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Short Communication

Colonization of intraradical structures of arbuscular mycorrhizal fungi by dark septate endophytic fungi

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Abstract

Extraradical spores of arbuscular mycorrhizal (AM) fungi are often parasitized by a wide range of soil microorganisms. However, reports on the parasitization of intraradical structures of AM fungi are very rare. While examining AM colonization in roots of *Cuminum cyminum* and *Sambucus nigra* growing in the medicinal plants garden of the Centre of Medicinal Plants Research in Homeopathy of the Nilgiris, we found that AM fungal hyphal coils, arbusculate coils, vesicles, and intraradical spores are colonized by dark septate endophytic (DSE) fungi which were co-occurring with AM fungi. The AM fungal hyphae were significantly wider than the DSE fungi. Colonization of different AM fungal structures significantly varied between the two plant species. Moreover, the percentage of colonized AM fungal structures was linearly correlated to the abundance of these structures. The colonization of the intraradical structures of AM fungi is illustrated and the significance of this phenomenon needs further elucidation.

Key words: arbusculate coils, hyphal coils, intraradical spores, microsclerotia, vesicles.

Resumen

Las esporas extrarradicales de hongos micorrícicos arbusculares (AM) a menudo son parasitados por una amplia gama de microorganismos del suelo. Sin embargo, los informes sobre la parasitación de estructuras intrarradicales de hongos AM son muy raros. Al examinar la colonización de AM en raíces de *Cuminum cyminum* y *Sambucus nigra* que crecen en el jardín de plantas medicinales del Centro de Investigación de Plantas Medicinales en Homeopatía de Nilgiris, encontramos bobinas de hifas de hongos AM, bobinas arbusculadas, vesículas y esporas intrarradicales colonizadas por hongos endófitos septados oscuros (DSE) que coexistían con hongos AM. Las hifas de hongos AM eran significativamente más anchas que los hongos DSE. La colonización de diferentes estructuras fúngicas AM varió significativamente entre las dos especies de plantas. Además, el porcentaje de estructuras fúngicas AM colonizadas se correlacionó linealmente con la abundancia de estas estructuras. Se ilustra la colonización de las estructuras intrarradicales de hongos AM y la importancia de este fenómeno necesita mayor aclaración.

Palabras clave: espirales arbusculadas, espirales de hifas, esporas intrarradicales, microesclerocios, vesículas.

Arbuscular mycorrhizal (AM) fungi belonging to the subphylum Glomeromycotina of the phylum Mucromycota are soil-borne and are an important component of many soil processes (Spatafora *et al*. 2016). These fungi associate with roots of a wide range of plant species growing in natural and agroecosystems. The AM symbiosis enables plants to thrive in nutrient and water-stressed soils and also protects plants from various biological stresses (Smith & Read 2008). Further, AM fungi alter the soil structure through the secretion of glycoproteins like glomalin. The common mycelial network of AM fungi connecting different root systems act as highways for the exchange of resources between plants in a plant community (Smith & Read 2008). A wide group of ascomycetous fungi with melanized or hyaline hyphae that are regularly septate and designated as dark septate endophytic (DSE) fungi

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also colonize plant roots. These fungi often cooccur with AM fungi and are shown to improve the growth and fitness of plants in different ecosystems (He *et al.* 2019).

In plant roots, AM fungi produce different types of structures like linear hyphae that are inter and/or intracellular, hyphal coils, arbuscules, arbusculate coils, and vesicles (Smith & Read 2008). Moreover, the extraradical AM fungal hyphae extending from the roots into the rhizosphere and the bulk soil acquire soil resources and can also act as propagules and bear spores. Purin & Rilling (2008) suggested that AM fungal spores could be a potential target for parasitic attacks as they are filled with energy-rich lipids. This could be easily observed in field explorations where most of the field-collected AM fungal spores are often parasitized by soil microorganisms or exist as spore cases (Lee & Koske 1994). If this is true then the intraradical structures like the vesicles and the hyphae could also be a potential target for parasites since they contain more resources than the root tissues (Rufyikiri *et al.* 2003). Besides, several AM fungi can also sporulate within plant roots. Although Purin & Rilling (2008) pointed out that studies have not addressed the parasitism of intraradical structures of AM fungi in roots there are a couple of observations from the fossil records (Harper *et al.* 2017; Krings & Harper 2018) and one field observation (Mandyam & Jumpponen 2008) where intraradical structures of AM fungi were shown to be colonized by septate fungi. Nonetheless, Harper *et al.* (2017) also noted that the reports of intraradical AM fungal vesicles or hyphae of extant plant species colonized by other fungi are extremely rare. The study (Godfrey 1957) cited by Harper *et al.* (2017) as an example for the colonization of intraradical structures of AM fungi pertains exclusively to the parasitization of soil-borne sporocarps and spores of AM fungi.

