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TERRESTRIAL ORCHID CONSERVATION BY SYMBIOTIC SEED GERMINATION: TECHNIQUES AND PERSPECTIVES

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ABSTRACT. Terrestrial orchids are generally regarded as one of the most vulnerable groups of higher plants, because they are difficult to grow from seed in the absence of mycorrhizal fungi. *In vitro* symbiotic seed germination often provides a reliable means to cultivate orchids from seed using naturally-occurring fungi, but its use in North America has been largely ignored. Mycorrhizal fungi should be included in propagation efforts to ensure that orchids persist in the natural setting. This paper summarizes the techniques associated with symbiotic seed germination (fungal isolation, identification and storage; seed collection and storage; seed pre-treatment, seed sowing and incubation; post-germination—*in vitro* to soil). It is anticipated that terrestrial orchid conservation will benefit from the techniques described herein.

INTRODUCTION

Mycorrhizal fungi are associated with the root systems of most terrestrial plants, and play a vital role in the ecological prosperity of the host (Arnolds 1991). These fungi receive sugars (energy) from photosynthesis by the host plant in exchange for critically-essential ions, namely phosphate and nitrate. In contrast, for members of the Orchidaceae, this partnership is reversed, where the fungus supplies the energy for the host plant (Rasmussen 1995). Most workers agree that the orchid-fungus symbiosis is a form of parasitism (Harley & Smith 1983, Clements 1988), because the fungus does not appear to benefit from the association and is actually harmed but rarely killed completely. In particular, coils of fungal hyphae (pelotons) are digested by various enzymes in the cortical region of root systems. As such, orchids are mycotrophs (fungus-feeders). All orchids seem to depend on mycotrophy during some phase of their life cycle (Arditti 1966, Dressler 1981, Rasmussen 1995). Epiphytic species rely on mycotrophy to a lesser extent because their above-ground habit provides them with greater access to light for photosynthesis (autotrophy), in general (Rasmussen 1995). Mycotrophy appears to be an absolute requirement for terrestrial orchids, particularly during seed germination and seedling (protocorm) development, processes that normally take place below ground in darkness. Fungal hyphae may be digested through adulthood, with mycotrophy supplementing photosynthetic nutrition. The reliance on mycotrophic nutrition by terrestrial orchids makes them one of the more vulnerable groups of higher plants (Rasmussen 1995). Growing terrestrial orchids from seed is often considered problematic; for some taxa it is a very slow process. This dilemma is further exacerbated by a general lack of information on orchid reproduction. Compared to large-scale attempts directed at seed propagation of temperate terrestrial orchids (e.g., Sainsbury Orchid Conservation Project in the UK), progress in North America has been relatively slow (Zettler 1996). Before many orchid species become extinct, efforts to develop effective protocols for artificial (seed) propagation should be a priority.

One possibility is the use of symbiotic seed germination. This technique, though not a "cure all" for propagation of terrestrial orchids, may be a useful way to germinate those species that have generally resisted artificial attempts at propagation. It is the purpose of this paper to provide a general understanding of the symbiotic techniques used to grow terrestrial orchids from seed in hopes that native taxa may be preserved. In addition, the long-term conservation of existing orchid habitats is discussed.

Symbiotic Seed Germination: General Methodology

In vitro symbiotic seed germination allows for mycotrophy to occur under artificial conditions. Best results are obtained by selecting a suitable fungus and by providing optimal seed pre-treatment for a given species. Unfortunately, this is a "hit or miss" affair; little or no germination could imply that the fungus or pre-treatment, or both were ineffective. Alternatively, a "shotgun approach" is most often preferred, where an arsenal of different fungal isolates and seed pre-treatments are tested. Such an approach is not practical for threatened and endangered species, where seed quantity is limited. I offer several suggestions to help maximize the probability of success with a minimum of effort and resources.

