Confronting assumptions about spontaneous autogamy in populations of *Eulophia alta* (Orchidaceae) in south Florida: assessing the effect of pollination treatments on seed formation, seed germination and seedling development

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The breeding system of the terrestrial orchid *Eulophia alta* was investigated in south Florida where it has previously been reported as an auto-pollinated species. The effect of breeding system on seed viability and germinability and seedling development was also investigated. Incidences of spontaneous autogamy in *E. alta* were rare at the study site, resulting in only 7.1% of observed flowers forming capsules. In addition, hand pollination resulted in significantly greater capsule formation when flowers were subjected to induced autogamy (46.4%), artificial geitonogamy (64.3%) and xenogamy at both short (pollen source 10–100 m away; 42.9%) and long (pollen source > 10 km away; 67.9%) distances. Pollen source had little effect on seed viability and germinability or seedling growth rates. However, seed resulting from spontaneous autogamy developed more slowly than seed originating from the other treatments. These data indicate that spontaneous autogamy is rare in *E. alta* and that naturally forming capsules may be the result of unobserved pollination events. © 2009 The Linnean Society of London, *Botanical Journal of the Linnean Society*, 2009, 161, 78–88.


INTRODUCTION

Pollination can be a limiting stage in the life cycles of flowering plants, especially for those for which fitness depends on pollination by insect vectors (Weekley & Brothers, 2006). Recent evidence of pollinator decline attributed to anthropogenic habitat loss (Biesmeijer et al., 2006; Taki & Kevan, 2007) has drawn attention from conservation and pollination biologists alike. Understanding threats to the reproductive success of plants is vital to conservation planning and implementation. The importance of maintaining functional breeding systems may be particularly relevant in Orchidaceae as pollinators have played a critical role in the diversification and speciation of the family (Darwin, 1885; Dressler, 1981; Tremblay et al., 2005).

Orchids have also evolved many mechanisms to enhance out-crossing and prevent or reduce incidents of self-pollination, but genetically regulated self-incompatibility appears to be rare in the family, or at least rarely reported (see Tremblay et al., 2005). This may reflect the effectiveness with which orchid floral structures, such as pollinia and rostellae, prevent...
self-fertilization by effectively separating pollen from the stigma on the same flower during flower maturation and immediately after pollen removal (Dressler, 1981). Other structural, phenological and evolutionary mechanisms that promote out-crossing without the need for genetic incompatibility include delayed caudicle taxis, shrinkage of pollinia after removal, incomplete flowers, dichogamy and attraction of pollinators with large foraging ranges (Dressler, 1981; Borba & Semir, 1999; Johnson & Edwards, 2000; Tremblay et al., 2005; Tremblay, Pomales-Hernandez & Mendez-Cintron, 2006). Evidence from nectar addition studies with orchid species also indicates that the evolution of deceptive pollination strategies, strategies in which pollinators are attracted to flowers with the promise of a reward without one being supplied, may have evolved to enhance out-crossing, as nectar rewards increase pollinator visitation time and lead to increased self-pollination (Johnson & Nilsson, 1999; Johnson, Peter & Agren, 2004; Cozzolino & Widmer, 2005; Jersáková & Johnson, 2006; Jersáková et al., 2008).

Early studies of orchid pollination systems in Florida and elsewhere have emphasized the identification of visitors but, in many cases, do not report capsule formation data (see, for example, Dodson & Frymire, 1961; Dodson, 1962). Undoubtedly, identification of visitors and pollinators is an important aspect of integrated orchid conservation, as protecting breeding systems is vital to the in situ persistence of populations and species (Swarts & Dixon, 2009). However, confirming successful breeding by quantifying capsule formation and identifying potential post-pollination reproductive barriers, such as genetic incompatibility, inbreeding depression and/or out-breeding depression, is equally as important. Given many recent reports of successful asymbiotic and symbiotic culture of rare, threatened and endangered orchid taxa (see, for example, Stenberg & Kane, 1998; Huynh et al., 2004; Zettler et al., 2005, 2007; Znaniecka et al., 2005; Stewart & Kane, 2006, 2007; Dutra, 2008; Dutra et al., 2008; Kauth et al., 2008; Dutra, Kane & Richardson, 2009b), examining the effects of breeding system studies on effective capsule formation, seed viability and germinability and seedling vigour could be particular illuminating in investigations of orchid breeding systems. Although self-fertilization has been shown to decrease the seed production and seed weight of some orchid species (Beardsell et al., 1986; Gonzalez-Diaz & Ackerman, 1988; Peakall, 1989; Robertson & Wyatt, 1990), virtually nothing is known about how breeding system affects germinability and early seedling development in orchids. Such integrated data would help to clarify possible effects of different pollination systems on reproductive success and stand establishment, leading to more informed decisions about the status of orchid populations, conservation concerns and conservation management.

