Symbiotic vs. asymbiotic seed germination in epiphytic orchids

Germinación simbiótica y asimbiótica en semillas de orquídeas epifitas

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Abstract

We compared seedling growth of the epiphytic orchid, *Tolumnia variegata,* in agar media with and without inoculated mycorrhizal fungi. Symbiotic germination produced more developed embryos than asymbiotic germination. Differences were highly significant, although some isolates of *Rhizoctonia*-like fungi were parasitic on seeds. Control seeds in a cellulose medium without *Rhizoctonia*-like fungi did not germinate. Seeds of *Epidendrum ramosum*, *Lepanthes rupestris* and *Psychilis monensis* showed no significant differences between asymbiotic and symbiotic germination using mycorrhizal fungi isolated from *T. variegata* roots, suggesting high mycorrhizal specificity. Our data suggest that the relationship between epiphytic orchids and the mycorrhizal fungi is more specific than previously thought. The use of the right fungal strain may enhance germination performance. Orchid growers may achieve better results in the propagation of some epiphytic orchids using symbiotic germination.

Key words: *Tolumnia variegata*, *Epidendrum ramosum*, *Lepanthes rupestris* and *Psychilis monensis*, Orchidaceae, germination, mycorrhizal fungi

Resumen

Se compara el crecimiento de plántulas de la orquídeas epifita *Tolumnia variegata* en medios de agar con y sin hongos micorrízicos. Los métodos de germinación simbiótica produjeron embriones de *T. variegata* más desarrollados que los métodos de germinación asimbiótica y las diferencias fueron estadísticamente significativas. Las semillas en los controles en medio de celulosa sin hongos micorrízicos no germinaron. Las semillas de *Epidendrum ramosum*, *Lepanthes rupestris* y *Psychilis monensis* no mostraron diferencias significativas en germinación entre métodos simbiótica y asimbiótica utilizando hongos aislados de raíces de *T. variegata*, incluso algunos aislados del grupo *Rhizoctonia* parasitaron las semillas. Los resultados sugieren que las semillas de orquídeas epifitas y sus hongos micorrízicos son más específicos de lo que anteriormente se había creído y por tanto es necesario utilizar los hongos específicos para cada especie de orquídea. Además, se sugiere que los cultivadores de orquídeas podrían obtener mejores resultados en la propagación de orquídeas epifitas si utilizan metodologías de germinación simbiótica apropiados.

Palabras clave: *Tolumnia variegata*, *Epidendrum ramosum*, *Lepanthes rupestres*, *Psychilis monensis*. Orchidaceae, germinación, hongos micorrízicos

Introduction

Since the pioneering studies of Knudson (1921), horticulturalists have germinated orchid seeds using nutrient combinations in sterile conditions (Arditti 1992). Only when these methods fail do orchid growers resort to techniques for seed germination using naturally occurring symbionts (Bernard 1899).

All orchids require a mycorrhizal association for the germination of their seeds, mainly the basidiomycete fungi in the group *Rhizoctonia* (Rasmussen, 1995). *Rhizoctonia sensu latu* is an artificial group that includes fungi whose sexual stages belong to the genera *Thanatephorus*, *Ceratobasidium*, *Sebacina* and *Tulasnella* (Sneh et al. 1991). This symbiotic dependence is due to the minimal quantity of nutrients stored in the tiny orchid seeds (Arditti and Ghani 2000). However, the fungi from the group *Rhizoctonia* are also well known for their pathogenicity in many different groups of plants.

Artificial germination of terrestrial orchid seeds in temperate zones is extremely difficult and requires very specific nutrient formulations in aseptic germination media (Arditti, 1992). Thus, many growers and researchers use symbiotic fungi for propagation. This contrasts with the cultivation of many epiphytic tropical orchids, which, in general are relatively easy to grow in asymbiotic conditions. In consequence, the growers of tropical orchids do not use symbiotic germination for propagation. For these reasons, the symbiotic germination interactions of terrestrial orchids from temperate regions are much better known than those of tropical epiphytic orchids. However, the techniques for symbiotic germination of tropical epiphytic orchid seeds could improve the cultivation success of these orchids, for both commercial and conservation purposes. The objective of this study was to compare the success in tropical epiphytic orchid propagation programs of symbiotic and asymbiotic germination techniques.

