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ARTÍCULO DE INVESTIGACIÓN / ORIGINAL RESEARCH PAPER

SEED GERMINATION AND PLANT DEVELOPMENT IN Escobedia grandiflora (OROBANCHACEAE): EVIDENCE OF OBLIGATE **HEMIPARASITISM?**

Germinación de semillas y desarrollo de plantas en Escobedia grandiflora (Orobanchaceae): ¿Evidencia de hemiparasitismo obligado?

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ABSTRACT

Root parasitic plants can be facultative or obligate. Facultative parasites are able to complete their life cycle and their seeds can germinate without a host. Escobedia grandiflora is a poorly studied species in spite of its ancestral importance as dye of foods and medicinal use. The present study evaluates the states of seed, seedlings and mature plants, under presence and absence of possible hosts, for inferring the type of parasitism exhibited by E. grandiflora. Seeds were evaluated using two conditions each of light (12 and 0 hours) and temperature (20 °C and 25 °C); percentage germination, and germination speed were determined. The seeds did not require a host to germinate, as is typical of facultative parasitic plants. Percentage of germination varied between 66 % and 85.3 % and was not affected by light or temperature although germination speed was greater at 25 °C. Larger seeds had a higher percentage of germination and produced larger seedlings. The seedlings planted without a host did not survive, while those planted with Paspalum notatum had a 45 % survival rate, demonstrating that this is a critical stage of development, even with a host. Escobedia grandiflora plants sowed with grasses began the reproductive stage at the 28th week, and those planted with Pennisetum purpureum showed better performance, expressed in more haustoria, higher dry matter of total plant, rhizome and aerial stems. Plants sowed alone lived for more than six months, but they did not produce flowers or fruits. According to the behavior of seedlings and plants, E. grandiflora is an obligate parasite.

Keywords: Escobedia, obligate hemiparasite, parasitic plant, root parasitism, seed germination.

RESUMEN

Las plantas parásitas de raíces pueden ser facultativas u obligadas, las primeras pueden completar su ciclo de vida y sus semillas pueden germinar sin un hospedero. Escobedia grandiflora es una especie poco estudiada, a pesar de su importancia ancestral como colorante de alimentos y uso medicinal. Este estudio evaluó los estados de semilla, plántula y planta adulta, en presencia y ausencia de posibles hospederos para inferir sobre su tipo de parasitismo. En las semillas se evaluaron dos condiciones de luz (12 y 0 horas) y temperatura (20 °C y 25 °C), el porcentaje y velocidad de germinación. Las semillas no requirieron la presencia del hospedero para germinar. El porcentaje de germinación osciló entre 66 y 85,3 % y no fue afectado por la luz o la temperatura, aunque la velocidad de germinación fue mayor a 25 °C. Las semillas con mayor tamaño presentaron mayor porcentaje de germinación y produjeron plántulas más grandes. Las plántulas sembradas sin hospedero no sobrevivieron, mientras que las sembradas con Paspalum notatum, tuvieron una sobrevivencia del 45 %, evidenciando que este estado es crítico, aún con hospedero. Las plantas de Escobedia grandiflora sembradas con pastos, iniciaron la etapa reproductiva en la semana 28, y aquellas sembradas con Pennisetum purpureum presentaron más haustorios, y mayor materia seca en la planta total, rizoma y tallos aéreos. Las plantas sembradas solas vivieron más de seis meses, pero ellas no desarrollaron flores y ni frutos. Según el comportamiento de las plántulas y las plantas, E. grandiflora es parásita obligada.