In contrast with the numerous examples of floral adaptations in orchids that promote out-crossing, some orchid species have evolved various methods of ensuring pollination in the absence of pollinators. In auto-pollination (= spontaneous autogamy) systems, pollinia are deposited on the stigmatic surface without a pollinator through a number of mechanisms, including development of powdery pollinia, expansion of the pollinia, malformation of the rostellum and bending of the caudicle (Gonzalez-Diaz & Ackerman, 1988; Catling & Lefkovitch, 1989; Catling, 1990; Gale, 2007; Micheneau et al., 2008). Autogamy is thought to facilitate (or to be a consequence of) range expansion into areas in which pollinators are rare or absent (Catling, 1990). Autogamy may be more widespread than previously thought; Fenster & Martén-Rodriguez (2007) suggested that pollinator specificity and auto-pollination may have co-evolved as adaptations to the same selection pressures: pollinator limitation and the uncertainty of seed set. In this scenario, auto-pollination could be a means of reproductive assurance given the ‘risky’ strategy of specialized pollinator attraction exhibited by many orchid species.

Reports of auto-pollination are not uncommon in Orchidaceae. Over 350 species have been reported to be autogamous in some part of their range, and estimates of the number of autogamous orchid species of some regional floras, such as Ecuador and Puerto Rico, are 20% or greater (Ackerman, 1985; Catling, 1990). Several species of orchids in peninsular Florida are reportedly autogamous (Leur, 1971; Leur, 1972; Goss, 1973; Dressler, 1981; McCartney, 1985; Catling, 1987, 1990; Calvo, 1990). However, most reports of autogamy do not include investigations of capsule formation under experimental conditions. Such studies are needed to confirm that auto-pollination is a successful mechanism of fruit production.

To quantify the rates of spontaneous autogamy and post-pollination reproductive isolating mechanisms, the pollination system of Eulophia alta (L.) Fawc. & Rendle (subfamily Epidendroideae, tribe Cymbidieae; Cameron et al., 1999) was studied. This terrestrial species is distributed in Africa, the neotropics and Florida, USA. It has been reported to be auto-pollinated in Mexico, South Africa, Zambia and Florida (Goss, 1973; Williamson, 1984; Catling, 1990), but its breeding system has not yet been fully studied. Consequently, several questions remain. (1) How common is spontaneous autogamy in this species in a single population? (2) How efficient is spontaneous autogamy as a mode of fertilization and capsule formation? (3) Does out-breeding enhance capsule
formation frequency? (4) Are there indications of genetic post-pollination reproductive barriers, such as self-incompatibility, in this species? In order to examine these questions, several experiments were designed with the objective of describing the pollination mechanisms of *E. alta* in south Florida. In addition, pollination treatment effects on seed viability, germination and early seedling development were also examined.

**MATERIALS AND METHODS**

**STUDY SPECIES**

*Eulophia alta* (common name: wild coco; Fig. 1A) is a terrestrial orchid found in Africa, the West Indies and the American tropics and subtropics. The occurrence of *E. alta* in the USA is restricted to south Florida, where it is found in moist to saturated soils of pine flatwoods (Brown, 2005). Although not listed as state threatened, *E. alta* is state protected, listed by the Federal Government as a wetlands indicator species (USDA, 2009) and described as commercially exploitable (Brown, 2005). At the study location, the Florida Panther National Wildlife Refuge (FPNWR), *E. alta* is locally abundant, but patchy in distribution (Stewart & Richardson, 2008). Concentrated populations are found in only five of 54 burn units on FPNWR, totaling approximately 2000 plants (S.L. Stewart, pers. observ.). Plants are found in the understorey of pine flatwoods, especially along mowed access roads.

