MYCORRHIZA IN OPHIOGLOSSACEAE : PART II—ISOLATION AND RE-INOCULATION OF THE ENDOPHYTES AND TESTING THEIR PATHOGENICITY.

LIZZIE N. NAIR AND T. S. MAHABALE

**Department of Botany, University of Poona, Pune*-411007, *INDIA*

ABSTRACT

The endophytes from the roots of a species of *Botrychium* and two species of *Ophioglossum* were isolated. *Fusarium oxysporum* Schlect. was isolated from the roots of *Ophioglossum reticulatum* L. and *Botrychium virginianum* var. *daucifolium* Bedd. *F. solani* (Mart.) Appel. & Wr. was isolated from the roots of *O. penduncolosum sensu non*-Desv. These fungi were proved to be the mycorrhizal forms by reinoculating them in young plants. As *Fusarium oxysporum* and *F. solani* are known to be pathogenic, they were tested for pathogenicity, but proved otherwise.

INTRODUCTION

The term mycorrhiza was coined by FRANK (1880) indicating the association of fungi with roots of higher plants. The early history of mycorrhiza has been fully given by RAYNER (1927) in his monumental work "*Mycorrhiza*". After RAYNER, BURGEFF (1938) published his views on mycorrhiza. This was followed by an excellent review on "The Biology of Mycorrhiza" by HARLEY (1959). Further work on the isolation of endophyte was done by BARRETT (1959). In India important work on mycorrhiza was done by the members of the Mycology Division of the Forest mycologists such as BAKSHI (1974). Prof. Kashyap and several others had noticed mycorrhiza in liverworts and ferns, but no systematic attempt was made to study them in Indian species of the Psilophytaceae, Lycopodiaceae, Ophioglossaceae, Marattiaceae, etc. which are known to harbour them.

Mycorrhiza in Ophioglossaceae was reported by NISHIDA (1956) and HEPDEN (1960). In India, its occurrence has been studied in detail by MAHABALE (1925, 1939) and NAIR AND MAHABALE (1975). The perennation of the mycorrhizal fungus in soil in natural condition also was studied by them (NAIR & MAHABALE, 1975).

MATERIAL AND METHODS

Plants of *Ophioglossum reticulatum* L. were collected from Mahabaleshwar and Purandar Hill stations near Poona, *O. penduncolosum sensu non* -Desv. from Poona and *Botrychium virginianum* var. *daucifolium* Bedd. from Kodaikanal, S. India.

The fungus was successfully isolated on Waksman's medium*. Fresh roots were

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Waksman's Medium :

Glucose	10 gms.
Peptone	5 gms.
KH_2PO_4	1 gm.
$MgSO_4$ $7H_2O$	0.5 gm
Agar	25 gms.
Water	1000 ml.
pH	5.5

Autoclave at 15 lb. for 20-minutes.

excised from the healthy plants, washed well with tap water and surface sterilized with 1 in 1000 mercuric chloride solution for 3-5 minutes. They were washed further with sterile distilled water 5-6 times until all traces of mercuric chloride were removed. Then they were cut into bits under aseptic conditions and inoculated on Waksman's slants.

For reinoculation, root buds after surface sterilizing, were grown in flasks with Knop's medium, after dipping them in spore suspensions of the respective endophytes already prepared. In some, the young roots were scratched with the help of a sterile needle so as to expedite the entry of the endophytes into them. Plants were also grown along with spore suspension of the endophytes in Knop's solution.

Another method followed was to grow the young surface sterilized *Ophioglossum* buds in pots containing sterilized soil mixed with spore suspensions of the respective endophytes. Pathogenicity tests were carried out in the same way by growing seeds of Cotton, *Vigna*, *Phaseolus* and *Lycopersicum* susceptible to the respective fungi in sterile pots containing sterile soil mixed with suspensions of the endophytic fungi.

