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DEVELOPMENT OF MYCORRHIZAL ASSOCIATIONS IN *CALADENIA TENTACULATA*

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ABSTRACT. A greater understanding of the orchid-mycorrhizal relationship will assist in the development of improved ex-situ propagation and cultivation methods and appropriate in-situ management techniques for the many threatened Australian terrestrial orchids. The development of mycorrhizal infection in the Australian terrestrial orchid, *Caladenia tentaculata,* was studied by comparing the in-vitro colonization of embryos infected by one of two fungal isolates prepared from collars of the same orchid growing in-situ. One fungal isolate stimulated germination of the orchid seed and supported subsequent growth (the compatible isolate), but the other did not (the incompatible isolate). Inoculated and un-inoculated orchid embryos and protocorms were sampled frequently after propagation, fresh mounted and visualized with ultraviolet (UV) light, fixed and either cleared or resin-embedded for light microscopy and hand sectioned for scanning electron microscopy (SEM). The compatible isolate characteristically infected the seed through the suspensor cells resulting in healthy seedlings (protocorms). This infection was restricted to the basal cells. The meristematic, starch storage and vascular tissues remained uninfected. Two layers of cells containing morphologically distinct fungal coils (pelotons) formed under the epidermal cells during compatible colonization. The pelotons within the inner-most layer were digested and the cells harboring these pelotons were re-infected with new hyphae. Multicellular rhizoids developed from the basal epidermal cells. The incompatible isolate either failed to infect the seed or penetrated the epidermal cells or developing rhizoids. Chance suspensor infection stimulated distorted protocorm growth, although infection was not restricted to basal cells, and the starch storage and meristematic tissues also were colonized. The distribution of the developing rhizoids was irregular unlike that during compatible infection.

Key words: mycorrhizal colonization, orchid, microscopy, *Caladenia,* protocorm infection

INTRODUCTION

Terrestrial orchids rely on mycotrophy for survival especially during seed germination (Zettler 1997) and do not become autotrophic for months or even years after the initiation of germination (Rasmussen 1995). Mature plants lack an extensive root system and have few leaves (Harley & Smith 1983) and are limited in their ability to store and synthesize food reserves. Terrestrial orchid species can experience annual periods of dormancy in which they are exclusively mycotrophic (Warcup 1971, 1990; Jones 1988; Masuhara & Katsuya 1992). This obligatory mycotrophy makes terrestrial orchids vulnerable to extinction (Rasmussen 1995) for their habitat also must support the proliferation of their fungal partner(s). Conservation and reintroduction methods require efficient propagation and cultivation techniques for these species and would be aided greatly by an improved understanding of the mycorrhizal associations of these orchids.

A considerable body of work exists on the infection and subsequent colonization of fungal symbiosis in North American and European terrestrial orchid species (Rasmussen 1990, Zelmer & Currah 1997, Uetake & Peterson 1997). Fungal isolates have been found to enter the orchid tissue either through the suspensor cells before germination (see Curtis 1939 for Bernard 1903 and Burgeff 1909, Mollison 1943, Richardson et al. 1992, Uetake & Peterson 1997) or through the rhizoids after germination (Harvais & Hadley 1967, Williamson & Hadley 1970, Anderson 1991). Rassmussen (1995) suggests that if initial infection occurs through the suspensor cells before germination has commenced, the mycorrhiza facilitates seed germination.

Many studies have found that not all endophytic fungi isolated from terrestrial orchids stimulate germination and growth (Harvais & Hadley 1967, Harvais 1973, Clements 1988, Smreciu & Currah 1989). Incompatible fungi may (1) fail to infect orchid seed that remains

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viable without germinating (Clements 1988); (2) penetrate the epidermis, infecting all cells of the embryo, including the meristem, resulting in death (Clements 1988); (3) parasitize the placental tissues (the suspensor cell), then infect the rhizoids resulting in a compatible association (Beyrle et al. 1991). These studies do not include detailed observations of the microscopic development of the incompatible infection, which would give significant insight into orchid mycorrhizal symbiosis.

The control mechanisms that limit infection by the mycorrhizal symbiont are poorly understood. Orchids may deploy defense mechanisms to restrict mycorrhizal colonization to specialized mycotrophic tissue. Rasmussen (1990) observed tannins within the suspensor cells of *Dactylorhiza majalis* and suggested that they inhibit peloton formation. The phytoalexin orchinol and other related defense compounds accumulate in unsuccessfully inoculated orchid tubers, inhibiting the growth of orchid mycorrhizae.

Little work has been conducted on the development of mycorrhizal associations within Australian orchid taxa. Clements (1988) describes colonization patterns primarily for *Pterostylis* species, although he also describes the specificity of mycorrhizal associations in *Caladenia* (*C. menziesii*) and other genera. *Caladenia* contains the largest number of threatened terrestrial orchid taxa of Australia (Department of the Environment and Heritage 2004) and are notoriously difficult to propagate (Richards et al. 1984). Asymbiotic propagation requires a very precise mix of nutrients to be developed for the genus, with high losses when protocorms are deflasked (R. Thompson pers. comm.). The specific nature of the mycorrhizal fungi to facilitate seed germination creates difficulties for orchid growers.

Caladenia are not colony forming, and reproduction is virtually restricted to seed (Richards et al. 1984), which form obligatory associations with specific mycorrhizae, at least during germination (Warcup 1981, Clements 1988). The successful conservation of *Caladenia* depends on understanding the orchid-mycorrhiza relationship from initial infection, through germination to stable protocorm development. *Caladenia tentaculata* is a relatively common species with a large distribution. Its use in in-vitro trials avoids additional pressures on already threatened species.

We describe the progression of fungal colonization in *Caladenia tentaculata,* following inoculation by either the compatible or incompatible mycorrhizal fungi, with compatible inoculation leading to stable protocorm formation in vitro.

Material and Methods

Caladenia tentaculata seeds (stored in paper bags at 4°C) were inoculated with compatible and incompatible mycorrhizal fungi isolated from naturally growing *C. tentaculata* collars collected near Inverleigh, Victoria, Australia, in November 1999. Inoculated seeds were sampled frequently during a 70-day period and prepared for light microscopy. Protocorms were sampled 28 and 54 days after propagation for scanning electron microscopy (SEM). The sites of infection and subsequent colonization of the embryos were observed. Un-inoculated embryos also were sampled for 14 days and prepared for fluorescent microscopy.

