

Review article

Fungal Symbioses with Orchid Protocorms

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Abstract

Orchid species have minute seeds that in nature and *in vitro*, if complex polysaccharides are used as a carbon source, must be invaded by symbiotic fungi in order for germination to occur. Subsequent to germination, a protocorm develops from the simple embryo contained in the mature seed. A shoot apical meristem and adventitious roots are initiated at the chalazal end of the protocorm, events that are necessary for seedling establishment. Most of the information pertaining to the interaction between symbiotic fungi and orchid protocorms has been obtained from laboratory experiments using terrestrial orchid species germinated *in vitro* in combination with fungal species that have been isolated either from roots of orchid species or from diseased non-host plant species. Fungal isolates were previously grouped in the form-genus *Rhizoctonia* although anamorphs and teleomorphs have now been placed in several genera. Symbiotic fungal hyphae enter the embryo of imbibed seeds either through the suspensor or epidermal hairs and form coiled, branched structures called pelotons in parenchyma cells of the embryo. The peloton is separated from the parenchyma cell cytoplasm by a perifungal membrane and interfacial matrix material. The perifungal membrane shares some characteristics with the peripheral plasma membrane but differs in that it does not show adenylate cyclase activity. Colonization of embryo cells induces nuclear hypertrophy, partly due to an increase in DNA synthesis, and changes in both microtubules and actin filaments. Pelotons undergo degradation and during this phase pectins, β -1,3 glucans

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and cellulose are deposited in the interfacial matrix surrounding senescing hyphae and hyphal clumps. The orchid protocorm-symbiotic fungal system shows promise for studies of plant cell-fungal interactions at the molecular level.

Keywords: Orchids, mycorrhizas, protocorms, structure

1. Introduction

Orchidaceae is one of the largest angiosperm families and the many species included within it occupy a variety of habitats globally. Although there is a huge diversity in morphological and reproductive characteristics, a unifying feature is the production of minute seeds, "dust seeds", that must under natural conditions become associated with fungi in order for seedling establishment to occur (Arditti, 1979; Benzing, 1981; Rasmussen, 1995). Orchid species also show a unique feature among angiosperms in that a parenchymatous structure, the protocorm, is intercalated between the seed and the seedling. This structure develops from a simple embryo contained within the mature seed and as it increases in size, a shoot apical meristem and adventitious roots are initiated and subsequently a seedling is established (see Fig. 1). Although many orchid species are chlorophyllous and, therefore, become autotrophic during seedling establishment, they are heterotrophic during the protocorm stage. These orchid species develop a symbiotic fungal association during seed germination and protocorm formation as well as with adventitious roots as they are formed. Achlorophyllous orchid species are mycoheterotrophic during their entire life cycle and as expected, therefore, are dependent on their fungal symbionts for nutrients during protocorm formation, seedling establishment and during their adult phase. In spite of the ubiquitous association of fungi with the various stages of the orchid life cycle, only recently have the systematics, structure, physiology and ecology of these fungal symbionts been studied. The emphasis in studies of orchid-fungal symbiotic associations has been biased in favour of the orchid, primarily because of the interest in propagation of commercially important and endangered species and because researchers studying pathogenic fungi do not usually study symbioses between orchids and fungi. This review will consider some aspects of the fungal symbionts but the main focus will be on the events that occur as terrestrial orchid seeds germinate and protocorms are established; particular emphasis will be placed on the interactions with symbiotic fungi during these processes. The significance of the symbiotic association with fungi during seedling establishment of terrestrial orchid species has been discussed in detail recently (Smith and Read, 1997). It is clear from the evidence now available that the fungal species involved in this association are capable of degrading complex carbohydrates that exist in the

soil and providing the heterotrophic protocorm stage with simple sugars required for growth and differentiation of a shoot and root system.

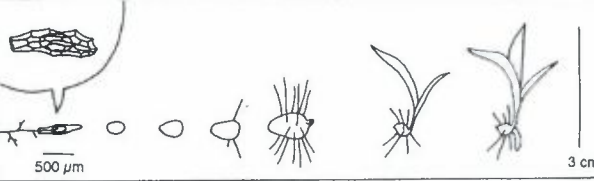

Carbon source	Fungus	
Complex - oatmeal - starch - cellulose	<i>Ceratobasidium cornigerum</i>	
Simple sugar - sucrose - glucose	—	

Figure 1. Comparison of symbiotic and asymbiotic culture methods of *Spiranthes sinensis*.

Fungal symbionts

The fungal species involved in orchid seed germination and protocorm development in nature are not well known. Due to their minute size, the sowing of orchid seeds is most easily accomplished *in vitro* under sterile conditions (Arditti, 1992). The fungi found in the environment of wild germinating seeds of most orchid species are difficult to isolate and identify; the experimenter must, therefore, choose fungi from those isolates available for use in seed germination in the laboratory. Most studies of seed germination, therefore, utilize easily-isolated endophytes from the roots of mature orchids. Our knowledge of the role of fungi in the symbiotic germination and development of orchids is derived almost entirely from these studies.

The appropriateness of fungi from these sources for the study of certain aspects (especially specificity) of the symbiotic germination of orchid seeds has been questioned (Masuhara et al., 1993; Zelmer et al., 1997), because the fungi associated with roots, for example, are not always the same as those associated with developing seedlings (protocorms) in nature and there may be several different fungi associated with roots of any given species. A degree of

fidelity between orchid protocorms and plants and particular species of symbiotic fungi has been reported, however, for a few orchid species (Masuhara and Katsuya, 1994; Currah et al., 1996). Although a few researchers have isolated and described symbiotic fungi from field-collected protocorms (Masuhara and Katsuya, 1994; Zelmer et al., 1997; Zelmer and Currah, 1997; Rasmussen, 1993; Currah et al., 1996), our knowledge of the fungi associated with naturally germinating orchid seeds is limited. The discussion that follows is, therefore, necessarily based upon isolates from orchid roots.

Many fungal taxa have been isolated from orchid roots (see the review by Currah and Zelmer, 1992; and papers by Richardson et al., 1993; Richardson and Currah, 1995; Bayman et al., 1997). Fungal hyphae are present on the root surface and also within the cortex, where the coiled or anastomosed 'pelotons' characteristic of orchid mycorrhizal associations form (Burgeff, 1936). Fungal hyphae and spores are also found within the root velamen (non-living, multiple epidermis) of epiphytic species (Richardson and Currah, 1995). Although only peloton forming fungi are presently considered to be the mycorrhizal fungi of mature plants and of protocorms, many of the fungi associated with the root surface have cellulolytic or lignolytic activity, and may also be important symbionts (Richardson and Currah, 1995; Bayman et al., 1997). Fungi are the best recognized of orchid symbionts, but other organisms, especially bacteria, may also be important (Wilkinson et al., 1989).

