Journal of Plant Ecology Advance Access published January 9, 2012

Journal of
Plant Ecology
PAGES 1–7
doi: 10.1093/jpe/rtr047

Altitudinal variation in the reproductive performance of the Mediterranean shrub *Rhamnus lycioides* L.

Javier Gulías[1,]* *and Anna Traveset*[2]

[1] *Research group on Plant Biology under Mediterranean Conditions, Department of Biology, Universitat de les Illes Balears, Carretera de Valldemossa km 7.5, 07122-Palma de Mallorca, Balearic Islands, Spain*
[2] *Institut Mediterrani d'Estudis Avançats (CSIC-UIB), C/ Miquel Marqués 21, 07190 Esporles, Mallorca, Balearic Islands, Spain*
*Correspondence address. Research group on Plant Biology under Mediterranean Conditions, Department of Biology, Universitat de les Illes Balears, Carretera de Valldemossa km 7.5, 07122-Palma de Mallorca, Balearic Islands, Spain. Tel: +34-9-71-17-27-10; Fax: +34-9-71-17-31-84; E-mail: dbajgl4@uib.es

Abstract

Aims
Rhamnus lycioides L. subsp. *Oleoides* (Rhamnaceae) is a perennial shrub native to the Mediterranean Basin distributed along an altitudinal gradient, from sea level up to 1 000 m a.s.l. The specific goals of our study were (i) to compare plant morphology between two contrasting populations, (ii) to determine the reproductive system of *R. lycioides*, quantifying the relative importance of insects and wind as pollen vectors, (iii) to test if pollen limitation differs between populations, (iv) to study the main factors influencing fruit set and (v) to compare plant reproductive performance (mass allocation to flowers, fruits and seeds) between the two habitats.

Methods
In the present study, we examined plant morphology and the reproductive performance of *R. lycioides* L. in contrasting environments in two populations located at the extremes of its altitudinal range in the island of Mallorca (Balearic Islands, Western Mediterranean Basin) along a 3-year period. Plant morphology, the relative importance of insects and wind as pollen vectors, the pollen limitation to seed production and the plant reproductive performance (mass allocation to flowers, fruits and seeds) were determined.

Important Findings
Rhamnus lycioides individuals showed a higher plant surface/plant height ratio at the mountain than at the coast. This species appeared to be ambophilous despite its inconspicuous flowers, although the relative importance of wind as a pollination vector was higher at the mountain than at the coastal site. Fruit set was much higher at the mountain, where pollen appeared to be a limiting factor. By contrast, fruit set was not limited by pollen availability at the coastal population, where resource (water and nutrients) limitation seemed to be more determining. Flower size was greater at the coast, in contrast to fresh fruit weight that was higher at the mountain. Despite the relatively few differences between sites in precipitation patterns along the study period, water availability appeared to be the key factor explaining not only fruit set but also the reproductive performance of this species in the study populations.

Keywords: ambophily • fruit set • Mediterranean vegetation • pollen limitation • reproductive performance

Received: 14 July 2011 Revised: 7 November 2011 Accepted: 19 November 2011

INTRODUCTION

The study of the limitations on different stages of the plant reproductive cycle in species living across a precipitation gradient (usually due to either altitudinal and/or latitudinal changes) can be very useful when predicting changes in plant performance in the face of global warming. Despite this, relatively few studies have focused on comparing such limitations in these wide-range species (but see, for instance, García-Camacho and Escudero 2009; Giménez-Benavides *et al.* 2007a, 2007b; Hampe and Petit 2005; Parmesan 2006; Ramírez *et al.* 2006). Mediterranean ecosystems, in particular, have an additional interest due to the fact that water supply has been shown to be strongly limiting (Lavorel *et al.* 1998; Michalet 2006; Sanz-Elorza *et al.* 2003). An increasing number of studies performed in the Mediterranean area have reported that low-altitude plant populations face especially harsher constraints on different stages of their reproductive cycle than mountain populations (García-Camacho and Escudero 2009; Giménez-Benavides *et al.* 2007a, 2007b; Ramírez *et al.* 2006). Such differences in performance have been attributed to both water shortage (Arrieta and Suarez 2006; Castro *et al.* 2004; García *et al.* 1999; Giménez-Benavides *et al.* 2007a, 2007b; Hampe and Petit 2005; Pigott and Pigott 1993) as well as to high temperatures (Peñuelas *et al.* 2002 and references therein).

