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Aspects of the reproductive biology of *Brassavola cebolleta* Rchb.f. (Orchidaceae)

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ABSTRACT. This survey assessed some aspects of the reproductive biology of *Brassavola cebolleta* Rchb. f. (Orchidaceae) in the Municipality of Dourados, Mato Grosso do Sul State, Brazil. Floral biology, breeding systems, floral visitors and seed germination were analyzed. Differential success and fruit production rate were calculated. Pollination system indicated the pollen vector dependence and absence of pre-zygotic barriers related to self-incompatibility. Pollination occurred at night, being the potential pollen vector a Lepidoptera-Notodontidae of the genus *Hemiceras*. Differential success of male was 19%, female 9%, and the rate of effective fructification was 6.3%. Fruits produced after pollinator visits were larger than those generated by manual cross pollination, and despite the lower number of potentially viable seeds produced by the first, they presented a higher germination rate in asymbiotic media. This study warns to the vulnerability of the studied species, since the habitat fragmentation associated with pollinator scarcity and seed predation may significantly decrease new recruitment into populations.

Key words: floral biology, fructification, pollination, breeding system.

RESUMO. Aspectos da biologia reprodutiva de *Brassavola cebolleta* Rchb. f. (Orchidaceae). Este trabalho teve como objetivo analisar alguns aspectos da biologia reprodutiva de *Brassavola cebolleta* Rchb. f. (Orchidaceae), em Dourados, Estado do Mato Grosso do Sul, Brasil. Avaliaram-se a biologia floral, o sistema reprodutivo, os visitantes florais e a germinação das sementes. Calcularam-se o sucesso diferencial e a taxa de frutificação. O sistema de polinização indicou a dependência de vetores de pólen e a inexistência de barreiras pré-zigóticas relacionadas à autopolinização. A polinização ocorreu à noite, sendo o potencial vetor de pólen um Lepidoptera-Notodontidae, do gênero *Hemiceras*. O sucesso diferencial masculino foi de 19%, o feminino de 9% e a taxa de frutificação efetiva foi de 6,3%. Os frutos gerados com auxílio do agente polinizador foram maiores que os produzidos por polinização cruzada manual e, embora o número de sementes potencialmente viáveis produzidas pelo primeiro tenha sido menor, elas apresentaram maior taxa de germinação em meio assimbiótico. Este estudo alerta para a vulnerabilidade da espécie estudada uma vez que a fragmentação de hábitat associado à escassez de polinizadores e à predação das sementes pode reduzir drasticamente os novos recrutamentos às populações.

Palavras-chave: biologia floral, frutificação, polinização, sistema reprodutivo.

Introduction

The floral biology of orchid flowers, their interactions with pollinators and diversification along the evolutionary time is a more fascinating topic than Darwin had ever suspected (TREMBLAY et al., 2005). Despite the fact that the number of surveys about this subject has increased in the last few years in Brazil, the great diversity of plants in the country, especially represented by families like Orchidaceae, remains little known concerning reproductive mechanisms of some genera.

Brassavola R. Br. presents flowers whose morphology indicates a possible sphingophilous pollination, once the floral structure allows the pollinators to feed fluttering in the air, inserting their proboscis into the cuniculus (CINGEL, 2001). The flowers, usually cream or pale green colored, exhale specific fragrances in the dusk or at night, and may have extrafloral nectaries that attract ants and other herbivorous (ROEBUCK; STEINHART, 1978).

Hummingbirds (Trochilidae) have been observed in Northern Brazil visiting flowers of *B. martiana* (BRAGA, 1977), and in Southern Brazil, flowers of *B. tuberculata* (CINGEL, 2001). In a study on the reproductive biology of *B. nodosa* it was observed the great dominance of male (pollinia removal) over the female success (deposition of pollinia on the stigmatic cavity) (SCHEMSKE, 1980).

