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Reproductive biology of *Boswellia serrata*, the source of salai guggul, an important gum-resin

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Detailed studies were carried out on the phenology, floral biology, pollination ecology and breeding system of *Boswellia serrata* Roxb. (Burseraceae) the source of 'salai guggul'. The trees remain leafless during the entire period of flowering and fruiting. The inflorescence is a terminal raceme and produces up to 90 bisexual, actinomorphic flowers. On average a flower produces 10 044 ± 1259 starch-filled pollen grains. About 85% of the fresh pollen grains are viable; the pollen to ovule ratio is 3348 : 1. The stigma is of the wet papillate type. The style is hollow with three flattened stylar canals filled with a secretion product. The stylar canals are bordered by a layer of glandular canal cells. The inner tangential wall of the canal cells shows cellulose thickenings. The ovary is trilocular and bears three ovules, one in each locule. Flowers offer nectar and pollen as rewards to floral visitors. The giant Asian honey bee (*Apis dorsata*) and *A. cerana* var. *indica* (Indian honey bee) are the effective pollinators. The species is self-incompatible and the selfed pollen tubes are inhibited soon after their entry into the stigma. Self-pollen tubes develop a characteristic 'isthmus' as a result of enlargement of the tube soon after emergence through the narrow germ pore. Cross-pollinated flowers allowed normal pollen germination and pollen tube growth, and resulted in fruit- and seed-set. Under open pollination fruit-set was only about 10%. Although manual cross-pollinations increased fruit set, it was only up to about 20%. Low fruit set appears to be the result of inadequate cross-pollination and other constraints, presumably limitation of available nutrients. *Botanical Journal of the Linnean Society*, 2005, **147**, 73–82.

ADDITIONAL KEYWORDS: breeding system – Burseraceae – pollen–pistil interaction – pollination ecology – self-incompatibility.

INTRODUCTION

Non-wood forest products (NWFPs) are important bioresources and provide livelihoods to a large number of people living in or around forests, particularly in developing countries. In the absence of scientific management and marketing, NWFP species are being overexploited and this has led to the depletion of genetic diversity. Conservation and sustainable utilization of NWFP species have become serious concerns for conservation biologists, foresters and small industrialists. One of the major constraints for developing effective strategies for their management is lack of information on their reproductive biology and yield data.

Gums and resins form a substantial proportion of the NWFPs in India. Many members of Burseraceae are well-known sources of gums and resins. Several members of this family are dioecious and produce small flowers. Information on reproductive biology is available on only a few species of this family. *Commiphora weightii*, the source of guggul, is composed largely of female plants in natural populations and is an obligate apomict with nucellar polyembryony and autonomous endosperm development (Gupta, Shivanna & Mohan Ram, 1996). *Bursera medranoana* is

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reported to be pollen sterile and is also apomictic (Cortez-Palomec, Nunez-Farfan & Marquez-Guzman, 1999). Information on some aspects of pollination biology is available on *Sentiria laevigata*; four species of stingless bees (*Trigona* spp.) are the regular pollinators in a lowland forest in Sarawak, Malaysia (Nagamitsu & Inouye, 1997).

The genus *Boswellia* comprises over 20 species, many of which yield important resins. Although a considerable number of studies are available on the uses of *Boswellia* resins, to our knowledge, there are no studies on details of reproductive biology for any species. *Boswellia serrata* Roxb. (Burseraceae) is the source of a most important gum-resin called 'salai guggul' or 'Indian olibanum' obtained from the bark after injury. The gum resin is fragrant and burns with a pleasant odour and is used as incense in religious ceremonies and worship. In recent years salai guggul has been used extensively in pharmaceutical formulations for relieving pains and aches, particularly associated with arthritis. Many commercial formulations of salai guggul in the form of ointments, creams and capsules are available on the market. The gum-resin is also reported to possess marked cholesterol- and triglyceride-lowering activity (Pachnanda *et al*., 1980). *B. serrata* is the only nonconiferous source of turpentine in India (Anonymous, 1988). Turpentine from *B. serrata* is superior to that obtained from chir pine (*Pinus roxburghii*) and is employed in the manufacture of paints and varnishes. Once abundantly available in the wild in Uttar Pradesh and Madhya

massie Brilliant Blue R as described by Heslop-Harrison, Knox & Heslop-Harrison (1974).

