Theor Appl Genet (2001) 102:375–378

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Geitonogamy: a mechanism responsible for high selfing rates in borage *(Borago officinalis* L.)

Received: 23 March 2000 / Accepted: 26 May 2000

Abstract Borage, a species traditionaly defined as allogamous, has revealed a high selfing rate although a mechanism of protandry has been confirmed in this plant. Studies investigating flower behaviour showed that several flowers open every day and that others are also receptive at the same time within a plant. Moreover, pollinator behaviour, mainly by bees, contributes to the selfing rate because it is demonstrated that these insects visit several flowers in a given plant before flying to other plants. The ecological studies reported here reveal the contribution of geitonogamous pollination to the high selfing rate detected in *Borago officinalis* L.

Keywords Borago officinalis · Geitonogamy · Selfing rate

Introduction

Borage (*Borago officinalis* L.) is an allogamous plant with an entomophilous pollination system. Bees, mainly, attracted by nectar, take pollen grains and scatter them on to neighbouring flowers (Alvarez and Villa 1992). Borage flowers are produced on scorpiod cymes. The cymes arise from the axils of the leaves at intervals on the stem. A plant has multiple stems that appear equally dominant. Each plant has a massive floral display and flowers are organised in inflorescences. Flowering proceeds basipetally in the inflorescence and each inflorescence develops several flowers. Two flower colours are found in borage. Wild genotypes have blue flowers whereas those cultivated in Spain have white ones. The flowers are continuously produced and mature over an extended period on time. The hermaphrodite, symetrical, and drooped flowers rise on a long pedicel. Flower structure consists of a calyx made up of five separate green sepals; a rotate corolla with a nectary in each of the five fused petals; an erect cone of five black (brown) stamens each born on a filament with a subtending purple flange, and a green, flat, four-lobed gynoecium with four ovules of axile placentation, a short style and a flat terminal stigma (Qinn et al. 1987).

Janick et al. (1989) refer to borage as a protandrous plant with pollen shed from the introrse anthers 12–24 h after flower opening, followed by the widening and flattening of the papillose-surfaced stigma 12–24 h after dehiscence in their environmental conditions.

As indicated by Snow et al. (1995), Darwin (1876) reported that the potential for selfing is greatest in species with a massive floral display, because having many flowers promotes the transfer of self-pollen to other flowers on the same genetic individual. This mechanism is called geitonogamy.

Several authors have suggested that geitonogamy leads to high rates of self-fertilization in self-compatible species, and reduces reproductive success in those that are self-incompatible (de Jong et al. 1993; Klinkhamer and de Jong 1993). In contrast, others have argued that the costs associated with geitonogamy are relatively minor because pollinators rarely visit more than a few flowers per plant, and pollen carryover allows some outcross pollen to reach most stigmas of these flowers (Geber 1985; Robertson 1992). The extent of geitonogamous pollination undoubtedly varies a great deal within and between species, depending on factors such as daily flower number (Stephenson 1982), plant densities (Klinkhamer and de Jong 1993) and pollinator behaviour (Schmitt 1980).

Although the breeding system in borage was originally reported as polygenic post-zygotic self-incompatibility (Crowe 1971), we recently demonstrated that borage is a self-compatible species (Montaner et al. 2000). If borage is self-compatible and its behaviour in Nothern latitudes is actually protandrous, it is of value to assess the influence of possible geitonogamy on the selfing rates of this massive floral-display species.

Communicated by H.C. Becker

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The aim of this work was to determine whether borage is a protandrous species under our conditions, to determine how daily flower number, floral longevity and pollinator behaviour affect geitonogamy, to assess the selfing rate in this species and how geitonogamy influences the reproductive success.

Materials and methods

Plant material

Two borage populations supplied by the Germplasm Bank of Zaragoza (Spain) were used:

- a wild blue-flowered population from Sweden, named BGHZ362,
- a local white-flowered population from a farmer of Luceni (Zaragoza)

A sample of each population was self-pollinated over four generations. In both cases we observed that the flower-colour character was fixed so we could consider that populations were homozygous for that trait.

Assays were made at the experimental fields and laboratories of Huesca University (UZ-Spain) from April to July of 1996.

