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Pollination ecology and reproductive biology of *Canarium strictum* Roxb. from evergreen forests of Central Western Ghats, India

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Abstract

Pollination and reproductive biology of a dioecious tree *Canarium strictum* Roxb. (Burseraceae) was extensively studied within the Agumbe forest range of Western Ghats, Karnataka to identify primary pollen vectors and to enumerate interrelationship with the pollinators. The study also investigated phenology, floral biology, pollen production, pollen viability, stigma receptivity and nectar production. Trees produced functionally unisexual flowers with white petals, organized densely on inflorescences. Staminate flowers produced high percentage of viable pollen and relatively abundant nectar (15.75 µl) as a reward to the pollinators, while pistillate flowers produced only nectar (12 µl). Successful fruit set with wind pollination was facilitated by synchronization of flowering male and female trees, long term receptivity of stigma in female flowers and extended lifespan of flowers. The highest mean percent of fruit set with hand cross-pollination (µ=91.06) suggests the influence of local male tree density, as well as, frequency and abundance of pollinator community on fruit set by open pollination.

Key words

Breeding systems, Dioecism, Heterostyly, Phenology

Introduction

The pollination and reproductive biology of several taxa in tropical forests has been extensively studied (Johnson and Steiner, 2000; Oliveira and Gibbs, 2000; Sigrist and Sazima, 2004) but the information on pollination and reproductive biology of many tropical tree species of Western Ghats is yet to be explored (Devy and Davidar, 2003). The reproductive success in dioecious systems is subject to diverse ecological constraints (Berry and Gorchov, 2004). Factors such as spatial distribution, sex ratio and sexual identity of neighbors have been shown to influence female reproductive success at local scales (Berry and Gorchov, 2004). Sexual reproduction in tropical lowland rainforests essentially requires a vector to transfer pollen between conspecifics (Berry and Gorchov, 2004; Bellusci *et al.*, 2009). These forests are also rich in dioecious taxa and are pollinated by several groups of animals (Molano-Flores, 2002; Klein *et al.*, 2003). Animal-pollinated species are bound by the ecological constraints such as foraging distance and availability of pollinators (Somanathan and Borges, 2000). Most pollination research in the tropics is focused on entomophilous plant species and experiences the local neighborhood effects as mediated through pollinator behavior (Mitchell *et al.*, 2009). Pollen flow in a natural population is influenced by self- and intra-morph incompatibility systems which are closely related with floral dimorphism and occur in most of the heterostylous taxa which are generally self-incompatible (De Castro and Araujo, 2004). Heterostylous species usually possess a sporophytic, diallelic incompatibility system which prevents self-and intra-morph fertilization (Ferrero *et al.*, 2012).

Canarium strictum Roxb. is a large canopy tree distributed across different parts of India, Myanmar and Yunnan province of China and considered as vulnerable in South India (Ravikumar and Ved, 2000). It can grow up to 40 m and is found in moist deciduous to evergreen forests at altitudes ranging from about 750 m to 1400 m (Ravikumar and Ved, 2000) and the trees are mainly utilized for their resin (Weeks, 2008). It naturally occurs at low density and are sparsely distributed (Mathachen *et al.*, 2005; Tambat *et al.*, 2005), with individuals growing together forming small clumps like patches (Varghese and Ticktin, 2008). The earlier studies by several workers have shown tapping practices as the major threat to *C. strictum* population by expressing concerns about decrease in their number (Augustine and Krishnan, 2006).

In spite of importance and wide distribution of species, information on pollination and reproductive biology is meagre. In the present study, field investigations were conducted within the sites of evergreen forests of Western Ghats, Karnataka to reveal floral phenology, pollination biology and breeding systems of dioecious tree *C. strictum.* The study also focused on the relationship between pollen vectors with the species.

Materials and Methods

The present study was carried out at lowland tropical forests of Agumbe region belonging to Someshwara Wild Life Sanctuary of Western Ghats, Karnataka. These were classified as tropical wet evergreen forests, rich in *Dipterocarpus indicus - Humboldtia brunonis – Poeciloneuron indicum* community association with an elevation range between 400-600 m above mean sea level harboring rich endemic flora. The mean annual rainfall was between 7000-7500 mm with a mean annual temperature of 22 °C.

