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**Seed germination and seedling allogamy in *Rosmarinus officinalis*: the costs of inbreeding**

Running head: Inbreeding in *Rosmarinus officinalis*

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**Abstract**

(1) Self-pollination by geitonogamy is likely in self-compatible plants that simultaneously expose large numbers of flowers to pollinators. However, the progeny of these plants is often highly allogamous. Although mechanisms to increase cross-pollination have been identified and studied, their relative importance has rarely been addressed simultaneously in plant populations.

(2) We used *Rosmarinus officinalis* to explore the factors that influence the probability of self-fertilization due to geitonogamy or that purge its consequences, focusing on their effect on seed germination and allogamy rate. For doing this, we experimentally tested the effects of geitonogamy on the proportion of filled seeds and how it influences germination rates. Then during two field seasons, we studied how life-history and flowering traits of individuals influence seed germination and allogamy rates of their progeny in wild populations at the extremes of the altitudinal range. The traits considered were plant size, population density, duration of the flowering season, number of open flowers, flowering synchrony among individuals within populations, and the proportion of male-sterile flowers.

(3) We found that most seeds obtained experimentally from self-pollinations were apparently healthy but in fact empty, and that the presence of filled seeds drove the differences in germination rates between self- and cross-pollination experiments. Plants from wild populations consistently showed low germination rates and high rates of allogamy as determined with microsatellites. Germination rates related positively to the length of the flowering season, flowering synchrony and the rate of male-sterile flowers whereas the rate of allogamous seedlings was positively related only to the rate of male-sterile flowers.

(4) Rosemary plants purge most of the inbreeding caused by its pollination system by aborting seeds. This study showed that the rates of seed germination and of the resulting allogamy are a function of a complex combination of factors that vary in space and time. Male sterility of flowers, length of the flowering season and flowering synchrony of individuals within populations all favor high rates of cross-pollination, therefore increasing germination and allogamy rates. These flowering traits appear to be highly plastic and respond to local and seasonal environmental conditions.

Keywords: Reproductive biology, pollination, geitonogamy, male-sterile flowers, seed germination, allogamy

**Introduction**

Self-compatible plants are faced with the challenge of reducing the potentially negative effects of pollen discounting and inbreeding-depression caused by self-pollination (Barrett 2002). Inbreeding is particularly likely in self-compatible insect-pollinated plants that bloom massively, where many flowers of the same plant or closely-related neighbours are exposed simultaneously to pollinators (Harder & Barrett 1995; Harder *et al*. 2004). When encountering a high amount of concentrated rewards, insect pollinators tend to remain within the same plant and potentially perform autogamous and geitonogamous pollinations (de Jong *et al.* 1993). For many plant species this selfing component of mating may represent a non-adaptive cost associated with the large floral displays needed to attract pollinators carrying pollen from other individuals (Barrett 2003).

However, most self-compatible plants produce outcrossed progenies at high rates (Goodwillie *et al*. 2005) by using multiple strategies that promote outcrossing or counteract the negative impacts of self-fertilization. Self-compatible plants, for example, may show mechanisms to reduce the chance of self-pollination by separating male and female functions in time (dichogamy) or space (herkogamy; Barret 2002). Also the pattern of flowering phenology of self-compatible plants influence the amount of self-pollination and the resulting outcrossing rates within populations (Kameyama & Kudo 2009). The duration of the flowering period, the number of open flowers at the same time, and the synchrony of individuals also influence the probability of geitonogamy (de Jong *et al.* 1993; Snow *et al.* 1996; Kudo 2006). Flowering synchronously enhances the attractiveness of populations to pollinators but large flowering displays of individuals can also have the disadvantage of causing more sequential pollinator visits within the same plant increasing selfing rates due to geitonogamy (Vrieling *et al.* 1999; Mitchell *et al*. 2004). Thus, reducing the number of flowers simultaneously exposed to pollinators but being highly synchronous with other individuals in the population could effectively reduce selfing rates (de Jong *et al.* 1993; Snow *et al.* 1996). In addition, extending the flowering period could allow individual plants to increase the chances of outcrossing, even with plants in more distant populations (Klinkhamer & de Jong 1990; Elzinga *et al*. 2007). Moreover, if the flowers have been self-pollinated, early inbreeding depression may cause plants purging the embryos produced by self-fertilization, therefore producing high rates of outcrossed progenies (Stephenson 1981; Husband & Schemske 1996; Snow *et al.* 1996; Wiens *et al.* 1987; Byers & Waller 1999). The extent and mode in which plants use one or several of these mechanisms simultaneously varies across species, among populations of the same species, during the life-cycle of a plant or even along the breeding season (Barret 2002; Eckert *et al.* 2006).

Persistent self-pollination because of geitonogamy in combination with strong inbreeding depression after selfing represents a disadvantage of hermaphroditism. In some plant lineages, this could favour the spread of male-sterile flowers mutants into populations, in a well-recognised evolutionary pathway to dioecy from gynodioecy (Thomson & Brunet 1990; Spigler & Ashman 2012). The advantage of male-sterile flowers over hermaphrodite flowers commonly involves one or more fecundity-related component (higher fecundity and/or lower inbreeding depression) that allow them to compensate for the loss of the male reproductive function (Jordano 1993; Wolfe & Shmida 1997; Dufay & Billard 2012).

