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Reproductive biology and seed germination of tropical evergreen tree *Canarium strictum* Roxb.

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Abstract

Canarium strictum Roxb. (Burseraceae) an evergreen tree was studied for its phenology, flower morphology, pollen viability, stigma receptivity, seed setting and seed germination. This species has got meager population and restricted distributed in the Kolli, Pacchamalai, Karanadamalai and Sirumalai hills of Eastern Ghats of Tamilnadu, India. Though there is enormous number of pollens (946/anther) produced and all of them are viable in X-gal test, their germination is very poor (12.5% only). Among the various methods evaluated for seed germination, osmopriming with Potassium dihydrogen phosphate proved to be efficient in breaking the dormancy (69.3 %) followed by GA_3 treatment (56 %) suggestive of possible sowing methods in the forest to enhance the population of this vulnerable species.

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Introduction

Understanding how reproductive traits evolved in the past leads to important insights into how organisms adapted. In sexually reproducing organisms, traits associated without crossing are thought to be under strong selection because of their direct effect on reproductive success; the radiation of floral morphologies in angiosperms represents a great example of this (Darwin, 1871; Lloyd and Webb, 1992). Specifically in the case of animal-pollinated plant species, pollinator preference represents a strong selective pressure to flower traits (Darwin, 1862; Fægri and van der Pijl, 1966; Stebbins, 1970; Schemske and Bradshaw, 1999). Evolutionary specialization leads to shifts in floral morphologies that are associated with the use of a subset of pollinators compared to those visiting the ancestral morphology (Armbruster *et al.*, 2000; Fenster *et al.*, 2004). The identification of specific shifts in floral morphologies allows us to establish when significant evolutionary changes took place, as well as to test specific hypotheses associated with the processes that may have led to such morphological shifts (Grant and Grant, 1968; Armbruster and Webster, 1982; Armbruster *et al.,* 1994; Johnson and Steiner, 1997; Hansen *et al.*, 2000; Fenster *et al.,* 2004).The number of seeds produced by a plant, the number of seeds it fathers with the pollen it produces and the proportion of these offspring which survive to reproductive maturity are factors which determine how many descendants left by a genotype expressing a particular life history pattern (Harper, 1977).

The seed is a dormant or resting stage in the life of a plant and the stage of the life cycle at which dispersal and colonization of new areas occurs. Seeds survive adverse conditions better than growing plants and thus plants "ride out" difficult environmental circumstances in the seed state with low levels of metabolic activity and resume active growth when more favourable conditions return (Hutchings, 1986). *Canarium strictum* Roxb. is an indigenous and endemic plant species of Eastern and Western Ghats. It is a large, resinous tree species, commercially harvested for dammar, throughout South and South East Asia. Due to its overexploitation and the loss of habitat, it was found to be an endangered species and, therefore, required urgent attention for its conservation. Its traditional medicinal and spiritual importance helps yield references that make us understand its links with the culture and tradition of our country (Meena *et al.,* 2012). *Canarium strictum* is a gigantic tree with a spherical crown having a clean bole of 30-35 meters length.

Based on technical inputs of conservation groups and forest agencies it has been observed that populations in the Eastern and Western Ghats are very different in their phenological behaviour (Kunhikannan *et al.*, 2004). It is poly-gamodioecious tree species and noted to be very rarely gregarious. Leaves are compound, imparipinnate, alternate, spiral, clustered at twig ends, rachis is ferruginous pubescent; leaflets 3-9 pair with odd one at apex, increasing in size towards apex; petiolule is 0.3-0.7 cm long; It shows with lamina 5-15 x 2.5-7 cm usually oblong, sometimes ovate, apex acuminate, base asymmetric-rounded; margin serrate or serrulate, coriaceous, rusty tomentose or pubescent beneath and glabrous above; secondary nerves are strong with 11-18 pairs; tertiary nerves are weakly percurrent (Meena *et al.*, 2012). *Canarium strictum* exudates a resin called as 'Sambrani' or 'Dammar' which has medicinal as well as commercial uses. Its usage among tribal and folk people for medicinal purposes in different parts of India has been explored through ethnobotanical studies. It is also used in Siddha system of medicine. It finds its usage in incense and varnish industries (Augustine and Krishnan, 2006) and also used as a substitute for burgundy pitch in making medicinal plasters.

