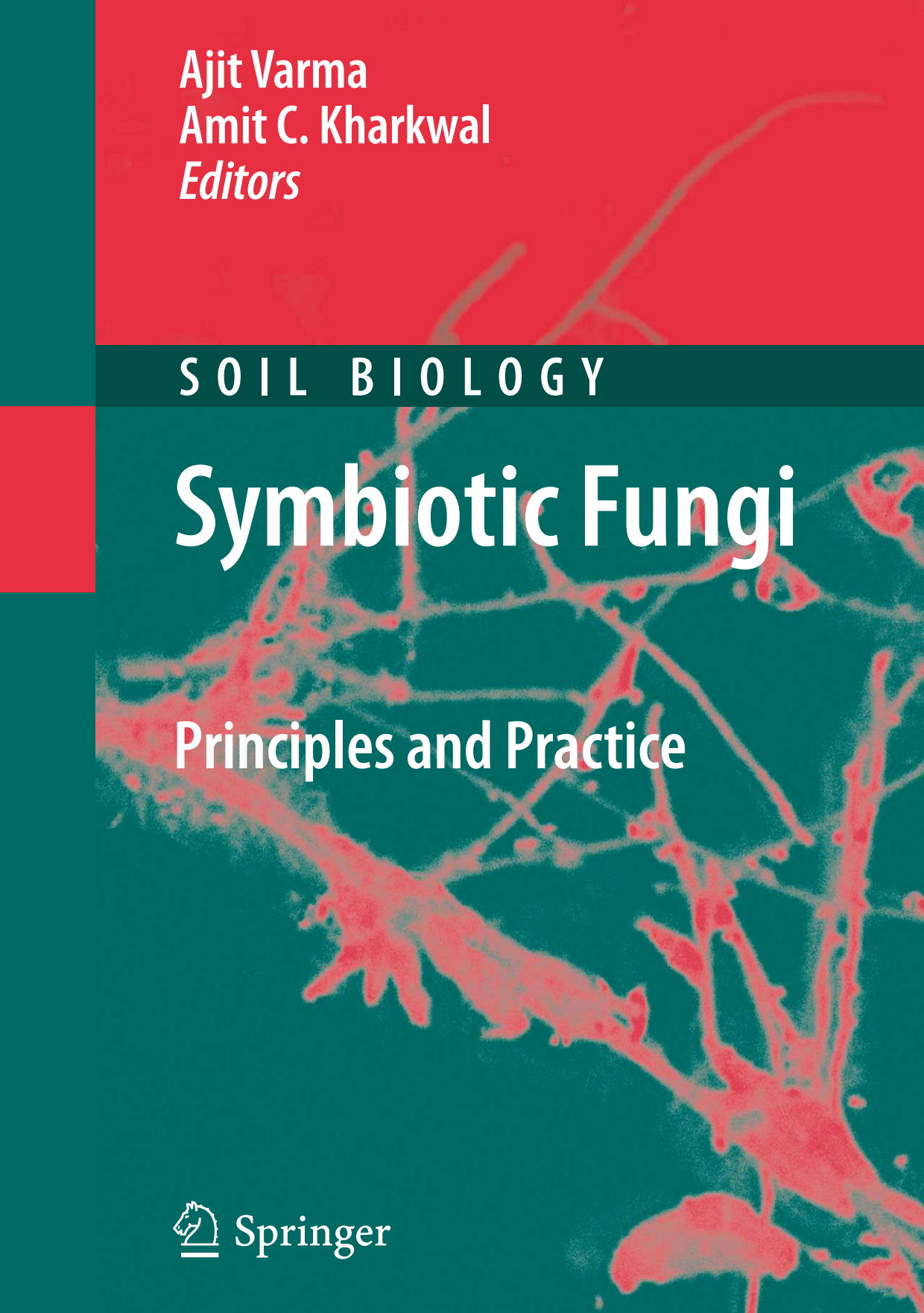
Ajit Varma
Amit C. Kharkwal
*Editors*

SOIL BIOLOGY

# Symbiotic Fungi

## Principles and Practice



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Editors

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## Principles and Practice

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# Foreword

So old, so new. . .

More than 450 million years ago, plants and fungi associated to produce a mutually beneficial symbiosis that assisted plants to invade the terrestrial environment, which was poor in nutrients and subject to desiccation and full sunlight. This is one of the main lessons that fossil records have transmitted to us, thanks to the reports of many paleontologists, starting with Kidston and Lang (1921). Their wonderful observations, which have been confirmed by many others, provided the evolutionary background to understand how mycorrhizas are a powerful driving force for the functioning of ecosystems, supplying land plants with phosphorus and nitrogen, as well as fungi with carbon, which finally accumulates in the soil.

On the other hand, few biological issues have entered the mainstream of biology in such a vigorous way as mycorrhizas. Mainly thanks to DNA technologies and genomics, new tools to discover symbiont communication, development and diversity and to reveal the contribution of each partner to the functioning of the association have been deciphered, thus offering breakthrough findings. Looking at the history of mycorrhizas, it can be seen that some very important events have marked recent years: the first sequenced genome of *Laccaria laccata* (Martin et al. 2008) opened a window on the secrets of ectomycorrhizal fungi thriving in forests and associated with woody plants; the identification of the plant genes that control the signal transduction pathways in arbuscular mycorrhizal (AM) legumes has allowed us to dissect the crucial steps of the fungal colonization process (Parniske 2008); the discovery that plant molecules, strigolactones, are perceived by AM fungi and act as "branching factors" (Akiyama et al. 2005) represents a landmark in our knowledge, but has also led to a second very recent discovery. Strigolactones have been found to impact the plant phenotype, representing a novel class of endogenous plant hormones that are present in a wide range of angiosperms from *Arabidopsis thaliana* to rice (Gomez-Roldan et al. 2008; Umehara et al. 2008).

But mycorrhizas also go beyond the issue of plant/fungal biology by occupying new fields, like that of environmental microbiology, and by pushing the development of new approaches, like those required for metagenomics. While the foundation set up by Craig Venter, the world-renowned genome research pioneer (www.jcvi.org/),

has the aim of exploring the microbial diversity in the world's oceans, the dream of soil microbiologists instead is to understand what the creatures that live in the soil are doing there (Dance 2008). However, in the rhizosphere, the thin soil layer where roots and soil microbes interact (Little et al. 2008), mycorrhizal fungi with their diverse guilds of associated microbes (bacteria, endophytes, saprotrophs) proliferate and dominate. To detect and quantify microbes in this environment, where researchers believe they can find the world's widest biodiversity (Dance 2008), will be one of the most exciting challenges for the scientific community working in the mycorrhizal field. This will mean overcoming some of the reductionisms related to laboratory practices and moving into the field, using updated high-throughput DNA sequencing technologies and large-scale genomic analysis. The development of such new platforms could lead to new paradigms, i.e. the description of mycorrhizas as the result of multiple organism interactions and functioning.

The chapters in this book mirror this mixture of old and new concepts, starting from the seminal concept of symbiosis, which is central in the evolution of complexity and is critical to the lifestyles of many animals and plants, and also to whole ecosystems, in which symbiotic organisms are key players (Moran 2006). The main aim of the book, however, is to combine the impetuous increase in knowledge concerning mycorrhizas with the best laboratory practices. Expert researchers illustrate the most updated techniques to deal with old/new questions.

Lastly, researchers enjoy investigating mycorrhizas since they are aware of their importance on the sustainability of our ecosystem. In this context, some chapters are devoted to technologies aimed at improving the quality of mycorrhizal inocula, thus increasing their application range in the frame of a more friendly agriculture.

This Soil Biology volume can surely offer many replies to the current questions concerning the way in which we can unlock the potential of mycorrhizal fungi, in order to make them a resource that will become available to everybody.

Torino
January 2009

*Paola Bonfante*

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# Preface

It has been a pleasure to edit this manual. The final outcome is the result of our painstaking efforts involving a passionate discussion with eminent scientists from across the globe working on symbiosis, students and fellow colleagues. The first edition of Mycorrhiza Manual edited by Ajit Varma was published in 1998 by Springer. This new edition is published as a volume under the banner of the Soil Biology series as *Symbiotic Fungi: Principles and Practice*. The timing of the present edition has coincided with tremendous technical progress that has been made in the area of fungal symbiosis. The third edition of Mycorrhiza — Genetics and Molecular Biology, Eco-Function, Biotechnology, Eco-Physiology, Structure and Systematics, edited by Ajit Varma, appeared in July 2008. This leads to enormous demand for the protocol book to carry out the laboratory exercises and field trials.

Growing on mineral particles and decaying organic matter, and living in the vicinity of or within plant roots, are a diverse array of fungal species, many of which form diverse symbiotic associations with plant roots. These symbiotic associations that form between the roots of most plant species and fungi are very well-known. They are characterized by bi-directional movement of nutrients where carbon flows to the fungus and inorganic nutrients move to the plant, thereby providing a critical linkage between the plant root and soil. In infertile soils, nutrients taken up by the fungi can lead to improved plant growth and reproduction. As a result, these associations are often more competitive and better able to tolerate environmental stresses than other plants. The book contains the current state of practice on many aspects of symbiotic fungi and associated microbes. It deals with organismic interactions, diversity of microbial communities, mycorrhizal bioassays, nutrient transfer techniques, restoration ecology, AM inoculum procedures, biotechnological application, microbial communication and mushroom technology. It will be of interest to a diverse audience of researchers and instructors, especially biologists, biochemists, agronomist, foresters, horticulturists, mycologists, soil scientists, plant physiologists, microbiologists and molecular biologists.

It is hoped that the protocols proposed by the authors will stimulate further research, as the information presented tends to highlight both the need for further

work in this challenging field and the lack of agreement on some fundamental issues.

In planning this volume, invitations for contributions were extended to leading international authorities working with symbiotic fungi. We would like to express our sincere appreciation to each contributor for his/her work, and for their patience and attention to detail during the entire production process. We sincerely hope these eminent contributors will encourage us in the future as well, in the greatest interest of academia.

The encouragement and inspiration received from Dr. Ashok K Chauhan (Founder President, Ritnand Balved Education Foundation), Sri Atul Chauhan (Chancellor, Amity University Uttar Pradesh), and Sri Aseem Chauhan (Chancellor, Amity University Rajasthan) need special mention. We are extremely grateful to the staff members of Springer Heidelberg, especially Dieter Czeschlik and Jutta Lindenborn, for their continued interest, critical evaluation, constructive criticism and support. We wish to acknowledge the help and support given to us by our students, faculty colleagues, family members and friends for their constant encouragement.

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January 2009 *Amit C. Kharkwal*

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# Chapter 1
# Symbiosis: The Art of Living

**Aparajita Das and Ajit Varma**

## 1.1 Introduction

The term symbiosis (from the Greek: sym, "with"; and biosis, "living") commonly describes close and mostly long-term interactions between different biological species. The term was first used in 1879 by the German mycologist, Heinrich Anton de Bary, who defined it as "the living together of unlike organisms".

In symbiosis, at least one member of the pair benefits from the relationship. The other member may be injured, i.e., have the parasitism association, or be relatively unaffected, i.e., have the commensalism association. Another type of relationship is mutualism; in this type both the partners get benefit from the associationship (Fig. 1.1). In this current chapter, we will discuss the term symbiosis only in relationship to these mutually beneficial interactions.

Endosymbiosis is any symbiotic relationship in which the symbionts lives within the tissues of the host, either in the intracellular space or extracellularly. Examples are nitrogen-fixing bacteria (called rhizobia) which live in root nodules on legume roots, Actinomycete nitrogen-bacteria called *Frankia* which live in alder tree root nodules, single-celled algae inside reef-building corals, and bacterial endosymbionts that provide essential nutrients to about 10–15% of insects.

Ectosymbiosis, also referred to as exosymbiosis, is any symbiotic relationship in which the symbiont lives on the body surface of the host, including the inner surface of the digestive tract or the ducts of exocrine glands. Examples of this include ectoparasites such as lice, commensal ectosymbionts, such as the barnacles that attach themselves to the jaw of baleen whales, and mutualist ectosymbionts such as cleaner fish.

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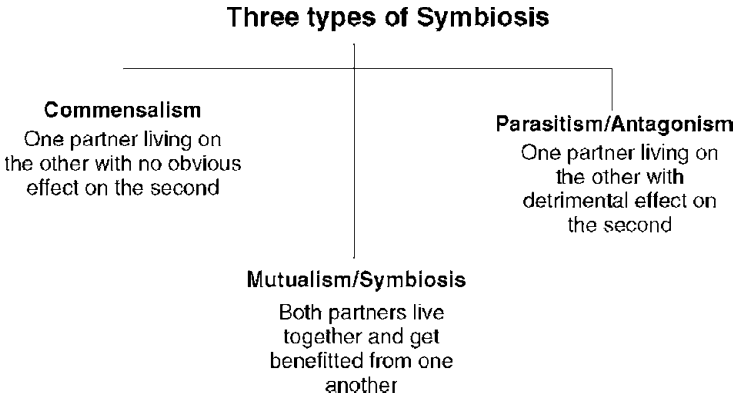


**Fig. 1.1** Types of symbiosis

All the divisions of the plant kingdom, namely bryophytes, pteridophytes, gymnosperms, and angiosperms, form symbiotic relationships with bacteria, cyanobacteria, actinomycetes, and fungi.

## 1.2 History of Symbiosis

Plants are the very foundations of life on the earth. A large proportion of the microbial population are present in the region immediately around plant roots, the rhizosphere. The surface and immediate neighborhood of a root provides a specialized environment for microorganisms where the microbial population is enhanced because of the root exudates. Almost 90% of all vascular plant families enjoy symbiotic relationships with microorganisms. Most symbiotic relationships probably started out as facultative. Over many generations, the organisms came to depend more on the symbiosis because natural selection favored those traits and not others. Eventually, the symbiosis became the sole source of the food, shelter, enzyme, or whatever else the symbionts derived from one another (http://science howstuffworks.com/symbiosis.htm).

Symbiotic relationships in which both the species of the association benefits are mutualistic. Mutualistic relations between plants and fungi are very common. The fungus helps the host plant absorb inorganic nitrogen and phosphorus from the soil. Some mycorrhizal fungi also secrete antibiotics which may help protect their host from invasion by parasitic fungi and bacteria. One of the most important examples of mutualism in the overall economy of the biosphere is the symbiotic relationship between certain nitrogen-fixing bacteria and their legume hosts. About 80% of all land plants have a symbiotic relationship with fungi of the phylum Glomeromycota. The fungus penetrates cells in the plant's roots, and provides the plant with

phosphates and other nutrients from the soil. This kind of symbiosis is called an arbuscular mycorrhiza. Fossil evidence (Remy et al. 1994) and DNA sequence analysis (Simon et al. 1993) have suggested that arbuscular mycorrhiza had appeared 400–460 million years ago, when the first plants were colonizing land. Remy et al. (1994) have reported the existence of arbuscules in the Early Devonian, which indicates that nutrient-transfer mutualism may have been in existence when plants invaded the land. The discovery of arbuscules in *Aglaophyton major*, an Early Devonian land plant, provides unequivocal evidence that mycorrhizae were established >400 million years ago. Nonseptate hyphae and arbuscules occur in a specialized meristematic region of the cortex that continually provided new cells for fungal infection. Arbuscules are morphologically identical to those of living arbuscular mycorrhizae, in consisting of a basal trunk and repeatedly branched bush-like tufts within the plant cell.

The arbuscular mycorrhiza play an important role in nature. Beneficial plant–microbe interactions in the rhizosphere are primary determinants of plant health and soil fertility. The carbohydrates produced by plants are translocated from their source location (usually leaves) to the root tissues and then to the fungal partners. In return, the plant gains the use of the mycelium's very large surface area to absorb water and mineral nutrients from the soil, thus improving the mineral absorption capabilities of the plant roots. Plant roots alone may be incapable of taking up phosphate ions that are immobilized, for example, in soils with basic pH. The mycelium of the mycorrhizal fungus can, however, access these phosphorus sources, and make them available to the plants they colonize. The mechanisms of increased absorption are both physical and chemical. Mycorrhizal mycelia are much smaller in diameter than the smallest root, and can explore a greater volume of soil, providing a larger surface area for absorption. Also, the cell membrane chemistry of fungi is different from that of plants. Mycorrhizae are especially beneficial for the plant partner in nutrient-poor soils. Arbuscular mycorrhizae are the most important microbial symbioses for the majority of plants and, under conditions of P-limitation, influence plant community development, nutrient uptake, water relations and above-ground productivity. They also act as bioprotectants against pathogens and toxic stresses. Other valuable effects of mycorrhiza in ecosystems are biological nitrogen fixation by *Rhizobium* in legume hosts, which can also be enhanced through co-infection with AMF (Xavier and Germida 2002).

A.B. Frank's observations and hypotheses about mycorrhizae in 1885 flew in the face of conventional thinking of the time. He reported that what we now term ectomycorrhizae were widespread on root systems of many woody plant species in a great diversity of habitats and soils. He hypothesized that mycorrhizae represent an all-encompassing mutualistic symbiosis in which fungus and host nutritionally rely on each other. He explained that the fungus extracts nutrients from both mineral soil and humus and translocates them to the tree host; and the host tree, in turn, nourishes the fungus. Today, with the help of modern scientific tools, it has been possible to achieve conclusive evidence to nearly Frank's entire hypothesis. Nonetheless, the revolution in thinking about plant and fungal evolution, ecology and physiology generated by Frank is still in the process of acceptance by much of

the scientific community, 120 years and tens of thousands of scientific papers since he coined the term mycorrhiza (Trappe 2005). Frank in 1885 gave the name "mycorhiza" to the peculiar association between tree roots and ectomycorrhizal fungi. In another publication, Frank in 1887 recognized a distinction between ectotrophic and endotrophic mycorrhizae, which included at the time only ericaceous and orchid mycorrhizas. A thorough discussion of the derivation of the word "mycorrhiza", including the incorporation of the second r, is given by Kelley (1931).

Koide and Mosse (2004) have reviewed the history of research on arbuscular mycorrhiza.

Arbuscular mycorrhizas may have been described as early as 1842 by Nageli, but most of Nageli's drawings only remotely resemble the arbuscular mycorrhiza. Trappe and Berch (1985) cite other early observations of the symbiosis during the period 1875–1895. Light and electron microscopical studies of arbuscular mycorrhizas were facilitated by the founding in 1950 of the Centro di Studio sulla Micologia del Terreno by Peyronel in Torino, Italy (Bonfante 1991).

Although there were already many independent descriptions of the arbuscular mycorrhiza in the late 1800s and early 1900s, the true identity of the fungi involved remained unknown for many decades. So unclear was their identity that at one point the possibility was circulated that a single fungus could form both ectomycorrhizas and arbuscular mycorrhizas (Lohman 1927).

Between 1952 and 1957 a fungus first isolated by Nicholls (1952) from surface-sterilized mycorrhizal onion roots was identified as a strain of *Pythium ultimum*. In 1955 Harrison, also from the Bristol group, isolated the organism again using the hanging drop technique of Magrou. Experiments to test whether inoculation with such isolates could produce typical arbuscular mycorrhizas were summarized by Hawker et al. (1957).

## 1.3 Symbiosis of Bacteria

### *1.3.1 Symbiotic Association of Bacteria with Leguminous Plants*

The symbiotic relationship of bacteria of the genus *Rhizobium* together with many members of the family Leguminosae, such as peas, beans, clovers, soyabean, forms an important nitrogen-fixing co-operative. An essential feature of the symbiotic fixation is the formation of nodules on the roots of the plants. Leguminous crops can fix nitrogen on earth through a symbiotic process with the gram-negative species of *Rhizobium* (Fig. 1.2).

*Rhizobium* has two types of strain:

(a) Fast growers that include *R. leguminosarum* or *Bacillus radicicola*, etc.
(b) Slow growers include *R. japonicum* and *R. lupinii*, etc.

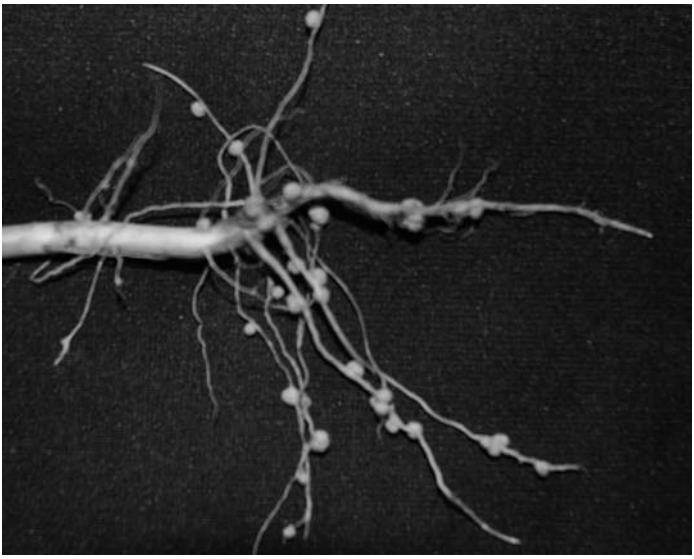

**Fig. 1.2** A typical root nodule caused by *Rhizobium* sp

*Rhizobia*(a collective name for representatives of the gram-negative genera *Azorhizobioum*, *Bradyrhizobium*, Mesorhizobium, Allorhizobium, *Rhizobium*, *Sinorhizobium*) are found in the soil of crop fields where bacteria and plant both specifically affect each other's growth. By and large known as rhizobia, they are grouped in different taxonomic families of the alpha-proteobacteria classified as Rhizobiaceae, Phylobacteriaceae and Bradyrhizobiaceae (Gonzalez et al. 2008). The bacterium which is microsymbiont infects the host root and forms nodules. Inside the nodules, the bacterium forms bacteroids in which it undergoes morphological and physiological transformations that can fix atmospheric nitrogen.

*Sesbania* species can establish symbiotic interactions with rhizobia from two taxonomically distant genera, including the *Sesbania rostrata* stem-nodulating *Azorhizobium* sp. and *Azorhizobium caulinodans* and the newly described *Sinorhizobium saheli* and *Sinorhizobium teranga* bv sesbaniae, isolated from the roots of various *Sesbania* species. Production of wheat typically requires intensive use of chemical fertilizers. Reliance on fertilizers may be decreased by exploitation of plant growth-promoting organisms. The work of Anyia et al. (2004) examines the responsiveness of Canadian cultivars of Hard Red Spring wheat to inoculation with *Azorhizobium caulinodans*, a diazotroph isolated from the legume *Sesbania rostrata*. This bacterium has been shown to colonize wheat roots through crack entry of the lateral roots. Inoculation of wheat cultivar CDC Teal grown in field soil caused increases in grain yield and total biomass of 34% and 49% respectively. Inoculated plants produced more tillers and had larger leaf area than un-inoculated plants.

### 1.3.2 Symbiotic Association of Bacteria with Nonleguminous Plants

Many species of as many as thirteen genera of nonleguminous angiosperms, which are all woody and dicots, bear root nodules that can fix nitrogen, e.g., *Casuarina*, *Alnus*. Some members of the family Rubiaceae develop nodule-like structures on the leaves, which contain nitrogen-fixing bacteria. Over 400 species of three genera of Rubiaceae and one genus of Myrsinaceae reportedly have bacterial leaf nodules. Light and/or electron microscope studies of a few species have shown that bacteria exist in spaces within buds filled with mucilage secreted by glands. These bacteria enter substomatal chambers (Rubiaceae) or marginal hydathodes(Myrsinaceae) and establish short-lived colonies, in intercellular spaces, that die out almost before full leaf expansion. Bacteria occur in seeds between endosperm and embryo, but only two studies have followed bacteria into flowers and ovules. Previous work on the physical relations of bacteria and host plants is discussed critically. Reviewing work done on isolation and identification of presumed endophytes leads to the conclusion that there is no agreement as to whether one or several bacterial taxa are the endophyte, and there are no unambiguous identifications, although four genera are suggested as possibilities (Lersten and Horner 1976).

### 1.3.3 Establishment of the Mutualistic Relationship Between Rhizobia and Legumes

#### 1.3.3.1 Presymbiosis Stage

A diverse group of rhizobia may inhabit the rhizosphere (root and soil area) of a specific legume, but only one or a limited number of species will interact with the legume. Assessment of rhizobial diversity indicates rhizobia are highly heterogeneous, as they differ in growth rates, biosynthetic pathways, habitats, catabolic activities, etc. (Gonzalez et al. 2008). The soil contains various bacteria and microorganisms. Each species of legume generally excretes a spectrum of flavonoids, stachydrines and aldenic acids into the rhizosphere, making the biochemical environment in which the rhizobia grow unique for each type of legume. This unique concentration gradient and spectrum of flavonoids is used to attract the appropriate rhizobial species to colonize the root and produce nodules. Some of these root exudates are also used as nutrients by some of the rhizobia, e.g., homoserine released by pea plants is the preferred nutrient (carbon and nitrogen source) for *R. leguminosarum biovar vicieae*, which forms a symbiotic relationship with pea plants (Van Egeraat 1975). Hence, a combination of preferential rhizobial population growth and movement, possibly by chemotaxis and/or electrotaxis (Miller et al. 1986) results in the appropriate rhizobial species colonizing the legume root and producing effective nodules.

#### 1.3.3.2 Rhizobial Attachment and Root Hair Deformation

The recognition factors between the host root and *Rhizobium* are shown to be proteins and sugars (Boogerda and Rossuma 1997). The proteins involved are the glycoproteins (carbohydrate containing proteins) also known as lectins. Such lectins can bind with several sugars. The lectins are present on the surface of the host root, while polysaccharides are present on the surface of the bacterium. The lectins have several attachment sites, each of which is specific to a particular type of polysaccharide. Consequently, the lectins of the host root recognize the polysaccharide receptors present on the surface of compatible *Rhizobium*. When such polysaccharides bind with a lectin molecule, this facilitates attachment of the bacterium to the root. Since lectins have several binding sites and the bacterium produces a range of sugars, this can ensure high specificity of recognition (Dazzo et al. 1988). Such lectins have been detected both in the seeds and nodulated plants of peanut and soybean (Kishinevsky et al. 1988). Leguminous plants release tryptophan into the soil, which is absorbed by *Rhizobium* and is metabolized to produce IAA (indole acetic acid).

The first random nonspecific interaction between the symbiotic partners is mediated by the bacterial microfibrili, rhicadhesin (14 kD, calcium binding protein), on individual plant cells and root hair tips (Smith et al. 1992). This is followed by more specific attachment in which multivalent lectins are involved and the attachment of the rhizobia becomes more secured with the aid of extracellular bacterial microfibrili(Dazzo and Wopereis 2000). Initial plant physiological responses to attachment of rhizobia (Felle et al. 1998) are:

1. Calcium influx (rapid intake of calcium causes a cascade of signal transduction that leads to the induction of nodulin genes)
2. Potassium/chloride efflux

In addition to plant lectins and calcium, the other four types of polysaccharides involved in the attachment process are:

1. Extracellular polysaccharides (EPS)
2. Capsular polysaccharides (CPS)
3. Lipopolysaccharides (LPS)
4. β-2 glucans

EPS and LPS are heteropolymers, which show species-specific variation and may react with lectins (Sprent 1989). The function of the CPS is that it mediates polar attachment to the legume host root hairs (Halverson and Stacey 1986).

As the bacteria attach to the cell wall of the root hair, a series of morphological and physiological changes occur in the root hair. Inhibition of cell expansion on one side of the root hair causes it to curl back on itself (root hair curl or "Shepard's crook") with the rhizobia attached to the inside surface of the curled root hair wall (Dazzo and Hubbell 1982). It is well established that lipo-chitin nodulation signals, produced by the bacterium, initiate these changes in the root hair cell. However, not

all the root hairs which get infected show curling (root curling factor). It is therefore not a necessary prerequisite for infection.

The root hair is covered with a mucilaginous substance in which the bacterium first becomes embedded. The cell wall of the root hair dissolves at the point of bacterial attachment, probably because of the secretion of enzymes like pectinases and polygalactouronases by the bacteria. Such dissolution of the root hair cell wall facilitates the entry of the bacterium into the root hair cell. This is the normal and common mode of infection. At times for instance in peanut, the bacteria may infect the root at the point of emergence of lateral root.

#### 1.3.3.3 Infection Thread Formation

The area on the plant cell wall where the bacteria are attached begins to grow inwards (invaginates), which forms a growing tube (infection thread) made of plant cell wall material and membrane with the rhizobia inside the tube (Dazzo and Wopereis 2000). When once the bacterium enters the root hair, certain structural and physiological changes occur in the root hair cell. Cytoplasmic streaming and respiration increase. The nucleus also increases in size. A new wall is laid down around the bacterium so that it is separated from the contents of the surrounding host cell. The new cell wall extends like a tube (infection thread), and the bacterium inside the tube starts dividing (Fig. 1.3). The infection thread further extends from the root hair cell toward the underlying cortical cells of the root (Devlin and Witham 1986).

The cells of the root cortex develop into large cells, each with an enlarged central nucleus. The infection thread intrudes, settles, and liberates its contents in cortical cells which are always polyploid (tetraploid) (Brewin 1991).

Bacterial cell surface polysaccharides appear to play a crucial role in infection thread growth. The bacteria divide as they move down the infection thread, either by gliding motility (since the bacterium does not have flagella inside the infection

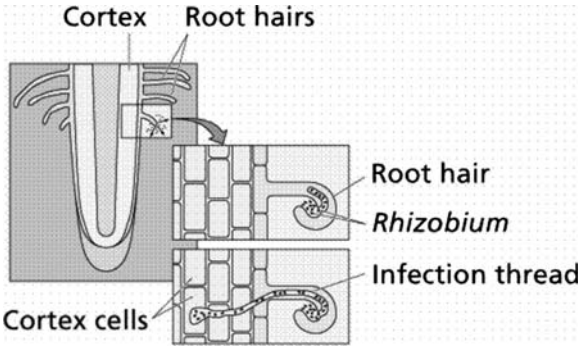


**Fig. 1.3** Infection thread formation on establishment of the mutualistic relationship between rhizobia and legume

thread) or by being somehow connected to the reoriented microtubule cytoskeleton. The infection thread eventually reaches the developing nodule and delivers the rhizobia into the appropriate nodule cell via endocytosis. Bacterial mutants defective in exopolysaccharide or lipopolysaccharide synthesis form infection threads that are blocked or fail to penetrate fully into the root. Similar mutants may also lead to defects in bacterial release from the infection thread (Becker et al. 2000).

#### 1.3.3.4 Nodule Formation

At the start of root hair cell deformation, root cortical cells begin to divide and differentiate into a nodule primordium. By the time the infection thread reaches the root cortex, the nodule has formed and the cells are ready to receive the rhizobial cells.

#### 1.3.3.5 Infection Thread Branching and Delivery of Rhizobia to the Nodule

As the infection thread (tube) gets near the cortical cells (nodules), it forms various side tubes (branches), each of which eventually comes to a stop at the cell wall of a nodule cell. At the point of contact, the cell wall dissolves and the rhizobia (10–100 bacterial cells) are delivered into the nodule cell by endocytosis.

#### 1.3.3.6 Bacteroids and Symbiosomes

Each rhizobial cell becomes enclosed in host plant cell membrane to form a vacuole-like structure, which is called a symbiosome. The rhizobial cell differentiates into a bacteroid capable of nitrogen fixation. The symbiosome is a quasiorganelle specialized for symbiotic nitrogen fixation.

#### 1.3.3.7 Nodule Organogenesis

As the nodule forms, the vascular cells of the plant extend into the nodule to form vascular tissue through which the two partners exchange nutrients and fixed nitrogen.

As the root hair initiates deformation, the cells of the inner cortex, mostly opposite the protoxylem elements, are stimulated by Nod signals to divide and proliferate, resulting in the development of the nodule. As more cells in the vicinity re-enter the cell division cycle, a nodule meristem develops which eventually becomes the nodule primordium.

The cells of the nodule are tetraploid, while the surrounding cortical cells are diploid. It is these tetraploid cells which become infected with bacteroids. There are two views regarding the formation of polyploid cells:

1. Such polyploidy cells may be already present in the root along with the diploid cell. However, with the infection by rhizobia these polyploid cells divide and form the nodule.
2. The other view is that upon infection by rhizobia, probably because of the secretion of some hormones, endomitosis occurs and the cells become polyploidy.

Usually the nodules originate opposite the protoxylem elements in the root. In this respect, these resemble the lateral roots. However, ontogenetically, lateral roots and nodules are quite different. While the roots originate in the pericycle, the nodules develop from the cells of the inner cortex. Therefore, the nodules are not analogous to lateral roots.

The nodules of different legumes differ in their anatomy and shape. As the nodule meristem cells divide, some arrest their cell division and begin to differentiate, leading to the zones of specialized cells and peripheral tissues. Depending on the host plant, the nodule can develop as the determinate or indeterminate type (Table 1.1).

Within the host cell cytoplasm, the bacteria become surrounded by the plasmalemma. This means that the bacterium is separated from the cytoplasm of the host cell. This plasmalemma surrounding the bacteria is known as peribacteroid membrane (symbiosome membrane), which forms a small vesicle called a symbiosome. The symbiosome is the active unit of nitrogen fixation. The bacteria divide and each bacterium in turn is surrounded by a separate peribacteroid membrane. Often within the same peribacteroid membrane there may be more than one bacterium, i.e., several bacteria may be surrounded by a common peribacteroid membrane.

After release, the bacteria stop dividing. Usually they enlarge in size and become pea-shaped. Such transformed bacteria present within the host cell are called bacteroids. Plant-derived environmental and/or bacterial-produced chemical signals are likely to be involved in triggering this differentiation process.

**Table 1.1** Difference between determinate and indeterminate nodule

| Determinate nodule | Indeterminate nodule |
|---|---|
| Does not possess a meristem and hence is capable of only limited growth | Presence of a meristem facilitates continuous growth of the nodule |
| Cells of the cortex divide only after being infected by bacteria | The bacteria are released only after the cell has ceased to divide |
| Nodules are spherical | Nodules are elongated and branched |
| In the nodules, either the infection threads are not formed or, if formed, they do not branch extensively. Consequently, spread of infection is by the division of infected cell itself. The vasculature of the nodule fuses at the tip | The vasculature of the nodule remains open at the tip |
| e.g., soybean, French bean, mungbean | e.g., pea |

Morphological changes in bacteria as they become bacteroids (Vassa et al. 1990):

- Type I — when rhizobia are released into the nodule cell, they still resemble the free-living bacteria without the flagella.
- Type II — become more elongated, and their nucleoid region is not discernable. Bacteria stop dividing. Their DNA continues to divide, hence they have a few more copies of DNA than the free-living bacteria.
- Type III — finish elongating and begin the transcription of nitrogen fixation (nif and fix) genes.
- Type IV — competent to begin nitrogen fixation as soon as the nodule becomes mature and a microaerobic environment is produced.
- Type V — begin to vary in morphology, show progressive changes in their cytoplasmic content, decrease in numbers and stop their nitrogen-fixing ability.
- Certain biochemical changes are also initiated both in the bacterium and in the host cell. Now the host cell starts producing the red-pigmented protein called leghemoglobin for which the red pigment, heme is supplied by the bacterium while the globulin part is synthesized by the legume host. Some new cytochromes, viz. cytochrome P-552 and P-420, which are not present in the free-living rhizobia appear in the bacteroids. The nif genes become derepressed, resulting in the synthesis of the enzyme nitrogenase. At this stage, the nodule becomes fully symbiotic, receiving the supply of carbohydrates from the host and exporting combined nitrogen in turn to the host.

When once the host cell becomes filled with bacteroids, mitochondria and other cell organelles of the host are pushed to the periphery of the cell. Usually it takes about 2 weeks after inoculation for the functional nodules to appear as in *Trifolium*.

#### 1.3.3.8 Leghemoglobin Production

Very early in the establishment of this relationship, plant cells begin to produce the oxygen-binding protein, leghemoglobin, which helps in producing the microaerobic environment necessary for nitrogenase activity. This leghemoglobin found in the root nodules of leguminous plants is a red pigment similar to that of hemoglobin of red blood corpuscles. Leghemoglobin is considered to be a product of the *Rhizobium*–legume complex. It is synthesized on plant genome, located in the peribacteroid membrane and serves the purpose of oxygen carrier in the nodule cell. Although correlation has been found between the concentration of leghemoglobin and the rate of nitrogen fixation, the pigment does not play a direct role in nitrogen fixation. It protects the nitrogenase inside the bacteroid from detrimental effect of $O_2$. It maintains adequate supply of $O_2$ in the bacteroid membrane, so that through respiration ATPs continue to be regenerated which are required for nitrogen fixation.

#### 1.3.3.9 Nitrogenase Production

Once the conditions are appropriate for nitrogen fixation, the bacteroids produce nitrogenase enzyme, the enzyme that converts $N_2$ to $NH_3$. The enzyme nitrogenase is reported to be present in all the nitrogenfixing organisms, viz. bacteroids, heterocysts of cyanophyceae and vesicles of *Frankia*.

#### 1.3.3.10 Senescence of Nodule and Release of Rhizobia

The ageing (senescence) process of the nodule is genetically programmed, and leads to the release of the bacteroids and loss of nodules.

## 1.4 Symbiosis of Actinomycetes

Actinomycetes are gram-positive bacteria that can form branching filaments. They may form true mycelia or produce conidiospores. *Frankia* is one of the representative genera of Actinomycetes.

Characteristics of *Frankia* are as follows:

1. It is a type IIID cell wall type.
2. It forms nonmotile sporangiospores in sporogeneous body.
3. It grows in symbiotic association with the roots of at least eight families of higher nonleguminous plants (e.g., alder trees).
4. It is a microaerophile, able to fix nitrogen.

The roots of the infected plants develop nodules that fix nitrogen so efficiently that a plant such as an alder can grow in the absence of combined nitrogen when nodulated. Within the nodule cells, *Frankia* forms branching hyphae with globular vesicles at their ends. These vesicles may be the sites of nitrogen fixation. The nitrogen fixation process resembles that of *Rhizobium*, in that it is oxygen-sensitive and requires molybdenum and cobalt. Some plants (*Alnus*, *Ceanothus*) have nodules as large as baseballs. The nodules of *Casuarina* approach soccer-ball size (Prescott et al. 1996).

The actinomycetes infect the root system and forms nodules in shrubs and trees belonging to eight dicot families except Leguminosae (Table 1.2). However, this is not of much economic significance, as none of the crop plants is infected with *Frankia*. The development of *Frankia* and the mechanism of nitrogen fixation is less well-understood than legume–rhizobium symbiosis. *Casurina, Eleagnus,* and *Alnus* are some of the important genera which form *Frankia* nodules. Such nodules are long-lived when compared with rhizobium nodules of legumes. *Frankia* is a free filamentous form which can also be cultured on nutrient media. The tip of the filament is enlarged, forming the vesicle which contains the enzyme nitrogenase.

**Table 1.2** Nonleguminous nodule bearing plants with *Frankia* symbioses (Prescott et al. 1996)

| Family | Genus |
|---|---|
| Casuarinaceae | *Allocasuarina* |
| | *Casuarina* |
| | *Ceuthostoma* |
| | *Gymnostoma* |
| Coriariaceae | *Coriaria* |
| Datiscaceae | *Datisca* |
| Betulaceae | *Alnus* |
| Myricaceae | *Comptonia* |
| | *Myrica* |
| Elaeagnaceae | *Elaeagnus* |
| | *Hippophae* |
| | *Shepherdia* |
| Rhamnaceae | *Ceanothus* |
| | *Colletia* |
| | *Discaria* |
| | *Kentrothamnus* |
| | *Retanilla* |
| | *Trevoa* |
| Rosaceae | *Cercocarpus* |
| | *Chaemabatia* |
| | *Cowania* |
| | *Dryas* |
| | *Purshia* |

This species can fix nitrogen, both symbiotically with the host plant and also independently when cultured *in vitro*. The host–symbiont specificity in *Frankia* is broad. The same organisms can often nodulate in a wide spectrum of species.

## 1.5 Symbiosis Between Blue–Green Alga and Fungus: *Geosiphon pyriforme*

*Geosiphon pyriforme* v. Wettstein is a coenocytic soil fungus, and till now the only known example of a fungus living in endocytobiotic association with a cyanobacterium, i.e., with *Nostoc punctiforme*. F.V. Wettstein described it as a symbiosis between a heterotrophic siphonal chlorophyceaen alga and *Nostoc*. The fungus lives together with the cyanobacterium on the surface and in the upper layer of wet soils poor in inorganic nutrients, particularly in phosphate. When a fungal hypha comes into contact with free-living *Nostoc* cells, the latter are incorporated by the fungus at the hyphal tip, which thereafter swells and forms a unicellular “bladder”,

about 1–2 mm in size and appearing on the soil surface. Inside this bladder, the cyanobacteria are physiologically active and dividing (Schubler and Kluge 2001). It has been suggested that fungus could provide an important model system for another symbiosis, the arbuscular mycorrhiza, see Fig. 1.4. Significant differences between *G. pyriforme* and lichen are tabulated in Table 1.3.

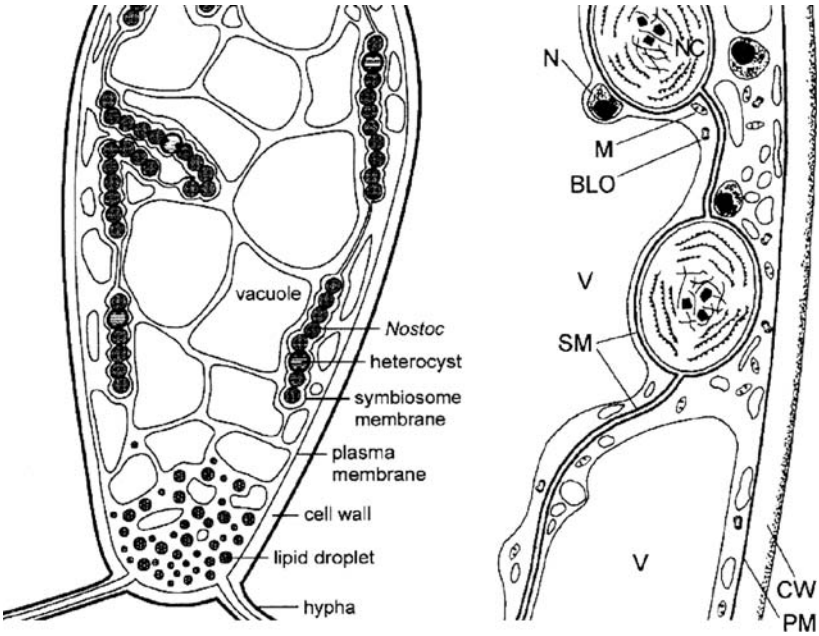


**Fig. 1.4** Schematic drawings of *Geosiphon* bladder compartmentation. Overview (*left*) and detail (*right*). *BLO* bacteria like organism, *M* Mitochondrium, *CW* cell wall, *N* nucleus, *NC Nostoc* cell, *PM* plasma membrane, *SM* symbiosome membrane, *V* vacuole (cf. Schubler and Kluge 2001)

**Table 1.3** Difference between *Geosiphon* and lichen (Kluge and Schubler 2002)

| *Geosiphon* | Lichen |
|---|---|
| It represents an endocytobiotic consortium, with the photobiont living inside the fungal cell | There is contact between the two partners (mycosymbiont and phycosymbioant) in lichens |
| Mechanism of vegetative propagation of the entire symbiotic system of *Geosiphon* does not exist | Vegetative reproduction through soredia and isidia by spreading both symbiotic partners together |
| *Geosiphon* does not survive water loss | Lichens are robust toward dehydration |
| *Geosiphon* is very sensitive toward high temperature | Many lichens are resistant to high temperatures |
| *Geosiphon* grows only in moderate light | It can tolerant high light irradiance |

## 1.6 Symbiosis Between Algae and Fungi: Lichens

Lichen is an association between an alga and a fungus in which the two organisms jointly form a thallus that is distinct from the either partners. The fungal partner is known as the mycobiont and the algal partner as the phycobiont. Both the partners are benefited by this symbiotic association. The fungus derives nutrition from the alga, which in turn is protected by the fungus (Backora et al. 2006). Lichens take the external shape of the fungal partner, and hence are named based on the fungus. The fungus most commonly forms the majority of the lichen's bulk, though in filamentous and gelatinous lichens this may not always be the case. The lichen fungus is typically a member of the Ascomycota (termed ascolichens), and rarely a member of the Basidiomycota (termed basidiolichens). Neither the ascolichens nor the basidiolichens form monophyletic lineages in their respective fungal phyla, but they do form several major solely or primarily lichen-forming groups within each phylum (Lutzoni et al. 2004).

The algal partner in lichens may be members of blue–green algae (*Anabaena*, *Nostoc*), green algae or yellow–green algae. In lichen associations, the blue–green alga performs the dual function of both photosynthesis and nitrogen fixation. However, if the lichen contains an additional partner, namely a green alga, then the blue–green alga becomes relieved of its function of photosynthesis and is geared to fix nitrogen exclusively. This is evidenced by the fact that in the presence of green alga, in a three-member association, the blue–green alga produces more heterocysts, which are the sites of nitrogen fixation.

Lichens are extremely slow-growing, showing about 1 mm growth per year, but are highly tolerant to xeric conditions such as desiccation and heat. Consequently, one can find them growing on tree trunks and rocks. However, these are very sensitive to atmospheric pollutants. As a result, they are rarely seen near human habitations where automobile fumes and factory effluents prevail.

## 1.7 Symbiosis in Bryophytes

Liverworts such as *Anthoceros*, *Notothylas* and *Blassia* possess blue–green algae as symbiotic partners. Here, the endophyte is cyanobacterium *Nostoc*. In liverworts there are mucilage cavities on the ventral side of the thallus. This is due to the breakdown of the cells and their replacement by mucilage. With the maturity of the thallus, the mucilage of these cavities dries out, resulting in an air-filled chamber. Such cavities become invaded with *Nostoc,* which produces more heterocysts than in the free-living condition, thereby indicating that these are quite active in nitrogen fixation. Secondly, within the cavity, *Anthoceros* produces branched hypha-like filaments which become interspersed with algal colony. Such filaments are also warted. As a result, the contact of the alga with the tissues of *Anthoceros* becomes increased, and facilitates transport and exchange of materials between the two symbiotic partners. While photosynthate is transported to *Nostoc* by bryophytes,

fixed nitrogen is transferred in the reverse sequence from *Nostoc* to bryophytes (Rashid 1998).

## 1.8 Symbiosis in Pteridophytes

*Azolla*, a floating fern, floats on the surface of water by means of numerous, small, closely-overlapping scale-like leaves, with their roots hanging in the water. When a section of *Azolla* is examined under a microscope, filaments of *Anabaena* living within ovoid cavities inside the leaves *Azolla* can be seen. This association of *Azolla* with the filaments of the blue–green alga *Anabaena* forms a symbiotic relationship, which fixes atmospheric nitrogen, giving the plant access to the essential nutrient (Peters 1977).

When free-living, the alga develops only 5–10% of its cells into heterocysts. However, the number of heterocysts increases to 25–30% when it lives symbiotically in association with *Azolla*. The epidermal cells inside the leaf cavity of *Azolla* project inside, to form numerous multicellular hairs with warty outgrowths. These probably serve as transfer cells for free exchange of nutrients between *Azolla* and *Anabaena*. *Azolla* can absorb nitrates from the water; it can also absorb ammonia secreted by *Anabaena* within the leaf cavities (http://waynesword.palomar.edu/plnov98.htm).

Since *Azolla* is easy to maintain in aquarium cultures, it is an excellent source of prokaryotic cells and heterocysts for general biology laboratory exercises on cell structure and function. It also has an interesting heterosporous life cycle, and can readily be adapted to laboratory exercises on symbiosis. In addition, this little fern and its algal partner provide an important contribution toward the production of rice for a hungry world.

A heterocyst is a differentiated cyanobacterial cell that carries out nitrogen fixation. The heterocysts function as the sites for nitrogen fixation under aerobic conditions. They are formed in response to a lack of fixed nitrogen ($NH_4^+$ or $NO_3^-$). The morphological differentiation is accompanied by biochemical alterations. The mature heterocysts contain no functional photosystem II, and cannot produce oxygen. Instead, they contain only photosystem I, which enables them to carry out cyclic photophosphorylation and ATP regeneration. These changes provide the appropriate conditions for the functioning of the oxygen-sensitive nitrogenase.

*Azolla* is grown in rice fields and ploughed as green manure, and this can supply up to 50–75% of the nitrogen requirement of the crop. This practice is extensively employed in China and Japan as a source of biologically fixed nitrogen in rice cultivation.

## 1.9 Symbiosis in Gymnosperms

*Cycas* is a gymnosperm, and it belongs to Cycadales. *Cycas* produces two types of roots. The normal roots grow downwards in the soil, and some of the branches of lateral roots come just above the soil and become aerial. These roots are

apogeotropic roots that grow toward the soil surface and repeatedly branch dichotomously. These are known as coralloid roots. The coralloid roots become infected with bacteria and blue–green algae like *Anabaena* and *Nostoc*. The endophyte is found in a single layer in the intercellular spaces between the outer cortex and inner cortex of the root.

### *1.9.1 Coralloid Roots of Cycas*

A coralloid root is formed at the surface of the soil. Bacteria of the soil enter and rapidly multiply in the cells of the apical portion. As a result, the cells disorganize and the intercellular spaces are also considerably enlarged. Then, algae such as *Anabaena* and *Nostoc* gain entry into the disorganized zone of the cortex, through apertures formed by the bacteroid infection. These algae are found in the midcortical zone as a conspicuous ring. This root, which is thus invaded by the algae, becomes negatively geotropic, and comes just above the surface or remains a little below the surface of the soil. They are dichotomously branched in a profuse manner, and form the so-called "root tubercles". Superficially, they form coralloid masses, and hence they are known as coralloid roots (Sambamurty 2005). Coralloid roots are only known to occur in cycads, and in turn cycads are the only known gymnosperms to form symbiotic relationships with nitrogen-fixing organisms (http://www.livingdesert.org).

## 1.10 Symbiosis in Angiosperms

The roots of almost all higher plants are known to form mutualistic symbioses with fungi. These latter are termed mycorrhizas. As well as with fungus, higher plants have symbiotic associations with blue–green algae, bacteria, and actinomycetes. Amongst the angiosperms it is only *Gunnera*, belonging to the family Haloragaceae, which is found to have symbiosis with *Nostoc*. The endophyte is situated in the glands situated on the stem at the point of attachment of the petiole. *Nostoc* filaments enter the cells of the gland (intracellular) and produce a large number of heterocysts. Here also, *Nostoc* is heterotrophic, dependent upon the host cells for the carbohydrate supply, as not much photosynthesis is possible in the absence of light in the intracellular environment of host cells.

### *1.10.1 Mycorrhizal Symbiosis*

Mycorrhizae are symbiotic associations that form between the roots of most plant species and fungi. The term mycorrhizas (fungus roots, from the Greek: mykes = mushroom or fungus and rhiza = root).

Mycorrhizae can also be defined as "a mutualistic symbiosis between plant and fungus, localised in a root or root-like structure in which energy moves primarily from plant to fungus and inorganic resources move from fungus to plant".

These symbioses are characterized by bi-directional movement of nutrients, where carbon flows to the fungus and inorganic nutrients move to the plant, thereby providing a critical linkage between the plant root and soil. In infertile soils, nutrients taken up by the mycorrhizal fungi can lead to improved plant growth and reproduction. As a result, mycorrhizal plants are often more competitive and better able to tolerate environmental stresses than are nonmycorrhizal plants (Fig. 1.5).

The term mycorrhiza, which literally means "fungus–root", was first applied to fungus–tree associations described in 1885 by the German forest pathologist A.B. Frank. The vast majority of land plants form symbiotic associations with fungi: an estimated 95% of all plant species belong to diverse genera that characteristically form mycorrhizae.

Depending on the environment in which they are growing, plants may divert up to 80% or more of the net energy fixed as sunlight to below-ground processes. Some of this energy goes into root growth; but a high proportion may be used to feed mycorrhizal fungi and other soil organisms. This is not energy that is lost to the plant. On the contrary, soil organisms living in the root zone greatly influence the ability of plants to establish themselves, through effects on nutrient cycling, pathogens, soil aeration, and soil water uptake. Of the various soil organisms that benefit plant establishment, the most is known about mycorrhizal fungi (http://www.fungi.com/mycogrow/amaranthus.html).

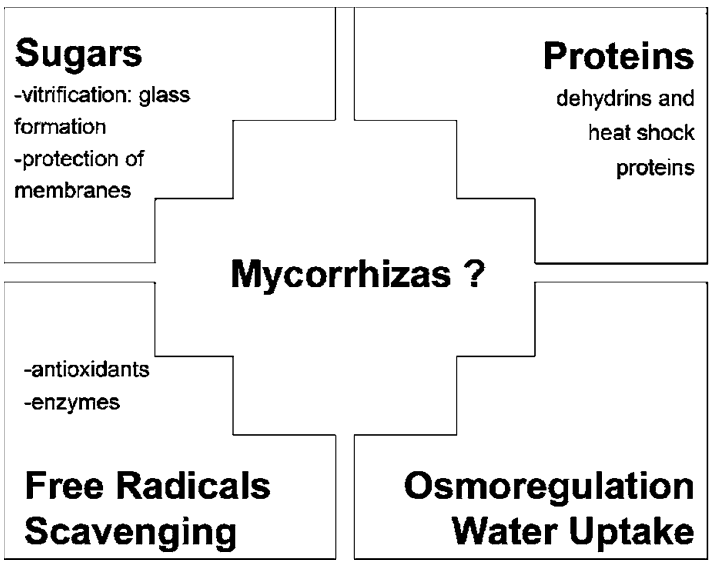


**Fig. 1.5** Mycorrhiza are considered to play vital role in overcoming drought stress

#### 1.10.1.1 Types of Mycorrhizal Fungi

So far seven types of mycorrhizae have come into general use over the years, on the basis of morphology and anatomy but also of either host-plant taxonomy or fungal taxonomy (Srivastava et al. 1996; Smith and Read 1997). These are: ectomycorrhiza, endomycorrhiza or arbuscular mycorrhiza, ericoid mycorrhiza, arbutoid mycorrhiza, monotropoid mycorrhiza, ect-endomycorrhiza, and orchidaceous mycorrhiza.

Ectomycorrhiza

Hyphae surround but do not penetrate the root cells. Ectomycorrhizae are commonly found in trees growing in temperate regions. The plant symbionts include both Gymnosperms and Angiosperms. Some have been found in the tropics also. The willow family (Salicaceae), birch family (Betulaceae), beech family (Fagaceae) and pine family (Pinaceae) have ectomycorrhizal associations. This possibly makes the trees more resistant to cold, dry conditions.

The hyphae grow in between the cortical and epidermal cells of the root, forming a network called the "Hartig net". A mantle of hyphae covers the root surface, and mycelium extends from the mantle into the soil. It provides a large surface area for the interchange of nutrients between host and the fungi. Most ectomycorrhizal fungi are basidiomycetes, but ascomycetes are also involved.

Arbuscular Mycorrhiza

The term refers to the presence of intracellular structures — vesicles and arbuscules — that form in the root during various phases of development. These mycorrhizae are the most commonly recorded group, since they occur on a vast taxonomic range of plants, both herbaceous and woody species. The plant symbiont ranges from Bryophytes to Angiosperms. Aseptate hyphae enter the root cortical cells and form characteristic vesicles and arbuscules. The plasmalemma of the host cell invaginates and encloses the arbuscules. Arbuscular mycorrhizal (AM) fungi belong to nine genera: *Gigaspora*, *Scutellospora*, *Glomus*, *Acaulospora*, *Entrophospora*, *Archaeospora*, *Gerdemannia*, *Paraglomus* and *Geosiphon*, the only known fungal endosymbiosis with cyanobacteria (Fig. 1.6).

Ericoid Mycorrhiza

In the Ericaceae, the heather family, the ectomycorrhizal hyphae form a web surrounding the roots. The ericoid mycorrhizae are endomycorrhizae in the general sense, since the fungal symbiont penetrates and establishes into the cortical cells.

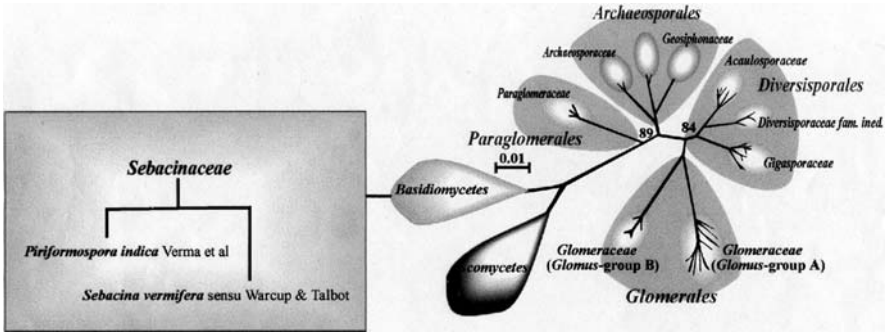


**Fig. 1.6** Diagramatic representation of molecular taxonomic position of symbiotic fungi (cf. Das et al. 2007)

Infection of each cortical cell takes place from the outer cortical wall; lateral spread from cell to cell does not occur. Infected cells appear to be fully packed with fungal hyphae. In the ericoid mycorrhizae, the host cell dies as the association disintegrates, thereby restricting the functional life (i.e., nutrient absorption) of these epidermal cells to the period prior to breakdown of the infected cell.

### Arbutoid Mycorrhiza

The arbutoid mycorrhizae have characteristics which are found in both ECM and other endomycorrhizae. Intracellular penetration of cortical cells and formation of a sheath can occur, and a "Hartig's net" is present. A feature distinguishing them from ericoid mycorrhizae is the presence of dolipore septum in internal hyphae. Fungal associates in arbutoid mycorrhizae belong to basidiomycetes.

### Monotropoid Mycorrhiza

This group of mycorrhiza is associated with the achlorophyllous plants in the family Monotropaceae. These mycorrhizae are very similar to the ECM, and form a distinct sheath and "Hartig's net". However, they exhibit a distinctive type of intracellular penetration in cortical cells that is unlike other endomycorrhizal types. The fungus forms a peg, into the cell wall.

### Ect-endomycorrhiza

These are formed with the members of the Pinaceae. These mycorrhizae form a "Hartig's net" in the cortex of the root, but develop little or no sheath. Intracellular

penetration of cortical cells takes place, and thus they are similar to the arbutoid type. Ect-endomycorrhizae in Pinaceae seem to be limited to forest nurseries, and are formed by a group of fungi called E-strain. These fungi are most likely to be the imperfect stage of ascomycetes; they may cause ect-endomycorrhizae in some tree species, and ECM in other tree species.

Orchidaceous Mycorrhiza

The fungal association is of the endomycorrhizal type, where the fungus penetrates the cell wall and invaginates the plasmalemma and forms hyphal coils within the cell. Once the plant is invaded, spread of the fungus may occur from cell to cell internally. The internal hyphae eventually collapse and are digested by the host cell. Since the symbiosis forms an external network of hyphae, it would seem probable that the fungal hyphae function in nutrient uptake as with other mycorrhizae, and that the coarse root system of orchids would be supplemented by the increased absorbing surface area of the hyphae (Smith and Read 1997). A number of basidiomycete genera have been shown to be involved in the symbiosis, although many reports on isolation of the symbiotic fungus from the roots of orchids have placed the symbionts in the form genus *Rhizoctonia* when the perfect stage was not known or the isolate was not induced to fruit in culture. Orchid seed germinates only in the presence of suitable fungus.

The closest relatives of *Piriformospora indica* are members of *Rhizoctina* group (Ceratobasidales). Preliminary studies indicated that *P. indica* was able to interact with orchids (*Dactylorhiza purpurella* and *D. majalis*) as a mycorrhizal partner. In this respect, more scientific studies are needed to be performed to quantify the promoting effect, and to study the interacting structures formed during the early stages of plant development.

#### 1.10.1.2 How Do Mycorrhizae Work?

Mycorrhizal root systems increase the absorptive area of roots 10–1,000 times, thereby greatly improving the ability of the plants to utilize the soil resource. Mycorrhizal fungi are able to absorb and transfer all of the 15 major macro- and micronutrients necessary for plant growth. Mycorrhizal fungi release powerful chemicals into the soil that dissolve hard to capture nutrients such as phosphorous, iron, and other "tightly bound" soil nutrients (Fig. 1.7). This extraction process is particularly important in plant nutrition, and explains why non-mycorrhizal plants require high levels of fertility to maintain their health. Mycorrhizal fungi form a complex web that captures and assimilates nutrients, conserving the nutrient capital in soils. In non-mycorrhizal conditions, much of this fertility is wasted or lost from the system.

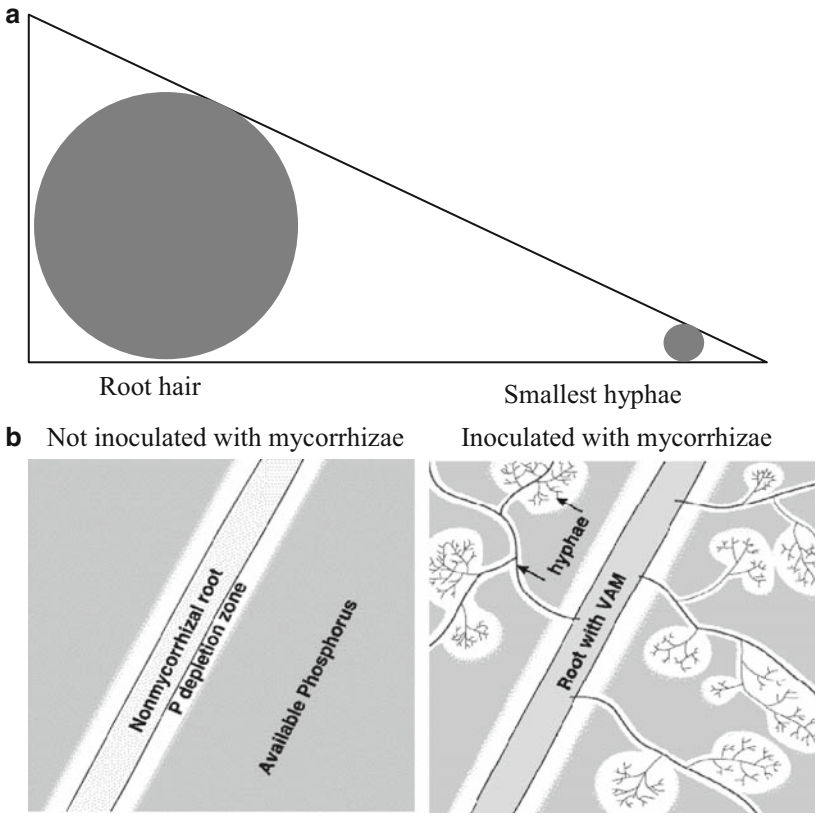


**Fig. 1.7** (**a**) Comparison of size of diameter of a fungal hypae with a root hair. (**b**) Difference between nonmycorrhizal and mycorrhizal root. Formation of arbuscules in roots inoculated with arbuscular mycorrhiza which facilitates the absorbtion of soil nutrients

#### 1.10.1.3 Interactions with Other Soil Organisms

Mycorrhizal fungi interact with a wide assortment of organisms in the rhizosphere. The result can be positive, neutral, or negative on the mycorrhizal association or a particular component of the rhizosphere. For example, specific bacteria stimulate EM formation in conifer nurseries, and are called mycorrhization helper bacteria. In certain cases, these bacteria eliminate the need for soil fumigation (Garbaye 1994).

The interaction between rhizobia and arbuscular mycorrhiza (AM) fungi has received considerable attention because of the relatively high phosphorus demand of nitrogen fixation. The two symbioses typically act synergistically, resulting in greater nitrogen and phosphorus content in combination than when each is inoculated onto the legume alone. Legumes are typically coarse-rooted and therefore inefficient in extracting phosphorus from the soil. The AM fungi associated

with legumes are an essential link for adequate phosphorus nutrition, leading to enhanced nitrogenase activity that in turn promotes root and mycorrhizal growth.

Mycorrhizal fungi colonize feeder roots, and thereby interact with root pathogens that parasitize this same tissue. In a natural ecosystem where the uptake of phosphorus is low, a major role of mycorrhizal fungi may be protection of the root system from endemic pathogens such as *Fusarium* spp. Mycorrhizae may stimulate root colonization by selected biocontrol agents, but our understanding of these interactions is meager. Much more research has been conducted on the potential effects of mycorrhizal colonization on root pathogens. Mycorrhizal fungi may reduce the incidence and severity of root diseases. The mechanisms proposed to explain this protective effect include: (1) development of a mechanical barrier — especially the mantle of the EM — to infection by pathogens, (2) production of antibiotic compounds that suppress the pathogen, (3) competition for nutrients with the pathogen, including production of siderophores, and (4) induction of generalized host defense mechanisms.

#### 1.10.1.4 Novel Symbiosis: *Piriformospora indica* with Higher Plants

Mycorrhizas, which are well-known for their exchange of nutrients and water between the partners in the symbiotic association, was discovered way back in 1898. However, despite their important role in agriculture and other related areas, their cultivation on culture media has not been possible to date. This restricts the exploitation of the biotechnological applications of this mycorrhiza, as they can not be grown in the absence of a living plant.

*Piriformospora indica* (Hymenomycetes, Basidiomycota) is the only cultivable endophyte that colonizes roots. This mycorrhizal fungus can grow on an artificial medium. *P. indica* can be multiplied in mass scale on cheap and industrially produced simplified nutrient medium. Inoculation with the fungus and application of fungal culture filtrate promotes plant growth and biomass production. Due to its ease of culture, this fungus provides a model organism for the study of beneficial plant–microbe interactions and a new tool for improving plant production systems (Varma et al. 1998; Varma et al. 1999; Varma et al. 2001).

*P. indica* vastly improves the growth and overall biomass production of diverse hosts, including legumes, medicinally and economically important. A pronounced growth-promotional effect has also been seen with terrestrial orchids. The medicinal plants which have been tested in laboratory conditions as well as in the extensive field trial are *Bacopa moniera* (Sahay and Varma 1999, 2000), *Azadirachta indica*, (Singh et al. 2002, 2003), *Withania somnifera*, *Spilanthes calva* (Rai et al. 2001), *Adhatoda vasica* (Rai and Varma 2005), and *Chlorophytum borivilianum* (Mathur et al. 2008). Rai et al. (2001) reported growth increase in *Withania somnifera* and *Spilanths calva* when they were interacted with the fungus *P. indica*. The fungus also provides protection when inoculated into tissue culture-raised plantlets, by overcoming the "transient transplant shock" on transfer to the field, leading to almost 100% survival (Mathur et al. 2008). The fungus forms inter- and

intracellular hyphae in the root cortex, often differentiating into dense hyphal coils (arbuscule-like structures), spore and vesicle-like structures. As with AM fungi, hyphae multiply within the host cortical tissues and never traverse through the endodermis. Likewise, they also do not invade the aerial portion of the plant (stem and leaves).

*P. indica* is a potential candidate to serve as biofertilizer, bioprotector, bioregulator, bioherbicide/weedicide, and to combat environmental stresses (chemical, thermal, and physical). The fungus also preserves soil fertility, and improves plant health as well. Similarly to arbuscular mycorrhizal fungi, *P. indica* stimulates nitrate assimilation in the roots and solubilizes insoluble phosphatic components in the soil. Recent experiments have amply demonstrated that *P. indica* provides resistance against heavy-metal contamination in the soil. *Piriformospora indica*, promotes growth of *Arabidopsis* and tobacco seedlings, and stimulates nitrogen accumulation and the expression of the genes for nitrate reductase and the starch-degrading enzyme glucan–water dikinase (*SEX1*) in roots (Sherameti et al. 2005).

*P. indica* is been reported to induce resistance to fungal diseases in the monocotyledonous plant barley, along with tolerance to salt stress. The beneficial effect on the defense status is detected in distal leaves demonstrating a systemic induction of resistance by a root-endophytic fungus. The systemically altered "defense readiness" is associated with an elevated antioxidative capacity due to an activation of the glutathione–ascorbate cycle and an overall increase in grain yield. Since *P. indica* can be easily propagated in the absence of a host plant, we conclude that the fungus could be exploited to increase disease resistance as well as yield in crop plants (Waller et al. 2005). The axenically cultivable root endophyte *Piriformospora indica* has also been described as a model organism to be used as a potential for biocontrol strategies. It is able to increase biomass and grain yield of crop plants. In barley, the endophyte induces local and systemic resistance to fungal diseases and to abiotic stress. To elucidate the lifestyle of *P. indica*, scientists have analyzed its symbiotic interaction and endophytic development in barley roots. It was found that fungal colonization increases with root tissue maturation. The root tip meristem showed no colonization, and the elongation zone showed mainly intercellular colonization. In contrast, the differentiation zone was heavily infested by inter- and intracellular hyphae and intracellular chlamydospores. The majority of hyphae were present in dead rhizodermal and cortical cells that became completely filled with chlamydospores. In some cases, hyphae penetrated cells and built a meshwork around plasmolyzed protoplasts, suggesting that the fungus either actively kills cells or senses cells undergoing endogenous programmed cell death. Seven days after inoculation, expression of barley BAX inhibitor-1 (HvBI-1), a gene capable of inhibiting plant cell death, was attenuated. Consistently, fungal proliferation was strongly inhibited in transgenic barley overexpressing GFP-tagged HvBI-1, which shows that *P. indica* requires host cell death for proliferation in differentiated barley roots. It has been suggested that the endophyte interferes with the host cell death program to form a mutualistic interaction with plants (Deshmukh et al. 2006).

*Piriformospora indica*, a basidiomycete of the Sebacinaceae family, promotes the growth, development, and seed production of a variety of plant species too.

*Arabidopsis* plants colonized with the fungus produce 22% more seeds than uncolonized plants. Deactivating the *Arabidopsis* single-copy gene DMI-1, which encodes an ion carrier, required for mycorrhiza formation in legumes, does not affect the beneficial interaction between the two symbiotic partners. A leucine-rich repeat protein is required for growth promotion and enhanced seed production mediated by the endophytic fungus *Piriformospora indica* in Arabidopsis *thaliana* (Shahollari et al. 2007).

The fungus has great potential for application in agro-forestry, flori-horticulture, arboriculture, viticulture and especially for better establishment of tissue culture raised plants — much needed for the application in plant industry. This would open up numerous opportunities for the optimization of plant productivity in both managed and natural ecosystems, while minimizing risks of environmental damage.

## 1.11 Conclusions

In this chapter, an attempt has been made to give an overview of the symbiotic relationship between microorganisms and also between plants and microbes. In recent years, great progress has been made in understanding the genetic interplay involved in plant–microbe symbiosis as well as between different microbes. In both cases, mankind has been able to benefit from the fascinating symbiotic relationship. However, there are areas which need to be investigated to get a better perception of the symbiotic relationship before we get to enjoy the full benefits. The recent application of genomics in this field has been successful in discovering several aspects of the symbiotic association which could be useful in the near future. It is expected that discoveries in this field would lead to its eventual application for the improvement of agriculture and eradication of life-threatening diseases, as well as providing a positive solution to our environmental problems.

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# Chapter 2
# Analysis of Rhizosphere Fungal Communities Using rRNA and rDNA

**Ari Jumpponen**

## 2.1 Introduction

Soils and soil-borne microbial communities are probably the greatest source of organismal diversity on Earth. In this soil matrix, plant roots and the soil impacted by those roots – the rhizosphere – host a great diversity of fungi. Fungal community composition in soil and rhizosphere has been assessed by growing fungi from environmental samples in different pure culture media. These studies have provided some impressive and comprehensive volumes of fungi (Domsch et al. 1980; Rambelli et al. 1983). However, such culture-based studies may suffer from some serious limitations because many soil-borne organisms can be difficult to bring into pure culture, or because the diversity is shadowed by few fast-growing organisms that hamper detection of others. The application of molecular techniques has resulted in great improvements in our understanding of the rhizosphere fungal ecology and revolutionized the tools available for exploring environmental fungal communities (Horton and Bruns 2001). A majority of these fungal community studies use PCR-based tools that specifically target the fungal ribosomal RNA (rRNA)-encoding genes (rDNA) or the non-coding regions of the rRNA gene repeats. The rDNA is a convenient target as the fungal rDNA is arranged in tandem repeats in multiple copies that contain both coding regions for primary rRNAs and non-coding regions with various levels of sequence conservation.

The rRNA genes and their spacer regions are extremely useful for detecting and identifying the fungi in complex environmental samples. However, rDNA may persist in the environmental DNA pools for organisms that maintain no metabolic activity and are not necessarily participating in the ecosystem functions at the time of assessment. This results in a fundamental difficulty of interpreting rDNA assays: the environmental DNA pools can maintain a large component of organisms that

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were once active in the environment but which presently remain dormant and inactive community components or are present as residual naked DNA detectable via PCR-based approaches. Use of rRNA molecules extracted directly from the environment presents an exciting alternative which specifically targets those communities that are active at the time of sampling (Girvan et al. 2004). The underlying assumptions are that rRNA is less stable than rDNA in the environment and that the metabolically active organisms maintain higher numbers of rRNA, or transcribe and process the rRNA precursors at a higher rate (Anderson and Parkin 2007; Prosser 2002).

This chapter describes a relatively rapid, kit-based application of rRNA-based community analysis from *Andropogon gerardii* rhizosphere in a tallgrass prairie ecosystem. Although one should be careful in extrapolating rRNA-based community assessment into enumeration of active cells in the environment, rRNA assays do provide a different view of the community when compared to rDNA-based assays (Duineveld et al. 2001). In the effort described here, the extracted rRNAs were reverse-transcribed, PCR-amplified, cloned and sequenced to assay the rhizosphere community composition at one snapshot in this environment. To test congruence among the rRNA- and rDNA-inferred community compositions, rDNA was also extracted from the same samples and subjected to community analyses through PCR-amplicon sequencing. The primary hypotheses included: (1) rRNA serves as a convenient target for molecular fungal community analysis and (2) fungal communities inferred from rRNA data include taxa that are a subset of those observed in the fungal communities inferred from rDNA. A further inference from the latter hypothesis implies that the species richness in the rDNA-assayed communities is higher than that in the rRNA-assayed ones.

## 2.2 Materials

### *2.2.1 Equipment*

Liquid nitrogen vapor cryoshipper
FastPrep instrument
Microcentrifuge (refrigerated and nonrefrigerated)
Rotating shaker
Micropipettors
−80°C Ultrafreezer
Thermocycler
Micropipettors
Power supply
Horizontal gel electrophoresis apparatus
UV illuminator
NanoDrop Spectrophotometer
42°C Water bath
37°C Incubator with a shaker

## 2.2.2 Materials

FastRNA pro soil-direct kit
FastDNA spin kit for soil
Nuclease-free water
Thermoscript RT-PCR two-step system
Primer for reverse transcription (NS8)
RNAse inhibitor (RNaseOUT)
10× PCR buffer
25 mM $MgCl_2$
2 mM dNTPs
Forward (nu-SSU-0817-5′) and reverse (nu-SSU-1536-3′) fungus-specific primers
*Taq* polymerase
TOPO-TA cloning kit for sequencing with chemically competent *E. coli*
Luriani Broth medium with 60 μg $ml^{-1}$ ampicillin (LBA)
60% Glycerol
Liquid $N_2$

## 2.2.3 Procedure

### 2.2.3.1 Sampling of the Rhizosphere Tissues

The plant materials were collected at the Konza Prairie Biological Station (KPBS, 39°05′ N, 96°35′ W), a Long-Term Ecological Research (LTER) site that represents a native tallgrass prairie in the Flint Hills of eastern Kansas, USA. The vegetation is dominated by big blue stem (*Andropogon gerardii* Vitman), indian grass [*Sorghastrum nutans* (L.) Nash.], little bluestem [*Schizachyrium scoparium* (Michx.) Nash.], and switch grass (*Panicum virgatum* L). The soil parent material is chert-bearing limestone with the soil bulk density of 1.0 g $cm^{-3}$. January mean temperature is −3°C (range −9°C to 3°C) and the July mean temperature is 27°C (range 20–33°C). Annual precipitation averages 835 mm, 75% of which falls in the growing season.

A total of three intact *A. gerardii* plants with their roots attached were excavated in early growing season (May) from an annually burned watershed (1D) at the KPBS. The focal watershed is a part of the regular LTER program that aims to study the impacts of fire frequency on ecosystem structure and dynamics. The watershed represents tallgrass prairies under a frequent fire cycle. The plant roots were shaken free of loose soil and ca. 10 cm of the root material still attached to the identifiable *A. gerardii* aboveground tissues were transferred to the Lysing Matrix E of the FastRNA Pro Soil-Direct kit (Qbiogene, Carlsbad, CA, USA). The tubes with the Lysing Matrix and the *A. gerardii* tissues were immediately flash-frozen in a liquid nitrogen cryoshipper (Mini-Moover Vapor Shipper, Chart Biomedical, Marietta,

GA, USA) to avoid degradation of the RNA. The samples were maintained frozen in the cryoshipper until the nucleic acid extraction.

#### 2.2.3.2 Nucleic Acid Extraction

The *A. gerardii* roots and the soil adhering to the roots were homogenized in the FastPrep instrument (Q-BioGene, Irvine, CA, USA) at setting 6 for 40 s in 1 ml RNApro Soil Lysis Solution and total RNA extracted using FastRNA Pro Soil-Direct kit as outlined by the kit manufacturer. Note that use of nuclease-free plastic disposables, cleaning the work surfaces with nuclease eliminators and keeping the extracts on ice will minimize the total RNA loss during the extractions. To obtain the genomic or total environmental DNA from the same samples, the nucleic acids that were eluted in nuclease-free water after the phenol-chloroform clean-up and the ethanol precipitation included in the FastRNA Pro Soil-Direct protocol were divided into two 200 µl aliquots. One of the two aliquots was cleaned up as instructed by the manufacturer of the FastRNA Pro Soil-Direct kit and eluted in 100 µl nuclease-free water with 100 units of RNaseOUT (Invitrogen, Carlsbad, CA, USA) to inhibit RNA degradation. The second aliquot was stored on ice until DNA was isolated using components of the FastDNA Spin Kit for Soil (Q-BioGene, Carlsbad, CA, USA). The DNA aliquot was combined with 978 µl of the Sodium Phosphate Buffer and 122 µl of the MP Buffer, mixed with 250 µl of the PPS reagent by frequent vortexing over a period of 10 min. After pelleting the precipitant at 14,000 g for 5 min, the supernatant was treated as instructed by the FastDNA Spin Kit manufacturer and eluted in 100 µl of nuclease-free water. Both nucleic acid extracts were stored on ice or in −80°C until further processed.

#### 2.2.3.3 Reverse Transcription of the rRNA

The extracted rRNAs were reverse-transcribed using Thermoscript RT-PCR two-step system (Invitrogen, Carlsbad, CA, USA). To acquire a near full-length cDNA of the small subunit (SSU) of the rRNA, NS8 primer (White et al. 1990) located near the 3′-end of the SSU was chosen. This primer choice for reverse transcription also permits use of a number of nested priming sites within the SSU. The rRNAs were denatured prior to the first strand cDNA synthesis. For denaturing, 2 µl of each RNA template was combined with 1 µl of nuclease-free 10 µM NS8 primer, 2 µl of the 10 mM dNTP mix and 7 µl of nuclease free $H_2O$ and incubated at 65°C for 5 min in an Eppendorf Mastercycler (Hamburg, Germany). The denatured RNAs were transferred to ice and combined with 1 µl of 0.1 M DTT, 1 µl of RNaseOUT (40 U $\mu l^{-1}$; Invitrogen), 1 µl nuclease-free $H_2O$, and 1 µl ThermoScript Reverse Transcriptase (Invitrogen). Reverse transcription was carried out in an Eppendorf

Mastercycler at 55°C for 60 min and the synthesized cDNAs returned to ice until PCR amplification. Note that the relatively temperature-stable reverse transcriptase used here allows higher reverse transcription temperatures and thus provides more stringent reaction conditions for target-specific primers during the reverse transcription.

#### 2.2.3.4 PCR Amplification

The reverse-transcribed cDNAs and the environmental DNAs extracted from the *A. gerardii* rhizospheres were PCR-amplified with Platinum *Taq* polymerase (Invitrogen) using primers targeting an approximately 760bp region within the SSU of the fungal rRNA gene (Borneman and Hartin 2000). For each 50 μl PCR reaction, 5 μl 10× PCR buffer, 5 μl 25 mM $MgCl_2$, 5 μl 2 mM dNTPs, 1 μl of 10 μM forward and reverse primers, 0.4 μl polymerase (5 U $\mu l^{-1}$) and 2 μl of the cDNA or environmental DNA plus 30.6 μl of nuclease-free water were combined. PCR reactions were carried out with initial 3 min denaturation at 93°C followed by 30 cycles of 1 min at 93°C, 1 min at 56°C, 2 min at 72°C and a terminal elongation at 72°C for 8 min. Longer extension steps may be necessary for long amplicons in complex environmental samples to minimize or eliminate the generation of artifactual PCR products (Jumpponen 2007). Presence of the target-sized amplicons was confirmed by visualizing the products by horizontal gel electrophoresis using 1.5% agarose gels in 0.5× TBE. The PCR products were quantitated using an ND-1,000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) in a 1 μl volume.

#### 2.2.3.5 Control Reactions

To account for contaminating DNA in the samples, three controls were included. First, to account for DNA contamination from the extraction system, a blank extraction without a sample was carried through the extraction protocol. Second, to account for PCR reagent-borne contaminants, a PCR control without template cDNA or DNA was included in the PCR. Third, to account for DNA carry-over through the RNA extraction, a control where Thermoscript reverse transcriptase was replaced with Platinum *Taq* polymerase was included. All these controls remained free of contaminants and yielded no visible PCR amplicons, indicating absence of DNA carry-through in the RNA extraction, absence of contaminating DNA in the DNA/RNA extraction, and absence of contaminating DNA in the PCR reagents. Note that many rRNA extraction protocols may fail to exclude DNA, and DNA removal with RNAse-free DNAse may be necessary.

#### 2.2.3.6 Cloning, Sequencing, and Analysis of the cDNA and Environmental DNA Amplicons

The mixed populations of PCR products were cloned using TOPO-TA cloning system (Invitrogen). A total of 40 ng of each PCR product from cDNA or environmental DNA template was ligated into 10 ng of linearized pCR4 vector (Invitrogen) and the circularized plasmids were transformed into competent TOP10 cells (Invitrogen) by a 30 s heat shock in a 42°C water bath following the protocol for TOPO-TA cloning. Bacteria were grown for an hour at 37°C under 200 rpm agitation in 250 μl of SOC medium. A total of 10 and 100 μl of the incubated bacteria were plated on LB agar with 60 μg $ml^{-1}$ ampicillin and grown at 37°C overnight to confirm putative positive transformants. A random sample of the transformants was screened for presence of an insert by PCR in 15 μl reaction volumes, using conditions and cycling parameters outlined above, and the products visualized on 1.5% TBE agarose gels with EtBr on a UV illuminator. The clone libraries were combined with an equal volume of 60% glycerol, flash-frozen in liquid $N_2$ and shipped for plasmid prep and sequencing at University of Washington High Throughput Genomics Unit (Seattle, WA, USA). A total of 96 clones from each of the six clone libraries were randomly sampled and sequenced. Vector contamination was removed using the automated vector trimming function in Sequencher (Version 4.6, GeneCodes, Ann Arbor, MI, USA). The similarities to existing rDNA sequences in the GenBank database were determined using Blast at the National Center for Biotechnology Information (Altschul et al. 1997).

## 2.3 Results

### *2.3.1 Nucleic Acid Extraction and Reverse Transcription from the Andropogon gerardii Rhizosphere*

This chapter describes simple, kit-based protocols for use of rRNA in fungal community analysis. The RNA extraction and cDNA synthesis should be completed expediently following the environmental sampling. Otherwise, the preservation and storage of RNA from environmental samples require special caution. The approach taken in this work included immediate sample preservation in an $N_2$-vapor cryoshipper that should ensure sample maintenance with minimal RNA degradation. Furthermore, the addition of RNAse inhibitors in the final elutes aims to minimize RNA template loss. Finally, use of extraction kits expedites the protocol and maximizes the RNA yields, as well as usually improves RNA quality for downstream applications.

The simple FastPrep-kit method successfully isolated reverse-transcribable and PCR-amplifiable nucleic acids from *A. gerardii* rhizosphere. DNA contamination is a serious concern in PCR-based applications – especially if cDNA is used as a

primary template. Should DNA carry-through occur, the extracted total RNAs must be further cleaned with RNAse-free DNAse. The three included controls behaved as expected: (1) The controls with the reverse-transcriptase substituted with a polymerase failed to produce visible PCR amplicons, indicating absence of contaminating DNA in the rRNA samples. (2) The extraction controls without the environmental samples remained clean of PCR amplicons regardless of whether or not the reverse-transcription step was included. (3) Finally, the regular PCR controls indicated that no false PCR amplicons were generated in absence of the template.

Universal primer NS8 was chosen for reverse transcription. NS8 provides a near full-length SSU cDNA and thus allows use of a number of priming sites for downstream PCR-applications. This work targeted the fungal community as a whole by use of fungus-specific primers that have been shown to amplify fungal targets across various fungal phyla (Borneman and Hartin 2000; Jumpponen 2003). Other sets of SSU-targeting primers would similarly be compatible with the use of a universal reverse transcription primer. For example, primer sets with strong bias toward Ascomycota and Basidiomycota (Smit et al. 1999) as well as toward Glomeromycota (Helgason et al. 1998) are readily available and should allow target-specific amplification using the same cDNAs.

### 2.3.2 *Community Assessment Using Reverse-Transcribed cDNAs and Environmental rDNAs*

The communities observed in the *A. gerardii* rhizosphere contained fungi from three phyla: Ascomycota, Basidiomycota and Glomeromycota (Table 2.1). The abundances of the phyla varied among the libraries. While one of the three cDNA libraries was comprised nearly exclusively of Glomeromycotan sequences (closest affinity to *Glomus mosseae*), two additional cDNA libraries had a minimal Glomeromycotan component and were dominated by various ascomycetes. Similar results on a phylum level were also observed in the rDNA libraries: only one of the three libraries was dominated by various Glomeromycotan sequences (*Glomus proliferum* and an unknown *Glomus* species). The low frequency of Glomeromycotan sequences as well as incongruences among the libraries are most likely attributable to temporal and spatial dynamics in the tallgrass prairie ecosystem. Ongoing, yet unpublished, results indicate that *A. gerardii* roots maintain low levels of Glomeromycotan colonization early during the growing season, whereas other, septate fungi are more common (Mandyam and Jumpponen, unpublished). These results corroborate observations of mycorrhizal colonization made by others at the KPBS (Bentivenga and Hetrick 1992).

The initial hypothesis in assessing the two different nucleic acid pools in the rhizosphere was that the cDNA community should be a subset of the rDNA community. Contrary to this hypothesis, the cDNA and rDNA clone libraries

**Table 2.1** Fungi detected in the *Andropogon gerardii* roots and rhizosphere by cloning and sequencing the PCR-amplified reverse transcribed ribosomal RNAs (cDNAs) and the PCR-amplified environmental DNAs. The totals refer to the total number of sequenced clones in each of the six clone libraries, *SR* indicates the species richness observed in each of the six clone libraries. Sequences from cDNA and DNA libraries that bin together at 98% similarity using Sequencher are highlighted in italics and bold

| Species (accession) | Order (Phylum) | Similarity (%) | cDNAs (SR) | DNAs (SR) |
|---|---|---|---|---|
| Sample 1 | | | | |
| *Ceramothyrium linnaeae* (AF022715) | Incertae Sedis (Asc) | 99 | 19 | |
| *Coniosporium* sp. (AJ972863) | Incertae Sedis (Asc) | 97 | 8 | |
| *Cordyceps konnoana* (AB031192) | Hypocreales (Asc) | 99 | | 26 |
| *Exidiopsis calcea* (AY293130) | Auriculariales (Bas) | 93 | 30 | |
| *Exidiopsis calcea* (AY293130) | Auriculariales (Bas) | 93 | 1 | |
| *Fusarium oxysporum* (DQ916150) | Hypocreales (Asc) | 98 | | 10 |
| *Fusarium oxysporum* (DQ916150) | Hypocreales (Asc) | 98 | | 2 |
| *Glomus intraradices* (AY635831) | Glomales (Glo) | 99 | | 12 |
| *Glomus intraradices* (AY635831) | Glomales (Glo) | 99 | 1 | |
| *Helicodendron paradoxum* (AY856945) | Helotiales (Asc) | 99 | 4 | |
| *Lithothelium septemseptatum* (AY584662) | Pyrenulales (Asc) | 99 | 25 | |
| *Lithothelium septemseptatum* (AY584662) | Pyrenulales (Asc) | 99 | 1 | |
| *Tubeufia pezizula* (AY856950) | Incertae Sedis (Asc) | 98 | | 45 |
| *Zopfia rhizophila* (L76622) | Incertae Sedis (Asc) | 95 | 7 | |
| Total | | | **96** (**9**) | **95** (**5**) |
| Sample 2 | | | | |
| *Campanella* sp. (AY916675) | Agaricales (Bas) | 98 | ***8*** | ***15*** |
| *Campanella* sp. (AY916675) | Agaricales (Bas) | 93 | | 1 |
| *Campanella* sp. (AY916675) | Agaricales (Bas) | 94 | | 1 |
| *Campanella* sp. (AY916675) | Agaricales (Bas) | 89 | | 1 |

*(continued)*

**Table 2.1** (continued)

| Species (accession) | Order (Phylum) | Similarity (%) | cDNAs (SR) | DNAs (SR) |
|---|---|---|---|---|
| *Glomus mosseae* (AY635833) | Glomales (Glo) | 88 | 86 | |
| *Glomus proliferum* (AF213462) | Glomales (Glo) | 99 | | 33 |
| *Glomus proliferum* (AF213462) | Glomales (Glo) | 98 | | 1 |
| *Glomus proliferum* (AF213462 | Glomales (Glo) | 98 | | 1 |
| *Glomus* sp. (AF480155) | Glomales (Glo) | 99 | **1** | **41** |
| Total | | | ***95 (3)*** | **94 (8)** |
| Sample 3 | | | | |
| *Asteromassaria olivaceohirta* (AY313953) | Pleosporales (Asc) | 99 | 7 | |
| *Coniosporium* sp. (AJ972863) | Incertae Sedis (Asc) | 99 | 7 | |
| *Endoxyla cirrhosa* (AY761089) | Boliniales (Asc) | 99 | 46 | |
| *Endoxyla cirrhosa* (AY761089) | Boliniales (Asc) | 98 | 12 | |
| *Endoxyla cirrhosa* (AY761089) | Boliniales (Asc) | 99 | 1 | |
| *Helicodendron paradoxum* (AY856945) | Helotiales (Asc) | 98 | ***5*** | ***27*** |
| *Helicodendron paradoxum* (AY856945) | Helotiales (Asc) | 99 | ***4*** | ***8*** |
| *Helicodendron paradoxum* (AY856945) | Helotiales (Asc) | 92 | 1 | |
| *Thanatephorus cucumeris* (DQ917659) | Ceratobasidiales (Bas) | 88 | | 5 |
| Total | | | **86 (8)** | **40 (3)** |

contained discrete taxa and the overlapping similar sequences were rare. To exemplify, while a single sequence type (*Glomus mosseae*) dominated one of the cDNA libraries, the rDNA library from that sample was dominated by two different Glomeromycotan sequences (one most similar to *Glomus proliferum*, and another most similar to an unidentified *Glomus* sp.). The reasons for this disparity remain unclear and open to speculation. It is possible that sampling of 96 cloned PCR products is inadequate, represents a gross undersampling for complex and diverse fungal communities, and thus fails to detect copies that are present in soil and rhizosphere in different numbers among the two nucleic acid extracts. The organisms that are present in high numbers in rRNA-derived communities should also be present in the rDNA-derived communities. Greater sampling intensity that would saturate the diversity in a soil environment should provide a more reliable estimate

of the community structure. It is also possible that the methods to isolate the different nucleic acids result in serious biases (von Wintzingerode et al. 1997): the template stability and the rate of template acquisition may differ among targets due to different cell resistance to homogenization or different nucleic acid folding structures that may preferably maintain some templates through the extraction protocols whereas others may be lost. Finally, in addition to these biases, reverse transcription and cDNA synthesis add steps and sources of possible incongruence to the data acquisition for community analysis. The cDNA synthesis in addition to the RNA isolation may be a source of bias (Kowalchuk et al. 2006) and, as such, a possible explanation for the observed differences among the cDNA- and rDNA-inferred fungal communities.

The analyses of reverse-transcribed and PCR-amplified rRNAs and PCR-amplified rDNAs indicated presence of relatively low diversity in the *A. gerardii* rhizosphere. On average, the rRNA-derived cDNA clone libraries had 6.7 taxa, while the rDNA-derived clone libraries had slightly lower species richness with 5.7 taxa. Both approaches provided comparable estimates of the diversity and evenness (Shannon's diversity index $H = 1.17$ and 1.15 and evenness $E_H = 0.59$ and 0.71; Simpson's diversity index $D = 2.86$ and 2.67 and evenness $E_D = 0.41$ and 0.51 for the cDNA and rDNA libraries, respectively). Note that none of these differences is significant at $\alpha = 0.05$ (Student's *t*-test). Again, contrary to the initial hypotheses, the rDNA libraries did not contain a higher species richness or diversity than the cDNA libraries.

## 2.4 Conclusions

The kit-based extraction method proved successful in producing reverse-transcribable and PCR-amplifiable rRNA templates. A major drawback of the procedure described here is that FastRNA extraction systems require relatively costly equipment (FastPrep instrument). However, other, alternative nucleic acid purification systems can be adopted for environmental samples (e.g., AllPrep RNA and DNA purification system from QiaGen, Valencia, CA, USA or PowerSoil RNA system from MoBio, Carlsbad, CA, USA). In adopting the former product, one must solve issues of tissue homogenization.

Although RNA isolation from environmental samples is relatively simple, the sample maintenance and inhibition of RNA degradation can be problematic. During sampling, the samples need to be maintained frozen or specific RNA stabilization reagents (e.g., RNAlater from QiaGen) may be necessary to minimize RNA degradation. Both approaches may have their caveats. While the immediate flash-freezing in a cryoshipper proved successful here, the transport of a bulky container with residual liquid $N_2$ can become problematic if travel by air or by foot is necessary during the sampling. Use of RNAse inhibitors (such as RNAlater) only extends the longevity of the environmental RNA to a limited extent, and this time is temperature-dependent.

The rRNA provides a target that may allow an improved understanding of the microbial communities (Poulsen et al. 1993). However, one should be cautious in considering the obtained data as a true reflection of active organisms in the environment. Many soil-inhabiting organisms may be able to maintain high ribosome levels through dormancy in order to be able to regain activity when favorable environmental conditions emerge (Kowalchuk et al. 2006). Furthermore, while the use of rRNA may circumvent the problems of using rDNA (non-active cells, naked DNA in soil), the reverse transcription necessitates additional steps in the data acquisition that may introduce additional biases that complicate community analyses. It remains unclear what is the impact of use of the universal primer for the reverse transcription, and how that choice impacts observed species-richness estimators.

Finally, the reasons for the observed incongruences among the cDNA- and rDNA-derived communities remain unclear. However, one should keep in mind that both community compositions may be equally true. While the cDNA community may provide a snapshot of the organisms that are present in the current active and/or dormant pools, the rDNA community carries a historical trace, and thus provides a view of that community and of those present in the recent past. As pointed out by Duineveld et al. (2001), the two views of the same community may differ. One just needs to be cautious about the interpretation of those results.

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# Chapter 3
# Use of Mycorrhiza Bioassays in Ecological Studies

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## 3.1 Introduction

It is well known that arbuscular mycorrhizal (AM) fungi are ubiquitous in agricultural and natural ecosystems (Brundrett 1991, 2002) and that most plant species form symbiotic associations with these fungi (Newman and Reddell 1987). The symbiosis can increase the growth and development of plants by enhancing nutrient uptake, especially phosphorus (Ortas 1996; Smith et al. 2004), into plants and by improving water relations of plants (Auge 2001; Boomsma and Vyn 2008; Jacobson 1997; Kyllo et al. 2003; Mohammad et al. 1995). Plants differ in their growth response to mycorrhizal colonization (Monzon and Azcon 1996; Ortas et al. 2002; Pankova et al. 2008). Explanations for differences in growth responses to mycorrhizal colonisation over the full life cycle of plants are complex (Gazey et al. 1992) and new explanations of mycorrhizal function are emerging (Li et al. 2008). Mycorrhizal dependency (Menge et al. 1978; Gerdemann 1975) varies among plant species (Abbott et al. 1995; Pankova et al. 2008), and may influence the abundance and community structure of AM fungi in soil and their infectivity (Crush 1978; Egerton-Warburton et al. 2007; Püschel et al. 2007; Zangaro et al. 2000). AM fungi can also contribute to improving soil physical fertility by enhancing soil aggregation (e.g. Moreno-Espíndolaa et al. 2007; Rillig and Mummery 2006) and protect crops against plant disease (see Douds and Johnson 2003). The soil environment, particularly factors that control soil fertility, strongly influences mycorrhizal function (Abbott and Robson 1982, 1991b; Cardoso and Kuyper 2006).

The diversity of AM fungi is generally low in agriculture compared with indigenous plant communities (Boerner et al. 1996; Helgason et al. 1998; Sieverding

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1991). Crop and rotation history also influence the abundance and diversity of AM fungi in agricultural soil (Johnson and Gehring 2007; Douds and Johnson 2003). Therefore, the capacity of AM fungi to form mycorrhizas that contribute substantially to plant nutrition, plant health or soil fertility depends on soil management. Within this context, calibrated bioassays of infectivity have the potential to enable interpretation of mycorrhizal function. However, due to the complexity, diversity and dynamics of mycorrhizal associations in space and time, measurements of mycorrhizal status of roots at one point in time is unlikely to enable full understanding of the interactions between AM fungi and host plants over their life cycle.

## 3.2 Assessment of Infectivity of AM Fungi in Soil

The term "soil infectivity" was used to define the ability of soil containing propagules of pathogens to initiate infection of host plants (Hornby 1990). Based on this plant pathology concept, "infectivity" of AM fungi in soil has been used with reference to the ability of AM fungi to colonise roots of a host plant (Abbott and Robson 1981) and explored further within the framework of mycorrhiza bioassays (e.g. Gianinazzi-Pearson et al. 1985; Plenchette et al. 1989). The infectivity of AM fungi can differ among host plants even when grown together in the same soil (Abbott et al. 1995). It is also important to be aware that bioassay measurements of infectivity of AM fungi can vary with soil environment, including differences in soil fertility, soil disturbance, host plant type and architecture, temperature, and water availability (Abbott et al. 1995).

Knowledge of the abundance and distribution of AM fungi in soil is acquired from measurements of the length of mycorrhizal root, the proportion of the root length colonised, the length of hyphae in soil, the number and types of spores and other propagules in soil, and the infectivity of the community as a whole (Abbott and Robson 1991a). Colonisation of roots by AM fungi can be estimated directly using microscopic observation of roots and indirectly using bioassays (Abbott and Robson 1981) or molecular assays (Jacquot et al. 2000; Stukenbrock and Rosendahl 2005).

### *3.2.1 Direct Assessment of AM Fungi*

Direct measurement of mycorrhizal colonisation of roots by length of root colonised and percentage of root length colonised is usually based on staining to distinguish the fungi from root tissues (see Brundrett et al. 1984). Colonisation can also be observed in unstained roots (Arias et al. 1987), but most histological methods have limitations because some fungi are not easily visible even after roots are cleared and the fungi stained (Saito et al. 1993). Most assessments of AM fungi

in roots do not take into account changes in the intensity of fungal colonisation within the root, but McGonigle et al. (1990) overcame this limitation to some extent by developing a magnified intersection method for assessing the colonisation of roots. This procedure assesses the proportion of root length containing mycorrhizal fungal structures (arbuscules, vesicles and hyphae). In-depth microscopic analysis of colonisation intensity is necessary for more complete understanding of relationships between root colonisation and mycorrhizal function (Gazey et al. 1992), and this will differ for plants with different root structures (Merryweather and Fitter 1998; van der Heijden et al. 1998).

None of the measurements mentioned above provide information about the activity of the fungi present inside roots or in soil, so histochemical procedures are required to determine whether the fungi are living or dead. Hyphae and vesicles can be observed inside roots, even in dead roots, and enzyme assays can be used to determine whether the fungi are alive (e.g. MacDonald and Lewis 1978; Saito 1995; Saito et al. 1993).

As root systems develop, changes occur in the abundance of fungi in the root system either as a whole or in terms of the relative abundance of different AM fungi; sequential sampling may be necessary. As suggested above, the architecture of the root system also needs to be considered when interpreting colonisation data. For example, calculation of the proportion of root length colonised for the whole of a woody root system is of little significance compared with a measurement that is restricted to the fine (most active) roots of the same plant. Similarly, calculations of the proportion of root length colonised on root systems of plants with highly differentiated root systems might be of little value without quantifying the relative abundance of different types and ages of roots. Mycorrhizal colonisation of plants that shed roots extensively may be difficult to determine accurately.

Morphological structures of AM fungi inside roots can be distinctive within some communities of fungi (Abbott 1982; Merryweather and Fitter 1998) but overall the fungi are structurally diverse (Dickson et al. 2007; Dodd et al. 2000; Smith and Smith 1997). The recognition of morphological similarities among AM fungi occurring in a soil community requires extensive experience based on observations of mycorrhizas formed in pure pot cultures of fungi isolated from one location and studied on a standard host plant (Abbott 1982). The morphology of a fungus within roots of different host plants can differ (Lackie et al. 1987; Smith and Smith 1997), so, while morphological characteristics may provide a tool for understanding the dynamics of colonisation of some root systems by distinct morphotypes (e.g. Abbott and Robson 1982; Merryweather and Fitter 1998; Scheltema et al. 1987), molecular characterisation is required to distinguish among fungi within morphotypes (e.g. Jacquot et al. 2000; Stukenbrock and Rosendahl 2005).

Combined with methods for determining physiological activity within hyphae (e.g. MacDonald and Lewis 1978; Saito 1995), direct morphological and molecular features of AM fungi can be used to determine the abundance, dynamics, diversity and activity of AM fungi inside roots (Solaiman and Abbott 2004; Wubet et al. 2004).

### 3.2.2 *Indirect Assessment: Mycorrhiza Bioassay*

Mycorrhiza bioassays involve use of bait plants grown in a soil sample; roots are examined for the presence of AM fungi after a set time. Glasshouse bioassays have been used to determine the infectivity of AM fungi in a range of situations (Table 3.1).

**Table 3.1** Examples of studies using bioassays to assess the infectivity of AM fungi (after Djuuna 2006)

| Purpose | Bioassay technique | Example references |
|---|---|---|
| Infectivity and effectiveness of AM fungi | Infectivity of AM fungi was assessed from field soil using subterranean clover | Abbott and Robson (1981) |
| Infectivity and effectiveness of AM fungi | Glasshouse trial using bait plants | Gianinazzi-Pearson et al. (1985) |
| The concept of soil infectivity and a method for its determination as applied to AM fungi | Soil infectivity was assessed by a standard bioassay method using Leek (*Allium porum*) as host plant | Plenchette et al. (1989) |
| Seasonal variation in the infectivity of AM fungi in an annual pasture in a Mediterranean environment | Undisturbed soil cores sown with *Trifollium subterraneum* L. in the glasshouse. Colonisation by AM fungi was measured 3 and 6 weeks after sowing | Scheltema et al. (1987) |
| The total inoculum potential of AM fungi | The rate of colonisation by AM fungi assessed by growing bait plants in intact cores of soil | Brundrett (1991) |
| Spatial variation in inoculum potential of AM fungi in mine site and agricultural soil | Infectivity of AM fungi in intact cores soil from disturbed and undisturbed sites | Jasper et al. (1991) |
| Abundance and frequency of mycorrhizal propagules | AM fungi assessed by growing bait plants in intact soil cores | Brundrett et al. (1996) |
| Infectivity of AM fungi exposed to long term saline conditions | Infectivity of AM fungi in saline soils studied after trap pot culture and multiple and single spore inoculation | Rafique (2005) |
| Infectivity of AM fungi in jarrah forest rehabilitated site after bauxite mining | Infectivity of AM fungi in intact soil cores collected from jarrah forest | Solaiman and Abbott (2003) |

Generally, bioassay plants are grown in defined conditions using soil collected from the field, and the extent of mycorrhizal colonisation is assessed after a predetermined period. Bioassays of this type have been used to show differences among soils in the infectivity of AM fungi present (e.g. Abbott and Robson 1982; Gianinazzi-Pearson et al. 1985). Few studies have evaluated the relationship between the infectivity assessed in a bioassay and the development of mycorrhizas *in situ* at the site from which the bioassay soil was collected (see Abbott and Robson 1991a). Steps in each mycorrhiza bioassay (soil sampling, bioassay conditions, calibration and testing field predictions) all need to be selected with the purpose of the analysis well defined in advance (Abbott et al. 1995).

*Soil sampling*: Soil samples can be collected as bulk soil (disturbed soil samples) or as soil cores (undisturbed soil). Sampling needs to be representative of each sampling point. Soil samples should be used quickly or stored at low temperature until used. Consideration should be given to whether or not to allow the soil to dry, and this will depend on the biology of the AM fungi expected to be present. Prior understanding of the fungi present is therefore advantageous.

*Bait plants*: Host plants such as grass or legume species are good bait plants for bioassays, but other species are suitable if they are highly mycorrhizal. Plants with fast-growing roots in the soil and environmental conditions used in the bioassay, and plants with roots which are easy to clear and stain for AM fungi are most useful as bait plants (Brundrett et al. 1996).

*Duration of bioassays*: Brundrett and Abbott (1995) noted that the duration of bioassays should not exceed the growth stage required for an adequate level of mycorrhizas to form. If a longer time is chosen before the bait plants are assessed, the plateau level of colonisation may mask differences in rate of colonisation (Abbott and Robson 1981; Sanders et al. 1977). While it is necessary to stop the bioassay before colonisation reaches the plateau, this time might depend on the communities of AM fungi, soil conditions, growing conditions and species of bait plant, highlighting the need for customised bioassays.

*Predicting mycorrhizal colonisation in the field*: An additional value of mycorrhiza bioassays is that they may be used to predict mycorrhiza development in agricultural or horticultural plants (Abbott et al. 1995). However, management practices imposed after collection of the soil can influence the subsequent field colonisation (Abbott and Robson 1991b). Therefore, if the intention is to use the bioassay to predict colonisation later in the season, a calibration is needed to take into account any effect of management practices imposed after soil is collected for the bioassay on expected colonisation in the field.

A bioassay for predicting mycorrhiza formation could use soil collected before the start of the season combined with (1) information about the proposed agricultural practice, (2) soil conditions, and (3) knowledge of how the AM fungi in this soil are expected to respond to these conditions and practices. Furthermore, in order to consider using a mycorrhiza bioassay to predict the rate and extent of colonisation of roots by AM fungi, the bioassay needs to be conducted under defined conditions.

Mycorrhiza bioassays have been used to quantify seasonal and spatial variations in inoculum potential of AM fungi (Brundrett and Abbott 1995; Brundrett et al. 1996; Jasper et al. 1991; Scheltema et al. 1987). Bioassay plants grown in undisturbed soil cores are likely to closely represent field conditions, but mixed soil samples can be used as part of a standardized bioassay procedure. However, if field soil is disturbed, infectivity of the fungi present may be altered (Miller et al. 1995), so interpretation of the data could be complicated by the conditions under which the assessment is conducted.