Concerning the flower visitors of the Orchidaceae, there is a need for specific pollinators, mainly in species where all the pollen are concentrated in pollinia (TREMBLAY et al., 2005). Thus, the attraction of non effective or unspecific pollinators leads to a negative selection due to the considerable loss of reproductive success (PANSARIN, 2003; WASER; OLLERTON, 2006). Morphological adaptations to specific pollinators ensure the predominantly cross pollination (COZZOLINO; WIDMER, 2005). This characteristic and the mycorrhizal association represent preponderant agents in the diversification and maintenance of the great number of species of Orchidaceae (OTERO; FLANAGAN, 2006). However, Tremblay et al. (2005) argue that the driving mechanism of diversity in Orchidaceae is based on the predominance of pollination limitation, which significantly favors well succeeded plant-pollinator interactions, since the pollination of the number of produced seeds is usually very expressive.

In this way, this survey assessed aspects of the floral biology, pollination systems, fruit set and seed germination in *Brassavola cebolleta* Rchb f. (Orchidaceae).

Material and methods

This study was carried out from June to September from 2006 to 2007 in Dourados Municipality, 22º21'03"S and 54º47'07"W, Mato Grosso do Sul State, Brazil, in a private reserve (Estância Aurora) on the margin of the Dourados river, in an altitude about 458 m. The climate of the region is humid with dry winter; average precipitation is 1,500 mm, and an annual average temperature of 22ºC. The vegetation is seasonally semi-deciduous forest.

We studied 32 individuals of *Brassavola cebolleta*, spread over two distinct populations that occur in a 10 m belt of riparian forest, about 3 km from each other, both on the same margin of the river, and the host trees were about 15 m far from each other. The study of floral biology and pollination ecology was done through uninterrupted observations of the clumps with flowers of B. *cebolleta*, from 4:00 to 9:00h, and from 17:00 to 22:00h, during five days in each population totaling up 200 hours, after the two years of the study. We assessed foraging behavior of floral visitors, time and legitimacy of the visitation and parts of the contact with reproductive structures. Twenty floral buds in five individuals were marked during pre-anthesis in order to evaluate the anthesis sequence, and the occurrence of visitations (removal and deposition of pollinia) between 9:00 and 17:00h, and between 22:00 and 4:00h, when focal observations were not conducted. For nighttime focal observation "black light" illumination was utilized.

Morphometric characterization of the floral parts was done with 30 flowers from 15 individuals, using a digital caliper. The presence of osmophores was tested by immersing eight flowers in a solution of 1% neutral red for 10 min. and then washing them with a solution of 5% glacial acetic acid (WIEMER et al., 2009). The presence of pigments was verified through the setting of eight flowers in ammonium hydroxide atmosphere for 5 min. (SCOGIN et al., 1977), in order to detect flavonoids. The stigmatic receptivity was tested over the study period by verifying the peroxidase activity, using V10 hydrogen peroxide (oxygenated water) directly on the stigmatic surface of isolated flowers (DAFNI, 1992). The receptivity was tested at 4:00, 6:00, 8:00, 18:00, 20:00, and 22:00h. The subjective odor assessment was obtained from 12 flowers placed in odorless glass bottles (four flowers per bottle) that were closed for 2 hours and afterwards opened, following olfactory characterization. The sugar content was measured with a refractometer after obtaining nectar with a micro syringe.

To quantify the differential success along the two years, we marked 277 flowers in the 32 studied individuals that were monitored until the end of the flowering period. These marked flowers were tracked over in order to assess the number of removed pollinia from anthers, and those deposited on the stigmatic cavities. Male success was obtained from the ratio between the removed pollinia and the total number of marked flowers. Feminine success was obtained from the ratio of the number of deposited pollinia in a stigmatic cavity and the total number of marked flowers (PARRA-TABLA et al., 2000). Fruit set rate, in percentage, which here represents the real fertilization and embryo development, was obtained from the ratio of number of existing fruits eight months after the end of the flowering period and the 277 marked flowers for this purpose (GARCIA-CRUZ; SOSA, 2008). We also calculated the efficiency index as the proportion of removed pollinia in relation to the fruit set (TREMBLAY et al., 2005).