*Eulophia alta* is among the largest North American terrestrial orchids. Indeterminate, unbranched racemes can be up to 2 m in height and contain over 50 flowers (although some carry as few as five flowers; Fig. 1A) that are 3.5–4.5 cm wide (Brown, 2005). Flowers open in succession and often have red hues (pink, red and maroon flowers are common). Green flowering phenotypes can also be found. The pollen of *E. alta* flowers is coalesced into two hard pollinia joined by a single motile stipe. The base of the pollinarium has a sticky viscidium that readily attaches to contacting objects. Flowers have no discernible scent.

**DETERMINATION OF POLLINATION MECHANISM**

Pollination experiments following the methods of Wong & Sun (1999) were conducted at the FPNWR (Collier County, FL, USA). In order to assess the breeding system of *E. alta*, the effects of seven pollination treatments (Table 1) on capsule formation, capsule length and capsule width were examined. Pollinia were harvested by touching a toothpick to the viscidium of flower pollinaria (to which the viscidium readily adheres), removing the anther cap and placing the harvested pollinia into a plastic bag for transport. Harvested pollinia were used within 8 h of collection. To test for asexual fruit set (agamospermy), flowers were emasculated and covered (bagged) with pollinator exclusion netting (2 mm mesh; Fig. 1B). The ability of flowers to transfer pollinia to the stigma without a pollination vector (spontaneous autogamy) was tested by bagging flowers with their pollinia left in place and without hand pollination. Within-flower self-incompatibility was tested for by emasculating flowers and pollinating them with their own pollinia (induced autogamy) before bagging. Self-incompatibility among flowers of the same inflorescence was tested for by pollinating flowers with pollinia from another flower on the same inflorescence (induced geitonogamy) before bagging. The effectiveness of outbreeding at great distances on capsule formation (artificial xenogamy) was examined by emasculating flowers and pollinating them with pollinia harvested from plants > 10 km from the study location.

![Figure 1. Study of the breeding system of Eulophia alta.](image)

(A) Flower. Scale bar, 1 cm. (B) Inflorescence with pollinator exclusion netting. (C) Infructescence. (D) Capsule. Scale bar, 1.5 cm. (E) Seeds stained with tetrazolium chloride. Scale bar, 250 μm. (F) Germinated seeds at week 8. Scale bar, 250 μm.
site before bagging. The effect of out-crossing at short
distances (induced xenogamy) was examined by pol-
linating emasculated flowers with pollinia collected
from plants at a distance of 10–100 m away from
manipulated plants.

Fourteen inflorescences from 14 individual plants
were selected with at least seven open, unpollinated
flowers. Each inflorescence received all seven previ-
ously described pollination treatments. Treatments
were randomly assigned to seven flowers on each
inflorescence. Senesced lower flowers were removed
as they were encountered and 0–5 flowers immedi-
ately above manipulated flowers were removed as
needed for the attachment of pollinator exclusion
netting. Upper flowers that opened later in the season
were not removed. For each treatment, \( n = 14 \) for each
of two study years, the first beginning on 9 October
2006 and then again on 11 November 2008. Potential
pollinators were excluded by securing 2 mm\(^2\) mesh
fabric around all flowers, except those in treatment 1
(Fig. 1B), the open pollination control. Capsules
(Fig. 1C, D) were examined 3, 6 and 12 weeks after
pollination treatments were initiated.

Inflorescences were used as block treatments. This
design was chosen because of the limited number of
flowering individuals and to avoid possible effects of
individual plant genotypes on the results. Logistic
regression was used to assess the effect of pollination
treatment on capsule formation using proc glimmix in
SAS v.9.1 (SAS Institute, 2003). Only capsules that
developed to at least week 11 were used in this
analysis. Least-squares means were used for mean
separation (\( \alpha = 0.05 \)). The effect of pollination treat-
ment on capsule dimensions (length and diameter)
was analysed using general linear modelling and
Waller–Duncan mean separation (\( \alpha = 0.05 \)). All data
were analysed using SAS v.9.1 (SAS Institute).

