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Floral and pollination biology, breeding system and nectar traits of *Callistemon citrinus* (Myrtaceae) cultivated in India

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ABSTRACT

This study reports the influence of habitat on floral (flower and nectar characteristics, phenology) and pollination biology (flower visitors and breeding system) on *Callistemon citrinus* (syn. *Callistemon lanceolatus*), an Australian species of family Myrtaceae. In India, this small ornamental tree is cultivated in gardens, avenues and road sides and flowers throughout the year. At Agra (Uttar Pradesh, northern India) this species flowers twice a year (February–May and August–November). Flowers open early in the morning and can be characterized as protandrous for a brief period. Fresh open flowers presented ca. 25% of total produced nectar. Nectar sugar composition consisted of only glucose and fructose. A wide array of visitors (honey bees (*Apis dorsata*), butterflies, wasps, ants, moth, hover flies, several birds and Indian palm squirrel) visit flowers either for pollen or nectar or for facilitating self- and cross-pollination by their intra- and inter-tree movements. Among these, honey bees are dominant in number and in the amount of pollen on their body, while butterflies, ants, wasps, sunbirds, parrots, oriental white-eye sparrow and squirrels forage only for nectar and can be considered occasional pollinators or nectar thieves. Although flowers are highly visited, nectar in standing crop showed that flower visitors did not consume the total nectar produced. Nectar replenishment decreased with age in both bagged- and exposed flowers, showing the capability of secreting nectar after removal during the entire flower lifetime. Experimental hand-pollinations showed that naturalized *C. citrinus* fruits matured through autogamy, geitonogamy or xenogamy as was reported for native Australian populations. *C. citrinus* displays an interesting reproductive strategy, shows phenotypic plasticity of flowering periodicity and interacts with a generalized pollinator system, attracts a wide array of animal species with a conspicuous amount or replenished nectar after removals, and uses both compatibility strategies – xenogamy and autogamy – to ensure successful pollination and seed production at native or naturalized habitats.

1. Introduction

Understanding the details of reproduction in plants has been a fascinating area of multidisciplinary research with immense value for extending not only the frontiers of fundamental knowledge (Tandon et al., 2005, Kaul-Moza and Bhatnagar, 2007) but also for genetic improvement of plants and optimal utilization and conservation of various plant species. Studies on flowering times stress the role of interactions between plant species which share pollinators or predators (Fenner, 1998). Local biota interact with foreign species, and many studies on biological invasions highlight that interactions among species may be positive (e.g., Ghazoul, 2004, Simberloff, 2006, Molina-Montenegro et al., 2008). Thus, it is interesting to document animal–plant interactions of foreign populations of plant species, particularly in their pollination interactions.

The dynamic nature of plant–pollinator interactions may be a function of the abundance of floral resources, and plants producing high amounts of nectar are attractive to floral visitors. Nectar production can stop after successive nectar removal or it can be maintained until the end of the flower's lifetime (e.g., Galetto and Bernardello, 1993; Bernardello et al., 1994; Rivera et al., 1996; Torres and Galetto, 1998). Variation in floral reward after removal (i.e., nectar standing crop after animal visits) can affect pollinator behavior, pollen movement, and consequently fruit and seed production (Torres and Galetto, 1998). Self-compatible species with an extended flowering phenology and flowers offering a large volume of nectar are expected to be visited by a wide arrange of floral visitors and to produce a large number of fruits and seeds. The study of invasion ecology is usually focused on the negative impacts of foreign species and potential positive impacts are often overlooked (Goodenough, 2010).

Callistemon, commonly called bottlebrush, is a genus of 40 species of shrubs of the family Myrtaceae, which are endemic to Australia (Anonymous, 2009). Most bottlebrushes have their native distribution

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in New South Wales, Victoria and southern Queensland in south-eastern Australia (Spencer and Lumley, 2012). They often grow in damp or wet conditions e.g., along creek beds or in areas which are prone to floods. *Callistemon citrinus* (Curtis) Skeels was earlier called by the name of *Callistemon lanceolatus* DC. (Anonymous, 2009). Celebrezze (2002) characterized this species as fully self-compatible and capable of setting seeds by autogamy when studied in native populations in Australia. Data of nectar sugar composition could not be found for the Australian population. Native population of this species showed a flowering peak between November and December (although it can occur sporadically year round) and they are visited by wasps, honeybees, and ants. Birds, such as honeyeaters, brush wattlebirds, and eastern spine bills (Celebrezze, 2002) were the predominant native visitors.