This study was carried out to investigate the phenology, pollen counting, pollen viability, pollen germination, stigma receptivity, seed setting and seed germination of *Canarium strictum* a tropical evergreen tree that are distributed in Eastern Ghats of Tamilnadu.

Materials and methods

Reproductive Biology

Phenology

Information on the date and month of flowering, fruiting, shedding of leaves and spring of new leaves were collected from the Flora of Tamilnadu Carnatic (Matthew, 1983) and Flora of Palni hills (Matthew, 1998). Besides, this information also was verified from the field survey and affirmed and taken record in the field notes (Sharma *et al*., 2011).*Information on Inflorescence*

Ten inflorescences at random from different individuals were sampled for the number of flowers per each inflorescence.

Flower morphology

Fresh flowers were collected and preserved in 5% formaldehyde solution for further study. Features such as the size of flower, number of bracts, sepals, petals, filaments, anthers, styles, carpels and ovules were counted and recorded (Koning, 1994; Banu *et al*., 2009).

Number of anther/flower

Information on the number of anther per flower and number of anther lobe per filament were counted and recorded. *Number of carpel, ovule/flower*

Transverse and longitudinal section of ovary was prepared and the number of carpel and ovule per carpel were counted and recorded. *Time of opening of flower*

In the field the opening time of flower was recorded in the observation note book.

Number of pollen/anther

Anthers are stored in 0.5 ml of ethanol in an eppendorf tube to release the pollen grain (Kearns & Inouye, 1993). The anthers must be transferred into 1 normal hydrochloric acid the day before; the anthers will sit in the HCl overnight. Place 0.5 ml of 3:1 lactic acid, glycerin solution into the tube of the tissue homogenizer. Remove the anthers from the HCl vial and place them in the tissue homogenizer without transferring any of the HCl. The next step is to place the small part of the homogenizer into the larger tube of the homogenizer to break the anthers. Crush the anthers so that there are little to no remnants of the anthers remaining. Place one drop on each section of the haemocytometer and cover the solution with a cover slip making sure that only one cover slip is used (more than 1 cover slip will make it hard to count the pollen because the lines of the haemocytometer will not be visible through the microscope).Place the haemocytometer on the microscope. The key to seeing the lines of the haemocytometer is to adjust the contrast. Once the lines of the haemocytometer are visible, counting can begin. Counting the pollen is done by counting the pollen grains in each small box that makes up 1 big box. 16 small boxes (4 by 4) make 1 large box. Once all of the small boxes that make up 1 large box are counted the slide can be moved to view a different large box and the pollen must be counted in that one as well. This should be repeated for about ten large boxes. Make sure a box is not counted twice; a good way to prevent this from happening is to count the small boxes in order from left to right then move down a row and count from right to left and repeating this until all 16 small boxes are counted. Furthermore, if a pollen grain is on the outer line of the large box, only count it if half of it or more is inside the box. Once the counting is completed an average of the pollen grains must be calculated. This is the average pollen grain count per large box. This is them multiplied by 2500 to find the average pollen count per flower. All data in the experiment were subjected to analysis of variance and the mean separation was done by Turkey's MRT at $P \leq 0.01$.

Pollen viability

Pollen has a very important role in the flow of genes in plants, especially in plants that are out crossing. The first method for testing pollen involves determining viability by staining. The X-gal test to determine the content of β-galactosidase (an enzyme involved in the lactose degradation). The X-gal test consists of a solution of 1 mg Xgal (5-bromo-4-chloro-3-indoyle-β-galactoside) that is dissolved in 50 µL N,N-dimethyl formamide and 1 mL acetate buffer (50 mmol with pH 4.8). Viable pollen turns blue (Wang *et al.*, 2012).