### SEED COLLECTION, STORAGE AND GERMINATION

In order to examine the effect of pollination treatment
on seed viability, germinability and seedling develop-
ment, capsules formed in 2006 as the result of the
pollination treatments were collected after 12 weeks
and dried over silica gel desiccant at room tempera-
ture until the capsules dehisced. At least one capsule
was collected from six of the pollination treatments.
Seed from each capsule was stored separately over
silica gel desiccant at room temperature for up to 2
months prior to experimentation. For each of the
treatments that produced more than one capsule,
5.0 mg samples from all capsules were collected and
pooled to create a proportional representative sample.
Approximately 5.0 mg of bulked seed from each treat-
ment was surface sterilized for 45 s in a solution of
100% ethanol–6.0% NaOCl–sterile distilled deionized
water (1 : 1 : 20), and then rinsed three times in
sterile distilled water.

PhytoTechnology Orchid Seed Sowing Medium
(P723) was used for asymbiotic germination. This has
been shown previously to support germination and
early seedling development of *E. alta* (Johnson et al.,
2007). The medium was adjusted to pH 5.7 with 1.0 M
NaOH and autoclaved for 40 min at 121 °C and
117.1 kPa. Sterilized medium was dispensed as
30 mL aliquots into Petri plates (diameter, 9.0 cm).
Approximately 30 surface-sterilized seeds from each
pollination treatment were sown separately onto

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**Table 1.** Pollination treatments tested in the study of the pollination mechanism of *Eulophia alta*

<table>
<thead>
<tr>
<th>Treatment number</th>
<th>Pollination treatment</th>
<th>Pollinators excluded</th>
<th>Pollinia removed</th>
<th>Pollen treatment</th>
<th>Assessment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control (open pollination)</td>
<td>No</td>
<td>No</td>
<td>No pollination</td>
<td>Capsule set under natural conditions</td>
</tr>
<tr>
<td>2</td>
<td>Agamospermy</td>
<td>Yes</td>
<td>Yes</td>
<td>No pollination</td>
<td>Frequency of non-sexual capsule set</td>
</tr>
<tr>
<td>3</td>
<td>Spontaneous autogamy</td>
<td>Yes</td>
<td>No</td>
<td>No pollination</td>
<td>Need for pollinators for reproduction</td>
</tr>
<tr>
<td>4</td>
<td>Induced autogamy</td>
<td>Yes</td>
<td>Yes</td>
<td>Same flower</td>
<td>Self-compatibility of flowers</td>
</tr>
<tr>
<td>5</td>
<td>Artificial geitonogamy</td>
<td>Yes</td>
<td>Yes</td>
<td>Different flower on same plant</td>
<td>Self-compatibility of plants</td>
</tr>
<tr>
<td>6</td>
<td>Artificial xenogamy</td>
<td>Yes</td>
<td>Yes</td>
<td>Flower from a distant population</td>
<td>Potential for out-breeding at long distances (pollen source &gt; 10 km)</td>
</tr>
<tr>
<td>7</td>
<td>Induced xenogamy</td>
<td>Yes</td>
<td>Yes</td>
<td>Distant plant in the same population</td>
<td>Potential for out-breeding at short distances (pollen source 10–100 m)</td>
</tr>
</tbody>
</table>
eight replicate Petri plates. The plates were then sealed with a single layer of Nesco film (Karlan Research Products Corp.), wrapped in two layers of aluminium foil to exclude light and incubated at 22 ± 3°C for 12 weeks. Seeds were exposed to short periods of light (less than 30 min) during data collection. Germination and seedling development was scored on a scale of 0–5 (Table 2) with a dissecting microscope after 4, 8 and 12 weeks of culture. The percentage seed and seedlings in each developmental stage and the percentage germination were calculated. Germination and developmental data were arcsine transformed and analysed using general linear modelling and Waller–Duncan mean separation (α = 0.05).

SEED VIABILITY TESTING

Tetrazolium chloride staining was used to assess seed viability by pretreating three replicates of 100–200 seeds from each pollen treatment with 0.5% calcium hypochlorite for 3 h. The seed was then rinsed three times in sterile distilled deionized water and soaked for 24 h in darkness at 22 ± 2°C. The seed was then soaked for 24 h in 1.0% tetrazolium chloride at 30 ± 2°C in darkness. The percentage viability was calculated for each replicate by dividing the number of pink/red-stained embryos (viable; Fig. 1E) by the total number of embryos counted. Three replications were performed for each treatment and this experiment was repeated once (n = 6). Statistical analysis was conducted as described for the germination data.

