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Studies on the mycorrhizal association of *Rhododendron arboreum* Sm. ssp *nilagiricum* (Zenker) Tagg

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Abstract: A study of the mycorrhizal system in *Rhododendron arboreum* ssp. *nilagiricum,* a member of the Ericaceae, was carried out. The root system of *R. arboreum* ssp. *nilagiricum* is dimorphic, with framework roots and hair roots. The framework roots exhibit secondary growth with differentiation of the cambium and phellogen. The hair roots have simple anatomical features, lacking secondary growth. The hair roots persist throughout the year; however, their abundance is seasonal. The ericoid fungal hyphae penetrate the epidermal cells of hair roots and form pelotons that fill the cell. Nutrient exchange is thought to occur through these pelotons. The pelotons produce hyphae that spread all over the host cells. The extent of fungal colonization in hair roots is higher than for framework roots. Fungal infection in the roots is found throughout the year; however, there is a decrease in percentage of fungal colonization during the dry season. This study is the first report of ericoid mycorrhizal fungi in *R. arboreum* ssp. *nilagiricum* in India.

Resumen: Se llevó a cabo un estudio del sistema micorrícico en *Rhododendron arboreum* ssp. *nilagiricum*, un miembro of las Ericaceae. El sistema radical de *R. arboreum* ssp. *nilagiricum* es dimórfico, con raíces estructurales o primarias y raíces pilosas o finas. Las raíces estructurales exhiben crecimiento secundario con diferenciación del cámbium y felógeno. Las raíces finas poseen rasgos anatómicos simples y carecen de crecimiento secundario. Las raíces finas persisten durante todo el año; sin embargo, su abundancia es estacional. Las hifas de los hongos ericoides penetran la epidermis celular de las raíces finas y forman pelotones que llenan la célula. Se cree que el intercambio de nutrientes tiene lugar a través de estos pelotones. Los pelotones producen hifas que se extienden completamente sobre las células de la planta infectada. La extensión de la colonización fúngica en las raíces finas es mayor que en las raíces estructurales. La infección fúngica en las raíces se encuentra durante todo el año; sin embargo, hay un decremento en el porcentaje de la colonización fúngica durante la estación seca. Este estudio constituye el primer reporte de hongos micorrícicos ericoides en *R. arboreum* ssp. *nilagiricum* en la India.

Resumo: Um estudo do sistema micorrízico em *Rhododendron arboreum* ssp. *nilagiricum*, um membro da Ericaceae, foi levada a efeito. O sistema radicular da *R. arboreum* ssp. *nilagiricum* é dimórfico, com raízes estruturais e raízes finas. As raízes estruturais apresentam crescimento secundário com diferenciação do câmbio e do felogénio. As raízes finas têm características anatómicas simples, sem crescimento secundário. As raízes finas persistem ao longo do ano; no entanto, a sua abundância é sazonal. As hifas fúngicas ericóides penetram nas células epidérmicas das raízes finas e formam novelos que enchem a célula. A troca de nutrientes é pensado ocorrer através destes novelos. Os novelos produzem hifas que se espalham por todas as células hospedeiras. A extensão da colonização fúngica nas raízes finas é maior do que para as raízes de estruturais. A infecção fúngica nas raízes é encontrada durante todo o ano; no entanto, há uma diminuição na percentagem de colonização fúngica durante a

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estação seca. Este estudo é o primeiro relato de fungos micorrízicos ericóides em *R. arboreum* ssp. *nilagiricum* na Índia.

Key words: Ericoid fungi, hair root, mycorrhizae, pelotons, *Rhododendron*.