Pollen tube germination

In the second method for testing the viability, germination tests were carried out to measure pollen viability. There are two major tests, which can be divided in two different parts. *In vitro* germination, pollen is grown on a specific media. *In vivo* germination pollen is grown on the stigma of the plant. In the present study the *in vitro* germination was conducted.

In vitro germination of pollen for viability

Fresh harvested pollen is grown on a medium containing 1% agar, 20% sucrose, 0.01% boric acid and 0.01% calcium nitrate. These compounds have been shown to be very important for pollen germination in different species. The pollen is grown in a humid environment and at room temperature (~20°C) for 8 hrs. The pollen is considered mature when the pollen tube length is longer than the diameter of the pollen grain. Germination was scored by a light microscope (x 100) in four random fields (about 50 grains / field) (Wang *et al.*, 2004).

Stigma receptivity Baker's procedure (Dafni, 1992)

This test detects the presence of alcohol dehydrogenase. The test solution consists of 10 ml of 1 M phosphate buffer (pH 7.3–7.5), diluted (1 part buffer to 2 parts distilled water); 5–10 mg nitroblue-tetrazolium to give a slight yellow color; 6 mg of nicotinamide adenine dinucleotide; and 1 ml of ethanol (95%). The fresh stigma was cut and removed in the field directly into a large droplet of this test solution on a slide and incubated at room temperature in a closed petri dish containing a moist filter paper in the bottom. The stigmas were inspected after 20–40 min under a magnifier (×20) or a microscope (×200) to locate the stained area.

Fruit and seed characters

The weight of fruit and seed were measured and the number of seed per fruit was also calculated.

Seed Germination

Seeds of *Canarium strictum* were collected from Pacchamalai hills in March 2013 and in lab, the immature seeds and those damaged by insects were removed. Seeds were surface sterilized by soaking in 5% Sodium hypochlorite (NaOCl) for 5 min and subsequently rinsed thoroughly with sterilized water prior to further treatments. All germination experiments were conducted using three replications of 25 seeds each. After following treatments seeds were placed on double layered filter paper in petri dishes, moistened frequently with distilled water and incubated at 25°C with a 16-h light/8-h dark photoperiod.

Chemical Scarification

Seeds were soaked in H_2SO_4 for 8 min, washed thoroughly with distilled water and incubated.

GA_3 Treatment

Seeds were soaked in Gibberellic acid at various concentrations (1000 ppm, 1500 ppm and 2000 ppm) for 72 h followed by thorough washing prior to incubation.

Heat Treatment

Seeds were treated at 50°C, 75°C, 100°C for 6 h, cooled for 10 min at room temperature and sterilized in sodium hypochlorite solution and washed thoroughly in distilled water before incubation.

Osmotic Potential Treatment

Prepare -10 bar potential value potassium dihydrogen phosphate at 15-25°C and the seeds were soaked for 8 days and then surface sterilized with 5 % sodium hypochlorite solution and rinsed in distilled water and finally incubated at25°C.

Mechanical Scarification

Seeds of *Canarium strictum* was scarified with sterile knife and soaked in water for 24 h.

Cow Dung Treatment

Seed were soaked in slurry of cow dung with water and kept in hot oven at 40ºC for 3 days.

After each treatment the seeds were sterilized with sodium hypochlorite solution for 5 min, immersed in1% bavistin and then washed thoroughly with distilled water and finally incubated at 25°C.

Field Test

1´x1´x1´ pits were made in the reserved forests of Paccahimalai hills, the locality from where the seed were collected. 1 x half feet long and broad Mosquito nets were placed in the pits and the pits were filled with the same soil. Seeds were soaked in distilled water for 24 h and then sowed in the above said pits to test its potency to germinate. Latter frequent visits were made to assess the growth of seedlings.