Isolation of Symbiotic Fungi (Endophytes)

Proper isolation of orchid endophytes (fungi) utilizes mycological concepts as well as good

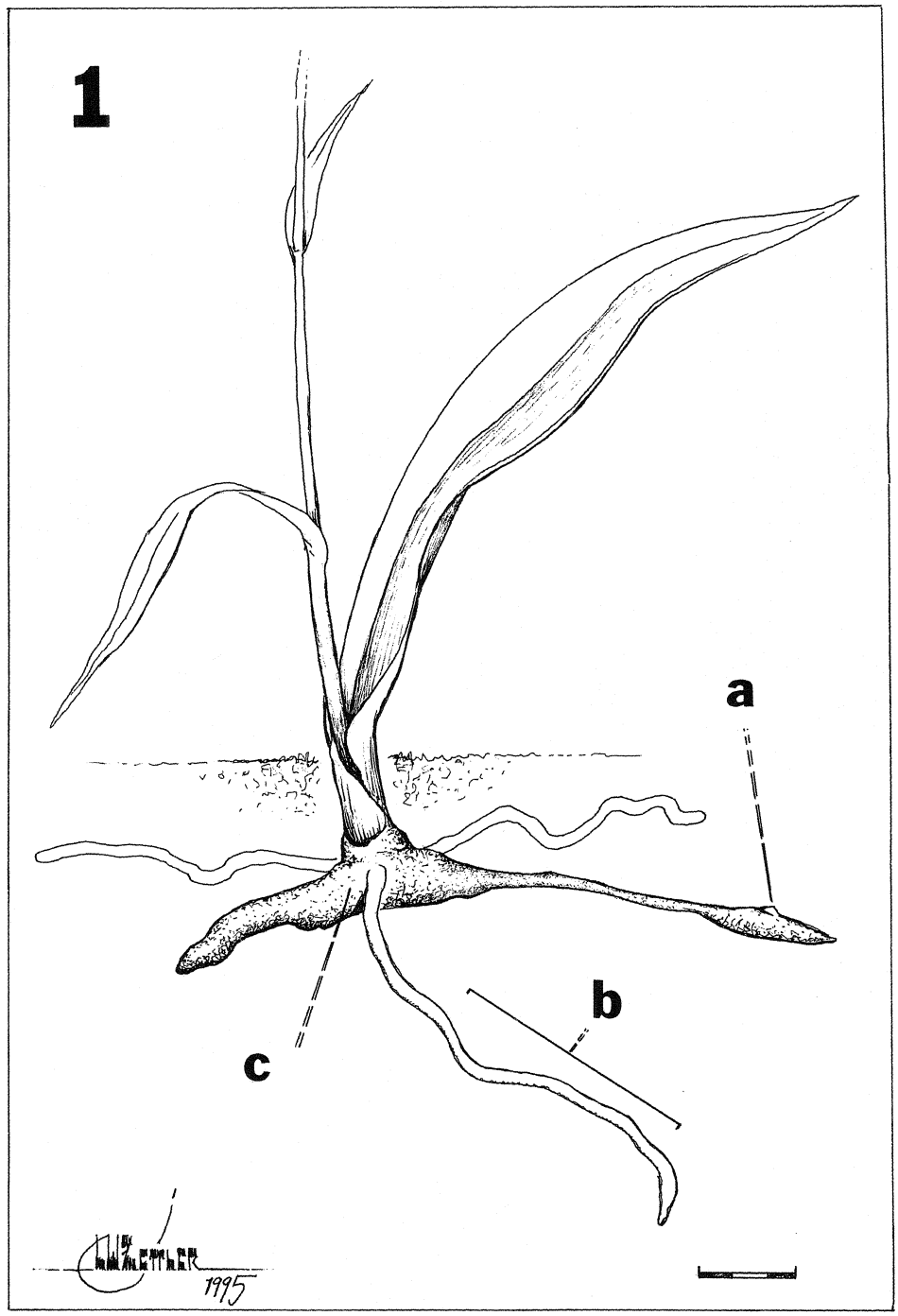

FIGURE 1. Generalized root system of *Platanthera* spp. showing next year's shoot (a), a portion of a lateral (branch) root that usually harbors viable pelotons (6), and the tuberous organ (c) that only rarely yields pelotons. Scale bar = 3 cm.

