Sex expression and breeding strategy in *Commelina benghalensis* L.

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This paper describes the results of a series of experiments conducted to unravel the patterns of sex expression and reproductive output in a fascinating species with high variation in sexuality. *Commelina benghalensis L.,* an andromonoecious rainy season weed, bears male and bisexual flowers in axillary spathes of all the plants investigated. Bisexual flowers are of two types; chasmogamous (CH) and cleistogamous (CL). The former are borne on subaerial and the latter on subterranean shoots, in addition to those on aerial spathes. Three populations of the species, designated JU1, JU2 and JU3, were scanned for three consecutive years from 1996 to 1998, and the number and distribution of male, CH and CL flowers per plant were found to vary. The mere number of CH/CL flowers per plant is by itself not an accurate measure of mixed mating. It is necessary to confirm that CH flowers actually outcross and, if they do so, to what extent. Comparison of the pollen/ovule (P/O) ratio and percentage pollen germination on the stigmas of the CH and CL flowers have been used as indices of the pollination system. Confirmation of this was sought from the fruit and seed sets obtained after manual pollination of emasculated flowers with self- and cross-pollen. Results so obtained were compared with those of natural pollination. In the majority of CH flowers, the male and female reproductive phases (i.e. anther dehiscence and stigma receptivity) overlap, providing for self-pollination. However, two exceptions to this general behaviour were found in some plants of all the three populations. In some CH flowers, the female phase matures prior to anther dehiscence while in others, the anthers are sterile. Such plants, designated as variants 1 and 2, respectively, facilitate cross-pollination. While the CL flowers contribute to the production of selfed progeny, the variants of CH ones permit formation of outcrossed progeny, indicating a mixed mating strategy in *C. benghalensis.*

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1. Introduction

Cleistogamy is reported in 693 angiosperm species distributed over 50 families (Culley and Klooster 2007). The widespread presence of this phenomenon in nature across mono- and dicot families suggests that this breeding system must have evolved repeatedly and independently many times during evolution. Culley and Klooster (2007) opine that on a family basis, this breeding system must have evolved approximately 34–41 times. Many factors thought to influence the evolution of cleistogamy include exposure to heterogeneous conditions, inbreeding depression, geitonogamy, differential expenditure, differential seed dispersal, plant size and various ecological factors (*see* Culley and Klooster 2007 and references therein). Relative to other breeding systems, however, this aspect has received little attention (Culley and Klooster 2007). To understand the evolutionary routes that this breeding system may have taken, more information on the reproductive attributes of cleistogamous taxa is needed. The present investigation is an initiative in this direction. Usually, species with this breeding system produce cleistogamous (CL) and chasmogamous (CH) flowers, though the time and site of their differentiation may vary.

The presence of CL and CH flowers on the same individual is considered a contrivance for 'mixed' reproductive strategy (Darwin 1877; Lord 1981; Schoen 1984; Le Corff 1996). It can, however, be so if both the floral types are actually

Keywords. Chasmogamous; cleistogamous; reproductive output; sex expression.

Abbreviations used: A, autogamy; AP, apomixis; CH, chasmogamous; CL, cleistogamous; MCP. manual cross-pollination; MSP, manual self-pollination; OP, open pollination; P/O, pollen/ovule; PGS, pollen germination on stigma surface; PTI, pollen transfer by insects

functional. Whereas cleistogamy is an assured contrivance for self-pollination (Kannenberg and Allard 1967; Levin 1972; Schemske 1978; Waller 1979, 1980, 1984; Lord 1980, 1981; Schoen 1982), chasmogamy by itself does not guarantee outcrossing. The degree of outcrossing in CH flowers varies from zero to very high, depending upon the temporal and spatial relationship between the male and female phases, mode of pollination and environmental conditions (Moore and Lewis 1965; Schoen 1982; Schoen and Clegg 1985; Holtsford and Ellstrand 1989).

Production of CH and CL flowers on a single plant is considered a stable mixed mating system (Culley 2000). This contradicts the theory which predicts that mating systems consisting of both selfing and outcrossing should not be evolutionarily stable (Charlesworth and Charlesworth 1987). Mixed mating may be stable if selfed and outcrossed progeny differ in the patterns of seed dispersal or if the overall level of inbreeding depression is low enough to enable selfed progeny to survive (Schoen 1984; Holsinger 1986, 1988; Le Corff 1996; Culley 2000). Unless CH flowers are not known to fully outcross, it is difficult to accurately measure inbreeding depression. On the other hand, measuring inbreeding depression would serve no purpose if fitness differences between CL and CH progeny are on account of flower-type instead of mating-type differences (*see also* Culley 2000).

Commelina benghalensis L., of the Commelinaceae family, is native to the tropical regions of Asia (Takematsu and Ichizen 1997), and a noxious weed of 25 different crops in 28 countries (Wilson 1981; Holm *et al.* 1991). Being a prolific rainy season weed, it is widely spread across the Indian subcontinent. However, its reproductive biology remains unexplored despite the fact that it exhibits extensive intra-plant diversity in foliar and floral features. First, it exhibits andromonoecy, an unusual pattern of sex expression represented in only 1.7% of known flowering plants (Yampolsky and Yampolsky 1922; Richards 1997). Known to be influenced by several environmental conditions (Solomon 1985; Diggle 1991; Emms 1993, 1996; Narbona *et al.* 2002), the evolutionary significance of andromonoecy is still not established. Second, it also bears CH and CL flowers that vary in structure and distribution on the plant. Plants have three kinds of floriferous branches: the negatively geotropic (aerial), the positively geotropic (subterranean) and the diageotropic (subaerial) types (Kaul *et al.* 2002). The subterranean and subaerial spathes carry a single bisexual flower each, but the aerial spathes have an average of three, one male and two bisexual; one bisexual each CH and CL. The subaerial flowers are invariably CH and the subterranean flowers always CL (Kaul *et al.* 2002). As such, an individual plant of the species produces three types of flowers; male CH, bisexual CH and bisexual CL, hereafter referred to as male, CH and CL, respectively. Third, the CH flowers in aerial spathes also vary in their sex expression; some are weakly protogynous (variant 1) and some functionally female (variant 2). No attempt has ever been made to relate the enormous floral variability of the plants to the breeding systems they follow. The present study was undertaken with a view to determine which features of floral variation influence the breeding system and to what extent. More specific objectives of the study were: (a) to compare reproduction through CH and CL flowers and attempt to accurately quantify the mixed reproductive strategy of this species, (b) to measure the level of inbreeding depression and quantify patterns of sex expression in CH flowers, (c) to find out how pollen germination compares between self and outcross pollen on emasculated hand-pollinated CH flowers, and (d) to determine whether differences in percentage fruit set, percentage seed set and percentage seed germinability are on account of differences associated with flower type or mating type, and (e) whether female flowers (variant 2) are equally likely as bisexual flowers to set fruit.