Phenological events were studied by randomly selecting 10 trees, which were free from biotic disturbances. Observations made on phenophases included: leaf initiation and drop; flowering and anthesis and fruit setting and dispersal during the period between January to December 2013. Phenological records were observed at weekly intervals during high activity period of flowering season, until fruit maturation and dispersal and, three week intervals during the rest of the year. Floral phenology of the species was studied by tagging five inflorescences each on 10 randomly selected individual trees and flowering stages were observed from bud until senescence. Flowers were monitored at every three hour interval to determine the timing of anther dehiscence and pollen release. Flowers with different stages, including the unopened matured buds were selected and tested regularly for stigma receptivity using peroxidase test and observed until floral senescence, using 10x hand lenses. The pollen count was made by taking 15 randomly selected anthers from 10 randomly selected flowers to avoid any bias, using a haemocytometer (Nanda *et al.*, 2006) and the number of ovules were counted by taking the cross sections of ovary (Pias and Guitian, 2001). *In-vitro* pollen germination was studied using Brew-Baker media; pollen viability using 0.5% of 2,3,5-Triphenyl-tetrazolium chloride solution (Shivanna and Rangaswamy, 1992) and by fluorescent method (Atlagic *et al.*, 2012). The volume and concentration of nectar was quantified separately on staminate and pistillate flowers four times during different hours of a day. The volume of nectar available to the floral visitors was quantified on three randomly selected flowers each time during 06:00, 09:00, 12:00 and 15:00 hours of a day by using calibrated capillary micropipettes. The volume and concentration of available nectar was quantified from three flowers each time and for four times a day. Total sugar or sucrose equivalents were quantified by using a hand held refractometer from three different flowers selected from different inflorescences each time (Molina-Freaner and Eguiarte, 2003). The atmospheric temperature was recorded each time during quantification of nectar concentration since nectar viscosity varied with variation in atmospheric temperature. The corresponding error values due to temperature changes were obtained from the standard table and added to each measurement (Nicolson and Thornburg, 2007).

Pollination and breeding systems : Breeding experiments were carried out by selecting five male and female trees each. Inflorescences were tagged and left for open pollination (103 flowers) without bagging and efficacy was confirmed upon fruit set through natural pollination. Manual hand pollination experiments included hand self-pollination (90 flowers), hand cross-pollination (65 flowers) performed by counting the number of matured, unopened flower buds on inflorescences followed by emasculation, manual pollen transfer to the stigmatic surface, followed by bagging with a nylon mesh cloth bag to control further pollination (Duffy *et al.*, 2009). The efficiency of fruit set upon a pollinator visit was assessed by bagging the flowers immediately after one time visit of at least any one of the pollinator species (Nayak and Davidar, 2010). Pollination experiments were conducted on both male and female trees separately to confirm the possibility of fruit set. Xenogamy was performed by collecting pollen from male parent (staminate) trees located at least 10 km away. Apomixis (39 flowers) was observed by emasculating androecium followed by immediate bagging to avoid further pollination (Borges *et al.*, 2009). Pollinator observations were carried out throughout the day between 06:00-18:00 hours on both male and female trees separately. Pollinators were differentiated from floral visitors by keen observation of their foraging activity and landing site on trees (Fenster *et al.*, 2004) and auto correlation between pollinator visitation frequency of male and female trees was tested with Durbin-Watson test.

Statistical analysis : Variations in the breeding experiments were compared using Student's *t*- test for comparing the mean percentage of fruit set. Linear regression and Durban-Watson tests were performed to compare the frequency of pollinators and floral visitors. Data was analyzed using statistical package PAST (Hammer *et al.*, 2001).

Results and Discussion

The peak leaf fall observed during second week of February was followed by fresh leaf sprouting by third week of same month. Leaves at initial stage were dark brownish-red, turned into yellowish green and finally attained green color at maturation by second week of March. Flowering started succeeding fresh leaf maturation during third week of March followed by initiation of fruit set during the last week. Fruit maturation was observed during the last week of June followed by dispersal. Flowers on individual tree showed either male or female flowers and were devoid of polygamous inflorescence among the selected population.

