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Reproductive biology of a medicinally important plant *Leonurus cardiaca* (Lamiaceae)

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Abstract. Motherwort (*Leonurus cardiaca* L.) is an annual species indigenous to central Europe and Scandinavia and has been used in traditional medicine because of its health benefits. The reproductive biology of *L. cardiaca* was investigated to provide a basis for the genetic improvement of secondary metabolites or extracts that could have human health benefits. *L. cardiaca* is self-compatible but bears protandrous flowers, which promote cross-pollination. The stigma becomes receptive 2 or 3 days after anthesis and anther dehiscence. Phenological observations revealed that the mean flowering duration was approximately 2 weeks within each inflorescence and 2 months within a plant. The timing of anthesis of flowers within each inflorescence and flowers of different inflorescences is usually synchronous, leading to the possibility of geitonogamous pollination. A high degree of synchronisation in flowering was observed among the plants within each of the populations studied. Among the populations, Khansar was the first to flower, whereas Dargaz was the last. A modified Brewbaker and Kwack (BK) medium optimised for *in vitro* germination of *L. cardiaca* pollen was used to establish a rapid and simple test that generally correlated with seed set. The optimised medium comprised 15% sucrose and 2.5% polyethylene glycol 4000. Data from staining with iodine–potassium iodide solution (IKI) and Alexander's stain were positively correlated with *in vitro* germination and, therefore, could be used in rapid pollen-viability assays for *L. cardiaca*.

Additional keywords: flower structure, pollen, pollination biology, stigmatic receptivity.

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Introduction

The motherwort genus (*Leonurus*) belongs to the mint family (Lamiaceae) and most species in the genus are native to Europe and Asia within the North Temperate Zone (Wu and Li 1977). *Leonurus* includes 24 species with three divisions and five subdivisions (Erdtman 1945; Hedge *et al.* 1990; Li and Hedge 1994; Chao and Zhou 1998; Ahvazi *et al.* 2012). The common motherwort (*Leonurus cardiaca* L.) is the only species of *Leonurus* found in Iran (Mozaffarian 1996; Ahvazi *et al.* 2012).

For more than 2000 years the Chinese have used some *Leonurus* species as medicinal herbs to invigorate blood circulation, regulate menstrual function, dissolve blood stasis, generate new tissues, promote urine excretion and reduce inflammation (Chinese Pharmacopoeia Commission 2010; Yin *et al.* 2010; Borna *et al.* 2016). The chemical constituents of *L. cardiaca* include flavonoids (apigenin, genkwanin), triterpenoids (ursolic acid and corosolic acid), alkaloids (stachydrine, leonurine), isomerestrine and betonicine (Tomás-Barberán *et al.* 1993; Ulubelen *et al.* 2005; Janicsák *et al.* 2006; Ali *et al.* 2007; Borna *et al.* 2016). Motherwort is also rich in epi-cedrol, a-humulene, germacrene-D and spathulenol for

medicinal use (Morteza-Semnani *et al.* 2008). Plant extracts from Motherwort have been used to treat psychological disorders, heart ailments, premenstrual syndrome (PMS), high blood pressure, stresses and labour pain. All these health benefits are attributed to the extracted chemicals that strengthen the nervous system, reduce spasms, increase blood circulation and reduce thyroid hormone production (Arber 1938; Chao and Zhou 1998; Mills and Bone 2000; Popescu *et al.* 2009; EMA/HMPC 2010; Quattrocchi 2012; Borna *et al.* 2016).

Reproductive-biological studies are essential for successful cultivation, conservation and genetic improvement of plants (Baskorowati 2006; Moza and Bhatnagar 2007; Baskorowati *et al.* 2010). Knowledge of flower structure and breeding system is essential for controlled pollination and crossing of any species. Inflorescence structure influences the foraging efficiency of insects, degree of outcrossing and other aspects of plant reproductive success (Wyatt 1982). Inflorescence architecture is known to significantly influence the probability of fruit maturation. The physical location of a flower within an inflorescence influences the probability of producing fruit because of vascular connection to the assimilate source and

competition with adjacent fruit for limited space and resource. The production of many few-flowered inflorescences distributes reproductive demands more evenly over an individual plant than does that of a few, many-flowered inflorescences (Stebbins 1974). An indeterminate inflorescence such as a raceme also spreads the demand for resources over a longer period of time, thus improving fruiting efficiency. Indeterminate inflorescences may also extend the length of the flowering period, so that more crosses with different individuals in the population are possible. Thus, the architecture of inflorescences may strongly affect the levels of outcrossing in natural populations and these effects manifest themselves in both time and space (Wyatt 1982).

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 (Tomás-Barberán *et al.* 1993; Ulubelen *et al.* 2005; Janicsák *et al.* 2006; Ali *et al.* 2007) as well as the species distribution of motherwort (Soorni *et al.* 2013a, 2013b; Khadivi-Khub and Soorni 2014; Soorni *et al.* 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. cardiaca*. An adequate understanding of the reproductive strategy of this plant requires detailed studies of its floral biology, flowering phenology, pollination and breeding systems. Therefore, the present study on *L. cardiaca* was undertaken to (1) ascertain its functional floral morphology, (2) study its floral phenology and sexuality including pollen and stigma biology, (3) evaluate its self- and cross-compatibilities *in vitro*, (4) optimise its *in vitro*

pollen germination by testing the effects of sucrose and polyethylene glycol (PEG) concentrations on pollen germination and pollen-tube growth, and (5) identify a pollen-staining method that correlates well with *in vitro* germination.

Materials and methods

Plant material and study site

Seeds of three populations of motherwort collected in Iran from three regions over three provinces were used in the study: Dargaz, Khansar and Sarab (from the provinces of South Khorasan, Isfahan and Ardabil respectively). The global distribution of *L. cardiaca* is shown in Fig. 1 (Discover Life 2016) and the sites of the three populations collected in Iran are shown in Fig. 2. The seeds were stratified at 4°C for 8–10 weeks, and, subsequently, sown in culture trays containing peat moss. The seedlings were transplanted at the 6–8-leaf stage to a tunnel house at the University of Sydney Plant Breeding Institute, Cobbitty (34.02°S, 150.67°E, altitude 87 m). These plants were grown to maturity and maintained in a hydroponic system at the experimental site.

During the course of the study, temperature, relative humidity and photosynthetically active radiation (PAR) in the tunnel house were recorded using a CR200X data logger (Campbell Scientific Australia, Townsville, Qld, Australia), as shown in Fig. 3.

Floral structure and phenology

Fourteen morphological traits of floral structure of the three populations (Dargaz, Khansar and Sarab) were studied. These traits included number of verticillasters per inflorescence, number of flowers per inflorescence, number of inflorescences per plant, length of floral-axis internode, length of inflorescence, flower length, calyx length, corolla length, pistil length at anthesis, stamen length, number of anthers per flower, average



Fig. 1. Global geographical distribution of *Leonurus cardiaca*. Map generated from 'Discover life' online database, January 2016.



Fig. 2. Geographical distribution of *Leonurus cardiaca* populations in Iran and their collection sites.

pollen grain number per flower, number of ovules per ovary and pollen : ovule ratio.

The structure and morphology of the inflorescences, floral buds, flowers and fruits were recorded daily from January to mid-March 2015. The morphological features of freshly opened flowers ($n=20$) were analysed under a binocular stereomicroscope (Zeiss Stemi 2000-C with KL1500 LCD light source, Carl Zeiss Oberkochen, Germany) and a digital caliper was used to measure the length and width of floral buds and flowers.

Flowering phenology was studied on single flowers, inflorescences as well as plants, and timing, duration, sequence and intensity of flowering were assessed. The phenological observations recorded on the inflorescences were (1) rate of flowering, (2) duration of flowering and (3) index of flowering magnitude. Ten inflorescences were tagged and observed daily for 1 month and the sequence of flowering was recorded when the general pattern had been established.

