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Pollination induced embryological studies in *Aerides multiflora* (Roxb.)

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Abstract
Pollination related embryological data is of outmost importance in predicting a breeding system and the knowledge of the breeding system is must in taxon like Orchidaceae where consistent improvement of ornamental traits through hybridization is a regular practice. Incidentally, the orchids provide a suitable material for embryological studies because of the presence of large nuclei and numerous ovules within the same ovary at different stages of development. Notwithstanding, orchids, known for their myriad of shapes, colors and sizes are of immense commercially potential as cut flowers and pot plant and fetch high price at National and International market. The taxon under study namely *Aerides multiflora* (Roxb.), belongs to a family, Orchidaceae. In the present investigations, an account of changes in morphology and reproductive biology, following pollination has been presented. The ovule is bitegmic, tenuinucleate and anatropous in present taxa. The total time taken to reach the organized embryo sac following pollination took between 55-60 days. The process of syngamy and triple fusion was accomplished between 46-50 days after pollination (DAP).

Keywords: *Aerides multiflora*, embryo sac, hybrids, megaspore mother cell, orchids, pollination

1. Introduction
Orchidaceae, a family of Flowers with myriad of shapes, colors and sizes embody an order of aristocracy among the flowering plants. These extraordinary plants with a spectrum of floral characteristics and intricate pollination mechanisms represent a fairly young (geologically), highly diverse and successful family of angiosperms with an estimated 28,000 species [1, 2].
They are found as terrestrials, epiphytes, lithophytes and subterraneans. Nearly 73% species are epiphytic which are distributed in tropical and subtropical climates (e.g. *Cymbidium*, *Dendrobium*, *Rhynchostylis*, *Vanda* etc.). *Gastrodia elata* is a saprophytic orchid whereas *Rhyzanthiella*, a genus of Australian orchids, is sub-terranean in habit. *Schomburgkia tibicinis* harbours ants in its roots and feeds upon them to augment its nutritional status, a carnivorous orchidt. Shrubby or even climbing habit is also found in some taxa e.g. *Vanilla planifolia*. Orchids range from a few centimeter e.g. *Bulbophylum minutissimum* to several meters in height e.g. *Galeola foliata*, *Grammatophylum speciosum.* Many orchids enjoy the status of the national flower in several countries e.g. Panama (*Peristeria elata*), Columbia (*Cattleya trianaei*), Singapore (*Vanda* miss Joaquim), Costa Rica (*Cattleya skinneri*).
The orchids have out-maneuvered their counterparts by evolving ingenuity and higher levels of specialization in both the vegetative and reproductive traits. One character shared by nearly all the orchids is the presence of dust likes tunicate seeds [3], i.e. only the uniseriate epidermis of the outer integument persists in the mature seed and forms a hollow space in which the embryo occurs. They still continue to be in an evolutionary flux due to (i) poorly developed b
Arriers of reproductive isolation, which promote free gene flow across the taxonomic limits and (ii) high survival frequency of neotypes and more than 1 lacs natural and manmade hybrids, at levels from interspecific to pleurigeneric have so far been listed [4] and many more are in the offing.
Due to their complex biology, notably their interactions with mycorrhizal fungi, pollinators and host trees, orchids present particular challenges for conservation, and this is compounded by non-sustainable and often illegal collection for horticulture, medicine and food and by climate change [5].
Heavy pressures of commercial collection and habitat destruction have detrimentally affected the size and frequency of their natural population, which is progressively on the decline. In fact, the orchids now figure prominently in the Red Data Book prepared by the International Union for Conservation of Nature and Natural Resources [6]. Some of them have already extinct and a similar fate awaits the others in the absence of remedial measures.
The present studies deal with post pollination embryological changes in *Aerides multiflora* (Roxb.) where the perianth members, ovule initiation and development, growth of the pollen tube, megasporogenesis, megagametogenesis and post fertilization changes following pollination under greenhouse conditions had been investigated (Figure 1A-D). Additionally, Importance of the embryological data in predicting a breeding system is well known and the knowledge of the breeding system is a must in taxon like Orchidaceae where consistent improvement of ornamental traits through hybridization is the need. Incidentally, the orchids provide a suitable material for embryological studies because of the presence of large nuclei and numerous ovules within the same ovary at different stages of development.

