

Delayed selfing and low levels of inbreeding depression in *Hibiscus trionum* (Malvaceae)

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Abstract. We used experimental pollinations to examine the breeding system and inbreeding depression in *Hibiscus trionum* L., an annual herb of cultivated and disturbed sites in eastern Australia. Seeds were not produced asexually. Flowers were fully self-compatible and autonomously self-pollinating. Autonomous self-pollination was due to a delayed selfing mechanism that gave precedence to cross pollen but ensured that stigmas contacted the anthers at the end of floral life. Using selfed and crossed progeny from 10 maternal plants, we examined the magnitude and timing of inbreeding depression over a range of life-cycle stages, including seed production by maternal plants, and seed germination, seedling growth, survival, flowering and seed production by F₁ plants. Average cumulative inbreeding depression was 0.15, although there was considerable variation among maternal families ($\delta = -0.07$ – 0.43). Inbreeding depression was not uniform across all life stages and was most prevalent late in the life cycle, affecting days to flowering and fruiting and flower production. Our results indicate that major lethal alleles have been purged from this population, probably through high levels of recurrent self-fertilisation. The flexibility in plant mating afforded by delayed selfing is likely to contribute to the invasiveness of *H. trionum*, particularly in annual cropping situations.

Introduction

Plant breeding systems represent the sum of sexual characteristics that directly influence the proportion of selfed and outcrossed progeny in the next generation (Richards 1997). Breeding system characteristics have a variety of demographic and genetic effects on populations. For example, in self-compatible species autonomous self-pollination can ensure a population's reproductive output in the absence of pollinator visits (Motten 1982; Rathcke and Real 1993; Fishman and Wyatt 1999). The benefits of reproductive assurance are considered to be important in selecting for autonomous selfing in annual herbs and colonising species (Baker 1955; Stebbins 1957; Lloyd 1979, 1992). However, persistent self-fertilisation increases homozygosity, which may reduce genetic variation and limit the ability of populations to adapt to changing environmental conditions (Silvertown *et al.* 2002). In addition, selfing may result in inbreeding depression, reducing the fitness of selfed progeny compared with outcrossed progeny (Charlesworth and Charlesworth 1987). Inbreeding depression may limit the growth and viability of populations by having an impact on critical demographic processes, especially when populations are small (Ellstrand and Elam 1993; Newman and Pilson 1997; Keller and Waller 2002).

Inbreeding depression coevolves with the frequency of self-fertilisation in populations (Lande and Schemske 1985; Schemske and Lande 1985; Barrett and Eckert 1990). The most important cause of inbreeding depression is considered to be the expression of deleterious recessive mutations in individuals made homozygous by inbreeding (Charlesworth and Charlesworth 1987). Because genetic load is likely to be substantial in primarily outcrossing populations, strong inbreeding depression is expected following selfing. However, with enforced selfing due to pollinator failure or population bottlenecks, deleterious alleles are expressed and may be purged from populations by selection (Barrett and Charlesworth 1991). Purging is expected to decrease the severity of inbreeding depression and should be most efficient at reducing the frequency of major lethal alleles affecting early life-cycle stages. In support of this view, a survey by Husband and Schemske (1996) showed that primarily selfing populations had lower levels of inbreeding depression than did primarily outcrossing populations. Their study also revealed a difference in the timing of inbreeding depression during the life cycle. Selfers typically show effects during later life-cycle stages, whereas outcrossers exhibit effects early in the life cycle. On the other hand, Byers and Waller (1999) surveyed inbreeding depression in populations that differed in inbreeding history and found

only limited evidence of purging, with some species showing high inbreeding depression for early traits in selfing populations.

The evolution of autonomous selfing in populations depends on the timing of selfing relative to outcrossing. Unlike prior and competing selfing, delayed selfing occurs after opportunities for outcrossing (Lloyd and Schoen 1992). Because ovules are not usurped by self-pollen, delayed selfing allows opportunities for outcrossing when conditions are suitable but ensures seed production when mating partners or pollinators are absent or limited. Delayed selfing is expected to evolve even when levels of inbreeding depression are high because ovules that are not outcrossed would otherwise remain unfertilised (Lloyd 1979, 1992). Depending on the extent of cross-pollination, plants with delayed selfing produce a mixture of selfed and outcrossed progeny, resulting in a mixed mating system. Delayed selfing is not uncommon and has been reported in a wide variety of species (e.g. *Mimulus guttatus*, Dole 1990, 1992; *Kalmia latifolia*, Rathcke and Real 1993; *Aquilegia canadensis*, Eckert and Schaefer 1998; *Collinsia verna*, Kalisz *et al.* 1999).