Stigma and style of different developmental stages were fixed in 2.5% glutaraldehyde-paraformaldehyde fixative (Karnovsky, 1965) prepared in 0.1 M cacodylate buffer pH 7.2 for 4 h at 4 °C and dehydrated through a graded series of ethanol (Feder & O'Brien, 1968). Semi-thin sections (1 or 2 μm) were cut using glass knives made with a LKB glass knife maker. For understanding general cytochemistry, sections were stained with 0.05% toluidine blue O (TBO) (O'Brien & McCully, 1981). Insoluble polysaccharides were localized by staining sections with periodic acid Schiff's (PAS) reagent (McGukin & McKenzie, 1958). Proteins were localized by staining with 0.25% Coomassie Brilliant Blue R prepared in methanol:acetic acid:distilled water (5 : 7 : 88 v/v) (Fisher, 1968). Lipoidal material was localized by staining the sections in a saturated solution of Sudan Black B or Sudan III (Bronner, 1975). For localization of pectins, sections were treated with 0.02% aqueous ruthenium red (Heslop-Harrison, 1979).

Scanning electron microscopy

Stigmas from freshly opened flowers and pollen grains from freshly dehisced anthers were fixed in Karnovsky/formalin acetic alcohol (FAA) fixative. They were dehydrated by passing them through an ascending series of cold acetone (30–100% for 15 min each), critical point dried, mounted on aluminium stubs using double-sided adhesive tape and coated with gold using a sputter coating unit. The stubs were observed using a Leo 435VP scanning electron microscope. Details of interest were photographed using Kodak TMX 100 ASA B/W film.

Breeding system

RESULTS

Figure 1 outlines the phenoevents. The trees remain leafless for 2–3 weeks prior to flowering and during the entire period of flowering and fruiting. There were no differences in the phenoevents in the three populations studied (GG, SV and SH).

FLORAL BIOLOGY

The racemes are borne at the tips of new branches. On average, each branch produces 7.2 ± 2.3 inflorescences (Fig. 2) and each inflorescence produces 20–90 flowers. During the peak of the flowering period an average of 5.9 ± 4 flowers open per inflorescence each day.

Flowers are bracteate, pedicellate, bisexual and actinomorphic. They are pinkish-white and measure 8.0 mm in length (from the base of sepals to the tip of petals). Flowers open between 12.30 and 15.00h. The sepals are reduced to five small scales. The corolla is represented by five petals. There are ten stamens arranged in two whorls of five each. The stamens of the outer whorl are larger (4.2 ± 0.2 mm long) than those of the inner whorl (1.1 ± 0.3 mm long) (Fig. 3). The anthers are dithecous and adnate (connective running the entire length of the anther). Anthers dehisce longitudinally along the theca and release the pollen grains. Flowers in three trees in the Ghatti population were found to have empty anthers during all years of study.

