

RESEARCH PAPER

Breeding system and factors limiting fruit production in the nectarless orchid *Broughtonia lindenii*

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Keywords

Cuba; inbreeding depression; Laeliinae; *Melissodes*; pollen limitation; post-removal senescence; self-compatibility; subequal pollinia.

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Editor

J. Arroyo

Received: 16 December 2009; Accepted: 16 April 2010

doi:10.1111/j.1438-8677.2010.00366.x

ABSTRACT

Low fruit set values in most orchids (especially epiphytic and tropical species) are normally thought to be the consequence of pollination constraints and limited resources. In particular, pollination constraints are modulated by pollinator visitation rates, pollinator visitation behaviour (promoting crossing or selfing), the type and number of pollinia deposited on stigmas (in the case of orchids with subequal pollinia) and the amount of pollen loaded per inflorescence. In order to assess to what extent these factors can affect fruit set in specific orchid–pollinator systems, the repercussions of some of these aspects on reproduction of *Broughtonia lindenii* were examined in a coastal population in western Cuba. The study focused on plant breeding system, importance of pollen load and type of pollinia on subsequent fruit and seed, limiting factors of seed production and interaction with pollinators. This species presents long-lasting flowers that senesce after all forms of effective visit. Pollinator dependence for fruit production was demonstrated, while hand-pollination experiments revealed self-compatibility and inbreeding depression at seed level. More pollinia on stigmas enhance the proportion of well-developed seeds. In contrast, the pollinia type used in pollination is not important for seed quality of fruits, suggesting that small pollinia are not rudimentary. Natural fruit set in two consecutive years was substantially affected by pollinator activity, and also by systematic depredatory activity of ants and a caterpillar. Considering that this orchid completely lacks nectar and that the local assemblage of pollinators and predators influenced its reproduction, a minor importance of resource constraints in this epiphyte (with long-lasting reserve structures) is confirmed at least for a short time.

INTRODUCTION

Most orchids produce far more flowers than fruits (Tremblay *et al.* 2005). This situation, although critical for epiphytic species (Neiland & Wilcock 1998), has been scarcely researched within this guild of plants, although extensively investigated in terrestrial orchids (Huda & Wilcock 2008). In general, low fruit set in orchids is the result of several factors, mainly limitation by pollination and by resources (in this order, *sensu* Tremblay *et al.* 2005; Jersáková *et al.* 2006). Most studies on this subject have assumed a dichotomy between these two causes, supposing that higher levels of fruit production are supported by sufficient availability of resources for the plants (Zimmerman & Aide 1989; Calvo & Horvitz 1990). Recently, this approach has been considered an over-simplification (Tremblay *et al.* 2005) considering that some plant populations, limited mainly by pollination, can also offer evidence of resource constraints (Montalvo & Ackerman 1987; Salguero-Faría & Ackerman 1999). Nevertheless, this approximation is still a good way to evaluate the importance of resource constraints, at least at a short temporal scale (Zimmerman & Aide 1989). In addition, environmental and biotic disturbances can drastically reduce fruit set (Stephenson 1980). This could happen indirectly by affecting

pollinator availability (Rathcke 2000) or directly by destroying part of the produced flowers or the already initiated fruits (Rathcke 2001). Consequently, in dry habitats, such as insular coastal ecosystems subject to strong water stress, severe meteorological events and high levels of herbivory, performance of experiments to contrast the incidence of resource *versus* pollination limitation must include a study of pollinator efficiency, mechanical damage to reproductive structures and activity of herbivores (Ackerman & Montalvo 1990; Rathcke 2001).

However, in a chasmogamous plant with pollination constraints, the ultimate origin of these limitations may be infrequent or ineffective pollinator visits, a high level of incompatible pollen loads on the stigma or both (Ackerman & Montalvo 1990). Thus, to define the type of pollination constraint that operates, previous knowledge of the breeding system is essential (Sutherland 1986; Wilcock & Neiland 2002; Humaña *et al.* 2008). Another important aspect of pollination limitations is the amount of pollen received by the stigma. Consequently, the study of how pollen production is fragmented and distributed by its vectors is very important (Huda & Wilcock 2008). In Orchidaceae, the evolution of pollen presentation ranges from sticky pollen grains, through sectile pollinia subdivided into small massules, to pollinaria

with very few hard and waxy pollinia (Dressler 1993). Along this gradual variation, some groups with waxy and compact pollinia present two or three sizes of pollinium co-existing in the same pollinarium (e.g. Burns-Balogh & Bernhardt 1985; Singer & Koehler 2004; Dathe & Dietrich 2006). Subequal pollinia have been interpreted as evidence of the pollinia reduction tendency mentioned above. Consequently, smaller pollinia within irregular pollinaria have been considered as probably rudimentary or vestigial (Dressler 1981, 1993). However, no experimental study has evaluated the apparently rudimentary condition of smaller pollinia, and the ecological role, if any, of subequal pollinia with regard to seed and fruit set is unknown. In contrast, the implications of sectile pollinia have been presented as an advantage that maximises the number of effective visits of each pollinator on subsequent conspecific plants (Darwin 1877; Johnson and Edwards 2000; Huda & Wilcock 2008). Thus, in orchids growing in environments where pollinators are very scarce, this mechanism could have great adaptive value for the orchid (Neiland & Wilcock 1995; Johnson and Edwards 2000).

The three Cuban species of the genus *Broughtonia* present eight subequal pollinia (four big and four small), while the remaining species of this genus have four equal, large pollinia (Sauleda & Adams 1984; Díaz 1997). Díaz (1997) suggested the vestigial nature of smaller pollinia in the Cuban taxa (including *Broughtonia lindenii*). Here, we tested whether the pollinia are functional and the reproductive consequences in relation to characteristics, behaviour and efficiency of the pollinators observed. The aims of this study were to characterise the breeding system and identify factors limiting fruit production in *B. lindenii*. With these aims, the following questions were addressed: (i) Do flowers of *B. lindenii* produce nectar? (ii) How is flower longevity affected after being effectively visited by pollinators? (iii) How important are pollinators to fruit production of this species? (iv) How do the two types of pollinia and pollination intensity affect female fitness? (v) What are the main limiting factors of seed and fruit production in this population?

