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ARTICLE

Endophytic fungal association in roots of exotic medicinal plants cultivated in the Nilgiris (Western Ghats, Peninsular India)

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Resumen

Asociación de hongos endofíticos en raíces de plantas medicinales exóticas cultivadas en Nilgiris (Western Ghats, India Peninsular)

Algunos microbios beneficiosos del suelo ayudan al establecimiento y crecimiento de plantas medicinales exóticas. Evaluamos la presencia y el estado de la asociación de endófitos de raíces [hongos micorrízicos arbusculares (AM) y hongos endofíticos septados oscuros (DSE)] en diez especies de plantas medicinales exóticas cultivadas en Nilgiris (Ghats Occidentales). Los hongos AM colonizaron todas las especies examinadas y ocho plantas tuvieron la co-ocurrencia de hongos DSE. El alcance de las variables de los hongos endofíticos y las características del pelo radicular difirieron significativamente entre las plantas. Se identificaron seis morfotipos de esporas de hongos AM. Por lo tanto, este estudio indicó la asociación de plantas medicinales exóticas con hongos nativos AM y DSE que podrían explotarse para promover el crecimiento y aumentar la producción de metabolitos secundarios en estas especies de plantas.

Palabras clave: Micorrizas arbusculares; Hongos endofíticos septados oscuros; Pelo radicular; Morfotipo de esporas

Abstract

Some soil beneficial microbes help in the establishment and growth of exotic medicinal plants. We evaluated the presence and status of root endophyte [arbuscular mycorrhizal (AM) fungi and dark septate endophytic (DSE) fungi] association in ten exotic medicinal plant species cultivated in the Nilgiris (Western Ghats). The AM fungi colonized all the examined plant species, and eight plants had the co-occurrence of DSE fungi. The extent of fungal endophyte variables and root hair characteristics significantly differed among the plants. Six AM fungal spore morphotypes were identified. Thus, this study indicated the association of exotic medicinal plants with native AM and DSE fungi which could be exploited to promote growth and increase secondary metabolite production in these plant species.

Key words: Arbuscular mycorrhiza; Dark septate endophytic fungi; Root hair; Spore morphotype.

Introduction

Medicinal plants are widely recognized with high healing activity worldwide. According to the World Health Organization (WHO), 80% of the world's people depend on traditional medicine for their primary healthcare needs (Ahvazi *et al.* 2012). Medicinal plants play a dynamic role in traditional medicines (Kumarasamyraja *et al.* 2012) as well as in trading commodities (Vasisht *et al.* 2016). Due to their popularity and application, there is a scarcity and also increasing demand for medicinal plants and their products (Nishteswar 2014). Also, the exploitation of medicinal plants growing in nature leads to the loss of natural populations that may ultimately lead to their extinction. This has promoted the cultivation of native and exotic medicinal plants to meet the demand for raw materials in the pharmaceutical sector.

In the current scenario, different systems of medicine are practiced worldwide in addition to their place of origin. This has greatly increased the demand for raw materials for drug preparation and many medicinal plants are cultivated apart from their native regions to increase their availability (Alencar *et al.* 2014, Medeiros *et al.* 2017). The medicinal plants that are introduced into newer regions other than their native habitats by humans are called exotics. Many exotic medicinal plants have the potential to grow and thrive in different ecological regions (Alencar *et al.* 2014, Nguanchoo *et al.* 2019). Successful growth and survival of exotic medicinal plants into an introduced area may be aided by several microorganisms that are present in the root and rhizosphere soil (Rajkumar *et al.* 2012, Zubek *et al.* 2012). Among the soil microbiome, fungal endophytes have a critical role in plant growth and health.

Endophytic fungi are non-pathogenic symbiotic soil fungi that provide habitat fitness to genetically distant host plants and also confer tolerance against various abiotic stresses (Begum *et al.* 2019, Diagne *et al.* 2020). Endophytic fungi influence the dynamics of the plant community through many mechanisms like nutrient uptake by plants, nutrient redistribution, and interplant competition (McGee *et al.* 2019). Among the different types of endophytic fungal symbiosis, arbuscular mycorrhizal (AM) and dark septate endophytic (DSE) fungal symbioses are of great ecological importance.

The ubiquitous AM fungi belonging to the phylum Glomeromycota (Terdosoo *et al.* 2018) colonize almost 72% of the plant species growing in the natural ecosystem (Brundrett & Tedersoo 2018). The AM symbiosis aids in plant growth by increasing the availability as well as translocation of various nutrients especially phosphorous (P) (Rouphael *et al.* 2015). The AM fungi form various structures like hyphae, arbuscules, hyphal and arbusculate coils, and vesicles in plant roots (Muthukumar *et al.* 2016, Muthuraja & Muthukumar 2019). In addition, there exist an arbuscule-forming fungus known as fine root endophyte (FRE) belonging to the subphylum Mucoromycotina of Mucoromycota (Orchard *et al.* 2017) which also co-occur in roots along with other fungal endophytes. Moreover, there is a great difference in the distribution or pattern of AM fungal structures in plant roots (Muthukumar *et al.* 2016). The morphology of AM fungi chiefly falls into major types based on the colonization patterns: *Arum*-, *Paris*- and intermediate types. The *Arum*-type is represented by intercellular hyphae and arbuscules, on contrary, *Paris*-type is characterized by intracellular linear hyphae, hyphal coils, and arbusculate coils. The intermediate type of AM morphology shares both the characters of *Arum*- and *Paris*- types (Dickson 2004).

In addition to the widely associated AM fungi, roots of many plants especially the non-mycorrhizal plants are colonized by septate conidial fungi with melanized hyphae that forms moniliform or microsclerotia within plant roots known as DSE fungi (Peterson *et al.* 2008). They are worldwide in distribution and interact with other soil biota both functionally and ecologically (Mayerhofer *et al.* 2013). The DSE fungi often co-occur with different types of mycorrhizal fungi and are reported in several aromatic plants, crops and horticultural plants (Muthukumar *et al.* 2018, Piszczek *et al.* 2019). Like AM fungi, DSE fungi also benefit plants by improving growth, nutrient uptake, and tolerance against different environmental stresses (Vergara *et al.* 2018, Farias *et al.* 2020).

