Floral biology and embryological studies are important for conservation of threatened plants having reproductive bottlenecks: a case study of *Illicium griffithii* Hook. f. & Thomson

E. J. Marbaniang[1], N. Venugopal[1,*], Susheel Verma[2], Ravinder Raina[3], Ankush Khajuria[2] and Kamini Gautam[3]

[1]Department of Botany, Centre of Advanced Studies in Botany, North Eastern Hill University, Shillong 793 022, India
[2]Centre for Biodiversity Studies, Baba Ghulam Shah Badshah University, Rajouri 185 234, India
[3]Department of Forest Products, Dr Y.S. Parmar University of Horticulture and Forestry, Solan 173 230, India

Information on reproductive biology of threatened plant species could be useful for conservation, particularly when the species fails to perpetuate in nature due to regeneration failure. In flowering plants, the domain of reproductive biology includes structural details of reproductive units such as flower or inflorescence, formation of viable gametes, pollination dynamics, role of pollinators, pollen–pistil interactions as determined through compatibility, breeding system and mating strategies, fertilization and embryogeny, seed development, dispersal and germination. The importance of reproductive biology in species conservation has been demonstrated through a case study of *Illicium griffithii* Hook f. & Thomson, a threatened plant species from Arunachal Pradesh. *I. griffithii* (Illiciaceae) is a member of the ANITA clade and a representative taxon of the three most basal angiosperms. The flowers of *I. griffithii* are obligate xenogamous, and hence compatible pollen grains only germinate on the stigmatic papillae. Esterase and phosphatase enzymatic activities are absent in most stigmatic surfaces. Therefore, stigmatic receptivity is absent in many carpels. The ovules are anatropous and bitegmic with a four-celled/four-nucleate structure of embryo sac at maturity. Antipodals and filiform apparatus are absent. Presence of mucilage cells in the embryo sac facilitates the entry of pollen tubes into it. Embryolike organization of the endosperm is present, with one pole globular having smaller cells and the other pole roughly filamentous with larger cells resembling that of a suspensor. Extragynoecial compitum is not observed in *I. griffithii*. The flowers are brooding sites for the midges and the young ovules are eaten by larvae of the midges. Only 10% of the flowers mature into 13-seeded fruits and the carpels of the remaining 90% of the flowers have seeds ranging from 1 to 5. The seedling survival rate is only 7%. In addition to the above-mentioned reproductive bottlenecks, the species is also being over-harvested. It is now categorized as endangered. The findings of the present study on reproductive biology of *I. griffithii* should help in improving its conservation status.

Keywords: Embryological studies, floral biology, *Illicium griffithii*, reproductive bottlenecks, threatened species conservation.

Introduction

ALTHOUGH several attempts are being made for conservation of threatened plant species globally through a combination of *in situ* and *ex situ* methods, in several cases success is limited due to intrinsic reproductive constraints of the target species[1–5]. A successful conservation programme for threatened taxa must incorporate the basic information on their reproductive biology, particularly for those which are not affected by any exogenous and management factor. Reproductive biology encompasses structures, mechanisms and biotic interactions that are involved in the generation of progeny leading to the perpetuation of species. Studies on reproductive biology of a taxon generate information on its life-history events from birth to death, modes of reproduction, state of generation and distribution of genetic diversity, and its influence on fitness and evolution of taxa.

The domain of reproductive biology encompasses several processes that range from gametogenesis to germination of seeds. The parameters studied under reproductive biology of angiosperm taxa include structural details of reproductive unit(s), i.e. flower or inflorescence, generation of viable gametes, pollination dynamics, role of pollinators, pollen–pistil interactions as determined by compatibility, breeding system and mating strategies, fertilization and embryogeny, and seed development, dispersal and germination.

Constraint in sexual reproduction in plants is the result of anomalies at any of the above stages. Gametogenesis is

the first and fundamental process of sexual reproduction underlying the formation of haploid and genetically recombined gametes, which act as the carriers of genetic variability to the next generation. Anomalies during gametogenesis in male[6] as well as female[7] have been reported to form non-functional gametes causing partial to complete failure of sexual reproduction. Pollination biology and breeding system constitute the two interdependent areas of sexual reproduction in angiosperms that have also been widely investigated for different species[8–12]. While pollination in a species serves to disperse pollen and is responsible for gene flow, the breeding system determines the direction and pattern thereof.

Studies in a number of threatened taxa having small populations have shown that pollination failure, resulting mostly from disruption of mutualistic plant–pollinator interactions, leads to failure of seed set[10]. Effects of genetic stochasticity such as inbreeding depression in small and fragmented populations of threatened plant species are also due to altered patterns of mating strategies and pollinator behaviour. Self-incompatibility in some threatened species also poses survival threat under conditions of unavailability of pollen from suitable mating type. In case of fragmented habitats, especially in clonally propagating species where all the plants in a habitat patch may belong to the same type, self-incompatibility becomes more common. There are some species where seed or fruit abortion leads to low reproductive output, whereas in other cases poor seed dispersal proves detrimental. A plant species may have either a single or multiple bottlenecks in reproduction, thus threatening its perpetuation across generations. Therefore, significance of different steps in sexual reproduction in maintaining a viable population with ample genetic variability and heterozygosity for its survival in nature, fitness and evolution cannot be undermined. A case study on reproductive biology of *Illicium griffithii* Hook f. & Thomson, a threatened plant species is presented here to demonstrate the role of reproductive bottlenecks in making a taxon threatened. Addressing the challenge(s) identified through the study of reproductive biology can improve the conservation status of the species.

Materials and methods

I. griffithii (family Illiciaceae) is one of the 42 species of the genus *Illicium* found worldwide, and three species found in India. 'Illicium' is derived from the Latin word 'Illicere', meaning to attract or entice, referring to the pleasant fragrance of the fruits of *Illicium*. In India, *I. simonsii* Maxim. and *I. arunachalensis* N. Venugopal & Ester Jones Marbaniang are the other two species[13,14]. Flowers of *Illicium* are hermaphrodite, small to medium-sized and mostly white, yellow or reddish-purple. Fruitlets are flattened with woody fruits when mature. The fruit follicles are arranged in a star-shaped pattern, hence *Illicium* is commonly known as 'star anise'[15]. The family Illiciaceae

is a member of the ANITA clade that consists of the three most basal angiosperm lineages, viz. the Amborellaceae, the Nymphaeales (Nymphaeaceae and Cabombaceae), and the Austrobaileyales (Austrobaileyaceae, Trimeniaceae, Illiciaceae and Schisandraceae)[1–3,16–18].

In the present study, *I. griffithii* was collected from Bomdila area, Arunachal Pradesh for four consecutive years (2011–14). The collection area lies between 27°05′19.7″N and 92°35′29.1″E at an altitude of about 2598 m amsl.

Stigma receptiveness was studied by assaying esterase and phosphatase enzyme activities. On the stigmatic surface, non-specific esterase and phosphatase were localized using α-naphthyl acetate[19,20] and α-naphthyl acid phosphate[21] as substrates. Cytochemical localization of non-specific esterases and phosphatases is based on hydrolysis of the substrate α-naphthyl acetate and α-naphthyl acid phosphate respectively. The resulting product α-naphthol is colourless, and forms a reddish insoluble complex with the coupling agent fast blue B and fast garnet GBC respectively. Stigmatic receptivity and temporal details of post-pollination events were recorded[22]. Floral herbivory was studied through close observation of the whole flower as well as individual floral parts by repeated monitoring every day during the flowering period. Each flower was monitored continuously for five days, i.e. from the day it opens till it sheds its tepals. Three consecutive flowering periods spread over 15 days were monitored taking 100 flowers as sample each time. Seed production was assessed by direct counting of seeds in 100 fruits and calculating the mean value per fruit. Seedling survival was assessed by tagging 500 seedlings and monitoring their fate over a period of three years, i.e. 2012–14.

Flowers of different developmental stages of *I. griffithii* were collected from Bomdila area and fixed in FAA (formalin – 5 ml : acetic acid – 5 ml : 70% alcohol – 90 ml). The collected plant samples were then subjected to microtomy by the usual dehydration method using tertiary butyl alcohol series followed by impregnation with paraffin wax[11,12,23]. The paraffin blocks were trimmed and sectioned at a thickness of 7–10 μm using Leica RM 2125 RT rotary microtome. The sections were then stained using safranin and fast green FCF[23]. Photomicrographs were obtained using Olympus BX 43 microscope and Olympus U-LH50HG Epifluorescence microscope. Scanning electron microscope (SEM) was also used in the study at the Sophisticated Analytical Instrument Facility (SAIF), NEHU, Shillong[24,25].