MATERIALS AND METHODS

Studied species

Broughtonia lindenii (Lindl.) Dressler is an epiphytic (rarely lithophytic) orchid, living mainly in dry vegetation in coastal areas of the Cuban and Bahamian archipelagos and on Cuban *mogotes* (abrupt elevations of various karstic landscapes dominated by steep or vertical-sided limestone towers). In the study site, *B. lindenii* grows mainly on *Gochnatia sagraeana* (Asteraceae) (85.2% of censused individuals). Each orchid annually produces one new pseudobulb at tips of the rhizomes with one to three coriaceous leaves. Large inflorescences are produced terminal between the leaf bases (Fig. 1E). Flowers are slightly scented, but there is no information on the composition of the aroma. All flowers are pinkish with a bell-like labellum (Fig. 1B) generally of darker pink to purple. The inner tube surface is white and has five to seven villous, green-yellow to orange, longitudinally oriented lamella, ending as purple veins near to the margin (Díaz 1997) (Fig. 1A and D). The column is white, with two lateral appendages at its base. At the bottom of the tube, the column and lip form

a cuniculus (ca. 4 mm in depth) with the appearance of a nectar container (Fig. 1C). The general aspect of the column is that which Dressler (1961) described for *Cattleya*. In *B. lindenii* the pollinarium is formed of eight subequal pollinia, four large and four small (Fig. 1F and G). The flowering period of *B. lindenii* is reported to be between March and July (Díaz 1997) or between May and September (Sauleda & Adams 1984), depending of location.

Study site

Boca de Canasí (23°08' N, 81°46' W), the outlet of the Canasí River, 62 km east of Havana, has an abrupt limestone relief of 50–80 m a.s.l. Local weather is warm and dry, with mean relative humidity of 78%, although seasonally humid from May to October (rainy season), and strongly influenced by marine breezes throughout the year. Mean annual temperature is 25.5 °C and annual rainfall is about 1300 mm. The vegetation is dry, littoral evergreen microphyllous forest (Capote & Berazaín 1984). Shrubs growing on pure limestone or thin soil are part of the dominant vegetation, particularly *G. sagraeana* (Asteraceae).

Floral traits, pollinators and breeding system

A set of 107 plants was chosen to analyse production of reproductive organs over two consecutive years (2005 and 2006). Moreover, in 2006 a further 27 flowers from different individuals were bagged and marked to examine nectar production 48 h later. Each flower was half-dissected and the cuniculus carefully examined for the presence of nectar.

Although the pollinarium is sessile in this species, we studied any possible post-removal changes in the relative position of pollinia for better understanding of the pollination system. Thus, before dissection, the pollinarium was removed with a toothpick. Variations in distance between pollinia (pollinia of the same size touch each other or are in contact with pollinia of the other group) and changes in the general shape of pollinaria (i.e. orientation of pollinia with regard to the toothpick axis: erect, oblique or decumbent) were assessed by eye. These observations were made just after removal, and 5 h later.

Also in 2006, 11 flowers (one per plant, tagged at the bud stage) were examined daily to assess mean floral lifespan. To determine the effect of pollination on floral lifespan, another 125 tagged buds were chosen and manipulated as follows: (i) pollinia removed (58 flowers in 11 plants) and (ii) pollinia removed and flowers hand-pollinated (67 flowers in 12 plants). In all cases, flowers were inspected 5 and 15 h after manipulation. Treatments were carried out when flowers had recently opened (1–3 days after anthesis, which was considered to be a similar stage, given the total flower lifetime). Flower withering was considered to occur when all tepals (including the labellum) had lost turgescence and consequently formed a closed wrapper that completely blocked the entrance to the floral tube, anthers and stigma.

To analyse pollinator dependence (or spontaneous capacity for autogamy) for fruit production, 40 randomly selected plants having only unopened buds were bagged. Nylon bags (excluding animals, water and wind) were used on five plants (72 flowers) in 2005. Net bags with 2-mm mesh (excluding