Isolation of most orchid mycorrhizal fungi is easily accomplished by plating either surface sterilized root segments or individual fungal pelotons onto a medium, such as tap water agar (Currah and Zelmer, 1992). Most peloton-forming fungi lack clamp connections and asexual spores (conidia), branch at roughly right angles to the main axis (Figs. 4 and 5), are binucleate (Fig. 5) to multinucleate, and form monilioid cells (Fig. 4) (Andersen, 1990). Anastomosis groupings have also been used extensively for the characterization of plant pathogenic and orchid-associated *Rhizoctonia* (sensu lato) (Ogoshi, 1987; Sneh et al., 1991; Ramsay et al., 1987). Table 1 shows the relationships between these morphological and anastomosis-based classification systems, and with known teleomorphic connections. Historically, these fungi have been placed into the form genus *Rhizoctonia* (Andersen and Stalpers, 1994). Moore (1987) distributed the species within *Rhizoctonia* into several form genera based on numbers of nuclei per cell, septal pore ultrastructure and known or suspected teleomorph connections. Among the orchid endophytes these groupings agree well with cultural characteristics.

The most commonly isolated symbiotic fungi from terrestrial orchids are species in the genera *Ceratorhiza* (Figs. 2 and 5), *Epulorhiza* (Figs. 3 and 4) and *Moniliopsis*. Although these genera are well defined, species concepts within the genera are developing more slowly.

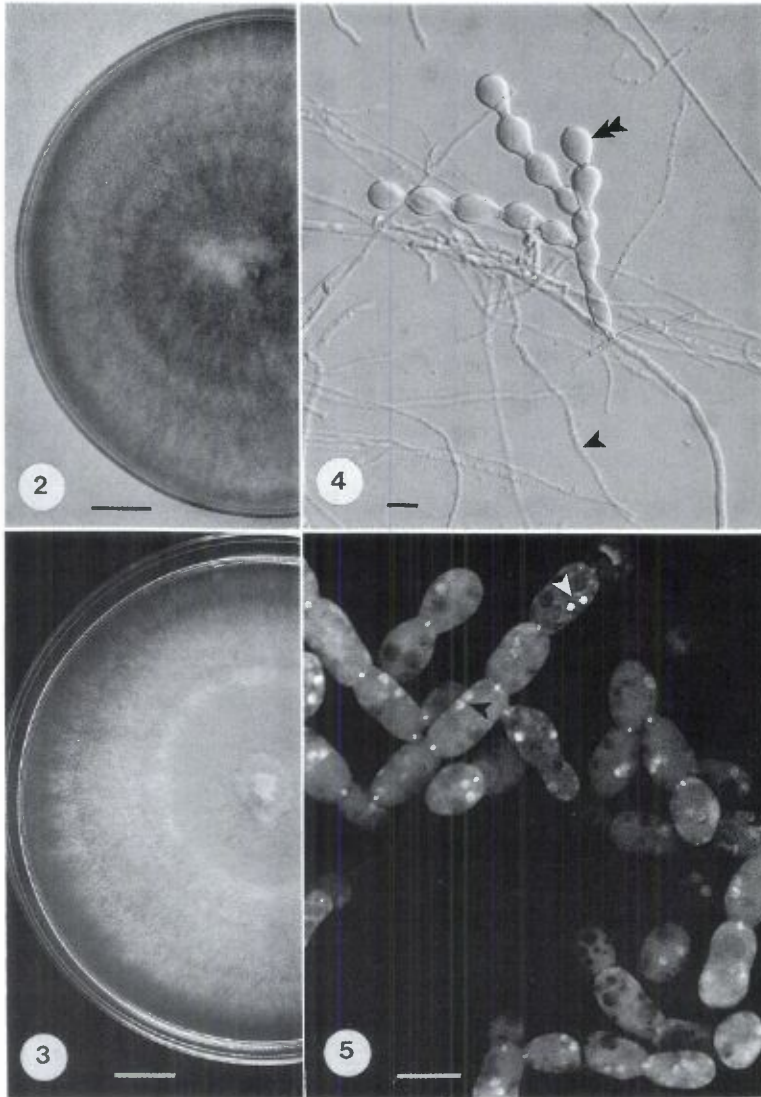
Table 1. Classification of orchid mycorrhizal fungi

Anamorph		Anastomosis groups	Teleomorph
de Candolle (1815)	Moore (1987)		
Rhizoctonia	Ceratorhiza	Binucleate e.g. AG-C, AG-I	Ceratobasidium
	Epulorhiza	In part binucleate e.g. R.r.1, R.r.2	Tulasnalla & Sebacina
	Moniliopsis	Multinucleate e.g. G-5, AG-6	Thanatephorus

The use of form genera and anastomosis groupings for these fungi associated with orchids has been necessary due to the infrequency with which they form sexual stages in culture, and the paucity of stable and useful taxonomic features in the asexual state. The application of molecular tools may clarify the taxonomy of these fungi in the future (Cubeta et al., 1991; Vilgalys and Gonzalez, 1990).

Several other fungi have also been suggested or demonstrated to be important symbionts of certain orchid species. The achlorophyllous (mycoheterotrophic) species are completely dependent upon fungal symbionts for their nutrition, and appear to form symbioses with fungi that are culturally and taxonomically distinct from those of most chlorophyllous orchids (Umata, 1995; Zelmer and Currah, 1995; Taylor and Bruns, 1996; Terashita and Chuman, 1987). Some of these orchid species apparently share mutualistic fungi with other plant species, leading to triple symbioses (Rasmussen, 1995). This relationship would allow the achlorophyllous orchids to extract photosynthates from ectomycorrhizal green plants via a common symbiont. Such a symbiosis has been proposed for *Corallorhiza trifida* Chat., whose fungal symbiont, an unidentified, bright yellow isolate with clamp connections, can form both orchid and ectomycorrhizal associations (Zelmer and Currah, 1995). Other *Corallorhiza* species have been linked to ectomycorrhizal fungi such as *Russula* and *Tomentella* (Taylor and Bruns, 1996). Although triple symbioses usually do not utilize *Rhizoctonia*-like fungi, there is evidence that some *Rhizoctonia*-like strains may be able to form ectomycorrhizas as well (Salmia, 1988; Warcup, 1991).

The ecological roles of most orchid symbionts are still unknown (Zelmer and Currah, 1997). In culture, *Epulorhiza* species can produce cellulases, while



