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The *Bletia catenulata* ornamental orchid is self-compatible but pollinator-dependent for reproduction[1]

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RESUMO

A orquídea ornamental *Bletia catenulata* é autocompatível, mas dependente de polinizador para reprodução

Bletia catenulata é uma orquídea nativa do Brasil, cujas populações têm sofrido coleta predatória. Objetivando conhecer aspectos da biologia reprodutiva e sua relação com a germinação de sementes, diferentes tipos de polinização foram realizados em flores de plantas cultivadas em estufa agrícola. A polinização cruzada e a autopolinização manuais resultaram em altos percentuais de frutificação (76,7-86,7 %), sendo de apenas 26,7 % na polinização natural, na qual a ausência de polinizadores, por isolamento das flores, resultou na ausência de frutos. A germinação *in vitro* de sementes de todos os frutos resultantes das diferentes polinizações foi baixa, sendo 8,6 % o maior valor percentual de protocormos obtidos. Portanto, *B. catenulata* é autocompatível, gerando sementes viáveis, mas dependente de polinizador para reprodução sexuada.

PALAVRAS-CHAVE: Germinação *in vitro*; aclimatização; Cerrado.

ABSTRACT

Bletia catenulata is a native Brazilian orchid whose populations have suffered from predatory collection. Aiming at elucidating aspects of its reproductive biology and the relation with seed germination, pollination tests were carried out in flowers of plants cultivated in a greenhouse. Manual cross and self-pollination resulted in high percentage of fructification (76.7-86.7 %), being only 26.7 % for natural pollination, in which the absence of pollinators, due to flower isolation, resulted in absence of fruits. *In vitro* seed germination for all fruits from the different pollination processes was low, being 8.6 % the highest percentage for protocorms. Thus, *B. catenulata* is self-compatible, resulting in viable seeds, but pollinator-dependent for sexual reproduction.

KEY-WORDS: *In vitro* germination; acclimatization; Brazilian Savannah.

INTRODUCTION

Bletia Ruiz & Pav. is a neotropical orchid genus consisting of about 40 species (Brown 2005). However, in Brazil, only two species have been registered (Barros et al. 2015).

Bletia catenulata Ruiz & Pav. is a common species in the Brazilian Savannah, Amazonia and Atlantic Forest, being also found in the seashore of the Rio de Janeiro State, at the Massambaba sandbank (Cepemar 2004).

Some authors reported that *B. catenulata* was found in different Brazilian States, such as Tocantins and Maranhão (Silva et al. 1995), Minas Gerais (Araújo et al. 2002), Distrito Federal (Batista et al. 2005) and São Paulo (Ferreira et al. 2010), and also in other countries, such as Bolivia (Vásquez et al. 2003) and Paraguay (Schinini 2010). We have recently identified this species in the Mato Grosso do Sul State, Brazil.

The distribution of *Bletia* specimens is determined by factors such as the degree of disturbance of their environment, adaptability of the species and diversity of morphotypes of the available mycorrhizal fungus, which is associated with their dispersibility (Beltrán-Nambo et al. 2012), among others.

In the northeast region of the Mato Grosso do Sul State, *B. catenulata* was found in four different sites, always with low frequency. In some sites, it is exposed to environments that undergo human disturbance. In addition, this orchid has terrestrial habits, superficial roots and often occurs near watercourses.

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Plant collection and environmental disturbance have threatened the species, intensifying its risk of extinction. Swarts & Dixon (2009) consider that orchids are primarily threatened by anthropogenic causes, such as habitat destruction, modification and fragmentation, or even over-collection.

Dressler (1968) stated that *Bletia* flowers are adapted to bee pollination. Self-pollination is frequent in *B. campanulata*, *B. macristhmochila*, *B. purpurea* and *B. urbana*, and nearly universal in *B. rosea*. Yet, observations of *B. campanulata* and *B. purpurea* indicate that many plants are facultative autogamous, according to the plant condition and probably to the habitat condition (Dressler 1968). Other plants, sometimes in the same populations, are always autogamous. However, except for the fact that some authors have reported *Xylocopa* sp. as the pollinator of *B. catenulata* (Dodson 1962, Van der Cingel 2001), little information related to floral and reproductive biology and no information on seed germination of this species are found in the literature.

