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Chapter 2.3

ARBUSCULAR MYCORRHIZA

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Symbiotic root colonisation by arbuscular mycorrhizal (AM) fungi plays a critical role in the capture of nutrients such as phosphorous from the soil. The roots of almost all vascular plants can interact with AM fungi with the notable exception of many brassicas, including the model plant Arabidopsis thaliana*. Legumes, which also form symbiotic nitrogen-fixing nodules, have been intensively studied over the past decade, revealing a surprising genetic overlap between the early stages of nodulation and AM formation. For this reason, legumes have become useful model systems for AM research. Here we present some basic techniques for rapid and consistent AM infection and for the visualisation of AM structures in plant roots. These techniques are useful for molecular and cellular studies and have been adapted to the specific requirements of* Lotus japonicus*.*

INTRODUCTION

Arbuscular mycorrhiza

Arbuscular mycorrhiza (AM) is an ecologically important and widespread symbiotic association occurring between roughly 200 species of microscopic soil fungi, the *Glomeromycota* (Schüssler et al., 2001), and over 90 % of vascular plant species (Smith and Read, 1997). Both partners benefit from this association; the plant supplies the fungal partner with hexose and the fungus delivers phosphates and other mineral nutrients to the plant. Benefits to plant growth, disease resistance, and survival, as well as soil particulate structure and microbial nutrient cycling make AM of fundamental importance to most ecosystems. AM may play a particularly significant role when nutrient availability is low due to rapid nutrient cycling and leaching in tropical soils (Bowman and Panton, 1993) or after soil disturbance and depletion in agricultural soils (Gianinazzi et al., 1995). It is also critical under

conditions where plant survival is restricted due to climate or contamination as in arctic (Dalpé and Aiken, 1998), saline (Al-Karaki and Hammad, 2001), desert (Bohrer et al., 2003), and polluted soils (Vosatka and Dodd, 1998).

Despite the ecological and potential agronomic importance of AM, study of the symbiosis at the molecular and genetic level has been hampered by technical problems in manipulating the AM fungal partner. First, AM fungi cannot complete their life cycle in the absence of a plant host and are therefore difficult to grow in axenic culture, a prerequisite for the production of standardised AM inoculum. Second, AM infection events are relatively rare and non-synchronous as the infection process occurs over several days with new infections occurring as new roots grow. Once arbuscules and vesicles have formed, all stages of infection can be present at once making gene expression studies based on total RNA extractions difficult to interpret. Finally, although transient marker gene expression using a biolistic approach has been successful in an AM fungus, the multinucleate spores and coenocytic hyphal growth of AM fungi make establishment of a robust, stable transformation system for the fungus unlikely in the near future. Full genome sequencing of *Glomus intraradices* will soon be in progress however, a suitable transformation system is a requirement for further studies in functional genomics.

On the plant side, AM research has benefited over the past decade from the discovery that some plant genes required for the formation of symbiotic nodules on legume roots by nitrogen-fixing Rhizobium bacteria are also required for AM infection (Duc et al., 1989). The mutants defective for both nodulation (Nod$^-$) and AM infection (Myc$^-$) that have been identified in *L. japonicus* indicate that at least six independent genes are common to the two root symbioses and are referred to as the common *SYM* genes (Kistner and Parniske, 2002). Nodulation and mycorrhization are very different processes, involving unrelated microsymbionts and giving rise to completely different symbiotic structures in the host root. However, the striking genetic overlap between these root symbioses in legumes and the fact that AM predates nodulation by roughly 300 M years, suggest that some plant functions from the ancient AM symbiosis may have been recruited in the evolution of root nodulation (LaRue and Weeden, 1994).

Stages of AM infection

AM-fungi are obligate symbionts that are present in the soil as multinucleate spores surrounded by thick cell walls. When temperature and humidity are favourable, spores germinate, forming hyphae that grow through the soil and begin to ramify. Hyphal growth is further stimulated by CO_2 and compounds secreted by the roots of potential host plants (Buée et al,. 2000). In turn, plant roots respond to diffusible signals from the AM fungus with changes to gene expression (Kosuta et al. 2003). At the surface of host roots, hyphae attach themselves and form slight swellings called appressoria, often in the groove between adjacent epidermal cells. Under the appressorium, fungal hyphae penetrate the epidermal cell, a process associated with the highly localised expression of specific plant genes (Blilou et al., 2000; Chabaud et al., 2002). Hyphae then exit the epidermal cell and grow intracellularly to the inner cortex. Here, the fungus forms long intercellular runner hyphae, from which

hyphal branches penetrate cortical cells to form tree-like intracellular structures called arbuscules.