Materials and methods

Tolumnia variegata (Sw.) Braem (Orchidaceae; subfamily Epidendroideae; tribe Maxillarieae) is a tropical epiphytic orchid endemic to the Greater Antilles, with the exception of Jamaica. Its name refers to its high variation in floral characteristics (Photo 1) (Ackerman 1995). The seeds for the present study were collected from a population located in the zone adjacent to the Natural Reserve Laguna de Tortuguero in Manatí (18° 27' 37'' N, 66° 25' 30'' O), Puerto Rico, between 5 and 10 m.s.n.m. Average annual precipitation is 1.688 mm, with a temperature of 25.4 °C. The zone is classified as tropical rain forest on the Holdridge Scale (Ewel and Whitmore 1973).

Foto 1: Planta adulta de *Tolumnia variegata* en su hábitat natural.

Photo 1: **Adult plant of *Tolumnia variegata* in its natural habitat**.

Forty-eight isolations of *Rhizoctonia* (Figure 1) were made from both adult and juvenile plants from different Puerto Rican populations of *T. variegata* (Otero et al., 2002, 2004, 2005, 2007). The roots of epiphytic plants found on bushes of *Randia aculeata* L. (Rubiaceae) and *Psidium guajaba* L. (Myrtaceae) were washed with Tween 20 under running water, and surface sterilized using serial baths of ethanol (70%, 1 min), sodium hypochlorite (2.5%, 2 min) and ethanol (70%, 1min). Root segments were inoculated in aseptic conditions in potato dextrose agar (PDA) and malt extract agar (MEA). Endophytic fungi were identified under the microscope using the guide of Sneh et al. (1991). Fungi with microscopic characteristics of

Rhizoctonia were transferred to new PDA plates, and characterized to morpho-species. Molecular characterizations are available in the literature (Otero et al. 2002, 2004, 2005, 2007).

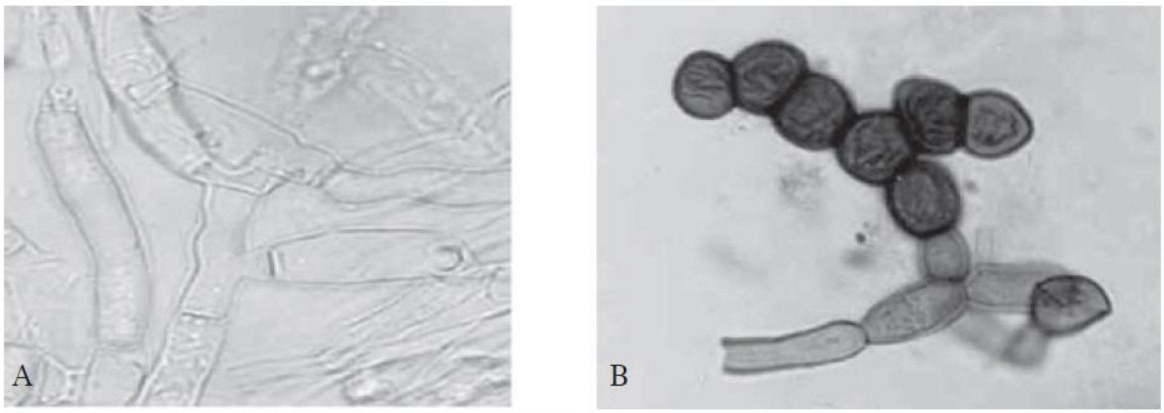

Figura 1: *Rhizoctonia* sp., cepa aislada de raíces de *Tolumnia variegata*. (A) Constricción en la ramificación de las hifas de Rhizoctonia. (B) Células monilioides.

Figure 1: *Rhizoctonia sp.*, strain isolated from roots of *Tolumnia variegata.* (A) Constrictions in the hyphal branches characteristic of *Rhizoctonia*. (B) Monilioloid cells.

The fruits of *T. variegata* were collected from the study site and surface sterilized using the same procedures as used for the roots. The seeds were extracted from the fruits under sterile conditions and their viability was evaluated with triphenyl tetrazolium chloride stain (TTC) (Otero et al., 2005).

