Two complementary forest-originated *Gigaspora* spp. shifted shoot-to-root ratio for growth improvement in *Cryptomeria japonica* seedlings

Akotchiffor Kevin Geoffroy DJOTAN [*1,3], Yosuke MATSUDA [1,4], Norihisa MATSUSHITA [*2,5], Kenji FUKUDA [2,6]

[1]Mie University, Graduate School of Bioresources, [2]University of Tokyo, Graduate School of Agricultural and Life Sciences

ORCID: [3]https://orcid.org/0000-0002-3726-9826, [4]https://orcid.org/0000-0002-7001-3101, [5]https://orcid.org/0000-0003-3281-8846, [6]https://orcid.org/0000-0002-9980-3107

[*]*Corresponding author, E-mail: geoffroydjotan@yahoo.fr, nmatsushita@g.ecc.u-tokyo.ac.jp*

Abstract

Arbuscular mycorrhizal fungi (AMF) are potential bioinoculants to grow healthy plants in healthy soils. However, most available AMF isolates originated from nonforest environments and AMF contributions to tree seedlings remain unclear. Here, our objective was to clarify the functions of forest-inhabiting AMF in tree seedling production. To achieve this, we first identified two *Gigaspora* AMF (LFB-4 and LFB-A1) previously isolated from *Cryptomeria japonica* (Cupressaceae) trees and characterized them using morphological and molecular analyzes. We then carried out inoculation assays to clarify the cohabitation and functions of LFB-4 and LFB-A1 in *C. japonica* seedlings. We identified LFB-4 and LFB-A1 as *Gigaspora rosea* and *Gigaspora margarita*, respectively. They produced spores inside host root cells, simultaneously developed multiple germ tubes during germination, and showed presymbiotic sporulation. LFB-A1 produces spores as large as 500 µm in diameter. LFB-4 and LFB-A1 were both beneficial AMF with different functions and complementary effects on the growth of *C. japonica* seedlings. In their cohabitation, while LFB-4 boosted water uptake and height growth, LFB-A1 improved biomass production. Together, they encouraged carbon release into the soil and increased the shoot-to-root biomass ratio for faster seedling growth, although without increasing root colonization. We concluded that, despite erratic root colonization, *G. rosea* and *G. margarita* worked synergistically to improve the growth of *C. japonica* seedlings by modulating root development, likely for carbon acquisition. Root colonization assessed by microscopy or metabarcoding may overlook AMF functions.

Keywords: Forest AMF, Inoculation assays, *Gigaspora rosea*, *Gigaspora margarita*, AMF cohabitation

Introduction

Arbuscular mycorrhizal fungi (AMF) are beneficial microorganisms that live in roots and soils simultaneously. They play crucial roles in sustainable agriculture and forestry, with major effects on plant growth by aiding their nitrogen and phosphorus uptake (Smith & Read, 2008). Meanwhile, little is known about their biology, diversity, ecology and how their functions are realized (Hodkinson & Murphy, 2019; Kuila & Ghosh, 2022; Young, 2012). Obviously, insufficient sampling and inadequate isolation of AMF from unexplored ecosystems limit our knowledge of the functions of the plant – AMF association (Bullington et al., 2021). Therefore, it is necessary to collect, characterize, and investigate AMF that inhabit forest ecosystems, where plant-AMF interactions are still poorly understood.

AMF diversity promotes the acquisition of phosphorus in plant communities and reduces carbon costs per unit of phosphorus (Weber et al., 2025). However, a meta-analysis revealed that commercial mycorrhizal inoculants sourced globally do not promote the growth of inoculated plants (Koziol et al., 2024). These inconsistent results could be related to different currently unresolved levels of compatibility and interactions between AMF species, other microbes, and host plants during their co-habitation. In this cohabitation, AMF can (1) be functionally complementary with positive effects on each other (Bunn et al., 2024; Jansa et al., 2008; Koide, 2000; Steidinger, 2024), (2) interfere with each other by competing over resources or space within a root system (Engelmoer et al., 2014; Thonar et al., 2014), or (3) be neutral neighbors without interference and complementarity. Therefore, the AMF association functions are a partially host plant-mediated mutualism-to-parasitism continuum where positive, neutral, and negative outcomes are possible (Jansa et al., 2008; Johnson et al., 1997; Merckx et al., 2024). However, it remains unclear whether and how these three types of cohabitation explain and align with host and symbiont traits in AMF associations. Therefore, the mechanisms by which AMF, within or between species, interfere or complement each other to affect plant communities, ecosystems, and biomes remain unclear.