Even while inside the plant cell, the fungus does not penetrate the cytoplasm. In the arbuscule-containing cell, a plant-derived membrane is formed that surrounds the arbuscule. The fungal cell wall becomes progressively thinner as the arbuscule develops, leading to the establishment of an extensive intracellular interface, which is where the phosphate and carbon are thought to be transferred between the plant and the fungus. In the intercellular spaces in the inner cortex, many *Glomus sp.* fungi also form vesicles: thin-walled, lipid-filled structures thought to be connected to energy storage.

TECHNIQUES FOR THE STUDY OF ARBUSCULAR MYCORRHIZA IN LOTUS

Glomus intraradices infection using a chive nurse pot system

The method described below can be used for both propagating *G. intraradices* and for infecting *L. japonicus* or any other susceptible plant of choice. The system delivers relatively synchronous infection as well as a high infection pressure.

Initially, when setting up the system, fungal inoculum (dried AM-infected substrate or surface-sterilised spores) is required to infect a young chive plant grown from seed. Subsequently, the infected chive plant is transferred into a small pot (10 cm^3) containing substrate composed of a 1:1 v/v mixture of Terragreen (Oil-Dri Ltd., Wisbech Cambs, UK) and sand (Double Arches 16/30 Sand, Hepworth Minerals and Chemicals, Cheshire, UK). The infected chive serves as nurse plant, allowing a dense AM hyphal network to be established throughout the entire substrate, which usually takes six to eight weeks. More chive plants are then infected by sowing chive seeds into the substrate around the initial nurse plant. Five to six weeks after germination, once a significant level of AM infection has been confirmed by staining some of the young chive roots, these young chive plants are individually transferred into fresh substrate in smaller pots (8.5 cm^3). To minimise the risk of transferring an un-infected plant, two young chives may be transferred to each smaller pot. After four to six weeks, these new chive nurse pots are in turn ready to be used for co-cultivation with *L. japonicus*. Pre-germinated seedlings are carefully transferred into the chive nurse pots. To minimize damage to the fragile hyphal network in the substrate, seedlings are placed into carefully dug, small holes not deeper than 2 cm. Scarified, non-germinated seeds can be sown directly into the chive nurse pots. It is advisable not to use chive nurse pots much older than six weeks for co-cultivation, as nutrient competition between the growing chive plants and the young seedlings may limit growth of the latter. After four to six weeks, smaller-than-average chive plants may not be sufficiently infected.

The maintenance and propagation of the chive nurse pots should be kept separate from *L. japonicus* plants in order to avoid contamination with *Mesorhizobium loti* (ideally in different glasshouses, growth chambers etc.). Moreover, older chive nurse pots should be discarded regularly in order to avoid potential accumulation of pathogens within the substrate.

Chive nurse pot nutrient solution
(Thilo Winzer, unpublished)

Macro and micro elements	Final concentration (mg/L)
$MgSO_4.7H_2O$	24.65
KNO_3	5.05
KH_2PO_4	0.115
K_2HPO_4	0.725
$CaCl_2.2H_2O$	18.55
H_3BO_3	0.0715
$MnSO_4.4H_2O$	0.051
$ZnSO_4.7H_2O$	0.011
$CuSO_4.5H_2O$	0.004
$Na_2MoO_4.5H_2O$	0.0025
$CoCl_2.4H_2O$	0.005
Fe-EDTA-di-hydroxyphenylacetate	0.835

Soak chive pots with this nutrient solution instead of water once a week.

Glomus intraradices infection using an axenic nurse culture system

The use of an axenic system is advantageous for gene expression studies and a prerequisite for detailed cell biology. However, possibly due to reduced plant vigour, AM infection can be slower and less reliable in the Petri dish than in the greenhouse. None the less, consistent AM infection of *L. japonicus* can be achieved under sterile conditions, with high infection pressure and relatively synchronous infection, using the nurse culture system based on Ri T-DNA transformed roots described below.