Another type of studies, mostly carried out within the Mediterranean area, have compared breeding systems, reproductive allocation, pollination success or plant–pollinator interactions across altitudinal gradients. In this regard, Malo and Baonza (2002) reported larger flowers at high-altitude populations than at low-altitude ones as a consequence of larger insect body sizes due to thermal constraints. Moreover, the sex ratio (male:female or hermaphrodite:female) in dioecious (Ortiz *et al.* 2002) or gynodioecious (Alonso 2005) species has been shown to increase with elevation. Differences in the pollination assemblage across altitudes have been also reported (De la Bandera and Traveset 2006; Gómez and Zamora 1999).

For some insect-pollinated plant species, the additional role of wind as pollen vector can ensure fruit set in stressful environments, compensating for the usually low frequency of insect visits in them (e.g. Arroyo *et al.* 1982; Gómez *et al.* 1996; Torres-Díaz *et al.* 2007). This mixed-mating system, named ambophily, has indeed been shown to be more common than previously thought (e.g. Bullock 1994; Dafni and Dukas 1986; De la Bandera and Traveset 2006; Gulías *et al.* 2004; Lázaro and Traveset 2006), although it is unknown yet to what extent it is a stable system or is a transient stage towards absolute anemophily or entomophily (Culley *et al.* 2002). Likewise, insects may play an important role on the reproduction of species with typically anemophilous flowers, i.e. unisexual, small in size, rather inconspicuous and with a low reward for pollinators (e.g. Gulías *et al.* 2004).

In the present study, we examined the reproductive performance of *Rhamnus lycioides* L. in contrasting environments at the two extremes of its altitudinal range in the island of Mallorca (Balearic Islands, Western Mediterranean Sea). This species has the particularity of occurring in two types of habitats, at coastal shrublands and in open shrublands at mountain areas. The specific goals of our study were (i) to compare plant morphology between two contrasting populations, (ii) to determine the reproductive system of *R. lycioides*, quantifying the relative importance of insects and wind as pollen vectors, (iii) to test if pollen limitation differs between populations, (iv) to study the main factors influencing fruit set and (v) to compare plant reproductive performance (mass allocation to flowers, fruits and seeds) between the two habitats.

MATERIALS AND METHODS

Study sites

The study was performed in two localities where *R. lycioides* is rather common and that differ in climate conditions (rainfall and temperature), type of habitat and predominant vegetation. The coastal population is located in the south of Mallorca island, at a site named Sa Bassa Plana, at ~75 m above sea level, 39°23′N 2°50′E. Mean annual precipitation at this site is 389 mm, mean maximum temperature of the warmest month (August) is 30.4°C and mean minimum temperature of the coldest (January) is 4.6°C (Guijarro 1986). The predominant vegetation here includes: *Olea europaea*, *Pistacia lentiscus*, *Cistus monspelliensis* and *Pinus halepensis*. A total of 38 males and 37 females of *R. lycioides* were recorded in this locality (and thus the sex ratio does not vary from 1:1). The mountain population is located at the south slope of the Tramuntana Mountain chain, at the northwest of Mallorca, in a mountain named Alfàbia at ~ 800 m above sea level, 39°44′N 2°42′E. Mean annual precipitation here is 875 mm and monthly mean maximum and minimum temperatures are 24.8 and 1.8°C, respectively (Guijarro 1986). The vegetation at this locality includes: *P. lentiscus*, *O. europaea*, *Cistus monspelliensis*, *Cistus albidus*, *Phlomis italica*, *Ampelodesmos mauritanica*, *Calicotome spinosa* and some individuals of *P. halepensis* and *Quercus ilex*. The number of *R. lycioides* individuals is lower here than at the coastal site, although we also found a similar number of males and females (13 and 16, respectively).