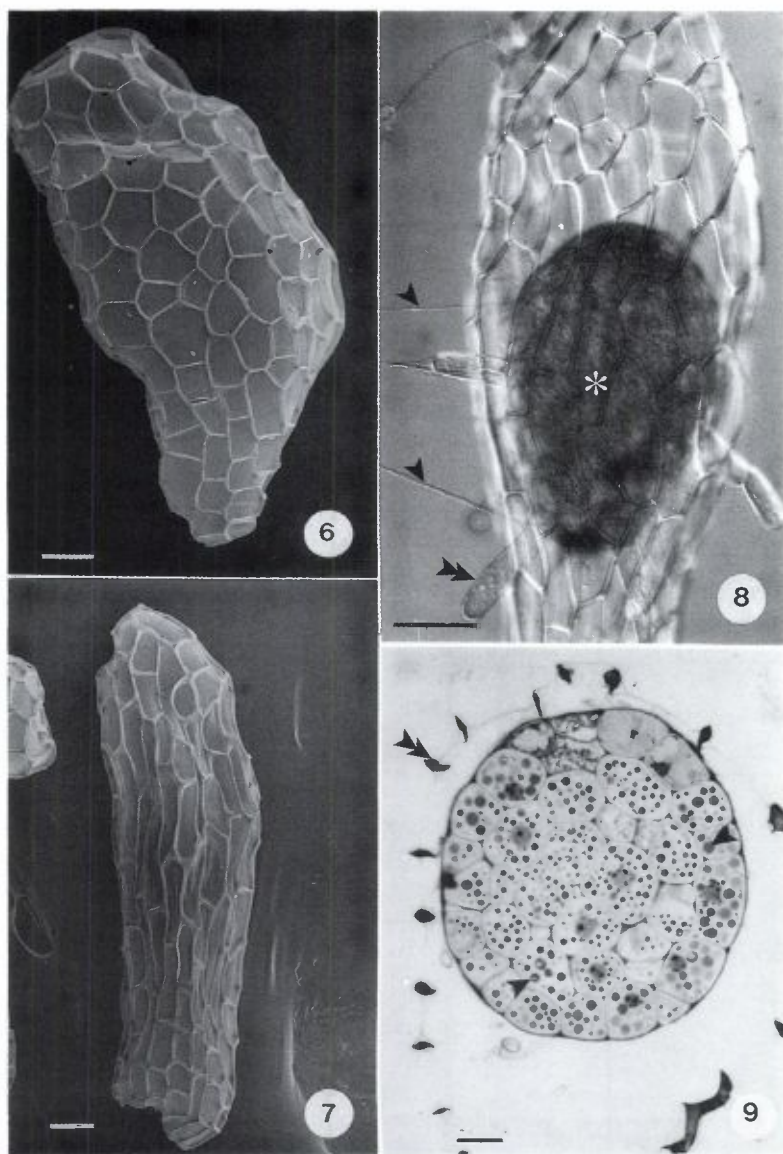
Figures 2-5. Cultures of symbiotic fungal isolates.

Figure 2. Mycelium of an isolate of *Ceratobasidium cornigerum* (anastomosis group C) as it appears on potato dextrose agar medium (PDA). Bar = 1.0 cm.

Figure 3. Mycelium of an isolate of *Epulorhiza* grown on PDA medium. Bar = 1.0 cm.

Figure 4. Hyphae (arrowhead) and monilioid cells (double arrowhead) of *Epulorhiza* viewed with differential interference contrast microscopy. Bar = 15 μ m.

Figure 5. Monilioid cells of *Ceratorhiza* stained with acridine orange and viewed with laser scanning confocal microscopy. The binucleate nature of these cells is evident (arrowheads). Bar = 15 μ m.



Figures 6–9. Bars = 25 μ m.

Figure 6. SEM of testa surface of *Listera australis*.

Figure 7. SEM of testa surface of *Platanthera hyperborea*.

Figure 8. Seed of *Platanthera hyperborea* showing embryo (*), epidermal hairs (double arrowhead) and fungal hyphae (arrowheads).

Figure 9. Transverse section through embryo of *Platanthera hyperborea*. Embryo cells have numerous protein bodies (arrowheads). Portions of the testa (double arrowheads) are evident.

many *Ceratorhiza* isolates produce both cellulases and polyphenol oxidases (Zelmer et al., 1997). Species of *Ceratorhiza*, *Moniliopsis*, and *Epulorhiza* can be isolated from soil and plant debris. Some strains of these fungi are also known as plant pathogens (Saksena and Vaartaja, 1961). It is unknown if these pathogenic strains can function as orchid mycorrhizal symbionts in nature. Seed germination using strains isolated from diseased non-orchid plants have proven useful in the *in vitro* germination and development of some terrestrial orchids (Smreciu and Currah, 1989; Masuhara et al., 1993). Other orchid symbionts, such as *Armillaria* (Terashita and Chuman, 1987) can be both pathogens of trees and wood degraders. *Erythromyces*, associated with the achlorophyllous orchid *Erythrorchis*, is also a wood decay fungus (Umata, 1995).

There is considerable scope for future research into the ecological significance of the various orchid-fungal associations that have been described.

Seed germination

Orchid seeds vary in size but in most species measure less than 2 mm along their longest axis and a fraction of a mm in width (Arditti, 1979). The lipophilic testa surface is generally sculptured (Figs. 6 and 7), (Arditti, 1979), a feature that may aid in the trapping of air bubbles for water dispersal of some species (Rasmussen, 1995). The bulk of the seed is airspace with the rudimentary embryo representing a small fraction of the overall seed volume (Fig. 8). The presence of air trapped between the testa and embryo provides seeds of some terrestrial orchid species with considerable buoyancy in water and also may aid in dispersal by wind.

Mature seeds (Fig. 8) of the majority of orchid species have a simple embryo consisting of parenchyma cells filled with lipid and protein bodies (Fig. 9) (Harrison, 1977; Manning and van Staden, 1987; Rasmussen, 1990; Richardson et al., 1992). The deposition of two or more discrete storage substances in seeds of a specific species is a common phenomenon among angiosperms (Bewley and Black, 1994). The protein bodies may contain globoid inclusions (Manning and van Staden, 1987; Rasmussen, 1990) that have the characteristics of phytin based on energy dispersive X-ray analysis (Richardson et al., 1992). This is important in that even in these very small seeds, there is a store of various minerals that can be utilized during seed germination (Lott et al., 1995). Plastids may contain small amounts of starch (Harrison, 1977) but this reserve substance is usually of minor importance in species examined to date (Rasmussen, 1995); *Calypso bulbosa* (L.) Oakes, however, may contain considerable starch in the mature embryo (Yeung and Law, 1992). Other

cellular organelles are present in mature, desiccated embryos but these are often difficult to demonstrate by microscopy because of the large amounts of lipid and protein present (Richardson et al., 1992).

Embryos may vary in size and complexity from species to species but none differentiates a shoot apical meristem or root apical meristem by the time seed maturation occurs (Veyret, 1974). In a few species, a "cotyledon-like" structure may form at the chalazal end of the embryo; this structure may either develop into a leaf-like organ or remain as a small protrusion (Nishimura, 1991), but nutritive tissue such as endosperm or perisperm is lacking. Storage reserves are, therefore, confined to the embryo and because of the minute size of this structure, exogenous sources of energy are required for seedling establishment.

Asymbiotic seed germination

Orchid seeds can be induced to germinate in a number of ways (Fig. 10) and considerable effort has gone into developing *in vitro*, asymbiotic methods for the commercial production of many species (Arditti, 1992; Rasmussen, 1995). This type of germination will be discussed only as a comparison to *in vitro* and *in situ* symbiotic germination when the information gained from this approach is relevant to the main thrust of the review. A thorough discussion of factors affecting germination and seedling establishment can be found in Arditti and Ernst (1984) and among these simple sugars are of major importance for asymbiotic germination.