Palabras clave: Escobedia, hemiparasíta obligada, planta parasítica, Parasitismo de raíz, germinación de semillas



INTRODUCTION

Root parasitic plants are classified according to their degree of host dependence. They are considered either holoparasites or hemiparasites. Holoparasites are almost completely lacking in chlorophyll while hemiparasites have chlorophyll, are photosynthetically active, and derive some resources from their host through haustoria, the organ of parasitism (Yoder, 2001; Watling and Press, 2001; Nickrent, 2002; Bennett and Mathews, 2006). According to the degree of dependence upon host plant(s) for completing the life cycle, root hemiparasitic plants can be facultative or obligate. Facultative hemiparasites can survive in autotrophic form, independent of the host, for at least part or all of their life cycle. They can reproduce without a connection to a host, and when available, parasitize opportunistically on neighboring plants (Fay et al., 2010; Westwood et al., 2010). In contrast, obligate parasites must parasitize a host in order to complete their life cycle (Westwood et al., 2010).

One of the characteristics differentiating obligate and facultative plants has to do with seed germination behavior. In order to germinate, seeds of obligate parasitic plants require the presence of the host plant whose roots secrete substances that are perceived by the seed of the parasite and thus stimulate its germination (Bouwmeester et al., 2007; Westwood et al., 2010; Cardoso et al., 2011). For example, in species of Orobanche L. (Plakhine et al., 2012), Phelipanche Pomel (Matusova et al., 2004) and Striga Lour. (Matusova et al., 2004), a very low germination rate (<10 %) has been found in the absence of hosts in all cases. The system of chemical recognition assures that germination begins only when the root of the potential host is available in the immediate vicinity (Mohamed et al., 2001; Joel et al., 2011). The formation of haustoria also occurs in answer to chemical and tactile stimuli provided by the host roots (Westwood et al., 2010). The seeds of other species such as Rhamphicarpa fistulosa (Hochst.) Benth do not require the exudates of the host root to stimulate germination and are able to complete their life cycle without a host, albeit with reduced fitness evidenced by the production of only a few seeds relative to those grown with a host (Ouedraogo et al., 1999). Nevertheless, seeds of these species require light, and because they are very small and have little food reserve for the embryo, they must be very close to the surface of the ground in order to quickly begin photosynthesis (Ouedraogo et al., 1999).

Orobanchaceae is a cosmopolitan family consisting of approximately 90 genera and over 2060 species approximately (McNeal et al., 2013); 21 genera and around 300-330 species are native to the Neotropics, especially the Andean Region and higher altitudes in Brazil (Souza, 2012). This family is morphologically diverse and includes the greatest number of root parasite species in the Plant Kingdom, but only one non-parasitic genus Lindenbergia Lehm., which is phylogenetically supported as sister to the remainder of the family (Young et al., 1999; Phoenix and Press, 2005; Wolfe et al., 2005). Many

species of this family are components of natural vegetation communities in different parts of the world, appreciated for their colorful flowers (Bouwmeester et al., 2007) and some, such as Euphrasia officinalis (L.) and Escobedia grandiflora (L.f) Kuntze, for their medicinal and food uses (Pennell, 1931; Trovato et al., 1999; Bussman et al., 2010). Nevertheless, many species are more well-known for their damaging effects when parasitizing economically important agricultural plants (Parker, 2009; Bouwmeester et al., 2007). In particular, species of the genus Alectra Thunb., Striga and Orobanche are considered noxious weeds of agriculture causing low productivity in a wide spectrum of economically important plants, especially in Africa and Asia (Wolfe et al., 2005; Van Ast and Bastiaans, 2006; Morawetz et al., 2010).

The degree of host specificity of species of Orobanchaceae is variable. There are records of simultaneous parasitism of various hosts, even of distant families (Yeo, 1964; Phoenix and Press, 2005; Parker, 2009; Qasem, 2009). For example, species of *Euphrasia* parasitize both monocot (Poaceae) and eudicot (Fabaceae) plants (Yeo, 1964). In contrast, some cases of high host specificity have also been documented (Ren *et al.*, 2010). Studies carried out to evaluate the possibility of establishing some parasitic species of *Euphrasia* (Yeo, 1961; 1964) under culture conditions found that the plants can grow when planted as seeds or seedlings in pots in which a host has previously been established. Yet, as a result of autoparasitism, they were also able to achieve the flowering stage when planted very close together (Yeo, 1961; Yeo, 1964).

