

## **Reproductive success of the endangered shrub *Buxus balearica* Lam. (Buxaceae): pollen limitation, and inbreeding and outbreeding depression**

**A. Lázaro and A. Traveset**

Institut Mediterrani d'Estudis Avançats (C.S.I.C.-U.I.B.), Esporles, Balears, Spain

Received August 15, 2005; accepted January 31, 2006

Published online: June 21, 2006

© Springer-Verlag 2006

**Abstract.** The increasingly common phenomenon of habitat fragmentation raises the probability of pollination failure in a number of species, as both pollen quantity and quality often decrease as populations become isolated. We experimentally investigated whether pollen was limiting reproductive success of the endangered shrub *Buxus balearica* in five populations, two continental and three insular, during 2002 and 2003. Pollen limitation varied among populations and years, but such variation was not related to density or degree of isolation. All populations showed inbreeding depression at different phases of the reproductive cycle, although its effects differed greatly among sites. Between-population outcrossing did not have a consistent effect on several components of fitness. The highest levels of inbreeding depression – detected at the level of fruit and seed set – occurred at the smallest and least fecund populations from each region. This indicates that further fragmentation of the populations of this already endangered species could certainly threaten their survival.

**Key words:** Balearic Islands, between-population crosses, fragmented populations, Iberian Peninsula, pollination failure, Mediterranean relict.

Plants typically produce many more flowers than fruits due to limitations pollen

availability, scarcity and/or inefficiency of pollination vectors, and poor pollen quality, among other factors (Burd 1994, Wilcock and Neiland 2002). During the last decade, the effects of pollen quantity and quality on reproductive success have become an important issue in plant conservation (Dudash and Fenster 2000, Hedrick and Kalinowski 2000, Rocha and Aguilar 2001, Byers 2004, Colling et al. 2004) as they are closely related to demographic and genetic factors that affect population decline and extinction.

Most endangered plant species exist as small and isolated populations, and even natural populations of widespread species are becoming increasingly fragmented. Pollen limitation has been widely reported in fragmented, isolated and sparse populations, both for anemophilous (Knapp et al. 2001, Koenig and Ashley 2003) and entomophilous taxa (Moody-Weis and Heywood 2001, Rocha and Aguilar 2001). Other consequences of fragmentation involve reductions in the number of breeding individuals within a population, gene flow among populations (Dudash and Fenster 2000) and pollination

efficiency (Jarne and Charlesworth 1993, Duncan et al. 2004). As a result, individuals in fragmented populations may be more likely to self, or cross with related individuals, than in non-fragmented ones, with subsequent negative consequences for fitness (Buza et al. 2000, Kéry et al. 2000). Moreover, small and isolated populations usually lose genetic diversity and become increasingly differentiated due to founder effects and increased genetic drift (Buza et al. 2000). Crossing among isolated populations can lead to outbreeding depression due to the loss of local adaptations in the hybrid progeny and/or the disruption of coadapted gene complexes (Dudash and Fenster 2000). Such outbreeding effects can be relevant to plan strategies for the recovery and conservation of endangered species. Both inbreeding and outbreeding depression can act at different life cycle stages of a plant and their effects may differ markedly among such stages (Ågren and Schamske 1993, Husband and Schamske 1996, Dudash and Fenster 2000).

In the present study we evaluated, by means of controlled crosses with pollen from different sources, the effect of pollen limitation, inbreeding and outbreeding on the fitness of *B. balearica*. This relict shrub is endemic to the Mediterranean Basin and its distribution has drastically decreased due to both climate change (5.000 years b.p.) and human activity during several centuries (Bonafè 1979, Yll et al. 1997). We studied fragmented populations of this species, with contrasting density and degree of isolation, at five localities in Spain: three in the Balearic Islands (Mallorca) and two in the southeastern Iberian Peninsula (eastern Andalucía). We specifically examined the levels of pollen limitation and quantified the effects of inbreeding and outbreeding in different phases of the plant reproductive cycle. We hypothesized that pollen limitation and inbreeding depression would be higher in smaller and more isolated populations.

## Materials and methods

**Study species.** *Buxus balearica* is an evergreen monoecious shrub up to 6 m tall, currently found in southern Turkey, southern Sardinia, Morocco, south-eastern part of the Iberian Peninsula and the Balearic Islands (western Mediterranean). In Spain, it is considered at risk of extinction in the Iberian Peninsula (Blanca et al. 1999) and several populations of the Balearic Islands are known to have considerably reduced their size in recent times (Bonafè 1979). The plant occurs in limestone, often on rocks, under subhumid conditions, between sea level and 1000 m (Benedí 1997). It is monoecious and a single individual can produce over 30.000 inflorescences. Each inflorescence contains one female flower surrounded by a variable number (one to six, mainly four) of male flowers, which bear an average of four anthers each; a variable number of male inflorescences can also be found in some individuals (Lázaro and Méndez, unpubl. data). The species is ambophilous (i.e. its pollination is mediated by both insects and wind), the importance of biotic pollination varying among populations (Lázaro and Traveset 2005). The range of P/O ratios was 86,058–34,209 (Lázaro, unpubl. data), indicative of xenogamous species (Cruden 1977). However, selfing is not irrelevant in this species and can occur both by geitonogamy mediated by insects and wind, and by self-deposition of pollen (Lázaro and Traveset 2005). It alternates years of high and low fecundity and, thus, behaves as a masting species. In addition, the species can also reproduce asexually, resprouting by burying its branches (Blanca et al. 1999).

