



## Does selfing provide reproductive assurance in the perennial herb *Bulbine vagans* (Asphodelaceae)?

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Selection for selfing to provide reproductive assurance depends on the balance between increased reproductive output when pollinators or potential mates are scarce and the extent that inbreeding depression erodes such fertility gains. We use glasshouse and field experiments to examine the benefits of autonomous and facilitated selfing in *Bulbine vagans*. Autonomous selfing was delayed until after opportunities for outcrossing and reproductive output was 0.67 relative to manual selfing and open pollination. Values less than one probably reflected insufficient autonomous deposition of self pollen. In the field, reproductive output of emasculated flowers was 0.50 relative to intact flowers that could both outcross and self, indicating that outcross pollen was limited and that selfing boosted reproductive output. Because all pollen was removed from anthers before intact flowers closed, facilitated selfing rather than autonomous selfing occurred. In the glasshouse, inbreeding depression was 0.45, but under natural conditions would probably exceed 0.5. Values greater than 0.5 negate the automatic gene transmission advantage afforded by selfing and increasingly erode the benefits of reproductive assurance. We conclude that in *B. vagans* delayed and facilitated selfing can confer reproductive assurance, providing the latter does not usurp ovules that could be outcrossed.

The transition in mating system from outcrossing to self-fertilization has occurred repeatedly in flowering plants, and perhaps a third of hermaphroditic species exhibit mixed mating systems, combining both outcrossing and selfing (Stebbins 1974, Barrett 2003, Goodwillie et al. 2005). Central to the understanding of such transitions are the adaptive values of self- and cross-fertilization under different ecological conditions. While recognizing that individual traits evolve in context with the entire phenotype, the evolution of selfing appears to be influenced predominantly by a few large benefits and costs (Eckert and Herlihy 2004). Two benefits provided by selfing are a 50% gene transmission advantage and reproductive assurance, and major costs include inbreeding depression and pollen and seed discounting (Fisher 1941, Baker 1955, Lande and Schemske 1985, Charlesworth and Charlesworth 1987, Uyenoyama et al. 1993). The transmission advantage occurs because selfing plants contribute both pollen and ovules to their selfed progeny as well as pollen to progeny on other plants, whereas outcrossing plants contribute either ovules or pollen to their progeny (Fisher 1941). This substantial advantage can be reduced or negated by inbreeding depression, the inferior performance of selfed relative to outcrossed progeny (Charlesworth and Charlesworth 1987). Considering these factors alone, theoretical models predict that populations should evolve towards either predominant outcrossing when inbreeding depression exceeds 0.5 or predominant selfing when inbreeding depression is less than 0.5 (Lande and Schemske 1985, Charlesworth and Charles-

worth 1987). The fact that many hermaphroditic plants exhibit mixed mating indicates that additional factors must contribute to the evolution of self-fertilization in natural plant populations (Holsinger 1996, Goodwillie et al. 2005).

Reproductive assurance, the second benefit of selfing, enables seed production when pollinators or mates are scarce or unavailable, and outcross pollen is insufficient for full fertilization of ovules (Darwin 1876, Baker 1955, Pannell and Barrett 1998, Moeller and Geber 2005). However, not all selfing is equivalent in terms of reproductive assurance. Lloyd (1979) pioneered models that considered reproductive assurance and the selective importance of how and when selfing occurs. Subsequent theory has modeled how pollen and seed discounting, inbreeding depression and its purging, spatial and temporal variability in pollination environments, and life-history influences the evolution of selfing and reproductive assurance (Lloyd 1992, Cheptou and Schoen 2007, reviewed by Goodwillie et al. 2005, Eckert et al. 2006). What is clear from this theory is that the role of reproductive assurance in the evolution of mating systems is complicated, despite seemingly obvious benefits.

Lloyd (1979, 1992) recognized three modes of autonomous self-pollination based on when each occurs relative to outcrossing (prior, competing and delayed). Potentially, all modes can provide reproductive assurance, although advantages depend upon the level of pollinator activity. When outcrossing is possible, prior and competing selfing can cause pollen and seed discounting, which reduces the

level of inbreeding depression that is required to negate advantages (Lloyd 1992, Morgan et al. 2005). By contrast, delayed selfing occurs after opportunities for outcrossing and does not usurp pollen and ovules. Delayed selfing can thus provide advantages at high levels of inbreeding depression, particularly in annuals. In perennials, trade-offs between production of selfed seeds and future survival and fecundity dictate the magnitude of advantages (Lloyd 1979, Morgan et al. 1997). Within-flower selfing can also be mediated by pollinators (facilitated selfing) and can provide reproductive assurance when outcrossing occurs but is insufficient to cause full seed set. In this scenario, pollen and seed discounting should be minimal and facilitated selfing is advantageous at high levels of inbreeding depression (Lloyd 1992). Selfing that occurs between flowers on the same plant (geitonogamy) is not expected to provide reproductive assurance because pollinators are required to transfer pollen, and gametes that could be otherwise involved in outcrossing are usurped (pollen and seed discounting; Lloyd and Schoen 1992).

Schoen and Lloyd (1992) highlight two complementary approaches to assess the reproductive assurance provided by within-flower modes of selfing. First, indirect information on the contribution of autonomous selfing to reproductive assurance can be gained by comparing seed set of plants that have been isolated from pollinators with that of open-pollinated plants. Seed set of isolated plants tests for autofertility and reflects the potential rather than the actual rate of autonomous selfing. Nevertheless, autofertility can provide useful insights into some of the ecological and evolutionary correlates of within-flower selfing (Lloyd and Schoen 1992, Schoen and Lloyd 1992). Second comparing seed set of emasculated flowers with that of naturally pollinated flowers provides information on maximal outcrossing rates and the amount of reproductive assurance afforded by facilitated plus autonomous selfing. This second approach assumes that removing anthers does not negatively affect pollinator behavior by rendering flowers less attractive, or does not damage flowers in such a way that reduces seed set (Schoen and Lloyd 1992, Eckert et al. 2006).

