Assisted pollination and seed formation for conservation of *Eulophia flava* (Orchidaceae): a case study from Himalayan Foothills, India[a]

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Key Words/mots clés : artificial pollination / pollinisation artificielle, conservation, *Eulophia flava*, seed viability test / test de viabilité de graines, Shivalik range, taxonomy / taxinomie, TTC test.

Abstract

A small, wild population of *Eulophia flava* (Lindley) Hooker f. was found on August 2002 in the Shivalik Ranges among Chandrabani forests that lies in the northern fringe of Rajaji National Park (Uttarakhand state). This was an authentic record of the occurrence of this species from the Shivaliks after a gap of around fifty years. Plants were healthy but they didn't flower until 2008, after making slight modifications in their habitat by providing an open canopy to the dormant underground corms in the early summer season. Flowering was induced successfully but, due to lack of pollinators, artificial pollination experiments were conducted and fruits were borne on the plant successfully. Seed viability testing was undertaken using TTC solution. The manuscript deals with the *in situ* conservation efforts for this species along with its updated taxonomy, description, flowering phenology, successful flowering induction, artificial pollination, successful fruit set and seed viability test for the species.

Résumé

Pollinisation assistée et formation de graines pour la conservation de *Eulophia flava* (Orchidaceae) : une étude de cas dans les contreforts himalayens, Inde – Une petite population sauvage de *Eulophia flava* (Lindley) Hooker f. a été découverte en août 2002 dans les Chaînes Shivalik, dans les forêts de Chanbrabani qui s'étendent sur la frange nord

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du Parc National Rajaji (Etat d'Uttarakhand). C'est un authentique enregistrement de l'occurence de cette espèce dans les Shivaliks, après un intervale d'environ cinquante ans. Les plantes étaient en bonne santé mais n'ont fleuri qu'en 2008, après qu'on eut apporté de légères modifications dans leur habitat qui ont permis de fournir aux bulbes dormants souterrains une canopée ouverte au début de la saison d'été. La floraison fut effectivement induite mais, du fait de l'absence de pollinisateurs, des expériences de pollinisation artificielle furent menées et des fruits furent ainsi produits. La viabilité des graines fut testée à l'aide d'une solution TTC. Le présent article présente les efforts de conservation *in situ* de cette espèce, ainsi qu'une mise à jour de sa taxinomie, sa description morphologique, la phénologie de sa floraison, son induction florale, sa pollinisation artificielle aboutissant à la fructification et enfin le test de viabilité des graines ainsi produites.

Introduction

Orchids are one of the largest and most advanced group of the Angiosperms. They have always attracted people due to their complex floral morphology and beautiful flowers. Dressler (2006) has reported that there may be around 27,135 species of orchids in the world (www.theplantlist.org). However, earlier estimates of species in the Orchidaceae family by different botanists varied considerably from 12,000 to 35,000 (Fiveash, 1974; Sanford, 1974; Hunt, 1984; Heywood, 1985; Dressler, 1993).

Orchids are highly susceptible to changes in the environment and their populations are dwindling rapidly due to habitat degradation or disintegration. This calls for immediate *in situ* and *ex situ* conservation measures. One of the important habitat requirements in the case of orchids is the availability of suitable pollinators which are often species specific. Most orchid species are self-compatible (Gill, 1989), but under natural conditions pollination and fruit setting greatly depends on pollinators. The orchids are pollinated by a variety of agents including bees, wasps, flies and gnats, butterflies, moths, birds and ants, which are attracted in different ways. Decline or local extinction of pollinators can thus adversely affect orchid populations.

During a routine survey of orchids in Doon Valley, Uttarakhand (India), we encountered a few individuals of a ground orchid. It had two large, plicate leaves with chain of obconical to irregular, slightly inflated, underground rhizomes. Despite repeated visits to the site, subsequent to its first discovery (August 2002 until 2007) during different seasons we did not notice any sign of flowering. The plant was tentatively identified as species of *Eulophia* which are known to prefer canopy gaps and flower during the summer. However, these plants were found under a dense canopy of shrubs. In order to allow more sunlight to the plants the canopy was slightly cleared in early March 2008, so as to allow light to fall on the area where the underground rhizomes were present, expecting the flowering to be induced on the availability of appropriate amounts of sunlight. The first flowering stalk was observed on the plants in May 2008 which emerged from the underground rhizomes resulting in full bloom within a month. On anthesis, the species was identified as *Eulophia flava* (Lindley) Hooker f. Interestingly, even during full bloom there were no signs of pollinators. Hence a few flowers were artificially pollinated that resulted in successful production of fruits and seeds. This article deals with the study of flowering phenology, assisted pollination and seed formation in case of *Eulophia flava* in Doon Valley.