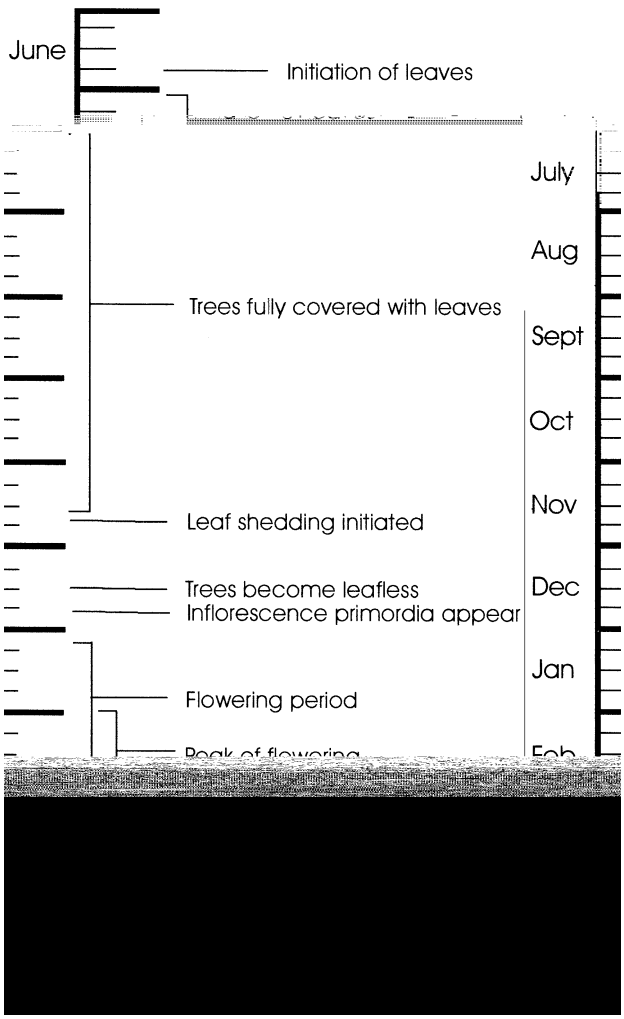


Figure 1. Various phenoevents in *Boswellia serrata*. Leaves are present only for over four months.

POLLEN GRAINS

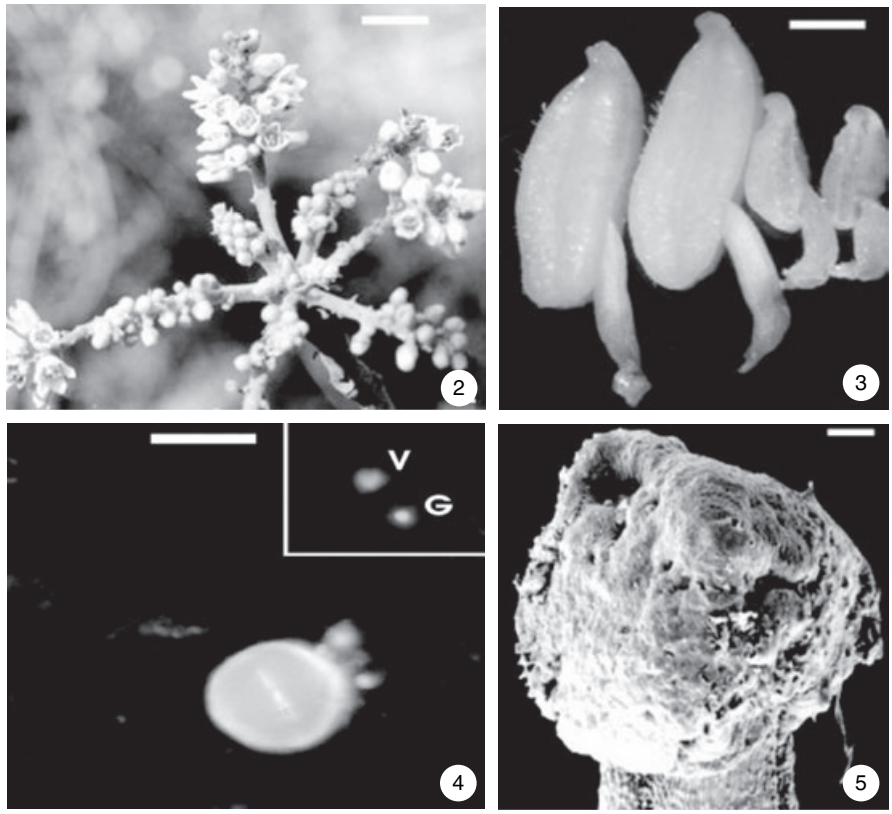
Pollen grains are spherical to oval in equatorial view. They are trizonocolporate with a faintly rugulose exine. The average diameter of the pollen grains from large anthers is 68.5 ± 2.0 mm and that from the smaller anthers is 60.5 ± 1.7 mm. Total pollen production in a flower is 10 044 ± 1259. Each large anther from the outer whorl produces 1624 ± 286 pollen grains whereas each small anther from the inner whorl contains 474.5 ± 119 pollen grains. The pollen to ovule ratio is 3348 : 1.