2. Materials and methods

2.1 *Materials*

Plants of *C. benghalensis* grow between May and October when day and night temperatures vary between 27–40°C and 19–29°C, respectively, and the relative humidity ranges from 55% to 100%. The flowering period of the plants extends from the last week of June to the end of October.

Three populations occupying diverse habitats, all in Jammu (Jammu and Kashmir, India), designated JU1 (rich in organic matter), JU2 (exposed and comparatively drier patches under the canopy of *Lagerstroemia* trees) and JU3 (moist and sunny patches in the Botanical Garden, Jammu), were sampled at random. In 1995, 25 seedlings of JU1 and 50 seedlings each of JU2 and JU3 populations were transplanted from the field to the experimental beds (1.8 X 1.2 m^2) in the Botanical Garden of the University of Jammu (Jammu and Kashmir, India). All transplanted seedlings were at the 4–5 leaf stage and similar in phenotype. Watered every alternate day, the transplants adapted well, produced flowers and set fruits. Seeds collected from these fruits germinated within 3–5 days when sown in the next season. These seedlings were transferred to beds and earthen pots (10” depth and 12” diameter) filled with garden soil, sand and manure mixed in a ratio of 1:1:1 by weight.

All flowers are nectarless, zygomorphic, hypogynous and trimerous with three vestigial and three fertile stamens and, except for the male flower, the others have a tricarpellary, syncarpous pistil (Maheshwari and Maheshwari 1955; Kaul *et al.* 2002). The vestigial anthers are bright yellow, 6-lobed (Maheshwari and Singh 1934; Maheshwari and Maheshwari 1955; Kaul *et al*. 2002) and indehiscent, with only a few

(0–68) pollen grains in two of its lobes (Kaul *et al*. 2001, 2002, 2007). Anthers of two of the three fertile stamens are light grey; that of the third central stamen is yellow. The former contain fewer pollen grains than the latter (for details, *see* Kaul *et al*. 2002). The number of ovules per pistil is five in the aerial and subaerial flowers, and three in the subterranean ones (Kaul 1998; Kaul *et al.* 2001, 2002, 2007).

2.2 *Methods*

All observations on floral phenology, floral biology and pollinator activity were made on the plants raised in the Botanical Garden of the University of Jammu. Data were collected for three consecutive years, 1996, 1997 and 1998. Five spathes each of 15 plants per population were marked to record the time of anthesis, anther dehiscence and stigma receptivity on alternate days. The time taken by pollen grains to germinate on the stigma following their release from anthers was recorded from 15 plants of each population. Stigma receptivity was estimated following controlled pollination as described in Gopinathan and Babu (1987). Only those stigmas which carried germinating pollen grains on their surface were considered receptive. Pollen load was estimated under the microscope from stigmas collected 4–5 h after anthesis and mounted in Lewis (1979) stain. Pollen grains that got detached from the stigmas during staining and floated in the stain were included in pollen load estimates.

The time of pollen discharge from anthers and of pollen germination on stigma were also recorded separately from 25 plants each of JU1 and JU2, and 27 plants of JU3. Variation in floral structure, other than the ones normally found such as unisexuality or andromonoecy, cleisto- and chasmogamy, wherever present, was scored and plants showing these variations were tagged and their numbers recorded. These studies were also conducted on 75 plants randomly selected from the populations growing in nature.

Pollen and ovule counts per flower type were calculated as described in Kaul *et al.* (2002). The pollen/ovule (P/O) ratio was estimated by dividing the average pollen count per flower by the ovule count of the same flower, as proposed by Cruden (1977). Since pollen count and P/O ratio per flower were exponential rather than linear, their logarithmic values were calculated.

To determine the type of breeding system operative in *C. benghalensis*, log (P/O) and pollen germination on stigma surface (PGS) were correlated using Karl Pearson coefficient of correlation (*see* Plitmann and Levin 1990). The results obtained were subjected to the '*t*'-test for estimating the significance of correlation coefficients (Gupta1993). Since P/O ratio and PGS could not be determined from the same flowers, flowers of different spathes from the same branch were used.

Data on the number and behaviour of insect visitors were collected through visual observations made between 5:30 and 12:00 h on sunny as well as cloudy days (total number of days = 18). For this purpose, 25 plants of each population were used, and three plants monitored per day. Frequency of pollinator visitation was determined from the total number of pollinators found visiting flowers at different time intervals during the months of July, August and September. The average number of flowers visited by each pollinator type in one minute was taken as the measure of pollinator efficiency.

Three specimens of each insect visitor were trapped in a net, anaesthetized, mounted on paper pins and identified with help from the Division of Entomology, Indian Agricultural Research Institute, New Delhi, India. The identity of one pollinator designated P_2 remains to be determined. Two or three insects of each type were examined under a light microscope to estimate the pollen load on different body parts. The results obtained were compared to distinguish pollinators from casual visitors.

2.3 *Pollination*

The aerial CH flowers distributed over the three populations were randomly marked and subjected to one of the following six treatments.

I. Control (natural or open pollination, OP): Flowers were tagged and checked for pollination and fruit set as they occur in nature. Such flowers represented the control.
II. Autogamy (spontaneous selfing, A): Flowers were bagged prior to anthesis and left undisturbed to check for unassisted or spontaneous self-pollination or autogamy.
III. Geitonogamy (manual self-pollination, MSP): Flowers were emasculated and pollinated with pollen collected from the anthers of other flowers of the same plant.
IV. Xenogamy (manual cross-pollination, MCP): Flowers were emasculated and pollinated with pollen collected from plants of a different population. Pollen was collected randomly, maintaining the same source of pollen for plants of each population.
V. Apomixis (AP): Flowers were emasculated and bagged to test for non-pseudogamous apomixis.
VI. Pollen transfer by insects (PTI): Flowers were emasculated, tagged and left untreated to record pollen transfer by insects, if any.

These treatments were tried on 3–5 flowers of 6–10 plants of each population. The total sample sizes per treatment are indicated in table 2.

The anthers along with their filaments belonging both to stamens and staminodes were removed 12–14 h before anthesis. Pollination was undertaken at anthesis when the stigma becomes receptive, anthers dehisce and pollen viability exceeds 90%. Pollen was applied to each stigma twice, with a gap of 15 min between the two applications. The manually pollinated flowers were bagged with butter-paper bags (15 cm × 10 cm). Stigmas of control flowers and those that received MSP and MCP treatments were studied under light and fluorescence microscopes 1/2, 1, 2, 3, 4 and 5 h after pollination (after anthesis in control flowers) to record pollen load and germination. Pollen tube growth was monitored in MSP and MCP flowers under a fluorescence microscope as per the protocol described by Shivanna and Rangaswamy (1992), with minor modifications.

Percentages of fruit and seed set, and seed germination were estimated separately for all treatments. Barring 3 treatments (one per population), the total sample size was 30 flowers per treatment per population. However, fruit and seed sets were not recorded in all 30. Seeds harvested from each treatment were spread for germination on moist filter paper in Petri plates at room temperature (28–32°C). The development of a seedling up to at least 2 cm length was considered an index of successful germination.

