

Effect of population combinations on the reproductive success and germination of seeds of *Bromus auleticus* (Poaceae)

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Abstract

Bromus auleticus is known for its qualities as forage but its domestication has limitations relating to seed production technology and farm-scale seed propagation. The effect of different combinations of population on the reproductive success and seed germination of a relict population of Argentina was studied. The tests used a population of Santa Fe (SF) as the recipient of pollen and three populations as donors (LP: La Pampa, ER: Entre Ríos and BA: Buenos Aires). Reproductive success was determined in plants from five pollination treatments: (i) (SFxSF), (ii) LP, (iii) ER, (iv) BA and (v) mixture of donor. Three temperature conditions were evaluated for germination: (i) 15°C, (ii) 28°C and (iii) alternating 15–28°C (12 h each). There were significant differences in reproductive success depending on the source of pollen. Seeds obtained from new genotypic combinations did not increase their weight in relation to the pollen donor. Germination behaviour was more affected by extrinsic factors (temperature) than by their genetic origin. However, under non-optimal germination conditions, seeds originating from new genotypic combinations showed a significant increase in germination percentage and germination rate. This work contributes additional knowledge to previous studies regarding the effect of pollen quality limitation on seed production and quality of *B. auleticus*. The results confirm the hypothesis that a mixture of genotypes from a diverse origin increases the quantity of flowers that become fruits and seed quality, which is of great importance to improved seed production and field establishment of *B. auleticus*.

Keywords: fruit/flower ratio, Pooideae, self-infertility

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Received 4 July 2012; revised 2 September 2013

Introduction

Bromus auleticus Trin. ex Nees belongs to the Pooideae subfamily and the Bromeae tribe (Clayton and Renvoize, 1986), and is commonly known as ‘cebadilla chaqueña’ (Argentina and Uruguay) or ‘cevadilha vacariana’ (Brazil). It is a South American native perennial grass with an autumn–winter–spring cycle (Longhi, 1977; Gutiérrez and Pensiero, 1998). It has been considered a potentially promising forage species (Burkart, 1969; Covas, 1978) because of its high forage quality (Oliveira and Moraes, 1993; Gasser *et al.*, 1996) in a critical period of the year (e.g. winter) and a yearly dry-matter production similar to that of other forage species like tall fescue (*Festuca arundinacea* Schreb) (Romero and Ruíz, 1997). In Argentina, this species occurs in a fragmented fashion in the central region (Gutiérrez and Pensiero, 2012), with some populations undergoing a process of drastic reduction in size due to anthropic action.

Even though *B. auleticus* is remarkable for its forage qualities, the quantity of seeds produced in natural populations and cultivars is variable (Burkart, 1969; Millot *et al.*, 1990; Ruíz and Covas, 2004). Millot *et al.* (1990) determined in 100 accessions an average seed production of 376 kg ha⁻¹, with extremes ranging from 21 to 853 kg ha⁻¹. Medium seed production (400 kg ha⁻¹) was obtained even when applying nitrogen fertilizer equivalent to 200 kg urea per hectare (De Batista and Costa, 1997). Similarly, seed production was also close to the average value when using nitrogen fertilizer in *Bromus auleticus* cv. ‘Pampera INTA’ in Argentina (Ruíz and Covas, 2004). Within yield components, the percentage of fruit-forming flowers also shows considerable variability, with reported values ranging from 30 to 70% (Ruíz and Covas, 2004).

A relict population of *B. auleticus* with a low number of individuals was found on the edge of a forest in the Province of Santa Fe (Argentina) (population SF, Table 1). Although this population stands out for its forage quality (leaf/stem ratio), it presents a low

Table 1 Origin of the populations studied.

