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Mycovitality and mycoheterotrophy: Where lies dormancy in terrestrial orchid and plants with minute seeds?

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Abstract

This article emphasizes the new concept of "mycovitalism": the relationship between fungi and seeds, maintaining their vitality and leading to germination. An innovative fungal bioassay to address the question of orchid seed viability is described in which viability is evaluated using co-culture with *Fusarium semitectum*. In viable seeds, this leads to seed coloration and germination. This *Fusarium* strain was isolated using myclobutanil agar and identified. Its phylogenetical status was defined based on sequences of the EF-1 alpha gene. The culture-independent PCR-DGGE fingerprinting method was optimized to assess *Fusarium* diversity *in situ* and select promising strains for orchid seed viability testing in orchid production using biotechnology. The *Orchidaceae* were selected as a model system for future studies on plants with minute seeds.

Keywords: Mycovitalism, PCR-DGGE molecular fingerprinting, EF-1 alpha, PCR, *Fusarium*, mycoheterotrophism, *Orchidaceae*

1. Orchid Seeds

Germination is an important indicator of seed viability, but it cannot be used as the only criterion in plants like terrestrial orchids which have minute (Szendrak, 1997) viable seeds that may not germinate. Foley (2001) emphasized the importance of physiological genetics and genes that regulate dormancy. However, physiological, morphological, chemical and mechanical barriers play a part as do other seed characteristics (Baskin and Baskin, 1998; Bewley and Black, 1994). Defining the environmental conditions that induce changes in seeds and their release from dormancy is a major challenge for germination ecologists.

Orchid species possess a puzzling diversity of seed germination responses for which there is no universally accepted classification (Rasmussen, 1995). Van Waes and Debergh (1986a) hypothesized that orchid seeds stay dormant because of unfavourable conditions. According to Silverton (1999) part of the problem lies in the fact that the mechanisms that control "dormancy" in plants are poorly understood. Most genera (e.g. *Cypripedium* and *Platanthera*) have minute/dwarf seeds of micro size <0.2 mm long. The embryo is undifferentiated: it consists simply of a mass of cells (from 25 to 100 cells) at the time of dispersal. Both differentiation and growth must take place in order for germination to occur. The cells have no food reserve, so that germination, hallmarked by protocorm formation (the cotyledon and radical are not formed) is dependant on fungi (Vujanovic and Vujanovic, 2006).

Biological vitality of living cells in orchid seeds (or their capacity to reach full-life functions) is in part myco-determined. We propose the term of "mycovitalism" to describe the phenomenon whereby compatible fungi in association with plant seeds lead to seed viability and germination. Another term "mycoheterotrophism" describes the situation that pertains in orchid protocorms (Fast, 1982; Peterson et al., 1998). It is evident at a more advanced stage of development than seed maturation and germination (Bruns and Read, 2000). The fungal partner(s) that can promote seed germination like *Fusarium* (Vujanovic et al.,

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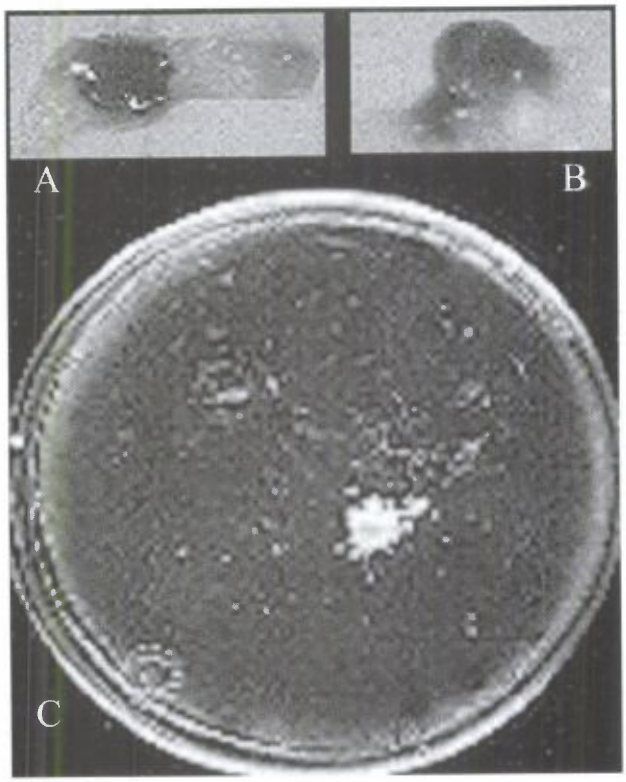

Figure 1. (A) *Cypripedium reginae* seed coloration, and (B) germination in co-culture, with (C) *Fusarium semitectum* wild strain *in vitro* (reprinted from Vujanovic et al., 2000).

