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Isolation of Endophytic Fungi and Their Mycorrhizal Potential for the Tropical Epiphytic Orchids *Cattleya skinneri*, *C. aurantiaca* and *Brassavola nodosa*

[1]Isidro Ovando, [1]Anne Damon, [1]Ricardo Bello, [2]Dolores Ambrosio,
[2]Víctor Albores, [2]Lourdes Adriano and [2]Miguel Salvador
[1]El Colegio de la Frontera Sur, Carretera Antiguo Aeropuerto Km 2.5, A.P. 36,
Tapachula, 30700, Chiapas, Mexico
[2]Departamento de Biotecnología, Facultad de Ciencias Químicas, Universidad Autónoma de Chiapas,
Carretera a Puerto Madero Km 2.0, Tapachula, 30700, Chiapas, México

Abstract: In the present study, we screened endophytic fungi in roots of mature plants of the epiphytic orchid *Cattleya skinneri* to test their potential to improve seed germination in *C. skinneri* and growth and development in acclimatizing plantlets of *C. aurantiaca* and *Brassavola nodosa*. We isolated 108 fungal colonies belonging to 11 genera and statistical differences were not found between samples according to season or orchid substrate. Germination experiments were carried out using three methods and eight strains that proved to be manageable under laboratory conditions. None of these strains proved to be promoters of seed germination in *C. skinneri*. For *ex vitro* plants of *C. aurantiaca,* three strains (*Trichoderma* IBTZ-0200F, *Epulorhiza* IBTS-0200G and *Botrytis* IBTZ-0200A) showed mycorrhizal characteristics, whereas none of the evaluated strains promoted plant growth in *B. nodosa*. Fungal strain specificity and their function as mutualists, neutral or latent pathogens are discussed in the light of the results obtained.

Key words: Epiphyte, orchid, mycorrhiza, germination, *ex vitro* plants

INTRODUCTION

Soconusco, in Chiapas, Southern Mexico, is a typical humid tropical region characterized by high plant diversity, within which vascular epiphytes, such as orchids, are an interesting guild because of their ecological, economic and social importance[1]. Although the orchidaceous flora of Soconusco region has been poorly studied, inventories exist reporting approximately 150, mostly epiphytic species[2,3]. However, changes in land use, industrialized agriculture, contamination and uncontrolled collection for ornamental and commercial purposes, among other complex factors, have led to local extinction and the classification of 33 native orchid species as rare or endangered; *Cattleya skinneri* Bateman, the best known and sought after orchid species in Soconusco region, is included within this list[4].

As a contribution to the conservation of the more exploitable or endangered Soconuscan orchids, our group has set up a program for the *in vitro* propagation of orchids using seeds and the cultivation of the resultant plants in coffee and cocoa plantations. A frequent problem with *in vitro* orchid propagation is the lack of ability of *ex vitro* plantlets for adequate acclimatization to field conditions[5-7], particularly in the hostile, warm, humid and pathogen propitious environments of the humid tropics. It is, therefore, necessary to develop low cost methods for hardening off orchid plantlets that permit improved growth and development of seedlings and higher rates of *ex vitro* survival. One such method is the inoculation of plantlets with Plant Growth Promoter Microorganisms (PGPM), currently used in various micropropagated plant species[8].

Isolates of potentially mycorrhizal endophytic fungi from orchid roots are the most studied PGPM in orchids[9,10], although bacteria have also been mentioned in this context. Orchid mycorrhizal fungi are deuteromycetes or basidiomycetes with the *Rhizoctonia* complex being most representative[11]. When a true mutualist mycorrhizal relation is established, several benefits are obtained for the orchid, including enhanced access to nutrients and water, accelerated growth and development of protocorms and protection of seedlings against root pathogens[12].