DESCRIPTION

1. Isolation of the Endophytes

A number of fungi occur close to the roots of *Ophioglossum* and *Botrychium* in soil, but only one of them appeared to be endophytic in each species. It had to be isolated and identified, wherever possible. But it could be isolated only from the roots of *O. reticulatum* L., *O. penduncolosum sensu non*-Desv. and *Botrychium virginianum* var. *daucifolium* Bedd. Procedure followed for isolation and description of the endophytes isolated are given below. It must be noted here, however, that the endophyte did not yield to the techniques in the case of other species such as *Ophioglossum nudicaule* L., *O. gramineum* Willd., *O. aitchisoni* d'Alm., *O. firbosum* Schum. and *Helminthostachys zeylanica* L.

Potato Dextrose Agar and Czapek's medium were used in the beginning, but they did not give successful results. HARRISON's (1955) section embedding technique also was tried but it also did not give any result.

For isolation of the endophyte, old dark brown roots were selected in *Botrychium virginianum* var. *daucifolium* Bedd., *O. reticulatum* L., *O. penduncolosum sensu non*-Desv. and of other species. These were then sectioned and examined to confirm that fungus was present in them. In the case of *B. virginianum* var. *daucifolium* Bedd. the presence of mycorrhiza was easily visible by naked eye as a continuous yellowish zone in the outer cortex of the root.

The fresh roots were washed under tap water to remove the soil and sand particles and then with distilled water. The washed roots were kept for five minutes in 1 in 1000 mercuric chloride solution. After this, they were washed with sterile distilled water 5-6 times to remove the last traces of mercuric chloride. They were cut obliquely with sterile scissors under aseptic conditions in an aseptic chamber. The pieces cut were transferred with sterile forceps to slants having Waksman's medium.

The roots were pressed gently into the slants so that the cut surface was in contact with the medium. Obliquely cut roots exposed greater surface of the mycorrhizal area. Inoculated tubes were kept at room temperature (25-27°C) for about 4-5 days. On the 6th day, white mycelia started growing out from the cut surface of the roots. They covered the slant surface in about next 2-3 days.

Sub-culturing of these fungi was done on freshly prepared slants of the Waksman's medium and observations were made on them. The following fungi emerged :

Host	*Endophyte*
O. reticulatum L.	*Fusarium oxysporum* Schlect.
O. pedunculosum sensu non Desv.	*Fusarium solani* (Mart.) Appel. & Wr.
B. virginianum var. *daucifolium* Bedd.	*Fusarium oxysporum* Schlect.

