'Prepackaged symbioses': propagules on roots of the myco-heterotrophic plant *Arachnitis uniflora*

Laura Domínguez[1], Alicia Sérsic[1], Lewis Melville[2] and R. Larry Peterson[2]

[1]Instituto Multidisciplinario de Biología Vegetal, C.C. 495, Córdoba 5000, Argentina; [2]Department of Botany, University of Guelph, Guelph, Ontario N1G 2W1, Canada

Author for correspondence:
Dr Laura Domínguez
Tel: +54 3514331056
Fax: +54 3514332104
Email: laura@imbiv.unc.edu.ar

Received: *18 May 2005*
Accepted: *16 July 2005*

Summary

• *Arachnitis uniflora*, a myco-heterotrophic plant species, has fleshy tuberous roots colonized by the arbuscular mycorrhizal fungal genus *Glomus* (Phylum Glomeromycota). These roots produce apical and lateral propagules, both reported here for the first time. The objective of the study was to characterize the ontogeny and structure of the propagules, and to determine their function.

• Scanning electron microscopy, laser scanning confocal microscopy and light microscopy were used to study the ontogeny and structure of the propagules.

• Propagules developed either from cortical parenchyma cells or from cells immediately beneath the root cap; they developed a shoot meristem and cells in the basal region which were colonized by various fungal structures including hyphae and vesicles.

• These propagules may detach from the roots, establishing new plants.

Key words: *Arachnitis*, hyphae, myco-heterotroph, vegetative propagules, vesicles.

New Phytologist (2005) **doi**: 10.1111/j.1469-8137.2005.01559.x

Introduction

The importance of symbioses between plants and fungi (mycorrhizas) in nutrient acquisition, water relations and disease resistance of plants is well known (Smith & Read, 1997). Recently, the role played by mycorrhizas in regulating biodiversity in ecosystems has received more attention from ecologists who are recognizing the complex interactions that occur between plant roots and soil organisms (Hartnett & Wilson, 2002; Leake *et al.*, 2004; Read *et al.*, 2004). Among the most complex interactions are those involving achlorophyllous plant species, which lack the ability to carry out photosynthesis and therefore rely either on fungal connections with autotrophic plants or on saprotrophic fungi for their source of carbon compounds. These plants, known as myco-heterotrophs, occupy various niches in diverse habitats (Leake, 1994, 2004). As the fungus does not appear to gain in any appreciable way from the association, Brundrett (2004) refers to these mycorrhizal associations as 'exploitive mycorrhizas'. Many of the approximately 400 species of myco-heterotrophs are associated with basidiomycete and ascomycete fungi and have received considerable attention as regards their specialized relationships with particular fungal species (Leake, 1994, 2004).

Arachnitis uniflora (Corsiaceae) is a nonchlorophyllous species that is restricted in its distribution to areas in Argentina, Chile, the Falkland Islands and Bolivia (Dimitri, 1972; Cribb *et al.*, 1995; Ibisch *et al.*, 1996). The roots of this species, along with several species of *Voyria* (Gentianaceae) and *Voryiella parviflora* (Gentianaceae), are associated with the genus *Glomus* in the ancient fungal group Glomeromycota (Bidartondo *et al.*, 2002). The colonization pattern of *A. uniflora* roots (Domínguez & Sérsic, 2004) is similar to the colonization patterns of the roots of other myco-heterotrophic species in the Gentianaceae (Imhof & Weber, 1997; Imhof, 1997), and Triuridaceae (Imhof, 1998, 2003).

In our studies of *A. uniflora* collected from various sites in Argentina, we have examined the fleshy tuberous roots in detail and have discovered an undescribed method of plant propagation and the continuation of a symbiotic relationship in this unusual plant. Although many plants, including myco-heterotrophic species (Champagnat & Champagnat, 1965;

Champagnat, 1971; Leake, 1994), are capable of forming adventitious shoots (sometimes called root buds) on their roots as a means of vegetative propagation, and many of the most pernicious weeds are able to spread by this means (Peterson, 1975), none has the characteristics shown here. The objectives of this study were therefore to determine the ontogeny of root-derived propagules, their relationship with root fungi, and their possible function.