**Study areas.** The two mainland populations are Cerro Gordo (Granada) and Rágol (Almería) in Andalucía (Iberian Peninsula) and the three insular populations are Sant Vicenç, Lluc, and Galatzó. Cerro Gordo is at sea level whereas Rágol is located in a mountain canyon of the Sierra de Gádor Mountains. The three Mallorcan localities are found within the Serra de Tramuntana, a mountain range oriented from northwest to southwest. Sant Vicenç occurs in a limestone cliff, Lluc in an Aleppo pine forest and Galatzó in mountain limestone cliffs facing south; these insular populations are not much separated from each other and constitute patches of different sizes. Traits of all these localities are given in Table 1.

**Pollination experiments.** To study pollen limitation and the effects of inbreeding and/or

**Table 1.** Traits of the populations of *B. balearica* examined in this study and number of individuals used for pollination experiments N. indiv. pollination exp. in each study population and year (all Selfing, Xenogamy I, Xenogamy II, and Control II treatments were conducted on the same individuals). UTM, region, elevation (H), approximate distance to the nearest population (Near. Dis), density (estimated as mean distance among individuals in the population), approximate patch sizes

POPULATION	[UTM]	REGION	H (m)	NEAR. DIS (km)	DENSITY (m)	PATCH SIZE (km <sup>2</sup> )	N. INDIV. POLLINATION EXP.	
							2002	2003
Cerro Gordo	30SVF3068	Iberian Peninsula	[0]	[5]	[1.2]	0.006	[7]	[10]
Rágol	30SWF2792	Iberian Peninsula	440	[70]	[2.1]	0.088	[12]	[12]
Sant Vicenç	31SEE0420	Balearic Islands	40	[< 1]	[0.6]	20	[10]	[10]
Lluc	31SDE9106	Balearic Islands	320	[1.5]	[1.5]	23	[9]	[10]
Galatzó	31SDD5687	Balearic Islands	900	[1.5]	[3.5]	8	[6]	[8]

outbreeding depression, we performed pollination experiments in the five populations during 2002 and 2003. In 2002, we marked 6–12 randomly-selected individuals per site (depending upon availability of reproductive individuals). In 2003, we used those same individuals that flowered again and added new individuals in some populations to increase sample size. The number of individuals used in each population and year is shown in Table 1. Pollination experiments were performed from February to March in Andalucía and from April to May in Mallorca, as the flowering period is several weeks later in the latter.

The levels of spontaneous selfing were evaluated by bagging inflorescences with gauze which prevented the passage of both insects and airborne pollen. As inflorescences have an open structure, with very high P/O ratios (see Study Species) and large overlapping between female and male phases, and because clusters of 5–10 inflorescences were bagged together, we assumed that levels of spontaneous selfing by autodeposition would be sufficient to pollinate all available ovules; for this reason we did not consider it necessary to include a treatment of induced selfing. A group of inflorescences of each individual was used as a control (open pollination;

Control I). Hand-pollination experiments were performed on previously emasculated inflorescences, using two different pollen sources: (1) from other individuals the own population (Xenogamy I), both in 2002 and in 2003, and (2) from individuals of the nearest studied population (Xenogamy II), used to detect any inbreeding/outbreeding depression at a larger spatial scale, in 2003. During the second year we also hand-pollinated using pollen from individuals the own population gathered the previous day (we called this Control II); such procedural control to Xenogamy II was used to account for the possible loss of pollen viability in the time elapsed between collection in the donor population and application in the receptor population. It was done at both mainland populations and at Lluc, where –for logistic reasons– pollen for Xenogamy II treatment was gathered also the day before hand-pollination. To perform Xenogamy II in Mallorca, we used pollen from Sant Vicenç to pollinate individuals at Lluc, and vice versa; Galatzó was pollinated with pollen from Lluc, both sites being separated by a distance of about 25 Km. Mainland populations were crossed between them (distance ca. 90 km).

In all cases, we used a mixture of pollen from 5–15 individuals, excluding pollen from the target

individual. In each individual, we selected 15–35 inflorescences distributed in 3–10 branches for each treatment and control. Branches of each treatment were pooled for data analyses.

Fruit initiation and fruit maturation (before dehiscence) were recorded during mid-May and June in Andalucía and during mid-June and July in Mallorca. We separately considered both phases to discern the effect of pollen limitation on fertilization from that on subsequent fruit abortion. Fruit set (proportion of female flowers that become fruits) was calculated in both cases. Ripe fruits were dissected in the laboratory, and the number of seeds in each of them was recorded to obtain a value of the seed set.

We calculated a pollen limitation index for each individual as  $PL = 1 - C/XI$ , where  $C$  and  $XI$  represent fruit/seed set of the control and of Xenogamy I, respectively (following Tamura and Kudo, 2000). We estimated the total production of inflorescences per individual (by counting them in a sector of the canopy and extrapolating to the entire shrub) to assess a possible association with levels of pollen limitation.

**Effect of pollen source on seed mass, viability and germination.** To detect any effect of inbreeding or outbreeding depression on seed weight, seeds obtained from the hand-pollination experiment were weighted to the nearest 0.1 mg, both in 2002 and 2003. In the fall of 2003, we also assessed depression at the seed germination stage. We separately placed seeds in trays of 60 pots, in a germination chamber at 17°C and 12 h photoperiod following Alomar and García-Delgado (2000). Trays were periodically watered to ensure permanent humidity. For each population, we used 5–20 seeds per treatment and individual depending upon availability. Germination (assessed as emergence of the hypocotyl) was monitored for the next six months until no more seeds had germinated for at least five weeks. Only seeds from one population (Rágol) germinated during the study period; hence, we performed an additional test in a sample of non-germinated seeds (two per individual and treatment) from each population in order to evaluate seed viability. For that, we introduced the seeds in a 1% solution of 2,3,5-Triphenyl-2H-tetrazolium chloride (TTC test), for 24 hours and in the dark. After this period, we dissected the seeds and scored their viability under a binocular scope. Only red-stained embryos were scored as viable (see details in Scharpf 1970).