Study species

Rhamnus lycioides L. subsp. *Oleoides* (Rhamnaceae) is a perennial shrub, up to 3 m tall, naturally occurring in the Western Mediterranean Basin (de Bolòs *et al.* 2005). It can be found from sea level up to 1 000 m a.s.l., usually in Mediterranean shrublands (Al. *Oleo–Ceratonion*) occurring at shallow soil areas (Tutin *et al.* 1996). *Rhamnus lycioides* is a dioecious species that flowers along the spring, with substantial variation in flowering time, from 30 to 45 days, among populations that usually depend on meso and microclimatic site characteristics. It produces fleshy fruits (berries) that ripen during late spring and early summer and thus represents an important water and nutrient source for birds and small mammals during the dry summer, as is the case with other congeneric species, *Rhamnus alaternus* (Gulías *et al.* 2004) and *Rhamnus ludovici–salvatoris* (Traveset *et al.* 2003). The fruits usually bear one to three seeds covered by an endocarp that opens when the fruit pulp is removed.

Plant morphology

Plant height was measured in all recorded plant individuals in each population. In those individuals, plant surface was estimated and then the ratio plant surface/plant height was calculated in order to characterize the plant morphology at each site. Moreover, female flower production was estimated in all female plants at each site.

In addition to these characteristics, and in order to assess how reproductive success might be related to the abiotic conditions of the populations, we analysed annual rainfall and maximum and minimum temperature at the two localities during the study period (Fig. 1), highlighting the large variation from year to year usually observed in Mediterranean climate areas.

Reproductive performance and pollen limitation

Buds from all the flowering female individuals, seven females at the coastal population and five females at the mountain, were bagged in 1999 with the following objectives: (i) to assess whether pollen arrival limits fruit set (flowers were hand pollinated at anthesis with pollen collected from different males at the same population) and (ii) to determine the potential role of wind as a pollinating vector (bags allowed pollen to pass through but not insects). A minimum of three branches per plant for each treatment were used. Another group of non-manipulated (open pollinated) flowers, also from a minimum of three branches per plant, served as controls. A similar number of flowers were used for treatments and control.

Fruit set was also in two subsequent years, in 10 females from each population in 2000 and in 8 females from each population in 2001. Flower crop was estimated and the distance to the nearest reproductive male for each female individual considered in the study was measured to determine the influence of these parameters on fruit set.

Flower characteristics, i.e. flower weight and length, number of stamens and carpels, as well as stamen and style length, were determined at both sites in 1999 by considering 40 flowers of each sex per site. That year, fruit fresh weight, seed number per fruit and seed dimensions (weight, length and width) were also measured in 50 fruits per site.

Data analyses

Differences in the breeding system and pollen limitation between the two localities were tested by means of a two-way analysis of variance (ANOVA) in which fruit set, normalized by means of the angular transformation, was the response variable and locality and year were the predictor variables. Pearson correlations were used to assess the association between fruit set data and precipitation as well as the association between fruit set and different plant traits such as flower crop, plant volume and distance to the nearest male.

Lastly, differences between localities in mass allocation to flowers, fruits and seeds were tested by means of a one-way ANOVA.

The statistical package employed in all cases was Statistica 5.5 (StatSoft Inc.).

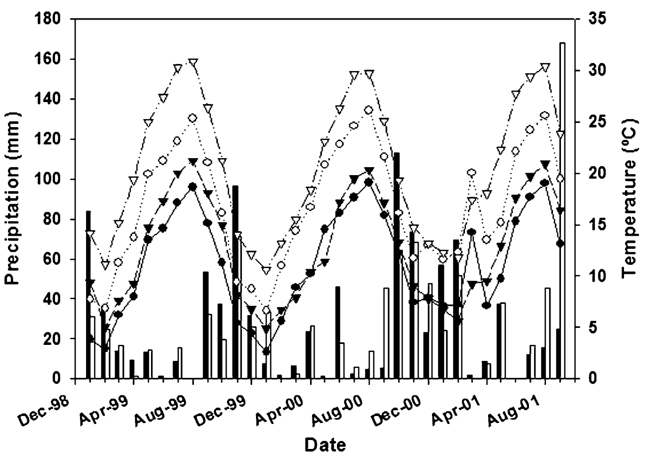

Figure 1: meteorological data at the mountain (Alfàbia) and at the coastal (Sa Bassa Plana) sites along the experimental period. Monthly precipitation at the mountain (black bars) and at the coast (white bars); monthly mean maximum temperature at the mountain (open circles) and at the coast (open triangles); monthly mean minimum temperature at the mountain (filled circles) and at the coast (filled triangles).