Estimation of fruit set in nature was based on the assumption that all the bisexual flowers inside the aerial spathe are capable of setting fruit.

The cumulative relative fitness method (*see* Culley 2000 for details) was utilized to measure the mating-type and floral-type differences in *C. benghalensis*. For this purpose, relative fitness values were first calculated for three stages in the life cycle – fruit set, seed set and seed germination. The three were assumed to be independent of each other and chosen because of their overall importance in the life cycle of a plant. Cumulative relative fitness was obtained by multiplying the relative fitness values for these life cycle stages. Relative fitness values were calculated as:

1. for mating-type differences;

$$\frac{\text{Fitness of selfed CH progeny (treatment III)}}{\text{Fitness of outcrossed CH progeny (treatment IV)}}$$

2. for floral-type differences;

a. $$\frac{\text{Fitness of aerial CL progeny}}{\text{Fitness of CH progeny (treatment II)}}$$

b. $$\frac{\text{Fitness of subterranean CL progeny}}{\text{Fitness of CH progeny (treatment II)}}$$

c. $$\frac{\text{Fitness of subterranean CL progeny}}{\text{Fitness of aerial CL progeny}}$$

A value of 1 indicates equal fitness and values close to 1 denote similar fitness. Inbreeding depression was calculated as 1– cumulative relative fitness for formula 1. A value of zero indicates the absence of inbreeding depression, positive values indicate that selfed progeny performed better than outcrossed ones, and negatives values the opposite (*see* Culley 2000).

2.4 *Data analyses*

Arcsine transformations were made to normalize some of the data before applying one-way and two-way ANOVAs (Sokal and Rohlf 1973). One-way ANOVA was conducted

(1) to analyse the frequency of normal and variant CH flower types among plants of the three populations;
(2) to compare the percentage of pollen germination on the stigmas following treatment VI with those of the control; and
(3) to determine the magnitude of difference between the maturation time of the male and female phases in variant 1 flowers. Pair-wise analysis between JU1 and JU2; JU1 and JU3; JU2 and JU3; was done by using the *t*-test.

Two-way ANOVA was employed to determine:

(1) the effect of flower count per aerial spathe and sex expression on the type of population;
(2) the effect of pollen source (self, cross) and population type on the percentage (a) fruit set, (b) seed set and (c) seed germination;
(3) the impact of time and flower type (bisexual, variant and emasculated bisexual) on (a) the frequency of pollinator visitation, and (b) efficiency of different pollinators; and
(4) the comparative efficiency of pollinators on sunny and cloudy days.

3. Results

3.1 *Variation in the frequency of different types of flowers*

Plants of *C. benghalensis* are weakly andromonoecious. The ratio of male to bisexual flowers per plant averages 0.21 (0.09–0.4) and that of CH to CL flowers averages 1.6 (0.78–3.8). These estimates exclude flowers borne underground.

Subaerial and subterranean spathes always have a single flower each. The former is always bisexual and CH, and the latter CL. On the contrary, spathes of the aerial branches vary in the number and sex expression of flowers. Depending upon the stage in the life cycle of a plant at which the spathe differentiates, it will have 1, 2, 3 or 4 flowers (figure 1). Spathes that differentiate first are larger and bear four flowers, one male and three bisexual. The youngest bisexual flower of the spathe is mostly CL while the other

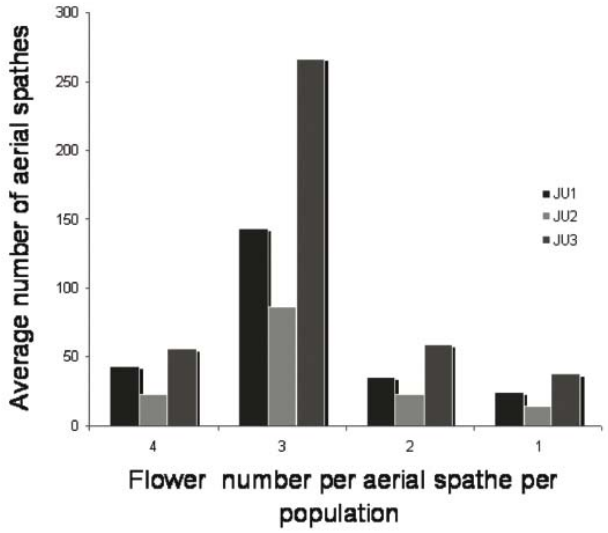


Figure 1. Average number of aerial spathes with variable flower count per plant in the three populations

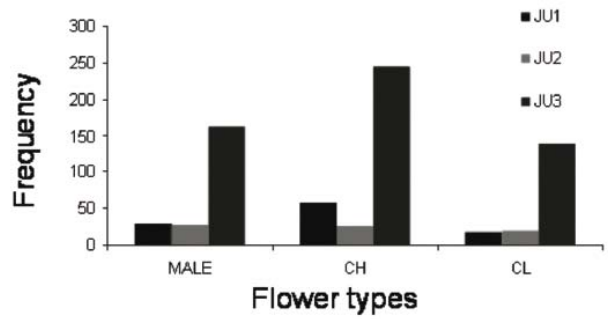


Figure 2. Frequency of male, chasmogamous (CH) and cleistogamous (CL) flowers in aerial spathes per plant in three populations, namely JU1, JU2 and JU3.

two are CH. Spathes that differentiate late are smaller, with only one or two bisexual flowers each; male and CL flowers are lacking.

The three populations revealed significant differences in the frequency of male, CH and CL flowers ($F_{(2,4)}$ =37.25; P<0.01) in their aerial spathes. However, within populations, the three floral types remained consistent ($F_{(2,4)}$ =3.95; P>0.01, figure 2).

3.2 *Floral biology*

In the majority of aerial spathes belonging to 75 plants (25 from each population), the male flower is the first to bloom. Occasionally (228 out of 1844 spathes), a CH flower blooms along with the male flower. Very rarely (in 31 out of 1844 spathes) does the CH flower bloom first. Whatever their structure, all flowers are ephemeral. They open fully at dawn and by the afternoon their petals senesce and shrivel, irrespective of whether the flowers have been pollinated or not (personal observation, unpublished).

Flowers of the aerial spathe bloomed within 1–3 days in acropetal succession. The subaerial flowers bloomed simultaneously or immediately after the aerial ones.

Anthesis and anther dehiscence were synchronous in CH flowers. All the anthers, yellow as well as light grey, dehisced along a lateral longitudinal slit. Close proximity of the anthers with the stigma at the time of floral expansion (figure 3a) ensured transfer of self-pollen to the already receptive stigma. This was verified by the heavy pollen load on these stigmas. Following self-pollination, the anthers and stigma diverged by more than 5 mm (figure 3b). No significant interpopulation difference was observed in this regard ($F_{(2,74)}$ = 1.298; P>0.01) indicating a uniform preponderance of such flowers across the three populations (table 1).

