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POLLINATION ECOLOGY AND BREEDING SYSTEM OF *ECBOLIUM LIGUSTRINUM* (ACANTHACEAE): A TRANSITION FROM AUTOGAMY TO XENOGAMY THROUGH SPECIALISED PLANT-POLLINATOR INTERACTIONS

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In view of the ongoing rarity of *Ecbolium ligustrinum* there is an urgent need for conservation of the species. For this, a detailed work was carried out regarding the untold story of its reproductive ecology. The work was done for three consecutive years (2015–2017) at Midnapore, West Bengal over three different populations collected from three different areas of West Bengal. Field data were also recorded from these three wild populations. The species produces gullet flowers with bi-labiate corolla having long slender tubes. The flowers exhibit one day of longevity. The flowers are visited by 10 species of insects. Among those, four species viz. *Eristalis tenax*, a Dipteran member and three ant species of Hymenoptera such as *Camponotus* sp., *Formica* sp. and *Monomorium* sp. are the effective pollinators. As per pollination efficiency, *Eristalis tenax* (PE*i* = 0.76) is the most successful one. The flowers are shortly protandrous (dichogamous) and passed by three distinct reproductive (male, bisexual and female) phases. The breeding system clearly depicts that the species is facultatively xenogamous supported by myophilous mode of pollination. However, geitonogamous type of pollination is also observed through myrmecophily, an atypical instance found in plants. Lastly, the plant retained some sort of autogamy through 'fail-safe' mechanism of pollination, an adaptation which might be developed in absence of pollinators. Therefore, undoubtedly it can be concluded that *E. ligustrinum* is a partially self-incompatible (ISI = 0.27) species having a mixed mating system, adapted for xenogamy through specialised mode of plant-pollinator interactions.

Key words: allogamy, myophily, myrmecophily, out-crossing, self-incompatibility, self pollination

INTRODUCTION

In order to suggest the suitable methods regarding survivability and conservation, information about reproductive ecology of rare plants is indeed crucial (Rodriguez-Perez 2005). Such kind of studies may facilitate to recognise the factors affecting the reproduction of individual species and the successive maintenance of populations. Various works have advocated that the maintenance of plant populations is strongly controlled by both demographic (reproductive success) and genetic (inbreeding depression, evolutionary potential)

mechanisms (Frankham and Ralls 1998, Saccheri *et al.* 1998). Pollination plays a key role for successful reproduction of plants, which is often dependent on mutualistic interactions with animals. Pollination ecology can provide almost unparalleled insights into evolution, ecology, animal learning and foraging behaviour. Flowers are often visited by a number of insect species, however, all such floral visitors of the plant are not essentially its pollinators (Inouye 1980*a*, *b*). Animal visitors are very effective particularly in pollen limited, certain specialised and generalised flowers. In course of evolution such flowers become largely dependent on insect pollinators for their reproductive success (Gómez 2002, Herrera 1988). Breeding system represents the mode of transmission of genes from parental generation to their progenies by means of sexual reproduction. The event of sexual reproduction is extremely variable and flexible. As per their necessity, present situation current status and availability of effective pollinators, plants have evolved a wide range of options starting from autogamy to obligate xenogamy. The system is responsive to different selection pressures, both intrinsic and environmental. Occasionally, truly xenogamous species tend to retain autogamy and/or geitonogamy for their sustenance in absence of pollinators. That could be an adaptive survival strategy for such out-crossed species and considered an important co-evolution as mixed mating system in flowering plants (Eckert *et al.* 2006, Kalisz and Volger 2003). However, extreme selfing may restrict to maintain the genetic variability of plants that causes inbreeding depression. Thus, it diminishes the evolutionary potential of such population and augmenting the genetic drift (Buza *et al.* 2000, Charlesworth and Charlesworth 1995). Many studies have suggested that rare plants exhibit slightly higher levels of self-compatibility than common plants (Saunders and Sedonia 2006). Thus the system is dynamic.

Ecbolium ligustrinum (Vahl) Vollesen (= *E. linneanum* Kurz var *dentata* Clarke, *E. viride* (Forssk) Alston) is a well-known medicinal plant for its diverse ethno-medicinal properties (Datta and Maiti 1968, Kirtikar and Basu 1987), such as positive cardio-vascular activity (Asolkar *et al.* 1992), free radical scavenging activity (Ashoka Babu *et al.* 2011), and anti-cancerous properties (Chaudhuri and Murugan 2012). The species is known to distribute in the Arabian Peninsula, Somalia, Kenya and tropical Asian countries including India often in isolated patches (Cecilia *et al.* 2012, Hooker 1885). In West Bengal, the plant is reported to grow wildly throughout the warmer areas (Prain 1903). However, recent survey entails that the species is becoming rarer and going to be regionally threatened. Therefore, conservation of the species is much needed. Based on different experimental conditions and observations, we addressed the following questions: (i) Is *E. ligustrinum* self-compatible? (ii) What type of breeding system does the species exhibit? (iii) Who are the potent pollinators of the species? (iv) What is the mode of plant-pollinator interactions exhibited by the species, generalised or specialised?