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Introduction

Ericoid mycorrhizas are diverse groups of soil fungi that establish a distinctive type of mycorrhizal association with ericaceous plants (Bonfante & Gianinazzi-Pearson 1979; Perotto *et al.* 2002). These fungi profusely colonize the roots of ericaceous plants, which are widespread in arctic, temperate and tropical climates, and are dominant in geographically and climatically disparate heath land and forest habitats (Read 1996). Soils in such habitats typically have low nutrient status, with slow mineralization of nitrogen, phosphorus and accumulation of organic polymeric compounds and thereby soil habitats are stressful to plants by factors such as pH, availability of metal ions, water and high or low temperatures (Cairney & Meharg 2003; Read 1996). The establishment of these mycorrhizal fungal associations is regarded as a key factor in the success of ericaceous plants on nutrient-deficient soils, and allows them to successfully compete with other plants (Read 1991, 1996). The fungi produce extracellular proteolytic enzymes that provide access to organic compounds, thereby enhancing nitrogen and phosphorus supply to the host plants (Smith & Read 1997). These fungi are also considered to be resistant to acidic metal-contaminated sites such as abandoned mine sites because they have a high tolerance to heavy metals (Bradley *et al.* 1982; Cairney *et al.* 2001; Denny & Ridge 1995; Martino *et al.* 2000b; Sharples *et al.* 2001) and possess mechanisms that allow them to utilize nutrients under these conditions (Gibson & Mitchell 2004, 2005; Martino *et al.* 2000a, 2003). Worldwide, there are approximately 125 genera and 4500 species of Ericaceae, with the five largest genera being *Rhododendron, Erica*, *Vaccinium*, *Cavendishia* and *Gaultheria* (Luteyn 2002). The genus *Rhododendron* has about 800 widely-distributed species, with a major concentration of diversity occurring in the Sino-Himalayan Mountains of Southeast Asia, and other significant areas of diversity in the mountains of Indo-China, Japan and Taiwan. In India, the genus *Rhododendron* is distributed in Himachal Pradesh, North Eastern States, and parts of Western Ghats in the south, where the areas are at an altitude of 1000 m above the sea level and have an annual average rainfall of about 20 cm.

Although there are about five genera and eleven species of Ericaceae found in India, few studies have focused on their mycorrhizal system. Thus there is a need for more research on this aspect, especially since there is currently great interest in the conservation and understanding of the symbiotic association of Ericaceae in the Indian context (Mao *et al.* 2002; Sastry & Hajra 1983; Singh *et al.* 2003; Yumnam 2008). In view of these lacunae, the present investigation was undertaken, focusing on *Rhododendron arboreum* Sm. ssp *nilagiricum* (Zenker) Tagg, a member of the Ericaceae family, as the study species.

Materials and methods

The study species, *R. arboreum* ssp. *nilagiricum* is a small tree distributed along the shola (evergreen forest) borders and slopes of the Palni Hills, an eastward spur of the Western Ghats. It is between 1900 and 2300 m above sea level, with mean annual rainfall of 135 cm and a mean annual temperature of 14.0 °C. The climate in this location is characterized by four distinct seasons: dry (December - March), warm (April - June), Southwest monsoon (June - September), and Northeast monsoon (October - November) (Matthew 1994). For the present investigation five different localities in the Palni Hills - Shembaganur, Kodaikanal lake, Bear shola, Pamban shola and Pillar rocks - were selected. From each study site roots and soil samples were collected during the four different seasons between 2006 and 2009 and brought to the laboratory in the Department of Plant Biology and Plant Biotechnology, St. Joseph's College, Tiruchirappalli, Tamil Nadu, India. The root samples were washed thoroughly with water, cut into small pieces and fixed in a formalin (10 ml) - acetic acid (5 ml) - alcohol (50 ml) - distilled water (35 ml) (FAA) solution. After

overnight fixation, the root pieces were transferred to a 70 % alcohol solution and stored at room temperature for further investigations. Fresh root materials were examined under a dissecting microscope immediately after arriving laboratory to determine root morphological characteristics. Transverse sections of roots were obtained by freehand sectioning and photomicrographs taken with a Nikon fluorescent microscope (model E400, Tokyo Japan). For mycorrhizal studies, 1-cm long root segments were stained with trypan blue (Phillips & Hayman 1970) or celluflour (Gahan 1984) before observation under the microscope. Infection density was calculated as a ratio of number of infected cells to total number of cells (Hadley & Williamson 1972). This involved analysis of 100 randomly selected transections of mycorrhizal roots.

Soil samples were mixed thoroughly and analyzed for pH (1:1 soil to water ratio), using a digital pH meter (LI 127 model, Elico Instruments, India). Analysis of organic carbon (Walkley & Black 1934) and soil organic nitrogen (Sankaram 1966) were carried out by Soil Testing and Technology Advisory Centre, Department of Soil Science and Agricultural Chemistry, Tamil Nadu Agricultural University, Coimbatore.

