to deposit collections of fungi in a registered herbarium (see Section 2.5), because names are always changing and, for example, a currently well-known fungus may be assigned to several different species in the future — when more is known about its biology.

2.3. DESCRIBING THE MACROSCOPIC MORPHOLOGY OF FUNGI

Observing and recording morphological details of the fresh specimens are very important in allowing immediate identification of ectomycorrhizal fungi in the field during collection and in the future. Fungal taxonomists will often require such details to make an accurate determination. A hand magnifying lens and good lighting are essential for observing fine morphological details such as surface scales. For mushroom-like and truffle-like fruit bodies each of the various parts such as the cap, stem and gills should be described systematically by observing:

1. *size*
2. *shape*
3. *colour*
4. *texture/consistency*
5. *special features.*

The morphology of young, expanding and mature specimens should be noted as changes often occur in texture, colour and other features with age. Fungal morphology is extremely diverse and in some cases extra features may need special detailed attention. Conversely, one fungus may not necessarily have all the parts that another fungus has, e.g. some fungi do not have a stem. Figure 2.3 shows a schematic representation of the main features of some mushroom-like fungi and a truffle-like fungus.

Terminology

Detailed and consistent descriptions of macroscopic and microscopic characters of ectomycorrhizal fungi require the use of specialised terminology. The reader is referred to a book by Largent (1977) for further illustrations and definitions of this terminology. Some of the main terms are also listed and defined in the outline of macroscopic characters below. It should be remembered that, although descriptive terms designate specific character states, a continuum of forms occurs among many fungi that sometimes clouds the distinction between terms and makes the choice of their application difficult.

Equipment for describing fungi

- hand lens (10–20X magnification)
- standardised data sheets for detailed notes
- pens/pencils
- ruler for measuring fungi
- white paper squares for spore prints
- floodlight to help examine specimens at night
- brush for cleaning specimens
- knife and razor blades for slicing specimens
- reference colour chart
- reference books (field guide type)
- chemicals in leak-proof small dropper bottles for spot tests

MACROSCOPIC CHARACTERISTICS OF FUNGI

EPIGEOUS FUNGI				
Feature	Size	Shape (type)	Colour	Texture
Cap (A)				
Hymenium (B)				
Stipe (Stem - C)				
Partial veil (D)				
Base (E)				
Universal veil (F)				
Flesh (G)				
HYPOGEOUS FUNGI				
Peridium(H)				
Gleba (I)				
Locules (J)				
Columella (K)				
ALL FUNGI				
Attached hyphae (L)				
Bruising / exudates (M)				
Odour/ taste				

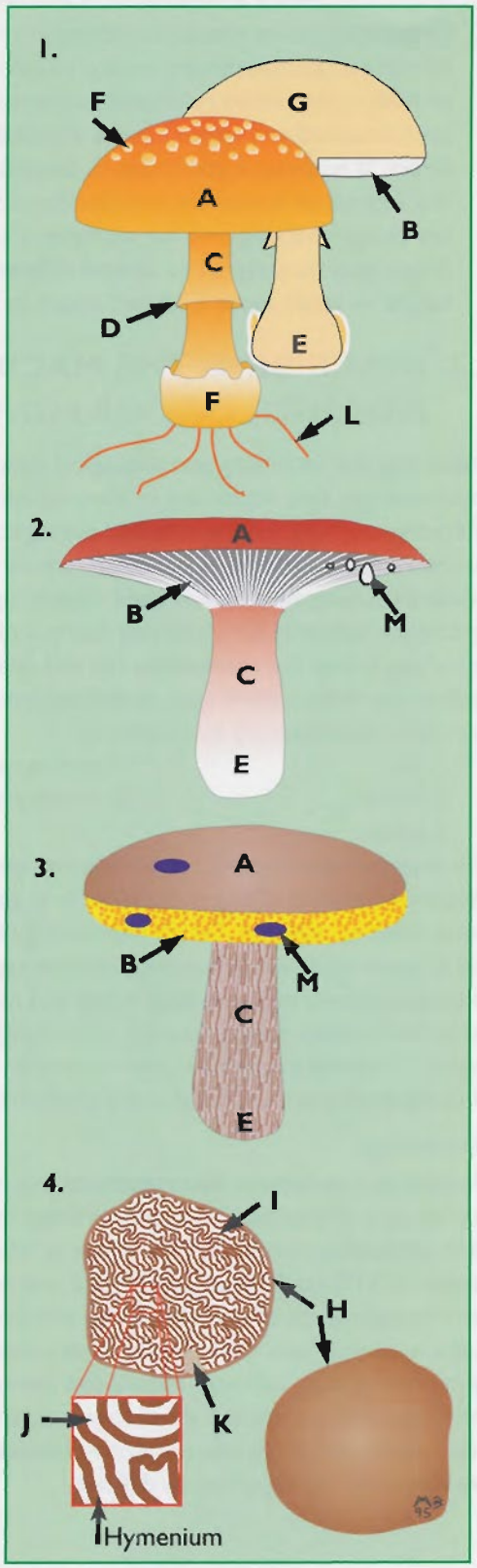


Figure 2.3. *General morphological characteristics of mushroom-like and truffle-like fungi. Fungus 1 is similar to an* Amanita, *fungus 2 is similar to a* Russula, *fungus 3 represents a bolete, fungus 4 represents a truffle-like form. The broad descriptive categories necessarily vary for each part (e.g. size of the stem refers to length and breadth, whereas size and shape of the bruising/exudates refer to occurrence.*

Table 2.3. Basic macroscopic data for fruit bodies of (A) mushroom-like fungi and (B) truffle-like fungi.

A. Mushrooms

Cap (pileus) — size, shape, colour, surface texture and moisture
Gills (lamellae) — colour, attachment, lamellules.
Stem (stipe) — size, shape, colour, surface texture and moisture
Partial veil (annulus or cortina)— presence or absence, form
Universal veil — presence or absence, form
Flesh — colour, texture, bruising
Chemical reactions — 15% KOH, 10% $FeSO_4$ (w/v), etc.
Odour and taste
Spore print colour

B. Truffles

Fruit body — size, shape, colour, surface texture and moisture
Skin (peridium) — thickness, layers, texture, bruising
Internal fertile tissue (gleba) — colour, texture, size and orientation of chambers and whether these are full or empty
Sterile tissue (columella) — presence or absence, form
Sterile base — presence or absence, form, basal mycelium or rhizoids
Chemical reactions — 15% KOH, 10% $FeSO_4$, etc.
Odour and taste

A. Describing colours of fungi



The instructions provided here apply to all the parts of fungi which are each described separately.

1. Colours of fruit bodies should be accurately described by referring to colour charts such as those in the *Methuen Handbook of Colour* (Kornerup & Wanscher 1978) or CYMK colour charts (see Chapter 3). The code numbers obtained from colour charts should be given along with a written description of the colour. If colour charts are not available, general common terms for colours may be recorded (e.g. pale brown, pink, red-orange, bright yellow, etc.). Try to use terms that will be meaningful to others (e.g. 'soil brown', 'sky blue', 'leaf green' are not helpful as they have different meanings to different people).
2. The colours of young and old fruit bodies, and whether colours change or fade with time should be noted. Colours of the cap, hymenium, stem, veils, etc. of mushroom-like fungi and the peridium, gleba, columella, etc. of truffle-like fungi and all other structures should be recorded (see Fig. 2.3).
3. It must be noted that colours differ when viewed under artificial light or sunlight. Sunlight is preferable. The lighting conditions and the time since the collection was made should be noted when recording colours. It is important that the colour guide and specimen are exposed to the same lighting conditions (i.e. do not mix incandescent, fluorescent, or daylight sources).

NOTE: It is important to compare immature and mature specimens of a fungus to observe developmental changes to structures. The appearance of fungi can also be substantially affected by changes in their moisture content

B. Recording details of the main morphological characters of fungi

Essential terminology and instructions for describing the morphology of various fungal organs are provided in the following boxes and diagrams. However, mycologists may use many additional terms in published descriptions of fungi, so readers may also have to refer to mycological textbooks such as Largent (1977) and *Ainsworth and Bisby's Dictionary of the Fungi* (Hawksworth et al. 1995). The following topics refer mainly to mushroom-like fruit bodies, but many are also applicable to truffle-like fungi (which are dealt with further below). These terms can also be used to describe club and coral fungi, etc.

1. Mushroom caps

The cap (pileus) of a mushroom is described by the following observations (refer to Figs 2.4 and 2.5).

Size — the cap width and height (at centre) of the smallest and largest mature fruit body in a collection (mm).

Shape — young and mature fruit bodies should be assessed (Fig. 2.4) in side view and top view. At the centre of the pileus there may be a depression (*centrally depressed*), or a protrusion (*umbonate*) (Fig. 2.4).

Margin — The cap margin should be assessed in terms of side view when cut in half, shape of the edge outline, and surface view of the edge (Fig. 2.5).

Moisture — the cap surface may be dry, or moist. If the cap is gelatinised it may be glutinous (slimy), or viscid (sticky).

Hygrophany — colour change in the cap resulting from desiccation occurring rapidly or slowly after collection. Often the cap is translucent-striate when moist but becomes opaque and paler from either the centre outwards or from the margin inwards as it dries out.

Universal veil remnants — see box 4.

Texture — The cap surface may be smooth or variously uneven. It should be noted whether the texture is due to superficial structures that are easily removed (often derived from the veil), or are part of the cap surface itself. A large range of terms applies to surface texture:

pits — include the following:
- *scrobiculate* — shallow pits
- *areolate* — deep pits
- *lacunose* — deep pits surrounded by ridges

splits — include the following:
- *rimose* — radially split
- *areolate* — split into polygonal or irregular shaped blocks

wrinkles — include the following:
- *rivulose* — with meandering or wavy lines
- *rugulose* — with undulating wrinkles
- *rugose* — with coarse wrinkles

Surface powder, hairs, scales — three extreme general categories are:
- *glabrous* — bald, wax-like surface
- *hispid* — covered with stiff hairs
- *scabrous* — surface roughened due to scales

Orientation of particles, fibrils, hairs or scales — may be:
- *appressed* — scales and fibrils flat on the cap surface
- *recurved* — base appressed but tips of the scales/hairs or fibrils turn upwards
- *erect* — perpendicular to the cap surface

Particles on the surface — include:
- *atomate* — minute shining particles
- *micaceous* — glistening minute particles
- *pruinose (pulverulent)* — covered with a fine powder
- *furfuraceous (scurfy)* — bran-sized scales like dandruff
- *granulose* — powder composed of particles as big as grains of salt

Fibrils lying on the surface — include the following:
- *fibrillose* — hyphae forming visible fibrils on the surface
- *downy* or *canescent* — soft downy surface
- *floccose* — coarser, loosely cotton-like surface
- *tomentose* — densely matted and woolly surface
- *matted fibrillose* — fibrils matted or interwoven

Fibrils/hairs perpendicular to the surface — include the following:
- *pubescent* — very short, soft hairs
- *velutinous* — short, fine, velvety flexible hairs
- *villose* — long fine hairs
- *hirsute* — abundant, stiff, inflexible hairs

Fibrils combining to form scales — include the following:
- *squamose* — scaly surface, large scales
- *squamulose* — small scales
- *squarrose* — erect scales
- *punctate* — dotted with minute scales
- *scabrous* — surface roughened

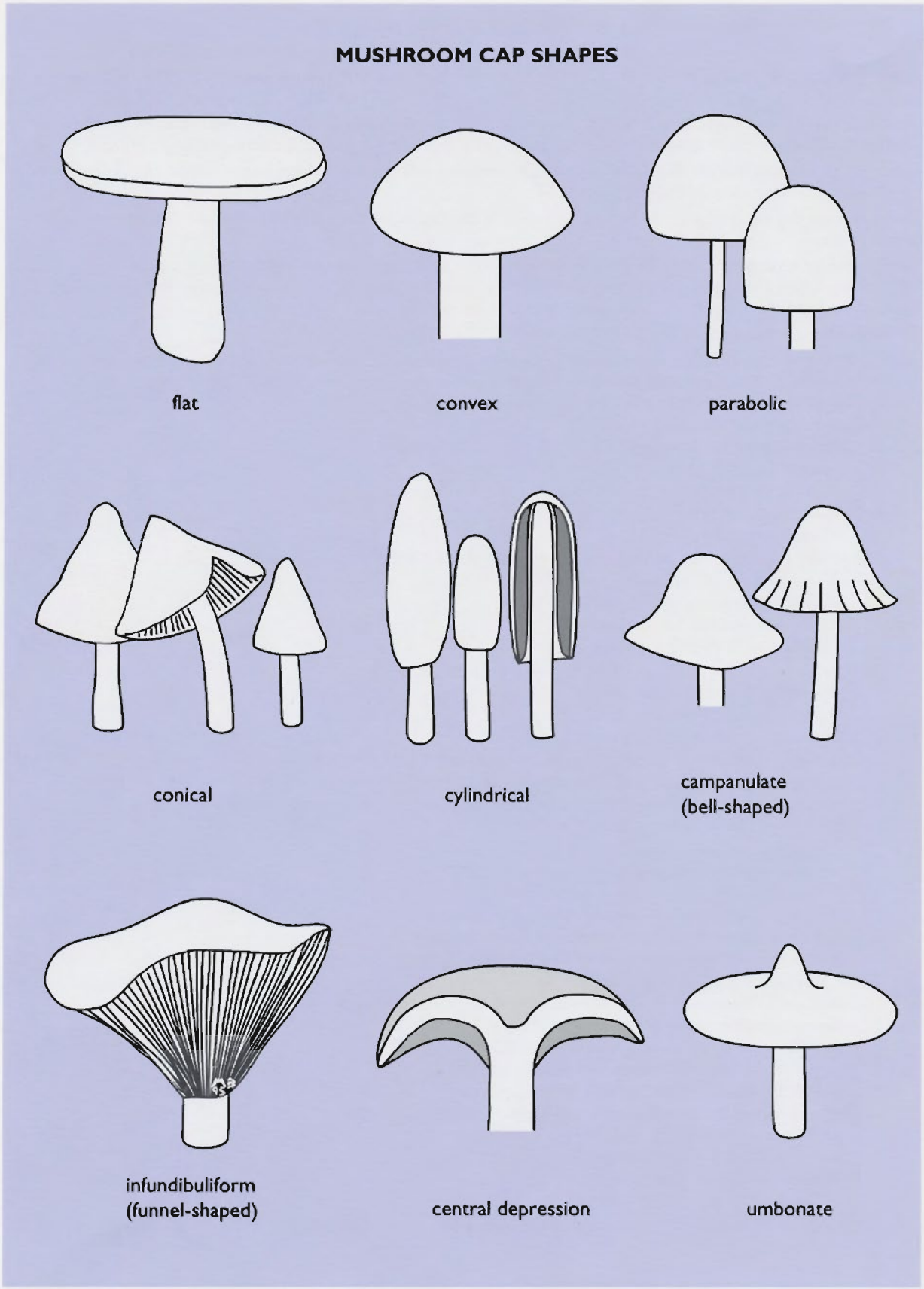


Figure 2.4. *Main cap (pileus) shapes of mushroom-like fruit bodies.*

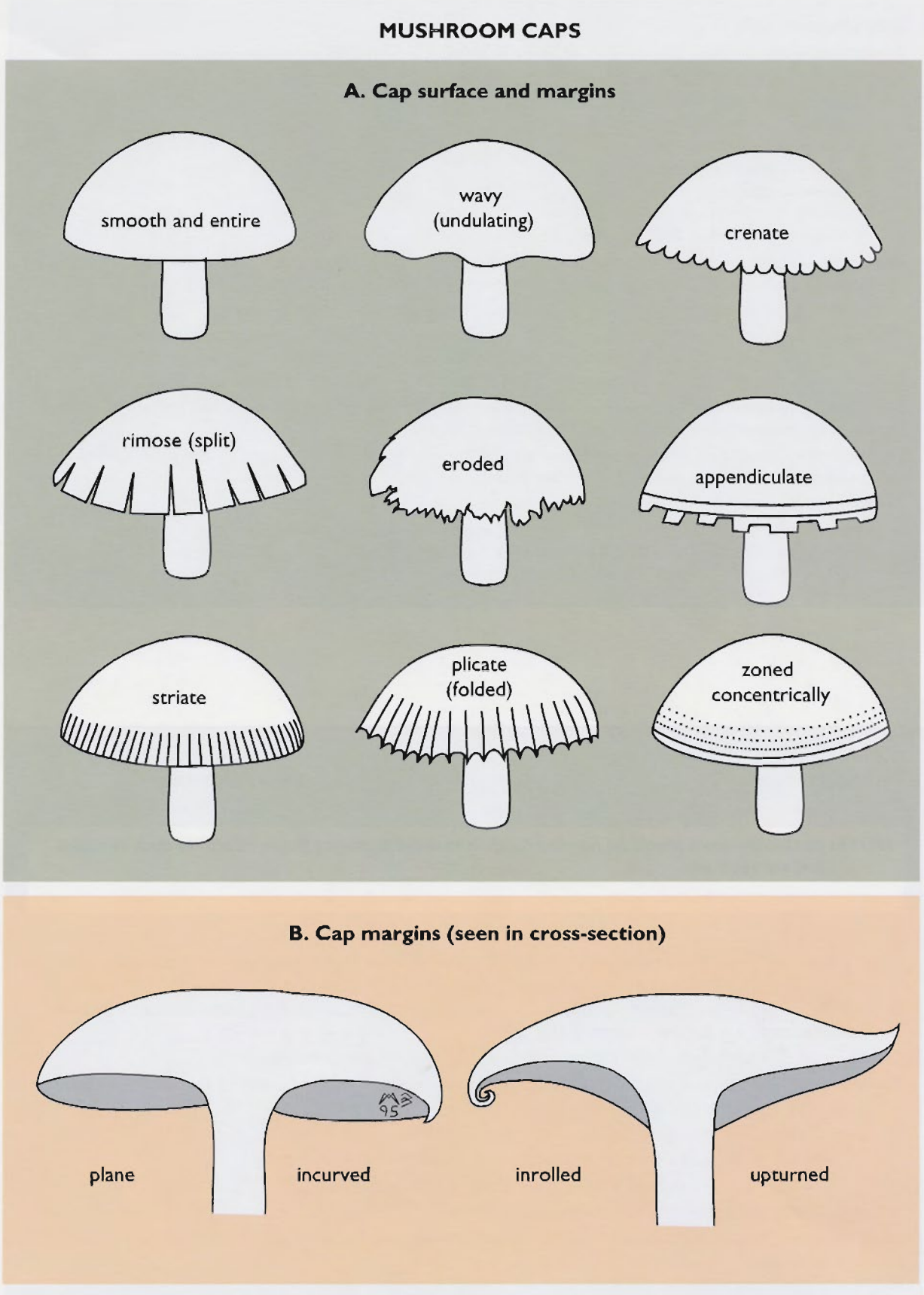


Figure 2.5. *Cap (pileus) margins of mushroom-like fruit bodies.*

2. Mushroom gills

The following observations are used to describe the shape, orientation, arrangement, spacing and attachment of gills (lamellae, fertile surface) of mushrooms (Fig. 2.6).

Attachment to stipe — described as follows: (see Fig. 2.6A)

decurrent — extending down the stem
adnate — broadly attached
adnexed — partially attached
free — not attached
sinuate — notched near the attachment
receding — attached at first becoming free later.

Spacing — cut off the stem and observe the radial pattern of the gills to see if they are crowded, well spaced apart (*distant*), or arbitrarily somewhat in between (*close or subdistant*) (Fig. 2.6B).

Thickness — in side view, the gills may be *thin*, *broad* or *ventricose* (swollen in middle).

Margin — the edge of the gills may be smooth or variously uneven as seen through a hand lens, e.g. *crenate*, *serrate* or *eroded* (Fig. 2.6C). The edge may differ in colour to the face, and may be *glistening* or *fimbriate* (minutely fringed, often due to presence of cystidia — then referred to as *cystidiate*).

Branching and anastomoses — the gills may be unbranched, dichotomously branched (*bifurcate*) or may be joined by anastomosing interconnections (*costate*).

Lamellulae — short gills which extend only part of the way from the cap margin to the stipe. Lamellules may be *absent* or *abundant* and of two or more sizes.

Tiers — the number of distinct lengths of lamellulae.

Number — the number of gills per cap varies between fungi and is largely related to the spacing. A standard way of recording this data is to count the number of gills (L) and lamellules (l) per half a cap, resulting in a notation for each collection (such as L = 10–14, l = 22–28).

3. Mushroom stems

The shape and tissue composition of stems (stipes) is described by the following parameters (Fig. 2.7).

NOTE: *mushroom stems should be handled carefully to avoid damaging fragile structures such as scales and veil remnants.*

Size — diameter at the base, middle and apex of the smallest and largest fruit body in a collection (mm).

Shape — in cross-section, e.g. if circular (*terete*) or *compressed*. The stipe shape in longitudinal view can be extremely variable, e.g. *cylindrical*, *clavate*, *tapering* (Fig. 2.7A). The base of the stipe must also be taken into consideration, e.g. *bulbous*, *radicating* (Fig. 2.7B).

Attachment — whether the stem has central or lateral attachment to the cap.

Texture — similar terms as used for pileus are applied to stipes. The distribution of any surface features along the stipe should be noted. A few extra terms include:

reticulate — interlocking pattern of ridges or wrinkles,
glandular — coloured dots or small scales, especially near the stipe apex.
Other options — longitudinally striate, ridged, veined, etc.

Interior — *solid*, *stuffed*, *chambered* (lacunose), or *hollow*.

Consistency — fragile, robust, cartilaginous, fibrous, woody, corky, chalky, etc. Some fungi have a tough outer layer or rind.

Veils on stipe — see boxes 4 and 5.

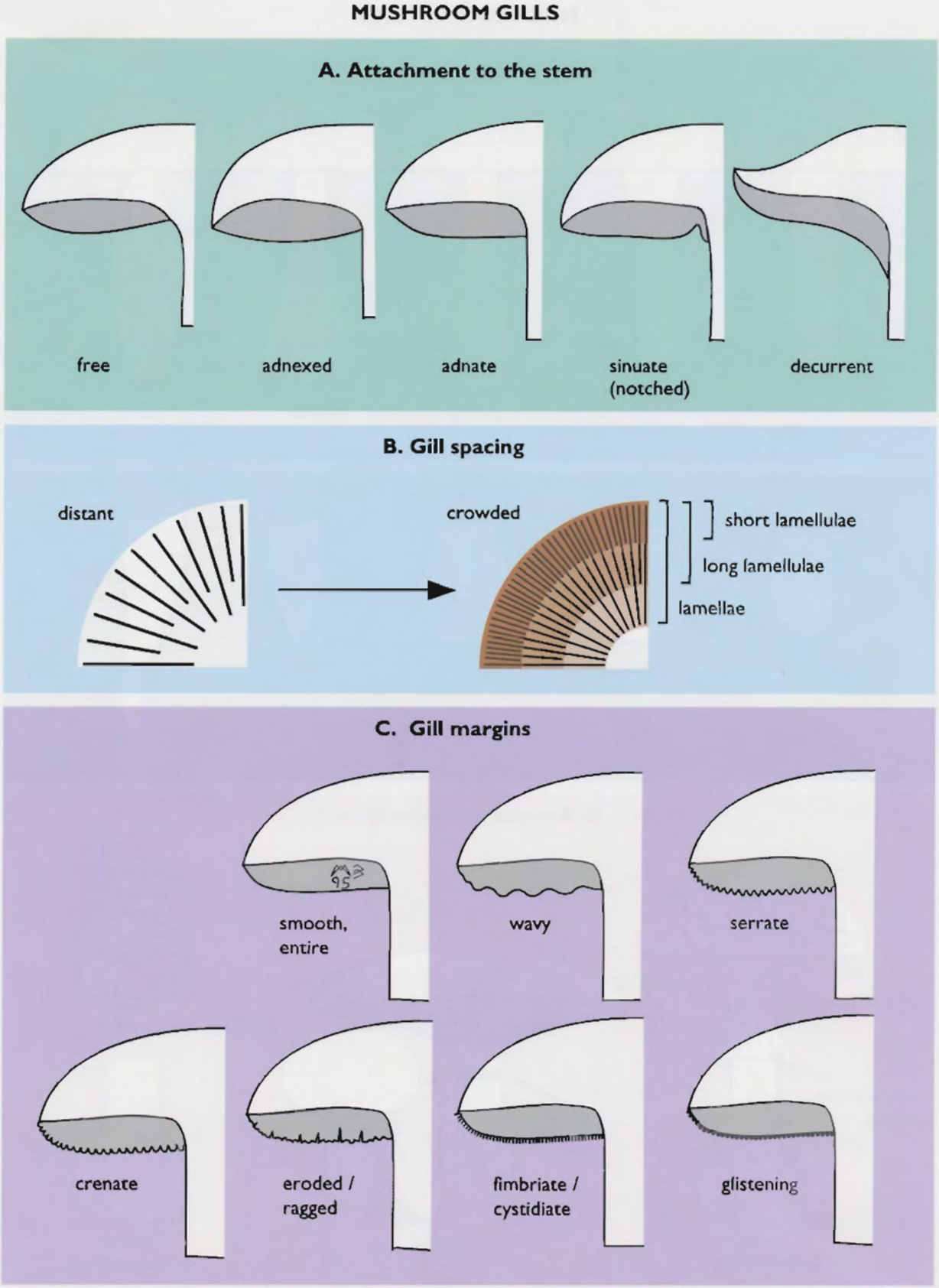


Figure 2.6. *Morphology of the gills (lamellae) of mushroom-like fruit bodies:* **A.** *Attachment of the gills to the stem.* **B.** *Spacing of the gills.* **C.** *Types of gill margins.*

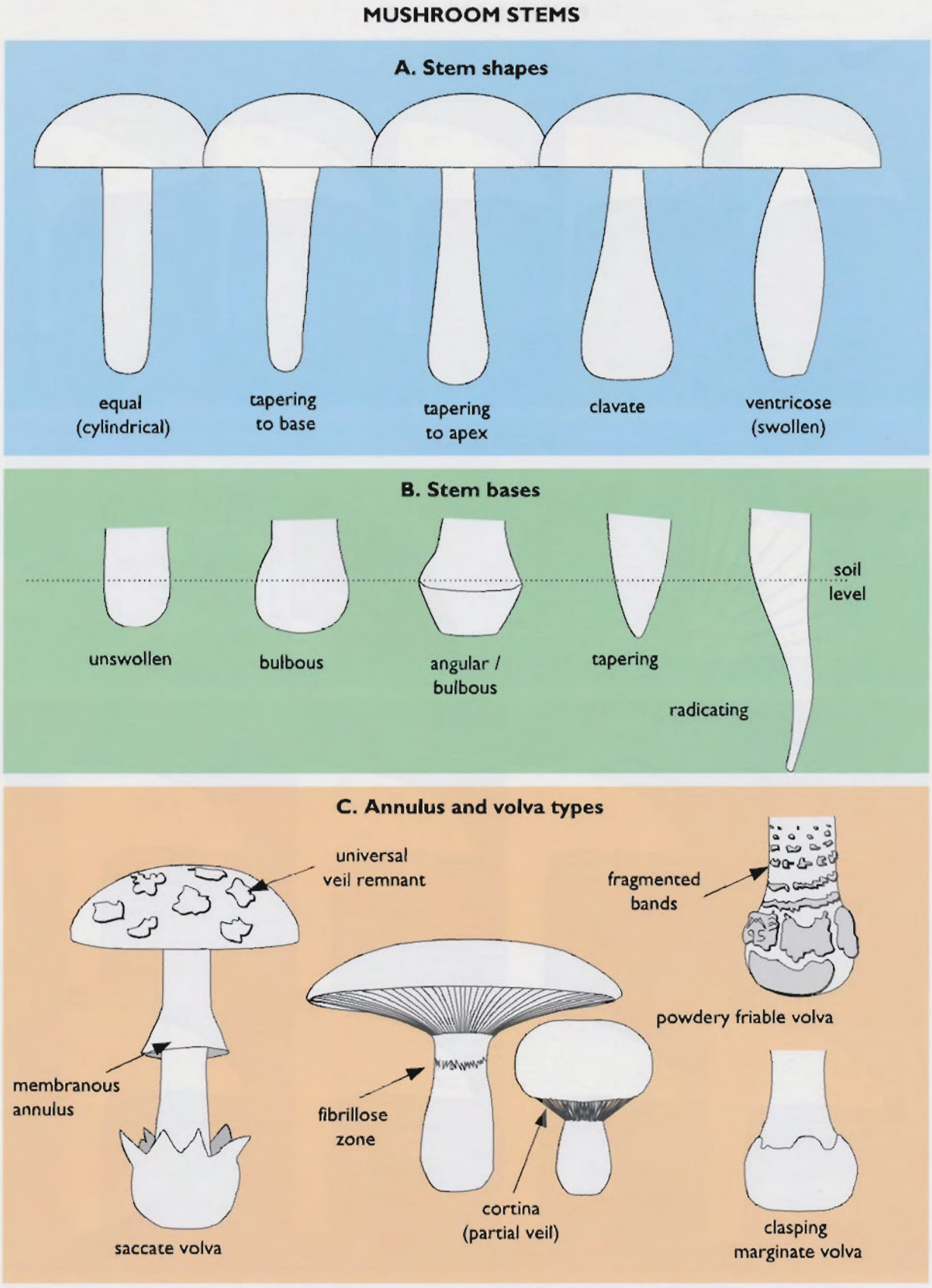


Figure 2.7. *Morphology of the stems of mushroom-like fruit bodies:* **A.** *Main shapes of the stems (stipes) of mushroom-like fruit bodies.* **B.** *Some types of stem bases.* **C.** *Some annulus and volva types.*

4. The universal veil

The universal veil completely encloses young fruit bodies of some mushroom-like fruit bodies. As the mushroom expands the universal veil ruptures. In mature fruit bodies it may be absent or remain as scales on the pileus surface and/or as a volva. The lower stipe may also have multiple zones of universal veil tissue (Fig. 2.7C).

Scales — patches of veil remnants on the cap surface. The texture of scales can be:

powdery,
appressed,
pyramidal warts, etc.

Volva — a cup-shaped structure at the base of the stipe formed from the universal veil. The volva may occur as a separate tissue (loose sack) or may be attached to a bulbous base, indicated only by the presence of a rim (see Fig. 2.7C). Terms used to describe the volva include:

membranous — thin, free from base
attached — inconspicuous tissue on base
saccate — like a loose bag
powdery, friable, ragged bands, etc.

5. The partial veil

In young fruit bodies the partial veil extends from the cap margin to the stipe and covers the gills during development, later breaking to expose the mature gills (see Fig. 2.7C). A partial veil is absent in many mushroom-like fungi. Both partial and universal veil remnants may form multiple bands on the stem.

Texture — of the intact partial veil varies between the following forms:

membranous — a thin, uniform sheath.
cortina — a cobweb-like structure. Cortinoid-type veils either soon disappear (i.e. they are *evanescent*), or remain as an indistinct *fibrillose zone* on the stipe.

Annulus *(ring)* — a persistent veil present on the stipe of mature fruit bodies.

Appendiculate cap margin — a rim on the cap margin formed from the partial veil.

Annulus position — rings may occur in the following locations:

superior position — near the stipe apex,
central, or
basal (inferior).

6. Other features

Fruit bodies should also be examined for these additional features (Fig. 2.3).

Flesh — The colour and consistency of the flesh comprising the cap and stem. Particularly any *colour changes* or *bruising* that occurs after cutting open a fruit body should be noted. Note how rapidly changes occur and the sequence of colours of structures.

Bruising — The surface and flesh of some fruit bodies may change colour when damaged or upon exposure to air, e.g. the bluing flesh of some boletes.

Exudations — Some fungi have droplets of clear, white or coloured *latex* on the cap, gills or stipe. If broken the flesh may yield more latex. The latex may change colour when dry or stain the gills.

Basal mycelium — The colour and abundance of hyphae at the base of the stipe vary greatly between species. If the strands are particularly thick (more than 0.5 mm) they are called *mycelial strands*, or *rhizomorphs* (if hyphae are differentiated). Sometimes the mycelium extends up the stipe for a short way — the *basal tomentum*.

Odour — Some fungi have distinct odours. Use freshly picked specimens and avoid over-mature or rotting specimens.

Taste — Taste is an important character for distinguishing species of some fungi. Take a small piece of the flesh and place on the tongue without swallowing. Note whether the taste is *immediate* or *latent*, i.e. is slow to take effect. Spit out the sample after testing. The taste of any latex should also be determined. Gills should also be tasted.

7. Chemical tests

These are performed by placing a drop of the chemical directly on the tissue (usually cap, stem or hymenium surface or flesh) and observing immediate and longer (e.g. 3–5 minutes) colour changes. There are many chemical spot tests used with fungal fruit bodies, but only those most commonly used are listed below (Largent et al. 1977):

— *15% (w/v) aqueous KOH*

— *30% (v/v) aqueous ammonia*

— *10% (w/v) aqueous* $FeSO_4$.

8. Additional characters for truffle-like fungi

Data from 6 and 7 above should be applicable to most truffle-like fungi. The first five points apply only to truffle-like fungi which have some resemblance to mushrooms, e.g. truffle-like *sequestrate* (closed) species that have a distinct cap and stem and/or a lamellate gleba. In addition, there are some unique characteristics of truffle-like fungi that should be noted. These characteristics also apply to Ascomycete truffles. More information on truffle-like fungi is provided in Section 2.6H.

Fruit body — The range of overall shape and size (length and breadth) of whole fruit bodies should be recorded. Common shapes for truffle-like fungi include *spherical, oval, pyriform*, etc.

Peridium — the skin of truffle-like fruit bodies. Describe in a similar way to the cap surface of mushroom-like fungi (as outlined above). An extra character of significance is the thickness, colour and layers of the peridium in cross-section.

Gleba — the interior of truffle-like fruit bodies containing the spore-bearing tissue (*hymenium*) and supporting sterile tissues (*trama*). The colour and consistency of young and mature fruit bodies should be described. The pattern of the locules (see below) should also be noted, e.g. if they are *radiating outwards* from a central point, or *distributed randomly*.

Locules — chambers within the gleba where spores are produced. Describe the size and shape of individual locules, and whether they are *empty* or *filled*. The constituency and colour of locule contents should be noted. Truffle-like fungi with readily apparent locules are called loculate.

Columella — sterile tissue representing a rudimentary stipe (see Fig. 2.35). Note whether the columella extends to the top of the gleba (*percurrent columella*), part way (*truncate columella*), or is branched into many thin intrusions throughout the gleba (*dendritic columella*). Sterile tissue may be completely absent or reduced to a small *basal pad* at the base of the gleba. The columella may also be emergent outside below the fruit body (*stipitate columella*).

2.4. MICROSCOPIC MORPHOLOGY OF FUNGAL FRUIT BODIES

Detailed information about the shape, size, colour and other features of microscopic structures such as spores and the specialised types of hyphae that compose various parts of fruit bodies such as the gills are often needed for accurately identifying the genus and/or species of ectomycorrhizal fungi. This information can be obtained from fresh material, or dried herbarium specimens. Generally, specialised histological techniques, such as those presented in Chapter 4, are not required during taxonomic studies of fungi.

Fungi that have similar macroscopic morphology may not necessarily have similar microscopic characters (e.g. Fig. 2.8). Some of the basic techniques for observing, describing, measuring and illustrating microscopic characters are outlined below. Although these are outlined here for Basidiomycetes — particularly mushroom or toadstool types of fruit bodies — the basic principles are applicable to truffle-like or other forms of fruit bodies produced by ectomycorrhizal fungi (Basidiomycetes and Ascomycetes).

A. Microscopic structures

Microscopic characters may be observed in fresh or rehydrated pieces of air-dried specimens. Only general characteristics can be observed with low power microscope objectives, so a 100X oil immersion lens is essential for observing minute details such as spore ornamentation and other characters that are important taxonomic criteria. The most important parts of a mushroom-like fruit body that need to be examined under the microscope include the following in order of levels of importance:

1. Spores are often the most important feature.
2. The hymenium (fertile layer — basidia and cystidia) and the pileipellis or peridium (cap cuticle) are also important.
3. For thorough examinations, other structures are observed such as the hymenial trama, stipitopellis, caulocystidia, veil remnants, and the pileus and stipe trama.

For a rapid assessment, only a single mature fruit body may need to be examined. For more detailed examinations, it is usually necessary to sample tissue from a range of fruit bodies representing the collection in order to observe immature and mature structures and to more accurately record the range of characters such as spore size. Techniques for observing the structures listed below are explained in this section and illustrated in Figures 2.9 to 2.15.

Equipment and reagents

- Compound microscope preferably with the following:
  100X oil immersion objective
  eyepiece micrometer
  microscope drawing attachment
  interference contrast capability (see 1.7)
- Sharp, two-sided razor blades for sectioning
- Fine watchmaker's forceps, dissecting needles, etc.
- Sharp drawing pencil
- Standardised data sheets (see Table 2.4)
- Glass microscope slides and coverslips
- Mounting solutions:
  — 3% aqueous (w/v) KOH
  — 10% aqueous (v/v) ammonia
  — Melzer's Reagent (see below)
  — 1% aqueous (w/v) Congo red
  — 1% (w/v) Cotton blue in lactic acid
  — semi-permanent PVLAG mountant (see 3.3B)
  — water alone
- **Melzer's reagent with chloral hydrate:**

1.5 g	iodine
100 g	chloral hydrate
5 g	potassium iodide
100 mL	water

NOTE: ***Chloral hydrate is toxic and must be handled carefully***



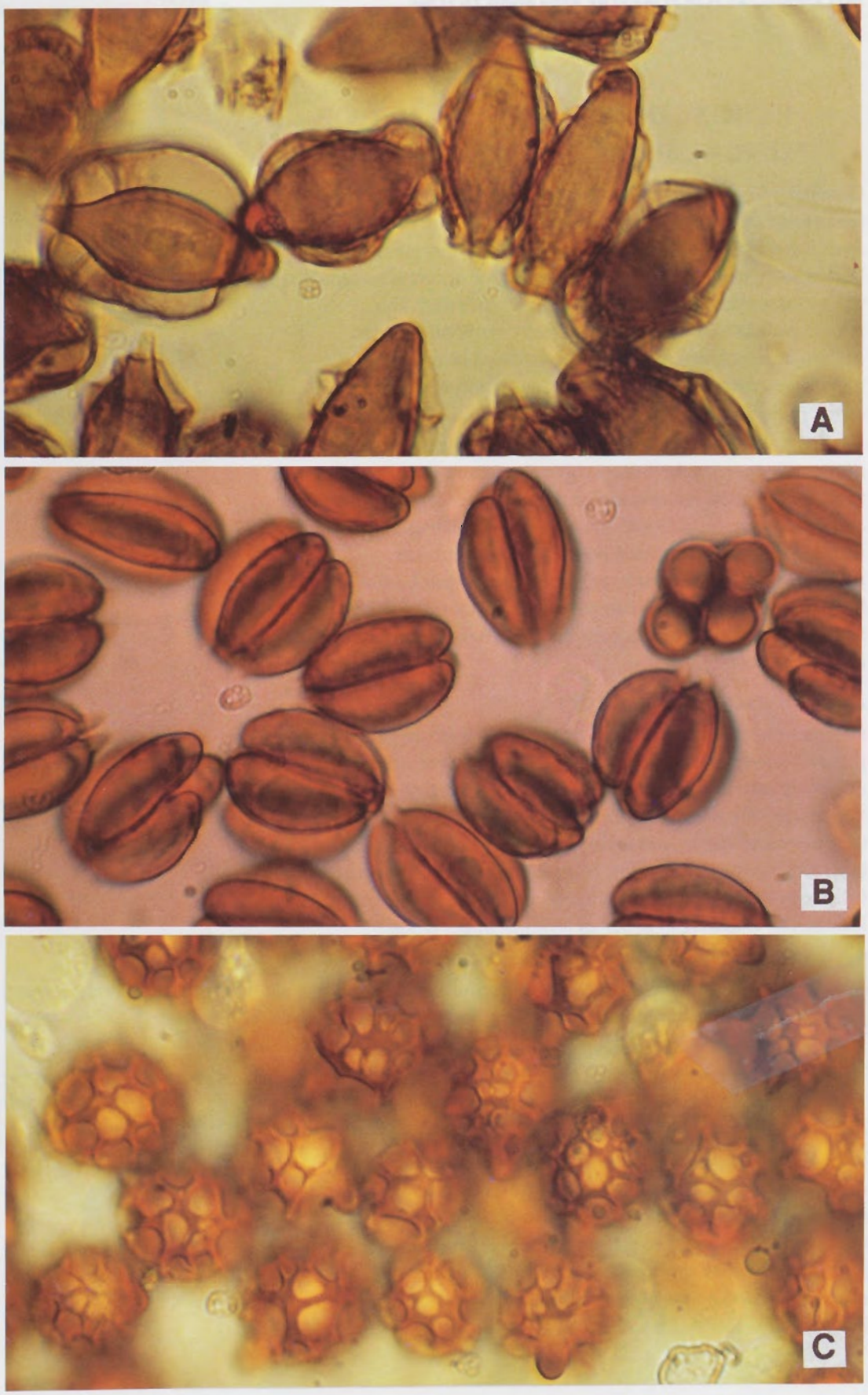


Figure 2.8. *Many fungi appear very similar macroscopically, but microscopic characters can reveal large differences. In the case of the spores illustrated here, all three of these truffle-like Australian fungi were originally placed in the genus* Hymenogaster *due to their similar macroscopic characters. However, spore morphology reveals that they represent three separate genera* **(A)** Descomyces, **(B)** Quadrispora *and* **(C)** Timgrovea.

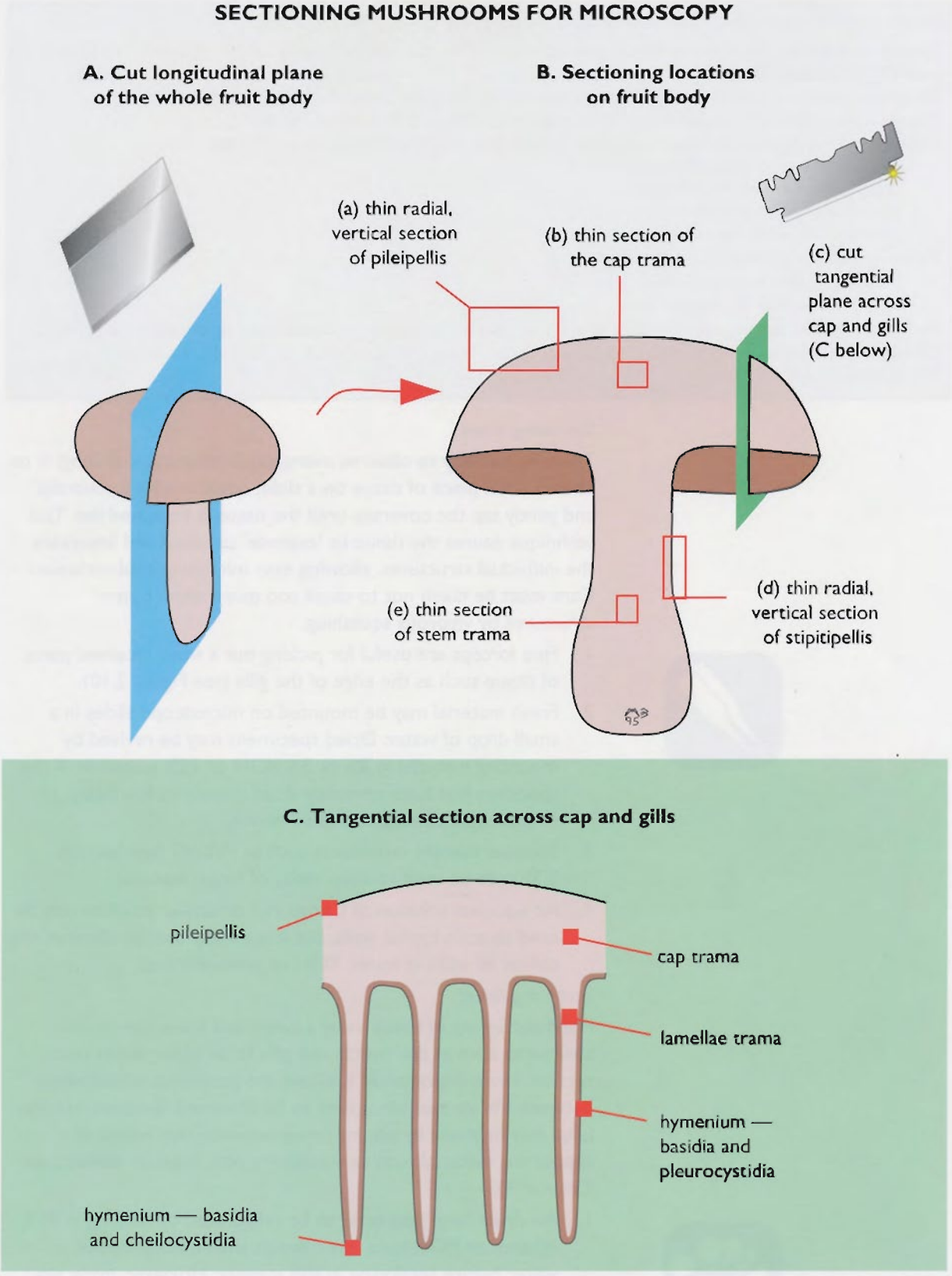


Figure 2.9. *How to dissect mushroom-like fruit bodies for observing the main microscopic characters. See text for detailed outline of procedure.*

A. *Cut the fruit body in half longitudinally.*

B. *Make thin sections, then make a tangential plane of the cap and gills.*

C. *Use the tangential plane now created to observe tissues.*

Basic microscopic data for fungal fruit bodies

Spores — note the colour, size, shape, wall ornamentation and thickness, amyloid or dextrinoid staining with Melzer's reagent.
Basidia — observe colour, size, shape, and number of sterigma (spore-bearing projections).
Trama — composed of hyphae which form patterns and layers in the gills, cap and stem.
Cystidia — swollen hyphal tips. Note their colour, size, shape and abundance. These include:
Pleurocystidia — on the gill faces
Cheilocystidia — on the gill edges
Pileocystidia — on the cap stem
Caulocystidia — on the stem
Pellis — skin (or cuticle) of a fungal organ
Pileipellis — skin on cap (pileus)
Stipitipellis — skin on stem (stipe)
Veil remnants — composed of hyphae which can have a characteristic colour, size, shape, or arrangement.
Clamp connections — often occur on fungal hyphae. Note their presence, or absence, abundance and distribution in different tissues.

Squashing tissue

The simplest way to observe microscopic characters of fungi is to place a small piece of tissue on a slide, cover it with a coverslip and gently tap the coverslip until the tissue is squashed flat. This technique causes the tissue to 'explode' outward and separates the individual structures, allowing easy microscopic observation. Care must be taken not to cause too much distortion of structures by vigorous squashing.



1. Fine forceps are useful for picking out a small localised piece of tissue such as the edge of the gills (see Figure 2.10).
2. Fresh material may be mounted on microscope slides in a small drop of water. Dried specimens may be revived by mounting material in 3% or 5% KOH or 10% ammonia. If the specimen had been correctly dried (slowly at low heat), all microscopic structures should revive.
3. Semi-permanent mountants such as PVLAG (see Section 3.3B) can be used to keep slides of fungal material.
4. An aqueous solution of Congo red or similar solution may be used to stain hyphal walls, but it is usually best to observe the colour of walls in water, KOH or ammonia first.

Sectioning tissue

Hand sectioning of tissue using a two-sided blade can enable structures such as the cuticle and gills to be observed in cross-section. More importantly, it allows the positional relationships between the various structures to be observed. Sections of fresh fungi may be made by placing tissue between two halves of a supporting material such as elderberry pith, foam or carrot (see Chapter 4).



1. Air-dried fungi may need to be rehydrated by soaking in 95% ethanol or KOH for a few minutes then quickly dipped in water before sectioning in this manner. However, many air-dried fungi, particularly truffle-like types, can easily be sectioned without rehydration.
2. First cut the fruit body in half longitudinally (Fig. 2.9A). Make thin radial sections of the cap cuticle (pileipellis) and trama,

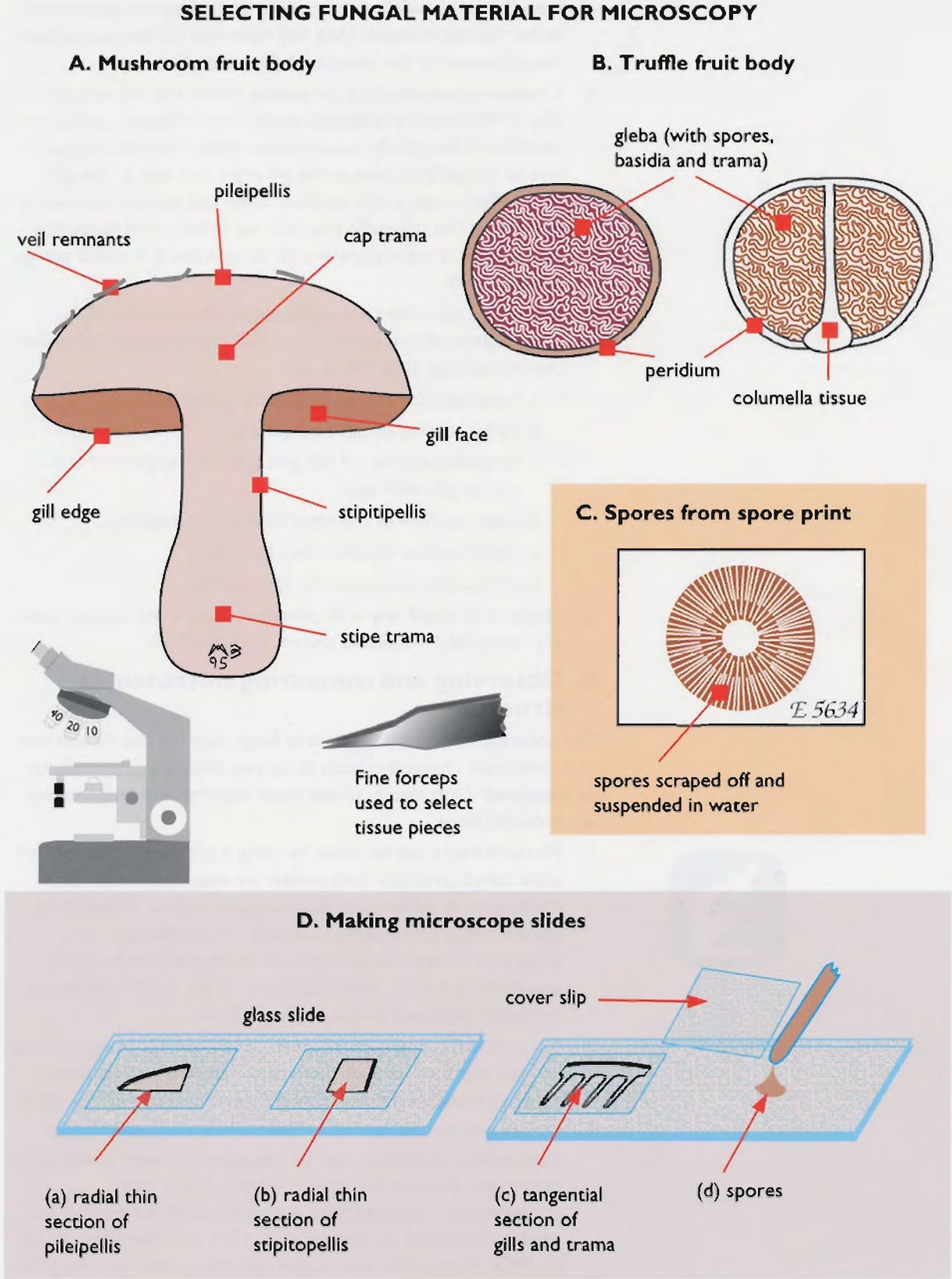


Figure 2.10. A–C. *Main parts of fruit bodies required for examining microscopic characters of ectomycorrhizal fungi, including spore prints. The diagrammatic fruit bodies shown here are sectioned in half longitudinally.* ***D.*** *Four main types of slide preparations.*

then the stem cuticle (stipitopellis) and trama for examination under the microscope. (Any veil remnants on the cap surface should appear in the section of the pileipellis.)

3. Create a tangential plane by cutting across the cap and gills (Fig. 2.9B). Use the tangential plane now created to make thin sections of the gills for examination under the microscope — one at the gill face, one at the gill edge, and one at the gill trama. Also make a thin section of the cap trama if wanted in this plane. The pileipellis may also be examined in tangential plane, but it is usually preferable to examine it in radial plane (as in step 3).
4. The stages described above should result in 6 main slide preparations of thin tissue slices, that can be examined under the microscope (Figs 2.9, 2.10):
   a. radial section of the cap cuticle (pileipellis),
   b. radial section of cap flesh (trama),
   c. tangential section of the gills (face and edge) and trama of the gills and cap,
   d. radial section of the stem cuticle (stipitopellis),
   e. radial section of stem flesh (trama),
   f. spores obtained from the spore print.
5. Sections of fungal tissue or spores scraped from a spore print are mounted on slides as shown in Figure 2.10.

B. Observing and measuring microscopic structures

The accurate identification of many fungi requires the dimensions of microscopic characters such as spores, basidia and cystidia to be measured. Only details of the most important measurements are provided here.



1. Measurements can be made by using a special eyepiece with a scale called an ocular micrometer or eyepiece micrometer. Calibration is achieved by counting the number of eyepiece divisions that correspond to usually 10 µm divisions on a stage micrometer which is placed in the position normally occupied by a slide. 1 µm = 0.000001 m ($m \times 10^{-6}$). Sizes are normally measured to the nearest 0.5 µm.
2. If the collection consists of more than one fruit body it is best to base measurement of characters on observations from a range of fruit bodies rather than from only a single fruit body.
3. *Shapes and ornamentations* — the surface texture of fungal microscopic characters can be described by many hundreds of terms (see glossary for some of them). For a more comprehensive account than is presented in this section the reader is referred to Largent et al. 1977 and Hawksworth et al. 1995. Many of the terms that are widely used in botany are applicable to fungi. For ectomycorrhizal fungi some of the main descriptive terms that apply to spores are outlined below and these can also be applied to other structures such as cystidia.

Table 2.4. Measurement parameters for microscopic structures used in fungal taxonomy. These categories can be used to make a microscope data sheet.

Structure	*Observation details*			
Spores	Colour when mounted in: water KOH Melzer's reagent other	size range*	shape ornamentation perisporium apex base/appendix	spore wall: structure, thickness, layers
Basidia	Colour	size range*	shape	hyphal wall, sterigmata, clamp connections
Gills	Hymenium structure	subhymenium	trama	arrangement of hyphae
Cap	Pileipellis	trama	veil remnants	
Stem	Stipitopellis	trama	veil remnants	
Cystidia Pleurocystidia Cheilocystidia Pileocystidia Caulocystidia	Presence, abundance, distributions	size range*	shape	colour, hyphal wall structure, clamp connections
*** For all size measurements**	sample size usually at least 20 to 25 per fruit body	Shortest and longest Narrowest and broadest	Average length Average breadth	Average ratio of length to breadth

4. *Colours and chemical reactions* — for all microscopic characters in water, KOH and Melzer's reagent, record the following.
   a. Some tissues (particularly spores) may react to Melzer's reagent by becoming blue (amyloid reaction) or brown (dextrinoid reaction). The reaction may be enhanced by gentle warming.
   b. In 1% cotton blue in lactic acid, the wall of spores or other structures may stain dark blue. This is called a positive cyanophilic reaction.
   c. Many fungi have distinct pigmentation and it is important to observe the location of these pigments. For example, pigmentation may occur inside cells (intracellular), between the cells (intercellular), or on cell walls (encrusting).
   d. There is a range of other chemical reactions associated with microscopic characters, but as these are not as frequently or widely used, they are not mentioned here.
5. *Data sheets* — a standardised data sheet is recommended for recording microscopic characters of fungi in a systematic way. Table 2.4 lists data categories which should be used for most fungi. It will not always be possible to record data for all of these categories. It is very important to record the name or number of the collection being examined and the magnification or scale of any drawings of microscopic features on every data sheet.

MEASURING BASIDIOSPORES

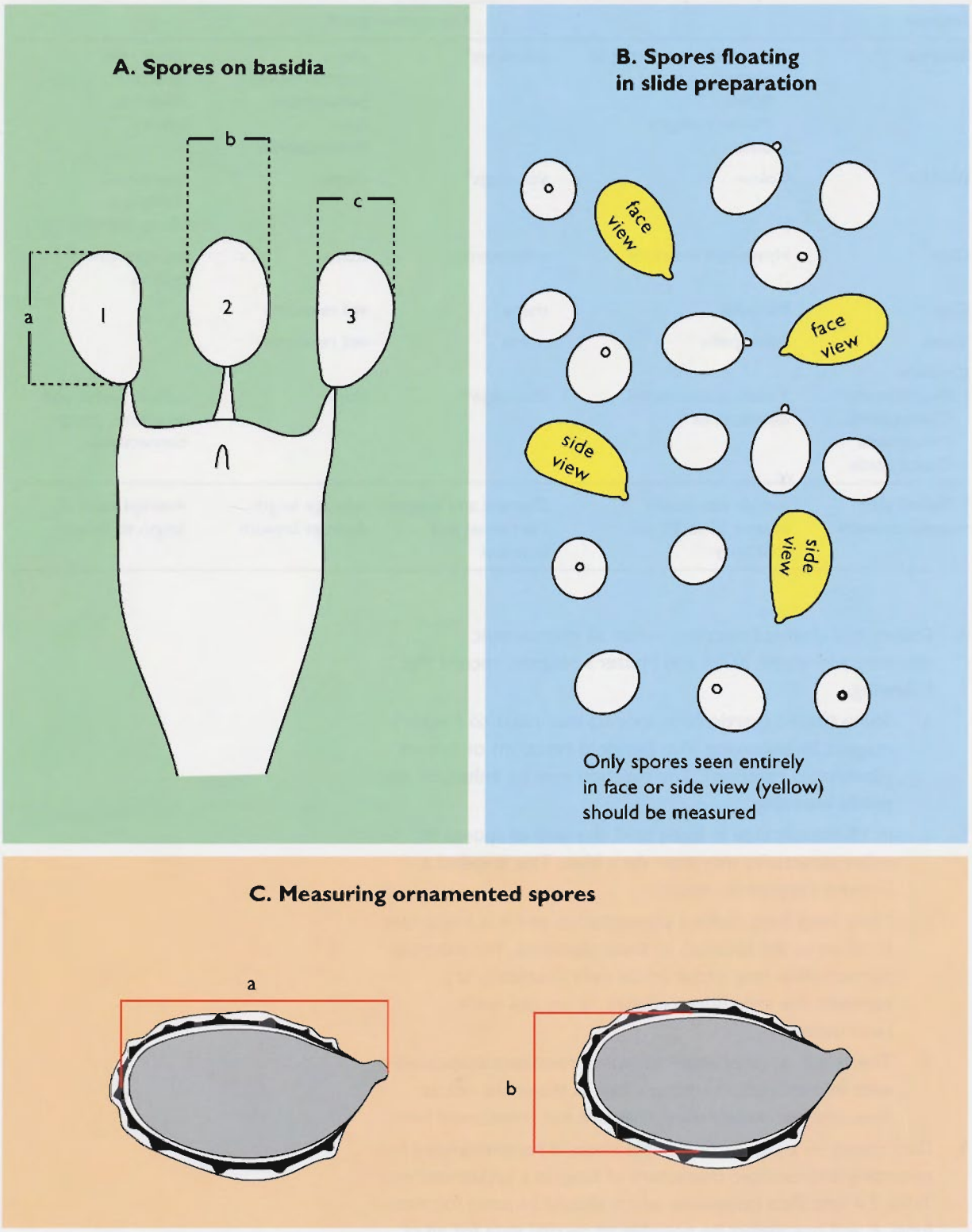


Figure 2.11. A. *View of a basidium with three of the four spores attached. Spores 1 and 3 are seen in side view. Spore 2 is seen in face view. Spore length is represented by a. Spore breadth in face view is represented by b, and spore breadth in side view is represented by c.*

B. *Measurement of spores floating in a microscope preparation.*

C. *Measurement of ornamented spores.*

C. Spore size and shape

The sexual spores produced by Basidiomycetes (basidiospores) and Ascomycetes (ascospores) are perhaps the simplest to observe and often the most distinctive microscopic character of ectomycorrhizal fungi. Spores are not simply circular propagules lacking distinct structure. Rather, each fungal species has unique spores with many characteristics that need to be recognised and described. ECM fungi have an extremely wide variety of spore colours, shapes, sizes and walls (see Figs 2.8, 2.14 for some examples).



1. *Mounting spores* — spores can be observed in mounts of gill tissue but this is not recommended because they will often include immature spores. Mature spores for describing or illustrating should be obtained from spore prints by scraping a small amount off the print with a blade or forceps. These spores are then mounted in water, 3% KOH, 10% ammonia, or PVLAG.
2. *Spore colour* — the colour of the spores under the microscope in water and in KOH or ammonia is usually recorded (Table 2.4). Also the reaction to Melzer's reagent is observed to determine if the spores are amyloid (blue reaction) or dextrinoid (red/brown reaction).
3. *Measuring spores* — a minimum of 20 to 25 spores per specimen is measured to the nearest 0.5µm: shortest and longest, average length, narrowest and broadest, average breadth, average ratio of length to breadth (as in Table 2.4). The dimensions of spores should be measured in face and side (profile) view (see Fig. 2.11A). *Note that spores floating in a mounting medium will have random orientations and only those seen completely in face or profile view should be measured* (Fig. 2.11B). This rule does not apply to many truffle-like fungi, which have symmetrical spores.
4. *Spore sizes* — the spores of ECM fungi vary in length from about 4 µm to about 30 µm (most are around 8–15 µm). Truffle-like fungi tend to have larger spores than mushroom-like fungi, and ectomycorrhizal Ascomycetes often have larger spores than Basidiomycetes. Record the medium in which measurements are made, as this can effect spore sizes (Oliver et al. 1987). It is usual to include the apiculus but not any ornamentation or perisporium in spore size measurements (Fig. 2.11C), but it is important to record what structures were measured.
5. *Spore symmetry* — as shown in Figure 2.11A, ECM fungi that produce above-ground fruit bodies usually have spores that are bilaterally asymmetrical (inequilateral). These spores are known as ballistospores as they are forcibly discharged from the basidium for dispersal in the air currents. They are also referred to as heterotrophic spores because during development they are borne obliquely on the sterigmata to facilitate discharge. Gasteromycetes and truffle-like fungi

usually have spores that are bilaterally symmetrical (equilateral), thus, if viewed in face, side, or end views the spore can be divided into mirror images. These symmetrical spores are called statismospores because they generally are not forcibly discharged from the basidium. During development statismospores are usually centred upon the sterigmata and are referred to as orthotropic spores which are later passively released.

6. *Spore shape* — can be described by many descriptive terms (Table 2.5, Fig. 2.12B). For more terms the reader is once again referred to Largent et al. 1977. Shapes that are in between these broad categories may be described by applying the prefix 'sub', e.g. subglobose is not quite globose but close to it. The shape and size of the hilar appendix (point of attachment to the basidium before separation) should be recorded, e.g. rounded, beaked, broken or entire. Also the spore apex (opposite end to the hilar appendix) may be rounded or attenuated (mucronate or rostrate).

Table 2.5. Main shapes of spores of ectomycorrhizal fungi (see Fig. 2.12B).

- *Ellipsoid* — sides are curved and ends rounded
- *Ovoid* — egg-shaped, one end larger than the other
- *Globose* — spherical
- *Oblong/cylindrical* — ends round, sides parallel
- *Fusiform* — tapering of both ends
- *Citriniform* — lemon-shaped, ends of the spores with a beak-like projection
- *Amygdaliform* — almond-shaped
- *Phaseoliform* — bean-shaped
- *Angular* — various angular spore shapes

D. Spore wall structure and ornamentation

The spore wall is an important diagnostic character for distinguishing ectomycorrhizal fungi, as they vary from thin and smooth to complex and ornamented (see Figs 2.13, 2.14). For example, some families such as the Amanitaceae have entirely smooth spores, while species of other families may have unique patterns of spore ornamentation.



1. Details of spore walls and ornamentation must be observed under the high power microscope objective (100X). In some cases, high power light microscopy will reveal fine ornamentation of spores which appear smooth at lower magnifications. More details of the surface topography of spore ornamentations are revealed with the scanning electron microscope (Fig. 2.14).
2. For all spores regardless of whether they are smooth or ornamented, the spore wall thickness and the number of distinct layers should be noted. Usually it is sufficient to note whether it is less than 0.5µm thick or not, but some spores may have walls up to 4 or 5µm thick. At the apex of the spore, the wall may be conspicuously thin and modified into a

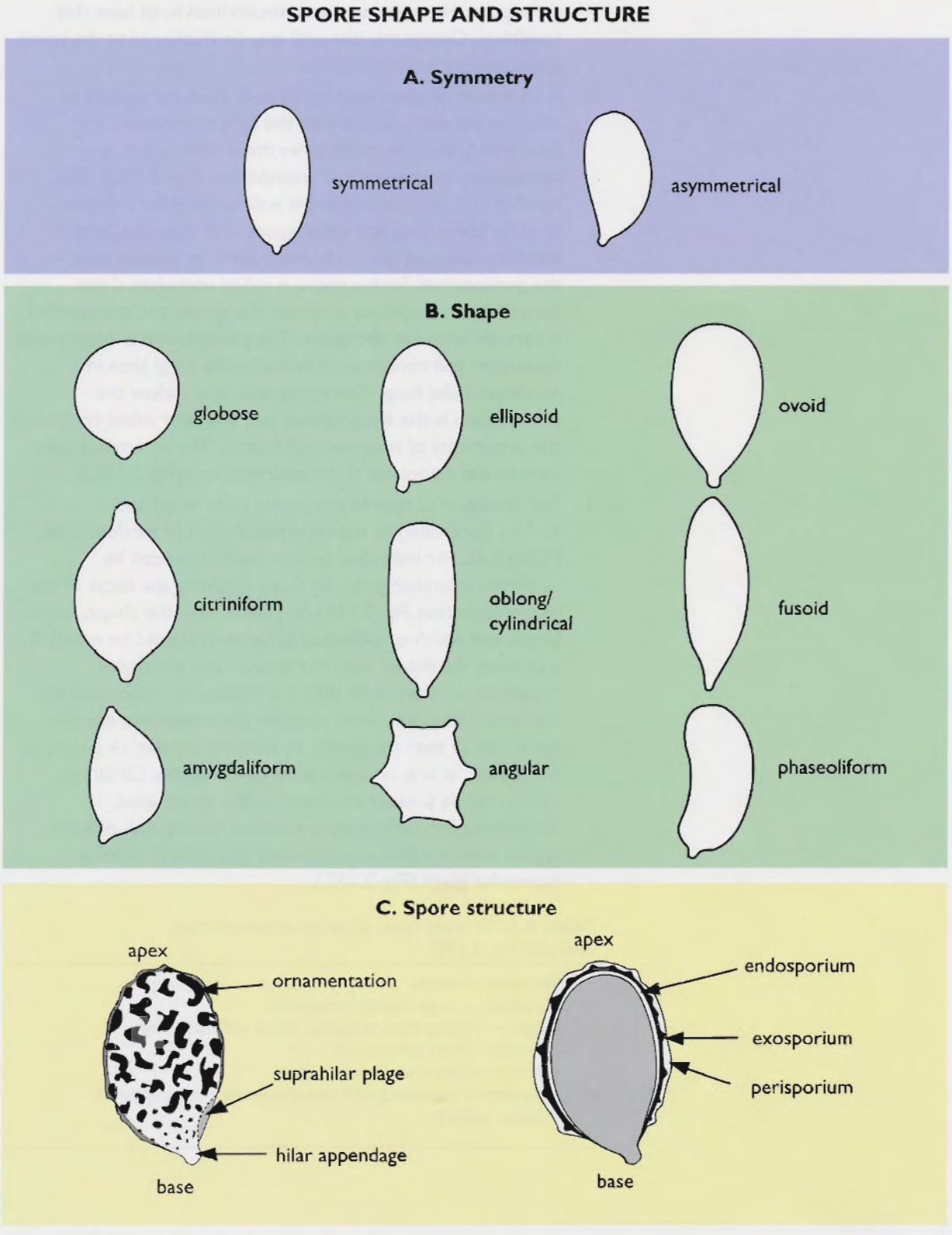


Figure 2.12. Spore morphology.

A. *Symmetry of spores (note, both spores are shown in profile (side) view. Many asymmetrical spores can appear symmetrical in face view while being asymmetrical in side view).*

B. *Some main spore shapes of fungi.*

C. *Main structures and wall layers of spores. Note that the spore shown is loosely based on a rough-spored Cortinariaceae species. Spores of other fungi may not have all these structures.*

germ pore. However, few ectomycorrhizal fungi have this condition. Conversely, the wall may be thickened at the spore apex, forming a distinct callus.

3. A multitude of spore wall layers have been recognised by electron microscopy, but with the light microscope it is usually only possible to observe three main layers, the *perisporium, exosporium and endosporium* (Fig. 2.12C). The number and thickness of spore wall layers differ between fungi. In some fungi the outer spore wall layer can form a distinct separated layer that may appear as a loose sack — the perisporium. Such a spore is called utriculate if the perisporium completely encloses the spore, and calyptrate if it partially encloses the spore. The perisporium is often more developed and conspicuous in truffle-like fungi than in mushroom-like fungi. The spore wall layer below the perisporium is the exosporium, and this layer often comprises the ornaments of rough-walled spores. The innermost layer next to the cytoplasm is the endosporium (Fig. 2.12C).
4. For ornamented spores the profile (side view) and surface (face view) of the ornaments need to be described (Table 2.6). For individual spores, both views can be observed interchangeably by finely adjusting the focus of the microscope (see Fig. 2.13A). In profile view the shape, apex, length and width of individual ornaments should be noted. In face view, the shape, size, distribution and density of ornaments and whether they are isolated or connected are important features. Note whether the ornaments are the same size all over the spore, or become smaller, or larger, or are absent, in one or more parts of the spore. Ornamentation may be present or absent at the spore apex. Sometimes the ornaments are absent from a well-defined region near the hilar appendix and this zone is called a suprahilar plage (Fig. 2.12C).

Table 2.6. Six main types of spore ornamentation (see also Fig. 2.13B).

- Verrucose — warts
- Nodulose — large knobbly ornaments
- Ridged — ranging from striate to ribbed with large ridges
- Punctate — small projections
- Echinate — long sharp pointed spines
- Reticulate— ornaments with interconnections in a regular or irregular pattern

SPORE ORNAMENTATION

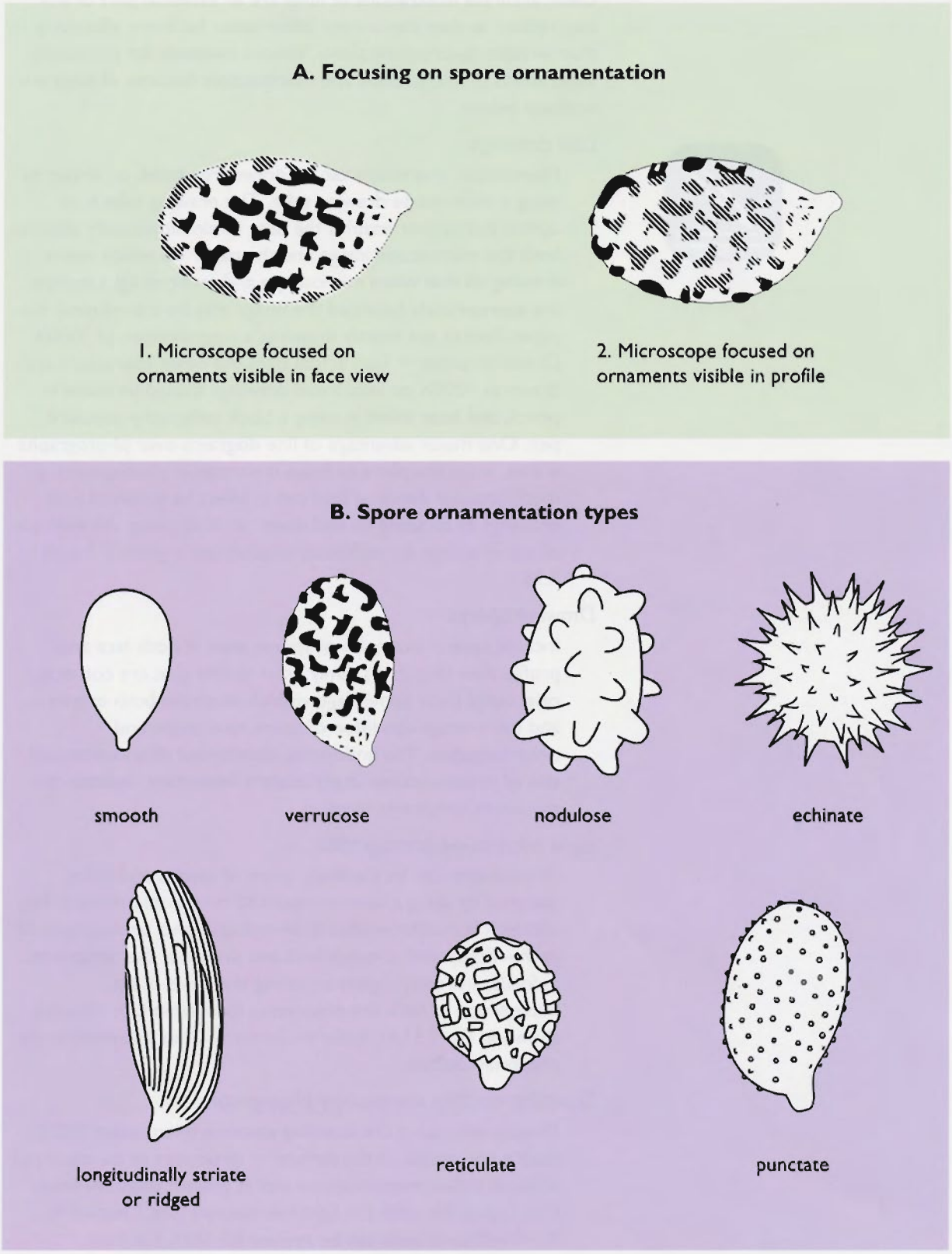


Figure 2.13. *Spore ornamentation:*

A. *Different views of spore ornamentation obtained by fine adjustment of microscope focus.*

B. *Some common types of spore ornamentation.*

E. Illustration of microscopic structures

Clear, accurate illustrations of fungi are an essential part of any description, as they can convey information far more effectively than written descriptions alone. Various methods for producing illustrations of microscopic and macroscopic features of fungi are outlined below.



Line drawings

Microscopic characters can be drawn free-hand, or drawn by using a microscope drawing tube. The drawing tube is an optical device that enables the user to simultaneously observe both the microscope image and the paper on which one is drawing so that when microscope and external light sources are appropriately balanced the image may be traced onto the paper. Spores are mostly drawn at a magnification of 2000X (2 mm on paper = 1µm actual size). All other characters are drawn at 1000X or less. Initial drawings should be made in pencil, and later inked in using a black calligraphy-standard pen. One major advantage of line diagrams over photographs is that, while the plane of focus is narrow in photographs, a much broader depth of field can in effect be achieved with drawings by focusing up and down while drawing. An example of line drawings for *Hebeloma westraliense* is given in Figure 2.15.

Drawing Spores

Include several examples of spores seen in both face and profile view (Fig. 2.15). Only draw spores that are correctly orientated (hilar appendage visible). Illustrate both extremes and the average condition of spore size, shape and ornamentation. The uniformity, density and distribution and size of ornamentation is particularly important. Indicate the mountant which was used.

Light microscope photographs

Photographs can be routinely taken of spores and other features, by using a camera mounted on the microscope. For spores it is recommended to always take two photographs of each – firstly with the side wall and profile of the ornaments in focus, and then slightly adjusting the focus of the microscope to have the ornaments focused in face view (as shown in Fig. 2.13A). Refer to Section 1.7 for information on photomicroscopy.

Scanning electron microscope photographs

Photographs using the scanning electron microscope (SEM) enable fine details of the surface of structures to be observed at much higher magnifications and at greater depth of focus than is possible with the light microscope (see Chapter 4). Air-dried specimens can be revived for SEM, but fresh

material fixed in buffered glutaraldehyde may give better results with delicate structures. Spores of ectomycorrhizal fungi are particularly suitable candidates for SEM (see Fig. 2.14, and Pegler & Young 1971), although other structures such as cystidia and clamp connections can be observed. More information on SEM procedures is provided in Chapter 4. Three methods of handling fungal spores for SEM are listed below.

1. Use a portion of a spore print on paper. Put paper through whole dehydration series and then stick it to SEM stub after critical point drying either with 2-sided tape, silver paint or nail varnish.
2. Use gill material. Put gill through whole series then after critical point drying, scrape spore 'dust' onto round coverslip, mounted on stub with silver paint.
3. Use a gelatine-based film — moisten with water, scrape on spores. Mount onto stub with silver paint after critical point drying.

F. Compiling detailed descriptions of fungi

Publishing taxonomic data on ectomycorrhizal fungi

A number of written and unwritten rules apply when publishing taxonomic data on named or new taxa. Specifically, the latest rules of the International Code of Botanical Nomenclature (ICBN) must be adhered to for valid publication. The Code covers rules such as the necessity of providing a Latin description, nominating a type specimens (the nomenclatural type on which the name is based), synonymy and priority rules. Priority principles and synonyms are particularly important.

Priority principles and synonyms — in cases where two or more different names have been independently given to the same taxon, the oldest name available must be used. All of the more recent names become synonyms. Names given to fungi before a certain date are invalid and it is not necessary to consider any names published before the nominated dates, as follows: for some fungi including Gasteromycetes the starting point is taken as 31 December 1801 — *Synopsis Methodica Fungorum* by Persoon. For other fungi including Hymenomycetes and Ascomycetes the starting point is taken as 1 January 1821 — *Systema Mycologicum* Volume 1, by Fries.

Publishing descriptive data about named species

In taxonomic works it is necessary to provide a list of synonyms for the species, i.e. mostly invalid names incorrectly applied to a species after it had already been validly published as a legitimate name initially, and their place and date of publication, i.e. indicate whether the synonym was based on the same specimen as the initial name was applied to (indicate by ≡), i.e. a nomenclatural synonym, or based on another specimen (=), i.e. a taxonomic synonym.

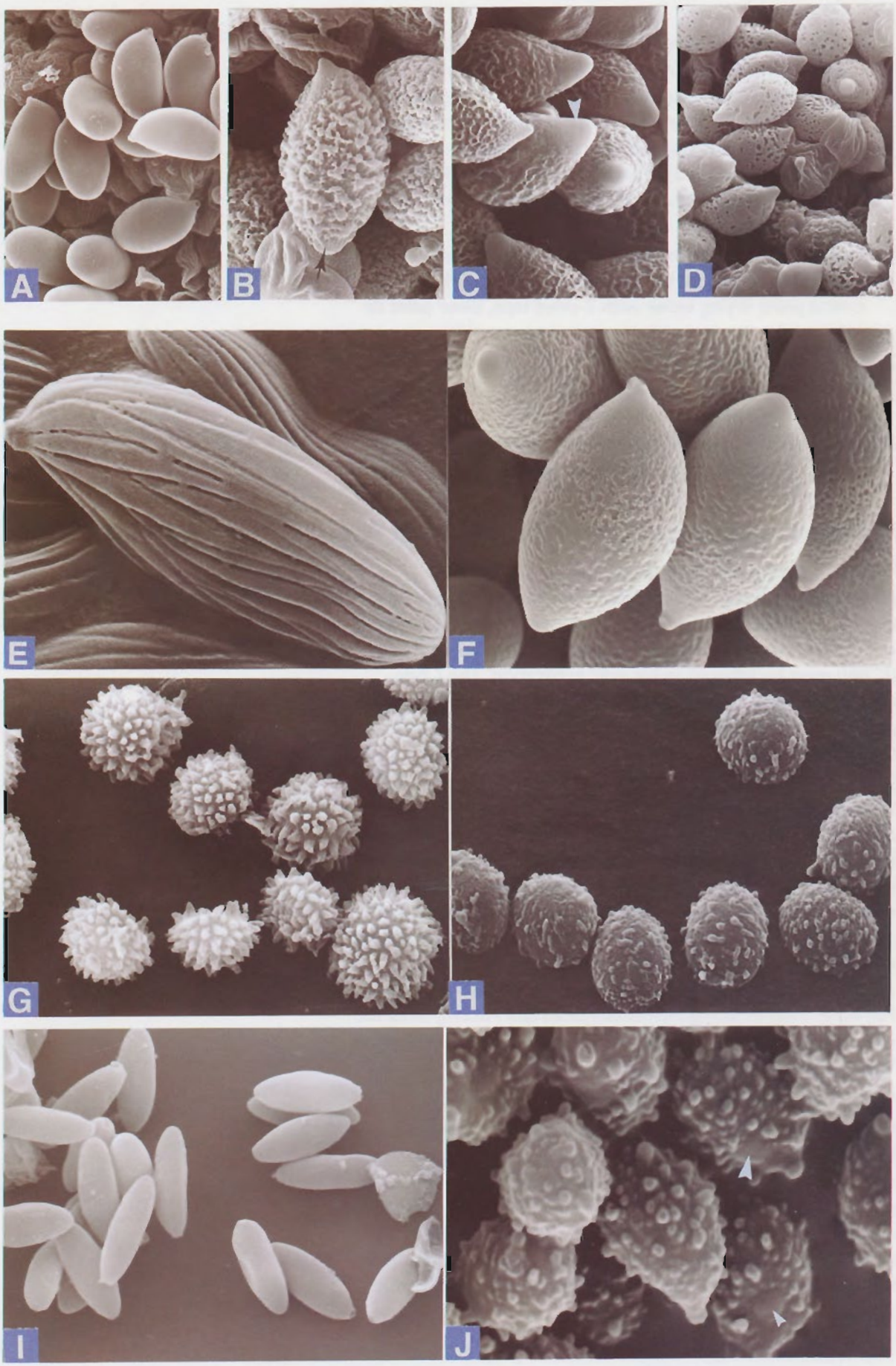

A
B
C
D
E
F
G
H
I
J

Figure 2.14. Scanning electron microscope photographs of fungus spores.

A. Pholiotina aporos — *smooth, ellipsoid.*

B. Descolea majestatica — *coarsely verrucose, ellipsoid, with roughened callus at apex (arrow).*

C. Descolea recedens — *verrucose, amygdaliform, with smooth attenuated apex (arrow).*

D. Descolea flavoannulata — *verrucose with perisporium partially covering surface, ellipsoid.*

E. Boletellus obscurecoccineus — *longitudinally ridged, fusoid-cylindrical.*

F. Descolea maculata — *finely verrucose, amygdaliform.*

G. Pisolithus tinctorius — *echinate, globose.*

H. Russula sp. — *warty-nodulose, broadly ovoid.*

I. Hysterangium sp. — *smooth, fusoid-oblong, with truncate base.*

J. Leucopaxillus lilacinus — *nodulose, ellipsoid-ovoid, with smooth suprahilar plage (arrows).*

Sample showing synonyms for a fungus

Scleroderma areolatum Ehrenb. (1818). *Sylv. Myc. Berol.*, p. 27.

= *Scleroderma lycoperdoides* Schw. (1882). *Schrift. Naturf.* Ges. Leip. 1, 61.

= *S. verrucosum* (Vaill.) Pers. subsp. *typicum* Sebek var. *violacens* Herink ex Sebek (1953). Sydowia 7, 176.

= *S. verrucosum* Pers., sensu Guzman (1967). *Ciencia* 25, 199.

After providing a description of your specimens (similar to the description provided below for a new species), it is useful to outline any differences between your specimens and previously published data on the species.

Publishing new species

Delimitation of a new species should not usually be based only on differences in a single character but at least several characters. Macroscopic characters such as fruit body colour and size can vary according to environmental conditions and are not always reliable for determining differences between species. It is therefore best to have several different collections from different locations before attempting to describe a new species. Also microscopic characters must be very distinct to separate species. For example, if one collection has larger spores than another, but there is some overlap in size, this difference does not define separate species unless other characters differ markedly between the collections.

In cases where specimens appear to be different to all named species, a thorough literature search must be conducted to obtain comparative taxonomic data of all potentially similar fungi. Furthermore, it is often desirable, if not essential, to obtain a loan of type or other authenticated material of named species from herbaria, in order to directly compare with specimens of the potentially new species. Another step in the process may be to consult a taxonomic mycologist who is familiar with the fungi being investigated.

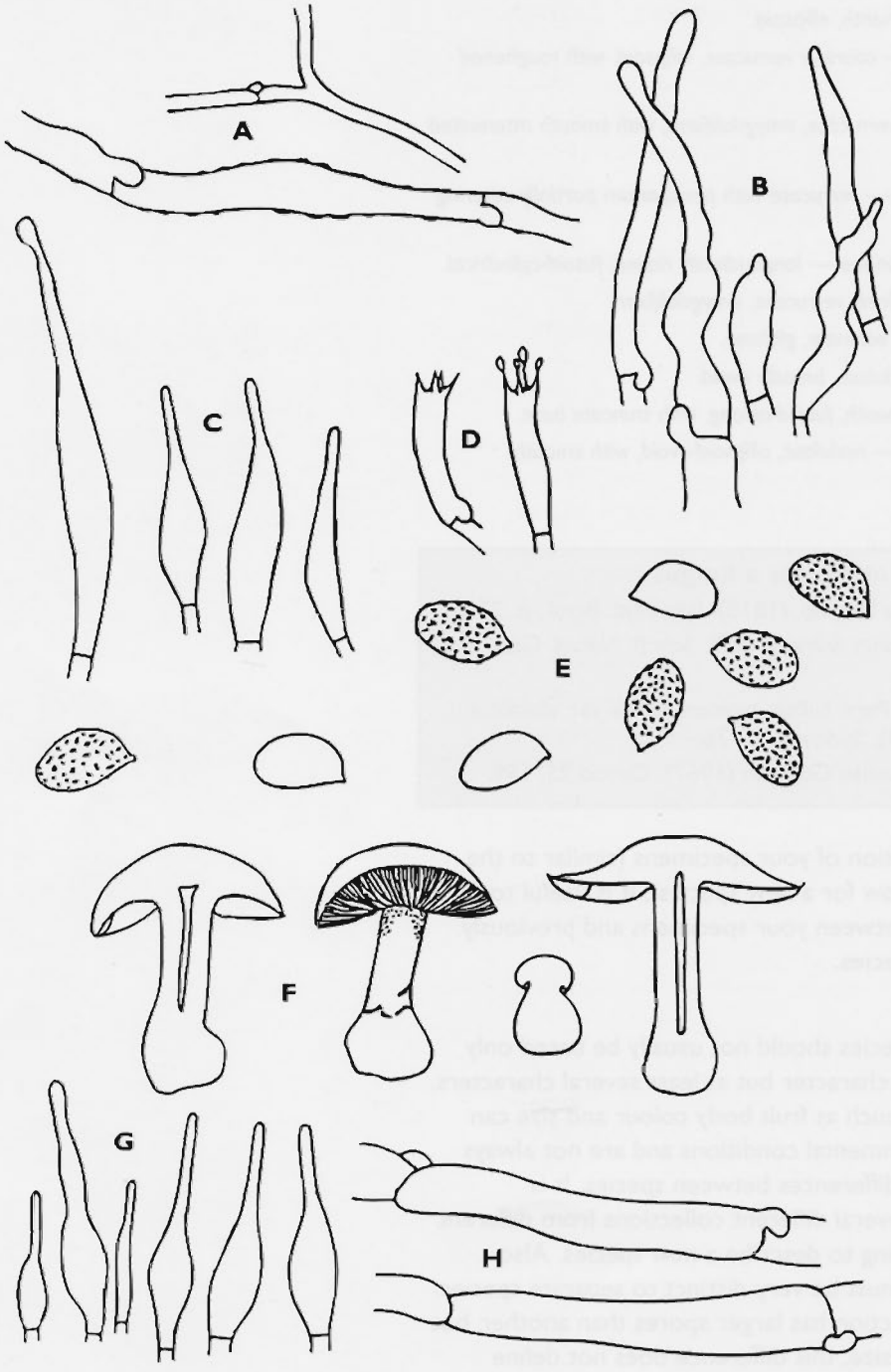


Figure 2.15. An example of line drawings of microscopic features of Hebeloma westraliense.

A. *Hyphae from the pileipellis.*

B. *Caulocystidia from the stem surface.*

C. *Cheilocystidia from the gill margins.*

D. *Basidia from the gill hymenium, showing four sterigmata.*

E. *Spores shown with and without ornamentation in face and profile views.*

F. *Young and mature fruit bodies of the species in side view and cross-section.*

G. *Pleurocystidia from the gill face.*

H. *Hyphae from the gill trama with clamp connections.*

If thorough literature searches and direct comparative investigations show that the specimens cannot be assigned to any described species, a new species may be proposed by valid publication preferably in an internationally circulated journal. The author should aim to convince the readers that the species being published is indeed newly discovered and unique. Generally, a comprehensive description with clear illustrations and logical evidence of the uniqueness of the proposed species is needed. For each proposed new species, the author needs to follow the following steps.



1. State the name of the new species and its genus (the binomial), and its author. Clearly state that the name is proposed as a new taxon.
2. Provide a description or diagnosis in Latin.
3. Compile a comprehensive macroscopic and microscopic description of all characters in English or the favoured language of the journal.
4. Provide illustrations of macroscopic and microscopic features, and a photograph of the fruit bodies where possible.
5. Clearly nominate a single collection to represent the holotype (nomenclatural type) of the species and give its herbarium location (and number) (isotypes may also be nominated). Collections must be lodged in recognised, safe herbaria (preferably those with an Index Herbariorum acronym).
6. Provide full details of collections examined.
7. Discuss the unique characters that define the new species.
8. Provide evidence that specimens (preferably type material) of closely related taxa have been examined, or, if not, that the differences are beyond doubt based on published data in the literature.

The following example of a published description of a new species of a mushroom-like ectomycorrhizal fungus is from Bougher et al. (1994). Line drawings and photographs of macroscopic and microscopic structures for each fungus were also provided in the publication.

Example of a published new species description

Rozites metallica Bougher, Fuhrer & Horak **sp. nov.**

Pileus ad 140 mm, primo convexus dein umbonato-applanatus, primo griseoazureus maturitate pallide griseus, zonis argillaceis vel cremeoluteis in umbone, glutinosus, radialiter rugulosus, squamulis albis evanescentibus reliquiis veli obtectus. Lamellae adnexae vel subdecurrentes, primo cremeae vel pallide virides, dein argillaceae, acie concolori subfimbriata. Stipes ad 150 x ad 15 mm, cylindricus, subclavatus vel apicem versus attenuatus, albus, siccus, minute squamulosus. Annulus membranaceus, albus, persistens. Odor saporque nulli. Caro subazurea ad apicem stipitis. Sporae 9.5–12 x 7.5–8.5 µm, amygdaliformes vel ellipsoideae, grosse verrucosae. Septa hypharum fibulata. Ad terram in sylvis nothofagineis, Australia (Tasmania), 22. v. 1993. Holotypus HO.

Etymology (derivation of species name)

Metallica refers to the distinctive bluish-grey colour of young basidiomes.

Pileus 40–140 mm broad, at first convex or broadly parabolic with plane, entire margin (sometimes with adhering velar remnants), slowly expanding via a campanulate stage to applanate at maturity with translucent striate, eroded margin; when young uniformly bluish grey (19E6), markedly hygrophanous, fading to pale grey (19D2) with tan or cream yellow central zone (near 4A4) and grey near pileus margin (19F3), brown tints eventually becoming more evident with age, surface smooth at first but radially wrinkled with age, strongly glutinous when wet and innately radially streaked as drying out, not bruising, context cream (a bluish tint sometimes evident in young specimens). *Lamellae* (L: 22–38, l: 44–64) 5–10 mm broad, adnexed with small decurrent tooth at first remaining adnexed at maturity, crowded then close, with abundant lamellulae (1 or 2 between each pair of lamellae), cream with slight greenish tinge at first (near 4A2), becoming pale tan (5B5 to 6C4) then darker tan (5D6) at maturity, edges concolorous, minutely fimbriate, not bruising. *Stipe* 50–150 x 7–15 mm, variable from cylindrical, slightly tapering towards apex, to having a swollen base up to 20 mm broad, solid, white, dry, shiny and longitudinally appressed fibrillose, overlain by superficial (easily removed on handling), abundant white small squamules and hyphal bundles (recurved near stipe base), attaining watery brown stains upon handling/bruising, context cream (bluish at apex in youngest specimens), basal mycelium white and may extend partially along the stipe base as an appressed covering. *Annulus* superior or central, membranous, smooth or faintly striate, flanging, white on surface and underside except where discoloured by spore deposit, persistent but sometimes fragmenting with age. *Universal veil* white, appressed, matted, fibrillose, squamules on the pileus, conspicuous at first but evanescent.

Macrochemical test 15% KOH reddish purple on pileipellis, 10% $FeSO_4$ no reaction on pileipellis, pileus context, or stipe context. *Odour* mushroom. *Taste* mild. *Spore print* brown (6D7 to 6D8) when fresh drying also brown (near 6E6).

Basidiospores side view 9.5–12 (12.5) x (7) 7.5–8.5 µm (n = 30), mean 10.5 x 7.8 µm, mean L/B ratio 1.3. Face view (9) 9.5–12 x (7) 7.5–8.5 µm (n = 21), mean 10.4 x 7.8 µm, mean L/B ratio 1.3. Yellow brown in 3% KOH. Broadly amygdaliform to pip-shaped in side view, broad ellipsoid in face view, mucro absent, coarsely verrucose. *Basidia* 32–41 x 10–14 µm, clavate, hyaline, thin-walled, sterigmata to 5 µm in length, 4-spored, clamped at base. *Lamellae trama* parallel, hyphae to 5 µm broad, hyaline, smooth-walled, septa clamped. *Subhymenium* undifferentiated. *Sterile marginal cells* 15–30 x 4–10 µm, clavate to filiform, hyaline, smooth-walled, with 1 or multiple septa, infrequent along lamella edge, not emerging beyond hymenium. *Sterile facial cells* undifferentiated. *Caulocystidia* absent. *Pileipellis* inner pellis subcellular, thick-walled, cells up to 30 µm broad, with golden encrusted walls less than 1 µm broad. Outer pellis of hyaline, thin-walled, smooth, clamped, hyphae (5–15 µm broad), with walls intact, loosely entangled in a hyaline matrix. *Stipitopellis* not differentiated. *Stipe trama* longitudinally arranged hyaline, thin-walled, clamped, hyaline hyphae (3–9 µm broad). *Pileus trama* hyaline, thin-walled, clamped hyphae with constricted septa, some inflated to 30 µm broad. *Clamp connections* present on all septa.

Habit and Habitat

Among litter in *Nothofagus* forests in Tasmania and Victoria. Locally abundant, with large numbers of basidiomes occurring within small areas.