Hibiscus trionum var. *trionum* L. (Malvaceae) is a non-endemic summer-growing annual herb of cultivated and disturbed sites (Harden 2000). Although plants maintain large showy flowers that are attractive to cross-pollinating insects, flowers are capable of delayed selfing. The stylar branches progressively reflex from an upright position when flowers first open to an inverted position at the end of floral life, ensuring that the stigmas contact the anthers (Buttrose *et al.* 1977; M. Ramsey, pers. obs.). These observations suggest a mixed mating system, although levels of self-compatibility, autonomous self-pollination and inbreeding depression have not been examined previously. Knowledge of these factors may help us to better understand the population biology of this weedy species and provide insight into the evolution of delayed selfing.

Here we use experimental hand-pollinations to investigate the breeding system and inbreeding depression in *H. trionum*. We first examine whether plants are capable of asexual seed production and the extent to which plants are self-compatible and capable of autonomous self-pollination. We then examine the performance of selfed and outcrossed progeny across a range of life-cycle stages to determine the magnitude and timing of inbreeding depression.

Methods

Study species

Plants of *Hibiscus trionum* var. *trionum* (bladder ketmia) have a sprawling habit and can grow up to 1 m in height under good conditions. Flowers are large (c. 45 mm in diameter) and have showy petals that are pale in colour with a dark crimson centre. At our study site, introduced honeybees visit flowers (M. Ramsey, pers. obs.). The numerous

stamens are fused to form a tube that is united to the base of the corolla. There are five prominent stylar branches with capitate stigmas (Harden 2000). Flowers open in the morning and close by mid-afternoon. The stylar branches recurve during the day so that by early afternoon the stigmas contact the anthers (Buttrose *et al.* 1977). Fruits are globular capsules with many seeds. Dormancy is enforced by a hard seed coat.

Breeding system

We established a bulk seed collection by harvesting one fruit from approximately 50 plants growing in a cultivated paddock near Armidale, NSW (30°30'S, 151°39'E, 1054 m a.s.l.). Seeds were stored for about 12 months in paper bags in the laboratory. We nicked the dry seeds in the chalazal region with a scalpel to break the seed-coat imposed dormancy. Fifteen plants were established by planting nicked seeds in soil-filled pots. Pots were placed in a glasshouse and plants were fertilised every 3 weeks with liquid fertiliser (Aquasol) and were watered regularly.

Flowers on each plant were randomly allocated to one of following pollination treatments: hand-crossing, hand-selfing, autonomous selfing (i.e. autofertility) and agamospermy. Pollination treatments were randomised in time. Two plants were attacked by whitefly and were omitted from the experiment. In each treatment, the average number of flowers per plant was 7.0 (range 2–10). There were a total of 110, 81, 124 and 47 flowers in the crossed, selfed, autofertility and agamospermy treatments, respectively. Cross-pollinated flowers received pollen from one to three other plants. Self-pollinated flowers received pollen from the same flower. Flowers in the autofertility treatment were left untouched. During the experiment, the glasshouse was free of pollinating insects and any seeds in the autofertility treatment resulted from autonomous self-pollination. In the agamospermy treatment, we emasculated flowers by removing the anthers. Treatments were carried out in the first 1–2 h after flowers opened. Mature fruits were harvested about 5 weeks later and seeds were counted.

We used a two-way ANOVA to compare the number of seeds per fruit among pollination treatments. Pollination treatment and maternal plant were considered fixed and random factors, respectively. The agamospermy treatment was excluded from analyses because no fruits were produced (see Results).

Inbreeding depression

Ten of the above plants were also used to generate selfed and crossed seeds to assess inbreeding depression. In addition to the flowers pollinated for the breeding-system experiment, we randomly allocated a further 10 flowers per plant to either a cross-pollination or an autofertility treatment; the breeding system analysis indicated that seed production did not differ significantly between the hand self-pollination and autofertility treatments (see results). Pollinations were performed as described above and seeds were counted.