MATERIAL AND METHODS

Plant species and study site. The study was conducted on *E. ligustrinum* in three wild populations (germplasms) of three different locations *viz.*, Midnapore Sadar area (22° 43′ 09″ N; 87° 32′ 15″ E) of West Midnapore District, Chandannagar area (22° 51′ 53″ N; 88° 21′ 47″ E) of Hooghly District and Monteswar Village (23° 25′ 21″ N; 88° 06′ 27″ E) of East Burdwan District, West Bengal, India (Fig. 1). Each natural population consists of 30–57 individuals restricted in 4–5 m^2 area. The breeding experiment was carried out on 30 individuals for each three populations grown at different plots in the research garden at Vidyasagar University campus. In each plot, 30 plants were grown in five rows, two metres apart and in each row six plants were planted with a gap of two meters. Three different plots were separated from each other by 10 m distance.

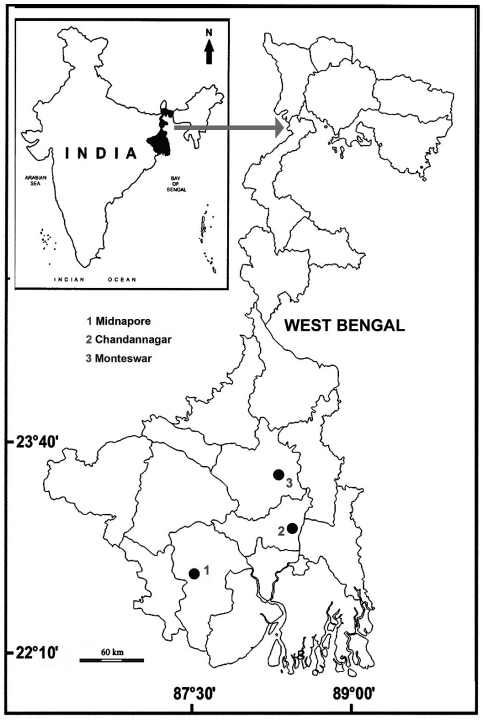

Fig. 1. Map: Geographic location of our study area and sampling sites. 1 = Midnapore town of West Midnapore District, 2 = Chandannagar area of Hooghly District and 3 = Monteswar village of East Burdwan District of West Bengal, India (Study site: Midnapore. Sampling sites: Midnapore, Chandannagar and Monteswar)

Flowering phenology and floral display size. Detail record of flower production of three wild populations at 7 days interval throughout the entire flowering season was noted for three consecutive years (2015–2017). The floral display size (mean no. of open flowers in any day of flowering season per plant) and flowering strategy was deduced as per the terminologies used by Gentry (1974) and Opler *et al.* (1980).

Flower architecture and floral events. Floral biology including flower morphology and different floral events (flower opening, anther dehiscence, onset of stigma receptivity and flower longevity) were studied in detail using a 10× hand lens, a Leica DMLB compound bright field light microscope and a Leica WILD M3B Stereo-binocular microscope. The observations were recorded on 180 flowers belonging to 15 individuals of three different populations through random selection. To identify the olfactory attractants sensible to human if any present in flowers, "closed vial

test" (Faegri and van der Pijl 1979) was performed. For detail analysis of floral volatiles, freshly opened flowers were put into a 20 ml Thomson Fisher Scientific glass vial in sealed condition and kept for 6–12 h at room temperature. The vial was placed into TriPlus RSH, an Autosampler for GC-MS analysis to inject the sample into a 1300 Gas Chromatography (GC) by a split less injector. This GC was connected to an ISQ QD single Quadrupole Mass Spectrometer (MS). Volatile compounds were separated in GC by using an EC-WAX column and helium as carrier gas. An empty glass vial was also used for the analysis as control. Volatile compounds were identified by comparing the SI, RSI values and mass spectra with the computerised MS-data base using NIST 2017 library. Secreting tissues were identified through sectional anatomy of the floral parts, which are again confirmed by neural red staining (Esau 1965). To confirm the presence of soluble sugar in the secretion, anthrone test was performed (McCready *et al.* 1950). To estimate the volume of nectar accumulation and solute concentration, flowers were bagged before opening and remain undisturbed from all visitors. Nectar samples were measured at 2 h intervals from commencement of flower opening up to corolla abscission (05.00 am – 5.00 pm). For collection of nectar 5 μl calibrated micro-capillaries (microcaps, Drummonds) were used. The concentration of solutes (%, Weight/Weight) was measured by using a Hanna Digital Refractometer (Model No: H1 96800).

Pollen production and pollen dispersal. Pollen production was measured by direct counting method (Cruden 1977). The percentage of pollen dispersal from anthers was measured from time to time since completion of their dehiscence at an interval of one hour till the anthers become empty. Estimation was done in each hour on 20 anthers once in each month of the flowering season and the same procedure was repeated for several times (Ghosh and Pal 2017). Pollen dispersal percentage was determined based on the mean value of initial pollen content in both the anthers of a single flower as deduced during the study of pollen production and the remnant pollen in anthers at particular time.

Stigma receptivity, pollen fertility and pollen-ovule ratio. Onset of stigma receptivity and its longevity was primarily judged by studying the detail morphological changes of stigma. This was also confirmed by in vivo pollen germination experiment as well as through dehydrogenase activity test by H_2O_2 (Galen *et al.* 1986, Zeisler 1933) at an interval of 30 min. The time of pollen deposition on the stigmatic surface was ascertained by examining the excised stigma during the floral longevity at an interval of one hour (Kearns and Inouye 1993). For staining purposes, lactophenol cotton-blue solution (0.08%) was used to determine the percentage of fertile pollen grains per flower (Darlington and La Cour 1960). Pollen fertility was judged in every 30 min interval. To find out the pollen-ovule ratio the mean number of pollen grains produced by a flower was divided by the mean number of ovules present in the ovary (Cruden 1977, Urbanska 1989).