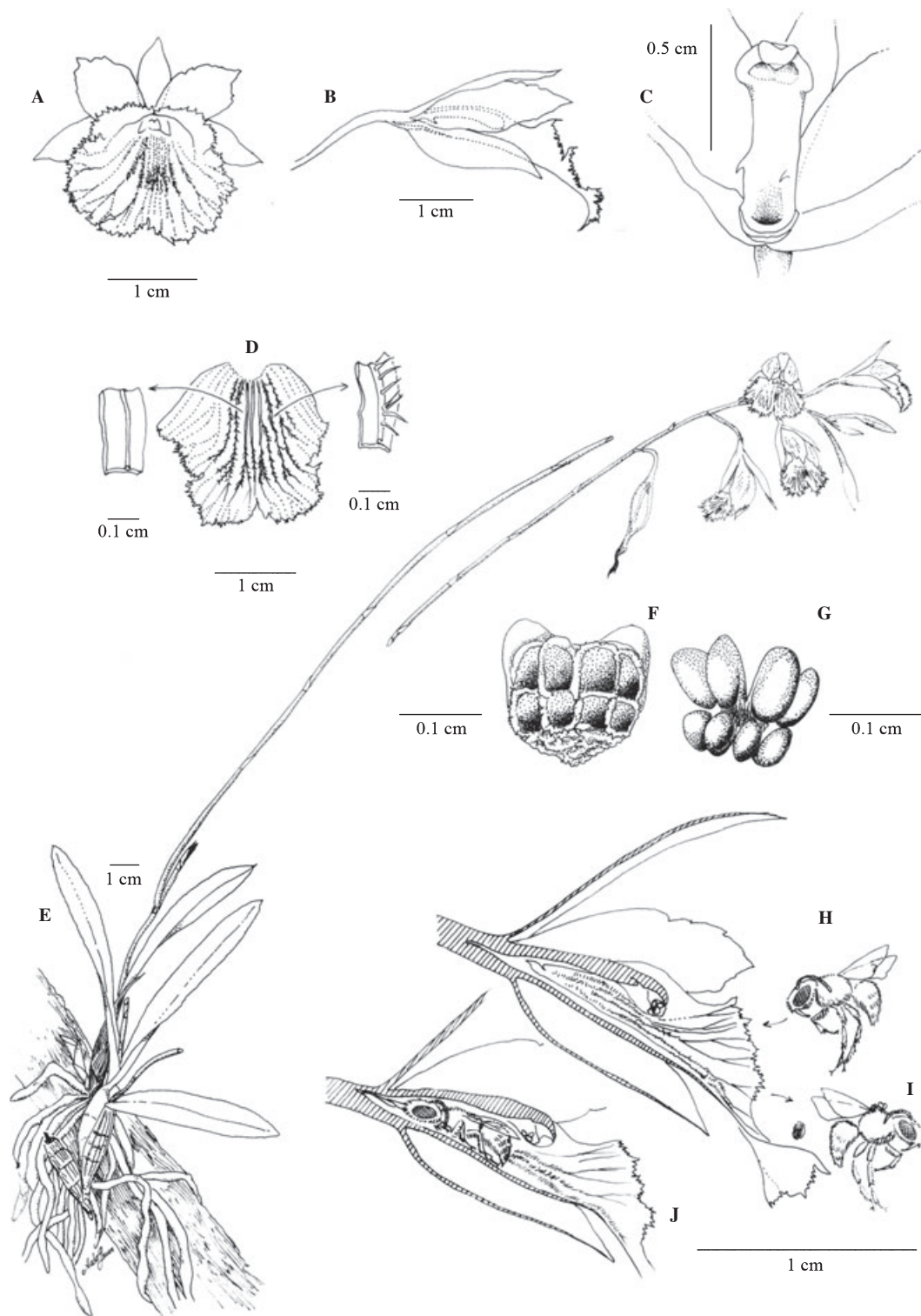


Fig. 1. Characteristics of the orchid *Broughtonia lindenii*: (A) frontal view, (B) lateral view of dissected flower, (C) detail of flower with lip retired, showing the cuniculus, (D) flower dissected and detail of the labellum showing the hairy lamella, (E) plant habit, (F) anther cap, (G) pollinarium, (H) a male of the bee *Melissodes leprieuri* landing at a not previously visited flower, (I) bee leaving the flower once it had removed and received the pollinarium on its thorax, (J) bee examining the cuniculus on a new, previously visited flower after depositing pollinia on the stigma.

only animal activity) were used on five plants (87 flowers) in 2005 and on 30 plants (74 flowers) in 2006. Observations of visitors were made in May 2005, during the flowering peak

from 08:00 to 17:00 (Cuba summer time). Each visitor was recorded in the area where 14 plants of *B. lindenii* presented an average of 53–62 flowers. At each visit, we collected data

on species and sex (when possible) of the visitor, and the number and type of interaction. Each interaction was classified as: (i) attempt to visit flower (when an agent flies close to flowers without touching), (ii) direct contact but without entering the flower tube, (iii) entry into floral tube, and (iv) pollinarium removed and/or deposition of pollinia on stigma. Floral visitors were captured for identification in the entomological collection of the Cuban Institute of Ecology and Systematics (CZACC).

In order to study the plant breeding system, in addition to the above-described spontaneous autogamy experiment, 28 plants were hand-pollinated in 2006. For all treatments, a half pollinarium (two small and two large pollinia) was used. The treatments applied included manual autogamy (hand-pollination with pollen from the same flower to test for self-compatibility), geitonogamy (hand-pollination with pollen of other flowers of the same plant) and allogamy (hand-pollination with pollen from a plant at least 5 m from the receiving flower). These treatments were randomly assigned to the three middle flowers of each raceme. The production, length and diameter of fruits and the proportion of seeds that contained well-developed embryos were measured. Approximately 10 weeks after the treatments were applied and fruits matured (but just before dehiscence), all treated inflorescences were cut and fruits digitally photographed on millimetre paper, using a Nikon Coolpix L1 camera (Nikon Inc., Japan). The photographs were used to measure fruit diameter and length using the measurement tool of the software Adobe Photoshop. The contents of each fruit were collected in individual glasses, and four sub-samples were obtained per glass by touching the seeds mass four times with the tip of a damp dropper (cleaned between collections). Seed samples were put on slides with a drop of water and observed under a microscope (at 100×) at one optical field per subsample. Only seeds with visible, rounded, but not black, embryos were considered well developed. In each field, we recorded the 'proportion of well-developed seeds'. An average from the four seed samples was calculated for each fruit. Inbreeding depression index (δ) was calculated as $1 - (\text{proportion of well-developed seeds after geitonogamous pollination} / \text{proportion of well-developed seeds after allogamous hand-pollination})$ (Charlesworth & Charlesworth 1987; see also Buide & Guitián 2002).

Effect of number and type of pollinia on fruit and seed production

To examine whether small pollinia from *B. lindenii* are functional or rudimentary, we examined the quality of pollen from small pollinia in relation to that of pollen from larger

pollinia. One flower from each of 12 plants was selected and any of the four combinations of pollinia size and number applied. Thus, three flowers from three different plants were pollinated each with one small pollinium, another three flowers received one large pollinium, three were pollinated with four small pollinia, and three received four large pollinia. All the manipulated racemes were net bagged to avoid herbivory. Fruit size and proportion of well-developed seeds were measured as previously described.