Materials and Methods

Plant material

Plants of *Arachnitis uniflora* Phil. were collected from two sites in Argentina: Chubut, Dpto. Cushamen, Lake Cholila, in December 2003, and Neuquén, Dpto. Los Lagos, Villa La Angostura, Laguna Verde, in December 2002. Plants were washed free of soil and fixed in the field in either 70% ethanol or 2.5% glutaraldehyde.

Scanning electron microscopy

Roots were excised from plants that had been fixed in 70% ethanol, dehydrated in a graded ethanol series to 100% ethanol, critical point dried, and mounted on aluminum stubs with two-sided sticky tape. Mounted samples were coated with gold before being examined in a Phillips scanning electron microscope (Phillips, Eindhoven, the Netherlands). Images were recorded with a digital camera.

Light microscopy

Roots for light microscopy were excised from plants that had been fixed in the field in 2.5% glutaraldehyde and stored

Fig. 1 Plants of *Arachnitis uniflora*. (a) Plants flowering in the field at Laguna Verde, Neuquén, Argentina. Bar, 15 mm. (b) Plants each with a single flower, an elongated stalk, and a cluster of fleshy, tuberous roots. Bar, 15 mm. (c) Fleshy tuberous roots showing apical propagules (arrows) and a lateral propagule (arrowhead). Bar, 1.0 mm. (d) Tuberous root with a lateral propagule (arrowhead). Bar, 1.0 mm. (e) Tuberous root with a well-developed lateral propagule with several adventitious roots. The arrowhead indicates an early stage in the initiation of an apical propagule. Bar, 1.0 mm.

in 70% ethanol until further processing in the laboratory. Samples were dehydrated in a graded ethanol series and embedded in LR White resin (Canemco, Lachine, Quebec, Canada). Sections (0.5–1.0 μm thick) were cut with glass knives on a Reichert Ultramicrotome (Reichert, Vienna, Austria), heat-fixed to microscope slides and stained with 0.05% toluidine blue O (Sigma-Aldrich, St Louis, MO, USA) in 1.0% sodium borate. Stained sections were viewed with a Leitz Orthoplan microscope (Wild Leitz Canada, Ottawa, Ontario, Canada) and images were captured with a Nikon digital camera (Nikon Canada, Mississauga, Ontario, Canada).

Laser scanning confocal microscopy

Roots that had been fixed in 70% ethanol were hand-sectioned with a sharp two-sided razor blade to a thickness of approx. 1.0 mm, cleared in 1.5% KOH by warming on a heating plate for approximately 1 h, rinsed with water, treated with 0.1 N HCl for 10 min, and then stained with acid fuchsin-lactoglycerol [equal parts of 0.01% aqueous acid fuchsin (weight/volume), 85% lactic acid and glycerol] by warming in an oven at 50°C for 1 h. After staining, root sections were mounted in glycerol under a cover glass and examined with a Bio-Rad MRC-600 laser scanning confocal microscope (Bio-Rad, Hemel Hempstead, UK) mounted on a Nikon Optiphot-2 upright microscope and equipped with a krypton/argon mixed gas laser. Images were captured using an excitation wavelength filter of 488/586 nm and a photomultiplier (PMT) detector filter of 590/600 nm. Serial optical sections taken at 1.0-μm intervals were compiled and images for the plates in this manuscript were prepared using Adobe Photoshop.

Results

Arachnitis uniflora is only visible above the soil surface at the time of flowering (Fig. 1a). The single flower is borne on an elongated stalk with a few scale-like bracts and is subtended by a cluster of fleshy, tuberous roots (Fig. 1b). The number of fleshy roots per plant varies (Fig. 1b); all lack laterals and root hairs but develop apical and lateral protuberances (Fig. 1c and d) that develop into propagules. These structures may detach at various stages but often those in a lateral position remain attached, forming new plants with several fleshy adventitious roots (Fig. 1e). Additional propagules may, in turn, develop from these newly formed roots (Figs 1e and 2a). Both apical and lateral propagules detach from the parent root at various stages of development, and these can often be found in large numbers in the soil in the vicinity of flowering plants (Fig. 2b). The larger of these structures often show bract primordia and outgrowths that may be the first roots or more likely additional propagules (Fig. 2b).

Some of the structures that occur in the soil adjacent to adult plants are derived from seeds (Fig. 2c), but these can be differentiated from root-derived propagules based on their shape and the presence of remnants of the testa (Fig. 2d and e).