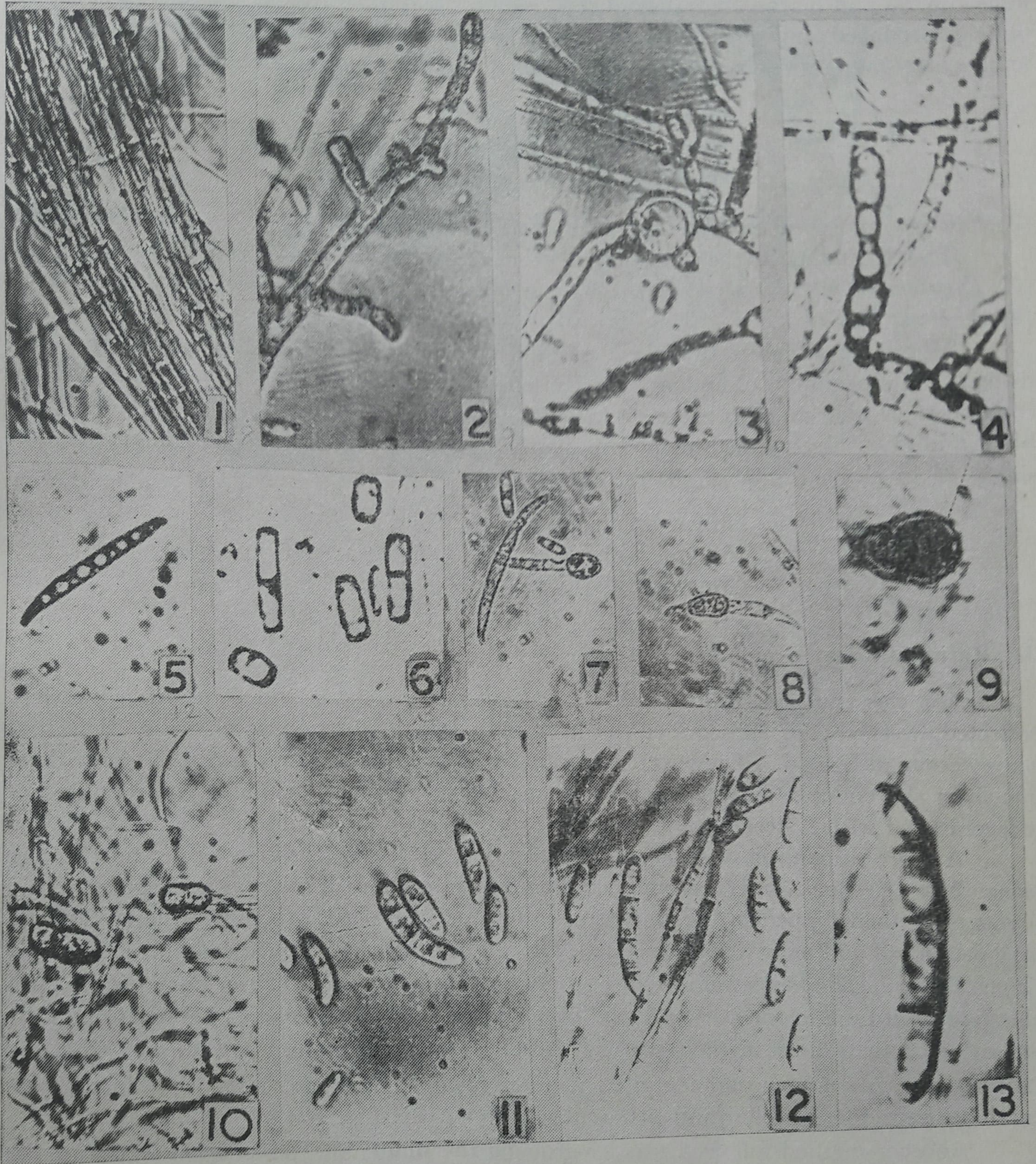


Plate 1. Figs. 1-13. Marphology of endophytes *Fusarium oxysporum* Schlet and *F. solani* (Mart.) Appel. & Wr. Figs. 1-9. *F. oxysporum* × 157. Fig. 1. Mycelium. Fig. 2. Single hypha. Figs. 3, 4. Young hyphal swellings. Fig. 5. Single macroconidium with thick walled cells. Fig. 9. Mature chlamydospore. Fig. 10-13. *F. solani*. Fig. 10. Mycelium and Microconidia and Macroconidia × 157. Chlamydospores × 315. Figs. 11-12. Microconidia and Macroconidia × 315. Fig. 13. Single macroconidia × 275.

2. Morphology of the Endophytes isolated *in vitro*.

(A) *Fusarium oxysporum* Schlect. from *Ophiloglossum reticulatum* L. Pl. 1, Figs. 1-9.

Conidia produced typically in sporodochia, conidiophores simple or branched, microconidia and macroconidia produced. Microconidia mostly one celled, hyaline, oval, oblong, produced singly from the tips of phialides. Macroconidia 3-4 septate, rarely 5-septate narrowed towards the tip and base. Chlaymydospores abundant.

Microconidia 4-14 $\mu m \times 2\text{-}5$ μm
Macroconidia 3 septate 20-25 $\times$ 2.5-5 μm
Macroconidia 5 septate 30-65 $\times$ 3-6 μm

(B) *Fusarium oxysporum* Schlect. isolated from *Botrychium virginianum* var. *daucifolium* Bedd.

This is similar to the one isolated from *O. reticulatum* L. described above.

(C) *Fusarium solani* (Mart.) Appel. & Wr. isolated from *O. pedunculosum sensu non* -Desv. (Pl. 1, Figs. 10-13).