In India, *C. citrinus* is cultivated as a small ornamental tree in gardens, avenues and on road sides (Gupta and Kumar, 1993). Sharanya et al. (2014) reported that this species flowers throughout the year at Calcut (India). Flowers presented 25–40% nectar concentration, and sugar composition is dominated by hexose, glucose and fructose at Nauni (Solan, India) (Gupta and Kumar, 1993). Flowers showed higher visitation frequency of honeybees at Nauni (Gupta and Kumar, 1993) and a large number of floral visitors at Calcut (Sharanya et al., 2014). Sharanya et al. (2014) reported that only some of them are pollinators while others are nectar robbers.

The present study has been undertaken with three main objectives of (a) characterizing floral biology (floral characteristics, phenology, and nectar characteristics); (b) characterizing pollination biology (floral visitors, breeding system); and (c) comparing data obtained under objectives 1 and 2 between native and naturalized populations of *C. citrinus* at Agra, Uttar Pradesh, Northern India, in order to deduce a habitat's influence on recorded characteristics.

2. Materials and methods

2.1. Plant material

The present investigation was conducted on 25 plants of *C. citrinus* (Curtis) Skeels marked from a large number of plants cultivated in five gardens (Table 1) of similar habitat in different parts of Agra city (26° 44′ N to 27° 25′ N and 77° 26′ E to 78° 32′ E and 171 m above sea level), Uttar Pradesh, India. The gardens have loam soil which is irrigated at regular intervals as required. The selected plants were more or less of the same age and cultivated through cuttings. Thus, they were of the same clone. Three sets of herbarium specimen of five selected plants (one each from each garden) were prepared and were sent to the Botanical Survey of India (BSI), Salt Lake, Kolkata and Regional Station, BSI, Kaulagarh Road, Dehra Dun for identification and one was deposited as voucher specimen No. Agra-211/2010 in the herbarium of Academy of Life Sciences, Agra.

2.2. Floral biology

2.2.1. Phenology

Phenological events (time of leaf fall, leaf renewal, flowering period – commencement, peak and decline in flowering – fruiting period, as well as number of open flowers and times of daily anthesis and anther dehiscence) were recorded by semi-quantitative (SQT) method based on monthly estimations of each phenophase incidence through a visual inspection of 25 plants in different parts of Agra city as described by Castro-Diez et al. (2003). Observations were recorded every day between 0500 and 1800 h in both flowering periods (February–May and August–November) for two consecutive years (2009–2010 and 2010–2011). Flowering intensity (average number of flowers/inflorescence × average number inflorescences/plant) was recorded according to Dafni (1992). To avoid recounting, open flowers were removed immediately after counting. Observations during flower lifetime were made at intervals of 2 h for five days.

2.2.2. Flower characteristics

Evaluation of pollen morphology and nectar quantity and quality and observations on the visitors behavior, were made following the procedure of Faegri and van Der Pijl (1979) and Dafni (1992). Number of ovules/flower was recorded after Stelly et al. (1984). Pollen–ovule ratio was obtained after Cruden (1977) and Barrett (1985). Pollen germinability and stigma receptivity were obtained after Martin (1959) and Mattson et al. (1974), respectively.

Size and shape of pollen, surface, aperture (shape, number and position) and exine sculpture was recorded in acetolyzed pollen grains under a light microscope. The samples were dehydrated through aqueous acetone series, dried with CO_2 in a Hitachi critical point dryer (HCP-2) and then coated with gold (20 mm) in a SCD 020 sputter coating unit (Polorn Equipment Ltd., Welford, England) and observed in a Philips EM 50, SEM at All India Institute of Medical science, New Delhi. Pollen size was measured after the method of McKone and Webb (1988). Pollen viability was assessed by in vitro pollen germination by the hanging drop culture method using Brewbaker and Kwack's (1963) medium, FCR (fluorochromatic reaction) test after Heslop-Harrison and Heslop-Harrison (1970) and 1% TTC (2,3,5-triphenyl tetrazolium chloride) in 0.15 M Tris buffer at pH 7 (Hauser and Morrison, 1964).