**Inbreeding and outbreeding depression indexes.** For each individual and data gathered in 2003, inbreeding depression was estimated as  $RP_i = (w_o - w_s)/w_{\text{maximum}}$ , where  $w_o$  is the mean performance of Xenogamy I progeny,  $w_s$  is the mean performance of selfed progeny, and  $w_{\text{maximum}} = w_o$  when  $w_o \geq w_s$  and  $w_{\text{maximum}} = w_s$  when  $w_s \geq w_o$  (Ågren and Schemske 1993). The index ranges from -1 to 1, with positive values indicating inbreeding depression. Similarly, we estimated outbreeding depression as  $RP_o = (w_o - w_{op})/w_{\text{maximum}}$ , where  $w_{op}$  is the mean performance of Xenogamy II and  $w_{\text{maximum}} = w_o$  when  $w_o \geq w_{op}$ , and  $w_{\text{maximum}} = w_{op}$  when  $w_{op} \geq w_o$ . As for  $RP_i$ , positive values of the index indicate outbreeding depression. Pollen was less viable one day after collection ( $\chi^2_2 = 7.33, P = 0.026$ ), and this was consistent among populations ( $\chi^2_4 = 5.67, P \gg 0.05$ ). For the calculation of the outbreeding index in Cerro Gordo, Rágol and Lluc we thus used the Control II instead of Xenogamy I.

We estimated the inbreeding and outbreeding effects for: fruit set (1) and seed set (2), seed mass (3), seed viability, and germination for each population. As such effects may be cumulative across the plant life cycle, we further calculated a multiplicative estimate of inbreeding and outbreeding depression as  $RP_{\text{TOT}} = (w_{o123} - w_{s123})/w_{\text{maximum}}$ , and  $RP_{o\text{TOT}} = (w_{o123} - w_{op123})/w_{\text{maximum}}$ , respectively (following Affre and Thompson, 1997 and 1999). In such estimate, however, we could only include the three former variables due to insufficient data on seed viability and germination on an individual basis.

**Data analysis.** Fruit and seed set, and viability of seeds from open pollinations were compared among populations by using Generalized Linear Models (GLIMs; GENMOD proc., SAS) with binomial distributions and logit link functions. Seed mass from open pollinations was compared among populations using General linear modelling (GLM proc., SAS).

In order to detect pollen limitation and/or inbreeding or outbreeding effects, the indexes of pollen limitation, inbreeding and outbreeding depression for each population were compared to zero by means of t-tests. When indexes differed from zero, their values among populations were compared by using the error distributions (either normal or gamma) and link functions that best fitted to the observed data, based on the whole-model deviances

**Table 2.** Pollen limitation per population and year. The index of pollen limitation ( $PL = 1 - C/XI$ ) for each population was compared with zero (see text for details) by means of t-tests. Significant p-values are shown in bold. Populations with the same letter did not differ significantly

[YEAR POPULATION]	[INITIATED FRUIT SET]			[FRUIT SET]			[SEED SET]			
	MEAN ± SD	df	[P]	MEAN ± SD	df	[P]	MEAN ± SD	df	[P]	
2002 RÁGOL	0.26 ± 0.42	11	<b>0.029</b>	0.10 ± 0.75	10	0.44	0.336	9	-0.23	0.587
SANT VICENÇ	-0.83 ± 2.57	9	0.834							
LLUC	-0.19 ± 2.15	7	0.595							
GALATZÓ	0.40 ± 0.40	5	<b>0.027</b>							
2003 CERRO GORDO	0.28 ± 1.22	8	0.253	0.44 ± 0.94	8	1.39	0.101	2	0.92	0.192
RÁGOL	-0.17 ± 0.68	11	0.801	-0.12 ± 0.72	11	-0.56	0.706	11	2.18	<b>0.026</b>
SANT VICENÇ	0.53 ± 0.27	9	<b>0.0001</b>	0.57 ± 0.32	9	5.68	<b>0.0002</b>	9	2.81	<b>0.010</b>
LLUC	0.02 ± 0.23	9	0.416	-0.03 ± 0.36	9	-0.29	0.612	9	-0.94	0.814
GALATZÓ	0.28 ± 0.31	6	<b>0.027</b>	0.44 ± 0.48	6	2.40	<b>0.027</b>	2	1.00	0.178

(GENMOD proc., SAS); populations with  $n < 6$  were not included in the analyses. The association between pollen limitation and number of inflorescences was tested with a Pearson correlation.

The effects of pollination treatment and seed mass on germination rate (speed) were tested using failure-time analysis. A Cox proportional hazard regression model (Fox 2001) was fitted to the number of days between sowing and seedling emergence for each seed. Only germinated seeds were included in this analysis. Seed mass was included as a covariate. Ties were estimated with the exact method, using the program S-Plus 2000 (Mathsoft 1999). The effect of different treatments was compared to controls.

All contrasts were corrected with the sequential Bonferroni's test (Rice 1989). Means are accompanied by their standard deviations unless otherwise indicated.

## Results

**Open pollinations. Fruit and seed set.** Fruit initiation was quite low in 2002 in all populations, especially in Cerro Gordo (4.55%), where only three of the monitored individuals produced fruits. Nevertheless, there were differences among populations in initiated fruit set (71.67% at Sant Vicenç vs. 21.11% in the others;  $\chi^2_4 = 35.07, P < 0.001$ ). Final fruit set and seed set were very low in all populations except Rágol (Rágol: 27.31 % and 36.88 % vs. mean of all other populations: 3.49 % and 19.38 %, for fruit and seed set respectively). A lack of resources in this non-masting year may be responsible for the low proportion of fruits that reached maturity and this in turn may preclude detecting differences among populations.

