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Orchid Mycorrhizal Studies of *Nervilia crociformis*

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Abstract

Orchid mycorrhizal associations involve a plethora of distinctive nutrient transport systems, structures and phenomena which have only been observed in the family Orchidaceae. Orchid mycorrhizae are symbiotic relationships between the roots of plants of the family Orchidaceae and a variety of fungi. Hence, current study aimed to isolate and purify the mycorrhizal fungi of orchid from natural habitats of Western Ghats. The terrestrial orchid *Nervilia crociformis,* was collected from natural habitats from various locations of Kemmangundi and Sagar, Karnataka State and were investigated for mycorrhizal association. Freshly collected root/tuber of *Nervilia crociformis* was used for isolation, identification and purification. The fungal isolates were purified by hyphal tip method. Classical identification techniques used in the identification of fungi include colony characters and microscopic characters of hyphae and spores**.** One fungal isolate was obtained from the tuber of *Nervilia crociformis* which was identified as *Colletotrichum fruticola.*

Keywords
Terrestrial orchid, *Nervilia crociformis, Colletotrichum fruticola,* fungal isolate

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INTRODUCTION

The term *mycorrhiza* was coined by Frank for the fungus infested roots [1]. Mycorrhizae are symbiotic associations between specialized soil fungi and plants. They are primarily responsible for nutrient transfer and are essential for one or both the partners. Most plants in natural ecosystems have mycorrhizal association when symbiotic fungi inhabit healthy tissues of the root of terrestrial plants and play an important role in their successful colonization. Bernard was the first to isolate *Rhizoctonia*- like fungi by surface sterilizing the root fragments and aseptically plating on nutrient agar medium [2]. Recently isolation from single peloton was attempted which is a tedious process [3].

Isolates of Rhizoctonia sp. obtained from terrestrial orchids *Dactylorhiza sambucina, D. maculata* and *Platanthera bifolia* collected from Italy were studied for macro and microscopic morphology, growth rate, ultrastructure of septal pore apparatus. On the basis of cultural and morphometric characters the isolates were divided into 3 groups. No perfect stage was produced in culture; group I were identified as species of Ceratobasidium, group II as species of Thanetophorus and group III as species of Tulasnella or Sebacina. According to Currah et al *Rhizoctonia panaicula* Currah, sp. now represented by 5 different isolates had a unique morphology of monilioid cells was isolated from *Platanthera obtusata*, *Ceratobasidium obscurum* from *Amerorchis*

rotundifolia, Phialocephala fortini from *Calypso bulbosa* [4]. Two new species of fungi mycorrhizal with terrestrial orchids native to Canadian priaries have been reported viz. *Ceratorhiza pernacatena sp.nov*. from mycorrhiza of Platanthera *praeclara. Epulorhiza calendulina sp.nov*. from mycorrhiza of *Amerorchis rotundifolia* [5].

These fungi were generally identified based on culture characters, microscopic study of hyphae, their associated structures. A key to the genera of fungi mycorrhizal with orchids was provided by Currah & Zelmer [6]. Sneh et al have given a guide to identify *Rhizoctonia* species [7]. A check list of *Rhizoctonia* epithets was provided by Anderson [8]. Recently orchid mycobionts being identified using molecular biology technique by sequencing the internal transcribed spacer (ITS) of the nuclear ribosomal DNA after PCR amplification using a variety of primer combinations has been the method routinely used to identify orchid mycobionts from cultured fungi or directly from orchid protocorms, roots, tubers and rhizomes [9,10]. Both universal fungal ITS primers and Tulasnaceae- specific PCR primers were being used [11,12]. Suarez *et al*., (2006), Martos et al used 5.8 S- Tul to amplify the 5' part of 28 S rDNA to work on range of clades of Tulasnaceae and Ascomycete mycobiontts [13,14]. ITS sequencing and cloning also revealed many endophytes associated with orchid roots [15-18]. Review of literature revealed that there are only a few reports on the study of orchid mycorrhiza especially from India [19-21]. Hence in the present work we attempted to study terrestrial orchid mycorrhizae of Karnataka province.