RESULTS

POLLINATION SYSTEM AND CAPSULE FORMATION

In 2008, a late frost destroyed developing capsules on all but five plants 11 weeks after the initiation of experimentation. All capsules resulting from agamospermy (1) and artificial xenogamy (4) were damaged, as well as six of seven induced autogamy capsules, seven of 10 artificial geitonogamy capsules and seven of nine artificial xenogamy capsules. The presence of capsules at the time of frost 11 weeks after experimentation began was confirmed by observing frost-damaged capsules at week 12. Undamaged capsules were used in the analysis of the effect of pollination on capsule dimensions.

Statistical analysis indicated that pollination treatment had a significant effect on capsule formation (F6,6 = 6.29, P = 0.02). Data on the effect of pollination treatment on capsule formation from both observation years were combined because significant differences were not observed between years (F1,6 = 0.28, P = 0.62). All pollen treatments resulted in capsule formation in 2006, but capsules that formed from spontaneous agamospermy aborted before 6 weeks of development. Similarly, the spontaneous agamospermy treatment resulted in only a single capsule being formed in 2008. Overall, agamospermy only produced a single mature capsule (Fig. 2; 3.6% ± 3.6%; mean ± 3.6%). Open pollination resulted in 14.3% ± 6.7% capsule formation and was not significantly different from the observed capsule formation from spontaneous autogamy (7.1% ± 5.0%) or agamospermy. A greater proportion of capsules formed from induced xenogamy (42.9% ± 9.5%) than spontaneous autogamy or agamospermy. Rates of capsule formation resulting from induced xenogamy, induced autogamy (46.4% ± 9.6%), geitonogamy (64.3% ± 9.2%)
and artificial xenogamy (67.9% ± 9.0%) were not significantly different.

The study year did not have a significant effect on capsule length or capsule diameter at week 3 (length: $F_{1,72} = 0.00; P = 0.99$; diameter: $F_{1,72} = 1.62; P = 0.21$) or week 12 (length: $F_{1,72} = 1.13; P = 0.30$; diameter: $F_{1,72} = 1.33; P = 0.26$). Significant differences were detected between study years during week 6 observations (length: $F_{1,57} = 15.32; P = 0.00$; diameter: $F_{1,57} = 26.31; P = 0.00$). However, when the capsule length and capsule diameter data for each study year were analysed separately, the same result was obtained for both study years; no significant differences were detected among treatments in 2006 ($F_{6,30} = 0.60; P = 0.79$; diameter: $F_{6,30} = 0.46; P = 0.80$) or 2008 ($F_{6,27} = 0.88; P = 0.52$; diameter: $F_{6,27} = 0.66; P = 0.68$) at week six. Because of these results, capsule dimension data for both study years were combined.

Pollination treatment did not have a significant effect on capsule length or capsule diameter at weeks 3 (length: $F_{5,72} = 0.92; P = 0.48$; diameter: $F_{5,72} = 0.54; P = 0.77$), 6 (length: $F_{6,72} = 1.33; P = 0.26$; diameter: $F_{6,30} = 0.65; P = 0.69$) or 12 (length: $F_{6,33} = 0.82; P = 0.55$; diameter: $F_{6,33} = 1.00; P = 0.43$). Capsule lengths were not significantly different among treatments at 6 and 12 weeks (Fig. 3A). At week 3, the capsule diameters of the agamospermous treatment were not statistically significantly different from those of capsules resulting from spontaneous autogamy treatment, but were significantly lower than those of all other treatments. The diameters of capsules resulting from different pollination treatments were not significantly different at any time examined (Fig. 3B).

**EFFECT OF POLLINATION TREATMENT ON SEED VIABILITY AND EARLY SEEDLING DEVELOPMENT**

In 2006, seed was generated from all pollination treatments except agamospermy. Seed viability was not significantly affected by pollination treatment ($F_{5,30} = 0.57; P = 0.57$) and ranged from 88.2% ± 2.4% to 92.7% ± 1.8%. Pollination treatment had a significant effect on germination after 12 weeks of culture ($F_{4,37} = 2.88, P = 0.03$) and ranged from 94.7% ± 1.4% to 99.3% ± 0.5% (Fig. 4). At week 4, most seeds were observed at stage 1 (swollen embryo; 74.2% ± 3.4% to 86.8% ± 2.4%) and were considered to have germinated. Pollination treatment had a significant affect on the percentage of seedlings at stage 2 at this time.