In 2003, populations also differed in initiated fruit set ( $\chi^2_4 = 57.84, P < 0.0001$ ), final fruit set ( $\chi^2_4 = 44.40, P < 0.0001$ ), and seed set ( $F_{3,354} = 6.45, P = 0.0003$ ; data from Cerro Gordo not included: 33.33%,  $n = 2$ ). Cerro Gordo produced significantly less fruits (initiated: 8.42%; final fruit set: 7.35%) than the rest, although for final fruit set differences with Galatzó were not significant. On the other hand, Lluc produced the highest initiated fruit set (68.45%), final fruit set (61.91%), and seed

set (69.60%), although its fruit production was not significantly different from that in Rágol. The other populations showed intermediate values (initiated: 41.14%; final fruit set: 30.01%; seed set: 49.53%).

**Seed mass, viability and germination.** Seeds from Rágol were significantly heavier ( $9.92 \pm 2.63$  mg) than those from Lluc ( $5.52 \pm 1.41$  mg) and Galatzó ( $5.59 \pm 1.63$  mg), whereas Sant Vicenç ( $7.33 \pm 1.38$  mg) presented intermediate values ( $F_{3,290} = 86.43, P < 0.0001$ ). Data from Cerro Gordo were not included in the analysis due to small sample size.

Only 3.14% of control seeds germinated during the study period in Rágol, whereas no control seeds from the other populations germinated. TTC test showed that no control seeds from Cerro Gordo and Galatzó were viable in 2003. Percentage of viable seeds did not differ significantly among the other populations ( $\chi^2_2 = 2.33, P = 0.311$ ), ranging between 25% and 56%.

**Pollen limitation.** Pollen addition increased fruit and seed set in *B. balearica* by an average of 13% and 5%, respectively. In 2002, only Rágol and Galatzó showed to be pollen limited at the time of fruit initiation (Table 2). These populations did not differ significantly in pollen limitation indexes ( $\chi^2 = 0.52, P = 0.473$ ). Due to low flower and fruit production, pollen limitation on both fruit and seed set could only be estimated for Rágol, where pollen appeared to be not limiting (Table 2). The differences in pollen limitation between initiated and final fruit set in this population and year are due to the fruit abortion taking place before maturation.

In 2003, fruit initiation, fruit set and seed set were all limited by pollen only at Sant Vicenç; pollen limited initial and mature fruit set at Galatzó and seed set at Rágol; in contrast, pollen was not limiting in the other populations (Table 2). Sant Vicenç and Galatzó had similar indexes of pollen limitation for both initiated fruits and final fruit set ( $\chi^2 = 2.28, P = 0.131$  and  $\chi^2 = 0.48, P = 0.491$ , respectively; Table 2). Finally, pollen limitation estimated with seed set data did not differ between Rágol and

Sant Vicenç ( $\chi^2 = 0.02, P = 0.885$ ; Table 2). At Rágol, no differences between years were found for either fruit or seed set ( $\chi^2 = 0.05$ , and  $\chi^2 = 0.12, P \gg 0.05$ , respectively).

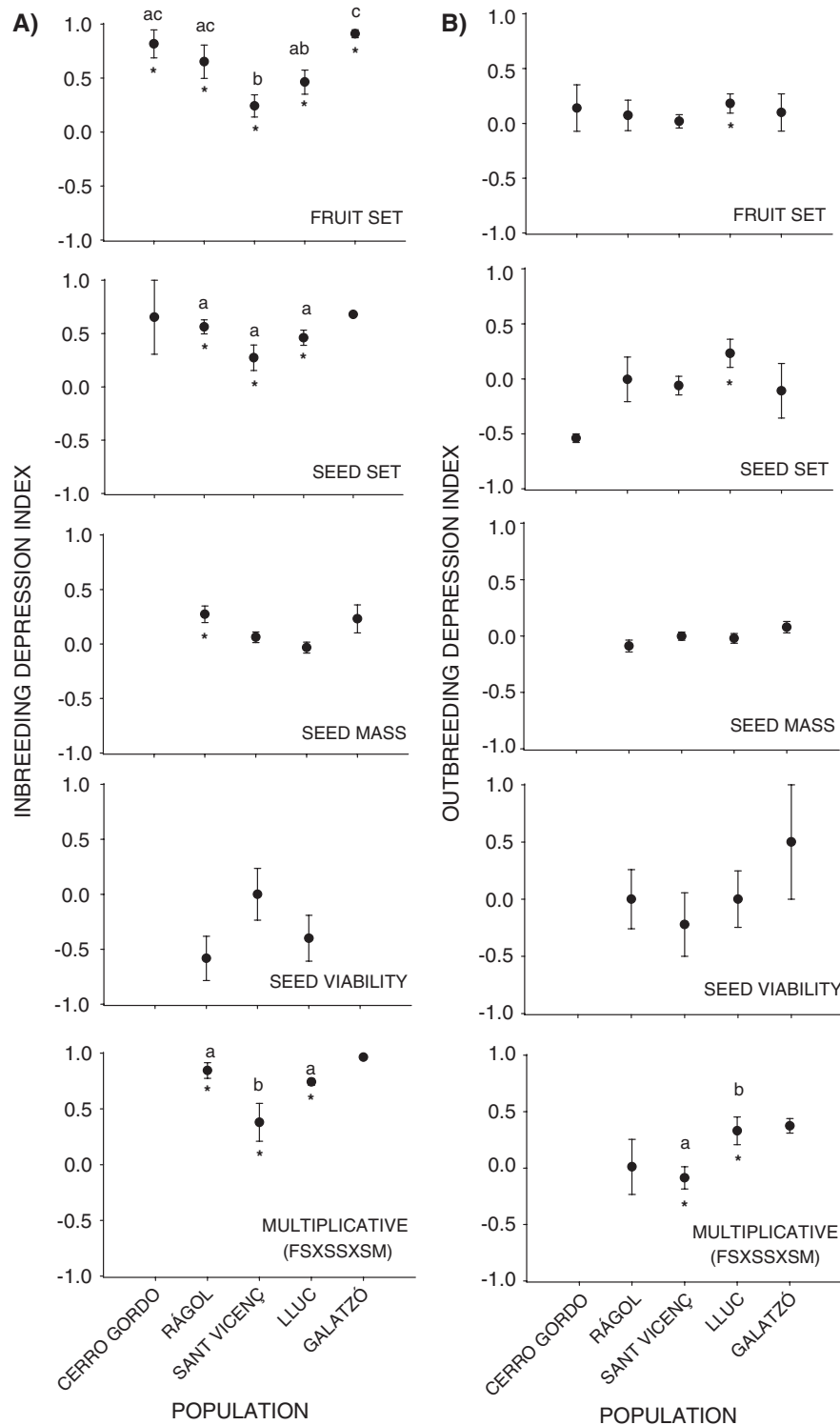
When all populations were pooled, pollen limitation indexes (using fruit set data) showed a marginally significant negative correlation with the number of inflorescences per plant ( $r = -0.280, P = 0.054, n = 48$ ), suggesting that more fecund individuals are less pollen-limited than less fecund ones.

