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## Effects of pollen quality and quantity on pollen limitation in *Crataegus monogyna* (Rosaceae) in NW Spain

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### Abstract

Pollen limitation occurs when plants produce less fruits and/or seeds than they would with adequate pollen receipt. If the addition of cross-pollen to stigmas increases fruit/seed production, it is interpreted as an evidence of pollen limitation. Much of the limitation may be associated with the quality rather than quantity of pollen; however, most studies do not discriminate between the two, which may lead to misinterpretation of the results. We studied the effects of quality and quantity of pollen on the reproduction of a northern Spanish population of *Crataegus monogyna*. The treatments included self- and cross-pollination, and supplementation to open and bagged flowers. The response variables considered were number of pollen grains per stigma, pollen tubes per style, and initial and final fruit set. In the Cantabrian range, *C. monogyna* requires insect pollinators to set fruit and is partially self-incompatible. We found that the number of pollen tubes did not differ between cross- and self-pollination treatments; however, self-pollinated flowers set less fruits than flowers that received pure cross-pollen or were supplemented with both cross- and self-pollen. The experimental design allowed us to infer qualitative rather than quantitative pollen limitation. Comparison of the number of pollen grains and tubes, and initial and final fruit set among pollination treatments suggested post-zygotic embryo selection against selfed progeny.

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**Keywords:** Fruit set; Pollen limitation; Pollination requirements; Pollen supplementation experiments; Pollen tube growth

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### Introduction

Fewer fruits and seeds usually mature on flowering plants than the flowers and ovules that the plants produce. In many angiosperm species, some ovules and flowers do not develop into seeds and fruits because of

pollen limitation (Burd, 1994), limited resources for maturation of fruits and seeds (Ashman et al., 2004; Haig and Westoby, 1988), or because of a the self-incompatibility mechanism (Nettancourt, 1997). The results of a recent survey suggest that insufficient pollen receipt may affect fruit and/or seed production in many plant populations, as 284 of 482 studies (63%) reported pollen limitation at some sites or during some years (Knight et al., 2005). However, there is evidence that this magnitude of pollen limitation may be influenced by publication bias, experimental design and the response variable chosen (Knight et al., 2006).

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Pollen limitation occurs when the seed production is less than that would be achieved if the overall “quantity” or “quality” of pollen deposited onto stigmas were increased, and limitation is demonstrated empirically when supplemental pollination of flowers increases their female fertility compared with open-pollinated flowers. Pollen “quantity” may be limiting if plants are isolated, pollinators are rare, or if plants compete for the services of pollinators (Anderson and Hill, 2002; Bierzychudek, 1981; Johnston, 1991). Pollen “quality” may be limiting, despite adequate pollination, if pollinators deposit self- or incompatible pollen on stigmas, or if they deposit closely related pollen, and early-acting inbreeding depression lowers seed set (Herrera, 1987; Pflugshaupt et al., 2002; Waser and Price, 1991). Thus, the limitations exerted by quantity and quality of pollen are quite different. Pollen supplementation experiments are unable to distinguish between these, and much of the estimated pollen limitation may be due to qualitative rather than quantitative limitations (Aizen and Harder, 2007). Such experiments are therefore of doubtful use for estimating pollen limitation. Furthermore, in supplementation experiments flowers remain open to natural pollination and thus may receive pollen before or after the experiment, so that the plants may receive a mixture of pollen. Another criticism of supplemental studies is that they usually focus on a single variable, commonly final fruit set or seed set, and seldom include intermediate stages such as pollen germination, pollen-tube growth, initial fruit set and seed set (Knight et al., 2006).

We used a detailed pollen supplementation experiment to examine the relative importance of pollen quantity and quality on fruit production, and extended the comparisons beyond cross-supplementation (the classical experiment) to pure self- and pure cross-pollen and mixtures of different qualities of pollen donors. The responses were examined in relation to pollen loads, number of pollen tubes, fruit production and fruit weight in *Crataegus monogyna* in NW Spain. The fruit set was assessed at both the beginning and end of the fruit-development period to detect possible effects of post-zygotic abortion. *C. monogyna*, commonly known as hawthorn, is a well-studied species with hermaphroditic flowers and single-seeded fruits. Although the species is reported as self-compatible (Yeboah Gyan and Woodell, 1987), the extent of autogamy shows certain variability, and cross-pollination may increase fruit set (Gutián and Fuentes, 1992), so that pollen quality may limit fruit production. We also used manipulative pollen experiments to test pollinator requirements, and evaluated the ability of the plants to self-pollinate autonomously and to set seeds by apomixis, i.e., without the need for pollen to set fruits or seeds.