Here we focus on a widespread Mediterranean plant to explore the relative importance of factors that potentially reduce selfing in natural populations. *Rosmarinus officinalis* L. (rosemary), of the Lamiaceae is a shrub that grows in dense populations and can produce hundreds or thousands of flowers simultaneously all along the breeding season. Flowers are self-compatible and attract a wide array of non-specialist insect pollinators looking for nectar and pollen rewards (Herrera 1987a; Herrera 2004). Because flowers are strongly protandrous, self-pollination due to autogamy seems to be unimportant (Herrera 1987a; Hidalgo-Fernández & Ubera 2001). In rosemary populations, less than one third of the open pollinated flowers produce seeds (Herrera 1986, 1987a, 1987b; Hidalgo-Fernández & Ubera 2001), and there is evidence that seed set is sensitive to inbreeding depression (Hidalgo-Fernández & Ubera 2001). Although there are no data available on the effect of inbreeding depression on the quality of seeds, Madeiras *et al.* (2009) related the consistently low germination rates in this species with the high rates of empty seeds they found. All these features suggest a mixed mating in rosemary plants, with insect-mediated geitonogamy causing high rates of self-fertilization but, at the same time, with a strong post-zygotic purging of inbred embryos that produce many apparently healthy but empty seeds. Consistently, a recent study on population genetics showed high levels of genetic diversity in 18 wild populations (Segarra-Moragues *et al.* 2016), supporting the possibility that inbreeding depression acts at early stages of seed development. It can be then expected that most, if not all, the seedlings that rosemary plants produce in the field have originated from cross-pollination.

Another important feature in *R. officinalis* is the variable production of male-sterile flowers intermixed with hermaphrodite flowers in individual plants (Roiz & Dulberger 1988). Although gynodioecy in the Lamiaceae has been frequently reported (Owens & Ubera 1992), unstable ginodioecy, the incomplete and variable production of male-sterile flowers in individual plant, is much less frequent. It has been reported so far for some *Origanum* L. and *Teucrium* L. species as well as for *R. officinalis* (Rodriguez-Riaño & Dafni 2007 and references therein). In *R. officinalis*,

populations range from those consisting of all individuals with 100% pure hermaphrodite flowers to a mixture of plants with variable proportions of male-sterile flowers (Herrera 1987a; Roiz & Dulberger 1988; Ubera-Jiménez & Hidalgo-Fernández 1992); in addition, the proportion of male-sterile flowers in individual rosemary plants varies throughout the breeding season (Ubera-Jiménez & Hidalgo-Fernández 1992).

In this study we aimed to explore the factors that potentially reduce the probability of self-fertilization due to geitonogamy or that purge its consequences in *R. officinalis* plants, focusing on their effect on seed germination and allogamy rate of the seedlings. Specifically, i) we compared experimentally the rates of viable seeds and germination between geitonogamous and cross-pollination treatments; ii) in six rosemary populations with contrasting environmental characteristics we measured plant density and plant size, and monitored along two breeding seasons the flowering phenology of individual plants, the number of flowers opened, the flowering synchrony of individuals within populations and the proportion of male-sterile flowers, and iii) we then related these variables to the germination rates of seeds collected from the same individuals and also to the rate of allogamy of the seedlings produced from the germinating seeds, estimated from microsatellite analysis.

**Material and Methods**

*Study species*

*Rosmarinus officinalis* is a very common plant in scrublands and open woodlands along the western Mediterranean Basin. It grows under very diverse climate and soil conditions and exhibits an extraordinary morphological and biochemical variation (e.g., growth habit, leaf size, flower size and colour, volatile compounds, etc.). Such phenotypic variability has been proposed to derive from the high phenotypic plasticity of plants and from the high genetic diversity within and among populations (Rosselló *et al*. 2006; Morales 2010; Mateu-Andrés *et al*. 2013). Significant levels of phenotypic plasticity have been long described in this species (Maffei *et al*. 1993; Munné-Bosch & Alegre 2000, Alarcón *et al*. 2006; Zunzunegui *et al*. 2011) and a recent population genetics study in 18 wild rosemary populations in eastern Spain showed high levels of genetic diversity and low genetic differentiation between populations even across the entire altitudinal distribution of the species and different soil types in a 6000 km$^2$ region (Segarra-Moragues *et al*. 2016).

The flowering period in *R. officinalis* fluctuates from only a few weeks in spring at the uppermost limit of its altitudinal range (around 1200 m a.s.l.) and far from the sea to almost seven months from autumn to late spring at sea level (Arroyo 1990; Herrera 1986). This flowering period is the consequence of equally long flowering periods of individuals and not to the sum of short flowering periods of different individuals within populations along the season (Herrera 1986), and its length is related to the duration of favourable temperature and moisture conditions (Petadinou *et al.* 1995 and references therein).

Flowers have four ovules, each potentially producing an independent single-seeded nutlet that remains enclosed by the calyx until maturation. Nutlets can develop even if the seed aborts, and seed-full or seed-less nutlets are indistinguishable in appearance but may differ in weight (Hidalgo-Fernández & Ubera, 2001). For convenience, we refer to ripe nutlets as seeds along the paper.

*Consequences of geitonogamy on the rates of germination and filled seeds*

In September 2010 we performed an experiment to test the consequences of geitonogamy on the rate of seed germination and the relationship between germination and the proportion of filled seeds. We used five 2-year old potted clones obtained from cuttings of each of three plants (R-10, R-13 and R-15, for a total of 15 experimental pots) from a single population (Porta-Coeli; Table 1). All receptive flowers in each plant were hand-pollinated with pollen from one of two sources: pollen from the same plant (geitonogamous self-pollination), or with a mixture of pollen from flowers from the two other individuals (cross-pollination). These hand-pollinations were applied to flowers during six weeks in order to obtain enough seeds. Plants were kept in a greenhouse inaccessible to potential pollinators and herbivores and they were homogeneously and regularly watered along the experiment.