Mycorrhizal Fungi

Two fungal isolates, R1 and R16 were isolated from pelotons from the collar region of mature *Caladenia tentaculata* plants collected from the same population near Inverleigh. *Caladenia tentaculata* seed inoculated with R1 resulted in 100% germination, and the isolate was described as being compatible (R. Raleigh pers. comm.). *Caladenia tentaculata* seed inoculated with R16 resulted in less than 1% germination, and the isolate was described as being incompatible (R. Raleigh pers. comm.). These isolates were incubated at 20°C in the dark on 2.5% oatmeal agar and subcultured every 2 months.

Fungal Inoculation of Seed

Caladenia tentaculata seed was surface-sterilized in 0.5% NaOCl for 3 min., rinsed with sterile deionized water three times and plated on sterile filter paper squares (20 × 20 mm; Whatman No. 1) on 2.5% oatmeal agar. A drop of a suspension of the fungal isolate (R1 or R16), in sterile deionized water, was placed on each of the four sides of the filter paper. Orchid seed also was plated without inoculum as a negative control. The plates were incubated in the dark for the first 21 days in a Conviron-controlled cabinet with a daily cycle of 17 hours at 20°C, 1 hour at 15°C, and 6 hours at 10°C and subsequently with a daily cycle of 17 hours at 20°C, 1 hour at 15°C, and 6 hours in the dark at 10°C.

Light, Electron, and Fluorescent Microscopy

More than 7600 protocorms from each treatment (the un-inoculated control, the R1 inoculated treatment and the R16 inoculated treat-

ment) were observed macroscopically during a period of 70 days. Three embryos were sampled for light microscopy from each treatment each day for the first 14 days after inoculation, and three samples from each treatment at 7-day intervals subsequently, for a total of 70 days. The experiment was repeated twice, although the sampling was restricted to the first 14 days. Five protocorms were sampled at 28 and 54 days after propagation for SEM. Ten embryos sampled daily for 14 days after propagation were prepared for fluorescent microscopy.

Light Microscopy

Three embryos from each treatment were sampled daily for 14 days, fixed overnight in 2.5% glutaraldehyde (in pH 7.4 phosphate buffer) then cleared in 10% KOH at 90°C for 3 hours. These embryos were stained under vacuum with Chlorozol Black E for 15 min. to visualize fungal hyphae within the protocorms. Three embryos from each treatment sampled each day for 14 days and at 7-day intervals for 70 days were then fixed as above and resin-embedded by dehydrating fixed embryos in an ethanol series (5%, 10%, 20% 30%, 50%, 70%, 90%, and 100%). They then were infiltrated with a series of dilutions of LR white resin in 100% ethanol (25%, 50%, 75%, and 100%) and were embedded in gel capsules at 60°C overnight. The specimens were sectioned at 1 μm and stained with warm Toluidine Blue pH 4.4. Iodine was used to identify starch granules. All specimens were observed with an Olympus BH-2 compound microscope, and the images were recorded with an Olympus D P10 digital camera.

Scanning Electron Microscopy

Five protocorms (from the R1 inoculated treatment and the R16 inoculated treatment), sampled at 28 days and 54 days, were cut longitudinally and fixed with 2.5% glutaraldehyde (in 7.4 pH phosphate buffer), then post-fixed in 2% OsO_4 for SEM. They were then dehydrated in an ethanol series (5%, 10%, 20% 30%, 50%, 70%, 90%, and 100%). The specimens were dried in a critical point dryer, using CO_2 as the transition liquid, and mounted on stubs with double-sided sticky tabs, then sputter-coated with gold for visualization with a Phillips XL30 field emission scanning electron microscope.

Fluorescent Microscopy

Ten unfixed embryos from the un-inoculated treatment were sampled daily for 14 days after propagation, mounted in distilled water and visualized using a 365 nm excitation filter (UV light) with an Olympus series BH-2 fluorescent microscope (with an Olympus mercury lamp).

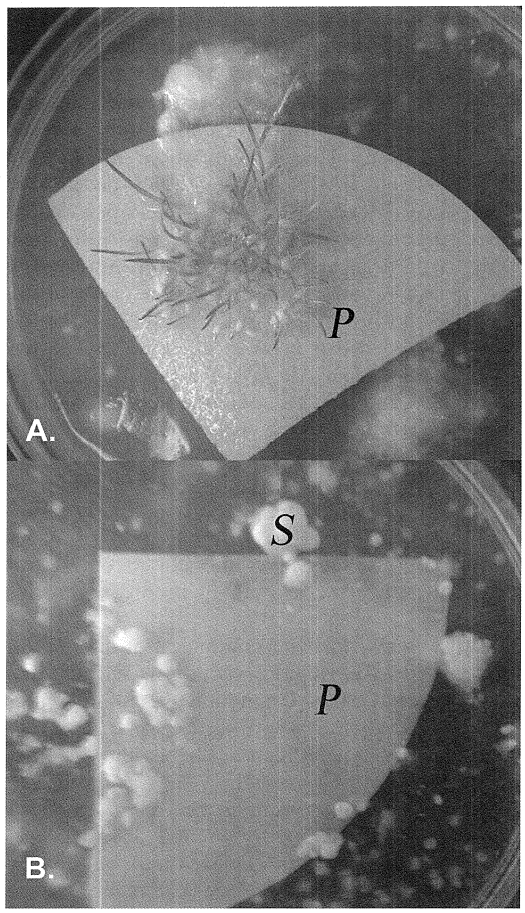

FIGURE 1. *Caladenia tentaculata.* Protocorm (P). Sclerotia (S). **A.** Protocorms 2 months after inoculation with R1 isolate. **B.** Protocorms 2 months after inoculation with R16 isolate.

Images were recorded with a Kodak Ektachrome 400 film using an Olympus C-35AD-4 camera controlled by an Olympus exposure control unit.

RESULTS

Fungal Isolates and Germination

Macroscopic observations revealed that R1 stimulated uniform germination and growth of healthy *Caladenia tentaculata* protocorms produced photosynthetic shoots 28 days after propagation. Healthy protocorms infected with R1, 54 days after propagation, are shown in FIGURE 1A. R16 failed to induce uniform germination, the few resulting protocorms were small, and photosynthetic shoots developed from only 0.02% of embryos (FIGURE 1B). No protocorms formed in the un-inoculated embryos.