2.2.3. Nectar characteristics

Volume of nectar from individual flowers (25 from each marked plant) was measured using 20 μL microcapillary tubes during both flowering periods. Nectar volume and concentration were calculated using the procedure of Cruden and Herman (1983). Amount of sugar (mg of sugars/μL nectar) was calculated after Galetto and Bernardello (2005). Sugars were separated using a Whatman No. 1 filter paper (Kearns and Inouye, 1993).

2.3. Pollination biology

2.3.1. Floral visitors

Observations on the floral visitors and their foraging behavior were made following the procedure of Faegri and van Der Pijl (1979) and Dafni (1992). Flower visitors were observed as potential pollinators using a binocular. Their behavior on the flowers, flying pattern across inflorescences, time of visit and type of resource collected were recorded. Pollination efficiency of different insects was checked by observing the pollen load on their body parts under the light microscope as per the procedure described by Kearns and Inouye (1993). Captured individuals were pinned and identified by Dr. Girish Maheshwari, School of Entomology, St John's College, Agra. Visiting birds detected by binocular observation were identified through comparison with the Indian main ornithology data resource "The Book of Indian Birds" (Ali, 2002).

2.3.2. Compatibility system

Autogamy (bagged and hand pollinated), geitonogamy and xenogamy were tested through controlled pollination studies. In order to observe the rate of fruit-set from flowers open to flower visitors, fifty inflorescences on different trees were tagged and were followed until fruit development. The daily foraging schedules, forage collected and probing behavior of different foragers were recorded.

Table 1
Study sites at Agra (26° 44′ N to 27° 25′ N and 77° 26′ E to 78° 32′ E and 171 m above sea level), Uttar Pradesh, India.

Sites	Place
1.	Motilal Nehru Park, near Taj Mahal
2.	Campany Garden, Cantonment area
3.	Paliwal Park, In the heart of the town
4.	Botanical Garden, DEI Deemed University, Dayalbagh
5.	Bharatpur House, (Posh colony)

2.4. Statistical data analysis

Data obtained from different floral variables were compared between flowering periods through *t* tests and those between pollination treatments were subjected to analysis of variance (ANOVA) after Snedecor and Cochram (1967).

3. Results

3.1. Floral biology

3.1.1. Phenology

C. citrinus is a small tree (Fig. 1a). Leaf fall and leaf renewal take place simultaneously throughout the year. Before the commencement of flowering, at the apex of the drooping branches, a cluster of 7 ± 3 young light green leaves develops (Fig. 1b, c, g). Floral buds appear just behind these leaves in the form of spikes (Fig. 1c). At Agra, *C. citrinus* has two distinct flowering periods in a year and their main characteristics are summarized in Table 2. During the first flowering period, fruiting starts in the last week of February and the fruits mature within 6–7 weeks dispersing its seeds during the last week of May, while in the second flowering period fruiting starts in the first week of September dispersing its seeds during the last week of November. The fruits are capsule, cup shaped, hard, woody, grayish brown and about 0.5 cm long (Fig. 1e), 3-celled and dehiscent with minute seeds and they remain attached to the tree until the following flowering season commences.

Propagation is both by seeds and cuttings. Fresh cuttings of 10–15 cm with 2–3 leaves are usually placed in the pots in the months of July/August, but only 27–38% develop into seedlings.