The identification tests of pollination systems were performed in the first study year. For this purpose, 60 floral buds in pre-anthesis, located at least in 10 different individuals, and always on the inflorescence basis, were bagged in order to avoid any contact with possible pollination agents. We performed the following five treatments: self-pollination, spontaneous self-pollination, geitonogamy, xenogamy and agamospermy. Not all treatments co-occurred in the same individual. For all systems n = 12 was used. Except for the self-pollination, all flowers were emasculated. Capsules that were naturally formed by the action of the pollinator agent were marked (n = 12), to evaluate natural pollination.

We accomplished 44 monitoring visits (approx. 1 hour each) to verify phenology aspects, biotic and abiotic features that could eventually interfere in the reproductive success of the studied species. When predation events were registered, the phytophagous were collected and observed in laboratory conditions in order to describe some interactions traits. The summed monitoring time to the direct observations resulted in a total of 244 hours of observations. The capsules descending from the tests for pollination systems that reached physiological maturity (color change) were collected and their length (CC), diameter (DC) and capsule weight (PC) measured, which basically represent their size and number of viable seeds (NSV). These variables underwent analyses of variance, and then mean tests (Tukey 5%).

The number of potentially viable seeds was evaluated through the method of coloration with tetrazolium solution adapted from Lakon (1949). We measured 0.005 g of seed from each capsule (n:10), which descended from two pollination systems (natural and xenogamy). These seeds were placed in a test tube, and we added 3 mL of the aqueous solution of 2,3,5 trifeniltetrazolium (p.a.) chloride 0.5%. This material was kept in total darkness for 24 hours. Afterwards, to the tetrazolium suspensions were added to 7 mL of distilled water, and 1 mL of this suspension transferred to the Peter's counting chamber. Using a binocular loupe we counted the number of seeds, which were carmine colored, considered potentially viable by the respiratory activity of the tissues. The number of potentially viable seeds per milligram of seeds from each pollination system was proportionally estimated.

After evaluating the viability, 0.010 g of seeds from each type of pollination were disinfected with a solution of 3 mL of sodium hypochlorite and 6 mL of distilled water for 15 minutes, and later diluted to 60 mL with sterilized distilled water for later in vitro sowing, in order to confirm the viability percentage through direct assessment of germination, which was monitored until the eight month after sowing. For in vitro sowing we used the asymbiotic culture medium suggested by Campos (2002), and closed glass bottles were acclimatized in a chamber with controlled photoperiod and temperature (12 hours and 23 ± 2ºC) during eight months for germination evaluation. Ten replications were used for this analysis. The observed results were quantitatively compared.

The voucher specimen (4604) of *Brassavola cebolleta* (A. R. Rech, 30) was deposited in the herbarium from the Universidade Federal da Grande Dourados (DDMS) and *Hemiceras* sp. (A. R. Rech, 01/2006) in the Museu de Zoologia of the Faculdade de Ciências Biológicas e Ambientais from the same university.

Results and discussion

Brassavola cebolleta is an annual species (NEWSTROM et al., 1994), similarly to most of the Orchidaceae (DAMON; SALAS-ROBLERO, 2007). Its flowering period began in June and extends until September, with the flowering peak in mid August (visual impression). Flowers were condensed in racemes with 5 to 7 flowers, spread along a 5.5 ± 3 cm long raquis (Figures 1 and 2). The flowers opening is sequential with two open flowers per day, whose stigmas are already receptive during ressupination until withering, 10 to 15 days later. When pollination occurs, the flower parts immediately senesce and dry. Measurements of the flowers are found in Table 1.

Figure 1. *Brassavola cebolleta* Rchb. f. in the study environment in Dourados, Mato Grosso do Sul State, Brazil.