**Inbreeding and outbreeding depression in different phases.** All populations showed inbreeding depression ( $RP_i$ ) for fruit set (all  $P < 0.02$ ) and seed set (all  $P < 0.04$ ; Fig. 1A). However,  $RP_i$  for seed mass was found only in Rágol ( $t_{11} = 3.60, P = 0.002$ ; Fig. 1A), while  $RP_i$  for seed viability was not detected in any population (all  $P \gg 0.05$ ; Fig. 1A). The multiplicative inbreeding index ( $RP_{i\ 123}$ ) showed depression in all populations (all  $P < 0.03$ ; Fig. 1A).

At the level of fruit set,  $RP_i$  was significantly lower at Sant Vicenç than at the two mainland populations (Cerro Gordo and Rágol) and Galatzó ( $\chi^2_4 = 2.27, P < 0.0001$ ; Fig. 1A). In contrast,  $RP_i$  for seed set did not differ among populations ( $\chi^2_2 = 1.05, P = 0.59$ ). Differences were found among populations ( $\chi^2_2 = 5.70, P = 0.048$ ; Fig. 1A), when we estimated the cumulative inbreeding depression, Sant Vicenç showing the lowest values.

Outbreeding depression ( $RP_o$ ) was found for fruit set ( $t_9 = 2.06, P = 0.035$ ; Fig. 1B), seed set (differences marginally significant;  $t_9 = 1.81, P = 0.052$ ; Fig. 1B) and for the multiplicative estimate ( $RP_{o\ 123}$ ;  $t_9 = 2.70, P = 0.012$ ; Fig. 1B) only in Lluc. In Sant Vicenç,  $RP_{o\ 123}$  also differed from zero, but in this case xenogamous pollen increased fitness (i.e. the index had a negative significant value) ( $t_6 = 5.82, P = 0.0006$ ; Fig. 1B). These two populations differed in  $RP_o$  ( $\chi^2_2 = 6.06, P = 0.014$ ; Fig. 1B). In contrast, the other populations did not show outbreeding depression in any of the phases (all  $P > 0.05$ ).

$RP_i$  and  $RP_o$  for germination could be obtained only from seeds of Rágol and were calculated on a treatment basis, pooling all



**Fig. 1.** Indexes of Inbreeding and Outbreeding Depression. MULTIPLICATIVE (FS\*SS\*SM) means the multiplicative estimate calculated with the fruit set, seed set, and seed mass performances. Only populations with  $n > 6$  were considered in the analyses. Asterisks indicate that the index was significantly different from zero. For each variable, values with the same letter were not significantly different among populations (populations were compared only when the indexes were significantly different from zero)

individuals in the population. The index of inbreeding depression was maximum for germination whereas that of outbreeding depression had a negative value, what suggests a better germination of crosses between than within populations.

Germination rate (speed) was tested for Rágol in 2003, comparing pollination treatments with controls. Seeds from Xenogamy I germinated faster than control seeds ( $\chi^2 = 2.00, P = 0.046$ ; Fig. 2), whereas all the other treatments did not differ significantly from controls. Heavier seeds were also found to germinate faster ( $\chi^2 = 2.84, P = 0.005$ ).

## Discussion

**Pollen limitation.** Pollen limitation has been documented for a large number of species and has been found to vary much both at a spatial and temporal scale even within a species (Wilcock and Neiland 2002, Ashman et al. 2004). There may be at least two ultimate causes of such pollen limitation: (1) recent ecological perturbations (for instance, habitat fragmentation or decrease in population size) and (2) stochastic rather than a constant pollination environment (Ashman et al. 2004). Regarding the former, an association between pollen limitation and plant density or plant isolation has been frequently reported for species either pollinated by insects (Kéry et al. 2000, Rocha and Aguilar 2001, Colling et al. 2004) or by wind (Allison 1990, Knapp et al. 2001, Sork et al. 2002, Koenig and Ashley 2003). Such association may be due to a decline in pollinator visits to sparse and isolated patches and/or to a decrease in the reception of conspecific pollen grains. In *B. balearica*, however, pollen limitation does not appear to be related either to isolation or density. At least at Sant Vicenç and Rágol, pollination limitation seems to depend more upon the particular and stochastic pollination environment than on fragmentation characteristics. The former is exposed to strong winds and has few insect visits to flowers (indeed, not a single insect was observed