## Materials and methods

### The plant species

*C. monogyna* Jacq. (Rosaceae, subfamily Maloideae), is a shrub or small tree up to 10 m. Flowers are actinomorphic, perfect and possess a hypanthium on which perianth and androecium are inserted. A nectary is present on the inner surface of the hypanthium (Evans and Dickinson, 2005). The corollas comprise five free white to pinkish petals that, when open, are perpendicular to the long axis of the flower at anthesis. Flowers grow in small clusters of 9–18 (Tutin et al., 1968) and are entomophilous of the ‘generalist’ bowl form (Proctor et al., 1996). *Crataegus* species are unique in the Maloideae in having two ovules that are typically superposed within the locule (Dickinson et al., 1996; Sterling, 1964). In northern Spain, the plant flowering season is April–June. Fruits are single-seeded drupes, red when ripe (September–October). Ripe fruits remain attached to the trees for a long period during autumn and winter (Tutin et al., 1968).

The subfamily Maloideae is versatile in its reproductive mode, as vegetative spreading, self-incompatibility, self-compatibility and apomixis, all occur (Campbell et al., 1991). The reported evidence of apomixis includes polyembryony, complete pollen sterility and seed set in the absence of pollination (Dickinson and Phipps, 1986; Tutin et al., 1968). *C. monogyna* has been described as apomictic in North America (Dickinson and Phipps, 1986), autogamous in Britain (Yeboah Gyan and Woodell, 1987) and partially self-compatible in Spain (Gutián and Fuentes, 1992).

In the flowering season of 2005, we performed 94 (each 10 min) censuses of flowering branches and observed 310 insects visiting *C. monogyna* flowers. We observed insect visits to flowers between 10:00 and 18:30 on sunny and slightly cloudy days with low wind velocity. The main visitors were flies, which performed nearly 88% of the visits; Calliphoridae and Muscidae accounted for 89% of the visits, while Syrphidae (mainly *Eristalis tenax* L.) and Empididae accounted for 12% and 9% of the visits, respectively. Honeybees performed 10% of the visits and some vespids and beetles were occasionally seen in flowers (1%). Visits were only considered when a visitor contacted a reproductive part of the flower (anthers and/or stigma).

### Study site

The experiments were carried out in the Sierra Peña Mayor-Trigueiro (43°17'N, 5°30'W, province of Asturias, NW Spain), a medium-altitude (ca. 900 m a.s.l.) mountain belonging to the Cantabrian Range. The climate of the region is Atlantic, with mean annual

temperature of 13 °C and rainfall (ca. 1300 mm) distributed throughout the year.

The landscape is dominated (ca. 75% cover) by a non-forest matrix of pastures surrounded by small fragments of secondary-growth forest dominated by *Ilex aquifolium* L., *C. monogyna* L., *Taxus baccata* L., *Sorbus* spp., and *Corylus avellana* L. (see detailed information about the region in García and Obeso, 2003; García et al., 2005). *C. monogyna* occurs frequently in forest patches and as isolated trees scattered throughout the non-forest matrix.

## Experimental design

We performed seven treatments:

- (1) *Apomixis*: Flowers were emasculated and bagged;
- (2) *Spontaneous autogamy*: Flowers were only bagged;
- (3) *Pure self-pollination*: Flowers were bagged and hand pollinated with geitonogamous pollen;
- (4) *Pure cross-pollination*: Flowers were emasculated, bagged and hand pollinated with xenogamous pollen;
- (5) *Self-pollen supplementation*: Flowers were left unbagged (open to natural pollination) and hand pollinated with geitonogamous pollen;
- (6) *Cross-pollen supplementation*: Flowers were left unbagged and hand pollinated with xenogamous pollen; and
- (7) *Natural pollination (control)*: Open flowers left unmanipulated (open to flower visitors and no pollen supplementation).

We selected 10 trees and on each, we tagged 17 branches with unopened flowers. The first six treatments were repeated twice (12 branches) and the *natural pollination* treatment repeated in five branches. The total number of flowers used per treatment was: 481 for *apomixis*, 854 for *spontaneous autogamy*, 543 for *pure self-pollination*, 338 for *pure cross-pollination*, 222 for *self-pollen supplementation*, 215 for the *cross-pollen supplementation* and 2261 for the *natural pollination* treatment.