Specimens Examined

Tasmania, Mount Field National Park, near Lake Fenton in dense, entangled, low forest dominated by *Nothofagus gunnii*, 22 May 1993, coll. N. Bougher, B. Fuhrer & S. Bolsenbroek *s.n.* (holotype HO 307262, isotypes at ZT and CSIRO E4929). Tasmania, Cradle Mountain — Lake St Clair National Park, Hounslow Heath, Waldheim in forest with *Nothofagus gunnii*, 20 May 1993, coll. N. Bougher & B. Fuhrer *s.n.* (paratype CSIRO E4911, HO 307263). Victoria, Donna Buang Road near road to summit of Mt Donna Buang, around base of *Nothofagus cunninghamii*, 18 May 1985, coll. T. May & B. Fuhrer *B343* (MEL, CSIRO E5053).

Further Notes

R. metallica is readily recognised in the field by the bluish-grey colour and conspicuous white veil fragments of young, near mature, and sometimes mature basidiomes. Weather conditions during development largely determine how long the juvenile colours persist, and individual basidiomes within the same location may vary in the rate at which they transform to grey and brown tints with maturity. The macroscopic appearance and the coarsely ornamented, broad spores separate this species from all others in the genus.

G. Glossary of terminology

Amyloid(y) — blue reaction of spores, hyphae or other structures to Melzer's reagent.
Asci — sack or tube-like structures containing the developing ascospores of Ascomycetes (singular = ascus).
Ascospores — sexual spores of Ascomycetes, borne in asci.
Asymmetrical spore — a spore which is not bilaterally symmetrical, i.e. cannot be divided axially into mirror images.
Ballistospore — spore which is forcibly discharged from the basidium. The spores are usually asymmetrical.
Basidia — club-like structures bearing the developing basidiospores of Basidiomycetes (singular = basidium).
Basidiospores — sexual spores of Basidiomycetes, borne on basidia.
Calyptrate — spores partially enclosed by an outer spore wall in the form of loose sack.
Caulocystidia — cystidia on the stem of a mushroom-like fruit body.
Cellular hyphae — inflated and packed together to resemble plant parenchyma.
Clamp connection — swollen structure or loop located at the septa (cross-walls) of some Basidiomycetes hyphae, involved in allowing nuclei to migrate into new cells after mitotic division.
Cyanophilic — spore or hyphal wall staining dark blue in cotton blue.
Cystidia — sterile cells of distinct shape, colour and/or size in the hymenium or other locations (see also caulocystidia, pileocystidia).
Dextrinoidy — brown reaction of spores, hyphae or other structures to Melzer's reagent.
Endosporium — the innermost spore wall layer next to the cytoplasm.
Exosporium — the spore wall layer immediately below the perisporium. This layer often comprises the ornaments of rough-walled spores.
Germ pore — a zone of reduced wall thickness at the spore apex (opposite end to the hilar appendage).
Heterotrophic — a spore which is borne obliquely on the sterigma. This type of attachment facilitates spore discharge. The spores are asymmetrical.
Hilar appendage — a projection at the base of basidiospores where the spore was attached to the sterigma. Sometimes referred to as the hilar appendix or apiculus.
Hyaline — clear and uncoloured as seen under the microscope.
Hymenial trama — tissue composed of sterile hyphae which support and nourish the hymenium, e.g. located in between the hymenial layers of the gill in mushroom-like fungi.
Hymenium — an organised layer that supports and includes the fertile structures bearing ascospores or basidiospores.
Hypha — microscopic tube forming fungal mycelium and fruit bodies. Each hypha may be separated or not into cells by septa (plural = hyphae).
Melzer's reagent — iodine solution used to test amyloidy and dextrinoidy of spores and other structures.
Mycelium — aggregation of hyphae (see Section 1.3C).
Orthotropic — a spore which is borne centred on the sterigma. Discharge is not forcible. The spores are usually symmetrical.
Pellis — outer edge (skin) of fungal fruit bodies.
Peridium — outer edge (skin) of truffle-like fruit bodies.
Perisporium — outermost spore wall layer, often evanescent, but sometimes persisting in mature spores and inflating out to form a loose calyptrate or utriculate sack.
Pileipellis — outer edge of the pileus (cap cuticle) of mushroom-like fungi.
Pileocystidia — cystidia in the pileipellis of mushroom-like fungi.
Pileus — cap of mushroom-like fungi.
Septa — cross-walls delimiting cells that comprise hyphae.
Statismospore — spores which are not forcibly discharged from the basidium. These spores are usually axially symmetrical.
Sterigma — projections on basidia to which developing basidiospores are attached before separation. Most often 4 per basidia, but may be any number ranging from 1 to about 8.
Stipe — stem of mushroom-like fungi.
Stipitopellis — outer layer of the stipe.
Suprahilar plage — a depressed or flattened spot near the hilar appendage of spores often distinguished by the absence of ornaments.
Symmetrical spore — if a spore viewed in face, side, and end views can be divided into mirror images it is considered symmetrical.
Trama — sterile tissue of the gills, cap or stem of fruit bodies, e.g. the cap trama constitutes and supports the bulk of a mushroom-like fruit body.
Utriculate — spores entirely enclosed by an outer spore wall in the form of loose sack.
Veil — outer layers of tissue that protect developing fruit bodies of some Basidiomycetes.
Veil remnants — hyphae or cells overlying the pellis, particularly pileipellis, usually derived from the universal veil.

The main aims of a herbarium collection

1. *Safe maintenance of specimens into the long-term future with up-to-date nomenclature.*
2. *High level of associated descriptive and illustrative data associated with the specimens.*
3. *Accessibility of physical data (i.e. the specimens) and electronic data (i.e. database) for rapid and flexible retrieval.*

2.5. MANAGEMENT OF COLLECTIONS AS A GENETIC RESOURCE

Representatives of all fungi collected should be retained for future reference. If retained, collections of ectomycorrhizal fungi undertaken over a period of time can result in the accumulation of a valuable genetic resource. The amount of accumulated finance, time and effort put into collecting the fungi is invariably large, and if specimens were discarded the cost of undertaking the collection phase would be enormous, i.e. each specimen has a high monetary value. The cost of maintaining a herbarium as a perpetual reference source of data is in comparison small (Bridson & Forman 1992).

A. Herbaria

Taxonomy, which provides names data about identity, biology and relationships, is ultimately based on data derived from specimens. Herbaria are the perpetual source of specimen-based information. Modern day specimen-based information is of two main types — physical, i.e. the specimens available for direct examination, and electronic, i.e. database having recorded information about the specimens. Specimens are an indispensable component of herbaria. They are essential for continual checking, confirmation and revision of taxa and names applied in published taxonomic and non-taxonomic works. Inaccuracies in published descriptions and other studies based on named organisms can be revealed by direct examination of specimens. If specimens were not lodged in herbaria but instead discarded soon after collecting, taxonomy then would entirely depend on a literature or electronic-based system. This would be totally inadequate for future purposes, as even the most comprehensive written notes and published descriptions will not cover all future requirements. For example, characters that were not recognised or recorded before the specimens were discarded may in the future become critical to studies using more advanced or different analyses. The actual specimens will be required to clarify and interpret taxonomic information in the literature long into the future.

The herbarium should aim to function as a service to provide accurate and rapid specimen data and identifications for research such as mycorrhizal and biodiversity work. Additionally it can form the basis of taxonomic revisions of selected taxa of ectomycorrhizal fungi by direct examination of locally housed specimens and comparison with specimens housed in other herbaria throughout the world obtained by two-way exchange.

It is highly desirable for herbaria to accumulate large numbers of specimens. Type specimens are important as they are the material on which species names are based. However, taxonomic research cannot be carried out on Type specimens alone, as this would ignore the variation that occurs in species. To assess spatial and temporal variation, specimens of a species need to be obtained from many points throughout its geographic range, and

over a long time-scale. The need to maintain historical collections is exemplified by old collections made in areas that have since been modified in some way either by natural succession, or by urbanisation. The old specimens now represent and contain significant ecological and taxonomic data about what was in that location in the past before the change. Similarly taxa may have become locally or globally extinct since collection. In all cases data about the taxon can be retrieved from the herbarium. Historical collections enable the accumulation of long-term data of geographic distributions, seasonal occurrences, and evolutionary variation in taxa over time.

Physical structure of a herbarium

Herbaria range from totally dedicated purpose-built buildings to drawers or cupboards located in a general laboratory. Cabinets with drawers suitable for standard mycological herbarium packets may be custom-built, or normal cupboards may have to suffice, at least when beginning a new herbarium. The larger the number of collections, the greater the need for specialised housing for specimens as the level of required accessibility and value of the specimens increase. In all cases, an insect-free situation needs to be established and maintained. If fumigation is used to control insects (see discussion below), the building itself and the drawers or cupboards may need to be specially sealed. Timber construction should be avoided especially in the tropics as it encourages unwanted fungal growth and insects.

Arrangement of collections

Specimens may be arranged in various ways.

1. Alphabetical — not recommended, as names of taxa change, and the arrangement does not reflect relationships. However, it is an easy way for non-mycologists to find taxa and to lodge collections in the herbarium.
2. Chronologically in order of collection time — suitable for herbaria not primarily for systematic research but those used mainly for a multidisciplinary group of researchers to consult. Difficult to find taxa by name.
3. Taxonomically in order of taxonomic groups — the best arrangement for herbaria in which systematic research is being carried out, because the arrangement does reflect relationships and all similar fungi will be physically grouped together.

Type collections

Specimens of a new taxon which have been nominated by the author of the taxon as representing the new name constitute type material of that new taxon. The holotype is the principal type to which the name of a taxon is attached. An isotype is any duplicate specimen from the holotype collection and is usually lodged at a different herbarium to the holotype for safety reasons, e.g. if the holotype is lost or destroyed, an isotype becomes the lectotype (new holotype). Type material requires special protection and some herbaria house it separately from the main collection in

Fundamental requirements of a Herbarium

- Insect-free, low-humidity room (dedicated to herbarium-only use if possible).
- Drawers or cupboards for housing specimens.
- Packets for filing specimens.
- Labels for data on specimen packets.
- Freezer for controlling insects (see below) or method of fumigating.
- Facilities for air-drying specimens.
- Computer for database specimen data.

fire-proof conditions. If the safety of the herbarium is in doubt, e.g. unsafe old building, or uncertain funding to continue into the future, type material (at least the holotype) should be lodged in a larger herbarium. As a rule, herbaria without an Index Herbariorum acronym (see below) should not hold holotype collections.

Herbarium procedures

A herbarium requires funding for basic curation to maintain the quality of specimens without deterioration. Furthermore, the herbarium ideally should be curated by a trained mycologist who is familiar with the taxonomy of fungi and is actively carrying out systematic research. The curator should also provide services such as loans to other herbaria, facilities to visiting scientists and distributing duplicate specimens, and be able to identify fungi brought in by researchers and the public. Exchange of specimens with other herbaria may not be possible unless a high level of curation can be proved or guaranteed.

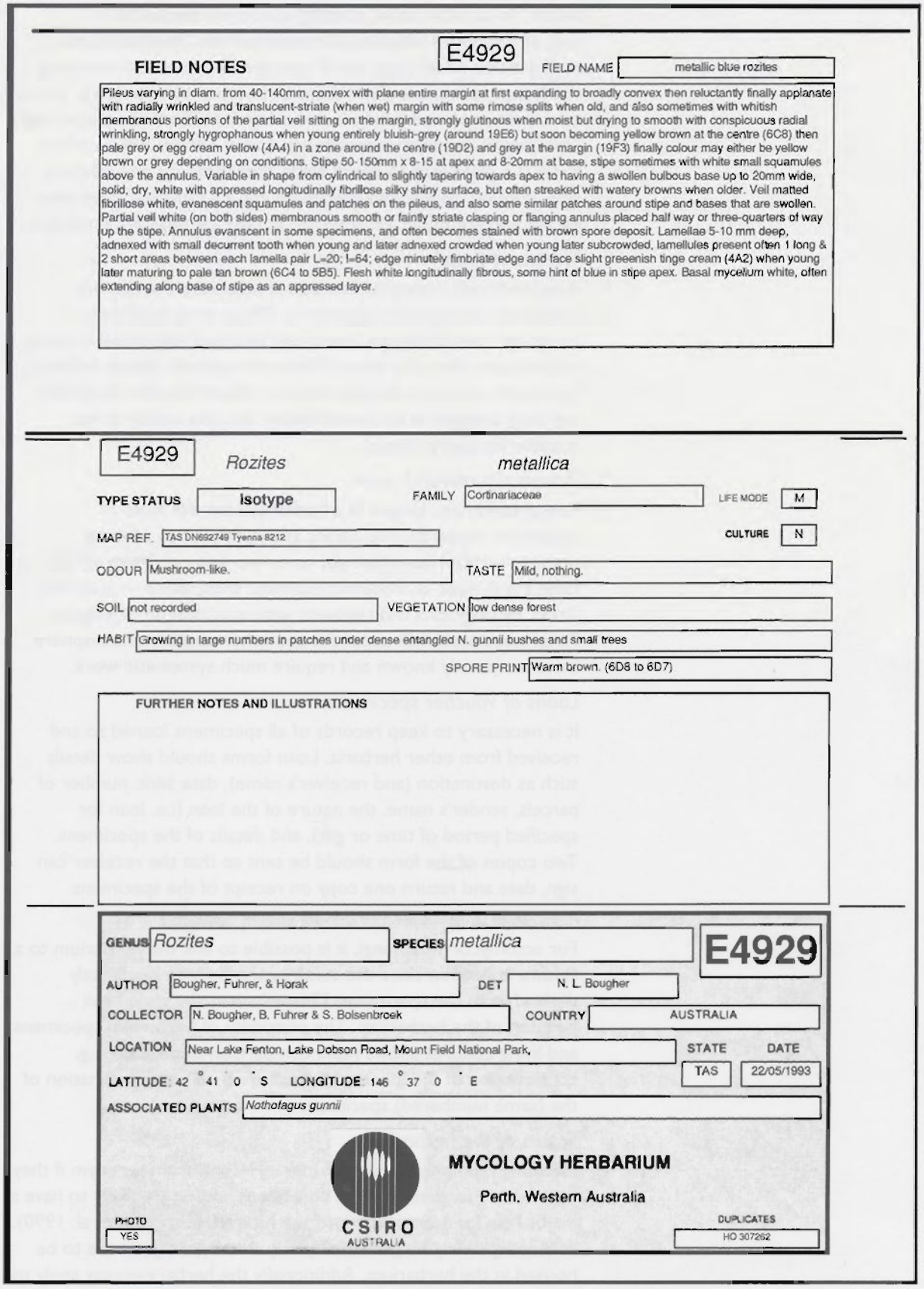
Processing and lodgment of collections from the field



1. Fresh specimens brought in from the field should have been processed as described above, in a location that is well separated from the herbarium. When that process is completed the dried specimens and associated data should be ready for further processing to allow their lodgment in the herbarium. A herbarium collection may consist of a single fruit body, but preferably at least several fruit bodies.
2. After removing any excess soil or dirt, specimens should placed in paper packets labelled with the accession number, taxa identity and some site details (Fig. 2.16). Labels may be written in ink by hand or generated by computer (see discussion of database, below). The packets may be constructed by folding A4-sized sheets of plain paper (Fig. 2.16). Acid-free paper is preferable but very expensive. Within the packets, air-dried specimens together with spore prints (where appropriate) and a tag with the accession number should be sealed in zip-lock type plastic bags. Alternatively, paper envelopes can be used, and must be used if fumigation is preferred to freezing as the method for insect control. For extra large collections, use boxes but also keep a sample in the normal packet with a note reading 'more specimens in box'.
3. Specimens and their packets need to be frozen or fumigated before lodgment in the herbarium to kill any potential insect pests (see discussion of insects, below).

Insect prevention

A regular routine for preventing insect damage to specimens must be undertaken. Methods used in herbaria include enclosure of insecticides such as naphthalene in the packets containing specimens, and/or periodic fumigation of the whole herbarium with chemicals such as methyl bromide. These methods are not well favoured now due to their detrimental effect on human



FIELD NOTES

E4929

FIELD NAME: metallic blue rozites

Pileus varying in diam. from 40-140mm, convex with plane entire margin at first expanding to broadly convex then reluctantly finally applanate with radially wrinkled and translucent-striate (when wet) margin with some rimose splits when old, and also sometimes with whitish membranous portions of the partial veil sitting on the margin, strongly glutinous when moist but drying to smooth with conspicuous radial wrinkling, strongly hygrophanous when young entirely bluish-grey (around 19E6) but soon becoming yellow brown at the centre (6C8) then pale grey or egg cream yellow (4A4) in a zone around the centre (19D2) and grey at the margin (19F3) finally colour may either be yellow brown or grey depending on conditions. Stipe 50-150mm x 8-15 at apex and 8-20mm at base, stipe sometimes with white small squamules above the annulus. Variable in shape from cylindrical to slightly tapering towards apex to having a swollen bulbous base up to 20mm wide, solid, dry, white with appressed longitudinally fibrillose silky shiny surface, but often streaked with watery browns when older. Veil matted fibrillose white, evanescent squamules and patches on the pileus, and also some similar patches around stipe and bases that are swollen. Partial veil white (on both sides) membranous smooth or faintly striate clasping or flanging annulus placed half way or three-quarters of way up the stipe. Annulus evanscent in some specimens, and often becomes stained with brown spore deposit. Lamellae 5-10 mm deep, adnexed with small decurrent tooth when young and later adnexed crowded when young later subcrowded, lamellules present often 1 long & 2 short areas between each lamella pair L=20; l=64; edge minutely fimbriate edge and face slight greeenish tinge cream (4A2) when young later maturing to pale tan brown (6C4 to 5B5). Flesh white longitudinally fibrous, some hint of blue in stipe apex. Basal mycelium white, often extending along base of stipe as an appressed layer.

E4929 *Rozites* *metallica*

TYPE STATUS: isotype

FAMILY: Cortinariaceae

LIFE MODE: M

CULTURE: N

MAP REF.: TAS DN692749 Tyenna 8212

ODOUR: Mushroom-like.

TASTE: Mild, nothing.

SOIL: not recorded

VEGETATION: low dense forest

HABIT: Growing in large numbers in patches under dense entangled N. gunnii bushes and small trees

SPORE PRINT: Warm brown. (6D8 to 6D7)

FURTHER NOTES AND ILLUSTRATIONS

GENUS: *Rozites* SPECIES: *metallica* **E4929**

AUTHOR: Bougher, Fuhrer, & Horak DET: N. L. Bougher

COLLECTOR: N. Bougher, B. Fuhrer & S. Bolsenbroek COUNTRY: AUSTRALIA

LOCATION: Near Lake Fenton, Lake Dobson Road, Mount Field National Park. STATE: TAS DATE: 22/05/1993

LATITUDE: 42 ° 41 ' 0 " S LONGITUDE: 146 ° 37 ' 0 " E

ASSOCIATED PLANTS: *Nothofagus gunnii*

CSIRO AUSTRALIA

MYCOLOGY HERBARIUM

Perth, Western Australia

PHOTO: YES

DUPLICATES: HO 307262

Figure 2.16. *Example of a herbarium packet and a front label suitable for ectomycorrhizal fungi. Note that the packet is made from an A4-size sheet and has been reduced in size in this figure.*

health. As an alternative, freezing specimens periodically (e.g. at 6-monthly intervals) is recommended. Specimens are sealed inside plastic bags and frozen for 72 hours. On removing the frozen specimens allow them an hour or two to slowly warm to room temperature before removing them from the plastic bag. This should ensure that specimens do not absorb any moisture, but it is a good idea to check some specimens for any rehydration. If found, rehydrated specimens can be removed from their packet and put on an air drier for an hour or until dry once again.

Names of fungi

A second basic curatorial function is to ensure the correct application of names to specimens. This is done by initially accurately identifying specimens and also applying modern names as these are often in a state of flux and regularly change following systematic revisions. Ideally a curator should be able to identify any fungi brought in for identification, but this usually is not possible for every fungus.

Systematic research work

Fungal specimens lodged in a herbarium are the basis of systematic research. The names and relationships of many ectomycorrhizal fungi have yet to be worked out. Many groups of fungi are in need of modern systematic treatments — including floras, monographs and revisions with emphasis on phylogeny. Fungi associated with eucalypts and other southern hemisphere trees are poorly known and require much systematic work.

Loans of voucher specimens

It is necessary to keep records of all specimens loaned to and received from other herbaria. Loan forms should show details such as destination (and receiver's name), date sent, number of parcels, sender's name, the nature of the loan (i.e. loan for specified period of time or gift), and details of the specimens. Two copies of the form should be sent so that the receiver can sign, date and return one copy on receipt of the specimens.

Association of herbarium to a living culture collection

For ectomycorrhizal fungi, it is possible to link the herbarium to a culture collection since the cultures are likely to be directly derived from the specimens. This enhances the gene bank function of the herbarium. The proximity of herbarium specimens and living cultures allows rapid comparison of the two, e.g. confirmation of the identity of a culture by direct examination of the (same numbered) specimen.

Status of the herbarium

Herbaria can apply to have an Index Herbariorum acronym if they have a fairly large number of collections and/or are likely to have a major role for a long time into the future (Holmgrem et al. 1990). An acronym may be particularly required if holotypes are to be housed in the herbarium. Additionally the herbarium may apply to have a statutory protection or similar government bill passed to increase the chances of its long-term future and secure finance.

Many herbaria throughout the world will not lend out their specimens unless the receiving herbarium has Index Herbariorum recognition and/or some form of statutory Index protection.

B. Databases

An electronic database is central to all modern herbaria with large numbers of collections. A database can vastly improve accessibility of the specimen data, e.g. for indexing and retrieval of selected groups of data. It can be used for producing packet labels. Specimen data such as scientific names can be easily updated. All information concerning macroscopic morphology, site details, and host associations can be entered from field sheets. Any subsequent data, e.g. microscopic and culture data, can be added to the database at any time. Colour photographs and illustrations of fruit bodies and microscopic characters can be scanned into the database.

Notes on data entry

1. Be consistent with font types, e.g. capital and small letters.
2. Use the same site name when there are many collections from what is obviously the same site even though they may have been written differently on the field sheets. Be consistent, e.g. always use 'National Park', not 'Nat. Park' or 'N.P'.
3. Check the correct spelling of genera in the mycological dictionary by Hawksworth et al. (1995).
4. If data are not given, leave the entry blank.

In addition to standard search and listings, the database is capable of producing customised data outputs including illustrations (Fig. 2.17). The data can be networked locally (multiple desk-top station users), throughout an organisation, or internationally for wide access. The choice of software is important to allow compatibility with larger or network systems. Another consideration is that the structure of a database may need to be compatible with interactive programs such as the internationally accepted DELTA system for taxonomic data and interactive keys (Dallwitz et al. 1993). This program handles taxonomic data to produce standardised descriptions, traditional and interactive keys, and a range of other applications such as distance and cladistic matrices.

Fields and data entry for herbarium database (ectomycorrhizal fungi)

Ideally, the database fields should correspond to entries on the data sheets used when recording specimen data in the field and laboratory. However, since herbaria of the world conform to an agreed standard, certain field designations may need to be included in a fashion more suited to plants than to fungi (see box).

List of fields of data for database in herbarium with ectomycorrhizal fungi.

Minimal or initial data input

1. **CODE**
2. **GENUS**
3. **SPECIES**
4. **COLLECTOR**
5. **DATE OF COLLECTION**
6. **LOCATION**
7. **STATE**
8. **COUNTRY**
9. **HABIT**
10. **VEGETATION TYPE**
11. **ASSOCIATED PLANTS**
12. **SOIL TYPE**
13. **CULTURE**

Other data fields to complete the database

14. FIELD NAME
15. AUTHOR OF SPECIES
16. FAMILY
17. DETERMINED BY
18. LAT. DEGREES
19. LATITUDE MINS
20. LAT. SECONDS
21. LAT. DIRECTION
22. LONGITUDE DEGREES
23. LONG. MINS
24. LONG. SECONDS
25. LONG. DIRECTION
26. MAP REFERENCE
27. DESCRIPTION OF SPECIES
28. SPORE PRINT COLOUR
29. MACROCHEMICAL TESTS
30. MODE OF LIFE
31. OTHER CODE/DUPLICATES
32. TYPE STATUS
33. PHOTOGRAPH
34. OTHER NOTES

E4929 — Mycology Herbarium: CSIRO Perth, Western Australia — E4929

GENUS *Rozites* SPECIES *metallica* AUTHOR Bougher, Fuhrer, & Horak DET N. L. Bougher

COLLECTOR N. Bougher, B. Fuhrer & S. Bolsenbroek DATE 22/05/1993 STATE TAS COUNTRY AUSTRALIA

LOCATION Near Lake Fenton, Lake Dobson Road, Mount Field National Park, MAP TAS DN692749 Tyenna 8212

ASSOCIATED PLANTS *Nothofagus gunnii*

SOIL not recorded

SPORE PRINT Warm brown. (6D8 to 6D7) CULTURE N

ODOUR Mushroom-like. TASTE Mild, nothing.

CHEMICAL TESTS Tissue tested Pileipellis, stipe, pileus flesh, lamellae. 15% KOH TEST: Pileipellis changes to reddish purple FeSO4: No reaction.

LIFE MODE M FAMILY Cortinariaceae OTHER CODES HO 307262

FIELD NOTES

Pileus varying in diam. from 40-140mm, convex with plane entire margin at first expanding to broadly convex then reluctantly finally applanate with radially wrinkled and translucent-striate (when wet) margin with some rimose splits when old, and also sometimes with whitish membranous portions of the partial veil sitting on the margin, strongly glutinous when moist but drying to smooth with conspicuous radial wrinkling, strongly hygrophanous when young entirely bluish-grey (around 19E6) but soon becoming yellow brown at the centre (6C8) then pale grey or egg cream yellow (4A4) in a zone around the centre (19D2) and grey at the margin (19F3) finally colour may either be yellow brown or grey depending on conditions. Stipe 50-150mm x 8-15 at apex and 8-20mm at base, stipe sometimes with white small squamules above the annulus. Variable in shape from cylindrical to slightly tapering towards apex to having a swollen bulbous base up to 20mm wide, solid, dry, white with appressed longitudinally fibrillose silky shiny surface, but often streaked with watery browns when older. Veil matted fibrillose white, evanescent squamules and patches on the pileus, and also some similar patches around stipe and bases that are swollen. Partial veil white (on both sides) membranous smooth or faintly striate clasping or flanging annulus placed half way or three-quarters of way up the stipe. Annulus evanscent in some specimens, and often becomes stained with brown spore deposit. Lamellae 5-10 mm deep, adnexed with small decurrent tooth when young and later adnexed crowded when young later subcrowded, lamellules present often 1 long & 2 short areas between each lamella pair L=20; l=64; edge minutely fimbriate edge and face slight greenish tinge cream (4A2) when young later maturing to pale tan brown (6C4 to 5B5). Flesh white longitudinally fibrous, some hint of blue in stipe apex. Basal mycelium white, often extending along base of stipe as an appressed layer.

FURTHER NOTES & ILLUSTRATIONS



Figure 2.17. An example of a customised output from the CSIRO Division of Forestry Herbarium database showing selected specimen data for collection E4929.

Recommended formats for herbarium database entries

1. CODE
Herbarium acronym and collection number.

2. GENUS
First letter capital. e.g. *Hebeloma*. (Use italics if possible.)

3. SPECIES
All in small letters. e.g. *westraliense*. (Use italics if possible.)

4. COLLECTOR
The name of the person or persons who collected the specimens in the field. Be consistent, e.g. one initial is entered first, then a space then the surname, e.g. J. Trappe. If no collector is given, enter 'anonymous'. If two or more collectors are given, enter as e.g.: J. Trappe & R. Young, or e.g. J. Trappe, R. Young & S. Joyce.

5. DATE
Enter date of collection in the form: DD/MM/YYYY , e.g. 05/09/1992 for 5 September 1992. If only partial data are given, enter as much as possible, e.g. / /1992.

6. LOCATION
Enter a place name, e.g. a town, National Park, Homestead or Station, River... also the distance (metric) and direction from the place, e.g. 5 km south of Manjimup, in Diamond National Park.

7. STATE
For Australian records, use capital letters: WA, SA, VIC, NSW, TAS, QLD, NT, ACT.

8. COUNTRY
Enter name of country in full in capital letters, e.g. AUSTRALIA, NEW GUINEA, NEW ZEALAND, CHINA, PHILIPPINES, NIGERIA.

9. HABIT
Enter any details given on the field sheets concerning the abundance of fruit bodies, their grouping, and their position in the site, e.g. abundant in caespitose groups, half-buried in leaf litter and on decaying wood at edge of lake.

10. VEGETATION NOTES
Enter any details given on the field sheets concerning the vegetation types, e.g. forest, heath.

11. ASSOCIATED PLANTS
Enter the major plant species present, preferably plants associated with the fungus, in full, e.g. *Eucalyptus marginata*, *Eucalyptus* sp., *Eucalyptus* spp., *Acacia* sp.

12. SOIL NOTES
Enter any details given on the field sheets concerning the soil, e.g. red earth, sand.

13. CULTURE
Enter whether the fungus is kept in a culture collection or not. Y or N or NA (not attempted). This will need to be updated at regular intervals.

14. FIELD NAME
A common name given to a fungus by workers in the field (e.g. Dave's golden cortinarius).

15. AUTHOR OF SPECIES
Enter name of author of the species. e.g. E. Horak. When there are two or more authors, connect with an ampersand (&).

16. FAMILY
Enter the family to which the genus belongs, e.g. Cortinariaceae.

17. DETERMINED BY
Enter in the name of the person who has identified the fungus as the named species, e.g. N.L. Bougher.

18 - 25. LATITUDE AND LONGITUDE
Enter two digit values, e.g. 15, 07. For direction, enter N or S.

26. MAP REFERENCE
Include a map reference if available.

27. DESCRIPTION OF SPECIES
Enter any descriptive data given about the fungus, e.g. pileus, lamellae, stipe.

28. SPORE PRINT COLOUR
Record the spore print colour (and code if a colour chart was used).

29. MACROCHEMICAL TESTS
Record the results of any chemical spot tests carried out on the fruit bodies.

30. MODE OF LIFE
Enter whether the fungus is considered to be saprophytic, mycorrhizal, or pathogenic (S, M, P).

31. OTHER CODES/DUPLICATES
Enter if a split of the specimens is kept at another Herbarium, e.g. BRIP2233. Enter if the collection has any alternative collection numbers, e.g. field collector numbers.

32. TYPE STATUS
Enter only if the collection represents a holotype, isotype or other designation.

33. PHOTOGRAPH
Indicate if a photograph of the collection was taken, Y (yes) or N (no).

34. OTHER NOTES
Enter any other data not included in the above fields.

Three examples of fungal classification schemes

A. Hawksworth et al. (1995)
Kingdom Fungi
Division Basidomycota
Division Ascomycota
Division Zygomycota

B. Margulis and Schwartz (1988)
Kingdom Fungi
Phylum Zygomycotina
Phylum Ascomycotina
Phylum Basidiomycotina

C. Kendrick (1992)
Kingdom Fungi
Division Zygomycota
Division Dikaryomycota
Subdivision Ascomycotina
Subdivision Basidiomycotina

2.6. AN INTRODUCTION TO THE AUSTRALIAN ECTOMYCORRHIZAL FUNGI

As outlined at the beginning of this chapter (Section 2.1), ECM fungi belong to the Ascomycetes, Basidiomycetes, and to a lesser extent the Zygomycetes. The definition of many taxonomic groups and their relationships with each other is the subject of much debate (see below). Many ECM fungi are very closely related to non-mycorrhizal (saprophytic and parasitic) fungi, and because of these similarities both mycorrhizal and non-mycorrhizal larger fungi are considered for each of the groups described below.

Stages in the fungal identification process

The identification of fungi requires a sequence of logical steps to be followed, each of which narrows down the groups of fungi to which it may belong. An example of how this process works is provided below. This follows the scheme provided in Figure 2.18. Some steps in the process outlined below may be difficult initially, but will soon become easier, once one has gained sufficient experience to readily recognise the major groups of fungi (e.g. the difference between Ascomycetes and Basidiomycetes, or between Aphyllophorales and Agaricales).

Higher classification of ectomycorrhizal fungi

The higher classification of fungi is unsettled. Many fungi having flagella at some stage of their life cycle such as the Chytridiomycetes and Oomycetes are now placed in a separate Kingdom — Protoctista (Margulis & Schwartz 1988). All ectomycorrhizal fungi belong to the Kingdom Eumycota (Fungi) which encompasses the Zygomycetes, Ascomycetes and Basidiomycetes. Three examples of higher classification schemes involving ectomycorrhizal fungi are shown above. Regardless of which classification is followed, the fundamental characteristics of the three large groups are outlined below and illustrated in Figure 2.19.

An example showing questions which could be used to identify a fungus

1. Does the fungus fruit above or below the soil surface? Note that both Ascomycetes and Basidiomycetes contain epigeous and hypogeous fungi.
2. If the fungus fruits above ground — does it resemble an Ascomycete (i.e. cup fungus, lichen), or a basidiomycete (i.e. mushroom, puffball, bracket fungus). In some cases this may have to be confirmed by looking for asci or basidia using a microscope.
3. If the fungus is an epigeous Basidiomycete — then is it mushroom-like (Agaricales and Boletales), polypore-like (Aphyllophorales), or puffball-like (Gasteromycetes)?
4. If the fungus is mushroom-like — then does it have gills or tubes under the cap?
5. If the fungus has gills — then what colour spore print does it have?

For this example we have used a fungus which is an epigeous Basidiomycete belonging to the Agaricales (gilled fungi). Further steps may allow this fungus to be assigned to a family or genus, by using other information included later in this Section.

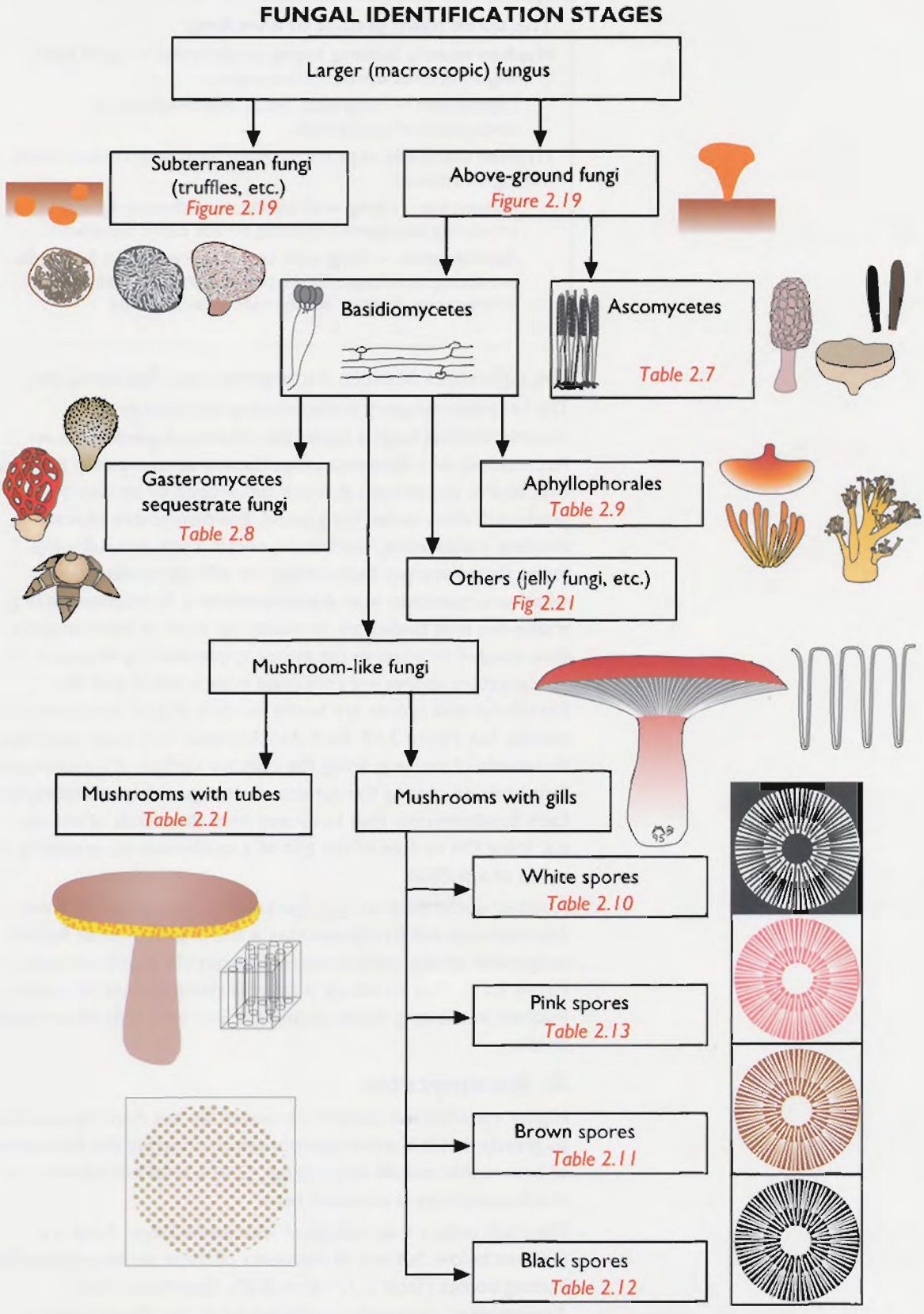


Figure 2.18. *A simplified scheme showing key stages in the process for identifying larger fungi. Note that this scheme applies to both mycorrhizal and non-mycorrhizal fungi.*

The three main groups of true fungi

Hyphae usually lacking septa (cross-walls) — small fungi (fruiting structures usually microscopic).

Zygomycetes — fungi with sexual reproduction by conjugation of gametangia.

Hyphae normally septate — many fungi large (macroscopic fruiting structures).

Ascomycetes — fungi with sexual reproduction by asci producing ascospores. Fruiting bodies called ascocarps.

Basidiomycetes — fungi with sexual reproduction by basidia producing basidiospores. Hyphae often with clamp connections. Fruiting bodies called basidiocarps.

The differences between Ascomycetes and Basidiomycetes

The broadest category in determining the identity of ectomycorrhizal fungi is to decide whether a specimen is an Ascomycete or a Basidiomycete. Ascomycetes typically produce fruit bodies (ascocarps) that are cup-shaped or similar, or produce truffles under the ground. Basidiomycetes typically produce mushrooms, toadstools, corals, clubs or truffle-like forms (basidiocarps). Sometimes it is difficult to determine whether a specimen is an Ascomycete or a Basidiomycete, e.g. truffle-like fruit bodies are produced by both. A microscope is then needed to observe the sexual spore-bearing structure. In Ascomycetes spores are contained in sacs called asci. In Basidiomycetes spores are borne on club-shaped structures called basidia. See Figure 2.19. Each Ascomycetes fruit body may have thousands of asci, e.g. lining the concave surface of a cup-shaped fruit body or coating the surface of a tongue-shaped fruiting body. Each Basidiomycete fruit body may have thousands of basidia, e.g. lining the surface of the gills of a mushroom or occupying the inside of a puffball.

Another useful microscopic character to distinguish between Ascomycetes and Basidiomycetes is the presence of an hyphal outgrowth on the septa of many (but not all) Basidiomycetes (see Figure 2.19). This structure is called a clamp connection, and is involved in allowing nuclei to migrate into new cells after mitotic division.

A. Ascomycetes

Higher classification systems proposed for the Ascomycetes vary so greatly that it is more appropriate to consider the two series of orders that include larger fungi. Only a brief introduction to the Ascomycetes is provided here.

The main orders that include at least some larger fungi are outlined below, but not all members of these series produce large fruiting bodies (Table 2.7, Figure 2.20). Ectomycorrhizal Ascomycetes apparently mainly belong in the Discomycetes orders Pezizales and Elaphomycetales (Trappe 1979) and a few in the Leotiales (Maia et al. 1996). There is relatively little information about mycorrhizal Ascomycetes, so fungi may be

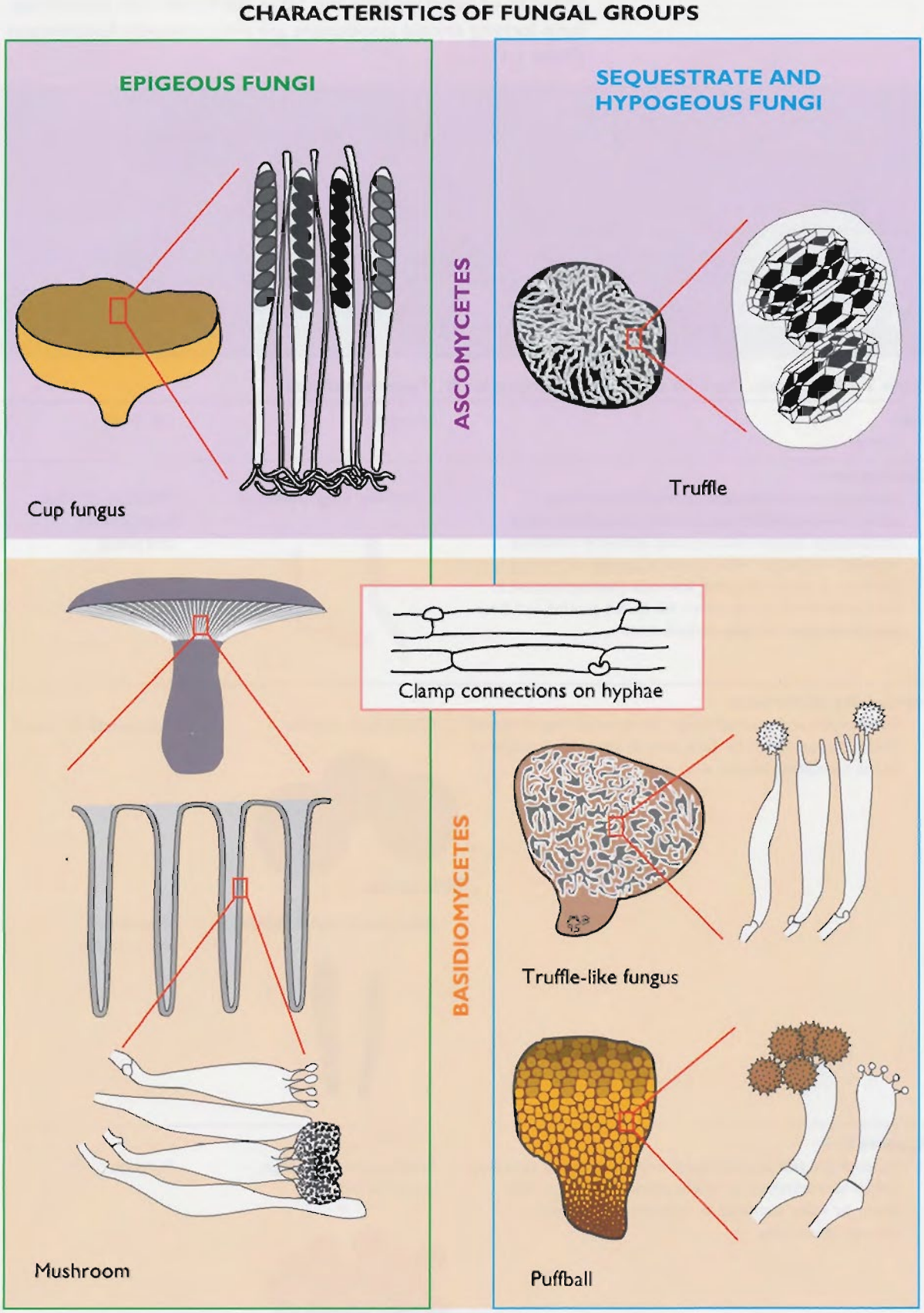


Figure 2.19. *Asci and basidia are the key difference between Ascomycetes and Basidiomycetes. Clamp connections on hyphae are a characteristic of many (but not all) Basidiomycetes, and are not found in Ascomycetes.*

added to or removed from this list in the future. The mycorrhizal fungi forming ericoid associations are predominantly Ascomycetes (Table 1.1).

Some Ascomycete groups

Pyrenomycetes — asci arranged in flask-shaped structures (perithecia) which in many members occur singly and are not visible to the naked eye. However, some other members have many perithecia embedded in a common sterile tissue (stroma) and in this way form large fruiting bodies.

Discomycetes — asci arranged in cup-shaped structures (apothecia) which may be modified and convoluted into a range of other shapes, e.g. tongues or truffles.

Lichens — include many Ascomycetes which form macroscopic vegetative and fruiting structures, but these can be distinguished by the presence of photosynthetic algae within the thallus and growth habit.

Hyphomycetes — many microscopic fungi which are saprophytes, plant pathogens, etc. often have ascomycete sexual states, but these are generally too small to be considered here.

Table 2.7A. Synoptic chart for orders of Ascomycetes: **A. Pyrenomycetes**

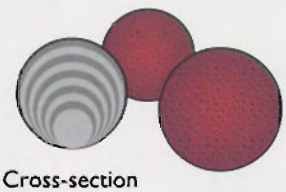
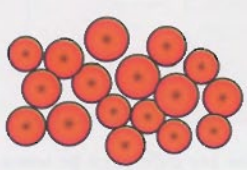
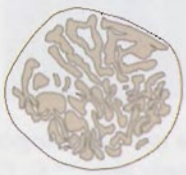
Order *Details*	*Examples*	*Life mode*
Clavicipitales Includes fungi that encase bodies of caterpillars, other insects, spiders and sometimes truffles before producing a stalked fruiting body. Also the infamous ergot of rye fungus with alkaloids capable of causing human sickness. It produces small sclerotia (resting bodies) in the flower head of rye which fall to the ground and later germinate into multiple-stalked stroma.	Caterpillar fungi *Cordyceps* 	Parasites of other fungi, insects and plants
Sphaeriales (Xylariales) Saprophytic and parasitic fungi mainly occurring on wood. Often characterised by dark, woody stromata (masses of tissue containing locules with asci).	Cramp balls *Daldinia*  Cross-section	Saprophytic on wood
	Dead men's fingers *Xylaria* 	Saprophytic and parasitic
Hypocreales Saprophytic and parasitic fungi occurring mainly on wood. Often characterised by bright yellow, orange or red, fleshy or waxy stromata in contrast to the dark, woody Sphaeriales.	Bright coloured canker fungi *Nectria* 	Parasitic

Table 2.7B. Synoptic chart for orders of Ascomycetes: **B. Discomycetes**

Order *Details*	*Example*	*Life mode*
Cyttariales Parasitic on southern beeches (*Nothofagus*) forming galls on the wood from which emerge spherical fruiting bodies with numerous small embedded cups (apothecia).	Beech orange or tree morel *Cyttaria* 	Parasitic on wood
Leotiales (Helotiales) Mainly saprophytes occurring on the ground. Asci line the surface of stalked fruiting bodies shaped like clubs or tongues.	Earth tongues *Geoglossum* 	Saprophytic on ground (some mycorrhizal?)
Pezizales Saprophytic on wood, soil or dung. Also some above-ground members and many below-ground members are mycorrhizal. Fruiting bodies are often large and conspicuous such as cups, morels and truffles.	Cup fungi *Peziza* 	Saprophytic and mycorrhizal
	Morels *Morchella* 	Saprophytic and mycorrhizal
	Truffles *Tuber*, *Labyrinthomyces* 	Mycorrhizal
Note: Underground fruiting Ascomycetes formerly placed in the Tuberales have now been assigned to the Pezizales (Trappe 1979).		
Elaphomycetales Includes a single genus of dark-coloured truffles distinguished from other truffles by having asci arranged randomly — not in an organised hymenium.	Deer truffles *Elaphomyces* 	Mycorrhizal

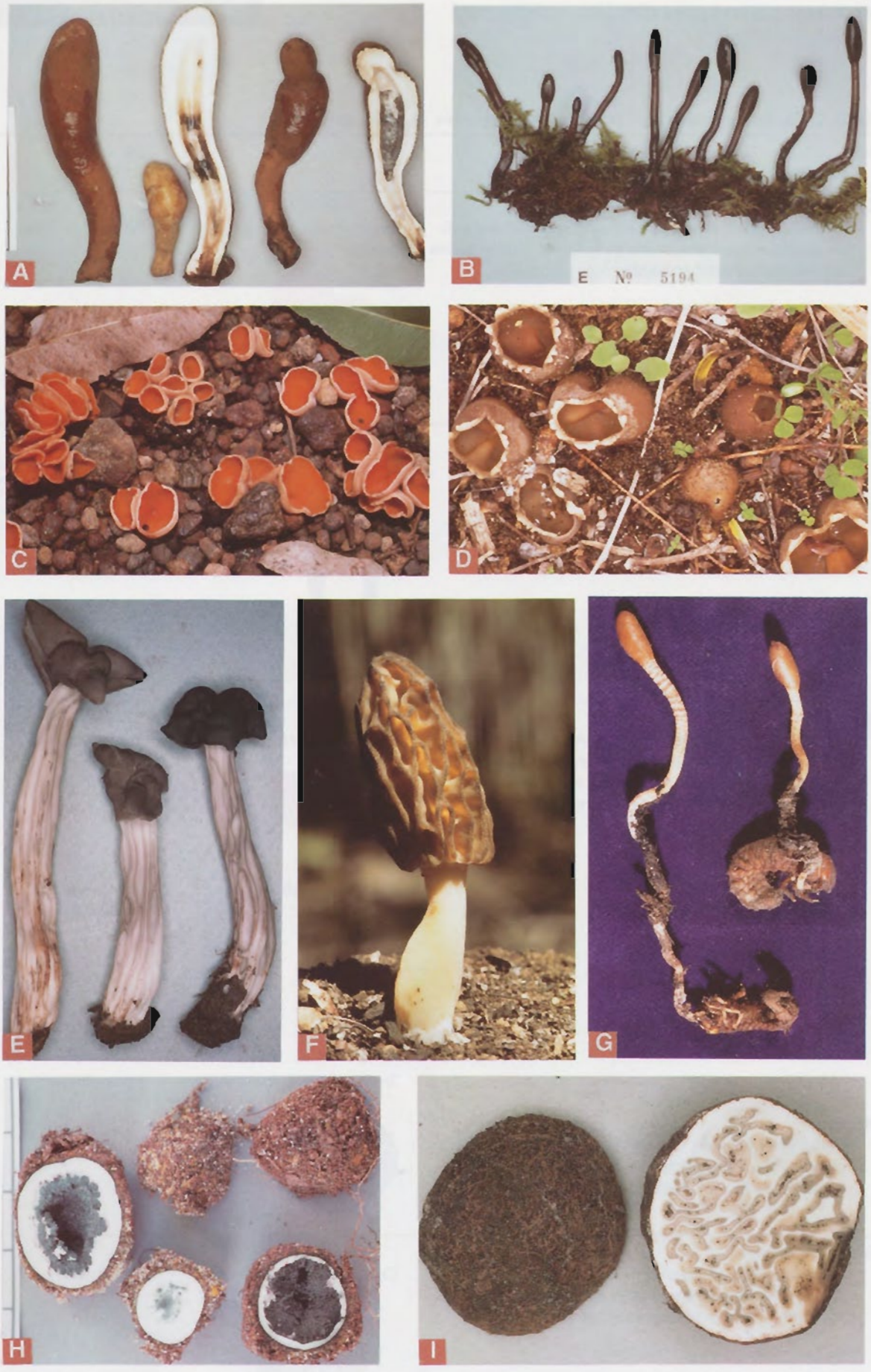


Figure 2.20. *Ascomycetes:* **A.** Xylaria *sp.* ** **B.** Geoglossum glutinosum **? **C.** Aleuria rhenana. ** **D.** Peziza *sp. (mostly* **) **E.** Helvella *sp.* * **F.** Morchella elata ** **G.** Cordyceps *sp.* ** **H.** Elaphomyces *sp.* * **I.** Labyrinthomyces varius *. *(* = mycorrhizal, ** = not mycorrhizal)*

B. Basidiomycetes

An introduction to Basidiomycete classification is provided below with explanations of how to distinguish the major groups. Most ectomycorrhizal fungi belong to the basidiomycetes.

Broad Basidiomycete classification

(following Kendrick 1992)

Basidiomycotina
- Class Teliomycetes
- Class Phragmobasidiomycetes
  - Order Tremellales
  - Order Auriculariales
  - Order Septobasidiales
- Class Holobasidiomycetes
  - Series Gasteromycetes
    - Order Sclerodermatales (and other orders)
  - Series Hymenomycetes
    - Order Exobasidiales
    - Order Dacrymycetales
    - Order Aphyllophorales
    - Order Agaricales

Key groups of Basidiomycetes

Teliomycetes — class of Basidiomycetes which usually do not produce large fruiting bodies. These include the rusts (Uredinales) and smuts (Ustilaginales) which are obligate plant pathogens.

Phragmobasidiomycetes — class of Basidiomycetes with basidia divided by septae (Figure 2.21). Phragmobasidiomycetes are generally non-mycorrhizal.

Tremellales — order of fungi with longitudinally divided basidia (Fig. 2.21). Non-mycorrhizal.

Auriculariales — order of fungi with horizontally divided basidia (Fig. 2.21). Non-mycorrhizal.

Septobasidiales — order of fungi with horizontally divided basidia with a thick-walled basal cell (Fig. 2.21). Non-mycorrhizal.

Tulasnellales— order of fungi where sterigma swell up and develop septa (Fig. 2.21). Non-mycorrhizal.

Holobasidiomycetes — class of Basidiomycetes with undivided basidia (Figure 2.21). A proportion of Holobasidiomycetes are mycorrhizal. Includes two informal groupings — Gasteromycetes and Hymenomycetes.

Gasteromycetes — series characterised by enclosed development of spores (sequestrate basidiomata) which when mature are eventually exposed in the case of puffballs as a powdery spore mass at a late stage in the development of the fruit body. Spores not forcibly discharged. These include the puffballs and truffle-like fungi.

Hymenomycetes — series where spores are usually exposed before maturity and forcibly discharged from the basidia. These include most fungi called mushrooms, toadstools, brackets, corals and clubs.

Exobasidiales — order of plant parasites that do not produce large fruiting structures. Non-mycorrhizal.

Dacrymycetales — order of jelly fungi with tuning-fork type of basidia (Fig. 2.21). All grow on wood and are non-mycorrhizal.

Aphyllophorales — order of flattened, club-like, coral-like, bracket-shaped, or less often mushroom-like fungi usually with a tough or woody consistency. Hymenium smooth, ridged, toothed, pored or sometimes gilled. Mycorrhizal and non-mycorrhizal.

Agaricales — order of mushroom-like fruit bodies with a fleshy consistency, i.e. they have a stalk and a cap. Also truffle-like fruit bodies underground. Hymenium of mushrooms gilled or pored. Mycorrhizal and non-mycorrhizal.

C. Gasteromycetes

The Gasteromycetes are a poorly defined group (an artificial series of orders) of Basidiomycetes which do not forcibly discharge their spores from their basidia but rely upon other means of dispersal such as wind, raindrops and animals. They include the puffballs, stinkhorns, earthstars, and many truffle-like fungi (Table 2.8, Figure 2.22). Many Basidiomycetes which were formerly placed in the Gasteromycetes order Hymenogastrales because they produce gastroid (puffball or truffle-like) basidiocarps are now recognised to have close taxonomic relationships with agaricoid (mushroom-like) genera (Hymenomycetes) or other groups of Gasteromycetes. Most are now placed in the Agaricales and Aphyllophorales or in orders of Gasteromycetes previously only comprising fungi which fruit above ground. An example is *Rhizopogon* which is now considered by most authors as aligned with the Boletales. The Hymenogastrales remain only as an artificial grouping of hypogeous fungi for which no relationships to Hymenomycetes or other Gasteromycetes have been recognised (see notes on truffle-like Agaricales below). Although many of the Gasteromycetes are saprophytes, ectomycorrhizal taxa are also abundant, e.g. Mesophelliaceae — *Castoreum* and *Mesophellia*, and the Sclerodermatales — *Pisolithus* and *Scleroderma*.

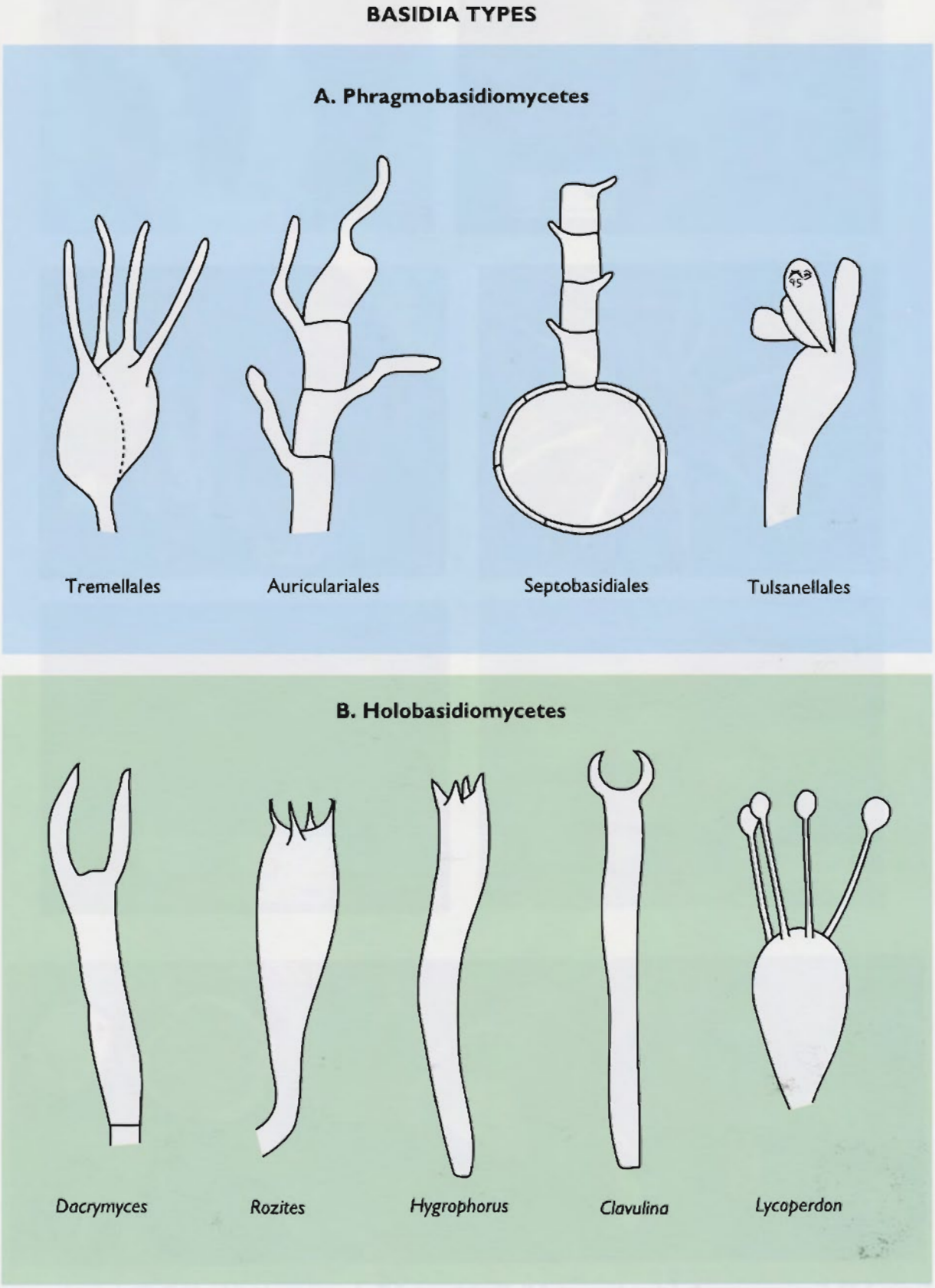
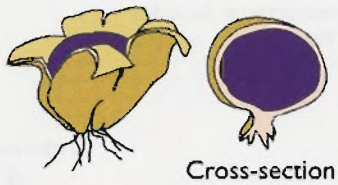
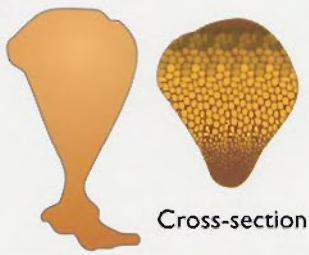
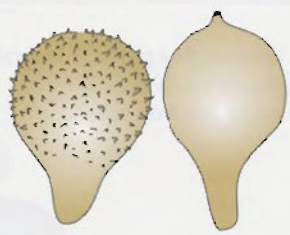
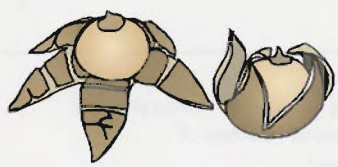


Figure 2.21. *Types of basidia produced by fungi which belong to some major groups of Basidiomycetes:* **A.** *Phargmobasidiomycetes.* **B.** *Holobasidiomycetes.*



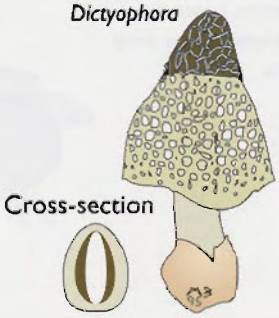
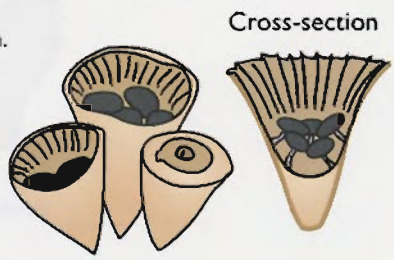
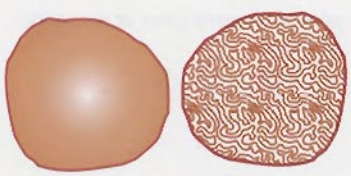
Figure 2.22. *Gasteromycetes.* **A.** Scleroderma *sp.* * **B.** Pisolithus *sp.* * **C.** Aseroe arachnoidea ** **D.** Clathrus cf. ruber. ** **E.** Podaxis pistillaris ** **F.** Rhizopogon occidentalis * *(now in Boletales).* **G.** Melanogaster euryspermus *. **H.** Hysterangium *sp.* * **I.** Mesophellia tropica * **J.** Castoreum *sp.* * *(* = mycorrhizal, ** = not mycorrhizal).*

Table 2.8. Synoptic chart for main orders of **Gasteromycetes.**

Order *Details*	*Examples*	*Life mode*
Sclerodermatales Earthballs with chambered immature spore mass and powdery mature spore mass. Includes some very abundant ectomycorrhizal genera.	*Scleroderma*  Cross-section	Mycorrhizal
	Pisolithus  Cross-section	Mycorrhizal
Lycoperdales Puffballs, earthstars and some truffle-like fungi with powdery spore mass at maturity. Mainly saprophytic but truffle-like taxa such as *Castoreum* and *Mesophellia* are probably mostly mycorrhizal.	Puffballs *Lycoperdon* 	Saprophytic (and some mycorrhizal)
	Earthstars *Geastrum* 	Saprophytic (and some mycorrhizal)
Tulostomatales Stalked puffballs with powdery spore mass at maturity.	Stalked puffball *Tulostoma* 	Saprophytic

continued over

Table 2.8. Synoptic chart for main orders of **Gasteromycetes** *(continued)*.

Order *Details*	*Examples*	*Life mode*
Phallales Stinkhorns. For dispersal they produce a slimy, smelly coating containing spores that attracts insects. Mainly saprophytic but some may be mycorrhizal. Includes some truffle-like fungi such as *Protubera*.	*Dictyophora*  	Saprophytic
	Clathrus 	Saprophytic
Nidulariales Birds nest fungi, so named because they produce several spore packages (peridioles) inside a cup-shaped structure which are dispersed by raindrop splash.	*Cyathus*  	Saprophytic
Hymenogastrales Truffle-like fungi producing hypogeous (underground) fruit bodies. Mostly hypogeous relatives of Hymenomycetes (see below). Most of the truffle-like fungi in the Hymenogastrales have now been transferred to other orders such as the Gautieriales, Leucogastrales, Melanogastrales, Boletales and Phallales.	 Cross-section (typical)	Mainly mycorrhizal

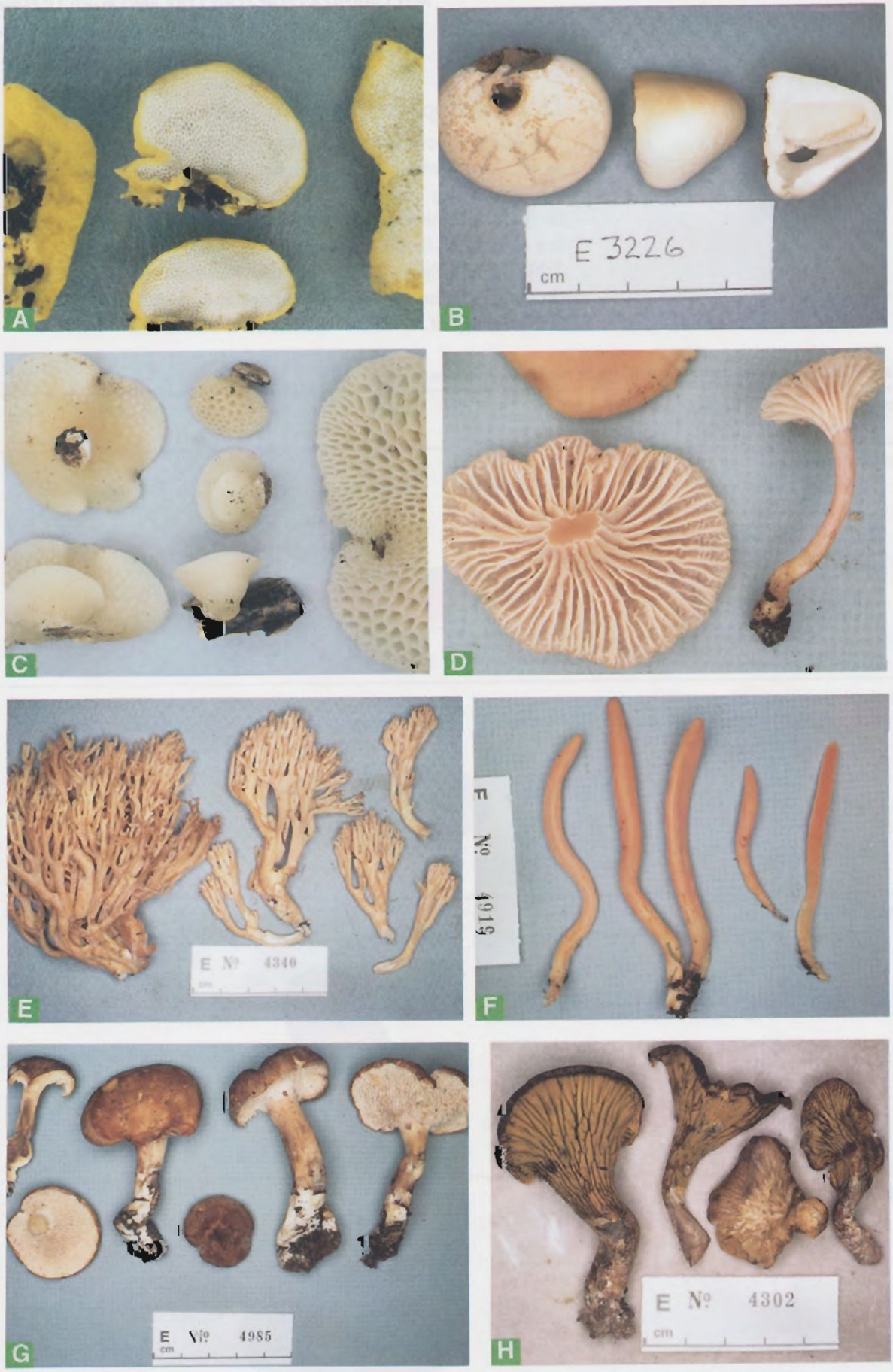


Figure 2.23. *Aphyllophorales:* **A.** Polyporus citreus. ** **B.** Cryptoporus volvatus *** *(not Australian).* **C.** Favolus sp. * **D.** Cantharellus sp. * **E.** Ramaria sp. * **F.** Clavulinopsis sp. * **G.** Hydnum sp. * **H.** Gomphus sp. * *(*=mycorrhizal, **=not mycorrhizal).*

D. Aphyllophorales

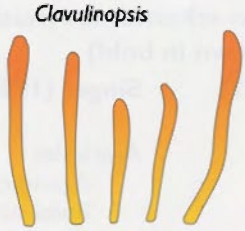
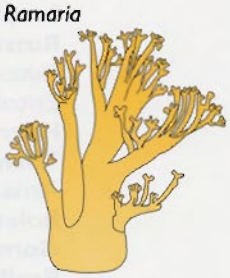
The Aphyllophorales include many non-mycorrhizal fungi such as the Polyporaceae which form bracket or shelf-like fruit bodies on wood. Many of the families are distinguished on the basis of fairly difficult microscopic characters. Some of the main families are recognised by their distinctive fruit body morphology (see Table 2.9 and Figure 2.23). Note that Hawksworth et al. (1995) do not recognise the Aphyllophorales as a natural order, and elevate many of its former families to order status.

Table 2.9. Synoptic chart for families of **Aphyllophorales.**

Family *Details*	*Examples*	*Life mode*
Polyporaceae Shelf or bracket-like fungi usually with a pored hymenium.	*Ganoderma* 	Parasitic and saprophytic
Thelephoraceae Resupinate (flattened skin-like) or fan-shaped fungi.	*Thelephora* 	Saprophytic and mycorrhizal
Corticiaceae Resupinate (flattened skin-like) fungi often on wood.	*Corticium* 	Saprophytic and mycorrhizal
Schizophyllaceae Fungi with split gills, on wood.	*Schizophyllum* 	Saprophytic on wood

continued over

Table 2.9. Synoptic chart for families of **Aphyllophorales** *(continued)*.

Family *Details*	*Example*	*Life mode*
Cantharellaceae Basidia borne on blunt ridges on the underside of funnel-shaped fruit bodies.	*Cantharellus* 	Mycorrhizal
Clavariaceae Club and coral fungi, often brightly coloured	*Clavulinopsis* 	Mycorrhizal
	Ramaria 	Mycorrhizal
Hydnaceae Basidia borne on teeth-like spines.	*Hydnum* 	Mycorrhizal

E. Agaricales

Mushrooms and toadstools are perhaps the most commonly observed type of fruit body in many forests including eucalypt forests, and are dealt with in some detail below. The classification of mushroom-like fungi (i.e. those with a cap and a stalk), has received different treatments by various authors (e.g. Julich 1981, Kühner 1980, Singer 1986). Families recognised by one author may be elevated to order level by another. For example, some authors elevate the family Boletaceae to an order level (Boletales), and similarly Russulaceae to Russulales (e.g. Kühner 1980, Watling 1982). The reader is referred to comprehensive books on mushroom-like fungi by Kühner (1980) and Singer (1986) for details of classifications. See also outline below with three types of schemes.

Three types of classification schemes for mushroom-like fungi (families with at least some mycorrhizal species are shown in bold)

Kuhner (1980)/Watling (1982)	**Singer (1986)**	**Hawksworth et al. (1995)**
Agaricales		
Agaricaceae	Agaricales	Agaricales
Bolbitiaceae	Agaricaceae	15 families e.g.
Coprinaceae	Bolbitiaceae	Agaricaceae
Cortinariaceae	Coprinaceae	**Amanitaceae**
Lepiotaceae	**Cortinariaceae**	Bolbitiaceae
Strophariaceae	Lepiotaceae	Coprinaceae
Russulales	Strophariaceae	**Entolomataceae**
Russulaceae	**Russulaceae**	**Hygrophoraceae**
Pluteales	Pluteaceae	Pluteaceae
Pluteaceae	**Entolomataceae**	Strophariaceae
Entolomataceae	**Hygrophoraceae**	**Tricholomataceae**
Tricholomatales	**Tricholomataceae**	Boletales
Hygrophoraceae	**Amanitaceae**	11 families e.g.
Pleurotaceae	**Boletaceae**	**Boletaceae**
Tricholomataceae	**Gomphidiaceae**	**Gomphidiaceae**
Amanitales	**Paxillaceae**	**Paxillaceae**
Amanitaceae	Crepidotaceae	Cortinariales
Boletales	Bondarzewiaceae	4 families e.g.
Boletaceae		**Cortinariaceae**
Strobilomycetaceae		Crepidotaceae
Gomphidiaceae		Russulales
Paxillaceae		**Elasmomycetaceae**
Coniophoraceae		**Russulaceae**

Although many of the Agaricales are ectomycorrhizal fungi, the order also includes many saprophytes and parasites (families listed above that are not in bold). Saprophytic and parasitic Agaricales are also included here to aid the distinction of these fungi from mycorrhizal taxa. Mushroom-like fungi with gills under the cap (many families) are separated from those with a sponge-like surface under the cap (the family Boletaceae). For further details of the various families outlined below, numerous books may be consulted. These include a book by Largent and Baroni (1988) which give outlines of the genera and various keys and

charts based on different character types, e.g. emphasising stature, spore colour, macroscopic features and growth substrate. There are very few books on Australian fungi. They include Cleland (1934), Young (1982), Fuhrer (1985), Fuhrer & Robinson (1992), and Bougher & Syme (1996).

Here we group all the mushroom-like fungi within the order Agaricales according to a system based on that of Singer (1986). An artificial grouping of the Agaricales families is presented to permit identification without the need to address classification schemes that are in a state of flux. The 15 or so well-established Agaricales families were originally classified largely on the basis of spore colour by Fries in the nineteenth century. This character is the easiest way of recognising families, although it must be remembered that there are exceptions to spore colour rules, e.g. although most pink-spored fungi are in the families Pluteaceae and Entolomataceae, some members of other families have pink spores such as some Tricholomataceae and some Amanitaceae. Spore colour is determined by making a spore print (as described in Section 2.2). Note that the colour of the gills cannot be reliably used as an indicator of spore print colour as they are not always the same.

F. Mushrooms with gills

The four following charts (Tables 2.10–2.13) are based on spore print colours — white, brown, black or pink. They display some of the main differences between families of mushroom-like fungi. Numbers of genera given for each family are approximate only in most cases, and only selected genera are listed (e.g. truffle-like genera are not listed). Photographs of fruit bodies for the main genera are presented in Figures 2.24–2.30.

Table 2.10. Some families of mushroom-like fungi with predominantly **white spore prints**. Mycorrhizal white-spored families are indicated in bold. For more information about fungi with white spore prints see Tables 2.16–2.17 for details of genera and Figures 2.24–2.27 for photographs of fruit bodies.

Family and selected genera	*Gill attachment*	*Volva*	*Ring*	*Spores*	*Other features*
Amanitaceae 2 genera: *Amanita* *Limacella*	free	often present	often present	smooth, amyloid or non-amyloid	divergent gill trama
Hygrophoraceae About 10 genera: *Hygrophorus, Hygrocybe, Camarophyllus*	attached — adnexed to decurrent	absent	absent	smooth, mostly non-amyloid	long basidia
Lepiotaceae About 8 genera: *Lepiota, Chlorophyllum, Leucocoprinus*	free	absent	present	smooth, often dextrinoid	parallel or interwoven gill trama
Russulaceae 2 epigeous genera: *Russula, Lactarius*	attached	absent	absent	ornamented, amyloid	composed of cells and filaments
Tricholomataceae Over 75 genera: *Armillaria, Tricholoma, Laccaria, Leucopaxillus, Clitocybe*	attached	absent	present or absent	smooth or ornamented, amyloid or non-amyloid	



Figure 2.24. *Photographs of mushroom-like Agaricales* **1: Amanitaceae.**
A. Amanita *sp. cf.* hemibapha. * **B.** Amanita *sp.* * **C.** Amanita *sp.* * *(vaginatae group), note the thick fleshy saccate volva at the base of the stem.* **D.** Amanita muscaria. * **E.** Amanita brunneibulbosa. * **F.** Limacella *cf.* illinata **
(= mycorrhizal, ** = not mycorrhizal).*

Table 2.11. Some differences between families of mushroom-like fungi with predominantly **brown spore prints**. Mycorrhizal brown-spored families are indicated in bold. For more information about fungi with brown spore prints see Tables 2.18–2.19 for details of genera and Figures 2.27–2.30 for photographs of fruit bodies.

Family and selected genera	*Gill attachment*	*Ring*	*Cap cuticle*	*Spores*	*Spore print*
Agaricaceae 2 genera: *Agaricus, Melanophyllum*	free	present	filamentous	smooth, with apical pore	chocolate-brown
Bolbitiaceae About 5 genera: *Agrocybe, Bolbitius, Conocybe*	usually free	present or absent	cellular	usually smooth, with apical pore	rust to cinnamon-brown
Cortinariaceae About 20 genera: *Cortinarius, Descolea, Hebeloma, Inocybe*	usually attached	present or absent, cortina often present	usually filamentous	smooth or rough, usually without apical pore	rust to cinnamon-brown
Paxillaceae 3 or 4 genera: *Paxillus, Phylloporus*	decurrent	usually absent	filamentous	smooth, without apical pore	rust to clay-brown
Strophariaceae About 10 genera: *Hypholoma, Pholiota, Psilocybe, Stropharia*	attached	often present	filamentous	usually smooth, with apical pore	purplish-brown

Table 2.12. Some differences between families of mushroom-like fungi with predominantly **black spore prints**. Mycorrhizal black-spored families are indicated in bold. For more information about fungi with black spore prints see Table 2.20 for details of genera and Figure 2.30 for photographs of fruit bodies.

Family and selected genera	*Gill attachment*	*Gills*	*Cap cuticle*	*Spores*
Coprinaceae About 7 genera: *Coprinus, Panaeolus, Psathyrella, Lacrimaria*	attached	thin, deliquescent	cellular	not subfusiform, with apical pore
Gomphidiaceae 3 genera: *Chroogomphus, Cystogomphus, Gomphidius*	decurrent	thick, not deliquescent	filamentous	subfusiform, without apical pore

Table 2.13. Some differences between families of mushroom-like fungi with predominantly **pink spore prints**. For more information about fungi with pink spore prints see Figure 2.30 for photographs of fruit bodies.

Family and selected genera	*Gill attachment*	*Volva*	*Spores*	*Habit*
Entolomataceae About 10 genera: *Clitopilus, Entoloma, Leptonia, Nolanea, Rhodocybe*	attached	absent	angular or striate	on ground
Pluteaceae About 3 genera: *Chamaeota, Pluteus, Volvariella*	free	present or absent	smooth and not angular	often on wood

G. Fungi with predominantly white spore prints

Amanitaceae

Members of this family are recognised by the often large fruit bodies with free white gills, a universal veil enclosing young specimens and often (but not always) remaining as a volva (cup at the base of the stem) and annulus (ring on the stem). The spore print is usually white and the spores are smooth.

Table 2.14. Genera of the **Amanitaceae**. *Amanita* species are obligately mycorrhizal, while species of *Limacella* may be ectomycorrhizal or saprophytic. See Figure 2.24 for photographs of fruit bodies.

Genera	*Volva*	*Annulus*	*Pileus*	*Stipe*	*Habit*
Amanita	present as a loose cup, rim, collar, concentric scales, or powdery zone	present as a membranous ring, or absent (sect. Vaginatae)	often warty, not usually viscid	usually not viscid	on ground
Limacella	usually absent, slimy if present	distinct annulus absent	not warty, usually viscid or glutinous	usually viscid or glutinous	on ground

Spores of the Amanitaceae

Spore print — white, cream, rarely grey, yellowish, greenish or pinkish.

Under microscope — hyaline, thin-walled, smooth-walled, non-amyloid (*Amanita* subgenus *Amanita*) or amyloid (*Amanita* subgenus *Lepidella*), mostly subglobose to ellipsoid.

Hygrophoraceae

Members of this family are recognised by the often bright coloured, small to medium-sized fruit bodies with thick, waxy lamellae. The spore print is usually white and the spores are smooth. The basidia are typically very long – over 5 times as long as the spores. Three genera are described in Table 2.15, but there are numerous other genera now recognised by some but not all authors in the Hygrophoraceae (e.g. *Aeruginospora*, *Bertrandia*, *Gliophorus*, *Humidicutis*, *Neohygrocybe*). See Singer (1986) and Horak (1990) for keys and descriptions of Hygrophoraceae genera.

Table 2.15. Some main genera of the **Hygrophoraceae**. *Hygrophorus* includes some mycorrhizal fungi, but most members of other genera are saprophytes. See Figure 2.25 for photographs of fruit bodies.

Genera	*Colour of fruit bodies*	*Gill trama*	*Veil*	*Gills*	*Habit*
Hygrophorus	usually dull-white, grey, brown	bilateral (ie. divergent from a central strand)	present or absent	adnate to decurrent	on ground
Hygrocybe	usually bright — orange, red, yellow	regular, parallel	absent	free to adnexed to decurrent	on ground
Camarophyllus	usually dull — white, grey, brown	irregular, interwoven	absent	usually decurrent	on ground

Spores of the Hygrophoraceae

Spore print — white, rarely tinged grey, magenta or violet.

Under microscope — hyaline, thin-walled, smooth-walled, non-amyloid, mostly ovoid-ellipsoid.

Russulaceae

Members of this family are recognised by the usual absence of a veil (some tropical species have an annulus), brittle flesh, and bright colours. Russulaceae is distinguished from other families of mushroom-like fungi with gills by the presence of cells (swollen hyphae called sphaerocysts) and filaments which constitute the fruit bodies and can be observed under the microscope in the gill trama and other parts. All other families lack sphaerocysts. The spore print is usually white and the spores have amyloid ornaments. The Russulaceae usually lack clamp connections in the hyphae of the fruit bodies.

Table 2.16. Main genera of the **Russulaceae**. These genera are mostly obligately ectomycorrhizal. See Figure 2.26 for photographs of fruit bodies.

Genera	*Latex*	*Gill trama*	*Habit*
Lactarius	present as clear, white, or coloured exudations from damaged gills or flesh	sphaerocysts absent or rare	on ground
Russula	absent	sphaerocysts abundant	on ground

Spores of the Russulaceae

Spore print — usually white, cream, or yellowish, less often with a brown tinge or ochraceous.

Under microscope — hyaline, but with amyloid ornamentation (blue in Melzer's reagent) such as warts, spines or a network of ridges thin-walled, mostly subglobose to ellipsoid.

Tricholomataceae

This is the largest family of mushroom-like fungi and the members are extremely diverse. The family is best determined by exclusion rather than by a succinct set of unique characters, e.g. fungi without long basidia, and without sphaerocysts. The spore print is usually white (sometimes cream, grey, pinkish, or yellowish) and the spores vary from smooth to ornamented, and may be amyloid or non-amyloid. The family includes many non-mycorrhizal genera such as wood decay fungi, but there are some ectomycorrhizal genera. Four of the main genera are outlined below.



Figure 2.25. *(above) Photographs of mushroom-like Agaricales* **2: Hygrophoraceae — A.** Hygrocybe *sp.* **? **B.** Hygrophorus lewelliana **? **C.** Bertrandia astatogala **? **D.** Hygrocybe graminicolor **? **Lepiotaceae — E.** Lepiota rachodes **. **F.** Leucocoprinus *sp.* ** *(* = mycorrhizal, ** = not mycorrhizal).*

Figure 2.26. *(Right) Photographs of mushroom-like Agaricales* **3: Russulaceae — A.** Russula rosacea *(not Australian)* * **B.** Russula *sp.* * **C.** Russula *sp.* * **D.** Russula *cf.* albonigra * *(note the blackening colouration).* **E.** Russula *sp.* * **F.** Lactarius eucalypti * *(note the white latex on the small cap near the bottom of the picture).* **G.** Lactarius *sp.* * **H.** Lactarius *sp. (not Australian)* * *(note this species has been gathered for food in Yunnan Province, China) (* = mycorrhizal).*


E3210
A


B


E № 4555
C


E № 4990
D


4730
E


E № 4442
cm
F


E № 4552
G


H

Table 2.17. Some main ectomycorrhizal genera of the **Tricholomataceae**. This table includes only four selected ectomycorrhizal genera. Most of the other Tricholomataceae genera are probably non-mycorrhizal. See Figure 2.27 for photographs of fruit bodies.

Genera	*Fruit bodies*	*Colour of fruit bodies*	*Annulus*	*Gill attachment*	*Spores*
Catathelasma	large, robust	white	double annulus	adnate to decurrent, not waxy	smooth, elongate, amyloid
Laccaria	small,robust or fragile	pink, lilac, or purplish	absent	adnate to decurrent, thick, waxy	echinulate or roughened, globose to ellipsoid, non-amyloid
Leucopaxillus	large, robust	mostly white	absent	adnate to decurrent, not waxy	warty or smooth, subglobose to ellipsoid, amyloid
Tricholoma	medium to large, robust	mostly white, less often coloured	present or absent	sinuate or adnexed, not waxy	smooth, subglobose to ellipsoid, non-amyloid

Spores of the Tricholomataceae

Spore print — usually white, otherwise cream, grey, pinkish, or yellowish.

Under microscope — hyaline, smooth-walled or ornamented, amyloid or non-amyloid, all shapes.

H. Fungi with predominantly brown spore prints

Cortinariaceae

The family is characterised by smooth or ornamented brown spores, lacking a germ pore. The genus *Cortinarius* is one of the largest genera of ECM fungi and is extremely diverse in Australia. *Cortinarius* is loosely characterised by having a rapidly disappearing cobweb-like partial veil called a cortina.

Figure 2.27. *Photographs of mushroom-like Agaricales* **4: Tricholomataceae. A.** Tricholoma coactatum *. **B.** Laccaria canaliculata *. **C.** Laccaria sp. * **D.** Lepista nuda **. **E.** Leucopaxillus amarus *(not Australian)* *. **F.** Termitomyces microcarpus *(not Australian)* **. **Agaricaceae** — **G.** Agaricus placomyces **. **Bolbitiaceae** — **H.** Bolbitius vitellinus ** *(* = mycorrhizal, ** = not mycorrhizal).*


E Nº 4292
cm
A


E Nº 4835
cm
B


E Nº 4998
cm
C


E 3209
D


E 3167
cm
E


F


E3164
cm
G


E Nº 5085
cm
H

Table 2.18. Some main ectomycorrhizal genera of the **Cortinariaceae**. This table includes only six selected ectomycorrhizal genera. Most of the other Cortinariaceae genera are probably non-mycorrhizal. See Figures 2.28 and 2.29 for photographs of fruit bodies.

Genera	*Partial veil*	*Cap surface*	*Cap cuticle*	*Cystidia on gill edge*	*Spores*
Cortinarius	cobweb or silky cortina, rarely persistent	dry or viscid, without veil scales	filamentous	infrequent	verrucose; ellipsoid, amygdaliform; spore print cinnamon to rust
Cuphocybe	absent	dry or glutinous, with pigmented veil scales	filamentous	absent	verrucose; ovoid, ellipsoid, amygdaliform; spore print cinnamon to rust
Descolea	membranous, persistent, striate annulus	dry or viscid with pigmented veil scales	cellular	frequent	verrucose; amygdaliform with attenuated smooth apex; spore print ochraceous
Hebeloma	absent or as evanescent cortina	viscid, smooth, without veil scales	filamentous (gelatinised)	abundant, conspicuous	smooth or verrucose; fusoid to ellipsoid; spore print dull brown to pinkish brown
Inocybe	absent or as evanescent cortina	dry, silky or radially fibrillose	filamentous	abundant, conspicuous, some thick-walled with crystals	smooth, nodulose or angular; ellipsoid, reniform; spore print dull brown
Rozites	membranous, persistent, striate annulus	dry or viscid with pigmented veil scales	filamentous	infrequent	verrucose; ellipsoid to amygdaliform with roughened non-attenuated apex; spore print rust brown

Spores of the Cortinariaceae

Spore print — brown — ranging from clay to ferrugineous.

Under microscope — smooth or ornamented, usually lacking a germ pore.



Figure 2.28. *Photographs of mushroom-like Agaricales* **5: Cortinariaceae — *Cortinarius species*. A.** Cortinarius (Myxacium) archeri *. **B.** C. (Myxacium) sinapicolor. **C.** C. (Myxacium) sp. * **D.** C. (Dermocybe) splendida *? **E.** C. (Dermocybe) austrovenetus *? **F.** C. (Hydrocybe) *sp.* *? *(* = mycorrhizal).*



Figure 2.29. *Photographs of mushroom-like Agaricales* **6: Cortinariaceae — *genera other than Cortinarius.***
A. Hebeloma westraliense *. **B.** Inocybe *sp.* *. **C.** Descolea phlebophora *. **D.** Rozites metallica *. **E.** Cuphocybe *sp.* *. **F.** Galerina *sp. cf.* unicolor** *(* = mycorrhizal, ** = not mycorrhizal).*

Paxillaceae

Members of this genus are closely related to the boletes (see outline of these genera below). Many authors place *Phylloporus* in the Boletaceae due to characters such as blue reaction of fruit bodies to bruising, gills easily separated from the cap, and subfusiform (bolete-like) spores. *Phyllobolites* with a single species is another ectomycorrhizal genus in the Paxillaceae. Other genera such as *Hygrophoropsis* are probably not ectomycorrhizal. Some non-mycorrhizal species of *Paxillus*, including *P. panuoides* are now placed in the genus *Tapinella*.

Table 2.19. Some main genera of the **Paxillaceae**. Taken broadly, these genera include mycorrhizal and non-mycorrhizal species. See Figure 2.30 for photographs of fruit bodies.

Genera	*Gill pattern*	*Gill colour*	*Spore print*	*Spores*	*Reaction of ammonia on flesh*
Paxillus	decurrent, forked, poroid-fusing near stipe, narrow, closely arranged	brown to ochre, bruising brown	brown without olive tinge	smooth, echinate or verrucose; globose, ovoid, ellipsoid or oblong	not blue
Phylloporus	decurrent, not forked or poroid-fusing, thick, wider spacing	bright yellow, bruising blue	brown with olive tinge	smooth, subfusiform	blue

Spores of the Paxillaceae

Spore print — dark yellow brown, cocoa, olive brown, rusty brown.

Under microscope — smooth or ornamented globose to fusiform.


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Figure 2.30. *Photographs of mushroom-like Agaricales* **7: Paxillaceae. A.** Paxillus muelleri *. **B.** Phylloporus *sp.* * **Strophariaceae — C.** Pholiota mulicingulata **. **D.** Hypholoma sublateritium **. **E.** Psilocybe subaeruginosa. **. **F.** Stropharia ambigua *(not Australian)* **. **Coprinaceae — G.** Coprinus curtus **. **H.** Panaeolus *sp.* ** **Gomphidiaceae — I.** Gomphidius subroseus *(not Australian)* *. **Pluteaceae — J.** Volvariella speciosa *(var.* gliocephala*)* *(* = mycorrhizal, ** = not mycorrhizal).*

I. Fungi with predominantly black spore prints

Gomphidiaceae

Members of this family are recognised by decurrent, thick gills which turn grey or black when mature, and a slimy or fibrillose partial veil. The spore print is usually smoky grey to black. These fungi are mostly associated with conifers, and probably not compatible with eucalypts. Note: * *Cystogomphus* is known only as one species from conifer plantations near Paris, France.

Table 2.20. Some main genera of the **Gomphidiaceae**. See Figure 2.30 for photograph of fruit bodies.

Genera	*Colour of young gills*	*Colour of young flesh*	*Texture of partial veil*	*Hyphae of the flesh*	*Composition of partial veil*
Chroogomphus	pinkish, brown, or ochre	brown, ochre or orange	fibrillose	amyloid	hyphae and sphaerocysts
Cystogomphus*	*	*	*	*	sphaerocysts only
Gomphidius	white	white	slimy	non-amyloid	hyphae and sphaerocysts

Spores of the Gomphidiaceae

Spore print — smoky grey to black (may attain a rusty tinge after lengthy storage).

Under microscope— grey under the microscope, smooth-walled, non-amyloid, fusoid, subcylindric.

J. Mushrooms with pores

Boletaceae

Some mushroom-like fungi have a sponge-like surface under the cap instead of gills. These are the boletes, many of which are ectomycorrhizal. The sponge can be easily and cleanly separated from the cap. The spores are produced on basidia that line the inside of the tubes of the sponge. Many of the boletes have a strong colour reaction when the flesh or tubes are damaged, such as a blue colour. Older classifications recognise few genera of boletes, or even only *Boletus* itself. However, up to 35 or so major genera are now recognised including those selected below. Note that many Aphyllophoralean fungi also have pores (Part D above), but these generally have fruit bodies with a harder consistency and longer life span than the boletes.

Spores of the boletes

Spore print — colours include olive, black, pink, brown, yellow, etc.

Under microscope — mainly smooth spores, but a wide range of shapes and ornamentations are represented.

Table 2.21. Some differences between a selection of genera of mushroom-like fungi with a sponge-like surface under the cap — **the boletes**. Most bolete genera are considered to be ectomycorrhizal. Photographs of fruit bodies are presented in Figures 2.31–2.32.