Flowers were small, actinomorphic borne on axillary, indeterminate panicles of racems. Sepals 3, basally connate and valvate; petals 3, white, arranged with induplicate-valvate aestivation. Stamens 6, in one whorl, filaments were connate at the base; anthers slightly basifixed and introrse with intra-staminal, cupular nectary disc. A gynoecium with 3 united carpels showed a 5-lobed capitate stigma. Ovary tri-locular with 2 epitropous, bitegmic ovules per locule with ventral raphe and upwardly and outwardly directed micropyle. The species was strictly dioecious, staminate and pistillate flowers were borne on different individuals with thrum and pin-morph respectively; possessed type-I unisexuality. Flowers with greenish trumpet calyx and white leathery corolla exhibited a cup shaped structure within which nectar was collected. It was observed that the flowers opened with a small opening at the top that facilitated foraging by floral visitors. Flowers thus exhibited classic pollination syndromes such as strong odour, rich nectar, pollen availability, flower anthesis, and particular shapes to attract certain groups of pollinators (Fenster *et al.*, 2004). The receptive stigmas on female flowers were wet, glistening and turgid. Formation of air bubbles after peroxidase activity on stigmatic surface confirmed their receptivity.

The mean pollen count taken from male flowers with functional pollen using haemocytometer indicated count per flower as 29904 with a mean pollen-ovule ratio of 4984. The mean percentage of *in-vitro* germination of pollen was 55.82. The mean percentage pollen viability with TTC and fluorescent analysis was 90.82 and 93.13. The mean percentage of nectar quantified on staminate and pistillate flowers were 27.26 and 26.58. The average volume of nectar was 15.75±2.39 μl and 12±1.22 μl in staminate and pistillate flowers, respectively (Fig.1). The mean nectar concentration and volume of nectar produced in both types of flowers measured during different hours of the day were significantly not different (α=0.05, p=0.12).

The manual breeding experiment (Fig.2) confirmed highest mean percentage of fruit set with hand cross pollination (μ=91.06±3.01%) followed by a onetime pollinator visit (μ=63. 38±9.80%) and open pollination (μ=52.05±3.07%). The Student's *t*-tests between open/natural pollination (control) with other treatments like hand self-pollination, hand cross-pollination, were significantly different ($p<0.05$, $\alpha=0.05$), while fruit set with one time pollinator visit was also insignificant ($p\geq 0.23$, $\alpha=0.05$). Fruit set was evident in emasculated and bagged female flowers on female trees, with no provision for internal or external pollination (μ=20.67±16.52%) confirming the possibility of fruit set through facultative apomixis.

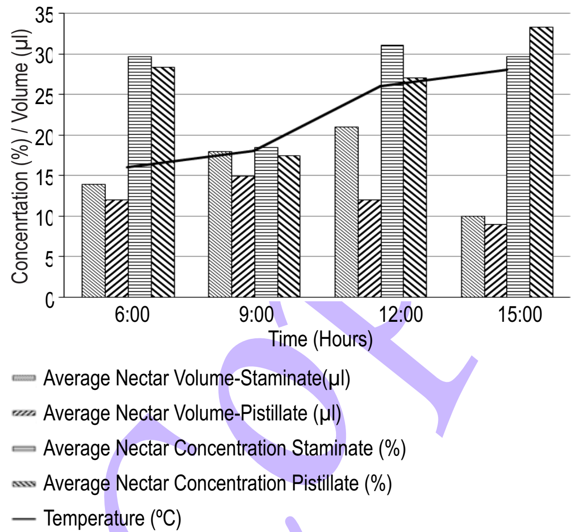

Fig. 1 : Volume and concentration of nectar pistillate and staminate flowers

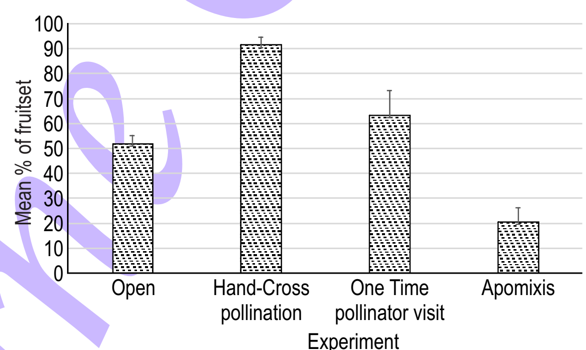

Fig. 2 : Breeding experiments showing mean percentage of fruit set. (Open- open pollination, HC- Manual hand cross pollination, OT-pollination upon onetime pollinator

