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Short communication

Effect of a dark septate fungal endophyte on seed germination and protocorm development in a terrestrial orchid

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Abstract

Phialocephala fortinii, a dark septate fungal endophyte, is capable of breaking down complex carbohydrates and has the potential, therefore, of providing simple sugars to an achlorophyllous plant host. In nature, orchid seeds require a fungal symbiont to provide sugars for germination and protocorm development. To investigate the potential of *P. fortinii* to function in this manner, seeds of the terrestrial species *Dactylorhiza praetermissa* [Druce] Soo were cultured with plugs of *P. fortinii* on media with ground oats as a complex carbohydrate. Seeds were also cultured on plates containing oats only, simple sugars only, and a combination of the two. Germination and protocorm development were judged by imbibition and epidermal hair growth. While seeds in the oats plus fungus treatment did not develop as quickly as those on simple sugars, overall protocorm formation rates were higher. Both germination and development rates were significantly higher in seeds grown in oats plus fungus than in those grown with oats alone. Hyphae and microsclerotia developed in embryo cells in the oats plus fungus treatment. *P. fortinii* may have a positive effect on seed germination and protocorm development of terrestrial orchids in nature by providing simple sugars. A preliminary experiment with epiphytic orchid species indicated the *P. fortinii* had a deleterious effect on germinating seeds when mineral nutrients were lacking in the medium.

Keywords: *Phialocephala*, *Dactylorhiza*, hyphal coils, microsclerotia, *in vitro*

1. Introduction

Dark septate endophytes

Dark septate endophytes (DSEs) are a ubiquitous group of anamorphic, root-inhabiting fungi which are characterized by darkly pigmented, septate hyphae (Jumpponen and Trappe, 1998; Peterson et al., 2004). Two DSEs, *Phialophora finlandia* and *Phialocephala fortinii*, have been shown to utilize cellulose, starch, laminarin, and xylan as sole carbon sources, and to hydrolyze proteins and ribonucleic acids as sole nitrogen and phosphorus sources, respectively, when grown *in vitro* (Caldwell et al., 2000). *P. fortinii* is also able to utilize uric acid and guanine as nitrogen sources (Caldwell and Jumpponen, 2003a) and to hydrolyze organic sulphate (Caldwell and Jumpponen 2003b). Hyphae of DSEs colonize root tissues both intercellularly and intracellularly and frequently form microsclerotia, consisting of modified hyphae in which proteins, polysaccharides, and polyphosphates are stored (Yu et al., 2001).

Dark septate endophytes affect their hosts along a mutualistic to parasitic gradient, often depending on the conditions under which the plants are grown (Jumpponen, 2001; Mandyam and Jumpponen, 2005). While there is no direct experimental evidence that DSEs improve their hosts' nutrient uptake, the results of several studies imply that this may be the case. Jumpponen et al. (1998) showed that *Pinus contorta* plants, inoculated with *P. fortinii*, showed an increase in shoot phosphorus. Similar results have been seen in sedges (Haselwandter and Read, 1982) and annual grasses (Newsham, 1999) which were colonized by dark septate endophytes.

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Phialocephala fortinii

Phialocephala fortinii Wang & Wilcox is typical of dark septate endophytes, exhibiting melanized hyphae and forming microsclerotia. Although *P. fortinii* can be found in many ecosystems, it is especially prevalent in the roots of plants living in cool boreal and alpine soils (Haselwandter and Read, 1980). The species was initially identified through morphological characteristics in culture (Currah and Tsuneda, 1993) and more recently by using genetic markers (Grünig et al., 2001). Like other DSEs, *P. fortinii* is sterile in culture, but produces conidia, which act as spores.

Orchid seed germination

Orchid seeds, which occur in the tens of thousands per capsule, are characterized by the lack of an endosperm and by an undifferentiated embryo consisting of parenchyma cells containing lipids and proteins as storage compounds but lacking a shoot and root meristem (Rasmussen, 1995; Peterson et al., 1998). In nature, orchid seeds depend on symbiotic fungi, usually basidiomycetes, for the acquisition of simple sugars needed for the development of a protocorm from which a shoot meristem and adventitious roots develop (Rasmussen, 1995; Peterson et al., 1998). Typical colonization of the embryo by symbiotic fungi occurs either through the suspensor cells or through epidermal hairs. Once inside, hyphae penetrate the parenchyma cells and form coiled structures called pelotons, which eventually fill most of an occupied cell (Peterson et al., 1998). The structure of the pelotons provides a large common surface area between the cell and the fungus, allowing for efficient nutrient exchange.