During a study on the incidence and abundance of AM symbiosis in exotic medicinal plants cultivated at the Centre of Medicinal Plants Research in Homeopathy (CMPRH), Emerald, the Nilgiris (Bharathy *et al.* 2021) we noticed that the AM fungal structures in roots of medicinal plants *Cuminum cyminum* L. (Apiaceae) and *Sambucus nigra* L. (Adoxaceae) were colonized by DSE fungi. Of the two plant species, *C. cyminum* had intermediate-3 type AM morphology characterized by inter and intracellular linear hyphae, hyphal coils and arbuscules, while *S. nigra* had *Arum-Paris* type morphology with inter and intracellular linear hyphae, hyphal and arbusculate coils, and vesicles (Bharathy *et al.* 2021). As this phenomenon was reported only once in the extant AM symbiosis before, we documented the incidence and morphology of DSE fungal colonization of AM fungal structures associated with roots of medicinal plants cultivated at CMPRH. In addition, we also assessed if the extent of colonization of AM fungal structures by DSE fungal structure was related to the abundance of these structures.

Healthy fine feeder roots of *C. cyminum* and *S. nigra* were collected from the medicinal plants garden of CMPRH, Emerald, The Nilgiris (11°19'30.30"N and 76°37'1.54"E; 1995 m a.s.l.), Tamilnadu, India during December 2019 for the examination of endophytic fungal association. The annual rainfall of the Nilgiris is 192 cm and the average temperature ranges from 10–25 °C during summer and 0–20 °C during winter. The feeder roots were washed, preserved in formalin-acetic acid-alcohol solution (5 ml: 5 ml: 90 ml), and transported to the laboratory for processing. The fixed roots were washed thoroughly to remove the traces of the fixative, cut into 1-cm long bits, cleared (2.5% KOH), acidified (5N HCl), and stained with trypan blue according to Koske & Gemma (1989). Squashes of stained roots were prepared and observed under an Olympus BX51 compound microscope (Japan) for the presence of fungal structures. The percentage of root length containing different AM fungal structures and these fungal structures colonized by DSE fungi were estimated according to the magnified intersection method (McGonigle *et al.* 1990). The width of the AM and DSE fungal hyphae were measured using a calibrated ocular scale (n = 50). The microscopic images were captured with a ProgRes CT3 camera (Jenoptik, Germany) attached to the microscope. The significance of variation between the DSE uncolonized and colonized AM fungal structures were determined using paired t-test after testing the data for homogeneity (Levene's test for equality of variance). The box plot analysis was used to compare the hyphal width of AM and DSE fungi in plant roots. The data on DSE colonized and uncolonized AM fungal variables were subjected to regression analysis to determine the relationship between the variables. Statistical analysis was performed using SPSS (version 9.0). Values are presented as mean ± standard error.

Roots of *C. cyminum* and *S. nigra* were colonized by AM and DSE fungi (Fig. 1). The hyphae of DSE fungi were found in the arbusculate coils (Fig. 1a), hyphal coils (Fig. 1c-e), and vesicles (Fig. 1f-g) of AM fungi. However, DSE fungi did