Methodology

Morphological studies were made on the live plants in both vegetative as well as floral stage and identification was carried out with the help of the type description in Hooker (1890) and regional orchid flora (Deva and Naithani, 1986) and also with the help of the type specimen from K (Royal Botanic Gardens, Kew).

Flowering studies were based on continuous observations made on the live plants in the wild.

For pollination studies, the floral spike was divided into three groups of flowers (8+8+9). The primary aim was to study the first eight flowers from the bottom, so as to experiment on the middle eight flowers and leave the upper most nine flowers untouched hoping that the plant would be pollinated by the natural pollinators if present. The blooming period and the ideal time for pollination was estimated based on observations of the floral visits by the insects. For this, a regular vigil of the first eight flowers was carried out. The time period at which maximum number of bees visited the flower was considered to be the ideal blooming period. This is the time when the stigma is fully mature; the flower is comparatively in the best conditions of health and the fragrance at its maximum. It is an ideal time for transfer of pollinia.

Artificial pollination was attempted, predicting that the area has not natural pollinators. Pollination was performed by removing the pollinia from the rostellum using sterile forceps and placing the pollinia on the stigma of different flower.

Seed viability testing was done by subjecting seeds to positive red staining test of viable embryos with Triphenyl Tertrazolium Chloride solution (TTC) (Van Waes & Deberg, 1986). This technique is internationally recognised for testing the seed viability and has been successfully used for Orchids by Vujanovic *et al.* (2000).

Study area

The Shivalik Range, also known as the lower or sub-Himalayas, is the southernmost and geologically youngest east-west mountain chain of the Himalayan System. The range extends to around 2,000 km from the Teesta River in Sikkim, westward through Nepal and Uttarakhand, Himachal Pradesh, Haryana, continuing into Kashmir and Northern Pakistan. There are vast networks of small rills and channels that form streams which are ephemeral (transient) in nature. The Shivalik ridges consist basically of the material eroded from the Himalayas and deposited at its base. This unconsolidated deposits of sandstone and conglomerates has recently (2-12 million years ago) been uplifted to an altitude of 1,200 masl (meters above sea level) in varying degrees from Pakistan (Salt ranges) to West Bengal. The elevation ranges from 900 to 1,200 m and at places width can be as low as 16 m. There are significant differences in the geological and vegetational characteristics of the middle and upper Siwaliks and the north and south facing slopes. The upper Shivalik are largely composed of conglomerates with alternate layers of sandstone and grits whereas the middle Shivalik are composed primarily of sandstone with alternate layers of gravels and clays. The soils of the region are usually acidic with pH between 5.2 and 6.5. Temperatures vary from a maximum of 45°C in May (with a monthly average of 39°C) to a minimum of 3°C in winter. The overall texture of the soil is coarse, sandy with higher internal drainage and with moisture deficiency. On the higher slopes the soil has a thinner profile lying over a strong bouldery sub-soil. The more stable sites of the deeper and gentle slopes have deeper and better formed soils with satisfactory drainage and water holding capacity.

The study area, Chandrabani forests, lies in the Northern fringe of Rajaji National Park and extends inside the WII campus. It is a small patch of natural forests, fragmented from the Shivaliks and is dominated by sal (*Shorea robusta*). The present study was conducted in one such forest in the Doon valley at an elevation of around 530 masl.

Results and observations

Description

***Eulophia* R.Brown ex Lindley, *Botanical Register* 7: t. 573 (1821), nom. cons.**
Terrestrial or ground orchids, rarely lithophytes. Leaves and flowers simultaneous or succeeding each other. Inflorescence lateral, terminal on root stock, erect, racemose or sometimes paniculate, of many large, variously coloured flowers. Sepals connate with foot of column. Lip saccate or spurred at the base, tri-lobed, rarely subentire; disc variously crested or lamellate, rarely naked. Column often provided with a foot, with or without lateral wings. Anther terminal, 2-loculed, sometimes with two apical processes. Pollinia two, globose, with simple stipes; viscidium oblong, elliptic or lunate.

