International Journal of Life science & Pharma Research

CONFOCAL STUDIES OF ARBUSCULAR MYCORRHIZAL FUNGI COLONIZING THE SPOROPHYTE OF *BOTRYCHIUM DAUCIFOLIUM* Wall., A RARE FERN OF INDIA

R.SARALA* AND S.R.SENTHILKUMAR[1]

[*] Department of Botany, Periyar E.V.R. College (A), Tiruchirappalli -23, Tamil Nadu, India.
[1] Department of Plant Biology and Plant Biotechnology, St.Joseph's College (A), Tiruchirappalli – 620 002, Tamil Nadu, India.

ABSTRACT

The arbuscular mycorrhizal (AM) fungi colonizing the sporophyte of the rare fern, *Botrychium daucifolium*, Ophioglossaceae were investigated in the present study. The roots of the sporophyte were analyzed for the presence of AM fungi. The confocal analysis of mycorrhizal association revealed the different stages of fungal lysis and degradation which served as the nutrients for the host cells. The AM fungi associated with the fern was identified to be *Glomus* species.

Key words : Arbuscular mycorrhiza, *Botrychium daucifolium*, confocal analysis, fern, sporophyte.

INTRODUCTION

The genus *Botrychium* is represented by 35 species worldwide and restricted to six species in India. In the world, the distribution of *Botrychium* is restricted to North temperate zone whereas in India, it is found in the Himalayas, Eastern Ghats and Western Ghats (Dixit, 1984). In India, the distribution of *Botrychium* is becoming limited. The six species of *Botrychium* found in India are *Botrychium virginianum* (L.) Sw., *B. lanuginosum* Wall., *B. lunaria* (L.) Sw., *B.multifidum* (Gmel.) Rupr., *B. daucifolium* Wall. and *B. ternatum* Thunb. Among these six species, two species namely *B. virginianum* and B. *daucifolium* are distributed in South India (Manickam and Irudayaraj, 1992). *B. daucifolium* has been included in the rare species list prepared by Chandra *et al*. (2008).

CONFOCAL MICROSCOPY

Confocal microscopy was pioneered by Marvin Minsky in 1955 while he was a Junior Fellow at Harvard University (Minsky, 1988). Modern confocal microscopes have kept the key elements of Minsky's design: the pinhole apertures and point-by-point illumination of the specimen. Advances in optics and electronics have been incorporated into current designs and provide improvements in speed (Wilson and Carlini, 1988). Confocal microscope generates information from a well-defined optical section rather than from the entire specimen, thereby eliminating the out-of-focus glare and increasing the contrast, clarity and detection sensitivity (Shotton, 1989; Squarzoni *et al.* 1994).

The confocal microscope provides novel and better understanding of cellular structures and processes. These include:

i) cellular localization of organelles, cytoskeletal elements and macromolecules such as proteins, RNA and DNA,
ii) tracing specific cells through a tissue,

iii) Producing optical sections for stereo image production and three-dimension reconstruction,
iv) imaging in four dimensions, and
v) ion imaging and fluorescence recovery after photo bleaching (Storrie *et al.* 1994).

The confocal microscope can also be used in the reflectance mode, which allows the reduction in out-of-focus blur from non-fluorescent labels such as the diaminobenzidine reaction products formed in the Cytochemical detection or the silver grains present in auto radiograms during *in situ* hybridization (Robinson and Batten, 1989).Further developments in the design of sensitive dyes and specific probes for use in confocal microscope will permit cell biologists to explore suitable relationships between cellular structures and function quantitatively in a dynamic volume (Singh and Gopinathan, 1998).

The main aim of the work presented here was to carry out the mycorrhizal association of the rare fern *B.daucifolium*, using confocal microscopy.

MATERIALS AND METHODS

SAMPLE

B.daucifolium is placed in the Division: Filicophyta/Pterophyta; Class: Eusporangiopsida; Order: Ophioglossales; and Family: Ophioglossaceae. The genus *Botrychium* has been derived from the Greek word '*botryos*' (= bunch of grapes) meaning 'grape fern'. The prominent clusters of round spore cases of the fertile sporophyte resemble the miniature cluster of grapes. *B. daucifolium* is a stout plant of about 25-60 cm high and the entire plant is hairy to glabrescent with lax pinnules. The rhizome is small and erect and bears thick fleshy, glabrous roots. The common stalk grows up to 32 cm and ensheathed at the base by the base of withered stalk and branches into a fertile or sterile segment. The sterile stalk grows up to 7 cm long and it is forked twice to result in three branches. Each branch bear bipinnatifid, transversely broad angulate-ovate blade. The fertile stalk arises from the common stalk 6 cm below the first forking point. The fertile segment grows up to 16 cm and bear 6-19 cm long tripinnate spike and the ultimate segment bears two compact rows of spherical sporangia. The spores are trilete and bear dense minute protuberances.