The obtained seeds per individual and pollination treatment were germinated in plastic petri-dishes over moist filter-paper in a growth chamber (12 h light at 20º C/12 h darkness at 10º C). Petri-dishes contained 7-25 seeds and were checked for germination every two days for 28 days. After this time, seeds that failed to germinate were dissected under a microscope. Empty seeds, seeds without an embryo and seeds with a dried embryo or cotyledons were considered as aborted seeds whereas filled seeds with healthy embryos were considered as viable. We did not study the effects of the treatments on seed set as they haves been documented previously (see Introduction).

*Influence of flowering traits and population characteristics on seed germination and allogamy of seedlings*

Along the breeding seasons of 2010-2011 and 2011-2012 we monitored several flowering traits, together with seed germination and the allogamy rate of the seedlings obtained from these seeds, in 150 individuals in six wild populations of *R. officinalis* in eastern Spain (Table 1). In order to maximize differences in the environmental variables that potentially influence flowering patterns, populations were located from near the sea level to inland areas at the highest altitudinal limit of the species (Table 1). Populations were at least 8 km apart from each other and as far as 75 km. All populations were in the same range of hillslope angle (25-15º) and aspect (southeast to southwest), and grew on calcareous soils under Mediterranean climate. The sampled populations have experienced the same type of land use during the last centuries, including extensive grazing, charcoal and honey production, and hunting. The last forest fires occurred in 1980 in Porta-Coeli and in 1988 in Pedralba, whereas there were no recorded fires for the remaining localities at least in the 40 years previous to our observations. In March 2010 we assessed the density of rosemary plants by counting the number of individuals in three 20 × 20 m plots in each population.

In March 2010 we labelled 25 individuals per population that were at least 5 m apart from each other and determined their size, estimated as the volume of a cone. Leaves from all individuals were sampled and dried in silica gel until DNA extraction. Individuals were genotyped using six *R. officinalis* polymorphic microsatellite loci (Segarra-Moragues & Gleiser 2009; see Segarra-Moragues *et al.* 2016 for detailed genotyping methods). These genotypes were used to estimate the allogamy rates of the progeny of mother plants (see below).

From September 2010 until June 2012 we visited all labelled plants biweekly. On each visit we counted all open flowers and recorded each flower as hermaphrodite or male-sterile (femaleness hereafter) depending on the presence of aborted stamens. Four to five weeks after a plant started flowering we began to collect in each visit up to 20 calyces containing mature seeds in each monitored plant. This period was considered to be the minimum time required for seed maturation according to previous field observations. Flower counts and calyx collection continued for as long as the flowering and fruiting season continued in each population. Because we visited the populations biweekly, it could occur that plants that we recorded as flowering for the first or the last time in a visit, really started or finished flowering sometime within the fifteen days previous or following to the visit. To prevent this from causing underestimation of the number of days flowering, we added to all plants 7 days before and after the first and last visit. We did not systematically census

pollinators in this study, but we observed that *Apis mellifera* L. was the most common and abundant pollinator in all populations.

From these data, for each individual plant and breeding season we then calculated the number of days flowering, the total number of flowers produced (i.e. the sum of all the flowers counted), the maximum number of flowers open in a day (i.e. the maximum number counted in any census day in the season), femaleness ratios (i.e. the ratio of male-sterile to the total number of flowers produced in a season) and the degree of flowering synchrony with the other monitored individuals in the population using the Augspurger's Synchrony rate index (Augspurger 1983). All these variables were selected because of their potential effect on the rates of geitonogamy of the plants.

Collected calyces from each plant and date were taken to the laboratory where the seeds were separated and stored in paper bags in the dark and constant room temperature until seed germination trials started. All the seeds of each individual along the season were combined for germination. In total we collected 11576 seeds in the 2010-2011 season and 19171 seeds in the 2011-2012 season. For the 2010-2011 season, it varied from 730 seeds in Llíria to 4263 in Porta-Coeli, and, in the 2011-2012 season, it varied from 1100 seeds in Pedralba to 6638 in Porta-Coeli. For the seeds collected along the 2010-2011 season, germination was carried out in plastic petri-dishes as in the pollination experiment. The method was changed the following year, because seedlings obtained in petri-dishes were often too small to successfully extract and amplify DNA. Therefore, germination for the 2011-2012 seeds was carried out in cultivation trays with a mixture of perlite, coconut fibre and peat in a greenhouse (9.5-25º C; data from temperature sensors inside the greenhouse) until seedlings were of sufficient size for DNA extraction.

Outcrossing rates of the seedlings produced by each plant and season were calculated from microsatellite analysis. We compared the genotypes at six polymorphic microsatellite loci between the mother plants and their corresponding offspring. A seedling was considered as generated by outcrossing when at least one of the microsatellite alleles was not shared between the seedling and its mother plant. In the case of complete matching between them we considered that autogamy cannot be fully discarded. We then obtained a minimum allogamy rate for each mother plant and breeding season as the proportion of the allogamous seedlings produced by the plant in relation to all the seedlings produced in the germination trials. In total, we genotyped 229 seedlings in the 2010-2011 season and 2071 seedlings in the 2011-2012 season.

*Statistical analysis*

Logistic generalized linear mixed models via PQL were fitted to the data of the hand-pollination experiment with the *glmmPQL* command (MASS package v. 7.3-45) in R (R Core Team 2017) with the binomial family distribution and cloglog link because of the high proportion of zeros (Zuur *et al*. 2009). The seeds per plant that were filled and germinated relative to the collected seeds and also the seeds that germinated relative to the seeds that were filled were used as dependent variables. Pollination treatment was used as fixed factor and plant individual as random factor.

