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SEED PROPAGATION OF THE EPIPHYTIC GREEN-FLY ORCHID, *EPIDENDRUM CONOPSEUM* R. BROWN, USING ITS ENDOPHYTIC FUNGUS

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ABSTRACT. Seed propagation of the epiphytic orchid, *Epidendrum conopseum,* using a naturally occurring fungus is reported for the first time. The fungus was isolated from roots of a mature *E. conopseum* specimen, and was identified as an *Epulorhiza* sp.—a genus rarely documented in epiphytic orchids. The symbiotic nature of the isolate was confirmed using the technique of symbiotic seed germination. Leaf-bearing seedlings of *E. conopseum* were obtained 71 days after seeds were inoculated with the fungus in vitro. Our study has practical applications for orchid conservation (i.e., restoration ecology) because we utilized the often ignored fungal component to propagate an orchid.

INTRODUCTION

Orchids are the only large group of plants that consistently utilize fungi as an alternative means to obtain energy (Rasmussen 1995). Mycotrophy (Clements 1988, Rasmussen 1995) may, in part, be responsible for the success of the Orchidaceae (30,000+ species: Smith 1977). Compatible fungi appear to be an absolute requirement for seed germination and seedling (protocorm) development in nature (Arditti 1966, Dressler 1981, Clements 1988), although the epiphytes may rely on mycotrophy less than the terrestrials (Rasmussen 1995). Horticulturists routinely propagate orchids from seed on aseptic media following procedures initially perfected by Lewis Knudson in the 1920's (Arditti 1990). As a result, the orchid mycorrhizal symbiosis remains poorly understood relative to other fundamental aspects of orchid biology (i.e., taxonomy) (Rasmussen 1995). Reports that describe the endophytic fungi of orchids of the New World are rare, particularly for the epiphytic taxa (Benzing & Friedman 1981, Benzing 1982, Richardson *et al.* 1993, Richardson & Currah 1995). *Epulorhiza* Moore appears to be one of the more common of the form-genera of Basidiomycotina isolated from terrestrial orchids (Currah *et al.* 1997), but has been less frequently encountered among the epiphytic species (Richardson *et al.* 1993, Richardson & Currah 1995). One goal of this study was to isolate endophytic fungi from the mycorrhizas of the epiphytic Green-fly Orchid, *Epidendrum conopseum* R. Brown. This goal is of interest because a fungus from this orchid has yet to be described. Another aim of the present study was to germinate the seeds of this orchid using the technique of symbiotic seed germination (Zettler 1997a).

MATERIALS AND METHODS

SEED AND FUNGUS COLLECTION. A mature yellowing capsule from *Epidendrum conopseum* was collected prior to dehiscence on 2 January 1996. The specimen grew in association with resurrection fern (*Polypodium polypodioides* (L.) Watt) on the horizontal limb of a ca. 200+ year-old *Quercus virginiana* Miller oak tree located 7 mi. SW of Gainesville (Alachua Co.), Florida. The capsule was stored in a plastic bag for two days and then dried at 22–24°C over $CaSO_4$ (Drierite). After 10 days at ca. 0% RH (22–24°C), seeds were harvested from the capsule, stored in a sealed sterile glass vial at ca. 0% RH, and at 6 ± 2°C in total darkness for 13.5 months until use. Fungi were isolated from orchid root systems according to the methods of Currah *et al.* (1987). One isolate (Pcil-154) originated from terrestrial *Platanthera ciliaris* (L.) Lindl., native to the southern Appalachians. This fungus (strain UAMH 7633; University of Alberta Microfungus Collection and Herbarium) was tentatively assigned to the anamorphic genus *Epulorhiza* (Moore 1987) and has been used in previous studies to germinate orchid seeds (Zettler & McInnis 1993, Zettler *et al.* 1995). Other tested fungi included *Ceratorhiza goodyera-repentis* (Costantin & Dufour) Moore (strain UAMH 6440) from *Platanthera obtusata* (Banks ex Pursh) Lindl. growing in Alberta, Canada, and *Moniliopsis anomala* Burgeff ex