Quantification of the rate of colonisation of bioassay plants by different morphotypes or genetically distinguishable groups of AM fungi in field soil could be used to estimate the potential of the components of the community to develop mycorrhizas (Abbott et al. 1995). Alternatively, it is possible that this may not represent the true potential infectivity of each group of fungi if there were no competing fungi present. Observations can be complicated by competition among the AM fungi during colonisation of roots (Solaiman and Abbott 2003).

## 3.3 Purpose of Bioassays for Infectivity Assessment of AM Fungi

The majority of ecological studies of AM fungi include a measure of mycorrhizal colonisation. However, colonisation of roots by AM fungi is a complex process and it is difficult to represent such a dynamic process in a single measurement made at one point in time. The complex characteristics of mycorrhizal associations depend on root growth and architecture as well as fungal diversity in the soil and inside roots (Abbott and Robson 1984).

The infectivity of AM fungi changes with spore maturation or dormancy (Tommerup 1983), in response to the presence of roots of different forms and age (e.g. associated with the nature and quantity of root exudates) and as the fungi proceed through stages in their life cycle (Pearson and Schweiger 1993). In the latter case, the infectivity of AM fungi associated with mycorrhizal roots paralleled the pattern of sporulation (and transfer of energy resources), despite the presence of fungi in the roots detected by staining. In natural ecosystems with little physical disturbance of soil, roots may be colonised by hyphae associated with adjacent plant species or by new hyphae from germinated spores. When soil is disturbed (e.g. with farming practices), the infectivity of AM fungi can be altered (Evans and Miller 1990). The extent of change may depend on the abundance of propagules of AM fungi present in the soil (Jasper et al. 1989). Soil disturbance in natural ecosystems can reduce the infectivity of AM fungi, especially where propagule abundance is low (Jasper et al. 1987).

A direct measurement of the presence of AM fungi in roots at a particular time results from colonisation events that have taken place during the life of the plant. In contrast, a measure of infectivity of AM fungi using a bioassay bait plant represents

the capacity of the hyphae to colonise roots at a particular point in time. A measure of infectivity of AM fungi assessed in a bioassay can be made at different stages in the plant's growth cycle and interpreted in terms of the ability of the hyphae in soil or in the roots to colonise new roots.

## 3.4 Conclusions

Mycorrhiza bioassays measure the infectivity in soil at a point in time, representing the potential of AM fungal propagules present in soil to colonise roots. However, the conditions of the bioassay may not be the same as those under field conditions; therefore, it is possible that fungi are present in soil in an infective state but not able to colonise roots for some reason, perhaps related to the physiological state of the roots or due to competition from other AM fungi. Bioassays of infectivity of AM fungi have potential to assist in predicting how roots might become colonised, but calibrations are necessary that take into account an understanding of the relationships between mycorrhizal colonisation under the expected soil conditions (i.e. that take into account intervening management practices such as fertilizer addition, crop or tillage practice). Therefore, several factors need to be considered before conducting a mycorrhizal bioassay, including the time of soil sampling, choice of bait plant, handling of soil samples, and length of the bioassay, and precautions are necessary when interpreting mycorrhiza bioassay data.

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# Chapter 4
# *In Vivo* Model Systems for Visualisation, Quantification and Experimental Studies of Intact Arbuscular Mycorrhizal Networks

**Manuela Giovannetti, Luciano Avio, Cristiana Sbrana, and Paola Fortuna**

## 4.1 Introduction

The root system of most land plants is colonised by obligately biotrophic fungi — arbuscular mycorrhizal (AM) fungi (Glomeromycota) — which, after establishing mutualistic symbioses, are able to develop extensive, belowground extraradical mycelium (ERM) fundamental to the uptake of nutrients from soil and their transfer to the host plant (Smith and Read 1997). Such mycorrhizal networks were first visualised and quantified *in vivo* by means of two-dimensional experimental systems, and were shown to be able to spread from colonised roots into the surrounding environment at growth rates ranging from 738 to 1,067 mm per day, depending on the host plant, and to reach hyphal extents of 10–40 mm per mm of root length (Giovannetti et al. 2001). Since AM fungi (AMF) have a wide host range, AM extraradical networks may interconnect different plants, by means of anastomosis, whose formation depends on a highly regulated mechanism of self-recognition between compatible hyphae. Successful anastomoses occur between hyphae belonging to the same individual and to different individuals of the same isolate, during the pre-symbiotic growth of AMF (Giovannetti et al. 1999). By contrast, hyphae of individuals belonging to different genera and species, and even to isolates of the same species originating from geographically different areas, are unable to fuse, and show rejection responses, either before or after anastomoses, revealing AMF hyphal ability to discriminate against nonself (Giovannetti et al. 2003). Extraradical mycorrhizal networks maintain the capacity of self-recognition,

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evidenced by the high frequency of anastomoses between hyphae originating from the same and different root systems colonised by a single AM fungal isolate (Giovannetti et al. 2004).

In this chapter we review the methods devised for the visualisation and quantification of anastomosis formation and for the study of mycorrhizal networks formed by AMF both during the pre-symbiotic and the symbiotic stages of their life cycle. Moreover, we describe experimental model systems showing that root systems of plants belonging to different species, genera and families may become connected by means of anastomosis formation between mycorrhizal networks, which can create indefinitely large numbers of fungal linkages among plants in a community.

## 4.2 Structure of Pre-symbiotic Mycelium of AM Fungi: Visualisation and Quantification of Anastomosis

### *4.2.1 Occurrence and Frequency of Anastomosis*

Anastomosis can be detected in mycelia originating from individually germinated spores or between mycelia originating from different germlings. Spores belonging either to the same or to different AM fungal isolates are surface-sterilised with 2% chloramine T, supplemented with streptomycin (400 μg $l^{-1}$) for 20 min, rinsed five times in sterile distilled water (SDW), and allowed to germinate individually in SDW in microtiter plates. Germinated spores are then transferred onto mixed cellulose esters membranes (0.45 μm diameter pores), which are placed on moist sterile quartz sand in 9 cm diameter Petri dishes, sealed with Parafilm and incubated at 25°C in the dark (Fig. 4.1a). To detect anastomoses between different individuals, pairings of different germinated spores are made by placing germlings approximately 1 cm apart. Occurrence of anastomoses is assessed by using a double staining method, in order to detect viable mycelia and protoplasmic continuity between fusing hyphae. First, germlings are stained for the presence of succinate dehydrogenase (SDH) activity (Smith and Gianinazzi-Pearson 1990), revealed by the deposition of formazan salts in viable hyphae. Second, membranes bearing SDH-stained germlings are mounted on microscope slides and stained with 0.05% Trypan blue in lactic acid, in order to visualise hyphal walls of the whole mycelium produced by each germling (Fig. 4.1b–d). Hyphal length is assessed by using image analysis software or a microscopic gridline eyepiece. All hyphal contacts are observed under a light microscope, counted at magnifications of ×125 to ×500 and verified at a magnification of ×1,250. Frequency of anastomoses is calculated by dividing the number of hyphal contacts leading to hyphal fusions by the total number of hyphal contacts. Chi-square analysis is used to determine the homogeneity of anastomosis frequency data, and the chi-square test of independence is performed to detect significant differences in anastomosis frequency between hyphae from the same spores and hyphae from different spores.

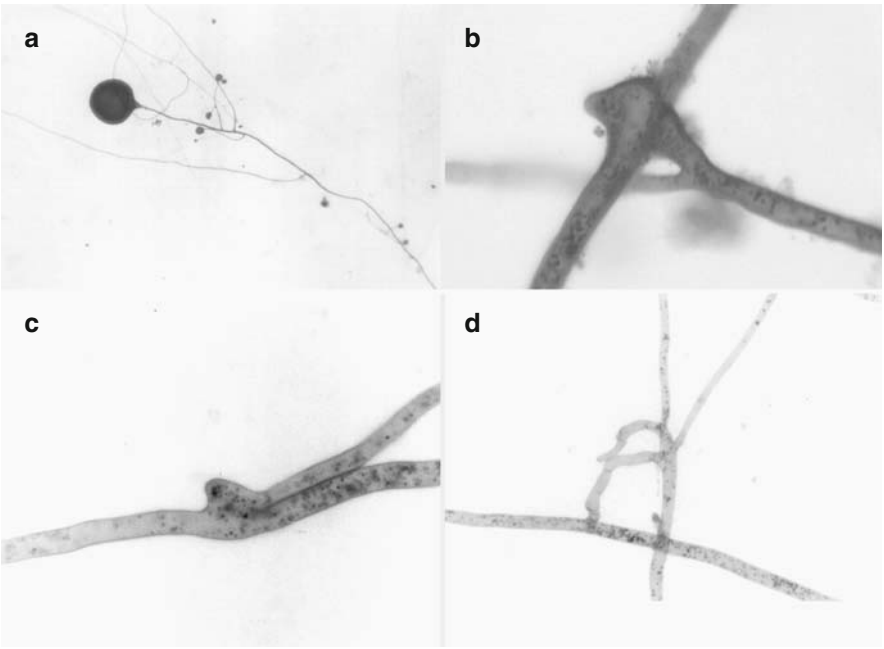


**Fig. 4.1** Light micrographs showing: (**a**) a germinated spore of *G. mosseae* grown on a cellulose ester membrane, (**b**) *G. mosseae* anastomosed hyphae after staining for SDH activity, (**c**) *G. mosseae* anastomosed hyphae after staining for SDH activity and mounting in Trypan blue, and (**d**) *G. mosseae* hyphae showing incompatible responses, after staining for SDH activity and mounting in Trypan blue

### *4.2.2 Dynamics of Anastomosis Formation in Living Hyphae*

Hyphal growth and anastomoses formation can be monitored in living hyphae by means of microchambers (Logi et al. 1998; Giovannetti et al. 2000) (Fig. 4.2). Surface-sterilised spores are germinated on 20 mm × 20 mm sterile cellophane membranes placed on a thin layer of 1% water agar (WA) in 5.5 cm diameter Petri dishes, for direct observations of spore germination and hyphal growth under the dissecting microscope. Cellophane membranes bearing germinated spores are placed on microscope slides in SDW. The coverslip is sealed with 1% WA, and periodically wetted with SDW. Alternatively, Petrislide plates, bearing the cellophane membrane placed on a 2-mm WA layer poured on the lid, may be used both to germinate spores and as imaging chambers. The microchambers obtained are then transferred to the light microscope and contacting hyphae can be observed for periods of several hours, in order to monitor the entire process of anastomoses formation, during which organelle flow between anastomosing hyphae can be monitored and images can be captured with a video camera and then recorded.

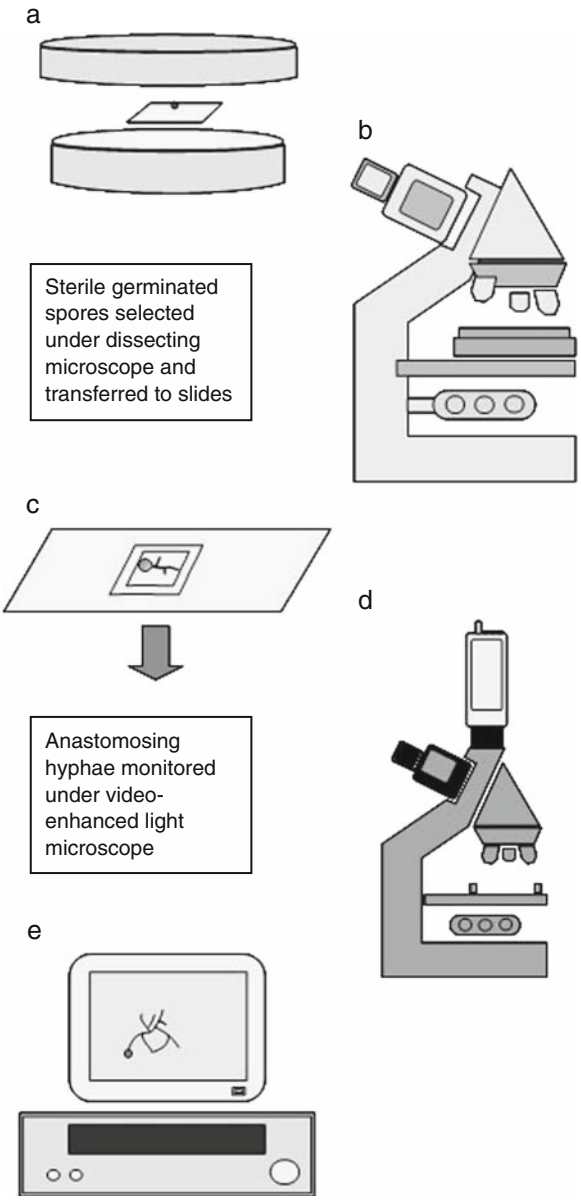


**Fig. 4.2** Schematic representation of the culture system used to monitor the dynamics of anastomosis formation in living hyphae of AM fungi. The system allows the selection of germinated spores and the monitoring of anastomosis formation under video-enhanced microscope

Bright contrast images are acquired after regulation of the condenser diaphragm. The experiments can be carried out at room temperature.

To confirm the establishment of protoplasmic continuity and the viability of anastomosed hyphae, cellophane membranes bearing germinated spores are transferred from microchambers and mounted on microscope slides, where, after SDH staining, they are observed for the presence of formazan salt depositions in hyphal bridges.

### *4.2.3 Cytochemical Analyses of Anastomosing Hyphae*

To visualise the occurrence and location of nuclei in anastomosis bridges, spores are germinated on polycarbonate black membranes, in order to obtain a better visualisation of nuclei under epifluorescence. The membranes are mounted on microscope slides, stained with Diamidinophenylindole (DAPI) (5 μg $ml^{-1}$ in water to glycerol 1:1 solution) and observed under epifluorescence using UV light (Fig. 4.3a). To visualise hyphal compatibility/incompatibility, some membranes are first stained with DAPI and then mounted in a 0.01% (w/v) solution of Calcofluor White and observed under epifluorescence using UV light (Fig. 4.3b). Calcofluor staining can be performed also on mixed cellulose esters membranes without DAPI staining (Fig. 4.3c, d).

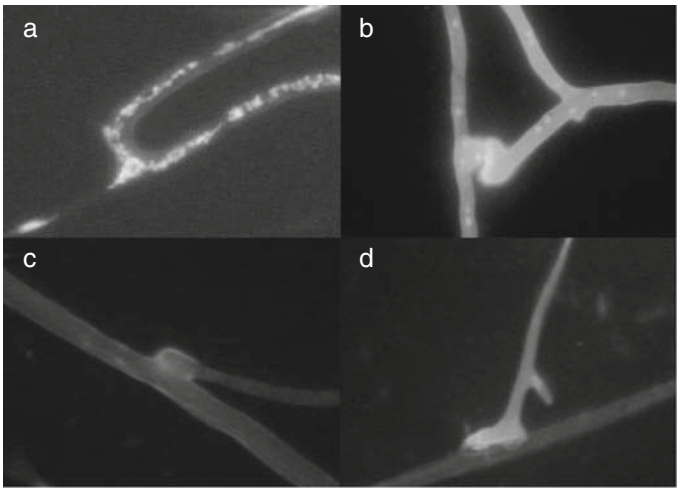


**Fig. 4.3** Epifluorescence micrographs showing: (**a**) DAPI and (**b**) DAPI Calcofluor-stained hyphae in, (**a**) compatible and (**b**) incompatible interactions in *G. mosseae* (courtesy of Applied Environmental Microbiology); (**c**) Calcofluor stained hyphae in compatible and (**d**) incompatible interactions in *G. mosseae*

### 4.2.4 Remarks

The methods described allowed the visualisation, for the first time, of anastomoses in different species of the genus *Glomus*. Time-course experiments showed that hyphal tips were able to fuse with hyphae growing nearby in about 35 min, and that a bidirectional flow of particles (vacuoles, mitochondria, nuclei, and fat droplets) moved at the speed of $1.8 \pm 0.06$ μm $s^{-1}$ through hyphal bridges formed during anastomosis (Giovannetti et al. 1999, 2000). Protoplasmic continuity, the characteristic feature of successful hyphal fusions, was evidenced by the complete disappearance of hyphal walls and visualised by histochemical localisation of formazan salts in hyphal fusions, after SDH staining. The established protoplasmic flow was further demonstrated by the detection of nuclei in hyphal bridges, evidenced by DAPI staining. Moreover, the described methods revealed that interactions between hyphae of species of the genera *Gigaspora* and *Scutellospora* never led to anastomosis formation (Giovannetti et al. 1999). This evidence was confirmed by other authors in *in vitro* monoxenic cultures (de la Providencia et al. 2005).

The described methods allowed the detection of nonself incompatibility in presymbiotic mycelial networks of AMF (Giovannetti and Sbrana 2001; Giovannetti et al. 2003). These findings, suggesting that AMF can recognise self-entities and discriminate self from nonself, opened the way to vegetative compatibility tests, already used for the identification of genetically different isolates of pathogenic, saprophytic and ectomycorrhizal fungi (Fries 1987; Sen 1990; Leslie 1993; Dahlberg and Stenlid 1994; Cortesi et al. 1996; Milgroom and Cortesi 1999; Glass et al. 2000). Such tests, carried out on geographically different isolates of *Glomus mosseae*, showed that hyphal interactions between different isolates never produce anastomoses, suggesting their genetic isolation (Giovannetti et al. 2003).

## 4.3 Visualisation and Quantification of Intact Mycelial Networks Spreading from Mycorrhizal Roots

### 4.3.1 Experimental System

This experimental model system has been devised for visualising and quantifying intact extraradical mycelia growing from mycorrhizal roots into the surrounding environment (Fig. 4.4). Surface-sterilised seeds of experimental plants are germinated in moist sterile quartz grit. After 15 days the root system of each seedling is sandwiched between mixed cellulose esters membranes containing germinated spores (obtained as described in Sect. 4.2). The seedlings with the sandwiched root systems are placed into pots filled with sterile quartz grit. Pots are closed in transparent polyethylene bags and maintained in a growth chamber with 24°C day and 21°C night temperature and 16/8 light/dark cycle. After 30 days' growth in the sandwich system, the plants are harvested, the roots are gently removed from

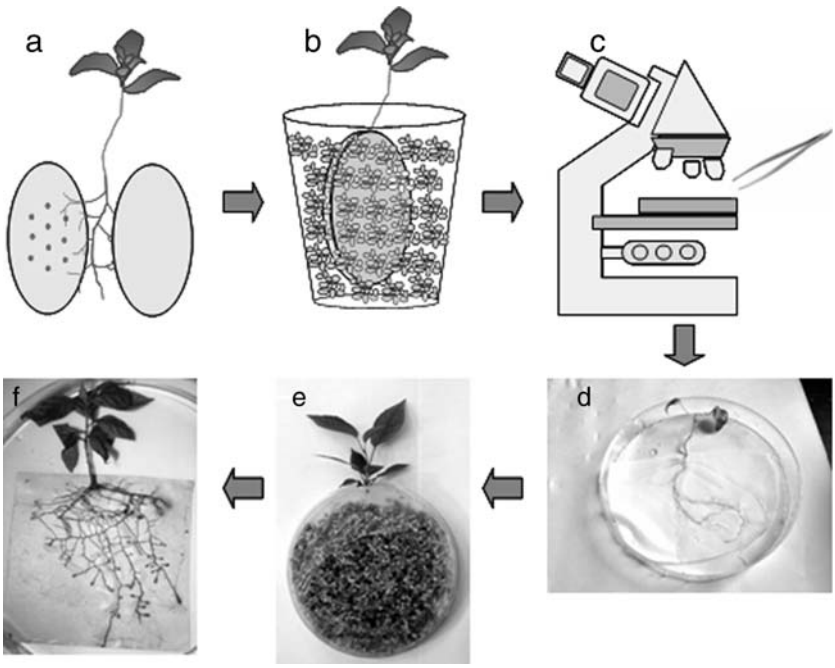


**Fig. 4.4** Schematic representation of the culture system used to study the extraradical mycorrhizal network. Plants are sandwiched with germinated spores grown on membranes and maintained in pots for root colonisation; root-adhering mycelium is plucked from the root system, the plant is transferred onto a new membrane, maintained in petri dish for at least 1 week and eventually stained for extraradical mycelium visualisation and quantification

sandwiches by immersion in water and checked for the occurrence of root-adhering mycelium, which is carefully plucked with forceps under a dissecting microscope. Thus, the experimental system allows the assessment of growth rate and extent of ex novo produced extraradical mycelium. The occurrence of mycorrhizal colonisation is assessed by autofluorescence of intraradical fungal structures in fresh whole roots mounted in water and observed under a light microscope equipped with epifluorescence optics by using blue light. Mycorrhizal colonisation may be confirmed on sample plants by clearing and staining with Trypan blue in lactic acid (Phillips and Hayman 1970).

The roots of each plant are then placed between two mixed cellulose esters membranes, transferred into 14 cm diameter Petri dishes containing sterile quartz grit and maintained in a growth chamber as described above. Seven to 21 days after removing the external mycelium and transplanting, plants are harvested, the root sandwiches are carefully opened and roots and extraradical mycelium growing from the roots on the membranes are stained with Trypan blue in lactic acid (0.05%). Such staining makes the fine network of hyphae extending from colonised roots visible to the naked eye (Fig. 4.5a).

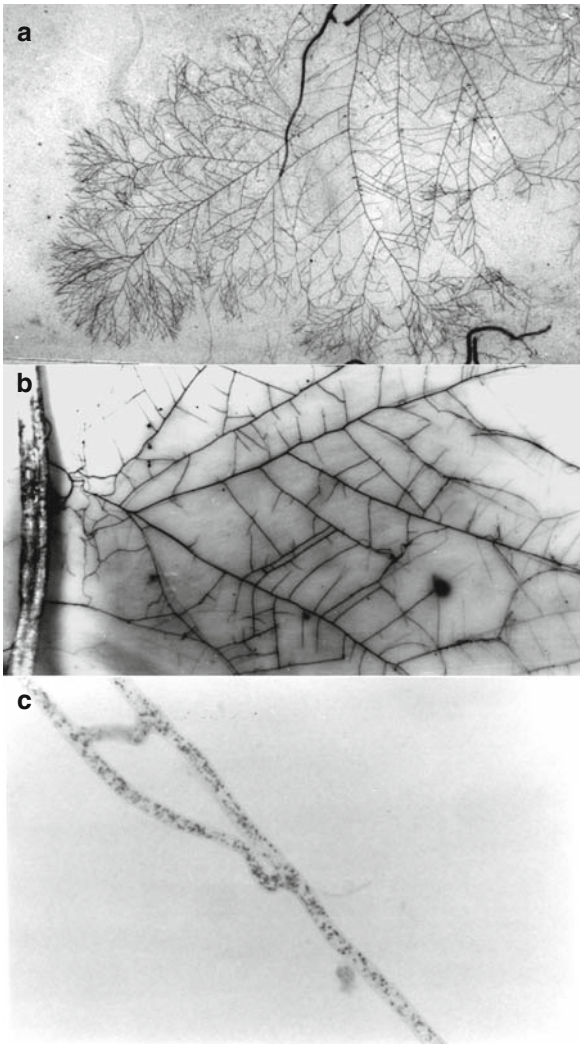


**Fig. 4.5** Light micrographs showing structure and viability (after SDH activity localisation) of the extraradical hyphal network originating from *G. mosseae* colonised plants

### *4.3.2 Quantification of the Extent and Structure of the Mycorrhizal Network*

Hyphal density (hyphal length per square millimetre) is estimated with the gridline intersect method by measuring the length of hyphae in sample areas of the membrane, using a grid eyepiece, under a dissecting microscope (Giovannetti and Mosse 1980) (Fig. 4.5b). Numbers of hyphal contacts, anastomosis and hyphal branches

are counted in sample areas of known size under a light microscope. Frequency of anastomoses is calculated by determining the proportion of anastomosing hyphal contacts. Membrane surface covered by mycelium is determined by using a transparent millimetric grid. Total hyphal length is calculated by multiplying hyphal density by the area covered by the mycelial network. Eventually, the root systems may be removed from the membranes, cleared and stained to assess the percentage of AM colonisation and the total length of the whole root system, by using the gridline intersect method (Giovannetti and Mosse 1980).

### 4.3.3 Viability of the Mycorrhizal Network

To assess the viability of the extraradical mycelium and the establishment of protoplasmic continuity in anastomosed hyphae, SDH activity may be assessed on sample membranes, as described in Sect. 4.2. After SDH staining, the extraradical mycelium is observed under the dissecting microscope, selected areas of membranes are cut, mounted on microscope slides and observed under a light microscope for the presence of formazan salt depositions in hyphal bridges (Fig. 4.5c). Total length of viable mycelium is assessed by using the gridline intersect method.

Occurrence and localisation of nuclei in hyphae is observed by DAPI staining. The root systems of sample mycorrhizal plants are sandwiched between two polycarbonate black membranes placed between two mixed cellulose esters membranes and transferred into Petri dishes containing sterile quartz grit, as described above. On these membranes fungal growth is slower, and an extensive mycorrhizal network may be detected only after 3–4 weeks. After opening the root sandwiches, selected areas of the membranes bearing extraradical mycelium are mounted on microscope slides in DAPI and observed under epifluorescence, by using the UV light.

### 4.3.4 Remarks

The bidimensional model system described above allowed the first visualisation of intact AM mycelium of *G. mosseae* extending from mycorrhizal roots into the extraradical environment: after only 7 days' growth, a fine network of extramatrical hyphae growing on the membranes was visible to the naked eye, and its length extended from 5 to 7 m (hyphal length), in *Thymus vulgaris* and *Allium porrum*, respectively (Giovannetti et al. 2001). Moreover it allowed the assessment of mean growth rate, which ranged from 0.7 to 1.0 m per day, depending on the host plant. Interestingly, a host plant effect has been detected on the development of extraradical mycelium, since hyphal density within network originating from cotton was 6.8 mm $mm^{-2}$, a value statistically different from those of other plant species — 2.9

to 4.1 mm $mm^{-2}$ — in lettuce and eggplant, respectively (Giovannetti et al. 2004). The experimental system evidenced not only that the mechanism involved in the formation of the network was represented by self-recognition and hyphal anastomosis, but it also allowed the determination of different parameters concerning the structure of the mycorrhizal network, such as number of branches (8.6–9.7 per cm of hypha), number of anastomoses per cm of hypha (4.6–5.1), as well as their frequency, 75–78% of hyphal contacts (Giovannetti et al. 2001, 2004). SDH staining showed that the mycorrhizal network was 100% viable after 7 days' growth, confirming previous reports (Schubert et al. 1987; Sylvia 1988; Hamel et al. 1990; Jones et al. 1998). DAPI staining evidenced the viability of the whole network, suggesting that protoplasmic continuity in hyphal bridges may involve also an information flow.

## 4.4 Visualisation of Belowground Links Between Plants of Different Species, Genera and Families

### *4.4.1 Experimental System*

The bidimensional model system devised for visualising intact extraradical mycelia can be modified to show interconnections between mycorrhizal networks originating from different host plants. Mycorrhizal plants, whose extraradical mycelium has been plucked, are obtained as described in Sect. 4.2, their root systems are paired approximately 3 cm apart, and sandwiched between two mixed cellulose esters membranes (Fig. 4.6a). They are then transferred into 14 cm diameter Petri dishes containing sterile quartz grit and maintained in a growth chamber as described. At intervals, the sandwiches are carefully opened, and the development of symbiotic extraradical mycelium spreading from the roots on the membranes is checked, to detect harvest time, i.e. the day when the two mycorrhizal networks come into contact.

### *4.4.2 Occurrence and Frequency of Anastomoses Within and Between Mycorrhizal Networks*

The root sandwiches are opened and the extraradical mycelium growing from the roots on the membranes is stained for the presence of SDH activity. After the secondary Trypan blue staining, different parameters related to the formation of mycorrhizal networks and its structure are assessed (Fig. 4.6b, c). Hyphal density (hyphal length per square millimetre) and number of hyphal contacts and frequency of anastomoses are estimated, as described in Sect. 4.3.

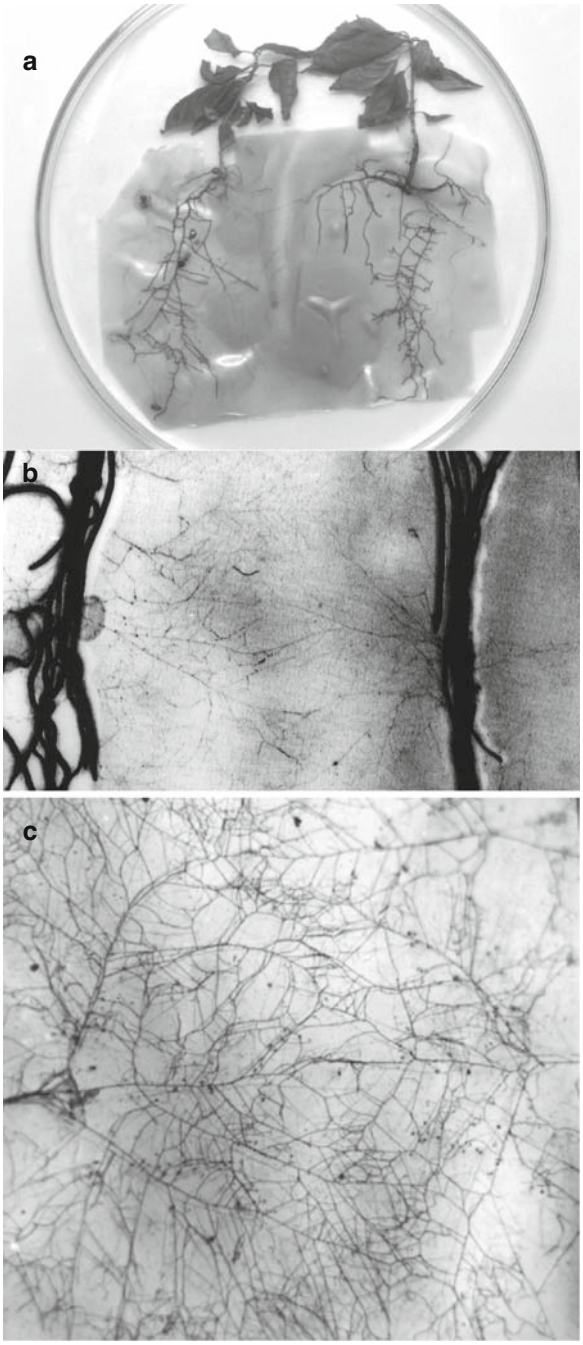


**Fig. 4.6** Schematic representation of the culture system used to study plant interconnections by means of AMF extraradical networks. Colonised host plant roots are paired on membranes (**a**) and the mycelial interconnections are visualised after SDH and Trypan blue stainings (**b**, **c**: courtesy of New Phytologist)

The detection of nuclei in hyphal bridges connecting different mycorrhizal networks can be performed on the mixed cellulose esters membranes utilised in this system, by using DAPI staining as previously described. Alternatively, membranes can be stained with 50 nM solution of Sytox Green (Molecular Probes Europe BV, Leiden, The Netherlands) and observed under epifluorescence using blue light. To detect incompatibility reactions in hyphal contacts, the presence of wall thickenings and retraction septa is investigated on membranes stained with DAPI, followed by Calcofluor White and washed in SDW.

### *4.4.3 Remarks*

The bidimensional experimental system utilised allowed the visualisation and quantification of fusions between mycorrhizal networks spreading from *Allium porrum* (leek) root systems and those originating from *Daucus carota* (carrot) *Gossypium hirsutum* (cotton), *Lactuca sativa* (lettuce), *Solanum melongena* (egg-plant) — after inoculation with the same AM symbiont *Glomus mosseae*. The frequency of anastomosis between mycorrhizal networks originating from the different plant species was high, ranging from 44% in the pairing leek-eggplant to 49% in the pairing leek-cotton, even though lower than that between networks spreading from the same species, leek (62%). No hyphal incompatibility reactions were found in interactions between hyphae connecting different mycorrhizal networks (Giovannetti et al. 2004). The high rate of anastomoses formation between extraradical hyphae spreading from the root systems of different plants suggests that plant interconnectedness may be greater than previously thought (Chiariello et al. 1982; Francis and Read 1984; Grime et al. 1987; Watkins et al. 1996; Graves et al. 1997; Lerat et al. 2002).

## 4.5 Conclusions

In this chapter, we have reviewed the main methods which can be utilised to investigate the structure of pre-symbiotic and symbiotic AM mycelium, to monitor anastomoses formation and to quantify hyphal interconnections in *in vivo* culture systems. Such systems also represent powerful tools for the detection of genetically different individuals within AMF and for investigating the population structure and genetics of these obligately symbiotic ancient asexuals (Gandolfi et al. 2003). The bidimensional experimental system devised could be further implemented to detect and quantify nutrient and carbon transfer in the large network of hyphae interconnecting contiguous plants, which represents a major factor in the distribution of resources in natural plant communities (Perry et al. 1989; Newman and Eason 1993; Pearson and Jakobsen 1993; Simard et al. 1997; Robinson and Fitter 1999).

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# Chapter 5
# Measurement of Net Ion Fluxes Using Ion-Selective Microelectrodes at the Surface of Ectomycorrhizal Roots

**A. Gobert and C. Plassard**

## 5.1 Introduction

Ion-selective microelectrodes are used to measure ion gradients across membranes, but they can serve various purposes (Newman 2001). They are used in animal, plant and even bacteria studies (McClure et al. 1990; Shabala et al. 2001; Kang et al. 2003). Two different kinds of ion-selective microelectrodes can be produced: microelectrodes for net flux measurements — non-invasive — (Gobert and Plassard 2002) and microelectrodes for intracellular studies — impalement needed (Miller et al. 2001). Although we have knowledge of double-barrelled microelectrodes for intracellular studies of ectomycorrhizas, this chapter is aimed at describing the technique of ion flux measurement outside the tissues in plants and ectomycorrhizal roots.

This technique applied to ion fluxes originating from plants is not widely used and arose in the late 1980s and early 1990s (Newman et al. 1987; Henriksen et al. 1990). Ion-selective microelectrodes give access to net flux measurements originating from a few micrometers at the surface of the root of an undisturbed plant. The technique can be easily applied to study the effects of gene mutation or differences between ecotypes or fungal strains. In addition, the effect of biotic (phytotoxins, myc factors) and abiotic factors (temperature, osmotic stress) can be assessed. The technique has been applied on plants for cation measurements such as $K^+$, $H^+$, $NH_4^+$ and $Ca^{2+}$ (Kochian et al. 1989; McClure et al. 1990; Huang et al. 1992; Henriksen et al. 1992) and anion measurements such as $NO_3^-$ (McClure et al. 1990). In our laboratory, we have developed this method to quantify net $H^+$, $K^+$ and

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$NO_3^-$ fluxes occurring along the roots of woody species and ectomycorrhizal roots (Gobert and Plassard 2002, 2007; Plassard et al. 2002). In this chapter, we will present the theory and the practice behind the microelectrodes, followed by some examples of local net flux measurements.

## 5.2 Principle of Ion Activities Measurement with Ion-Selective Microelectrodes

### *5.2.1 What Is an Ion-Selective Microelectrode?*

In our system, ion-selective microelectrodes are made from a glass capillary which has been pulled and silanized. Then the tip is filled with a minimal amount of a liquid membrane (the cocktail) that contains the ionophore selective for an ion. Finally, the remainder of the capillary is filled with a saline solution (the back solution) (Fig. 5.1). The microelectrodes are then connected to an amplifier via a silver wire to measure the electrochemical potential difference of the studied ion in the solution, setting out and inside the microelectrode across the cocktail. Voltage values given by the amplifier are only due to the ion under study because the cocktails are highly specific.

The main component of the cocktail solution is a complexing agent (ligand) responsible for ion selectivity. This agent belongs to the category of ionophores, comprising ion channels and transporters, although only ion transporters are used to make the cocktail (Ammann 1986). The transporter, which is dissolved in an organic phase, diffuses throughout the membrane as complexes with the ion under study, thus carrying the ion from the outside solution (the measuring solution) into the inside solution (the back solution of the microelectrode).

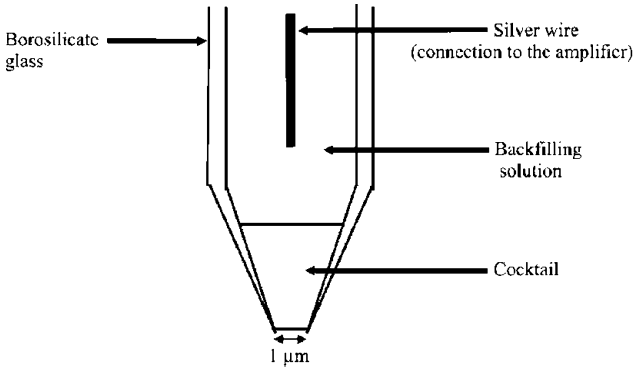


**Fig. 5.1** Schematic representation of an ion-selective microelectrode, with the cocktail containing the ionophore at the tip and the backfilling solution creating the electric connection with the silver chloride wire

The back solution is a salt solution containing chloride ions ($Cl^-$) and the ion under study. Its concentration should be very high to avoid possible variation and to minimize possible errors in ion activity determinations. Generally, KCl is used, but it is possible to use another salt if anion and cation mobilities are as close as possible.

The measurement of the electrochemical potential difference requires movements of small amounts of electrical charges between the aqueous phase (back solution) and the metal phase (the silver wire) constituting an electrode. It is necessary for the charge movement to occur through a reversible, reproducible and dominant chemical reaction at the interface solution/solid metal. The chemical reaction which is generally used is the one occurring at the interface silver/silver chloride, which is as follows:

$$\mathrm{AgCl(s)} + \mathrm{e}^- \rightarrow \mathrm{Ag(s)} + \mathrm{Cl}^-.$$

This reaction supplies a good electrical contact between the silver wire and the solution (Thain 1995). As indicated by the equation given above, the electrical potential measured with the silver/silver chloride electrode will depend on the concentration of available chloride anions in the solution. It is therefore important that this concentration remains constant during the experiment.

### 5.2.2 Expression of the Voltage Difference Across the Cocktail

The system has two distinct compartments: the internal compartment (back solution) with a concentration $C_\mathrm{i}$ and the external compartment (the solution to be measured) with a concentration $C_\mathrm{e}$ In each compartment, the ion electrochemical potential will be

$$\mu = \mu^\circ + V^\circ(P_\mathrm{a} - P^\circ) + RT\ln(C\gamma) + Mgh + nFV, \tag{5.1}$$

with $\mu$ electrochemical potential, $\mu^\circ$ reference electrochemical potential, $V$ voltage, $P_\mathrm{a} - P^\circ$ pressure difference, $R$ constant of perfect gas, $T$ temperature, $C$ concentration, $\gamma$ activity coefficient, $g$ earth force coefficient and $n$ ion valence. In addition, we know that at equilibrium the electrochemical potential of the ion has the same value in both compartments; thus,

$$\mu_\mathrm{i} = \mu_\mathrm{e} \tag{5.2}$$

and

$$\Delta\mu = \mu_\mathrm{i} - \mu_\mathrm{e} = 0. \tag{5.3}$$

After simplification of (5.3) (for detail of calculation, see Ammann 1986), we obtain Nernst's equation, which gives the voltage difference across the two compartments as a function of the concentrations:

$$\Delta V = (RT/nF) \ln (C_e/C_i). \tag{5.4}$$

Therefore, the ideal relationship between electrode output (mV) and the activity ($a_i$) of the ion under study (i) is log-linear, and should give an ideal slope of 59 mV per decade change in the activity of a monovalent ion at 25°C when the electrode is calibrated in the appropriate solution. However, in practice, the situation is more complicated than this, because no ion-selective electrode has ideal selectivity for one particular ion, and under most conditions there is more than one ion present in the sample solution. Hence, contributions to the overall electro-motive force (EMF) made by each interfering ion, $J$, must be taken into account (Ammann 1986). Major interfering ions for each selective membrane are given in the Fluka catalogue. If the measuring solution contains known interfering ions, the selectivity coefficient of the membrane should be determined during the calibration step. For that, the fixed interference method is most commonly used to calculate the selectivity coefficient, and it is the method recommended by the International Union of Pure and Applied Chemistry (Ammann 1986). However, very simplified solutions, with a minimal concentration of interfering ions, can be used when measuring ion fluxes at the root surface, thus minimizing the lack of sensitivity of a given membrane, if it exists.

## 5.3 Principle of Flux Measurement

### *5.3.1 Expression of Local Diffusion Flux in a Solution*

If a coloured solution is poured very gently into a non-coloured solution, we will see the delimited surface between the two liquids which disappears with time. This is due to the diffusion of the coloured solution into the non-coloured one, and vice versa. Similarly, across a transversal section, there are always particles running in opposite directions. The movement of these particles occurs in both directions and will stop only when the mixing is perfect. However, the diffusion vector $J$ is orientated towards the decreasing potentials. The equation that describes the diffusion is Fick's law:

$$J = -D(C_1 - C_0)/(x_1 - x_0) = -D\Delta C/\Delta x, \tag{5.5}$$

with $J$ local flux of diffusion, $D$ diffusion coefficient of the ion of interest, $C_1$ ion concentration at the distance $x_1$, $C_0$ ion concentrations at the distance $x_0$.

### 5.3.2 Estimation of Ion Fluxes at the Root Surface

When a root is placed in a solution where ions are moving by diffusion and where the coordinates of radial symmetry apply (Fig. 5.2), the net radial ion flux is given by the following equation (Newman et al. 1987; Henriksen et al. 1992):

$$J = \frac{2\pi D(C_2 - C_1)}{\ln(r_2/r_1)} \quad (\text{in mmol cm}^{-1}\ \text{s}^{-1}), \tag{5.6}$$

with $D$ diffusion coefficient ($\text{cm}^2\ \text{s}^{-1}$), $C_1$ concentration at a radial distance $r_1$, $C_2$ concentration at a radial distance $r_2$, $r_1$ and $r_2$ radial distances from the cylinder centre.

The ion flux crossing the transversal root section of area $A$ and with a root density $\rho$ (that we take by default equal to 1) for 1 h will be

$$J = \frac{k(2\pi D/A\rho)(C_2/C_1)}{\ln(r_2/r_1)}, \tag{5.7}$$

$$J = \frac{k(2D/r^2)(C_2/C_1)}{\ln(r_2/r_1)}. \tag{5.8}$$

Finally, we obtain the following equation used to calculate the ion flux occurring at the root surface:

$$J = \frac{k(2D/r^2)(C_2/C_1)}{\ln[(d_2 + r)/(d_1 + r)]} \quad (\text{in } \mu\text{mol}\, g^{-1}\ \text{root fwt}^{-1}\ \text{h}^{-1}), \tag{5.9}$$

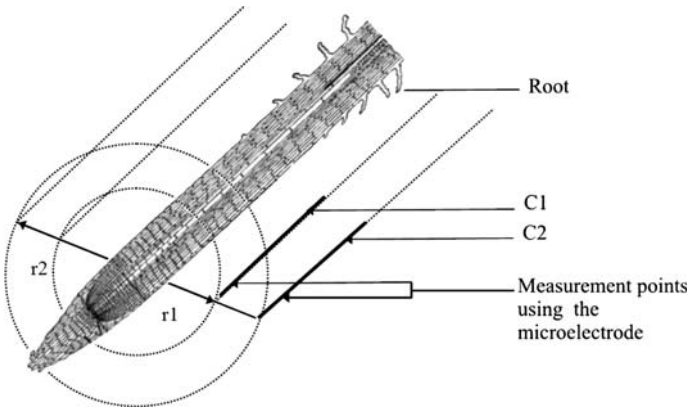


**Fig. 5.2** Schematic representation of the radial symmetry of ion diffusion occurring in the solution at the surface of the root

with $k$ coefficient for unit conversion, $r$ root radius, $d_1$ and $d_2$ measuring distances from the root surface (Fig. 5.2).

## 5.4 Equipment and Microelectrode Fabrication

### *5.4.1 Experimental Set-up*

#### 5.4.1.1 Electric Circuit

The use of ion-selective microelectrodes needs a controlled environment against electric, electromagnetic and vibration interference, because the measurements are in the pico-Amp value range. The measurements are realised in a Faraday cage on an antivibration table in order to reduce background noise. The measurement set-up is composed of an electric circuit with several resistances (Fig. 5.3). The main resistance is included in the high impedance amplifier (A) (1,015 Ω). The potential difference between the flowing solution and the backfilling solution of the microelectrode is the generator. The microelectrode ($M_e$, whose resistance is $R_e$) is fixed on a headstage giving a gain of 0.0001 M (Rpa) before entering the amplifier with resistance $R_a$. The circuit is closed by the pH meter electrode, which acts as a reference electrode ($M_{ref}$).

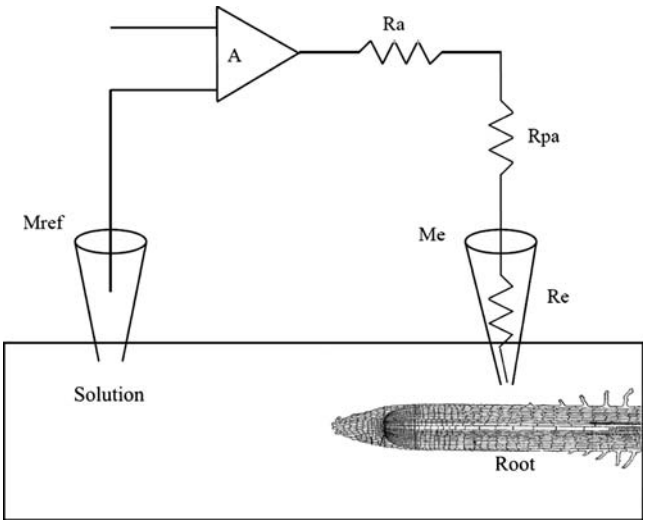


**Fig. 5.3** The measurement set-up of an ion-selective microelectrode

#### 5.4.1.2 Equipment Set-up

As described above, specific equipment is needed in order to carry out the flux measurements. Indeed, the current throughout the cocktail is very small and a high impedance electrometer amplifier and background noise protection are needed.

#### 5.4.1.3 Equipment Needed

Faraday cage sitting over an "antivibration" table (home-made)
Microscope (e.g. ×180, from Ealing, http://www.ealingcatalog.com/) with a fitted live camera fixed on the table (i.e. from Sony, http://www.sonybiz.net/)
Manual micromanipulators (where the microelectrode holders are fixed, e.g. from Narishige, http://www.narishige.co.jp/)
Computer-assisted stage (where the measuring chamber is placed; e.g. from Ealing)
Amplifiers (e.g. Axoprobe-1A, Axon Instruments, now available at http://www.moleculardevices.com/)
Interface (e.g. MacLab/8e, ADInstruments, http://www.adinstruments.com/)
Computer with acquisition software (e.g. Chart V3.3.7, ADIntruments)
Monitor (live feed from the camera fixed to the microscope; e.g. Sony)
pH meter and electrode (e.g. Metrohm)
Optic fibre light source
Bottles and tubing for the solution entrance and exit from the chamber
The set-up is virtually described in Fig. 5.4, with equipment placed inside and outside of the Faraday cage.

#### 5.4.1.4 Inside the Faraday Cage

On the "antivibration" table, the microscope and the computer-assisted stage are the main features. A camera is fixed at the back of the microscope. The table supports some posts with clamps to fix the microelectrode holders (HS-2 Headstage, Axon Instruments, 0.0001 M gain). All the cables are earthed.

The chamber holding the root is mounted on the computer-assisted stage. It is designed so that the root system can be continuously perfused with the experimental nutrient solution throughout the measurement. The waste solution coming out of the chamber is collected in a plastic beaker in the cage.

#### 5.4.1.5 Outside the Faraday Cage

All the electronic equipment is outside the cage. The electric signal coming from the electrodes is sent to a high-impedance electrometer amplifier (1,015 Ω) (Axon Instruments, Inc. 1993). Then, the electrometer output is directed via an A/D

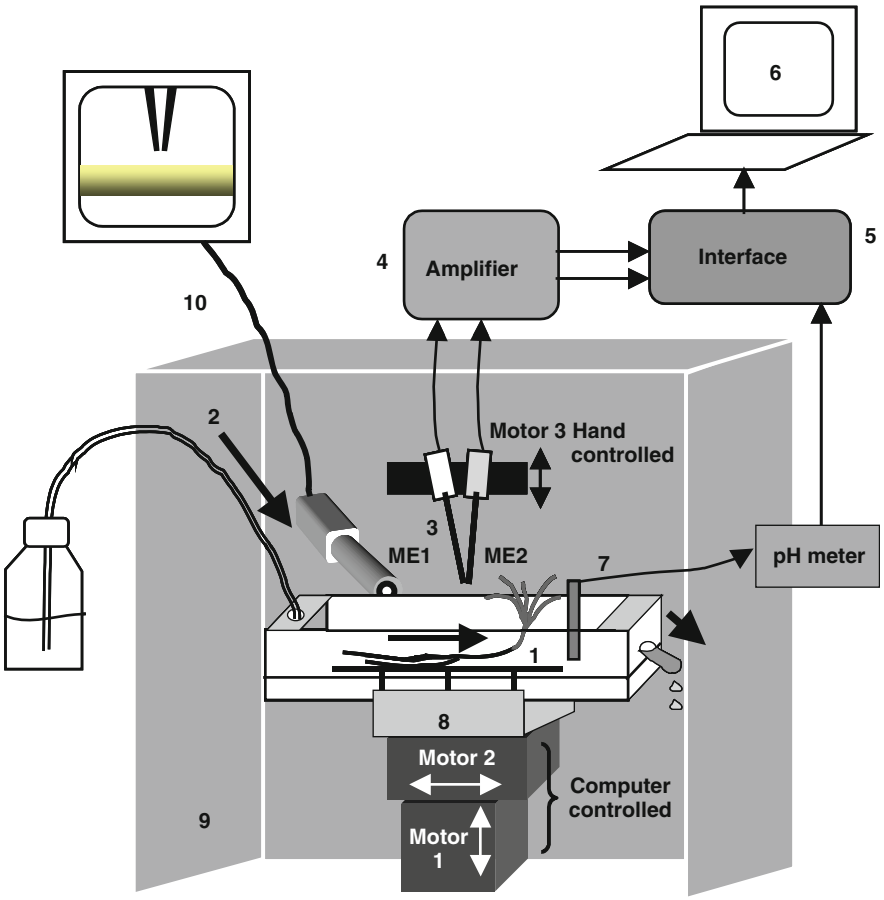


**Fig. 5.4** Experimental set-up for continuous monitoring of ion fluxes using ion-selective microelectrodes: (*1*) Intact plant in a perspex cuvette, (*2*) flowing solution, (*3*) ion-selective microelectrodes plugged in headstages and moved by a hand-controlled motor, (*4*) amplifier, (*5*) interface (McLab), (*6*) computer (MacIntosh), (*7*) combined pH-macroelectrode, (*8*) mobile plate moving the cuvette using computer-controlled motors, (*9*) Faraday cage, (*10*) monitor connected to the camera

converter to a computer. The circuitry is terminated through a pH meter submerged in the chamber.

In addition, the camera monitor and the light supply are outside the cage.

Finally, the perfusing solution is made just prior to use (the pH is carefully set in order to avoid discrepancy between the different concentrations). The solution is bubbled with decarbonated air (air going though soda lime and boiled water) to obtain an $HCO_3^-$ free solution. The bottles of solution stand outside the cage at a higher position than the chamber, and the liquid moves with gravity towards the chamber (into tubing with a tap). The flow is reduced using a low-diameter needle plunged into the chamber.

## 5.4.2 Making the Microelectrodes

### 5.4.2.1 Pulling of Glass Micropipettes

In our set-up, the microelectrodes are made from borosilicate glass. The borosilicate glass tubes have to be pulled into two equal pieces to create the microelectrodes.

### 5.4.2.2 Equipment Needed

Borosilicate capillaries (GC150F-10, Clark Electromedical Instruments, Pangbourne, UK) two-step puller (provider).

One extremity of the borosilicate tube is fixed at each side of the puller, the bottom mortar being lifted.

Then, a two-step protocol is applied to produce microelectrodes of the desired size (1–2 μm diameter in our case). An estimate of the tip size of the microelectrode is determined by measuring its electrical resistance when filled with 3 M KCl. Smaller tips have higher resistances than larger tips, but the dimensions of the microelectrodes are usually a compromise between obtaining a stable membrane potential and a good calibration response (detection limit).

## 5.4.3 Internal Silanization of the Pulled Borosilicate Tube or Microelectrode

The inside of the glass micropipettes must be given a hydrophobic coating, to allow the formation of a high resistance seal between the glass and the hydrophobic ion-selective membrane.

### 5.4.3.1 Equipment Needed

Fume hood (for all silanizing agent and solvent use)
Oven reaching 180°C (in a aerated area or in the fume hood)
Micropipette holder (home-made in our case, Fig. 5.5)
100-μl glass syringe
Silanizing agent (e.g. *N*, *N*-dimethyltrimethylsilylamine or tributylchlorosilane, from Fluka)
Tetrahydrofurane (for rinsing the syringe after silicone use)

First, carefully place the extremity of the microelectrodes in the hole in the holder (tip to the sky). The holder has an inside cavity where the silanizing agent can be poured; this cavity is linked only to the inside of the microelectrodes. When placing

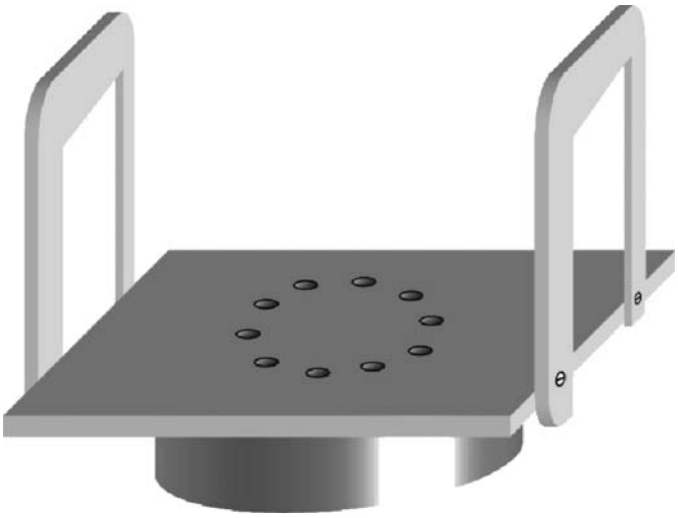

**Fig. 5.5** Home-craft microelectrode handler for silanization of the internal surface of the microelectrodes

the microelectrodes, one hole remains empty of microelectrode in order to pour the silicon. It may be closed with a nail. The holder is then placed for 1 h into an oven set at 150°C in order to get rid of any traces of humidity that could interfere with the process of silanization. In the fume hood, 40 µl of silanizing agent is sucked into a glass syringe. The holder is taken from the oven to the fume hood and the silicon-based compound is injected into the hole by lifting the nail with forceps (beware — the holder is very hot). The hole is immediately sealed with the nail once again, and the holder placed into the oven set at 130°C for 1 h. The silicone vapours condense on the wall of the pipettes. The holder can be removed from the oven and left to cool on the bench.

## *5.4.4 Backfilling the Pipette*

The microelectrodes are filled at the tip with the cocktail containing the ion-selective ionophore and then 24 h later backfilled with an aqueous solution. The microelectrodes are then ready for use.

### 5.4.4.1 Equipment Needed

Dissecting microscope
Flame-elongated borosilicate tubes with very thin diameter in order to rich the tip of the microelectrode from its other end

10-ml syringe extended with a silicon-based tube whose inner diameter matches the outer diameter of the borosilicate tubes
Ion-selective cocktails (ready-to-use, e.g. Fluka or home-made)
Salt solution ($KNO_3$, KCl, . . .), ultrapure water and pH meter

In our laboratory, we use $H^+$, $K^+$ and $NO_3^-$ microelectrodes. $H^+$ microelectrodes are filled with a ready-to-use cocktail (Fluka 95297), as are our $K^+$ microelectrodes (Fluka 60398). $NO_3^-$ microelectrodes are filled with a home-made cocktail comprising MTDDA $NO_3^-$ (8.3%), MTPPB (1.4%) and NPOE (90.3%).

These cocktail solutions are sucked up into the rod from the flame-elongated tube with the syringe (the borosilicate tube being connected to the silicon base tube). You need to pull the syringe hard to fill only a few micrometers of the rod. This rod is then inserted into the extremity of the microelectrode (it is better to use a magnifier) and moved towards the tip of the microelectrode. When it has reached the tip, push the syringe to expel a minimum amount of cocktail into the tip, and remove the rod carefully. The microelectrode is placed almost horizontally (tip part being slightly upward) into a silica-gel-dried sealed container in the dark in order to avoid dust and preserve the quality of the cocktail. The cocktail is going to move upward to the tip by capillarity (you need to wait overnight at least).

Next day, the backfilling solutions can be added (usually just before use). The solutions are buffered at pH 6 and comprise 0.3 M KCl, 0.01 M TRIS/MES for $H^+$, KCl 3 M for $K^+$ et $KNO_3$ 0.1 M, KCl 0.1 M for $NO_3^-$. Before using the microelectrodes, we break the tip carefully in order to increase the tip diameter to 5 µm. It gives a better and faster response from the microelectrodes even if their lifetime is reduced. The microelectrodes are finished and ready for calibration.

## *5.4.5 Calibration*

Ion-selective microelectrodes can be calibrated using concentration or activity; the electrodes actually respond to changes in activity. For these reasons, the calibration of microelectrodes generally uses solutions that match the extracellular environment which is going to be used during the measurements. In our case, the ion concentrations are low and the activity coefficient can be approximated to 1. The ideal relationship between electrode output (mV) and the concentration (mM) of the ion under study is log-linear, and is described mathematically by the Nernst equation (see Sect. 5.2.1).

### 5.4.5.1 Equipment Needed

Salt solutions ($KNO_3$, $CaSO_4$) and pH buffers (5, 6, 7)
Electrophysiology equipment (see Sect. 5.5)
Ag/AgCl metal electrodes (in the base of the microelectrode holder)

First, chloride must be added on the surface of the silver rod in order to create a good electric connection. The silver rod is immersed into an HCl 0.1 M bath while

connected to a 1.5 V battery. A second silver rod in the solution ends the loop. The silver rod connected the positive side is recovered by a chloride coating.

When the Ag/AgCl electrode is ready, place the microelectrode into its holder. Then, you need to dip the electrode into the "zero" solution, which is basal $CaSO_4$ 0.2 mM in our case (the pH electrode ends the loop). The voltage can be recorded (value 1). Then, we record the voltage values for $KNO_3$ 0.05, 0.25 and 0.5 M (values 2, 3 and 4). The data are plotted ($V = fct \log_{10} [KNO_3]$ for $K^+$ and $NO_3^-$) to get *y*-intercept and slope values. The slope of the linear regression should be close to 59 mV for a perfect microelectrode at 25°C. Microelectrodes can be discarded if the slope is less than 55 mV. However, as slopes are temperature-sensitive, always carry out the calibration at the same temperature as the measurement. For calibration of pH microelectrodes, a set of standard pH buffers can be used and simply checked with a pH meter.

### *5.4.6 Microelectrode Selectivity*

In order to determine the selectivity of the microelectrodes produced, they were subjected to a gradient of ion concentration. In the following example (Fig. 5.6), the microelectrodes were immersed into an increasing concentration of $KNO_3$ (from 1 µM to 0.1 M) with $CaSO_4$ 0.2 mM as background solution. The pH was set at 5.8 in each solution. The voltage values obtained at the voltmeter amplifier were plotted against the log values of the concentrations. The response of the microelectrode should be linear. The limit of detection is where the measurement shifts from the linear range. In our case, the detection limits for our $K^+$ and $NO_3^-$ microelectrodes are between 1 and 10 µM. Our studies are therefore realised in a concentration range above 10 µM $KNO_3$ in the solution.

The microelectrodes are ready for net ion flux measurements at the surface of the roots or mycorrhizal roots.

## 5.5 Setting up the Electrophysiological Measurements

The measurement of the ion net fluxes at the surface of the root is determined by (5.9), as described in Sect. 5.3 of this chapter, which is

$$J = \frac{k(2D/r^2)(\mathrm{C}_2 - \mathrm{C}_1)}{\ln[(d_2 + r)/(d_1 + r)]}.$$

With the microelectrodes, we measure the ion concentrations $C_1$ and $C_2$ at the distances $d_1$ and $d_2$ from the root surface. The radius of the root is determined by measuring the root diameter on the monitor linked to the camera. The conversion factor is known to translate into the actual root diameter, which is subsequently divided by two. The coefficients $k$ and $D$ are invariable in our conditions of

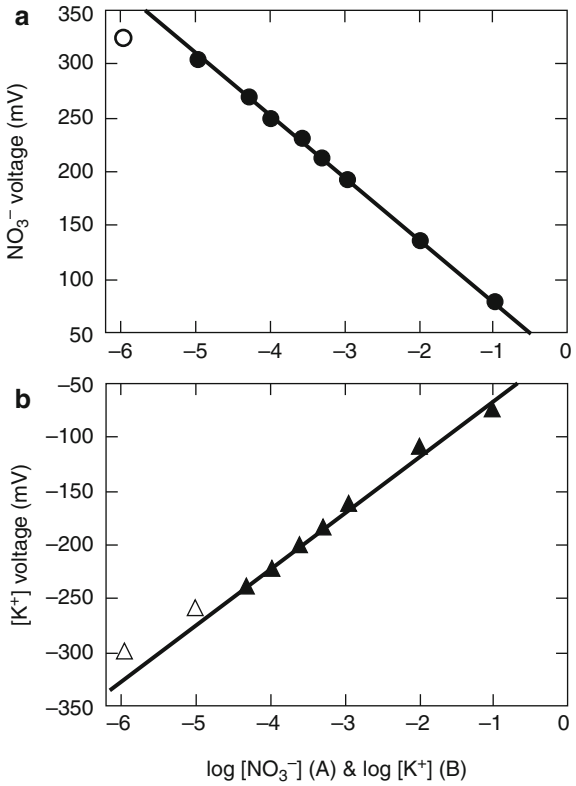


**Fig. 5.6** Selectivity of the $NO_3^-$ (**a**) and $K^+$ (**b**) microelectrodes. The microelectrodes were immersed in $CaSO_4$ 0.2 mM solutions containing increasing concentrations of $KNO_3$. The potential values were then plotted vs the log [ion] in the solution. A linear regression was plotted on the points to determine the selectivity of the microelectrodes

experimentation. The only factors modifying the flux values are the distance from the root and the concentration (microelectrodes).

So, which distances from the root surface to choose?

And which parameters can affect the calculation of the ion concentrations?

## *5.5.1 Determination of the Distances from the Root Surface for the Measurement Points*

### 5.5.1.1 Concept of the Undisturbed Layer and the Ionic Gradient

In our system, the woody seedling lies at the bottom of a small container (see Fig. 5.4). This small container has a solution entry on one side and a solution exit at the other side. The solution flows on top of the seedling and can be changed at

any time (i.e. by increasing the ion concentration). The flow has to be small enough in order to replace the solution far away from the root without disturbing the solution layer at the surface of the root. The solution at the surface of the root does not flow, or does so at a negligible speed due to friction forces (like the wind with an object). The undisturbed layer size has to be determined using the microelectrodes and the net flux measurements have to occur in this undisturbed layer of solution.

In order to determine the size of the undisturbed layer, the microelectrodes were moved from 10 to 1,510 µm with 100 µm steps. In the undisturbed layer of solution, the ion concentrations form a linear gradient (Fig. 5.7). If the solution flow slowly increased from the root surface (no layer), the points would form a curve. The limit of the gradient can be calculated from the intersect between the final concentration and the slope of the gradient (Fig. 5.7).

Then, we measured the average distance at which the gradient disappears (Table 5.1) along the taproot of a Corsican pine seedling. The shortest distance from the root where the gradient disappeared was around 600 µm. Measurement along the root of maritime pine gave the same order of values (data not shown).

The limit of the gradient determined, two points in this radius are needed in order to determine the net flux. Of course, the distance between the two measurement points is important for the calculation of the net flux [$d_1$ and $d_2$ in (5.9), Sect. 5.3.2]. The greater the distance, the greater the chance that a difference of concentrations can be detected. The larger the difference in concentration, the more accurate is the final value of the net flux.

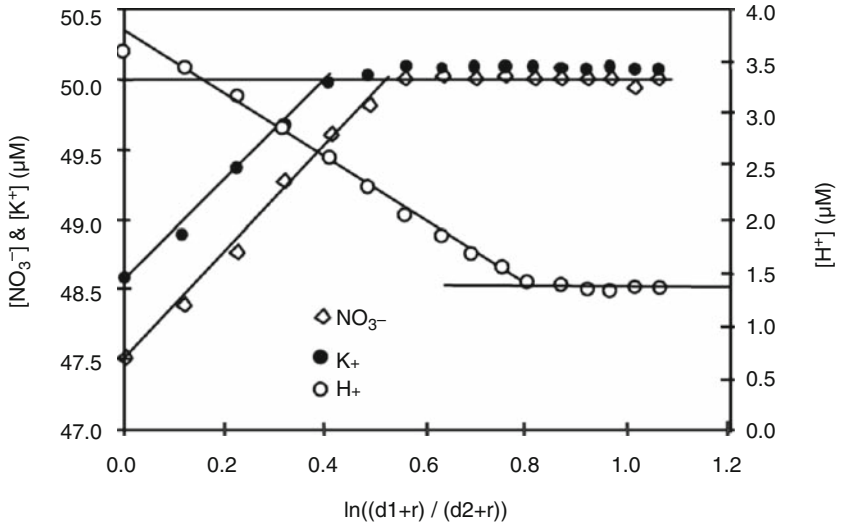


**Fig. 5.7** Gradients of $NO_3^-$, $K^+$ and $H^+$ concentrations from the surface of the root (~0.01 mm) to the bulk solution (~2 mm)

#### 5.5.1.2 What is the Distance from the Root to Choose?

Our next task was to determine which distance from the root was right to determine the net fluxes. Of course, the points of measurement are localized in the undisturbed layer of solution (<600 μm; Table 5.1). The distances 300, 400 and 500 μm were tested all along the first 10 mm of the taproot of a Corsican pine seedling.

As shown in Fig. 5.8, the first 2 millimetres of the root provide a huge variation in the net flux measurement for all the microelectrodes. Thus, for $NO_3^-$, the values for 300 μm are divergent from the values taken at 400 and 500 μm. For $H^+$ and $K^+$, values are very similar to each other whatever the distance from the root. We can observe a huge efflux of $H^+$ at the tip of the pine root which reduces strongly after 1–2 mm from the apex. For $K^+$, a strong efflux appears at 2 mm and reduces gradually towards 6 mm from the apex.

### 5.5.2 *Factors Affecting the Calculation of the Concentration*

The calculation of the ion concentration is determined by (5.4) described in Sect. 5.2.2 of this chapter, and this can be transformed to

$$C_e = C_i \exp(-nF\Delta V/RT). \tag{5.10}$$

Therfore, the potential at the microelectrode is the main factor to be studied. $C_e$ can be $C_1$ and $C_2$ depending on the distance from the root surface. The microelectrodes have a time response to changes in the solution. Also, this time response is fast, making an average of the concentration over a short length of time when the potential values are stable more acceptable as a basis for calculating the concentration at a given distance.

**Table 5.1** Size of the undisturbed layer and/or size of the gradient at the surface of the first 10 mm of the taproot of a Corsican pine seedling

| | Size of the undisturbed layer (μm) | |
|---|---|---|
| Distance from the root tip (mm) | $NO_3^-$ | $K^+$ |
| 0.2 | 784 ± 163 | 822 ± 203 |
| 1 | 813 ± 149 | 1,068 ± 181 |
| 2 | 645 ± 121 | 716 ± 129 |
| 3 | 853 ± 213 | 837 ± 170 |
| 4 | 762 ± 183 | 729 ± 278 |
| 5 | 1,086 ± 201 | 744 ± 174 |
| 7 | 758 ± 183 | 602 ± 116 |
| 10 | 1,284 ± 107 | 1,179 ± 302 |
| Average | 879 ± 79 | 837 ± 73 |

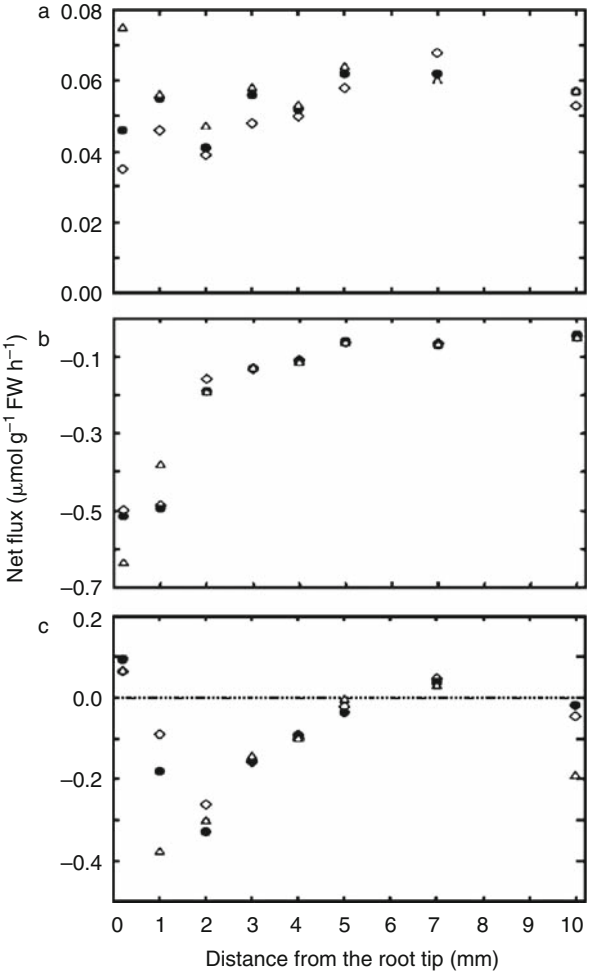


**Fig. 5.8** Net ion fluxes of $NO_3^-$ (**a**), $H^+$ (**b**) and $K^+$ (**c**) determined from measurement points 10–310 µm (*diamonds*), 410 µm (*filled circles*) or 510 µm (*triangles*) along the first 10 mm of the taproot of a Corsican pine seedling

#### 5.5.2.1 Setting up the Recording Program

A stable voltage reading is needed at one given measurement point. In our calculation, we always take the average of several values (A in Fig. 5.9) at one point (2 in Fig. 5.9) and the average of four passages at one point (1–4 in Fig. 5.9).

From this chart, we can observe that the experimenter can increase the number of concentration values at each point and the number of passages at each point. If a discrepancy in the concentrations between the four point measurements appears,

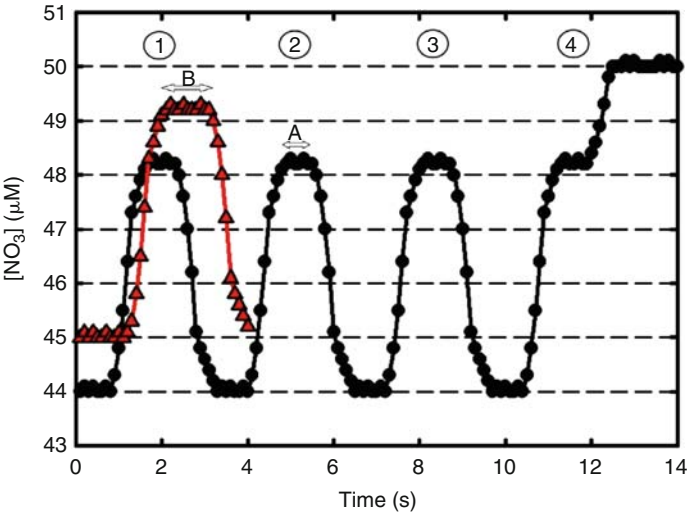


**Fig. 5.9** Chart recording plotting the concentration vs time (each plateau correspond to a measurement point, here 10, 410 and 2,010 μm). *Closed circles:* a full plot with a time of measurement (*A*); *grey triangles:* a partial plot with a time of measurement ($B > A$). In the full plot (*A*), we can observe the four time measurements at 410 μm from the root (labelled from *1* to *4*)

there is obviously something wrong (e.g. the microelectrode might have touched the root, creating a leakage from the broken cells at the surface of the root).

#### 5.5.2.2 Effect of the Recording Time on the Flux Calculation

When small variations in the ionic concentrations are measured, a small error in the concentration difference ($C_2 - C_1$) results in a large variation in the net flux value *J* (5.9). We tested two different durations of measurement at one point, 15 and 20 s, when measuring $NO_3^-$ and $K^+$ net fluxes at the surface of the first 10 millimetres of the taproot of a Corsican pine seedling (Fig. 5.10). Ion fluxes at the root surface of this species are usually smaller than for the maritime pine, and a variation in the concentration difference ($C_2 - C_1$) is surely going to affect the resulting net flux.

The ratios of the net $NO_3^-$ (A) and $K^+$ (B) fluxes between 15 and 20 s are plotted in Fig. 5.10. The variations of $NO_3^-$ net fluxes are generally low (except for one point) and the data are not significantly different from one another, indicating that increasing the recording time did not improve the flux calculation. For $K^+$, we always observed a large variation of ion fluxes which probably explains the large variation of ratios shown in Fig. 5.10b. These variations could be due to a periodicity in intensity at one given point along the root, as shown in maize roots for $H^+$ and $Ca^{2+}$ (Shabala et al. 1997). Also, we noticed that $K^+$ microelectrodes have a much shorter life than $H^+$ or $NO_3^-$ microelectrodes, hours ($K^+$) compared with days ($H^+$ and $NO_3^-$).

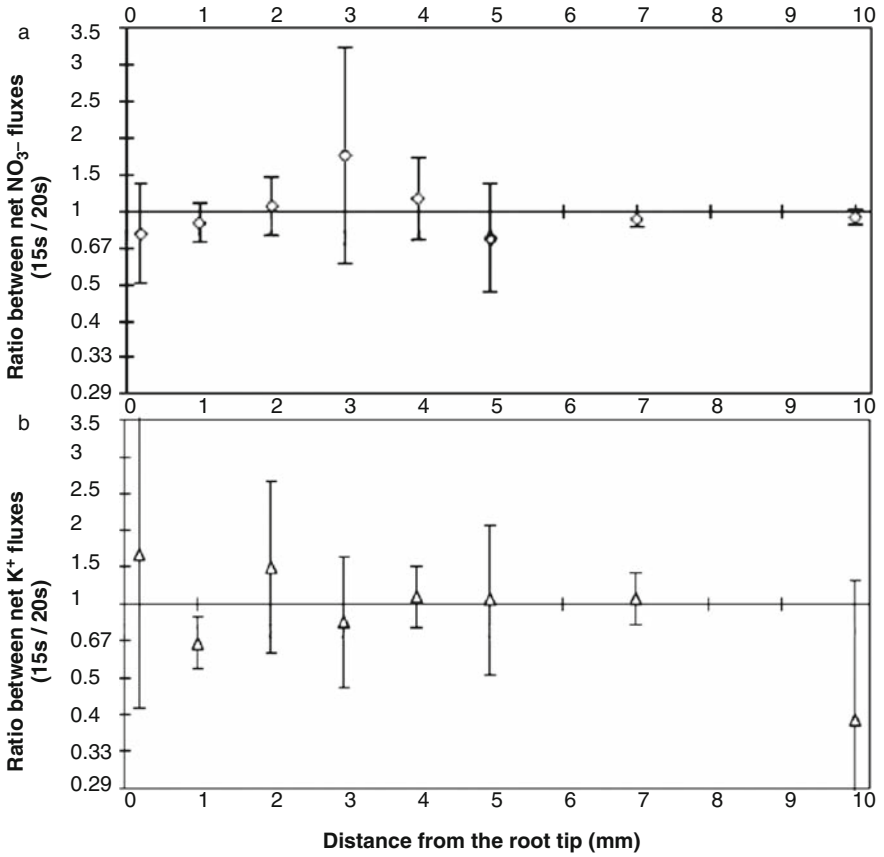


**Fig. 5.10** Effect of the duration of the measurement (15 or 20 s) on the calculation of the $NO_3^-$ (**a**) and $K^+$ (**b**) net fluxes. The values are expressed as the ratio between the values obtained with a 15-s step and the values obtained with a 20-s step as a function of the distance from the root tip of the Corsican pine seedling

Obviously, the longer the duration at one point, the more accurate should be the average of the potential values at this point but this matter has to be tested at least once for an experience condition given for the different type of microelectrode used.

### *5.5.3 Using a Complex Perfusing Solution*

In our system, we always use a simple perfusing solution containing $CaSO_4$ 0.2 mM, and then we add the required concentration of $KNO_3$ (in order to measure the $H^+$, $NO_3^-$ and $K^+$ net fluxes). Of course, we have tested the effect of the pH on the responses of the $NO_3^-$ and $K^+$ microelectrodes (data not shown). Extreme

pH affects the responses, but our solutions are always set around 5.8–6. If other salts have to be added to the perfusing solution, the response of the microelectrodes to these additional ions has to be tested and the microelectrode should be calibrated in a solution containing these additional ions.

## 5.6 A Case Study: Measurement of $NO_3^-$ Net Fluxes into Ectomycorrhizal Short Roots

### *5.6.1 Validation of Flux Measurements into Coniferous Plants*

When using ion-selective microelectrodes for measuring net fluxes occurring into the roots, one of the main problems is to find some ways to evaluate the validity of this methodology. We did this in young maritime pine seedlings exhibiting only one main tap root (Plassard et al. 2002). These non-mycorrhizal young roots exhibited highly differentiated root zones together with highly different nitrate and potassium net fluxes measured using ion-selective microelectrodes. Potassium net uptake was characterised by a maximum net influx in the subapical zone (the "pink" zone), together with a high net efflux or no net flux occurring in the first millimetres of apex and in the browning zone far away from the apex, respectively. Nitrate net uptake was also maximal in the subapical root zone, but no net efflux was observed in the apical part, and low values of net influx were measurable in the browning root zone (Plassard et al. 2002). To validate these local measurements we measured nitrate and potassium uptake by the whole seedlings incubated in the same conditions using conventional methods (bulk medium depletion). The net uptake rates calculated from the integration of net ion fluxes with microelectrodes were found to be remarkably similar to those calculated from medium depletion (Fig. 5.11), validating our measurement conditions.

Another validation of this methodology was obtained at the level of ectomycorrhizal short roots and $^{15}N$ accumulation in *Pinus pinaster* plants associated with the ectomycorrhizal fungus *Rhizopogon roseolus*. Net $NO_3^-$ fluxes measured at the surface of *R. roseolus* mycorrhizal short roots were twice as high as those measured at the surface of non-mycorrhizal short roots in plants incubated with a flowing solution containing $KNO_3$ 50 µM (Gobert and Plassard 2007). This enhancement of $NO_3^-$ uptake into mycorrhizal roots was confirmed by incubating NM and M plants for 6 h in a solution containing $^{15}NO_3^-$ 50 µM. In these conditions, the $^{15}N$ rates measured were 1.2 ± 0.2 and 0.7 ± 0.15 µmol $^{15}NO_3^-$ $g^{-1}$ root dry. wt $h^{-1}$, respectively, in M and NM plants. Total amounts of $^{15}N$ measured in mycorrhizal plants were linearly correlated with the concentration of ergosterol, a sterol extracted from living fungal cells (Fig. 5.12). The contribution of ectomycorrhizal roots to the total uptake of $^{15}NO_3^-$ into the intact plants was then calculated from the value of the slope of the linear regression fitted to these data. This contribution was estimated to be 2.5 µmol $^{15}NO_3^-$ accumulated $mg^{-1}$ of ergosterol $h^{-1}$ whereas

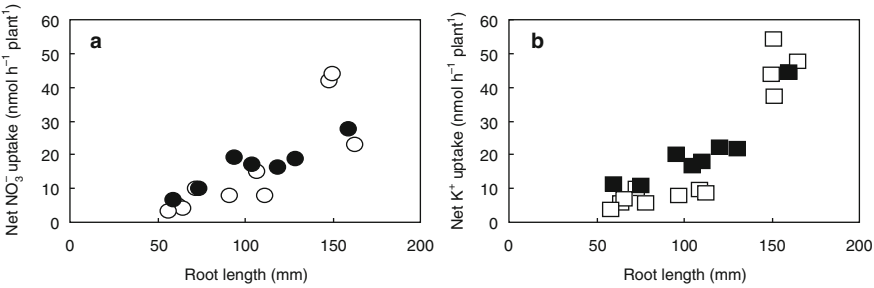


**Fig. 5.11** Net uptake rates of $NO_3^-$ (**a**) and $K^+$ (**b**) determined in whole roots of young non-mycorrhizal *P. pinaster*. Three-week-old plants presenting only one root, whose length varied from *c.* 50 to *c.*160 mm, were incubated in a solution containing 0·2 mM $CaSO_4$ and 20 µM $KNO_3$ (pH 5·7). Uptake rates were measured either from ion depletion from the medium (*open symbols*) or using ion-selective microelectrodes (*closed symbols*). In the latter case, local flux data obtained along the root were integrated over the whole root length to obtain net uptake rates per plant (redrawn from Plassard et al. 2002, with permission from Blakwell Publishing)

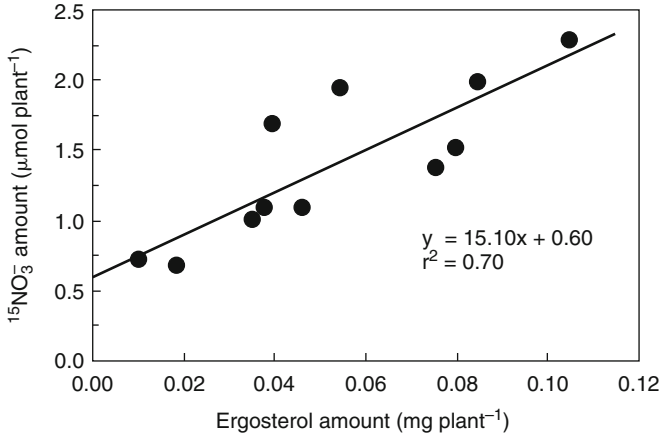


**Fig. 5.12** Relationship between root ergosterol and total $^{15}N$ amounts measured in *P. pinaster* associated with the ectomycorrhizal fungus *R. roseolus*. The plants were pre-incubated for 3 days in 1 mM $NO_3^-$ solution before incubation for 6 h into the labelling solution containing 50 µM $^{15}KNO_3$. The linear regression fitted to the points was highly significant ($P$ = 0.0012) (redrawn from Gobert and Plassard 2007, with permission from Blackwell Publishing)

the contribution of the remaining root system was calculated to be 0.6 µmol $^{15}NO_3^-$ per plant or 0.5 µmol $^{15}NO_3^-$ $g^{-1}$ root dry. wt $h^{-1}$ (the *y*-intercept). The $NO_3^-$ uptake rate into ectomycorrhizal roots ($V$, µmol $NO_3^-$ $g^{-1}$ f wt $h^{-1}$) was thus calculated using the equation:

$$V = (vE)(F),$$

where $v$ is the uptake rate per unit of ergosterol (μmol $NO_3^-$ $mg^{-1}$ ergosterol $h^{-1}$), $E$ is the average concentration of ergosterol in the fungus in pure culture (0.34 mg $g^{-1}$ f wt, Plassard, unpublished data) and $F$ is the percentage of fungal tissue in ectomycorrhizal roots (62%, Plassard et al. 2002). Using the parameters in the above equation, the estimated value of $NO_3^-$ uptake rate into mycorrhizal short roots was calculated to be 0.53 μmol $NO_3^-$ $g^{-1}$ f wt $h^{-1}$, a value that was very close to the one measured with an ion-selective microelectrode that was 0.6 μmol $NO_3^-$ $g^{-1}$ f wt $h^{-1}$ (Gobert and Plassard 2007).

### 5.6.2 *Variation of $NO_3^-$ Net Fluxes into Ectomycorrhizal Short Roots: Effect of N Source Supplied to the Plants*

Several studies have indicated that $NO_3^-$ uptake by forest trees and their fungal partners may be affected differently by variable conditions of $NO_3^-$ availability that can occur in natural conditions, e.g. temporary variations of soil nitrate or ammonium concentrations and their ratios. We addressed this question by carrying out a comparative study of $NO_3^-$ uptake induction under various conditions, with or without $NH_4^+$ supplied at 50 and 500 μM, two concentrations close to the actual N level in forest soil. For this study, we used *P. pinaster* plants, whether associated or not with the ectomycorrhizal basidiomycete *R. roseolus*. Three-month-old plants were pre-incubated without nitrogen for 7 days (−N plants) and then 3 days with 50 μM $NO_3^-$ (+$NO_3^-$) with or without 50 or 500 μM $NH_4^+$ (+$NH_4^+$ 50, + $NH_4^+$ 500) before net $NO_3^-$ flux measurements. We applied $NO_3^-$ concentrations ranging from 0 to 500 μM as $KNO_3$ in the flowing solution. Depending on the pre-incubation solution, we obtained different kinetics in non-mycorrhizal- and mycorrhizal short roots (Gobert and Plassard 2007). As shown in Table 5.2, which gives a summary of the values of apparent maximal net flux ($J_{max}$) and affinity ($\kappa_m$) calculated from these kinetics, nitrate net fluxes into non-mycorrhizal short roots from N-starved plants were very low. In these conditions, the measurement of $NO_3^-$ net fluxes presented a limit, since it was not possible to measure low fluxes at high external ion concentrations. Indeed, the voltage difference measured between the solution and the root becomes very low in these conditions (Gobert and Plassard 2007). Given the precision of the reading on the amplifier (0.1 mV), we chose to calculate flux values when the average voltage difference was higher than 0.5 mV. This situation was encountered again when plants, whether mycorrhizal or not, were incubated with high $NH_4^+$ concentration (500 μM). However, measurement of $NO_3^-$ net fluxes with $NO_3^-$ selective microelectrodes enabled us to show that a complex kinetic process was occurring in non-mycorrhizal short roots fully induced for $NO_3^-$ uptake, resulting in different values of apparent $J_{max}$ and affinity $\kappa_m$ (Table 5.2). In contrast, mycorrhizal short roots always displayed higher $NO_3^-$ net fluxes than non-mycorrhizal short roots, together with simple kinetics (Table 5.2). Nevertheless, both types of short roots exhibited the same sensitivity to the $NH_4^+$

**Table 5.2** Apparent values of $J_{max}$ and $\kappa_m$ for $NO_3^-$ uptake of −N, $+NO_3^-$ and $+NH_4^+$ pre-treated *P. pinaster* seedlings, whether associated or not with the ectomycorrhizal basidiomycete *R. roseolus*. Net $NO_3^-$ fluxes were measured using $NO_3^-$ selective microelectrodes placed at the surface of non-mycorrhizal short roots (NMSR) of non-inoculated plants or mycorrhizal short roots (MSR) of inoculated plants. The plants (3 months old) were pre-incubated without nitrogen for 7 days (−N plants) and then 3 days with 50 μM $NO_3^-$ ($+NO_3^-$) with or without 50 or 500 μM $NH_4^+$ ($+NH_4^+50$, $+NH_4^+500$) before net $NO_3^-$ flux measurements. Parameters are given ± confidence interval at $P = 0.05$ ($n \geq 6$) and with $P$ value of the determination (from Gobert and Plassard 2007, with permission from Blackwell Publishing)

| Pre-treatment | Short root type | $[NO_3^-]$ range (μM) | $J_{max}$(μmol $g^{-1}$ root fr. wt $h^{-1}$) | $\kappa_m$ (μM) | $r^2$ |
|---|---|---|---|---|---|
| −N | NMSR | 0–70 | 0.29 ± 0.02 | 20 ± 2.9 | 0.99 |
| | *P* | | <0.0001 | <0.0001 | |
| | MSR | 0–500 | 1.32 ± 0.07 | 33 ± 6.7 | 0.99 |
| | *P* | | <0.0001 | <0.0001 | |
| $+NO_3^-$ | NMSR | 0–70 | 0.54 ± 0.08 | 21 ± 6.7 | 0.98 |
| | *P* | | <0.0001 | <0.0001 | |
| | | 70–200 | 1.17 ± 0.29 | 111 ± 58 | 0.97 |
| | *P* | | <0.0001 | 0.0034 | |
| | MSR | 0–500 | 1.48 ± 0.04 | 82 ± 6.7 | 0.99 |
| | *P* | | <0.0001 | <0.0001 | |
| $+NH_4^+50$ | NMSR | 0–70 | 0.39 ± 0.08 | 20 ± 8.5 | 0.99 |
| | *P* | | 0.0002 | 0.0136 | |
| | | 70–200 | 0.75 ± 0.22 | 73 ± 58 | 0.83 |
| | *P* | | 0.0007 | 0.0416 | |
| | MSR | 0–500 | 2.1 ± 0.22 | 117.5 ± 33 | 0.97 |
| | *P* | | <0.0001 | <0.0001 | |
| $+NH_4^+500$ | NMSR | 0–100 | 0.36 ± 0.09 | 57 ± 14 | 0.99 |
| | *P* | | <0.0001 | 0.0003 | |
| | MSR | 0–100 | 0.35 ± 0.09 | 66 ± 29 | 0.99 |
| | *P* | | <0.0001 | 0.0021 | |

supply, with a decreasing effect appearing only with high $NH_4^+$ supply (Table 5.2). Taken together, these measurements showed us that the regulation of nitrate uptake into the host plant and its ectomycorrhizal fungus could be different. This also strongly suggests that the ectomycorrhizal symbiosis could greatly help the plant to cope with changing $NO_3^-$ availability in the soil, especially when it is low.

## 5.7 Conclusions

In this chapter we have described the technique of ion fluxes measurement outside the tissues in woody plants and ectomycorrhizal roots. We demonstrated that this technique could be a really powerful methodology to assess the actual role of ectomycorrhizal roots in highly heterogeneous root system. In our laboratory, we

have made ion-selective microelectrodes only for three ions, but it is possible to extend the range of these microelectrodes to many other ions. However, among the numerous ionophores/cocktail solutions that are now available commercially, an ionophore for orthophosphate activities is still missing, despite recent papers on this topic (Wang and Bishop 2005; Zou et al. 2007). Indeed, the main problem of this anion is that its net valence (*F* in Nernst's equation) will depend on $H^+$ concentration in the solution, thus restricting its use to extremely well-defined pH conditions. Such pH stability will not be compatible with pH changes mediated by the root activity that will occur at the root surface. To us, the main advantage of the microelectrode technique is that net fluxes can be recorded for several hours, allowing the variation of ionic conditions of the flowing solution and the measurement of net fluxes at the same spot of the root. From an experimental point of view, its main limitation is the impossibility of measuring low fluxes at high external ion concentrations. However, it can be argued that in soil conditions, concentrations of available mineral nutrients will probably be most of the time in the range that can be used for measurements with ion-selective microelectrodes.

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# Chapter 6
# Assessment of Phosphatase Activity Associated with Mycorrhizal Fungi by Epi-Fluorescent Microscopy

**Ingrid M. van Aarle**

## 6.1 Introduction

The use of the ELF-97 endogenous phosphatase detection kit, a fluorescence-based method from Molecular Probes (Leiden, the Netherlands) for the assessment of phosphatase activity associated with mycorrhizal fungi is detailed. The kit consists of an ELF-97 phosphatase substrate [2-(5-chloro-2-phosphoryloxyphenyl)-6-chloro-4-(3H)quinazolinone (CPPCQ)], an alkaline detection buffer and a mounting medium, enabling the detection of alkaline phosphatase activity. However, use of the substrate in combination with an acid buffer (such as citrate buffer or acetate buffer) enables the detection of acid phosphatase activity.

The ELF-97 substrate is a fluorogenic substrate that has been used for location of phosphatase activity in (for example) fixed, cultured cells from rat (Telford et al. 1999) and zebrafish (Cox and Singer 1999), marine phytoplankton (González-Gil et al. 1998), and mycorrhizal fungi (Van Aarle et al. 2001, 2005; Alvarez et al. 2004). The substrate is normally slightly fluorescent in the blue range. However, once its phosphate is enzymatically removed the substrate forms a crystalline precipitate, which is bright green fluorescent, indicating the site of activity (Huang et al. 1993; Larison et al. 1995). Hence the process is called enzyme-labelled fluorescence (ELF). The precipitate has a maximum emission at 530 nm, and is separated by more than 100 nm from its maximum excitation at 345 nm (Molecular Probes 2004). This specificity of the ELF substrate makes the signal clearly distinguishable from the autofluorescence of biological material such

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as mycorrhizal hyphae. The ELF precipitate is yellow-green against the blue background of the hyphae when visualised with a Hoechst/DAPI longpass filter set.

## 6.2 Phosphatase Activity

Phosphatase activity released or excreted in the growth substrate as well as the extracellular activity of roots and hyphae can be quantified spectrophotometrically with the *p*-nitrophenyl phosphate method. Location of phosphatase activity in mycorrhizal fungal tissues has been studied with colorimetric methods such as staining with Fast Blue RR salt in the presence of α-naphthyl phosphate (Tisserant et al. 1993; Saito 1995). The activity can be observed as a dark stain using light microscopy. Alkaline phosphatase activity of extraradical mycelium of mycorrhizal fungi, as well as the alkaline phosphatase activity associated with the intraradical structures of arbuscular mycorrhizal (AM) fungi, can thus be visualised (Boddington and Dodd 1998; Tisserant et al. 1993).

The Fast Blue method has been used by Saito (1995) and by Van Aarle et al. (2002b) to visualise acid phosphatase activity associated with intraradical mycelium that has been extracted from roots with the enzyme digestion method (see Chap. 11, Solaiman for method). However, the high background of acid phosphatases in all plant roots prevents an observation of the mycorrhizal acid phosphatase activity in roots or root sections with this method. Due to the higher sensitivity of the ELF substrate than that of non-fluorogenic substrates, the ELF substrate is suitable for visualizing both alkaline and acid phosphatase activity associated with mycorrhizal fungi, even in root sections (Van Aarle et al. 2005). Examples of possibilities of this method are given in Sect. 6.4, whereas the advantages and limitations of the method are discussed in Sect. 6.5.

## 6.3 Equipment and Laboratory Material

### *6.3.1 Equipment*

- Epi-fluorescent microscope equipped with a Hoechst/DAPI longpass filter set
- Micropipettes and tips
- Vortex mixer

### *6.3.2 Laboratory Material*

- ELF-97 endogenous phosphatase detection kit (E6601 – Molecular Probes, Leiden, The Netherlands)
- Eppendorf tubes
- Syringes
- 0.22 μm syringe filters (millex-GV, Millipore)

- Slides and cover glasses
- Fine forceps
- Nail varnish

### 6.3.3 Solutions

A couple of solutions are needed before starting an ELF-assay. Some can be purchased, whereas others should be prepared in advance.

In order to visualise alkaline phosphatase an alkaline buffer is needed. Most appropriate is to use the alkaline detection buffer from the ELF kit. However, if another alkaline buffer is used (such as, e.g., Tris-HCl) one should pay attention that the precipitate dissolves at a pH above 8.0–8.5. In order to visualise acid phosphatase a buffer with a low pH should be used. It is possible to use a commercial citrate buffer, such as the one from Sigma with pH 4.8 (90 mM). Otherwise an acetate or citrate buffer can be used and in that case the pH can be set at a specific value. These buffers are prepared as follows:

1. Acetate buffer, pH 5.5, 0.2M:
   - 0.2M acetic acid solution by dissolution of 12.10 g acetic acid in $dH_2O$, fill up to 1 l
   - 0.2M sodium acetate solution by dissolution of 16.46 g sodium acetate in $dH_2O$, fill up to 1 l
   - Mix 0.2M acetic acid solution into 0.2M sodium acetate solution until pH 5.5 is reached
2. Citrate buffer, pH 4.0–6.0, 0.1M:
   - 0.1M solution of citric acid by dissolution of 21.01 g citric acid in $dH_2O$, fill up to 1 l
   - 0.1M solution of sodium citrate by dissolution of 29.41 g sodium citrate ($C_6H_5O_7Na_3 \cdot 2H_2O$) in $dH_2O$, fill up to 1 l
   - Mix x ml of citric acid solution with *y* mL of sodium citrate solution according to Table 6.1
   - Confirm pH

After the samples have been incubated in the ELF substrate-buffer solution and before mounting the samples on the slide, it is recommended to wash the samples in

**Table 6.1** The proportion by which the two components ($x$ = 0.1 M citric acid solution, $y$ = 0.1 M sodium citrate solution) of the citrate buffer should be mixed to obtain a pH between 4.0 and 6.0

| pH | *x* (ml) | *y* (ml) |
|---|---|---|
| 4.0 | 33.0 | 17.0 |
| 4.4 | 28.0 | 22.0 |
| 4.8 | 23.0 | 27.0 |
| 5.2 | 18.0 | 32.0 |
| 5.6 | 13.7 | 36.3 |
| 6.0 | 9.5 | 41.5 |

a wash buffer. This will reduce the unspecific precipitation of the substrate and it will increase the time that samples can be stored. If the samples are not properly washed this may result in large crystals distributed throughout the sample and possibly also in the mounting medium. In the first protocol provided by Molecular Probes (1994) a wash buffer based on Tris and NaCl was used. Later revised protocols (see, e.g., Molecular Probes 2004) use a wash buffer based on PBS, EDTA (25 mM) and levamisol (5 mM), pH 8.0. However, levamisol is not a specific inhibitor of fungal phosphatases. It is possible to construct other wash buffers containing a specific inhibitor for fungal alkaline phosphatases or fungal and plant acid phosphatases. Here however, only the preparation of the original wash buffer is detailed:

1. Tris wash buffer, pH 8.0 (30 mM Tris, 1.5M NaCl, 0.05% w/v Triton X-100):

   - Add 3.635 g of Tris to 750 ml $dH_2O$
   - Add 87.7 g of NaCl to the Tris solution
   - Dissolve, set pH at 8.0 with HCl and fill up to 1 l
   - Add 0.5 mg Triton X-100 to the buffer
   - Confirm pH

### *6.3.4 Protocol for Assessment of Phosphatase Activity with ELF-97 Substrate*

It is important to prepare all buffers, reagents and materials before starting, since the reaction is relatively fast. The microscope should also be fitted with the proper filter set before starting the reaction. According to Molecular Probes (2004) the reaction occurs within 30–90 s; however, for fungal tissues the optimal incubation time seems to be approximately 15–30 min (Van Aarle et al. 2001; Alvarez et al. 2006). With epi-fluorescent microscopy, often no precipitate associated with mycorrhizal fungi is observed in the first 10 min of the reaction. Fixation and especially permeabilisation of the samples could possibly influence the reaction time. Permeabilisation does, however, increase the risk of leaking of cytoplasm and phosphatase enzymes out of the fungal structures, which could pose a problem, especially for non-septate AM fungal hyphae.

#### 6.3.4.1 Collection and Processing of Material

1. *Roots and Intraradical Mycelium (AM fungi)*:

   - Collect roots or mycorrhizal root tips from the growth medium and remove all remaining soil, medium and/or debris. Wash with $dH_2O$ and keep humid and preferably cold until use.
   - Rapidly process roots (cut, section or digest) so that they can be transferred to the ELF substrate-buffer solution, or fix the roots in ethanol:

- Cut root sections with a razor blade or with a microtome. Different protocols exist that do not destruct the phosphatase enzymes (see, e.g., Van Aarle et al. 2005).
- Cut roots in small pieces of ca. 0.5 cm and put under vacuum so that air bubbles at the extension are removed and stain can better penetrate.
- Digest roots partially or fully with an enzyme digestion solution to either facilitate the substrate to penetrate and to improve contact with the intraradical structures or to carefully collect intraradical mycelium (see chap. 11, Solaiman, for method).

2. *Extraradical mycelium*:

- Carefully collect the extraradical mycelium with fine forceps from the growth medium (mini-rhizotrons, liquid cultures, AM fungal root organ cultures with liquid medium) or extract the mycelium according to standard protocols from the growth substrate (pot cultures, field soil, mesh bags, AM fungal root organ cultures with solid medium).
- Wash the mycelium clean from all media or debris.
- Keep humid and cold until use or fix in ethanol.

#### 6.3.4.2 Detailed Protocol (based on Van Aarle et al. 2001, 2005 and Olsson et al. 2002)

1. Dilute the ELF substrate in the appropriate buffer (see Sect. 6.3.3.):

- Normally a 20-fold dilution of the ELF substrate is suitable; however, depending on the samples or material used, dilutions between 1:10–1:100 should be tested. Too high concentration might lead to over-labelling, whereas too low a concentration might lead to abundant background crystals due to the prolonged reaction time needed.

2. Vortex the solution for several seconds at high speed and pass it through a 0.22 μm syringe filter (millex-GV, Millipore) in order to dissolve and to remove any crystals that might have been formed in the substrate during storage:

- Use syringe filters with a small diameter in order to limit the loss of the ELF substrate-buffer solution.
- Preferably prepare the ELF substrate-buffer solution fresh each day. However, if a large amount remains at the end of the day it could be stored in the fridge until the next day. Overnight storage has so far not shown to have a significant effect on the reaction or the amount of background staining, at least not if the solution is vortexed at high speed for at least 1 min and subsequently filtered through a 0.22 μm syringe filter.

3. Divide the filtered ELF substrate-buffer solution over Eppendorf tubes, add a sample to each tube and note the time of start of the reaction for each sample:

- Usually 20–50 μl is used for mycelium, 40–100 μl for root sections and 100–500 μl for root pieces.

4. Let the reaction develop for 20 min:
   - Optimal reaction times should be determined for each type of samples. Ideally this is done by immersion of the sample in a couple of drops of ELF substrate-buffer solution on a glass microscopy slide. The sample is monitored continuously under the microscope for the occurrence of ELF crystals (bright yellow-green fluorescence), and the time of appearance of first specific ELF crystals as well as that of background staining in the medium is measured. The fluorescent precipitate is very photostable and supports long periods of visualisation.
5. Wash the sample with wash buffer in order to remove excess substrate. This washing increases the preservation time of the slides:
   - Normally three changes of wash buffer from 5 min each with gentle agitation are sufficient, but sometimes more changes are needed in order to remove all excess substrate.
   - The pH of the wash buffer must not be above 8.0 otherwise the crystals may dissolve.
   - In order not to lose too many hyphae or not to damage the root sections during this washing step the samples could be washed in mini-sieves. Such sieves can be made from 2 ml Eppendorf tubes, by cutting off the bottom, heating the cut edge in a flame and immediately put the heated edge on a piece of nylon mesh. Once the edge is cooled the mesh is sealed to the tube, resulting in a mini-sieve.
6. Carefully remove the wash buffer without drying the samples.
7. Mount the sample in the mounting medium provided with the kit. Put one or two drops of mounting medium on the microscopy slide, put the sample in the mounting medium. Hyphae and intraradical mycelium can be carefully distributed with fine forceps. Cover the samples with a cover slip:
   - Leave the mounted slides on a flat surface to dry. After several hours the slides can be sealed with nail varnish.
   - The fluorescent signal of the ELF precipitate is stable for a long time; however, it is advisable to store the samples in the dark. After several days of storage the mounting medium might start to crack, especially if the mounting medium was viscous/aged at the time the slides were made.
   - Store the mounting medium upside down. In this way the tube does not have to be inversed when applying the mounting medium. Inversion of the tubes readily leads to the formation of air bubbles, especially when the mounting medium gets older and more viscous.
   - Do not use glycerol to mount the samples, since the ELF crystals will dissolve within hours.

8. Visualise the ELF-stained samples through a standard Hoechst/DAPI longpass filter set:
   - The ELF precipitates appear as yellow-green fluorescent crystals. Mycorrhizal fungi and roots without enzyme activity have a light blue fluorescence.

## 6.4 Various Applications of ELF-97 Substrate in Mycorrhizal Research

The ELF-97 phosphatase substrate is a novel fluorogenic substrate that recently has been applied in mycorrhizal research. Several publications have appeared in which this substrate has been used to visualise and quantify acid and alkaline phosphatase activity associated with arbuscular and ectomycorrhizal fungi (Van Aarle et al. 2001, 2002a, 2005; Olsson et al. 2002, 2005; Alvarez et al. 2004, 2005, 2006).

### *6.4.1 Phosphatase Activity Associated with Ectomycorrhizal Fungi*

The ELF method has been used to quantify the acid and alkaline phosphatase activity associated with extramatrical hyphae of *Hebeloma cylindrosporum* in association with *Pinus pinaster* grown in different soil types (Fig. 6.1). Also the phosphatase activity associated with pure culture of *H. cylindrosporum* grown in liquid medium rich or poor in phosphorus was studied in this way. In all cases the acid phosphatase activity associated with the mycorrhizal hyphae was much more pronounced than the alkaline phosphatase activity (Van Aarle and Plassard, unpublished).

Alvarez et al. (2004, 2005, 2006) combined the ELF method with confocal laser scanning microscopy to study surface-bound phosphatases of ectomycorrhizal fungi. They were able to localise and quantify the phosphatase activity of the mycelium of five ectomycorrhizal fungi (Alvarez et al. 2004). Furthermore, the same authors demonstrated acid phosphatase activity associated with the mantle and the Hartig net (Alvarez et al. 2005). They stated that the staining procedure can be performed easily and reliably in living ectomycorrhizal material (Alvarez et al. 2004).

### *6.4.2 Phosphatase Activity Associated with Arbuscular Mycorrhizal Fungi*

Quantification of the phosphatase activity of AM fungal extraradical mycelium can be determined with the ELF method. For *Glomus intraradices* the alkaline phosphatase activity is normally higher than the acid phosphatase activity (Olsson et al.

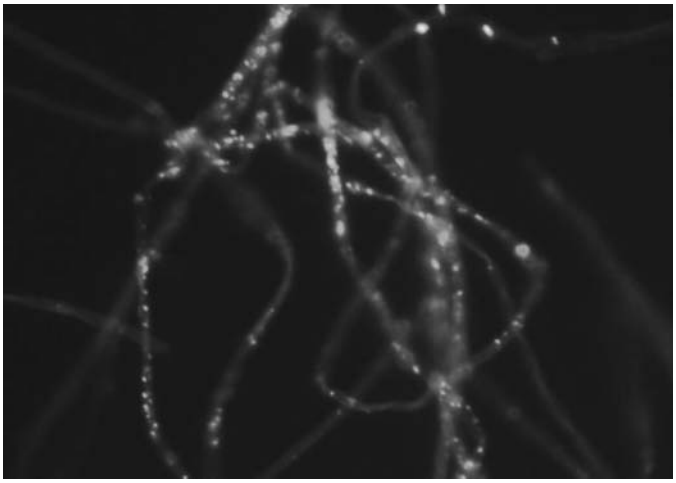

**Fig. 6.1** Extramatrical hyphae of *Hebeloma cylindrosporum* incubated in ELF substrate-alkaline buffer solution showing alkaline phosphatase activity (bright spots)

2002, 2005; Van Aarle et al. 2002a), whereas for *Scutellospora calospora* the inverse has been shown (Van Aarle et al. 2002a). Furthermore, the acid phosphatase activity of *G. intraradices* did not respond to the external P content, whereas the alkaline phosphatase activity was increased at high P content (Olsson et al. 2002).

The phosphatase activity of the extraradical mycelium of *S. calospora* was larger than that of *G. intraradices*; however, it appeared that the intraradical mycelium of *G. intraradices* showed more activity than that of *S. calospora* (Van Aarle et al. 2002a). *Glomus intraradices* showed higher acid than alkaline phosphatase activity for the intraradical structures, as visualised after staining with ELF (Van Aarle et al. 2005). This fungus forms arbuscules when in association with *Allium porrum* (Fig. 6.2) and coils when in association with *Asphodelus fistulosus*. These two AM associations were compared in order to study the phosphatase activity of different symbiotic interfaces. More arbuscules showed phosphatase activity than coils; however, intercellular hyphae had the lowest phosphatase activity. From this study it was suggested that not only arbuscules but probably also coils are a site for P transfer to the host plant (Van Aarle et al. 2005).

