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Root fungal associations in *Gaultheria fragrantissima*

Das, P[1*]. and Kayang, H.[2]

[1]Microbiology Laboratory, Department of Botany, Tripura University, Suryamaninagar- 799 130, Tripura, India
[2]Microbial Ecology Laboratory, Department of Botany, North Eastern Hill University Shillong-793 022, India

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The mycorrhizal type of *Gaultheria fragrantissima* was investigated from northeast India. The root was colonized by dark ericoid mycorrhizal fungi (DERMF), non dark ericoid fungi (NDERMF), arbuscular mycorrhizal fungi (AMF) and dark septate endophytic fungi (DSEF). The co-colonization of DERMF and AMF association were observed in three sites; NDERMF and DSEF were recorded in the entire site. The highest colonization by DERMF, NDERMF, AMF and DSEF recorded were 62.0, 72.0, 34.0 and 74.0%, respectively. Positive statistical correlation was found between DERMF colonization with AMF and DSEF colonization. The study revealed a multiple mycorrhizal structural occupancy with co-existence of ericoid coils, arbuscules, vesicles, hyphal coils, arbuscule-like, vesicle-like, round bodies and microsclerotia in the host root.

Key words: *Gaultheria fragrantissima*, dark ericoid mycorrhizal fungi, non dark ericoid mycorrhizal fungi, dark septate endophyte, arbuscular mycorrhizal fungi

Introduction

Gaultheria fragrantissima Wall. (Ericaceae) is a much-branched, evergreen, aromatic shrub, 1-3 m height, with orange-brown bark, commonly found in Khasi Hills and Western Ghats, India at altitudes of above 1500 m. The oil from leaves is used as anti-rheumatic, anti-sciatica, painkiller, stimulant, carminative, antiseptic and vermicide (Kayang *et al*., 2008).

Mycorrhizal fungi can be classified as ectomycorrhizal and endomycorrhizal depending on their morphological connection with the host. The former envelop the root with a hyphal sheathing structure and grow between root cortical cells to form the Hartig net, whereas the latter enter the root cell to form intracellular arbuscules (arbuscular mycorrhizal fungi) or coils (ericoid and orchid fungi). An intermediate behaviour is displayed by fungi that

* Corresponding author: Das, P; e-mail: panna11d@yahoo.co.in

produce ectoendomycorrhizal colonizations, i.e. a sheathing structure, a rudimentary Hartig net and intracellular structures such as pegs (monotropoid fungi) or coils (arbutoid fungi) (Smith and Read, 1997).

In natural ecosystems, plant roots can establish mycorrhizal associations with several fungal partners belonging to different taxa (Bergero *et al*., 2000). The co-occurrence of both arbuscular mycorrhizal fungi (AMF) and ectomycorrhizal fungi (EMF) colonization is common in several arborescent angiosperms, however, roots of Pinaceae members are co-colonized by EMF, AMF and dark septate endophytic fungi (DSEF) (Wagg *et al*., 2008). The members of the Ericaceae often show association with ericoid mycorrhizal fungi; some have arbutoid mycorrhizas and ectomycorrhizal and few have arbuscular mycorrhizal fungi (Urcelay, 2002). The roots of rhododendrons possess arbuscular mycorrhizal colonization in field condition was reported earlier (Chaurasi *et al*., 2005). It can be expected that at natural sites with ericaceous plant species, both ERMF and DSEF occur together in the soil and interact. The nature of these interactions is, however, unknown. Direct observations in roots have confirmed numerous colonization of ericaceous species (Vohník *et al*., 2005). Moreover, molecular methods proved the simultaneous presence of ERMF (*Hymenoscyphus ericae*-like and *Oidiodendrons*) and DSEF (*Phialocephala fortinii*) within the same root system (Midgley *et al*., 2004). In *Epacris pulchella*, rDNA internal transcribed spacer (ITS) restriction fragment length polymorphisms (RFLPs) and sequences for the cultured isolate assemblage with fungi identified in DNA extracted directly from the same root systems by cloning or denaturing gradient gel electrophoresis (DGGE). The most abundant RFLP types in the cultured isolate assemblage were identified as putative ericoid mycorrhizal ascomycete endophytes and in addition, Glomerales taxa and probable basidiomycetes were also identified (Bougoure and Cairney, 2005).