It is important to note that: a) almost all work on orchid mycorrhizae has been carried out with temperate

Corresponding Author: Anne Damon, El Colegio de la Frontera Sur, Carretera Antiguo Aeropuerto Km 2.5, A.P. 36, Tapachula, 30700, Chiapas, Mexico
Tel: 962 6 28 10 77 5200 Fax: 962 6 28 10 15 E-mail: adamon@tap-ecosur.edu.mx

terrestrial orchids and studies upon tropical epiphytic orchids have been modest[13]; b) orchid root endophytic fungi are currently mentioned as mycorrhizal without confirming bioassays; it is, therefore, more accurate to use the term endophytic[14,15]; c) orchid-fungi interactions have been shown to be variable and unstable[16], hence, although is possible to extrapolate results from one orchid-fungus system to anothe[17], it is necessary to study each system individually, emphasizing the environmental influences on the relationship and to determine simple, measurable parameters indicating the mycorrhizal potential of fungal isolates.

In this study, we identified eleven genera of endophytic fungi, isolated from mature plant roots of the epiphytic orchid *Cattleya skinneri* growing in the Soconusco region. Under Soconuscan summer conditions, we then evaluated the mycorrhizal potential of eight selected strains upon *ex vitro* plants of the epiphytic orchids *C. aurantiaca* (Bateman ex. Lindley) P.N. Don and *Brassavola nodosa* (L.) Lindley, as well as on *C. skinneri* seeds. We discuss fungal effect based on growth promotion ability and influence on plant health.

MATERIALS AND METHODS

Study site: The screening of orchid root endophytic fungi and their evaluation on *ex vitro* plants was conducted at the Regional Botanical Garden El Soconusco of ECOSUR (El Colegio de la Frontera Sur), situated at 80 masl in the municipality of Tuzantán, Chiapas, Southern Mexico (15°07'03 N, 92°24'53 W)[18] and the orchidarium Santo Domingo (850 masl, 15°01'48 N, 92°06'21 W)[18]. The climatic and vegetation characteristics of these two sites are typical of Soconusco region[3].

Fungi isolation: Using a sterile scalpel, apparently healthy roots were excised from two mature plants of the epiphytic orchid *C. skinneri*, growing on each of four different substrates (Table 2) in the Botanical Garden, in February (dry season) and July (rainy season) in 2000. In order to avoid dehydration, the roots were placed in sterile glass test tubes with the cut edges submerged in 0.89% NaCl solution, then cooled and carried to the laboratory for processing as soon as possible[19]. Roots were superficially disinfected by soaking in 0.5% NaClO solution for five minutes, followed by three rinses in distilled water. Under aseptic conditions roots were divided onto three sections, root apex, medium zone (photosynthesing) and mature zone (with white velamen). Each section was then sliced into 1 mm thick discs, plated onto Potato Dextrose Agar (PDA) and incubated in darkness for eight days at 27°C. Resulting fungal colonies were isolated, purified and then identified using the morphology of fructiferous bodies and spores[20,21]. Slants and spores were conserved in Ringer solution and stored at 4°C until use.

Fungal effect on seed germination: Semi-mature capsules of *C. skinneri* were collected from the Botanical Garden and the field in January 2001, cleaned and stored at 25°C in bottles containing anhydrous calcium and magnesium chlorides (Easy Dry™, OSMAR, Mexico) to prevent high humidity and rotting. During spring and summer 2001 seed-fungi co-cultures were set up testing three germination methods. First, following Masuhara and Katsuya[22], filter paper disks on PDA plates were aseptically inoculated with approximately 100 randomly distributed seeds and a fragment of fungal colony was placed in the center of the paper. Second, two or three seeds encapsulated in alginate/chitosan spheres (4 mm diameter)[23] were placed on PDA plates pre-inoculated with fungus one week in advance, as in Khor *et al.*[17]. For the third method we used Petri dishes containing one 20 cm^2, 48% humidity, fragment of cedar bark (*Cedrela odorata* L. (Meliaceae)), on which natural germination of some orchids is occurring in the region. Previous experiments on the influence of bark humidity on seed germination, without fungi, showed that 45-48% humidity and vertically positioned bark results in 75±11% initial germination, which we defined as the swelling of the seed and the acquisition of a pale green color. Bark fragments were autoclaved and inoculated with 0.5 mL of a solution of $1x10^3$ spores/mL. Approximately 100 seeds were then distributed on each fragment and petri dishes were vertically positioned. For each of the three methods, five replications of nine treatments (eight fungal strains and a blank; Table 1) were carried out, under conditions of 12/12 h photoperiod (~3000 lux, full spectrum fluorescent light) and 26°C. After six weeks, we evaluated percentage germination, defined as mentioned above.

