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The reproductive biology of motherwort (*Leonurus cardiaca* L.)

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Abstract

Leonurus cardiaca (Lamiaceae) is an important medicinal plant, growing wildly in many parts of Iran. It has been used to cure cardiovascular diseases, stress, anxiety, and nervous irritability. There has been no report on the breeding system of this species. This experiment was accordingly conducted to investigate the flower biology, pollination system, pollinators, and breeding system. The results show protandry is the dominant form, and the stigma reaches its most receptivity 48 hours after anthesis, while the highest in-vitro pollen germination is observed within two hours after anthesis. The examination of different types of pollination in this plant indicates that the highest seed set percentage, seed weight, and seed viability can be obtained by open pollination, but the existence of pollinators improves the reproduction significantly. Based on our results, *L. cardiaca*, with 27.48 ± 1.45% autogamy, is a self-compatible plant, whose reproduction is improved by cross-pollination. Honey bees (*Apis melleifera*) were the most important and common pollinators.

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Introduction

Reproduction is one of the crucial steps determining the abundance and distribution of living organisms. Reproductive strategies determine the quantity and quality of the offspring and, consequently, the movement of the genes in time and space (Barrett 2010; Charlesworth 2006). The reproductive strategy assumes a special relevance in rare, endemic and/or narrowly distributed species, as it will act in a reduced number of individuals/populations directly determining their fitness and genetic composition, and affecting population dynamics and long-term persistence (Oostermeijer 2003). Understanding pollination and breeding systems that regulate the genetic structure of populations is important for determining the reproductive constraints in conservation and management (Kaye, 1999; Koul and Bhatnagar, 2007). Besides quality improvement of medicinal plants, cultivation of medicinal plants is a challenging task because less is known about their reproductive and seed biology (Anderson *et al.*, 2006; Neal and Anderson, 2005). Equally important is the pollination behavior of the species which is an integral part of reproductive biology. Breeding systems are influenced by a variety of flower traits (e.g., flower size and shape and degree of dichogamy), self-incompatibility, floral display, and the arrangement of flowers on the plant (Harder and Barrett, 1996; Tandon *et al.*, 2003). The timing of flowering can strongly influence the reproductive success of a plant in several ways (Rathcke and Lacey, 1985). Such effects may be mediated by abiotic factors and factors operating within plants, within populations, and between species (McIntosh, 2002).

Lamiaceae is a large family, ca. 220 genera and 4000 species, with a cosmopolitan distribution, which is particularly well represented in warm temperate regions (Cronquist, 1981; Hedge 1992). Allogamy is a usual reproductive process in the plants. There is still no wholly convincing evidence that self-incompatibility occurs in every species in Lamiaceae. Protandry is common, and self-pollination is therefore almost impossible. Geitenogamy and cross-pollination are dominant in the family (Hidalgo and Ubera, 2001; Rodriguez-Riano and Dafni, 2007). The reproductive biology of several species of Lamiaceae has been widely studied; for example, *Origanum majorana* and *Teucrium capitatum* (Rodriguez-Riano and Dafni, 2007), *Rosmarinus officinalis* (Hidalgo and Ubera, 2001), and *S. smyrneae* (Subashi and Guvensen, 2011). However, little is known about the genus *Leonurus*. Yeo *et al.* (2006) described flower and inflorescence developments of *L. sibiricus* and reported natural self-pollination and artificial cross-pollination.

Leonurus cardiaca, commonly known as motherwort, is a member of the Lamiaceae family that has been consumed in Asian countries as a traditional remedy against nervous and functional cardiac disorders (Wojtyniak *et al.*, 2013). *Leonurus cardiaca* belongs to the genus *Leonurus* and family Lamiaceae, previously called Labiatae. It is a perennial herb prevalent in Europe, usually found in country areas throughout the plains and hills, as well as in East Asia to the Himalayas and eastern Siberia, Northern Africa, and North America (Milkowska-Leyck *et al.*, 2002). Its chemical compounds such as alkaloids, iridoids, flavonoids, saponins, cardenolid-like glycosides, and diterpenoids have been isolated from the leaves and flowers (Milkowska-Leyck *et al.*, 2002). The healing of heart diseases is mainly connected with the flavonoids (Mockute *et al.*, 2006).

Improving the quantity and quality of the active ingredients of motherwort would be the objective of a *L. cardiaca* plant selection and breeding program. Numerous research papers have been published on the active constituent and pharmacological activities (Ulubelen *et al.* 2005; Janicsák *et al.* 2006; Ali *et al.* 2007) as well as the species distribution of motherwort (Soorni *et al.* 2013; Khadivi Khub and Soorni 2014). However, the reproductive biology and constitution of *Leonurus* spp. have been less studied (Popescu *et al.* 2009; Heuberger *et al.* 2010). Heuberger *et al.* (2010) reported that L. japonicus is mainly a cross-pollinating species and intensively visited by bees and bumble bees during flowering, whereas self-fertilisation was observed under isolation conditions.

