SYMBIOTIC GERMINATION OF THREE SPECIES OF EPIPHYTIC ORCHIDS SUSCEPTIBLE TO GENETIC EROSION, FROM SOCONUSCO (CHIAPAS, MEXICO)

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ABSTRACT

The Soconusco region of southeast Mexico has almost a quarter of the orchid species registered in Mexico and 37 threatened species (NOM-059-SEMARNAT-2001), many with severely reduced and non-viable populations. We chose two of the most threatened species, *Rossioglossum grande* (Lindl.) Garay and G. C. Kenn. and *Cuitlauzina convallarioides* (Schltr.) Dressler and N. H. Williams and a rare species recently discovered in the region, *Rhynchostele bictoniensis* (Bateman) Soto Arenas and Salazar, to study the mycorrhizal fungi associated with the roots, isolate them and use them to induce seed germination and promote development in asymbiotically produced protocorms, in the laboratory. We isolated ten strains of *Rhizoctonia*-like orchid mycorrhizal fungi from *Rossioglossum grande* and three from *Cuitlauzina convallarioides*. Using selected fungal strains from the same species, we tested for the promotion of further development of asymbiotically pre-germinated protocorms of *R. grande* and the promotion of seed germination of *C. convallarioides*. In the case of *R. bictoniensis*, we studied the effects on seed germination of nine strains of *Rhizoctonia*-like fungi isolated from other orchid species. For *R. grande*, after 10 months, one strain of *Rhizoctonia* promoted development of the pre-germinated protocorms, and almost 90% of the protocorms produced rhizoids. For *C. convallarioides*, after 3 months, one fungal strain promoted protocorm development to the stage where they produced green tissue under illumination, suggesting the onset of photosynthesis. For *R. bictoniensis* three of the fungal strains (from other orchid species) promoted germination and, after 4 months, autotrophic protocorms.

Keywords: symbiotic seed germination, orchid mycorrhizal fungi, protocorms, inoculated orchid protocorms, threatened species

Introduction

The Tacaná-Boquerón Biological Corridor, which includes the Priority Terrestrial Region 135 (according to CONABIO. Arriaga et al. 2000) and the Volcán Tacaná Biosphere Reserve is the second most orchid species-rich region in Mexico, with 295 species registered so far (Damon 2011). This figure represents almost half of the 600 species registered for the whole of the biodiverse, tropical state of Chiapas (Cabrera 1999) and includes 37 threatened species mentioned in the NOM-ECOL-059-2001 (Semarnat 2002), such as *Rossioglossum grande* (Lindl.) Garay and G. C. Kenn. (in danger of extinction), *Cuitlauzina convallarioides* (Schltr.) Dressler and N. H. Williams (threatened) and *Rhynchostele bictoniensis* (Bateman) Soto Arenas and Salazar, the latter a new record for Soconusco for which a few individuals were discovered in 2010 (A. Damon pers. comm.). Many species registered historically in the region have already disappeared, whilst the populations of several others are severely depleted due to habitat loss and illegal trafficking (López 1980; Pérez 1994; Soto-Arenas et al. 2007).

From the very first attempts at *in vitro* germination (Knudson 1922), we have evidence that fungi and bacteria are associated with the germination of orchid seeds. Orchid seeds usually lack, or at best have limited nutritive reserves and often depend upon mycorrhizal fungi for germination (Rasmussen 1995). Normally, colonization of the seed by the fungus starts when hyphae enter cells within the seed and form three dimensional structures, termed pelotons, and stimulate development and differentiation of the seed. In some cases, colonization of asymbiotically produced protocorms may occur via the rhizoids and recolonization may also occur via the rhizoids (Williamson and Hadley 1970; Peterson et al. 2004). Evidence suggests that the orchid effectively parasitizes the fungus by digestion of the pelotons, and the benefits of this interaction with fungi may include increased absorption of water and nutrients, the production of plant hormones and protection against pathogenic fungi (Rasmussen 1995). However, the benefits of the orchid-fungal interaction during the process of germination are far more complex than the simple production of IAA (indoleacetic acid) or sugars etc. (Hayes 1969) and further studies are needed.

Although photosynthetic orchids are considered as mycoheterotrophs in their juvenile stage (Dearnaley 2007), it is possible that fungal interactions during the earliest stages of germination are not obligate. Smith and Read (1997) suggest three reasons for supporting the non-obligatory hypothesis: 1) some orchid seeds contain sufficient lipids and proteins to complete the process of germination (Davis 1966; Arditti 1967; Zettler et al. 2003), 2) rapid assimilation of nutrients is observed in asymbiotic *in vitro* cultures (Arditti 1992) and 3) the seeds of epiphytic orchids are exposed to light, and once they have imbibed water proceed rapidly to a photosynthetic state (Arditti 1992). None the less, studies of symbiotic *in vitro*

germination suggest that dependence is more complex and that the fungus also intervenes in the process of differentiation and development of the different stages from seed to juvenile plant (Zettler et al. 1998). However, it is clear that the seeds of many orchid species cease development and eventually die if not colonized by a suitable mycorrhizal symbiont (Zettler et al. 1998; Markovina and McGee 2000; Pereira et al. 2005).

In the majority of cases, OM (orchid mycorhizae) are considered as a separate group of mycorrhizae that conform to the *Rhizoctonia* group (Otero et al. 2007). In almost all cases reported, mycorrhizae isolated from orchids often do not yield a perfect state under *in vitro* conditions, and have binucleate hyphae (Mosquera-Espinosa et al. 2010), making classification beyond the genus level difficult without DNA analysis.