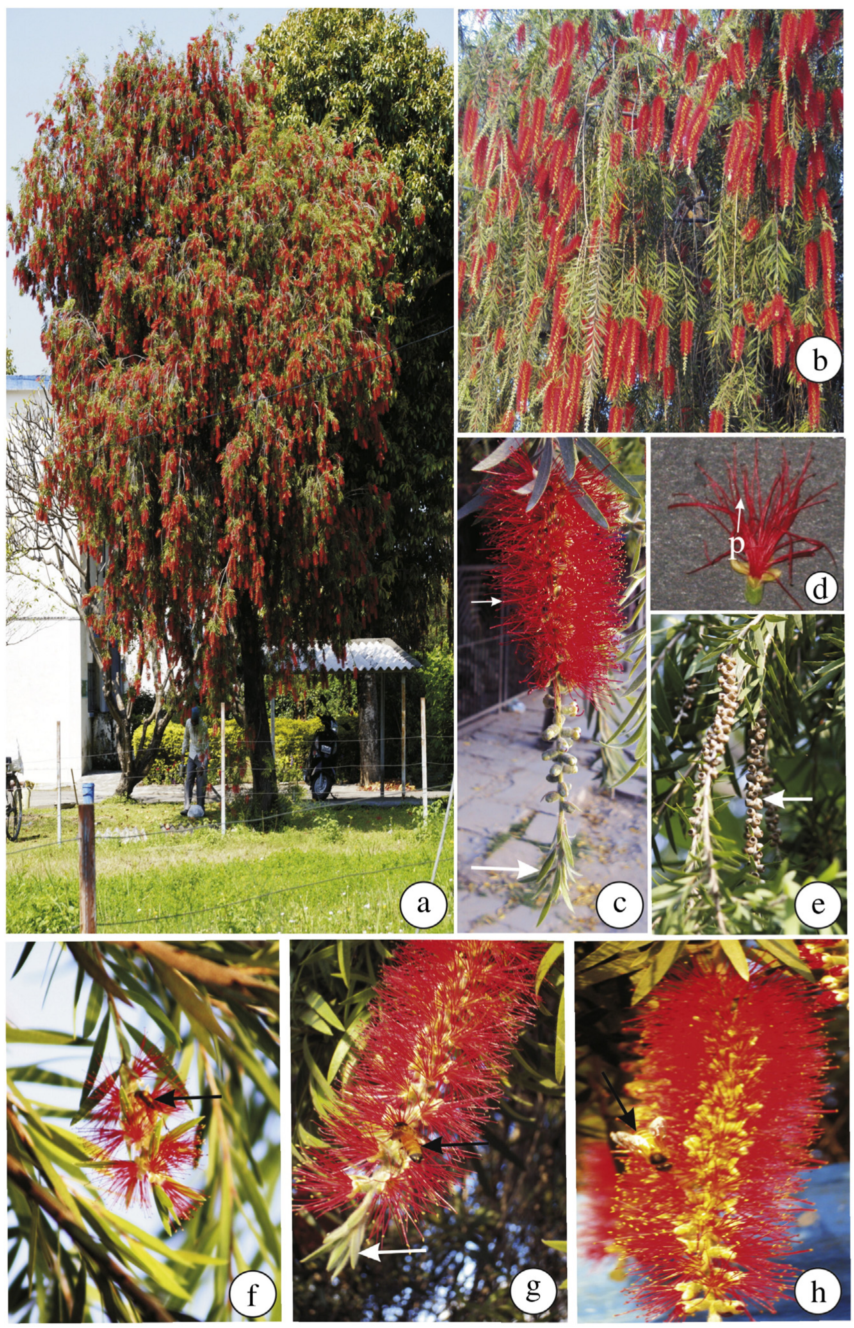

Fig. 1. *Callistemon citrinus* (Curtis) Skeels. a. Mature flowering tree. b. Plant in full bloom. c. Inflorescence with young buds and bunch of young leaves (arrow); receptive stigma (arrow). d. Single flower. e. Mature fruits (arrow). f. Wasp on flowers (arrow). g. Honey bee on flowers (arrow). h. Hoverfly on flowers (arrow).

Table 2
Qualitative and quantitative floral characteristics of *Callistemon citrinus*.

Floral characters	Flowering periods	
	First	Second
Flowering period	February–May	August–November
Optimum flowering	March	October
Temperature régime	33–44 °C	22–32 °C
Relative humidity (%)	20–40	60–80
Inflorescence	Terminal spike	
No. of flowers/inflorescence	60 ± 4.5*	52 ± 2.5
Flower	Hermaphrodite, Actinomorphic, Epigynous	
No. of stamens/flower	42 ± 3.0*	38 ± 5.0
Calyx	5 sepals, gamosepalous	
Corolla	5 petals, polypetalous	
Anthesis	0500–0630 h	0530–0700 h
Anther dehiscence	0600–0730 h	0630–0800 h
Pollen/anther	920 ± 245*	890 ± 123
Pollen/flower	38640 ± 825*	35540 ± 265
Pollen viability(%)	98 ± 8*	80 ± 5
Stigma receptivity	0800–1200 h	0830–1230 h
Gynoceium	Tricarpellary	
Ovary	Inferior, unilocular	
No. of ovules/locule	Two on axile placenta	
Pollen–ovule ratio	19320:1*	17770:1

± Standard deviation.
* Significant at 5%.