Some CH flowers (referred to as variant 1) are weakly protogynous and herkogamous. Their anthers dehisced 2–45 min after the flowers opened and the stigma was receptive. In the fully opened flowers, the anthers and stigma were 5 mm or more apart, leaving no chance for self-pollination. Flowers of this type constituted 9.5%, 10.3% and 13.1% of the total flowers produced by plants of the JU1, JU2 and JU3 populations, respectively. The difference in the frequency of these flowers among the three populations is statistically significant ($F_{(2,57)}$ =3.158; P<0.05). Herkogamy, i.e. difference in time between the maturation of the male and female phases, was also statistically significant ($F_{(2,163)}$ =3.0107; P<0.05). However, it was more pronounced among plants of JU1 ($\bar{X}$=17±1.9 min) than those of the other two populations (JU2, $\bar{X}$=12.2±1.2 min and JU3, $\bar{X}$=12.8±1.002

Table 1. Frequency of occurrence of aerial CH flower types in the three populations

Flower type	Average flower number per plant in each population		
	JU1	JU2	JU3
Normal	68.96±4.9*	81.16±5.89	76.11±6.88
	(29–111)**	(34–133)	(32–203)
	N = 25	N = 25	N = 27
Variant 1	7.8±1.04	10.2±1.75	12.8±1.61
	(1–18)	(1–29)	(1–21)
	N = 18	N = 20	N = 13
Variant 2	5.2±1.28	7.5±1.50	8.5±2.08
	(1–22)	(1–23)	(4–31)
	N = 20	N = 18	N = 18

*mean ± SE; **range; *N,* number of plants

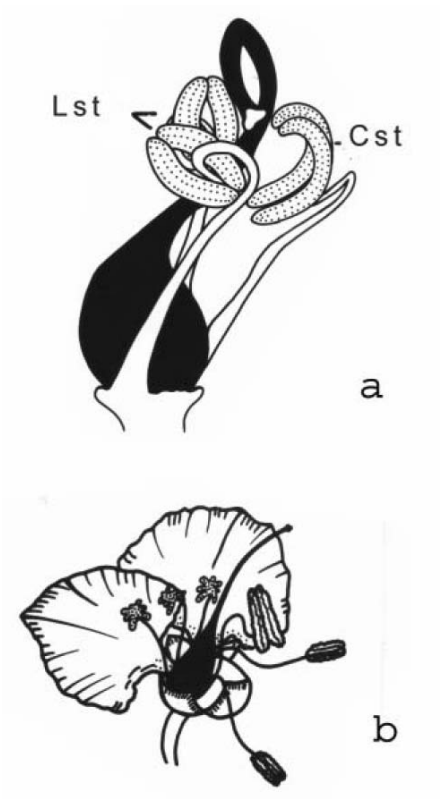


Figure 3. (a) Diagrammatic representation of flower bud depicting the relative position of the two types of fertile anthers and stigma x 22. Note that sepals, petals and three staminodes have been removed. (b) An open flower x 5.4.

min). Results of the *t*-test indicate that this difference between plants of JU1 and JU2 ($t_{(88)}$ =20.285; P<0.001), and JU1 and JU3 populations was significant ($t_{(112)}$ =2.3402; P<0.05) but the difference between JU2 and JU3 was not ($t_{(128)}$ =0.3806; P>0.01).

In variant 2 flowers, the anthers were sterile. The male sterile flowers constituted 6.3%, 7.6% and 8.73% of the total flowers produced by plants of JU1, JU2 and JU3 populations, respectively (table 1). Male sterility was imposed either by failure of the anthers to dehisce or by their precocious degeneration characterized by discoloration and blackening. Such anthers, squashed in 1% acetocarmine and examined under the microscope, were found to contain only non-viable pollen. These anthers did not reveal any bacterial or fungal infection (personal observation). Although the number of such flowers was higher in plants of the JU3 population, the difference between the other two populations was not significant ($F_{(2,48)}$=0.4834; P>0.01).

Stigma receptivity lasted for 2–4 h after anthesis. Thereafter, the stigma coiled around the staminal filaments. In nature (non-experimental conditions), the stigmas of still-closed flowers carry no pollen, indicating that pollination is not effected prior to anthesis. Anthesis initiated with the expansion of floral parts is accompanied by anther dehiscence and onset of stigma receptivity. Since the anthers and stigma are in close proximity, 1–123 pollen grains get transferred within 30 min from the former to the latter. Some of these pollen grains started germinating almost immediately after settling on the receptive stigma. Pollen deposition on stigma picked up gradually and so did pollen germination. It was maximum 3–4 h after anthesis (table 2). While the majority of flowers (86.6%) were of this type, variants 1 and 2 comprised 7.6% and 5.8%, respectively, of the total number (6710) observed (figure 4).

In emasculated and non-bagged flowers (PTI), stigmas received 10–53 pollen grains, of which 52% germinated.

3.3 *Pollen/ovule ratio and pollen germination on stigma*

Pollen production varied among the anthers of a flower, and also among flowers of different branch systems: the number was highest in male (24, 274) and least in subterranean (6915) flowers (Kaul *et al.* 2002). Ovule number was five in all above-ground and three in underground flowers. The P/O ratio and its logarithmic value varied between flowers of different branch systems. The above-ground bisexual flowers had a higher P/O ratio compared with subterranean flowers (table 3; Kaul *et al.* 2002).

Pollen germination on stigma (PGS) was monitored 4 h post anthesis in all types of flowers. No correlation was found to exist between PGS and log P/O in the aerial flowers. Negative correlation was found between the two in the subaerial and subterranean flowers, but it was not statistically significant ($t_{(6)}$ = –0.38 and $t_{(7)}$ = –1.74, P>0.01, respectively; *see* table 3).

3.4 *Pollination in nature*

Flowers were visited by a variety of hymenopteran insects, namely *Andrena* sp. (P_7) , *Anthophora* sp. (P_8), *Bombus* sp.

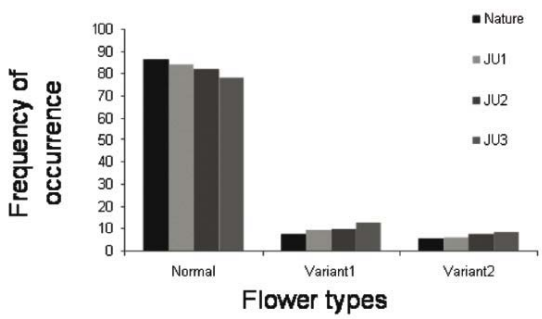


Figure 4. Frequency of occurrence of normal and variant (1 and 2) chasmogamous (CH) flower types in plants of natural and experimental (JU1, JU2 and JU3) populations.