Positive results have been registered using selected *Rhizoctonia*-like strains to promote the symbiotic germination of some species of epiphytic orchids, paving the way for significant advances in this area (Otero and Bayman 2009). The possibility of obtaining plantlets of epiphytic orchids inoculated with mycorrhizal fungi shows promise as a component of strategies for the restoration of natural populations, as these plants should be better able to survive natural environmental conditions and resist attack by pathogens. In this way, we should be able to develop viable and efficient techniques for the mass production of endangered and overexploited orchid species and reduce genetic erosion in both natural and cultivated populations (Zettler et al. 2007).

The Project "Ecology and sustainable cultivation of the orchids of Soconusco" is dedicated to develop mechanisms for the conservation and restoration of natural populations of native orchid species. In this study, our goal was to symbiotically propagate three species of rare/endangered orchids, using strains of *Rhizoctonia*-like fungi isolated from the same or other species of orchid found in the same area. In the case of *Rossioglossum grande* we also sought to investigate the possibility of the inoculation of asymbiotically, *in vitro*, pregerminated protocorms to increase the survival, quality and numbers of plants produced and to shorten production time. For *Cuitlauzina convallarioides* and *Rhynchostele bictoniensis* we studied the possibility of germinating seeds using *Rhizoctonia*-like fungi isolated from other species.

Materials and Methods

The study area was the Tacaná-Boquerón Biological Corridor in the extreme southeast of the state of Chiapas (Mexico) between: 14°53′24″–15°21′36″N, and 92°04′12″–92°22′12″W (Arriaga et al. 2000). All samples were collected during the dry season (November to May). We collected roots and a mature fruit of *Rossioglossum grande* (Lindl.) Garay and G. C. Kenn. in March, roots and mature fruits of *Rhynchostele bictoniensis* (Bateman) Soto Arenas and Salazar and *Cuitlauzina convallarioides* (Schltr.) Dressler and N. H. Williams were collected in January, and roots of *Rhynchostele cordata* (Lindl.) Soto Arenas and Salazar were collected from February to May.

Isolation of endophytic fungi from orchid roots

Three root samples were taken from plants of each species growing *in situ*, *R. grande*, *R. cordata*, *R. uroskinneri* and *C. convallarioides*. The roots were taken to the laboratory (ECOSUR-Tapachula) in sealed plastic bags in an ice box with synthetic ice, to avoid decomposition and oxidation. Within 48 hrs of collection, the roots were washed to eliminate surface impurities and the velamen was removed. Using a scalpel, transverse sections (every 2 cm) of the roots were prepared and observed for the presence of fungal pelotons to permit selection of the sections with the highest levels of colonization and lowest levels of digestion of the pelotons. The selected sections were sterilized by immersion in calcium hypochlorite (Cloralex®, Mexico) at 1% for 3 min. Under aseptic conditions in a laminar flow hood, 9 segments of roots (2 mm length) were sown onto 10 g/l of Bactoagar DIFCO® (WA: water agar) in 9 cm Petri dishes. Incubation was carried out in the dark, at a temperature of 25 ± 2 °C (Shimura and Koda 2005) and fungal hyphae began to emerge after 24–48 hrs. Hyphal tips were recovered from the cultures and the whole process was repeated two or three times to obtain pure cultures. The pure strains were sown onto potato dextrose agar (PDA. BIOXON®) at 39 g/l and maintained in the dark at 25 ± 2 °C to observe the morphological characteristics of the developing fungal colony.

Study of the morphological characteristics, and tannin digestion test, of the endophytic fungal strains isolated

We studied the following parameters of the purified fungal strains cultured in PDA (BIOXON® 39 g/l, pH adjusted to 6.8), in darkness, at 25 ± 2 °C: morphological characteristics of mycelial growth and hyphal structure, growth rate, characteristics of the fungal colony in terms of color, topology (ring patterns), texture (fluffy, smooth, lumpy), fluorescence of nuclear DNA with 4′,6-diamidino-2-phenylindole (DAPI), width of principal hypha, length of hyphal bifurcation up to the dividing sector and width at the dividing sector.

We also applied the polyphenol oxidase enzymatic test. This is a test for the ability to digest tannin, which is a characteristic of some groups of fungi and also indicates the potential of the fungus to colonize orchids that grow on tannin-rich substrates. For this test, 7.5 g of agar and 5 g of malt were dissolved in 425 ml of distilled water and autoclaved without adjusting pH. Under aseptic conditions 2 g of tannic acid was dissolved in 75 ml of distilled water, through a syringe fitted with an acrodisc filter GELMAN® with mesh size 0.45 µm, to avoid con-

tamination. After three days, media free of contamination were inoculated with the fungal strains and incubated in the dark at a temperature of 25 ± 2 °C. A change of colour, from beige to intense dark brown indicates digestion of the tannins.

Disinfection of seeds

Due to the scarcity of these orchids, and their habitual low rates of pollination, only one fruit was collected for each species, each containing several thousand seeds. The capsules of both *R. grande* and *R. bictoniensis* were already dehiscing when collected, and the seeds had to be sterilized before sowing. The seeds were submerged in a solution of calcium hypochlorite at 1% for 3 min, after which the solution was passed through a filter to recover the seeds, which were then rinsed in distilled water and filtered, which was repeated a further three times. The capsule of *C. convallarioides* was still closed and was washed with commercial detergent and sterilized externally by the same process, rinsed in alcohol and flamed before opening and extracting the seeds.