Floral visitors. The observations were made from three different wild populations of three different study sites during the entire work period. A detail record regarding incidence of visitation and activity of each visitor was considered during the period of anthesis. The floral visitors were captured at 1 hour interval and critically observed by 10× hand lens. Altogether 200 specimens were captured and preserved separately in dry condition as well as in 70% ethyl alcohol. The specimens were identified with the help of entomologists, Zoological Survey of India, Ministry of Environment and Forestry, Govt. of India and with the help of available literature. The visitors were carefully studied under a Leica WILD M3B Stereobinocular microscope to detect the presence of pollen (if any) on their body parts and mode of pollen deposition. On the basis of these observations and their activities floral visitors were classified into three different categories *viz.*, (1). Category-I: Pollinator (2). Category-II: Pollen thief and (3). Category-III: Nectar forager (Kundu *et al.* 2018).Visitors' frequency was also recorded in percentage all through the day. Pollination efficiency was evaluated by using Spear's pollination efficiency index (Spears 1983): PE*i* = (P*i* – *Z*) / (*U* – *Z*), where P*i* = average no. of seeds in the fruit that received only one visit by the pollinator '*i*'; Z = average no. of seeds in the fruit that received no visit by the pollinator '*i*'; U = average no. of seeds in the fruit that received unrestricted visits.

Breeding system. Following experiments were performed in the research garden of Vidysagar University at every 15 days interval during entire flowering season: (i) autogamous pollination (ii) geitonogamous self-pollination (iii) cross pollination (iv) open pollination and (v) emasculation. To study the presence of self-dependent autogamy, flowers (n = 10 for each population) were bagged with fine silken cloth from flower opening up to senescence and fruit set was counted. For geitonogamous self-pollination and cross pollination, emasculation was done carefully just before the opening of flowers and then bagged with fine silken cloth. During midday, when stigmas of the emasculated flowers became receptive those were pollinated artificially by the pollen grains from the dehisced anthers of the same plant (geitonogamy) as well as from different plant (xenogamy). After pollinating the stigmas the emasculated flowers were bagged again. The experiments were performed on flowers (n = 10 for each population) for geitonogamous and xenogamous pollination in all possible combinations using flowers from different populations. To observe the situation in open pollination, flowers (n = 10 for each population) remained undisturbed up to fruit and seed set. To study agamospermy, flowers were emasculated followed by bagging.

We also used the index of self-incompatibilty (ISI) (Zapata and Arroyo 1978) for calculation of self-incompatibility using following formula:

ISI = (fruit set in self-pollinated flowers) / (fruit set in cross-pollinated flowers)

Fruit set was assessed on the basis of percent fruit set/pollination treatment. The species is considered fully self-compatible when the ISI is 1 or > 1, partially self-incompatible when ISI is > 0.2 but < 1 and fully self-incompatible when ISI is < 0.2 or 0.

An auto-fertility index (AFI) was also calculated from fruit set and seed production through autonomous self-pollination/hand self-pollination (Eckert *et al.* 2006, Lloyd and Schoen 1992). A Sony DSC-H70 digital camera and Nikon D5000 SLR camera are used during field photography.

Statistical analyses. Statistical analyses of the data were conducted to obtain arithmetic mean and standard error. Analysis of variance (ANOVA) was performed to evaluate the differences between fruit/flower and seed(s)/flower for different pollination treatments in different populations followed by Duncan's multiple range test (DMRT) and $P \leq 0.05$ was considered statistically significant.

RESULTS

Morphology of flowers and inflorescence

Ecbolium ligustrinum produces moderate-sized bilabiate flowers with bluish green corolla in axillary and terminal spikes (Fig. 2A). The length of individual spike ranges from 5.2 cm to 15.1 cm and number of flowers varies from 14 to 51. A short spike with a length of 5.2–7.8 cm contains 14–26 flowers and takes 26–37 days for complete opening of all the flowers, while a medium sized spike (8.1–11.8 cm) shows 28–39 flowers and takes 43–61 days to bloom all the flowers. A large spike (12.0–15.1 cm) contains 40–51 flowers and takes a much longer period of time (64–77 days) to reach in its full blooming stage. Total number of inflorescence per plant varies from 4 to15, though 10 to 14 inflorescences are more common. Individual flower is subtended by a foliaceous green bract. Bracts are persistent in nature even after maturation and dehiscence of fruits.

Flowering phenology and floral display size

The species flowers once in a year (June to December) with 1–14 flowers open per day per inflorescence. The species is perennial and remains unbranched in its first reproductive year and produces only 4–6 short to medium sized spikes. In subsequent years, the number of lateral branches increases gradually. Therefore, daily flower production as well as floral display size of the species varies widely with the age of the plant. The individual plant produces 1.63 ± 0.33 (mean ± SE, n = 90) flowers per day and altogether 351 ± 18.68 (mean ± SE, n = 90) flowers per flowering season.