We applied generalized linear mixed models to the field data to assess the influence of the flowering variables on the proportion of seeds that germinated per plant and the rate of allogamy of the seedlings per plant. Plant identity was used nested within population as a random term in the models because the clustering structure of the design (Zuur *et al.* 2009). The interaction between breeding season and all the flowering characteristics were included in the fixed terms in order to test for the influence of the flowering variables each season. We did not include altitude as a fixed factor because altitude was used in the experimental design to increase the variation in the flowering traits of the plants. Therefore, including altitude as a variable in the fixed term of the model would incur in a twofold influence because altitude would affect both the response variable and the variables in the fixed term. To avoid this, prior to building the fixed term, we checked for the effect of altitude on the flowering variables. For this, we used linear mixed models with the *lme* command (nlme package, v. 3.1-128 implemented in R) including altitude and season as fixed factors and population as random factor. In the models for plant size and plant density we did not include season since these variables were measured once along the two seasons, and in the model for density we did not include population because all individuals in a population shared their values. For those models where population was not included we fitted linear models with the *lm* command (R package stats, v.3.3.2). Plant size, the number of flowers and the maximum number of open flowers were log-transformed before the analyses.

Previous to the analyses, we tested the variables included in the fixed term of the models and their interaction for multi-collinearity using the *vif* command (car package, v. 2.1-4 in R) and dropped those terms with variance inflation values of 2.5 or higher. The final model with the random and fixed term was analysed with the *glmmPQL* command with binomial distribution of errors and logit link.

To estimate the contribution of the fixed and random terms in the mixed models to the total R squared value we used the *r.squaredGLMM* command (MuMIn package, v 1.15.6) that returned the values of the marginal R squared (variance explained by the fixed factor) and conditional R squared (variance explained by both the fixed and random factors; Nakagawa & Schielzeth 2013). Graphical model validation was used in all analyses to check for homoscedasticity and normality of residuals (Zuur *et al.* 2009).

**Results**

*Consequences of geitonogamy on the rates of germination and filled seeds*

Seeds from the cross-pollination treatment were filled and germinated with a higher probability than seeds obtained from geitonogamy (Table 2). The model for the rate of seed germination accounted for 57% of the variance (conditional $R^2$) and an important part of this variance was explained by the fixed term (marginal $R^2$= 41%) but still plant identity played a role explaining differences in germination. The model showed that seeds from cross-pollination had 1.99±0.23 times higher probability to germinate than seeds from geitonogamy (t-value = 8.739; p-value < 0.001). Concerning the filled seeds, most of the variance was explained by the treatment (conditional $R^2$= 38% and conditional $R^2$= 47%), indicating that plant identity had little effect on it. The probability of seeds being filled was 1.72±0.20 times higher for the cross-pollination than for the geitonogamy treatment (t-value = 8.542; p-value < 0.001). When we analysed the rate of seed germination relative to the number of seeds that were filled, the model accounted for 13% of the deviance but now the pollination treatment and plant identity contributed in a similar amount to the variation in seed germination (marginal $R^2$= 7%), indicating that seeds from cross-pollination had 0.619±0.25 times higher probability to germinate than seeds from geitonogamy (t-value = 2.438; p-value = 0.016).

*Influence of flowering traits and population characteristics on seed germination and allogamy of seedlings*

Tables 1 and 3 show the values of the studied variables for each population and season of study. We found that plant size was affected by the population but not by altitude (Table 4). However, altitude negatively influenced plant density, suggesting that soil conditions in populations and time from the last perturbation were more important than altitude in determining plant size

(Table 4). Analyses of the flowering variables of plants showed that the range of altitudes in our design widely influenced them, singly or in interaction with season, but also that population still explained part of the variation in all the variables (Table 4). So the number of days that plants flowered in the season decreased with altitude and was shorter in the second season of study, the driest one (Figures S1 and S2, Tables 3 and 4). Flowering synchrony within populations was higher in the second than in the first season but altitude had no effect on it (Tables 3 and 4). The total number of flowers that plants produced in a season and the number of flowers opened in a census day increased with altitude but only on the first season of the study. In the second season the large reduction in the number of flowers of plants at the highest populations vanished that effect in the total number of flowers (Tables 3 and 4). The proportion of male-sterile flowers that a plant produced in a season varied along the season and also between years and it was negatively affected by altitude but was not significantly affected by the season (Tables 3 and 4). We also found that along the first study season all plants in all populations had some male-sterile flowers (Tables 3 and 4), but on the second season we found that 33% and 66% of individuals in Remedio and Manzaneruela, respectively, the two populations at highest altitudes, had only hermaphrodite flowers along the season.

Seed germination rates of plants were very low in all studied populations (Table 3), reaching its maximum value at the Porta-Coeli population in both seasons, with 0.34 and 0.35 respectively, and its minimum value in Remedio in the 2011-2012 season, the highest altitude and less dense population, with a rate of 0.01.

For the initial model of seed germination, variance inflation analyses indicated that the number of flowers that plants produced in a season and the maximum number of flowers opened in a census day were highly correlated and so we decided to retain in the model the latter variable because of its higher potential relevance for geitonogamy. Variance inflation analyses indicated also that all the interactions among the variables were highly collinear and therefore they were also removed from the model.

The resulting generalized linear mixed model for seed germination explained ca. 23% of the variation (Table 5) and most of this variance was accounted for the fixed factor suggesting that plant identity and the population have a low contribution to the variation in seed germination. All the variables in the fixed factor, except for the maximum number of flowers opened in a census day, had an influence on the germination rate of plants in one or both seasons (Table 5). The number of days that plants flowered and flowering synchrony positively affected seed germination in both seasons. Plant density and the proportion of male-sterile flowers of the plants also positively affected seed

germination but they did so only the second season. Finally, plant size had a negative influence on germination rate in the first season but had non-significant effect in the second season (Table 5).