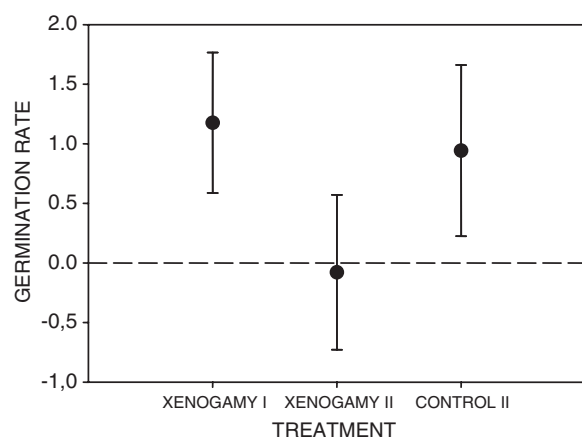
during our censuses; Lázaro and Traveset 2005), what could partly explain the pollen limitation detected at this site; the humidity in this coastal site may further increase pollen limitation, as found for other wind-pollinated species (Wilcock and Neiland 2002). At Rágol, in contrast, insect flower visitation positively contributes to seed set (Lázaro and Traveset 2005), and temporal variations in insect abundance might be responsible for the temporal variations in pollen limitation.

Larger flower crops of *B. balearica* tended to be less-pollen limited than small ones, as reported for other species (Dudash 1993, Lawrence 1993). This fact might be due to floral display effects on pollinator attraction, as observed in other species (e.g. Devlin et al. 1992, Torres et al. 2002, Holland et al. 2004). Indeed, we found a higher frequency of insect visits in larger crops at Rágol –the population with most insect visits–, although this was not consistent in all populations (Lázaro and Traveset 2005). Alternatively, larger flower crops might have relatively greater levels of geitonogamy, which could act as reproductive assurance against pollen limitation, as proposed for *Scorzonera humilis* by Colling et al. (2004).

Nevertheless, the limitations of our experimental approach make us to be cautious interpreting these results. First, the low availability of reproductive individuals in some populations of this rare species necessarily makes decrease sample size. In addition, to more accurately assess a relationship between fragmentation and pollen limitation we would need to examine a higher number of populations. A study encompassing more years would also allow testing the consistency of our results. Moreover, the unknown level of pollen manipulation, the possible quality differences between supplement and control treatments, and the probable effect of reallocation of resources, are intrinsic limitations in this type of experimental approach that could affect the results (Ashman et al. 2004).

**Inbreeding and outbreeding depression in *Buxus balearica*.** Decrease in different components of fitness, such as fruit and seed set,





**Fig. 2.** Germination rate of seeds from each treatment in a germination chamber (data are from Rágol, the only population from which seeds germinated). Parameter estimates were obtained from separate Cox regressions (mean  $\pm$  SE). Data are expressed setting control treatments to zero

seed mass, and seed germination, promoted by self-pollination compared to outcross pollination has been reported for many species (e.g. Waser and Price 1991, Hauser and Loeschke 1996, Olesen et al. 1998, Affre and Thompson 1999). In *B. balearica*, we found evidence of inbreeding depression at the level of fruit and seed set in all populations, and at the level of seed mass and germination in one of them (Rágol). Moreover, the addition of xenogamous pollen within a population significantly increased the quality of seeds what suggests that, at least the qualitative components of fitness of the naturally pollinated individuals may be limited due to high levels of self-fertilization.

The finding that the levels of inbreeding depression varied much among populations of *B. balearica* is interesting, both from an ecological and conservation viewpoint. In general, endangered species show greater variation in inbreeding depression across populations than common species (Hedrick and Kalinowski 2000), which is attributed to genetic drift and to the variable ability of purging deleterious alleles (Byers and Waller 1999). Moreover, levels of inbreeding

depression may also depend upon factors such as dominance and/or overdominance (Dudash and Fenster 2000) and upon the breeding history of the population (Lande and Schemske 1985, Husband and Schemske 1996). The extent of inbreeding depression is usually greater in small populations (Byers 2004, Buza et al. 2000, Kéry et al. 2000). However, over time, purging of genetic load may restore or maintain the mean individual fitness to the level found in larger populations, and thus, it can lead to a reduction in inbreeding depression (Hauser and Loeschke 1994, and references therein). We did find the highest levels of inbreeding depression –at the level of fruit and seed set– at the smallest and least fecund populations from each region (Cerro Gordo and Galatzó, whereas the lowest levels at the densest population (Sant Vicenç). Nonetheless, there was no clear pattern relating inbreeding depression with population density or isolation, what could be attributed to the relatively recent decrease in size of some of these populations, i.e. such effects may have not become apparent yet, as suggested for *Lychnis flos-cuculi* by Hauser and Loeschke (1994).

The effects of outbreeding in *B. balearica* were much more limited, appearing only in two of the five studied populations (specifically at Lluc and at Sant Vicenç) and not as consistent as those found for inbreeding. Outbreeding depression has been found at very different scales (within populations as well as between populations separated by different distances; Dudash and Fenster 2000, and references therein), and rather contrasting results have been reported in the literature for long-distance pollinations: a decreased fitness (Fenster and Galloway 2000), a similar fitness (Schlichting and Devlin 1992, Sork and Schemske 1992), and an increased fitness (Hauser and Loeschke 1994, Colling et al. 2004). We suggest that results for outbreeding success in *B. balearica* are due to (1) the greater genetic variation within than among populations and (2) to the different pollen performances among populations, as observed in *Silene nutans* (Hauser and

Siegismund 2000). Indeed, pollen performance (germination and size) differs among populations of *B. balearica* (Lázaro and Méndez, unpubl. data) and the effect of exogenous pollen on small populations or little fecund populations, as Cerro Gordo or Galatzó, seem to have a positive effect on fruit set, in contrast to the tendency observed in the larger and more fecund populations, Rágol and Lluc (Lázaro and Traveset 2005). A more comprehensive assessment of offspring fitness (Waser and Price 1989) would certainly be desirable to assess the effect of outbreeding depression in this species.