Several inflorescences approaching anthesis were also selected so as to study floral phenology. Flowers at the bud

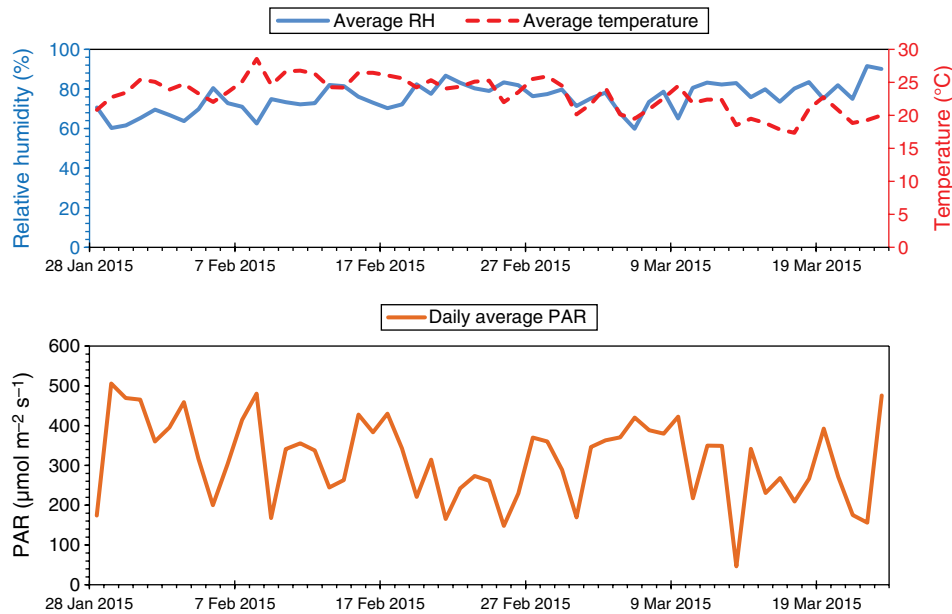


Fig. 3. Temperature, relative humidity and photosynthetically active radiation (PAR) recorded inside the tunnel house on a daily basis during the period from 28 January to 23 March 2015.

stage on different plants were tagged (coloured tags) for further identification and observation. Flowering progress under the tunnel-house conditions was observed every day until flower opening and then every 2–3 h. On the day of anthesis, observations were made throughout the day at shorter intervals (every 15 min). Using physical examination and digital imagery, sequential growth was recorded from the day a new floral bud appeared until all developmental stages were complete.

Pollen and stigma biology

Pollen biology

Optimisation of the pollen germination medium. Culture media with different components were screened for optimal pollen germination. The components of the pollen-germination medium (PGM) were optimised using a modified Brewbaker and Kwack (BK) basal medium enriched with different concentrations of sucrose and polyethylene glycol 4000 (PEG; Brewbaker and Kwack 1963). The experiment was conducted using sucrose at six concentrations (0%, 5%, 10%, 15%, 20 and 25%, w/v) and PEG at three concentrations (0%, 2.5% and 5% w/v; Table 1). The basic BK medium contains $100 \text{ mg L}^{-1} \text{ H}_3\text{BO}_3$, $300 \text{ mg L}^{-1} \text{ Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, $200 \text{ mg L}^{-1} \text{ MgSO}_4 \cdot 7\text{H}_2\text{O}$, $100 \text{ mg L}^{-1} \text{ KNO}_3$ and 10% sucrose (all w/v). Solutions containing the BK medium and all possible combinations of sucrose and PEG were adjusted to pH 6.2.

Pollen was collected from flowers at anthesis and distributed on glass slides with one drop of medium placed on each slide. As suggested by Stanley and Linskens (1974), the slides were inverted (hanging-drop technique) and maintained in Petri plates lined with moist filter paper (RH > 95%) for 2 h at 22.5°C. A pollen grain was considered germinated when the length of its tube was equal to or greater than the diameter of the grain. The pollen grains were scored for germination by using

randomly selected microscopic fields in a completely randomised design within a factorial treatment structure. Each treatment contained three replicates, and three fields of view were assessed in each replicate. Pollen grains in at least three randomly selected microscopic fields on each slide were observed after 2 h of incubation at 22.5°C. It was convenient to move the preparation under the microscope in consecutive rows, and score one or two fields in each row. This eliminated the probability of scoring the same pollen grains. For each field, the total number of pollen grains and number of germinated grains were recorded. Pollen rehydration was unnecessary because preliminary results showed no rehydration effect on pollen germination.

Assessments of pollen viability and stainability Pollen viability was assessed using fluorescein diacetate (FDA) test (Heslop-Harrison and Heslop-Harrison 1970; Heslop-Harrison *et al.* 1984) and pollen fertility was assessed using various non-vital staining methods, such as 1% acetocarmine (Nassar *et al.* 2000), 0.5% lactophenol cotton blue (Sigma 61335, Sigma-Aldrich, Sydney, Australia) modified from Hauser and Morrison (1964), 1% iodine in potassium iodide solution (IKI) and Alexander's stain (Alexander 1969, 1980).

Pollen was collected in Petri dishes immediately after anthesis and subsequently stained on microscope slides. For each of the vital and non-vital stains, three replicated slides were prepared and, in total, 200–300 pollen grains were counted and scored on the basis of their colour, in three fields of view per slide by using a light microscope. Pollen grains of abnormal size, light colouring and with reduced or lack of protoplasm were considered non-viable, whereas those with intact exines and strongly coloured protoplasm with homogeneous distribution were classified as viable. In the FDA test, preparations were observed under a fluorescence microscope (Leica DMIL, Leica Microsystems CMS GmbH, Wetzlar, Germany) with fluorescence

Table 1. *In vitro* pollen germination of *Leonurus cardiaca* (Khansar) using Brewbaker and Kwack (BK) basic media enriched with 18 combinations of sucrose and polyethylene glycol (PEG) at different concentrationsValues within the 4th column followed by the same letter are not significantly different at $P=0.05$ (l.s.d. test)

Medium	Sucrose (%)	PEG (%)	Germination (%)	Comment
1	0	0	0.00f	High frequency of pollen burst
2	0	2.5	0.00f	High frequency of pollen burst
3	0	5	0.00f	Pollen burst
4	10	0	81.67bc	Pollen and pollen-tube burst
5	10	2.5	82.57bc	Pollen-tube burst
6	10	5	89.40ab	Pollen-tube burst
7	15	0	83.87bc	Pollen and pollen-tube burst
8	15	2.5	94.80a	Long pollen tube, minimum burst
9	15	5	78.27c	Long pollen tube, minimum burst
10	20	0	95.57a	Short pollen tubes and high frequency of tube burst
11	20	2.5	87.80abc	Pollen-tube burst, short pollen tubes
12	20	5	91.67ab	Pollen-tube burst, short pollen tubes
13	25	0	10.33ef	Pollen and pollen-tube burst
14	25	2.5	11.63e	Pollen and pollen-tube burst, short pollen tubes
15	25	5	55.67d	Pollen and pollen-tube burst, short pollen tubes
16	30	0	16.73e	High frequency of pollen and pollen-tube burst
17	30	2.5	0.00f	Pollen burst
18	30	5	18.33e	High frequency of pollen burst, short pollen

filters. Pollen grains showing bright fluorescence were considered viable. An *in vitro* pollen-germination test was conducted concurrently to benchmark the supplemented modified BK germination medium (supplemented with sucrose and PEG as described earlier).

Pollen quantification For estimating the average number of pollen grains produced by each flower, each mature but undehisced anther was randomly selected individually from 10 different plants in each population. The selected anthers were individually squashed in two drops of aniline blue in lactophenol, and to calculate the average pollen number for each flower, two counts on the Fuchs–Rosenthal clinical haemocytometers grids (E. Hartnack, Berlin, Germany) were used.

Stigma receptivity

Stigma receptivity in the present study was determined experimentally by pollen germination, pollen-tube growth on stigmas, seed set after pollination at different times relative to anthesis and enzymatic-activity testing using Peroxtesmo KO (Machery-Nagel, Duren, Germany; Dafni and Maués 1998). The stigma receptivity of 35 fresh inflorescences with flowers between pre-anthesis and senescence were tested by soaking one test paper (15 × 15 mm) in 1 mL of distilled water, and then applying a droplet of the solution directly onto the stigma using a 10- μ L pipette, and inspecting after several minutes for blue coloration. In each inflorescence, flowers in three areas (lower, middle and upper third) were tested and the combined results were recorded as the peak of receptivity. All stigmas examined were checked under a magnifier ($\times 30$) for the presence of pollen as well as damage to the surface of the stigma because either of these factors can cause enzymatic activity regardless of the stigma receptivity (Kearns and Inouye 1993).