However, a recent report indicates that *G. fragrantissima* is found to be colonized by ericoid mycorrhizal fungi (Bagyalakshmi *et al*., 2010). Moreover, G. *poeppigi* was found to colonize by three fungal symbionts. Therefore, we investigated the roots of this medicinal plant to assess the co occurrence of mycorrhizal types.

Materials and methods

Collection sites

Four locations were selected for collection of root and soil samples of *G. fragrantissima*. Site 1 & 2 (25°32'N 91°50'E, 1830 m.a.s.l. and 25°32'N 91°51'E, 1870 m.a.s.l., respectively) were situated on the way to Laitkor Peak,

Meghalaya, India and site 3 & 4 (25°31'N 91°48'E, 1703 m.a.s.l. and 25°29'N 91°49'E, 1687 m.a.s.l., respectively) were located on the way to Cherrapunjee, Meghalaya, India. The plant at site 2 was growing on the edges of sub-tropical forest and plants in other three sites were found growing on the vertical slope of the hill on rocky crevices and sandy substratum.

Sampling

Three plants from each location were uprooted for collection of roots along with the adhering soils which were then placed in polythene bags, labeled and processed for further analysis in the laboratory. The soil was removed by careful rinsing under running tap water for 15 min. Any remaining soil, litter or debris was gently removed with forceps. The root samples from three plants from each site were bulked to form a composite soil sample. The composite samples of about 100 g adhering soils to root were prepared from all the sites for soil properties analysis and AMF spore extraction.

Root processing

For the determination of percent root colonization, fine hairy and thicker root portions were processed to investigate mycorrhizal structures. Root segments of approximately 1 cm were stained with Trypan blue as described by Phillips and Hayman (1970) and examined for mycorrhizal structures under light microscope (Olympus 41209). Roots with hyphal coils which was hyaline and stained blue were considered as non dark ericoid mycorrhizal fungi (NDERMF) and which were dark brown were measured as dark ericoid mycorrhizal fungi (DERMF), both classified as ERMF. When coexistence of all the mycorrhizal types were observed, AMF, NDERMF, DERMF and DSEF; colonization were recorded independently as 1 for the intensity of colonization. The estimation of mycorrhizal colonization was quantified using the following method:

$$\text{Mycorrhizal colonization (\%)} = \frac{\text{Number of root colonized by fungi}}{\text{Total number of root}} \times 100$$

AMF spore analysis

The spores were isolated from 10 g soil by modified wet sieving and decanting method (Muthukumar *et al*., 2006). The isolated spores were picked up with needle in 1-2 drops polyvinyl alcohol-lactoglycerol under a dissecting microscope. The spores were examined using a compound microscope.

Taxonomic identification of spores was done by matching original descriptions (http://www.invam.caf.wvu.edu & http://www.lrz-muenchen.de/~schuessler /amphylo). The root segments colonized by fungi and spores of AMF were photographed with the help of Leica DM 1000 microscope (Switzerland). Abundance was measured as the number of identified AMF spores.

Soil properties assessment

The soil samples were air dried after analysis of pH and moisture content. Then they were cleaned, ground, sieved with a 2 mm sieve, stored at 4°C and processed for further soil physical and chemical properties. For moisture content (%), 10g sample of soil from each site was oven dried and weight was determined. Measurement of pH and electrical conductivity was done using digital pH meter (Systronics). Soil texture was analyzed using sodium hexametaphosphate method and soil available phosphorus was determined following molybdenum-blue method (Allen *et al*., 1974). The soil organic carbon was estimated using colorimetric method (Anderson and Ingram, 1993).

Data analysis

Standard errors of means were calculated and analysis of variance (ANOVA) was carried out and means were separated by Fisher test. Pearson correlation was conducted to evaluate the relation between soil properties and mycorrhizal colonization. The analyses were done with the help of Statistica 9.0 software.

Results

The physical and chemical properties are presented in Table 1. Highest amount of sand was found in sites 1, 3 and 4. Site 3 has the highest silt content and site 2 has the highest amount of clay and moisture content. The highest electrical conductivity and lowest pH was recorded in site 1. The lowest quantity of organic carbon and available phosphorus were found in site 3. There was no significant difference in soil properties among the four sites ($p<0.05$).

