

## RESEARCH PAPER

# Reproductive versatility in legumes: the case of amphicarp in *Trifolium polymorphum*

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**ABSTRACT**

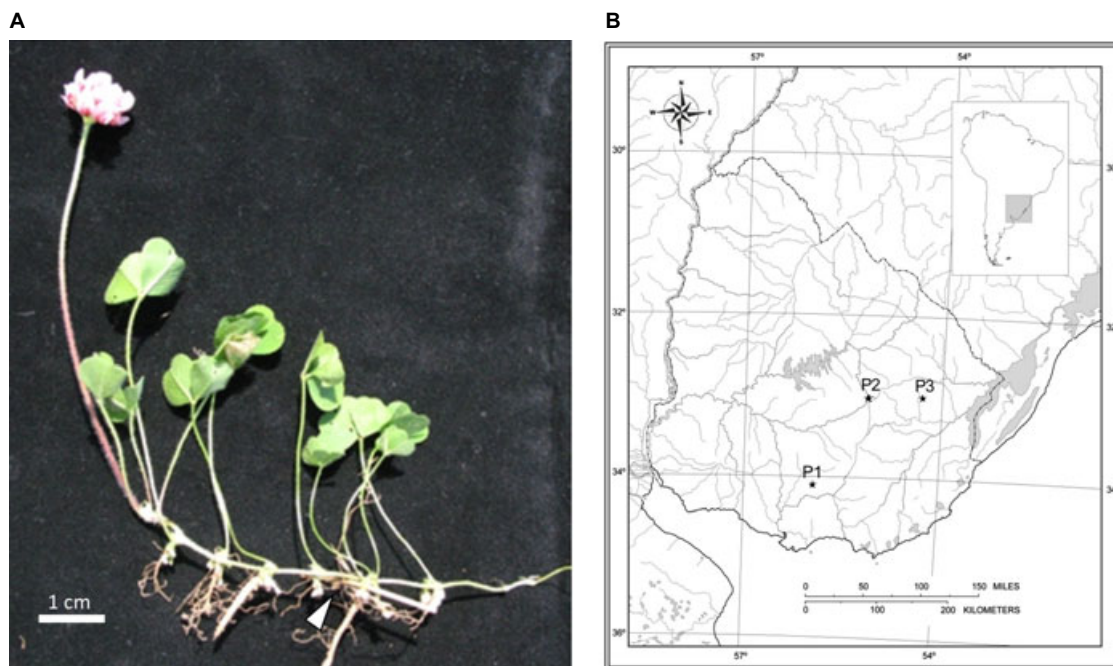
Amphicarp is a fascinating reproductive strategy, defined as fruit produced both below the soil surface and as aerial fruit on the same plant. *Trifolium polymorphum* is a grassland species subject to herbivory that combines amphicarp with vegetative reproduction through stolons. Underground flowers have been described as obligate autogamous and aerial ones as self-compatible allogamous, with aerial floral traits favouring cross-pollination. In the present work we performed different pollination treatments on aerial flowers to analyse rates of pollen tube development and offspring fitness, measured as fruit set, seed production and germination percentage. This last variable was compared to that of seeds produced underground. No significant differences were found between fruit set in self- and cross-pollinations. Seed production was higher in self-pollinations, which is consistent with the higher rate of pollen tube development observed in self-crosses. Spontaneous self-pollination is limited in aerial flowers; thus pollen transfer by means of a vector is required even within the same flower. Germination tests showed that aerial seeds produced after self- and cross-pollination did not differ in fitness, but underground seeds had higher germination percentage than aerial ones. Thus, we conclude that *T. polymorphum* has a mixed mating system. In grasslands with heavy grazing pressure, clonal propagation and underground seed production ensure persistence in the field. An intermediate level of selfing in aerial flowers ensures offspring, but morphological (herkogamy) and functional (dicogamy) floral traits maintain a window to incorporate genetic variability, allowing the species to tolerate temporal and spatial pressures.