Population	Province (Department)	Town	Collection site (Lat./Long.)
SF	Santa Fe (Castellanos)	Lehmann	S 31° 09'W 61° 18'
LP	La Pampa (Realicó)	Realicó	S 34° 56'W 63° 38'
ER	Entre Ríos (Uruguay)	Concepción del Uruguay	S 32° 28'W 58° 13'
BA	Buenos Aires (Pdo. de la Costa)	San Bernardo	S 36° 38'W 56° 42'

percentage of seed set (Vegetti, 1997; Gutiérrez *et al.*, 2006b). This population was the only one detected in the Province of Santa Fe (133 007 km²) despite the numerous scientific expeditions conducted. Greenhouse tests on supplementation with pollen from different sources were performed for the said population, and although the contribution made by donors increased population fertility, seed production could not be raised beyond 40% (Gutiérrez *et al.*, 2006b). These data, along with the values reported in the literature (regarding seed production and fruit-forming flowers), allowed us to assume that limitation by pollen is one of the reasons for low seed production.

Aizen and Harder (2007) indicated that limitation by pollen has two components: limitation by pollen quantity and quality, the latter being more common than thought. In the case of wind pollination of allogamous species, the quantity of pollen arriving at the stigma is related to the density of donors (number of individuals and distance to the source), whereas the quality of pollen is directly linked to its genetic constitution, the ends of which are represented by inbreeding depression (Charlesworth and Charlesworth, 1987) and outbreeding depression (Waser and Price, 1991). The former implies the deposition of the species' own pollen or that coming from genetically related donors, while the latter entails cross-pollination with very distant plants (Ashmann *et al.*, 2004; Aizen and Harder, 2007). Seed production may be reduced in both cases.

Another aspect relating to limitation by pollen quality is that, due to habitat fragmentation, plants could generate seeds of a lower quality because small and isolated populations are more likely to experience cross-breeding in the same plant or among neighbouring plants that are usually related (Ellstrand and Elam, 1993; Routley *et al.*, 1999). In this case, limitation by pollen would lead to inbreeding depression, which manifests as a reduction in the viability, size or germination capacity of the seeds produced (Henriquez, 2004). In the case of *B. auleticus*, a poor establishment in open fields was reported (Silva *et al.*, 2007) as related to seed quality. Thus, the seed production of *B. auleticus* is variable, not only in yield but also in quality (Ruíz *et al.*, 2006). Therefore, it is important to be able to determine seed quality to obtain a correct field establishment (Olmos, 1993). This has led to

research on germination requirements (Ruíz *et al.*, 2006), seed dormancy (Silva *et al.*, 2002), optimum harvest time (Ruíz *et al.*, 2003) and the effect of seed weight on germination (Silva *et al.*, 2007). For this species, most reports indicate optimal germination temperatures close to 15–20°C (Ruíz *et al.*, 2006) and the presence of dormancy induced by temperatures of higher than 28°C (Olmos, 1993; Silva *et al.*, 2001).

The present work had the objective to quantify the limitation by pollen quality of a relict population of *B. auleticus*, assessing the increase in fertility and/or germination of the seeds obtained from cross-breeding with different pollen donors. Explicitly, we sought answers to the following questions: (i) What effect does limitation by pollen quality have on the fruit/flower ratio? (ii) Is there a variation in the remaining yield components? and (iii) Do seeds obtained from cross-breeding with different donors differ in quality (higher weight) or have a different germination behaviour?

Materials and methods

Plant materials

Trials were carried out in Esperanza, Argentina (31° 27'S, 60° 56'W) with populations from four different Argentine origins (Table 1). A population from Santa Fe (SF) was used as the recipient of pollen, and three populations were used as donors (LP: La Pampa, ER: Entre Ríos, and BA: Buenos Aires).

Ripened inflorescences were harvested in mass in every population of no less than 50 plants per place of origin separated by a minimum of 5 m to ensure that a representative sample was obtained from each population. Inflorescences were threshed by hand, and caryopses were germinated at 15°C in plastic trays containing three Whatman paper discs and moistened with distilled water. Seedlings were transplanted, placed in 5-L pots and then kept in the vegetative stage by means of periodic cuttings up to the moment of cloning and transplanting.