Being a crucial element of soil microbial community in the terrestrial environment, both AM and DSE fungi could be an important characteristic in the exotic plant process by promoting local adaptation, decrease environmental stresses, and their influence on plant competition (Gucwa-Przepióra *et al.* 2016). Moreover, investigation on

mycorrhizal status and identification of AM fungal spores associated with them could be useful in the formulation of bioinoculants for cultivating exotic medicinal plants (Zubek *et al.* 2012). Though the mycorrhizal status of many medicinal plants has been investigated (Muthukumar *et al.* 2006, Sharma & Jha 2012, Safari Sinegani & Yeganeh 2017), reports on beneficial endophyte fungal association in the cultivated exotic medicinal plants species are very limited. Moreover, only ~1% of the global medicinal plants have been investigated in terms of their association with soil-borne fungi (Piszczek *et al.* 2019). Therefore, the present study was undertaken to examine the occurrence and extent of AM and DSE fungal association, AM colonization patterns, and diversity of AM fungi associated with ten cultivated exotic medicinal plant species belonging to different families. In addition, the root hair morphology of exotic medicinal plants and its relationship with the colonization levels of AM and DSE fungal structures were also assessed.

Materials and methods

Site description and examined exotic medicinal plant species

Root and soil samples were collected from 10 exotic medicinal plant species (Supplementary Table S1) cultivated at the Central Survey of Medicinal Plants Collection (CSMPC), Emerald (11º33' N, 76º61' E), the Nilgiris district of Tamilnadu, India during December 2019. The site is located at an altitude of 1995 m above sea level with a relative humidity of 70-75%. The average rainfall is about 2,778 mm/year and the mean temperature of the coldest month is 15 ºC.

Collection of samples

The soil samples from the root zone region at three points around each plant were collected using a hand trowel, composited, and stored in polybags. The fine feeder roots of each plant were also collected during soil sampling. The collected roots were thoroughly washed with tap water and stored in small plastic vials containing FAA (Alcohol: Formaldehyde: Acetic acid/ 90 ml: 5 ml: 5 ml) for the assessment of root fungal association and root hair characteristics. The collected root and soil samples were labelled and brought to the laboratory in an icebox within 12 hrs of collection. The soil samples were air-dried and stored at 4 ºC for further analysis.

Chemical analysis of soil

The characteristics of the soil samples collected from each of the ten exotic medicinal plant species such as pH, electrical conductivity (EC), and macronutrients were analyzed. The soil extract was prepared by mixing the soil sample with distilled water (1:1), pH and EC were measured using respective digital meters. The total nitrogen (N) was estimated by the micro Kjeldahl method and available P was determined by the colorimetric method as described by Jackson (1971). Exchangeable potassium (K) in soil was extracted using ammonium acetate solution and measured with a digital flame photometer (Jackson 1971).

Enumeration, isolation, and characterization of AM fungal spores

The AM fungal spores were extracted from 50 g of each soil sample by modified wet sieving and decanting technique and enumerated (Muthukumar *et al.* 2006). Only intact AM fungal spores were counted and isolated. The morphological and subcellular characters of the isolated spores were determined by mounting them in polyvinyl alcohol/lactic acid/glycerol (PVLG) reagent using an Olympus BX51 microscope. The AM fungal spores were identified by comparing the spore characteristics with the original descriptions available at the AM phylogeny web page (http://www.amf-phylogeny.com).

Processing of root samples for endophytic fungal colonization

Root samples stored in the FAA were washed thoroughly with distilled water and cut into 1 cm long pieces and clarified with 2.5% KOH at 90 ºC for about 13 hours. Further, the root pieces were rinsed with water and soaked in 5 N HCl for 15 minutes. After acidification, the roots were placed in 0.05% trypan blue staining solution and left undisturbed overnight for staining (Koske & Gemma 1989). The excess stain from 30 randomly selected root pieces were removed by placing them in clear lactoglycerol and mounted on clean microscopic slides, covered with cover glasses, squashed gently, and observed under Olympus BX51 microscope. The images were

captured using a ProgRes 3 digital camera. The percentage of total root length colonization by fungal endophyte was determined by observing one hundred intersections for each root sample at 400 × using the magnified intersection method according to McGonigle *et al.* (1990). The type of AM morphology was inferred according to Dickson (2004).

Assessment of root hair morphology

For assessment of root hair characteristics, approximately 15 mm long root pieces were mounted in the water on a microscope slide and observed under an Olympus BX51 compound microscope fitted with a calibrated ocular scale (×40-100). The root hair number, length, and width were measured according to Ma *et al.* (2001).

Statistical analysis

The data of soil analysis, endophytic fungal colonization, and root hair parameters were tested for homogeneity (Levene's test) and subjected to one-way analysis of variance (ANOVA). Mean separations were performed using Duncan's multiple range test ($p<0.05$). The relationship between endophytic fungal colonization variables and root hair characteristics and the number of AM fungal spores were determined by Pearson's correlation analysis. All the statistical analysis was performed using SPSS (version 16.0).

Results

Soil characteristics

The soil pH of the exotic medicinal plants was slightly acidic to marginally alkaline ranging from 5.97 [*Jacobaea maritima* (L.) Pelser & Meijden] to 7.04 (*Cuminum cyminum* L. and *Sambucus nigra* L.) (Supplementary Table S2). The EC of the soil ranged between 0.19 dS/m (*C. cyminum*) and 0.91 dS/m (*J. maritima* and *S. nigra*) and the total N ranged from 87 kg/ha [*Rosmarinus officinalis* L. (= *Salvia rosmarinus* Schleid.)] to 170 kg/ha (*Hypericum hookerianum* Wight & Arnott, *C. cyminum*, *Acacia mearnsii* De Wild and *S. nigra*). The maximum available P in soil was recorded in *C. cyminum*, (26 kg/ha) and minimum in *Achillea millefolium* L., *Melaleuca alternifolia* (Maiden & Betche) Cheel and *J. maritima* (7 kg/ha). Similarly, exchangeable K was higher (311 kg/ha) in soil under *C. cyminum* and lower (114 kg/ha) in *A. millifolium* (Supplementary Table S2).

Root hair characteristics

The root hair morphology including the number, length, and width of root hairs varied significantly ($p<0.01$) among the different plant species. The maximum number of root hairs was recorded in *A. millifolium* when compared to other exotic medicinal plants. The root hair length ranged from 27.76 µm (*H. hookerianium*) to 51.83 µm (*M. alternifolia* and *R. officinalis*). Nevertheless, the root hairs of *Aloe ferox* Mill. was wider when compared to other plant species examined (Table 1).