This genus is comprised of 217 species including 10 varieties, 2 subspecies and 2 hybrids (Govaerts *et al.*, 2011). In India the genus is represented by 24 species (Misra, 2007). The name "*Eulophia*" was derived from the Greek words "*eu*" and "*lophos*", referring to the crested ridges of the labellum, present in most of the species.

***Eulophia flava* (Lindley) Hooker f., *Flora of British India* 6: 7 (1890).**
Cyrtopera flava Lindley, *Genera and Species of Orchidaceous Plants:* 189 (1833).
Cyrtopodium flavum (Lindley) Bentham, *Journal of the Linnean Society, Botanics* 18: 320 (1881), *nom. illeg.*
Graphorkis flava (Lindley) Kuntze, *Revisio Generum Plantarum* 2: 662 (1891).
Lissochilus flavus (Lindley) Schlechter, *Repertorium Specierum Novarum Regni Vegetabilis, Beihefte* 4: 260 (1919).
Cyrtopera cullenii Wight, *Icones Plantarum Indiae Orientalis* 5: t. 1754 (1851).
Eulophia cullenii (Wight) Blume, *Collection des Orchidées le plus remarquables de l'Archipel Indien et du Japon*: 182 (1859).

Type: *Wallich Cat.* 7364, Morang Hills (**K**- holo !*)

Plant tall, 30-140 cm, robust, terrestrial orchid with underground corms. *Corms* dirty white coloured, rough, obconical, 3-6 × 4.5-8 cm, in chain, may or may not be branched, with vertical striations and horizontal nodes, roots vermiform, white. *Leaves* 2, large, alternate, distichous, oblong-lanceolate, acuminate, 10 × 20 cm to 40 × 80 cm, plicate, many veined, sheathing at the base, sheaths long and tubular. Inflorescence arises from the base of the last year's corm, 80-100 cm long, 1.5 cm thick; peduncle terete with few wide, oblong bracts, acute sheaths; raceme with up to 25 flowers laxly clustered on the upper half. Bracts pale green with greenish-white base, linear-lanceolate, acuminate, 1.7 × 0.35 cm. *Flowers* bright yellow, pedicellate, sweetly odorous, ca 7.5 × 4 cm, floral bracts thick. *Sepals* yellow, spreading, unequal; dorsal sepal 3.8 × 1.4 cm, oblong-lanceolate, broader towards the upper half, acute; lateral sepals 4.2 × 1.6 cm, base and apex oblique, apiculate. *Petals* yellow, 3.1 × 2 cm, obovate, obtuse, base slightly oblique and thickened, apex apiculate. *Labellum* yellow, 3.3 × 3.5 cm, trilobed, with red spots on the inner surface near the spur, 3 crenate lamellae on the inner $2/3^{rd}$ of the length, running parallel towards the tip, base tending to be connivent making the base saccate; midlobe orbicular, obovate with constricting base, margin slightly crenate, apex rounded; side lobes broad with rounded apex, entire. *Spur* broad, saccate. *Column* clavate, 2.2 × 0.5 cm, greenish-white; clinandrium apiculate at the back; foot 0.4 cm long. *Stigma* obovate; stigmatic cavity reniform. *Anthers* yellow, subglobose, ca 0.35 × 0.32 cm, deeply cleft at the base; pollinia waxy, trapezoid, 0.17 × 0.18 cm; *stipe* short, triangular, 0.2cm, hyaline; viscidium short, 0.4 × 0.04 cm, tips filiform. *Capsules* drooping, spindle shaped, strongly 3-ribbed, 6 × 1.5 cm. (Fig. 1 and colour plate on page 37)

Flowering: May-June; Fruiting: June-October.

Specimens examined: WII: *Pankaj Kumar 081001*, Chandrabani forests, Dehradun, elevation: 530 masl. K: *Wallich Cat.* 7364 (Type), Morang Hills; *Strachey and Winterbottom 20*, India; *Inayat* 24142, Upper Gangetic Plain, India; H.F.Mooney 113, Jashpur, Chhattisgarh, 10.06.1936 and *E.F.Hilton sine no.*; Shivalik Hills, Dehradun; BSD: K.M.M.Dakshini 8050 & 5535, Mohtronwala, Dehradun.

Distribution: China, India (Uttarakhand, Madhya Pradesh, Maharastra and Kerala), Nepal, Laos, Myanmar, Thailand and Vietnam (Govaerts *et al.*, 2011; Shrivastava, 2004).
Note: The plant is very rare locally. This is the first authentic collection of the species after a gap of fifty years.