2000) is not necessarily the fungal symbiont that is active in the "mycoheterotrophism" stage in the protocorm (Fig. 1).

At the "mycovitalism" stage, the fungal partner(s) seems to be functionally less specific than at the "mycoheterotrophism" stage (Shefferson et al., 2005; Taylor et al., 2002). This may provide an evolutionary advantage for the seeds, allowing them to meet an omnipresent fungal partner(s) such as *Fusarium* in the critical period of seed dispersal. This could be important for terrestrial orchids' adaptability and ability to colonize poor soil or disturbed land as a member of the pioneer vegetation. Some true mycorrhizal (*Rhizoctonia*-like) fungi could act at both mycovitalism and mycoheterotrophism stages in an orchid's developmental chronosequences, from seed germination to protocorm formation and full plant maturity (Fig. 2). Thus, under conductive environment conditions (Shimura and Koda, 2005), whether seed from a same population or genotype come in contact with a compatible fungal strain(s) *in situ* could determine if germination will occur in a single season or after several years.

From a biotechnological standpoint, it is important to concentrate on the fungal partners involved in mycovitality or germination events, as they have never been studied appropriately. Using orchids as a model system should enable a better understanding of the co-evolutionary processes involving mycovitalism in plants with small or minute seeds.

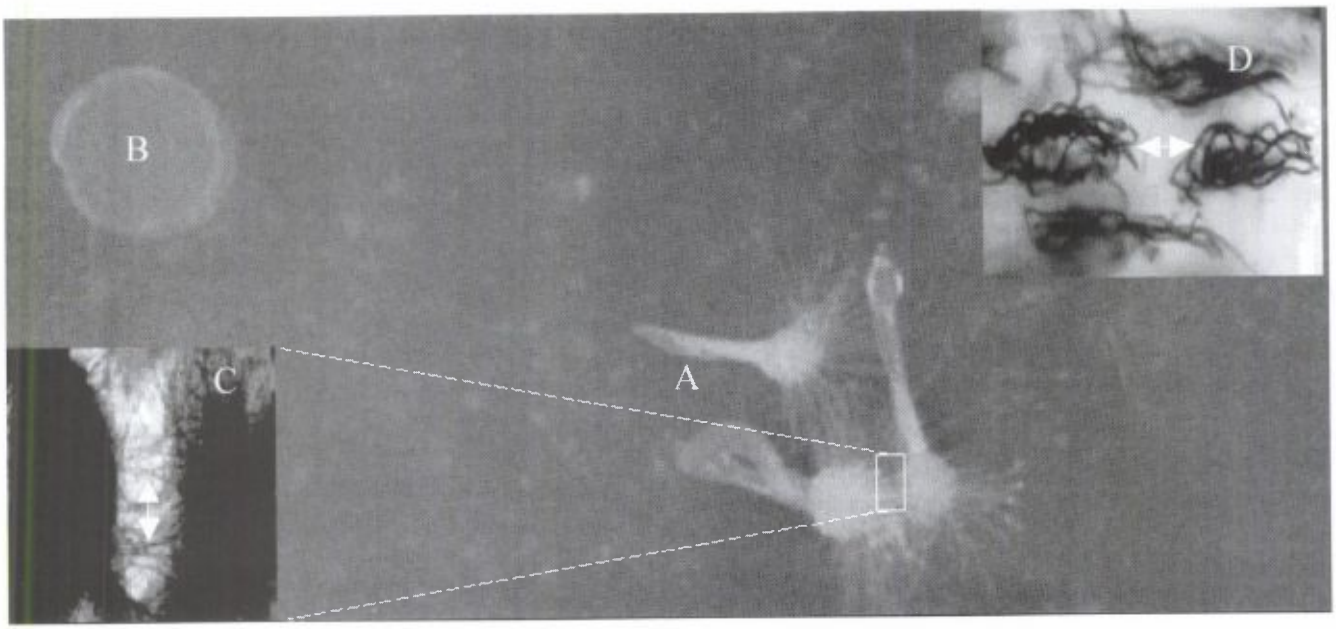

Figure 2. (A) Development of protocorms and young seedlings in co-culture, with (B) compatible *Rhizoctonia* fungal partner, (C) Anastomosis between hyphae and fusion between plant roots (arrows), and (D) details of fungal pelotons formation (arrows) in roots of *Cypripedium in vitro*.

2. Seed Germination in Orchids

The assessment of seed viability using fungi that colour seeds (Mycock and Berjak, 1995) was investigated and applied in biotechnology for the first time by Vujanovic et al. (2000). Mycorrhizologists demonstrated for some orchids that seed germination *in vitro* could easily be brought about by culturing seeds with specific fungi, e.g. *Rhizoctonia* sp. isolated from orchid mycorrhiza, roots and protocorms (Smreciu and Currah, 1989; Zelmer and Currah, 1997; Zettler, 1998). However, this co-culture germination method did not greatly increase the rapidity or proportion of seeds that germinated, reflected by the seed germination count (SGC). Symbiotic germination could take from a month or two to a year depending on the orchid species or seed lot (Arditti et al., 1990) and the ability of the specific fungal isolate to induce germination (Zettler, 1997). Other saprophytic, endophytic or non-mycorrhizal fungi are frequently isolated from orchid roots and protocorms. These include seed-born *Alternaria, Cladosporium, Cylindrocarpon, Fusarium, Trichoderma*, etc. but their role in orchid germination is unclear (Bernard, 1900; Currah et al., 1989; Zelmer, 1994).