### *6.4.3 Other Applications of the ELF-97 Substrate*

Samples that are stained for phosphatase activity with the ELF substrate can also be observed with a laser scanning confocal microscope (Alvarez 2004, 2005, 2006; Van Aarle et al. 2005). Alvarez et al. used it for quantification and localisation of surface-bound phosphatases of ectomycorrhizal fungi (see above), whereas

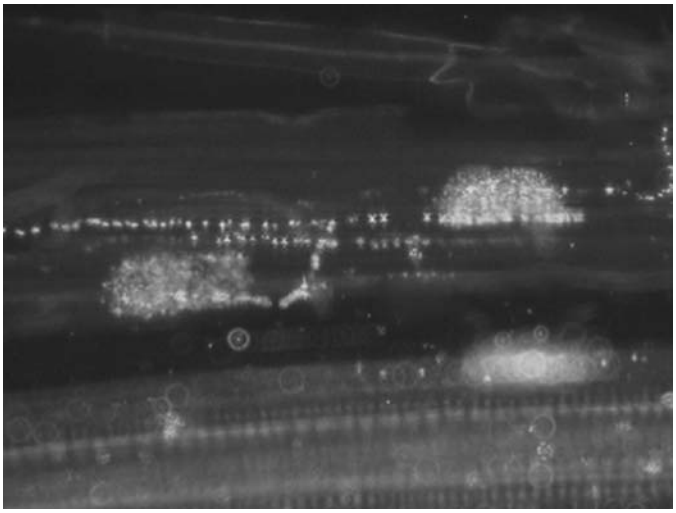

**Fig. 6.2** AM root (*A. porrum* with *G. intraradices*) incubated in ELF substrate-alkaline buffer solution showing alkaline phosphatase active arbuscules and hyphae

Van Aarle et al. (2005) used it to visualise alkaline and acid phosphatase activity associated with AM fungal structures such as arbusculated coils.

Another application of the ELF substrate in mycorrhizal research is the visualisation of an alkaline phosphatase-conjugated antibody that had been used in *in situ* RT-PCR studies on ectomycorrhizal mycelium (Van Aarle et al. 2007). In this way the upregulation of a phosphate transporter gene of *H. cylindrosporum* under P starvation could be visualised. This fluorescent *in situ* RT-PCR has great potential and could possibly also be used to localise genes and their expression in darkly coloured plant and fungal tissues.

## 6.5 Advantages and Limitation of ELF-97 Substrate

The above examples show that the ELF-97 substrate can be used to assess the phosphatase activity associated with mycorrhizal fungal structures. The high sensitivity of the ELF method for assessment of phosphatase activity is an advantage. Furthermore, the protocol is fast to perform and allows detection of both alkaline and acid phosphatase activity associated with the mycorrhizal fungus, even inside root tissues. The fluorescence of the precipitate allows visualisation of phosphatase activity even in darkly stained tissues such as those of pine roots. The yellow-green fluorescence of the ELF precipitate also avoids interference with the autofluorescence of soil particles and of plant and fungal material. Another additional advantage of the ELF method is that it can be used for observations with confocal laser scanning microscopy.

A disadvantage of the ELF method is the relative high cost of the kit, which also prevents the use of relatively large quantities. Sometimes the reaction is too sensitive, resulting in a very intensive precipitation and a blur of fluorescence. In that case, non-fluorescent and less sensitive methods, such as the Fast Blue method, are more suitable. The kit has a relative short shelf-life, according to the producer ca. 6 months, but if handled correctly it can be used up to 1 year. The shelf-life of the kit seems mainly limited by the quality and ageing of the mounting medium.

## 6.6 Conclusions

The ELF-97 substrate is a suitable substrate for the location, visualisation and quantification of acid and alkaline phosphatase activity associated with mycorrhizal fungal structures. The ELF method is fast to perform and has a high sensitivity. So far it has successfully been used to assess the acid and alkaline phosphatase activity associated with different ectomycorrhizal and arbuscular mycorrhizal fungi.

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# Chapter 7
# *In Vitro* Compartmented Systems to Study Transport in Arbuscular Mycorrhizal Symbiosis

**H. Dupré de Boulois, L. Voets, and S. Declerck**

## 7.1 Introduction

At the end of the 1960s up to the beginning of the 1970s, numerous studies investigated the mechanisms behind the higher biomass and phosphorus (P) content of arbuscular mycorrhizal (AM) plants (e.g. Daft and Nicolson 1966; Sanders and Tinker 1971, 1973; Hayman and Mosse 1972). However, the essential role of AM fungi in the uptake of P and its subsequent translocation and transfer to their host plants awaited the studies of Hattingh et al. (1973), Pearson and Tinker (1975), Rhodes and Gerdemann (1975) and Cooper and Tinker (1978, 1981) to be convincingly demonstrated. This major finding was obtained using ingenious bi-compartmented pot culture systems and isotopic tracers. In these systems, roots and mycorrhizal fungi were allowed to develop in one compartment (i.e. the root compartment, RC) while the other compartment (i.e. the hyphal compartment, HC) was restricted to the exclusive development of the extraradical mycelium (ERM) of the AM fungi. Over the years, numerous other compartmented pot systems based on the same concept were developed to study AM fungal transport (see for instance Ames et al. 1983; Frey and Schuepp 1992; Mäder et al. 1993; Schweiger and Jakobsen 2000; Jansa et al. 2003; Smith et al. 2003; Tanaka and Yano 2005).

Although these compartmented pot systems have led to striking results on element transport by mycorrhizal fungi (see for references Marschner 1995; Schweiger and Jakobsen 2000), they presented some important drawbacks: (1) the presence of undesirable micro-organisms which could influence element bio-availability or

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transport processes, (2) the difficulty of visualizing the development of the two partners of the symbiosis and of recovering roots and extraradical fungal mycelium, (3) the possible interaction between the element and the soil matrix, (4) the potential direct uptake by the roots of the element under study due to tracer leakage towards the root compartment caused by diffusion or mass-flow, and (5) the complexity of performing physiological and molecular studies on extraradical fungal mycelium and mycorrhizal roots (Schweiger and Jakobsen 2000; Rufyikiri et al. 2005). For these reasons, compartmented *in vitro* (monoxenic) culture systems became widely used (Rufyikiri et al. 2005).

The aim of this chapter is to provide methodologies to perform transport studies using bi-compartmented *in vitro* culture systems, with root-organs or whole plants as host, and to give a detailed description of the advantages and disadvantages of these systems.

## 7.2 Equipment and Laboratory Material

### *7.2.1 Equipment*

- Horizontal or vertical laminar flow hood
- Stereo-microscope (magnification ×10–×40)
- Bunsen burner or bead sterilizer
- Cooled heated incubator
- Climatic chamber
- Rotating agitator
- Thermo-fusible glue applicator

### *7.2.2 Laboratory Material*

- Bi-compartmented Petri plates (usual diameter: 90 mm)
- Polypropylene centrifugation tubes (50 ml)
- Scalpels and forceps
- Set of cork-borers
- Needles
- Manual or motorized pipettors adapted to 5–20 ml sterile pipettes
- 1–1,000 µl micropipettes with corresponding sterile tips
- Cellophane wrap (roll of 2 cm-large) and Parafilm (note that Seal View, Petri Seal™ or other products can also be used)
- Thermo-fusible glue
- Silicon grease
- Black plastic bags (12 × 12 cm)
- Inclined (0–45°) support for Petri plates

**Remark**: All manipulations of plant, AM fungi, medium and isotopic tracers should be conducted under sterile conditions (i.e. under a laminar flow hood) and with sterile or sterilized laboratory material. Equipment and laboratory material that have no direct contact with the AM cultures do not require to be placed in a sterile environment or to be sterile or sterilized.

## 7.3 Culture Media

### *7.3.1 Composition*

In Table 7.1, the compositions of the modified Strullu-Romand (MSR – Declerck et al. 1998, modified from Strullu and Romand 1986) medium and the minimum (M) medium (Bécard and Fortin 1988) are given. These media are the most widely used (see Table 7.1 in Cranenbrouck et al. 2005) for the *in vitro* culture of AM fungi.

**Table 7.1** Mineral and vitamin composition of the modified Strullu-Romand (MSR) medium and minimal (M) medium

| | MSR medium | M medium |
|---|---|---|
| $N(NO_3^-)$ (μM) | 3,800 | 3,200 |
| $N(NH_4^+)$ (μM) | 180 | – |
| P (μM) | 30 | 30 |
| K (μM) | 1,650 | 1,735 |
| Ca (μM) | 1,520 | 1,200 |
| Mg (μM) | 3,000 | 3,000 |
| S (μM) | 3,013 | 3,000 |
| Cl (μM) | 870 | 870 |
| Na (μM) | 20 | 20 |
| Fe (μM) | 20 | 20 |
| Mn (μM) | 11 | 30 |
| Zn (μM) | 1 | 9 |
| B (μM) | 30 | 24 |
| I (μM) | – | 4.5 |
| Mo (μM) | 0.22 | 0.01 |
| Cu (μM) | 0.96 | 0.96 |
| Ca Panthotenate (μM) | 1.88 | – |
| Biotin (μM) | 0.004 | – |
| Pyridoxine (μM) | 4.38 | 0.49 |
| Thiamine (μM) | 2.96 | 0.3 |
| Cyanocobalamine (μM) | 0.29 | – |
| Nicotinic acid (μM) | 8.10 | 4 |
| Glycine (mg/l) | – | 3 |
| Myo-inositol (mg/l) | – | 50 |

### 7.3.2 Stock Solutions

The preparation of the MSR and M medium requires the preparation of stock solutions (see Table 7.2).

### 7.3.3 Preparation of Culture Media

For 1 l of MSR or M medium:

1. Mix together:
   - 10 ml of the macroelement stock solution
   - 10 ml of the calcium nitrate stock solution
   - 5 ml of the NaFeEDTA stock solution
   - 1 ml of the microelement stock solution
   - 5 ml of vitamin stock solution to prepare MSR medium or 1 ml for M medium

**Table 7.2** Concentration of stock solutions ($g.l^{-1}$) to prepare the MSR and M medium

| | | MSR medium | M medium |
|---|---|---|---|
| Macroelements | | | |
| | $KNO_3$ | 7.6 | 8 |
| | KCl | 6.5 | 6.5 |
| | $KH_2PO_4$ | 0.41 | 0.48 |
| | $MgSO_4{\cdot}7H_2O$ | 73.9 | 73.1 |
| Calcium Nitrate | $Ca(NO_3)_2{\cdot}4H_2O$ | 35.9 | 28.8 |
| NaFeEDTA | | 1.6 | 1.6 |
| Microelements | | | |
| | $MnSO_4 \cdot 4H_2O$ | 2.45 | 6 |
| | $ZnSO_4{\cdot}7H_2O$ | 0.28 | 2.65 |
| | $H_3BO_3$ | 1.85 | 1.5 |
| | $CuSO_4{\cdot}5H_2O$ | 0.22 | 0.130 |
| | $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ | 0.034 | – |
| | $Na_2MoO_4{\cdot}2H_2O$ | 0.0024 | 0.002 |
| | KI | – | 0.75 |
| Vitamins | | | |
| | Ca panthotenate | 0.18 | – |
| | Biotine | $0.18\ 10^{-3}$ | – |
| | Pyridoxine | 0.18 | 0.1 |
| | Thiamine | 0.2 | 0.1 |
| | Cyanocobalamine | 0.08 | – |
| | Nicotinic acid | 0.2 | 0.5 |
| | Glycine | – | 3 |
| | Myo-inositol | – | 50 |

2. Add 10 g of sucrose
3. After dissolution of sucrose, adjust the volume to 1 l
4. Adjust the pH to 5.5 with NaOH or KOH and HCl
5. Add 3 $g.l^{-1}$ Gelgro™ (ICN Biochemicals, Cleveland, OH), 3 $g.l^{-1}$ Phytagel or 8 $g.l^{-1}$ agar.
6. Autoclave for 15 min at 121°C under 1bar pressure
7. After autoclaving, the medium can be directly used but can also be stored for a few hours at 40–60°C

**Remark:** Gelling agents may contain diverse macro-, micro- and trace-elements. It can therefore be critical to determine their presence and concentration.

E.g. Gel-gro™ and Phytagel contain large quantities of K and P. Declerck et al. (2003) showed for instance that MSR medium solidified by 3 $g.l^{-1}$ Gelgro™ actually had a concentration of 4.38 mM K and not 1.65 mM (see Table 7.1). The difference (i.e. 2.73 mM K) was coming from the gelling agent.

## 7.4 Transport Studies with Root-Organ Cultures (ROC)

While Hattingh et al. (1973) demonstrated for the first time that AM fungi can transport P, Mosse and Hepper (1975) reported the first *in vitro* co-culture between a root-organ and an AM fungus. However, in the mid 1970s, this finding did not receive much consideration. Indeed, AM monoxenic cultures awaited the end of the 1980s and the beginning of the 1990s to be used and developed (Bago and Cano 2005) when (1) the experimental conditions for AM monoxenic cultivation were reformulated by the use of transformed root-organs to allow the cultures to be easily maintained (Bécard and Fortin 1988), and (2) bi-compartmented Petri plates were used to allow the separation of the ERM from the mycorrhizal roots (St-Arnaud et al. 1995, 1996). These improvements participated significantly in the success of this cultivation system (Bago and Cano 2005) and in particular for transport studies (Rufyikiri et al. 2005).

At present, most of the monoxenic cultures of AM fungi are maintained on Ri T-DNA transformed root-organs as host and, in particular, on Ri T-DNA transformed carrot (*Daucus carota* L.) roots (see Table 7.1 in Cranenbrouck et al. 2005). These transformed roots offer the advantage of presenting a greater growth potential than non-transformed roots in culture media with limited supply of nutrients (Fortin et al. 2002). In addition, AM fungi in association with Ri T-DNA transformed roots show greater AM intraradical colonization and sustain higher ERM development than non-transformed root-organs.

Theoretically any AM fungal strain should be cultivable under *in vitro* conditions on root-organs. However, for a large set of AM fungal species, this is still complicated, explaining why a limited number of strains (e.g. *G. intraradices*, DAOM 197198, MUCL 41833; *G. proliferum,* MUCL 41827, *Glomus* sp., MUCL 43195) have been used for *in vitro* transport studies.

Preparation of ROC to perform transport studies comprises three major steps:

- Preparation of the Petri plates, i.e. adding medium in the RC and HC
- Selection of the root
- Inoculation of the root with AM fungal propagules.

### *7.4.1 Adding Medium to the Petri Plates*

In Figs. 7.1 and 7.2, different methods of adding medium in bi-compartmented Petri plates are presented. In the RC, solid medium is added, while in the HC, either solid or liquid medium can be used. For each of the different methods, it is recommended to add medium in the RC at least to the level of the top of the partition wall in order to facilitate the ERM spreading from the RC into the HC (Figs. 7.1a–c and 7.2a–d). Identically, the level of medium in the HC should also be high enough to help the ERM cross the partition wall. However, if liquid medium is added in the HC (Fig. 7.1a–c), manipulations may lead to spilling of medium to the RC, so the level of medium in the HC should be cautiously chosen (typically between 5 and 20 ml for a 90 mm diameter Petri plate). If solid medium is used in the HC (Fig. 7.2a–d), the level can be as high as in the RC (see Fig. 7.2a, b).

Logically, the type of medium (i.e. MSR or M) used in the RC should be identical to the one in the HC. However, it is advised not to add sucrose in the HC. Indeed, if no strict comparison has been made yet on the ERM growing with or without sucrose in the HC, it was suggested that the lack of sucrose could increase ERM and spore production (St-Arnaud et al. 1996). The absence of gelling agent in the medium of the HC can be justified as it enables an easy manipulation of the environment surrounding the ERM (e.g. modification of element composition, pH), and an easy access and harvest (Bago and Cano 2005). However, if using liquid medium in the HC is extremely useful for transport studies, it should be noted that

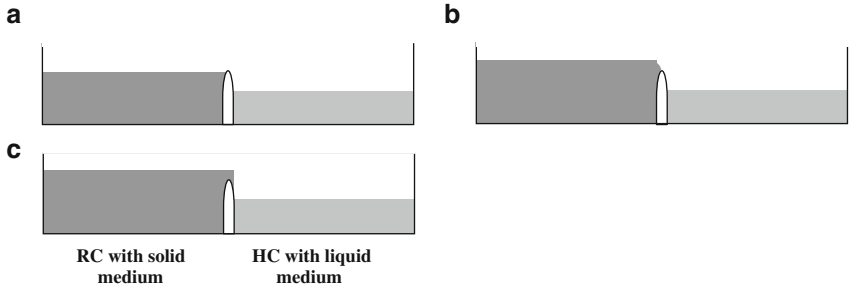


**Fig. 7.1** Bi-compartmented Petri plates with solid medium in the RC and liquid medium in the HC. The medium in the RC (**a**) reaches the top of the partition wall, (**b**) extends above the partition wall (i.e. convex meniscus) thanks to surface tensions, (**c**) is added in the entire Petri plate and extends 1–3 mm above the partition wall; after solidification, the medium in the HC is removed using a scalpel and spatula (Dupré de Boulois et al. 2005). In the HC, liquid medium is added, and its level can be adapted to facilitate hyphal crossing or to restrict the possibility of spilling in the RC

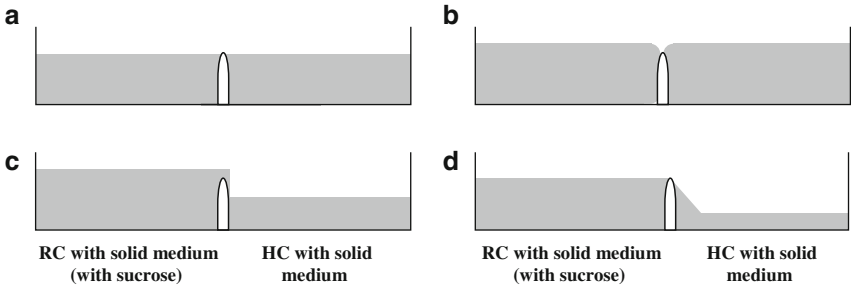


**Fig. 7.2** Bi-compartmented Petri plates with solid medium in both the RC and HC. The medium in the RC can be added as described in Fig. 7.1. (**a**) The medium in the HC reaches the top of the partition wall. (**b**) The medium in the HC extends above the partition wall (i.e. convex meniscus) thanks to surface tensions. (**c**) Solid medium is added in the entire Petri plate and extends 1–3 mm above the partition wall; after solidification, the medium in the HC is removed using a scalpel and spatula and new solid medium is added to the HC (Dupré de Boulois et al. 2005). (**d**) System of St-Arnaud et al. (1996), where a slope of medium against the partition wall is made by inclining the Petri plate at 45° using a support for Petri plates. Medium is then added in the HC so that it reaches the top of the partition wall. When the medium is solid, the Petri plate is placed horizontally and medium is added in the rest of HC

morphology of the ERM may differ from when the ERM develops into a solid medium (Bago and Cano 2005). For instance, the regular pattern of development, with runner hyphae radially extending from the fungal colony, and producing BAS at regular intervals, might be impaired in liquid medium, in which runner hyphae are predominant and BAS are scanty and appear much less branched. These morphological differences should be taken into account as they may reveal other cytological or functional differences that may be important in the study of AM fungal transport. These modifications could influence (1) cell wall structure for adsorption/immobilization, (2) transport protein distribution and abundance for uptake, and (3) cytoskeleton organization for translocation. However, if it is still unknown whether these modifications could have an impact on transport by AM fungi, their consequences are probably minimal, as several studies indicate that the symbiosis is functioning normally in nutrient and C transport (Pfeffer et al. 2004 and references therein).

### *7.4.2 Selection of a Root-Organ*

Following medium addition to the Petri plates, root-organs need to be placed in the center of the RC. This could be achieved with:

- A host root (60–100 mm long, presenting secondary roots and/or secondary root primordia)
- A mycorrhizal root apex (20–40 mm)

#### 7.4.2.1 Remarks

Root-Organ Selection

Root choice is essential for the successful establishment of the AM fungal symbiosis (Fortin et al. 2002). Ideally, a Ri T-DNA transformed carrot root should be 2 weeks old and present a vigorous taproot and a pyramidal pattern of lateral root development. Avoid selection of:

- Yellow or yellowing roots
- Roots exceeding 1 mm in diameter
- Roots presenting swellings (especially at the level of the meristem) or deformations
- Roots presenting grey-translucent tips
- Roots presenting lateral roots close to the primary root tip (<2 cm)
- Roots growing on the surface of the medium
- Punctured or broken roots

Mycorrhizal Root-Organ Selection

If mycorrhizal root apexes are chosen it is important to verify that ERM is extending from them under stereo-microscope. This will provide an indication that AM symbiosis is established within these roots. Further inoculation with fungal propagules is not necessary.

Notice that a section of medium (corresponding approximately of 5–10 ml) containing root apexes and ERM can also be inserted in the RC (Cranenbrouck et al. 2005). However, we would not recommend this methodology as the concentration of the elements present in the RC will be strongly modified.

### *7.4.3 Inoculation of the Root with AM Fungal Propagules*

Fungal propagules should be inoculated onto the lateral roots and their primordia as well as in their direct vicinity, at a distance generally not exceeding 5 mm.

At present, only *Glomus* species producing an extensive ERM bearing several thousand spores under ROC conditions have been used to perform transport studies (e.g. *G. intraradices*, DAOM 197198, MUCL 41833; *G. proliferum,* MUCL 41827, *Glomus* sp., MUCL 43195).

The isolation and inoculation of AM fungal spores from *Glomus* species producing several thousand spores are achieved as follows:

1. Extract a section of medium containing the number of spores desired from a well-established culture (3–6-month-old culture) and transfer it to an empty Petri plate.

2. Add more or less twice the volume of filter sterilized citrate buffer (pH 6, 10 mM: 1.8 mM citric acid + 8.2 mM sodium citrate).
3. Agitate the Petri plate slowly on a rotating agitator (50 rotation $min^{-1}$) at 25–27°C until dissolution of the medium.
4. Transfer the spores to a new Petri plate containing sterile water using a 1,000 µL micropipette.
5. Separate the spores attached to the extraradical hyphae with needles in order to have clusters containing approximately 5–10 spores.
6. Inoculate approximately 100 spores on the host root apexes or branched roots using a 200 µl micropipette (try to minimize the volume of water necessary to take up the spores).
7. Leave the Petri plate open to evaporate excess water ( $\pm$ 5 min).
8. Seal the Petri plate.

After inoculation, the Petri plates are incubated in a cooled heated incubator at 25–27°C in the dark. If the root-organs used have a negative geotropism, e.g. Ri T-DNA transformed carrot roots of the clone DC1, Petri plates should be placed in an inverted position. It is advised to check each Petri plate every week during the first month to verify that no roots are growing into the HC. When roots are crossing the partition wall, they should be trimmed and removed from the HC. This can be easily achieved with forceps and scalpel under laminar flow.

When the extraradical hyphae start to cross the partition wall (typically 2–4 weeks after initiation of the cultures), the Petri plate can be reverted and MSR or M medium (liquid or solid) lacking sucrose filled into the HC (see Sect. 7.4.1). Isotopic labelling can then be performed (see Sect. 7.6).

Notice that a section of medium (corresponding approximately of 100–250 µl) containing ERM harbouring approximately 100 spores can also be inserted in the RC as inoculum in the vicinity of the root-organs. This methodology is also very efficient to inoculate AM fungi, but the volume of medium has to be minimal to have the least effect on the concentration of the elements present in the RC.

## 7.5 Transport Studies with Autotrophic Plants

The bi-compartmented ROC systems have been successfully used for numerous transport studies over the past 15 years, as previously mentioned, but they suffered from several major drawbacks. These can be summarized as follows: (1) the absence of photosynthetic tissues which prevent the determination of element transfer from AM fungi to root cells (except if using autoradiography or NMR (see Nielsen et al. 2002 and Pfeffer et al. 2004)) and photosynthate transport to the AM fungi, (2) the incomplete source-sink relationships between AM fungi and host, and root and shoot which could influence bi-directional transfers at the AM fungal-host interface and (3) the presence of sucrose in the culture medium and the lack of a normal hormonal balance which could alter plant-fungal symbiotic interactions including bi-directional exchanges (Fortin et al. 2002).

In an attempt to obtain both an autotrophic plant and AM fungi under *in vitro* conditions, numerous systems have been developed with more or less success over the last 20 years but have never been used thereafter (see reference in Voets et al. 2005). Indeed, these systems were too difficult to reproduce or utilize and the data generated on the growth and development of the symbiotic partners were limited, questioning the actual establishment of the symbiosis (see Voets et al. 2005). It should nonetheless be noticed that the tripartite system developed by Elmeskaoui et al. (1995) has allowed successful mycorrhization of strawberry and potato plantlets (see Louche-Tessandier et al. 1999; Hernández-Sebastià et al. 1999, 2000). However, in this system (1) the plants were grown in mixotrophic conditions, and (2) mycorrhizal excised or transformed roots were present in the system. Recently, Voets et al. (2005) and Dupré de Boulois et al. (2006) proposed two new *in vitro* culture systems in which an AM fungus and a plant could grow in association on gelled MSR medium lacking both sucrose and vitamins. These *in vitro* culture systems have now been successfully used with *G. intraradices* (MUCL 43194 and 43204), *G. clarum* (MUCL 46238), *G. claroideum* (MUCL 46102), and *Glomus* sp. (MUCL 41833, 43195) grown in association with at least one of the following host plants: *Medicago truncatula* and *Solanum tuberosum* (five cultivars), *Solanum phureja*, *Plantago lanceolata*, *Nicotiana plumbaginifolia*, *Centaurium erythraea, Gentiana verna and Musa* spp. It is also worth noticing that specific adaptations of the systems of Voets et al. (2005) and Dupré de Boulois et al. (2006) to some host plants can be necessary (e.g. due to plant size as in the case of *Musa* spp.).

In the section below, we will present the procedures to obtain AM fungus-plant associations in the systems of Voets et al. (2005) and Dupré de Boulois et al. (2006) with seeds of *Medicago truncatula* as plant starting material. Note however that micro-cuttings from *in vitro* micro-propagated plantlets (see Voets et al. 2005) can also be used and offer the advantage that all plantlets have the same genetic profile.

### *7.5.1 Surface-Sterilization/Scarification*

Various methods have been developed to sterilize/scarify seeds. They can involve, for instance, concentrated sulphuric acid, sodium/calcium hypochlorite, or mercuric chloride. The procedure described here can greatly vary between plant seeds due to the thickness of the seed coat or sensibility of the seeds.

Here we will present the sterilization/scarification of *M. truncatula* seeds using sodium hypochlorite as described by Dupré de Boulois et al. (2006).

1. Place seeds into a disposable sterile 50 ml Falcon tube.
2. Add 25 ml of concentrated sodium hypochlorite (8% active chlorine).
3. Shake the falcon tube for 10 minutes. The seeds will slightly swell and the outer hard seed coat will break.
4. Remove the sodium hypochlorite.
5. Add 25 ml of sterile distilled water to the seeds and mix gently for 10 minutes.

6. Remove the water and repeat the rinsing step twice.
7. Place the surface-sterilized seeds in Petri plates (10–15 seeds/Petri plate) containing 40 ml solidified (3 $g.l^{-1}$ GelGro ) MSR or M medium lacking both sucrose and vitamins.
8. Place the Petri plates in a cool-heated incubator (20°C) in the dark.
9. The seeds are expected to germinate within 3–5 days.

Note that surfactant such as Tween-20 or 80 can be added to sodium hypochlorite to reduce surface tension and allow better surface contact.

Stratification (cold treatment) can be performed to homogenize germination and growth, but this procedure is not compulsory and not necessary with *M. truncatula* as all seeds germinate within a short period of time.

1. After sterilization/scarification, place the Petri plate at 4°C instead of 20°C in the dark.
2. After 14 days, place the Petri plates at 20°C in the dark.
3. The seeds will germinate within 2–3 days.

### 7.5.2 Medium

Neither sucrose nor vitamins need to be included in the culture medium as the plants are photosynthetically active. The host plants therefore grow in total autotrophy.

In the works of Voets et al. (2005) and Dupré de Boulois et al. (2006), MSR medium or an MSR-based medium (MSR medium with low K content to study Cs transport) have been used, but K. Fernandez (pers. comm.) has successfully used other media based either on the MSR or MS medium to associate *S. tuberosum* with *G. clarum* in the system developed by Voets et al. (2005).

In the systems developed by Voets et al. (2005) and Dupré de Boulois et al. (2006), medium needs to be added in the RC during the course of the experiment to support plant and AM fungal growth, but also to restrict medium depletion due to plant transpiration. Therefore, the RC of the systems cannot be filled as described for bi-compartmented ROC systems (see Sect. 7.4.1.). In the following paragraphs (Sects. 7.5.3. and 7.5.4.), detailed explanation will be given for the two types of system.

### 7.5.3 The Half-Closed Arbuscular Mycorrhizal–Plant (HAM–P) In Vitro Culture System (Voets et al. 2005)

Originally, the half-closed AM–P (HAM–P) *in vitro* culture system (Fig. 7.3) consisted of one compartment, i.e. a 90 mm diameter Petri plate, in which the roots of an autotrophic plant and an AM fungus were associated (Voets et al. 2005),

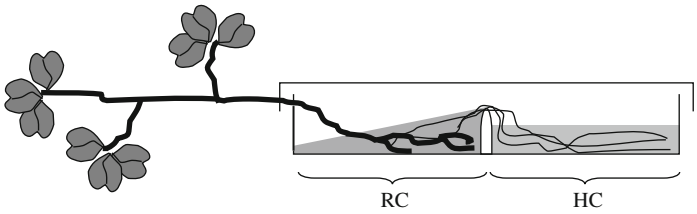


**Fig. 7.3** Schematic representation of the half-closed arbuscular mycorrhizal–plant (HAM–P) *in vitro* culture system viewed from the side. The system allows the spatial separation of a root compartment (*RC*) where roots and an AM fungus grow and a hyphal compartment (*HC*) in which only the extraradical hyphae are allowed to proliferate. The shoot develops in open-air conditions while the roots and AM fungus are maintained *in vitro* in the RC and HC of a bi-compartmented Petri plate (90 mm diam)

while the shoot developed outside this compartment under open-air conditions. Using this system, Voets et al. (2005) obtained several thousand spores, an extensive ERM and abundant root colonization. The spores produced in this system were able to colonize new plantlets under the same conditions and therefore underlined the capacity of this autotrophic culture system to continuously culture AM fungi. The HAM–P *in vitro* culture system was thereafter adapted to perform transport studies by using a bi-compartmented Petri plate instead of a mono-compartmented Petri plate, as shown in Fig. 7.3 with a root compartment (RC) and a hyphal compartment (HC).

The set-up of this system is as follows:

1. A hole (±2 mm in diameter) is made using heated scalpel by melting (a) the edge of the lid to its top (2 mm wide) and (b) the base of the Petri plate 2 mm downwards (2 mm wide). This hole is made in the middle of the RC, perpendicularly to the partition wall.
2. In the RC, 20 ml of solid culture medium lacking both sucrose and vitamins is added at an angle of ±4°, using a support for Petri plates, so that the medium reaches the top of the partition wall while on the opposite side of the RC the medium reaches a height of 2-3 mm (see Fig. 7.2).
3. A plantlet presenting vigorous growth and a strong primary root is transferred to the system. The roots are placed on the surface of the medium in the RC and the shoot is inserted in the hole previously made in the base of the Petri plate.
4. Approximately 100 spores are inoculated along the roots of the plantlet after their isolation as described in Sect. 7.4.3.
5. The lid is placed on the Petri plate so that the cut edge of the lid is above the plantlet shoot. The hole is then carefully plastered with sterilized (121°C for 15 min) silicon grease to avoid contamination.
6. The Petri plate is sealed with Parafilm.
7. The Petri plate is covered with an opaque black plastic bag and incubated horizontally in a growth chamber set at 20°C with 70% relative humidity and 16/8 h photoperiod. The photosynthetic photon flux should ideally comprise between 200 and 400 $\mu mol.m^{-2}.s^{-1}$.

8. Medium is regularly added (about 5 ml once every one to three weeks) to the RC to guarantee plant and fungal growth, but also to guarantee that medium does not dry out. The medium is added at a temperature of ±40°C in order to reduce the risk of damaging the roots and AM fungi.
9. When extensive ERM is reached, liquid or solid medium is added into the HC to ensure mycelium growth in this compartment (see Sect. 7.4.1.).
10. Isotopic labelling and harvest are described in Sects. 7.6.2. and 7.6.4.

#### 7.5.3.1 Remarks

Sterility of the System

This system does not allow growth of both plant and AM fungi under strict *in vitro* conditions as the shoot develops in open-air conditions. The shoot can therefore be subjected to various abiotic stresses and to possible contaminations that could lead to the development of micro-organisms within the Petri plate (Dupré de Boulois et al. 2006).

Depletion of the Medium

Due to the transpiration of the host plant, rapid depletion of the medium in the RC occurs while the plant is actively growing. Regular addition of new medium is therefore necessary. However, as plants may not deplete the medium at a similar rate, it is difficult to add the same amount of medium to each HAM–P *in vitro* culture system. The rapid depletion of the medium in the RC is problematic as it may cause (1) difficulties for the ERM to cross the partition wall, (2) numerous extraradical hyphae to break after they have successfully crossed the partition wall, and eventually (3) the necessity to add medium in the RC during the labelling period.

Labelling

As the shoot develops in open-air conditions, it can easily be labelled with gases (e.g. $^{13,14}CO_2$, $^{15}NO_2$) or any labelled molecules or elements that can be absorbed by the leaves or stem (e.g. sugars, fungicides, Cs, U, Zn, Sr). This represents a major advantage compared to the system developed by Dupré de Boulois et al. (2006).

### *7.5.4 The Arbuscular Mycorrhizal–Plant (AM–P) In Vitro Culture System (Dupré de Boulois et al. 2006)*

To overcome the limitations of the HAM–P *in vitro* culture system (i.e. sterility and medium depletion), Dupré de Boulois et al. (2006) developed a complete arbuscular mycorrhizal–plant (AM–P) *in vitro* culture system, in which both AM fungi and

plant are maintained under strict *in vitro* conditions. The arbuscular mycorrhizal-plant (AM–P) *in vitro* culture system (Fig. 7.4) consists of three compartments, i.e. a shoot compartment (SC) where the shoot develops, a root compartment (RC) where roots and AM fungi grow and a hyphal compartment (HC) where only the ERM can proliferate. Using this system, Dupré de Boulois et al. (2006) showed that AM fungi can take up, translocate and transfer Cs to *M. truncatula*. Transport of P was also studied and the results showed that 98.3% of the initial supply of $^{33}$P in the HC was taken up by the ERM developing in the HC. Translocation to *M. truncatula* represented 91.7% of the $^{33}$P taken up, and the distribution of $^{33}$P in the mycorrhizal plantlets was 76.9% in the roots and 23.1% in the shoot. AM fungal growth parameters were high and similar to the ones obtained by Voets et al. (2005) using the HAM–P *in vitro* culture system. Spores produced in the AM–P *in vitro* culture system were viable and able to colonize new plantlets under the same conditions (unpublished data).

### 7.5.4.1 Preparation of the AM–P *In Vitro* Culture System

1. A hole (5 mm diameter) is made in the lid of the Petri plate using a cork-borer and the lower part of a Falcon tube (50 ml, Sarstedt Aktiengesellschaft, Nümbrecht, Germany) is cut diagonally (hole of about 5 mm diameter) with a

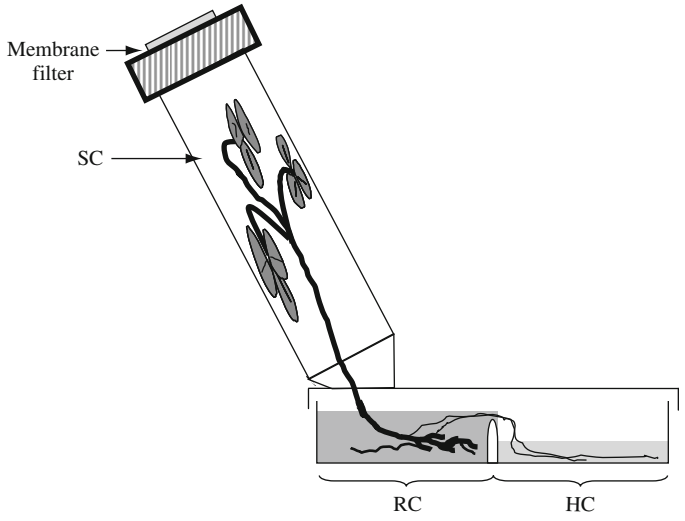


**Fig. 7.4** Schematic representation of the arbuscular mycorrhizal–plant (AM–P) *in vitro* culture system. The system allows the spatial separation of a shoot compartment (*SC*) where the stem and leaves develop, a root compartment (*RC*) where roots and hyphae grow, and a hyphal compartment (*HC*) in which only the hyphae are allowed to proliferate (from Dupré de Boulois et al. 2006). The RC and HC are constituted of a bi-compartmented Petri plate (90 mm diam) and SC of a 50 ml Falcon tube. A membrane filter fixed on the SC allows gaz exchange while preventing microbial contaminations

scalpel to obtain an angle of 65 ± 5° when placed on the lid of the Petri plate. The cork-borer and scalpel are heated with the flame of a Bunsen burner to facilitate the cuttings.
2. The Falcon tube and the Petri plate are then glued together using plastic thermo-fusible glue adapted to plastic gluing.
3. A hole (10 mm diameter) is made in the cap of the Falcon tube using a cork-borer, and a filter (18.6 mm diameter with inner efficient gas exchange capacity of 10 mm diameter and surface of 78.5 $mm^2$) is fixed on the cap. The filter used by Dupré de Boulois et al. (2006) is an Adhesive Microfiltration Disc (AMD) in polypropylene-laminated PTFE (Tissue Quick Plant Laboratory, Hampshire, UK) which prevents microbial contamination but allows gas exchange (Nominal Pore Size (NPS) of 0.25 μm). Note that other ventilation filters may be used such as the one made of cellulose or polyvinyl chloride.
4. The AM–P *in vitro* culture system is then sterilized at 25 kGy by gamma irradiation. Note that other sterilization procedures using, for instance, electron-beam (E-Beam) can also be used, but it is not recommended to use ethylene oxide (EtO) sterilization as EtO can be absorbed by some plastics and must then be treated to eliminate any EtO before use. Note that autoclaving the AM–P *in vitro* culture system is not possible as thermofusible glue could melt and systems be deformed by the heat.

#### 7.5.4.2 Set Up

In the work of Dupré de Boulois et al. (2006), solid $MSR^{Cs}$ medium (Declerck et al. 2003) lacking vitamins and sucrose was added in the Petri plate as described in Fig. 7.1c, but two holes were made in the medium with a cork borer to allow addition of new medium during the course of the experiment to guarantee adequate mineral nutrition of plant and AM fungi. However, if this procedure was efficient (Dupré de Boulois et al. 2006), it has to be noticed that due to water uptake by the mycorrhizal roots and transpiration, a slight medium depletion was observed (eq. to about 10 ml of medium in 10 weeks with *M. truncatula*) and probably restricted the capacity of fungal hyphae to cross the partition wall and thus develop in the HC. Therefore, we propose here an alternative procedure.

1. In the RC, add 20 ml of MSR medium lacking sucrose and vitamins.
2. A germinated plantlet presenting vigorous growth and a strong primary root presenting eventually lateral roots is transferred to the system. The roots are placed on the surface of the medium in the RC and the shoot is inserted into the SC.
3. Approximately 100 spores are inoculated along the roots of the plantlet after their isolation, as described in Sect. 7.4.3.
4. The Petri plate is sealed with Parafilm or plastic wrap.
5. The Petri plate is then covered with an opaque black plastic bag and incubated horizontally in a growth chamber set at 20°C with 60% relative humidity and

16/8 h photoperiod. The photosynthetic photon flux should comprise between 200 and 400 μmol $m^{-2}$ $s^{-1}$.
6. Medium is regularly added (about 5 ml once every 2 to 4 weeks) to the RC to guarantee plant and fungal growth. The medium is added at a temperature of ±40°C in order to reduce the risk of damaging the roots and AM fungi.
7. When an extensive ERM is reached, liquid or solid medium is added into the HC to ensure mycelium growth in this compartment (see Sect. 7.4.1).
8. Isotopic labelling and harvest are described in Sects. 7.6.3 and 7.6.5.

Remarks

*Depletion of the Medium*

Due to the transpiration of the host plant, depletion of the medium in the RC occurs while the plant is actively growing. However, contrarily to the HAM–P *in vitro* culture system, the depletion of the medium is limited. Indeed, as the whole plant is maintained under *in vitro* conditions, the transpiration of the plant is highly reduced. It is nonetheless necessary to regularly check the level of medium in the AM–P *in vitro* culture system and, even if no medium depletion is visible, medium should be added regularly to provide nutrients to the plant and the AM fungus. It is thus advised to add medium depending on the nutritional needs of the plant and AM fungus. Concentrated medium (2–10-times) can also be used (see for instance Dupré de Boulois et al. 2006) to provide nutrients. If other ventilation membranes or Petri plate sizes are used, it is necessary to assess medium depletion to ensure that the extraradical hyphae can cross the partition wall between the RC and HC.

*Plant Transpiration*

As the plants develop fully *in vitro* with only a membrane allowing gas exchange, it is possible that the relative humidity within the AM–P *in vitro* culture system is very high and limits plant transpiration. If this is advantageous as medium depletion in the RC is low, it may have significant impact on plant physiology such as plant photosynthesis, nutrient translocation from root to shoot or plant abscisic acid content.

## 7.6 Labelling with Isotopic Tracers

Isotopic labelling is performed by using an isotopic tracer to observe the movement of certain elements in chemical, biological, or physical processes. The term tracer is applied commonly to any stable or radioactive isotope used to trace the course of non-radioactive elements or biological substances (e.g. proteins, sugars, DNA), and also radioactive elements such as radionuclide contaminants. The observations may

be conducted by the measurement of the relative abundance of isotopes if stable isotopes are used as tracers or of radioactivity in the case of radioactive tracers. Instruments used to detect stable isotopes include principally mass spectrographs and nuclear magnetic resonance (NMR) spectrometers. Instruments used to detect radiation may include liquid scintillation counters, gamma counters and (micro)-autoradiographs.

The isotopic tracer should be sterilized before addition to the Petri plate. Filter-sterilization using a 0.2 μm filter is recommended.

### *7.6.1 Stable or Radio-Isotopic Tracer?*

The choice between stable and radioactive isotopic labeling depends on:

- The purpose of the experiment. If the goal is to demonstrate the presence of an element, both stable and radioactive isotopes can be used. However, if the goal is to study a metabolic process, a stable isotope should be used in combination with NMR spectrometry or mass spectrometry (MS).
- The availability of the stable and suitability of the radioactive isotope. Some elements have no stable isotope (i.e. technetium, tungsten and promethium and all elements with an atomic number greater than 82). Therefore, all tracers used to investigate these elements must be their radioactive forms. Conversely, some elements have only radioactive isotopes with a very short half-life (i.e. inferior to a few days). Therefore, they cannot, or only with difficulty, be used for transport studies. In this case the tracer must be a low-abundance stable isotope prepared in enriched form. Also, not all stable isotopes can be used with NMR spectrometry (i.e. isotopes with a zero nuclear spin) or mass spectrometry (i.e. isobars). Note that isobars and isobar molecules can be distinguished using Accelerator Mass spectrometry (AMS) or Resonance-Ionization Mass Spectrometry (RIMS), but these instruments are only available in some highly specialized laboratories.
- The detection limit of the instruments. The choice between stable and radioactive isotopes can be determined by the so-called "dilution factor", which is a measure of the concentration of tracer required for detection. Generally, radioactive tracers may be detected in much lower quantities than stable tracers. For example, a pure $^{13}C$ tracer would be detectable after being diluted 100,000 to 1 million times with natural $^{12}C$. Radioactive $^{14}C$, however, can be detected after being diluted 25 billion times with $^{12,13}C$.

For many studies, it is possible to choose between stable-isotope tracers and radioactive-isotope tracers. For example, it is possible to choose between $^{13}C$ (stable) and $^{14}C$ (radioactive) or $^{31}P$ (stable) and $^{32,33}P$ (radioactive) isotopes.

### 7.6.2 Adding the Isotopic Tracer

After the addition of medium in the HC, extraradical hyphae will develop rapidly and extend in the whole compartment. Generally, within 1–4 weeks the density of the ERM will be sufficient to expose it to the isotopic tracer. However, it is also possible to expose the ERM to the labelled medium contained in the HC as soon as it crosses the partition wall (see Table 7.3).

The isotopic tracer can be added to the HC either directly in/on the medium contained in this compartment depending on whether liquid or solid medium is used, or after renewing it. Note that if the isotopic tracer is added on solid medium, its diffusion into the medium should be verified (using autoradiography for instance, Gray et al. 1995). Renewing liquid medium is done by gently removing the old medium using a pipette and adding new medium. In the case of solid medium, a scalpel and forceps are used to cut and remove the medium. Thereafter, new medium is added in the HC. Solid medium should be cooled at a temperature of $\pm 40°C$ before its addition in order to reduce the risk of damaging the roots and AM fungi. The temperature of the liquid medium should be 25–27°C.

Renewing solid medium in the HC means that the AM fungal mycelium growing in this compartment will be removed. However, rapid development of AM fungal mycelium is usually observed. This operation might be even useful to synchronize ERM growth in the HC.

It should be noticed that it is also possible to mix the isotopic tracer directly with the medium before addition in the HC. For some experiments, for instance on C transport, the isotopic tracer should be placed in the RC. As the medium cannot be removed, the isotopic tracer must then be added on the solidified medium.

To get a more accurate measurement of the transport and metabolism processes, it can be useful to suppress from the medium the element (or a chemical analog) under study. For instance, Declerck et al. (2003) strongly reduced the concentration of K in the RC and had no K in the HC to study Cs transport. Identically, Jin et al. (2005) used medium lacking N but amended with $^{15}N$-labelled substrates to study N transport and metabolism in AM symbiosis. However, it should be taken into consideration that removing or modifying the concentration of one element from the culture medium will perturb the concentration of at least one other element and the balances between elements. This may therefore have an impact on the growth of the AM fungus and host.

### 7.6.3 Exposure Time

There is no predefined time for contact between the ERM and the isotopic tracer. AM transport and biosynthetic pathways vary depending on the element or substances under study but also on the fungal strain, age of the culture and ERM density in the HC. In Table 7.3, a few representative examples are given for five isotopic tracers and with the four major techniques used to detect them.

**Table 7.3** Examples of use of the ROC and AM-P *in vitro* culture systems to track element transport and metabolism by AM fungi. Time between initiation of ERM development in the HC and labeling ($T_{between}$), exposure of the ERM and/or roots to an isotopic tracer are given, as well as the *in vitro* model used and technique to detect the tracer

| Element or molecule studied | ($T_{between}$) | Exposure time | *In vitro* model used | Technique used | Reference |
|---|---|---|---|---|---|
| $^{134}Cs$ ($^{134}CsNO_3$), $^{33}P$ ($H_3{}^{33}PO_4$) | 2 weeks | 2 weeks | AM-P | Gamma counter, LSC | Dupré de Boulois et al. (2006) |
| $^{134}Cs$ ($^{134}CsNO_3$) | 2 weeks | 2 weeks | ROC | Gamma counter | Dupré de Boulois et al. (2005) |
| $^{15}N$ ($^{15}NO_3$, $^{15}NH_4{}^+$, arginine-guanido-$^{15}N$.HCl), $^{13}C$ ($^{13}C_{U6}$ arginine, $^{13}C_2$ acetate) | No | 6 weeks | ROC | GC-MS | Govindarajulu et al. (2005) |
| $^{15}N$ ($^{15}NH_4Cl$, arginine-guanido-$^{15}N$.HCl, $^{13}C_{U6}$ arginine) | No | 1, 3 and 6 weeks | ROC | GC-MS | Jin et al. (2005) |
| $^{13}C$ ($^{13}C_1$ glucose) | 11–13 days | 8 weeks | ROC | NMR & GC-MS | Pfeffer et al. (2004) |
| $^{14}C_U$ glucose | 2 weeks | 1, 2, 4, 6 and 8 weeks | ROC | LSC | Pfeffer et al. (2004) |
| $^{137}Cs$ ($^{137}CsNO_3$) | 1 weeks | 4 weeks | ROC | Gamma counter | Declerck et al. (2003) |
| $^{33}P$ ($H_3PO_4$), $^{233}U$ ($^{233}UO_2(NO_3)_2$) | 1 week | 2 weeks | ROC | LSC | Rufyikiri et al. (2002, 2003) |
| $^{32}P$ ($H_3{}^{33}PO_4$, α-$^{32}P$-CTP) | 3 weeks | 7 hours to 20 days | ROC | Autoradiography & LSC | Nielsen et al. (2002) |
| $^{33}P$ ($H_3{}^{33}PO_4$) | 4 weeks | 6 to 48 hours | ROC | LSC | Maldonado-Mendoza et al. (2001) |
| $^{32,33}P$ ($H_3{}^{33}PO_4$, 5′ [α-$^{32}P$] AMP) | Not mentioned | 3 days | ROC | LSC | Joner et al. (2000) |

*LSC* liquid scintillation counting; *GC-MS* gas chromatography–mass spectrometry; *NMR* nuclear mass spectrometry

### 7.6.4 Harvest

Before collecting the ERM from the HC containing liquid medium, the medium is removed using a manual or motorized pipettor, and the compartment is rinsed 2–3 times with water. The extraradical hyphae can then be harvested easily by shearing the extraradical hyphae along the partition wall using forceps and scalpel under the stereomicroscope.

If the HC contains solid medium, the harvest of the ERM is more arduous as separation can only be performed by dissolving the gel using citrate buffer (pH 6, 10 mM: 1.8 mM citric acid + 8.2 mM sodium citrate) with the risk of cytoplasm leakage.

To harvest the roots and ERM in the RC, it is possible to either:

- Remove the roots from the solid medium using forceps and eventually rinse them with citrate buffer if medium remains attached to the roots. The ERM can then be separated from the solid medium using citrate buffer as described above.

  or

- Place the medium containing the roots and ERM directly in citrate buffer. In this case, the ERM will still be attached to the root, so hyphae will need to be sheared from the root using scalpel and forceps. Roots and ERM are then rinsed in water.

## 7.7 Conclusions

In this chapter, we have presented the bases for conducting transport studies using either the ROC or the newly developed HAM–P and AM–P *in vitro* culture systems. These systems represent powerful tools for investigating nutrient, trace element and C transport and metabolism by AM fungi. However, the utility of these systems should not be limited to transport studies. Indeed, as the HAM-P and AM–P *in vitro* culture systems have major advantages over the ROC, these systems will most probably become important models for investigating AM biology and AM fungus-plant interactions.

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# Chapter 8
# Use of the Autofluorescence Properties of AM Fungi for AM Assessment and Handling

**B. Dreyer and A. Morte**

## 8.1 Introduction

Different methods for the visualization of arbuscular mycorrhizal (AM) fungal structures in roots have been described (Dickson and Smith 1998; Vierheilig et al. 2005) most based on non-vital and vital staining, thus making the biological material useless for further analysis. Moreover, these methods present the disadvantages of using toxic chemical compounds and the possible loss of information concerning the colonization percentage (Gange et al. 1999). One technique that is free from such disadvantages is the autofluorescence detection of AM fungal structures in roots. The method was first described by Ames et al. (1982) and involves subjecting roots to illumination, under which the arbuscules autofluoresce.

Only a few studies have made use of the autofluorescence of AM fungal structures as a technique for evaluating mycorrhizal colonization (Ames et al. 1982; Jabaji-Hare et al. 1984; Gange et al. 1999; Dreyer et al. 2006). Ames et al. (1982) found that the extent of colonization detected by this method correlated well with the colonization level detected by the staining method described by Phillips and Hayman (1970). In contrast, Gange et al. (1999) found that autofluorescence of roots in most plant species examined consistently produced a higher arbuscule count than did trypan blue, acid fuchsin or chlorazol black E stainings. They also found that stained preparations gave variable results, whereas autofluorescence gave much more consistent data.

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Recently, in light of criticisms that suggested that autofluorescence is attributable to the dead state of the arbuscules (Vierheilig et al .1999, 2001), we reassessed the autofluorescence method (Dreyer et al. 2006). The autofluorescence of AM structures within, and isolated from mycorrhizal palm roots, was compared with their succinate dehydrogenase (SDH) activity. In contrast to the previous reports, we found that all fungal structures, both intra- and extraradical, autofluoresced under blue light excitation, regardless of their state (dead or alive). The results supported the use of autofluorescence for the evaluation of AM colonization, at least in palm species. Further, it was shown that AM spores can be detected by flow cytometry, providing an example of how the fluorescence properties of AM fungal structures could be exploited (Dreyer et al. 2006).

The aim of this chapter is to provide a detailed description of the methodologies that make use of the autofluorescence properties of AM fungal structures for their assessment. The advantages and disadvantages of the techniques are discussed in relation to other methods used for visualizing AM fungal structures.

## 8.2 Equipment and Laboratory Material

### *8.2.1 Equipment*

- Stereo-microscope
- Epifluorescence microscope
- Confocal microscope
- Centrifuge
- Electronic balance
- Bunsen burner or bead sterilizer
- Incubator
- Rotating shaker
- Polytron
- Benchtop centrifuge for microtubes
- Sort flow cytometer

### *8.2.2 Laboratory Material*

- Petri dishes (usual diameter: 90 mm)
- Scalpels
- Forceps
- Razor blades
- Needles
- Paint brushes
- Pasteur pipettes

- Flasks
- Dropper bottles
- Microscope slides and cover glasses
- Manual or motorized pipettors adapted from 5 to 20 ml sterile pipettes
- 1–1,000 µl micropipettes with corresponding sterile tips
- Falcon tubes (50 ml)
- Glass centrifugation tubes (50 ml)
- Sieves (50, 125, 250 and 1,000 µm)
- 1,000 ml Beakers
- Glass rods
- Cheese cloth
- Microtubes

## 8.3 Sample Preparation

### *8.3.1 Whole Root Samples*

Roots of plants with fine root systems are mounted directly as whole roots in deionized water on slides for examination by fluorescence microscopy. For example, the roots of *Medicago sativa*.

Conversely, thicker roots must be sectioned for observation under the epifluorescence microscope (see below). Thick roots can also be digested in 10% (w/v) potassium hydroxide for 1 h at 100°C and mounted on slides with water, pressing the roots under the cover glass for examination. However, we have observed that the digestion of palm roots with potassium hydroxide removes the autofluorescence of arbuscules (Dreyer et al. 2006). But, as Jabaji-Hare et al. (1984) still detected autofluorescence after root digestion, it would be advisable to test this aspect when working with new plant material.

### *8.3.2 Root Section Samples*

As mentioned, in the case of thick roots (e.g. palm roots), root sections, rather than whole roots have to be used because the autofluorescence of arbuscules and other intraradical AM fungal structures is not visible in unsectioned whole roots due both to their thickness and/or to the high autofluorescence of extremely thickened, lignified outer cell walls of the rhizodermis of some plant species (Dreyer et al. 2006).

For analysis, root samples are cut by hand with the help of a razor blade into longitudinal and transverse root sections. By means of a paintbrush, the root sections are transferred onto slides and mounted in deionized water for immediate examination by fluorescence or confocal microscopy and afterwards subjected to the different staining procedures (see Sects. 8.5, 8.6 and 8.7). Some of the root sections are stored in ethanol in microtubes as a control for the non-viability of AM fungal structures.

### 8.3.3 Isolation of Intraradical Fungal Structures

The intraradical fungal structures of the roots are isolated following the protocol described by Saito (1995), with minor modifications.

The following solutions are prepared:

- Enzyme digestion solution:
  - 20 g $l^{-1}$ cellulase
  - 1 g $l^{-1}$ pectolyase
  - 1 g $l^{-1}$ bovine serum albumin
  - 1 mM dithiothreitol (DTT)
  - 0.3 M mannitol
  - 0.01 M MES-NaOH buffer (pH 5.5)
- Washing buffer:
  - 0.3 M mannitol
  - 1 mM DTT
  - 0.01 M Tris-HCl, pH 7.4

Approximately 5 g fresh mycorrhizal roots are harvested and washed first with water and then with 0.5 mM $CaSO_4$ for several minutes. The roots are cut into 0.5 cm root segments and incubated under shaking in the enzyme digestion solution at 30°C for 2 h. The digested roots are collected on a 50 µm sieve and washed with washing buffer. The root segments are then transferred to tubes with 20 ml washing buffer for tissue homogenization using a Polytron (30 s, lowest speed). The resulting homogenate is filtered through two layers of cheesecloth, the filtrate is kept in reserve, and the residue is collected in the tubes and homogenized again with 20 ml washing buffer. This process of homogenization and filtration is repeated twice more. The filtrates are combined and centrifuged at 1,000 g for 10 min. The pellet is suspended in 2 ml washing buffer and loaded onto a Percoll gradient consisting of 40, 20, 15 or 10% Percoll in 0.3 M mannitol, 1 mM DTT and 0.01 M Tris-HCl, pH 7.4. The Percoll gradient is prepared by successive loading of the Percoll solutions in a 50 ml glass centrifuge tube. The centrifugation is performed at 430 g for 30 min. The fractions enriched in AM fungal structures are collected from the interfaces between the Percoll bands with a Pasteur pipette. These fractions are mixed, diluted with 20 ml washing buffer and centrifuged again at 1,000 g for 5 min. The pellet is transferred to a 50 µm sieve and washed with washing buffer before being centrifuged again in 10 ml washing buffer at 1,000 g for 10 min. The resulting pellet contains the AM fungal structures.

**Remark:** All steps during the enzyme digestion are performed at 4°C.

The isolated fungal structures can be directly subjected to autofluorescence and/or SDH-activity staining. The process performed for isolating AM fungal structures does not presumably affect the enzymatic SDH-activity (Saito 1995; Dreyer et al. 2006).

### 8.3.4 *Spores*

The most frequently used techniques for inoculum production in most of laboratories working with mycorrhiza are the propagation of AM fungi in pot cultures with trap plants, or in monoaxenic cultures with transformed carrot roots, according to Bécard and Fortin (1988). Consistently, only these two methods are considered here. The methods for producing AM fungus inocula in pot cultures in association with trap plants have been extensively described (Menge 1984; Jarstfer and Sylvia 1993; Brundrett et al. 1996), while several reviews are devoted to inoculum production by means of root organ cultures (Bécard and Piché 1992; Fortin et al. 2002; Declerck et al. 2005).

In the case of pot cultures, spores are isolated by wet sieving and decanting (Gerdemann and Nicolson 1963). In the case of monoaxenic cultures, the method of Doner and Bécard (1991) is followed.

After these isolation steps, the spores are transferred to microtubes and subjected to several washing steps in sterile deionized water to eliminate as much debris as possible. Some spore samples are mounted on slides with the help of a Pasteur pipette and examined immediately under the epifluorescence microscope. They can then be subjected to staining directly afterwards.

Other spore solutions are prepared for flow cytometry. It is recommended that the mean spore number be determined under the stereomicroscope when setting up this experiment. Additionally, it is important to determine the mean diameter of the spores of the AM fungus species to be studied. The spore solutions of species with spore sizes bigger than 200 µm should be sieved through a 200 µm sieve to fit the particle size that can be handled by the flow cytometer to prevent the tubes of the fluid system from obstruction. This is not necessary, for example, for *Glomus intraradices* since the spores of this species range between 40 and 150 µm in size, while *Glomus clarum* spore diameters range between 100 and 260 µm, making sieving advisable.

## 8.4 Bright-Field Microscopy, Epifluorescence Microscopy and Lambda-Scan

All the root sections, isolated intra-radical structures and isolated spores prepared as stated above can be observed by bright-field and epifluorescence microscopy. In our laboratory, we use a Leica Leitz DMRD epifluorescence microscope fitted with a mercury lamp (Leica, Wetzlar, Germany), which permits easy switching between the different settings and filter cubes.

Unstained fresh material should always be observed under bright-field settings before subjection to illumination.

The autofluorescence of AM fungal structures is observed with our microscope under epifluorescence settings with the filter cubes IR (excitation filter BP 450–490,

dichroic mirror RKP 510, barrier filter LP 515), and N2.1 (excitation filter BP 515–560, dichroic mirror RKP 580, barrier filter LP 590) for blue and green light excitation, respectively. Most of the epifluorescence microscopes on the market allow similar settings, as these are the most commonly used settings for biological samples. However, the terminology applied to the different filters by the different manufacturers may be confusing. Basically, there are three categories of filter: exciter filters, barrier filters and dichromatic beam splitters (dichroic mirrors). Exciter filters permit only selected wavelengths to pass through on the way toward the specimen. Barrier filters are designed to suppress or block the excitation wavelengths and only permit selected emission wavelengths to pass towards the eye (or the detector). Dichroic mirrors are specialized filters which are designed to efficiently reflect excitation wavelengths and allow emission wavelengths to pass. As an example, our filter cube IR has a 450–490 excitation filter, meaning that a high percentage of the excitation light falls between 450 and 490 nm in wavelength. The dichroic mirror, RKP 510, shows high transmission at wavelengths above 510 nm and maximum reflectivity to the left of 510 nm. The IR filter cube has a LP 515 barrier filter which transmits a high percentage of wavelengths above 515 nm, from green to far red. The optics of the microscope should be checked before starting the experiment, carefully choosing the filters in accordance with the maxima of the absorption-emission spectra of the material to be examined. In case of doubt, it is advisable to consult the manufacturer about the characteristics of the particular filters. Microscope companies supply the transmission curves for their excitation and barrier filters as well as for their dichroic mirrors.

Although we observed no autofluorescence when the AM fungal structures were excited with UV-light using the filter cube A (BP 340–380 excitation filter, RKP 400 dichroic mirror, LP 425 barrier filter) (Dreyer et al. 2006), this should be checked before working with a new sample for the first time.

Immediately after examination, all the samples observed for autofluorescence are subjected to different vital and non-vital staining procedures (see Sects. 8.6 and 8.7). These are then observed under bright-field settings. In the case of sections and spores stained with calcofluor white M2R "new" (CFW), these are examined under UV excitation, while the sections treated with 0.1 M ammonium hydroxide are observed under both UV and blue light excitation.

**Remark:** Micrographs should be taken from the same root section for autofluorescence and for staining, to allow proper comparison.

If available in the laboratory, a confocal laser scanning microscope should be used for examining unstained root sections and spores. In our case, we count with a Leica TCS-SP2 confocal laser scanning microscope (Leica Microsystems Inc., Wetzlar, Germany). Samples are examined by excitation with an argon/krypton (Ar/ArKr) laser at 488 nm and by using a reflection short pass (RSP) 500 filter. The spores can be additionally excited by the 543 nm line of a green helium/neon (GreNe) laser. Emission is observed with a water immersion lens ×20 (PL APO CS) using an FITC band pass filter.

**Remark:** An interesting feature of some confocal laser scanning microscopes is that a lambda-scan can be conducted. This allows the recording of the emission

spectra of the structures being observed. We conducted a lambda-scan each 5 nm for emission spectrum recording of arbuscules, vesicles and spores (Table 8.1; Fig. 8.1; Dreyer et al. 2006). The maxima of the emission spectra could serve to define appropriate settings when using confocal laser scanning microscopes, epifluorescence microscopes or flow cytometers. For example, when observing the arbuscules by epifluorescence with two emission maxima at 534.7 and 552.5 nm (Fig. 8.1), their autofluorescence can be observed by blue excitation, but the emitted wavelengths can be restricted to green emission only, using a BP 515–560 barrier filter.

**Table 8.1** Emission maxima for the different AM fungal structures measured using the λ-scan of the confocal laser scanning microscope. (Dreyer et al. 2006)

| AM fungal structure | Emission maxima | | |
|---|---|---|---|
| | 1 | 2 | 3 |
| Vesicles | 515–529 | 572–600 | |
| Arbuscules | 526–536 | 550–576 | |
| Spores | 503–527 | 559–608 | 655–680 |

The excitation wavelength used for λ-scan leading to the emission maxima for arbuscules and vesicles was 488 nm and for spores were 488 nm, for maxima 1 and 2, and 543 nm for maximum 3

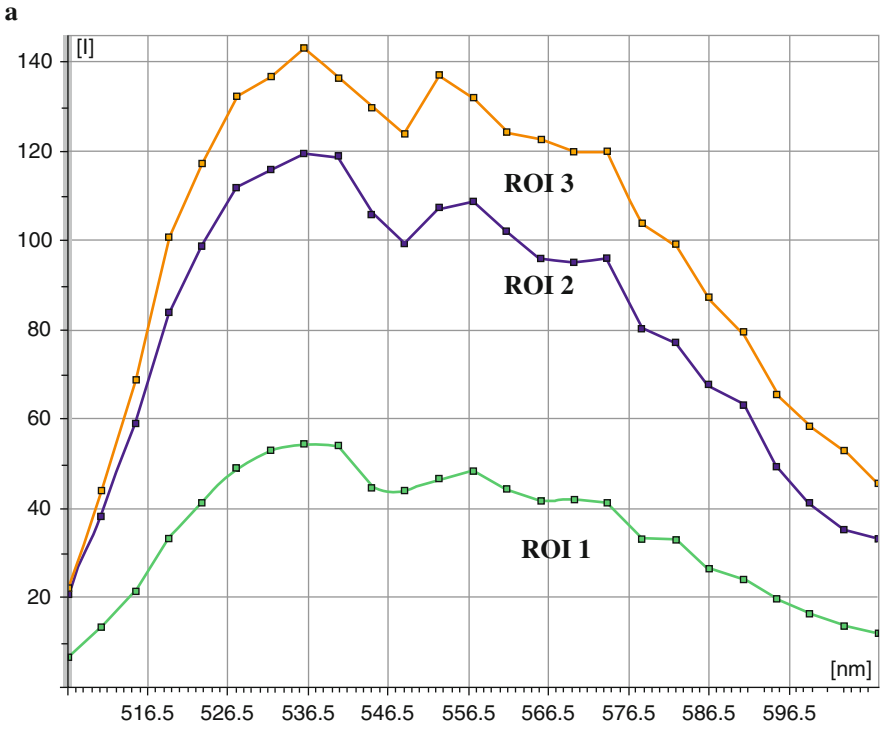


**Fig. 8.1** (Continued)

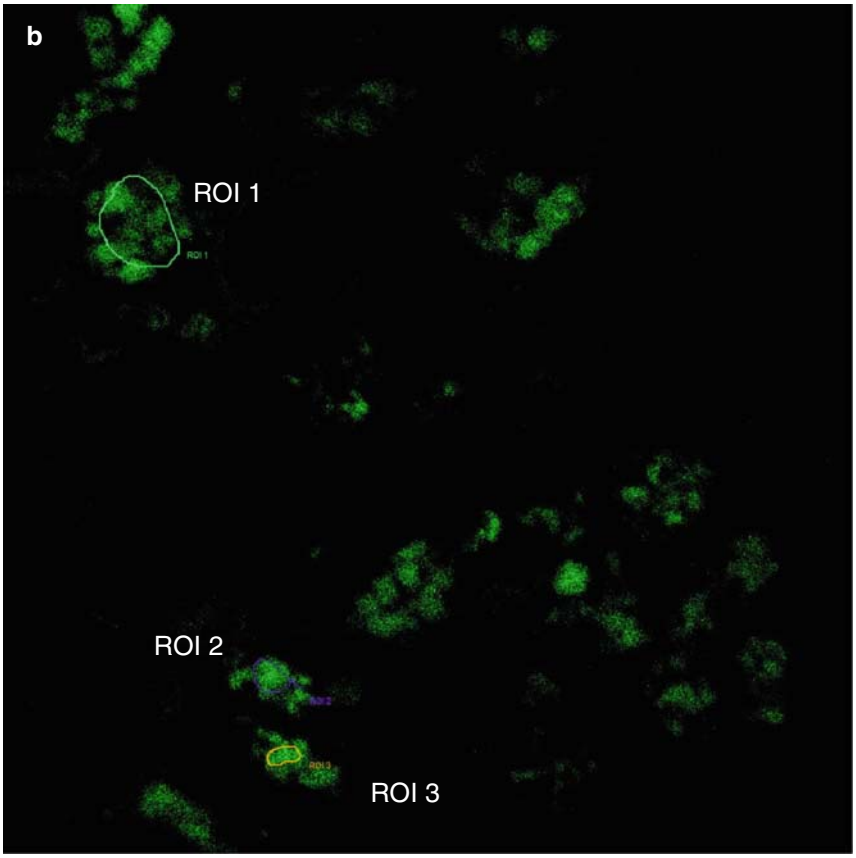


**Fig. 8.1** Fluorescence spectra of three different arbuscules. (**a**) The two emission peaks were observed at 534.7 nm and 552.5 nm. The λ scanning of the confocal microscope was made each 5 nm. (**b**) Confocal image of the autofluorecing arbuscules, showing the area for which the λ scan was conducted, the objective ×20

## 8.5 Autofluorescence of AM Fungal Structures

The process by which material, whether living or non-living, organic or inorganic, absorbs and subsequently re-radiates light, is described as photoluminescence. If the light emission persists only during the absorption of the excitation light, the phenomenon is known as fluorescence. When the studied material is made to fluoresce in its natural form, the process is known as primary fluorescence or autofluorescence, but when the studied material is treated with fluorochrome stains, the process is called secondary fluorescence (Abramovitz 1993). Many specimens, e.g. microminerals, crystals, resins, chlorophyll, phenolic compounds, etc., autofluoresce when irradiated with ultraviolet or blue light. This is also the case when

arbuscules, formed during AM colonization as characteristic fungal structures in roots, are irradiated with blue light (Ames et al. 1982).

Although the method of autofluorescence detection of AM fungal structures has been known for more than 25 years (Ames et al. 1982), it has not been widely used and its advantages for the assessment and management of AM fungi have not been fully explored.

Ames et al. (1982), tested five different plant species colonized by different AM fungi, including species of the genera *Glomus*, *Gigaspora* and *Acaulospora*, and observed that the arbuscules autofluoresced when illuminated with blue light and that the AM fungal species, host plant and growth conditions had no effect on the autofluorescence pattern. The study of Gange et al. (1999) confirmed that the autofluorescence of AM fungal structures was a common phenomenon, since it was observed in eight different plant species colonized by native AM fungi. Table 8.2 shows the plant species studied until now by means of autofluorescence detection of AM fungal structures.

There are several possible reasons why autofluorescence detection has not been more widely used as a method for visualizing AM fungal structures. One reason may be that the title of the article by Ames et al. (1982) was misleading, since the authors described the autofluorescence as being induced by ultraviolet light. If some

**Table 8.2** Plant species studied by autofluorescence detection for visualizing AM fungal structures

| Plant species[a] | Material examined | Reference |
|---|---|---|
| *Allium porrum* | Root sections | Jabaji-Hare et al. (1984) |
| *Allium textile* | Whole-root segments | Ames et al. (1982) |
| *Artemisia frigida* | Whole-root segments | Ames et al. (1982) |
| *Bouteloua gracilis* | Whole-root segments | Ames et al. (1982) |
| *Brachypodium pinnatum* | Whole-root segments | Gange et al. (1999) |
| *Brahea armata* | Root sections | Dreyer et al. (2006) |
| *Chamaerops humilis* | Root sections | Dreyer et al. (2006) |
| *Dactylis glomerata* | Whole-root segments | Gange et al. (1999) |
| *Fragaria vesca* | Whole-root segments | Ames et al. (1982) |
| *Holcus lanatus* | Whole-root segments | Gange et al. (1999) |
| *Hyacinthoides non-scripta* | Whole-root segments | Gange et al. (1999) |
| *Lolium perenne* | Whole-root segments | Vierheilig et al. (1999) |
| *Medicago sativa* | Whole-root segments | Dreyer et al. (2006) |
| *Nicotiana tabacum* | Whole-root segments | Vierheilig et al. (2001) |
| *Phoenix canariensis* | Root sections | Dreyer et al. (2006) |
| *Phoenix dactylifera* | Root sections | Dreyer et al. (2006) |
| *Plantago lanceolata* | Whole-root segments | Gange et al. (1999) |
| *Scutellaria brittonii* | Whole-root segments | Ames et al. (1982) |
| *Senecio vulgaris,* | Whole-root segments | Gange et al. (1999) |
| *Senecio jacobaea* | Whole-root segments | Gange et al. (1999) |
| *Veronica persica* | Whole-root segments | Gange et al. (1999) |

[a]Gange et al. (1999) also examined the plant species *Capsella bursa-pastoris* and *Poa annua*, but as no arbuscules were seen, they were omitted from further analysis

researchers followed these indications and excited their root samples with UV-light, they would surely not have achieved any results, as the AM fungal structures do not autofluoresce under UV-light excitation but under blue light excitation (Dreyer et al. 2006). Another reason may be that the literature contains some contradictions concerning the reasons for autofluorescence. Some studies attribute autofluorescence to the dead state of the arbuscules (Vierheilig et al. 1999, 2001), while others show that all AM fungal structures autofluoresce regardless of their metabolic state (Dreyer et al. 2006; see Sect. 8.6). Furthermore, it is not known whether the autofluorescence is due to plant or fungal cell-wall components (see Sect. 8.7). Finally, it has also to be noted that many laboratories do not possess epifluorescence microscopes and that non-vital staining is much cheaper, which could explain why most mycorrhizologists still use non-vital staining for visualization.

To measure autofluorescence, the whole roots, root sections, as well as the isolated AM fungal structures are mounted in deionized water (pH 7) on slides for observation at $\times 100$, $\times 200$ and $\times 400$ under the epifluorescence microscope, as described above.

Figure 8.2 shows some images obtained with the epifluorescence microscope, where it can be seen that all intra- and extra-radical AM fungal structures, e.g. hyphae, vesicles, arbuscules and spores, autofluoresce (Fig. 8.2a). The same autofluorescence pattern is observed in AM fungal structures isolated from roots (Fig. 8.2b).

Switching between the different possible filter settings of the microscope is recommended since, although all AM fungal structures autofluoresce under blue light excitation, some, such as mature spores and some arbuscules, have been shown to autofluoresce when excited with green light (Fig. 8.2c; Dreyer et al. 2006). No autofluorescence was found when the sample was irradiated with UV light (Fig. 8.2d; Dreyer et al. 2006). The only study in which excitation wavelengths other than those in the blue range were assayed, showed strong emission in all AM fungal structures under UV, blue and green light irradiation (Jabaji-Hare et al. 1984), emphasising the need to use all possible filter combinations when examining new material.

**Remark:** When using whole roots, the autofluorescence detection method is accurate only for detecting arbuscules and extraradical hyphae and spores (Ames et al. 1982; Gange et al. 1999; Dreyer et al. 2006). However, when root sections are used, all AM fungal structures can be detected, with differences, of course, in their emission intensity (strong autofluorescence of the arbuscules, weak autofluorescence of intra-radical hyphae and vesicles).

Even though it is a time-consuming task to evaluate the degree of mycorrhization in a complete root system by producing many root sections (independently of the visualization method chosen, i.e. staining or autofluorescence), for some non-annual plants there may be no way of avoiding this, as not all plants are characterized by thin root systems that can be easily stained and/or mounted on slides for microscopy. In addition, it should be remembered that data obtained from whole-root observations represent qualitative results, while an approach using sectioned material results in quantitative data (Toth and Toth 1982). Studies dealing

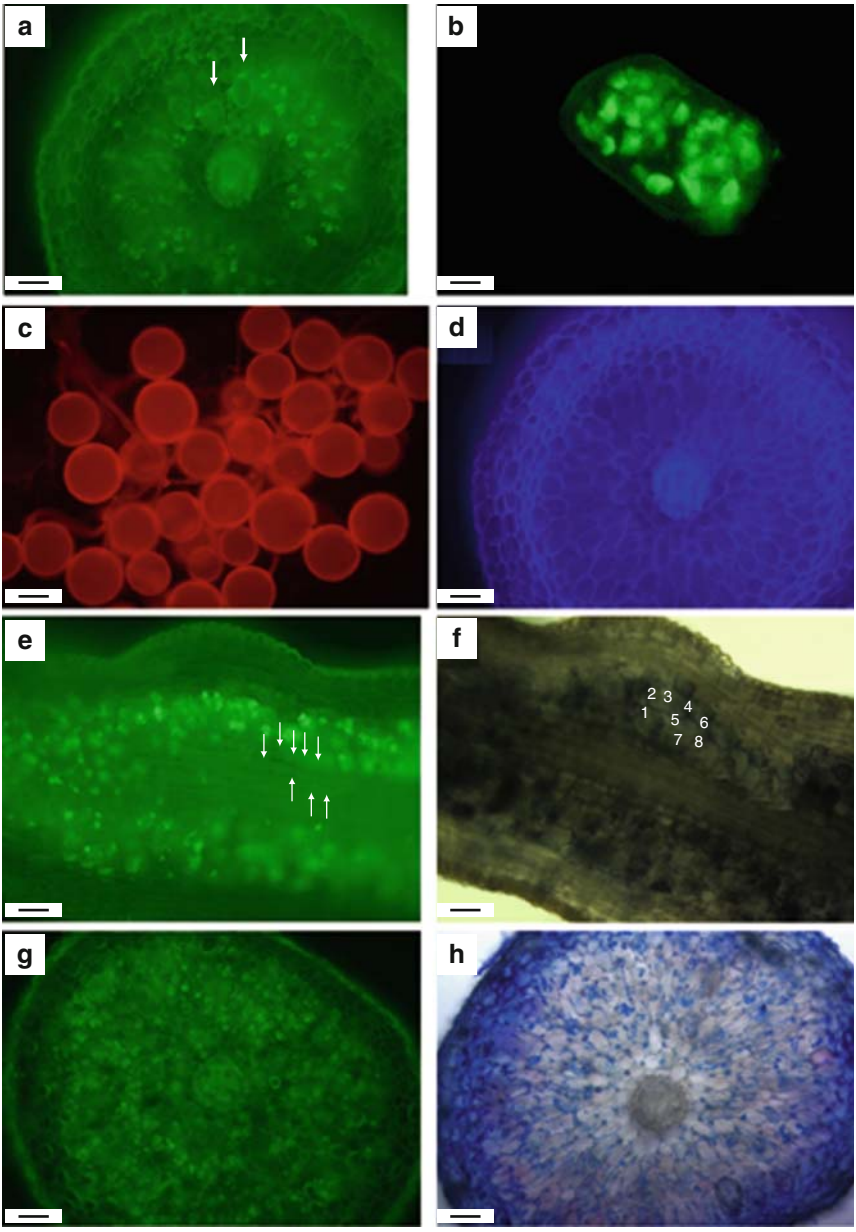


**Fig. 8.2** (**a**) Cross root section of *Phoenix canariensis* irradiated with blue light, showing autofluorescing arbuscules and vesicles (*arrows*). (**b**) Isolated arbuscule autofluorescing under blue light excitation. (**c**) Spores of *Glomus intraradices* observed under green light excitation. (**d**) Same root section as in **a**, but irradiated with UV light showing no autofluorescence of AM fungal structures. (**e**) Longitudinal third-order root section of *Phoenix canariensis* under blue light excitation showing autofluorescing arbuscules; *arrows* correspond to numbers *1–8* from **f**. (**f**) Same section as in **e** after NBT-staining; numbers *1–8* correspond to *arrows* in **e**. (**g**) Transverse root section of *Phoenix dactylifera* showing autofluorescing arbuscules. (**h**) Same section as in **e** stained with trypan blue. *Bar* = 100 μm. (Dreyer et al. 2006)

with the efficiency of the association, e.g. experiments on phosphorus uptake, require a more quantitative approach. In such cases autofluorescence detection could be advantageous, not only because staining with harmful stains is avoided, but because it permits the observation of structures of AM fungi *in vivo* (Dreyer et al. 2006).

## 8.6 Viability of Fungal Structures

The quantity of viable AM fungal structures has been determined by a variety of methods (Dickson and Smith 1998; Vierheilig et al. 2005). Some of them use tetrazolium salts that act as electron acceptors for coenzyme-linked cellular dehydrogenases, which, upon reduction, form highly coloured insoluble compounds, called formazans. In the case of AM roots, nitroblue tetrazolium (NBT) has frequently been used (MacDonald and Lewis 1978; Smith and Dickson 1991; Schaffer and Peterson 1993; Vierheilig et al. 2001; Dreyer et al. 2006). NBT is coupled to the activity of succinate dehydrogenase (SDH), a mitochondrial enzyme, to produce the purple product formazan. However, colour reactions can range from purple in isolated AM fungal structures to brown in root sections (Dreyer et al. 2006). The reason for the different staining of the SDH is not known. McGee and Smith (1990) indicated that both colours were indicators of SDH activity occurring in the fungal mitochondria and that the blue-purple colour indicated the full reduction of the tetrazolium salt, while the red-brown colour indicated its partial reduction.

The staining for succinate dehydrogenase (SDH) activity is performed as follows:

The stock incubation solution for the determination of the succinate dehydrogenase activity consists of (MacDonald and Lewis, 1978):

- Tris buffer, 0,2 M, pH 7,4 2.5 ml
- Nitro-blue tetrazolium (4 mg $ml^{-1}$) 2.5 ml
- $MgCl_2$ 0,05 M 1 ml
- Distilled Water 3 ml

Prior to use, the stock incubation solution is amended with 2.5 M disodium succinate as follows:

- Stock incubation solution 9 ml
- Disodium succinate solution 1 ml

**Remark:** The disodium succinate solution is prepared fresh every time, stirring the solution on a warm plate to dissolve the crystals. All material and samples used must be stored in the dark as formazan is a photosensitive compound.

Root sections and isolated AM fungal structures are stained directly with this NBT solution, without further digestion or clearing. The incubation times are 16 and 2 h for root sections and AM fungal structures, respectively (Dreyer et al. 2006).

**Remark:** Clearing is necessary in the case of whole roots (Schaffer and Peterson 1993; Brundrett et al. 1994). If samples are counterstained with acid fuchsin then the viable and total (viable and non-viable) root colonisation can be assessed (Brundrett et al. 1994; Dickson and Smith 2001).

After incubation in NBT, the fungal structures are washed with deionized water and mounted on slides for observation. The percentage of arbuscules showing SDH activity can be calculated.

Figure 8.2e, f show root sections observed by epifluorescence microscopy and immediately afterwards incubated in an NBT solution. It can be clearly observed that the arbuscules that autofluoresced were also stained by NBT, from which it follows that metabolically active arbuscules autofluoresce. In Table 8.3 data of the percentage of autofluorescing arbuscules showing SDH activity are listed for four palm species. More than 80% of the autofluorescing arbuscules present in root sections were metabolically active. In the case of arbuscules isolated from roots of *Phoenix dactylifera*, 94% stained positively with NBT (Dreyer et al. 2006). These results show that both dead and living arbuscules autofluoresced.

For comparison with the results obtained with SDH staining, further root sections, after their examination with fluorescence microscopy, can be stained overnight with 0.05% trypan blue in lactic acid without previous digestion or clearing, and then examined again under bright-field microscopy. Trypan blue is recommended for comparative studies, as it is the most widely used non-vital stain in AM studies (Gange et al. 1999). The non-vital staining results normally show that the extent of arbuscular colonization is better detected by autofluorescence than by trypan blue staining (Figs. 8.2 g, h; Gange et al. 1999). Vierheilig et al. (2001) showed that clumped autofluorescing arbuscules were not stained by trypan blue. This could explain why fewer arbuscules are detected by trypan blue staining than by autofluorescence and indicates further that both dead and living arbuscules can be detected by autofluorescence. However, the fact that some arbuscules are not stained by trypan blue could be due to the differential stain penetrability of the roots of different plant species, and the extent to which the stain is taken up by the fungus, as demonstrated by Gange et al. (1999). This aspect merits further study, as if collapsed arbuscules can not be stained by currently used non-vital stains, it would

**Table 8.3** Percentage of autofluorescing arbuscules that were metabolically active. (Dreyer et al. 2006)

| Root sections | Percent of succinate dehydrogenase-active arbuscules[a] | Total number of arbuscules |
|---|---|---|
| *B. armata* | 88.2 ± 2.4 | 348 |
| *C. humilis* | 83.9 ± 3.6 | 967 |
| *P. canariensis* | 89.0 ± 1.9 | 889 |
| *P. dactylifera* | 82.7 ± 5.3 | 482 |
| Isolated fungal structures of *P. dactylifera* | 93.5 ± 2.4 | 400 |

[a]Means with standard error

mean that the counterstaining used in combination with the vital staining in AM studies, e.g. in Dickson and Smith (2001), would not function either.

## 8.7 Autofluorescence Localization

The literature contains many contradictions concerning the reasons for autofluorescence. It is not known, for example, whether the autofluorescence is due to plant or fungal cell-wall components. The source of autofluorescence has been shown to be localized in the fungal cell wall of both hyphae and spores (Dreyer et al. 2006). However, in arbuscules it was almost impossible to determine whether the fungal cell wall is the cause of the autofluorescence because of the strong emission intensity and the small diameter of the arbuscular branches. Although we have shown that arbuscules isolated from roots still show autofluorescence (see Sect. 8.5) and thus it can be indirectly inferred that the autofluorescence is due to fungal material, it is possible that some plant material is still attached or linked to the arbuscules in spite of the multiple washing steps.

Chitin (Jabaji-Hare et al. 1984) and plant phenolic compounds (Vierheilig et al. 1999, 2001) have been mentioned as candidates for the fluorescing components. Staining root sections and AM fungal structures for these compounds after autofluorescence detection could therefore serve to characterize the source of autofluorescence.

Two methods are presented here that may help visualize these compounds:

Calcofluor white M2R "new" (CFW) is a fluorochrome that stains 1, 4-linked polymers such as chitin and cellulose (Jensen et al. 1998). For CFW staining, a drop of a 10% (w/v) potassium hydroxide solution is mixed with a drop of a 0.1% (w/v) CFW solution on a microscope slide and the root sections or spores are placed in this mixture. After 1 min, the slides are observed under epifluorescence microscopy. Fluorescence of CFW-stained AM fungal structures is observed when the sample is excited with UV-light which, in our microscope, corresponds to the filter cube A (excitation filter BP 340–380, dichroic mirror RKP 400, barrier filter LP 425). The CFW-stained structures emit an intense chalk-white colour when illuminated with UV-light. It has been shown that CFW stains the same parts of the AM fungal structures that autofluoresce (Dreyer et al. 2006), indicating that chitin might be the cause of the observed autofluorescence. Unfortunately, CFW stains the cell wall of both AM fungal structures and plant cells, which hinders documentation by micrography of the stained fungal structures in root sections (Dreyer et al. 2006). It should also be considered that fluorescence may depend on molecules other than chitin or on the interaction of chitin with other cell wall components.

It is known that treatment with ammonium hydroxide can shift the fluorescent properties of phenolic acids, flavonoids and their derivatives (Harris and Hartley 1976; Mathesius et al. 1998). After examination for autofluorescence at pH 7, fresh cross-sections can be treated with 0.1 M ammonium hydroxide (pH 10) for 5 min and then observed for changes in fluorescence intensity and colour under UV- and

green-illumination. Such a treatment has been shown to cause the fluorescence intensity of arbuscules to diminish (Dreyer et al. 2006). It can be assumed from this that the autofluorescing compounds are easily hydrolyzed.

Although it has been shown that the source of autofluorescence is located in the fungal cell wall of AM fungi, there is still no information on the exact nature of the compounds of the AM fungal structures responsible for their autofluorescence patterns. Further studies should be devoted to clarifying this aspect.

## 8.8 Spore Autofluorescence and Flow Cytometry

Spores of AM fungi also autofluoresced under blue light and green light excitation (Fig. 8.2c; Dreyer et al. 2006). This spore autofluorescence can be exploited for counting spore numbers, determining spore size, as well as for detecting and separating a small number of particular spores from a large mixed population based on their different autofluorescence patterns (e.g. young versus mature spores) by means of flow cytometry.

By definition, cytometry means cell measurement. Flow cytometry measures and analyzes many different physical characteristics of single cells as they move in a fluid stream and are excited by a laser beam. As cells are exposed to the laser light they scatter light in different directions and emit fluorescence. Scattered light and fluorescence are recorded by a coupled electronics system which converts the detected light signals into electronic signals that can be processed by the computer. From these, cell properties, such as relative size, relative granularity or internal complexity and relative fluorescence intensity, can be calculated.

This technique had never been used before to assess AM spores, but preliminary results with spores of *Glomus intraradices* and *Glomus clarum* show that the above technique may be a very promising tool for evaluating inocula produced on a large scale (Fig. 8.3; Dreyer et al. 2006). As laser irradiation takes a very short time (about 2–5 μs spore$^{-1}$), spores do not suffer any alteration or fluorescence bleaching and could therefore be used subsequently for inoculations or further analysis.

Solutions containing 1,000–10,000 spores per ml are prepared as stated above, and analysed with a flow cytometer. In our laboratory, we work with a Becton Dickinson FACSort flow cytometer (Becton Dickinson, Franklin Lakes, NJ) powered by an argon-ion laser (488 nm). Data are collected for right-angled light scatter and fluorescence signals in the green (530/30 band pass filter), orange (585/42 band pass filter) and red (650 long pass filter) regions of the spectrum. Instrument settings are chosen so that 10,000 events are analysed per sample. Spontaneous settling of the spores should be minimized by continuous shaking of the sample tubes.

**Remark:** Some flow cytometry instruments may be additionally equipped with a sorting feature that permits the physical separation of subpopulations of cells based on specific cellular characteristics. Thus, it is possible to rapidly separate spore aliquots into different vials, facilitating the preparation of spore inocula with exact propagule numbers and physical characteristics. Cell sorting could be performed

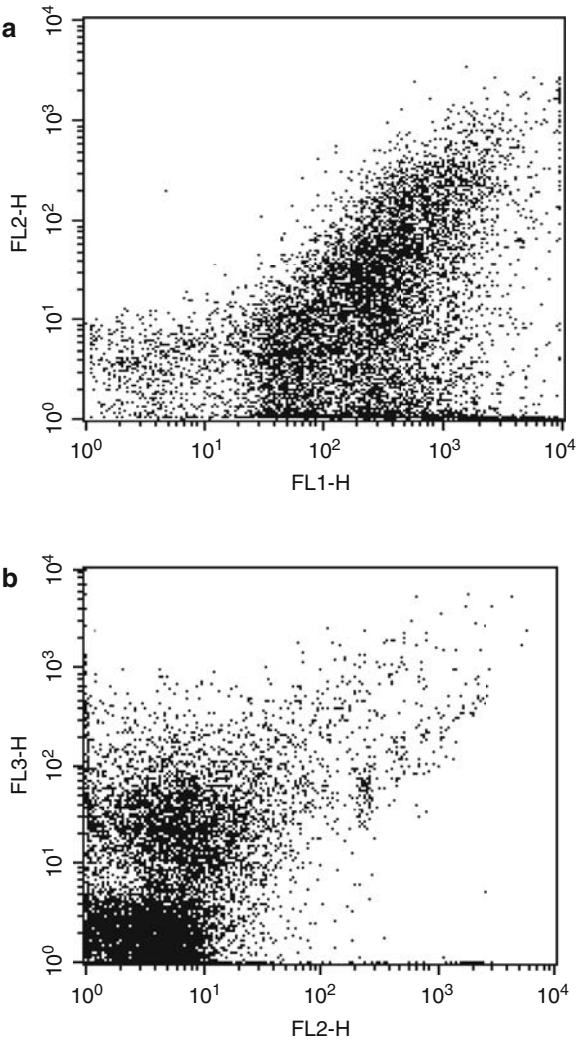


**Fig. 8.3** Spore populations of *Glomus clarum* analysed by flow cytometry. (**a**) *FL1-H*: green fluorescence, *FL2-H*: orange fluorescence. (**b**) *FL2-H*: orange fluorescence, *FL3-H*: red fluorescence. (Dreyer et al. 2006)

under sterile conditions and the cells could be collected into various types of container (Eppendorf tubes or microscope slides).

The usefulness of flow cytometry for detecting AM fungi spores is currently limited only by the diameter of the fluid system tubes, as the currently available flow cytometers are only suitable for the analysis of particles or cells ranging from 0.2 to 200 μm in size. Some AM fungal species possess spore sizes bigger than 200 μm, which can not be managed by flow cytometry.

## 8.9 Conclusions

This chapter provides information on the different methods tested until now that exploit the autofluorescence properties of AM fungi for their assessment and handling. The autofluorescence detection method is probably the only method that permits AM colonization of roots to be measured *in vivo* in a non-destructive manner. The use of this method in conjunction with confocal laser scanning microscopy may open up a field of multiple opportunities. Spore autofluorescence can be further used for determining the number, size and other physical characterisitics of spores of AM fungi by flow cytometry.

The fluorescent patterns of AM fungal structures could be used for characterizing their optical properties and hence their chemical components by optical spectroscopy. As we have seen, there are differences in the emission maxima of the different structures and also between different developmental stages (additional red light emission coinciding with the mature stage of spores).

It is unfortunate that the advantages of the method of fluorescence detection of AM fungi have not been fully recognized, but as more laboratories investigate the possibilities we can expect to see further improvement in the technology and a wider acceptance of the method.

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# Chapter 9
# Role of Root Exudates and Rhizosphere Microflora in the Arbuscular Mycorrhizal Fungi-Mediated Biocontrol of *Phytophthora nicotianae* in Tomato

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## 9.1 Introduction

Pesticides are still intensively used in modern agriculture in order to counterbalance yield losses due to disease outbreaks. Fumigants are highly volatile and toxic to human and environmental health. Other pesticides, also pollutants, are poorly efficient against soilborne diseases and their use often leads to pathogen resistance. These products are thus more and more banned. On the other hand, techniques such as cultural rotation, solarization and the management of biological control agents constitute simple and environmentally respectful alternatives that would make it possible to fight soilborne diseases with fewer unwanted side effects. Biological control (or biocontrol) consists in the reduction of disease damages or pathogen propagation through the application of antagonistic agents. Arbuscular mycorrhizal fungi (AMF) are ubiquitous and form a mutualistic symbiosis with most land plants through which growth and resistance to abiotic and biotic stresses are then generally increased (Smith and Read 1997). Bioprotection by these fungi has been shown in many agricultural systems invaded not only by pathogenic soilborne protists, fungi and nematodes (St-Arnaud and Vujanovic 2007; St-Arnaud et al. 1995a) but also by insects (Gange 2001; Gange and Nice 1997). This biological control can be explained through the contribution of diverse, interrelated and often synergistic mechanisms (St-Arnaud and Vujanovic 2007). The understanding of these mechanisms is a prerequisite in order to optimize AMF management in agriculture.

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Repercussions on the host plant physiology and on the soil microbial communities after the spread of mycorrhizal inocula or during natural mycorrhizal population management in the field must be understood and controlled in order to efficiently use this promising approach of plant disease control.

This chapter focuses particularly on the mechanisms implicated in the biocontrol which AMF induce in tomato plants infected by the soilborne pathogen *Phytophthora nicotianae*. The present paper focuses particularly on the role of root exudates. *P. nicotianae* mainly proliferates through asexual reproduction that leads to the massive liberation of mobile spores, making it a sensitive tool allowing dissecting the effect of AMF on pathogen development in the soil, before root infection.

## 9.2 Zoospore Chemotaxy in *P. nicotianae*

*P. nicotianae* Breda de Haan, 1896, is a filamentous stramenopile of the order *Oomycete*, family *Pythiaceae*. Its virulence was shown over 72 genera and 40 families of angiosperms (Satour and Butler 1967) and it is responsible for important yield losses of various agricultural crops and fruit trees, including tomato. This pathogen essentially invades roots, but necroses, fades and biomass decreases have also been reported on shoots of sensitive plants. Strategies to fight its propagation mainly involve cultural rotation, soil disinfection, and fungicide drenches.

The genus *Phytophthora* possesses a diploid life cycle (meiosis happens when reproductive cells are formed). It proliferates mainly by the spread of mobile spores (zoospores), formed during asexual reproduction (Erwin and Ribeiro 1996). The reduction in the number of zoospores attracted by roots, their encystment and germination and then ability to form appressoria on root surface allow the plant to efficiently activate defense pathways and to limit infection. Conversely, if these preinfection steps are not hampered, many cycles of zoospore production occur and the pathogen proliferates and rapidly induces diseases symptoms.

The conditions that lead to zoospore formation are high temperature (between 20°C and 25°C) and humidity. Sporangia, formed at the extremities of hyphae, differentiate zoospores that swim towards roots, in the soil solution. Zoospores are mostly attracted by the subapices of roots where most exudates are liberated, and their lifespan is strongly influenced by environmental conditions (Ho and Hickman 1967a, b).

Chemotaxy of zoospores by root exudates (Deacon 1996; Deacon and Donaldson 1993) and by ethanol, sugars, amino acids, organic acids, secondary metabolites, and volatiles (Allen and Newhook 1973; Donaldson and Deacon 1993a, b; Halsall 1975; Hickman 1970; Jones et al. 1991; Leaño et al. 1998) has been shown *in vitro*. Attraction depends on pH (Morris et al. 1995), on the experimental conditions (Deacon and Donaldson 1993), and is strain- and species-dependent: the more a species is host-specific, the more its zoospores are attracted by specific molecules. For example, the zoospores liberated by *Phytophthora sojae*, which is virulent

on soybean only, were only attracted by the flavonoids dadzeine and genisteine (Connolly et al. 1999; Morris and Ward 1992). Zoospores of *P. nicotianae* were shown to be attracted by various sugars (sucrose, dextrose, fructose, rhamnose, and maltose) but also to a lower degree by amino acids (asparagine and glutamine especially) (Dukes and Apple 1961; Halsall 1975). The activation of receptors would be implicated in chemotaxy: zoospores do not take up nutrients (Deacon and Donaldson 1993) and their aggregation is induced by antibodies (Addepalli and Fujita 2001; Estrada-Garcia et al. 1990; Hardham et al. 1994). Nonetheless, they have been shown to be attracted by the anode in an electric field (Khew and Zentmyer 1973; Morris et al. 1995; van West et al. 2003) and to autoaggregate (Reid et al. 1995).

## 9.3 Biocontrol Mediated by AMF on *P. nicotianae* Infecting Tomato

The AMF-mediated biocontrol of the root disease caused by *P. nicotianae* on tomato plants has been the object of much interest in the last decade. Colonization with *Glomus mosseae* (Nicol. and Gerd.) Gerdemann and Trappe (BEG 12) was shown to protect tomato plants subsequently infected by *P. nicotianae*. Reductions of the number of infection loci, of the level of root necroses, of intraradical pathogen development and of the biomass loss caused by the pathogen as compared to nonmycorrhizal plants were documented (Cordier et al. 1996; Pozo et al. 2002a; Trotta et al. 1996; Vigo et al. 2000). Using a compartmented soil system in which tomato plants were first colonized with either *Glomus mosseae* or *Glomus intraradices* Schenck and Smith (DAOM 181,602) and then exposed to infection with *P. nicotianae*, we recently showed that *G. intraradices* express a similar biocontrol ability to that of *G. mosseae* (Lioussanne et al. 2006a), contrary to the observations of Pozo et al. (2002a), who used another *G. intraradices* strain (BEG 72). The pathogen biomass was significantly reduced within tomato roots precolonized with both AM fungi in comparison to nonmycorrhizal plants. In plants colonized with *G. mosseae*, the reduction of pathogen development was shown to be systemic and to happen not only in mycorrhizal root tissues but also in non-colonized parts of the root system (Pozo et al. 2002b).

## 9.4 Induction of Tomato Plant Defense Mechanisms Following Mycorrhizal Colonization

The stimulation of tomato plant defense pathways has been shown. Constitutive synthesis of β-1,3-glucanase, and new isoforms of β-1,3-glucanase and chitinase have been detected within roots colonized with *G. mosseae* (Pozo et al. 1996, 1998, 1999). However, these changes were shown not to be systemic (Pozo et al. 2002a).

Nonetheless, Cordier et al. (1998) identified in nonmycorrhizal roots of plants colonized with *G. mosseae* and infected with *P. nicotianae*, not only local cell wall modifications such as the accumulation of callose around cortical cells containing arbuscules, but also the accumulation of PR-1 proteins and cell wall thickenings rich in pectin characteristic of induced systemic resistance (ISR). The stimulation of defense pathways seems therefore to play an important role in AMF biocontrol of plant diseases, and is supported by a large body of evidence (Pozo and Azcón-Aguilar 2007; St-Arnaud and Vujanovic 2007). Meanwhile, the activity of a new isoform of superoxide dismutase was detected after colonization not only with *G. mosseae* but also with *G. intraradices* (BEG 72), which did not induce biocontrol (Pozo et al. 2002a). Moreover, the reduction of the total number of infection loci in tomato plants inoculated with *P. nicotianae* (Vigo et al. 2000) suggests that the proliferation of the pathogen would be inhibited before it penetrates mycorrhizal roots within the soil.

Jasmonic acid (JA) is an essential hormone implicated in the ISR provoked by rhizobacteria (Pozo et al. 2004) that also accumulates within mycorrhizal roots (Hause et al. 2002, 2007; Isayenkov et al. 2005; Stumpe et al. 2005), in a nonsystemic manner (Meixner et al. 2005). The application of JA on *Tropaeolum majus* and on *Carica papaya* leaves strongly reduced colonization by *G. mosseae* (Ludwig-Müller et al. 2002). The overexpression of the gene *MtAOC1* coding for the JA-biosynthetic enzyme allene oxide cyclase increased the level of this hormone but reduced the level of mycorrhizal colonization (Hause et al. 2007). JA is involved in defense responses after biotic and abiotic stresses and would permit the regulation of the mycorrhizal symbiosis. Its accumulation within cortical cells containing arbuscules (Hause et al. 2002) could also play a role in the reduction of pathogen proliferation, especially within colonized cells where hyphae of *P. nicotianae* were never observed in the same cells as those containing *G. mosseae* (Cordier et al. 1996). However, a demonstration that AMF induce biocontrol via reactions implicating JA has yet to be published.

## 9.5 Effect of Mycorrhizal Root Exudates on *P. nicotianae* Zoospore Chemotaxy *In Vitro*

Transformation of plants with *Agrobacterium rhizogenes* allowed isolated root lines to grow *in vitro* (Labour et al. 2003), and greatly simplified the study of interactions between roots, AMF and pathogens (Fortin et al. 2002; St-Arnaud et al. 1995b). Taking advantage of this approach, an *in vitro* bi-compartmental Magenta box system was designed to collect exudates liberated by transformed tomato roots colonized or not with the *G. intraradices* DAOM 181,602, without interference of other soil microorganisms (Lioussanne et al. 2008). Capillaries were then filled with exudates and exposed to zoospore suspensions of *P. nicotianae* (ATCC 13 196). Exudates collected from mature mycorrhizal roots attracted significantly

less zoospores than water or exudates from nonmycorrhizal roots. The opposite was observed with actively growing roots, with exudates from mycorrhizal roots being more attractive than exudates from nonmycorrhizal roots. These results suggested that higher amounts of attractive molecules may be released by young mycorrhizal roots compared to noncolonized roots, while older mycorrhizal roots would contain repulsive molecules. Accumulation of the secondary metabolite blumenin within barley and wheat roots colonized with *G. intraradices* has been shown to be low when roots were 2 weeks old, reaching a maximum level when roots were 3–4 weeks old and being detected only in trace amounts when roots were older than 5 weeks (Fester et al. 1999). The liberation of attractive or repulsive compounds could also vary according to the roots and the AMF development stage.

Quantitative and qualitative changes in root exudation after mycorrhizal colonization have often been reported (Azaizeh et al. 1995; Bansal and Mukerji 1994; Graham et al. 1981; Kapoor et al. 2000; Marschner et al. 1997; Sood 2003). However, among 27 sugars, amino acids and organic acids quantified within root exudates, only proline and isocitric acid concentrations differed between mycorrhizal and nonmycorrhizal roots. Proline concentration in exudates liberated by mature mycorrhizal roots was higher than in exudates from nonmycorrhizal roots and from younger roots (Lioussanne et al. 2008). Proline accumulates and is involved in plant protection against water and salt stresses (Kishor et al. 2005; Kuznetsov and Shevyakova 1999; Rai 2002; Sharma and Dietz 2006). It also accumulated in tomato leaves following infection with *P. nicotianae* (Grote and Claussen 2001) and in the cortex of *Theobroma cacao* after infection with *P. megakarya* (Omokolo et al. 2002). The accumulation of proline after colonization with AMF has been reported (Azcón et al. 1996; Diouf et al. 2005; Ruiz-Lozano et al. 1995; Vazquez et al. 2001; Wu and Xia 2006). These results suggest that proline may be implicated in the biocontrol induced by AMF on tomato plants infected by *P. nicotianae*, reducing the accumulation of zoospores in the vicinity of roots.

Glomalin is yet the only protein detected from AMF structures that favors soil aggregation (Wright and Upadhyaya 1998; Wright et al. 1996, 1999). Its impact on pathogen has nonetheless never been studied. Root colonization with AMF has been shown to inhibit further mycorrhizal colonization systemically (Vierheilig 2004; Vierheilig et al. 2000a). Modification of root exudation after mycorrhizal colonization would be at the origin of this regulation (Pinior et al. 1999; Vierheilig 2004; Vierheilig and Piché 2002; Vierheilig et al. 2003). The flavonoids acacetin and rhamnetin (Scervino et al. 2005), and the carotenoid-derived isoprenoids blumenin, mycorradicin and nicoblumin have been shown to be accumulated within mycorrhizal roots (Fester et al. 1999, 2002a, b, 2005; Strack and Fester 2006; Vierheilig et al. 2000b). Blumenin applied on barley split-root systems resulted in the systemic suppression of colonization of already mycorrhizal roots (Strack and Fester 2006). Moreover, some carotenoid-derived compounds isolated from maize exudates have been shown to be responsible for the inhibition of *Fusarium oxysporum* f. sp. *melongenae* induced by resistant maize plants (Park et al. 2004). Thus, the molecules regulating mycorrhizal symbiosis may also play a significant role in the inhibition of soil-borne pathogens within the mycorrhizosphere or colonized roots.

## 9.6 Effect of Mycorrhizal Root Exudates on Tomato Infection by *P. nicotianae* in Soil

Our group recently studied the role of mycorrhizal root exudates in the biocontrol of *P. nicotianae* in tomato plants in soil. Tomato plants with different mycorrhizal inoculation or root exudate treatments were grown individually in a compartmentalized system and placed equidistant from a central unit inoculated with *P. nicotianae*. Half of the plants were filled with root exudates collected from tomato plants colonized with either *G. mosseae* or *G. intraradices* or from nonmycorrhizal plants. The other half was not supplied with root exudates but was inoculated with the same two AMF species or left noninoculated. As expected, direct tomato root colonization significantly reduced the intraradical growth of *P. nicotianae*, while the application of mycorrhizal root exudates did not affect the growth of the pathogen which colonized roots to the same extent as in the nonmycorrhizal plants (Lioussanne et al. 2006a). While a differential attraction of zoospores by mycorrhizal root exudates was noted *in vitro*, root infection was not affected by the application of mycorrhizal root exudates in a more complex soil system. Other authors also observed that exudates from AMF structures or from mycorrhizal roots affected the formation and/or the germination of pathogen propagules. It was observed that germination and hyphal growth of *F. oxysporum* f. sp. *chrysanthemi* was stimulated when conidia were inoculated directly onto a *G. intraradices* mycelium *in vitro* (St-Arnaud et al. 1995b). On the other hand, Filion et al. (1999) showed that crude extracts of *in vitro*-grown *G. intraradices* mycelium reduced germination of *F. oxysporum* conidia. Analogous inhibitive effects were also reported with exudates liberated by strawberry roots colonized by *G. etunicatum* or *G. monosporum*, on the sporangia production of the pathogen *Phytophthora fragariae in vitro* (Norman and Hooker 2000). More recently, microconidia germination of *F. oxysporum* f. sp. *lycopersici* was shown to be more than doubled in the presence of root exudates from tomatoes colonized with *G. mosseae* compared to nonmycorrhizal plants. The more the tomato roots were colonized by the AMF, the more microconidia germination was increased, suggesting a relation between the level of root colonization and the alteration of exudation pattern (Scheffknecht et al. 2006). A similar stimulatory effect was exhibited by root exudates of twelve *F. oxysporum lycopersici* nonhost species from eight plant families, showing that mycorrhization-induced changes in the root exudates were unrelated to the pathogen sensitivity of the plant (Scheffknecht et al. 2007).