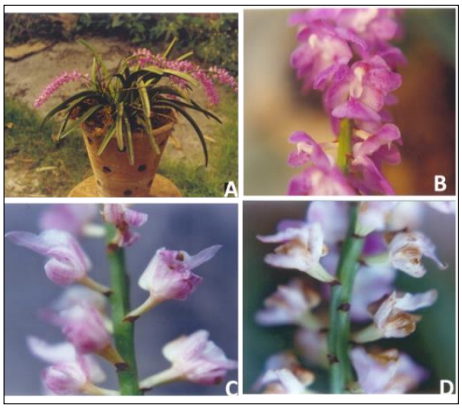

Fig 1: (A) An inforescence of *A. multiflora*; (B) Magnify view of flowers; (C) Start of wilting 3-4 DAP; (D) Wilting advances stage at 4-5 DAP

2. Material and Methods

2.1. Fixation

The flowers were hand pollinated at anthesis and fixed in FAA (70% Ethanol: Glacial acetic acid: Formalin; 18: 1: 1) for 36 hours and subsequently stored in 70% ethanol for further use. Initially, the flowers were fixed after 24 hours of pollination to observe the time of pollen germination and to trace the path of pollen tube, thereafter, the fixation was done at 4 days intervals to study the post pollination developmental changes in the ovule and embryogenesis.

2.2. Section cutting and Staining

The fixed material was dehydrated in ethanol-tertiary butyl alcohol series. The dehydrated samples were embedded in paraffin wax (58-60°C) and sectioned on a Spencer '820' rotary microtome (American Optical Company, USA) at 7-12pm. The sections were stained with Safranin and fast-green combination [7]. Hand sections were also cut with the help of a razor blade and stained in Safranin to observe the structure of pericarp. Whole mounts of ovules were also prepared after clearing in 1N KOH and stained in safranin to study the development of female gametophyte.

2.3. Aniline Blue- Fluorescence Test [8]

2.3.1. Solution Required: 0.1% w/v solution of aniline blue (water soluble) in 0.1 M of disodium hydrogen phosphate (1.7816 g of disodium hydrogen phosphate-dihydrate was prepared in enough of distilled water to make 100 ml solution). The pH was adjusted at 9.5.

2.3.2. Staining procedure: The sections of the material fixed after 24 hours interval were hydrated. It was stained for 1-2 hours in aniline blue solution. The sections were mounted in the same aniline blue solution and observed under Nikkon fluorescent microscope and photographed. Callose fluoresced bright yellow in pollen tubes and ovules.

3. Results and Discussion

The species under study is *Aerides mulltiflora* (Roxb.) is an extensive genus contains more than 60 species of monopodial epiphytes or lithophytes. It is a widely distributed species of Fox-tail orchids, extending from India to Thailand through Nepal and Burma. In India, it is met within Himalayan, peninsular and Andaman and Nicobar regions [9]. It was collected from its natural habitat Kangra and Palampur (H.P.) and maintained in the Orchid House, Department of Botany, Panjab University, Chandigarh (Figure 1 A-D). The orchid classification as given by Dressler [10] has been followed.

The hand pollination was accomplished around 9 a.m. in plants maintained under green-house conditions. Both self and cross pollination was conducted. The pollen grains germinated within 24 hours after pollination. First visible change was shown by the lip, which turned darker from its original color after one day of pollination. Thereafter, the color started fading with the passage of time. The lip started turning towards the column and ultimately the lip shrank and became blackish brown and persisted as a crumpled mass on the top of the inferior ovary/ fruit (Fig.1). The other perianth members showed signs of wilting at 2-3 DAP, which gradually increased at 3-4 days after pollination (DAP) (Figure 1C, D.) and subsequently, the perianth turned blackish mass after 10 DAP. It became crumpled structure around 30-34 DAP and persisted on the top of the fruit as such (Figure 2 A-B).

The findings, in general, revealed that unpollinated flowers of *Aerides multiflorum* (Am) 17 days to show senescence but the pollinated flowers attained senescence in 7 DAP suggesting a rapid progression of senescent related events triggered by pollination. These observations are in agreement with some of the earlier ones observed in other orchid species where for example, flowers of *Phalaenopsis* and *Cymbidium* species were found to live up to 8 weeks, if unpollinated but died within 7 days after pollination. Likewise, *Paphiopedilum* blossoms lasted for 3 months in unpollinated state but senesced within 3 weeks after pollination [11], 6-8 days in *C. pendulum*, *C. Aloifolium* and *Rhynchostylus retusa* [12, 17].

The first detectable symptom after pollination was alteration in lip color of the lip which turned darker due to elevation of anthocyanins that continue to increase till the perianth wilted and shrunk to its minimum size. The change in lip colour happened 1 DAP as reported earlier reported[12-17,18,19] and It has been reported that over 74-angiospermic family exhibit floral pigmentation changes in response to pollination or flower ageing and proposed that pollinators recognize color change and visit preferentially previously unpollinated flowers [20, 21].

It has been suggested that pollination-regulated color changes evolved independently in angiosperms many different times [21]. The perianth of flowers showed wilting following pollination that was one of the quickest symptom observed in pollinated flowers. In *Phalaenopsis* cv. 'Herbet Hager' flowers having longevity of 2-3 weeks showed rapid acceleration of the wilting process, beginning after only 24 h of pollination [22] while in *C. pendulum*, *C. aloifolium* and *R. retusa*, it was 6-7 days after pollination, which otherwise 16-20 days when unpollinated [12, 17].