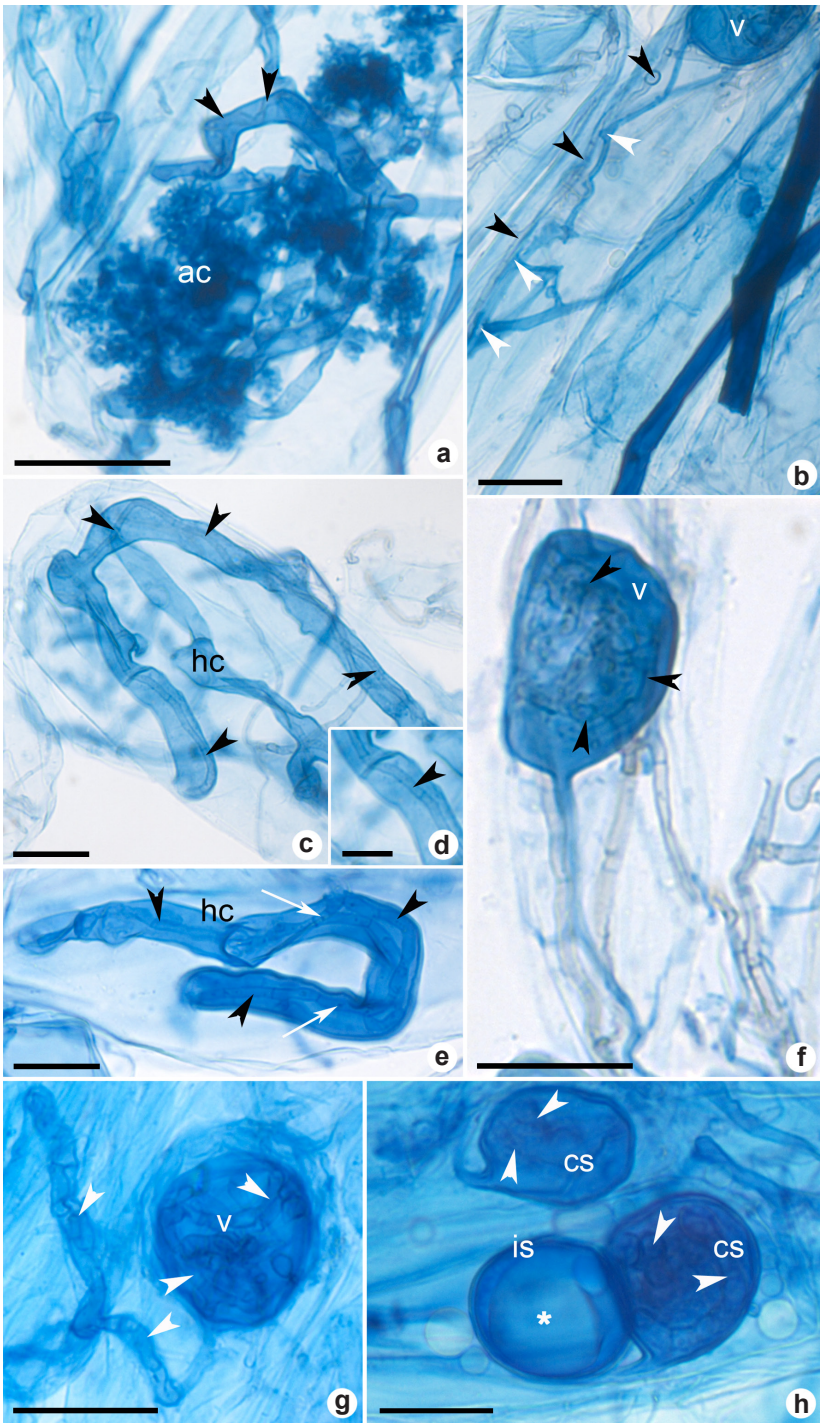

Figure 1 – a-h. Colonization of intraradical structures of arbuscular mycorrhizal (AM) fungi by dark septate endophyte (DSE) fungi in *Cuminum cyminum* (a, g) and *Sambucus nigra* (b-f, h) – a. arbusculate coil (ac) in root of *C. cyminum* colonized by DSE fungi (black arrow heads); b. intracellular hyphae of AM (white arrow heads) and DSE (black arrow heads) fungi and vesicle in root of *S. nigra*; c-e. colonization of AM fungal hyphal coil (hc) by DSE fungi (black arrow heads) containing uninucleate (white arrows) cells in *S. nigra*; f. hyphae of DSE fungi (black arrow heads) in vesicle (v) of *S. nigra*; g. coiled hyphae of septate endophytic fungi (white arrow heads) in vesicle (v) and hyphae of *C. cyminum*; h. coiled hyphae of DSE fungi (white arrow heads) in colonized spores (cs) and intact intraradical spore (is) of *S. nigra*; asterick indicate the oil globules in the intact spore. Scale bars: a-c, e-h = 30 µm; d = 10 µm.