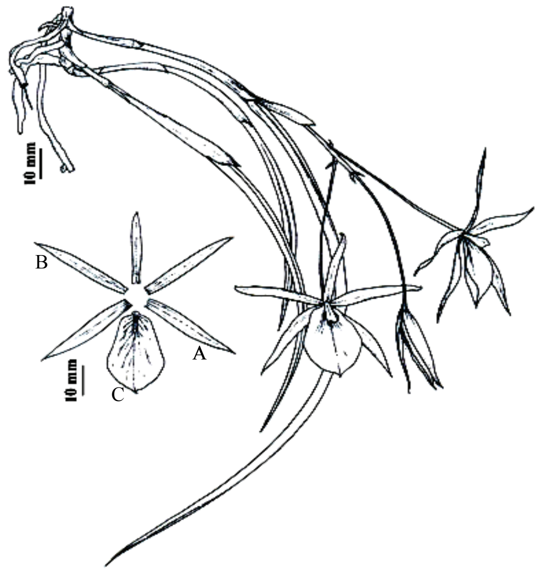

Figure 2. Illustration of *Brassavola cebolleta* Rchb. f., sepals (A), lateral petals (B) and the labellum (C).

The osmophores are mostly located in the labellum, with sporadic occurrence in the sepals. Odor was characterized as slightly sweet, being predominantly released in the vespertine twilight, which is very similar to previously described to other species of *Brassavola* by Roebuck and Steinhart (1978). The nectary is of the cuniculus type and produces an insufficient amount of nectar for quantification of total sugar content.

Table 1. Morphometric characteristics of flowers of *Brassavola cebolleta* Rcbh. f. (N:30) studied in Dourados, Mato Grosso do Sul State, Brazil.

	Length (mm)	Width (mm)	Color
Petals			
2 free	35 ± 2	3.0 ± 0.1	White, slightly yellowish
1 labellum	17.0 ± 1.1	27.0 ± 2.1	White, with lime-green nectar guides at the base
Sepals			
3 free	35 ± 3	5.0 ± 0.4	White, slightly yellowish
Gynostemium	8.0 ± 0.9	4.5 ± 0.5	White
Peduncle + ovary	6.0 ± 0.5	2.0 ± 0.2	Green

We registered 31 flower visitations, from which we infer that pollination occurs predominantly in twilight. The collected insect with potential characteristic (size and behavior) to be a pollinator was a Lepidoptera that belongs to the family Notodontidae Stephens, 1829, genus *Hemiceras* Guenée, 1852. However, no deposition of pollinia into stigmatic cavities was observed. The visitation begins with the insect flying in front of the inflorescence, then it chooses a flower and inserts the proboscis into the nectar at the moment it lands on the labellum, and stays there for about 15 sec. exploiting the resource, then its thorax get contact with the stigmatic cavity. When it leaves the flower, the visitor touches its thoraxic surface in the anther and usually heads for another inflorescence. During the day we only registered visits of the small bee *Plebeia remota* Holmberg, 1903, which did not visit the flower legitimately.

The pollinators' sharing rate in the Orchidaceae is very low. Nevertheless, species that present food resources (nectar) have greater chance of being visited by more species of potential pollinators (SCHIESTL; SCHLÜTER, 2009). Although legitimated visits have been registered for just one visitor species, *B. cebolleta* has nectar; hence we believe that more time of observation is needed to infer a species-specific relationship. In a study performed in Chiapas (Mexico) Damon and Salas-Roblero (2007) show that several species of Lepidoptera were seen visiting flowers of *Brassavola nodosa* (L.) Lindl during the day, however the visitor with characteristics to be a potential pollinator was a Sphyngidae, only seen at night and not captured.