Multiple mycorrhizal structural occupancy in the root of *G. fragrantissima* was observed (Fig. 1). The root was found to be co-colonized by DERMF, NDERMF, AMF and DSEF. The colonization range of NDERMF, DERMF, AMF and DSEF were 8.00-72.00 %, 0.00-62.00 %, 0.00-34.00 % and 14.00-74.00 %, respectively (Table 2). Hyphal coils of AMF colonizers were frequent in wider root segments than vesicles and arbuscules (Fig. 1e). The presence of intracellular hyphal coils from one cell to another reveals *Paris* type

of AMF morphology. The lowest colonization was performed by AMF and highest was DSEF. DERMF and AMF colonization was absent in the roots from site 1. AMF colonization did not significantly differ (P<0.05) among the sites (Table 2). There is statistical significant correlation between DERMF and DSEF colonization (r=0.99, p=0.003).

Table 1. Soil physical and chemical properties from four location of *Gaultheria fragrantissima*

	Soil texture (%)			Moisture content (%)	Electrical conductivity (mv)	pH	Organic carbon (%)	Available phosphorus µg/g
	Sand	Silt	Clay					
Site 1	73.86	1.07	12.58	31.77±0.50	146.67±3.18	4.87±0.07	1.16±0.03	7.47±0.41
Site 2	53.2	3.78	43.2	44.97±1.12	125.33±3.38	5.04±0.04	1.06±0.01	8.73±0.96
Site 3	78.04	11.73	10.23	15.90±0.60	99.00±1.15	5.54±0.09	0.71±0.03	5.13±0.27
Site 4	94.29	0.45	5.26	6.43±0.62	108.67±1.20	5.21±0.05	1.12±0.03	8.33±0.53

Table 2. Mycorrhizal and dark septate endophyte fungal colonization percentage from four location of *Gaultheria fragrantissima*

	NDEC	DEC	AMF	DSE
Site 1	8.0±3.88a	0.0a	0.0a	14.0±4.96a
Site 2	64.0±6.86b	62.0±6.93b	3.0±6.55b	74.0±6.27b
Site 3	32.0±6.66c	48.0±7.18b	2.0±5.71b	64.0±6.86b
Site 4	72.0±6.41b	62.0±6.93b	34.0±6.77b	72.0±6.41b

Means in a column followed by different letter are significantly (p<0.05) different according Fisher LSD

Two types of microsclerotia were observed (Fig. 1g and h) in few root segments. In addition, numerous round bodies (Fig. 1k) were noticed in few root segments. The extraction of AMF species shows five morphotypes. *Glomus* sp 1, *G*. sp 2, *Acaulospora* sp 1, *Pacispora boliviana* Sieverding & Oehl and one remain unidentified. All the five morphotypes were found from site 2, one from site 3 and three from site 4.