Results

Morphological characters

I. griffithii is an evergreen, medium-sized tree (Figure 1 *a*) with 10–15 days leaf fall during April. The flowers

are creamy-white to white in colour, aromatic, axile, solitary, erect to drooping and hermaphrodite (Figure 1 *b*). An individual flower has 23–25 tepals (3 mm–1.6 cm × 3–5 mm) which are arranged in three whorls; the outer whorl is strap to tongue-like, the middle is tongue-like to subulate, and the inner whorl is subulate to lanceolate (Figure 1 *c*).

Androecium consists of 27–29 stamens arranged in two whorls encircling the carpels (Figure 1 *c*) with flat filaments. Anther lobes are yellowish-green, dorsifixed with lateral dehiscence of tricolpate pollen grains having reticulate exine (Figure 1 *f*).

Gynoecium consists of 13–20 carpels arranged in a single whorl around the thalamus. The carpels are conduplicate, open and apocarpous. The appressed surfaces of carpel valves form the horizontal stigmatic surface. The stigmatic surfaces cover about three-fourth of the carpel, and are pappilate (wet stigma) and recurved (Figure 2 *a*). Transverse section of a flower shows the presence of a single ovule within each carpel (Figure 2 *b*). The flower usually remains open only for 3–5 days, after which the anthers and tepals drop leaving the carpels to develop into fruits. The carpels enlarge in size after fertilization and the vasculature is greatly augmented. Expansion of carpels during fruit development and post-genital fusion of the walls of the lateral carpel take place. The receptacle also expands vertically and radially to accommodate maturation of the carpels. The stigmas are separated, but in the floral centre there is a hump with secretory surface.
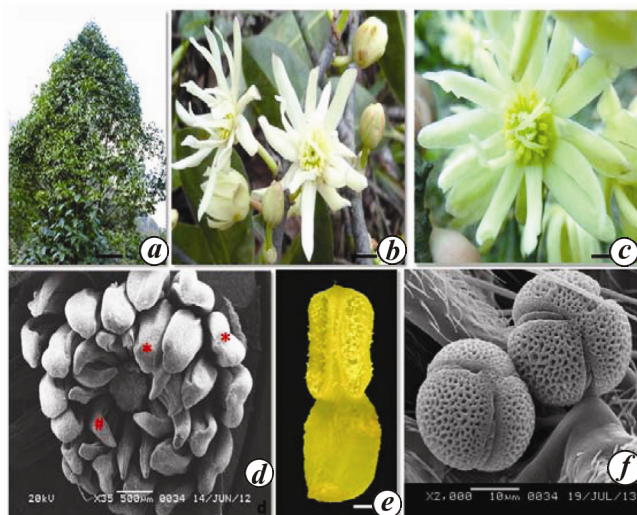
Carpels and central hump are contiguous but not fused with each other (Figure 2 *b*).

The flowers of *I. griffithii* are obligate xenogamous and hence compatible pollen grains only germinate on the stigmatic papillae. Esterase and phosphatase enzymatic activities are absent in most stigmatic surfaces. Therefore, stigmatic receptivity is absent in many carpels.

Embryological development

In *I. griffithii*, the ovules are anatropous, solitary and bitegmic (Figure 2 *c*). During the initiation of the integuments, a single hypodermal archesporial cell becomes differentiated at the apex of the nucellus. This archesporial cell divides to form the primary parietal cell and the primary sporogenous cell. The latter does not undergo further division, instead it increases in size and becomes more prominent, and then functions directly as the megaspore mother cell (Figure 2 *d*). The megaspore mother cell undergoes division to form a linear row of four megaspores, which collectively constitute the tetrad (Figure 2 *e*).

The chalazal megaspore maintains a relatively large size, undergoes enlargement, and is the functional megaspore (Figure 2 *f* and *g*). While the functional megaspore
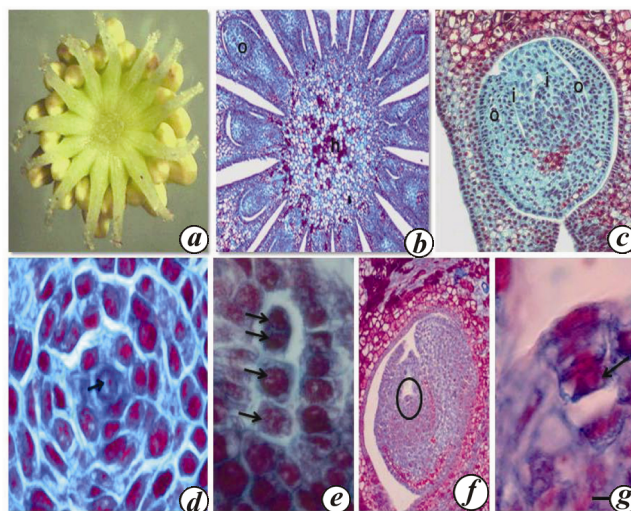

Figure 1. *a*, An evergreen, medium-sized tree in its natural habitat (bar = 1.2 m). *b*, A flowering branch showing the solitary, erect to drooping and hermaphrodite creamy-white to white-coloured flowers with flower buds (bar = 1.4 cm). *c*, Enlarged view of a single flower depicting the spiral arrangement of carpels, yellowish anthers and white tepals (bar = 0.5 cm). *d*, SEM of a young flower bud showing the two whorls arrangement of the anthers (*) surrounding the carpels (#). *e*, Enlarged view of a single yellowish and matured stamen with lateral dehiscence of pollen grains (bar = 0.5 cm). *f*, SEM of tricolpate pollen grains with reticulate ornamentation of the exine. Colpi are narrow at the poles and are joined at the distal pole.

Figure 2. *a*, Enlarged view of conduplicate, open and apocarpous carpels in which the appressed surfaces of carpel valves form horizontal stigmatic surface. Recurved and pappilate stigmatic surfaces (wet stigma) covered to about three-fourth of the carpel. Note the protogynous and glistening nature of the stigmatic surfaces (bar = 0.1 cm). *b*, L.S. of a young carpel showing the presence of a single ovule (o) within each carpel (bar = 400 μm). *c*, L.S. of a carpel showing the bitegmic ovule (bar = 100 μm) (o, outer integument; i, inner integument). *d*, Enlarged view of the functional megaspore mother cell (arrow) which increases in size, becomes more prominent with dense cytoplasm and distinct nucleus (bar = 68 μm). *e*, L.S. of an ovule showing the four linear megaspores (bar = 33.64 μm). *f*, L.S. of an ovule showing the chalazal megaspore (circle) which is the functional megaspore (bar = 200 μm). *g*, Enlarge view of the chalazal megaspore which is relatively larger in size (bar = 50 μm).

enlarges, its nucleus moves towards the micropylar portion of the cell and undergoes two mitotic divisions. The first mitotic division results in the formation of two nuclei. The two nuclei remain in close proximity to one another within the micropylar region instead of migrating to the opposite poles (Figure 3 *a* and *b*).

Further elongation of the female gametophyte is accompanied by broadening of the micropylar region, so that the female gametophyte appears club-shaped in later stages. After the second mitosis (Figure 3 *c*), four free nuclei are present within the micropylar region. The four-nucleate stage is quickly followed by cellularization, in which three uninucleate cells are formed at the micropylar pole (Figure 3 *d* and *e*), and the fourth nucleus is situated at the centre of the female gametophyte forming the central cell (Figure 3 *f*). At maturity, the female gametophyte is a four-celled/four-nucleate structure with a single nucleus positioned in the central region of the embryo sac. Antipodal cells are absent and the chalazal region remains unfilled.