Cryptomeria japonica D. Don (Cupressaceae) is the most planted tree species in Japan. Although its roots and surrounding soils have been intensively investigated in plantations in various environments and seasons in Japan (Djotan et al., 2022, 2023, 2024a, 2024b, 2025; Matsuda et al., 2021; Yustikasari et al., 2025), molecular detection of *Gigaspora* spp. in and under trees is very rare; for example, 0.21% of total AMF sequences from 369 samples (Djotan, 2024). These figures question the association of

Gigaspora spp. with the *C. japonica* tree. Here, our objective was to investigate the functions of two AMFs of *Gigaspora* (LFB-4 and LFB-A1) previously isolated from *C. japonica* to the seedlings of the tree and to assess how their co-habitation drives these functions. Therefore, we first identified LFB-4 and LFB-A1 and characterized them using morphological and molecular analyzes. Then, their functions were elucidated in *C. japonica* seedlings using inoculation assays. Due to the less frequent detection and very small relative abundances of *Gigaspora* DNA in roots of *C. japonica* and surrounding soils, we hypothesized that LFB-4 and LFB-A1 are not beneficial to the plant. Additionally, because *Gigaspora* spp. are thought to be edaphophilic, they are assumed to have fewer hyphae in roots and more in soils (Hart & Reader, 2002) and that AMF root colonization increases with taxon richness (Verbruggen et al., 2013), we expected that inoculation with the two *Gigaspora* isolates would increase root colonization.

Materials and Methods

Replication Statement

Scale of inference	Scale at which the factor of interest is applied	Number of replicates at the appropriate scale
Species	Species	2 arbuscular mycorrhizal fungal species (AMF) used as inoculants in manipulated inoculation assays
Species	Species	2 host plant species used as subjects in manipulated inoculation assays
Plant (mycorrhizal responses)	Pot	Treatment of AMF inoculation on individual plants: Plant species 1: 4 replicates of Control, 12 replicates of Inoculation (4 replicates of each treatment with AMF1, AMF2 and AMF1 + AMF2) Plant species 2: 10 replicates of Control, 30 replicates of Inoculation (10 replicates of each treatment with AMF1, AMF2 and AMF1 + AMF2)
Soil (mycorrhizal responses)	Pot	Treatment of AMF inoculation in individual plant cultivation pots: Pots with plant species 1: 5 replicates of Control, 15 replicates of Inoculation (5 replicates of each treatment with AMF1, AMF2 and AMF1 + AMF2); Pots with plant species 2: 10 replicates of Control, 30 replicates of Inoculation (10 replicates of each treatment with AMF1, AMF2, and AMF1 + AMF2)

Biological material and AMF isolate identification

Two *Gigaspora* isolates (MAFF 520098 and MAFF 520099; LFB-4 and LFB-A1 hereafter) collected from a *C. japonica* forest, *G. rosea* T.H. Nicolson & N.C. Schenck (MAFF 520062), *G. margarita* W.N. Becker & I.R. Hall (MAFF 520052), non-*Gigaspora* AMF taxa F-1 (*Acaulospora longula* Spain & N.C. Schenck, MAFF 520060), TSU-2 (*Rhizophagus clarus*, (T.H. Nicolson & N.C. Schenck) C. Walker & A. Schüßler, MAFF 520089), and YC-1 (*Paraglomus occultum* (C. Walker) J.B. Morton & D. Redecker, MAFF 520091) obtained from the National Agricultural Research Organization (NARO) in Japan were used in this study (see Supplementary Materials and Methods, SMM1). Molecular and morphological analyzes were used to identify LFB-4 and LFB-A1 (see SMM2). We further observed, monitored, and described spore germination and presymbiotic mycelium growth by incubating them in 1.5% gellan gum medium at 25 ° C in the dark in an incubator (MIR-554; Sanyo, Osaka, Japan), following Djotan (2024).