cernua. Monilioid cells are seemingly absent from pelotons in other orchids, but will readily form in pure culture. Of the three form-genera, *Epulorhiza* appears to be the most prevalent in our terrestrial orchids. Most members of the genus are cream-colored to dull orange on PDA or comparable media (FIGURES 3 & 4), and they generally have little aerial mycelia. Most grow readily on various media and are easy to subculture and maintain. Repeated subculturing over a period of several months or years runs the risk of contamination. One report (Alexander & Hadley 1983) proposed that repeated subculturing reduces a culture's effectiveness at seed germination, but I have found *E. inquilina* to be equally effective at germinating orchid seeds after 2 years of subculturing at 3 month intervals.

Long-term storage of endophytes for repeated use is most effective by growing the culture on a low nutrient medium such as water agar (0.7%) or oat meal agar (0.25% rolled oats in water agar) at cool temperature (ca. 5 C). I have observed viability >1 year under such conditions; other techniques (addition of mineral oil, lyophilization) that extend viability >1 year may also apply.

Seed Collection and Storage

Proper collection and storage of orchid seed has a profound impact on successful symbiotic seed germination. Unlike epiphytic species, terrestrial orchids produce far fewer seeds per capsule, many species' seeds are large (up to 1 mm in length), and most North American species are dependent on insect pollination for fruit set to occur; few (*Isotria medeoloides, Platanthera clavellata*) are auto-pollinated. The fact that percent germination was greater for seeds from a large population of *P. integrilabia,* compared to seeds obtained from two smaller populations (Zettler & McInnis 1992) implies that inbreeding depression may exist in smaller populations.

Seed collections should be made prior to capsule dehiscence (usually preceding first frost) because the interior of the capsule is relatively sterile and embryos are no longer dependent on parental photosynthetic material. For many species, especially members of *Platanthera,* yellowing capsules often yield ideal seed. Within 24 hrs of collection, capsules should be dried thoroughly using a desiccant such as Drierite ($CaSO_4$) to minimize invasion by potential opportunistic microbes. Reducing the moisture content to, and maintaining it, at about 5% extends viability of orchid seeds several years when exposed to low or sub-freezing temperatures (Rasmussen, 1995). Indeed, my *P. clavellata* seeds have remained viable >5 years (ca. 0% RH @ 6 and −7 C). Many terrestrial orchid species require a period of prolonged cold (stratification) that normally lasts several months for breaking dormancy and optimal germination. *Spiranthes cernua,* however, germinates readily without cold (<4 C) treatment. It is recommended that seeds be stored in complete and continuous darkness because embryos are light sensitive.

Pre-treatment / Seed Sowing / Incubation

Resistance to water uptake is more common for seeds of terrestrial orchids than for epiphytes, in general (Stoutamire 1983). Many orchid embryos must take up free water to prompt development; the most likely scenario is that the impermeable nature of the hydrophobic testa that encases the embryo, is stripped away (scarification) by natural weathering. Rinsing the seeds in a 5% solution of hypochlorite, preferably $Ca(OCl)_2$, artificially weathers the testa and surface-sterilizes the seeds for symbiotic seed germination. Exposure to calcium hypochlorite varies between 1 min to <10 hrs, though overexposure may actually reduce the potential of some seeds to germinate. Recommended treatment for *Epidendrum, Goodyera, Platanthera,* and *Spiranthes* is ethanol: 5.25% NaOCl (Clorox): water (1:1:18, v/v/v) for 1 min, and rinsed twice for 1 min in water without the use of a detergent.