Pollen germination commenced within 24 –48 hours after pollination that coincided with change in lip color that eventually showed fading. The stimulus of pollination induces growth and the size of the column and ovary started increasing 24 hours after pollination here. This appears to be a general feature of the most of the epidendroid orchids where the ovule development is a post-pollination phenomenon [23, 24]

The pollen tubes reached the base of the column (6-10 DAP) and entered the ovarian locule between 8-16 DAP. In a *Phalaenopsis* cultivar also, the pollen tubes took about 14 days to reach the locule [24] and 8-16 days in *C. pendulum*, *C. aloifoilum* and *R, retusa* [12, 17]. After entering the locule,

the pollen tubes grew on either side of the each placenta so that in a transection of the ovary, six groups were discernible as reported by earlier worker [25, 26].

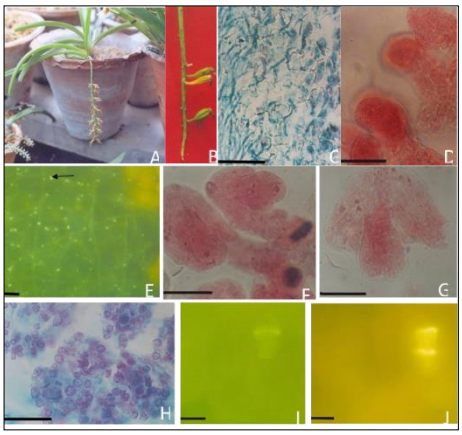

Fig 2: (A) Crumpled perianth; (B) Fruit Formation; (C) Pollen tubes; (D) Nucellar filaments with sub epidermal cell differentiated in to Archesporial cell (20-26 DAP); (E) Pollen tubes in locules; (F-H) Anatropous ovule with MMC (30-36 DAP); (I) First Meiotic division 36-40 DAP; (J) Triad (Two callose partitions at 36-40 DAP) ; Scale bars= 50 um

In epidendroid orchids, the placentae usually become active only after the stimulus of pollination e.g. *Arundina graminifolia* [27], *Dendrobium* species [25]. In the representatives of the subfamilies Cypripedioideae e.g. Spiranthoideae e.g *Spiranthes*, *Zeuxine*, and *Goodyera* [27], the ovary exhibits either poorly developed ovules or may possess an archesporial cell or a megaspore mother cell. Some of the primitive epidendroid taxa e.g. *Neottia*, also show ovule primordia with an archesporial cell on a megaspore mother cell [27] while *Epipogium roseum* seems unique in possessing ovules ready for fertilization at the time of anthesis [28]. After 24 hours of pollination, the three placental ridges become meristematically active, undergo branching several times and the ultimate ones develop into finger- shaped ovule primordia 6-8 DAP. This period has been reported even longer in *Phalaenopsis*, where the ovule primordia are formed at 42 DAP [24].

The ovule primordia multiply to produce nucellar filaments consisting of 5-7 cells in the axial row surrounded by a nucellar epidermis, the terminal cell of the filament differentiated into an archesporial cell (Figure 2 A-J). A similar feature has been recorded in a number of orchid taxa studied so far e.g. *Cymbidium bicolor* [29], *C.sinense* [30], *Epipactis helleborine*, *E.veratrifolia*, *Hebenaria sp.* [27], *Microstylis wallichii* [29], *Phalaenopsis sp*, [24] etc. Irregularly arranged biseriate filaments have been reported in *Vanilla planifolia* [32] and *Blettila striata* [33].

The ovule underwent bending due to unilateral growth and the inner integument was initiated as a ring near the base of the megasporocyte. The outer integument arouse soon after just below the inner one. The inner integument is single layered and form the micropyle at the time of completion of megasporogenesis. The ovule became anatropous at this stage. The outer integument is two layered and it always remains a little behind the inner integument. The ovule is thus bitegmic, tenuinucleate and anatropous (Figure 2 F-H). The ovule has been reported to be bitegmic, tenuinucleate and anatropous in all the orchids so far investigated [24, 30, 34, 35]. The micropyle may be formed by the inner integument alone e.g. *Dendrobium microbulibon* [23], species of *Epipactis* [35, 36] or both the integuments may organize a micropyle e.g. *Eulophia epidendraea* [38], *Bulbophylum mysorense* [23] and *Rhynchostylis retusa* [39].