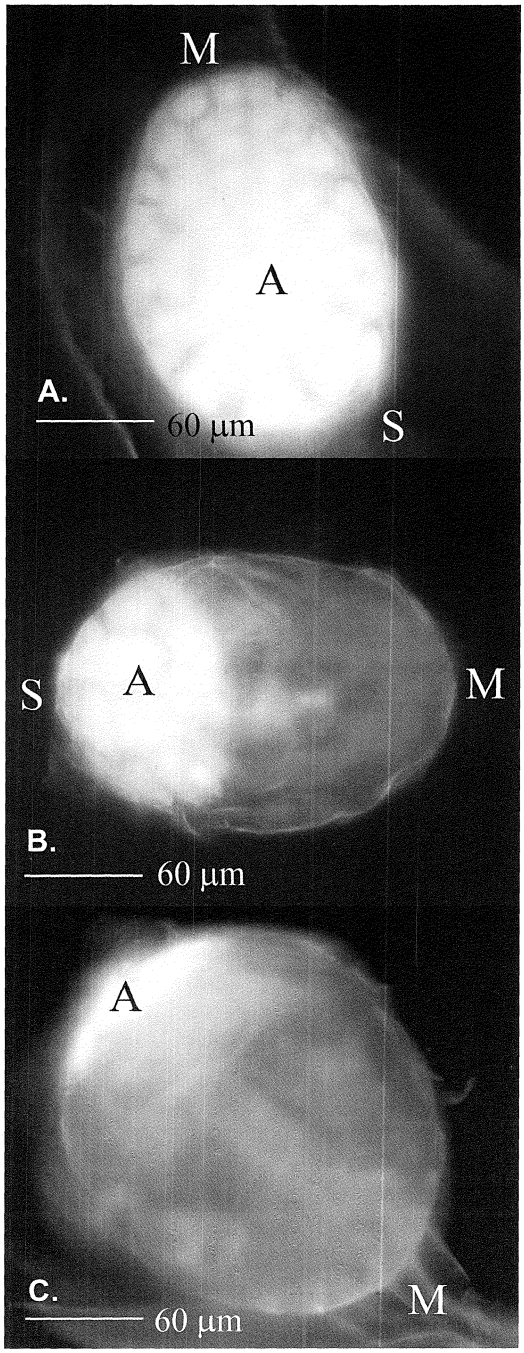

FIGURE 2. *Caladenia tentaculata.* Autofluorescence (A). Meristem (M). Suspensor (S). **A.** Uninoculated embryo 1 day after propagation, visualized with UV light (365 nm). Cells of the whole embryo contain autofluorescent compounds. **B.** Uninoculated embryo 2 days after propagation, visualized with UV light (365 nm). Autofluorescent compounds have contracted towards the suspensor end of the embryo. **C.** Uninoculated embryo 4 days after propagation, visualized with UV light (365 nm). Autofluorscencing compounds remain only in the suspensor cell.

Imbibition and Development of Embryos

UV autofluorescence was observed in the cytoplasm of the entire cells of the pre-imbibed embryos. During imbibition (swelling of seed with water absorption), autofluorescence receded towards the suspensor region (FIGURE 2A, B).At the completion of imbibition, the autofluorescence was observed only in the suspensor cell (FIGURE 2C).

Starch accumulation and meristematic division were observed in sectioned imbibed, inoculated, and un-inoculated embryos. The cortical cells of the embryos infected with R1 (FIGURE 3A) had more starch than the un-inoculated embryos 14 days after propagation (FIGURE 3B). They also were larger and had a more developed meristem. Rhizoids formed from single enlarged epidermal cells, which elongated as early as 6 days after propagation in all treatments (the R1 inoculated treatment, the R16 inoculated treatment, and the un-inoculated treatment). These rhizoids developed into symmetrical multicellular structures in the mature protocorms inoculated with R1. No R16 infection was observed until 35 days after propagation, and the effects on growth and development were variable.

Infection Pathway

Fungal hyphae of R1 were observed clustering at the base of the embryos, penetrating their suspensor cells as early as 5 days after propagation (FIGURE 3C). Hyphae grew straight through to the adjacent cortical cells and formed pelotons in these cells.

In the older infected cortical cells of sectioned embryos, collapsed fungal hyphae were observed in the center of the cell, as remnants of empty hyphal cell wall material (FIGURE 3D). These cortical cells were re-infected and contained healthy as well as collapsed fungal hyphae. R1 hyphae grew through the rhizoids of the protocorms, although the direction of growth could not be distinguished (FIGURE 4A). No peloton formation was observed within the rhizoids. Protocorms resulting from R1 infection were pear-shaped, with prolific multicellular rhizoids emerging from the basal epidermal cells (FIGURE 4B).

Of the R16-inoculated embryos, 67% remained un-infected. In 74% of the infection events observed, penetration occurred through the epidermal cells (FIGURE 4C). This pathway