The visit frequency was considered very low during all the sampling period, which may be due to the number of observation hours, since the visits occurred when light availability was very low (despite the used illumination) and it was difficult to observe a sufficient number of flowers to allow us a better quantification of visitors. There was no pollinia removal apart from the observed period in the flowers marked for this purpose, which leads to the conclusion that the foraging time coincides with the observed time. Other important feature to be considered is habitat fragmentation and massive utilization of pesticides, common in the region. These features significantly contribute to the pollinator fauna reduction (DONALDSON et al., 2002; KEVAN; IMPERATRIZ-FONSECA, 2006). In a meta-analysis Aguilar et al. (2006) evidenced that plants whose pollination depends on a biotic vector (like most Orchidaceae), are strongly affected by habitat fragmentation which may significantly decrease their reproductive success.

Out of the 277 flowers evaluated for differential success, 53 (19% of male success) had pollinia removed, and 27 (10% of feminine success) had pollinia deposition on the stigmatic cavity. The male or feminine success did not vary as a function of size or nesting clumps, found by Schemske (1980) in *B. nodosa*. In the present study the fruit production rate, evaluated one month after the end of flowering period was 6.1%, and the efficiency index was 3.11:1 (removed pollinia: formed fruits). Pollinia deposition rate was 2.9 higher than the effective fruit formation. The fructification rate of this survey was similar to that found in *Rodriguezia bahiensis* Rchb. f. (6.57%) (CARVALHO; MACHADO, 2006), in *Myrmecophila christinae* (3%) (PARRA-TABLA; VARGAS, 2007). However, this rate may be considered low after studies from Schemske (1980) and Damon and Salas-Roblero (2007), which found rates higher than 10% in *B. nodosa*, and Tremblay et al. (2005), in a meta-analysis, indicated an average fructification rate for tropical Orchidaceae of 17.0 ± 2.1% (N = 91).

Flowers that were isolated for verification of spontaneous self-pollination and agamospermy did not initiate fruit development, which indicates the dependence of *B. cebolleta* on pollen vectors to execute natural pollination, which is a common phenomenon in the Orchidaceae due to hercogamy (HUMANA et al., 2008; VIEIRA et al., 2007). All flowers used for the study of manual self-pollination, geitonogamy and xenogamy initiated the ovary development, though in some treatments there was subsequent abortion. We believed that the initial development probably occurs due to the activity of auxines released by the pollen grain (TAIZ; ZEIGER, 2004). The rate of fruit production when there is pollen supplementation, like in some of the tests of pollination systems performed, is always higher that that found in natural conditions, revealing a pollination deficit in such conditions (PRITCHARD; EDWARDS, 2006; PELLEGRINO et al. 2005; YU et al., 2008). This difference corroborates Tremblay et al. (2005), as it follows the deficit in the pattern of pollination found in the Orchidaceae, which according to the author may be the main mechanism able to select more efficient pollinators, and thus enabling the appearance of mega-diverse plant groups.

Four months after performing the tests to evaluate pollination system, half of the fruits from self-pollination and geitonogamy were aborted, meanwhile those produced from xenogamy or natural pollination continued developing. In April 2007 all fruits from self-pollination or geitonogamy were aborted, while only one fruit, descendant from xenogamy was lost. We believe there was no preferential abortion of self-pollinated fruits, since in individuals whose the only treatment was self-pollination, all the fruits were also aborted.

Fruits from natural pollination were not aborted, different from Ackerman and Oliver (1985) and also Pansarin and Amaral (2009). These authors suggest that the high abortion rates for the Orchidaceae under natural conditions may be due to hydric stress, which is a strong abortive feature. However, it is pointed out that plants assessed here were not subject to water stress in their habitats, since the branches of their host trees in the riparian forest lean towards the Dourados river. Our results suggest that there are no mechanisms of prezygotic self-incompatibility in *B. cebolleta*. Nonetheless, late abortions exclude *B. cebolleta* from the majoritary orchid group completely self-compatible (SINGER; SAZIMA, 1999; JOHNSON; MORITA, 2006; DUFFY et al., 2008; PANSARIN, 2008; WANG et al., 2008; SHI et al., 2009), and actually differ from *B. nodosa,* which according to Schemske (1980) is strongly self-compatible, though this author had not monitored the seed behavior until germination.