Escobedia Ruiz and Pav. is an American hemiparasitic genus (Bennett and Mathews, 2006; Carranza and Medina, 2008). Two phylogenetic analyses of the family placed this genus in the same clade of obligate parasitic species such as Striga, and as sister to the clade of Melasma P.J. Bergius and Alectra (Bennett and Mathews, 2006; Morawetz et al., 2010). In contrast to other genera of the Orobanchaceae family, there are no studies on the parasitic behavior of Escobedia species, nor records that relate it to the loss of economic crops.

Escobedia grandiflora is found from Central America (Mexico, Costa Rica, Panama) to South America (Colombia, Venezuela, Ecuador, Peru, Brazil, Paraguay and Argentina) and is widely distributed in Colombia, from 0 to 3500 m (Tropicos.org, 2013; review of JAUM, MEDEL, HUA and TOLI herbariums). This species has been important to ancestral communities, especially in the north of South America because of the use of its orange-colored roots as a natural food coloring (Pennell, 1931), and until a few decades ago, for medicinal purposes (Uribe, 2011). Even today, E. grandiflora is sold in local markets in Colombia, and it is considered a native species that can generate economic value, about which there is little information. It is thought that its use has been mainly extractive since no record of its cultivation has been found. This species, abundant in the past (Uribe, 2011), is now found sporadically in small patches especially along roads and in abandoned pastures.

Escobedia seeds are small and linear, similar to those of Melasma and Alectra (Falçao, 1980), and translucent in color except at the center (Souza and Giulietti, 2009). Over 300 seeds are formed in each capsule (Falçao, 1980). There have been no studies related to the germination capacity of E. grandiflora seeds, nor the effect of different hosts on the germination and development of the plants, except for a reference indicating a low percentage of viability of the seed (Castro et al., 2012).

The proposed objective of this study was to evaluate *E. grandiflora* seed germination, seedling, and plant development, with or without hosts of the Poaceae family, in order to make inferences about the degree of parasitism for this species.

MATERIALS AND METHODS

Study site

Escobedia grandiflora seeds were collected from capsules of individuals found in four municipalities in Antioquia (Colombia): Yarumal (07°09' N, 75°22' W), Gomez Plata (06°38' N, 75°11' W), Yolombo (06°34' N, 75°08' W) and Copacabana (06°18' N, 75°30' W) (Fig. 1).

The seeds were extracted manually and then selected with the aid of a stereoscope, in order to reject deformed ones. The plants were collected from the same natural population located in the municipality of Copacabana. The monitoring of the plants was carried out at the *Politecnico Colombiano Jaime Isaza Cadavid* laboratories in the municipality of Bello, Antioquia.

Seed germination

All of the seeds were submerged in distilled water for 24 hours and were then divided into four germination treatments each with four repetitions. Variables in treatments were light and temperature. Two temperatures were evaluated: 20 °C and 25 °C, corresponding to values frequent on sites with wild populations of the species. The light treatments were 12 hours light/12 hours of darkness, and 0 hours light/24 hours of darkness.

Fifty seeds were set on moistened absorbent paper placed in plastic trays, and then covered with a thin, porous white veiling to allow for aeration and avoid a rapid loss of humidity. The seeds were watered periodically with distilled water and observed every 24 hours, for 31 days. They were considered to have germinated when the radicle became visible.