**INTRODUCTION**

Diversity in plant reproductive biology proves the wide adaptive ability among plants (Barrett 2010). A large variety of anatomical/morphological (e.g. gynodioecy, heterostyly) and physiological (e.g. self-compatibility) flower/plant adaptations have been described as breeding systems in angiosperms (Neal & Anderson 2005). These adaptations determine matches between individuals, promote levels of selfing or outcrossing, and are known as mating systems (Neal & Anderson 2005). In addition, plant species combine different reproductive strategies or reproduction modes (e.g. set of different dispersal units, combined sexual and asexual reproduction) to ensure persistence over time and space. One of the most interesting reproductive strategies in plants is amphicarp, which was defined by Cheplick (1987) as fruits produced below the soil surface on specialised structures, together with aerial fruit production on the same plant. Barker (2005) further elaborated this definition, describing aerial flowers as chasmogamous, with fruits differing from those produced from underground cleistogamous flowers (a condition referred to as amphi-geocarpy). This phenomenon has been described so far in around 50 angiosperm species (Lev-Yadun 2000), ten of which are Leguminosae (Arroyo 1981; Kaul *et al.* 2000). Amphicarp has been associated with

ecological adaptation to adverse growth conditions, where underground seed production ensures persistence in a more stable environment (Rivals 1953; Koller & Roth 1964; Plitman 1973; Fevereiro-Barbosa 1987; Kaul *et al.* 2000; Lev-Yadun 2000; Abd El Moneim & Elias 2003; Zhang *et al.* 2005, 2006; Culley & Klooster 2007; Sadeh *et al.* 2009; Tan *et al.* 2010).

*Trifolium polymorphum* Poir. is a stoloniferous, amphicarpic legume occurring in southern South America: Argentina, Brazil, Paraguay, Chile and Uruguay (Burkart 1987; Rossow 1999). During winter, it is an important qualitative component in natural pastures for its good quality fodder, especially for sheep (Speroni & Izaguirre 2003). Although it can be heavily grazed, *T. polymorphum* is highly persistent in the herb layer, because it combines the amphicarpic condition with vegetative propagation through stolon pieces separated from the mother plant (Fig. 1). Underground flowers are highly modified and adapted to the subterranean environment and never open (Speroni & Izaguirre 2001; Speroni *et al.* 2010). The corolla is reduced to three petals and the androecium has only three stamens, whose anthers lean on the stigma. The anthers never open but pollen tubes grow through the anther wall. Ontogenetic studies have not revealed apomictic embryo sac development (Speroni & Izaguirre 2001; Speroni *et al.* 2010), hence underground seeds are produced through obligate self-pollination. However, aerial



**Fig. 1.** *Trifolium polymorphum* Poir. (A) Flowering plant with aerial and underground flowers (arrow). (B) Location of the three studied populations P1 (34°09'52.6''S, 55°52'51.8''W), P2 (33°00'46''S, 55°04'22''W) and P3 (33°10'38''S, 54°24'66''W).

flowers are papilionaceous, with morphological features common to entomophilous flowers (e.g. bilateral symmetry, pink colour, scent emission, presence of nectar at the base of the ovary). These aerial flowers have low fruit set and have been reported as cross-pollinated (Burkart 1952; Coll & Zarza 1992). However, zygote formation occurs before anthesis, according to anatomical studies on embryo sac ontogeny and egg cell development (Speroni & Izaguirre 2001), suggesting that self-pollination or apomixis might occur. Having analysed offspring from different pollination treatments using molecular markers, Real *et al.* (2007) concluded that *T. polymorphum* is an allogamous, self-compatible species (60% selfing was found), which benefits from pollinator presence for seed production. Studies of intrafloral phenology carried out on Uruguayan populations of *T. polymorphum* (Speroni *et al.* 2009, 2012) indicated that aerial flowers exhibit morphological and functional characteristics promoting outcrossing and delaying selfing. In pre-anthesis, when the anthers dehisce, the stigma is positioned above the anthers, preventing self-pollination (thus, flowers are herkogamous). Flower anthesis lasts 4–5 days, and maximum pollen viability does not occur when anthers dehisce but 1–2 days after the start of anthesis. Similarly, highest stigmatic receptivity occurs 3 days after the start of anthesis (Speroni *et al.* 2009, 2012). This time mismatch generates what Lloyd & Webb (1986) defined as incomplete functional dichogamy.

Considering that underground flowers of *T. polymorphum* are obligate self-pollinated, and aerial ones are described as allogamous self-compatible with phenological adaptations that promote cross-pollination and delay selfing, in this work we address the following questions: (i) are there differences in the growth of self- and cross-pollination pollen tubes in aerial flowers; (ii) how does pollination affect reproductive success; and (iii) are there any fitness differences between seeds produced on aerial and underground fruits?

