

# Breeding system of the Iberian endemic shrub *Colutea hispanica* (Leguminosae)

by

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## Abstract

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Plant breeding systems have been viewed as mechanisms to promote outcrossing in order to increase genetic variability and prevent inbreeding depression. However, reduced fitness after crossing, outbreeding depression, has been often observed between individuals from different populations and more rarely, within populations. We studied the breeding system of *Colutea hispanica* using experimental hand pollinations in field conditions in one population in Central Spain in 2004 and 2006. Pollination experiments showed that *C. hispanica* set fruits from both self and cross-pollinations, suggesting that the species is highly self-compatible. In addition, fruit set was highest following geitonogamy in both years and no flowers set fruits from xenogamy in 2004. Although such enhanced reproductive success following selfing rather than crossing has been largely ignored in many studies on plant reproduction, it seems to be not as rare in plants as previously thought.

**Keywords:** fruit set, hand pollination, inbreeding, outbreeding, self-compatibility.

## Introduction

Knowledge of the breeding system is critical to understand the reproductive success of plants. Plant breeding systems are viewed as mechanisms to promote outcrossing, increase genetic variability and prevent inbreeding depression (Darwin, 1876; Charlesworth & Charlesworth, 1987; Barrett, 2003) so the evolution of plant mating systems has been

## Resumen

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Los sistemas reproductivos en las plantas se han considerado como mecanismos que promueven los cruzamientos con la finalidad de aumentar la variabilidad genética y evitar la endogamia. Sin embargo se ha observado con frecuencia un reducido éxito reproductor tras cruzamientos entre individuos de diferentes poblaciones, lo que se ha denominado "depresión exogámica" e incluso más raramente entre individuos de la misma población. Se estudió el sistema de cruzamiento de *Colutea hispanica* mediante la aplicación de polinizaciones manuales en el campo en una población situada en Madrid (España) durante los años 2004 y 2006. Se obtuvieron frutos tanto por autopolinización como por polinización cruzada, sugiriendo que la especie posee un sistema altamente compatible. La alta autocompatibilidad observada podría deberse a una adaptación a condiciones de escasez de polinizadores y de "parejas reproductoras". El máximo valor de fruit set se alcanzó en los cruzamientos geitonogámicos en ambos años, y en el año 2004 los cruzamientos xenogámicos no produjeron ningún fruto. Este último fenómeno parece ser más común entre plantas de lo que en principio se podría pensar, pero ha sido ignorado en la mayor parte de los estudios de biología reproductora.

**Palabras clave:** tasa de fructificación, polinización manual, endogamia, exogamia, autocompatibilidad.

channelled by the so called avoidance strategy. However, breeding systems in plants range from complete self-compatibility, usually with self and cross pollination being equally successful, to complete self-incompatibility, with no fruits or viable seeds produced by self-pollination, with partial self-incompatibility (Lloyd, 1992) as an intermediate strategy. It has been long proposed that self-fertilization can provide re-

productive assurance when cross-fertilization is unable to do so (Darwin, 1876). However, although theory predicts that either complete self-fertilization or complete cross-fertilization systems should be selected as stable strategies (Lloyd, 1992), several reviews have concluded that plants with mixed systems, with both selfing and crossing, are common between plants (Goodwillie & al., 2005). Such a mechanism may be advantageous to ensure reproductive success in unpredictable environments.

Inbreeder species could suffer depression resulting in reduced fertility. However, some species have purged deleterious alleles by selection, minimizing the risk of inbreeding depression (Husband & Schemske, 1996; Goodwillie & al., 2005). Moreover, in selfing taxa, adaptation to local environmental conditions or coadaptation of different genes may cause reduced fitness of progeny when outcrossing occurs between individuals from different populations, giving so-called outbreeding depression (Quilichini & al., 2001).

Breeding systems have important ecological and evolutionary consequences (Richards, 1997). For instance, they have been proposed as an important factor affecting vulnerability of plant species to habitat fragmentation, in such a way that self-compatible species may be less vulnerable to the effects of fragmentation (Aguilar & al., 2006).

We studied the mating system of *Colutea hispanica* Talavera & Arista (Leguminosae) an endemic and rare shrub, which is very patchily distributed in Central Spain. A previous evaluation of its female reproductive success suggested that habitat fragmentation may have improved the reproductive output of this plant, since in some years higher fruit set values were observed in smaller and isolated populations (Rabasa & al., 2009). We hypothesized that selfing could contribute to explain this surprising result. To determine the breeding system of *C. hispanica*, we conducted experimental pollinations in the field during 2004 and 2006 flowering seasons in one population in Central Spain.