The variables evaluated were percent of germination and vigor, the latter measurement through speed of seed germination and seedling length (Gupta, 1993). Variables were determined in the following manner:

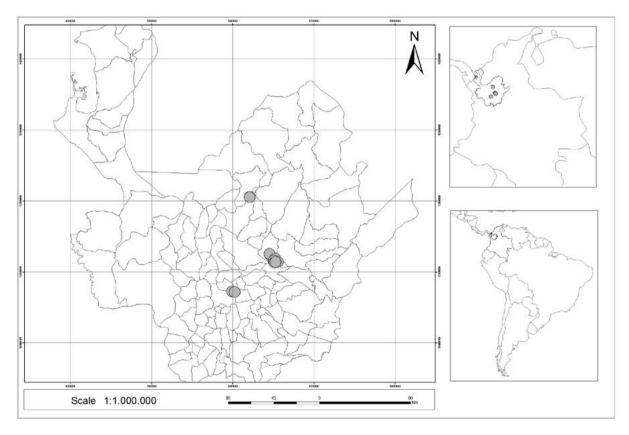


Figure 1. Location of collection sites of Escobedia. grandiflora in Antioquia-Colombia.

Percent of germination: $\%G = \binom{n_i}{n_T}$, where n_i is the

number of seeds germinated and n_T is the total number of seeds. Germination speed was determined according to Raizada and Raghubanshi (2010):

$$GV = \frac{N_1}{1} + \frac{N_2}{2} + \frac{N_3}{3} + \frac{N_4}{4} + \dots + \frac{N_n}{n}$$

where N_n = number of germinated seeds on days 1, 2, 3, n etc.

Variance analysis was used to analyze the data after verifying fulfillment of the assumptions of homoscedasticity and distribution of residuals. The data on percentage of germination was transformed using the arcsine function. The Tukey Test was used as an *a posteriori* test.

One hundred and twenty seeds were used to evaluate the length of the *E. grandiflora* seedlings. Seeds had previously been hydrated in water for 24 hours at a temperature of 25 °C and with 12 hours of light. When the radicle emerged of the seed, it was then planted in a germinator containing a substrate taken from the four localities and mixed with soil rich in organic matter in a proportion of 2:1. During a 40-day period, five seedlings were measured every seven days. Different plants were chosen for each measurement because the stress produced by their extraction could also cause a delay in growth. The information was analyzed by means of multiple regression analysis using SAS System (SAS Institute Inc., Cary, North Carolina).

Seedling development

Seeds from two grasses previously planted in a germination tray were used. Eight days after their germination, a recently germinated *E. grandiflora* seedling was planted. The seedlings were divided into three treatments: *E. grandiflora* alone, *E. grandiflora* with *Andropogon bicornis* L., and *E. grandiflora* with *Paspalum notatum* Alain ex Flüggé. The plants were observed for 16 weeks, and every four weeks the variables of percentage, survival and length of the *E. grandiflora* seedlings were measured.

Plant development

Sixty small plants were collected from a natural population of E. grandiflora, selected according to the following characteristics: height between 4.0 and 9.5 cm, a minimum of two leaves and root length of between 3.0 and 9.0 cm. The plants were then taken to the laboratory, washed with water and planted in nursery bags (43 cm long and 18 cm wide) using a substrate consisting of sand, organic material and soil from areas where the plant was found. The plants were divided into three treatments of 20 plants each consisting of E. grandiflora alone (T1); E. grandiflora in association with the narrow-leafed grasses Calamagrostis viridiflavecens (Poir.) Steud., Sporobolus jacquemontii Kunth, and A. bicornis (T2); and E. grandiflora with Pennisetum purpureum (T3) (Fig. 2). T2 included three grasses because, although the initial collection was identified as a single species, three different species were later recognized. Thus, the three were included in all T2 repetitions. The species on the collection site were naturally associated with E. grandiflora. All bags received an