Propagules initiated from the root apex are covered initially by sloughing root cap cells of the parent root (Fig. 3a); these loose cells are lost when tissue is embedded for light microscopy (Fig. 3b). Sections through the root apex showed that

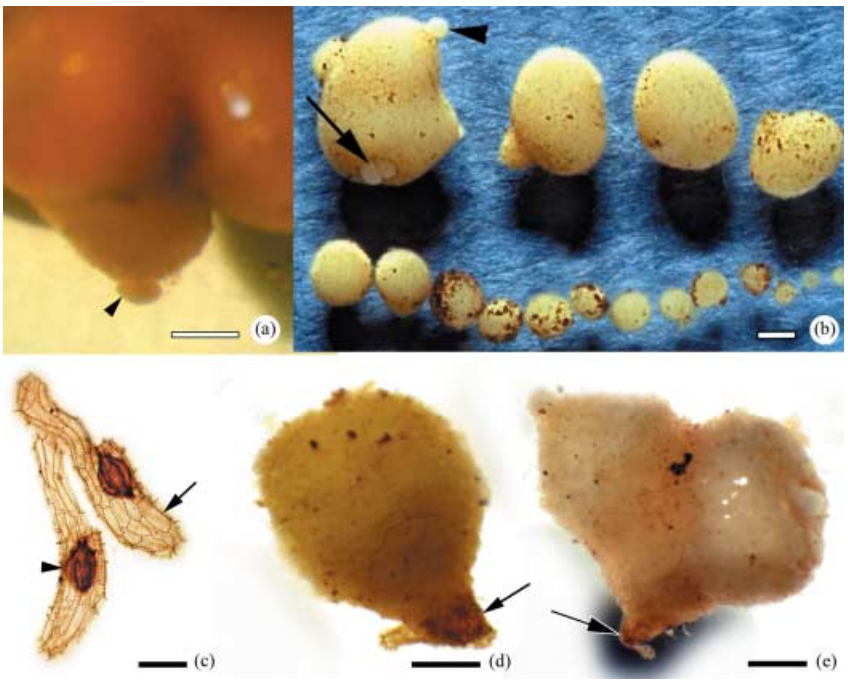

Fig. 2 Propagules, seeds and seedlings of *Arachnitis uniflora*. (a) Adventitious roots from a lateral propagule similar to that shown in Fig. 1e, showing an apical propagule (arrowhead). Bar, 1.0 mm. (b) Propagules at different stages of development obtained from the soil adjacent to plants. Arrow, developing shoot; arrowhead, developing root or propagule. Bar, 1.0 mm. (c) Seeds, each showing the reticulate nature of the testa (arrow) and an undifferentiated embryo (arrowhead). Bar, 100 μm. (d, e) Stages in the development of new plants from seeds. Remnants of the testa are visible at the elongated base of each specimen (arrows).