## Material and methods

### Species

*Colutea hispanica* (Leguminosae) is a perennial leguminous shrub that occurs on limestone or occasionally gypsum soils in the Iberian Peninsula (Talavera & Arista, 1998). Plants are mostly summer semideciduous shrubs, that reach 2-3 m in height and usually flower between April and June. The hermaphrodite, papilionoid-type flowers have standard and keel

petals of around 2 cm. The big size of flowers might be a mechanism to attract pollinators. They are visited by butterflies and hymenopterans, including honey bees (*Apis mellifera* Linnaeus (1758)), and particularly by the carpenter bee *Xylocopa violacea* Linnaeus (1758), which is probably the only effective pollinator (pers. obs.). Flowers and fruits are consumed by several insect predators, including larvae of hymenopterans, microlepidopterans and two lycaenid lepidopterans (*Iolana iolas* Ochsenheimer (1816) and *Lampides boeticus* Linnaeus (1767)), which may destroy all seeds in a fruit (Rabasa & al., 2009, report the proportion of damaged fruits ranged 72-98% in 2003-2005).

### Study area

The study was conducted in 2004 and 2006 in one population of *C. hispanica* located in a gypsum soil area near Chinchón, approximately 45 km southeast of Madrid, Central Spain (centred on 40°08' N, 3°28' W). *C. hispanica* is patchily distributed in this area, with small populations ranging from tens to a few hundred individuals, which allows the easy delineation of populations in the field. We conducted all of our experiments in one of the larger populations in the area, with several hundreds of individuals, so as to avoid bias due to small population sizes.

The climate is semi-arid Mediterranean with a mean annual temperature of 14.2°C and average annual rainfall of 438 mm (data from Arganda del Rey thermopluviometric station,  $N = 29$  years). Mean daily maximum and minimum temperatures are, respectively, 10.6°C and 0.2°C in January, and 33.1°C and 15.5°C in July. There is a pronounced summer drought from May to September. The average rainfall in spring (from April to June) in 2004 and 2006 was 210.5 mm and 68.8 mm respectively.

### Pollination experiments

We performed crossing experiments with samples of 20 plants in 2004 and 8 plants in 2006. We could not obtain more plants with sufficient available flowers in 2006 due to low flowering intensity coupled to an extreme summer drought (see Study area above). We selected 5 flowers per treatment in each plant, totalling 400 flowers in 2004 and 160 in 2006. Flowers were selected before anthesis and assigned to one of four treatments: (1) Control: open-pollination; (2) Autonomous selfing (autogamy): flowers non-manipulated; (3) hand pollinated with pollen from other flowers in the same plant (geitonogamy); and (4) hand pollinated with pollen from other plants (xenogamy).

To avoid contamination of the stigmas with self-pollen, we previously removed all anthers from the flowers in treatments 3 and 4. Previously, we had carried out an emasculating test in all plants to examine the effectiveness of this treatment. No fruits were produced in emasculated flowers. All flowers in treatments 2, 3 and 4 were bagged with cellophane bags until fruit maturation to exclude pollinators. Pollen for xenogamous crossings was collected from a variable sample of flowers (ranging from 5 to 10 flowers per plant) of at least 5 different adjacent plants (separated from each other at least 2-3 meters) in the same population. Pollen for geitonogamous crossings came from at least five flowers in the same plant. Hand pollinations were made by rubbing the stigmas with a little paint brush.

Although we did not test the viability of pollen, it was thoroughly mixed before treatment in an Eppendorf tube. Thus, if differences in quality existed, those differences would be similarly partitioned across all treatments and would not affect the results. We collected fruits before completing seed maturation to avoid predation as much as possible. For this reason, we could not estimate seed set. We defined fruit set as the proportion of flowers setting fruit per plant on each treatment.

### Data analyses

We performed separate analyses for each year using generalized linear mixed models (GLMM; McCullagh & Nelder, 1989; Breslow & Clayton, 1993) using a randomized block design. We did not analyze fruit set per individual flower since binary data with few replicates (5) on each subject can produce biased results (Breslow & Clayton, 1993). Instead, we considered the response variable fruit set per treatment and plant. It was performed assuming a binomial distribution of errors with logit link. We used the restricted maximum-likelihood (REML) method (Patterson & Thompson, 1971) and Satterthwaite's method to determine the approximate denominator degrees of freedom for tests (Verbeke & Molenberghs, 1997). We considered treatment, with 4 levels (control, autonomous selfing, geitonogamy and xenogamy) as fixed variable. To take into account the variability on fruit set between plants we considered plant as random variable (block). We carried out post-hoc analyses of least-squared means to identify significant differences among levels of treatment. Analyses were performed using SAS Macro program GLIMMIX (GLIMMIX v. 8 for SAS/STAT; available on the

World Wide Web at <http://www.sas.com.techsup/download/stat/>).