Post germination development test

Rossioglossum grande. Seeds were pre-germinated asymbiotically in Dalla Rosa and Laneri culture medium (1977) and when they had reached stage 1 protocorms (Zettler and McInnis 1994) they were transferred to nutrient poor basic oat culture medium SIGMA® (USA) (Mitchel 1989), sowing 30 protocorms per Petri dish. Each Petri dish was inoculated with one of the fungal strains previously isolated. A total of 10 treatments were set up, each one with one of the fungal strains from the same species, a control with uninoculated nutrient poor basic oat medium and another control with uninoculated nutrient rich Dalla Rosa and Laneri medium.

Variables analyzed: Monthly, frequency of surviving protocorms was recorded, and the diameter of the protocorms were measured non-destructively using a microscope. The survival frequencies at 3 and at 10 months after sowing, were compared using Pearson's χ^2 test and the Likelihood Ratio χ^2 test per treatment. Diameters of protocorms were compared with a One-way ANOVA and the Tukey's HSD test ($p = 0.05$) (software Minitab 15®).

Germination test

Rhynchostele bictoniensis. Approximately 200 seeds per Petri dish were sown onto basic oat culture medium. At three days after sowing, Petri dishes with no signs of contamination were inoculated with one of 7 selected fungal strains: 1 strain isolated from the roots of *C. convallarioides*, two strains from the roots of *Rhynchostele uroskinneri* (Lindl.) Soto Arenas and Salazar, two strains from *R. cordata* and two from *R. grande*, plus a control of

Table 1 Morphometrics of the hyphae of fungal strains: average, SE and CV of the hyphal width of the principal axis and at the bifurcation, and the distance between the bifurcation and septum.

Inoculated orchid species	Fungal strain	Origen of fungal strain	Width of principal hypha (µm)			Hyphal width at bifurcation (µm)			Length from bifurcation to septum (µm)		
			Average	SE	CV	Average	SE	CV	Average	SE	CV
R. bictoniensis	ru21	*R. uroskinneri*	4.966	0.152	0.150	3.718	0.122	0.161	2.998	0.195	0.319
	ru5	*R. uroskinneri*	5.151	0.195	0.186	3.783	0.142	0.183	3.085	0.209	0.332
	rc18	*R. cordata*	5.499	0.132	0.123	3.911	0.097	0.127	2.706	0.243	0.457
	cc.pa	*C. convallarioides*	2.662	0.095	0.182	2.234	0.075	0.171	3.380	0.382	0.575
	rc24	*R. uroskinneri*	4.954	0.225	0.222	3.914	0.139	0.174	3.516	0.364	0.507
	rg26	*R. grande*	5.012	0.082	0.114	4.457	0.133	0.140	3.956	0.238	0.288
C. convallarioides	cc.pa	*C. convallarioides*	2.662	0.095	0.182	2.234	0.075	0.171	3.380	0.382	0.575
	cc9	*C. convallarioides*	4.076	0.162	0.202	3.868	0.128	0.169	4.784	0.554	0.591
	cc4	*C. convallarioides*	4.917	0.092	0.116	4.479	0.090	0.096	5.030	0.362	0.345
R. grande	rg1	*R. grande*	4.830	0.150	0.181	3.975	0.115	0.126	3.109	0.257	0.370
	rg4	*R. grande*	3.694	0.182	0.251	3.069	0.097	0.161	3.123	0.177	0.288
	rg7	*R. grande*	3.952	0.149	0.189	3.625	0.132	0.182	3.897	0.258	0.331
	rg8	*R. grande*	3.642	0.111	0.149	3.18	0.132	0.204	3.583	0.272	0.372
	rg11	*R. grande*	5.464	0.137	0.177	4.569	0.114	0.116	4.010	0.247	0.288
	rg14	*R. grande*	4.836	0.094	0.127	4.144	0.066	0.086	4.299	0.385	0.482
	rg24	*R. grande*	5.604	0.116	0.124	4.4108	0.105	0.118	3.412	0.230	0.33
	rg25	*R. grande*	4.946	0.103	0.149	4.162	0.109	0.143	3.257	0.220	0.478
	rg26	*R. grande*	5.012	0.082	0.114	4.457	0.133	0.140	3.956	0.238	0.288
	rg27	*R. grande*	3.802	0.123	0.158	3.13	0.112	0.175	4.165	0.369	0.434

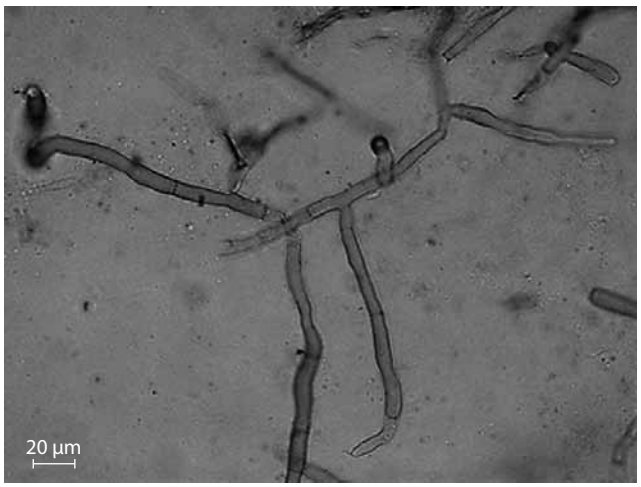

Fig. 1 Hyphae of the fungal strain cc4 stained with Acid Fuchsine. Scale bar 20 µm.