Inoculation assays

The seeds of *C. japonica* (Ichishi cultivar, Mie Prefecture, Japan) were surface sterilized and grown under axenic conditions to test the responses of its seedlings to inoculation with LFB-4 and LFB-A1 (see SMM3). On 17 June 2024, 2 cm tall seedlings or more were inoculated with water (control), LFB-4, LFB-A1 or both AMF and cultured in a plant room at 20 ° C with a light cycle of 12 h per day (8:00–22:00; light intensity of 65 µmol m^{-2} s^{-1}). Throughout the plant-AMF co-culture period spanning 204 days, we monitored seedling growth by measuring plant height. Other sets of *C. japonica* seedlings inoculated with and without the isolates were grown separately for 10 months to further evaluate the long-term response of the host to the inoculation. To evaluate the contribution of forest-inhabiting AMF to crop plants in agricultural systems, we also tested LFB-4 and LFB-A1 in carrot plants (*Daucus carota* L.) in inoculation assays spanning 140 days of cultivation (SMM3). Briefly, each of the AMF treatments described above was applied to 10 pots containing 10 *D. carota* plants each. In different assays carried out under the same conditions as described earlier, *C. japonica* seedlings were inoculated with F-1, TSU-2 and YC-1 (n = 10 per treatment) and grown for 88 days to compare the responses of *C. japonica* to these non-*Gigaspora* AMF relative to *Gigaspora* isolates LFB-4 and LFB-A1.

Harvest and sample processing

After harvesting *C. japonica* seedlings on 17 March 2025, we scanned the whole plant (Online Resource 1) and then measured the fresh weight of the plant (FW_{plant}), the height of the plant (H), the fresh weight of the shoot (FW_{shoot}), the fresh weight of the root (FW_{root}) and the dry weight of the shoot (DW_{shoot}). Total root length (L_{root}) was measured using WinRHIZO (WinRHIZO, 2025). The shoots were dried in a drying sterilizer (SH600; Yamato Scientific, Tokyo, Japan) at 65 ° C for 18 days. For DNA extraction, half of the root samples were kept frozen until lyophilization using a freeze-dryer (FDU-2000; EYELA, Tokyo, Japan) and the lyophilized samples were milled using a smasher (MicroSmash^{TM} MS100; TOMY, Tokyo, Japan). We added 10% KOH to the other half of the root samples for the morphological analysis of root colonization. Growth media (soils) were air dried at room temperature for pH and C/N measurements using a pH meter (MP220; Mettler-Toledo, Greifensee, Switzerland) and element analyzer (vario EL cube element; DKSH Japan, Tokyo, Japan), respectively. To compare the mycorrhizal growth response of *C. japonica* to F-1, TSU-2, and YC-1 relative to the forest-originating *Gigaspora* isolates LFB-4 and LFB-A1, H was measured every time in both assays.

Upon the harvest of *D. carota* plants, the most vigorous individual was selected in each replicate pot, resulting in 10 *D. carota* plants per treatment (40 plants in total). We measured the H, FW_{shoot} and fresh weight of the taproot ($FW_{taproot}$, consumable carrot) of the selected *D. carota* plants.

Analysis of mycorrhization and root colonization

Mycorrhization and root colonization were evaluated using morphological and molecular analyzes. We counted the number of spores and checked for the presence of AMF hyphae in 30 g of air-dried experimental soils passed through a 1 mm mesh by wet sieving and decanting (Brundrett et al., 1996). Observations and counts were performed using a stereoscopic microscope (SZX16; Olympus, Tokyo, Japan). Root samples previously stored in 10% KOH were stained with Trypan blue in lactoglycerol (Phillips & Hayman, 1970) and a total length of 80 cm (5 samples × 4 treatments × 4 root fragments × 1 cm) of root fragments were arbitrarily selected and analyzed under a light microscope (BX53; Olympus) following McGonigle et al. (1990) to record detection of AMF characteristic, arbuscules or coils, hyphae, spores and vesicles. We further amplified partial SSU rDNA to confirm root colonization of isolates in *C. japonica* (see SMM3).

Statistical Analysis

We calculated the relative water content in the shoots (WC_{shoot}, %) as 100 × (FW_{shoot} – DW_{shoot}) / FW_{shoot} and the fresh shoot-to-root weight ratio (S/R_{fresh}). After validating the assumptions of homogeneity of variances and normality in measured plant and soil variables with the Levene and Shapiro tests at $p > 0.05$, we analyzed the effects of AMF inoculation on the variables using one-way analysis of variance (ANOVA). Differences between treatments were tested using Tukey's test at $p < 0.05$. Upon violation of at least one of the assumptions mentioned above for parametric tests, we used the Kruskal-Wallis test followed by *the post hoc t-student* test with the Bonferroni *p*-value adjustment method to check the effect of inoculation treatments at $p < 0.05$ and compare variables between treatments. For *C. japonica* assays, to understand how AMF inoculation treatment affected plants and soils, we calculated and tested the significance of Pearson correlations between plant and soil variables at $p < 0.05$ using the *Hmisc* R package (Harrell, 2025). We performed a local polynomial regression (Cleveland et al., 1992) fitted to the Gaussian family using the Loess method in the R package *stats* v. 4.3.2 (R Core Team, 2023) on H growth rates of *C. japonica* seedlings for each treatment as a function of time.