### Results

Fruit set was relatively low in all cases in both years, in spite of following the geitonogamy treatment. Five and one plants in 2004 and 2006 respectively produced no fruit in any treatment (control, autonomous selfing, geitonogamy or xenogamy). These data were removed from subsequent analyses to avoid confusion between effects of treatment and other factors affecting plant reproduction. The average ( $\pm$  SD) values of fruit set per treatment are summarized in Table 1. These results are in concordance with the low fruit set reported in this population in several years in natural conditions (Rabasa & al., 2009).

The GLMM results showed that there was a significant effect of treatment on fruit set in both years (Table 2). Fruit set varied widely among plants in 2004 but not in 2006. Although a randomized block design does not allow for testing interactions, a higher fruit set by geitonogamous crossings was consistent among plants in 2004 and 2006 (Fig. 1). Post-hoc test showed that fruit set varied significantly between treatments with more fruits produced by geitonogamous crossings in comparison with control, autonomous selfing and xenogamy in 2004 (Table 1). In 2006, geitonogamy also had the highest fruit set, but it was not significantly different from that found for xenogamy. Control and autonomous selfing treatments achieved significantly lower fruit set than geitonogamy and xenogamy (Table 1).

### Discussion

*Colutea hispanica* has a self-compatible breeding system, with the highest fruit set produced mainly by geitonogamous crossings in hand pollinations but it also has a considerable capacity for autonomous selfing. The close position of mature anthers and stigmas suggests that autonomous selfing would occur even

**Table 1.** Results of hand pollination experiments on *Colutea hispanica* in 2004 and 2006 (Mean fruit set value  $\pm$  SD). Values followed with different letters are different at  $P < 0.05$ .

Treatment	2004	2006
Control	0.19 $\pm$ 0.21a	0.08 $\pm$ 0.16a
Autonomous selfing	0.11 $\pm$ 0.13b	0.21 $\pm$ 0.17ab
Geitonogamy	70.56 $\pm$ 0.23c	0.41 $\pm$ 0.23c
Xenogamy	0 $\pm$ 0d	0.31 $\pm$ 0.29bc

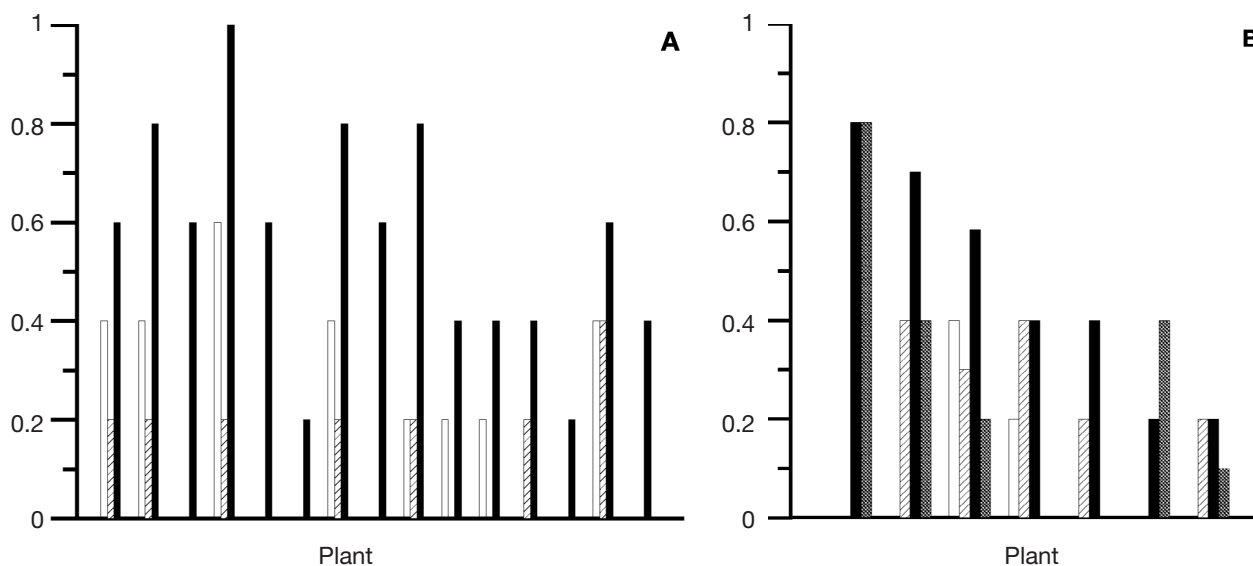
**Table 2.** Generalized linear mixed models for fruit set for 2004 and 2006. Analyses were based on a GLMM with binomial errors and logit link using REML estimation. Random effects were tested with Wald tests and fixed effects with Type III *F*-tests.