Genus	*Spore print colour*	*Spore wall*	*Spore shape*	*Cap*	*Veil*	*Stipe surface*	*Other characters*
Austroboletus	vinaceous pink, purple-brown, chocolate brown	warty, reticulate pitted	fusoid, amyg-daliform	smooth, viscid or dry	present, usually remaining on margin of cap	smooth, reticulate, lacunose, or ridged	hyphae mainly without clamp connections
Boletus	olive brown, cinnamon dull yellow brown	smooth	fusoid elongate	smooth or cracking, dry or less often viscid	absent	reticulate, finely floccose, no scabers	hyphae without clamp connections, flesh may blue if damaged
Boletellus	olive brown deep olivaceous	striate costate	fusoid elongate	scaly or smooth, dry or viscid	present, usually as a ring or on margin of cap	smooth, pruinose, or coarsely reticulate	probably mostly not mycorrhizal
Boletinus	olivaceous	smooth	fusoid elongate		present, usually forming an annulus	smooth, fibrillose, no glandular dots	hymenium decurrent, pores radially elongated
Fistulinella	pinkish brown ferrugineous	smooth	fusoid elongate	smooth to tomentose, viscid	absent	smooth, scurfy, rarely reticulate	slender basidiomes, long stipe
Fuscoboletinus	greyish-brown dull purplish	smooth	fusoid elongate	usually smooth, viscid or dry	present		similar to *Suillus* except spore colour
Gyroporus	pale yellow	smooth	short ellipsoid	smooth to squamulose, dry	absent	usually smooth	firm but often hollow stem
Heimiella	olive brown	reticulate without adaxial patch	ellipsoid	smooth or cratered, dry or viscid	absent	furfuraceous, squamulose, reticulate	no red reaction when flesh is damaged
Leccinum	olive brown pinkish brown	smooth	fusoid elongate	smooth to tomentose, dry or viscid	mostly absent	with scales or dots pale at first then dark	long stipe, large basidiomes
Pulveroboletus	olive brown	smooth	fusoid to subglobose	smooth or powdery, dry or viscid or glutinous	present usually as a powder on cap and stem or a ring	smooth, finely fibrillose reticulate, powdery	pores and flesh usually blueing if damaged
Strobilomyces	black blackish-brown	reticulate with adaxial patch	ellipsoid	scaly, dry	present on cap margin and/or a ring	squamose, reticulate	red reaction when flesh is damaged
Suillus	cinnamon brown olivaceous	smooth	fusoid elongate	smooth usually viscid or glutinous	present on cap margin or as a ring	with glandular dots	cystidia in fascicles (clumps) form dark dots on the stem
Tylopilus	grey-brown pink vinaceous vinaceous-brown	smooth	fusoid to ellipsoid	smooth to tomentose, usually dry	absent	smooth, pubescent, reticulate	flesh may not blue but may become brown if damaged
Xanthoconium	bright yellow	smooth	fusoid elongate	usually smooth	absent	smooth, finely pruinose	small pores, closely related to *Boletus*


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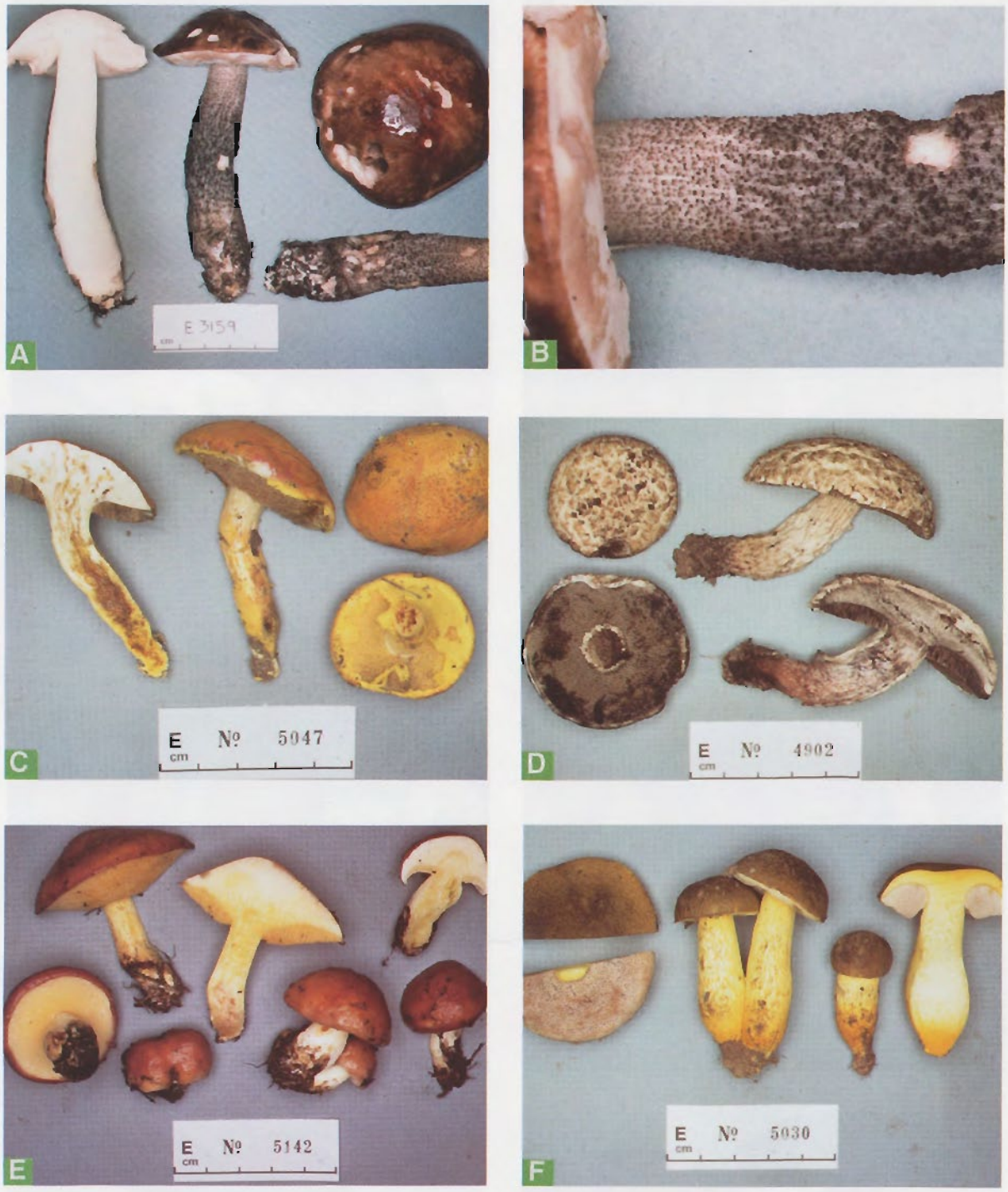


Figure 2.32. *(above)* **Boletaceae 2. A** and **B.** Leccinum scabrum * *(not Australian) note the dark scales on the stem — a distinctive character of the genus.* **C.** Pulveroboletus *sp.* * **D.** Strobilomyces *sp.* * **E.** Suillus granulatus *.
F. Tylopilus chromapes * *(* = mycorrhizal).*

Figure 2.31. *(previous page)* **Boletaceae 1. A.** Austroboletus occidentalis *. **B.** Boletus *sp.* * **C.** Boletus edulis * *(not Australian).* **D.** Boletus (Xerocomus) *sp.* * **E.** Boletus *cf.* ballouii group *. **F.** Boletellus obscure-coccineus **?
G. Gyroporus *sp. cf.* cyanescens* **H.** Heimiella *sp.* * *(* = mycorrhizal, ** = not mycorrhizal).*

K. Truffle-like Agaricales

Many sequestrate fungi form underground, truffle-like fruit bodies, but have characteristics that link them with agaricoid (above-ground, mushroom-like) Agaricales. Most of these truffle-like fungi were formerly placed in the Hymenogastrales and other Gasteromycetes orders, but are now recognised as belonging to families in the Agaricales (see table 2.22). An example is *Zelleromyces* with hypogeous gastroid basidiocarps which produce latex and have microscopic characteristics corresponding with the epigeous agaricoid genus *Lactarius* (Fig. 2.33). Note: there are also some truffle-like fungi related to the Aphyllophorales (see notes on Gasteromycetes above). Nearly all truffle-like fungi are considered to be ectomycorrhizal and many are also important food sources for Australian animals (Claridge & May 1994).

Another important group of ECM fungi represents an intermediate taxonomic link between the hypogeous gastroid and truly agaricoid Agaricales. These are the sequestrate fungi which produce subagaricoid basidiocarps characterised by a reduction in the stipe, and a convoluted hymenium which remains enclosed during all stages of development. For example, in the family Cortinariaceae, *Setchelliogaster* (subagaricoid basidiocarp) is an intermediate genus linking *Descomyces* (gastroid basidiocarp) and *Descolea* (agaricoid basidiocarp) (Bougher & Castellano 1993) (Figs 2.33, 2.34). The sequestrate or truffle-like Agaricales occur worldwide, but are particularly abundant in native Australian *Eucalyptus* forests (Beaton et al. 1985, Castellano & Bougher 1994), including the tropical regions. A few truffle-like Agaricales are extremely abundant in eucalypt plantations within Australia and overseas, e.g. *Hydnangium*, and *Descomyces* (Fig. 2.33).

Table 2.22. Linkages between selected genera of truffle-like (sequestrate) fungi associated with *Eucalyptus* and above-ground mushroom genera of Agaricales.

Truffle-like genus	*Family*	*Related above-ground genus*
Arcangeliella	Russulaceae	*Lactarius*
Descomyces	Cortinariaceae	*Descolea*
Gastroboletus	Boletaceae	*Boletus*
Hydnangium	Tricholomataceae	*Laccaria*
Macowanites	Russulaceae	*Russula*
Martellia	Elasmomycetaceae	*Russula*
Paxillogaster	Paxillaceae	*Paxillus*
Podohydnangium	Tricholomataceae	*Laccaria*
Protoglossum (Cortinomyces)	Cortinariaceae	*Cortinarius*
Richoniella	Entolomataceae	*Entoloma/Leptonia*
Setchelliogaster	Cortinariaceae	*Descolea*
Thaxterogaster	Cortinariaceae	*Cortinarius*
Torrendia	Torrendiaceae	*Amanita*
Zelleromyces	Elasmomycetaceae	*Lactarius*

Identification of truffle-like fungi

The process of describing macroscopic and microscopic features of hypogeous fungi is generally similar to that for epigeous fungi (Sections 2.4, 2.5). However, some specialised terminology is required (see Section 2.4 B — part 8).

Truffle-like fungi often have relatively few structures to use as taxonomic features, when compared to epigeous fungi. Consequently, much emphasis is placed on microscopic features of fruit body structure, especially the structure of spores (Castellano et al. 1989). Information about a few key genera of Australian truffle-like fungi is provided in Table 2.23, and some illustrations of truffle spores are provided in Figures 2.8 and 2.34.

Truffle-like fungi often have a remnant stalk, known as a columella, which provides further evidence of evolutionary linkages with the Agaricales. Columella structures vary from well-developed stems (stipitate columella), to small pads of sterile tissue at the base of fruit bodies (Fig. 2.35).

***Figure 2.33.* Truffle-like (sequestrate) Agaricales.**
A. *Phylogenetic series from left to right showing the gastroid genus* Descomyces, *the intermediate secotioid genus* Setchelliogaster *and the agaricoid genus* Descolea. * **B.** *Phylogenetic series from right to left showing the gastroid genus* Protoglossum (Cortinomyces), *the intermediate secotioid genus* Thaxterogaster, *and the agaricoid genus* Cortinarius. * **C.** *The closely related fungi* Podohydnangium *(left) and* Laccaria *(right).* * **D.** *The closely related fungi* Martellia *(left) and* Macowanites *(right)* *. **E.** Zelleromyces *sp., a gastroid relative of* Lactarius, *note the white latex.* * **F.** Hydnangium *sp., a gastroid representative of the phylogenetic series that also includes* Podohydnangium *and* Laccaria. * **G.** Gastroboletus turbinatus *(not Australian).* * **H.** Paxillogaster *sp.* * **I.** Cortinarius globuliformis, *a hypogeous fungus morphologically placed between* Thaxterogaster *and epigeous* Cortinarius. * **J.** Macowanites *luteiroseus, a secotioid relative of* Russula * *(* = mycorrhizal).*


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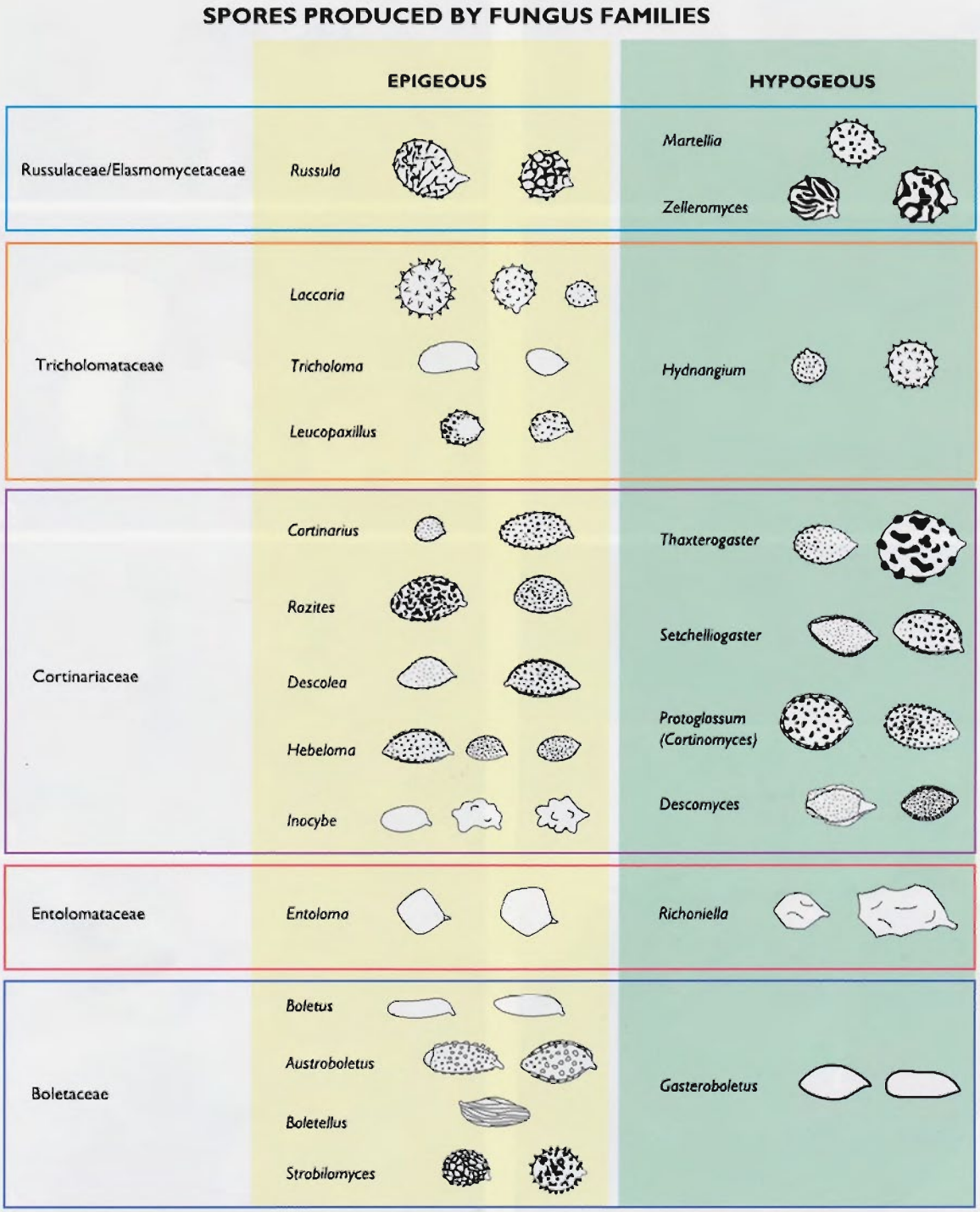


Figure 2.34. *Representative basidiospores of some mushroom-like and truffle-like ectomycorrhizal fungi that belong to the same families. Note that many other forms of spores occur among the ectomycorrhizal fungi and cannot be illustrated here.*

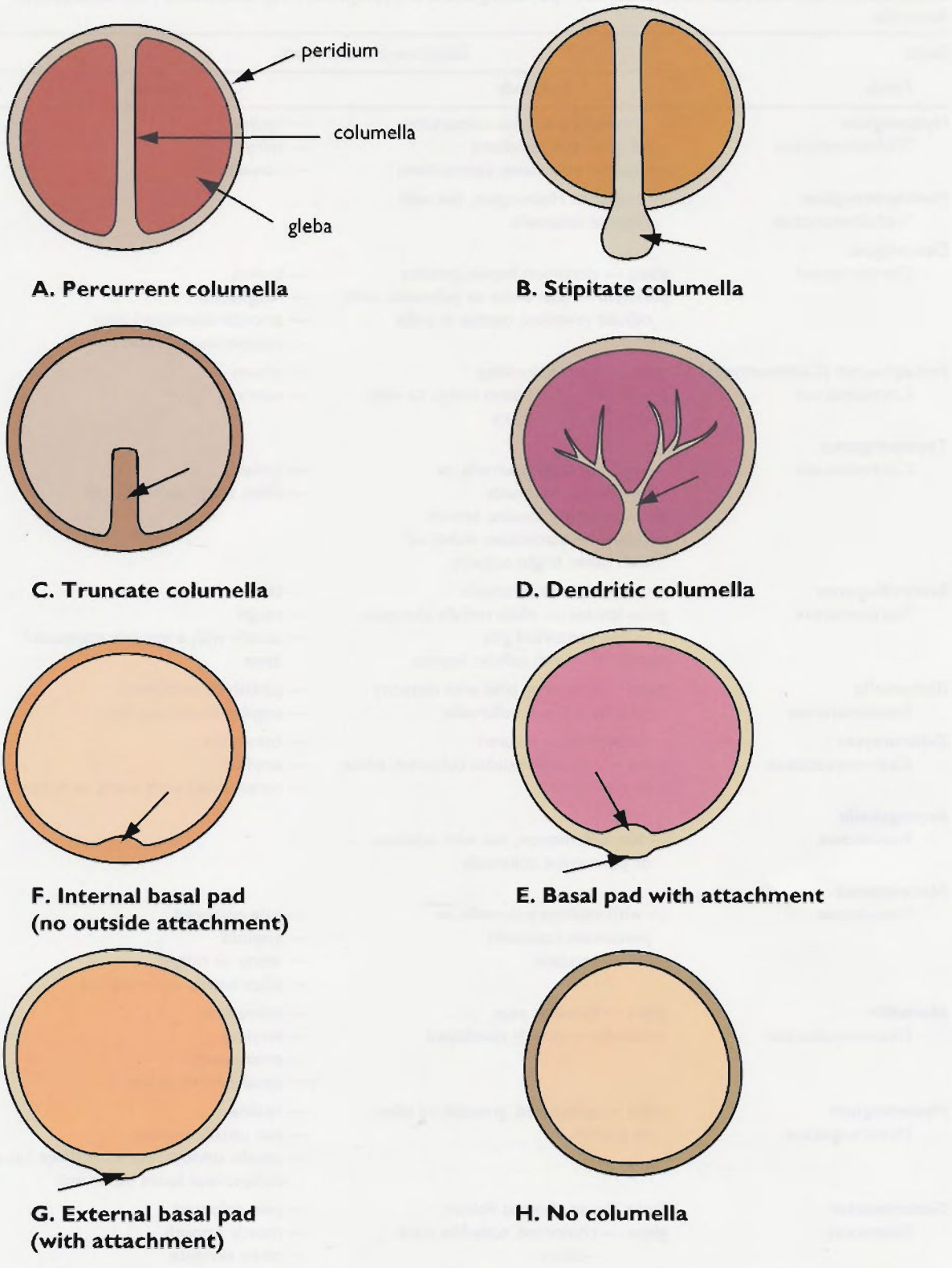


Figure 2.35. *Columella shapes found in truffle-like fungi.*

Table 2.23. Main characteristics of some important genera of hypogeous fungi associated with eucalypts in Australia.

Genus / *Family*	*Distinctive characteristics*	
	Fruit body	*Spores*
Hydnangium Tricholomataceae	— generally with pink colouration (of gleba and peridium) — hyphae with clamp connections	— hyaline — spiny — non-amyloid
Podohydnangium Tricholomataceae	— similar to *Hydnangium*, but with stipitate columella	
Descomyces Cortinariaceae	gleba — cinnamon brown loculate peridium — thin white or yellowish, with cellular (swollen) hyphae in pellis	— brown — roughened — smooth-attenuated apex — conspicuous perisporium
Protoglossum (Cortinomyces) Cortinariaceae	gleba — brown, loculate peridium — sometimes violet, or with other bright colours	— brown — warty
Thaxterogaster Cortinariaceae	— with stipitate columella, or percurrent columella gleba — empty locules, brown peridium — sometimes violet, or with other bright colours	— brown — often axially symmetrical
Setchelliogaster Cortinariaceae	— with stipitate columella gleba locules — often radially elongate, or like contorted gills peridium — with cellular hyphae	— brown — rough — usually with a smooth attenuated apex
Richoniella Entolomataceae	gleba — becoming pink with maturity — usually without columella	— pinkish to colourless — angular, Entoloma- like
Zelleromyces Elasmomycetaceae	— usually pale-coloured gleba — loculate, exudes coloured, white, or clear latex	— colourless — amyloid — ornamented with warts or ridges
Arcangeliella Russulaceae	— like *Zelleromyces*, but with stipitate, or percurrent columella	
Macowanites Russulaceae	— with stipitate columella, or percurrent columella gleba — loculate	— pale-coloured — amyloid — warty or ridged — often axially asymmetrical
Martellia Elasmomycetaceae	gleba — loculate, pale columella — poorly developed	— colourless — amyloid — ornamented — usually symmetrical
Hysterangium Hysterangiaceae	gleba — gelatinised, greenish to olive or pinkish	— hyaline — not usually amyloid — usually smooth, but sometimes has a conspicuous loose outer wall
Gastroboletus Boletaceae	looks like an aborted *Boletus* gleba — contorted, tube-like stipe —short	— pale-coloured — mostly smooth — often elongate

Chapter 3

WORKING WITH GLOMALEAN FUNGI

3.1 INTRODUCTION

An introduction to VAM associations produced by Glomalean fungi and the terminology used to describe them are provided in Chapter 1 (Section 1.4). These associations involve fungi in the Zygomycete order Glomales which occupy the roots of a wide diversity of plants. They consist of hyphae, which are generally non-septate, that ramify within the cortex of roots forming intricately branched haustoria called arbuscules, and in many cases also forming storage structures called vesicles (see Figs 1.10, 3.6, 3.7). Glomalean fungi produce a network of hyphae in the soil with relatively thin, highly branched hyphae, which are thought to absorb nutrients, and thicker hyphae which interconnect roots, spores and absorptive hyphae (Fig 1.8). These fungi also produce large asexual spores (20–1000+ µm diameter) on the soil hyphae, or within roots, and features of these spores are usually used to identify Glomalean fungi (Fig. 3.1).

The main procedures used to examine Glomalean fungi are listed in Table 3.1. Glomalean fungi are obligate mutualists that can only be propagated in association with the roots of a growing host plant. Accurate identification of mycorrhizal fungi is required to allow information about their biology to be exchanged, but is not always possible, for the reasons discussed below. Thus it is essential that good-quality specimens of any fungus used in experiments are lodged in a herbarium to allow the identity of fungi to be reassessed in the future (Section 3.3D).

Methods for separating spores of Glomalean fungi from soil samples and making slide preparations are presented in Section 3.3. However, the accurate identification of spores from field samples can be difficult if spores are old or damaged by microbial activity (Morton 1993, Walker 1992). It may be necessary to first establish pot cultures of VAM fungi (Section 3.4) to allow their accurate identification (and confirm that they are mycorrhizal fungi).

Table 3.1. Procedures and objectives for experiments with VAM fungi (with reference to sections of this manual).

1. *Spore extraction from soil (3.3)*
   - — identification of fungi (3.2)
   - — spore density measurements
2. *Other propagules*
   - — bioassay measurements (4.4)
   - — extraction of hyphae (not considered)
3. *Examination of mycorrhizal roots*
   - — clearing and staining procedures (4.2)
   - — other microscopy procedures (4.5, 4.6)
   - — quantifying associations (4.3)
   - — identifying fungi in roots (3.2)
4. *Propagation of fungi*
   - — isolation from spores or other propagules (3.4)
   - — pot-culturing procedures (3.4)

Diversity and population ecology of Glomalean fungi

Glomalean fungi consist of large asexual spores and coenocytic hyphae distributed throughout the soil, but we have little idea of the size of individuals of these fungi or how much genetic diversity occurs within their multinucleate organs (Tommerup 1988). Many species of Glomalean fungi have worldwide distribution patterns and have apparently adapted to diverse habitats. However, it is known that soil factors such as pH restrict the distribution of some taxa (Abbott & Robson 1991), and some of these widespread taxa are now known to comprise more than one species (Morton 1988). Information about the soil and climatic conditions where isolates used in experiments were originally obtained should be provided to allow correlations between the taxonomy and physiology of Glomalean fungi to be established. Much of the functional diversity of Glomalean fungi occurs at the isolate level rather than species level (Brundrett 1991, Morton & Bentivenga 1994). Consequently, habitat information is as important as knowledge of the taxonomic identity of fungi, for comparing the results of experiments, or the selection of isolates for practical use.

Populations of mycorrhizal fungi are thought to have occupied the same soil habitats for millions of years, slowly adapting to changes in site conditions (Trappe & Molina 1986). Changes in populations of Glomalean fungi have been observed when ecosystems are converted to monocultures or severely disturbed, providing indirect evidence for habitat preferences by these fungi (Abbott & Robson 1991, Brundrett 1991). There is also limited experimental evidence that the performance (measured as increased host plant growth) of fungal isolates is related to the factors outlined in Table 1.4 (Brundrett 1991, Abbott et al. 1992).

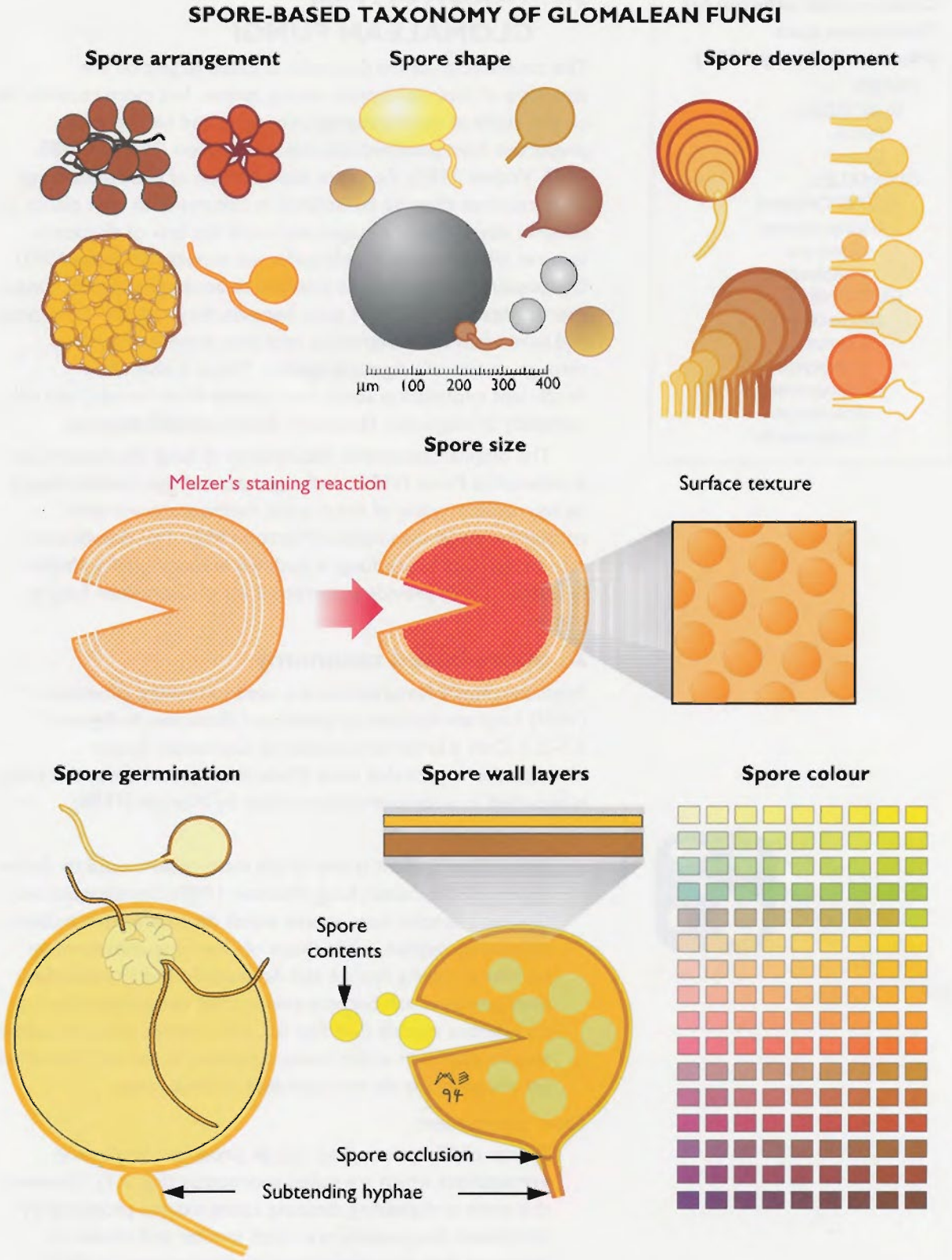


Figure 3.1. *Taxonomic criteria which can help to identify Glomalean fungi include various aspects of spore structure, as well as details of spore production, external hyphae and mycorrhizal associations within roots. Important features include spore colour, surface texture, size, shape, subtending hyphae, contents, wall layers and germination processes. Details of spore wall structure can be examined only after making microscope preparations of squashed spores and comparing unstained spores to those stained with Melzer's reagent.*

Classification scheme for Glomalean taxa (Morton & Benny 1990)

ORDER
SUBORDER
Family
Genus
GLOMALES
GIGASPORINAE
Gigasporaceae
Gigaspora
Scutellospora
GLOMINEAE
Glomaceae
Glomus
Sclerocystis
Acaulosporaceae
Acaulospora
Entrophospora

3.2. TAXONOMY OF GLOMALEAN FUNGI

The classification of the Glomales is based largely on the structure of their soil-borne resting spores, but more recently the careful study of developmental processes and biochemical properties have provided valuable information (Morton 1988, 1993, Walker 1992). Accurate identification of Glomalean fungi often requires them to be isolated in cultures with host plants, to observe developmental stages and avoid the loss of diagnostic features which occurs in field-collected material (Morton 1993). Consequently, it may not be possible to accurately identify fungal spores obtained from field soils, because they are of unknown age and have often been altered by microbial activity or passage through an animal's digestive system. There is also often insufficient information about how spores were formed, and the variability of diagnostic features in field-collected material.

The original taxonomic descriptions of fungi are reproduced in Schenck & Perez (1990), although users of this manual should be warned that many of the original descriptions are now considered to be incomplete (Morton 1993). The classification scheme for Glomalean fungi is outlined in margin (left). Walker & Trappe (1993) provide a corrected list of Glomalean fungus names.

A. Spore-based taxonomy

Important spore features that are used to identify Glomalean (VAM) fungi are considered below and illustrated in Figures 3.1–3.7. Only a brief introduction to Glomalean fungus identification is provided here. More information about this topic is provided in a comprehensive review by Morton (1988).



Spore development

Spore development is one of the main criteria used to define genera of Glomalean fungi (Morton 1988). *Scutellospora* and *Gigaspora* species have spores which develop from a bulbous subtending hyphae, while those of *Glomus* species form on narrow or flaring hyphae and *Acaulospora* and *Entrophospora* have spores which become sessile after detachment from a sporiferous saccule (see Figs 3.2, 3.4). Spores of many *Glomus* species can form within roots, as well as in the soil, but other genera generally do not sporulate in living roots.

Spore arrangement

Spores of Glomalean fungi can be produced singly or in aggregations which are called sporocarps (Fig. 3.1). However, this term is misleading, because spore masses produced by Glomalean fungi usually are much smaller and simpler in structure than the sporocarps (mushrooms and truffles) produced by Ascomycetes and Basidiomycetes (Chapter 2). Glomalean fungus spore aggregations often contain soil materials, may not contain many specialised hyphae, but may have a peridium (outer covering of hyphae). The genus

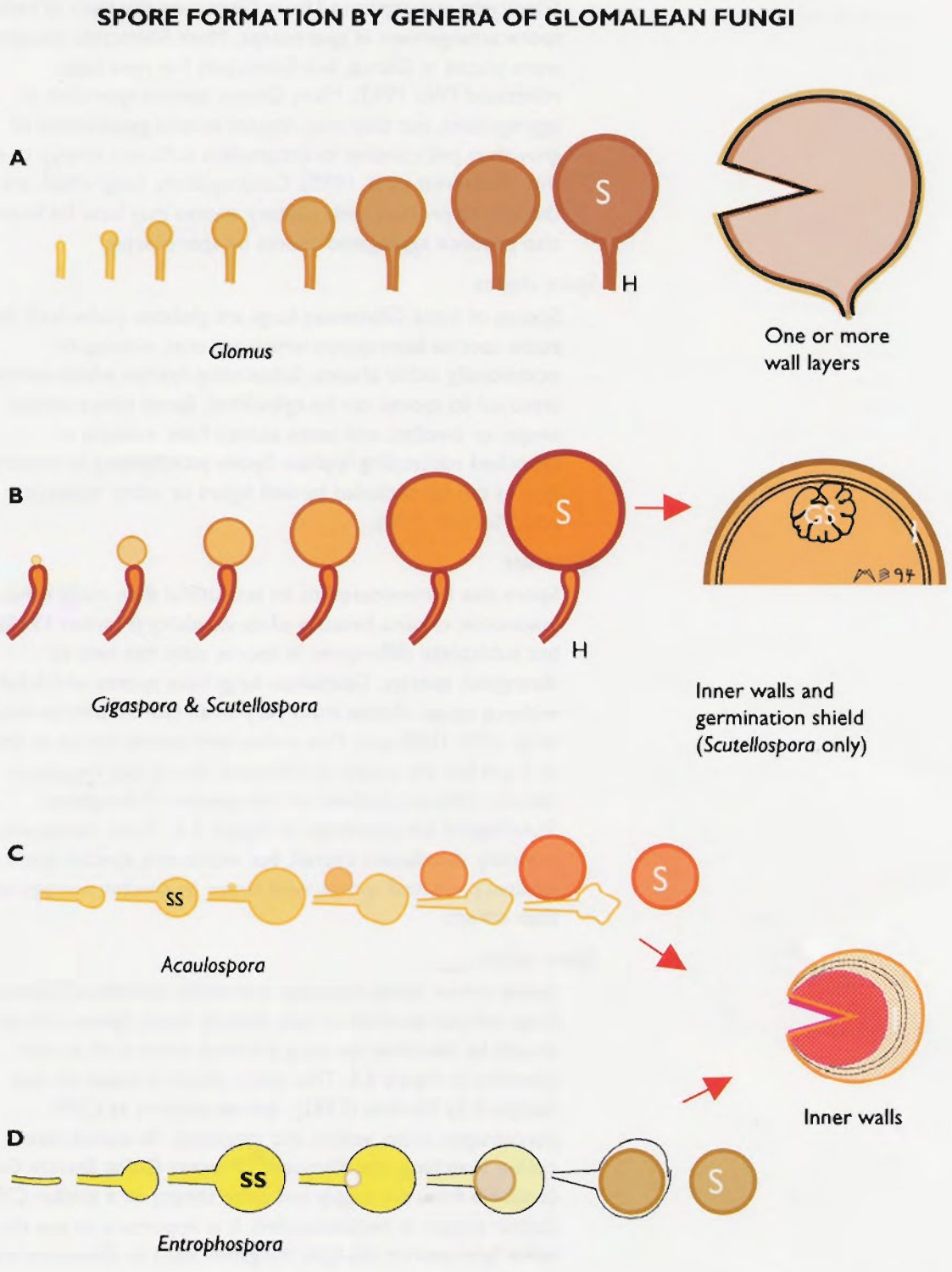


Figure 3.2. *Developmental processes during spore formation by different genera of Glomalean fungi.* **A.** Glomus *spores are relatively simple structures that form from hyphal swellings, which develop thickened walls that may be multi-layered.* **B.** Scutellospora *and* Gigaspora *spores form on a swollen subtending hypha.* Scutellospora *spores develop various inner wall layers and have a germination shield.* **C,D.** Acaulospora *and* Entrophospora *spores are produced at the base of or within a sporiferous saccule which collapses after transferring its contents into the developing spore. These spores develop multiple wall layers and may have complex ornamentation.*

Sclerocystis was separated from *Glomus* on the basis of radial spore arrangement in sporocarps. Most *Sclerocystis* species were placed in *Glomus*, but *Sclerocystis* has now been reinstated (Wu 1993). Many *Glomus* species sporulate in aggregations, but they may require several generations of growth in pot cultures to accumulate sufficient energy to do this (Brundrett et al. 1995). Consequently, fungi which are thought to produce only solitary spores may later be found to also produce aggregated spores or sporocarps.

Spore shapes

Spores of most Glomalean fungi are globose (spherical), but some species have spores which are oval, oblong, or occasionally other shapes. Subtending hyphae which remain attached to spores can be cylindrical, flared into a conical shape, or swollen, and some spores have multiple or branched subtending hyphae. Spore attachments of mature spores can be occluded by wall layers or other material (see Morton 1988).

Spore size

Spore size is considered to be less useful than many other taxonomic criteria because of its variablity (Morton 1988), but substantial differences in spores sizes can help to distinguish species. Glomalean fungi have spores which fall within a range of sizes from very small (20–50 µm) to very large (200–1000 µm). Fine endophyte spores can be as small as 5 µm but are usually overlooked. Spore size frequency data for different isolates of one species of the genus *Scutellospora* are presented in Figure 3.3. These values were normally distributed overall, but within this species some isolates produced spores with larger or smaller average sizes than others.

Spore colour

Spore colour varies between and within isolates of Glomalean fungi and can be used to help identify them. Spore colours should be identified by using a colour chart such as that provided in Figure 3.5. This chart, which is based on one designed by Morton (1992), defines colours as CYM percentages (cyan, yellow and magenta). To standardise colour matching, the 'Pantone® Process Color System Guide' (available from art supply and print shops) or a similar CYMK colour system is recommended. It is important to use the same light source (daylight, tungsten, etc.) to illuminate both the subject and colour guide.

Spore ornamentation

Examples of spore surface features include pits, reticulations, spines and papillae (Fig. 3.4), and these are most often found on *Scutellospora* and *Acaulospora* spores. Scanning-electron microscope (SEM) observations can reveal details of spore surface features more clearly than the light microscope (Fig. 3.4L–N). Observation of spores which appear dull under

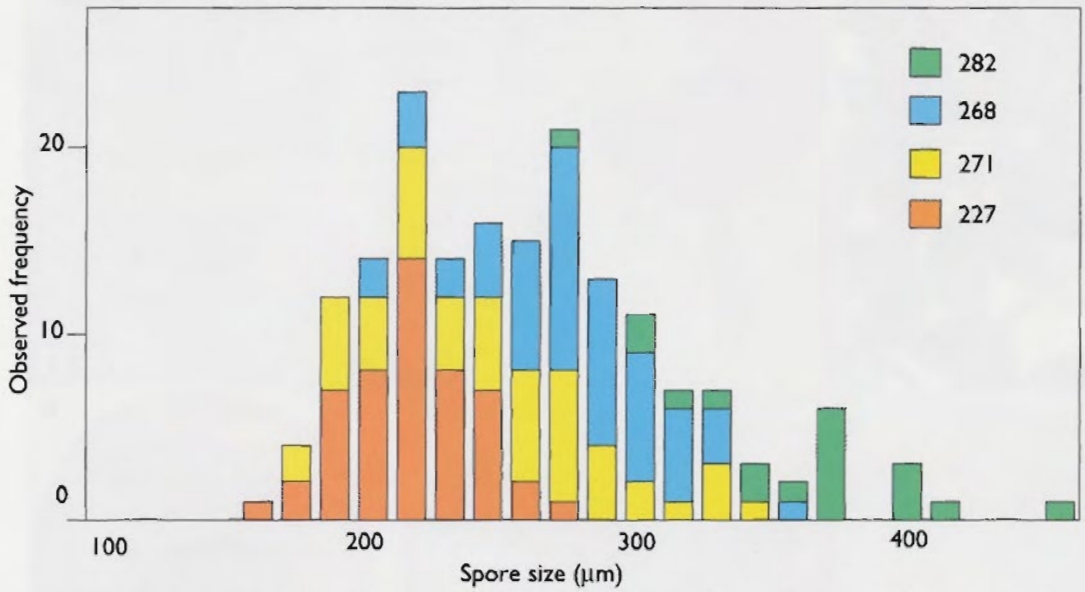


Figure 3.3. *Spore size ranges for 4 isolates of a* Scutellospora *species from different habitats in a region of tropical Australia (Brundrett et al. 1995).*

the dissecting microscope often have papillae or other surface ornamentations which diffract light, and can be seen with the SEM or with the 100X oil immersion objective on a light microscope, while shiny spores are likely to have smooth outer walls.

Spore wall layers and staining reactions

Walls of Glomalean fungus spores have one or more layers, that vary in their thickness, structure, appearance and staining reactions, and can be described using standardised terminology or diagrams (murographs) (see Walker 1983, Morton 1988). *Acaulospora, Entrophospora* and *Scutellospora* species typically have a complex wall structure consisting of a thicker outer wall and one or more thin inner wall layers. These wall layers can be seen only when crushed spores are observed with a compound microscope (Section 3.3D). One or more wall layers may stain red or purple with Melzer's reagent (Morton 1988). Melzer's staining reactions may occur in inner or outer wall layers of spores in all genera, but typical staining reactions may not occur in spores that are old, damaged or have been stored in preservatives. *Glomus* or *Gigaspora* spores are generally simpler in structure than those of other genera, but *Glomus* spores often have several wall layers (Fig. 3.4). Immature *Glomus* spores may have a weak Melzer's staining reaction that is absent in older spores. Young *Glomus* spores often have a fragile outer wall layer which is lost as spores age (Fig. 3.4B).

Spore contents

Spores contain lipids and other contents, which vary in colour and may be arranged in large or small droplets or granules. The size or arrangement of lipid droplets can help identify fungi, but will change when spores age. Spores of Glomalean

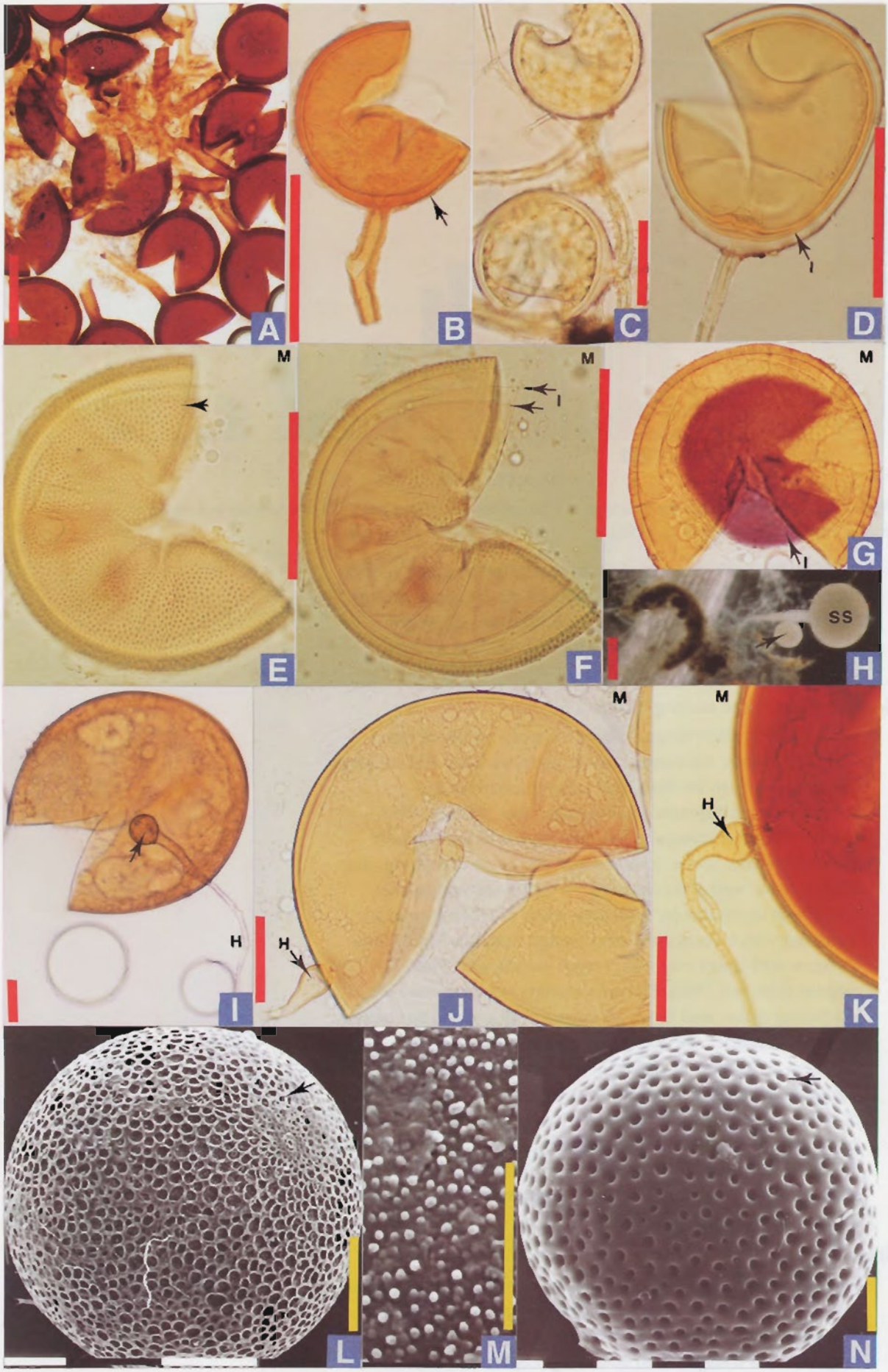

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Figure 3.4. *Examples of spores of* Glomus, Acaulospora *and* Scutellospora *showing spore wall structures, as well as developmental and germination processes.* **A–K** *are light microscope photographs.* **L–N** *show details of spore surface texture revealed with the scanning electron microscope.*

Abbreviations: **M** = *stained with Melzer's reagent,* **A** = *arbuscule,* **V** = *vesicle,* **AV** = *auxiliary vesicle,* **S** = *spore,* **H** = *subtending hypha,* **GS** = *germination shield,* **SS** = *sporiferous saccule,* **I** = *inner-wall layers. Orange scale bars are 100 µm long. Yellow scale bars are 10 µm long.*

A–D.* Glomus *spores.

A. *Mounted whole spores of* Glomus invermaium *from a spore aggregation. These old, dead spores are typical of those found in field soils.*

B. *Young spore of the same* Glomus *sp. from a pot culture, squashed to show the thin irregular outer wall layer found on young spores (arrow), the thick brown unit wall and subtending hyphal attachment.*

C,D. Glomus clarum *spores.* **C.** *Spores produced individually on soil hyphae.* **D.** *Squashed spore showing striated yellow inner wall layer (arrow), the thicker white outer layer and subtending hyphal attachment.*

E–H. *Acaulospora spores.*

E,F. *Squashed spore of an* Acaulospora *sp. stained with Melzer's reagent.*

E. *Focused to show pits in outer wall (arrow).*

F. *Same spore focused to show inner wall layers (arrows).*

G. *Squashed spore of another* Acaulospora *sp. stained with Melzer's reagent. The orange outer wall layer, purple staining innermost wall layer (arrow), and a thin intermediate wall layer can be seen.*

H. *Spore (arrow) forming at the base of a sporiferous saccule outside a clover root.*

I–K. Gigaspora *and* Scutellospora *spores.*

I. *Squashed spore of* Gigaspora *sp. with swollen subtending hypha (arrow).*

J,K. *Crushed spores of a* Scutellospora *species showing bulbous subtending hyphae and wall layers revealed by Melzer's staining.*

L–N. *SEM views of spore surface texture.*

L. *Reticulate outer wall ornamentation on a spore of* Scutellospora nigra.

M. *Papillae on the surface of a* Scutellospora *sp. spore.*

N. *Pits on an* Acaulospora *sp. spore.*

fungi often contain parasitic organisms, especially if collected in the field, and these result in the formation of holes in spore walls and/or cytoplasmic changes (Lee & Koske 1994).

Spore germination

Spore germination mechanisms can also be used to distinguish Glomalean fungi, especially *Scutellospora* species which have germination shields with complex infoldings on their inner walls (Fig. 3.6B). When *Scutellospora* spores germinate, hyphae arise from compartments within these shields and then grow through the outer wall (Fig. 3.6A). There are also characteristic features of spore germination of *Acaulospora* (germination shields) and *Gigaspora* (warts inside the spore wall) spores.

Soil hyphae

Substantial differences between isolates of Glomalean fungi in the appearance of soil mycelial systems (wall thickness, staining, associated structures, etc.) can be observed (M. Brundrett, unpublished data). Unfortunately, these features have rarely been considered in taxonomic studies. Large-spored *Scutellospora* species can have distinctive melanized hyphae that remain brown even after clearing and staining processes (Fig. 3.6F), while the hyphae of other fungi are hyaline or less darkly pigmented (yellow or brown).

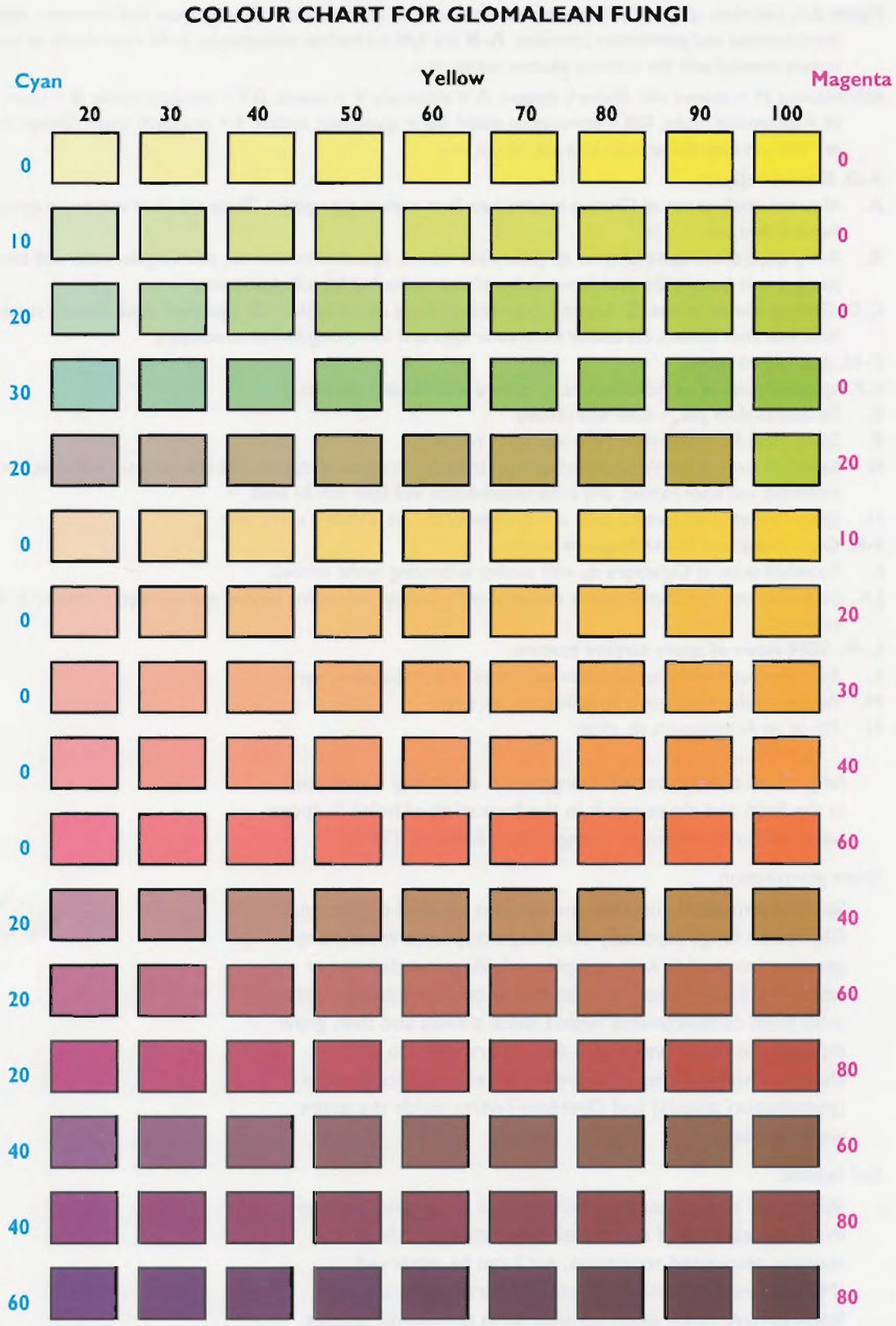


Figure 3.5. *Colour chart which can be used to help describe spores of Glomalean fungi. Note that colours in the final version may have changed. Colours can be described as % CYM values (cyan, yellow, magenta). This chart is a modified version of the INVAM colour chart (Morton 1992).*

Glomalean fungi produce coarse distributive 'runner' hyphae and finer branched 'absorptive' hyphae. The thickness of the coarsest hyphae produced by Glomalean fungi varies considerably from up to 20 µm for some *Glomus* species to 5 µm or less for some *Acaulospora* species, while 'fine endophytes' have hyphae which are 2 µm or less in diameter. The hyphal walls of some *Glomus* species are substantially thickened and stain very strongly (Fig. 3.6G).

Structures associated with soil hyphae

Auxiliary vesicles (also called auxiliary bodies or cells) are clustered structures produced by the soil hyphae of *Scutellospora* and *Gigaspora* species, that can be used to help identify them (R. E. Koske, pers. comm.). Several types of these cells are shown in Figure 3.6. External hyphae of *Glomus* and *Acaulospora* species often also produce small, round 'vesicles' in the soil.

B. Identifying Glomalean fungi by root colonisation patterns



A technique introduced by Abbott (1982) allows the contribution of individual Glomalean fungi to total mycorrhizal root colonisation to be determined, by recognising characteristic root morphology patterns produced by different fungi. This allows the relative activities of different types of Glomalean fungi present in soil to be determined, and should be more reliable than estimates of spore production (which varies between fungi). Identification of endophytes within roots is important for pot-culture quality control (Section 3.4E), because contaminating fungi can be identified months before they sporulate. This procedure can also be used to determine the mycorrhizal inoculum potential of different fungi in bioassay experiments (Section 4.4).

Examples of keys that can be made to distinguish fungi present in an area are provided by Abbott (1982) and McGee (1989). However, the best way to identify Glomalean fungus colonisation in roots is to get to know them by examining single-isolate pot cultures. Routine assessment of the quality of pot cultures provides an opportunity to become familiar with the distinguishing features of fungi that are important in the soils. Mycorrhizal morphology is also influenced by host root structure (Brundrett & Kendrick 1990b), so it is best to work with one plant species. It is usually easier to identify fungi in roots with a thick cortex than in species with narrow roots, and species with heavily pigmented roots that are difficult to clear should be avoided if possible.

It is generally easy to recognise genera of VAM fungi by their root colonisation patterns, but it is also often possible to separate species (especially within *Glomus*). Morphological features that are important are listed in Table 3.2 and include variations in vesicles (size, shape, wall thickness, wall layers, position and abundance), hyphal branching patterns, the diameter and structure of hyphae (especially near entry points), and the staining intensity of hyphae (dark or faint).

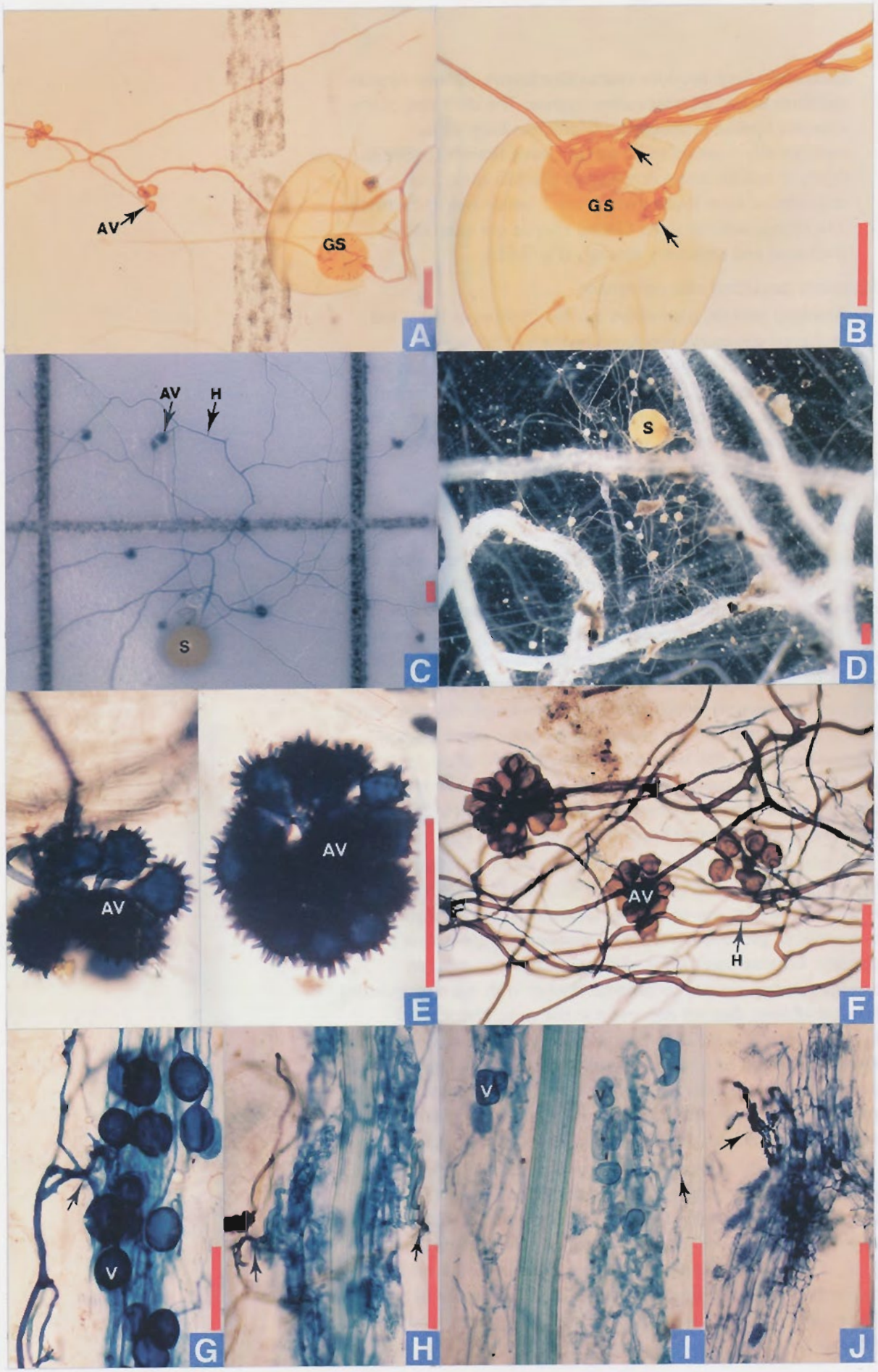

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Figure 3.6. *Spore germination, soil hyphal structures and mycorrhizal associations by Glomalean fungi in the genera* Glomus, Acaulospora *and* Scutellospora.

A–F. *Germinated spores and hyphae of Glomalean fungi.*

A,B. *Germinating hyphae emerging through the outer wall (arrows) from the germination shield of a* Scutellospora *species. Auxiliary vesicles (AV) are also present.*

C. *Germinated spore of a* Gigaspora *species on a membrane filter showing germinated hyphae with auxiliary vesicles.*

D. *Hyphae, auxiliary vesicles and mycorrhizal roots produced by a single germinated spore of a* Gigaspora *species. This single spore pot culture has been washed gently to keep hyphae attached to roots.*

E. *Large auxiliary vesicle clusters consisting of a globular arrangement of cells with numerous finger-like projections produced by a* Gigaspora *species.*

F. *External hyphae with large brown-pigmented auxiliary vesicles with relatively broad projections associated with a large-spored* Scutellospora *species.*

G–J. *Mycorrhizal formation by Glomalean fungi.*

G and J *show VAM in clover roots and H and I, in sorghum roots.*

G. Glomus *species VAM colony with darkly staining hyphae and vesicles in the root cortex and irregularly thickened soil hyphae connected to the entry point (arrow).*

H. *VAM colonies of a* Scutellospora *species showing entry points (arrows) and the lack of internal vesicles.*

I. Acaulospora *species VAM colonies with characteristic irregularly lobed internal vesicles.*

J. *Fine endophyte VAM in a root showing typical colonisation pattern and hyphal swellings near entry point (arrow).*

There are also variations in VAM morphology within genera. For example, substantial differences in the diameter of hyphae, the size and wall structure of vesicles, vesicle production, etc. can often be used to destinguish species of *Glomus*. Further research will undoubtedly uncover more useful features. Characteristics of genera of Glomalean fungi are outlined below and illustrated in Figures 3.6 and 3.7.

Table 3.2. Characteristic root colonising patterns which can be used to identify different genera of Glomalean fungi.

Genus	*Characteristics*
Glomus	• Relatively straight hyphae ramify along the root cortex (if root anatomy permits), often producing 'H' branches which result in simultaneous growth in two directions. Staining of these hyphae is usually relatively dark. • Oval vesicles, which usually form between root cortex cells, are present in many cases. These vesicles persist in roots and often develop thickened and/or multilayered walls.
Acaulospora	• Entry-point hyphae have characteristic branching patterns. Hyphae in the outer cortex generally are more irregularly branched, looped or coiled than for *Glomus*. • Internal hyphae are thin-walled, often stain weakly and thus may be very hard to see. They are often made more conspicuous by rows of lipid droplets. • Intracellular oil-filled vesicles, that are initially rectangular, but often become irregularly lobed due to expansion into adjacent cells, are a characteristic feature of most isolates. These vesicles have thin walls and do not persist in roots.
Scutellospora	• Looping hyphae are often present near entry points. The hyphal branching pattern for this genus is similar to *Acaulospora*, but hyphae in the cortex generally are thick-walled and stain darkly. • Internal vesicles are not present. • Arbuscular trunk hyphae normally are much longer and thicker than those of *Glomus*. Arbuscules may appear 'wispy' because of relatively long curving branches.
Gigaspora	• The root colonisation pattern for these fungi is very similar to that of *Scutellospora*, but hyphae are thicker than those of most other VAM fungi.
Fine endophyte	• These unusual fungi have been called *Glomus tenue*, but these endophytes are different from other *Glomus* species. Fine endophytes can easily be distinguished by their very narrow hyphae (<1 µm in diameter) and net-like growth pattern in roots. • Small hyphal swelling (<5 µm) can occur near entry points and may be analogous to vesicles.

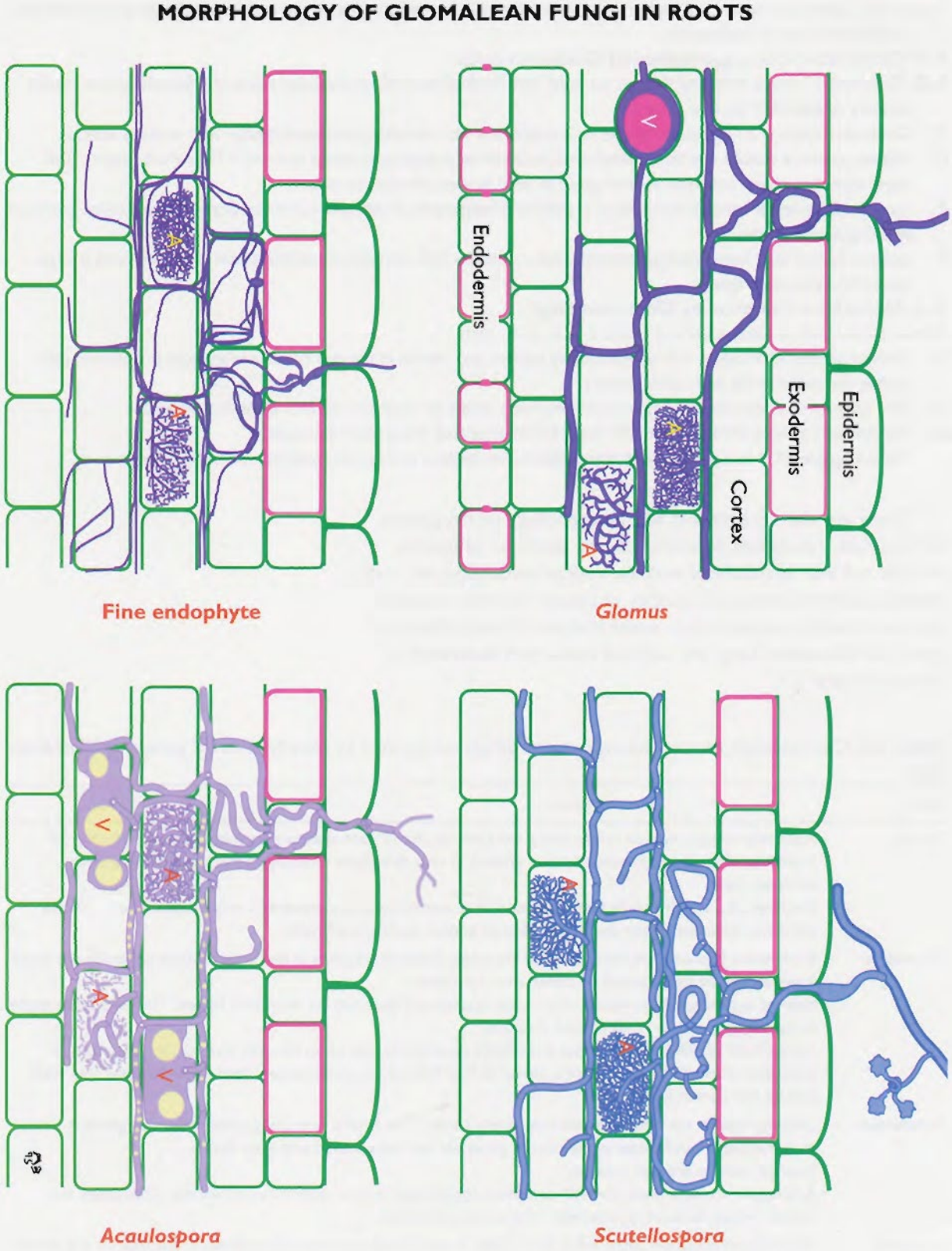


Figure 3.7. *Mycorrhizal morphology features that can be used to identify genera of Glomalean fungi. Note characteristic differences in hyphal branching patterns and orientation within roots and vesicle production.*

3.3. SEPARATING SPORES FROM SOIL

Spore density data can be obtained by counting spores in a soil sample. However, spore numbers may be poorly correlated with mycorrhizal fungus activity in soils — which can best be measured by bioassays (Section 4.4). It should not be assumed that spores present in soils are viable, unless their germination has been tested, as spores may be dormant (Tommerup 1992), and mortality due to parasitism of spores by other soil microbes is common (Lee & Koske 1994).

When comparisons are made, spore numbers can be used directly, or converted to bio-volume estimates (using the formula Volume = $4/3\pi r^3$, where r is the average radius) to account for variations in size. There is a strong correlation between the size of spores of soil fungi and the numbers they produce in a volume of soil (Chuang & Ko 1981). Consequently, Glomalean fungi with small spores should have higher spore densities in soil than those with large spores, but their capacity to colonise roots may be similar. In a study of Australian tropical ecosystems spore density data were better correlated with mycorrhizal formation in bioassays than spore bio-volume data (M. Brundrett, unpublished data).

The standard procedure for separating Glomalean (VAM) fungus spores from soil samples (Daniels & Skipper 1982, Tommerup 1992) is presented here. Other methods involving sucrose density layers or gradients can also be used. The method presented has been successfully used with a wide range of habitats and soil types in Australia (Fig. 3.8). However, it is a good idea to occasionally check the efficiency of your procedure by looking for spores in the fractions that would normally be discarded, especially those containing roots or organic matter.

A. Sieving and centrifugation

1. Weigh soil samples and determine their moisture content by oven-drying (90°C overnight) a small subsample in a pre-weighed container. This will allow spore numbers to be expressed relative to dry soil weight. You may wish to remove coarse debris and rocks from samples with a 2-mm sieve. Ensure that dry soil samples have been thoroughly wetted for at least 30 minutes before sieving them. The size of soil samples that can be processed will depend on spore numbers and soil texture (clay soils tend to clog finer sieves). Generally less than 100 g of soil is best, but larger samples of up to 1 kg of sandy soils can be used if care is taken to ensure that fine screens do not become clogged.
2. Soil is mixed in a substantial volume of water and decanted through a series of sieves after allowing heavy soil particles to settle for a few seconds (Fig. 3.9). This washing and decanting process is repeated until the water is clear. Roots and coarse debris are collected on a coarse (750 µm or 1 mm) screen, while spores are captured on one or more finer screens. Ensure that soil aggregates have broken apart. Figure 3.9 shows an example where 50, 100 and 250 µm fractions from

Equipment and reagents

- Two or more interlocking stacking sieves with nylon or stainless steel mesh and a range of pore sizes.
  Sieves can be purchased or made from plastic cylinders and nylon mesh.
  Sieve sizes:
  — 40–50 µm (for small spores)
  — 100 µm (medium-sized spores)
  — 250 µm (very large spores and sporocarps)
  — 750–1000 µm (roots and coarse organic matter)
- Wash bottles containing water and 50% sucrose (w/v)
  (use finely granulated food-grade white sugar)
- Centrifuge (2000 RPM) with tubes and caps
- Vacuum filter apparatus
  (Buchner funnel, side-arm flask and vacuum source)
- Glass fibre (Whatman GF/A) or paper filters (11 cm diameter)
- Petri dishes to hold filters (reusable)



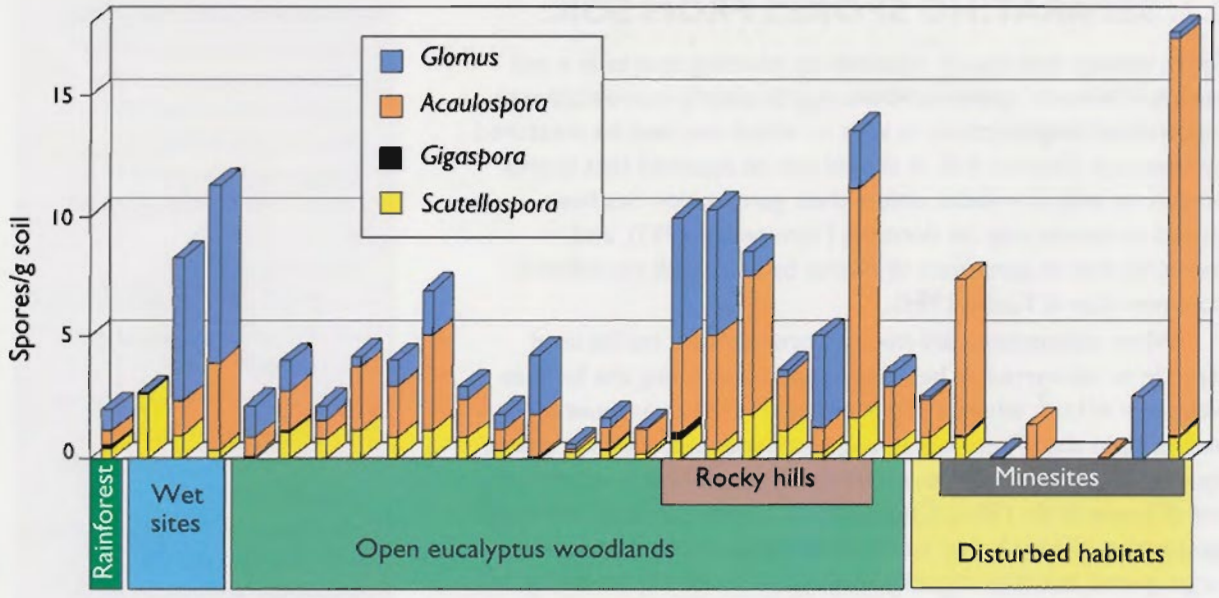


Figure 3.8. *Variations in spore numbers of different genera of Glomalean fungi in soil samples from different natural habitats and disturbed mine sites in a tropical Australian region (Brundrett et al. 1995).*

a soil are processed separately, but you may wish to use fewer fractions or other screen sizes. A dispersant such as 'calgon' (sodium hexameta phosphate) can be used with clay soils, but may affect the viability of spores. Vigorous washing with water or hand mixing may be necessary to free spores from aggregates of clay or organic materials.

3. The first water centrifugation is optional, but removes substantial amounts of floating organic debris from many soils. After this step the supernatant and floating debris is discarded. A small amount (± 100 mg) of finely powdered kaolin clay (from an art supplier) can be added to water with sievings to help form a stable pellet during centrifugation. **Ensure that the centrifuge is properly balanced before switching it on.**
4. The next step involves re-suspending the pellet in 50% sucrose by vigorously shaking tightly stoppered tubes. The samples are then centrifuged for 1 minute at 2000 RPM to separate spores (and any remaining organic debris) from denser soil components (Fig. 3.9). Immediately after centrifugation, spores in the sucrose supernatant are poured onto the finest sieve (40–50 µm) and carefully washed with water to remove the sucrose.
5. After rinsing the spores, wash them onto a pre-wetted filter paper in a Buchner funnel before vacuum filtration (Fig. 3.9). Glass fibre filters (Whatman GF/A) are superior to paper ones, but both are adequate. Filter papers can be marked with parallel lines ± 7 mm apart to separate microscope fields for spore counting. Inverted Petri dishes can be used to store filter papers with spores.

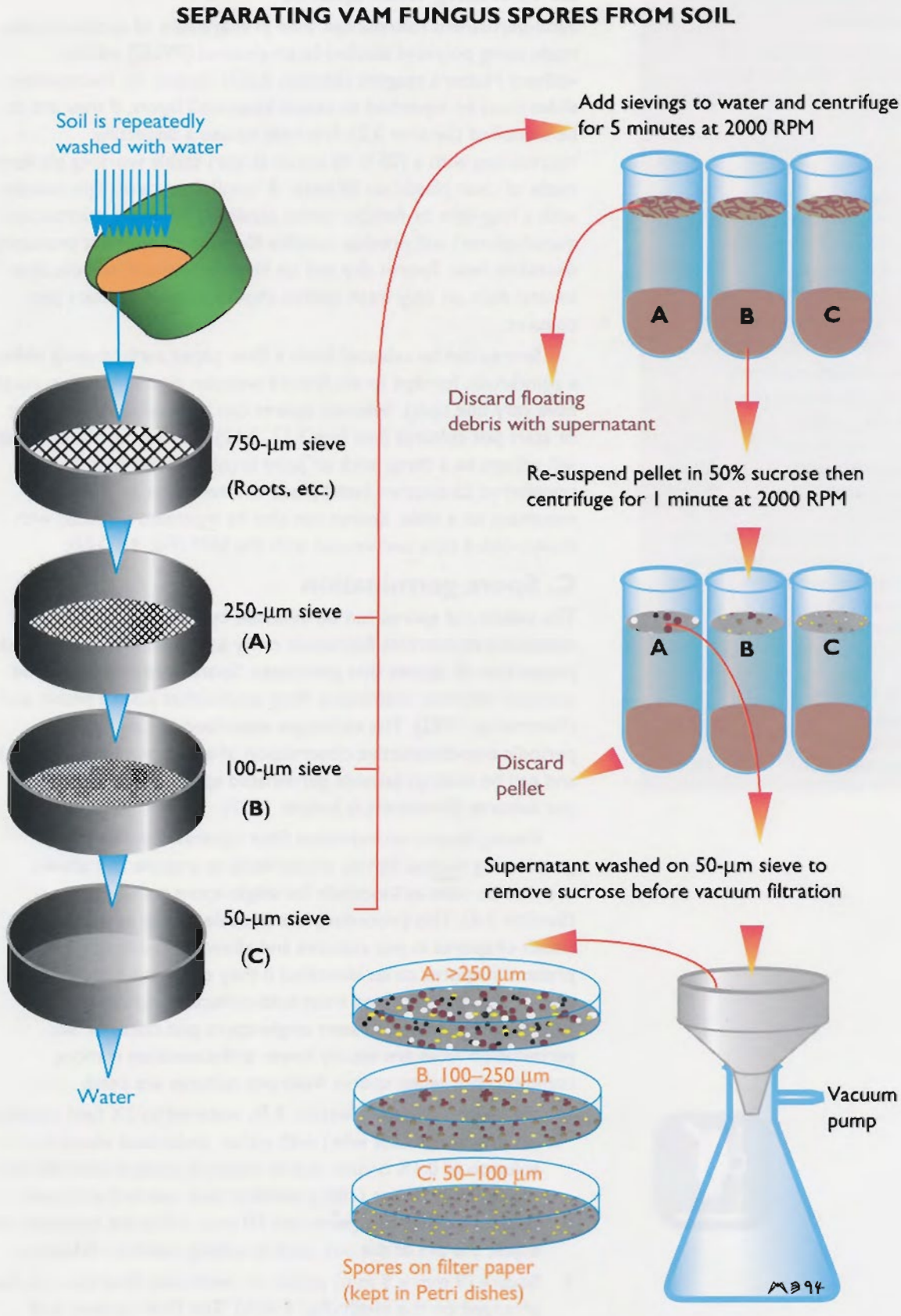


Figure 3.9. *Separating Glomalean fungus spores from soil using the wet sieving and 50% sucrose centrifugation methods.*

Spore mounting supplies and media

- Paint brush, sharpened wooden dowel or forceps with very fine tips for spores (Fig. 3.11)
- Spore germination on membrane filters:
  - — squares cut from nitrocellulose membrane filters (0.45 µm)
  - — Petri dishes (square 10 × 10 cm)
  - — 11 × 11 cm pieces of nylon mesh (50 µm)
  - — steamed sandy soil
- PVLG mountant (Koske & Tessier 1983)

polyvinyl alcohol*	8.33 g
distilled water	50 mL
lactic acid	50 mL
glycerine	5 mL

  * 24–32 centripose viscosity polyvinyl alcohol should be used and can be dissolved in water by heating (90° C) overnight.
- Melzer's reagent – mixed 1:1 (v/v) with PVLG (Morton 1991, 1992)

iodine	1.5 g
potassium iodide	5 g
distilled water	100mL

B. Working with spores

Semi-permanent microscope slide preparations of spores can be made using polyvinyl alcohol-lacto-glycerol (PVLG) with or without Melzer's reagent (Section 3.3D). Spores on microscope slides must be squashed to reveal inner-wall layers, if they are to be identified (Section 3.2). It is best to use a dissecting microscope with a (25 × 40 cm or larger) stable working platform made of clear plastic on its base. A 'cool' fibre-optic light source with a ring-light or flexible cables (available from the microscope manufacturer) will provide suitable illumination without producing excessive heat. Spores dry out on filter papers and will die after several days, so only fresh spores should be used to start pot cultures.

Spores can be selected from a filter paper surface using either a paintbrush, forceps or sharpened wooden dowel, but they must have very fine tip(s). Selected spores can be used to make slides, or start pot cultures (see Figs 3.11, 3.12). Spores on a filter paper will adhere to a damp stick or paint brush and can then be transferred to another (wet) piece of filter paper or a drop of mountant on a slide. Spores can also be mounted on stubs with double-sided tape and viewed with the SEM (Fig. 3.4L–N).

C. Spore germination

The viability of spores can be assessed by inoculating plants and measuring mycorrhiza formation or by assays which measure the proportion of spores that germinate. Spore germination can be assessed between membrane filter sandwiches placed within soil (Tommerup 1992). The technique described below allows periodic non-destructive observation of spore germination events and can be used to provide germinated spores for single-spore pot cultures (Brundrett & Juniper 1995).

Placing spores on individual filter squares ensures that germinating hyphae can be traced back to a spore and allows them to be used as inoculum for single-spore pot cultures (Section 3.4). This procedure is a valuable way of assessing the health of spores in pot cultures and allows parasitic organisms present in spores to be identified if they sporulate on dead spores. Spores separated from field-collected soil can also be germinated and used to start single-spore pot cultures, but germination rates are usually lower and parasitism is more common than when spores from pot cultures are used.



1. Steamed sandy soil (Section 3.3), watered to 2X field capacity (usually about 20% w/w) with either de-ionised water or a solution of 0.1% trypan blue in water, is packed into 100-mm square Petri dishes (100 g soil/dish) and overlaid with one layer of nylon mesh (pore size 50 µm). It may be necessary to adjust the pH of the soil used by adding calcium carbonate.
2. Square (9 mm x 9 mm) pieces of membrane filter are cut then arranged on the mesh (Fig. 3.10A). The filter squares and nylon mesh are sterilised in 70% ethanol and rinsed with distilled water before use. A washed spore (or 1-mm piece of

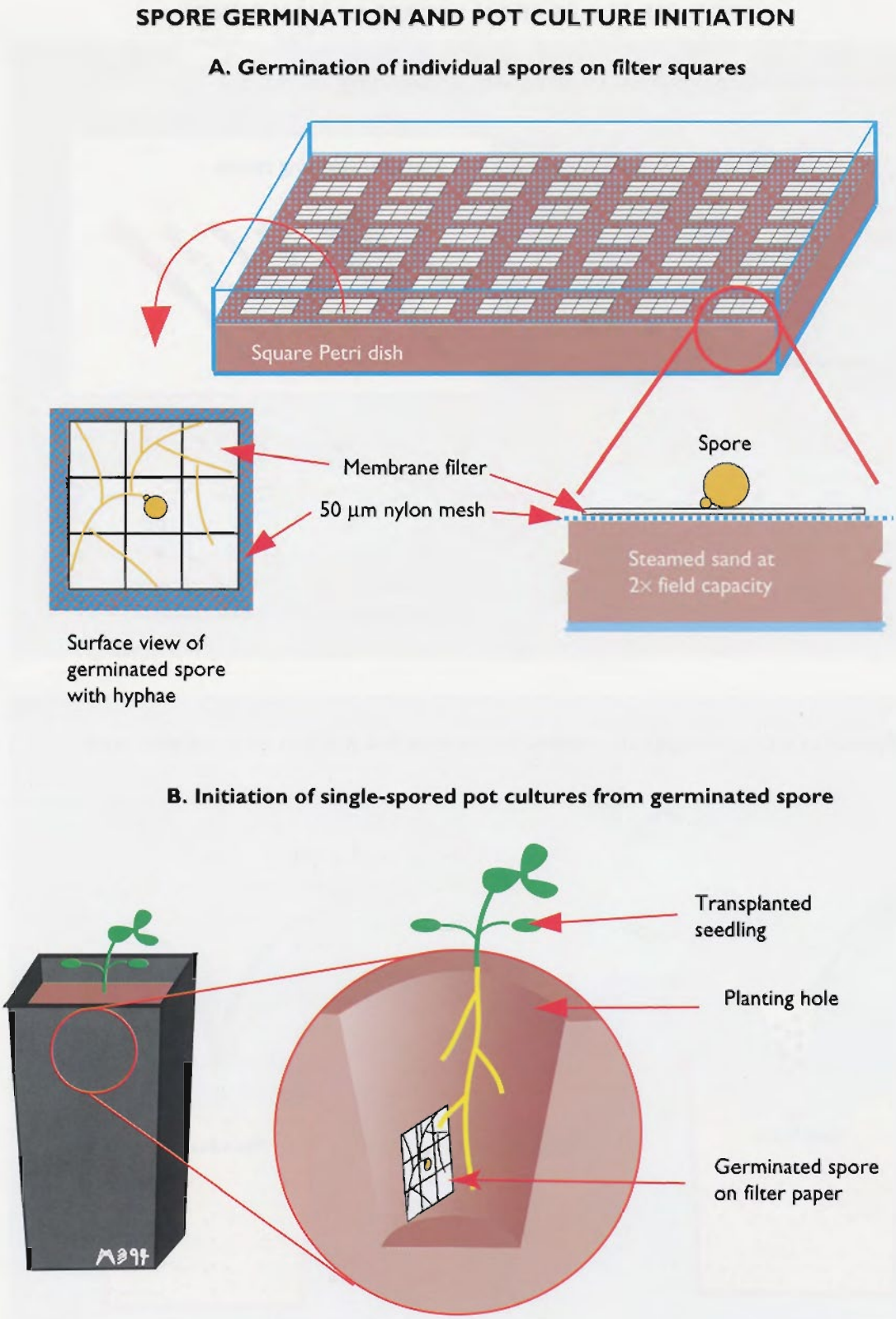


Figure 3.10. *Spore germination (**A**) and pot culture initiation (**B**).*

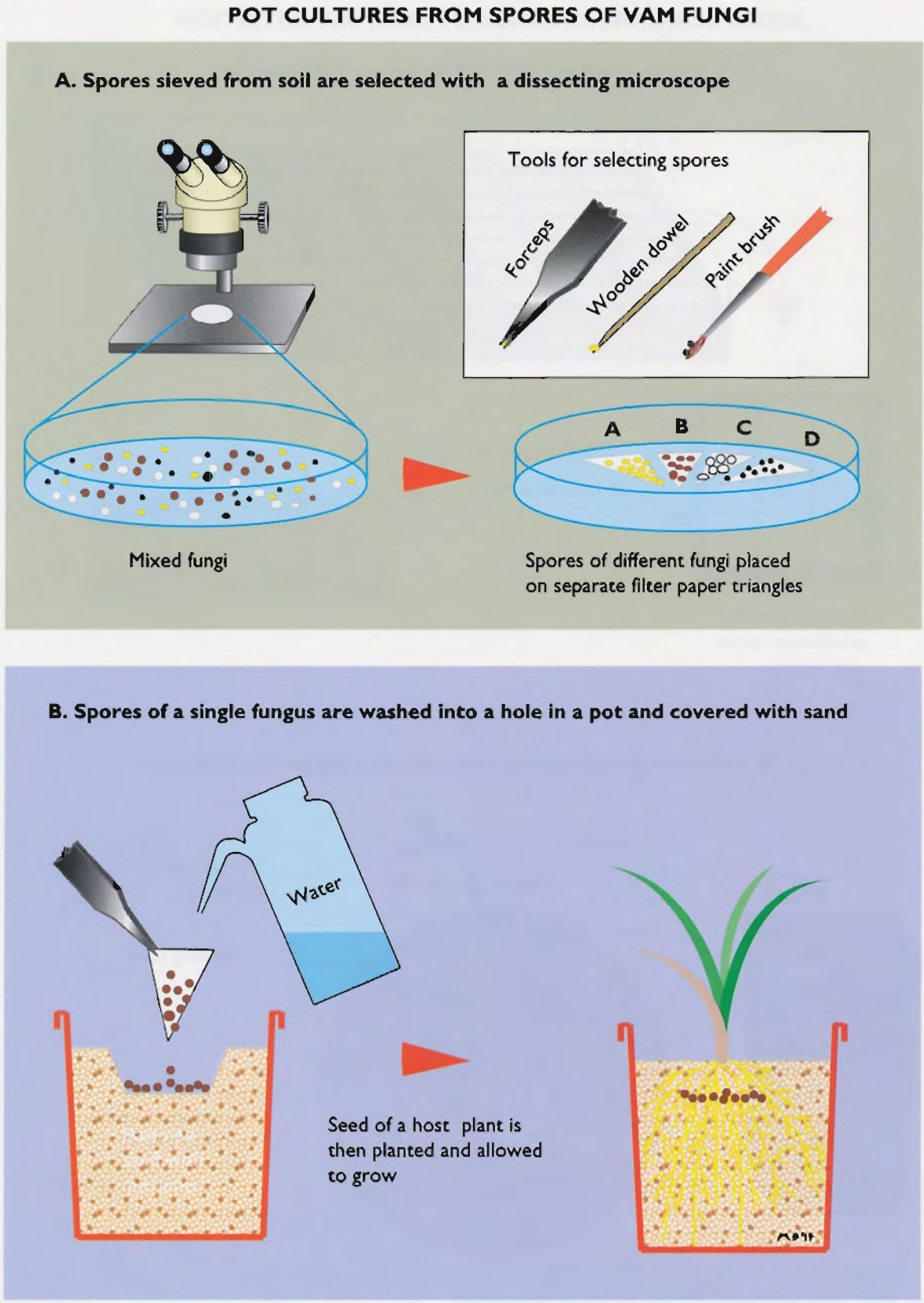


Figure 3.11. Selecting spores of individual Glomalean fungi from a mixed sample **(A)** *and using them to start pot cultures* **(B).**

root) obtained from a pure pot culture or field soil is then placed in the centre of each filter square. It is usually not necessary to surface-sterilise spores, as parasitic organisms are more likely to grow from inside spores.

3. Petri dishes are sealed and incubated in the dark at an appropriate temperature (i.e. 20°C for temperate fungi, 30°C for tropical fungi). At 1–4 day intervals, the dishes are placed under a dissecting microscope with fibre-optic illumination to observe the number of spores at different stages of germination. Frequent observations may result in the death of some germinating hyphae, but most will not be affected by brief exposure to light and desiccation.
4. Spores at different germination stages can be mounted on slides to make observations of spore germination mechanisms, or used for histological or physiological studies. The development and structure of external hyphae and associated structures can also be documented using this procedure (see Fig. 3.6CD).

D. Herbarium specimens and slide preparations



Fungal material of sufficient quality and quantity to allow future taxonomic studies of all isolates used in mycorrhizal research should be submitted to an internationally accredited herbarium. This will ensure that research results remain valid if the names of fungi are changed in future. You may also wish to keep your own collection of fungal material. Information about the storage of fungal material in herbaria is provided in Section 2.5.

Specimens of living (pot culture) material of Glomalean fungi can be sent to an international collection (see below). Inclusion of fungal isolates in these collections will allow their identity to be confirmed and provide material for future taxonomic and physiological research by an international network of mycorrhizal scientists.

Spores on filter paper, sporocarps, or dry soil from a pot culture can be used as herbarium specimens of a Glomalean fungus. Spores of Glomalean fungi can also be stored in a chemical preservative (5% glutaraldehyde, 3.5% formalin, or 0.025% sodium azide solutions are recommended by INVAM and BEG, **but these solutions should be used carefully as they are highly toxic**). The preservatives FAA (formalin: acetic acid: alcohol) and lactophenol **should not** be used because they influence the wall structure and staining reactions of spores (Morton 1987). The ultimate life span of chemically preserved spores is not known, so dry material of fungi should always also be kept.

Several different slides of each Glomalean fungus must be prepared to identify fungi, or for use as herbarium specimens. These slide preparations should include: (i) mounts of uncrushed spores, (ii) crushed spores, (iii) spores which have been crushed a second time after a few minutes, (iv) heavily crushed spores, and

International culture collections of Glomalean fungi

INVAM — Curator: Dr J. Morton, Division of Plant and Soil Sciences, 401 Brooks Hall, West Virginia University, Morgantown, WV 26506-6057, USA.

Bank European de Glomales (BEG) — contact: Dr C. Walker, Forestry Authority, Northern Research Station, Roslin, Midlothian, EH25 9SY, UK.

(v) crushed spores mounted in Melzer's reagent. These different treatments are required to reveal all the wall layers present in spores, especially for *Scutellospora* and *Acaulospora* species (Walker 1983, Morton 1988). Slides of stained roots should also be included if they are available. Slide preparations made with PVLAG are self-sealing and will last for years. However, it is important to also provide unmounted spores of each fungus as herbarium specimens, so that new staining, biochemical, or DNA procedures can be used in the future.

3.4. ISOLATING AND PROPAGATING GLOMALEAN FUNGI

Glomalean (VAM) fungi are usually propagated by growing them with a living host plant in soil pot cultures (Figs 3.12, 3.13). These pot cultures, which consist of soil, spores, root pieces and hyphal fragments, can be used as inoculum for experiments or to introduce fungi into soils. Pot culture isolation procedures often help to identify VAM fungi from soils collected in the field, when these do not contain spores of sufficient quality or quantity to allow fungi to be accurately identified. Many different methods have been used to establish and maintain living cultures of Glomalean fungi (see Sieverding 1991, Jarstfer & Sylvia 1993). The methods presented here, which utilise plants grown in non-draining pots of steamed sand supplemented with a complete

Figure 3.12. *Procedures for pot-culturing Glomalean fungi.*

A. *Dissecting microscope view of spores of Glomalean fungi selected from a tropical Australian soil sample after wet sieving and sucrose centrifugation. These spores of* Scutellospora *and* Acaulospora *species were sorted into nine putative species to start individual pot cultures (spores range from ≈ 50 to 400 µm in diameter).*

B. *Starting a pot culture using carefully selected spores (S). Water (W) is used to wash spores into steamed sand containing nutrients.*

C. *Pot cultures used to propagate single isolates of Glomalean fungi by growing sorghum plants in steamed sand. Non-draining pots (1-L buckets and 2-L cartons) are used to avoid cross-contamination.*

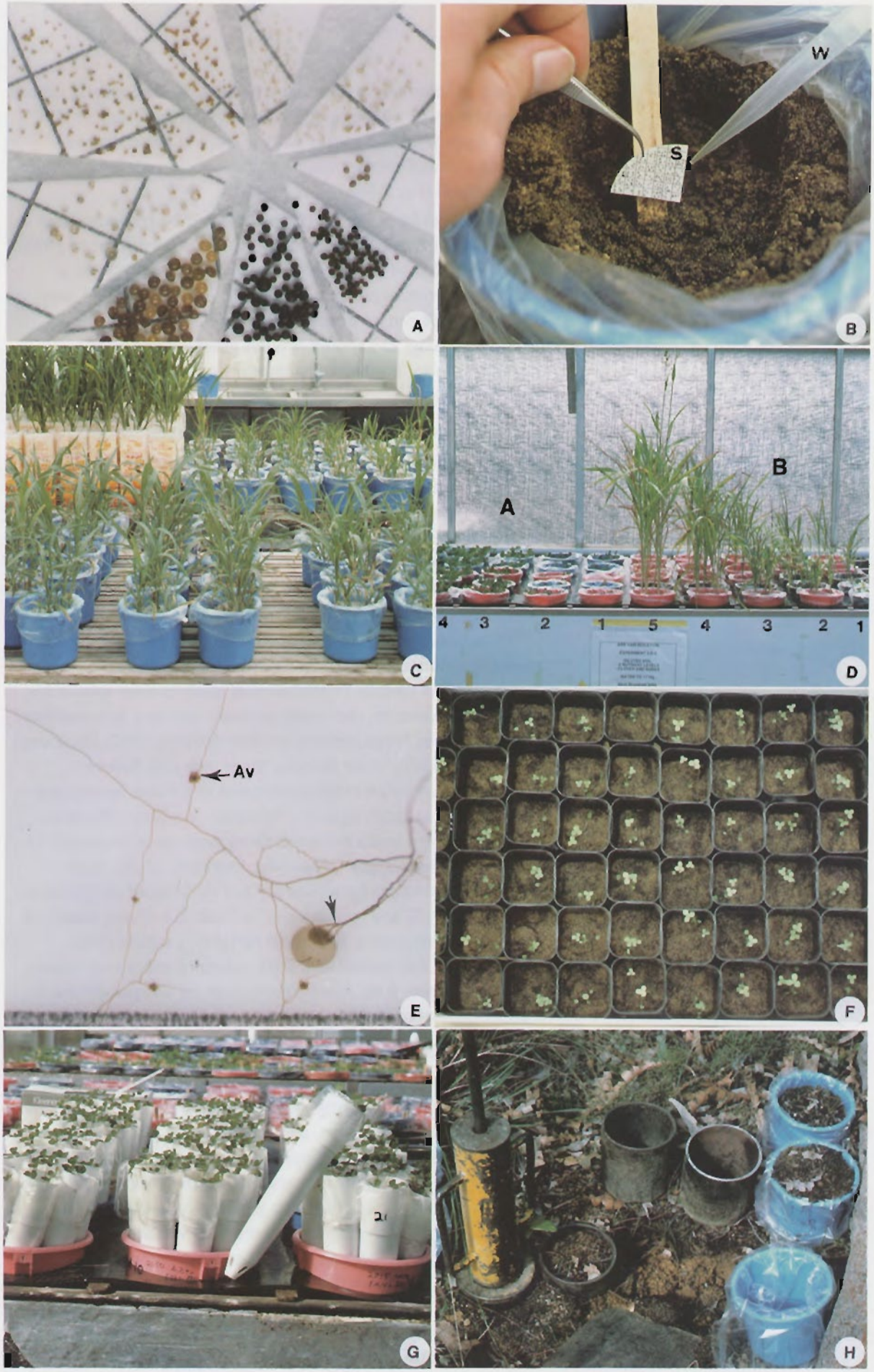
D. *Clover (A) and Sorghum (B) soil-trap cultures growing in sand supplemented with different rates of P fertilizer (1–5). Plants grown in buckets are immersed in a water bath to control soil temperature.*

E. *Dissecting microscope view of a single germinated spore of a* Scutellospora *species suitable for starting a single-spore pot culture. Germinating hyphae (arrow) and accessory vesicles (Av) are present on the surface of a membrane filter square.*

F. *Single-spore pot cultures in small (150 mL) pots. A slow-growing clover species (*Trifolium resupinatum*) has been used.*

G. *Pot-cultures started by transplanting clover seedlings which were grown in intact cores of soil from field sites. Small conical pots (conetainers) allow many replicate seedlings to be used.*

H. *Taking 1-L intact cores of soil from a field site in a West Australian eucalypt forest. These cores can be used for bioassays (see Chapter 4) and for growing bait plants which are transplanted to start pot cultures.*


A
W
S
B
C
A
B
4 3 2 1 5 4 3 2 1
D
Av
E
F
G
H

nutrient solution, were developed by Dr L. K. Abbott and others at the University of Western Australia in Perth (Gazey et al. 1992). A study of Glomalean fungi in tropical Australia has allowed new and existing methods for isolation of these fungi to be compared (Fig. 3.14).