Table 2. Comparison of averages of pollen load, percentage pollen germination and tube length in manually self- and cross-pollinated flowers with the control

Time post pollination (h)	Open pollination (control)		Manual self-pollination			Manual cross-pollination		
	Pollen load	% Pollen germ.	Pollen load	% Pollen germ.	Pollen tube length (μm)	Pollen load	% Pollen germ.	Pollen tube length (μm)
½	22 (0–123) *N*=94	21.1 (0.0–48.7) *N*=94	97.35 (18–311) *N*=20	71.0±5.8* (0.0–100.0)** *N*=20	38.7±2.15 (24.94–54.61) *N*=16	106.53 (21–211) *N*=15	74.3±4.3 (43.8–96.8) *N*=15	43.86±2.58 (33.54–71.81) *N*=15
1	49 (24–147) *N*=15	40.7 (0.0–54.7) *N*=15	101.28 (14–179) *N*=21	84.1±7.3 (0.0–100.0) *N*=7	39.99±1.72 (35.26–61.06) *N*=5	79.75 (15–251) *N*=12	85.2±2.5 (72.1–100) *N*=12	57.19±3.01 (44.72–80.41) *N*=12
2	65 (21–161) *N*=25	67.7 (37.0–82.4) *N*=25	136.9 (18–309) *N*=11	85.5±4.9 (0.0–100.0) *N*=11	61.49±4.73 (39.13–87.29) *N*=10	132.06 (17–437) *N*=15	84.07±2.1 (70.3–100) *N*=15	61.92±3.44 (42.57–93.31) *N*=15
3	89 (27–201) *N*=15	79.8 (68.9–89.7) *N*=15	93.15 (16–172) *N*=13	81.6±2.2 (0.0–95.0) *N*=13	73.1±2.58 (56.33–82.13) *N*=11	112.5 (27–208) *N*=17	85.8±2.6 (70.9–100) *N*=16	78.26±2.58 (49.02–86.0) *N*=16
4	123 (39–351) *N*=41	86.8 (58.2–92.9) *N*=41	76 (19–129) *N*=7	86.4±3.9 (72.4–95.0) *N*=6	76.97±2.58 (67.51–82.13) *N*-=5	113.4 (16–309) *N*=11	86.5±4.5 (53.9–100) *N*=13	81.27±1.29 (76.54–89.01) *N*=12
5	119 (54–246) *N*=46	86.3 (71.2–94.3) *N*=46	111.25 (49–159) *N*=8	86.3±3.4 (73.8–95.6) *N*=5	82.99±3.01 (75.66–94.6) *N*=5	130.2 (78–205) *N*=10	88.1±4.9 (62.5–100.0) *N*=9	86.86±3.87 (64.5–102.77) *N*=8

*Mean ± S E; **Range; *N* sample size.

Table 3. Pollen count, log (pollen/ovule [P/O]) and percentage pollen germination on stigma (PGS)

Type of flower	Pollen count per flower	P/O ratio	Log (P/O)	PGS (%)
Male	24 274	--	--	--
Aerial CH	17 302	3 460	3.539	86.3
Aerial CL	14 623	2 295	3.466	79.2
Sub-aerial	11 723	2 345	3.370	76.4
Subterranean	6 915	2 305	3.362	79.5

(P_9), *Halictus* sp. (P_1), *Nomia eburnigera* (P_3) Cockerell and two other (P_4 and P_6) species of *Nomia* (all belonging to Apidae), *Steganomus* sp. (P_5) (of subfamily Megachilidae) and an unidentified species (P_2) visitor. *Apis cerana indica* (Apidae) foraged the flowers occasionally. In addition, *Ceratina sexmaculata* and *C. heiroglyphica* (Anthophoridae) also visited the flowers intermittently during September. The insects seemed to get attracted to the colourful petals, anthers and stigma. In return, the flowers rewarded the visiting insects with large quantities of pollen. Pollen count, listed separately for different flower types, was fairly high (table 3, Kaul *et al.* 2002).

Insect visits to the flowers extended over a maximum period of 6 h a day. The flowers opened around 6:00 h, the

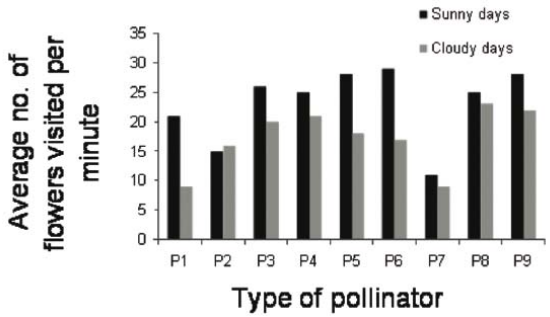


Figure 5. Efficiency of different pollinators visiting flowers of *Commelina benghalensis* on sunny and cloudy days.

frequency of the visitors picked up gradually and peaked between 7:00 and 8:00 h. Some variation was recorded during different months (table 4). Species of *Nomia* and *Steganomus* were the most frequent and brisk visitors. The rate of visitation as well as foraging by insect visitors was significantly higher on sunny days ($F_{(1,8)}$ =14.44; P<0.01). This was true for all the pollinators ($F_{(8,8)}$ =5.138; P<0.025; figure 5). Notwithstanding the differences in their sizes, the behaviour of most visitors was alike. Generally, they alighted on the male flowers first and then moved to the bisexual flowers. Once seated on the flower, the insects first approached the yellow anther, probed it with their proboscis

Table 4. Frequency of insect visitors at different times of the day during different months

Time of visitation (h)	Total number of insect visitors at different times of the day during		
	June	August	September
7:00	22	23	13
8:00	19	18	11
9:00	9	11	13
10:00	8	11	10
11:00	2	3	6
12:00	-	1	-

and legs, and then repeated the same behaviour with the grey anthers. In the process, parts of the insect body got loaded with pollen. Later, when these insects visited another bisexual flower; the ventral side of their abdomen and legs brushed against the stigma, pollinating it in the process. All insects collected from open flowers carried large quantities of pollen on different body parts. The load was highest on the hind legs (377–2855) followed by the forelegs (54–404).

In comparison with normal CH flowers, pollinator visits to emasculated CH flowers (PTI) and variant 2 were significantly low over all time periods (flower type $F_{(2,10)}$ = 10.04 and time interval $F_{(5,10)}$ = 7.88; P<0.01, *see* figure 6). Their efficiency in terms of the average number of flowers foraged per minute was drastically low (flower type $F_{(2,16)}$ = 91.36; P<0.01 and pollinator type $F_{(8,16)}$ = 2.598; P<0.05, figure 7).

3.5 *Pollination treatments*

Plants of *C. benghalensis* were self- as well as cross-compatible. The initial response of self- and cross-pollen on stigma was alike; both took 20–30 min to germinate and produce pollen tubes. Initially, percentages of pollen germination were also comparable (figure 8). Subsequently, cross-pollen germinated in higher numbers, and their tubes grew faster (figure 9; table 2). From among the 30 emasculated and non-bagged flowers (PTI), pistils of only 21 carried pollen. Their pollen load ($\bar{X}$ = 25.19), determined 3 h after anthesis was, however, much lower than that in the control ($\bar{X}$ = 89). The percentage pollen germination was also significantly lower than the control; 27.3–69.2% against 68.9–89.7% in the control ($F_{(1,34)}$ = 90.6; P<0.001).