Temperate terrestrial orchids have germination requirements which can often make *in vitro* culture quite challenging. Because germination and much of the early growth of temperate orchids occurs underground under entirely mycotrophic conditions, seeds generally require darkness to germinate (Rasmussen, 1995).

Dark septate fungal endophytes and orchids

Studies of the interactions of DSEs with orchids have been restricted to terrestrial orchids. Currah et al. (1988) isolated *P. fortinii* from the temperate orchid *Calypso bulbosa*. While the study was inconclusive, the authors suggested that the fungus may be involved in the nutrition of the plant. A closely related DSE, *P. victorinii*, was isolated from the temperate terrestrial orchid, *Cypripedium parviflorum* by Vujanovic et al. (2000a), but these authors made no reference to the effect of the fungus on its host. The effect of DSEs on orchid seed germination and protocorm development has not been studied.

The objective of this study was to investigate the effect of one dark septate fungal endophyte, *P. fortinii*, on orchid seed germination and protocorm development. We tested the hypothesis that *P. fortinii* would colonize orchid seeds resulting in protocorm formation.

2. Materials and Methods

Fungal culture

Phialocephala fortinii (isolate UAMH 10266, isolated from *Salix arctica* GenBank AY 237604) was obtained from the University of Alberta Microfungus Collection and Herbarium and grown on half-strength potato dextrose agar (PDA). Plugs measuring 1 cm × 1 cm were cut from these cultures and incubated under sterile conditions in Petri dishes containing half-strength PDA. Subcultures were allowed to grow at approximately 20°C for one month before being used to inoculate seed plates.

Plant material and fungal inoculation

To test the ability of *P. fortinii* to initiate seed germination and promote protocorm development, seeds of the terrestrial orchid *Dactylorhiza praetermissa* (Druce) Soo were used (provided by Mr. Peter Croezen, Green Canyon Orchids, Kitchener, Ontario). Seeds were surface sterilized for ten minutes in an aqueous solution of 10% household bleach containing several drops of Tween 20 detergent to break surface tension. The bleach solution was removed by drawing off liquid with a pipette and replacing it with sterile distilled water several times. Seeds were then placed in a small flask of sterilized water and quickly drawn up in small aliquots with a pipette after swirling the flask. Seeds were dispensed on the media plates and the plates shaken from side to side in order to disperse the seeds. Once seeds had been added, all plates were sealed with Parafilm and stored at room temperature.

Seeds were subjected to four treatments: 1. Sucrose (0.2%), a simple sugar which allows orchid seeds to germinate asymbiotically. This treatment acted as a control to ensure seed viability; 2. Ground oat powder (0.2%), which contains complex carbohydrates and does not allow asymbiotic germination. This treatment would provide a baseline for developmental capacity in the absence of simple sugars; 3. Ground oat powder + sucrose (both at 0.2%). This treatment ensured that the oatmeal itself had no detrimental effect on seed germination; 4. Ground oat powder (0.2%) onto which one 1 cm × 1 cm plug per plate containing fungal hyphae was introduced after seeding.

All media were solidified with 1% agar containing 1/10 strength Long Ashton nutrient solution (Hewitt, 1966). The nutrients were added to ensure that fungal parasitism did not occur due to a lack of available nutrients. A preliminary experiment in which seeds of three epiphytic orchid species

were plated onto water agar without additional nutrients resulted in destruction of the embryos by *P. fortinii* (see Results). Thirty replicates were included per treatment for a total of 120 plates. Following seeding and inoculation, all plates were stored in a Fisher Isotemp 200 series incubator at 25°C in complete darkness, to aid in germination. After six months of incubation, plates were moved for a further two months to a laboratory bench at a temperature of 22°C and ambient lighting for approximately eight hours per day.

Seed germination and protocorm development

Eight months after seeding, plates with any level of contamination were discarded and of the remaining plates, 25 were selected at random from each treatment. These were unsealed and scored for germination under a Carl Zeiss stereo binocular microscope using a multi-channel counter. To increase seed visibility during counting, a small amount of dilute aqueous Ruthenium Red stain was spread over the surface of each plate just prior to counting, highlighting fine epidermal hairs against overlying fungal hyphae. To score seeds for germination, a grid was drawn on the lid of a Petri dish and nine random 1 cm squares per plate were counted. In each square, counts were taken of the number of non-imbibed seeds, the number of imbibed seeds with no epidermal hairs, and the numbers of seeds with fewer than five hairs, five to ten hairs, and greater than ten hairs. Data from each category of germination scoring was relativized and tested for differences between treatments using a one-way analysis of variance (ANOVA). If an ANOVA showed significant differences between groups, a Tukey post-hoc test was then used. The above tests were also used to determine significance in data showing the presence of any number of epidermal hairs across the four treatment groups. Statistical tests were conducted using Systat 8.0.