For each maternal plant, we pooled fruits for each pollination treatment separately (i.e. 10 family lines per treatment). About 2 months later, we examined seed mass by individually weighing 20 selfed and 20 crossed seeds from each family to the nearest 0.1 mg. We nicked seeds and placed them in Petri dishes on germination pads moistened with distilled water. Dishes were positioned in a germination cabinet with an alternating temperature regime of 25 and 15°C and a corresponding 12 h light and 12 h dark photoperiod. These germination conditions would be similar to those experienced under natural field conditions. The number of seeds germinating each day was recorded until germination was complete or seeds had decayed; 99% of seeds germinated within 3 days. Germination was defined as emergence of the radicle from the testa.

Two days after seeds had germinated, 10 selfed and 10 crossed seedlings from each family were randomly chosen and planted into separate 460-cm³ pots with a 1:1:1 mixture of sand, loam and peat. Pots were randomly placed into two separate family trays, each containing five selfed and five crossed progeny. Trays were then assigned to random positions on glasshouse benches and were reassigned every 2–3 days to avoid the effects of micro-environmental variation on seedling growth. Because we used two trays per family and regularly reassigned tray positions, differences among families should reflect genetic differences rather than tray or bench position effects. Plants received 8 h per day supplementary lighting (Osram HQI-R 250W/NDL metal halide lamps) until they were 20 days old. Pots were regularly watered and were fertilised twice with 25 mL of Aquasol.

When seedlings were 17 days old, we measured the length and width of the first leaf that was fully expanded. Length was measured along the mid-vein from the base of the lamina to the tip and width was measured at the widest point. We calculated leaf area as

$$A = \pi(L/2 \times W/2),$$

where L = leaf length and W = leaf width. When seedlings were 21 days old, we measured seedling height as the distance from the soil surface to the first leaf. The number of plants surviving to flowering was recorded.

For each plant, we recorded the date that the first flower opened and left these flowers untouched to assess autofertility. The number of ovules in the second flower was determined by dissecting immature fruits and counting ovules under a stereo microscope. The mass of the third flower was determined after drying at 65°C for 6 days. The date that the first fruit matured was recorded when the dried capsule began to split open. We harvested these fruits and counted the number of seeds in each. When plants were 78 days old, we counted the total number of open flowers per plant and determined above-ground mass after drying plants at 65°C for 14 days.

We used two-way ANOVAs to compare traits between the two pollination treatments. Pollination and maternal family were considered fixed and random factors, respectively. Because equal or almost equal numbers of progeny germinated and survived in each treatment, we did not statistically analyse these traits.

We estimated the relative performance of selfed progeny for all traits by using the means of the 10 maternal plants. For most traits, we calculated population level relative self performance as

$$RP = w_s/w_c,$$

where w_s and w_c are the mean performances of selfed and crossed progeny, respectively. Higher values represent better performance for most traits (e.g. number of seeds), but for traits such as days to flowering and fruiting, higher values generally represent poorer performance. To address this, we calculated relative self-performance for these traits as $RP = w_c/w_s$ (Ramsey and Vaughton 1996).

To estimate inbreeding depression for individual maternal families we used a subset of the above traits, including the number of seeds per fruit for the maternal plants and for the F₁-selfed and -crossed progeny, percentage seed germination, percentage survival to flowering, flower number and seed number per fruit. These traits were chosen because they are related to overall fitness and are probably independent from one another. Relative performance for each maternal family was calculated as described above. Cumulative relative performance was calculated for each maternal family as the product of the relative performances of the above five traits. Inbreeding depression was then calculated as

$$\delta = 1 - W_s/W_c,$$

where W_s/W_c is the cumulative relative performance (Charlesworth and Charlesworth 1987).

Statistical analyses

In all analyses, F -ratios for the effect of pollination and the effect of maternal plant or family were calculated with the interaction mean square and the error mean square, respectively, as denominators. The conclusions of the study would be unaltered if the non-significant interactions were pooled with the error terms. Flower and plant masses were ln-transformed and days to first flower and days to first fruit were square-root transformed. Transformations improved homoscedasticity and normality. Other traits did not require transformation. Means (\pm s.e.) are presented. We used the statistical package JMP (version 5, SAS Institute Inc.) for all analyses.