Here we investigate whether selfing provides reproductive assurance and whether inbreeding depression offsets any benefits in *Bulbine vagans*, a self-compatible perennial herb. In natural populations, plants often occur at low densities and bloom during spring and summer, a period of frequent storms and inclement weather. Pollinator unpredictability owing to inclement weather could favour delayed selfing. On the other hand, lack of available mates owing to low plant density might favour either delayed or facilitated selfing, providing pollinators are present during suitable conditions. Because most plants open only one flower/day that closes after 8 h, any pollinator-mediated selfing is likely to occur within flowers rather than between flowers on the same plant (Vaughton unpubl.).

We first use experimental manipulations in a glasshouse to examine the relative reproductive output and mechanism of autonomous selfing. Then, we examine the performance of selfed and outcrossed progeny at several life-cycle stages to estimate inbreeding depression and potential costs of selfing. We next quantify the potential reproductive output afforded by autonomous selfing and pollinator-mediated

selfing in the field using 1) pollinator exclusion experiments, and 2) flower emasculation experiments. Finally, we integrate estimates of inbreeding depression and reproductive output to assess the amount of reproductive assurance provided by the different modes of selfing.

## Material and methods

### Study species and sites

Populations of *Bulbine vagans* (Asphodelaceae) are restricted to the escarpment of the tablelands in northern New South Wales and southern Queensland, Australia (Godden 1993). The yellow flowers (15–25 mm diameter) appear adapted for pollination by native bees, but do not produce nectar. Clavate hairs surround the anthers and may play some role in attracting pollinators. No fruits are produced asexually (Ramsey unpubl.). We studied two populations located near Armidale, NSW (LP: 30°39'S, 151°57'E, 944 m a.s.l.; DF: 30°40'S, 151°43'E, 947 m a.s.l.). Populations contain several hundred plants sparsely arranged in rock crevices along cliff tops and are dangerous to access. Consequently, we used established potted plants for our glasshouse and field studies. We maintained potted plants in a fully screened glasshouse that was free of pollinating insects. We conducted field studies in the LP population only and mimicked the natural arrangement of plants by placing pots 2 m apart about 5 m upslope from where *B. vagans* occurred naturally.

### Autonomous self-pollination: reproductive output

To assess autonomous self-pollination, we assigned 5–10 newly opened flowers on each of 10 and 12 plants from LP and DF, respectively, to either an autonomously selfing or a manual selfing treatment. We left flowers in the autonomous selfing treatment untouched. We brushed anthers from the same flower across stigmas to self-pollinate flowers manually. After four weeks, we harvested fruits and counted ovules and seeds of five fruits/treatment/plant. Ovules were smaller than seeds. We calculated fruit set as mature fruits/pollinated flowers and seed set as seeds/ovules. Following Lloyd and Schoen (1992), we estimated an index of autofertility for each plant as: (autonomous fruit set  $\times$  seed set)/(selfed fruit set  $\times$  seed set). For each population, we used one-sample t-tests to test if autofertility differed from 1.0.

For fruit set, we computed logistic models and analyses of deviance to examine the effects of population, plant nested within population, pollination and the population  $\times$  pollination interaction. We used a binomial error structure and logit link function, and scaled deviances to remove overdispersion (Crawley 1993, Beath 2004). For seed set, we used a split-plot ANOVA with population a fixed between-subject factor, plant a random factor nested within population, and pollination a fixed within-subject factor. We used JMP to compute ANOVAs (ver. 5.01a, SAS Inst.). Means ( $\pm$  SE) are given.

## Autonomous self-pollination: mechanism

In a glasshouse, we determined when autonomous selfing occurred relative to opportunities for outcrossing. We first visually examined five flowers/population between flower opening and closing to assess when anthers and stigmas make contact. We then assessed stigmatic pollen loads on 63 flowers on five DF and seven LP plants just as flowers closed. Stigmas were excised and placed on microscope slides with small cubes of glycerine gel. We melted the gel and counted pollen grains on stigmas at 100 × magnification. Few or no pollen grains on stigmas indicate that autonomous selfing is delayed until after opportunities for outcrossing.

Flowers have three short and three long stamens. We examined the role of stamen height in autonomous selfing by allocating flowers on each of seven plants/population to four treatments: anthers removed from long stamens; anthers removed from short stamens; no anthers removed; and all six anthers removed ( $13.8 \pm 1.3$  flowers/treatment/plant). Flowers were emasculated as they opened. We counted fruits and seeds of 4–5 fruits/treatment/plant. Removing all anthers tested for inadvertent self-pollination when we emasculated flowers. Inadvertent selfing was low; only 6/106 flowers set fruits with  $1.4 \pm 0.7$  seeds/fruit.

Low fruit and seed set following autonomous selfing can be caused by insufficient pollination or reduced pollen and stigma viability at the end of floral life. We examined the effect of floral age by manipulating the timing of self-pollination. We allocated 5–13 flowers on seven plants/population to three treatments: autonomous selfing; early manual selfing as flowers opened; and late manual selfing as flowers closed. We pollinated flowers as described above and counted fruits and seeds of five fruits/treatment/plant. Less fruit and seed set by late vs early selfed flowers indicates that older flowers have reduced pollen and stigma viability. Greater fruit and seed set by late vs autonomously selfed flowers indicates that the amount of pollen deposited autonomously limits reproductive assurance.