MATERIALS AND METHODS

The materials and methods used in the present investigation on the study of mycorrhizae in terrestrial orchids included collection of orchid plants root and tuber of selected ground orchids.

Isolation of associated mycorrhizal fungi

Standard procedures were used for isolation of mycorrhizal fungi by plating portions of surface-sterilized roots/tubers on suitable media [22]. Fresh root/tuber samples of terrestrial orchids were collected from their natural habitats by carefully removing the soil so as to keep the underground system intact. Roots and tuber (if present) of the orchid plants were used separately for the purpose of isolation. The sample surface was first washed with running tap water several times carefully to get rid of soil particles and organic debris. The roots were then surface sterilized by submerging them in 0.1% mercuric chloride solution for 3-5 minutes washed in three changes of sterile distilled water, 5-10 mm portions of the roots were then cut, aseptically, plated on Potato dextrose agar (PDA) medium containing streptomycin (50µg/mL) and incubated at 25°C. Fungi grown from the cut ends were used for obtaining the pure culture. These isolates were further grown on PDA at 25°C for 4 weeks under normal diurnal conditions to allow sclerotia and chlamydospores to form and mature before identification was attempted.

Identification of associated mycorrhizal fungi by classical method

The taxonomic identification of an organism is an essential part of the investigation of a new species or isolate of a known species. It is imperative that the organism is correctly identified so that when reference is made to published material, the experimental data becomes valid and may be used and compared by other investigators. Classical identification techniques used in the identification of fungi include colony characters and microscopic characters of hyphae and spores**.** In the present work, attempts were made to identify all the isolates obtained by studying the similarities and differences between the isolates in the following characters: (a) colony characters- including growth rate, colour, shape and texture of fungal colony; sclerotia present or absent; colour changes in the substrate (medium) caused by the fungus (b) Microscopic characters involving colour, size, septation, branching of the hyphae, formation of rhizomorphs, sclerotia, and sporulation if any. Of these characters, the size and shape of chlamydospores, the size and texture of sclerotia, characteristics of hyphae and spores as described by Barnett & Hunter were given special consideration [23].

Purification and maintenance of fungal isolates

The fungal isolates were purified by hyphal tip method. They were maintained on PDA slants in the refrigerator for further studies. This technique was used for species which frequently develop degenerate cultural variants from germinated conidia, or fungi such as *Rhizoctonia* which do not sporulate in culture.

RESULTS AND DISCUSSION

The terrestrial orchid, selected for mycorrhizal study was *Nervilia crociformis* 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 [24-26].