Even if exudates from mycorrhizal plants were shown to affect the formation, the attraction and/or the germination of various pathogen propagules, the mycorrhizal-induced modifications would not interfere with the proliferation of *P. nicotianae* within host tissues (Lioussanne et al. 2006a). Moreover, it was shown that application of carbenzamine, a fungicide reducing mycorrhizal colonization, had also the effect of increasing the *Aphanomyces euteiches* oospores number in pea roots (Bødker et al. 2002). In this experiment, mycorrhizal colonization extent was not correlated with disease severity. From this, the authors suggested that AMF may not

impact on the vegetative growth, causing root necrosis, of the *P. nicotianae*-related pathogen *A. euteiches*, but only on its capacity to form reproductive structures.

## 9.7 Effect of Mycorrhizal Root Exudates on the Rhizosphere Bacterial Community Structure

Mycorrhizal colonization was shown to modify the bacterial community structure of the rhizosphere (Marschner and Timonen 2005; Marschner et al. 2001; Wamberg et al. 2003). Among the possible mechanisms involved in this process, modification in root exudation has been suggested (Bansal and Mukerji 1994; Marschner et al. 1997). Filion et al. (1999) observed *in vitro* that extracts from *G. intraradices* mycelium had differential effects on soil microbes, stimulating the growth of *Pseudomonas chlororaphis* and *Trichoderma harzianum*, reducing germination of *F. oxysporum*, and having no effect on the growth of *Clavibacter michiganensis*. Sood (2003) also reported that the chemotactic response of the plant-growth-promoting rhizobacteria *Azotobacter chroococcum* and *Pseudomonas fluorescens* were significantly stronger towards exudates of tomatoes colonized with *G. fasciculatum* than towards exudates of nonmycorrhizal roots. The utilization of the PCR-DGGE technique on the ribosomal gene 16S permitted Lioussanne et al. (2006a) to efficiently characterize the bacterial community structure within the rhizosphere of tomato exposed to mycorrhizal root exudates or direct AMF inoculation. As shown before, root colonization with either *G. mosseae* or *G. intraradices* had a significant impact. However, the bacterial community structure of rhizosphere supplied with exudates from tomatoes colonized with the same AMF isolates was not significantly different from the one of tomatoes supplied with exudates from nonmycorrhizal-tomatoes. These results suggest that the modification of the rhizosphere bacterial community structure induced by mycorrhizal colonization would not be mediated by root exudation modification. If bacterial community structure changes in mycorrhizal rhizosphere were involved in the AMF-mediated biocontrol, it would not depend on changes in root exudation pattern.

The high microbial activity present in the rhizosphere in comparison to the bulk-soil was often believed to be due to the supply of nutrients liberated by the roots in the form of exudates. Soil or rhizosphere enrichment with artificial exudates shifted the microbial community structure more and more consistently as substrate concentration load increased (Baudoin et al. 2003; Griffiths et al. 1999; Kozdrój and van Elsas 2000; Pennanen et al. 2004). The modification of the amount of root exudate after mycorrhizal colonization would not be important enough to induce significant bacterial community changes. Lynch and Whipps (1990) calculated that exudates contained only 9–10% of the amount of substrate required to explain the quantified microbial biomass in the rhizosphere of barley and maize. Lugtenberg and colleagues (1999) reported that the ability of the biocontrol bacteria

*P. fluorescens* WCS365 to use sugars, an important exudate constituent, does not play a major role in tomato root colonization. They showed that the mutant PCL1083 from WCS365, impaired in the ability to grow on simple sugars, reached the same population level at the root tip as the wild-type strain, when inoculated on germinated tomato seeds. Additionally, it was demonstrated that the bioavailability of some amino acids detected in tomato exudates is too low to support root tip colonization by auxotrophic mutants of *P. fluorescens* strain WCS365. The genes required for amino acid synthesis are therefore necessary for root colonization (Simons et al. 1997).

On the other hand, it has been shown that roots constitute a physical support essential for rhizosphere competence of specific soil bacteria. Mutants of *P. chlororaphis* strain PCL1391 impaired in the known tomato root colonization traits (motility and production of the site-specific recombinase) were not able to control *F. oxysporum* f. sp. *radicis-lycopersici* contrarily to the wild-type strain (Chin et al. 2000). Root colonization thus plays a crucial role in biocontrol for bacteria. The presence of AMF structures may also be essential for bacterial competence within the mycorrhizosphere and, if so, for their contribution to biocontrol. They may extend the soil area colonized by an AMF-modified bacterial community to the mycorrhizosphere which is far larger than the rhizosphere, and in this manner favor their competition on soilborne pathogens.

Physical interactions between bacteria and AMF have been described. The capacity of rhizobia and pseudomonads to adhere to *Gigaspora margarita* spores and hyphae under sterile conditions has been reported to be strain-dependent (Bianciotto et al. 1996a). The first stages of attachment (nonreceptor-dependent) would be governed by general physiochemical parameters, such as electrostatic attraction, and then later secured by specific bacterial cell surface components. The capacity to adhere to *G. intraradices* structures by different bacterial species was shown to depend on the capacity to form biofilms as mutants affected in the production of extracellular polysaccharides (EPS) essential for biofilm formation were strongly impaired in their capacity to attach to both mycorrhizal roots and AMF mycelium (Bianciotto et al. 2001a). Furthermore, mucoid mutants of the biocontrol strain *P. fluorescens* CHAO (with an alginate biosynthesis activation) adhered more importantly to the surface of this fungus than the wild type strain (Bianciotto et al. 2001b). Toljander et al. (2006) also reported that the attachment of different bacteria to AMF extraradical hyphae depended on the bacterial species and on the fungal species and vitality. Levy et al. (2003) reported the colonization of both hyphae and spores of *G. decipiens* by *Burkholderia* spp. The spore's outer layer of *G. geosporum* was shown to be eroded and covered by mucilaginous products, which suggests that AMF are directly consumed by bacteria (Roesti et al. 2005). AMF may specifically favor the proliferation of some bacteria, serving as substrate or interacting with them, favoring the formation of biofilms. *G. mosseae* was shown to increase the population of the biocontrol agent *P. fluorescens* within the tomato and leek rhizosphere (Edwards et al. 1998). Nonetheless, AMF have negative effects on the biomass of some soil bacteria (Bansal and Mukerji 1994; Cavagnaro et al. 2006; Christensen and Jakobsen

1993; Ravnskov et al. 1999), probably because of competition for inorganic nutrients. Bacteria able to form biofilms and/or to use AMF structures as substrate would then establish within the mycorrhizosphere. Some of them could act as antagonists among soilborne pathogens, as many soil bacteria have been shown to act as biocontrol agents (Bowen and Rovira 1999).

Contrarily to the two AMF species, the inoculation of *P. nicotianae* did not significantly modify bacterial community structure of tomato rhizosphere (Lioussanne et al. 2006a). Moreover, the impact of AMF on the bacterial community structure was not significantly affected by the inoculation of *P. nicotianae*. Similarly, the bacterial community analyzed using PCR-DGGE in hydroponic systems used for tomato growth was not much perturbed by the introduction of *P. cryptogea* or *Pythium aphanidermatum* (Calvo-Bado et al. 2006). The bacterial community associated with the rhizosphere of avocado infected with the pathogen *P. cinnamomi* and receiving repetitive applications of the biocontrol agent *P. fluorescens* was also shown to be similar to the rhizosphere of healthy roots, but to be significantly different from the rhizosphere of plants infected with the pathogen only (Yang et al. 2001). Therefore, pathogens such as *P. nicotianae* would weakly impact on the rhizosphere microflora in comparison to biocontrol agents. If AMF stimulate biocontrol agents within the mycorrhizosphere, their establishment, which would then efficiently antagonize the pathogen proliferation, would be poorly affected by pathogen infection.

### 9.7.1 Antagonistic Potential of Bacteria Associated with Spores of G. mosseae

Through surface disinfection of spores of *G. mosseae* and inoculation on a standard culture medium, Lioussanne et al. (2006b) obtained 18 bacterial isolates. Sequencing of the 16Sr RNA gene permitted their classification among nine different clusters. These isolates belonged to three genera: *Paenibacillus*, *Bacillus,* and *Methylobacterium*. Comparatively, 34 sequence variants were observed from direct DNA extracts of identically treated spores of *G. mosseae*, using PCR-DGGE of the 16S rRNA gene. Isolates identified as *B. simplex*, *B. niacini*, *B. drententis*, *Paenibacillus* spp. and *Bacillus* sp. were, *in vitro*, antagonistic against *P. nicotianae*, but also against *F. solani* and *F. oxysporum*. A *Paenibacillus* sp. strain B2 that was previously isolated from the mycorrhizosphere of *G. mosseae* also induced antagonism against *P. nicotianae*, *in vitro* and *in vivo*, and was shown to reduce the root necroses formed by this pathogen (Budi et al. 1999). By electron microscopy, strain B2 was shown to disorganize the cell walls of *P. parasitica* and *F. oxysporum* (Budi et al. 2000). The antibiotic polymyxine B1 and other analogue compounds (antagonistic among *F. solani* and *F. acuminatum*) were further identified within exudates from the same *Paenibacillus* strain (Selim et al. 2005).

*Paenibacillus*, *Bacillus,* and *Methylobacterium* taxa were previously identified in plant mycorrhizosphere or from soil AMF structures. About 80–92% of the

bacteria isolated from decontaminated spores of *G. clarum* NT4 were *Bacillus* (Xavier and Germida 2003). Using bromodeoxydurine immunocapture in soil known to contain AMF and through RFLP analysis, Artursson and Jansson (2003) mostly identified *Bacillus* and *Paenibacillus* taxa. *Paenibacillus* was also largely identified by FAME analysis in sterilized root-free sand extracts after *G. intraradices* inoculation but not in the AMF mycelium-free control (Mansfeld-Giese et al. 2002).

Other bacteria were isolated from extraradical and intraradical AMF structures or from the hyphosphere (the zone of soil influenced by AMF) (Andrade et al. 1997; Artursson and Jansson 2003; Filippi et al. 1998; MacDonald et al. 1982; Mansfeld-Giese et al. 2002; Mayo et al. 1986; Secilia and Bagyaraj 1987; Xavier and Germida 2003). Moreover, *Candidatus* Glomeribacter gigasporarum (*Burkholderiaceae*), a noncultivable (thus obligatory) bacterial endosymbiot was described from spores (within the vacuoles), mycelium and clover intraradical hyphae of *G. margarita* (Bianciotto and Bonfante 2002; Bianciotto et al. 2003; Bianciotto et al. 1996b; Bianciotto et al. 2004). This endobacterium was later phenotypically described in detail (Jargeat et al. 2004) and shown to be widespread within *Gigasporaceae* (Bonfante 2003). AMF inoculation from nonaxenically produced inocula is thus accompanied by associated bacteria that would establish within the mycorrhizosphere. These bacteria may have a significant contribution in the biocontrol that AMF induce on soilborne pathogens. They would also be implicated in the modifications of the bacterial community structure detected within the rhizosphere after AMF inoculation (Lioussanne et al. 2006a).

### 9.7.2 *Other Mechanisms Contributing to the Biocontrol Induced by AMF on P. nicotianae*

Through PLFA profile analysis, Larsen and Bødker (2001) showed a decrease in the biomass and energy reserves of both *G. mosseae* and *A. euteiches* coinoculated on pea roots. It has been observed that *P. nicotianae* and *G. mosseae* never occupied simultaneously the same root tissues (Cordier et al. 1996). Decrease in the extent of mycorrhizal colonization driven by different pathogens has also been reported (Bååth and Hayman 1983; Davis and Menge 1980; Krishna and Bagyaraj 1983). AMF may thus compete with pathogens for space and nutrients.

*P. nicotianae* is a soilborne pathogen causing root necrosis. In conditions of water or nutrient deficiencies, the decreased number of functional roots normally encountered in diseased plants hampers the plant's ability to access nutrients and water, making it more prone to death if drought conditions happen. It is well known that AMF increase plants' access to nutrients and water, reducing damages caused by water stress (Smith and Read 1997). Therefore, higher biomass and ramification measured in mycorrhizal versus nonmycorrhizal plants (Norman et al. 1996) exposed to pathogenic infections may be related to the increased capacity of the

mycorrhizal roots to access resources, counterbalancing weight loss caused by the pathogen. Increasing inorganic phosphate supply to tomato plants inoculated with *P. nicotianae*, Trotta et al. (1996) did not observe a reduction of disease symptoms similar to that induced by *G. mosseae* inoculation, showing that the increase in phosphorus nutrition by mycorrhizal colonization, often suggested as being importantly implicated in the biocontrol induced by AMF, would not be involved in the biocontrol in this system.

## 9.8 Conclusions

*G. mosseae* but also *G. intraradices* (DAOM 181 602) have been shown to induce biocontrol on tomato plants inoculated with *P. nicotianae* locally (within root tissues colonized with AMF), and also systemically (in the case of *G. mosseae*). Local and systemic plant defense reactions are obviously important factors which hamper the pathogen proliferation within host tissues and decrease the extent of disease symptoms. As our understanding of signalling pathways and of the roles of molecules such as jasmonic acid increases, we should be able to unravel the complex cascade of events leading to the AMF-mediated biocontrol. The quicker and upper activation of host defense pathways does not however allow us to explain every characteristic of biocontrol. Changes in root exudates after mycorrhizal colonization would not directly or indirectly (by modification of the rhizosphere bacterial community structure) diminish the pathogen ability to infect roots. However, bacteria identified within or onto the surface of mycorrhizal structures may help to reduce the pathogen proliferation not only within the soil but also within roots. These bacteria could contribute to the biocontrol conferred by AMF through several mechanisms such as the formation of a suppressive area near the mycorrhizal network and/or the stimulation of the plant defense reactions, but also to the overall plant health through other beneficial effects on plant nutrition, growth and metabolism.

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# Chapter 10
# Assessing the Mycorrhizal Diversity of Soils and Identification of Fungus Fruiting Bodies and Axenic Cultures

**Dirk Krüger, Manisha Sharma, and Ajit Varma**

## 10.1 Introduction

Symbiotic mycorrhizal fungi play a pivotal role in biological interactions and biogeochemical cycles because the carbon they obtain from their photosynthetic plant hosts is allocated through the mycorrhizal mycelium to the soil ecosystem. In addition to these interactions with their host plant's roots, the mycelia also interact with a range of organic and inorganic substrates, as well as with different organisms such as bacteria, other fungi, soil micro- and mesofauna and the roots of secondary host plants (Finlay 2005).

Progress has been made in recent decades in the knowledge of root and mycorrhizae formation and turnover and its impacts on soil ecosystems; soil biota, exudations, secretions and soil aggregation phenomena; the biology of invasive species in soils; soil biodiversity, legacies and linkages to soil processes; and ecosystem functional responses (Coleman 2008).

The advances and cost reduction in DNA-based identification of biological material has been greatly improving the catalog of methods available to soil ecologists (Anderson and Cairney 2004). While we here describe the work with specimens and cultures, it is noteworthy that direct application of molecular methods to environmental material can detect many more, yet often different, sets of fungal taxa, as for example shown for the large basidiomycete diversity in agricultural soil (Lynch and Thorn 2006).

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## 10.2 General Characteristics of Mycorrhizae

There can be no clear morphological, phylogenetic or ecological definition of soil fungi as these concepts are very difficult to implement since the geographically unbounded soil ecosystem harbors a diverse plethora of fungi with great morphological, genetic, and functional diversity. These fungi include yeasts and filamentous fungi, ascomycetes and basidiomycetes, ectomycorrhizal fungi (EMF) and arbuscular mycorrhizal fungi (AMF), anamorphic fungi and teleomorphic fungi. Mycorrhizal fungi (Fig. 10.1) have the ability to interact with the roots of more than 80% of land plants (Newman and Reddle 1987) and form symbiotic associations termed mycorrhizae. On the basis of the colonization pattern of host cells, two major types of mycorrhizae can be identified: ectomycorrhizae and arbuscular mycorrhizae. In the ectomycorrhiza the fungus does not penetrate the host cells, but forms a sheath around the roots and only traverses the cortical layers of the roots in the intercellular spaces, forming an interface called Hartig's Net. However, in arbuscular mycorrhiza the fungal hyphae penetrate cells and form intracellular structures such as coils or arbuscules. Mycorrhizal fungi provide improved access to limited soil sources such as phosphorus and nitrogen to the host plant. In exchange, mycorrhizal fungi receive carbon compounds from host plants to sustain their metabolism and complete their life cycle; they also receive protection from other microbes in the rhizosphere, and hence form a multipartite symbiotic interaction (Jeewon and Hyde 2007). A novel endophytic, root-interacting fungus, *Piriformospora indica* (Hymenomycetes, Basidiomycota) has been isolated and found to mimic the capabilities of a typical mycorrhizal fungus (Verma et al. 1998, Varma et al. 1999, 2001).

## 10.3 Classical Fungal Processing and Identification

### *10.3.1 Field Notes, Processing, Fungal Identification*

The field label of a specimen should include (a) field identification, (b) collector's name, (c) collection number and date, (d) detailed locality information, including coordinates and elevation (best by GPS), (e) a small description of the habitat, substratum and host. If possible, photographs should be taken on site, showing the habitat of the specimen, or on a neutral background to better show colors and details. A digital camera is of great help. Such photographic evidence can later help memorizing ephemeral characteristics required for identification and description.

The microscopic shape, size, color, manner of arrangement of spores on the fruiting bodies (sporophores), the hyphal characteristics of mycelium, as well as detailed information on fruiting bodies themselves, are the characteristics which would suggest themselves to someone somewhat experienced in the taxonomy of fungi, the class, order, family, and genus to which the particular fungus belongs.

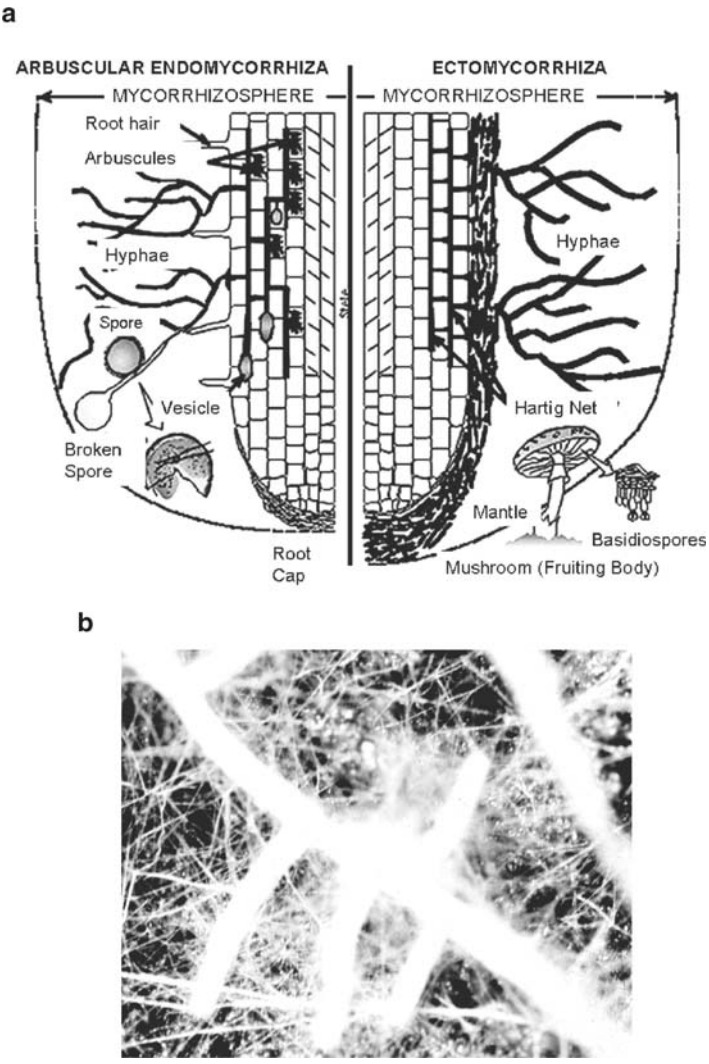



**Fig. 10.1** (**a**) Schematic presentation of mycorrhizosphere http://invam.caf.wvu.edu/collection/pubs/abstracts/mycorhiz.jpg (accessed 06 Sept. 2008). (**b**) Root hair and fungal association http://www2.warwick.ac.uk/fac/sci/whri/research/soilmicrobialdiversity/mycorrhizaldiversity_/paxillus_involutus_root.jpg (accessed 06 Sept. 2008)

Microscopic characteristics are best examined under a compound microscope, perhaps adding various stains such as lactophenol cotton blue, toluidine blue, Giemsa stain, etc., and vital stain such as Janus green. In any case, these characteristics can be utilized to trace the fungus through published analytical, often dichotomous, keys to the genus, and perhaps finally to the species to which it belongs. Published color photograph guides and web-based keys and photographs can help further. Detailed descriptions of the known species are found in monographs of

genera or in individual publications in research journals. The specimen is often kept moist for a few days to promote spore development for the production of spore print on paper or a glass slide, e.g., for help in microscopic identification or inclusion in the herbarium (see below). Alternatively, the fungus may be isolated as a culture from soil, root, sporophore or germinating spores (see below) and grown on artificial medium and identified on the basis of spores produced on the media. For some fungi, special nutrient media have been developed that allow selective growth only of the particular fungus.

The advent of molecular techniques, particularly the polymerase chain reaction (PCR), quick and inexpensive sequencing of DNA, and the accumulation of large databanks of DNA sequences have revolutionized fungal systematics and ecology (Agrios 2005).

### *10.3.2 Herbarium Facilities*

Herbaria serve as conservatories for voucher specimens. Preservation of vouchers in publicly accessible herbaria is extremely important for further scientific studies (Agerer et al. 2000), not just because of the type requirements of nomenclature codes (McNeill et al. 2006). Herbarium specimens and their accompanying field notes document the existence of a fungus at a given place and time and provide the raw data from which taxonomic concepts are constructed. The examination of voucher specimens also provides a reliable way of verifying or correcting the identity of organisms recorded in cytological, ecological, populational, morphological, phylogenetic, and molecular studies. Scientific specimens are also a source of DNA and other compounds for phylogenetic, ecological, and other studies. Spore prints, if there are any, should be placed carefully in packets and stored with the specimens. They are also a source of DNA, identification of fungal characteristics, growing new cultures, etc.

For scientific studies specimens collected must be accompanied with informative, reproducible details:

- A loose label and/or field tag with the collector's initial and collection number should be associated with the specimen at all times to facilitate their retrieval and identification.
- Ideally, color photographs should be deposited with the specimen. In addition the collector should provide detailed notes on the appearance of the specimen when fresh, including pertinent information about the color (e.g., using a color guide, Kornerup and Wanscher 1978, http://www.bio.utk.edu/mycology/Color/color-intro.htm), stature, shape and general ecology.
- Field notes should be written on archival-quality paper using permanent ink. Some collectors enter field data directly into a computer, in which case back-up files should be kept separately from the computer, and a hard copy should be printed as soon as possible. Copies generated using a laser printer and archival-quality paper is adequate for long-term preservation.

- A specimen container (e.g., jeweler's box or folded paper, with or without attached herbarium sheet) should be large enough to accommodate fungal specimens of various shapes and sizes to provide appropriate protection. Unpacked specimens should never be mounted directly on herbarium sheets.
- Specimens may originate from staff fieldwork or as gifts, exchanges, or loans. All incoming specimens, but especially those coming directly from the field, should be checked on arrival to determine if they are completely dry. Damp specimens must be dried thoroughly. Incoming specimens must also be disinfected (to kill insects) before they are stored in herbarium. Disinfestation can be achieved by deep freezing. Packages of specimens are wrapped in plastic bags and placed in a freezer at or below −20°C for 7 days. Bagging is essential for preventing condensation of moisture on a specimen during the process. Double plastic bags may be used to prevent air leakage. After freezing specimens should remain in their plastic bags until the whole package regains room temperature.
- Taxonomic and sometimes other information about a specimen increases as the specimen is studied over time. Such information enhances the value of the specimen and should be recorded by the investigators on an annotation label provided by the herbarium. An annotation label should include: (a) the identification number of the specimen (i.e., collector and/or herbarium number) in case the label becomes separated from the specimen, (b) annotations for any scientific name newly applied to the organism, (c) authorship of the scientific name, (d) relevant literature citation(s), (e) the name of the annotator, (f) annotation date, and (g) other observations. Annotation labels should also be made of archival-quality paper and written with permanent ink. Policies regarding attachment of annotation labels vary among herbaria. It is recommended that investigators place annotation labels inside the packet, box, or mounting sheets with a paper clip or rubber band. A herbarium curator or technician is then notified of their existence and can attach them more securely, if necessary (Wu et al. 2004).
- Phalloid, clathroid and other delicate fungi can be fixed and stored in liquid preservatives so that the forms and fragile textures of the fungi are not distorted. However, chemical fixation alters the DNA of an organism, so specimens fixed in that manner are not suitable for molecular studies. Consequently, warm-air drying is the preferred method of preserving fungal specimens. Freshly collected material is submerged in a fixative for 2–7 days and then transferred to storage solutions such as 70% ethanol, or they can be more complex mixtures of chemicals. Bridson and Forman (1992) recommended the Kew mixture and Copenhagen solution for fixation and storage, respectively. Both contain glycerol, which keeps the specimen from hardening. Collections stored in ethanol must be monitored closely to observe that fluid loss through evaporation can be replaced. Specimens are stored in glass jars or vials with neoprene caps or stoppers. Caps may be sealed further with sealing wax, Parafilm, and other materials to limit evaporation. Because chemical preservatives are harmful to human health, safety procedures must be followed when fixative and storage media are handled, both in the field and the laboratory. Investigators should wear gloves and

safety goggles and should avoid breathing fumes by wearing masks or working in a chemical fume hood (Wu et al. 2004).

## 10.4 Isolation of Fungal Cultures from Soils, Mycorrhizosphere and Sporophores

### *10.4.1 Preparations*

Before isolation, one must gather sterilized plastic items or pre-sterilize glassware, such as Petri dishes, test tubes and pipettes, by dry heat (150-160°C for 1 h or more), autoclaving, or dipping for one minute or more in 70–80% ethanol (Agrios 2005). There are many culture media available. The media have to be prepared in advance. General fungal media are malt extract agar, potato dextrose agar or Sabouraud dextrose agar. Selective agar media are designed to isolate specific groups of fungi such as cellulose agar to isolate microbes which are able to utilize long complex carbohydrates. Czapek yeast agar is used for the isolation and culture of saprobic soil microorganisms and Rose Bengal agar is used for the selective enumeration of yeast and molds. Rose Bengal is present in the media to restrict (not inhibit) the growth of *Rhizopus* and *Mucor* sp. that often overgrow culture plates. Some media are entirely synthetic, i.e., made up of known amounts of chemicals. Some are broth or semi-liquid apart from solid media (Pacioni 1992). Solutions of culture media are prepared in flasks, which are plugged and placed in an autoclave at 121°C at 15 psi pressure for 20 min. Sterilized media are allowed to cool until touchable (~55°C) and are subsequently poured from the flask into sterilized Petri dishes, test tubes, or other appropriate containers. Pouring of the culture medium into the containers is carried out aseptically either in a separate culture room or in a clean room free from dust and contaminants. In either case the work table must be wiped with 70% ethanol, hands should be clean, and tools such as scalpels, forceps, and needles should be dipped in alcohol and flamed to prevent introduction of contaminating microorganism. Working in a laminar airflow hood greatly helps to grow the desired fungi free of airborne contaminants.

### *10.4.2 Isolation from Fruiting Bodies and Spores*

Fungal cultures can be obtained by placing aseptically removed internal tissue of sporophores onto media. Polyspore (thus heterokaryotic) cultures can be derived from sporophore fragments glued onto Petri dish lids and sporulate onto the medium. Cutting out individual germinating spores onto separate dishes can yield monokaryotic single spore isolates. Sometimes the use of spore prints, or

plating out sterilized water in which hymenophore (the spore-producing part of sporophores) fragments were vortexed, suffices to produce cultures.

### *10.4.3 Indirect Methods for Screening*

Sporulating fungi are isolated by indirect techniques. The soil-dilution plate technique is the most common method (Fig. 10.2). In serial dilution methods, the spread plate technique is advantageous over the pour plate technique as the latter may eliminate some heat-sensitive fungi; also, some fungal spores do not germinate if submerged. Techniques such as serial root washings, soil washings, sedimentation and sieving are known for good recovery, as these techniques involve agitation in soil aggregates, and thus the spores can be easily released.

### *10.4.4 Soil Dilution Technique for Enumeration of Fungi*

1. To enumerate the total population of fungi, serial dilution and subsequent plating of the rhizosphere soil is done on the specified medium. This will estimate the number of viable fungal propagules present per gram of soil capable of growing in the specified medium, as shown in Fig. 10.2.
2. Suspend 1 g of soil in 9 ml of sterile dilution blank made up of distilled water, saline, or phosphate buffer; this produces a 1:10 dilution.

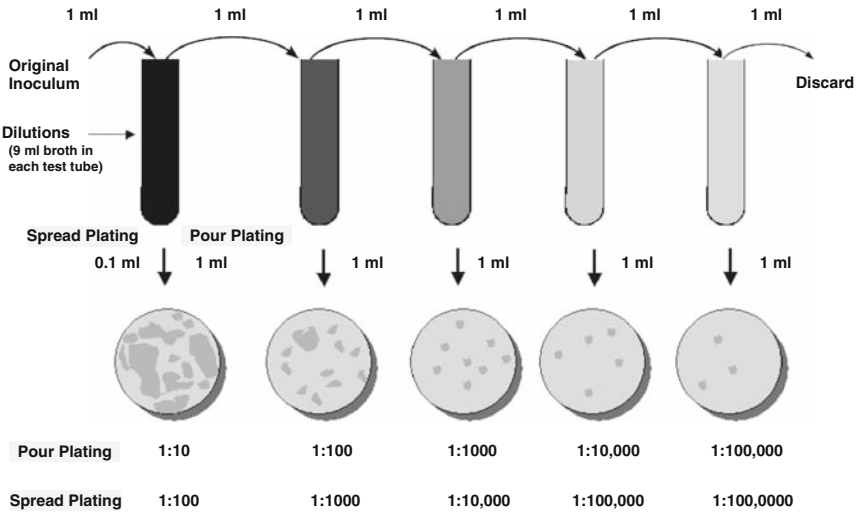



**Fig. 10.2** Serial dilution method: *Calculation*: Number of colonies on plate X reciprocal of dilution of sample = number of colony-forming units CFU/ml. For example: 32 colonies are on a plate of $^{1}/_{10,000}$ dilution, then the count is 32 × 10,000 = 320,000/g in given soil sample. The colonies obtained in Petri plates can be further subcultured and identified

3. From the first dilution after proper mixing, take a further 1 ml and transfer to a fresh dilution blank, hence getting a 1:100 dilution. Continue the serial dilution until the desired dilution is obtained.
4. Each suspension is shaken by hand for a few seconds and is drawn into a pipette and pour-plated or spread-plated (Fig. 10.3). 1 ml of the desired dilution is transferred to the Petri dish by micropipette or glass pipette. 15–20 ml medium is added into the Petri plate, cooled just above solidifying temperatures. The Petri dishes are swirled clockwise and anti-clockwise in order to disperse diluted soil sample in medium.
5. In case of spread plating for isolating surface-growing fungi 0.1–0.5 ml of the desired dilution is transferred onto the solidified agar plates. The suspension is spread over the surface of agar with help of a spreader or sterilized 2–3 mm diameter glass beads.
6. Incubate at 24–30°C for 6–14 days. The colonies are counted on colony counter and average number of colonies per dish is multiplied by dilution factor to obtain the number of microorganisms per gram of the original soil sample (Mukerji et al. 1998).

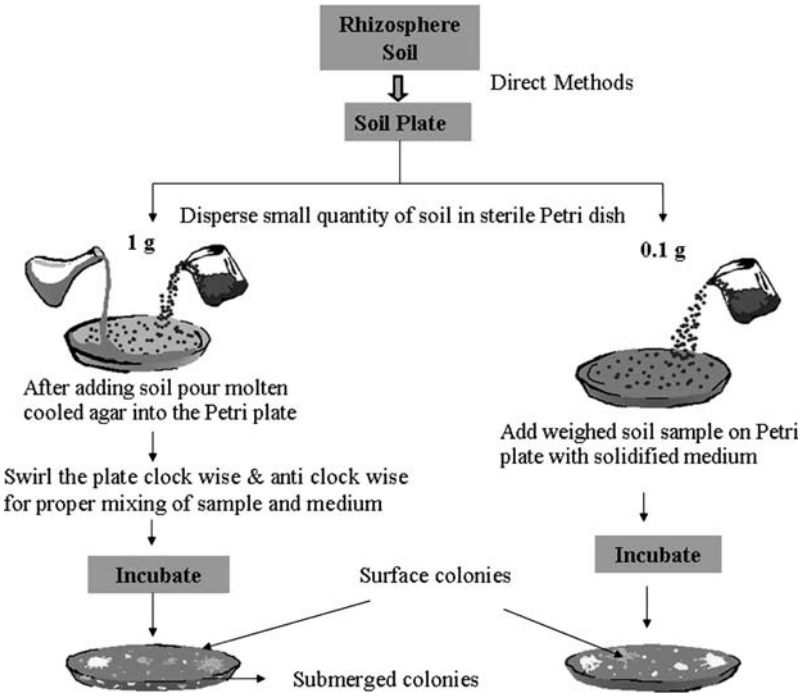



**Fig. 10.3** Schematic presentation showing pour plate method and direct plate method for isolation of fungi

### 10.4.5 Direct Plate Technique

For direct observation a soil profile is created in the rhizosphere zone and for other techniques, such as soil plate and soil dilution techniques, soils are collected at different depths by using a soil auger.

Direct methods are useful for quick scanning of soil fungi and are more appropriate for isolating fungi that do not sporulate and exist as mycelium attached to soil humus. Plants under study can also be uprooted carefully and either total rhizosphere soil or soil from different root zones is collected. Isolation is done by preparing a number of replicate series and after the required period of incubation. Other direct observation methods include immersion technique and soil box, with removable microscopic slides for zonal sampling at desired depth. A recent and more appropriate method to observe the rhizosphere effect is using a rhizotron, a root observation and sampling chamber (James et al. 1985).

To isolate specific fungal groups, sample pretreatment and the use of selective media have to be considered. Re-treatment involves heat shock by steaming and subsequent immersion in ethanol. Selective medium is added with enrichment compounds and certain growth retardants such as Rose Bengal and streptomycin, etc., inhibiting Actinobacteria, other bacteria, and fast-growing molds. These are used to curtail the interference and to facilitate the slow-growing fungi (Ranganayaki et al. 2006).

### 10.4.6 Wet–Sieving and Decanting Technique for the Extraction of Spores of AMF (Fig. 10.4)

1. An optimal ratio of soil/water was found to be 1/10, that is 100 ml of soil in 1 l of water.
2. The suspension should be stirred with a magnetic stirrer or by hand using a rod. Stirring time varies according to the nature of soil; usually 10 min is sufficient.
3. To avoid foam that could retain debris, add an antifoam agent, for example Tween 80, 0.1–0.5%.
4. Pass the suspension through the sieve with a 1 mm-wide mesh, keeping the filtrate.
5. If the soil contains clay that yields a suspension blocking the sieve, precipitate the particles in 0.1 M sodium pyrophosphate.
6. Debris retained by this sieve must be re-suspended into the kept suspension, stirred again, or washed under a stream of water, saving the suspension.
7. Decant all suspensions through a sieve series ranging from 1 mm to 40 μm.
8. With the aid of a jet of water directed at both sides of the sieve, the content of each sieve is transferred to a Petri dish.
9. The shallow suspension can be observed with a stereo-dissecting microscope, and spores and microsporocarps picked up with a flattened needle or a Pasteur pipette.
10. Small concave watch glasses or slides can be used for further observations.


**Fig. 10.4** Steps in the wet-sieving technique: (**a**) metallic sieve, (**b**) decanting through a sieve series, (**c**) removal of debris

Further modification of the filtering apparatus can be introduced particularly when working with either humic or clay soils and where the efficiency of the filter is lowered by the presence of foam or suspended particles. The removal of spores from metallic sieves can be difficult, particularly when working with soil where predominantly rare, single spores occur:

- Nylon filters with standard meshes of the kind used in palynology or with cellular cultures for the separation of protoplasts can be used.
- A series of filters, decreasing in pore size (1 mm–40 μm), are attached to each other by plastic tubing of 20 cm diameter, cut in lengths of 20 cm.
- Once filled, the filters are placed onto a transparent Plexiglas grid-lined sheet and placed under a stereoscope; the spores are supported by the filter meshes where they can be counted accurately and manipulated easily. Using a transmitting light stereoscope, the transparency of the supports (nylon filter and Plexiglas sheet) allows easy observation of spores.

### *10.4.7 Techniques for Large Volumes of Soil*

The techniques mentioned above are suitable for small-scale studies, involving up to 100 g of soil at a time. If kilogram quantities of soil are to be examined, a drum, for example a gasoline barrel without cover but with a lateral overflow pipe, can be utilized. The filtering apparatus is made up of a metallic sieve with meshes of 1 mm for retaining larger debris and a nylon filter bag held by strings or tapes. The size of the mesh is selected with the dimensions of the spores in mind:

1. Place a plastic pipe or insert a fixed water source at the bottom of the drum.
2. Place the filtering apparatus to catch the water suspension.

3. Fill the drum, suspend the soil, stir with a rod, treat as necessary and then turn on the water, leaving the suspension to flow through the filtering apparatus.

## 10.5 Preservation and Maintenance

### *10.5.1 Culture Collections*

Primary methods of culture preservation are continuous growth, drying, and freezing. Continuous growth methods, in which cultures are grown on agar, typically are used for short-term storage. Such cultures are stored at temperatures of 5–20°C, or they may be frozen to increase the interval between subculturing. The methods are simple and inexpensive because specialized equipment is not required. Freezing methods, including cryopreservation, are versatile and widely applicable. Most fungi can be preserved, with or without cryoprotectants, in liquid nitrogen or in standard home freezers. With freeze-drying, or lyophilization, the fungal cultures are frozen and subsequently dried under vacuum. The method is highly successful with cultures that produce mitospores. Freeze-drying and freezing below −135°C are excellent methods for permanent preservation, and we highly recommend them. However, both methods require specialized and expensive equipment. Permanent preservation is essential for strains with critically important characteristics and for type specimens. Cultures that are permanently preserved in metabolically inactive states can serve as type specimens (Nakasone et al. 2004).

In long-term preservation, sclerotization is an important method. Some fungi develop sclerotia or other long-term survival propagules in culture as well as in nature. Preserving such structures, usually at 3–5°C, is a good way of maintaining fungal strains. Two important methods are the oil overlay method or storage in distilled, sterilized water (Burdsall and Dorworth 1994; Nakasone et al. 2004). They are low-cost and low-maintenance methods for preserving cultures, either growing on agar slants and covered with mineral oil, or placed on cut-out pieces of solid medium in vials of sterile water. These cultures can be kept for several years or, in exceptional cases, up to 32 years at room temperature or 15−20°C. This method is especially appropriate for mycelial or nonsporulating cultures that are not amenable to freezing or freeze-drying, or when lacking suitable lab facilities (Nakasone et al. 2004).

Traditional methods for assessing fungal diversity in the soil environment rely mainly on the dilution-plating technique (coupled with use of selective media) and microscopy to identify sporulating fruiting bodies. The traditional methods tend to overestimate species that sporulate in soil, while those in mycelial state or those that have slow growth in culture are largely overlooked. In addition, most of these methods result in isolation of only the most common and abundant fungi (often referred to as generalists) such as the asexual ascomycetous molds *Fusarium, Penicillium* and *Trichoderma*, Zygomycota (especially *Mucor*), and the Oomycota

(e.g., *Pythium*). These cultivated microorganisms are those that can utilize the energy source under the physical and chemical limitations of the growth medium. This clearly indicates that many other fungi do not respond readily to cultural techniques. Therefore the diversity data cannot be considered as accurate. Altered and optimized growth media, coupled with advanced molecular biology techniques, have demonstrated that a larger proportion of uncultured fungi belonging to novel fungal lineages could be isolated and identified (Jeewon and Hyde 2007).

Public culture collections are repositories of often published or of economically critical fungi. Some of the larger culture collections are ATCC (http://www.atcc.org), CBS (http://www.cbs.knaw.nl), BCCM-MUCL http://bccm.belspo.be), DSMZ (http://www.dsmz.de/), INVAM (http://invam.caf.wvu.edu), and IFO (http://www.nbrc.nite.go.jp).

### *10.5.2 Cultures in Herbaria*

The choice of preservation methods depends on the species of concern, the resources available, and the goal of the project. Therefore it can be a long-term or short-term preservation. Fungal cultures can be preserved as voucher specimens by drying. The mature fungal colony, in its Petri plate, is placed in a frost-free freezer. The freezer must be frost-free or the preparation will not dry properly. Data are written on the plate bottom. After 4–6 weeks the colony should be sufficiently dry so that it is loose in the plate. The colony is then transferred either to an archival-paper envelope or to a thin (e.g., 8 mm) Petri plate and placed in a herbarium packet, and a label with all accompanying data is prepared. The old plate and lid are discarded. The paper envelopes can be used for most pyrenomycetes, many discomycetes and all loculomycetes. Delicate taxa such as zygomycetes, hyphomyctes, coelomycetes and some ascomycetes are best stored in thin Petri plates. The material is then placed in a 100% cotton rag packet to which the label is attached with acid-free glue (Wu et al. 2004). Drying is the most useful method of preservation for cultures that produce spores or other resting structures.

## 10.6 Modern Molecular Methods Used in Fungal Identification

The initial task towards molecular identification of a fungal specimen or culture is to make nucleic acid available for amplification by the PCR (Saiki et al. 1985). While there is a plethora of modifications and personal preferences, all at some step must include the leaking of nucleic acid into a buffer that preserves it but allows further purification to remove it from PCR-interfering cell wall components and

compounds. A workflow of steps possible in molecular identification is given with Figs. 10.5–10.8.

While for some time the direct amplification from bacteria, especially *Escherichia coli* but also others, such as Actinobacteria (e.g., Ishikawa et al. 2000) has been routine in molecular biology labs, commercialized and home-made "extraction-free" methods for other biological organisms have become available and can be inquired with your local sales representative. To ease the identification of soil fungi, we suggest starting with such a simple method here. It involves the unique activity of liquid polyethylene glycol 200 (PEG200) in cell disruption and DNA stabilization (Chomczynski and Rymaszewski 2006). In order to allow adjustment to the equipment available, we are intentionally vague in the protocol given below.

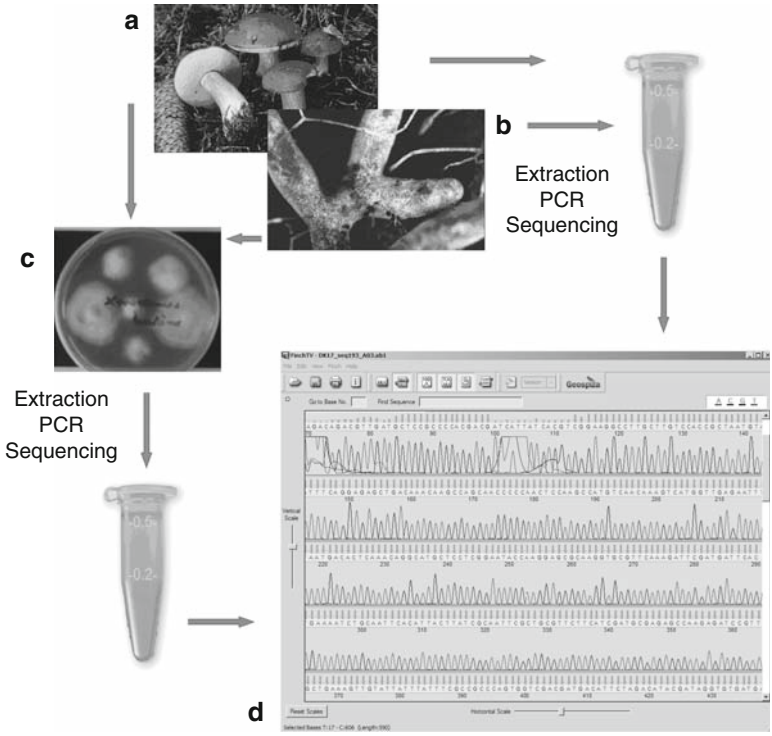



**Fig. 10.5** Workflow of bioinformatic processes in identifying an unknown fungal object (process step query DNA sequence) Part 1. Process step **a**: example *Xerocomus badius* (http://www.mtsn.tn.it/bresadola/gallery.asp?code = 31) specimens, or **b**: *Xerocomus badius* mycorrhiza on *Pinus* roots (http://www.ektomykorrhiza.de/mycorh1.gif) serve in the production of a **c**: culture on malt extract agar. The biological material is extracted, resulting DNA cleaned, amplified, and sequenced, yielding **d**: electropherogram (e.g., FinchTV, Geospiza, Inc, Seattle, WA, USA) — note the potential need to edit the data, as e.g., there are "dye blobs" that are introduced by the sequence cleaning method with ethanol precipitation

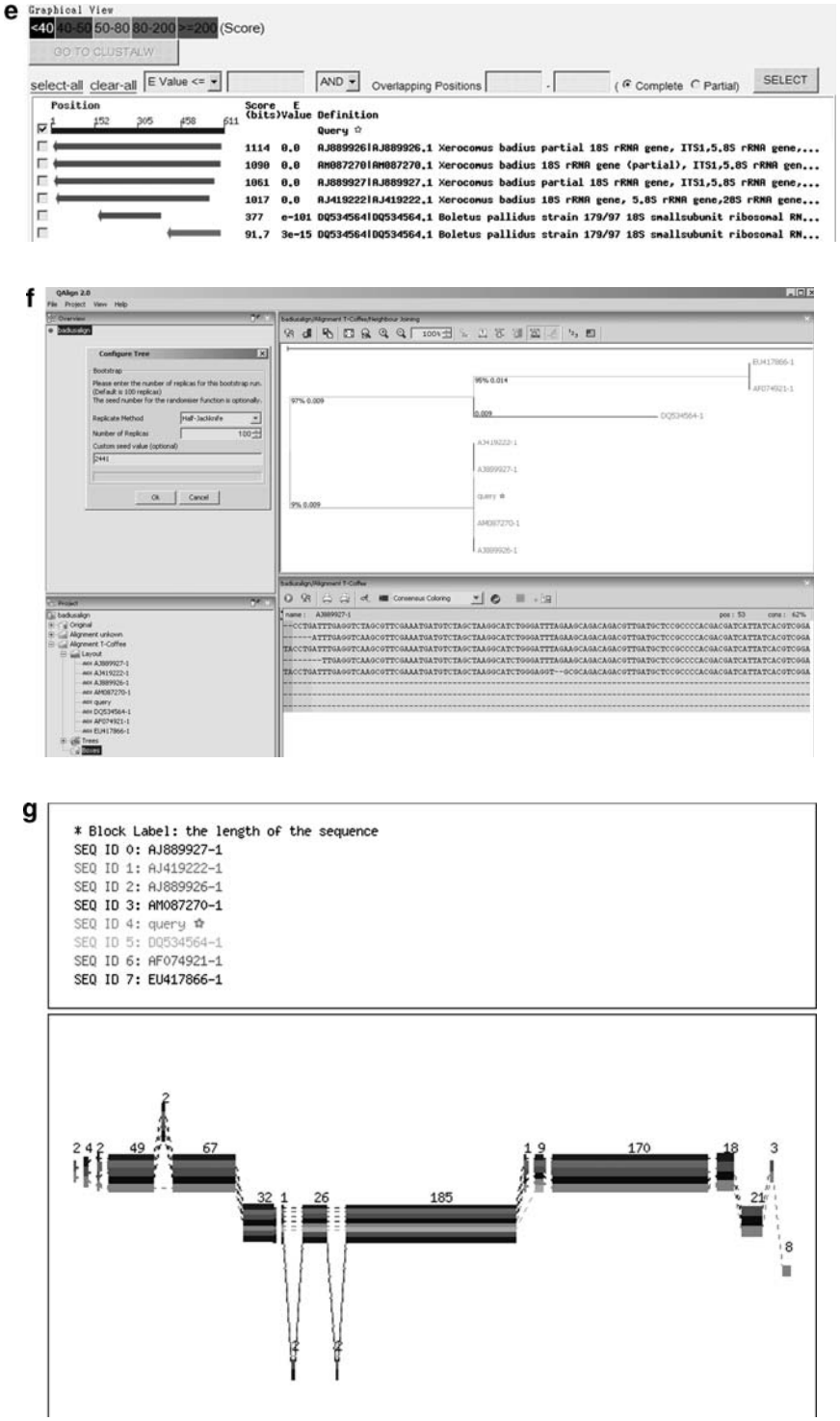



**Fig. 10.6** Workflow of bioinformatic processes in identifying an unknown fungal object (process step query DNA sequence) Part 2. Process Step **e**: graphical result of BLAST-n search on the

### 10.6.1 "Extraction-Free" Preparation of PCR-Ready Material

1. Prepare 2 ml screw-cap microtubes by adding 300 µl PEG200-KOH (adjusted with KOH to pH 13.3–13.5 and autoclaved) and a sterile glass bead of 2–3 mm diameter.
2. Take a loop full of yeast culture or a few $mm^3$ of fungal fruiting body or a small amount of fungal aerial hyphae from a Petri dish and mix into the PEG200. Freeze if not used right away.
3. Before going to PCR, vortex (the glass bead here supports homogenization) and spin down.

The use of sterile sand, a commercial grinding resin, or a microtube pestle to help disrupt the material is optional, e.g., in place of the glass bead.

### 10.6.2 PEX Extraction

For tough fungal herbarium specimens (Krüger 2002) or from mycorrhizal root tips (Martin and Rygiewicz 2005) the xanthogenate (PEX) protocol is a useful and time-saving method. This can be implemented as an alternative to the above PEG200 protocol.

Buffers needed:

(a) 100 ml TEx buffer stock:

10 mM Tris-HCl (starting from 0.5 ml 2 M Tris-HCl pH 7.4)
1 mM EDTA (starting from 0.2 ml 0.5 M EDTA pH 8.0)
0.5 M $CaCl_2$ (5.5 g)
Autoclave

(b) 100 ml XT buffer stock:

100 mM Tris-HCl (starting from 5 ml 2 M Tris-HCl pH 7.4)
20 mM EDTA (starting from 4 ml of 0.5 M pH 8.0)
5 ml Tween 20
800 mM sodium acetate (starting from 6.16 g or 16 ml 5 M stock)
Autoclave

(c) 0.1X TE pH 8.0:

=1 mM Tris-HCl, 0.1 mM EDTA, autoclaved.

**Fig.10.6** (continued) website of the DNA Databank of Japan (DDBJ), allowing query and anchoring sequences to be sent to ClustalW for alignment. Process step **f**: the program QAlign 2 (Panta Rhei) aligns sequences and can also compute simple phylogenetic trees, here a jackknife resampling, T-Coffee alignment with associated Kimura 2-parameter Neighbour-Joining tree. **g**: graphical representation of highly similar (*top lines*) and dissimilar (*bottom lines*) areas of alignment in POAVIZ (Grasso et al 2003)

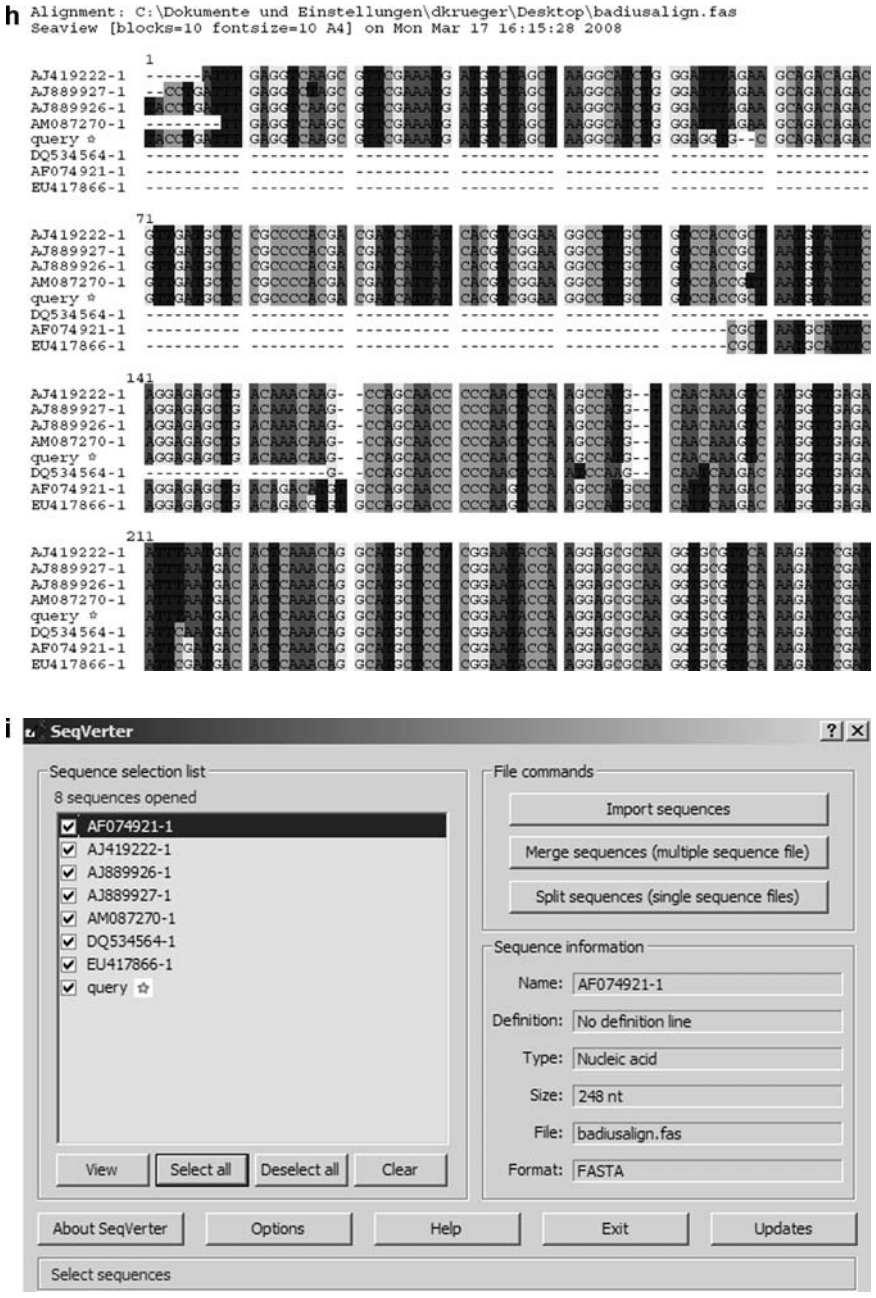



**Fig. 10.7** Workflow of bioinformatic processes in identifying an unknown fungal object (process step query DNA sequence) Part 3. Process step **h**: visualization of the FASTA formatted alignment as a PDF output from SeaView (Galtier et al 1996). Process step **i**: using the software Seqverter (GeneStudio, Inc., Suwanee, GA, USA) data can be converted, e.g., from FASTA to the NEXUS

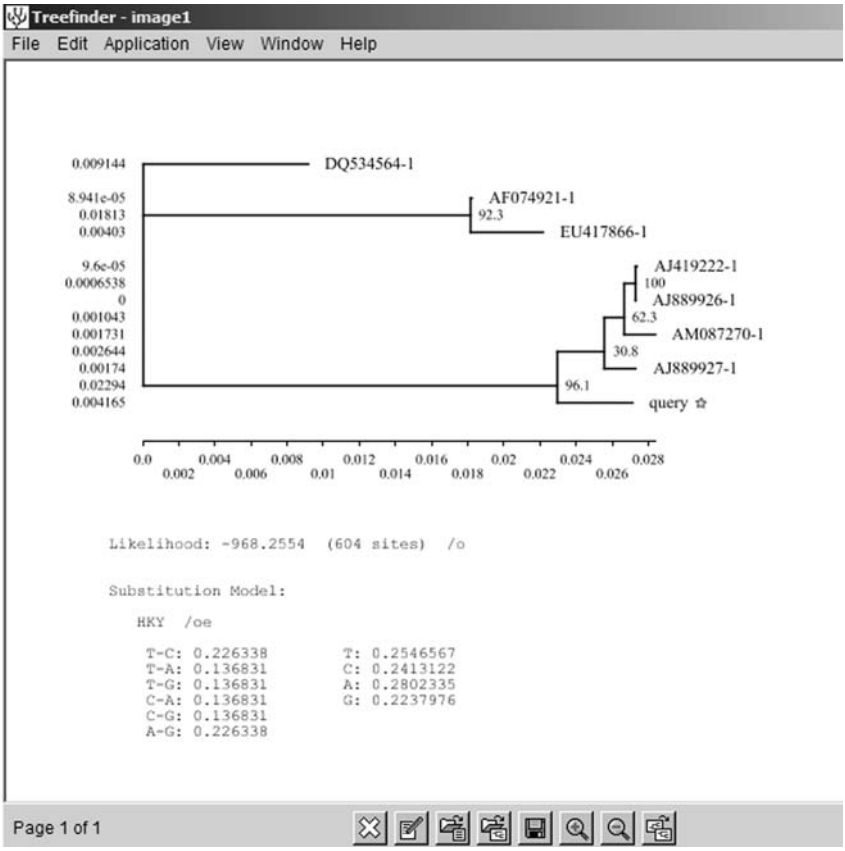
j


**Fig. 10.7** (Continued) format **j** as for example used in PAUP*, Mesquite (Maddison & Maddison 2007a), MacClade (Maddison & Maddison 2007b), or MrBayes

Prior to extraction, for each sample dissolve 7.5 mg potassium ethyl xanthogenate (*O*-ethyl xanthic acid, PEX) in 0.75 ml XT buffer. Possibly heat it slightly. Do not inhale vapours:

1. Place the fungal material, some sterile sand, and 50 µl phosphate buffered saline–Tween 20 (10%) or 1% sodium pyrophosphate with 10% Tween 20 in a 1.5 ml microtube. Mesh the material with a sterile microtube pestle. Add 50 µl TEx. Prepare a heating block to 70°C.
2. Add 750 µl XT buffer with added PEX and vortex vigorously.
3. Incubate 60 min at 70°C, shaking occasionally.
4. Incubate 30 min on ice.
5. Centrifuge 10 min at full speed.

k




l

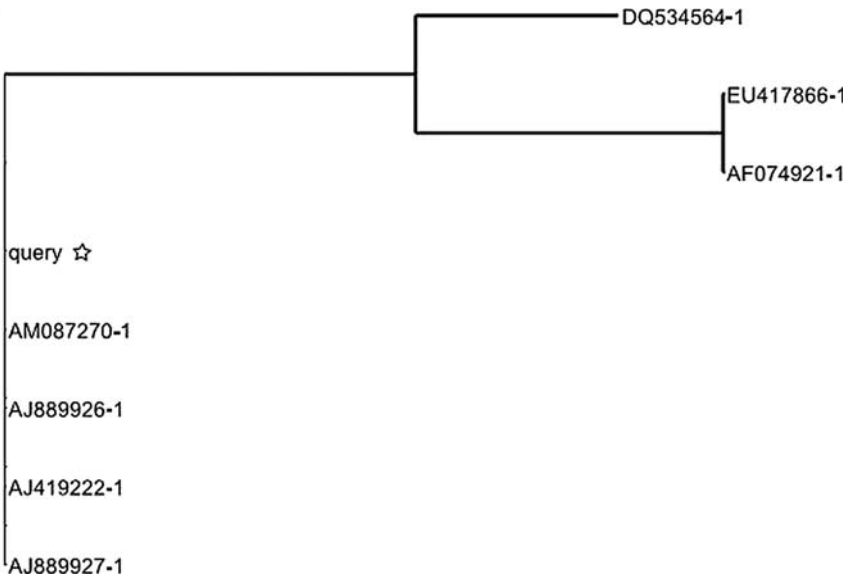



**Fig. 10.8** (Continued)

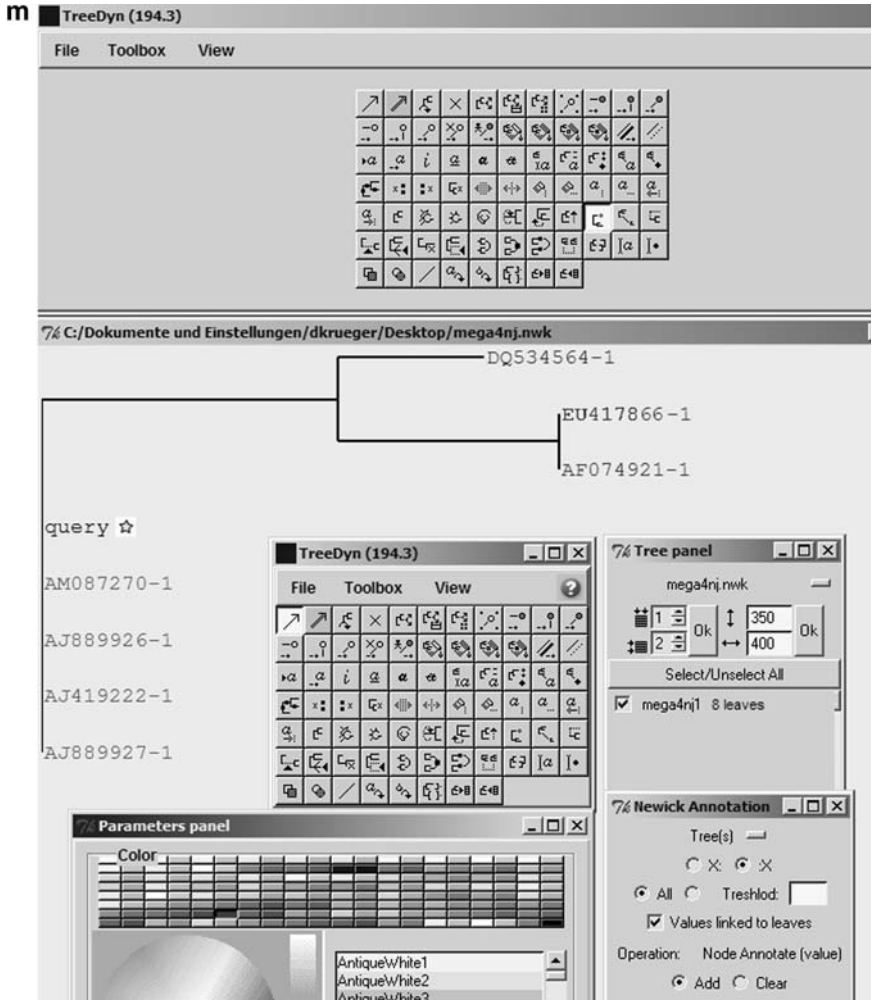



**Fig. 10.8** Workflow of bioinformatic processes in identifying an unknown fungal object (process step query DNA sequence) Part 4. Process step **k**: treefinder Maximum-Likelihood phylogeny, and Process step **l**: minimum-evolution phylogeny from software MEGA4. Process step **m**: a general or specialized graphics program such as TreeDyn (Chevenet et al. 2006) can be used to beautify the phylogenetic trees

6. Mix the supernatant in a new microtube with 80% cold isopropyl alcohol. Potentially use a coloured co-precipitant from a commercial vendor – it will improve visibility of small pellets as a precaution against loss. Prepare heating block to 90°C.
7. Centrifuge 10 min at ca. 10,000 rpm. Remove the alcohol.

8. Wash the pellet with 100 µl cold ethanol (100%), scraping the tube on a rack to allow the alcohol to reach all sides.
9. Centrifuge 10 min at full speed.
10. Pipette off alcohol, then incubate 1 min at 90°C. Air dry the pellet.
11. Dissolve the pellet in 50 µl 0.1X TE, placing it 1 min at 90°C.
12. Add 0.5 µl RNAse A and spin down. Store at −20°C.

If PCR is inhibited by too much pigment or carbohydrates, clean/re-extract further with additional chloroform-isoamyl alcohol extraction, a commercial kit, polyvinylpyrrolidone (Berthelet et al. 1996, Young et al. 1993), polyethylene glycol 8,000 (Howeler et al. 2003), glassmilk and guanidine (Saltikov and Olson 2002), or hydroxyapatite (Purdy et al. 1996).

### *10.6.3 Choice of PCR Target*

Because the fungal ITS rDNA barcoding region is the most commonly used genetic marker deposited in databases, usually exhibiting high variability descriptive at the species level, this is the target of choice. In general, the highly repetitive nature of the ribosomal DNA genes (Fig. 10.9) by itself makes them easier amplification loci, offering a higher amount of template than single-copy genes. However, it is possible that not all loci are created equal, and also noteworthy is that a single gene may not accurately represent the phylogenetic background of the organism (Rokas and Carroll 2005). Using ribosomal DNA genes complicates this matter because it codes for RNA and not protein, and thus the standard models of sequence evolution may not be adequate (Gesell and von Haeseler 2006). However, for molecular identification and measuring diversity, precise branching order in phylogenetic trees is not troublesome, but rather of interest to hardcore molecular systematists. Cloning PCR products even from heterogeneous copies of rDNA from pure cultures or specimens is no more a necessity than if amplifying from polygenomic extractions. PCR errors and natural sequence heterogeneity within an organism will play no major role in further analyses explained below.

For the PCR amplification we recommend using a ready mastermix that already contains the nucleotides, *Taq* polymerase, and loading dye for subsequent electrophoresis. More and more companies provide such solutions which help decrease cost, workload, and contamination potential. With one such mastermix, we use the following standard protocol with primers ITS5 and ITS4 (White et al. 1990) (see Table 10.1). Ideally, some sterile environment and gamma-irradiated reaction tubes will also decrease chances of contamination.

For ITS5 (GGAAGTAAAAGTCGTAACAAGG) and ITS4 (TCCTCCGCTT ATTGATATGC) we use the following cycling conditions: initial denaturation 94°C/10 min, 32 cycles of denaturation 94°C/40 s, primer-annealing 54°C/30 s, extension 72°C/40 s, followed by final extension 72°C/4 min, and a hold at 10°C. Generally, the annealing temperature is to be about 5°C below the melting temperature of the lower one of a primer pair. The melting temperature is given by the

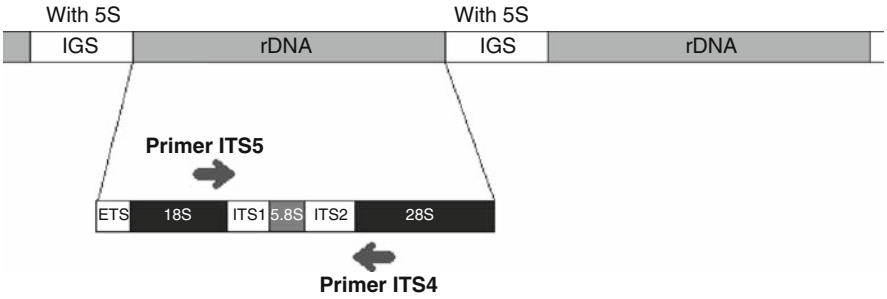



**Fig. 10.9** Scheme of the tandemly arrayed ribosomal RNA gene cluster (rDNA). *IGS* Intergenic Spacer; *ETS* External Transcribed Spacer; *ITS* Internal Transcribed Spacer (ITS1, ITS2). 18S rDNA is also called the SSU (small subunit) gene, 28S rDNA the LSU (large subunit) gene. Note the unfortunate naming of standard primers, e.g., ITS5 and ITS4 (yes, there are primers ITS1 and ITS2 as well)

manufacturer of the primers, or can be calculated at their website. Other primer information is available from, e.g., http://www.biology.duke.edu/fungi/mycolab/primers.htm.

### *10.6.4 PCR Troubleshoot*

There are many variants of PCR, but for extractions from pure cultures it should normally be possible to use standard settings. Sometimes it is necessary to dilute the samples, increase primer concentrations, or to optimize the program. Of good use is a temperature-gradient thermocycler to judge susceptibility to annealing temperature variation. PCR additives can facilitate the success of PCR, yet this needs to be optimized (Rådström et al. 2004). Besides choosing a commercial, proprietary product, freely available enhancer compounds include

- 0.2–0.6 M trehalose (Spiess et al. 2004)
- The alcohols glycerol at 5–20% (Nagai et al. 1998) or polyethylene glycol (PEG) of various molecular weights at 0.75–15% (Giordano et al. 2001; Rådström et al. 2004; Lareu et al. 2007)
- Polyamines such as 0.1–2 M spermidine (Wan and Wilkins 1993; Ahokas and Erkkila 1993)
- Amides such as 0.4 M 2 pyrrolidone (Chakrabarti and Schutt 2001a)
- Methylammonia such as 1 M monohydrate of betaine (carboxymethyl trimethylammonium) (Baskaran et al. 1996; Rees et al. 1993), tetramethylammonium chloride (TMAC, Chevet et al. 1995), tetramethylammonium oxalate (Kovárová and Dráber 2000)

**Table 10.1** Typical PCR contents

| | 1x |
|---|---|
| 2X commercial mastermix | 9 µl |
| Sterile water | 8.6 µl |
| Primer 1 50 µM | 0.2 µl |
| Primer 2 50 µM | 0.2 µl |
| PER WELL w/o template | 18 µl |
| Template (DNA extract) | 2 µl |

- 0.5% non-ionic detergents Triton X-100, Tween 20 or Nonidet P-40 (Demeke and Adams 1992)
- Solvents such as 1–5% formamide (Sarkar et al. 1990), 0.15–0.4% tetramethylene sulfone (Chakrabarti and Schutt 2001b), 2–10% dimethyl sulfoxide (DMSO, Bookstein et al. 1990)
- Proteins 0.1-0.8 µg $\mu l^{-1}$ BSA or T4 gp32 (Kreader et al. 1996), 0.01–1% gelatin (Ohler and Rose 1992)
- 10–20 mM ammonium sulfate

### *10.6.5 Sequencing and Editing Sequences*

In the modern molecular biology lab, the dye-dideoxy variant of the enzymatic method by Sanger (Sanger and Coulson 1975) is the standard. Here, a special PCR reaction containing only one primer synthesizes the DNA from the template, the prior PCR product. A certain number of deoxy nucleotides are modified to interrupt chain elongation upon being randomly incorporated in the growing DNA strands. These also carry different fluorescence labels for each of the four different bases. Hence, all lengths are produced, and the final nucleotide will be known when the cycle-sequencing product is electrophoresed on the sequencer. There, it is separated on either a polyacrylamide gel or a capillary. It is necessary to clean the PCR product prior to cycle sequencing, and after. The former can be achieved by enzymatic means (exonuclease/shrimp alkaline phosphatase), or centrifugation through commercially available columns. After cycle sequencing, one uses columns, alcohol precipitation (perhaps modified with magnetic beads or coprecipitants), or centrifugation through self-prepared Sephadex columns.

Sequence output files (electropherograms) can be visualized and edited in a variety of free software programs, such as Chromas Lite (Technelysium Pty Ltd, Australia), Staden Package trev.exe (https://sourceforge.net/projects/staden), Ridom TraceEdit (Ridom GmbH, Würzburg, Germany), FinchTV (Geospiza, Inc., Seattle, WA, USA), BioEdit (Hall 1999; Tippmann 2004) or one of the commercial products. It is necessary to decide on nucleotide composition where there are artefacts such as “dye blobs” caused by alcohol precipitation. Unreliable

ends need to be trimmed off. In addition, it might be necessary to repeat the sequencing, sequence with another primer, or clone the PCR products if they appear to be mixed, rather then having occasional point errors or single nucleotide polymorphisms (SNPs). DNA analysis software such as used for phylogenetics, as well as BLAST-n (Altschul et al. 1997) can deal with the ambiguity codes for nucleotides (see Table 10.2, Cornish-Bowden 1985).

### *10.6.6 Cloning*

If no good sequence can be obtained, e.g., because the specimen or culture is impure or the ribosomal DNA is heterogeneous, cloning is necessary. It is simplest to use a T/A or U/A cloning kit (Mead et al. 1991) where the PCR products, which should have A overhangs due to the peculiarity of *Taq* polymerase, are ligated into a special cloning vector for the bacterium *E. coli*. In that case, one can then directly add a little pipette tip full of *E. coli* single colony material into a PCR mix such as above (barely touch individual colony on plate!), but using primers anchored in the vector, such as recommended by the cloning kit manufacturer. The sequencing of the colony PCR product also has the advantage of getting a clean sequence from the start, as the otherwise usually "messy" start and the region where dye blobs would

**Table 10.2** Meaning of letters (IUPAC codes) and symbols in nucleotide sequences

| Code | Meaning | Complement | Ribonucleotide type |
|---|---|---|---|
| **A** | A (adenine) | T | purine |
| **C** | C (cytosine) | G | pyrimidine |
| **G** | G (guanine) | C | purine |
| **T** | T (thymine) | A | pyrimidine |
| **U** | U (uracil) | A | pyrimidine (in RNA only) |
| **I** | I (inosine) | N, X | purine (RNA; can cost-efficiently replace N in primers, Candrian et al 1991) |
| **M** | A or C | K | |
| **R** | A or G | Y | purine |
| **W** | A or T | W | |
| **S** | C or G | S | |
| **Y** | C or T | R | pyrimidine |
| **K** | G or T | M | |
| **V** | A or C or G | B | |
| **H** | A or C or T | D | |
| **D** | A or G or T | H | |
| **B** | C or G or T | V | |
| **N, X** | G or A or T or C | N, X | |
| **– (in alignment)** | Indel (insertion/deletion) | | |
| *** (in alignment)** | Identical | | |

appear are in the vector sequence area. It is necessary to find out in what region of the DNA sequence is the vector sequence, which can be done with the aid of commercial tools, as well as by special BLAST (e.g., EMVEC database query, http://www.ebi.ac.uk/blastall/vectors.html).

The edited DNA data must also be oriented in the correct way, which can be gauged by BLAST search. Sequences can then be "reverse complemented." Such manipulations can be done within the program BioEdit or with the BCM Search Launcher (http://searchlauncher.bcm.tmc.edu).

### *10.6.7 Database Queries and Alignment*

A good way to BLAST is to use the site of the Japanese data repository DDBJ (http://www.ddbj.nig.ac.jp/). Here, it is possible to BLAST multiple sequences together, and also to align query sequences with similar sequences. BLAST outputs contain two numerical values, one score correlating with similarity of query and database entry, and the other being the E-value that reflects the probability of finding matches with equal scores. Hence, one looks for high scores and low E-values. More information can be found in Korf et al. (2003). The data maintained at DDBJ are synchronized with the American NCBI GenBank and the European EMBL database. Alignment can be performed with the ClustalW algorithm (Thompson et al. 1994), which in DDBJ can also be used to obtain sequences for anchoring and new sequences for rooting the tree with an outgroup. ClustalW is also available within BioEdit, or comes with its own graphical interface that can even produce simple phylogenetic trees (Thompson et al. 1997). There are other, alternative alignment programs such as Dialign-T (Morgenstern 2004, Subramanian et al. 2005), Divide-and-Conquer (Stoye et al. 1997), Align-m (Van walle et al. 2004), MAVID (Bray and Pachter 2004), MUSCLE (Edgar 2004), POA (Lee et al. 2002), MAFFT (Katoh et al. 2005), multalign (Corpet 1988), T-Coffee (Notredame et al. 2000), or combinations in the program QAlign (Sammeth et al. 2003, 2006). A variety of these alignment programs is available for online use at EMBL (http://www.ebi.ac.uk/Tools/sequence.html). Local databases of sequence data can be well-managed with the programs ARB (Ludwig et al. 2004), BioEdit, GeneDoc (Nicholas et al. 1997), or the sequence editor of MEGA (Tamura et al. 2007).

It is also possible to align sequences to the specialized database mor (Hibbett et al. 2005), which contains the curated data of a large fungal phylogenetic project (AFTOL, Lutzoni et al. 2004), or to use seeded alignments against the SILVA database, a progression of ARB and the European Small Subunit Ribosomal RNA database (http://www.arb-silva.de/, Pruesse et al. 2007). A specialized database for ectomycorrhizal fungal sequences exists in UNITE (http://www.unite.ut.ee). The molecular approach to systematics has even led people to propose new classifications entirely based on cladistic principles (PhyloCode, Donoghue and Gauthier 2004).

Alignment, which also decides on positional homology, is a prerequisite for phylogenetic analysis showing the putative evolutionary relationship between the culture or specimen to be identified and database comparison sequences. For the ITS spacers, alignment can be very difficult beyond closely related taxa. Thus, for uncertain homology, stretches of sequence should be excluded in the phylogenetic analysis, although of course the complete data without potential vector sequence would be deposited in the public repositories upon publication. SequIn (http://www.ncbi.nlm.nih.gov/Sequin/) at NCBI GenBank can be used when publishing own sequences. Secondary structure of the ribosomal RNA transcript can sometimes help in alignment, yet when recoding data for structure features information content is lost (Krüger and Gargas 2004).

### *10.6.8 Phylogenetic Placement*

To simply identify fungi, a phenetics-based distance method such as neighbor-joining NJ (Kimura 1980) or minimal-evolution ME (Rzhetsky and Nei 1993) suffices. These are implemented in the package PHYLIP (Felsenstein 2005), or in MEGA, and rudimentary in ARB, ClustalX and QAlign. Some of these programs allow specification of a model of DNA sequence evolution. For simple identification, this may not be so important. Otherwise, the use of the popular program PAUP* (Swofford 2002) together with modeltest (Posada and Crandall 1998) is needed to decide on the model of sequence evolution. More sophisticated phylogenetic analysis based on different search strategies, e.g., on the principles of maximum-parsimony MP (available for example in MEGA and PAUP*), maximum-likelihood ML (programs PUZZLE, PHYML, or Treefinder; Schmidt et al. 2002, Guindon and Gascuel 2003, Jobb et al. 2004 respectively), or Bayesian inference (program MrBayes, Ronquist and Huelsenbeck 2003), is often not needed. One is advised to read Hall (2004) for instructions. Conflicting signals in phylogenetic data can be visualized as reticulogram (e.g., Makarenkov 2001, Huson and Bryant 2006). For likelihood approaches, including Bayesian, more computing power than for NJ is advised. Commonly, a random resampling/reanalysis strategy is used to gauge consistency of a dataset and the support for the branching order. The two most common forms of frequentist support of data-to-tree topology are the jackknife and the bootstrap over characters (Krüger and Gargas 2006). For example, a clade on a phylogenetic tree with 67% bootstrap support was found in 67 of 100 resampled datasets. Branch lengths in phylograms can variably depict the distance, likelihood, or steps of mutations (the latter in parsimony). A consensus tree usually collapses all branches with less than 50% support, though perhaps 90% support is considered as significant in the relationship of the branches. The root of the tree is usually a sequence or group of sequences a priori known to be closely related to yet outside the sequences of interest. The programs TreeView (Page 1996), PhyloDraw (Choi et al. 2000) and Dendroscope (Huson et al. 2007) are three of many for visualization of phylogenetic trees. Because file types are not always compatible, a conversion

tool such as ForCon (Raes and Van de Peer 1999) is useful. A website that contains several programs to cover various steps in phylogenetic analysis is in development in France (http://www.phylogeny.fr/) and hopefully will be further expanded.

## 10.7 Conclusions

Mycorrhizal fungi have the ability to interact with roots and form symbiotic associations termed mycorrhizae. For fungal identification, field notes, drawings, and photographs are essential tools. A duplicate set of field notes should be kept separately although original field notes should be placed with the specimen and, if available, spore print, both deposited in a publicly accessible herbarium. There are several traditional methods such as the isolation of fungi from soil samples by serial dilution, culture plating, microscopic identifications, etc. which form the preliminary part of fungal identification. Cultures should be kept at a publicly accessible culture collection. A variety of extraction methods for fungal material yields the DNA template identification by sequencing, subsequent database comparison, alignment, and phylogenetic placement of the fruiting bodies and cultures.

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# Chapter 11
# Isolation of Metabolically Active Arbuscules and Intraradical Hyphae from Mycorrhizal Roots

**Zakaria M. Solaiman**

## 11.1 Introduction

Arbuscular mycorrhizal (AM) fungi are obligate symbionts and colonize more than 80% terrestrial plant roots and fungi belonging to the Glomales. The mutualistic nutrient exchange in this symbiosis is characterized by the transfer of phosphorus from fungi to host plant in exchange for carbon compounds derived from photosynthesis (Smith and Read 1997; Solaiman and Saito 1997). It is now well-reported that phosphate present in soil is taken up into the extraradical hyphae by a phosphate transporter, subsequently condensed into polyphosphate, and translocated by protoplasmic streaming into the intraradical hyphae (Saito 2000). The arbuscule is supposed to be the main site for the nutrient exchange. Alkaline phosphatase activity has been expressed in arbuscules, and it is suggested that this relates to the efficiency of phosphorous uptake (Tisserant et al. 1992) and sugar metabolism (Ezawa et al. 1999; Solaiman and Saito 1997) of AM. However, the carbon and phosphorus metabolism involved in the nutrient exchange at the arbuscular interface remain to be investigated.

The isolation of arbuscules from the host tissue and subsequent examination of biochemical activities are required to clarify the mechanism occurring in nutrient exchange in this symbiotic system. The isolation of arbuscules from host tissue is a powerful process, especially because this endo-symbiont cannot be independently cultured *in vitro*. However, it is not easy to isolate the arbuscules from host tissue because of the complex penetration of hyphae into cortical cells. Intraradical

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hyphae have been isolated from the host by enzymic digestion of root tissue with cellulase and pectinase, followed by hand-sorting of the hyphae under a dissecting microscope (Capaccio and Callow 1982; Smith et al. 1985; Hepper et al. 1986). But this method was laborious, and enzymic digestion of the mycorrhizal roots for more than 12 h reduced the metabolic activity of the hyphae, as evaluated by histochemical staining for succinate dehydrogenase (SDH) activity (McGee and Smith 1990). Later on, Saito (1995) reported a method for isolation of metabolically active intraradical hyphae from AM onion roots after 1–2 h of enzymic digestion. The metabolic activity of the isolated hyphae, based upon the evaluation of SDH staining, was not significantly affected by this enzymic separation procedure. In addition, a mass of intraradical hyphae which are almost free from plant debris can be collected after Percoll gradient centrifugation. But this method is still laborious, and 2–3 h are needed to complete the isolation of the intraradical hyphae or arbuscules. A simple and rapid method is now available for the isolation of arbuscules (Senoo et al. 2007). But it needs highly colonized plant roots from which we can isolate arbuscules without intensive labor.

We now have an increased arbuscule-forming mutant *har1* (previously known as *Ljsym78*) of *Lotus japonicus* L. (Nishimura et al. 2002; Solaiman et al. 2000); this has shown increased colonization by arbuscules on its roots compared to the wild-type 'Gifu'. Most of the arbuscules on the mutant root were SDH-active, well-developed and tough in morphology. The isolation procedure of arbuscules from the mutant roots, and the quantity and quality of the isolated arbuscule fraction have been described elsewhere (Senoo et al. 2007).

## 11.2 Isolation

### *11.2.1 Isolation of Arbuscules and Intraradical Hyphae with Enzymatic Digestion*

#### 11.2.1.1 Materials

Waring blender, microscope, slides, reciprocal shaker, centrifuge machine, tubes.

#### 11.2.1.2 Chemicals

(a) Enzymic digestion solution

Mix 1% cellulase, 0.2% pectolase, 0.1% bobin serum albumin, 1 mM dithiothreitol (DTT), 0.01 M Mesh-NaOH buffer of pH 5.5, 0.3 M mannitol

(b) Washing buffer (WB)

Mix 0.3 M mannitol, 1 mM DTT, 0.01 M Tris-HCl of pH 7.4

(c) Percoll gradient solution

Prepare 80% Percoll stock solution (prepare 50 ml of 0.05 M Tris-HCl solution, mix mannitol @1.5 M and DTT @ 5 mM, add 200 ml Percoll, finally adjust pH to 7.4); then prepare gradient Percoll solution by mixing Percoll stock solution (80%) with washing buffer as 40% (25 ml stock + 25 ml WB), 20% (12.5 ml stock + 37.5 ml WB), and 10% (6.25 ml stock + 43.75 ml WB).

#### 11.2.1.3 Protocol

Isolation of arbuscules is outlined here according to the method of Saito (1995) used for isolation of intraradical hyphae from onion roots:

1. Immerse fresh onion roots in 0.5 mM $CaSO_4$ for a few minutes. Take out and remove access $CaSO_4$ using paper towel.
2. Cut into 5-mm segments and weigh 1g, then add 10 ml of enzymic digestion solution and incubate for 1–2 h at 30°C in a reciprocal shaker at 110–120 strokes per minute.
3. Decant digestion solution on 50 μm nylon mesh, and then wash with washing buffer.
4. Collect roots in homogeniser in Waring blender and add 10–20 ml, washing buffer and homogenise for 1 min at a speed of 6,000 rpm.
5. Collect suspension in a beaker on ice and homogenate filter through two layers of cheesecloth and repeat two more times.
6. Combine filtrates in centrifuge tubes and centrifuge for 10 min at 3,000g.
7. Collect pellet and re-suspend with washing buffer before pouring 5–10 ml of suspension into Percoll discontinuous gradient solution (prepare by adding 8 ml of each 40%, 20% and 10% Percoll solution respectively from bottom to top in a centrifuge tube).
8. Centrifuge at 400*g* for 30 min. Collect arbuscules and hyphal fraction from layers between 40% and 20% and also between 20% and 10% in a new tube.
9. Add washing buffer and centrifuge again at 1,000g for 10 min. Filter precipitates on 50 μm nylon mesh and wash with washing buffer.
10. Collect in an Eppendorf tube, centrifuge for 10 min at 1,000g. Store precipitates and count under microscope by preparing using following methods.

Fix isolated arbuscules and intraradical hyphae with 1 m formaldehyde solution and stain with trypan blue (0.5 mg per ml w/v). Filter an aliquot (100 μl) of the stained hyphae on nitrocellulose membrane filter; then air-dry and immerse in microscopic oil to render them transparent. Measure hyphal length by the gridline intersect method (Newman 1966) using an eyepiece with a total magnification of ×100. Thick hyphae including intracellular hyphae and arbuscular trunks should be measured, but it may not be possible to assess the very fine arbuscular branches.

## 11.2.2 Isolation of Arbuscules and Intraradical Hyphae without Enzymatic Digestion

### 11.2.2.1 Materials

Waring blender, microscope, slides, centrifuge machine, tubes.

### 11.2.2.2 Chemicals

Washing buffer: Prepare by mixing 0.3 M mannitol, 0.01 M Tris-HCl of pH 7.4, 1 mM DTT

### 11.2.2.3 Protocol

Isolation of arbuscules outlined here according to the method of Senoo et al (2007).

1. Immerse fresh roots of mutant (*har1*) of *Lotus japonicus* L. in a cold solution of 0.5 mM $CaSO_4$ for a few minutes, and then cut into 5 mm length. Collect 5g of the root pieces on nylon mesh (50 μm), and wash with washing buffer.
2. Transfer washed root pieces into a 100 ml homogenizer of Waring blender with 40 ml of cold washing buffer, and homogenize for 30 s at 6,000 rpm.
3. Filter homogenate through two layers of cheesecloth, and again homogenize the residue with 40 ml of cold washing buffer. Repeat this homogenization/filtration process two more times.
4. Filter through 50 μm and then 30 μm nylon mesh. Collect residue on the 30 μm nylon mesh with a Pasteur pipette as the arbuscule and hyphae fractions. Do all the procedures at 0–4°C if possible.
5. Store precipitate and count under microscope by staining using appropriate methods.

## 11.2.3 Measurement of Metabolic Activity of Isolated Arbuscules and Hyphae

### 11.2.3.1 Materials

Microscope, slides, needles.

### 11.2.3.2 Chemicals

0.25 M sodium succinate, 0.5 mM $MgCl_2$, nitroblue tetrazolium (1 mg $ml^{-1}$), 4 mM α-naphtyl acid phosphate, Fast Blue RR (0.6 mg $ml^{-1}$), 0.1 M Tris-HCl buffer (pH 8.5), 0.1 M sodium acetate buffer.

#### 11.2.3.3 Protocol

The isolated arbuscule and hyphal fraction are subjected to the assessment based on histochemical observation of SDH, alkaline phosphatase (ALP) and acid phosphatase (ACP) according to the methods described elsewhere (McDonald and Lewis 1978; Saito et al. 1993; Saito 1995).

1. Incubate arbuscules and hyphae at 35°C for 2 h in the following solutions: SDH; 0.25 M sodium succinate, 0.05 M Tris–HCl buffer (pH 7.6), 0.5 mM $MgCl_2$, 1 mg $ml^{-1}$ Nitroblue tetrazolium; ALP; 4 mM α-naphtyl acid phosphate, 0.6 mg ml−1 Fast Blue RR, 0.1 M Tris–HCl buffer (pH 8.5); ACP; as ALP but using the buffer 0.1 M sodium acetate.
2. Wash arbuscules with deionized water after incubation of the arbuscules, treat the arbuscule fractions for SDH, ALP and ACP staining by counter staining with 0.5 mg $ml^{-1}$ acid fuchsin, transfer to lactoglycerol and mount on glass slides.
3. Count the number of arbuscules and hyphae under the light microscope. Then count the number of arbuscules with SDH, ALP or ACP activity, and those without activity separately.
4. To evaluate the loss in the SDH, ACP and ALP activity of arbuscule during the isolation process, the percentage of SDH, ACP and ALP active arbuscules in the roots can be compared.
5. Squash the stained root pieces on a slide glass and measure the percentage of the arbuscules showing SDH, ACP or ALP activity.

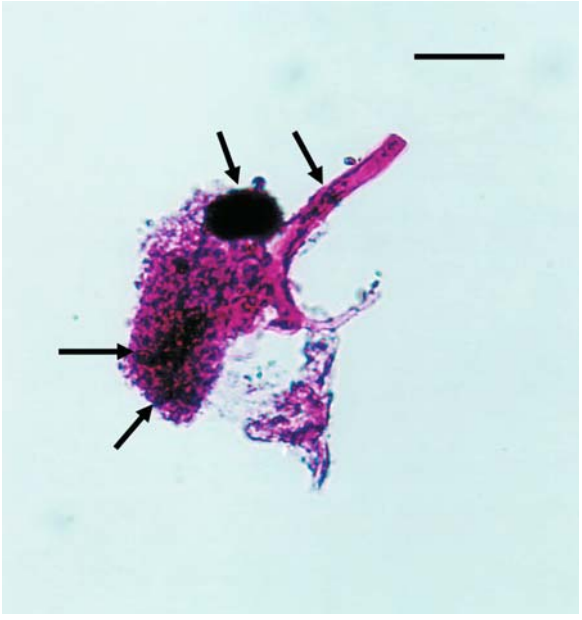

**Fig. 11.1** Arbuscules isolated from root of a mutant *har1* colonized by *Glomus* sp. and stained for succinate dehydrogenase (SDH) activity. SDH activity of arbuscules indicated with *arrows*. *Bar* indicates 50 μm

## 11.3 Conclusions

A simple and rapid method for isolation of metabolically active arbuscules from onion root after enzymatic digestion, and from the root of increased arbuscule-forming mutant (*har1*) of *Lotus japonicus* L. without enzymatic digestion is described. Enzyme histochemical staining showed that the collected arbuscules had succinate dehydrogenase (SDH), alkaline phosphatase (ALP), and acid phosphatase (ACP) activities. It was observed that the SDH activity of arbuscules was retained after the isolation process (Fig. 11.1). The development of a simple and quick method for isolation of metabolically active arbuscules will shed light for possible investigation of the biochemical mechanisms in nutrient (phosphorus and carbon) exchange occurring at the arbuscular interface.

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# Chapter 12
# Interaction with Soil Microorganisms

**R. Hampp and M. T. Tarkka**

## 12.1 Introduction

Roots constitute important plant organs for water and nutrient uptake. However, they also release a wide range of carbon compounds of low molecular weight. These can amount to between 10 and 20% of total net fixed carbon (Rovira 1991) and form the basis for an environment rich in diversified microbiological populations, the rhizosphere (Hiltner 1904). Its specific conditions lead to the selection of distinct microbial communities (Smalla et al. 2001), where fungi play an important role (Linderman 1988; Andrade et al. 1997; Frey-Klett et al. 2005; de Boer et al. 2005). This is due to the fact that the fungal hyphae, which emanate from mycorrhizas, release a substantial amount of the acquired plant carbon to the soil. The energy-rich plant compounds promote bacterial growth and survival (Hobbie 1992), and the soil volume filled with mycorrhizal fungal hyphae is therefore often depicted as the mycorrhizosphere (Foster and Marks 1967).

The mycorrhizosphere effect leads to the enrichment of microorganisms that improve plant fitness (Frey-Klett et al. 2005). Some of the mycorrhizosphere organisms directly influence the development and physiology of the plants through the production of plant growth regulators (Azcon et al. 1978), by increasing the root branching rate or root permeability. Others may interact in a more indirect way that supports plant growth. For example, the interacting organisms may improve nitrogen or phosphate availability, lead to better survival of the hyphae and the plants in a contaminated soil, assist the plant resistance against pathogens by

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biological control, or show direct effects on soil quality (Barea et al. 2005; Frey-Klett et al. 2005).

The most investigated group of microorganisms interacting with mycorrhizal fungi and plants are the mycorrhizosphere bacteria (de Boer et al. 2005). These include intrahyphal bacteria in ectomycorrhizal (Bertaux et al. 2003) and intra-spore bacteria in arbuscular mycorrhizal (Bianciotto et al. 1996) fungi, as well as bacterial species that colonize the surfaces of fungal hyphae and mycorrhizal roots (Foster and Marks 1967; Nurmiaho-Lassila et al.1997; de Boer et al. 2005; Artursson et al. 2006). Some of the mycorrhizosphere bacteria, classified as Mycorrhiza Helper Bacteria (MHB, Garbaye 1994), can promote mycorrhiza formation. These include a variety of Gram-negative (Gryndler and Vosatka 1996; Founoune et al. 2002) and Gram-positive (Ames 1989; Abdel-Fattah and Mohamedin 2000; Maier et al. 2004; Schrey et al. 2005) species.

We have worked on a collection of actinomycetes (Gram-positive) that were isolated from the rhizosphere of a Norway spruce (*Picea abies*) stand. One of the isolates, *Streptomyces* sp. AcH 505, significantly promoted the mycelial growth and mycorrhization rate of the symbiotic fungus *Amanita muscaria* but suppressed the mycelial extension of the plant pathogens *Armillariella obscura* and *Heterobasidion annosum* (Maier et al. 2004). This indicates an important application for such mycorrhization helper bacteria: the simultaneous promotion of mycorrhizal symbiosis as well as the suppression of pathogenic fungi. For more information on this topic see Schrey et al. (2005), Tarkka et al. (2006), Riedlinger et al. (2006), and Keller et al. (2006).

In this chapter we present some methods which we use to investigate the interactions between filamentous Gram-positive bacteria from the genus *Streptomyces* with mycorrhizal and plant-pathogenic fungi, and plants. In practice we have investigated the interaction between two to three organisms at once under *in vitro* conditions. This has enabled us to dissect some mechanisms of action of streptomycetes (reviewed in Tarkka and Hampp 2008).

## 12.2 Fungal Growth Promotion in Bacterium–Fungus Co-Cultures: An Indication of Mycorrhiza Helper Function

Due to the ease in handling, dual cultures on agar media are commonly used to screen for interactions between fungi and bacteria. A strong correlation exists between the induction of fungal growth in dual cultures and mycorrhization helper effect (Garbaye 1994; Frey-Klett et al. 2007).

Three methods are commonly used for co-culturing fungi and bacteria on solid media. One approach is to grow the bacteria next to the fungus on agar medium, allowing diffusible molecules to interact with the organisms. Another approach includes the use of a cellophane membrane (CM) below the fungal material (Fig. 12.1). This way, fungal growth into the agar is prohibited, i.e., fungal growth

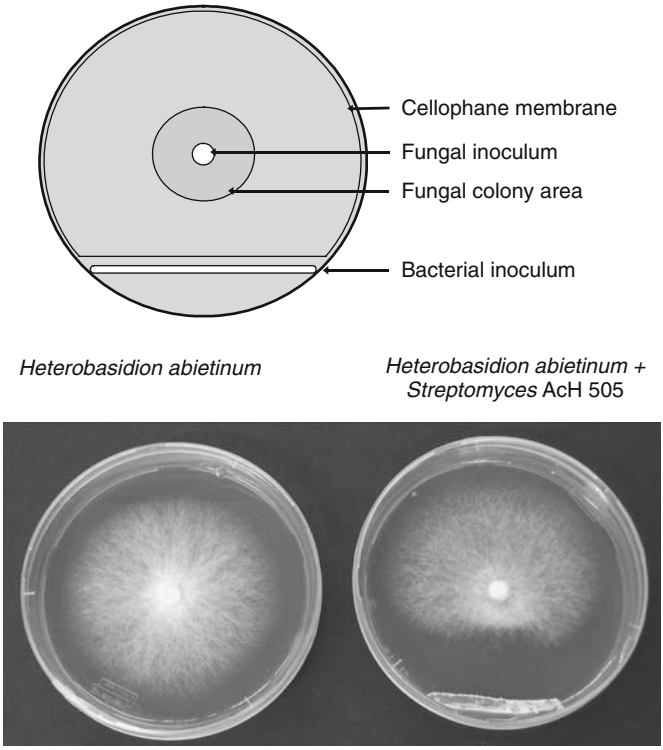


**Fig. 12.1** Fungus–bacterium co-culture

can be determined as the change in the area covered by mycelium, or as a change in mycelial biomass. It also facilitates easy harvesting of mycelia for further analyses (Maier et al. 2004; Schrey et al. 2007). The exclusion of macromolecules due to the cut-off of the CM (our case 10 kDa) means in practice that bioactive bacterial metabolites have to be either small soluble metabolites (e.g., secondary metabolites; Riedlinger et al. 2006) or volatiles. The latter can be easily confirmed by the use of cross-walled Petri dishes (Duponnois and Kisa 2006; see Procedures). The use of the three methods may provide a preliminary picture of the groups of substances involved in the bacterium–fungus interaction (soluble or volatile).

Abiotic and biotic factors influence the communication and competition between fungi and bacteria (de Boer et al. 2005; Artursson et al. 2006). Media composition and the distance between, and the size of microbial inocula are of critical importance; microorganisms commonly activate their secondary metabolism during stress or as a response to competition (Slattery et al. 2001; de Boer et al. 2007). If an adequate medium supplies both organisms with ample carbon and mineral sources and growth factors, the organisms are under stressful conditions only when competing with each other, i.e., during direct contact. This may result in strong production of substances relevant for the interaction only at the time of intimate contact (de Boer et al. 2007). However, if the organisms are stressed due to

suboptimal medium composition, continuous production of at least some secondary metabolites can be expected. Among others we have observed that changes in media composition lead to different outcomes in the interactions between organisms. For example, the mutualistic interaction between *Streptomyces* AcH 505 and *Amanita muscaria,* changes to an antagonistic one if the organisms are cultivated on ISP2 medium instead of the MMN medium. AcH 505 grows extremely well on ISP2 and sporulates much more strongly than on MMN, indicating that the concentrations of antifungal metabolites dominate on ISP2 but not on MMN. The fungal response is also related to the growth rate of the mycelium. We and others have observed that if the growth rate of a fungus is high due to optimal growth conditions, the growth-promoting influence by a bacterium may no longer be apparent. In line with this, the strain of *Amanita muscaria* growing most slowly on MMN was most responsive to the mycorrhization helper streptomycetes, AcH 505 and AcH 1003 (Schrey et al. 2005). This suggests that bacteria may compensate for poor growth, but may not increase the maximum growth of faster growing fungal isolates under optimal growth conditions.

Further crucial factors influencing the bacterium–fungus interactions are the quality and the extent of bacterial or fungal inocula. Both the bacteria and the fungi may lose some of their characteristics over time. This includes alterations in secondary metabolite production in bacteria and decreased mycorrhization capacity in the fungi. Therefore it is advisable to have a set of glycerol stocks of the bacteria at −80°C and a good stock of fungal cultures at +4°C. As far as the mycorrhiza helper bacteria are concerned, a fine balance exists between too small (no effect), adequate (fungal growth promotion), and excess (no influence or antagonism) amounts of bacterial inocula. It is thus necessary to inoculate equal amounts of equally fresh bacteria from the same culture density, holding on to the same culturing conditions. Growth conditions may also influence the interactions. Deveau et al. (2007) used 10°C to maximize the reproducibility of *Laccaria bicolor–Pseudomonas fluorescens* dual cultures for analyses of the fungal transcriptome. Correctly they argued that instead of artificial temperatures used normally for dual cultures (around 20–25°C), 10°C is a normal temperature in temperate or boreal forest soils, and should thus be more commonly used for interaction studies.

## 12.3 Rapid Fungal Responses to Bacteria and Their Metabolites: Bacterium–Fungus Suspension Cultures

Detailed information of interaction mechanisms between microbes may be acquired by the treatment of one microbe with culture supernatant or growth regulator of another microbe (Melin et al. 1999; Agarwal et al. 2003). The tip regions of fungal hyphae respond most strongly to interacting organisms (Schrey et al. 2007). As only the extreme front of the mycelium in solid agar cultures is metabolically active, harvesting the entire mycelium from solid agar cultures leads to the risk of masking the active responses occurring in the extending hyphal tips. One option to prevent this

is to collect the mycelium from as short a distance from the mycelial front as possible (Deveau et al. 2007). A second option is the use of suspension cultures; when these are homogenized regularly, the cultures consist entirely of actively growing hyphal tips. In our opinion the suspension cultures are thus most suitable for the investigation of rapid fungal responses to organisms and their bioactive metabolites. The isolation of bioactive metabolites is also much easier from suspension cultures than from solid media (Riedlinger et al. 2006). However, the fungal responses may differ between submerged cultures and those grown on solid media.

## 12.4 Materials

### *12.4.1 Reagents for Culture*

#### 12.4.1.1 CMS Medium

| | |
|---|---|
| Citric acid | 2 mM |
| $MgCl_2 \times 6H_2O$ | 1.25 mM |
| KCl | 10 mM |
| $Na_2SO_4$ | 2 mM |
| $NaH_2PO_4 \times H_2O$ | 10 mM |
| $NH_4Cl$ | 100 mM |
| Glucose | 200 mM |
| MES-Buffer, pH 7.2 | 200 mM |
| Trace elements solution (TE) | 1% v/v |
| Agar | 2% w/v |

#### 12.4.1.2 Trace Elements (TE, in 0.1 N HCl)

| | |
|---|---|
| $FeCl_3 \times 6H_2O$ | 1 mM |
| $MnSO_4 \times H_2O$ | 2.5 mM |
| $CuSO_4 \times 5H_2O$ | 0.2 mM |
| $CaCl_2 \times 2H_2O$ | 10 mM |
| $H_3BO_3$ | 1 mM |
| $CoCl_2 \times 6H_2O$ | 0.1 mM |
| ZnCl | 0.5 mM |
| $Na_2MoO_4 \times H_2O$ | 0.1 mM |

#### 12.4.1.3 ISP2 Agar (Shirling and Gottlieb 1966)

| | |
|---|---|
| Yeast extract | 4 g/l |
| Malt extract | 10 g/l |
| Glucose | 4 g/l |

| | |
|---|---|
| Agar-agar | 20 g/l |
| Distilled water | at 1 l |
| pH | 7.3 |

#### 12.4.1.4 MMN Agar (Molina and Palmer 1982)

| | |
|---|---|
| $CaCl_2 \times 2H_2O$ | 0.05 g/l |
| NaCl | 0.025 g/l |
| $NaH_2PO_4 \times H_2O$ | 0.5 g/l |
| $MgSO_4 \times 7\ H_2O$ | 0.15 g/l |
| $FeCl_3 \times 6H_2O$ | 1 mg/l |
| Thiamine hydrochloride | 100 µg/l |
| Trace elements (TE) | 10 ml/l |
| pH | 6.6 |
| Agar-agar | 2% (w/v) |

#### 12.4.1.5 Malt Agar (1.5%)

| | |
|---|---|
| Malt extract | 10 g/l |
| Agar-agar | 1.5% |

### *12.4.2 Organisms*

#### 12.4.2.1 Fungi

*Amanita muscaria* strains were isolated from fruiting bodies collected in the Schönbuch forest near Tübingen, Germany. They were cultivated in the dark at 20°C on MMN agar with 1% glucose. *Heterobasidion* strains were obtained from K. Korhonen (Finish Institute of Forest Research, Finland) and maintained on malt agar.

#### 12.4.2.2 Soil Bacteria (Actinomycetes)

As a source for bacteria we used a spruce stand with well-mycorrhized roots and extensive fruiting body formation by *Amanita muscaria*. As described in Maier et al. (2004), soil samples were taken from the respective "hyphosphere" which was defined as the fungal hyphae-containing organic layer after removal of the uppermost undigested litter layer; between 3–8 cm from the surface and at a distance of ~2 m from the next spruce trunk. Five samples from an area covering 1.5 $m^2$ of surface area were mixed. The soil samples were kept at 60°C for 40 min. Bacteria were isolated from soil by membrane filtration according to Hirsch & Christensen (1983). The procedure was as follows:

For growth of bacteria, synthetic CMS medium was used. To suppress the growth of fungi, the medium contained 100 µg $ml^{-1}$ each of cycloheximide

and candicidin. The antibiotics, glucose, magnesium chloride, and TS were sterilized by filtration through a 0.2 µm membrane and added separately to autoclaved and cooled CMS before the plates were poured. The solidified medium was covered by a cellulose ester membrane (mesh size 0.3 µm; Millipore). Soil samples (0.5 g) were suspended in 100 ml sterilized, distilled water and vigorously shaken for 30 min. The membrane surface was inoculated with 200, 100, and 50 µl aliquots.

This approach selects for actinomycetes; only these are able to penetrate the membrane pores in order to grow into the underlying agar. In contrast, growth of other bacteria is restricted to the membrane surface. The purity of optically distinguishable colonies was verified by repeated culture of diluted samples on ISP 2 agar in the dark at 20°C (Shirling and Gottlieb 1966).