For fruit set, we used analysis of deviance to examine the effects of population, plant nested within population, pollination and the population × pollination interaction. For seed set, we used a split-plot ANOVA with population a fixed between-subject factor, plant a random factor nested within population, and pollination a fixed within-subject factor. We used non-orthogonal planned comparisons and Bonferroni corrections to compare early vs late and late vs autonomous selfing treatments.

## Inbreeding depression

In a glasshouse, we manually self- or cross-pollinated 5–10 newly opened flowers on each of 10 plants population<sup>-1</sup>. We counted fruits and seeds and individually weighed five selfed and five outcrossed seeds from each family to the nearest 0.1 mg. We placed 15 selfed and crossed seeds per family on moist germination pads in Petri dishes arranged randomly in an incubator with 12 h days (20°C) and 12 h nights (10°C). We recorded germination for 12 weeks after which no further seeds germinated. We randomly chose six selfed and crossed seedlings per family and planted them

into individual 460 cm<sup>3</sup> pots containing sand, loam and peat (1:1:1). We arranged pots on benches, with two selfed and crossed seedlings per family in each of three blocks. After eight weeks, we counted surviving plants and assessed plant size by counting leaves/plant as an indicator of future reproductive potential and survival.

For all traits, we calculated relative self-performance of each maternal family as:  $rp = w_s/w_c$ , where  $w_s$  and  $w_c$  are the performances of selfed and crossed progeny, respectively. We estimated cumulative performance of selfed and crossed progeny as the product of the traits most likely to be related to fitness (fruit set, seed set, seed germination, plant survival and size), and then estimated family cumulative RP and inbreeding depression as:  $\delta = 1 - RP$ .

For each population, we used analyses of deviance to assess the effects of pollen type and family on fruit set, seed germination and seedling survival; we also included block as a factor for survival. For seed set and seed mass, we used two-way ANOVAs, with pollen type (fixed) and family (random) as factors. For seedling size, we included a covariate, days since planting, and block in the analysis. Seedling size required square root transformation.

## Pollinator exclusion experiment

In the field, we quantified reproductive output following autonomous selfing by allocating 22 plants to either pollinator exclusion or open-pollination treatments. We excluded pollinators using metal cages (apertures, 5 × 3 cm) covered with flywire (0.5 × 0.5 mm). Open-pollinated plants were caged only, allowing access to pollinators but excluding wallabies and kangaroos that ate plants. After 12 d, 15–24 flowers plant<sup>-1</sup> had opened and we removed the flywire. After four weeks, we counted fruits and seeds of five fruits plant<sup>-1</sup>. Flywire did not adversely affect autonomous fruit and seed set, because fruit set of bagged plants in the field was similar to that of unbagged plants in the glasshouse (both 55%).

We compared fruit set between treatments using analysis of deviance with plants as replicates. We compared seed set using nested ANOVA with treatment a fixed factor and plants nested within treatment a random factor. We estimated relative reproductive output of each bagged plant as: (fruit set × seed set)/(mean [open fruit set × open seed set]).

## Does emasculation affect pollinator visitation?

Over three days, we assigned flowers haphazardly to three treatments: anthers and clavate hairs left intact (intact); anthers removed but hairs left intact (emasculated); and both anthers and hairs removed (hairless). We removed anthers before they dehisced, taking care not to damage the hairs. Each day, we assigned plants to two plots located 50 m apart and haphazardly allocated flowers on plants such that there were similar numbers of flowers per treatment. We thus treated flowers as replicates rather than plants. In total, we used 24 plants and observed 53, 53 and 62 flowers in the intact, emasculated and hairless treatments, respectively. We simultaneously monitored plots during four 15-min periods between 10:00 and

14:00 h on each day and counted pollinator visits to each flower. We also timed the duration of individual visits by pollinators to intact and emasculated flowers (both  $n = 80$  visits).

We compared pollinator visits between treatments with a split-plot ANOVA, with plot (fixed) and day (random) as between-subject factors, and treatment (fixed) a within-subject factor. The three-way interaction was not significant and was omitted from the final analysis ( $p = 0.900$ ). We used non-orthogonal planned comparisons and Bonferroni corrections to compare intact vs emasculated and emasculated vs hairless treatments. For duration of visits, we used a two-way ANOVA with day and treatment as fixed factors. Number of visits was square-root and duration of visits was  $\ln$  transformed. Similar visitation to intact and emasculated flowers validates the use of emasculation experiments.

## Emasculation experiment

Over 10 d, we evaluated whether selfing contributed to reproductive output by assigning 22 plants to either emasculated or intact treatments in the field (12–22 flowers/plant/treatment). We counted fruits and seeds of 5 fruits/plant/treatment. We compared fruit set using analysis of deviance with plants as replicates and seed set using ANOVA with treatment (fixed) and plant (random) nested within treatment as factors. We estimated reproductive assurance as:  $1 - [(\text{emasculated fruit set} \times \text{seed set}) / (\text{intact fruit set} \times \text{seed set})]$  (Eckert et al. 2006). Because emasculated flowers only outcross, but intact flowers can self and outcross, greater overall seed set by intact plants demonstrates reproductive assurance. We also assessed the likelihood of autonomous vs facilitated selfing by examining anthers for pollen in closing intact flowers with a  $10 \times$  hand lens. The absence of pollen indicates facilitated selfing.