Microscopy

Representative samples were taken from each treatment group, fixed overnight in 2.5% glutaraldehyde in 0.1 M HEPES buffer, dehydrated in a graded ethanol series, and embedded in LR White resin (Canemco, Lachine, Quebec, Canada). Embedded samples were sectioned using glass knives on a Sorvall Porter-Blum MT-1 Ultra-microtome. Sections were heat-fixed to microscope slides and stained with 0.05% toluidine blue O (Sigma-Aldrich, St. Louis, MO, USA) in 0.1 M phosphate buffer (pH 6.6), then photographed using a Nikon digital camera under a Leitz Wetzlar compound light microscope.

Germinated seeds from plates inoculated with *P. fortinii* were cleared for one hour in 10% potassium hydroxide over low heat, acidified by soaking in 1 M HCl for one hour, and stained overnight with 0.01% acid fuchsin (Sigma-Aldrich, St. Louis, MO, USA) in lactoglycerol. These samples were then mounted in 50% glycerin on glass slides and viewed under the compound light microscope described above equipped with Nomarski optics. Some inoculated seeds were stained with dilute aqueous crystal violet (Carolina Biological Supply Company, Burlington, NC, USA) and viewed under the compound microscope with or without the seed coat intact.

3. Results

Seed germination and protocorm development

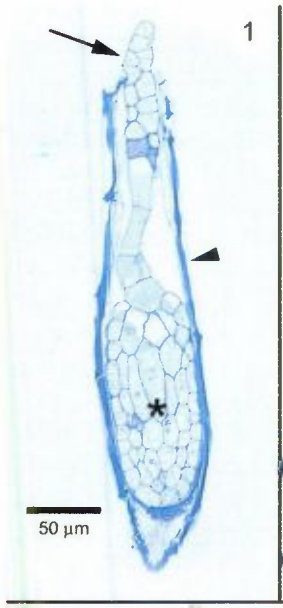
In all cases, the ANOVA test showed significant differences between groups ($F_{3,96}$= 104.737; $p<0.001$). When overall epidermal hair development was considered, combining data for all hair classes, a significantly greater (Tukey test: $p<0.001$) number of protocorms from the oats plus inoculum treatment initiated hairs compared to any other treatment (Table 1). There was no significant difference (Tukey test: $p>0.05$) between oats plus sucrose and sucrose only treatments. Developing protocorms in the oats only treatment had significantly lower (Tukey test: $p<0.001$) rates of hair development than all other treatments.

When hair development was broken down into protocorms with fewer than five hairs, five to ten hairs and more than ten hairs, further differences between treatments became obvious (Table 1). In counts of protocorms with one to four epidermal hairs, the oats plus inoculum treatment showed much higher (Tukey test: $p<0.001$) percentages than any other group, while the sucrose treatment showed significantly lower (Tukey test: $p<0.05$)

Table 1. Effect of growth medium on epidermal hair growth in *Dactylorhiza praetermissa* protocorms.

Treatment	% with hair growth	% with 1–4 hairs	% with 5–10 hairs	% with >10 hairs	Total seeds counted
Oats	6.60 a	6.13 d	0.47 g	0.00 j	1,696
Sucrose	22.55 b	2.33 e	5.47 h	14.75 k	3,911
Oats/Sucrose	19.74 b	4.73 d	4.91 h	10.09 l	2,645
Inoculated	30.01 c	18.03 f	11.80 i	0.18 j	4,881