Results and discussion

Edaphic characteristics of samples collected from study site indicated that the soil was highly acidic (pH 4.86). This is due to the deposition of plant litter on the ground and subsequent transformation of organic debris by soil microbes, especially fungi, into organic acids like amino acids, humic acids and so on (Oades 1988). Because of the high organic acid content, the soil pH became very low (Jalal & Read 1983). In the study site the organic carbon content is 1.8 g kg^{-1} and organic nitrogen content is 493 kg ha^{-1}. The organic form of nitrogen is normally unavailable to higher plants. However, ericoid mycorrhizal symbiosis is considered to be critical to success of the study species in this low-nutrient soil, by enabling the use organic nutrients. Bajwa & Read (1986) and Leake & Read (1990) demonstrated that ericaceous fungi can obtain nitrogen from a range of acidic, neutral, and basic amino acids, and release acid proteases that mediate utilization of simple proteins. The ericaceous fungi of Northern Hemisphere appear to possess similar abilities to access, at least, organic forms of N (Xiao & Berch 1999). Chen *et al.* (1999) and Whittaker & Cairney (2001) observed that ericoid mycorrhizal fungi in a dry sclerophyll forest can utilize NO_3^- and NH_4^+ along with a broad range of amino acids and simple proteins as a sole source of nitrogen. These mycorrhizal fungi also produce phosphomonoesterases and phosphodiesterases that mobilize phosphorus from inositol phosphates and other phosphomonoesters, along with phosphodiesters such as nucleic acids (Leake & Miles 1996; Straker & Mitchell 1986).

The root system of *R. arboreum* ssp. *nilagiricum* is dimorphic (Fig. 1a). It includes large 'framework roots' and a minute 'fine nutrient roots' or hair roots (Cairney & Ashford 2002). Bell & Pate (1996) have described the root systems of epacrid genera from South Australia which showed four different types of root morphology. Similarly, Allaway & Ashford (1996) described a dimorphic root system in *Lysinema ciliatum*. In *R. arboreum* ssp. *nilagiricum* the framework roots were relatively large (diameter ranges from 450 µm to 660 µm), branched profusely, with indeterminate growth and gave rise to hair roots. The hair roots were extremely fragile, much smaller, and with determinate growth (short roots). In addition, the framework roots have the presence of secondary growth with differentiation of the cambium and phellogen (Fig. 2a,b). However, the hair roots have simple anatomical features lacking secondary growth. In *L. ciliatum* the mean length of hair roots was 3.4 mm, the hair roots branched, and there were three orders of them i.e. primary, secondary and tertiary branches, ranging from 70 µm in diameter to < 50 µm (Allaway & Ashford 1996).

The present study was carried out over three years (2006 - 2009). During this time it was observed that the hair roots in *R. arboreum* ssp. *nilagiricum* persisted throughout the year; however, the abundance of hair roots was seasonal. In the study species, the total hair root length rapidly declined to a low level during December to March (Fig. 1b). During these months it was Dry season in the collection sites and, as a result, the soil dried out (Mathew 1994). The hair roots reappeared during the Southwest monsoon (June - September) and progressively increased in length during the Northeast monsoon, when the rate of rainfall was maximum (October - November) (Fig. 1c,d). Bell & Pate (1996) and Hutton *et al.* (1994) reported that there were seasonal variations in the abundance of hair roots and mycorrhizal infection in several species in southwest Australia. They further found that hair root length was reduced during summer and reappeared in autumn (April) in all the species they examined. Read (1996) reported that different