Results

Presymbiotic growth of LFB-4 and LFB-A1 and root colonization

Based on molecular and morphological analyzes, we identified LFB-4 and LFB-A1 as *G. rosea* and *G. margarita,* respectively (Online Resources 1~3). On 1.5% gellan gum medium, it took up to two weeks for the LFB-A1 spores to germinate, while it took less than a week for the LFB-4 spores. One to three germ tubes developed simultaneously from the germinal layer (L3) through the spore wall, generally near the sporogenous cell, during germination (Online Resources 2 & 3). During the presymbiotic stage of some individuals of LFB-4 and LFB-A1, immature spores developed intercalary at the tip of the growing mycelium and an anastomosis-like connection of hyphae was also observed. LFB-4 and LFB-A1 formed mycorrhizae with lab grown clover (*Trifolium repens* L.), sorghum (*Sorghum bicolor* (L.) Moench) (propagation assay), *C. japonica* and *D. carota* (mycorrhizal response assay). Mycelial coils and spores formed sporadically within the roots of *C. japonica* but were more often formed in *T. repens* and *S. bicolor* (Online Resources 2 & 3). Examination of the stained roots of inoculated *T. repens* showed intensive root colonization, where the root cells were filled with arbuscules, coiled hyphae, and sometimes spores. In addition, multiple spores were produced on the root surface. However, examination of the stained roots of *C. japonica* roots revealed

a very erratic distribution of coiled intracellular hyphae, intercellular hyphae and spores in the root cells (Table S1). Hyphae and spores of the corresponding AMF isolates were present in the soils of the inoculated pots, but not in the control pots (Table S1). In the mixed treatment pots, spores of either isolate were observed. Based on PCR, AMF DNA was detected in all *C. japonica* subjects inoculated, but not in control subjects (no inoculation). For *D. carota*, we confirmed that the cultivation soils contained freshly produced spores.

Response of soil and plants to LFB-4 and LFB-A1 inoculation

Total soil C, but not N, C/N, and pH, was significantly different between inoculated and non-inoculated pots (Table 1, Table S2). For the two tested plant species (*C. japonica* and *D. carota*), growth acceleration was observed in all inoculated plants compared to control plants, particularly for *C. japonica* seedlings (Online Resources 4 & 5). In *C. japonica*, S/R_{fresh}, FW_{plant}, DW_{shoot}, FW_{shoot}, H, and WC_{shoot} were higher in inoculated than in non-inoculated control seedlings, and these differences were significant ($p < 0.05$), except for FW_{plant} (Table 1). However, L_{root} was significantly higher in the uninoculated control than in the inoculated seedlings ($p = 0.03$). When grown for 10 months, the root architecture differed between treatments, but the root biomass was comparable (Online Resource 6). At 88 days after inoculation, *C. japonica* seedlings with LFB-4 and LFB-A1 grew taller (H) than those with F-1, TSU-2 and YC-1 (Online Resource 7). In *D. carota*, all measured plant properties (FW_{shoot}, H, and $FW_{taproot}$) were also higher in inoculated plants than in non-inoculated control plants, but the difference was significant only for FW_{shoot} and H ($p < 0.05$).

When comparing soil properties among the four treatments (control, treatments with LFB-4, LFB-A1 and LFB-4 + LFB-A1), no significant effect of treatment was observed ($p > 0.05$; Table 1, Table S2). However, for plant properties, we observed significant differences ($p < 0.05$) between treatments in S/R_{fresh}, H, and WC_{shoot} (*C. japonica*), and in H and $FW_{taproot}$ (*D. carota*). For *C. japonica*, non-inoculated subjects had the highest FW_{root} and L_{root}; the highest values of FW_{plant} and DW_{shoot} were observed in seedlings with LFB-A1 and with the mixture of LFB-4 and LFB-A1, H, S/R_{fresh}, FW_{shoot}, and WC_{shoot} were the highest (Table 1). Although not significant ($p > 0.05$), LFB-4 inoculated seedlings had a higher H than LFB-A1-inoculated seedlings. For *D. carota*, FW_{shoot} and $FW_{taproot}$ were highest with LFB-4, and H was highest with the mixture of LFB-4 and LFB-A1.