SAMPLE COLLECTION SITE

The sample collection site is located in Salem District, the Kolli Hills of Eastern Ghats. Its elevation ranges from 700-1500 m from the sea level.

COLLECTION PROCEDURE AND FIXATION

Roots and rhizosphere soil samples were collected from the study site, the Kolli Hills. The roots along with the soil were brought to the laboratory in sealed polythene bags. The collected roots were soaked in water for about one hour and then washed thoroughly to remove the soil from the roots. The root system of each plant were cut into bits of equal size and fixed in Formalin, Acetic acid and Alcohol (Krishnamurthy, 1999).After overnight fixation, the root bits were transferred to 70% alcohol and stored at room temperature for further investigations. Fresh root materials were used for various analyses.

CONFOCAL ANALYSIS

Confocal studies were carried out using Zeiss LSM 10 META confocal system at the Centre for Cellular and Molecular Biology (CCMB), Hyderabad, India.

RESULTS AND DISCUSSION

During the recent years, the role of AM fungal association in fern and fern-allies has received increasing attention as 414 species of ferns have been reported to be in the rare and threatened species list (Chandra *et al*. 2008). Several physiological studies have shown that nutrient exchanges occur between the endophyte and the host plant (Bonfante-fasolo and Gianinazzi-Pearson, 1979). The mycorrhizal fungus appears to obtain carbohydrates from the host plant and it can benefit the latter by transporting soil nutrients to the roots (Stribly and Read, 1976).

Lysis and digestion of the fungus has generally been regarded as the mechanism whereby these nutrients are subsequently transferred into the host cell (Stribley and Read, 1976). Apart from the nutrient exchange, the establishment of compatible contacts between the fungus and root and signal exchanges requires walls, which are permeable to molecules exchanged between the partners. Since plasmodesmata do not occur between plant and fungi, the only working pathway would be the apoplastic material that separates the plant and fungal cell membranes, consisting of the host membrane, interfacial material, the fungal wall and membrane (Bonfante and Perotto, 1995).

Smith and Smith (1996) and Smith *et al*. (1994) have reported that the interface compartments are the sites of the two-way nutrient transfer. The two-way transfer between plants and AM fungi also involves fungal membranes, the important component of the interface. They have consistent H^+/ATPase activity and based on differences in the cytochemical distribution of the enzyme between arbuscules and intercellular hyphae, Gianinazzi-Pearson *et al*. (1991) have suggested that exchanges occur across both the arbuscule interface, and the interface produced by cortical cell walls and intercellular hyphae.

The confocal analysis of the roots of *B.daucifolium* revealed the fungal colonization in the middle cortical cells and the fluoresced starch grains in the inner cortical region. The fungal colonization was localized in the fourth to sixth layers of the cortex. The outer and the inner cortical cells lacked the fungal colonization (Fig.1). The cells that lacked fungal colonization were rich in starch grains. This suggests that the cells of the middle cortex contained starch grains prior to fungal colonization.

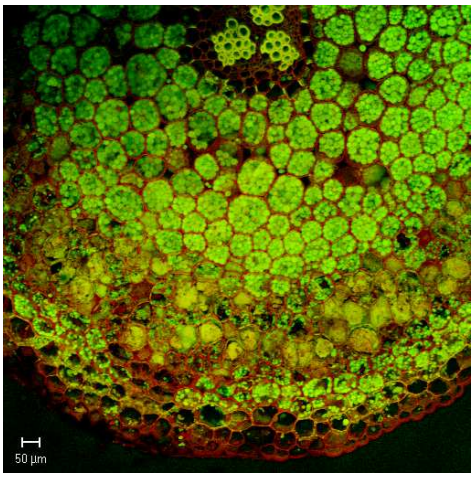

Fig. 1. ***Confocal image of Botrychium daucifolium root illustrating the fungal colonization in the middle cortex.***

The colonization of fungi has led to the absence of starch grains which is an indication that the starch grains must have been the direct source of carbon that has been utilized by the fungi for its development. As the fungi entered the middle cortical cells, the hypha began its development utilizing the carbon in the starch grains and developed into hyphal coils, arbuscules and vesicles.

The matrix material with filaments appearing around the arbuscules was found to be considerably thin whereas Schmid and Oberwinkler (1996) have reported that a high amount of matrix material was found surrounding the arbuscules in *Ophioglossum reticulatum*. Starch was not found in cells colonized by arbuscules even after the arbuscules had completely collapsed whereas Schmid and Oberwinkler (1996) reported that starch again appeared in the host cells with the degradation of arbuscules in *O. reticulatum*.

Confocal studies have revealed the different stages of lysis of the fungal hyphae. The cell with the live hyphae and hyphal coils showed the presence of starch grains whereas the arbuscule containing cells were devoid of starch grains. The entire cell has been filled with the lysed fragments of the hyphae.