Fungal effect on *ex vitro* plants: We used *ex vitro* one-year-old plants of *C. aurantiaca* and *B. nodosa* resulting from the program for the propagation of seeds of several native orchid species; at this time, plantlets of *C. skinneri* were not ready for planting out. From June to October 2001, we evaluated fungal effect on plant growth and health, under Botanical Garden conditions. 30-40 plants per treatment were sown in trays containing 1 cm^3 fragments of clay tiles, which had proved to be the best substrate for the developing plants (Damon, unpublished). One month later, the young roots were inoculated with 1 mL of a solution of $1x10^4$ spores mL^{-1}

Table 1: Fungal strains tested on *Cattleya skinneri* seeds and *ex vitro* plants of *C. aurantiaca* and *B. nodosa*[*]

Genera	Strain	Isolation season	Isolation site
Botrytis	IBTZ-0200A	Dry	Botanical Garden
Pestalotiopsis	IBTS-0200B	Dry	El Soconusco Orchidarium
Verticillium	IBTZ-0200C	Dry	Santo Domingo
Aspergillus	IBTZ-0200D	Dry	El Soconusco
Fusarium	IBTS-0200E	Dry	El Soconusco
Trichoderma	IBTZ-0200F	Dry	Santo Domingo
Penicillifer	IBTZ-0600A	Rainy	El Soconusco
Epulorhiza	IBTS-0200G	Dry	El Soconusco

[*]Other isolated strains were not ready for tests

of each fungal strain (Table 1); distilled water was used as a blank. Plants were not fertilized. Data for plant height (from base to apex for *C. aurantiaca* and longest leaf length for *B. nodosa*), leaf number, health and mortality were collected at the beginning of the experiment and monthly thereafter during three months. At end of the experimental period, three plants per treatment were randomly sampled for fresh and dry weight determinations. Root preparations for light microscopy were made as recommended by Phillips and Hayman[24] to verify fungal infection.

Data analysis: To compare the effect of substrates and season upon the number of fungal colonies within the samples, a student's t test was used. For analysis of fungal effect on *ex vitro* plants, data of randomly assigned treatments were analyzed by ANOVA and means compared by Tukey`s test (α=0.05). A Plant Health Index (PHI), which combines pathogenicity/mortality data of n plants in relation to time (cumulative data) and to the blank (not inoculated), was constructed using the following expression:

$$PHI = \sum_{i}^{n} S_{treatment}/n - \sum_{i}^{n} S_{blank}/n$$

Values for S (individual sanitary status) were: 2.0 for totally healthy plant, 1.5 when a little rot or localized yellowing was present, 1.0 for two to more areas of rot or generalized yellowing/browning and 0.0 for a dead plant. A fungal strain was considered as a plant health promoter if PHI was a positive number, neutral if zero and pathogenic when negative.

RESULTS

Isolated fungi: One hundred and eight isolates of endophytic fungi were obtained from root tissues of the epiphytic orchid *C. skinneri* growing in the Soconusco region, being possible to identify the majority to genus level (11 genera), except for those isolates that failed to fruit even in agar-water medium. Variations occurred between substrates and seasons, as shown in Table 2. More isolates were obtained from live substrates in the dry season, but no statistical differences were found either between substrates (Student's t, $p>0.05$) or seasons (Student's t, $p=0.0608$). Analysis per root zone revealed that the root apex is not a host to endophytic fungi in the dry season and supports only light infections (5.5%) in the rainy season; whereas 41.6 and 52.7% of the fungi were isolated from the root medium zone and mature zone, respectively.