Effect of resources versus pollen limitation on fruit and seed production

Twelve plants were used to compare the effect of two levels of pollination intensity on fruit and seed production. Six racemes from different plants received low pollination (only two flowers per raceme) while another six were fully pollinated (all flowers). In order to avoid the influence of other factors on the response variable, the treatments were assigned to plants of similar vegetative and reproductive development (*i.e.* similar number of leaves, pseudobulbs, flowers, anthers and length of inflorescences when treatments were applied). To conduct pollinations, a half-pollinarium (two small and two large pollinia) per flower was used from plants that were at least 5 m apart. Treatments were also compared with open-pollinated flowers in plants of similar reproductive and vegetative condition. Plants assigned to these three treatments belong to the same statistical population (Table 1). Finally, all these inflorescences were also bagged to avoid herbivory of fruits. The response variables were fruit production, fruit size and proportion of well-developed seeds.

Natural limiting factors on fitness

In a plot of 625 m², all detected plants of *B. lindenii* were marked with numbered acrylic tags. These individuals were not manipulated and were visited weekly from April to July in 2005 and 2006. At each field visit, the following variables were recorded for each control plant: (i) flowers produced, (ii) flowers with any physical damage, (iii) flowers without pollinaria (as a measure of male success), (iv) flowers with pollinia on stigmatic surface (an initial measure of female success), (v) withered flowers, (vi) initiated fruits, (vii) fruits eaten or damaged in any way, and (viii) mature or dehiscent fruits. Caterpillars found eating reproductive organs of the orchids were collected, properly sheltered and fed with flowers of *Broughtonia* up to metamorphosis, after which adult individuals were identified and deposited at CZACC.

Table 1. Vegetative and reproductive conditions in the 18 plants used in the experiment of resource versus pollen limitation in the orchid *Broughtonia lindenii*. Length of inflorescences in cm; the other variables are counts. The values are given as arithmetic mean \pm standard deviation ($n = 6$ for each treatment). Results of the Kruskal–Wallis test are presented.

variable	full-pollinated	low-pollinated	open-pollinated	pooled groups	<i>H</i>	df	<i>P</i>
number of pseudobulbs	12.0 \pm 1.4	11.4 \pm 1.2	12.0 \pm 1.7	11.9 \pm 1.4	0.193	2	0.908
number of leaves	17.0 \pm 5.8	16.5 \pm 6.6	16.5 \pm 5.0	16.7 \pm 5.5	0.288	2	0.866
length of inflorescence	36.1 \pm 15.5	34.8 \pm 14.3	36.0 \pm 15.7	35.6 \pm 14.3	0.082	2	0.960
flowers produced	18.7 \pm 3.1	17.8 \pm 3.4	19.0 \pm 3.5	18.5 \pm 3.2	0.328	2	0.849
flowers in anthesis	7.7 \pm 2.2	7.5 \pm 1.9	7.0 \pm 1.8	7.4 \pm 1.9	0.387	2	0.824

Data analysis

The numeric values of the proportion of well-developed seeds were arcsine square-root-transformed. Data for all variables were normally distributed (Kolmogorov–Smirnov test, $P > 0.05$) and presented homogeneity of variances (Brown–Forsythe test, $P > 0.05$). For response variables of the breeding system, a two-factorial analysis of variance (ANOVA) of mixed effects without replication was applied. Lost data due to herbivory were estimated with an iterative method, and mean squares of treatments and degrees of freedom of the total and the residual were corrected (Zar 1999).

For response variables of the pollen loading experiment, a two-factorial ANOVA of fixed effects was applied as a PERMANOVA (Anderson 2005). Permutation of raw data (9999 permutations) was used with each variable on the basis of Bray–Curtis dissimilarities. Due to the small sample size ($n = 3$), the Monte Carlo asymptotic P-values are provided instead of the permutation P-values, as recommended by Anderson (2005). Hence, crude values of the response variables are also provided instead of the arithmetic mean and standard deviation.

For the pollination intensity experiment, a one-way ANOVA model of fixed effects was used, and followed when necessary by a Tukey test for multiple comparisons. As critical values for this test, the arithmetic mean of critical values of the Tukey and Newman–Keuls tests, were used as suggested by Zar (1999). The level of significance of all hypotheses tested was 0.05.

RESULTS

Floral traits, pollinators and breeding system

In *Broughtonia lindenii*, only the most recent pseudobulbs produce new inflorescences each year. Most adults (88.9% in 2005, and 88.0% in 2006) bloomed with only one raceme per plant each year (93% of active plants in 2005, and 97% in 2006). Undamaged racemes were mainly pauciflorous (median = 7 flowers, range 1–63, $n = 92$ plants). Among all inspected flowers ($n = 27$), none presented intrafloral rewards at the cuniculus. Flowers of *B. lindenii* were long lasting (median \pm SE = 19.6 ± 1.3 days, $n = 11$) and senesced both after pollinarium removal and after pollinarium

removal + pollination. Within 5 h of pollinarium removal, 86% of flowers wilted (58 flowers from 11 plants). Similarly, 91% of flowers (67 flowers from 12 plants) lasted <5 h once pollinated. The remaining flowers that did not immediately wilt, wilted 15 h later. The pollinarium, once removed, did not suffer any evident reconfiguration of pollinia, and immediately after removal lost the protective anther cap.

Bees constituted 33.3% of observed visitors and had 73.7% of observed interactions with flowers of *B. lindenii*. The only efficient pollinators detected were male bees of *Melissodes leprieuri* (Apidae) (Figs 1H–J and 2). Their activity was characterised by 6.2-times more records than all other visitors, but was dominated by no-landing events (Fig. 2). These male bees displayed marked territorial behaviour, patrolling landmarks on local blossoms including *B. lindenii*. These insects flew for hours around some groups of plants, and alternated hovering behaviour with scarce and rapid visits to orchid blossoms, fighting each other and spraying pheromones sporadically when near their flowers. However, during these rare visits, <3 s was required to dislocate the whole pollinarium, causing its attachment to the dorsal middle thorax surface of the solitary bee (Fig. 1H–J). In addition, and completely out-with the observation programme, other males of *M. leprieuri* were observed with pollinaria or parts of pollinaria while visiting flowers of other reward-producing plant species.