Economic Importance: A potential garden plant, corms consumed by pigs and the plant is used against spider poison (Shrivastava, 2004).

Blooming period
First noticeable floral spike was seen on 05.05.2008. First flower opened on 01.06.2008 and after five days of a regular vigil the blooming period was found to be 11:30AM to 12:00PM. This time was chosen for artificial pollination. The ideal flower for pollination was chosen by an assumed indicator crab spider (*Thomisus* sp.) which resided on the floral parts since the opening of first flower and tended to shift to the next flower. The tendency of this spider to shift to next flower was assumed to be based on the availability of maximum prey insects visiting the flower. Unfortunately, none of visiting insects were the natural pollinators because none of the first eight flowers were pollinated, and secondly because the size of the visiting insects as well as the spider was comparatively smaller than the pollinia as well as the operculum of this particular flower. Unpollinated flowers could be identified by the wrinkled ovary as well as black marking at the place of attachment of pedicel with the floral stalk. These flowers shrivelled and fell off within 2-3 days, whereas pollinated flowers remained for more duration, with more stout and wrinkle-free ovary.

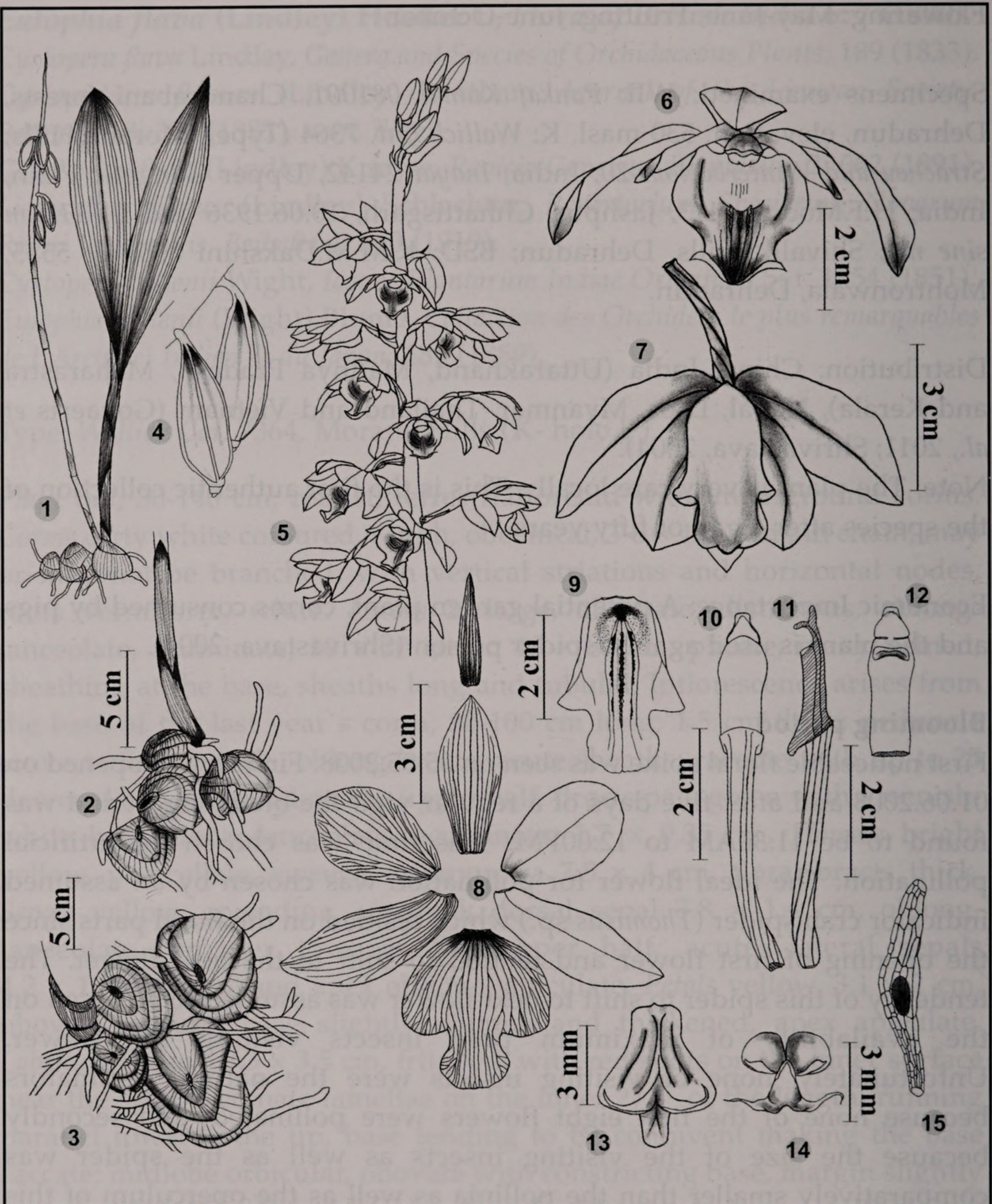