Plant species	Root hair		
	number (mm^{-1}/plant)	length (µm/plant)	width (µm/plant)
Acacia mearnsii	25.60±9.62^{ab}	31.00±3.00^{b}	1.00±0.00^{b}
Achillea millefolium	38.20±13.89^{a}	48.67±3.05^{a}	1.00±0.00^{b}
Aloe ferox	5.80±1.24^{c}	44.67±2.78^{a}	1.27±0.12^{a}
Cuminum cyminum	16.20±3.46^{bc}	47.17±4.18^{a}	1.00±0.00^{b}
Digitalis purpurea	4.80±0.66^{c}	32.00±1.91^{b}	1.00±0.00^{b}
Hypericum hookerianum	11.60±1.78^{bc}	27.67±2.05^{b}	1.00±0.00^{b}
Jacobaea maritima	18.20±1.46^{bc}	51.17±5.24^{a}	1.00±0.00^{b}
Melaleuca alternifolia	12.20±1.66^{bc}	51.83±5.97^{a}	1.00±0.00^{b}
Rosmarinus officinalis	25.80±6.11^{ab}	51.83±4.45^{a}	1.00±0.00^{b}
Sambucus nigra	6.60±1.54^{bc}	30.83±2.12^{b}	1.00±0.00^{b}
F-statistics	3.314***	7.261***	5.091***
df	9,49	9,149	9,149

Tabla 1. Características de los pelos radicales de especies de plantas medicinales exóticas cultivadas. Las medias en una columna seguidas de una (s) misma (s) letra (s) no son significativamente diferentes ($p> 0.05$) según la Prueba de rango múltiple de Duncan.

*** Significativo al nivel del 0,1%. df- Grado de libertad.

Table 1. Root hair characteristics of cultivated exotic medicinal plant species. Means in a column followed by the same superscript letter(s) are not significantly ($p>0.05$) different according to Duncan's Multiple Range Test.

***Significant at 0.1% level. df- Degree of freedom.

Endophytic fungal association

Roots of all the examined medicinal plant species were colonized by both AM and DSE fungi except *A. ferox* and *M. alternifolia* which were colonized only by the former. The FRE was observed only in the roots of *Digitalis purpurea* L. The AM, DSE and FRE fungi were characterized by the presence of an appressorium on the root surface as

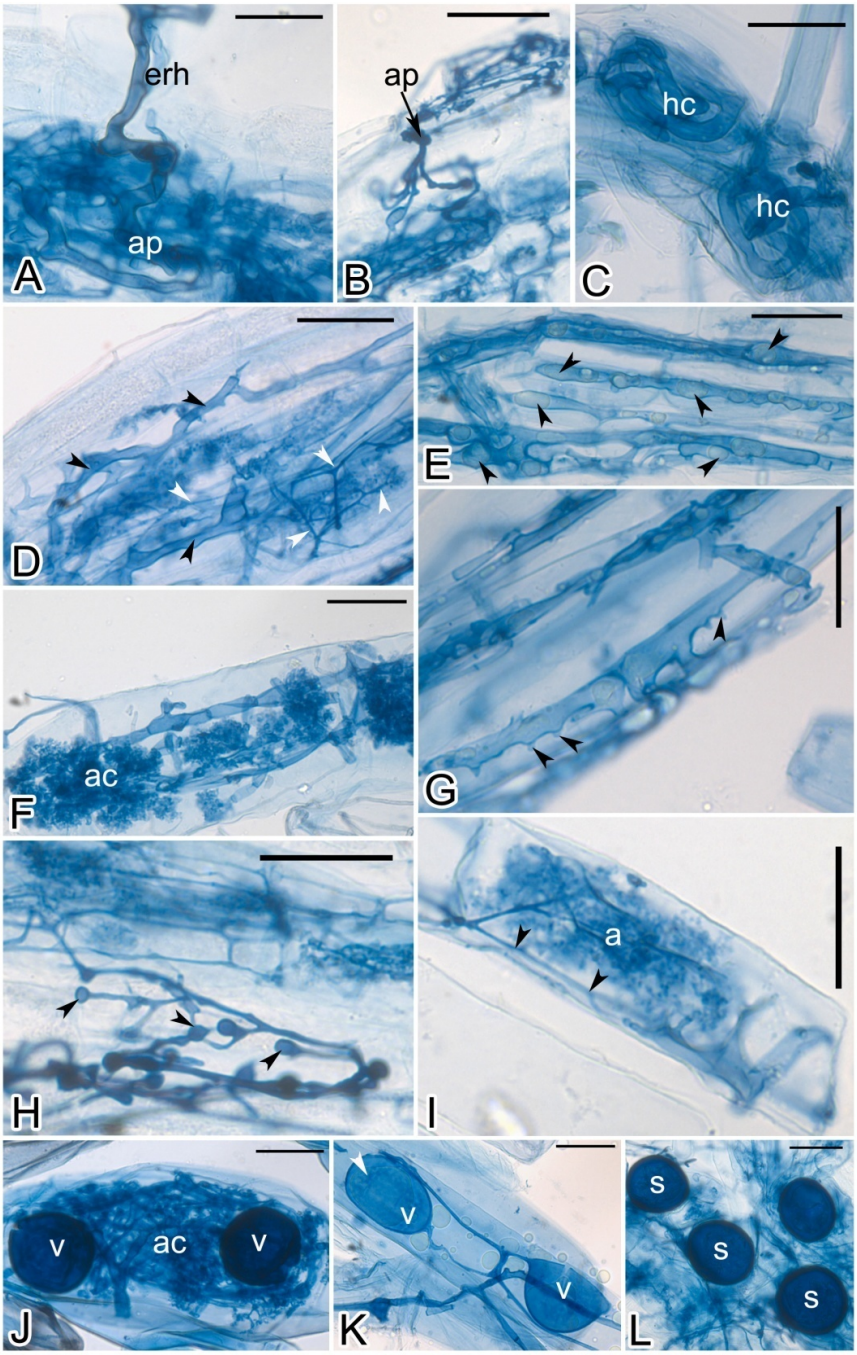

Figura 1. Colonización de micorrizas arbusculares (AM) y endófitos de raíces finas (FRE) en plantas medicinales cultivadas en el jardín de plantas medicinales de la colección central de muestras de plantas medicinales. **A:** Hifas extrarradicales (erh) y apresorio (ap) de hongos AM en la superficie de la raíz de *Digitalis purpurea*; **B:** Apresorio (ap) de FRE en la superficie de la raíz de *D. purpurea*; **C:** Espirales hifales (hc) de hongos AM en raíces de *Melaleuca alternifolia*; **D:** Presencia de hifas AM (puntas de flecha negras) y FRE (puntas de flecha blancas) en la raíz de *D. purpurea*; **E:** Gotas de aceite (puntas de flecha negras) en las hifas AM de *Rosmarinus officinalis*; **F:** Espiral arbusculada (ac) en la célula cortical de la raíz de *Sambucus nigra*; **G:** Clavijas hifas (puntas de flecha negras) en el micelio intrarradical de *R. officinalis*; **H:** Inflamaciones hifales intercalares y terminales (puntas de flecha negras) de FRE en *D. purpurea*; **I:** Hifas intracelulares (puntas de flecha negras) y arbúsculo (a) de FRE en *D. purpurea*; **J:** Espiral arbusculada (ac) y vesículas intracelulares (v) en *Aloe ferox*; **K:** Vesículas intracelulares (v) y gotita de aceite en *S. nigra* (punta de flecha blanca); **L:** Esporas intrarradicales en *D. purpurea*. Barras de escala = 30 µm.