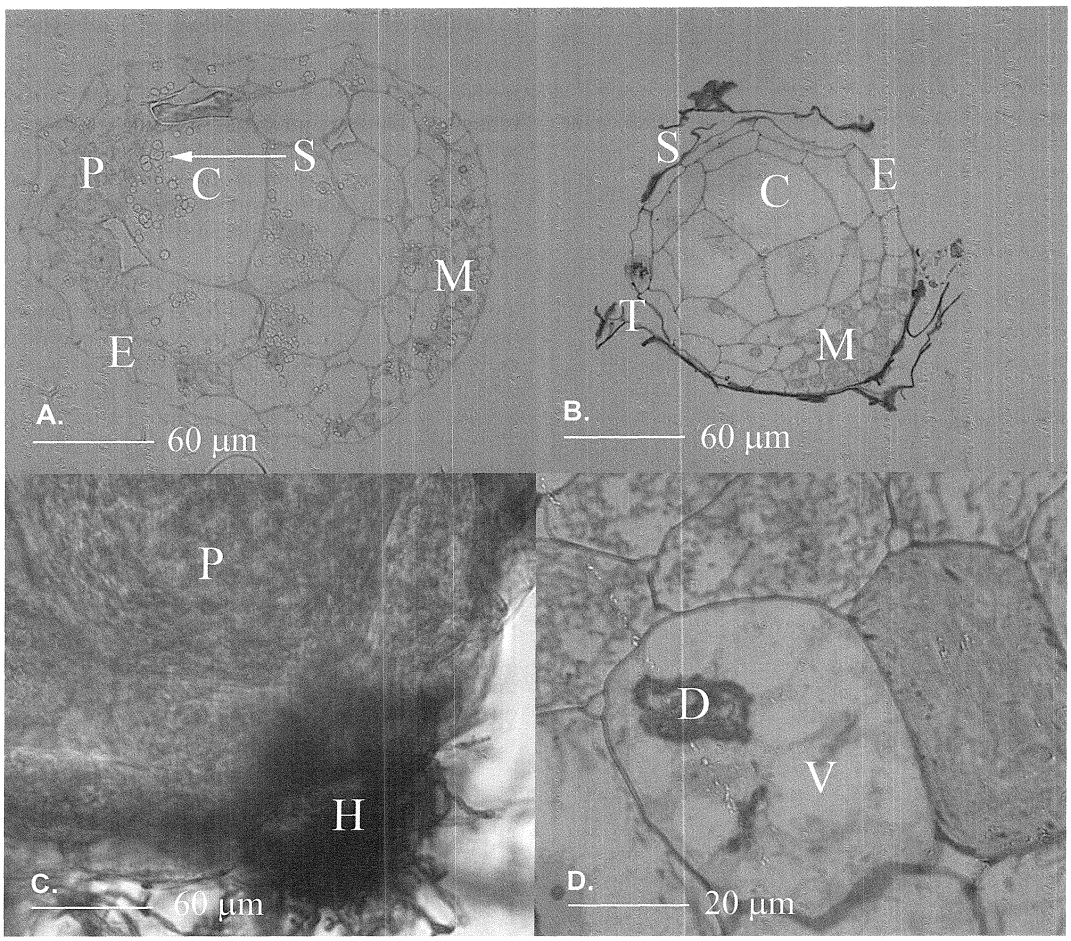

FIGURE 3. *Caladenia tentaculata.* Cortical cells (C). Digested peloton (D). Epidermal cell (E). Hyphae (H). Meristem (M). Pelotons (P). Starch (S). Testa (T). Orchid cell vacuole (V). **A.** Longitudinal section through an embryo infected with R1 isolate 14 days after propagation stained with Toludine blue pH 4.4. **B.** Longitudinal section through an uninoculated embryo 14 days after propagation stained with Toludine blue pH 4.4. **C.** Cleared protocorm 21 days after propagation infected with R1 stained with Chorozol Black. **D.** Transverse section through the basal cortical cells of a protocorm infected with R1 isolate 42 days after propagation stained with Toludine blue pH 4.4.

resulted in extensive colonization and collapse of infected cortical cells (FIGURE 4D). Of the infection events, 23% penetrated through the rhizoid initials (FIGURE 4E). Rhizoid infection resulted in sparse colonization of the adjacent cortical cells with no peloton formation.

In only one embryo infected with R16 was suspensor penetration microscopically observed (FIGURE 4E). Fungal hyphae clustered around the penetration site, as seen with R1 infection. Suspensor infection resulted in protocorm formation. Of the 7600 embryos inoculated with R16, only 20 protocorms developed. Protocorms prepared for SEM varied in shape, and most had reduced meristem formation (FIGURE 4F). Rhizoid formation was not restricted to the basal epidermal cells of these protocorms, and formation of the multicellular rhizoids was not symmetrical. These were termed "intermediate protocorms."

Colonization Patterns

Colonization with R1 was restricted to basal cortical cells of the protocorm. The meristematic, vascular, and storage cells remained uninfected (FIGURE 5A). Starch grains accumulated in the cortical cells located near the vascular bundle (FIGURE 5B). Storage cells at the periphery of the infected region contained limited un-