Estimation of protandry

Study of floral phenology

To determine whether protandry occurs under borage in our conditions, we studied the floral phenology to establish the flower evolution from flower-bud opening to senescence. We subdivided this process into three stages:

stage 1: determined by the pollen shed; this stage develops 24 h after flower opening,
stage 2: determined by the pistil growing, widening and flattening of stigma; this stage develops 48 h after flower opening,
stage 3: characterised by corolla senescence with the pistil still visible similar to flowers at stage 2; this stage develops 72 h after flower opening.

Diallel cross

A diallel cross involving the three stages described above was designed. Ten flowers per cross, were hand-pollinated. Three repetitions per cross were established. Emasculations were made the day before of anthesis. The mean number of ripe seeds per flower and per cross was calculated. Means were compared using a Fisher LSD test.

Estimation of daily flower number

To estimate the daily flower number, all flowers which opened every day were labelled, indicating the date and the inflorescence where they developed. This procedure was repeated over a period of 60 days. The number of flowers that opened every day per inflorescence was recorded.

Pollinator’s behaviour

The flight and the foraging behaviour of insects were studied to assess how insect activity can influence self-pollination. Determination of the predominant pollinator species, the pattern of the insect’s flight and the number of flowers visited per plant by pollinators, were made. To determine the main pollinator species, a field of 200 plants was studied using the transect method (Dafni 1992). The species of insects on the flower were recorded. Data were recorded manually. To study the pattern of an insect’s flight, a bee was chosen and its flight followed visually. The number of flowers foraged by a honeybee within a plant was registered and represented in a histogram. This procedure was repeated ten times every day for 5 different days.

How within-plant visits affect self- and outcross-fertilization

Because pollination insects forage in different plants, it is difficult to decide whether fertilization of a flower is carried out by pollen from the same plant or by pollen from a neighbouring plant. In order to determine whether borage prefers self- or cross-pollen, when both arrive on the stigmatic surface, we designed the following experiment assuming that flower colour in this species was a monogenic trait showing blue to be dominant over white (Montaner 1997). Pollen from white- and blue-flowered plants was separately collected. The same quantities (in weight) of both types of pollen were mixed. Previous to hand-pollination the viability of pollen was assessed using the “in vitro” germination method described by Montaner et al. (1995). White-flowered plants were emasculated on the day of flower opening. A total of 150 emasculated flowers were pollinated with mixed pollen. Harvested seeds were grown and the flower colour of the resulting plants was recorded. Previously we confirmed the compatibility between both genotypes.

Assessing the extent of selfing due to geitonogamy

One way to assess the extent of selfing due to geitonogamy is to determine the proportion of selfed vs outcrossed progeny produced from a number of flowers over a period of time. We used flower colour as a marker in a bee-mediated pollination experiment. We have already shown that the bees foraging behaviour is not dependent on flower colour. This trait is also a good marker because flower colour is determined by a pair of alleles and blue colour is dominant to white flower-colour. In a progeny it is very easy to distinguish plants produced from crossing and those produced by selfing.

The selfing rate (S) due to geitonogamy was easier to calculate using the estimation of crossing rate because of the special floral biology of borage. For that purpose an experiment involving white- and blue-flowered cross-compatible plants was designed. White-flowered plants were grown mixed with blue-flowered ones, to establish that pollinator foraging in a blue and a white flower was the same. Previously we checked that flowering period, flower attraction and plant development were similar in both genotypes, because it was necessary to avoid the possibility that one particular genotype attracts more pollinators than another.

Seeds ripen 25–30 days after pollination at which time fruit-dehiscence occurs. Plants were exposed to insect pollination over a period of 20 days. Normally, borage-fruit dehisce at maturity and seeds are scattered on the ground. To avoid the possibility of mixing seeds from white flowers with those from the blue ones next to them, the blue flowers were removed after 20 days of exposure to insects. Ripe seeds from white flowers were harvested every day after the blue-flowered plants had been removed. Two hundred seeds were sown and finally 178 seedlings were grown to flowering. The number of white-flowered plants was recorded and the selfing rate was estimated using the following expression:

$[1-(\text{number of plants from crossing}/\text{total number of plants grown})]\times 100.$

We could assume that, because of protandry, the blue progeny from white-flowered plants is the result of a white×blue cross. Also, because of the compatibility between both lines, we could expect that the rate of white×white plants is the same as white×blue ones. This feature was taken into account to calculate the selfing rate.