The pollen transfer experiment was also carried out on plants growing naturally inside the Botanical Garden. About 490 flowers (2 had withered) were randomly selected for the purpose. While 44 pistils carried pollen on their stigma, only 18 showed germination. However, the percentage germination recorded was higher (47.5–82.1%) than among those in the beds.

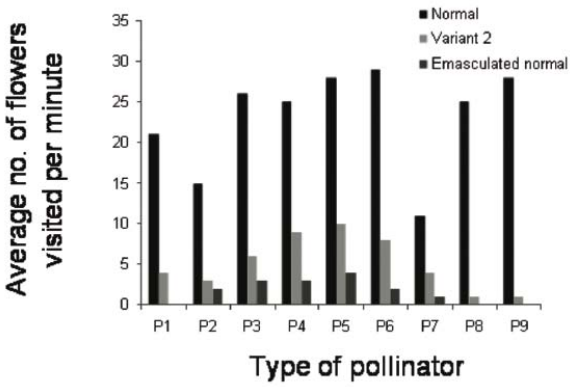


Figure 6. Rate of visitation of different pollinators to normal, variant 2 and emasculated normal chasmogamous (CH) flowers of *C. benghalensis*.

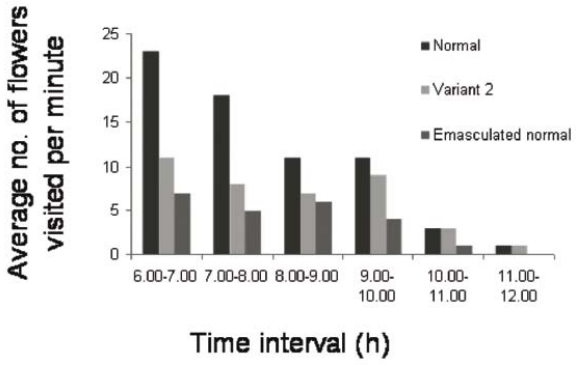


Figure 7. Frequency of different pollinators visiting normal, variant 2 and emasculated normal chasmogamous (CH) flowers during different time periods of the day.

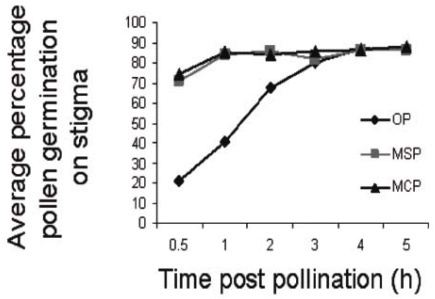


Figure 8. Comparison of pollen germination (in percentage) on open (OP), manual self- (MSP) and manual cross-pollinated (MCP) flowers of *C. benghalensis.*

3.6 *Fruit set*

In nature (i.e. non-experimental conditions), fruit set per plant varied between 45.8 and 90.7 ($\bar{X}$=68.4±3.1%) for aerial, 39.3–100 ($\bar{X}$=60.0±2.9%) for subaerial and 27.3–100% ($\bar{X}$=64.0±2.8%) for subterranean shoots. However,

within a spathe, the number of capsules formed was more or less commensurate with the number of bisexual flowers. One to three capsules developed per aerial spathe, and one each in the subaerial and subterranean spathes. Fruit set on the aerial branches segregated into 72.9±2.4% (62.9–90.5) for CH and 73.9±4.2% (50.0–100) for CL flowers present within the aerial spathes.

Fruit set also varied with pollination treatment (figure 10). Fruit set was 100% following MSP and MCP. It was lower following open pollination in all the three populations. Results of two-way ANOVA highlight the significant impact that pollination treatment had on fruit set ($F_{(4,8)}$ =11.65; P<0.01). The difference was, however, uniform within each of the three populations ($F_{(4,8)}$ =0.495; P>0.01). No fruit set was ever recorded in emasculated and bagged flowers, ruling out the involvement of non-pseudogamous AP in fruit/seed development in the species. In emasculated and non-bagged flowers, the fruit set for JU1, JU2 and JU3 was 66.7%, 73.3% and 70.0%, respectively.

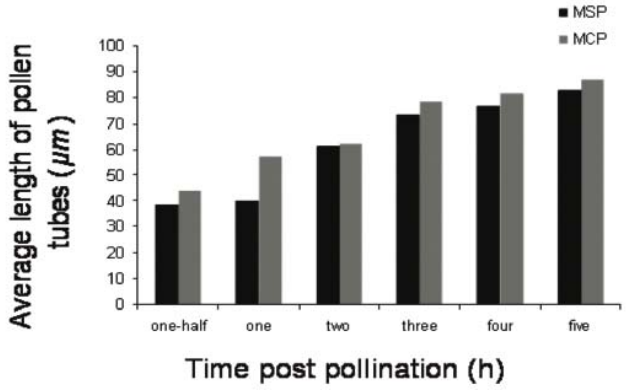


Figure 9. Periodic average length of pollen tubes (μm) following manual self- (MSP) and manual cross-pollination (MCP).

3.7 *Seed set*

The seed set exceeded 50%, irrespective of the pollination treatment used (figure 11). It was, however, significantly influenced by the quality of pollen the stigma received ($F_{(4,8)}$ =34.34; P<0.01) in all populations ($F_{(2,8)}$ =1.104; P>0.01). The number of seeds set per fruit increased considerably after MCP. The seed output following OP was almost comparable with that recorded from MSP and bagging. In variant 2 flowers, though the seed set was the least, it was above 50% (51.3%, 58.2% and 53.2% in JU1, JU2 and JU3, respectively) despite the fact that it was largely or exclusively the result of cross-pollination. In emasculated non-bagged flowers, seed set approached 53% (52.8 ± 6.8%) for JU3 only. Unfortunately, the fruits harvested from JU1 and JU2 were destroyed and could not be compared.

3.8 *Seed germination*

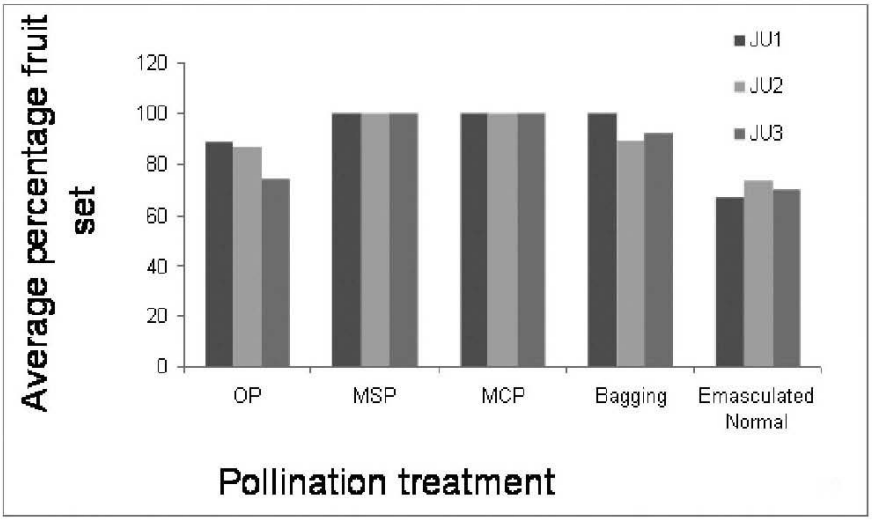
Seeds harvested following different pollination treatments were tried for germination. The highest germination (93.6–96.4%) was recorded for MCP seeds and the least (64.4–72.5%) in those formed after bagging (figure 12). The difference in percentage seed germination was statistically significant ($F_{(2,6)}$ =36.11, P<0.01) across all populations ($F_{(2,6)}$ =0.953, P>0.01). However, seeds resulting from treatment VI showed 90.3% germination (JU3).