Most orchids are self-compatible (Van der Pijl & Dodson 1966). However, spontaneous self-pollination tends to be avoided by the presence of some floral mechanisms (Van der Pijl & Dodson 1966, Catling & Catling 1991). Additionally, orchids tend to produce more viable seeds after cross-pollination (Stort 1983 and 1986, Smidt et al. 2006). Pollination carried out under experimental conditions also promotes increased fruit production in many Orchidaceae species, when compared with populations in the natural environment (Pansarin 2003, Mickeliunas et al. 2006, Storti et al. 2011). In their habitat, herbivory and fruit and seed predation can negatively influence its reproductive success.

The study of orchid reproductive biology is essential for propagation and breeding. In general, seed viability tests are carried out by using tetrazolium (Suzuki et al. 2012), fluorescent diacetate staining (Chen et al. 2015) and/or *in vitro* asymbiotic germination (Suzuki et al. 2012, Chen et al. 2015). Asymbiotic seed germination has been used for the multiplication of commercially important orchids, being an effective tool for orchid propagation, concerning conservation and reintroduction purposes (McKendrick 1995, 1996a and 1996b, Stenberg & Kane 1998, Kauth et al. 2006, Stewart & Kane 2006, Johnson & Kane 2012, Wu et al. 2014).

In vitro seed germination studies can provide insights into *in situ* plant responses to environmental conditions and basic information on early plant growth and development. Several *in vitro* seed germination studies on *B. purpurea* can be found in the literature (Dutra et al. 2008, Johnson & Kane 2012 and 2013). However, no information has been found about *B. catenulata*.

This study aimed at obtaining initial information on ornamental potential and influence of pollination type on seed viability of the *B. catenulata* orchid, in order to enable future preservation programs.

MATERIAL AND METHODS

Eight plants of *B. catenulata* were collected in December 2013, in Cachoeira da Rapadura (18º25’36.54”S; 52º56’57.23”W), in Costa Rica, Mato Grosso do Sul State, Brazil.

Plants were cultivated in plastic pots, using Plantmax® - HT substrate, in a greenhouse, with daily irrigation. For the species identification procedures, the specimens were deposited in the herbarium of the Instituto de Botânica, in São Paulo, Brazil.

During the flowering stage of the collected plants, the height of the inflorescence stalk (measured from the base of the stalk to the topmost flower insertion point) and number of flowers/inflorescence were measured to calculate the mean number and standard deviation. Additionally, floral longevity was estimated by daily observation to obtain the date of the first anthesis and of the last senescent flower. Plants were monitored from July 1st to October 10th, in 2014.

To analyze the reproductive biology, ninety flowers in six inflorescences of six plants maintained in a greenhouse were used to carry out manual pollinations. After flower anthesis, self-pollination was carried out in thirty flowers. Cross-pollinations among flowers of the same individual (geitonogamy) and different individuals (xenogamy) were performed using thirty flowers for each pollination treatment. In all three treatments, pollinia were dislodged from the gynostemium by applying slight upward pressure to the bottom of the anther cap. After removal, pollinia were gently transferred into the stigmatic surface of the same (geitonogamy) or another (xenogamy) flower. Hand pollinated flowers were closely monitored for flower senescence, capsule development and capsule maturity.

To analyze the natural pollination, thirty flowers were maintained inside the greenhouse to test the spontaneous pollination without pollinators contact. Another plant with thirty flowers was maintained outside the greenhouse to verify open pollination with possible pollinator presence. In all previous cases, each group of ten flowers was considered a replication. The assessment of the pollination was carried out by counting the number of fruits obtained from the initial number of flowers.

After fruits were ripened, *in vitro* seed germination was carried out. Fruits were sterilized for twenty minutes in a solution of sodium hypochlorite (2 % active Chlorine), followed by five minutes in 70 % ethanol, and finally rinsed three times in sterile distilled water. Seeds were sown in Petri dishes containing 20 mL of Knudson C medium (Arditti & Ernst 1993), supplemented with MS vitamins, 58.4 mM of sucrose, 100 mg L^{-1} of myo-inositol and 0 g L^{-1} or 2 g L^{-1} of activated charcoal, pH of 5.6 ± 0.1, and solidified with 5.0 g L^{-1} of agar (HiMedia®).