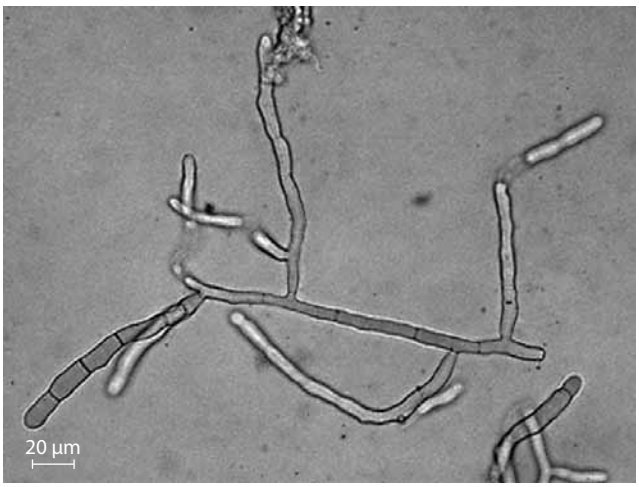

Fig. 2 Hyphae of the fungal strain rc18 stained with Acid Fuchsine. Scale bar 20 µm.

uninoculated nutrient rich Knudson C culture medium (1946). There were three replicates per treatment.

Cuitlauzina convallarioides. Applying the same process as described for *R. bictoniensis*, in this case, three fungal strains isolated from the same species were used.

Variables analyzed: Data were taken of the frequency of surviving protocorms per treatment. The frequency of healthy spherical (as compared with ovoid) protocorms and their colour, was noted as indications of development, given that rhizoids had still not formed. Frequencies of surviving protocorms, spherical and ovoid, were compared using Pearson's χ^2 test and the Likelihood Ratio χ^2 test (software Minitab 15), at 4 (*R. bictoniensis*) and 3 (*C. convallarioides*) months after sowing.

All cultures were maintained under a photoperiod of 16/8 h light/dark, with ca. 14.1 µmol m^2 s^{-1}, at a temperature of 25 ± 2 °C.

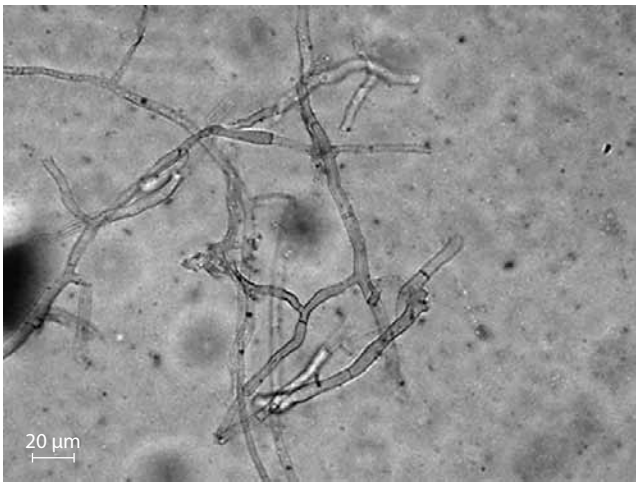

Fig. 3 Hyphae of the fungal strain rg27 stained with Acid Fuchsine. Scale bar 20 µm.

Results and Discussion

Endophytic fungi

The morphological characteristics of the purified fungal strains are resumed in Tables 1 and 2. For the determination of the colour of each strain, we consulted the fungal colour reference of the Royal Botanic Garden (2003). All the strains presented binucleate hyphae and most had a width of 2 to 5 µm. All of the fungal strains isolated from *R. grande* and most of those from *R. cordata* (rc18, rc24) had marked rings. Figures 1, 2 and 3, show the characteristic form of the hyphae of *Rhizoctonia*-like fungi, taken from three of the most effective strains in this study.

An interesting discovery was that some of the strains in this study produced polyphenol oxidase, implying the capacity to live within high concentrations of tannin and suggesting decomposing organic material. These strains were isolated from: *R. grande* (rg7, rg 8, rg24), *C. convallarioides* (cc9, cc.pa) and *R. uroskinneri* (ru21) (Table 2). Epiphytic orchids feed upon decaying organic material on the bark surface, and furthermore, *Rhynchostele cordata*, *R. uroskinneri* and in a few cases *R. grande*, were observed to colonize the basal part of the trunk of the host tree, with their roots, therefore, in contact with leaf litter.

Under laboratory conditions, in PDA with a pH of 6.8, the fastest-growing strain was from *C. convallarioides* (cc9; 11.5 mm/24h), and the slowest growing from *R. grande* (rg8, rg25, rg27; 3.5 mm/24h) (Table 2).

Post germination development test with *R. grande*

After one month of inoculation, pelotons were found inside the cortex of the protocormos in every treatment (examples in Fig. 5). After 3 months there were differences in mortality, with 86.6% survival of the protocorms of *R. grande* that had been inoculated with fungal strain rg27, as compared to 63.3% survival of protocorms in the control (non-inoculated Dalla Rosa and Laneri) and only 53.3% survival in basic oat medium (Table 3).

Table 2 Development characteristics of the fungal colonies in PDA at pH 6.8, and tannin digestion test (polyphenol oxidase).

Fungal strain	Growth (mm/24h)	Polyphenol oxidase	Description of the fungal colony	Colour
ru21	6.0	+	Partially fluffy and lumpy, aerial older mycelia.	2B
ru5	5.0	–	No ring development, fluffy from the beginning, slightly lumpy.	30 Clay Pink
rc18	4.5	–	Notable rings (almost 7), partially fluffy, slightly lumpy, aerial mycelia.	4D/White
rc24	5.5	–	Notable rings (almost 7), partially fluffy, slightly lumpy, aerial mycelia.	4D/White
cc.pa	9.0	+	Fluffy, no obvious rings, few aerial mycelium	Green
cc9	11.5	+	Partially fluffy, aerial mycelia, few lumps.	2B
cc4	3.8	–	Partially fluffy, aerial mycelia with few lumps.	2B
rg1	4.0	–	Very fluffy, aerial mycelia, obvious rings.	7White/2B
rg4	4.0	–	Very fluffy, aerial mycelia, obvious rings.	7White/2B
rg7	4.0	+	Very fluffy, aerial mycelia, obvious rings.	7White/2B
rg8	3.5	+	Very fluffy, aerial mycelia, obvious rings.	7White/2B
rg11	4.5	–	Very fluffy, aerial mycelia, obvious rings.	7White/2B
rg14	4.0	–	Very fluffy, aerial mycelia, obvious rings.	7White/2B
rg24	4.0	+	Very fluffy, aerial mycelia, obvious rings.	7White/2B
rg25	3.5	–	Very fluffy, aerial mycelia, obvious rings.	7White/2B
rg26	4.0	–	Very fluffy, aerial mycelia, obvious rings.	7White/2B
rg27	3.5	–	Very fluffy, aerial mycelia, obvious rings.	7White/2B

