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Anatomical Studies of *Nervilia crociformis*

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Abstract: To study the anatomical features of terrestrial orchid (*Nervilia crociformis*) collected from natural habitats of western ghats, Karnataka, India. The terrestrial orchid, *Nervilia crociformis,* was collected from natural habitats from various locations of Kemmangundi and Sagar, Karnataka State. Freshly collected root/tuber of *Nervilia crociformis* was used for anatomical studies. Free hand sections were taken, stained with tryphan blue and viewed under the light microscope. Ultra-thin sections of roots of *Nervilia crociformis* were taken and viewed under transmission electron microscopy (TEM) and the colonized cells was studied. **L**ight microscopic study revealed varied distribution of intact and lysed pelotons in the root cortex and the tubers were sparsely colonized. The extent of peloton formation was assessed in all the species. TEM studies *Nervilia crociformis* revealed fine structure of pelotonic hyphae, their lysis and interfacial membrane. Anatomical studies of *Nervilia crociformis* by light and transmission microscopy revealed the presence of mycorrhizal association.

Keywords - Nervilia crociformis, Anatomical studies, Light microscopy, TEM, Mycorrhizal association

I. INTRODUCTION

Orchidaceae is a cosmopolitan with 27135 accepted species in 925 genera mainly distributed in the tropical and sub-tropical regions of the world. Majority of the adult orchids are green and photosynthetic.[1] However, about 200 species are achlorophyllous and myco-heterotrophic.[2] Orchids are mostly dependent on mycorrhizal fungi in their early stage of development or throughout their life cycle. The orchids are mycorrhizal have been known for over 125 years by the works of Wahrlich and Janse who surveyed a large number of temperate and tropical orchid species and noted the regular occurrence of fungal colonization of the roots.[3,4] Many orchid products are known to have medicinal properties and are used as aphrodisiacs, treatment of sores, vermifuges.[5,6] Orchid endophytic fungi were first observed by Reissek much before the term mycorrhiza coined by Frank.[7] Both Wahrlich and Janse surveyed many cultivated and wild orchid species and found fungal infection in each of them.[3,4] Major experimental work on isolation, identification of associated fungi, its role in orchid seed germination, nature of symbiosis, plant defense response and application of such studies in the multiplication of orchids was initiated by Bernard.[8-17] Subsequently, several research investigators have shown mycorrhizae to be ubiquitous in terrestrial and many epiphytic orchids.[18-25]

Mycorrhizal colonization is found to be sporadic in most epiphytes but is consistent in terrestrial orchids.[19.26] Different genera of terrestrial orchids have distinctive colonization patterns within their roots or stems. These mycorrhizal infection patterns in the whole plant (roots, collar, stem, rhizome, etc…) may be associated with particular fungal types.[23] Colonization patterns of mycorrhizal fungi within plants are primarily determined by host cell properties, but mycorrhizal morphological features can also be correlated with the presence of certain fungi. Orchid mycorrhiza has been critically reviewed by various researchers.[26-28]

A review on the studies of mycorrhizal fungi of Chinese orchids and similar region wise reviews published by various workers revealed limited work on orchid mycorhhiza. Work on orchid mycorrhizae is very much limited when compared to other parts of the world.[29] Senthilkumar and Krishnamurthy worked on the mycorrhizal association of *Spathoglottis plicata* colonized with *Rhizoctonia*.[30,31] Cytochemical studies of fungal pelotons and the associated hyphae were made. They also discussed the role of root hairs in the reproduction of associated fungal hyphae.[32] Kaliomurthy et al. studied the pattern of natural mycorrhizal infection in epiphytic and terrestrial orchid species growing in peninsular India.[33] *Aerides maculosum*, an epiphytic orchid and *Calanthe triplicate*, a terrestrial orchid was investigated. Sathiyadash et al analysed the mycorrhizal associations of 31 adult wild green orchids (22 epiphytic, 8 terrestrials, 1 both epiphytic and lithophytic) from different vegetation types of Western Ghats, South India.[34]