Figure 1. Arbuscular mycorrhizal (AM) and fine root endophyte (FRE) colonization in medicinal plants cultivated at the medicinal plants garden of the central survey of medicinal plants collection. **A:** Extraradical hyphae (erh) and appressorium (ap) of AM fungi on the root surface of *Digitalis purpurea*; **B:** Appressorium (ap) of FRE on the root surface of *D. purpurea*; **C:** Hyphal coils (hc) of AM fungi in roots of *Melaleuca alternifolia*; **D:** Presence of AM (black arrowheads) and FRE (white arrowheads) hyphae in root of *D. purpurea*; **E:** Oil droplets (black arrowheads) in AM hyphae of *Rosmarinus officinalis*; **F.** Arbusculate coil (ac) in root cortical cell of *Sambucus nigra*; **G.** Hyphal pegs (black arrowheads) in the intraradical mycelium of *R. officinalis*; **H:** Intercalary and terminal hyphal swellings (black arrowheads) of FRE in *D. purpurea*; **I:** Intracellular hyphae (black arrowheads) and arbuscule (a) of FRE in *D. purpurea*; **J:** Arbusculate coil (ac) and intracellular vesicles (v) in *Aloe ferox*; **K:** Intracellular vesicles (v) and oil droplet in *S. nigra* (white arrowhead); **L:** Intraradical spores (s) in *D. purpurea*. Scale bars = 30µm.

a point of fungal entry arising from extraradical hyphae (Figs. 1A, B, 2A). The linear hyphae of AM fungi and FRE were respectively >4 and <2 µm in diameter and were inter-and intracellular (Fig. 1D). The AM fungi also formed coiled hyphae in all the examined plant species (Fig. 1C). Numerous oil droplets were visible in the AM fungal hyphae (Fig. 1E). Arbusculate coils (Figs. 1F, J) and intracellular vesicles were observed in the root cortical cells (Figs. 1J, K). Intraradical hyphae of AM fungi consisted of hyphal pegs in the roots of *R. officinalis* (Fig. 1G). Both intercalary and terminal swellings of FRE hyphae occurred in the roots of *D. purpurea* (Fig. 1H). In addition, AM fungi were also represented by the presence of intracellular hyphae, arbuscules, and intraradical spores (Figs. 1I, L). The DSE fungi were characterized by the presence of regularly septate melanized hyphae and microsclerotia (Figs. 2B-G).

AM morphology

Roots of all examined exotic medicinal plants colonized by AM fungi had different types of AM morphology. Of the ten exotic medicinal plant species, four plant species exhibited *Arum-Paris* type, whereas, the other three plants had intermediate type 3. However, *M. alternifolia* and *A. millifolium* formed intermediate type 4 and *R. officinalis* had intermediate type 1 respectively (Table 2).

The extent of AM and DSE fungal colonization

The percentage of root length containing both different AM and DSE fungal structures as well as total root length colonization varied significantly among all the exotic medicinal plant species examined. The percentage of root length containing hyphae (%RLH) and hyphal coils (%RLHC) ranged from 18.88% (*R. officinalis*) to 61.06% (*A. millifolium*) and 1.20% (*J. maritima*) to 11.22% (*S. nigra*) respectively. However, the hyphal coils were not observed in the roots of *R. officinalis*. The percentage of root length containing arbuscules (%RLAR) was maximum in *S. nigra* (24.03%) and minimum in *C. cyminum* (0.52%) but it was not observed in the roots of *A. millifolium*, *M. alternifolia* and *A. mearnsii*. The percentage of root length with arbusculate coils (%RLAC) was 62.70%–97.32% higher in the roots of *D. purpurea* when compared to other plant species. Nevertheless, the arbusculate coils were absent in the roots of *A. millifolium*, *C. cyminum*, *M. alternifolia*, *R. officinalis* and *J. maritima*. The percentage of root length with vesicles (%RLV) ranged between 0.60% (*J. maritima*) and 5.95% (*D. purpurea*). Nevertheless, vesicles were not seen in *M. alternifolia* and *R. officinalis* roots. Overall, the total root length colonization with AM fungi (TRLCAM) was maximum in *S. nigra* (89.79%) and the minimum in *R. officinalis* (21.55%).

The roots of *A. ferox* and *M. alternifolia* were not colonized by DSE fungi. Nevertheless, the percentage of root length colonized by dark septate fungal hyphae (%RLDSH) was 48.22%–89.37% higher in *R. officinalis* than in other examined plant species. Likewise, the percentage of root length containing microsclerotia (%RLMS) ranged from 0.54 (*H. hookerianium*) to 5.84 (*C. cyminum*). But microsclerotia were absent in *A. ferox* and *J. maritima* roots. The moniliform hyphae were present only in the roots of *A. millifolium* (0.65%) and *R. officinalis* (2.31%). Overall, *R. officinalis* (45.31%) had the maximum total root length colonized by DSE fungi (TRLCD) and minimum occurred in *S.*

Plant species	Linear hyphae		Hyphal coils	Arbuscules	Arbusculate coils	Vesicles		AM type
	Inter	Intra				Inter	Intra	
Acacia mearnsii	+	+	+	+	—	+	—	Intermediate 3
Achillea millefolium	+	+	+	—	—	+	+	Intermediate 4
Aloe ferox	+	+	+	+	+	+	+	*Arum* & *Paris*
Cuminum cyminum	+	+	+	+	—	+	+	Intermediate 3
Digitalis purpurea	+	+	+	+	+	—	+	*Arum* & *Paris*
Hypericum hookerianum	+	+	+	+	+	—	+	*Arum* & *Paris*
Jacobaea maritima	+	+	+	+	—	+	+	Intermediate 3
Melaleuca alternifolia	+	+	+	—	—	—	—	Intermediate 4
Rosmarinus officinalis	+	+	—	+	—	—	—	Intermediate 1
Sambucus nigra	+	+	+	+	+	+	+	*Arum* & *Paris*

Presence: +; Absence: —

Tabla 2. Distribución de diversas estructuras fúngicas micorrízicas arbusculares (MA) en diferentes especies de plantas medicinales exóticas.
Table 2. Distribution of various arbuscular mycorrhizal (AM) fungal structures in different exotic medicinal plant species.