Mycelium profusely branched, septate, conidia produced in false heads, or sporodochia. Microconidia abundant, mostly one celled, small, oval to oblong. Macroconidia subcylindrical with more or less uniform width, slightly curved at both the ends, blunt and smoothly rounded at the tip, mostly 3-5-septate, thick walled. Chlamydospores singly or in pairs, rarely in chains.

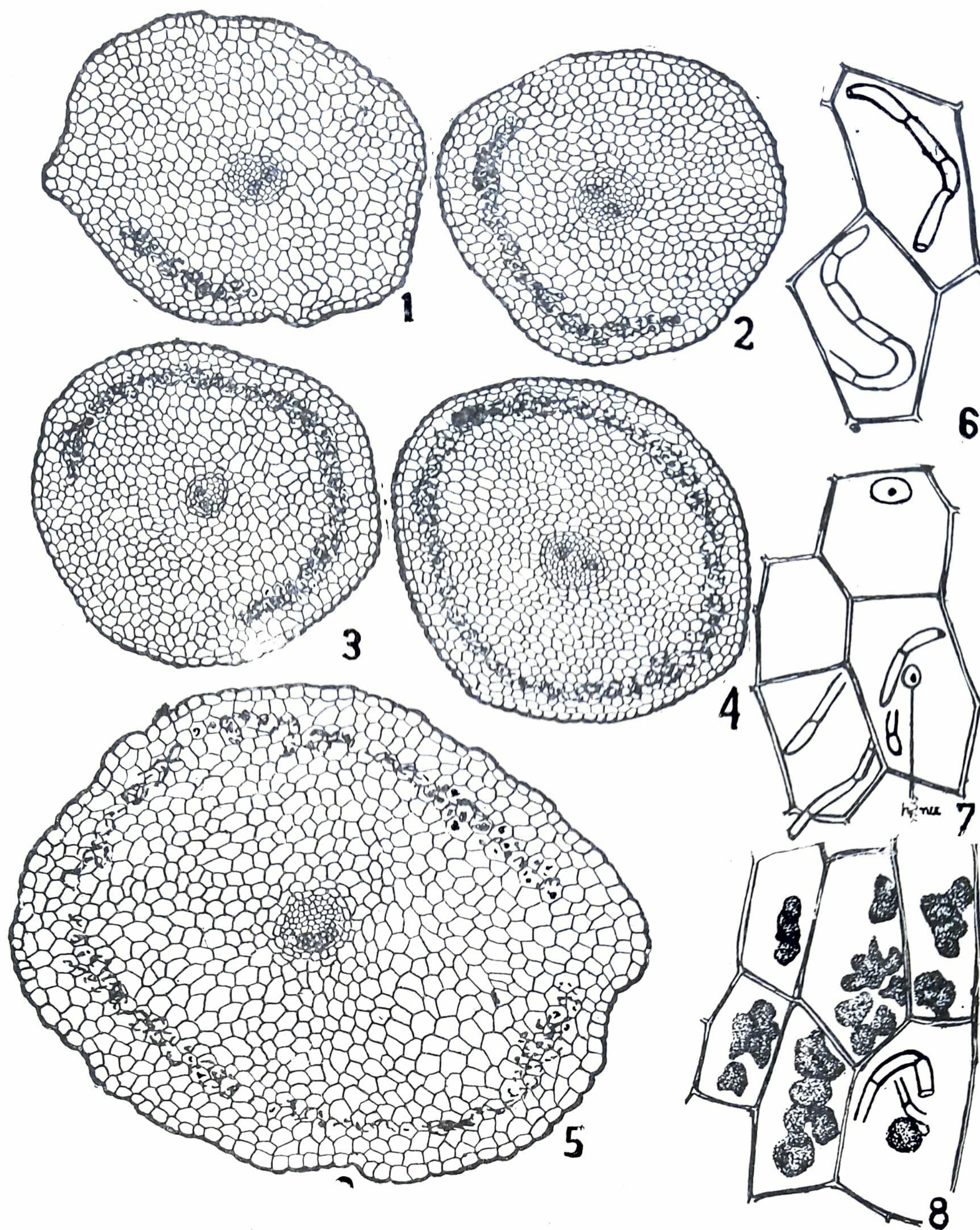
Microconidia 5-12 $\mu m \times 2\text{-}5$ μm
Macroconidia 3 septate 20-63 $\mu m \times 3.5\text{-}6$ μm
Macroconidia 5 septate 22-75 $\mu m \times 4\text{-}8$ μm