Breeding system

The following six pollination treatments were given: (1) bagging inflorescences with intact flowers without emasculation, so as

to test for spontaneous self-pollination because individual flowers were not amenable for bagging; (2) to prevent position effect, manual self-pollination of unemasculated randomised flowers within inflorescences was performed with their own pollen when the stigmas became receptive; (3) hand-pollination of emasculated flowers with pollen from other flowers of the same plant to test for geitonogamy; (4) manual emasculation and cross-pollination to test for cross-compatibility among plants of the same population and also among plants of the three different populations; (5) emasculation of 10 pre-anthesis flowers to check for the possibility of apomixes; and (6) free pollination without bagging. Each of these treatments was based on 30 flowers repeated thrice and applied to three randomly selected plants from each *L. cardiaca* population. So as to avoid contamination by cross-pollination, self-pollinated flowers were allowed to self only within perforated wax-coated paper bags that were secured at their open ends with thread. Open-pollinated flowers were scored from the same plants to ensure that flower bagging had no deleterious effect on the seed set. In another procedure, pollinations were performed on excised pistils implanted in 1% agar in Petri dishes as outlined by Lundqvist (1961). The pollen-tube growth was observed after staining the pistils with 0.05% aniline blue in 0.1 M phosphate buffer (pH 7) for 20 min at 25°C. The stigmas, styles and ovaries were viewed using a Leica DMIL compound microscope using fluorescence optics with short-wavelength ultraviolet light and photographed with a Nikon Coolpix (Tokyo, Japan) photo camera mounted on the same microscope. The fluorescence of callose deposits in the germ-pore and tube tip on the stigmatic surface together with the deposition of callose plugs in the elongating pollen tubes within the stigmatic tissue, style or ovary were recorded as an indication of pollen–pistil compatibility and the travel distance of the pollen tube (Martin 1959). Fruit setting for 30 flowers per pollination treatment were counted on individual plants to calculate fruit-setting percentages (number of fruits/total number of pollinated

flowers). Fruit development was recorded when nutlets emerged. The number of nutlets formed within each calyx was also recorded.

Pollinators

During the flowering period, inflorescences of 15 plants from the three populations (5 plants from each population) were labelled and positioned for floral visitors. Floral visitors and their behavioural patterns were recorded from 0800 hours to 1500 hours for 5 days. The species of the floral visitors and their interactions with flowers (e.g. attempt to visit the flower, close to or hovering over the flowers without touching, direct contact and effective pollination, time on each flower, duration on each inflorescence, duration on each plant and frequency on each population) were properly recorded. Some floral visitors were photographed where possible with a Canon D500 SLR camera (Tokyo, Japan) and representative specimens of visitors were captured with a net for identification.

Light-microscopy methods

Floral buds, flowers and fruits were examined under the binocular microscope and photographed with a digital camera. Floral structures at the various stages of development were fixed in formalin acetic alcohol (FAA, 5 parts formalin : 5 parts glacial acetic acid : 90 parts 50% ethanol, v/v/v) and stored in 70% ethanol for histological observations. They were then dehydrated through an ethanol series and embedded in paraffin with a 58–60°C melting point for microtoming. Serial sections were cut using a rotary microtome (Spencer 820, American Optical Co., Buffalo, NY, USA) at 5 µm, stained with safranin-O and Fast Green FCF (Sass 1958) and, subsequently, dehydrated through an alcohol series to absolute ethanol and mounted in DPX (BDH, Poole, UK). The samples were examined using normal brightfield optics on a Leica DM 2500M light microscope and photographed with a Leica DFC500 12-megapixel digital colour camera mounted on the same microscope, using Leica Application Suite software Version 4.0.0.

Data analysis

The standard error was calculated for the measured floral-structure parameters. Data were statistically analysed using an ANOVA for *in vitro* pollen-germination test. Means were separated using Fisher's protected least significant difference at the 0.05% level of probability. All analyses were performed using SPSS 19.0 statistical software (SPSS Inc., Chicago, IL, USA). Relationships between the individual staining assays and the *in vitro* germination were analysed using the linear regression function within the SPSS software.

Results

Floral structure and development

Morphological characteristics of the floral structure of *L. cardiaca* among three populations are presented in Table 2. There were considerable variations in the number of inflorescences produced per plant among the populations studied. On average, plants at Dargaz were the least prolific, producing ~7.5 inflorescences per plant. This was greatly exceeded by plants in the Sarab and Khansar populations that produced 18.6 and 22.4 inflorescences per plant respectively. Each inflorescence in Sarab, Khansar and Dargaz comprised an average of 9.5, 9.0 and 8.0 verticillasters and ~181, 298 and 76 flowers per inflorescence respectively. The average inflorescence length was lowest in the Sarab population (12.5 cm) followed by Khansar (14.7 cm) and Dargaz (21.6 cm). Likewise, the internode length on the floral axis was longest in Dargaz (3.85 cm) and shortest in Sarab (2.63 cm; Table 2).

At the individual-flower level, reproductive organs (stamens and pistil) and the accessory organs (perianth) showed less variation than did the inflorescence in the populations studied. The average pistil length at anthesis was 8.3 mm, whereas the average long-stamen length at the same stage was 5.21 mm. For the accessory organs, the average length of the corolla was 9.17 mm and that of the calyx 5.58 mm (Table 2).

Each pair of opposite axillary sessile cymes is a seeming whorl called a verticillaster, as shown in Fig. 4a–f. Flowering progression and maturation in each inflorescence was acropetal.

Table 2. Morphological characteristics of floral structure of three *Leonurus cardiaca* populations

Values are means ± s.e. of 10 randomly selected inflorescences

Character	Sarab	Khansar	Dargaz
Average number of verticillasters per inflorescence	9.50 ± 0.56	9.00 ± 1.11	8.00 ± 1.66
Average number of flowers per inflorescence	181 ± 46.15	298 ± 14.0	76 ± 22.66
Average number of inflorescences per plant	18.60 ± 4.65	22.40 ± 3.16	7.50 ± 2.19
Average length of floral axis internode (cm)	2.63 ± 0.22	2.95 ± 0.66	3.85 ± 0.41
Average length of inflorescence (cm)	12.5 ± 1.6	14.7 ± 2.1	21.6 ± 3.8
Average flower length (mm) ^A		9.17 ± 0.83	
Average calyx length (mm) ^A		5.58 ± 0.58	
Average corolla length (mm) ^A		9.17 ± 0.83	
Average pistil length at anthesis (mm) ^A		8.3 ± 0.64	
Average stamen length (mm) ^A		5.21 ± 0.44	
Number of anthers per lower ^A		4	
Average pollen grain number per flower ^A		37 400 ± 545	
Number of ovules per ovary ^A		4	
Pollen : ovule ratio ^A		9350 : 1	

^ACharacter with no significant variation among the studied populations; value is the average over all three populations.



Fig. 4. Inflorescence sequence and structure in *Leonurus cardiaca*. (a) Five developmental stages of the inflorescence from unexpanded floral stalk holding floral buds at an early stage of development before anthesis in Stage I to a fully expanded stalk holding floral structures at various developmental stages in V. (b–e) Inflorescences showing asynchronous anthesis among flowers of the same verticillaster in b and c, and synchronous anthesis within the same verticillaster in d and e. (f) Fruit setting in flowers of one verticillaster, showing an upper view of a dichasium structure in the axils of opposite leaves. Scale graduations are in mm. Photographs by Nabil Ahmad.

In all plants, nodes closer to the apex of the inflorescence were less developed with shorter internodes at bud stage, whereas nodes closer to the base had fully developed flowers (Stage IV, Fig. 4a) or even fruits (Stage V, Fig. 4a). Floral buds on each verticillaster generally do not exhibit synchronised anthesis (Fig. 4b, c). However, in a few instances, the floral buds of one or two consecutive verticillasters reached maturity at the same time and were, therefore, synchronised at full bloom (Fig. 4d, e).

The mean flowering duration for *L. cardiaca* was 2 weeks within each inflorescence and 2 months within the individual plants. However, there was variation among the populations with respect to timing and duration of flowering. The earliest flowering occurred in the Khansar population, followed 1 week later by Sarab, and 14 days later by Dargaz.

The cumulative percentage of *L. cardiaca* flowers at anthesis over time is given in Fig. 5 for the Sarab population. There was an overlap of flowering in three or four verticillasters for ~75%

of the total flowering duration. The index of flowering magnitude (IFM) was calculated as a measure of floral resources within the inflorescence and expressed as the number of flowers open simultaneously in a given time, presented as a percentage of the total number of flowers (Fig. 5). Nearly 40% of flowering occurred during the first 5 days after anthesis. The middle verticillasters were the most asynchronous because anthesis can spread over 8–9 days, whereas the flowers of the first and last verticillasters were the most synchronous, completing their flowering in just a few days (Fig. 5).