As the pollen tube makes its way between the stigmatic papillae of the carpel and reaches the embryo sac, the contents of the pollen tube are discharged into the embryo sac. At fertilization, one male gamete fuses with the egg and the other with the polar nucleus to form the zygote (Figure 4 *a* and *b*) and primary endosperm nucleus (PEN) (Figure 4 *a* and *c*) respectively.
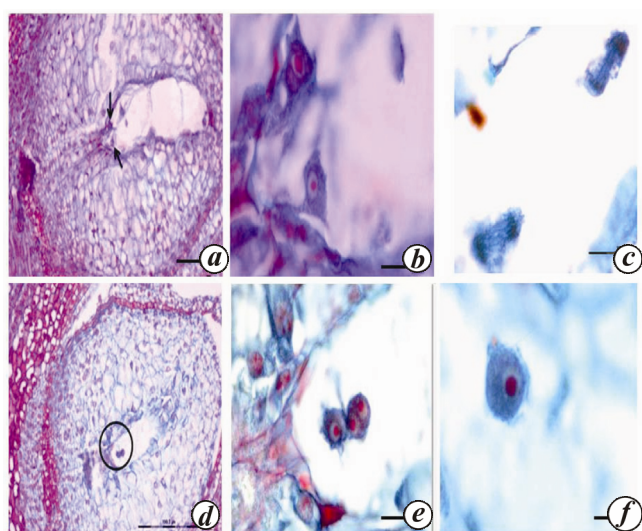
The zygote is elongated ovoid and located at the micropylar pole of the embryo sac with its basal portion attached to the embryo sac wall and the apical portion projected into the central cell. The apical end of the zygote contains the nucleus and cytoplasm, while the basal portion is slender and vacuolated. The first asymmetric division of the zygote yields an apical cell (Ca) and a basal cell (Cb) (Figure 4 *d*).

The apical cell divides transversely to form a four-celled filamentous proembryo (Figure 5 *a* and *b*), while the basal cell divides to form the prominent linear suspensor which is 4–5-celled (Figure 5 *c* and *d*). The growth of the suspensor is rapid between the globular and filamentous stages of development. Thereafter, further increase in length ceases, and during later stages, the suspensor begins to degenerate and is apparently absorbed by the embryo. Further development of the embryo takes place following numerous cell divisions in various planes and causes the proembryo to assume a globular configuration (Figure 5 *c*). The globular proembryo passes through a phase before cotyledons and epicotyls become outwardly evident at specified loci, and there is transition from radial symmetry to bilateral symmetry (Figure 5 *d* and *e*). At the heart-shaped stage, the vascular initials that connect the root and shoot apices are formed and cotyledon development begins (Figure 5 *f*). During development of the heart-shaped embryo, the suspensor is entirely absorbed by the embryo and becomes degenerated and disorganized.
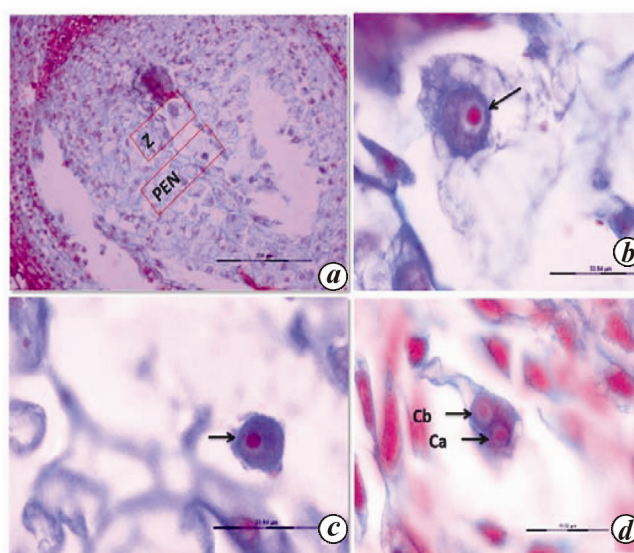

Figure 3. *a*, L.S. of a mature ovule showing two nuclei at the micropylar region (arrows) that remain in close proximity to one another (bar = 200 μm). *b*, Enlarged view of the two nuclei at the micropylar region, each with a prominent nucleus (bar = 33.64 μm). *c*, Second mitotic division of the two nuclei within the embryo sac. Late anaphase with distinct spindle fibres (bar = 33.64 μm). *d*, Three nuclei at the micropylar region formed after the second mitotic division (bar = 300 μm). *e*, Enlarged view of the three nuclei at the micropylar region which form egg apparatus (bar = 33.64 μm). *f*, Enlarged view of a single polar nucleus at the centre of the embryo sac which is four-nucleate (bar = 33.64 μm).

Figure 4. *a*, Zygote (Z) and primary endosperm nucleus (PEN) formed after double fertilization in the embryo sac (bar = 400 μm). *b*, Enlarged view of zygote (arrow) which is larger in size, consisting of a prominent nucleus and dense cytoplasm situated at the chalazal region of the embryo sac (bar = 68 μm). *c*, Enlarged view of the primary endosperm nucleus (arrow) formed at the centre of the embryo sac (bar = 68 μm). *d*, First division of the zygote leads to the formation of two small cells – the basal cell (Cb) and the apical cell (Ca) (bar = 68 μm).

Endosperm in *I. griffithii* shows a distinctly bipolar nature. It has two clear-cut regions, viz. chalazal region and micropylar region separated by the cell wall (Figure 6 *a*). The two regions show independent patterns of development. The micropylar domain initiates a free nuclear pattern of development, while the chalazal domain undergoes cellular development (Figure 6 *b*). Later, with the development of the globular embryo the endosperm becomes cellular, except that surrounding the embryo which remains nuclear. As the embryo becomes heart-shaped, the endosperm becomes completely cellular (Figure 6 *b*).

Embryo-like organization of the endosperm exists in *I. griffithii*, in which one pole is globular with smaller cells and the other pole is roughly filamentous with larger cells resembling that of a suspensor (Figure 6 *c* and *d*).

Seed maturation, herbivory and seedling survival

Seeds are matured as indicated by glossy brown colour, ~30 days after pollination. Only 10% of the flowers mature into 13-seeded fruits and the carpels of the remaining 90% of the flowers have seeds ranging from 1 to 5. The flowers are the brooding sites for the gall midges (Cecidomyiidae) and the young ovules are often damaged by larvae of the midges. The survival rate of seedlings is very low, i.e. only 7%.
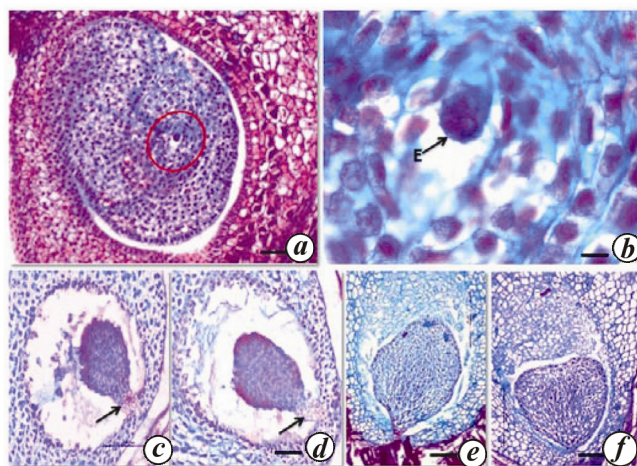
Discussion

Formation of viable gametes is fundamental to the success of sexual reproduction in plants. Pollen grains and embryo sac represent male and female gametophytes produced by meiosis in the micro- and mega-spore mother cells inside the anther and ovule respectively. Meiosis during the life cycle of a plant signals the transition from sporophytic to gametophytic generation as gametes are the specialized cells with haploid genome and altered gene combinations resulting from gene recombination between homologous chromosomes. Fusion of gametes not only restores original chromosome number in zygote of the species, but also creates new genotypes adding to the genetic variability of the species. Though crucial, this aspect of reproductive biology is relatively less explored in the case of threatened plant species, and the information available focuses mainly on male gametophyte development. Meiotic irregularities have been reported in some taxa, which directly affect pollen fertility and ultimately the success of reproduction. *Manglietia aromatica* (Magnoliaceae), a threatened plant species of China, faced severe depletion due to human activities and was found to have very poor regeneration due to reproductive constraints[26]. Pollen in this species had very poor germinability of 0.01%; however, primary mother cells (PMCs) meiosis was normal. Moreover, development of female gametophyte in this species showed anomalies and degeneration of megaspore and/or egg apparatus. The low seed set in this species has been attributed to the production of abnormal gametes and has also been accepted as
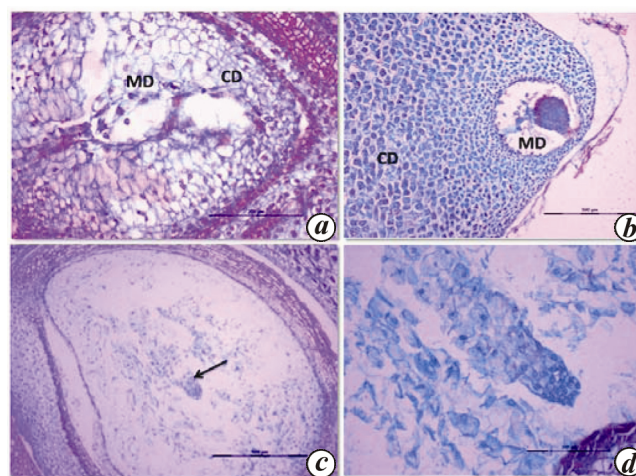

Figure 5. *a*, Micropylar position of proembryo (circle) (bar = 500 μm). *b*, Enlarged view of the quadrant proembryo (bar = 200 μm). *c*, Enlarged view of the globular embryo with well-developed suspensor cells (arrow). The suspensor is prominent, linear with 4–5 cells (bar = 800 μm). *d*, Transition stage of the embryo between globular and heart-shaped. Note only the endosperm surrounding the embryo is nuclear, while the rest is cellular. The suspensor is 4–5 cells (bar = 800 μm). *e*, Enlarged view of a late globular embryo in which most of the suspensor cells are absorbed by the developing embryo and begin to degenerate. Note the endosperm is almost cellular (bar = 500 μm). *f*, Enlarged view of a heart-shaped embryo with no suspensor cells and endosperm is now cellular (bar = 500 μm).