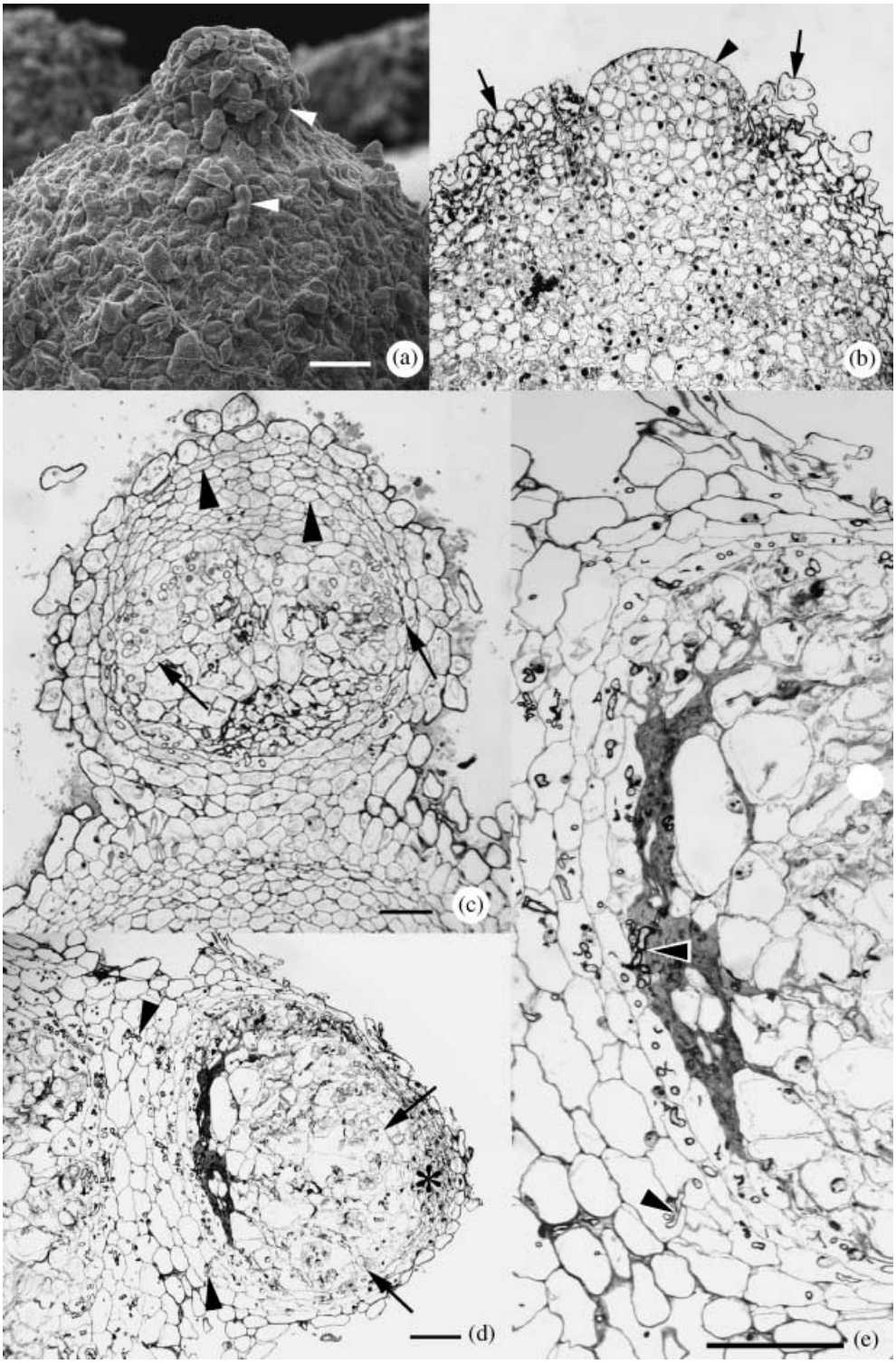

Fig. 3 Sections of *Arachnitis uniflora* propagules. (a) Scanning electron micrograph of a propagule originating from the root apex. Loose root cap cells (arrow) are visible on the surface. Bar, 100 μm. (b) A section of the apex of a fleshy root showing an early stage in the development of a propagule. An epidermal layer (arrowhead) has developed. Loose root cap cells (arrows) are evident. Bar, 100 μm. (c) A later stage in the development of an apical propagule. Middle and basal cells contain fungal structures (arrows). A region of small, dividing cells (arrowheads) has differentiated. Bar, 100 μm. (d) A section through a lateral propagule. A shoot meristem (*) is present and many cells are colonized by fungal structures (hyphae, and vesicles in bundles) (arrows). Arrowheads indicate fungal hyphae at the base of the propagule that have originated from the cortex of the parent root. Bar, 100 μm. (e) An enlarged view of cells at the base of the lateral propagule shown in (d), showing fungal hyphae (arrowheads) that have originated from the cortex of the parent root. Bar, 100 μm.

initiation occurs in cells below the root cap and the propagule develops a distinct epidermal layer early in ontogeny (Fig. 3b). At this early stage, none of the cells of the developing propagule contains fungal structures but, as development proceeds and as a region of meristematic cells differentiates, cells in the basal portion are colonized by fungal hyphae and other fungal structures (Fig. 3c). From serial sections through these apical propagules, it appears that fungi do not pass directly into them from the parent root.

Lateral propagules are initiated from the cortex of parent roots and these also develop a meristematic region along with basal cells occupied by fungal structures (Fig. 3d). Serial sections of lateral outgrowths showed that fungal hyphae enter the basal region of the protuberance from the cortical cells of the parent root (Fig. 3d and e). Lateral propagules often remain attached until considerable colonization of basal cells has occurred and a well-defined shoot apical meristem region has developed (Fig. 4a). In Fig. 4b, an enlargement of a region of the basal portion of a lateral protuberance shows the various fungal structures (hyphae, and vesicles in bundles) present in these cells. The vascular system of the developing shoot never joins the vascular tissue of the parent root (Fig. 4c).