## MATERIAL AND METHODS

Plants of *T. polymorphum* were collected from three Uruguayan wild populations (wild populations, WP) and maintained in cultivation in the Facultad de Agronomía (Montevideo, Uruguay) experimental garden (experimental crop, EC). As this species has an intricate stolon system and plant limits are difficult to verify, manual pollinations were carried out with pollen from populations separated by long distances (P1, P2 and P3; Fig. 1) to ensure genetic differences between them. Previous studies in these same populations showed that maximum pollen viability occurs 1–2 days after the start of anthesis and highest stigmatic receptivity occurs 3 days after the start of anthesis (Speroni *et al.* 2009, 2012). These features were strictly considered in manual pollination treatments. We carried out self- and cross-hand-pollinations in aerial flowers of EC plants in order to evaluate pollen tube growth. In order to rule out endogenous factors that could affect experimental results, we carried out hand-pollinations only on the two peripheral flower rows that actually attain anthesis, avoiding central flowers that usually fail to develop. Furthermore, we only used up to four flowers per head to avoid disturbance due to manipulation. In EC plants, we isolated young heads with paper bags to prevent foreign pollen arrival. In each population, in order to allow quantification of pollen tube growth, 35 previously emasculated flowers were pollinated with pollen from another genetically distinct population (cross-pollination), and a further set of 35 flowers were pollinated with pollen from their own flowers (self-pollination). Five flowers from each treatment were fixed in FAA (formalin, glacial acetic acid, 70% alcohol, 5:5:90) at each of the following time periods: 3, 6, 9, 12, 15, 24 and 48 h post-pollination (hpp). Flowers were later processed for pollen tube observation through fluorochromatic reactions using methyl blue as fluorochrome (Kearns & Inouye

1993). Observations were performed with an epifluorescence microscope (Olympus Vanox AH-3) using a BV filter. Pictures were taken with an Olympus DP 71 digital camera and processed using DPManager. Pollen tube development rate was calculated as the ratio of pollen tube distance (mm) from the stigma to the length of time post-pollination (h).

In order to determine how pollination affects reproductive success, we carried out several pollination treatments: (i) manual self-pollination in EC (as described above); (ii) manual cross-pollination in EC (as described above); (iii) spontaneous self-pollination in EC (bagged flowers without manual transport of pollen, therefore we measured spontaneous self-pollination within the same flower); (iv) open pollination in EC (unbagged flowering heads in cultivated plants, exposed to pollinators present in cultivation conditions); and (v) open pollination in WP (unbagged flowering heads in WP, exposed to natural pollinators; performed only on P2 and P3). In each pollination treatment, reproductive success was measured as fruit set (fruits per flowers proportion), seed production (seeds per flower proportion), seed fresh weight and germination percentage. Seed weight was measured on Eurotherm-Gibertini precision scales.

Germination tests were performed after aerial seeds were exposed to mechanical scarification. Seeds were placed in Petri dishes with wet paper in a germination chamber with 16 h light–8 h dark, at *ca.* 20 °C (Ellis *et al.* 1985). The percentage of seed germination was evaluated between 7 and 15 days after sowing, as suggested by the International Board for Plant Genetic Resources (IBPGR) protocol for *Trifolium*, and then the observation period was extended up to 27 days. Considering that germination percentage was low during this period, an additional cold treatment was performed (4 °C for 1 week), and then seeds were subjected to with the initial germination conditions.

To compare treatment results of fruits per flowers and seeds per flower proportions, the following generalised linear model (McCullagh & Nelder 1983) was used, assuming a Poisson distribution for counts, with a log-link function and an auxiliary variable (OFFSET = base count, by example when COUNT = number of seeds/flowers then OFFSET = number of flowers) to estimate the proportions.

$$\log(\text{COUNT})_{ij} = \log(\text{OFFSET}) + \tau_i + \varepsilon_{ij}$$

$$\log(\text{COUNT}) - \log(\text{OFFSET}) = \tau_i + \varepsilon_{ij}$$

$$\log\left(\frac{\text{COUNT}}{\text{OFFSET}}\right) = \tau_i + \varepsilon_{ij}$$

This model allows comparisons between pairs of treatments by approximating  $\chi^2$  of the likelihood ratio test statistic. The GENMOD procedure in SAS version 9.01 (SAS Institute, Cary, NC, USA) was used.