Furthermore, Simmi Aggarwal et al have investigated *in-vitro* symbiotic seed germination and molecular characterization of associated fungus, *Rhizoctonia zeae* (*Thanatephorus cucumeris)* with *Vanda coerulea* growing at Imphal, Manipur.[35]

Literature study revealed that there are only few reports available on the study of orchid mycorrhiza especially from India.[36-38] Hence, the present study was designed to conduct with the main aim to study the anatomical features of terrestrial orchid (*Nervilia crociformis*) collected from various parts of Karnataka state.

II. MATERIALS AND METHODS

The *Nervilia crociformis* samples used in the present investigation included collection of orchid plant parts *viz.* root and tuber of the selected ground orchid.

Free hand sectioning and microscopic study of *Nervilia crociformis*

Thin free hand sections of the *Nervilia crociformis plant parts* such as root / tuber were taken for colonization in the cortical cells. Colonization density was calculated as described by Hadley and Williamson.[20] The plant material was stained with tryphan blue (0.05% in lactophenol) and observed under the light microscope for the presence of fungal colonization in the form of hyphal coils in the cortical cells. Highly coiled hyphal pelotons (intact and digested) were observed and assessed in each section. The number of colonized cells in the cortex was expressed as a percentage of cells available for colonization.

Transmission Electron Microscopic (TEM) Studies of *Nervilia crociformis*

Collection of specimens

Healthy root samples of *Nervilia crociformis* were used for TEM studies. The sample was rinsed to remove the soil from the roots with cold water. These rinses were performed quickly and gently, thorough enough to remove adherent soil particles which would interfere with microtomy later. The cleaned root was exposed immediately to the chosen fixative and reduced to pieces not larger than 1mm in diameter as small sample size exerts a profound effect upon the quality of fixation achieved, and is a major factor influencing the length of exposure intervals required during dehydration and embedding.

Fixation techniques

Primary fixation of the root bits was fixed in 3% glutaraldehyde in 0.1M Cacodylate buffer at pH 7.2. It was later washed in phosphate buffer to completely remove glutaraldehyde. Fixation was performed at 4ºC for 1-4 hrs and was followed by post fixation for 1-4 hrs at 4ºC in 1-2% Osmium tetroxide (OsO_4) in the same buffer used with the aldehyde.

Dehydration

The primary concern of dehydration is to remove water from the sample rapidly to minimize extraction of cytoplasmic components and to achieve complete dehydration and ethanol was used for this purpose. To facilitate infiltration with liquid resin for embedding, dehydration was done. The roots were transferred to different concentrations of ethanol *i.e.*, in 30%, 50%, 70%, 90% and 100% sequentially for 20 mins each at room temperature.

Infiltration

Dehydrated roots were transferred to an infiltration medium of 100% propylene oxide in glass vials. Fresh resin was prepared and the old infiltration medium was replaced with new infiltration medium containing a mixture of propylene oxide and resin. The vials were placed in rotary shaker overnight at room temperature for complete infiltration.

Embedding

Vials containing the samples and resin were poured into beam capsules and were labeled. The root fragments were oriented under the stereomicroscope and kept in the oven for polymerization at 60ºC for 48hrs. After complete polymerization the blocks were ready for sectioning. The blocks were stored in a desiccator to prevent softening.

Trimming

The blocks after polymerization were trimmed using a glass knife to have a small cutting face free from extra resin (embedding medium) mounted on a specimen holder on an ultramicrotome.

Semi-thin sections

The trimmed blocks were sectioned using glass knives and semi-thin sections were obtained. Selected semi-thin sections were placed on a glass slide and kept on a hot plate at 80ºC and dried. The sections were stained with 1% toluidine blue for one minute, washed in running water, dried and mounted with a mixture of distyrene, a plasticizer and xylene (DPX). The slides were observed under light microscope.