Canarium strictum showed mixed pollination syndrome by diverse species of honey bees (*Apis mellifera*), carpenter bees (*Xylocopa violacea*), butterfly (*Junonia spp.*) and small flies (Calliphoridae) wasp (*Vespa cincta*) and ants (*Camponotus sericeus*) (Fig.3) with the carpenter bee showing highest frequency to both staminate and pistillate flowers. The frequency of each pollinator visit to male and female inflorescences (Fig.4) were strongly correlated with high Durbin-Watson positive auto-correlation index (N=12, $\alpha=0.05$, $r^2=0.95$, d=0.98).

Flowers are adapted to distyly with staminate and pistillate flowers developing thrum and pin morph respectively, which are genetically self-incompatible (Kang and Bawa, 2003). Retention of sterile sex organs in flowers: genetic correlation between androecium and gynoecium that cause suppression of one to impact the other (Yu *et al.*, 2011), or importance of such

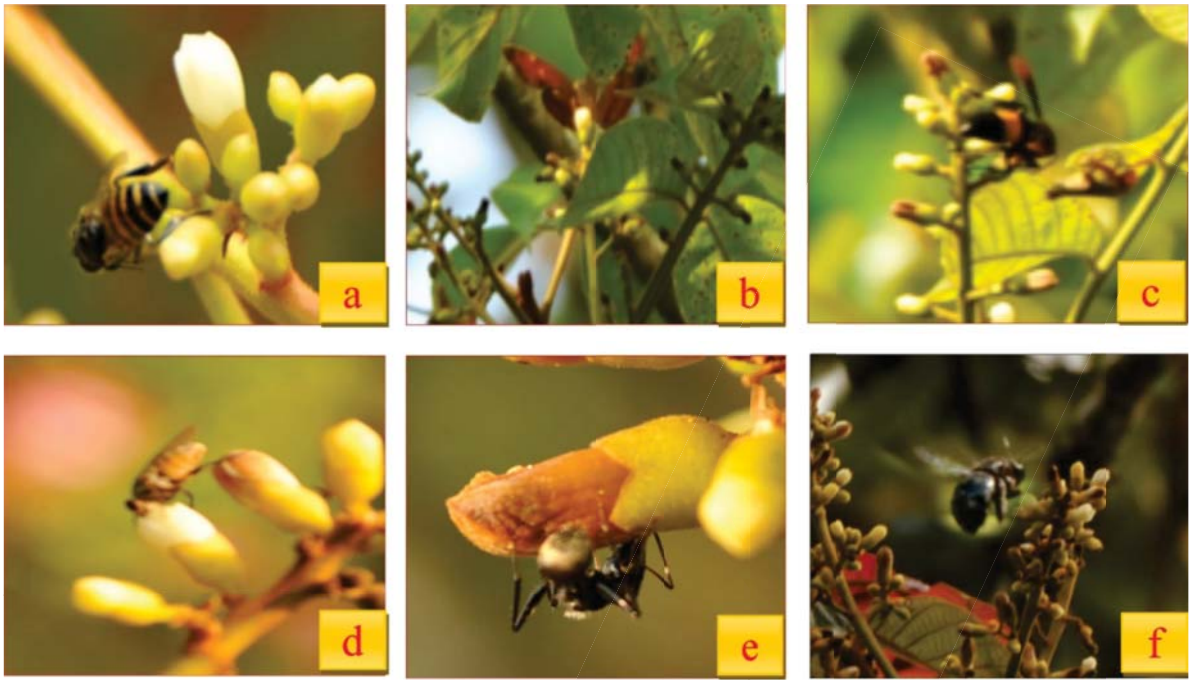

Fig. 3 : Pollinators of *Canarium strictum* (a) *Apis dorsata;* (b) *Junonia spp.*; (c) *Vespa cincta;* (d) *Calliphoridae fly;* (e) *Camponotus sericeus* and (f) *Xylocopa spp.*