In July 2007 when capsules of *B. cebolleta* reached physiological maturity, the size of those from xenogamy tests was smaller than those from natural pollination. However, the average number of potentially viable seed per milligram of seeds was lower in natural fruits (124.1 seeds mg^{-1}) than those from xenogamy (190.4 seeds mg^{-1}) (Tables 2 and 3). Nevertheless, in the germination analysis, the number of seed that developed was clearly higher for plants whose pollination occurred via natural pollinator. We quantified an average germination of 95 ± 5 seedlings in the ten samples whose seed proceeded from pollination by a pollinator agent, in comparison with an average of 3 ± 3 seedlings for seeds from the xenogamy test.

Table 2. Result from the analysis of variance with values of length (CC), diameter (DC) and weight of capsule (PC) and number of potentially viable seeds (PVS) for *Brassavola cebolleta* studied in Dourados, Mato Grosso do Sul State.

Source	GL	CC	DC	PC	PVS
Treatment	1	84.04**	19.99**	2.00*	50.62*
Residual	18	9.68	1.62	0.30	11.30
CV(%)		12.89	10.44	28.57	27.87

**: F significant to 1% *: F significant to 5%.

Table 3. Average measurements of length (CC), diameter (DC) and weight of capsule (PC) and number of potentially viable seeds (PVS) obtained for fruits of *Brassavola cebolleta* from tests of xenogamy and natural pollination in Dourados, Mato Grosso do Sul State.

Treatment	CC	DC	PC	NSV
Xenogamy	22.10b	11.20b	1.60b	190.40a
Natural	26.20a	13.20a	2.23a	124.10b

F: 1 and 5% of probability.

We inferred that the data obtained from tests of viability and germination have a relation with the vigor of the seeds and arise from characteristic of embryos and capsules development. In *Cleistes divaricata* (L.) Ames, the effect of varying pollen deposition over seed development was investigated, and as expected, seed production was positively correlated with increasing pollen input (GREGG, 1991). However, unlike to observed in the present study, seed fertility remained unchanged. According to Kerbauy (2004), in many cases positive correlations of seed mass and germination capacity, vigor and/or seedlings survivorship are described, and though not being a general rule, explains the higher number of germinated seedlings descending from seed produced by natural pollination, where the lower seed number, produced in larger fruits, would result in embryos with better germination capacity as possible.

Meanwhile, concerning reproductive success, in field we verified the predation on fruits and seeds by larvae of *Hyphilaria thasus* (Stoll, 1780) (Lepidoptera: Riodinidae), as already described by Rech et al. (2008). In natural conditions we found plants with eggs, and up to ten larvae feeding on fruits and seeds. In laboratory we found that three individuals are able to predate one fruit per day. Some surveys already have related cases of phytophagy of fruits and seed of orchids by beetles of the family Curculionideae on *Oncidium* Sw. (BONDAR, 1948) and *Grobya amherstiae* (MICKELIUNAS et al., 2006). In the last research, the authors highlight the beetle from the genus *Montella* Bondar which promotes self-pollination followed by oviposition in the flower ovary. However, the species *Eulophia foliosa* has adaptation in the cap anther that prevents self-pollination by elaterid beetles, favoring crossed pollination (PETER; JOHNSON, 2006).

Concerning the low rate of visitation, and consequent low fructification rate, abortions caused by self-pollination, and fruit and seed predation, we discern a danger situation for the maintenance of *B. cebolleta* populations in the Dourados region. Despite the fact that we did not monitor populations of adults and young individuals, we point out that we almost never observed non-adult individuals. The scenario in which *B. cebolleta* is placed in Mato Grosso do Sul State calls the attention for the need of studies with taxonomic approach and reproductive biology in the region, in order to justify the need of implementing permanent preservation areas and public policies focusing on sustainability to ensure species perpetuation.

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