As is true for seeds in general (Bewley and Black, 1994), imbibition of orchid seeds triggers a series of physiological processes resulting in non-reversible changes in the storage reserves and cellular organelles in the embryo. Some of these changes have been monitored in both asymbiotically and symbiotically-germinated seeds. In seeds of *Cattleya aurantica* (Batem. ex Lindl.) P.N. Don germinated asymbiotically, Harrison (1977) showed that within the first five days of germination, protein bodies became less dense and some seemed to fuse, mitochondria became evident, but lipid bodies did not change in appearance or quantity. Glyoxysomes, identified by structure only and not by biochemical methods, were not evident at any stage of seed germination up to 15 days post-imbibition and lipid reserves did not change over 1-2 months of culture. In several South African orchid species with lipids and proteins as the major storage reserves, protein breakdown was rapid after imbibition but lipids were not mobilized unless simple sugars were included in the culture medium (Manning and van Staden, 1987). Correlated with this, it was observed that glyoxysomes appeared in embryo cells as early as four days post-imbibition on medium containing sugars but even after 30 days on medium without sugars, they were not apparent. Subsequent to the hydrolysis of proteins and lipids,

SYMBIOTIC		ASYMBIOTIC
<i>in situ</i>	<i>in vitro</i>	<i>in vitro</i>
Seed 'packets' in soil	Medium with complex polysaccharide and mineral nutrients; seeds and fungus	Medium with nutrients and simple sugars; seeds
↓	↓	↓
Colonization of embryos by compatible indigenous fungal species	Colonization of embryos by compatible fungus	Growth of embryos
⇒	↓ Protocorms ↓ Seedlings	⇐

Figure 10. Methods of orchid seed germination.

plastids in embryo cells stored considerable starch (Manning and van Staden, 1987). During symbiotic germination of *Spiranthes sinensis* (Pers.) Ames seeds, however, glyoxysomes were detected at all stages in embryos of seeds germinated on medium containing oat powder as a complex carbon source (Uetake et al., 1993), and many of these showed a positive reaction for malate synthase activity, a key enzyme in the glyoxylate cycle in fatty seeds (Bewley and Black, 1994). There appears to be some discrepancy, therefore, concerning whether or not glyoxysomes are present in mature orchid seeds and whether lipids can be metabolized without the presence of an exogenous source of sugars. Protocorms and young seedlings of *Cattleya aurantica* grown without exogenous sugars were unable to convert acetate to sugars as shown by labelling experiments (Harrison and Arditti, 1978). Since so few orchid species have been studied with respect to mobilization of storage reserves it is difficult to come to a definitive conclusion at this time. What is clear is that without an exogenous supply of simple sugars imbibed seeds germinated asymbiotically do

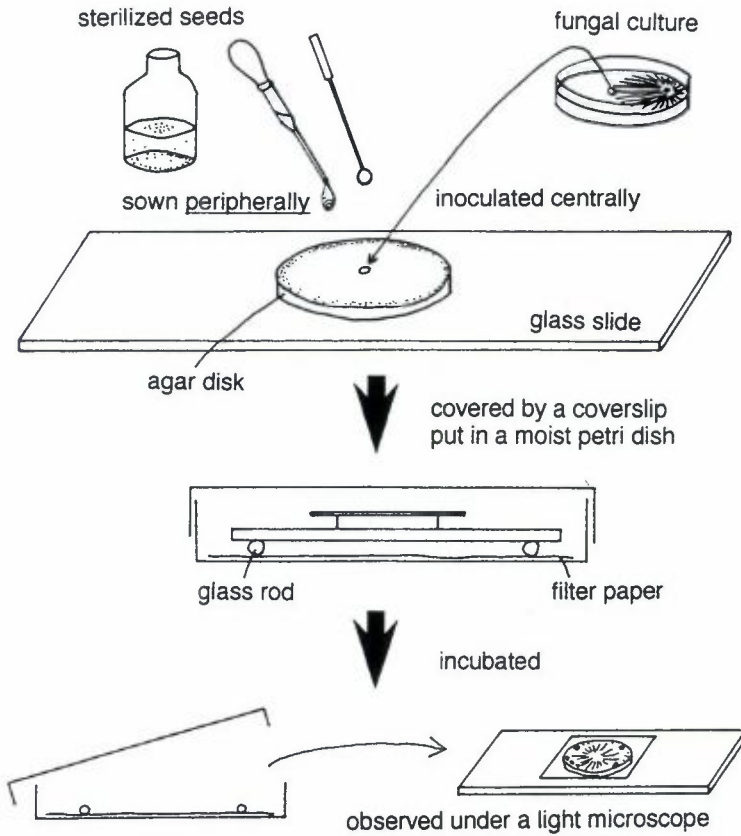


Figure 11. Slide method for symbiotic seed germination.

not show further growth. Manning and van Staden (1987) have suggested that one of the primary roles of symbiotic fungi during early stages of seed germination under natural conditions may be to provide simple sugars for induction of the process of gluconeogenesis; this idea is worth exploring further.

Symbiotic germination

Although methods have now been developed to follow the germination of orchid seeds *in situ* by the use of seed packets buried in the soil (Fig. 10) (Masuhara and Katsuya, 1994; Rasmussen, 1995; Zelmer et al., 1997; Zelmer and Currah, 1997), most of the information available to date on seed-fungal interactions comes from *in vitro* methods (Fig. 10). To obtain colonization of

seeds in symbiotic germination methods, sterilized seeds are imbibed on agar medium to which a complex polysaccharide is included as a source of carbon. Various substrates including starch, cellulose (Warcup, 1973) and powdered oats (Uetake et al., 1992) have been used. Inoculum of a compatible fungal strain is added either at the time of initial imbibition or shortly thereafter and as fungal hyphae grow they hydrolyze the complex polysaccharide and subsequent to colonization of the embryo, transport simple sugars to the developing protocorm. Because of the small size of orchid seeds hundreds can be sown on the surface of medium within Petri plates or on microscope slides (Williamson and Hadley, 1970; Uetake et al., 1992). The latter method, illustrated in Fig. 11, is particularly useful to monitor the growth of imbibed seeds (Uetake et al., 1992), the penetration of epidermal hairs (Williamson and Hadley, 1970) and for obtaining stages in the colonization process for cytological studies (Uetake et al., 1997).

There are few observations on the entry of fungal hyphae into orchid seeds. Clements (1988) observed that micropores and gaps between the plates of the integument are sites of fungal ingress, although colonization via the open end of the testa is also probable. Once within the testa, fungal hyphae usually enter the embryo through the suspensor (Burgeff, 1959; Clements, 1988; Peterson and Currah, 1990; Richardson et al., 1992) and from there grow into living cells of the embryo. Fungal hyphae could potentially enter seeds before any imbibition-induced changes have occurred in embryo cells and therefore substances produced by these hyphae could influence early changes in the embryo (Hadley, 1982; Rasmussen, 1990). In *Dactylorhiza purpurella* (T. and T.A. Steph.) Soo (Williamson and Hadley, 1970; Hadley and Williamson, 1971), *Dactylorhiza majalis* (Rchb. f.) Hunt & Summerh. (Rasmussen, 1990), and some *Platanthera* and *Coeloglossum* species, hyphae enter hairs (sometimes called rhizoids) that develop from epidermal cells of developing protocorms. In *D. majalis*, hyphae were observed also in the suspensor end of the embryo but were inhibited from further development presumably by the presence of tannins in these cells (Rasmussen, 1990). Careful observations over time showed clearly that epidermal hairs of *D. purpurella* protocorms are the sites of hyphal entry, with several hairs frequently invaded simultaneously (Williamson and Hadley, 1970). Hyphae swelled slightly on contact with a hair, formed a narrow penetration peg that penetrated the cell wall and then grew, sometimes with branching, towards the base of the hair. Cytoplasmic streaming continued in the hair as the intracellular hypha developed. These authors also observed that hyphae within colonized protocorms could enter the base of an epidermal hair and exit the hair at some distance along the length of the hair; this has been observed for *Pterostylis* species as well (Clements, 1988). Recently, it has been shown that actin filaments are retained in