Pollen was applied every day or every other day (with toothpicks when necessary) to receptive stigmas in whole flowers, as flowers dehisced. For the hand-self treatments (treatments 3 and 5), the pollen applied was obtained from flowers from the same plant. For the hand-cross treatments (treatments 4 and 6 with xenogamous pollen), the donor pollen was obtained from flowers from three different plants growing at least 10 m away (the typical distance between trees was between 5 and 80 m).

One week after pollination, styles were collected just before they started falling naturally, to estimate pollen-

tube density. From the tagged flowering branches of each treatment we collected ca. 30% of the styles; overall we collected a mean number ( $\pm 1$  SE) of 133 ( $\pm 16$ ) styles per treatment. Styles were fixed and stored in individual microcentrifuge tubes containing FAA (formalin:acetic acid:ethyl alcohol, 5:5:90). In the laboratory, styles were cleared in a 10 ml/l NaOH solution for 3 h and stained with 0.1% aniline blue in 0.1 mol/l  $K_3PO_4$  (Martin, 1959). Squashed preparations were examined with an epifluorescence microscope at  $100\times$ . For each style, we counted the number of pollen grains germinating in the stigma and the number of pollen tubes at the base of the style. We also counted and collected all ripe fruits from pollination treatments, dried them in an oven at 20 °C for 1 week and weighed them. From these data we determined the fruit set (i.e., number of fruits/number of flowers) and fruit weight.

We evaluated the effects of pollination treatments on the following variables: number of pollen grains germinating in the stigma, number of pollen tubes per style, initial and final fruit set (number of fruits/flower) and fruit weight. We performed pollination treatments between 13th May and 8th June 2005, collected styles during the same period and assessed initial fruit formation on 27th June and final fruit set on 2nd August, when we also collected fruits.

## Data analysis

The effect of the pollinator requirements and pollen limitation (quality and/or quantity) was tested by fitting generalized and general linear mixed models to the data. The application of generalized linear models (McCullagh and Nelder, 1989) is justified by the nature of the dependent variables under consideration, as only fruit weight followed a normal distribution, whereas the other variables showed Poisson or binomial error distributions, as they are, respectively, counts (pollen grains and pollen tubes) and proportions (fruit set). Since conventional general linear models consider all the effects in the model as fixed effects, we preferred to use a mixed model that allowed us to incorporate the plants as random factors (Bennington and Thayne, 1994). Satterthwaite's approximation method was used to estimate degrees of freedom of the model and thus to identify the denominator of the *F*-tests (Littell et al., 1996).

We performed three different analyses to the data:

- (1) To assess the pollinator requirements we compared *apomixis* and *spontaneous autogamy* with the *natural pollination* treatment, by fitting a model in which the *pollination* treatment was considered as a fixed effect. We performed contrasts to assess significant differences among experimental treatments and the natural pollination treatment, using Dunnett's comparison procedure that controls the error rate

associated with the experiment (Littell et al., 2002; SAS, 2004).

- (2) To assess pollen limitation we compared *pure cross*- and *supplemental cross*-pollination with *natural pollination*, with the *pollination* treatment as a fixed effect. We performed contrasts to assess differences between treatments.
- (3) To assess the importance of “quantitative” compared with “qualitative” pollen limitation, we compared *hand pollination* treatments: *pure self*, *pure cross*, *supplemented self* and *supplemented cross*, with the *pollination* treatment as a fixed effect. We performed contrasts to assess significant differences among treatments, and used the False Discovery rate (FDR) to control the expected proportion of falsely rejected hypothesis, rather than the Bonferroni correction (Benjamini and Hochberg, 1995).

The computations were performed by the MIXED procedure (SAS version 9, SAS, 2004) for the variable fruit weight, as it was normally distributed, and the GLIMMIX procedure for the remaining variables (Glimmix experimental procedure of SAS version 9, SAS, 2004).

## Results

Naturally pollinated flowers of *C. monogyna* contained a mean number ( $\pm 1$  SE,  $N$ ) of 136 ( $\pm 49$ , 193 styles) pollen grains in the stigma, but only 5 ( $\pm 1$ , 182 styles) pollen tubes were counted at the base of the style, near the ovary. Of the 2261 open-pollinated flowers, 25% of them set initial fruits and 11% set final fruits. The average weight of the fruits was 132 mg ( $\pm 10.0$ , 253 fruits).