Year	Variable	Estimate $\pm$ SE	df	Test-value	P
2004	Random				
	Plant	2.40 $\pm$ 1.08		2.23	0.0129
2004	Fixed				
	Residual	0.11 $\pm$ 0.02		5	<0.0001
2006	Random				
	Plant	0.58 $\pm$ 0.58		1	0.1585
2006	Fixed				
	Residual	0.23 $\pm$ 0.07		3.15	0.0008
	Treatment		4, 32.5	20.83	<0.0001
	Treatment		4, 18.4	5.48	0.004

before flower opening (personal observation). However, the investment in relatively large flowers and nectar suggests that *C. hispanica* has evolved to attract pollinators. In fact, artificial pollination increased fruit set compared to open pollination, indicating that pollinators actually are needed to increase fruit set and that some pollen limitation occurs. The main pollinator of *C. hispanica*, *Xylocopa violacea*, was relatively scarce in the study area (Rabasa, personal observation). The highly self-compatible reproductive system of *C. hispanica* may be advantageous to conditions of scarce pollinators and potential mates in this area, as a strategy to ensure reproduction (Kalisz & Vogler, 2003). Nevertheless, a high proportion of self-pollina-

tion might also result in a loss of genetic diversity that might preclude potential adaptation to changing environments. Initially, an increase of selfing may also lead to unviable seeds, reduced germinability of these seeds, or reduced fitness of the progeny. Nevertheless, in a previous study that focused on the effects of habitat fragmentation on the reproduction of *Colutea hispanica* (unpublished data), we found a high rate of germination of seeds from very isolated plants whose seeds were probably produced by autogamy.

An unexpected finding was the lack of fruit set produced by xenogamous crossings in 2004. In 2006, the proportion of fruits produced by xenogamy was also lower than by geitonogamy (fruit set 0.41 and 0.31 for



**Fig. 1.** Fruit set produced by control (open bars), autonomous selfing (hatched bars), geitonogamy (closed bars) and xenogamy (dotted bars) by each plant in **A** 2004, and **B** 2006. Plants that produced no fruits in any treatment (5 in 2004, 1 in 2006) are not shown.



geitonogamy and xenogamy, respectively), but not significantly so. It is possible that the lack of difference in this year was due to the small sample size (8 plants) (Fig. 1).

The possibility that self-compatible individuals may produce greater seed set or fruit set by selfing rather than xenogamous crossing, has been largely ignored in the literature, although this phenomenon does not seem to be uncommon in plant species. In their review of self- and cross- fertilization in plants, Lloyd & Schoen (1992) found that almost 40% of their self-compatible species produced a higher fruit set or seed set by selfing than by cross-pollination, but surprisingly they attributed those results to experimental errors, arguing that there are no biological grounds for explaining them. This conclusion is probably a consequence of the difficulties for an adequate explanation under the current paradigm. We did not perform any test for viability of pollen or receptivity of stigmas, but flowers were randomly assigned to treatments, and pollen of both geitonogamous and xenogamous crossings were treated equally. In addition, experiments were repeated in two different years. Thus, there is apparently no reason to believe that results are due to errors in experimental manipulation. Moreover, there are some other studies providing similar results. Hand-pollination experiments in *Cassia aphylla* Cav. (Leguminosae), *Hypoxis decumbens* (Hypoxidaceae) and *Talinum paniculatum* (Portulacaceae) showed a reduced fitness from xenogamous crossings (Aizen & Feinsinger, 1994; Raimúndez & Ramírez, 1998; Valerio & Ramírez, 2003). Such “outbreeding depression”, or incompatibility between individuals of the same species that reduces fitness, has been widely described in plants among isolated populations as a mechanism for local adaptation (e.g., Richards, 1986; Waser & Price, 1994; Fischer & Matthies, 1997), but it has rarely been studied among individuals within populations. However, outbreeding depression may also occur over very small distances within plant populations, even at less than 20 cm in the rare endemic species *Anchusa crispa* (Boraginaceae) (Quilichini & al., 2001), and in *Amphicarpea bracteata* (Leguminosae) Parker (1992). Our results provide further evidence for the occurrence of these negative effects of outcrossing on a very small local scale. This phenomenon is probably more common in plants than previously reported, and further research is necessary in order to understand the genetic and evolutionary mechanisms operating therein.

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