Table 3 Frecuency of survival of protocorms at 3 months from inoculation, in *Rossioglossum grande* postgermination test, with Chi-square.

Fungal strain	Dead	Alive	Total
rg1	19	11	30
rg11	16	14	30
rg14	20	10	30
rg24	20	10	30
rg25	12	18	30
rg26	11	19	30
rg27	4	26	30
rg4	16	14	30
rg7	19	11	30
rg8	22	8	30
Control Dalla Rosa	11	19	30
Control oatmeal	14	16	30
Total	184	176	360

Pearson Chi-Square = 39.308, DF = 11, P = 0.000.
Likelihood Ratio Chi-Square = 41.686, DF = 11, P = 0.000

After 10 months, comparing the frequency of live protocorms, there was significantly higher survival of the protocorms inoculated with rg27 (Pearson's $\chi^2 = 85.415$ and Likelihood Ratio $\chi^2 = 87.230$, both with d.f. 11, P = 0.000). To confirm the results we compared strain rg27 with both controls (Dalla Rosa and Laneri and basic oat media), applying Bonferroni's Correction and also obtained a significant difference (Pearson's $\chi^2 = 26.809$ and a Likelihood Ratio $\chi^2 = 29.415$, both with d.f. = 2, P = 0.000. Finally, a direct comparison between rg27 and the control in Dalla Rosa and Laneri, applying Bonferroni's Correction, also gave a significant difference (Pearson's $\chi^2 = 9.320$ and Likelihood Ratio $\chi^2 = 9.770$, both with d.f. = 1, P = 0.002).

For protocorm diameter (Table 4), there were significant differences between treatments. The basic oat medium control showed the least growth and protocorm development, with an average diameter of 1.38 mm at 10 months, as compared to the Dalla Rosa and Laneri control which had an average diameter of 2.62 mm,

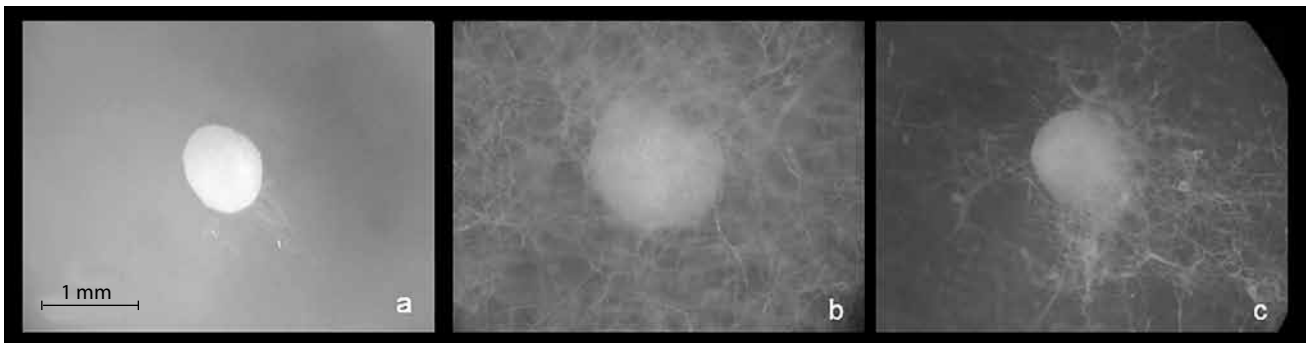

Fig. 4 *Rossioglossum grande* postgermination test; protocorms one month after inoculation (a: Control oatmeal; b: rg27 strain; c: rg14 strain). Scale bar 1 mm.

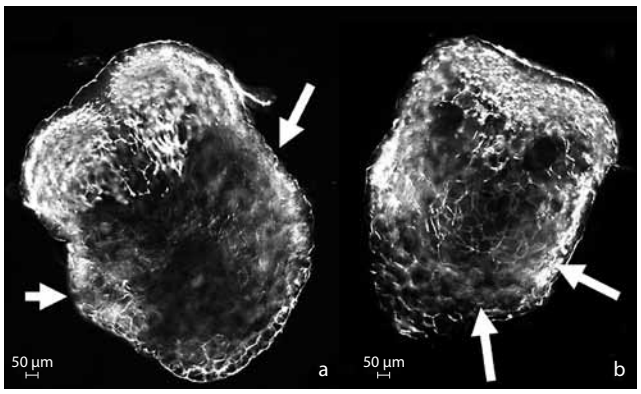

Fig. 5 *Rossioglossum grande* postgermination test; longitudinal section of the protocorms one month after inoculation. The arrows show the pelotons inside the cortical tissue of the protocorms (a: rg27 strain; b: rg14 strain). Scale bar 50 µm.