Experimental design

The plants of the SF population were used as pollen receptors in all treatments (i.e. with the purpose of

evaluating the same genotypes in all treatments). The experimental design allowed every experimental unit (SF) that acted as a parent (pollen receptor) to have a high probability of being fertilized by any pollen from five different sources (SF-LP-ER-BA or a mixture of them). The SF \times SF treatment was regarded as the control treatment. In open field, the receptor genotypes were placed in the centre of a hexagon with its vertices placed at the donor genotypes. Donor plants were 30 cm from the receptor plant. When the successive hexagons of the same trial were joined, a spatial configuration (similar to a bee hive) was obtained (Figure 1). Each of the five treatments consisted of 14 genotypes of SF that acted as pollen receptors and 42 donor plants (two clones of 21 genotypes) (Table 2). The experimental unit was the pollen receptor plant. This design was similar to one used in work with other species like *Agrostis* sp. (Belanger *et al.*, 2003), *Avena fatua* (Murray *et al.*, 2003), *Amsinckia* sp. (Chep-tou and Schoen, 2003), *Eucalyptus* sp. (Campinhos *et al.*, 1998) and *Datura stramonium* (Motten and Antono- vics, 1992).

Due to the fact that this species is wind pollinated and the flowering phenology of these precedences coincide in time (Gutiérrez *et al.*, 2006a), the plots for crossing were placed at a distance no less than 600 m and in the direction of the predominant wind so as to avoid pollen contact among treatments. Once the transplanting process was finished, the plots were periodically watered and fertilized with urea (N) every 15 d and superphosphate (P), up to 3 weeks after the

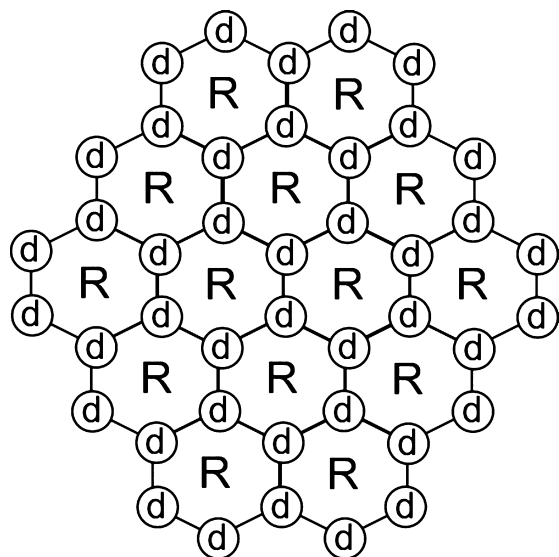


Figure 1 Spatial configuration of the treatments, where 14 receptor genotypes (R) are surrounded by 42 donor plants (d) (2 clones from 21 genotypes).

Table 2 Treatment configuration with different pollen donors.

Treatment	Receptors (cloned SF genotypes)	Pollen donors genotypes (clones)			
		SF	LP	ER	BA
Control	14	21 (42)*	–	–	–
Donor LP	14	–	21 (42)	–	–
Donor ER	14	–	–	21 (42)	–
Donor BA	14	–	–	–	21 (42)
Donors mixture	14	–	14	14	14

*In Control (SF \times SF), the 21 genotypes used as pollen donors were different from the 14 used as pollen receptors.

beginning of flowering (flower anthesis), to avoid limitation in reproductive success due to a lack of nutrients.

The reproductive success of the SF genotypes and the fruits produced in relation to the flowers (florets) that made each spikelet was determined for every pollination treatment. Spikelets are multiflowered in *B. auleticus* with every floret having an hermaphrodite flower. Following Wiens *et al.* (1987), the pre-emergent reproductive success of one species can be assessed as the product of the relationships fruit/flower and seed/ovule. As in the grass family fruits are uniovulated, the fraction corresponding to the seed/ovule ratio is equal to one, which is the reason why the pre-emergent reproductive success for the species is defined by the fruit/flower ratio.