not invade the *Arum*-type arbuscular structures of *C. cyminum*. In the portion of roots where both AM and DSE fungi were present, the hyphae of the later grew in close oppression before entering the structures of the former (Fig. 1b). No swellings or appressorium of DSE fungi were evident at the point of entry of AM fungal structures. The DSE fungal hyphae were unbranched or branched and spread within the AM fungal hyphae (Fig. 1c-d). The cells of the DSE fungal hyphae were uninucleate (Fig. 1e). The DSE fungi also formed tight coils or aggregates in the AM hyphae, and vesicles (Fig. 1f-g) of AM fungi. The hyphae of DSE fungi in the present study closely appressed the AM fungal hyphae, and no visual disruption, coiling of the host hyphae by the DSE fungal hyphae or physical damage were evident as exhibited by a more aggressive necrotrophic parasite. The unidentified intraradical spores in roots of *S. nigra* were also colonized by DSE fungi (Fig. 1h). The hyphae of AM fungi (7.20 ± 0.17 µm) was significantly wider ($t = 16.898$, $P < 0.001$) than the DSE fungi (3.30 ± 0.16 µm) (Fig. 2). The extent of colonization of AM fungal structures like hyphal coils ($t = -4.840$; $P < 0.05$), arbusculate coils ($t = -11.604$; $P < 0.01$), and vesicles ($t = -4.330$; $P < 0.05$) by DSE fungi was significantly higher in roots of *S. nigra* than in *C. cyminum* (Fig. 3). The root length with colonized AM fungal structures was linearly related to the total root length containing these structures (Fig. 4).

The presence of AM and DSE fungi in the roots of *C. cyminum* and *S. nigra* is in accordance with studies where the colonization of these plant roots by endophytic fungi has been reported (Walter *et al.* 2016; Zamani *et al.* 2019; Bharathy *et al.* 2021). Nevertheless, the colonization of intraradical structures of AM fungi by DSE fungi to our knowledge is the second report on the colonization of these structures by any type of fungi in extant plant species. In the present study diverse intraradical AM fungal structures like hyphal and arbusculate coils, vesicles, and spores were colonized by DSE fungal structures. This extends the observations of Mandyam & Jumpponen (2008) where melanized septate fungal were present only in the hyphal coils of AM fungi in plants roots growing in an undisturbed native tallgrass prairie, in eastern Kansas of USA. Moreover, the presence of DSE fungi in AM vesicles furthers the observations of this phenomenon in fossil specimens from Early Devonian Rhynie Chert to modern AM symbiosis (Harper *et al.* 2017; Krings & Harper 2018). Nevertheless, the structures produced by the colonizing DSE fungi within AM fungal structures in the present study were entirely different from those reported in the fossil specimens. For example, we did not observe the various types of colonizing structures like the spheroidal propagules of different sizes developed at the tip of branched hyphae or within a tenous mycelium, and thin-walled structures up to 10 µm in diameter

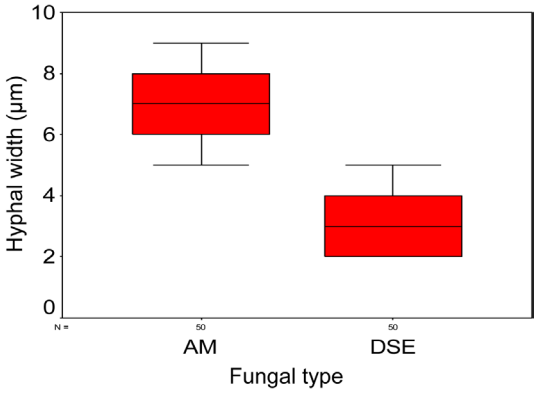

Figure 2 – Box plot comparing the hyphal width of arbuscular mycorrhizal (AM) and dark septate endophytic (DSE) fungi.

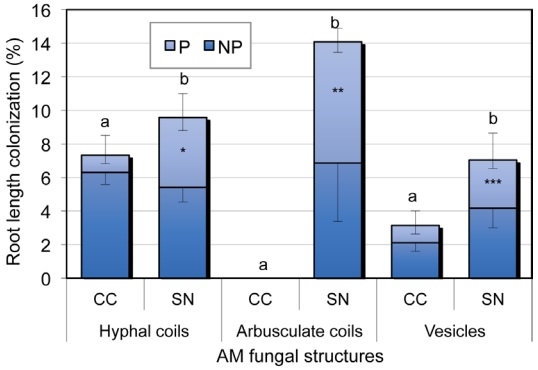

Figure 3 – Percentage of root length containing different noncolonized (NP) and colonized (P) arbuscular mycorrhizal (AM) fungal structures in *Cuminum cyminum* (CC) and *Sambucus nigra* (SN). *, **, ***, ns: significant at $P < 0.05$; $P < 0.01$, $P < 0.001$ and non-significant respectively. Minus error bars for P and NP indicate 1 standard error for the variable and the plus error bars indicate 1 standard error for the total root length containing the AM fungal structure (colonized + noncolonized). Bars bearing different letter for an AM fungal structure are significantly different ($P < 0.05$) according to students paired t-test.

in inflated hyphae of non-AM fungi colonizing AM fungal structures as observed by Harper *et al.* (2017) in any of the specimens examined. These variations could be due to the different fungal communities involved in the colonization of AM fungal structures. Further, the observations of the present and other studies confirm Purin & Rilling (2008) speculation that root tissues may contain specialized microorganisms that could parasitize AM fungal hyphae. As the concentration of carbon and other elements are higher in the intraradical than the extraradical structures of AM fungi, it is not surprising for these intraradical structures to be specific targets for parasitic attack (Parihar *et al.* 2020).