Cultures were maintained under a 16-hour photoperiod, with irradiance of 36 μmol m^{-2} s^{-1} provided by two fluorescent tubes of 20 W (Luz do Dia Especial, Osram, Brazil), and temperature of 27 ± 2 ºC, in a growth room. After 45 days, seed germination data were obtained by counting protocorms and unfertile seeds using a light optical microscope. The seeds and protocorms counts were conducted in three sampling areas (2.0 cm in diameter) of each Petri dish.

After vitroplants elongation, they were used to evaluate the acclimatization phase. Thirty vitroplants were individually placed in black plastic pots (7 cm height × 7 cm diameter) containing Plantmax® substrate. Pots were placed on benches and kept for 90 days in a greenhouse, with intermittent irrigation system comprised of micro-sprinklers activated by a timer, with two daily irrigation cycles of two minutes each. Plants received foliar fertilizer application (Nipokan®) at weekly intervals according to the manufacturer recommendation (75 mL/ 100 L). After the acclimatization period, plants were evaluated in terms of survival percentage.

The pollination experiment was carried out using three replicates with ten flowers each. Due to the limitation in the number of collected plants supported by the SISBIO 22570-2 authorization, replicates were from the same plant containing at least 30 flowers. Thus, morphological and pollination data were used to calculate means and standard deviation. The *in vitro* experiment was performed using five replicates (plates) for each treatment, and it was arranged using a completely randomized design. Percentage data were arcsine transformed prior to the statistical analysis, and the Anova and Tukey test were used to compare the treatment ($\alpha = 0.05$), using the Sisvar software (Ferreira 2011).

RESULTS AND DISCUSSION

The inflorescence stalk mean length of 75.4 ± 10.2 cm, combined with the attractive and intense violet color, size and shape of flowers (Figure 1B), provides great potential for economic and ornamental exploitation of this species.

The *B. catenulata* flowering period extended from July to October, and racemes had 20.9 ± 6.1 flowers each. The total inflorescence longevity was 35 ± 4 days, considering the period between the first anthesis and the senescence of the last flower, resulting in a long lifetime of raceme and a long life-span flower, showing the ornamental potential of this species. Additionally, this could ensure better pollination success under scarce or uncertain pollinator visits, and consequently increase the time that each inflorescence is exposed to potential effective visitors to pollination (Flores-Palacios & Garcia-Franco 2003, Castro et al. 2008).

Collected data show that *B. catenulata* presents successful fecundation, regardless of the pollen origin. Thus, all the pollination processes [geitonogamy, xenogamy, auto-pollination and open pollination (outside greenhouse)] resulted in fruit formation, and also in viable seeds. However, when flowers were protected against pollinator visits or spontaneous pollination (inside greenhouse), neither fruits and consequently nor seeds were detected (Table 1). These results indicate that *B. catenulata* is a self-compatible, pollinator-dependent species.

Most orchids are self-compatible (Van der Pijl & Dodson 1966). However, spontaneous self-pollination tends to be avoided by the presence of special floral arrangements (Van der Pijl & Dodson 1966, Catling & Catling 1991). Manual pollination carried out under experimental conditions results in increased fruit production in many Orchidaceae species, if compared with populations in the natural environment (Pansarin 2003, Mickeliunas et al. 2006, Storti et al. 2011, Suetsugu 2015). Herbivory and fruits and seeds predation may negatively influence the reproductive success in orchids' natural habitat.

The fruit formation percentage in flowers submitted to open pollination (outside greenhouse) was considerably lower than in those submitted to manual pollination (Table 1), since, in many cases, pollination agents are a limiting factor. Similar results were found in other orchids genera. Smidt et al. (2006) and Silva-Pereira et al. (2007) studied the reproductive biology of four species from Chapada Diamantina, Bahia State, Brazil, and all were self-compatible and pollinator-dependent. On the other hand, Micheneau et al. (2008) found that *Jumellea stenophylla* is clearly able to produce seeds in the

Table 1. Number of evaluated flowers, fruit formation (mean ± standard deviation) percentage and *in vitro* seed germination, in relation to total seeds, from different pollination treatments carried out in *Bletia catenulata* Ruiz & Pav. (Costa Rica, Mato Grosso do Sul State, Brazil, 2014).