Table 5. Relative fitness values in *Commelina benghalensis* to measure mating-type and floral-type differences

Population	Relative fitness values using percentage			Cumulative relative	Inbreeding depression
	Fruit set	Seed set	Seed germination	frequency (crf)	(1–crf)
Mating-type differences					
JU 1	1	0.816	0.971	0.7923	0.207
JU 2	1	0.869	1.014	0.8807	0.119
JU 3	1	0.879	0.96	0.8438	0.156
Floral-type differences*					
a. JU 1	0.739	1.038	0.994	0.7241	-
JU 2	0.829	1.086	1.0	0.9003	-
JU 3	0.801	0.976	0.888	0.6942	-
b. JU 1	0.64	0.929	1.063	0.632	-
JU 2	0.718	0.972	1.126	0.786	-
JU 3	0.693	0.874	1.0	0.606	-
c. -	0.866	0.895	1.126	0.873	-

* *See* Materials and methods for details.
crf, cumulative relative frequency

(10)



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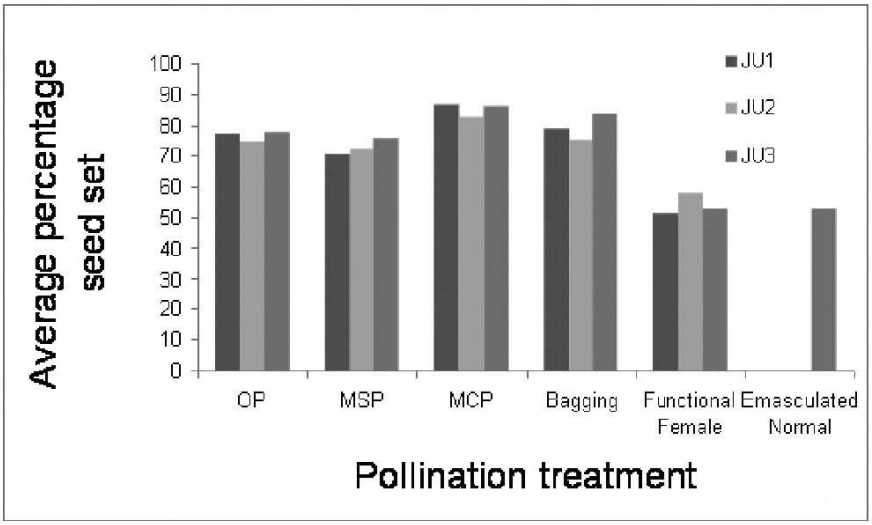
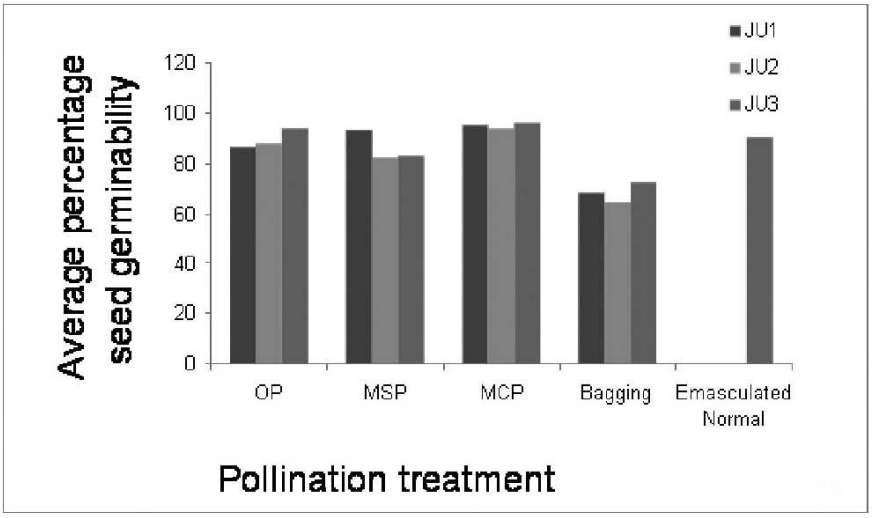


(12)



Figures 10–12. The effect of various pollination treatments on percentage fruit (**10**) and seed sets (**11**) and seed germination (**12**) in three populations; JU1, JU2 and JU3 of *Commelina benghalensis.* OP, open pollination; MSP, manual self-pollination; MCP, manual cross-pollination.

3.9 *Mating-type and floral-type differences*

Individual fitness components for the three life cycle stages of manually selfed and manually outcrossed progeny of the three populations were similar, with values close to or equal to 1 (*see* table 5). The cumulative relative frequency showed a similar trend. The values for inbreeding depression were positive, indicating that the outcrossed progeny performed better than the selfed one, with an overall low level of inbreeding depression. Fitness differences associated with flower type (CL vs CH) across the three life cycle stages did not vary much. Apart from a few values around 0.1, most others were close to 1, indicating that both cross-types had more or less similar fitness. The same was true of aerial CL and subterranean CL flowers.

4. Discussion

Commelina benghalensis is distinct from other species of the genus *Commelina* for the foliar and floral variability that it exhibits. An individual plant has three branch systems, and structurally 4 and functionally 5 types of flowers. Floral diversity of this magnitude creates the impression that *C. benghalensis* has a mixed mating system. To what extent this holds true in this weedy species is likely to emerge from the discussion and analyses of the results of observations and experiments conducted by the authors for three consecutive years, between 1996 and 1998.

The flowers are of CL and CH types. The latter are uni- or bisexual. Unisexuality is due to structural reasons in the majority of flowers and functional reasons in some flowers. Every plant bears unisexual and bisexual flowers in its aerial spathes. The unisexual flowers are structurally male in that they uniformly lack a pistil. In the functionally unisexual flowers, stamens are sterile while the pistil is functional – a feature not common to every plant.