To examine the effect of *Rhizoctonia* isolates on orchid seed germination, a mix of at least 100 seeds from the three plants with greatest viability was inoculated onto Petri dishes with cellulose agar (CA), on each of which a different isolate of *Rhizoctonia* had been inoculated three days beforehand. Seven fungal strains were evaluated. Control plates were inoculated with seed, but not fungus. The asymbiotic germination of each orchid species was evaluated with five plates with Knudson C commercial media (KC, Sigma Aldrich Inc.). Data were collected at two points: after two weeks; and after two months after inoculation. Data were collected by measuring ten randomly chosen plantlets on each Petri dish. Additionally, each seed was assigned a developmental state according to Stenberg and Kane (1998). In the absence of any signs of seed germination the state registered was zero (0) (Figure 2B). With an embryo < 1000 µm the state was 1 (Figure 2C), with the presence of absorbent hairs, the state was 2, with a primordial leaf, the state was 3 (Figure 2D), with a leaf in development and a primordial leaf, the state was 4, and with two leaves the state was 5 (Figure 2E). Finally, the small plantlets were registered as state 6 (Figure 2F). Parasitized seeds, determined by the growth of hyphae from the seeds (Figure 2A), registered a negative value (-1).

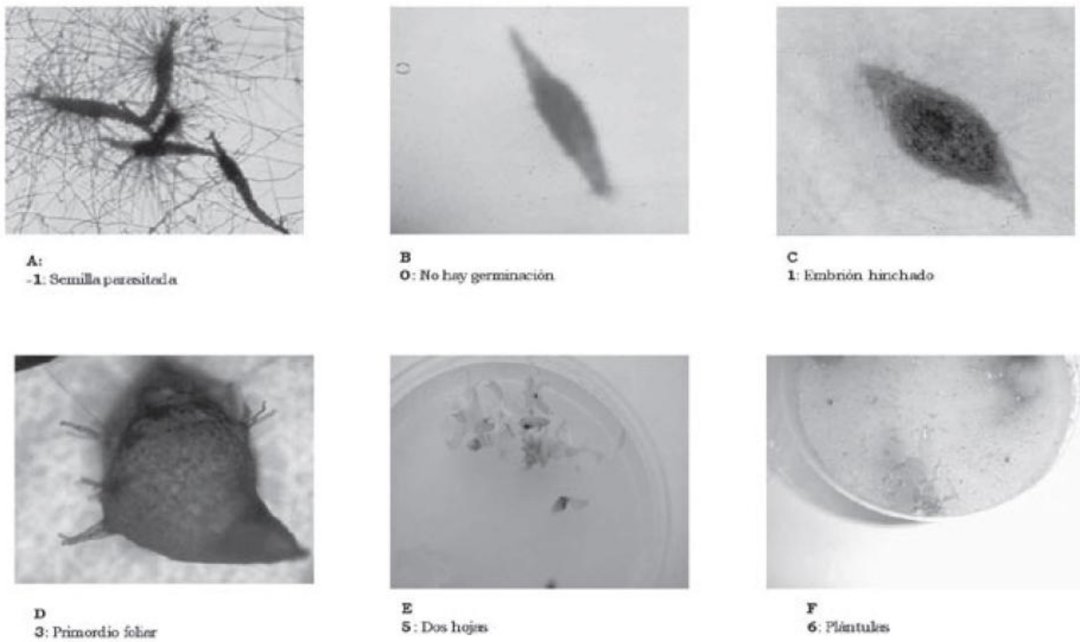

Figura 2: Estados de germinación de semillas y desarrollo de protocormos en Tolumnia variegata.

Figure 2. States of seed germination and protocorm development in *Tolumnia variegata.* A. -1: Parasitized seed. B. 0: no germination. C. 1: swollen embryo. D. 3: primordial leaf. E. 5: two leaves. F. 6: Plantlets.

The study also evaluated the effect of fungi isolated from *T. variegata* on the seed germination of the orchids *Epidendrum ramosum* Jacq. (subfamily Epidendroideae), *Lepanthes rupestris* Stimson (subfamily Pleurotallidiinae) and *Psychilis monensis* Sauleda (subfamily Epidendroideae). The data were collected two weeks after inoculating the seeds, in the same way as described for the experiments on seed germination of *T. variegata*. Data analysis was performed using one-way analysis of variance, as the data complied with the requirements of normality for the analysis.