As reported by Smith and Smith (1996), the hypha was separated from the host protoplasm by an interfacial matrix. The matrix had membranous configuration and fibrillar material, contributed by the host and the fungus. The interfaces between the partners were always limited on one side by the fungal membrane and on the other side by the plasma lemma of the host plant. The intracellular type of interface was found in the present study. This intracellular interface plays a major role in the two-way exchange of signal and nutrients.

In host cells with cytoplasmic arbuscules, cytoplasm and organelles increased and starch disappeared from plastids. This corresponded to the reports on the development of arbuscular host-fungus interactions within roots (Scannerini and Bonfante-Fasolo, 1983) and suggested an active symbiosis with nutrient transfer.

The confocal microscopic analysis revealed the presence of different stages of fungal degradation and lysis. Live hyphae, hyphae at different levels of degeneration and collapsed arbuscule were seen together in the same cell (Fig.2). The lysed fungi were fragmented and were utilized by the host.

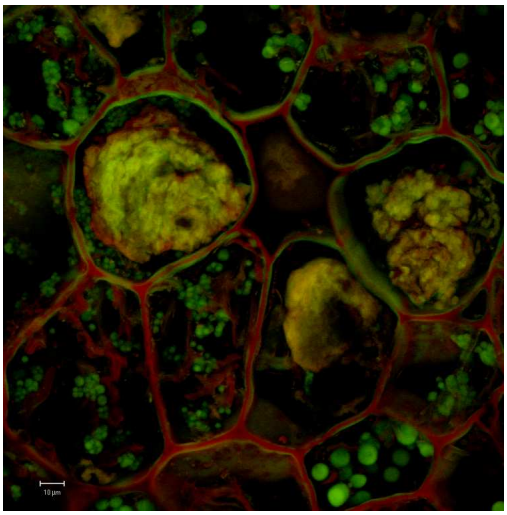

Fig. 2. ***Confocal image of B.daucifolium root illustrating the different stages of fungal lysis and degradation.***

FUNGAL ENDOPHYTE

Mycorrhizae between pteridophytes and fungi have been found in the fossil rhizomes of *Rhynia* and *Asteroxylon*, which were common in the Devonian to Carboniferous periods in the Paleozoic era, and these mycorrhizae were considered to be the earliest arbuscular mycorrhizae (Hass *et al*. 1994). Although having persisted through evolution for 300-400 million years, the arbuscular mycorrhizal status of extant pteridophytes is still not clear.

West *et al*. (2009) have identified 30 potential ribotypes from eight fern species using gradient gel electrophoresis. Sequence analysis of fungal isolates from three species of fern indicated that the primers were generally highly specific for *Glomus* species but some non-target DNA was also amplified. Cloned polymerase chain reaction (PCR)

products from *Polystichum acrostichoides* and *Osmunda regalis* revealed several phylogenetically distinct *Glomus* species. A single *Glomus* species was identified in the cloned PCR products from *Botrychium virginianum*.

Evidence indicates that plants often benefit from the presence of mycorrhizal associates via a variety of mechanisms including the mobilization of essential minerals, enhancement of desiccation resistance and protection from pathogens and herbivores (Smith and Read, 2008). The presence of arbuscular mycorrhizal fungi reduces the number of plant species required to achieve equivalent levels of biomass production compared to communities that lack AMF (Maherali and Klironomos, 2007).

Two recent studies employed molecular strategies for studying fern-fung

al symbioses and both focused on the genus *Botrychium* (Kovacs *et al*., 2007; Winther and Friedman, 2007). Kovacs *et al*. (2007) have reported that several AM fungal lineages were associated with the sporophyte of *B. virginianum*. These phylotypes were not distinct and they grouped with widely distributed taxa and with AM fungal sequences of different geographic and host origin. Winther and Friedman (2007) examined mycoheterotrophic and autotrophic plants of *B.lanceolatum* and *B.crenulatum* and found that mycoheterotrophic plants appear to be more selective in the types of AMF symbionts with which they interact.

The hyphae of the endophyte have no septa, with thin wall and the fungal protoplasm was found to be rich in organelles like mitochondria, small nuclei, small vacuoles, abundant rough or smooth endoplasmic reticulum and many electron dense deposits. The roots of *B.daucifolium* showed the presence of *Glomus* species belonging to the order *Glomales.*

CONCLUSION

The confocal microscopic analysis of *B.daucifolium* revealed the presence of different stages of fungal degradation and lysis. Live hyphae, hyphae at different levels of degeneration and collapsed arbuscule are seen together in the same cell. The lysed fungal cells are fragmented into different stages and are utilized by the host. The rhizosphere revealed 12 different species of AM spores belonging to three genera namely *Glomus, Acaulospora* and *Sclerocystis*. Among the identified 12 spores of AM fungi, the AM fungi that colonized the *Botrychium daucifolium* root belong to *Glomus* which shows the specificity of the host to the fungus.