Flower architecture and floral events

The plant produces a typical tube flower with bilabiate corolla lobe (Fig. 2C). Flowers are complete, bisexual, zygomorphic and hypogynous. The persistent, campanulate, gamosepalous calyx consists of five greenish sepals, proximally fused up to ± 1/5th (1–1.5 mm) of their length (Fig. 2B). In open

Fig. 2. Different floral events, reproductive parts and pollinators of *Ecbolium ligustrinum*: (a) = an inflorescence with terminal and axillary spikes; (b) = calyx; (c) = a typical flower; (d) = bluish corolla of the flower created a sharp contrast against the anther lobes; (e) = bilobed stigma with pollen grains; (f) = commencement of anther dehiscence; (g) = *Eristalis tenax*; (h) = *Formica* sp.; (i) = *Eristalis tenax* with adhered pollen grains on its dorsal surface of body parts

flower condition a slimy white secretory substance comes out from cells of the ventral surface and is stored in the calyx cup. The tissue responsible for such secretion was visualised all through the calyx under neutral-red staining.

The greenish, gamosepalous, bilabiate corolla with whitish, slender corolla tube is composed of five petal lobes organised into a two-lipped structure (Fig. 2C). The two posterior petal lobes are linear in shape, 15–17 mm long and 1.5–2.0 mm wide, and fused throughout their inner margins leaving only a small (±1 mm) terminal cleft giving rise to a bifid appearance. The anterior lip is composed of three petals joined together forming a landing platform for the incoming visitors. In a freshly opened flower the corolla lobes remained greenish and with time gradually transformed into bluish (Fig. 2D).

Male reproductive organ consists of two epipetalous stamens. The free portions of filaments are 6–9 mm long, projecting the terminal anthers beyond the throat of the corolla tube (Fig. 2C). The anthers are basifixed, bilobed with longitudinal dehiscence slit facing interior of flower i.e. introrse (Fig. 2C, D). Gynoecium consists of a superior ovary which is obconical, light green, 3.0–3.5 mm long and 1.5–2.0 mm wide with a basal annular disc. Upper portion of the ovary consists with multicellular hairs. Ovary is two chambered with two anatropous ovules in each chamber. Basal disc of the ovary bears large number of secretory glands. The style is slender, terminal, whitish, 38–42 mm long and flexible in nature. The distal part of the style beyond the corolla tube is about 6–9 mm long running through the middle of the two anthers (Fig. 2D) along their ventral surfaces. The stigma is bilobed, spathulate forming a central groove (Fig. 2E). In a freshly opened flower, the stigmatic lobes are creamish white, papillate and dry-type. The whole gynoecium is persistent with marscecent type and after corolla senescence the style remains in a hyperbolic curvature for 2 days followed by a shrivelled condition. The commencement of flower opening was seen at dawn nearly 05.15–05.30 am by the formation of a slit in-between the two corolla halves. The completion of flower opening requires 1 h to 1.15 h and by 7.00 am all the flowers remained open. The corolla together with epipetalous stamens abscised during late afternoon of the same day.

'Close vial test' revealed that the flowers of *E. ligustrinum* have no such specific odour sensible to human. Though the GC-MS analysis showed eight compounds with highest concentration (peak area %) *viz.* carbamic acid; phthalic acid, di (2-propylpentyl) ester; pyrrole [1,2-a] pyrazine-1,4-dione, hexahydro-; propanamide, N-(aminocarbonyl)-; butyl isobutyl isobutal; ethyne, fluoro-; phenol, 4-[2-(methylamino) ethyl]-; and amphetamine-3-methyl (Fig. 3). The maximum amount of secretion stored in the calyx cup and within the base of the corolla tube is 2.74 ± 0.09 μl (n = 15) with solute concentration of 24.71±2.46% was recorded at 11.00 h. The secretion was viscous and showed positive result by anthrone test which confirmed the presence of soluble sugars.

Pollen production and pollen dispersal

Anther dehiscence is a gradual process and it starts at 5.15–5.30 am in the morning and completed by 8.30 am. The commencement of anther dehiscence is marked by the appearance of a longitudinal dehiscence-slit along the anther lobes (Fig. 2F). At about 08.00–08.30 am the anthers become fully dehisced, bringing about full exposure of the loose masses of bluish green pollen grains. A single anther produces 1,107–1,353 pollen grains. Individual flower having such two anthers produces 2,394–2,623 pollen grains with a mean value 2,502.9 ± 19.23 (n = 20). *E. ligustrinum* exhibits maximum pollen dispersal in the middle of the day (11.30 am – 03.30 pm) (Fig. 4). The anthers became almost emptied having negligible amount of pollens by the end of the day (04.30 pm – 05.30 pm) and finally senesced along with the corolla during 5.15 pm – 5.45 pm.

Stigma receptivity, pollen fertility and pollen-ovule ratio

In a freshly opened flower the creamish white bifid stigmatic lobes remained almost adpressed with each other. The stigmatic lobes diverge gradually and at around 10.30 am maximum divergence occurs. The stigmatic lobes were light brown in colour with a lustrous look which may indicate the onset of stigma receptivity. Dehydrogenase activity test also gives positive result, which was again confirmed by in vivo pollen germination test. The receptivity of the stigma continues up to 5.00 pm and at around 5.30 pm stigma completely loses its receptivity.