Figure 6. *a*, Distinct bipolar nature of endosperm development having a clear-cut micropylar region (MD) and chalazal region (CD) separated by a cell wall (bar = 400 μm). *b*, MD initiates a free nuclear pattern of development, while CD undergoes cellularization (bar = 1000 μm). *c*, Embryo-like organization of the endosperm (bar = 1000 μm). *d*, Enlarged view of the embryo like organization of the endosperm in which one pole is globular with smaller cells and the other pole is roughly filamentous with larger cells, resembling a suspensor (bar = 400 μm).

the reason for poor regeneration and endangerment of this taxon along with anthropogenic causes.

PMC meiosis studied in *Cephaelis ipecacuanha* (Rubiaceae), a threatened medicinal plant species of Brazil, has revealed several irregularities like ascending chromosomes, laggards and chromosome sets leading to pollen sterility. Although this species also multiplies vegetatively, low pollen viability affects sexuality of the species, and also increases the frequency of genetically uniform individuals in populations[27].

Very low reproductive output in *Camellia grijsii* (Theaceae), an endangered tree species has been attributed to a high percentage of abortive ovules as revealed by histological studies[28]. A critically endangered species of the Northwestern Himalayan region, *Inula racemoses* has also been reported with meiotic irregularities like inter-bivalent connections, chromosomal stickiness and unequal segregation that lead to high pollen sterility[29].

At least three species of medicinally important genus *Valeriana* (Valerianaceae) have been reported with a significant level of pollen sterility[30]. In *V. officinalis* and *V. scandens*, sterile pollen grains are produced due to early degeneration of tapetum[30]. In *V. nitida*, aneuploidy has been described as the reason for pollen sterility[31]. In *V. wallichii* plants with a high percentage of sterile pollen have been detected with meiotic irregularities like non-disjunction of homologues during the first meiotic division[32,33]. *V. wallichii* has been declared endangered due to overexploitation for pharmacological purpose.

Gentiana kurrooa, a threatened medicinal herb of temperate Himalaya shows fast decline in pollen viability with only 3.7% pollen remaining viable after 108 h from anther dehiscence stage[34]. These case studies indicate that several threatened species suffer reproductive stress during and after the gametogenic process, and are bound to have low reproductive outcome which limits their regeneration potential.

Successful pollination is a pre-condition for fertilization and sexual seed set in angiosperms. This step in sexual reproduction of angiosperms involves active mutualistic interactions between plants and animals, mostly insects. Bees, flies, butterflies, moths, beetles, ants, snails, birds and bats are the well-known pollinators[35–39]. Plant–pollinator interactions can be specific where a particular species of plant is pollinated only by some specific animal species or vice versa, or diffuse where there is a wide spectrum of pollinators for a plant species and vice versa[35–39]. Not only do the pollinators transfer pollen among plants and effect fertilization, but their foraging behaviour and visiting range also determines the genetic structure of populations and has been an important factor in devising mating patterns of species[40].

In context of the reproductive biology of threatened plant species, the role of successful pollination is of utmost importance in ensuring reproductive success, as a number of case studies in rare and threatened plants have highlighted the pollinator limitation in small populations as an impinging factor for low seed set[41]. Small populations are known to suffer a drop in pollinator visitation rate, which is attributed to their being less attractive to the pollinators than the large aggregated populations[42]. *Primula vulgaris*, a threatened herbaceous species shows a comparative decrease in reproductive output in smaller fragmented populations due to a decreased rate of pollinator visitation[43]. A threatened alpine species of Hawaii, *Argyroxiphium sandwicense* subsp. *macrocephalum* (Asteraceae) has been investigated to assess the impact of its declining populations on pollinator visitation rates and consequently on the seed set. A number of studies on this taxon revealed that the species has an unspecialized pollinator system consisting of many entomophilous species with the chief pollinator being *Hylaeus* bees. The isolated patches receive much less pollinator visits than those having large aggregation of plants, which directly influences seed set in the former. Species possess cross-incompatibility in the case of pollen from half and full siblings. Due to poor seed dispersal much of the plants in a close aggregation belong to the same genetic stock and have the same kind of incompatible alleles. Since *Hylaeus* bees were found effective in only short-distance cross-pollination, habitat fragmentation and large interplant distances hamper seed set. However, populations at some sites having abundance of other pollinators like beetles were able to overcome such limitation. Therefore, strategies to protect native arthropod pollinators were suggested as an effective measure to improve seed set to recover and replenish the species[44].

Caldesia parnassifolia, a threatened plant species from China suffers a drop in pollinator visitation rates in populations found near agricultural fields due to the use of insecticides. The fruit set in such populations was lower than the other studied populations of the species receiving sufficient pollinator services. Moreover, the rate of production of vegetative propagules was also higher in these populations than the latter. Higher pollen–ovule ratio and early flowering are also reported in these populations, which reflects strategies to overcome pollen scarcity[45].

Disruption in the foraging range of pollinators due to fragmentation of a continuous habitat into smaller patches is the main reason for the drop in pollinator frequency in small populations. Moreover, such small populations with scattered individuals also fail to attract pollinators. This isolation effect was observed in *Picrorhiza kurrooa*, a threatened medicinal plant species found in the alpine Himalaya. The species multiplies vegetatively and forms thick mats over the ground, but due to large-scale exploitation from natural habitat, the naturally long patches are transformed to very small scattered patches often surrounded by many other flowering annuals. The patches become too inconspicuous to be noticed by the already scarce pollinator fauna of alpines, mainly

consisting of *Bombus* species. This has a direct bearing on the percentage of spikes with fruits and also percentage seed set per capsule. Hand-pollinations revealed the species to be cross- as well as self-compatible[32]. Effects of pollinator limitation are even more drastic in case the species in question is self-incompatible[46,47]. In such cases, scarcity of suitable mate with appropriate S-allele is encountered due to depletion of S-allele diversity in small populations.

In self-incompatible systems, restriction of pollinators foraging range to small patches may lead to no seed set if the patches consist of individuals with the same S-allele as in the case of vegetatively propagating species. Even if the compatible pollens are available, excess of self-pollen deposition over stigma may interfere with the germination of outcrossed pollens and reduce the seed set[48]. Many medicinal plants like *Stevia rebaudiana*, *Aconitum heterophyllum*, *Sausurrea costus*, *Inula racemosa* are self-incompatible, depending completely either on pollinators or human interventions for successful seed set/reproduction[49–52].