## 12.5 Procedures

### *12.5.1 Culture on Solid Media*

For testing the effect of bacteria on fungal growth, dual cultures were used. The fungal inoculum was excised from the actively growing edge of a fungal colony using the wide end of a Pasteur pipette and transferred to the center of a sterile cellophane sheet (exclusion limit 10 kDa; Folia, Wendelstein, Germany) on top of ISP 2 agar and contained in a 9-cm-diameter Petri dish (compare Fig. 12.1; Maier 2003). Bacterial isolates were applied to the edge of the Petri dish outside the cellophane layer as a thin line of about 4 cm in length. The distance between the inocula was approximately 3.5 cm. In order to provide sufficient gas exchange, the Petri dishes were not sealed with Parafilm. Contamination was kept to a minimum by placing the Petri dishes in miniature plant culture chambers at 20°C in darkness. For preliminary screening, two independent trials with four parallels each were performed. With bacterial isolates which had been shown to promote or suppress fungal growth, culture experiments were extended for up to 11 weeks (slow growing *Amanita muscaria*) with three trials each consisting of 20 parallels.

Stock solutions of bioactive secondary metabolites from AcH 505 culture supernatant, the growth-promoting compound auxofuran and antifungal compound WS-5995 B (Riedlinger et al. 2006), were prepared by dissolving the substances in MeOH at concentrations of 50 mM for auxofuran and 30 mM for WS-5995 B. 7-dehydroxyauxofuran, which is liquid at room temperature, was diluted to obtain a concentration of 50 mM in MeOH. After a short incubation at room temperature, the stock solutions were vortexed vigorously to ensure that the compounds were completely dissolved before use. For all substances, concentrations ranging from 150 nM to 150 µM were used to assess their impact on fungal growth.

For the analysis of mycelial growth on solid media, Petri dishes were filled with 35 ml MMN medium. The compounds were added to 40°C warm medium in 100 µl

of MeOH. Introduction of specific substances directly into 40°C culture medium gave results comparable to the results obtained when substances were applied to the top of the agar. A cellophane sheet was placed on the MMN medium, and four fungal inocula were positioned equally spaced from the Petri dish margin. The radii of fungal colonies were determined after 3 and 6 weeks of growth. Fungal growth was recorded by means of a digital image analysis system (DIAS, Bachofer, Reutlingen, Germany). The density of the fungal mycelium in DC was determined as the ratio between the weight and the surface area of the outermost zone of the circular mycelium (zone width 1 cm).

To harvest the mycelium for biochemical analyses, the outermost area of 1 cm width was rapidly separated from the older tissue using a sterile scalpel, weighed and immediately frozen in liquid nitrogen and stored at −80°C. For immediate light microscopy of the mycelium, a 1 × 1 cm section of the cellophane sheet including the mycelial front was cut out with a scalpel, placed on a microscope slide, and covered with a cover slip.

### *12.5.2 Suspension Cultures*

Suspension cultures of *Amanita muscaria* were started from 8-week old mycelium growing on MMN agar. The fungal mycelium was finely cut, transferred to MMN medium and homogenized with an Ultra-Thurrax (Janke and Kunkel, Germany). The MMN medium was exchanged once a week. Streptomycete cultures were started by adding three loopfuls of bacterial spores and filaments to 80 ml MMN medium in a 300 ml Erlenmeyer flask with a metal spiral on the bottom of the vials. All cultures were incubated in the dark at 20°C on a rotary shaker at 80 rpm. If not otherwise stated, fungal suspension cultures (5 days after the change of the MMN medium) were used for the different treatments described below. After 5 days of incubation, the fungal mycelium is actively growing and most of the nutrients have been depleted from the culture medium.

To determine the effect of streptomycete culture supernatants on fungal gene expression, each bacterium was grown until an $OD_{600}$ of 0.2 in MMN medium. Fifteen ml of streptomycete suspension were filtered with a 0.22 μm mesh and added to 45 ml of fungal suspension culture (1/4; v/v). Auxofuran and WS-5995 B were applied on the mycelium of *A. muscaria* to test their influence on fungal gene expression. Prior to substance application to submerged fungal mycelia, the fungal suspension cultures were washed twice with MMN, and the substances were added in 100 μl of MeOH to the culture medium. The final concentration of auxofuran was 15 μM, corresponding to a concentration that promoted *A. muscaria* growth to a comparable extent as with AcH 505, and that of WS-5995 B was 60 μM, corresponding to a concentration that significantly retarded *A. muscaria* growth. A MeOH-treated fungal suspension culture served as a control. Influence of heat shock on the mycelia (Riedlinger et al. 2006) was investigated by transferring the fungal suspension cultures from 20 to 30°C for 3 h. Carbon-based induction of gene expression was

investigated according to Nehls et al. (1999). MMN medium supplemented with 40 mM glucose was added to mycelia that were carbohydrate-depleted for 1 week.

Bacterium–fungus dual cultures were performed according to Riedlinger et al. (2006) in magnetically stirred glass fermenters containing modified MMN medium. To inoculate a fermenter, the pellet from a 200-ml shake flask preculture of strain AcH 505 grown for 120 h and the pellet from a 500-ml shake flask preculture of *A. muscaria* grown for 1 month were resuspended in 500 ml modified MMN medium. Fermentation was carried out with an aeration rate of 0.25 v $v^{-1}$ $min^{-1}$ and agitation at 100 rpm. At harvest, suspension cultures were filtered through a 100 μm nylon mesh (Seidengazefabrik, Thal, Switzerland). The material was immediately frozen in liquid nitrogen and stored at −80°C.

## *12.5.3 Co-Cultures*

### 12.5.3.1 Mycorrhizal Synthesis with Mycorrhization Helper Actinomycetes

We commonly use modified MMN agar media without glucose for the synthesis of ectomycorrhizas (Schaeffer et al. 1996). Alternatively, we have used a sterile culture system based on moss peat and perlite that has been adapted from Poole et al. (2001) and is explained here. Seeds of Norway spruce [*Picea abies* (L.) Karst] were obtained from the Staatsklenge Nagold (Nagold, Germany). Seedlings are grown under sterile conditions in Petri dishes for 4 weeks at 22°C (16 hrs light, 200 μE $m^{-2}$ $s^{-1}$) on MMN medium, containing 1% glucose and 0.8% agar. Ectomycorrhizas were synthesized using a Petri dish method modified from Poole et al. (2001). Moss peat (Botanical Garden, Tübingen, Germany) and perlite (Knauf Perlite GmbH, Dortmund, Germany) were mixed in 1:1 ratio, crushed, moistened with double distilled water (10 g water: 15 g mixture) and autoclaved. A slit was cut into the side of the Petri dishes and their covers using a hot scalpel. One part MMN medium (without glucose) was mixed to four parts peat-perlite mixture and evenly spread onto the Petri dishes. Plants and fungal inocula (*A. muscaria* 6, or *S. bovinus* K3) were positioned according to Poole et al. (2001). Briefly, plants were laid into the slit, placing the root systems on the moss-perlite mixture and shoots exposed to the atmosphere. Three fungal inocula, consisting of 5 mm diameter discs cut from the growing margin of an MMN agar culture, were placed upside-down along each side of the root at 15 mm intervals, at a distance of 5 mm from the root.

For bacterial inoculations, 1-week-old AcH 505 or AcH 1003 cultures in MMN medium were centrifuged (1,500 g, 15 min) and the pellets were resuspended at OD 0.6 in MMN medium without glucose. Two 500-μl aliquots of bacteria were pipetted between the roots and the fungal plugs. Four-month-old seedlings were harvested for analysis. For all inoculations, 15 replicates were used, out of which ten were used for analyses of mycorrhization and the development of seedlings.

### 12.5.4 Simple Culture System for the Inoculation of Norway Spruce Roots with Actinomycetes

We use this highly reproducible culture system to analyze young Norway spruce seedlings under the influence of actinomycetes and pathogenic fungi. It suits the analysis well if a mycorrhization helper bacterium has a local (root) or a distal (shoot) influence on disease development in Norway spruce (Lehr et al. 2007). Seedlings are grown until 4 weeks as explained before. Cross-walled Petri dishes are filled from one side with 25 ml MMN medium without glucose, containing 2% agar. The agar is then covered by Cellophan. The bacteria are pre-cultured on ISP2 agar as described above. About ten colonies are evenly suspended in 1 ml of sterile distilled water. A 25 µl aliquot of the bacterial suspension is evenly distributed on the agar. The roots of three seedlings are spread on the Cellophan and covered by wet filter paper which serves two purposes: it fixes the root and keeps them humid. The shoots extend into the agar-free second half of the dish. After adding the top plate, the Petri dishes are secured with a layer of Parafilm (Fig. 12.2). The dishes are stored vertically and the culture continued at 22°C (16 h light, 200 µE $m^{-2}$ $s^{-1}$) for 7 days prior to inoculation with pathogenic fungi.

### 12.5.5 Impact of Pre-Inoculation with Mycorrhization Helper Bacteria on Heterobasidion Root Rot

We have used *Heterobasidion annosum* as a root pathogen to test how actinomycetes influence disease development in Norway spruce (Lehr et al. 2007).


**Fig. 12.2** Culture system to analyze the influence of mycorrhization helper bacteria on plant pathogens

*H. annosum* strains were precultured on malt agar (1.5% malt extract, 1% agar). Conidiospores were washed off from 2-week-old cultures by pipetting back and forth with 0.05% Tween solution. Inoculum density was adjusted to $1 \times 10^5$ conidia per ml. Seedlings either pre-treated with bacteria or water, as indicated above, were treated with fungal spores: filter paper on the roots was set aside, 50 µl of conidia suspension were pipetted directly on the roots, and filter paper was placed on the roots again. Petri dishes were closed and secured with Parafilm, and the culture was continued in the upright position.

### 12.5.6 Impact of Volatiles of Mycorrhization Helper Bacteria on Heterobasidion Root Rot

Mycorrhization helper bacteria may exert their function by the production of volatiles (Garbaye 1994), and we have observed that the volatiles of a mycorrhiza helper bacterium have a positive impact on spruce main root colonization by *H. abietinum*. For these experiments, spruce seedlings and fungi were cocultured in Petri dishes, as explained above. Bacteria, precultivated on HA agar, were cut out as agar blocks of about 25 $mm^2$, and positioned in the agar-free part of the Petri dish in the vicinity of the shoot (Fig. 12.3). Communication between bacteria and plant or fungus was thus only possible by air space.

### 12.5.7 Physiological Screening of Host Plant Viability

Chlorophyll fluorescence of photosystem II is an indicator of quantum yield of photosynthesis and can be used as a measure of plant viability (Schreiber 2004). We used a system provided by Walz, Effeltrich, Germany (Imaging PAM) which

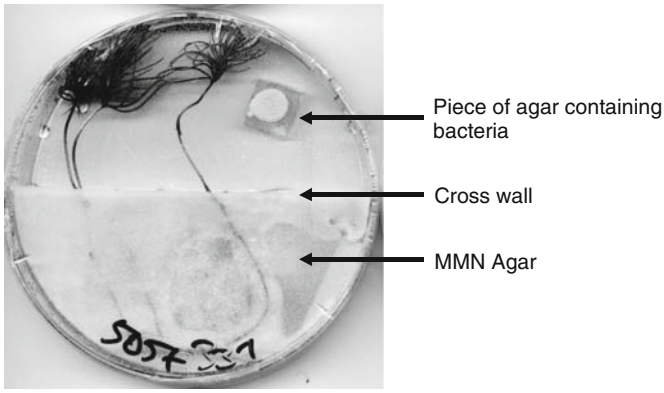


**Fig. 12.3** Culture system for the analysis of volatile-based signaling by mycorrhization helper bacteria

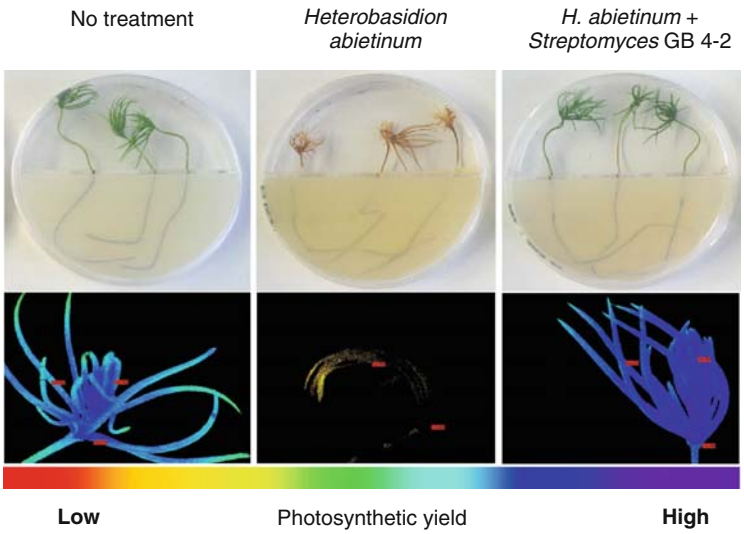


**Fig. 12.4** The use of chlorophyll fluorescence of photosystem II as an indicator of plant viability

employs a CCD camera for fluorescence analysis. This way, leaves of small plants such as young seedlings (spruce, *Arabidopsis thaliana* etc.) can be measured inside Petri dishes without the need to open the lid of sterile cultures. Figure 12.4 gives an example for the quantum yield of photosystem II of spruce seedlings alone, and after coculture with the pathogenic fungus, *Heterobasidion abietinum*, or both the fungus and *streptomyces AcH 505*. The photographs clearly show how the detrimental effect of the fungus is completely compensated by the bacterium.

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# Chapter 13
# Isolation, Cultivation and *In Planta* Visualization of Bacterial Endophytes in Hanging Roots of Banyan Tree (*Ficus bengalensis*)

**Khyati Pathak, Haresh Keharia, and Amit C. Kharkwal**

## 13.1 Introduction

Endophytic bacteria live in the plant tissue without causing substantive harm to the host plant. They may either benefit the host or benefits may be reciprocal (Bacon et al. 2002).

Interaction between plant and beneficial bacteria can have profound effects on crop health, crop yield and soil fertility (Kloepper et al. 1989). These microorganisms can improve plant quality in several ways: competition for habitat by production of antibiotics, induction of plant defense mechanisms, production of plant growth stimulators, and improving soil quality (Ciccillo et al. 2002; Glick 1995; Kloepper et al. 1989). Endophytes are a poorly investigated group of microorganisms that represent an abundant, dependable source of biologically important and chemically novel bioactive compounds with potential for exploitation in a pharmaceutical and agricultural fields (Strobel and Daisy 2003). The use of microbial endophytic flora opens up a new arena of biotechnological exploitation which requires us to understand the endophytic flora, their existence, and their response to the environment in which they exist (Bacon et al. 2002). To better understand the endophytes, it is necessary to isolate and cultivate these microorganisms.

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## 13.2 Isolation and Cultivation of Endophytes from Plant Roots

The most important and critical step for working with microbial endophytes is to isolate them. The procedure should be sensitive enough to recover a maximum number of endophytes and strong enough to eliminate the epiphytes present on the root surface.

### *13.2.1 Selection of Plant Material*

Around 30,000 plant species exist on the earth. Since each individual plant is a host to one or more endophytes, creative and imaginative strategies must be used to quickly narrow the search for the host plant for bioprospective endophytes (Strobel and Daisy 2003; Mittermeier et al. 1999). While selecting a plant for investigation of endophytes capable of producing novel bioactive molecules, the following criteria need to be considered (Strobel and Daisy 2003):

1. Plants from a unique environmental niche, especially those with an unusual biology and possessing novel strategies for survival are seriously considered for study.
2. Plants that have an ethnobotanical history (use by indigenous peoples) that are related to some specific application of interest are selected for study.
3. Plants that are endemic, that have an unusual longevity, or have occupied a certain ancient land mass, are appropriate for study.
4. Plants growing in areas of great biodiversity also have the potential for housing endophytes with great diversity.

It is important to select healthy and disease-free plant material for isolation work, to prevent the isolation of localized pathogenic endophytic microorganisms. It is always desirable to select the procedure that allows recovery of several species of bacteria from the selected plant material.

### *13.2.2 Isolation of Endophytic Microorganisms*

The first step in the isolation of microorganisms residing in plant tissues is surface sterilization of explants. To achieve complete surface sterilization, various steps should be followed; the method varies according to the type of tissue as well as its location.

#### 13.2.2.1 Pre-Washing and Cleaning

Thorough pre-washing of plant material is necessary to remove the adhering soil particles and dust on the root surfaces in order to reduce the load of surface flora.

Pre-washing can increase the effectiveness of the surface-sterilizing agents. The root tissue selected for isolation should be disease-free and freshly collected. Immediately after collection, the sample should be processed for surface sterilization. Pre-washing can be carried out by vigorous washing with tap water, distilled water or one of several dilute saline buffers of pH-7.2 (Bacon et al. 2002). Sonication may also be used to dislodge soil and organic matter from the root tissue before surface sterilization (Hallmann et al. 2006).

#### 13.2.2.2 Surface Sterilization

Sterilizing agents are the chemicals used to kill the microflora on the surface of plant material. The most commonly employed surface-sterilizing agents are sodium hypochlorite, mercuric chloride, ethanol, hydrogen peroxide, and chlormine T (Dong et al. 1994; Gardner et al. 1982; Gagne et al. 1987; McInroy and Kloepper 1994). Propylene oxide vapor (Sardi et al. 1992) and formaldehyde (Cao et al. 2002) are less frequently used chemical sterilizing agents. More effective sterilization can be achieved using a combination of agents or combined chemical and physical agents. For example, root tissue of *Citrus jambhiri* was sterilized by dipping in 90% ethanol and flamed to remove surface micro flora by Gardner (1982). To achieve effective surface sterilization, nonionic detergents like Tween 20, Triton X-100, Tween-80 can sometimes be used along with surface-sterilizing agents, in order to improve penetration of surface sterilizers to niches and grooves beyond the epidermal cells (Bills 1996; Hallmann et al. 2006). Concentration of sterilizing agents, combination treatment, and incubation period for sterilization may vary depending on plant as well as tissue material.

Most common methods used for sterilization include three steps: 70% ethanol treatment followed by sodium hypochlorite and again 70% ethanol treatment, or ethanol treatment followed by mercuric chloride and ethanol treatment.

During surface sterilization after following each step it is recommended to wash the tissue with sterile water to remove and prevent the entry of sterilizing agents inside the root tissue. Sometimes the sterilizing agents penetrate the internal tissue of plant and can kill the endophytic flora, resulting in lower recovery of endophytic flora. So, depending upon the plant species, tissue and age, concentration and combination of sterilizing agents as well as incubation period needsto be optimized for each condition in order to achieve maximum recovery of endophytes without contamination of epiphytes.

The efficiency of surface sterilization can be ascertained by rolling the surface-sterilized tissue onto the surface of agar-containing media, by dipping the roots into nutrient broth (Gagne et al. 1987), or by streaking the water collected after washing the explants with surface-sterilizing agent onto the nutrient agar media (McInroy and Kloepper 1994).

### 13.2.3 Cultivationof Endophytes

Surface-sterilized root tissue is subjected for isolation procedure. Most commonly employed procedures are cutting of the surface-sterilized plant tissue and maceration. The root material is cut aseptically into small pieces (7–8 mm) and subjected for cultivation on nutrient media (Hallmann et al. 1997). Plant secretions such as latex and wax also contain microflora, so these secretions should be collected aseptically and streaked onto nutrient agar media to cultivate the endophytic bacteria. During cultivation the organism can grow fast, and hence limitation of nutrients can affect the isolation of slow-growing microflora. Maceration is the method by which the slow- and fast-growing cultures can be isolated. Maceration requires sterilized mortar and pestle, homogenizer or blender (Parmeela and Johri 2004; Hallmann et al. 2006). Sterile buffer or sterile saline is used during homogenization of plant tissue in order to maintain the integrity of endophytic organisms. The whole procedure should be performed aseptically to avoid contamination. However, heat generation during the maceration may kill the endophytes, so care should be taken while processing to have minimum heat generation. All the equipment required for maceration should be sterilized properly. If not, there is a considerable risk of airborne contamination. Certain plant enzymes or toxic material released during maceration from plant cells can kill the microorganisms, so to prevent this it is advised to use polyvinyl pyrrolidone (PVP) or EDTA (Hallmann et al. 2006).

Most endophytic bacteria can grow on routine laboratory bacterial growth media in the laboratory, viz. Luria broth, glucose yeast extract agar, tryptic soy agar, King's B medium and MacConkey agar which support the growth of a broad range of bacteria (Balandreau 1983; Bagley and Seidler 1978).

## 13.3 Protocol for Isolation and Cultivation of Bacterial Endophytes from Hanging Roots of Banyan Tree

### 13.3.1 Requirements

1. Sample collection bags
2. Tap water
3. Sterile distilled water
4. Containers for pre-washing and pre-treatment.
5. Sterile containers for each surface-sterilization step and washing steps
6. Labolene – Neutral liquid detergent (Qualigens)
7. 10% Savlon
8. 70% ethanol
9. 0.1% mercuric chloride

10. Sterile forceps
11. Sterile petri dishes
12. Sterile blade
13. Laminar air flow unit
14. BOD incubator

### 13.3.2 Media for Cultivation

1. Luria agar
2. Potato dextose agar

### 13.3.3 Method

1. *Collection*. Collect a disease-free, growing part of the hanging roots of banyan tree aseptically, and proceed to isolation on the day of collection.
2. *Cleaning and pre-washing*. Clean the hanging roots by washing vigorously under tap water to remove dust adhered to the surface of aerial roots.
3. *Pre-treatment*. The cleaned hanging roots are then subjected to the detergent treatment. Commercially available neutral liquid detergent Labolene (Qualigens) is used to remove hydrophobic substances on the roots surface in order to improve accessibility of sterilizing agents. Roots are treated with 10% Labolene for 15 min.
4. *Surface sterilization*. Surface sterilization of banyan hanging roots requires three steps:

Pre-treated hanging roots are kept in the commercially available antiseptic liquid solution of 10% (v/v) Savlon for 15 min. Savlon consists of key components such as cetrimide and chlorhexidine, these broad-spectrum antiseptics having antibacterial and fungicidal action. Treatment with Savlon helps to eliminate most of the bacterial and fungal plant surface flora.

The Savlon-treated roots are then washed with sterile distilled water to remove the remaining traces of cetrimide and chlorhexidine from the root surface. The washed roots are then treated with 70% Ethanol solution, twice each for 2 min.

The roots are then immersed in 0.1% $HgCl_2$ (w/v) for 30 s followed by repeated (at least 6–8 times) washes with sterile distilled water to remove excess of $HgCl_2$ from the root surface. The surface-sterilized roots thus obtained are then placed in a sterile Petri dish and cut about 5 mm from both ends using a sterile surgical blade. The central portion of the root is then cut into small segments of about 8 mm.

5. *Cultivation*.

   (a) Surface-sterilized small segments of hanging roots of banyan tree are placed on the surface of sterile Luria agar as well as on sterile potato dextrose agar plates. The root segments are placed on media, keeping at

least 2 cm space between two root segments so as to provide the area for the bacterial growth from and around the root segments.

(b) The plates are incubated at 30 ± 2°C in a BOD incubator and observed every day for growth.

(c) During incubation, the endophytes from the root tissue migrate outside towards media and start colonizing.

(d) The bacterial growth thus obtained is then subcultured onto sterile Luria agar or potato dextrose agar media using a sterile nichrome wire loop to get isolated colonies.

(e) The isolated colonies thus obtained are repeatedly subcultured on respective media to ascertain purity and then screened for production of bioactive compounds.

*Remarks*. All the steps for surface sterilization should be carried out in aseptic conditions in a laminar air flow unit; all the containers and glassware required for surface sterilization should be autoclaved at 15 lbs pressure for 15 min.

## 13.4 *In Planta* Visualization/Localization of Endophytes

To study plant-interacting bacteria such as endophytes and their behavior in the plant system it is necessary to demonstrate their internal colonization ability, the establishment of endophytic strain in the plant. Isolation procedures are the simplest way to demonstrate the establishment of microflora in the internal tissue of a plant and give a rough estimate of the bacterial population density. However, isolation procedures do not allow an exact localization and often detect only minor naturally occurring microorganisms. In general, the two types of investigation of bacterial endophytic behavioral study are those that are concerned with the indigenous populations, and those that monitor the colonization of an introduced endophyte (Hallmann et al. 1997).

### *13.4.1 Vital Staining Method*

Various bacterial staining techniques cannot distinguish between living or dead bacterial cells and moreover most of the stains which are used for bacterial staining can stain plant cells and cell materials, which makes it difficult to distinguish bacterial cells inside the plant tissue. Viable staining techniques overcome the problems associated with non-cultivable microorganisms and can also be applied for bacterial detection *in situ*. Therefore, vital stains might provide a more realistic tool for enumeration and localization of microbial communities (Hallmann et al. 1997).

### *13.4.2 Electron Microscopy*

Electron microscopy is a powerful tool for identifying and placing endophytic bacteria in plant tissue. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) have been successfully employed to detect the endophytic bacteria in various plants. For SEM the sample needs to be cut, so the risk of bacterial contamination from the root surface is always there. In order to avoid such contamination, the plant tissue with endophytes should be fixed first and then sectioned. TEM can reduce such contamination because it requires sample fixation and sectioning. For electron microscopy the processing of tissue is very much important. Some problems, such as access of fixatives or resins to uncut intercellular spaces due to heavily suberized and lignified parenchyma cells or to oozing out of bacteria from the apoplast fluid of cut spaces, create difficulties in detecting bacteria in the plant tissue (Dong et al. 1994). Preparation of plant tissue for EM requires much time compared to animal tissue, and requires expertise in sample preparation, ultra thin sectioning and electron microscopy to differentiate between plant organelles and bacterial cells.

## 13.5 Protocol for Detection of Endophytic Bacteria by Vital Staining

Use of Tetrazolium dyes as vital stain for demonstration of bacteria inside the plant tissue was introduced by Patriquin and Dobereiner (1978). Bacteria inside the surface-sterilized hanging root tissues of Banyan tree utilize malic acid as carbon source and reduce 2,3,5- triphenyl tetrazolium chloride salt (TTC) to form water-insoluble red-colored formazans. The actively respiring (viable) bacterial cells inside the plant tissue reduce TTC and deposit formazans as discrete dark red to purple granules in the cells and these stained bacterial cells can be visualized by light microscopy.

Acridine orange is a fluorescent cationic dye. It is cell-permeable, and interacts with DNA and RNA intercalation or electrostatic attraction. It is used to stain microorganisms in unfixed specimen, for vital staining and differential staining of DNA and RNA. It is used for the direct counting of endophytes *in situ* in combination with epifluorescence microscopy. Sometimes small particles are difficult to distinguish from bacteria, so by counterstaining Tetrazolium-treated cells with Acridine orange one can differentiate bacteria and small particles (Bacon et al. 2002).

### *13.5.1 Equipment*

(a) Incubator
(b) Compound microscope

(c) Cryotome Leica
(d) Sterile forcep
(e) Conical Flask (250 ml)
(f) Sterile cotton-plugged sugar tubes
(g) Sterile screw-cap tubes (5 ml).
(h) Glass micro slides

### 13.5.2 Chemicals and Reagents

(i) 50 mM Potassium phosphate buffer (pH-7)
(j) 2, 3, 5 – triphenyl tetrazolium chloride salts
(k) Malic acid
(l) Conical flask (250 ml)
(m) Sterile cotton-plugged sugar tubes
(n) Sterile screw-cap tubes (5 ml)
(o) Glass micro slides
(p) Surface-sterilized hanging roots of banyan tree
(q) 1% aniline blue
(r) Acridine orange reagent: Dissolve 0.1 g acridine orange in 100 ml of distilled water and store it at 4°C in the dark, and from the stock prepare working solution by diluting stock acridine orange with 50 mM of potassium phosphate buffer in the ratio of 1:10.

### 13.5.3 Procedure

1. Take 100 ml 50 mM potassium phosphate buffer (pH-7) and add 0.0625 g malic acid into that in a conical flask and autoclave it for 15 min at 15 lbs pressure.
2. Take 0.15 g 2, 3, 5- triphenyl tetrazolium chloride (TTC) and add into sterile 50 mM malate potassium phosphate buffer (pH-7) in aseptic condition.
3. Dip surface-sterilized hanging roots of banyan tree into 20 ml of above sterile TTC solution and incubate overnight at 30 ± 2°C in incubator.
4. Take longitudinal or cross sections of TTC-treated roots either by hand or using cryotome.
5. Place sections on glass microscopic slides and prepare wet mount of the sections with phosphate buffer, place a glass cover slip and seal the edges with colorless nail polish or DPX mounting media and examine the sections under compound microscope at 100× magnification.
6. The bacterial cells stain red to purple (Fig. 13.1).
7. To enhance the TTC-stained tissue, counterstain the TTC-treated sections with 1% Aniline blue for 1 min and then remove the excess of stain and prepare wet mount and observe it immediately under the microscope.
8. Stain cross-sectional or longitudinal sections of root tissue with working solution of Acridine orange for 3–5 min at room temperature and allow excess dye to run off.

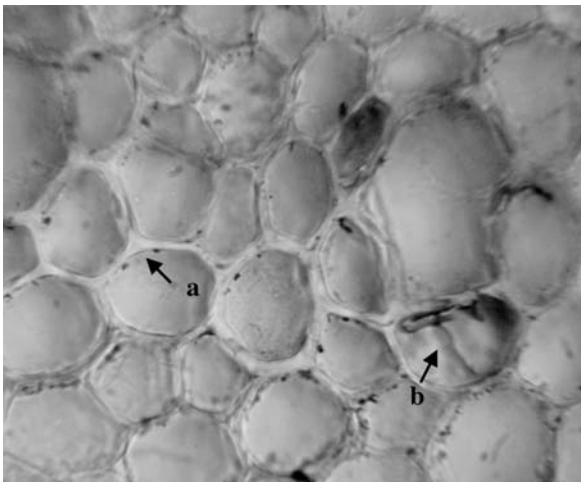


**Fig. 13.1** Light micrograph of transverse section of hanging roots of Banyan tree treated with tetrazolium dye, showing the presence of endophytic bacteria (magnification ×100). **a** *Arrow* points at the bacterial cell located at inner side of the wall of root cortical cell. **b** Fungal hyphae located inside the root cortical cell

9. Rinse the sections with phosphate buffer for 2 min.
10. Prepare wet mount and observe under epifluorescence microscope.
11. Counterstain TTC-treated root sections with acridine orange by previously described method and mount in phosphate buffer.
12. Observe each microscope field first using epifluorescence illumination and count all fluorescing bacteria (vital) and without moving the stage switch to phase contrast count all bacteria stained red to purple. By viewing in epifluorescence illumination and phase contrast one can confirm the presence of living bacteria in the plant root tissue by practice.

## 13.6 Protocol for Detection of Bacterial Endophytes by Transmission Electron Microscopy

The following methodology can be followed for fixation of tissue specimen, ultrathin sectioning and staining of sections for observing bacterial cells in sections using TEM (Rensing 2002; Roland 1978).

### *13.6.1 Requirements*

#### 13.6.1.1 For Fixation

(a) 0.1 M sodium phosphate buffer pH-6.8
(b) Commercial 8% glutaraldehyde

(c) 1N sodium hydroxide
(d) Deionized water
(e) Storage glass vials (5 ml)
(f) 10% (W/V) para formaldehyde (10 g of para formaldehyde powder are dissolved in 100 ml of deionized water by heating to 60–70°C and stirring. Solution becomes milky white in color; to that, add 2–3 drops of 1N NaOH until the solution becomes transparent. The solution is cooled before use.

#### 13.6.1.2 Preparation of Combined Glutaraldehyde–Formaldehyde Fixative

| | |
|---|---|
| 0.1 M sodium phosphate buffer pH-6.8 | 10 ml |
| Deionized water | 4 ml |
| 10% para formaldehyde | 4 ml |
| 8% glutaraldehyde | 4 ml |
| Total volume of fixative | 22 ml |

(g) 2% osmium tetroxide ($OSO_4$) (EM grade osmium tetroxide is available in either crystal or liquid form. Prepare 2% osmium tetroxide in water in fume hood. The solution can remain stable for months; store it in dark in tight container)
(h) Fume hood
(i) Ultramicrotome
(j) Wooden applicator sticks
(k) Uranyl acetate
(l) Acetone
(m) Spurr resin
(n) Rubber casting tray for block preparation
(o) 5% toludine blue
(p) Formvar solution: 0.4–0.6 g of formvar in 100 ml chloroform or dichloromethane. Store in refrigerator
(q) 0.1% NaOH
(r) Sato's Lead stain

| | |
|---|---|
| Lead acetate | 1.0 g |
| Lead nitrate | 1.0 g |
| Lead citrate | 1.0 g |
| Sodium citrate | 2.0 g |
| Deionized water (pre-boiled) | 82 ml |

Mix all chemicals for 2 min, than add 18 ml of 4% NaOH.

**Note**: All the chemicals required for the EM processing should be of EM grade. Commercially available 8% glutaraldehyde is colorless; do not use if it appears pale in color. Spurr resin is toxic so avoid contact with skin.

## 13.6.2 Procedure

1. *Fixation*. Clean the roots by thoroughly washing under tap water followed by distilled water. Cut roots 3–5 mm and transfer the roots into vial containing 3 ml of formaldehyde-glutaraldehyde fixative. Roots should be completely immersed in the fixative. If roots are floating in the fixative, degas the vial and incubate the tissue in fixative for 40–60 min at room temperature. It can be continued upto 2–5 h also. Then store this tissue in refrigerator for 2 h. Decant the fixative from the vials.
2. *Washing with buffer (cold)*. Wash the root pieces with cold 0.1 M sodium phosphate buffer pH-6.8 for 10 min. Repeat this step three times.
3. *Transfer to 2% osmium tetroxide ($OSO_4$)*. Incubate the root segments in 2% $OSO_4$ overnight or for desired time according to material.
4. *Buffer wash*. Repeat step 2.
5. *Water wash*. Wash the root pieces with cold deionized water for 2 min. Repeat this step one more time.
6. *1% Uranyl acetate*. Soak the root tissues in cold 1% aqueous uranyl acetate for 30 min.
7. *Water wash*. Repeat step 5.
8. *Dehydration*. After fixation the root segments undergo dehydration treatment.
9. Dehydration is carried out by using acetone-water series to remove water from the root tissue. Roots are treated for each of the following acetone-water solutions in sequence for 20 min:.

   (a) Cold 10% acetone
   (b) Cold 25% acetone
   (c) Cold 50% acetone
   (d) Cold 70% acetone
   (e) Cold 95% acetone

Henceforth the treatment is given with acetone stabilized at ambient temperature:

   (f) 95% acetone
   (g) 100% acetone
   (h) 100% acetone
   (i) 100% acetone

### 13.6.2.1 Infiltration with Spurr Resin

Treat dehydrated root tissues in each composition mixture of Spurr resin given below:

1. 3 volume of Acetone: 1volume of Spurr for 1–2 h
2. 2 volume of Acetone: 2 volume of Spurr for 12 h

3. 1 volume of Acetone: 3 volume of Spurr for 24 h
4. Pure Spurr for 24 h

#### 13.6.2.2 Embedding

Pour resin and put root pieces in the small slots of a rubber casting tray and pour resin in each slot to submerge the roots pieces in it. Incubate the tray containing root pieces in the oven at 70°C for 12–48 h so that spurr gets polymerized and becomes hard. Spurr blocks of hard polymer having root segments are now ready for sectioning.

The significance of using formaldehyde-glutaraldehyde combined fixative is that formaldehyde penetrates faster than glutaraldehyde and temporarily stabilizes structures which are subsequently more permanently stabilized by glutaraldehyde. Osmium tetroxide can provide electron density to the material which can help in electron microscopy by creating contrast. Treatment with osmium tetroxide leads to blackening of the tissue. Uranyl acetate can stain the tissue and provide an electron-dense area; it also prevents injuries to the plant material. Uranyl acetate treatment can also be given after embedding and sectioning, depending upon the requirement.

#### 13.6.2.3 Ultrathin Sectioning

Trim the block and use it for ultrathin sectioning for light microscopy to check the proper infiltration and embedding and locate the area of interest:

1. Cut the sections 0.6–6 μm thick with an ultramicrotome using adiamond knife.
2. Transfer the sections to a drop of water on a glass slide and heat it on a hot plate at 60°C until the water has evaporated.
3. Stain the sections with 5% Toludine blue in aqueous NaOH. Cover the sections with a drop of stain, heat on the 60°C hot plate for 5–10 s. Then wash off the excess stain from the slide with a gentle stream of water.
4. Evaluate the sections with an LM. Fig. 13.2 shows the picture of Toludine blue-stained cross section of banyan root.
5. Cut the thin sections (at 70 nm) only from the best blocks and mount them on formvar-coated grids. Stain grids by immersing each grid in a drop of 1% uranyl acetate in 50% ethanol for 10 min. Wash these grids with distilled water three times.
6. Immerse each grid in a drop of Sato's lead stain (Sato 1967) for 5 min, rinse three times with water. This produces sufficient contrast in a short time. Allow the formvar-coated grids to dry in a desiccator. Coated grids can be used directly for TEM (Fig. 13.3).

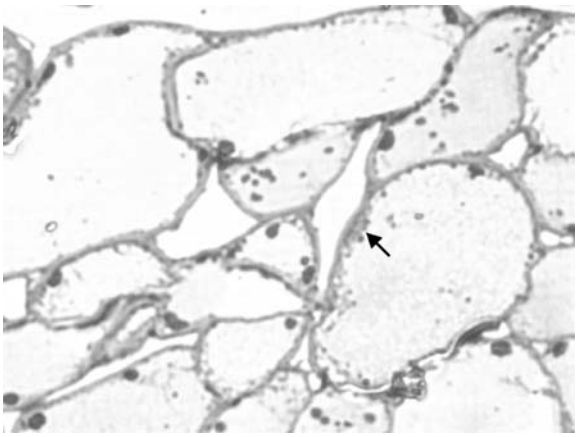

**Fig. 13.2** Light microscopy of 5% Toluidine blue stained cross-section of spurr embedded Banyan aerial roots. *Arrow* points at the uranyl acetate stained bacterial cell located near inner side of the cell wall of Banyan root cortical cell

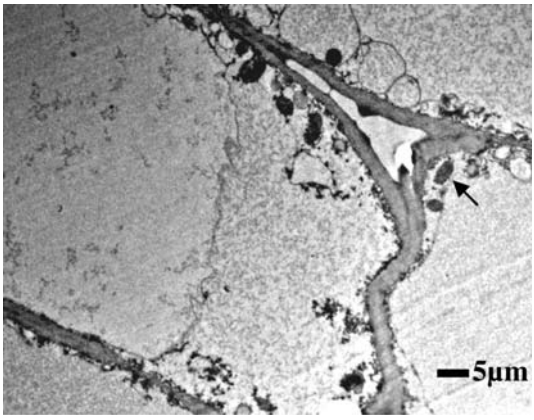


**Fig. 13.3** Electron micrograph of transverse section of spurr embedded Banyan aerial root. *Arrow* points at the uranyl acetate stained bacterial cell located near inner side of the cell wall of Banyan root cortical cell

## 13.7 Conclusions

Endophytes are an agriculturally and industrially important group of microorganism, with the ability to produce important chemically novel and biologically active compounds. It is necessary to understand their mechanism for producing biotechnologically important compounds in order to exploit them commercially. To exploit the endophytes it is important to isolate and cultivate them on the artificial media so one can grow them and produce the bioactive compounds in bulk for its

industrial usage. These groups of microorganism have generated research interest because of their various properties, such as the ability to colonize the internal plant tissue, and their interaction with the host tissue, producing compounds which can protect the plant from other infectious organisms and from physical stress, provide help in growth, and maintain plant tissue. To understand the plant-microbe interaction and endophytic behavior, it is important to localize them in plant tissue. In this chapter, we have tried to cover the complete methodology of isolation, cultivation and *in situ* detection of microbes in plant tissues.

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# Chapter 14
# Micro-PIXE Analysis for Localization and Quantification of Elements in Roots of Mycorrhizal Metal-Tolerant Plants

**Katarina Vogel-Mikuš, Paula Pongrac, Primož Pelicon, Primož Vavpetič, Bogdan Povh, Hermann Bothe and Marjana Regvar**

## 14.1 Introduction

Many fundamental processes of plant and fungal physiology are affected or regulated by mineral nutrients (Marschner 1995). The mechanisms of nutrient uptake, and their roles in plant and fungal metabolism are therefore of great importance for our understanding of symbiotic interactions and their functioning. In addition, there is a great demand for methods that can document the responses of plants and fungi to environmental stresses that can be caused by either factors present in the natural environment or by anthropogenic pollution. Particle-induced X-ray emission with a focused proton beam (micro-PIXE) is a powerful tool for these types of studies (Scheloske et al. 2004; Przybyłowicz et al. 2004; Vogel-Mikuš et al. 2007, 2008a, b). All elements encompassing the list of macro- and micro-nutrients, as well as elements considered as toxic pollutants, can be measured simultaneously by micro-PIXE. Maps of element distribution can provide valuable information that is not possible to obtain using point analyses or linear scans. The localization and quantification data on toxic elements in plants and fungi that have been gained using this technique have indicated possible pathways of detoxification mechanisms, and can therefore improve our understanding of plant and fungal adaptations to various environmental conditions. However, tracking element distribution can

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only be achieved through the appropriate preparation of specimens, making this the most important step in the analysis (Mesjasz-Przybyłowicz and Przybyłowicz 2002; Schneider et al. 2002). This chapter is therefore mainly dedicated to micro-PIXE specimen preparation, which has been developed in our laboratory in collaboration with colleagues from the Max Planck Institute for Nuclear Physics, Heidelberg.

## 14.2 Materials and Procedures

### *14.2.1 Equipment for Sample Preparation*

- Thermo-block, for rapid freezing of specimens
- Cryo-microtome
- Freeze-drier

### *14.2.2 Laboratory Materials*

- Scalpels and forceps
- Needles with polished tips
- Aluminium foil for making tissue-freezing medium beds
- Beakers
- Tissue-freezing medium
- Liquid nitrogen
- Propane
- Pioloform in chloroform
- Microscope object-glass
- Aluminium sample holders
- Glue (two-component Araldite)

### *14.2.3 Specimen Preparation*

The aim of the preparation of the biological material is the preservation of the element distribution as close as possible to its native (*in vivo*) state. The high-resolution capability of modern microprobe equipment places stringent demands on the tissue preparation for intracellular and tissue element-distribution studies. Any measurement can thus be meaningless and misleading unless changes in tissue morphology and chemical redistribution have been limited to dimensions that are smaller than the resolution of the microprobe (Schneider et al. 2002).

Incomplete protocols can cause artefacts that can lead to the removal or redistribution of elements, and hence to errors far exceeding those inherent to a measurement technique. It is generally accepted that only low-temperature methods can eliminate such artefacts, since it is evident that with chemical preparations some elements may be added, washed out or redistributed (Schneider et al. 2002;

Mesjasz-Przybyłowicz and Przybyłowicz 2002). Therefore, specimen cryo-fixation is typically followed by specimen sectioning at low temperatures and finally by freeze-drying (Frey et al. 2000; Schneider et al. 2002; Vogel-Mikuš et al. 2008b).

Even though cryo-sectioning is accepted as a routine preparation technique for animal tissues, the number of studies that have used cryo-sections of fully differentiated plant material for analytical purposes remains small. The primary reasons for this are technical problems during freezing, due to the poor thermal conductivity of plant tissues arising from the cellulose cell wall, a unique feature of plant cells, and to the intercellular spaces filled with gas. Additionally, the alternating sequences of different materials, e.g., the rigid cell walls, vacuolar ice crystals and gas-filled intercellular spaces of the plant tissues, tend to result in sample crumbling during either sectioning or freeze-drying, which makes it particularly difficult to obtain cryo-sections with well-preserved morphology (Schneider et al. 2002).

The details of the more established cryo-preparation protocols for electron microscopy cannot be easily adapted here. The sections required for micro-PIXE analysis have to be much thicker (e.g., 60 μm) to obtain sufficient X-ray yield (Vogel-Mikuš et al. 2008b), which in the case of micro-PIXE is acquired from the entire depth of the specimen. Cryo-fixation must also be as rapid as possible to avoid the formation of larger ice crystals, which could damage plant and fungal cells. In addition, nuclear microprobe analyses are performed under vacuum conditions ($10^{-5}$ mbar or lower), and therefore the specimens must be dry and immobilized in their preanalysis functional state (Vogel-Mikuš et al. 2007, 2008a, b).

#### 14.2.3.1 Plant Material

Care should be taken regarding the physiological state of the material harvested for micro-PIXE analysis, and therefore the use only of freshly harvested roots is recommended, to avoid artificial element redistribution resulting from wilting and senescence. The roots should be cleansed with tap and distilled water to remove all of the soil particles. The secondary roots hosting mycorrhizal fungi should be excised with stainless steel scissors to avoid sample contamination with metals.

#### 14.2.3.2 Specimen Freezing

The freezing of the specimens should be done as rapidly as possible to reduce the growth of ice crystals. Excised root fragments (cca. 0.5 cm long) are inserted into stainless steel needles with polished tips (Schneider et al. 2002; Scheloske et al. 2004; Vogel-Mikuš et al. 2008b), which are carefully chosen according to the root diameter, to ensure a tight hold of the root during sectioning (Fig. 14.1). Alternatively, the excised root fragments can be transferred into aluminium foil beds (0.5 × 0.5 × 0.5 cm) that are filled with tissue-freezing medium. Afterwards the specimens are dipped into propane cooled with liquid nitrogen, which provides a superior cryogen when compared to liquid nitrogen, due to its higher cooling rate. A specially designed thermo-block is used for this (Fig. 14.1), which should be

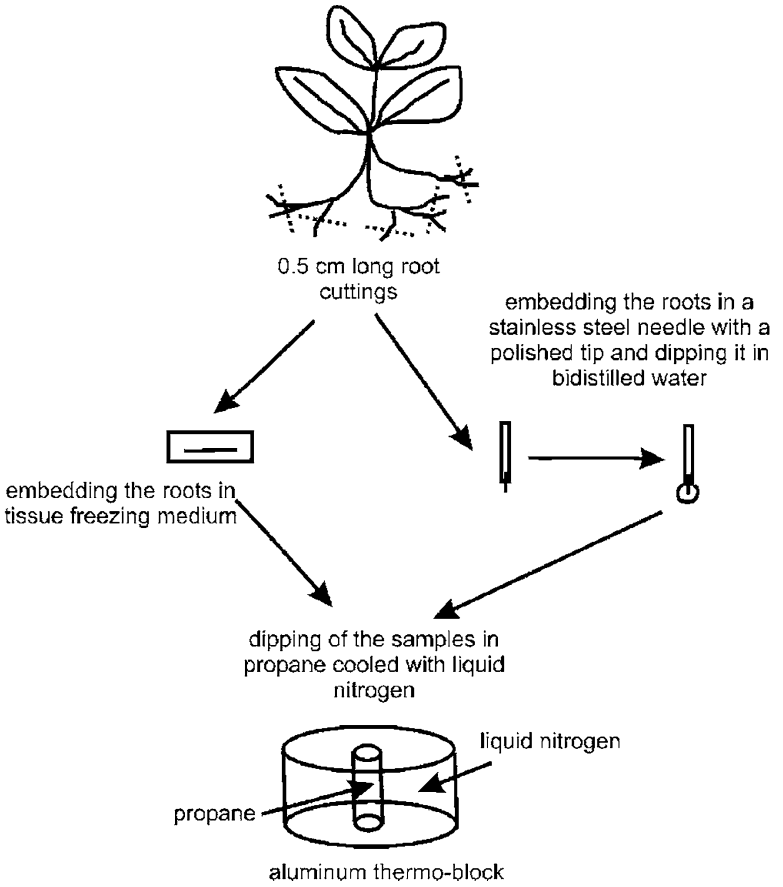



**Fig. 14.1** Schematic representation of root sample preparation and rapid freezing

additionally isolated with a polystyrene bed to avoid rapid evaporation of the liquid nitrogen. Great care needs to be taken when handling propane and liquid nitrogen: a mixture of propane and air can be explosive, and handling liquid nitrogen in a closed room can cause asphyxiation. Make sure that this is carried out in a well-ventilated room (door and windows always open) so that the concentrations of both gases in the air are kept to a minimum.

After freezing, the specimens in the propane (middle thermo-block chamber) are rapidly transferred to a liquid nitrogen (bigger thermo-block chamber) (Fig. 14.1). Then all of the prepared specimens together with the thermo-block are transferred directly into the cryo-microtome chamber to avoid the thawing of the samples.

In general, root-specimen preparation using polished needles (Schneider 2002; Scheloske et al. 2004; Vogel-Mikuš et al. 2008b) enables more rapid freezing, due to the direct contact of the specimen with the cryogen, and thus the ice crystals formed are smaller, providing superior preservation of cell morphology.

However, for scans of the specimens with a lateral resolution of 1–3 μm, which enables element mapping at the tissue level, the embedding of the specimens in tissue-freezing medium appears to be just as adequate. In addition, embedding the roots in the medium ensures that the holding of the specimen is more stable, making the cryo-sectioning easier (Fig. 14.2). Moreover, this way of specimen mounting can also be applied to longitudinal sections. On the other hand, using cryo-sectioning with needles is more demanding, because of the loose root support within the needle which can easily break during sectioning. A substantial improvement to this protocol can be achieved by dipping the inserted root into bidistilled water in a vertical position, and afterwards dipping it into a cryogen embedded with a water droplet, which then freezes. In this way, amorphous ice is formed from the droplet, which embeds the root and provides support for cryo-sectioning. In addition, a specially designed adapter is needed to fix the needle with a specimen into the head of the cryo-microtome (Schneider et al. 2002).

#### 14.2.3.3 Cryo-Sectioning

When cryo-sectioning, it is of vital importance that the cryo-microtome temperature set-up, the cutting velocity, and the section thickness are optimized for each particular plant species and tissue in question (Schneider et al. 2002). Only after optimization of these parameters can promising results be expected. The temperature of the cryo-microtome head and chamber usually varies between −40°C to −20°C, depending on the tissue water content. As a general rule, smoother sectioning of tissues with high water contents can be obtained at lower temperatures. When ideally frozen, plant tissues resemble amorphous glass, and sections obtained from

**Fig. 14.2** Cryo-microtome chamber with a sample embedded in tissue-freezing media

such specimens ensure the best possible level of preservation of tissue morphology, and thus the most reliable results. In addition, to control the quality of the sections, a dissecting binocular should be provided with the cryo-microtome. The specimens should be sectioned using disposable stainless steel cryo-microtome blades, which are superior to the standard steel or diamond knives (Schneider et al. 2002). The sections of the specimens are then put on pre-cooled filter paper into specially designed pre-cooled aluminium beakers with a cover, and kept in liquid nitrogen for freeze-drying.

#### 14.2.3.4 Freeze-Drying

Freeze-drying is a very sensitive step in the whole procedure, since the samples can wilt and shrink drastically due to the large amounts of water in plant tissues. The samples should therefore be freeze-dried at the lowest temperature possible (they are best put in the freeze-dryer in liquid nitrogen) and at low pressure ($10^{-5}$ bar). To ensure the flatness of the sections, they should be laid between two layers of precooled filter paper and fixed with a pre-cooled heavy object (e.g., a part of microscope object glass).

#### 14.2.3.5 Mounting of the Samples into Holders

The freeze-dried samples are mounted into aluminium holders that are covered with a thin foil (cca. 300 nm thick) of Pioloform (SPI Chem) (Fig. 14.3). The foil is prepared by dissolving 1 g of Pioloform in 75 ml chloroform (Vogel-Mikuš et al. 2007), which can be kept in a dark flask for cca. 6 months, with the dissolved solution then poured into a beaker. The easiest way of making the foils is to dip a clean microscope object glass into the foil solution for 2 s and then let it dry for 3 min. Then the edges of the foil are scraped with another clean object glass, to enable the detachment of the foil from the object-glass. The foil is then floated from the object glass by dipping it into bidistilled water and picking out the foil on a specially designed aluminium section holder, as schematically represented in Fig. 14.3. The sections should be carefully put on a holder with forceps and covered with another holder covered with Pioloform foil, to fix the section into a sandwich (Vogel-Mikuš et al. 2007, 2008b). The sections can also be mounted on foil with special two-component glue (e.g., Araldite), being sure that the areas of interest for scanning remain clean.

### *14.2.4 Micro-PIXE Analysis*

Micro-PIXE permits quantitative studies of element distributions, with lateral resolution of the order of 1 μm for elements from Na to U. Relatively few nuclear microprobe set-ups for the analysis of biological material exist worldwide because

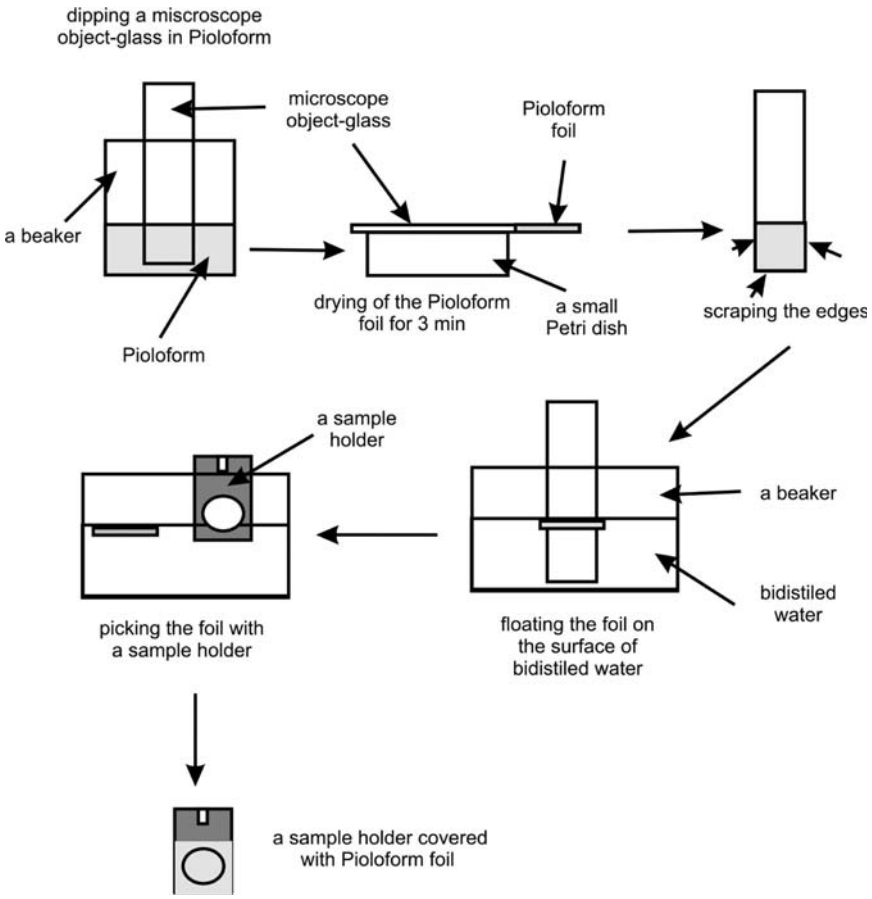



**Fig. 14.3** Schematic representation of the preparation of Pioloform foil for covering the sample holders

of financial constraints, and they vary in their technical characteristics. The most common configuration consists of an ion accelerator, object and collimating slits, ion lens and measuring station. Fig. 14.4 shows a schematic set-up of the nuclear microprobe at the Jožef Stefan Institute (JSI) (Simčič et al. 2002; Pelicon et al. 2005).

For the analysis of thin biological samples at the nuclear microprobe of the JSI, high- and low-current modes are applied sequentially to the same sample region of interest. In the high-current mode used for micro-PIXE analysis, a proton beam with an energy of 3 MeV with a diameter varying from 1 to 3 μm at ion currents ranging from 60 to 500 pA is formed, depending on the required lateral resolution. In the low-energy mode, the object slits are closed to reduce the beam flux to cca. 500 protons per second. The passivated implanted planar silicon detector is positioned directly in the beam, to obtain the best contrast in scanning transmission ion

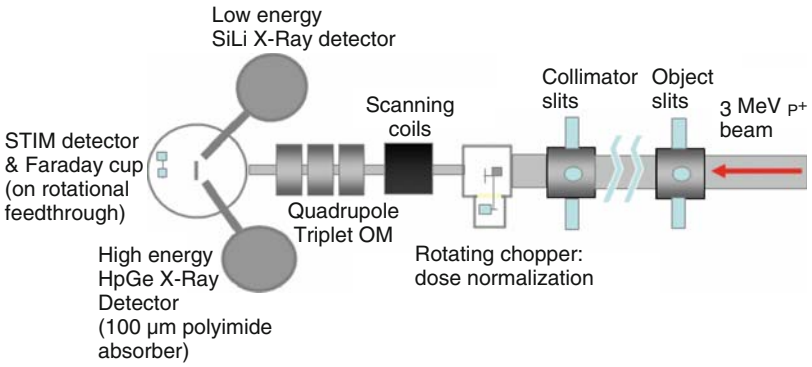



**Fig. 14.4** Schematic diagram of the nuclear microprobe at JSI

microscopy (STIM), which is used for the determination of specimen thickness (Vogel-Mikuš et al. 2008b).

The detection of X-ray energies from 1 keV up to 25 keV is provided by a pair of X-ray detectors. These include a high-purity germanium X-ray detector with an active area of 95 $mm^2$, a 25-µm-thick beryllium window and a 100-µm-thick polyimide absorber positioned at an angle of 135° with respect to the beam direction. Simultaneously, a Si(Li) detector with an area of 10 $mm^2$ and an 8-µm-thick Be window is installed at the angle of 125° with respect to the beam direction, for the detection of low-energy X-rays, in the energy range from 0.8 to 4 keV. At the JSI, the samples are sprayed with low-energy electrons from a hot tungsten filament during the measurements, to avoid sample charging, thus efficiently avoiding time-consuming specimen carbon coating.

Precise proton-dose determination is required for quantitative micro-PIXE analysis. For this reason, an in-beam chopping device is positioned in the beam line after the last collimation of the beam before it hits the sample. The rotating chopper is of gold-plated graphite, and periodically intersects the beam with a frequency of ca. 10 Hz, which makes the method insensitive to beam-intensity fluctuations. The spectrum of back-scattered protons from the chopper is recorded in parallel with the PIXE spectra in the list mode. The high-energy part of the spectrum consists of protons scattered from the Au layer, and it appears as a separate peak, the area of which is proportional to the proton flux. During the off-line data processing, the proton dose corresponding to an arbitrary scanning area selection can be extracted from the list-mode results simultaneously with the PIXE spectra (Vogel-Mikuš et al. 2007, 2008b).

The regions of interest on the samples are preselected by short PIXE mapping in high-current mode. After the final sample positioning and scan size selection, the object slits are closed and the STIM maps are measured in the list mode. A partially depleted planar silicon detector is positioned directly in the beam to obtain the best contrast for the STIM. This is followed by high-current mode, during which the PIXE maps of the same region are measured in list mode over a longer period of

time, lasting from 1 up to 30 h, depending on the concentration and atomic numbers of the elements under consideration. As a third and last step, the STIM map is again measured over the same sample area for a control of sample consistency, thinning, and possible shrinking.

In combination with the assumed cellulose matrix composition, proton exit energy measured by STIM is used for determination of sample thickness. The stopping power of 3 MeV protons in cellulose is 114 keV (mg $cm^{-2}$)$^{-1}$. Frozen hydrated plant tissue is usually sectioned on the cryo-microtome to a thickness of 60 μm. The pass of the proton beam through a freeze-dried tissue sample results in an energy loss of ca. 120–150 keV. This energy loss corresponds to a cellulose area density of 1.05–1.31 mg $cm^{-2}$. Assuming a cellulose bulk density of 1.6 g $cm^{-3}$, this gives 6.5–8.1 μm of equivalent bulk cellulose thickness. The heterogeneous morphology of the sample results in an uneven area density after freeze-drying. To quantify the element concentrations of a particular morphological structure correctly, its thickness is extracted in the form of the exit energy from the STIM data, and it is fed into the trace calculation of the GUPIXWIN code, which is dedicated to the fitting and quantification of PIXE spectra (Campbell et al. 2000).

The calibration of the PIXE method is usually verified by analysis of the multi-element standard reference materials NIST SRM 1573a (tomato leaves, homogenized powder, analyzed in a form of a pressed pellet), NIST SRM 1107 (naval brass B, alloy), and NIST SRM 620 (soda-lime flat glass) (Nečemer et al. 2008). The inter-calibration of PIXE and STIM is usually verified by thin mono-element metallic foils (Vogel-Mikuš et al. 2008b).

An example of a detailed study of the element distribution in mycorrhizal and nonmycorrhizal *Aster tripolium* can be found in Scheloske et al. (2004). In addition, micro-PIXE mapping of the element distribution in arbuscular mycorrhizal roots of the grass *Cynodon dactylon* is presented in Weiersbye et al. (1999).

## 14.3 Example of Micro-PIXE Analysis

For the purpose of this manuscript, a selected sample application in studies of plant/fungal interactions in a metal-polluted environment is presented. Only a small amount of plant material (few leaves or roots) is needed for the micro-PIXE analysis, which is highly appreciated in plant physiology studies and a prerequisite for the following study with the endangered zinc violets. The data were obtained using a nuclear microprobe at the Microanalytical Centre of the JSI, Ljubljana, Slovenia.

### *14.3.1 Sample Preparation and Micro-PIXE Analysis*

Zinc violets (*Viola lutea* ssp. *westfalica*; for the nomenclature see Hildebrandt et al. (2006)) were collected at a nonpolluted site (garden, D-50374 Erftstadt-Bliesheim, Germany) and at a Zn- and Pb-polluted site (D-33165 Blankenrode, Germany), and

transferred to the laboratory. The collected plants were colonized by arbuscular mycorrhizal fungi with a frequency of $F(\%) = 31\%$ for the garden material and $F(\%) = 42\%$ for the plants from Blankenrode, as determined by the method of Trouvelot et al. (1986) after staining with Trypan Blue (Philips and Haymann 1970).

The root samples for micro-PIXE analysis were prepared using cryo-fixation in tissue-freezing medium, cryo-sectioning and freeze-drying, all as described above. Element maps were generated using a 3 MeV proton micro-beam (Fig. 14.5). Afterwards, the areas of interest (epidermis, cortex, endodermis, and vascular tissues) were encircled and their corresponding spectra extracted (Tables 14.1 and 14.2; Fig. 14.6). The element concentrations were calculated on the basis of

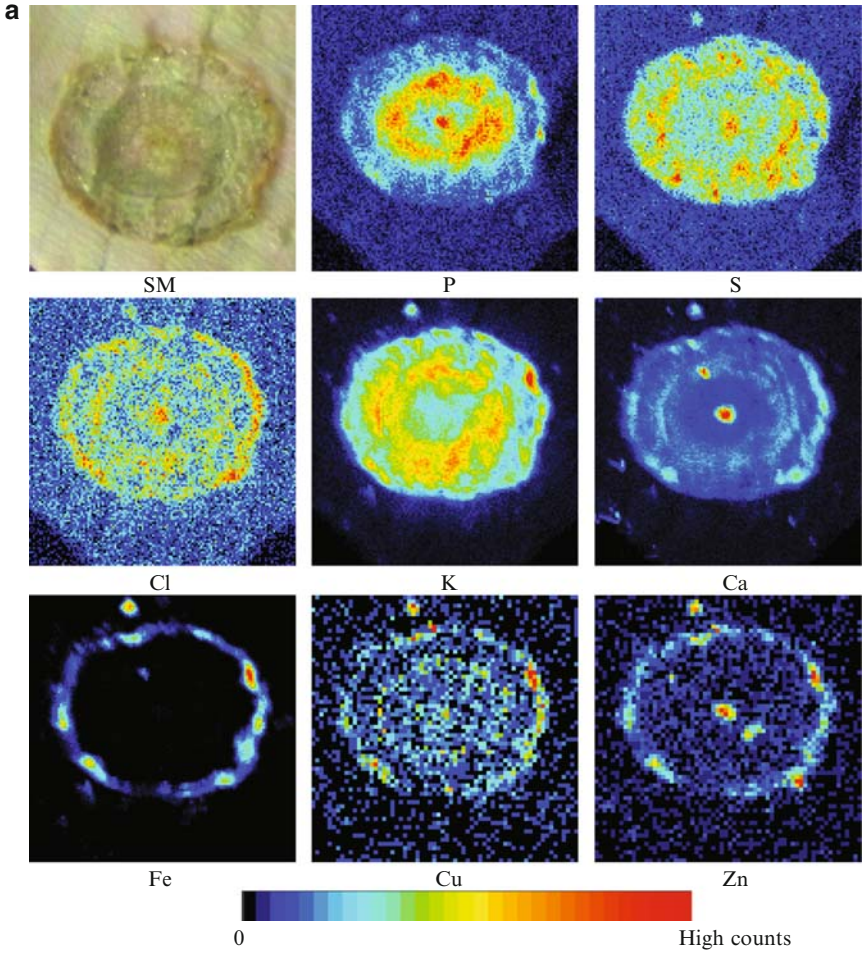



**Fig. 14.5a** (continued)

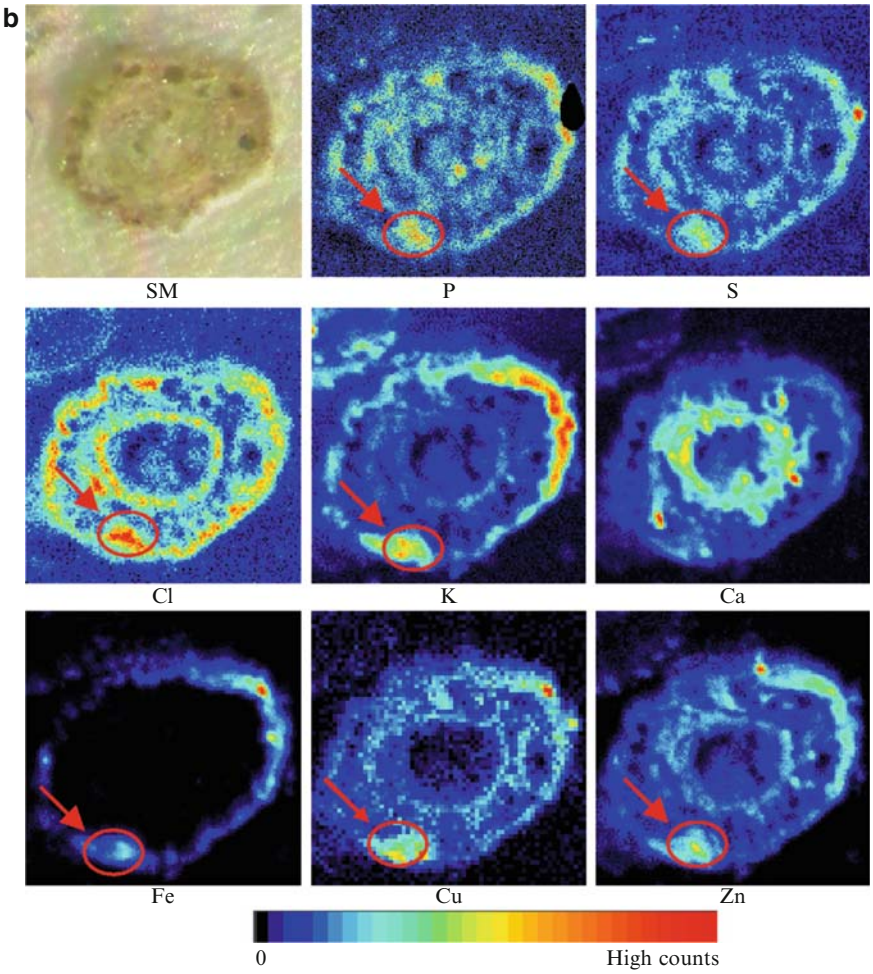
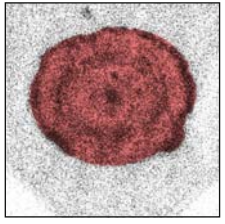
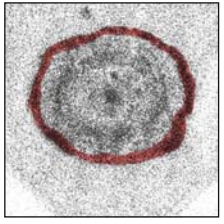
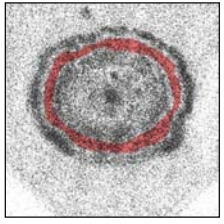
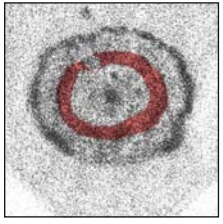
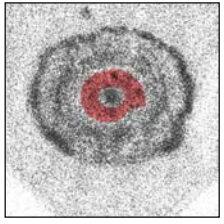



**Fig. 14.5** (**a**) Element maps of *Viola lutea* ssp. *westfalica* root samples collected at the nonpolluted site. Scan size 250 × 250 μm$^2$. *SM* – photograph of the sample taken under an optical stereo-microscope. (**b**) Element maps of *Viola lutea* ssp. *westfalica* root samples collected at the metal-polluted site. Scan size 250 × 250 μm$^2$. *SM* – photograph of the sample taken under an optical stereo-microscope. An arbuscular mycorrhizal structure is indicated by an *arrow*

characteristic X-ray counts (PIXE spectra), proton dose, sample matrix density (cellulose), and thickness of the sample as determined by STIM.

The determination of concentrations in selected plant tissues allows one to compare the element distributions in differently treated plants, and/or to assess shifts in element depositions caused by mycorrhization, in a similar way to that described by Scheloske et al. (2004).

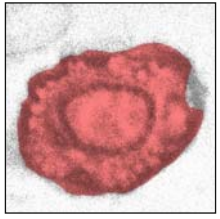
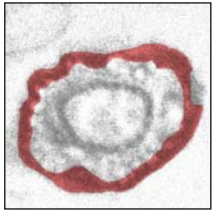
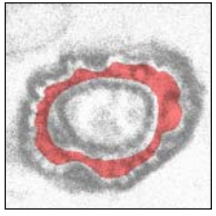
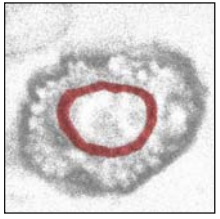
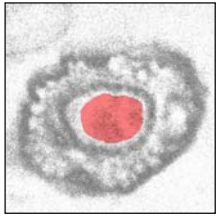
**Table 14.1** Element concentrations with measurement errors (Stat.err.) and limits of detection (lod) of selected regions within *Viola lutea* ssp. *westfalica* roots from the nonpolluted site. Scan size 250 × 250 μm$^2$

| | Whole area | | | Epidermis | | | Cortex | | | Endodermis | | | Vascular tissues | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Elements | Conc (μg.g$^{-1}$) | Stat.err. (%) | lod (μg.g$^{-1}$) | Conc (μg.g$^{-1}$) | Stat.err. (%) | lod (μg.g$^{-1}$) | Conc (μg.g$^{-1}$) | Stat.err. (%) | lod (μg.g$^{-1}$) | Conc (μg.g$^{-1}$) | Stat.err. (%) | lod (μg.g$^{-1}$) | Conc (μg.g$^{-1}$) | Stat.err. (%) | lod (μg.g$^{-1}$) |
| **P** | **6125** | 0.57 | 35.5 | **1709** | 4.85 | 84.0 | **3197** | 1.30 | 61.3 | **9909** | 0.58 | 71.3 | **9031** | 0.76 | 85.8 |
| **S** | **4013** | 0.49 | 23.7 | **2857** | 1.17 | 50.0 | **4726** | 0.82 | 45.5 | **4668** | 0.93 | 52.3 | **3093** | 1.48 | 58.5 |
| **Cl** | **2274** | 0.73 | 22.0 | **2903** | 1.15 | 46.7 | **1842** | 1.69 | 42.6 | **2547** | 1.34 | 46.0 | **1429** | 2.47 | 52.8 |
| **K** | **26458** | 0.15 | 22.0 | **21443** | 0.34 | 56.1 | **24686** | 0.34 | 50.8 | **33751** | 0.28 | 44.5 | **21786** | 0.44 | 49.2 |
| **Ca** | **5293** | 1.05 | 98.4 | **6444** | 1.18 | 120.7 | **4031** | 1.93 | 136.9 | **5638** | 1.67 | 166.3 | **1943** | 3.84 | 138.9 |
| **Mn** | **170.2** | 1.07 | 2.5 | **596.5** | 1.07 | 7.8 | **13.8** | 14.75 | 3.6 | **37** | 6.31 | 3.8 | **14.3** | 18.46 | 4.7 |
| **Fe** | **3066** | 0.19 | 2.3 | **12111** | 0.19 | 8.4 | **106.3** | 2.54 | 2.3 | **85.5** | 3.25 | 3.9 | **43.5** | 6.05 | 3.6 |
| **Cu** | **35.3** | 3.18 | 1.6 | **38.1** | 7.69 | 4.7 | **18.8** | 10.33 | 2.4 | **54.6** | 4.45 | 2.0 | **39.8** | 6.65 | 1.7 |
| **Zn** | **65.5** | 2.21 | 1.5 | **139.3** | 3.08 | 4.2 | **35.7** | 6.34 | 2.3 | **29** | 7.67 | 1.9 | **26.9** | 10.50 | 2.4 |

The X-ray spectra corresponding to particular root morphological structures (whole root area, epidermis, cortex, endodermis, and vascular tissues) were extracted from the encircled regions and analysed using GUPIX software

**Table 14.2** Element concentrations with measurement errors (Stat.err.) and limits of detection (lod) of selected regions within *Viola lutea* ssp. *westfalica* roots from the metal-polluted site. Scan size 250 × 250 μm$^2$

| | Whole area | | | Epidermis | | | Cortex | | | Endodermis | | | Vascular tissues | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Elements | Conc (μg.g$^{-1}$) | Stat.err. (%) | lod (μg.g$^{-1}$) | Conc (μg.g$^{-1}$) | Stat.err. (%) | lod (μg.g$^{-1}$) | Conc (μg.g$^{-1}$) | Stat.err. (%) | lod (μg.g$^{-1}$) | Conc (μg.g$^{-1}$) | Stat.err. (%) | lod (μg.g$^{-1}$) | Conc (μg.g$^{-1}$) | Stat.err. (%) | lod (μg.g$^{-1}$) |
| **P** | **1453** | 1.72 | 24.2 | **1494** | 4.53 | 52.1 | **1275** | 1.64 | 35.4 | **1583** | 2.36 | 68.6 | **1826** | 1.67 | 48.3 |
| **S** | **1255** | 1.23 | 30.2 | **1582** | 1.58 | 47.9 | **1023** | 2.27 | 45.8 | **1316** | 2.90 | 75.2 | **1073** | 2.26 | 40.9 |
| **Cl** | **4342** | 0.30 | 14.3 | **6248** | 0.39 | 27.6 | **2991** | 0.68 | 23.7 | **6124** | 0.64 | 41.4 | **1606** | 1.40 | 29.3 |
| **K** | **4632** | 0.27 | 13.4 | **9224** | 0.31 | 26.4 | **2492** | 0.77 | 22.2 | **3803** | 0.95 | 44.3 | **1664** | 1.50 | 32.5 |
| **Ca** | **8828** | 0.25 | 23.9 | **4601** | 0.75 | 51.3 | **8381** | 0.41 | 26.8 | **18748** | 0.39 | 48.6 | **8116** | 0.59 | 31.9 |
| **Mn** | **18.8** | 4.78 | 1.7 | **41.2** | 5.48 | 4.1 | **10** | 10.94 | 2.0 | **10.9** | 17.23 | 3.4 | **0.0** | / | 2.6 |
| **Fe** | **2341** | 0.14 | 1.2 | **7594** | 0.13 | 2.3 | **137.5** | 1.28 | 1.6 | **64.8** | 3.43 | 2.9 | **35** | 4.58 | 2.1 |
| **Cu** | **18.8** | 7.36 | 2.4 | **34.1** | 7.16 | 4.2 | **11.2** | 17.18 | 3.4 | **17.1** | 22.27 | 7.0 | **8.6** | 23.35 | 3.7 |
| **Zn** | **8801** | 0.10 | 2.3 | **11846** | 0.15 | 4.2 | **6970** | 0.22 | 1.7 | **12031** | 0.25 | 7.0 | **3913** | 0.44 | 5.6 |
| **Pb** | **1192** | 0.64 | 1.9 | **1642** | 0.96 | 8.8 | **1231** | 1.26 | 5.1 | **1234** | 1.91 | 15.5 | **214.4** | 5.00 | 8.8 |

The X-ray spectra corresponding to particular root morphological structures (whole root area, epidermis, cortex, endodermis, and vascular tissues) were extracted from the encircled regions and analysed using GUPIX software

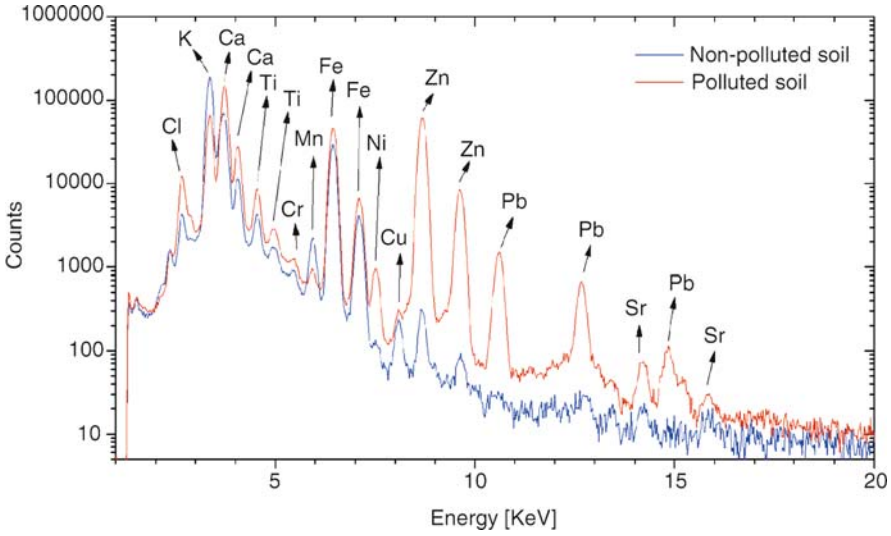



**Fig. 14.6** Micro-PIXE spectra of the whole root area (Table 14.1), from the nonpolluted and Zn- and Pb-polluted sites

### *14.3.2 Results*

Significantly higher Zn and Pb concentrations were seen in *Viola lutea* ssp. *westfalica* roots collected at the polluted site (Tables 14.1 and 14.2), presumably as a consequence of soil contamination with these elements. The concentrations of P, S, K, Mn, Fe, and Cu were, on the other hand, higher in the violet roots from the nonpolluted garden soil (Tables 14.1 and 14.2). The root element concentrations correlate well with the element concentrations of the soil (Marschner 1995). Higher amounts of nutrients are usually found in gardens, which might mainly come from sediments of the small river Erft in the proximity, as well as from fertilization in this particular allotment. Metal-polluted sites are characterized by enhanced metal concentrations and, remarkably, also by low concentrations of mineral nutrients (Ernst 2006).

Comparison of the element distribution between the root samples from nonpolluted and polluted sites using the element distribution maps (Fig. 14.5) and the element concentrations of particular root regions (Tables 14.1 and 14.2) showed that some elements, like Fe, Zn, Mn, Cu, and Pb, accumulated typically in root epidermis. Enhanced Zn and Pb concentrations were also seen in the cortex and endodermis of the roots of the plants from the metal-polluted site. Only small amounts of metals were, however, detected in the vascular tissue beyond the endodermis, as already recognized in other studies (Seregin and Ivanov 2001), indicating metal exclusion as a tolerance strategy (Baker 1981) also for *Viola lutea* ssp. *westfalica*. The highest concentrations of Cl for both samples were in the epidermis and endodermis, and of P in endodermis and vascular tissues (Tables 14.1 and 14.2).

However, the distribution of K was different, with the highest concentration seen in the cortex and endodermis in the roots of violets collected in the nonpolluted garden, and in the epidermis in the roots collected at the metal-polluted site (Tables 14.1 and 14.2). Ca was concentrated in the endodermis in the roots from the polluted site, while in the roots from the nonpolluted soil the Ca concentration gradually decreased from epidermis to central cylinder (Table 14.1).

PIXE analysis does not allow one to detect arbuscular mycorrhizal fungal structures in the root cells directly. However, in the root sample collected at the polluted site, a P-, S-, Cl-, K-, Fe-, Zn-, and Pb-rich structure was seen in the root cortex (Fig. 14.5b), which could be attributed to an arbuscule of AM fungi. In the study by Weiersbye et al. (1999) on element maps generated by micro-PIXE, arbuscules were seen as localized accumulations of P and Fe in and around cortical cells. These might be linked to the highly efficient P and Fe sequestration mechanisms characteristic of AM fungi (Smith and Read 1997), while vesicles usually accumulate higher Mn, Cu, and Ni than cortical cells (Weiersbye et al. 1999). In addition, the accumulation of Zn and Pb in AM fungal structures is consistent with the high metal-binding capacity of AM mycelium (Joner et al. 2000). In line with this, arbuscular mycorrhizal fungal structures only occur in the outer tissues, where they might bind heavy metals, whereas they cannot surpass the tight Casparian strip that is deposited on the walls of the endodermal cells.

## 14.4 Conclusions

Micro-PIXE is a powerful tool for qualitative and quantitative investigations of element distributions in plant and fungal tissues, and it also provides especially attractive element-mapping capabilities. To date, the appropriate morphology preservation of specimens during sample preparation appears to be one of the main obstacles to obtaining high-resolution qualitative and quantitative element maps primarily on the cellular, tissue and organ levels. The element localization maps obtained by this method can contribute significantly to our current knowledge of plant physiology, stress responses, and the complexity of plant–microbe interactions, as well as having broad applications in biotechnology. However, a prerequisite for success in this broad-spectrum interdisciplinary research field is close collaboration between physics, chemistry, biochemistry, biotechnology, and plant sciences researchers.

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# Chapter 15
# Functional Genomic of Arbuscular Mycorrhizal Symbiosis: Why and How Using Proteomics

**Eliane Dumas-Gaudot, Ghislaine Recorbet, Franck Robert, Benoit Valot, and Nardjis Amiour**

## Abbreviations

| | |
|---|---|
| *AB* | Amersham biosciences |
| *APS* | Ammonium persulfate |
| *AM* | Arbuscular mycorrhiza |
| *CHAPS* | 3-[(3-cholamidopropyl) dimethyl-ammonio]-1-propanesulfonate |
| *DEPC* | diethylpyrocarbonate |
| *DTT* | 1,4-dithio-DL-threitol |
| *2-DE* | Two-dimensional electrophoresis |
| *ESI* | Electrospray ionization |
| *ESTs* | Expressed sequence tags |
| *IEF* | Isoelectrofocusing |
| *IPGs* | Strips of immobilized pH gradients |
| *MS* | Mass spectrometry |
| *MS-MS* | Tandem mass spectrometry |
| *MALDI-TOF* | Matrix assisted laser desorption/ionization-time of flight |
| *NCBI* | National Center for Biotechnology Information |
| *PDA* | Piperazine diacrylamide |
| *PMSF* | Phenylmethanesulfonyl fluoride |
| *SDS* | Sodium dodecyl sulfate |
| *sqf* | Sufficient quantity for |

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DOI: 10.1007/978-3-540-95894-9_15, 

| | |
|---|---|
| *TEMED* | N,N,N′,N′-Tetramethylethylenediamine |
| *Tris* | Tris-[hydroxymethyl] aminomethane |
| *US DOE* | US Department of Energy |

## 15.1 Introduction

The achievement of genome sequencing programs launched for numerous organisms, including those of several plant, bacteria and fungal species (http://www.ncbi.nih.gov/, http://www.energy.gov/), together with the increase in available ESTs corresponding to various biological situations of plants, bacterial and fungal models, have opened the way for functional genomic analyses. Among them, proteomics looks very promising, as it gives a direct access to the gene effectors, i.e., proteins.

Proteomics, according to its first definition by Wilkins et al. (1996), designs the new strategies aimed at researching global protein expression in different organisms. Thanks to the great improvements in protein separation methods, development of mass spectrometry techniques and advances in bioinformatic tools, it has become more and more popular during the last decade. Proteomics relies to a major extent on experimental analyses to identify and elucidate proteins. One of the major experimental methods used in protein identification is two-dimensional gel electrophoresis (2-DE) coupled with mass spectrometry (MS). 2-DE/MS systems have the ability to identify a large number of proteins from a single sample. Studies have shown that 2-DE/MS systems could identify somewhere in the region of thousands of proteins per sample (Harry et al. 2000; Görg et al. 2000). Their possible use for revealing protein modifications in response to root colonisation by arbuscular mycorrhizal (AM) fungi has already been reported several times (Bestel-Corre et al. 2004a; Canovas et al. 2004; Jorrin et al. 2006). Surprisingly, to date, for the other well-known symbiosis, i.e., the ectomycorrhizal interaction, use appears much more restricted (listed in Canovas et al. 2004). This situation could change very quickly, due to the fact that genomes of *Populus trichocarpa,* the first perennial plant to be tackled, and its microcosm-associated partners are being fully sequenced (Martin et al. 2004). While the general protocols for successfully performing plant and/or microbe proteomics have been largely described (Chen et al. 2005; Giavalisco et al. 2003; Grinyer et al. 2004; Hajheidari et al. 2005; Rose et al. 2004; Saravanan and Rose 2004), the aim of this chapter will be to address the specific points that have to be considered when applying a proteomic approach to the study of mycorrhizal symbioses.

AM fungi colonize the vast majority of plant species, yet unlike the ectomycorrhizal fungi, are incapable of growth without a symbiotic host. Therefore, a first challenge will deal with the fact that the fungal partner is an uncultivable microorganism. The second relates to the lack of genomic sequences in database, a hitch that will soon be bypassed (Lammers et al. 2004). The general strategy we are using routinely in our laboratory is presented on Fig. 15.1.

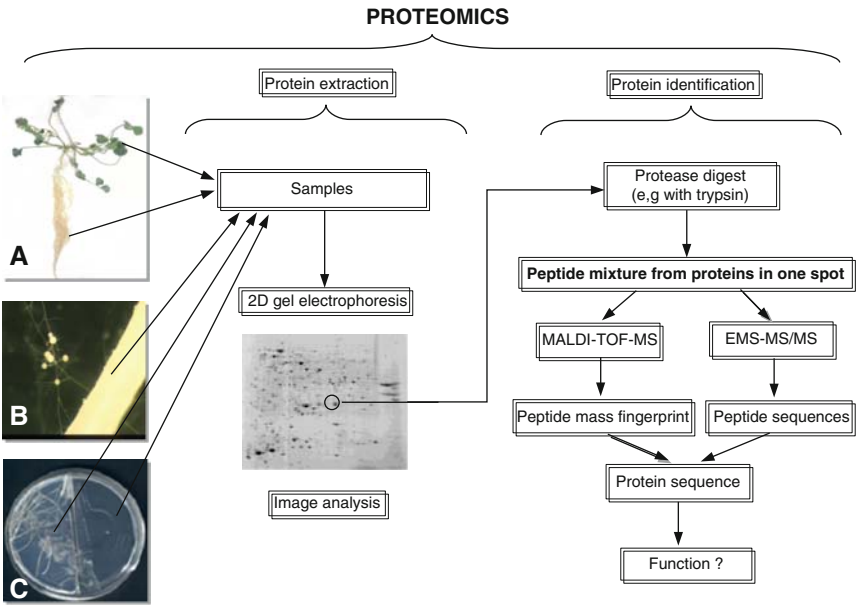


**Fig. 15.1** Typical flow-chart for the analysis of proteomes by mass spectrometry

## 15.2 General Considerations About the Biological Material

One should keep in mind that proteomics would make it possible to reveal most of the protein modifications in response to any environmental change. Therefore, because this approach may be tedious and in some way expensive, it is strongly recommended to apply a proteomic approach only to well-defined and characterized biological samples. In our case, it has only been applied to plants grown under specific conditions in growth cabinets (Bestel-Corre et al. 2002, 2004a, b; Dumas-Gaudot et al. 2004a; Repetto et al. 2003; Amiour et al. 2006) as well as on monoxenic mycorrhizal cultures of transformed roots (Dumas-Gaudot et al. 2004b).

### *15.2.1 Root Handling for Protein Extraction Protocols of*

#### 15.2.1.1 Soil-Grown Roots Inoculated or not with an AM Fungus

When plants have been grown in any soil-containing substrate, at sampling date, roots are carefully removed from the soil mix by immersing pots in tap water. Then roots are gently rinsed to eliminate any remaining soil particles, first with running tap water, and then with deionised water. At this step, part of the root system may be checked for mycorrhizal infections with an appropriate test. The remaining root

system is weighted, and either immediately frozen in liquid nitrogen and conserved at −80°C until protein extraction or directly submitted to the selected protein extraction process.

#### 15.2.1.2 Monoxenic Root-Inducing Transferred-DNA (Ri T-DNA) Transformed Roots Inoculated or not with an AM Fungus

*Glomus intraradices*, as well as some other AM fungi (http://emma.agro.ucl.ac.be/ginco-bel/), can be grown *in vitro* in dual culture with transformed roots. Of particular importance is the development of a split-plate system in which a separate fungal compartment allows a large range of manipulations and observations to be made on the extraradical mycelium in the absence of other organisms (St-Arnaud et al. 1996). The growth of mycorrhizal roots is restricted to one compartment (proximal) containing modified minimal (M) medium in such a way that only the endosymbiont grows on the second compartment (distal) containing the same medium lacking sugar. At a selected time, control, AM-inoculated Ri-T-DNA transformed roots and extraradical fungal material (spores and hyphae) can be harvested for protein extraction as follows:

1. Select Petri dishes, for their compartment colonization status by the fungus, by assessment with a magnifying binocular (when hyphae cover over 50% or more of the distal compartment areas, they can be used for fungal protein extraction). Usually, five independent samples are prepared from the mix of colonized roots from *n* Petri dishes per sample ("*n*" will depend on the plant species). For non-colonized roots, the root material from more Petri dishes is generally required for obtaining an equivalent amount of root tissue dry weight.
2. Separate control, AM-inoculated transformed roots and AM extraradical material from the medium. The remaining phytagel can be solved by immersion of samples in sterile 10 mM sodium citrate buffer (pH 6.0, 4°C) according to Doner and Bécard (1991).
3. The AM extraradical material, after phytagel dissolution, can be collected on a 45 μM sieve, washed several times with sterilized deionized water, weighted and frozen in liquid nitrogen ($N_2$). All samples (roots and mycelium) can be either lyophilized/stored at −80°C until protein extraction, or directly used for protein extraction.

## 15.3 Materials and Apparatus

### *15.3.1 Products and Buffers for Protein Extraction*

Extraction buffer (see Table 15.1)
Tris pH 8 saturated phenols (Biosolve or any other chemical company)
Cold 0.1 M ammonium acetate in methanol (kept at −20°C)

**Table 15.1** Extraction buffer (100 ml) for total and soluble proteins

| | |
|---|---|
| Tris 0.5 M | 6.055 g |
| Milli-Q water | Adjust the pH to 7.5 and complete to 50 ml |
| Sucrose 0.7 M | 23.900 g |
| KCl 0.1 M | 0.750 g |
| Thiourea 10 mM | 0.076 g |
| EDTA 5 mM | 50 mM 10 ml |
| β-mercaptoethanol 2%[a] | 2 ml |
| PMSF 1 mM in DMSO | 0.017 g. DMSO $ml^{-1}$ |
| Milli Q water | sqf 100 ml |

[a] To be added just before using the solution

**Table 15.2** Solubilization buffer for soluble proteins[a]

| | |
|---|---|
| Urea 9 M | 5.4 g |
| CHAPS 4% (w/v) | 0.4 g |
| Triton X-100 0.5% (v/v) | 50 µl |
| DTT 100 mM | 0.154 g |
| IPG buffer 3–10 2% (w/v) | 200 µl |
| Milli Q water | sqf 10 ml |

[a]Can be stored at −20°C

Cold methanol and acetone
Solubilizing buffer (see Table 15.2) [modification of O'Farrell lysis buffer (O'Farrell, 1975)]
Liquid nitrogen $N_2$
Gas nitrogen

### *15.3.2 Products and Buffers for 2DE*

**Note 1**: All chemical for electrophoresis must be high quality grade IPG strips (ready-made IPG gels Immobiline) such as Immobiline DryStrips 3–10 NL, Immobiline DryStrips 4–7 (or any other restricted gradient)

IPG buffers in the same gradient range as IPGs
Mineral oil (Sigma)
Acrylamide
Agarose
Urea
SDS
Iodoacetamide
TEMED
Ammonium persulfate

Bromophenol blue
CHAPS
DTT
Glycine

## *15.3.3 Products for Protein Staining*

### 15.3.3.1 For Silver Staining

Ethanol
Acetic acid
Glutaraldehyde
Potassium tetrathionate
Sodium acetate trihydrate
Sodium thiosulphate
Formaldehyde
Potassium carbonate anhydrous
Silver nitrate
HEPES
Milli Q water (resistivity 18,000 Ohms)

### 15.3.3.2 For Coomassie Blue Staining

Methanol
Coomassie blue G-250
Ammonium sulfate
85% phosphoric acid

### 15.3.3.3 For Fluorescence Staining

Sypro orange, Sypro red, Sypro ruby, etc.

## *15.3.4 Equipment*

### 15.3.4.1 Isoelectrofocusing (IEF)

Can be performed with IPG strips on

- Multiphor II horizontal electrophoresis apparatus (Amersham Pharmacia), IPGphor (GE Healthcare, formerly Pharmacia Biotech/Amersham Company/ Amersham Biosciences) or IEF cell apparatus (Bio-Rad Laboratories)

- IPG strip holders
- Immobiline DryStrip Kit
- Reswelling cassette (125 × 260 mm2)
- Clamps
- Reswelling tray
- IEF sample applicator strip (AB)
- Parafilm (roll, 50 cm × 15 m)
- Milli-Q System (Millipore)
- EPS 3,500 XL power supply (3,500 V min)
- Multitemp II thermostatic circulator

#### 15.3.4.2 2D-Electrophoresis Apparatus

- They are available from several companies (GE Healthcare, Bio-Rad, etc.).
- Hoefer DALT, Ettan DALT, etc.

**Note 2**: Although several groups have produced good results when using small systems for 2-DE, we strongly recommend that larger gel separation be performed and that for preference analytical devices allowing multi-gel separations be chosen. This will greatly improve both the quality of the protein profiles together with the reproducibility among batches of experiments.

#### 15.3.4.3 Staining Apparatus

- Hoefer Processor Plus (GE)
- Dodeca Stainer (Bio-Rad),

**Note 3**: Although in theory plastic trays, rotary shaker and classical glass laboratory equipments will permit the staining of gels, we strongly recommend that the laboratory be equipped with systems allowing semi-automatic staining of gels such as the two listed above. We have very satisfying results with the Dodeca stainer, which has the great advantage of staining 12 gels at the same time with a unique batch of stain (moreover, some stains can be re-used several times).

#### 15.3.4.4 Scanning System and Image Analysis Software

Automated computer analysis systems are needed for a rigorous qualitative and quantitative analysis of complex 2-DE protein patterns:

- A flatbed scanning laser densitometer providing high resolution (down to 50 μm) combined with a high dynamic range (up to 4 OD)

**Note 4**: The current generation of desktop scanners can also achieve high resolution (600dpi is equivalent to 42 μm) and a high dynamic range (12bits), but care must be

taken to ensure the linearity of such devices. One can also prepare film images of radiolabeled 2-DE separations using such devices, but accurate quantitation is complicated both by the limited dynamic range and the non-linearity of film response. This problem can be overcome by using phosphorimaging screens together with a 'phosphorimaging' scanner. The major advantages of this approach compared to conventional autoradiography are: (1) relatively short exposure times required, (2) high dynamic range, and (3) good linearity of response. The only disadvantage is the high capital cost of the phosphorimaging screens and the dedicated imaging device.

- For fluorescently labeled proteins, a dedicated densitometer is required for the imaging of 2-DE profiles [a convenient and versatile device is the Odyssey apparatus (Li-Cor)].
- All of the current generation of commercial 2-DE analysis software systems (Melanie II, Bio-Rad Laboratories; BioImage, BioImage Systems; Phoretix, Phoretix International; Kepler, LSB, etc.) can be used on desktop workstations, such as Unix, PC or Mac.

**Note 5**: These systems mean that we can derive qualitative and quantitative information from individual 2-DE gels, to match the protein separation profiles from large numbers of 2-DE gels and construct comprehensive databases of quantitative protein expression for cells, tissues and whole organisms.

#### 15.3.4.5 Protein Identification Strategies

- The various strategies for protein identification are briefly summarized in Fig. 15.1. It is obvious that during the two last decades a major breakthrough in rapid protein identification has been achieved through the use of mass spectrometry (MS) together with bioinformatics. We are not going to consider either the different techniques of MS or the various apparatus. This is a domain in constant evolution, and readers are invited to consult specific reviews (Aebersold and Mann 2003; Rabilloud 2000a, b; Mann and Wilm 1995; Wilm et al. 1996; James 1997; Shevchenko et al. 1996; Patterson 1994; Patterson and Aebershold 1995).
- 2-D gel protein database: The final requirement for proteomic technology is that we must be able to store all the data that are generated in a database that we can interrogate effectively in the laboratory and also, when possible, make available to other scientists worldwide. Our best approach at present is to use the World Wide Web (WWW). In order to provide optimal interconnectivity between the 2-D gel protein database and other databases of related information available via the WWW, it has been suggested that 2-D gel databases may be constructed according to a set of fundamental rules. Databases conforming to these rules are

said to be 'federated 2-D databases', while many other databases are in agreement with at least some of the rules. A list of the 2-D protein databases can be viewed at *World2Dpage* (www.expasy.ch/ch2d/2d-index.html).

## 15.4 Procedure

Plant cells are rich in compounds that interfere with the 2-DE separation method, such as salts, organic acids, phenolics, pigments, terpenes, among others. A common protocol used in our lab for extracting total and soluble proteins from plant tissues consists in the homogenization of mortar-grounded material in $N_2$ with the extraction buffer (Table 15.1) and the quick addition of phenol-Tris buffered (Bestel-Corre et al. 2002). This protocol has several advantages over others (Rose et al. 2004; Saravanan and Rose 2004; Wang et al. 2003), including its good yield for protein extraction and high preservation of extracts from endogenous proteases. Because they have been the subject of specific publications (Valot et al. 2004, 2005, 2006), alternative protocols directed toward organelle and/or membrane proteins will not be described here.

### *15.4.1 Extraction of Total and Soluble Proteins*

1. Grind the roots in liquid nitrogen. Usually, 1 g (fresh weight) of *M. truncatula* roots is ground in a cold mortar with $N_2$, but the amount can be adjusted when required. When the root sample looks like a very thin powder, transfer in a second mortar to be homogenized with the extraction buffer (Table 15.1) (1/10 w/v of fresh root material).
2. Transfer the resulting mix to centrifuge tubes (take care to choose tubes resistant to organic solvents).
3. Add immediately an equal volume of Tris pH 8 saturated phenols (Biosolve). Mix thoroughly for 30 min in a shaker in a cold room.
4. Centrifuge for 30 min at 12,000 g (4°C), re-extract the phenolic phase with the same volume of the extraction buffer and centrifuge for 30 min at 12,000 g (4°C).
5. Precipitate the proteins overnight at −20°C with five volumes of 0.1 M ammonium acetate in methanol.
6. Next day, collect by swing centrifugation at 16,000 g for 30 min (4°C). Rinse the pellets first with methanol, and second with acetone at −20°C, and dry under nitrogen gas.
7. Dissolve the pellets 4 h at 20°C in O'Farrell lysis buffer (Table 15.2) and centrifuge for 30 min at 170,000 g (20°C). Supernatants may be either directly analyzed or stored at −80°C for further analysis.

**Note 6**: Steps 1 and 2 are realized in a cold room, while during steps 3 and 4, for safety reasons, the Tris-saturated phenol is added under a hood.

## *15.4.2 Combined Extraction of RNA and Proteins*

We have developed a protocol in which proteins can be analyzed from a same root sample as a mRNA population, in order to make simultaneous proteome and transcriptome profiling possible. This protocol was validated to analyze the early stages of AM symbiosis (Dumas-Gaudot et al. 2004a). It has the added advantage that it circumvents the problems inherent to a limited amount of biological material.

### 15.4.2.1 First Day

1. Take 1 g of roots from plantlets freshly harvested or stored at −80°C.
2. Crush the roots in a cold mortar with liquid $N_2$ into a very thin white powder.
3. Pour this material in a $N_2$-cooled tube, wash the mortar with a little liquid $N_2$ to collect all the root material, and leave the liquid $N_2$ to evaporate without letting the powder dry.
4. Add 2 ml of NTES buffer (Table 15.3)+ 2µl β-mercaptoethanol per g of roots.
5. Shake with a vortex until a very viscous liquid is obtained.
6. Add 1 volume of phenol pH 4–5/chloroform/isoamylic alcohol (25/24/1), and shake again with a vortex until a whitish liquid is obtained. Sample four times 1,000 µl in Eppendorf tubes.
7. Centrifuge at 12,000 g for 15 min at 20°C.
8. Collect the supernatant (400–500 µl). Avoid taking the intermediary phase, and keep the phenolic phase on ice for the protein extraction.
9. Repeat steps 6–8 twice.
10. Keep the supernatant (about 200 µl) to proceed to a chloroform extraction of RNAs, and put aside all the phenolic phases at −20°C.
11. Centrifuge 12,000 g for 10–15 min at 20°C.
12. Collect the aqueous phase and add 0.05 v acetic acid (1 M) and 0.7 cold ethanol 96% (kept at −20°C).
13. Mix carefully and precipitate nucleic acids overnight at −20°C.

**Table 15.3** NTES buffer for RNA extraction

| | Initial concentration | Final concentration | per ml |
|---|---|---|---|
| NaCl | 5 M | 150 mM | 30 µl |
| EDTA | 0.5 M | 5 mM | 10 µl |
| Tris HCL pH 9 | 1 M | 50 mM | 50 µl |
| SDS | 10% | 5% | 500 µl |
| DEPC water | | | 410 µl |

#### 15.4.2.2 Second Day

1. Centrifuge at 12,000 g for 35 min at 4°C.
2. Throw away the supernatant and suspend the pellet in 200 µl DEPC water.
3. Shake the tubes upside down and leave on ice 5 min.
4. Add 1 volume of LiCL (4 M) to precipitate RNAs, mix carefully and leave for 4-5 h in a cold room (4°C).
5. Centrifuge 12,000 g for 35 min at 4°C.
6. Throw away the supernatant and suspend the pellet in 200 µl DEPC water.
7. Shake the tubes and leave on ice for 10 min.
8. Precipitate RNAs overnight at −20°C with 0.1 volume sodium acetate (3 M) and 3 volumes of cold ethanol 96% (kept at −20°C).

#### 15.4.2.3 Third Day

1. Centrifuge at 12,000 g for 35 min at 4°C.
2. Throw away the supernatant and re-suspend the pellet in 200 µl of cold ethanol 75% (kept at −20°C).
3. Centrifuge at 12,000 g for 15 min at 4°C.
4. Dry the pellet (about 1 h) and suspend in 50 µl of DEPC water (leave about half a day at 4°C and mix from time to time).
5. Leave overnight at −20°C (may also be kept at −80°C).

#### 15.4.2.4 Fourth Day

Quantify the RNAs with a spectrophotometer and check their quality on an agarose gel (Sambrook and Russel 2001).

### *15.4.3 Protein Extraction*

1. Pool all the phenolic phases and then complete with phenol up to 10 ml.
2. Homogenize in 10 ml of extraction buffer (Table 15.1) and proceed as previously described. Proteins can be then analyzed by 2-DE.

**Note 7**: The NTES buffer may be prepared in advance in Eppendorf tubes, but add extemporaneously β-mercapto-ethanol (10 µl.10–1 ml NTES buffer).

### *15.4.4 Measure of Protein Content in Samples*

This step is crucial for further 2-DE analysis, as the determination of the amount of proteins, effectively solubilized and applied to gels, is required to accurately and quantitatively evaluate protein maps. The protein content of the supernatant is determined

by Bradford's assay (Bradford 1976), with the modifications suggested by Ramagli and Rodrigez (1985). This allows protein quantification even in samples containing components that usually interfere with the reactive of Bradford's assay.

## *15.4.5 Two-Dimensional Electrophoresis*

### 15.4.5.1 First Dimension

Isoelectric focusing (IEF) is an electrophoresis method for separating proteins according to their charge in a specific pH gradient. Proteins are separated within an electrical field until they reach the position in the gel where their own charge is zero (isoelectric point, *pI*).

We strongly suggest to readers to have a look to the following websites for additional information

http://www.expasy.ch/ch2d/protocols/protocols.fm6.html

http://www.weihenstephan.de/blm/deg/manual/manualwork2html02test.htm

**Note 7**: Several companies are delivering high-quality IPGs (ready-made Immobiline DryStrips) available now in a large scale of pI gradients. They offer high resolution and great reproducibility and allow high protein loads. As a general rule, we suggest beginning with non-linear or linear 3–10 pH gradients, and depending on the protein repartition to the pI scale and/or to the proteins under study (acidic/basic ones), selecting narrower gradients.

**Note 8**: It is strongly recommended that the experiments be planned in advance (included repetitions), so that IPGs from the same batch of IPGs can be ordered from the manufacturer. This will minimize gel-to-gel variability.

The sample loading can be realized either by in-gel rehydratation or sample cup loading.

### 15.4.5.2 In-Gel Rehydratation

Incubate the precast 18 cm nonlinear pH 3–10 IPG strips overnight with 350 µl of the following rehydratation solution (9 M urea, 4%, w/v CHAPS, 13 mM DTT, 2%, v/v IPG buffer pH 3–10 and bromophenol blue) containing the accurate amount of proteins.

**Note 9**: The rehydratation solution may be prepared in advance without IPG buffer and stored in 2 ml aliquots at −20°C.

### 15.4.5.3 Sample Cup Loading

1. Incubate the IEF gels (18 cm) overnight in 350 µl of the same rehydratation solution as above.
2. Prior to the separation, place the sample cups onto the gel surface at the anodic side of the gel.
3. Then apply the protein solution (20–100 µl) into the cups.

**Note 10**: Overlay the gels with mineral oil to control the correct positioning of the sample cups

4. Carry out the IEF at 20°C with the Multiphor II system (AB) as recommended by the manufacturer
5. Store the strips in clean plastic trays at −80°C, or equilibrate them according to Görg et al. (1987) for immediate separation on SDS-PAGE.

In order to determine the *Mw* and *pI* of the polypeptides separated in the gels, add 10 µl of standard proteins (2-D, Bio-Rad, AB, etc.) for co-migration with samples. Alternatively, the standard proteins may be loaded on a small piece of Whatman paper placed on the border of the 2D gels.

#### 15.4.5.4 Electric Focusing

1. For analytical separations, 100 µg of total or cytosolic proteins are usually loaded and focused at 20°C for 50 kVh using a gradually increasing voltage.
2. For micropreparative analysis, up to 600 µg of proteins can be loaded and the focusing extended to 71 kVh (Valot et al. 2005).
3. Carry out the focusing at 20°C with the Multiphor II system (AB) under mineral oil as follows:

   - Pre-electrophoresis with successive steps (150, 300 and 1,500 V)
   - Then electrophoresis at 3,500 V overnight at 20°C according to the following programme

| | | | | |
|---|---|---|---|---|
| 1/1 | 150 V | 1 mA | 5 W | 0:01 h |
| 1/2 | 150 V | 1 mA | 5 W | 0:30 h |
| 1/3 | 300 V | 1 mA | 5 W | 0:01 h |
| 1/4 | 300 V | 1 mA | 5 W | 1:00 h |
| 1/5 | 1,500 V | 1 mA | 5 W | 1:00 h |
| 1/6 | 1,500 V | 1 mA | 5 W | 1:00 h |
| 1/7 | 3,500 V | 1 mA | 5 W | 3:00 h |
| 1/8 | 3,500 V | 5 mA | 5 W | 20:00 h |

   - The last steps needs at least 12 h for analytical gels and 18 h for the micro preparative ones

#### 15.4.5.5 IEF with the IEF Cell

1. Connect the IEF Cell. Launch the “rehydratation” program: that will allow the system to equilibrate at 20°C.
2. Place the electrode strips (previously wetted with milli Q water) over the platinum electrodes at the extreme part of each channel of the tray (12 maximum).