## Results

### Autonomous self-pollination: reproductive output and mechanism

Plants in both populations selfed autonomously, but fruit and seed set were significantly greater following manual selfing (Table 1; fruit set, DF:  $91.5\% \pm 4.5$  vs  $70.0\% \pm 4.3$ , LP:  $90.7\% \pm 3.3$  vs  $56.0 \pm 5.4$ ; seed set, DF:  $59.6\% \pm 2.8$  vs  $56.7\% \pm 3.3$ , LP:  $56.8\% \pm 3.1$  vs  $49.1\% \pm 3.5$ ). Relative

Table 1. Results of an analysis of deviance ( $\chi^2$ ) and a split-plot ANOVA (F) examining differences in fruit and seed set following manual selfing or autonomous selfing in *Bulbine vagans*. For the ANOVA, numerator and denominator degrees of freedom are given.

Source	Fruit set			Seed set		
	DF	$\chi^2$	p	DF	F	p
Population	1	2.67	0.102	1,20	1.44	0.242
Plant (population)	20	27.47	0.123	20,181	3.92	<0.001
Pollination	1	34.17	<0.001	1,181	4.56	0.034
Pollination $\times$ Pop	1	0.61	0.433	1,181	0.70	0.403
Error	20	18.97	0.524	181		

autofertility was significantly less than one in both populations (DF:  $0.75 \pm 0.08$ ,  $t_{12} = 3.17$ ,  $p = 0.004$ , LP:  $0.58 \pm 0.08$ ,  $t_{10} = 5.24$ ,  $p < 0.001$ ).

Autonomous self-pollination was delayed until after opportunities for outcrossing and occurred as flowers closed. When flowers first opened the three long and three short stamens were clustered together on one side of the flower, while the style curved away in the opposite direction of the anthers. As flowers closed, styles straightened and filaments wilted, bringing the anthers and stigma into contact. Of the 63 stigmas collected just before flowers closed, 59 had no pollen grains and the remaining stigmas had only 3.7 pollen grains on average.

We assessed the importance of stamen height on autonomous selfing by removing either the short or long stamens, or leaving all intact. Removal of short stamens significantly reduced fruit set by about 33% compared with removing long stamens or leaving all stamens intact. Seed set did not differ among the three treatments (Fig. 1, Table 2).

Pollen deposition, rather than reduced pollen viability and stigma receptivity, limited reproductive output following autonomous selfing. Fruit and seed set were similarly high following early and late manual selfing. By contrast, fruit and seed set following autonomous selfing were significantly less than following late selfing (Fig. 2, Table 3).

In all ANOVAs, population and treatment  $\times$  population interactions were not significant, and all treatments affected plants similarly in both populations (Table 1, 2, 3).

### Inbreeding depression

Crossed progeny outperformed selfed progeny in both populations for seed set, seedling survival and seedling size, and for seed mass in DF and seed germination in LP (Table 4, 5). Fruit set did not differ in either population. Pollination  $\times$  family interactions were significant only for seed mass in DF and seedling survival in DF and LP, indicating that the effect of selfing varied among families. Maternal families exhibited significant variation for some of the traits. For seedling survival, block was significant in LP ( $p = 0.023$ ) but not in DF ( $p = 0.641$ ). For seedling size, block and the covariate were significant in both populations (all  $p < 0.013$ ). Relative self-performance of traits varied between 0.68–1.02 (Table 4). Mean cumulative relative performance did not differ between populations ( $F_{1,18} = 0.31$ ,  $p = 0.583$ ). Overall, selfed progeny were about 55% as fit as crossed progeny.

### Pollinator exclusion experiment

Plants from which we excluded pollinators produced fewer fruits than did open-pollinated plants ( $54.3\% \pm 7.9$  vs  $79.6\% \pm 5.9$ ;  $\chi^2 = 6.89$ , DF = 1,  $p = 0.009$ ). Seed set did not differ ( $75.5\% \pm 3.1$  vs  $75.0\% \pm 2.9\%$ ;  $F_{1,20} = 0.04$ ,  $p = 0.835$ ), but plants nested within treatment varied significantly ( $F_{20,75} = 2.73$ ,  $p = 0.001$ ). Relative reproductive output of plants from which pollinators were excluded was  $0.68 \pm 0.11$ .