Results

Canarium strictum Roxb. is collected from Pacchamalai hills (11°16'28" N and 78°37'59" E), part of the Eastern Ghats of Tamilnadu in the month of 4th August 2012. Voucher collections for each were made and deposited in the herbarium of the Department of Botany, St. Joseph's College, Tiruchirappalli.

Reproductive Biology

Flowering period

Flowering of *Canarium strictum* Roxb. was observed in the month of March-April while fruiting was observed in the month of April onwards. On a random subset of ten panicles were monitored to observe the time of flowering. The flowering time was noted 11.30hr in the month of 10 March 2013. The phenomenon of flowering and fruiting is determined by the photoperiod of a specific region which again is influenced by the variation in seasons such as monsoon, winter and summer, hence always differs from one geographical region to other.

Information on Inflorescence

On each twig 5-8 panicles are noticed. Each inflorescence consists of 7 to 12 flowers and an average of ten flowers. An average of 2-3 flowers open per peduncle. Inflorescense is axillary or terminal panicle.

Flower morphology

Flowers 3-merous, *polygamous*, to 8 mm across. Male: *Calyx*-tube campanulate, pubescent without, 5 mm; lobes 3, triangular, 1mm. *Petals* 3, pale yellow, oblong, 7 × 3.5 mm, concave, apiculate. *Disc* annular, ca. 6-lobed, apically pilose, intrastaminal. *Staminal tube* to 3 mm. *Stamens* 6, free from disc; filaments 1 to 2 mm; anthers oblong, subequal; pistillode short. *Bisexual*: *Calyx*-tube urceolate, 4 mm, pubescent without; lobes 3, triangular, 0.5 mm. *Petals* 3, oblong, to 8 mm. pubescent without. *Disc* obscurely lobed, pilose above. *Staminal tube* to 3 mm; filaments 0.5 mm; anthers subequal. Ovary to 3.5 mm, pilose, 3-celled; ovule 1 per cell; style to 1.5 mm; stigma capitates. Drupe oblong, 4 × 1.5 cm.

Number of ovule/flower

Ovary 3 celled. 1 ovule per cell, in each drupe contains 1 or 2 ovules that are matured and another one is immature.

Number of pollen/Anther counting

The number of pollen ranged from 828 to 1206, mean number of pollen being 946.

Pollen viability

The study revealed that the range of pollen fertility in 100 percentage.

In vitro germination of pollen

Pollen germination was observed at 40 x. Out of 50 pollens only 6 have germinated in this experiment.

Stigma receptivity

There was no color change in the stigma.

Fruit characters and Seed setting

Out of 100 seeds randomly selected 47 seeds were damaged, and 53 seeds were healthy. Seed weight ranged from 2.634 to 6.453 gm. Fruit weight ranged from 6.028 to 12.740gm.

Seed germination

Canarium strictum seeds, when given different treatments separately (control, scarification, heat, cow dung, osmotic potential, H_2SO_4, GA_3, field) gave varied percentage of germination at different time duration (Table1).

Table 1. Influence of various dormancy breaking methods on *Canarium strictum.*

Treatment	Treated Seeds	Germinated seeds			Germination Percentage (%)
Control	25 x 3	-	-	-	-
Scarified	25 x 3	6	2	7	20
Heat 50˚C	25 x 3	-	-	-	-
Heat 75˚C	25 x 3	-	-	-	-
Heat 100˚C	25 x 3	-	-	-	-
Osmotic potential	25 x 3	21	11	20	69.3
Cow dung	25 x 3	12	14	13	52
GA_3 (1000ppm)	25 x 3	11	17	14	56
GA_3 (2000ppm)	25 x 3	-	-	-	-
H_2SO_4	25 x 3	-	-	-	-
Field test	25 x 3	-	1	2	2

Scarified seeds germinated on 21th day but the percentage of germination was 20%. Osmoprimed seeds started to germinate after 21 days of incubation and it continued for prolonged periods and at the end 69.3% germination was recorded. Among the three concentrations of GA_3 treatments only one seed at 1000 ppm concentration germinated (5.6%) on 17th day. Cow dung slurry treated seeds germinated on 44th day with 52% germination significantly. In other treatment like acid treatment, heat treatment seed germination was nil.