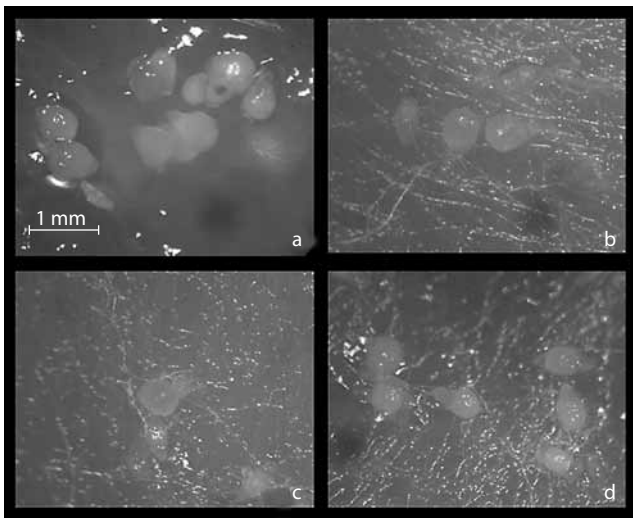

Fig. 6 *Rhynchostele bictoniensis* germination test; protocorms 4 months after inoculation (a: Control Knudson C; b: ru21 strain; c: rc18 strain; d: rg26 strain). Scale bar 1 mm.

which was not significantly different than the diameters of protocorms obtained with fungal strains rg1, rg25, rg4 and rg7. The diameter of protocorms in the Dalla Rosa and Laneri control was not significantly different from the diameter of protocorms growing with fungal strains rg1, rg25, rg4 and rg7, but was significantly greater that protocorms exposed to fungal strain rg27, which, in turn was superior to the basic oat meal control. We consider strain rg27, to be the most effective fungus in supporting growth and survival of protocorms of *R. grande* (Fig. 4).

Table 4 Statistical difference between average protocorm diameter 10 months after inoculation in *Rossioglossum grande* postgermination test. Values were compared by One-Way ANOVA and Tukey's HSD test (*different letters mean significant differences at p = 0.05).

Source	DF	SS	MS	F	P
Treat.	10	7.923	0.792	5.21	0.000
Error	86	13.078	0.152		
Total	96	21.002			

S = 0.3900, R-Sq = 37.73%, R-Sq(adj) = 30.49%

Fungal strain	N	Average (mm)	SE
rg1	7	2.2429^{bc*}	0.055
rg11	10	1.97^{b}	0.018
rg14	10	2.03^{b}	0.040
rg24	5	2.06^{b}	0.052
rg25	3	2^{abc}	0.000
rg26	4	1.875^{ab}	0.063
rg27	26	2.0154^{b}	0.010
rg4	6	2.1333^{bc}	0.072
rg7	5	2.1^{bc}	0.045
Control Dalla Rosa	15	2.62^{c}	0.046
Control oatmeal	6	1.3833^{a}	0.054

Germination test with *Rhynchostele bictoniensis*

Symbiotic germination was promoted by fungal strains from *R. uroskinneri* (ru21), *R. cordata* (rc18, rc24) and *R. grande* (rg26) (Fig. 6), as evidenced by healthy, spherical protocorms and the probable development of chlorophyll, as shown by the green colour (Table 5).

Table 5 Germination, protocorm development and colour, 4 months after inoculation in *Rhynchostele bictoniensis* germination test.

Fungal strain		Ovoid	Spherical	Color
ru5	dish 1	37	0	white to pale green
	dish 2	40	0	
	dish 3	24	0	
ru21	dish 1	72	2	white-green (approx. 15% oxidation)
	dish 2	80	20	
	dish 3	40	3	
rc18	dish 1	98	0	white-green
	dish 2	77	32	
	dish 3	94	8	
rc24	dish 1	45	2	white-green
	dish 2	27	0	
	dish 3	77	0	
cc.pa	dish 1	47	0	white (20% oxidation)
	dish 2	22	0	
	dish 3	41	0	
rg26	dish 1	34	0	white-green
	dish 2	51	34	
	dish 3	63	0	
Control Knudson C	dish 1	57	45	green (approx. 30% oxidation)
	dish 2	64	22	
	dish 3	61	23	

There was a significant difference in percentage germination, considering frequencies of ovoid and spherical protocormos (Pearson $\chi^2 = 139.526$ and Likelihood Ratio $\chi^2 = 163.103$, both with d.f. = 6, P = 0.000). The highest

germination was in the asymbiotic Knudson C control, but there was evidence of oxidation, possibly due to a reduction in pH promoted by the development of the protocorms themselves. In contrast, the treatment with fungal strain rc18 had pale green protocoms with no trace of oxidation, and this strain had the highest germination of all the fungal strain treatments, but a lower value than that obtained with the asymbiotic Knudson C control. We consider the presence of ovoid protocorms as positive as a preliminary stage of germination, before advancing to a spherical shape and producing rhizoids. Good results were also obtained with fungal strains ru21 and rg26, with most of the protocorms having a healthy colour and spherical shape (Table 5).

No protocorms with rhizoids were observed during the 4 months in any of the treatments or Knudson C medium control.

Germination test with *C. convallarioides*

There was a significant difference in seed germination among treatments (Pearson $\chi^2 = 1124.552$ and Likelihood Ratio $\chi^2 = 1334.756$, both with d.f. = 3, P = 0.000), where the Knudson C control was shown to be the most efficient in terms of development of spherical protocorms (Table 6).

Table 6 Germination, protocorm development and colour, 3 months after inoculation in *Cuitlauzina convallarioides* germination test.