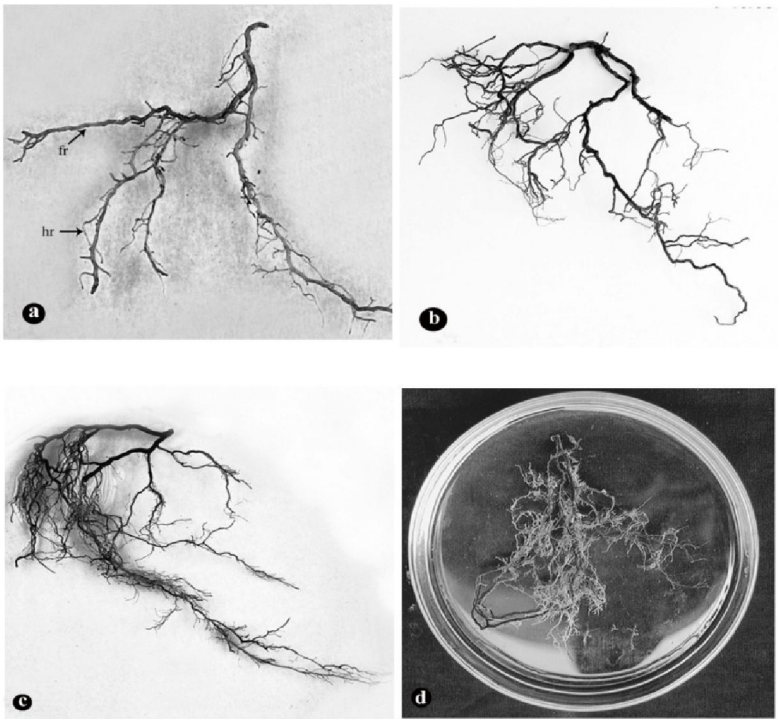

Fig. 1. Roots showing different morphological features: (a) dimorphic root showing framework root (fr) and hair root (hr), (b) root collected during dry season (December to March), (c) root collected during the warm season (April to June), (d) root collected during the rainy season (June to November).

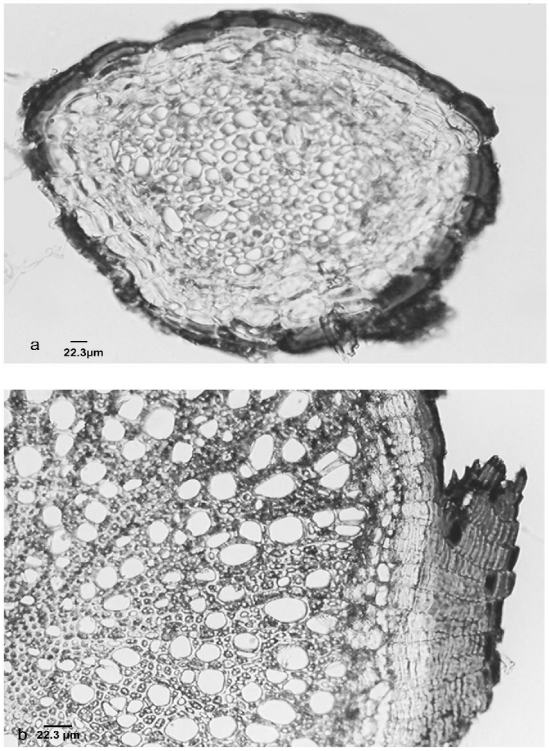

Fig. 2. Anatomy of framework roots: (a) transverse section of framework root, (b) framework root showing secondary growth.

moisture regimes will result in distinctive patterns of seasonal hair root development.

The present investigation revealed that the ericoid fungal mycelia were abundantly present on the surface of the hair roots and in the adjacent rhizosphere. These fungi form a loose hyphal network over the surface of the hair roots (Fig. 3a). By contrast, in *Pinus* the mycorrhizae form a mantle of fungal cells that completely sheaths the short feeder roots of the host (Zelmer & Currah 1995). The symbiotic association between the fungus and the host species is initiated when a fungal hypha contacts a compatible region of the hair root (Cairney & Ashford 2002). In the present study it was found that a hypha which oriented longitudinally along the surface of a hair root produced short, perpendicular branches towards the root surface, called penetration hyphae (Fig. 3b), that penetrate the epidermal cells of hair roots. All epidermal cells were penetrated, often at several points in each cell. There was little evidence of extensive enzymatic degradation at the sites of wall penetration during the establishment of the mycorrhizal symbiosis (Perotto *et al.* 1995). These fungi grew through the outer tangential wall and entered the periplasmic space of the epidermal cell, where it widened and formed a fungal coil also

Table 1. Micrometry analysis of infected and uninfected root cells from *R. arboretum* ssp. *Nilagiricum*. Values are means ± standard deviation (n = 100).

Root location #	Uninfected root		Infected root	
	Cell length (µm)	Cell width (µm)	Cell length (µm)	Cell width (µm)
1	61.44 ± 0.48	21.22 ± 0.33	66.44 ± 0.41	25.94 ± 0.18
2	63.84 ± 0.46	23.46 ± 0.29	65.20 ± 0.41	25.46 ± 0.57
3	61.72 ± 0.38	22.36 ± 0.25	65.64 ± 0.37	25.52 ± 0.40
4	61.76 ± 0.45	22.66 ± 0.58	65.66 ± 0.87	26.46 ± 0.16
5	63.12 ± 0.29	23.82 ± 0.42	65.26 ± 0.42	25.04 ± 0.15

Five different microscopic fields of root samples.