The characteristics of the above inflorescences are a consequence of the rate at which the flowers open sequentially (anthesis rate), their longevity and the extent to which receptive stigmas and viable pollen are asynchronous in such bisexual flowers (dichogamy). The reproductive parts (stamens and pistil) of each flower of *L. cardiaca* are enclosed by enlarged white–pink petals before anthesis. The upper lip of the petals open first, thus causing the long filaments and style to uncurl, eventually

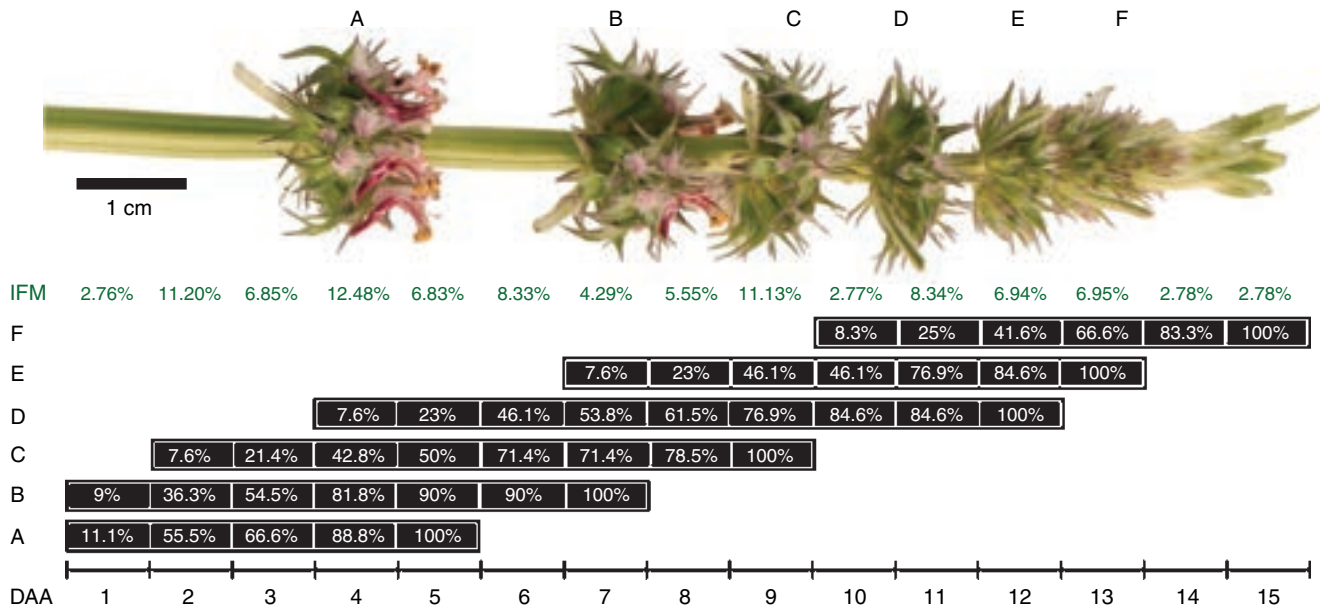


Fig. 5. Cumulative percentages of flowers in anthesis represented by the timing of flower anthesis on different verticillasters within the same inflorescence, and index of flowering magnitude. Accumulated percentages of floral buds at or after anthesis within each whorl are shown on the horizontal bars with time. A, basal inflorescence whorl; DAA, days after the onset of anthesis (flowering commencement) within the inflorescence; F, top whorl; and IFM, index of flowering magnitude. Values are means of 10 inflorescences.

exposing the dehiscing anthers and stigma to pollinators (Fig. 6a). Anthesis usually occurred between 0600 hours and 1030 hours, and was affected by temperature, relative humidity and light intensity; in fact, higher temperature, lower relative humidity and higher light intensity promoted early anthesis.

The flowering progress and duration of individual flowers is given in Fig. 6a. In the morning of the day of anthesis (Fig. 6a, Stage 0), the anthers began to dehiscence by longitudinal slits from 1000 hours to 1200 hours (Fig. 6b–d); however, overcast or rainy weather delayed anthesis and pollen presentation. Stamens slowly dried out, turned brown and began to wilt 2 days after anthesis and eventually abscised with the corolla 6 days after anthesis (Fig. 6a). A change in the shape of the stigma and style indicated readiness of the stigma to receive pollen. At an early stage of development, the stigma had lobe tips curved downward at an obtuse angle between the two lobes (Fig. 6b); however, by anthesis, the two lobes become less curved, with a more acute angle between them (Fig. 6b, d). The style at this stage was long enough to bring the stigma to the same height as the upper pair of anthers (Fig. 6b). With time after anthesis, the angle between the stigmatic lobes widens during the second and third day and, simultaneously, the distal ends of the stigmatic lobes gradually bend until they eventually contact the anthers of the longer stamen pair (Fig. 6a, e). Morphological analysis of the stigmatic surface using scanning electron microscopy revealed distinguished receptive papillae in the distal portion of the stigmatic lobes 2 days after anthesis (Fig. 6f–h). This was also confirmed by the enzymatic activity indicated by the blue coloration at the distal ends of the stigmatic lobes (Fig. 6i). Pollen adhesion and germination were limited to the small receptive area, as shown in Fig. 6g, h. Histological sections of the flower bud before anthesis confirmed differences in maturation time between

the male and female organs (Fig. 6j, k) when anthers mature earlier and, therefore, stigmas become receptive 2–3 days after flower anthesis.

Optimisation of the pollen-germination medium

In the present study, 15% sucrose and 2.5% PEG 4000 were observed to be the most effective modification to the BK basic medium, permitting high-quality germination of *L. cardiaca* pollen (Table 1). The standardised concentrations of sucrose and PEG allowed 94.80% pollen germination and produced the longest pollen tubes with minimal pollen and pollen-tube bursting. There were no significant differences in germination percentages between this combination of sucrose and PEG (15%, 2.5%) and other such combinations (10%, 5%), (20%, 0%), (20%, 2.5%), (20%, 5%); however, high incidences of shorter and ruptured pollen tubes in most media combinations indicated that the (15%, 2.5%) medium was the optimum combination for *in vitro* pollen germination. Variations in the effect of different sucrose concentrations are associated with different osmotic potentials, because pollen-tube bursting is a common feature in all media with very low or very high osmotic concentrations. In our study, no germination occurred with sucrose concentrations lower than 10% (Table 1), thus confirming that sucrose plays a nutritive role in pollen germination.

The PEG was effective in promoting pollen germination and reducing bursting at its highest concentration (5%) when less than 15% sucrose was used (Table 1). However, 2.5% PEG was significantly better than 5% in combination with 15% sucrose in inducing maximum germination and germination rates (longest pollen tubes within 30 min), thereby