To find out if there were differences in fitness between aerial and underground seeds, we collected underground seeds from wild population P1, and their weight and germination percentage (as an estimate of fitness) were compared in the same way as the seeds from P2 and P3 WP aerial flowers. To compare the proportions of germinated seed, we used a 'binomial proportions comparison', assuming binomial distribution, and a Z-test.

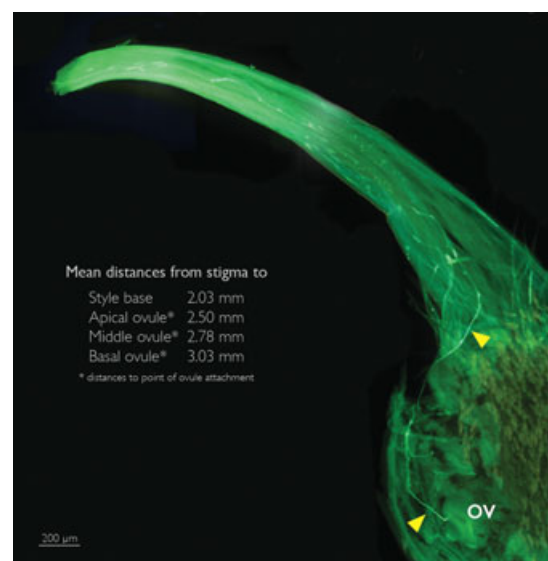
## RESULTS

### Pollination and pollen tube growth

Pollen tubes showed homogeneous yellow fluorescence and an intercellular pathway of growth (Fig. 2). Signals of rejection of pollen tubes (*e.g.* lenticular callose deposition in stigmatic papillae, callose plugs at the tip of developing tubes or exploded tubes) were not observed in any of the pistils analysed, and therefore we did not consider incompatibility reactions. The number of germinated pollen grain was low. From a total of 210 hand-pollinated aerial flowers, only eight were self-pollinated and seven cross-pollinated flowers showed developed pollen tubes (Table 1). Pollen tube growth rate was higher after self-pollinations (Table 1). Six hours after pollination, self-pollen tube growth did not exceed stigmatic papillae length, but reached the apical, middle and basal ovules at 12, 15 and 24 hpp, respectively. Cross-pollination treatments exhibited an even lower rate of pollen tube growth, with pollen tubes reaching apical ovules only at 24 hpp.

### Pollination and reproductive success: fruits, seeds and germination

Fruit set and seed production in all treatments were very low (Table 2). There were no significant differences between fruit set in hand-outcrossing and selfing treatments, thus fruit were set whenever pollen was applied to the stigma, regardless of the pollen source (Table 2). Seed production, however, was significantly higher in hand self-pollinations (Table 2), which is consistent with the higher rate of pollen tube growth observed in this pollination treatment. Spontaneous self-pollination in aerial flowers is limited in this species, and pollen transfer *via* an intervening vector is necessary in order to increase fruit set and seed production (Table 2). The need for pollinators was also evident in open-pollination, since fruit set in the WP (where natural pollinators occur) was higher than in unbagged heads in the EC (Table 3). On the other hand, the reverse condition



**Fig. 2.** *Trifolium polymorphum* pollen tube growth as seen in fluorochromatic reactions. Pollen tube pathways (arrows) from stigma up to ovule penetration (ov: ovule).

**Table 1.** Pollen tube length (mm) and growth rate (mm·h<sup>-1</sup>) in self- and cross-pollinations of *Trifolium polymorphum* aerial flowers (hpp: hours post-pollination; pa: pollen tubes do not exceed stigmatic papillae; -: without pollen tube development).

flower self-pollinated (n = 105)				flower cross-pollinated (n = 105)			
hpp							
3	-	-	-	-	-	-	-
6	pa						
9	1.05 mm 0.12 mm·h <sup>-1</sup>	2.88 mm 0.32 mm·h <sup>-1</sup>	1.52 mm 0.17 mm·h <sup>-1</sup>	2.94 mm 0.32 mm·h <sup>-1</sup>	2.03 mm 0.22 mm·h <sup>-1</sup>		
12	2.53 mm 0.31 mm·h <sup>-1</sup>	-	-	-	1.35 mm 0.11 mm·h <sup>-1</sup>		
15	2.78 mm 0.19 mm·h <sup>-1</sup>	-	-	-	0.81 mm 0.05 mm·h <sup>-1</sup>		
24	3.03 mm 0.13 mm·h <sup>-1</sup>	-	-	-	2.53 mm 0.11 mm·h <sup>-1</sup>	1.07 mm	0.04 mm·h <sup>-1</sup>
48	3.03 mm	pa	pa	-	3.03 mm 0.06 mm·h <sup>-1</sup>	2.78 mm	

was observed in seed production, where EC seed production was higher than that of WP (Table 3). Seed germination, one of the variables that describe offspring fitness, showed no significant differences among treatments.