Pollination process	Flowers (n)	Fruits (%)	Germination (%)
Spontaneous self-pollination	30	00.0	-
Manual self-pollination	30	86.7 ± 12.5	8.6 a
Manual cross-pollination (geitonogamy)	30	76.7 ± 9.4	4.5 a
Manual cross-pollination (xenogamy)	30	83.3 ± 9.4	2.0 ab
Open pollination	30	26.7 ± 4.7	0.9 b

Means followed by the same letter in the column do not differ by the Tukey test, at 5 %.

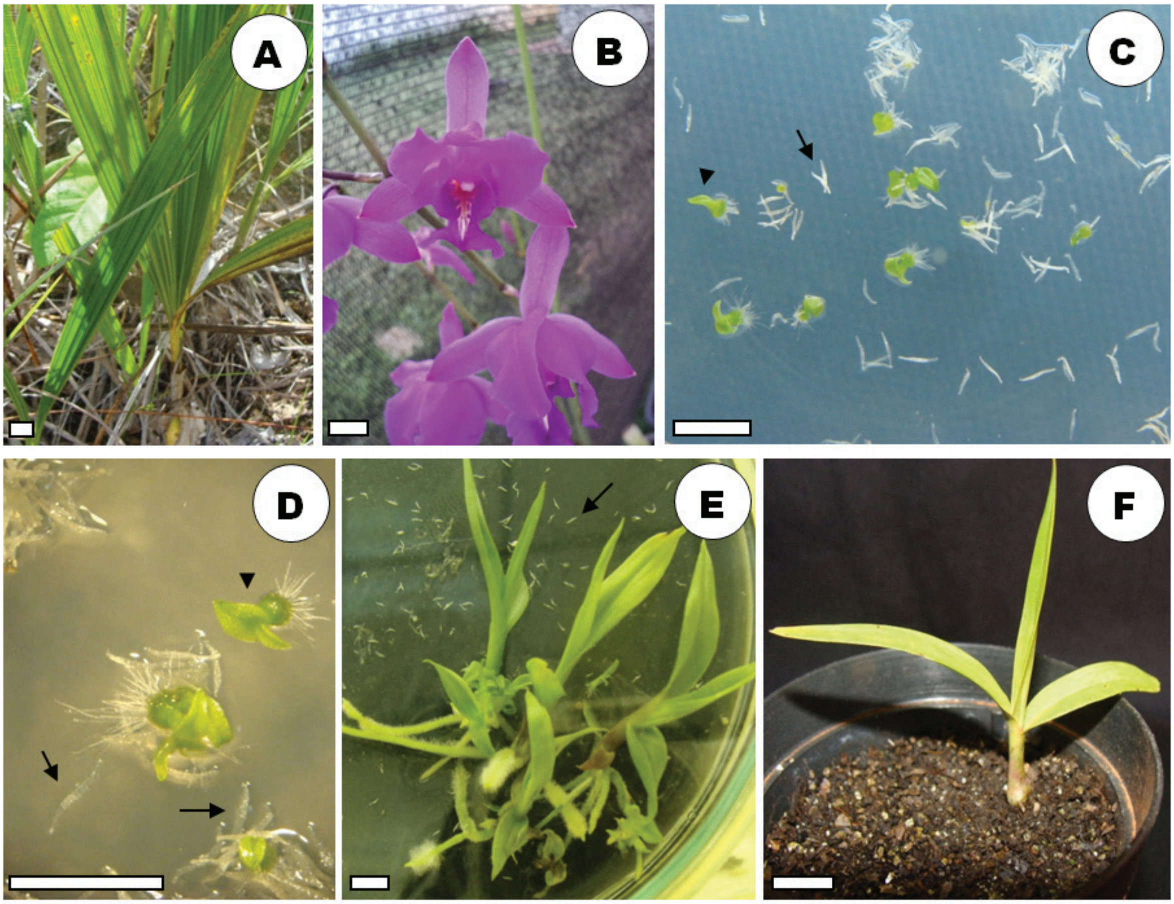

Figure 1. *Bletia catenulata* Ruiz & Pav. in different cultivation stages: adult plant (A), flower after anthesis (B), vitroplants obtained from viable seeds (arrow heads) and unfertile seeds (arrows) on germination medium (C and D), elongated vitroplants and unfertile seeds (arrows) (E) and acclimatized vitroplant (F). Bar = 1.0 cm (Costa Rica, Mato Grosso do Sul State, Brazil, 2014).

absence of pollinators, and the fruiting success of bagged flowers reached 66.7 %.