REFERENCES

1. Bonfante P and Perotto S. Strategies of arbuscular mycorrhizal fungi when infecting host plants.New Phytologist. 1995: 130: 3-21.
2. Chandra S, Fraser-Jenkins CR, Alka Kumari and Archana Srinivastava . A Summary of the Status of Threatened Pteridophytes of India.Taiwania. 2008: 53: 170-209.
3. Dixit RO. A census of the Indian Pteridophytes. Flora of India-Series IV. B.S.I., Howrah. 1984.
4. Gianinazzi-Pearson V, Gianinazzi S,Guillemin JP,Trouvelet A and Duc G. Genetic and cellular analysis of resistance to vesicular arbuscular (VA) mycorrhizal fungi in pea mutants.Advances in molecular genetics of plant microbe interaction.Kluwer.Dordecht. Netherlands. 1991:1:336-342.
5. Hass H, Taylor TN and Remy W.Fungi from the lower Devonian Rhynie Chert:Mycoparasitism. American Journal of Botany.1994: 81: 29-37.
6. Kovacs GM, Balazs T and Penzes Z. Molecular study of arbuscular-mycorrhizal fungi colonizing the sporophyte of the eusporangiate rattle snake fern (*Botrychium*

virginianum, Ophioglossaceae). Mycorrhiza. 2007:17: 597-605.
7. Krishnamurthy KV. Methods in Cell Wall Cytochemistry. CRC Press, USA. 1999: p. 1-320.
8. Maherali H and Klironomos JN. Influence of phylogeny on fungal community assembly and ecosystem functioning. Science. 2007: 316: 1746-1748.
9. Manickam VS and Irudayaraj V. Pteridophyte flora of the Western Ghats, South India. B. I. Publications Pvt. Ltd., New Delhi. 1992.
10. Minsky M.Memoir on inventing the confocal microscope. Scanning. 1988:10: 128-138.
11. Robinson JM and Batten BE. Detection of diaminobenzidine reactions using scanning laser confocal reflectance microscopy. J Histochem Cytochem. 1989:37: 1761-1765.
12. Scannerini S and Bonfante-Fasolo P. Comparative ultrastructural analysis of mycorrhizal association.Can.J.Bot. 1983:61 : 917-943.
13. Schmid E and Oberwinkler F. Light and electron microscopy of a distinctive VA mycorrhiza in mature sporophyte of *Ophioglossum reticulatum*.New Phytologist. 1996:165: 567-579.
14. Shotton DM. Confocal scanning optical microscopy and its applications for biological specimens. J Cell Science. 1989: 94: 175-206.
15. Singh A and Gopinathan KP. Confocal microscopy: A powerful tool for biological research. Current Science.1998: 74: 841-851.
16. Smith FA and Smith SE. Mutualism and Parasitism: Diversity in function and structure in the 'arbuscular' (VA) mycorrhizal symbiosis. Adv Bot Res. 1996: 22: 1-43.
17. Smith SE and Read DJ. Mycorrhizal symbiosis. 3rd ed. Academic, San Diego. 2008
18. Smith GE, Gianinazzi-Pearson V, Koide R and Cairney JWG. Nutrient transport in mycorrhizas:Structure,physiology and consequences for efficiency of the symbiosis.Plant Soil. 1994: 159: 103-113.
19. Squarzoni S, Cinti C, Santi S,Valmori A and Maraldi NM. Preparation of chromosome spreads for electron (TEM, SEM, STEM) light and electron microscopy.Chromosoma.1994:103: 381-392.
20. Storrie B, Pepperkok R, Stelzer EHK and Kreis TE. The intracellular mobility of a viral membrane glycoproteins measured by confocal microscope fluorescence recovery after photobleaching. Journal of Cell Science. 1994:107: 1309-1319.
21. Stribley DP and Read DJ. The biology of mycorrhiza in the Ericaceae. VI. The effects of mycorrhizal infection and concentration of ammonium nitrogen on growth of cranberry (*Vaccinum macrocarpon* Ait) in sand culture. New Phytologist.1976:77: 63-72.
22. West B, Brandt J, Holstein K, Hill A and Hill M.. Fern associated arbuscular mycorrhizal fungi are represented by multiple *Glomus* spp. Do environmental factors influence partner identity? Mycorrhiza. 2009.
23. Wilson T and Carlini AR. Three dimensional miaging in confocal imaging systems with finite-sized detectors. J Microsc 1988: 141: 51-66.
24. Winther JL and Friedman WE.Arbuscular mycorrhizal symbionts in *Botrychium* (Ophioglossaceae). American Journal of Botany. 2007: 94:1248-1255.