3.1.2. Flower characteristics

The flowers are crimson red, sessile, bracteates, hermaphrodite, actinomorphic, epigynous and complete (Fig. 1d). The calyx is gamosepalous with five green sepals arranged in quincuncial aestivation and the corolla is polypetalous with five whitish green petals also arranged in quincuncial aestivation.

Flowers traits and variations between flowering periods are presented in Table 2. The filaments are very long, crimson red, slightly connate at the base and the purple anthers are dorsifixed, versatile, bi-celled and dark brown (Fig. 1c, g, h). Pollen grains are triangular, parasyncolpate, tricolpate and 30 ± 2.5 μm in diameter with psilate and scabrate surface (Fig. 3a, b). Pollen viability is high (Table 2) and declines gradually after anther dehiscence in both periods and is completely lost after 12 h. The stigma is wet and globular (Fig. 3c), with a small depression (Fig. 3d), and covered with several globular papillae and long tapering trichomes (Fig. 3c, d, e).

3.1.3. Nectar characteristics

The quantity of nectar from fresh open flowers represents ca. 25 and 20%, respectively, of the total nectar production (Fig. 4a, c; in terms of volume or sugar content). Nectar concentration showed an increase during flower lifetime (Fig. 4b). Nectar standing crop and total nectar production after removal was significantly higher during the first period

Fig. 2. Floral visitors recorded in *Callistemon citrinus*. a. Hanging inflorescence with black ants (arrow) and a hovering moth (circle). b& c. Oriental white-eye sparrow (*Zosterops palpebrosus*). d. Sun bird (*Nectarinia asiatica*), e & f. parrots (*Psittacula krameri*), g. Indian palm squirrel (*Funambulu spalmarum*).

Table 3
Floral visitors and pollen load on their body parts during first and second periods of flowering in *Callistemon citrinus* (+: Present; −: Absent).

Visitor species	Flowering periods				Carrier of pollen (P), nectar (N) or nectar robbers (NR)
	I		II		
	P/A	Pollen load	P/A	Pollen load	
1. *Apis dorsata*	+	477 ± 170	+	388 ± 123	P/N
2. *Vespa* spp.	+	415 ± 89	+	375 ± 78	P/N
3. *Pieris brassicae*	+	330 ± 72	+	295 ± 81	NR
4. *Danaus obesa*	+	309 ± 59	−	−	NR
5. *Campnotus compressus*	+	210 ± 51	−	−	NR
6. *Ornida obese*	+	152 ± 25	−	−	NR
7. *Nectarinia asiática*	+	−	−	−	NR
8. *Psittacula krameri*	+	−	+	−	NR
9. *Pycnonotus attrieps*	+	−	+	−	NR
10. *Zosteropus palpebrosus*	+	−	+	−	NR
11. *Funambulus palmaru*	+	−	−	−	NR

(ca. 40% in terms of volume or sugar content) of flowering as compared to that of the second period (Fig. 4c). The quantity of replenished nectar/flower declined gradually in terms of volume (Fig. 4a) or solutes (Fig. 4c). Only fructose and glucose were identified in the nectar of fresh open flowers during both flowering periods.

3.2. Pollination biology

3.2.1. Floral visitors

The floral visitors in the first flowering period were honey bees (*Apis dorsata*) (Fig. 1g), butterflies (*Pieris brassicae* and *Danaus plexippus*), wasps (*Vespa* spp.) (Fig. 1f), ants (*Campnotus compressus*), hover flies (*Ornida obesa*) (Fig. 1h), and moth (Fig. 2a), sunbirds (*Nectarinia asiatica*) (Fig. 2d), parrots (*Psittacula krameri*) (Fig. 2e, f), black-headed bulbul (*Pycnonotus atrieps*), oriental white-eye sparrow (*Zosterops palpebrosus*) (Fig. 2b, c), and Indian palm squirrel (*Funambulus palmarum*) (Fig. 2g) (Table 3).