Because the available data on the allogamy rate of the seedlings was very low for the first season for model fitting we used only those data for the 2011-2012 season (Table 3). We found that for the 2011-2012 breeding season, the allogamy rate of the seedlings of each plant was very high (Table 3) in almost all populations, with most of the values greater than 80%.

Variance inflation analyses for the allogamy model indicated again a high correlation between the number of flowers that plants produce in a season and the maximum number of flowers opened in a census day and thus, we retained the later variable in this case as well. The interactions among all the variables were also removed from the fixed term because their high variance inflation values. The final model for the allogamy rate of seedlings explained 68% of the variance, but still plant identity and population retained a substantial proportion of the variance (13%, Table 6). The femaleness ratio was the only significant variable in the fixed factor; Figure 1 shows the effect of the femaleness on the allogamy rate of the seedlings at the plant level. It indicates that plants with a proportion of male-sterile flowers of 50% or higher along the season guarantee that almost none of the seedlings that a plant produces is the consequence of self-pollination.

**Discussion**

A reduction in germination rate as a consequence of geitonogamy seems to be the rule in the mass flowering shrub *R. officinalis*. This study shows that the impact of inbreeding depression, measured here as the rates of seed germination and of the resulting allogamy, is a function of a complex combination of factors that vary in space and time.

We confirmed experimentally that geitonogamy strongly reduced the probability of seed germination in *R. officinalis*, and also that this reduction was mainly the consequence of the similarly high rate of empty seeds caused by geitonogamy. Our experimental results are based on a few individuals of a single population and could have been affected by a particular inbreeding level of that population; however, we found from genetic indices based on 11 microsatellite loci that the source population did not differ from 18 other populations of rosemary in eastern Spain (Segarra-Moragues *et al.* 2016). On the other hand, a previous pollination experiment with individuals of a different population not included in this study similarly yielded a strong reduction in the proportion of filled seeds after geitonogamy (Garcerá & García-Fayos unpublished).

Despite the severe reduction in germination, plants produce many seeds after geitonogamous self-pollination and a few of these seeds germinated and developed seedlings, supporting a mixed mating system in *R. officinalis* (Goodwillie *et al*. 2005). In addition to geitonogamy, another source of reduction in seed germination of the seeds collected in the field could come from empty seeds resulting from biparental inbreeding or mating between relatives. Neighbouring rosemary shrubs are likely close relatives, because this species lacks mechanisms for long-distance seed dispersal and the single-seeded dry indehiscent fruits (schizocarps) or entire calyces containing the fruits are gravity dispersed, below or close to the maternal plants (Bouman & Meeuse 1992). Furthermore, when moistened or wetted, the pericarp mucilage hydrates, gluing the fruits to the soil surface and preventing or reducing both secondary dispersal and predation by ants (Engelbrecht & García-Fayos 2012). Accordingly with these features, high inbreeding coefficients have been found in these populations (Segarra-Moragues *et al*. 2016).

The results of field observations strongly suggest that the conspicuous floral display of rosemary plants within populations is successful in attracting pollinators but also that it causes high rates of geitonogamy, as predicted (Charlesworth & Charlesworth 1987). However, the experimental and observational results also indicate that rosemary plants purge most of the inbreeding caused by selfing by aborting seeds or by reducing their probability of germinating, as occurs in other species (Wiens *et al.* 1987; Husband & Schemske 1996). Although this finding can explain the reported high proportions of empty seeds in this species (Clemente *et al.* 2007; Madeiras *et al.* 2009), we cannot discard the influence of other potential factors inducing seed abortion, like environmental stress (Stephenson 1981). Previous experiments with rosemary clones showed a decrease in the rate of filled seeds from 68% to 49% after reducing 1/3 water availability of plants, independently of other controlled factors (pollen amount and source, plant size, etc., Garcerá & García-Fayos unpublished).

In addition to the self-pollination consequences of geitonogamy because of the highly attractive floral display of *R. officinalis* to pollinators, our study also confirms that several flowering characteristics of this display also correlate with cross-pollination (Klinkhamer & de Jong 1990; Ison *et al.* 2014). Plant density and the degree of flowering synchrony of plants both had a positive influence on the proportion of seeds per plant that actually germinated. The magnitude of the floral display, represented by plant size and the number of flowers that plants exposed simultaneously to pollinators, both of which were previously proposed to increase geitonogamy and attractiveness to pollinators (de Jong *et al*. 1993; Albert *et al*. 2008), had no effect on the rates of germination and allogamy in rosemary plants, except for plant size that negatively influenced seed germination the first season (Table 3). This suggests that the potential increase of geitonogamy in plants with large

floral displays is partially compensated by the simultaneous influence of other flowering characteristics of this display promoting cross-pollination. This supports that the size and other characteristics of the floral display of plants cannot be fully understood by examining them in isolation (Harder *et al*. 2004).

Other flowering characteristics that we did not expect to have an influence directly on the attractiveness of plants to pollinators, such as the length of the flowering period and the proportion of male-sterile flowers in plants, played a role in reducing the rate of geitonogamous pollinations (Table 6). The length of the flowering period had to co-act with other flowering characteristics to have an influence in reducing geitonogamy. Other studies have shown that extending the flowering period is a good strategy to reduce geitonogamy only if it combines with each individual opening only a few flowers every day and the is a high flowering synchrony of other individuals in the neighbourhood (de Jong et al. 1993; Kuning 1993; Bris *et al*. 2008). Notwithstanding, this was not observed in our study. We found a negative relationship between the number of days the plants flowered and flowering synchrony in rosemary plants ($r_p$ =-0.540, p<0.001 for the 2010-2011 season and $r_p$ = -0.201, p = 0.030) and also that altitude did not influence on the number of flowers open at a time, on flowering synchrony or plant density in our studied populations or it only did in one of the study seasons (Tables 3 and 4). However, the length of the flowering period is a very plastic trait and is influenced by environmental properties that also can influence on the other flowering traits. So altitude strongly reduced the number of days that plants flowered in their respective populations in both seasons (Tables 3 and 4), but at the same time, the length of the flowering season of plants living at the highest and the lowest altitude populations overlapped when clones from these plants were grown in a common garden (Castellanos & García-Fayos, unpublished).