#### Aerial versus underground seeds

Seed weight from underground seeds harvested in P1 WP was nearly three times higher than that of aerial seeds harvested in P2 and P3 WP (Table 4). Similarly, underground seed germination was significantly higher than that of aerial seeds (Table 4). Underground seeds obtained from P1 WP had different development performance. Nine seeds showed complete development and regular morphology, while the other 63

had irregular morphology, possibly due to terminal developmental difficulties. However, the germination ratio of irregular seeds exceeded that of well-formed seeds.

## DISCUSSION

### *Trifolium polymorphum* mating system and reproductive strategies

No aerial pistils showed incompatibility reactions between sporophyte maternal tissue and sporophyte/gametophyte paternal tissue, as described in Dumas & Knox (1983). There was a high growth rate of self-pollen tubes, ruling out cryptic incompatibility (Busch *et al.* 2010); this higher growth rate agrees with the detected increased self-seed production. These features,

**Table 2.** Values obtained for each treatment in the three populations of *Trifolium polymorphum* (P: population; N: treated flowers; N°fr: fruit number obtained; fr/fl: fruit per flower proportion; N°s: seed number obtained; s/fl: seeds per flower proportion; sw: seed average weight (g); N°sg: number of germinated seeds; %g: germination percentage).

P	treatment	N	N°fr	fruits			seeds		
				fr/fl mean ± SE left SE right	N°s	s/fl mean ± SE left SE right	sw	N°sg	%g mean ± SE left SE right
1	cross-pollination	33	1	3.0ab 1.9 5.2	1	3.0a 1.9 5.2	0.0020	1	100a 63 0
	self-pollination	34	4	11.8a 4.6 7.6	8	23.5b 7.0 10.0	0.0006	3	38a 16 29
	spontaneous selfing	335	6	1.8b 0.6 0.9	10	3.0a 0.8 1.1	0.0011	1	10a 6 17
2	cross-pollination	37	3	8.1ab 3.6 6.3	4	10.8a 4.2 7.0	0.0015	4	100a 39 0
	self-pollination	48	12	25.0a 6.3 8.4	16	33.3b 7.4 9.5	0.0008	9	56a 16 22
	spontaneous selfing	485	19	3.9b 1.0 0.9	21	4.3a 0.9 1.1	0.0007	10	48a 13 18
3	cross-pollination	32	0	-	-	-	-	-	-
	self-pollination	70	5	7.1a 2.6 4.0	6	8.6b 2.9 4.3	0.0015	6	100a 34 0
	spontaneous selfing	676	21	3.1a 0.6 0.8	22	3.3a 0.6 0.8	0.0010	5	23b 8 13

a, b: means followed by different letter are statistically significant ( $P < 0.05$ ). Because of data skewness the confidence intervals are not symmetric, with different SE on the left and right.

**Table 3.** Values obtained for open-pollinations in experimental crop and wild populations 2 and 3 of *Trifolium polymorphum* (P: population; N: observed flowers; N°fr: fruit number obtained; fr/fl: fruit per flowers proportion; N°s: seed number obtained; s/fl: seeds per flower proportion; sw: seed average weight (g); N°sg: number of germinated seeds; %g: germination percentage).

P	treatment	N	fruits			seeds			
			N°fr	fr/fl mean ± SE <sub>left</sub> SE <sub>right</sub>	N°s	s/fl mean ± SE <sub>left</sub> SE <sub>right</sub>	sw	N°sg	%g mean ± SE <sub>left</sub> SE <sub>right</sub>
2	open poll. - exp. crop	645	6	0.9b 0.3 0.5	6	0.9a 0.3 0.5	0.0009	5	83b 30 47
	open poll. - wild pop.	1086	75	6.9a 0.8 0.9	20	1.8a 0.4 0.4	0.0007	4	20a 8 13
3	open poll. - exp. crop	202	19	9.4a 1.9 2.4	16	7.9b 1.8 2.3	0.0010	9	56a 16 22
	open poll. - wild pop.	1632	194	11.9a 0.8 0.9	77	4.7a 0.5 0.6	0.0008	24	31a 6 7

a,b: means followed by different letter are statistically significant ( $P < 0.05$ ). Because of data skewness the confidence intervals are not symmetric, with different SE on the left and right.