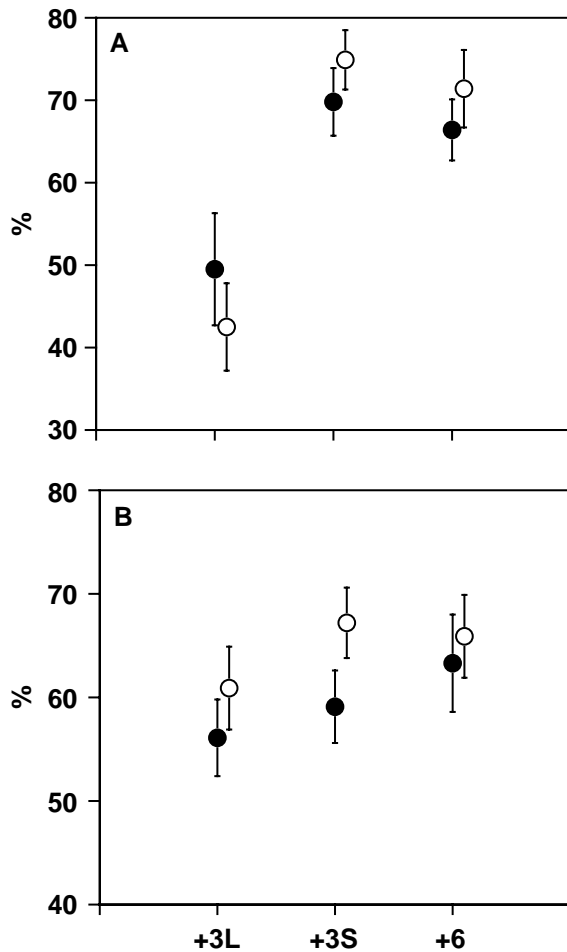


Fig. 1. Mean ( $\pm$ SE) percent fruit set (A) and seed set (B) when anthers from the three short stamens were removed (+3L), anthers from the three long stamens were removed (+3S) or all six anthers were left intact (+6). Filled and open circles represent the LP and DF populations of *Bulbine vagans*, respectively.

### Does emasculation affect pollinator visitation?

Visitation to intact and emasculated flowers was similar, but hairless flowers received fewer visits than did emasculated flowers (Table 6). Plots and days also differed, indicating that visitation varied both spatially and temporally.

Table 2. Results of an analysis of deviance ( $\chi^2$ ) and a split-plot ANOVA (F) examining the effects of removing the three short anthers (+3L), the three long anthers (+3S) or leaving all six anthers (+6) intact on fruit and seed set in *Bulbine vagans*. For the ANOVA, numerator and denominator degrees of freedom are given. Significant pairwise comparisons following Bonferroni correction are shown in bold. Data are given in Fig. 1.

Source	Fruit set			Seed set		
	DF	$\chi^2$	p	DF	F	p
Population	1	0.49	0.322	1,12	0.65	0.435
Plant (population)	12	23.36	0.025	12,189	4.50	<0.001
Removal	2	49.07	<0.001	2,189	1.57	0.211
+3L vs +3S	1	<b>32.11</b>	<b>&lt;0.001</b>	1,189	1.69	0.195
+3S vs +6	1	0.10	0.756	1,189	0.16	0.688
Removal $\times$ Pop	2	2.01	0.132	2,189	0.31	0.728
Error	24	14.96	0.922	189		

Although the treatment  $\times$  plot interaction, but not the treatment  $\times$  day interaction, was significant, hairless flowers always received fewer visits than did intact and emasculated flowers (intact,  $10.3 \pm 1.0$ ; emasculated,  $10.1 \pm 0.9$ ; hairless,  $4.7 \pm 0.4$  visits  $h^{-1}$  of observation). The duration of pollinator visits to intact and emasculated flowers did not differ (day:  $F_{1,156} = 2.12$ ,  $p = 0.147$ ; floral treatment:  $F_{1,156} = 0.001$ ,  $p = 0.985$ ; day  $\times$  floral treatment:  $F_{1,156} = 1.17$ ,  $p = 0.280$ ). Visits to intact and emasculated flowers lasted  $17.5 \pm 1.9$  and  $18.5 \pm 2.6$  seconds, respectively.

### Emasculation experiment

No pollen was observed in anthers of intact flowers just prior to their closing. Plants with emasculated flowers produced only 67% as many fruits and 76% as many seeds per fruit as did plants with intact flowers (Fig. 3, % fruit set:  $\chi^2 = 35.13$ ,  $DF = 1$ ,  $p = 0.004$ ; % seed set:  $F_{1,20} = 10.02$ ,  $p = 0.004$ ). For seed set, plants also varied significantly ( $F_{20,78} = 1.80$ ,  $p = 0.035$ ). Reproductive output, estimated as the proportion of ovules that set seeds in all treated plants (i.e. fruit set  $\times$  seed set), was  $0.732 \pm 0.046$  for intact plants but only  $0.373 \pm 0.063$  for emasculated plants. Using these values, reproductive assurance was 0.49.

### Discussion

Our study demonstrates that selfing can provide reproductive assurance in *Bulbine vagans*, but inbreeding depression erodes the magnitude of any benefit. These findings augment growing evidence that selfing mechanisms can elevate seed production in plant populations (Herlihy and Eckert 2002, Elle and Carney 2003, Kalisz and Vogler 2003, Moeller and Geber 2005; but see Eckert et al. 2006 for exceptions). In *B. vagans*, flowers can autonomously self-pollinate after opportunities for outcrossing, but also undergo pollinator-mediated selfing. Inbreeding depression was about 0.45, but would exceed 0.5 over the entire life cycle. Models predict that if inbreeding depression is less than 1.0, then delayed autonomous selfing can confer reproductive assurance (Lloyd 1979, 1992, Morgan et al. 1997). By contrast, the benefit of pollinator-mediated selfing will depend upon the extent that selfing usurps ovules that could otherwise be outcrossed as well as the degree that inbreeding depression exceeds 0.5. Below we discuss how autonomous selfing occurs in *B. vagans* and consider potential benefits and costs associated with delayed and pollinator-mediated selfing.