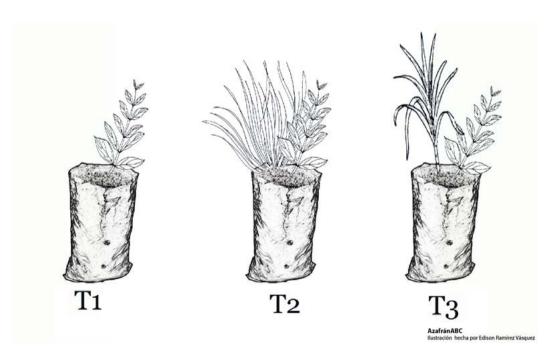


Figure 2. Evaluation of parasitism of *Escobedia grandiflora* on grasses. T1: *E. grandiflora*, T2: *E. grandiflora* + *C. viridiflavecens*, *S. jacquemontii* and *A. bicornis*, T3: *E. grandiflora* + *Paspalum purpureum*.

application of diluted inorganic fertilizer (BP-150, Safer Agrobiológicos, Registro ICA 6870) every two weeks in order to guarantee the input of nutrients from the grasses.

The E. grandiflora plants were observed for 28 weeks, and measured every two weeks for variables of stem length, number of leaves and width-length of leaves in the middle third of the plant. On the last two dates, the distance between internodes, number of internodes, number of stems and thickness of stems were measured. At the end of this period, the plants were extracted from the bags in order to measure final root length and determine presence and number of haustoria. A repeated analysis measurement was used to analyze the data: the SAS System (SAS Institute Inc., Cary, North Carolina) and MIXED procedure. Comparison of measurements was carried out using the Tukey test. The Wilcoxon rank test was used to compare the variables measured on the last two dates.

RESULTS

Seed germination

Germination of the E. grandiflora seeds ranged between 66 % and 85.3 % (Table 1). The root emerged from the narrowest area of the seed and, later, the plumule with a pair of pubescent cotyledons although three cotyledons were observed in a few seeds. In cases where the plumule emerged first, the seedlings did not survive. The two conditions of temperature and luminosity had no effect on seed germination percentage.

In contrast, germination speed was affected by temperature (F = 18.09; p = 0.003), being greater in seeds submitted to a temperature of 25 °C. The two conditions of light evaluated had no differential effect on rate of germination. The size of the seed determined the size of the seedling (R-adjusted = 63.42 %, p = 0.001). Thus, the longest and heaviest seeds showed the largest percentage of germination and produced larger seedlings.

During the germination tests, some seeds were affected by the Fusarium sp. fungus, which were discarded. The genus Fusarium have been reported on seeds of Striga and Alectra (Abbasher and Sauerborn, 1992; Marley et al., 2005).

Seedling development

During the first four weeks, the survival of E. grandiflora seedlings from sexual seeds was 100 %. At eight weeks, E. grandiflora without host had a survival percentage of 7.5 %, E. grandiflora with P. notatum of 95%, and E. grandiflora with A. bicornis of 60 %. At the end of the test, only E. grandiflora seedlings with P. notatum survived at 45 %. In general, the growth rate of the seedlings was slow: they grew an average of 1 mm and developed two leaves every four weeks. At 16 weeks, the roots began to exhibit the orange color typical of the species.

Plant development

In contrast to survival of seedlings, at four weeks the percentage of survival of small plants was 93.3 %. All of the plants, independent of the treatment, showed a slow growth rate and aerial stems formed from buds of the rhizome. The formation of branches of the aerial stem from axillary buds was an occasional event that could possibly be induced by the breaking of the main stem through wind action, or by damage or weakening of the main stem. In many apparently healthy plants, independent of treatment, the apical bud of the main stem would usually die and the aerial stem would then wither completely. Later, another stem would form from the new buds on the rhizome. This behavior led to a great variation in the data from all of the treatments as seen in the high values in standard deviation. Nevertheless, clear differences between treatments began to be noted by the 16th week. Between weeks 22 and 26, T3 (E. grandiflora+P. purpureum) presented a greater number of leaves, a greater length of aerial stems and larger leaves (Table 2). However in the 28th week, T2 (E. grandiflora+narrow-leafed grasses) caught up with T3 regarding number of leaves, length of aerial stem, size of leaves and length of internodes, both being superior to T1 (E. grandiflora alone). The more significant event was that T2 and T3 initiated formation of reproductive structures, but T1 did not (Table 2). There were a greater number of internodes in T3 than in the other two treatments, but no differences among treatments as far as the variables of internode distance, number of aerial stems and stem thickness (Table 2).