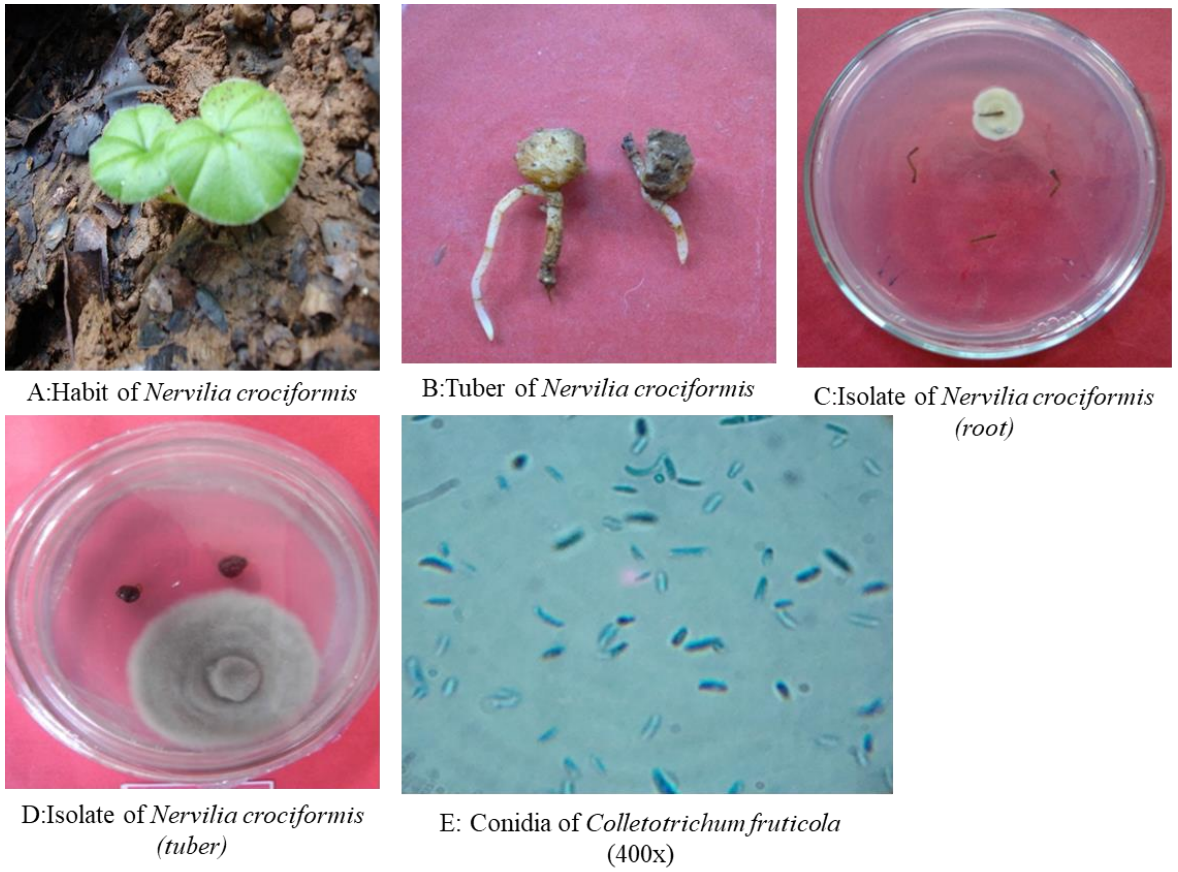
Isolation of associated mycorrhizal fungi

The fungal isolates were obtained from the root/tuber/ of the terrestrial orchid. The fungi grew from the cut ends of the root/tuber as white cottony

growth. Pure cultures were obtained by hyphal tip method and were maintained in PDA slants for further studies. These were preliminarily identified using colony characters and morphological characteristics of hyphae and spores if any as described by Barnett & Hunter [23].

Identification of associated mycorrhizal fungal isolates by classical method

The fungal isolate from the tuber of *Nervilia crociformis* showed *Colletotrichum fruticola* (Plate 1 A-E). Colonies on PDA at first white, becoming grey to dark grey at the centre with age, in reverse greyish green with white halo becoming black with age, maximum of 8.3cm in diam. in 7 days. Aerial mycelium pale grey, dense, cottony, without visible conidial masses. Sclerotia absent. Conidia 9.5-14 x 3-4.5µm, one- cell, smooth walled with a large guttule at the centre and surrounded by smaller guttules, hyaline, cylindrical with obtuse to slightly rounded cells, sometimes oblong.



A:Habit of *Nervilia crociformis*

B:Tuber of *Nervilia crociformis*

C:Isolate of *Nervilia crociformis (root)*

D:Isolate of *Nervilia crociformis (tuber)*

E: Conidia of *Colletotrichum fruticola* (400x)

Plate 1

CONCLUSION

In conclusion, the terrestrial orchid identified as *Nervilia crociformis,* was collected from natural habitats from various locations of Kemmangundi and Sagar, Karnataka State and were investigated for mycorrhizal association. The fungal isolate from the tuber of *Nervilia crociformis* was identified as *Colletotrichum fruticola.*

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