Effect of isolates on germination: None of the fungal strains showed symbiotic (mycorrhizal) effects upon seeds of *C. skinneri*. With the first method, all fungal strains overgrew in a few days and their effect was detrimental for all seeds; while in the blank (without inoculum) 50.5±6.2% of seeds developed into green protocorms. When encapsulated seeds were used, fungal mycelia penetrated the spheres and made contact with the seeds more slowly that in the first method; their pigments and the fungi per se caused seed inhibition; 49±10% of fungus-free encapsulated seeds developed into protocorms. On the other hand, seed-fungi co-cultures on cedar bark revealed initial germination with *Botrytis* IBTZ-0200A (10.4±4.2%), *Epulorhiza* IBTS-0200G (7.6±2%) and *Pestalotiopsis* IBTS-0200B (6.5±2.5%), but this was minimal in relation to the blank (62±7.3%). We left the protocorms on the fungus-free bark fragments, undisturbed, to check for continued development and observed that, after 14 weeks, the majority of individuals had acquired a dark green color and had formed foliar primordia.

Effect of isolates on *ex vitro* plants of *C. aurantiaca*: Plant height was the most representative variable of plant growth. Other variables were not used for analysis because of their high Standard Deviation (almost 50% for number of leaves) yet they were helpful for other analyses. The growth kinetic of *ex vitro* plants of *C. aurantiaca* inoculated with eight fungal strains isolated from *C. skinneri* is showed in Table 3. From the second month, the height of plants was clearly greater for plants inoculated with *Trichoderma* IBTZ-0200F (Table 3). In contrast, the blank showed the least plant growth during the three-month experimental period. Statistical analysis revealed no significant differences between the non-inoculated plants and *Aspergillus*, *Penicillifer* and *Verticillium* treated plants (Table 3).

Numerical analysis of fresh weight revealed that *Trichoderma*-treated plants were no different to the *Botrytis*, *Epulorhiza* and *Fusarium* treatments, which

Table 2: Fungi isolated from *Cattleya skinneri* roots in the Botanical Garden El Soconusco, Southern Mexico

	Dry season		Rainy season	
Substrate	#Isolates	Genera	#Isolates	Genera
Mangifera indica branches	13	*Trichoderma, Verticillium* and *Aspergilus*	17	*Epulorhiza, Penicillium* and *Verticillium*
Apocynaceae tree	17	*Epulorhiza*, *Fusarium*, *Monilliopsis* and *Pestalotiopsis*	18	*Penicillifer* and *Tetracladium*
Dead branches	7	*Botrytis*, *Epulorhiza* and *Verticillium*	26	*Epulorhiza* and *Trichoderma*
Clay	0	--------	10	Epulorhiza

Table 3: Plant height, fresh and dry weight and water content of *ex vitro* plants of *Cattleya aurantiaca* inoculated with fungal strains

Strain	Plant height increase (%)	Fresh weight/ plant (g)	Dry weight/ plant (g)	Water content (%)
Trichoderma	30.34a*	1.21a	0.07d	94.5a
Pestalotiopsis	23.06b	0.68c	0.09c	86.7c
Epulorhiza	23.01b	1.09b	0.06e	94.4a
Botrytis	22.72b	1.27a	0.16a	87.4c
Fusarium	21.89b	1.05b	0.10b	90.4b
Aspergillus	11.69c	0.29e	0.09b	68.9e
Penicillifer	08.43c	0.56c	0.08c	85.7c
Verticillium	07.70c	0.49e	0.07d	85.7c
Non inoculated	06.17c	0.46e	0.11b	76.1d

*Values followed by the same letter denote no significant difference at 95% significant level (ANOVA, Tukey test)

Table 4: Plant height, fresh and dry weight and water content of *ex vitro* plants of *Brassavola nodosa* inoculated with fungal strains