Results and discussion

Estimation of protandry

Table 1 shows the number of ripe seeds per flower after a diallel cross of the three floral stages defined in borage.

Table 1 Mean number of seeds per flower after crosses. Data followed by different letters a–d are significantly different at the 5% level according to the Fisher LSD test

Crosses	Mean number of seeds/flower
Pollen stage1×pistil stage2	3.30 a
Pollen stage1×pistil stage1	2.10 b
Pollen stage2×pistil stage1	1.65 b
Pollen stage1×pistil stage3	1.55 b
Pollen stage2×pistil stage3	0.95 c
Pollen stage2×pistil stage2	0.85 c
Pollen stage3×pistil stage2	0.40 dc
Pollen stage3×pistil stage3	0.40 dc
Pollen stage3×pistil stage1	0.00 d

As the data show, when pollen from flowers at stage 1 is used to pollinate flowers at stage 2 the best results were obtained. These results enable us to confirm the description made by Janick et al. (1989) of the floral biology of borage. These authors report that borage is a protandrous species. In this case self-pollination is avoided by temporal separation of pollen ripeness and stigma receptivity in the hermaphrodite borage flower. When we did the first experimental work with borage, we observed that, in an insect-proof greenhouse, borage did not produce seeds (Montaner 1997). This may explain why that happened, although we had observed pollen grains on the stigma surface after passing the pistil through the stamens. Nevertheless, protandry in borage would not prevent geitonogamous self-pollination, because geitonogamy in protandrous plants would be influenced by features such as the daily flower number, the pollinator's behaviour or the pollination process.

Estimation of daily flower number

Data obtained in this experiment show that in a borage plant several new flowers open every day, although we observed that only one flower per inflorescence opens. This result agrees with that of Quinn et al. (1987) who made the same observation. The total number of flowers that opened every day equals the number of inflorescences producing flowers at a given time. The number of inflorescences increases over the flowering period of the plant. On the first day, only one flower (from one inflorescence) opens, but this number increases geometrically in a few weeks. The final number of flowers that a borage plant can produce is related to the number of branches and varies according to the genotype. Our experience working with borage shows that cultivated lines or genotypes produce more branches than wild borage germplasm. Each branch has several inflorescences, and so several flowers are available to be pollinated by insects on the same day. The manner in which these insects forage in a borage plant will determine whether self-pollination by geitonogamy is possible in this species.

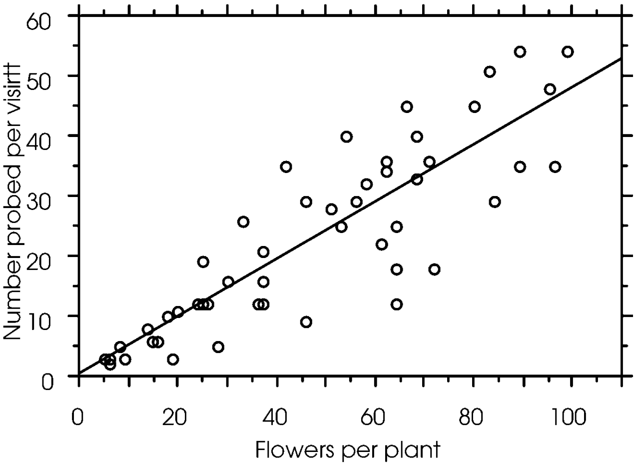

Fig. 1 Number of flowers probed by a honeybee within a plant relative to total number of flowers per plant (r^2=0.75; *P*-value <0.0001)

Pollinator behaviour

When we studied pollinator behaviour, visual observations allowed us to draw the following conclusions

- Honeybees are the main borage pollinators in our experimental fields although it is also possible to find some *Bombus* species.
- In the plants analysed, the number of approaches by insects increases with the number of opened flowers (range: 3 to 54 approaches made by a bee inside a plant).
- Bees minimize the flight distance when foraging.
- The number of flowers probed within a plant increase with the total number of flowers per plant (Fig. 1). In this sense we agree with Crawford (1984) who argues that bees apparently visit more flowers in succession when more flowers are available within a plant.