Light is also an important factor. Seeds of most temperate terrestrial orchids are extremely light-sensitive especially during incubation; thus exposure to total darkness is recommended immediately after sowing. At least two species (*Dactylorhiza majalis* and *P. integrilabia*) whose seeds were pre-treated with light responded favorably with significantly higher germination (Rasmussen et al. 1990, and Zettler & McInnis 1994, respectively), though this is probably the exception rather than the rule. Therefore, seeds in short supply (rare species) should not initially be pre-treated with light.

Terrestrial orchids also appear to vary in pH preference with optimal germination between pH 5–8 or lower for bog-inhabiting species and higher pHs for those that inhabit alkaline soils. I typically try to match the pH of the agar medium to that of the original soil.

The seed is sowed as described (Dixon 1987). Briefly, freshly-scarified seeds are broadcast onto the agar surface of low nutrient rolled oat medium (Dixon 1987) followed by fungal inoculation. Media rich in simple sugars (sucrose, glucose, and others) display prolific fungal growth that may reduce germination, or make germination difficult to assess. A "stable" me-

dium such as oatmeal agar restricts fungus growth, offers a steady source of carbohydrate to the embryos, and changes in the chemistry of the agar from fungal metabolism are slower, enhancing embryonic development. Sowing seeds directly onto filter paper strips placed on the agar surface provides for greater oxygen exposure for the developing protocorms. Sealing of Petri plates (Parafilm, Am. National Can) maintains a high internal relative humidity and reduces the risk of contamination. Subsequent incubation should take place in darkness at a temperature between 22–25 C (cooler for *Cypripedium* spp.). Exposure to light can be considerably minimized by wrapping Petri plates in aluminum foil.

Post-germination Treatment (*in vitro* to soil)

Germination is achieved when an embryo swells and ruptures the testa and single-celled root-like rhizoids are evident, a distinguishing feature that represents true germination from size increase due to imbibition. Many seeds will germinate within 6 months. *Spiranthes* are notorious for germinating rapidly; seeds of *S. odorata,* for example, germinate within two weeks; and certain seeds, (e.g. *Isotria medeoloides),* refuse to germinate despite repeated treatments, and this implies that some dormancy mechanisms remain to be broken.

Seldom does germination and initial development culminate in plantlets (leaf-bearing protocorms). In *Platanthera* spp. (*P. integrilabia, P. clavellata,* and *P. cristata)* for example, <3 % germinated seeds continue development to plantlets suitable for soil transfer. Exposure to white light (30–60 μmol $m^{-2}s^{-1}$, LD 14h:16h) initiates the formation of photosynthetic tissues in many species, but rarely will it accelerate development. Developmentally-arrested protocorms may be incapable of developing further. I expose protocorms to light while in the Petri dish so that they rely less on mycotrophy once transplanted to soil.

Transfer of photosynthetic seedlings from *in vitro* to soil is a critical step. Because symbiotically-grown seedlings harbor a compatible fungus, they tend to have a higher survivability compared to seedlings grown in the absence of the fungus (FIGURE 5). Pre-inoculation of soil with the symbiotic fungus prior to the introduction of seedlings is highly recommended (Dixon 1987), which favors survivorship. Regular misting or spraying water helps prevent dehydration in the greenhouse, and this is particularly important for bog-inhabiting species such as *Platanthera.* Successfully transplanted seedlings can now be removed from the greenhouse and introduced into native areas.

General Interpretations

Focusing on seed propagation for long-term preservation of native terrestrial orchids provides for the expression of genetic variability that could not be achieved by asexual cloning. This means the preservation of large orchid populations with diverse gene pools as well as the habitats of insect-pollinators. There appears to be a critical requirement by terrestrial orchids for fungi to complete their life cycles, and the fungi of common orchids may play a heretofore unrecognized dimension in orchid ecology that involves below-ground infection patterns between different kinds of orchids. These considerations argue for the preservation of large areas to include all orchids, their fungi, and their insect pollinators. These efforts are anticipated to be considerably enhanced by the technique of symbiotic seed germination.

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