Sections of detached propagules similar to the larger ones depicted in Fig. 2a showed that they have a small shoot apical meristem that initiates the bract primordia; beneath the meristematic region the cells are heavily colonized by fungal structures (Fig. 4d).

Laser scanning confocal microscopy of sections of these structures showed more details of the fungi that colonize the basal region. Clusters of vesicles develop from trunk hyphae (Fig. 5a) and cells close to the surface contain branching hyphal complexes (Fig. 5b). Host cell nuclei are very large and often have more than one nucleolus (Fig. 5a). Collapsing fungal hyphae and vesicles also occur in some regions of detached structures (Fig. 5c).

Discussion

The myco-heterotrophic species *A. uniflora* is unique in that propagules are initiated from the root apex as well as from lateral positions along the root axis. The initiation of propagules from the root apex is similar to that reported for the orchid species *Neottia nidus-avis* (Champagnat & Champagnat, 1965) but the propagules in the latter do not become colonized by fungi from the parent root; instead, it has been suggested that fungi colonizing roots of the new plant arise from the soil (Champagnat, 1971). In a recent study on *Voyria flavescens* (Gentianaceae), Franke (2002) mentioned that this species forms new plants from its root tips; however, no anatomical details were provided and there was no mention of fungal colonization of these plants.

Several fern species, including the eusporangiate fern *Ophioglossum petiolatum*, form new shoots from root tips, but these are usually initiated from a recent derivative of the root apical cell (Peterson, 1970). In these cases, however, Glomalean fungi that are known to be typical mycorrhizal fungi in roots of this genus (Read *et al.*, 2000) are not passed into the vegetative propagules.

In most Angiosperms, adventitious shoot buds occurring on roots are initiated from cells in the cortex, pericycle and vascular parenchyma, and occasionally from tissue derived from the phellogen (Peterson, 1975; Bosela & Ewers, 1997). Although the lateral propagules of *A. uniflora* are initiated from the cortex of the parent roots, they differ significantly from the shoot buds that are initiated in the root cortex of other Angiosperms. In the latter, the site of bud initiation is usually in close proximity to the lateral roots (Peterson, 1975; Polowick & Raju, 1982; Sharma *et al.*, 1993) and vascular connections occur between the developing shoot bud and the vascular system of the parent root (Peterson, 1975; Polowick & Raju, 1982). Neither of these features is shown by *A. uniflora*. In *A. uniflora*, the lack of vascular connections contributes to the ability of propagules to be detached from roots at various stages in their development.

Although it is not clear how the apical propagules of *A. uniflora* become colonized by fungi, they are usually colonized before they detach from the parent root. It is possible that fungi pass directly from the parent root, although our sections do not seem to show this, or these propagules may become colonized either from extraradical hyphae attached to the parent root or from hyphae in the soil. This remains to be determined. Sections of lateral propagules show a continuity of colonization between cortical cells of the parent root and the basal region of developing propagules. Some of these propagules may therefore carry a symbiotic fungus from the mother plant and this may be an important method of assuring the continuity of this symbiotic association from one generation to the next.

The fungal structures present in the propagules are similar to those reported previously for roots of *A. uniflora* (Domínguez & Sérsic, 2004), suggesting that arbuscular mycorrhizal fungi in the genus *Glomus* (Bidartondo *et al.*, 2002) are involved. Molecular work needs to be performed to confirm this. In both roots and propagules, the morphological features of the mycorrhizal association, including the presence of coiled hyphae, the lack of arbuscules and the development of vesicle-like structures, are similar to those of other achlorophyllous species in the Gentianaceae (Imhof & Weber, 1997; Imhof, 1997) and Triuridaceae (Imhof, 1998, 2003). A detailed analysis of the development and morphological features of the fungi associated with roots and propagules in *A. uniflora*, using both confocal microscopy and transmission electron microscopy is in progress.

In future work, it would be of interest to use the root-produced propagules in microcosms to test whether the fungi present are able to colonize seedlings of species found in the vicinity of *A. uniflora* plants in the field that have been shown, by molecular analysis of roots, to share the same fungus (Bidartondo *et al.*, 2002).