epidermal hairs of *Dactylorhiza aristata* (Fisch) Soó and *Spiranthes sinensis* that had been invaded by hyphae of *Ceratobasidium cornigerum* (Bourdot) Rogers (Uetake and Peterson, 1997). This observation combined with the observation that cytoplasmic streaming is retained may indicate that compatible fungal species do not elicit defence reactions during early stages of colonization; this needs further research. There is no information either on the effects of intracellular hyphae on other cytoplasmic structures and organelles in protocorm epidermal hairs or whether these hyphae are separated from the cytoplasm by a membrane.

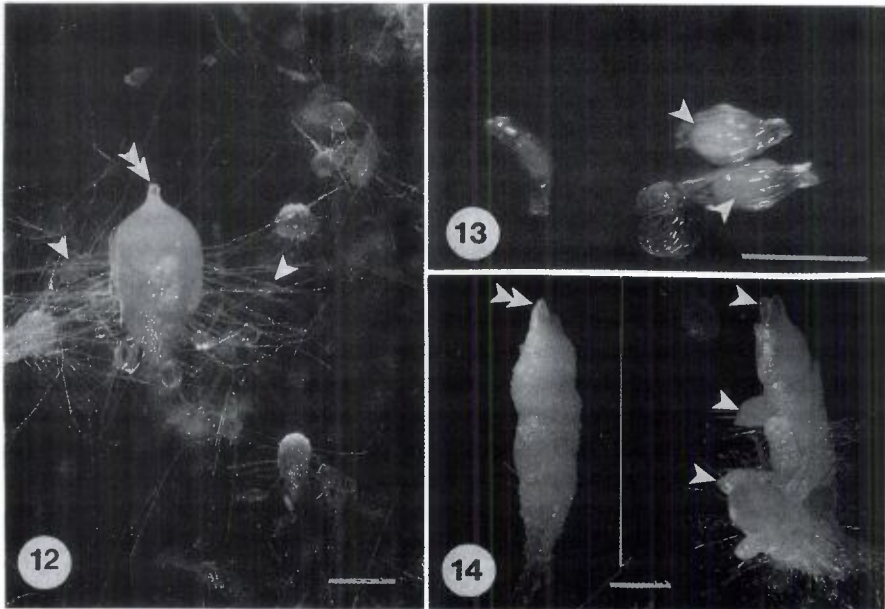
Colonization of embryo/protocorm cells

The dramatic effect that a symbiotic fungus has on embryo development into a protocorm is obvious when comparisons are made of *S. sinensis* protocorms on oat agar medium in the presence of *C. cornigerum* (Fig. 12), oat agar medium without *C. cornigerum* (Fig. 13) and medium with sucrose (Fig. 14).

During symbiotic germination, parenchyma cells of the embryo are penetrated by hyphae originating either from the suspensor cells (Fig. 15), the base of an epidermal hair, or from adjacent colonized cells during later stages of development. A single hypha normally enters a cell and from this point major changes occur in both the hypha and the invaded orchid cell, indicating that there is a close interaction between the symbionts during the establishment of the mycorrhizal association. Not all cells of the embryo/protocorm are colonized; small cells at the chalazal end of the embryo from which a shoot apical meristem becomes established are not invaded by hyphae (Fig. 16). Hyphae in subepidermal cells may be the source of hyphae for reinvasion of central parenchyma cells.

Arditti et al. (1990) have suggested that the fungus may trigger germination either by producing elicitors or by carrying plasmids with genes needed for germination events. It is known that the presence of fungal hyphae does elicit phytoalexin production in protocorms of *Orchis morio* L. (Beyrle et al., 1995).

Hyphae of compatible fungal species undergo rapid growth within parenchyma cells to form a coiled structure called a peloton (Figs. 17 and 18). Hyphae may show branching and anastomoses in these cells (Hadley et al., 1971) and when fully developed may occupy most of the cell volume (Fig. 18). The coiling and branching provides a large shared surface area between the symbionts (Hadley, 1982). The cell wall of hyphae forming pelotons is generally thin (Uetake et al., 1992) and may appear as a two-layered structure (Hadley et al., 1971). The latter authors also observed protuberances on the outer surface of intracellular hyphae which were in contact with host plasma membrane; these structures have not been reported in other ultrastructural



Figures 12–14. Protocorms of *Spiranthes sinensis* grown *in vitro* on agar medium.

- Figure 12. Oat agar inoculated with an isolate of *Ceratohriza*. Protocorms of various size at 72 days are evident. The large protocorm has developed numerous epidermal hairs (arrowheads) and a young shoot (double arrowhead). Bar = 1.0mm.
- Figure 13. Oat agar without fungal inoculum. Embryos (arrowheads) have enlarged in some seeds but are still enclosed in the testa after 1 year on medium. Bar = 0.5 mm
- Figure 14. Medium containing sucrose without fungal inoculum. Protocorms of various shapes have formed in this 1 year old culture. The protocorm on the left had a single shoot apex (double arrowhead) and few epidermal hairs, whereas the protocorm on the right developed multiple shoot apices (arrowheads) and many epidermal hairs. Bar = 1.0 mm.

studies. The cytoplasm contains many mitochondria, ribosomes, and other subcellular structures (Hadley et al., 1971), and consequently these hyphae appear electron dense when viewed by electron microscopy (Peterson and Currah, 1990; Uetake et al., 1992) and densely stained with general stains used for light microscopy (Hadley et al., 1971; Peterson and Currah, 1990; Uetake et al., 1992). Often there is a marked increase in diameter of portions of the peloton hyphae and these regions may accumulate proteins (Rasmussen, 1990). It is common in sectioned protocorms to observe cells in various stages of peloton formation and degeneration. Also, within a protocorm cell, portions of the