Values within columns with the same letter not significantly different (Tukey test, $p<0.001$ across all groups).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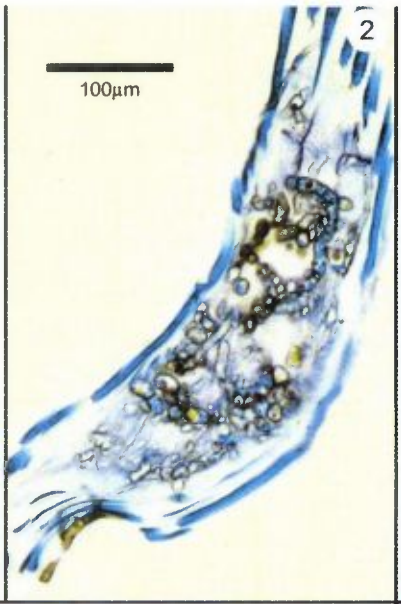
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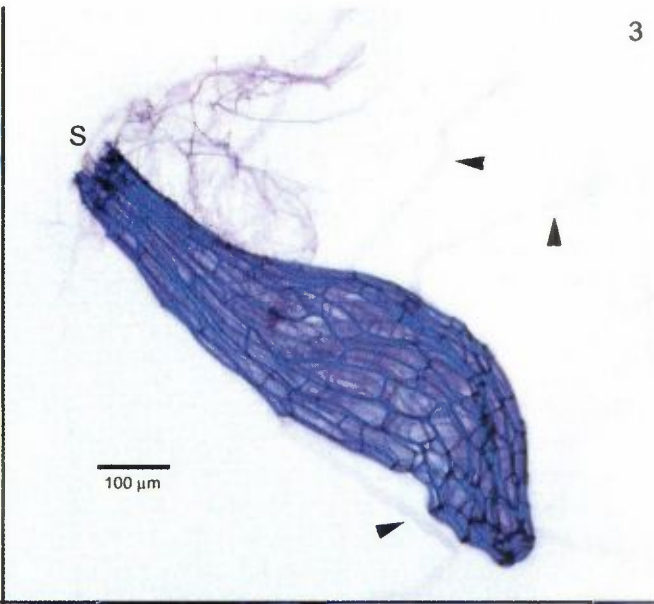
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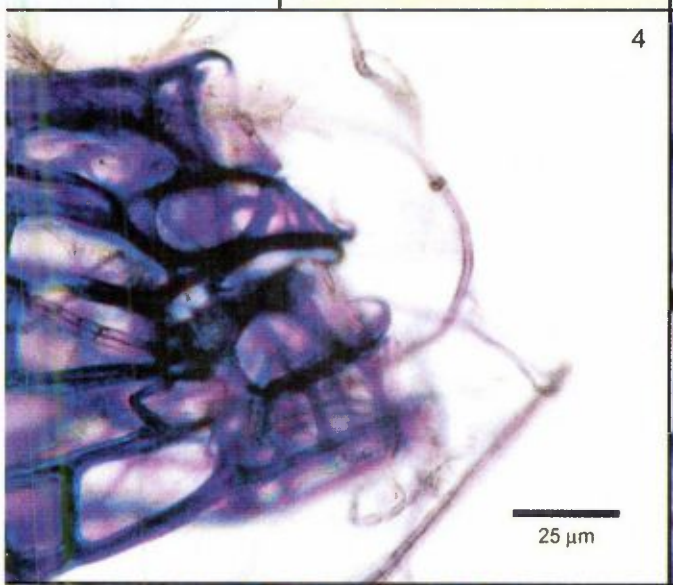
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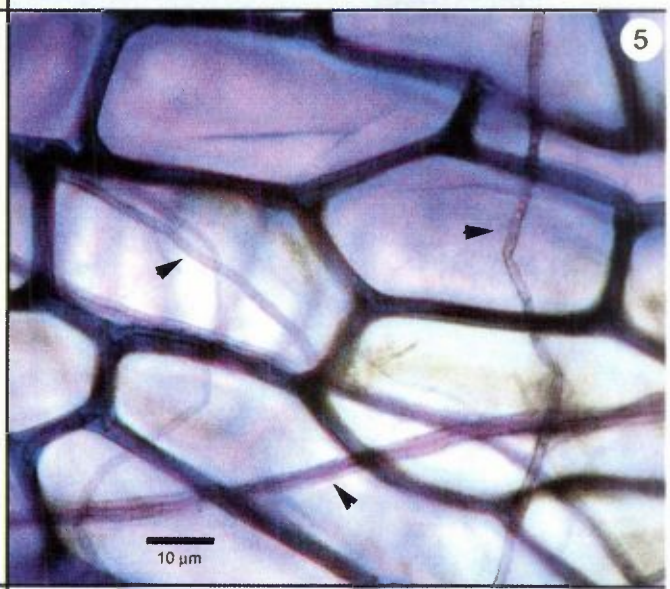
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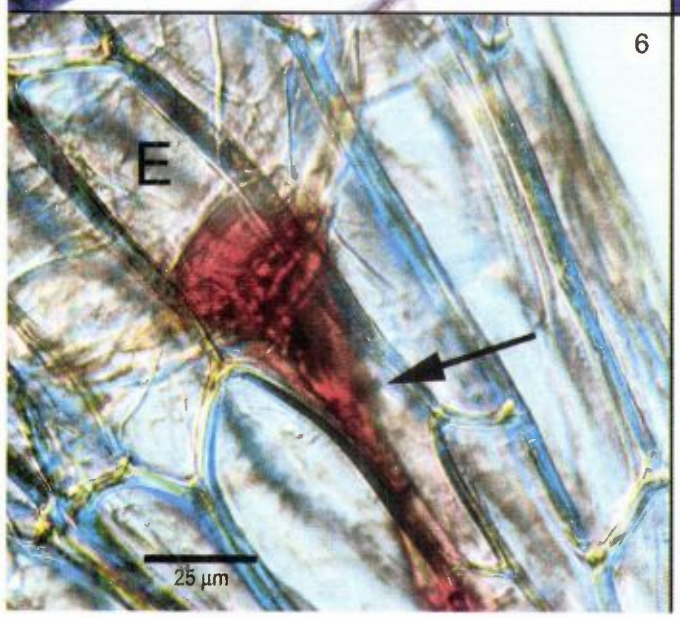
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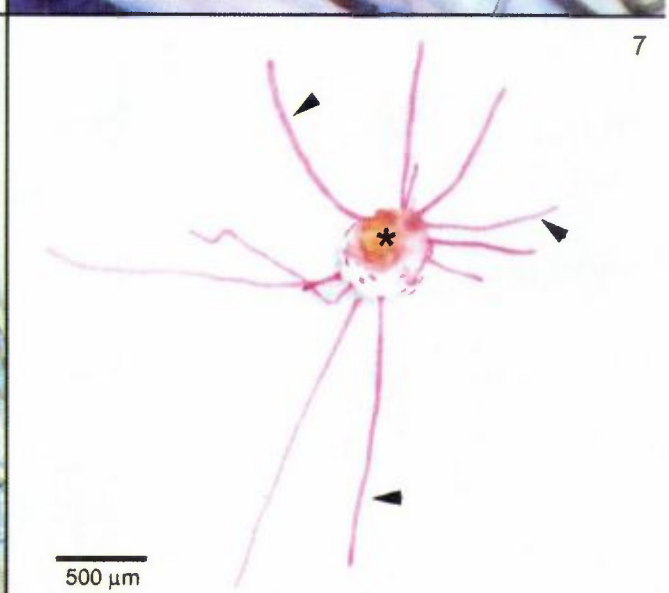
7
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rates compared to all other treatments. In protocorms with five to ten epidermal hairs, the oats plus inoculum treatment again showed significantly higher percentages (Tukey test: $p<0.001$) than all other treatments, but levels in the oats only treatment had become quite low. Percentages in the sucrose only treatment had risen somewhat, but were still low compared to the oats plus inoculum treatment. When protocorms with greater than ten epidermal hairs were counted, both the oats plus inoculum and oats only treatments had dropped to negligible amounts, while the sucrose only and oats with sucrose treatments had risen, with the sucrose only treatment significantly higher (Tukey test: $p<0.001$) than any other group.