3. Take the hydrated IPGs, rinse them with milli Q water and eliminate the excess of liquid with clean paper (on the plastic side).
4. Place each IPG strip, gel down, into the channel in the right direction for polarity. The two extreme parts of the gel must lie on the electrode strips.
5. Cover each IPG strip with mineral oil.
6. Stop the "rehydratation" program and place the tray into the IEF cell.
7. Select the required program, which has to be determined in advance following the manufacturer's instructions.
8. Enter the number of strips and then run the program.

#### 15.4.5.6 Second Dimension

Casting SDS Gels

During SDS-PAGE, proteins are separated according to their *Mw* in a polyacrylamide gel of specific concentration in acrylamide/bis-acrylamide. Prior to SDS-PAGE, the proteins are saturated with the strong anionic detergent SDS in order to mask their own charge, which might disturb the separation. SDS forms complexes with the proteins and "overrides" their charge. The combination of IEF and SDS-PAGE allows the separation of proteins based on two different parameters (charge, molecular mass) and results in a 2-D protein pattern, which theoretically allows the analysis of thousands of protein spots within one single 2-D gel at the same time. A protocol for using the Hoefer DALT device is very briefly described below, but if another multi-gel system is used, follow the manufacturer's instructions.

1. Prepare the buffer for the separation in the second dimension (Table 15.4).
2. Carefully clean the casting cassettes before setting them into the gel caster device.
3. Make the solutions for the gel caster prior to gel preparation. For slab gels (approx 23 × 20 × 0.1 cm) prepare the solutions according to Table 15.5.
4. Quickly cast this solution through a tube into the gel caster.
5. Overlay each gel with about 1 ml of water-saturated N-butanol to reach a uniform gel surface.
6. Slab gels polymerise after 2–3 h at room temperature.

**Table 15.4** Electrophoresis buffer[a]

| Eau milli Q | 20 l |
|---|---|
| Tris | 60.5 g |
| Glycine | 288 g |
| SDS | 20 g |

[a]The buffer may be prepared directly into the tank of the DALT system and re-used three times for analytical silver-stained gels and twice for preparative Coomassie Blue-stained gels. In that case, it is stored at 4°C in containers

**Table 15.5** Composition of 12% SDS polyacrylamide gel

| | Gel number | | |
|---|---|---|---|
| | 10 | 12 | 18 |
| Acrylamide-PDA[a] | 267 ml | 310 ml | 440 ml |
| Tris-HCl 1.5 M pH 8.8 | 167 ml | 194 ml | 275 ml |
| Eau milli Q | 219 ml | 254 ml | 360 ml |
| Degases on ice | | | |
| SDS 10% (w/v) | 6.7 ml | 7.8 ml | 11 ml |
| APS 10% (w/v) | 6.7 ml | 7.8 ml | 11 ml |
| TEMED 10% (v/v) | 950 µl | 1.1 ml | 1.55 ml |

[a] We find that the addition of PDA as cross linker instead of bis-acrylamide adds significant tensile strength and decreases background when using silver staining

**Table 15.6** Equilibration buffer

| Solution A | For 100 ml |
|---|---|
| 1.5 M Tris/HCl pH 8.8 | 3.34 ml |
| Urea | 36 g |
| Glycerol | 35 ml |
| SDS | 2 g |
| DTT | 1 g |
| Solution B | For 100 ml |
| 1.5 M Tris/HCl pH 8.8 | 3.34 ml |
| Urea | 36 g |
| Glycerol | 35 ml |
| SDS | 2 g |
| Iodoacetamide | 5 g |
| | Bromophenol blue |

**Note 10**: APS and TEMED should be added extemporaneously.

#### 15.4.5.7 Running 2D-Electrophoresis

1. Prior to separation in the second dimension, equilibrate the strips (either just after IEF or stored at −80°C) according to Görg et al. (1987). Briefly, equilibrate the IEF gels (IPGs) successively in solutions A and B under slight shaking for 10 to 15 min in 3–4 ml equilibration buffer (Table 15.6).
2. During equilibration, remove the casting cassettes (containing the polymerized gels) and keep them in milli Q water.
3. Remove the excess of equilibration solution by briefly washing the IPGs with milli Q water.
4. Directly place the IEF gels onto the gel surface and carefully remove any air bubbles between the two gels. Stick the IPG gels with hot (40°C) agarose,

solubilized in electrophoresis buffer and previously stained with a little bromophenol blue.
5. Place each gel cassette into the tank (Hoefer DALT II system, AB) and fill with the electrophoresis SDS buffer up to cover the whole gel surface.
6. Run electrophoresis at 10°C for 30 min at 30 V, then 30 min at 60 V and finally at 100 V overnight until the dye front reaches the bottom of the gels.

### *15.4.6 Protein Detection*

The application of the 2-DE technology to separate, analyze and characterize proteins of biological samples would not have been possible without the development of complementary detection methods. The following non-radioactive stains can be employed for protein detection: metal stain (zinc/copper), Coomassie blue, silver and fluorescent dies. Perhaps one of the most popular is the silver-staining method, which is 100-fold more sensitive than Coomassie Brilliant Blue staining (Chevalier et al. 2004; Lopez et al. 2000; Rabilloud 2001). However, there can be some problems using this stain as a quantitative procedure, because it is known to be non-stoechiometric and prone to saturation and negative staining effects, where regions of very high protein concentration do not stain and appear as 'holes' in the pattern of stained spots. Moreover, only a few silver-staining methods are compatible with further MS analyses (Rabilloud 2001). Finally, detection methods based on fluorescent compounds promise to overcome these problems because of their excellent linearity and high dynamic range (i.e., they can be used over a wide range of protein concentrations) (Chevalier et al. 2004).

#### 15.4.6.1 Colloidal Coomassie Blue Staining

This stain is not specific, and will also detect non-protein components such as polysaccharides. Its sensitivity is fairly good, and it is compatible with mass spectroscopy. We use the protocol of Mathesius et al. (2001), and find it easy to use and quite sensitive enough. It can detect 36–47 ng of protein.

1. After electrophoresis rinse gels briefly (<1 min) with milli Q water.
2. Stain gels for 18–20 h with Coomassie blue solution (Table 15.7).
3. Wash gels for 1–3 min with 0.1 M Tris-H3PO4 pH 6.5.
4. Rinse gels briefly (<1 min) with 25% methanol.
5. Wash gels for 24–72 h with 20% ammonium sulfate.
6. Repeat this staining procedure one or twice.
7. Store gels in 20% ammonium sulfate. Avoid alcohol-containing solutions.

**Note 11**: Let this above solution cool, and then add 0.2 g $l^{-1}$ sodium azide as a preservative. Store at room temperature. It can be re-used at least four times.

**Table 15.7** Coomassie Blue staining solutions

| | |
|---|---|
| Solution 1 | |
| Ammonium sulfate | 80 g |
| Phosphoric acid 85% | 22.5 ml |
| Milli Q water | qsf 700 ml |
| Solution 2 (hot) | |
| Coomassie blue G250 | 1 g |
| Hot milli Q water | 20 ml |
| Mix solutions 1 and 2 | |
| Methanol | 200 ml |
| Milli Q water | qsf 1 l |

**Note 12**: The main limitation, even for colloidal staining, lies in its rather limited sensitivity. Nevertheless, this can be partly solved by scanning gels with the Odyssey Infrared Imaging System.

**Note 13**: There are a large number of protocols for Coomassie staining and different Coomassie stains. Generally, like the above staining technique, they are quick, easy and cheap, although making up the stain can be a little messy. For those protocols, it is important to make up fresh stain regularly, as it lose its sensitivity with age. You can also opt for the more expensive ready-made stains such as Bio-safe Coomassie (Bio-Rad). Most companies (Pierce, Sigma) make these, and suggest that they are more sensitive than conventional Coomassie stain; they also require much less destaining and are kinder to the environment.

#### 15.4.6.2 Silver Staining

The great advantage of this stain is its sensitivity (0.5–1.2 ng protein). However, like Coomassie stain it suffers from a lack of specificity, together with a lack of dynamic range. Although the protocol requiring good-quality substrates may be judged to be a time-consuming and expensive staining technique, it gives excellent results (Rabilloud 2000 a, b). Moreover, several new protocols have been described as being compatible with mass spectroscopy. However, great care should be paid not only to the quality of substrates but also to the quality of the water used all through the protocol. If an accurate quality of water cannot be guaranteed, we strongly suggest using a technique such as the one described by Mathesius et al. (2001), which gives good staining and reproducible results.

#### 15.4.6.3 Silver Staining Protocol (Mathesius et al. 2001)

**Note 14**: All steps are performed at room temperature on an orbital shaker at 36 rpm or in the Dodeca Stainer.

**Table 15.8** Sensitization solution

| | |
|---|---|
| Ethanol | 30% |
| Sodium acetate trihydrate (or anhydrous) | 68 g $l^{-1}$ (41 g $l^{-1}$ anhydrous) |
| Potassium tetrathionate | 2.75 g $l^{-1}$ |
| Adjust to 990 ml and filter | |
| 50% glutaraldehyde | 10 ml |

**Table 15.9** Developing solution

| | |
|---|---|
| Potassium carbonate anhydrous | 30 g $l^{-1}$ |
| Sodium thiosulphate | 11–12 mg $l^{-1}$ |
| Adjust to 1,000 ml and filter | |
| Formaldehyde | 250 µl |

**Table 15.10** Silver staining solution

| | |
|---|---|
| Silver nitrate | 2 g $l^{-1}$ |
| HEPES | 1.246 g $l^{-1}$ |
| Adjust to 1,000 ml and filter | |
| Formaldehyde | 700 µl |

**Note 15:** to be made 30–45 min before use, keep away from light

1. At the end of the second dimension run, remove the gels from the casting cassettes.
2. Soak in ethanol/acetic acid/water (40/10/50) for 3 × 30 min.
3. Soak overnight under hood in the sensitization solution (Table 15.8).
4. Wash six times in milli Q water for 20 min.
5. Incubate into the silver-staining solution (Table 15.9) in the dark.
6. Wash in milli Q water for 10 s.
7. Develop in the developing solution (Table 15.10).
8. When a slight background stain appears, stop by incubation for 30 min in the stop solution containing 50 g $l^{-1}$ Tris and 20 ml $l^{-1}$ acetic acid.

**Note 16**: To be made fresh 30–45 min before use

#### 15.4.6.4 Silver Staining Protocol Compatible with MS Analysis

1. Fix the gels by soaking in methanol/acetic acid/water (40/10/50) for 30 min.
2. Soak the gel in the sensitization solution, i.e., methanol/sodium thiosulfate/sodium acetate (30%/0.02%/6.8%) for 30 min.
3. Wash with milli Q water 3 × 5 min.

4. Stain the gels with the silver stain (0.25% in water) for a period of 20 min.
5. Wash with milli Q water 2 × 1 min.
6. Add the developing solution, i.e., sodium carbonate: formaldehyde* (2.5/0.04) in water. (*: to be added extemporaneously)
7. Stop staining by immersing gels in 5% glacial acetic acid.
8. Wash with milli Q water 3 × 5 min.

**Note 17**: The gels must be scanned, the interesting spots sampled and destained as soon as possible in order to avoid blocking of proteins that will hamper subsequent MS analysis (T. Rabilloud, personal communication).

#### 15.4.6.5 Fluorescent Stains (Detect 1–2 ng)

There are many different types of fluorescent stain, and most are easy and relatively quick to use. Some are fairly cheap and can be used more than once; others, like SYPRO ruby, are shockingly expensive. These dyes are said to be both selective and quantitative. That means that unlike silver, the intensity of the protein spot can be directly correlated to the amount of protein. SYPRO ruby is also said to be more sensitive than silver staining (Chevalier et al. 2004). Obviously, you will need some sort of gel viewing system, as these stains can't be seen with the naked eye. There are disadvantages of this stain, as once more, with the use of SYPRO ruby, the sensitivity issue puts the onus onto the MS. Also, we often manually cut out our spots, and to do this, it is easier if you can see them.

#### 15.4.6.6 Image Analysis

Although there are innumerable methods for creating an image of a gel (including flatbed scanners, fluoro- and phosphoimagers, and densitometers), the ability to use data relies on image analysis software packages, such as those listed in Table 15.11.

All these 2-DE imaging systems provide powerful and innovative solutions for shortening the path from data acquisition to protein information. Spot detection and matching algorithms facilitate the extraction of statistically valid differences between groups of 2-D gels, while requiring reduced user intervention and therefore speeding up analysis time. Their applications integrate filtering, querying, reporting, statistical and graphic options so that it becomes easier to view, compare, analyze and present 2-DE data.

Briefly,

1. Digitise gel images using an appropriate scanner with accurate scan software.
2. Proceed to image analysis following the tutorial instructions of the available software.
3. The first step consists of detecting and quantifying spots. The user must establish background thresholds. This is especially critical when trying to identify spots of low intensity. Many programs then filter the image and raise the contrast to further highlight the smallest spots.

**Table 15.11** 2-DE imaging systems

| Analysis package | Source | Web |
|---|---|---|
| Gellab-II 2D | Scanalytics | www.lmmb.ncifcrf.gov/gellab/ |
| ImageMaster | Amersham Pharmacia Biotech | www.apbiotech.com |
| ImageMaster 2D Platinum 6.0 | GE Healthcare | www.gehealthcare.com |
| Impressionist | Gene Data | www.genedata.com |
| Kepler | Large Scale Proteomics | www.lsbc.com |
| Melanie III | Geneva Bioinformatics SA | www.genebio.com |
| PDQuest | Bio-Rad | www.discover.bio-rad.com |
| Phoretix 2D | Phoretix International | www.phoretix.com |
| Progenesis | Nonlinear Dynamics | www.nonlinear.com |
| ProteinMine | Scimagix | www.scimagix.com |
| Z3 | Compugen | www.2dgels.com |

4. A spot identifier isolates individual spots on the gel, typically by analyzing the intensities of individual and groups of pixels, and gives them codes. Then, it will be necessary to identify protein spot patterns between gels (i.e., comparative analysis).
5. In matching gel images, the program performs automatically what the human eye does naturally: it looks for pairs of features that have the same or similar spatial distributions. If there is a doublet in the top right corner near a smeared singlet in gel 1, the program searches for a similar doublet–singlet combination in gel 2. This process is then repeated thousands of times until the program has accounted for all of the spots that it can identify.
6. When comparing several gels, one gel is established as the reference gel, and all spot combinations on the other gels are compared with it.
7. With analysis software, researchers can examine several gels of the same sample and average the locations of individual defects, creating a "perfect" gel in the process. Alternatively, many of the newer programs allow for localized perturbations by keeping the larger picture in mind.
8. When proceeding to comparative analysis, we should be aware that perturbations from an event such as protein phosphorylation or glycosilation, etc. can adversely affect the comparative process.
9. Ideally, in response to a treatment (AM colonization, any stress, etc.), changes will occur only in a few spots and other protein expression levels will be maintained. One might use these proteinaceous standards as landmarks (e.g., the housekeeping proteins that maintain a cell's internal environment).

#### 15.4.6.7 Drying Gels

1. Remove the storage solution from your 2-D gels.
2. Wash the gels with milli Q water until the storage solution has been completely removed.

3. Just before drying 2-D gels, put them in the drying solution (30% ethanol/5% glycerol/65% milli Q water) for 30–60 min and shake slowly in a rotary shaker.
4. Wet the cellophane papers (2 per gel) in milli Q water for at least 15 min and then proceed with the drying steps, placing both the cellophane and gel into the drying plastic frame and clamps, taking care to avoid all air bubbles between the cellophane papers.
5. Dry gels over night at room temperature.

**Note 18**: 2-DE gels can be dried between two sheets of cellophane as above. This will help during the image analysis procedure, since at any time one can refer back to dry gels. Moreover, dry gels can also be used to sample spots for MS analysis, assuming all the precautions for avoiding any keratin contamination have been taken during the whole process. In that case, dried gels can be gently and carefully washed with ethanol to eliminate any external contamination; then the spots are picked up and placed to rehydrate in an Eppendorf tube in a small volume of milli Q water, to help the elimination of cellophane with clean tweezers.

#### 15.4.6.8 Mass Spectrometry Analyses

As already mentioned, our purpose is not to review the different MS strategies. We will only introduce a few arguments that have to be taken into account when it is time to choose among the various possibilities of MS. First of all, one should take into consideration the quantities of data in databases in regard to the AM interaction studied. If the genomes of two organisms (plant and fungus) are about to be fully sequenced (as is the case of *M. truncatula* and *G. intraradices*), MALDI-TOF analysis and peptide mass fingerprinting will certainly provide more throughput and be the least expensive method to perform. In other cases, partial peptide sequence data will be an extremely powerful adjunct to identify proteins by peptide mass profiling. Several MS-based approaches can be used, which take advantage of the ability of the different mass spectrometers (MALDI-TOF-TOF, ESI-MS/MS triple-quadrupole or ion trap instruments) to induce fragmentation of peptide bonds. Automated interpretation of ESI-MS/MS fragmentation data may be used to search sequence databases directly. However, in several cases, de novo sequencing will require human intervention. A second point to be considered is the proximity of a proteomic platform and/or the facilities to set up collaboration between mycorrhiza and mass spectrometer teams. Last but not least, one should consider the number of protein identifications that have to be performed in a project in respect to the budget allocated for this item.

#### 15.4.6.9 2DE Spot Sampling for MS Analysis

As this step will strongly depend on the equipment with which MS analyses are carried out, only very general rules are presented below.

Following extensive washing of a micro preparative gel with water, excise the spots into small pieces with tips, dry with a speed vac apparatus and store at room temperature until subsequent MS analysis.

#### 15.4.6.10 Distaining of Colloidal Coomassie Blue Stained Spots

1. Distain each spot in 100 µl of 25 mM ammonium bicarbonate/5% acetonitrile. Incubate 30 min under agitation then remove the buffer.
2. Add 100 µl of 25 mM ammonium bicarbonate/50% acetonitrile. Incubate 30 min then remove the buffer.
3. Add 200 µl of acetonitrile 100% and incubate for 10 min.
4. Remove acetonitrile and dry spots under vacuum and heat.

**Note 19**: Great care should be paid to avoid any keratin and dust contamination. Therefore, we strongly suggest assigning a place in the lab for this step, and to wear lab coat, gloves, cuffs and cap to prevent as much as possible skin and hair contamination.
**Note 20**: Depending on its staining, if a spot seems to correspond to a very small amount of protein, one can collect the same spot for more than one gel.
**Note 21**: A certain number of proteomic platforms are now equipped with robotic systems for picking and digesting spots. In this latter case, a special type of 96-hole plastic tray may be required and, therefore, one should take care to follow the technical rules of the platform before collecting the spots for MS.

#### 15.4.6.11 Bioinformatics Tools

Generally, mass spectrometry apparatus is delivered equipped with its own software for querying and assigning protein identities. In addition, a large number of Internet sites have been established that provide tools for identifying proteins, using either MALDI peptide mass fingerprinting data (PMF) or LC-ESI-tandem mass spectrometry data (MS/MS). Table 15.12 lists some of the most frequently used tools currently available.

## 15.5 A Case Study Applied to Dissect the Early Stages of *Medicago Truncatula* Mycorrhizal Symbiosis: When Transcriptomics and Proteomics are Working Together

During AM symbiosis, two well-defined key steps are most suitable for proteomic investigations: the early stages, corresponding to appressorium formation at the root surface, and the late arbuscule stages, in which the fungus has fully colonized roots to establish a functional symbiosis. Transcriptomics and proteomics have so far

**Table 15.12** Websites for the tools most frequently used for mass spectrometry analysis

| Sites | Useful utilities | Websites |
|---|---|---|
| NCBI | Homology search | http://www.ncbi.nlm.nih.gov/ |
| ExPASy | Proteomic links repository | http://www.expasy.org/ |
| Blast/EBI | Homology search | http://www.ebi.ac.uk/Tools/similarity.html |
| Aldente | Search databases with PMF data | http://www.expasy.org/tools/aldente/ |
| PROWL | Search databases with PMF data or a sequence tag | http://prowl.rockefeller.edu/ |
| Mascot | Search databases with PMF and MS/MS data and sequence tags | http://www.matrixscience.com/ |
| Protein prospector | Search databases with PMF and MS/MS data | http://prospector.ucsf.edu/Phenix |
| Protein prospector | Search databases with MS/MS data | http://www.phenyx-ms.com/ |

mainly targeted the late stages of AM symbiosis (Brechenmacher et al. 2004; Liu et al. 2003; Bestel-Corre et al. 2002, 2004b; Hohnjec et al. 2005), for which the amount of root material is not limiting. There are very few reports on the appressorial stage, for which only limited amounts of root material are available, thus making proteomics a particularly difficult task (Amiour et al. 2006). For both stages, a simultaneous comparison of gene expression and protein profiles has not yet been assayed. We report here on the application of a protocol for combined extraction of mRNAs and proteins to study the early stages of the *M. truncatula-Glomus mosseae* root interaction.

### *15.5.1 In Silico Analysis*

By comparing *G. mosseae*-inoculated roots to their respective non-inoculated control roots, 29 ESTs were found to be over-expressed in the wild genotype cv. Jemalong J5 in response to inoculation. On the other hand, no over-expression was observed in the case of the *G. mosseae*-inoculated mycorrhiza deficient mutant *dmi3* (*Mtsym*13, TRV25) (Weidmann et al. 2004). To compare mRNA expression to that of their corresponding proteins, in silico analyses were carried out prior to proteomic analysis. So far, the international sequencing project on *M. truncatula* has generated more than 220,000 ESTs, available online (http://www.tigr.org/tdb/tgi/mtgi/). For each nucleotide sequence corresponding to an EST previously reported as being over-expressed in response to *G. mosseae* inoculation (Weidmann et al. 2004), the longest open reading frame (ORF) was selected to identify the corresponding protein. Translation did not always correspond to a single ORF, since from the 29 ESTs only 15 homologies were translated from a single ORF. The other homologies were issued from the translation of a cluster of sequences of the three first ORFs.

Once homologies were determined, p*I* and M*r* of 25 proteins out of 29 were calculated. Based on these parameters, visual examination of 2-DE gels was carried out to localize these proteins. Spots corresponding to the p*I*/M*r* of 13 proteins out of the 25 cited above were putatively localised on 2-DE gels of *G. mosseae*-inoculated root extracts.

### *15.5.2 Proteomic Analysis*

When a blind image analysis was carried out on these 2-DE gels, other spot modifications were revealed in addition to those already mentioned above. In total, 19 modifications were observed in response to AM inoculation (Fig. 15.2

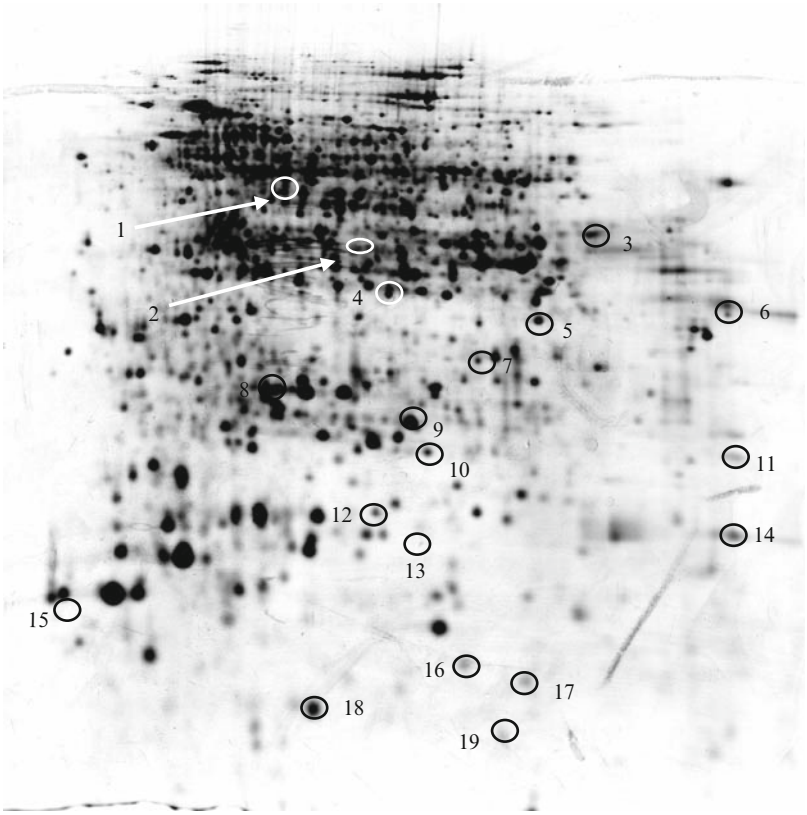


**Fig. 15.2** Protein profile corresponding to a micropreparative Coomassie Blue-stained gel of *Medicago truncatula* jemalong J5 roots indicating the 19 *circled spots* that showed modifications in their amount in response to *Glomus mosseae* inoculation. Protein extracts were obtained as described by Dumas-Gaudot et al. (2004a) on biological material produced by Weidman et al. (2004)

*NB* depending on the cloudy spot environment, the 19 circled spots are indicated in *black* or *white* characters (circles and/or numbers)

and Table 15.13), with seven and 12 spots whose abundance were decreased and increased in *G. mosseae*-inoculated roots respectively. All of them were excised from a micropreparative Coomassie Blue-stained gel and subjected to PMF search following MALDI-TOF spectrometry. Among the 13 proteins, only 11 spots could be excised from the gel. Six out of these 11 spots, indicated by asterisks in Table 15.13, gave positive hits after PMF search.

Five ribosomal genes have been previously reported as being over-expressed at the transcript level, among which four were predicted to encode proteins potentially located on 2-DE gels after in silico analysis. When these spots were submitted to PMF search, only spot 11* was confirmed as being a ribosomal protein. None of the other predicted proteins (spots 3*, 5*, 6* and 7*) could be confirmed. Instead of the in silico-predicted proteins, a probable peroxidase, a guanine nucleotide binding protein, a probable peroxidase precursor and a probable GTP-binding protein were identified (Table 15.13).

Spots 3* and 6*, whose amount increased in response to *G. mosseae* inoculation, gave hits with a probable peroxidase (MtC40023) and a probable peroxidase precursor (MtC10717). Since the first publication of Spanu and Bonfante-Fasolo (1988) several groups have reported an increase of peroxidase activities in response

**Table 15.13** Proteins differentially displayed upon *Glomus mosseae* inoculation of *Medicago truncatula* roots

| Spot[a] | Accession number | Matched peptide number | Coverage/score | Identification |
|---|---|---|---|---|
| 1 | MtC00229 | 7 | 48%/3.28e + 003 | Alanine aminotransferase |
| 2 | MtC50061 | 6 | 23%/9.13e + 003 | Serine/threonine kinase |
| 3[b] | MtC40023 | 10 | 40%/2.13e + 004 | Probable peroxidase |
| 4 | MtC00294 | 10 | 38%/5.29e + 004 | Chalcone reductase |
| 5[b] | MtC00498 | 9 | 40%/2.37e + 005 | Guanine nucleotide binding protein |
| 6[b] | MtC10717 | 10 | 34%/8.46e + 004 | Probable peroxidase precursor |
| 7[b] | MtC00087 | 12 | 71%/2.01e + 006 | Probable GTP-binding protein |
| 8 | MtC00681 | 8 | 25%/3.02e + 004 | Glutathione-S-transferase |
| 9[b] | MtC10018 | 20 | 70%/1.79e + 005 | Probable GST |
| 10 | – | – | – | Nd |
| 11[b] | MtC00128 | 12 | 41%/1e + 005 | 40 S ribosomal protein S5 |
| 12 | – | – | – | Nd |
| 13 | – | – | – | Nd |
| 14 | – | – | – | Nd |
| 15 | – | – | – | Nd |
| 16 | – | – | – | Nd |
| 17 | – | – | – | Nd |
| 18 | – | – | – | Nd |
| 19 | – | – | – | Nd |

[a]Numbers correspond to those in Fig. 1

[b]Indicates proteins which were deduced from in silico analyses of ESTs over-expressed in response to mycorrhizal inoculation (Weidmann et al. 2004)

*Nd* no protein identification was obtained after MALDI-TOF analysis and PMF search

to both ecto- and endo- mycorrhizal symbioses (Albrecht et al. 1994; Lambais et al. 2003; Guenoune et al. 2001). Spots 5* and 7* gave a positive hit with MtC00498 and MtC00087 respectively. These clusters correspond to guanine nucleotide binding like proteins (GTP-binding proteins). GTP proteins are glycoproteins anchored on the cytoplasm cell membrane acting as mediators in many cellular processes, including signal transduction, protein transport, growth regulation and polypeptide chain elongation. While a gene encoding a *M. truncatula* mitogen-activated protein (MAP) kinase was previously identified at the transcript level prior to and during appressorium formation of *G. mosseae*, the putative in silico-predicted protein corresponded to the MtC00294 EST cluster encoding a NAD(P)H Dependent 6′-deoxychalocone synthase, also called chalcone reductase. Recently, such a protein was also identified as being up-regulated in *G. intraradices*-inoculated roots of both *M. truncatula* J5 and *dmi3* mutant (Amiour et al. 2006). It appears to respond to early events of elicitor-mediated signaling. It has now been repeatedly claimed that some events, including signal perception, signal transduction and defense gene activation, similar to those found in plant/pathogen interactions, have occurred in AM symbioses (Dumas-Gaudot et al. 2000; Pozo et al. 2002). The induction of defense gene expression could result from fungal elicitor recognition and signal transduction pathway activation. The weak and transient character of plant defense responses could be a consequence of the low capacity of the fungus to trigger such a response and/or to induce a plant mechanism that suppresses an already activated defense response at several levels, allowing fungal growth within the plant tissue (Dumas-Gaudot et al. 2000; Garcia-Garrido et al. 2000).

Among the spots revealed by the blind proteomic analysis that accumulated in response to *G. mosseae*-inoculation, spot 1 matches to an alanine aminotransferase, an enzyme of the primary metabolism, while spot 2 matches to the MtC50061 cluster, classified in the signal transduction protein category (Journet et al. 2002). In plants, protein kinases are involved in the adaptation to various changing environmental conditions (Chinnusamy et al. 2004). To date, at least three MAP kinases have been reported to be up-regulated during early root colonisation of *M. truncatula* (Weidmann et al. 2004; Liu et al. 2003). The increased amount observed in the present work is therefore in agreement with the previous data gained from transcriptomic investigations. Two glutathione-S-transferase (GST) were identified in the present work (spots 8 and 9). Up to now, GST expression in mycorrhiza has mainly been addressed on arbuscule development. Both activation of GST encoding genes and accumulation of a corresponding protein were reported in fully colonised root tissues of potato and *M. truncatula* (Strittmatter et al. 1996; Wulf et al. 2003; Brechenmacher et al. 2004; Hohnjec et al. 2005; Bestel-Corre et al. 2002). It was proposed that in mature mycorrhiza, GST expression may be part of a plant defense reaction or involved in arbuscule degradation (Brechenmacher et al. 2004). To our knowledge, this is the first report of an accumulation of GST at the very early stages of the AM symbiosis, i.e., prior to fungal colonization. For the down-regulated spot 9, PMF search gave a hit with the MtC10018 cluster, which belongs to the Tau class of GSTs. Interestingly, such a protein was previously reported as being also down-regulated in response to *G. intraradices* inoculation of both the hyper-mycorrhizal

mutant TR122 and the J5 wild-type line genotypes (Amiour et al. 2006). Plant GSTs form a large family of catalytic and binding proteins that have long been associated with stress tolerance, including toxic chemicals, environmental stresses and diseases (Frova 2003). Moreover, for auxin-inducible GSTs, a time-dependent increase in their expression at the transcript level was previously recorded in *M. truncatula* roots upon inoculation with *G. mosseae* (Brechenmacher et al. 2004), suggesting that auxin-inducible GSTs might be developmentally regulated when roots are colonized by AM fungi. Spots 10 to 19 did not give positive hits following PMF search, a fact that could be related (1) to the low molecular weight of some of the proteins resulting in a low number of trypsic digestion peptides, (2) to insufficient protein amount in each spot, or (3) to the absence of the corresponding protein in the database.

## 15.6 Conclusions

While investigating the early stages of the *M. truncatula*/*G. mosseae* interaction, we might have expected, by using a protocol designed for extracting simultaneously mRNAs and proteins, to increase the correspondence between transcripts and proteins. In fact, while in silico prediction of transcripts previously reported as being up-regulated in response to *G. mosseae* inoculation gives rise to 13/29 proteins potentially detected on 2-DE, only a few results were common at the transcript and protein level. Such a discrepancy between predictive data and experimental ones could be related to spot overlapping in 2-DE. Behind one spot we can have more proteins and the interesting one is not identified because of its lower number of copies or just because this protein was not detectable with the staining procedure. Spot overlapping is a phenomenon demonstrated very recently to occur to a much higher extent than previously envisaged (Campostrini et al. 2005). Moreover, it should be emphasised that transcriptomics based on the construction of a SSH library was more particularly designed to enrich the samples in rare mRNAs. No such enrichment can be achieved by a classical proteomic approach, for which it is well-known that, without sub-cellular purifications, only major proteins can be visualized on classical 2-DE gels. In addition, incorrect assignment of the start site of the correct frame or (as in our study), where a number of homologies were derived from a cluster of more than one ORF, may also be responsible for this lack of correspondence. Post-translational modifications also alter protein charges, and this phenomenon frequently occurs in signaling events where cellular changes, including influx of $Ca^{2+}$ and activation of kinases are shown in response to plant biotic and abiotic stresses. Breakdowns of the protein by altering the M*r* parameter should also be considered as factors potentially affecting the 2-DE gel location. Some proteins can be excluded from the analysis if we consider their 2-DE characteristics. Indeed, while a certain number of the in silico-predicted proteins could be separated on 3–10 NL IPG strips, some of them

were theoretically excluded following the second dimension. Finally, it is also well known that membrane proteins are resistant to 2-DE separation (Valot et al. 2004, 2005).

## 15.7 New Insights

Proteomics interfaces with and complements genomics to provide information on quantitative protein expression in any biological system. In the particular field of plant–microorganism interactions (including the mycorrhizal symbioses), its potential is still far from being fully explored. To obtain mycorrhiza-related protein identification at a higher throughput, attempts should be made to define more precisely both the mycorrhizal stage under study and the particular cell compartment. In this respect, recent comparative sub-cellular proteomics directed towards microsomes (Valot et al. 2004, 2005) and plasmalemma (Valot et al. 2006) have proven their usefulness to identify proteins previously not reported as being expressed in AM symbiosis. Comparative proteomics of several other cell compartments such as, for example, the cell wall (for improving our understanding of the recognition events accompanying the first stage of mycorrhizal infection), or the mitochondrion and/or vacuolar compartments (for decrypting parts of the regulation events during the mycorrhizal process), need to be investigated. Recently, we have initiated a comparative root plastid proteomic approach (Daher et al. 2006). New insights into the complex cellular process accompanying the mycorrhizal symbiosis may also be expected from the use of more sophisticated proteomic strategies, together with the exponential increase of data in general database. So clearly, more refined proteomic tools that have recently emerged could be applied to the study of symbioses. Fluorescence-based differential in gel electrophoresis (DIGE) allows the differential analysis of two samples concomitantly run within the same gel, circumventing reproducibility problems and image analysis (Ünlü et al. 1997). More revolutionarily, the multidimensional protein identification (MudPIT), (Whashburn et al. 2001) and isotope-coded affinity tag (ICAT) (Gygi et al. 1999) methods bypass 2-DE by directly analysing protein mixtures by mass spectrometry. Laser dissection microscopy coupled to MALDI-TOF analyses could also gives rise to important knowledge in direct relation to the localization of the proteins in any cell compartment. Likewise, AM fungal proteomics will certainly soon benefit from the full genome sequencing of *G. intraradices*, together with the possible use of MS apparatus of higher sensitivity such as MALDI-TOF/TOF.

High-throughput microbial proteomics will also improve our understanding of molecular responses to external stimuli, as it promises to yield information about cell responses to disease and/or stress processes. A targeted proteomic approach allowed us a few years ago to identify proteins whose accumulation was modified in response to cadmium stress in mycorrhizal pea roots. In a more recent investigation, because of the use of the model plant *M. truncatula* in symbiosis with *G. intraradices*, we are expecting to get a broader view of the molecular mechanisms involved in the responses of mycorrhizal plant to cadmium stress (Aloui et al. 2006).

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# Chapter 16
# Using Stable Carbon Isotope Labelling in Signature Fatty Acids to Track Carbon Allocation in Arbuscular Mycorrhiza

**Pål Axel Olsson**

## 16.1 Introduction

A technique based on stable carbon (C) isotope labelling and subsequent compound specific isotope ratio mass spectrometry can be used to estimate C flow between different soil organism groups (Olsson et al. 2005). The C flow from atmosphere to green biomass can be estimated by traditional techniques and the allocation below ground into roots and transfer to specific molecules can be determined with compound-specific isotope ratio mass spectrometry.

In soil and roots different organisms occur closely associated with each other. It is therefore not always possible to separate soil organisms mechanically from each other. Above-ground tissues such as plant leaves and fruitbodies of fungi can be sampled as relatively pure examples of single organisms. In soil, the use of signature compounds is a great advantage both for the estimation of microbial biomass and for the study of C flow in the rhizosphere. In particular the neutral lipid fatty acid 16:1ω5 is useful for detecting and estimating C flow to the arbuscular mycorrhizal (AM) fungi in roots and soil (Olsson et al. 2005). Furthermore, the C transferred to saprophytic microorganisms can be estimated by use of other fatty acids (Olsson and Johnson 2005).

By use of lipid analysis the C flow in the rhizosphere can be described according to the conceptual model proposed in Fig. 16.1. The amount of recently assimilated C retained in each box in Fig. 16.1 can be estimated, and the total C flow, represented by the arrows, can be indicated by these measurements. The amount

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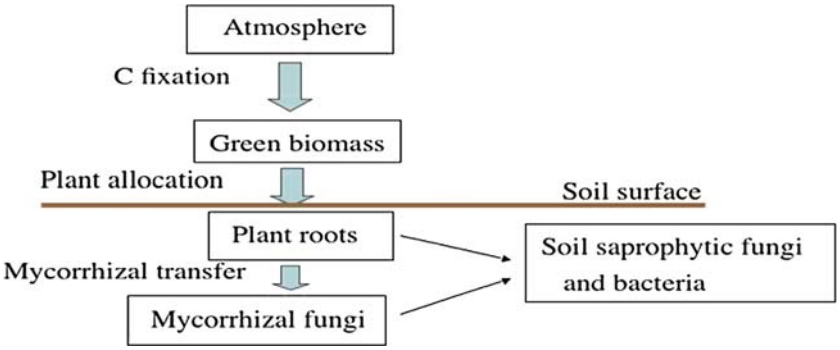


**Fig. 16.1** Schematic presentation of C pools (*boxes*) that can be estimated by use of $^{13}$C-labelling and the flow of C between these different pools

of C respired away from each box can not be determined, but during microbial growth an added C substrate is normally used to almost 50% for energy production and the rest is used for biomass components such as storage (for example Sylvia et al. 1998). At the same time the biomass C in each box can be estimated and be compared with the recent uptake of plant-assimilated C.

## 16.2 Microbial Biomass Distribution

Biomass of different groups of organisms in the soil can be determined by use of biochemical markers and biomass conversion factors obtained from pure cultured microorganisms or field samples of hyphae and spores. By use of various signature compounds (Olsson 1999), the relative biomass of saprophytic fungi [ergosterol and phospholipid fatty acid (PLFA 18:2ω6,9)], AM fungi (fatty acid 16:1ω5, no ergosterol) and bacteria (10 specific PLFAs) can be determined.

Neutral lipids are storage compounds that may comprise around 20% of the biomass of hyphae, vesicles, and spores of AM fungi (Olsson and Johansen 2000). The phosphate group of the phospholipids is easily and rapidly released through enzymatic actions in soil (White et al. 1979) and thus PLFAs reflect the occurrence of mainly living organisms, and also neutral lipids can be expected to be degraded rapidly when fungal hyphae senesce.

Consequently, the dynamics of $^{13}$C-labelled signature fatty acids can be used to track carbon flux from plants to intra- and extraradical AM fungal tissues and to other rhizosphere microorganisms (Olsson et al. 2002; Gavito and Olsson 2003). Since total $^{13}$C incorporation in AM fungal hyphae is correlated to that in the neutral lipid fatty acid (NLFA) 16:1ω5 (Olsson et al. 2005), it is possible to make an estimate of C uptake in AM fungi by analysing this compound.

## 16.3 $^{13}C$ Labelling

### *16.3.1 Timing of Measurements*

Timing is extremely important in all labelling experiments, particularly for studies of C allocation, because C is continually respired. However, a large proportion of AM fungal C is contained in lipids in vesicles inside plant roots and in spores on the external mycelium where they accumulate and may be stored for long periods of time. As long as the mycelium is expanding and spores are formed, there should be a continuous allocation of carbon to long-term storage. In field-labelled AM fungal mycelium, labelled respiration ceased within seven days after pulse-labelling (Johnson et al. 2002), indicating that only labelled stored material remained at that time. We observed a similar level of enrichment in the extraradical and intraradical mycelia after seven days (Olsson et al. 2005; Olsson and Johnson 2005), indicating that there was enough time for C translocation between different parts of the mycelium. We also found that recently assimilated C in *Plantago lanceolata* appeared in the extraradical Am fungal hyphae within a few days and remained to a large part there for at least 30 days (Olsson and Johnson 2005). Higher $^{13}C$ enrichment in lipids of the intraradical mycelia than in plant root lipids shows that lipid metabolism is more active in the intraradical mycelia than in the plant (Olsson et al. 2005). Such a difference was particularly evident in the monoxenic experiments with $^{13}C$-glucose.

### *16.3.2 Labelling in Monoxenic AM Cultures*

Monoxenic AM cultures are now well-established as experimental systems for the investigations of AM fungal biology (Bécard and Fortin 1988; Fortin et al. 2002). Established cultures in 1- or multi-compartmental Petri dishes can be labelled. The plates are supplied with sterile filtered [$^{13}C$] D-glucose solution (7 mg glucose per dish, U-$^{13}C_6$, 99% $^{13}C$) in a ring, 5 mm outside the initial inoculum plug. Solid MM medium (Fortin et al. 2002) with only hyphae can be cut out from the plates and dissolved in 10 mM Na citrate (Doner and Becard 1991) by mixing on a magnetic stirrer for 1 h at low speed at 20°C to leave extraradical mycelia and spores. The mycelia are collected on a 25 μm nylon mesh and washed in deionised water in a glass beaker. Roots are collected with forceps from the plates and washed in 10 mM Na citrate to remove any remaining solid medium. Using two-compartmental dishes with established root cultures in solid medium and with a liquid medium compartment for hyphal growth (Maldonado-Mendoza et al. 2001) is recommended for various reasons. First of all the mycelium can be subjected to treatments that do not directly influence the roots (Gavito et al. 2005; Olsson et al. 2006).

Furthermore, C translocation by mycelium themselves can be estimated and the sampling is easier when mycelium is growing in liquid medium. Mycelia and roots are stored at −20°C until analysed for $^{13}C$ enrichment and fatty acid content and composition.

### 16.3.3 Labelling in Pot Experiments

Controlled labelling of experimental pots can be obtained by transferring them to a greenhouse seedling propagation box (volume around 25 $dm^2$). The transparent lid of the greenhouse box can be adapted with a fan, a thermometer, and a gas-sampling tubing connected to an infra-red gas analyser (IRGA) and a small hole with a septum. The bottom tray and the lid of the box are sealed with paraffin and the initial $CO_2$ concentration recorded. Labelling is done when the fan is turned on by injection of, for example, 100 ml of $^{13}CO_2$ (99% $^{13}CO_2$) with a gas-tight syringe through the septum. The $CO_2$ concentration inside the box increases usually from 240–270 to 500–1,000 ppm after such an injection. A pulse period of 2–4 h is usually appropriate. We have observed that assimilation may be very low the first hour after labelling, and therefore a longer period than 1 h is recommended. It is best to investigate the assimilation using the IRGA. The lid is removed after the labelling time.

### 16.3.4 Labelling in the Field

$^{13}C$ labelling in the field can be done by enclosing a piece of vegetation, or a single shoot, in a plastic chamber and then injecting the labelled gas. Basically the labelling in the field works in the same way as for pot cultures. Almost all incorporation goes via the autotrophic shoots. The chamber may first be emptied for non-labelled $CO_2$, which can be done by letting the plants themselves empty $CO_2$ in an enclosed room. Usually carbon dioxide content goes down rapidly after enclosing in a closed chamber. The initial carbon dioxide, and increase due to labelling, can be measured using an IRGA coupled to the chamber. Each system is unique and it is wise to make a pilot study for each type of plant, or plant species. The fraction of C that moves below ground to the roots may in some cases be small and a rather high label in roots is needed to be able to track the C further to AM fungi and rhizosphere bacteria. The initial labelling can be checked by sampling shoots directly after the pulse period. If the labelling is too small, it is very difficult to trace the C later on after the chase period. If the initial labelling results in a delta $^{13}C$ value of around 1,000 (corresponding to around 2% $^{13}C$, which means around 1% enrichment), there is a good chance of tracing the C further. If it is around delta 100, the chances are small.

The weather during field labelling is crucial. Too hot and sunny weather may cause very high temperature in the labelling chamber, something that many plants are sensitive to. Too low light intensity may also reduce the assimilation. Furthermore, it is important to do labelling during a period (season) when the plants are active (if this is not the research question itself).

## 16.4 Analytical Techniques

### *16.4.1 Homogenization of Samples*

In order to obtain an efficient extraction of lipids from mycelial and root samples it is recommended to use some way of homogenizing samples. Mycelial samples can be milled with stainless steel balls by shaking by hand in Teflon tubes, and roots can be ball-milled (15 s, 300 strokes $min^{-1}$) in stainless steel beakers. Too harsh milling may heat samples too much, which can damage the lipids. Another way to homogenize mycelium or small amounts of roots is putting them in Eppendorf tubes together with a few grains of pure sand, and then grinding them with a pestle. Soil samples are usually not mechanically homogenized before lipid extraction, but to be sure to extract all lipids from mature spores, ball-milling is recommended (Olsson and Johansen 2000). There are tungsten beakers available that will not be damaged by sand particles.

### *16.4.2 Lipid Analysis*

The lipids from mycelia (0.5–1 mg dry mass), roots (15–30 mg), soil (0.5–3 g) or sand (10 g) are extracted by vortex mixing (1 min) in a one-phase mixture of citrate buffer, methanol and chloroform (0.8:2:1, v/v/v, pH 4.0) (8). The lipids are fractionated into neutral lipids, glycolipids and phospholipids on pre-packed silica columns by eluting with 1.5 ml chloroform, 6 ml acetone, and 1.5 ml methanol, respectively. The fatty acid residues in the neutral lipids and the phospholipids, transformed into free fatty acid methyl esters, are analysed by gas chromatography using a 50 m HP5 capillary-fused silica column with $H_2$ as carrier gas (Frostegård et al. 1993). The fatty acids are identified from their retention times relative to that of the internal standard (fatty acid methyl ester 19:0). These are compared with those earlier determined by gas chromatography-mass spectrometry.

### *16.4.3 Signature Fatty Acids*

NLFA 16:1ω5 is a sensitive signature of AM fungi in both roots and soil (Graham et al. 1995; Olsson et al. 1995). PLFA 16:1ω5 is a constituent of AM fungal membranes, with rather low specificity as a signature due to relatively low content

in AM fungi and a high background in soil originating from bacteria (Olsson et al. 1995). NLFA 16:0 is a general NLFA which often increases in concentration in plant roots following AM colonization due to the high content of storage lipids in AM fungi (Olsson et al. 1995). NLFA 18:2ω6, 9 is present in plant storage lipids. It is also the dominant fatty acid in most fungi, but occurs at very low levels in AM fungi (Graham et al. 1995; Olsson and Johansen 2000). PLFAs i15:0, a15:0 and cy19:0 are three bacteria-specific PLFAs that can be used as indicators of bacterial biomass (Frostegård and Bååth 1996). Both the neutral lipid fatty acid (NLFA) and the phospholipid fatty acid (PLFA) 16:1ω5 are effective signatures for AM fungi (Olsson 1999; van Aarle and Olsson 2003), while other PLFAs (i15:0, a15:0, i16:0, 10Me16:0, i17:0, a17:0, cy17:0, 10Me17:0, 10Me18:0 and cy19:0) are effective signatures for rhizosphere bacteria (Frostegård and Bååth 1996).

### *16.4.4 Determination of $^{13}C$ Enrichment in Crude Tissue Samples and Fatty Acids*

Freeze-dried mycelia (approx. 20–100 μg) or ball-milled root material (approx. 100 μg) are placed in tin capsules (crude tissue samples) and analysed by continuous-flow isotope ratio mass spectrometry (IRMS) using a stable isotope analyser interfaced to a solid/liquid preparation module. The $^{13}C/^{12}C$ ratios of $CO_2$ of the combusted samples (total C) are determined with a 0.01% precision. Data are expressed as atom % $^{13}C$ with reference to a sucrose standard, calibrated against the Pee Dee Belemite (PDB) standard (the limestone fossil *Belemnitella americana* from the Cretaceous Pee Dee formation in South Carolina, USA; International Atomic Energy Agency; Dowson and Brooks 2001).

The $^{13}C$ enrichment in fatty acid methyl esters was determined in the IRMS interfaced with a gas chromatograph equipped with a split/splitless injector, flame ionization detector, a high temperature reaction furnace mounted, and a sampler unit. The chromatographic conditions are as described for the lipid analysis except that He was used as the carrier gas. The effluent from the capillary column passes through an Al tube with CuO wires at 860°C, where the fatty acids are converted to $CO_2$. Co-generated water vapour is removed via a Nafion membrane and the purified $CO_2$ released into the IRMS.

The atom % $^{13}C$ values are calculated based on atom $^{13}C$ of the reference $CO_2$ gas, injected three times at the beginning and end of a chromatographic run. The reference $CO_2$ is standardized with the PDB standard using solid/liquid preparation module. Integration for each peak needs to be checked and corrected manually. The $^{13}C$ enrichment of fatty acids is calculated after correction for the C added in the methanolysis step of the fatty acid analysis procedure.

### 16.4.5 Quantification of Transferred Carbon

The correspondence between $^{13}C$ enrichment in NLFA 16:1ω5 and $^{13}C$ enrichment in total mycelium, shown by Olsson et al. 2005, suggests that the flow of C from host plants to fungal mycelia can be calculated from $^{13}C$ enrichment in NLFA 16:1ω5. In this specific study it was found that for every C incorporated into NLFA 16:1ω5 in mycelia, 2.7 atoms of C was incorporated into the total mycelium mass.

The flow of C from the host plant to the NLFA 16:1ω5 can be calculated from measurements of NLFA 16:1ω5 in the mycelia and its $^{13}C$ enrichment:

$$\text{NLFA16:1}\omega5\text{-C}(\mu g) \times {}^{13}\text{C-enrichment in NLFA16:1}\omega5(\%/100) = \text{C-flow to NLFA16:1}\omega5$$

Similarly, the flow of total C to the mycelia can be calculated by:

$$\text{Mycelium biomass-C}(\mu g) \times \text{total}^{13}\text{C-enrichment}(\%/100) = \text{C-flow to mycelium}$$

The flow of plant C to the AM fungal mycelia can be estimated based on measurements of the signature NLFA 16:1ω5:

$$\text{C-flow to mycelium} = \text{C-flow to NLFA16:1}\omega5 \times 2.7$$

Thus, for every C atom incorporated in NLFA 16:1ω5, 2.7 C atoms are incorporated into the fungal mycelia. This calculation can be used when pure mycelium cannot be extracted, such as from colonised roots or mycelium-containing soil.

## 16.5 Sensitivity and Specificity of the Method

Natural abundance of $^{13}C$ ($\delta^{13}C$) is generally around δ -30 (corresponds to 1.15% $^{13}C$) in C3 plants, and the $^{13}C$ abundance in AM fungi is similar to that of their host plant (Nakano et al. 1999, 2001; Staddon et al. 1999). Abraham et al. (1998) provided various substrates to different microorganisms and observed that the $^{13}C$ abundance in the fatty acid 16:0 extracted from the microorganisms resembled that of the substrate. With our method, using a $^{13}C$-labelled substrate, we reach a much higher sensitivity when tracing the C metabolism than by just using the natural differences in natural $^{13}C$ abundance between different substrates.

The dominance of the fatty acid 16:1ω5 in AM fungi and its rareness in other fungi (Müller et al. 1994; Stahl and Klug 1996) make it a useful biomarker probably for all *Glomus* species (Graham et al. 1995) and also for *Scutellospora* species

(Graham et al. 1995; van Aarle and Olsson 2003), but not for *Gigaspora* species (Graham et al. 1995). Neutral lipids are important for energy storage, and 16:1ω5 is particularly common in this fraction of AM fungal lipids (Olsson et al. 1995). Triacylglycerols are the main type of neutral lipids found in AM fungal spores and vesicles (Beilby and Kidby 1980; Cooper and Lösel 1978; Nagy et al. 1980), although other fractions, e.g. diacylglycerols and free fatty acids, are also important in *Glomus*. All of them are, however, dominated by 16:1ω5.

16:1ω5 is suitable for labelling because the amount of this fatty acid is correlated with total $^{13}C$ enrichment in hyphae and because this fatty acid represents a significant portion of the AM fungal biomass, which is consistent with the hypothesis of Bago et al. (2002) that up to 50% of the hyphal volume may be lipid bodies. That a high proportion of AM fungal biomass is lipids is probably the main reason for the strong correlation between $^{13}C$ enrichment in hyphae and in NLFA 16:1ω5 extracted from the same hyphae.

## 16.6 Conclusions

In a recent study we investigated the C turnover in mycorrhizal fungi in a pot experiment (Olsson and Johnson 2005). We used $^{13}C$ incorporation into signature fatty acids to study carbon dynamics in an AM fungus in symbiosis with *Plantago lanceolata*. Most carbon assimilated by intra- and extraradical AM fungal structures remained 32 days after labelling. Furthermore, $^{13}C$ enrichment of signature fatty acids showed a gradual release of carbon from roots to rhizosphere bacteria, but at a much lower rate than direct transfer of plant assimilates to AM fungi. These findings indicated that retention of carbon in AM fungal mycelium may contribute significantly to soil-organic C; and that $^{13}C$-labelled fatty acids can be used to track carbon flux from the atmosphere to the rhizosphere.

The method described here provides an objective way to compare C allocation to intraradical and extraradical AM hyphae and to track C allocation in plant roots to fungal symbionts. Plant regulation of C allocation can be followed by this method after the symbiosis has been established. The regulation of colonization establishment in AM has been well-studied, while the regulation of the C transfer in established symbiosis has been little studied due to methodological difficulties. The method may be used to test hypotheses regarding the relative C allocation to AM fungi under different environmental conditions and to estimate how much different plant species allocate to the AM symbiosis.

The method may also be used to calculate carbon fixation in AM fungi in field ecosystems, but this needs further evaluation. The $^{13}C$-labelling technique has the potential to contribute to understanding a significant part of the global carbon cycle. The rhizosphere dynamics and pH effects studied are of general importance for interpreting dynamics of all types of grasslands and can also help to understand the C fixation in different ecosystems and how it is influenced by successions. Today it is often believed that AM fungi contribute to a great extent to C cycling in any type

of grassland. By this technique it is possible to investigate if this is true for all kinds of grasslands.

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# Chapter 17
# $^{15}$N Enrichment Methods to Quantify Two-Way Nitrogen Transfer Between Plants Linked by Mychorrhizal Networks

**X.H. He, C. Critchley, K. Nara, D. Southworth, and C.S. Bledsoe**

## 17.1 Introduction

Nitrogen (N) movement or transfer from one plant (donor) to another (receiver) is of fundamental importance in $N_2$-fixing plant-based agricultural and natural ecosystems (Stern 1993; Chalk 1998; Graham and Vance 2003; Forrester et al. 2006). Nitrogen transfer is relevant to global concerns about N excess and N limitation in terrestrial ecosystems (Vitousek et al. 1997; Moffat 1998; Sanchez 2002; Nosengo 2003). Numerous studies over the past three decades have demonstrated that plant-to-plant N transfer from the donor to the receiver is not restricted to mass flow and diffusion through soil pathways, and can take place directly through mychorrhizal hyphae in common mychorrhizal networks (CMNs) that interconnect roots. Such mycorrhizal-mediated N movement between plants could have practical implications for plant performance, especially in N-limited habitats (Sprent 2005; Forrester et al. 2006; Nara 2006). However, recapture of decomposed and exuded plant and fungal materials by the receiver may occur simultaneously in the soil.

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In many cases, N is transferred from $N_2$-fixing mychorrhizal plants to non-$N_2$-fixing mychorrhizal plants via a CMN (unidirectional or one-way N transfer) (Newman 1988; He et al. 2003). In a few cases, N is also transferred from non-$N_2$-fixing mychorrhizal plants to $N_2$-fixing mychorrhizal plants via a CMN (bidirectional, two-way or net N transfer) (Johansen and Jensen 1996; He et al. 2004, 2005; Moyer-Henry et al. 2006). Thus, the $N_2$-fixing plant is not always warranted to be the N-donor. Furthermore, limited data show that the net N gains through CMNs in the recipient may play beneficial roles in affecting their fitness (Newman 1988; Johansen and Jensen 1996; He et al. 2004, 2005; Moyer-Henry et al. 2006).

Understanding the directions and magnitude of N transfer between plants through CMNs helps clarify the agricultural and ecological importance of CMNs (He et al. 2005; Hogh-Jensen 2006). Studies are therefore needed to quantify net N transfer between plants, and to determine whether mychorrhizal hyphae facilitate N transfer. However, it is experimentally difficult to distinguish N transfer between the soil and the mychorrhizal pathway. We suggest that the importance of mycorrhizas in N transfer may be demonstrated by separation of the root systems of plants so that only hyphae connect the plants, and there is no root contact. In doing so, 25–37 μm nylon or metal mesh has been used in most studies (Newman 1988; Johansen and Jensen 1996; He et al. 2003). A polytetrafluoroethylene (PTFE) hydrophobic membrane has been applied in some studies (Frey et al. 1998). A narrow air gap between fine meshes separating plants can further prevent interplant nutrient movement through the soil pathway (Faber et al. 1991). However, $^{15}N$ could cross the air gap in hyphae, then leak into the soil and be taken up again as an indirect step following the direct transfer through hyphae. This sort of $^{15}N$ movement or translocation could be minimized by severing the hyphae within the air gap to see if transfer is disrupted and whether the flow, once established, stops.

## 17.2 Use of $^{15}N$ Isotopes for Investigating Nitrogen Transfer Between Plants

Nitrogen has two stable isotopes, $^{14}N$ and $^{15}N$, and four radioactive isotopes, $^{12}N$, $^{13}N$, $^{16}N$ and $^{17}N$. The short half-life time (0.01 s → 10 min) of radioactive isotopes of N makes them unsuitable for investigating most plant physiological or ecological processes (Knowles and Blackburn 1993). Of the stable isotopes, $^{14}N$ is more abundant, accounting for ~99.6337% of atmospheric N; $^{15}N$ is rare, accounting for ~0.3663% of atmospheric N. Since the ratio of $^{15}N/^{14}N$ (0.0036765) in the atmosphere is constant, atmospheric $N_2$ is used as the standard for $^{15}N$ analysis by continuous flow isotope ratio mass spectrometer (IRMS) (Mariotti 1983; Knowles and Blackburn 1993; Unkovich et al. 2001).

Two methods have been used for quantifying N transfer between plants: $^{15}N$ enrichment and $^{15}N$ natural abundance (He et al. 2003; Hogh-Jensen 2006). With the $^{15}N$ enrichment or labeling method, an external source of $^{15}N$-enriched

inorganic chemicals or organic compounds (up to 99.90 atom $^{15}$N, %) is applied to plant tissues, growth media or soils, and the events of N metabolism or N translocation are followed over short-term periods (days to weeks). The $^{15}$N labeling method involves a large enrichment of $^{15}$N over background, making measurement of isotopic effects easy because the difference between the isotopic compositions of the source and the plant or the soil is large. In the $^{15}$N natural abundance or $\delta^{15}$N method, plant tissues are analyzed to determine $^{15}$N at naturally occurring levels ($\delta^{15}$N values, %). In the $\delta^{15}$N method, there is a small enrichment of $^{15}$N over background, making measurement of isotopic effects difficult. The difference between isotopic compositions of the source and the plant or soil is small, but does reflect naturally occurring $^{15}$N variations over long-term periods (years to decades) (Hobbie and Hobbie 2006). However, variations of $\delta^{15}$N values are often small (~10%) among AM or EM plant species that have unique physiological processes in different habitats (Handley and Scrimgeour 1997; Högberg 1997; Hobbie and Hobbie 2006). As a consequence, it is hard to demonstrate plant-to-plant N transfer through CMNs by $\delta^{15}$N variations of mychorrhizal plants, at least at face value, although they may demonstrate the magnitude of $^{15}$N fractionations during N translocation in long-term natural conditions.

## 17.3 Experimental Design for Investigating Two-Way Nitrogen Transfer Between Plants

Here we explore the $^{15}$N enrichment method to determine two-way N transfer between plants through mychorrhizal connections and to measure the net benefit to the partners. With either the $N_2$-fixing or the non-$N_2$-fixing plant as the N-donor, four reciprocal pairings are created to study two-way plant-to-plant N transfer, and to distinguish N transfer between the soil and the mychorrhizal pathway (Table 17.1). First, as a control, the non-nodulated/non-mychorrhizal pair monitors N transfer in the absence of both hyphal connections and $N_2$ fixation. Second, the nodulated/non-mychorrhizal pair tests effects of $N_2$ fixation on N transfer in the absence of hyphal connections. Third, the non-nodulated/mychorrhizal pair determines effects of hyphal connections on N transfer in the absence of $N_2$-fixation. Fourth, the nodulated/mychorrhizal pair examines effects of both mychorrhizal and $N_2$ fixation on N transfer. To minimize mass flow and diffusion through soil pathways, two perforated Perspex plates (2.5 mm thick) are inserted in the middle of a 5 l plastic box (300 × 12 × 150 cm) to divide the box into two chambers with a 5-mm air gap (Fig. 17.1). On one side of the two plates, sheets of 25–37 μm nylon mesh are inserted on the inner side of the plates to allow only hyphal connections. To further minimize water movement across the air gap between the two chambers, high water holding capacity crystals [0.5% (w/w), *RainSaver*, Hortex Australia Pty. Ltd., NSW, Australia] are incorporated in the potting mix. External $^{15}$N may be added directly to the N-donor side.

**Table 17.1** Experimental design for two-way N transfer between $N_2$-fixing and non-$N_2$-fixing plants grown in two-chambered pots. Enriched $^{15}N$ compounds are supplied to the growth medium or the leaves of the N-donor plant. Time period of $^{15}N$ labeling (days to months) depends on physiological activity of plants species and experimental purposes

| Plant pair name | Plant status | | Treatment status | |
|---|---|---|---|---|
| | N-donor | N-receiver | Myc | Nod |
| Non-nodulated/non-mycorrhizal pairs | $N_2$-fixing | Non-$N_2$-fixing | – | – |
| | Non-$N_2$-fixing | $N_2$-fixing | – | – |
| Nodulated/non-mycorrhizal pairs | $N_2$-fixing | Non-$N_2$-fixing | – | + |
| | Non-$N_2$-fixing | $N_2$-fixing | – | + |
| Non-nodulated/mycorrhizal pairs | $N_2$-fixing | Non-$N_2$-fixing | + | – |
| | Non-$N_2$-fixing | $N_2$-fixing | + | – |
| Nodulated/mycorrhizal pairs | $N_2$-fixing | Non-$N_2$-fixing | + | + |
| | Non-$N_2$-fixing | $N_2$-fixing | + | + |

*Myc* Mycorrhizal status; *Nod* Nodulation status

### *17.3.1 Quantification of Nitrogen Transfer Between Plants*

Nitrogen transfer has been quantified in three ways: percentage of $N_{transfer}$ (% $N_{transfer}$, (17.1–17.3)); amount of N transferred (mg plant$^{-1}$, (17.4); and percentage of NDFT (% NDFT, N in the *N*-receiver *d*erived *f*rom *t*ransfer, (17.5) and (17.6)) (Ledgard et al. 1985; Giller et al. 1991; Johansen and Jensen 1996).

$$\%N_{transfer} = {}^{15}Ncontent_{receiver} \times 100/({}^{15}Ncontent_{receiver} + {}^{15}Ncontent_{donor}) \quad (17.1)$$

where

$${}^{15}Ncontent_{plant} = atom\%{}^{15}N\ excess_{plant} \times total\ N_{plant}/atom\%{}^{15}\ N\ excess_{labeled\ N} \quad (17.2)$$

and

$$atom\%\,{}^{15}N\,excess_{plant} = atom\%\,{}^{15}N_{plant\ after\ labeling} - atom\%\,{}^{15}N_{plant\ background} \quad (17.3)$$

$$N_{transfer} = \%N_{transfer} \times total\ N_{donor}/(100 - \%\ N_{transfer}) \quad (17.4)$$

$$\%\,NDFT = N_{transfer} \times 100/total\ N_{receiver} \quad (17.5)$$

In addition, % NDFT values can also be calculated as follows (Tomm et al. 1994):

$$\%NDFT = (atom\,\%{}^{15}N\ excess_{receiver}/atom\,\%{}^{15}N\,excess_{donor}) \times 100 \quad (17.6)$$

Equations (17.5) and (17.6) are equal if the 0.3663%$^{15}$N of atmospheric $N_2$ is subtracted from the measured atom %$^{15}$N in (17.5).

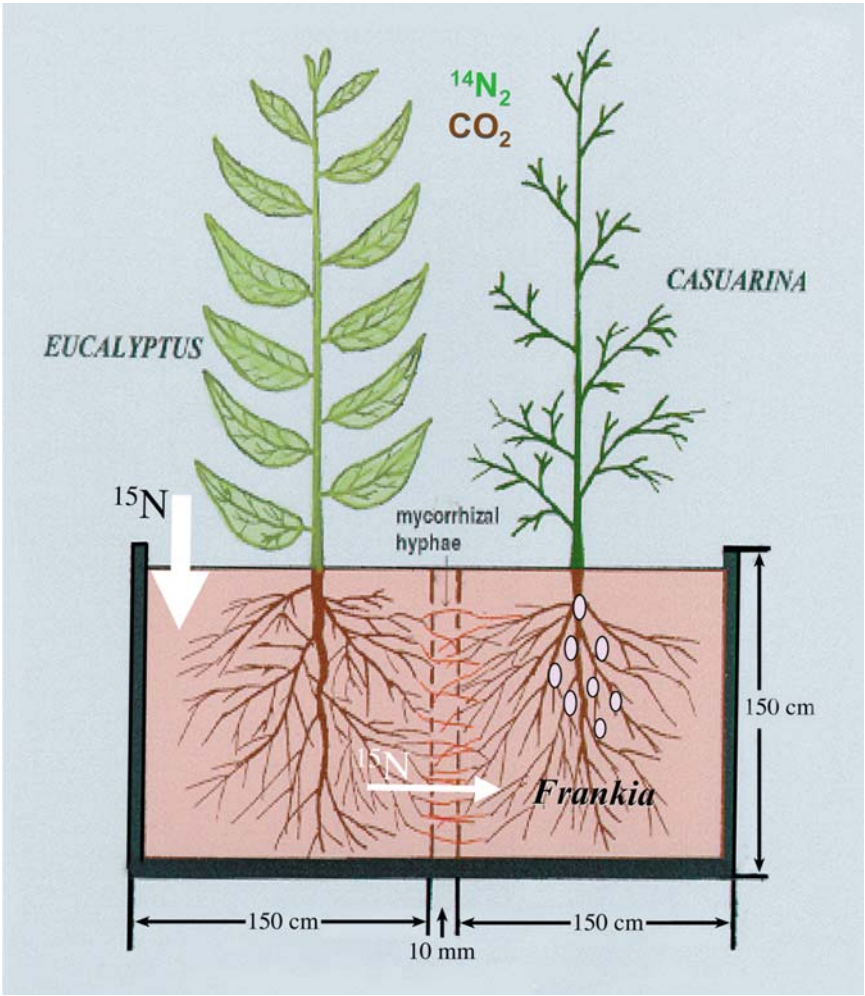


**Fig. 17.1** Diagram (longitudinal section) of a two-chambered growth pot. Chambers are separated by a 5-mm air gap created by two Perspex plates to minimize nutrient movement through the soil pathway. Plates are covered with μm nylon mesh to prevent root, but allow hyphal penetration. Each chamber contains one plant, either $N_2$-fixing (e.g. *Casuarina*) or non-$N_2$-fixing (e.g. *Eucalyptus*). Either plant can be the donor to which $^{15}NH_4^+$ or $^{15}NO_3^-$ is supplied to the growth medium or to the leaves to study two-way N transfer

Two-way (bidirectional) or net N transfer can be calculated as the difference between N transfer from plant 1 to plant 2, and the reverse. Then net N transfer could benefit one of the two species, when that plant received more N than it donated through the transfer (Newman 1988; Johansen and Jensen 1996; He et al. 2004, 2005; Moyer-Henry et al. 2006).

#### 17.3.1.1 Comments

The $^{15}N$ enrichment or labeling method has been widely used to investigate N transfer between plants (Stern 1993; Chalk 1998; He et al. 2003). However, the addition of $^{15}N$ to quantify N-transfer has limitations, especially in fertile soils. Compared to the large volume of soil N, which is available to plants along with the additional $^{15}N$-labeled N, the amount of N transferred from the N-donor to the N-receiver may be much less than the amount of N taken up from the soil. Also, root depth and pattern of N uptake of the N-receiver may be dissimilar in mixed species and single species situations, which may cause the ratio of unlabeled-to-labeled N to be different in such growth systems. Either can invalidate the assumptions for estimating N transfer.

Furthermore, the above experimental design and calculations to study two-way or net N transfer are based on separate, reciprocal, experiments — one where $^{15}N$ is applied to plant 1, another where $^{15}N$ is applied to plant 2. A more accurate detection of net N transfer would be to use dual isotopes so that concurrent N transfer could be investigated in a single experiment. Obviously, $^{15}N$ is not suitable, since no technique is available to distinguish the side from which the detected $^{15}N$ comes. One possible method is to supply the stable isotope of $^{15}N$ to one plant and the radioactive isotope of $^{13}N$ to another, but the short half-life time (10 min) of $^{13}N$ may not be suitable for tracing N translocation in long-term plant and/or fungal biological processes (Knowles and Blackburn 1993).

## 17.4 Conclusions

Both $^{15}N$ enrichment and $^{15}N$ natural abundance have been employed to study N transfer between plants linked by mychorrhizal networks. By tracing translocation of external high-enriched $^{15}N$ from one plant to another over short-term periods (days to weeks), the above experimental design allows a two-way or net N transfer through CMNs to be investigated by the $^{15}N$ enrichment method. Considering that N movement is crucial in most terrestrial ecosystems (Vitousek et al. 1997; Moffat 1998; Sanchez 2002; Graham and Vance 2003; Nosengo 2003), and that the potential benefits of N transfer are great, particularly in N-limited environments (Chalk 1998; Sanchez 2002; Sprent, 2005; Forrester et al. 2006; Nara 2006), more research is warranted on two-way N transfers mediated by mychorrhizal networks with different species and under field conditions.

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# Chapter 18
# Analyses of Ecophysiological Traits of Tropical Rain Forest Seedlings Under Arbuscular Mycorrhization: Implications in Ecological Restoration

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## 18.1 Introduction

Deforestation is one of the greatest problems in terms of environmental deterioration, especially in tropical rain forests, given the possibility of their disappearance (Dirzo and Raven 2003).

The causes of deforestation are primarily anthropogenic, including forest exploitation, clearing for crop and livestock use, and the development of urban infrastructure, among others (Geist and Lambin 2001, 2002). Socio-economic factors such as land ownership inequality and livestock subsidies have been key factors in land use and deforestation changes for 50 years in Mexico, as in many other developing countries (Masera et al. 1997).

The tropical rain forest is losing forest coverage at a rate of 2% annually (Masera et al. 1997), which has contributed to an increase in carbon emissions and led to reduced carbon sequestering. This is very important considering that a Mexican rain forest stores an estimate of 60% of C in soil and constitutes 37% of net primary production (Hughes et al. 2000).

### *18.1.1 Fragmentation*

Fragmentation is the direct result of the deforestation process, and can increase population mortalities and reduce reproduction rates (Fahring 2003). Pollinator and seed disperser populations are reduced and, thus, the presence of some species in the seedling bank (Benítez-Malvido and Martínez-Ramos 2003). Light, heat, and

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wind exposure can also be increased, which can cause tree damage or death (Laurance et al. 2000; Benítez-Malvido and Martínez-Ramos 2003).

These changes also produce alterations in soil microbiota, of which one of the most important components are the mycorrhiza-forming fungi and nitrogen-fixing bacteria. Hart and Reader (2004) observed that the arbuscular mycorrhizal fungi of the suborder Gigasporineae were significantly less affected by soil disturbance than Glomineae, considering root colonization and spore density.

### *18.1.2 Restoration*

One of the principal objectives of restoration programs should be the facilitation of system function reestablishment, maintaining long-term stability. This could require the restoration of soils using a native microbiotic community (Haselwandter 1997; Requena et al. 2001), especially arbuscular mycorrhizal fungi which allow soil retention by forming aggregates and retaining organic material. This improves soil structure and reduces or prevents water and wind erosion, along with their respective nutrient loss (Jasper 1994; Haselwandter 1997).

Facing rapid rain forest deterioration, restoration ecology practices are extremely necessary as they allow ecosystem recuperation. Restoration ecology is defined as a process where the objective is to recuperate one or more ecosystem functions or attributes. This allows the recuperation of species composition as well as their interactions and relationships until a close resemblance to the original community is obtained (SER 2004; Hobbs 2005). Restoration is based on successional models in order to reconstruct the abiotic environment, and, in turn, helping natural succession processes and a return to the original system conditions (Suding et al. 2004).

In the tropical rain forest, regeneration and succession occur naturally due to the life histories of the species, which are adaptive responses to environmental conditions. Light-demanding species (pioneers) occupy the first successional stages, grow in high light environments, have rapid growth rates, produce many small fruits, and can have invasive growth patterns and low herbivore defense capacities. Shade-tolerant species (late successional) have slower growth rates, produce larger fruits in smaller quantities, develop more herbivore defense and can form seedling banks (Martínez-Ramos 1994). Other species traits are shown in Table 18.1.

### *18.1.3 What is the Role of Arbuscular Mycorrhizal Fungi in Habitat Recovery?*

In the last decade, the use of arbuscular mycorrhizal fungi (AMF) as an additional tool in restoration has been proved to be effective. Maintaining or reestablishing a native community of these fungi can contribute to the recuperation of some soil properties (Allen et al. 2003).

**Table 18.1** Life history traits of plant species of the tropical rain forest at Los Tuxtlas, Veracruz, Mexico

| Variable | Light demanding species | Shade tolerant species |
|---|---|---|
| Light demands | High | Low |
| Relative growth rate | High | Low |
| Photosynthetic rate | High | Low |
| Reproduction | Early; high | Late; low |
| Seed dormancy | Induced | Innate |
| Seed size | Small | Large |
| Seed Bank | Yes | No |
| AMF response | Non-mycorrhizal or facultative | Facultative or obligate |

Pattinson et al. (2004) suggest that AMF are essential in the return from disturbed states to complex communities with close to original conditions. Camargo-Ricalde (2002) emphasizes that AMF are important in restoration ecology not only in terms of plant establishment but also in the function and diversity of ecosystems. If a plant community fundamentally consists of non-mycotrophic plants and the reentry of AMF propagates is slow, natural succession can be retarded and the restoration of that area can be difficult because some plants of late successional species depend on these fungi (Cuenca et al. 2002).

AMF inoculation has started to be common practice in many restoration projects; however, in the tropics few studies have used AMF as a practical restoration tool. In the Great Savanna, Venezuela, Cuenca et al. (1998) studied the establishment of introduced plants and found that plants with AMF and fertilizer grew better. Siqueira et al. (1998) have proposed the use of AMF in southeastern Brazil as a necessary practice for improving plant growth and development in tropical reforestation, specifically due to the high pioneer species responsiveness to AMF. In another study, Cuenca et al. (2002) observed that mycorrhizae and certain quantities of P help the recruitment of native species and recommend their use as a strategy for restarting succession in degraded areas.

On the other hand, Allen et al. (2003) found that AMF inoculum from early successional stages in a seasonal tropical forest provoked significant differences in the growth of arboreal species. Finally, studies carried out by our work group in a Mexican tropical rain forest have shown that pioneer species with AMF have a tendency to survive more than non-inoculated plants. Also, shade-tolerant species respond better to transplant in terms of growth variables if they are inoculated (Álvarez-Sánchez et al. 2007).

## 18.2 Objective

The objective of this work is to provide a general protocol for rainforest restoration, using AMF, based on life history traits of plant species. Our focus considers the responses of plant species to inoculation by using growth analyses that reflect the

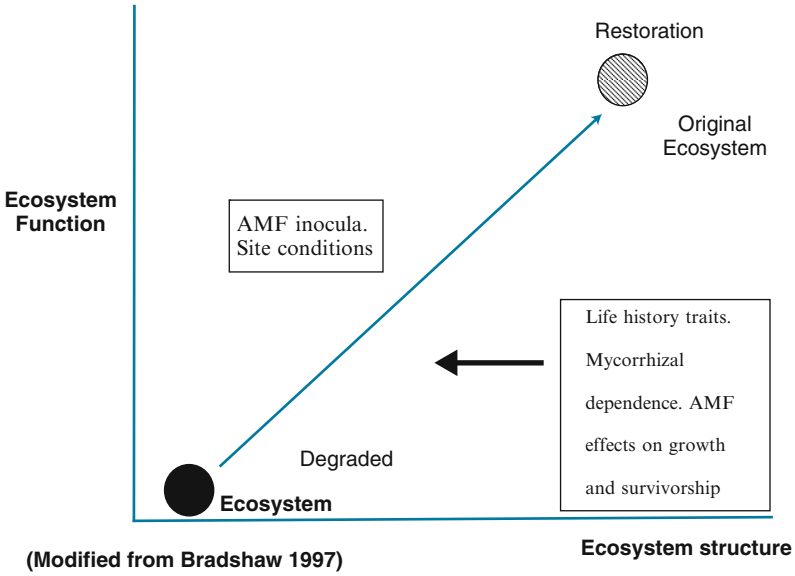


**Fig. 18.1** Use of ecological theory and specific projects (ecological restoration) with arbuscular mycorrhizal fungi for improving ecosystem restoration (modified from Bradshaw 1997)

assignation of biomass and leaf area as well as survival. All are important components in plant adaptation and, thus, determine their establishment success in deteriorated environments. Our proposal is based on the fact that to succeed in rain forest restoration two simultaneous activities are necessary.

1. Continue AMF ecology studies with greenhouse and field experiments. This has allowed us to understand their role in plant species growth, the variation in space and time, and other aspects such as mycorrhizae dependency in light-demanding species. Such is the case of *Heliocarpus appendiculatus* in a Mexican tropical rainforest (Guadarrama et al. 2004), and of *Oyedaea verbesinoides* in Venezuela (Flores and Cuenca 2004) (Fig. 18.1).
2. Carry out specific restoration projects considering anthropogenic effects and local social participation. This allows the acceleration of secondary succession by an integral ecosystem management process (Castillo et al. 2005) which includes human impact (Fig. 18.1).

## 18.3 Case Study

### *18.3.1 Study Site*

The study was carried out at a pastureland near "Los Tuxtlas" Tropical Biology Station that belongs to the Universidad Nacional Autónoma de México, located in

Veracruz State SE, Mexico. The climate is classified as a humid warm tropical type and has three slightly marked seasons, dry, rainy and *nortes*, with 4,725.2 mm of total annual precipitation and 24.3°C of mean annual temperature (García 1988).

Soils of volcanic origin present few horizons, low concentrations of N, P and K, Al and Mn toxicity, and Ca and Mg deficiency. The soils have a clayish-crumbly texture with organic matter accumulations in the upper layers (from 1.64 to 11.11%); pH varies between 5.3 and 6.8. Cationic exchange capacity fluctuates from 5.2 to 46.9 (Sommers–Cervantes et al. 2003). Original vegetation is a tropical rainforest, with a mixture of patches in varying successional stages. On the other hand, pastures used for cattle grazing, remnant and fruit trees characterize deforested land.

## *18.3.2 Methods*

We collected seed from various individuals of each selected species. They were germinated in the greenhouse and inoculated when they reached a height of about 10 cm.

### 18.3.2.1 Transplant and Inoculation

To determine the quantity of spores for plant inoculation, ten samples of 100 g of soil were obtained randomly from the rainforest. These samples were extracted using the wet sifting and decanting method proposed by Gerdemann and Nicolson (1963) and modified by Daniels and Skipper (1982). The samples were observed in a stereoscopic microscope (5×); counting the quantity of spores present in the soil determined the use of 150 spores per 200 g of soil. The root-dying technique of Phillips and Hayman (1970) was used to determine the AMF percentage colonization, which is important for monitoring the inoculation state at the beginning and end of the experiment.

Two factors were analyzed: (1) mycorrhization [three levels: tropical rain forest inoculum (TRF M+), pasture (P M+) and the control without inoculum (M−)], and (2) type of soil [tropical rain forest (TRF) and pasture (P)]. For each factor we used five repetitions for each treatment and each species. It is convenient to inoculate at the beginning of the rainy season, which favors plant growth. After 3 months of inoculation, we transplanted in two parcels abandoned for 6 months using a random plot design.

We registered stem height, leaf area, stem, root, leaf, and total dry weight in the initial harvest in December 2003. In the field, stem height and diameter (DS, 5 cm from the soil surface) was measured along with survivorship. After 7 months in the field, the final harvest was carried out. We measured leaf area (LA) (with a Digital Image Analysis System, version 1.06), dry stem (S), root (R), leaf (L) and total (TDW) weight (after placing them in an oven for a minimum of 72 h at

70°C). We also calculated root/shoot ratio (R/S), leaf area ratio (LAR), specific leaf area (SLA), net assimilation rate (NAR) and relative growth rate (RGR) (Hunt 1982):

Dry leaf weight L; g
Dry stem weight S; g
Total dry weight TDW; g
TDW = R + S + L*
Relative growth rate RGR; g $g^{-1}$ $day^{-1}$

$$RGR = \frac{(\ln(TDW_2) - \ln(TDW_1))}{T_2 - T_1}**$$

Leaf area ratio LAR; $cm^2$ $g^{-1}$
LAR = LA/TDW ***
Specific leaf area SLA; $cm^2$ $g^{-1}$; SLA = LA/L
Net assimilation rate NAR; g $cm^{-2}$ $day^{-1}$

$$NAR = \left[\frac{TDW_2 - TDW_1}{T_2 - T_1}\right]\left[\frac{\log LA_2 - \log LA_1}{LA_2 - LA_1}\right]****$$

*R = dry root weight (this measurement was not taken into account in the field work)
**$T_1$ = initial day, $T_2$ = final day.
***Where LA = leaf area; $cm^2$
****$TDW_2$ and $TDW_1$ correspond to initial total dry weight and final total dry weight, respectively; $T_1$ = initial day, $T_2$ = final day; $LA_2$ and $LA_1$ correspond to initial and final leaf area, respectively.

## *18.3.3 Results*

### 18.3.3.1 Growth Analysis

In this chapter we present only RGR in height and biomass and survival.

In light-demanding species, mean $RGR_{height}$ varied between 0.0032 and 0.0042 cm $cm^{-1}$ $day^{-1}$, while shade-tolerant species varied between 0.0022 and 0.0025 cm $cm^{-1}$ $day^{-1}$ (Fig. 18.2). There were significant differences between species ($p < 0.001$), and the interaction soil × AMF × species was also significant ($p < 0.027$) where *Heliocarpus appendiculatus* height, growing without mycorrhizae and in tropical rain forest soil, was significantly different from the other interactions.

$RGR_{biomass}$ fluctuated between 0.0093 and 0.0129 g $g^{-1}$ $day^{-1}$ for light-demanding species, while for shade-tolerant species t fluctuated from 0.0014 to 0.0059 g $g^{-1}day^{-1}$ (Fig. 18.3). Significant differences for all factors were registered: soil ($p < 0.002$), AMF ($p < 0.027$), species ($p < 0.001$). In this case, *Heliocarpus donnellsmithii* was the one with the highest growth rate, and all interactions were significant: soil × AMF ($p < 0.001$) being the soil from pastureland without AMF the interaction with the lowest values of RGR based on biomass, soil × species ($p < 0.001$), species × AMF ($p < 0.001$), soil × AMF × species ($p < 0.001$). The interaction *Ficus yoponensis* × TRF soil × M + presented the highest growth rate, and it was significantly different from all shade-tolerant species growing in pastureland soil without AMF.

#### 18.3.3.2 Survivorship

Survival percentage rates were between 19 and 51% for light-demanding species; survivorship curve comparisons are showed in Table 18.2. For shade-tolerant species survival percentages were between 20 and 33%, and survivorship curve comparisons are showed in Table 18.3.

### *18.3.4 Remarks*

In general, RGR based on height or biomass, and survivorship values were higher for pioneer species than for shade-tolerant ones. All species suffered a high mortality, mainly because of the dry season and the transplanting.

It is very interesting that in most species, the treatment with the highest RGR response was not highest for survivorship. This probably has to do with the idea of resource allocation: one individual can not allocate all resources to just one of its functions, and certain functions always receive more resources. When resources are allocated to height or biomass, survival is disadvantaged and vice versa.

Most species, independently of their life history traits, clearly improved their growth or survivorship in tropical rain forest soil compared with pastureland, which can probably be explained by soil quality. Quality implies a better structure and a higher AMF diversity, etc. The former result is supported by the hypothesis that AMF determine aboveground diversity (van der Heijden et al. 1998; Klironomos et al. 2000; Hart and Klironomos 2002; van der Heijden 2002) and every forest plant species depends on certain fungi species that are only found in forest soil.

Grouping species by life history traits was insufficient because some of the plant species broke with these classifications. We expected low growth rates and higher response to AMF for shade-tolerant species, but we found that *Ficus yoponensis* and *F. insipida* did not respond in that way; whereas we supposed that pioneer species would have high growth rates and low response to AMF, but

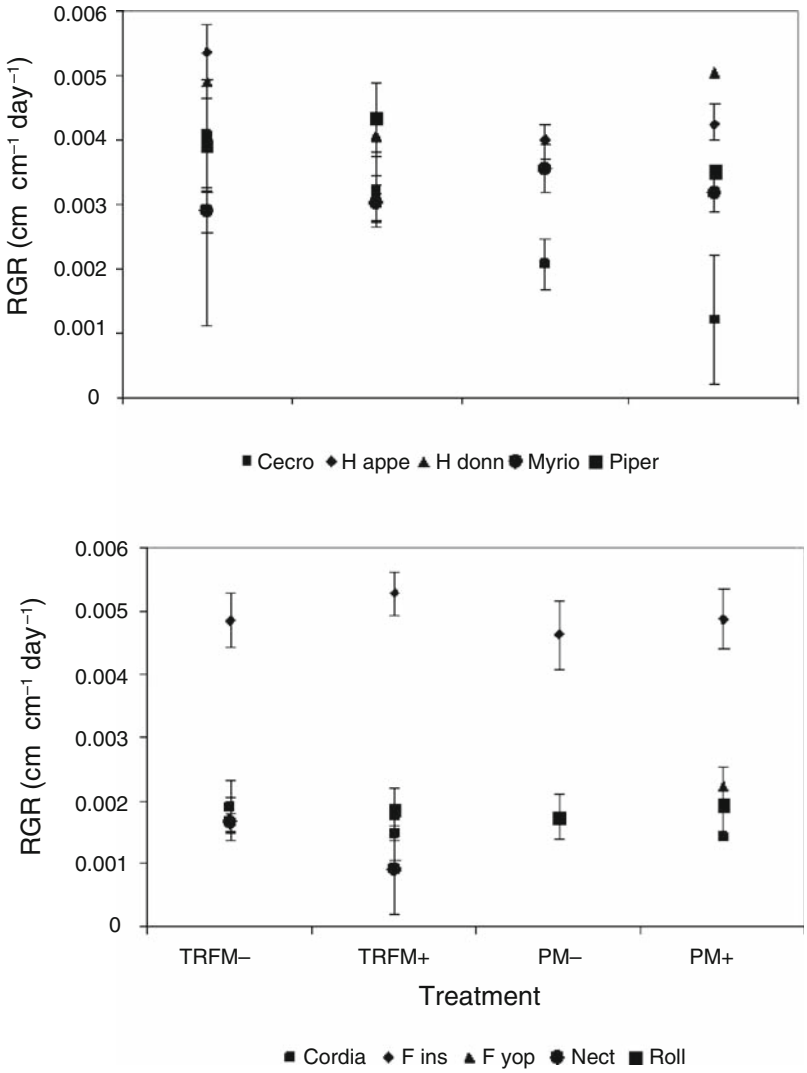


**Fig. 18.2** RGR based on height for light demanding and shade tolerant species. Cecro: *Cecropia obtusifolia*; H appe: *Heliocarpus appendiculatus*; H donn: *H. donnellsmithii*; Myrio: *Myriocarpa longipes*; Piper: *Piper auritum*, Cordia: *Cordia megalantha*; F ins: *Ficus insipida*; F yop: *F. yoponensis*; Nect: *Nectandra ambigens*; Roll: *Rollinia jimenezii*. *TRF:* Tropical rain forest soil; *P* pastureland soil. *M*+: with AMF; *M*−: control

the behavior of *Piper auritum*, *Myriocarpa longipes*, and *Cecropia obtusifolia* was contrary to our expectations.

The use of AMF to restore degraded places seems to be useful for increasing biomass; however, survivorship can be decreased. It is also recommended that soil

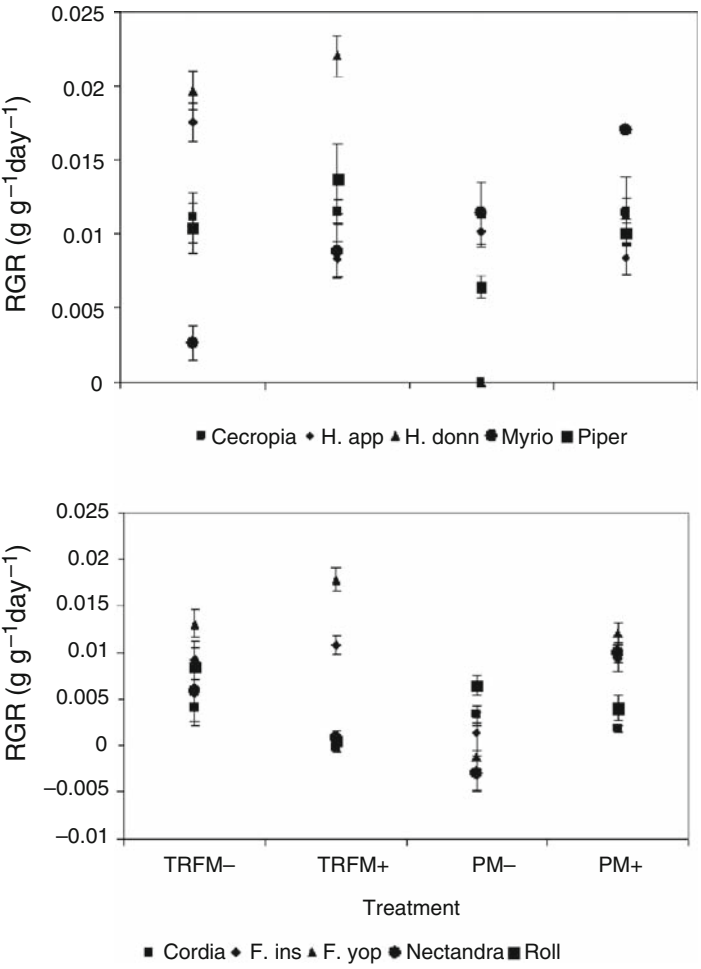


**Fig. 18.3** RGR based on biomass for light demanding and shade tolerant species. Cecropia: *Cecropia obtusifolia*; H. app: *Heliocarpus appendiculatus*; H. donn: *H. donnellsmithii*; Myrio: *Myriocarpa longipes*; Piper: *Piper auritum*, Cordia: *Cordia megalantha*; F. ins: *Ficus insipida*; F. yop: *F. yoponensis*; Nectandra: *Nectandra ambigens*; Roll: *Rollinia jimenezii*. *TRF*: tropical rain forest soil; *P*: pastureland soil. *M*+: with AMF; *M*−: control

from sites where the study species grow naturally is used. In this study, pioneer species survived more than shade-tolerant species.

Mycorrhizal inoculation has the potential to help establish seedlings in deforested sites (Onguene and Kuyper 2005). The increase in height growth with AMF and the mycorrhizal dependence of *H. appendiculatus* (Guadarrama et al. 2004) reinforce the evidence that AMF can favor pioneer growth.

**Table 18.2** Survivorship curves comparisons by pair of treatments for pioneer species. Calculated values of Chi-square were compared to $\chi^2_{df=1,\alpha=0.05} = 3.84$. Where: *X* = there were not enough data for the analysis; *n.s.* no significant differences. When significant differences exist, the name of the highest survivorship treatment is mentioned

| | TRF + M vs TRF−M | P + M vs P−M | TRF + M vs P + M | TRF + M vs P−M | TRF−M vs P + M | TRF−M vs P−M |
|---|---|---|---|---|---|---|
| *Myriocarpa longipes* | n.s. | n.s. | n.s. | n.s. | n.s. | 4.75 TRF−M |
| *Piper auritum* | n.s. | 4.17 P + M | n.s. | n.s. | n.s. | 4.42 TRF−M |
| *Cecropia obtusifolia* | 4.21 TRF−M | n.s. | X | n.s. | n.s. | X |
| *Heliocarpus appendiculatus* | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. |
| *H. donnellsmithii* | n.s. | n.s. | X | n.s. | n.s. | X |

**Table 18.3** Survivorship curves comparisons by pair of treatments for shade-tolerant species. Calculated values of Chi-square were compared to $\chi^2_{df=1,\alpha=0.05} = 3.84$. Where: *n.s.* no significant differences. When significant differences exist, the name of the highest survivorship treatment is mentioned

| | TRF + M vs TRF−M | P + M vs P−M | TRF + M vs P + M | TRF + M vs P−M | TRF−M vs P + M | TRF−M vs P−M |
|---|---|---|---|---|---|---|
| *Cordia megalantha* | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. |
| *Ficus insipida* | n.s. | n.s. | n.s. | n.s. | 5.56 TRF−M | n.s. |
| *F. yoponensis* | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. |
| *Nectandra ambigens* | n.s. | n.s. | n.s. | n.s. | n.s. | 4.94 TFR−M |
| *Rollinia jimenezii* | 3.93 TRF + M | 5.37 P + M | 4.28 TRF + M | n.s. | 6.56 P + M | 5.01 P−M |

## 18.4 Restoration and Arbuscular Mycorrhizae Fungi

Harris et al. (2005) point out that there are various mechanisms which prove that soil biota is critical in ecosystem restoration; at least three of them can be applied to AMF:

1. Establishment of late successional species could only occur when the soil biota contributed sufficient nutrients as a consequence of the successional process.
2. The presence of symbiont propagules can facilitate the establishment of late successional species as suggested by Connell and Slatyer (1977). Allen and Allen (1988) demonstrated this in harsh or very disturbed sites.
3. Symbionts offer competitive advantages to plants and inhibit late successional species establishment, also as suggested by Connell and Slatyer (1977). Requena

et al. (2001) showed that AMF and nitrogen-fixing bacteria caused what they called trophic barriers in experiments in semi-arid Mediterranean ecosystems.

The use of AMF in restoration practices should be seen as an element of ecosystem management. Harris et al. (2005) mentioned that introducing pathogen spores could promote plant diversity; thus the manipulation of organic material or physical soil condition, including fertilizer addition, is not sufficient. However, Maluf de Souza and Ferreira (2004) found that plant community composition and structure changes can depend on forest age and structure. Lekberg and Koide (2005) found that mycorrhizal fungi colonization is affected by agricultural practices.

Cuenca and Lovera (1992) demonstrated that AMF-inoculate recuperation augmented the colonization of native plants in reclamation programs in nutrient-poor areas. However, the response of plant species to AMF inoculate in degraded areas depends on the cost of maintaining the mutual association, principally of the plant in accordance with the trade-offs and life history traits. Hart and Reader (2004) results, where plant biomass and AMF were positively correlated, and Álvarez-Sánchez et al. (2007) with divergent responses, point in this direction.

However, for a tropical rain forest such as that on the eastern coast of Mexico, it is possible to make generalizations about native AMF inoculum use as a favorable response in survival and growth factor is expected.

## 18.5 Conclusions

Finally, restoration success evaluation depends on long-term monitoring of plant and AMF community responses. Because of their fragmented landscapes, tropical rainforests with high connectivity are appropriate for reforestation programs (Hérault et al. 2005). Patterson and Álvarez-Sánchez (unpublished data) have recently discovered that the restoration with fragment edges with *Pleurantodendron lindenii* and *Pimenta dioica* is possible and that the use of inoculum from distinct (small or large) rainforest patches creates a highly complex interaction plant-inoculum.

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# Chapter 19
# Techniques for Arbuscular Mycorrhiza Inoculum Reduction

**Isabel Brito, Mário de Carvalho, and Michael J Goss**

## 19.1 Introduction

It is well-established that arbuscular mycorrhizal (AM) fungi can play a significant role in sustainable crop production and environmental conservation. With the increasing awareness of the ecological significance of mycorrhizas and their diversity, research needs to be directed away from simple records of their occurrence or casual speculation of their function (Smith and Read 1997). Rather, the need is for empirical studies and investigations of the quantitative aspects of the distribution of different types and their contribution to the function of ecosystems.

There is no such thing as a fungal effect or a plant effect, but there is an interaction between both symbionts. This results from the AM fungi and plant community size and structure, soil and climatic conditions, and the interplay between all these factors (Kahiluoto et al. 2000). Consequently, it is readily understood that it is the problems associated with methodology that limit our understanding of the functioning and effects of AM fungi within field communities.

Given the ubiquitous presence of AM fungi, a major constraint on the evaluation of the activity of AM colonization has been the need to account for the indigenous soil native inoculum. This has to be controlled (i.e. reduced or eliminated) if we are to obtain a true control treatment for analysis of AM in natural substrates. There are various procedures possible for achieving such an objective, and the purpose of this chapter is to provide details of a number of techniques and present some evaluation of their advantages and disadvantages.

Although there have been a large number of experiments to investigate the effectiveness of different sterilization procedures for reducing pathogenic soil

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fungi, little information is available on their impact on beneficial organisms such as AM fungi. Furthermore, some of the techniques have been shown to affect physical and chemical soil characteristics as well as eliminate soil microorganisms that can interfere with the development of mycorrhizas, and this creates difficulties in the interpretation of results simply in terms of possible mycorrhizal activity.

An important subject is the differentiation of methods that involve sterilization from those focused on indigenous inoculum reduction. Soil sterilization aims to destroy or eliminate microbial cells while maintaining the existing chemical and physical characteristics of the soil (Wolf and Skipper 1994). Consequently, it is often used for experiments focused on specific AM fungi, or to establish a negative control in some other types of study. In contrast, the purpose of inoculum reduction techniques is to create a perturbation that will interfere with mycorrhizal formation, although not necessarily eliminating any component group within the inoculum. Such an approach allows the establishment of different degrees of mycorrhizal formation between treatments and the study of relative effects.

Frequently the basic techniques used to achieve complete sterilization or just an inoculum reduction may be similar, but the desired outcome is accomplished by adjustments of the dosage or intensity of the treatment. The ultimate choice of methodology for establishing an adequate non-mycorrhizal control depends on the design of the particular experiments, the facilities available and the amount of soil requiring treatment.

## 19.2 Solarization

Solarization is the process of heating soil by covering the land with clear plastic. It is used mainly for control of weeds and plant diseases in regions receiving high levels of solar radiation. In the appropriate climatic regions, a layer of clear plastic film is generally applied to the soil prior to planting and is left in place for 4–6 weeks during the hottest part of the year. Because solarization is a hydrothermal process, its success also depends on appropriate levels of moisture to achieve maximum heat transfer.

Schreiner et al. (2001) reported an increase in the average daily soil temperature of 6–10°C and a maximum daily temperature between 10 and 16°C at 5–20 cm depth. Al-Momani et al. (1988) reported that solarization led to the complete elimination of endomycorrhizal fungi at 10 and 20 cm soil depths, whereas Afek et al. (1991) reported mycorrhizal colonization of cotton roots still occurred after soil solarization.

Bendavid-Val et al. (1997) recognized the uncertainty around whether AM fungi can or cannot survive solarization treatments and developed an extensive study on the subject. Field experiments were carried out in loamy sand and a silt soil. In both cases the presence of indigenous AM fungal populations was investigated using the most probable number (MPN) method. Indigenous fungal populations were reduced to zero after 2 or 4 weeks of solarization treatments; however the *Glomus*

*intraradices* introduced into the field was not affected, probably due to its tolerance of changing environmental conditions. The dramatic reduction in the number of infective propagules of sensitive species was found to be more pronounced in the upper soil layers then in lower ones. In both experiments, plants (wheat, onion and carrot) sown on solarized plots were colonized within 5 or 6 weeks after emergence. The authors suggested that some of the AM inoculum potential in the soil was in the form of hyphae and was thus particularly sensitive to the high temperatures developed during the solarization treatments; a longer time was required for the few remaining propagules (many as spores) to colonize plant roots. They also concluded that the effect of solarization varied according to AM fungal species, inoculum form, density and host crop together with the duration of solarization.

Schreiner et al. (2001) monitored the infectivity of AM fungi before and after solarization of two fields using a greenhouse bioassay with *Sorghum bicolour*. Infectivity was greatly reduced in solarized plots 8 months after solarization (over winter) in both years tested. Results were similar for greenhouse or in-field bioassays. These authors concluded that solarization indirectly reduced AM fungi in soil by restricting the weed populations that maintained infective propagules over winter.

As with other forms of soil heating, solarization results in complex changes in soil physical, chemical and biological properties. Availability of many mineral nutrients is increased following solarization, particularly those mainly associated with organic matter, such as $NH_4^+$and $NO_3^-$. Amounts of these ions can vary considerably depending on the aeration of the soil, which is a function of the soil physical properties and moisture content, as well as the presence of nitrifying microorganisms. Extractable P, K, Ca and Mg may be present in greater concentration after solarization (Stapleton 1990), together with Cu, but Zn may decrease (Baptista et al. 2006).

The increase in soluble mineral nutrients and the various different effects on AM colonization dependent on AM fungal species and soil conditions combine to make solarization a tool that needs very careful consideration before use in mycorrhizal research. When AM fungi are re-inoculated after solarization, colonization rates can be 20% higher (Afek et al. 1991; Nair et al. 1990).

## 19.3 Steam Sterilization

Autoclaving is widely used to sterilize soil samples, as the equipment is readily available in most microbiology research and teaching laboratories. Soil is autoclaved at 121°C at 1.1 atm for a minimum of 20–30 min. The length of time has to be increased when large amounts of soil are used, but treatment of big volumes at one time should be avoided, and the soil separated into several smaller volume containers to establish an effective distribution of the heat. For the same reasons the soil should not be packed or compressed onto containers, but left unconsolidated to allow the steam to permeate. Cotton material bags perfectly satisfy this requirement. Soil should be air-dried or with a water content of less than about

60% of moisture-holding capacity to permit better sterilization (Trevors 1996). Sterilization results in the destruction of both microbial cells and spores. However, some resistant spores may germinate after a first cycle of sterilization, so that a second cycle might be recommended after a 24 h interval.

Steam sterilization is an efficient method of eliminating the indigenous population of AM fungi (Smith and Smith 1981; Thompson 1990; Vosátka 1995); however, the process may alter the structure and physicochemical properties of the soil (Gantotti and Rangaswami 1971). The effects on the chemical and mineralogical properties, although seemingly not that obvious (Egli et al. 2006), can result in the release of nutrients, which may affect the growth of non-mycorrhizal control plants. Furthermore, if there is a re-inoculation with mycorrhizal propagules, the elevated nutrient content may hamper root infection by mycorrhizal fungi (Blanke et al. 2005).

Net mineralization ($NH_4^+$) levels increase but nitrification is inhibited because of the elimination of nitrifying bacteria, as a result of steam sterilization. There is also an enhancement of the extractable P content and can be a slight increase in pH (Thompson 1990; Alphei and Scheu 1993). Depending on the soil, release of trace elements such as Mn, Fe and Cu is also promoted by steam sterilization (Aryabod et al. 2006).

The changes resulting from steam sterilization has limited its adoption as a technique for establishing negative AM control. However, Smith and Smith (1981) studied the effect of early endomycorrhizal infection on nodulation and growth of *Trifolium subterraneum* L. in non-sterilized and steam-sterilized soil. They concluded that growth differences were a classical mycorrhizal response, and did not reflect toxic effects of sterilization. Significantly, Smith and Smith (1981) reported that the growth of *Brassica oleracea* (broccoli), which does not form mycorrhiza, was better on sterilized soil.

## 19.4 Pasteurization

The pasteurization process involves the application of heat, but raises the temperature of the soil for shorter periods of time than required for steam sterilization. Consequently, chemical changes in the soil are also not as great.

Heating the soil to 60°C for 4 h had no significant effect on soil nutrient concentrations and reduced AM colonization of *Plantago lanceolata* roots by less than 1% (Endlweber and Scheu 2006), suggesting that moderate heating is preferable to other methods, at least for experiments to investigate effects of AM and their interactions with decomposer organisms on plant growth.

The only differences found between the nutrients of pasteurized (soil slowly heated to 80°C, maintained at this temperature for 2 h, and then allowed to cool) and non-pasteurized soils were increased extractable $NH_4^+$-N and $NO_3^-$-N (McGonigle and Miller 1996). There were no changes to concentration of P, Mg or K or to pH,

but arbuscules in roots were reduced to trace numbers. Scagel (2004) reported only trace AM colonization of Brodiaea "Queen Fabiola" in pasteurized soil.

## 19.5 Gamma (γ)-Irradiation

A number of studies have suggested that γ-irradiation is highly effective as a biocide and preferable to other methods of treating soil, because it can have less of an impact on soil chemical and physical properties, including little effect on particle size or aggregate stability (Bowen and Rovira 1961; McLaren 1969). The advantage of γ-radiation over other similar techniques is that it does not induce sample radioactivity, making handling safe; on the other hand, it requires special equipment not readily available in most laboratories.

Generally the greater the size or complexity of an organism the more susceptible to radiation it is: fungi seem to be more sensitive than bacteria (McLaren 1969). The larger the sample, the more likely that a variable dose may be delivered due to internal shielding from irradiation (Yardin et al. 2000). An irradiation source strength of 3 kGy, smaller than the 10 kGy usually recommended, may be enough to eliminate AM fungi infectivity, and have little impact on soil conditions (Kahiluoto et al. 2000; Thompson 1990). However, radiation requirements depend upon the soil type, moisture content and former management (Powlson and Jenkinson 1976; Parekh et al. 2005).