Plant species	AM colonization (%)						DSE colonization (%)			
	RLH	RLHC	RLA	RLAC	RLV	TRLCAM	RLDSH	RLMS	RLMH	TRLCD
Acacia mearnsii	31.22±1.58^{d}	7.33±1.18^{ab}	0.52±0.52^{e}	0.00±0.00^{c}	3.14±0.87^{abc}	42.21±1.87^{e}	3.64±0.97^{de}	5.84±0.61^{a}	0.00±0.00^{b}	9.49±0.62^{c}
Achillea millefolium	61.06±1.59^{a}	3.16±2.39^{bc}	0.00±0.00^{e}	0.00±0.00^{c}	1.85±1.14^{bcd}	66.07±4.11^{c}	19.30±3.64^{b}	2.37±0.78$^{ab c}$	0.65±0.65^{b}	22.32±2.68^{b}
Aloe ferox	45.04±2.80$^{b c}$	10.12±1.12^{a}	14.75±3.08^{b}	5.25±1.18^{bc}	4.21±0.88^{ab}	79.37±3.72^{b}	0.00±0.00^{e}	0.00±0.00^{c}	0.00±0.00^{b}	0.00±0.00^{d}
Cuminum cyminum	31.22±1.58^{d}	7.33±1.18^{ab}	0.52±0.52^{e}	0.00±0.00^{c}	3.14±0.87^{abc}	42.21±1.87^{e}	3.64±0.97^{de}	5.84±0.61^{a}	0.00±0.00^{b}	9.49±0.62^{c}
Digitalis purpurea	43.26±3.06^{c}	3.34±1.04^{bc}	13.32±0.63^{b}	24.29±2.16^{a}	4.91±1.50^{a}	89.12±2.03^{a}	6.69±1.71^{d}	0.94±0.56^{bc}	0.00±0.00^{b}	7.63±1.91^{c}
Hypericum hookerianum	40.19±2.36^{c}	3.18±0.54^{bc}	10.13±0.69$^{b c}$	2.35±1.40^{c}	1.22±0.62^{cd}	57.06±1.09^{d}	7.69±0.82^{cd}	0.54±0.54^{cb}	0.00±0.00^{b}	8.23±1.08^{c}
Jacobaea maritima	48.50±1.66^{b}	1.20±0.60^{c}	5.97±2.37^{cd}	0.00±0.00^{c}	0.60±0.60^{cd}	56.27±3.13^{d}	13.19±1.67^{bc}	0.00±0.00^{c}	0.00±0.00^{b}	13.19±1.67^{c}
Melaleuca alternifolia	31.14±0.86^{d}	0.65±0.65^{cd}	0.00±0.00^{e}	0.00±0.00^{c}	0.00±0.00^{d}	31.79±0.21^{f}	0.00±0.00^{e}	0.00±0.00^{c}	0.00±0.00^{b}	0.00±0.00^{d}
Rosmarinus officinalis	18.88±1.94^{e}	0.00±0.00^{c}	2.66±1.45^{de}	0.00±0.00^{c}	0.00±0.00^{d}	21.55±1.19^{g}	37.28±2.24^{a}	5.72±2.46^{a}	2.31±0.73^{a}	45.31±4.62^{a}
Sambucus nigra	39.53±2.28^{c}	11.22±4.10^{a}	24.03±2.86^{a}	9.06±4.61^{b}	5.95±1.59^{a}	89.79±1.79^{a}	6.46±1.70^{d}	0.62±0.62^{bc}	0.00±0.00^{b}	7.08±1.66^{c}
$F_{9,29}$	22.814***	5.549***	25.460***	19.948***	5.939***	81.597***	31.227***	4.204**	5.632***	44.036***

Tabla 3. Asociación de hongos micorrízicos arbusculares (AM) y endofiticos septados oscuros (DSE) en especies de plantas medicinales exóticas.
RLH-Longitud de raíz con hifas, RLHC-Longitud de raíz con hifas hifas, RLA-Longitud de raíz con arbuscules, RLAC-Longitud de raíz con espirales arbusculadas, RLV-Longitud de raíz con vesículas, RLDSH-Longitud de raíz con hifas septadas oscuras, RLMS-Longitud de raíz con microesclerocios, RLMH-Longitud de la raíz con hifas moniliformes, TRLCAM- Colonización de la longitud total de la raíz por hongos AM, TRLCD- Colonización de la longitud total de la raíz por hongos DSE. Las medias en una columna seguidas por la misma (s) letra (s) en superíndice no son significativamente (p> 0.05) diferentes según la Prueba de rango múltiple de Duncan. ** Significativo al nivel del 1%, *** Significativo al nivel del 0,1%.

Table 3. Arbuscular mycorrhizal (AM) and dark septate endophytic (DSE) fungal association in exotic medical plant species.
RLH-Root length with hyphae, RLHC-Root length with hyphal coils, RLA-Root length with arbuscules, RLAC-Root length with arbusculate coils, RLV-Root length with vesicles, RLDSH-Root length with dark septate hyphae, RLMS-Root length with microsclerotia, RLMH-Root length with moniliform hyphae, TRLCAM- Total root length colonization by AM fungi, TRLCD- Total root length colonization by DSE fungi. Means in a column followed by same superscript letter(s) are not significantly (p>0.05) different according to Duncan's Multiple Range Test. ** Significant at 1% level, ***Significant at 0.1% level.