Glomalean fungi can also be propagated by growing plants in aeroponics or other hydroponic systems (Jarstfer & Sylvia 1993), in turface (calcined montmorillonite clay — Plenchette et al. 1982), or in root organ culture (Bécard & Piché 1992). These other methods can be used to propagate existing isolates of mycorrhizal fungi and should provide greater control of inoculum quality, but are not practical for isolating new fungi from field soils.

A. Soils and nutrient supplements

For pot cultures, most researchers use a coarse textured (sandy) soil with moderate nutrient levels, supplemented with mineral nutrients. Sandy soils can be pasteurised by steaming for 1 hour at 90°C on two consecutive days, or by other techniques such as solar heating (Section 6). Soil pH should be similar to that of the soil from which fungi are isolated (the pH of acidic soils can be raised by adding the appropriate amount of lime ($CaCO_3$)).

Mineral nutrient supplements are usually provided, but can adversely influence fungal development if they are too high (Fig. 3.13). The control of phosphorus levels is most critical for inoculum production, but optimum levels can vary between host plants, soils and fungal isolates (Jarstfer & Sylvia 1993). Nutrients can be provided by incorporating them into soils before experiments or by periodic applications of a dilute hydroponics solution (in free-draining pots). However, it will be difficult to regulate nutrient levels in free-draining pots, since there will be uncontrolled losses due to leaching. Examples of nutrition supplements that are optimised for pot cultures of two hosts in an infertile sandy soil are provided in Table 3.3. Plants which do not fix nitrogen require additional NH_4NO_3 supplements (i.e. 30 mg/kg soil applied as a 3 g/L solution) every two weeks. Refer to Chapter 6 for more information on the glasshouse management of mycorrhizal plants.

B. Host plants

The choice of host plant used to propagate Glomalean fungi in pot cultures can have a large influence on fungal sporulation and mycorrhizal root formation, and consequently on resulting inoculum levels (Struble & Skipper 1988, Simpson & Daft 1990). However, the most important considerations for choosing host plants concern their tolerance to growing conditions in the glasshouse or growth chamber where they will be grown (temperature, light levels and drought stress), which must also be favourable to the mycorrhizal fungus. Grasses such as *Sorghum* species, corn (maize) and bahiagrass (*Paspalum* sp.) are often used as host plants because their extensive root systems result in more mycorrhiza formation (Struble & Skipper 1988,

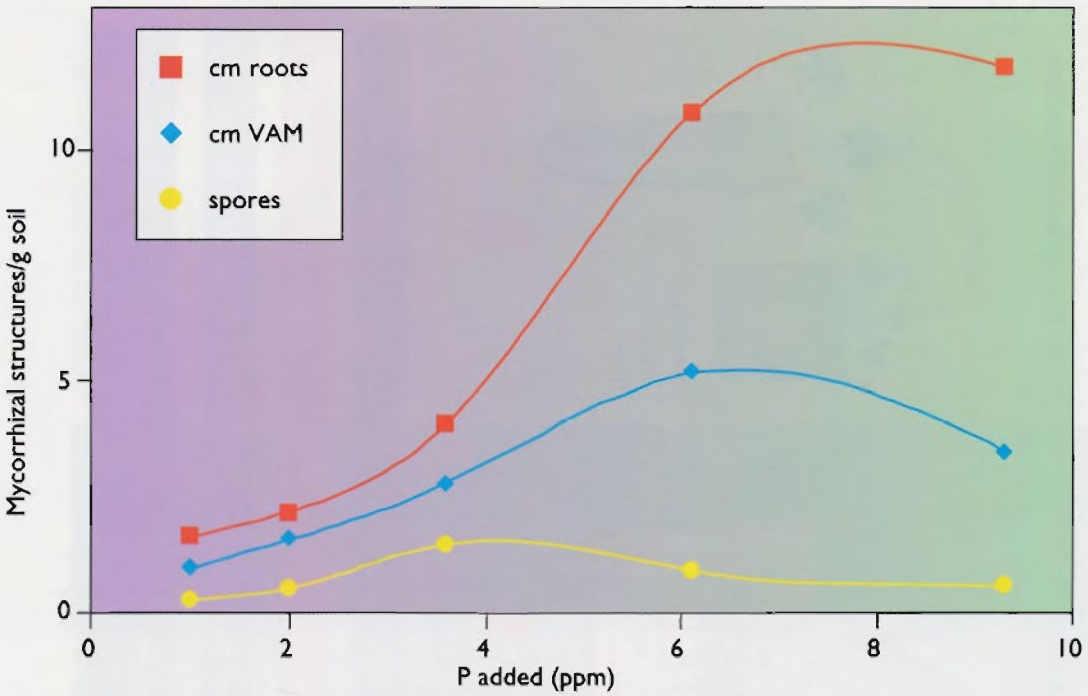


Figure 3.13. *The effect of soil phosphorus levels on root growth, mycorrhizal formation and sporulation by VAM fungi in soil-trap cultures. Sorghum plants were grown in sand using a standard nutrient solution (see text) supplemented with five levels of phosphorus (see Fig. 3.12. D).*

Simpson & Daft 1990). These tropical grasses will often provide the best results in warm environments such as summer glasshouse conditions, but temperate species such as *Trifolium* or *Allium* species may be preferable if temperatures or light levels are lower.

C. Pot culture initiation

Five different methods for starting pot cultures are outlined below and results are compared using data from a field survey in tropical Australia where all these methods were used (Brundrett et al. 1995). This comparative information is summarised in Figure 3.14. For all methods, seed of a host plant such as sorghum or clover (with rhizobium inoculum) is sown into pots with VAM fungus inoculum (Fig. 3.11). Inoculum may be in the form of spores (2–4 below), single germinated spores (5), soil (6), root samples from the field (7) or the mycorrhizal roots of transplanted seedlings (8).

1. For all the methods used to start pot cultures, steamed sand is watered to field capacity with a nutrient solution (Table 3.3) after adding the inoculum to pots. In all cases pots are maintained in a glasshouse by regular watering to weight as described in Section 3.3D below, and sampled periodically to assess fungal development as described in Section 3.3E.
2. Spores are separated from a soil sample by wet-sieving and centrifugation (Section 3.3). It is best to avoid the use of dispersants such as calgon, as these can kill spores. Fifty to 1000 g of field soil is usually required to obtain sufficient



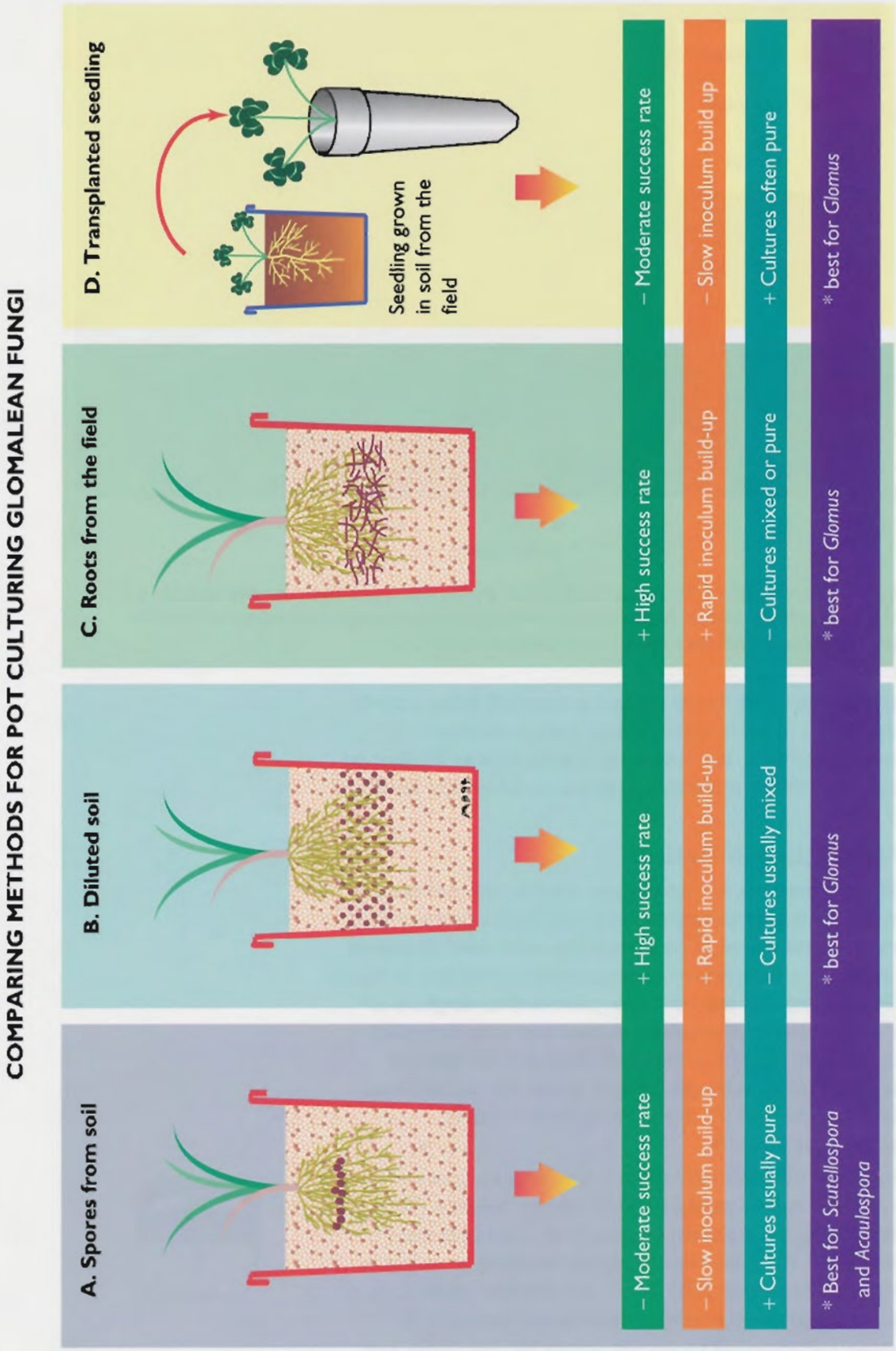


Figure 3.14. *The relative advantages and disadvantages of four methods for starting pot cultures of Glomalean fungi from field soils. Compiled from data obtained during a study of Glomalean fungi in tropical Australia (Brundrett et al. 1995).*

spores of a fungus isolate, depending on their density in a soil sample.

3. Healthy spores of uniform appearance are selected using a dissecting microscope and transferred to small filter paper triangles as described in Section 3.3B (Fig. 3.11A). Five to 100 large or 50–500 small spores are used to initiate a pot culture. Additional spores of each type should be mounted in PVLG with Melzer's reagent (Section 3.3D) to observe diagnostic features with a compound microscope.
4. Spores on the filter paper triangles are washed into a wide, 3-cm-deep hole in a pot (Fig. 3.11B). These spores are then covered with sand and watered with nutrients before host plant seeds are planted over them. This procedure is relatively time-consuming, but will often result in good single-isolate pot cultures if sufficient spores of a fungus can be found. However, this method will not work if fungi do not produce many spores, if spores have low viability, or are dormant when collected.
5. Pot cultures can also be started using single spores that have been germinated on a membrane filter square (Fig. 3.10). Healthy, vigorous spores are selected and carefully planted below a transplanted seedling of a slow-growing species (such as *Trifolium resupinatum*) in steamed sand with nutrients (Brundrett & Juniper 1995). Starting these cultures with one spore from an existing culture will ensure that only one species of fungus is present, and will greatly reduce the possibility of transferring parasitic organisms in unhealthy spores.
6. Trap pot cultures can also be started using soil samples from the field. This procedure has been used to obtain many fungi for the INVAM culture collection (Morton et al. 1993). This method can be expected to result in mixed cultures, which would have to be further purified by identifying and separating spores of different fungi. However, trap cultures generally contain many more viable spores than the field soils from which they were derived. For this procedure 100–250 g of dry soil is mixed with an equal amount of sand and then layered within more steamed sand in 1-L plastic pots (see Fig. 3.14B).
7. Pot cultures can be started using root samples collected from potential host plants as shown in Figure 3.14C. This procedure can be used to identify non-sporulating fungi from natural habitats (Liberta et al. 1983), but *Glomus* species are predominant in the resulting cultures (Brundrett et al. 1995). Root samples used can be fresh or dry, but should not have been exposed to rapid changes in temperature or humidity.
8. VAM fungi can be isolated from transplanted seedlings that have been grown in intact soil cores or soil samples from the field (Gilmore 1968). These seedlings act as bait plants and transfer active fungi in their roots when they are transplanted

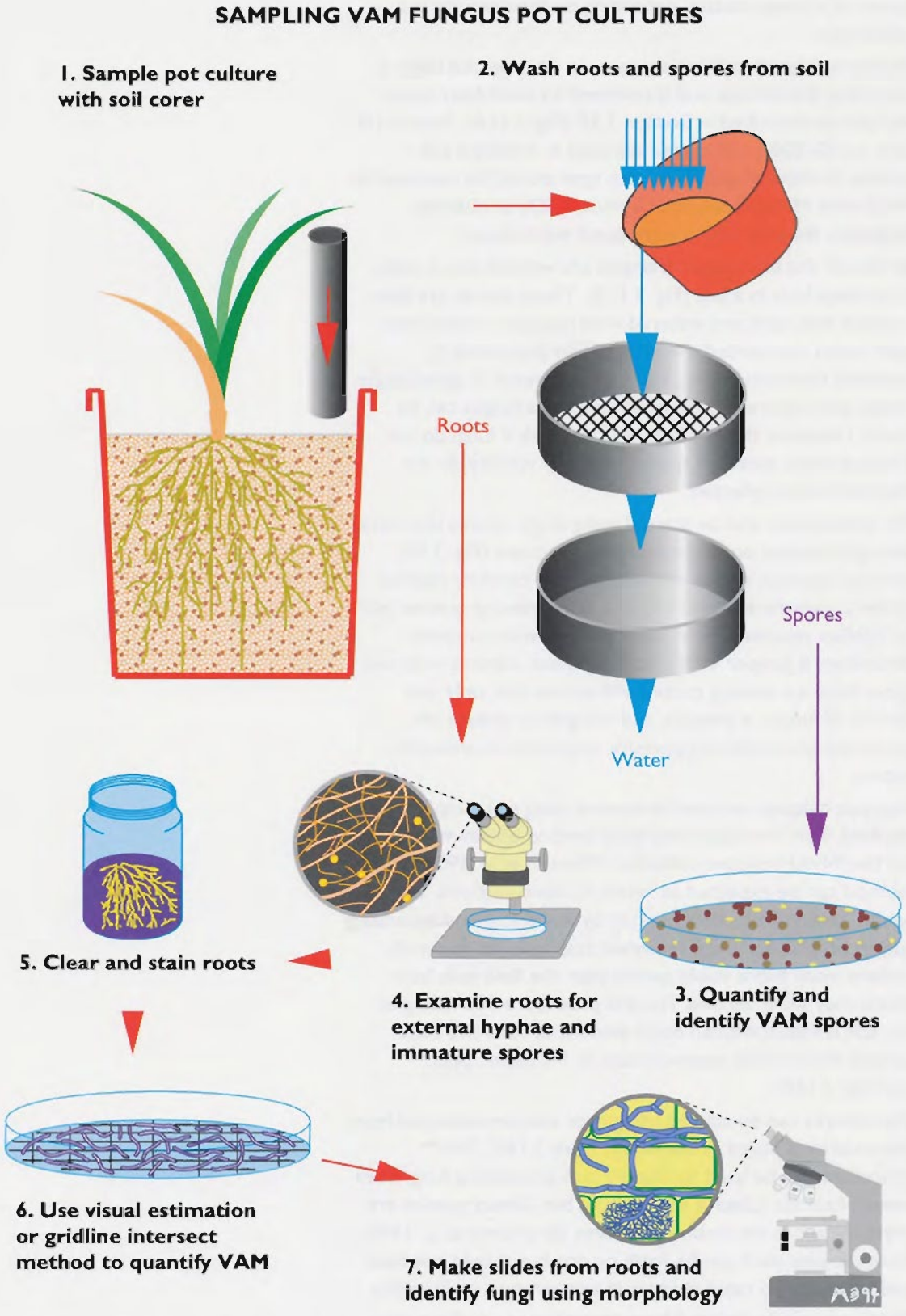


Figure 3.15. *Sampling procedures to assess the quality of Glomalean fungus pot cultures. Examination of mycorrhizal roots allows fungal development to be quantified and contaminating fungi to be detected. Numbers of mycorrhizal propagules can be estimated by counting spores and measuring the length of mycorrhizal roots.*

(M. C. Brundrett & G. Murase, unpublished data). It is important to handle seedling roots carefully, keep them moist and replant them as soon as possible. The use of a relatively slow-growing host plant and small pots (such as conetainers) is recommended to allow numerous replicate plants to be grown in a small space (Fig. 3.14D). This procedure is less successful than most others, because not all mycorrhizal fungi survive the transplant procedure. However, this method produces relatively pure cultures of *Glomus* species which could not be isolated from spores.

Table 3.3. Nutrient supplements for pot cultures grown in infertile sandy soil, using clover or sorghum as host plants.

Compound	*Added to pot (mg/kg soil)*		*Stock solution for sorghum*	
	Clover[1]	*Sorghum*[2]	*(g/L)*[3]	*No.*
KH_2PO4	36	36	10.8	A
K_2SO_4	71	150	45	A
NH_4NO_3/2 weeks		50	15	A
$CaCl_2.2H_2O$	94	150	45	B
$MgSO_4.7H_2O$	20	20	6	C
$MnSO_4.H_2O$	10	10	3	C
$ZnSO_4.7H_2O$	5	10	3	C
$CuSO_4.5H_2O$	2.1	5	1.5	C
H_3BO_3	0.8	0.8	0.24	C
$CoSO_4.7H_2O$	0.36	0.4	0.12	C
$Na_2MoO_4.2H_2O$	0.18	0.3	0.09	C

Notes:

1. P level adjusted to provide 60% of maximum clover growth, while other nutrients are optimal for a very infertile sandy soil (Gazey et al. 1992). Clover seedlings are inoculated with an appropriate strain of rhizobium so they do not require nitrogen supplements.
2. Based on the nutrition requirements of cereals grown in a very infertile sandy soil (Snowball & Robson 1984). This P level has been adjusted to limit plant growth, while promoting VAM fungus formation and sporulation. Fe EDTA (25mg/kg) may be required for some soils.
3. These nutrients are dissolved in water to form three stock solutions (A, B, C) which are further diluted into a nutrient solution (by adding 33 mL of each stock/L final volume). This solution is applied to soil by watering it to field capacity (i.e. add 100 mL solution/kg of dry sand to get 10% water content). These values will have to be adjusted for soils with other field capacities. This method of applying fertilizers works very well for sandy soils that have a limited capacity to fix nutrients. For other soils, nutrients may have to be applied in a dry form and mixed through the soil (see Section 6.2).

The maintenance of a clean dust-free glasshouse environment is essential to prevent contamination of pot cultures by foreign mycorrhizal fungi or pathogenic organisms. The use of non-draining pots is recommended. Glasshouses should be regularly cleaned by hosing them down with water, and dry floors should never be swept clean as this generates dust. Take care when using fertilizers, pesticides or soils near glasshouses as these may ruin an experiment.

D. Maintenance of pot cultures

Procedures used to care for pot cultures and the glasshouse environment where they are kept are of the upmost importance if Glomalean fungus propagation is to be successful (see below). Pot cultures can be grown in free-draining pots or non-draining buckets (which are watered to weight). It is easier to water draining pots, but water dripping from them may cause cross-contamination between isolates, so the use of non-draining pots is recommended. Non-draining pots are also required for glasshouse experiments where nutrient levels must be carefully regulated (Section 6). Non-draining pots require individual watering on a balance (to their calculated field-capacity weight) once or twice a week, while the amount of water they require can be estimated at other times. The water use of plants can be moderated by avoiding the use of excessive amounts of fertilizers, especially phosphorus. Polyethylene beads (available from a plastics manufacturer) can be added to the surface of pots (approximately 50 g) to reduce evaporation and the risk of cross-contamination. Plastic bags that allow gaseous exchange and reduce water loss can be used to enclose pot cultures to prevent contamination (Walker & Vestberg 1994).

It is very important to set quality control standards concerning spore numbers, root colonisation levels, isolate purity and contamination by other organisms in pot cultures, and check these factors using the procedures outlined here.



E. Quality control sampling of pot cultures

It is recommended that quality control samples should be taken to monitor the build-up of inoculum (colonised roots and spores) several times during the 4–8 month life of a pot culture (every 1–2 months). This sampling protocol can also be used in mycorrhizal experiments to provide information about the time-course of mycorrhizal formation without destructively harvesting entire pots.

1. Pot cultures are sampled by removing a small sample with a cylindrical (10–15 mm diameter) stainless steel corer (Fig. 3.15). It is best to take several cores from opposite sides of the pot and combine them in a sample (±30 g) which contains an appropriate amount of roots for clearing and staining procedures (Section 4.2). Tap the side of the corer to loosen soil with a stainless steel rod then use it to push out the soil.
2. *The sterility of the corer and other implements used during sampling must be maintained to avoid cross-contamination of pot cultures by mycorrhizal fungi or pathogenic organisms.* This can be done by immersing them in 70% (v/v) ethanol for several minutes and wiping them with a clean tissue before each use. Soil taken in cores can be replaced with new steamed sand so the weight of pots does not change.
3. Roots are separated from these samples by washing the soil through a coarse screen, and then cleared and stained to assess the degree of mycorrhiza formation (Section 4.2).
4. If desired, spores can simultaneously be collected on fine screens and concentrated by the wet-sieving procedure (Section 3.3). External hyphae and young spores remain

attached to roots, if they are gently washed free of sand, and can be examined under a dissecting microscope before roots are cleared (Fig. 3.15). This will provide valuable information about soil hyphae and early stages of spore formation.

5. The number and types of VAM fungi present in cultures should be established by observing characteristic features of their root colonisation patterns (Fig. 3.7) as well as spores and external hyphae. Pot cultures without any VAM should be discarded after four months, while successful cultures will require 4–8 months for adequate levels of mature spores and other propagules to form.

F. Inoculum of Glomalean fungi

Resilient propagules of VAM fungi include spores, mycorrhizal root pieces, and organic matter containing hyphae. The inoculum potential of mycorrhizal pot cultures can be tested by bioassay experiments as described in Section 4.4.

Inoculum storage



Pot cultures, which consist of sand, roots and spores, are placed in a sealed container for storage. Pot cultures are usually stored by refrigerating damp inoculum at 4°C, or by air drying inoculum, but the responses of individual fungi to these treatments may vary (Jarstfer & Sylvia 1993). At Perth, Western Australia, fungi from seasonally dry climates have been maintained in storage (at room temperature) for years after allowing pot cultures to dry gradually (by withholding water). Refrigerated, moist inoculum apparently has a shorter shelf-life. Inoculum can also be cryopreserved in liquid nitrogen for long-term storage (Morton et al. 1993).

Multiplying inoculum

New pot cultures can be started from mixed inoculum, root fragments, or spores from a previous pot culture. However, the use of carefully selected spores of uniform appearance is recommended to ensure that only one fungus is present. Pot cultures that have been restarted from bulk soil inoculum for several generations should be checked carefully to ensure they are pure.

Several cycles of pot cultures are usually required to obtain highly infective and pure inoculum of a new isolate. Pot cultures may have to be stored for up to six months to overcome dormancy requirements of some fungi (Tommerup 1992). Thus it takes several years to develop good-quality inoculum of a new VAM fungus isolate.

Using inoculum

Pot culture inoculum consisting of spores, mycorrhizal roots, etc. can be applied to seedlings grown in a nursery or broadcast in the field, using similar procedures to those used to inoculate plants with ectomycorrhizal fungi (Chapter 5). Screening criteria and procedures for mycorrhizal fungal isolates are considered in Chapter 6.

Chapter 4

EXAMINING MYCORRHIZAL ASSOCIATIONS

This section contains information on methods commonly used to sample roots and to examine their mycorrhizal associations. A general introduction to root sampling, processing and microscopy methods used to examine mycorrhizal associations is provided. Further information is available in standard botanical microtechnique and histology references such as Jensen (1962) and O'Brien & McCully (1981). The book *Practical Methods in Mycorrhiza Research* (Brundrett et al. 1994), contains additional information about microscopy techniques used to study mycorrhizas.

The study of plant roots has received much less attention than it deserves, due to the technical difficulties of studying events within the soil (Lyr & Hoffmann 1967, Harper et al. 1991). Data concerning root systems and mycorrhizal associations can be of great value in forestry and agriculture for the reasons outlined in Chapter 1. There is also a need for more information about the role of mycorrhizal associations in various plant communities (St John & Coleman 1983, Brundrett 1991). This information can be provided by sampling roots in natural ecosystems, forest plantations, agricultural situations and disturbed habitats by growing seedlings of plants in intact cores of soil from their natural habitats, or by inoculating them with particular mycorrhizal fungi. These alternative procedures were compared during a survey of mycorrhizal associations of jarrah forest plants (Brundrett & Abbott 1991). Experiments with seedlings were found to be more time-consuming (due to the time required for plant growth and mycorrhiza formation), but resulted in root samples of superior quality to those collected from the field (where it is difficult to obtain unmixed samples of fine, young roots). Root parameters that can be used to evaluate the performance of mycorrhizal and non-mycorrhizal root systems are outlined in Table 4.1.

Table 4.1. Root system information that could be used in mycorrhizal studies.

- fresh or dry root mass (g/plant)
- root length (m/plant) and density (m root/L soil)
- specific root length (m root/g root)
- branching orders and branching frequency of laterals
- root hair length (mm) and frequency (root hairs/mm root)
- growth rates (mm/day)
- phenology (seasonal changes in activity)
- life span (weeks, years) and turnover rates (m/week)
- mycorrhizal roots:
  - total length (m/plant, m/L soil)
  - proportion of root length (%)
  - biomass (% of root biomass, mg/kg soil)
- nutrient contents (Chapter 6)

Equipment

- Root washing facility
  - large sink or outdoor basin and hose for large volumes of water
  - soil collection system to prevent clogged drains
  - 2 or 4 mm screen to catch roots
  - fine sieve (<1 mm) to catch fine lateral roots.
- Fine forceps, dissecting needles, etc. to pick up roots
- Containers and preservative to store roots
  - jars with tight sealing lids
  - 50% ethanol (v/v) root preservative



4.1. MYCORRHIZAL ROOT SAMPLES

Roots samples can be obtained from the glasshouse, nursery or field (Fig. 4.1A). Separating roots from soil may be relatively difficult or easy depending on soil texture and the nature of root systems. It is generally easy to remove roots from sandy soils, but soils with a high clay or organic matter content may cause problems. *The loss of fine laterals must be avoided so that measurements of mycorrhizal formation are not biased.*

A. Washing and sampling roots

Optimal root washing procedures will vary depending on the relative importance of maintaining intact root systems, obtaining clean root samples and the time required to process numerous samples efficiently (Fig. 4.1B). Once separated from the soil, root samples can be used to obtain root length and biomass data, and the staining procedures outlined in the following sections can be used to examine mycorrhizal associations. Root weight and length data can be used to calculate root:shoot ratios, specific root length (root length:weight ratios), root production rates, etc. (Table 4.1). The presence or absence of other root features such as root hairs and root nodules, should also be noted.

Cleaning roots

1. Intact root systems, with attached hyphae (Section 3.3), can be separated from blocks of soil by immersing them in a tub or bucket of water and gently agitating them to remove most of the soil. A mechanical shaker or ultrasonic water bath can be used to further clean intact root samples.
2. Cleaner samples of fragmented roots can be obtained by vigorously washing with water from a hose over a 1–2 mm screen (to catch roots).
3. Extra care must be taken for plants with fine lateral roots, such as the ECM roots of many species, as these may easily be lost, and in some cases these contain the majority of mycorrhizal structures. It is important to wash roots carefully over a fine sieve to ensure that finest laterals are not lost.
4. After washing from soil, root samples can be kept moist in plastic bags and refrigerated (approx. 5°C) for several days if necessary. They can then be processed or preserved in 50% ethanol in tightly sealed plastic vials for transport and storage (for months or even years).

Subsampling roots

There are practical limits to the amount of roots that can be processed during clearing and staining steps, or measured during assessment procedures, so subsampling is often required. Roots can be subsampled by one of the methods listed below. However, it is best to avoid subsampling by collecting smaller samples in the first place. When sampling soil or mycorrhizal roots, the use of many small samples, instead of a few large ones, allows valuable information about spatial variability in mycorrhizal fungus activity to be obtained (Brundrett & Abbott 1995).

1. Suspend roots in a large volume of water, stir them, then randomly select volumes of water until sufficient roots have been obtained.
2. Roots can be subsampled by mixing soil and randomly selecting soil samples before separating root pieces.
3. After washing roots, they can be chopped, mixed, and random subsamples taken to make a sample of the specified weight, or volume.
4. Washed roots can be sorted into fine and coarse root categories, if only the fine roots have mycorrhizas. The coarse roots can be dried for biomass determination or nutrient analysis.

B. Glasshouse investigations of mycorrhizal relationships

Mycorrhizal associations are often studied in glasshouse or growth chamber experiments to provide some level of control of environmental conditions and reduce the risk of contamination by airborne spores. The following protocols were used to investigate the mycorrhizal associations of plants which occur in an Australian forest community (Brundrett and Abbott 1991). In this study, the growth of seedlings in forest soil, or steamed sand containing mycorrhizal inoculum, produced higher quality root samples than field sampling techniques (**C.** below) — which often produced roots which were too old to have active mycorrhizas. However, some field sampling was required to confirm the results of glasshouse studies.



1. Seeds of the species to be tested are germinated in steamed sand to provide seedlings of adequate size (after a few weeks or several months). Seedlings are then transplanted into soil collected from habitat where these species occur naturally, in a similar manner to mycorrhizal propagule bioassays (Section 4.4). Intact cores of soil are superior, because they contain intact networks of hyphae, but mixed soil samples can also be used.
2. Alternatively, seedlings can be inoculated with known mycorrhizal fungi from VAM fungus pot cultures (Chapter 3) or ECM fungi — using spores, or mycelia from sterile cultures (Chapter 5). In this type of experiment, mycorrhizal formation will demonstrate the capacity for hosts to form associations, but their failure to establish mycorrhizas will not rule out the possibility that other inoculum forms or fungal species might have been successful (because of potential problems with host specificity and inoculum efficacy).
3. After 1–2 months of growth in soil, intact seedling root systems are carefully washed free of soil cores then cleared and stained to reveal mycorrhizal associations (Section 4.2). The time required for mycorrhizal formation depends on the root growth rates of species and the quantities of mycorrhizal propagules in soil. Most plants will form VAM after 2–4 weeks, but ECM formation may require 1–3 months. Consequently, several harvest times may be required.

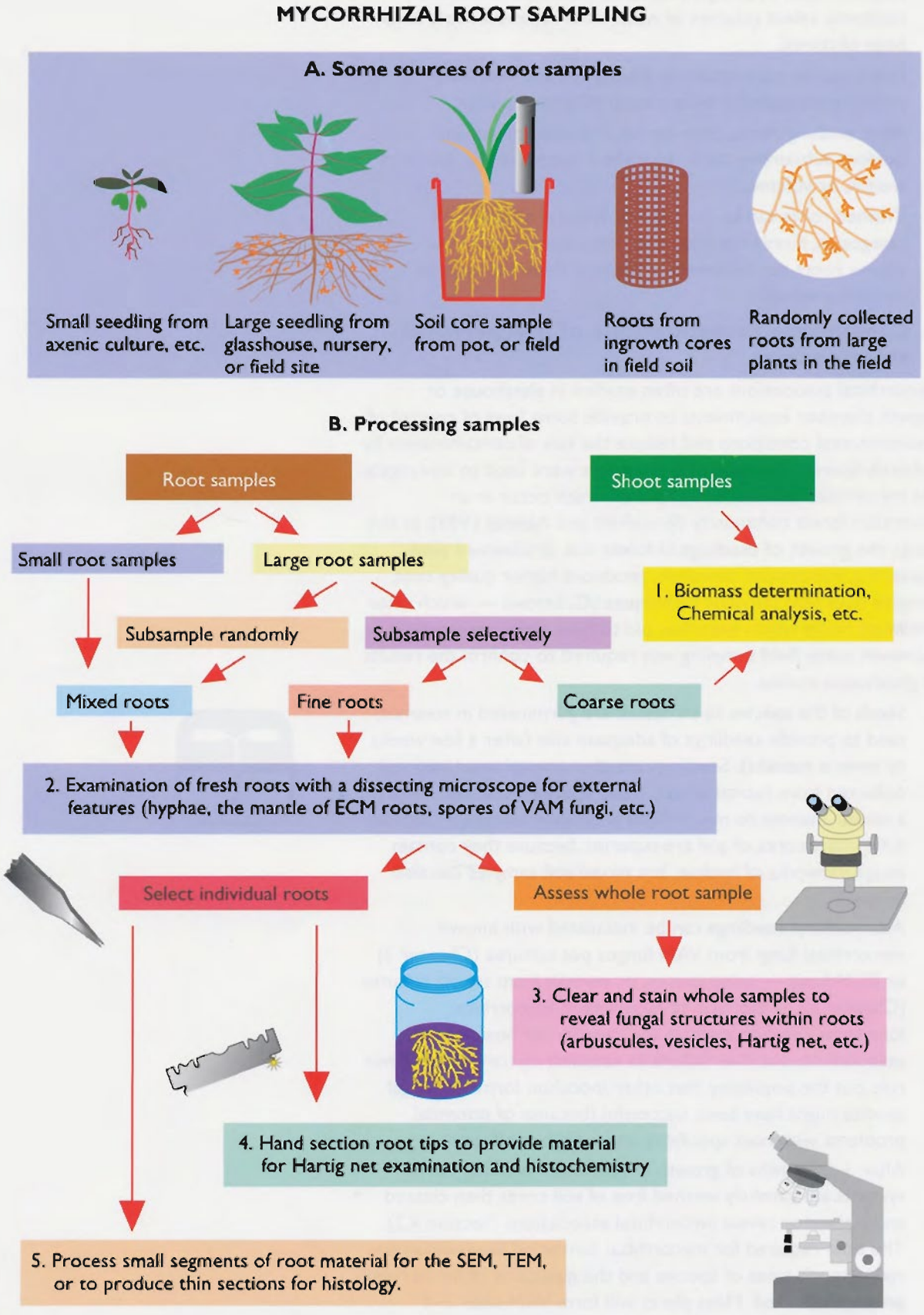


Figure 4.1. Mycorrhizal root sampling.

C. Field collections of roots

Special problems arise when mycorrhizal studies utilise roots of plants from cultivated crops, forest plantations, or natural ecosystems. *The most important consideration is that sampled roots are not contaminated by other plants and are young enough to have mycorrhizas.* It is also important to examine sufficient quantities of roots (from different individuals of a species) and to use rigorously applied definitions of mycorrhizal association types (see Chapter 1).



1. Root systems of target plant species are excavated taking care to ensure that fine roots are well represented in samples and to exclude entangled roots of other species, if these can be recognised. When excavating small plants, it is best to use roots which are attached to the base of a plant, but this is usually not possible for shrubs and trees.
2. Samples of different individuals of a plant species should be taken to determine if there is variation in the consistency and degree of mycorrhizal fungus colonisation of their roots between or within sample sites. Separate samples taken from different habitats or locations should be used if possible.
3. Plants should be accurately identified to the species level using a standard taxonomic reference (regional flora). It is important to state in publications which reference books were used. Herbarium voucher specimens should be collected if there is any doubt about plant identifications.
4. Samples are cleaned (Section 4.1A), cleared and stained using standard procedures (Section 4.2), mycorrhizal colonisation quantified and types of associations established by careful microscopic examination (Section 4.3).
5. Even carefully collected root samples will often contain roots of more than one species. Contaminating roots can usually be identified by differences in appearance (due to colour, size, structural features, etc.) and should be ignored when root samples are examined. This task becomes easier once you have gained some experience observing the roots of common plants in a community.
6. It is recommended that species be examined at different times of the year to find active associations with arbuscules and observe seasonal fluctuations in colonisation levels. In seasonal climates there may be certain times of the year (such as the start of the wet season, or early spring) when root growth commences and mycorrhizal associations will likely become active (Lyr & Hoffmann 1967, Brundrett & Kendrick 1988).

D. Other methods of studying roots

In situ observations of roots

Valuable information about the development of mycorrhizal associations can be obtained by observing the growth of living roots in soil. Roots growing in the field can be observed growing in soil contained in glass-walled root chambers, under glass panels placed on the soil surface and covered with a layer of insulating material, or from within rhizotrons that can be excavated, to observe events in the field (Skinner & Bowen 1974, Reddell & Malajczuk 1984, Mackie-Dawson & Atkinson 1991, Egli & Kälin 1991). Figures 1.6A and 4.6A show examples of ECM roots of eucalypts formed in forest soil in observation chambers.

Ingrowth soil cores

These are mesh cylinders filled with soil which has been sieved to remove existing roots, which are used to measure root production and turnover rates (Persson 1983, Graham et al. 1991). Ingrowth cores are buried in topsoil in the field near plants which are being studied, and excavated at various time intervals to obtain root samples of a known maximum age (Fig. 7.15G). These cores can be made by forming coarse, sturdy mesh (2–5 mm) into cylinders (the plastic mesh used to cover house rain gutters is suitable).

Taking small cores from pot experiments

The removal of small (1–1.5 cm diameter) cores from pots in glasshouse experiments allows mycorrhizal formation to be examined without harvesting plants. This procedure is described in Section 3.4E and illustrated in Figure 3.15. When working with different fungi, great care must be taken to prevent cross-contamination by the transfer of fungal spores or hyphae between pots. Periodic sampling using soil cores allows the time-course of root and mycorrhiza development to be studied without substantial damage to root systems. Several cores that extend the full depth of a pot should be taken and the final volume or roots should be small enough to be processed without subsampling. These cores should contain sufficient roots to quantify mycorrhizal activity. The resulting holes can be filled with fresh sterile soil and their location marked to prevent resampling the same spot.

4.2. CLEARING AND STAINING MYCORRHIZAL ROOTS

Structures produced by VAM fungi are often not visible when fresh roots are observed, because internal structures are obscured by the natural pigments and cell contents within roots. While whole ECM roots can often be identified by observation with a dissecting microscope, internal details of these associations are revealed by removing pigments in root cells and mantle hyphae. Clearing procedures, which use chemical agents to remove cell contents and cell wall pigments, are a valuable method for viewing internal features in plant tissues (Gardner 1975). Fungal structures in plant tissues can be observed by the use of stains which bind to fungal hyphae without much background staining of the cleared plant material. Trypan blue or Chlorazol black E (CBE) in lactoglycerol are generally used to stain mycorrhizal structures in roots that have been cleared by heating in KOH (Bevege 1968, Phillips & Hayman 1970, Kormanik & McGraw 1982, Brundrett et al. 1984). This procedure is outlined in Figure 4.2.

Root samples

Clearing and staining procedures require root samples that have been washed free of soil (Section 4.1). It is imperative that KOH or staining solution volumes are sufficient for the amount of roots being processed and that roots are not tightly clumped together — for uniform contact with solutions. It is often best to chop roots into 2–4 cm long segments before clearing them. It may be necessary to sub-divide or sub-sample large volumes of roots to obtain good results. The fine roots of woody plants can also be separated from coarse roots, after determining their proportion of the total root system.

A. Clearing roots with KOH

1. KOH 10% w/v is normally used to clear roots. Clearing procedures generally do not work well with root samples that are more than 1–2 g, otherwise a subsample of roots should be used. Many published micrographs of VAM show roots that were insufficiently cleared, perhaps due to low temperatures, short times, or large samples. Roots that are insufficiently cleared will still have cell contents which obscure mycorrhizal structures, while over-cleared roots may disintegrate.
2. Root clearing is fastest in an autoclave, which provides an efficient means of processing samples. An autoclave liquids cycle of 15–20 minutes at 121°C is sufficient for most roots. Samples containing old roots, roots with abundant phenolics, or field-collected roots often require longer clearing times (25–60 minutes). Samples should be in autoclave-resistant glass containers that are less than one-third full, or they may overflow in the autoclave. Wide containers work better than tall narrow tubes which may overflow.
3. Roots can also be cleared in a water bath by heating the KOH to 60–90°C. The time required for adequate clearing with

Safety warnings!!!

1. *10% w/v **KOH** is used to clear roots. **Care should be taken to avoid skin contact with this caustic chemical !** Use gloves, safety glasses, etc.*
2. *The toxic chemical **phenol**, which has traditionally been included in some stain preparations, **should not be used!** — its omission will have little influence on staining quality.*
3. ***Chlorazol black E and trypan blue are suspected to be carcinogens** (as are many other biological stains — Coombes & Haveland-Smith 1982)! Use gloves to protect your hands when using stain solutions and take care to avoid breathing dust, or getting any in your eyes, when handling dye powders.*
4. ***Formalin-based preservatives** are bad for your health. Preservation of plant samples with 50% ethanol works as well and is much safer.*
5. *Refer to safety data sheets for the latest information on the chemicals you use.*



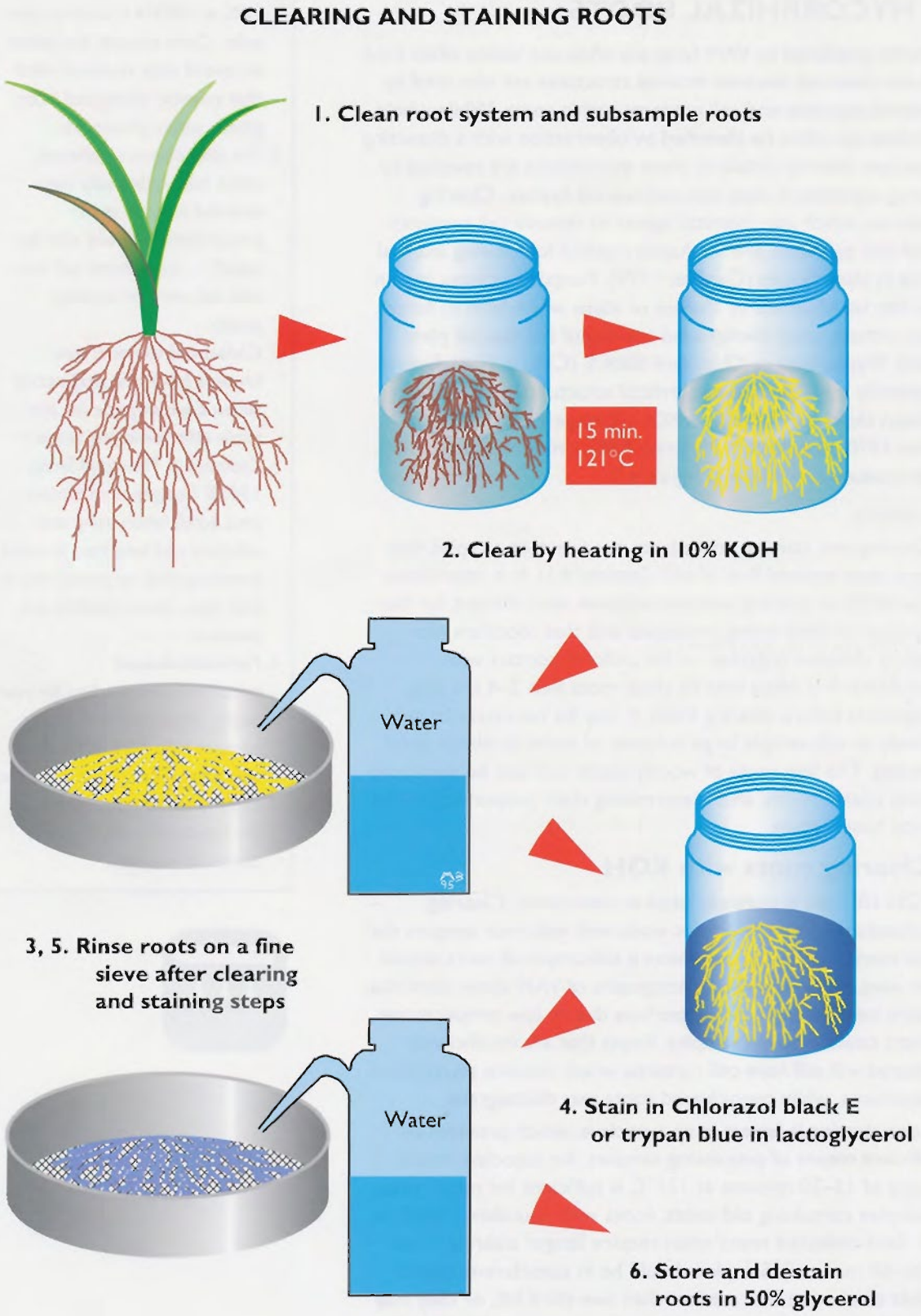


Figure 4.2. Root clearing and staining.

this method varies widely — roots from young plants will usually only require 2–4 hours, but some samples of older plants, field-collected roots, or heavily pigmented roots such as those belonging to many trees or shrubs require much longer to be cleared (from 5 hours to several days). One hour of clearing at 60°C is approximately equivalent to 5 minutes in an autoclave at 121°C.

4. Cleared roots are captured on a fine sieve and rinsed with water before transferring them into the staining solution (Fig. 4.2).

B. Staining roots with Chlorazol black E (CBE) or trypan blue

1. Cleared roots are stained with CBE in a lactoglycerol solution (Brundrett et al. 1984). The optimum stain concentration will depend on the dye source and microscope procedures used. CBE of 0.03% w/v is best in most cases, but a higher concentration may enhance morphological observations, while a lower concentration may be best for low-magnification assessment of roots. It is best to try a range of concentrations (0.1%, 0.03%, 0.01%) when using this procedure with a new type of roots, or when using a new source of dye.
2. Roots are stained by heating for several hours at 90°C, or 15 minutes in an autoclave using a liquids cycle at 121°C, or by leaving them in the solution for several days. The staining solution may be reused several times if filtered through folded cheesecloth or 50 µm nylon screen after each use (to remove root fragments). Staining solutions become translucent when too weak for further use.
3. Roots can also be stained with trypan blue (Bevege 1968, Phillips & Hayman 1970, Kormanik & McGraw 1982). A concentration of 0.05% w/v in lactoglycerol is often used to stain cleared roots as described above. Trypan blue staining is not recommended for studies involving morphological observations or photomicroscopy, because the resulting images have lower contrast than those resulting from CBE staining (Brundrett et al. 1994). However, trypan blue staining is adequate for assessment at low magnifications, where colour contrast may be beneficial. Acid fuchsin and cotton blue can also be used to stain fungi in roots, but destain rapidly and produce low-contrast images (Brundrett et al. 1984).

C. Working with darkly pigmented roots

1. Prolonged clearing times with KOH will often remove more phenolic pigments from roots, but may not be appropriate if root samples tend to disintegrate or contain wall-bound secondary metabolites which are resistant to clearing.
2. Post-clearing bleaching with alkaline hydrogen peroxide (0.5% NH_4OH and 0.5% H_2O_2 v/v in water) effectively removes any phenolic compounds left in cleared roots (Bevege 1968,

Equipment and reagents

- 50% ethanol (v/v) root preservative
- 10% KOH (w/v potassium hydroxide) dissolved in water. *This is an exothermic reaction — use a heat resistant container!*
- 0.03% w/v Chlorazol black E in (CBE) in lactoglycerol (1:1:1 lactic acid, glycerol and water). Dissolve CBE in water before adding equal volumes of lactic acid and glycerol
- 0.05% w/v trypan blue in lactoglycerol (1:1:1 lactic acid, glycerol and water)
- 50% glycerol–water (v/v) solution for destaining and storage of stained roots
- food grade lactic acid and glycerol are adequate and can be purchased in bulk
- autoclave (121°C), water bath, or oven (60–90°C) to heat root samples for clearing and staining
- autoclave-resistant glass jars or tubes to hold samples
- fine nylon mesh screen (±100 µm) to prevent root loss when changing solutions
- fine forceps and dissecting needles to transfer roots
- plastic vials with tight-sealing lids for storage of stained samples in 50% glycerol

Kormanik & McGraw 1982). The time required for roots to discolour in this solution varies between samples. This procedure should be used cautiously because subsequent staining of mycorrhizal fungus hyphae will be reduced and may even be eliminated. Hydrogen peroxide bleaching of cleared roots is particularly useful for revealing the Hartig net in whole ECM roots (Nylund et al. 1982, Brundrett et al. 1990).

3. CBE and trypan blue stain lignified or suberized cell walls in roots, especially xylem, endodermal and exodermal cells, as well as fungal hyphae. Staining of modified plant cell walls results from the nature of cell wall components, and cannot be completely eliminated by clearing or bleaching steps. Staining of these structures can conceal mycorrhizal details in some roots unless they are squashed under a cover slip (Brundrett & Kendrick 1988).

D. Alternative methods

1. Vital staining procedures that measure succinate dehydrogenase activity can be used to confirm that mycorrhizal fungus hyphae which are enumerated are metabolically active (Schaffer & Peterson 1993, Tisserant et al. 1993). However, the assessment of arbuscular colonisation levels should provide similar information (see 4.3).
2. Modifications to standard clearing and staining procedures have been proposed for safety reasons. Grace and Stribley (1991) suggest that methyl blue or aniline blue can be used as less toxic replacements for Chlorazol black E or trypan blue. However, there is insufficient evidence to confirm that these other dyes are non-toxic — so they must also be handled carefully. A lower concentration of KOH (2.5%) can be used to reduce the risk of injury (Koske & Gemma 1989), but this may not be feasible with roots that are hard to clear.
3. The stain acid fuchsin can be used, in combination with fluorescence microscopy, to stain fungal structures in roots (Merryweather & Fitter 1991), but this requires specialised microscope equipment.

E. Sample storage and slide preparation

1. Staining quality is substantially improved by destaining roots in 50% glycerol for several days prior to observation to allow excess stain to leach from roots.
2. Roots stained with CBE can be stored in 50% glycerol, but trypan blue staining is less permanent, unless samples are stored in lactoglycerol or kept in the staining solution.
3. Semi-permanent slides of stained roots can be made with PVLG mountant (Section 3.2), or a gum arabic based mounting media (Cunningham 1972). A slide mounting procedure is shown in Figure 4.4.
4. Interference-contrast microscopy substantially enhances the contrast of stained fungal structures, especially when photographs are taken (Brundrett et al. 1984).

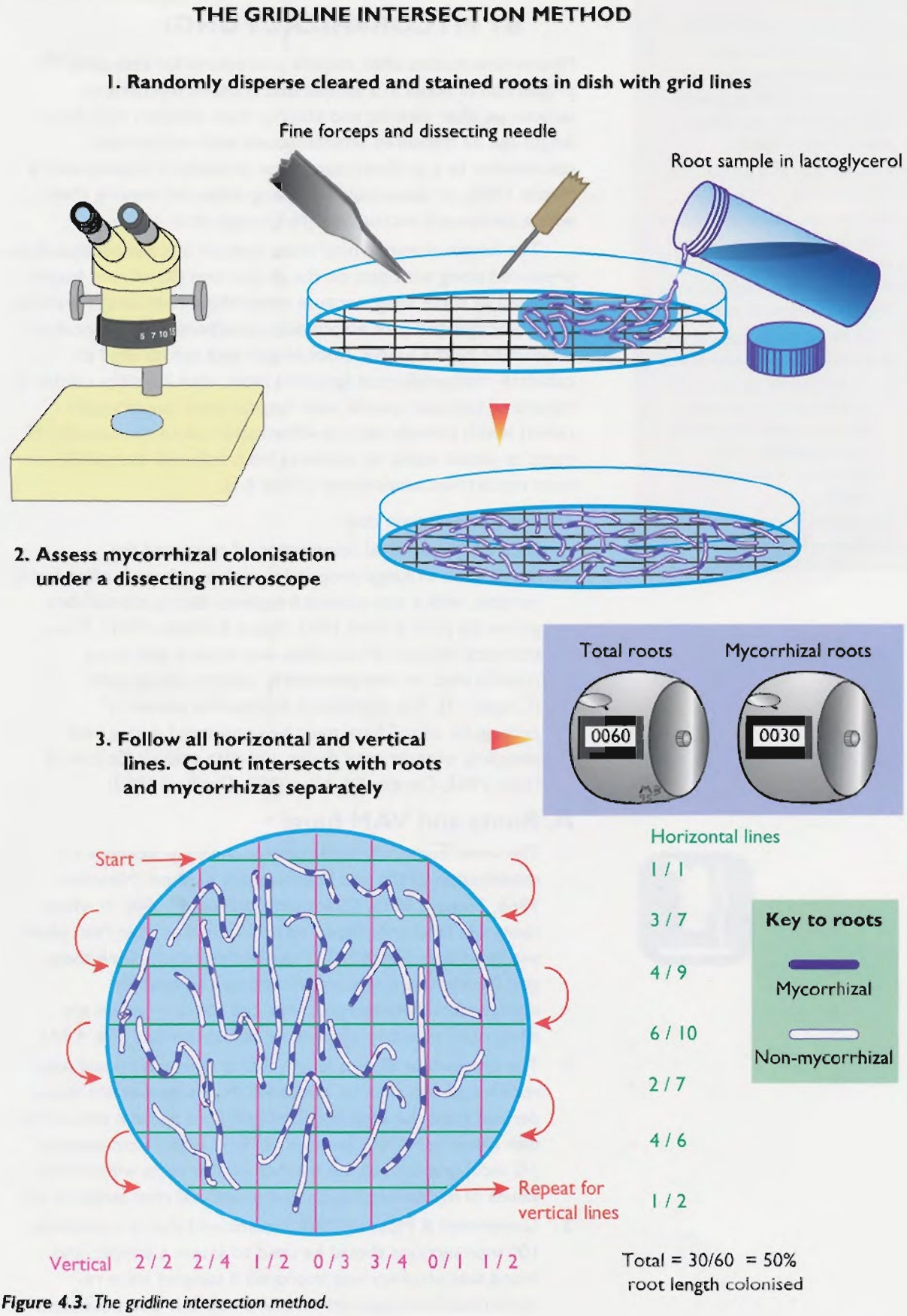


Figure 4.3. *The gridline intersection method.*

Equipment

- Clear plastic dishes with inscribed grid lines to measure colonisation
- Fine screen (100 µm) with nylon mesh for transferring roots from solutions
- Fine forceps and probes for manipulating roots
- Microscope slides, long cover slips and PVLG mountant (Section 3.3)
- Plastic vials with tight-sealing lids for storage of samples
- Dissecting microscope with a transmitted light illumination
  - a clear plastic panel over the microscope base is recommended to provide a stable platform and protection from spilled liquids
- Compound microscope with an eyepiece cross-hair

4.3. MEASURING ROOT COLONISATION BY MYCORRHIZAL FUNGI

Mycorrhizal studies often require procedures for estimating the proportion of roots in a sample that contains mycorrhizal structures, after clearing and staining them (Section 4.2). Root length can be measured simultaneously with mycorrhizal colonisation by a gridline intersection procedure (Giovannetti & Mosse 1980), or separately by making slides and viewing them with a compound microscope (McGonigle et al. 1990).

The length of mycorrhizal roots present in a sample should be presented along with data on the proportion (%) of root length occupied by these fungi, because mycorrhizal root length is more directly correlated with association costs/benefits and inoculum production by the fungus. Root-length data can be used to calculate root production (growth) rates, root densities (within a volume of soil) and specific root lengths (root length:weight ratios) which provide valuable information about the capacity of roots to obtain water or nutrients from soils and their ability to form mycorrhizal associations (Table 4.1).

Analysis of colonisation data

Data on mycorrhizal colonisation of roots and the distribution of fungal propagules such as spores is often highly variable, with a non-normal frequency distribution of data points (St John & Hunt 1983, Friese & Koske 1991). Thus statistical analysis of such data may require data to be transformed, or non-parametric statistics to be used (Chapter 7). The aggregated distribution pattern of propagules of soil fungi must be considered during field sampling, experimental design and data analysis (St John & Hunt 1983, Campbell & Noe 1985, Dutilleul 1993).

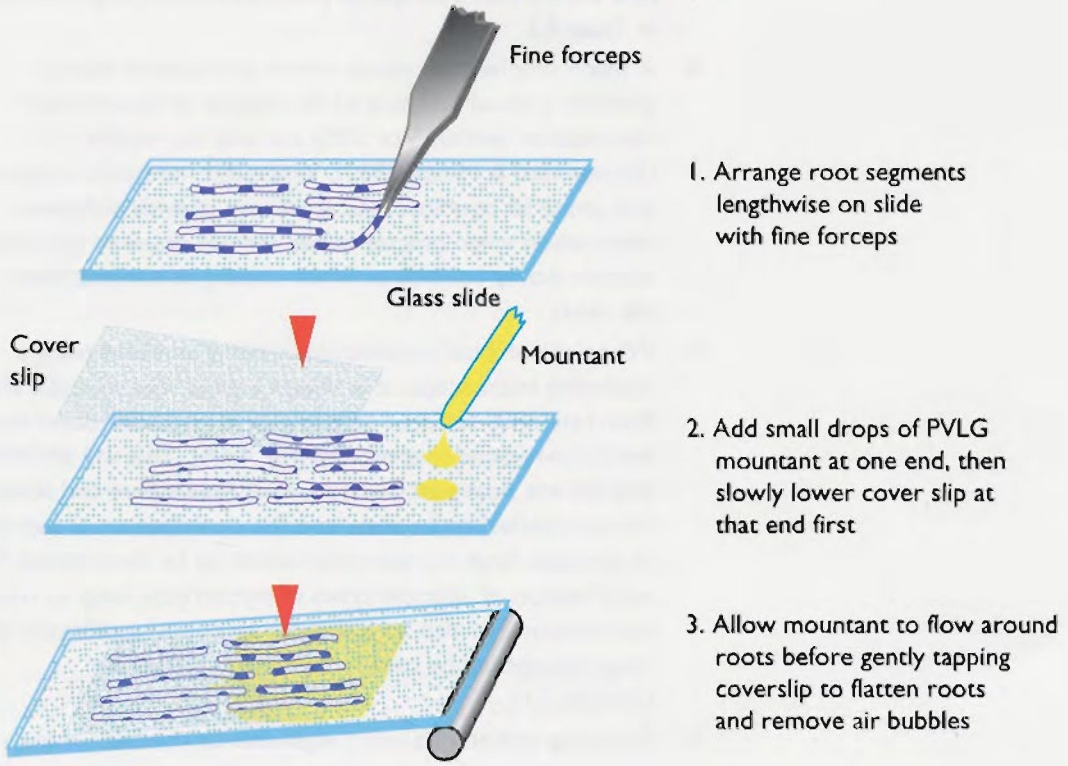
A. Roots and VAM fungi



1. The most frequently used root measuring procedure is a modification of the grid line intersect method (Newman 1966, Tennant 1975, Giovannetti & Mosse 1980), in which roots are randomly dispersed in a 9-cm diameter Petri plate with grid lines (Fig. 4.3A). The observer scans along these grid lines with a dissecting microscope to quantify intersections between grid lines and roots — which are designated as either *colonised* or *non-mycorrhizal* (Fig. 4.3A).
2. The proportion of root length that is mycorrhizal and total root length can then be calculated from a conversion factor derived from the total length of grid lines and the area of the dish (Newman 1966, Tennant 1975). If a 14/11 cm (approx. 1/2 inch) grid is used the number of intersects will provide values of mycorrhizal and non-mycorrhizal root length in cm.
3. Giovannetti & Mosse (1980) recommend that at a minimum 100 intersections should be used to assess a sample, and found that accuracy was improved if samples were re-randomised and counted several times. It is also possible to

MICROSCOPIC EXAMINATION OF ROOTS

A. Mounting roots on slides



1. Arrange root segments lengthwise on slide with fine forceps

2. Add small drops of PVLG mountant at one end, then slowly lower cover slip at that end first

3. Allow mountant to flow around roots before gently tapping coverslip to flatten roots and remove air bubbles

B. Assessing mycorrhizas mounted on slides

Randomly selected microscope field of view and cross-hair positions

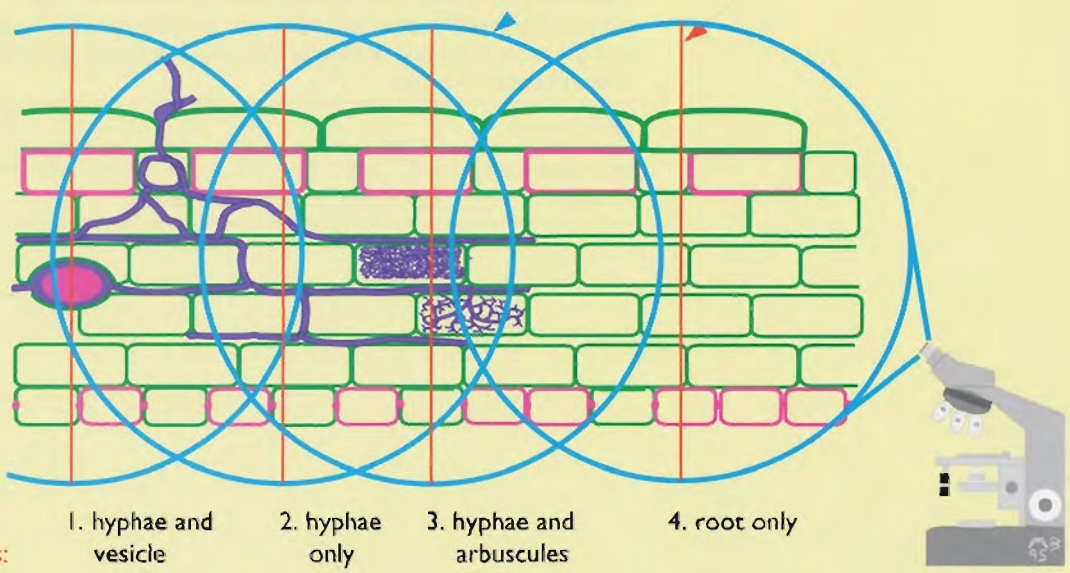


Figure 4.4. Microscopic examination of roots.

use this method with larger circular or rectangular dishes. However, with rectangular dishes care must be taken to ensure that the number of vertical and horizontal lines are fully representative of the area of the dish. It is best to test new dishes with a sample of precisely known length, as shown in Table 4.2.

4. A much simpler procedure, where an observer simply provides a visual estimate of the degree of mycorrhizal colonisation (within 5 or 10%) can also be reliable (Giovannetti & Mosse 1980). While this method is subjective and prone to operator bias, it still can provide sufficient information when precise values are not required (for pot culture quality control, or when looking at samples from the field).
5. When mycorrhizal colonisation is being assessed using a dissecting microscope, it is always a good idea to make slides from randomly selected subsamples of roots for observation with a compound microscope (Fig. 4.4A). This will allow fungi that do not stain well (such as many *Acaulospora* and some *Glomus* species) to be seen, and the contribution of saprobic or parasitic fungi to root colonisation to be determined. The contribution of different types of mycorrhizal fungi to root colonisation can also be estimated by counting different VAM fungi (recognised by root morphology) separately (Section 3.1).
6. Assessing mycorrhizal root segments can be done using a compound microscope with an eye-piece cross-hair which is moved to randomly selected positions (McGonigle et al. 1990). This allows the length of arbuscules, vesicles and internal hyphae within roots to be separately determined (Fig. 4.4B).

Table 4.2. Gridline intersection method example using an 8.5 cm-diameter round Petri dish with a 1/2 inch (14/11 cm) grid and a 1-m test sample of thread cut into fragments and randomly redistributed 10 times.

Redistribution	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*
Intersects (cm)	102	107	91	98	92	114	108	99	104	94
Average	100.9 cm ± 2.5 (standard error)									

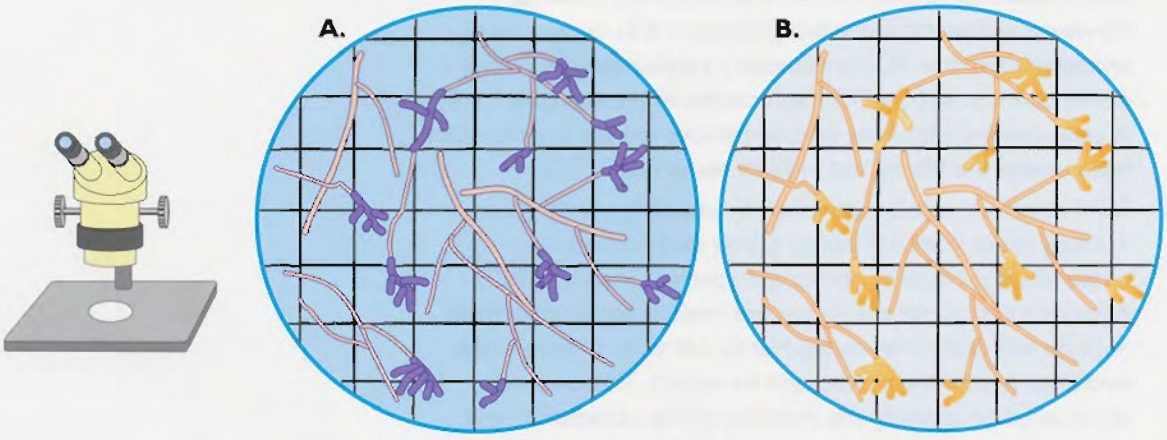
B. Quantifying ectomycorrhizal associations



1. A variety of methods has been used to quantify ECM roots (Grand & Harvey 1982). Unstained ECM roots can usually be distinguished from non-mycorrhizal roots by differences in their colour, thickness, texture and branching patterns. However, a clearing and staining (Section 4.2) or sectioning procedure (Section 4.5) is necessary to visualise the Hartig net to confirm that an ECM association is present (see 1.6 B). A post-clearing bleaching step to remove excess tannins often helps reveal the Hartig net in ECM roots (see 4.2C).
2. ECM roots are usually quantified by sampling seedlings, or washing roots from soil cores, taking care to exclude contaminating roots of non-target species (Section 4.2C). Assuming that roots are young and healthy, each mycorrhizal root tip will contain an active Hartig net zone (where active exchange processes are thought to occur). These tips can be counted to quantify the intensity of the association, and their numbers should be expressed relative to root length and soil volume.
3. The root length of a sample can be measured with the gridline intersect method, while either simultaneously measuring the length of mycorrhizal roots or separately counting the total number of mycorrhizal tips (Fig. 4.5).
4. For some mycorrhizas, the intensity of branching within a mycorrhizal cluster varies considerably (Fig. 4.5), and can be quantified with a branching density index that was developed for pine roots (Marx 1969). However, branching intensity will be taken into account by the gridline intersection method if ECM root tips are counted.
5. Ectomycorrhizal association of eucalypts may be more difficult to recognise than those typical of conifer roots, in cases where there are minimal changes to the host root (see 1.6 B). This occurs when mycorrhizal colonisation results in minimal root swelling, limited root branching, a shallow Hartig net, and a thin 'superficial' mantle. These associations can be recognised with practice, but require more careful examination of roots.

A. Using the gridline intersection method to count ECM roots

1. Measure root length of stained (A) or unstained (B) roots with a dissecting microscope using the gridline intersect method



2. Count the length of mycorrhizal roots or the number of mycorrhizal root tips



Results: 65 = Root length (cm)
24 = Mycorrhizal root length (cm)
36 = Mycorrhizal root tips

B. Classifying ECM roots by branching patterns and appearance

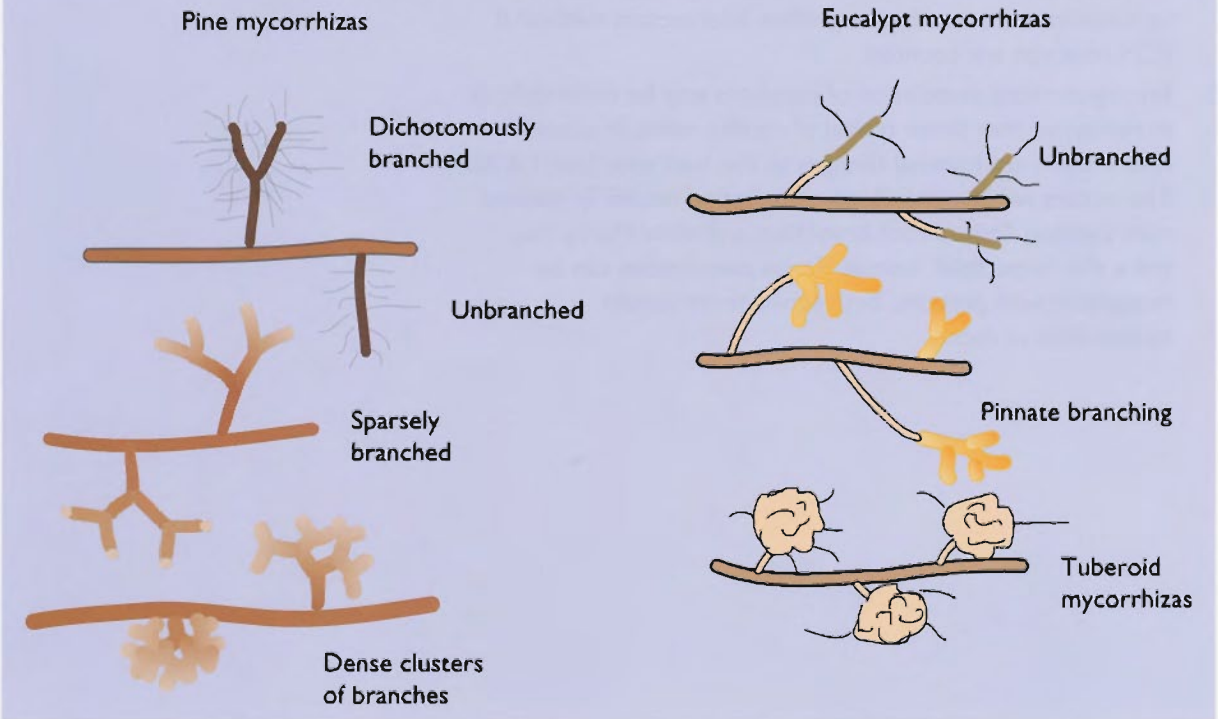


Figure 4.5. **A.** *Using the gridline intersection method to count ECM roots,* **B.** *Classifying ECM roots by branching patterns and appearance.*

C. Identifying ectomycorrhizal fungi

Characteristic features of fresh samples of ECM root tips can be used to identify particular ECM fungi (Figs 4.6, 4.7). Some of these characteristics are outlined in Table 4.3, and include features which are also used to identify fungal fruiting bodies (Chapter 2). Many features in Table 4.3 can be observed with a dissecting microscope, but more information can be obtained by sectioning roots, using procedures described in the following sections. Atlases illustrating ECM root types which are associated with particular fungi in some habitats have been produced (Agerer 1986, Ingleby et al. 1990, Haug & Pritsch 1992). Before mycorrhizal fungi can be recognised on roots from the field, they must be characterised by observations of roots which are colonised by known ECM fungi as described below.



1. Roots can be obtained from synthesis experiments by using inoculum of a particular fungus (Chapter 5) and excluding other fungi by pasteurising soil (Chapter 6). Examples of mycorrhizal roots from syn-thesis experiments are shown in Figures 4.6 and 4.7.
2. Alternatively, roots can be collected from under a particular fungal sporocarp in the field as described by Agerer (1986, 1991). First, an undisturbed block of soil (up to 10 cm^3) is excavated from under a sporocarp and transported back to the laboratory. Next, a dissecting microscope is used to trace hyphal connections between that sporocarp and mycorrhizal roots presumed to be formed by that fungus. A voucher collection of fruit bodies of fungi should also be made and their identity confirmed by microscopic investigations (Chapter 2).
3. A uniform collection of ECM roots can be characterised using anatomical details (Table 4.3) which can be photographically recorded (Ingleby et al. 1990, Agerer 1991, Haug & Pritsch 1992). External features are observed with a dissecting microscope, while internal features are documented with a compound microscope by progressively squashing roots or by making thin longitudinal sections through the inner and outer mantle and the Hartig net layers (Ingleby et al. 1990, Agerer 1991).

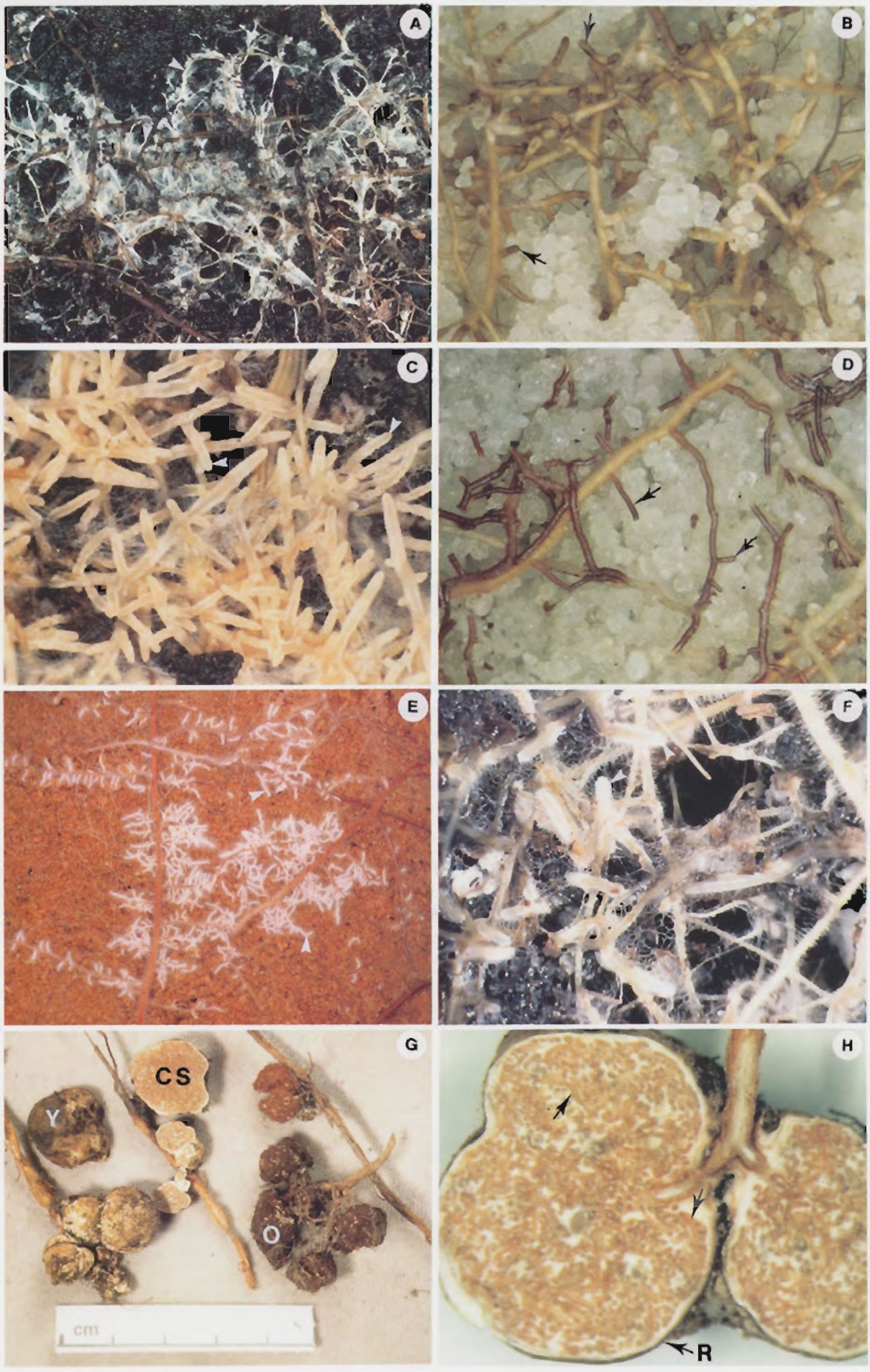

A
B
C
D
E
F
G
CS
Y
O
cm
H
R

Figure 4.6. *Examples of ECM root systems of eucalypt species.*

A. *Roots of* Eucalyptus marginata *growing in forest soil amended with burnt litter in a root chamber. Abundant extramatrical mycelium of a* Hysterangium *species can be seen (arrows).*

B–F. *In situ examples of synthesised ECM associations of eucalypt seedlings grown in the glasshouse (B,D,E), or nursery (C,F).*

B. Pisolithus *sp. and* E. diversicolor *in white sand.*

C. Laccaria laccata *and* E. globulus *in a potting mix.*

D. Setchelliogaster *sp. and* E. globulus *in white sand.*

E. Scleroderma *sp. and* E. globulus *in yellow sand.*

F. Laccaria fraterna *and* E. regnans *in a potting mix.*

G, H. *Tuberculate ECM of* Eucalyptus pilularis *collected in Queensland.*

G. *Young (Y) and old (O) tuberculate ECM attached to woody roots.*

H. *Closeup of tuberculate mycorrhizas showing numerous ECM roots (arrows) encased with an outer rind (R).*

Table 4.3. Diagnostic features of ectomycorrhizal roots that may be associated with particular mycorrhizal fungi.

Mycorrhizal development
- root branching patterns and density
- root thickening
- reduction in root elongation

External hyphae
- presence, abundance, distribution
- arrangement – single, strands, rhizomorphs
- structure – colour, wall, thickness, clamp connections, crystals
- sclerotia

Mantle
- thickness
- colour, changes with bruising, or age
- hyphal organisation in surface and in deeper layers
  - loose or compact hyphae or pseudo-parenchyma, prosenchyma
- hyphae – thickness, wall structure, clamp connections, septae
- cystidia, crystals, exudates
- reactions to chemicals (Melzer's reagent, KOH, etc.)
- staining reactions
- odour
- fluorescence

Hartig net
- presence, thickness
- hyphal organisation
- hyphal structure

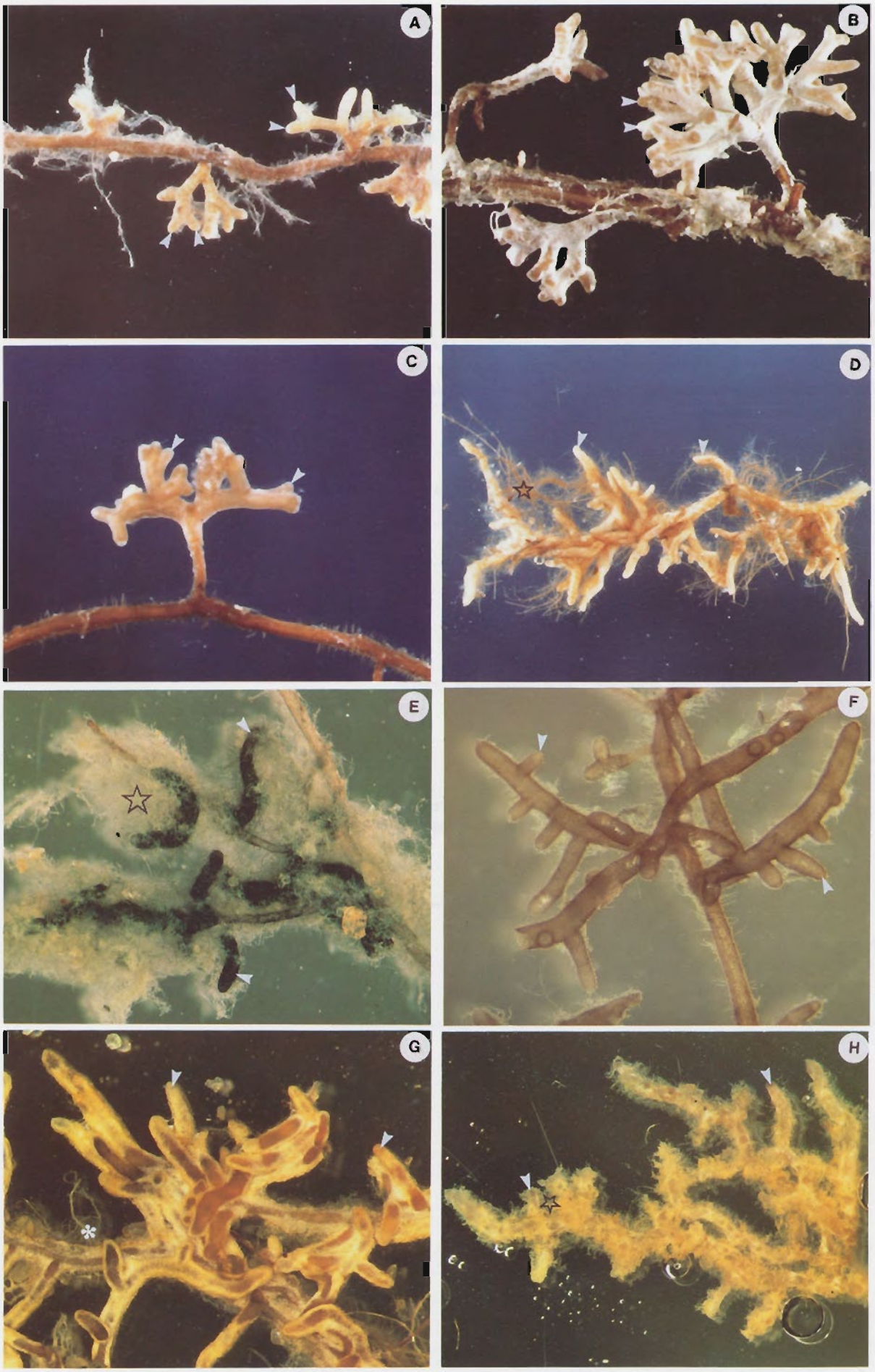

A
B
C
D
E
F
G
H

Figure 4.7. Ectomycorrhizal roots washed from soil, illustrating characteristic features of different inoculated fungi.

A–C. Pinus radiata *roots with* Suillus brevipes *(A),* Amanita mascaria *(B) and* Boletus edulis *(C). Dichotomously branched root tips are shown (arrows).*

D. Eucalyptus maculata *and* Astraeus pteridis *association with numerous mycelial strands (star).*

E. Tylopilus *sp. and* E. urophylla *association with black roots (arrows) and abundant soil mycelium (star).*

F. Reddellomyces *sp. and* E. grandis *association with shiny brown roots (arrows).*

G. Pisolithus tinctorius *and* E. urophylla *with yellow-orange roots (arrows) and mycelial strands (asterisk).*

H. Amanita *sp. and* E. urophylla *with yellow roots (arrows) covered with a dense covering of short hyphae (star).*

4.4. BIOASSAY MEASUREMENTS OF MYCORRHIZAL INOCULUM IN SOILS

Propagules of vesicular-arbuscular mycorrhizal (VAM) fungi are thought to include spores, dead root fragments and other colonised organic material, as well as networks of hyphae in soil (Brundrett 1991). Hyphal networks are considered to be especially important in natural ecosystems, where they can survive drought conditions, but are more sensitive than other propagules to soil disturbance (Jasper et al. 1989, 1991). Propagules of ectomycorrhizal (ECM) fungi include networks of mycelial strands, old mycorrhizal roots, sclerotia and spores (Skinner & Bowen 1974, Ba et al. 1991).

Inoculum potential is defined as the energy for growth of an organism at the surface of its host, and is a consequence of the numbers of active propagules of that organism and their nutritional status (Garrett 1956). Indirect estimations of the inoculum potential of mycorrhizal fungi have been obtained by measuring mycorrhizal formation after serial dilutions of soil, or by counting propagules such as spores, but results of these procedures do not always correspond with mycorrhiza formation in the same soil (Abbott & Robson 1991, Brundrett 1991). The total mycorrhizal inoculum potential of soils can be determined by growing bait plants in intact cores of soil to measure the rate of mycorrhiza formation (Brundrett 1991, Abbott & Robson 1991). This method is superior to 'most probable number' assay procedures (which involve serial dilutions of soil), because it will measure infectivity due to intact hyphal networks, as well as that from disturbance-tolerant propagules such as spores.

Intact soil core bioassays have been used to quantify seasonal and spatial variations in VAM inoculum potential and the relative contribution of different VAM fungi to these levels (by comparing mycorrhizal morphology within roots), in a wide range of

Equipment list

- Intact soil coring system (Figs 3.12H, 4.6)
  - corers made from sharpened steel cylinders
  - weight, guide rod and adaptor plate
- Buckets or pots the same size as soil cores
  - plastic bags to line pots
- Seeds of appropriate host plants for use in bioassays
  - seeds of fast-growing plants pregerminated by soaking in aerated water for 12–24 hrs.
  - seedlings of slow-growing species transplanted after germination in pasteurised sand



Australian natural ecosystems, mine sites and agricultural soils (Jasper et al. 1991, Scheltema et al. 1987, Brundrett et al. 1995). Results of bioassay experiments vary with the use of different bait plants or environmental conditions, but can be relied upon to provide a relative measure of mycorrhizal fungus inoculum, which can be compared with the results of other assays done using similar procedures. The results of 'most probable number' assays also depend on the host plant used, soil and environmental conditions (Adelman & Morton 1986, An et al. 1990, Wilson & Trinick 1982).

Experiment duration

It is recommended that the duration of bioassay experiments should not greatly exceed the time required for adequate levels of mycorrhiza formation, to minimise changes in fungal activity within soil cores that could be caused by the presence of host roots or changes in soil conditions. Longer duration bioassays can be used to assess the potential of field soils to support mycorrhizal formation, but provide less information about the rate of mycorrhizal formation, which is due to initial propagule numbers (Brundrett & Abbott 1995).

Bait plants

Plants with roots that grow rapidly in the soils and environmental conditions used and which are easy to clear should be selected as bait plants for bioassay experiments. It is also a good idea to include one or more species which are native to the soils used, especially if ECM fungus activity is being measured. In Western Australia, we often use germinating clover seeds inoculated with rhizobium to assay VAM, and seedlings of an appropriate *Eucalyptus* species for ECM.

1. Figure 4.8 shows how a soil corer can be used to sample relatively undisturbed volumes of soil. In this case, 1-L cylinders of soil are taken by driving an 12-cm diameter × 14 cm deep steel tube into the ground with a 10-kg weight that slides along a steel rod. The guiding rod ensures that only vertical force is transferred to the corer.
2. Soil cores are immediately pushed out of the steel ring into plastic bag-lined 1-L buckets, where they remain for storage, transport and subsequent bioassays (Figs 3.12H). These buckets are not free-draining and can be maintained at a constant temperature by immersion in a controlled temperature water tank (Fig. 3.12D).
3. It is best to avoid exposing soil cores to extremes in temperature during transport or storage and to use them as soon as possible. No additional nutrients are required.
4. Additions of water to non-draining pots should be regulated by watering them to a specified weight using a balance (see Chapter 6). Field capacity (% water) levels and soil moisture levels (determined by drying small samples of soil) can be used to estimate the weight of each core at field capacity.

INTACT SOIL CORE BIOASSAYS

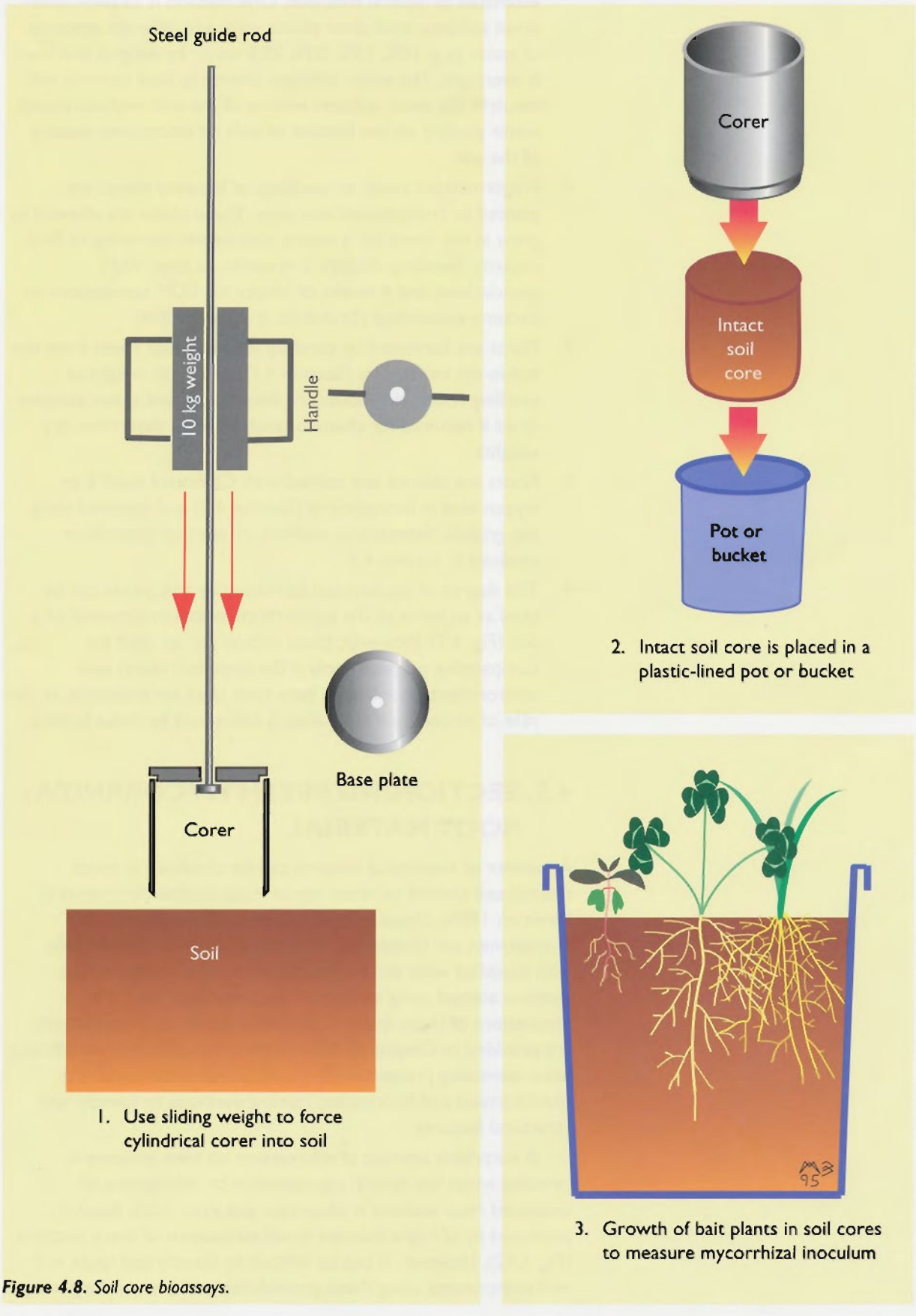


Figure 4.8. *Soil core bioassays.*

5. Field capacity values for a particular soil type can be estimated by several methods. One method is to pack oven-dried soil into small clear plastic vials, add different amounts of water (e.g. 10%, 15%, 20%, 25% water by weight) and leave it overnight. The water addition closest to field capacity will result in the most uniform wetting of the soil, without excess water pooling on the bottom of vials or incomplete wetting of the soil.
6. Pregerminated seeds or seedlings of bioassay plants are planted or transplanted into pots. These plants are allowed to grow in the cores for 4 weeks with regular watering to field-capacity. Seedlings require 2–4 weeks to form VAM associations, and 4 weeks or longer for ECM associations to become established (Brundrett & Abbott 1994).
7. Plants are harvested by carefully washing their roots from the soil in the intact core (Section 4.1). The fresh weight of seedling roots and shoots are determined and shoot samples dried if required for chemical analysis or to determine dry weights.
8. Roots are cleared and stained with Chlorazol black E or trypan blue in lactoglycerol (Section 4.2) and assessed using the gridline intersection method, or another procedure outlined in Section 4.3.
9. The degree of mycorrhizal formation by bait plants can be used as an index of the mycorrhizal inoculum potential of a soil (Fig. 4.9). However, these indices can be used for comparative purposes only if the same bait plants and environmental conditions have been used for bioassays, as the rate of mycorrhizal formation is influenced by these factors.

4.5. SECTIONING FRESH MYCORRHIZAL ROOT MATERIAL

A number of anatomical features can be observed in roots cleared and stained to reveal mycorrhizal hyphae (Brundrett & Kendrick 1988). Details of the structure of exodermis, endodermis, phi thickenings, xylem, periderm, and other cells with modified walls can be observed in whole roots or root sections stained using various procedures (Figs 1.3, 1.12). Illustrations of these features and information on root anatomy are provided in Chapter 1. Root sections produced by an efficient hand-sectioning procedure (Frohlich 1984) can be used with histochemical and fluorescent staining methods to identify wall structural features.