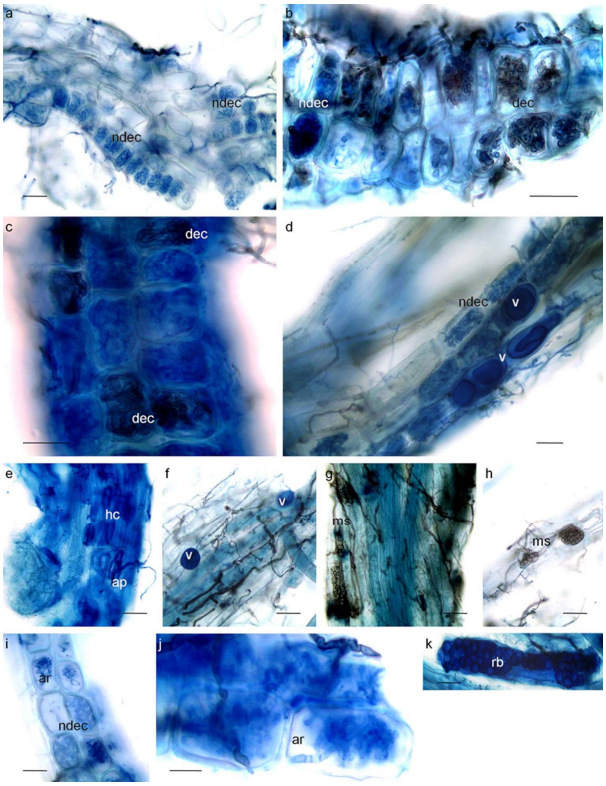

Fig. 1. (a-k) Mycorrhizal and dark septate endophyte fungal colonization in the root of *G. fragrantissima*. (a) Root portion showing colonization by non-dark ericoid coils (ndec). Bar scale= 300 µm. (b) Root segments showing co-colonization by ndec and dark ericoid coils (dec). Bar scale=100 µm. (c) Root segments showing dark ericoid coils (dec). Bar scale=100 µm. (d) Vesicle-like (v) structures and ndec in the portion of root. Bar scale=300 µm. (e) Root tip showing appressoria (ap) and aseptate hyphal coils of AMF. Bar scale= 250 µm. (f) Vesicles (v) in the root fragment. Bar scale=300 µm. (g & h) Microsclerotia (ms) in the root pieces. Bar scale=250 & 300 µm, respectively. (i) Presence of arbusculate coils (ar) and ndec. Bar scale=300 µm. (j) Root section illustrating arbuscules. Bar scale=300 µm. (k) Round bodies (rb) in the root segment. Bar scale=300 µm.

Discussion

The co-colonization of mycorrhizal associates in this species is the first report in this ericoid plant species. The numerous round bodies (Fig. 1k) indicate *Pirisformospora indica* colonizing the fewer root segments (Verma *et al*., 1999). However, there is previous report of occurrence of ericoid mycorrhizal fungi in this species from India (Bagyalakshmi *et al*., 2010).

The hyphal coils of ERMF appears granular, brownish and poorly stained, but remains clearly distinguishable at maturity (Urcelay, 2010), however, we have divided ERMF into NDERMF and DERMF where DERMF appears brownish but it was not granular. In addition, there could be possibility of

DSEF association in forming DERMF. Moreover, in the present investigation, the positive correlation between DERMF and DSEF could support the division of DERMF from ERMF and DSEF could possibly form ericoid like coils in the roots of *G. fragrantissima*. Additional supports could be drawn from the study (Usuki and Narisawa, 2005) where *H. chaetospira* is able to form an ericoid structure which is a root endophyte and were consistent with the DSEF complex resembling mycorrhizas within the roots of *Rhododendron obtusum*. In addition, both light and dark sterile mycelia have been associated with ericoid mycorrhiza (Monreal *et al*., 1999). Nonetheless, endophytic species *P. fortinii* was clearly not a typical ericoid mycorrhizal fungus, although it could easily be detected in roots and is associated with roots of various Ericaceae. Since these endophytic species generate distinct RFLP patterns, they can be distinguished from ericoid mycorrhizal fungi that may inhabit the same roots (Monreal *et al*., 1999). The colonization pattern of AMF, ERMF and DSEF was concurred with the report (Urcelay, 2002). A basidiomycete strain of RFLP formed coil-like mycorrhizal structures in the roots of *Rhododendron fortunei* in vitro and showed positively effects on the seedlings of *R. fortunei*. These results confirm for the first time the basidiomycetes strain as putative ericoid mycorrhizal fungi (Zhang *et al*., 2009). In our study, presence of round bodies indicate colonization by *P. Indica* which is also basidiomycetes could possibly form ERMF coils; however, attempts were failed to isolate axenically *P. indica* from the roots of *G. fragrantissima*. This is in accord with the study conducted by Zhang *et al*. (2009) and reported that mycorrhizal infection rate of the basidiomycete strain was quite low in an inoculation test and they presumed that this might be one reason why basidiomycete strains are usually isolated at a very low frequency.

Furthermore, a number of reports have also suggested earlier that ericoid mycorrhizae might also be formed by basidiomycetes but attempts to synthesize ericoid mycorrhizae with basidiomycetes have failed (Monreal *et al*., 1999).

Glomus and *Acaulospora* of AMF species isolated in the study (Urcelay, 2002) was similar except *Scutellospora* was not found in this study. DSE colonization type was similar as reported (Urcelay, 2002) i.e., *Phialocephala* Medlar type (Fig. 1g) and *Rhizoctonia* DC type (Fig. 1h).

The present study reveals that multiple mycorrhizal occupancy with co-existence of ericoid coils, arbuscules, hyphal coils, arbuscule-like, vesicles, vesicle-like, round bodies and microsclerotia in the host root. Ericoid coils suggests ERMF association, arbuscules, vesicles, and aseptate hyphal coils indicate AMF association, microsclerotia suggests DSEF colonization and round bodies in root segments possibly colonized by *P. indica*.

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