Results

In total 55 fungi (classified in 26 morphospecies) were isolated from the roots of *T. variegata*, with 48 (21 morphospecies) having the characteristics of *Rhizoctonia* (87.3%), 31 of these (in 13 morphospecies) were evaluated as mycorrhizal. Some isolates were mycorrhizal, but others showed seed parasitism. Thirteen (6 morpho-species) showed a positive effect on seed germination.

The embryos of *T. variegata* that germinated in the presence of one of the 13 *Rhizoctonia* isolates from *T. variegata* were significantly larger than those that germinated in the Knudson medium (one-way ANOVA, $F_{(1,9)}$ = 63,00, P < 0.0001) two weeks after having been inoculated (Figure 3). Significant differences were also found (one-way ANOVA, $F_{(1,9)}$ = 62,32, P < 0.0001) two months after inoculation (Figure 4).

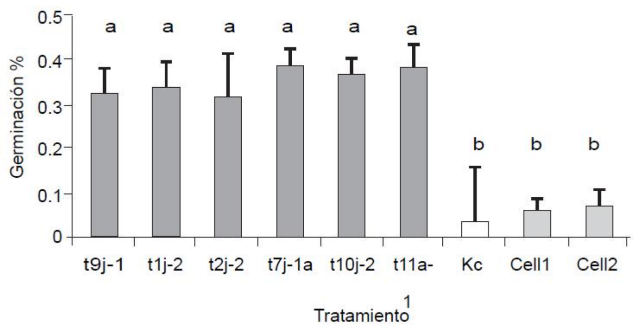

Figura 3: Germinación de semillas de *Tolumnia variegata* dos semanas después de la inoculación con diferentes hongos y con controles. Kc Medio Knudson C. Cell: Agar de celulosa sin hongo micorrizico.

Figure 3: Seed germination of *Tolumnia variegata* two weeks after inoculation with different fungi and in the control. Kc – Knudson C medium. Cell – Cellulose agar with no mycorrhizal fungi.

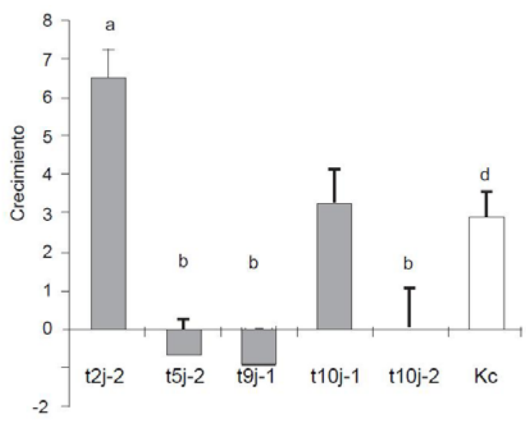

Figura 4: Germinación de semillas de *Tolumnia variegata* 2 meses después de la inoculación con diferentes aislados y con controles. Kc Medio Knudson C. Cell: Medio de celulosa sin hongo micorrizico.

Figure 4: Seed germination of *Tolumnia variegata* two months after inoculation with different fungi and in the control. Kc – Knudson C medium.

The germination of the other orchid species with mycorrhizal fungi originating from *T. variegata* did not show the same advantages with respect to the symbiotic germination (one-way ANOVA, *E. ramosum* $F_{(1,7)}$ = 1,15, P = 0.32; *L. rupestris* $F_{1,7}$ P = 0.32; & *P. monensis* $F_{1,7}$ = 0,53 P = 0.50). The seeds of *L. rupestris* and *P. monensis* initiated germination with all the fungi, while for those of *E. ramosum* germination was induced by all but two fungi (Figure 5).