We thank Amparo Castillo, Javier Rodríguez, and Vicente Fernández for their help during field work. John Thompson, Marcos Méndez, Miguel Verdú, and Luis Santamaría critically commented an early version of this manuscript. J. M. Gutiérrez provided data on patch size of the Balearic populations. We thank the 'Consejería de Medio Ambiente de la Junta de Andalucía' for allowing our field work in the Andalusian populations. This study was supported by a FPU fellowship from the Spanish Ministry of Education conceded to A. L. and by the project BOS2001–0610 from the Spanish Ministry of Science financed to A. T.

## References

- Affre L., Thompson J. D. (1997) Population genetic structure and levels of inbreeding depression in the Mediterranean island endemic *Cyclamen creticum* (Primulaceae). *Biol. J. Linn. Soc.* 60: 527–549.
- Affre L., Thompson J. D. (1999) Variation in self-fertility, inbreeding depression and levels of inbreeding in four *Cyclamen* species. *J. Evol. Biol.* 12: 113–122.
- Ågren J., Schemske D. W. (1993) Outcrossing rate and inbreeding depression in two annual monoecious herbs, *Begonia hirsuta* and *B. semiovata*. *Evolution* 47: 125–135.
- Allison T. D. (1990) Pollen production and plant density affect pollination and seed production in *Taxus canadensis*. *Ecology* 71: 516–522.
- Alomar G., García-Delgado A. (2000) Reproducció de planta autòctona per a l'ús en repoblacions forestals, paisagisme i jardineria. Govern de les Illes Balears. Conselleria de Medi Ambient.
- Direcció General de Biodiversitat, Servei de Gestió Forestal i Protecció del Sòl.
- Ashman T. L., Knight T. M., Steets J. A., Amarasekare P., Burd M., Campbell D. R., Dudash M. R., Johnston M. O., Mazer S. J., Mitchell R. J., Morgan M. T., Wilson W. G. (2004) Pollen limitation of plant reproduction: ecological and evolutionary causes and consequences. *Ecology* 85: 2408–2421.
- Benedí C. (1997) *Buxus balearica*. In: Castroviejo S., Aedo C., Benedí C., Laínz M., Muñoz Garmendia F., Nieto Feliner G., Paiva J. (eds.) *Flora Iberica*. Vol. VIII. Real Jardín Botánico, CSIC, pp. 187–189.
- Blanca G., Cabezudo B., Hernández-Bermejo J. E., Herrera C. M., Molero Mesa J., Muñoz J., Valdés B. (1999) Consejería de Medio Ambiente. Junta de Andalucía. Libro Rojo de la flora silvestre amenazada de Andalucía. Tomo I: especies en peligro de extinción, pp. 75–78.
- Bonafè F. (1979) *Flora de Mallorca* Vol. III Editorial Moll, Mallorca, p. 149.
- Burd M. (1994) Bateman's principle and plant reproduction: the role of pollen limitation in fruit and seed set. *Bot. Rev.* 60: 83–139.
- Buza L., Young A., Thrall P. (2000) Genetic erosion, inbreeding and reduced fitness in fragmented populations of the endangered tetraploid pea *Swainsona recta*. *Biol. Conserv.* 93: 177–186.
- Byers D. L., Waller D. M. (1999) Do plant populations purge their genetic load? Effects of population size and mating history on inbreeding depression. *Annual Rev. Ecol. Syst.* 30: 479–513.
- Byers D. L. (2004) Pollen quantity and quality as explanations for low seed set in small populations exemplified by *Eupatorium* (Asteraceae). *Amer. J. Bot.* 82: 1000–1006.
- Colling G., Reckinger C., Matthies D. (2004) Effects of pollen quantity and quality on reproduction and offspring vigor in the rare plant *Scorzonera humilis* (Asteraceae). *Amer. J. Bot.* 91: 1774–1782.
- Cruden R. W. (1977) Pollen-ovule ratios: a conservative indicator of breeding systems in flowering plants. *Evolution* 31: 32–46.
- Devlin B., Clegg J., Ellstrand N. C. (1992) The effect of flower production on male reproductive success in wild radish populations. *Evolution* 46: 1030–1042.
- Dudash M. R. (1993) Variation in pollen limitation among individuals of *Sabatia angularis* (Gentianaceae). *Ecology* 74: 959–962.