The variation in reproductive success was determined as the proportion of flowers able to give fruits (fruit/flower ratio) in two panicles per experimental unit and per treatment. In all cases, harvested inflorescences belonged to the first three reproductive tillers, responsible for 75% of the final yield (Maia *et al.*, 2006). So as to evaluate the variability in other yield components, we determined the: (i) number of panicles per plant, (ii) number of spikelets per panicle, (iii) number of florets per spikelet and (iv) weight of 1000 seeds. The physiological maturity of receptor genotypes was established in agreement with the criterion proposed by Ruíz *et al.* (2003). The variation in number of panicles per plant, as the average number of inflorescences from the receptor genotypes for every analysed treatment ($n = 70$), was also determined at that time. A harvest of all the inflorescences from the receptor plants was made 20 d after physiological maturity and kept in labelled envelopes. The variation

in number of spikelets per panicle was determined in three inflorescences per experimental unit and per treatment ($n = 210$). This value was expressed as the average number of spikelets of the receptor genotypes for every analysed treatment. The variation in number of florets per spikelet was determined for two other panicles per experimental unit and per treatment. Ten spikelets placed at the apical, middle and basal sites of the inflorescence were taken from every panicle ($n = 1400$). This value was expressed as the average of the number of florets from the receptor genotypes for every analysed treatment. The variation in weight of 1000 seeds was quantified as an indirect indicator of seed size. Consequently, all harvested fruits (seeds) were placed in envelopes so as to make seed pools for treatments from which eight samples of 500 seeds per population were weighed. The values obtained were expressed in grams as the weight of 1000 seeds.

An independent trial was performed to test self-infertility. It consisted of isolating three receptor genotypes of SF from other conspecific pollen pools by a distance not <600 m. From every 'isolated plant', two inflorescences were randomly taken, and 15 spikelets from different sectors ($n = 90$ spikelets) were analysed. The quantity of caryopses present per spikelet was recorded.

Germination behaviour was evaluated in seeds from SF plants (pollen receptors) under five treatments of pollen donors. Caryopses were gathered in a pool per pollination treatment and kept in paper bags at room temperature for up to 3 months after harvest. Before sowing harvested caryopses in petri dishes, they were disinfected with 1% sodium hypochlorite for 1 min and then rinsed in abundant water. Every sample consisted of 250 caryopses per treatment (five repetitions of 50 caryopses per treatment). Three temperature conditions were evaluated in a germination chamber without supplementary light, at two constant (15 and 28°C) and one alternating temperatures (15 and 28°C, 12 h each). Counts were carried out at 7, 14 and 21 d after germination (Maia *et al.*, 2006). When the radicle appeared with a minimum length of 2 mm, germination was considered to have begun. Germination percentage and germination rate were the variables analysed. Germination rate was calculated according to the Maguire index (Maguire, 1962). Caryopses that did not germinate at the end of the trial but were intact were considered dormant seeds (Maia *et al.*, 2006).

Statistical analysis

The variables of reproductive success, number of panicles per plant, average number of spikelets per panicle, quantity of florets per spikelet and the weight of 1000

seeds were analysed with the statistical program Info-stat (2009) by analysis of variance (ANOVA). Differences between mean values were tested for significance using Tukey's test ($P < 0.05$). The statistical model used to determine reproductive success was

$$Y_{ij} = \mu + \text{treat}_i + e_{ij}$$

where:

Y_{ij} = stands for the observation in treatment i in plant j ;

μ = general mean;

treat_i = effect of the pollination treatment i ;

e_{ij} = effect associated with the experimental error.

The statistical model used to evaluate the yield components was

$$Y_{ijr} = \mu + \text{treat}_i + \text{pop}_j + \text{treat} * \text{pop}_{(ij)} + e_{ijr}$$

where:

Y_{ijr} = observation in treatment i in plant j ;

μ = general mean;

treat_i = effect of treatment i ;

pop_j = effect of population j ;

$\text{treat} * \text{pop}_{(ij)}$ = effect of the interaction between treatment i and population j ;

$e_{(ijr)}$ = experimental error attributed to Y_{ijr} observation.