### Mechanism of delayed autonomous self-pollination

Both stamens and styles move to effect delayed autonomous selfing in *B. vagans*. When flowers of *B. vagans* open they are strongly herkogamous: stamens are clustered together on one side of a flower and styles are curved in the opposite direction. As flowers close, filaments wilt and styles straighten, bringing anthers and stigmas into contact. We observed negligible pollen loads on stigmas prior to flowers closing, indicating that herkogamy prevents autonomous selfing. Our anther removal experiment demonstrated that

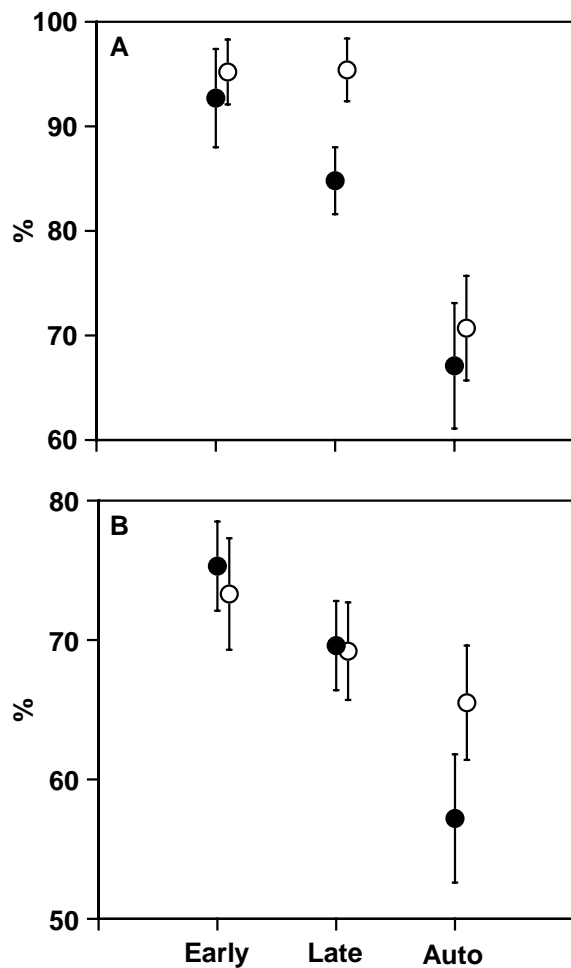


Fig. 2. Mean ( $\pm$ SE) percent fruit set (A) and seed set (B) following early selfing, late selfing and autonomous selfing in the LP (filled circles) and DF (open circles) populations of *Bulbine vagans*.

anthers of short stamens contribute more to autonomous selfing than do anthers of long stamens. Whether anthers of these stamens differ in pollen production or are otherwise specialised for different functions is unknown.

In the glasshouse, autofertility was substantial, but less than 1.0 in both populations (average, 0.67). The difference in reproductive output between autonomous selfing and

Table 3. Results of an analysis of deviance ( $\chi^2$ ) and a split-plot ANOVA (F) examining differences in fruit and seed set following early selfing, late selfing or autonomous selfing in *Bulbine vagans*. For the ANOVA, numerator and denominator degrees of freedom are given. Significant pairwise comparisons following Bonferroni correction are shown in bold. Data are given in Fig. 2.

Source	Fruit set			Seed set		
	DF	$\chi^2$	p	DF	F	p
Population	1	0.75	0.387	1,12	0.09	0.759
Plant (population)	12	15.00	0.242	12,192	4.87	<0.001
Pollination	2	24.19	<0.001	2,192	7.18	0.001
Early vs late	1	0.96	0.326	1,192	1.99	0.160
Late vs auto	1	<b>12.61</b>	<b>&lt;0.001</b>	1,192	<b>5.48</b>	<b>0.020</b>
Pollination $\times$ Pop	2	1.66	0.436	2,192	1.29	0.276
Error	24	20.68	0.657	192		

late manual selfing as flowers closed was probably caused by insufficient autonomous pollen deposition rather than substantial declines in pollen viability and stigma/ovule receptivity. By contrast, autofertility is near zero for the closely related *B. bulbosa*. In this species, flowers possess long stamens only, herkogamy is maintained throughout floral life and stigmas exhibit reduced receptivity as flowers close (Owen et al. 2007). Our autofertility estimate for *B. vagans* is intermediate to those taxa for which reproductive assurance has been quantified in previous studies. In these taxa, autofertility was not correlated with reproductive assurance as estimated using emasculation experiments (Eckert et al. 2006).

### Reproductive assurance

In the field, reproductive output of bagged plants was almost 0.70 that of open-pollinated plants, corroborating the high autofertility that we found in the glasshouse. These findings demonstrate that delayed selfing should be beneficial, ensuring seed production when outcrossing mates or pollinators are scarce or absent. Such conditions may be common in field populations of *B. vagans*. Populations occur in steep, rocky habitats and individual plants can be isolated. In addition, plants flower during late spring and summer when frequent storms cause pollinator inactivity. Because delayed selfing does not usurp pollen and ovules, reproduction is assured as long as some selfed seeds survive to reproduce (i.e. inbreeding depression,  $\delta < 1$ ), and the production of selfed seeds does not aggravate resource competition among developing seeds or affect subsequent adult survival or reproduction (Lloyd 1979, Lloyd and Schoen 1992, Harder and Routley 2006).

We compared emasculated and intact flowers under natural pollination conditions and found that outcross pollen receipt was limited and that selfing strongly boosted fruit and seed set. We emasculated flowers by carefully removing anthers without damaging the clavate hairs, filaments, stigma or tepals. Emasculation did not affect either the number or duration of visits by insect pollinators. Conversely, removal of both anthers and hairs reduced visitation by over 50%, indicating that hairs have some undetermined role in attracting pollinators. Thus, emasculation had no confounding effect on our estimates of reproductive assurance in *B. vagans*. Eckert et al. (2006, appendix 10.1) list 16 taxa for which reproductive assurance was quantified using emasculation techniques that did not damage flowers or reduce pollinator visits. Our estimate of reproductive assurance (0.49) was marginally greater than the median of these taxa, although individual estimates varied widely (0.31; range 0–0.97).