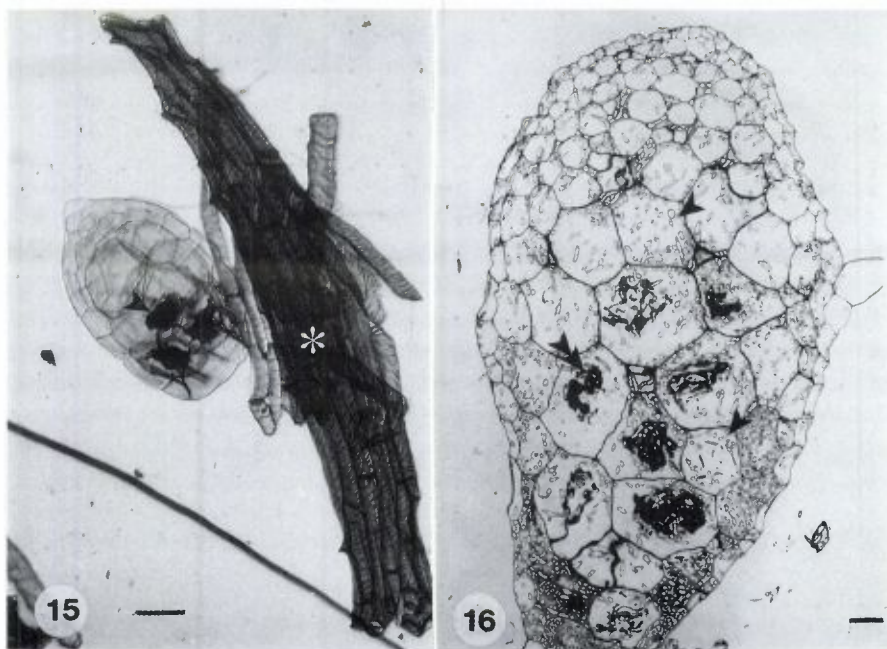
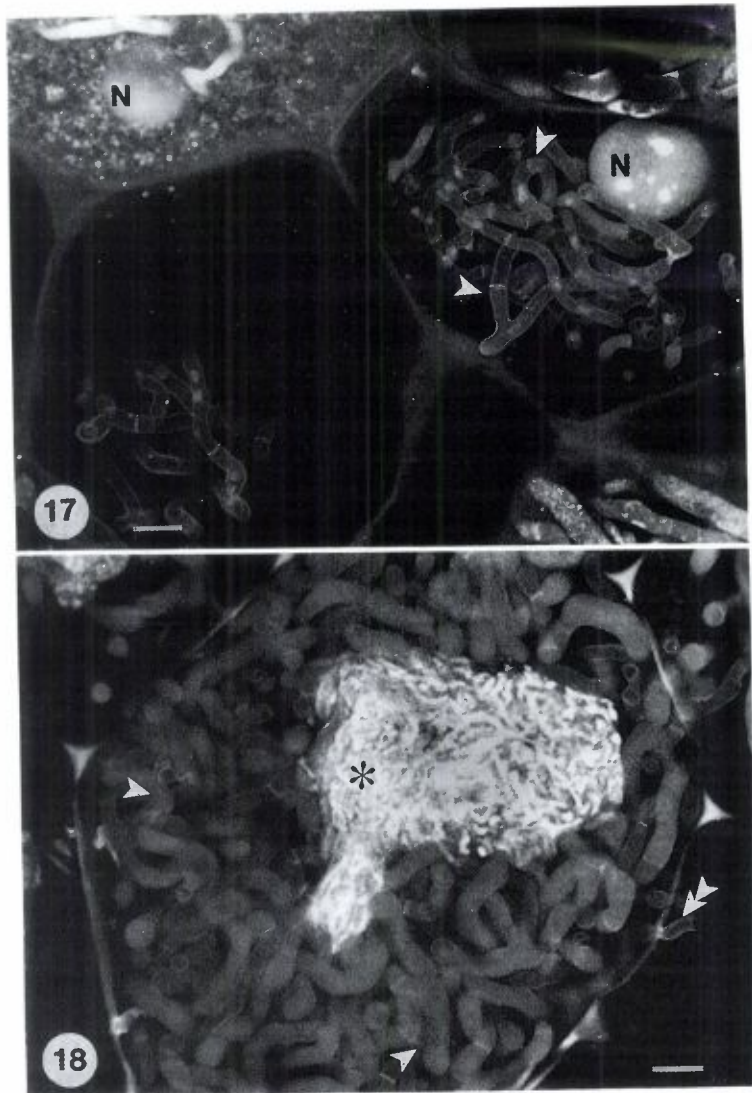


Figure 15. Embryo of *Amerorchis rotundifolia* colonized at the suspensor end by symbiotic fungal hyphae (arrowhead). Remains of the testa (*) are evident. Bar = 25 μ m.

Figure 16. Longitudinal section of developing protocorm of *Goodyera repens* colonized by *Ceratobasidium cereale*. Cells containing sectioned pelotons (arrowheads) and degraded pelotons (double arrowheads) are evident. The extensive development of fungal hyphae towards the chalazal end of the protocorm indicates that the association is probably becoming parasitic. Bar = 50 μ m.

peloton hypha may appear viable while other portions are vacuolated and undergoing collapse (Rasmussen, 1990; Peterson and Currah, 1990). Eventually, the entire peloton degenerates and a mass of hyphal cell wall material interspersed with remains of fungal cytoplasm and protocorm cell-derived substances remains in the cell (Figs. 16 and 18) (Hadley, 1982; Uetake et al., 1992). It is not clear whether the breakdown of the peloton is a result of autolysis or activation of hydrolytic enzymes within the protocorm cell (Williamson, 1973). It is common for protocorm cells with a mass of degenerated hyphal material to be reinvaded by hyphae from neighbouring cells; this is followed by the formation of a secondary peloton (Fig. 18) (Peterson and Currah, 1990; Uetake et al., 1992).



Figures 17–18. Laser scanning confocal microscopy of *Spiranthes sinensis* protocorm cells colonized by an isolate of *Ceratobasidium cornigerum*. Scale bars = 10 μm .

Figure 17. Early stages of peloton formation of acridine orange-stained hand section of protocorm. Hyphae (arrowheads) have branched and coiled. Protocorm cell nuclei (N) are evident.

Figure 18. Protocorm cell with collapsed hyphal mass (*) from an initial colonization. A peloton (arrowheads) from a secondary colonization has filled the protocorm cell. The penetration site for the secondary colonization is still evident (double arrowhead). Tissue unfixed, hand-sectioned and viewed unstained.

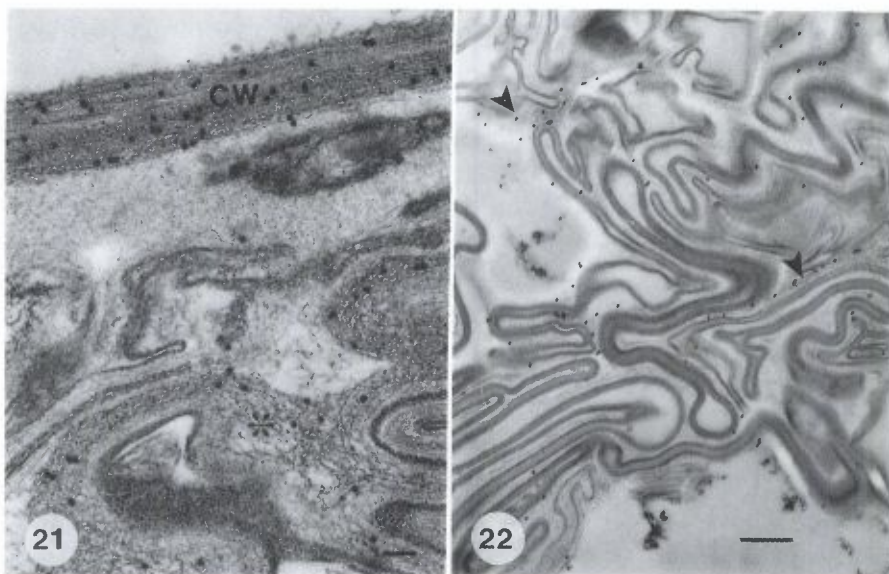


Figures 19–22. Transmission electron microscopy (TEM) of intact hyphae or degenerated hyphal masses.