Bagged inflorescences did not produce any fruits (*i.e.* spontaneous autogamy did not occur, Table 2), while control inflorescences had a low fruit set both in 2005 (4.2%, $n = 451$ flowers) and in 2006 (3.3%, $n = 612$ flowers). There was not fruit abortion among hand-pollinated flowers, nor significant differences among sizes of fruits obtained from manual autogamy, geitonogamy or allogamy (Table 2). However, significant statistical differences were found in the proportion of well-developed seeds among treatments. Both kinds of self-pollination produced the same proportion of well-developed seeds, but had significantly less well-developed seeds with regard to allogamy (Tukey test, $P < 0.001$). This last indicates the presence of inbreeding depression at the seed level ($\delta = 0.52$).

Effect of pollinia type and number on fruit and seed production

All flowers treated with the four combinations of size and number of pollinia produced fruits. According to the results

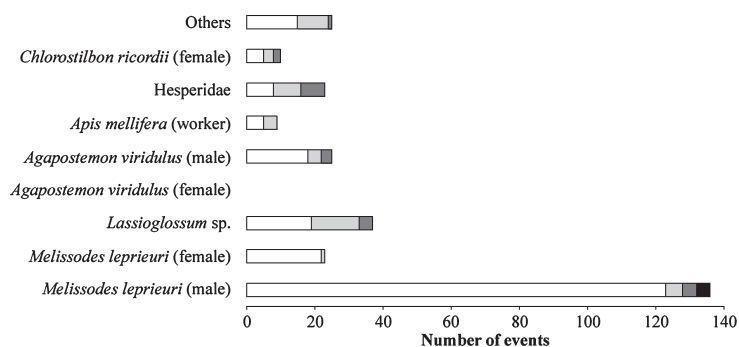


Fig. 2. Main floral visitors of the orchid *Broughtonia lindenii* at Boca de Canasí, western Cuba. Bars show the total number of interactions of each visitor. Different colours represent the kind of activity detected: contact attempts (white), contact without entering the flower tube (light grey), entry into the flower gullet (dark grey), pollinium removal and/or pollination (black). The group *Others* contains visitors that made any kind of contact with flowers at a frequency of <1% (includes coleopterans, dipterans, bees, butterflies and orthopterans).

Table 2. Effect of bagging experiment (spontaneous autogamy) and pollination type (manual autogamy, geitonogamy and allogamy) on fruit set (%), fruit dimensions (diameter and length, in mm) and the proportion of well-developed seeds (%) in the population of *Broughtonia lindenii* in Boca de Canasí. The values are given as arithmetic mean \pm standard deviation (bagging: n = 233 flowers from 40 plants; manual autogamy: n = 27 plants, geitonogamy: n = 28 plants, allogamy: n = 24 plants).

	spontaneous autogamy	manual autogamy	geitonogamy	allogamy	F _{2,49}	P
fruit set	0	1.00	1.00	1.00	–	–
fruit diameter	0	11.72 \pm 2.19	12.02 \pm 1.38	12.74 \pm 1.27	0.03	>0.05
fruit length	0	20.88 \pm 3.46	20.63 \pm 4.61	22.23 \pm 3.52	0.18	>0.05
proportion of well developed seeds	0	19.4 \pm 22.2 ^a	28.3 \pm 23.8 ^a	58.9 \pm 19.3 ^b	51.39	<0.0005

The letters represent the results of the Tukey test (P < 0.001): different letters show statistical differences.

Table 3. Effect of number and type of pollinia on fruit and seed production. Fruit dimensions are in mm. Crude values are provided instead of central tendency and dispersion values due to the small sample size (see Materials and Methods for details).

treatment	fruit diameter	fruit length	proportion of well-developed seeds
one large pollinium	10.25	18.29	0.167
	9.13	17.62	0.084
	12.11	16.43	0.262
one small pollinium	7.34	14.05	0.211
	9.20	19.84	0.637
	10.01	13.75	0.429
four large pollinia	12.66	24.07	0.761
	11.07	23.00	0.214
	11.40	19.73	0.702
four small pollinia	11.88	20.07	0.874
	12.38	22.59	0.633
	12.26	21.13	0.489

Table 4. Effect of pollen loading on fruit dimensions (diameter and length, in cm) and the proportion of well-developed seeds in the population of *Broughtonia lindenii* in Boca de Canasí in 2006. The P values correspond to Monte Carlo asymptotic P values as estimated in PERMANOVA (9999 permutations, analysis based on Bray–Curtis dissimilarities). Significant effects (P < 0.05) are highlighted in bold.

response variable	source	df	SS	MS	F	P
fruit diameter	number	1	354.4655	354.4655	11.1651	0.0088
	size	1	31.8804	31.8804	1.0042	0.3384
	number	1	81.0870	81.0870	2.5541	0.1434
	\times size					
	residual	8	253.9807	31.7476		
	total	11	721.4136			
fruit length	number	1	541.0349	541.0349	14.7432	0.0039
	size	1	40.1833	40.1833	1.0950	0.3170
	number	1	11.2730	11.2730	0.3072	0.6006
	\times size					
	residual	8	293.5785	36.6973		
	total	11	886.0698			
proportion of well-developed seeds	number	1	1563.3616	1563.3616	5.9919	0.0303
	size	1	762.9851	762.9851	2.9243	0.1130
	number	1	193.2850	193.2850	0.7408	0.4226
	\times size					
	residual	8	2087.3170	260.9145		
	total	11	4606.9478			

Table 5. Effect of pollination intensity on fruit set (%), fruit size (diameter and length, in mm) and the proportion of well developed seeds (%) in the population of *Broughtonia lindenii* in Boca de Canasí. The values are given as arithmetic mean \pm standard deviation (n = 6 for all treatments).

	full pollination	low pollination	open pollination	F _{2,15}	P
fruit set	1.00	1.00	1.00	–	–
fruit diameter	11.40 \pm 1.09 ^a	14.49 \pm 0.71 ^b	12.23 \pm 1.88 ^a	8.79	<0.005
fruit length	18.90 \pm 1.93	23.56 \pm 3.16	21.16 \pm 4.75	2.70	>0.05
proportion of well developed seeds	78.0 \pm 8.5 ^b	51.9 \pm 15.2 ^{ab}	37.0 \pm 24.9 ^a	8.28	<0.005

The letters represent the results of the Tukey test (P < 0.05): different letters show statistical differences.