The uppermost cells of the nucellar filament differentiated into an archesporial cell at 20-26 DAP. In most of the orchids studied so far, it is the terminal cell which increases in size and differentiates into the archesporial cell [23, 24, 37, 40, 41]. There are a few taxa where the second or the third cell or even any cell in the nucellar filament form an archesporial cell [27], suggesting an equipotential nature of these cells. The archesporial cell increases in size and in a span of four days it differentiates into a megasporocyte. In *C. sinense*, it has been shown that the increase in size of the archesporial cell to form the megasporocyte and subsequently the dyad and triad cells after meiosis-I is due to an increase in the number of cell organelles rather than vacuolation [42] (Figures 3 A-D).

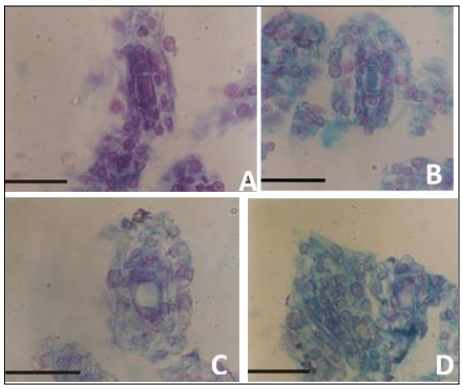

Fig 3: (A) Degenerating Megaspore mother cell and upper dyad (38-42 DAP); (B) Functional Megaspore; (Early telophase in megaspore (40-44 DAP); (C) 2-nucleated embryo sac (40-44 DAP); (D) 40 nucleate embryo sac (40-44 DAP); Scale bars=50 um

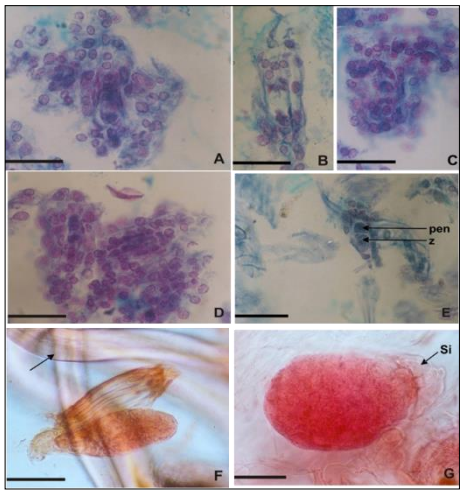

Fig 4: (A) 4-nuclaeate embryo sac (40-44 DAP); (B) 8-nucleate embryo sac (42-46 DAP); (C-D) Organized embryo sac with egg cell, Central cell and antipodal cells (46-50 DAP); (E) Zygote (z) and Pri. Endospermic nuclei (pen) (46-50 DAP); (F) Embryo with seed coat; (G) Embryo sac with suspensor; scale bars= 50 um

The megaspore mother cell undergoes meiosis resulting in the formation of a triad of megaspore (the upper most cell being the dyad cell). The chalazal megaspore became functional and formed an eight-nucleate polygonum type of embryo sac. The meiotic divisions are accomplished in a

period of four days, while it requires a period of another ten to twelve days to reach at the organized embryo sac stage. Thus, the total time taken to reach the organized embryo sac is between 55-60 days. The time taken to achieve this stage in *Dendrobium* sp. and *Phalaenopsis pulcherrima* has been reported to be 50-65 days [25, 26].

The development of embryo sac in most of the epidendroid and spiranthoid and orchidoid taxa corresponds to the Polygonum type [23, 24, 37, 40, 43].

The process of syngamy and triple fusion was accomplished between 46-50 DAP in present studies (Figure A-F) as reported earlier in *C. pendulum* and *C. aloifolium* as reported [12, 34]**.** There is a good deal of variation in the time interval between pollination and fertilization in the orchidaceae. The period is generally short in the terrestrial orchid of the subfamily Spiranthoideae and Orchidoideae this duration is only eight to ten days in *Habenaria* sp. and *satyrium napalense* while this duration varies from five days to two to three months in the epidendroid taxa e.g. fifteen days in *Spathoglottis plicata*, thirty days in *Vanilla planifolia*, forty five days in *Cymbidium bicolor* and *Eulophia epidendrea*e, sixty to seventy five days in *Bulbophyllum mysorense*, *Dendrobium* sp., *Geodorum densiflorum* [23] and *Phalaenopsis* sp [24]. Each orchid thus seems to have stabilized its time interval for different developmental stages in relation to the environment and the availability of species –specific pollinators.

4. Conclusions

The present studies deal with post pollination embryological changes in *Aerides multiflora* (Roxb.) changes had been investigated. It was observed that developmental changes are pollination directed. Orchid seems to have stabilized its time interval for different developmental stages in relation to the environment and the availability of species –specific pollinators.

More such studies should be carried out to gather the maximum data related to developmental biology, which seems halted from quite some time as very less reports are encountered as far as embryology is concerned. From the present paper and earlier reports too, it is well established that Pollination related developmental studies are of immensely useful for plant breeders to understand the nature of taxon so as to develop intergeneric and interspecific hybrids.

5. Acknowledgment

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