179 FW_{shoot} and DW_{shoot} were not significantly correlated with WC_{shoot} ($p > 0.05$), while the latter showed
180 significant positive and negative correlations with H (Pearson correlation = 0.65, $p = 0.01$) and FW_{root}
181 (Pearson correlation = -0.61, $p = 0.01$), respectively (Table S3). Furthermore, L_{root} was negatively correlated
182 with WC_{shoot} (Pearson correlation = -0.58, $p = 0.02$). Total soil C was significantly correlated with WC_{shoot}
183 (Pearson correlation = 0.55, $p = 0.03$) and C/N was significantly correlated with DW_{shoot} (Pearson correlation
184 = 0.51, $p = 0.04$).

185 Table 1 Soil feedbacks and responses of *Cryptomeria japonica* and *Daucus carota* to inoculation of different arbuscular mycorrhizal fungi and their
186 combination

Soil properties	**Control (n = 5)**	**LFB-4 (n = 5)**	**LFB-A1 (n = 5)**	**Mix (n = 5)**	**AMF inoculated (n = 15)**
pH	5.87 ± 0.04 a A	5.90 ± 0.05 a	5.84 ± 0.04 a	5.12 ± 0.05 a	5.88 ± 0.06 A
C (%)	0.007 ± 0.001 a B	0.008 ± 0.001 a	0.009 ± 0.001 a	0.009 ± 0.002 a	0.009 ± 0.001 A
N (%)	0.0003 ± 0.0001 a A	0.0004 ± 0.0001 a	0.0004 ± 0.0001 a	0.0004 ± 0.0001 a	0.0004 ± 0.0001 A
C/N	21.3 ± 5.4 a A	22.0 ± 6.2 a	23.1 ± 7.6 a	20.6 ± 1.4 a	21.9 ± 5.4 A
Plant properties - *Cryptomeria japonica*	**Control (n = 4)**	**LFB-4 (n = 4)**	**LFB-A1 (n = 4)**	**Mix (n = 4)**	**AMF inoculated (n = 12)**
S/R_{fresh}	1.76 ± 0.56 b B	4.34 ± 1.16 ab	3.78 ± 0.76 ab	5.73 ± 2.47 a	4.60 ± 1.70 A
FW_{plant} (mg)	639.8 ± 204.7 a A	790.3 ± 141.3 a	828.0 ± 480.0 a	809.3 ± 262.4 a	809.2 ± 295.5 A
FW_{root} (mg)	189.0 ± 60.5 a A	145.8 ± 50.7 a	160.8 ± 88.9 a	122.0 ± 49.8 a	142.8 ± 61.7 A
FW_{shoot} (mg)	312.8 ± 66.8 a B	589.8 ± 71.3 a	617.3 ± 360.8 a	646.3 ± 208.6 a	617.8 ± 222.1 A
DW_{shoot} (mg)	59.6 ± 8.8 a B	98.2 ± 15.8 a	107.6 ± 64.1 a	106.4 ± 38.1 a	104.1 ± 40.0 A
H (cm)	2.1 ± 1.2 b B	7.2 ± 1.0 a	5.0 ± 1.6 ab	7.3 ± 2.4 a	6.5 ± 1.9 A
WC_{shoot} (%)	80.7 ± 2.0 b B	83.4 ± 0.9 a	82.6 ± 0.6 ab	83.7 ± 0.8 a	83.2 ± 0.9 A
L_{root} (cm)	82.2 ± 12.7 a A	54.6 ± 17.5 a	57.9 ± 32.2 a	52.2 ± 17.2 a	54.9 ± 21.3 B
Plant properties - *Daucus carota*	**Control (n = 10)**	**LFB-4 (n = 10)**	**LFB-A1 (n = 10)**	**Mix (n = 10)**	**AMF inoculated (n = 30)**
FW_{shoot} (mg)	234.1 ± 173.0 a B	349.1 ± 173.0 a	293.8 ± 153.1 a	343.6 ± 145.1 a	328.8 ± 154.0 A
H (cm)	13.0 ± 3.3 b B	16.2 ± 3.0 ab	16.4 ± 3.1 ab	17.6 ± 1.4 a	16.7 ± 2.6 A
$FW_{taproot}$ (mg)	1130.2 ± 571.9 b A	1878.2 ± 818.5 a	1153.9 ± 479.8 b	970.8 ± 287.2 b	1334.3 ± 681.0 A