**Table 4.** Underground and aerial seeds of *Trifolium polymorphum* (P: population; N: observed seeds; sw: seed average weight (g); N°sg: number of germinated seeds; %g: germination percentage; in parentheses 95% confidence interval for percentage).

P	seed development	N	sw	N°sg	%g
1	underground	72	0.0020	64	89a (82, 96)
2	aerial	20	0.0007	4	20b (25, 38)
3	aerial	77	0.0008	24	31b (21, 42)

a,b: percentages followed by different letters are statistically significant ( $P < 0.05$ ) with the Z-test.

along with the obligate self-fertilisation of underground flowers (Speroni & Izaguirre 2001; Speroni *et al.* 2010), indicate that *T. polymorphum* is a highly self-compatible species. These results agree with Real *et al.* (2007), who examined molecular markers in offspring obtained by open-pollination and found 60% autogamy. However, spontaneous self-pollinations are very low, and a pollinator vector is necessary for pollen transport. This is here confirmed in the low fruit set of spontaneous self-pollination and the increased fruit set and seed production of WP, where natural pollinators are present. In the same way, *T. polymorphum* aerial flowers have features (herkogamy and functional dichogamy) that prevent pollen transfer from anthers to the stigma of the same flower and facilitate foreign pollen load by pollinators (Speroni *et al.* 2009, 2012). Self-pollination is also hindered by the presence of a stigmatic cuticle that prevents early pollen germination on the stigma before anthesis (Speroni *et al.* 2012). This feature, common in entomophilous and self-compatible legumes (Heslop-Harrison & Heslop-Harrison 1983; Small 1986; Bruneau & Anderson 1988; De las Heras *et al.* 2001; Galloni *et al.* 2007; Etcheverry *et al.* 2008; Sahai 2009; Basso-Alves *et al.* 2011), is associated with 'tripping' pollination, where mechanical pollinator action is needed to break down the cuticle, allowing pollen grain adhesion and germination (Frankel & Galum 1977).

Therefore, *T. polymorphum* aerial flowers present a mixed mating system (MMS), the most common system found in flowering plants (*ca.* 42%; Goodwillie *et al.* 2005, 2009). The

MMS is interpreted as one of the most advantageous reproductive strategies when combined with mechanisms that favour outcrossing and delay selfing (Lloyd 1992; Lloyd & Schoen 1992). The MMS has evolved as a way to ensure offspring production in scenarios of uncertain cross-pollination (Goodwillie *et al.* 2005, 2009; Morgan & Wilson 2005; Busch & Delph 2012). Given the inherent complexity of plant mating systems, it is not easy to formulate a general pattern to predict them. Nevertheless, it is possible to identify several variables that affect mating systems, such as inbreeding depression, resource allocation, pollinator availability, floral advertising and rewards, seed protection and defence against herbivory (Ivey & Carr 2005; Charlesworth 2006). Of all these variables, defence against herbivory might be the most important in promoting the *T. polymorphum* mating system. This legume combines MMS in aerial flowers, obligate self-fertilisation in underground flowers and clonal reproduction by stolons. Clonal reproduction is widespread (70–80%) in angiosperms (Honnay & Jacquemyn 2010; Vallejo-Marín *et al.* 2010) and presents advantages such as persistence in unfavourable habitats for sexual reproduction, search for resources in heterogeneous environments and reduction in the risk of ramet loss (Vallejo-Marín *et al.* 2010).

Adaptation to unstable environments has also been linked to the evolution of amphicarp, particularly in habitats where the presence or activity of pollinators is reduced (Rivals 1953; Koller & Roth 1964; Plitman 1973; Feveiro-Barbosa 1987; Kaul *et al.* 2000; Abd El Moneim & Elias 2003; Zhang *et al.* 2005, 2006; Sadeh *et al.* 2009; Tan *et al.* 2010). However, neither the grassland habitat where *T. polymorphum* occurs nor its latitudinal range is considered as extreme environmental conditions leading to serious reproductive instability. The highest disturbance faced by grassland species is herbivory, which *T. polymorphum* overcomes by combining amphicarp and clonal reproduction. Herbivory has also been suggested as one of the main forces acting on the evolution of plant mating system, along with soil type, dispersal mechanisms and pollinator activity (Ivey & Carr 2005; Steets *et al.* 2006, 2007; Karron *et al.* 2012).