RESULTS

Differences in plant morphology between the two populations

Plant morphology differed between sites and plants appeared to be shorter but more extensive at the mountain than at the coast, showing a significant higher surface/height ratio, 1.81 and 1.03 $m^2\ m^{-1}$, respectively (Table 1). By contrast, female flower production was not significantly different between populations ($P > 0.05$, data not shown).

Differences in reproductive system and pollen limitation at the two populations

Rhamnus lycioides showed to be an ambophilous species, being pollinated by both insects (mainly dipterans) and wind. Both male and female flowers produce small amounts of nectar. Wind played a very important role as a pollen vector at the mountain whereas it showed a relatively lower relevance at the coastal population. Some pollen limitation was detected at the mountain site, as fruit set was slightly higher in the hand-pollination treatment than in the control group (Fig. 2). By contrast, pollen did not appear to be limiting at the coastal site, where fruit set was actually slightly lower when pollen was manually added—possibly due to a stigma clogging effect.

Factors influencing fruit set at the two localities

Fruit set was consistently higher at the mountain than at the lowland site in the three studied years (Fig. 3). Along the experimental period, fruit set varied from 53 to 76% at the mountain and from 9 to 19% at the lowland. Moreover, it is noticeable that a high variability of fruit set among individuals was observed at the coastal site, where many plants did not set any fruit regardless of treatment.

Table 1: plant characteristics at each population: plant height, plant surface and the ratio plant surface/plant height

	Coast (Sa Bassa Plana)	Mountain (Alfàbia)
Plant height (m)	1.12 ± 0.06**	0.78 ± 0.06
Plant surface (m^2)	1.31 ± 0.19	1.51 ± 0.23
Plant surface/plant height ($m^2\ m^{-1}$)	1.03 ± 0.12*	1.81 ± 0.20

Values are means of all the recorded individuals at each population ± standard error.

*Statistically significant differences at $P < 0.05$ between sites, **statistically significant differences at $P < 0.001$ between sites.

The strong differences in fruit set between the two localities might be related to the different climatic conditions (Fig. 1). The period 1999–2001 was especially dry and warm at both sites. Nevertheless, differences from a typical year were much higher at the mountain site than at the coast (Fig. 1). In fact, annual precipitation was less than half that of a normal year at the mountain in the 3 years, while such a reduction was not higher than 35% at the coastal site. By contrast, precipitation of the winter-spring period (from January to May) was notably reduced in 1999 and 2000 at both sites. The driest year was 2000, with only 41 and 63 mm at the mountain and coastal sites, respectively, compared with 1999 (156 vs. 88 mm) and to 2001 (174 vs. 121 mm). At both sites, temperatures registered during the experimental period were higher than average (between 1 and 2°C). Differences between both sites were higher in maximum than in minimum temperatures. In this regard, at the mountain site, annual average maximum and minimum temperatures were 16.8 and 11.2°C, respectively, whereas at the coast they were 20.6 and 12.6°C, respectively.

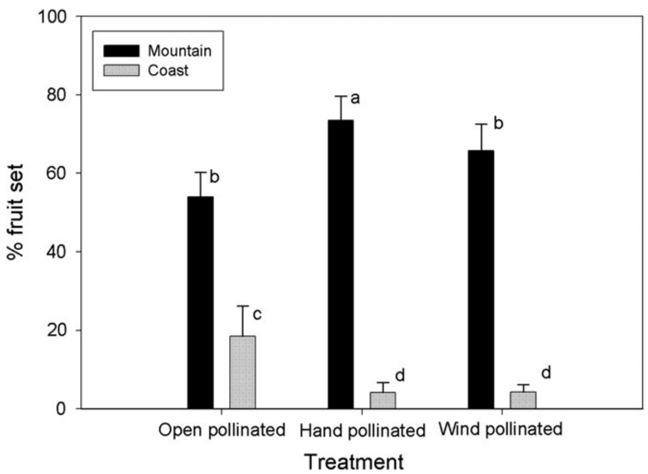

Figure 2: fruit set of *Rhamnus lycioides* at the mountain (Alfàbia) and at the coastal (Sa Bassa Plana) sites in 1999. Three treatments were applied: (**a**) open pollinated, flowers were left to be naturally pollinated, (**b**) hand pollinated, flowers were pollinated by adding pollen from nearby male individuals and (**c**) wind pollinated, flowers were bagged in order to avoid insect pollination but allowing the arrival of wind transported pollen. Different letters indicate significant differences at $P < 0.05$.