Table 1. Percentage of seed germination of *Escobedia grandiflora* under two conditions of light and temperature.

Treatment	% Germination	Germination speed
T1 (20 °C, 0 hours light)	85.3 a	1.7 a
T2 (25 °C, 0 hours light)	82.0 a	3.7 b
T3 (20 °C, 12 hours light)	66.0 a	2.3 a
T4 (25 °C, 12 hours light)	84.0 a	3.9 b
Control	74.7 a	2.8 a

Different letters indicate statistically significant differences (p < 0.05) (Test de Kruskal-Wallis).

At the end of the experiment T3 *E. grandiflora* plants showed a greater number of haustoria, greater accumulation of total dry matter of the plant, rhizome dry matter, and of aerial stems than in T1 and T2 plants (Table 2). Root and leaf weight, as well as increased root length, was similar in the three treatments (Table 2). In T2 haustoria were observed in the three narrow-leafed grasses and, in some replicates simultaneous parasitism was also observed of two or three species. In general, the most parasitized hosts were *S. jacquemontii* and *A. bicornis*. In T1, haustoria sometimes formed without the presence of a host, and there were haustoria in T2 that were not parasitizing roots, but in any case auto-parasitism was not observed.

DISCUSSION

Escobedia grandiflora seeds do not require the presence of the host in order to germinate, which is typical of facultative parasitic plants (Těšitel et al., 2011). Although no studies on Escobedia seed germination are known, studies on seeds of related obligately parasitic genera, Alectra and Aeginetia L., recorded a lower percentage of germination than that of those found here (French and Sherman, 1976; Bennett and Mathews, 2006). So it can be argued that E. grandiflora seeds have a high percentage of germination. The 25 °C

temperature at which the seeds had a greater germination speed is similar to the average temperature of locations where the plant is most abundant in Colombia. The two conditions of light evaluated were not decisive in germination.

Although the largest and heaviest seeds produced more vigorous seedlings, survival after four weeks was not successful without the host. Seedlings even with the host, were few in number and showed slow growth afterwards. It is possible that, as in other hemiparasitic species of the family, the state of the seedling is critical (Těšitel et al., 2011) although it initially seems to depend on the food reserve of the seeds, and on their own photosynthetic activity. Germinated seeds need to quickly parasitize a host in order to obtain nutrition. The food reserve within the seed is scarce given its morphology and small size. Although each capsule produces 300 seeds or more (Falçao, 1980) that are easily disbursed to new sites by wind, a low survival rate and a slow growth rate of the seedlings could imply serious consequences for the population and be a vulnerability factor for the species. This may explain the scarce disbursed natural populations that are presently observed in the Colombian Andean region, although each population is locally abundant.

Propagation of *E. grandiflora* from small plants between 4 and 9 cm in height produced an initially high rate of survival,

Table 2. Statistical analysis of growth and development variables of *Escobedia grandiflora* in absence and presence of the hosts.