187 Values are presented as mean ± standard deviation. C: Total carbon, N: Total nitrogen, C/N: C to N ratio, S/R_{fresh}: Fresh shoot to root weight ratio,
188 FW_{plant}: Plant fresh weight, FW_{root}: Root fresh weight, DW_{shoot}: Shoot dry weight, FW_{shoot}: Shoot fresh weight, H: Plant height, WC_{shoot}: Shoot water
189 content, L_{root}: Total root length, $FW_{taproot}$: Fresh weight of the taproot. We performed a Tukey honestly significant difference test after one-way analysis
190 of variance or a post hoc analysis with the Bonferroni probability adjustment method following the Kruskal-Wallis test (see Table S2). Treatments
191 with the same letter were not significantly different. Lowercase letters compare values between control and inoculated plants by AMF treatment (df
192 = 3). Uppercase letters compare values between control and inoculated plants regardless of AMF species (df = 1)

Discussion

In this study, we identified two isolates of *Gigaspora* AMF (LFB-4 and LFB-A1) previously collected from *C. japonica* trees in central Japan, *G. rosea and G. margarita* (see Supplementary Results and Discussion), and investigated their biology, ecology and function. Because of the very low relative abundance of *Gigaspora* spp. DNA detected in the roots and surrounding soils of *C. japonica* over five years of investigation (Djotan et al., 2022, 2023, 2024a, 2024b, 2025), we hypothesized that LFB-4 and LFB-A1 are not beneficial to the plant. Furthermore, because *Gigaspora* spp. are thought to be edaphophilic (Hart & Reader, 2002) and root colonization increases with taxon richness (Verbruggen et al., 2013), we expected that inoculation with the two isolates would increase root colonization compared to inoculation with a single isolate. The two AMF isolates, LBF-4 (*G. rosea*) and LFB-A1 (*G. margarita*), showed considerable growth promotion in *C. japonica* seedlings and were complementary in their cohabitation in the host plant. However, co-inoculation did not increase root colonization, although it provided the best growth-promoting benefits for *C. japonica*. These results suggest that (1) environmental metabarcoding (eDNA) may overlook some beneficial taxonomic groups of AMF of a given host plant species, and (2) root colonization is not necessarily proportional to the amount of benefits that AMF can provide to their host plant.

Importance of characterizing AMF as independent living organisms

In the present study, *G. rosea* (LFB-4) and *G. margarita* (LFB-A1) sporadically colonized the roots of *C. japonica,* producing spores in some root cells. However, the colonization of *T. repens* roots, where spores are produced not only in root cells but also on the surface of the roots, was not sporadic. The differential colonization of *C. japonica* (a tree) and *T. repens* (an herb) could be related to the suggested inverse proportionality of AMF richness in roots and the lifespan of the corresponding host plants (Djotan et al., 2025; Torrecillas et al., 2012). Our findings suggest that *Gigaspora* spp. may colonize herbaceous plants more easily than they can colonize the roots of woody plants. Consequently, the colonization of woody and herbaceous plants could differ within the same AMF species. To date, our observations of spore production inside host plant cells in *Gigaspora*, simultaneous development of multiple germ tubes during spore germination, and intercalary spore production at the tip of running mycelium in a gellan gum medium during the presymbiotic stage have not been previously reported. Our observations underscore the

importance of studying and characterizing AMF as living organisms, like other macrofungi, rather than just as plant symbionts. Taken together, our findings represent a good guide to how *G. rosea* and *G. margarita* inocula can be successfully produced and applied in the field.

Despite having an erratic root colonization, *G. rosea* and *G. margarita* inhabiting forest ecosystems are plant-beneficial AMF

Gigaspora rosea and *G. margarita* had less fungal biomass (spores and hyphae) inside plant roots but showed growth promotion in *C. japonica*. Based on previous functional classifications of AMF families into three guilds, that is, rhizophilic, edaphophilic, and ancestral, root and soil biomass allocations in AMF vary between taxa (Hart & Reader, 2002; Powell et al., 2009; Varela-Cervero et al., 2015; Weber et al., 2019). Rhizophilic guild (high intra- and low extraradical hyphae) includes Glomeraceae, Claroideoglomeraceae, and Paraglomeraceae; edaphophilic guild (low intra- and high extraradical hyphae) includes Gigasporaceae and Diversisporaceae; and the third guild, dubbed ancestral (low intra- and extraradical hyphae), includes Archaeosporaceae, Ambisporaceae, Pacisporaceae, and Acaulosporaceae. We found that, in the roots of fast-growing *C. japonica* seedlings inoculated with LFB-4 or LFB-A1 (compared to control seedlings without AMF), the presence of hyphal coils was very unpredictable. This finding supports the above functional classification of AMF describing *Gigaspora* spp. as edaphophilic AMF and suggests that a high density of hyphae might not be required for a functional mycorrhizal association. However, while extensive root colonization by rhizophilic AMF can protect host plants against pathogen infection, extensive extraradical mycelia by edaphophilic AMF (eg, *Gigaspora* spp.) are suitable for nutrient uptake (Sikes et al., 2010). With few infection points, it can be assumed that *Gigaspora* spp. are vulnerable to soil disturbance, but the taxon appears to be less affected (Hart & Reader, 2004). Soil disturbances may break hyphae-root connections, requiring partners to reestablish symbiosis. Thus, *Gigaspora* spp. being reported to be less vulnerable to disturbances questions how they overcome soil disturbances. Our findings question the common practice of using the proportion of root length colonized by fungi as a proxy for assessing mycorrhizal function (McGonigle et al., 1990; Smith & Smith, 2011). While the proportion of colonized root length and similar measures remain useful and easily obtained metrics, colonization–benefit relationships are context dependent and far from straightforward (Frew, 2025; Hoeksema et al., 2010).