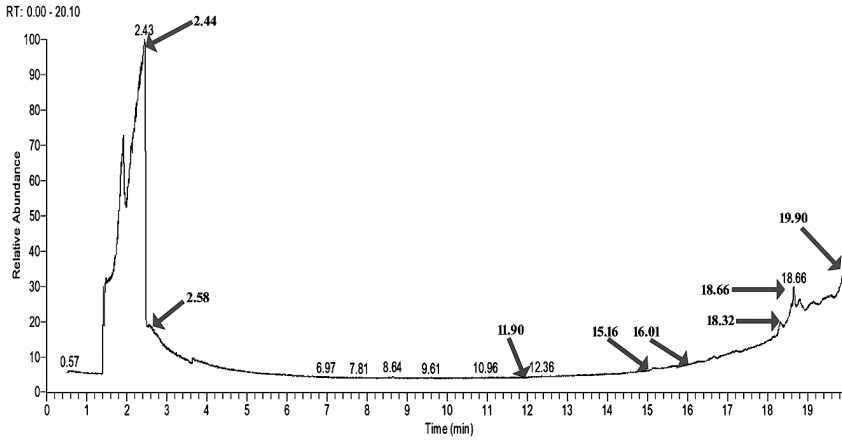

Fig. 3. Gas chromatography-mass spectrometry chromatogram of floral scent of *Ecbolium ligustrinum* (RT: 2.44 – carbamic acid; 2.58 – ethyne, fluoro-; 11.90 – amphetamine-3-methyl; 15.16 – butyl isobutylisobutal; 16.01 – phenol, 4-[2-(methylamino) ethyl]- ; 18.32 – propanamide, N-(aminocarbonyl)- ; 18.66 – pyrrolo [1,2-a] pyrazine – 1,4-dione, hexahydro-; 19.90 – phthalic acid, di (2-propylpentyl) ester]. USIC section, Vidysagar University, Midnpore, India

The observation on pollen fertility status with 0.08% lactophenol cotton-blue solution revealed that 80.54 ± 1.30% of the pollen grains were fertile and rest are sterile or abortive in nature at the time of anther dehiscence. Fertility of pollens decreases gradually. After 3.30 pm, fertility falls rapidly (31.36±0.70) and by 4.30–5.00 pm fertility of the pollen grains completely lost. Parallel observations performed under in-vivo germination experiments exhibit a similar result. The details of different floral events with distinct reproductive phases are mentioned in Table 1.

The individual flower produced a mean number of 2,503 pollen grains. The ovary contains four ovules. Therefore, the pollen-ovule ratio for the species is 626 : 1.

The different floral events of the species are more or less synchronous not only among the individuals but also among the different populations in a particular habitat.

Floral visitors

In *E. ligustrinum*, the flowers are visited by ten insect species and can be classified into three different categories as per their activities. Category–I: A fly species *viz. Eristalis tenax* (Fig. 2G) and three ant genera *viz. Camponotus*, *Formica* (Fig. 2H) and *Monomorium* belong to this category. The visitors of category–I are the effective pollinators of the plant. Category–II: *Crypto-*

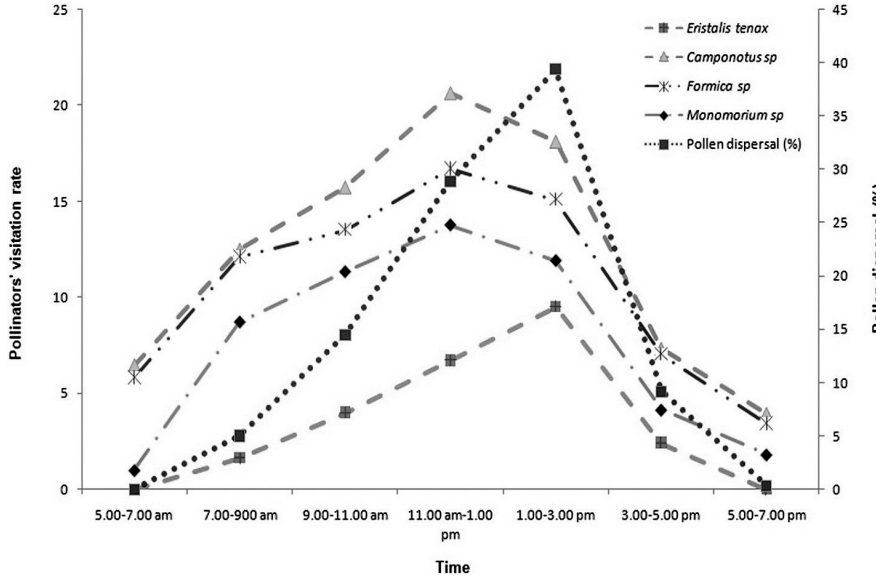

Fig. 4. Pollinators' visitation rate (number of visits per flower per two hours) and percentage of pollen dispersal from anthers during the period of anthesis in *Ecbolium ligustrinum*

Table 1
Floral events in relation to temporal reproductive phases of *Ecbolium ligustrinum* during the period of anthesis