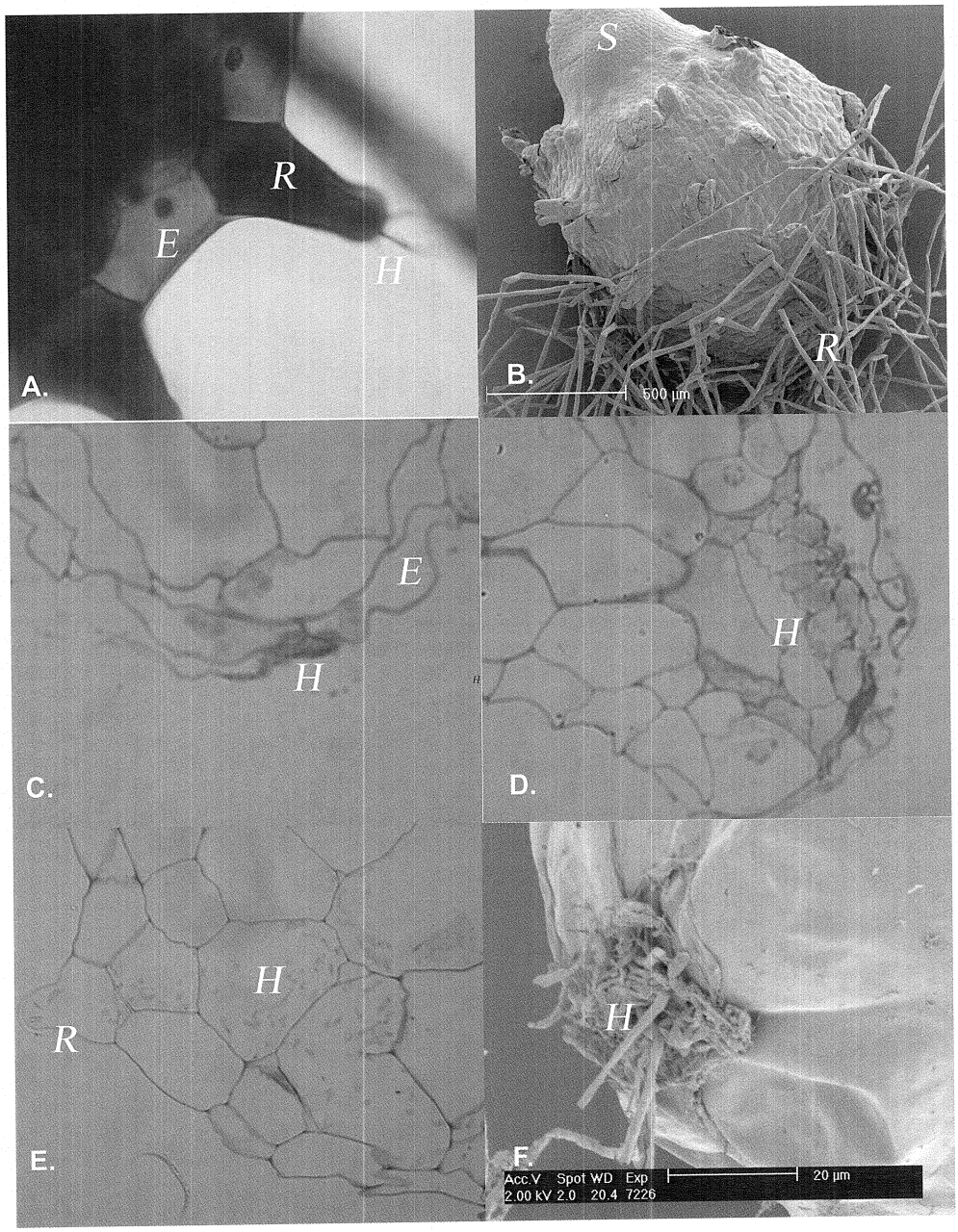

FIGURE 4. *Caladenia tentaculata.* Epidermal cells (E). Hyphae (H). Rhizoid (R). Photosynthetic shoot (S). **A.** Developing rhizoid of a cleared protocorm 14 days after propagation infected with R1 stained with Chorozol Black. Fungal hyphae are shown growing through the cell wall of the rhizoid initial. **B.** Scanning electron micrograph of an R1-infected protocorm 28 days after propagation. **C.** Longitudinal section through an embryo inoculated with R16 isolate, 49 days after propagation stained with Toludine blue pH 4.4. **D.** Longitudinal section through an embryo inoculated with R16 isolate 28 days after propagation stained with Toludine blue pH 4.4, showing extensive colonization and collapse of cortical cells. **E.** Longitudinal section through an embryo inoculated with R16 isolate 21 days after propagation stained with Toludine blue pH 4.4, showing infection through a rhizoid. **F.** Scanning electron micrograph of the basal end of a protocorm shows suspensor infection by R16 isolate 56 days after propagation.