Germination analysis consisted of a factorial design with two factors, pollination treatment (five levels), germination temperature (three levels) and their interactions. The data obtained were analysed by ANOVA. The statistical model for evaluating germination behaviour was:

$$Y_{ijr} = \mu + \text{treat}_i + \text{temp}_j + \text{treat} * \text{temp}_{(ij)} + e_{ijr}$$

where

Y_{ijr} = stands for the observation in the treatment in the plant j ;

μ = general mean;

treat_i = effect of the pollination treatment i ;

temp_j = effect of the germination temperature j ;

$\text{treat} * \text{temp}_{(ij)}$ = effect of the interaction between treatment i and temperature j ;

e_{ijr} = experimental error attributed to Y_{ijr} observation.

Results

The reproductive success (fruit/flower ratio) of *B. auleticus* was the only component of the yield that

showed significant differences as a function of the origin of the genotypes used as pollen donors ($P < 0.0001$) (Figure 2).

The differences in reproductive success among treatments showed three defined groups. One group constituted of the donor treatments LP and ER, and showed the highest values for the fruit/flower ratio (mean of 68%). Another group, the one formed by the treatment mixture of donors and donors BA, showed intermediate values (mean of 60%), and a third group, composed of the control treatment (donor SF), exhibited the lowest reproductive success (mean of 45%). The spatial isolation of genotypes from other conspecific pollen sources (self-infertility test) reduced fruit production significantly. The range of values obtained was 4.9–14.3%, with a mean of 10.6 ± 2.7 (mean \pm standard deviation) fruits every 100 flowers.

The variation in germination percentage and germination rate was significant for temperature

($P < 0.0001$), pollination system ($P < 0.0001$) and their interactions ($P < 0.0019$). Germination percentage and germination index were significantly higher at constant 15°C than alternating temperatures and constant 28°C in all pollination treatments. The significant interactions indicated that the seeds from different populations varied in germination responses depending on the temperature treatment applied. Because of these significant interactions, it was concluded that temperature treatments had to be analysed separately. There were significant differences among populations ($P < 0.01$) at all the tested temperatures (Table 3). All populations subjected to 15°C showed a germination percentage value higher than 80%, and the highest differences in germination between lines were found in the 15/28 and 28°C treatments. The lowest germination percentage and germination index were found in the control group for the 15/28 and 28°C treatments (Table 3).