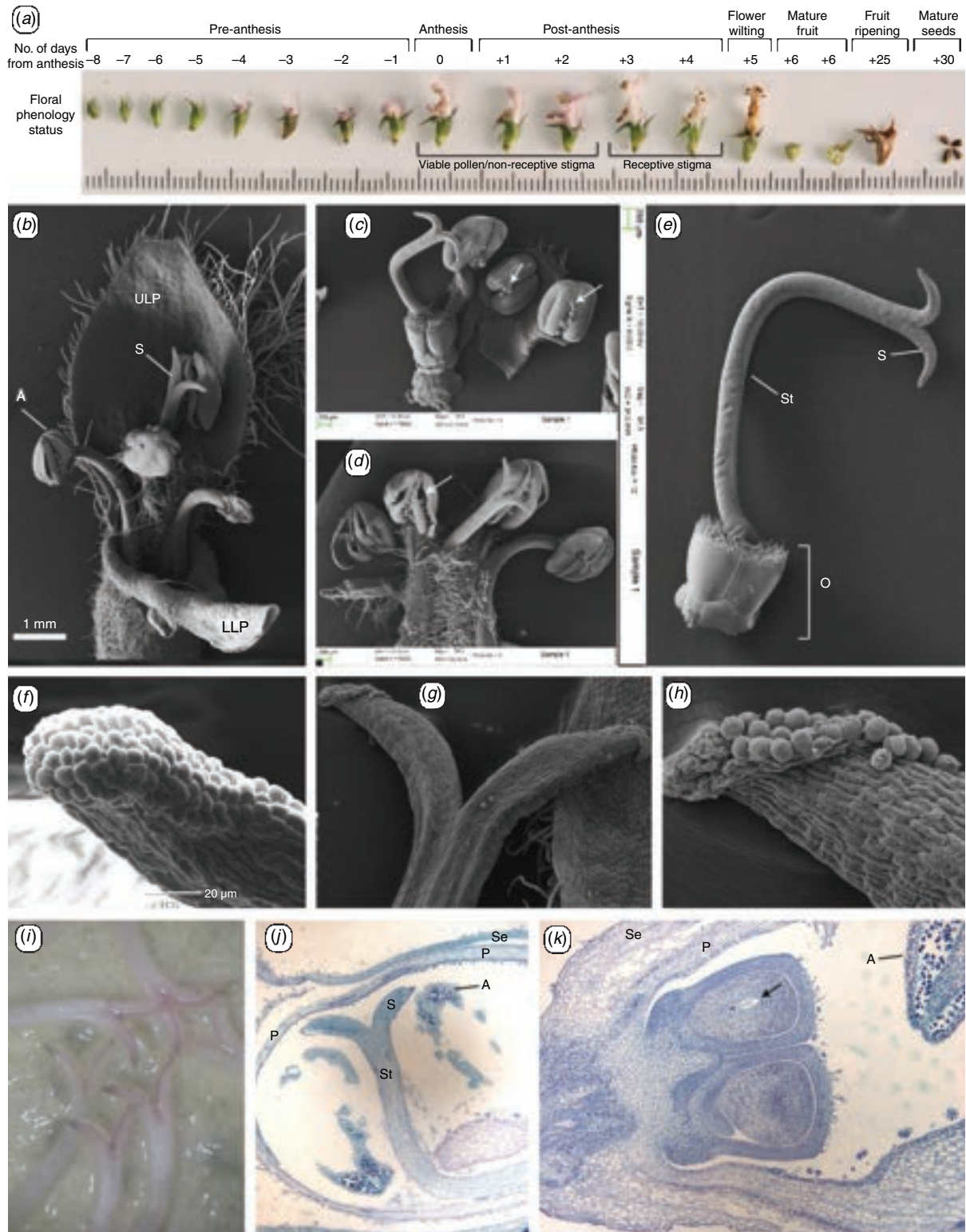


Fig. 6. Flower morphology and structure in *Leonurus cardiaca*. (a) Morphological floral-developmental sequence. (b–h) Scanning electron microscopy images showing the flower at anthesis with dehiscent anthers (arrow) and non-receptive stigma in b, difference in timing of maturity between male and female organs in c and d, and receptive stigma in e–h, showing the obtuse angle between the two lobes of the stigma and downward curving of the lobe tips. (i) Stigma receptivity testing based on enzymatic activity (Peroxtesmo KO test) indicated by blue coloration. (j, k) Histological sections of the floral bud, showing a non-receptive stigma in f and less advanced ovary in g next to a more advanced developmental stage of the anther. A, anther; LLP, lower lip petal; O, ovary; S, stigma; Se, sepal; St, style; and ULP, upper-lip petal. Photographs (a, f–k) by Nabil Ahmad; (b–e) by Sue Lindsay.

underscoring the importance of the osmotic pressure of the medium. However, using PEG, especially at the 5% level, with more than 15% sucrose, resulted in shorter pollen tubes, which might be related to the physical resistance of the medium to pollen-tube growth.

Pollen viability and stainability

Alexander's stain and 1% IKI produced the highest positive correlations with the *in vitro* germination test, giving 0.942 and 0.960 coefficients of determination respectively (Fig. 7, Table 3). The coefficient frequency for FDA and lactophenol cotton blue were 0.726 and 0.648 respectively. However, the acetocarmine stainability test had the lowest correlation with the *in vitro* germination tests.

Stigma receptivity

Leonurus cardiaca has a dry-type stigma, with receptive cells concentrated on the inner side of the distal ends of the two stigmatic lobes (Fig. 6*f–h*). During anthesis, the stigmatic surfaces of the two lobes are smooth and curved inward at the lobe tips. However, the stigmatic lobes change in shape from downward curving at their far ends before anthesis, to a less curving at anthesis and, subsequently, return to a downward curve 2 days post-anthesis. The angle between both lobes varied from an obtuse angle before anthesis, to an acute angle during anthesis and returning to an obtuse angle 2–3 days after anthesis, with their distal ends gradually bending until they eventually come in contact with the anthers of the longer stamen pair.

The peak phase of stigma receptivity was confirmed chemically by testing the enzymatic activity using a Peroxtesmo KO test. The highly receptive portion of the stigmatic surface turned blue, indicating a high activity of the peroxidase enzyme (Fig. 6*i*). Moreover, experimental tests to observe the pollen-tube growth within the pistil (after pollinating the stigmas at different times relative to anthesis) also confirmed the time when the stigmatic tissue is highly receptive. In this experimental test, no pollen germination or pollen-tube growth was observed until 2 days after anthesis.

Breeding system and plant–pollinator interaction

Pollination treatments showed that *L. cardiaca* is self-compatible (Table 4, Fig. 8). Morpho-histological features (Fig. 6) and chemical tests showed that there is asynchrony between pollen exposure and stigma receptivity (maturity of the sexual elements within each flower). Anther dehiscence and dispersion of pollen grains in each flower occurred before the stigma became receptive, indicating the prevalence of protandry in this species.

Spontaneous autogamy in *L. cardiaca* populations resulted in ~50% fruit set, whereas fruit set following manually assisted self-pollination was 30% and ~60% in geitonogamy treatments (Table 4). The difference in fruit setting between spontaneous and assisted autogamy could be the result of incidental geitonogamous pollination, most likely being a result of viable fresh pollen falling from opening flowers. There was low variation among the studied populations in their fruit setting using their own pollen. However, controlled cross-pollination within these populations represented by the assisted xenogamy

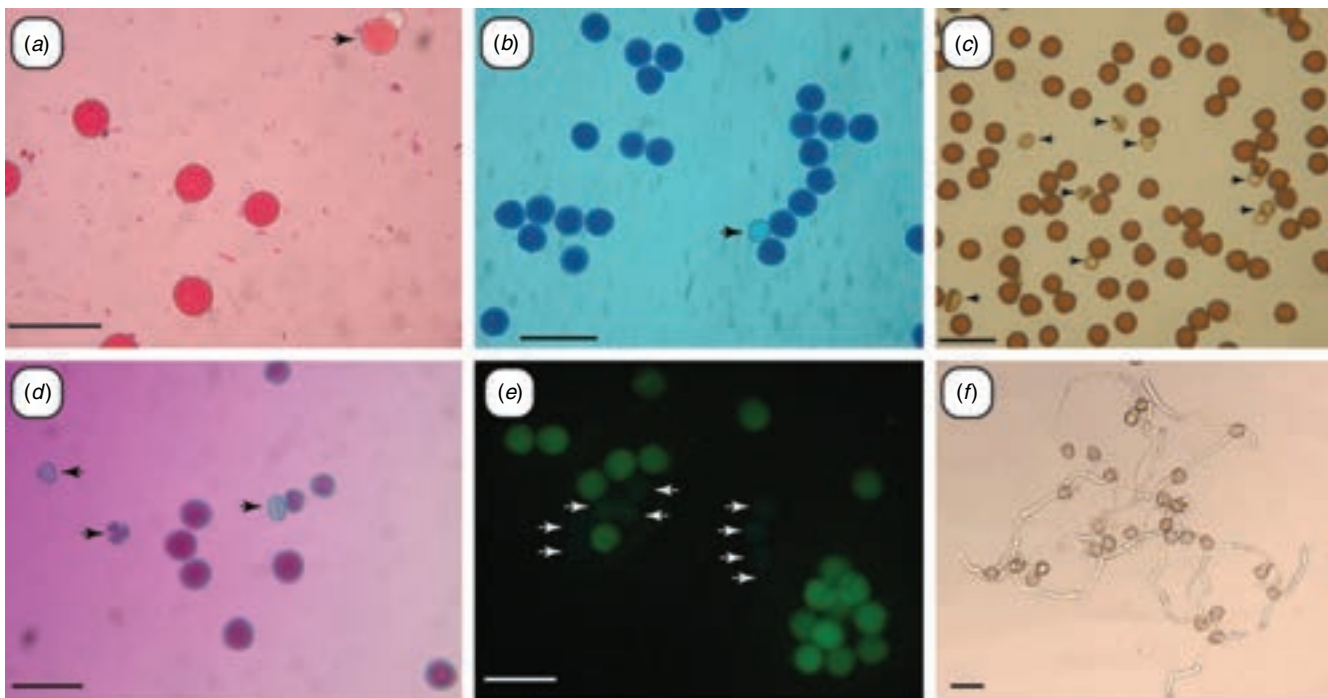


Fig. 7. Pollen-viability staining methods and *in vitro* pollen germination in *Leonurus cardiaca*: (a) 1% acetocarmine; (b) 0.5% lactophenol cotton blue; (c) 1% iodine in potassium iodide solution (IKI); (d) Alexander's stain; (e) fluorescein diacetate (FDA) test. (f) *In vitro* germination in optimised *in vitro* Brewbaker and Kwack (BK) medium. Arrows indicate non-viable pollen grains. Scale bars = 50 μ m.

gave a fruit-setting result similar to that of selfing. These results indicated that *L. cardiaca* is a self-compatible species.