In this scenario, as a result of herbivory, clonal propagation and underground seed set would ensure persistence of this legume in the grassland herb layer. Aerial flowers play a major

role in long-distance dispersal, and through a MMS strategy, these flowers would incorporate genetic variability.

### Aerial and underground investment

*Trifolium polymorphum* makes a large energy investment in aerial and underground flowering. However, most aerial flowers are consumed by livestock and the remaining flowers show low fruit set. Many species invest energy in flowering even though fruit set accounts for a small percentage. This is a very common feature in woody species (Bawa & Webb 1984; Gibbs & Sasaki 1998), and has also been reported for several legumes: *Hedysarum glomeratum* (De las Heras *et al.* 2001), *Lupinus luteus* and *L. texensis* (Stephenson 1981). This depletion of female fertility has been associated with genetic parameters such as the genetic load originated from selfing (Charlesworth 1989). In clonal species with a high degree of geitonogamy, seed abortion could be interpreted as an early expression of inbreeding depression (Vallejo-Marín *et al.* 2010). Nevertheless, generally four ovules are present in each aerial ovary and embryo sacs are formed in every ovule of *T. polymorphum* (Speroni *et al.* 2010), the proportion of seeds per fruit being 1:1 (Table 2), even in hand-pollination where pollen load is not a limiting factor. However, Speroni *et al.* (2009) analysed the same three populations studied here and found that pollen viability varied widely among flowers of the same population. Low individual values were recorded, which probably agrees with the irregularities in microspore tetrad formation observed by Speroni & Izaguirre (2001). Even though pollen source does not affect seed production and germination since there were no differences between self- and cross-pollination treatments, variability of pollen viability could affect seed production. Therefore, we can conclude that *T. polymorphum* is a species with naturally low female fertility in aerial flowers.

Reproductive investment in underground flowers represents a significant persistence strategy, improving competition with other species, avoiding predation and favouring the symbiotic establishment with *Rhizobia* already present in the mother plant. Heteromorphic seed production is considered a bet-hedging strategy that reduces parental investment risk, distributing offspring in space and/or time to avoid variations in the environmental (Imbert 2002). The size difference between aerial and underground seeds of *T. polymorphum* and the higher germination percentage of underground seeds highlights the differential energy investment on the two flower types, as also reported for other amphicarpic species (Imbert 2002; Zhang *et al.* 2005; Conterato *et al.* 2010). Underground flowers are noticeably smaller and each ovary usually contains two ovules that generally develop into two larger seeds (Speroni & Izaguirre

2001; Speroni *et al.* 2010). Braza *et al.* (2010) observed that availability of both maternal and environmental resources are the two variables affecting fertility proportion and quality in *Plantago coronopus* (Plantaginaceae). Seed size is one of the most effective adaptations ensuring germination and seedling establishment, being related to embryo nutrient storage destined to nurture the first developmental stages until the embryo achieves nutritional independence (Fenner 1985). In the amphicarpic species *Commelina benghalensis* (Commelinaceae), seedlings produced by underground seeds are more vigorous than those produced from aerial seeds (probably because they are produced from larger and heavier seeds), leading to increased competitiveness of the former (Kaul *et al.* 2000). Seed size has also been associated with vegetation type and presence of herbivores. Species that inhabit dense vegetation types tend to produce larger seeds than those living in open habitats, a condition that probably constitutes an adaptation to establishment under shade (Fenner 1985; Westoby *et al.* 1992).

### CONCLUDING REMARKS

*Trifolium polymorphum* ensures persistence by means of underground seeds and vegetative reproduction and attains long-distance dispersal by means of aerial seeds. Underground seed set is high and ensures regeneration by means of storing a seed bank away from the harvest horizon of herbivores. Future studies are needed to quantify the richness of the underground seed bank and determine which parameters affect its productivity. Aerial flowers have a mixed mating system, with floral traits promoting allogamy, but have self-pollen tubes with a higher growth rate. Therefore, for a species with a natural strong bias towards homozygosity through selfing processes and clonal reproduction, cross-pollination constitutes an opportunity to incorporate genetic variability. Genetic variation studies at the population level are underway, with the objective of interpreting how different combinations of reproductive strategies affect the genetic structure of wild populations of *T. polymorphum*.

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