Results

Breeding system

No fruits were produced in the agamospermy treatment, indicating that plants are unable to produce seeds asexually. In the other pollination treatments all flowers set fruits. For seed number per fruit, a significant treatment \times plant interaction indicated that the effect of pollination treatment varied among maternal plants ($F_{22, 279} = 2.10$, $P = 0.003$). Variation among maternal plants was also significant ($F_{11, 279} = 2.05$, $P = 0.024$), but there was no overall effect of pollination treatment on seed number per fruit ($F_{2, 22} = 0.32$, $P = 0.726$). The mean number of seeds per fruit was 22.7 ± 0.7 , 24.0 ± 1.1 and 24.4 ± 0.8 in the autofertility, hand self-pollination and hand cross-pollination treatments, respectively.

Inbreeding depression

Maternal families differed significantly for all traits, indicating genetic variation among plants in the population (Tables 1, 2). For several traits, a significant pollination \times family interaction indicated that the effect of selfing v.

Table 1. Performance following self- and cross-pollinations across a range of life-cycle stages in *Hibiscus trionum*

Data are means \pm s.e., pooled over 10 maternal families. Relative self-performance (RP) was calculated as the ratio of self:cross-performance, except for days to first flower and first fruit, which were calculated as the ratio of cross:self-performance

Trait	Self	Cross	RP
Maternal plant			
Seeds per fruit	23.3 \pm 0.8	24.6 \pm 0.9	0.95
F ₁ progeny			
Seed mass (mg)	4.69 \pm 0.03	4.75 \pm 0.03	0.99
Germination (%)	99.5 \pm 0.5	100.0 \pm 0.0	1.0
Area of first leaf (cm ²)	3.40 \pm 0.09	3.59 \pm 0.10	0.95
Seedling height (cm)	4.01 \pm 0.06	4.11 \pm 0.06	0.98
Survival to flowering (%)	98.0 \pm 1.3	98.0 \pm 1.3	1.0
Days to first flower	42.8 \pm 0.3	41.6 \pm 0.3	0.97
Flower mass (mg)	39.0 \pm 0.6	38.7 \pm 0.5	1.01
Ovules per flower	48.7 \pm 0.2	48.3 \pm 0.3	1.01
Days to first fruit	72.4 \pm 0.2	70.9 \pm 0.2	0.98
Seeds per fruit	41.4 \pm 0.5	42.3 \pm 0.4	0.98
Flowers per plant	6.2 \pm 0.1	6.8 \pm 0.1	0.91
Plant mass (g)	4.39 \pm 0.08	4.53 \pm 0.08	0.97

crossing varied among maternal families. Pollination treatment was only significant for the later life-cycle stages of days to first flower, days to first fruit and flower number (Tables 1, 2). Days to first flower and days to first fruit were on average 2–3% more rapid for crossed progeny than for selfed progeny. Not unexpectedly, these traits were correlated (Pearson correlation: $r = + 0.74$, d.f. = 194, $P < 0.001$). For flower number, crossed progeny produced on average 9% more flowers than did selfed progeny. Population-level relative performances for these three traits varied from 0.91 to 0.98 (Table 1).

For the five fitness-related traits that we examined at the individual family level, only flower number showed consistent superior relative performance of crossed progeny (all family RP < 0.98; Table 3). Relative performance for seeds per maternal plant showed the greatest variability, with individual values ranging from 0.72 to 1.21. Relative performances for the other traits were less variable and were generally ≤ 1.0 . Inbreeding depression values for the

10 maternal families ranged from -0.07 to 0.43 (Table 3). Mean inbreeding depression was low, but was significantly greater than zero (0.15 ± 0.05 ; one-tailed t -test, $t_9 = 2.91$, $P = 0.009$).