Ultrathin sections

After scanning the sections under the light microscope, blocks were further trimmed and ultrathin sections of 60 nm were taken using fresh glass knives to obtain high resolution. The ultrathin sections were collected on metal grids and the grids were stored in grid boxes.

Staining

To obtain contrast, the root tissues were stained with heavy metals. Double staining was done using Uranyl acetate followed by lead acetate. The sections were observed under Transmission Electron Microscope FEI Tecnai 12 G^2 operating at 80kV. The electron photomicrographs were obtained with fine structural details using TEM facility at NIMHANS, Bangalore.

III. RESULTS

The terrestrial orchid *Nervilia crociformis* selected for anatomical studies in the current study, was collected from Sagar, Shimoga district, Karnataka. They were identified using taxonomic keys published in various Floras like Abraham & Vatsala, Santapau & Kapadia and Ananda Rao.[39-41]

Light microscopic study of *Nervilia crociformis*

Transverse sections of the underground part (root/tuber) of the terrestrial orchid were taken and stained with tryphan blue and observed under a binocular compound microscope. The percentage of colonization in the form of pelotons was calculated. The diameter of the roots varied between 2-8 mm in test species. Parenchymatous cortex was wider than the vascular bundles, a feature seen in most of the *Nervilia crociformis* root samples collected from various parts of Karnataka state.

Transverse section of the root showed fungal colonization in the cortical region. The fungus gained entry into the root through the root hairs (Plate 1A). Pelotons were observed as loosely arranged clumps of hyphae within the cortical cells of the root and in the superficial cells of the tuber (Plate 1B). Colonization of the fungal hyphae is a continuous process and all the cells of the cortex were not colonized at the same time. There was generally a mix up of the pelotonic and non-pelotonic hyphal colonization in the cortex (Plate 1C).