Fusarium spp. are very common, occurring in many plants and soils (Nelson et al., 1981). They are red-pigment producers (Gordon, 1952). Some cause plant diseases while other are competitive with pathogenic fungi (Garcia-Romera et al., 1998) and some produce plant growth-promoting substances like gibberelin (Domsch et al., 1980). Red-pigment producing *Fusarium* sp. are reported to induce red staining and enlargement of healthy soybean seeds in the field (Clear et al., 1989). During a survey of microorganisms associated with terrestrial orchid species, a *Fusarium* isolate was found associated with red staining in the protocorms of *Cypripedium reginae, in situ*. The possibility that this isolate could stain viable orchid embryos *in vitro* and stimulate their seed germination was hypothesized. A study by Vujanovic et al. (2000) demonstrated the ability of this *Fusarium* to promote orchid seed coloration and germination (Fig. 1).

A bioassay was developed, using the *Fusarium* isolate (MT-O/258) from the protocorm of *C. reginae,* for assessing orchid seed viability. This bioassay was compared with the standard seed staining that used chemical procedures *in vitro*, i.e. TTC and acid Fuchsin (AF). The efficiency of the bioassay was similar to those of AF for *C. reginae*, *C. parviflorum* and *P. grandiflora*; and both were superior to TTC (Vujanovic et al., 2000). Our results clearly showed that the Red *Fusarium* bioassay, that involves the staining of viable terrestrial orchid seeds, could be an alternative to the standard chemical methods (Van Waes and Debergh, 1986b; Singh, 1981; Lauzer et al., 1994). Moreover, the bioassay is more appropriate for embryo viability estimation after prolonged chemical pretreatment of orchid seeds, which is needed to promote germination of dormant seeds (Malmgrem, 1996). We also obtained *in vitro* seed germination of *C. reginae* using the same *Fusarium* isolate. These studies confirmed Bernard's (1900) early results, which indicated that *Fusarium* sp. could promote orchid seed germination. Our results confirm that in addition to *Rhizoctonia*, non-mycorrhizal fungi may also be involved in germination.

3. Identity of the Fusarium Isolate

To study *Fusarium* biodiversity, Vujanovic et al. (2002) developed a genus-specific (selective) myclobutanil agar (MBA) medium for the isolation of *Fusarium* species occurring in plant roots or soil samples. The high phenotypic plasticity and variations in morphological characters render the task of species identification difficult (Gerlach and Nirenberg, 1982; Nelson et al., 1983). Therefore, it was necessary to develop a simple and reliable method to characterize *Fusarium* biodiversity samples.