Discussion

Our study shows that *H. trionum* is fully self-compatible and autonomously self-pollinating, but because autonomous selfing is delayed, flowers have the capacity to be cross-pollinated. Seed set following autonomous self-pollination was equivalent to that resulting from manual cross- and self-pollination and would ensure seed production in the absence of mating partners or pollinator visits. In *H. trionum*, delayed autonomous selfing is accomplished by the progressive recurvature of the stylar branches, such that at the end of floral life the stigmas contact the anthers. Interestingly, this recurvature may be facultative. Buttrose *et al.* (1977) examined clones of a single genotype and found that if stigmas were pollinated when flowers first opened,

Table 2. Results of two-way ANOVAs showing the effects of pollination, maternal family and their interaction on plant traits in *Hibiscus trionum*
* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Trait	Pollination		Family		Pollination \times family	
	d.f.	<i>F</i> -value	d.f.	<i>F</i> -value	d.f.	<i>F</i> -value
Maternal plant seeds per fruit	1, 9	1.06	9, 180	2.74 **	9, 180	1.18
Seed mass	1, 9	0.84	9, 380	16.69***	9, 380	3.22***
Area of first leaf	1, 9	1.24	9, 177	6.31***	9, 177	2.86**
Seedling height	1, 9	1.09	9, 177	11.69***	9, 177	3.05**
Days to first flower	1, 9	10.11*	9, 177	2.45*	9, 177	1.18
Flower mass	1, 9	0.07	9, 177	9.19***	9, 176	1.92
Ovules per flower	1, 9	0.23	9, 177	3.64***	9, 176	2.93**
Days to first fruit	1, 9	15.20 **	9, 176	7.01***	9, 176	2.33*
F ₁ progeny seeds per fruit	1, 9	2.02	9, 176	3.46***	9, 176	1.14
Flowers per plant	1, 9	54.96***	9, 176	9.26***	9, 176	0.56
Plant mass	1, 9	1.08	9, 176	8.29***	9, 176	1.31

Table 3. Relative self-performances, cumulative performance and inbreeding depression in *Hibiscus trionum*

Cumulative performance (W_S/W_C) was calculated as the product of the relative self-performances of the five fitness traits measured on each of 10 maternal families. Inbreeding depression was calculated as $1 - W_S/W_C$. Mean (\pm s.e.) inbreeding depression was 0.15 ± 0.05

Family	Maternal plant seeds per fruit	Germination	Survival to flowering	No. of flowers	F ₁ progeny seeds per fruit	Cumulative performance	Inbreeding depression
1	1.07	1.00	0.90	0.89	0.88	0.75	0.25
2	1.19	1.00	1.00	0.94	0.96	1.07	-0.07
3	0.86	1.00	1.10	0.90	0.93	0.79	0.21
4	1.03	1.00	1.00	0.93	1.04	0.99	0.01
5	0.80	0.95	1.00	0.98	0.93	0.69	0.31
6	0.89	1.00	1.10	0.93	0.99	0.90	0.10
7	1.21	1.00	1.00	0.88	0.98	1.04	-0.04
8	0.91	1.00	1.00	0.88	1.01	0.81	0.19
9	0.72	1.00	0.90	0.88	1.00	0.57	0.43
10	0.95	1.00	1.00	0.93	1.01	0.89	0.11

then movement of the stylar branches was prevented. Prevention of stylar movement would ensure that if cross pollen was deposited onto the stigma, then ovules would be cross-fertilised. Overall, these results indicate that *H. trionum* has a very flexible mating system.

Autofertility has been reported in the perennial herb *H. laevis* and occurs by a similar mechanism as in *H. trionum*, although fewer seeds result from autonomous pollination than from manual self-pollination (Klips and Snow 1997). In contrast, the perennial herb *H. moscheutos* is self-compatible but lacks the ability to autonomously self-pollinate (Spira 1989). Autofertility is a notable feature of many weedy species (Baker 1974). The advantages of autofertility include the ability of a single immigrant to establish a seed-producing population and the capacity of a single plant to regenerate a population when weed-control measures reduce population size. However, the ability to cross-pollinate is also considered advantageous because occasional outcrossing maintains genetic diversity within populations. Plants of *H. trionum* were fully autofertile but our study population maintained substantial genetic variation as evidenced by variation in fitness-related traits among maternal families. Genetic diversity is considered a critical factor in determining whether weedy invasive species are able to adapt and become established in new habitats (Sakai *et al.* 2001).