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Currah (strain UAMH 6451) from *Coeloglossum viride* (L.) Hartm. from Alberta. These cultures were chosen to determine whether or not *E. conopseum* displays specificity for fungi. A fourth fungus (Econ-242), isolated on 7 June 1995 from the same *E. conopseum* specimen that yielded seed, was identified as a member of *Epulorhiza* (C. Zelmer pers. comm.) on the basis of cultural characteristics and TEM observation of imperforate parenthesomes (C. Zelmer pers. comm.). A voucher specimen of that orchid is in The Illinois College Herbarium (LWZ #854). All fungal isolates were maintained at 6 ± 2°C on modified oats medium (Dixon 1987).

SEED SOWING, FUNGAL INOCULATION AND INCUBATION. Seeds were surface-sterilized 1 min. in a vial containing absolute ethanol: 5.25% NaOCl (Chlorox): DI water (1:1:1 v/v/v); after this time, an equal amount of water was added to the solution vial and seeds were soaked an additional minute. Some seeds remained in this diluted solution for up to 10 min. during seed sowings. One ml of the solution containing seed was pipetted onto 20 ml of modified oats medium (Dixon 1987) within a 9 cm diam. Petri dish; the medium was adjusted to pH 7.4 prior to autoclaving. Approximately 40 seeds were spread on each 1 × 4 cm filter paper strip (Whatman No. 1) using a wire loop, and the strip was placed on the medium. Additional seeds were broadcast over the agar surface. Each Petri dish was then inoculated with a block (about 1 cm^3) of agar containing mycelium of a single fungus. Five replicate plates were prepared per isolate. Uninoculated Petri dishes served as controls. Petri dishes were sealed with Parafilm (Am. National Can Co., Greenwich, CT), and stored (3 weeks) at 22–24°C.

Seed germination was monitored for 5 months at 2 week intervals. Dishes were removed from darkness and seed germination/seedling development were scored on a scale of 0–5 where 0 = no germination; 1 = production of rhizoids (i.e., germination); 2 = rupture of the testa by enlarged embryo; 3 = appearance of promeristem; 4 = appearance of first true leaf; and 5 = elongation of true leaf; 6 = length >0.5 cm—suitable for removal from *in vitro* conditions onto new substrate. Plates containing seedlings >0.5 cm in length (Stage 6) were removed from darkness and illuminated by natural, filtered light at 22–24°C. Following the appearance of pigmented (green) tissues upon illumination, one Stage 6 seedling was stained to reveal the extent of fungal infection (Phillips & Hayman 1970), and mounted in a drop of glycerin. Pelotons were detected using a light microscope.

RESULTS AND DISCUSSION

Advanced-stage *E. conopseum* seedlings were obtained using the *Epulorhiza* endophyte (Econ-242) isolated from the same orchid that yielded seed. The other three endophytes (UAMH 7633, 6440, 6451) and the control (aseptic media) failed to promote seed germination. Seed embryos incubated in the presence of the three ineffective endophytes did not undergo any observable changes in size or color. Until now, it was not known that *E. conopseum* could be cultivated from seed using a naturally-occurring fungus. Our study also represents one of the few reports for an epiphytic orchid. Overall, 4.8 % of the seeds inoculated with *Epulorhiza* endophyte (Econ-242) had germinated 119 days after sowing; 2.0 % of these seed embryos developed to Stage 6 (FIGURE 1). These percentages albeit small, may be indicative of a generally ineffective endophyte, or may reflect low seed viability. The latter possibility seems reasonable because the seeds used in this study were derived from a single capsule. Seed germination and protocorm development to Stage 3 occurred 51 days after sowing. Emergence of the first true leaf (Stage 4) was observed 71 days after inoculation. The largest (Stage 6) seedlings were 0.9 cm long by day 119. All Stage 6 seedlings developed pigmented (green) tissues, particularly at the leaf-bearing end (FIGURE 2). Seedling (protocorm) morphology was similar in appearance to that observed for terrestrial species grown in symbiotic culture (Zettler & McInnis 1994, Zettler *et al.* 1995, Zettler & Hofer 1998). Pelotons indicated a parasitic symbiosis on the part of the orchid on the fungus had ensued (Clements 1988). We presume that the development of leaves and pelotons, advanced developmental landmarks, would render the seedlings suitable for transplantation to a substrate.