Measurement variable	F- value	Pr > F	Differences
N° of leaves	2.23	0.007	From week 16, T2 and T3 had a greater number of leaves than T1. In week 24, the three treatments differed, number of leaves in T3 being greater, and less in T1. T1 plants maintained the same number of leaves during the last six weeks, T3 had a greater number of leaves in week 24, but by week 28 T2 and T3 had a similar number of leaves.
Length of aerial stem (cm)	2.67	< 0.001	T2 and T3 plants had longer stems than T1 from week 22 on. T1 plants did not vary in length over the last 4 weeks. T2 plants varied progressively, passing through three different sizes and only equaling T3 in the last week.
Leaf width (cm)	2.11	0.001	In week 18, T2 and T3 had wider leaves than T1. In weeks 22, 24 and 26, T3 was significantly wider. In week 28, T2 and T3 were equal with values superior to those of T1.
Leaf length (cm)	2.92	< 0.001	Same as description of the last variable.
N° of internodes	2.94	0.024	In T3, a greater number of internodes was observed in weeks 26 y 28 than in the other two treatments.
Distance of internodes (cm)	0.68	0.60	Similar in all treatments.
N° of aerial stems	0.66	0.62	Similar in all of the treatments.
Thickness of aerial stems (cm)	0.69	0.60	Thickness of aerial stems was similar in all of the treatments.
N° of haustoria	8.21	0.009	T3 had more haustoria than T1 and T2.
Total plant weight (mg)	5.79	0.024	T3 had more dry matter than T1 and T2.
Rhizome weight (mg)	7.30	0.013	T3 plants had rhizomes with more dry matter than those of T1, but similar to T2.
Aerial stem weight (mg)	5.69	0.025	T3 plants had aerial stems with more dry matter than T1 and T2.
Root weight (mg)	1.95	0.20	Plants with the three treatments had root with equal amount of dry matter.
Increase in root length (cm)	4.09	0.09	Plants with the three treatments had roots of equal length
Weight of leaves (mg)	3.8	0.063	T3 had heavier leaves than those of T1, and similar to T2.

T1: E. grandiflora, T2: E. grandiflora + C. viridiflavecens, S. jacquemontii y A. bicornis, T3: E. grandiflora + P. purpureum.

93 %, even without the presence of the host. This study found that the species was able to live without a host for over 28 weeks, and that only after week 18 is a clear disadvantage observed in plants alone with respect to plants with a host. The differences between treatments in the accumulation of dry matter from the rhizome, number of haustoria and dry matter from the aerial stems suggest that the plant initially accumulates an important food reserve in the rhizome. This permits it to survive for a time until parasitism is successfully established. Additionally, after the plant has been established it is able to form new functional roots even without a host. This is suggested by the similarity in root length and the accumulation of dry matter of this organ. The plants without host did not produce flowers or fruits/seeds, and, conversely the tendency in their development indicates that they will die after 28 weeks, while those formed with hosts will begin the reproductive phase.

Of the species considered in this study, the best hosts for *E. grandiflora* were *P. purpureum*, *S. jacquemontii* and *A. bicornis*. The first of these permitted rapid growth and there was a strong attachment of haustoria in all three. In order to evaluate the reproductive phase, the application of this study over longer periods of time is recommended. Hemiparasitic plants have an ambiguous relation with their host since they take nutrients from it but also compete with it for light thus affecting photosynthetic activity (Těšitel *et al.*, 2011). In this experiment, the leaves of the host were pruned to favor the photosynthetic activity of the *E. grandiflora*. So it is possible that the results obtained will be modified if the host is allowed to develop completely.

The obligate hemiparasitic nature of *E. grandiflora* is confirmed, an aspect unknown until now intropical American countries, where it is used for coloring foods (Pennell, 1931; Ulloa, 2006). Biological knowledge of the species is also increased in key aspects such as seed germination, seedling development and the recording of four hosts under natural conditions. Studies must be continued in order to evaluate the feasibility of cultivating this parasitic species of ethnobotanical importance in Central and South America.

CONCLUSION

Escobedia grandiflora demonstrates attributes of facultative hemiparasitic plants such as a high rate of seed germination in the absence of a host, and prolonged survival of plants with rhizomes in the absence of a host. Nevertheless, two important factors support its habit as an obligate hemiparasite. First, the seedling cannot survive alone and presents a low survival rate even with a host. Second, the reproductive stage only begins in plants with a host. This paper is the first revision of parasitism type presented by this species and by Genus Escobedia, which may be important to understand why E. grandiflora has been disappearing from the Colombian Andes.

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