Fig. 1: *Eulophia flava* (Lindley) Hooker f.

1. Habit; 2, 3. Corms; 4. Fruits; 5. Inflorescence; 6. Flower, front view; 7. Flower, ventral view; 8. Dissected floral whorls; 9. Labellum; 10. Column, dorsal view; 11. Column, lateral view of V.L.S.; 12. Column, ventral view; 13. Operculum, ventral view; 14. Pollinia; 15. Seed [Illustrations by Dr. Pankaj Kumar]

Pollination

When the spider was found sitting on the 12th flower, the artificial pollination procedure of eight flowers was carried out on 05.06.2008. Pollinia were removed from the flowers with the help of sterilized forceps (12th-19th flowers), reshuffled among the same eight flowers and placed on the stigmatic cavity. Care was taken firstly to check if the stigma had any pollinia on it and secondly, not to place the pollinia in a flower from which it had been removed. No naturally transferred pollinia were seen in any of the flowers.

It was found that out of 25 flowers borne by the plant, only 5 out of the eight artificially pollinated flowers developed as fruits. The rest of the flowers fell off gradually. Fully mature and partly dehisced fruits were collected after 4 months (159 days) on 10.10.2008. The graphic representation of the procedure is presented in Fig. 2.

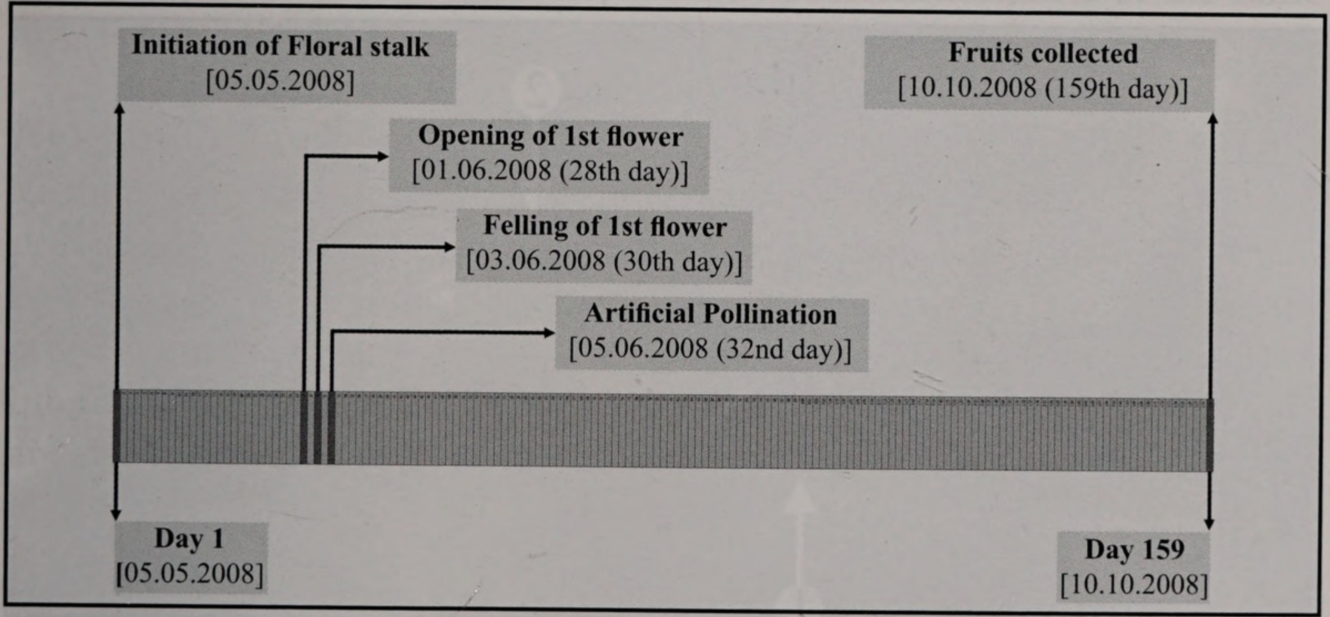

Fig. 2: Graphical representation of flowering and fruiting phenology of E*ulophia flava*.