(Table 3), fruit size and proportion of well-developed seeds were significantly affected by number of pollinia, while the type (small or large) of pollinium did not affect these variables (Table 4). Fruits resulting from both of the one-pollinium deposition treatments generally had shorter and fewer well-developed seeds (Table 3).

Effect of resources versus pollen limitation on fruit and seed production

Fruits developed from fully pollinated inflorescences or control inflorescences had significantly smaller diameters than those from two low-pollinated inflorescences (Table 5). No differences were found in fruit set or fruit length. The proportion of well-developed seeds was significantly higher in fully pollinated inflorescences than in controls, but did not differ from that in low-pollinated inflorescences. Similarly, there were no statistical differences in the proportion of well-developed seeds between low-pollinated and control inflorescences (Table 5).

Natural limiting factors on fitness

In general, the reduced reproductive potential in 2005 and 2006 followed a similar pattern (Fig. 3). Each year, many inflorescences (50% in 2005, 32% in 2006) were damaged by herbivory and mechanical causes before anthesis. Consequently, 35.9% and 18.6% of flowers produced during both reproductive periods were not available to pollinators. In

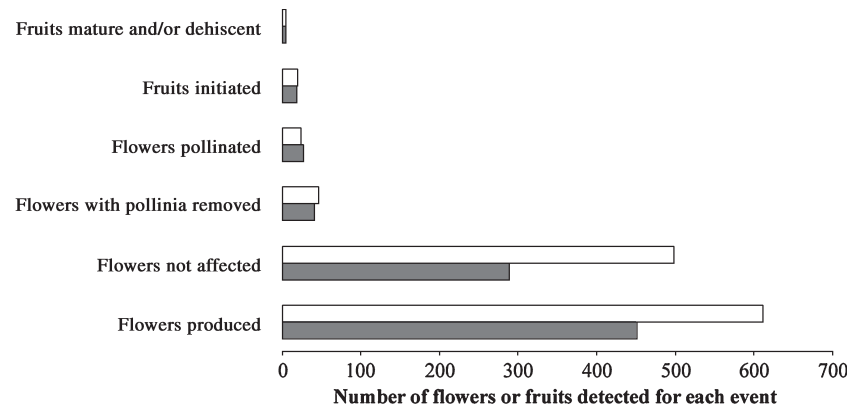


Fig. 3. Patterns of loss of reproductive potential in a natural population of *Broughtonia lindenii* at Boca de Canasí, western Cuba, during two reproductive periods: 2005 (grey bars) and 2006 (white bars).

both seasons, the scarce activity of pollinators was the most important limiting factor for fruit and seed production, as observed in control plants. Natural pollinarium removal rates, pollinia deposition rates and fruit set were very low and similar in both years among unaffected blossoms (pollinarium removal rate: 9.1% in 2005 and 7.5% in 2006, pollinia deposition rate: 6.0% in 2005 and 3.9% in 2006, fruit set: 4.2% in 2005 and 3.3% in 2006). There was also a reduction in reproductive potential due to predation of fruits in both years (26.3% in 2005 and 25.0% in 2006). Larvae of the nocturnal moth *Platynota rostrana* and the leaf-cutting ant *Atta* sp. were identified as agents affecting both floral parts and seeds of early fruits (Fig. 3).

DISCUSSION

Floral strategies and pollinators

As predicted by Dressler (1981) for orchids with gullet flowers, in *Broughtonia lindenii* bees were the dominant visitors. In this case, only males of *Melissodes leprieuri* were observed carrying and depositing pollinia on the stigma (Figs 1I, J and 2), consistent with this insect being the legitimate pollinator (Dressler 1981). This is the first report of pollination of orchids by this genus of bees, in particular by males. During our study, males of *M. leprieuri* performed constant and similar flights within the same forest area, including sites where many plants of *B. lindenii* were in bloom. During these flights, the male bees usually spread pheromones on the flowers. This behaviour is apparently more related to territoriality than to foraging, as reported in other orchid-bee systems: *Cephalanthera rubra* (Nilsson 1983), *Orchis papilionacea* (Vogel 1972) and *Disa tenuifolia* (Johnson & Steiner 1994). Previous studies have associated this phenomenon with a syndrome in which the orchid exploits the sexual drive of male bees during mate-seeking flights (*i.e.* rendezvous flowers: Vogel 1972; van der Cingel 1995, 2001; Jersáková *et al.* 2006). The release of pheromones over flowers of *B. lindenii* by hovering males of *M. leprieuri* (taking the flowers as focal odour points for its flights) also supports this hypothesis.

Breeding system and lifespan

According to our results, *B. lindenii* is pollinator-dependent and self-compatible, a combination broadly reported among

tropical nectarless orchids, especially among the Laeliinae (Smidt *et al.* 2006). In addition, the species was strongly pollinator-limited as reported for most deceptive orchids (Tremblay *et al.* 2005; Jersáková *et al.* 2006). Its floral lifespan (*ca.* 20 days) was quite long, and this could ensure better pollination success under scarce or uncertain pollinator visits, by increasing the time each flower is exposed to potential effective visitors (Flores-Palacios & García-Franco 2003; Castro *et al.* 2008). Another way to increase effective visits could be senescence of the flower after pollinaria removal and pollination, as reported here. This mechanism controls that each flower receives only one charge of pollen while others remain completely unvisited, and is common in Orchidaceae (Abdala-Roberts *et al.* 2007).