As well as reducing genetic variation, another potential disadvantage of self-fertilisation is inbreeding depression. We examined inbreeding depression in *H. trionum* by assessing the fitness of selfed and crossed progeny at a variety of life-cycle stages and found evidence of superior performance of crossed progeny. The average level of inbreeding depression over all life-cycle stages examined was 0.15, which is similar to that reported for predominantly selfing populations ($\delta = 0.23$; Husband and Schemske 1996). In contrast, predominantly outcrossing populations tend to have higher overall levels of inbreeding depression ($\delta = 0.53$; Husband and Schemske 1996). Our results for *H. trionum* indicate that most individuals have been largely purged of deleterious recessive alleles, probably as a result of recurrent bouts of self-fertilisation, perhaps during past colonisation events or population bottlenecks. Purging in *H. trionum* appears to have been efficient at eliminating major lethal alleles acting at early life-cycle stages as has been found for predominantly selfing species (Husband and Schemske 1996). In *H. trionum*, we detected inbreeding depression at some later life-cycle stages. This is consistent with the theory predicting that mildly deleterious alleles acting at later life-cycle stages are difficult to purge, even when levels of selfing are high (Husband and Schemske 1996; Byers and Waller 1999; Keller and Waller 2002).

Inbreeding depression in *H. trionum* was most pronounced for flower number and on average crossed progeny produced 9% more flowers than did selfed progeny.

Crossed progeny also produced their first flower and fruit earlier than did selfed progeny, although differences between pollination treatments were only in the order of 2–3%. Flower number is likely to be highly correlated with fitness in selfing annual species such as *H. trionum*, while individuals that reproduce more quickly are likely to have an advantage in environments where the timing of disturbance is unpredictable. Studies of inbreeding depression in other *Hibiscus* species have only considered early life-cycle stages. In both *H. laevis* and *H. moscheutos*, inbreeding depression was found for early seedling growth. Similar to our results for *H. trionum*, seed production and seed germination were unaffected by pollination type (Snow and Spira 1993; Klips and Snow 1997).

We found considerable variation in the magnitude of inbreeding depression among maternal families. One family had relatively high inbreeding depression ($\delta = 0.43$), while in three families inbreeding depression was absent ($\delta = -0.07, -0.04, 0.01$). Similar among-family differences in the magnitude of inbreeding depression were detected in *H. moscheutos* (Snow and Spira 1993) and such variation is a common feature of other inbreeding-depression studies (Dudash 1990; Byers and Waller 1999). Variation in the level of inbreeding depression among maternal families may reflect differences in the history of selfing and opportunities for purging among families that may be related to variation in floral traits. For example, in *Ipomoea purpurea* and *Gilia achilleifolia*, among-plant variation in stigma-anther distance influences selfing rates, which in turn affects levels of inbreeding depression (Chang and Rausher 1999; Takebayashi and Delph 2000). In *H. trionum*, variation in the extent or speed of stylar movement may have an impact on levels of autonomous selfing that could contribute to variation in inbreeding depression among families. Alternatively, variation in inbreeding depression among families may be the result of past mutational history rather than selection (Schultz and Willis 1995).

Although we examined performance of selfed and crossed progeny at a range of life-cycle stages, we may have nevertheless underestimated inbreeding depression because we did not examine lifetime seed production. The timing of senescence varies among *H. trionum* plants growing under glasshouse conditions and if this variation impacts on lifetime seed production and is related to the degree of inbreeding, then actual levels of inbreeding depression may be higher than our results indicate. Also our study was conducted under non-competitive conditions in the glasshouse and it is possible that the magnitude of inbreeding depression may be greater under more stressful or competitive conditions in the field (Dudash 1990; Ramsey and Vaughton 1998; Cheptou *et al.* 2000). Finally, we did not perform crosses among populations to assess the possibility of heterosis. Inbreeding resulting from population isolation and genetic drift can result in deleterious alleles becoming

fixed in populations so that only progeny from crosses among populations show increased fitness (Keller and Waller 2002).

In conclusion, the delayed selfing mechanism in *H. trionum* is likely to result in considerable mating flexibility and may have contributed to the invasiveness of this species in cropping systems of eastern Australia and elsewhere. Besides increasing the likelihood of successful colonisation of new habitats and ensuring high seed production, the delayed selfing allows outcrossing to occur when conditions are suitable, maintaining genetic diversity within populations. We found no differences in performance between selfed and crossed progeny at early life-cycle stages and the effects of inbreeding depression at later stages were small in magnitude. Because overall levels of inbreeding depression were low, it seems unlikely that this factor will limit population growth or persistence if plants are forced to self-fertilise.

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