These visitors forage during the day (first between 0500 and 1300 h and again during 1600–1800 h) for nectar and/or pollen with peak frequency during 0500–1100 h. Honey bees make 40%, butterflies 20%, wasps 20%, birds 16%, and ants 4% of the total frequency of visitors. Among these only honey bees and wasps forage for both floral rewards, while others forage for nectar only. Although all insects act as pollen carriers facilitating both self- and cross-pollination by their inter- and

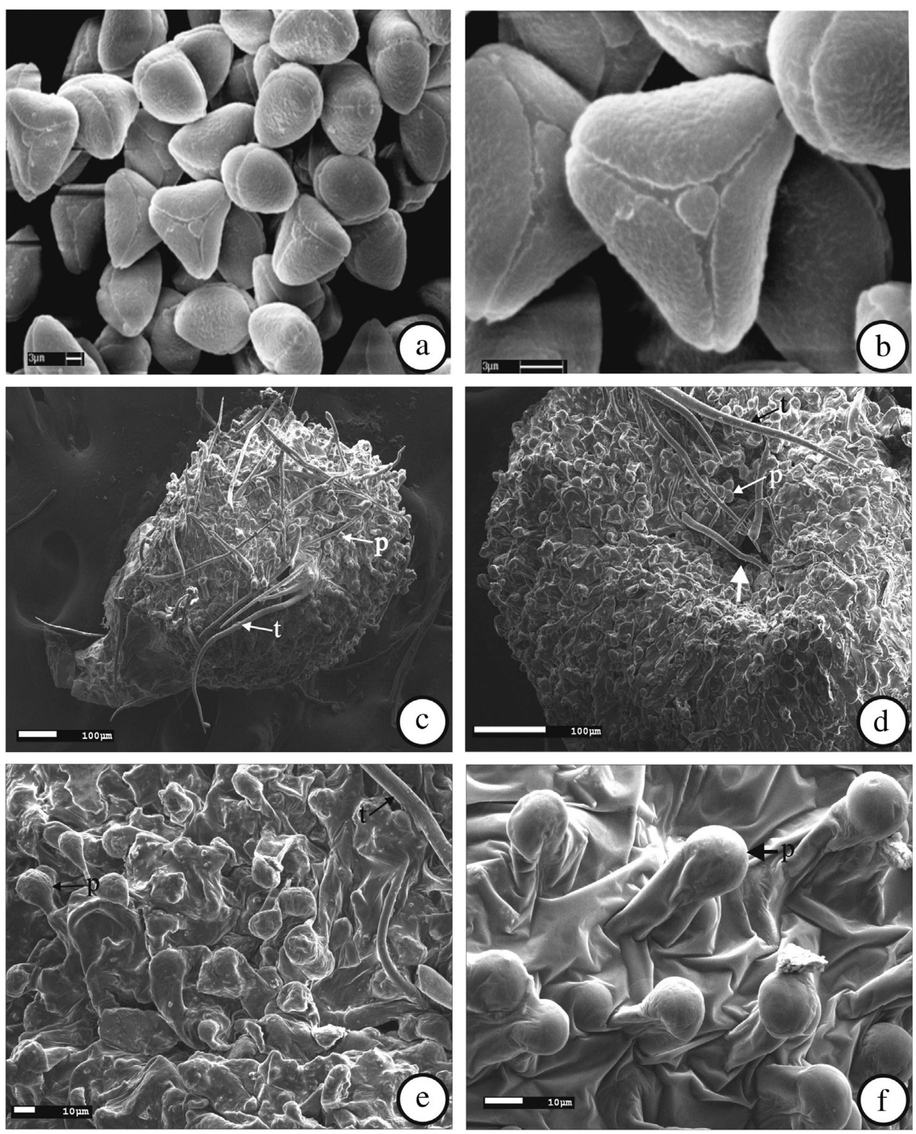

Fig. 3. Pollen grains and pistil of *Callistemon citrinus*. a. Mature pollen grains. b. Magnified view of triangular, tricoplate pollen grains. c. Globular stigma covered with capitate papillae and long tapering trichomes. d. Upper view of stigma with a depression (arrow), covered with capitates papillae and long tapering trichomes. e & f. Magnified view of stigmatic surface showing capitates papillae and trichomes. (p: papillae; t: trichomes). Magnification: a & b: 3 μm; c & d: 100 μm and e & f: 10 μm.