There are no previously published studies on the reproductive biology of *L. cardiac*. The present work on *L. cardiaca* covers: (a) pollination biology (b) the breeding system and (c) pollinators.

Materials and methods

Experimental site and plant materials

The present investigation was carried out at Research Center for Department of Horticulture, University of Tehran, Karaj, Iran, during 2015-2016. Its geographical coordinates are N 36°19'0" and E 50°59'29" with an altitude of 1320 meters above sea level. This area is a mountainous region with a cold and wet winter and a mild summer. The present experiment was conducted on 80 random plants (three-year old) originated from seeds collected from their natural habitat in Isfahan. The soil texture was clay loam.

Pollen germination and stigma receptivity

To evaluate pollen germination at different stages of flower development, pollens were collected at four developmental stages, including 2, 24, 48, and 72h after anthesis. To determine the germination, pollen grains were sowed with a clean brush in petri dishes containing culture medium (15% sucrose, 100ppm boric acid (H_3BO_3) and 1% agar) according to Dane's *et al.* (2004) method. The petri dishes were incubated at the constant temperature of 25°C under usual light conditions (500 lux) for 24h. The pollen grains were considered germinated when the pollen tube length was greater than the diameter of the pollen grain. A minimum of 100-150 pollens were counted per petri dishes with three replicates. Germination percentage was determined by dividing the number of germinated pollen grains by the total number of pollen per field of view. A light microscope was used to determine the pollen germination.

Timing of stigma receptivity was investigated by assessing seed set following controlled pollination. 35 flowers were emasculated early in the morning before anthesis. Hand pollination was performed with freshly collected pollens at three different times: 24, 48, and 72h after flower opening. Flowers were bagged and left until seed production.

Breeding system

To assess the breeding or reproductive system in *Leonurus cardiaca*, six experiments were conducted in two flowering seasons, April to September 2014 and 2015. The breeding system type was evaluated by determining the level of seed set. Observations were made on 100 flowers from 10 randomly selected plants. Hand pollinations were performed during 8 to 10am. Measurements were performed on 50 mature seeds (10 seeds in 5 replicates) for each experiment, and means of seed weight and seed viability were calculated. Seeds were cut, and by the presence or absence of embryo, they were considered healthy and viable or dead and aborted, respectively. The experiments were run as follows:

1) Autogamy (self-pollination): flower buds were bagged before anthesis to avoid being visited by insects and left for seed set. 2) Apomixy: flowers were emasculated before anthers dehisced and then bagged and left for seed set. 3) Geitonogamy: flowers were emasculated and then artificially hand-pollinated with pollens taken from the older flowers on the same plant by shaking ruptured anthers on the stigma to test self-compatibility. 4) Natural outbreeding (xenogamy by insects): flowers were emasculated and then left open 5) Artificial outbreeding (xenogamy by hand): flowers were emasculated and pollinated by fresh pollens collected from a donor plant from the same population; and 6) Open pollination: plants were left open and exposed to pollinators without any treatments (control plants).

Self-incompatibility index (ISI) was calculated by the formula proposed by Zapata and Arroyo (1978), which measures the self-incompatibility of a plant species: ISI = (self-fruit set) / (cross fruit set), where self-fruit set and cross-fruit set are data obtained from controlled pollination experiments. Value 1 indicates self-compatibility; values $0.2 > ISI < 1$ indicate partial self-compatibility; $ISI < 0.2$ indicates mostly self-incompatible plants, and ISI = 0 indicates complete self-incompatibility.

Floral visitors

Insects observed visiting the flowers were captured using a standard net at three different times: 9-10a.m, 12-13p.m, and 16-17p.m, when 50-70 percent of flowers were opened. Insects were placed into separate vials containing cyanide solution and then transferred to 70% ethanol for further identification. The floral visitors were identified by the experts in the entomology lab (University of Tehran).

Statistical analyses

Pearson correlation coefficients were calculated to investigate the relationships between the style length, stigma reception, and pollen germination. Analysis of variance was performed by One-way ANOVA using SPSS v.22 software (IBM Corp., 2013), and means were compared according to Duncan's multiple range test.