In the fragmented populations of self-compatible plant species, restricted foraging range of pollinators may lead to increased chances of geitonogamy or autogamy. This leads to increased selfing rate in such small populations[53]. An important consequence of such short-distance pollination is inbreeding depression observed in some species[54]. Absence of pollinators from a particular habitat may be due to a number of factors, including variation in temperature, extent of pollution, pesticides or herbicides exposing the species to pollen stress and many a times, if self-compatible, may resolve to autogamy by delayed selfing in order to ensure reproductive success, although to lesser extent. This has been demonstrated in case of threatened species *Valeriana wallichii*, with gynodioecious populations and a mixed breeding system[55]. Several species of *Apis* effect pollination. However, pollen stress is imposed under conditions of rainfall during peak flowering season or rise in temperature during the later part of the season leading to almost negligible pollinator visitation. During this period in the hermaphrodite plants, stigma approaches the self-anthers and lies very close to them or even touches them by the specific curvature or bend in the style. This has been termed as stylar bending[55] and helps to achieve some degree of reproductive success. On the other hand, stigma of female flowers carries no pollen load under such conditions. In some species, insects not only act as pollen carriers but also affect anther dehiscence by exerting mechanical pressure on specialized structures located on the anther wall so that pollens get dispersed. This observation has been reported in *Incarvellia emodii*[56]. In some cases insects affect pollination by making the stamens move towards the receptive stigma, so that dehisced anthers get stuck to it and deposit pollen there. This has been observed in *Berberis lyceum*, where pollination and consequently reproduction are ensured through the intervention of insects, which do not affect pollen transfer to the stigma directly but by touching the base of the staminal filament while foraging nectar secreted by nectarines at the base of the corolla, thus leading to stamina movement[57]. *P. kurrooa*, a cross-pollinating threatened Himalayan medicinal herb producing only up to 9% fruit set under geitonogamous/self-pollination, requires pollination vectors in nature to facilitate successful pollination and seed set[58]. *Nardostachys grandiflora*, a critically endangered medicinal and aromatic species of the Himalaya shows infrequent flowering with only 8%–10% plants in a population bearing flowers in a season, thereby limiting seed set and reproduction[59]. *Gloriosa superba*, a threatened medicinal herb depends entirely on pollinators, especially birds, for reproduction because of the presence of herkogamous flowers that avoid passive autogamy; however, geitonogamy or active autogamy is also possible which can ensure successful pollination[60].

Therefore, pollination studies are vital to understanding the low reproductive rates in several threatened plant species. Plant–pollinator interactions ensure both reproduction of plants and food availability to the insects; but the former gets more benefit from these interactions as patterns of gene flow through pollen are also guided by the visiting range and foraging behaviour of the pollinator, and as such they become an important determinant of the genetic structure and evolutionary potential of the populations. Restoration of habitat to protect native pollinator fauna has been suggested as a measure to not only ensure plant reproduction, but also for restoring and maintaining natural patterns of gene flow between populations[61].

Seed is the ultimate outcome of sexual reproduction and the propagule for the future generations. Seed dispersal not only leads to the spread of species to new areas establishing new populations, but also facilitates flow of genes from one population to another. Seeds are thus considered as important determinants of metapopulation dynamics and species composition in ecological communities[62]. Poor germination, mortality and poor recruitment of seedlings are often studied as a measure of fitness and indicators of inbreeding depression in small populations. Poor seedling recruitment also causes hurdles in sustaining populations of plants[63]. It may be due to various inherent as well as ecological reasons like poor viability, mortality, competition, poor site quality, susceptibility to insect pest attack, weeds and herbivory[63]. Seedling establishment is a major reproductive bottleneck in *Swertia chirayita*, a threatened medicinal herb of the Himalaya, as the seeds are very light which leads to their non-uniform dispersal in nature[64–67]. As the seeds are small and light, they are easily blown away by wind and water causing dense settling on sites, which leads to inter-sibling competition and finally poor seedling establishment.

Loss of seed-dispersing animals through habitat loss or hunting and introduced mammal species has been reported to adversely affect seed dispersal and have been spread of some threatened tree species of New Zealand[68].

In some species, animal intervention in seed dispersal and germination has been found so crucial that it cannot overcome dormancy and start germination unless it passes through the gut of a specific animal[69,70]. *Elaeocarpus blascoi*, an endemic and threatened tree species of the Western Ghats of India has been studied for reproductive biology. It revealed very low germinability (5% only) with 70% of seeds losing viability within a year[71]. Low seed germination has also been reported in threatened *Physoplexis comosa* and *Primula glaucescens* of the Italian Alps[72], and in *Loxopterygium guasango* a threatened tree of the Coastal region in northwestern South America[73]. Poor seed set (up to 8%) has been observed in *Nardostachys grandiflora*, a critically endangered high-altitude Himalayan herb, although plants favour cross-pollination[59]. *Artemisia glauca*, another threatened Himalayan herb showed poor (8%) seed germination[74]. *Podophyllum hexandrum*, a high-altitude, critically endangered Himalayan herb also exhibits seed dormancy with poor germination (7–45%) beginning after 1–2 years[75].

Herbivory is the consumption of fruits, seeds or flowers by animals. Time and magnitude of herbivory are important with respect to the outcome of reproduction, i.e. what proportion of seeds is actually available for germination and dispersal so as to effect the number and distribution range of species population[76]. Comprehensive studies have been conducted on the ecology of plant–herbivore relationship, diversity of herbivores and plant predators, and their effect on the evolution of population dynamics[77].

Despite the works cited, it is evident that not enough information is available on the direct relevance and impact of such interactions on the reproductive success of threatened species. Predispersal herbivory has been noticed in *Ferula jaeschkeana* (Apiaceae), which is categorized vulnerable to extinction due to anthropogenic causes. The umbellate inflorescences of this species are infested by a beetle species. Soon after fruit set, it lays eggs inside the ovary by piercing the ovary wall and after hatching, the larvae feed on the developing seed tissues. About 90% of the seeds are damaged due to herbivory in this species[32]. Similarly, about 73% seed damage due to predation has been recorded in the orchid, *Cypripedium candidum*[78].

In *I. griffithii*, based on the present floral biology and embryological studies, several reproductive bottlenecks have been identified. Embryological characters in basal angiosperms have been the subject of numerous recent studies[4,5,10,79,80], and have also become the focus of the study on the origin of flowering plants. The monosporic seven-celled/eight-nucleate polygonum-type female gametophyte has long been associated with the origin of flowering[6–8,81].

In recent years, there have been studies on female gametophyte development in basal angiosperm lineages, which found variations in the development of female gametophyte even within the clade. The *Amborella* female gametophyte was found to be monosporic and seven-celled/eight-nucleate[8]. In contrast, the *Nuphar* (Nymphaeaceae) female gametophyte was found to be monosporic, but four-celled/four-nucleate; and the finding was true for almost all members of Nymphaeales[80]. Austrobaileyales, Schisandraceae[9] and Illiciaceae[10] have four-celled and four-nucleate female gametophytes. An extragynoecial compitum, though common in the basalmost angiosperms[82], was not observed in *I. griffithii*. In extragynoecial compitum, the apocarpous carpels have contiguous secretory stigmas that allow crossing of pollen tubes between carpels. An extragynoecial compitum that enables pollen tube growth between carpels has been observed as an advantage for fertilization in many species, thus enhancing their reproductive potential[83]. Absence of extragynoecial compitum is thus a major reproductive bottleneck in *I. griffithii*.

The four-celled/four-nucleate development of female gametophyte represents the origin of embryo sac development in angiosperms and the four-celled/four-nucleate embryo sac gives rise to the more common seven-celled/eight-nucleate-type of embryo sac[80].

I. griffithii being a member of the basal angiosperm has primitive characters such as spiral arrangement of the tepals and stamens on the flat thalamus and conduplicate carpels. These features are also seen in the other ANITA clade[84,85]. The stigmas are far apart, but in the floral centre there is a protuberance or a hump-like structure with secretory surface, a characteristic feature found in all the members of basal angiosperm. This protuberance together with the lower ventral parts of the carpels act like a junction where pollen tubes can cross between carpels to fertilize the ovules. This is also a primitive character of the plant. Absence of an extragynoecial compitum, as observed in *I. griffithii*, was also reported in Schisandraceae, Amborellaceae, Austrobaileyaceae, Schisandraceae, Illiciaceae and in some Nymphaeaceae[86].

Pollen grains are tricolpate in *I. griffithii* with a uniformly dense reticulate ornamentation of the exine. This confirms the findings that the pollen grains in the family Illiciaceae have tricolpate apertures[87]. In this study, we found that *Illicium* could be categorized into two groups based on the shape, colour of the tepals, and the type of pollen grains. *Illicium* species with obovate, ovate to suborbicular tepals and pinkish, reddish or purplish-red in colour having trisyncolpate pollen grains may be categorized under one group. On the other hand, *Illicium* species with elliptic, narrowly oblong tepals and whitish or yellowish-green, pale yellow to creamy-white in colour having tricolpate pollens may be categorized under

another group[13]. Pollen grains of some species in the genus were also examined and showed the presence of both trisyncolpate and tricolpate pollen grains with reticulate sculpture of exine[88,89].