### Pollinator requirements

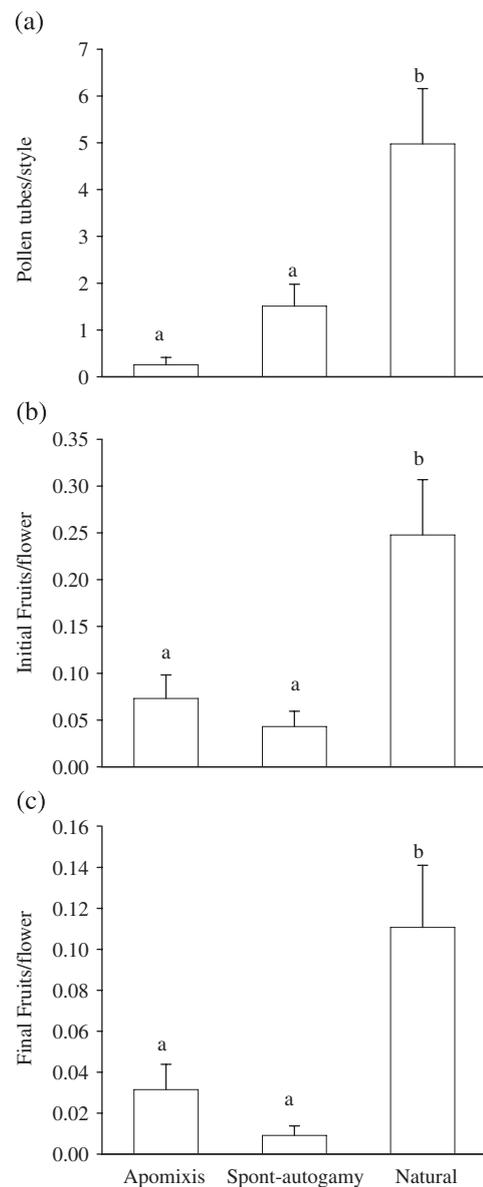
#### Apomixis and spontaneous autogamy

More pollen tubes were found in the styles of naturally pollinated flowers than in emasculated flowers ( $t = -4.88$ ,  $df = 18$ ,  $P_{adj} = 0.0002$ ) and spontaneously self-pollinated flowers ( $t = -4.28$ ,  $df = 18$ ,  $P_{adj} = 0.0009$ , Table 1). Styles from *naturally pollinated* flowers had 19 and 3 times more pollen tubes than in the *apomixis* and *spontaneous autogamy* treatments, respectively (Fig. 1a). Also, *naturally pollinated* flowers initially set 4 and 6 times more fruits than those in the *apomixis* ( $t = -3.28$ ,  $df = 16.2$ ,  $P_{adj} = 0.008$ ) and *spontaneous autogamy* treatments ( $t = -4.40$ ,  $df = 17.3$ ,  $P_{adj} = 0.0007$ , Table 1, Fig. 1b). Similar differences between treatments occurred when the final fruit set was considered (*naturally pollinated* compared with *apomixis*  $t = -2.63$ ,  $df = 23.99$ ,  $P_{adj} = 0.027$  and *naturally pollinated* compared with

**Table 1.** Results of pollinator requirements, ANOVA comparing pollen tubes, initial and final fruit set and fruit weight in apomixis, spontaneous autogamy and natural pollination treatments

Dependent variable	df <sup>a</sup>	F	P
Pollen tubes	2, 18	19.07	<.0001
Initial fruit set	2, 17.9	11.15	0.0007
Final fruit set	2, 26	10.06	0.0006
Fruit weight	2, 10	0.83	0.4657

<sup>a</sup>Numerator, denominator degrees of freedom.



**Fig. 1.** Least-squares means of number of pollen tubes, initial and final fruit set and fruit weight ( $\pm 1$  SE) for different treatments: *apomixis*, *spontaneous autogamy* and *natural pollination*. Different letters indicate significant differences between treatments ( $P < 0.05$ ) according to the scheme of Dunnett's contrasts (see data analysis and Table 1).