Our experiments do not allow us to assess the relative contributions of autonomous selfing or pollinator-mediated selfing to reproductive assurance (Herlihy and Eckert 2004). However, when we visually examined anthers as flowers closed all pollen had been removed, strongly indicating that autonomous selfing was unlikely during our study and that pollinators probably mediated selfing. Further, most selfing would have occurred within flowers because plants usually open only one flower day<sup>-1</sup> ( $1.4 \pm 0.3$ ;  $n = 185$  plants; Vaughton unpubl.), rendering

Table 4. Effects of self- or cross-pollination on performance in the DF and LP populations of *Bulbine vagans*. Ten maternal plants were examined from each population. Relative self-performances ( $rp = w_s/w_c$ ) are means ( $\pm$ SE) of the maternal plants from each population. Cumulative relative performance and inbreeding depression estimates include all traits except seed mass, and were estimated as the mean of the 10 maternal families.

Trait	DF			LP		
	Self	Cross	rp	Self	Cross	rp
Fruit set (%)	91.4 $\pm$ 3.1	89.8 $\pm$ 2.7	1.02 $\pm$ 0.97	88.5 $\pm$ 3.3	91.9 $\pm$ 3.0	0.97 $\pm$ 0.03
Seed set (%)	58.3 $\pm$ 2.6	65.1 $\pm$ 2.8	0.93 $\pm$ 0.05	56.3 $\pm$ 3.0	67.0 $\pm$ 2.5	0.82 $\pm$ 0.06
Seed mass (mg)	2.2 $\pm$ 0.1	2.5 $\pm$ 0.1	0.89 $\pm$ 0.04	2.3 $\pm$ 0.1	2.5 $\pm$ 0.1	0.95 $\pm$ 0.05
Seed germination (%)	58.0 $\pm$ 6.6	63.5 $\pm$ 5.8	0.99 $\pm$ 0.13	38.7 $\pm$ 6.1	59.3 $\pm$ 5.4	0.72 $\pm$ 0.15
Seedling survival (%)	61.8 $\pm$ 8.9	92.1 $\pm$ 5.5	0.68 $\pm$ 0.09	70.7 $\pm$ 8.9	82.0 $\pm$ 5.1	0.89 $\pm$ 0.12
Number of leaves	16.8 $\pm$ 0.8	19.8 $\pm$ 0.9	0.85 $\pm$ 0.04	15.7 $\pm$ 0.8	18.5 $\pm$ 0.9	0.87 $\pm$ 0.06
Cumulative RP			0.59 $\pm$ 0.14			0.51 $\pm$ 0.19
Inbreeding depression			0.41 $\pm$ 0.14			0.49 $\pm$ 0.19

geitonogamy unlikely. We propose that delayed selfing provides reproductive assurance when pollinators are absent, but when active, they mediate facilitated selfing.

### Does facilitated selfing provide reproductive assurance?

Whether facilitated selfing provides reproductive assurance will depend upon inbreeding depression, selfing rate and the proportion of ovules usurped by selfing that could otherwise be outcrossed. Inbreeding depression in both populations was about 0.45, but this is an underestimate because we examined early life stages in a glasshouse rather than the entire life cycle under natural conditions (Hayes et al. 2005). We estimated a minimal selfing rate of 0.50 from the emasculated plants that only outcross and intact plants that both outcross and self (i.e. 1-emasculated/intact). This assumes that the reproductive output of emasculated plants represents the maximum outcrossing rate and that facilitated selfing only fertilizes ovules that are not outcrossed. If facilitated selfing usurps ovules, then the actual selfing rate of intact plants could exceed 0.5. Selfing rates now need to be verified using genetic markers.

Inspired by Cheptou and Schoen (2007), we estimate the amount of reproductive assurance provided by facilitated selfing as the number of gene copies transmitted to the next generation rather than the numbers of seeds. Counting gene copies allows the effects of the selfing transmission advantage and inbreeding depression to be incorporated into an estimate of reproductive assurance. The number of gene copies transmitted to the next generation by maternal

plants can be estimated as:  $sn2(1 - \delta) + (1 - s)n$ , where  $s$ ,  $\delta$  and  $n$  are the selfing rate, inbreeding depression and ovule number per plant, respectively. The left and right hand parts of this equation represent the number of genes transmitted by selfing and outcrossing, respectively, and recognises that parents provide two gene copies for selfed seeds but only one copy for outcrossed seeds (Fisher 1941). We omit a term for siring outcrossed seeds and assume that pollen export is independent of facilitated selfing (i.e. pollen discounting = 0; but see Porcher and Lande 2005, Harder and Routley 2006). If  $0.5 < \delta < 1.0$ , then the gene advantage of selfing is negated but reproductive assurance is provided, although the benefit is eroded as  $\delta$  increases. If  $\delta = 1.0$ , then reproductive assurance is also negated.

In *B. vagans*, inbreeding depression probably exceeds 0.5, and if  $\delta < 1.0$ , then facilitated selfing would confer maximal reproductive assurance, providing that selfing augments outcrossing and does not usurp ovules. Findings from our emasculation experiment indicate that this should occur when  $s = 0.5$ . If, however, ovules are usurped (i.e.  $s > 0.5$ ), such that fewer outcrossed seeds are produced, then the benefit of reproductive assurance would be eroded, even though similar numbers of seeds are produced. This is a consequence of more selfed seeds, which are less fit than outcrossed seeds, being produced.