Seed viability tests showed that the germination rate was low in all types of obtained fruits (Table 1). These low germination values can be attributed to the high percentage of seeds lacking embryos present in the fruits (Figures 1C, 1D, 1E), always higher than 90 %.

The proportion of seeds containing viable embryos is critical to the ongoing maintenance of orchid population (Alexander et al. 2010). Bowles & Jacobs (2002) showed that outcrossing by hand pollination produced higher percentage of viable seeds than with natural pollination in the *Platanthera leucophaea* orchid. According to Borba & Braga (2003), the lower percentage of viable seeds from autogamous and geitonogamic pollinations in *Pseudolaelia corcovadensis* populations indicates the occurrence of endogamic depression in the early stages of development (embryo abortion). That was also frequently observed in other orchids from the Laeliinae subtribe (Stort & Galdino 1984, Matias et al. 1996), or even in other orchids groups (Catling 1982, Borba et al. 2001), and seems to be widely distributed in the family.

The endogamic depression observed in *P. corcovadensis* is usually found in predominantly allogamous plant populations (Wiens et al. 1987, Husband & Schemske 1996, Byers & Waller 1999). Here, despite the intense presence of unviable seeds or infertile ovules, higher germination percentages were obtained by self-pollination and geitonogamy originated seeds. So, the occurrence of endogamic depression in *B. catenulata* is an unclear phenomenon and requires additional research.

The absence of fruits in the spontaneous self-pollination treatment (Table 1), when flower was spatially isolated from insects, showed that the pollinator presence is absolutely necessary for the *B. catenulata* sexual reproduction. *Broughtonia lindenii*, another species belonging to the Laeliinae subtribe, is pollinator-dependent and self-compatible (Vale et al. 2011). This reproductive combination of traits is broadly reported among nectarless tropical orchids, especially among the Laeliinae (Smidt et al. 2006). Although some orchid species are known to be self-incompatible, many are self-compatible and show varying levels of natural self-pollination or autogamy (Weston et al. 2005). Obligated autogamy is found in a few species (e.g. *Orthoceras strictum*), presumably as an adaptational response to scarcity or absence of pollinators. Completely autogamous species tend to produce smaller, less attractive/colorful flowers than closely related outcrossers. This difference may be an evolutionary response to the loss of selective pressure for pollinator attraction. Conversely, we found that *B. catenulata* has characteristics of a pollinator-dependent species, such as relatively big, intense purple-colored flowers (Figure 1B). Thus, manual pollination can enhance propagation and preservation efforts.

Vitroplants resulting from protocorms (Figures 1C, 1D) showed good growth and development during the *in vitro* phase (Figure 1E). The acclimatization process was successful for 55 % of the vitroplants (Figure 1F), and did not differ between treatments. Thus, the vitroplants acclimatization capacity is not dependent on the pollination type.

Knowledge on the reproductive biology of *Bletia catenulata* is important for *in situ* preservation programs which attempt to develop pollination strategies to enhance propagation success in areas where the species presents reduced number of plants. Furthermore, the commercial exploitation using manual pollination and *in vitro* seed germination could reduce the ongoing pressure originating from predatory collections of *B. catenulata* native plants.

CONCLUSIONS

1. Manual pollination increases the number of fruits and consequently the seed production, in relation to natural pollination.
2. *Bletia catenulata* seeds can be produced both by cross and self-pollination.
3. *Bletia catenulata* pollination is insect-dependent under natural conditions.
4. Fruits resulting from pollinated flowers offer viable seeds capable of producing normal plants by using the *in vitro* germination methodology.

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