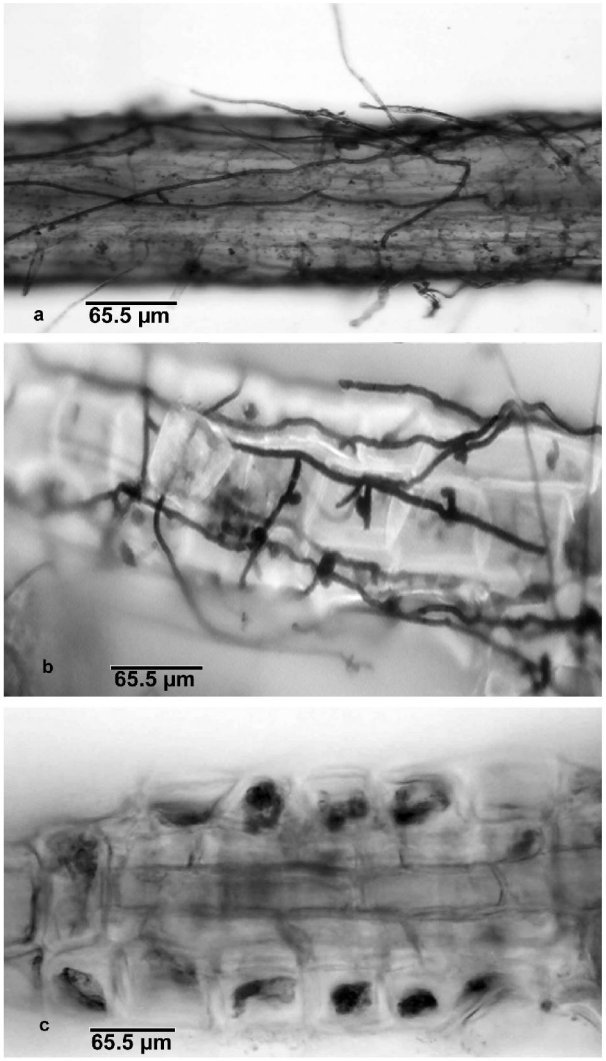

Fig. 3. Various images of whole hair roots: (a) distribution of fungal hyphae on the root surface, (b) hyphae with series of side branches (penetration hyphae), (c) well-colonized mature cells. Images (a) and (c) were stained in trypan blue; image (b) was stained in celluflour.

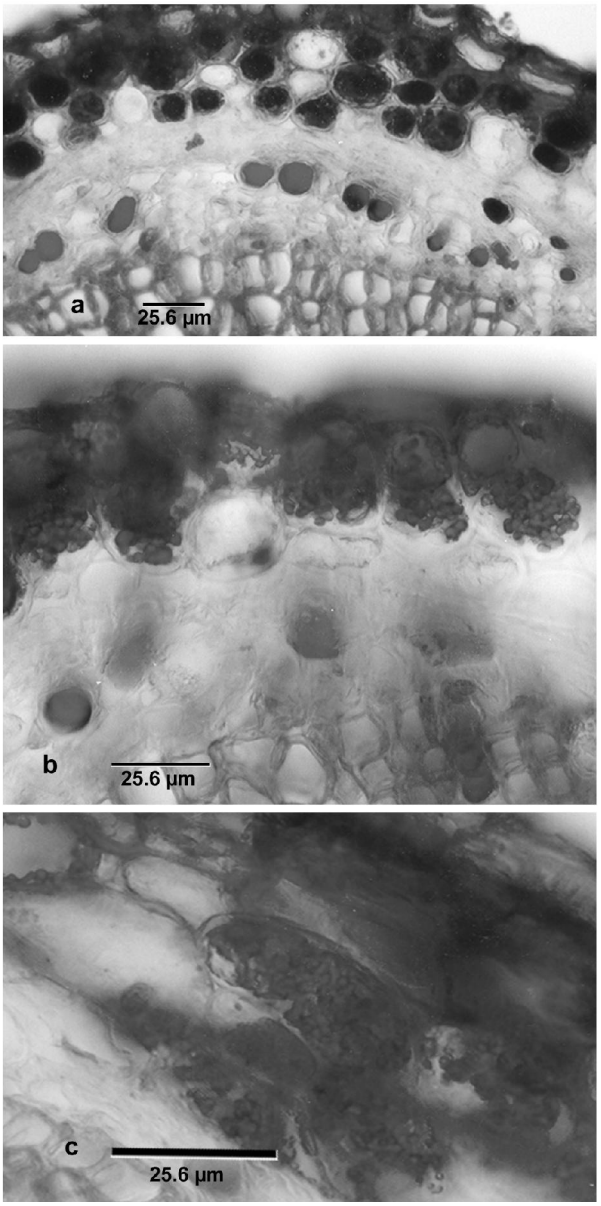

Fig. 4. Pattern of colonization in framework roots: (a - c) well-colonized cortical cells of framework roots.