Thus, all plants of *C. benghalensis* bear male and bisexual flowers and are therefore andromonoecious. Considered a contrivance for outcrossing, the presence of staminate flowers does not preclude the possibility of selfing in bisexual flowers unless it is accompanied by spatial or temporal separation of the two phases (Primack and Lloyd 1980; Muller 1983; Koul 1985; Emms 1993). Evolutionarily, it is argued that the staminate flowers are either a result of hemisterilization of bisexual flowers or increase in the absolute number of staminate flowers in the inflorescence. There is evidence in favour of both hypotheses (Carr *et al.* 1971; Carr and Carr 1972; Primack and Lloyd 1980). As far as *C. benghalensis* is concerned, there is sufficient evidence to suggest that historically, the male flower was bisexual from which female function has been lost (V Kaul, unpublished). It is not uncommon to still find many male flowers carrying a pistillode that functions once in a while and gets transformed into fruit (V Kaul, unpublished).

In strong contrast, cleistogamy ensures selfing (Kannenberg and Allard 1967; Levin 1972; Lord 1981; Schoen 1984; Le Corff 1996; Culley 2000; Culley and Klooster 2007). CL flowers are produced on both above- and underground shoots in the species under study. Despite their practising the same mode of pollination, they exhibit several differences. These are associated with the differences in their location on the plant, overall size, P/O and sex allocation ratios, seed count and/or age. Variation in P/O ratios among flowers of the same plant of *Lamium amplexicaule* has been explained on similar grounds (Lord 1980). In *C. benghalensis*, subterranean flowers differentiate very early in the life cycle, when the seedlings have 5–7 leaves (Kaul 1998; Kaul *et al.* 2000, 2002) and the resources available are few. Therefore, these flowers are small with less P/O and sex allocation ratios (*see* Kaul *et al.* 2002).

Aerial CL flowers exhibit a male-biased sex allocation ratio (*see* Kaul *et al.* 2002), despite the general view that a higher selfing rate leads to a female-biased ratio. This anomaly can be attributed to the fact that in the not too distant past, these flowers may have been CH. Failure of anthesis was probably selected to ensure fertilization and seed set. Regression in size and P/O ratio are subsequent changes that have followed the shift from chasmo- to cleistogamy. This reduction is not yet complete. Furthermore, greater fruit and seed set in aerial CL flowers is possibly a reflection of the previous heterotic effect.

Performance of self CH progeny and outcross CH progeny is almost similar in all fitness measures. This is indicative of the low levels of inbreeding depression in *C. benghalensis.* It would therefore be safe to conclude that selfing does not have a negative impact on the fitness of the species. These results agree with the prediction that the level of inbreeding depression should be considerably low if cleistogamy is to be maintained (Schoen and Lloyd 1984). Results similar to ours have been reported by Culley (2000) in *Viola canadensis*.

In CH flowers that are protogynous (variant 1), the stigma becomes receptive and therefore available for pollination before the dehiscence of anthers. Spatial and temporal separation of the male and female reproductive phases in these flowers enhances the chances of cross-pollination. But since male flowers are the first to open, therefore, geitonogamy cannot be ruled out completely. The same is true of variant 2 flowers, which are functionally female due to the sterility of their anthers. Therefore, such flowers indulge in geitono- and allogamy. Pollen transfer from male and bisexual flowers of one plant to female and bisexual flowers of the same or another plant is carried out by insects which get attracted to the colourful corolla, stamens and stigma, and are rewarded with copious pollen.

Events of floral biology demonstrate that the CH flowers receive a mixture of self- and cross-pollen. Intrafloral

pollination precedes cross-pollination in the majority of flowers. This is ensured by the close proximity between the dehiscing anthers and pistil, and synchrony between pollen dispersal and stigma receptivity. As the flowers open fully, the already self-pollinated stigmas get loaded further with cross-pollen brought from male flowers of the same or different spathes by the visiting insects. That insect visitors to *C. benghalensis* flowers carry pollen from one flower to another is testified by microscopic examination of the insects, which carry a lot of pollen on their body parts; pollen load (10–53) on the stigmas of emasculated flowers (treatment VI) and presence of *C. benghalensis* pollen on stigmas of *C. caroliniana* in sympatric populations of the two species and vice versa (Kaul 1998; Kaul and Koul 2008). The basic contention is that, if pollinators are able to transfer interspecies pollen, there is every likelihood that intraspecies pollen is also transferred.

Using Plitmann and Levin's (1990) indices of P/O and PGS, *C. benghalensis* falls in the category of self-compatible taxa with a 'mixed' breeding system.

Whether mixed pollination leads to mixed mating is not easy to establish in the present case, because the self- and cross-pollen could not be distinguished for want of specific markers. However, some indirect evidence is of significance. Studies on floral biology reveal that intrafloral pollination precedes cross-pollination. This puts self-pollen at an advantage for fertilizing ovules. The only factor that is likely to neutralize this advantage to some extent is the faster rate of germination of cross-pollen and faster growth of their tubes (*see* table 2). In pollination experiments conducted separately with self- and cross-pollen, the latter exhibits superiority in terms of time taken to germinate and rate of pollen tube growth. Whether or not they retain this superiority even in mixed pollination is difficult to assess. In case they do so to the extent of neutralizing the advantage of prior pollination by self-pollen, it will impose cryptic self-incompatibility (Snow and Spira 1991). Such pistils mostly yield out-crossed seed (Weller and Ornduff 1977; Bowman 1987; Jones 1994; Smith-Huerta 1996). One cannot rule out the competition between self- and cross-pollen and their pollen tubes, which is inherent in natural mixed pollination. As of now, it is not possible to say with certainty whether or not mixed pollination of CH flowers ends in mixed mating. It is, however, clear from table 2 that outcross pollen tubes were consistently longer over several time periods. It is likely that this superiority of cross-pollen is maintained even during mixed pollination.

The average fruit set (100%) in manual self- and manual cross-pollination was surprisingly higher than that in control (88.5%, 86.9% and 74.2% in JU1, JU2 and JU3, respectively). Emasculated flowers yielded fewer fruits, while natural variant 2 yielded fewer seeds than the control. Unfortunately, pollinators avoided the emasculated flowers because they forage for pollen. Non-availability of pollen not only reduced the number of visitors, they also drastically cut down the duration of individual visits. The reduced reproductive output could thus be due to pollen limitation caused by low pollinator visitation. Results of two-way ANOVA indicated significant differences. As a result, the stigmas are likely to have received a suboptimum pollen load leading to little or no competition. Even low-quality pollen grains get the opportunity to sire ovules. Such fertilization is likely to result in the production of seed with poor germination.

No apparent floral-type differences were detected (*see* table 5). However, the progeny obtained from each type need to be raised and compared to conclude whether the differences in life-cycle traits are due to floral-type (CH vs CL) or mating-type (self vs outcross) differences. Such studies would help in determining the genetic basis of cleistogamy in this species.

To sum up, it follows that floral heteromorphy of *C. benghalensis* leads to variability in the breeding system. An individual plant of the species practises auto-, geitono- and allogamy through different floral types, each specialized by specific functional or structural contrivance for a particular breeding system. The ability of a plant to use all types of sexual systems to reproduce puts it at an advantage. For weeds such as *C. benghalensis*, mixed mating imparts the flexibility to accumulate as well as fix variation. This dual advantage helps them to invade new niches and colonize them.

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