Since Mosse and Hepper (1975) first established monoxenic mycorrhiza with *Glomus mosseae*, at least 27 AM fungal species have been successfully cultured monoxenically in association with *Agrobacterium rhizogenes* T-DNA transformed or non-transformed excised host roots of many plant species (Fortin et al. 2002). The most widely-used plant-fungus combination is carrot (*Daucus carota*) and *G. intraradices* (Bécard and Piche, 1992) because of the vigour of this system and the ease with which it can be propagated. Sterile starter cultures of several AM fungal species to be used for research purposes can be obtained from the Glomales *in vitro* Collection (GINCO) (see www.mbla.ucl.ac.be/ginco-bel or http://res2.agr.ca/ecorc/ginco-can/ for a list of available species and ordering instructions) and various protocols for *A. rhizogenes* transformation have been published (Stougaard et al., 1987; Hansen et al., 1989; Boisson-Dernier et al., 2001).

To infect Ri T-DNA transformed roots with AM fungi, place sterile spores within 1 cm of roots, close enough so that AM-stimulatory compounds produced by roots will reach the fungus. While infection can be achieved on moist sterile filter paper, long-term cultures require a nutrient-rich culture medium. To maximise infection,

the medium described by Bécard and Fortin (1988) contains minimal phosphorus and has low pH (5.5). Furthermore, the use of gellan gum (Phytagel, Sigma, St Louis, USA) as a gelling agent provides a completely transparent medium, which allows growth of the microscopic AM fungal hyphae to be followed and photographed with ease. Infection can take 2-3 weeks to become established, after which time the extraradical hyphae will begin to grow throughout the Petri dish, forming a dense hyphal mat throughout the medium over a period of 4-6 weeks. The negative geotropism of Ri T-DNA transformed carrot roots can be further exploited by turning the Petri dish on its side so that roots remain confined to the upper third of the Petri dish. AM co-cultures grow well in the dark at 22-28°C, in Petri dishes sealed with parafilm to conserve moisture. The direct transfer of a portion of the *G. intraradices*-infected carrot roots to a new Petri dish containing fresh medium will propagate the co-culture.

M medium (pH 5.5)
(Bécard and Fortin 1988)

Macro and micro elements	Final concentration (mg/L)
$MgSO_47H_2O$	731
KNO_3	80
KCl	65
KH_2PO_4	4.8
$Ca(NO_3)_24H_2O$	288
NaFeEDTA	8
KI	0.75
$MnCl_24H_2O$	6
$ZnCl_27H_2O$	2.65
H_3BO_3	1.5
$CuSO_44H_2O$	0.13
$Na_2MoO_45H_2O$	0.0024
Vitamins	
Glycine	3
Thiamine HCl	0.1
Pyridoxine HCl	0.1
Nicotinic acid	0.5
Myo inositol	50
Sucrose	10,000
Phytagel	
Horizontal cultures	4,000
Vertical cultures (Petri dishes turned on their side)	5,000

To infect *L. japonicus*, pre-germinated surface-sterilised seeds are placed directly into the Petri dish on a root-free part of the hyphal mat and grown in a well-lighted growth chamber (16 hr photoperiod, 3200 cd m^{-2} light intensity) at 22-28°C. Petri

dishes containing seedlings should be sealed with breathable medical tape (Micropore, 3M Health Care, St Paul, MN), propped at an angle of 70° and the bottom portion of the Petri dish covered with black plastic to protect the *L. japonicus* seedling roots from the light. The first signs of AM infection (appressorium formation) will be visible on *L. japonicus* roots after 5-10 days, and infection sites with arbuscules and vesicles should be present by 15 days after planting. Seedlings can be grown under these conditions for 4-6 weeks before nutrients in the medium become limiting.

Staining protocol to visualise AM fungi in and on roots

This procedure is a modification of common mycological staining techniques, in which roots are cleared by heating in potassium hydroxide, then acidified and stained. Protocols for AM staining with a variety of colorants, including the most commonly used Chlorazol Black E, Trypan Blue, and Acid Fuschin have been described in detail (Brundrett et al., 1994; Brundrett et al. 1996). Ink staining is preferable because of the rapidity of this procedure and the relative non-toxicity of ink compared to some of the other colorants.