A surprising amount of information on root anatomy is revealed when the natural pigmentation or refringence of unstained root sections is observed, and even more detail is produced by UV-light induced autofluorescence of these sections (Fig. 1.12). However, it can be difficult to identify cell types and wall components using these procedures, and the resulting images

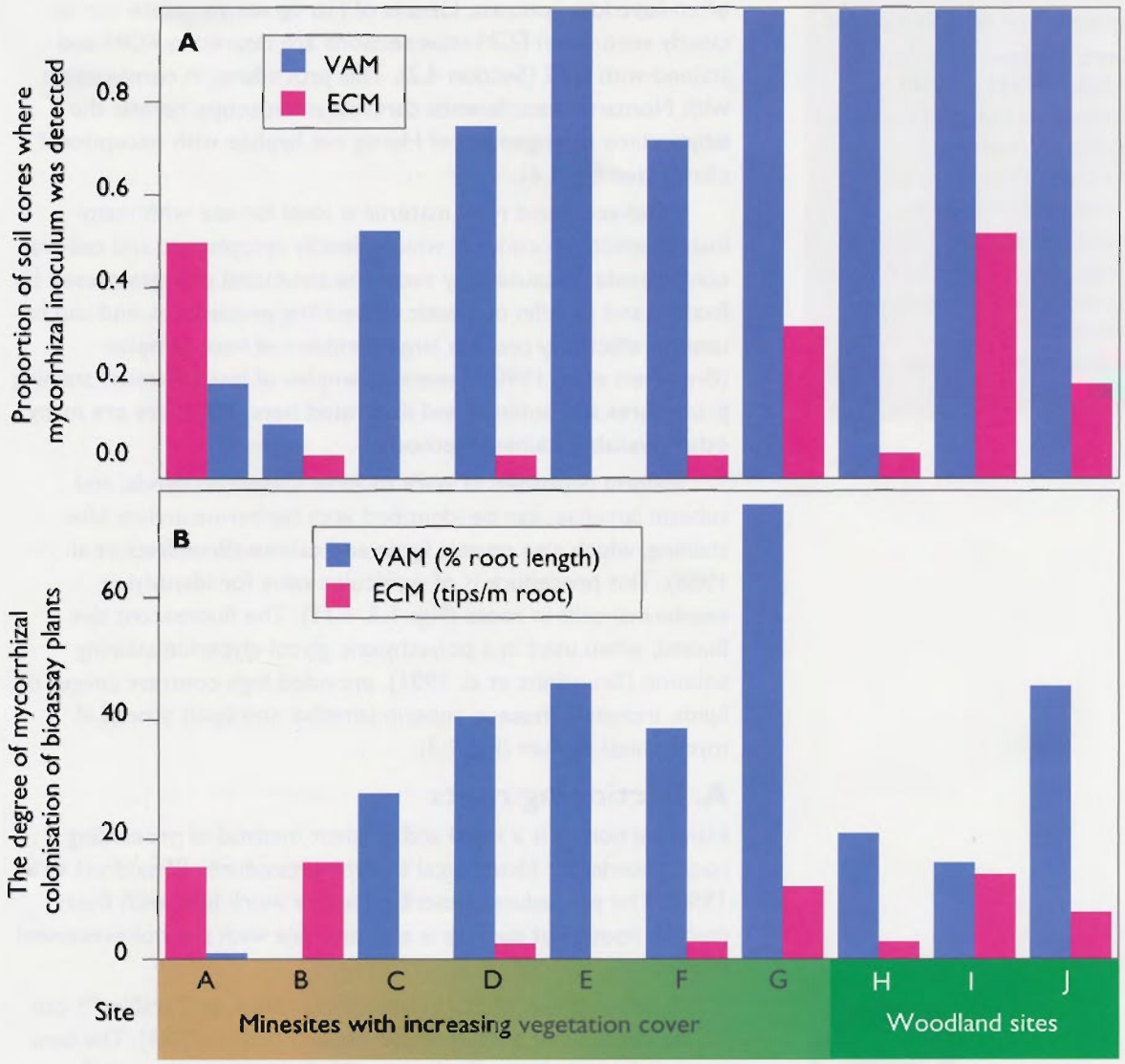


Figure 4.9. *Mycorrhizal bioassay results using clover (*Trifolium subteranneum*) and eucalypt (*Eucalyptus miniata*) seedlings grown in intact soil cores to provide estimates of mycorrhizal inoculum potential for VAM and ECM fungi respectively. This procedure provided information on both (**A**) the frequency of occurrence of mycorrhizas (number of soil cores with mycorrhizal seedlings) and (**B**) the average values for mycorrhizal colonisation across each site. For this experiment, 20 soil cores were collected along a 100-m transect from seven minesite habitats (arranged in order of increasing vegetation cover) and three savanna woodland sites (data from Brundrett et al. 1995).*

Equipment and reagents

- Sharp 2-edged razor blades **(USE WITH CARE)**
- Material to hold plant material during sectioning
  - Parafilm™ squares and plastic Petri dish lids
  - styrofoam or pith pieces
- Dissecting needle, fine forceps or paintbrush to manipulate sections
- Stains, section holders, slides, coverslips, etc.

often have low contrast. Details of Hartig net structure can be clearly seen when ECM root sections are cleared in KOH and stained with CBE (Section 4.2). This procedure, in combination with Nomarski interference contrast microscopy, reveals the labyrinthine arrangement of Hartig net hyphae with exceptional clarity (see Fig. 1.6).

Hand-sectioned root material is ideal for use with many histochemical procedures which identify cytoplasmic and cell-wall components, because they avoid the structural changes caused by fixation and paraffin or plastic embedding procedures, and can be used to efficiently process large numbers of root samples (Brundrett et al. 1990). Several examples of histochemical staining procedures are outlined and illustrated here, but there are many other available staining methods.

Suberin deposited in walls to form Casparian bands, and suberin lamellae, can be identified with berberine-aniline blue staining, which also reveals lignin and callose (Brundrett et al. 1988). This procedure is of particular value for identifying exodermal cells in roots (Figs 1.3, 1.11). The fluorescent dye fluorol, when used in a polyethylene glycol-glycerine staining solution (Brundrett et al. 1991), provided high contrast images of lipids, including those in suberin lamellae and lipids stored in mycorrhizal hyphae (Fig. 1.3).

A. Sectioning roots

Hand sectioning is a rapid and efficient method of processing root material for histological staining procedures (Brundrett et al. 1990). The procedures described below work best with fresh (turgid) roots, but success is also possible with alcohol-preserved roots.



1. Cross-sections of ECM roots immobilised by Parafilm™ can be made using a sharp razor blade (Frohlich 1984). The best results are obtained when the blade is drawn repeatedly across a bundle of roots to produce numerous sections (Fig. 4.10A). Many sections of variable thickness should be produced rapidly, as it is impossible to precisely regulate the thickness of sections.
2. Thin, uniform sections are then selected with fine forceps or a pipette while observing sections floating in water under a dissecting microscope. Selected sections can be placed in sample holders (Fig. 4.11), or transferred between solutions with a pipette.
3. Similar results can be obtained using styrofoam pieces, pith, or other similar materials to immobilise roots during hand sectioning (Fig. 4.10B).

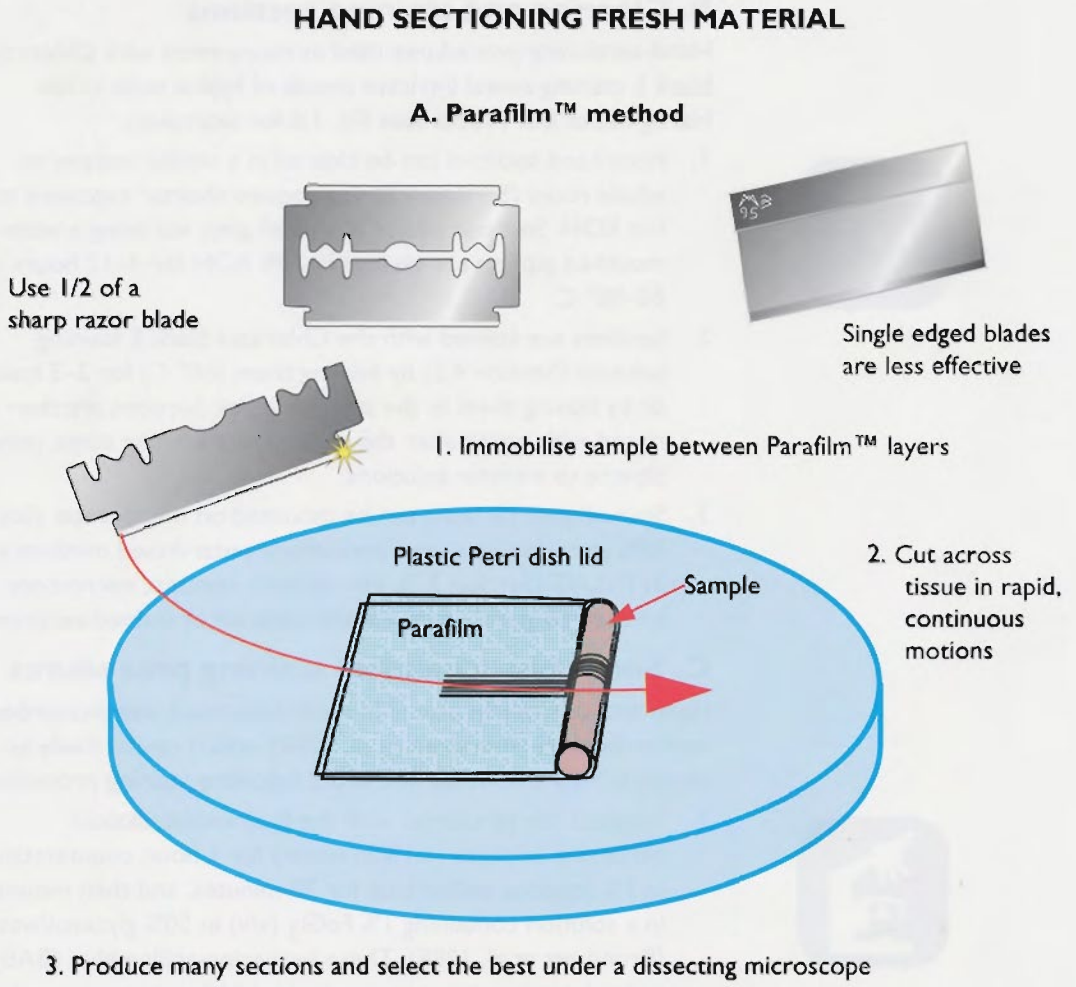


Figure 4.10. Two methods for hand sectioning fresh material.

B. Clearing and staining sections

Hand-sectioning procedures used in conjunction with Chlorazol black E staining reveal intricate details of hyphal walls in the Hartig net of ECM roots (see Fig. 1.6 for examples).



1. Root hand sections can be cleared in a similar manner to whole roots (Section 4.2), but require shorter exposure to hot KOH. Sections placed in a small glass vial using a wide-mouthed pipette are cleared in 10% KOH for 4–12 hours at 60–90° C.
2. Sections are stained with the Chlorazol black E staining solution (Section 4.2) by heating them (60° C) for 2–3 hours, or by leaving them in the stain for a day. Sections are then rinsed with water after the clearing and staining steps, using a pipette to transfer solutions.
3. Stained root sections can be mounted on microscope slides in 50% glycerine or a semi-permanent water-based medium such as PVLAG (Section 3.2). Interference contrast microscopy can be used to greatly enhance the contrast of stained sections.

C. Some histochemical staining procedures

Hand sections can be placed in mesh-bottomed, multi-chambered section holders (Brundrett et al. 1988), which can be made as shown in Figure 4.11, for use in the following staining procedures.



1. Sections can be stained with the fluorescent alkaloid berberine sulphate (0.1% in water) for 1 hour, counterstained in 1% aqueous aniline blue for 30 minutes, and then mounted in a solution containing 1% $FeCl_3$ (v/v) in 50% glycerol/water (Brundrett et al. 1988). These berberine-aniline blue (BAB) stained sections are examined with UV-fluorescence to show cell walls containing Casparian bands, lamellar suberin or lignin (see Figs 1.3ACE, 1.12E–G).

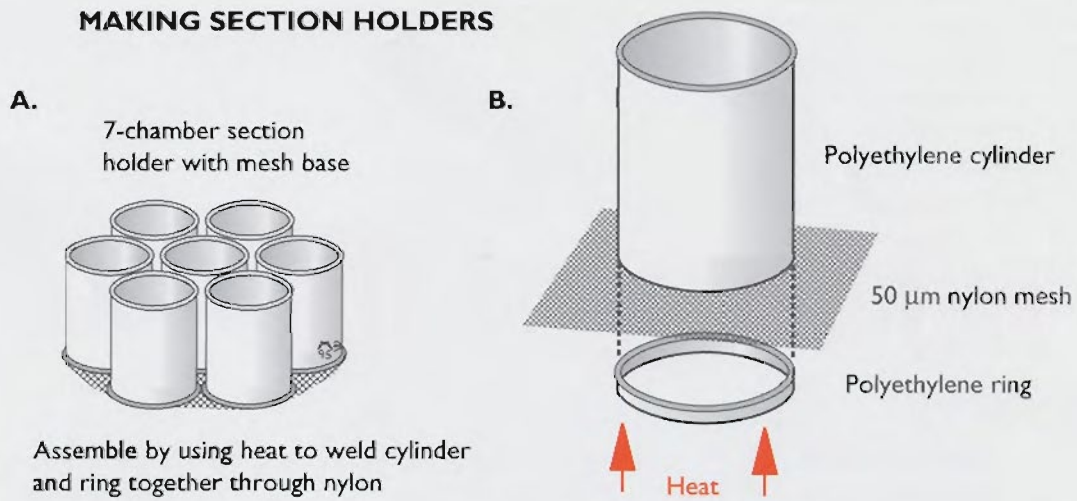


Figure 4.11. A. *Multi-chambered section holders made from polyethylene tubing and nylon mesh.* ***B.*** *The polyethylene ring and cylinder are melted together through the nylon mesh (using a glass slide placed on a hot-plate).*

2. The fluorescent dye fluorol (fluorol yellow 088, solvent green 4, obtained from SIGMA or BASF) can be used as a specific fluorochrome to stain lipids (Brundrett et al. 1991). This dye is used at 0.01% in a staining solution consisting of 1:1 polyethylene glycol (average molecular weight = 400) and 90% glycerol. Sections in holders are stained for one hour, rinsed briefly, then mounted in glycerol. This stain provides high-contrast UV-fluorescent images of non-polar substances such as lamellar suberin and lipids (see Figs 1.3BDF, 1.12H).
3. Sudan red 7B (Solvent red 19, fat red) is the most effective of the non-fluorescent lipid stains and can be used with the polyethylene glycol/glycerol solution described above (Brundrett et al. 1991). Specimens can be mounted directly in an 0.1% solution of this stain, and heated gently to reverse any stain precipitation which occurs. Other fat dyes such as Sudan III, IV, or Sudan black B can also be used (O'Brien & McCully 1981).
4. Phloroglucinol in HCl (a saturated solution in 20% v/v HCl/water) can be used to confirm the presence of lignin in cell walls (Jensen 1962).
5. The vital stain fluorescein diacetate can be used to indicate living fungal hyphae and spores under ultraviolet light with a fluorescence microscope (Schubert et al. 1987, Hamel et al. 1990). Material is mounted in a 50 µg/mL (w/v) solution in water or buffer (made by first dissolving 5 mg of FDA in 1 mL of acetone).
6. The florescent nuclear stain DAPI (4',6-diamidino-2-phenylindole — 5 µg/mL w/v in water or buffer) effectively stains nuclei in mycorrhizal fungi (Cooke et al. 1987, Balestrini et al. 1992).
7. Ling Lee et al. (1977) list additional histochemical stains for phenolics, carbohydrates, etc. O'Brien & McCully (1981) provide details of many additional histochemical procedures which can be used on fresh or plastic embedded plant materials.
8. Many fluorescent stains have been used to study the structure and development of nuclear, cell wall and cytoplasmic components of fungi (Butt et al. 1989). These include lectin and antibody conjugates for the specific detection of proteins, carbohydrates, etc.
9. Calcofluor white M2R new (0.01% w/v in distilled water) is a very bright fluorescent stain of cellulose in plant cell walls (Hugues & McCully 1975).

4.6. ADVANCED STAINING AND MICROSCOPY PROCEDURES

This manual contains only introductory information about microscopy, histology and histochemistry. More information can be obtained from histology books and microscopy manuals. The use of dissecting microscopes to view whole objects with reflected or transmitted light and the compound microscope to view thin preparations of objects with transmitted light is introduced in Section 1.7.

A. Comparison of methods

Table 4.5 compares advantages and disadvantages of different microscope techniques. Light microscopes can be adapted by the use of specialised illumination and optical systems to allow more specialised microscopic techniques to be utilised. Polarised light, phase contrast and Nomarski interference contrast microscopy can be used to enhance the contrast of unstained or stained materials. Fluorescence microscopy provides the opportunity to observe auto-fluorescent details of tissues that are otherwise invisible and utilises fluorescent stains which stain tissue constituents with much higher contrast than regular stains.

Embedding material in plastic resins such as epoxies or methacrylates allows uniformly thin sections to be obtained with a microtome. Tissue components must first be preserved by a fixative such as glutaraldehyde and dehydrated using an organic solvent. Plastic sections are often stained with the metachromatic stain toluidine blue O (TBO) which differentiates lignified cell walls, but many histochemical staining procedures can also be used (O'Brien & McCully 1981). Examples of thin sections of ECM roots stained with TBO are shown in Figures 4.13 and 4.14.

The scanning electron microscope (SEM) has been used to study the surface morphology of mycorrhizal roots, and has provided valuable information about changes in hyphal morphogenesis after root contact (Jacobs et al. 1989, Massicotte et al. 1987). Examples of SEM photographs of ECM roots are shown in Figure 4.12. Surface features of spores, etc. can be valuable for identifying ECM and VAM fungi (see Figs 2.14, 3.3). The SEM requires tissues to be dehydrated and their stability is usually improved by fixation. Surface contrast is often enhanced by coating with a metallic film.

Transmission electron microscopy (TEM) examinations have resolved many details in the formation of VAM and ECM associations. This procedure usually involves material which has been fixed, dehydrated, embedded in plastic resin and sectioned with a microtome which is capable of taking ultra-thin sections. An introduction to electron microscopy procedures and specific staining techniques is provided below. Some examples of TEM photographs of ECM roots and fungal spores are shown in Figure 4.15. The TEM is particularly valuable for observing cytoplasmic and cell wall changes in the host and fungus, that occur during

mycorrhiza formation and are likely correlated with functioning of associations (see reviews by, for example, Peterson & Bonfante 1994 and Kottke & Oberwinkler 1986).

Table 4.5. Comparison of microscopy procedures with their relative advantages and disadvantages compared with other procedures.

Procedure	*Advantages*	*Disadvantages*
A. Basic procedures		
Dissecting microscope	— convenient, inexpensive — non-destructive observation of living, unstained material — reflected or transmitted light	— low contrast resolution, magnification — shallow depth of field (compared to SEM)
Compound microscope (transmitted light)	— convenient, inexpensive — higher magnification and resolution — many general and specific staining procedures available	— shallow depth of field — contrast may be low — destructive sampling usually required
B. Specialised compound microscope procedures		
Phase contrast or polarised light	— enhanced contrast of unstained material — easy and inexpensive	— halos form around objects — walls must be refringent
Interference contrast	— high contrast images of unstained or stained material	— harder to use — equipment expensive and hard to adjust correctly
Fluorescence	— very high contrast images — relatively fast and easy — specific stains and probes are available	— equipment expensive — many stains fade rapidly — long exposure times often required
C. Electron microscope procedures		
Scanning Electron Microscope (SEM)	— high resolution — very large depth of field	— surface details only — expensive processing and equipment
Transmission Electron Microscope (TEM)	— very high resolution — cytological details can be seen — specific stains and probes are available	— very time-consuming and expensive — limited observations — sample size limited — fixation artefacts can occur

Microscopy reagents

Fixatives (see Table 4.7)

Buffers

— Phosphate buffer (pH 7.0) made from two stock solutions:
(a) KH_2PO_4 and (b) Na_2HPO_4 combined at the correct molarity

— HEPES (N-2-Hydroxyethylpiperazine-N-2-ethanesulfonic acid)

— dissolved in water at required molarity and pH adjusted with HCl

Solvents (for dehydration) in a graduated series:

— 10%, 30%, 50%, 75%, 90%, 100%

— 100% dried by adding molecular sieves

— ethanol, methanol, acetone or amyl acetate are commonly used

Safety notes

All fixatives by their nature can also fix human tissue. The eyes and nasal membranes are vulnerable. Avoid breathing vapours and wear gloves.

OsO_4 is extremely hazardous and samples must be prepared and used in a fume cupboard. Do not dispose of waste solutions down the sink.

Some resin components may be carcinogens and unpolymerised resin should always be treated with care — use disposable gloves and a fume cupboard, and dispose of unused resin by polymerisation.

Refer to safety data sheets for the latest information on the chemicals you use.

B. Electron microscopy

In electron microscopy, beams of electrons are used either to bombard the surface of a specimen (SEM) or the components of thin sections (TEM). Traditionally, specimens are prepared from fixed and dehydrated organs and tissues. However, some modern microscopes are fitted with cryostages which allow living material (in bulk, sectioned on a cryotome or freeze fractured) to be examined.

Electron microscopes are extremely expensive pieces of equipment and therefore they mostly exist only in specialised histology laboratories. However, fixed, embedded or dried material can be dispatched to collaborating laboratories for examination. Electron microscopy should be undertaken only after light microscopy has been exhausted and where specific information is required (Table 4.6).

Table 4.6. Examples of purposes of the use of electron microscopy for the study of mycorrhizas.

Objective of study	*Technique*
A. Three-dimensional detail of interactions between fungus and host	SEM — whole mount of sectioned mycorrhizas
B. Ultrastructure and histopathology of cells at interface	TEM — ultra-thin section of mycorrhiza
C. Elemental composition of mycorrhizal tissues	SEM — X-ray dispersion analysis
D. Elemental composition of cellular components of mycorrhiza	TEM — X-ray dispersion analysis
E. Immunolocalisation of proteins	TEM — immuno-gold labelling

Figure 4.12. *Scanning electron microscope (SEM) images of ectomycorrhizal roots. A, B. and F, G are whole root samples from the field, others have been synthesised in glasshouse experiments.*

A. *Branched jarrah (*Eucalyptus marginata*) ECM appressed to the surface of a decomposing leaf.*

B. *Superficial ECM formed by* Cortinarius globuliformis *on* E. marginata *with abundant attached mycelium.*

C,D. *High magnification views of fractured ECM roots.*

C. *Longitudinal section of an* E. diversicolor *ECM root showing mantle (M) hyphae and epidermal cells (E) with Hartig net hyphae (arrows).*

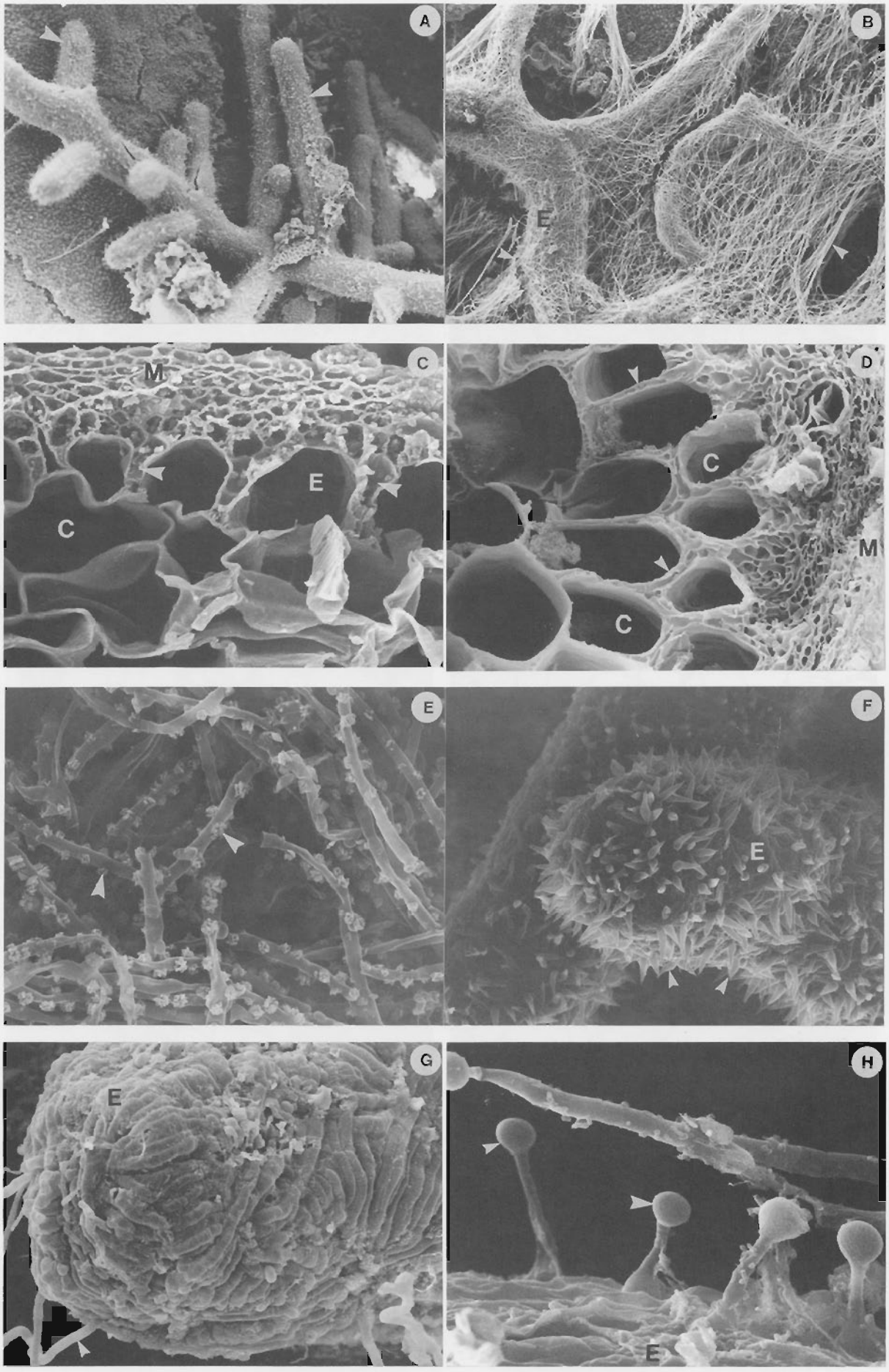
D. *Cross-section of* Pinus radiata *ECM root from the field showing mantle (M) and Hartig net hyphae (arrows) between cortex cells (C).*

E. *Crystals of calcium oxalate (arrows) on hyphae of* Hysterangium *sp. associated with* E. diversicolor.

F. *Surface of a brown ECM root of jarrah (*E. marginata*) from forest soil, showing characteristic cystidia (arrows).*

G. *Surface of a black ECM root of jarrah (*E. marginata*) from forest soil, showing distinctive pattern of the mantle surface and hyphae (arrow).*

H. *Cystidia (arrows) of* Descolea maculata *arising from the mantle of an* E. diversicolor *mycorrhizal root.*


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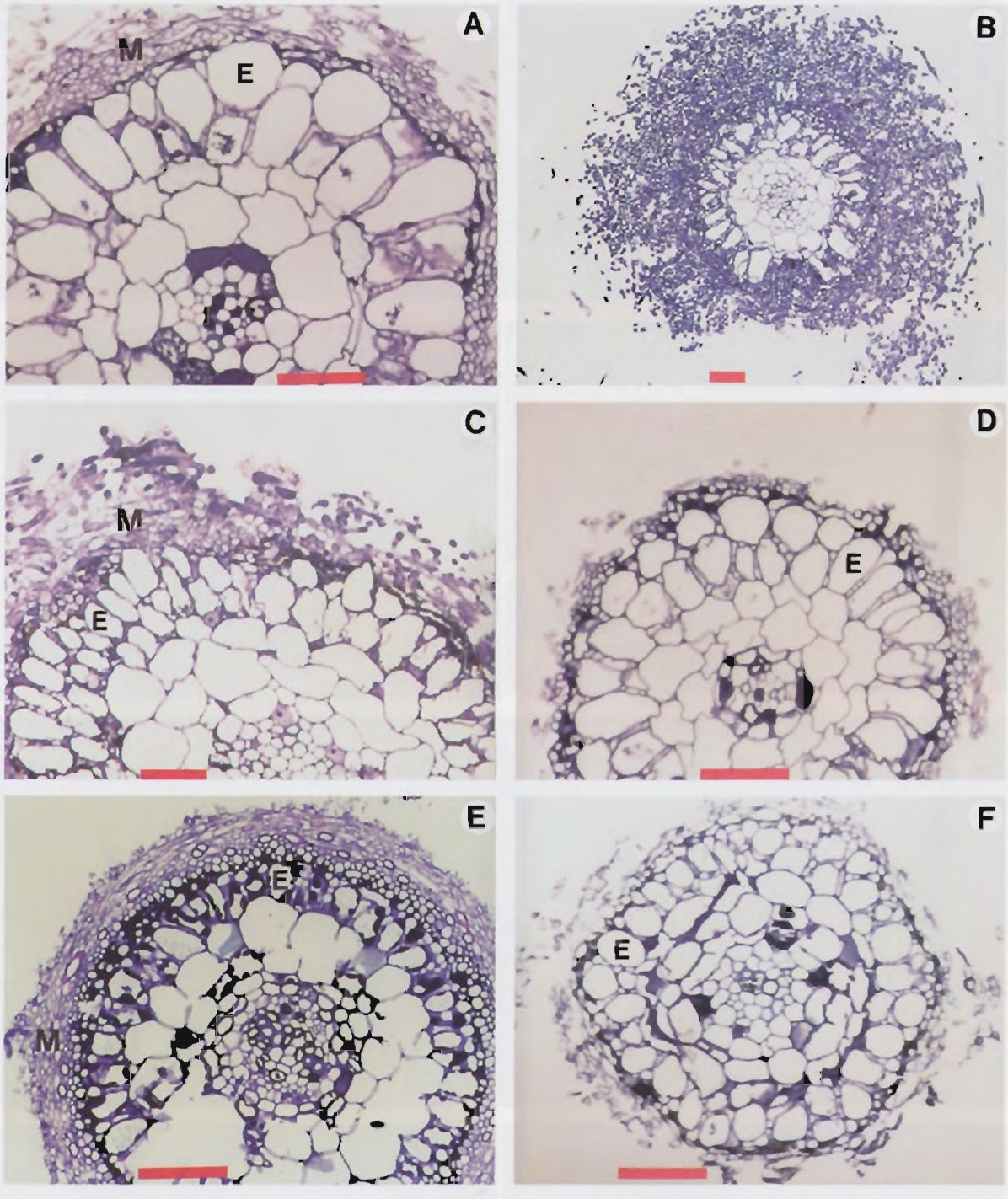

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Figure 4.13. *Semi-thin plastic sections of ectomycorrhizal (ECM) roots. These roots have been embedded in plastic resin sectioned with a microtome, and stained with toluidine blue O. All scale bars are 100 µm long,* **E** *= epidermal cell,* **M** *= mantle.*

A. *Cross-section of an ECM association of* Pisolithus *sp. on* Eucalyptus grandis. *This association has a thin mantle and a well-developed Hartig net between elongated epidermal cells.*

B. *ECM association formed by* Pisolithus *sp. and* E. grandis *with a very thick mantle.*

C. *Pisolithus isolate which is incompatible with* E. urophylla. *A mantle has formed, but there is no epidermal Hartig net.*

D. *More compatible isolate of* Pisolithus *forming ECM roots with a thin mantle and intermediate Hartig net development.*

E. *Field-collected dipterocarp (*Shorea *sp.) root with two-layered mantle and epidermal Hartig net.*

F. *A superficial ECM association between* E. marginata *and a* Cortinarius *species. Note the very thin mantle and shallow Hartig net due to lack of epidermal cell expansion.*

C. Scanning electron microscopy

Examples of SEM images are presented in Figure 4.12. A beam of electrons is scanned across the specimen as in a television picture. Secondary electrons emitted from the specimen on impact are attracted towards a positively charged grill and detection system. The amplified signals are visualised on a cathode-ray tube screen that can be focused (Hayat 1981).

The most essential requirements for SEM preparations are that the specimens are dry and the surface is electrically conductive. A standard protocol for fixing, dehydrating and critical point drying specimens is outlined below. Some roots should be sectioned with a clean sharp razor blade if internal structures such as fungal hyphae are to be seen.



1. Fix plant and fungal tissues (from 1 to 8 mm in diameter and up to 4 mm in height) in 3% glutaraldehyde in 0.025 M phosphate buffer, overnight at 4°C. If small segments of mycorrhiza are being processed, they can be post-fixed in OsO_4 to stabilise lipids (see below). Alternatively, specimens can be freeze-dried without fixation. Air-drying will cause excessive collapse of hyphae but can be useful for observing some features such as spores and crystals. Samples stored without proper fixation in 50% ethanol can be processed through an alcohol dehydration series but the cytoplasm and other delicate structures will be poorly preserved.
2. Rinse with buffer three times.
3. Dehydrate in an series of increasing concentration of ethanol, 10%, 30%, 50%, 75%, 90%, 3x100% for 30–60 minutes (depending on size) at each step.
4. Transfer specimens into amyl acetate or dry acetone in 25%, 50%, 75% each for 30 min, then use several changes of 100% amyl acetate or acetone.

5. Process through a critical point dryer. Once dry, keep specimens in a desiccator with fresh desiccant.
6. Attach specimens using silver/carbon paste or double-sided adhesive tape to aluminium stubs so that the part to be observed is uppermost.
7. Sputter or evaporative coat the specimens with a good conductor (gold, gold–palladium, or carbon if samples are for elemental analysis).
8. Examine specimens in a SEM. Take adequate notes. Images can be obtained on photographic film, or on computer disc. Note that specialised information on operation of a SEM is available from the manufacturer and is not included in this manual.

D. Plastic sections for light and transmission electron microscopy

In the TEM, a series of electromagnetic lenses focuses a beam of electrons in an evacuated column onto a thin tissue section normally embedded in an epoxy or acrylic resin supported on a metal grid. To achieve contrast, sections are treated with electron-dense materials, usually heavy metals such as uranium, lead and tungsten, to achieve differential scattering of electrons (Hayat 1981). This process is referred to as staining. Staining is necessary because there is very little difference in electron opacity of the various components in a mycorrhiza. Electrons which do not strike the electron-dense particles or structures in the section are not deflected and pass through to interact with a phosphorescent viewing screen to produce an image. The screen is replaced by photographic film to form a negative.

The ability to distinguish between two objects is known as resolution. In a TEM, the working magnification range for biological materials 60–70 nm in thickness is about 400X to 100 000X with a maximum resolution of 1–2 nm. This compares with a resolution in a compound light microscope of 0.2 µm and 0.1 mm for the unaided eye.

It is critical that great care is taken to ensure samples are adequately fixed and as free as possible from artefacts such as plasmolysed cells, broken membranes and lost lipids. An example of poor fixation is given in Fig. 4.15. Specimens must also be fully infiltrated with resins. To achieve this, protocols must be strictly adhered to and specimens should be fresh and divided with a sharp, new razor blade into small pieces, approximately 1–2 mm cubes. Glauert (1980) contains much relevant information.



1. Cut mycorrhizas into segments 1–2 mm in length and place into the primary fixative (Table 4.7) in small labelled (by pencil on paper inside) glass vials. Loosely fit the lids and place under a vacuum in a plastic or shielded glass container for 2–3 hours at room temperature or until bubbles emerge and specimens sink. Do not allow samples to become dry. Continue fixation overnight at 4°C or proceed immediately to step 2 .
2. Rinse in the same buffer contained in the fixative (three times, for several minutes).

3. Post-fix in 1% OsO_4 in the same buffer for 2 hours at room temperature. The specimens will go black. This step should be omitted if sections are to be used for light microscope histology.
4. Rinse with buffer (as in 2 above).
5. Dehydrate in an acetone series 10%, 30%, 50%, 75%, 90%, 3x100% for 20 minutes at each step. Molecular sieves should be added to the 100% acetone to remove any traces of water.
6. Infiltrate with resin. Epoxy resins (e.g. Spurrs, Araldite®, epon) are widely used. Alternatively, polyester (e.g. LR White, LR Gold) and methacrylate resins can be used for special purposes such as immunohistochemistry with the TEM or histochemistry with the light microscope. For epoxy resins, the specimens should be infiltrated by increasing the resin concentration (complete mix) in the 100% acetone by 5% increments, each step taking a minimum of 3 hours at room temperature on a shaker (rotating inclined plane is best). This slow infiltration is essential to minimise specimen shrinkage.
7. Finally, the specimens are transferred into embedding moulds (e.g. flat rubber with elongated wells) with 100% resin and polymerised under mild heat (see specifications with commercial embedding kits).
8. Material in hardened plastic is exposed by trimming blocks with a sharp razor blade and glass knives. Blocks are then sectioned on an ultramicrotome using glass or diamond knives. See Reid (1980) and Brundrett et al. (1994) for details of procedures to be followed.
9. Resin blocks can also be sectioned on microtomes with glass knives producing sections from 0.5 mm to 2 mm in thickness. These semi-thin sections are transferred onto to glass slides, affixed by heating, then stained for light microscopy (see Table 4.8). For epoxy sections, it is often best to remove the resin before staining by flooding the sections in saturated KOH in ethanol for 20 to 30 seconds.
10. For the TEM, sections are required to have a thickness from 50–70 nm (silver to gold). These sections are supported on copper grid and stained with electron stains (heavy metals). The selective deposition of heavy metal atoms increases contrast in the photographic image obtainable with the TEM. Recipes and procedures are available in Lewis & Knight (1980) and Hayat (1981).

Table 4.7. Buffered fixatives for TEM. More information on fixative recipes is given in Glauert (1980) and Hayat (1981).

Fixative and buffer	*Comments*
A. 3% glutaraldehyde, 0.025 M phosphate buffer at pH 7.0	Widely used fixative with poor buffering capacity and slow tissue penetration
B. 2% paraformaldehyde + 2.5% glutaraldehyde in phosphate, or cacodylate buffer at pH 7.0	Faster penetration of fixative into difficult tissues than with glutaraldehyde alone
C. 2.5% glutaraldehyde in 0.1 M HEPES buffer	Better fixation than phosphate buffer (Massicotte et al. 1985)
D. Osmium tetroxide post-fixative 2% in water	Used to help stabilise SEM and TEM samples and increase the contrast of TEM images



Table 4.8. A brief introduction to stains for semi-thin plastic sections.

Stain	*Comments*	*References*
Toluidine blue O (TBO) 0.05% (w/v) in water	Metachromatic stain commonly used for plant materials	O'Brien et al. (1964)
Sudan black B in 70% ethanol	Very effective lipid stain	Bronner (1975)
Various staining procedures	General and specific stains for carbohydrates, lipids, protein, etc.	see Appendix B in Brundrett et al. (1994), O'Brien & McCully (1981)
Staining procedures for phenolics	Various procedures can be used, but results differ	Ling Lee et al. (1977)

Figure 4.14. *Semi-thin plastic sections of ectomycorrhizal (ECM) roots. These roots have been embedded in plastic resin sectioned with a microtome, and stained with toluidine blue O. E = epidermal cell, M = mantle.*

A. *Cross-section of an ECM association of* Laccaria laccata *on* Eucalyptus marginata. *This association has a thick mantle (M) and a well-developed epidermal Hartig net (arrows).*

B. Pisolithus sp.–E. grandis *association with a deep Hartig net due to epidermal cell elongation.*

C. *Longitudinal section of a 7-day-old lateral root tip of* E. grandis *inoculated with a compatible* Pisolithus *isolate. Note the apical meristem (A) and the zone where Hartig net initiation occurs (arrows).*

D. *Section of a tuberculate ECM showing hyphae and mycorrhizal roots (arrows) enclosed by a rind (R).*

E. *Higher magnification view of ECM roots in a tuberculate mycorrhiza. These roots have a thin mantle and a well-developed Hartig net (H).*

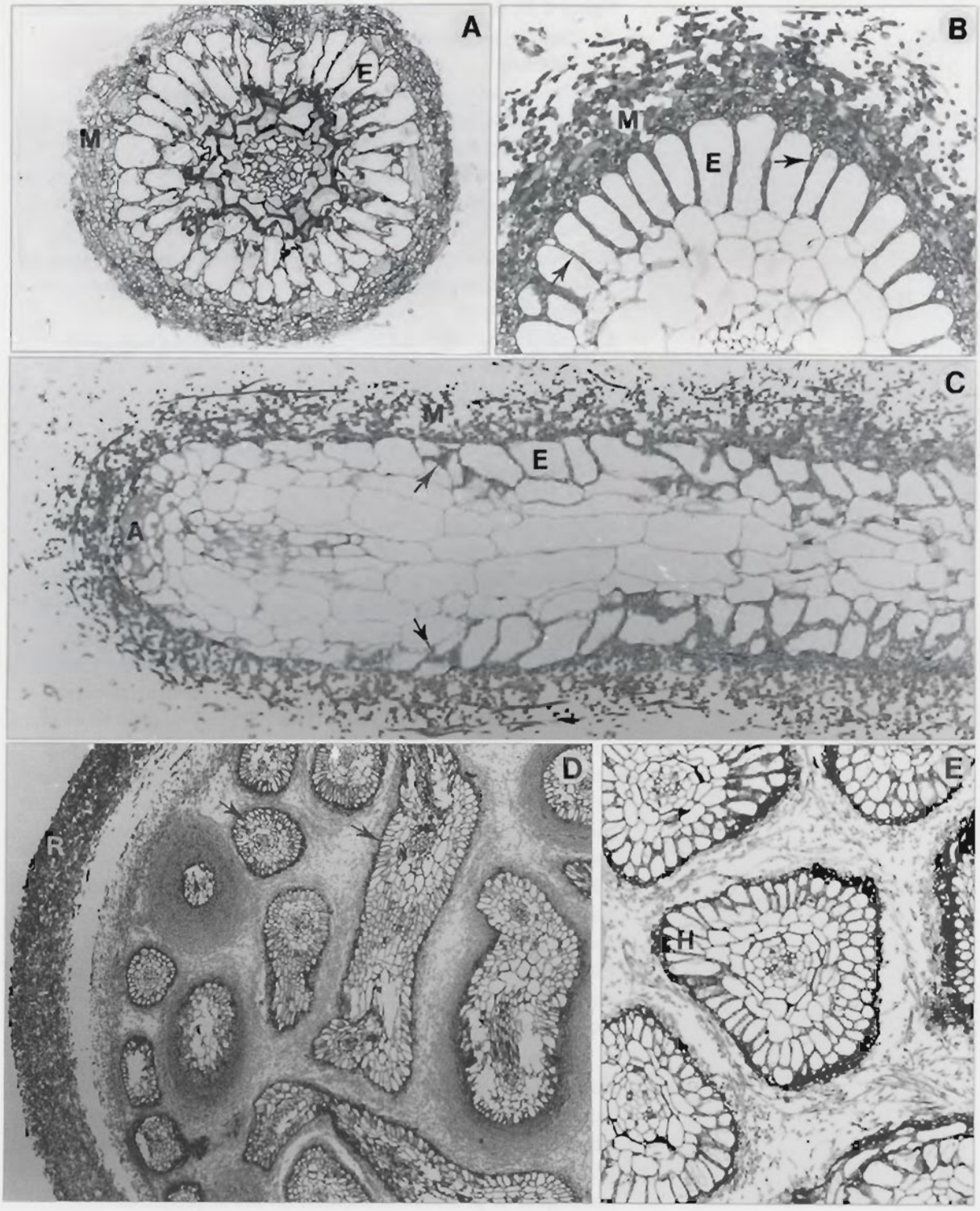

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Figure 4.15. Transmission electron microscope (TEM) images of ECM roots.

A. *Cross-section of an* Amanita *sp.–*Eucalyptus microcorys *ECM association.*

B. *Cross-section of an ECM root from a* Cortinarius globuliformis–E. marginata *superficial association showing fungal hyphae in the Hartig net (arrows) and thin mantle (M).*

C. Scleroderma verrucosa *and* E. grandis *ECM. Note peripheral cytoplasm of living epidermal cells (arrow) and extracellular material on mantle hyphae.*

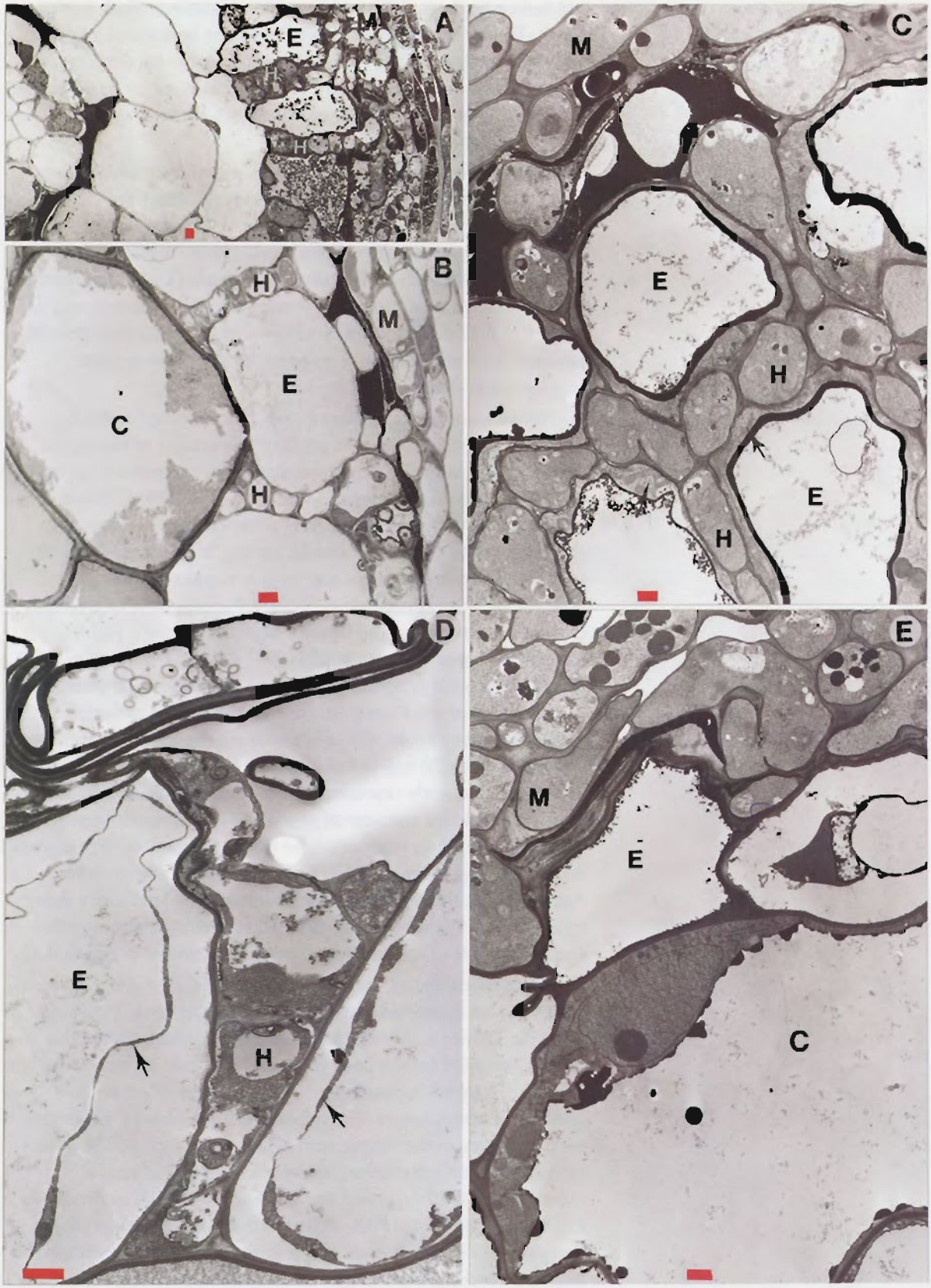
D. Hysterangium *sp.–*E. marginata *ECM. The inner mantle and Hartig net can be seen, but cytoplasm has pulled away from cell walls due to poor fixation (arrows).*

E. Scleroderma verrucosa *and* Casuarina equisetifolia *incompatible reaction with no Hartig net and dead epidermal cells over a living cortex.*

4.7. THE VALUE OF HISTOLOGICAL INFORMATION

Information obtained from histological investigations can be of great value in mycorrhizal studies. Careful observations of cleared and stained roots or sectioned root material are required to confirm that mycorrhizal associations are present, and that they conform to the definitions of association types provided in Chapter 1. The SEM is especially useful for studying early stages of the association, when fungus hyphae first colonise the root surface (Massicotte et al. 1987). The TEM can be used to examine sub-cellular details of the host–fungus interface that could not otherwise be observed. Such details include changes to cellular organisation which occur as mycorrhizas develop and age — and thus can be used to indicate zones where active exchange between the symbionts is likely to occur (Kottke & Oberwinkler 1986, Massicotte et al. 1987). In some cases host cells develop as *transfer cells*, by forming wall ingrowths which increase the surface area of the interface (Massicotte et al. 1987, Ashford et al. 1989). Microscopic examinations of fresh ECM roots or cleared and stained VAM roots can also be used to determine the identity of fungal associates.

There are serious limitations to the use of electron microscope procedures, including artefacts of fixation which often occur, and logistical constraints which limit numbers of observations and the site of root pieces that can be examined (Table 4.5). Thus, preliminary studies using light microscope techniques to provide information on the sequence of mycorrhizal development are an essential prerequisite to any ultrastructural study. Mycorrhizal synthesis techniques, such as axenic culture methods, the use of growth pouches, or root observation chambers, are recommended to produce material for EM studies, as they allow root development to be monitored and typical roots of a known age to be sampled (Chapter 5). Development of the host–fungus interface in VAM associations also follows a cycle of rapid synthesis of arbuscular branch hyphae


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and host cytoplasm, followed by their senescence (Toth & Miller 1984). Thus it is also important to use root samples of a known age when examining VAM associations. This can be done by root-organ culture synthesis (Bécard & Piché 1992), or by periodically harvesting seedlings transplanted into pots containing active associations (Brundrett et al. 1995).

Histochemical staining procedures, many of which are only possible with fresh material, are required to obtain an understanding of root anatomy features which can influence mycorrhiza formation (Brundrett et al. 1990). Histochemical studies have allowed permeability barriers that can delineate the interface zone to be detected by using stains for suberin and dye tracers (Ashford et al. 1989, Brundrett et al. 1990). Interactions between root structure and mycorrhizal development are considered in detail in Section 1.5.

When plants are inoculated with specific fungal isolates, correlation between morphological observations of Hartig net development and host growth responses to the same fungi have been reported (Burgess et al. 1994, Dell et al. 1994). These results suggest that morphological observations may provide a valuable tool to assist in isolate screening, but further research with a wider range of hosts and fungi is required to determine how robust this correlation is. Table 4.9 shows how microscopy techniques can be used to identify different eucalypt ECM association types. Burgess et al. (1994) defined five types of ECM associations, with progressively thicker mantles and greater development of the Hartig net (due to the extent of fungal growth between epidermal cells and their degree of elongation), when screening 20 isolates of *Pisolithus* with *E. grandis*. These catxories apparently represent levels of compatibility, which perhaps should be considered as part of a continuum between fungi which are fully compatible or fully incompatible with a given host. Superficial eucalypt ECM associations are common in Australian natural ecosystems (Malajczuk et al. 1987), but their role requires further investigation. Illustrations of incompatible and compatible eucalypt associations are provided in Figure 4.13.

It has often been assumed that arbuscule production in roots, which can be quantified by microscopic procedures (McGonigle et al. 1990, Toth et al. 1990), can be used as an indication of the capacity of VAM associations to supply nutrients to the host plant. However, internal hyphae, which occupy roots of perennial species for much longer than arbuscules, may also be important exchange sites in the longer term (Brundrett et al. 1990). There typically is good correlation between the length of roots colonised by VAM fungi and the number of arbuscules produced in a host (Toth et al. 1990). When data from field studies in the literature are compared, there generally is a poor correlation between levels of VAM fungus colonisation in roots and plant yields (McGonigle 1988). However, most of these studies measured VAM colonisation once, after colonisation levels had already peaked, so they do not allow mycorrhizal inoculum levels

to be compared (Abbott et al. 1992). Gazey & Abbott (pers. comm.) observed that there is good correlation between the rate of root colonisation by different isolates of VAM fungi and subsequent plant growth responses in a glasshouse study. It is likely that correlations between the rate of mycorrhizal development and plant growth will be detected in the field in future studies.

Table 4.9. Types of eucalypt ECM association and procedures required for their diagnosis (DLM = Dissecting Light Microscope, CLM = Compound Light Microscope).

Association	*Diagnosis*			*Definition*
(Illustrations)	*Eye*	*DLM*	*CLM*	*(References)*
Typical compatible (Fig. 1.6, 4.14A–C)	• mantle • thicker, branched roots	• mantle • hyphae	• Hartig net	— well-developed mantle and Hartig net with elongated epidermal cells, or hyphae extending into cortex (see Chapter 1)
Incompatible (Fig. 4.13C, 4.15E)	• thin roots	± mantle • hyphae	– Hartig net • wound reaction	—mantle poorly developed —Hartig net absent or abnormal —excessive phenolic production (Malajczuk et al. 1982, Molina & Trappe 1982, Burgess et al. 1994)
Partially compatible (Fig 4.13D)	± thicker, branched roots	± mantle • hyphae	± Hartig net	—limited hyphal penetration between epidermal cells, which remain compact —thin mantle due to limited hyphal proliferation (Burgess et al. 1994)
Superficial (Fig. 4.13F, 4.15B)	• thin ECM roots	• thin mantle • hyphae	• Hartig net	- differs from typical ECM by having a thin Hartig net, due to the lack of expansion of epidermal cells and thin mantle (Malajczuk et al. 1987)
Mesophellia type	• fruit bodies in soil	• ECM roots in fruit body	• Hartig net	—ECM roots occupy a zone inside the peridium of hypogeous sporocarps of *Mesophellia* and *Castorium* species (Dell et al. 1990)
Tuberculate (Fig. 4.6GH, 4.14DE)	• large structures in soil	• ECM roots in rind	• Hartig net	—compact clusters of ECM roots within a rind of fungal tissue (Dell et al. 1990) May be mistaken for a hypogeous fruit body unless sectioned

Chapter 5

PURE CULTURE ISOLATION OF FUNGI AND THE PRODUCTION OF INOCULUM

5.1. INTRODUCTION

In this manual, Chapters 5, 6 and 7 concern methods used to manipulate mycorrhizal associations, with the eventual aim of producing mycorrhizal plants and evaluating their performance in the glasshouse or field. This information is organised so that laboratory-based activities are covered in this chapter, glasshouse and nursery activities in Chapter 6 and field experimentation in Chapter 7. However, mycorrhizal research programs will often incorporate several of these levels of organisation, and will also require information about mycorrhizal roots and fungi to be obtained using methods described in earlier chapters.

Procedures for sterile culture isolation, isolate selection and inoculum production of ECM fungi are essential parts of research programs which aim to harness the potential of these fungi for plantation forestry (Palmer 1971, Molina & Palmer 1982). Such a program often aims to acquire and maintain isolates of ECM fungi in a pure culture collection, for one or more of the following reasons.

1. To allow physiological experiments with particular fungi, where the chemical and physical environment for fungus growth is carefully regulated. This allows the impact of specific conditions on fungi to be examined and provides information which may be required for large-scale inoculum production.
2. To produce large or small amounts of fungal inoculum which contains actively growing fungal mycelium and is free of contamination by other organisms, for use in experiments or for practical applications.
3. To perform axenic culture synthesis experiments to test host–fungus compatibility under conditions where no other organisms are present. This also allows clean root material of a known age to be produced for morphological and physiological studies.
4. To maintain the biodiversity of ECM fungi, particularly of fungi which are associated with rare plant species, or where large-scale habitat loss is of major concern.

Figure 5.1 is a flow chart which shows how a research program which aims to produce inoculum of ECM fungi could be organised. This scheme demonstrates how preliminary studies which concern optimising culture conditions for particular fungi and comparing inoculum forms are a prerequisite for large-scale inoculum production for use in forestry, horticulture or agriculture. Many of the procedures described here were developed by researchers in the CSIRO Forestry Laboratory in Perth, Western Australia, including Nick Malajczuk,

Shirley Snelling, Neale Bougher, Susan Bolsenbroek, Janine Catchpole, Trena Burgess, Inez Tommerup and Tatsuya Shiraishi. This chapter only concerns methods used to produce axenic cultures and inoculum of ECM fungi. Information on the propagation of VAM fungi is provided in Chapter 3.

There is a trade-off between the simple conditions in laboratory experiments, where factors can be controlled but results may not be relevant to the real world, and more complex field experiments where there is less control of experimental factors, but results are likely to be more relevant (Fig. 5.2). From Table 5.1 it can be seen that the advantages of sterile culture methods are generally due to the simplicity of axenic systems, which allow easy observation and manipulation of mycorrhizas, while their disadvantages are due to the highly artificial nature of these systems.

Axenic culture experiments have been used to examine the tolerance of fungal isolates to adverse factors such as pH extremes, toxic levels of metal ions, water stress, temperature extremes and calcium availability (Brundrett 1991). However, there may be poor correlation between in vitro responses to particular factors and responses to the same factors in the field (Hung & Trappe 1983, Coleman et al. 1989). Thus we must be careful when interpreting the results of simplified experiments, as fungal physiology may be influenced by growth in a highly artificial environment (Table 5.1).

Axenic culture synthesis experiments have been frequently used, especially to test host–fungus compatibility and to provide material for anatomical studies, but host–fungus interactions may also be influenced by these artificial conditions (Table 5.1). Further research comparing the structure and function of artificially synthesised mycorrhizal associations with those occurring in the field is required before the relevance of these experiments can be fully established (Peterson & Chakravarty 1991).

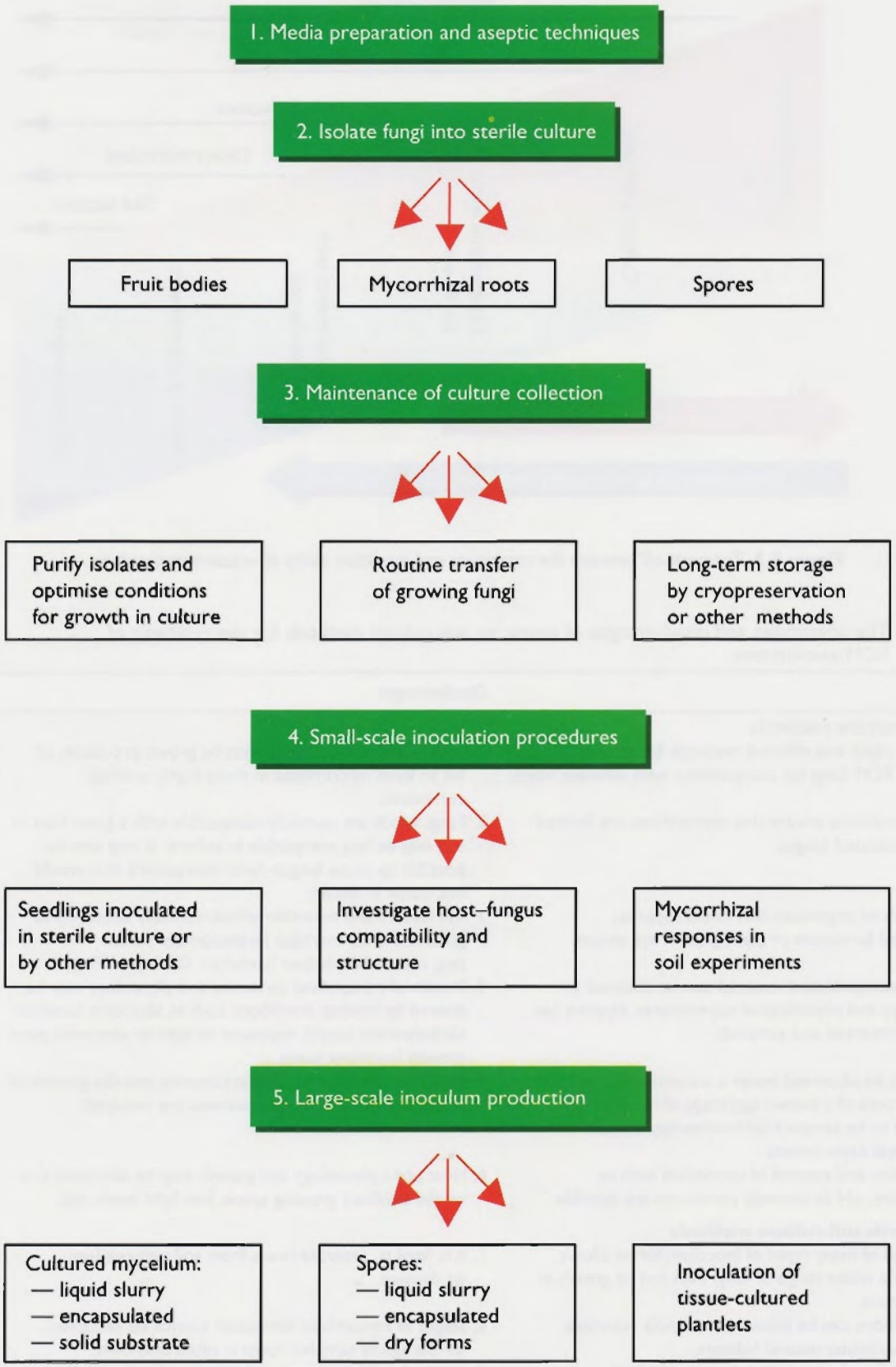


Figure 5.1. *Flow chart illustrating stages in a research program designed to investigate methods for the isolation of ECM fungi, selection of superior isolates and development of effective methods for large-scale inoculation with them.*

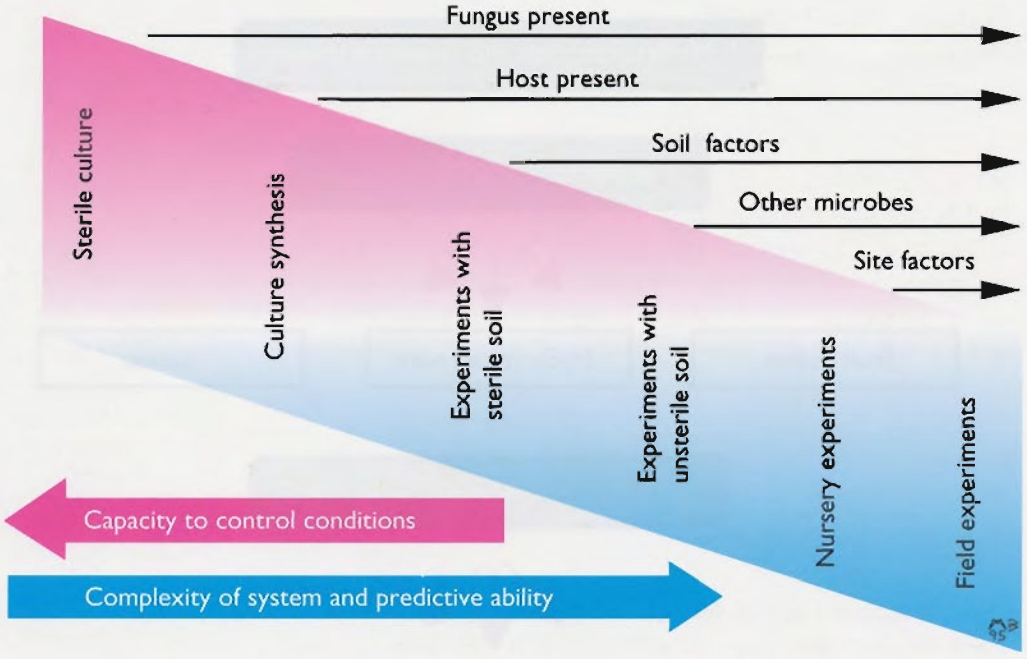


Figure 5.2. *The trade-off between the complexity and predictive ability of experimental systems.*

Table 5.1. The advantages and disadvantages of axenic vs. soil culture methods for the synthesis of ECM associations.

Advantages	*Disadvantages*
1. Axenic culture methods	
1. Relatively rapid and effective methods for screening amenable ECM fungi for compatibility with different hosts.	1. Some compatible fungi cannot be grown in culture, or fail to form mycorrhizas in these highly artificial conditions.
2. Axenic conditions ensure that mycorrhizas are formed by the inoculated fungus.	2. Fungi which are normally compatible with a given host in soil may be less compatible in culture. It may also be possible to cause fungus–host interactions that would not occur in nature.
3. Soil factors or organisms that could suppress mycorrhizal formation or plant growth are absent.	3. Soil factors and microbes which normally assist hyphal growth and mycorrhizal formation are absent (e.g. mycorrhiza helper bacteria – Garbaye 1994)
4. Clean, uncontaminated material can be obtained for microscopy and physiological experiments. Hyphae can easily be observed and sampled.	4. Details of mycorrhizal structure and physiology may be altered by unusual conditions such as abundant external carbohydrate supply, exposure to light or abnormal plant growth hormone levels.
5. Events can be observed under a dissecting microscope, to allow roots of a known age/stage of mycorrhizal formation to be sampled for microscopy or used in physiological experiments.	5. Expensive facilities for axenic culturing and the growth of plants in a controlled environment are required.
6. Manipulation and control of conditions such as temperature, pH or osmotic conditions are possible.	6. Host plant physiology and growth may be abnormal due to the confined growing space, low light levels, etc.
2. Non-sterile soil-culture methods	
1. The choice of many types of inoculum forms allows the use of a wider range of fungi than can be grown in sterile culture.	1. It is hard to separate roots from soil without loss or damage.
2. Soil conditions can be adjusted to closely resemble those in particular natural habitats.	2. Stages in mycorrhizal formation cannot be observed, so the age of sampled roots is often unknown.
3. Extrapolation of experimental results to field conditions is less difficult.	3. It is impossible to control contamination by unwanted microbes and soil animals
4. Plant growth is more natural in a less stressful environment.	4. Soil hyphae are difficult to observe or sample.

A. Aseptic techniques

Basic equipment for laboratory-based studies of ECM fungi are listed and an introduction to basic procedures provided below.

Creating a sterile working environment

Some procedures, such as initial isolation from fungus fruit bodies, can be carried out over any clean surface. However, it is best to do this in a laboratory laminar flow hood (Fig 5.4), or indoors where air-borne contamination due to dust, etc. can be minimised. The routine transfer of fungal isolates in a culture collection should be done only in a laminar flow hood to minimise the chance of loss due to contamination (Fig. 5.3). Laminar flow hoods must be maintained by sterilising the working surface before each use and by routine inspections to ensure that air-flow is adequate and filtration has removed all microbes.

Sterilising equipment

Utensils such as scalpels, forceps and probes must be sterilised each time they are used (Fig 5.3D). The most convenient way of doing this is to dip them in 70% ethanol and then heat them to incandescence over a flame (their tip should have a red glow). The utensil must then allowed to cool (in a laminar flow hood) before use. Utensils must be sterilised before each operation carried out to initiate a sterile culture or transfer fungal material between two sterile culture vessels.

B. Culture media

Table 5.2 lists components of some of the more common media which are used to culture ECM fungi. These general purpose media are useful for isolating fungi and maintaining culture collections, but many other media types which are optimised for particular fungi have been developed and can be found in the scientific literature (Molina & Palmer 1982, Fries 1983a,b, Ohta 1990).

Making media

Culture media for ECM fungi must be accurately formulated so that the results of experiments can be replicated in the future. It is best to use purified sources of inorganic salts and organic nutrient supplements (Fig. 5.3A). An accurate analytical balance is required to weigh ingredients and a pH meter should be used to adjust the final pH by adding drops of acid (10% HCl) or alkali solution (10% KOH). Agar is added to solidify media to the degree required (0.8% w/v is usually sufficient, but 1.0% or 1.5% w/v agar is often used) before sterilising in an autoclave for 15–20 min. at 121°C.

Preparing culture vessels

Culture vessels are filled with sterilised agar media in a laminar flow hood using a calibrated dispenser, or by pouring by hand. Some typical volumes are 20 mL for 90 mm Petri

Equipment list

Reagents
- sterile distilled water
- culture media ingredients:
  - agar
  - essential inorganic nutrients in pure chemical forms
  - organic nutrient supplements (vitamins, amino acids, etc.)
  - additives to control contamination (antibiotics, etc.)

Equipment
- laminar flow hood (which maintains a strong, uniform flow of air that has been filtered to remove microbes over a sterile work surface)
- forceps, scalpel, dissecting needles, wire loops, etc. for manipulation
- cylindrical cork borers for cutting plugs from fungal cultures (5 mm, 7 mm, 10 mm, etc.)
- strips of plastic film (food wrap) or tape for sealing plates
- marking pens for labelling and colour-coding media.

Sterilisation
- 70% ethanol in a jar
- controlled flame from a gas bunsen burner, alcohol lamp, etc.
- autoclave for sterilising media, etc. (at 121°C for 20–60 min.)

Making media
- large flasks, beakers or bottles (to mix and autoclave media)
- sensitive balance to weigh ingredients
- agar
- pure mineral salts (chemical reagents)
- other ingredients (vitamins, antibiotics, etc.)
- water bath (to keep media at correct temperature for pouring)
- pH meter to correct media pH (10% HCl and 10% KOH in dropper bottles)
- adjustable flow-through syringe to dispense media

Culture vessels (purchased as sterile, or autoclaved)
- Petri dishes (small – 65 x 15 mm and large – 90 x 15 mm).
- isolation tubes (5 mL volume with screw top lid)
- flasks for liquid media
- plant tissue culture jars, etc.

Storing cultures
- temperature-controlled incubators or rooms
- incubator with shaker for liquid cultures

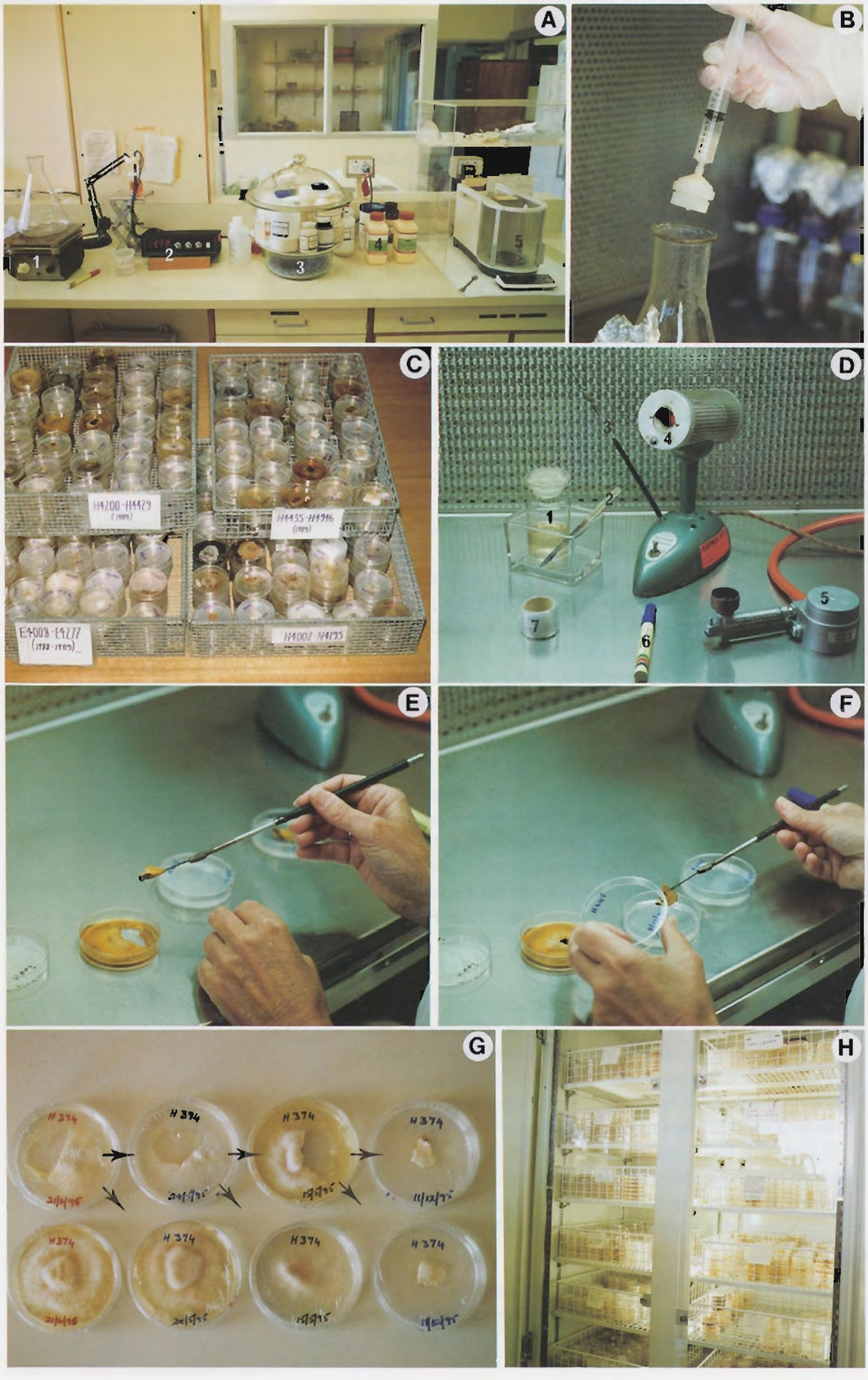

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Figure 5.3 *Equipment and methods for the maintenance of fungal cultures.*

A. *Media preparation area with: 1—magnetic stirrer, 2—pH meter, 3—desiccator for storage of hygroscopic media components, 4—pure chemical reagents and 5—accurate balance.*

B. *Filter-sterilising an antibiotic which must be added after autoclaving media.*

C. *Examples of fungal isolates in a culture collection which are maintained at 20°C and transferred onto new nutrient agar every three months.*

D. *Equipment for transferring fungi in cultures in a laminar flow hood: 1—70% ethanol, 2—scalpel, 3—dissecting needle, 4—electrical heater for sterilising equipment (alternative to 5), 5—bunsen burner, 6—marker pen to label cultures and 7—plastic film used to seal plates.*

E–F. *Transferring a segment of agar with mycelia from the original culture onto a fresh agar plate to start a new culture.*

G. *Periodic culture transfers used to maintain a fungal isolate. Segments of culture were transferred onto two plates with different media types every three months (arrows). Older plates were kept for nine months as backups.*

H. *Temperature-controlled incubator opened to show fungal cultures.*

dishes, 10 mL for 65 mm Petri dishes and 2 mL for 5 mL field isolation tubes. Agar media should be allowed to cool to below 60°C before it is poured, or excessive condensation will result. Media can be kept in a water bath (60°C) if it cannot be poured before it solidifies in the flask.

Potato dextrose agar (PDA) medium

This commonly used mycological media is not listed in Table 5.1, as it can be easily prepared from a commercially available powder (39.0 g/L Bacto Potato Dextrose Medium from Difco Laboratories), as directed by the container labelling. Otherwise, the filtered extract from 200g of potatoes boiled in water, plus 20 g dextrose and 16 g agar can be used to make one litre of media solution (Difco Laboratories 1953).

Table 5.2. Composition of some media which are commonly used for the isolation and culture of mycorrhizal fungi.

Ingredient	*MMN*[1]	*PACH*[2]	*FDA*[3]	*Gamborg*[4]	*Fries*[5]
Mineral nutrients (mg/L w/v)					
$(NH_4)_2HPO_4$	250				
NH_4Cl			500		
$(NH_4)_2SO_4$				1652	
$C_4H_{12}N_2O_6$*		500			1000
KH_2PO_4	500	1000	500	163	200
$MgSO_47H_2O$	150	500	500	246	100
$CaCl_22H_2O$	50	50		147	26
NaCl	25				20
Fe EDTA	20	20			
$FeSO_47H_2O$				28	1
H_3BO_3		2.8		3.1	
$MnCl_22H_2O$		3.0			
$MnSO_4H_2O$				10.1	0.81
$ZnSO_47H_2O$		2.3		2.0	0.88
$CuCl_22H_2O$		0.63			
$CuSO_4$				3.0	
$Na_2Mo_42H_2O$		0.27		2.0	
$CoCl_26H_2O$				0.025	
KI				0.75	
Carbohydrate source (g/L w/v)					
Maltose		5			
Glucose	10	20	20		4
Malt extract	3		5		1
Dextrose				5	
Vitamins (µg/L)					
Thiamine HCl	0.1	0.1	see Table 5.3		
Agar (g/L w/v)					
Range			8.0–15.0		
pH					
Adjusted pH to	5.8	5.4	5.0	5.5	5.5

*Notes: 1 = Modified Melin Norkans medium (Marx 1969), 2 = Pachlewski medium (Pachlewski & Pachlewski 1974), 3 = Ferry & Das (1968), 4 = modified Gamborg medium (Gamborg et al. 1968, Tatsuya Shiraishi, pers. comm.), 5 = Fries medium for spore germination (Fries 1978), * = ammonium tartrate.*

C. Media additions

Solutions of vitamins, growth promoters and antibiotics are added to the media in varying quantities and mixtures (Table 5.3). Some fungi make their own vitamins, but many are deficient (partially or absolutely) in thiamine (B_1), biotin (B_7), riboflavin (B_2), pyridoxine (B_6), nicotinic acid (B_3) and others (Kendrick 1992, Griffin 1994). The addition of vitamins to media may improve the success of isolation attempts, or the growth and health of fungal isolates being maintained in culture (Molina & Palmer 1982).

Soil micro-organisms closely associated with fungi are potential contaminants, e.g. bacterial contaminants from isolation attempts of *Laccaria* are often found to be predominantly Gram negative. Antibiotics can be added to isolation media to reduce the risk of bacterial contamination (Table 5.4). The fungicide benomyl can also be added to media, using the same method as antibiotics, to inhibit the growth of Ascomycetes (including many saprophytic conidial fungi), when the ECM fungi being cultured are known to be Basidiomycetes (Erland & Söderström 1990a). However, ECM fungi vary widely in their capacity to tolerate antifungal chemicals such as benomyl (Hutchinson 1990), so it is advisable to always also use media without antibiotics or fungitoxic compounds when isolating fungi.

Cultures of ECM fungi vary in their responses to the pH of nutrient media (Hung & Trappe 1983). Non-toxic buffers such as HEPES (Section 4.6) can be added to media to help stabilise pH (Erland & Söderström 1990a, Ohta 1990).

Vitamins



Vitamins can be added to fungus isolation and culture media to promote the growth of fungi (Table 5.3). It is usually best to make a stock solution containing higher concentrations of all vitamins and add a small volume of this solution to media (i.e. make a stock solution with 100X the final concentrations of all vitamins and add 10 mL/L of media). Some vitamins may have to be dissolved in a small volume of an appropriate solvent before being added to the media.

Antibiotics

A filter sterilised antibiotic solution containing a mixture of antibiotics can be added to media as described below (Table 5.4). Only a few antibiotics should be used together as some combinations can produce cytotoxic effects. Many antibiotics are heat labile and should be added to media by filter sterilisation, after autoclaving (see below).

Filter sterilisation

Membrane filters (0.2 µm Millipore) attached to a syringe with a special adaptor are used to filter sterilise antibiotic solutions or other media additives which cannot be autoclaved (see Figure 5.4B). Filtered antibiotics should be added to autoclaved media only after it has cooled to approximately 45°C (i.e. not hot enough to denature the antibiotics, or cool enough to solidify).

Storing media

Media with vitamin supplements should be used promptly or stored in a refrigerator, as many vitamins are light and heat sensitive, becoming ineffective after a few weeks.

Table 5.3. Examples of vitamins and nutrient supplements which can be added to fungal culture media.

	Concentration (mg/L)			
Vitamin solution	*Fries (1978)*	*Gamborg (et al. 1968)*	*Molina & Palmer (1982)*	*Ohta (1990)*
Thiamine HCl	0.1	10.0	0.5	3.0
Pyridoxine HCl	0.1	1.0		0.05
Riboflavine	0.1			
Biotin	0.025		0.01	0.05
Nicotinic acid	0.1	1.0		0.05
Folic Acid				0.03
p-Amino benzoic acid	0.1			
Panthonic acid	0.1			
Myoinositol	10.0			
Carnitine chloride				0.01
Adenine $H_2SO_4 2H_2O$				0.03
Choline chloride				0.03

Table 5.4. Examples of antibiotic or fungistatic components that have been added to isolation media to help control microbial contamination.

Antibiotics	*Concentration (mg/L)*	*Susceptible organisms*	*Reference*
Streptomycin	0.133	Bacteria	Chu-Chou (1979)
Chlortetracycline	10	Most bacteria	Tommerup & Malajczuk (unpublished data)
Streptomycin	10		
Colistin sulphate	10		
Trimethoprim	10		
Chlortetracycline	30	Bacteria	
Benomyl	1	Ascomycete fungi	Erland & Soderstrom (1990a)

Figure 5.4. *Equipment and methods for sterile culture isolation of fungi and examples of cultures.*

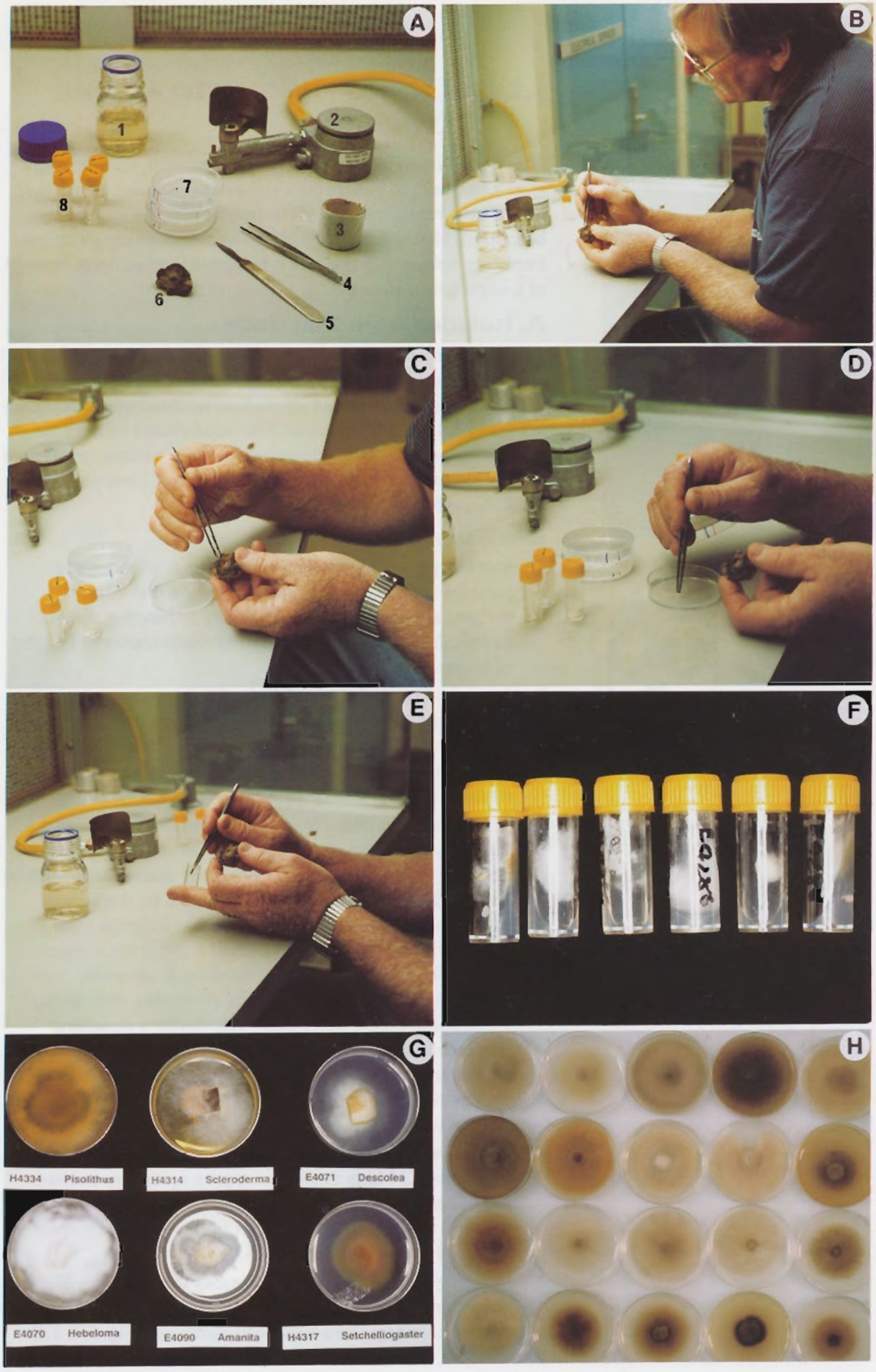
A. *Equipment for isolating fungi in a laminar flow hood: 1—70% ethanol, 2—bunsen burner, 3—cellophane wrap for sealing plates, 4—forceps, 5—scalpel, 6—fungal fruit body, 7—Petri dishes with nutrient agar, 8—isolation tubes (5 mL) with agar slants.*

B–E. *Stages in the isolation process using a fruit body* (Pisolithus *sp.) fractured to expose internal tissues.*

F. *Isolation tubes with initiated cultures from a fungal fruit body after incubation for several weeks.*

G. *Some examples of cultures showing differences in the appearance of the mycelia of different fungi.*

H. *Variations in the culture morphology of isolates within the genus* Pisolithus.


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H4334 Pisolithus
H4314 Scleroderma
E4071 Descolea
E4070 Hebeloma
E4090 Amanita
H4317 Setchelliogaster
H

Key points

- Generally, the younger the fruit body of a fungus is, the greater the probability of successful isolation of that fungus. The tissue of fruit bodies tends to differentiate or degenerate with age and also becomes more susceptible to bacterial and other contamination.
- Some fungal genera are particularly difficult to isolate into pure culture from fruit body tissue (e.g. *Russula, Inocybe*), while others are more amenable to culturing (e.g. *Pisolithus, Scleroderma, Laccaria* and others in Tables 5.3, 5.4).



5.2. ISOLATING FUNGI INTO AXENIC CULTURE

Fruit bodies of ectomycorrhizal fungi collected in the field are most often used to initiate pure cultures on nutrient media, but mycorrhizal roots, sclerotia, rhizomorphs and spores have also been used (Molina & Palmer 1982). The aim of isolating cultures from fungi collected in the field is to obtain purified living material of a single fungus isolate.

A. Isolation from fruit bodies

The most successful method involves aseptically removing a small portion of tissue from the fruit body with forceps and placing it on agar in specially prepared sterile field-isolation tubes, as shown in Figures 5.4 and 5.5. A laminar flow hood should be used for this procedure if available. However, any clean surface can be used and we have successfully isolated fungi in hotels, campsites, and stationary vehicles during field trips.

Isolation procedure

1. Sporocarps should first be brushed free of adhering soil particles and fractured carefully to ensure that internal surfaces are not contaminated by dirt or handling. Avoid insect-infected tissue as indicated by the presence of bruising or burrowings.
2. After the fruit body is carefully broken open, a small amount of tissue (approximately 2 mm^3) is removed with fine forceps that have been flame sterilised after immersion in 70% ethanol using an alcohol lamp or bunsen burner and placed on agar in a Petri dish or tube (see Figs 5.4, 5.5).
3. For mushrooms, tissue from the apex of the stem or in the cap itself, just above the gills, is often the most successful for obtaining cultures, but tissue from other places should also be used. Isolation from immature fruit bodies (at the button stage) is more likely to be successful than isolation from mature fruit bodies. For puffballs and truffles, the immature internal fertile tissue (before it becomes powdery at maturity) is most suitable. Delicate specimens, such as tiny mushrooms, should be carefully split (under a dissecting microscope if necessary) to find the largest areas of relatively undifferentiated tissue (often at the base of the stipe). Alternatively, a surface layer can be peeled off to expose clean internal tissue.
4. A number of different media should be used when attempting to isolate ECM fungi as growth requirements vary between fungi (Table 5.2). We often use FDA, PACH and MMN media, with and without broad spectrum antibiotics to help control microbial contamination (Table 5.4).
5. Isolates growing in field-isolation tubes should be transferred onto Petri dishes and repeatedly subcultured until all contaminant organisms have been eliminated (Figs 5.3, 5.5).

Several different media can be used to select which one is best for growth in culture. Many ECM fungi are very slow growing and need to be regularly (daily) monitored for emerging hyphae. These can be observed by placing a sealed Petri dish or isolation tube under a dissecting microscope, or the low powered (10X) objective of a compound microscope.

6. Cultures are usually incubated at 20–25°C, but growth rates at this temperature usually necessitate fresh subcultures to be made every 8 to 12 weeks. Growth rates of mycelium on agar vary greatly between fungi, and slower growing isolates may not continue to grow after transfer from isolation tubes.
7. Other soil fungi or bacteria may cause serious contamination of fledgling mycorrhizal cultures due to their rapid growth and sporulation. Contamination may be detected macroscopically (if they are fast-growing or have contrasting colours), but it is important also to check cultures under the microscope for contaminant structures such as sporulating conidial fungi or bacterial colonies (see D below). Contaminated isolation tubes should be discarded immediately unless the isolate contained was growing well and the other tubes had failed. Isolates with fungal contamination restricted to some part of the tube can be rescued by carefully subculturing the uncontaminated section onto new plates with antibiotics.

B. Isolation of fungi from within mycorrhizal roots

Mycorrhizal fungi have successfully been isolated from surface sterilised mycorrhizal root tips (Zak & Bryan 1963, Chu-Chou 1979, Erland & Söderström 1990a). Apparently, this procedure has only been used with pine roots, but it should also be possible with roots from other hosts. The procedure outlined below, which is based on Erland & Söderström (1990a), uses an agar medium with antibiotics and a fungicide to reduce the frequency of contamination. Chu-Chou (1979) compared different concentrations of calcium hypochlorite (0, 0.7%, 1.0%, 1.5% w/v) and found that surface sterilisation with this chemical adversely affected recovery rates, especially at the two highest concentrations. Surface sterilisation with H_2O_2 may be less detrimental to fungal growth.



1. Selected mycorrhizal roots of uniform appearance are washed with running tap water until free of soil particles. An ultrasonic water bath or mechanical shaker can be used to help clean roots by vigorous agitation. Roots can be enclosed in a nylon mesh bag (300 µm mesh) to facilitate transfer between solutions. Many roots should be used as the success rate may be very low.
2. Roots are pre-treated with a wetting agent by immersion in a 0.2% aqueous solution of Tween 80 (v/v) or another detergent then rinsed in tap water.

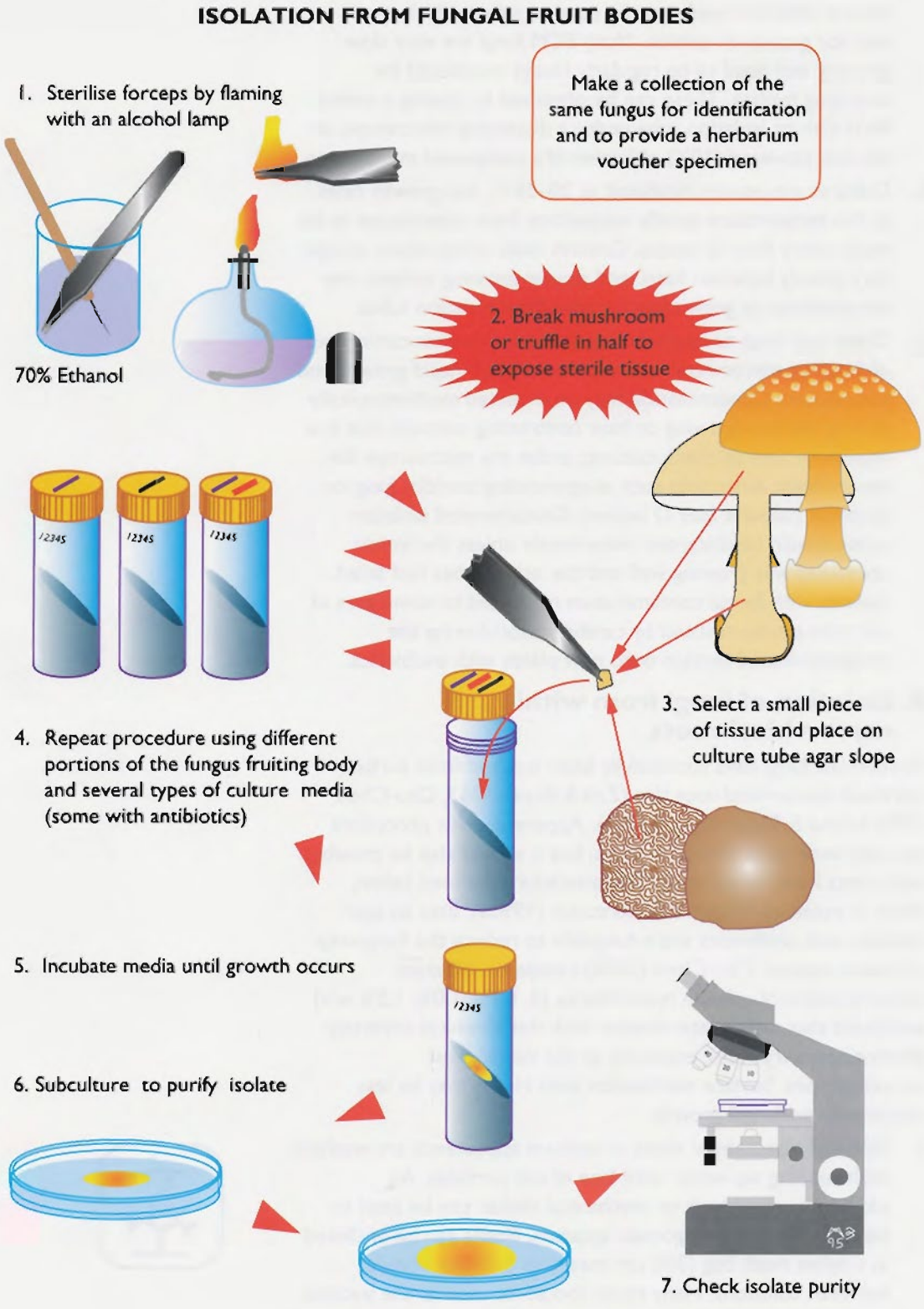


Figure S.5. *Isolation of ECM fungi from fruit bodies. Sterile forceps (1) are used to select tissue from fractured fruit bodies of a fungus (2). Selected material is then placed on nutrient agar (3). This process is repeated using different media types and portions of the fungus (4). The media is incubated for several weeks (5), then any resulting mycelial cultures are subcultured (6) and checked for contamination (7).*

3. Roots are surface sterilised in 30% aqueous H_2O_2 (v/v) for 20 seconds then immediately transferred into 1 L of sterile water for further rinsing. All subsequent steps should be carried out under sterile conditions (in a laminar flow cabinet).
4. The bag containing surface-sterilised roots is placed on agar media in a Petri dish and opened to remove the roots. Erland & Söderström (1990a) used 1/2 strength MMN agar (Table 5.2) with chlortetracycline and Benomyl (Table 5.4). Root tips can be dissected into small pieces using sterilised fine pointed tweezers. Ideally, segments of root and fungus tissue from the inner mantle or Hartig net region should be used, to ensure that only one organism is isolated, but isolation from whole root tips may also be successful.
5. Root pieces are individually arranged on nutrient agar on Petri plates (5–10 per plate) then incubated and examined daily to remove contaminants and monitor the development of fungal colonies. Some growing mycorrhizal fungus colonies, recognised by their appearance under a dissecting microscope (see D below), are then transferred into fresh agar media.
6. Alternatively, root pieces can be washed by vigorous agitation in 10 changes of sterile distilled water (for 2 min. each), before root tips are excised, blotted dry and plated out under axenic conditions (Chu-Chou 1979).

C. Spore germination

Spore germination in axenic culture is generally much easier to achieve with saprophytic fungi than with ECM fungi, but it has been possible to germinate spores of some species of ECM fungi (Fries 1987). One example of a spore germination media is provided in Table 5.2. For some genera of ECM fungi, spore germination may be promoted by the presence of (1) other micro-organisms (yeasts, fungi, etc.), (2) growing hyphae of the same species, (3) growing roots of a host seedling, or (4) activated charcoal, which absorbs inhibitory substances in the media (Fries 1983a, 1987). The production of culture isolates from a single germinated spore is a valuable tool for studying the genetics and biology of fungi (Fries 1983b). A method of spore germination described by Fries (1978) is outlined below.

1. Spores are obtained from a spore print by suspending a fungus fruit body over a sterile Petri dish for several hours (petroleum jelly can be used to stick the top of a mushroom cap to the inside of the Petri dish lid). This procedure is repeated several times to increase the probability that a sample of sterile spores has been obtained.
2. Working in a laminar flow cabinet, spores are suspended in 0.5 mL of sterile distilled water. The suspension is diluted with additional sterile distilled water to obtain a concentration between 0.5×10^6 and 2×10^6 spores/mL. A haemocytometer should be used to count a subsample of the spore suspension to determine how much dilution is required.



3. 50 µl of diluted suspension is spread evenly over the surface of a Petri dish containing spore germination agar media (Table 5.2). Plates are sealed with Parafilm™ or cellophane, after leaving them overnight in the flow hood so excess water can evaporate.
4. Spore germination is visibly assessed twice a week and the percentage of germinated spores estimated. Hyphal colonies can be observed under a dissecting microscope.