As a consequence of soil γ-irradiation, short-term increases in temperature and nutrient release can occur (Yardin et al. 2000). More $NH_4^+$-N, organic N, small amounts of Mn, soluble C and exchangeable S and P has been reported after γ-irradiation of soil (Alphei and Scheu 1993; McLaren 1969, Thompson 1990). $NO_3^-$-N generally declines after γ-irradiation, but pH variation showed no consistent trends (McNamara et al. 2003). The release of soil-bound residues is not modified by γ-irradiation compared with autoclaving (Nakagawa and Andrea 1997).

One of the clear advantages of γ-irradiation is that it is highly effective at sterilization and leaves no chemical contamination post-treatment, and re-inoculation experiments can take advantage of this. Of particular interest is the potential for γ-irradiation to be used as a tool for selectively manipulating biodiversity in soils while causing minimal disruption (McNamara et al. 2003). Comparing AM-inoculated and non-inoculated plants in partially sterilized soil at 10 kGy γ-irradiation, Thompson (1990) unreservedly recommended the method for use in nutritional studies.

## 19.6 Chemicals

Various gaseous chemicals (ethylene oxide, propylene oxide, chloroforms) have been used to fumigate soil. Methyl bromide ($CH_3Br$), an extremely poisonous gas, appears to be especially toxic to AM fungi and many researchers have used it as a fumigant to eradicate AM fungi from experimental soils (Plenchette et al. 1983;

Thompson 1990; Vosátka 1995); however, toxic effects of inorganic bromide residues in the soil and phytoxicity symptoms resulting from Br concentrations in plant tissue may occur (Alphei and Scheu 1993; Thompson 1990). Use of methyl bromide is being banned due to its adverse effect on the ozone layer and is scheduled to be phased out in the second decade of the new century (Bell 2000).

Ethylene oxide ($C_2H_4O$) is a colorless, flammable gas at room temperature and pressure. To sterilize soil, it is introduced under reduced pressure to containers holding the soil in pots or trays. Changes in the soil physical and chemical properties seem to be minor (Rose and Bailey 1952).

Propylene oxide ($C_3H_6O$) is a colorless, extremely flammable liquid that can alkylate functional groups of proteins. In a comparison of propylene-oxide-treated soil with untreated material, Alphei and Scheu (1993) reported a marked increase in the $CO_2$ release throughout the experiment and smaller mineral nitrogen content, indicating immobilization of N for microbial growth.

Both ethylene and propylene oxide can increase soil pH during fumigation and produce residues that hinder plant growth (Trevors 1996).

Chloroform ($CHCl_3$) is highly volatile and has been used to fumigate soils for the estimation of microbial biomass. Chloroform fumigation can defaunate the soil, not necessarily eliminating microbial populations completely (Alphei and Scheu 1993). Endlweber and Scheu (2006) reported a massive reduction in AM colonization of *Plantago lanceolata* roots by more than 99% in plants from chloroform treated soil, but there was also an effect on plant growth and nutrient concentrations within plant tissue.

Other fungicidal chemicals can also be used to prevent the development of AM. Mostly these chemicals adversely affect AM fungi (Manjunath and Bagyaraj 1984; Salem et al. 2003) although the degree of toxicity varies with the active ingredient, the application rate (Habte and Manjunath 1992) and specific AM fungal isolate (Schreiner and Bethlenfalvay 1997). The disadvantages of using fungicides to create an indigenous inoculum-free soil relate to the fungicide residues left in the soil that might be toxic to re-inoculated microbes or to the plants. According to Kahiluoto et al. (2000) Benomyl (carbendazin) incorporation in the soil, besides being most effective in suppressing AM fungi, is the most appropriate method currently available for creating a non-mycorrhizal control for AM fungi community in the field, irrespective of soil type and management history since Benomyl treatment showed no ecologically significant effects on soil pH or K, Ca and Mg contents or phyto-toxicity agents like Al, Fe, Cu or Mn in the experiments they performed.

The use of formaldehyde to suppress AM fungi was tested by Covacevich and Echeverria (2003) and they concluded that the concentration range of 1,67 to 5% (formaldehyde-water) effectively eliminated indigenous mycorrhizal colonization without restricting plant growth and allowed the development of inoculated AM isolates.

An extensive review made by Menge (1982), on the effects of many other fumigants and fungicides specifically on AM fungi, is highly recommended despite the passage of time since it was published.

## 19.7 Soil Disturbance

The direct effects of soil disturbance on mycorrhization are related to physical disruption of the soil hyphal network and to the mixing of surface residues within the soil profile, affecting the effectiveness of AM symbiosis.

When host plants are present and the soil is not disturbed, hyphae from colonized roots and mycelia network are the main source of inoculum, they are more rapid and efficient at initiating colonization (Martins and Read 1997) than are spores. Spores are considered to be "long-term" propagules (Kabir 2005), mainly because it would take longer for spores to germinate and for the hypha to make contact with roots as opposed to runner hyphae infection from well-developed extra-radical mycelium (Klironomos and Hart 2002). Evans and Miller (1990) demonstrated that disruption of the hyphal network was directly responsible for much of the effect of soil disturbance on mycorrhizal colonization, leading to differences in AM colonization of almost 50% in pot trials and Brito et al. (2006), under field conditions, reported 20% differences in AM colonization of wheat.

In addition, deep ploughing (to more than 15 cm) hinders subsequent mycorrhiza formation by reducing propagule density in the rooting zone (Kabir et al. 1998). Abbott and Robson (1991) observed that no-tilled soil had more spores in the top 8 cm whereas tilled soils had more spores in the 8–15 cm depth.

Fairchild and Miller (1988) developed a "Cycles Technique" to study the differences in AM colonization and P absorption in disturbed and undisturbed soil. Air-dried soil was sieved (5 or 4 mm), packed into the pots to a natural bulk density of approximate 1,2 g $cm^{-3}$ and sown with the desired plant. Three weeks after emergence, plant shoots were excised and measured. Half of the pots were then taken, the soil removed as two layers and passed separately through a 4 mm sieve. All root material separated on the sieve was cut into 2 cm-long segments and mixed into the soil of the appropriate layer. Soil was repacked in the pots and arranged in the same two layers. In the other half of the pots the soil remained undisturbed. The pots were reseeded and a new cycle initiated. The authors argued that the possible microbial flush of N (mineralization) in the soil caused by disturbance ought to be negligible compared with the relatively large concentrations of N (100 μg $g^{-1}$) added to the soil at the start of the experiment and further insist, this small amount of N will mainly be released in the initial phase of the experiment. The effect of soil disturbance on AM infection could be mediated through changes in soil physical properties; however, bulk density measurements were unable to discern any significant differences between the two soil treatments.

Using this technique, and after three or four cycles of disturbance, greater colonization rates are observed consistently in plants coming from undisturbed soil pots (Goss and de Varennes 2002; McGonigle et al. 2003; Antunes et al. 2006; Brito et al. 2006).

A number of advantages are associated with this technique, namely that it does not make use of any toxic compounds, causes little nutrient release, exploits the naturally occurring inoculum and allows a common history of inoculum and host plant throughout the successive cycles.

## 19.8 Crop Rotation

Although most crops are dependent upon mycorrhizal fungi, roots of some crops such as, for example, the ones belonging to the Chenopodiaceae and Brassicaceae families, do not form mycorrhiza. When such crops are used in rotation, they tend to lead to a reduction in mycorrhizal propagules. Arihara and Karasawa (2000) studied the effects of fallow and the previous cultivation of sunflower, maize, soybean, potato, sugar beet and canola (oilseed rape) on AM colonization of maize, and found that shoot weight and grain yield of maize were much greater in the plots following sunflower, maize, soybean and potato than those after canola or sugar beet (non-mycorhizal crops) or fallow. The cultivation of a non-AM host such as sugar beet or canola reduces the mycorrhizal propagules and consequently AM colonization of the following crop (Arihara and Karasawa 2000; Gollner et al. 2004) even with no alteration to the availability of P in the soil induced by the previous crop (Karasawa et al. 2001).

Reduction of AM propagules is also associated with the practice of bare-fallow. Because AM fungi are strictly biotrophic, their survival depends on the presence of host plants. Harinikumar and Bagyaraj (1988) reported that leaving the land fallow decreased the mycorrhizal propagules in 40%, while growing a non-mycorrhizal host reduced it by 13%.

## 19.9 Other Methods

Ozone, possessing strong oxidative and germicidal properties, has a very short half-life of minutes or less in soil and decomposes to simple diatomic oxygen. Takayama et al. (2006) developed an ozonation technology based on the generation of electrical discharges by applying high voltage to soil placed between two electrodes. Soil treatments of 20 g $O_3$ $m^{-3}$ for 10 min almost killed *Fusarium oxysporum* and with a 20 min treatment over 80% of the soil bacteria were eliminated.

Although not directly interfering with the AM inoculum the use of isogenic $myc^-$ mutants of AM hosts as a non-mycorrhizal control may be useful to avoid disruptive soil treatments and the safety and environmental problems caused by most of the chemicals. The use of $myc^-$ mutants was investigated by Kahiluoto et al. (2000), but problems with AM dependence, compatibility with indigenous AM fungi communities and agricultural relevance, due to the limited selection of $myc^-$ mutants available, are difficult to overcome. AM mutants currently available have all been isolated from pre-existing $nod^-$ pools. As a result these mutants have modified genes that play a role in both mycorrhiza formation and nodulation. Given the fact that nodulation is essentially restricted to one plant family, whereas AM are widespread, a significant number of mycorrhiza-specific genes must exist (Marsh

**Table 19.1** Techniques for AM inoculum reduction and main implications for soil properties

| Method | AM inoculum | | Soil chemical and physical effects | | Comments |
|---|---|---|---|---|---|
| | Elimination | Reduction | Nutrient release | Soil structure | |
| Solarization | | X | Yes | kept | Appropriate climatic conditions |
| Steam sterilization | X | | Yes | Changed | Easily available, small volumes of soil treated at any one time |
| Pasteurization | | X | Only N | Kept | Less destructive then sterilization |
| γ Irradiation | X | | Very few | Kept | No post-treatment chemicals, small volumes of soil treated at any one time |
| Chemicals | X | | Yes | Kept | Toxicity |
| Soil disturbance | | X | No | Changed | Time-consuming |
| Crop rotation | | X | No | Kept | Time-consuming |
| Ozone, myc$^-$plants | – | – | – | – | – |

and Schultze 2001). Once they are identified, myc$^-$ mutants may became a more helpful tool for non-mycorrhizal controls.

## 19.10 Conclusions

No method developed for reducing the competition from indigenous AM fungi is ideal for every application, and some still require a full evaluation. Table 19.1 summarizes the techniques described and their effect on AM inoculum, and indicates some of the implications for soil physical (structure) and chemical (nutrient release) characteristics.

Judicious use of crop rotations provides an important opportunity to minimize the competition to introduced inoculum from indigenous AM fungal species on a field scale without having major impacts on the general nutrient status of the soil or on the structure of the soil. Weeds and volunteer plants could reduce its efficacy. Furthermore, the time frame for preparing the land requires long-term planning. Most rapid approaches have limitations because of the volume of soil that can be treated at one time, or because of changes in nutrient availability or structural properties. As we improve our ability to characterize species diversity and quantify the number of individuals in real time, techniques that encourage the use of local indigenous beneficial species may be of greatest benefit.

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# Chapter 20
# Best Production Practice of Arbuscular Mycorrhizal Inoculum

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## 20.1 Introduction

The application of quality assessment standards to arbuscular mycorrhizal inoculum (AM) mainly requires that the product is 'fit for purpose'. The product must meet or exceed the customer's requirements. It is the customer who sets "quality standards" in terms of his expectations. Customers may define different quality characteristics for mycorrhizal inoculum: formulation, handling, weight, safety, functionality or others. Socio-Economic Impact Analysis and adequate Environmental Risk Assessments carried out along with Life Cycle Assessment are measures for energy, material flows and impact estimates associated with all stages of a product from inoculum producer to the customer. Finally, the Cost-Benefit Analysis Process (on the basis of the Business Ethics Assessment) involves monetary calculations of initial and ongoing expenses vs expected return (Compare Feldmann 2007).

All these instruments should be common practice in a company producing inoculum for national or international market. Only the application of all instruments of concurrent quality control procedures results in a traceable and reliable supply chain with the consequence of reliability of the whole product chain as basis for sustainability. This pre-requisite prevents the customer from buying some expensive, non-effective instead of high-quality AM inoculum.

The basis for production of high-quality AM inoculum is the understanding of biological principles of the population biology of AM fungi (AMF). But we shall not go into too much detail when documenting this biological basis, the working hypotheses or the procedures chosen. In this chapter we describe how to design an

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inoculum of an AMF generalist and how to produce it on a large scale (>8 × $10^9$ spores per year).

## 20.2 Working Hypotheses (Following Feldmann 1998)

- AMF are phenotypically highly variable due to their multicaryotic and heterocaryotic spores.
- Hosts temporarily canalize functional genotypes depending on environmental and endogen plant factors.
- Under uniform conditions AMF spore multiplication results in widely reproducible effectiveness only for maximally three multiplication cycles.

## 20.3 Basic Assumptions for the Inoculum Production (Following Feldmann and Grotkass 2002)

- A plant does not aim for colonisation by mycorrhizal fungi. It depends on the host and fungus genotypes, on their coherence and on favourable environmental conditions to develop a mycorrhiza (Allen 1991). This offers the possibility of choosing a large amount of suitable host/fungus combinations for inoculum production. The relevant taxonomic units for specific phenomenon are plant variety and fungal strain.
- The process of colonisation by mycorrhizal fungi means stress for the host and the AM symbiosis is a parasitism-mutualism continuum. This can easily be observed after inoculation of young seedlings expressing growth depression. Following the stress theory of Stocker (1947) this can be overcompensated after the "alarm phase", resulting in a desired host response (e.g. better growth), and can be stabilised by product exchanges (e.g. carbohydrates vs water and nutrients) with concurrent mutualism. In inoculum production we prefer and force the host plants to allocate as much carbohydrate as possible to the fungus. We balance the developing parasitism-mutualism continuum (Johnson et al. 1997; compare Feldmann, 1998b) by nutrients and irrigation to favour fungal sporulation.
- Ecological characteristics of AM inoculum can be designed by pre-adaption processes (Feldmann and Grotkass 2002). This offers the possibility of replacing expensive, time-consuming screenings of isolates with a short shelf life. Biological basics to be considered are the problem of the ecological niche of AMF and hosts, limiting factors of plant growth and phenotypic plasticity of both.

- Phenotypical characteristics of inoculum are often expressed on the basis of fungal population composition (Feldmann 1998). This requires stepwise multiplication cycles during up-scaling procedure, concurrent quality control and advanced mixing techniques which take into account the phenotypic variation of mycorrhizal fungi.

On the basis of these working hypotheses, four steps are demonstrated:

1. In the planning phase, the requirements of the inoculum production are outlined.
2. In the analytical phase, the tuning of inoculum is described, and the immanent functional variability of inoculum is tested.
3. In the adaption phase, how to extend the abilities of inoculum is defined.
4. Finally, the up-scaling phase of inoculum production is described

This chapter will be completed by describing concurrent quality control procedures.

### *20.3.1 The Planning Phase: Define what you Need*

The aims which an inoculum should meet, which organisms are involved and the purpose of the product dominate the planning phase, and result in a biological data sheet. Following our experience, the design of an inoculum for very general use is possible, but requires much more attention, knowledge and experience than a more limited design. Nevertheless, the commercial exploitation of a generalist's inoculum is much more attractive than restricted inocula. As an example, we describe the planning phase for an inoculum to be used in ornamentals in greenhouses (as previously developed for German gardeners; see Feldmann et al. 1999).

The Biological Data Sheet (BDA) should contain specifications about host and fungus (Table 20.1). Details concerning the desired target plants are fixed. The evaluation of this commercial BDA should lead to discussion of desired inoculum characteristics including desired carrier materials, formulations and acceptable pricing (maximum price). In our example it was vital that the fungus should colonize a range of different ornamental plant species within 2 weeks (between potting the first and second time) and developing growth responses within 8 weeks after the second potting (before sale to the customer). This inoculum had to tolerate high dosages of fertilizer. Furthermore, the price had to be very low because the user was not the one to expect the benefit, but buying interest was based on added value regarding inner quality of plants.

At this point you have to decide on a starter inoculum. The more detailed the knowledge available on the isolates, the easier it is to select AMF from one's own gene bank, or from an *in situ* conservation area (Feldmann and Grotkass 2002), to isolate fungi or to buy them.

**Table 20.1** Biological data sheet for inoculum design and planning

| Parameter | Macrosymbiont (Host) | Microsymbiont (AMF) |
|---|---|---|
| Species, varieties/strains | *Heliotropium arborescens* cv. "Marine", *Bidens ferulifolia* cv. "Goldmarie", *Brachycome iberidifolia; Chrysanthemum* cv. "Maja Bofinger", *Lobelia erinus* cv. "Cobalt Blue", *Lantana camara* cv. "Feston Rose", *Sutera cordata* cv. "Snowflake", *Sanvitalia procumbens* cv. "Gold Braid", *Pelargonium cv.* "Butterfly", "Leuchtkaskade", "Grand Prix", *Verbena x hybrida cv.* "Imagination" and "Romance" | *Glomus etunicatum* |
| Range of intended use | Ornamental greenhouses; mycorrhization from January to March | |
| Desired mycorrhizal effect | Shortening of the "standing period" of ornamentals in greenhouses, earlier flowering and therefore earlier sale Longer flowering under balcony conditions Underlying effect: quicker growth | |
| Care requirements after installed (water weekly, water once, plant protection etc.) | Temperature: 14–21°C; fertilizer: max. 2 g/l × week of NPP 18/12/18; use of diverse pesticides, sprayed and in the irrigation water | |
| Natural occurrence (where, how common) | Not relevant | Cosmopolitan, not in sterilized substrates |
| Habitat preferences | Warm, moderate | Very variable |
| Strategy type/successional stage (stress-tolerator, competitor, weedy/ colonizer, etc.) | r- and k-strategists | r-strategist; strain selected for rapid colonisation; generalist |
| Potential ecological main factors under natural conditions | – | High tolerance to high fertilizer concentrations |
| Associated species | No | No |
| Material provided by/ collected as/from | Plants available from production companies | Gene bank |
| Propagation | Cuttings | DIPP with two steps (medium scale) To be adapted to pH 5.0 |

(*continued*)

**Table 20.1** (continued)

| Parameter | Macrosymbiont (Host) | Microsymbiont (AMF) |
|---|---|---|
| Soil, substrate or medium requirements for cultivation | Humus-rich, moist soil, with pH 5.0 to 6.5 (mildly acidic) | |
| Specific growth, spread; or lifespan conditions (later host plant) | Introduction very early after preparation of cuttings or seedlings; three changes of users: seedling company, nursery, selling company, consumer | |
| Desired carrier material for plant material or propagules; technical requirements | Standard soil, potted | Expanded clay particles (1–2 mm diameter, broken), maximally 0.5% of later pot volume (defined by plant producer); suitability for automatic potting |
| Restrictions or guidelines | Free of oomycetes, free of weed seeds, free of fertilizers | |
| Maximum price | 0.12 Euro/cutting in average | 0.01 Euro/cutting in average |
| References (own experiences) | Feldmann et al. (1999); Weissenhorn and Feldmann (1999) | |

### *20.3.2 The Analytical Phase: Make your AMF Isolate a Strain and Describe its Abilities*

In the literature, a large number of examples for positive mycorrhizal effectiveness in scientific experiments are published. Most AMF users do not apply AMF strains, but inocula on the species level, undefined AMF or mixtures of different sources. Experience shows that offering host plants any mycorrhizal symbiont results in some effectiveness if the plant has a need for symbionts.

The important point is that the predictability of effectiveness is of crucial importance for commercialisation: no plant producer will buy and introduce AM inoculum if the outcome is not to some extent predictable. Only the description of the inoculum, possibly followed by selection processes or mixing procedures offers the possibility of preparing inoculum with desired characteristics and predictable effectiveness. Against that background we recommend that a strain is developed from field collections, or that a starter inoculum with exactly described content is sought.

When developing a strain, we start with the description of phenotypic variability in test plant populations resulting from the cloning process of single spores.

Here, the microsymbiont was represented by single spore descendants of a taxonomically not distinguished Glomus sp. (probably *Glomus etunicatum*, Table 20.1, compare Feldmann 1998b). The spores were produced on *Petroselinum crispum* in sand and used for inoculation after extraction from the soil by wet sieving and decanting techniques (Schenck 1984). For inoculation, single spores were separated with micropipettes and placed near the rhizosphere of the host plant. Sand was used as substrate and the plants were kept in 2.5 ml plastic tubes first and

transferred to 25 ml plastic tubes after two weeks under controlled greenhouse conditions according to Feldmann et al. (1998a): illumination by SON-T AGRO 400 Philipps lamps (360 μE. m-2. s-1), 14 h per day; 60–80% relative humidity; 18–20°C night, 22–26°C daytime; irrigation below field capacity; fertilisation once per week with 1% pot volume of a commercial fertiliser solution (1 g fertiliser/l solution), pH 5.5. The fertiliser contained 15% N (10% nitrate, 5% ammonium), 7% $P_2O_5$, 22% $K_2O$, 6% MgO, 0.03% B, 0.05% Mn, and 0.01% Zn.

For the analysis of AM phenotype frequencies in the developed strain, we chose test plants with a broad ecological niche and easy to cultivate: *Anagallis arvensis* and *Plantago lanceolata*. The selected plant species occur on arable lands, on open, sandy or rocky habitats or wasteland, and even polluted areas. They can be found on soils with pH between 4.5 and 8.0. Soils may be poor or rich in nutrients, variable temperature and light is tolerated. The ecological niche of these plant species covers most of the factors important for agricultural and horticultural practice; both are intensively colonized by mycorrhizal fungi (Weissenhorn and Feldmann 1999).

For inoculation, single spores were separated with micropipettes and placed near the rhizosphere of the host plant. At that time cuttings (*Anagallis arvensis*) had a root system of approximately 6–7 cm length and the upper plant parts were at homogeneous developmental stage (i.e. the variation of shoot length, leaf number and leaf size was not larger than 5%).

Plants were inoculated with single spores and plant fresh weight was measured after 8 weeks of cultivation (C1). After that, from three colonized host plants of significantly different fresh weight each time ten single spores were isolated and inoculated to new host plant individuals. After another 2 months the fresh weight was measured (C2). All sub-strains of C2 deriving from one single spore (C1) were mixed and 15 single spores each isolated from this mixed population and afterwards inoculated. The third propagation cycle was carried out within the next 2 months.

Mycorrhizal colonization was qualitatively determined after cleaning the roots in 10% KOH for 15 min, neutralisation with HCl, washing and staining three times for 25 min each time in 0.05% trypan blue in lactic acid/glycerin (10:1 vol/vol). For estimating the degree of colonization the whole root system was used.

The mycorrhizal efficiency (MEI) index was estimated according to Bagyaraj (1994):

$$\mathrm{MEI} = \frac{\text{weight of inoculated plant} - \text{weight of uninoculated plant}}{\text{weight of inoculated plant}} \times 100$$

Statistical evaluation of the data was carried out by the one-way analysis of variance (ANOVA) for the respective factors with a significance level at 5%.

Following these methods it was observed that inoculation with single AMF spores from cloned strain shows a variability of effectiveness from slightly effective to medium to highly effective (Fig. 20.1, C1). The multiplication of single spores from sub-populations with distinct effectiveness conserved the characteristics in the next propagation cycle (C2), though the variability of effectiveness increased after a further propagation cycle (C3). Distinct characteristics of the sub-populations no longer existed after C3.

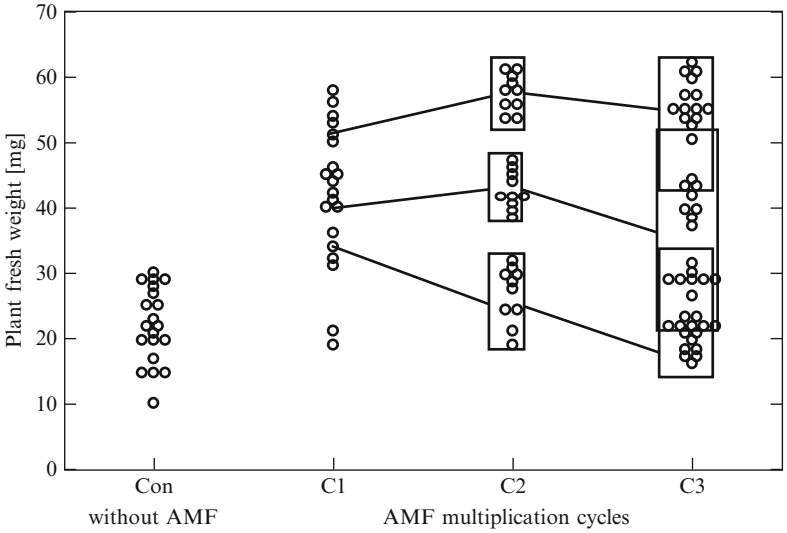


**Fig. 20.1** Mycorrhizal effectiveness of AMF single-spore descendants (*Glomus sp.*) on the biomass of *Anagallis arvensis*. See distinct sub-population characteristics in C2 and overlapping effectiveness in C3

The reproducible response of the clonal host under standard conditions caused by AMF descendants of single spore isolates verified the existence of genotypic differences in the initial spore population. The slight variability of effectiveness during the first propagation process reflects the still-existing variability of the plant material and experimental errors. If the variability of effectiveness observed in C1 had been the result of phenotypic plasticity of only one fungal genotype, the same variability would have had to occur in C2.

After the second propagation cycle the distinct characteristics of the genotypes start to become modified because there is an increase of variability in effectiveness in C3 (Fig. 20.1). The basic mechanism for the enhanced variability in effectiveness of genotypes still remains unclear. Host gene/AMF gene adaptations are as possible as high mutation rates of the fungus.

For practical application, the findings are of special importance: if genetically fixed characteristics of AMF spores are stable for only one or two propagation cycles, AMF inoculum production should not be based on past inoculum charge but on fresh spore material from stock cultures. This complicates up-scaling in inoculum production, because slight differences, as shown for the effectiveness of C3 (Fig. 20.1), can create considerable changes in effectiveness of an inoculum produced by this method (Feldmann et al. 1999).

Based on this system a wide range of parameters can be tested: velocity of colonization, salt content, and heavy metal stress (Feldmann and Grotkass 2002). Phenotypic variability exists in every case and we recently described, using quantitative molecular genetical methods, which percentage of the variability might be genetically fixed and heritable.

### 20.3.3 The Adaption Phase: Direction Instead of Screenings

The best sub-strains of C2 for adaption of inoculum to lower soil pH (or P-content stressors such as drought, salt or heavy metals and others) are chosen (Fig. 20.2b).

Ten cuttings of *Anagallis arvensis* per treatment in three parallel repetitions are grown until they develop a considerable root system (conditions as above). Before inoculation the soil is infiltered with nutrient solution of changed pH (pH 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0) until the run-off has the same pH as the infiltrated solution. For inoculation approximately 100 spores are transferred into the substrate near the roots of *Anagallis arvensis*. After 21 days the plants are carefully extracted from substrate, the roots washed to remove old spores and planted to a larger pot (50 ml) with fresh substrate. The time period may be chosen following your requirements. Here it is short because of the need to select "rapid" genotypes in addition to pH-tolerant ones. The plants remain in that pot for approximately another 4 weeks until sporulation of the fungus. Plants are harvested, shoot fresh-weight determined and mycorrhizal status of roots analysed.

An analogous experiment with different phosphate concentrations in the substrate at pH 5.5 was carried out (5 ppm, 15 ppm, 30 ppm, 60 ppm, 90 ppm, and 120 ppm) and has already been published (Feldmann and Grotkass 2002).

At extreme soil pH the colonization of the host plants initially may be low (Fig. 20.2a). But the percentage of spores within the tested inoculum able to colonize under extreme conditions can be enhanced by separate propagation and by later mixing in the freshly produced spores. Consequently, the effectiveness of the tuned inoculum is enhanced under extreme conditions, in comparison with the initial start inoculum. This is a further indication for the existence of different genotypes within a strain and an important step on the way to directing the inoculum production process successfully.

Under variable environmental conditions probably the physiological status of the host is the main factor that expresses dependency of or independency of mycorrhizal fungi. Therefore, the *directed inoculum production process* (DIPP) will be especially successful if the relationship between later target plants and desired target mycorrhizal effect is clearly defined before the inoculum production starts.

In summary, there is a possibility of influencing the genotype composition of an AMF population by directed processing of the inoculum production. Abiotic environmental factors can be used to select and canalize AMF genotypes. But the chosen plant species with its specific mycorrhizal dependency seems to have special importance for the result of the process as well. Finally, it has to be pointed out that inoculum adaptation to stressors (salt, heavy metals) lasts only one to two multiplication cycles (Feldmann and Grotkass 2002).

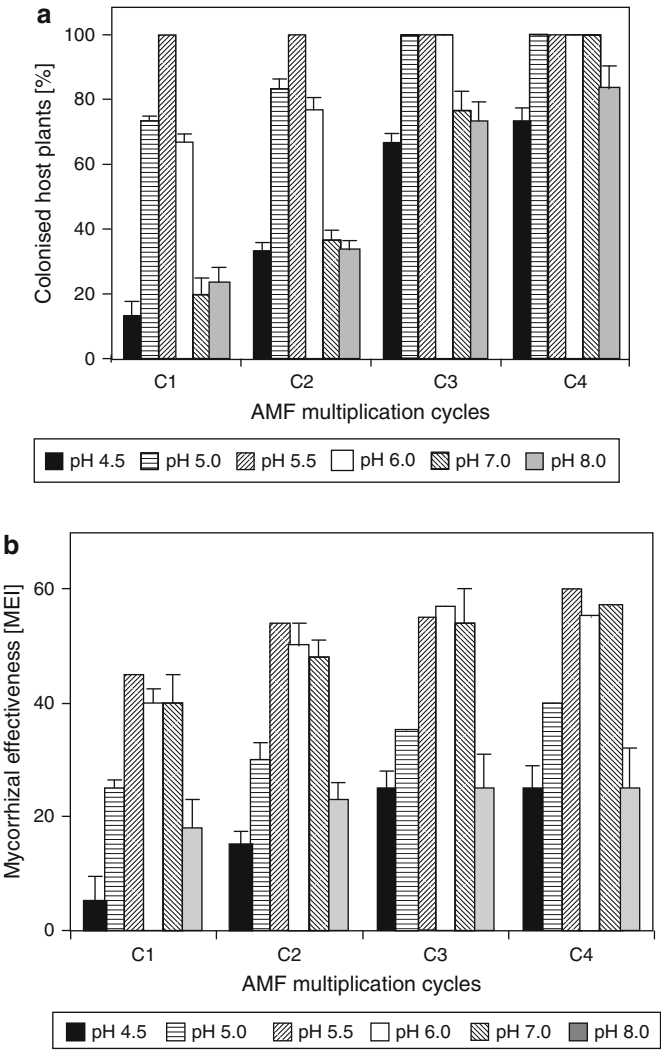


**Fig. 20.2** Root colonization ability (**a**) and mycorrhizal effectiveness (**b**) of AMF populations (*Glomus sp*. GK 12 on *Anagallis arvensis*) with technically modified genotype composition (Selection factor "soil-pH", details see text). *Bars*: SD

### *20.3.4 The Up-Scaling Phase: One Further Step Only*

As mentioned above, strain or inoculum characteristics are stable minimally one, normally two or — depending on the desired effect — three multiplication cycles.

Mass production of AMF means the production of up to several thousands of litres of inoculum containing approximately 80,000 infection units per litre.

Inoculum is normally produced in pots of different sizes with one or two, sometimes four host individuals.

Without nutrient limitation the growth of the host plants in pots is quite homogeneous due to limited space for root development. Therefore, differences in AMF effectiveness of sub-populations were rarely observed or interpreted as a result of the genetic differences between host individuals. The AMF action and the host growth were found to be different in larger plots without space limitation of root development. We calculate on the following basis: one single plant individuum (here: *Zea mays*) produces, depending on the substrate type, approximately 400,000–1,600,000 spores in 6 l. How long it will take depends on the quantity of starter inoculum. One should inoculate not less than 1 spore $ml^{-1}$ and not more than 10 spores $ml^{-1}$ starter inoculum to the seedling initially.

The mass inoculum production should be carried out in greenhouses in ground beds (Fig. 20.3). Up to 50 host individuals are involved in the AMF multiplication in one unit. The ground should be covered with foil, permeable for water but not for soil fauna. The seedlings are planted with a spacing of 50 cm × 50 cm. Light, temperature and nutrients should not limit the growth of the host. Select a host variety which is susceptible to mycorrhiza under optimal growing conditions and let the plants grow for up to 5 months in the substrate under optimal conditions.

At the end of the inoculum production the host plant has to be dried within less than 2 weeks without removal of green plant parts. Observe the fungus sporulating in this time period. As soon as the spore number not longer varies, the process is over and drying of the inoculum can start.

As regards effectiveness, it may be observed that the unlimited root growth of hosts in plots may lead to further segregation of pre-selected strains with high effectiveness into new sub-strains with neutral to high effectiveness during mass production (Fig. 20.4). Nevertheless, more than 90% of the inoculum caused positive growth response in the host (*Zea mays*) during inoculum mass production. This quota seems to be reproducible and would make it economically feasible to select sub-strains with special effectiveness and to discard eventual sub-populations of lower effectiveness after mass production.

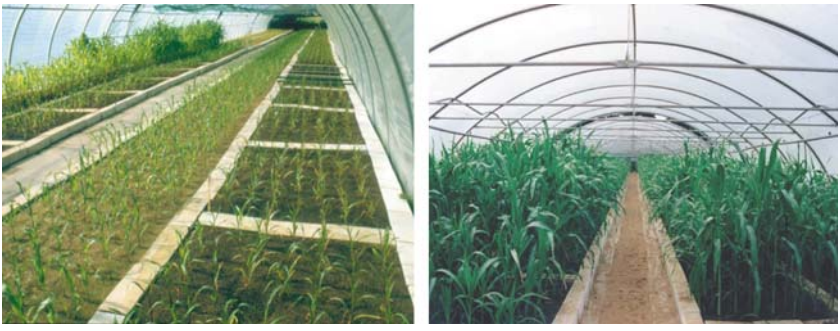

**Fig. 20.3** Ground-bed plots in the up-scaling phase of inoculum production

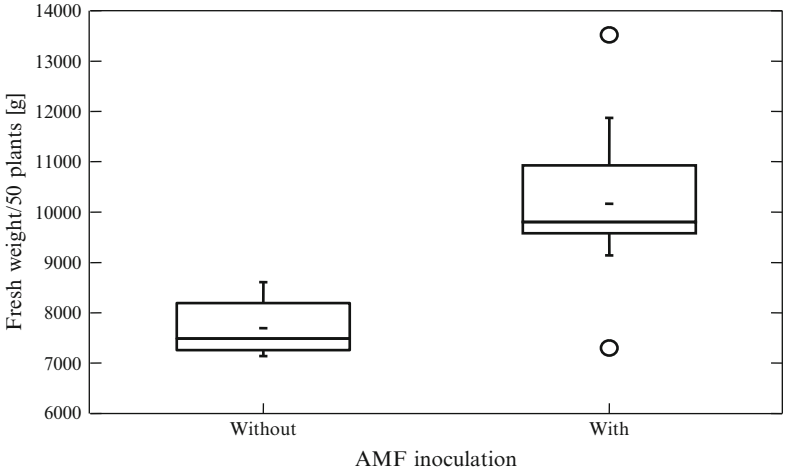


**Fig. 20.4** Variability of effectiveness of an AMF strain (*Glomus sp.*) during inoculum mass production in plots with 50 host individuals (*Zea mays*)

#### 20.3.4.1 Concurrent Quality Control: You Should know what is Going on

Samples are taken every 3 to 4 weeks to measure the principal components in relation to the developing symbiosis. Soil nutrient analysis is carried out by commercial labs, faunistic and microbiological analysis by molecular genetic analysis (DNA multiscan). The Most Probable Number of infective propagules is measured only at the end of the production according to Feldmann and Idczak (1994).

All results are analysed together in a multi-variety plot to investigate whether negative interrelationships developed (Fig. 20.5). Based on this analysis, modifications to the system are processed. Be aware that we are not speaking about the final quality control procedure for the inoculum before being released on the market (compare von Alten et al. 2002), but process control of the production process.

#### 20.3.4.2 Testing the Inoculum in Practice: Did it Achieve the Desired Effects?

The inoculum was introduced to the plant production process under practical conditions. Later plants were sold or studied until their death caused by the end of the growing season at their growing site (balconies, gardens). Mycorrhizal plants were for preference sold on average 5 days earlier (Fig. 20.6). This effect was observed in ten of 15 plant cultivars tested, reflecting the achievement of the desired effects (better flowering or better growth). The effect was measured as a multifactorial trait by the customer. Out of a mixed plant population in a plant shop customers selected plants without knowing that AMF were used. If they preferred AM plants the desired effect was called “realised” (Fig. 20.7).

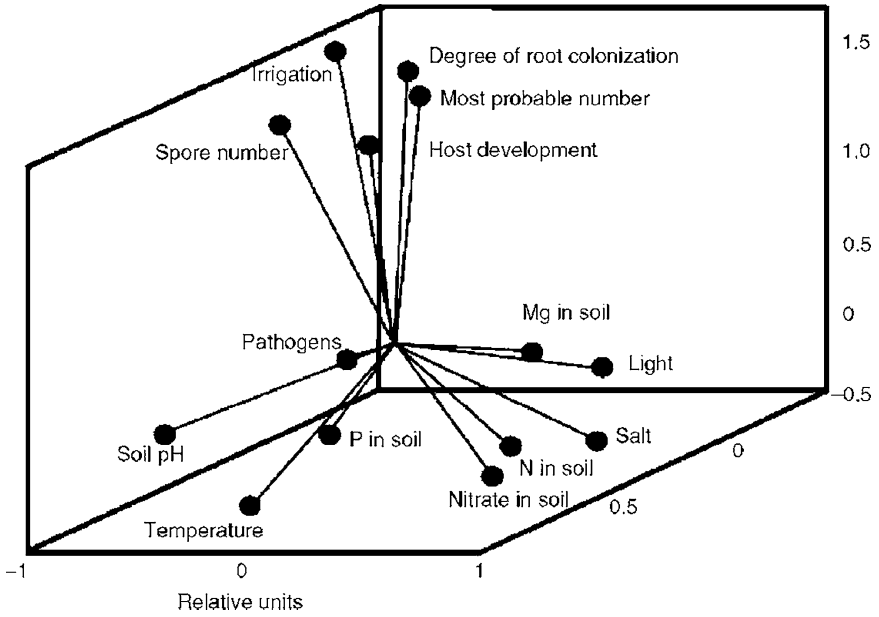


**Fig. 20.5** Principal component factoring of growth conditions

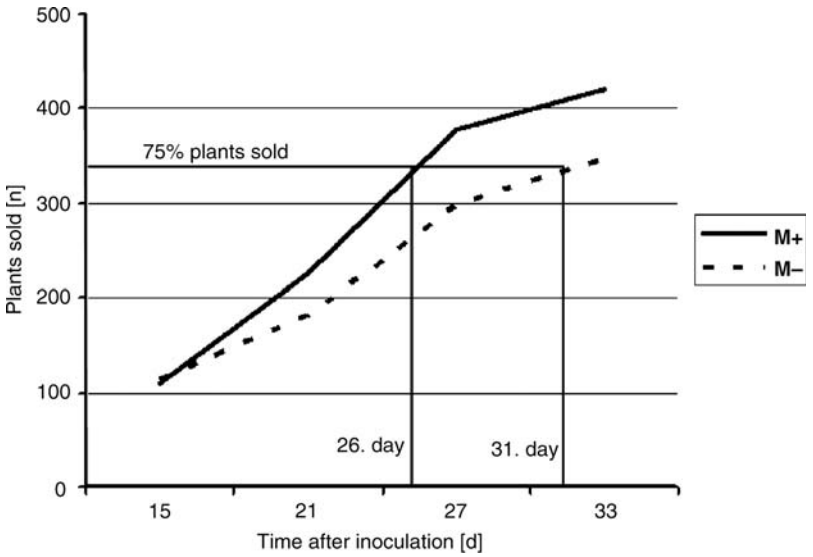


**Fig. 20.6** Preferential sale of mycorrhizal vs. non-mycorrhizal plants: the action was made economic sense, and the desired effect was achieved after selling 75% of the plants in a shorter time period (Figure from Feldmann et al. 1999)

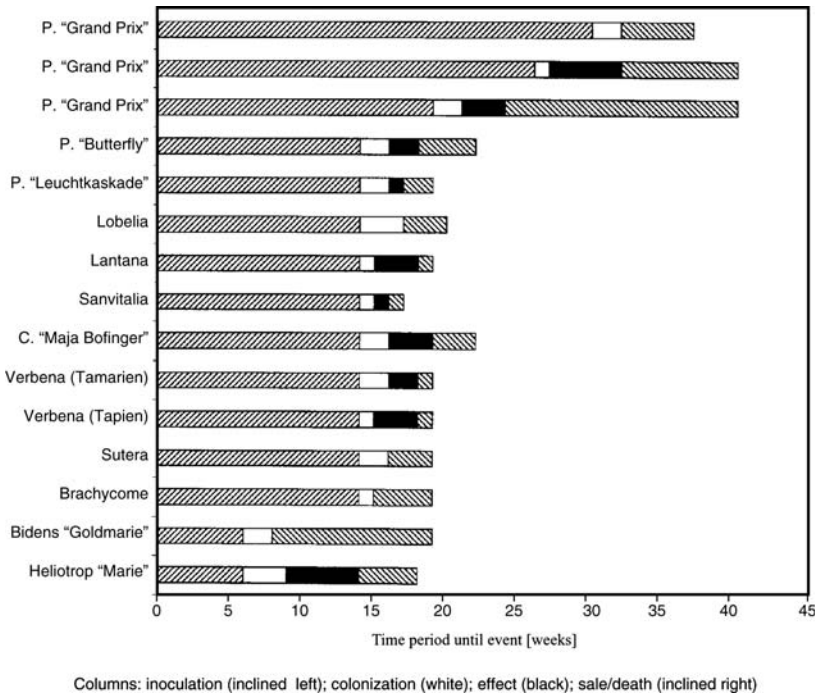


**Fig. 20.7** Effectiveness of directed AMF inoculum in practice: *black columns* show the time period until the AM plants were preferentially sold to customers out of a plant population mixed with non-mycorrhizal individuals. (Figure from Feldmann et al. 1999)

**Table 20.2** Increase of predictability of mycorrhizal effectiveness with *directed inoculum production process* (DIPP). "Constant environments" are greenhouse or growth chamber conditions; field or garden experiments were carried out under "variable environments"

| Inoculum production | Experiments [*n*] | | Predicted success [% experiments] | |
|---|---|---|---|---|
| | Environment | | Environment | |
| | Constant | Variable | Constant (%) | Variable (%) |
| With DIPP | 16 | 35 | 87.5% | 68.6 |
| Without DIPP | 59 | 41 | 52.5% | 36.6 |

To demonstrate the impact of *Directed Inoculum Production Process* (DIPP) many experiments with the same AMF strain but different host species, different inoculum quantities, environmental conditions, scales and effects were designed. In practice, a threshold value of MEI $> 30$ must be exceeded to create the interest of a potential customer in mycorrhizal technology. The positive outcome of an inoculation was called "predicted" if that MEI value was clearly passed under the commercial conditions of plant producers.

DIPP was introduced to the plant production and optimized in the company IFP starting in 1996. Defining "predictability of AMF effectiveness" as quantitative

value for the frequency of expected host growth response to symbiosis we can compare experiments before and after the introduction of DIPP. The results (Table 20.2) showed that predictability could be clearly increased. DIPP is a promising way of providing guaranteed thresholds of effectiveness.

## 20.4 Conclusions

All over the world there are efforts to include arbuscular mycorrhizal technology in processes of plant production. Benefits caused by arbuscular mycorrhizal fungi (AMF) are used in the weaning stage of *in vitro* cultivated plants. Inoculation of seeds, seedlings, cuttings or completely developed plants (Chang 1994) is recommended. The introduction of AMF to target plants is carried out under greenhouse conditions (Miller et al. 1986), in nurseries (Nemec 1987) and in the field (Thompson 1994). One single AMF species can be inoculated to dicotelidons, monocotelidons and ferns (e.g. Feldmann 1998a). Furthermore, the same AMF species can be used in humid tropics (Sieverding 1991) and in temperate climates (Baltruschat 1993).

In spite of such a spectrum of different environmental and cultivation conditions, there is one unique expectation in the case of AMF inoculation: the developing symbioses have to work successfully, must provide advantages to the target plant and fulfil the customer's requirements. "Symbiontal effectiveness" is a multifactorial phenomenon. Host and fungal genotype both together influenced by abiotic and biotic environmental conditions express the phenotype of the specific, relevant symbiosis. "Positive effectiveness" in agricultural or horticultural sense is judged as a "positive response" of the host when considering plant growth, yield or stress tolerance.

There are several possibilities of influencinge the phenotype expression of the symbioses in practice, e.g. deciding the time of inoculation with respect to the developmental stage of the host, quantifying the inoculum potential or changing the culture conditions.

Nevertheless, before introduction of DIPP there was only a low predictability of the quantitative aspect of an effect (i.e. the effectiveness) a mycorrhizal symbiosis might have in practice. In fact, AMF effectiveness following artificial inoculation ranged from positive to negative (Varma and Schuepp 1994) in a mutualism-parasitism continuum (Johnson et al. 1997).

To deal with that problem, screening processes for AMF strains (Dodd and Thomson 1994) in order to find the "best" mycorrhizal strain (e.g. Baltruschat 1993) or effective AMF mixtures (e.g. Sieverding 1991) have been developed. The results of all those efforts were disillusioning. The predictability of AMF effectiveness remained too low for the sustainable use of AMF in commercial horticultural and agricultural practice, especially in moderate climates. Industrial interest in the use of AMF in plant-production processes still bears no relation to the potential of the technology (compare Feldmann 1998a).

At present, there are two fundamental questions to be answered for understanding the basis of mycorrhizal effectiveness:

(a) The "mycorrhizal dependency" of a host is genetically fixed (Azcon and Ocampo 1981) and the degree of mycorrhizal dependency is expressed at the level of an individuum, as a gradient within the host?s ecological niche and relevant environmental conditions (Feldmann 1998a). But are we able to predict mycorrhizal dependency under specific conditions? Predicted success of the symbiosis is still based on practical experiences and not on the knowledge of the basic mechanisms for host dependency. Only if we learn to describe the limiting factors of host growth in much more detail will predictability of effectiveness be enhanced even more.

(b) AMF inoculum was thought to be genetically homogeneous in a wide range, because of the mitotic reproduction of spores. Ignoring that, the initial inoculum multiplication was often processed using a multispore start inoculum. The assumed genetic homogeneity of AMF inoculum was the basis for all screening projects on AMF strains. But the genetic homogeneity of an AMF strain does obviously not exist: recent experiments on the variability of mycorrhizal phenotypes demonstrated that the mutualism-parasitism continuum of mycorrhizal effectiveness is even found within one single strain of an AMF containing only single spore descendants (Feldmann et al. 1998; Feldmann 1998b).

In those experiments it remained unclear whether the mutualism-parasitism continuum was based on the action of different AMF genotypes or showed genetic differences between individual host seedlings, i.e. the reaction norm of the host population to a genetically homogeneous AMF strain. It was of special importance to clarify whether different genotypes occur within an AMF strain and whether their action results in changes in mycorrhizal effectiveness.

In the results shown in this report and in earlier studies (Feldmann and Grotkass 2002) we focussed on the second question. We assumed that spores or AMF infection units are able to colonize a host root-system without respect to their later effectiveness (Feldmann 1998b) and that more than one infection unit of the AMF population will be successful in infecting the roots. To prove the hypothesis of different genotypes within an AMF population we therefore worked with distinct fungal units, with single spores.

In our definition a "genotype" is a functional one, reacting to a given environment in a reproducible, predictable way for one propagation cycle of the spores as a minimum. That means that the phenotypic characteristic of a symbiosis arising from the inoculation of single spores must be reproduced when descendants of these spores are inoculated to homogeneous plant material in a subsequent experiment. A functional description of a genotype does not of course describe the actual genetic differences between AMF units on the DNA level but is focussed on active functional genes for specific interactions. Nevertheless, the chosen way reflects genotypes as targets for eco-factor actions and therefore gives a strict orientation for practice of mycorrhizal technology.

We here present a procedure for handling potential genetic differences of an inoculum by directing the variability of effectiveness via the technical modification

of abiotic and biotic selection factors during the inoculum production process. This procedure, called the directed inoculum production process (DIPP), increased the predictability of the qualitative and quantitative output of the symbioses. DIPP might serve as a prototype for process optimizations which finally lead to the achievement of AMF inoculum with predictable characteristics.

Producing AMF inoculum is no longer a "black box" process. Defining an AMF "genotype" we focussed on phenotypic effects which were pronounced in the hosts by single-spore inoculation, and could be reproduced after replication of single spore descendants (compare Tommerup 1988, who defined the AMF species level as AMF genotype). Nevertheless, the stability of the characteristics was very low, indicating that there might be a mechanism involved which can change the strain characteristics rapidly to a certain extent. To us, such changes do not occur spontaneously but are triggered by abiotic or biotic ecofactors including the host itself. If we assume that gene/gene interactions of host and fungus establish and perform a symbiosis (Krishna et al. 1985; Lackie et al. 1988; Gallotte et al. 1993), and if we accept that the quantitative effects of the symbiosis depend on polygenic characters of the host, any increasing or decreasing variability of the host phenotype may be due to a large amount of mycorrhiza-induced changes to the host physiology.

Of special importance is the multinuclear character of AMF spores (Peterson and Bonfante 1994; Genre and Bonfante 1997; Lingua et al. 1999). We still do not know how many and which nuclei of an AMF spore are active, how they are activated and what influence the heterocaryosis within a spore would have on the effects observed. Does caryogamy exist? Does a population biological process exist favouring the selection of specially adapted nuclei within the population of single spore descendants of an AMF strain? Are strain characteristics mixed under the control of the host? Due to the relative stability of AMF effectiveness after one propagation cycle there is no arbitrary exchange of information between spores of a spore population colonizing a host during this process but a competition between genotypes being controlled by the host or not.

This hypothesis means that a 100% predictability of mycorrhizal effectiveness cannot be achieved. This information is necessary for the selection of target areas, target effects, target plants, and design of inoculum.

The directed inoculum production process presented here integrates many aspects resulting from the practical extrapolation of theoretical hypothesis, and is already leading to more than 85% predictability under commercial conditions. That means that we solved a general problem to an extent which probably reaches the biological limitations of the system. In future we will turn to technical applications of DIPP, e.g. in bioreactors and *in vitro* techniques. But clarifying the basis of mycorrhizal dependency of host plant species (compare Tewari et al. 1993; Boyetchko and Tewari 1995) will be of special importance for the economically successful application of mycorrhizal technology in agriculture and horticulture in the future.

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# Chapter 21
# The Use of AMF and PGPR Inoculants Singly and Combined to Promote Microplant Establishment, Growth and Health

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## 21.1 Introduction

Micropropagation is an alternative to seed and vegetative propagation for the production of planting material (Hartman et al. 2001; Cassells 2005). Micropropagation is often cited as the most successful example of a laboratory technique that has become an important commercial industry (Sahay and Varma 2000). Tissue culture or laboratory-based methods of propagation involve higher capital, recurrent and labour costs than conventional propagation methods. Labour, for example, has been estimated at approximately 60% of the total cost of production of the micropropagules. Consequently, the competitiveness of micropropagation is improved if production is located in a low labour cost economy. Alternatively or additionally, the competitiveness of micropropagation can be further increased by automation of the labour-intensive stages. Nevertheless, micropropagation can only compete directly on price with expensive seed and higher value vegetative propagules. Micropropagation can become more competitive in its niche markets by reducing production losses and by adding value to micropropagules, e.g. guaranteeing freedom from specified pathogens, having novel genotypes and by establishing and growing on the microplants before selling them as liners or finished plants (Cassells 2005). Value can also be added potentially by exploiting the benefits of biological inoculants to protect the plants at establishment, thereby reducing production losses and promoting the growth of the microplants. Additionally, if the

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benefits of inoculant are carried forward to improve the field performance of the microplants this should command a higher selling price.

Malfunctional stomata and thin cuticles where the deposition of cuticular wax has been impaired have been proposed as contributing to poor establishment of microplants through failure of these systems to control water relations in the microplant, leading to desiccation of the tissues (Davies and Santamaria 2000). Stage 3 of micropropagation (Cassells and Gahan 2006) is used to prepare the microplants for weaning (termed *in vitro* weaning), which involves strategies to induce functionality in leaf and root function (Debergh et al. 2000). This problem, which may be covert, conventionally has been addressed by establishing the plants in a fogged or misted tunnel with shading to prevent photobleaching (see below). Sensitivity of the poorly developed photosystems of microplants to high ambient light intensity has been addressed by shading of the establishment bench. It is possible that PGPR and/or AMF inoculants added in Stage 3 may improve microplant establishment and early post-establishment growth.

Another problem encountered at micropropagule establishment is that the aseptically produced tissues, which may be 'soft', i.e. hypolignified and 'juvenile', may have poorly developed constitutive pest and disease resistance akin to seedlings which, when combined with the absence of a protective rhizosphere flora and adverse reaction to some pesticides (Werbrouck and Debergh 1996), makes them susceptible to pests and pathogens (Williamson et al. 1997). This problem may be compounded when poor physiological quality results in a growth check while the autotrophic state and stomatal and root functionality are achieved (Davies and Santamaria 2000). Limited energy reserves in the micropropagules (Van Huylenbroeck et al. 1998) may also restrict the expression of inducible defences (Strange 2003).

Micropropagules are produced in a biological vacuum in sterile laboratory conditions in the absence of microorganisms (Vestberg et al. 2002). However, upon transplanting to a growing medium for weaning, they will immediately be exposed to various kinds of microorganisms. These include pathogens and facultative pathogens that cause damping-off of the unprotected microplants (Williamson et al. 1997). The weaning substrates are usually devoid of beneficial microorganisms. Adding such microorganisms to the weaning substrate may therefore protect the micropropagules against biotic and abiotic stress. Microorganisms such as arbuscular mycorrhizal fungi (AMF), plant growth-promoting rhizobacteria (PGPR), *Rhizobium*, *Frankia*, biocontrol fungi and bacteria, etc. have been studied in this respect. All these organisms have their own mode of action, which has to be taken into account when using them.

Several reviews have been written on the potential use of microorganims for creating the beneficial rhizosphere in microplants (Vestberg and Estaún 1994; Varma and Schüepp 1995; Lovato et al. 1996; Azcón-Aguilar and Barea 1997; Budi et al. 1998; Rai 2001; Vestberg et al. 2002). Here, the potential of biological inoculants to achieve an incremental improvement in microplant establishment and to add value to micropropagules by improving post-establishment growth and health, including pest and disease resistance, is discussed. Much of the evidence for the beneficial effects of AMF and PGPR inoculants comes from their use in seed

and vegetative propagation and it is hypothesised that, in addition to addressing the above problems which are unique to microplants, results obtained with conventional propagules can, in principle, be extrapolated to micropropagules. It is appreciated that for biological inoculants to be commercially attractive they should offer at least a 4-fold return on the inoculum cost (Fravel et al. 1999). For a recent review on the commercialisation of biological control inoculants see Fravel (2005).

## 21.2 Mode of Action and Safety of Inoculants

Many microorganisms have been investigated for their potential to directly control pests and diseases of plants, and for their ability to form beneficial associations with plants promoting growth and increasing pest and disease resistance based on priming ('biopriming') of plant resistance mechanisms (Bloemberg and Lugtenberg 2001; Conrath et al. 2002; Nowak and Shulaev 2003). While many microorganisms are known to form beneficial associations with plants, those that have been investigated most include the mycorrhizal fungi, and the plant growth-promoting and nitrogen-fixing rhizobacteria (Bashan 1998). Here, discussion will be restricted to arbuscular mycorrhizal and non-nitrogen-fixing rhizobacteria, specifically to *Pseudomonas* and *Bacillus* species, as these are seen to have potential to address the general problems associated with weaning and post-establishment growth of micropropagules. From an applied perspective, it should be noted that in spite of the large number of journal articles on biocontrol inoculants, very few have advanced to the stage of being formally approved in the US for commercial use (Fravel 2005). The EPA-approved microorganisms include the fungal species *Aspergillus flavus* (2 isolates), *Candida oleophila* (1 isolate), *Coniothyrium minitans* (1 isolate), *Gliocladium* (2 species; 2 isolates), *Trichoderma harzianum* (3 isolates) for direct pathogen control and bacterial species *Agrobacterium radiobacter* (2 isolates), *Ampelomyces quisqualis* (1 isolate), *Bacillus* (3 species; 6 isolates), *Pseudomonas* (5 species, 5 isolates), *Streptomyces griseoviridis* (1 isolate) (Fravel 2005).

### *21.2.1 Arbuscular Mycorrhizal Fungi*

The AMF, belonging taxonomically to the phylum Glomeromycota (Schüßler et al. 2001), form a symbiotic relationship with more than 80% of plant species living on land. Symbiosis plays an equally important role in natural ecosystems and in agroecosystems. Mycorrhizae have been characterized as an ecosystem service to humanity. In a narrow sense, symbiosis means a mutualistic relationship between AMF fungus and host. Benefits attributed to a functional AMF symbiosis include increased plant growth due to improved nutrient uptake, especially phosphorus (P), (Marschner and Dell 1994) and water (Al-Karaki and Clark 1998; Augé et al. 2004; George et al. 1992), alleviation of biotic (Linderman 2000) and abiotic (Barea et al. 1993) stress, and improved soil aggregation (Miller and Jastrow, 1990; Rillig and

Mummey 2006). AMF may also interact with plant hormonal systems (Allen et al. 1980; Duan et al. 1996). Thus, AMF have three main ways of function as improvers of nutrient and water uptake (often erroneously termed biofertilizers), as biocontrol agents and as bioregulators (Lovato et al. 1996). In addition to increasing the absorptive surface area of their host plant root systems, the hyphae of AMF provide an increased area for interactions with other soil microorganisms, and an important pathway for the translocation of energy-rich plant assimilates to the soil (Johansson et al. 2004). Since plant roots in most ecosystems are mycorrhizal the rhizosphere concept has been widened to include also the fungal structures around plant roots, resulting in the concept "mycorrhizosphere" (Johansson et al. 2004; Rambelli 1973). The zone surrounding individual mycorrhizal hyphae is called the "hyphosphere".

For the beneficial effects, the plant must however also pay a price. Between 10 and 20% of the net photosynthates are required for formation, maintenance and function of mycorrhizal structures (Jakobsen and Rosendahl 1990). A large amount of this carbon eventually ends up in soil, which strongly affects microbial interactions in the rhizosphere (Johansson et al. 2004). A presymbiotic and a symbiotic phase can be recognised in the AMF symbiosis. The former phase can further be developed into three steps including spore germination, host recognition and host colonisation (Giovannetti 1997). The presymbiotic phase can also be characterized as a parasitic phase, although this term is not always accepted in the mycorrhizal society. Mycorrhizal associations have generally been classified as mutualisms, but an increasing number of studies show that this is not always the case. Upon closer analysis, there appears to be a continuum of plant responses to mycorrhizal colonisation ranging from positive (mutualism) to neutral (commensalism) and negative (parasitism) (Smith and Smith 1996; Johnson et al. 1997). Negative impacts of AMF on plant growth have been shown, for example, at high soil P (Abbott and Robson 1984; Liu et al. 2000), in peaty substrates (Linderman and Davis 2003; Vestberg et al. 2000), with cultivars of low relative mycorrhizal dependency (Declerck et al. 1995) and in micropropagation systems (Azcón-Aguilar et al. 1994; Uosukainen and Vestberg 1994).

### *21.2.2 Plant Growth Promoting Rhizobacteria (PGPR)*

As first defined, the PGPR are bacteria, which either promote plant growth or control plant disease (Kloepper and Schroth 1978). Those which have been most extensively investigated for beneficial effects on seedlings and vegetative propagules are principally *Pseudomonas* (Ramamoorthy et al. 2001) and *Bacillus* species (Kloepper et al. 2004). Many isolates have been selected for their ability to produce iron-chelating siderophores, and/or antibiotics, and/or to a lesser extent, plant growth regulators (Handelsman and Stabb 1996; Walling 2001). Feeding on root exudates, the PGPR produce siderophores and antibiotics to protect their niche, thereby antagonising pathogens and/or excluding pathogens from the rhizoplane

(Walsh et al. 2001). Control of minor or subliminal root pathogens (Suslow and Schroth 1982), as reported when systemic fungicides were introduced, may also result in yield increases, i.e. may be the expression of the 'plant growth promotion' effects. Other PGPR have been shown to produce plant growth regulators, which affect plant growth (Schroth and Hancock 1982; Arshad and Frankenberger 1998; Bloemberg and Lugtenberg 2001) or reduce the inhibitory effects of ethylene on root growth (Glick et al. 1998). Some PGPR are nitrogen-fixing, e.g. *Psuedomonas putida* GR12–2 (Lifshitz et al. 1986), whereas most, like AMF, are involved in nutrient cycling in the rhizosphere and exercise a general 'biofertilizer' effect (Bashan 1998).

Some PGPR are known to induce systemic resistance (ISR – see systemic acquired resistance (SAR); van Loon et al. 1998; Heil and Bostock 2002). Such plants are considered to have their defences primed such that, on subsequent infection, they are rapidly up-regulated compared with an non-inoculated, non-primed plant (Kuc 2001). The determinants of ISR induction in *Pseudomonas* spp. include lipopolysaccharides, siderophores and either salicyclic acid or jasmonic acid (Ramamoorthy et al. 2001; Kloepper et al. 2004). It is interesting to note here that *Bacillus* spp. commonly both promote plant growth and induce systemic resistance, whereas ISR is not as frequently associated with growth promotion by *Pseudomonas* spp. (Kloepper et al. 2004).

PGPR inoculants developed for use in the seed and vegetative propagations sectors have also been used by micropropagators (e.g. Duffy et al. 1999; Sharma and Nowak 1998; Barka et al. 2000; Cordier et al. 2000; Sturz et al. 2000; Srinath et al. 2003; Vestberg et al. 2002; Vestberg et al. 2004; Mia et al. 2005; Rodriquez-Romero et al. 2005; Tahmatsidou et al. 2006). Unlike the reported plant genotype – AMF isolate specificity reported (Mark and Cassells 1996, see above), PGPR appear to exhibit less host specificity but, like AMF inoculants, which are strongly influenced by the substrate in which the plants are established and grown, similar problems may be anticipated for some micropropagule growing systems (Hoitink and Boehm 1999; Krause et al. 2001). Microplants are generally established in greenhouses in hydroponics or artificial substrates. Such substrates may include peat-based formulations where available, or peat substitutes such as coir. This is in contrast with conventional propagules, which are commonly planted into intensively cultivated soils with a depleted microflora (generally low competition favours inoculants) at one extreme, and into highly fertile soils (containing antagonists and competitors of the inoculant) at the other extreme (Hoitink and Boehm 1999). In contrast to AMF, PGPR are less sensitive to substrate phosphate concentration.

Safety guidelines for the evaluation of inoculant have been established (Cook et al. 1996). Of paramount concern is the risk to users and consumers reflected in the withdrawal of *Pseudomonas* (*Burkholderia*) *cepacia,* which is associated with a human health risk (Parke and Gurian-Sherman 2001). Unlike AMF inoculants, PGPR pose potentially greater human health risks because of their relatedness to human (and animal) pathogenic bacteria and the ease with which bacteria exchange genes. Another concern is associated with the release of non-native organisms into

a new quarantine zone; this however has not prevented the commercialisation of European PGPR inoculants in North America (Fravel 2005) and vice versa.

## 21.3 Selection, Production and Formulation of Inoculants

Some, on ecological grounds, have suggested that microorganisms should be sought in pathogen-suppressive soils, or at least from soils and environments similar to those in which the inoculant will be used (Cook and Baker 1983). However, economic constraints may influence the methods used to select potential inoculants as inoculum producers seek to reduce costs by seeking 'universal' strains which have wide soil and environmental tolerance (Schisler and Slininger 1997; Fravel 2005). In general, AMF inoculants show high host genotype-environment sensitivity, whereas PGPR inoculants have a broad host range and wide environmental tolerance.

### *21.3.1 Arbuscular Mycorrhizal Fungi*

Many species of AMF can infect a wide range of plant hosts. In early studies AMF were therefore regarded non-specific also in terms of symbiotic functionality. In the 1970s and 1980s research with AMF was much focussed on finding some super strains capable of increasing plant biomass under any environmental and soil conditions (Vosatka and Dodd 2002). However, studies done during the last decade have shown a high degree of AMF/host genotype and environmental specificity. It is now recommended that AMF strains intended for commercial use should be selected from the environments similar to those of their intended use. In an experiment at the English Channel tunnel, this principle was demonstrated by Dodd et al. (2002) who showed the superior effects of inoculation with native AMF on the growth of *Elymus pycnantus* in comparison with commercially produced non-mycorrhizal plants.

For screening and producing AMF inoculum, the first basic requirement is to have a large collection of characterized AMF. This is very laborious since special care has to be taken to keep the strains uncontaminated with environmental microorganisms. Inoculum quality control is still one of the biggest bottlenecks in AMF utilisation. Prediction of symbiotic effectiveness in terms of increased growth or stress tolerance of the host is another obstacle to the successful commercial use of AMF. Feldmann and Grotkass (2002) discussed this problem in connexion with a directed inoculum production process (DIPP). They claim that in practice the Mycorrhizal Effectivity Index (MEI, Bagyaraj 1994) should exceed 30% for creating an interest in potential customers. The challenges of mycorrhiza technology are treated in more detail by Vosátka et al. (2008).

### 21.3.2 Plant Growth-Promoting Rhizobacteria

In most cases, the potential of a PGPR isolate to control a pathogen has been based on inhibition of the pathogen in *in vitro* co-cultivation tests, i.e. on testing for antibiotic production (Han et al. 2000). Screening for growth promotion by inoculation of plants, e.g. *in vitro* (Cassells 2000) and *in vivo* (Cordier et al. 2000), have also been described. Methods have also been described for screening for ISR (Han et al. 2000). Table 21.1 summarises the methods used to characterise inoculants and plant responses. However, such isolates may not give economic control under field conditions, particularly when introduced into soils of high fertility, such as land converted to organic production, where they may not be able to achieve the critical population density (Hoitink and Boehm 1999). In general, it is essential that inoculants are tested in greenhouses or in the field production system in which they are to be used to determine the host genotype–inoculant–pathogen isolate–environment interaction, that is, to confirm their reliability (Fravel 2005). In the case of application to micropropagules, they should be tested in the establishment growth substrate (Krause et al. 2001).

PGPR are readily cultivable on a large scale in fermenters using established technology (Eyal et al. 1997). The facilities are widely available on a contract basis. Media details are in the public domain. Inoculum formulation may be based on imbibing a carrier such as talc or methylcellulose with washed cells to achieve a target density expressed as cfu/g of product. The carrier is then air-dried to set moisture content, milled to a fine powder and stored under dry conditions in a coldroom. More complex formulations are discussed in Burges (1998); Harris and Adkins (1999); Walling (2001); Bashan et al. (2002).

A critical commercial issue is stability where inoculum must survive at point of sale, survive shipment generally under ambient conditions, survive intermediate storage and at point of use, being exposed to a wide range of temperatures along the chain. It has been argued that while *Pseudonomas* species are quick colonisers of roots, *Bacillus* species have the advantage of having heat-resistant spores, which give good product shelf life. Current release of new isolates and sales of *Bacillus* inoculants would tend to support this view (Kloepper et al. 2004).

Product quality is major cause of concern to both producers and users of biocontrol inoculants and it is essential that producers have an adequate quality control system (Jenkins and Grzywacz 2000). In the case of PGPR inoculants, product cfu/g can be determined by plate counts following serial dilutions, and purity may be determined by examination of colony morphology. However, confirming the identity of the microorganism(s) in a formulation and inoculant persistence in the growth substrate may be more problematic. This can be done using molecular methods but at considerable cost (Stead et al. 2000). Emerging techniques such as MALDI-TOF mass spectrometry of intact bacteria may offer rapid identification with limited preparation and consumable costs (Cassells and Doyle 2006; Tahmatsidou et al. 2006).

**Table 21.1** The characterization of PGPR and plant responses to inoculants and activators. The expression of ISR and SAR is characterised by the detection of the genes PDF1.2 and PR1, respectively. Unless these proteins or their respective mRNAs or other selective marker gene are detected (Hammond-Kosack and Parker 2003; Reymond and Farmer 1998), the expression of the response should be referred to as increased disease protection

| Character | Expression | Test method | Reference |
|---|---|---|---|
| Pathogen antagonism | Inhibition of pathogen growth | PGPR spotted at three points on a culture plate and sprayed with pathogen spores, inhibition zones measured | van Wees et al. (1999) |
| Plant growth promotion | Stimulation of plant growth | Measurement of plant height/ biomass | Srinath et al. (2003) |
| Induction of ISR | Increased disease protection | Root inoculation/challenge of seedlings | Han et al. (2000); van Wees et al. (2000) |
| | | Inoculation of detached leaves with PGPR followed by spraying of leaves with pathogen spores | Zhang et al. (2002) |
| | | Detection of PDF1.2 (defensin) by RNA gel blot analysis | Spoel et al. (2003) |
| Induction of SAR | Increased disease protection | Foliar inoculation with necrotising pathogen/challenge inoculation with second pathogen | Van Wees et al. (2000) |
| | | Detection of PR1 by RNA gel blot analysis | Spoel et al. (2003) |
| Priming of plant defences | Increased disease protection | Spraying/drenching of plants with elicitor or plant activator followed by pathogen challenge | Cohen (2002) |
| | | Floating of leaves on plant activator/challenge inoculation with pathogen | Ton et al. (2002) |

## 21.4 Use of Inoculants in Micropropagation

### *21.4.1 Arbuscular Mycorrhizal Fungi*

AM fungi have successfully been used for improving the establishment and growth of microplants. As is the case for plants propagated from seeds or cuttings, a range of factors affect the success of AMF inoculation in micropropagation systems. These factors include timing of inoculation, properties of weaning and later substrates, physiological state of micropropagules and host/AMF combinations. Moreover, in the last few years it has become evident that higher benefits from inoculation can be obtained through combinations of AMF with other beneficial soil organisms such as plant growth-promoting rhizobacteria (PGPRs) or biocontrol fungi (Gianinazzi et al. 2003; Vestberg et al. 2004).

#### 21.4.1.1 *In Vitro* Vs *In Vivo* Inoculation

Microplants can be inoculated with AMF both at the *in vitro* and post vitrum phase of the micropropagation cycle. Sometimes an even later timing of inoculation is necessary to avoid negative effects in microplants, which have a prolonged phase of photoheterotrophic growth (Vestberg et al. 2002).

Pioneer work concerning *in vitro* AMF colonisation in root organ cultures originates from the early 1960s (Mosse 1962). At that time the presence of *Pseudomonas* was considered necessary for ensuring colonisation, but later on bacteria-free cultures were established (Phillips 1971). After these early studies the technique has been much improved. AMF growth in root organ cultures has been found to be influenced by, for example, $CO_2$ (Bécard et al. 1992; Gianinazzi-Pearson et al. 1989), plant flavonoids (Chabot et al. 1992) and root exudates (Gianinazzi-Pearson et al. 1989). AMF spore production *in vitro* of *G. intraradices* was further greatly increased by St-Arnaud et al. (1996) in a two-compartment system. They counted up to 15,000 mostly viable spores per agar plate in a sugar-free compartment. The potential of this technique not only for research purposes but also for large-scale inoculum production is obvious (St-Arnaud et al. 1996). In the 2000s AMF *in vitro* monoxenic culture has been further developed. An international *in vitro* collection of AMF has also been established (GINCO, http://emma.agro.ucl.ac.be/ginco-bel/index.php). Several species of *Glomus* can now be produced and maintained *in vitro* in monoxenic culture. Such purified AMF strains provide excellent possibilities for genetic, taxonomical, molecular biological, physiological, etc. studies of AMF. The potential benefits of using aseptically produced AMF inoculum for improving the growth of micropropagated banana were demonstrated by Declerck et al. (2002).

AMF have been successfully introduced not only into root organ cultures but also into the *in vitro* multiplication phase of micropropagation (Pons et al. 1983; Ravolanirina et al. 1989). However, the specific techniques involved here are

difficult to apply for mass production of micropropagated plants (Lovato et al. 1996; Vestberg and Estaún 1994). In addition, the extra benefit of an *in vitro* AMF inoculation does not exceed that of an early post vitrum inoculation (Ravolanirina et al. 1989; Branzanti et al. 1992). Another possibility is to use AMF in autotrophic culture systems (Cassells 2000). Autotrophic culture has the advantage that it is sugar-free and is carried out in simple mineral nutrient solutions, including low-phosphate formulations (Vestberg et al. 2002). These conditions favour mycorrhizal symbiosis.

Because weaning substrates normally lack indigenous AMF, *in vivo* inoculation done at the weaning stage has been shown to decrease weaning stress and increase early shoot growth in a large number of micropropagated horticultural high-value crops. These include for example *Annona cherimola* (Azcón-Aguilar et al. 1994), globe artichoke (Gianinazzi et al. 2003) banana (Jaizme-Vega et al. 1998; Yano-Melo et al. 1999), cassava (Azcón-Aguilar et al. 1997), coffee (Vaast et al. 1996), garlic (Lubraco et al. 2000), grapevine (Schubert et al. 1990) hortensia (Varma and Schüepp 1994a) *Leuceana leucosephala* (Puthur et al. 1998), peach and pear (Rapparini et al. 1994), potato (Vosatka and Gryndler 2000; Yao et al. 2002), and strawberry (Vestberg 1992; Vestberg et al. 2004; Sharma and Adholeya 2004). However, AMF utilization in microplants is not always a straightforward process because of the great variety of micropropagated plants with different growth requirements. Vestberg et al. (2002) divide the microplants into two groups including those that are "easy" to acclimatize and those that are "difficult" to acclimatize. These concepts agree well with the degree of photoautotrophy at the early weaning stage. Strawberry is an example of a plant having a early start of photoautotrophy at weaning including also fast formation of new roots. According to Vestberg et al. (2002) certain cultivars of apple belong to the other extreme with slowly developing autophototrophy. AMF utilization proved non-problematic in strawberry but much more complicated in apple.

In some cases, AMF have not increased weaning survival but rather decreased it. This phenomenon has been observed when studying several host/AMF combinations. Uosukainen and Vestberg (1994) observed strongly decreased growth of weaning survival of apple microcuttings with one AMF strain out of three, but all strains improved subsequent growth. Similar results were obtained by Varma and Schüepp (1994b) who observed species and cultivar-specific results in three micropropagated plant species. The effects ranged from mutualistic (hortensia), through neutral (strawberry 'Avanta', raspberry Zeva I') to negative (strawberry 'Elsanta', raspberry 'Himboqueen'). However, when the plants were transplanted into the field AMF-inoculated plants were healthier and inoculated strawberry produced more runners than uninoculated ones. Keller et al. (1997), working with two clones of the *in vitro*-propagated legume *Baptisia tinctoria,* found strongly decreased weaning survival in one clone but slightly increased plant survival in the other after AMF inoculation, but no such results were obtained following *Rhizobium* inoculation. Balla et al. (1998) observed decreased growth of peach at early weaning, indicating a carbon drain effect, but after 5 months positive growth effects due to AMF were also observed.

#### 21.4.1.2 Impact of Growing Medium

The growing media chosen for weaning and subsequent growth of micropropagated plants are important not only for establishment and growth of the microplants but also for the effectiveness of the mycorrhizal symbiosis. The amount of extractable P in the growing medium has a great impact on growth responses in microplants following inoculation (Vaast et al. 1996; Shashikala et al. 1999). However, physical properties of the growing medium also affect results obtained by mycorrhizal inoculation. Growers favour peat-based growing media although a trend towards larger use of renewable materials is evident. Incompatibility between peat and AMF, sometimes even inhibitory effects on AMF, has been observed in several studies (Calvet et al. 1992; Estaún et al. 1994; Vestberg et al. 2000), but the nature of this phenomenon is still not fully understood. Certain peat grades seem to be more incompatible with AMF than others (Biermann and Linderman 1983; Calvet et al. 1992; Linderman and Davis 2003). Often it has been found that AMF colonisation can develop to acceptable levels in peat-based mixes but in mineral soil the mycorrhizal symbiosis functions better, resulting in higher growth. (Schubert et al. 1990; Vestberg et al. 2000; Vidal et al. 1992).

The incompatibility between peat and AMF is arguably the biggest bottleneck for the large-scale utilization of AMF in horticultural substrates. The reasons behind this phenomenon are still not defined. However, it is a known fact that most peat types have a very low anion exchange capacity, which easily results in high levels of extractable P in peaty substrates. The most important organic constituents of peat are lipids (sometimes called also bitumen), such as resins and waxes; humic substances, such as humines, fulvic and humic acids; and carbohydrates, such as pectins, hemicelluloses and cellulose. One or several of these components may have a negative impact on AMF. On the other hand, AMF have also been shown to be stimulated by humic substances (Gryndler et al. 2005). The reason for the peat/AMF incompatibility may be in the rich microbial communities of the peat. Some of these microorganisms have even been developed for use in plant disease control (Tahvonen and Lahdenperä 1988). A multidisciplinary approach including biologists and chemists is needed to further elucidate this problem in utilization of mycorrhizae.

#### 21.4.1.3 Effect Against Pathogens and Pests

AMF not only have an impact on nutrient uptake of microplants but also have the potential to protect plants against biotic stress caused by nematodes and soil-borne pathogens. This phenomenon is likely to be of greater importance in micropropagation systems than in other plant propagation systems. Microplants are susceptible to pathogens because of their poor cuticular and root development and consequent need to be maintained within a high humidity environment. The application of AMF therefore offers not only an opportunity to reduce fertiliser inputs but also a possibility of reducing the use of pesticides (Hooker et al. 1994a).

Most studies concerning the use of AMF for controlling soil-borne disease in microplants have been done with pathogens belonging to the genus *Phytophthora*. AMF have shown promising biocontrol properties against strawberry red core disease caused by *P. fragariae* (Mark and Cassells 1996; Norman et al. 1996; Norman and Hooker 2000). In micropropagated pineapple, AMF prevented growth depressions caused by *P. cinnamomi* (Guillemin et al. 1994). In *Prunus avium* *G. mosseae* had a protective effect against *P. cinnamomi* in one clone, but *G. intraradices* had no such effect (Cordier et al. 1996). Similar results were obtained by Yao et al. (2002) according to whom the control effect of AMF against *Rhizoctonia solani* in potato was dependent on both AMF strain and potato cultivar. Strawberry crown rot caused by *P. cactorum* rot was partly controlled by AMF in one study (Vestberg et al. 2004), but the disease was increased in another study (Vestberg et al. 1994). Two species of *Glomus* reduced both internal and external symptoms of the Panama disease (caused by *Fusarium oxysporum* f. sp. *cubense*) in micropropagated banana (Jaizme-Vega et al. 1998).

The mechanisms behind the protection against soil-borne fungal pathogens are summarized by Azcón-Aguilar et al. (2002). They say that the AMF-induced protection is probably a consequence of several interacting mechanisms and that the relative contribution of different mechanisms is directly related to the AMF/plant genotype combination and to the environmental conditions. Six possible mechanisms are identified (Azcón-Aguilar et al. 2002): (1) improvement of plant nutrient status/damage compensation, (2) competition for host photosynthates and colonisation sites, (3) changes in the anatomy and architecture of the root system, (4) microbial changes in the rhizosphere, (5) activation of local plant defence mechanisms, and (6) systemic effects of AMF colonisation, i.e. induced systemic resistance (ISR)

AMF have also been shown to protect plants against soil-borne nematodes (Hooker et al. 1994b; Linderman 1994; Pinochet et al. 1996; Elsen et al. 2003). In banana, *G. mosseae* suppressed root galling and nematode build-up in the roots of *Melodogyne incognita* (Jaizme-Vega et al. 1997). Numbers of *Pratylenchus brachyurus* nematodes per g of root were significantly decreased in mycorrhizal pinapple microplants when the pathogen was introduced at planting out or 1 month later. Both *G. intraradices* and *G. mosseae* suppressed build-up of *P. vulnus* in a pear rootstock (Lopez et al. 1997). In a dixenic root organ culture including both the AMF *Glomus intraradices* and the burrowing nematode *Radopholos similes*, Elsen et al. (2001) demonstrated that AMF suppressed the *R. similis* population by almost 50% and thus increased protection of the root against the nematode. The mechanisms causing reduction in nematode numbers due to mycorrhizal inoculation is still uncertain. Factors such as improved nutrient status, microbial changes in the rhizosphere, competition for penetration sites and nutrients, biochemical changes in the plant and anatomical changes in the roots have been proposed as possible mechanisms (Linderman 1994). In connection with their study in dixenic roots where many of the above alternatives can be ignored, Elsen et al. (2001) discussed whether the effect of AMF on nematode numbers is connected to that of newly

formed plant products such as symbiosis-related proteins which can also be involved in plant defence at the site of infection.

In contrast to the above studies, nematode numbers have been reported to increase due to AMF inoculation in some other studies. Calvet et al. (1995) observed increased growth of micropropagated quince rootstock after inoculation with *G. intraradices* and the nematode *P. vulnus* although a twofold increase in final nematode populations was recovered from plants with combined inoculations of pathogen and symbiont. They concluded that mycorrhizal rootstocks had increased tolerance to the presence of nematodes despite the increased total numbers of nematodes. Studying the impact of co-inoculation with the AMF *G. mosseae* and the nematode *P. vulnus* on growth and nematode populations in plum rootstocks Camprubi et al. (1993) observed a considerably higher final nematode population in the mycorrhizal cultivar Marianna than in non-mycorrhizal 'Marianna'. However, shoot dry weights were higher in the majority of the AMF-inoculated plants after both growing seasons.

### *21.4.2 Plant Growth-Promoting Rhizobacteria*

Inoculation of conventional non-aseptic seed and vegetative propagules faces competition from environmental microorganisms (Hoitink and Boehm 1999). Consequently, in an attempt to ensure colonization with the inoculant the tissues or growth substrate is 'inundated' with the inoculant, i.e. the inoculant is formulated at high cfu (colony forming units). There is much research reported in the literature on the use of bacterial inoculants as seed coatings and in formulations for application to seed, to plant roots, to tubers as dips, in powder form for inclusion in the potting compost or in the planting hole, or for watering onto the soil (Fravel 2005).

Inoculants for micropropagules are, generally, similarly applied (Sharma and Nowak 1998; Duffy et al. 1999; Barka et al. 2000; Cordier et al. 2000; Sturz et al. 2000; Vestberg et al. 2002; Srinath et al. 2003; Vestberg et al. 2004; Mia et al. 2005; Rodriquez-Romero et al. 2005; Tahmatsidou et al. 2006); the difference is that, in the case of micropropagules, the inoculant does not have to compete with or displace propagule resident microorganisms. Ideally, this aspect should be exploited by applying the inoculum directly to the micropropagules before it is planted in the substrate. In addition, as microplants are aseptic, gnotobiotic cultures may be established by inoculation *in vitro* with PGPR (Cassells 2000) at relatively low cfu (see benefits of *in vitro* inoculation with AMF (Cassells et al. 1996)). To avoid the risk of the PGPR inoculant overrunning the micropropagules and becoming vitropathogens (Cassells and Doyle 2006), inoculation is recommended under autotrophic culture conditions where sucrose and other organic components are removed from the medium (Cassells 2000). Inoculation is carried out in Stage 3 (preparation of micropropagules for return to the open environment). PGPR growth and root colonization *in vitro* depends on the leakage of nutrients from the roots.

## 21.5 Holistic Strategies for the Use of Inoculants

Often, inoculants fail to reproduce in commercial production the beneficial results obtained in greenhouse microcosm experiments (Gurr and Wratten 2000). This has led to the evaluation of a range of strategies for the deployment of inoculants. These vary from the use of mixed formulations of inoculants, e.g. AMF or PGPR formulations containing two or more AMF species or isolates or PGPR species (Yan et al. 2002; Mia et al. 2005; Domenech et al. 2006) or isolates (Bora et al. 2004); combinations of AMF and PGPR (Rodriquez-Romero et al. 2005); multiple combinations of AMF and PGPR (Vestberg et al. 2004); combinations of AMF, PGPR and *Trichoderma harzianum* (Srinath et al. 2003); AMF, PGPR and other bacteria (Bashan 1998); AMF, PGPR and nitrogen-fixing rhizobacteria, with stimulants of AMF spore germination and plant colonization, plant stimulants and fertilizers (Handelsman and Stabb 1996). The results of the use of multiple/combined inoculants have been variable. Either the inoculants, or some combinations thereof, may interact positively (Vestberg et al. 2004; Domenech et al. 2006) or show no additive effects (Tahmatsidou et al. 2006), or sometimes the plants may suffer from biological overload and show reduced yield (Cordier et al. 2000).

Another alternative strategy to the use of single inoculants, which approaches the use of complex mixtures of inoculants, has been the attempt to establish a 'beneficial microflora'. This may be through the use of relatively specific soil adjuvants, e.g. chitin, which is a substrate for lytic enzyme-producing microorganisms (including the widely used *Trichoderma* inoculants) (Rafferty et al. 2003) or the use of compost, green manure and other organic amendments (Hoitink and Boehm 1999; Rafferty and Cassells 2003). The concept of the beneficial microflora is the essence of the buffered microflora characteristic of traditional farming systems (Altieri 1995) and aspired to by farmers converting to organic farming, which results in pathogen-suppressive soils (Cook and Baker 1983).

The main emphasis in the use of biocontrol inoculants has been for the control of soil-borne pathogens, which are relatively unaffected by foliar fungicides (Handelsman and Stabb 1996). Indeed, the market niche for inoculants has expanded with the withdrawal of the soil sterilant methyl bromide (Ristaino and Thomas 1997; USDA 2000; Tahmatsidou et al. 2006). The withdrawal of methyl bromide is but one aspect of the general debate on the sustainability of intensive agriculture/horticulture. On energy, food safety and environmental pollution grounds there is increasing pressure to adopt more sustainable, environmentally friendly production methods (Chrispeels and Sadava 2003). The fungicide industry has responded by developing synthetic 'plant activators' (Strange 2003; Agrios 2004). Commercial plant activators include reactive oxygen species, analogues of the SAR (systemic acquired resistance) signalling-compounds salicylic acid and of ISR-signalling compounds jasmonic acid and ethylene (for reviews see Oostendorp et al. 2001; Gozzo 2003). For reviews on fungal and bacterial elicitors of plant defence responses, and for the signalling pathways involved see: Gatz (1997); Chang and

Shockey (1999); Pieterse and van Loon (1999); Clarke et al. (2000); Kunkel and Brooks (2002); Strange (2003); Laloi et al. (2004).

The use of AMF and/or PGPR to prime the plant's ISR/SAR response, sequentially with the use of plant activators and elicitors of SAR, may offer an effective strategy for controlling both soil-borne and foliar diseases of plants (see Fig. 21.1). Preliminary evaluation of this hypothesis has indicated that PGPR or AMF combined with foliar spraying with an elicitor can be used to control late blight of potato caused by *Phytophthora infestans* (O'Herlihy et al. 2001).

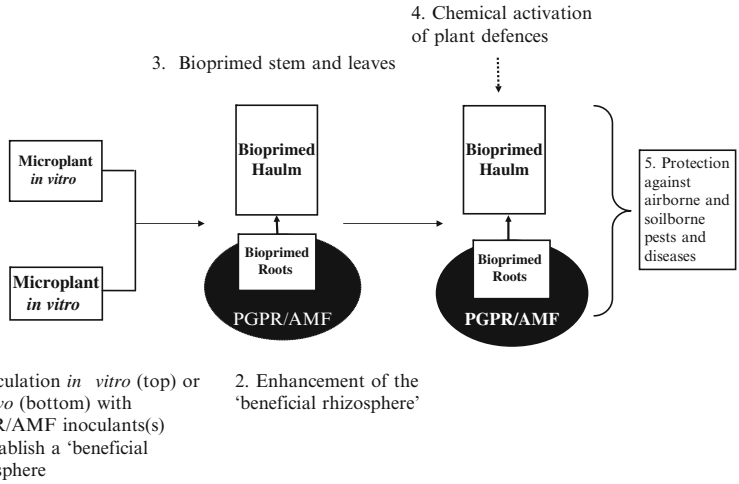


**Fig. 21.1** An illustration of the proposed strategy for holistic biocontrol of plant pests and diseases of the root system and above-ground parts of the plant. Step 1 involves inoculation with selected PGPR and/or AMF, with the objective of introducing pathogen antagonism, plant growth promotion and induction of ISR (see text for selection criteria, also Table 21.1). This may be carried out in autotrophic culture *in vitro*, or at establishment of the microplants. Step 2 may involve soil inoculation with additional microorganisms such as *Trichoderma* and free-living nitrogen-fixing bacteria and/or the addition of soil amendment such as chitin to promote the Step 1 inoculants and other pathogen antagonists. Step 3 'Biopriming' is the consequence of inoculation with ISR-inducing inoculants. Prime plant defences are expressed on pathogen or pest attack more strongly than non-primed defences. Step 4 Activation of (primed) plant defences involves spraying with 'chemical activators'; the term in the broad sense includes elicitors and signalling compounds such as nitric oxide, reactive oxygen species, salicylic acid, jasmonic acids and ethylene and their analogues (Cohen 2002). Broad spectrum defence responses may be expressed following spraying with inducers of both SAR and ISR (Ton et al. 2003), but it has been argued that enhancement of the defence response appropriate to the specific pathogen may give better control (Kunkel and Brooks 2002). The efficiency of the spray application may be improved when based on forecast of the disease specific risk(s). Some treatment may result in increased susceptibility to non-target pests and diseases (Thaler et al. 1999)

## 21.6 Conclusions

In view of the high cost of micropropagation, there is a need to improve the quality and disease resistance of microplants to ensure 100% establishment, rapid growing on and improved resistance to pests and diseases, especially damping-off diseases. There is evidence from the literature (see above) of the beneficial effects of inoculants for both growth promotion and improved disease resistance, but not all inoculants or more complex formulations of inoculants have been shown to be positive (e.g. see Vestberg et al. 2004).

Given the high cost of micropropagules, they are usually grown on in intensive propagation systems. There has been much concern about the use of pesticides in intensive greenhouse production and there is the universal concern about the sustainability of agriculture, problems of chemical residues in produce and the environmental impact of pesticides. This poses the challenge as to whether biological strategies can reduce pesticide usage or, indeed, substitute for pesticides. Holistic biocontrol strategies, involving the use of inoculants to protect against soil-borne diseases and to promote plant growth and bioprime the plants defences, combined with the use of plant activators, ideally natural elicitors, to activate the primed defences for the control of haulm diseases, have been proposed (O'Herlihy et al. 2003). These strategies require the development of methods for screening potential inoculants for the traditional traits of pathogen suppression, for growth promotion and for their ability to prime plant defences. Also, it will be necessary to screen plant activators for their ability to augment primed defence response (Table 21.1). In the latter regard, it is important to recognise that salicylate and its analogues, signal specific components of the biotic stress response, i.e. SAR proteins, whereas optimisation of the defence response may require elicitation of a broader response (McDowell and Dangl 2000; Conrath et al. 2002).

The combination of adding value to the micropropagules by improving establishment and to the plants derived from them by influencing ontogenetic development (Duffy and Cassells 1999) and by improving disease resistance has to be offset against the cost of the inoculants and saving in the cost of pesticides and, possibly, fertilizers. It remains to be determined which isolates or combination of isolates, and which plant activators, will provide these benefits and whether pathogen- and genotype-independent strategies can be developed to enable access to a large market for the products.

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# Chapter 22
# Co-Culture of *Linum album* Cells and *Piriformospora indica* for Improved Production of Phytopharmaceuticals

**A. Baldi, A. Jain, N. Gupta, A.K. Srivastava, and V.S. Bisaria**

## 22.1 Introduction

It is estimated that there are about 250,000 species of higher plants, but six times as many species of fungi. The interaction between plants and fungi is very complex and may be very specific to a given combination of plant and fungus (Grayer and Kokubun 2001). The association of fungus and part of any vascular plant is called a mycorrhiza. Mycorrhiza, the nutritional symbiosis between fungi and plants, has been found in 85% of higher plant genera (Chauhan et al. 2006). In these associations, the plant benefits from more efficient water and mineral (nitrogen and phosphorus) uptake and also through resistance against some pathogens. The fungus benefits through carbon source (sugar) which is translocated to the roots by the plants (Das et al. 2006). The most common and prevalent association with a plant is by arbuscular mycorrhizal fungi. These fungi belong to the order Glomerales (Morton and Benny 1990), which include all species capable of living symbiotically with plants. But these fungi are not able to grow in pure cultures without living host roots. Understanding this inability is one of the most challenging goals because the field of studying these symbiotic relationships, their mechanisms and effects still remains unexplored. The cultivation of root-organ cultures with AM fungi aseptically was developed as an alternative system to study the symbiosis in detail (Becard and Fortin 1988). But a new vista for scientists working on the study of plant-microbe interaction has opened up with the advent of *Piriformospora indica,* "fungus of the millennium" (Kumari et al. 2005). This fungus mimics

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the capabilities of a typical AM fungus and is also axenically cultivable. This is also a potential candidate to serve as biofertilizer, bioprotector, bioregulator, and bio-herbicide, and an excellent source for the hardening of tissue culture-raised plants (Chauhan et al. 2006).

In addition to essential primary metabolites, plants produce a wide variety of secondary metabolites. Plant secondary metabolites can be defined as compounds that have no recognized role in the fundamental life processes in the plants that synthesize them, but have an important role in the interaction of the plants with their environment. Almost 100,000 secondary metabolites have been discovered from the plant kingdom but the structures of only half of them have been fully elucidated (Verpoorte 1998; Dixon 2001). An extensive array of secondary metabolites have also been produced by plant cell suspension cultures as an alternative strategy, but to date their limited commercial exploitation has been a major concern. This is because only small amounts of these compounds are found in whole plants and cell culture systems. A combined application of biotic elicitation along with other yield-enhancement strategies can develop techniques for large-scale production of commercially important compounds by plant cell cultures.

*Piriformospora indica*, an endophyte, isolated from the soil of Rajasthan (India), has been reported to show growth-promotional and secondary metabolite-enhancer activities with diverse medicinal and economically important plants such as *Bacopa moniera* (Sahay and Varma 1999, 2000), *Azadirachta indica* (Singh et al. 2002, 2003), *Withania somnifera*, *Spilanthes calva* (Rai et al. 2001), *Tridex procumbans*, *Abrus procatorius* (Kumari et al. 2004), *Adhatoda vasica* (Rai and Varma 2005) and *Chlorophytum borivilianum* (Chauhan et al. 2006). All of these reports are on tissue culture-raised or field-grown plants. Therefore, for enhanced production of phytopharmaceuticals it is worthwhile to study the interaction between symbiotic fungi like *Piriformospora indica* and the plant cells in a co-culture system.

Podophyllotoxin is the most important aryltetralin lignan pharmaceutically due to its cytotoxic and antiviral properties along with its use in treatment of genital warts (Imbert 1998). It is used as a raw material for synthesis of cytostatics such as etoposide, teniposide and etopophos. Currently, podophyllotoxin is extracted from the roots of *Podophyllum hexandrum* and *P. peltatum* plants. Supply of this lignan by the conventional solvent extraction method has its limitations, due to the endangered status of *P. hexandrum* and uneconomical chemical synthesis (Van Uden 1992). For these reasons, production of podophyllotoxin by biotechnological means remains a favored goal for researchers. Very low content of podophyllotoxin has been reported in cell cultures of *Podophyllum* species (Chattopadhyay et al. 2001, 2002a, b, 2003a–c, Farakya et al. 2004). On the other hand, cell cultures of *Linum* spp. have been reported to produce podophyllotoxin with higher productivity in comparison with cell cultures of other plants (Baldi et al. 2007). Biosynthesis of these lignans via the phenylpropanoid pathway involves a bridge reaction of primary and secondary metabolism, i.e. conversion of phenylalanine to cinnamic acid by deamination. This reaction, catalyzed by the phenylalanine ammonia lyase (PAL) enzyme, has been reported as a rate-limiting step of lignan biosynthesis (Nicholas et al. 1994). So measurement of PAL enzyme activity has been an

important parameter in understanding the mechanism of yield enhancement of lignans.

This chapter reports a detailed procedure for the development of a successful co-culture system between plant cells of *Linum album* and fungal cells of *P. indica* in suspension cultures to produce the anticancer compound podophyllotoxin at significantly enhanced levels (Baldi et al. 2008).

## 22.2 Development of Plant Cell Cultures

### *22.2.1 Germination of Seeds*

1. Treat seeds of *L. album* with 1% Savlon (Johnson and Johnson, USA) for 5 min.
2. Rinse seeds four to five times with sterile double-distilled water.
3. Transfer seeds to a 100 ml Erlenmeyer flask containing 50 ml of 70% ethanol and treat for 1 min.
4. Remove ethanol after treatment and rinse the seeds with sterile double-distilled water three times.
5. Transfer seeds in a 100 ml Erlenmeyer flask containing 25 ml of 0.01% mercuric chloride and treat for 2 min.
6. Remove mercuric chloride solution, and rinse the seeds with sterile double-distilled water three times.
7. Transfer one seed/culture tube containing 20 ml of Murashige and Skoog (MS) medium (Murashige and Skoog 1962) solidified with 1% agar for germination using sterile forceps over flame. Gently press the explants into media for good contact.
8. Plug the culture tubes with sterile cotton plug over flame.
9. Transfer the tubes to a culture room at 25 ± 2°C under complete darkness and allow the seeds to germinate.

### *22.2.2 Initiation of Callus Cultures*

1. Collect stem portions from 30-day-old *in vitro* germinated plants of *L. album* and place the explants (stem) in a sterile Petri dish.
2. Cut stem portions to 1 cm × 1 cm size using a sterile blade.
3. Place one explant in one Petri dish containing 15 ml of MS medium solidified with 1% agar and supplemented with 0.4 mg $l^{-1}$ NAA (naphthalene acetic acid) for callus initiation using sterile forceps.
4. Cover the Petri dish with lid and seal it with parafilm.
5. Transfer Petri dishes in a culture room at 25 ± 2°C under 16 h/8 h light/dark cycle with a light intensity of 1,200 lux.
6. Observe the callus initiation.

### *22.2.3 Initiation of Suspension Cultures*

#### 22.2.3.1 Inoculum Preparation

1. Transfer fresh and friable cells (20-day-old) from callus culture (5 g $l^{-1}$ on dry cell weight basis) in to 250 ml Erlenmeyer flask containing 50 ml MS media containing 0.4 mg $l^{-1}$ NAA with the help of sterile spatula over flame.
2. Plug the culture tubes with sterile cotton plug over flame.
3. Place inoculated flasks on a gyratory shaker rotating at 125 rpm in a culture room at 25 ± 2°C under 16 h/8 h light/dark cycle with a light intensity of 1,200 lux.
4. Allow the cells to grow in suspension culture for 12 days.
5. Centrifuge the grown cells in sterile centrifuge tubes at 5,000 rpm for 10 min.
6. Decant the spent media and use cells to inoculate suspension cultures for elicitation set-up.

#### 22.2.3.2 Development of Suspension Culture

1. Transfer these cells (5 g $l^{-1}$ on dry cell weight basis) in to 250 ml Erlenmeyer flask containing 50 ml MS media containing 0.4 mg $l^{-1}$ NAA with the help of sterile spatula over flame.
2. Place inoculated flasks on a gyratory shaker rotating at 125 rpm in a culture room at 25 ± 2°C under 16 h/8 h light/dark cycle with a light intensity of 1,200 lux and allow the cells to grow for 12 days.

## 22.3 Development of Fungal Culture

### *22.3.1 Maintenance of Piriformospora indica*

1. Transfer inoculum disk of arbuscular myccorhiza-like fungus, *Piriformospora indica*, to a sterile Petri dish containing 20 ml MYPG media of following composition:

   Malt extract: 3 g $l^{-1}$
   Yeast extract: 3 g $l^{-1}$
   Peptone: 5 g $l^{-1}$
   Glucose: 10 g $l^{-1}$
   Agar: 20 g $l^{-1}$
   pH: 6.2

2. Incubate the inoculated Petri dish in an incubator at 25 ± 2°C.

3. Subculture the fungus on the same medium every month for maintenance of the culture.

## *22.3.2 Initiation of Fungal Culture*

### 22.3.2.1 Inoculum Preparation

1. Transfer 5-day-old fungal inoculum (3 × 1 cm squares of grown fungi) from solid MYPG media plates into a 250 ml Erlenmeyer flask containing 50 ml Hill and Kaefer's medium (Hill and Kaefer 2001).
2. Place inoculated flasks on incubator shaker rotating at 200 rpm and maintain at 30 ± 2°C.
3. Allow cells to grow in suspension culture for 5 days.
4. Centrifuge the grown cells in sterile centrifuge tubes at 5,000 rpm for 10 min.
5. Decant the spent media and use cells to initiate suspension cultures of *P. indica*.

Preparation of Hill and Kaefer Medium

1. The components of Hill and Kaefer medium are divided into two parts: A and B (Compositions are given in Tables 22.1 and 22.2).
   (a) The concentrations of various components mentioned in part A and part B are the final concentrations of components after mixing part A and part B.
   (b) Prepare $FeCl_3$ and $FeSO_4 \cdot 7H_2O$ solutions in 1 N $H_2SO_4$.
   (c) Separately prepare the stock solutions of all vitamins in distilled water. Bring the pH of solution to 12.5 in order to solubilize them except Nicotinamide.
2. Autoclave both stock solutions separately. After adding both the solutions, bring pH to 6.5 by adding required amount of autoclaved 2 M NaOH under aseptic conditions.
3. Store all the stocks at 4°C except vitamins.
4. Store stock solution of vitamins at −20°C.

### 22.3.2.2 Development of Fungal Culture for Co-Culture Setup

1. Transfer these fungal cells (0.6 g $l^{-1}$ on dry cell weight basis) to a 250 ml Erlenmeyer flask containing 50 ml Hill and Kaefer's medium aseptically with the help of a sterile spatula over flame.

2. Place inoculated flasks on a gyratory shaker rotating at 200 rpm on an incubator shaker at 30 ± 2°C.
3. Allow cells to grow for 5 days in suspension cultures.
4. Centrifuge the grown cells in sterile centrifuge tubes at 5,000 rpm for 10 min.
5. Decant the spent media and crush fungal cells with the help of a sterile magnetic bead, rotating for 15 min at 100 rpm on a magnetic stirrer under aseptic conditions.

**Table 22.1** The composition of part A of Hill and Kaefer Medium (2001)

| Components | Concentration (ml $l^{-1}$) |
|---|---|
| Peptone | 1.0 g $l^{-1}$ |
| Yeast extract | 0.1 g $l^{-1}$ |
| Casamino acid hydrolysate | 0.1 g $l^{-1}$ |
| Macro elements stock solution (A) | 50.0 |
| Micro elements stock solution (B) | 2.5 |
| Biotin (0.05%) | 1.0 |
| p-Amino benzoic acid (0.1%) | 1.0 |
| Nicotinamide (0.5%) | 1.0 |
| Pyridoxal phosphate (0.1%) | 1.0 |
| Riboflavin (0.25%) | 1.0 |
| **(a) Macro elements stock solution** | |
| | Concentration (g $l^{-1}$) |
| $NaNO_3$ | 120.0 |
| KCl | 10.4 |
| $MgSO_4 \cdot 7H_2O$ | 10.4 |
| $KH_2PO_4$ | 30.4 |
| **(b) Micro elements stock solution** | |
| $ZnSO_4 \cdot 7H2O$ | 22.0 |
| $H_3BO_3$ | 11.0 |
| $MnCl_2 \cdot 4H2O$ | 5.0 |
| $CoCl_2 \cdot 6H2O$ | 1.6 |
| $CuSO_4 \cdot 5H2O$ | 1.6 |
| $(NH_4)_6Mo_7O_{27} \cdot 4H_2O$ | 1.1 |
| $Na_2EDTA$ | 5.0 |

**Table 22.2** The composition of part B of Hill and Kaefer Medium (2001)

| Components | Concentration (ml $l^{-1}$) |
|---|---|
| Glucose | 20.0 g $l^{-1}$ |
| $CaCl_2$ (0.1 M) | 1.0 |
| $FeCl_3$ (0.1 M) | 1.0 |
| $FeSO_4 \cdot 7H_2O$ (0.5%) | 2.5 |

6. Use these fungal cells of *P. indica* to develop co-culture with cells of *L. album* in suspension cultures.

## 22.4 Establishment of Co-Culture of *L. album* with *P. indica*

### *22.4.1 Development of Co-Culture (Fig. 22.1)*

1. Add viable fungal cells to suspension culture of plant cells on a %, w/v basis (generally in a range of 0.5–10%, w/v) to suspension cultures of *L. album* at different phases of growth of plant cells.

   Note that the time of addition can be at the start of cultivation, end of lag phase, middle/late log phase or early stationary phase.

   Depending on time of addition of fungal cells to the medium and harvest time of culture, one can also test the effect of various times of co-cultivation; e.g. if a known concentration of fungal cells are added on the 8th, 10th and 11th days of a cultivation that ends on the 12th day, the time of incubation will be 96 h, 48 h and 24 h respectively.

2. Harvest the flasks, in duplicate, at the end of cultivation and analyze for effect of co-cultivation on growth and product accumulation.
3. Measure responses in terms of biomass of plant cells (g $l^{-1}$, on dry cell weight basis), podophyllotoxin accumulation (mg $l^{-1}$), overall volumetric productivity of podophyllotoxin (mg $l^{-1}d^{-1}$) and phenylalanine ammonia lyase enzyme activity (μkat/kg protein).

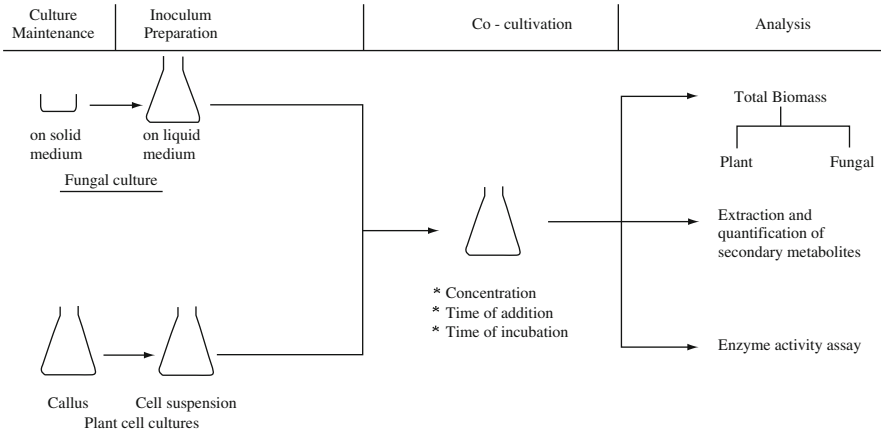


**Fig. 22.1** Development of co-culture of plant and fungal cells for enhanced production of secondary metabolites

## 22.5 Analysis

### *22.5.1 Growth in Terms of Dry Cell Weight*

1. Harvest individual flasks, in duplicate, at the end of cultivation.
2. Filter the cell suspension through Whatman No. 1 filter paper on a Buchner funnel under slight vacuum.
3. Wash the cells with double-distilled water.
4. Drain fully under vacuum.
5. Transfer the cells to pre-weighed Petri dish. Dry cells at 25 ± 2°C to a constant weight (approx. 48 h).
6. Reweigh the cells with Petri plate.
7. Calculate biomass produced/50 ml media by subtracting weight of empty Petri dish from total weight of Petri dish and dried cells. Express the weight of cell biomass as g $l^{-1}$ dry cell weight.