A culture-independent PCR-denaturing gradient gel electrophoresis (DGGE) assay to assess *Fusarium* biodiversity from environmental samples was developed by our research group (Yergeau et al., 2005). *Fusarium*-specific PCR primers (Alfie1 and Alfie 2) targeting a specific region of EF-1 alpha gene were designed. Their specificity against genomic DNA extracted from a large collection of closely related and distant organisms was tested (Vujanovic et al., 2006; Yergeau et al., 2005). The accuracy and separation potential of DGGE was tested over a range of twenty-one *Fusarium* species composed of 40 different isolates. The technique distinguished between most species and also between different *formae speciales* of *F. oxysporum*. Genomic DNA was extracted from field-grown plants naturally infested with *Fusarium* species, submitted to PCR amplification and DGGE analysis. Results indicated that the obtained DNA sequences were all affiliated with *Fusarium* species, clearly supporting the specificity and usefulness of this approach to study *Fusarium* species diversity from environmental samples.

The red-pigment producing strain of *Fusarium* (MT-O/258) that promotes germination in orchids has been identified as *F. semitectum* (Vujanovic and Vujanovic, 2006). Using the approach described by Vujanovic et al. (2006), the phylogenetical position of this *Fusarium* strain based on the EF-1 alpha gene (Fig. 3) was determined (Vujanovic and Vujanovic, 2006). The *F. semitectum* MT-O/258 forms a separate clade which is close to the clade of sister *F. acuminatum* and *F. sambucinum,* for which a phenotypic distinction was difficult. Following the procedure of PCR-DGGE methods described by Yergeau et al. (2005), the fungus was then recognized from roots of *C. regine*. The distinctive band of *F. semitectum* was specifically identified by its different migration position on the gel in comparison with *F. sambucinum*, *F. scirpi*,