The proportion of male-sterile flowers in *R. officinalis* plants showed an extensive temporal variation along the breeding season (see Fig. S2), confirming the findings reported by Ubera-Jiménez & Hidalgo-Fernández (1992), but it also was negatively affected by altitude (Tables 3 and 4). We can confirm the suggested positive influence of the femaleness ratio on the seed germination rates. At the same time, we found that the femaleness ratio was the only trait influencing the outcrossing rate of the seedlings. That is, after discounting for the effects of the flowering characteristics of the plants and populations on the seed germination rate, the ratio of male-sterile flowers still revealed a positive influence on the outcrossing rate. These findings are coherent with the idea that the joint presence of female-only and hermaphrodite flowers within individuals will reduce the chance of geitonogamous pollination compared to individuals producing only hermaphrodite flowers (Albert *et*

*al.* 2008; Dufay & Billard 2012) and it strongly suggests that femaleness plays a central role mediating in the conflict between attracting pollinators and reducing geitonogamy in *R. officinalis*.

We found that a proportion of 50% of male-sterile flowers is enough to guarantee that almost all the progeny of *R. officinalis* plants originate from cross-pollination (Fig. 1). This result is coherent with the suggestion that geitonogamy could be a driver towards ginodioecy. This is true for self-compatible plants incurring in high flowering costs to attract pollinators and simultaneously suffering from strong inbreeding depression because of self-pollination (de Jong & Geritz 2001; Sinclair *et al*. 2013). However, femaleness in rosemary plants does not seem to be a long-term stable strategy since to date no wild plants of this species have been described as female. In our study we only found 1 plant (out of 150 individuals) that behaved exclusively as a female; however, it did only along one season. Within individual instability in male-sterility may be the result of genetically-controlled gender variation in interaction with the environment (Wolfe & Shmida 1997; McCauley& Bailey 2009; Harkess & Leebens-Mack 2016). On the one hand, Hidalgo-Fernández *et al.* (1999) found that rosemary plants with some proportion of male-sterile flowers had higher mitochondrial genome variability than plants with all flowers hermaphrodite, suggesting some kind of genetic control as occurs in other Lamiaceae (Belhassen *et al*. 1991). But on the other hand, the strong and persistent negative effect of altitude on the proportion of male-sterile flowers we found in our study is coherent with the finding that warmer temperatures favour the expression of female flowers (Koelewijn & Van Damm 1996), and point more towards an abnormal development of flowers (Richards 1997, pp. 320) than to genetically controlled variation

Our study provides a careful study case of the effects of inbreeding on germination and allogamy and the importance of a large number of factors influencing outcrossing. The seedlings produced by plants of *R. officinalis* in natural conditions were mostly outcrossed and their rate was strong and positively influenced by the proportion of male-sterile flowers within individuals. These high outcrossing rates are, at least partially, the consequence of plants purging most of the potential inbreeding produced by geitonogamy by aborting most of the inbred seeds or reducing their rate of germination. Among the flowering traits promoting cross-pollination in rosemary, long flowering spans and high flowering synchrony among individuals in a population emerged as the most important factors at both altitudinal levels, plant density also played a role on it, and apparently counteract the potential negative effects of intense flower displays attracting pollinators. However, we found that all these flowering characteristics are highly plastic and further experimental studies would be needed to test their role as adaptations of rosemary plants to reduce selfing. Rosemary plants can thus grow and reproduce in a variety of conditions across an altitudinal gradient that

combined with high levels of gene flow (Segarra-Moragues *et al*. 2016) can explain the high levels of genetic variation of populations of this species.

**Acknowledgements**

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Tables captions:

Table 1. Name, location and main characteristics of the studied *Rosmarinus officinalis* populations. Climatic data are year average values from the nearest meteorological stations along the 1961-1990 period (taken from Pérez 1994).

Table 2. Number of seeds obtained from each plant in the hand-pollination experiment and number (and average rate ± SE) of filled seeds relative to all seeds or that germinated relative to all seeds and, in italics, *germinated seeds relative to filled seeds*. Values of germination for individual R-15 were not tested because of the low numbers of filled seeds.

Table 3: Population average values ± SE of the reproductive variables for *Rosmarinus officinalis* for the two study seasons. Numbers in brackets indicate sample sizes.

Table 4. Synthesis of the generalized linear mixed models testing the effect of population (random term), altitude, season of study and their interaction (fixed terms) on the flowering variables. The first two columns indicate the proportion of the variance explained by the random and fixed terms. The columns for altitude, season and their interaction indicate their respective coefficients (± SE) in the model and their significance (bold numbers indicate significant coefficients). Plant size, number of flowers and maximum number of flowers open at a time were log-transformed ($\log_{10}$). The symbol # denotes that that term was not included in the model.

Table 5. Synthesis of the generalized linear mixed model testing the effect of plant size, plant density and flowering characteristics (fixed term) on the germination rate of seeds in *R. officinalis* Individual identities were included as a random term nested to populations. Significant partial coefficients are in bold.

Table 6. Synthesis of the generalized linear mixed model testing the effect of plant size, plant density and flowering characteristics (fixed term) on the allogamy rate of the seedlings in *R. officinalis*. The model was fitted with data of the breeding season 2011-2012 only (see text for model details). Individual identities were included as a random term nested to populations. Significant partial coefficients are in bold.