Whether the combination of delayed selfing, facilitated selfing and outcrossing in *B. vagans* is evolutionarily stable depends upon the variability in outcross pollen receipt and requires further study. The stability of mixed mating requires that the cost of selfing is sufficiently high to negate the gene transmission advantage, but is sufficiently low to

Table 5. Values for analyses of deviance ( $\chi^2$ ) and two-way ANOVAs (F) examining the effects of self- or cross-pollination and maternal family on plant traits in the DF and LP populations of *Bulbine vagans*. For analyses of deviance, degrees of freedom were: 1 for pollination; and 9 for family and pollination  $\times$  family interactions. For ANOVAs, degrees of freedom were: 1,9 for pollination; 9,9 for family; and between 9,56 and 9,80 for the pollination  $\times$  family interactions. Data are given in Table 4.

Source	Fruit set <sup>a</sup>		Seed set <sup>b</sup>		Seed mass <sup>b</sup>		Seed germination <sup>a</sup>		Seedling survival <sup>a</sup>		Seedling size <sup>b</sup>	
	DF	LP	DF	LP	DF	LP	DF	LP	DF	LP	DF	LP
Pollination	0.06	0.78	3.32 <sup>†</sup>	10.05 <sup>*</sup>	6.99 <sup>*</sup>	2.38	0.58	7.96 <sup>**</sup>	22.35 <sup>***</sup>	12.95 <sup>***</sup>	9.30 <sup>*</sup>	5.61 <sup>*</sup>
Family	8.67	16.54 <sup>*</sup>	3.80 <sup>*</sup>	6.65 <sup>**</sup>	1.82	5.10 <sup>*</sup>	16.68 <sup>*</sup>	9.79	39.98 <sup>***</sup>	11.83	3.21 <sup>*</sup>	1.64
Poll $\times$ Family	–	–	0.91	1.27	2.04 <sup>*</sup>	1.08	–	–	17.65 <sup>*</sup>	16.23 <sup>†</sup>	0.70	1.33

<sup>†</sup>  $p \leq 0.10$ ; <sup>\*</sup>  $p \leq 0.05$ ; <sup>\*\*</sup>  $p < 0.01$ ; <sup>\*\*\*</sup>  $p < 0.001$

<sup>a</sup>  $\chi^2$ -value

<sup>b</sup> F-value

Table 6. Results of a split-plot ANOVA examining differences in the number of visits to intact, emasculated or emasculated+hairless flowers in *Bulbine vagans*. Numerator and denominator degrees of freedom are given. Significant pairwise comparisons following Bonferroni correction are shown in bold. Data are given in text.

Source	DF	F	p
Plot	1,2	35.88	0.024
Day	2,2	33.09	0.036
Plot × Day	2,154	0.52	0.594
Floral treatment	2,4	18.30	0.009
intact vs emasculated	1,4	<1.0	0.996
emasculated vs hairless	1,4	<b>26.75</b>	<b>0.006</b>
Floral treatment × Plot	2,154	4.97	0.008
Floral treatment × Day	4,154	1.78	0.135
Error	154		

allow reproductive assurance (i.e.  $0.5 < \delta < 1.0$ ). Two detailed studies have documented that variation in cross-pollination favours reproductive assurance, but that paradoxically mixed mating is probably unstable. In *Collinsia verna*, selfing rates were lower than expected considering that inbreeding depression was very weak ( $s = 0.73$ – $1.0$ ,  $\delta < 0.1$ ; Kalisz and Vogler 2003, Kalisz et al. 2004). By contrast, the high selfing rates in *Aquilegia canadensis* ( $s > 0.6$ ) were inconsistent with the very strong inbreeding depression ( $\delta > 0.9$ ; Herlihy and Eckert 2002, 2004, Eckert and Herlihy 2004). These surprising findings underscore the need for further experimental analyses in a spectrum of plants to better understand the role of reproductive assurance in the evolution of mixed mating (Eckert et al. 2006).

In conclusion, *B. vagans* exhibits a flexible best-of-both-worlds strategy, in which selfing provides reproductive assurance when cross pollen is limited, resulting in mixed mating (Becerra and Lloyd 1992, Kalisz and Vogler 2003). We demonstrated that delayed selfing can occur, but that under the natural pollination conditions of our study pollinators mediated selfing. We propose that facilitated selfing can confer reproductive assurance, if selfing does not usurp ovules such that outcrossing is reduced. We are presently examining reproductive assurance under a wider

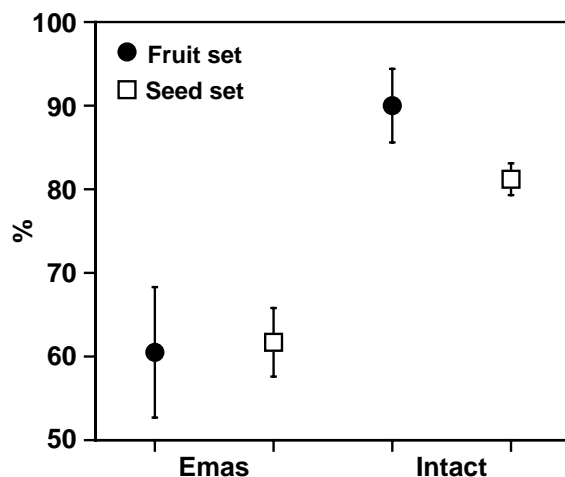


Fig. 3. Mean ( $\pm$ SE) percent fruit set and seed set of emasculated and intact plants in the LP population of *Bulbine vagans*.

range of ecological conditions to assess how outcrossing and delayed vs facilitated selfing contribute to plant mating.

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