A high percentage of pollen grains (84.26 ± 4.5) were fertile from both the large and small anthers as ascertained from acetocarmine staining. Viability of pollen grains as determined through a FDA test was also high (85 ± 4.89) in both types of anthers. The pollen sample maintained under laboratory conditions lost viability gradually and reached zero within 7 days. There was no difference in per cent fertility or viability in the three populations studied.

Pollen grains stained intensely with I_2KI solution indicating the presence of starch as the reserve material. Sudan III or Sudan Black did not reveal the presence of lipids. Pollen grains are 2-celled at the time of shedding (Fig. 4).

THE PISTIL

The pistil is well demarcated into ovary, style and stigma. The ovary is superior and trilocular; each locule bears a single ovule borne on an axile placenta. There is a prominent bright-red annular nectary disc between the stamens and ovary. The stigma is capitate and is of the wet papillate type (Figs 5, 6). The papillae are unicellular, up to 273 mm long, and contain numerous small vacuoles. The stigma surface is covered with a copious amount of exudate at maturity (Fig. 5). The exudate stains positively for insoluble polysaccharides and lipids. Non-specific esterases are also localized in the exudate.



Figures 2–5. Fig. 2. A tip of a branch (top view) showing inflorescences at various stages of development. Scale bar = 15 mm. Fig. 3. Two stamens each from the outer and inner whorls, respectively, excised before dehiscence. Scale bar = 0.76 mm. Fig. 4. Fluorescence micrograph of a pollen squash in a drop of bis-benzimide to show pollen nuclei. A large vegetative (V) and a small generative (G) nucleus are clearly seen in the inset. Scale bar = 46 mm. Fig. 5. Scanning electron micrograph of the stigma on the day of anthesis. The stigma is covered with a copious amount of exudate which has obscured the visibility of the papillae. Scale bar = 110 mm.

The style in mature flowers is 3.0 mm long and is trilobed in transverse section. The style is hollow with three flattened stylar canals that traverse the entire length of the style (Fig. 7). There are two vascular bundles in each lobe on either side of the stylar canal. The stylar canal is bordered by a layer of well-defined, compactly arranged canal cells (Figs 7, 8). The canal cells are radially elongated, contain starch grains and stain intensely with toluidine blue and Coomassie Brilliant Blue. The inner tangential wall of the canal cells is thick (Fig. 8), similar to that seen in transfer cells. The stylar canal is filled with a secretion product which stains for insoluble polysaccharides, lipids, pectins and proteins.

Stigma receptivity

To determine stigma receptivity, pistils of different developmental stages (48 h before to 48 h after anthesis) were pollinated by cross-pollen (20 pistils for each stage) and studied for pollen adhesion and germination. Stigmas pollinated 48 h before anthesis did not support pollen adhesion and germination; those pollinated 24 h before anthesis and up to 48 h after anthesis supported pollen adhesion and germination. The maximum receptivity of the stigma extended from anthesis up to 24 h.

Breeding system

To ascertain whether or not apomixis is prevalent, six trees from each population were used. A total of 200 flower buds from each tree (one day before anthesis) were retained on the inflorescences; the remaining buds and older flowers were removed. The retained flower buds were emasculated and the inflorescences were bagged. The bags were opened after 15 days. There was no fruit set in any of the flowers; all flowers had abscised ruling out apomixis.

Details of the breeding system were studied through controlled field pollinations and subsequent studies on pollen germination and pollen tube growth as well as

'isthmus' region showed accumulation of callose as revealed through aniline blue fluorescence (Fig. 11). None of the selfed flowers set fruits and they abscised within 3 days.

POLLINATION AGENTS

The possibility of wind pollination was studied by suspending glycerine jelly coated slides at various heights in and around the canopy of the trees. Microscopic examination of these slides failed to show the presence of any pollen grains. This study ruled out wind pollination in *B. serrata*.

Several insects visited the flowers. Some (*Oxycetonia versicolor* and *Mylabris* sp.) visited the flowers occasionally and carried no pollen load on them. *Apis dorsata* (the giant Asian honey bee) and *A. cerana* var. *indica* (Indian honey bee) were the only active foragers. These bees came regularly to the flowers and inserted their head inside the petals to locate the nectar present inside the disc. Maximum activity of the bees was observed between 12:00 and 13.00 h. Bees visited only freshly opened flowers and avoided flowers that had opened the previous day which showed slight withering of the corolla. During foraging activity a large number of pollen grains were transferred to the body parts of the bees. The pollen loads recorded on *A. dorsata* and *A. cerana* var. *indica* were, respectively, 714 ± 187 and 472 ± 98.