Parasitic fungi are known to display a wide range of host-parasite interfaces like close contact to the total disruption of the hyphae as observed in the present study (Jeffries 1995). Penetration of the AM fungal structures by DSE fungi without the formation of an appressorium or hyphal swelling as observed in the present study is also reported in the colonization of plant-pathogen *Fusarium solani* hyphae by the antagonistic *Trichoderma harzianum* (Melo & Faull 2000). Instead, the complete occupation of the fungal storage structures like the vesicles and spores indicates that the DSE fungi could reduce the fitness of the AM fungi through the draining of the resources. In addition, colonization of AM fungal hyphal coils or arbusculate coils by DSE fungi could reduce the exchange of carbon and nutrients between AM fungi and plants thereby reducing the symbiotic efficiency (Giesemann *et al.* 2021). However, the nature of the relationship between these fungal groups needs to be elucidated through an experimental approach. Another interesting aspect that was noticed in the present study was that the AM fungal structures colonized were in fact living structures. This is evidenced by the presence of intact arbusculate coils in *C. cyminum*.

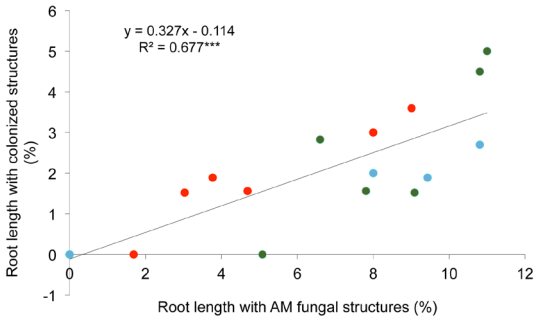

Figure 4 – Relationship between root length with different arbuscular mycorrhizal (AM) fungal structures and root length with colonized structures. Blue circles = arbusculate coils; Green circles = hyphal coils; Red circles = vesicles.

Most studies on the colonization of AM fungi by parasitic microorganisms are restricted to the soil-borne spores as they were considered to be the chief perennating structures in the absence of host roots. Limited studies in this line indicate that parasitic fungi isolated from the parasitized AM spores or soils, were able to colonize healthy and dead spores of different AM fungi (Purin & Rilling 2008). The observations of the present study and those of Mandyam & Jumpponen (2008) shows that the intraradical structures of AM fungi are also vulnerable to colonization by septate endophytic like the extraradical structures. The positive linear relationship between the proportion of colonized and uncolonized AM fungal structures are also in line with the observations of Mandyam & Jumpponen (2008) where the infected AM fungal hyphal coils in roots were correlated to the overall abundance of the intact coils. The linear increase in the proportion of colonized and uncolonized AM fungal structures suggest that colonization of AM fungal structures increases with the abundance of these structures. It is well known that the parasitization of soil-borne spores reduces the propagules of AM fungi in the soil (Purin & Rilling 2008). For instance, *T. harzianum* colonized spores of *Rhizophagus intraradices* (= *Glomus intraradices*) by producing spore wall degrading chitinolytic enzymes, proliferated abundantly in the spores and hyphae and emerged through moribound AM fungal structures (Rousseau *et al.* 1996). The negative influence of antagonistic fungi like *Trichoderma pseudokoningii* on AM fungi is exemplified in the studies, where these fungi were shown to reduce spore germination and mycorrhizal colonization in different plant species (*e.g.*, Martínez *et al.* 2004). Like spores, mycorrhizal roots also act as propagules of AM fungi, and it is not clear if the colonization of intraradical fungal structures could affect the capacity of mycorrhizal roots acting as propagules of AM fungi. Further experimental studies in this line are necessary to ascertain if the colonization of DSE fungi actually reduces the fitness of the AM symbiosis or the capability of AM fungal roots to act as propagules. This is important both from the ecological and application point of view as both these fungi coexist in the roots of plants in the studied ecosystem.

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