Angiosperms exhibit many different patterns of female gametophyte development. During cell differentiation, the nuclei at the micropylar end develop into the egg cell, the micropylar polar nucleus and the synergid cells, while the chalazal nuclei develop into the three antipodal cells and the chalazal polar nucleus. This type of female gametophyte development is common in most of the angiosperms. In *I. griffithii*, organization of the embryo sac is four-celled/four-nucleate without the antipodal cells. This type of female gametophyte development is the *Schisandra*-type, because the first record of this mode of formation of the embryo sac was described in *Schisandra chinensis* of Schisandraceae[90,91]. Most embryological reports in Austrobaileyales are also consistent with the four-celled/four-nucleate pattern of *Illicium*[91–97] and have concluded that this is the ancestral condition.

On the other hand, some authors considered the *Schisandra*-type as a subtype of the *Oenothera*-type of embryo sac. The difference is that in the *Schisandra*-type embryo sac, the chalazal megaspore of the tetrad of megaspores is functional, whereas in the *Oenothera*-type embryo sac, the micropylar megaspore in the tetrad is functional[36]. In addition, the former is restricted to Austrobaileyales and Nymphaeaceae in the basal angiosperms, whereas the latter is restricted to Onagraceae in the Myrtales of the eudicots[98,99].

Mucilage cells found inside the embryo sac of *I. griffithii* are another peculiar characteristic feature of the plant. These mucilaginous cells may direct the pollen tube into the embryo sac during fertilization, but this fact needs further reconfirmation as the development and detailed functioning of these mucilage cells have not been studied here. However, presence of mucilage cells is another characteristic feature of some basal angiosperms. Among the basal-most angiosperms, surface oil cells were found only in the carpels but not in the embryo sac of members of Trimeniaceae, Schisandraceae (*Sarcandra*), Illiciaceae and Chloranthaceae (*Chloranthus*)[86]. The presence of such mucilage cells inside the embryo sac is reported in this study in *I. griffithii* as well as in the family Illiciaceae.

In *I. griffithii*, there is no filiform apparatus. So far, the absence of hooks and indentation on the synergids has been described only in *Plumbagella*, *Ditepalanthus*, *Helosis*, and in a few members of Ulmaceae and Urticaceae[100]. Hence, this is also another novel finding for the family Illiciaceae. No record in this respect was available within the ANITA clade.

The embryo-like nature of endosperms found in *I. griffithii* and among basal angiosperms may have important indications for analysing the evolution of endosperms, and debate has been focused on whether the endosperm represents a highly modified supernumerary embryo or a sexualized modification of a component of female gametophyte development[101–103]. Comparative information on the basic molecular programmes of endosperm and embryo development among basal angiosperms is critical for the evaluation of these alternative hypotheses.

Having obligate xenogamy flowers, absence of stigmatic receptivity in many carpels, and herbivory by the larvae of midges, seed production in the species is extremely restricted. Extragynoecial compita, commonly occurring in basal angiosperms, have been proposed to have the potential to increase the number of offspring in apocarpous species through the intercarpellary growth of pollen tubes[104]. However, absence of extragynoecial compita in *I. griffithii* also explains its poor seed production ability. In addition to the above mentioned reproductive bottlenecks, the species is also being over-harvested. The species is now categorized as endangered.

Conclusion

Study of reproductive biology of threatened plants like *I. griffithii*, identifies the bottlenecks in the process of sexual reproduction. This helps the researchers in designing appropriate species-specific conservation programmes. Despite the significant role of studies on reproductive biology of threatened species, this area has been largely neglected. Scanty reports on the aspect are testimony to this. Therefore, this area of conservation biology needs to be vigorously pursued to achieve the goals of threatened species conservation.

1. Qiu, Y. L. *et al.*, The earliest angiosperms: evidence from mitochondrial plastid and nuclear genomes. *Nature*, 1999, **402**, 404–407.
2. Qiu, Y. L. *et al.*, Phylogeny of basal angiosperms: analyses of five genes from three genomes. *Int. J. Plant Sci.*, 2000, **161**, S3–S27.
3. Renner, S. S., Circumscription and phylogeny of the Laurales: evidence from molecular and morphological data. *Am. J. Bot.*, 1999, **86**, 1301–I315.
4. Friedman, W. E. and Ryerson, K. C., Reconstructing the ancestral female gametophyte of angiosperms: Insights from *Amborella* and other ancient lineages of flowering plants. *Am. J. Bot.*, 2009, **96**, 129–143.
5. Williams, J. H., Novelties of the flowering plant pollen tube underlie diversification of a key life history stage. *Proc. Natl. Acad. Sci. USA*, 2008.
6. Johri, B. M., Recent advances in the embryology of angiosperms. In *Embryology and Taxonomy* (ed. Maheshwari, P.), International Society of Plant Morphologists, Department of Botany, University of Delhi, Delhi, 1963, pp. 395–444.
7. Cronquist, A., *An Integrated System of Classification of Flowering Plants*, Columbia University Press, New York, USA, 1981.
8. Tobe. H., Jaffre, T., Raven, P. H., Embryology of *Amborella* (Amborellaceae): descriptions and polarity of character states. *J. Plant Res.*, 2000, **113**, 271–280.
9. Friedman, W. E., Gallup, W. N. and Williams, J. H., Female gametophyte development in Kadsura: implications for

Schisandraceae, Austrobaileyales, and the early evolution of flowering plants. *Int. J. Plant Sci.*, 2003, **164**(5), S293–S305.

10. Friedman, W. E. and Williams, J. H., Modularity of the angiosperm female gametophyte and its bearing on the early evolution of endosperm in flowering plants. *Evolution*, 2003, **57**, 216–230.

11. Berlyn, G. P. and Miksche, J. P., *Botanical Micro-technique and Cytochemistry*, Iowa State University Press, Ames, Iowa, USA, 1976.

12. Sass, J. E., In *Botanical Microtechnique*, Iowa State College Press, Ames, Iowa, USA, 1958.

13. Marbaniang, E. J. and Venugopal, N., A new species of *Illicium* (*Illicium arunachalensis*) from Arunachal Pradesh, India. *Res. J. Sci. IT Manage.*, 2015, **4**, 36–43.

14. Xia, N. and Saunders, R. M. K., Flora of China. 2008, **7**, 32–38.

15. Keng, H., Illiciaceae. The families and genera of vascular plants. II. In *Flowering Plants: Dicotyledons*, *Magnoliid*, *Hamamelid and Caryophyllid Families* (eds Kubitzki, K., Rohwer, J. G. and Bittrich, V.), Springer, Berlin, 1993, pp. 344–347.

16. Qiu, D., L., Liu, X. H. and Guo, S. Z., Effects of simulated acid rain on fertility of litchi. *J. Environ. Sci.*, 2005, **17**(6), 1034–1037.

17. Borsch, T. K., Hilu, W., Quandt, D., Wilde, V., Neinhuis, C. and Barthlott, W., Non-coding plastid *trnT–trnF* sequences reveal a well supported phylogeny of basal angiosperms. *J. Evol. Biol.*, 2003, **16**, 558–576.

18. Soltis, D. E., Soltis, P. S., Chase, M. W., Mort, M. E., Albach, D. C., Zanis, M. and Savolainen, V., Angiosperm phylogeny inferred from a combined data set of 18S rDNA, rbcL, and atpB sequences. *Bot. J. Linn. Soc.*, 2000, **133**, 381–461.

19. Mattsson, O., Knox, R. B., Heslop-Harrison, J. and Heslop-Harrison, Y., Protein pellicle of stigmatic papillae as a probable recognition site in incompatibility reactions. *Nature*, 1974, **247**, 298–300.

20. Ghosh, S. and Shivanna, K. R., Structure and cytochemistry of the stigma and pollen-pistil interaction in Zephyranthes. *Ann. Bot.*, 1984, **53**, 91–105.

21. Scandlios, J. G., Genetic control of multiple molecular forms of enzymes in plants: a review. *Biochem. Genet.*, 1969, **3**, 37–79.

22. Shivanna, K. R. and Rangaswamy, N. S., *Pollen Biology: A Laboratory Manual*, Springer Verlag, Berlin, 1992.

23. Johansen, D. A., *Plant Microtechnique*, McGraw Hill, New York, USA, 1940.

24. Dashek, W. V., *Methods in Plant Electron Microscopy and Cytochemistry*, Humana Press, New Jersey, USA, 2000.

25. Dey. S., Basu, B. T. S., Roy, B. and Dey, D., A new rapid method of air drying for scanning electron microscopy using tetra methyl silane. *J. Microsc.* (*Oxford*), 1989, **156**, 259–261.

26. Zou1, F., Yuan, D., Tan, X., Xie, P., Liao, T., Fan, X. and Zhang, L., Megasporogensis and female gametophyte development of *Camellia grijsii* Hance. *J. Chem. Pharm. Res.*, 2013, **5**(11), 484–488.