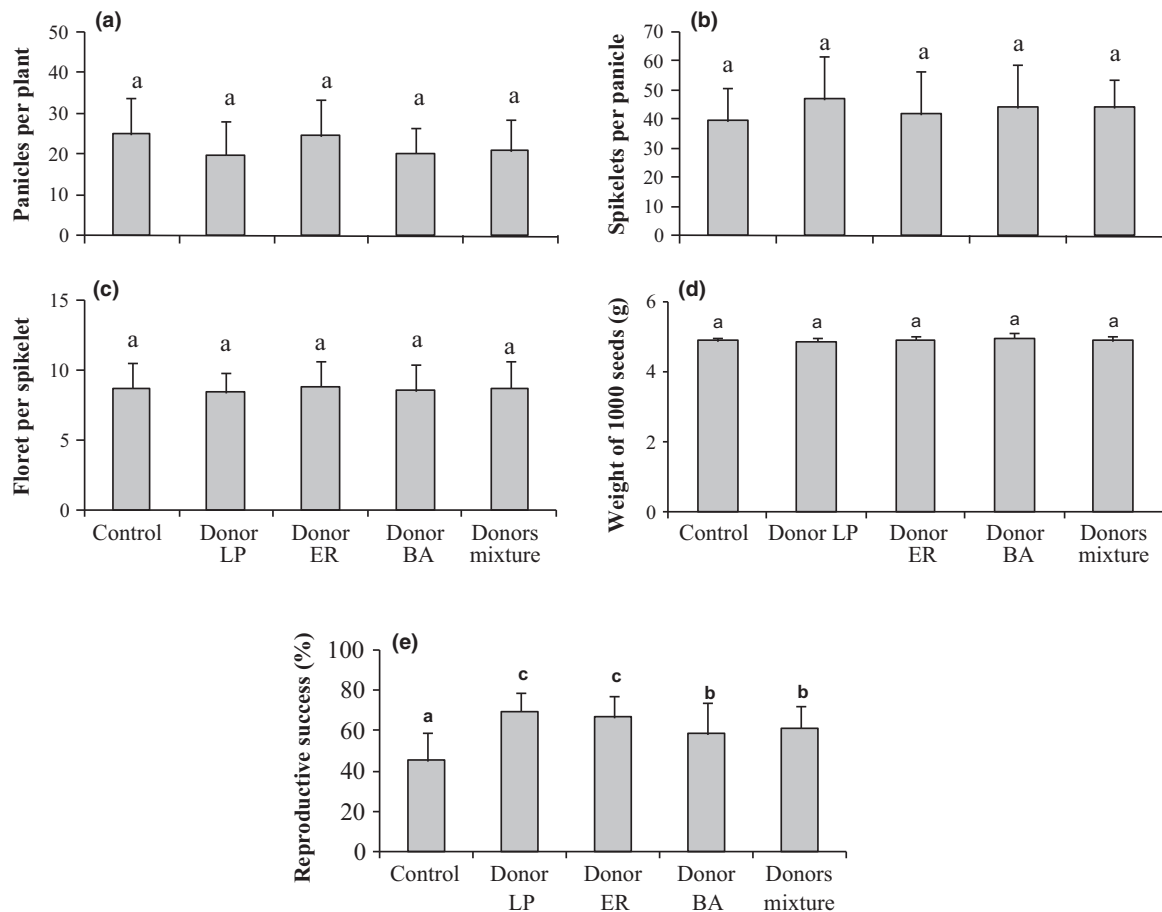


Figure 2 Yield components of the genotypes of *Bromus auleticus*, under different pollen donors. (a) Quantity of panicles per plant; (b) Quantity of spikelets per panicle; (c) Quantity of florets per spikelet; (d) Weight of 1000 seeds and (e) Reproductive success. Means with different letters are different at $P < 0.05$ (Tukey's Test).

Table 3 Percentage of germination and germination ratio of seeds of *B. auleticus* obtained by free pollination with different pollen donors for three temperature treatments.

Treatment	Percentage of germination ^{*,†}			Germination rate ^{*,†}		
	15°C	15–28°C	28°C	15°C	15–28°C	28°C
Control	87.6 ± 6.8 ^{ab}	59.6 ± 7.5 ^a	9.6 ± 3.9 ^a	3.8 ± 0.4 ^b	2.2 ± 0.5 ^a	0.3 ± 0.1 ^a
Donors LP	95.2 ± 4.2 ^b	80.4 ± 8.9 ^b	18.8 ± 5 ^{ab}	3.6 ± 0.2 ^b	3.6 ± 0.7 ^{bc}	0.6 ± 0.2 ^{ab}
Donors ER	94 ± 3.2 ^b	88.4 ± 8.3 ^b	28.8 ± 9.5 ^b	4 ± 0.4 ^b	4.6 ± 0.8 ^c	1.1 ± 0.4 ^b
Donors BA	81.2 ± 6.42 ^a	74.4 ± 9.21 ^{ab}	27.2 ± 6.4 ^b	2.7 ± 0.6 ^a	3.1 ± 0.7 ^{ab}	1.1 ± 0.3 ^b
Donors mixture	88.8 ± 8.7 ^{ab}	74.0 ± 10.9 ^{ab}	31.6 ± 13.8 ^b	3.8 ± 0.4 ^b	3.9 ± 0.6 ^{bc}	1.2 ± 0.4 ^b

^{*}Mean ± standard deviation. [†]Different letters show significant differences among treatments at different temperatures (Tukey's test, $P < 0.05$).

Discussion

Even though limitation by pollen occurs at the level of individuals, it transfers its effect to the level of populations (Ashmann *et al.*, 2004). Indeed, in a relict population, this effect is presented in the form of loss of fertility, which jeopardizes its survival in the long term by reducing the quantity of seeds for subsequent generations. The allogamous or auto-incompatible condition of a species makes limitation by pollen riskier (Burd, 1995). Fertility obtained by means of isolating plants from other sources of pollen represents what happens at the level of individuals, whereas fertility achieved by free pollination with donors of the same origin or with genotypes of a different origin represents what happens at the level of populations. In the present experiment, seed production obtained from donors 'SF' represented what occurs with an adequate density of donors (pollen quantity), while with donors of different origins, it represented potential seed set. Therefore, reduced population size in allogamous species, particularly in self-incompatible ones, leads to endogamy (crosses between related individuals) and inbreeding depression (Keller and Waller, 2002). Inbreeding depression causes a reduction in the mean value for quantitative traits associated with fertility, as in *Bromus inermis* (Christie and Kalton, 1960).