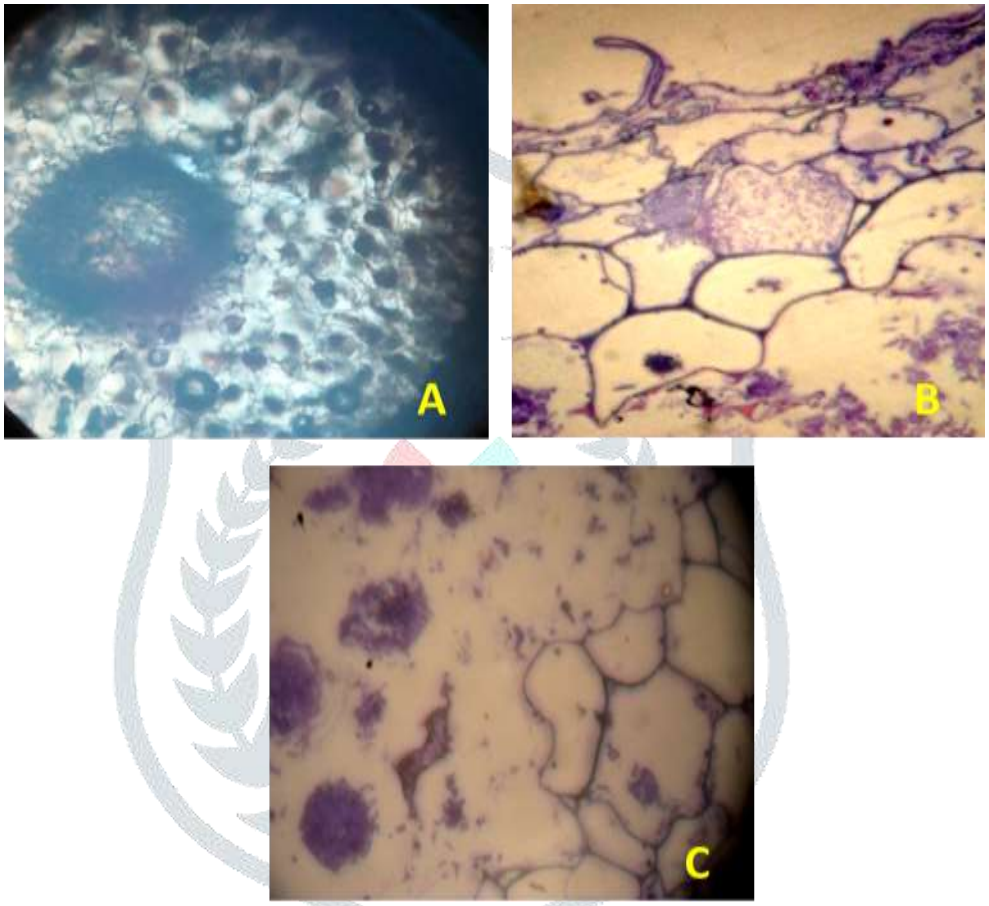

Plate 1

Rhizodermis uniseriate, thin walled, cortex 6-8 cells wide, parenchymatous. Pelotons observed in the inner cortex, extending up to 2-3 layers, both intact and digested pelotons were observed. Colonisation density was up to 40%. Pelotons were absent in the rhizodermis, endodermis and vascular region. The transverse section of the tuber showed few pelotons in the peripheral cells.

One of the significant events in the orchid-mycorrhizal association is the lysis of the pelotons.[42] Lysis is initiated sequentially in the oldest colonized cells followed by the cells that have been subsequently colonized.[43] Each intracellular peloton has a short span of life, lasting only for a few days, before it degenerates and is digested by the orchid cell. The older hyphae develop larger vacuoles, thick cell walls and the cytoplasm degenerates which eventually collapse and are consumed by the orchid cell. During this process cell remains functional and get re-colonized by any surviving hyphae or by fungi invading from adjacent cells.

Digested pelotons were observed randomly in the cortical cells of all other test plants and not to a defined zone of cells, which was recognized earlier in orchid root cortex by Hadley & Williamson.[20] Like many autotrophic orchids, the mycorrhiza in the orchid of present study were of tolypophagy type, in which the fungal hyphae get digested, and the organic products from the fungal mycelium transferred to the plant.[44]

Recolonisation of cortical cells of the root after peloton digestion was observed in *Satyrium nepalense* as reported in earlier studies by Senthilkumar et al in *Spathoglottis plicata* who reported the presence of pelotons up to three generations in the root cortical cells.[43] Generally, tubers and pseudobulb restrict the fungal colonization by accumulation of phenolic compounds, raphides and mucilage. The control of hyphal invasion in an orchid tissue may be due to the production of phytoalexins.[45,46] Raphides and phenolic substances were present in the cortical cells of root of *Nervilia crociformis*. The calcium oxalate crystals

(raphides) have different functions like storage forms of calcium and oxalic acid, ionic balance and osmotic regulation. These substances prevent the invasion of fungi in the deeper regions of the cortex.