FRUIT SET

Fruit set varied considerably from tree to tree (Table 1). Fruit set under open pollination was low and ranged from 2.6 to 10% in different trees (Table 1). The extent of fruit set in manual cross-pollinations ranged from 10 to 20% in all three populations. Thus, the percentage fruit set under open pollination was markedly lower than that resulting from manual pollination. To ascertain whether the low fruit set under open pollination was due to lack of pollination, 850 stigmas from different trees were excised two days after anthesis and examined for pollen load under the microscope. All the stigmas contained 12–122 pollen grains; 22–50 pollen grains were present in 13% of the stigmas, 51–100 in 21% and >100 pollen grains in 58% of the stigmas. As there are only three ovules per ovary, the number of pollen grains received on all the stigmas was sufficient to effect fertilization, and fruit and seed set.

To check if autogamy occurs, 150 inflorescences from different trees of GG population and 25 each from SV and SH populations were randomly selected and tagged. All opened flowers and developing fruits were removed retaining only unopened flower buds; the inflorescences were bagged without emasculation.

Table 1. Fruit set under open pollination and manual xenogamous cross-pollinations on 15 selected trees of the Ghatti (GG) population

Tree number	Per cent fruit set (open pollination)*	Per cent fruit set (manual cross-pollination)†
GG1	5.8	15.2
GG2	5.1	20.0
GG4	6.1	17.3
GG6	6.3	9.4
GG7	6.4	11.1
GG8	4.4	13.1
GG9	5.7	20.2
GG10	2.6	14.5
GG12	3.5	11.0
GG14	4.6	7.7
GG15	7.8	19.5
GG17	5.8	13.6
GG18	10.0	17.0
GG20	5.4	12.0
GG25	7.5	13.2

*Average of 15 inflorescences per tree.
†Average of at least 200 pollinations per tree.

There was no fruit set in any of the bagged inflorescences and all the flowers abscissed. Studies of the stigma of abscised flowers showed the presence of pollen grains indicating the occurrence of natural autogamous pollination. Although many pollen grains had germinated, pollen tubes were confined to only the stigma.

The fruit is a simple, dry, dehiscent, septicidal capsule. The fruit attains its maximum size (19 ± 4 ¥ 11.6 ± 2.5 mm) by 50 days after pollination. The fruits split open along the septa and release winged seeds. The average flower to fruit ratio was 18.35 : 1. All the three ovules in the ovary develop into seeds bringing the ovule to seed ratio to 1 : 1.

DISCUSSION

The present study is the first comprehensive study on the reproductive biology of *Boswellia serrata*. The trees remain leafless during the whole of the flowering and fruiting period. The only other species which showed broad synchrony in the timing of flowering and fruiting in all the three study sites was *Sterculia urens* (Sunnichan, Mohan Ram & Shivanna, 2004). An interesting feature was that three trees from the GG population were male sterile throughout the duration of this investigation. The underlying cause(s) for pollen sterility in the three trees are not clear.

Structure of the stigma and style

The stigma of *B. serrata* is of the wet papillate type and produces a copious amount of exudate. The structural features of the stigma and the composition of the stigmatic surface components are similar to those reported for other species (Raghavan, 1997; Shivanna, 2003). The papillae have numerous tiny vacuoles. It has been suggested that these vacuoles serve as storage sites for precursor materials of the stigmatic exudate (Tilton & Horner, 1980).

The style is hollow with three flattened stylar canals traversing the length of the style. As reported in other hollow-styled systems, the canal cells are glandular. The inner tangential wall shows thickenings similar to transfer cells, and this feature is believed to help in the secretion that accumulates in the stylar canals. Such thickenings of the inner tangential wall have been reported in *Lilium longiflorum* (Rosen & Thomas, 1970; Dickinson, Moriarty & Lawson, 1982) and *Citrus limon* (Ciampolini *et al*., 1981). The canal cells of many other hollow-styled species such as *Gladiolus* (Clarke *et al*., 1977) do not show such wall thickenings. In common with the other hollow-styled systems, the stylar canal is filled with secretion products containing carbohydrates, proteins and lipids.