Seed Viability Test

TTC solution was prepared by dissolving 1g of TTC in phosphate buffer. The solution was then left in dark by covering the flask with aluminium foil, and left for 48hr. Seeds were first kept in 10% NaOCl for 2 hr. This is thought to promote dormancy release in terrestrial orchid seeds (Lauzer *et al.*, 1994). Seeds were then transferred to TTC in small eppendorf tubes, covered with aluminium foil and left for 5 hr. Seeds were taken out after 5 hr, rinsed with distilled water and transferred to the Whatman-50 filter paper. Observations were made under compound and dissecting microscopes. Distinct dark reddish-pink colouration of the embryo was observed after 5 hr as mentioned by Vujanovic *et al.* (2000) providing positive test for seed viability (Fig. 3). Around 18% of the seeds were found to be viable. Apart from viable seeds, some non-viable seeds as well as embryoless seeds were also observed. As a part of the *in situ* conservation programme, remaining untouched seeds were scattered in the Chandrabani forests at known localities. These could take at least one rainy season to germinate and at least three years to be noticed as the young plants are too small to be found.

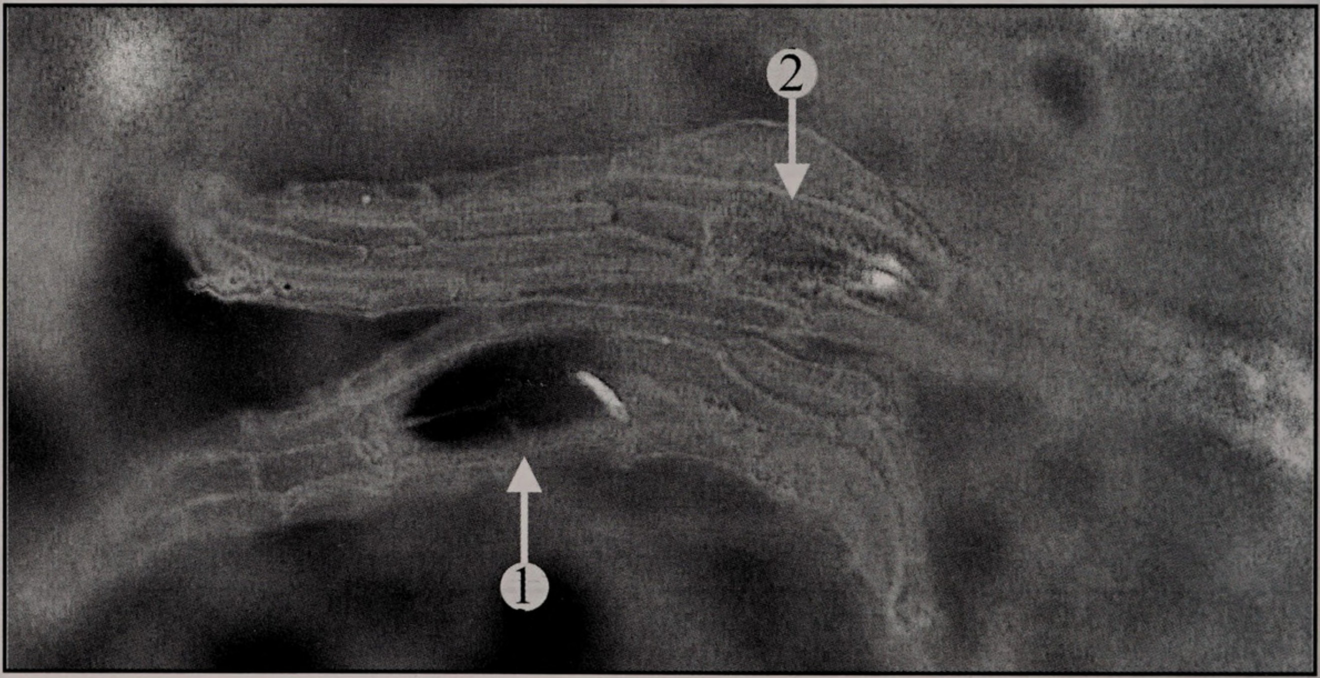

Fig. 3: Seeds of *Eulophia flava*

1. Viable embryo stained in dark red colour by TTC
2. Unstained non-viable embryo

[combined red and magenta channels of a colour view]