Figure caption:

Figure 1: Influence of the femaleness ratio (proportion of female flowers in relation to the total number of flowers) of *Rosmarinus officinalis* plants on the probability of plants producing allogamous seedlings.

Supplementary Information

(I split each supplementary figure into 2 subfigures (A & B) to maintain the quality level of each figure.

Figure S1: Temporal flowering pattern of the *Rosmarinus officinalis* plants during two years of study in a low altitude population (Lliria) and a high altitude population (Manzaneruela). Each line represents an individual plant; the lines for two of the individuals in each population were drawn differently to highlight the within and between season variability in the flowering pattern.

Figure S2: Temporal patterns in the femaleness ratio (proportion of male-sterile flowers) of the *Rosmarinus officinalis* plants during two years of study in a low altitude population (Lliria) and a high altitude population (Manzaneruela). Each line represents an individual plant to highlight the within- and between-season variability in femaleness ratio.

Table 1.

| Population | Longitude (W) | Latitude (N) | Altitude (m a.s.l.) | Distance to the sea (km) | Temperature (ºC) | Precipitation (mm) | Plant density (ind.ha$^{-1}$) | Plant size (dm$^3$) |
|---|---|---|---|---|---|---|---|---|
| Llíria | 39°38'18.99" | 0°36'38.08" | 200 | 28.3 | 16.5 | 410 | 9000 | 154.7 ± 32.5 |
| Pedralba | 39°35'05.27" | 0°41'30.76" | 210 | 33.4 | 16.5 | 434 | 11900 | 368.0 ± 64.7 |
| Porta-Coeli | 39°39'43.02" | 0°29'35.48" | 240 | 20.7 | 15.9 | 409 | 8933 | 456.0 ± 79.8 |
| Manzaneruela | 39°56'59.41" | 1°18'49.57" | 1050 | 66.8 | 11.1 | 495 | 9067 | 225.2 ± 21.7 |
| Remedio | 39°38'04.01" | 1°08'08.17" | 1240 | 71.1 | 11.9 | 564 | 1433 | 252.6 ± 51.2 |
| Sacañet | 39°51'27.14" | 0°42'45.58" | 995 | 47.2 | 11.6 | 485 | 9400 | 375.1 ± 48.5 |

Table2.

| Individual | Geitonogamous-pollination | | | Cross-pollination | | |
|---|---|---|---|---|---|---|
| | Seeds | Filled | Germinated | Seeds | Filled | Germinated |
| **R-10** | 27 | 10 (0.37±0.09) | 3 (0.11±0.06) *(0.30±0.14)* | 82 | 49 (0.60±0.05) | 42 (0.51±0.05) *(0.86±0.05)* |
| **R-13** | 212 | 32 (0.15±0.02) | 28 (0.13±0.02) *(0.87±0.06)* | 62 | 45 (0.72±0.05) | 42 (0.68±0.06) *(0.93±0.04)* |
| **R-15** | 21 | 1 (0.05±0.04) | 1 (0.05±0.04) | 29 | 9 (0.31±0.09) | 5 (0.17±0.07) |

Table 3.

| Population/season | Days flowering | Total number of flowers | Maximum number of flowers/c | Flowering Synchrony | Femaleness | Germination | Allogamy |
|---|---|---|---|---|---|---|---|
| **Llíria** | | | | | | | |
| 2010-2011 | 128.2 ± 11.8 (25) | 283.4 ± 45.4 (25) | 109.6 ± 15.3 (25) | 0.65 ± 0.02 (25) | 0.44 ± 0.05 (25) | 0.20 ± 0.03 (5) | n.a. (0) |
| 2011-2012 | 113.0 ± 4.7 (25) | 649.8 ± 138.6 (25) | 179.5 ± 35.9 (25) | 0.84 ± 0.02 (25) | 0.29 ± 0.04 (25) | 0.15 ± 0.02 (17) | 0.90 ± 0.02 (15) |
| **Pedralba** | | | | | | | |
| 2010-2011 | 124.1 ± 10.2 (25) | 406.2 ± 99.1 (25) | 136.2 ± 29.9 (25) | 0.69 ± 0.02 (25) | 0.35 ± 0.05 (25) | 0.17 ± 0.05 (7) | 0.80 ± 0.10 (6) |
| 2011-2012 | 71.3 ± 7.5 (24) | 219.0 ± 51.1 (24) | 93.4 ± 18.7 (24) | 0.67 ± 0.03 (24) | 0.63 ± 0.06 (24) | 0.07 ± 0.02 (12) | 0.98 ± 0.01 (9) |
| **Porta-Coeli** | | | | | | | |
| 2010-2011 | 152.6 ± 9.4 (25) | 1205.2 ± 183.6 (25) | 488.1 ± 84.5 (25) | 0.81 ± 0.02 (25) | 0.21 ± 0.04 (25) | 0.34 ± 0.02 (16) | 0.94 ± 0.06 (4) |
| 2011-2012 | 158.1 ± 4.6 (25) | 1227.9 ± 201.8 (25) | 318.4 ± 56.1 (25) | 0.91 ± 0.01 (25) | 0.29 ± 0.04 (25) | 0.35 ± 0.03 (23) | 0.87 ± 0.03 (23) |
| **Manzaneruela** | | | | | | | |
| 2010-2011 | 58.6 ± 2.4 (25) | 1406.5 ± 236.5 (25) | 820.6 ± 144.1 (25) | 0.88 ± 0.02 (25) | 0.08 ± 0.01 (25) | 0.14 ± 0.02 (9) | n.a. (0) |
| 2011-2012 | 50.9 ± 3.0 (25) | 215.9 ± 72.5 (25) | 121.3 ± 37.1 (25) | 0.85 ± 0.02 (25) | 0.02 ± 0.01 (25) | 0.02 ± 0.01 (17) | 0.76 ± 0.19 (5) |
| **Remedio** | | | | | | | |
| 2010-2011 | 64.2 ± 3.3 (25) | 3722.1 ± 693.2 (25) | 1976.1 ± 285.7 (25) | 0.84 ± 0.02 (25) | 0.06 ± 0.03 (25) | 0.09 ± 0.02 (12) | n.a. (0) |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 2011-2012 | 59.6 ± 1.4 (25) | 1152.2 ± 236.4 (25) | 573.1 ± 121.9 (25) | 0.97 ± 0.00 (25) | 0.05 ± 0.02 (25) | 0.01 ± 0.00 (24) | 0.67 ± 0.12 (11) |
| **Sacañet** | | | | | | | |
| 2010-2011 | 129.9 ± 8.1 (25) | 4050.2 ± 371.3 (25) | 1624.9 ± 194.5 (25) | 0.88 ± 0.02 (25) | 0.07 ± 0.01 (25) | 0.22 ± 0.02 (18) | 0.80 ± 0.10 (2) |
| 2011-2012 | 78.8 ± 3.3 (25) | 595.2 ± 113.9 (25) | 205.3 ± 38.3 (25) | 0.86 ± 0.01 (25) | 0.19 ± 0.04 (25) | 0.12 ± 0.02 (23) | 0.93 ± 0.20 (21) |