- Dudash M. R., Fenster C. B. (2000) Inbreeding and outbreeding depression in fragmented populations. In: Young A., Clarke G. (eds.) Genetics, demography and viability of fragmented populations. Cambridge University Press, Cambridge, United Kingdom, pp. 35–53.
- Duncan D. H., Nicotra A. B., Wood J. T., Cunningham S. A. (2004) Plant isolation reduces outcross pollen receipt in a partially self-compatible herb. *J. Ecol.* 92: 977–985.
- Fenster C. B., Galloway L. F. (2000) Inbreeding and outbreeding depression in natural populations of *Chamaecrista fasciculata*. *Conserv. Biol.* 14: 1406–1412.
- Fox G. A. (2001) Failure-time analysis: studying times to events and rates at which events occur. In: Scheiner S. M., Gurevitch J. (eds.) Design and analysis of ecological experiments. Oxford University Press, New York, pp. 235–266.
- Hauser T. P., Loeschke V. (1994) Inbreeding depression and mating-distance dependent offspring fitness in large and small populations of *Lychnis flos-cuculi* (Caryophyllaceae). *J. Evol. Biol.* 7: 609–622.
- Hauser T. P., Loeschke V. (1996) Drought stress and inbreeding depression in *Lychnis flos-cuculi* (Caryophyllaceae). *Evol.* 50: 1119–1126.
- Hauser T. P., Siegismund H. R. (2000) Inbreeding and outbreeding effects on pollen fitness and zygote survival in *Silene nutans* (Caryophyllaceae). *J. Evol. Biol.* 13: 446–454.
- Hedrick P. W., Kalinowski S. T. (2000) Inbreeding depression in conservation biology. *Annual Rev. Ecol. Syst.* 31: 139–162.
- Holland J. N., Bronstein J. L., DeAngelis D. L. (2004) Testing hypotheses for excess flower production and low fruit-to-flower ratios in a pollinating seed-consuming mutualism. *Oikos* 105: 633–640.
- Husband B. C., Schemske D. W. (1996) Evolution of the magnitude and timing of inbreeding depression in plants. *Evolution* 50: 54–70.
- Jarne P., Charlesworth D. (1993) The evolution of the selfing rate in functionally hermaphrodite plants and animals. *Annual Rev. Ecol. Syst.* 24: 441–466.
- Kéry M., Matthies D., Spillmann H. H. (2000) Reduced fecundity and offspring performance in small populations of the declining grassland plants *Primula veris* and *Gentiana lutea*. *J. Ecol.* 88: 17–30.
- Knapp E. E., Goedde M. A., Rice K. J. (2001) Pollen-limited reproduction in blue oak: implications for wind-pollination in fragmented populations. *Oecologia* 128: 48–55.
- Koenig W. D., Ashley M. V. (2003) Is pollen limited? The answer is blowin' in the wind. *Trends Ecol. Evol.* 18: 157–159.
- Lande R., Schemske D. W. (1985) The evolution of self-fertilization and inbreeding depression in plants. I. Genetic models. *Evolution* 39: 24–40.
- Lawrence W. S. (1993) Resource and pollen limitation: plant size-dependent reproductive patterns in *Physalis longifolia*. *Amer. Naturalist* 141: 296–313.
- Lázaro A., Traveset A. (2005) Spatio-temporal variation in the pollination mode of *Buxus balearica* (Buxaceae), an ambophilous and selfing species: mainland-island comparison. *Ecography* 28: 640–652.
- Mathsoft (1999) S-Plus 2000. Guide to Statistics, Vol. 2. Seattle: Mathsoft Inc.
- Moody-Weis J., Heywood J. S. (2001) Pollination limitation to reproductive success in the Missouri evening primrose, *Oenothera macrocarpa* (Onagraceae). *Amer. J. Bot.* 88: 1615–1622.
- Olesen J. M., Forfang A. S., Báez M. (1998) Stress-induced male sterility and mixed mating in the island plant *Cedronella canariensis* (Lamiaceae). *Pl. Syst. Evol.* 212: 159–176.
- Rice W. R. (1989) Analyzing tables of statistical tests. *Evolution* 43: 223–225.
- Rocha O. J., Aguilar G. (2001) Reproductive biology of the dry forest tree *Enterolobium cyclocarpum* (Guanacaste) in Costa Rica: a comparison between trees left in pastures and in continuous forest. *Amer. J. Bot.* 88: 1607–1614.
- Scharpf R. F. (1970) Seed viability germination and radicle growth of dwarf mistletoe in California. USDA Forest Service Research, Paper PSW-59, California
- Schlichting C. D., Devlin B. (1992). Pollen and ovule sources affect seed production of *Lobelia cardinalis* (Lobeliaceae). *Amer. J. Bot.* 79: 891–898.
- SAS Institute Inc. (1997) SAS/STAT Software: Changes and enhancements through release 6.12. SAS Institute, Cary, North Carolina, USA.
- Sork V. L., Schemske D. W. (1992) Fitness consequences of Mixed-Donor Pollen loads in the annual legume *Chamaerista fasciculata*. *Amer. J. Bot.* 79: 508–515.

- Sork V. L., Davis F. W., Smouse P. E., Apsit V. J., Dyer R. J., Fernandez M. F. J., Kuhn B. (2002) Pollen movement in declining populations of California Valley oak, *Quercus lobata*: where have all the fathers gone? *Molec. Ecol.* 11: 1657–1668.
- Tamura S., Kudo G. (2000) Wind pollination and insect pollination of two temperate willow species, *Salix miyabeana* and *Salix sachalinensis*. *Plant. Ecol.* 147: 185–192.
- Torres E., Iriondo J. M., Perez C. (2002) Vulnerability and determinants of reproductive success in the narrow endemic *Antirrhinum microphyllum* (Scrophulariaceae). *Amer. J. Bot.* 89: 1171–1179.
- Waser N. M., Price M. V. (1989) Optimal outcrossing in *Ipomopsis aggregata*: seed set and offspring fitness. *Evolution* 43: 1097–1109.
- Waser N. M., Price M. V. (1991) Reproductive costs of self-pollination in *Ipomopsis aggregata* (Polemoniaceae): are ovules usurped? *Amer. J. Bot.* 78: 1036–1043.
- Wilcock C., Neiland R. (2002) Pollination failure in plants: why it happens and when it matters. *Trends Ecol. Evol.* 7: 270–277.
- Yll R., Pérez-Obiol R., Pantaleón-Cano J., Roure J. M. (1997) Palynological evidence of climatic change and human activity during the Holocene on Minorca (Balearic Islands). *Quaternary Res.* 48: 339–347.

Address of the authors: Amparo Lázaro (e-mail: vieaal@uib.es) and Anna Traveset, Institut Mediterrani d'Estudis Avançats (C.S.I.C.-U.I.B.), C/Miquel Marqués n° 21, 07190 Esporles, Balears, Spain.