Although few of the 30,000+ species of orchids have been examined for endomycorrhizal fungi (C. Zelmer pers. comm.), *Epulorhiza* appears to be more prevalent in (temperate) terrestrial orchids (Currah & Zelmer 1992, Currah *et al.* 1997) than in epiphytic orchids (Richardson *et al.* 1993, Richardson & Currah 1995). The germination and development of *E. conopseum* using the *Epulorhiza* endophyte, and subsequent peloton formation observed in seedling tissues, confirms the endomycorrhizal abilities in the host. The symbiotic ability of the endophyte is further supported by a recent study that used the same fungus to germinate the seeds of another Florida epiphytic orchid, *Encyclia tampensis* (Lindl.) Small, in vitro (Burkhead *et al.* 1998). Interestingly, *E. conopseum* is exceptionally frost-hardy for an epiphytic orchid, and extends

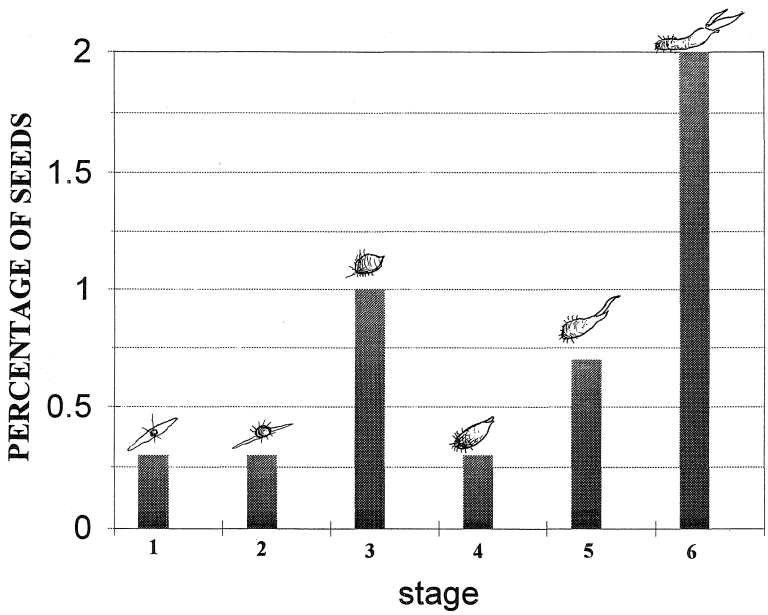

FIGURE 1. Percentage of *Epidendrum conopseum* seeds in a given developmental stage, 119 days after inoculation with fungal isolate Econ-242 (*Epulorhiza* sp.).

farther north than any other epiphytic species in the Western Hemisphere (Luer 1972). The northern range of this orchid suggests that *Epulorhiza* is a more cold-adapted genus than the other endophytic fungi associated with orchids. Considerably more orchid taxa should be examined for their endomycorrhizal fungi in order to determine the true distribution of *Epulorhiza* in temperate and tropical regions.

Epidendrum conopseum frequently grows on the large limbs of deciduous trees (i.e., *Quercus virginiana*) in association with resurrection fern (*Polypodium polypodioides*). Presumably, the fern's thick rhizome-root system intermixed with bark provides an ideal substrate for the orchid, its seedlings and fungi. It is conceivable that our strain of *Epulorhiza* (Econ-242) could be closely associated with resurrection fern, the host tree, or both. If true, this may partly account for the lack of the orchid on terrestrial substrates. That three other endophytes from terrestrial orchids failed to initiate germination supports the concept of fungal specificity by *E. conopseum* for its own endophyte. Although we did not isolate additional fungi from our root samples, we suspect that other symbiotic endophytes exist in *E. conopseum,* because fungal infection is reported to be sporadic in epiphytic orchids, in general (Richardson *et al.* 1993, C. Zelmer pers. comm.).