27. Shabir, P. A., Nawchoo, I. A. and Wani, A. A., Chromosomal stickiness and related meiotic irregularities in *Inula racemosa* – a critically endangered medicinal herb of North Western Himalayas. *Eur. J. Biosci.*, 2013, **7**, 41–46; http://dx.doi.org/10.5053/ejobios.2013.7.0.5).

28. Zou, F., Yuan, De-Yi., Tan, X.-F., Xie, P., Liao, T., Fan, X.-M. and Zhang, L., Megasporogensis and female gametophyte development of *Camellia grijsii* Hance. *J. Chem. Pharm. Res.*, 2013, **5**, 484–488.

29. Shugaeva, E. V., Male sterility of *Valeriana officinalis* L. s.l. *Sov. Genet.*, 1979, **15**, 138–143.

30. Erica, D.-S., André, L. L., Vanzela, J. and Mariath, E. A., Developmental and cytogenetic analyses of pollen sterility in *Valeriana scandens* L. *Sex Plant Rep.*, 2010, **23**, 105–113.

31. Shugaeva, E. V., Abnormalities of microsporogenesis in *Valeriana nitida* KR polyploids. *Sov. Genet.*, 1972, **8**, 37–46.

32. Khajuria, A., Conservation biology of three overexploited medicinal plants of North-West Himalayan region, Ph D thesis, BGSBU, Rajouri, 2013.

33. Raina, R. and Srivastava, L. J., Floral polymorphism in *Valeriana jatamansi*. *Indian J. Plant Genet. Resour.*, 1992, **5**, 93–94.

34. Raina, R., Behera, M. C., Chand, R. and Sharma, Y. P., Reproductive biology studies in *Gentiana Kurroo* Royle. *Curr. Sci.*, 2003, **85**, 667–670.

35. Jennersten, O., Pollination in *Dianthus deltoids* (Caryophyllaceae): effects of habitat fragmentation on visitation and seed set. *Conserv. Biol.*, 1988, **2**, 359–366.

36. Vikas, G. M., Tandon, R. and Mohan Ram, H. Y., Pollination biology and breeding system of *Oroxylum indicum* in Western Himalaya. *J. Trop. Ecol.*, 2009, **25**, 93–96.

37. Sarma, K., Tandon, R., Shivanna, K. R. and Mohan Ram, H. Y., Snail-pollination in *Volvulopsis nummularium*. *Curr. Sci.*, 2007, **93**, 826–831.

38. Tandon, R., Shivanna, K. R. and Mohan Ram, H. Y., Pollination biology and breeding system of *Acacia senegal*. *Bot. J. Linn. Soc.*, 2001, **135**, 251–262.

39. Tandon, R., Manohara, T. N., Nijalingappa, B. H. M. and Shivanna, K. R., Pollination and pollen–pistil interaction in oil palm, *Elaeis guineensis*. *Ann. Bot.*, 2001, **87**, 831–838.

40. Landry, C. L., Mighty mutualisms: the nature of plant–pollinator interactions. *Nat. Edu. Know.*, **3**, 37.

41. Rajkumar, K., Keshavanarayan, P. and Sivaram, V., Pollination biology and breeding system of *Eugenia discifera* Gamble – an endangered species of Western ghats, India. *Int. J. Sci. Nat.*, 2015, **6**, 1–11.

42. Sih, A. and Baltus, M. S., Patch size, pollinator behavior, and pollinator limitation in catnip. *Ecology*, 1987, **68**, 1679–1690.

43. Brys, R., Jacquemyn, H., Endels, P., Hermy, F. V. R. M., Triest, L., Bruyn, L. D. and Blust, G. D. E., Reduced reproductive success in small populations of the self-incompatible *Primula vulgaris*. *J. Ecol.*, 2004, **92**, 5–14.

44. Krushelnycky, P. D., Evaluating the interacting influences of pollination, seed predation, invasive species and isolation on reproductive success in a threatened alpine plant. *PLoS ONE*, 2014, **9**, 1–13.

45. Moza, M. K. and Bhatnagar, A. K., Plant reproductive biology studies crucial for conservation. *Curr. Sci.*, 2007, **9**, 1207.

46. Agren, J., Population size, pollinator limitation, and seed set in the self-incompatible herb *Lythrum salicaria* by the Ecological Society of America. *Ecology*, 1996, **77**(6), 1779–1790.

47. Snow, A. A., Spira, T. P., Simpson, R. and Klips, R. A., The ecology of geitonogamous pollination. In *Floral Biology: Studies on Floral Evolution in Animal-Pollinated Plants* (eds Lloyd, D. G. and Barrett, S. C. H.), Chapman and Hall, New York, 1996, pp. 191–216.

48. Young, A. G., Broadhurst, L. M. and Thrall, P. H., Non-additive effects of pollen limitation and self-incompatibility reduce plant reproductive success and population viability. *Ann. Bot.*, 2012, **109**, 643–653.

49. Raina, R., Bhandari, S. K., Chand, R. and Sharma, Y. P., Strategies to improve poor seed germination in *Stevia rebaudiana*: a low calorie sweetener. *J. Med. Plant Res.*, 2013, **7**, 1793–1799.

50. Nautiyal, B., Nautiyal, P., Mohan, C., Khanduri, V. P. and Rawat, N., Floral biology of *Aconitum heterophyllum* Wall.: a critically endangered alpine medicinal plant of Himalaya, India. *Turk. J. Bot.*, 2009, **33**, 13–20.

51. Wafai, B. A., Siddiqui, M. A. A., Nawachoo, I. A., Dar, N. A., Bhat, M. A. and Mohi-Ud-Din, G. G., Studies on reproductive biology of some endangered medicinal herbs as a prelude to their *ex situ* conservation. *J. Indian Bot. Soc.*, 2005, **84**, 88–106.

52. Wan, P. A., Ganaie, K. A., Nawchoo, I. A. and Wafai, B. A., Phenological episodes and reproductive strategies of *Inula*

recemosa (Astersaceae): critically endangered medicinal herb of NW Himalaya. *Int. J. Bot.*, 2006, **2**, 388–394.

53. Harder, L. D. and Barrett, S. C. H., Pollen dispersal and mating patterns in animal-pollinated plants. In *Flora Biology: Studies on Floral Evolution in Animal-Pollinated Plants* (eds Lloyd, D. G. and Barrett, S. C. H.), Chapman and Hall, New York, 1996, p. 140.

54. Heschel, M. S. and Paige, K. N., Inbreeding depression, environmental stress, and population size variation in scarlet gilia (*Ipomopsis aggregata*). *Conserv. Biol.*, 1995, **9**, 126–33.

55. Khajuria, A., Verma, S. and Sharma, P., Stylar movement in *Valeriana wallichii* DC. – a contrivance for reproductive assurance and species survival. *Curr. Sci.*, 2011, **100**, 1143–1144.

56. Verma, S., Kaul, V., Magotra, R. and Koul, A. K., Pollinator induced anther dehiscence in *Incarvillea emodi* (Bignoniaceae). *Curr. Sci.*, 2008, **94**, 1372–1374.

57. Sharma, S. and Verma, S., Male function for ensuring pollination and reproductive success in *Berberis Lycium* Royle: A novel mechanism. *J. Biosci.*, 2016, **41**, 21–25.

58. Raina, R., Mehera, T. S., Rana, R. C. and Sharma, Y. P., Reproductive biology of *Picrorrhiza kurrooa* – critically endangered high value temperate medicinal plant. *Open Access J. Med. Aromat. Plant.*, 2010, **1**, 40–43.

59. Gautam, K. and Raina, R., New insights into the phenology, genetics and breeding system of critically endangered *Nardostachys grandiflora* DC. *Caryologia*, 2016, **69**, 91–101.

60. Raina, R. and Gupta, L. M., Increasing seed yield in Glory Lily (*Gloriosa superba*) – experimental approaches. *Acta Hortic.*, 1999, **502**, 175–179.

61. Vaughan, M. and Black, S. F., Native pollinators: how to protect and enhance habitat for native bees, Xerces Society for Invertebrate Conservation, Portland, USA, OR97215, 2008.

62. Yao, H. T. P. and Linsenmair, K. E., Seed dispersal mechanisms and the vegetation of forest islands in a West African forest savanna mosaic (Comoe´ National Park, Ivory Coast). *Plant Ecol.*, 1999, **144**, 1–25.