Treatment		Ovoid	Spherical	Color
cc.pa	dish 1	280	0	white
	dish 2	372	0	
	dish 3	158	0	
cc9	dish 1	44	0	white
	dish 2	120	0	
	dish 3	180	0	
cc4	dish 1	100	75	white to green
	dish 2	97	43	
	dish 3	98	51	
Control Knudson C	dish 1	54	137	green-brown (oxidation approx. 70%)
	dish 2	35	152	
	dish 3	50	145	

The fungal strain that resulted in the highest germination rates after three months was cc4, but the results were not significantly different from the Knudson C control (Pearson $\chi^2 = 84.968$ and Likelihood Ratio $\chi^2 = 84.584$, both with d.f. = 1, P = 0.000, applying Bonferroni's Correction). However, as observed previously, significant oxidation occurred in the protocorms growing in the asymbiotic Knudson C control, which at three months were a brown-green colour, which was not observed in any of the inoculated treatments. These results suggest that the fungal inoculates may offer a competitive advantage to the orchid protocorms, in terms of survival and vigour (Fig. 7).

Conclusions

Germination of orchid seeds under natural conditions is promoted by mycorrhizal fungi largely assignable to the form-genus *Rhizoctonia*. The preliminary results of our research suggests that the use of fungal isolates from the same orchid species or other species growing in the same habitat may indeed promote health and vigour and greater long-term survival of protocorms, when compared with protocorms grown in asymbiotic low and high nutrient media.

We have obtained evidence that endophytic *Rhizoctonia*-like fungi isolated from orchid roots are compatible with the seeds of the same species of orchid and may promote greater health and survival of protocorms. In the specific case of *R. grande*, we offer evidence that inoculation may be successfully carried out after an asymbiotic pre-germination phase and offer observable advantages.

The results obtained with *C. convallarioides* and *R. bictoniensis* confirm that not all fungal isolates are equally effective in promoting orchid seed germination and development of protocorms. However, these preliminary results suggest that in most cases some benefit is afforded in terms of the health of protocorms obtained when compared with those obtained in asymbiotic,

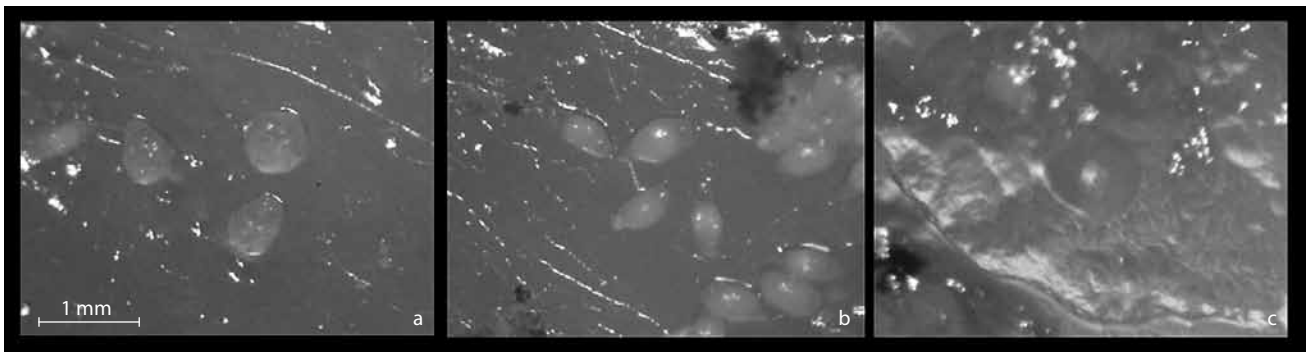

Fig. 7 *Cuitlauzina convallarioides* germination test; protocorms 3 months after inoculation (a: treatment cc4, protocorms acquire round shape and green colour; b: cc.pa, ovoid protocorms; c: Control Knudson C, development of first rhizoids, note oxidized green-brown protocorms). Scale bar 1 mm.

nutrient-rich media (Dalla Rosa and Laneri, Knudson C) and also nutrient-poor media (basic oat meal agar), which showed signs of oxidation and deterioration. The presence of the fungal inoculants may serve to maintain optimum conditions within the culture, particularly acting as a buffer to maintain suitable pH (between 5–5.5).

Some orchid species tested in this study (e.g., *R. bictoniensis*) may rely on a broader range of fungi to fulfil their mycotrophic needs fitting the profile of a "generalist" (Swartz and Dixon 2009), and may be compatible with certain fungal isolates from other orchid species albeit from the same habitat or region and/or the same genus. We have evidence of compatibility with fungi isolated from the roots of two other species of the same genera, *Rhynchostele* (*R. uroskinneri* and *R. cordata*), both of which promoted seed germination in *R. bictoniensis* and evidence of compatibility with fungal isolates from a different genus, *Rossioglossum* (*R. grande*) growing in the same region, which also demonstrated positive effects. However, fungal isolates from *Cuitlauzina* (*C. convallarioides*) did not promote germination in *Rhynchostele bictoniensis*.

Our results coincide with comments in the literature that a minimum of 6 months is required for significant development of protorms in symbiotic cultures, as observed by Hayes (1969) with *Odontoglossum* and *Miltonia* in symbiotic culture media with 1% starch.

These preliminary results are a contribution towards the in depth study of the symbiotic or perhaps parasitic relationship that epiphytic orchids maintain with endophytic mycorrhizal *Rhizoctonia*-like fungi, towards a future application of that relationship for the restoration of natural populations and sustainable exploitation of over exploited species. We are continuing with our study and plan to carry out DNA analysis of the most effective fungal strains, and determine the distribution of those strains amongst orchid species and genera, habitats and substrates. We hope eventually to employ fungal isolates for the mass production of vigorous plants capable of adapting to natural conditions for the restoration of endangered and overexploited epiphytic orchids in the Soconusco region of southeast Mexico.