organs in pollinator attraction (Irwin *et al.*, 2004). In case of *C. strictum* sterilization of androecium or gynoecium during post-initiation, possibly followed by evolutionary reduction of those organs but not leading to complete loss of organ at maturity showing type-I unisexuality as suggested by Mitchel (2009). The population of species was sparsely distributed, yet they showed aggregation of male and female trees within the population which accounted for successful pollination and fruit set. There was also a synchrony with flower anthesis, long flower longevity and stigma receptivity enabling the species to attract pollinators frequently leads to visit and successful fruit set.

Individual type of pollinators were recorded on both staminate and pistillate flowers including honey bees (*Apis dorsata*), wasp (*Vespa cincta*), butterfly (*Junonia spp.*), ant (*Camponotus sericeus*), beetle (*Xylocopa spp.*) and fly (Calliphoridae). Contrary to the report of Glaiim (2009) recording *Vespa cincta* as a serious pest and killer of honey bees in South India, but the investigations in case of *C. strictum* indicated their participation both as active foragers of flowers and effective performers of pollination. Raju *et al*. (2012) also reported *Vespa cincta* as a pollinator on *Terminalia pallida*. Butterflies feed on flowers since flowers are cup shaped with a strong odor (Cunningham *et al.*, 2004) and rich in nectar. Their visit is also facilitated by pollination syndrome of nocturnal opening of flowers (Somanathan and Borges, 2001; Amorim *et al.*, 2013). Ants also forage flowers, but they fail to perform successful pollination since trees are dioecious and produce either functional male or female flowers limiting pollen transfer. Carpenter bee (*Xylocopa spp.*) is recorded to be the most frequent visitor, effectively performing pollination because of its ability to travel long distances (Raju and Rao, 2006).

Many dioecious taxa with pistillate flowers mimic staminate flowers without producing any nectar dupe to the visiting pollinators. Nectar produced in both staminate and pistillate flowers of *C. strictum* is not significantly different from each other, but help in retaining the probability of each type of pollinating species enabling effective pollination. The highest percent of fruit set with hand cross pollination suggest a decreasing trend in the population of pollinator community besides influence by local male density. It is also influenced by synchronization of flowering of the neighborhood species *Ailanthus triphysa* (Dennst.), surrounding *C. strictum* population, which attracted good number of frequently visiting pollinators contributing to distress in successful pollination. Fruit set through controlled experiments confirmed the occurrence of apomixis and a similar phenomenon was reported in *Commiphora wightii* belonging to same family, Burseraceae (Geetha *et al.*, 2013). *Canarium strictum*, a threatened taxa, exhibits dioecious sexual system. Pollinators play a significant role in bringing successful fruit set, and local male tree density is an important criteria influencing successful pollination and fruit set through pollen vectors. Breeding results with highest fruit set through hand

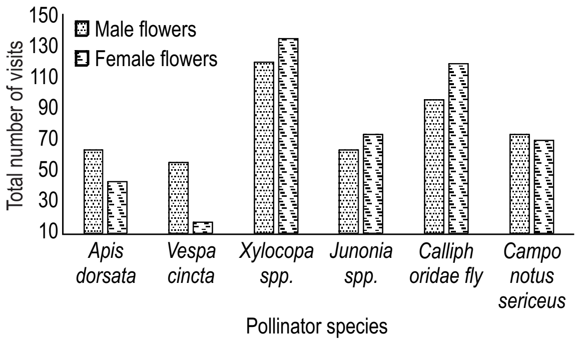

Fig. 4 : Pollinator visitation frequency to staminate and pistillate flowers observed between 06:00-18:00 hours

cross-pollination, and relatively good fruit set through one time pollinator visit, suggests the importance of pollinators in bringing successful fruit set through natural pollination.

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