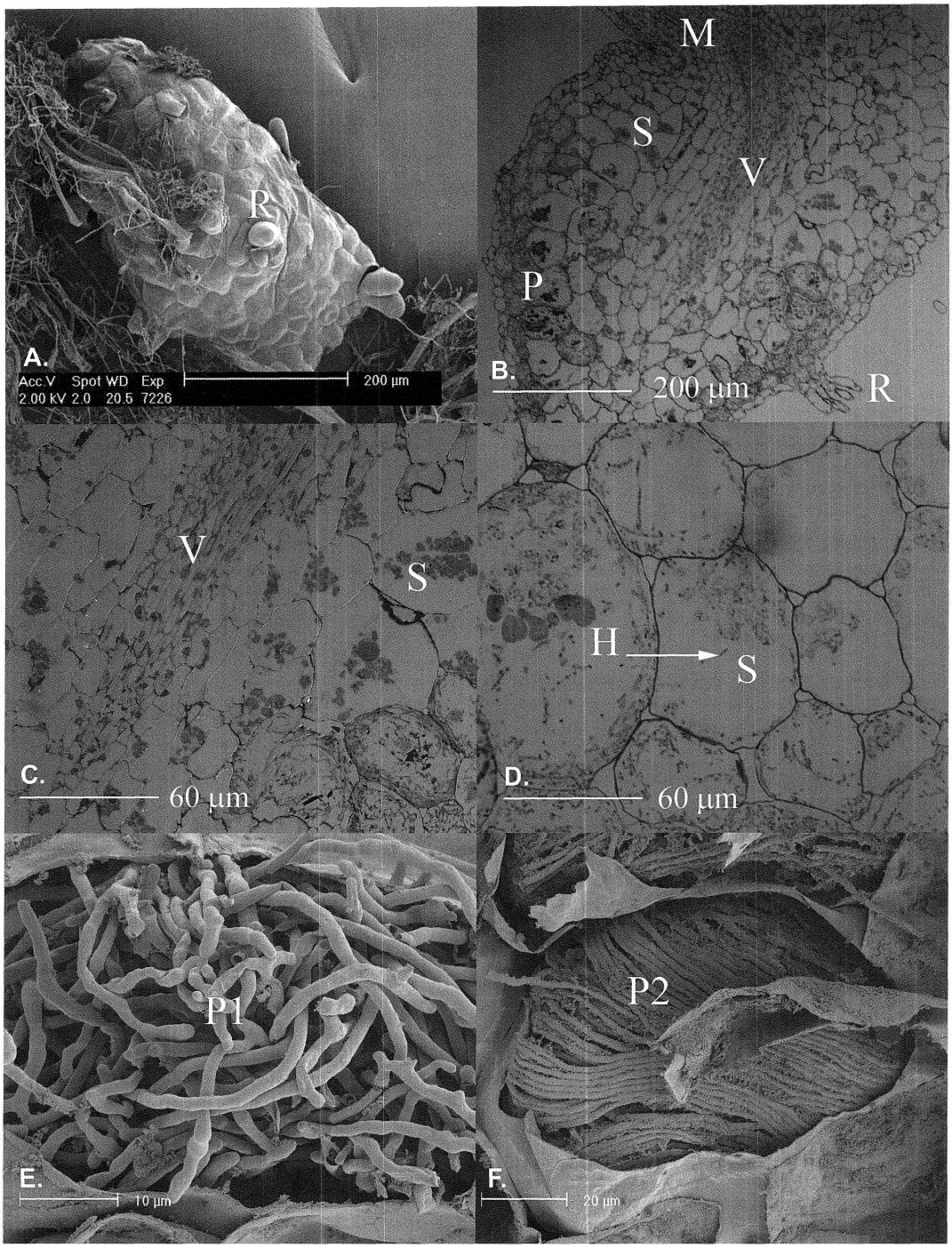

FIGURE 5. *Caladenia tentaculata.* Hyphae (H). Meristem (M). Pelotons (P). Peloton type 1 (P1). Peloton type 2 (P2). Rhizoid (R). Starch storage cells (S). Vascular tissue (V). **A.** Scanning electron micrograph of a protocorm infected with R16 isolate 56 days after propagation. **B.** Longitudinal section through a protocorm infected with R1 isolate 56 days after propagation stained with Toludine blue pH 4.4. **C.** Longitudinal section through a protocorm infected with R1 isolate 56 days after propagation stained with Toludine blue pH 4.4. **D.** Longitudinal section through a protocorm infected with R1 isolate 56 days after propagation stained with Toludine blue pH 4.4. **E.** Scanning electron micrograph of a protocorm infected with R1 isolate 28 days after propagation. **F.** Scanning electron micrograph of a protocorm infected with R1 isolate 28 days after propagation.

coiled fungal hyphae and less starch than the uninfected storage cells (FIGURE 5C).

Two peloton types were observed in protocorms infected with R1. The first type occurred in the outer layer of cortical cells and consisted of branched hyphae and loosely coiled monilioid hyphae (FIGURE 5D). These pelotons were not seen in stages of digestion. The second type consisted of unbranched hyphae, which coiled in one direction and were located in a deeper cortical layer (FIGURE 5E). In sectioned protocorms, these pelotons were seen in stages of digestion, and the cells containing them also were infected with healthy hyphae.

Infection with R16 extended from the basal cortical cells of the protocorm to cells near the meristem (FIGURE 5F). The starch storage cells contained more starch in comparison to those in protocorms infected with R1. All of these storage cells were infected with fungal hyphae (FIGURE 6A).

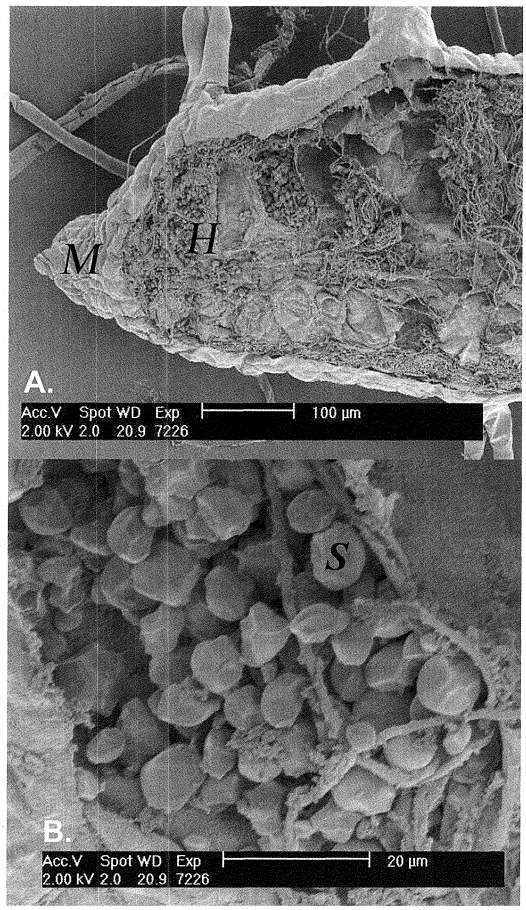

FIGURE 6. *Caladenia tentaculata.* Hyphae (H). Meristem (M). Starch storage cells (S). **A.** Scanning electron micrograph of a longitudinal cross section through a protocorm infected with R16 isolate 56 days after propagation. **B.** Scanning electron micrograph of a longitudinal cross section through a protocorm infected with R16 isolate 56 days after propagation.

DISCUSSION

The seed of *Caladenia tentaculata,* like that of Northern Hemisphere terrestrial orchid species, requires infection by a compatible symbiont for germination and healthy protocorm development to proceed. The stages of mycorrhizal infection and colonization in *C. tentaculata* are similar to those previously described for Northern Hemisphere species. Two unique observations were made in this study: (1) the presence of phenolic compounds in the pre-imbibed *C. tentaculata* seed and (2) the development of two morphologically distinct peloton types observed during infection with the compatible isolate, occurring in different layers of cortical cells in the protocorms.

Compatible Colonization

Initial infection of *Caladenia tentaculata* seed by the compatible isolate (R1) occurred through the suspensor cell similar to the most commonly observed initial infection pathway in Northern Hemisphere species of terrestrial orchids (Mollison 1943, Richardson et al. 1992, Rasmussen 1995, Uetake & Peterson 1997). This infection pathway also was observed in *C. menziesii* by Clements (1988). Rasmussen (1995) suggests that infection through the suspensor cell indicates that the fungal isolate facilitates germination.

The disappearance of starch grains in the cells of *Caladenia tentaculata* protocorms when infected by the compatible isolate is not unique to this species. Previously it has been observed in protocorms and roots of Northern Hemisphere orchid species (Hadley & Williamson 1971, Purves & Hadley 1975). A small proportion of the starch storage cells at the periphery of the mycotrophic tissue of *C. tentaculata* contained fungal hyphae, associated with fewer starch grains in these cells, similar to the report of Uetake et al. (1992) in *Spiranthes sinensis.* As the *C. tentaculata* protocorm enlarges during colonization, the mycorrhizal fungus either translocates or degrades the starch.

UV autofluorescence in the pre-imbibed embryos indicates the presence of phenolic compounds. This autofluorescence contracted towards the suspensor region during imbibition, suggesting that the phenolic compounds are water-soluble and are leached from the embryo via

its most permeable point, the suspensor cell. The seed of Australian terrestrial orchids has been shown to survive the dry summer months after dispersal in the soil in the pre-imbibed state (Batty et al. 2000). The presence of phenolic defense compounds in the pre-imbibed embryos could protect the seed from saprophytic fungi and bacteria. Tannins (phenolic compounds) have been observed in the suspensor cell of *Dactylorhiza majalis* (Rasmussen 1990), where they may inhibit peloton formation. *Caladenia* also may regulate the establishment of mycorrhizal fungi by accumulating inhibitory phenolic compounds.