Breeding system

Although apomixis is prevalent in some members of Burseraceae (Gupta *et al*., 1996; Cortez-Palomec *et al*., 1999), present investigations have clearly shown that apomixis is absent in *B. serrata*. Numerous studies have shown that outcrossing is a significant reproductive strategy in tropical trees (Zapata & Arroyo, 1978; Baker, Bawa & Opler, 1983; Frankie *et al*., 1983; Sunnichan *et al*., 2004). A number of tropical trees producing bisexual flowers are self-incompatible (Bawa, 1992). Studies communicated here have clearly shown that *B. serrata* is self-incompatible. Self-incompatibility (SI) is mediated through the inhibition of pollen tubes soon after their entry into the stigma.

In a majority of species showing gametophytic type of SI, pollen tubes are inhibited after their entry into the style (de Nettancourt, 2001). However, there are many species with gametophytic type of SI in which self-pollen tubes are inhibited in the stigma itself, soon after their entry (*Oenothera*, Dickinson & Lawson, 1975; *Commelina*, Owens, 1981). Thus, the zone of inhibition in *S. urens* is similar to that in gametophytic systems and not comparable to sporophytic systems in which pollen grains are inhibited strictly on the stigma before the entry of pollen tubes (Nasrallah & Nasrallah, 1993). Other cytological features of self-pollen tubes such as larger tubes and swellings at their tips are features characteristic of gametophytic SI systems (de Nettancourt, 2001). The zone of pollen tube inhibition and the cytology of pollen tubes clearly suggest that self-incompatibility in *B. serrata* is of the gametophytic type.

The formation of a characteristic 'isthmus' in self pollen is an interesting feature not reported in any other SI species. The significance of an 'isthmus' is not clear. Also it is yet to be ascertained whether the formation of an 'isthmus' is the cause for or the result of pollen tube inhibition. Further studies particularly on temporal details of its formation in relation to pollen tube growth and biochemical studies using *in vitro* assay as has been carried out in *Nicotiana* (Sharma & Shivanna, 1983) and *Papaver* (Franklin-Tong *et al*., 1988) would be useful in understanding the role of an 'isthmus' in SI.

Fruit set

Fruit set under open pollination in *B. serrata* is low although the quantity varies among individual trees (2.6–10%); the average flower to fruit ratio is 18.35 : 1. Many authors have reported low fruit set in a number of tree species (Lloyd, 1980; Stephenson, 1981; Rathcke, 1983; Bawa & Webb, 1984; Campbell, 1987; Sunnichan *et al*., 2004). Lack of successful pollination, adjustment of maternal investment to match available resources, and inherent conflicts in optimization of male and female reproductive success are proposed to account for low fruit set.

To determine the possible cause(s) of low fruit set in *B. serrata*, pollination efficiency was investigated. The stigmas examined two days after opening showed that all the flowers had been pollinated. Bagging of non-emasculated flowers invariably resulted in self-pollen deposition. In addition to autogamy, geitonogamous self-pollination is also a likely phenomenon as has been reported in many other insect-pollinated, mass flowering tree species (de Jong, Waser & Klinkhamer, 1993; Tandon, Shivanna & Mohan Ram, 2003). Availability of a large number of flowers on each tree facilitates the visits of insects to more flowers of the same tree in succession. As the species is strictly self-incompatible, both autogamous and geitonogamous self-pollinations do not result in fruit set. Recruitment of cross pollen through insect visits is likely to be infrequent. Anemophily is absent in this species. Thus, insufficient cross-pollination is likely to be a constraint for fruit set. However, the extent of increase in fruit set even in manually cross-pollinated pistils was rather low and never exceeded 21%. These results indicate that, apart from limitation of cross-pollination, there are other constraints to fruit set. As the distribution of fruits on the inflorescence is very sparse, space does not seem to be a constraint for fruit set. In the absence of leaves on the trees during the entire

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