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REFERENCES

Arditti J (1967) Factors affecting the germination of orchid seeds. Bot Rev 33: 1–97.

Arditti J (1992) Fundamentals of orchid biology. John Wiley and Sons, New York, USA.

Arriaga L, Espinoza JM, Aguilar C, Martínez E, Gómez L, Loa E (Coordinadores) (2000) Regiones Terrestres Prioritarias de México. Comisión Nacional para el Conocimiento y Uso de la Biodiversidad. México, pp 528–530.

Cabrera TG (1999) Orquídeas de Chiapas. Instituto de Historia Natural y Gobierno del Estado de Chiapas. Mexico.

Dalla Rosa M, Laneri U (1977) Modification of nutrient solutions for germination and growth "in vitro" of some cultivated orchids and for the vegetative propagation of *Cymbidium* cultivars. American Orchid Society Bulletin 46: 813–820.

Damon A (2011) Diversidad y conservación de las orquídeas del corredor biológico Tacaná-Boquerón. Final report. FONDOS MIXTOS-CHIAPAS CHIS-2006-C06-45802.2007-2010.

Davis G (1966) System embryology of the angiosperms. Wiley, NY, pp 194–196.

Dearnaley JD (2007) Further advances in orchid mycorrizal research. Mycorrhiza 17: 475–486.

Hayes AB (1969) Observation on orchid seed mycorrhizae. Mycopathologia 38: 139–144.

Knudson L (1922) Nonsymbiotic germination of orchid seeds. Bot Gaz 78: 1–25.

Knudson L (1946) A new nutrient solution for orchid seed germination. American Orchid Society Bulletin 15: 214–217.

López R (1980) Tipos de vegetación y su distribución en el estado de Tabasco y norte de Chiapas. Universidad Autónoma de Chapingo. México, pp 9–14.

Markovina AL, McGee PA (2000) Comparison of symbiontic seed germination and plantlet development in *Sarcochilus* (Vandae; Orchidaceae). Lindleyana 15: 68–72.

Mitchell RB (1989) Growing hardy orchids from seeds at Kew. The Plantsman 11: 152–169.

Mosquera-Espinosa AT, Bayman P, Otero JT (2010) *Ceratobasidium* como hongo micorrízico de orquídeas en Colombia. Acta Agronómica 59: 316–326.

Otero JT, Bayman P (2009) Germinación simbiótica y asimbiótica en semillas de orquídeas epifitas. Acta Agronómica 58: 270–276.

Otero JT, Flanagan N, Herre A, Ackerman J, Bayman P (2007) Widespread mycorrhizal specificity correlates to mycorrhizal function in the neotropical, epiphytic orchid *Ionopsis utricularioides* (Orchidaceae). Am J Bot 94: 1944–1950.

Pereira OL, Kasuya MCM, Rollemberg CL, Borges AC (2005) *In vitro* symbiotic seed germination of *Oncidium flexuosum* (Orchidaceae) by *Rhizoctonia*-like mycorrhizal fungi. R Bras Ci Solo 29: 199–206.

Pérez R (1994) Chiapas: recursos naturales y problemática ambiental. In: Armendáiz ML (ed) Chiapas una Monografía. Fondo Cultural Económico. Mexico, pp 38–48.

Peterson RL, Massicotte HB, Melville LH (2004) Mycorrhizas: Anatomy and cell biology. NRC-CNRC CABI. Ottawa, USA.

Rasmussen HN (1995) Terrestrial orchids: From seed to mycotrophic plants. Cambridge University Press, London, UK, pp 167–172.

Royal Botanic Garden, Edinburgh (2003) Flora of British Fungi: Colour Identification Chart. Stationery Office Books. London.

Semarnat (2002) Norma Oficial Mexicana NOM-059-SEMARNAT-2001, Protección ambiental – Especies nativas de

México de flora y fauna silvestres – Categorías de riesgo y especificaciones para su inclusión, exclusión o cambio – Lista de especies en riesgo. *Diario Oficial de la Federación*, 6 of March 2002.

Shimura H, Koda Y (2005) Enhanced symbiontic seed germination of *Cypripedium macranthus* var. *rebunuense* following inoculation cold treatment. Physiol Plantarum 123: 281–287.

Smith SE, Read DJ (1997) Mycorrhizal Symbiosis. Academic Press, London.

Soto-Arenas MA, Solano-Gómez R, Hágsater E (2007) Risk of extinction and patterns of diversity loss in Mexican orchids. Lankersteriana 7: 114–121.

Swartz ND, Dixon KW (2009) Terrestrial orchid conservation in the age of extinction. Annals of Botany, 104: 543–556.

Williamson B, Hadley G (1970) Penetration and infection of orchid protocorms by *Thanatephorus cucumis* and other *Rhizoctonia* isolates. Phytopathology 60: 1092–1096.

Zettler LW, Poulter SB, McDonald KI (2007) Conservation-driven propagation of an epiphytic orchid (*Epidendrum nocturnum*) with a mycorrhizal fungus. Hortscience 42: 135–139.

Zettler LW, Delaney TW, Sunley JA (1998) Seed propagation of the epiphytic green-fly orchid, *Epidendrum conopseum* R. Brown, using its endophytic fungus. Selbyana 19: 249–253.

Zettler LW, McInnis TM (1994) Light enhancement of symbiotic seed germination and development of an endangered terrestrial orchid (*Platanthera integrilabia*). Plant Sci 102: 133–138.

Zettler LW, Sharma J y Rasmussen FN (2003) Mycorrhizal diversity. In: Dixon KW, Kell SP, Barrett RL, Cribb PJ (eds) Orchid conservation. Natural History Publications, Borneo, pp 205–226.