*spontaneous autogamy*  $t = -4.26$ ,  $df = 26$ ,  $P_{\text{adj}} = 0.0005$ , Table 1, Fig. 1c). Nearly 50% of the fruits set from *naturally pollinated* flowers, 40% set from the flowers in the *apomixis* treatment and 25% from the flowers in the *spontaneous autogamy* treatment aborted during early ripening (Figs. 1b, c). Although the fruit set was very low in the *apomixis* and *spontaneous autogamy* treatments, the weight of the fruits did not differ from that of the fruits set by *naturally pollinated* flowers (Table 1).

### Pollen limitation

*Natural pollination* initially set 66% less fruits than the *cross-pollen supplementation* treatment (Initial fruit set from *natural pollination* compared with initial fruit set from *cross-pollen supplementation* treatment:  $t = -2.71$ ,  $df = 1, 12$ ,  $P = 0.02$ , Table 2). However, there were no differences between *naturally pollinated* and *pure cross-pollinated* flowers (Table 2). There were no significant differences between treatments in terms of final fruit set, although almost half the number of fruits were set by flowers in the *natural pollination* treatment than in the *cross-pollen supplementation* treatment ( $t = -1.34$ ,  $df = 1, 12$ ,  $P = 0.20$ , Table 2). There were no differences between the *pure cross* and the *supplemented cross* treatments. However, the greatest difference between *natural* and *pure cross-pollination* was in final fruit set ( $t = -1.56$ ,  $df = 12$ ,  $P = 0.14$ ), even higher than in the *cross-supplementation* treatment.

### Pollen quality and quantity

#### Pure self, pure cross and supplementation of self- and cross-pollen

There were differences among pollination treatments (treatments 3–6) in terms of the number of pollen grains germinating in the stigma and also fruit set, but not the number of pollen tubes and fruit weight (Table 3).

Flowers from the *pure cross-pollination* treatment received less pollen than the other pollination treatments (Fig. 2a, possibly due to an experimental error); however, there was no difference in the number of pollen tubes in the different treatments (Fig. 2b).

The *pure self-pollination* treatment initially set ca. 6 times less fruits than flowers that received *pure cross-pollen*, or flowers exposed to pollinators and that received additional pollen (self and cross) (Fig. 2c). Although fruit abortion was almost 50% for all treatments, the self-pollinated flowers (either pure or supplemented to open flowers) lost on average 69% of their fruits, while the cross-pollinated flowers lost nearly 42%, and the *pure cross* treatment lost the least amount of fruit (30% of the fruits aborted). Final fruit set was lower in the *pure self-pollination* treatment than in the flowers that received cross-pollen, either bagged or unbagged (Fig. 2d). Fruit weight did not differ among pollination treatments (Table 3).

### Discussion

The present results indicate that cross-pollinated plants produce more fruits than naturally pollinated plants, and therefore that *C. monogyna* may be pollen limited. There was no difference in the number of pollen tubes in *cross-* and *self-pollination* treatments; however, *pure cross-pollinated* flowers and the supplementation treatments (both cross- and self-) produced more fruits than the pure self-pollinated flowers, which suggests that pollen quality rather than quantity may be one of the limiting factors for fruit production in this species.

**Table 3.** Results of ANOVA comparing pollen grains, pollen tubes, fruit set, and fruit weight in the different hand pollination treatments

Dependent variable	df <sup>a</sup>	F
Pollen grains	3, 19.9	9.6**
Pollen tubes	3, 19.5	0.3
Initial fruit set	3, 25.4	12.4****
Final fruit set	3, 22.7	5.5**
Fruit weight <sup>b</sup>	2, 8.1	0.8

<sup>a</sup>Numerator, denominator degrees of freedom.

\*\* $P < 0.01$ .

\*\*\*\* $P < 0.0001$ .

<sup>b</sup>As there were no fruits for the *pure self* treatment, the numerator had two degrees of freedom.