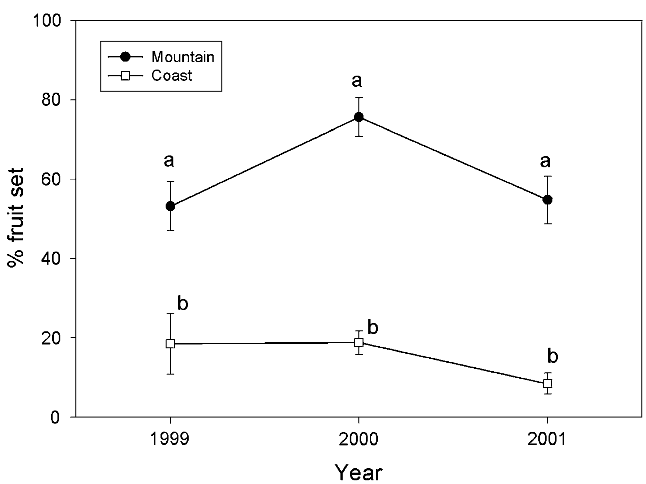

Figure 3: fruit set evolution of *Rhamnus lycioides* at the mountain (Alfàbia) and coastal site (Sa Bassa Plana), along the period 1999–2001. Different letters indicate significant differences at $P < 0.05$.

Despite the differences in precipitation across years between sites, fruit set did not show any relationship across the 3 years of the study with either annual or spring precipitation. Fruit set tended to be higher, though not significantly, at the mountain site during the driest year 2000, but, at the coast, it was quite similar to that found the previous year (Fig. 3).

Likewise, fruit set showed to be unrelated to plant volume and to flower production along the experimental period at both populations ($r^2 = 0.04$; $P > 0.05$ and $r^2 = 0.08$; $P > 0.05$, respectively). By contrast, fruit set was negatively correlated to male distance ($r^2 = -0.84$; $P < 0.01$); however, this was found only at the mountain site in 2000, and thus, such correlation was consistent neither in space nor in time (Fig. 4).

Plant reproductive allocation (mass allocation to flowers, fruits and seeds) at the two localities

Flower, seed and fruit characteristics showed few differences between sites. Flowers were heavier and longer at the coastal than at the mountain site, and such differences were higher in male than in female flowers (Table 2). By contrast, female flowers showed a significantly higher number of carpels and longer style at the mountain than at the coastal site (Table 2). Accordingly, the number of seeds per fruit tended to be higher at the mountain site. Fruit weight, but not seed weight, was also higher at the mountain (Table 3).

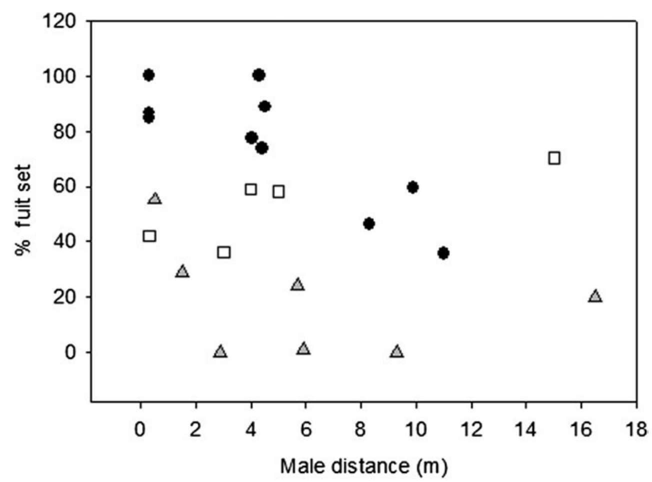

Figure 4: relationship between fruit set and male distance in *Rhamnus lycioides* at the mountain (Alfàbia) in 1999 (open squares) and 2000 (filled circles) and at the coastal site (Sa Bassa Plana) in 1999 (grey triangles). Significant correlation was only observed at the mountain site in 2000.