Mycorrhizal fungi play a vital role in the ecological prosperity of host plants (Arnolds 1991) and will be an important component of restoration efforts projected for the next century. Compared to other higher plants, orchids have a critical need for mycorrhizal fungi to initiate and sustain their life cycles in nature. The ability of any orchid population to perpetuate itself will ultimately depend on its ability to spawn seedlings—a process that requires fungi (Rasmussen 1995). Consequently, orchid endophytes should be recovered and preserved as quickly as possible to ensure that future conservation practices are rendered more effective (Zettler 1997b). The only reliable means to obtain such fungi is via their extraction from actual orchid tissues, but this will become increasingly difficult as orchid populations continue to decline (Rasmussen 1995). It has been proposed that epiphytic orchids are less dependent on endomycorrhizal fungi than temperate terrestrial species, in general (Rasmussen 1995). Indeed, mounting evidence suggests that mature epiphytic orchids harbor fewer pelotons in mycorrhizal roots; however, the physiological role of peloton-forming fungi in the epiphytic orchid life cycle remains largely unstudied (Richardson *et al.* 1993). Because our *Epulorhiza* endophyte promoted seedling development in vitro, it is conceivable that the same fungus could serve a physiological role in the mature host. For conservation purposes, it should be assumed that mature epiphytic orchids have a physiological

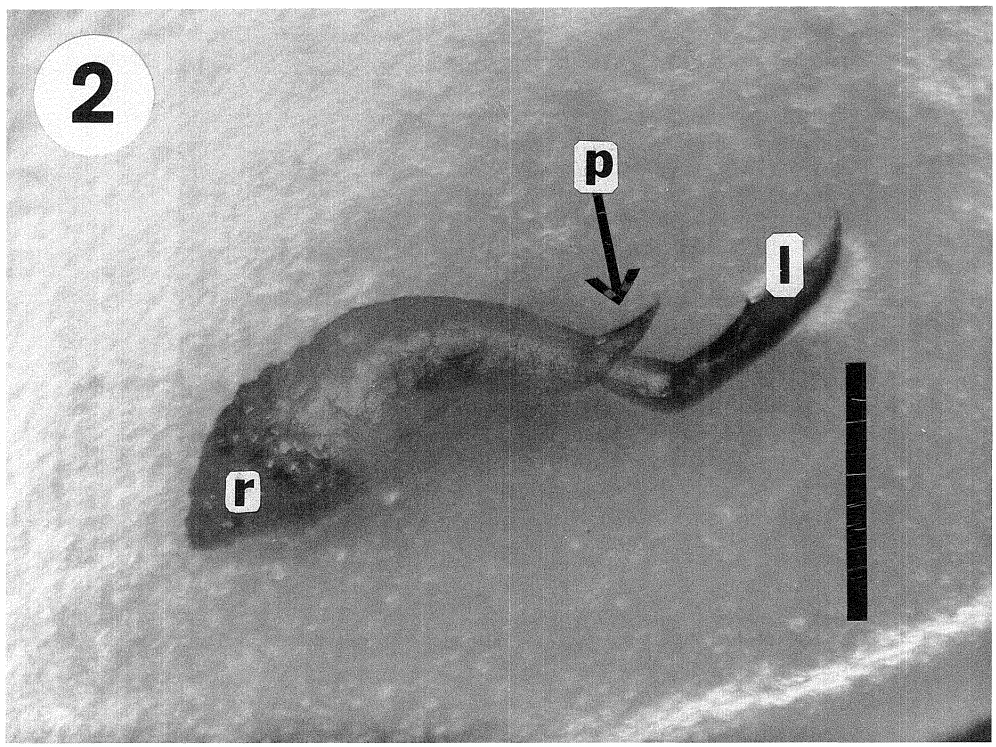

FIGURE 2. Differentiation of symbiotically-grown Stage 6 *Epidendrum conopseum* seedling in vitro, 130 days following fungal inoculation, and 19 days after exposure to light. Pigmented true leaf (l) is shown extending beyond the promeristem (p); traces of rhizoids are visible on lower portion of the seedling (r). Scale bar = ca. 2.5 mm.

need for endophytic fungi in nature, until more information becomes available.

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