Strain	Plant height increase (%)	Fresh weight/ plant (g)	Dry weight/ plant (g)	Water content (%)
Non inoculated	15.50a*	1.18b	0.10b	91.5b
Trichoderma	14.77a	0.69d	0.06c	91.3b
Fusarium	13.19a	0.68d	0.07c	89.7c
Epulorhiza	08.41ab	1.68a	0.15a	91.1b
Penicillifer	08.04ab	0.41e	0.03d	91.8a
Verticillium	04.50bc	0.41e	0.04d	90.3c
Pestalotiopsis	01.12c	0.68d	0.07c	89.7c
Botrytis	00.73c	0.93b	0.10b	92.8a
Aspergillus	00.13c	0.84c	0.07c	91.6b

*Values followed by the same letter denote no significant difference at 95% significant level (ANOVA, Tukey test).

indicates that plants inoculated with *Trichoderma* were taller, but less dense. Fresh weight values similar to the above-mentioned treatments were expected for *Pestalotiosis*-treated plants, but a lower value was observed, indicating lower density. On the other hand, dry weight analysis revealed that the *Trichoderma* and *Epulorhiza*-treated plants had higher growth rates but very low dry weights, indicating a relatively high water content (Table 3).

Effect of isolates on *ex vitro* plants of *B. nodosa*: Unlike *C. aurantiaca*, none of the fungal isolates promoted growth of *B. nodosa*. The heights of inoculated plants were slightly lower, but statistically similar to the blank (Tukey, α=0.05) (Table 4) for *Trichoderma*, *Fusarium*, *Epulorhiza* and *Penicillifer*-treated plants. As with *C. aurantiaca*, some treatments (e.g. *Epulorhiza* and *Botrytis*) produced short, but heavy plants, nonetheless, all plants of *B. nodosa* were similar in water content (Table 4).

Effect of fungal strains on plant health: As mentioned above, some fungal strains promoted plant growth by an increase in plant height, fresh or dry weight. Nevertheless, the results do not give sufficient evidence to support the hypothesis that the fungal strains have a positive effect upon the development of young orchid plants. Some inoculants causing enhanced plant height in surviving individuals were pathogenic or debilitating when analyzed in relation to the Plant Health Index (PHI) (Table 5). As explained above, PHI takes into consideration surviving and dead plants and compares them with the Non-inoculated plants. In this way, *Pestalotiopsis* IBTS-0200B and *Fusarium* IBTS-0200E were pathogenic or debilitating to plants of *C. aurantiaca* (negative values of PHI), even though plant height increases were higher in relation to the blank (Table 3). In contrast, the same *Fusarium* IBTS-0200E strain was the major plant health promoter in *B. nodosa* (Table 5).

Confirmation of fungal infection: Analysis of the roots of the young plants of *C. aurantiaca* and *B. nodosa* showed that both were fungal infected, most probably by the inoculated strains as environmental contamination was minimal during the experimental period. Intercellular and intracellular hyphae and/or pelotons were observed in the cortical zone and no correlation between heaviness of infection and plant growth or health promotion was found.

DISCUSSION

This study shows high incidence of root endophytic fungi in adult plants of the tropical epiphytic orchid *Cattleya skinneri* growing in the Botanical Garden "El Soconusco" in Chiapas, Southern Mexico. Although only two time periods were sampled, it is reasonable to assume that the pattern of infection of this orchid is more or less constant throughout the year, as previously suggested by Rivas *et al.*[13]. In contrast to data presented by Goh *et al.*[25], indicating that various tropical epiphytic orchids carry different fungal loads depending upon the substrate, we found that the orchids growing on the two living substrates sampled (*Mangifera indica* and a tree of

Table 5: Plant Health Index (PHI) found in *ex vitro* orchid plants inoculated with fungal strains