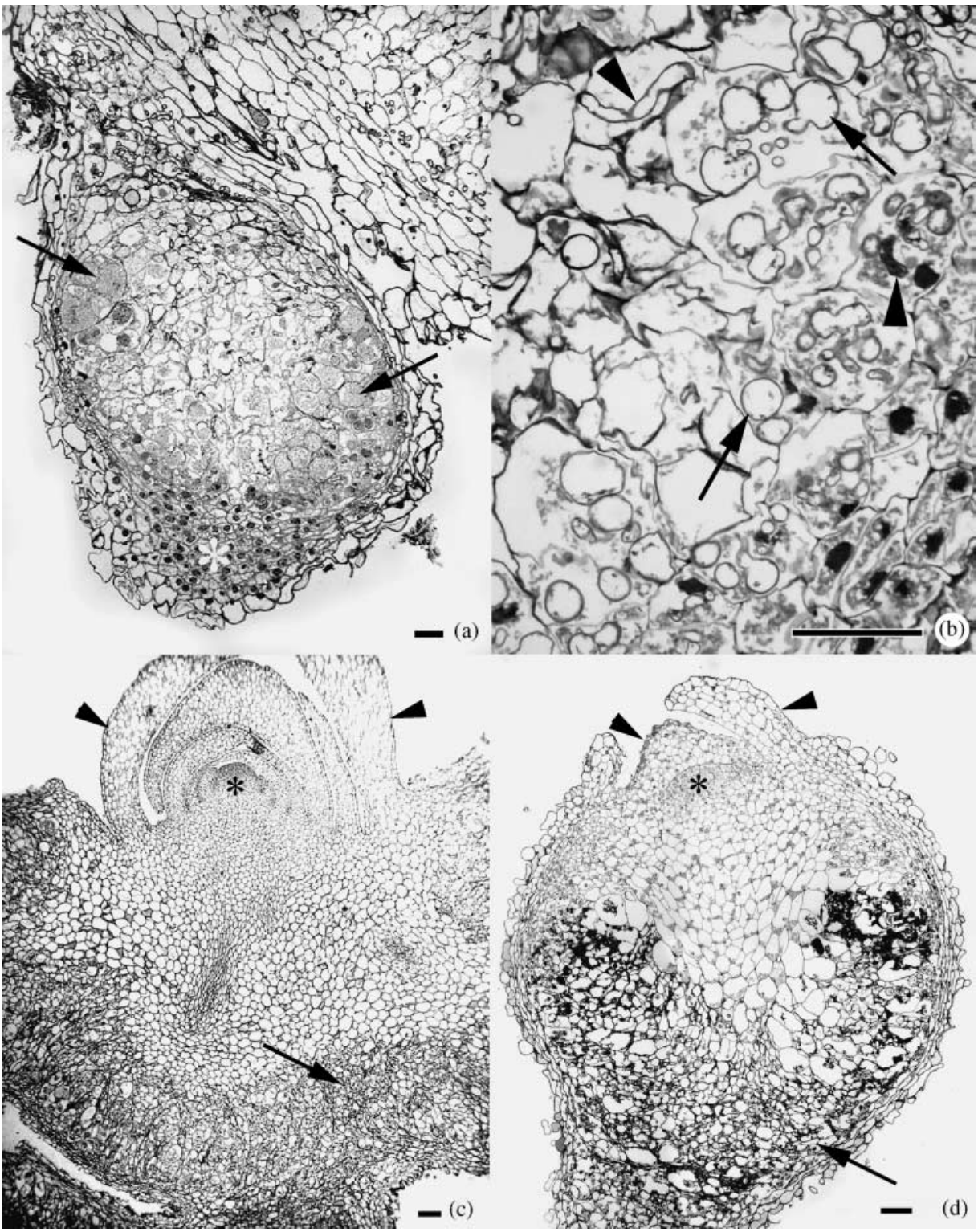

Fig. 4 Longitudinal sections of *Arachnitis uniflora* lateral propagules. (a) A lateral propagule of *Arachnitis uniflora* showing the development of a shoot apical meristem region (*) and many cells that are colonized by fungal structures (arrows). Bar, 50 μm. (b) Cells from the basal region of a lateral propagule showing fungal hyphae (arrowheads) and vesicle-like structures (arrows). Bar, 50 μm. (c) A section through a lateral propagule with a shoot apical meristem (*), bract primordia (arrowheads) and a basal region of cells colonized by fungi. There is a continuation of colonized cells from the parent root at the bottom of the photomicrograph. Material in this figure was processed by Trevor Wilson through paraffin and stained with safranin and alcian green. Bar, 50 μm. (d) A detached fungal propagule showing the presence of a shoot apical meristem (*), bract primordia (arrowheads) and extensive colonization by fungal structures in the basal region (arrow). Bar, 50 μm.