Microscopy

Epiphytic orchid seeds, which are similar structurally to terrestrial orchid seeds in that they are composed of a simple embryo and suspensor cells (Fig. 1), were used in preliminary tests in which no extra mineral nutrients were added to the media. In this case, strong fungal parasitism occurred in which hyphae degraded the embryo (Fig. 2).

When examined under the microscope, inoculated *Dactylorhiza* seeds had numerous fungal hyphae clustered around the suspensor pole of the testa (Fig. 3). Closer examination showed that hyphae entered the opening in the testa at this site (Fig. 4) and were present beneath it (Fig. 5). In seeds of all treatments, suspensor cells and the surrounding base of the embryo showed a visible build-up of tannins (Fig. 6).

In sucrose-grown seeds, which had developed further than other treatments, the testa split and remained attached to the tip of the embryo as the protocorm enlarged and numerous epidermal hairs protruded from the surface (Fig. 7). Sections of these seeds showed a well-developed shoot meristem at the chalazal pole of the embryo, characterized by enlarged nuclei (Fig. 8).

Evidence of fungal colonization was first observed in embryos which had their seed coats removed and were stained using Crystal Violet. These showed numerous lipid bodies of fungal origin within hyphal cells running along the embryo (Fig. 9). This finding was confirmed in sectioned seeds by the presence of low concentrations of hyphae within many cells, and the appearance of early coils in some, as well as the development of a meristem (Figs. 10, 11). Meristems in inoculated seeds did not appear as advanced as those seen in sucrose-grown seeds. In several seeds, fungal hyphae had formed microsclerotia which appeared to fill the embryonic cells in which they occurred (Figs. 12, 13). Both microsclerotia and fungal coils, as well as the majority of hyphae seen, occurred either at or near the surface of the embryo.