Discussion

Orchids are important part of our ecosystem and they need to be conserved. An attempt was made for the *in situ* conservation of *Eulophia flava* in the Chandrabani forests, doing some scientific study simultaneously. Orchids have specific pollinators and they may or may not have more than one pollinator and it is assumed that orchids and pollinators have co-evolved. Unlike many orchids, sexual deception or food deception (Ledford, 2007) is absent in *Eulophia flava,* and hence they attract pollinators by other means, like large and bright coloured flowers, strong fragrance and pollinating rewards in the form of nectar. Despite of all these, the plant could not be pollinated naturally. The main reason that could be attributed to this fact is the lack of suitable natural pollinators in the habitat. This is one of the reasons because of which most of the orchids are now a days getting extinct from wild. To counteract with the lack of pollinators, the plant was artificially pollinated and fruits were born with viable seeds. The TTC test for seed viability was found to be suitable for this species. Seeds were later dispersed in different localities of the same forests for *in situ* conservation.

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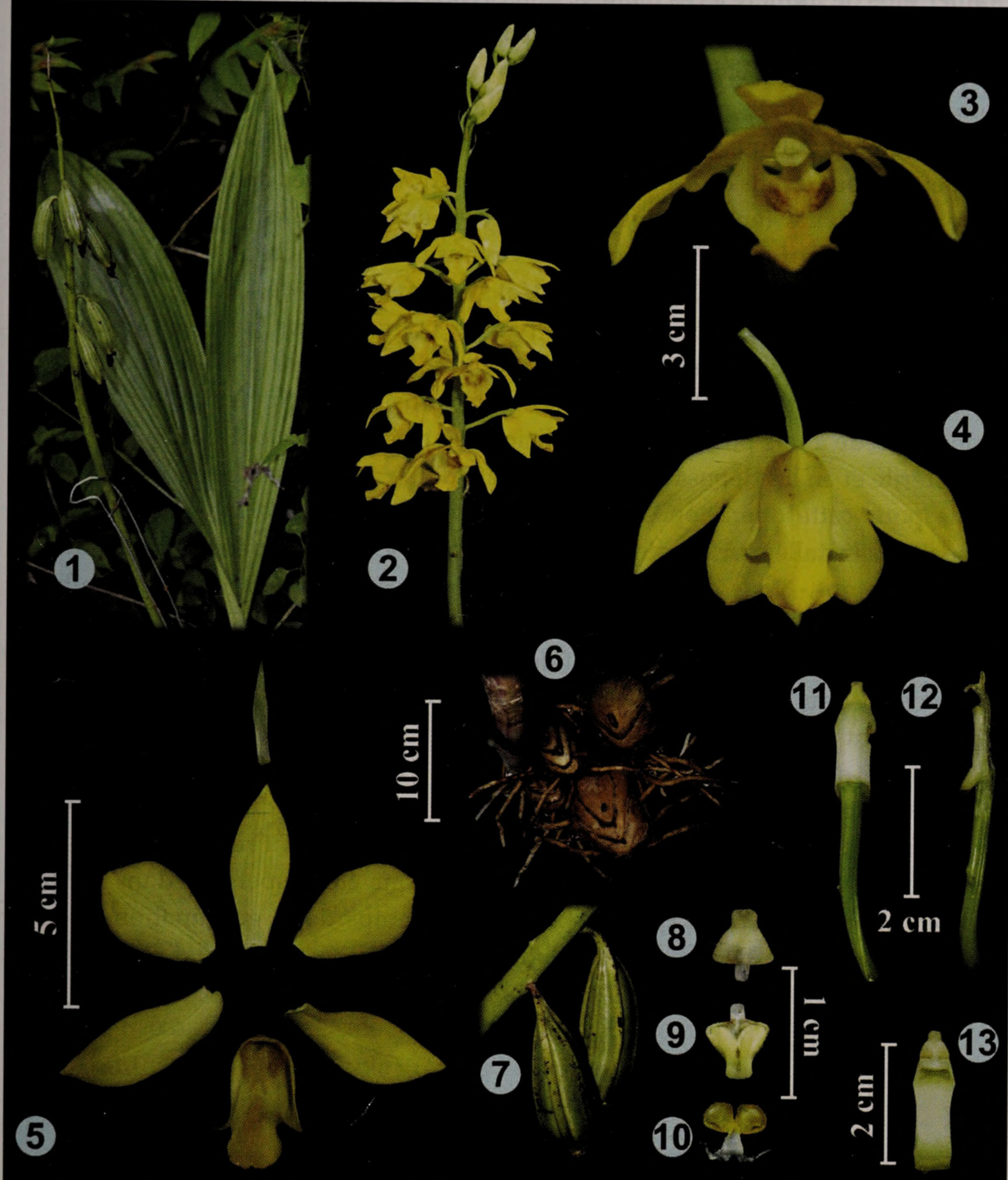
Les orchidées sont l'un des groupes d'Angiospermes les plus vastes et les plus avancés. Dressler (2006) évalue leur nombre à 27 135, mais les estimations antérieures varient de 12 000 à 35 000. Elles sont très sensibles aux modifications de leur environnement et leurs populations décroissent rapidement avec la dégradation de leurs habitats. Ceci appelle des actions immédiates de conservation *in situ* et *ex situ.* L'une des nécessités est la présence de pollinisateurs, qui sont souvent des espèces spécifiques. L'extinction locale d'un pollinisateur peut affecter une population d'orchidées. Au cours d'une observation de routine des orchidées dans la Vallée Doon, Uttarakhand, Inde, nous avons rencontré quelques individus d'une orchidée terrestre qui, au cours des visites répétées que nous leur avons faites entre 2002 et 2007, n'ont jamais montré le moindre signe de floraison. Cette orchidée ressemblait à un *Eulophia,* genre connu pour préférer des canopées ouvertes, alors que là elle poussait sous une canopée dense. Début mars 2008, pour