In a self-compatible species like *B. officinalis* L. (Montaner et al. 2000) with a massive floral display, and without a mechanism like dioecy, hercogamy or dicogamy present, selfing by geitonogamous pollination and later fertilization could only be avoided if self-pollen is rejected in favour of cross-pollen. The results of the following experiment clarified this point.

How within-plant visits affect self- and outcross-fertilization

Seeds harvested from mixed pollinations gave a total of 58 plants. Twenty seven of them showed blue flowers, and the remainder of 31 white flowers (no significant differences, with *df*=1, *P*-value=0.599, were detected after a χ^2 test: χ^2=0.28). These data show that borage shows no preference for cross-pollen, and both self- and cross-pollen are equally competitive. Epperson and Clegg (1987) described a similar feature in *Ipomoea purpurea*. Those authors claim that the time of self- and out-cross pollen deposition will also affect the selfing rate and that the earlier arriving pollen is favoured. The results of this assay, compared with the self-compatibility described in this species in a previous work (Montaner et al. 2000), lead us to assume that the borage response was similar to that of *I. purpurea*.

We also considered the possibility of a mentor effect when mixed self- and cross-pollen were deposited on the stigma. However, the results obtained in a previous study, where data from individual self- and cross-pollinations were compared, did not detect any difference between the processes (Montaner 1997). This suggests that the germination of self-pollen is not stimulated by foreign pollen, and allows us to confirm that borage does not select pollen of maternal origin.

If, as we mentioned previously, flowering structure, pollinator behaviour and pollen competition do not prevent geitonogamous pollination, it would be of interest to assess the extent of selfing in this species.

Assessing the extent of selfing due to geitonogamy

In this assay we counted 175 plants showing white flowers in a sample of the 178 plants grown. Only three plants showed blue flowers. In order to determine the selfing rate we considered that the probability of crossing between a white- and a blue-flowered plant is similar to the probability of crossing between two white-flowered plants. If this is the case then the total number of white plants coming from cross-pollination would be three, so that the estimation of selfing rate for borage is 96.63%. $[1-(3+3/178)]\times 100$.

The selfing rate expressed above shows the importance of geitonogamy in the pollination process and mating system of *B. officinalis* L. This result, obtained using a morphological trait as a marker, agrees with the results obtained by Leach et al. (1993). These authors, working with two different isozyme variants of Diaphorase, calculated a rate of 0.85% out-crossing after open pollination. Nevertheless these authors did not comment on the influence of geitonogamy in this species.

The fitness consequences of geitonogamous selfing depends on the extent of inbreeding depression, which is largely determined by the genetic load of a given population (Charlesworth and Charlesworth 1987). When inbreeding depression is especially strong, selfed embryos may abort prior to maturation, while in other species inbreeding depression appears to be negligible (Barret and Charlesworth 1991). We studied the effects of inbreeding depression in borage during five generations of selfing and observed that this allogamous species did not show any symptom of depression (Montaner et al. 2000). This fact let us think that the frequency of selfing in borage results in only minimal inbreeding depression, that so from a fitness point of view the cost of self-pollination due to geitonogamy is small. In this context we believe that the presence and effect of geitonogamous pollination in borage means that the genetical structure of borage populations is more similar to that of autogamous species than allogamous ones, particularly in cultivated varieties which have a greater number of flowers per plant than wild ones. We also believe that this feature should be taken into account in the design of future plant-breeding programs in relation to *B. officinalis* L. Until now the breeding methods used for borage were those employed for outbreeding species. The results of this work will allow applying other breeding methods and strategies common to autogamous species that have not been used before in borage.

Acknowledgements The authors are grateful to the Vegetable Gene Bank of Zaragoza for seeds and local cultivars. This work was supported by a grant from Gobierno de Aragón. All the experiments comply with the current laws of Spain.

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