4. Discussion

Early stages of seed germination and protocorm development in most orchid species are dependent on fungi for a source of carbon and therefore can be classified as being myco-heterotrophic (Leake, 1994). Chlorophyllous orchid species normally form associations with a few fungal genera in the Basidiomycetes (Currah and Zelmer, 1992; Currah et al., 1997) that are capable of degrading complex carbohydrates and thereby potentially providing the carbon needed for protocorm development (Zelmer et al., 1997). Recently, an isolate of *Fusarium*, a Hyphomycete, induced germination in seeds of the terrestrial orchid *Cypripedium reginae in vitro* (Vujanovic et al., 2000b), suggesting that a broader range of fungi may be capable of inducing orchid seed germination.

This is the first study to test the ability of a dark septate fungal endophyte (DSE), known to be able to utilize complex carbohydrates and organic nitrogen compounds (Caldwell et al., 2000), to trigger seed germination and initiate protocorm development in orchids. Our results support the initial hypothesis that the DSE, *Phialocephala fortinii*, an Ascomycete, would induce germination of orchid seeds.

In the present study, hyphae of *Phialocephala fortinii* initially entered seeds of *Dactylorhiza praetermissa*

See figures on previous page.

Figure 1. Longitudinal section of an unimbibed seed of the epiphytic orchid *Cattleya jenmanii* showing testa (arrowhead), embryo (*), and suspensor cells (arrow). Stained with TBO.

Figure 2. Longitudinal section of an inoculated seed of the epiphytic orchid *Phalaenopsis amabilis* showing heavy fungal infection causing embryo degradation. Stained with TBO.

Figures 3–5. Fresh seeds of *Dactylorhiza praetermissa* inoculated with *Phialocephala fortinii* and stained with Crystal Violet.

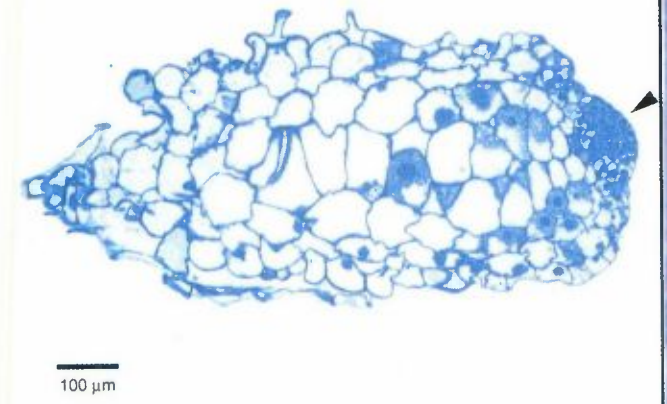
Figure 3. Seed with epidermal hairs (arrowheads) that have developed from the embryo and fungal hyphae entering via the suspensor region (S). *See cover illustrations.*

Figure 4. Detail of hyphae entering the testa through the suspensor region.

Figure 5. Close-up of seed showing fungal hyphae (arrowheads) between the testa and the embryo.

Figure 6. *D. praetermissa* seed stained with acid fuchsin and viewed using Nomarski optics. The base of the embryo (E) and remaining suspensor cells (arrow) appear red due to a build-up of tannins.