Features	Male phase	Bisexual phase	Female phase
Starting from anther dehiscence (± 1 h)	0–3 h	3–8 h	8–9 h
Period of each phase (± 1 h)	2–3 h	5–5.5 h	1–1.5 h
Nature of style and stigma	style more less straight and stigma distally pointed	style slightly curved downward	style completely bent downward, stigmatic lobes become slightly brownish in colour
Stigma receptivity	non-receptive	receptive	receptive
Nature of anthers	fresh, turgid, bright, bluish-green in colour with pollen grains	lustrous look declined, turgid, pollen grains lesser in number	dehydrated and dried, brownish, more or less emptied with pollen grains
Pollen fertility	± 80.54%	± 80.38–31.36%	non-fertile

cephalus sp., a member of Coleoptera and a Hemipteran visitor belongs to this category. Considering the activities, these visitors may be regarded as pollen thieves. They do not play any role in pollination. Category–III: Two genera of Hemiptera *viz. Acanthosoma* and *Cymus*; a hawk-moth species and a Lepidopteran member (*Pseudoborbo bevani*) remained under this category. The mem-

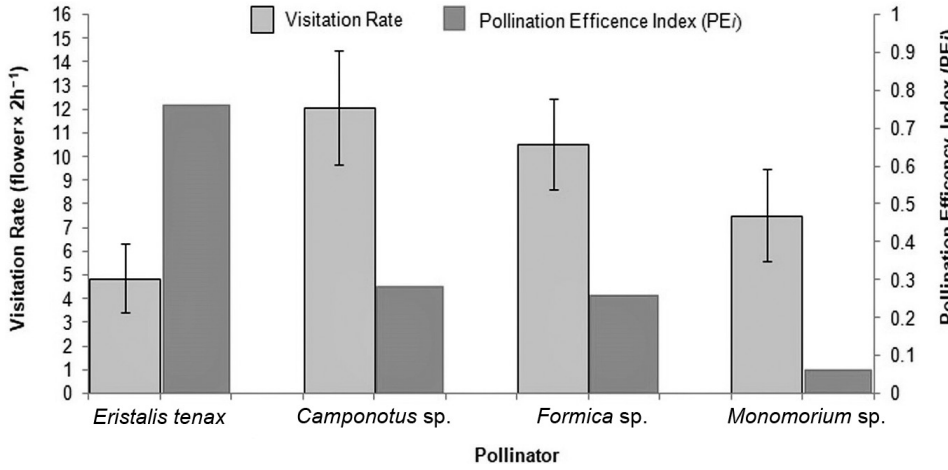

Fig. 5. Pollination efficiency index (PE*i*) and visitation rate (mean ± SE; number of visits per flower per two hours) of each pollinator since flower opening to senescence in *Ecbolium ligustrinum*

bers of this category are purely nectar foragers and did not perform any role in pollination. Among the insect visitors', *Eristalis tenax* pollinate the flowers nototribically i.e. through the dorsal surface of the body (Fig. 2I) and the three ants mainly perform pleurotribic (by the limbs and whole body surface) mode of pollination which were either autogamous and/or geitonogamous type. Among the pollinators, the peak visitation time of *E. tenax* was 11.00 am to 3.00 pm. For ants the foraging duration was between 8.00 am to 4.00 pm. The pollination efficiency index (PE*i*) of the pollinators is 0.76, 0.28, 0.26 and 0.006 for *E. tenax*, *Camponotus*, *Formica* and *Monomorium*, respectively. PE*i* of the pollinators is not always directly proportional to the visitation frequency e.g. in spite of low visitation frequency, *Eristalis tenax* exhibits the highest PE*i*. The visitation rate of those pollinators represented graphically in relation to their role in pollen dispersal (Fig. 4). A comparison is also drawn amongst the pollinators regarding their pollination efficiency and visitation rate (Fig. 5).

Breeding system

Artificial pollination tests confirmed that *E. ligustrinum* is a self-compatible and mostly out-crossed without any evidence of agamospermy. The percentage of fruit set in autogamy, geitonogamy and xenogamy are 19.24–24.76, 23.8–24.2 and 83.33–84.16, respectively. Emasculated bagged flowers showed no fruit set that means apomixes is absent. Fruit set in bagged con-

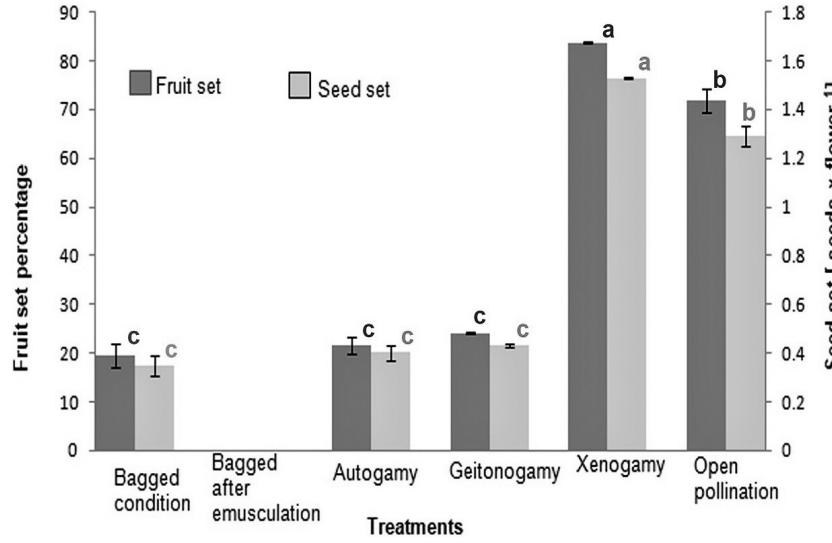

Fig. 6. Percentage of fruit set (%; mean ± SE) and seed set (seeds × flower^{-1}; mean ± SE) under different pollination treatments/conditions of *Ecbolium ligustrinum*