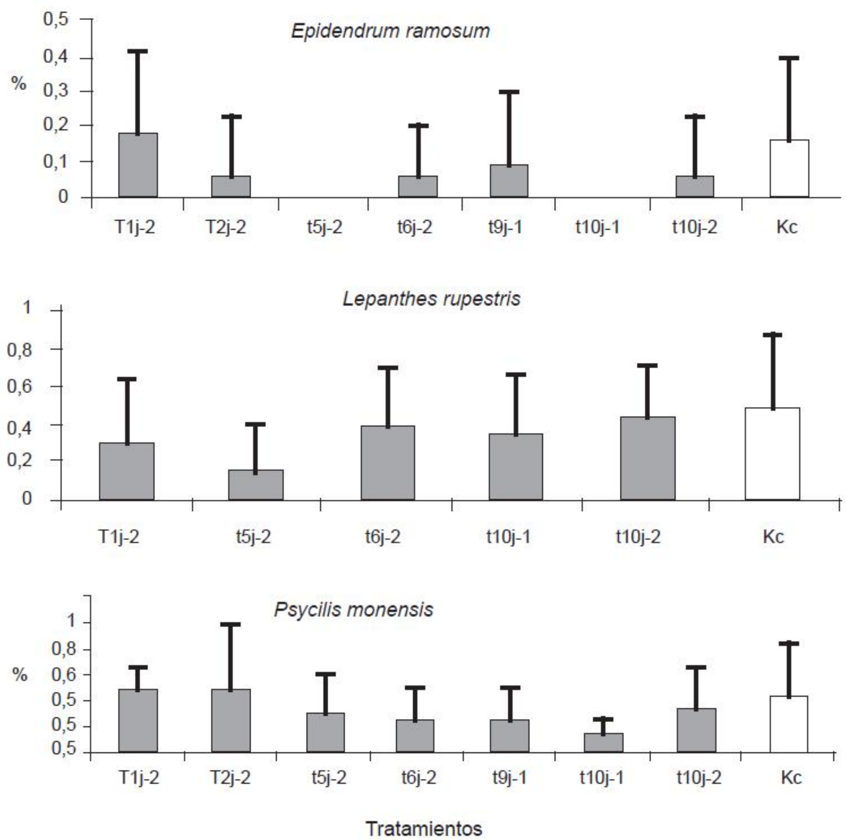

Figura 5: Germinación de semillas de *Epidendrum ramosum* (A), *Lepanthes rupestris* (B), y *Psychilis monensis* (C) 2 semanas después de la inoculación con diferentes hongos micorrízicos con controles. Kc Medio Knudson C.

Figure 5: Seed germination in *Epidendrum ramosum* , *Lepanthes rupestris* , and *Psychilis monensis* two weeks after inoculation with different mycorrhizal fungi, and with controls. Kc – Knudson C medium.

Discussion

The seeds of *T. variegata* germinated better in the presence of fungi isolated from plants of the same species than in the commercial Knudson C medium. Although mycorrhizal fungi are not commonly used in the *in vitro* germination of epiphytic seeds, the use of appropriate mycorrhizal strains could improve the cultivation of these plants from seed. Nonetheless, the mycorrhizal fungi isolated from *T. variegata* were not as beneficial in inducing germination in other epiphytic orchid species. The data from this study suggest that the relationships between epiphytic orchids and their mycorrhizae are more specific than was previously thought (Arditti et al. 1990). This specificity could complicate the commercial application of these fungi in the cultivation of orchids, as it implies that each species needs a particular mycorrhizal group. More studies are needed in order to identify the natural symbionts associated with economically-important orchid species, and also with those species threatened with extinction, with the aim to improve cultivation from seed. This challenge will be facilitated through the creation and *in vitro* analysis of a germplasm bank of orchid mycorrhizal strains, and the evaluation of the effects of germination in different orchid species. These results will improve efforts towards achieving a sustainable use of tropical epiphytic orchids, important resources in the flower industry.

Symbiotic germination of orchid seeds also offers another advantage compared with asymbiotic germination. The culture media for asymbiotic germination (such as the Knudson C medium used in this study) are rich in sugars and nutrients, and the plantlets take months to become established. This combination of conditions makes the plantlets susceptible to contamination with pathogenic fungi. The inoculation of seeds with mycorrhizal fungi contributes to inhibiting the growth of other, contaminating, fungi and bacteria (Porras and Bayman, 2007).

Conclusions

Symbiotic germination techniques using mycorrhizal fungi offer an effective alternative for the seed germination of tropical epiphytic orchids. In comparison with asymbiotic methods the use of natural symbionts may be more efficient if they employ appropriate fungi for germination. Unfortunately, very little is known about which are the most appropriate symbionts for the most economically important orchid species, and thus it is recommended that studies are undertaken in order to identify the best fungi to stimulate *in vitro* germination in these species.

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