Figure 3. Effect of pollination treatment on Eulophia alta capsule length (A) and diameter (B). Data from two study years are combined. The data for agamospermy (*) at week 6 are for the length and width of a single capsule (a single capsule formed in 2006, but aborted after week 3 and a single capsule that formed in 2008 was destroyed by frost).
significantly fewer seeds from the spontaneous autogamy treatment had developed to stage 2 (6.8% ± 2.5%) than all other treatments, except for artificial geitonogamy (11.5% ± 2.5%). At week 8 (Fig. 1F), pollination treatment had a significant effect on the percentage of seeds/seedlings at stage 0 ($F_{5,37} = 6.41; P = 0.00$) and stage 3 ($F_{5,37} = 7.85; P = 0.00$). Significantly more nongerminated seeds (stage 0) were observed from spontaneous autogamy (6.7% ± 1.7%) and artificial autogamy (6.7% ± 1.3%) treatments. Fewer seeds from the spontaneous autogamy treatment had developed to stage 3 (7.3% ± 2.5%) compared with other treatments. Statistically significant differences were not found among treatments at stages 1 ($F_{5,37} = 0.86; P = 0.51$), 2 ($F_{5,37} = 4.48; P = 0.07$) or 4 ($F_{5,37} = 0.86; P = 0.51$) after 8 weeks of culture. After 12 weeks of culture, pollination treatment was found to have a significant effect on the percentage of seedlings at stages 0 ($F_{5,37} = 3.10; P = 0.02$), 2 ($F_{5,37} = 7.10; P = 0.00$), 3 ($F_{5,37} = 5.96; P = 0.00$) and 4 ($F_{5,37} = 38.20; P = 0.00$), but not at stage 1 ($F_{5,37} = 0.37; P = 0.86$). At this time, signifi-
cantly fewer seedlings from the spontaneous autogamy treatment were observed at stage 4 (7.2% ± 1.2%) compared with other treatments. Seed generated from the spontaneous autogamy treatment also had significantly lower seedling development than other treatments, as evidenced by a significantly higher percentage of seedlings at stage 3 (56.2% ± 4.1%; F,3,37 = 5.96; P = 0.00).

**DISCUSSION**

*Eulophia alta* infrutescences with abundant capsules are often observed at FPNWR (T.R. Johnson pers. observ.; Fig. 1C, D). Goss (1973) reported that, soon after flowers open, the caudicle of the pollinarium of south Florida *E. alta* plants bends forwards bringing the pollinia into contact with the stigma. However, this mechanism was not observed during the course of our study. Given previous reports of an autogamous breeding mechanism of *E. alta* in Florida (Goss, 1973) and the lack of documented *E. alta* pollinators in North America, there has been little reason to investigate the pollination biology of this species. However, the low success rate of induced autogamy in forming capsules during this investigation indicates that field-observed capsules could be the result of unobserved pollinator visitation.

Further evidence of the scarcity or lack of auto-pollination in *E. alta* at the study site comes from the observation that glasshouse-grown plants do not produce capsules unless pollinated by hand (T.R. Johnson, pers. observ.). Discrepancies between this report and previous reports of autogamy in *E. alta* could be a result of differences in the frequency of auto-pollination of *E. alta* throughout its range in Florida, although this requires further study. Capsule formation in other auto-pollinated orchid species is typically much more efficient than observed for *E. alta* (Tremblay et al., 2005). The fruit set of *Nervilia nipponica* Makino under pollinator exclusion netting was 93% and that of *Jumellea stenophylla* Schltr. was 66.7%–83.9% (Gale, 2007; Micheneau et al., 2008). This is to be expected given the strong selection pressure that pollinator absence would impose.