Therefore, we need to review how we interpret our observations and how we assess the function of AMF associations.

We found that in single- or dual-species inoculation, *G. rosea* and *G. margarita* had remarkable growth promoting effects on a tree host plant (*C. japonica*) and an herb plant species (*D. carota*), in addition to the plants used to propagate them in a laboratory (*S. bicolor* and *T. repens*). This shows that forests inhabiting *G. rosea* and *G. margarita* are beneficial AMF and can be used in plant production. In a meta-analysis, Marro et al. (2022) reported that Gigasporales are beneficial to plants facing biotic stress, such as insects, microbial pathogens, and nematodes. Furthermore, because Gigasporales are assigned to an edaphophilic guild (Hart & Reader, 2002), their members are known to be good nutrient mobilizers (Sikes et al., 2010). In contrast, based on inoculation tests of *Allium vineale* L. plants, Bever et al. (2009) described *G. margarita* as a non-beneficial AMF. In addition, previous studies have reported that *Gigaspora* species poorly help plant nutrient uptake and nearly affect plants like parasites, rather than improving their growth (Reynolds et al., 2005; Reynolds et al., 2006). It was also shown that *G. margarita* can procure benefits to host plants only when in complementarity with *Claroideoglomus candidum* (Furrazola, Kaonongbua, & Bever) Oehl, G.A. Silva & Sieverd dubbed a beneficial AMF (Steidinger, 2024). These controversies reside in the context-dependent nature of the benefits of the plant-AMF association (Frew, 2025). Furthermore, these findings are consistent with the assertions that mycorrhizal symbioses function along a mutualism-parasitism continuum and thus can result in positive, neutral, and negative outcomes (Johnson et al., 1997; Merckx et al., 2024). We added that the origin could matter: the same species, if isolated from different environments, could induce different mycorrhizal growth responses.

Host- and trait-dependent cohabitation of LFB-4 and LFB-A1

We observed significant complementary and antagonistic effects of LFB-4 (*G. rosea*) and LFB-A1 (*G. margarita*) on plant growth dependent on host and plant traits. Although it was not clear whether both species colonized every host plant simultaneously in the mixed treatment, the observed changes in plant performance between the single species and co-inoculation treatments determined that LFB-4 and LFB-A1 co-colonized *C. japonica* and *D. carota*. We observed complementarity between LFB-4 and LFB-A1 in *C. japonica* for all measured plant properties. On the other hand, the cohabitation of LFB-4 and LFB-A1 was complementary to H and FW_{shoot} but antagonistic to $FW_{taproot}$ in *D. carota*. These plant responses support