Results and discussion

Pollen germination and stigma receptivity

The time of stigma receptivity was measured by seed production percentage. As the flower aged, the stigma receptivity increased significantly ($P < 0.01$; Fig. 1). The peak of seed production occurred within 48h after anthesis (three-day old flowers) and decreased dramatically in next 12h. With increasing the stigma receptivity, the style became longer, and the stigma's lobes opened. The stigma receptivity and style length were positively correlated. The highest percentage of in-vitro pollen germination occurred 2 hours after flower opening, synchronizing with anther dehiscence, and then reduced significantly, reaching its minimum after 72 hours ($P < 0.01$; Fig. 1). Pearson correlation coefficient showed that style length had a positive meaningful correlation with stigma receptivity, while it had negative correlations with flower age and pollen germination (table 1). In *L. cardiaca*, the release of pollen and stigma receptivity are asynchronous, and anther dehisces before the stigma becomes receptive. The time gap between male and female phases is about 2-3 days. The flowers pass through sequence of male, bisexual, and female phases.

Breeding system in *Leonurus cardiaca* favours outcrossing through the dehiscence of anthers before the stigma is receptive (protandry). In protandrous species are two sex phases (male and female), which were separated in terms of time. Pollen dispersal starts 2-3 h after flower opening, while the style is situated below the level of anthers, and the stigma branches are closed. At this stage, the flowers are totally male and disperse pollens. At female stage, the style increases in length, and stigma branches separate (after 2 days). It seems that autogamy can take place at the second day after anthesis, when flowers act as bisexual, which means both stigma and pollens are functional. Lloyd and Webb (1986) have suggested that the separation of pollen and stigma acts in general to reduce self-interference and often reduces self-fertilization. To avoid self-pollination, a negative correlation between stigma receptivity and pollen viability exists in protandrus species, although it cannot prevent the pollination of the flower by pollens of the other flowers on the same plant (geitenogamy). Rodriguez-Riano and Dafni (2007) pointed out that in *Origanum syriacum*, self-pollination is avoided by dichogamy (a negative correlation between stigma receptivity and pollen viability), and the style length is significantly positively correlated with stigma receptivity.

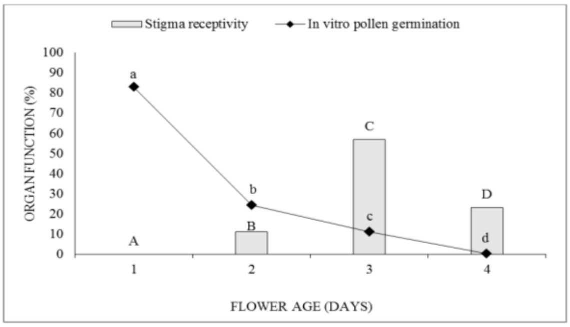

Fig. 1. Sexual functioning of flowers *Leonurus cardiaca*. Significant differences in female function and male according with flower age ($P < 0.01$).

Table 1. Correlation coefficients among style length, stigma receptivity and pollen germination in *Leonurus cardiaca.*

Stigma receptivity	Pollen germination	Style length	
		1	Style length
	1	-0.977 **	Pollen germination
1	-0.655 *	0.779 **	Stigmatic receptivity

Breeding system

Seed set was significantly different among treatments ($P < 0.01$) (table 2). The lowest figure (27.48 ± 1.45%) was observed in self-fertilization treatment (autogamy), while the highest occurred in open pollination (93.75 ± 1.53%), followed by xenogamy by insects and hand pollination, respectively. There was no seed production in the apomixy test. Again, the maximum amount of both seed weight and seed viability were recorded in the open pollination test while the minimum observed in xenogamy (by hand) and geitonogamy. Our experimental studies indicate that *L. cardiaca* is self-compatible (27.48% seed set in autogamy and 42.46% in geitenogamy). Self-compatibility was already presented in many species of Lamiaceae (Navarro, 1997; Jorge *et al.*, 2014; Subashi and Guvensen, 2011; Hidalgo and Ubera, 2001; and Rodriguez-Riano and Dafni, 2007).

Self-incompatibility index =0.45 (% of seeds produced by manual self-pollination [geitenogamy]/ % of seeds formed by crossed pollination) is characteristic of partially self-compatible species (Zapata and Arroyo, 1978). Although *L. cardiaca* is self-compatible, a higher seed production occurs following cross pollination, indicating facultative allogamy. High outcrossing levels can be maintained even in self-compatible species through floral strategies that favor some pollinator foraging strategies. The study concludes that even though *L. cardiaca* is a cross-pollinated species, self-pollination is still occurred in the absence of pollinators. Goodwillie *et al.* (2005) have shown that mixed mating systems are common in nature. Many observations also suggest that facultative allogamy is wide-spread in Lamiaceae. The flexibility in the breeding system to reproduce by both auto- and allogamy might have evolved as a response to a gradually changing environment. This breeding system alone would lead to quick propagation and colonization of the new habitat (Allards, 1965).