Free pollination treatment resulted in high fruit setting in all three populations (>87%), highlighting the importance of insect visitation in the natural pollination of *L. cardiaca*. Our results also showed that all three of the *L. cardiaca* populations were incapable of agamosperous reproduction because none of the emasculated tagged flowers set fruit (Table 4). Furthermore, successful fruit setting resulted in 100% seed set (4 nutlets per fruit) for all other treatments. The cross between Sarab and Dargaz produced more than 90% fruit set, whereas that between Dargaz and Khansar produced 50%. These results

Table 3. Coefficients of determination and regression of percentage pollen viability to *in vitro* pollen germination for five different pollen-viability and -stainability assays

Stain	FDA, fluorescein diacetate; IKI, iodine in potassium iodide solution	
	Coefficient of determination (r^2)	Regression equation
IKI	0.960	$Y = 53.468 + 0.410X$
Lactophenol cotton blue	0.648	$Y = -56.95 + 1.563X$
FDA	0.726	$Y = 90.38 + 0.054X$
Alexander's stain	0.942	$Y = 34.29 + 0.601X$
Acetocarmine	0.007	$Y = 75.65 + 0.175X$

Table 4. Details of pollination treatments and percentage fruit set in three populations of *Leonurus cardiaca*

Fruit-setting means \pm s.e. are based on 30 flowers per treatment, repeated three times

Treatment applied	Fruit setting (%)		
	Sarab	Khansar	Dargaz
Spontaneous autogamy	55.56 \pm 2.93	53.33 \pm 3.84	46.67 \pm 1.92
Assisted autogamy	32.22 \pm 2.94	30.00 \pm 3.33	27.78 \pm 2.94
Geitonogamy	60.00 \pm 3.33	61.11 \pm 2.94	57.78 \pm 2.22
Assisted xenogamy (within population)	67.78 \pm 2.94	63.33 \pm 5.08	62.22 \pm 4.00
Free pollination	88.89 \pm 2.94	91.11 \pm 2.22	87.78 \pm 2.22
Apomixis	0	0	0

indicated the existence of differences in compatibility for individual combinations among the three populations studied.

The flowers in *L. cardiaca* were visited by honeybees (*Apis mellifera*), ladybird beetles, adult blowfly, cabbage white butterfly (*Pieris rapae*) and European paper wasp (*Polistes dominula*) (Fig. 9). The bee lands on corollas and then pushes its head down slightly into the throat of the flower to access nectar at the base of the ovary by using its long proboscis (Fig. 9a). In pressing its head into the throat, the bee comes into contact with the dehiscent anthers and ends up with pollen grains visibly dusted on the top of its head. The visits are fast, with the bee spending 1–2 s per flower and visiting a series of flowers on the same plant before flying away to other plants. A second visitor, ladybird beetles (Fig. 9b, c), were observed moving slowly among flowers, causing some pollination. A third visitor, adult blowfly, was observed visiting the flowers (Fig. 9d). The fly's mode of visitation and pollen dusting are similar to those of the bee, although the visitation time is longer for each individual flower. Cabbage white butterfly was another flower visitor (Fig. 9e), spending 5–10 s on each flower. European paper wasps were also observed visiting the flowers (Fig. 9f). However, due to the long proboscis and long legs of the wasp, the insect's body and head had no contact with the anthers. Moreover, in a few cases, spiders have been spotted moving among the flowers hunting insects and possibly aiding in the pollination process (Fig. 9g).

The honeybees, cabbage white butterfly and the European paper wasp visited the flowers mainly in the morning after anthesis, whereas all other visitors were seen foraging flowers or moving among flowers throughout the day. An average number of floral visitors in 15 different plants studied during the peak flowering period (from 1000 hours to 1200 hours) was 21 honeybees, three ladybird beetles, two blowflies, three butterflies and three wasps. On the basis of our observations, it can be concluded that the effective pollinators of *L. cardiaca* are honeybees because they were observed visiting many flowers and returning every few minutes to each plant.

Any breeding strategy adopted to improve motherwort requires an understanding of its reproductive process. Emasculatation and cross-pollination techniques were developed

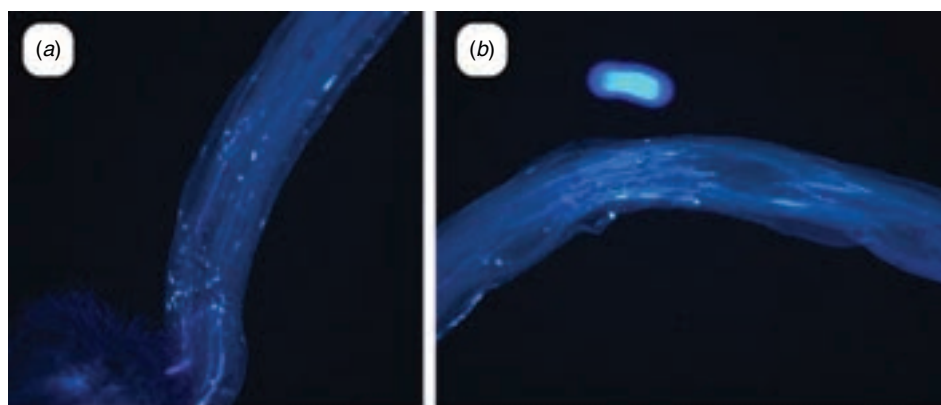


Fig. 8. Fluorescence microscopy images of the *Leonurus cardiaca* style, showing the pollen-style compatibility test. (a) Self-compatibility showing callose plugs in the style, indicating pollen-tube growth after self-pollination. (b) Cross-compatibility showing callose plugs in the style, indicating pollen-tube growth after cross-pollination of emasculated flowers.