Eulophia flava (Lindley) Hooker f.

1: Habit – 2: Inflorescence – 3: Flower (front view) – 4: Flower (ventral view) – 5: Dissected floral whorls – 6: Corms – 7: Fruits – 8: Operculum (dorsal view) – 9: Operculum (ventral view) – 10: Pollinia – 11: Column (dorsal view) - 12: Column (lateral view) – 13: Column (ventral view) donner plus de lumière à ces plantes, nous avons légèrement éclairci la canopée. La première inflorescence fut observée en mai de la même année. A l'anthèse, l'espèce fut identifiée comme *Eulophia flava*. Il est intéressant de noter que, même en pleine floraison, il n'y avait aucun signe de pollinisateurs. Nous avons donc pollinisé artificiellement quelques fleurs, qui ont produit des fruits et des graines.

A cet effet, nous avons divisé l'inflorescence en trois groupes de fleurs (8+8+9). Les 8 premières fleurs (en bas) ont été régulièrement observées pour déterminer le moment idéal pour la pollinisation. Nous avons pour cela noté les visites d'insectes et notamment d'abeilles. Ce moment idéal s'est révélé être de 11h30 à 12h00.

Supposant que la zone ne possédait pas de pollinisateurs naturels (et cela est conforté par le fait qu'aucune des huit premières fleurs n'a été fécondée et corroboré par la taille des insectes visiteurs, plus petits que les pollinies), nous avons procédé à une pollinisation artificielle en prélevant les pollinies à l'aide d'un outil stérile et en les déposant sur le stigmate d'une autre fleur. Le choix de la fleur idéale pour cette opération est basé sur l'observation d'une araignée, araignée-crabe, qui s'installe sur la première fleur à fleurir et passe d'une fleur à l'autre au cours du temps. Nous supposons que son déplacement est lié à la disponibilité d'un maximum d'insectes visitant les fleurs, qui sont des proies pour elle. Sur les huit fleurs ainsi pollinisées, cinq ont développé des fruits. Les fleurs du troisième groupe sont peu à peu tombées, sans avoir été fécondées. Des fruits matures et partiellement déhiscents ont été collectés quatre mois plus tard.

La viabilité des graines obtenues a été testée en soumettant les graines à une teinture rouge de Triphenyl-Tertrazolium-Chloride (TTC) qui ne teinte que les embryons viables. Environ 18% des graines collectées se sont révélées viables.

Les graines viables non utilisées pour l'étude ont été dispersées dans certains endroits de la forêt. Dans quelques années (le temps de la germination et de la croissance des plantules jusqu'à une taille observable), des plantes d'*Eulophia flava* devraient être observées.

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