Seed that formed from the autogamous treatment in 2006 exhibited a slower rate of germination and seedling development compared with seed resulting from other pollination treatments. This is an indication that rare spontaneous autogamy may produce seed of lower fitness than other pollination treatments. It is possible that the difference in capsule formation and seedling development between spontaneous autogamy and induced autogamy treatments is a consequence of pollination with aged pollen (Rosell, Galan Sauco & Herrero, 2006; Castro, Silveira & Navarro, 2008). More pronounced effects of inbreeding have been reported for other orchid genera. Seed germination of *Dactylorhiza sambucina* (L.) Soó and the subsequent survival of reintroduced plants produced from induced autogamy were lower than that of seeds and seedlings produced from out-crossing pollination treatments (Juillet, Dunand-Martin & Gigord, 2007). Induced autogamy also decreased the seed viability of *Barlia robertiana* (Loisel.) Greuter and *Anacamptis morio* (L.) R.M.Bateman, Princeon & M.W.Chase and reduced seed weight and the number of seeds with embryos of *Platanthera ciliaris* Lindl. (Robertson & Wyatt, 1990; Smithson, 2006).

Most pollination treatments did not have a significant effect on seed viability of *E. alta*. In all pollination treatments, tetrazolium chloride staining underestimated the observed germination by as much as 10.8% (artificial geitonogamy: estimated, 88.2% ± 2.4%; observed, 99.0% ± 0.7%), but provided a good relative estimate of germinability among treatments. This difference in tetrazolium chloride-estimated viability and germinability highlights the need to perform tests of both seed respiration and germinability.

One consequence of using a blocked design in the current study was the inability to control for the possible selective abortion of low-quality fruits. It is possible that low capsule formation in spontaneous autogamous treatments is reduced when higher quality pollen is available for fertilization (Bookman, 1984). However, the observations that glasshouse-grown plants do not form capsules without hand pollination suggests that resource limitation does not regulate spontaneous autogamy. In addition, self-pollination resulted in a high degree of capsule formation and vigorous seed on tested plants. These data indicate that *E. alta* populations at FPNWR are not autogamous, as has been reported previously for the species in Florida (Goss, 1973; Catling, 1990). Whether autogamy of *E. alta* is common throughout its range or limited to certain populations also remains to be studied.

Capsule formation resulting from induced autogamy in self-compatible orchids can be highly effective: 85% in *Listera ovata* (L.) R.Br. and 71.9–90% in *Platanthera ciliaris* at two study sites (Ackerman & Mesler, 1979; Robertson & Wyatt, 1990). Although induced autogamy in *E. alta* resulted in fewer capsules than did artificial geitonogamy and induced xenogamy, the means were not statistically different. These data indicate that there are no significant reproductive barriers to within-flower self-fertilization for *E. alta* in Florida. However, the transfer of pollinia to stigma may still be vital to capsule formation, given the significant difference in capsule formation between treatments that did not involve hand pollination (control, agamospermy and spontaneous autogamy) and those that did (induced

autogamy, artificial geitonogamy, artificial xenogamy and induced xenogamy).

Pollination by induced autogamy did not significantly reduce seed viability, germination or the development of seeds and seedlings relative to other pollen supplementation treatments. Agamospermy appears to be a rare event in this species as no capsules formed from emasculated flowers in 2006 and a single capsule formed in this treatment in 2008. Although only visibly unpollinated flowers were used during the experimental set-up, undetected pollination of some flowers could have occurred. Agamospermy appears to be rare in E. alta and is not expected to contribute to viable seed production.

The hypothesis of insect-mediated pollination of E. alta in Florida may be speculative, but there is evidence that plants could be pollinated by an unidentified vector. Comparison with other Eulophia spp. suggests that E. alta could be pollinated by bees (as is Eulophia foliosa Bolus) or Xylocopa bees (as are E. cristata Lindl. and E. rosea (Lindl.) A.D.Hawkes [= E. horsfallii (Bateman Summerh.)] (Kullenberg, 1961; Lock & Profta, 1975; Peter & Johnson, 2006). Scarce pollinators have been linked to poor capsule formation in Cyrtopodium punctatum (L.) Lindl., an orchid found in the vicinity of E. alta at FPNWR. Cyrtopodium punctatum is hypothesized to be pollinated by Xylocopa bees, populations of which may be declining in south Florida as a result of agricultural pesticide application (Dutra et al., 2009a). If Xylocopa bees are responsible for the pollination of E. alta in Florida, as they are for other Eulophia species in other parts of the world, pollinator observations would be expected to be exceedingly rare (Dutra et al., 2009a). As spontaneous autogamy (the previously reported mode of pollination) was inefficient in producing capsules, a new priority in efforts to manage and protect E. alta populations in south Florida is to determine whether a pollinator exists.

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