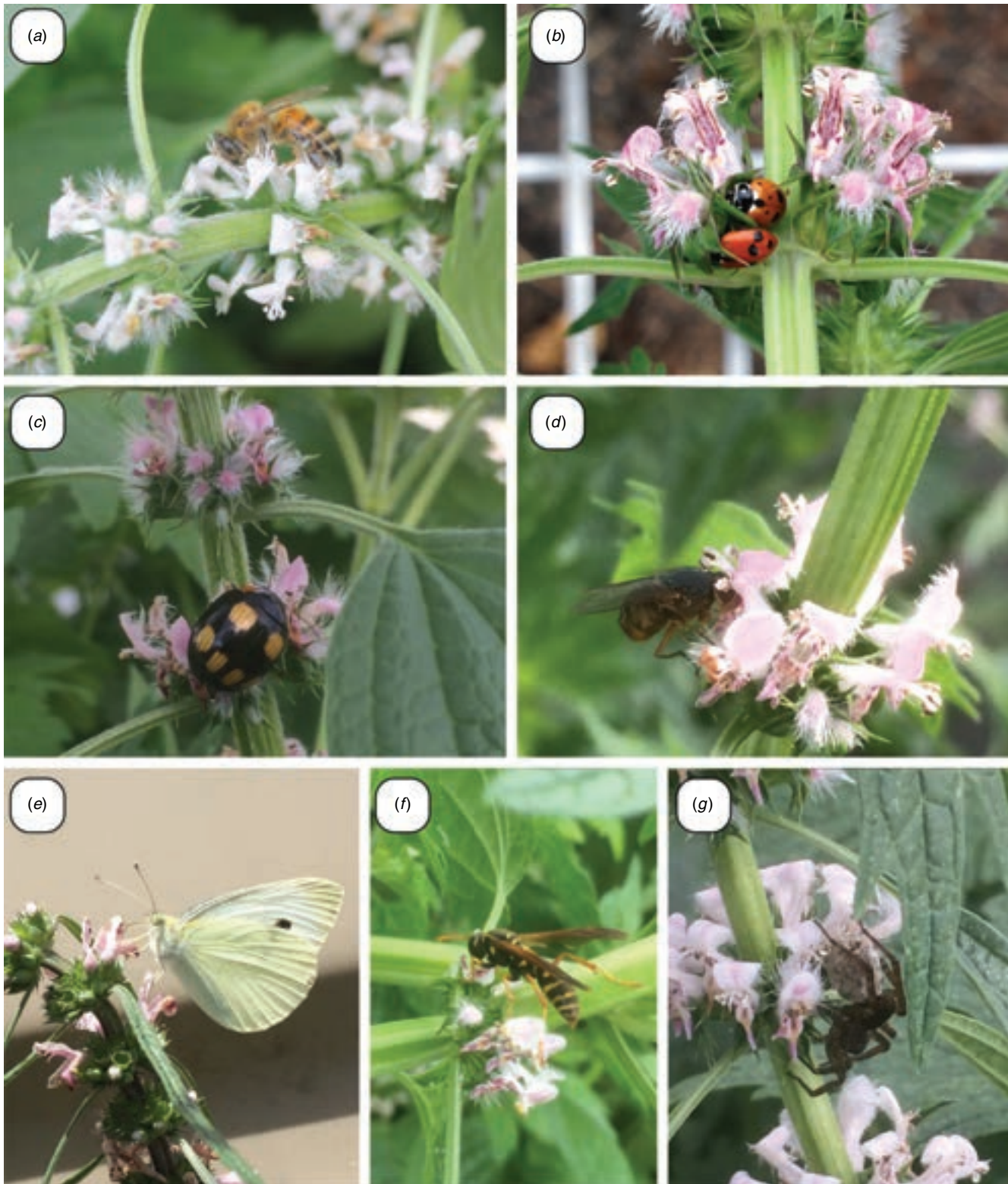


Fig. 9. Floral visitors to *Leonurus cardiaca* plants grown in an open greenhouse. (a) Honeybees (*Apis mellifera*) were the most frequent pollinators because they were attracted to nectar and/or pollen as a reward. (b, c) Ladybird beetles attracted to flowers to consume nectar or to look for prey. (d) Adult blowfly feeding on nectar. (e) Cabbage white butterfly (*Pieris rapae*). (f) European paper wasp (*Polistes dominula*) feeding on nectar. (g) Other non-insect such as spiders can cause pollination by movement around the flowers. Photographs by Nabil Ahmad and Fatemeh Borna.

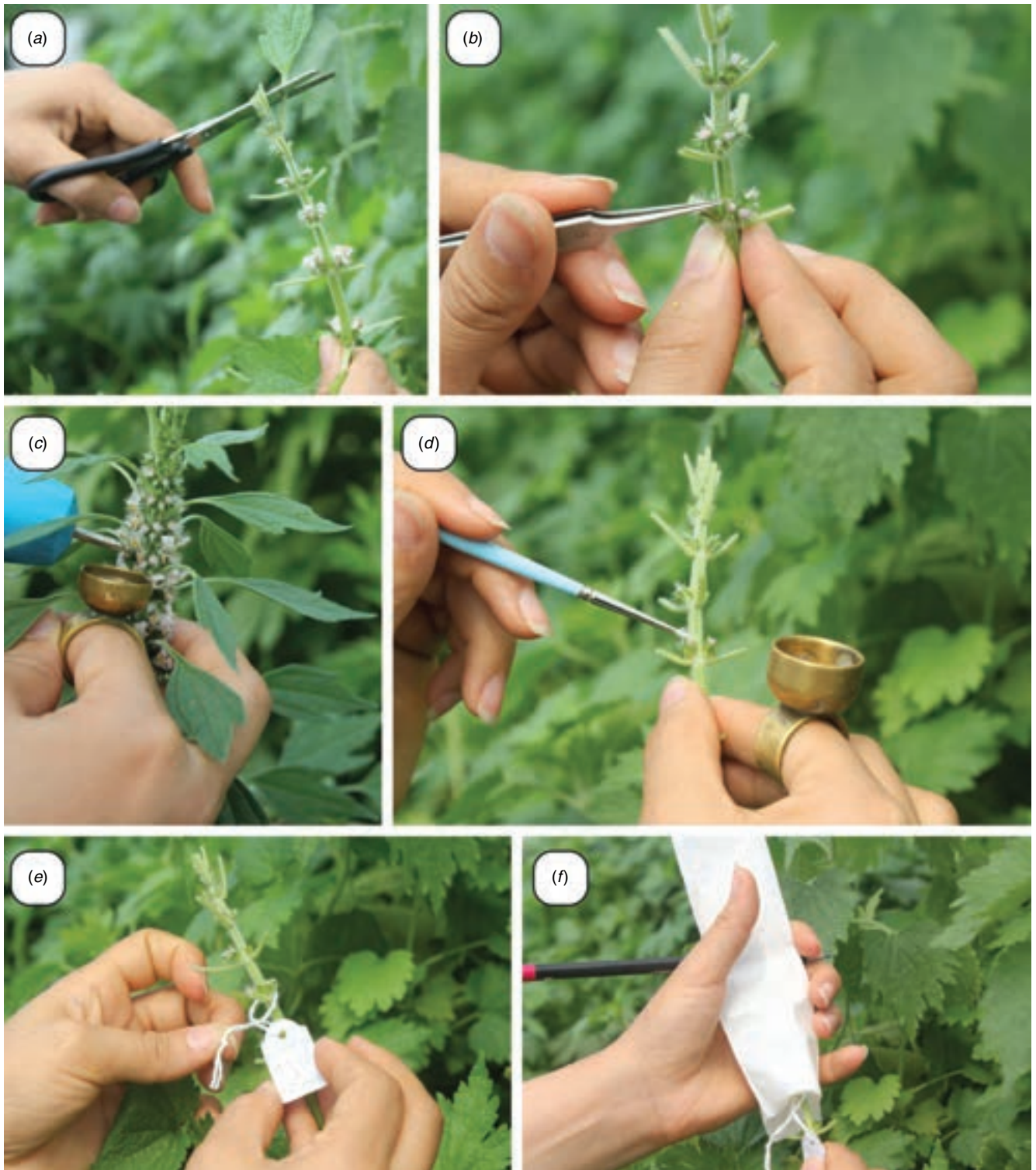


Fig. 10. Pollination procedures for *Leonurus cardiaca* flowers. (a) Removal of the leaves. (b) After removal of all opened flowers, emasculation takes place by the removal of anthers before dehiscence. (c) Pollen collection from the pollen-donor parent by using a hand-held battery-powered engraver to release the pollen into a brass collection ring. (d) Applying the pollen on the surface of the exposed stigmata 36 h after emasculation by using a fine brush. (e) Tagging pollinated flowers with all necessary information. (f) Bagging the inflorescence to avoid cross contamination by pollen from other sources. Photographs by Nabil Ahmad.

on the basis of pollen and stigma biology and breeding-system studies (Fig. 10). Inflorescences were prepared for controlled-pollination treatments by removing the leaves for easy access to the flowers (Fig. 10a). This was followed by emasculation (removal of undehisced anthers from flower buds before anthesis) with fine forceps (Fig. 10b). Removal of some flower buds next to the targeted ones was very important to minimise competition among the flowers for photosynthates and also to facilitate the manoeuvre around the targeted buds in this delicate procedure. Pollen from the pollen source can be collected from dehisced anthers of flowers at the anthesis stage by shaking the flowers with a hand-held battery-operated engraver (Fig. 10c). Freshly collected pollen was then used for pollinating the emasculated flowers 2 days post-anthesis by using a fine brush (Fig. 10d). The pollinated flowers were then tagged with all necessary information, which includes both parents and date of pollination (Fig. 10e). The whole inflorescence was then bagged to avoid cross-contamination by pollen from other sources (Fig. 10f).