DISCUSSION

Differences in plant morphology between the two populations

The two study populations of *R. lycioides* presented some clear differences in plant morphology. At the mountain, *Rhamnus* individuals presented lower plant height and greater plant surface/height ratio, which could be partially explained by the more intense and frequent winds (Lain *et al.* 2008; McArthur *et al.* 2010) registered at this site. Moreover, *Rhamnus* tends to grow isolated at the mountain, which increases plant exposure to wind. By contrast, at the coastal site, most *Rhamnus* plants were located besides *O. europaea* and *P. lentiscus* plants. These three species tend to form groups, providing a microhabitat suitable for plant vertical development. These groups were probably a consequence of the important perch effect at this site, where rainfall is scarce. The importance of the perch effect has already been described in the recruitment process of several Mediterranean shrubs (Gulías *et al.* 2004; Rey and Alcántara 2000; Traveset *et al.* 2003; Verdú and García-Fayos 1996), including *R. lycioides* (Barberà *et al.* 2006; Pausas *et al.* 2006).

Differences in reproductive system and pollen limitation at the two populations

Although the relative importance of insects and wind as pollen vectors differed among populations, *R. lycioides* behaved as an ambophilous species at the two studied sites. At the mountain site, both insects and wind showed similar efficiency as pollen vectors since open- and wind-pollinated flowers did not differ in fruit set (Fig. 2), highlighting the importance of wind as a pollination vector at mountain areas (Gómez and Zamora 1996). By contrast, open-pollinated flowers showed a greater fruit set than wind-pollinated ones at the lowland, suggesting the important role played by insects at this site (Fig. 2). Similar results, showing differences in the role played by wind as pollen vector among populations, have been reported for other congeneric and ambophilous *Rhamnus* species, like *R. ludovici–salvatoris* (Traveset *et al.* 2003) and *R. alaternus* (Gulías *et al.* 2004). However, De la Bandera and Traveset (2006) did not report any difference in the relative importance of insects and wind as pollination vectors in *Thymelaea velutina* between coastal and mountain populations.

Fruit set was only limited by pollen availability at the mountain site (Fig. 2), where hand-pollinated flowers showed a greater fruit set than the open-pollinated ones. Similar spatial variation in the limiting role of pollen availability has been observed in other Mediterranean shrubs (Aronne and Wilcock 1995; Gulías *et al.* 2004; Traveset *et al.* 2003). Although variation in insect abundance may be responsible for those spatial differences, resource (water and nutrients) availability may also play an important role in pollen limitation (Ashman *et al.* 2004; Cazares-Martínez *et al.* 2010; García-Camacho and Totland 2009). At the coast, male flower production was very low, which probably led to the extremely low fruit set observed at this site regardless of treatment. Hand-pollinated flowers showed a decrease in fruit set at that site, which might be attributable to the phenomenon of stigma clogging (e.g. Holland and Chamberlain 2007).

Factors influencing fruit set at the two localities

The high variability in annual precipitation is a well-described characteristic of Mediterranean climate (Rodó and Comín 2001), in which dry periods of several years used to be followed by wet periods. The period 1999–2001 registered ~30% lower

Table 3: seed and fruit characteristics at each population in 1999: number of seeds per fruit, seed weight, seed dimensions (length and width) and fruit fresh weight

	Coast (Sa Bassa Plana)	Mountain (Alfàbia)
No. of seeds per fruit	2.1 ± 0.14	2.3 ± 0.09
Seed weight (mg)	6.6 ± 0.24	6.2 ± 0.18
Seed length (mm)	4.0 ± 0.03	3.9 ± 0.06
Seed width (mm)	1.6 ± 0.02**	1.5 ± 0.02
Fruit fresh weight (mg)	90.9 ± 3.1*	127.1 ± 8.4

Values are means of 50 repetitions ± standard error.
*Statistically significant differences at $P < 0.05$ between sites, **statistically significant differences at $P < 0.001$ between sites.