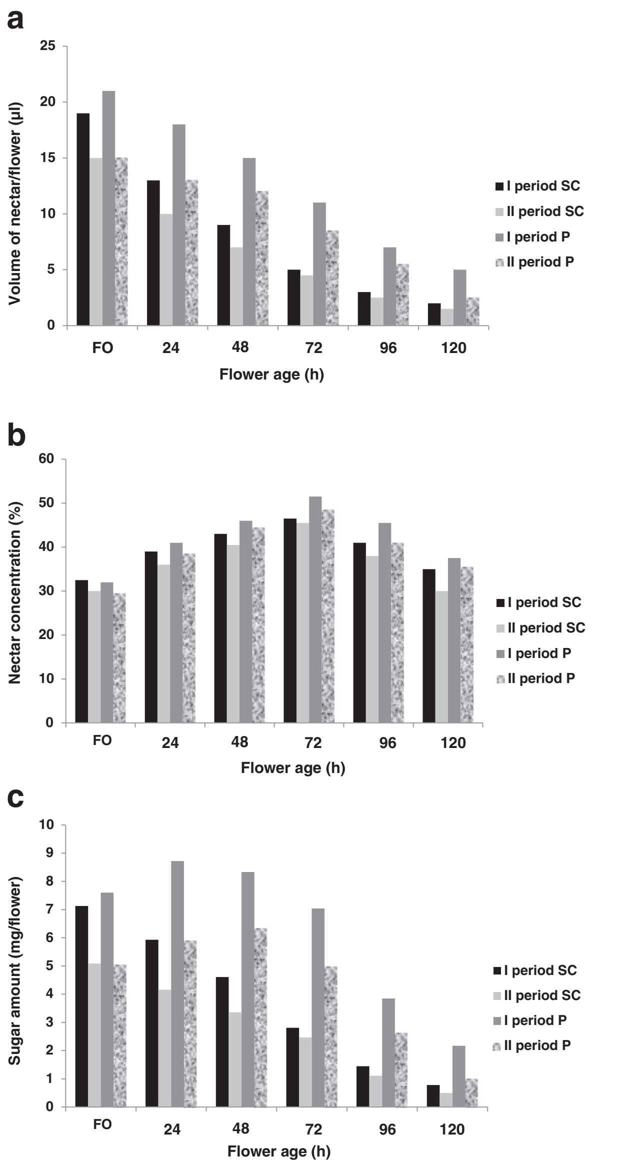

Fig. 4. Nectar traits through floral lifetime for flowers open to visitors in standing crop (SC) and protected flowers (P) during different flowering (I & II, see details in the Materials and methods section) periods of *Callistemon citrinus*. a. Volume of nectar/flower (μL). b Nectar concentration (%). c. Sugar amount (mg/flower).

intra-tree movements, in *C. citrinus*, *A. dorsata* is the main pollinator in both flowering periods not only because of its frequent inter- and intra-tree movements, but also because it has a higher amount of pollen on its body in comparison to other insects when observed under a microscope (Table 3). Other insects which were found to be important pollinators for this species are butterflies and wasps (Table 3). Interestingly, during the second flowering period these floral visitors were also acting as main pollinators (table 3). Sun birds (*N. asiatica*), parrots (*P. krameri*), oriental white-eye sparrow (*Zosterops palpebrosus*) and Indian palm squirrel (*Funambulus palmarum*) were found to act only as nectar thieves only during both flowering periods.

3.2.2. Compatibility system

Bagged flowers left for autonomous self-pollination failed to set fruits. Hand-self and cross-pollinations set fruits (Table 4). Results of experimental hand-pollinations for both flowering periods are shown in Table 4.

Table 4
Experimental details of breeding system during the periods of flowering.

S. No.	Type of breeding system flowering Period	No. of flowers pollinated	No. of flowers set fruits	Fruit-set (%)	No. of fruits dropped prematurely	Fruit drop (%)
1.	Autogamy					
	i. Bagged flowers					
	a. I Period	50	0	0	0	0
	b. II Period	50	0	0	0	0
	ii. Bagged & hand pollinated					
	a. I Period	50	3	68*	11	16.17
	b. II Period	50	23	46	14	30.43*
2.	Geitonogamy					
	a. I Period	50	41	82*	18	21.95
	b. II Period	50	33	66	21	31.81*
3.	Xenogamy					
	a. I Period	50	45	90*	5	5.55
	b. II Period	50	39	78	9	11.53*

± Standard deviation.
* Significant at 5% between flowering periods and pollination.