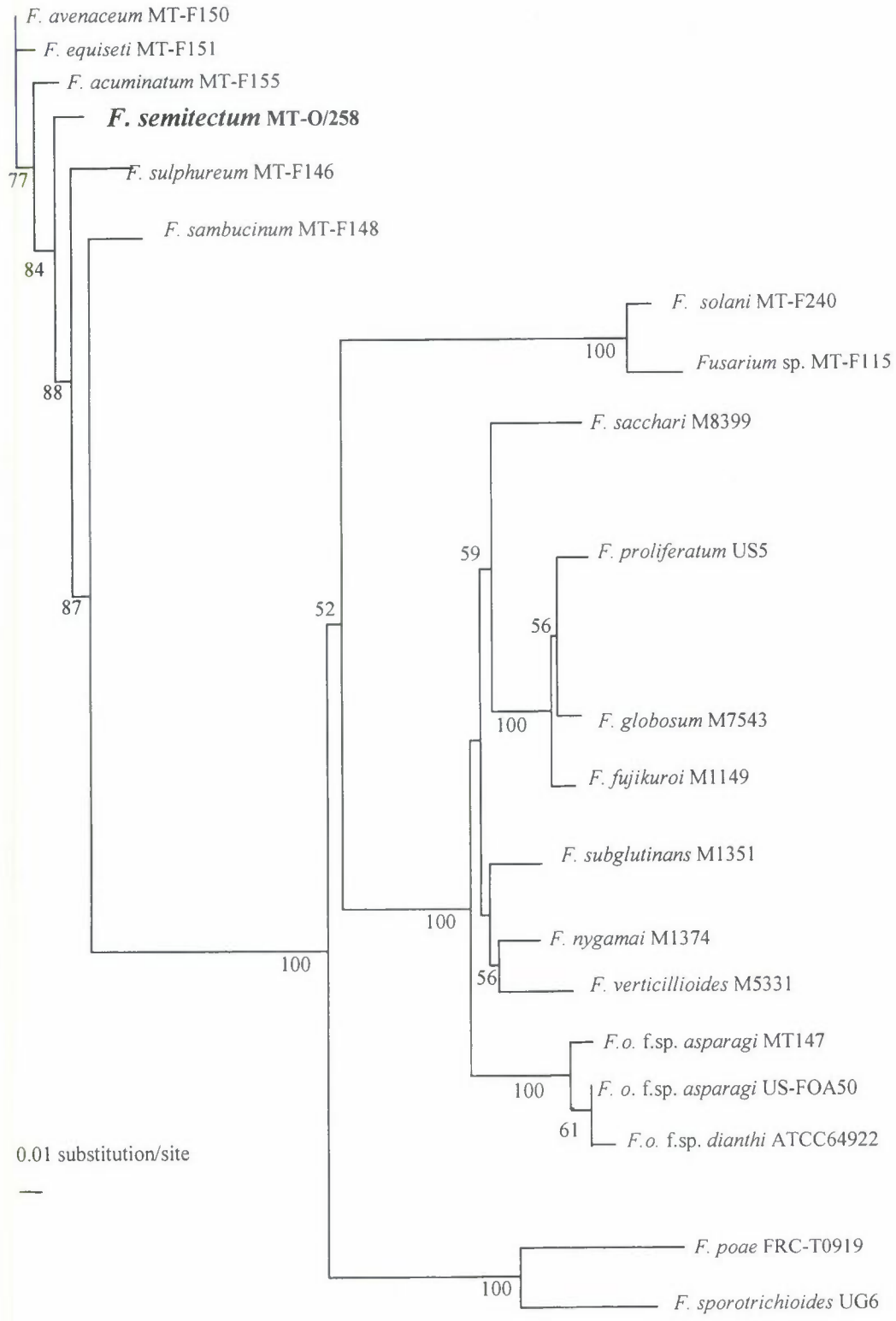

Figure 3. Unrooted distance tree showing the similarity between EF-1 alpha gene sequences obtained from 20 *Fusarium* strains and indicating the position of *F. semitectum* (MT-O/258) used in our bioassay. The values of the bootstrap analysis (1,000 repetitions) are given at the nodes. Molecular marker: (1) *F. subglutinans* (UG6), (2) *F. sambucinum* (University of Montreal MT-F148) and (3) *F. solani* (MT-F240). MT: University of Montreal, QC, Canada; FRC: Fusarium Research Center, Pennsylvania State University, Philadelphia, PA, USA; UG: University of Guelph, Guelph, Ont., Canada; ATCC: American Type Culture Collection, Manassas, VA, USA; M: McGill University, Montreal, QC, Canada; US: Connecticut Agricultural Experimental Station, New Haven, CT, USA.

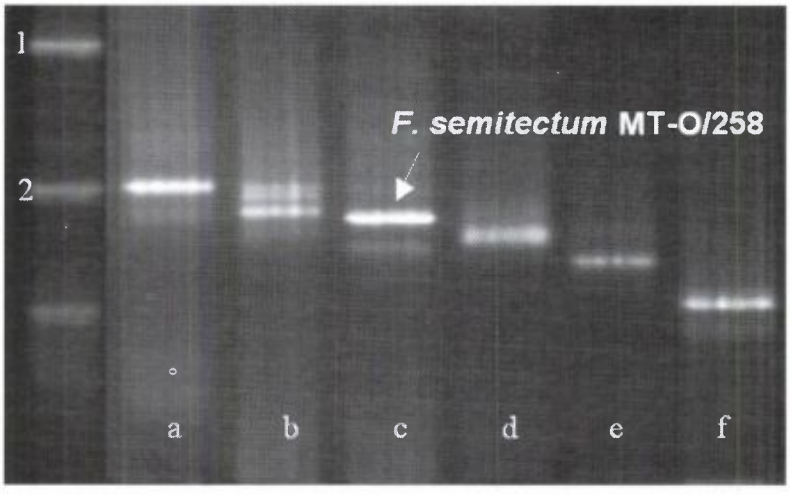

Figure 4. DGGE separation of EF-1 alpha gene amplicons for 6 *Fusarium* isolates: (a) *F. sambucinum* (MT-F149); (b) *F. scirpi* (MT-F153); (c) *F. semitectum* (MT-F144); (d) *F. verticillioides* (M-5331); (e) *Fusarium* sp. (MT-F115); (f) *F. solani* (MT-F240). Molecular marker: (1) *F. subglutinans* (UG6), (2) *F. sambucinum* (University of Montreal MT-F148), (3) *F. solani* (MT-F240). MT: University of Montreal, QC, Canada; FRC: Fusarium Research Center, Pennsylvania State University, Philadelphia, PA, USA; UG: University of Guelph, Guelph, Ont., Canada; ATCC: American Type Culture Collection, Manassas, VA, USA; M: McGill University, Montreal, QC, Canada; US: Connecticut Agricultural Experimental Station, New Haven, CT, USA.