TEM study of *Nervilia crociformis*

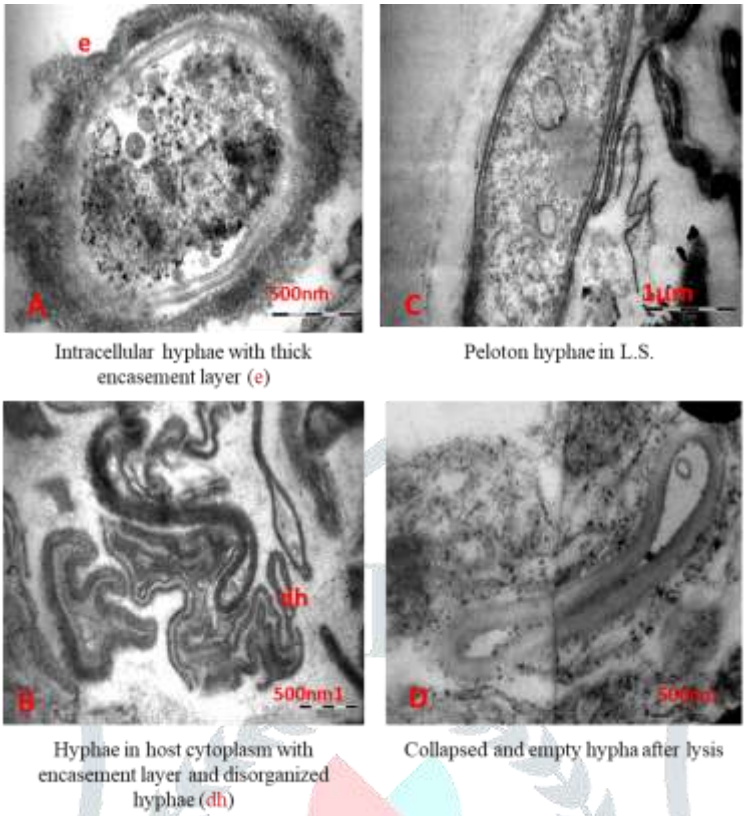

Plate 2

TEM study helps to decipher the fine structure of pelotons and to observe interfacial membrane seen in tolypophagous mycorrhizal association. The *Nervilia crociformis* with very thin roots were selected for the study. Both intact and digested pelotons were seen in all the roots. Pelotons were seen as lumps of hyphae of bizarre shape within the cortical cells of the root. The hyphae were seen to be thinly enveloped by host cytoplasm (Plate 2A). The walls of the intracellular hyphae consisted of two layers, the inner electron dense and normally 60nm thick, the outer rather granular or flocculose and from 100-200nm thick. The cytoplasm of the hyphae was often densely packed with ribosomes, mitochondria and vacuoles were fewer in old than young hyphae. The plasmalemma of the fungus invaginates to form tubes into the cytoplasm and in one section appears continuous with membranes and formed a system of tubules. Inclusions presumed to be reserve material were sometimes present (Plate 2C). The plasma membrane of the plant remained intact and was separated from the clumped fungal material by an electron-lucent layer.

During lysis (also called digestion) of pelotons the fungal contents become disorganized and the clump together, forming an irregular mass. As the hyphae lose turgor they presumably collapse, lose their soluble cell contents and become flattened (Plate 2D). The host plasmalemma draws away leaving a space between it and the encasement layer (Plate 6 A). In the ultimate phase of degeneration individual hyphae became indistinguishable as they congeal into a mass (Plate 2B). These masses are called as clots, clusters or digested pelotons. The electron dense strands are the remains of hyphae and they are interspersed with less dense zones of material originating from the encasement layers (Plate 2D). During this process the plant cells remain alive and active, and may be recolonized by hyphae either apparently surviving the lytic process or invasion from adjacent cell.

Hadley et al interpret this outer layer as originating from the fungus alone.[47] Similar features were seen in the species investigated for *Nervilia crociformis*. TEM studies of orchid mycorrhizae in these plant species have been investigated for the first time. They differ from TEM studies on mycoheterotrophic orchids; Elena et al on *Orchis militaris*, Martos et al on *Wullschlaegelia aphylla* and Roy et al on *Epipogium aphyllum* in that the digestion of pelotons is absent in mycoheterotrophic orchids.[48-50]

IV. Conclusion

The terrestrial orchid identified as *Nervilia crociformis*, was collected from natural habitats from various parts of Karnataka State and were investigated for mycorrhizal association. The anatomical studies of *Nervilia crociformis* by light and transmission electron microscopy revealed the presence of mycorrhizal association. The mycorrhizae in orchid were found to be quite less especially in terrestrial orchids. TEM studies of orchid mycorrhizae in these plant species have been investigated for the first time.

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