Figure 19. Conventional TEM showing a fungal hypha (H) surrounded by a perifungal membrane (arrowheads). A portion of a collapsed hypha (*) and host cell vacuole (V) are evident. *Spiranthes sinensis* protocorm colonized with *Ceratobasidium cornigerum*. Scale bar = 1.0 μ m.

Figure 20. Hyphae (H) of *Ceratobasidium cornigerum* within protocorm cells of *Spiranthes sinensis*. Labelling of hyphal cell walls (arrowheads) with polyclonal antibodies to localize β -1, 3 glucans is evident. Scale bar = 1.0 μ m.

Pelotons and degenerated hyphal masses are separated from the host cytoplasm by a membrane (referred to here as a perifungal membrane) (Fig. 19) and interfacial matrix material (Hadley, 1982; Peterson and Currah, 1990; Richardson et al., 1992; Uetake et al., 1992; Peterson et al., 1996). Little is known concerning the physiological characteristics of this membrane but recently Uetake and Ishizaka (1996) have shown that in *S. sinensis* protocorm cells colonized by *C. cornigerum* this membrane around peloton hyphae has some staining features in common with the peripheral plasma membrane in these cells indicating their relationship. However, unlike the peripheral plasma membrane that shows high adenylate cyclase activity, the perifungal

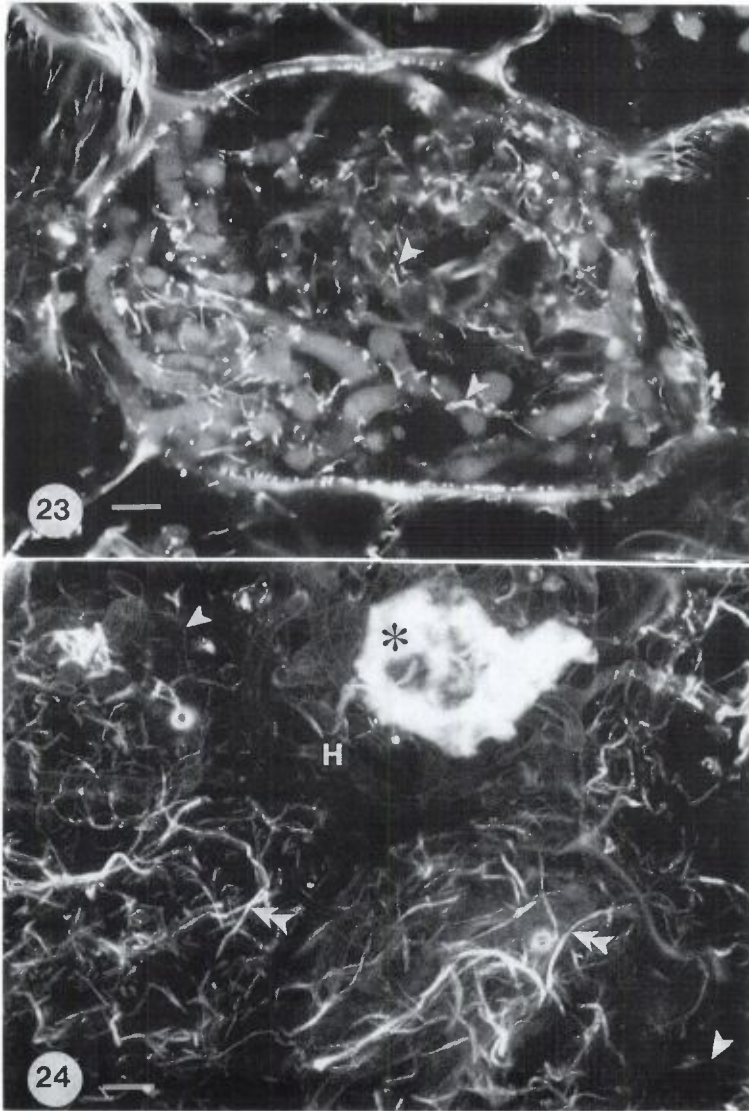


Figures 19–22. Transmission electron microscopy (TEM) of intact hyphae or degenerated hyphal masses.

Figure 21. Portion of protocorm cell of *Platanthera hyperborea* colonized by an isolate of *Ceratorhiza goodyera-repens* treated with cellobiohydrolase-I complexed to colloidal gold as a probe for cellulose. The protocorm cell wall (cw) as well as the interfacial matrix material (*) surrounding degenerated hyphae have labelled. Scale bar = 0.1 μm .

Figure 22. Same material as in Fig. 20 labelled with JIM-5 monoclonal antibodies conjugated to colloidal gold as a probe for non-esterified epitopes of pectin. The interfacial matrix (arrowheads) around collapsed hyphae has labelled. Scale bar = 1.0 μm .

membrane lacks activity of this enzyme. The authors conclude that this shows that there are some qualitative differences between the two membranes. Studies similar to that on intracellular hyphal structures in arbuscular mycorrhizae in which activity of ATPase and other enzymes has been demonstrated (Smith and Smith, 1990) are needed to clarify the possible physiological processes that may be occurring along this extensive membrane interface between the symbionts. The nature of the interfacial region between the perifungal membrane and the cell wall of intact peloton hyphae is also of importance in that substances being exchanged between the symbionts would have to move through this region. The most thorough study of this region (Peterson et al., 1996) has used various affinity techniques that had been used previously to characterize the interfacial matrix between arbuscules and root



Figures 23–24. Laser scanning confocal microscopy of *Spiranthes sinensis* protocorm cells colonized by an isolate of *Ceratobasidium cornigerum*. Scale bars = 10 μ m.

Figure 23. Protocorm cell treated with antibodies to β -tubulin and subsequently with secondary antibodies conjugated to CY3 fluorochrome. Microtubules (arrowheads) are closely associated with the peloton hypha.

Figure 24. Protocorm cells treated with phalloidin conjugated to rhodamine fluorochrome to localize actin. Actin filaments (arrowheads) and cables (double arrowheads) are present in colonized cells. H: fungal hyphae; *: collapsed hyphal mass.

cell cytoplasm in arbuscular mycorrhizae and hyphal coils and epidermal cell cytoplasm in ericoid mycorrhizae (see Bonfante and Perotto, 1995). Probes used for β -1,3 glucans, cellulose and pectins showed that the interface surrounding intact peloton hyphae did not contain these wall components (Peterson et al., 1996). The wall of peloton hyphae gave a positive reaction for β -1,3 glucans (Fig. 20). All of these compounds are present in the interface surrounding arbuscular branches in arbuscular mycorrhizae (Bonfante and Perotto, 1995) but are absent in the interface surrounding hyphal coils in ericoid mycorrhizae (Perotto et al., 1995). Interestingly, the interfacial matrix surrounding collapsing hyphae and degenerated hyphal masses in orchid protocorm cells, gave positive labelling with the same probes indicating the presence of β -1,3 glucans, cellulose (Fig. 21) and pectins (Fig. 22) (Peterson et al., 1996). These authors suggest that viable peloton hyphae may either utilize these cell wall components as they are synthesized or prevent the synthesis of these substances. As hyphae degenerate the interfacial matrix substances may be important to isolate hydrolytic enzymes from the protocorm cell cytoplasm (Richardson et al., 1992; Peterson et al., 1996).