Figure 7. Developing protocorm of *D. praetermissa* grown on sucrose-containing medium, stained with Ruthenium Red. Epidermal hairs (arrowheads) and remains of testa (*) are visible. *See cover illustrations.*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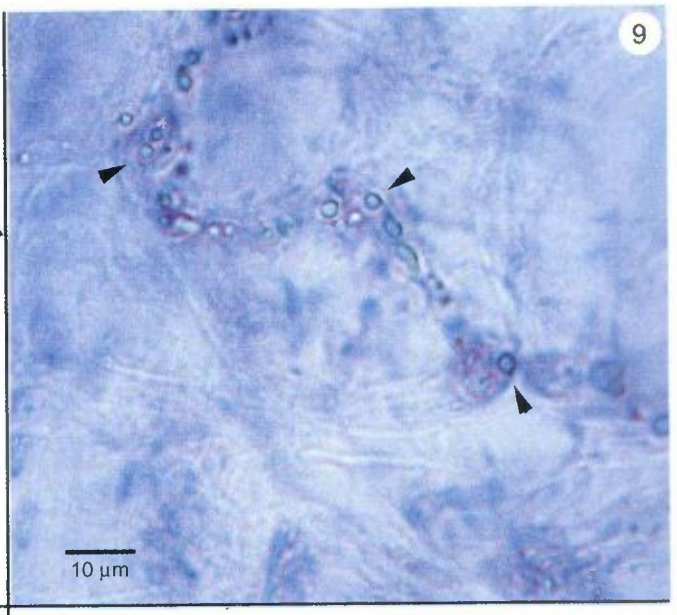
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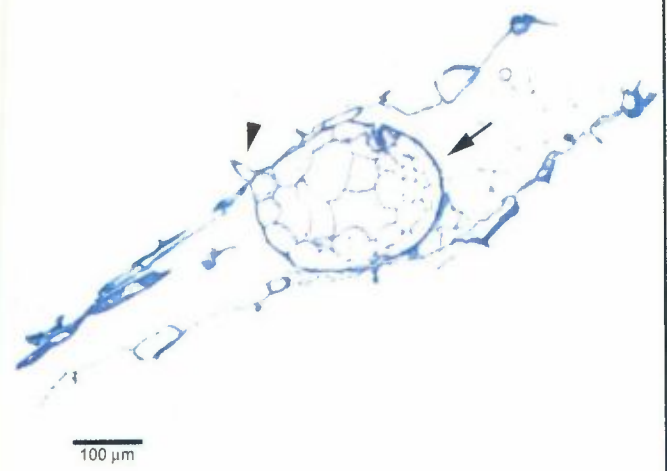
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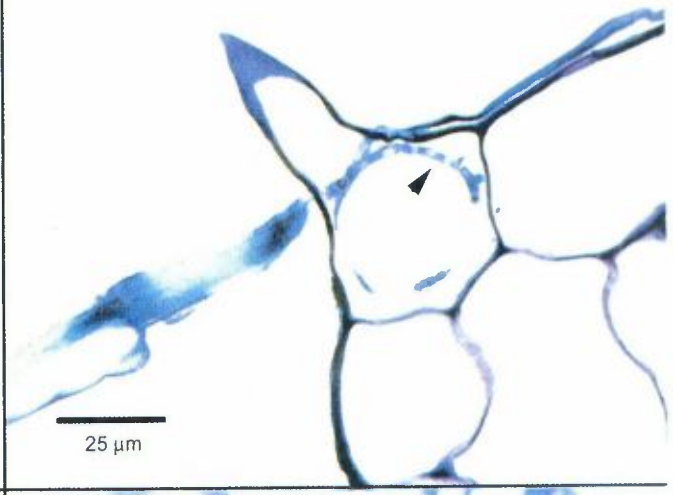
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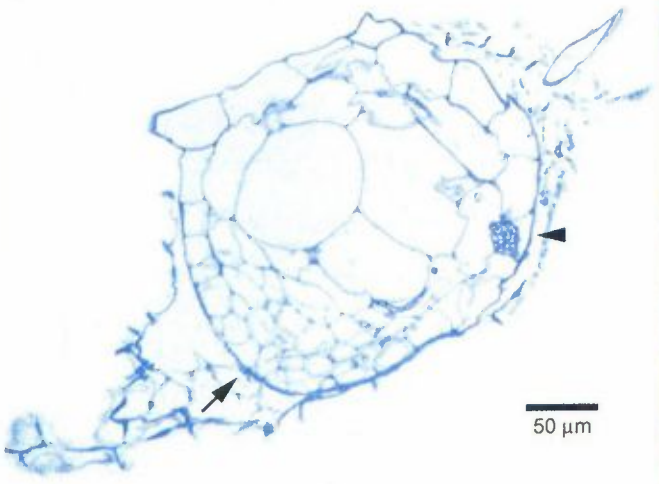
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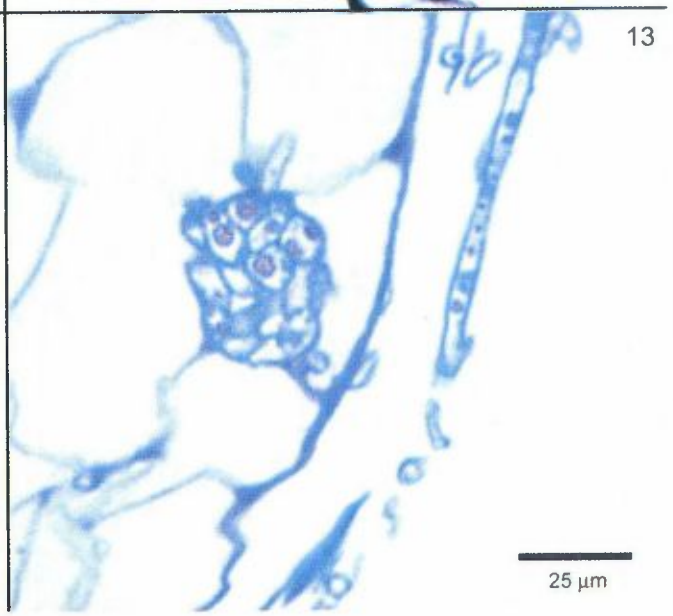
13
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through the break in the micropylar region of the testa and occasionally formed hyphal coils and microsclerotia. With the exception of the formation of microsclerotia, these events mirror the early stages of colonization by typical orchid mycorrhizal fungi (Peterson et al., 1998).