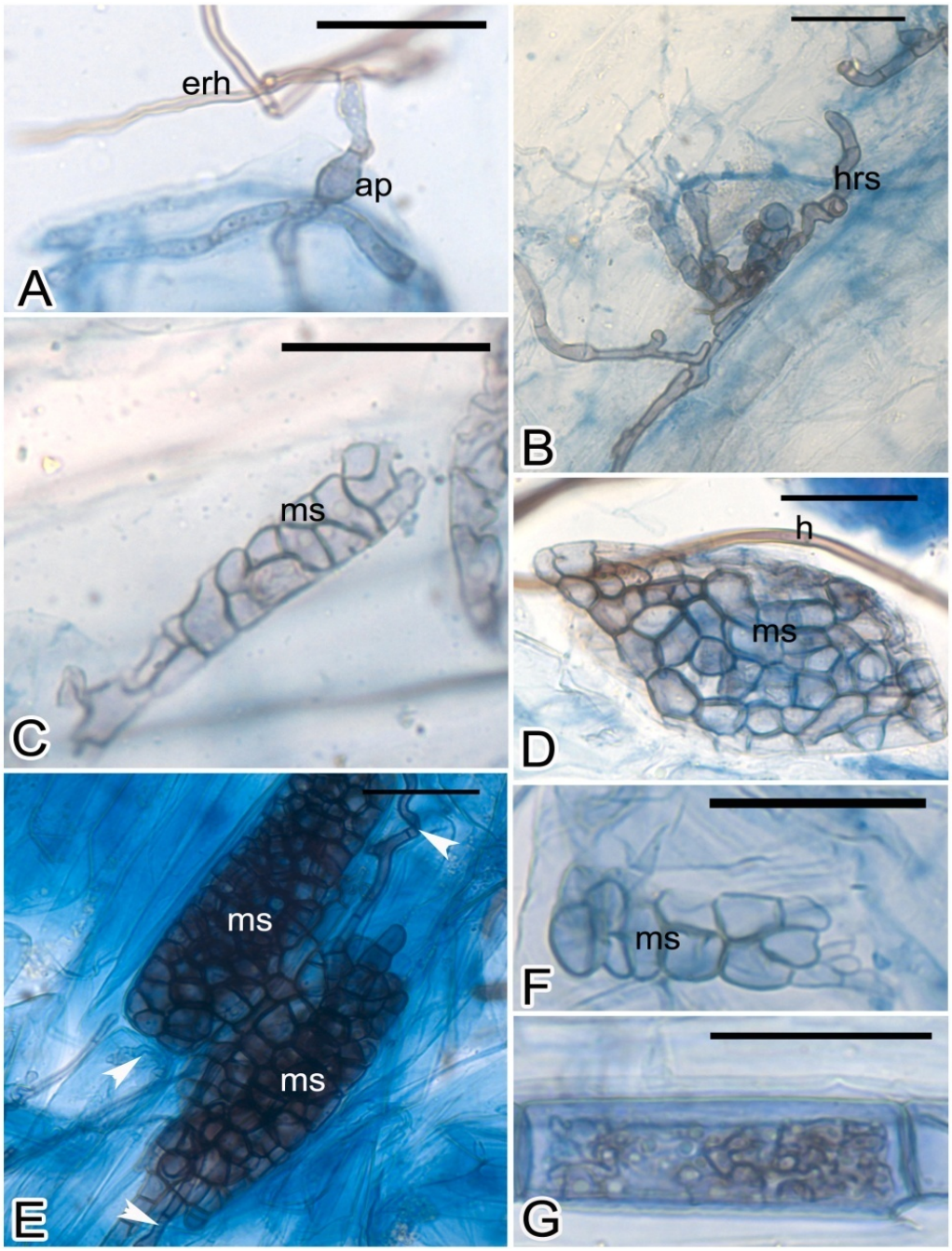

Figura 2. Colonización de hongos endófitos septados oscuros (DSE) en plantas medicinales cultivadas en el jardín de plantas medicinales del estudio central de la colección de plantas medicinales. **A:** Apresorio (ap) e hifas extrarradicales (erh) en *Hypericum hookerianum*; **B:** Hifas melanizadas de hongos DSE en la superficie de la raíz (h) en *H. hookerianum*; **C-G:** Microsclerotia (ms) e hifas (hy puntas de flecha blancas) en las células corticales de la raíz de *H. hookerianum* (D), *Rosmarinus officinalis* (E), *Melaleuca alternifolia* (F) y *Achillea millefolium* (G). Barras de escala = 30 µm.

Figure 2. Dark septate endophyte (DSE) fungal colonization in medicinal plants cultivated at the medicinal plants garden of the central survey of medicinal plants collection. **A:** Appressorium (ap) and extraradical hyphae (erh) in *Hypericum hookerianum*; **B:** Melanized hyphae of DSE fungi on the root surface (hrs) in *H. hookerianum*; **C-G:** Microsclerotia (ms) and hyphae (h and white arrowheads) in root cortical cells of *H. hookerianum* (D), *Rosmarinus officinalis* (E), *Melaleuca alternifolia* (F) and *Achillea millefolium* (G). Scale bars = 30µm.

nigra (7.08%) (Table 3).

AM fungal species diversity

The number of AM spores significantly varied ($F_{9,29}$=8.876; p<0.01) in the root zones of different exotic medicinal plant species. The average number of AM spores in exotic medicinal plant species per 50 grams of soil ranged from 96 (*H. hookerianium*, *A. ferox* and *C. cyminum*) to 1051 (*D. purpurea*) (Fig. 3). Spore morphotypes of six AM fungal species including *Acaulospora scro-*

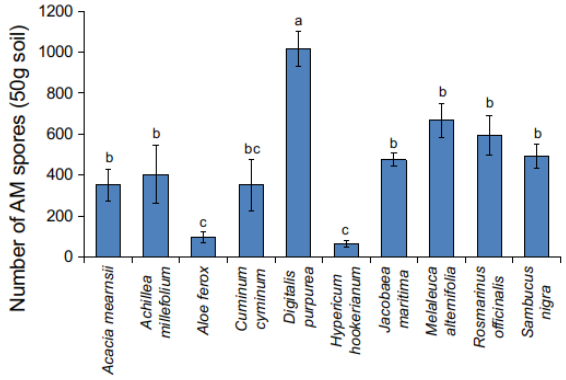

Figura 3. Número de esporas de hongos micorrízicos arbusculares (AM) presentes en los suelos de la zona radicular de especies exóticas de la colección de plantas medicinales Nilgiris. La barra de error representa ± error estándar. Las barras que llevan la(s) misma(s) letra(s) no son significativamente diferentes (p>0.05) según la Prueba de rango múltiple de Duncan.

Figure 3. Number of arbuscular mycorrhizal (AM) fungal spores present in the root zone soils of exotic species of medicinal plants collection, the Nilgiris. Error bar represents ± standard error. Bars bearing the same letter(s) are not significantly different (p>0.05) according to Duncan's Multiple Range Test.