Nonetheless, an explanation of the consequences of senescence after only pollen removal (also demonstrated for *B. lindenii* and once in the subtribe [Ackerman 1989]) seems less obvious, and is scarcely mentioned for orchids in general (*e.g.* Clayton & Aizen 1996; Luyt & Johnson 2001). At flower level, this kind of senescence could be seen as an additional reproductive limitation because, as a result, the flower reduces to <5 h the time available for a second visit (even when successful pollination has not been accomplished). However, it is necessary to consider that, unlike a flower without a pollinarium (which can only have female success), an intact flower can double successfully as mother and father because removal/adherence of a pollinarium only occurs when the bee leaves the flower (Dressler 1961). Consequently, among all possible alternative interactions between this orchid and bees, the only combination that can be completely unproductive for the plant is that of a bee that does not carry any pollinia and a flower with its pollinia already removed. Hence, it is possible that avoidance of such interactions could be a crucial evolutionary pressure, considering that male *M. leprieuri* rarely visit this orchid. Thus, the post-removal senescence could encourage the very rare visits to mostly flowers with pollinia. In this way, a visit could both ensure male success and indirectly favour female success, or even both sexual functions at once (when the visiting insect has pollinia attached to its thorax).

Thus any possible negative effect of post-removal senescence in orchid reproduction (at the flower level) could be rewarded by benefits at a population level, particularly if the longevity (>50 years, calculated from the number of pseudobulbs per plant in the larger individuals in Boca de Canasí)

and perennial habit of the plant are considered. These factors ensure a high probability of forming a fruit or fertilising other plants during the lifetime of the orchid (Aragón & Ackerman 2004; Zimmerman & Aide 1989). Although fruit set detected was low, more than 39.3% and 40.3% of individuals of *B. lindenii* that bloomed in Canasí received at least one effective visit in 2005 and 2006, respectively. Also, 21.3% and 28.4% of the orchids produced at least one fruit in each year (data from our control plants). The same situation (*i.e.* low fruit set but well-distributed fitness among individuals) was reported in Aragón & Ackerman (2004) for *Psychilis monensis* (a Caribbean species related to *B. lindenii*) in similar habitat conditions, but probably by means of different pollination strategies (long flowering periods and small floral display). A similar reproductive scenario has been described for a broad spectrum of orchids lacking a reward, studied across the tropics (Tremblay *et al.* 2005). However, in most of these studies, there are no data on floral lifespan to predict interaction patterns of flower duration and both forms of reproductive success. In fact, in most orchid surveys, male success is not reported despite its extraordinary importance for fruit set (Sutherland & Delph 1984), and its high relation with pollination constraints (Tremblay *et al.* 2005).

Moreover, wilting of flowers that exported pollen could favour secondary re-allocation of resources, as it reported elsewhere (Gutián & Navarro 1996; Gutián *et al.* 1996; Navarro 1996). This is more related to resource constraint regulation by the plant, with direct implications for optimum female success of pollinated and not visited flowers. In orchids, it has been suggested that this mechanism acts by transferring water, nutrients and energy from flowers that lose pollinaria to remaining unvisited flowers and/or those that have begun fruit development (Clayton & Aizen 1996; Luyt & Johnson 2001). This last proposal could be crucial for *B. lindenii*, considering the dryness of its habitats and intense predation of reproductive organs in the studied ecosystem (Fig. 3).

B. lindenii showed evidence of inbreeding depression at the seed production level ($\delta = 0.52$). This value was higher than the mean reported by Smithson (2006) for nectarless orchids worldwide ($\delta = 0.32 \pm 0.05$), and also higher than the critical value established by Charlesworth & Charlesworth (1987) for angiosperms, but was similar to that reported by Husband & Schemske (1996) for allogamous angiosperms ($\delta_{\text{allogamous}} = 0.53$ vs. $\delta_{\text{autogamous}} = 0.23$). In plants, inbreeding depression is considered the strongest selective pressure preventing evolution of self-pollination in species without physiological self-incompatibility (like *B. lindenii*) and so exposed to self-fertilisation events (Ortiz-Barney & Ackerman 1999; Navarro & Gutián 2002; Porcher *et al.* 2009). Therefore, in cases where inbreeding depression occurs, herkogamy, dichogamy and similar mechanisms may be selected to avoid its negative consequences (Buide & Gutián 2002). In Orchidaceae, deceptive pollination has been traditionally suggested as a very important mechanism to avoid inbreeding (Jersáková *et al.* 2006), but it is possibly associated with other complementary and specific traits (Smithson 2006). Among these, are the herkogamous condition of the column structure (Dressler 1993), delayed adherence of the pollinarium to the pollinator when it leaves the flower (Borba & Semir 1999), many position/size changes of pollinaria once removed (Borba & Semir

1999; Johnson & Edwards 2000; Johnson *et al.* 2004), and anther cap retention after pollinaria attachment to the pollinator (Peter & Johnson 2006).

Broughtonia lindenii presents only the first two and most basic (*sensu* Dressler 1993) mechanisms: herkogamy and delayed pollinarium adherence to the pollinator (which occurred in all pollinator effective visits observed during this survey). The pollinarium of *B. lindenii* is sessile and does not show any movement or size modification after its removal. In addition, the foraging behaviour of the pollinator could also favour autogamous or geitonogamous pollination. For several hours, the pollinator focuses activity on specific groups of inflorescences but visits only a few of them, which are used as focal points for releasing pheromones. Hence, the existence of some inbreeding depression could be particularly beneficial for this species to control the incidence of pollination-mediated geitonogamy episodes. The maintenance and expression of inbreeding depression is probably temporally modulated by fluctuations of many environmental factors (Porcher *et al.* 2009), among which pollination availability and variations in pollination behaviour and efficiency could be decisive. Then, even when inbreeding affects a certain number of seeds per fruit, this mechanism could permit pollination success by maintaining a mixed breeding system in species exposed to very rare pollinator activity, such as *B. lindenii*. This hypothesis is reinforced by the observation of less well-developed embryos among control plants as evidence of insufficient deposition of pollen or poor pollen flux mediated by the natural pollinator of this chasmogamous orchid.