Table 2

Breeding experiment of *Ecbolium ligustrinum* on three different populations viz. Midnapore, Chandannagar and Monteswar areas

Treatment		Plant of Midnapore locality (PMi)	Plant of Chandannagar locality (PCh)	Plant of Monteswar locality (PMo)	Mean percentage (%) of fruit set and average no. of seeds (seeds × flower^{-1})
Bagged condition	Percentage(%) of fruit set	15.71^{a} ± 2.02 (n = 70)	18.57^{a} ± 3.40 (n = 70)	24.28^{a} ± 4.28 (n = 70)	19.52 ± 2.51
	Average no. of seeds (seeds × flower^{-1})	0.28^{a} ± 0.03 (n = 70)	0.35^{a} ± 0.07 (n = 70)	0.42^{a} ± 0.07 (n = 70)	0.35 ± 0.04
Bagged after emasculation	Percentage(%) of fruit set	0.0 (n = 70)	0.0 (n = 70)	0.0 (n = 70)	0.0
	Average no. of seeds (seeds × flower^{-1})	0.0 (n = 70)	0.0 (n = 70)	0.0 (n = 70)	0.0
Autogamy	Percentage(%) of fruit set	19.24^{a} ± 2.41 (n = 57)	20.64^{a} ± 3.09 (n = 72)	24.76^{a} ± 3.53 (n = 68)	21.54 ± 1.65
	Average no. of seeds (seeds × flower^{-1})	0.35^{a} ± 0.05 (n = 57)	0.41^{a} ± 0.06 (n = 72)	0.46^{a} ± 0.06 (n = 68)	0.40 ± 0.03
Geitonogamy	Percentage(%) of fruit set	24.20^{a} ± 1.57 (n = 62)	23.80^{a} ± 4.23 (n = 84)	24.20^{a} ± 2.88 (n = 61)	24.06 ± 0.13
	Average no. of seeds (seeds × flower^{-1})	0.43^{a} ± 0.03 (n = 62)	0.43^{a} ± 0.09 (n = 84)	0.45^{a} ± 0.04 (n = 61)	0.43 ± 0.006
Xenogamy	Percentage(%) of fruit set	83.97^{a} ± 1.34	84.16^{a} ± 2.39	83.33^{a} ± 1.72	83.82 ± 0.25
	Average no. of seeds (seeds × flower^{-1})	1.53^{a} ± 0.04	1.53^{a} ± 0.05	1.53^{a} ± 0.02	1.53 ± 0.001
Open pollination	Percentage(%) of fruit set	67.14^{a} ± 3.59 (n = 70)	72.85^{a} ± 2.85 (n = 70)	75.71^{a} ± 3.68 (n = 70)	71.90 ± 2.51
	Average no. of seeds (seeds × flower^{-1})	1.21^{a} ± 0.07(n = 70)	1.30^{a} ± 0.05 (n = 70)	1.35^{a} ± 0.08 (n = 70)	1.29 ± 0.04

Values given as mean ± standard error. The means in the row with same lowercase letter indicating the means were not significantly different by DMRT ($P \leq 0.05$), among the germplasms of all three habitat types

dition and open condition showed 15.71–24.28% and 67.14–75.71%, respectively. The detail results of artificial pollination experiments are presented in Table 2. While considering the different types of pollination treatments, we found highly significant outcome both on fruit set (%) and seed set (seeds/flower) ($F_{5, 12} = 424.59$, $P < 0.0001$ for fruit set and $F_{5, 12} = 493.26$, $P < 0.0001$ for seed set, ANOVA). Bagged flowers (pollinators absent) without emasculation; hand pollinated (pollen supplemented) autogamy and hand pollinated geitonogamy showed $19.52^{c} \pm 2.51$, $21.54^{c} \pm 1.65$ and $24.06^{c} \pm 0.13$ mean percentage of fruit set and $0.35^{c} \pm 0.04$, $0.40^{c} \pm 0.03$ and $0.43^{c} \pm 0.006$ mean seed set respectively (Fig. 6). These three treatments did not show any significant differences (DMRT, $P \leq 0.05$). However, other two treatments like open pollination and hand pollinated xenogamy exhibit highly significant value in mean fruit set ($71.90^{b} \pm 2.51$, $83.82^{a} \pm 0.25$) and seed set ($1.29^{b} \pm 0.04$, $1.53^{a} \pm 0.001$) respectively (DMRT, $P \leq 0.05$) (Fig. 6).Thus, the above result indicates that the pollen supplemented xenogamous pollination exhibit much higher fruit set percentage and also higher seed set value than autogamous, geitonomous and open pollination condition. The index of self-incompatibility (ISI) was 0.27, which indicates that the species is partially self-incompatible. Therefore, it may be said that though *E. ligustrinum* is partially self compatible, but needs pollinators for a better fruit and seed set which leads to a healthier progeny.