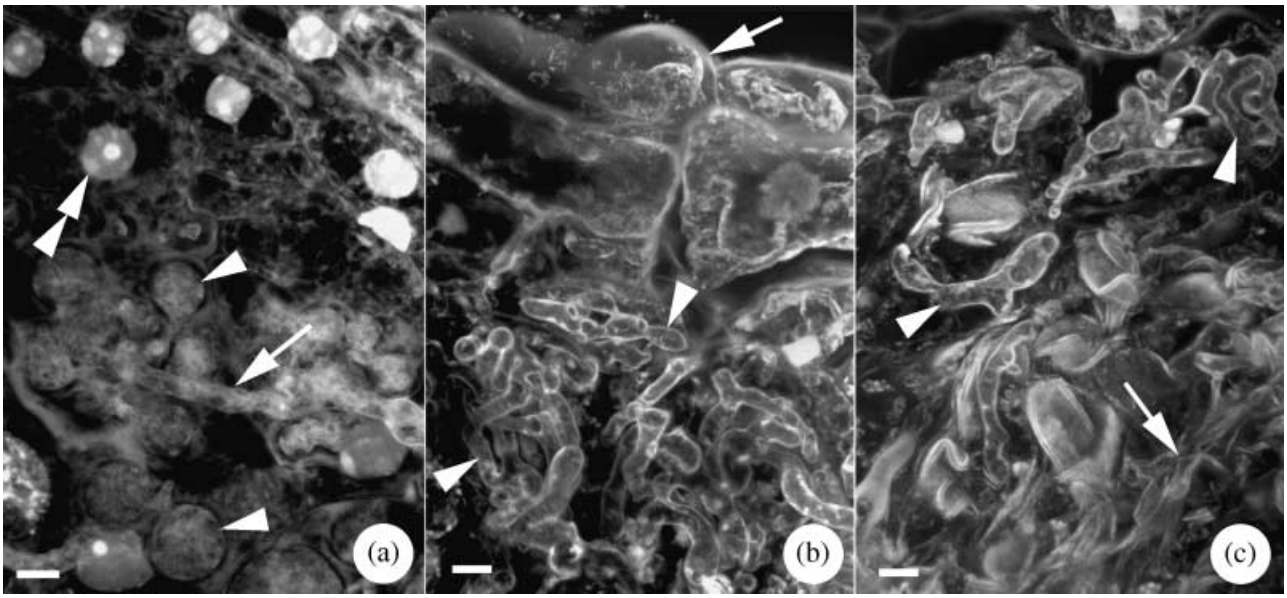

Fig. 5 Scanning laser confocal microscopy of fungal structures in propagules of *Arachnitis uniflora.* All bars, 10 μm. (a) Vesicle development (arrowheads) from a trunk hypha (arrow). Large nuclei (double arrowheads), most with several nucleoli, are evident. (b) Coiled and branched hyphae (arrowheads) in cells close to the periphery (arrow) of a propagule. (c) Coiled hyphae (arrowheads) and collapsing fungal structures (arrow).

Acknowledgements

This research was supported in part by a Discovery Grant from the Natural Sciences and Engineering Research Council of Canada to R.L.P. We thank the Myndel Pedersen Foundation for supporting the field trips and acknowledge support from CONICET, Argentina of which A.S. is a member.

References

Bidartondo MI, Redecker D, Hijrl I, Wiemken A, Bruns TD, Domínguez L, Sérsic A, Leake JR, Read DJ. 2002. Epiparasitic plants specialized on arbuscular mycorrhizal fungi. *Nature* **419**: 389–392.

Bosela MJ, Ewers FW. 1997. The mode of origin of root buds and root sprouts in the clonal tree *Sassafras albidum* (Lauraceae). *American Journal of Botany* **84**: 1466–1481.