Rapid ink staining (adapted from Vierheilig et al., 1998 by M Chabaud, INRA Toulouse)

1. Place root fragments (1-5 cm long) in an Eppendorf tube. Make a hole in the tube cover with a syringe to avoid the tube bursting open during heating. Separate large root samples into several smaller batches in several tubes. Packing large root samples into a single tube will result in inefficient clearing and staining.
2. Cover root fragments with 10% KOH (1 mL) and heat 7-10 minutes at 95°C using a block heater or water bath. Older roots are generally thicker, tougher and more highly-pigmented and thus require longer clearing with KOH. To determine the clearing time required, replace the discoloured KOH in the tube after 10 min with fresh KOH and heat for 10 min more. Repeat until KOH remains colourless after heating. Gloves, safety goggles, and extreme care are necessary when handling hot KOH.
3. Remove KOH and rinse roots twice with 5% acetic acid. Avoid touching roots as they are very fragile after heating in KOH and will be damaged by rough treatment.
4. Cover root fragments with 5% black ink (Sheaffer Manufacturing Co., Ft. Madison, IA) in 5% acetic acid and heat 5 min at 95°C.
5. Remove ink solution and rinse roots twice with 5% acetic acid. Ink solution can be filtered and re-used.
6. De-stain roots by soaking in 5% acetic acid for 20 min or longer, depending on the intensity of colour desired. Stained roots can be left in water for several days, but for a longer term should be stored in 1:1 glycerol:water.

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Adaptations for high throughput staining

A large volume of independent samples can be stained simultaneously using a multi-well support and a series of clearing, rinse, and staining baths. An appropriately sized support for root systems of individual 2-8 week old plants is a 0.8 mL, 96-well storage plate (ABgene House, Surrey, UK). A 1 mm diameter hole drilled into the bottom of each well allows it to drain and fill with various solutions while retaining root fragments. KOH and stain baths can be heated in any chemical- and heat-resistant plastic or glass container, such as a clear 174x115x60mm lunch box (Watkins & Doncaster the Naturalists, Kent, UK). These particular boxes are heat resistant only up to 90°C. The staining protocol is very similar to that described above, but the following adaptations have been made to accommodate the larger volume of samples.

1. Preheat KOH and ink stain baths to 90°C in a large water bath placed in the fume hood.
2. Place root samples/fragments into individual wells, curling the root pieces and adjusting them with forceps to ensure that they are wedged at the bottom of the well.
3. Lower the root support containing root samples into the hot KOH bath. The KOH level should be just high enough to ensure that roots are submerged. Cover the water bath and heat at least 30 min at 90°C.
4. Carefully remove root support from KOH bath using long forceps and allow KOH several minutes to drain away.
5. Rinse root samples in 5% acetic acid by lowering the root support into rinse baths of similar volume to the KOH bath, ensuring that roots are submerged each time, and allowing several minutes draining between rinses.
6. After draining away the final rinse, lower the root support into the hot ink stain bath, ensuring that roots are completely submerged in the stain. Cover the water bath and heat at least 15 min at 90°C.
7. Remove root support from ink bath using long forceps, allow the ink to drain, then rinse and de-stain 20 min in 5% acetic acid baths. Root samples can be stored in the support in a water bath for several days.

CONCLUSION

This review briefly describes some of the basic techniques for AM culture and study that have proven to be effective in *L. japonicus*. Further information about AM fungi, including helpful culture and staining protocols, can be obtained from the following organisations/sites:

- The Mycorrhiza Information Exchange http://mycorrhiza.ag.utk.edu/
- The International Culture Collection of Vesicular Arbuscular Mycorrhizal Fungi (INVAM) http://invam.caf.wvu.edu/
- The International Bank for the Glomeromycota (BEG) www.kent.ac.uk/bio/beg/

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- The Glomales In Vitro Collection (GINCO) www.mbla.ucl.ac.be/ginco-bel or www.agr.gc.ca/science/ecorc/ginco-can/index_e.htm

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