If it is considered that fruit production consists of defined components before pollination-fecundation (panicles per plant, spikelets per panicle and florets per spikelet) and components that are defined after that process (seed weight and fruit/flower ratio), it is worth highlighting that for trial conditions, the only yield component with significant differences among treatments was the fruit/flower ratio. The evaluated yield components showed values in the range of those obtained by different authors for both natural populations (Boggiano and Zanoniani, 2001; Traverso, 2001; Silva *et al.*, 2007) and commercial cultivars (Rivas, 2001; Ruíz and Covas, 2004). Some of these studies reported values 30–40% of empty florets,

even for commercial cultivars (Ruíz and Covas, 2004).

Under isolation conditions (spatial separation from other pollen sources), the reproductive success of *B. auleticus* was less meaningful than for the control group. Under such conditions, *B. auleticus* showed a very low frequency of self-fertility (less than 15%), as demonstrated previously (Gutiérrez *et al.*, 2006b). When pollen flow came from donors of a different origin, the fruit/flower ratio was higher than that of the control group and showed significant differences among origins. Donors LP and ER improved the fruit/flower ratio by 32 and 35%, respectively, while donors BA and the mixture increased this ratio by 23 and 26% respectively. The differences in reproductive success between the control and the donors could be due to the quantity of effective matings. For the control group, the values obtained are related to a decrease in effective matings, probably caused by a lower variability in incompatibility genes, as suggested by Marshall and Ludlum (1989) for ryegrass. Seeds obtained from new genotypic combinations did not increase their weight in relation to the pollen donor, in agreement with the findings by Silva *et al.* (2007). The weight values of 1000 seeds were similar to those obtained by Ruíz and Covas (2004) under conditions of high fertility. Germination behaviour was similar to that reported by other authors. Olmos (1993) and Silva *et al.* (2002) reported dormancy induced by high temperatures for this species, similar to other cool-season grasses (Zabala *et al.*, 2009).

Under non-optimal germination conditions, seeds originating from new genotypic combinations showed a significant increase in germination percentage and germination rate. In *Blandfordia grandiflora*, a partially self-fertile species, fewer self-seeded seeds germinated compared with out-crossed seeds, particularly in an adverse field environment (Ramsey and Vaughton, 1998).

This study evaluated only one *B. auleticus* population. However, it presented an innovative approach to studying factors affecting seed production, because it minimized the empirical errors made when analysing limitation by pollen (Ashmann *et al.*, 2004; Aizen and Harder, 2007). In wind-pollinated species, many such errors are related to the manipulation of flowers, the density of donors and the acquisition of resources. In the present study, flowers were under free pollination, with an adequate supply of resources and a steady density of donors among treatments. Furthermore, the fruit/flower ratio was evaluated, and the remaining yield components were studied as well. Boggiano and Zanoniani (2001) reported that the main causes of variability in *B. auleticus* seed production were the origin of populations and reproductive management (fertilization and harvest time). The present work showed that pollen-quality limitation affects seed production by reducing the fruit/flower ratio. Consequently, the fruit/flower ratio can be used as an indicator of pollen limitation, particularly in populations or ecotypes of low seed production. Besides, we provided evidence that the ability of seeds to germinate under non-optimal conditions was also affected by pollen quality. It is interesting then, that pollen quality can exert a positive and significant effect on two fitness components: the seed set and the capacity to germinate under a wider range of temperatures. This last item would allow a greater field germination period.

Acknowledgments

We would like to thank Sergio Puig for his valuable fieldwork. Financial support for this research was provided by Universidad Nacional del Litoral and Agencia Nacional de Promoción Científica y Tecnológica (PICTO 35727).

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