D. Identification of mycorrhizal fungi and microbial contaminants in culture

The success of attempts to isolate mycorrhizal fungi can only be judged by distinguishing between mycorrhizal fungi and contaminants in culture. This task is initially very difficult, but becomes easier with experience. Many genera of ECM fungi have a characteristic appearance or growth form in culture that allows them to be recognised (see Tables 5.5, 5.6). When isolating a new fungus it is best to make three or more attempts, using different fruit bodies, roots, etc., to allow the desired fungus to be recognised if several fungi emerge.

Various techniques can be used to ensure new fungal cultures are free of contaminants. Bacterial contamination is easily recognised under the microscope and can often be eliminated through the addition of antibiotics to culture media (Table 5.4). Suspected soil fungi can be verified, in many cases, microscopically because they produce conidial spores in culture, while most ECM fungi do not. Saprobic fungi often generally have relatively narrow hyphae without the clamp connections produced by many basidiomycetes in culture. Confirmation of successful isolation is not as simple for Ascomycetes and those Basidiomycetes that do not produce clamp connections. Thus it may be necessary to confirm that isolates are ECM fungi by inoculating a host plant with them under conditions suitable for ECM formation.

Morphological characteristics, such as the colour and texture of mycelial colonies, can often be used to distinguish particular isolates (Fig. 5.4GH). A wide range of information obtained by observation or by experimentation can be used to help identify or confirm the identity of ECM fungi growing in cultures (Hutchinson 1991). This information includes variations between fungi in their:



1. capacity to utilise different organic carbon and nitrogen sources,
2. growth rates at different temperatures,
3. tolerance to different fungistatic or fungitoxic chemicals,
4. polyphenol oxidase activity,
5. reactions of mycelia to stains such as Diazonium blue B,

6. culture morphology (texture, growth rates, colours, media pigmentation, and
7. microscopic morphology (clamp connections, the shape, thickness, branching, colour and growth patterns of hyphae, production of hyphal swellings, or strands, etc.).

This information has been used to produce a key which could be used to identify a wide range of fungal isolates obtained from ECM roots in North America (Hutchinson 1991). Other techniques which analyse chemical constituents such as proteins or DNA should also be of great value in identifying fungi isolated into pure culture in the future.

Table 5.5. Cultural characteristics of some epigeous genera of ECM fungi from Australia.

Genus	*Growth rate*	*Habit*	*Culturability*	*Appearance*
Amanita	variable, moderate	surface and aerial	amenable	white mycelium, or with slight colouration
Austroboletus	slow to moderate	submerged to aerial	moderate	white aerial mycelium which may become orange-brown when submerged
Boletellus	moderate	aerial and submerged	moderate	small white aerial cultures at first, developing a submerged system of hyphae later
Boletus	some rapid though most are moderate	mostly aerial	amenable	generally white and cotton wool-like but variable, sometimes light brown
Cortinarius	generally slow	surface to aerial	difficult	white cultures which may have varying degrees of purple tinge
Descolea	variable	centrally aerial, but submerged peripherally	difficult	varying from white to light orange brown in colour, centre darker than margins
Hebeloma	rapid	surface and aerial	amenable	white rumpled culture which may produce fruit bodies in culture
Laccaria	slow to moderate	submerged	difficult	white mycelium with clamps
Lactarius	slow	variable	difficult	variable
Leccinum	slow	some aerial, mostly submerged	difficult	some white aerial mycelium with extensive submerged mycelium
Paxillus	slow	submerged	some are difficult	white to light brown mycelium, agar may be discoloured
Phylloporus	variable	variable	variable	variable
Ramaria	moderate	surface	variable	thick woolly texture, orange-brown
Russula	very slow	surface	difficult	prefers liquid culture
Strobilomyces	slow	mostly submerged	difficult	some chocolate-brown aerial hyphae with pale submerged mycelium
Suillus	slow	submerged and aerial	difficult	light-coloured aerial mycelium, darker when submerged, discolouring PACH media
Thelephora	moderate	submerged and surface		hyaline and white mycelium
Tricholoma	slow	variable	moderate	variable
Tylopilus	variable	variable	variable	variable

Table 5.6. Cultural characteristics of some hypogeous genera of ECM fungi from Australia.

Genus	*Growth rate*	*Habit*	*Culturability*	*Appearance*
Castoreum	moderate	extensively submerged and surface	moderate	white mycelium, cultures have indistinct margins
Chamonixia	moderate	submerged	moderate	light to hyaline mycelium
Descomyces	slow	mostly aerial with some submerged hyphae	difficult	light-coloured cotton wool appearance, hyphae with clamps
Elaphomyces	moderate to rapid	submerged	moderate	cultures may be distinctively black or white
Gautieria	slow	submerged and surface	difficult	white to hyaline mycelium
Hydnangium	slow	submerged	difficult	yellow-cream and hyaline mycelium
Hysterangium	moderate	submerged and surface	moderate	white and hyaline mycelium
Labyrinthomyces	slow to moderate	submerged	moderate	cream and hyaline mycelium
Malajczukia	moderate	submerged with some surface growth		white and hyaline mycelium
Mesophellia	moderate	surface and submerged	moderate	white mycelium
Pisolithus	moderate to rapid	primarily aerial	amenable	orange-brown mycelium, discolouring media golden-brown
Pseudohysterangium	variable	variable	variable	variable
Scleroderma	moderate to rapid	surface, aerial and submerged	amenable	white to brown mycelium, cultures may discolour media to bright yellow
Setchelliogaster	slow	aerial with submerged margins	moderate	golden-brown cotton wool-like appearance
Thaxterogaster	moderate	aerial with submerged margins	moderate	rich golden-brown cultures with cotton wool-like appearance
Timgrovea	slow	variable	moderate	variable
Zelleromyces	very slow	submerged	difficult	cultures produce small amounts of brown and white mycelium

5.3. MAINTENANCE OF FUNGAL ISOLATES

Once isolates of ECM fungi have been obtained from fruit bodies, etc. (Section 5.2), they can be maintained on nutrient agar by subculturing them onto fresh media at regular intervals when their food supply is exhausted. Culture collection maintenance requires constant attention to avoid problems caused by the factors listed in Table 5.7. Choosing the best media for particular fungi requires trials with different media types (Table 5.2) and perhaps also different nutritional supplements (Table 5.3) to obtain adequate rates of growth in culture. Information on media preferences for some key genera of Australian ECM fungi is provided in Table 5.8. Various sizes and shapes of culture vessels can be used, but small (65 mm diameter) Petri dishes provide sufficient surface area for fungal growth, yet are small enough to be stacked efficiently in incubators (see Fig. 5.3C).

Routine subculturing of established isolates is necessary to maintain active mycelial growth. Changing the composition of media used to culture fungi can sometimes rejuvenate old cultures which have become weak. However, the characteristics of fungal cultures may well alter over time, due to physiological or genetic changes resulting from life in an artificial environment. The performance of fungal isolates may be improved after re-isolation using mycorrhizal roots from a tissue culture inoculation trial, or fruit bodies that have emerged from pots where plants inoculated with a fungal strain have been allowed to grow in soil for some time (Thomson et al. 1993). The protocol for transferring fungi outlined below and illustrated in Figure 5.3 has been used successfully to keep fungi for up to 16 years in our laboratory.

A. Transferring fungi to start new cultures



1. Using a scalpel or dissecting probe which has been flame sterilised, cut a wedge-shaped segment of agar from the edge of the parent colony which includes a substantial amount of new hyphae and a small segment of its starting (grandparent) culture (see Fig. 5.3E). If hyphal growth is limited use up to half of the parent colony.
2. Place one segment upright in the centre of a new 65 mm Petri dish containing agar media and seal well with plastic film (see Fig. 5.3F). Repeat with a second media type (see Fig. 5.3G). We generally use both MMN and PACH media, but use additional, more specialised media types for difficult fungi (Table 5.8).
3. Monitor new plates to ensure that fungi grow from plugs and plates are free of contamination. Keep several generations of older plates in reserve. Do not keep all the cultures of a fungus in the same location or incubator.
4. Cultures are usually incubated at 20–25°C, but growth rates at this temperature usually necessitate fresh subcultures to be made every 8 to 12 weeks, before nutrients in the media are completely utilised or inhibitory substances accumulate.

Table 5.7. Enemies of living fungus culture collections, the problems they cause and suggested remedies.

Enemy	*Problem*	*Countermeasures*
Mites	Traffic of tiny feet across plates causes contamination	Quarantine all soil and plant materials, as well as field equipment, by keeping them well away from cultures and seal plates (see below)
Desiccation	Shrinkage of growing media, osmotic stress	Seal plates by wrapping tightly with stretched plastic film or tape
Temperature fluctuations	Condensation (water droplets can affect fungi)	Keep cultures at a constant temperature
Time	Genetic or physiological changes in growth or mycorrhizal formation	Use cryopreservation, or re-isolation from mycorrhizas/ fruit bodies from pot cultures

Table 5.8. Some examples of culture media which have been successfully used to grow members of some important genera of Australian ECM fungi.

Fungal genera	*Media types (see Table 5.1)*
Pisolithus	MMN, PACH
Scleroderma	MMN, PACH
Laccaria	MMN, PACH, Gamborg
Hebeloma	PDA, MMN, PACH
Descolea	MMN, PACH
Lactarius	Yeast extract agar, PACH
Amanita, Boletus	Gamborg, MMN, PACH
Tricholoma	Yeast extract agar, PDA
Suillus	MMN, PACH
Rhizopogon	MMN, PACH
Hysterangium/Hydnangium	PDA, MMN, PACH
Labyrinthomyces	PDA

B. Preservation of fungal cultures

An alternative to maintaining growing cultures is long-term storage which may minimise loss of vigour and genetic changes that often occur after repeated subculturing. Long-term storage is needed to reduce the frequency of subculturing and preserve the phenotype of the original collection in culture. Methods which have been used for the long-term preservation of fungal isolates include storage at 20–25°C, refrigeration, covering cultures with oil or water, freezing, desiccation, freeze-drying, or cryopreservation in liquid nitrogen (Smith & Onions 1994). Smith & Onions (1994) compare these methods in terms of cost-effectiveness, culture longevity and genetic stability. They recommend liquid nitrogen storage as the most reliable for many fungi. A possible procedure is briefly introduced below, but more research into the cryopreservation of ECM fungi is required before a more detailed protocol can be provided. Information on other methods which have been used to store fungal isolates can be found in Smith & Onions (1994).

Cryogenic preservation of fungi

Preservation of fungal cultures by cryogenic methods (i.e. liquid nitrogen at −196°C) has many advantages. The long-term conservation of an isolate provides insurance against loss, contamination, genetic change and a more practical means of maintaining cultures. Published information indicates that methods enabling 100% recovery after cryofreezing are seldom attained for many species of plants, animals and fungi. The feasibility of cryostorage for most ECM fungi is as yet unknown, although some fungi have been successfully stored. The methods for investigating cryopreservation of ECM fungi are illustrated in the flow chart (Fig. 5.6). Factors such as the effect of cryoprotectant concentration and time of exposure to the cryoprotectant on the time taken to regrow cultures may be tested. Different freezing and thawing regimes also need to be tested. More than 65% recovery of subsamples after freezing and thawing may be considered as an acceptable result for preservation in liquid nitrogen.

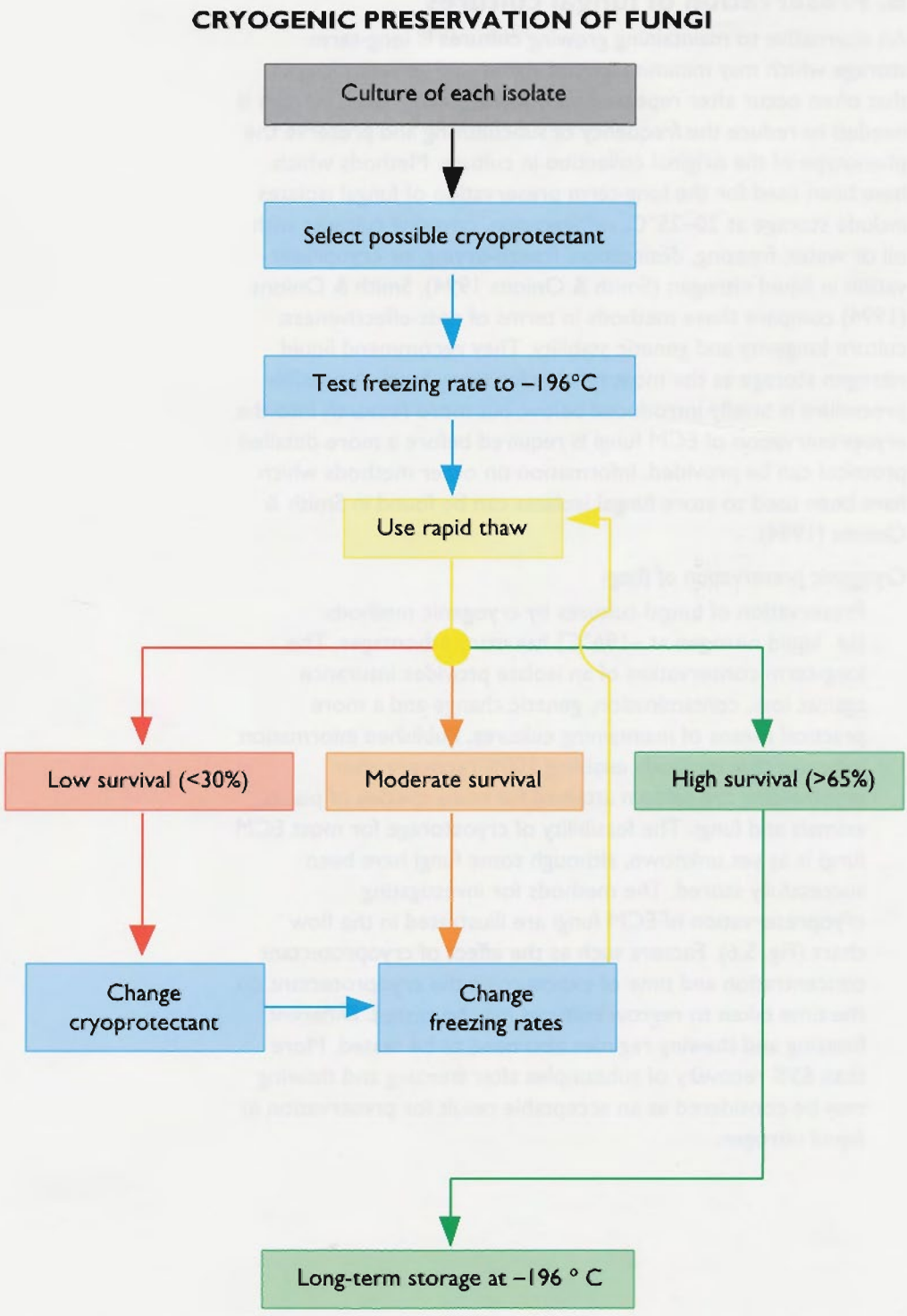


Figure 5.6. *Flow diagram of methods for optimisation and testing procedures for the cryopreservation of ECM fungi in liquid nitrogen (I. Tommerup, unpub. data).*

5.4. THE SYNTHESIS OF ECTOMYCORRHIZAL ASSOCIATIONS

Mycorrhizal associations can be synthesised by exposing seedlings of host plants to inoculum of ECM fungi in a favourable growth environment. An introduction to procedures for producing mycorrhizal plants on a small scale for research purposes is provided in this section. Methods which are more suitable for mycorrhizal inoculation on a larger scale are considered in Section 5.5.

Mycorrhizal synthesis experiments are most frequently used to test host–fungus compatibility, provide clean root material for structural investigations and conduct physiological investigations where growth conditions are rigidly controlled. However, there are advantages and disadvantages of sterile culture procedures relative to experiments using soil (Table 5.1). Sterile-culture synthesis techniques allow axenically germinated seedlings or tissue cultured plantlets to be inoculated with ECM fungi in vitro (Malajczuk & Hartney 1986, Burgess et al. 1994). Two of these procedures are outlined below, but other efficient methods for producing mycorrhizal seedlings are also available. These alternatives include plastic growth pouches where roots grow on a paper lining (Fortin et al. 1983), various types of large culture vessels which enclose mycorrhizal seedlings (Malajczuk et al. 1982, Chilvers & Gust 1982) and a large Petri dish method which encloses the roots but not the shoots of seedlings (Wong & Fortin 1989). Peterson & Chakravarty (1991) discuss the advantages and disadvantages of these procedures and a wide range of other synthesis techniques which have been used.

A. Seed surface sterilisation

Axenic culture procedures require surface sterilisation of seeds and their subsequent germination on nutrient agar, so that no unwanted microbes are present. A variety of chemicals has been successfully used to surface sterilise seeds. Normand & Fortin (1982) found that 30% hydrogen peroxide (H_2O_2) was the most effective at producing axenic germinants, but sodium hypochlorite is also commonly used to surface sterilise seeds.



1. Healthy, uniformly sized seeds of a host plant (such as a *Eucalyptus* species) are selected for use.
2. Seeds can be pre-treated with 70% ethanol containing a small amount of surfactant (such as 0.1% Tween) to ensure uniform wetting of seeds during surface sterilisation.
3. 5% sodium hypochlorite (NaOCl) can be used to surface sterilise seeds for 10 to 30 minutes (optimum times and concentrations vary with different plant species). The sterilised seeds are then thoroughly washed with three changes of sterile water.
4. Alternatively, seeds can be sterilised in a 30% hydrogen peroxide (H_2O_2) solution for 5 to 30 minutes before rinsing in sterile water.

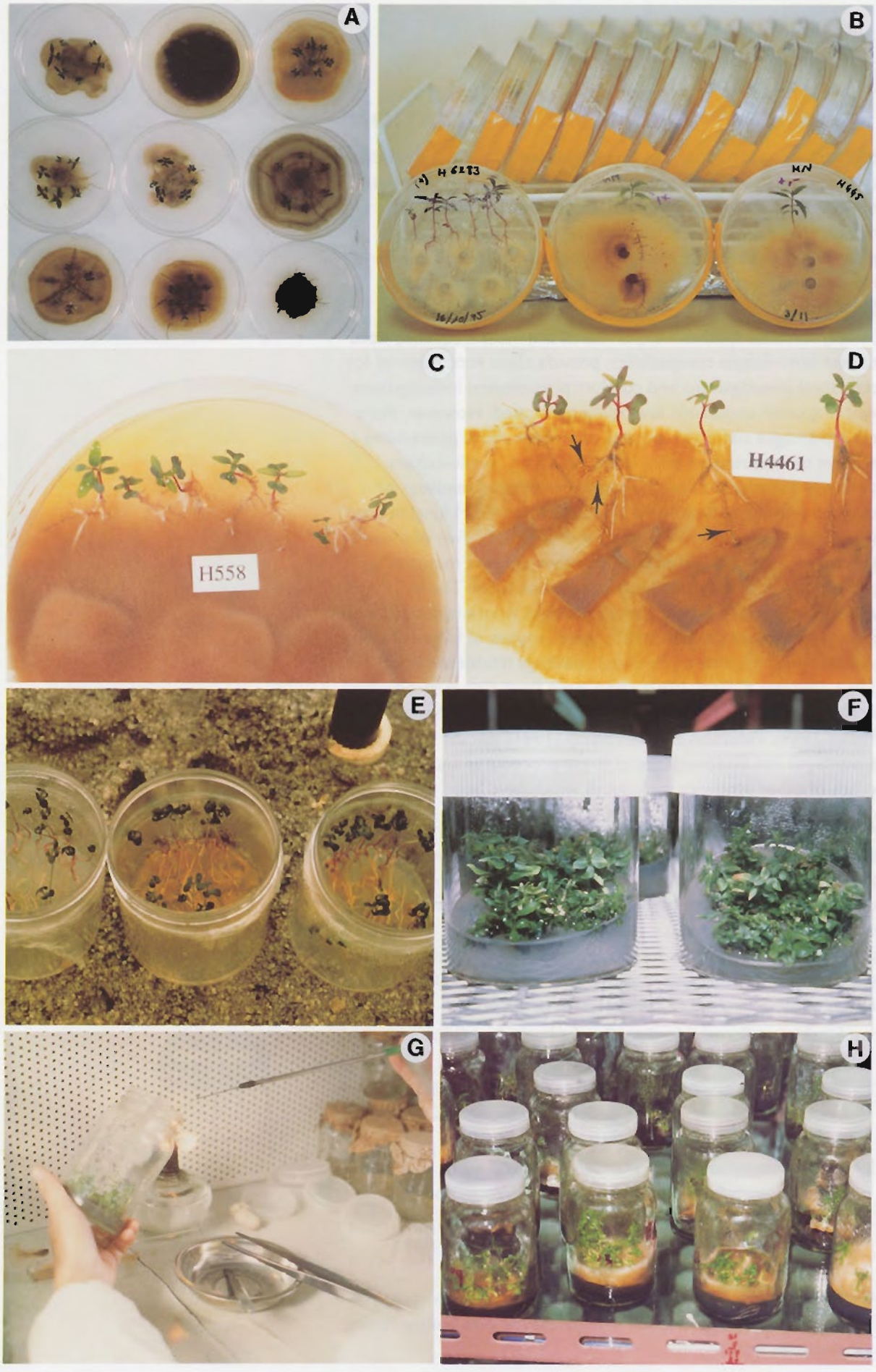

A
B
C
H558
D
H4461
E
F
G
H

Figure 5.7. *Axenic culture methods used for ECM synthesis for experimental purposes or the large-scale production of mycorrhizal seedlings.*

A. *Axenically germinated* Eucalyptus *seedlings placed onto cultures of ECM fungi. Root growth is disorganised on these horizontally incubated plates.*

B–D. *Mycorrhizal formation by axenically germinated eucalypt seedlings on fungus cultures which have been incubated in a slanted upright position.*

C–D. *Mycorrhiza formation by* Pisolithus *isolates (arrows).*

E. *Mycorrhizal inoculation of eucalypt seedlings grown with ECM fungi in tubs.*

F–H. *Mycorrhizal inoculation of tissue-cultured eucalypt plantlets for later use in the field in China.*

F. *Eucalypt plantlets growing in sterile culture.*

G. *Inoculation of plantlets with a mycorrhizal fungus.*

H. *Tissue-cultured plantlets after substantial growth of an inoculated mycorrhizal fungus (*Pisolithus *sp.).*

5. Seeds of different plants vary in their capacity to tolerate sterilising agents and the time required for them to become sterile. Consequently, it is best to try a range of different sterilisation times to determine which produces the most sterile germinants, when working with a new plant species.
6. Sterile seeds are individually placed on agar in Petri dishes for germination to begin and to check for contaminating microbes. The culture media should contain traces of B and Ca to help seeds germinate, and a carbohydrate source so that contaminants can be detected.

B. Mycorrhizal synthesis in sterile culture

This procedure requires a sterile environment such as a laminar flow hood, sterile equipment and careful axenic technique (see Equipment list). Plants with small seeds often provide the best results in axenic culture systems as they are less likely to rapidly outgrow their living space than plants with large seeds. We have used this procedure with *Eucalyptus* species and a range of *Pisolithus* isolates, but it should also work with many other ECM fungi and hosts.

The axenic-culture synthesis technique described below was developed to test the compatibility of host plants and mycorrhizal fungi and to provide mycorrhizal root material for microscopy (Burgess et al. 1994). The use of angled Petri plates with agar media provides superior results to horizontal plates or tissue culture vessels, because roots of seedlings are relatively straight, grow towards the fungal inoculum, and excess moisture flows away from fungal colonies (Fig. 5.7B–D). Agar media can be covered with a layer of porous cellophane to prevent fungal hyphae and roots from growing into the media.

1. Petri dishes (140 × 20 cm) are filled with 30 mL of complete nutrient agar based on a solution for plant nutrient culture that maximises mycorrhizal root development by eucalypts (Table 6.8). This media is solidified with 0.8% agar and supplemented with 0.01% glucose to support fungal growth.



The agar is overlain with cellophane disks that have been washed, boiled, rinsed, then autoclaved before use. These disks are cut from porous transparent cellophane sheets and are slightly smaller than the Petri dishes.

2. Hyphal plugs 5 mm in diameter are taken from the edge of 14-day-old colonies of ECM fungi growing in agar media in Petri dishes with a cork borer. These plugs are placed approximately 1.5 cm apart in 2 rows (4–5 plugs per row) in the centre of the Petri dish (see Fig. 5.7B–D). Plate edges are then sealed with plastic film or tape.
3. Plates are incubated at a constant temperature (20–25°C) in the dark, until sufficient hyphal growth has occurred to result in colonies which are 1–2 cm in diameter (1–2 weeks for most fungi, but some may take several months).
4. To synthesise mycorrhizas, axenically germinating seeds (see above) with a short radicle are placed in a row 1–3 cm above the level of outermost growing hyphae in the plates with fungi. The plates are then resealed and incubated on a slant (approx 20° from the vertical), so that seedling roots grow towards the fungus and adhere to the cellophane surface, while excess water drains away from the roots (see Fig 5.7B). Plates can be sealed with Parafilm™ at the top to allow some gas exchange, but must be tightly sealed with waterproof tape at the bottom.
5. The timing of initiation of plates with mycorrhizal fungus inoculum and germinating seedlings will depend on the rates of hyphal growth by the fungus and root elongation by the host. This information should be obtained from a preliminary experiment. These factors will also determine where seedlings should be placed on plates, so that lateral root production occurs near active hyphae and seedling shoots are not overwhelmed by the fungus.
6. Plates with fungi and seedlings are incubated in a controlled environment chamber with a temperature of 25°C and light levels which are adequate for seedling growth provided by fluorescent tubes (grow lights providing a light level of 200 $\mu E/m^2/s$ are adequate). Avoid excessive heating of plates by the light source (cooling can be provided by a fan).
7. Inoculation of tissue-cultured plantlets is possible by similar procedures. Plantlets produced by standard tissue culture propagation methods can be successfully inoculated with ECM fungi after transfer to root initiation media (Malajczuk & Hartney 1986, Tonkin et al. 1989). Examples of the use of this procedure for the commercial production of mycorrhizal eucalypts in China are shown in Figure 5.7F–H.

5.5. LARGE-SCALE INOCULUM PRODUCTION

Inoculation of eucalypt seedlings with ectomycorrhizal fungi should aim to ensure that seedlings have extensive infection at the time of outplanting from the nursery to the field. A range of inoculum forms is available for delivering ECM fungi to seedlings, which contain various forms of cultured mycelium and spores from fungal fruit bodies (Figure 5.8).

Inoculum of ECM fungi should satisfy the criteria listed below to be cost-effective to produce and efficient to use. To be practical, inoculum must also be relatively easy to produce consistently using available technology (Marx & Kenney 1982, Marx 1991, Castellano 1994, Kuek 1994). It is also important that inoculum is in a physical form which can be efficiently transported and stored before use. It must be possible to use fungal isolates which have been selected for their capacity to promote the growth of a particular host in the environment where it will be grown. Mycelium produced axenically or spores in inoculum should be in a physiological state which allows them to survive transport and storage, yet still allows rapid initiation of mycorrhizas after inoculation. Any carrier or bulking material used should protect fungal biomass against physiological stress during production. The choice of inoculum forms may involve a compromise between effectiveness and practicality.

A wide range of nursery conditions and techniques is used, so that the optimum procedure in one situation may not be applicable in another. Inoculation of eucalypt seedlings with ECM fungi should aim to ensure that seedlings have extensive root colonisation at the time of outplanting from the nursery to the field (Chapter 6). However, the ultimate test of the performance of fungal isolate or inoculum forms is the measurement of yield responses in field trials (Chapter 7).

Criteria for selecting inoculum forms

1. *Compatibility* with ECM fungi which have been selected for their capacity to increase tree growth and survival is essential.
2. *Physiological condition* of fungal propagules (mycelium or spores) should be maintained in a way which allows them to survive in storage and rapidly initiate mycorrhizas after application.
3. *Efficiency* of inoculum use is required for ease of handling, storage and uniform application.
4. *Consistency* of inoculum is required (i.e. different batches of the same fungus should have a similar capacity to initiate mycorrhizas).
5. *Purity* of inoculum is essential, so that unwanted organisms are not included.
6. *Cost-efficiency* of large-scale inoculum production must be possible (i.e. the labour and material costs of inoculum must be low relative to the cost of seedling production).

A. Types of inoculum

The relative advantages and disadvantages of different inoculum forms are summarised in Table 5.9. The effectiveness of an inoculum type is ultimately determined by its capacity to propagate the association, but other considerations such as quality control, ease of use and its capacity to be produced and used in a cost-effective manner are also important.

Soil inoculum containing one or more mycorrhizal fungi has been used successfully in the past, but is no longer considered to appropriate (Castellano 1994, Chalermpongse 1995). In particular, mycorrhizal formation can be irregular and inconsistent, fungi may not be appropriate for the early growth performance of seedlings, and there is a risk of introducing pathogens into the nursery.

Inoculation with spores has been widely practised, as spores are relatively easy to apply by mixing with nursery soils prior to planting, or watering onto nursery beds and seedling containers (Castellano et al. 1985, Castellano & Trappe 1985). Spore pellets

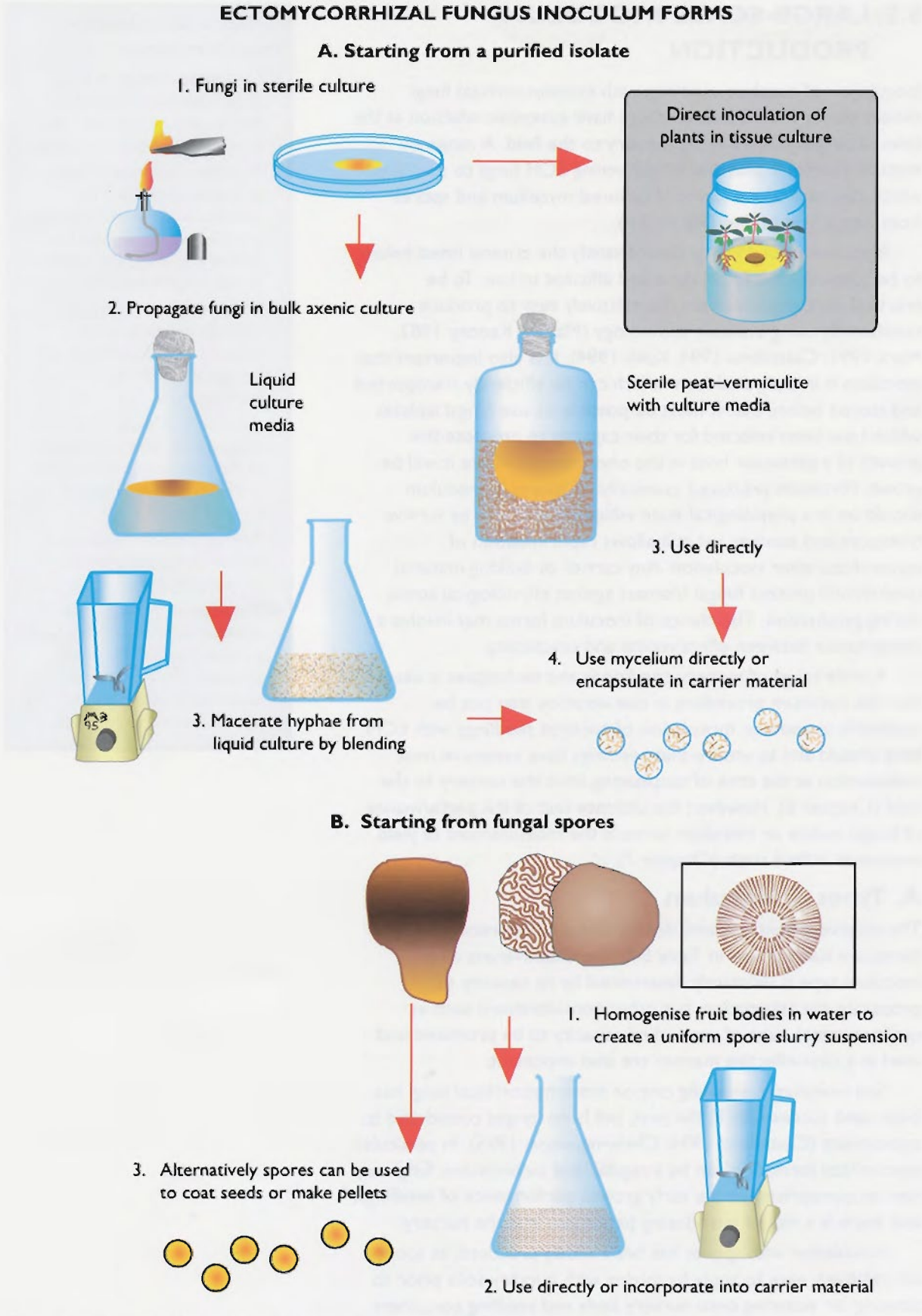


Figure 5.8. *Methods which can be used to produce inoculum of ECM fungi for use on a large scale.*

and spore coating of seeds have also been used as ECM inoculum (Theodorou & Bowen 1973, de la Cruz et al. 1988, Marx et al. 1984). Major drawbacks to the use of spore inoculum are the restrictions to fungal species that produce abundant sporocarps, the poor germination or low viability of spores of some species and that large spore numbers are often required for colonisation of roots (Fries 1983, Cordell et al. 1988).

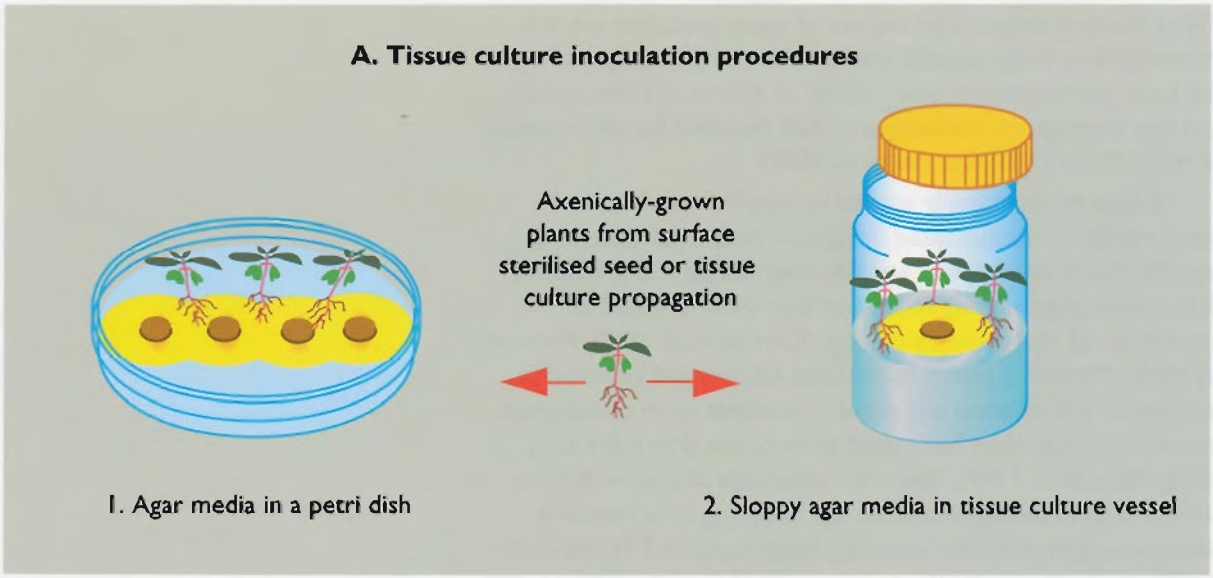
Mycelia are commonly applied in peat–vermiculite mixes, liquid media, or encapsulated in alginate beads. Advantages of mycelial forms of inoculum are that they can be more effective than spores in colonising roots and they allow large-scale production of single strains of fungi (Cordell et al. 1988, Marx et al. 1989). Mycelium grown in mixtures of peat and vermiculite moistened with mineral and organic nutrients or in cereal-grain based media has often been used as inoculum (Marx & Kenny 1982, Marx et al. 1989). The main advantage of solid-substrate culture is its relatively simple set-up, requiring only standard laboratory equipment for culturing fungi (Section 5.1). However, production is limited by the space required to grow cultures and the labour required to initiate and dispense inoculum (Marx & Kenny 1982, Kuek 1994). On a commercial scale, quality and an absence of microbial contamination are difficult to maintain (Kendrick & Berch 1985, Le Tacon et al. 1985).

Mycelial slurries can be applied directly to seedling roots (Boyle et al. 1987, Parladé et al. 1996), but must be used rapidly to ensure effective root colonisation. Cultured mycelium can be protected from desiccation and damage during storage and after application to soil by encapsulation in alginate beads. This technology has been used to produce inoculum of Australian isolates of *Laccaria, Hebeloma, Descolea, Elaphomyces* and *Pisolithus* (Kuek et al. 1992). Beaded forms of inoculum are highly effective per unit volume of inoculum, can be stored for several months and are suitable for application by mechanised planting systems such as fluid drills (Kuek et al. 1992).

B. Spore-based inoculum forms

Use of ECM spores is widely used in forest nurseries (Castellano 1994). However, use of spores as inoculum is most effective with species that produce sporocarps in large numbers, that are easily collected. For example, the puffballs *Pisolithus* and *Scleroderma* have been used extensively because they fruit abundantly in forests and plantations and contain large numbers of spores (Fig. 5.10A). Truffle-like fungi also make good spore inoculum, in that much of the sporocarps consist of spore-bearing tissue (Fig. 2.33), but they occur below the soil surface, so it may be difficult to excavate sufficient numbers for inoculation. While mushrooms accumulate fewer spores than truffles or puffballs, their caps can also be used as a source of spores for inoculation (Fig. 5.10B). Some spore inoculation procedures for nursery seedlings are described below.

MYCORRHIZAL INOCULATION PROCEDURES



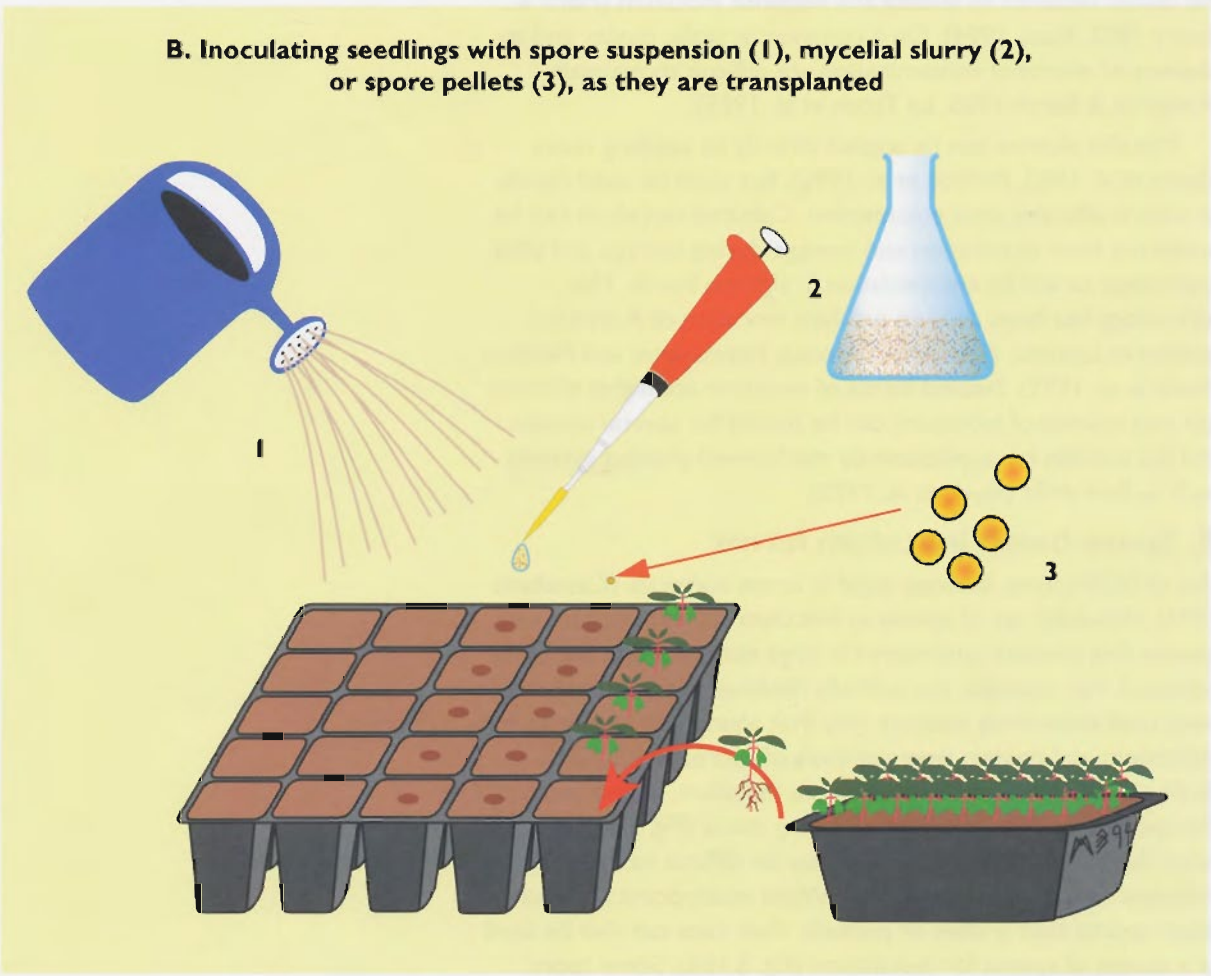


Figure 5.9. *Methods for applying inoculum of ECM fungi to seedlings in sterile culture (A) or in the nursery (B).*

Table 5.9. Comparison of the relative advantages and disadvantages of different forms of inoculum used to propagate ECM fungi.

Type of inoculum	*Advantages*	*Disadvantages*
A. Soil from under host	• inexpensive and easy to obtain • fungi should be well-adapted to local conditions	• soil bulky and labour-intensive to collect • high risk of introduction of pathogens and weeds • specific fungi cannot be selected • inoculum levels may be low
B. Spore slurries	• very efficient means of inoculating large numbers of seedlings • specialised equipment and procedures not required, but can be used for large-scale nursery inoculation	• fruit body collection may be difficult • may be ineffective due to slow germination or low spore viability • cross-contamination more likely
C. Spore pellets	• efficient method for storage, transport and application of spores • most effective with sequestrate fungi such as *Pisolithus* and *Scleroderma*	• only a few fungi have been used • expensive pelleting machine required • other micro-organisms may be present
D. Slurries of cultured mycelium	• pure inoculum of a selected fungus can be produced • relatively fast ECM formation can occur, as mycelium is ready for immediate growth	• facilities and supplies expensive • personnel must be highly trained • inoculum storage limited • only fungi which grow well in culture and survive fragmentation can be used
E. Encapsulated forms of cultured mycelium	• as in D above • protection of mycelium allows transport, storage and efficient application	• as in D above, but storage less of a problem
F. inoculation of seedlings or plantlets in sterile culture	• as in D above • ECM fungi occupy roots before seedlings are planted in soil	• as in D above, but may be even more expensive in terms of facilities and trained personnel required

Collecting spores



1. Most large sporocarps occur during late autumn in the northern and southern hemispheres or during the onset of the wet season in the tropics. Fungi should be located, collected and carefully identified as described in Chapter 2. Freshly collected sporocarps should be kept in paper bags for transport to the laboratory.
2. Sporocarps are brushed free of adhering debris. Larger sporocarps are usually cut into smaller pieces and any vegetative components such as the stipe can be discarded. Truffles are generally bisected before drying them. Some material of each fungus should be kept as a herbarium specimen (Chapter 2).
3. Sporocarps may be used to provide fresh or dry spore inoculum. If dry spores are required, forced air drying at temperatures below 35°C should be used to avoid loss of spore viability (Fig. 5.10C).
4. Dried sporocarps are crushed manually in plastic bags to ensure minimal loss of spores and cross-contamination between fungi. They are then gently sieved through 200–500 µm sieve to standardise the inoculum particle size (Fig. 5.10D). Dry spores can be stored by refrigeration at

4°C, but fungi may quickly lose viability (Fries 1984). These spores can then be used in the procedures described below.

Utilising wet or dry spores

Wet or dried spores can be utilised in a variety of formulations to inoculate seedlings in the nursery. Some of the procedures which have been used are outlined below.



1. Wet spore suspensions are made by homogenising fruit bodies in water, then filtering them through cheesecloth or a fine screen (approx. 200 µm). Suspensions can be used immediately or stored at 4°C spore for up to one year (Castellano et al. 1985). Stored suspensions should be re-filtered to remove saprophytic fungi which often develop on the surface. Wet spores are used as described below.
2. Spore slurries are made by suspending powdered sporocarps (measured by weight) in the required volume of water (Fig. 5.10F). The optimum method for applying spore slurries will depend on the scale of the operation (Fig 5.9). For glasshouse experiments spores can be added to individual pots as aliquots at the base of transplanted seedlings (Fig 5.10G). In small nurseries suspensions can be added manually by watering seedlings flats with spore suspension in a watering can (Fig 5.9B). In mechanised nurseries suspensions can be automatically applied to large numbers of seedlings by adding a water injection system to the potting and seeding machinery (Fig. 5.10H). This latter approach allows spore slurries to be efficiently applied without slowing seedling production.
3. Spore powder can be bulked out by mixing it with sterilised fine sand (e.g. 1:100 w/w ratio) to facilitate handling. This dry mixture can then be mixed throughout a potting soil, or applied at the base of seedlings.
4. Dried spores can be compressed into pellets using expanding clay as an inert carrier (Fig. 5.11H). These pellets should contain approximately 1–2% spores to ensure efficient mycorrhizal formation. One pellet is placed at the base of a seedling during planting (de la Cruz et al. 1990).
5. Spores can also be used to coat seeds by mixing them with a binding agent such as clay (Marx et al. 1984). This method works best with large-seeded species of plants such as pines.
6. For all methods of spore inoculation, spore concentrations should be determined by counting spores in a subsample of inoculum with a haemocytometer.

C. Mycelium-based inoculum forms

Fungal mycelium produced in sterile culture may be used directly as inoculum or it may be associated with carrier materials including hydrogels, peat–vermiculite or cereal grains (Mark & Kenney 1982). However, mycelial cultures may not be suitable for all types of ECM fungi, as some fungi are presently difficult or impossible to grow in culture, and many others are too slow for the economical production of inoculum.



1. Cultures may be produced by incubating large containers with a solid substrate inoculated with an ECM fungus, such as large plastic bags (Fig. 5.11G). A peat–vermiculite mixture saturated with one of the standard nutrient media (Table 5.2) can be used for bulk inoculum production of ECM fungi (Fig. 5.11G). Prior to its use, this inoculum should be wrapped in cheesecloth and washed with sterile distilled water to remove excess nutrients, to limit unwanted microbial growth. This inoculum should be used immediately and handling minimised so that unprotected mycelium remains viable. On a larger scale, rotary drum vessels can be used to propagate fungi in solid substrate cultures (Marx & Kenney 1982).
2. Segments of agar containing mycelium can also be used to inoculate seedlings grown in tissue culture on root initiation media (Fig. 5.7E).
3. Mycelial cultures can be grown in the laboratory in flasks or other large culture vessels in a media made with a standard nutrient solution (Table 5.2) partially solidified with 0.3% agar (Figs 5.11AB). Flasks should be shaken periodically to fragment mycelium, or can be incubated on a rotary shaker. Some ECM fungi can also be grown on an industrial scale using liquid medium which is stirred and/or supplied with air within a large microbial culture vessel (called a bio-reactor, or fermentor). Several forms of inocula based on vegetative mycelium that can be produced via this technology are described below.
4. Fungal biomass from liquid culture may be applied directly as an inoculum slurry in a similar manner to spores (Fig 5.10G). The mycelium is usually fragmented in a blender for 10–60 seconds (Fig. 5.11C) depending on the consistency of the culture. Mycelia of many ECM fungi survive this treatment but some do not (Boyle et al. 1987). Fragmented mycelial cultures can be applied directly to seedlings or encapsulated as described below. Some nutrients contained in culture media will be applied with mycelial inoculum and might encourage the growth of saprophytic organisms after inoculation. However, our experience suggests this is a minor problem, perhaps because most nutrients are depleted by the time sufficient fungal biomass is produced in culture. Excess nutrients can be removed by washing mycelia with sterile distilled water immediately prior to use. The viability of homogenised slurries may vary due to the fungi used or


A
T1
T2
T3
B
C
D
2
1
3
E
F
Hydrotaea
H80
H62
G
H
1
2
3

Figure 5.10. *Formulating and utilising ECM fungus inoculum from spores.*

A. *Examples of sporocarps of sequestrate ECM fungi in the genera* Pisolithus *(T1) and* Scleroderma *(T2,3) from Thailand. These fruit bodies contain vast numbers of spores that can be used as inoculum.*

B. *Caps of a mycorrhizal fungus (*Laccaria *sp.) from a* Eucalyptus globulus *plantation, placed on paper to make a spore print. Both the caps and spore print were used as spore inoculum.*

C. *A fungus dryer using forced warm air (35°C) to dry fruit bodies of ECM fungi (arrows) for use as spore inoculum or for preservation as herbarium specimens.*

D. *Processing dried fungal fruit bodies (1), by crushing them and sieving through a 200–500 µm mesh sieve (2), to make a powder containing spores (3).*

E. *Sorting dried* Boletus edulis *fruit bodies in Kunming, China. These were collected for food, but could also be used as source of spore inoculum.*

F. *Making spore slurries by mixing a given weight of powdered fruit bodies with a specified volume of water plus a fews drops of a wetting agent (detergent).*

G. *Small-scale application of spore slurries (arrow) to holes in pots where pre-germinated seedlings will be planted, for a glasshouse experiment.*

H. *Large-scale application of spore slurry inoculum to seedling flats in a nursery (Bunnings Treefarms, Manjimup, Western Australia). A venturi system (1) was used to draw up spore suspension (2), which was applied while watering flats after seeding (3).*

preparation methods, so it is essential that the inoculum potential of slurries be tested before use. This can be done by dispensing aliquots onto nutrient media in Petri dishes, but some isolates will not grow from fragmented hyphae without the presence of host roots.

5. Homogenised mycelium from aseptic culture can be mixed with a sodium alginate solution (2% w/v in sterile distilled water) and then solidified into beads by adding drops of 0.7M calcium chloride solution (Le Tacon et al. 1985, Mauperin et al. 1987). This results in the encapsulation of hyphal fragments within beads of alginate gel, that can vary in size from 0.5 to 2 mm in diameter (Fig. 5.11E). A more advanced form of this inoculum is where fungal hyphae are allowed to continue growth within these beads (Kuek et al. 1992). This allows mycelium to recover from fragmentation before application, so they are more effective propagules. A similar procedure has been used to produce alginate encapsulated root fragments containing inoculum of VAM fungi (Strullu & Plenchette 1991).


A
B
C
D
E
F
G
H
1000 ml
H1058
H1438
H1101R
E1209
H1023R
MYCOGROE
MYCORRHIZAL TABLETS

Figure 5.11. *Formulating and utilising ECM fungus inoculum produced from cultured mycelium, or spores.*

A. *Flasks containing fungal cultures grown in partially solidified nutrient solution (0.3% agar). These static cultures were periodically shaken to break up the mycelium into smaller segments.*

B. *Flasks containing ECM fungi in semi-liquid culture (as in A), incubated with continuous agitation by a rotary shaker. Note that fungal mycelium has formed into large or small balls, which can be directly used as inoculum.*

C. *Homogenising mycelium from a culture with sterile water to produce mycelial slurry inoculum.*

D. *Mycelial slurries, ready to inoculate eucalypt seedlings in the glasshouse or nursery.*

E. *Mycelium of an ECM fungus encapsulated in alginate gel to form beaded inoculum.*

F. *Growth of mycelium from a bead of inoculum (star) and mycorrhizal formation (arrow) by a eucalypt seedling growing on a Petri dish.*

G. *Large-scale production of mycelium of a* Pisolithus *species in sterile peat–vermiculate amended with nutrients. Note the spread of mycelium from the point of inoculation down through the substrate (arrows).*

H. *Spore pellets (arrows), a commercially available form of inoculum, formed by compressing expanding clay and powdered ECM fungus fruit bodies.*

D. Storage of inoculum

The shelf-life of ECM fungus inoculum must be sufficient to allow its storage and transport. Dry spores should last for months or years, as they are in a physiologically quiescent state, but they should be used quickly once mixed with water. The shelf life of spore slurries made from fresh fruit bodies would likely be shorter than dried spores, but wet spores have been reported to last for up to two years with refrigeration (Castellano 1994).

Unprotected fragmented mycelium from culture normally survives while maintained in a sterile environment with nutrients, but its viability rapidly declines once washed free of nutrients. Solid-substrate (peat–vermiculite) cultured inocula of *P. tinctorius* or *H. crustuliniforme* can be stored for a month at 4°C (Cordell et al. 1988, Hung & Molina 1986). Alginate beads containing *Hebeloma westraliense* and *Laccaria laccata* mycelium cultured within the beads retained their ability to form colonies on agar plates after 6 months storage in water at 4°C (Kuek et al. 1992).

At present, there is insufficient information to allow us to make safe predictions about the capacity of different ECM fungi to survive in storage. Thus it is necessary for each new combination of mycorrhizal fungus and inoculum form to be tested to determine optimal storage conditions and longevity.

Chapter 6

MANAGEMENT OF MYCORRHIZAL PLANTS

This chapter contains information on the management of mycorrhizal plants in the glasshouse and nursery for experimentation and production of mycorrhizal seedlings for use in the field (Fig. 6.1). References to mycorrhizas in this chapter refer primarily to ECM fungi associated with eucalypts grown for plantation forestry, but the information presented is also relevant to other hosts and the growth of plants with VAM associations. Methods for producing inoculum of VAM and ECM fungi are presented in Chapters 3 and 5, respectively.

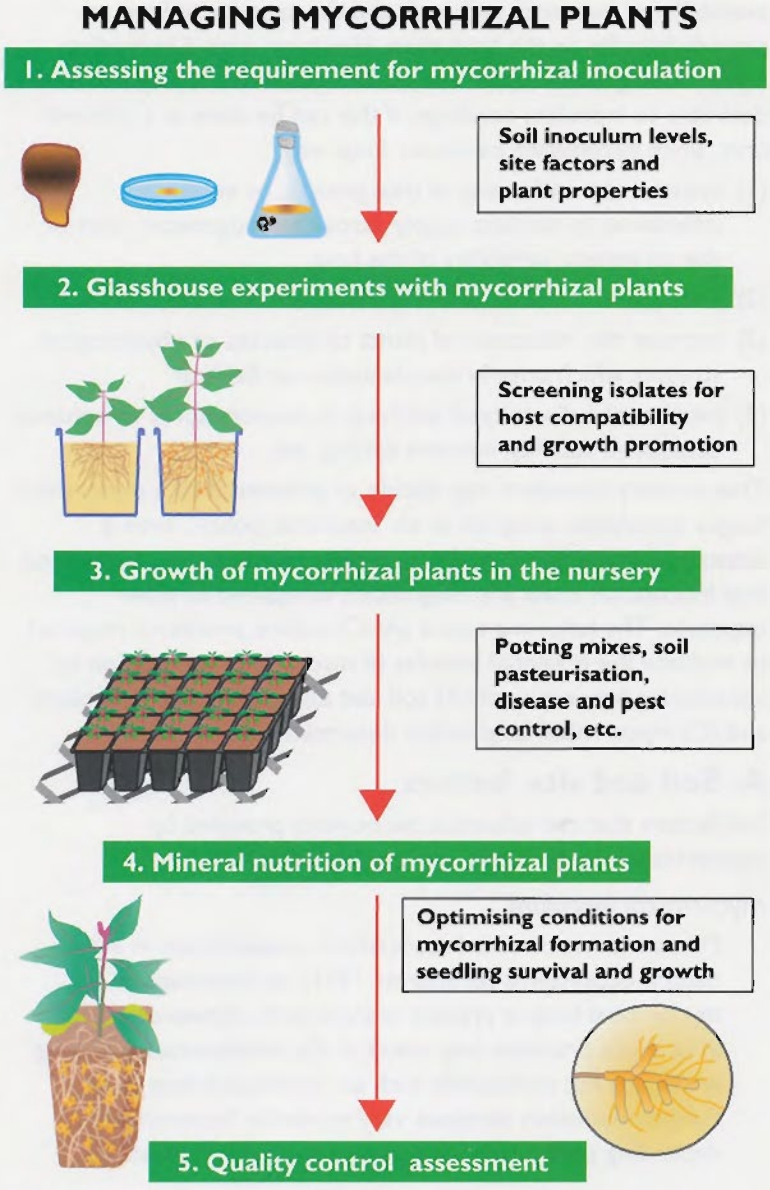


Figure 6.1. *Managing mycorrhizal plants.*

6.1 ASSESSING THE BENEFITS OF MYCORRHIZAL INOCULATION

Throughout this book, it has been necessary to assume that a decision to utilise mycorrhizal fungi has already been made, when presenting methods for inoculum production, field experiments, etc. However, the reality is that production of mycorrhizal seedlings is only likely if benefits from the manipulation of these fungi are expected. The potential benefits of mycorrhizal inoculation can be assessed by answering a series of questions concerning the availability of inoculum of appropriate fungi in soils and the benefits expected from mycorrhizal associations in a particular soil or habitat (Fig. 6.2).

The primary goal of mycorrhizal inoculation is to increase the yield of plants grown for plantation forestry, agriculture or horticulture. These growth responses depend on the mycorrhizal dependency of plant species, soil properties, especially the availability of nutrients such as P, and the capacity of fungi to provide benefits to the host plant. However, even if immediate mycorrhizal growth responses are not observed, it may still be desirable to inoculate seedlings, if this can be done at a minimal cost, since introducing particular fungi may:

(1) improve the uniformity of tree growth, by equalising differences in nutrient supply across heterogeneous sites or due to genetic variability of the host,

(2) produce fruit bodies which are desired for food or medicine,

(3) increase the resistance of plants to diseases or physiological stresses which arise in the plantation or field, or

(4) increase the diversity of soil fungi in monocultures to enhance ecosystem stability, nutrient cycling, etc.

Thus nursery managers may decide to proceed with a mycorrhizal fungus inoculation program as an 'insurance policy', even if substantial immediate benefits cannot be demonstrated, provided that inoculation costs are insignificant compared to other expenses. The following topics (A–C) outline processes required to evaluate the potential benefits of mycorrhizal inoculation by considering properties of (A) soil and site factors, (B) host plants and (C) mycorrhizal fungi which determine these benefits.

A. Soil and site factors

Soil factors that can influence the benefits provided by mycorrhizal fungi to plants are considered below.

Mycorrhizal inoculum

Plants with mycorrhizal associations predominate in most natural ecosystems (Brundrett 1991), so inoculum of mycorrhizal fungi is present in most soils. However, changes in land use practices may result in the predominance of fungi which are not compatible with an introduced host plant. Fungal population densities vary markedly between sites, depending particularly on previous land use. In Australia,

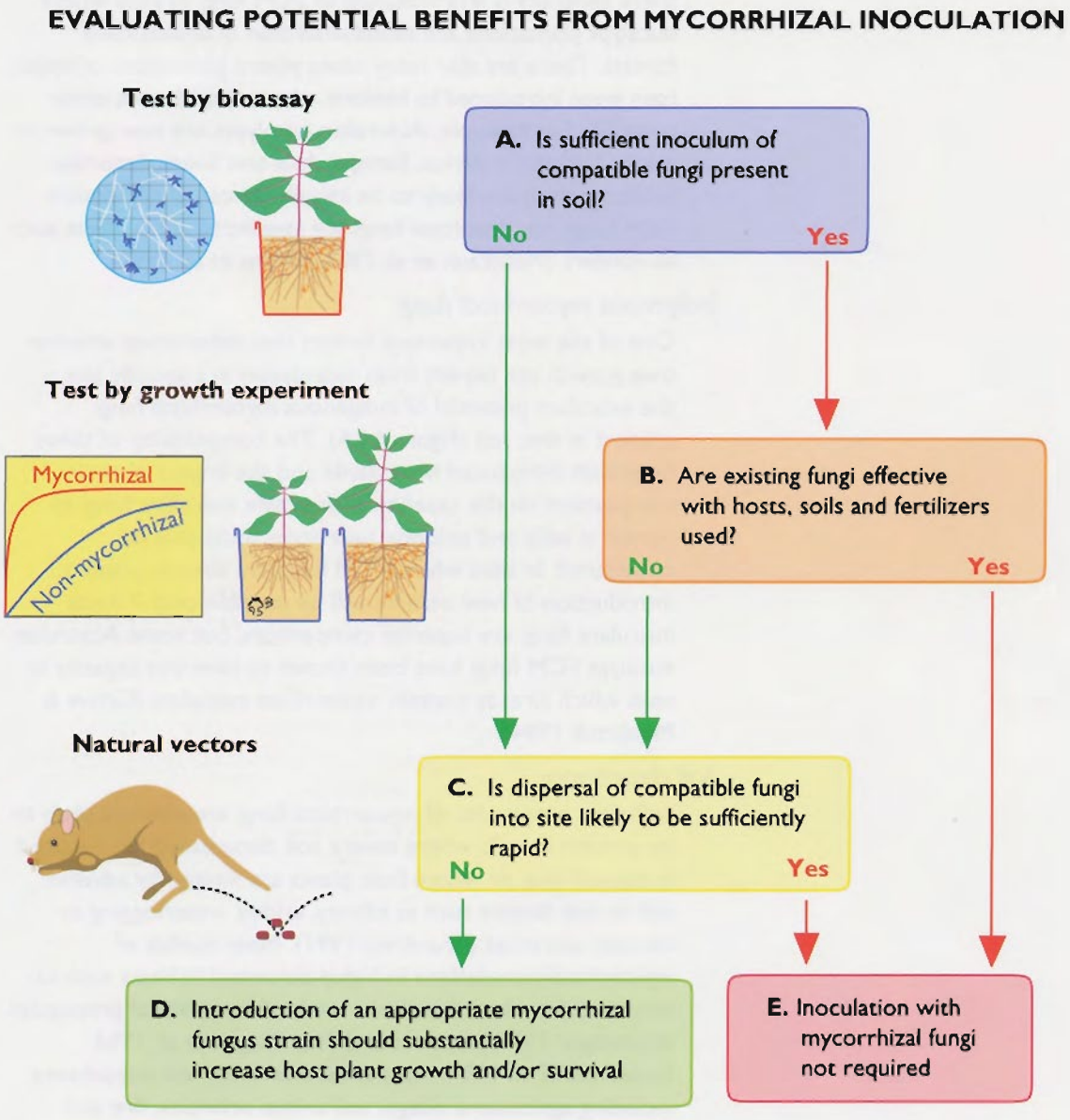


Figure 6.2. *Flow chart summarising decision-making steps which can be used to evaluate the potential benefits of mycorrhizal inoculation in a specific situation.*

A. *Inoculum levels of compatible mycorrhizal fungi should be determined by bioassay experiments where a particular host plant is grown in soil from the site.*

B. *Soil properties, especially nutrient availability and fertilizer applications, are important determinants of mycorrhizal responses. Mycorrhizal benefits can be tested by glasshouse experiments which measure plant growth responses in the appropriate soil with standard fertilizer applications.*

C. *Natural vectors such as fungus-feeding animals can effectively introduce desirable mycorrhizal fungi, but this process may be too slow to be effective.*

D. *In this case mycorrhizal fungus inoculum is likely to be effective if it can be applied cost-effectively and efficiently.*

E. *Alternatively, mycorrhizal inoculation might not be expected to provide substantial benefits, but might still be applied for other reasons.*

there generally is less inoculum of ECM fungi in sites where eucalypt plantations are established than in undisturbed forests. There are also many cases where plantations of trees have been introduced to habitats where they do not occur naturally. For example, Australian eucalypts are now grown in exotic habitats in Africa, Europe, Asia and South America, habitats which are likely to be initially devoid of compatible ECM fungi, because local fungi are specific to other hosts such as conifers (Malajczuk et al. 1982, Molina et al. 1992).

Indigenous mycorrhizal fungi

One of the most important factors that determines whether tree growth can benefit from inoculation in a specific site is the inoculum potential of indigenous mycorrhizal fungi present in that soil (Figure 6.2A). The compatibility of these fungi with introduced host plants and the impact of their competition on the capacity of desirable inoculant fungi to persist in soils and colonise new roots must also be considered. In sites where ECM fungi are already present, introduction of new isolates will be possible only if these inoculant fungi are superior competitors, but some Australian eucalypt ECM fungi have been shown to have this capacity in soils which already contain mycorrhizal inoculum (Grove & Malajczuk 1994).

Soil disturbance

Sufficient propagules of mycorrhizal fungi are also not likely to be present in soils where severe soil disturbance has resulted in topsoil loss, or where host plants are limited by adverse soil or site factors such as salinity, aridity, waterlogging or climatic extremes (Brundrett 1991). Most studies of mycorrhizal associations in highly disturbed habitats such as minesites have found reduced levels of mycorrhizal propagules (Danielson 1985, Jasper et al. 1992, Pfleger et al. 1994, Brundrett et al. 1995). Less severe forms of soil disturbance, including agricultural tillage, soil animal activities, fire and erosion, can also reduce levels of mycorrhizal fungus propagules (Habte et al. 1988, O'Halloran et al. 1986, Read & Birch 1988, Vilarino & Arines 1991).

Colonisation by mycorrhizal fungi

In soils which are initially devoid of suitable mycorrhizal fungi, their introduction will depend on the arrival of propagules such as wind-blown spores produced by fungi fruiting under host trees (Grove & Le Tacon 1993, Malajczuk et al. 1994b). Spores and other propagules of mycorrhizal fungi can also be dispersed by wind or water erosion or by the activity of animals which feed on hypogeous or epigeous fungi (Allen 1991, Brundrett 1991, Claridge & May 1994, McGee & Baczocha 1994). In a disturbed habitat, the effectiveness of these natural vectors will depend on the proximity of undisturbed habitats containing suitable fungi (and their associated animals) as well as the seasonality of fruiting of

fungi. Colonisation of disturbed habitats, such as minesites in Australia, by mycorrhizal fungi has been observed, but there is as yet insufficient information about the time required for this process to occur (Jasper et al. 1992, Brundrett et al. 1995).

Measuring inoculum

The quantity of inoculum of mycorrhizal fungi which is compatible with a host plant in soils can be determined by bioassay experiments (Fig. 6.2). In these experiments, seedlings are grown in intact soil cores or mixed soil samples for sufficient time to allow mycorrhizas to form, then roots are sampled, processed and assessed to measure mycorrhiza formation (see Section 4.4). Bioassay experiments have been used to quantify indigenous mycorrhizal fungi compatible with specific hosts, examine spatial variability in inoculum levels and test the ability of indigenous fungi to compete with inoculant fungi (Perry et al. 1982, Parke et al. 1984, McAfee & Fortin 1986, Brundrett & Abbott 1995). Typical results of a mycorrhizal bioassay experiment are presented in Figure 4.9.

Soil fertility

A key factor likely to determine the potential for mycorrhizal inoculation to improve tree growth in particular sites is the supply of phosphate and nitrogen in soil (Abbott & Robson 1991, Grove et al. 1991). High rates of P and N fertilizers suppress ectomycorrhiza development in the field (Menge et al. 1977, Newton & Pigott 1991) and high concentrations of soil N can also reduce the number and relative abundance of different ectomycorrhiza types (Alexander & Fairley 1983). The effect of high rates of P and N fertilizers on mycorrhiza development is demonstrated by Figures 3.13 and 6.11. The demand for a particular mineral nutrient depends on plant internal requirements, while the supply of that nutrient primarily depends on its availability and mobility in soils (Russell 1977, Marschner 1986). Phosphorus is generally considered to be the most important plant-growth limiting factor which can be supplied by mycorrhizal associations, because of the many abiotic and biotic factors which can restrict its mobility in soils (Harley & Smith 1983, Hayman 1983, Marschner 1986, Bolan 1991). Reductions in the benefit provided by mycorrhizal associations to plants caused by increasing soil phosphorus levels have often been observed (Bougher et al. 1990, Jones et al. 1990, Schweiger et al. 1995) (see Fig. 6.3). The effect of soil properties which influence the availability of nutrients such as P and N on plant responses to mycorrhizas requires further investigation.

Minor elements

Nutrient disorders involving elements other than P are common when eucalypts are planted in disturbed or nutrient-poor soils (Marschner & Dell 1994). There is limited experimental evidence that mycorrhizal fungi can help to alleviate other nutrition deficiencies by enhancing uptake of

N, K, Ca, S, Cu and Zn, while substantial contributions to the host supply of other nutrients (Mg, B, Fe, etc.) are suspected, but requires further study (Marschner & Dell 1994). In native forests, eucalypts rarely show symptoms of mineral nutrient deficiencies, but when they are planted on disturbed or infertile soils nutrient disorders are common, especially when rapid growth results from P and N application (Dell et al. 1995). Observations of the early growth of eucalypts in ECM field trials in Australia and China have revealed a range of micronutrient disorders. Boron can be a limiting factor in the establishment of plantation trees in southern China (Turnbull 1994, Dell & Malajczuk 1994), where many soils are considered to be deficient in this nutrient (Wenfu et al. 1991). The availability of both macronutrients and micronutrients in soils are likely to be important determinants of plant responses to mycorrhizal associations. Plantation forestry programs must consider responses to fertilizer applications on specific sites, to determine the magnitude of any nutrient deficiencies which occur, so that fertilizer applications can be used to remedy these problems.

Adverse soil conditions

Land degradation due to salinity, waterlogging, erosion, etc. is a serious and growing problem in Australia (Bell 1988, Scott 1992). There is increasing awareness that the growth of salt-tolerant tree species can combat salinity and waterlogging problems in cleared land (Marcar et al. 1991, Scott 1992). Some *Eucalyptus*, *Acacia*, *Melaleuca* and *Casuarina* genotypes can be grown in saline soils (Marcar et al. 1991). Excessive NaCl levels in soil inhibit mycorrhizal formation and restrict the activity of most mycorrhizal fungi, but some mycorrhizal fungi can tolerant these conditions (Malajczuk et al. 1989, Juniper & Abbott 1993). Observations in natural ecosystems have shown that plants with mycorrhizal associations may be less common than non-mycorrhizal species in soils which are waterlogged or saline, but that some mycorrhizal plants are normally present in even the worst soils (Brundrett 1991). There is some evidence that ECM fungi are highly sensitive to waterlogging of soils, but VAM fungi may be less sensitive (Theodorou 1978, Lodge 1989, Bougher & Malajczuk 1990). It seems likely that adverse soil conditions could reduce the benefits provided by mycorrhizal associations by restricting fungal activity in some situations, but in other situations the use of fungal isolates which are tolerant to these conditions could be highly beneficial, by helping to increase plant survival.

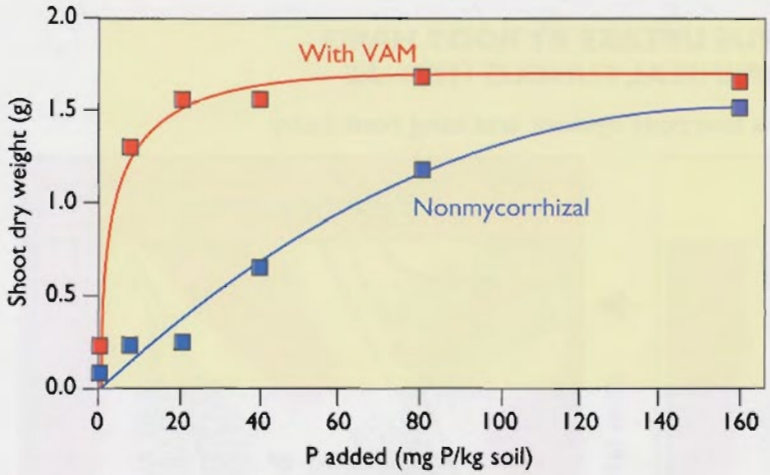


Figure 6.3. *Response curves produced by growing mycorrhizal and non-mycorrhizal plants of* Cassia pruinosa *at a range of soil P levels. Note that the benefit provided by mycorrhizal associations to this species is dependent on soil P levels and can be defined by comparing these two curves (data from Jasper et al. 1994).*

B. Plant root systems

Plants in natural ecosystems are known to have varying degrees of dependence on mycorrhizal associations, which have resulted in the designation of obligate and facultative associations (see Section 1.6). These categories are the result of inherent properties of the plants themselves, as well as the availability of nutrients in the soils in which they naturally occur. Host and soil factors which can be used to help predict the potential benefit of mycorrhizas are outlined in Table 6.1.

Table 6.1. Generalised relationships between features of root systems and the mycorrhizal dependency of plants (after Brundrett 1991).

Root feature	*Mycorrhizal dependency of plant*	
	High	*Low*
A. Root surface area	low	high
1. Root system surface area (specific root length)	low	high
2. Branching orders of lateral roots	few	many
3. Branching frequency (architecture)	sparse	frequent
4. Root hair abundance and length	few/short	many/long
B. Root activity	slow	fast
1. Root growth rate	slow	fast
2. Response to soil conditions	slow	fast
3. Primary root life span	long	short
4. Protective structural features	strong	weak
5. Root exudation	? less	? more
C. Mycorrhizal formation	efficient	inefficient or inhibited

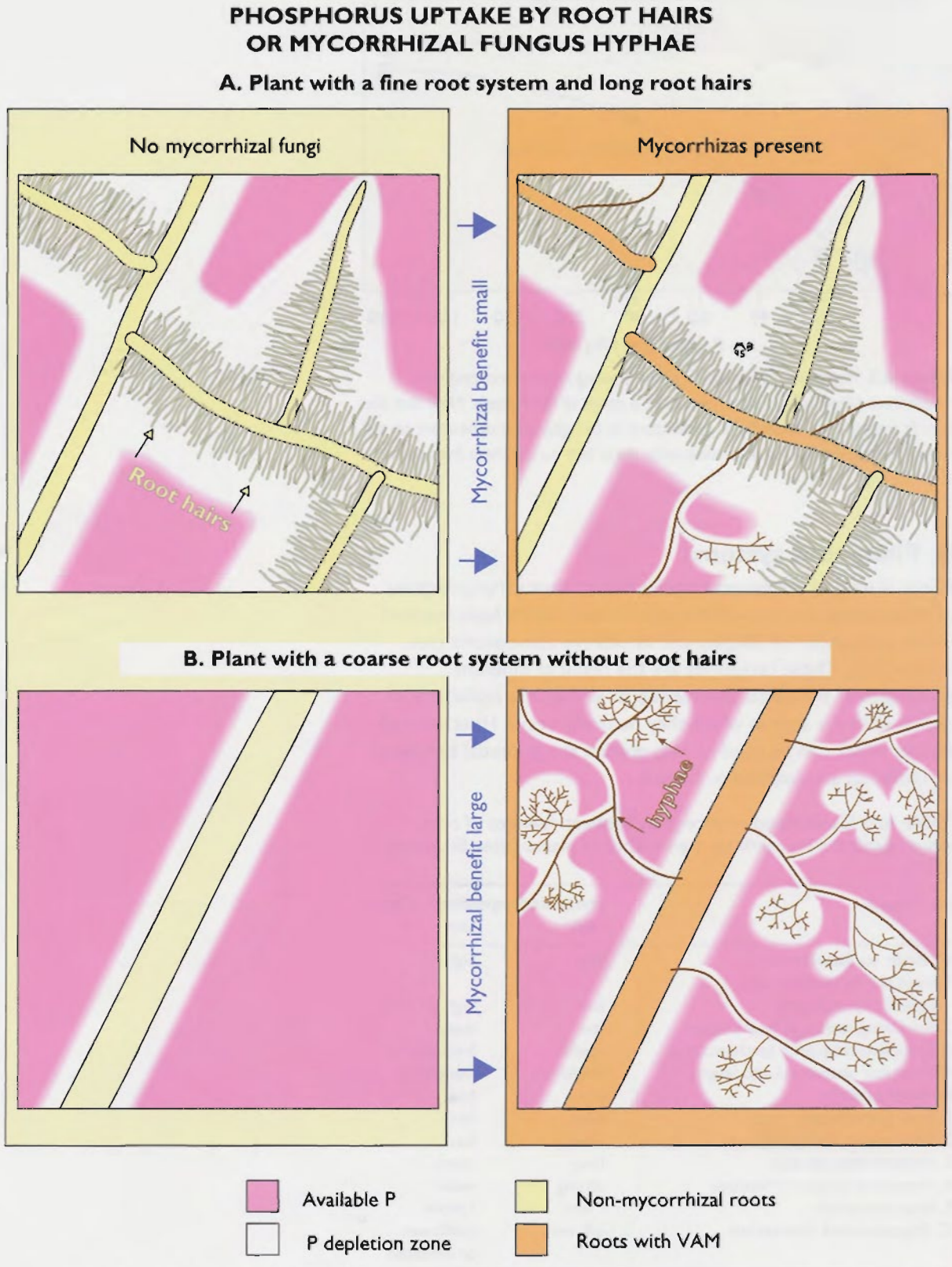


Figure 6.4. *The role of root hairs and mycorrhizal fungus hyphae in acquiring poorly mobile nutrients such as P from soils. This diagram demonstrates why a plant with fine, extensive roots and long root hairs (**A**) would normally receive much less benefit from the presence of mycorrhizas than a plant with coarse roots (**B**).*

Mycorrhizal dependency

The potential benefits resulting from the inoculation of plants with mycorrhizal fungi may be large or small depending on properties of the host plant, mycorrhizal fungi or soil where they are grown (Fig. 6.3). Mycorrhizal dependency is simply a measure of the benefit provided by mycorrhizas and will depend on relative contribution of root and mycorrhizal mediated nutrient uptake to plants (Janos 1980). Mycorrhizal dependency has often been quantified by calculating the yield ratio between mycorrhizal plants and uninoculated control plants grown in a particular soil at a single soil P level (Koide et al. 1988, Manjunath & Habte 1991, Hetrick et al. 1992). However, it is better to analyse mycorrhizal benefits across a range of soil P levels by producing nutrient response curves like that shown in Figure 6.3 (Abbott & Robson 1984). Mycorrhizal benefit can be estimated from P response curves by calculating the slopes of mycorrhizal and non-mycorrhizal curves at the crossover point (see Schweiger et al. 1995).

Nutrient depletion zones

Mineral nutrients such as phosphorus have very limited mobility in soils so that depletion zones — where all the available nutrient has been utilised — quickly form around roots (Bhat & Nye 1974, Russell 1977, Marschner 1986). Thus to obtain more phosphorus, plants must bypass the depletion zones surrounding existing roots by further root activity elsewhere in the soil (see Fig. 6.4). The outcome of this quest for phosphorus (and other relatively immobile soil resources) should largely be determined by the surface area of a plant's root system, which in turn is a product of the properties outlined below.

Root system form

Considerable variations in root system extensiveness, geometry, depth distribution and plasticity occur between plant species (Grime et al. 1986, Fitter 1987). Plants with extensive roots (highly branched, fine, long roots with numerous root hairs) have often been observed to derive little benefit from mycorrhizas in experiments (Baylis 1975, Koide et al. 1988, Manjunath & Habte 1991, Hetrick et al. 1992). In experiments, total biomass is the parameter most often used to quantify roots, but provides much less information than root length or specific root length (length/unit root weight) data (Fitter & Hay 1987, Eissenstat 1992). Parameters that could be used to predict nutrient absorption are: total root biomass < fine root biomass < root length < root surface area < rhizosphere volume, arranged in increasing order of predictive ability. When root parameters of plant species are compared, root hairs have been found to be most highly correlated with their ability to absorb P from soils (Itoh & Barber 1983, Föhse et al. 1988, Schweiger et al. 1995).

Root activity

There is a tendency for mycorrhizal plants to have roots which are less active (grow slowly, live longer, etc.) than species with little or no mycorrhizal colonisation of their roots (Table 6.1). The responsiveness or capacity of plants to exploit small-scale or short duration changes in water or nutrient availability by rapidly producing new roots is thought to be an important determinant of their success during competition for soil resources (St John et al. 1983, Grime et al. 1986, Fitter 1987). Some plants can also change rhizosphere conditions, such as pH, which may influence nutrient availability (Marschner 1986, Uren & Reisenaur 1988). Australian non-mycorrhizal plants in the family Proteaceae produce cluster roots that likely promote nutrient uptake by their large surface area and production of leachates which contain organic acids and other substances (Attiwill & Adams 1993, Lamont 1993). The capacity of plants to influence nutrient availability in soils will also depend on the extensiveness and activity of their root system, since young roots are the primary source of exudates (Curl & Truelove 1986, Uren & Reisenaur 1988).

The role of hyphae

It has been postulated that differences between fungi in the efficiency of their soil hyphae should be an important determinant of their mycorrhizal performance (Smith & Gianinazzi-Pearson 1988, Abbott et al. 1992). Mycorrhizal fungus hyphae function primarily by increasing the soil volume from which available forms of phosphorus are absorbed and provided to roots (Hayman 1983, Harley & Smith 1983). Hyphae of VAM fungi have also been observed to respond to localised sources of soil nutrients more rapidly than roots (St John et al. 1983, Warner 1984) and to produce fine highly-branched 'absorptive' hyphae in decomposing organic substrates (Mosse 1959, Nicholson 1959). The proliferation of hyphae of ECM fungi in decomposing soil organic matter has often been noted (Harvey et al. 1976, Reddell & Malajczuk 1984, Bending & Read 1995). It has often been stated that hyphae of VAM fungi can only utilise the same forms of nutrients that are accessible to roots (Harley & Smith 1983, Hayman 1983), but there is some evidence that these associations may have a greater benefit when phosphorus is present in less-soluble forms (Bolan 1991, Schweiger 1994). Hyphae of ECM fungi apparently can utilise both inorganic and simple organic sources of nitrogen and phosphorus (Abuzinadah & Read 1989, Hausling & Marschner 1989). There is also evidence of enhanced soil mineralisation by organic acids within ECM fungus mats (Griffiths & Caldwell 1992). Thus while mycorrhizal fungi may have access to some forms of nutrients which are not directly available to plants, the key role of mycorrhizal fungus hyphae seems to be to acquire nutrients which are spatially or chronologically separated from roots.

Roots vs. hyphae

As Figure 6.4 demonstrates, the main role of mycorrhizal associations is to acquire nutrients by exploring the soil volume with hyphae that are both more responsive and more extensive than the roots themselves. However, roots of some species of plants are also capable of effectively exploring large soil volumes and responding to temporary soil resources, and these species are consequently less likely to benefit from mycorrhizal associations. These alternative strategies both require plants to expend more metabolic energy on their root systems, but mycorrhizal fungus hyphae should be a more cost-effective means of exploring large soil volumes (Harley 1989). Because of the high metabolic cost that would result, plants generally do not support both high levels of mycorrhizal colonisation and root systems with fine/active roots (Table 6.1). *Assuming that soil nutrient levels are not unusually high and inoculum of appropriate mycorrhizal fungi is available, root strategy differences will primarily determine the magnitude of benefits from mycorrhizal associations.*

C. Characteristics of fungal isolates

The potential for the utilisation of mycorrhizal fungi in nursery and forestry practices to increase production has long been a goal both overseas and in Australia. The CSIRO Division of Forestry program of mycorrhizal research has shown that it is possible to increase the productivity of plantations of eucalypts by introducing selected ECM fungi to seedlings in the nursery in Australia and China (Grove et al. 1991, Ji et al. 1995, Malajczuk et al. 1994a). However, we have observed that field trials of eucalypts inoculated with ECM fungi in Australia have produced variable results on different sites. Since each species and isolate of an ECM fungus has specific ecological requirements, the screening of a wide diversity of fungi is required. Fungal selection requires an understanding of their effectiveness in relation to a range of environmental conditions. *Careful isolate selection is the key to successfully introducing ECM fungi into plantations.*

The importance of fungal diversity

High fungal diversity is a characteristic of most forests where ECM trees occur (Harley & Smith 1983, Meyer 1973, Castellano & Bougher 1994). In contrast, the diversity of ECM fungi occurring in man-made plantations of eucalypts is often much less, which may result in fewer options for physiological response to environmental changes. In Australia there are thousands of species of ECM fungi which can occur in eucalypt forests (Bougher 1995), but we have observed that there generally is a very low diversity of these fungi in eucalypt plantations. This may result because eucalypt plantations are often established on soils which have been used to grow pasture and crop plants with VAM associations, resulting in the decline of ECM fungi which were associated with trees before the land was cleared. The low fungal

diversity in plantations could also result because they are a much more uniform habitat than natural forests, or because many forest fungi prefer older trees or less disturbed soils. *The biological diversity of mycorrhizal fungi present in a soil is likely to be linked to their functional diversity.* This functional diversity would result from variations in the capacity of individual fungi to tolerate cold or hot temperatures, tolerate wet or dry soil conditions, utilise different forms of inorganic and organic nutrients, acquire ions from mineral soil components, or protect roots by detoxifying soil or antagonising pathogens (Table 1.4). *Thus it seems likely that there will be many benefits from increasing the diversity of mycorrhizal fungi associated with eucalypts in plantations.*

Host–fungus compatibility

It is essential that fungi used to inoculate plants are fully compatible with them. Ectomycorrhizal fungi may have narrow or broad host ranges with respect to host genera (Molina et al. 1992). Eucalypt-compatible fungi rarely associate with host trees from other geographic locations such as pines, poplars or willows and their fungi are not compatible with eucalypts (Malajczuk et al. 1982, 1994a). Histological investigations (Chapter 4) and sterile culture synthesis experiments (Chapter 5) have demonstrated that there are various degrees of compatibility between hosts and fungi associated with variations in mycorrhizal formation and benefits to the host (Section 4.7).

Matching fungi to sites

The selection of fungal isolates is based on the desirable characteristics listed in Table 6.2. Important roles of mycorrhizal fungi include enhancing seedling survival, promoting early tree growth and improving the uniformity of plantations (Burgess & Malajczuk 1989, Grove et al. 1991, Dodd & Thomson 1994). Some of the most important factors which may limit fungal growth are N and P supply to plants, and the pH, temperature and moisture of soil (Section 6.3). Considerable variation between and within species of fungi in their responses to these factors has been reported (Brundrett 1991). The capacity for fungi to produce soil hyphae is thought to be a major determinant of mycorrhizal fungus benefits to their host (Jones et al. 1990, Thomson et al. 1994) and would result from inherent properties of fungi and interactions with soil conditions. Knowledge of the characteristics of the site from which a fungal isolate is collected will provide some indication of the edaphic and climatic conditions suited to that fungus. *However, selection of fungi must occur at the isolate level, as generalisations about fungal performance made at the family, genus or species level are not possible with our current understanding of the population biology of mycorrhizal fungi* (Trappe & Molina 1986, Brundrett 1991, Morton & Bentivenga 1994).

Fungal persistence

An important factor to consider in any mycorrhizal inoculation program is whether the inoculant fungi can survive and persist in field conditions (O'Dell et al. 1992, Dodd & Thomson 1994). Thus the introduction of new strains of mycorrhizal fungi into new habitats requires thorough testing of their performance with the appropriate host in target soils. Comparisons of young vs. older trees in plantations, or recently disturbed habitats vs. older sites, have shown that successional changes in the populations of associated fungi occur (Mason et al. 1987, Gardner & Malajczuk 1988, Termorshuizen 1991). These successional changes, which parallel vegetation and/or soil development, suggest that some fungi which are very productive in young plantations may be replaced in time by other fungi.

Ameliorating soil conditions

Substantial variability between species and isolates of species of mycorrhizal fungi in their tolerance to adverse soil conditions such as salinity, waterlogging, low water activities or elevated heavy metals have been observed whenever comparisons are made (Robson & Abbott 1989, Danielson 1985, Brundrett 1991, Juniper & Abbott 1993, N. Aggangan, pers. comm.). While some tolerance mechanisms that allow fungi to grow in 'stressful' environments have been identified, these mechanisms have not been investigated for many fungi (Jennings 1993). The potential for the use of mycorrhizal fungus isolates which are tolerant to adverse soil conditions to help plants revegetate degraded habitats and toxic soils requires further study.

Edible fungi

Certain groups, especially the truffles and truffle-like fungi and the Boletaceae and Russulaceae, are important food for forest-dwelling mammals and for humans (Castellano et al. 1989, Claridge & May 1994). For example, edible fungi collected from forests are a highly prized but declining resource in China, so it would be desirable to supplement these indigenous fungi with introduced Australian fungi which grow in eucalypt plantations (N.L. Bougher, pers. comm.). Some of the most sought-after fungi in the world (truffles, chanterelles, matsutake, boletes, etc.) are mycorrhizal associates of trees that are, or potentially could be, produced in plantations. Indeed some fungi which could be harvested annually from a plantation could greatly exceed the commercial value of wood produced by the trees. Thus edible fungi should be given preference over other fungi, if they are equally good at promoting tree growth, and care must be taken to avoid the introduction of highly toxic fungi, such as some *Amanita* species, into new habitats.

Table 6.2. Criteria for selecting mycorrhizal fungi for practical use.

No.	*Selection criteria*	*Details*
1.	Compatibility with key host plant(s)	• usually tested by glasshouse trials using pasteurised soil or synthesis attempts in axenic culture • microscopy procedures are required to confirm mantle and Hartig net formation
2.	Compatibility with soils and climatic conditions	• isolates should be obtained from similar climates/soils to where they will be used • habitat data concerning climatic conditions, host trees and soil properties from herbarium databases may be used
3.	Growth promotion of host species	• tested by measuring growth responses in glasshouse experiments at realistic soil fertility levels
4.	Amelioration of adverse soil conditions	• specific fungal isolates may help plants tolerate adverse soil conditions or enhance resistance to pathogenic organisms
5.	Capacity to obtain or produce inoculum	• hyphal growth rates in sterile culture will determine the feasibility of producing mycelial inoculum • experiments may be required to optimise growth media composition • spore inoculum production depends on the ease of sporocarp acquisition and their quality
6.	Colonisation of seedling roots in the nursery	• standard nursery practices which cannot easily be changed (the composition of potting mixes, water regimes, fertilizer applications, pesticide use, etc.) may eliminate fungi
7.	Persistence and spread in the field	• fungi may fail to perform in the field due to factors such as competition with other fungi or lack of tolerance to soil conditions • soil conditions in plantations may become less or more favourable for a particular fungus with time
8.	Other values of fungi	• edible fungi may be harvested for food or medicine to provide extra income from forest plantations

6.2. MYCORRHIZAL PLANTS IN THE GLASSHOUSE

A. Glasshouse experimentation

Some typical objectives of mycorrhizal glasshouse experiments are listed in Table 6.3. Whatever the objectives of a particular experiment, basic protocols are often similar. Glasshouse screening trials can be used to establish the mycorrhizal status of host plants (Section 4.1B). Fungal performance in glasshouse experiments can be evaluated by measuring the growth and survival of associated plants, relative to control plants which are non-mycorrhizal or colonised by fungi already present in soils. Plant nutrient content measurements can also be used to compare uptake capacities of fungi and measure their capacity to exclude toxic elements present in soils. Anatomical investigations of the host–fungus interface (Hartig net or arbuscules) in mycorrhizal roots can be used to confirm mycorrhizal formation and will provide valuable information about host–fungus compatibility and the potential for nutrient exchange between partners.

Bioassay experiments with mixed soil or intact soil cores can be used to measure mycorrhizal inoculum in soils (Section 4.4). The capability of mycorrhizal fungi to compete with indigenous soil microbes in a particular soil can be established by comparing their colonisation ability and capacity to promote plant growth in pasteurised and unsterile soil (Aggangan et al. 1995). However, field trials provide the ultimate opportunity to evaluate the persistence and performance of fungi in the field (Chapter 7). Basic equipment for glasshouse experiments is listed here.

Table 6.3. Typical objectives of mycorrhizal glasshouse experiments.

No.	*Experiment*	*Primary objective*
1.	Mycorrhizal synthesis	Confirmation of the mycorrhizal status of host plants and mycorrhizal fungi and their compatibility
2.	Mycorrhizal dependency	Comparison of mycorrhizal and non-mycorrhizal plant growth responses to mycorrhizal inoculation at different soil fertility levels
3.	Bioassays	Estimation of the inoculum potential of compatible mycorrhizal fungi in a particular soil
4.	Isolate screening	Selection of superior mycorrhizal fungus isolates by comparing growth responses of host in particular soils
5.	Micronutrient research	Determine critical levels of trace elements for mycorrhizal and non-mycorrhizal plants
6.	Histology	To provide mycorrhizal root material of known fungi for microscopy
7.	Physiology	To allow testing plant/fungal response to various soil factors

Equipment list

Glasshouse or growth chamber

- light levels must be adequate for normal growth
- cooling and/or heating may be required
- raised benches
- clean, dust-free working environment

Growing plants

- clean, sterile pots
- soil or potting mix
- system to pasteurise or fumigate soil to remove microbial contaminants
- fertilizers which contain defined quantities of nutrients
- clean, uncontaminated water supply
- balance to weigh pots for field capacity watering
- pest control measures may be required

Plant harvesting facilities

- root washing
- plant drying
- tissue grinding

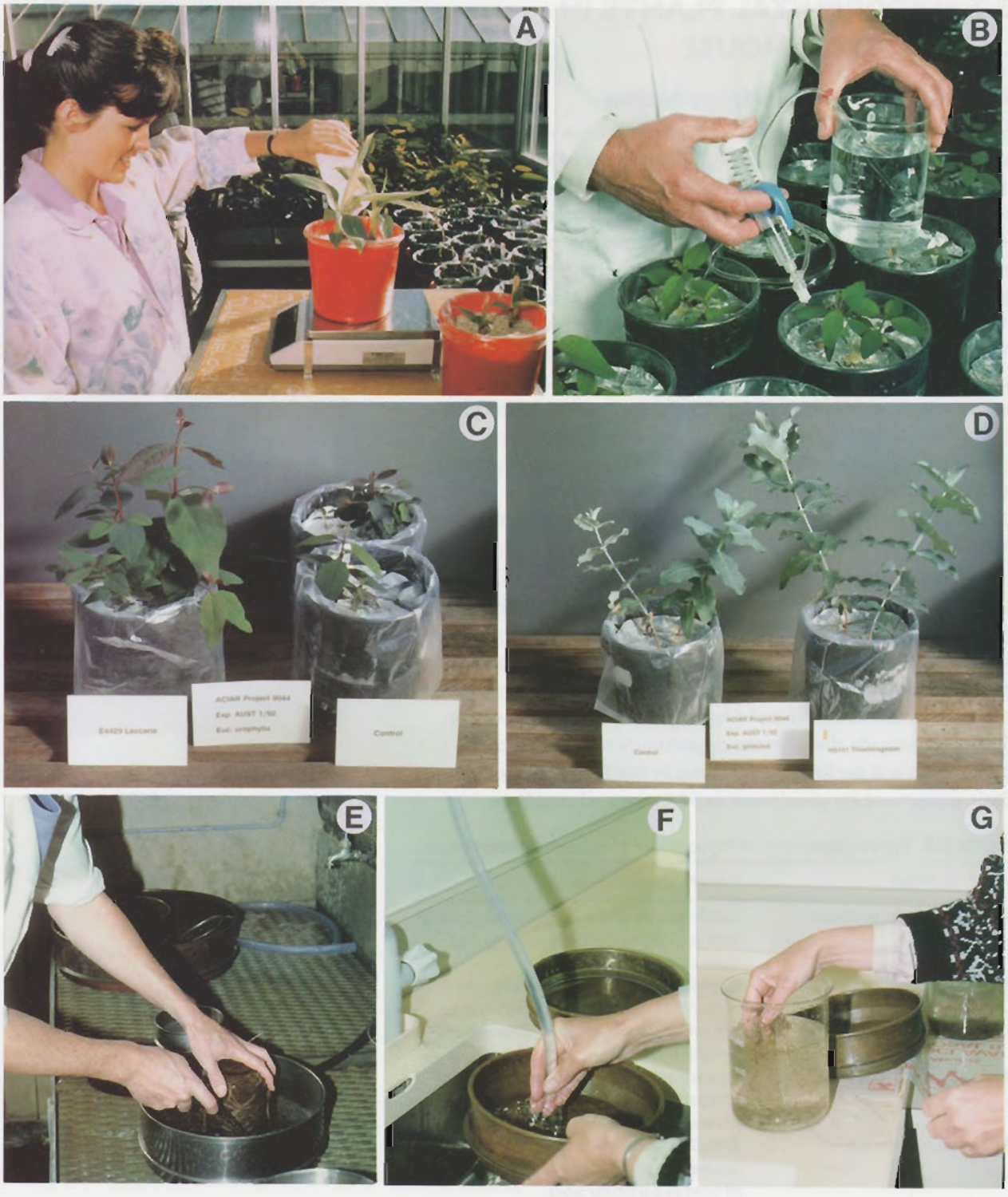

A
B
C
Control
D
E
F
G

Figure 6.5. *Management of glasshouse plants and harvesting roots for a mycorrhizal experiment.*

A. *Uniform watering of plants in non-draining pots by using a balance to add water to pots until they reach field capacity.*

B. *Uniform addition of a fertilizer supplement (N solution) to pots using a calibrated dispenser.*

C,D. *Photographic documentation of eucalypt seedling responses to mycorrhizal inoculation in a glasshouse experiment.*

E–G. *Washing roots after harvesting plants in a mycorrhizal glasshouse experiment.*

E. *Initial removal of bulk soil with a hose over a coarse (2 mm) screen.*

F. *Further cleaning of a root sample by washing over a 200 µm sieve.*

G. *Final cleaning of root mass by suspension in water to allow sand particles to settle, before roots are collected on a fine sieve. Root samples should then be clean enough to observe mycorrhizas under a dissecting microscope, or for staining procedures.*

Experimental design



An experimental plan (preschedule) for a typical glasshouse experiment would cover many of the same topics as a plan for a field experiment (Table 7.1). An experiment to compare several treatments must have both replication and randomisation. The simplest experimental design which incorporates these two principles only is the *completely randomised* layout. If it is known or suspected that there is another source of systematic variation in addition to and independent of the treatments, a *randomised complete block* design should be used. For example, in a nursery fertilizer trial, there may be a shading effect which can be compensated for by blocking. The layout of treatments must be random within each block, a fresh randomisation being used for each block. Experimental designs are considered in greater detail in Section 7.2.