Effects of quality and quantity of pollinia on fruit and seed production

A reduction in number of pollinia is considered a general evolutionary tendency in orchids. Dressler (1993) suggested that, in many cases, smaller pollinia tend to be rudimentary and virtually non-functional. Díaz (1997) also suggested this explanation for Cuban species of *Broughtonia*, but we do not find experimental support for a rudimentary or dysfunctional condition of smaller pollinia in this species. Similarly, Singer & Koehler (2004) presented the incidence of two types of unequal pollinia as a common character for most species of Brazilian Maxillariinae, although they did not find any particular bio-mechanistic implications for this feature. Perhaps small pollinia, or even the presence of eight pollinia (considered an ancestral feature within the subtribe Laeliinae according to Dressler 1993), are only an inherited character, without any dysfunctional implications. However, this subdivision of pollen into a higher number of unequal pollinia could favour pollen carryover, which is a good strategy to increase pollen dispersal in time and space and allow pollination of more flowers during visiting periods of the same pollinator, which later learns to avoid that orchid (Tremblay *et al.* 2005). This is a very common strategy among scitellid pollinia orchids but scarcely reported among orchids with hard pollinia (Johnson & Edwards 2000; Huda & Wilcock 2008). In this work, it was demonstrated that pollination of flowers of *B. lindenii* with four pollinaria produces fruits with a higher number of well-developed seed than produced after pollination with only one pollinium of any size. Also, it is

relevant that *M. lepriouri* normally deposited less than a complete pollinarium at each visit (Vale A., unpublished data). Hence, and considering that flowers of this orchid wilt once pollinated (excluding the arrival of additional pollinia), it could be important to evaluate the natural frequency of one-pollinium depositions on the stigma and its consequences. Furthermore, the occurrence of this kind of pollen deposition could act as an additional limiting factor, since many *B. lindenii* plants could receive insufficient pollen loads to fertilise all ovules.

Pollination and natural limiting factors on fruit and seed production

The experiments on resource *versus* pollen limitation in *B. lindenii* confirmed that more intense pollination does not imply a problem of resource limitation, because these plants were able to produce fruits with a diameter slightly (but not significantly) smaller than open-pollinated plants, and were able to produce a higher proportion of well-developed seeds. This agrees with the reviews of Neiland & Wilcock (1998) and Tremblay *et al.* (2005), which showed that tropical orchids are generally more limited by pollen than by resources. Nevertheless, it is important to consider the correct temporal scale for interpreting these results (Ackerman & Montalvo 1990). Consequently, the repercussions of intense pollination events on subsequent years should not be neglected, and could reveal a secondary limitation by resources at a longer time-scale (Montalvo & Ackerman 1987; Ackerman & Montalvo 1990).

Coincidentally, fruit set under natural conditions was very low (4.2% in 2005 and 3.3% in 2006) when compared to fruit set averages reported in other pollinator-dependent orchids lacking a reward ($10.8 \pm 11.2\%$ for tropical species and $19.8 \pm 18.6\%$ for species in all latitudes, calculated from Tremblay *et al.* 2005). According to our survey, this could be a multi-causal situation and could be attributed to florivory and fruit predation by caterpillars and leaf cutter ants, but mainly to the low activity of pollen vectors, characterised by very few effective visits and the patrolling of a particular fraction of the orchid population (see Fig. 3). In addition, the mechanism of post-removal senescence and its consequences for floral lifespan could negatively influence fruit set at the plant level, even when such a mechanism is beneficial at a population level (as discussed above). Finally, deception in *B. lindenii* could restrict potential pollinators only (or mainly) to males of *M. lepriouri* that are mate-seeking and use the flowers as meeting places. By contrast, female bees (of *M. lepriouri* and other species) visit flowers mainly looking for food, it being more important for them to learn how to avoid nectarless flowers of *B. lindenii*. Similar reproductive fates in relation to scarce pollinator activity have been reported for many temperate and tropical nectarless orchids (Gill 1989; van der Cingel 1995, 2001; Neiland & Wilcock 1998). In fact, most of the deceptive orchids studied to the date are strongly limited in pollinators, especially in the tropics (Tremblay *et al.* 2005).

In summary, within Orchidaceae, *B. lindenii* is the first report of pollination by bees of the Neotropical genus *Melissodes*. This orchid has serious reproductive limitations at a local and short-time scale in Canasí (east of Havana) due to

intrinsic features (self-compatibility but tendency to inbreeding depression at seed production level) and ecological aspects (scarce pollinator activity, tendency of pollinator to over-visit a given orchid cluster with a possible effect on breeding performance, and systematic predation of flowers and fruits by other insects). The study of these elements in the sister, endangered species *B. cubensis* and *B. negrilensis*, and in other taxa of the alliance is particularly relevant for its conservation, and for the potential novelty in explaining the evolution of pollination ecology of this group as a whole.

ACKNOWLEDGEMENTS

This research was supported by the Institute of Ecology and Systematics of Cuba and the Cuban Project 2043 of CITMA, the Spanish Dirección General de Investigación, Ciencia y Tecnología (CGL2006-13847-CO2-01-02), the Agencia Española de Cooperación Internacional (A/017570/08 and A/023710/09), CYTED (409AC0369) and the Xunta de Galicia (PGIDT04-PXIC31003PN). The programme MUTIS of MAE-AECID financed the work of A. Vale through a PhD scholarship. We thank Dr Julio Genaro (York University, Canada) and the entomologists of the Institute of Ecology and Systematics of Cuba for identification of the insects. We are also grateful to Rayner Núñez, Ilsa M. Fuentes, Iralys Ventosa, Elier Fonseca, Mercedes Montero and others for valuable help in the field. Finally, we thank the anonymous reviewers for their helpful suggestions.

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Conflicts of interest added after online publication: The authors have not declared any potential conflicts.