previous studies describing AMF complementarity (Bunn et al., 2024; Jansa et al., 2008; Koide, 2000; Steidinger, 2024) and add that the role of each partner and the outcome of their complementarity depend on the host plant identity and plant trait. At the inoculation time, despite having emerged from seeds sown on the same day and grown under the same conditions, *C. japonica* seedlings already had different growth, and thus different H, and potentially different L_{root}, S/R_{fresh}, FW_{plant}, FW_{root}, and WC_{shoot} due to differences between individuals. To avoid additional stress to plants during and after inoculation, only H was repeatedly measured from the day of inoculation to the end of the tests. The potential difference in the properties of the above-mentioned plant at the beginning of the inoculation assays potentially contributed to the statistically nonsignificant difference ($p > 0.05$) observed (1) between treated and untreated plants for FW_{plant} and FW_{root}, and (2) between AMF for all the properties of the plants measured. Although the presence of AMF restricted root development in *C. japonica* seedlings and encouraged carbon release into the soil (significantly higher soil C content in inoculated pots than in controls), it increased shoot biomass production by increasing water content, inducing height growth and branching, which could be attributed to improved AMF-induced nutrient acquisition (Hodge & Storer, 2015). This attribution was supported by the correlations obtained between various variables, particularly FW_{shoot}, DW_{shoot}, WC_{shoot}, and L_{root}. However, as the seedlings grew bigger, roots of inoculated subjects developed longer, while those of controls developed wider, but without significant biomass differences. These results showed that at the early stage of the development of *C. japonica* seedlings, AMF-associated seedlings rely on extraradical hyphae to absorb water and nutrients rather than develop their roots. As a result, they grow faster than seedlings without AMF because the latter need to develop extensive root systems for water and nutrient uptake. As they grow larger, inoculated seedlings extend their root system, although they are still associated with AMF, because they need a strong foundation as a physical support for the tree to evolve. When the mycorrhizal growth response was analyzed by inoculation treatment, we found with a significant difference ($p < 0.05$) that the complementarity of LFB-4 and LFB-A1 resulted in the lowest L_{root}, highest H, S/R_{fresh} and WC_{shoot}. In the absence of a significant difference ($p > 0.05$), biomass production (FW_{plant} and DW_{shoot}) was the highest with LFB-A1, and LFB-4 contributed more to plant elongation (growth in H) than LFB-A1. However, in *D. carota*, the application of isolate LFB-4 resulted in the highest $FW_{taproot}$ and FW_{shoot} while the complementary effects of LFB-4 and LFB-A1 were the most positive for H. From the above, it can be said

that different AMF colonizing the same root system contribute to the fitness of their host plants differently and in host and plant trait-specific ways. Thus, our data support the claim that the actual realized effects in plant-AMF associations are likely a composite of complementary and competitive effects (Steidinger, 2024).

Root colonization assessed by microscopy and environmental metabarcoding may overlook AMF functions

eDNA remains the most common method for estimating AMF diversity in soils and roots of host plants. With this approach, the co-occurrence patterns of AMF hint at their potential cohabitation traits within the roots and surrounding soils of their host plants (Djotan et al., 2023, 2024a, 2024b, 2025), raising multiple questions related to their basic life strategies, which remain largely unanswered (Hart et al., 2015). Meanwhile, the relationship between DNA counts and AMF functions is poorly understood, particularly when PCR is used. Metabarcoding can detect some taxa, while others are overlooked because of primer-based amplification (Hart et al., 2015; Krüger et al., 2009; Lee et al., 2008; Lekberg et al., 2018; Schlaeppi et al., 2016). In this situation, it is unclear whether taxa whose DNA is abundantly detected in the roots are beneficial to the host plant. For example, DNA from Glomeraceae AMF, particularly *Dominikia*, *Rhizophagus*, and *Glomus,* is generally abundantly detected in the roots of *C. japonica* and surrounding soils (Djotan, 2024), which is also the case in many studies tackling other host plant species, but we do not know how beneficial the corresponding AMF are to the host plant. For example, although DNA from *Gigaspora* spp. is always detected in very small relative abundances or is not detected at all in *C. japonica* roots, inoculation of *C. japonica* seedlings with *G. rosea* (LFB-4) and *G. margarita* (LFB-A1) resulted in a positive symbiont-dependent mycorrhizal growth response in the plant along with a complementary interaction between the two AMF species. Meanwhile, inoculation with rhizophilic Glomeraceae AMF, such as *Rhizophagus* and *Glomus* obtained from NARO, did not (Online Resource 7). Therefore, the colonization rate and DNA abundance do not necessarily matter, and we need to collect AMF from forest ecosystems and characterize them as living organisms rather than just plant symbionts.

In conclusion, despite erratic root colonization, AMF isolates LFB-4 (*G. rosea*) and LFB-A1 (*G. margarita*) worked synergistically to improve the growth of *C. japonica* seedlings by modulating root development, likely for carbon acquisition. Therefore, *G. rosea* and *G. margarita* from forest ecosystems

are beneficial AMF, suggesting that their origins are important. Root colonization evaluated by microscopy and metabarcoding may overlook AMF functions.

Statements and Declarations

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Competing Interests

The authors declare that they have no conflicts of interest.

Author Contributions

All authors conceived the study and the experimental design. A.K.G. Djotan implemented and conducted the experiments, collected and analyzed the data, and wrote the first draft of the manuscript. All authors commented on the previous versions of the manuscript and approved its final version.

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Data Availability

We deposited the data generated during this study in relevant publicly accessible databases, as described in the Materials and Methods and Results sections.

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