Growth substrates

Sandy soils are normally used for mycorrhizal glasshouse experiments, because their coarse texture facilitates washing out intact mycorrhizal roots after the experiment, while fine clay, silt or organic matter in soils make this much more difficult. However, other types of soils (specific soils from field sites, potting mixes, etc.) may be required in specific cases. Soil collected from the B horizon will contain less organic debris and microbial inoculum than soil from surface layers. The chemical properties of soils, especially pH, are also important (most Australian plants and fungi prefer moderately acidic soils, but alkaline soils may be required in other cases). After collecting soil, it should be sieved to remove coarse gravel and organic debris, dried using a drying facility, then stored in bags in a safe place. Unwanted microbes in soils should be removed by steam pasteurisation (2 × 60 min. at 80°C on consecutive days), or by using one of the other methods discussed in Section 6.3B.

Plant materials

Plants used in glasshouse experiments are typically grown from seeds. Uniformity in plant establishment is especially important, as variations in plants caused by erratic seed germination or a lack of genetic uniformity will interfere with treatment effects (Section 7.2E). Several strategies can be used to increase the uniformity of seedlings. Uniformly sized, undamaged seeds from a single seedlot (parent plant) or cultivar should be selected to reduce variability. Clonally propagated plants from a single parent would be more uniform than seedlings, if they are available. Sufficient quantities of seeds should be germinated on agar, steamed sand, or damp paper, to allow seedlings at a uniform stage of germination to be selected for planting. An alternative strategy is to sow extra seeds (2–3 × the required number) in each pot and thin seedlings after germination, to increase uniformity. It is often desirable to surface-sterilise seeds to reduce the risk of microbial contamination (Section 5.4A).

Fungal materials

Inoculum forms and inoculation procedures for ECM and VAM fungi are described in Sections 5.5 and 3.4, respectively. Uniformity in mycorrhizal root formation within fungal treatments is especially important in glasshouse experiments and should be tested in a preliminary trial of inoculum forms and dosages. A lack of uniformity may sometimes be corrected by using much higher inoculum levels than would be possible in the nursery, but any extra nutrients supplied with the mycorrhizal inoculum need to be taken into account (by control treatments). It is also essential that mycorrhizal propagules such as spores become active before root growth reaches a plateau, when plants are grown in a confined space.

Watering plants

Plants in glasshouse experiments can be grown in free-draining pots or non-draining buckets. Non-draining pots are preferred for many experiments, because they allow nutrient and water supply to be precisely regulated. These pots must be watered to a predetermined weight (e.g. 70% of field capacity) by weighing pots on a balance (Fig. 6.5A). These weights are calculated from the field capacity of the soil, which can be reduced by 10–30% to improve aeration, while taking the weight of pot, etc. into account. Small plants need to be watered accurately every 2–3 days and extra water inputs may be estimated at other times. Large plants may need to be watered accurately every day to limit differences in water supply between treatments. Water use can be recorded to provide an estimation of transpiration differences between treatments. The surface of soil in pots should be covered with aluminium foil disks, plastic beads, etc. to reduce transpiration, without restricting gas exchange. Mycorrhizal experiments can also be conducted in freely

draining pots, provided a dilute nutrient solution (such as 10% Long Ashton's or Hoagland's solution) is applied at regular intervals to replace leached nutrients, and the substrate used (coarse sand, hydroponic beads, etc.) does not accumulate nutrients (e.g. Table 6.8).

Nutrient supply

Since soil nutrient levels can have a large impact on the results of mycorrhizal experiments, it is essential that fertilizers are accurately and uniformly applied to soils. The most common methods of applying nutrients are outlined below.

A. Soil is prepared by sieving, thoroughly mixing and drying it, then uniform quantities are dispensed into pots (using an accurate balance).
B. Carefully measured quantities of concentrated stock solutions are dispensed onto the surface of soil in pots (a calibrated automatic pipette should be used and separate stock solutions should be applied in different areas).
C. The soil is allowed to dry for several days in a warm dust-free area.
D. The soil in pots is then thoroughly mixed by pouring it into a large clean tightly sealed plastic container, breaking up any lumps of soil, then mixing it in a uniform fashion (e.g. inverting the container 60 times). **Take care to avoid excessive manual handling of soils in one session as repetitive strain injuries can result.** A mechanical shaker should be used for this task.
E. Nutrients can also be applied by watering soils to field capacity with diluted nutrient solution, provided soils do not fix nutrients very rapidly (Table 3.3).

Supplemental fertilisation

Small nitrogen supplements should be applied at regular intervals (e.g. 20 mL of 5 g/L w/v NH_4NO_3 solution/kg soil every 2–3 weeks) as N is lost from the soil much more rapidly than other nutrients (Fig. 6.5B).

Environmental conditions

The glasshouse environment should be monitored to ensure light and temperature conditions are favourable to plant growth and mycorrhizal formation, and these conditions should be reported when publishing results. Standardising growing conditions as much as possible will increase the repeatability of experiments. Control of soil temperatures and moisture levels is especially important if the rate of mycorrhizal formation is to be determined. Root-cooling tanks, where temperature-regulated water is circulated around buckets containing soil, can be used to control soil temperatures (Fig. 3.12D).

Experiment duration

The optimal duration of experiments will depend on the growth rate of plants, soil nutrient levels and the activity of fungi. Prolonging experiments after the growth of larger plants stops due to root confinement, water restrictions, or lack of nutrients will reduce treatment effects. It is advisable to monitor mycorrhizal development by taking small core samples from pots following instructions in Section 3.4E (Fig. 3.15), to ensure that sufficient mycorrhizal development occurs before plants are harvested.

Measuring plants and harvesting experiments

Plant height measurements are commonly used in glasshouse experiments as they allow growth to be quantified periodically without damage to plants, but other growth parameters can also be used (see Section 7.3D). Height measurements can be used to reveal growth trends and determine when to stop the experiment. Once the experiment is over, plants can be harvested by cutting off shoots, which are weighed to determine fresh weight then dried to determine dry weight, and by washing roots free of soil. Other parameters used to measure plant growth are listed in Table 7.4.

Table 6.4. A complete nutrient solution which has been optimised for ECM root formation in sand culture experiments.

Ingredient	*Concentration mg/kg soil (w/w)*
NH_4NO_3	36*
K_2SO_4	111.6
$Ca(H_2PO_4)_2.H_2O$	40
$CaSO_4.2H_2O$	51.5
$MgSO_4.7H_2O$	33.7
FeEDTA	25
$MnSO_4.4H_2O$	16.9
$CuSO_4.5H_2O$	8.2
$ZnSO_4.7H_2O$	9.2
$Na_2B_4O_7.10H_2O$	1.1
$(NH_4)_6Mo_7O_{24}.4H_2O$	0.46
$CoCl_2.6H_2O$	0.34

* *N is added every 2 weeks*

B. Selecting superior fungal isolates

Glasshouse studies are used to compare the efficacy of mycorrhizal fungi under controlled temperature and moisture conditions (Table 6.2). These experiments can also be modified to examine the impact of soil and environmental factors (such as nutrient supply, pH, temperature, moisture) on the performance of specific fungi (Burgess et al. 1993, Malajczuk et al. 1994b). Experiments should utilise conditions which are as close to the 'real world' as possible (Section 5.1). However, some factors are much more difficult to manipulate in a glasshouse experiment, so their influence cannot be examined. Consequently, fungi selected in the glasshouse as superior isolates for use with particular host plants, soils, or environmental conditions may be less successful in

the field. In practice, selection of fungi which are tolerant to a wide range of conditions may be prudent. An example of a glasshouse ECM fungus isolate screening experiment (Example 1) is provided below. Criteria which could be used to help select fungal isolates are listed in Table 6.2.

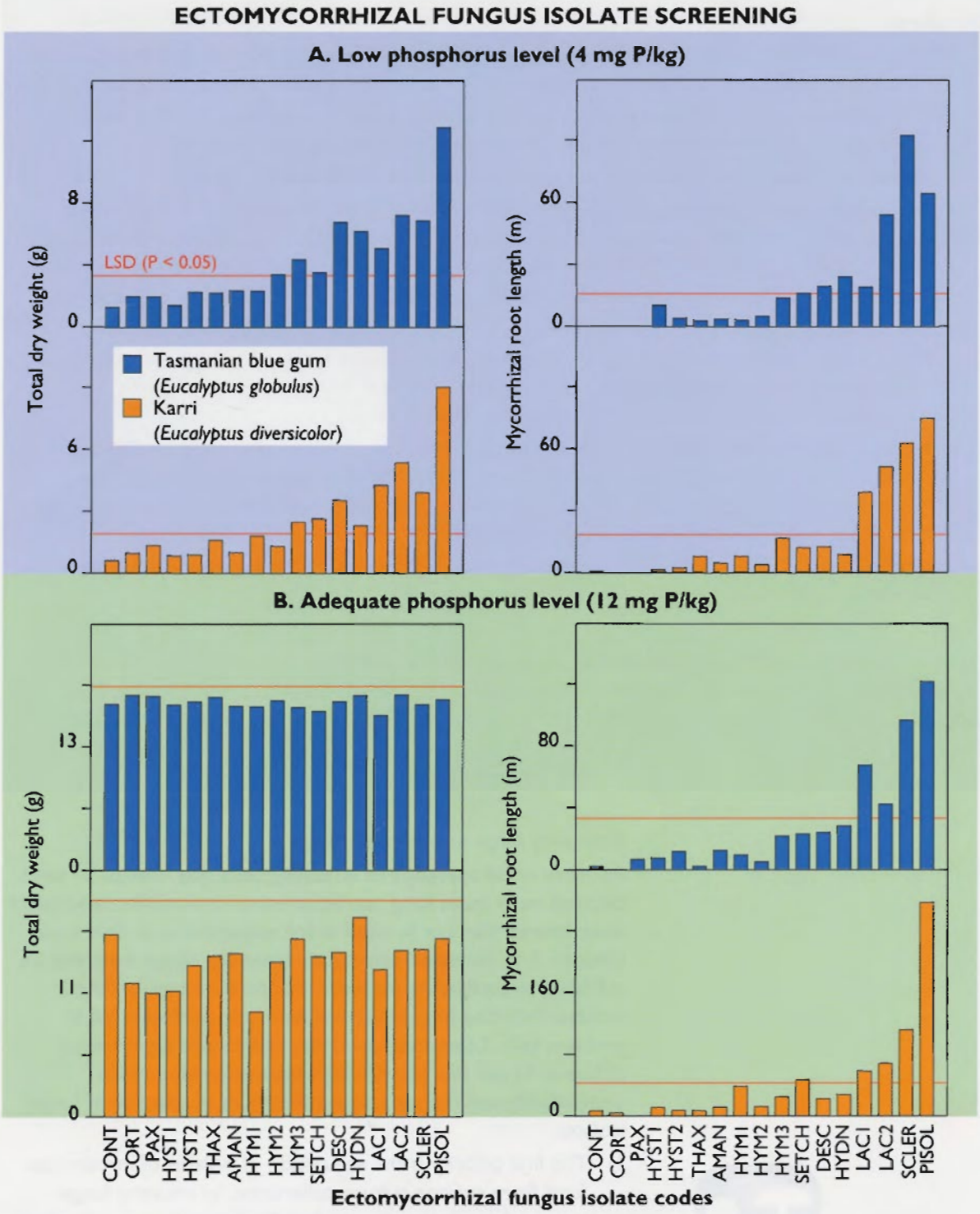


Figure 6.6. *Results of a mycorrhizal fungus screening experiment (Example 1) with 16 isolates of ECM fungi, 2 Eucalyptus species and 2 phosphorus levels (A, B). Note that many fungal treatments were significantly different from the control (CONT) at the low P level (A) but not at the higher level (B). Codes identifying fungal species are explained in Table 6.5.*

Example 1. Glasshouse ECM fungus isolate screening

Aim

To compare the effects of 16 fungal isolates on *Eucalyptus globulus* and *E. diversicolor* seedling growth.

Experimental design

A fully randomised factorial design was used (2 host trees × 17 fungal treatments × 2 P levels × 4 replicates=272 pots).

Procedure

Soil: A yellow sand (from the Spearwood dune system with pH 5.5 and less than 2 mg/kg available P) was pasteurised with steam (1 hr at 80°C) and 2.5 kg of sieved soil was placed in each 140-mm diameter non-draining plastic pot.

Fertilizers: an adequate supply of all nutrients was applied (Table 6.4), except for P which was applied as aerophos ($Ca(H_2PO_4)_2H_2O$) at 4 or 14 mg P/kg. Plants were given a nitrogen supplement every 2 weeks.

Plants: surface sterilised seeds of the two eucalypt species were germinated aseptically (Section 5.4A).

Fungi: germinated seedlings were placed in 65 mm diameter x 80 mm high polycarbonate jars containing MMN medium (Table 5.2) with 0.5% glucose and 0.3% malt extract. These jars were previously inoculated and contained active colonies of one of the ECM fungi listed in Table 6.5 (except for the control treatment). Seedlings were left in contact with fungi for 7–10 days in a growth cabinet (25°C, 16 hr light).

Planting and care: four seedlings with hyphae on their roots were transplanted into each pot of sand along with a small plug (0.2 g) of agar colonised with hyphae. Plants were grown on raised benches in a glasshouse and pots were randomly moved to new locations every 2–3 days. Plants were watered to field capacity (10% w/w water for this soil) by weighing them every 3 days initially, then daily when they grew larger.

Measurements and harvesting: height measurements were taken every 2 weeks starting in week 3. Seedlings were harvested 100 days after planting, before their growth began to slow. Shoots were dried in an oven (70°C) to determine shoot dry weight. Roots were separated into coarse root fractions, which were dried and weighed, and fine roots which were assessed to determine mycorrhizal root length (see Chapter 4).

Statistical analysis: ANOVA procedures were used to assess treatment effects and LSD values were calculated to allow comparison of treatments (Chapter 7).

Results

Results are presented in Figure 6.4. Many fungal treatments were significantly different from the control at the low P level but not when P supply was adequate for maximum growth.

Reference

Burgess et al. (1993)

Screening large numbers of fungi

An hierarchical approach to screening tests may have to be used, because many more fungi can be screened in simplified laboratory experiments than can be used in soil experiments or field trials (Section 5.4). However, laboratory screening assays must first be validated by comparing the performance of a range of fungal isolates, including fungi which are known to perform well in problem soils. Laboratory synthesis and culture experiments (Chapter 5) can also provide key information about host compatibility and the capacity for inoculum production of fungal isolates.



1. The first priority is the acquisition of isolates of mycorrhizal fungi from existing culture collections, by initiating fungal isolates from field-collected fruit bodies or by collecting fruit bodies to use as spore inoculum (Chapter 4).

Table 6.5. Origin of ectomycorrhizal isolates giving codes used in Figure 6.6, the fungal species, the area where the isolate was found. (TAS = Tasmania, WA = Western Australia) and the *Eucalyptus* sp. it was associated with when originally collected.

Code	*Species*	*Site*	*Host tree*
CORT	*Cortinarius globuliformis*	TAS	*E. globulus*
PAX	*Paxillus muelleri*	WA	*E. diversicolor*
HYST1	*Hysterangium inflatum*	WA	*E. globulus*
HYST2	*Hysterangium inflatum*	TAS	*E. globulus*
THAX	*Thaxterogaster* sp.	TAS	*E. globulus*
AMAN	*Amanita xanthocephala*	WA	*E. diversicolor*
HYM1	*Hymenogaster zeylanicus*	TAS	*E. globulus*
HYM2	*Hymenogaster viscidus*	TAS	*E. globulus*
HYM3	*Hymenogaster zeylanicus*	WA	*E. globulus*
SETCH	*Setchelliogaster* sp.	TAS	*E. globulus*
DESC	*Descolea maculata*	WA	*E. marginata*
HYDN	*Hydnangium carneum*	TAS	*E. globulus*
LAC1	*Laccaria laccata*	WA	*E. marginata*
LAC2	*Laccaria laccata*	TAS	*E. globulus*
SCLER	*Scleroderma verrucosum*	WA	*E. diversicolor*
PISOL	*Pisolithus* sp.	WA	*E. marginata*

2. It is desirable to target fungi which are likely to perform well in particular soils and climatic conditions. Herbarium database information on the distribution of fungi, host plants, climatic conditions and any soil factors likely to be correlated with distribution of fungi may be of value (Section 2.5), but taxonomic generalisations are often required as information about the habitat tolerances of a fungal species may not be relevant to particular isolates.
3. Dual-organism sterile culture experiments can be used to confirm the mycorrhizal status and compatibility of fungal isolates with key host plants (Chapter 5).
4. Glasshouse experiments such as the example provided can be used to test the capacity of fungal isolates to colonise roots in soils and the growth responses of host plants. Additional bioassay experiment could be used to measure (a) the inoculum potential of competing indigenous fungi in soils, (b) the capability of inoculant fungi to persist and spread in the presence of soil micro-organisms, and (c) the capacity of mycorrhizal associations to promote the growth of plants at realistic soil nutrient levels.
5. Anatomical investigations of the host–fungus interface (Hartig net or arbuscules) in mycorrhizal roots should be used to confirm that functional mycorrhizal associations are present (Chapter 4).
6. Field trials will provide the ultimate test of the capacity of fungal isolates to function in adverse soils (Chapter 7), but will only be possible for a limited number of fungi. The evaluation of results will involve statistical comparisons of habitat information and isolate characteristics revealed by different screening procedures.

6.3. MYCORRHIZAL SEEDLINGS IN THE NURSERY

Many forest managers are now requesting tree seedlings that are inoculated with efficient strains of mycorrhizal fungi before outplanting in the field, but there may be a need to improve nursery practices to ensure mycorrhizal plants can be delivered. The primary objective of inoculation in nurseries is to produce healthy seedlings, suitable for planting in the field, that have roots colonised with a selected fungus. Growth responses to inoculation in forest nurseries are generally of secondary importance although benefits can be obtained for slow-growing conifers where the time required before seedlings are planted in the field can be significantly reduced (Le Tacon & Bouchard 1986). Increased growth due to inoculation appears to be more common in bare-rooted seedlings than in containerised seedlings, possibly because ECM fungi can increase P uptake more from the soils of nursery beds than from the potting media used in containerised nurseries (Grove & Le Tacon 1993).

A wide range of practices can be encountered in nurseries producing containerised conifers or broad-leaved species for commercial forestry operations around the world (see Figs 6.7, 6.8, 6.9). Key issues for the management of mycorrhizal plants in nurseries include: the choice and preparation of potting mixes, the use of fertilizers, and nursery hygiene. The composition, pH, moisture content and temperature of the rooting media, fumigation and pasteurisation practices and the use of biocides and fungicides can be manipulated to provide conditions which are optimal to both root colonisation and seedling production (Marx et al. 1982, Cordell et al. 1989). However, in large-scale nursery operations, there may be less scope to control these factors than in smaller nurseries. Fertilizer use is given special emphasis (Section 6.4) because seedling nutrition has a major impact on mycorrhizal development. While the principles of good nursery management apply to all mycorrhizal plants, we focus mainly on eucalypts because a number of good nursery manuals are available for conifers in the northern hemisphere (e.g. Duryea & Landis 1984, Landis et al. 1989, 1990).

A. Potting mix components

No single soil can provide all the characteristics required to grow seedlings effectively, so nurseries generally combine several of the substrates described below to make a potting mix (Fig. 6.10). These ingredients can be mixed with soil or sand, but are most often combined to make a 'soil-less' potting mixture.

Peat

Peat is a major component of many potting mixtures providing an organic substrate for root growth. There are many variations in 'peat' formulations used by nursery managers and these include humus collected from under trees, shredded coconut, etc. However, the traditional peat

derived from sphagnum moss has the best physical and chemical formulation, being low in available nutrients and pH. For container mixes peat is often used as a 50% mix with other components such as vermiculite and perlite.

Vermiculite and perlite

These relatively cheap and readily available components are bulking agents that keep the potting mixes well aerated and prevent the growing medium from settling and compacting. Both are lightweight and pH-neutral. Perlite lacks any buffering capacity and is nutritionally inert, while vermiculite has some buffering capacity and can provide Mg and K for seedling growth (Goh & Haynes 1977).

Wood products

Sawdust and bark are used as natural substitutes for peat if supplies are limited or prohibitively expensive. The major disadvantage of these wood products is the high C:N ratio which immobilises nutrients, causing potential deficiency disorders in seedlings. They may also decompose rapidly, causing soluble nitrogen to be removed from the water in the potting mixes, and out of the reach of plant roots (Handreck & Black 1984). Some types of wood can be toxic to root growth, and preliminary testing of these products is essential prior to their commercial use. Aged and leached wood products are preferred (i.e. composts).

Composts

Compost is manufactured by combining plant material such as straw, wood products, plant residues and animal wastes in a pile, and maintaining it in a moist state to allow partial microbial decomposition (Hoitink 1980, Hardy & Sivasithamparam 1989). The final product is a humus-like soil amendment relatively high in nitrogen with a much reduced C:N ratio, relative to raw wood products. Compost has a high water retention quality, low weight, and mixes well with sand, soil, perlite or vermiculite. Potting mixes based on composted eucalypt bark have been found to suppress pathogens such as *Phytophthora* spp. (Hoitink 1980, Hardy & Sivasithamparam 1991). A potting mix based on composted bark has been found to be highly conducive to ECM formation on eucalypt roots in commercial nurseries in Western Australia (Hardy, pers. comm.).

Mixtures

The ratio of the substrates listed above in potting mixes varies according to the quality of these substrates used. A 1:1 mix of peat and vermiculite or perlite is generally a preferred ratio, allowing for maximum aeration and drainage, although these ratios can be changed to compensate for the availability and cost of components (Figure 6.10). Composted bark is usually combined with perlite and/or vermiculite in potting mixes.


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Figure 6.7. *Nursery practices in Kunming, China (Kunming Institute of Botany). This system, where seedlings are raised in 'polybags' in the ground, is common in China.*

A. *Preparation of growing medium by mixing burnt organic matter with topsoil.*

B. *Polybags filled with soil and arranged in a nursery bed.*

C. *Adding a seed and ECM fungus inoculum (arrow) to each polybag.*

D. *Young* Eucalyptus globulus *seedlings.*

E. *Seedlings ready for outplanting after 4 months.*

F. *Inoculating cuttings of* E. grandis × urophylla *by placing 'mycobeads' — encapsulated mycelium of an ECM fungus (star), in a hole near the base of each cutting.*

G. *Examination of roots of a eucalypt seedling for mycorrhizal formation in the nursery.*

B. Pasteurising potting mixes

Potting mixes with peat, perlite or vermiculite as major components often do not require pasteurisation, whereas mixes of sawdust, bark, raw humus or soil should be treated to reduce the potential threat from pathogens and other micro-organisms which would limit mycorrhizal fungal growth on the roots. It is best to pasteurise soil mixes when they are warm and moist. Peat, however, can be steamed when it is almost dry. Steam treatment, when carried out at 80°C for 30 minutes, is preferred because it is non-toxic and only partially sterilises mixes, eliminating harmful micro-organisms and weed seed while some beneficial microbes remain (Baker & Roistacher 1957). It is important that much of the saprophytic microflora remains. An additional goal in mycorrhizal nurseries is to eradicate less efficient strains of mycorrhizal fungi, such as *Thelephora* spp., which can reduce the success of controlled inoculation programs. A wide variety of steam pasteurisation equipment is available, but may be too expensive for small nurseries. Temperatures much over 80°C can be disadvantageous in soil mixes containing some types of acid soil because the release of toxic amounts of Mn can cause Fe deficiency in containerised plants (Sonneveld 1979).

A cheaper alternative to the use of steam is solar pasteurisation, where wet potting mixes are covered by, or enclosed in, clear polyethylene sheeting and exposed to direct sunlight for a period of time (Fig 6.10B). Temperature under the polyethylene sheet can rise to over 60°C during the summer months, and may be higher in the tropics. Solarisation results in continuous or repeated exposure to sublethal temperatures which will either kill the majority of the pests, weed seeds and pathogens, or weaken them so that they cannot survive (Stapleton et al. 1985, Porter & Merriman 1985). Solar pasteurisation can also be used in nurseries producing bare-root seedlings (Fig. 6.9EF). In this situation seedlings beds are prepared according to standard procedures being adopted by the grower at least 3 months prior to seeding (Duryea & Landis, 1984). It is advisable that during this period air temperatures are high. In

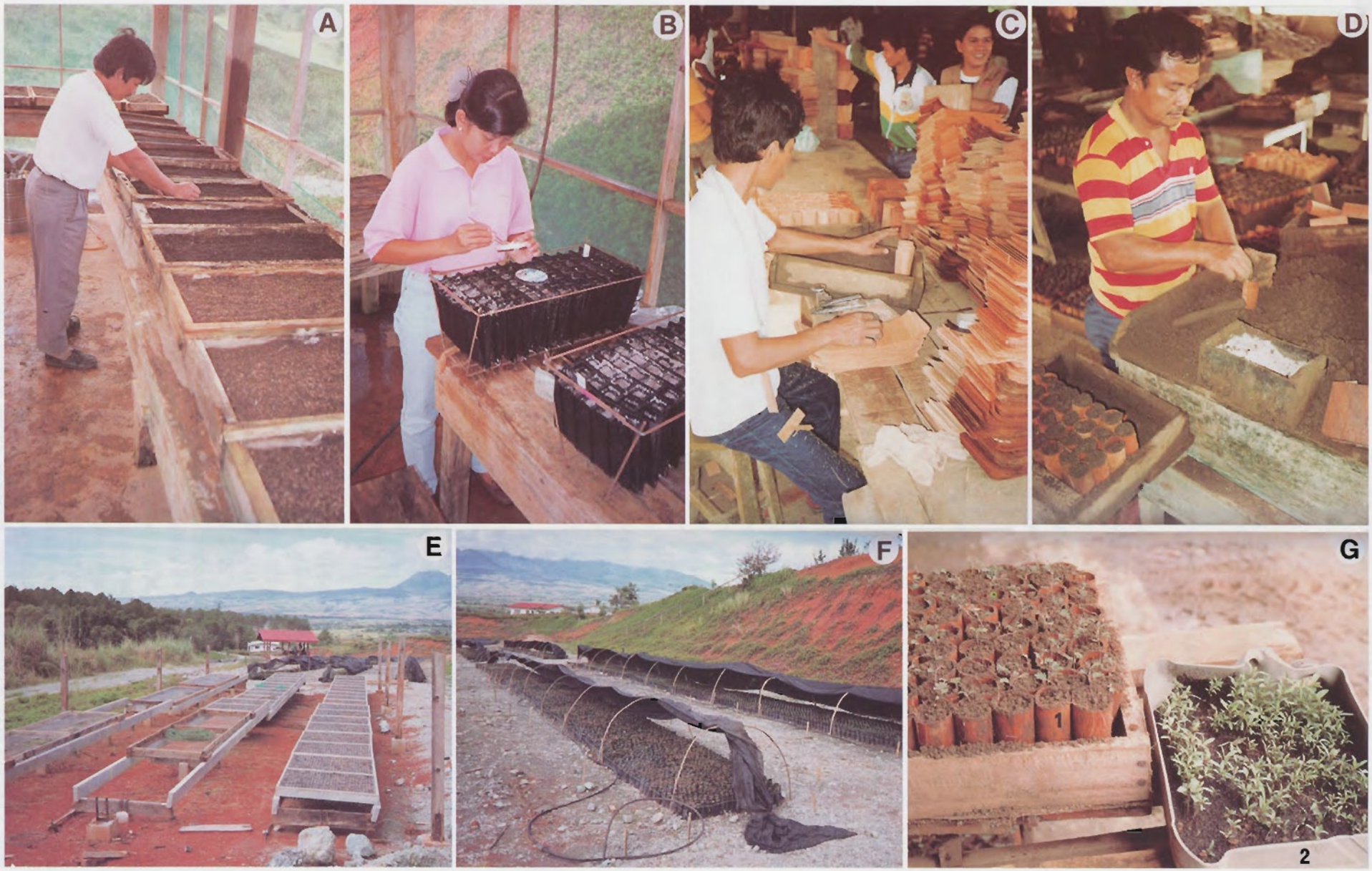

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Figure 6.8. Seedling production at two nurseries in the Philippines.

A. *Steamed and sieved forest soil placed in boxes for seed germination.*

B. *Transplanting seedlings into 'root trainer' pots while applying ECM fungus inoculum (segments of mycelium, axenically cultured on agar).*

C,D. *Forming cylindrical seedling containers out of wood veneer and filling them with soil.*

E. *Construction of raised nursery beds for seedling growth.*

F. *Seedlings grown on crushed rock and protected by shadecloth. This rock substrate was later found to contain toxic levels of heavy metal ions.*

G. Acacia *seedlings transplanted into wood veneer tubes (1) and* Eucalyptus *seedlings ready for transplanting (2).*

Australia, beds can be prepared during the beginning of summer. Seedlings beds are then well watered and sheeting of clear polyethylene sheets laid along the rows (Fig 6.9E). During the summer period we have recorded temperatures under these sheets as high as 60°C within the top 5 cm and 40°C at 20 cm. These temperatures not only control major root diseases (Porter & Merriman, 1985) but increase the availability of nutrients (Katan 1981, Stapleton et al. 1985). Removal of plastic sheets at the end of this period shows obvious weed reduction (Fig 6.9F). Inoculum is added prior to seeding the beds by raking in solid state vegetative inoculum (Fig.6.9G) or by watering in spores either before or after seeding.

Chemical sterilisation with fumigants can be simple and effective, but precautions indicated on canisters must be followed and advice sought from the local agricultural pest control agency. Methyl bromide-chloropicrin (MBC) fumigant is the more widely used, providing effective sterilisation of soils.

WARNING

Chemical sterilants are highly hazardous materials. Extreme care is essential in the handling and application of these chemicals and the instructions for use indicated on labels must be strictly followed.

An alternative to pasteurisation for some forest nurseries may be to use carefully controlled composted components for their nursery potting mixes. Hoitink & Fahy (1986) and Hardy & Sivasithamparam (1991) have demonstrated that composting can thermally eradicate some plant pathogens, and this is aided by the antibiotic action of thermostable micro-organisms which proliferate during the composting cycle.

C. Seedling containers

Mycorrhizal seedlings of trees raised for plantation forestry can be produced in open beds of soil (bare-root seedlings) or in specialised seedling containers in the nursery. Bare-root seedling production (Figs 6.9GH) will not be considered in detail here, as it is more commonly used for pines and other trees grown in colder climates than for eucalypts. Disease and pest management is simplified if seedlings are grown in containers which are raised off the ground (Figs 6.8E, 6.9D), since they are not exposed to soil pests and pathogens and will be well aerated. The control of soil and environmental factors which can influence mycorrhizal formation would also be easier in containerised systems than in bare-root nurseries. A variety of nursery systems based on


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Figure 6.9. *Examples of nursery management and mycorrhizal inoculation practices in Australia.*

A–D. *Bunnings Treefarms Forest Nursery in Manjimup, Western Australia.*

A. *A potting mix based on composted pine bark.*

B. *Machinery which fills seedling flats with potting mix and plants seeds in a continuous operation. This equipment was adapted to efficiently apply spore slurry inoculum of ECM fungi to seedlings.*

C. *Close-up of equipment in B, but showing the fluid drill seeder (arrow).*

D. *Seedlings growing on open raised beds.*

E–H. *Raising bare-root mycorrhizal seedlings in soil beds at the Department of Conservation and Land Management Nursery in Manjimup, Western Australia.*

E. *Clear plastic sheets placed over beds for solar pasteurisation of soil. Sheets were kept in position over damp soil for 1–2 months in summer, to raise the soil temperature above 50°C, to kill weeds and pathogens.*

F. *Same area as E some time after removal of the plastic, showing that soil in beds is free of weeds (arrows).*

G. *Applying mycorrhizal inoculum (peat–vermiculite solid substrate culture) to plots within beds. Inoculum was dispersed over beds then raked into the soil.*

H. *Inoculated* Eucalyptus diversicolor *seedlings after 6 months of growth.*

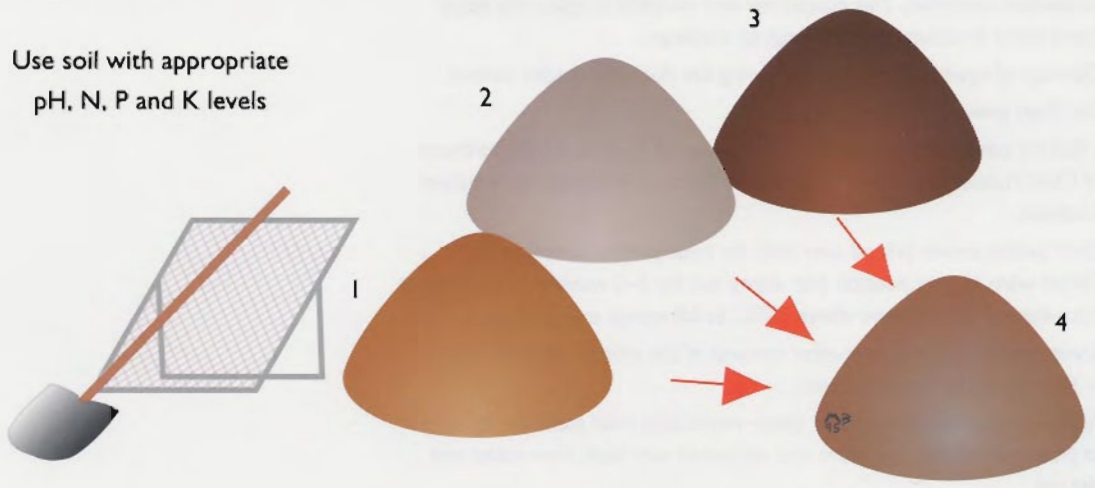
different types of growth containers for seedlings is compatible with mycorrhizal fungus inoculation (compare Figs 6.7, 6.8 and 6.9).

Soil-filled polybags have been used to produce seedlings throughout Asia for many years, and may be the only available alternative in some areas (see Fig. 6.7). They are relatively inexpensive and can be manufactured locally, so are appropriate for use in small, community-run nurseries. However, the use of polybags as nursery containers has a number of serious technical and logistical disadvantages. Polybags require large amounts of soil, are difficult to handle due to their large size and weight, are poorly aerated, preventing good root growth, and occupy large areas in the nursery. Nurseries using fine textured soil often have problems with waterlogging, especially if the polybags are partially buried in the soil (Fig. 6.7C). Polybags are also not reusable. More importantly, seedlings produced in polybags can have root systems that are twisted, knotted or coiled, so the resulting trees may be subjected to wind-throw and root strangulation years after they are outplanted. Wood veneer tubes are a similar system to the polybag that is used in some nurseries in the Philippines (Figs 6.8CD,G).

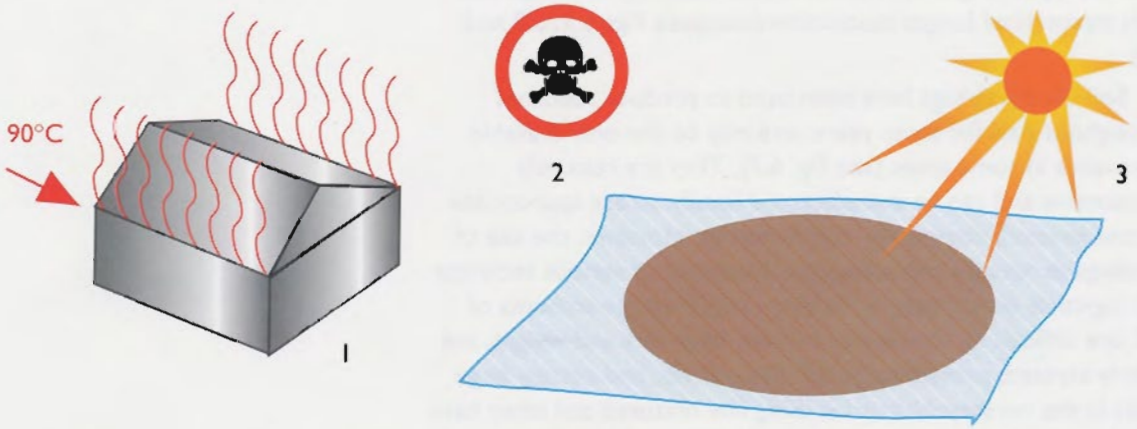
Fortunately, there are a number of good alternatives to the polybag. Modern containers that can be used include individual seedling containers (Fig. 6.8B) and compartmentalised seedling trays (Figs 6.9–D, 6.10C). Modern containers are designed so they lack sharp edges that could distort root growth, have vertical ribs inside to direct roots downwards to prevent spiralling and have an open base to allow for air pruning to encourage lateral root

SOIL MANAGEMENT IN NURSERIES

A. Combine (1) sieved soil or sand, (2) perlite and/or vermiculite and (3) peat or composted organic matter to make a potting mix (4)



B. Pasteurise potting mix using (1) steam heating, (2) chemical fumigation or (3) solar heating



C. Add potting mix to sterilised (1) polybags, (2) root training containers or (3) flats

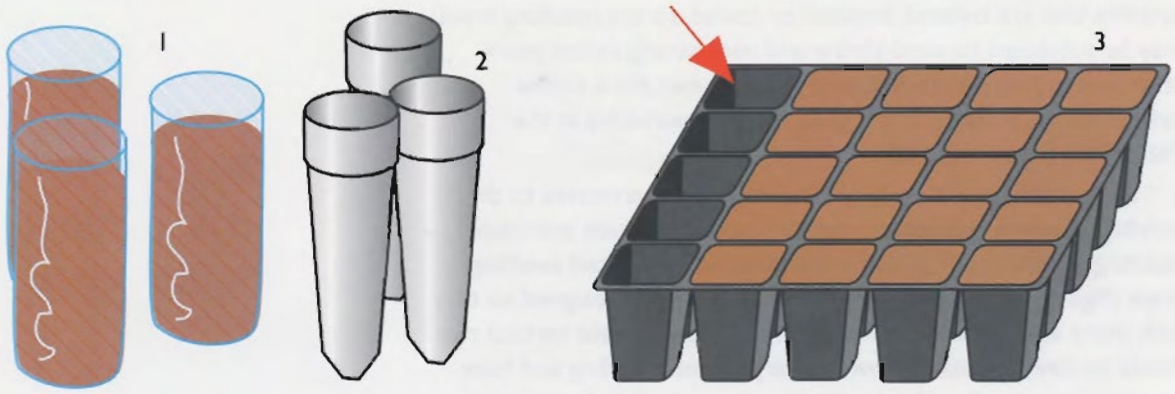


Figure 6.10. Nursery soil management.

formation. Containerised nursery production systems have evolved over the past 50 years and there are many products on the market which provide a range of costs, shapes and sizes (Hahn 1982). Many studies have demonstrated advantages of containerised seedlings after outplanting, such as faster growth and a superior capacity for early root elongation (Hahn 1982, Landis et al. 1989). More importantly, the use of lighter and smaller containers is less labour-intensive and allows greater numbers of seedlings to be transported to reafforestation sites. There is the added benefit of increasing the flexibility to scale seedling production up or down in response to the needs of the planting programs. Mechanised planting systems, which are now often used in containerised nurseries (Fig. 6.9BC), can be readily adapted to provide a more efficient delivery of fungal inoculum than is possible with manual planting systems (Section 5.5).

D. Disease and pest control

Intensive production of plants in nurseries creates opportunities for pests and diseases to flourish if not checked. These are exacerbated under conditions of poor hygiene or where plants are stressed due to poor management (e.g. over- or under-watering, inappropriate potting mixes or overfertilisation). All nurseries should have appropriate strategies for controlling pests and diseases which consider the interactions between the causal organism, the containerised mycorrhizal plant and environmental factors. Examples of integrated pest management strategies are given in most good books on disease control (e.g. Jarvis 1992).

Chemicals are widely used to control fungal diseases (fungicides), insect pests (insecticides) and nematodes (nematicides) in nurseries. In addition, some nurseries may use herbicides to control weeds. *In mycorrhizal nurseries, no chemicals should be used without first determining their effect on the development of mycorrhizas.* Much work has been undertaken in this area but the results can appear to be contradictory because different fungi can respond differently to a given chemical (Trappe et al. 1984). In general, fungicides that have been formulated specifically for basidiomycete fungi and copper-based fungicides should not be used in ectomycorrhizal nurseries. Where there is a need to control damping-off fungi such as *Pythium* then fungicides which target the Oomycetes should be used rather than broad range fungicides. These include Previcur® and Fongarid®. Our experience with ectomycorrhizal eucalypt seedlings is that the first few weeks of ECM development following inoculation are the most critical stage when fungicides can have their most damaging effect. Fungicides which are commonly used in nurseries in Australia, such as Benlate ®, Bravo®, Sumislex 275® and Delsene® have been found to have a limited impact on ECM fungi provided they are not applied frequently and recommended dosages are not exceeded (G. Hardy, pers. comm.). Herbicides should also be used with caution, as some may have negative short-term effects on ECM fungi (Chakravarty & Chatarpaul 1990).

6.4. MINERAL NUTRITION OF MYCORRHIZAL PLANTS

A. Nutrient requirements

Growth and performance of seedlings are primarily related to the nutrient content of the potting media. Nutrient uptake varies with each tree species and preliminary experiments should be carried out to determine the requirements for nitrogen and phosphorus. There is a characteristic relationship between the concentration of a mineral nutrient in seedling tissue and its growth. *When a nutrient concentration is low enough to have an adverse effect on a plant, it is said to be deficient.* At low nutrient levels, seedlings exhibit certain abnormalities called deficiency symptoms (Dell & Robinson 1993), which could include stunting and foliar discolouration (Fig 6.12). At slightly higher concentrations, the nutrient is still deficient, but as the nutrient supply is increased the seedlings reach an optimal range for maximum growth (Fig. 6.14). Even when mineral nutrients are present in surplus quantities, seedlings may still continue to take them up with no measurable increase in growth. This luxury consumption is common in forest nurseries, as many growers keep nutrient levels at high levels because fertilizers are relatively inexpensive relative to other costs. However, overapplication of nutrients can eventually lead to toxicity problems such as 'burning' of the foliage of seedlings and reduced growth(see B below).

Fertilizers



Ideally, fertilizer should be supplied in small amounts throughout the growing season to meet the plant's requirement for growth. The production of ECM plants, however, requires extra care in formulating fertilizer regimes and fertilizer rates. This is because the overriding objective is to produce well-inoculated seedlings, cuttings or tissue explants. Maximum growth in the nursery is not always necessary and is often undesirable for fast-growing trees such as eucalypts, because plants with large shoots do not transplant well. Trees and ECM fungi largely have similar requirements for specific mineral nutrients, the exceptions being the alleged ability of some fungi to grow without boron (Kendrick 1992, Griffin 1994). The total inorganic nutrient demand for hyphal growth in pots is small relative to that required to support the development of the plant body. Typically, the nutritional requirement for the growth of the inoculant fungus in nursery containers is less than 15% of the total nutrient demand of the plant. We recommend that both the plant and soil should be monitored by chemical analysis to ensure that soil fertility levels are appropriate for ECM development.

Effects of nutrients on mycorrhizal development

Inappropriate levels of fertilizer and imbalances in soil fertility can have detrimental effects on ECM root development through effects on the availability of assimilates to roots and on root growth (Hepper & O'Shea 1984, Beckford et al. 1985, Shaw et al. 1987, Gagnon et al. 1988, Ericsson & Kähr 1993, Göransson 1993, 1994). Both the supply and composition of nutrients can influence mycorrhizal fungus development in the nursery (Section 6.1B). Considerable research worldwide has been undertaken, especially on conifers, to develop bare-root and container nursery regimes which produce ECM seedlings. In containers, mycorrhizal development is greatest where the phosphorus supply to roots is limiting for the maximum growth of non-mycorrhizal plants under the same conditions, and there is no response to inoculation where the phosphorus supply is adequate (Bougher et al. 1990, Jones et al. 1990). High rates of fertilizer applications used to produce large non-mycorrhizal seedlings for outplanting have been shown to suppress mycorrhizal root formation, while reduced fertility levels result in good ECM root development of container seedlings (Marx & Barnett 1974, Molina 1979, Molina & Chamard 1982, Danielson et al. 1984a,b, Gagnon et al. 1987, Marx 1990). Gagnon *et al.* (1995) have determined N fertilizer rates that optimise both the ECM root development and growth of Douglas-fir seedlings.

Nitrogen forms

The form of nitrogen (ammonium vs. nitrate) applied can influence the growth of seedlings in pots and the mycorrhizal response to inoculation (Marschner & Dell 1994). For example, eucalypt seedlings utilise ammonium more efficiently than nitrate (Shedley et al. 1993).

Interactions between nutrients

There is a lack of quantitative information on the extent to which interactions among nutritional and non-nutritional factors affect root colonisation and growth responses to inoculation. Experiments have only examined effects of single nutrients, combined fertilizers containing fixed ratios of N and P (Ruehle & Wells 1984), or interactions among the effects of N, P and K at single ratios of addition (Menge et al. 1977, Newton & Pigott 1991). The supply of P, N and inoculated fungal isolates each has a marked effect on root colonisation of *Eucalyptus diversicolor* grown under glasshouse conditions (Fig. 6.11), but there is a marked interaction between the effects of all three factors on seedling growth. The effects of P and N supply on colonisation by ECM fungi are often linked to their effects on the concentration of soluble carbohydrates in roots (Harley & Smith 1983), although complex interactions between a number of regulatory mechanisms are likely. No doubt, further interactions between nutrients and mycorrhizal development will be uncovered with more experimentation.

Determining nutrient requirements through experimentation in the nursery

Since the levels of N and P in potting mixes can strongly influence mycorrhizal development, it is recommended that fertilizer trials should be undertaken to determine fertilizer levels that maximise mycorrhizal development. This should be carried out each time the composition of a potting mix is changed. The case study below (Example 2) gives a protocol for this purpose.

Example 2. Determining mycorrhizal plant nutrient requirements

Aim

To determine liquid N and P fertilizer rates for healthy ectomycorrhizal eucalypt seedlings in nursery containers.

Experimental design

Use a factorial design consisting of 3 nitrogen rates (e.g. 9, 27 and 81 µmoles N/plant/week supplied as urea or NH_4NO_3) by 3 phosphorus rates (e.g. 0.1, 0.5 and 2.5 µmoles P/plant/week supplied as KH_2PO_4). If specific N and P fertilizer rates are currently being used in the nursery operation, then include these as one of the N and P treatments.

Procedure

A. To the potting mix add:
   1. complete micronutrient mix
   2. gypsum (2 g/L potting mix)
   3. dolomite to adjust pH (in the range 5 to 6.5)

B. Mix thoroughly and pasteurise with steam

C. Place mix in containers, add imbibed seed and fungal inoculum

D. Randomise the treatments in 3 or 4 replicate blocks in the nursery

E. Two weeks after germination apply fertilizer treatments and a basal liquid fertilizer containing all essential nutrients except N and P (Table 6.8)

F. Repeat foliar fertilisation at regular intervals

G. Measure plant height weekly

H. After 8–12 weeks, harvest plants and quantify ECM development.

I. Subject the data to an analysis of variance to determine whether significant differences exist among the treatment means. If there is a significant difference, analyse the data further to explain the nature of the response in more detail

Results

Use results to recommend appropriate operational levels of N and P use for the nursery or undertake further experimentation. Figure 6.11 illustrates results from a glasshouse trial investigating the effect of N x P interactions on ECM development by *Eucalyptus diversicolor*.

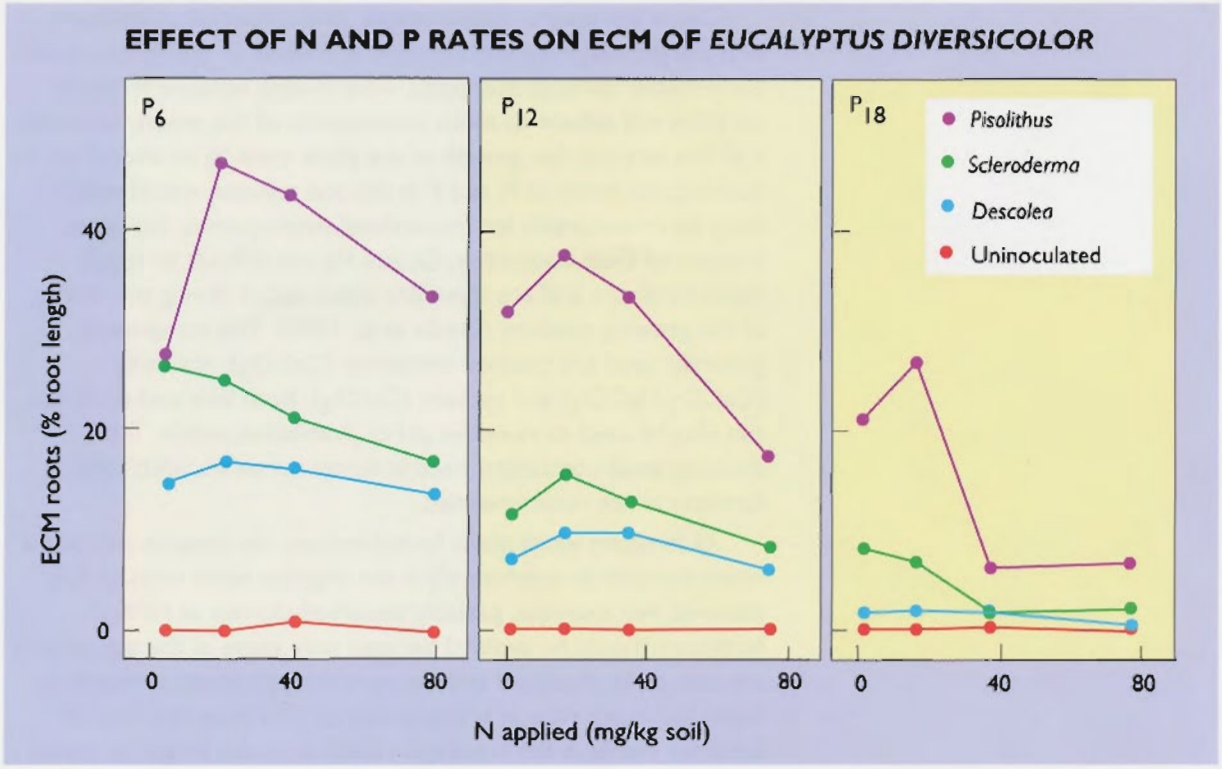


Figure 6.11. *The effect of three rates of phosphorus application (P_6, P_{12}, P_{18}) and variations in N supply on ECM root formation by three mycorrhizal fungi. P_6, P_{12}, P_{18} correspond to 6, 8 and 12 mg P applied per kg soil (data from Grove, Dell & Malajczuk, unpublished; Marschner & Dell 1994).*

B. Fertilising eucalypts in nursery containers

Many issues must be considered when formulating fertilisation schedules for containerised mycorrhizal tree seedlings. These include the type and rate of fertilizer and the application method and frequency (Table 6.6).

Fertilizers can be single or multi-elemental in composition and can be applied in either a dry or wet formulation. Complete fertilizers have: three primary macro-elements: nitrogen, phosphorus and potassium; the secondary nutrient elements Ca, Mg and S; and the micro-elements Fe, Mn, Zn, Cu, B, Mo, Ni and Cl. Legumes may also require Co for the N-fixing bacteria in root nodules. A wide range of fertilizer formulations exists including slow-release and controlled-release formulations (Sanderson 1987).

Method of application

There are four ways to apply fertilizers in a container nursery.

1. Application of dry fertilizer to the growing medium before planting.
2. Injecting liquid fertilizer into the irrigation water (fertigation).
3. Manual application of liquid fertilizer separate from the irrigation water.
4. Top dressing solid fertilizer to the container surface.

There are several disadvantages to incorporating fertilizers into the growing medium. Firstly, it is difficult to evenly distribute the fertilizer through the media while mixing because fertilizer particles will adhere to moist constituents of the media. Secondly, if all the fertilizer for growth of the plant were to be added before planting, the levels of N and P in the soil solution would most likely be unfavourable for mycorrhizal development. However, because of their insolubility, Ca and Mg are difficult to supply in liquid fertilizers and are therefore often added during the mixing of the growing medium (Landis et al. 1989). The compounds generally used are crushed limestone ($CaCO_3$), dolomite ($CaCO_3.MgCO_3$) and gypsum ($CaSO_4$). Both lime and dolomite can also be used to raise the pH of peat-based media. Top dressing small containers, used in forest nurseries, with solid fertilizer is not recommended.

All fertilizers which are to be applied over the containerised plants either manually or automatically in the irrigation water must be fully dissolved. For example, partially dissolved slurries of NPK fertilizers should be avoided because only some of the ingredients are able to be dissolved and the remaining particulate matter can cause leaf burn. Also, it is impossible to distribute this kind of fertilizer evenly in the nursery by hand or in the irrigation water.

- **Seedling containers should be regularly flushed with lots of water to prevent the build-up of fertilizer salts in the potting mix.** Accumulated salts can impair root and shoot growth and may inhibit the formation of ectomycorrhizas.
- **Liquid fertilizer application should be scheduled as early as possible in the day.** This is to prevent fertilizer burn in full sun and to reduce overnight leaf wetness which favours foliar diseases such as *Botrytis*.

Timing of liquid fertilizer application

Liquid fertilizers can be applied continuously each time the plants are irrigated or periodically according to a fixed schedule. Clearly, much lower concentrations of nutrients will be applied at each application in the former case. A further level of sophistication is to add nutrients according to the internal demand in the seedling for growth. In programmed nutrition addition, the amount of fertilizer applied will be low when the seedlings are small and larger when the seedlings have grown. However, since many forest nurseries producing mycorrhizal eucalypts do not have fertigation equipment, the fertilizer prescriptions given below are for period application at a fixed fertilizer rate.

Choice of fertilizers

The objective of liquid fertilisation is to provide a complete and balanced mixture of essential plant nutrients that will optimise the development of ECM plants in containers. Only inorganic fertilizers should be used for liquid application. Since no single compound contains all the essential nutrients, a mixture of compounds must be used. These dry fertilizers can be dissolved in water and mixed prior to use, or concentrated forms of commercial liquid fertilizers can be used. The choice of compounds to use in liquid fertilizers is limited because of **solubility**, **interaction of ions in solution**, and **salt toxicity**. Table 6.7 lists recommended compounds for making up liquid fertilizers.

Table 6.6. Key questions on fertilisation of mycorrhizal eucalypt seedlings in the nursery.

Question	*Answer*
1. What nutrient elements should be supplied in the fertilizer?	A complete fertilizer should be supplied containing all the essential nutrients for tree growth. These are the macronutrients N, P, K, Ca, Mg and S, and the micronutrients Cl, Fe, Cu, Zn, Mn, B and Ni. **Notes:** Mn should not be added if the potting mix contains acid forest soil high in Mn. If nitrogen is being added as NO_3^-, Mo should be included. For N-fixing trees such as *Acacia* and *Casuarina*, add Co and Mo in trace amounts. There are usually sufficient amounts of Ni in other fertilizer salts to maintain seedling growth.
2. Can standard commercial fertilizers be used?	Yes, provided they contain a balanced composition of the essential nutrients and these are in a form suitable for plant growth. **Notes:** Most commercial fertilizers labelled as complete fertilizer lack essential micronutrients. In many commercial NPK mixes, the P:N ratio is unfavourable for ECM development.
3. Are there forms of inorganic fertilizers that should be avoided?	Yes, many ectomycorrhizal fungi and some eucalypts grow better on NH_4^+ than on NO_3^-. Nitrogen is best supplied as urea or as NH_4NO_3. Do not use NH_4SO_4 as this can overly acidify the potting mix. Avoid using Cl^- salts as this can lead to chloride toxicity. **Notes:** Where the fertilizer is supplied to the plant in liquid form, use soluble salts. Filtered slurries of compound fertilizers should be avoided because some fertilizer components are difficult to dissolve and the distribution of fertilizer across nursery beds is often uneven.
4. Can animal manures be used in nursery containers?	Animal manures are best avoided because it is difficult to obtain controlled rates of application and uniform distribution of nutrients in nursery containers.
5. When should fertilizer be applied?	Do not add N and P to the potting mix before planting. Often there are enough starter nutrients in the sterilised potting mix to sustain plant growth for several weeks. A dose of micronutrients, gypsum and dolomite (if necessary to adjust pH) can be included in the potting mix, ensuring it is well mixed before potting up. Complete liquid fertilizer can then be added at 1–2 week intervals from the start of ECM formation (approx. 2 weeks for eucalypts) during the growing season according to plant growth.
6. What amount of fertilizer should be applied?	Firstly, the amount of P should be marginal to low in order to encourage good mycorrhizal development. This rate should be determined by undertaking a phosphorus rates trial (see Example 2). The amounts of the other nutrients should be sufficient to promote healthy plant growth. The amount of nutrients the plant needs will depend on the relative growth rate. Plant analysis (see Section C) can be used to calculate the internal nutrient requirement and can also be used to monitor the nutrient status of the containerised plants. Table 6.7 contains a recommended foliar fertilizer for eucalypt seedlings.
7. How should the fertilizer be applied?	The best way to supply a carefully regulated amount of nutrients is as a liquid, either in the irrigation water (fertigation) or manually with watering cans.

Table 6.7. Chemicals suitable for providing macronutrients for liquid fertilizer formulations.

Compound	*Formula*	*% Nutrient supplied*
Ammonium nitrate	NH_4NO_3	34% N
Ammonium sulfate	$(NH_4)_2SO_4$	21% N, 24% S
Calcium chloride	$CaCl_2$	36% Ca
Calcium nitrate	$CaNO_3$	17% Ca, 15% N
Di-ammonium phosphate	$(NH_4)_2HPO_4$	21% N, 24% P
Di-potassium phosphate	K_2HPO_4	45% K, 18% P
Magnesium sulfate	$MgSO_4$	10% Mg, 13% S
Mono-ammonium phosphate	$NH_4H_2PO_4$	11% N, 21% P
Mono-potassium phosphate	KH_2PO_4	28% K, 23% P
Potassium chloride	KCl	52% K
Potassium nitrate	KNO_3	37% K, 13% N
Potassium sulfate	K_2SO_4	44% K, 18% S
Urea	$CO(NH_2)_2$	45% N

Notes:

1. Be careful when using analyses given on fertilizer bags. By convention, concentrations appear as percentage of N but as percentage of the oxide of P, K, Ca and Mg. To convert oxide values to nutrient values: for P in P_2O_5 multiply by 0.437, for K in K_2O multiply by 0.83, for Ca in CaO multiply by 0.714, and for Mg in MgO multiply by 0.6. However, this convention does not apply in all countries so check the fine print on the label.

2. Ammonium sulfate can acidify the potting mix.

3. High rates of calcium chloride or potassium chloride can cause salt toxicity.

4. Because the following chemicals are incompatible in the concentrated fertilizer stock solutions, they must be dissolved separately:

— calcium nitrate or calcium chloride and any sulfate salts e.g. ammonium sulfate, potassium sulfate, magnesium sulfate, copper sulfate

— salts containing calcium and salts containing phosphate.

Fertilizer application rates

Although there are many commercial fertilizers currently available, the instructions indicated on the labels rarely indicate application rates suitable for mycorrhizal plants in containers. Generally the phosphorus levels in these fertilizers are excessive for good mycorrhizal development. We have achieved good ECM development in containers using well-aerated, pasteurised potting mixes where the inorganic fertilizer is applied in solution in small doses during the growing season. We recommend this approach because the amount of fertilizer can be easily regulated according to the growth rate and appearance of the plants. Furthermore, there is less risk in having levels of phosphorus and nitrogen which may inhibit ECM development when the plants are very small. Liquid fertilizer can be applied either in watering cans by hand or it may be added in the irrigation water if appropriate systems are available in the nursery. A recommended schedule is proposed in Table 6.8.

Table 6.8. Recommended nutrient concentrations in liquid fertilizer for promoting development of eucalypt ECM roots in nursery containers when applied weekly. Several potential sources are provided for each element, but only the most cost-effective or readily available source would be used.

Element	*Concentration (μM in applied water)*	*Recommended sources*
Nitrogen	4,000	urea NH_4NO_3
Potassium	800	KNO_3 K_2SO_4
Calcium	250	$CaNO_3$ $CaCl_2$
Magnesium	250	$MgSO_4.7H_2O$
Sulfur	250	$MgSO_4.7H_2O$ K_2SO_4
Phosphorus	40	KH_2PO_4 K_2HPO_4
Iron	10	NaFeEDTA NaFeHEDTA NaFeEDDHA $FeSO_4$
Boron	10	H_3BO_3 $Na_2B_4O_7$ B_2O_3
Zinc	2	$ZnSO_4.7H_2O$ $Na_2ZnEDTA$ NaZnHEDTA
Manganese	2	$MnSO_4.5H_2O$ MnEDTA
Copper	1	$CuSO_4.4H_2O$ $Na_2CuEDTA$ NaCuHEDTA
Molybdenum	0.1	$NaMoO_4.2H_2O$ $(NH_4)_6Mo_7O_{24}.4H_2O$

Notes:

1. *Stock solutions at about 1000 X the above dosages should be made up separately for each of the above compounds and then diluted to produce the working solution.*
2. *The working solution should be applied as a drench over the foliage and potting mix of containerised plants at weekly intervals from 2 weeks after seedling emergence.*
3. *Apply fertilizer in the early morning.*
4. *Use FeEDTA if the pH of the potting mix is below 5.6.*
5. *Although KCl can be used to balance the rates of K and S, care should be taken to avoid Cl^- toxicity.*
6. *EDTA = ethylenediaminetetracetic acid,*
   HEDTA = hydroxyethylethylenediaminetriacetic acid,
   EDDHA = ethylenediamine dihydroxyphenylacetic acid.

C. Fertilising eucalypts in field trials

Fertilizer trials are usually undertaken in conjunction with mycorrhizal fungus screening trials in the field to allow the benefit of inoculation to be realistically assessed. Chapter 7 provides information on trial design and analysis. Issues relating to fertilizer preparation and use are considered here. Great care is required to ensure that fertilizers are prepared and used correctly so that growth responses can be attributed correctly to a fertilizer variable being assessed (e.g. rate of supply or specific nutrient). The following points arise from our experience using fertilizers in field trials in Australasia.



1. Fertilizers that are distributed in the field must be accurately measured either by use of a balance (e.g. 0.5–10 kg), or volumetrically (e.g. 50–500 mL). When measuring fertilizer volumes, small plastic containers which are trimmed to a precise volume are particularly useful. We have often weighed fertilizers which are banded or applied on an area basis, but used volume measures when fertilising individual trees.
2. Many commercial fertilizers such as superphosphate form solid lumps. These should be separated by sieving (1 cm mesh), broken up and recombined with the fertilizer before use.
3. When calculating rates of micronutrients to apply, check the analysis on the bag (see Table 6.7). Be aware that different methods of expression of nutrient values can be used. It is a good idea to have samples of fertilizers analysed by a reliable laboratory, as concentrations can vary considerably between batches in some areas. Accurate determination of application rates for micronutrients such as B is very important, because there is a narrow range between deficient and toxic levels.
4. Working with micronutrients requires particular care because of the risk of contamination. The main sources of contamination are;
   - — micronutrients present as minor contaminants of other fertilizers such as superphosphate or NPK mixes,
   - — use of dirty equipment for sieving, mixing, weighing or distributing fertilizers,
   - — mixing or weighing fertilizers too close to the field trial,
   - — inadvertent movement of fertilizers with soil on workers' boots or implements, and
   - — uncontrollable events such as abundant rainfall or water buffalo traffic that may transport fertilizers at sites.
5. It is often necessary to make up special micronutrient mixes for field trials because the incidence of micronutrient deficiencies varies between sites. Weigh out components and mix thoroughly away from the field site, taking into account the following points;
   - — all fertilizer salts should be dry and without lumps, and
   - — components should be mixed as close to their time of use as possible, as chemical reactions may occur.

D. Identifying nutrient disorders in the nursery

Cause of disorders

Nutrient disorders in containerised tree seedlings can result from the following.

1. *Nutrient deficiency* due to plant growth exceeding nutrient supply in the potting media and irrigation water. This can be due to the potting mix having very low levels of particular nutrient elements or because nutrients are locked up in the potting mix due to soil reactions at high or low pH.
2. Nutrient toxicity due to excessive use of fertilizer, high salt concentrations in the irrigation water, use of organic potting components contaminated with heavy metals, and addition of unsuitable soils (e.g. serpentine soils, acid soils high in Mn, sands laden with sea salts) to potting mixes.
3. A *nutrient imbalance* caused by poor nursery management. Examples encountered in nurseries include excessive watering or poor drainage (e.g. insertion of plastic bags into the soil) resulting in poorly aerated or waterlogged soils, placement of containers on crushed ultramafic rock, herbicide drift resulting in Fe deficiency, application of macronutrient fertilizers (e.g. NPK-type fertilizer) without micronutrients, and outbreaks of disease.

Problem identification

Diagnosing nutrient disorders in the nursery is dependent on several of the following: careful observations of plants, a sound knowledge of the appearance of healthy plants, accurate records of nursery management, and detailed foliar, soil and water analysis.

Use of symptoms

Fortunately, most single element nutrient deficiencies result in plants showing external symptoms that can readily be identified when the deficiencies are severe (Fig. 6.12). The main characteristics of nutrient deficiencies in eucalypts are as follow.

1. Symptoms appear initially at the shoot tip and spread to older leaves *or* they may initially appear in old leaves and spread to younger leaves.
2. Symptoms may appear as chlorosis (yellowing due to impaired synthesis of chlorophyll or accelerated senescence), necrosis (drying due to cell death), leaf deformation (small leaves, malformed leaves) and shoot dieback. Generally, patterns of chlorosis are related to leaf venation.
3. The appearance of symptoms indicating nutrient deficiency is related to the function of the nutrient in the plant and its ability to move in the phloem out of old leaves to regions of active cell growth. For example, in Ca deficiency, symptoms of cell death occur first at the growing points because Ca cannot be retranslocated in the phloem from old leaves.

Nitrogen deficiency results in chlorosis of old leaves as proteins are broken down and soluble N reserves are redirected to actively growing parts of the shoot. Provided care is taken to exclude confounding factors, such as the occurrence of symptoms caused by non-nutritional factors, symptoms can be used to diagnose mineral nutrient disorders in tree seedlings. The following key (Table 6.9) was developed for eucalypt seedlings. Similar keys are available for coniferous species.

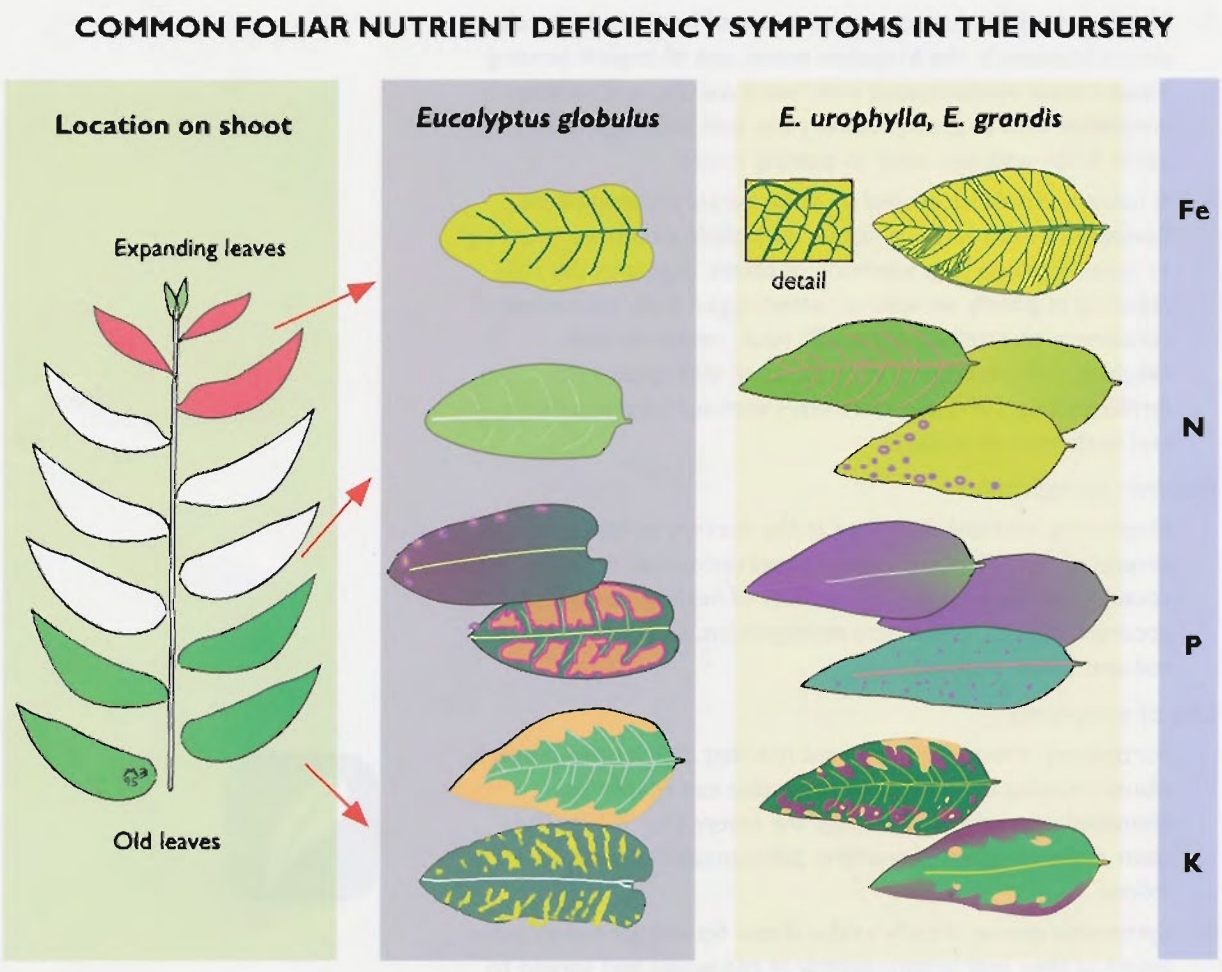


Figure 6.12. *Diagrammatic summary showing the appearance and location of the most common foliar nutrient deficiency symptoms for several* Eucalyptus *species.*

Table 6.9. Key for identifying deficiency symptoms in eucalypt seedlings.

	Symptoms	*Deficient element*
A1	**Symptoms appearing first on older leaves**	
	1. Leaves uniformly pale green, then turning yellow	N
	2. Leaves with reddish blotches or uniform reddish discolouration	P
	3. Leaf margins turn yellow then brown as tissues dry out	K
	4. Leaves yellow with broad dark green veins, especially the midrib	Mg
A2	**Symptoms appearing first on younger leaves**	
	1. Leaves uniformly yellow-green	S
	2. Leaves yellow with narrow green veins	Fe
	3. Leaves yellow-green with broad green veins	Mn
	4. Leaves small and crowded together on shoots	Zn
	5. Distorted leaves with corky veins, leaf tips pale green	B
	6. Leaves twisted, dark green	Cu
	7. Early death of shoot apex, leaves develop brown margins	Ca

Use of foliar analysis

Foliar analysis is particularly valuable because growth can be substantially reduced without the appearance of obvious symptoms in leaves. Foliar analysis can be used to:

a. verify diagnosis from visual symptoms,

b. identify nutrient deficiencies and toxicities, and

c. predict the future onset of a deficiency or toxicity.

The interpretation of plant analysis depends on the availability of nutrient standards or critical nutrient concentrations. Standard nutrient ranges are often obtained by compiling data for plants under conditions of known nutrient adequacy, nutrient deficiency, and more rarely, under nutrient toxicity. Table 6.10 gives an example of nutrient concentration ranges for *Eucalyptus urophylla*.

Table 6.10. Nutrient concentration ranges observed in the youngest fully expanded leaves of three-month-old *Eucalyptus urophylla* seedlings in a glasshouse (taken from Dell et al. 1995).

Nutrient	*Deficient seedlings*	*Adequate seedlings*
	mg/g dry weight	
N	6–11	25–40
P	0.3–0.4	1.9–4.0
K	3–5	15–18
Ca	0.8	3.1–4.3
Mg	0.7–0.8	2.1–2.3
S	0.9	2.2–2.6
	mg/kg dry weight	
Fe	20–32	52–97
Zn	8–10	13–31
Mn	5–16	43–109
Cu	0.4–1.0	3.4–4.5
B	8	12–27

Critical nutrient concentrations for diagnosis of deficiencies or toxicities are determined by using nutrient application rates trials to establish a relationship between plant yield and the nutrient concentration in a selected part of the tree or seedling (Fig. 6.13). An example of actual data for a Zn response is shown in Figure 6.14.

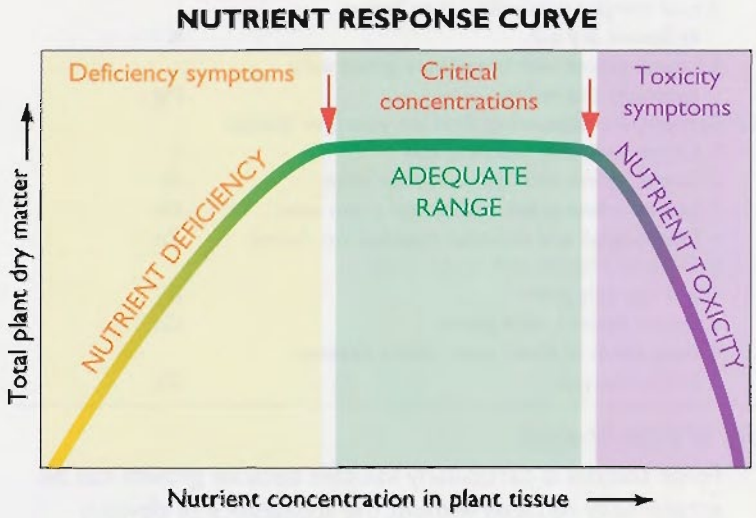


Figure 6.13. *A generalised relationship between nutrient concentrations in plant tissue and plant growth, showing where nutrient deficiency and toxicity symptoms would be expected to occur.*

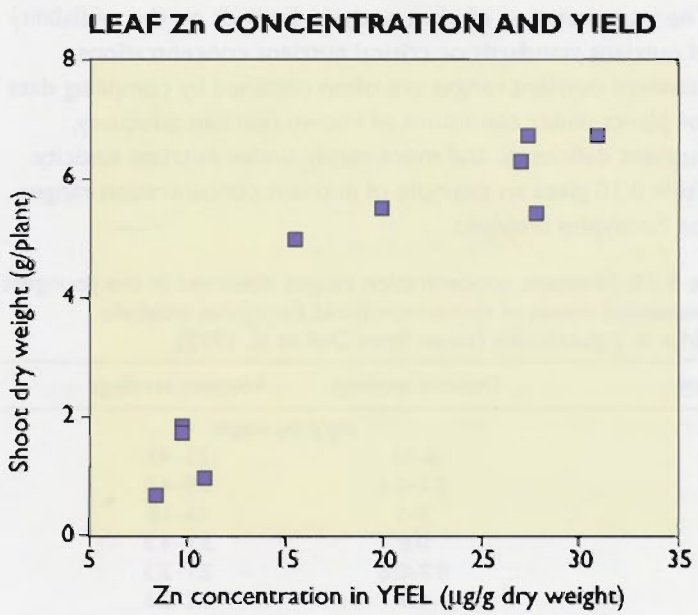


Figure 6.14. *Relationship between shoot dry weight and Zn concentration in the youngest fully expanded leaves of* Eucalyptus urophylla. *Data are from a glasshouse experiment where plants were grown in a grey sand fertilised with different amounts of Zn and adequate levels of all other nutrients (Dell & Xu Daping 1995).*

Sampling leaves for foliar analysis

Great care must be taken when sampling leaves for foliar analysis. The key points to consider are listed below.



1. *Age of leaf.* We recommend the youngest fully expanded leaf (YFEL) be sampled for routine plant analysis because this best reflects the overall nutrient status of the plant (see Fig. 7.14C). However, if micronutrient deficiencies are suspected the young expanding leaves should also be collected. If toxicities or deficiencies of N, P or K are implicated, sample mature but non-senescent leaves.
2. *Age of plant.* Do not combine samples across age cohorts because foliar nutrient concentration may alter with plant age.
3. *Number of samples.* It is best to collect 5–10 samples (approx. 1–5 g), each consisting of 10 bulked plants, randomly sampled in the nursery. If symptoms are present, collect healthy plants separately from unhealthy plants.
4. *Sample handling.* Wrap collected leaves in tissue paper, place in paper envelopes, label the envelopes and transfer to drying ovens at 70°C for 1–3 days. Remove plant material from ovens and store desiccated until samples can be ground for plant analysis. In hot climates, samples should be transported back to the laboratory in an ice box to prevent the leaves from sweating and turning black.
5. *Contamination.* The concern here is mainly the contamination of plant samples with metals. Avoid using hand creams (they can contain micronutrients), sampling leaves while wearing brass or copper ornaments, contaminating samples with cigarette ash, using galvanised metal containers for transporting samples, contaminating samples with nursery soil, etc.
6. *Washing.* Do not wash foliar samples as this can lead to the loss of some nutrients, e.g. K, B, Cl.

Preparation of plant samples for analysis

Samples are first ground then subjected to a wet acid digestion before analysis. As these procedures would normally be done in a separate laboratory with highly specialised analytical chemistry facilities, these processes are not considered in detail in this manual. Information on analysis procedures is given by reference books on these topics (e.g. Walsh & Beaton 1973, Rayment & Higginson 1992). Here, we outline some of the precautions to be undertaken when handling samples, assuming that analysis of the material will be done elsewhere.

1. *Grinding.* Small samples can be ground by hand using a mortar and pestle. Bulk samples can be ground in a hammer mill. Note that contamination can occur from oxides on the metal surfaces of grinding mills.
2. Contamination of the digests can occur due to impurities in the reagents, the laboratory equipment, or particles in the aerial environment.

3. Loss of nutrient elements in a sample can occur from mechanical dispersion (spitting of the reaction mix), volatilisation from excess heat, precipitation in particles such as silica and adsorption onto container surfaces.

Accuracy

Accuracy is the closeness of an observed value to the true value for the parameter being measured. It is important that recommendations (e.g. for nutrient application rates) are based on accurate measurements obtained from an analytical laboratory, because these values may be used to make management decisions in the nursery. Always ensure that a number of standard plant or soil samples, containing predetermined concentrations of the elements being measured, are included with each batch of samples sent for analysis.

E. Analysis of potting mix, soil, water and fertilizer

In addition to monitoring nutrient levels in seedlings in containerised nurseries, it is advisable to measure the parameters listed below. The pH and conductivity of solutions can be easily measured with meters, but details of other methods are not provided here. Table 6.11 lists some measures that can be taken to correct problems which have been identified. More information is given in nursery manuals such as Landis et al. (1989).



pH

The pH of irrigation water and growing media should be measured routinely while plants are growing in the nursery. The pH of water which has leached from the base of nursery containers should also be monitored.

Electrical conductivity

Measure the electrical conductivity of irrigation water and leachates from containers (see above) to ensure that total salt concentrations (resulting in osmotic water potential, or salinity) are not excessive for plant growth and mycorrhizal development.

Mineral nutrients

Chemical analysis of a potting medium before use and during plant growth may help to identify likely problems due to nutrient imbalances. Potential nutrient toxicity problems should also be investigated by plant analysis. The disadvantages of soil and potting mix analyses are that they can only be used to predict nutrient disorders, as measured values are influenced by soil properties and may not give an accurate measure of nutrients which are actually available to plants. Nevertheless, there are times when soil test data should be considered, especially if plant and water analysis does not identify problems of plant growth.

Fertilizers

The micronutrient content of macronutrient fertilizers can vary considerably with fertilizer form and origin. Researchers investigating micronutrient effects on mycorrhizal seedlings would be well advised to submit samples of fertilizers for chemical analysis. For example, we have found B levels in commercial fertilizers to range from 5 mg/kg in a sample of urea to 60 mg/kg in a batch of superphosphate. The concentrations of major nutrients such as N, P and K can also vary considerably in different batches or sources of fertilizers.

6.5. ASSESSMENT OF MYCORRHIZAL FORMATION

The success of mycorrhizal inoculation of seedlings should be examined both before and after seedlings are outplanted. A number of different approaches to these quality control assessments are possible, depending on the accuracy of information required.



1. Superficial visual assessment of in situ root systems for changes in colour and appearance due to mycelial systems and root tips will often be sufficient to detect mycorrhizas (Figs 6.15E–H). Mycelia produced by many fungi also have a characteristic odour which can be detected when handling roots (i.e. how strong is the fungus smell associated with seedlings).
2. Fruit body production under seedlings may occur in some cases, and as this requires a large accumulation of fungal biomass, it indicates that mycorrhizal inoculation was very successful and fungi were highly compatible with nursery conditions (Figs 6.15B–D,H). However, many fungi will not fruit in the nursery, so the absence of fruit bodies is insufficient evidence to indicate failure of inoculation.
3. Changes in the size or growth form of seedling, while usually not substantial, may help to separate mycorrhizal and non-mycorrhizal plants.
4. Microscopic examination of whole root systems lifted from containers is recommended to provide a quantitative assessment of mycorrhizal formation. Portable dissecting microscopes can be used for this purpose (Fig. 6.15A). A representative number of randomly selected seedlings should be examined for each inoculation treatment, including uninoculated control plants.
5. Random samples of root material can be stored in 50% ethanol and processed later in the laboratory for an accurate assessment of the degree of mycorrhizal formation, using methods presented in Chapter 4.
6. Microscopic examination of roots may also allow specific fungal identification to confirm that mycorrhizas were formed by the inoculated fungus, not a contaminant (see Section 4.3C).


A
B
C
D
E
F
G
H

Figure 6.15. *Evaluating ECM formation by* Eucalyptus *seedlings in the glasshouse (***B–D***) and nursery (***E–H***).*

A. *Checking ECM colonisation of seedling roots using a portable dissecting microscope in a nursery.*

B. *Mycelium (star) and fruit bodies produced by* Hebeloma westraliense *after inoculation of* E. grandis.

C. *Hypogeous fruitbodies and brown mycorrhizas (arrows) of* Setchelliogaster *sp. under* E. globulus.

D. Laccaria fraterna *fruit bodies and primordia (arrows) associated with* E. globulus.

E–H. *Mycorrhizal formation five months after spore slurry inoculation of* E. nitens, E. globulus *and* E. regnans *in the CSIRO Division of Forestry glasshouse in Hobart, Tasmania.*

E. *White mycorrhizas of* Laccaria fraterna *(arrows).*

F. *Mycorrhizas (arrows) and associated hyphae of* Laccaria laccata.

G. *Mycorrhizas of a* Pisolithus *species.*

H. *Fruiting of* Laccaria laccata.

7. Genera and species of mycorrhizal fungi can be identified by DNA fingerprinting using DNA samples extracted from mycorrhizal roots and fungal fruit bodies. Polymerase chain reaction (PCR) methods with specific probes and primers have been used to identify ECM and VAM fungi collected in the field (Bruns & Gardes 1993, Gardes & Bruns 1993, Henrion et al. 1992, 1994, Clapp et al. 1995). Randomly amplified polymorphic DNA (RAPD) methods can also be used to identify fungi, provided that care is taken to standardise procedures (Tommerup et al. 1995). We have used one- and two-dimensional PAGE electrophoresis of protein profiles to identify Australian fungi several years after they were introduced to a Chinese field site (Malajczuk et al. 1994a). These advances suggest that it may soon be possible to routinely use DNA or protein-based assay procedures to confirm that inoculated fungi are present in roots from the nursery or field.
8. The ultimate test of the persistence of inoculated mycorrhizal fungi is confirmation of their presence as mycorrhizal roots or fungal fruit bodies in plantations after seedlings are outplanted. Identification of fungal fruit bodies using standard taxonomic methods (Chapter 2) may be used, if indigenous fungi are substantially different. Alternatively, the morphological or DNA-based procedures listed above may be used.

Troubleshooting

Assuming that suitable fungi and inoculum forms have been used, failure of mycorrhizal roots to form in the nursery could occur as a result of various factors which can inhibit fungal activity. Table 6.11 considers some of these factors, along with corrective measures which can be taken if they are suspected to be responsible for poor seedling growth and mycorrhizal formation.

Table 6.10. Factors to consider if ECM root development or plant growth is poor in containerised nurseries.

Possible causal agent	*Recommendation*
1. Poor drainage	Check that the potting mix drains freely. The optimum air-filled porosity at potting should be 25–30%. The infiltration rate should be about 1.5 L/min. Change potting mix composition.
2. Overwatering	Reduce watering frequency so that the potting mix is not waterlogged.
3. Underwatering	Check that there are not dry patches in the potting mix or areas that are hydrophobic. Change the watering regime.
4. Unsuitable pH	Check that the potting mix has a pH in the range (for eucalypts) 5.0–6.5. Adjust potting mix if necessary with dolomite.
5. Unfavourable nutrient supply	Nutrient supply can be excessive (the more common) or deficient (less common). Check that N and P are not being added into the potting mix before inoculation and that fertilizer rates are not excessive. High levels of P and N at the time of inoculation can severely retard ECM formation. Check plants for symptoms of nutrient deficiencies, sample leaves and perform foliar analysis (see Section 6.4). Ensure that a complete basal fertilizer is being applied after ECM roots begin to develop.
6. Unfavourable potting mix	Check that wood-based or bark-based potting mixes are well aged, or better still, composted to prevent phytotoxic or fungitoxic reactions. Avoid the use of soil in potting mixes.
7. Poor water quality	Two common problems of water quality are the presence of dissolved salts and pathogenic water moulds. Check that the levels of dissolved salts (conductivity) are not too high. Check that the pH is near neutral (6–7). Hard water (pH>7) may have to be treated before use. Groundwater may have high concentrations of Fe which stain plants and can occlude the stomatal pores on leaves and prevent leaves from functioning. River water and dam water should be sterilised to reduce the incidence of fungal diseases which can impair plant growth, thus reducing the plant's ability to form mycorrhizas.
8. High plant density	If fungal diseases are prevalent reduce plant density in trays to prevent overcrowding and to enhance airflow around plants.
9. Fungicides	See Section 6.3D.
	Check that fungicides formulated for Basidiomycetes are not being used, especially during early ECM development. Generally fungicides specific for Ascomycetes are less of a problem and those formulated for Oomycetes can be used at any time. Recommended application rates should not be exceeded. If fungicides are used routinely, their effect on ECM formation should be tested. Copper-based compounds should not be used.
10. Temperature extremes	Some ECM fungi will not form mycorrhizas if soil temperatures are less than a critical value (e.g. 15°C). Extremely high temperatures could also be a problem.
11. Fungal inoculum	Check the quality and quantity of the fungal inoculum being used in the nursery inoculation program. For eucalypt seedlings, efficiency of inoculation is maximal where fungal inoculum is simultaneously placed into containers with primed and imbibed seeds using a fluid drill.

Chapter 7

FIELD EXPERIMENTS: PLANNING, DESIGN, MEASUREMENT AND DATA ANALYSIS

7.1. INTRODUCTION

This chapter provides an introduction to the concepts and procedures that would be required to plan, initiate, monitor and analyse experiments involving mycorrhizal inoculation or fertilizer application treatments. The following discussion is concerned primarily with larger field trials required in plantation forestry, but most principles apply equally to smaller experiments conducted in the glasshouse or nursery, and are also relevant to experiments with horticultural or agricultural crops, or native plants grown to restore degraded habitats. Sound experimental design can be more critical in field experiments than in nursery or glasshouse experiments, for the following reasons.

1. The establishment of large-scale field experiments is very labour-intensive, time-consuming, and expensive.
2. Biological resources required for mycorrhizal experiments (e.g. inoculum of appropriate fungi) can be difficult to produce or expensive to obtain.
3. Experiments are conducted on a long time-scale, due to the life span of trees and plantation rotation intervals, so several years of tree growth may be required before treatment differences are established.
4. Field experiments inherently have larger sources of variability than glasshouse experiments due to spatial variability in site factors, and are influenced by climatic factors which cannot be controlled.

Thus, while it is always important to take a great deal of care when designing experiments, this is even more critical for field experiments — as logistical and time constraints may make it impossible to replace an experiment which has failed.

There are many alternative approaches to designing, implementing and analysing experiments. The purpose of this section is not to explore all the possibilities, but to briefly describe the types of experiments we and others have used to examine response to mycorrhizal and nutrient treatments. A rather elementary approach is used initially as a basis for discussing some of the important aspects of design and analysis. Simple examples are presented to illustrate basic concepts. Principles used for the design and analysis of experiments have been described in a number of books, which should be consulted for further information (e.g. Zar 1984, Mead 1988, Snedecor & Cochran 1989, Williams & Matheson 1994).

Although much of the planning and analysis of experiments is based on commonsense, knowledge of techniques and experience of how to obtain maximum information and accuracy from experiments are also essential. The importance of good design can not be overemphasised. A poorly designed or poorly run experiment can give misleading information on treatment effects and may lead to incorrect conclusions and recommendations.

A. Experimental process

An important initial step is the development of a clear hypothesis as the basis for the experiment or series of experiments. Following this is a sequence of steps through the planning and establishment of the experiment, measurement and sampling, collation and analysis of the data and interpretation of results (Fig. 7.1). Results obtained from the experiment can then be used to modify the working hypothesis and help in defining specific objectives for subsequent experiments. The steps in this process are described in the remainder of this section.

B. Planning experiments

At an early stage in planning it is advisable to produce a document, referred to as an experimental plan or preschedule, detailing all aspects of the experiment. This will help to clarify ideas, decide on treatments and procedures, and assess statistical methods. Table 7.1 lists the topics which could be included in a typical preschedule. Such a document can be used to review and revise plans, explain procedures to other participants and reduce the likelihood of overlooking important procedures, and will help in report writing. Schedules and procedures can be modified during progress of the study as required. Detailed records of events (the dates when treatments were applied, etc.) and any changes to planned procedures which occur during the experiment should be kept. It is often important to acquire temperature and rainfall data and note any other factors which could influence an experiment.

C. The basis for comparing treatments

A brief and elementary discussion of some fundamental aspects of sampling distributions and treatment comparisons will be used here to provide a basis to consider the design and analysis of field experiments. This is perhaps best introduced by the simple examples provided below.

If we were to measure the height of 20 trees within a plot of 100 trees which had received uniform treatment and appeared to be on uniform soil, we would most likely find that tree height varied considerably among the individuals (Fig. 7.2A). This effect may be caused by many factors (e.g. genetic differences among individuals, variation in soil properties). If we determined a frequency distribution of height classes we might expect heights to be normally distributed (Fig. 7.2B). Two parameters used to describe this distribution are the mean and the standard deviation (**SD**), which is a measure of spread (i.e. dispersion of data around

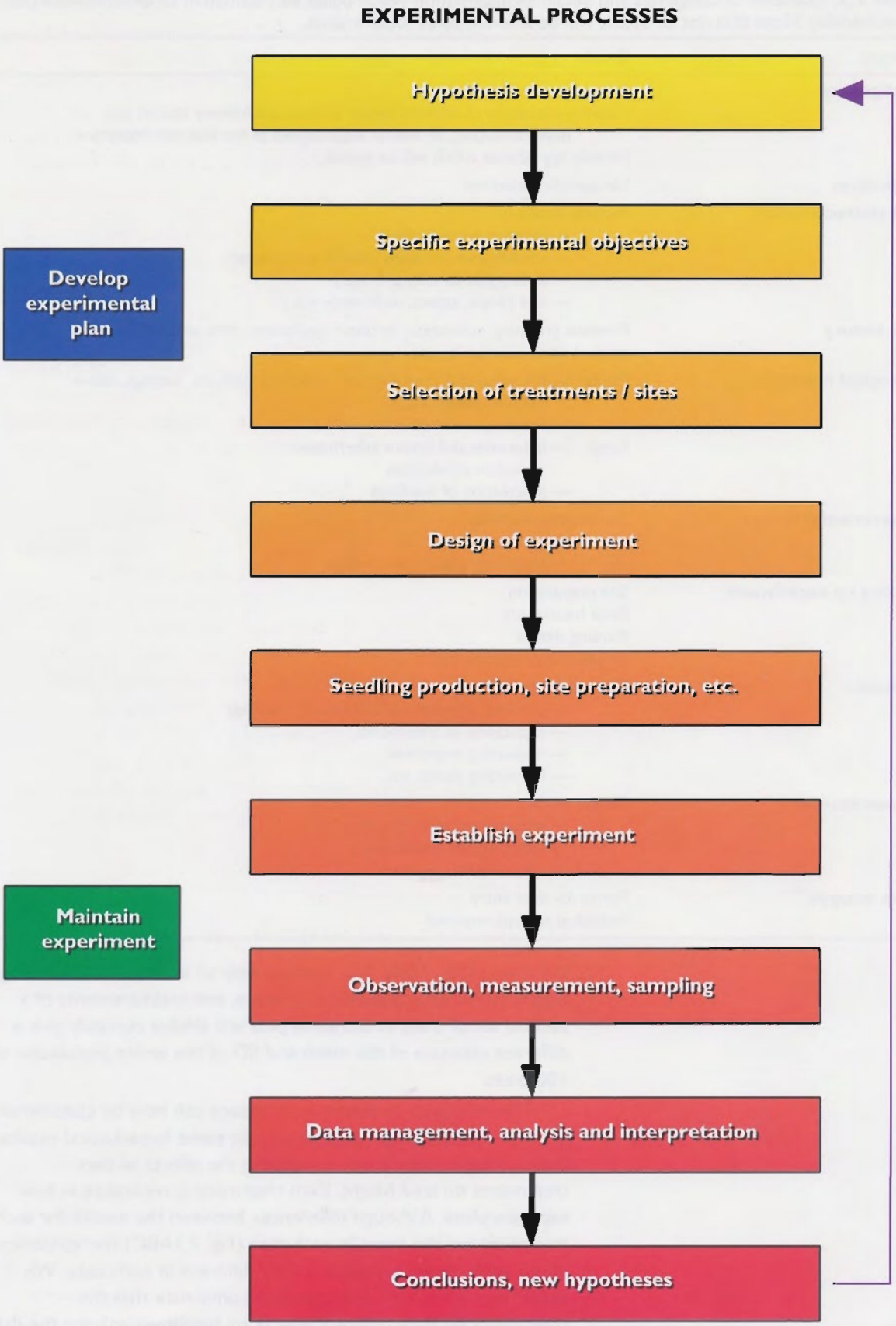


Figure 7.1. Steps in the experiment process.