		Orchid species	
Fungal strain		*Cattleya aurantiaca*	*Brassavola nodosa*
Epullorhiza	IBTS-0200G	0.493	0.540
Trichoderma	IBTZ-0200F	0.342	-0.009
Verticillium	IBTZ-0200C	0.330	-0.356
Botrytis	IBTZ-0200A	0.308	-0.423
Penicillifer	IBTZ-0600A	-0.177	0.551
Pestalotiopsis	IBTS-0200B	-0.314	0.287
Aspergillus	IBTZ-0200D	-0.328	-0.134
Fusarium	IBTS-0200E	-0.363	0.668

the Apocynaceae family) were similarly infected, according to the number of individual fungi isolated, although the species composition was distinct. Notably, the fungal microbiota of the non-living substrates (clay and dead branches) were dependant upon seasonally determined humidity levels.

The root fungal endophytic community of *C. skinneri* and other tropical epiphytic orchids has been poorly studied and Rivas *et al.*[13] reported that only one of five examined plants of *C. skinneri* growing in a Costa Rican Botanical Garden was colonized by root fungi, but those fungi were not identified and no tests were carried out to confirm mutualist activity. In contrast, we found that the roots of 100% of plants sampled were fungal infected, reinforcing Rivas *et al.*[13] who mentioned that, in general, tropical orchids are heavily associated with root fungi.

All fungal isolates belonged to deuteromycetes or the imperfect fungi group. The majority of the genera used for bioassays have been reported as root endophytes or mycorrhizal in other orchid species[11,26-28], but are also widely reported as saprophytic[29] or even phytopathogenic to many plant species[30]. None of the fungal strains showed symbiotic (mycorrhizal) effects upon the seeds of *C. skinneri*, to the contrary they were shown to be pathogenic, causing higher levels of seed mortality than the blank. It is possible that fungal strains associated with mature plants are not implicated in the symbiotic germination of orchid seeds[31] and *vice versa*.

Some fungal strains were pathogenic or debilitating to *ex vitro* plants of both tested orchid species (negative PHI values); those endophytic fungi may be latent pathogens, as observed in other plant species[32]. However, distinction between an endophyte and a pathogen is not always clear. A single mutation can change a pathogen to a nonpathogenic endophytic organism and *vice versa*[33].

We considered fungal isolates to have mycorrhizal potential if they promoted plant growth and showed positive PHI values in these experiments. Hence, only three out of eight fungal strains tested (*Trichoderma* IBTZ-0200F, *Epulorhiza* IBTS-0200G and *Botrytis* IBTZ-0200A) were considered mycorrhizal for *C. aurantiaca* plants and none for *B. nodosa*. To the contrary, *Fusarium* IBTS-0200E was the most detrimental strain for *C. aurantiaca* but was a plant health promoter in *B. nodosa*. We offer the explaination that this difference may be phylogenetically based. Although there are few reports in the literature of such a basis[19], *C. skinneri* is obviously very closely related to *C. aurantiaca*, but, conversely, is also relatively closely related to *B. nodosa*, both belong to the Orchidoideae: Epidendroid: Epidendreae: Laeliinae line[34].

Though the mechanisms for plant growth promotion by mycorrhizal fungi are unknown, the results indicate a relation to water metabolism, possibly that fungal presence improved access to water. It is important to mention that the *ex vitro* plants in these experiments were not fertilized and mycorrhizal fungi are often attributed the ability to canalize minerals towards the host plant, which may be particularly important for epiphytic orchids subsisting in a nutrient poor environment. Increased access to water and nutrients, coupled with the increased resistance to pests and diseases often mentioned in the literature, via fungal inoculation[35] could prove to be a suitable, low cost hardening off method for these plants, which typically suffer major losses in the transition from laboratory to the open environment[6]. Improved survival for *ex vitro* plants re-introduced to depleted or regenerating habitats could offer an important step forward for the conservation of tropical epiphytic orchids. However, there are indications in these experiments that seeds can reach the plantlet stage on sterile bark fragments without the presence of fungi, suggesting that the interaction is an option, not a necessity.

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