63. Ruxton, G. D. and Schaefer, H. M., The conservation physiology of seed dispersal. *Philos. Trans. R. Soc. Bot.*, 2012, **367**, 1708–1718.

64. Raina, R., Patil, P., Sharma, Y. P. and Rana, R. C., Reproductive biology of *Swertia chirayita* – a temperate critically endangered medicinal plant. *Caryologia*, 2013, **66**, 12–20.

65. Shah, I. A., Sharma, Y. P., Raina, R. and Rana, R., Pollination studies in *Swertia chirayita*: a critically endangered medicinal plant of Western Himalayas. *Open Access J. Med. Aromat. Plant*, 2011, **2**, 14–17.

66. Shah, I. A., Studies on seed germination and seed set in *Swertia chirayita* (Roxb. ex. Flem.) Karst., M Sc thesis, Department of Forest Products, Dr Y. S. Parmar University of Horticulture & Forestry, Solan, 2008.

67. Patil, P., Studies on morphological variation and breeding system in *Swertia chirayita* (Roxb. ex. Flem) Karst., M Sc thesis, Department of Forest Products, Dr Y. S. Parmar University of Horticulture & Forestry, Solan, 2011.

68. Wotton, D. M. and Kelly, D., Frugivore loss limits recruitment of large-seeded trees. *Proc. R. Soc. Bot.*, 2011, **278**, 3345–3354; doi:10.1098/rspb.2011.0185.

69. Robertson, A. W., Trass, A., Ladley, J. J. and Kelly, D., Assessing the benefits of frugivory for seed germination: the importance of the deinhibition effect. *Funct. Ecol.*, 2006, **20**, 58–66.

70. Samuels, I. A. and Levey, D. J., Effects of gut passage on seed germination: do experiments answer the questions they ask? *Funct. Ecol.*, 2005, **19**, 365–368.

71. Ramasubbu, R. and Irudhyaraj, F. D., Reproductive biology of *Elaeocarpus blascoi* Weibel, an endemic and endangered tree species of Palni Hills, Western Ghats, India. *Curr. Sci.*, 2016, **110**, 240.

72. Cerabolini, B., De Andreis, R., Ceriani, R. M., Pierce, S. and Raimondi, B., Seed germination and conservation of endangered species from Italian alps: *Physoplexis comosa* and *Primula glaucescens*. *Biol. Conserv.*, 2004, **117**, 351–356.

73. Agarwal, A. A., Seed germination of *Loxopterygium guasango* a threatened tree of coastal northwestern south America. *Trop. Ecol.*, **37**, 273–276.

74. Butola, J. S. and Badola, H. K., Effect of pre-sowing treatment on seed germination and seedling vigor in *Angelica glauca*: a threatened medicinal herb. *Curr. Sci.*, 2004, **87**, 796–799.

75. Badhwar, B. L. and Sharma, B. K., A note on the germination of *Podophyllum hexandrum* seeds. *Indian For.*, 1963, **89**, 445–447.

76. Bastrenta, B., Ebreton, J. L. and Thompson, J. D., Predicting demographic change in response to herbivory: a model of the effects of grazing and annual variation on the population dynamics of *Anthyllis vulneraria*. *J. Ecol.*, 1995, **83**, 603–610.

77. Huntly, N., Herbivores and the dynamics of communities and ecosystems. *Annu. Rev. Ecol. Syst.*, 1991, **22**, 477–503.

78. Walsh, R. P., Arnold, P. M. and Michaels, H. J., Effects of pollination limitation and seed predation on female reproductive success of a deceptive orchid. *AoB PLANTS*, 2014, **6**, plu031; doi:10.1093/aobpla/plu03.1.

79. Williams, J. H., *Amborella trichopoda* (Amborellaceae) and the evolutionary developmental origins of the angiosperm progamic phase. *Am. J. Bot.*, 2009, **96**, 144–165.

80. Batygina, T. B., Shamrov, I. I. and Kolesova, G. E., Embryology of the Nymphaeales and Nelumbonales. II. The development of the female embryonic structures. *Bot. Zh.* (*Leningrad*), 1982, **67**, 1179–1195.

81. Maheshwari, P., *An Introduction to the Embryology of Angiosperm*, Tata McGraw Hill Publishing Company Limited, New Delhi, 1950

82. Endress, P. K., The evolution of floral biology in basal angiosperms. *Philos. Trans. R. Soc. London, Ser. B*, 2010, **365**(1539), 411–421.

83. Lau, J. Y., Pang, C. C., Ramsden, L. and Saunders, R. M., Stigmatic exudate in the Annonaceae: Pollinator reward, pollen germination medium or extragynoecial compitum? *J. Integr. Plant Biol.*, 2017.

84. Oh, I. C., Denk, T. and Friis, E. M., Evolution of *Illicium* (Illiciaceae): mapping morphological characters on the molecular tree. *Plant Syst. Evol.*, 2003, **240**, 175–209.

85. Takhtajan, A L., The criteria used in evaluating the relative degree of their advancement. In *Flowering Plants: Main Vectors of Evolution in Flowering Plants*, Science and Business Media B.V., Springer, 2009, pp. 10–29.

86. Endress, P. K. and Igersheim, A., Gynoecium structure and evolution in basal angiosperms. *Int. J. Plant Sci.*, 2000, **161**, S211–S213.

87. Wodehouse, R. P., *Pollen Grains. Their Structure, Identification and Significance in Science and Medicine*, McGraw Hill, New York, 1935, p. 574.

88. Huang, T. C., *Pollen Flora of Taiwan*, Taiwan University Press, Taipei, 1972, vol. 143, pp. 10–16.

89. Lin, Q., A study of pollen morphology of genus *Illicium* L. *Bull. Bot. Res.*, 1989, **9**, 115–124.

90. Battaglia, E., Embryological questions: 7. Do new types of embryo sac occur in Schizandra? *Ann. Bot.*, 1986, **44**, 69–82.

91. Yoshida, O., Embryogenic studies in *Schisandra chinensis*. *J. Coll. Arts Sci.*, *Univ. Chiba*, 1962, **4**, 459–462.

92. Tobe, H. and Raven, P. H., Embryology of Onagraceae (Myrtales): characteristics, variation and relationships. *Telopea*, 1996, **8**, 667–688.

93. Friedman, W. E., Madrid, E. N. and Williams, J. H., Origin of the fittest and survival of the fittest: Relating female gametophyte development to endosperm genetics. *Int. J. Plant Sci.*, 2008, **169**, 79–92.

94. Endress, P. K. and Sampson, F. B., Floral structure and relationships of the Trimeniaceae (Laurales). *J. Arn. Arbor.*, 1983, **64**, 447–473.

95. Solntseva, M. P., Illiciales. In *Comparative Embryology of Flowering Plants* (ed. Yakovlev, M. S.), Nauka, Leningrad, Russia, 1981, pp. 51–54.

96. Endress, P. K., The reproductive structures and systematic position of the Austrobaileyaceae. *Bot. Jahr. Syst.*, 1980, **101**, 393–433.

97. Swamy, B. G. L., Macrogametophytic ontogeny in *Schisandra chinensis*. *J. Indian Bot. Soc.*, 1964, **43**, 391–396.

98. Batygina, T. B. and Vasilyeva, V. E., *Plant Reproduction*, University Press, St Petersburg, Russia, 2002.

99. Tobe, H. and Raven, P. H., An embryological analysis of myrtales: its definition and characteristics. *Ann. Mo. Bot. Gard.*, 1983, **70**, 71–94.

100. Fareglind, F., Vorkommen und entseehung von hakenleisten an synergiden und eizellen. *Sevensk Bot. Tidskrif.*, 1943, **35**, 157–176.

101. Friedman, W. E., Developmental and evolutionary hypotheses for the origin of double fertilization and endosperm. *C. R. Acad. Sci.–Ser. III*, 2001, **324**, 559–567.

102. Friedman, W. E., The evolution of double fertilization and endosperm: an 'historical' perspective. *Sex Plant Reprod.*, 1998, **11**, 6–16.

103. Friedman, W. E., Organismal duplication, inclusive fitness theory, and altruism: understanding the evolution of endosperm and the angiosperm reproductive syndrome. *Proc. Nat. Acad. Sci. USA*, 1995, **92**(9), 3913–3917.

104. Du, W. and Wang, X. F., Intercarpellary growth of pollen tubes in the extragynoecial compitum and its contribution to fruit set in an apocarpous species, *Schisandra sphenanthera* (Schisandraceae). *Am. J. Bot.*, 2012, **99**(5), 961–966.