(Note that this biomass concentration represents the cumulative biomass of plant cells and fungal cells grown during a particular co-cultivation set-up)

### *22.5.2 Determination of Fungal and Plant Biomass*

#### 22.5.2.1 Determination of Fungal and Plant Biomass

1. Subject 1 g of dried biomass samples (plant + fungal) for acid hydrolysis and subsequent colorimetric analysis, as described in subsequent sections, in order to estimate concentration of fungal biomass in terms of chitin.
2. Determine plant biomass produced in co-culture experiments by subtracting the fungal biomass from total biomass produced in respective co-culture set-ups.

#### 22.5.2.2 Acid Hydrolysis for Chitin Estimation

1. Digest a known amount of dried fungal/(plant + fungal) biomass from each sample with 50 ml of 5 M sulfuric acid under agitation at 100 rpm for 15 min.
2. Centrifuge it at 5,000 rpm for 40 min and discard the supernatant.
3. Rinse the biomass with distilled water twice.
4. Incubate the cells with 10 ml of 10 N HCl at 25 ± 2°C for 16 h.
5. Make up the volume to 100 ml with double-distilled water.
6. Autoclave each sample at 15 psig and 121°C for 2 h for hydrolysis.
7. Neutralize hydrolysate to pH 7 using 10 M NaOH and 0.5 M NaOH.
8. Subject this hydrolysate for estimation of chitin using MBTH (3-methyl-2-benzothiazolone hydrazone hydrochloride).

#### 22.5.2.3 Colorimetric Assay for Chitin Estimation (Smith and Gilkerson 1979)

1. Suspend 1 g of dried and powdered fungal biomass in 100 ml of double distilled water to get a stock solution of 10 g $l^{-1}$ concentration.
2. Prepare standard suspensions of fungal biomass in the concentration range of (0.15–1.5 g $l^{-1}$ on dry cell weight basis).
3. Hydrolyze these fungal biomass samples with sulfuric acid as described in earlier section.
4. Add 2 ml of 2.5% sodium nitrite to 1 ml of acid hydrolyzed fungal or total biomass sample.
5. Add 500 μl of 0.5 M HCl to the reaction mixture.
6. Vortex reaction mixture to liberate excess $NaNO_2$ and incubate at 25 ± 2°C for 15 min.
7. Then add 1 ml of 0.25%, w/v MBTH to this and vortex again.
8. Incubate the reaction mixture in water bath at 65°C for 60 min.
9. Add 1 ml of freshly prepared $FeCl_3$ (0.5%, w/v).
10. Measure absorbance of the final mixture (intense blue color) at 650 nm after 15 min.
11. Plot standard curve between different concentrations of fungal biomass (0.15–1.5 g $l^{-1}$ on dry cell weight basis) and respective $A_{650}$ values.
12. Estimate fungal biomass from the absorbance value of the respective sample from the standard curves.

### *22.5.3 Extraction and Estimation of Podophyllotoxin*

1. Suspend accurately weighed dried and powdered cells (100 mg) in 5 ml of methanol in 10 ml glass tube.
2. To release intracellular product, sonicate cells at 4–6°C for 15 min and allow it for complete extraction for 24 h.
3. Centrifuge the extract at 5,000 rpm for 10 min and collect the supernatant.
4. Allow methanol to evaporate at 25 ± 2°C (approx. 12–18 h).
5. Re-dissolve this extract in known volume of HPLC grade methanol and filter it through 0.22 μm filter.
6. Quantify podophyllotoxin in each sample on HPLC under following conditions:

   Column: Nova Pak RP-$C_{18}$ column (Waters, USA)
   Packing: 250 × 4.6 mm
   Mobile phase: 0.01% phosphoric acid in water: acetonitrile (72:28%, v/v)
   Flow rate: 0.8 ml $min^{-1}$
   Column temperature: 30°C
   Detection: Diode array detector at 290 nm
   Standard: Commercially available podophyllotoxin (Sigma, USA)

### 22.5.4 *Phenylalanine Ammonia Lyase (PAL) Enzyme Extraction and Assay*

1. Freeze fresh weight of plant cells (1 g) and homogenize using liquid nitrogen in a precooled mortar and pestle at 4°C.
2. Add 3 mL of borate buffer (0.1 M, pH 8) containing 5% glycerol and 50 mM β-mercaptoethanol to powdered cells.
3. Ultrasonicate samples at 4°C for 1 min.
4. Centrifuge the homogenate at 10,000 rpm for 30 min at 4°C and collect the supernatant.
5. For activity assay, add 1 ml of 50 mM phenylalanine to 0.5 ml of supernatant and allow to incubate at 40°C for 60 min.
6. Stop the reaction by addition of 0.2 mL 6 N HCl.
7. Extract the reaction mixture by addition of 4 ml toluene and vortex for 15 s.
8. Centrifuge enzyme extract at 1,000 rpm for 10 min to separate the phases.
9. Analyze the toluene phase for the trans-cinnamic acid recovered against a toluene blank at 290 nm.
10. Express PAL enzyme activity as μkat (μmoles of cinnamic acid formed per second) per kg protein. Total protein estimation based on the principle of protein-dye binding can be determined using bovine serum albumin as standard (Bradford, 1976).

## 22.6 Conclusions

Plant cell culture-based technologies with intervention of arbuscular mycorrhiza-like fungi such fungi can provide a suitable alternative for large-scale production of important secondary metabolites at a commercial level. In this regard, *Piriformospora indica* can be used as a very promising tool, as significant enhancement in plant cell biomass and podophyllotoxin productivity has been observed. Podophyllotoxin accumulation and PAL enzyme activity were found to be directly associated, as maximum lignan production resulted from maximum increment of enzyme activity in the present case study.

*Note:* Intellectual property rights of the present work by the authors are covered under Indian patent application no. 1266/DEL/2007 entitled “A process for enhanced production of bioactive compounds”

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# Chapter 23
# Fungal Elicitors for Enhanced Production of Secondary Metabolites in Plant Cell Suspension Cultures

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## 23.1 Introduction

Plant cell culture systems are viable alternatives for the production of secondary metabolites that are of commercial importance in food and pharmaceutical industries. However, relatively very few cultures synthesize these compounds over extended periods in amounts comparable to those found in whole plants. Various strategies have been employed to increase the production of secondary metabolites in cell cultures as well as in hairy root cultures for commercial exploitation. These include manipulation of culture media (hormonal and nutrient stress) and environmental conditions (temperature, pH and osmotic stress), precursor addition, elicitation and combination of these strategies. Nowadays, genetic manipulation of biosynthetic pathways by metabolic engineering has also become a powerful technique for enhanced production of desired metabolites.

The recent developments in elicitation of cell cultures have opened a new avenue for the production of these compounds. Secondary metabolite synthesis and accumulation in cell cultures can be triggered by the application of elicitors to the culture medium. Elicitors can be defined as signalling molecules triggering the formation of secondary metabolites in cell cultures by inducing plant defence, hypersensitive response and/or pathogenesis related proteins. Depending on the origin, elicitors can be classified in two classes: biotic and abiotic. Elicitors of biological origin are called biotic elicitors. These include polysaccharides, proteins, glycoproteins or cell-wall fragments derived from fungi, bacteria and even plants.

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Among these, fungal elicitors have been most widely studied for enhancement of synthesis of commercially important compounds from plant cell cultures. Elicitors of non-biological origin are called abiotic elicitors, which include metal ions, UV light and chemically defined compounds. Recently, the term 'abiotic stress' is also being used for abiotic elicitors.

Many studies have shown that both biotic elicitors and abiotic stresses enhance secondary metabolite synthesis in plant cell cultures. However, no elicitor has been found to have a general effect on many culture systems, and no system has been found to respond to all elicitors. So it is necessary to screen various elicitors for a particular system for production of a desired compound. Moreover, the concentration of elicitors, and the incubation time required for maximum elicitation, differ with the kind of elicitor and the culture system. Therefore, screening is a must to arrive at a suitable elicitor, and to determine its concentration and contact time for maximal response in terms of secondary metabolite accumulation. Another important aspect is the time of addition of a elicitor to the culture. Optimal induction occurs when the elicitors are added to cultures at late exponential or early stationary phase of plant cell growth. So the effect of any elicitor for maximum response depends on the age of culture, concentration of elicitor and incubation time with the elicitor. Keeping this in view, there has been a strong need for the discovery of useful elicitors and for novel screening methods that would allow substances having elicitation capability to be rapidly and easily screened.

In most of the studies done to date to enhance the synthesis of commercially important plant-derived compounds by the addition of fungal elicitors to cell cultures, pathogenic fungi have been used. Fungi, which mainly induce hypersensitive response in plant cells, result in activation of plant defence pathways and thereby increase phytoalexin production. Another significant plant–fungi interaction reported is the symbiotic relationship between arbuscular myccorhizal fungi and plant cells/organs. But due to the inability of these symbiotic fungi to grow in a synthetic media, it has not been possible to study their effect for elicitation, if any. With the discovery of *Piriformospora indica* (Varma and Franken 1997), a novel endophytic axenically cultivable fungus, which mimics the capability of arbuscular myccorhizal fungi, a new era has opened for enhanced production of plant-based secondary metabolites in cell cultures by elicitation with this fungus.

Podophyllotoxin is one of the most promising secondary metabolites from medicinal plant research, due to its pronounced cytotoxic activity. It is currently being used for the synthesis of anticancer drugs such as etoposide, teniposide, and etopophos, which are used for the treatment of testicular and lung cancers and certain leukemias (Stahelin and Wartburg 1991; Imbert 1998). The isolation of podophyllotoxin from the rhizomes of *Podophyllum peltatum* and *P. hexandrum* (Berberidaceae) plants is not a very ideal production system. The *P. hexandrum* rhizomes may contain ca. 4% of podophyllotoxin on a dry weight basis, while *P. peltatum* contains still lower amounts of it. The supply of this compound has become increasingly limited due to intensive collection, lack of cultivation, long juvenile phase and poor reproduction capabilities (van Uden 1992). The species *P. hexandrum* is also listed in Appendix II of CITES (Convention for International Trade in Endangered Species). This appendix lists species that are not necessarily

threatened now with extinction, but which may become so unless trade is closely controlled (World Conservation Monitoring Centre 2001).

The chemical synthesis of podophyllotoxin is possible (Hadimani et al. 1996), but largely hampered by the complicated stereochemical ring closure necessary to attain this compound. Synthetic production therefore only yields restricted quantities at high cost.

This supply problem forms the drive for a large number of scientists to search for alternative sources of podophyllotoxin. An ideal resource would be a fast-growing and easy to cultivate plant cell or organ culture with a high lignan content. Tissue cultures of *Podophyllum* species turned out to be quite recalcitrant or low-yielding (Chattopadhyay et al. 2001, 2002a, b, 2003a–c; Farakya et al. 2004); therefore, in recent years tissue culture of Iranian flax, *Linum album*, has become an attractive alternative. These cell cultures have been reported to produce lignans with highest productivity (Baldi et al. 2007). This chapter provides details for enhanced production of podophyllotoxin in cell suspension cultures of *Linum album* by elicitation with culture filtrate and cell extract of *P. indica*.

## 23.2 Development of Plant Cell Cultures

### *23.2.1 Germination of Seeds*

1. Treat seeds of *L. album* with 1% Savlon (Johnson and Johnson, USA) for 5 min.
2. Rinse seeds with sterile double distilled water four to five times.
3. Transfer seeds into a 100 ml Erlenmeyer flask containing 50 ml of 70% ethanol, and treat for 1 min.
4. Remove ethanol after treatment, and rinse the seeds with sterile double distilled water thrice.
5. Transfer seeds in a 100 ml Erlenmeyer flask containing 25 ml of 0.01% mercuric chloride, and treat for 2 min.
6. Remove mercuric chloride solution, and rinse the seeds with sterile double distilled water thrice.
7. Transfer one seed/culture tube containing 20 ml of Murashige and Skoog (MS) medium (Murashige and Skoog 1962) solidified with 1% agar for germination using a sterile forceps over flame. Gently press the explants into the media for good contact.
8. Plug the culture tubes with a sterile cotton plug over flame.
9. Transfer the tubes into a culture room at 25 ± 2°C under complete darkness, and allow the seeds to germinate.

### *23.2.2 Initiation of Callus Cultures*

1. Collect stem portions from 30-day-old *in vitro* germinated plants of *L. album*, and place the explants (stem) in a sterile Petri dish.
2. Cut stem portions to 1 cm × 1 cm size with the help of sterile blade.

3. Place one explant in one Petri dish containing 15 ml of MS medium (Murashige and Skoog 1962) solidified with 1% agar and supplemented with 0.4 mg $l^{-1}$ NAA (naphthalene acetic acid) for callus initiation using a sterile forceps.
4. Cover the petri dish with lid and seal it with parafilm.
5. Transfer Petri dishes in a culture room at 25 ± 2°C under 16 h/8 h light/dark cycle with a light intensity of 1,200 lux.
6. Observe the callus initiation.

### *23.2.3 Initiation of Suspension Cultures*

#### 23.2.3.1 Inoculum Preparation

1. Transfer fresh and friable cells (20 days old) from callus culture (5 g $l^{-1}$ on dry cell weight basis) into a 250 ml Erlenmeyer flask containing 50 ml MS media containing 0.4 mg $l^{-1}$ NAA with the help of sterile spatula over flame.
2. Plug the culture tubes with sterile cotton plug over flame.
3. Place inoculated flasks on a gyratory shaker rotating at 125 rpm in a culture room at 25 ± 2°C under 16 h/8 h light/dark cycle with a light intensity of 1,200 lux.
4. Allow the cells to grow in suspension culture for 12 days.
5. Centrifuge the grown cells in sterile centrifuge tubes at 5,000 rpm for 10 min.
6. Decant the spent media and use cells to inoculate suspension cultures for elicitation setup.

#### 23.2.3.2 Development of Suspension Culture

1. Transfer these cells (5 g $l^{-1}$ on dry cell weight basis) into a 250 ml Erlenmeyer flask containing 50 ml MS media containing 0.4 mg $l^{-1}$ NAA with the help of sterile spatula over flame.
2. Place inoculated flasks on a gyratory shaker rotating at 125 rpm in a culture room at 25 ± 2°C under 16 h/8 h light/dark cycle with a light intensity of 1,200 lux and allow the cells to grow for 12 days.

## 23.3 Development of Elicitors

### *23.3.1 Preparation of Elicitors*

1. Transfer cells of arbuscular myccorhiza like fungus, *Piriformospora indica*, to a 250 ml Erlenemeyer flask containing 50 ml of MYPG media of following composition:

Malt extract: 3 g $l^{-1}$
Yeast extract: 3 g $l^{-1}$
Peptone: 5 g $l^{-1}$
Glucose: 10 g $l^{-1}$
(pH: 6.2)

2. Incubate the inoculated flasks on an incubator shaker, rotating at 200 rpm, at 25 ± 2°C in 16 h/8 h light/dark cycle, and allow the cells to grow till the end of the log phase (6 days).
3. Collect the culture broth by passing the fungal culture through a Whatman No. 1 filter paper.
4. Centrifuge the culture broth at 5,000 rpm for 15 min and remove fungal cells, if any.
5. Farakya et al. (2005) suggested the following technique for preparation of fungal elicitors.

   (a) Divide the culture broth into two portions. Use one portion after passing it through 0.22 μm filter and designate it as 'filtered culture broth'.
   (b) Autoclave another portion of culture broth at 15 psig and 121°C for 20 min and designate it as 'autoclaved culture broth'.
   (c) Wash the fungal mat several times with sterile double distilled water and allow it to dry at 40 ± 2°C in a hot air oven to constant weight.
   (d) Crush the dry cells in a mortar pestle.
   (e) Suspend 10 g of dry cell powder in 100 ml of double distilled water (pH 5.7) and autoclave it at 15 psig and 121°C for 20 min for hydrolysis.
   (f) Centrifuge the hydrolysate at 5,000 rpm for 10 min
   (g) Collect the supernatant and designate it as 'cell extract'.
   (h) Store the fungal elicitor preparations at 4°C till further use.

   Methodology for preparation of fungal elicitors is represented as Fig. 23.1.

### *23.3.2 Addition of Elicitors*

1. Add all three fungal elicitors separately on v/v basis (generally in a range of 1–10%, v/v) to suspension cultures of *L. album* at different phases of growth with the help of sterile pipette.

(Note that the time of addition can be at the start of cultivation, end of lag phase, middle and/or late log phase or early stationary phase. Depending on the time of addition of elicitor to the medium and harvest time of culture, one can test the effect of various times of incubation also, e.g. if the elicitor is added on the 8th and 10th days of a cultivation that ends on the 12th day, the times of incubation will be 96 and 48 h respectively.)

2. Harvest the flasks, in duplicate, at the end of cultivation, and analyse for the effect of fungal elicitors on growth and product accumulation.
3. Measure the responses in terms of biomass (g $l^{-1}$, on dry cell weight basis), podophyllotoxin accumulation (mg $l^{-1}$) and overall volumetric productivity of podophyllotoxin (mg $l^{-1}d^{-1}$).

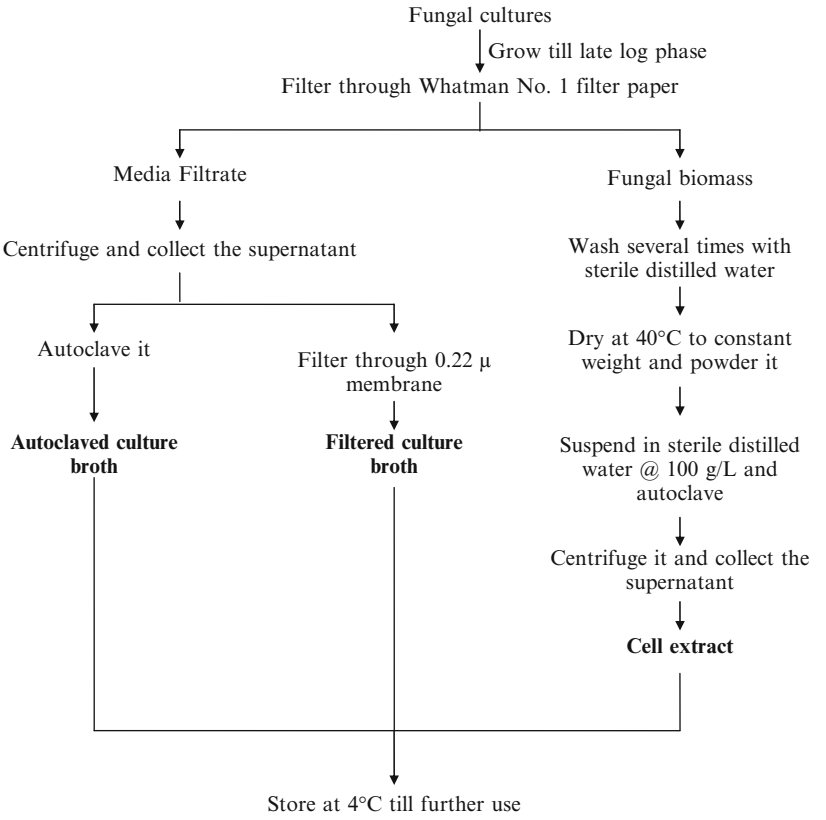


**Fig. 23.1** Preparation of fungal elicitors

## 23.4 Analysis

### *23.4.1 Growth in Terms of Dry Cell Weight*

1. Harvest individual flasks, in duplicate, at the end of cultivation.
2. Filter the cell suspension through Whatman No. 1 filter paper on a Buchner funnel under slight vacuum.
3. Wash the cells with double distilled water.
4. Drain fully under vacuum.
5. Transfer the cells to a pre-weighed Petri dish. Dry cells at $25 \pm 2°C$ to a constant weight (approx. 48 h).
6. Reweigh the cells with a Petri plate.
7. Calculate biomass produced/50 ml media by subtracting weight of the empty Petri dish from total weight of Petri dish and dried cells. Express the weight of cell biomass as g $l^{-1}$ dry cell weight.

### 23.4.2 Extraction and Estimation of Podophyllotoxin

1. Suspend accurately weighed dried and powdered cells (100 mg) in 5 ml of methanol in 10 ml glass tube.
2. To release intracellular product, sonicate cells at 4–6°C for 15 min and allow complete extraction for 24 h.
3. Centrifuge the extract at 5,000 rpm for 10 min and collect the supernatant.
4. Allow methanol to evaporate at 25 ± 2°C (approx. 12–18 h).
5. Re-dissolve this extract in a known volume of HPLC grade methanol and filter it through 0.22 μm filter.
6. Quantify podophyllotoxin in each sample on HPLC under following conditions:

   Column: Nova Pak RP-$C_{18}$ column (Waters, USA)
   Packing: 250 × 4.6 mm
   Mobile phase: 0.01% phosphoric acid in water: acetonitrile (72:28%, v/v)
   Flow rate: 0.8 ml $min^{-1}$
   Column temperature: 30°C
   Detection: Diode array detector at 290 nm
   Standard: Commercially available podophyllotoxin (Sigma, USA)

## 23.5 Conclusions

For elicitation studies in plant cell cultures, it is necessary to screen various elicitors and optimize their concentration and time of incubation. *P. indica* has been found to enhance podophyllotoxin in plant cell cultures by several-fold. This can provide a valuable technique in commercial production of plant-derived secondary metabolites.

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# Chapter 24
# Auxin Production by Symbiotic Fungi: Bioassay and HPLC-MS Analysis

**Anke Sirrenberg, Richard Splivallo, Astrid Ratzinger, Katharina Pawlowski, and Petr Karlovsky**

**Abbreviations**

| | |
|---|---|
| ESI | Electrospray ionization |
| HPLC | High performance liquid chromatography |
| IAA | Indole-3-acetic acid |
| MS | Mass spectrometry |
| MSK | Murashige and Skoog |
| NAA | α-naphthalene acetic acid |
| PTFE | Polytetrafluorethylene |

## 24.1 Introduction

Many soil bacteria and fungi, including many ectomycorrhizal fungi, produce indole-3-acetic acid (IAA) or similar derivatives, collectively termed auxins (Gruen 1959; Ek et al. 1983; Barker and Tagu 2000). IAA is a plant hormone responsible for the regulation of cell elongation; directed growth and apical dominance (see Taiz and Zeiger 2006). In a specific concentration range which may vary for different plant species, externally applied auxin increases root branching and hairy root formation, with concurrent reduction of main root length.

Auxin secreted by a fungus into the soil in the vicinity of plant roots may thus increase the overall root surface, improving the chance for the fungus to establish

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physical contact with a potential host. Apart from this mechanistic aspect, the secretion of auxin by plant symbionts and pathogens constitutes a chemical signal conditioning the plant for the interaction (Spaepen et al. 2007). The modification of plant root architecture by plant-associated microbes producing auxin may lead to the improvement of the water and mineral uptake capacity of the plant, and as such has a potential of improving the yield of cultivated plants.

Here we present a simple bioassay which can be conducted in any laboratory with basic microbiological equipment, and which may give a first hint as to whether a mycorrhizal or endophytic fungus produces auxin. We also present a detailed protocol for IAA determination by HPLC-MS. Part of the procedure has been published in Sirrenberg et al. (2007).

*Arabidopsis thaliana* is recommended as a test plant for the bioassay, although it is non mycorrhizal. *A. thaliana* seedlings are very suitable for *in vitro* tests in Petri dishes because they are small and the roots are very sensitive to auxin. Additionally, many mutants are available, including mutants impaired in auxin uptake or perception. Other plants have also been successfully tested, including *Lotus japonicus* (Fig. 24.2), *Medicago truncatula* (A. Sirrenberg, unpublished data) and *Cistus incanus* (R. Splivallo, unpublished data).

## 24.2 Equipment and Laboratory Material

### *24.2.1 Equipment*

For bioassays:

- Laminar flow bench or clean area for sterile work
- Pipettes 20–200 µl
- Growth chamber (or greenhouse with stable conditions)
- Autoclave

For HPLC-MS analysis:

- Rotary evaporator or SpeedVac vacuum concentrator
- Round-bottom flasks
- Rotary incubator (optional)
- Homogenizer or blender to grind the agar for IAA extraction
- HPLC-ESI-MS system, e.g.:
  - Autosampler ProStar 430, Varian, Germany
  - Binary pump system (ProStar 210, Varian, Germany)
  - Column oven
  - Reversed-phase column (Polaris C18-A, 150 × 2 mm; 5 µ, Varian, Germany) and a compatible guard column
  - Triple-quadrupole mass spectrometer with electrospray ionization interface (Varian 1200 L LC/MS, Varian, Germany)

### 24.2.2 Laboratory Material

- *Arabidopsis thaliana* seeds (wild type and/or mutants) or seeds of mycorrhizal plants
- Murashige and Skoog medium with vitamins (e.g. Duchefa, The Netherlands)
- Cultures of fungi to be tested
- Malt extract (optional)
- Sucrose
- Agar or Phytagel (Sigma-Aldrich, St Louis, MO, USA)
- Petri dishes (preferably square, 10 cm × 10 cm)
- Autoclaved calibrated cylinder or sterile plastic tube (50 ml)
- Ethanol
- Indole-3-acetic acid (IAA)
- Deuterated indole-3-acetic acid ($D_5$-IAA for HPLC-ESI/MS analysis)
- Filter paper discs (e.g. antibiotic assay discs, Ø 9 mm)
- Sterile aluminum foil (approx. 3 cm × 4 cm)
- Tweezers
- Sterile demineralized water
- Sterile filter paper (e.g. 6 cm diameter)
- Sterile toothpicks
- 0.1% sterile water agar (optional)
- Scalpel (optional)
- Spatula (optional)
- Ethyl acetate (for the diffusion bioassay see Sect. 24.4.5)
- Methanol, water, acetonitrile, acetic acid (for HPLC/MS analysis)

## 24.3 Interaction on the Same Medium: *Piriformospora*/*Arabidopsis*

### 24.3.1 Preparation of Slope Agar Plates

Prepare 1/2 MSK liquid medium by dissolving 2.2 g $l^{-1}$ Murashige and Skoog medium with vitamins and 15 g of sucrose in 800 ml of demineralised water. Adjust pH to 5.8. Adjust volume to 1,000 ml. Add 15 g agar or 3 g phytagel and autoclave.

Pour 40 ml of the autoclaved medium into a square Petri dish. Create a slope by carefully lifting the plate at one end and sliding the lid underneath (see Fig. 24.1). The rim of the lid should be placed at the middle of the Petri dish.

**Remark:** Choosing the gelling agent: Medium prepared with Phytagel is transparent and allows better observation of root development and colonization, even microscopical observation at low magnitude through the bottom of the closed Petri dish.

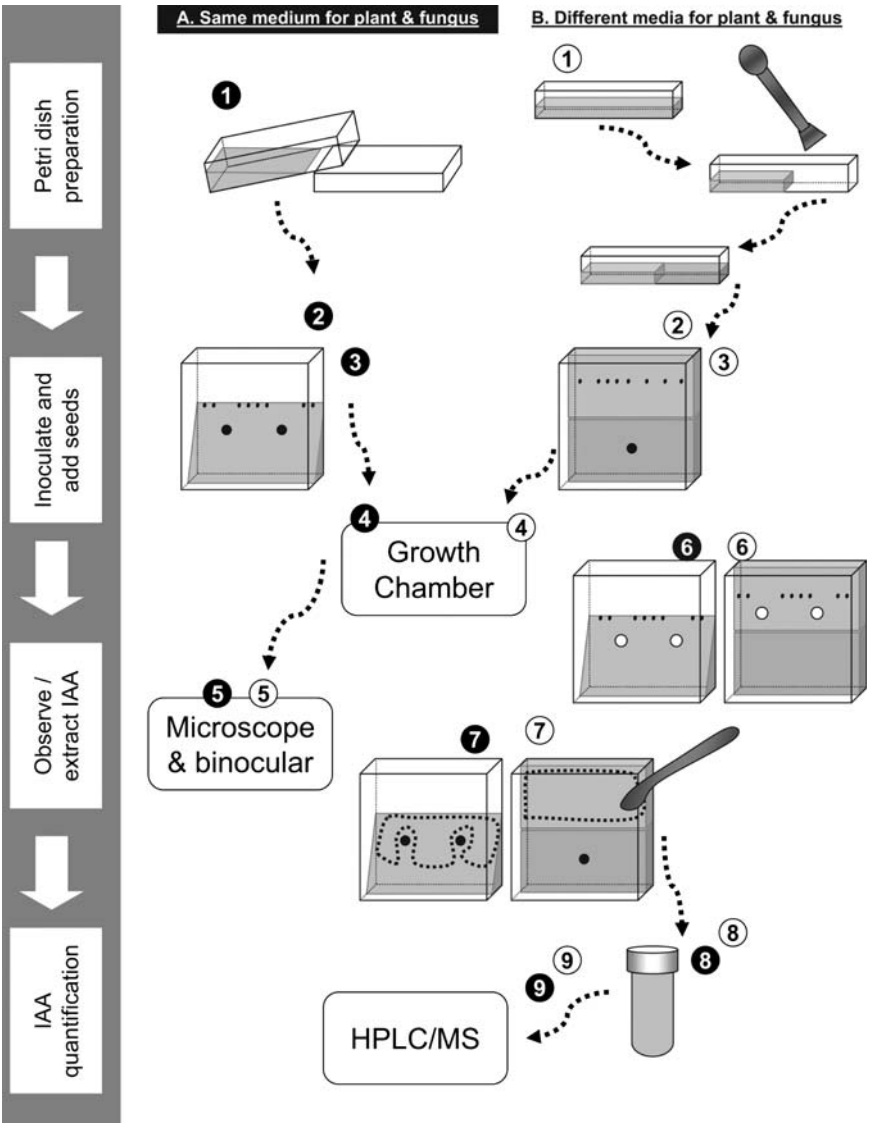


**Fig. 24.1** Step-by-step guide to IAA determination
Bioassay preparation (24.3.1, 24.4.1). Inoculate fungus (*the black larger dots*) (24.3.2, 24.4.2). Add seeds of test plants (24.3.3, 24.4.3). Grow plants (24.3.4, 24.4.4). Observe morphological changes of the roots (24.3.5). Bioassay with synthetic IAA or culture filtrates on the filter discs (*white circles*) (24.4.5). Collect agar with a scalpel for IAA quantification (24.4.6). Extract IAA with ethyl acetate (24.4.7). Quantify IAA by *HPLC-MS* (24.4.8)

In addition, roots may be harvested from phytagel without damage by dissolving the solid medium in three volumes of 100 mM sodium citrate buffer pH 6.

**Remark:** If buffering the pH of the medium is required, remember that an acidic medium will facilitate IAA uptake in the seedlings better than a neutral one, because of passive diffusion of of the protonated IAAH through the cell walls (Yang et al. 2006).

**Remark:** In mycorrhizal research, lower sucrose concentrations are often recommended to improve the colonization of the root by the fungus. We have tested sucrose concentrations in a range between 0.5 and 2%, and obtained similar results with regard to root branching of *A. thaliana* inoculated with *Piriformospora indica*.

**Remark:** Round Petri dishes (Ø 9.0 cm) can be used instead of square ones, but the development of the roots can then only be clearly observed in the middle of the plates, especially in the controls where the roots tend to be longer.

### *24.3.2 Inoculation of the Fungus*

Place mycelial plugs from the growing margin of a colony below the seeds, as indicated in Fig. 24.1.

### *24.3.3 Adding Arabidopsis Seeds to the Petri Dish*

Sterilize seeds as follows:

- 70% ethanol, 1 min
- 20 min in 1% sodium hypochloride
- Rinse three times with sterile water

Transfer sterilized seeds to a sterile wetted filter paper in a round Petri dish and if needed stratify them (2 days at 4°C) to synchronize germination (may be necessary especially for some mutants of *A. thaliana*).

To transfer seeds onto the agar, take them one by one with the tip of a sterile toothpick and gently place them at the surface of the agar, as illustrated in Fig. 24.1. Alternatively, suspend sterilized seeds in 0.1% sterile water agar and pipette seeds one by one onto the rim of the agar slope.

**Remark:** We recommend a maximum of 10 seeds of *A. thaliana* per Petri dish if precise microscopic observations need to be made on root branching and root length. The bioassay can be followed up and the root branching quantified until the rooting system becomes too entangled to distinguish primary from secondary roots. This time period depends on the fungus; for truffles (e.g. *Tuber melanosporum* or *T. borchii*) we were able to monitor root development of *A. thaliana* in this system for 15 days.

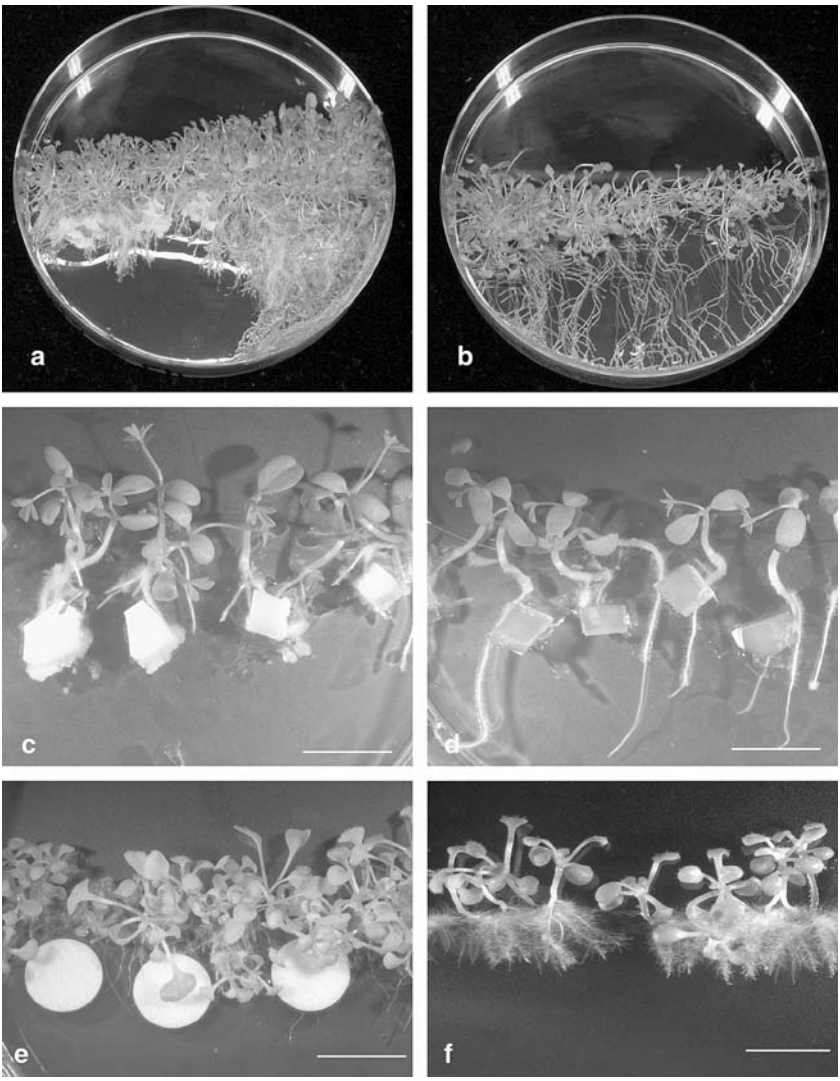


**Fig. 24.2** Effect of living mycelium of *P. indica* and externally applied auxin on *A. thaliana* **a** Bushy root growth of *A. thaliana* inoculated with *P. indica* (8 weeks). Even plants at a distance of some cm of the mycelium show increased root branching. **b** Control plants with normal (elongated) roots. **c** Increased root branching in *Lotus japonicus* inoculated with *P. indica*; **d** control (8 weeks). **e** Externally applied auxin induces bushy root phenotype in *A. thalina*, 250 µg IAA per filter disc (5 weeks); **f** 1 µM NAA in the medium (2 weeks). Scale bar denotes 1 cm

### 24.3.4 Growth Conditions

We recommend 20°C, 120 μmol $m^{-2}$ $s^{-1}$, 16 h light.

A yellow plastic shield may be used to protect auxins from light degradation during plant growth (Stasinopoulos and Hangarter 1990).

### 24.3.5 Evaluation of Roots

Branching: observe under a binocular and count secondary and tertiary roots. Root length: acquire an image of the Petri dishes by using a scanner or camera, measure root length. Root hairs: take pictures under a binocular, measure root hair length. Software such as the freeware Image J (available at http://rsb.info.nih.gov/ij/) may be used for determining root hair length and root length from digital images.

## 24.4 Interaction on Two Different Media: Truffles/*Arabidopsis*

Some mycorrhizal fungi are difficult to grow under laboratory conditions, and may require specific medium formulation. If no or poor growth is achieved with the fungus on MSK-medium, an alternative "dual-culture" bioassay can be used to test the strain for the production of IAA.

### 24.4.1 Preparation of Dual Bioassay Plates

Square Petri dishes (10 × 10 $cm^2$) are first filled with 50 ml of the fungal medium. Keep the Petri dishes horizontally in a sterile bench until the medium solidifies. Then remove half of the medium with a scalpel and spatula (see Fig. 24.1), and add 25 ml of melted MSK-agar (or another plant medium) to fill the space where the fungal medium has been removed (see the illustration in Fig. 24.3a).

### 24.4.2 Inoculation of the Fungus

Inoculate the fungus in the middle of the fungal medium, and if growth or IAA exudation is very slow, allow it to grow in the dark before plant seeds are added for

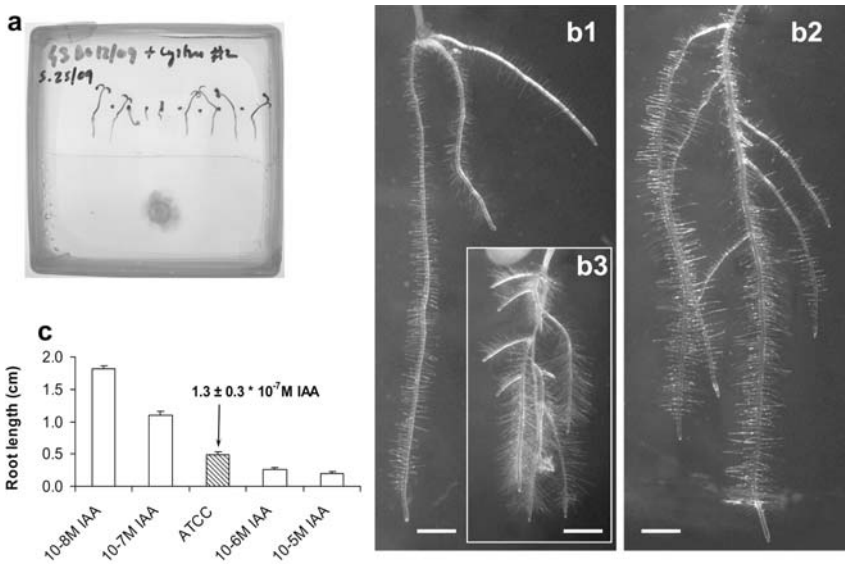


**Fig. 24.3** Dual bioassay setup and effect of truffle metabolites on *A. thaliana*
Bioassay setup with 10 days old *Cistus incanus* seedlings growing on MSK-agar (*upper part*) and truffle mycelium on malt extract agar (*T. borchii* strain 43BO, *lower part*). Scale: 10 × 10 $cm^2$ Petri dish. Root morphology of 15 days old *A. thaliana* seedlings of the control (no mycelium) (**b1**); or induced by mycelial exudates of *T. melanosporum* (strain Rey_t) (**b2**) or *T. borchii* (strain ATCC 96540) (**b3**). Note the root shortening induced by *T. borchii* as well as the increased branching and root hair length induced by both truffle species. Scale: 0.10 cm. Dose-response of *A. thaliana* primary root to IAA (*bars* = standard errors) (**c**). Note that the root shortening observed with strain ATCC correlates with the IAA quantification in the MSK-agar by HPLC/MS ($1.3\pm 0.3*10^{-7}$ *M* after 10 days of growth in the dark, $n = 3$ Petri dishes)

a period of time which needs to be determined empirically (for example, 10 days are suitable for truffles) (Fig. 24.3a).

### *24.4.3 Adding Arabidopsis Seeds to the Petri Dish*

Sterilize and stratify seeds as described under Sect. 24.3.3. After a sufficient amount of mycelial biomass has built, add the seeds of the test plant (e.g., *A. thaliana*) onto the MSK-side of the Petri dish, as depicted in Figs. 24.1 and 24.3a.

### *24.4.4 Growth Conditions and Evaluation*

The same as described under Sects. 24.3.4 and 24.3.5.

### 24.4.5 Estimation of the IAA Amount Produced by the Fungus

Establish a dose response of *A. thaliana* to synthetic IAA as described below. Prepare IAA standard solutions in ethanol, e.g. 1 µM, 10 µM, 100 µM. Apply 45 µl of the solution per filter disc (FD). Place the FDs on aluminum foil in a cleanbench, and allow the solvent to evaporate completely (this may take a few hours). Prepare FDs with pure solvent (ethanol) for the control Petri dishes.

Place the FD on the agar as indicated in Fig. 24.1 (2 FDs per Petri dish), add the seeds of the test plants as described earlier and grow the plants for ten to 15 days. Compare the morphological changes to the rooting system induced by the fungus to changes caused by known IAA amounts.

**Remark:** Alternatively, auxin may be added directly to the medium. In this case, a stock solution in ethanol is prepared, and a suitable volume is added to the medium after autoclaving when the medium has cooled down to a temperature of about 60°C. Mix by swinging the bottle gently. Instead of IAA, the more stable derivative α-naphtalene acetic acid (NAA) may be used (Fig. 24.2f), but care must be taken when working with auxin deficient mutants. NAA may enter the roots by diffusion, circumventing carrier mechanisms (Delbarre et al. 1996).

**Remark:** Fungal culture filtrate may also be used directly or after extraction with ethyl acetate to test the presence of IAA. In the case of *Piriformospora*, inoculate an appropriate medium, e.g. 100 ml M+ (4 g $l^{-1}$ yeast extract, 10 g $l^{-1}$ malt extract, 4 g $l^{-1}$ glucose, 20 g $l^{-1}$), with 20 mycelial plugs from the growing margin of a colony on solid medium. Mock-inoculate control flasks with plugs from solid medium without fungus. Be sure to use medium prepared the same day and stored under the same conditions for fungal cultures and controls. In a first experiment, cultures should be harvested at different time points, e.g. once a week (at least one fungal culture and one control), until the timepoint of maximal IAA concentration is known. Separate mycelium from medium by filtration through filter paper. Take 15 ml of the culture supernatant and of sterile medium as a control. Sterilize 5 ml by sterile filtration, apply to filter paper discs as described above (in experiments with culture filtrate of *Piriformospora indica*, 135 µl per disc were effective). Adjust the remaining 10 ml of medium to pH 3 with acetic acid, and extract with equal volume of ethyl acetate. Evaporate the organic phase to dryness under vacuum or nitrogen stream. Resolve the residue in 200 µl of ethyl acetate, apply to filter paper discs (45 µL on one disc) and repeat the bioassay.

**Remark:** The bioassays described above should not be considered as a proof that a fungus produces IAA, because other substances may cause similar effects, or inhibiting substances may interfere with the effect of IAA. It rather provides a quick indication that a fungus produces IAA or metabolite(s) with similar effects.

### 24.4.6 IAA Quantification in Agar Plates

Slope agar plates or dual bioassay plates are prepared as described above. Three Petri dishes are inoculated with the fungus (*Piriformospora* or truffle) as described

in Fig. 24.1, and three control plates are mock-inoculated with plugs taken from the same medium that served to pre-cultivate the fungus. The fungus is allowed to grow under the same conditions as with the bioassay described earlier. After the desired time, the agar zone where the plants were normally growing (~10 g agar: determine the exact weight) is harvested with a scalpel as depicted in Fig. 24.1 and stored in a 50 ml Falcon tube at −20°C until further extraction.

### *24.4.7 Extraction of the Agar Plates for IAA Determination*

- Avoid direct light to prevent IAA degradation.
- To each agar sample add 7.0 ml water/methanol mixture (1:1) containing the desired amount of internal standard (e.g. 250 ng deuterated IAA). Homogenize/crush with a blender.
- Add 50 µl 100% acetic acid (fuming).
- Add 7.0 ml ethyl acetate and vortex for 30 s followed by centrifugation at 4,500 rcf for 10 min.
- Remove upper phase (ethyl acetate), transfer to a new centrifuge tube (or 15 ml Falcon tube) and remove the solvent under vacuum at 40°C.
- Redissolve the residue in 1.0 ml water/methanol containing 7.0 mM acetic acid, filter through a 0.2 µm PTFE filter into a dark HPLC vial.

**Remark:** Alternatively, IAA concentration can be determined in liquid cultures described under 24.4.5. Add internal standard $D_5$-IAA (we used 250 ng of $D_5$-IAA for 100 ml of liquid culture) to the culture filtrate and to the control. Adjust to pH 3 with acetic acid. Extract twice with an equal volume of ethyl acetate, using a separating funnel. The separation of the two phases may be accelerated by centrifugation (3,200g, 10 min, 10°C). Collect organic phases, evaporate to dryness (SpeedVac or vacuum evaporator), redissolve in 1.0 ml water : methanol 1: 1 with 7 mM acetic acid, filter through a 0.2 µm PTFE filter into dark HPLC vials.

### *24.4.8 HPLC-ESI-MS/MS Determination of IAA*

#### 24.4.8.1 HPLC Conditons

Ten microliters of the HPLC sample are injected onto a RP-18 column and eluted isocratically with methanol/water/acetonitrile (50: 47.5: 2.5) containing 7 mM acetic acid at a flow rate of 0.2 ml $min^{-1}$.

#### 24.4.8.2 ESI-MS/MS System and Conditions

- Triple-quadrupole mass spectrometer (Varian 1200 L LC/MS, Varian, Germany) with electrospray ionization source

- Drying gas (nitrogen) pressure: 18 psi (pounds per square inch, equivalent to 124 kPa) at 250°C, nebulizing gas (air): 50 psi (345 kPa)
- Pressure of collision gas (argon): 1.4 mTorr
- Needle voltage: −4400 V
- Shield voltage: −600 V
- Capillary voltage: −40 V
- The mass spectrometer was operated with negative Electrospray ionization in multiple reaction monitoring mode.
- Mass transition for IAA: *m*/*z* 173.9/ *m*/*z* 130.0 (collision energy (CE) 9.0 eV)
- Mass transition for $D_5$-IAA: *m*/*z* 178.8/ *m*/*z* 134.0 (CE 11.5 eV)

A calibration curve of the ratio of peak areas of unlabeled standards to peak area of deuterium labeled IAA was used for quantification.

**Remark:** Because IAA occurs in common media components, extracts of media in which no fungus had been cultured should always be analyzed as negative controls.

If any IAA is found in the pure medium, its concentration has to be subtracted from the IAA concentrations in culture filtrates of the fungus. Obviously, the use of media which do not contain detectable amounts of IAA is preferable.

## 24.5 Examples of IAA Production by Fungi

### *24.5.1 Piriformospora indica*

*Piriformospora indica* is an endophytic fungus which increases plant growth in many plants of different families (Varma et al. 1999). In contrast to arbuscular mycorrhizal fungi, *P. indica* grows well on artificial media, and it is also able to colonize cruciferous plants including *A. thaliana*. In many interactions increased shoot growth was accompanied by increased root biomass. The mechanism by which this beneficial effect is achieved has long been debated. In our bioassay, *A. thaliana* inoculated with *P. indica* showed increased root branching and reduced main root length ("bushy roots"), whereas the roots of uninoculated plants reached the bottom of the Petri dish after 2 weeks (Figs. 24.2a, b). Similarly enhanced root branching was observed after inoculation of the mycorrhizal plant *Lotus japonicus* with *P. indica* (Figs. 24.2c, d). The phenotype resembled very much typical auxin effects. Therefore we compared the effect of pure externally applied auxin in different concentrations (Figs. 24.2e, f, for details see Sirrenberg et al. 2007) with the effect of *P. indica* culture filtrates and its ethyl acetate extracts. The pure culture filtrate as well as the extracts inhibited main root length while increasing root branching. The IAA content in the culture filtrate was determined by HPLC-MS and GC-MS to be 1.36 +/− 0.36 μM (after 4 weeks of growth in M+ medium at 23°C on a rotary shaker (Sirrenberg et al. 2007).

### 24.5.2 Truffles

Truffles are symbiotic fungi forming ectomycorrhizae with trees (e.g., oaks, hazels) and some shrubs (e.g., *Cistus*). Production of IAA by ectomycorrhizal fungi is well-known, and IAA, probably with other signal molecules, might drive the ectomycorrhiza genesis (Barker and Tagu 2000). Using the bioassay described in Fig. 24.3a, we demonstrated that the exudates of two truffle species (*Tuber borchii* and *T. melanosporum*) modify the root architecture of *Arabidopsis thaliana* (i.e., root shortening, increased branching and root hair length: see Figs. 24.3b1–b3). IAA was quantified by HPLC-MS directly from the MS agar zone of the bioassay (Fig. 24.3a). The mycelium of *T. borchii* (strain ATCC 96540) produced 1.3 $\pm$ 0.3 $*10^{-7}$ M IAA after 10 days of growth in the dark (about ten times more than was found in control Petri dishes containing no fungus). After establishing a dose response of *A. thaliana* root length to synthetic IAA, we concluded that the IAA exudated by *T. borchii* fully accounts for the root shortening of *A. thaliana* seedlings (Fig. 24.3c).

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# Chapter 25
# Siderophores of Mycorrhizal Fungi: Detection, Isolation and Identification

**K. Haselwandter and G. Winkelmann**

## 25.1 Introduction

Iron is an essential element for almost all forms of life. In an aerobic environment, iron in aqueous solution exists in the form of $Fe(H_2O)_6^{3+}$ and precipitates as an oxide–hydroxide polymer. With a solubility product of $K_S = 10^{-38}$ M for Fe $(OH)_3$, the maximum concentration of free ferric iron in aqueous solution is $10^{-17}$ M at pH 7. This renders iron unavailable for organisms in sufficient quantities, unless they produce a solubilization system. Under iron-limiting conditions most bacteria and fungi excrete siderophores as chelating agents which form soluble complexes with Fe (III) with very high formation constants, thus solubilizing ferric iron. Properties and functions of microbial siderophores have been compiled earlier in a comprehensive handbook on microbial iron chelates (Winkelmann 1991). The reader is also referred to recent reviews on: (1) the biosynthesis of siderophores and the identification of membrane transporters (Winkelmann 2001; Haas 2003), (2) the ecological significance of fungal siderophores (Winkelmann 2006), and (3) siderophores of symbiotic fungi (Haselwandter 2008; Johnson 2008; Haselwandter and Winkelmann 2007).

This chapter is designed as a collection of common fungal siderophore structures and useful methods for the identification and characterization of siderophores, and to be complementary to Haselwandter and Winkelmann 1998. Hence, for bioassays and most of the chemical assays, for example, the 1998 publication should be consulted. For elucidating the potential of mycorrhizal fungi to release siderophores, they have to be grown in a nutrient medium adequate not only for fungal

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growth but, most importantly, for maximum siderophore release into the nutrient medium. As the nutrient requirements of mycorrhizal fungi vary strongly, it is not attempted to list here the composition of different nutrient media suitable for cultivation of mycorrhizal fungi. Instead, standard literature ought to be consulted, and the composition of the nutrient medium modified for maximum siderophore production, which can be enhanced by, for example, the addition of specific presursors for their biosynthesis.

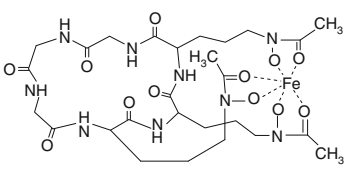
Siderophore production, in general, is stimulated by lowering the external concentration of ferric iron. Under some circumstances, not only omission of any iron compound, but also deferration of the nutrient medium may be required. This can be achieved by adding an excess of Chelex$^{R}$ 100 to the nutrient medium, and shaking for 15 min followed by filtration in order to remove the Chelex$^{R}$ 100 prior to the sterilization of the nutrient medium.

## 25.2 Isolation of Hydroxamate Siderophores from Fungal Culture Filtrates

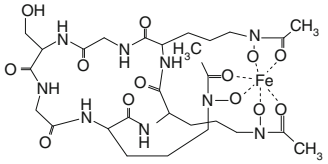
When hydroxamate siderophores are expected to occur in the culture filtrate, ferric chloride or ferrous sulfate can be added to obtain the ferric hydroxamate complexes, the latter under stirring for aeration. The amount of ferric salts added is critical, and has to be judged from the color formation. However, a surplus of ferric iron or resulting hydroxides are removed during the following adsorption procedure on Amberlite$^{R}$ XAD-2 or -16 which adsorbs a variety of lipophilic compounds and most of the ferric hydroxamates. Alternative methods employ an extraction of ferric hydroxamate siderophores with organic solvents, using chloroform, ether or benzylalcohol. Most ferric hydroxamates from aqueous culture filtrates can be adsorbed to Amberlite$^{R}$ -2 or -16 by using either a batch or a column procedure. The XAD-column procedure is performed as follows:

Wash the XAD material extensively first with methanol and then with water before use. Let the culture filtrate slowly percolate through the XAD column. Loading of the column can be visually followed by an increasing orange-brown color of the XAD material. To remove residual culture medium and surplus ferric hydroxides, wash with two volumes of distilled water. The adsorbed ferric hydroxamates are then desorbed with one or two volumes of methanol.

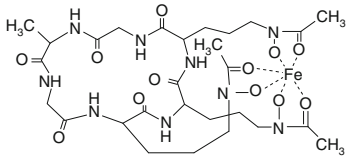
Separation and identification of fungal siderophores requires the application of various analytical methods which are discussed in this chapter in greater detail. In general, we observe two main groups of peptidic ferrichromes; those having N-acetyl side chains varying only in the amino acid residues of the hexa- or heptapeptide ring (Fig. 25.1), and those having a common ferrichrysin peptide ring but varying in the N-acyl side chains, such as ferrirubin, ferrirhodin or ferrichrome A (Fig. 25.2). The ester-containing coprogens and fusarinines represent the other group of hydroxamate siderophores (Fig. 25.3). The rhizoferrins (Fig. 25.4) do not belong to the hydroxamates, but are citrate-containing siderophores



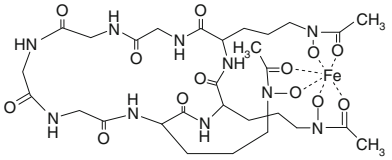
**Ferrichrome $M_r$ = 740**



**Ferricrocin $M_r$ = 770**

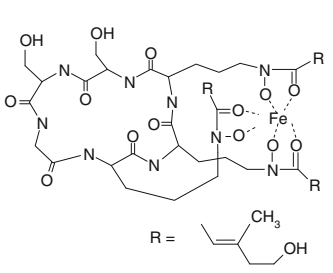


**Ferrichrome C $M_r$ = 754**

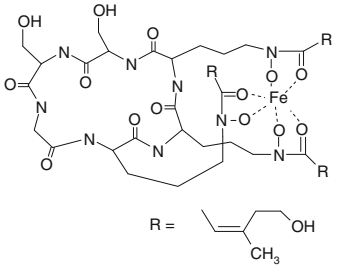


**Tetraglycylferrichrome $M_r$ = 797**

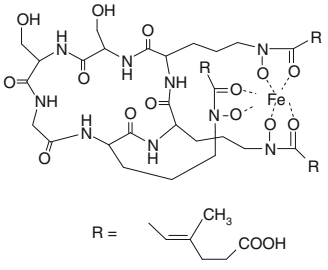
**Fig. 25.1** Fungal ferrichromes with N-acetyl side chains



**Ferrirubin $M_r$ = 1010**

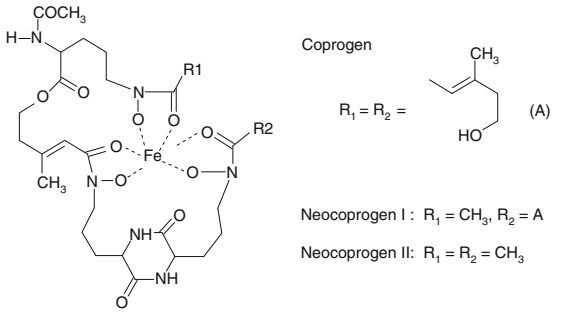


**Ferrirhodin $M_r$ = 1010**

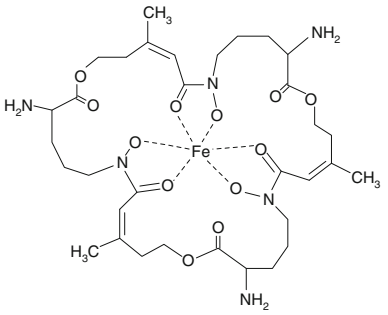


**Ferrichrome A $M_r$ = 999**

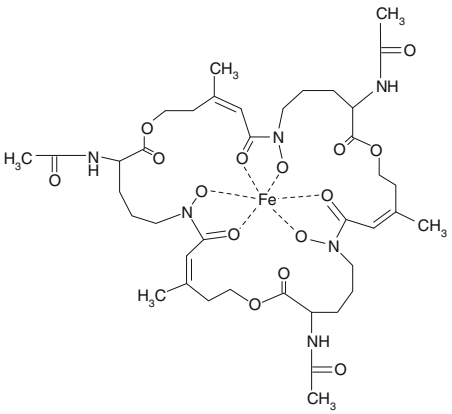
**Fig. 25.2** Fungal ferrichromes containing longer N-acyl chains




**Coprogen $M_r$ = 821**

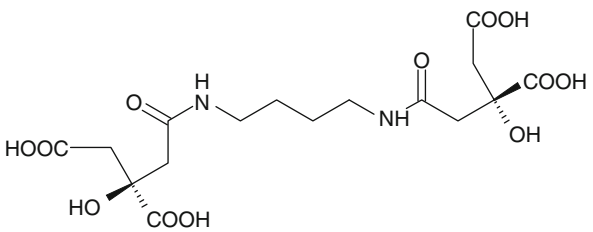



**Fusigen $M_r$ = 779**




**Triacetylfusarinine C $M_r$ = 905**

**Fig. 25.3** Fungal coprogens and fusarinines



**Rhizoferrin $M_r$ = 436**

**Fig. 25.4** Rhizoferrin

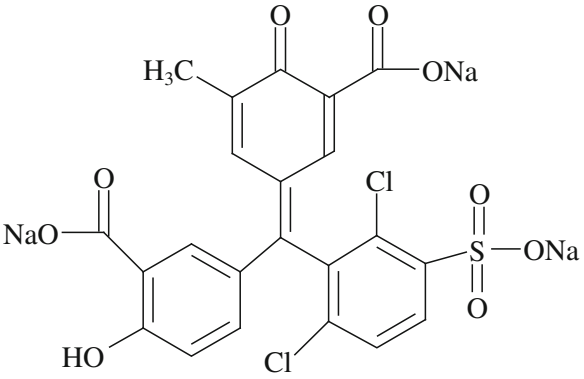
originally isolated from Zygomycetes (Drechsel et al. 1991, 1992; Winkelmann 1992). Whether some mycorrhizal fungi may produce, in addition to hydroxamates, also such polycarboxylate-type siderophores still remains an open question.

## 25.3 Chemical Assays

Several chemical assays have been developed for the detection of catechol and hydroxamate siderophores (see, for example, Neilands and Nakamura 1991; Payne 1994; Haselwandter and Winkelmann 1998). However, the CAS assay is the one which is still widely used. Therefore the CAS assay is selected for a more detailed description.

### *25.3.1 Chrome Azurol S (CAS) Assay (Modified After Schwyn and Neilands 1987)*

Chrome azurol S (Fig. 25.5) was developed as an indicator for metal titration, and turned out to be a useful compound for the detection of siderophores in the form of the so-called CAS assay. This powerful method uses the high formation constant of siderophores for ferric iron. Strong chelators, like siderophores, remove iron from the blue ternary complex of the chrome azurol S-iron(III)-detergent complex, and thereby decolorate the solution (Schwyn and Neilands 1987). The CAS assay can be applied for the determination of siderophores in solution or on agar plates, for detecting siderophore excretion into the culture medium. As indicated in the original description, chrome azurol S agar plates can be used directly to search for siderophore-producing colonies. This is also possible for mycelial cultures of mycorrhizal fungi, provided the agar is supplemented with the required nutrients and the detergent (HDTMA) does not inhibit the growth of fungal colonies. If the latter is the case, this problem can be dealt with by reducing the HDTMA concentration to the maximum which is tolerated by the micro-organism to be cultured.




**Chrome azurol S $M_r$ = 605**

**Fig. 25.5** Structure of chrome azurol S used for the CAS assay

#### 25.3.1.1 Dye Solution

(a) 6.0 mg CAS in 5 ml distilled water
(b) 1 ml Fe(III)-solution (1 mM $FeCl_3$ $6H_2O$, 10 mM HCl)
(c) Mix solution a and b to give the CAS–Fe solution
(d) 7.29 mg hexadecyltrimethylammonium bromide (HDTMA) in 4 ml distilled water
(e) Dye solution: mix d with c and sterilize

#### 25.3.1.2 Preparation of Chrome Azurol S Agar Plates (100 ml Solid Medium)

(a) PIPES salts solution: prepare the appropriate basal salts medium (without glucose and agar) and add 3.25 g PIPES which dissolves after adjusting to pH 6.8 with NaOH (50%)
(b) Add 1.5 g agar to the PIPES salts solution and autoclave. After cooling to 50–60°C add sterile solutions of carbon source and other required supplements such as vitamins or antibiotics.
(c) The dye solution is then added to the PIPES-salts solution containing agar, carbon source and other nutrient medium constituents.

## *25.4 Separation of Ferric Hydroxamates by HPLC*

Modern laboratory equipment allows to separate and identify most siderophores by HPLC, using reversed phase columns (4.6 × 250 mm, Nucleosil $C_{18}$, 5 µm), a gradient system containing acetonitrile/water (6–40%) plus 0.1% trifluoroacetic acid (TFA) or formic acid, pH 2, or gradients of acetonitrile/10 mM ammonium acetate, pH 3, and a detector wavelength of 220 nm or a hydroxamate specific wavelength of 435 nm (Konetschny-Rapp et al. 1988; Fernandez and Winkelmann 2005). Small amounts of hydroxamates can be purified on a semi-preparative reversed-phase column (8 × 250 mm, Nucleosil $C_{18}$, 7 µm) using a gradient of acetonitrile and a fraction collector system.

Ferrichromes (Fig. 25.6) as well as coprogens and fusarinines (Fig. 25.7) are well-separated under the conditions as indicated above, although discrimination between ferrichrome, ferricrocin and ferrichrysin requires careful construction of the gradient system. The more lipophilic siderophores ferrichrome A, ferrirubin, ferrirhodin and triacetylfusarinine C (triacetylfusigen) are generally easy to separate, and represent no problem for identification according to retention times. It is, however, recommended to use reference compounds which can be added in a co-chromatography mode (spiking). Siderophores as reference compounds can be ordered, for example, from EMC Microcollections GmbH, Tuebingen, Germany.

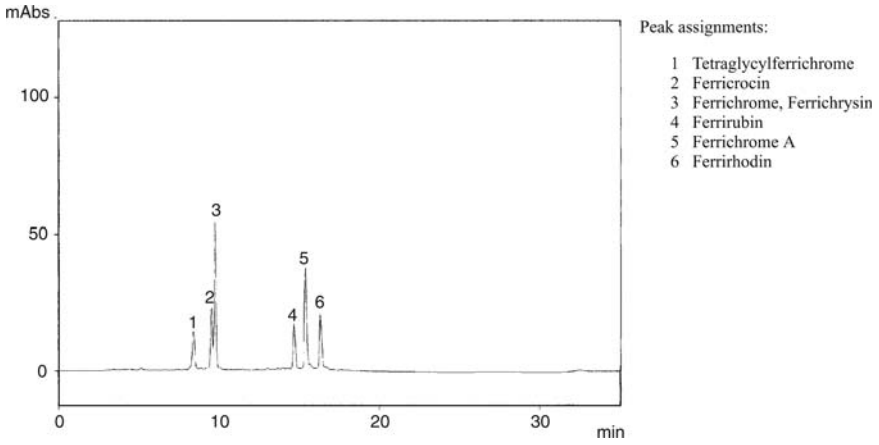



**Fig. 25.6** HPLC chromatogram of ferrichromes

## 25.5 Mass Spectrometry

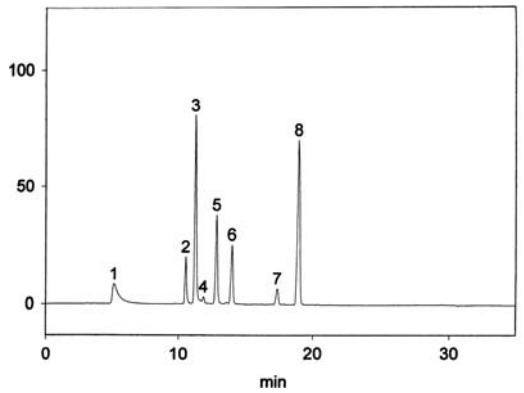
After purification by HPLC, hydroxamate siderophores can be identified by fast atom bombardment (FAB) mass spectrometry or by ion spray mass spectrometry. According to the authors' experience, a Sciex API III triple quadrupole mass spectrometer with 2,400 Da mass range equipped with an ion spray source (Sciex, Toronto, Canada), for example, works well for the determination of molecular mass equivalents ($MH^+$, $MNa^+$, $MK^+$). This method was used previously, for example, by Reissbrodt et al. (1990) and Drechsel et al. (1991). Most useful are Fourier transform ion cyclotron resonance–electrospray ionization–mass spectra (FTICR-ESI-MS; e.g., 4.7 T APEX II FTICR Bruker-Daltonics) for the identification of novel or yet unknown siderophores.

### *25.5.1 Gas Chromatography: Mass Spectrometry*

The constituents of siderophores are determined after acid hydrolysis and derivatization on basis of GC-MS experiments. This approach is successfully used to identify the amino acids and organic acids which constitute most of the hydroxamate siderophores. It is often required to reduce the N-OH group of $N^5$-OH-ornithine by using a reductive hydrolysis with HJ.

## 25.6 NMR Spectroscopy

$^1H$ NMR and $^{13}C$ NMR spectra are finally required to elucidate the chemical structure of isolated siderophores. Detailed descriptions of spectroscopic identification of fungal siderophores are given in Jalal and van der Helm (1991).




Peak assignments:

1 Fe-Dimerum acid
2 Fusigen (cyclic)
3 Neocoprogen II
4 Impurity
5 Neocoprogen I
6 Coprogen
7 Triacetylfusarinine C (linear)
8 Triacetylfusarinine C (cyclic)

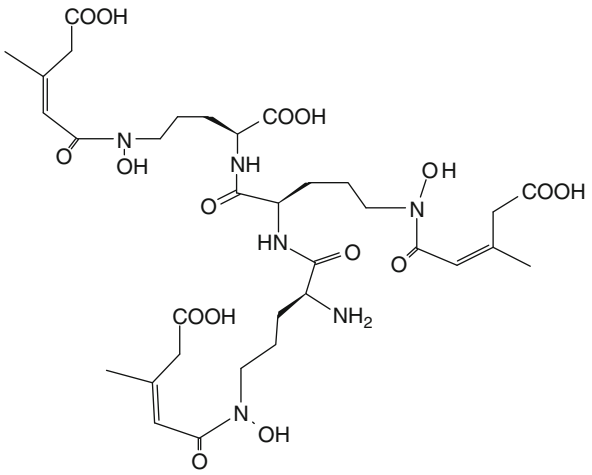
**Fig. 25.7** HPLC chromatogram of coprogens and fusarinines

For carboxylate structures, see Drechsel et al. (1992). The connectivity of siderophore constituents can be determined by using 2D-NMR techniques such as HSQC, HMBC, COSY and NOESY.

## 25.7 Conclusions

Ericoid mycorrhizal fungi produce as main siderophores ferricrocin or fusigen (Haselwandter et al. 1992). Ferricrocin has also been demonstrated to represent the main siderophore of the widespread ectomycorrhizal fungus *Cenococcum geophilum* (Haselwandter and Winkelmann 2002), some *Wilcoxina* spp forming ectendomycorrhizae (Prabhu et al. 1996), and *Phialocephala fortinii*, a dark septate root endophyte (Bartholdy et al. 2001). All the fungal species producing ferricrocin as listed above are ascomycetes; hence, it is of interest to note that ferricrocin seems to be also the main siderophore exuded by the extraradical mycelium of the basidiomycetous ectomycorrhizal fungus *Hebeloma crustuliniforme* (van Hees et al. 2006). Thus, the main siderophores released by mycorrhizal fungi covering most of the major kinds of mycorrhizal symbiosis have been identified, albeit for a limited number of species (see, for example, review by Haselwandter and Winkelmann 2007). However, there are still significant gaps in our knowledge. It is, for example, still unknown whether and what kind of siderophores are released by the Glomeromycota which form the widespread arbuscular mycorrhizae.

The methods described above can be employed for investigations of mycorrhizal fungi in which siderophores may be detected which are novel with regard to their chemical structure. It was hypothesized earlier that ‘a screening of mycorrhizal fungi for their potential to synthesize siderophores could lead to the discovery of natural, yet unknown iron-chelating agents’ (Haselwandter 1995). Recently this hypothesis was corroborated when a novel siderophore was detected in culture




**Basidiochrome (iron-free) $M_r$ = 787**

**Fig. 25.8** Basidiochrome, a novel hydroxamate siderophore isolated from the orchidaceous mycorrhizal fungi *Ceratobasidium* and *Rhizoctonia spp*

filtrates of mycorrhizal fungi of orchids, its precise chemical structure elucidated, and the novel siderophore described as basidiochrome (Fig. 25.8) which is the main iron-chelating agent released by a range of orchidaceous mycorrhizal fungi (Haselwandter et al. 2006). However, the methods compiled in this chapter can also lead to the detection of siderophores which are novel in terms of the taxonomic affiliation of the respective producer.

**Disclaimer** Reference to a company and/or product is for purposes of information only, and does not imply approval or recommendation of the product.

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# Chapter 26
# Biology and Molecular Approaches in Genetic Improvement of Cultivated Button Mushroom (*Agaricus Bisporus*)

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## 26.1 General Biology

### *26.1.1 Morphology and Life Cycle*

Practically all the cultivated species of mushrooms (including *Agaricus bisporus*) belong to a subdivision of fungal kingdom designated as Basidiomycota. *A. bisporus* is placed in the order Agaricales of Basidiomycota, and are characterized by carrying sexual spores externally on a structure called basidium (pl. basidia).

The vegetative phase of *A. bisporus* is mainly underground and made up of filaments called hyphae, which branch in all directions and constitute the mycelium. The reproductive phase is above ground and constitutes the fruiting body. Fruiting bodies may attain varied shapes in mushroom. However, the most common type of fruiting body, met mainly in Agaricales and to a lesser extent in other groups, is an umbrella type, consisting of Pileus (pl. pilei) or cap, supported by a stipe (stalk). The fruiting body of *A. bisporus* (Fig. 26.1) also consists of a pileus (cap) supported by a central stipe (stalk). The basidia are borne in a palisade-like layer called the hymenium on the lower surface of pileus, known as hymenophore. The hymenophore appear in the forms of gills or lamellae, which are radiating plates with hymenium covering the two faces.

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**Fig. 26.1** Mushroom fruiting body

The distinguishing features of *A. bisporus* fruiting body are: Chocolate-brown spores (as seen in spore print), gills not attached to the stipe (free) and a distinct ring on the stalk (stipe) termed as the 'annulus'.

The life cycle starts with the germination of spore discharged from basidium. *A. bisporus* is characterized by all basidia bearing only two basidiospores, as opposed to the four produced in other *Agaricus* species. On germination, spores of *A. bisporus* produce a mycelium, which is characteristically fertile. In the case of all the other four spore species of *Agaricus* mycelium arising from uninucleate spore, they are in the first instance sterile, and only on mating with a compatible homokaryotic mycelium produce a fertile diakaryotic mycelium. Mycelium in *A. bisporus* is septate, and each of the cells contains all necessary information for independent growth. If a mycelium is fragmented, each fragment can regenerate to form a new colony. All the cells are multinucleate, as opposed to most of the other basidiomycota, where the cells are uninucleate or binucleate. Mycelium in *A. bisporus* does not bear any clamp connection, which is characteristic of dikaryotic mycelia of the majority of Basidiomycota.

Mycelium developing from germinating spore continues to grow as long as the conditions are favorable. It ramifies, absorbing food from the substrate, until ready to fruit. Fruiting is initiated by a change in the environment. The fruiting body begins as a tiny knob of tissue arising from the underground intertwining mycelium, usually referred to as pinhead. These hyphal knots increase in size and form the button stage. As the development proceeds, the differentiation of pileus and stipe takes place. In further development, very thin radiating plate-like structures known as gills or lamellae are formed on the undersurface of the pileus. The gills bear

**Fig. 26.2** Section of gills and hymenium showing the basidium and basidiospores of *Agaricus bisporus*

spores and are covered by a structure called veil. Veil extends from the margin of the cap to the stalk.

On further development, the veil breaks, exposing the gills and allowing the spores to deposit on the surface below. The broken portion of the veil forms a ring on the stalk which is called annulus. A section of the gill (Fig. 26.2) under the microscope shows that the center of the gills is made up of bundles or of mycelial threads known as trama. Towards the outside of the trama, the cells form a thin layer of short cells called the sub-hymenium. The sub-hymenium gives rise to long club-shaped cells, which are at right angles to the surface of gills and are known as basidia. Basidia in *A. bisporus* have two spine-like projections, the sterigmata, on which basidiospores are borne. Interpersed in the basidia are many sterile cells known as cystidia. The two together constitute the hymenium.

There is a constant and progressive reduction in the number of nuclei per cell from the base of the stipe upwards, ultimately reduced to two in the young basidia. These two nuclei later fuse in the basidium, and the diploid nucleus is formed. The diploid nucleus undergoes meiosis, giving rise to four haploid nuclei. In *A. bisporus* these migrate in pairs to the two basidiospores, in contrast to most other mushrooms, where a single nucleus passes on to each of the four basidiospores, and on discharge starts the cycle again.

### *26.1.2 Taxonomy and Nomenclature*

The genus *Agaricus* belongs to family Agaricaceae in the order Agaricales of Basidiomycota. The family Agaricaceae is characterized by having a central stipe, pileus ordinarily readily separable from the stipe, lamellae free or nearly so and easily separable, spore deposits in some shades of chocolate color to blackish, and partial veil present, leaving more or less distinct annulus on the stipe. The genus *Agaricus* in addition, to the above family characteristics, possesses fibrillose cuticle on the pileus, with fibrils more or less radially arranged and at times broken into squamules (Smith 1978).

There is no taxonomic monograph on the genus, and no consensus on the number of species (Elliott 1985a). Singer (1975) recognized 35 species worldwide. However, other workers (Elliott 1985a) have put the number of species from 40 to 100. Three groups are generally recognized within the genus; two major groups, *Rubescentes* and *Flavescentes* have been differentiated on the basis of color reaction of the broken or cut flesh. In *Rubescentes,* the cut flesh turns more or less red, and in *Flavescentes* the cap turns more or less yellow when touched. There is a third minor group, also containing species with delicate fruiting bodies (Elliott 1985a).

Though there are a number of edible species in the genus, commercially cultivated species are only two — *A. bisporus* and *A. bitorquis*. Both of these belong to the *Rubescentes* group. As stated previously, of the two, the major volume of production is in *A. bisporus* only; *A. bitorquis* only came into commercial cultivation only during the 1970s and has only been cultivated to a minor extent. The production of *A. bisporus*, referred to earlier, constitutes about 37% of total world production of all cultivated species. It has been intensively investigated in all its aspects, cultivation having started in the West over three centuries back.

The nomenclature of *A. bisporus* has been changing. The cultivated white button mushroom was referred to as *Psalliota campestris* in earlier books. However, subsequently it was transferred to genus *Agaricus* for taxonomic reasons and appeared as *Agaricus campestris*, and was so till recent times. Subsequently, the mushroom in commercial cultivation was found distinct from *A. campestris* in a number of characteristics, especially two-spored basidia. Lange (Smith 1978; Hayes 1978) described the two-spored species as *Psalliota hortensis* Lange, but this was renamed *A. bisporus* (Lange) Singer by the International Botanical Congress at Paris in 1954. In some publications (Smith 1978) it has been referred to as *A. bisporus* (Lange) Imbach, based on Imbach's description, which is current in American literature. In Europe and most other countries the name *A. bisporus* (Lange) Singer based on Singer's description is adopted. *A. campestris*, the common field mushroom, is also very popular with mushroom-eaters.

Hayes (1978) has reproduced Singer's description. The salient points are given below:

*A. bisporus* (Lange) Sing. *Lilloa* 22: 431 (1951). *Pileus*: pure white to stramineous, or brown, almost naked to finely and almost to quite appressedly squamulose (finely scaly), dry, with thick projecting margins, the latter some times fibrillate-serrate or crenate, convex then convex with flattened center or becoming entirely flat in old specimens, mostly 35–100 mm wide. *Lamellae*: whitish, then a beautiful pink, finally concolorous with spore print, close to crowded, narrow or moderately broad, free, edge fimbriate.

*Spore print:* 'Sepia', close to 'mummy brown'.

*Stipe*: White or whitish, annulated, solid in age becoming hollow, equal or slightly tapering upwards or with a slight bulb at the base, 30–120 × 10–18 mm. Basal white rhizomorph often very distinct of variable diameter, veil superior.

*Context* (flesh): white when fresh, becoming red by auto oxidation, pinkish on exposure.

*Odor:* at first very pleasant and weak, eventually slightly acidulous or flesh.

*Basidia:* 20–28 × 6.7–7.7 μ, one-, two- or three-spored, almost all or the majority two-spored. Basidiospores 6.3–8.5 × 5.5–6.8 μ, short-ellipsoid, smooth, without a distinct germ-pore, brown.

Wild races are found on soil rich in nitrogen, spontaneously in hot beds, green houses, near manure or road sides, among scattered horse manure, in gardens and parks, etc. Fruiting from May to early November in northern temperate zone. Natural geographical area probably all over the northern hemisphere, outside the tropics and the Arctic.

Singer (1961) distinguishes three varieties of the species, namely: (1) var. *bisporus*, the type variety with a brown pileus but only rarely cultivated, (2) var. *avellaneus* (Lange) Sing. with a pale brown pileus that corresponds to the 'Brown' variety in cultivation, and (3) var. *albidus* (Lange) Sing., with a white pileus in youth which becomes cream as it matures and corresponds to the cream-colored varieties of cultivation.

It is commonly accepted, however, that all of the white strains now in cultivation were originally selected from natural color mutations and by a continuous process of selection. A wide range of strains are now available with different growth characteristics.

Some taxonomists are of the opinion that the correct name of the white button mush room is *Agaricus brunnescens* Peck on a priority basis. However, in view of the widespread usage of *A. bisporus*, the changeover has not been effected (Kapoor 1989).

In view of widespread commercial cultivation of the species, efforts have been made over the years to develop new strains with desirable characteristics. To augment these efforts, the *A. bisporus* recovery programme (ARP) (now named as *Agaricus* Resource Programme) was founded by Dr. Rick Kerrigan of Sylvan Research, USA 1988 (Rai and Ahlawat 2002). More than 206 *A. bisporus* strains, including the wild ones, are available with ARP. These were collected from spawn companies and research institutes all over the world, and have been identified using isozyme marker techniques. They are an excellent source of genetic variability. As a part of this effort, Callac et al. (1993) reported a tetrasporic variety from the Sonoran desert of California. These authors described a new variety of *A. bisporus* named as *A. bisporus* var. *burnettii*. This differs from the earlier variety designated as var. *bisporus*, in the sense that most of the basidia of this variety are tetrasporic, while the var. *bisporus* had 81% bisporic, 18% trisporic and 1% tetrasporic basidia; var. *burnettii* had 85% tetrasporic, 14% trisporic and 1% bisporic basidia. The tetrasporic variety holds promise for simplifying the process of homokaryon isolation, particularly for application in a hybrid breeding programme.

### *26.1.3 Nutritional Requirements*

*A. bisporus* being a heterotrophic organism, like all other saprophytic fungi, depends for its nutrition and subsequent metabolism on carbon compounds that have already been produced by green plants. In addition to the carbon compounds (often sugars), nitrogen compounds (either in organic or inorganic forms), essential mineral nutrients (calcium, magnesium, potassium, phosphorus, sulfur, copper, iron, etc.), vitamins (thiamine and biotin), and growth factors are essential for successful growth of the mushroom. Hence, a thorough knowledge of nutrition is essential for successful commercial cultivation procedures. Wood and Fermor (1985) have provided an exhaustive review of the nutritional requirements of *A. bisporus* both in pure culture and in commercial growth substrates. The salient points are presented below.

#### 26.1.3.1 Carbon

Investigations on carbon nutrition in pure culture for both mycelial growth and fruiting have been carried out and reviewed by a number of workers (Wood and Fermor 1985). The results obtained have led to the conclusion that in general, glucose, fructose and xylose are good carbon sources, and other sugars and organic acids less good. The ability to degrade and utilize various higher molecular weight carbohydrate polymers, particularly those found in green plants, has also been investigated. Starch, glycogen, mulin, xylan, cellulose (filter paper) and pectin are utilized to varying degrees. Utilization and degradation of cellulose and hemicellulose have also been investigated, and found to provide good growth (Hayes 1978; Wood and Fermor 1985; Mohammadi Goltapeh and Pourjam 2005).

Studies concerning the nutrition of *A. bisporus* in composted wheat straw have demonstrated a preference for utilizing the lignin and protein fractions of compost, but cellulose and hemicellulose fractions could also be utilized (Wood and Fermor 1985). It has also been shown that supplementation of compost with lipid- and protein-rich materials such as various seed meals, oil or defatted seeds leads to increased crop production (Hayes 1978; Wood and Fermor 1985; Mohammadi Goltapeh and Pourjam 2005).

#### 26.1.3.2 Nitrogen

*A. bisporus*, like all other fungi, requires a nitrogen source. Suitable nitrogen sources include urea, a range of ammonium salts and many amino acids — asparagine, alanine and glycine are especially good sources (Hayes 1978). Various types of protein are efficiently utilized by *A. bisporus* (Wood and Fermor 1985). The nitrogen sources available to *A. bisporus* in the compost are lignin–nitrogen fraction

of compost and microbial protein synthesized during the composting process (Wood and Fermor 1985).

#### 26.1.3.3 Essential Elements

Four macronutrients — magnesium, phosphorus, potassium and sulfur — and five micronutrients — boron, iron, manganese, molybdenum and zinc — are required for optimum mycelial growth of *A. bisporus*. However, in *A. bisporus*, in addition to the above, calcium is also required and has been shown to fulfill an important physiological role in the medium (Hayes 1978; Wood and Fermor 1985). Traditional horse manure compost at spawning and wheat straw can provide for all mineral nutrients, and in a properly formulated compost, none is likely to act as a limiting factor.

#### 26.1.3.4 Vitamins and Growth Factors

Biotin and thiamine have been shown to be required for mycelial growth (Hayes 1978). However, microbial flora in the compost produce B-complex vitamins, and composts (both horse manure and synthetic) would not be vitamin-deficient (Wood and Fermor 1985). Growth-promoting activity has been shown with the addition of indole acetic acid, esters of oleic and linoleic acid and amino acids — phenylalanine, methionine and proline (Hayes 1978).

#### 26.1.3.5 Role of Microbial Biomass

Microbial activity converts the components of straw and manure into mushroom compost. The total count of these micro-organisms (both living and dead) in composts has been termed microbial biomass. Various workers have suggested a role for this biomass in the nutrition of *A. bisporus*. Wood and Fermor (1985) have reviewed these studies. Biomass could also act as a concentrated source of nitrogen and minerals.

### *26.1.4 Environmental Requirements*

Environmental parameters have a definite role to play in commercial production of *A. bisporus*. At every stage in the production process, starting with composting, the environment has to be carefully manipulated and controlled. In seasonal or cottage-scale cultivation, a favorable environment prevailing during the season is exploited and obtained by manipulation of growing conditions. However, in highly controlled conditions, a precise environment is simulated for crop-raising, and in some farms

computerization has been introduced to automatically maintain and control the environmental parameters in the cropping room and in bulk chambers.

#### 26.1.4.1 Temperature

An optimum temperature is essential for growth and reproduction of any living organism, including the mushroom. In most of the cultivated types of mushrooms (including *A. bisporus*), the optimum temperature for vegetative growth is different from that required for fruiting (sexual phase). The optimum temperature for mycelial growth in *A. bisporus* is in the range of 23–25°C (air temperature). Limited mycelial growth takes place at temperatures $\leq$3 and $\geq$30°C. However, for fruiting, temperatures are in the range of 15–17°C. At temperatures $\geq$20° and $\leq$14°C, growth in the developing buttons is slowed.

#### 26.1.4.2 pH

Though vegetative growth is possible over an extended range of 3.5–9.0, optimum pH is around 6.0. A pH of 6.8–7.2 is considered ideal for fruiting, which is maintained in compost and casing for commercial cultivation.

#### 26.1.4.3 Light

Light does not play any role in vegetative growth or fruiting of *A. bisporus*. However, exposure of even smaller periods of light in off-white strains results in browning of the mushroom (Kapoor 1989).

#### 26.1.4.4 Moisture

Moisture content of the substrate and relative humidity of atmosphere in the crop room are of prime importance in growth and reproduction of *A. bisporus* as in other fungi. Fifty-five to 65 percent of water in the substrate has been found to be appropriate for initiating fruiting in *A. bisporus* (Manchere 1980). Flegg found that the best growth occurred when the water content of the compost ranged between 55% and 70% (Flegg and Wood 1985).

#### 26.1.4.5 Relative Humidity

Relative humidity of the atmosphere in the crop room plays a vital role during growth and reproduction of *A. bisporus*, especially during pinhead formation.

RH levels of 85% and 95% have been found to be optimum during initiation and development of fruiting bodies and cropping, respectively.

#### 26.1.4.6 Aeration

Relative concentration of oxygen and carbon dioxide, and presence or absence of toxic gases in the atmosphere, affects the growth and fruiting in *A. bisporus*. In consequence, the role of aeration assumes great importance, and is to be carefully controlled at various stages. Growth in pure culture is not affected at oxygen concentrations ranging from 21% to −0.6% (Hayes 1978).

The influence of carbon dioxide has been investigated, and mycelial growth is reduced at about 2% and is totally inhibited at 32%. Initiation of fruiting occurs, preferentially at a concentration ranging from 0.03% to 0.1%, and 0.5% to 1.0% is high enough to inhibit the premordia formation (Kaul 1978; Flegg and Wood 1985).

### *26.1.5 Sexuality and Breeding*

The nature of sexuality and breeding strategies adopted in edible mushrooms has been comprehensively dealt with in an earlier publication (Kaul 2002). An attempt will be made here to present in brief the situation in *A. bisporus*, with special focus on the molecular techniques used in genetic improvement of the crop.

The yield and quality of mushrooms produced by commercial growers is mainly determined by two factors: (1) the genetic make-up of the strain, and (2) the environmental condition in which the strain is grown. The genotype of the strain has a big role to play in the quality and quantity of mushrooms obtained, and any improvement is welcome. The goal of the mushroom breeder is the assembling of the best combination of genes into one individual stock for production of mushrooms of high quality. To achieve this, it is necessary to have a basic understanding of the sexuality of individual species and possible breeding strategies.

The life cycle, pattern of sexuality and sexual mechanism are three parameters of sexual behavior in *A. bisporus*. The life cycle in *A. bisporus* differs from nuclear phases of a typical basidiomycota in having a heterokaryotic (dikaryotic phase) originating directly from germinating spore. The majority of the basidia are bispored, having two compatible nuclei each, which on germination give rise to heterokaryotic dikaryon directly without plasmogamy (union of homokaryotic mycelia). In rare cases the basidia are four-spored, where spore gives rise to homokaryotic mycelia. Dikaryon in *A. bisporus* is multinucleic, unlike most other edible mushrooms. As in other basidiomycota, dikaryon is the predominant phase, leading to the production of fruiting bodies. Subsequently, the transient diploid phase is initiated by karyogamy (union of nuclei) in basidia. This is immediately followed by meiosis, resulting in four nuclei in the basidium. These, unlike those in other edible mushrooms, are transferred to two spores in pairs in *A. bisporus*.

Two basic patterns of sexuality recognized in Basidiomycota are: (1) homothallism, where mycelium from a single germinated spore is self-fertile, and (2) heterothallism, where a single basidiospore germinates to give rise to monokaryon, which must fuse to produce a fertile mycelium. However, the pattern in *A. bisporus* has been designated as secondary homothallic. In this pattern, though single spores are fertile, each spore has two compatible nuclei. Heterothallism here is concealed by the two-spored nature of basidia.

Patterns of sexuality are in general controlled by two mechanisms: (1) the distribution of four post-meiotic nuclei to the basidiospores, and (2) genetic factors of a mating system known as the incompatibility system, which may be either unifactorial or bifactorial. In *A. bisporus* in most cases two spores receive two nuclei: each of these forms a basidium which is of a different mating type and which has unifactorial control.

#### 26.1.5.1 Genetic Improvement

The main hurdle in serious scientific work on the cultivated mushroom has been the mystery about the life-cycle of fungi. Discovery of the aseptic technique and subsequent understanding of the life cycle opened up the possibilities of improvement in mushroom crops. The first germ-free spawn was made in 1894 at Pasteur Institute, Paris, and Duggar perfected the method in USA in 1905 (Singer 1961).

Though cultivation of *A. bisporus* is over three centuries old, still very little has been achieved in respect of improved strains. Even the appearance of the white strain in *A. bisporus* was a result of spontaneous mutation in a population of cream fruiting bodies. The cultivation technology has been thoroughly investigated, and taken to the level of computerization on large-scale commercial farms. However, the breeding programme has not advanced much. This has been mainly because of incomplete knowledge of the sexuality of this mushroom. This was elucidated only about two decades back, and Elliott (1985b) has traced the historical development in the understanding of sexuality in *A. bisporus*. *A. bisporus* was classified in the secondary homothallic group, with an unusually complex breeding system.

*A. bisporus* lacks two of the diagnostic features of Basidiomycota. Firstly, it has two-spored basidia rather than four-spored ones on its gills, and secondly its vegetative mycelium lacks clamp connection. The fertility of single spore (binucleate) isolate is because many spores receive nuclei from basidium which are of different mating types. Horgen (1992) is of the opinion that there appears to be a controlled biological mechanism which ensures that the basidiospores contain nuclei of a compatible mating type. The formation of two binucleate spores on each basidium is the normal pattern of development in this species, but occasionally (about 20%) a basidium may bear three or four spores. The latter receive only a single nucleus and are infertile. Secondary homothallism of *A. bisporus* where a single spore is fertile due to having two compatible nuclei has also been substantiated by genetic analysis conducted by Raper et al. (1972).

The fertile heterokaryotic mycelium that normally develops from the germinated basidiospore in *A. bisporus* is morphologically indistinguishable from the exceptionally occurring self-sterile homokaryotic mycelium. The cells of both mycelia are multikaryotic. The cells also lack clamp connections, which are a feature of fertile heterokaryotic mycelium in most other species. The creation of a fertile heterokaryon by crossing two compatible self-sterile homokaryons can therefore be detected only through the tedious process of a test for fruiting. This fact, together with the extreme mechanical difficulty of isolating the rare self sterile, cross fertile homokaryons, has presented serious problems in breeding in *A. bisporus*. The available systems for strain improvement in *A. bisporus* are: selection, breeding and gene transformation (Sodhi et al. 1997). Selection was the only course followed for obtaining improved strains prior to 1970.

#### 26.1.5.2 Selection

The selection procedure in *A. bisporus* (as in other edible mushrooms) has been utilized either in introduction and screening of new cultures from foreign countries, or in screening the existing commercial strains. The three procedures usually followed are tissue culture, multispore, or single spore.

Tissue culture taken from the stem or cap of a mushroom has been the oldest method of raising cultures from the wild type. Tissue culture raised from phenotypically healthy looking fruiting bodies for strain improvement has been suggested by some authors for improvement. However, the method has not been found to be of much promise in *A. bisporus* (Yadav et al. 2002).

In the multispore method, a spore print is obtained from a suitable fruiting body and mass of spores germinated together. Elliott (1985b) states that most commercial strains in *A. bisporus* prior to the release of hybrids were obtained by this method. However, other authors are of the opinion that multispore culturing does not generate significant variability in terms of genetic improvement (Mehta and Bhandal 1994).

The single-spore selection method is based on the premise that about 70% of spores produced in *A. bisporus* are fertile, and that selection can be practiced among these fertile spores. It has been widely followed for developing superior cultivars in *A. bisporus*. Isolation is a time-consuming process, but the selection of superior strains out of single-spore isolates is the most acceptable method in *A. bisporus* (Sodhi et al. 1997). However, other authors maintain that the method has perhaps a short-term role in strain improvement in *A. bisporus*, and only limited gains for genetic improvement are expected (Elliott 1985b; Mehta and Bhandal 1994).

#### 26.1.5.3 Hybridization

Subsequent to elucidation of the sexual cycle by Miller (1971) and Elliott (1972), efforts have been made to obtain improved stocks of *A. bisporus* by hybridization. It involves mating (anastomosis) of self-sterile and compatible homokaryotic lines.

Commercial successful hybrid spawn was achieved first in the Netherlands when Fritsche (Elliott 1985b) released such spawn under codes $U_1$ and $U_3$ in 1981. These hybrids were the result of a breeding programme to combine the desirable qualities of the off-white and pure white spawn types. The strains are highly productive, with better size, and have dominated commercial markets ever since. Another hybrid obtained with this procedure has been from Taiwan (Elliott 1985b). Other hybrids available are Amycel 208 and NCH-102, from USA and India respectively (Yadav et al. 2002).

The steps involved in the breeding programme of *A. bisporus* are as follows:

1. *Selection of parent fruit bodies* with desired characteristics and collection of spore print (parents with different characteristics are selected).
2. *Single spore isolation*. Spore prints obtained are serially diluted and finally plated. The single spores are located under microscope and transferred to plates with a suitable medium.
3. *Testing of single spore isolates for homokaryotic nature*. The majority of single spores are heterokaryotic, with a small minority being homokaryotic. Traditionally, the criteria of colony morphology (appressed type), slow mycelial growth and non-fruiting were used for identification of homokaryons. The procedure is quite cumbersome. However, modern tools of molecular biology — isozyme (allozyme) analysis, electrophoresis, restriction fragment length polymorphism (RFLP) and randomly amplified polymorphic DNA (RAPD) — are now available for homokaryon isolation in *A. bisporus*. With a combination of isozyme analysis, RFLPs and RAPDs, the identification of homokaryons is now a routine process in the *A. bisporus* breeding programme (Loftus et al. 1995).
4. *Selection for homokaryons* has also been made easy with the discovery of tetrasporic variety var. *burnettii* (mentioned earlier). The majority of basidia (85%) in the variety having four spores, isolation of homokaryons is easy.
5. *Homokaryons thus selected* are crossed in various combinations to locate compatible single-spore isolation, and in the success of such a cross-hybrid follows. . .
6. *Identification of hybrid*. The recognition of hybrids again poses a major problem in *A. bisporus*, unlike many in other edible mushrooms, as no clamp connections are formed. Identification was traditionally made by the microscopic observation that compatible homokaryons show heavier growth in the zone of confrontation. A fruiting test was necessary for confirmation. However, molecular markers are now mainly utilized for identification of hybrids.
7. *Grow out tests* of the hybrids developed in this way, for expression of the desired characteristics of the parents.

#### 26.1.5.4 Molecular Markers

*Alozyme (isozyme) markers*. The first genetic test for homokaryons and hybrids which became available to the breeder was isozyme analysis. Isozymes are different

forms of the same enzyme, differing in electrophoretic mobility as a result of allelic differences in a single gene. In isozyme analysis, mycelium is ground up and the cellular extract is run on electrophoretic gel. With the use of an enzyme-specific strain, homokaryons can be distinguished from heterokaryons. Staining gives characteristic banding patterns, and strains with different banding patterns can be crossed: hybrids are recognized by novel banding patterns (Elliott 1985b). More than a dozen isozyme markers have so far been identified in *A. bisporus* (Rai and Ahalawat 2002).

*Auxotrophic markers*. Auxotrophs are strains which have additional nutritional requirements for growth, and are the result of nutritional changes in individual genes (Elliott 1985b). These can be used to make hybrids. If a cross is made which is deficient for amino acid praline and capable of making methionine is mated with a strain deficient for methionine but capable of making praline, a hybrid will be produced which is capable of growth on a medium lacking both these amino acids, and can be easily recognized. Unfortunately, auxotrophs are not easily available due to the masking effect of multikaryotic nature of *A. bisporus* cells. Elliott (1985b) states that this procedure will not have an important role in any hybridization programme.

*Resistance markers*. In this, a strain resistant to a fungicide can be intermated with another strain resistant to another fungicide, and their hybrid can be identified by growing the intermated culture on a medium containing both fungicides. Several fungicide-resistant mutants of *A. bisporus* have been produced using UV mutagenesis, and are being evaluated for their insensitivity to fungicides during cropping (Elliott 1985b).

*RFLP markers (restriction fragment length polymorphism)*. In the mid 1980s, more powerful DNA-based techniques became available, and RFLP is one of them. RFLP markers show heritable differences in the length of DNA fragments which are generated when DNA is digested by a restriction endonuclease. If the DNAs of different strains are cut with the same restriction enzyme, any natural variation or polymorphism in the position of the site at which the enzyme cuts will result in DNA fragments which differ in length. DNA fragments generated by restriction endonucleases are separated by gel electrophoresis, and made single-stranded by their transfer onto a nitrocellulose-based membrane for hybridization (Rai and Ahlawat 2002). These can be visualized using labeled DNA probes (Challen et al. 1991; Clutterbuck 1994). RFLP markers have been used both for identification of homokaryons and confirmation of their hybrids in *A. bisporus* (Yadav et al. 2002). RFLP analysis measures genetic differences at the most fundamental level in an organism — the DNA. Some of the limitations in the use of these markers are the high recurring cost and the requirement for a large quantity of pure DNA (5–10 μg).

*RAPD markers (randomly amplified polymorphic DNA)*. Another method in widespread use to-day is RAPD analysis. RAPD is basically based on the amplification of random DNA segments by polymerase chain reaction. The polymerase chain reaction (PCR) is the pioneering technique for *in vitro* amplification of nucleic acids. The PCR produces greater than $10^6$-fold amplification of a target DNA sequence in 1–2 h (Keller and Manak 1993). The requirement for a small

quantity of DNA, fast speed and ability to handle multiple numbers of samples at a time, makes this technique more user-friendly than RFLP. RAPD markers have been utilized for homokaryon identification and confirmation of hybrids generated by crossing two compatible homokaryons. However, RAPD analysis is considered less robust than RFLP analysis — RFLP results being more consistent and reproducible from laboratory to laboratory (Loftus 1995).

RAPD and RFLD DNA marker systems are the means by which DNA fingerprints are made. DNA fingerprints are also made for patent applications, and being unique to an individual mushroom strain, they protect the strains from theft by competing spawn companies.

#### 26.1.5.5 Genetic Engineering

Genetic engineering, also referred to as transformation, is the technology that permits the transfer of specific genes within and between species in a way that is both precise and simple. It involves the isolation, cloning, sequencing and insertion of desirable gene(s) into the genome of a target organism to obtain expression of the desirable trait. The birth of recombinant DNA technology or genetic engineering occurred in the 1970s when a group of scientists succeeded in splicing viral and bacterial DNAs in test tube (Romaine 2002).

The method is based on the ability to cut DNA molecules precisely into specific pieces, and to recombine those pieces to produce new combinations. The procedure depends on the existence of restriction enzymes that break DNA at sites where specific nucleotide sequences occur. Any two fragments produced by the same restriction enzymes can be joined with one another in this way, using a sealing enzyme called ligase (Raven et al. 1986). Transfer of genes is affected through vector, electroporation or ballistic gun system.

Vector transformation is the preferred route, and if it fails then other methods are used. In the ballistic gun method, small fragments of DNA with desired genes are coated on a tungsten chip and bombarded onto any tissue of mushroom with very high speed.

In vector-mediated transformation, plasmids are used. Plasmid is a small fragment of DNA that exists free in the cytoplasm of a bacterium and can be integrated into and then replicated with a chromosome. These are common in bacteria but rare in Eukaryotes. Bacterial plasmid ($T_i$) associated with the crown gall bacterium *Agrobacterium tumefaciens* has been found useful in inserting foreign gene into plants and has subsequently been used in fungi too. Significant advances in the development of transgenic crop plants have been made. We have ‘golden rice’ where a gene from daffodil has been introduced to make the rice rich in beta carotene, and Bt cotton where insect-resistant genes have been imported from *Bacillus thuringensis* bacterium.

With the discovery in 1995 that *A. tumefaciens* can also operate in yeast fungi, ways were opened for its use as a vector in filamentons fungi. Since then, a lot of

work on genetic manipulation of *A. bisporus* and other mushroom species has been done through this vector. Romaine (2002) has provided a review of these efforts. He also outlined the steps in the *Agrobacterium*-mediated gene transfer method. These are:

1. Gill tissue is taken from mushroom with intact veils.
2. This is cut into 2–5 mm pieces.
3. The tissue pieces are vacuum infiltrated with a suspension of *Agrobacterium* carrying an antibiotic (hygromycin)-resistant gene.
   (a) The tissue pieces are placed on a medium amended with hygromycin.
   (b) Within several weeks the mushroom cells which received the resistance gene grow into visible culture.

Though transformation attempts have been successful in many species, e.g., *Coprinus* sp. and *Schizophyllum commune*, no significant success has been achieved in *A. bisporus* so far (Sodhi et al. 1997; Rai and Ahlawat 2002 and Mehta and Bhandal 1994). Efforts are, however, ongoing, and a long list of desirable clonable genes from *A. bisporus* and possible useful genes from other sources for incorporation in *A. bisporus* have been identified (Sodhi et al. 1997). Challen et al. (2000) have reported preliminary success in transformation of a number of edible mushrooms — *Agrocybe aegerita*, *A. bisporus*, *Lentinus edodes*, *Pleurotus ostreatus* and *Volvariella volvacea*. Though *Agrobacterium tumefaciens*-mediated transformation was successful in *A. bisporus*, yet further refinement is needed before transgenic strains can be developed.

Sodhi et al. (1999) have also constructed genomic libraries for *A. bisporus*. A genomic library is a set of recombinant clones which represent the complete DNA present in an individual organism, and its construction is a step toward mushroom strain improvement.

Loftus et al. (1995) maintained that the potential of genetically engineered mushroom is immense, and is readily apparent in three main areas: virus resistance, improved shelf life, and improved compost utilization.

#### 26.1.5.6 Mutation Breeding

Mutagenesis is attempted to create new variability for the selection and hybridization programme. A natural mutant was responsible for isolation of the white strain of *A. bisporus* from the cream strain in 1927, which is in widespread commercial culture (Kumar 1997). Though success in obtaining higher penicillin yielding strains of *Penicillium* has been achieved by cycles of mutation and selection, no such success has been achieved in *A. bisporus*. Mutagenesis may have value in production of markers for identification of hybrids (Elliott 1985b).

#### 26.1.5.7 Protoplasting

Protoplasts are created when the cell wall is digested away from mushroom mycelium, leaving 'naked cells'. Protoplasts can be plated out and cell walls regenerate. If a heterokaryon is protoplasted, a proportion of the mycelial colonies from the regenerated protoplasts will possess one nucleus, giving rise to homokaryons. Protoplasts are thus useful as a tool for isolating homokaryons in *A. bisporus*.

Protoplasts are also useful in genetic engineering experiments, whereby DNA can be introduced into the cell without the constraints provided by the cell barrier. Protoplast technology involves the following sequential steps (Yadav et al. 2002):

1. Isolation of protoplasts using cell wall digestive enzyme.
2. Fusion of protoplasts with polyethylene glycol and $CaCl_2$.
3. Regeneration and evaluation of somatic hybrids.

The fusion of protoplasts can also be achieved using the electrofusion technique. There has been no significant achievement in the improvement of *A. bisporus* strains by this method either.

Amycel Strains

A number of laboratories involved in the production of improved strains are making use of new technology. Amycel/Spawnmate biotechnological group research laboratories in Watsonville, CA, USA, has a major programme in strain improvement, and Loftus et al. (1995) have described in detail the technologies in use, achievements made and emerging technologies of the future. Systematic breeding of new mushroom cultivars through a combination of molecular breeding techniques, specific traits identified in wild *A. bisporus* and backcrossing strategy (similar to that used in plant breeding) is being attempted by Amycel.

Loftus (1995) concludes that future commercial mushroom spawns will be created through a range of new technologies (which include genetic engineering); novel strains protected through patents will be available to growers, and the days of dominance of the off-white hybrid strains are numbered.

## 26.2 Trouble Shooting

- Morphologically, *A. bisporus* resembles many poisonous mushrooms such as Amanita. Hence the identification of wild collections must be done by an expert, or otherwise the strains should be obtained from an authentic source.

- The cultivation of the mushroom is generally been done on composted wheat straw, so the formulation must be done in such a way that the carbon:nitrogen ratio is set at 35:1 at the start of the composting process, and the final C:N ratio must reach 18:1 after the microbial succession. Poor production or disease and pest infestations are generally the results of an improper C:N ratio at the end of the composting. Hence if the C:N ratio is higher than 18:1 at the end of the composting, the substrate should be composted for a few more days until it reaches 18:1.
- The fungal mycelium is very sensitive to ammonia, which is a byproduct of the composting process. Presence of even small amounts of ammonia may result in poor mycelial growth and reduced yield of the mushroom. To avoid such conditions, the presence of ammonia must be checked at the end of the composting process, either by smelling or by ammonia checking strips, and if even small traces of ammonia are detected, the compost must be reconditioned to release the traces of ammonia.
- Environmental requirements also affect the production of the mushrooms; hence, the pH of compost must be adjusted near to 7.0 with the use of gypsum. The temperature is another environmental consideration for the good yield of the mushroom; hence, the optimum temperatures for mycelial growth and fruiting must be maintained properly. The requirements of oxygen of the fungus during mycelial run and fruit body formation are different. The mushrooms require a high concentration of $CO_2$ during the mycelial run phase, while during fruiting a high concentration of oxygen is required to switch over from the vegetative to the reproductive phase of the fungus. As the mushroom contains 90% water, and this water is taken from compost/casing at the time of fruiting, hence the moisture content of the compost beds must be maintained close to 65% by regular water spray and maintaining relative humidity in the cropping rooms.
- Breeding new varieties in *A. bisporus* is a difficult task, because it is secondarily homothallic in nature, producing homokaryons as well as heterokaryons. Hence, before selection of the single-spore isolate for hybrid breeding, it is a must to ascertain the homokaryotic nature using molecular markers, and also the probable hybrids must be confirmed by DNA markers as well as fruiting trials.
- The hybrids produced in this way are always prone to segregate for traits; hence, the hybrid strains must be maintained vegetatively to maintain their hybrid nature, and also the culture medium must be alternated between poor and rich nutrient levels to maintain the vigor of the strain.

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# Index