Effects of fungal colonization on protocorm cells

Cytoskeleton

The cytoskeletal system of plant cells, comprised primarily of microtubules (MTs) and microfilaments (MFs), plays a major role in such basic processes as mitosis, cytokinesis, cell wall synthesis and movement of cellular organelles (Lloyd, 1991). Numerous studies have shown that this system is sensitive to various abiotic factors (Lloyd, 1991). Recently, several studies have focused on the effect of pathogenic fungi (see Kobayashi et al., 1995) and mycorrhizal fungi on both MTs and MFs. In ectomycorrhizae, Timonen et al. (1993) showed that colonization of pine roots by ectomycorrhizal fungi led to the disappearance of both MTs and MFs in cells adjacent to fungal hyphae. Similarly, Dearnaley and McGee (1996) showed that colonization of protocorms of the orchid species *Microtis parviflora* R. Br. by a symbiotic fungus resulted in a loss of all MTs in colonized protocorm cells. In contrast, Uetake et al. (1997) reported that although there were alterations in MTs in protocorm cells of the orchid *S. sinensis* colonized by the compatible fungal species, *C. cornigerum*, some were always retained. The population of MTs in the cortical region of protocorm cells disappeared following fungal invasion and peloton formation, but MTs were always closely associated with peloton hyphae and with the protocorm cell nucleus (Fig. 23). A population of MTs was always associated with collapsing hyphae and hyphal masses and cortical MTs reappeared as the peloton underwent degeneration. These authors suggest

that the MTs closely associated with viable peloton hyphae may be involved in some way with the perifungal membrane while those associated with degenerating hyphae and hyphal masses may be necessary for the synthesis of the interfacial matrix material known to be present during these stages of protocorm cell colonization (Peterson et al., 1996).

Microtubules are also retained during the colonization of root cortical cells of transgenic tobacco plants by arbuscular mycorrhizal fungi although, as in orchid protocorm cells, a change in organization followed fungal penetration; microtubules were closely associated with intracellular hyphae and with arbuscular branches (Bonfante et al., 1996). In this study there was a specific up-regulation of one α -tubulin gene in root cells containing arbuscules.

There has been less work on alterations in MFs during colonization of plant cells by mycorrhizal fungi. Using the same system described above (*S. sinensis* protocorms colonized by *C. cornigerum*), Uetake and Peterson (1997) have shown that MFs are retained during all stages of protocorm cell colonization but that they are not as closely associated with the fungus as the MTs (Fig. 24). These authors suggest that the change in pattern of MF arrays compared to uncolonized cells might reflect the reorganization of the protocorm cell cytoplasm as a consequence of fungal invasion and peloton formation in that the large central vacuole and peripheral cytoplasm typical of uncolonized cells are altered. As the peloton develops, cytoplasm surrounds the hyphae and many small vacuoles appear.

The retention of both MTs and MFs during colonization of *S. sinensis* protocorm cells by a compatible fungal species suggests that defence responses typical of plant cells invaded by pathogenic fungi i.e. the localization of these components of the cytoskeleton to the site of hyphal ingress (Kobayashi et al., 1995), are not induced. Recent work using inhibitors of tubulin or actin polymerization or depolymerization inhibitors (Kobayashi et al., 1997), has shown clearly that the cytoskeleton plays a significant role in the defence response in barley coleoptiles. It would be of considerable interest to assess the effects of fungal isolates showing differing degrees of aggressiveness (Masuhara and Katsuya, 1994) on the cytoskeleton of protocorm cells.

The orchid protocorm system should also prove useful to monitor the changes in both tubulin and actin polypeptides as a consequence of invasion by symbiotic fungal hyphae for comparison for the recent work on ectomycorrhizae (Timonen et al., 1993; 1996; Martin and Tagu, 1995; Niini et al., 1996) and arbuscular mycorrhizal fungi (Bonfante et al., 1996).

Nuclei

Nuclear hypertrophy has been described as a normal event in orchid embryo

cells following imbibition of mature seeds (Rasmussen, 1990) and in protocorm cells of many orchid species (Rasmussen, 1995). Embryo cells in seeds of *Vanda sanderiana* Reichb. f. germinated asymbiotically, showed increases in DNA content per nucleus as determined by microspectrophotometric measurements of Feulgen-stained material (Alvarez, 1968). An increase to 8C levels in central parenchyma cells of protocorms was common and occasionally in later stages of protocorm development, higher levels were recorded. Williamson and Hadley (1969) and Williamson (1970), also using microspectrophotometry, reported that nuclei of uncolonized *D. purpurella* protocorm cells could reach values of 64C but commonly they were at levels of 16C and lower. Nuclei of colonized cells, however, frequently attained levels as high as 128C. Neither of these studies included controls of known DNA level to standardize readings.

Rasmussen (1990) used nuclear volume as an indication of DNA level per nucleus, assuming a direct correlation between the two. She reported levels in colonized protocorm cells of *D. majalis* similar to those reported for *D. purpurella* (Williamson and Hadley, 1969). In an earlier study, however, Alvarez (1968) found that the relationship between nuclear volume and DNA level only holds for values up to 8C.

Using *S. sinensis* protocorms colonized by *C. cornigerum*, we have determined DNA levels using Feulgen-stained nuclei but including an internal standard of known DNA content/nucleus. An increase in DNA content was observed in colonized inner parenchyma cells compared to uncolonized cells but the levels reached were not as high as reported in previous studies (Punshon, personal communication). Nuclei in cells of the meristematic region of both uncolonized and colonized protocorms remained at 2C–4C levels.

Although Rasmussen (1990) found little structural change other than enlargement in nuclei as protocorm cells became colonized she did note that peloton hyphae often developed close to protocorm cell nuclei, a feature also noted in our studies, and, based on light microscopy, often appeared to pass through them. A detailed ultrastructural study is required to assess this observation and to monitor changes in nuclei and chromatin during nuclear enlargement.

2. Concluding Remarks

Studies of the interactions between symbiotic fungi and germinating orchid seeds are adding to the general understanding of how biotrophic fungal symbionts and higher plant cells are altered structurally during their intimate association to form mycorrhizae. The experimental results obtained with germinating orchid seeds are also providing the basic information needed to

researchers who are attempting to propagate and re-establish endangered orchid species in their natural habitats. Most of the research on fungal-orchid interactions, however, has involved a few terrestrial orchid species grown under laboratory conditions and using *in vitro* methods. Research needs to be expanded to consider epiphytic orchid species and to study orchid symbioses under more natural conditions.

The orchid protocorm-fungal symbiotic system is easy to manipulate experimentally compared to other symbiotic systems and may prove very useful in determining the molecular events that underlie the pronounced structural changes that occur in both symbionts during mycorrhiza formation. The system may also help to clarify the physiological roles played by both symbionts during various stages of the relationship.

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