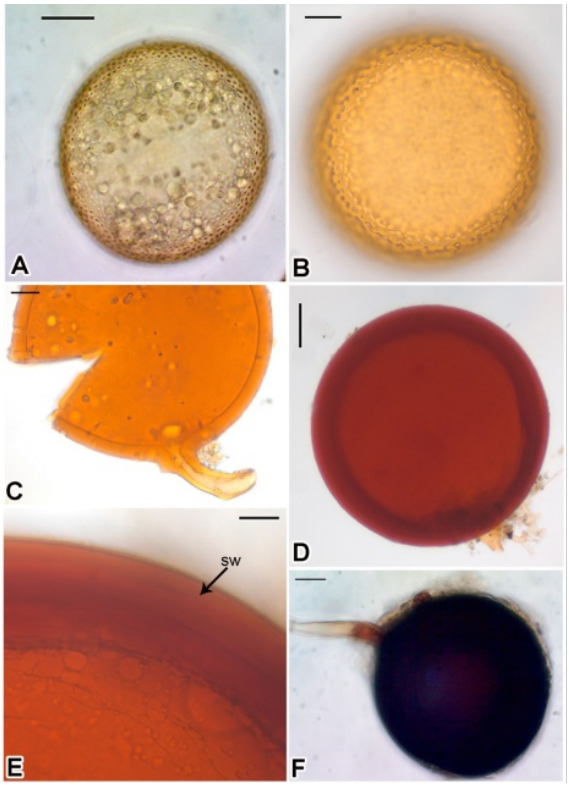

Figura 4. Esporas de hongos micorrízicos arbusculares (AM) aisladas de los suelos de la zona radicular de diferentes especies de plantas medicinales exóticas. **A:** *Acaulospora scrobiculata*; **B:** *Acaulospora tuberculata*; **C:** *Claroideoglomus etunicatum*; **D:** *Gigaspora decipiens*; **E:** Pared engrosada (sw) de *G. decipiens*; **F:** *Funneliformis geosporum*. Barras de escala = 20 µm.

Figure 4. Arbuscular mycorrhizal (AM) fungal spores isolated from the root zone soils of different exotic medicinal plant species. **A:** *Acaulospora scrobiculata;* **B:** *Acaulospora tuberculata;* **C:** *Claroideoglomus etunicatum;* **D:** *Gigaspora decipiens*; **E:** Thickened spore wall (sw) of *G. decipiens*; **F:** Spore of *Funneliformis geosporum*. Scale bars = 20 µm.

biculata Trappe, *Acaulospora tuberculata* Janos & Trappe, *Gigaspora decipiens* I.R. Hall & L.K. Abbott, *Claroideoglomus etunicatum* (W.N. Becker & Gerd.) C. Walker & A. Schüßler, *Funneliformis geosporum* (T.H. Nicolson & Gerd.) C. Walker & A. Schüßler and *Rhizophagus fasciculatus* (Thaxt.) C. Walker & A. Schüßler were isolated from the rhizosphere soil of different exotic medicinal plant species (Fig. 4). *Acaulospora* species was dominant in all the examined soil samples.

Relationship between root hair, AM and DSE fungal variables

Pearson's correlation indicated a significant negative correlation (r=-0.465; p<0.05; n=30) between %RLAC and spore number. Similarly, root hair number was significantly and negatively correlated to %RLHC (r=-0.377; p<0.05), %RLAR (r= -0.470; p<0.01), %RLAC (r=-0.405; p<0.05), %RLV (r=-0.502; p<0.01) and %TRLCAM (r= -0.474; p<0.01). On contrary, %RLDSH (r= 0.391; p<0.05) and %TRLCD (r= 0.404; p<0.05) had a significant and positive correlation with root hair number.

Discussion

The present study revealed the existence of fungal endophyte association in all the examined exotic medicinal plant species under cultivation. To the best of our knowledge, the endophytic fungal association is reported for the first time in six (*A. ferox*, *A. mearnsii*, *C. cyminum*, *H. hookerianium*, *J. maritima* and *M. alternifolia*) of the ten medicinal plant species examined. In this study, all the studied medicinal plants had a symbiotic association with AM fungi and eight plant species had the co-existence of DSE fungi. Similarly, previous studies have also reported the root fungal endophyte association in medicinal plant species (Muthukumar *et al.* 2006, Zubek & Błaszkowski 2009, Sharma & Jha 2012, Piszczek *et al.* 2019). The occurrence of AM fungi in all the studied plants may be due to the fact that almost all the vascular plants are naturally colonized by AM fungi (Wang & Qui 2006, Brundrett 2009).

All the studied plant species in the current study exhibited the intermediate type of AM morphology. The occurrence of intermediate AM morphology in *A. millifolium* in this study contradicts the observations of Zubek *et al.* (2011) and Safari

Sinegani & Yeganeh (2017) who reported typical *Arum*- or *Paris*-type of AM colonization in *A. millifolium*. The *Paris*-type AM is mostly prevalent in the plants or sites characterized by low-nutrient content and highly stressed habitat in the natural ecosystem (Ahulu *et al.* 2006). Moreover, most of the plant species of Fabaceae, Asteraceae, Lamiaceae, and Asclepidiaceae have typical *Arum*- or *Paris*-type morphology (Muthukumar *et al.* 2006, Zubek & Błaszkowski 2009, Safari Sinegani & Yeganeh 2017). The difference in the AM morphological types in this study with earlier studies could be attributed to the variations in environmental factors including soil moisture, light intensity, and temperature at different regions which affect the patterns of AM colonization in plant roots (Yamato 2004). Also, the AM morphology is affected by the characteristics of the host plant (Tominga *et al.* 2020). Host plant with sporadic intercellular air spaces generally has the intermediate type of AM morphology (Smith & Smith 1997).

The significant differences in the percentage of root length with different AM fungal structures could be due to the variations in their soil P level (Rożek *et al.* 2019). Though, the mycorrhizal status of four of the medicinal plant species examined in the current study (*A. millifolium*, *D. purpurea*, *R. officinalis* and *S. nigra*) has already been reported (Wang & Qiu 2006), the extent AM fungal structures occurring in roots of those plants have not been quantified. Moreover, most of the plants reviewed by Wang & Qiu (2006) have been examined from their native region in the natural ecosystem (Gucwa-Przepióra *et al.* 2016). Nevertheless, the medicinal plants analyzed in this study were exotic and are under cultivation.

To the best of our knowledge, FRE is reported in the roots of *D. purpurea* apart from AM and DSE association for the first time. These endophytic fungi have a very small hyphal diameter (<2.0μm), forms arbuscules and, often co-occur with AM fungi (Hoysted *et al.* 2019). The FRE is assumed to improve the P uptake in plants; however, their function along with AM fungi is not largely understood (Orchard *et al.* 2017). Moreover, the maximum %RLAR and %RLAC levels observed in the *D. purpurea* roots could be attributed to the presence of both FRE and AM fungi. In addition, the highest number of AM fungal spores was also associated with the soils of this plant species.

The occurrence of DSE fungi recorded in the current study is similar to those reported in other medicinal plants (Zubek *et al.* 2012, Piszczek *et al.* 2019). Also, there was a significant variation in the DSE fungal variables among the studied medicinal plants. The regularly septate melanized hyphae of the DSE fungi colonizing the cortical cells were more prevalent than the other structures like microsclerotia or moniliform hyphae in the roots examined. This is similar to the observations in other studies where DSE fungal hyphae were reported to be the major DSE fungal structure in medicinal plants (Zubek & Błaszkowski 2009). Although AM symbiosis in *A. millifolium* was reported in a previous study (Zubek *et al.* 2011), this study revealed the co-occurrence of DSE fungal association in this species. Previous studies have shown that DSE fungal symbiosis could stimulate metabolites production in medicinal plants (Zhu *et al.* 2015).