Therefore, *E. ligustrinum* exhibited mixed mating system without any occurrence of apomixis. The natural selfing (delayed autogamy) happened through fail-safe mechanism when pollination was hindered in absence of pollinators. During abscission, corolla with the epipetalous stamens slides down through the slender flexible style as a channel and remains in hanging condition for several minutes to few hours. At that time fertile pollen grains from dehisced anthers get stuck to both sides of the receptive stigma and thus ensure pollination. The efficiency of delayed-autogamy was calculated as auto-fertility index (AFI), which is 0.9058 for fruit set and 0.8724 for seed production. Besides, geitonogamous mode of pollination was performed by different genera of ants. Such kind of positive interactions between ant and plant is referred to as myrmecophily. Ants also protect the plants from herbivory.

DISCUSSION

Ecbolium ligustrinum flowers for nearly seven months (June–December) with a "Steady- state" flowering strategy. The bilabiate corolla with epipetalous stamens adpressed with the upper lip of the corolla and a long narrow tube shows an adaptation to specialised pollinators especially for bees or flies. This kind of specific mode of arrangement plays a significant role for the long term sustenance (Walsh *et al.* 2019). Flowers produce nectar stored in corolla

tube and extrafloral nectar stored in the calyx cup to attract legitimate and certain other illegitimate visitors respectively. The flowers are of single day longevity. The species exhibits both herkogamy and partial dichogamy (protandry). Pollen grains are produced in moderate numbers (2,502.9±19.23 per flower) and maximum pollen dispersal took place during midday (11.30 am – 03.30 pm). The stigma receptivity started at 10.30 –11.30 am and the optimum visitation of the visitors especially *Eristalis tenax* occurred between 11. 30 am – 3.00 pm. Therefore, maximum transfer of pollen grains from flower to flower was happened through dorsal surface of the fly during 11. 30 am – 3.00 pm and thus, ensured cross pollination. The gradual transformation from greenish to blue colour of the corolla lobes may increase the visitation frequency of the pollinators as blue is a preferred colour for insect visitors (Chittka *et al.* 2001). The organic volatiles emitted by the flowers may have a definite role for chemosensory communication with their pollinators (Kantsa *et al.* 2019) and such kind of specific floral volatiles exhibit differential attraction (Byers *et al.* 2014), that may favour *Eristalis tenax*. Identification of specific volatiles functioning as visitor attractants required further investigation. The dynamics of floral nectar production (2.74 ± 0.09 μl) and sugar concentration (24.71 ± 2.46%) is a phenomenon of co-evolution with the requirements and activity between plant and pollinators (Antoń *et al.* 2017, Layek *et al.* 2020, Souza *et al.* 2017). There also exists a significant positive correlation between nectar attributes and foraging behaviour of pollinators (Pyke *et al.* 2020). Here, geitonogamous mode of pollen transfer was done by three ant species. The fruit set percentage and seed set in pollen supplemented xenogamous treatment was significantly higher than open pollination (DMRT, $P \leq 0.05$). Therefore the species suffers from scarcity of effective pollination services. At the end of the day (3.30–4.00 pm), stigmas of some plants, which were yet to get any pollen grains, smeared with self pollens through 'fail-safe' mechanism and got fertilised. The delayed autogamy through fail-safe mechanism of pollination is an adaptation retained by certain plant taxa to maintain their progeny in absence of pollinators (Wolf and Stiles 1989). Here, the structural strategy of the flower promotes a specialised mode of plant-pollinator interactions rather than generalised ones. The auto-fertility index both for fruit set and seed set also establish that the species is capable to survive through autogamous sexual reproduction in absence or scarcity of pollinators (Jiang *et al.* 2010).

It was envisaged that prime selfing and principal out-crossing should be alternative stable outcomes of mating system evolution in the majority of plant population (Lande and Schemske 1985), which was corroborated by the existence of a number of species with balanced, mixed mating-systems in typical plant populations (Barrett 1998, Barrett and Harder 1996). The pollen-ovule ratio of the species is 626:1, which corresponds to the values reported

for facultative xenogamy (Cruden 1977). Our results illustrated that in the mating system of *E. ligustrinum* existed both selfing and out-crossing simultaneously. Although, *E. ligustrinum* mostly an out-crossed species, autonomous selfing just played a supportive role to assure fertilisation in the breeding system when circumstances for out-crossing are critical, such as effect of climate change, or the usual pollinators are scarce (Cogoni *et al.* 2019, Huang *et al.* 2006, Stebbins 1974, Whitehead *et al.* 2018).

CONCLUSIONS

Ecbolium ligustrinum is a partially self-incompatible species having facultatively developed xenogamous mode of pollination. Out-crossing is being done mainly by Dipteran fly. Long floral tube with small mouth appears to be specialised traits regarding pollination. The specialised pollination mutualism, especially for out-crossing is the significant key for its future existence. Besides, geitonogamy is seen in the plant aided by ants. Such ant-plant positive interactions are indeed significant for colonising the plant species. 'Fail safe' mechanism of autonomous selfing, another important phenomenon, was observed in the species. The mixed mating reproductive strategy with secure autonomous selfing is an encouraging behaviour. Though the plant species was visited by several groups of insects, the species is mainly cross pollinated by the fly *Eristalis tenax* and thus conforms the specialised mode of plant-pollinator interactions.

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