Discussion

In the present study on the impact of inflorescence architecture (flower number, arrangement, and phenology, including the timing and duration of floral exposure) on pollination and fruit set, our results are similar to those reported by Wyatt (1982), Harder and Prusinkiewicz (2013) and Schneemilch and Steggles (2010). According to Spira (1980), Davila and Wardle (2002), Barrett (2013) and Prenner (2013), the architecture of the inflorescence of an angiosperm influences reproductive success. The inflorescences in *L. cardiaca* are indeterminate thyrses forming axillary cymes, and this is the most common type of inflorescence structure found in the Lamiales (Weber 2013). The role of an inflorescence in pollination, beyond that served by individual flowers alone, depends on the duration of flowering, the number and arrangement of open flowers and the distribution of sex, all of which influence intra-inflorescence flowering phenology (Harder and Prusinkiewicz 2013).

The study showed that there were high degrees of overlap and synchronisation in flowering among the plants within each population of *L. cardiaca*, a feature that may facilitate outcrossing (transfer of pollen grains from the anther to the stigma of a different plant). Plants that display many flowers simultaneously attract more pollinators than do those with small displays (Ohashi and Yahara 2001), but are also more likely to experience among-flower self-pollination through geitonogamy (Barrett *et al.* 1994; Karron *et al.* 2004), an associated reduction in pollen export (pollen discounting) and siring success (Harder and Barrett 1995; Karron and Mitchell 2012). This is due to the low amounts of inter-plant movement by pollinators because they remain constant for long periods among the large number of flowers on a single plant (Augspurger 1980).

The present study looked at the flowering dynamics in *L. cardiaca* because these can be utilised by plants to save resources and optimise the fruit set. It is known that the timing and duration of floral exposure, specifically the rate at which flowers open and their longevity, determine the presentation of the flower canopy (Harder and Johnson 2005). Such a presentation typically changes over time, with the set of flowers that open

simultaneously being referred to as the floral display (Harder and Barrett 1996). In a few instances of *L. cardiaca*, the floral buds of one or two consecutive verticillasters reached maturity at the same time and were, therefore, synchronised at full bloom. These flowering dynamics were observed in all of the three populations of *L. cardiaca*, indicating that large numbers of hybrid seeds can be produced in controlled crosses. However, it is not known whether this flowering dynamic is controlled genetically or is an artefact of the environment. Earlier studies indicated that the components of the architecture of flowering inflorescences (including phenology, topology and geometry) are largely subject to quantitative genetic control (Harder and Prusinkiewicz 2013).

For the species *L. cardiaca*, it was found that anther dehiscence and dispersion of pollen grains in each flower occurred before the stigma became receptive. This indicates the prevalence of protandry in this species. Such a temporal separation between the male and the female reproductive elements promotes cross-pollination, thus enabling a mating strategy preventing exclusive self-pollination with its consequent inbreeding depression. This finding is similar to those of Haque and Ghoshal (1981), Huck (1992), Owens and Ubera Jimenez (1992) and Barrett *et al.* (2000) in their studies of pollen-presentation and stigma-receptivity systems in other genera.

In vitro pollen germination is a rapid method for assessing pollen viability (Dafni *et al.* 2005). The use of PEG to optimise *in vitro* germination conditions in the present study proved successful. Generally, PEG is inert metabolically and cannot enter cells but is thought to regulate the permeability of the plasma membrane and to give stability to the pollen-tube membrane (Read *et al.* 1993). Our results were similar to those found when PEG is added to the pollen-germination medium of other genera such as *Anacardium* (Subbaiah 1984), *Brassica* (Ferrari and Wallace 1975; Shivanna and Sawhney 1995), *Capsella* (Leduc *et al.* 1990), *Cicer* (Shivanna *et al.* 1997), *Nicotiana* (Read *et al.* 1993), *Petunia* (Zhang and Croes 1982) and *Pistacia* (Golagoldhirsh *et al.* 1991). Shivanna and Sawhney (1995) found that lowering sucrose concentration from 20% to 5% and adding PEG 4000 (15%) in the culture medium resulted in higher pollen germination and increased rate and duration of tube growth (10 times greater than that obtained in the standard medium) in three species of cultivated *Brassica*. Compared with the control, pollen tubes were straight and smooth without abnormality.

The present study found that the BK medium was optimised with 15% sucrose and 2.5% PEG for *in vitro* germination of pollen in *L. cardiaca*, whereas normally the BK medium contains only 10% sucrose. The role of sucrose as an osmoregulator and nutritional compound is currently widely accepted. Visser (1955) and Hoekstra *et al.* (1989) (see also review by Taylor and Hepler 1997) previously found that sucrose maintains the osmotic pressure of the medium as well as enhances pollen metabolism. These authors concluded that high sucrose concentrations in the growth medium enter the pollen and augment the already high endogenous concentrations. However, when sucrose concentrations in our study were above 15%, the rupture of pollen tubes increased, indicating that high sucrose concentrations may alter the permeability of the

growing pollen tube, ultimately resulting in the leaching of metabolites and ions into the media, as reported in other studies (Zhang and Croes 1982; Subbaiah 1984; Crowe *et al.* 1989; Golangoldhirsh *et al.* 1991). There is now the possibility that the optimised medium we have found in the present study could be used for further work on the reproductive biology of *L. cardiaca*.

In relation to self-pollination of the three *L. cardiaca* populations we studied, the stigmatic lobes are slightly exerted above the longer pair of stamens and there is no contact of these two sexual organs at the time of anthesis. This stigmatic structure and the timing of pollen export can minimise the chance of each flower being pollinated by its own pollen once the stigma is receptive. Furthermore, the flowers remain in this situation until the second day after the stigma becomes receptive, until the flower is visited by a pollen-bearing insect. In the absence of pollination, the angle between the stigmatic lobes widens during the second and third days and, simultaneously, the stigmatic lobes gradually bend until they eventually contact the anthers of the longer stamen pair, thus causing self-pollination if some viable pollen is still attached to the already dehiscent anthers. This would suggest that in future breeding programs of *L. cardiaca*, techniques to eliminate the possibility of self-pollination are required.

In addition to being protandrous, the flowers of *L. cardiaca* open in sequence from the bottom of the inflorescence upward (acropetal), which corresponds with the acropetal movement of the pollinator in an inflorescence, thus enhancing cross-pollination. Consequently, pollen is deposited on the stigmas at the bottom of the next plant visited by the pollinator. Such a system enhances the legitimate transfer of outcross-sourced pollen. The glandular trichomes on the anther surface can also affect the level of cross-pollination because the adhesive secretions they produce can prevent the pollen from being easily wiped off. This promotes adherence to the insect integument and transfer to the stigma. Our finding is similar to that reported by (Moyano *et al.* 2003).

There was considerable variation in the inflorescence structure, the number of flowers per inflorescence, and floral phenology among the three populations. For example, Khansar population flowered 20 days earlier than did the other two populations, allowing limited inter-population pollinations. The difference in the floral biology among populations also reflects the evolutionary history of this species (Collins *et al.* 2008). The observed reproductive system evolved from the existing genetic diversity in *L. cardiaca*. The genetic variation observed among populations provides a basis for future genetic improvement.

Regarding pollinators, Anderson and Symon (1988) and Davila and Wardle (2008) outlined some of the insect species and their roles in the reproduction of certain Australian native plants. The *L. cardiaca* in our study was occasionally visited by butterflies, adult blowfly, European paper wasps and some ladybird beetles. Even though spiders and ants seemingly took no part in pollination, their visits were probably accidental and are likely to be the result of a persistent search for prey or nutrients. However, honeybees, by effect of their abundance and efficiency, were the principal pollinators. In relation to controlled pollination, the small size of the *L. cardiaca* flowers makes hand-pollination difficult.

Conclusions

The reproductive biology of the medicinal plant *L. cardiaca* was investigated using controlled pollination, structural and phenological studies, at the level of single flowers, inflorescences, individual plants and populations. Although this species is self-compatible, dichogamy in the form of protandry increased opportunities for out-crossing. Pollen viability was evaluated using laboratory tests and *in vitro* germination. A modified BK medium with 15% sucrose and 2.5% PEG was optimised for the germination. The *in vitro* germination and staining protocols developed in the study will be useful for both basic and applied studies on the pollen biology of motherwort collections from different regions. Furthermore, IKI and Alexander's stain were found to have high positive correlations with the *in vitro* germination and, therefore, can be used for quick assays of pollen viability in *L. cardiaca*. These protocols will be particularly useful for motherwort breeding to optimise pollen viability and subsequent pollen storability. The results from the present study have the potential to optimise the outcomes of intraspecific crosses targeting an improvement in the quantity and quality of the medically active ingredients from *L. cardiaca*.

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