3. Reinoculation

For this purpose, fresh plants of *Ophioglossum reticulatum* and *O. pedunculosum* were collected, washed well and surface sterilized with 1 in 1000 mercuric chloride. The root buds were mainly used. These were tested to see if the roots had mycrorrhiza. Only those free from the endophyte were used for reinoculation. In certain plants the root surface was scratched with a sterile needle in order to facilitate the early entry.

A spore suspension of *Fusarium oxysporum* Schlect. from *O. reticulatum* L. and *Fusarium solani* (Mart.) Appel. & Wr. from *O. penduncularum sensu non*-Desv. was prepared. These plants were then dipped in the spore suspension and transferred to conical flasks containing both liquid and agar Knop's media. The whole operation was carried out under aseptic conditions. The flasks were kept at room temperature (25-27°C) for about 5-6 months checking from time to time. At the end of 5 months, it was noted that the roots had fully incorporated the fungus in their cortical tissue. These roots were removed, sectioned and stained with cotton blue and mounted in Lactophenol (Text-figs. 1-5). The fungus was found to have the myceliał and arbuscular stages (Text-figs. 6-8).

Another method followed was by growing the plants and endophytes in pots. For this *Fusarium oxysporum* 'A' isolated from *Ophioglossum reticulatum* L. were grown in surface cultures. These were mixed with sterile soil separately. In pots with sterile soil mixed with *Fuasrium oxysporum*, young plants of *Ophioglossum reticulatum* having roots not having mycorrhiza were planted. In the same way, pots containing soil mixed with *Fusarium solani* were planted with *O. penduncu losum* plants free of mycorrhiza in their roots. This took less time, 3-4-months only, for the endophytes to establish within the roots.



Figs. 1-8.

4. Testing the Pathogenicity

Some sterilized soil was taken and mixed with the *Fusarium oxysporum* 'A' isolated from *O. reticulatum*, *F. oxysporum* 'B' isolated from *Botrychium virginianum* var. *daucifolium* and *F. solani* from *O. pedunculosum*. For the first two isolated forms of *Fusarium oxysporum* 'A' and 'B' pathogenic to varieties of Cotton, *Vigna* and *Lycopersicum* susceptible seeds of these were used. For each of them six pots, each having 10 seeds, were used. For the third fungus *F. solani*, susceptible seeds of *Phaseolus mungo*, *P. radiatus* were selected. This also had 6 pots each with 10 seeds. These pots were kept watered under sterile conditions in a glass house. Observations were made every 3rd day.

It was observed that though *F. oxysporum* is reported to cause wilt in Cotton and other plants, the variety of this fungus isolated from *O. reticulatum* and *B. virginianum* var. *daucifolium* does not cause wilt in Cotton and other plants. Similarly, *F. solani* which causes diseases in species of *Phaseolus* also did not do so here.

CONCLUSION

Fusarium oxysporum Schlect. isolated from *O. reticulatum* L. and *B. virginianum* var. *daucifolium* Bedd. were possibly two different strains of the fungus. The third endophytic fungus was an entirely different species, *F. solani* (Mart.) Appel. & Wr. The chlamydospores in *O. reticulatum* roots as observed by MAHABALE (1935, 1939) and NAIR AND MAHABALE, (1975) are quite characteristic of the genus *Fusarium*. The reinoculation experiments clearly indicate the association of the respective endophyte with the particular plant and confirm its specificity.

Though both the species of *Fusarium* isolates are known to cause diseases in plants, they proved otherwise in the present cultures. This is due to the fact that these fungi have lost their pathogenicity on account of its long association with these plants. Hence, they have become non-pathogenic strains of *Fusarium* species known to be parasitic on other plants.

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