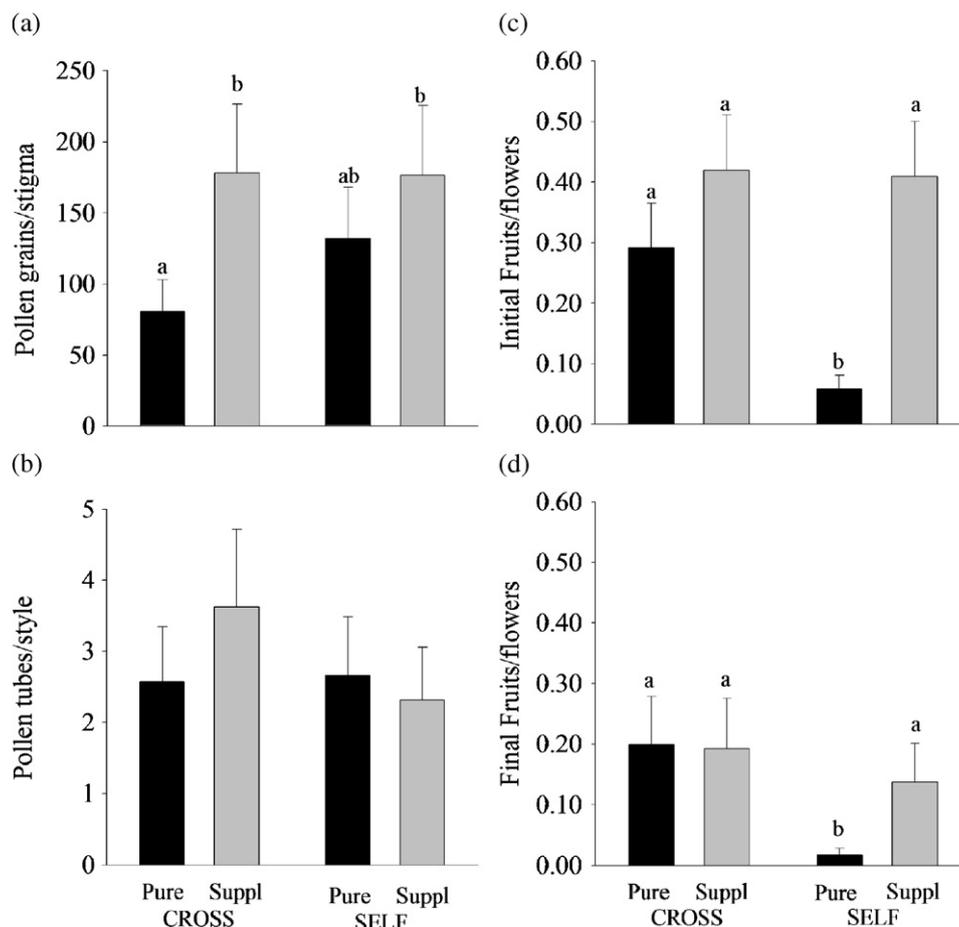
**Table 2.** Results of pollen limitation, ANOVA comparing initial and final fruit set in naturally pollinated, pure cross-pollinated and supplemented cross-pollinated flowers

Dependent Variable	df <sup>a</sup>	F	Natural pollinated	Pure cross-pollinated	Supplemented cross-pollinated
Initial fruit set	2, 15.5	3.69*	0.25 (0.05)a	0.30 (0.06)ab	0.42 (0.07)b
Final fruit set	2, 14.6	1.49	0.11 (0.04)	0.21 (0.07)	0.20 (0.07)

Means ( $\pm 1$  SE) for each treatment are given, different letters indicate statistically significant differences between pollination treatments.

<sup>a</sup>Numerator, denominator degrees of freedom.

\* $P < 0.05$ .



**Fig. 2.** Least-squares means of number of pollen grains (a), number of tubes (b), initial fruit set (c) and final fruit set (d) ( $\pm 1$  SE). Different letters indicate significant differences between treatments ( $P < 0.05$ ) according to the FDR correction.

The experimental design allowed us to infer a qualitative rather than a quantitative limitation for pollen. Comparison of the number of pollen grains and tubes, and initial and final fruit set among treatments revealed the effects of pollen donor identity during pollination and after the pollination stage.

### Pollinator requirements

Although *C. monogyna* is apomictic in North America (Dickinson and Phipps, 1986) we found that fruit set in emasculated flowers was almost inexistent, which indicates that apomixis without pollen induction is not common. In contrast with the results reported in a similar study carried out in North America (Campbell et al., 1991) the present results indicate the relevance of insect pollination, as in the absence of flower visitors we found negligible fruit production (less than 3% compared with 25% of flowers exposed to pollinators). We found some pollen grains germinating on stigmas and pollen tubes, which suggests that although emasculat

was performed before dehiscence a few pollen grains may have accidentally fallen onto the stigmas when anthers were removed. We must attribute this fruit set to pollen contamination rather than to apomixis.