Rasmussen (1990, 1995) has suggested that orchid seeds may become colonized more than once during early development, first through the suspensor cells and later through the epidermal hairs, and that a temporary build-up of tannins in the suspensor pole of the embryo eliminates the hyphae which initially colonized the seeds. The tannin build-up seen in seeds in this experiment may have served this same purpose, forcing the fungus already present between the seed coat and the embryo to recolonize the embryo via the epidermal hairs. While a distinct fungal entry point into the embryo could not be found, the frequent occurrence of fungal structures around the edges of the sectioned embryos may be indicative of epidermal hair as opposed to suspensor cell colonization. This would be consistent with the observations made with the *in vitro* germination of *Dactylorhiza majalis* (Rchb. f.) Hunt & Summerh. seeds inoculated with an *Epulorhiza* isolate (Rasmussen, 1990).

In the presence of *Phialocephala fortinii*, the lower number of epidermal hairs may indicate that fungal hyphae are replacing epidermal hairs as a means of nutrient uptake. There was a significant increase in overall epidermal hair development, taken here to indicate protocorm development, in inoculated seeds compared to those grown on oats alone, suggesting that simple sugars resulting from the breakdown of the complex polysaccharides in oats are likely provided to the embryos by the fungus. Although the experiment was not continued until leaves and adventitious roots developed from the embryos, it is noteworthy that a shoot apical meristem developed in the sucrose, sucrose plus oats, and the oats plus fungal inoculation treatments, indicating the potential for seedling establishment.

What remains to be demonstrated is the occurrence of dark septate endophytes that stimulate protocorm development in the embryos of seeds germinating in soil. This could be tested by using seed packets (Masuhara and Katsuya, 1994; Rasmussen, 1995) containing the seeds of various orchid species buried in soil in typical orchid habitats. It is possible that DSEs could provide the initial trigger for orchid seed germination but then be replaced by typical Basidiomycete fungal endophytes. Dark septate endophytes have been isolated from roots of orchid species (Currah et al., 1988; Vujanovic et al., 2000a; Kulikov and Filippov, 2001), indicating that they may play a role in the host's survival and growth. As in other situations in which DSEs have been described, no direct evidence exists for the transfer of nutrients between host and fungal partners (Mandyam and Jumpponen, 2005). Future work should also compare the effects of *P. fortinii* on seed germination with those of a Basidiomycete, ideally isolated from *Dactylorhiza praetermissa*, which is known to form mycorrhizas with orchids. A direct demonstration of the ability of *P. fortinii* to release soluble sugars from complex sources such as oats is still needed.

Acknowledgements

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See figures on previous page.

Figure 8. Longitudinal section of a sucrose-grown *D. praetermissa* seed, stained with TBO. A meristematic region (arrowhead) has developed and behind this, embryo cells contain enlarged nuclei.

Figure 9. Fresh *D. praetermissa* embryo viewed with Nomarski optics, showing the lipid bodies of a fungal hypha (arrowheads) running through the host tissue. Stained with Crystal Violet.

Figures 10–13. Resin-embedded and sectioned seeds of *Dactylorhiza praetermissa* inoculated with *Phialocephala fortinii*; stained with TBO.

Figure 10. Longitudinal section showing a meristem (arrow) beginning to develop; an epidermal hair (arrowhead) is present.

Figure 11. Detail of a hyphal coil (arrowhead) in the base of the epidermal hair seen in previous figure.

Figure 12. Longitudinal section showing the developing shoot meristem (arrow) and a microsclerotium formed by the fungus (arrowhead).

Figure 13. Detail of the microsclerotium shown in the previous figure.

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