Table 2: flower characteristics at each population in 1999: fresh weight, total flower length, number of stamens, stamen length, number of carpels and style length

	Coast (Sa Bassa Plana)		Mountain (Alfàbia)	
	Female flowers	Male flowers	Female flowers	Male flowers
Fresh weight (mg)	2.23 ± 0.08*	2.76 ± 0.10**	1.89 ± 0.08	2.19 ± 0.07
Total length (mm)	1.04 ± 0.05	2.38 ± 0.07**	1.00 ± 0.04	1.84 ± 0.09
No. of stamens	4.0 ± 0.02	4.0 ± 0.03	4.0 ± 0.03	4.0 ± 0.03
Stamen length (mm)	1.12 ± 0.05	2.09 ± 0.04	1.02 ± 0.04	2.06 ± 0.06
No. of carpels	2.1 ± 0.05*	2.3 ± 0.10*	2.4 ± 0.09	2.0 ± 0.03
Style length (mm)	2.59 ± 0.13*	1.80 ± 0.12**	3.01 ± 0.09	1.28 ± 0.06

Values are means of 40 flowers ± standard error.
*Statistically significant differences at $P < 0.05$ between sites, **statistically significant differences at $P < 0.001$ between sites.

precipitation than the average in the island of Mallorca, and the two sites considered in this study were not outliers to this general trend, although such a reduction was much higher at the mountain (60%) than at the coast (20%). In spite of this unusually similar precipitation between both sites during the experimental period, fruit set was significantly lower in all treatments and years at the coastal than at the mountain site (Figs 2 and 3). Such differences can also be attributed to a higher evapotranspiration at the coast as a consequence of the registered higher temperatures at this site (Fig. 1). Moreover, differences in soil water availability between sites due to soil characteristics, such as depth and stone presence, as well as to competition for water resources among plant species cannot be ruled out. In fact, as has already been pointed out, *R. lycioides* individuals tend to be grouped with other species at the coastal site, which might decrease availability of soil water. Previous studies have suggested water limitation as a factor determining fruit set in Mediterranean species (Aragón *et al.* 2008; Giménez-Benavides *et al.* 2008). Moreover, the lack of a consistent relationship between fruit set and the distance to the nearest reproductive male (Fig. 4) suggests that other factors, like water and/or nutrients, and not only pollen availability, are also responsible for fruit set variations along sites and years.

Plant reproductive allocation (mass allocation to flowers, fruits and seeds) at the two localities

The reproductive allocation did not differ as much as expected between sites probably as a consequence of the low-precipitation differences registered along the experimental period at both sites (Fig. 1; Tables 2 and 3). Nevertheless, the observed larger flowers at the coastal than at the mountain site suggest that biomass allocation to flower crop is not a limiting factor to fruit set. In fact, this species produces flowers during winter and early spring, when water availability is maximum under Mediterranean conditions and low temperatures are usually limiting photosynthesis and growth process, a limitation that tends to be higher at mountain sites (Gulías *et al.* 2009). By contrast, fresh fruit weight was higher at the mountain than at the coast, probably as a consequence of both the greater water content and the greater total seed biomass. These parameters are mainly determined at late spring, at the onset of the drought period, which usually starts earlier at the coast.

In conclusion, *R. lycioides* appeared to be an ambophilous species despite its inconspicuous flowers, in which the relative importance of insects and wind as pollination vectors depends on population characteristics. In this regard, plants at the mountain population showed a more prevalent role of wind as pollination vector than those at the coast. Fruit set was much higher at the mountain than at the coast. Although fruit set appeared to be limited by pollen availability in some situations, resource availability cannot be ruled out as an important limiting factor in dry years. In fact, water availability seems to play a key role not only determining fruit set but also the reproductive performance of this species at the two different localities.

FUNDING

This work was framed within the projects: FEDER IFD97-0551 funded by the European Union, and BOS2001-0610 funded by the Spanish Ministry of Science.

ACKNOWLEDGEMENTS

We are grateful to Maurici Mus for indicating to us the two populations and to Francesc Socias for allowing us to work in Alfàbia. Two anonymous reviewers significantly contributed to improvement of the manuscript.
Conflict of interest statement: None declared.

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