Table 4.

| | Random term | Fixed term | Variables in the fixed term | | |
| --- | --- | --- | --- | --- | --- |
| | | | ALTITUDE | SEASON | ALTITUDE x SEASON |
| Plant size | 13% | 0% | -0.0002 ± 0.0001 | # | # |
| Plant density in the population | # | 26% | -4.6370 ± 2.7940 | # | # |
| Days flowering in the season | 20% | 48% | **-0.0775 ± 0.0213** | **-30.4207 ± 8.3807** | 0.0089 ± 0.0098 |
| Number of flowers | 18% | 34% | 0.0006 ± 0.0003 | 0.0110 ± 0.1266 | **0.0008 ± 0.0002** |
| Maximum number of flowers opened at a time | 13% | 42% | **0.0008 ± 0.0002** | -0.0968 ± 0.1208 | **-0.0008 ± 0.0001** |
| Flowering synchrony with the other plants in the population | 39% | 25% | 0.0002 ± 0.0001 | **0.0965 ± 0.0287** | -0.0000 ± 0.0000 |
| Femaleness | 6% | 28% | **-0.0003 ± 0.0001** | 0.0709 ± 0.0557 | -0.0000 ± 0.0000 |

Table 5.

| GERMINATION | Variance (%) |
| --- | --- |
| Random factor | 4.85 |
| Fixed factor | 17.77 |

| Variables in the fixed factor | season | Coefficient | d.f. | t-value | p-value |
| --- | --- | --- | --- | --- | --- |
| Intercept | | -7.7198 ± 0.7693 | 122 | 10.0342 | <0.0001 |
| Plant size | **2010-2011** | **-0.6455 ± 0.2965** | **43** | **2.1767** | **0.0350** |
| | 2011-2012 | 0.1199 ± 0.2659 | 43 | 0.4510 | 0.6543 |
| Plant density at population | 2010-2011 | 0.0000 ± 0.0000 | 43 | 0.1669 | 0.8682 |
| | **2011-2012** | **0.0002 ± 0.0000** | **43** | **4.7374** | **<0.0001** |
| Number of days flowering | **2010-2011** | **0.0114 ± 0.0024** | **43** | **4.7710** | **<0.0001** |
| | **2011-2012** | **0.0182 ± 0.0020** | **43** | **9.3110** | **<0.0001** |
| Maximum number of flowers opened in a census day | 2010-2011 | 0.0000 ± 0.0000 | 43 | 0.3777 | 0.7075 |
| | 2011-2012 | 0.0002 ± 0.0003 | 43 | 0.9189 | 0.3633 |
| Flowering synchrony between individuals | **2010-2011** | **5.5042 ± 0.8213** | **43** | **6.7015** | **<0.0001** |
| | **2011-2012** | **2.2227 ± 0.7026** | **43** | **3.1637** | **0.0029** |
| Femaleness | 2010-2011 | 0.5384 ± 0.5080 | 43 | 1.0598 | 0.2951 |
| | **2011-2012** | **0.8885 ± 0.4008** | **43** | **2.2170** | **0.0320** |

Table 6.

| SEEDLING ALLOGAMY | Variance (%) |
|---|---|
| Random factor | 12.95 |
| Fixed factor | 55.04 |

| Variables in the fixed factor | Coefficient | d.f. | t-value | p-value |
|---|---|---|---|---|
| Intercept | 2.2194 ± 1.8621 | 77 | 1.1919 | 0.2370 |
| Plant size | 0.3974 ± 0.4552 | 77 | 0.8729 | 0.3854 |
| Plant density at population | 0.0001 ± 0.0000 | 77 | 1.1205 | 0.2660 |
| Number of days flowering | -0.0036 ± 0.0035 | 77 | 1.0307 | 0.3059 |
| Maximum number of flowers in a census day | -0.0005 ± 0.0004 | 77 | 1.1936 | 0.2363 |
| Flowering synchrony between individuals | -1.2879 ± 1.6638 | 77 | 0.7741 | 0.4413 |
| Femaleness | **4.7881 ± 1.0399** | **77** | **4.6043** | **<0.0001** |