F. verticillioides; *Fusarium* sp. (MT-F115) and *F. solani* also present in orchid roots, as shown in Fig. 4. Subsequent band extraction and sequencing (EF-1 alpha gene), as well as sequence analysis, when compared with GenBank databases, confirmed the efficacy of the two complementary (phylogenetical and DGGE) proposed methods to discriminate *Fusarium* species. This protocol confirmed clearly that *F. semitectum* in orchids belongs to the Red *Fusarium* group (Vujanovic et al., 2006), whose members are known as pigment producers (Gordon, 1952).

4. Discussion

Our results led to the conclusion that *Fusarium semitectum,* as an orchid endophyte, can recognize viable orchid embryos and induces staining and germination. By promoting *Cypripedium* germination, *F. semitectum* MT-O/258 compatible isolates have demonstrated the existence of mycovitalism. The percent of induced stain and germination was (for *C. reginae, C. parviflorum*, and *P. grandiflora*) indirectly proportional to the sodium hypochlorite pretreatment duration (Vujanovic et al., 2000). The advantage of the bioassay, compared to the chemical procedure, is that it can be used to study the pretreatment effects on seed germination, such as decreased embryo vigour during longer sterilizing treatments. In addition to being more sensitive, it is also a non-cancerigenic method contrarily to many chemical dyes. Furthermore, this non-destructive approach induces both viable seed coloration and germination and thus can differentiate with more specificity viable from non-viable seeds. The widespread use of the bioassay staining procedure for orchid seed viability testing requires research on factors such as fungal specificity and optimization of the test (Zettler, 1997). The efficiency and efficacy of the three available staining methods [*Fusarium*-bioassay (+) with TTC (+) and AF (–)] should be evaluated on various orchid taxa. Our new bioassay approach could also be applied to other plants with tiny seeds that are important in horticulture or agriculture.

The recent North American strategy for orchid conservation proposes the symbiotic method for orchid seed germination (Peterson et al., 1998; Zettler, 1998). In the light of our results, special attention should now be paid to saprophytic and endophytic fungi (including *Fusarium* sp.) that could be important in mycovitality. Like the symbiotic *Rhizoctonia,* they may play a role *in situ* and/or *in vitro* growth of orchids that will develop "mycoheterotrophism" later on.

5. Conclusions

This integrated approach, combining fungal isolation using *Fusarium*-specific myclobutanil agar (MBA) medium, fungal phylogenetical analyses using EF-1 alpha gene, fungal assessment using DGGE fingerprinting molecular analyses and bioassay using *F. semitectum* co-culture *in vitro*, is proposed as a powerful tool to: a) distinguish and select specific fungal strains, b) characterize potential fungal strains for orchid biotechnology production, c) to induce seed coloration and germination, d)

evaluate host-fungus relationships, seed *mycovitality* and *mycoheterotrophy* in plants, and e) assess their dispersion and dynamics *in situ*. These techniques could be optimized, enlarging the spectrum of fungal candidates valuable in current efforts to preserve orchids and develop an appropriate biotechnology for their *in vitro* germination, growth and production.

The mycoheterotrophism in orchid plants follows seed germination and appears during the first protocorm formation and lasts throughout plant maturation (McKendrick, 2002). However, it is unclear whether orchids at the mycovitalism stage are involved in a truly mutualistic symbiotic relationship. Nevertheless, it seems that seed-borne *Fusarium* sp. may be dispersed with minute orchid seeds, as vectors, and in a second phase maintain viability and enhance orchid seed germination. The ultimate question arises: "Where does dormancy lie in terrestrial orchid plants with minute seeds?". This question is still a bit of a "mystery" in orchids, a group of plants that has evolved an incredible diversity over a short evolutionary period, and has dispersed throughout all terrestrial ecosystems. This question awaits further scientific exploration.

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