On the other hand, spontaneous self-pollination is possible, but scarcely effective in reproductive terms, as demonstrated by the low number of pollen tubes and fruit set in the *spontaneous autogamy pollination* treatment. *C. monogyna* fruit set was less than 10% in bagged flowers relative to the natural treatments; species are defined as autogamous when more than 20% fruit is set in bagged relative to outcrossed treatments (as defined by Larson and Barrett, 2000). The degree of self-fertilization in *C. monogyna* may depend on environmental conditions and the vector species visiting each flower (Lloyd, 1992; Lloyd and Schoen, 1992). In fact, quite different results have been reported in two other studies of the breeding system of this species in Europe. Yeboah Gyan and Woodell (1987) suggested autogamy in populations in Britain, because they found no difference in fruit set among hand (cross) pollinated, open (exposed) and bagged flowers. Furthermore,

Gutián and Fuentes (1992) found different levels of autogamous pollination in populations of the species in NW Spain, and reported that fruit production in bagged and self-pollination treatments was nearly half that observed in naturally pollinated flowers. The present results also suggest such a trend, but with much greater differences between natural and bagged ( $\frac{1}{2}$ ) and natural and self ( $\frac{1}{6}$ ). The more rugged environment of the present study site relative to the other areas, at higher altitude and with shallower soils, may cause resource limitation in mother trees and explain the reported differences among sites (for other Maloideae trees in which fruit set varied depending on resource availability, see Pías and Gutián, 2006; Sperens, 1997).

### Pollen limitation and advantages of cross- compared with self-pollination

The present results showed that a mixture of pollen donors (cross- and self-pollen) and cross-pollen alone produced more fruits than flowers that only received self-pollen. Because pollen loads deposited naturally in the stigmas usually contain a mixture of self- and cross-pollen (Harder and Thomson, 1989), in this species, apparently, the opportunity for self-pollen to sire seeds may be low compared with the cross-pollen. In addition, there appears to be post-zygotic selection of outcross fruits, as suggested by the higher abortion rate in the predominantly self-pollinated fruits compared with cross-pollinated fruits.

Initial fruit set was higher in the cross-pollinated flowers than in the naturally and bagged self-pollinated flowers, which suggests a qualitative limitation of pollination. When sufficient pollen was delivered to the stigmas, both self- and cross-pollen germinated in the style without any apparent differences. Higher initial fruit production from cross- and mixed pollen (the supplementation treatments), may indicate that cross-pollen tubes germinate and fertilize the ovules faster (Aizen et al., 1990; Kao and McCubbin, 1996; Rigney et al., 1993). In addition, the fact that exposed and hand self-pollinated flowers initially set as many fruits as cross-pollinated flowers may indicate that the flowers naturally received some amounts of cross-pollen carried by pollinators.

Independently of any effect of pollen quality before fertilization, the present results suggest a clear advantage of cross-pollen for fruit set until ripening. In fact, there was a high abortion of developing fruits, and the greatest loss of embryos corresponded to those from self-pollinated flowers. This suggests post-zygotic selective embryo abortion, by which the mother plant selects between embryos or embryos compete for resources from the mother (Kobercka et al., 2002; Kozłowski and Stearns, 1989; Obeso, 2004). Whatever the mechanism in

*C. monogyna*, cross-pollinated flowers and those flowers exposed to natural pollination and receiving supplemental self-pollen initially set more fruits than the flowers that only received pure self-pollen. In addition, there was differential abortion of selfed fruits.

In summary, our results indicate that in the Cantabrian range *C. monogyna* needs insect pollinators to set fruit, is partially self-incompatible and suffers decreased fruit set due to post-zygotic embryo selection against selfed progeny. This idiosyncratic breeding system probably constrains the reproductive success and the population dynamics of this species in different ways. Firstly, it may hamper the ability to buffer interannual variability in pollen and pollinator resources, which is probably high in this system due to the short flowering period of the plant (ca. 20 days), the differences in flower production from year-to-year, the low abundance of high-quality pollinators such as bees or bumblebees and the unpredictability of the weather in these middle-mountain areas. Secondly, near-obligate cross-pollination may make this species more susceptible to habitat fragmentation processes that lead to reductions in the size of plant populations (Aizen et al., 2002; Ashworth et al., 2004; García and Chacoff, 2007), given that the fruit set depends more on the abundance of high-quality donors close to the mother plant than on individual flower production.

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