4. Discussion

4.1. Floral biology

Celebrezze (2002) studied native populations of this species in Australia and found that the flowering peak occurs between November and December, although *C. citrinus* can flower sporadically year round. Outside the native distribution of this species, Sharanya et al. (2014) have reported that this species flowers throughout the year at Calicut (India) and results presented here showed that this species has displayed two distinct flowering periods at Agra (north India). Particular variations in seasonal conditions (e.g., photoperiod, rainfall, soil water availability, droughts, temperature) are thought to modify the intensity of flowering by influencing bud formation and development (e.g., Law et al., 2000; Borchert et al., 2004). Moreover, flowering phenology in urban environments is modified compared to un-urbanized habitats (Neil and Wu, 2006). Available data for *C. citrinus* may suggest phenotypic plasticity of flowering periodicity in this species and that environmental changes can modify flowering when wide-ranging populations are considered.

We did not find nectar data from native populations of this species, thus we only discuss nectar variations of naturalized populations at India. Gupta and Kumar (1993) have recorded many nectar traits in plants of this species cultivated on roadside when in blooms during April–May at Nauni, Solan, India. Their data as compared to that recorded at Agra are more or less similar, but the main difference is related to total nectar sugar production per flower (only 6 as compared to 26–37 mg/flower; Gupta and Kumar, 1993 and our data, respectively). It is possible that this difference between studies can be explained on the basis that total sugar production per flower can vary considerably if it is calculated as nectar standing crop in open flowers or after repeated removals in bagged flowers (Galetto and Bernardello, 2005), as was in our case.

4.2. Pollination biology

Available data on floral visitors indicate that different groups of animals can pollinate successfully flowers of *C. citrinus* (Gupta and Kumar, 1993, Celebrezze, 2002, Sharanya et al., 2014, our data) and consequently the pollination system can be characterized as generalized. However, native Australian populations are bird pollinated (Celebrezze, 2002) but cultivated Indian populations are mainly pollinated by insects. These variations of the pollinators can be explained because brush flowers allow nectar access to different animal visitors and they produce abundant nectar that attracts different effective pollinators. This pattern was previously registered for other species with brush flowers (e.g., Bernardello et al., 1994).

Variations of nectar traits among populations can also be related with differences in pollinator visits. A comparative lower nectar volume and higher nectar sugar concentration are common to most entomophilous plants (Faegri and van Der Pijl, 1979; Pacini et al., 2003) than for perching birds (Bernardello et al. 1994 and references therein). In particular, Johnson and Nicolson (2008) have characterized general trends for different nectar properties in the plants that are pollinated by specialized and generalized birds. Nectar traits of *C. citrinus* can be related to a generalized bird pollinator system (except for the high nectar concentration). Nevertheless, *C. citrinus* seems to present versatility receiving different groups of pollinators because at Agra nectar replenishment was higher than nectar removal by animals during the entire floral lifetime and in both flowering periods. Thus, flowers of this species show a high production capability displaying nectar standing crops that can feed different animal floral visitors.

Our results from hand-pollination experiments indicate that *C. citrinus* shows facultative xenogamy which is in concordance with literature data for native and naturalized populations (Celebrezze, 2002; Sharanya et al., 2014). Further, our results suggest that self-pollen deposition does not take place without pollinators, which is supported by herkogamy of *C. citrinus* flowers in which anthers and stigma are spaciously separated. This facultative breeding system facilitates fruit-set through pollen mediation by different flower visitors (i.e. autogamy, geitonogamy and xenogamy) by providing adaptiveness of *C. citrinus* necessary for colonization of its naturalized habitat.

Although this species can be self- and cross-pollinated, it sheds most of the autogamous and geitonogamous fruits, while retaining all xenogamous fruits to maturity. This suggests that pollinators are important to set fruits in *C. citrinus* by cross-pollinating, but leaves open the possibilities for mediated self-pollination. *C. citrinus* displays an interesting reproductive strategy: shows phenotypic plasticity of flowering periodicity and interacts with a generalized pollinator system, attracts a wide array of animal species with a conspicuous amount or replenished nectar after removals, and uses both compatibility strategies – xenogamy and autogamy – to ensure successful pollination and seed production at native or naturalized habitats.

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