Table 7.1. Examples of categories and details of information which could be included in an experimental plan (preschedule). Note that not all details will be relevant to all experiments.

Category	*Details*
Background	Explain reasons for study Mention outcomes of relevant earlier studies: preliminary studies you have conducted, or similar experiments in the scientific literature Provide hypotheses which will be tested
Objectives	List specific objectives
Site characteristics	Provide details of: — location (accessibility) — climate (temperature, rainfall, seasonality) — soils (type, fertility pH, etc.), — site (slope, aspect, uniformity, etc.)
Site history	Previous cropping, cultivation, fertilizer application, time since clearing, original plant community, etc.
Biological materials	Plants: — full name and source (seeds, seedlots, cultivars, cuttings, tissue cultured plants, etc.) — Seedling production Fungi: — full names and isolate information inoculum production — inoculation of seedlings
Experimental design	Site diagram showing: — plot layout — treatment allocation to plots
Setting up experiment	Site preparation Basal treatments Planting details Fertilizer applications, etc.
Schedule	Planned times for: — planting, growing and inoculating seedlings — application of treatments — measuring responses — harvesting plants, etc.
Measurements	Details of: — measurements to be taken — sampling and processing — data analysis required
Data analysis	Forms for data entry Statistical analysis required

the mean) (Fig. 7.2A). The mean is only an *estimate* of the average height of the total population of trees, and measurements of a second set of trees in the same plot will almost certainly give a different estimate of the mean and SD of the entire population of 100 trees.

This variability in estimates of means can now be considered in a different way. Figure 7.3 illustrates some hypothetical results from a simple experiment comparing the effects of two treatments on tree height. Each treatment is replicated in four separate plots. Although differences between the means for each treatment are the same in each case (Fig. 7.3ABC) the variability about each treatment mean is very different in each case. We would therefore have much greater confidence that the treatments do have different effects on height growth for the data in Fig. 7.3B than in Fig. 7.3A. Figure 7.3C shows another example

where variability follows a (systematic) trend. This trend can be accounted for by using appropriate statistical methods which allow treatment differences to be tested (see Section 7.2E). The comparison of variability caused by different treatments and the variability associated with each treatment mean is the basis for testing for treatment differences in the *analysis of variance (ANOVA)*.

The model of analysis of variance can be expressed in the algebraic form (Equation 1) where $\mathbf{Y_{ij}}$ is the observed value (e.g. tree height) for the $\mathbf{i^{th}}$ treatment and $\mathbf{j^{th}}$ replicate, **m** is the overall mean, $\mathbf{t_i}$ is the effect of the $\mathbf{i^{th}}$ treatment on the overall mean and $\mathbf{e_{ij}}$ the residual (unexplained) variability for that observation. These components of the total variation are illustrated in Fig. 7.4. The square of each of the **t** and **e** components, summed over all observations, makes up the *Sums of Squares* (**SS**) for the treatment and residual term in an ANOVA table.

Equation 1

$$Y_{ij} = m + t_i + e_{ij}$$

An example of an ANOVA table for an experiment with 4 replicates of each of 5 fungal treatments is shown in Table 7.2. In this table, two sources of variability among observations (fungal treatments and residual terms) make up the *Total* **SS** component of variability — which is the variability about the grand mean. A *mean square* (**MS**) is derived by dividing the **SS** for each component by the *degrees of freedom* (**DF**) — this value is the number of independent parameters contributing to that component of variation. In Table 7.2, the **DF** values are one less than the total number of treatments for *Fungus* and one less than the number of observations for the *Total* **DF**, while the *Residual* **DF** is the difference (remainder) left after subtracting the *Fungus* **DF** from *Total* **DF**. The *variance ratio* (**VR**) is then calculated by dividing the **MS** value for treatments by the *residual* **MS**. The **VR** is then compared to a critical value obtained in a table of *critical values of the* **F** *distribution* or provided by a computer program, to estimate the probability that there are statistically significant differences among treatments in their effect on the variable being measured. Thus the ANOVA table allows an experimenter to quantify variability which is caused by treatments, relative to the random variability (unexplained variation caused by other factors) which is associated with all of the observations. This comparison of variability provides a probability value which is the basis for accepting or rejecting the hypothesis which is being tested (see Section 7.4 for examples).

The accuracy with which treatment effects are estimated and the confidence the experimenter has about differences among treatments increase as the amount of unexplained variability between experimental units (residual error) decreases relative to the variation between treatments. This is an important aim in selecting the most appropriate experimental design and using the most suitable statistical analytical techniques. A number of methods of reducing the size of experimental error are considered in the following sections.

VARIABILITY IN TREE HEIGHT DATA

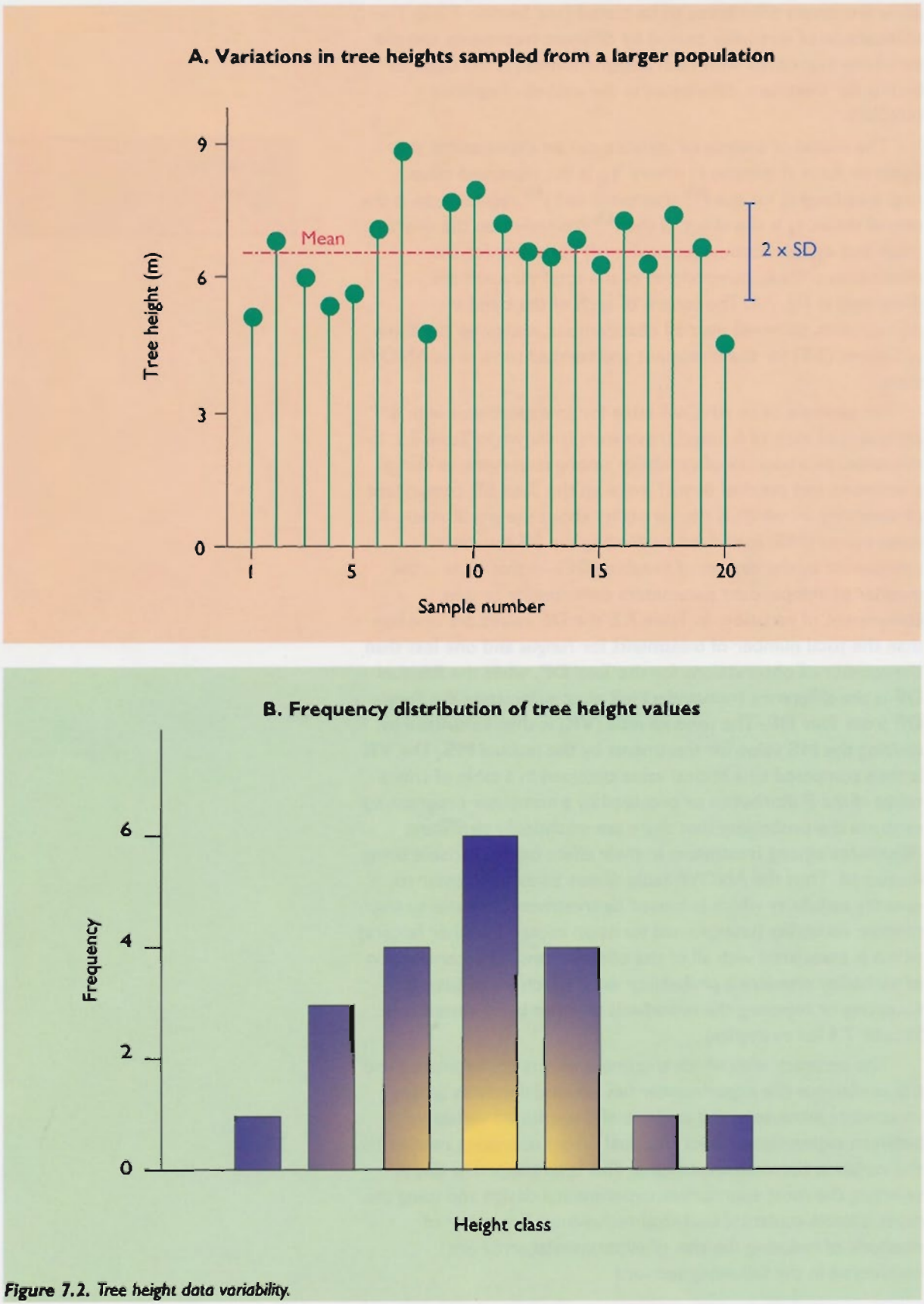


Figure 7.2. Tree height data variability.

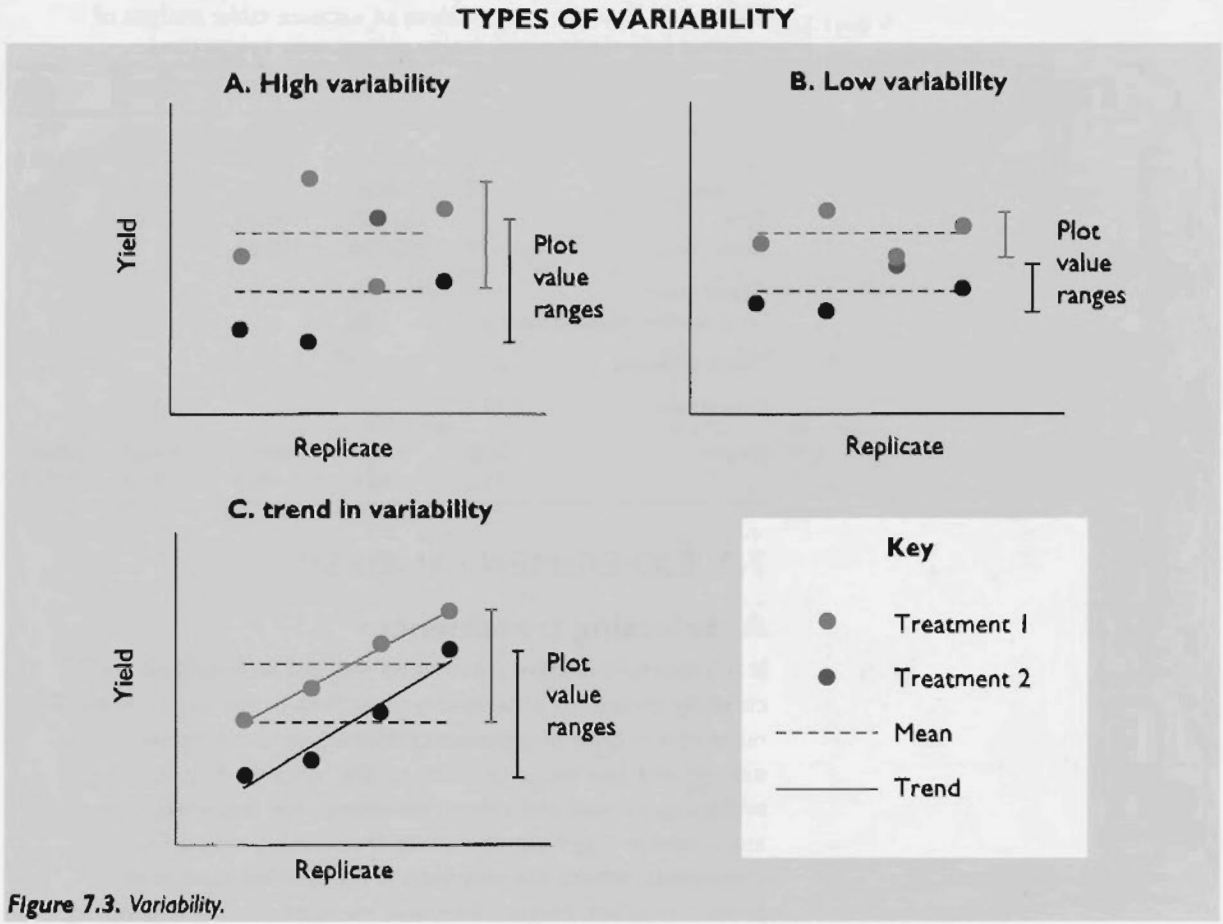


Figure 7.3. Variability.

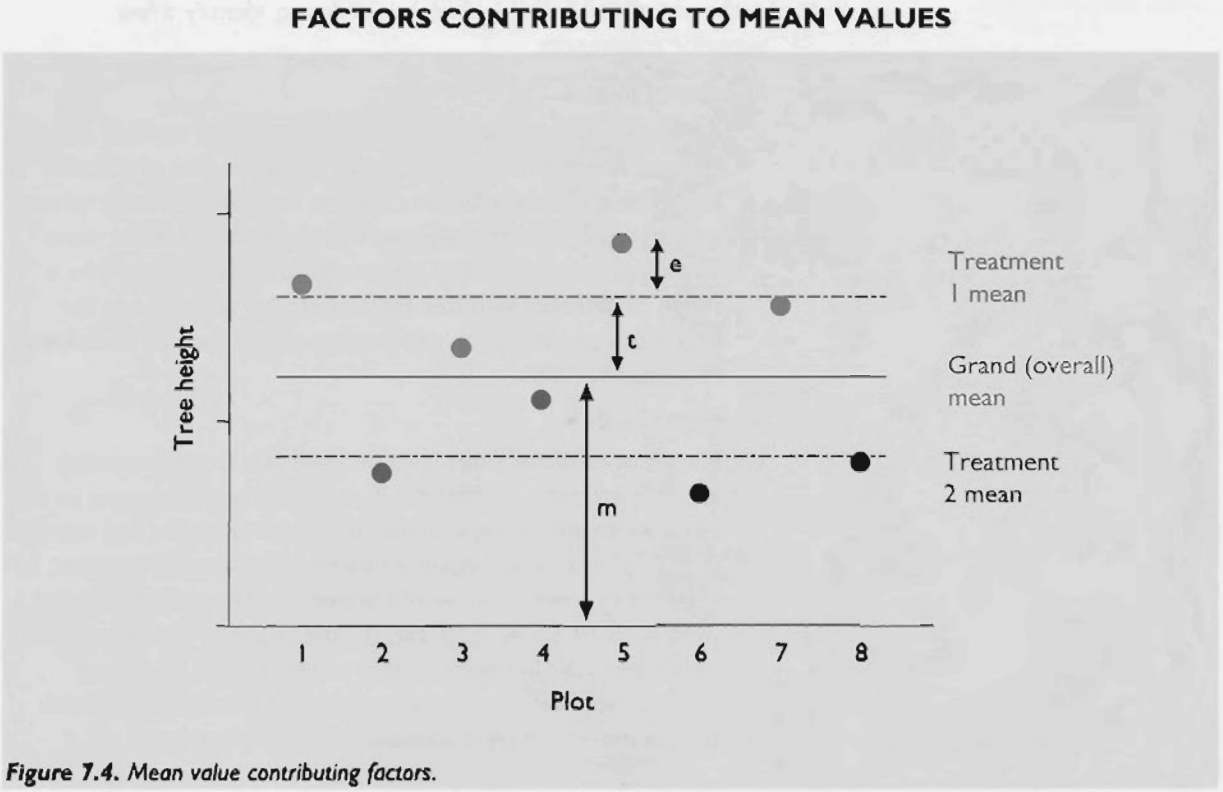


Figure 7.4. Mean value contributing factors.

Table 7.2. Example of an analysis of variance table: analysis of variance fully randomised design variate: tree height (cm).

Source of variation	*DF*	*SS*	*SS%*	*MS*	*VR*
Units stratum					
Fungus	4	1476.80	80.93	369.20	15.914
Residual	15	348.00	19.07	23.20	
Total	19	1824.80	100.00	96.04	
Grand total	19	1824.80	100.00		
Grand mean		73.6			
Total number of observations		20			
Tables of means					
Grand mean	73.6				
Fungus	Cont	Fung1	Fung2	Fung3	Fung4
	70.0	60.0	80.0	85.0	73.0

7.2. EXPERIMENTAL DESIGN

A. Selecting treatments

It is important to have treatments that are well defined and carefully chosen to achieve the objectives of the experiment. The number and type of treatments depend on the purpose of the experiment and on constraints to the research (e.g. funding, availability of land and other resources). For example, some experiments may have objectives that involve only a few treatments, where the emphasis is on detailed studies of processes which require intensive measurement and sampling. Alternatively, experiments may involve comparisons among a large number of treatments (e.g. screening of seedlots or ectomycorrhizal fungal isolates) in order to identify a few superior treatments.

Number of factors

An experiment can involve a single factor or a number of factors. A factor refers to a series of treatments of a similar kind. For example, a factor could be fungal treatments, where a number of different fungi were inoculated and there were also uninoculated control plants. Another factor could be a range of different fertilizer treatments (nil to adequate for maximum growth) in an experiment established to examine a response function.

Treatment levels

If a set of fertilizer rates is to be used as a factor, selecting appropriate rates is essential if maximum information is to be obtained from the experiment and treatments are not wasted (Fig. 7.5). In an experiment limited to five rates of fertilizer, an ideal set of treatments would provide information on (a) the yield without added fertilizer, (b) the shape of the response to added fertilizer in the deficiency range, (c) the minimum fertilizer application required to achieve maximum yield and (d) the maximum yield possible with adequate levels of

fertilizer. The example of treatment levels shown in Figure 7.5A provides information on only (a) and (c), while the rates used in Figure 7.5B provide maximum information. In order to select a suitable range of rates, the researcher could use any relevant information from previous studies indicating likely responses, sample and analyse soils to predict responses, or, if time permits, carry out a small pilot study prior to starting the main experiment.

Treatment selection

Treatments need not be confined to those that are likely to be economically feasible or practicable, when used on a larger scale. Some may be included to give greater understanding of the influence of factors or better information on processes. For example, inoculation of tree seedlings with a certain

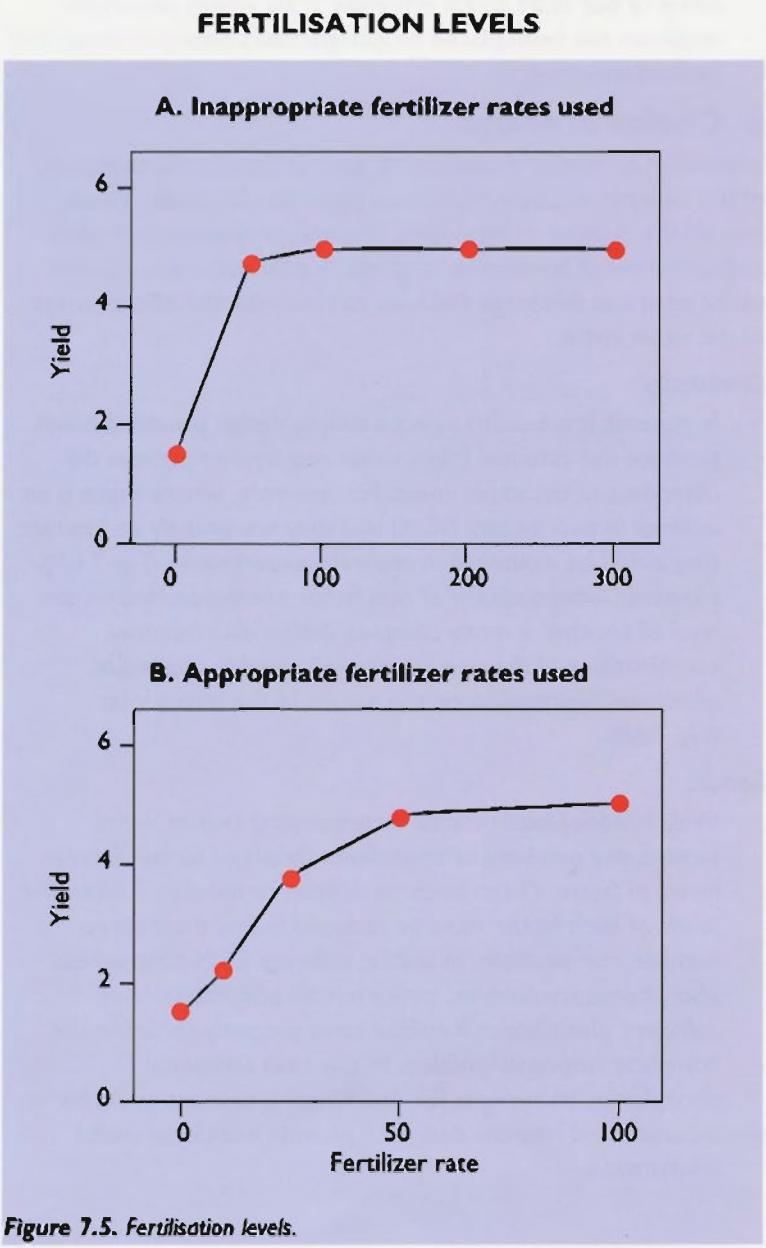


Figure 7.5. *Fertilisation levels.*

fungal isolate may not be expected to increase growth (e.g. conditions may not be suitable for that fungus), but it may still be included to test specific hypotheses. Where a sequence of experiments which follow a similar theme is being conducted, it can be worthwhile including one or more common treatments for comparison, even if they are not of primary interest in some of the experiments. This can provide an important linkage between experiments and contribute to a greater overall depth of knowledge.

Balanced designs

In most experiments, treatments are equally represented with the same numbers of replicates. However, if better information is required for a specific treatment, replication of this treatment may be increased. For example, we have incorporated additional control (uninoculated) treatments in some of our ECM fungal screening trials where particular emphasis has been placed on comparisons between fungal and control treatments.

B. Choice of design

In addition to selecting treatments appropriate to the objectives of the experiment, other decisions must also be made. These include the number of replicates, size and arrangement of plots and allocation of treatments to plots. A statistician can provide useful advice at this stage and help to maximise the effectiveness of the experiment.

Complexity

In general, it is best to select a simple design provided it will produce the essential information required to achieve the objectives of the experiment. For example, where there is an interest in two factors (X, Y) and they are unlikely to interact, they could be examined in separate experiments (Fig. 7.6A). However, where effects of one factor are dependent on the level of another, a more complex design with factorial combinations of the two factors will provide important additional information on the nature of the interaction (Fig. 7.6B).

Factors

With factorial experiments incorporating two or more factors, the numbers of treatments (levels of factor X times levels of factor Y) can become difficult to handle — often the levels of each factor must be reduced below the desired number. For example, in testing different ECM fungi across phosphorus treatments, it may not be possible to have sufficient phosphorus fertilizer rates properly to define the complete response function. In this case additional phosphorus treatments for one fungal treatment could be incorporated into the design to provide additional useful information.

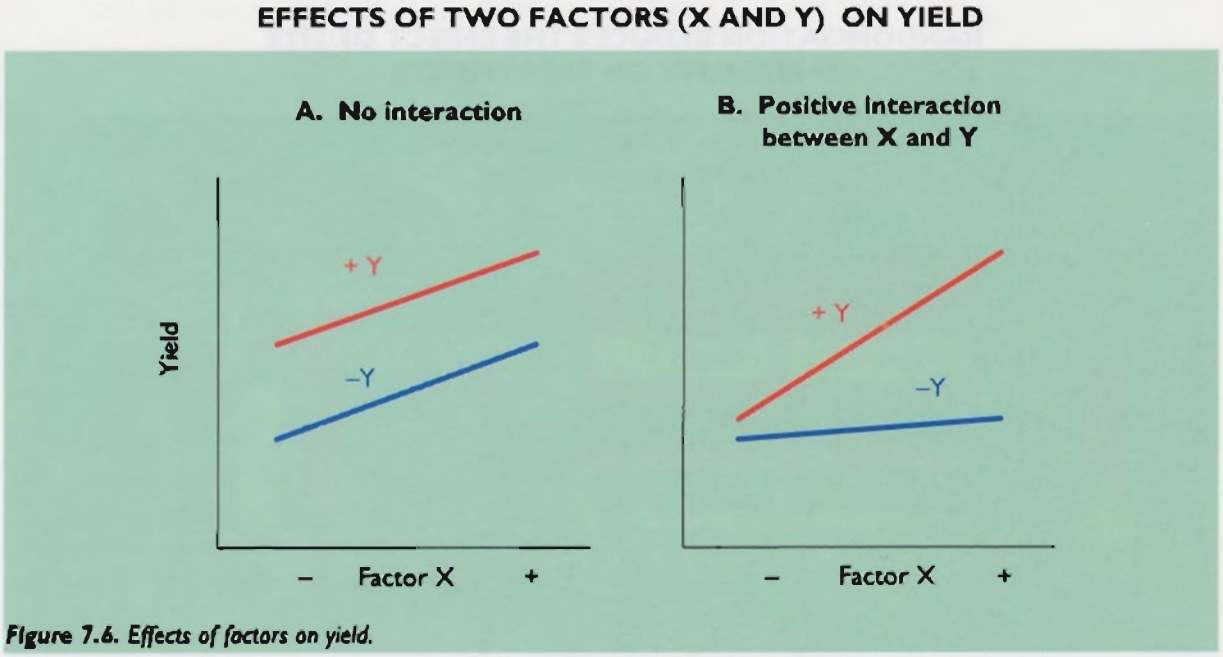


Figure 7.6. Effects of factors on yield.

Size

The selection of the design and the size and layout of the experiment will depend on the number of factors examined, the number and nature of treatments for each factor, the time-scale over which measurements are to be made, variability of the site and likely edge effects between treatment plots and logistical constraints. These aspects and examples of simple designs are discussed in the following sections.

C. Replication and randomisation

Replication is a most important aspect of experimental design. The correct balance must be reached between placing treatment trees in large plots with few replicates or small plots with many replicates. This will depend on factors such as available resources, the degree of variability over the site, the nature of treatments, the need for buffer areas between plots and the period over which measurements are to be made. Although within-plot variation cannot be used in testing effects of treatments, increasing plot size and the number of trees within the plot provides a better estimate of the mean and can contribute to lowering the between-plot variance. The optimal size of an experiment and amount of replication required to detect differences between means of a specified amount can be estimated if there is some prior knowledge of the likely variability that will be encountered, but most often this is not known.

Major problems can occur if treatments are placed in a systematic order in the field rather than being randomly allocated to plots. In the example in Figure 7.7, the systematic arrangement of treatments would lead to treatment effects being confounded with the effects of a fertility gradient across the site. Adequate randomisation of treatment plots and replicates is essential to avoid bias in estimating the effects of treatments.

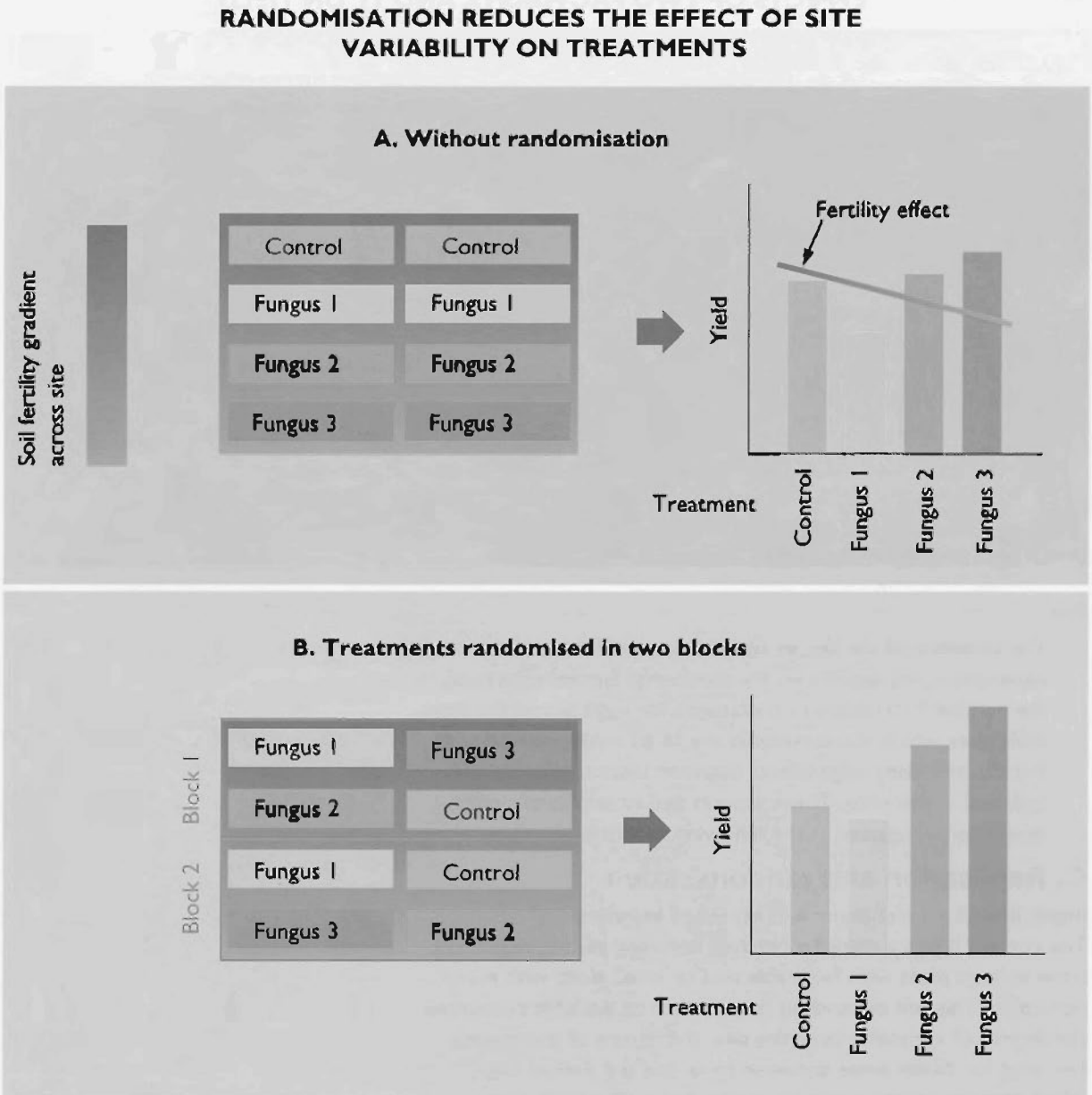


Figure 7.7. *The use of randomisation can reduce the effect of site variability on the measurement of treatment effects. In this example a soil fertility gradient (red-orange) masks yield responses if plots with different fungal treatments are uniformly arranged (A), but has less effect if plots are arranged randomly (B).*

D. Examples of designs and plot layouts

This section compares simple designs that may be used in field studies examining effects of mycorrhizal fungi and nutrient application on tree growth. More complex designs (e.g. incomplete block designs) are also used to manage or account for variability across experimental sites (Williams & Matheson 1994).

Randomised design

This is the simplest experimental design, where treatments and replicates of these treatments are randomised among all plots (Fig. 7.8A).

Randomised block design

A more commonly used design for field experiments is the randomised block design, where the full set of treatments is randomised within each replicate block (Fig. 7.8B). The advantage of this design over a fully randomised design is that, where there is expected to be variability in growth over the site (e.g. due to a gradient in soil fertility), arrangement of treatments into blocks can reduce the effect of this unwanted variability and improve the capacity of the experiment to test treatment effects. The model of analysis of variance (Equation 1) is modified to include a term for blocks (Equation 2) where b_j is the variation due to block differences.

Equation 2

$$Y_{ij} = m + b_j + e_{ij}$$

Factorial design

If two factors are included in the experiment, treatments may be factorial combinations of each factor. For example, we may be looking at the effects of two fungal treatments *(F)* at each of three levels of applied phosphorus *(P)* — each of the treatment combinations could be randomised within each block (Fig. 7.8C). In this case the t component in the above model (2) would be replaced by three new terms (e.g. *F, P, F×P*) which represent the main effects of each treatment and the effect of interaction between the two factors, respectively.

Split plot design

In an alternative design, the fungal treatments could be arranged as subplots within P fertilizer plots (Fig. 7.8D). In this case, the residual error value to test effects of P treatments is different to that used to test the effects of fungi and the interaction between fungal and P treatments. Furthermore, the fungal and interaction effects are tested more accurately than the P effect, but this may be consistent with the primary objective of the experiment.

Selecting designs

Advantages and disadvantages of different experimental designs are considered further in Figure 7.9, which shows various plot arrangements. The size of plots will depend on the length of time for which measurements are required. In experiments with small plots, edge effects may affect

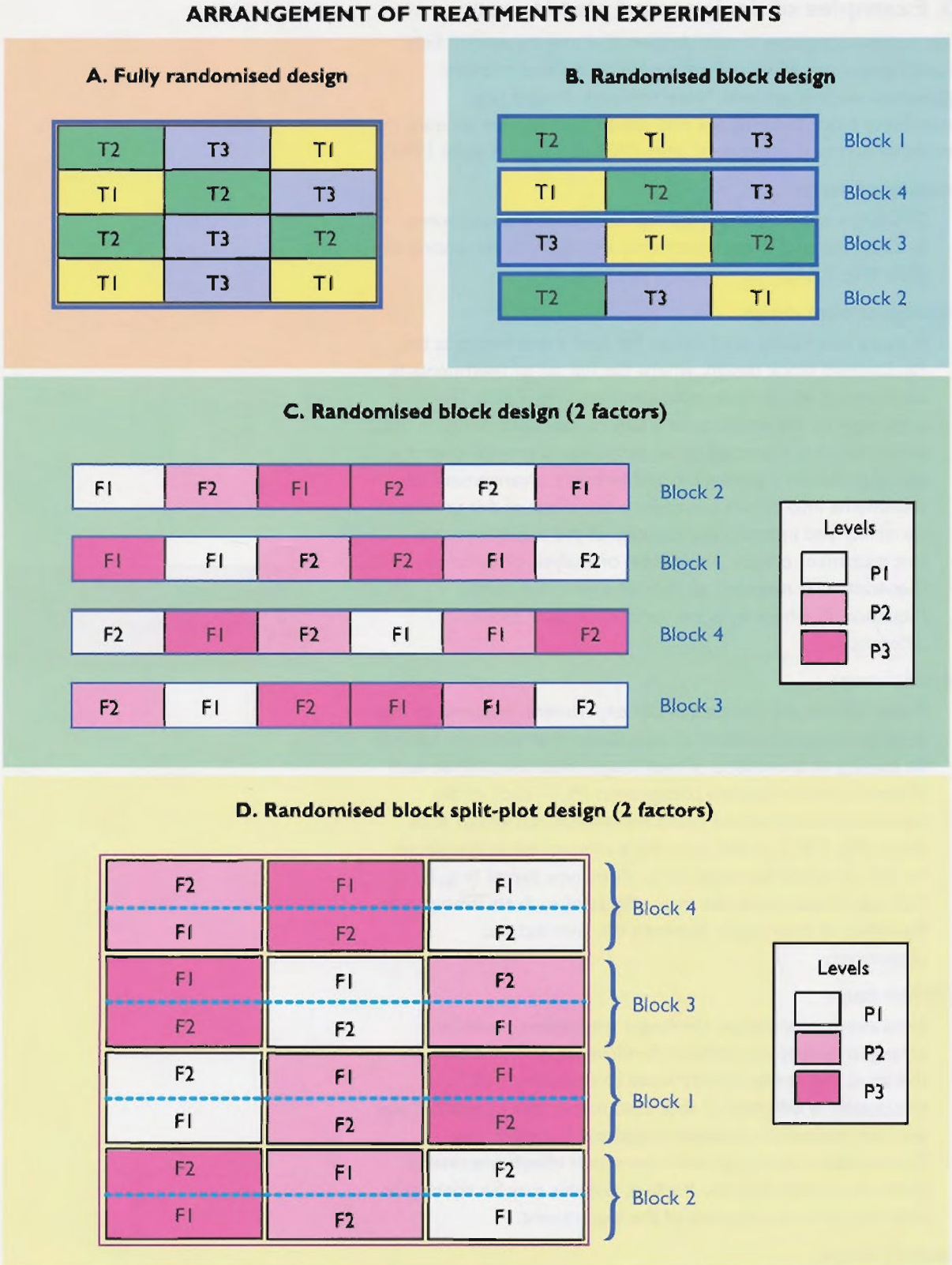


Figure 7.8. *Examples of plot layouts.*

A. *A fully randomised design with three treatment levels (T1–T3) shown by different colours and four replicates of each.*

B. *A similar experiment to A, but with treatments assigned to four blocks.*

C. *A randomised design with four blocks and two factors represented by letters (F1, F2) and colour shading (P1–P3).*

D. *A randomised split-plot design with the same two factors as C. In this case plots are assigned to one level of factor P, but are divided (dashed line) with two treatments of factor F applied to each.*

treatment trees at a relatively early stage. However, more treatments can generally be included and effects of spatial variability are likely to be less than in experiments with large plots (Fig. 7.9A). Buffer zones are often required to separate treatments, and split-plot designs may help to reduce the area required for these (Fig. 7.9B). In experiments involving both fungal and P treatments, we have often allocated the fungal treatments to subplots of main plots receiving different P treatments — this is because there is a greater need for a buffer zone between P treatments and there is greater accuracy in assessing the effects of fungal treatments.

Stratifying variability

Tree-to-tree variation in growth is normally lower over small areas than over larger areas, and this explains why the within-plot variability is not used in comparing between-plot differences and why different error terms must be used to test treatments in different strata (i.e. main plot vs. subplot) of the analysis of variance (Fig. 7.9C).

E. Minimising effects of extraneous variation

Various methods can be used to reduce or account for inherent (extraneous) variability over an experimental site.

Uniformity

A field experiment will produce results that are specific to the set of conditions in which the experiment was run. For some factors we have little control, but others will be influenced by basal treatments which can be applied uniformly across a site, or by selecting seedlings that are as uniform as possible (see Section 7.4).

Blocking

The importance of allocating treatments to replicate blocks to remove variability associated with fertility gradients has been described above. It may be possible to exclude disturbed or less uniform areas of the site from the experiment when plots are being marked out. The blocking of treatment plots is most often based on observed variation over the site (e.g. topography, aspect, soils). However, blocking may also be based on criteria not associated with the site. For example, if the seedlings to be planted out vary considerably in size we may grade these and allocate larger plants to one block, and so on. More complex designs such as incomplete block designs are often more successful than complete block designs in accounting for variability over sites (Williams & Matheson 1994).

Covariates

Measurement of other factors that are likely to be related to tree growth but are independent of treatment effects is often useful in explaining some of the variability and reducing the residual error in the analysis of variance. These measurements would need to be obtained for each plot in the

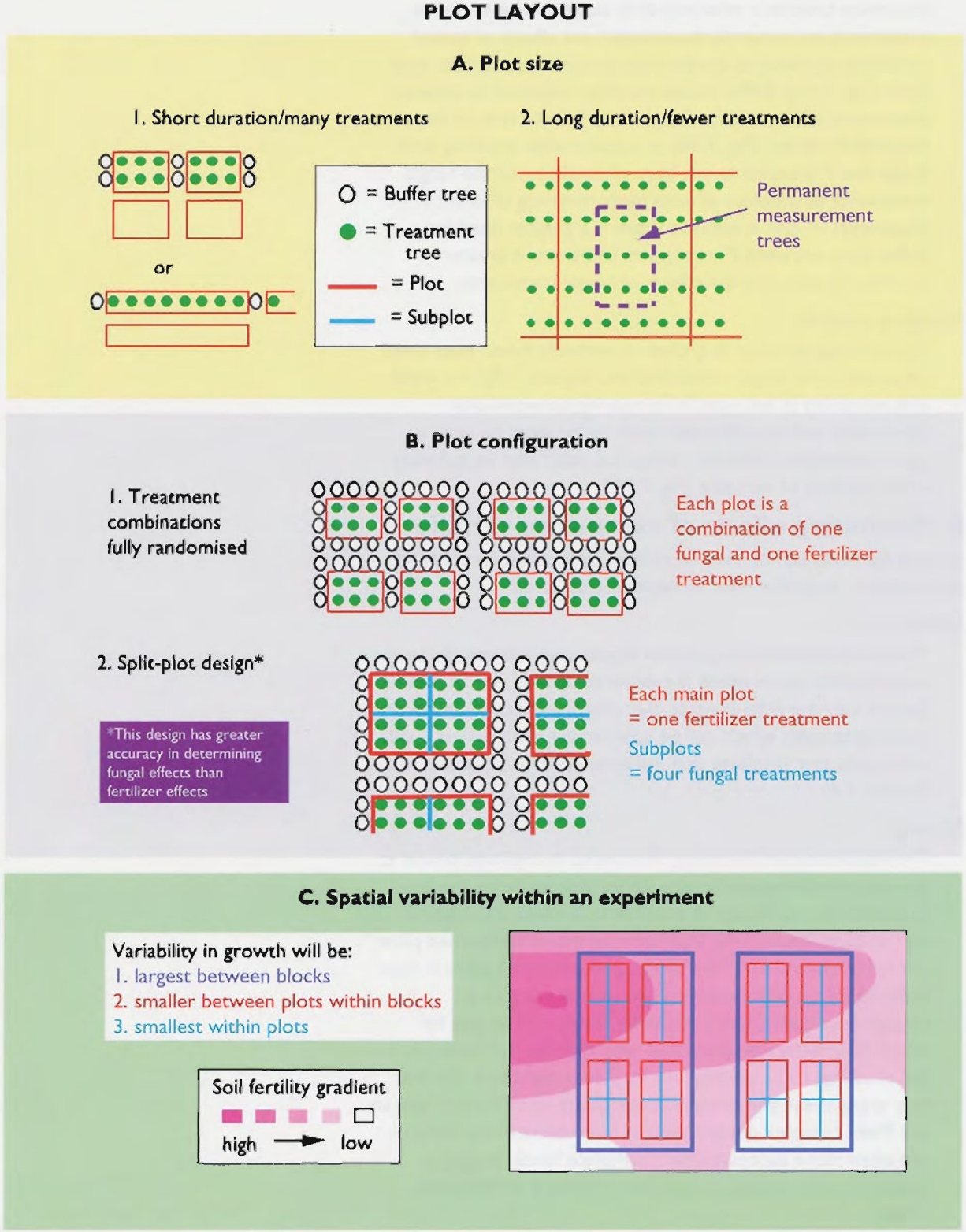


Figure 7.9. *Plot layouts in field experiments.*

A. *Plot size depends on the time-scale of experiments, as longer trials will require more space for trees to grow and larger buffers between treatments.*

B. *Plot layout depends on available space, the nature of treatments and research priorities. In this case a split-plot design (2) requires less space than a fully randomised (1) design with the same treatments, as extra rows of buffer trees are required for the fully randomised design.*

C. *The effect of spatial variability in a soil factor which influences plant growth can be partially overcome by the use of blocks to compensate for large-scale variability.*

experiment. The types of variables that might be used are the yield of a previous crop, nutrient status of the soil, or growth of non-treatment trees in buffer areas surrounding each plot. A variable such as these can be used as a covariate in the analysis of variance to account for some of the unexplained variability.

7.3. EXPERIMENTAL PROCEDURES

This section contains an outline of the procedures required to initiate, maintain and measure the results of a field experiment. A list of equipment which may be required is provided in Table 7.3.

A. Site selection

Selecting a suitable site is an important step in the experimental process. The aim is generally to select a site that is as uniform as possible in relation to characteristics such as slope, aspect, soil properties, vegetation and previous history. Processes that can be used to determine site characteristics, which may help predict plant responses to fungal inoculation or fertilizer application treatments, are considered in Chapter 6.



Soil and site factors

Examining excavated soil profiles can help in choosing a site with as little variation as possible in soil depth, physical or chemical characteristics (Fig. 7.14F). Steep topographic gradients can present particular problems with fertilizer treatments, where nutrients could be washed down the slope. This situation should be avoided by preparing the site to reduce the likelihood of nutrient movement (e.g. by terracing), or by selecting another site. An experimental site is often chosen to be representative of planting sites in the region, or there may be a series of experiments on different sites stratified to represent a range of major land units.

Site history

Consideration should be given to previous land use to ensure there has been uniform treatment over the site. Site conditions that could affect the outcome of an experiment need to be noted (Table 7.1). Important details of site history include previous crops, the original plant community and time since clearing, and management practices such as tillage, fertilizer applications, pesticide use or specific silvicultural methods. For example, the time which has elapsed since a site was cleared will affect the viability and abundance of mycorrhizal propagules in soils. Furthermore, past applications of fertilizers (especially P) can have a large influence on the outcome of mycorrhizal and nutrient experiments.

Environmental factors

Information about prevailing climatic factors that influence plant growth, especially temperature and rainfall, should also be used when selecting field sites. It is important that climatic

Table 7.3. Example of a field equipment and supplies list for field experiments.

Trip: _____ Date: _____

Category / *Item*	*Number* Required	Packed
A. Living materials		
Mycorrhizal fungus inoculum		
Mycorrhizal seedlings		
Seed or plants		
B. Fungal collecting		
Isolation tubes		
Equipment (see Chapter 2)		
C. Leaf collecting		
Tissue paper		
Small and large envelopes		
Secateurs, scissors, tree lopper		
Data sheets		
D. Soil collecting		
Corers, sledge hammer, soil auger, etc.		
Plastic or cloth bags		
Sieve (2 mm mesh)		
E. Tree measuring		
Calipers		
Height stick		
Data sheets		
F. Nursery experiments		
Trays, tubs, pots, etc.		
Peat, vermiculite, perlite		
Fertilizers		
G. Specimen collection for histology		
Gluteraldehyde in buffer and/or ampoule		
Ethanol to fix roots (50%)		
Jars and vials with tight-fitting lids		
Fine forceps, razor blades, probes, etc.		
Parafilm™, labels, etc.		
H. Photography		
Camera, lenses, tripod, etc.		
Film (slide and print)		
Card or cloth backgrounds, signs to show plots		
I. Starting field trials		
Maps, plot diagram, plan (preschedule)		
Macronutrient and micronutrient fertilizers		
Shovels, rakes, hoes, mattocks, etc.		
Plastic bags, buckets		
String, flagging tape, pegs, labels		
J. Computing		
Portable computer		
Printer, spare batteries, cables, adaptors		
K. Other		
Permits to collect or import plants, fungi, soil, etc.		
Compass, knife, water bottles, sturdy boots, etc.		
Pens, markers, pencils, clipboards, notebooks, etc.		

conditions are suitable for the plants (and fungi) which will be used. Climatic information can be obtained from a local weather station, or by installing on-site measuring devices which can be attached to a data-logging computer.

B. Site preparation and experiment establishment

Many forestry experiments are established on land that has been prepared for planting by standard forestry practices. However, if the area is chosen well in advance, greater care can be taken to ensure that preparation methods are uniformly applied across the site (e.g. even spacing of cultivation lines or terraces, minimising topsoil movement and compaction). Care at this stage will help to avoid introducing greater variability into the experiment.



Site preparation

Before planting, decisions must be made on how to prepare the site. For example, should slash from the previous forest plantation be retained, burnt or removed from the site, is there a need to spray for weed control, what type of cultivation should be carried out? Cultivation procedures such as mounding, deep ripping or trenching may be used to prepare soil for trees, and terraces may be required on steep sites (Figs 7.11A, 7.12AB)

Plot layout

A regular (or symmetrical) arrangement of replicate blocks and plots is often not possible. In marking out the experiment, some areas may need to be excluded due to soil disturbance, slash residues, rock outcrops, gullies or other factors (see Fig. 7.10). Most importantly, land within each replicate block should be relatively uniform.

Basal treatments

Basal treatments are any treatments that are uniformly applied over the whole experiment. The choice of these will depend very much on the objectives of the experiment and site conditions (see above). Examples of basal treatments include the method of site preparation (cultivation, deep ripping, etc.), methods used to control weeds, insect pests or plant diseases (pesticides, etc.), uniform applications of nutrients (types, rates, methods of application, timing) and the need for other soil amendments such as the application of lime to increase soil pH. Basal treatments can have a marked effect on the outcome of an experiment, particularly where marked interactions (Fig. 7.6) occur between a basal application and a treatment factor (e.g. soil N affects P supply to plants — see Chapter 6). If this type of interaction occurs and the experiment involves only one factor (e.g. P treatments), effects of different P treatments could depend on the level of the basal N treatment which is applied.

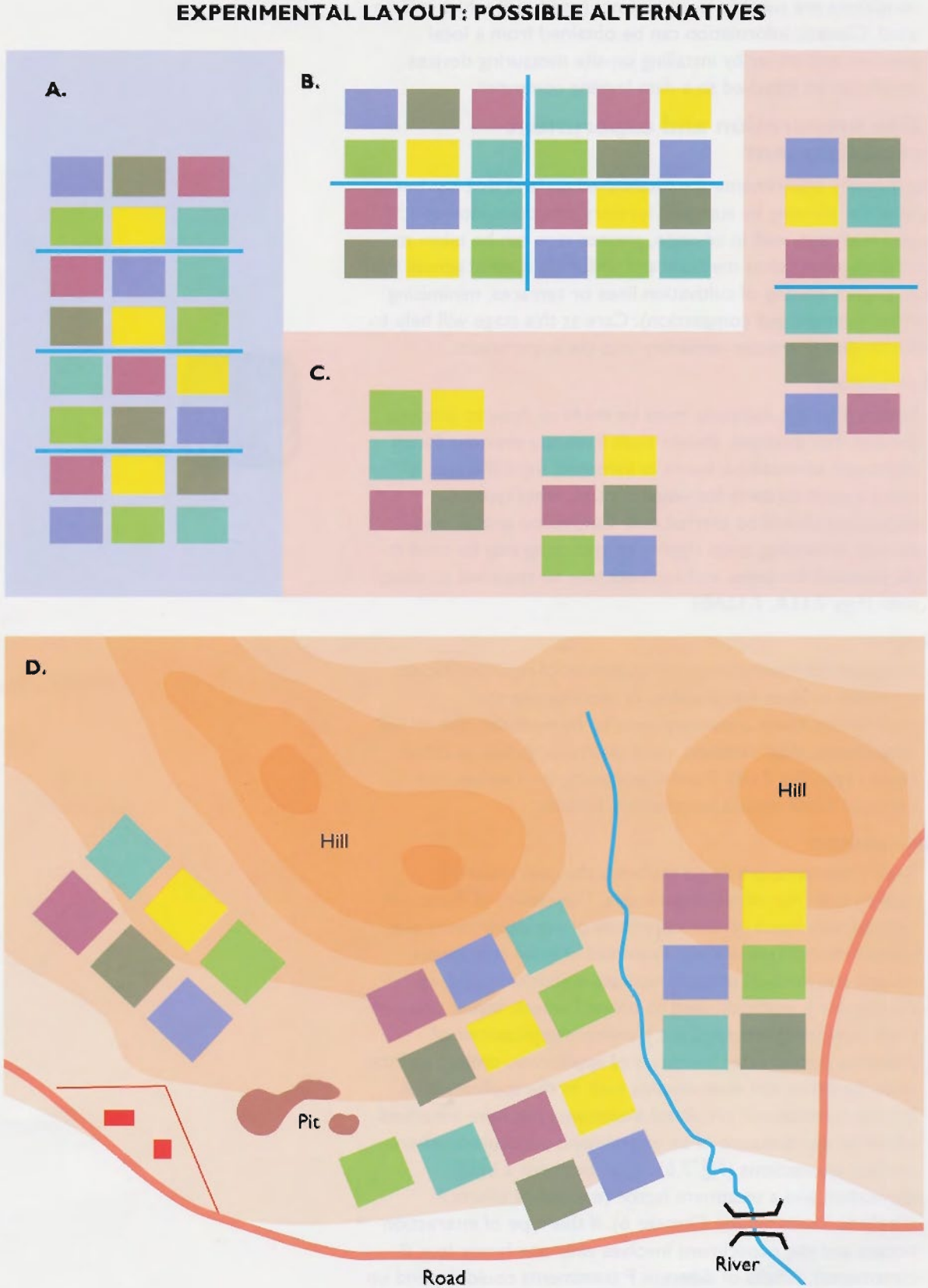


Figure 7.10. *Examples showing possible layouts (A–D) for an experimental trial with six treatments (represented by different colours) and four blocks. Site factors will determine how blocks should be arranged to minimise variability within each block.*

Planting

The time of year when planting is carried out can be critical in climates where trees must become established before the onset of drought or cold conditions. Care must be taken at planting to avoid damage to seedlings and to minimise stress (Figs 7.11B, 7.12CD). Seedlings should be well watered just prior to planting. With mycorrhizal seedlings, disturbance to the mycorrhizal roots and fungal hyphae must be minimised.

Fertilizer application

The types and rates of fertilizers applied both for experimental treatments and as basal dressings must be decided using information on soil fertility levels obtained from soil tests and/or measurements of plant growth responses to fertilisation from earlier glasshouse or field experiments (Chapter 6). Specific treatments that are different to standard silvicultural practices may be required to ensure that nutrient limitations do not affect growth, and to avoid uneven nutrient applications which can introduce variability into the experiment. Fertilizers may be applied as spot applications placed near each seedling (Fig. 7.11CD), but it is better to apply fertilizers uniformly to plots on an area basis (Fig. 12E–G).

Avoiding bias

When treatment trees are planted or treatments applied to field plots, precautions must be taken to avoid introducing bias. For example, when several people are involved in establishing an experiment, each person should be responsible for a task that is carried out over all treatments, rather than assigning specific treatments to each person. In the latter case, differences between people in planting technique or the method of applying fertilizers could introduce variation in tree growth that masks the effect of treatments.

Uniformity of experimental materials

The quality and uniformity of experimental materials such as tree seedlings and fertilizers is very important. Seedlings which are uniform in size, nutrient status and health should be selected for use. To allow for the culling of seedlings, many more seedlings should be planted in the nursery than are required for a field experiment. This can be much easier if seedlings are genetically uniform (i.e. from a single seed provenance, or clonally propagated). In mycorrhizal experiments, we would also try to select seedlings that are also similar in their extent of root colonisation by mycorrhizal fungi (see Chapter 6). The quality and uniformity of fertilizers used is also critical. The same batch (source) of a particular fertilizer should be used throughout an experiment and chemical analysis used to check the nutrient content of fertilizers.

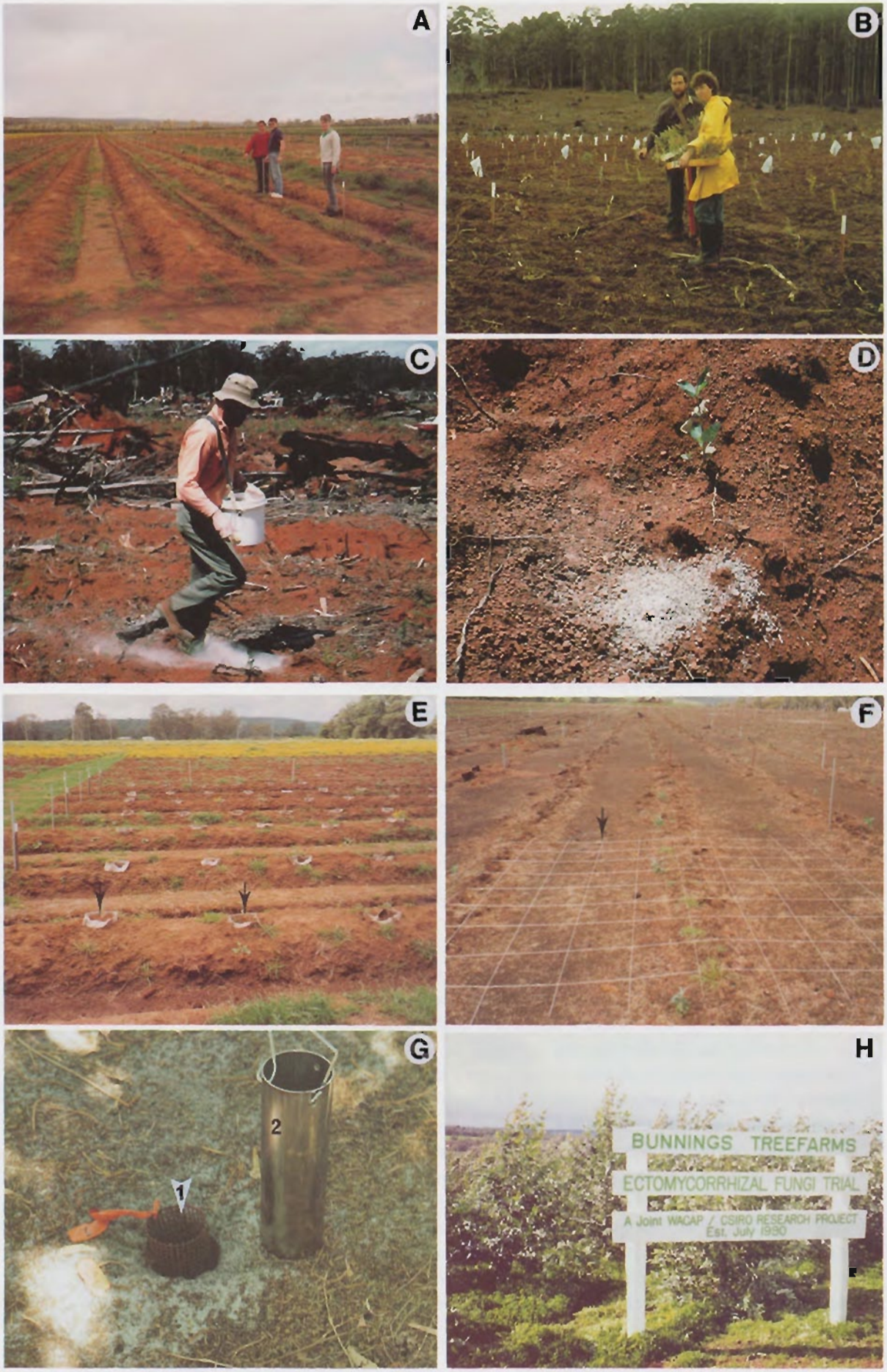

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ECTOMYCORRHIZAL FUNGI TRIAL
A Joint WACAP / CSIRO RESEARCH PROJECT
Est. July 1990

Figure 7.11. *Setting up mycorrhizal field experiments in Australia.*

A. *Site preparation by cultivation to loosen soil and create mounds for planting.*

B. *Planting mycorrhizal tree seedlings in marked plots according to the experimental design.*

C,D. *Spot application of a standardised volume of a complete fertilizer (N, P, K, etc.) to seedlings after planting.*

E. *Seedlings planted in field soil in porous woven-plastic bags (arrows), for later removal to test the persistence and spread of inoculated ECM fungi.*

F. *Placement of 30 cm^2 grids (arrow) during establishment of a field trial, for later observations of fungal fruiting.*

G. *Placement of ingrowth soil cores in a field site for measurement of mycorrhizal and non-mycorrhizal root growth and turnover. A plastic mesh cylinder (1) is placed in a hole made with a soil corer (2) and filled with sieved soil from the site.*

H. *Field trial in Western Australia with* Eucalyptus globulus *inoculated with ECM fungi, nine months after planting.*

Minimising variability

Procedures used at planting and for applying treatments should always aim to minimise variability within plots. Care must be taken to apply treatments uniformly. Variability may be introduced, for example, by the application of a basal phosphorus treatment to trees in a mycorrhiza screening trial. In situations where the applied P is not adequate for maximum tree growth, any variation in soil fertility caused by inexact fertilizer applications (ΔP in Fig. 7.13) will result in large differences in tree growth (ΔY_1 of Fig. 7.13). This unwanted variability would be much smaller in situations where P supply is adequate (ΔY_2). Distributing fertilizers evenly when they are being broadcast is difficult in large plots and it may be better to apply smaller, uniform amounts to subplots, individual rows within the plots, or individual trees (see Figs 7.11D, 7.12E–D).

Other considerations

If errors are made in the execution of experimental plans, it is important to try to rectify the problem. Anomalies that cannot be overcome need to be recorded and taken into account in the analysis of results from the experiment. Uncontrollable environmental factors may have a severe impact on experiments (Fig. 7.14G), requiring some trees to be excluded from analysis, and may even result in the loss of an entire trial.


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Figure 7.12. *Setting up mycorrhizal field experiments in China as part of a collaborative project between ACIAR (Australian Centre for International Agricultural Research) and the Chinese Academy of Forestry.*

A. *Use of terracing to control erosion, prevent fertilizer runoff and improve access to a steep site in Kaiping.*

B–H. *Stages in the establishment of a mycorrhizal trial with* Eucalyptus globulus *near Kunming.*

B. *Trenches that were dug along rows to loosen soil and allow deep placement of basal fertilizer treatments, before planting trees.*

C. *Planting trees in 30-cm diameter by 30-cm deep holes.*

D. *View of site after trenches were filled in and trees were planted.*

E. *Measuring fertilizers using a pan balance to control application rates.*

F. *Broadcast application of fertilizer to one plot of an experiment.*

G. *View of a site after fertilizer application, showing strings used to mark plot boundaries.*

H. *Trees after three months growth in the field.*

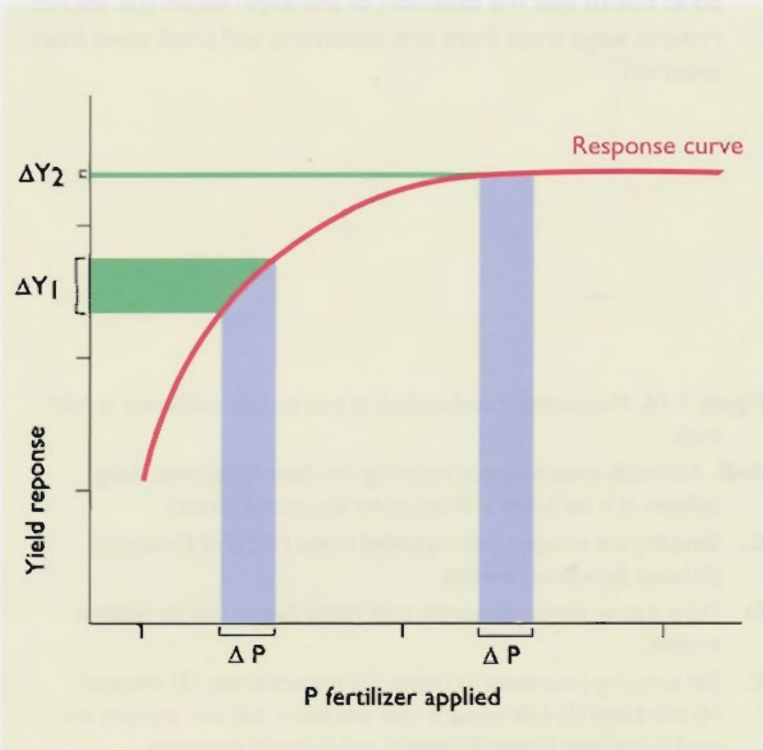


Figure 7.13. *Graph of a typical fertilizer response curve showing how the same fertilizer application (ΔP) can result in large (ΔY1) or small (ΔY2) yield responses depending on the amount of that nutrient already present in soils.*

C. Maintenance of the experiment

The experiment should be constantly monitored following planting. In the initial period after planting, some seedlings may die as a result of stress associated with transplanting or the impact of pathogens, pests or environmental factors. Where significant numbers of plants are lost it may be necessary to replace them to maintain a uniform density. Replacement seedlings should be noted and excluded from the data analysis.

Maintenance procedures

Constant observation of the experiment will help to identify any other problems as they arise (e.g. the need for weed, disease, or insect control). If a basal dressing of nutrients has been applied to the experiment at planting there is often a need to follow this up with later applications to ensure tree growth does not become limited by deficiencies of these nutrients. For example, if the trial is measuring the response to different rates of P application, regular applications of N and K may be required so that limitations of these elements do not affect the shape of the P-response function.

Thinning

Where trees have been planted at a closer spacing than the normal practice, they will have to be thinned. This can be done selectively to improve uniformity within the plots, but so as not to bias the outcome of the experiment (i.e. do not remove large trees from one treatment and small trees from another).

Figure 7.14. Measurement and analysis of tree and soil properties in field trials.

A–B. *Accurately measuring and recording the diameter of trees, using callipers at a set height (10 cm) above the ground (arrow).*

C. *Sampling the youngest fully expanded leaves (YFEL) of* Eucalyptus globulus *for nutrient analysis.*

D. *Using a corer (arrow) to sample bark layers from a tree for nutrient analysis.*

E. *Soil sampling procedures: (1) corer, (2) protective cap, (3) hammer, (4) pliers and (5) hole where a core was taken. Soil core samples are used to measure chemical, physical and biological properties.*

F. *Soil pit excavated to characterise the soil profile of a field site.*

G. *Observing typhoon damage to trees in a trial. Such deformed trees must be excluded from measurements in an experiment.*

H. *Using a computer to manipulate and analyse data from a field trial in China.*


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D. Measurements and observations

Various types of measurements can be made to quantify tree growth. Typically, measurements of tree height and/or stem diameter are taken, but many other parameters may be recorded in order to address specific experimental objectives (Table 7.4). Basic measurements such as stem diameter and height can be used to estimate yield parameters such as stem basal area, total above-ground dry matter, stem volume, stem weight and stem lengths for poles. Some details of measurement procedures are provided below and illustrated in Figures 7.14–7.16.

Table 7.4. Parameters which can be measured in mycorrhizal field experiments. Only a few of these categories would apply to a particular experiment. Many of these parameters can only be measured by limited destructive sampling.

Category	*Measurements*
Shoot growth	Height (stem length) Stem diameter (basal, breast height, stem volume, etc.) Biomass (branch, trunk, leaves, etc.)
Root growth	Root length, or density Biomass (coarse, fine, woody, etc.) Mycorrhizal formation
Nutrient content	Leaves, roots, bark, wood
Soil properties	Organic matter, pH, nutrient levels, etc.
Physiological properties	Photosynthesis, transpiration, etc.
Fungal growth	Sporocarp production Biomass of mycorrhizal roots, hyphae, etc.



Initial measurements

Seedling size (height or stem diameter) can be recorded immediately after planting. This provides a measure of any differences between treatments at the time of planting, and therefore indicates effects that occurred during growth in the forest nursery (e.g. as a result of mycorrhizal inoculation). This measure can be used as a covariate in the analysis of

Figure 7.15. *Observing results of a large factorial trial with mycorrhizal inoculation and phosphorus fertilisation treatments, established in 1992 at Zhenhai Forest Farm near Kaiping, China.*

A. *Two-year-old* Eucalyptus urophylla × grandis *trees showing substantial differences in the growth of trees in different plots, which are primarily due to P fertilizer application rates. In this split-plot design, there were different fungal inoculation treatments within each main fertilizer treatment plot.*

B–C. *Early growth responses (3 months), observed by measuring tree height.*

D. *Measuring the stem diameter at breast height (130 cm) of a tree at this site.*

E. *Fruiting of an inoculated Australian strain of* Pisolithus *that could be distinguished from related Chinese fungi by morphological characteristics. These observations were used to confirm the persistence of introduced fungi.*


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later measurements to separate effects that developed in the field from initial effects that occurred in the nursery.

Frequency of measurements

Measurement frequency will depend on the specific objectives of the experiment and on whether, or how rapidly, we expect responses to change over time. For mycorrhizal experiments we often measure growth three times in the first year, because responses to fungal inoculation can change during this time as a result of differences among fungi in their rate of spread on the root system and their ability to persist. The frequency of measuring is often decreased (to once or twice a year) in subsequent years.

Taking measurements

If stem measurements are to be used to estimate growth, stem diameter should be measured below major branches and where there is uniform taper (i.e. above any basal swelling). For young trees this may require measurements to be taken 5 or 10 cm above the ground (Fig. 7.14A,B). For older trees, stem diameters are generally measured at 130 cm above the ground (diameter at breast height over bark or DBHOB) (Fig. 7.15D). At the time treatment trees are being measured, notes should be made of any damaged or unusual trees, as this may justify leaving them out of the data assessment at a later stage. Measurements taken above major branching may not provide a good estimate of tree growth if there is considerable tree-to-tree variation in tree form (Fig. 7.16A). Where trees have more than one stem, the diameter of each stem can be measured and the equivalent diameter of a single stem calculated (Fig. 7.16B).

Allometric functions

Data that can be readily obtained from across an entire experiment can be used to provide estimates of important yield parameters which are harder to obtain and require destructive sampling. For example, tree diameter data can be used to estimate above-ground biomass (dry weight) by establishing allometric functions which relate diameters to weights of different tree components, or of whole shoots. A protocol for establishing these functions is outlined below and an example is shown in Figure 7.17 for two component parts of *Eucalyptus nitens*.

1. Allometric functions require destructive sampling a number of trees which cover the range of tree sizes which occur in the main experiment. They may be obtained from areas adjacent to the trial or in buffer areas between treatments.
2. Each tree is cut at ground level, its height recorded, and the diameter of its stem measured at various distances (e.g. 5, 10, 15, 20, 50 and 130 cm) from the base.
3. The tree is then sorted into various components (e.g. leaf, branch and stem). These components are then oven-dried and weighed to determine their biomass.

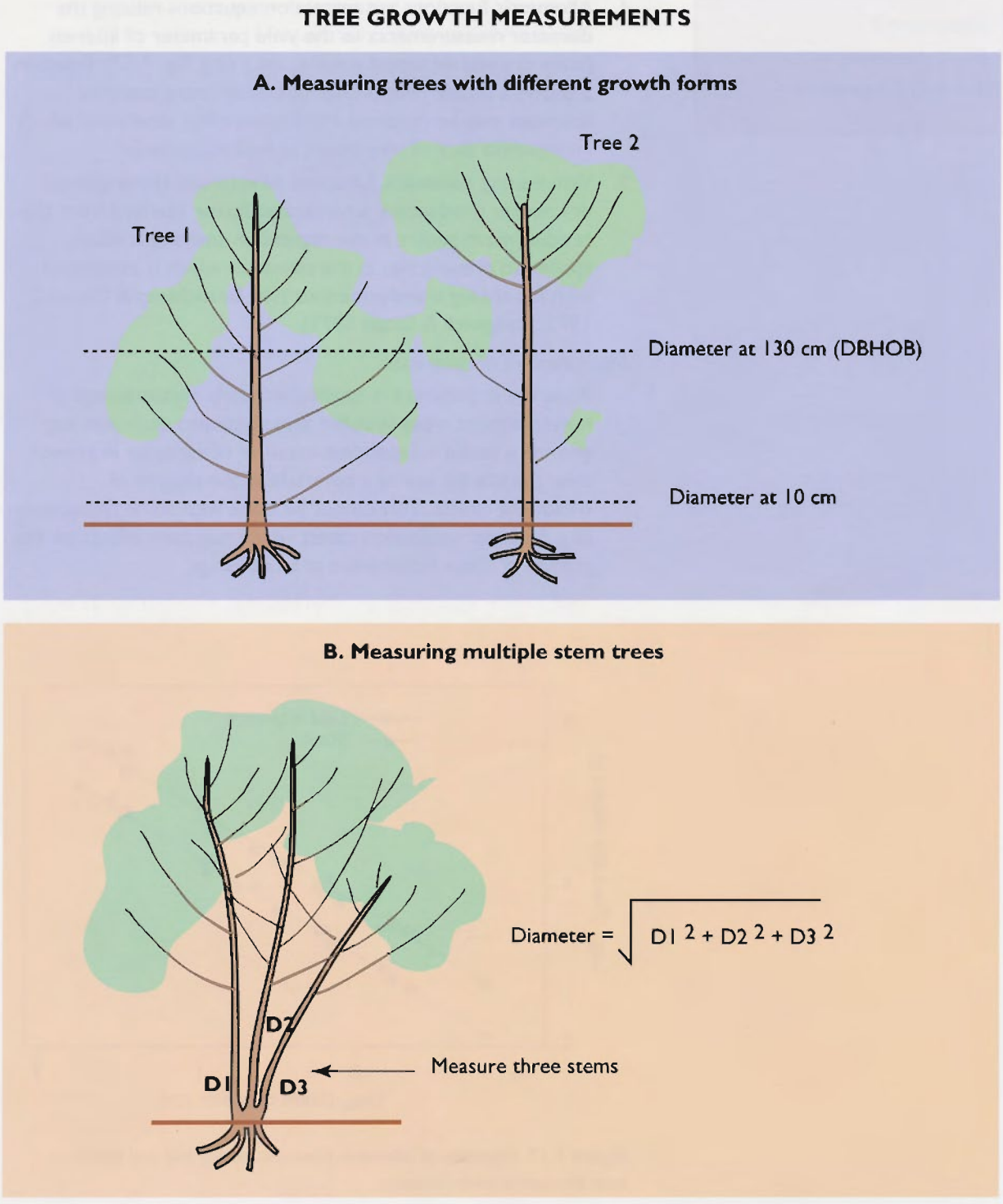


Figure 7.16. *Measuring tree diameter to quantify growth responses.*

A. *Trees should be measured below major branches to overcome problems due to variations in growth forms. In this example Tree 1 would have a greater biomass than Tree 2, but Tree 2 appears as large if they are both measured at 130 cm. Measuring both trees at 10 cm provides a more representative estimate of their dry mass.*

B. *For trees with multiple stems, each stem (D1–D3) should be measured separately and an equivalent diameter (D) for a single stem calculated from them.*

Equation 3
ln [dry matter] =
a + b ln [diameter]

4. Allometric functions are regression equations relating the diameter measurements to the yield parameter of interest (stem dry matter, wood volume, etc.) (e.g. Fig. 7.17). Equation 3 shows a simple relationship, but other more complex functions may be required. Models are often developed which incorporate data of tree height as well as diameter.
5. When using allometric functions to estimate above-ground dry matter production, a correction factor (derived from the residual mean square in the regression analysis) is often applied to prevent bias in the estimates which is associated with log-antilog transformations (see Beauchamp & Olson 1973, Madgwick & Satoo 1975).

Measurement of covariates

After the experiment is established, early measurement of non-treatment trees in buffer areas between each plot may provide a useful independent measure of variation in growth over the site for use as a covariate in the analysis of treatment effects. This cannot be done with some treatments (e.g. fertilizer application rates) which may have effects on the growth of these buffer trees at an early age.

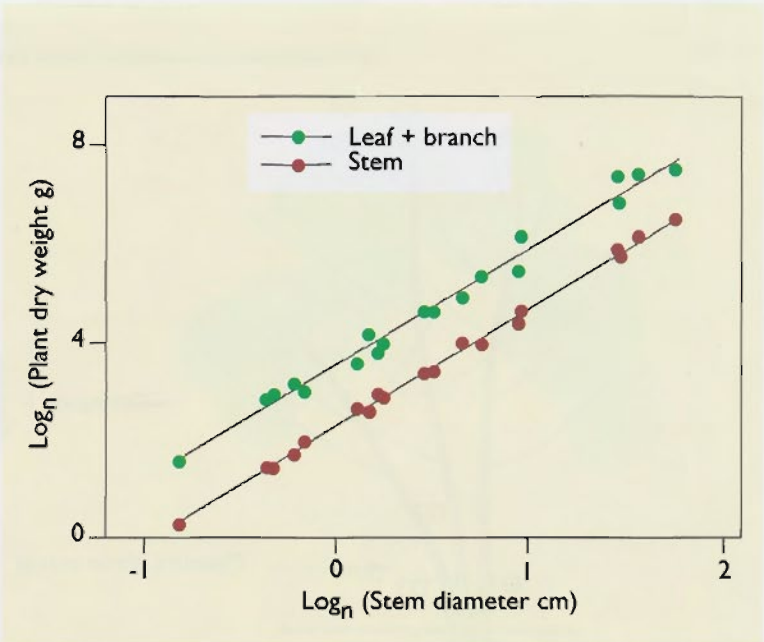


Figure 7.17. *Examples of allometric functions relating leaf and branch or stem biomass to stem diameter.*

Measures of fungal performance in mycorrhiza trials

Various measurement and sampling procedures may be used to indicate the success of inoculant fungi in persisting and spreading on roots. The most common procedures used are listed below.

1. Sporocarps (fruiting bodies) of ECM fungi can be identified and quantified using procedures for fungal identification presented in Chapter 2 (Fig. 7.15E). Spatial mapping of sporocarp occurrence can be used to follow changes in fruiting by different fungi (Fig. 7.11F).
2. Soil cores can be extracted (Fig. 7.14E) to obtain fine roots and assess mycorrhizal development associated with both endemic and introduced fungi (Chapter 4).
3. The persistence of inoculated fungi can also be examined by planting some extra seedlings in mesh bags for removal and assessment at intervals after establishment of the experiment (Fig. 7.11E).
4. Ingrowth soil cores (mesh cylinders filled with sieved soil) can be placed around trees and removed at intervals to quantify the growth and turnover of mycorrhizal and non-mycorrhizal roots (Fig. 7.11G).

Plant sampling and analysis

Plant tissues are often sampled and analysed to obtain information on the nutrient status of trees and nutrient limitations to growth and to examine the effects of treatments on nutrient uptake and distribution in plants (see Chapter 6). This information is relevant in nutrient response trials for establishing relationships between tissue nutrient concentrations and growth. These relationships may be useful in the diagnosis of nutrient deficiencies and in predicting fertilizer requirements for tree crops (Dell et al. 1995). Further information on plant nutrient analysis and the diagnosis of nutrient deficiencies is provided in Section 6.4.

Soil sampling

After the experiment is marked out, soil samples are often collected prior to treatments being applied. Analysis of these samples will characterise the soils and indicate the uniformity of soil properties over the site. Composite samples of several surface soil cores (Fig. 7.14E) of a standard depth may be obtained from each replicate block, or from each plot if it is felt that this intensity of sampling is warranted. The water content, pH, organic matter content, texture and nutrient status of soils can be established using standard procedures (Rose 1966, Rayment & Higginson 1992).

7.4. DATA PROCESSING AND STATISTICAL ANALYSIS

There are many techniques and approaches that can be used in compiling, summarising and statistically analysing the results of experiments (Zar 1984, Mead 1988, Snedecor & Cochran 1989, Williams & Matheson 1994). These will not be discussed in any detail here, but a few aspects will be mentioned and illustrated with examples. Many software programs are now available to process and analyse data. They include spreadsheets such as Microsoft Excel and Lotus 1-2-3, data tabulation and pre-processing programs such as Datachain (Roger & Muraya 1991) and programs for statistical analysis such as Genstat and Minitab (Anon. 1991, Payne et al. 1987). Stages in the handling of data from field experiments are outlined below.



1. Data are collected using a field data sheet or record book. A duplicate set of data (made using carbon paper or a photocopier) should always be kept in a safe place.
2. Data are typed into computer files using a spreadsheet program (Excel, Lotus 1-2-3, etc.), specialised data management software such as Datachain, a database program or a text editor.
3. A preliminary analysis of the data should be performed by calculating treatment means and making graphs using appropriate software.
4. The data are checked and arranged to ensure they are in the correct format for the statistical program, which is then used initially to identify any outlier points which may occur (e.g. damaged trees or incorrectly recorded or entered values). The homogeneity of variance of the data should also be tested (see below).
5. Calculation of new variables (e.g. biomass from diameter measurements) and plot means is done if required. It may also be necessary to transform the data if this is indicated during step 4.
6. The data can then be analysed statistically as described below. This allows the hypothesis to be tested and a summary table to be generated. The most common statistical procedures for field trial data include various types of ANOVA models as well as regression and correlation techniques.
7. It is also desirable to produce graphs which illustrate trends in the data. Further analysis of treatment means may also be used for specific comparisons of treatment effects.

One of the assumptions in the analysis of variance is that the variance is relatively homogenous. This assumption is met in Example 1 (Fig. 7.18A). However, in another situation (Fig. 7.18B) there is a need to transform the data (e.g. to a log or square-root scale) before analysis. These graphs are made by plotting residuals (calculated by subtracting treatment values from fitted values) against fitted values (Williams & Matheson 1994).

Another useful tool for checking variance homogeneity is a frequency histogram plot of residuals of all the replicates with their respective treatment means removed.

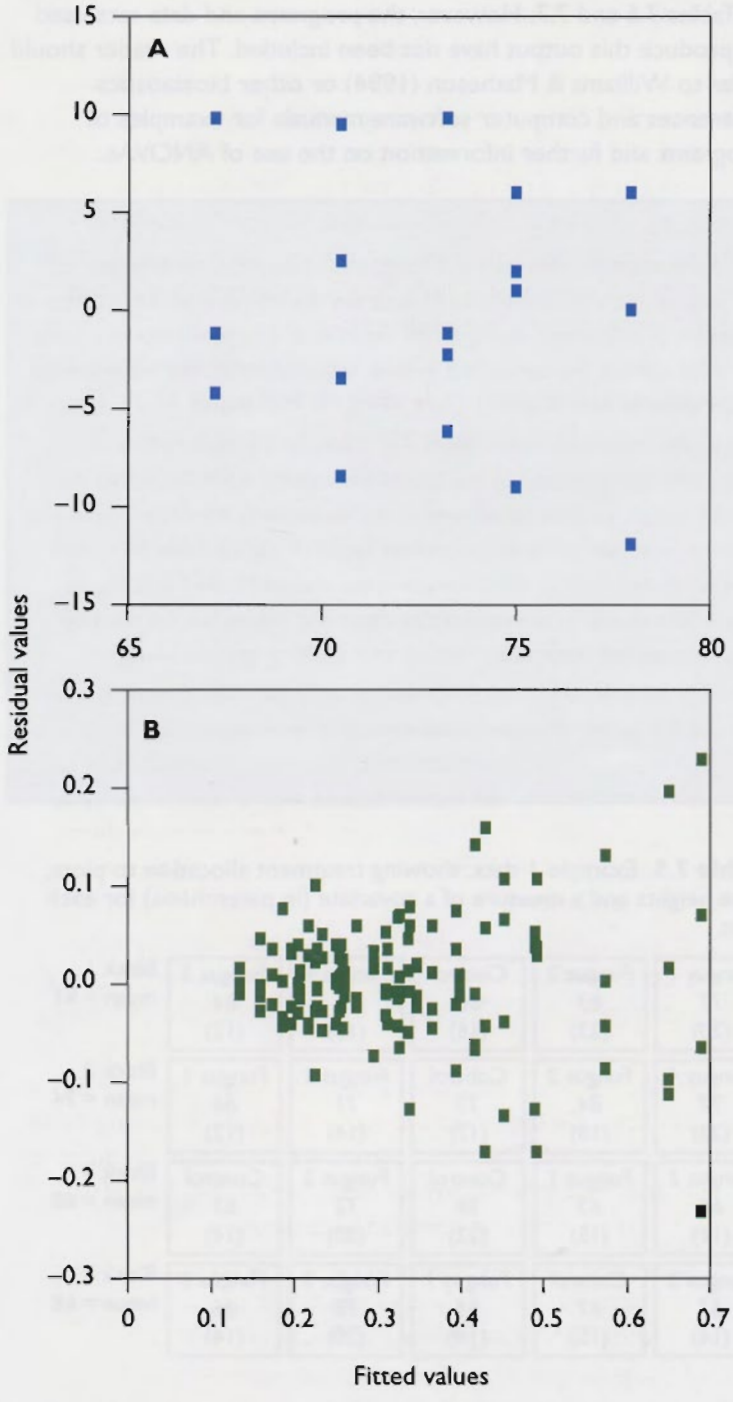


Figure 7.18. *Two examples of plots of residual vs. fitted data from ANOVA datasets.*

A. *An example where variation is uniform (homogenous) across experimental treatments.*

B. *Example with non-uniform variation, where transformation of data would be required.*

The structure of the ANOVA depends on the experimental design and layout of treatment plots. The following examples illustrate some aspects of analysis of variance and the use of a covariate. Typical examples of output generated by statistical analysis software packages such as Genstat and Minitab are shown in Tables 7.6 and 7.7. However, the programs and data sets used to produce this output have not been included. The reader should refer to Williams & Matheson (1994) or other biostatistics references and computer software manuals for examples of programs and further information on the use of ANOVAs.

Example 1.

In this case we will use tree height measurements from an experiment with five fungal treatments (Control, Fungus 1, Fungus 2, Fungus 3, Fungus 4) randomised within each of four replicate blocks to illustrate the effectiveness of blocking in reducing the residual error and improving the measure of differences among fungal treatments. The layout of treatments and fictitious plot values for heights of treatment trees and for a covariate (e.g. growth measures of border trees around plots that are not affected by fungal treatments) are given in Table 7.5.

If this experiment was set up and analysed as a fully randomised experiment (Fig. 7.8A), no significant effect of treatments would be found (Table 7.6A). Treatment effects are masked by the inherent variability in growth across the site. When analysed correctly as a randomised block design, a significant amount of the variability is accounted for by the variation between blocks, and fungal treatments are now shown to significantly affect height ($P \leq 0.05$; Table 7.6B). The resolution of the treatment effect is further improved by inclusion of the covariate in the analysis (Table 7.6C). Figure 7.19 shows that with each of these analyses there is little change in the estimate of treatment means, but the residual error with which the treatment effects are tested progressively decreases.

This example emphasises the need to choose a suitable experimental design and to apply the correct structure for an ANOVA. The potential advantages of blocking and the use of covariate measurements in resolving treatment differences are also demonstrated.

Table 7.5. Example 1 data, showing treatment allocation to plots, tree heights and a measure of a covariate (in parenthesis) for each plot.

Fungus 1 77 (20)	Fungus 2 83 (23)	Control 80 (16)	Fungus 4 81 (15)	Fungus 3 84 (12)	Block 1 mean = 81
Fungus 4 77 (22)	Fungus 3 84 (18)	Control 73 (17)	Fungus 2 71 (14)	Fungus 1 66 (12)	Block 2 mean = 74
Fungus 3 66 (11)	Fungus 1 63 (15)	Control 76 (22)	Fungus 2 72 (20)	Control 62 (14)	Block 3 mean = 68
Fungus 2 67 (14)	Control 67 (15)	Fungus 1 63 (14)	Fungus 3 78 (20)	Fungus 4 66 (14)	Block 4 mean = 68

Example 2.

This example shows the output from a more complex analysis of variance structure (Table 7.7), which could be used to analyse effects of two factors (fungal inoculation and P application treatments) on stem basal area. A split-plot, randomised block design was used for this example. Note that there are four strata (blocks, plots, subplots, units), each with a different error structure to ensure that each factor is tested by the appropriate error term.

The final step in the data analysis process is the interpretation and presentation of results. When compiling reports describing experiments, it is important to be selective with the results that are presented and concentrate on the information that addresses the primary objectives of the experiment. In experiments involving more than one factor, values for all treatment combinations would perhaps be summarised in tables or figures only where the interaction between these factors was significant. There is no need to clutter the paper with too much statistical data, but it is important to provide the essential statistical information to substantiate statements about treatment differences.

The above examples give only a very cursory look at some aspects of data analysis. Experimenters will often need to seek advice from experts in statistical analysis and/or books on this subject when designing and analysing results from field trials.

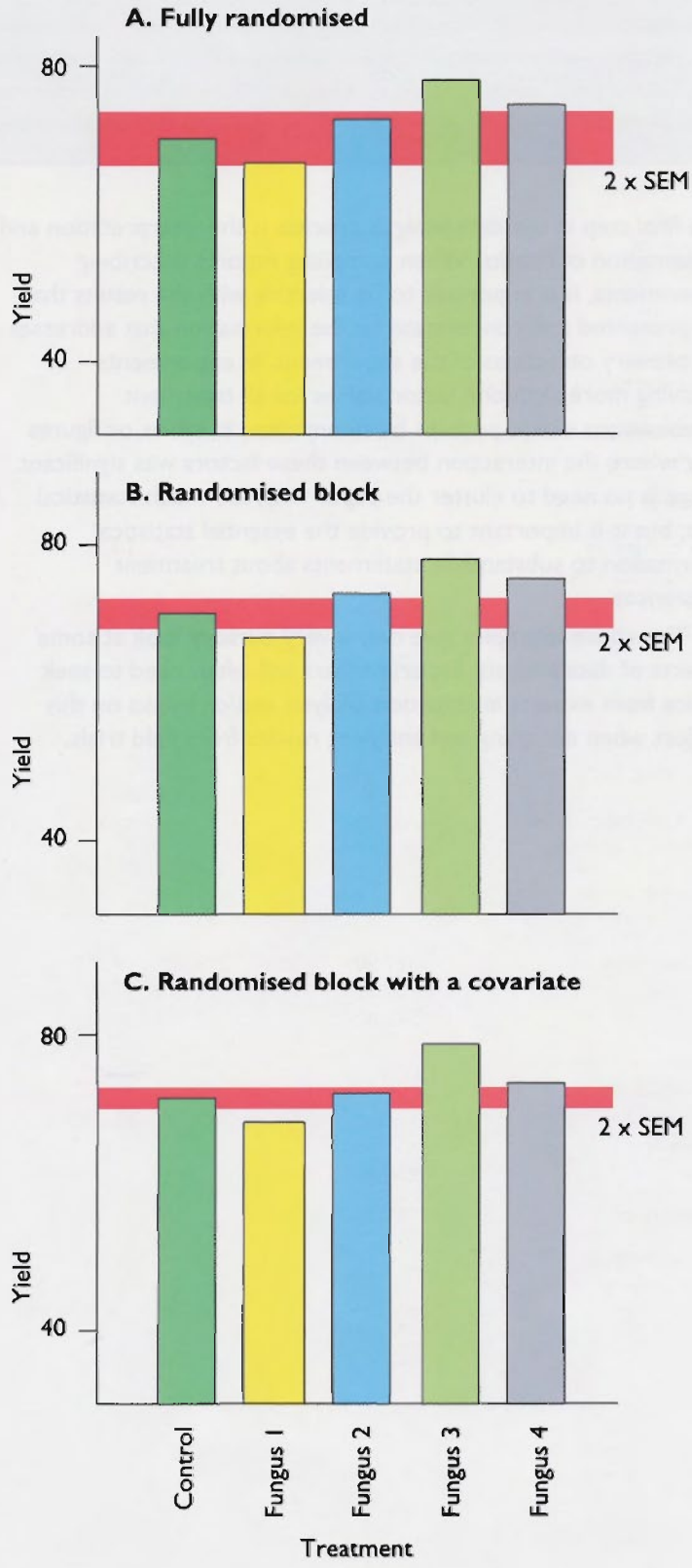


Figure 7.19. *Three examples of showing how changes to experimental designs and statistical analysis procedures can result in improved detection of treatment effects due to reductions to the residual variation (SEM).*

Table 7.6. Analysis of variance of the same tree data using different ANOVA structures generated by a statistical software package (Genstat).

A. Analysis of variance fully randomised design variate: tree height (cm)

Source of variation	*DF*	*SS*	*SS%*	*MS*	*VR*	*P*
Units stratum						
Fungus	4	272.70	25.60	68.18	1.290	ns
Residual	15	792.50	74.40	52.83		
Total	19	1065.20	100.00	56.06		
Grand total	19	1065.20	100.00			
Grand mean		72.8				
Total number of observations		20				

B. Analysis of variance fully randomised block design variate: tree height (cm)

Source of variation	*DF*	*SS*	*SS%*	*MS*	*VR*	*P*
Blocks stratum	3	576.80	54.15	192.27	10.696	≡0.001
Blocks units stratum						
Fungus	4	272.70	25.60	68.18	3.793	<0.05
Residual	12	215.70	20.25	17.98		
Total	16	488.40	45.85	30.53		
Grand total	19	1065.20	100.00			
Grand mean		72.80				
Total number of observations		20				

C. Analysis of variance (adjusted for covariates) — randomised block design using covariate data variate: tree height (cm)

Source of variation	*DF*	*SS*	*SS%*	*MS*	*VR*	*P*
Blocks stratum						
Covariates	1	389.505	36.57	389.505	4.159	
Residual	2	187.295	17.58	93.648	17.687	
Total	3	576.800	54.15	192.267	36.314	<0.001
Blocks units stratum						
Fungus	4	249.267	23.40	62.317	11.770	<0.001
Covariates	1	157.460	14.78	157.460	29.740	
Residual	11	58.240	5.47	5.295		
Total	16	464.967	43.65	29.060		
Grand total	19	1041.767	97.80			
Grand mean		72.80				
Total number of observations		20				

Table 7.7. Analysis of variance table for a split-plot randomised block design.

Analysis of variance variate: basal area (cm^2/tree)

Source of Variation	*DF*	*SS*	*SS%*	*MS*	*VR*	*P*
Blocks stratum	3	1858.32	4.55	619.44	55.776	<0.001
Blocks plots stratum						
P-level	5	25101.19	61.42	5020.24	34.940	<0.001
Residual	15	2155.20	5.27	143.68	12.937	
Total	20	27256.39	66.70	1362.82	122.712	
Blocks plots subplots stratum						
Fungus	7	508.80	1.25	72.69	3.054	<0.01
P-level × Fungus	35	1207.44	2.95	34.50	1.450	≅0.05
Residual	124(2)	2950.97	7.22	23.80	2.143	
Total	166	4667.21	11.42	28.12	2.532	
Blocks plots subplots units stratum						
	741(27)	8229.44	20.14	11.11		
Grand total	930	42011.36	102.80			

Estimated grand mean	10.39
Total number of observations	960
Number of missing values	29
Maximum number of iterations	2

Tables of means variate: basal area (cm^2/tree)
Grand mean 10.39

P-level	0	161	645	1290	2580	3871		
	1.09	6.64	12.59	11.33	14.57	16.15		
Fungus	control	H4937	H4240	H4014	E4100	E4070	CH8909	H4111
	11.41	9.21	10.62	10.41	10.67	11.27	9.50	10.08

Fungus	control	H4937	H4240	H4014	E4100	E4070	CH8909	H4111
P-level								
0	1.97	0.96	0.58	0.93	1.56	1.26	0.83	0.64
161	6.38	4.30	7.94	5.27	6.59	8.23	7.31	7.07
645	13.91	13.44	12.58	14.17	13.50	11.04	10.60	11.48
1290	12.83	10.55	10.16	10.71	12.01	13.21	9.05	12.14
2580	16.58	11.93	14.35	13.91	16.07	17.85	12.57	13.29
3871	16.80	14.06	18.08	17.43	14.28	16.05	16.63	15.84

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