Table 2. Colonization percentage by ericoid Mycorrhiza in *R. arboreum* ssp. *Nilagiricum* roots. Analysis carried out in each season for two consecutive years. Values are mean ± standard deviation (n = 200, 100 per year).

Season	Root colonization (%)	
	Hair root	Framework root
Dry (December-March)	20 ± 2.3	12 ± 1.2
Warm (April-June)	35 ± 3.3	12 ± 1.0
Southwest monsoon (June-September)	47 ± 3.3	16 ± 2.7
Northeast monsoon (October-November)	55 ± 3.9	18 ± 2.4

called a peloton. The peloton is the point of nutrient and information exchange between plant and fungus, and provides a large surface area of contact between the host and the fungal endophyte (Dickson & Kolesik 1999). Since the fungal pelotons occurred inside the periplasmic space, they were considered intracellular. The pelotons filled up the infected cells almost completely (Fig. 4c). Carlile & Watkinson (1994) reported that up to 80 % of root cell volume was filled by the fungal tissue. In the hair roots these fungi colonized the cells of both epidermis and cortex (Fig. 3c); however, the immature cells of root apices were not colonized by the fungi. The apices of the hair roots were not invaded by fungus, which were intact as previously described in the literature (Ashford *et al.* 1996; Briggs & Ashford 2001; McNabb 1961). The micrometry analysis of the root showed that the infected cells increased in length and width more than the uninfected cells (Table 1). Cairney & Ashford (2002) reported that the roots of ericaceous plants collected from the field showed thick-walled epidermal cells containing coils with live fungal hyphae, surrounded by collapsed, thin-walled uncolonized cells.

In the framework roots of *R. arboreum* ssp. *nilagiricum* the fungal colonization occurred in the intracellular spaces of the secondary cortex (Fig. 4a-c). Bonfante-Fasolo *et al.* (1984) observed similar results in an ericaceous species colonized with the fungus *Hymenoscyphus ericae*. It was interesting to note in the present investigation that in the cortex of the framework roots some of the cells were filled with secondary metabolites, and those cells were not colonized by the ericoid fungi, suggesting that ericoid fungi are very sensitive to the presence of secondary metabolites. Jalal *et al.* (1982) reported that the total phenolic content in the roots of *Calluna,* an ericaceous member, were remarkably high. In the present investigation the framework roots exhibited a lower percentage of colonization than the hair roots. Within the root, the intracellular fungal coil produced narrow hyphae that penetrated both radial and tangential walls of the root cells, and formed a network of hyphae within the root tissues.

Although *R. arboreum* ssp. *nilagiricum* was positive for ericoid mycorrhizal colonization, the infection density varied between framework and hair roots, and hair roots were more prone to fungal infection than the frame work roots. The degree of infection by the fungus was estimated by calculating the infection density according to Hadley & Williamson (1972). The extent of ericoid fungal colonization in hair roots was more than 55 % during the peak season (i.e. North East monsoon; Table 2), whereas the framework roots showed less than 20 % colonization. In other seasons the infection density was gradually reduced, and was lowest both in hair and framework roots during the dry season. The decrease in colonization during the dry season could be explained by the decrease in root biomass. It is possible to say from this experiment that the observed seasonal differences were consistent from year to year (Table 2). Reed (1989) found that the hair roots were persistent throughout the year in *L. juniperinus*; however, the incidence of mycorrhizal infection was much lower the dry season. Similar results were reported for *Woolsia pungens* (Cac.)F. Muell., in which the fungal infection persisted around the year (Cairney & Ashford 2002; Read & Kerley 1995). The above mentioned reports indicated that the ericoid mycorrhiza fungi are functional all the year round except in very dry conditions. Haselwandter (1987) and Read *et al.* (1976) reported that water and temperatures stresses influence the levels of mycorrhizal colonization.

This study is the first report of ericoid mycorrhizal fungi in *R. arboreum* ssp. *nilagiricum* in India. It provides information on the colonization and infection processes involved in the formation of compatible mycorrhizal associations. This baseline information should be helpful to future research on this species.

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