Two distinct peloton types formed in separate layers of the cortical cells of *Caladenia tentaculata* protocorms, as in developing protocorms of the Northern Hemisphere orchids *Dactylorhiza majalis* (Rasmussen 1990) and *Spiranthes sinensis* (Uetake et al. 1992). In *D. majalis* and *S. sinensis*, the two types were differentiated on functional grounds. Cortical cells containing permanently un-digested pelotons were termed "host cells," and those containing regularly digested pelotons, "digestion cells" (Rasmussen 1995). Huynh et al. (in press) found morphologically distinct pelotons in adult *Caladenia formosa* plants in-situ and concluded the presence of more than one fungus. The different peloton types in *C. formosa* did not appear in separate cortical layers, and this organization may differ between the protocorm and that of the adult plant.

The morphology of the pelotons in the outer layer suggests that they are more vigorous; and the branched hyphae, multi-directional growth, and presence of monoiliodal cells are similar to the morphology of the extracellular hyphae. The inner layer of cortical cells can only be re-infected by the intracellular hyphae in the first cortical cell layer and thus contain less vigorous mycorrhizal growth. The functions of nutrient deficiency, limited re-infection, and continual digestion may restrict fungal infection and protect the starch storage and vascular tissue from colonization.

Kristiansen et al. (2001) showed by DNA sequencing that two fungi were isolated from single pelotons in *Dactylorhiza majalis.* This finding sheds a different light on the distinct peloton morphology observed in protocorms inoculated with R1; they may be two different fungal strains, as reported in *Caladenia formosa* (Huynh et al. in press) The two peloton types observed in *C. tentaculata* may represent the two fungal organisms sequenced by Raleigh in the R1 isolate (R. Raleigh pers. comm.). The effectiveness of the R1 isolate to germinate *C. tentaculata* seed may be dependent on both fungal organisms being present, as the different peloton types appear to have different functional roles within the protocorm. This evidence suggests that more than one fungus is required to stimulate germination and growth in some terrestrial orchid species, including *Caladenia.*

Incompatible Colonization: A Comparison

The infection pathway and colonization pattern of *Caladenia tentaculata* varied widely following incompatible inoculation, ranging from protocorms bearing photosynthetic shoots indicating stable protocorm formation (Anderson 1991) to soft rot as a result of parasitism (Beyrle et al. 1991, Masuhara et al. 1993). Similar variation in protocorm formation has been observed in mycorrhizal associations with other species of terrestrial orchids (Clements 1988, Beyrle et al. 1991). Although rare, suspensor infection was inevitably successful, even with the incompatible isolate.

Most *Caladenia tentaculata* seed remained un-infected by the incompatible isolate, reflecting the resistance described by Clements (1988) in *Pterostylis* species. Such resistance is not apparent with the compatible isolate, where all viable *C. tentaculata* seed germinated and produced protocorms. The difference in resistance to the two isolates indicates recognition by the orchid of differences between the compatible and incompatible isolates. The suspensor may be the site of recognition, for it is the only infection pathway that leads to healthy protocorm formation. The importance of suspensor infection for *C. tentaculata* seed was highlighted by the rarity of this infection pathway with the incompatible isolate. The phenolic compounds located in the suspensor cell may control stable mycorrhizal establishment; if such is the case and initial infection occurs through another pathway, stable mycorrhizal establishment cannot be reached.

Rhizoid infection was observed during inoculation with both isolates, although it resulted in different outcomes. Rhizoid infection by the incompatible isolate occurred as primary infection resulting in a colonization pattern without peloton formation in the cortical cells, similar to pathogenic infection seen in *Dactylorhiza incarnata* by Beyrle et al. (1991). Hyphae of the compatible isolate also were observed penetrating the rhizoids, although this occurred secondarily to suspensor infection, and fungal colonization would be limited, because digestion already has been initiated in the cortical cells. This comparison again highlights the importance of initial infection through the suspensor cell in the development of a stable mycorrhizal association.

Incompatible infection did not inhibit starch assimilation in storage cells of the protocorms infected with the incompatible isolate, as these cells were densely packed with starch grains and hyphae. By comparison, starch reserves in cortical cells infected by the compatible isolate were limited. Incompatible fungal infection may increase the rate of starch deposit in infected cells, allowing the isolate access to the nutrient reserves of the orchid. This study was unable to elucidate the fate of starch grains in cells infected by either the compatible or the incompatible isolates. It follows, however, that if the isolate is parasitizing the protocorm (incompatible isolate), it is likely to be degrading the orchid's stored reserves.

Photosynthetic shoot formation is extremely rare during infection with the incompatible isolate. Most protocorms had greatly reduced meristematic regions, in which colonization was not restricted to the basal cells and reached the periphery of the meristematic tissue. Clements (1988) observed protocorms developing normally, until colonization reached the meristem, resulting in death of the seedling. Meristematic colonization by the incompatible isolate also may have occurred in the *Caladenia tentaculata* protocorms showing symptoms of soft rot; however, the disease symptoms technically prevented microscopic analysis. Meristematic tissues remain un-infected during compatible colonization, a phenomenon also observed in many Northern Hemisphere (Uetake et al. 1992, Rasmussen 1995) and Australian species (Clements 1988), including *C. tentaculata.*

Rhizoid development stimulated by incompatible infection was not restricted to the epidermal cells at the base of the protocorms. The rhizoids appeared to develop haphazardly, some forming multicellular structures and others not. This seemly random distribution of rhizoids over the epidermal surface gave the fungus access to the entire protocorm. The orchid therefore was unable to restrict the fungal invasion to the basal cells. Abnormal rhizoid development previously has not been implicated in incompatible infection of other terrestrial orchid species, but observations for *Caladenia tentaculata* illustrate that normal rhizoid development may be important in the production of a stable healthy protocorm. These observations implicate rhizoid development as a diagnostic feature in assessment of protocorm health. Rhizoid development may be used as a macroscopic feature to assess probability of survival for a given protocorm, and therefore time may be saved by discarding those that would not survive deflasking and re-introduction to field habitats.