The co-occurrence of AM and DSE fungi in the eight plant species of the current study is in line with observations of previous studies where the dynamic nature of these root colonizing fungal communities was reported (Muthukumar *et al.* 2006, Zubek & Błaszkowski 2009, Piszczek *et al.* 2019). The overall higher AM fungal colonization than the DSE fungal colonization in this study is in line with the findings of earlier studies where such an observation was made. For example, Zubek *et al.* (2011) reported AM colonization levels of 2.5%–77.9% compared to the 2.4%–47.3% of DSE fungal colonization levels in medicinal plants growing in the Jagiellonian University Botanical garden, Kraków, Poland. Similarly, Piszczek *et al.* (2019) also reported low levels of DSE fungal colonization (1.11%–21.97%) compared to AM fungal colonization (29.3%–100%) in medicinal plants cultivated in Poland. In this study, *S. nigra* with the highest %TRLCAM had the lowest %TRLCD and *R. officinalis* exhibited maximum %TRLCD but minimum total root length colonization by AM fungi. Some authors speculate that DSE fungi may play the function of AM fungi in its absence or rare occurrence in the roots as reported in some of the medicinal plants (Zubek & Błaszkowski 2009). Nevertheless, DSE symbiosis could not be considered as a substitute for AM fungi as DSE fungi do not form special interfaces like AM fungi for resource transfer. Though both these fungal associations aid in plant growth promotion and nutrient uptake (Rouphael

et al. 2015, He *et al.* 2019), but their role in the establishment of exotic/introduced plant species outside their native range needs an experimental approach to understand the functions of these fungi in the survival of these exotic medicinal plant species.

Root characteristics including root hair morphology play an important role in mycorrhizal dependency and colonization levels in plants (Maherali 2014, Zou *et al.* 2019). In the present study, root hair number, length and width significantly varied among the studied medicinal plant species. Muthukumar *et al.* (2004) suggested that plants growing in nutrient rich soils often consist of copious root hairs and low mycorrhizal colonization. Similarly, in the current study, plants with higher AM colonization levels had less root hairs. However, some of the studies showed that root hair traits (root hair number, length, and diameter) are independent of mycorrhizal colonization in plant roots (Novero *et al.* 2008). This is evidenced by the existence of a significant negative correlation between the root hair number and root length containing most of the AM fungal structures. This is in accordance with the observations of previous studies in citrus and tea plants (Wu *et al.* 2016, Shao *et al.* 2018). This negative correlation observed in this study could be due to the presence of abundant root hairs that could help in direct P acquisition by plants in P deficient soil. However, the positive correlations between DSE fungal variables (%DSH and %TRLCD) and root hair number could be due to the penetration of DSE hyphae mostly through the root hairs (Vergara *et al.* 2019).

The number of AM fungal spores reported in the present study (96 to 1050/50 g soil) is higher than the spore numbers from various vegetation types in Nilgiris Biosphere Reserve, southern Western Ghats (Lakshmipathy *et al.* 2012). Moreover, Wang & Jiang (2015) also reported a high prevalence of AM fungal spores (135-1430/50 g soil) in medicinal plants growing in southeast China. In contrast to the observations of the present study, Rajkumar *et al.* (2012) reported only 10-260 AM spores/50g of soil from different medicinal plants growing in Western Ghats region of Karnataka. The variation in the AM spore number could be due to the variations in edaphic factors, climatic conditions, vegetation, and host plant, intraspecific competition among fungi and the environmental factors, and sporulation ability (Song *et al.* 2019, Silva-Flores *et al.* 2019). The AM spore number was related to colonization levels in certain exotic medicinal plants. For example, the highest number of AM fungal spores was observed in *D. purpurea* which also had higher AM colonization levels. This observation was further supported by the fact that spore numbers were positively correlated with %RLAC in the present study.

In the present study, the soils of all the medicinal plant species were slightly acidic to neutral or slightly alkaline soil. It has been suggested that acidic soil (pH>4.5) decreases the number of AM spores and affects the germination of AM fungi (Isobe *et al.* 2007, Kohout *et al.* 2015). Moreover, a large number of AM fungal spores prevail in neutral soil pH (6.5) when compared to soils with pH less than 5.5 (Jamiołkowska *et al.* 2018). This supports the observations of this study, where slight acidic to marginally alkaline soil harboured a greater number of AM fungal spores.

Though the number of AM spores occurring in the soils was large, but these belonged to only six AM fungal morphotypes. Of these, spores of *Acaulospora* species was found in all the soil samples analyzed. Similarly, Zubek *et al.* (2012) also reported six AM fungal spore morphotypes from soils of 25 medicinal plants. In contrast, Radhika & Rodrigues (2010) and Rajkumar *et al.* (2012) reported more than 40 AM fungal spore morphotypes associated with different medicinal plants of southern Western Ghats, India. Similar to the observations of the current study, Wang & Jiang (2015) also reported the presence of *Acaulospora*, *Gigaspora*, *Funneliformis*, and *Rhizophagus* species in soils associated with the medicinal plant species. The dominance of *Acaulospora* species may be attributed to its high adaption to different soil conditions, endures environmental stresses (Chagnon *et al.* 2013, Vieira *et al.* 2020), and also it is assumed that species of *Acaulospora* could rapidly sporulate (Wang & Jiang 2015). The AM fungal diversity is affected by several factors including seasonal variations; soil characteristics host plant type, vegetation, and environmental factors (Bauer *et al.* 2020). Moreover, earlier studies have reported low AM fungal spore diversity at higher elevation or altitudes which could be the possible reason for less AM fungal species richness in the current study (Egan *et al.* 2017, Shi *et al.* 2019). The diversity of AM fungi associated with these exotic medicinal plant

species could be formulated into bioinoculums which would be helpful in increasing the growth and productivity of these medicinal plants.

Conclusion

The present study clearly indicated that the exotic medicinal plant species cultivated in the Nilgiris could form a strong symbiotic relationship with indigenous endophytic fungi. All the exotic medicinal plants harboured intermediate type of AM morphology. Among the different exotic plant species examined, *D. purpurea* was simultaneously colonized by AM, DSE, and FRE fungi. Moreover, root hair dimensions had a significant influence on AM and DSE fungal association in these cultivated exotic medicinal plants. It is clear from the study that native AM/DSE fungal communities help in the successful establishment and improve plant productivity of these exotic medicinal plant species. Further, experimental studies are required to investigate the influence of AM and DSE fungi association in phytochemical enhancement activity in these medicinal plants.

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