Brundrett M. 2004. Diversity and classification of mycorrhizal associations. *Biology Reviews* **79**: 473–495.

Champagnat M. 1971. Recherches sur la multiplication vegetative de *Neottia nidus-avis* Rich. *Annales des Sciences Naturelles* **12**: 209–248.

Champagnat M, Champagnat P. 1965. Vers une etude expérimentale des problèmes de morphogenèse pose par *Neottia nidus-avis* Rich. *Clermont-Ferrand Universite Faculte des Sciences Annales* **26**: 115–124.

Cribb PJ, Wilkin P, Clements M. 1995. Corsiaceae: a new family for the Falkland Islands. *Kew Bulletin* **50**: 171–172.

Dimitri MJ. 1972. Una nueva especie del género '*Arachnitis*' Phil. (Corsiaceae). *Revista Facultad de Agronomía de la Universidad Nacional de la Plata* **48**: 37–45.

Domínguez LS, Sérsic A. 2004. The southernmost myco-heterotrophic plant, *Arachnitis uniflora*: root morphology and anatomy. *Mycologia* **96**: 1141–1149.

Franke T. 2002. The myco-heterotrophic *Voyria flavescens* (Gentianaceae) and its associated fungus. *Mycological Progress* **1**: 367–376.

Hartnett DC, Wilson GWT. 2002. The role of mycorrhizas in plant community structure and dynamics: lessons from grasslands. *Plant and Soil* **244**: 319–331.

Ibisch PL, Neinhuis C, Rojas PN. 1996. On the biology, biogeography, and taxonomy of *Arachnitis* Phil. Nom. Cons. (Corsiaceae) in respect to a new record from Bolivia. *Willdenowia* **26**: 321–332.

Imhof S. 1997. Root anatomy and mycotrophy of the achlorophyllous *Voyria tenella* Hook. (Gentianaceae). *Botanica Acta* **110**: 298–305.

Imhof S. 1998. Subterranean structures and mycotrophy of the achlorophyllous *Triuris hyaline* (Triuridaceae). *Canadian Journal of Botany* **76**: 2011–2019.

Imhof S. 2003. A dorsiventral mycorrhizal root in the achlorophyllous *Sciaphila polygna* (Triuridaceae). *Mycorrhiza* **13**: 327–332.

Imhof S, Weber HC. 1997. Root anatomy and mycotrophy (AM) of the achlorophyllous *Voryia truncata* (Standley) Standley & Steyermark (Gentianaceae). *Botanica Acta* **110**: 127–134.

Leake JR. 1994. The biology of myco-heterotrophic ('saprophytic') plants. *New Phytologist* **127**: 171–216.

Leake JR. 2004. Myco-heterotroph/epiparasitic plant interactions with ectomycorrhizal and arbuscular mycorrhizal fungi. *Current Opinion in Plant Biology* **7**: 422–428.

Leake JR, Johnson D, Donnelly D, Muckle G, Boddy L, Read D. 2004. Networks of power and influence: the role of mycorrhizal mycelium in controlling plant communities and agroecosystem functioning. *Canadian Journal of Botany* **82**: 1016–1045.

Peterson RL. 1970. Bud development at the root apex of *Ophioglossum petiolatum. Phytomorphology* **20**: 183–190.

Peterson RL. 1975. The initiation and development of root buds. In: Torrey JG, Clarkson DT, eds. *The development and functioning of roots.* London: Academic Press, 125–161.

Polowick PL, Raju MVS. 1982. The origin and development of root buds in *Asclepias syriaca. Canadian Journal of Botany* **60**: 2119–2125.

Read DJ, Duckett JG, Francis R, Ligrone R, Russell A. 2000. Symbiotic fungal associations in 'lower' land plants. *Philosophical Transactions of the Royal Society of London* **355**: 815–831.

Read DJ, Leake JR, Perez-Moreno J. 2004. Mycorrhizal fungi as drivers of ecosystem processes in heathland and boreal forest biomes. *Canadian Journal of Botany* **82**: 1243–1263.

Sharma KK, Yeung EC, Thorpe TA. 1993. Histology of shoot bud ontogeny from seedling root segments of *Brassica napus* L. *Annals of Botany* **71**: 461–466.

Smith SE, Read DJ. 1997. *Mycorrhizal symbiosis.* San Diego, CA, USA: Academic Press.