Under natural conditions (open pollination), *L. cardiaca* showed the highest seed set percentage, seed weight, and seed vigor, which is consistent with the results of Navarro (1997), on *S. Verbenaca* and Jorge *et al* (2014), on *S. sclareoides*. It seems that the absence of pollinators reduces fruit set, and the presence of pollinators significantly enhances it by facilitating the pollen transfer. Low seed set observed in autogamy and geitonogamy tests is a consequence of pollinator limitation, which is perceived as a mechanism to promote cross-pollination (Marshall and Folsom, 1991). Outcrossing gives the opportunity for gene flow within and/or among populations, thus increasing genetic diversity and enhancing offspring performance and its potential for adaptation (Morran *et al.*, 2009). Under unpredictable pollination services or male availability, selfing can bear some advantages, as it can work as a reproductive assurance mechanism, and it can enable the preservation of well-adapted genotypes (Charlesworth, 2006; Kalisz and Vogler, 2003).

Table 2. Pollination treatments, percentage seed set and their seed weight and seed viability (mean ± S.E) in *L. Cardiaca*.

Treatment applied	Seed weight (%)	Seed set (%)	Seed viability (%)
Autogamy	9.80 ± 0.34^{b}	27.48 ± 1.45^{e}	76.96 ± 3.50^{b}
Geitonogamy	5.96 ± 0.59^{c}	42.46 ± 2.14^{d}	28.22 ± 4.57^{c}
Xenogamy (insect pollinated)	10.58 ± 0.26^{ab}	77.44 ± 3.27^{b}	80.99 ± 2.83^{b}
Xenogamy (hand pollinated)	6.16 ± 0.58^{c}	54.39 ± 2.27^{c}	27.53 ± 4.25^{c}
Open pollination	11.68 ± 0.43^{a}	93.75 ± 1.53^{a}	93.61 ± 2.14^{a}

Self-incompatibility index = 0.54.

Floral visitors

A total of 162 specimens belonging to four families and seven species were collected and identified (table 3). The most abundant insects belonged to Apidae and Megachilidae, examples of which were *Anthophora*, *Osmia*, *Megachile*, *Thyreus*, and *Apis*. Regarding the frequency of the visitor insects, it was seemed that *Apis mellifera* was the most effective species for the pollination of *Leonurus cardiaca*. *Apis mellifera* (honeybee) was recorded as the main pollinator because it frequently touched the flower reproductive organs during foraging activity. They were also the most abundant visitors among other pollinators.

Honeybees act as nectar collecting bees and during forage, the pollens stick to their feet and body. Thus, the pollens are transferred when the insects visit other flowers. Honeybees visit several flowers in the same plant before moving on to a different shrub. Insect pollination is thought to benefit the yields of 75% of globally important crop species and is responsible for approximately 35% of world crop production (Klein *et al.,* 2003). Although many species are known to provide pollination services, honey bees (*Apis mellifera* L.) are often assumed to provide the majority of these services to agriculture (Breeze *et al.,* 2011).

Members of Apidae were playing crucial roles for picking up and delivering of pollens in Lamiaceae. The bilabiate corolla is favored mainly by bees. The essential reproductive structures of the flower were arranged in the upper lip and the lower lip, acting as a landing place for insects. To access the nectar, the visitors sat on the lower lip and inserted proboscis deeply into the corolla tube. While probing nectar from flower, pollen grains were stuck in the insects back (Torezan-Silingardi and Del-Claro, 1998). Some studies have documented that honey bees have an important role in the pollination of the members of Lamiaceae such as *Salvia sclarea*, *Melissa officinalis*, *Salvia officinalis*, *Lavandula stoechas*, and *Hyssopus Officinali* (Bozek, 2003; Macukanovich-Jocich *et al.*, 2011).

Megachile is also reported as the principal pollinator in *Anisomeles indica* and *A. malabarica* (Lamiaceae) (Solomon Raju and Subba Reddi, 1989). *Thyreus* is a parasite bee that visits mostly long tube flowers and transfers their pollens.

Table 3. Flower visitors of *L. cardiaca* during pollination stage.

Family	Species	No. specimens	Relative percentage
Apidae	*Apis mellifera*	94	61.84
Apidae	*Anthophora zonata*	13	8.55
Megachilidae	*Osmia* sp.	17	11.8
Megachilidae	*Megachile* sp.	11	7.24
Apidae	*Thyreus* sp.	20	6.58
Coccinellidae	*Coccinella septempunctata*	3	1.97
Syrphidae	-	4	2.63
Total		152	100

Conclusion

This study has shown that *L. cardiaca*, is a facultative allogamous and protandrous species. Seeds could be produced through autogamy, geitenogamy, xenogamy, and open pollination but not through apomixy. Protandry is a dominant phenomenon in the pollination of this species. The adaptation of species to insect and to a flexible breeding system involving both selfing and crossing safeguards its survival under changing environments. These findings could be useful in developing efficient seed-regeneration protocols and breeding strategies for the species.

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