Discussion

Identification of the functional and adaptive significance of variation in flower morphology is fundamental to our understanding of the processes that shape patterns of seed production and floral evolution (Campbell, 1991; Galen and Cuba, 2001; Aigner, 2004). The position of the stigma within the flower is a key aspect of flower morphology which influences the efficiency of pollen transfer (Campbell *et al.*, 1996; Nishihiro *et al.*, 2000).There could be factors other than pollen fertility influencing seed setting percentage. The pre-fertilization stages like pollen germination, pollen tube elongation might be sensitive to lower minimum temperature resulting reduced seed set. Similar observations on higher pollen fertility and lower spikelet fertility were recorded in rice by Sampath (1964) and Sivasubramaniam *et al.*, (1972).Interestingly pollen fertility percentage had no association with seed set percentage, number of seeds per panicle and grain yield per plant in all the dates except 3rd date. This indicates that pollen fertility may not be related to spikelet fertility. Previous reports have indicated higher pollen fertility but lower seed set (Sampath, 1964). This suggested that the two aspects of sterility i.e., pollen sterility and spikelet sterility may have distinct causes. It is also possible that, environmental factors may influence at pollen germination and pollen tube growth stages and not at the pollen production level (Mukri *et al.*, 2010).In numerous plant species stigmatic receptivity decreases as the flower ages. At senescence, the stigmatic papillae in *Actinidia* lost their integrity, cellular content was released into the stigmatic fluid, and the secretion contained phenolic compounds which may regulate whether pollen germination occurs (González *et al.*, 1994; 1995).Based on seed germination Suthar *et al.*, (2009) tested various methods of mechanical scarification in *Solanum nigrum* and found that sand paper scarification produced better results, whereas mechanical scarification with chemical scarification enhanced the seed dormancy in *Albizia gummifera, A. grandibracteata* (Tigabu and Oden 2001). The seeds of several members of the family Fabaceae were found to be sensitive to heat and germination of their seeds was enhanced by temperature above 120˚C (Herranz *et al.*, 1998). However, treatment with optimum temperature was not much influenced by the duration of treatment applied. Rosello and Myol

(2002) reported that the seeds of *Lysimachia minoricensis* pretreated at 80˚C have germinated 90% showing less difference from the control. Scarification and heat treatments proved to be futile for *Aristolochia tagala*. Osmopriming was found to enhance germination ability in *Podophyllum hexandrum, Gentiana kurroo* and *Berberis aristata* especially *Berberis aristata* exhibited highest percentage of germination at -10 bar potassium dihydrogen phosphate (Thakur, 2008). Onion seeds osmotically primed in polyethylene glycol solution (342 g/kg water) for 14 days improved the rate of germination (Dearman *et al.*, 1986). Osmoprimed *Aristrolochia* seeds also exhibited similar results (Soosairaj *et al.*, 2013).

Gibberellin is required to overcome the germination constrains imposed by seed coat and abscisic acid related with embryo dormancy (Debeaujon and Koornneef 2000). In many cases dormancy breaking condition, gibberellins seem to be a must (Groot and Karssen 1987, Yan *et al.*, 2002). *Rhododendron maddenni* and *R. niveum* seeds responded well when treated with gibberellin (Tiwari and Chauhan 2007). Seeds of *Comparettia falcata* germinated (90%) when grown in medium added with Kinetin and GA_3 at the concentration of 15 µM (Pedroza-Manrique *et al.*, 2005) produced increased seed germination rate. Thus, the results of the present investigation clearly show that treatments with potassium di-hydrogen phosphate and GA_3 separately break the dormancy in seeds of *Canarium strictum*. Hence these treatments could be useful in enhancing its population under natural conditions.

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