Conclusions

This study revealed details of the obligate mycorrhizal associations with *Caladenia tentaculata* and confirmed that ex-situ conservation efforts for *Caladenia* require symbiotic germination with an appropriate mycorrhizal isolate, for normal protocorm development to proceed. Suspensor infection by the mycorrhizal fungi not only leads to normal protocorm development in *C. tentaculata* but may be important for controlling in which cells of the embryo mycorrhizal colonization occurs.

Future studies into the development of mycorrhizal associations in *Caladenia* species using UV autofluorescence during initial infection may offer an explanation for the apparent resistance to infection by incompatible isolates. The question of the origin of the two peloton types could be answered with RNA in situ hybridization with probes designed for the two organisms sequenced by R. Raleigh (pers. comm.).

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Literature Cited

Anderson, A. 1991. Symbiotic and asymbiotic germination and growth of *Spiranthes magnicamporum* (Orchidaceae). Lindleyana 6(4): 183–186.

Batty, A., K. Dixon, M. Brundrett, and K. Sivasithamparam. 2000. Soil seed-bank dynamics of terrestrial orchids. Lindleyana 15: 227–236.

Beyrle, H., F. Penningsfeld, and B. Hock. 1991. The role of nitrogen concentration in determining the outcome of interaction between *Dactylorhiza incarnata* (L.) Soo and *Rhizoctonia* sp. New Phytol. 117: 665–672.

Clements, M. 1988. Orchid mycorrhizal association. Lindleyana 3: 73–86.

Curtis, J. 1939. The relation of specificity of orchid mycorrhizal fungi to the problem of symbiosis. Amer. J. Bot. 26: 390–398.

Department of the Environment and Heritage. 2004. Environment Protection and Biodiversity Conservation Act 1999. Biodiversity, Threatened species. Published on the Internet at http://www.deh.gov.au/epbc/. Access date: 10/4/04.

Harley, J.L. and S.E. Smith. 1983. Mycorrhizal Symbiosis. Academic Press, New York.

Hadley, G. and B. Williamson. 1971. Analysis of the post-infection growth stimulus in orchid mycorrhiza. New Phytologist 70: 445–455.

Harvais, G. 1973. Notes on the biology of some native

orchids of Thunder Bay, their endophytes and symbionts. Canad. J. Bot. 52: 451–460.

Harvais, G. and G. Hadley. 1967. The development of *Orchis purpurella* in asymbiotic and inoculated cultures. New Phytol. 66: 217–230.

Huynh T.T., C.B. McLean, F. Coates, and A.C. Lawrie. (In press). Effect of the developmental stage and peloton morphology on success in isolation of mycorrhizal fungi in *Caladenia formosa* (Orchidaceae). Austral. J. Bot.

Jones, D. 1988. Native Orchids of Australia. Reed Books, Frenchs Forest, Australia.

Kristiansen, K.A., D.L. Taylor, R. Kjøller, H.N. Rasmussen, and S. Rosendahl. 2001. Identification of mycorrhizal fungi from single pelotons of *Dactylorhiza majalis* (Orchidaceae) using single strand conformation polymorphism and mitochondrial ribosomal large subunit DNA sequences. Molec. Ecol. 10: 2089–2093.

Masuhara, G. and K. Katsuya. 1992. Mycorrhizal differences between genuine roots and tuberous roots of adult plants of *Spiranthes sinensis* var. *amonea* (Orchidaceae). Bot. Mag. Tokyo 105: 453–460.

Masuhara, G., K. Katsuya, and K. Yamagushi. 1993. Potential for symbiosis of *Rhizoctonia* with seeds of *Spiranthes sinensis* var. *amonena* in vitro. Mycol. Res. 96: 199–206.

Mollison, J. 1943. *Goodyera repens* and its endophyte. Trans. Bot. Soc. Edinburgh 33: 391–403.

Purves, S. and G. Hadley. 1975. Movement of carbon compounds between the partners in orchid mycorrhiza. Pp. 175–194 *in* F.E. Sanders, B. Mosse and P.B. Tinker, eds. Endomycorrhizas. Academic Press, London.

Rasmussen, H. 1990. Cell differentiation and mycorrhizal infection in *Dactylorhiza majalis* (Rchb. f.) Hunt & Summerh. (Orchidaceae) during germination in vitro. New Phytol. 116: 137–147.

———. 1995. Fungi. Pp. 77–112 *in* Terrestrial Orchids from Seed to Mycotrophic Plant. Cambridge University Press, Cambridge.

Richards, H., R. Wotton, and R. Datodi. 1984. Cultivation of Australian Native Orchids. Australian Native Orchid Society, Victoria Group Inc.

Richardson, K., R. Peterson, and R. Currah. 1992. Seed reserves and early symbiotic protocorm development of *Platanthera hyperborea* (Orchidaceae). Canad. J. Bot. 70: 291–300.

Smreciu, E.A. and R.S. Currah. 1989. Symbiotic germination of seeds of terrestrial orchids of North America and Europe. Lindleyana 6(l): 6–15.

Uetake, Y. and L. Peterson. 1997. Changes in actin filament arrays in protocorm cells of the orchid species, *Spiranthes sinensis,* induced by the symbiotic fungus *Ceratobasidium cornigerium.* Canad. J. Bot. 75: 1661–1669.

Uetake, Y., K. Kobayashi, and A. Ogoshi. 1992. Ultrastructural changes during the symbiotic development of *Spiranthes sinesis* (Orchidaceae) protocorms associated with binucleate *Rhizoctonia anastomosis* group C. Mycolog. Res. 96: 199–206.

Warcup, J. 1971. Specificity of mycorrhizal associations in some Australian orchids. New Phytol. 70: 41–46.

———. 1981. The mycorrhizal relationships of Australian orchids. New Phytol. 87: 371–381.

———. 1990. Mycorrhiza. Pp. 21–26 *in* R. Bales and J. Webber, eds. Orchids of South Australia. Flora and Fauna of South Australia Handbook Committee, Adelaide.

Williamson, B. and G. Hadley. 1970. Penetration and infection of orchid protocorms. Phytopathology 60: 1092–1096.

Zelmer, C. and R. Currah. 1997. Symbiotic germination of *Spiranthes lancera* (Orchidaceae) with a naturally occurring endophyte. Lindleyana 12(3): 142–148.

Zettler, L. 1997. Terrestrial orchid conservation by symbiotic seed germination: techniques and perspective. Selbyana 18(2): 188–194.