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Research Article

Growth Characteristics of *Rhizophagus clarus* Strains and Their Effects on the Growth of Host Plants

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Abstract Arbuscular mycorrhizal fungi (AMF) are ubiquitous in the rhizosphere and form symbiotic relationships with most terrestrial plant roots. In this study, four strains of *Rhizophagus clarus* were cultured and variations in their growth characteristics owing to functional diversity and resultant effects on host plant were investigated. Growth characteristics of the studied *R. clarus* strains varied significantly, suggesting that AMF retain high genetic variability at the intraspecies level despite asexual lineage. Furthermore, host plant growth response to the *R. clarus* strains showed that genetic variability in AMF could cause significant differences in the growth of the host plant, which prefers particular genetic types of fungal strains. These results suggest that the intraspecific genetic diversity of AMF could be result of similar selective pressure and may be expressed at a functional level.

Keywords Arbuscular mycorrhzal fungi, Genetic variability, Intraspecific diversity, *In vitro culture*, *Rhizophagus clarus*

Arbuscular mycorrhizal fungi (AMF) form symbiotic relationships with the majority of land plants [1], and are known to influence not only individual plants but also the nutrient cycling, the biodiversity and productivity of plant communities, as well as ecological succession in the forest ecosystem [2, 3]. It is generally known that the symbiotic effects of AMF on the growth of individual host plants are influenced by such physiological characteristics as spore germination and hyphal growth of AMF [4], thus it can be said that the growth responses of host plants reflect differences in the physiological characteristics of AMF.

AMF have a long evolutionary history [5, 6], but to date only approximately 240 species have been recorded [7]. Considering that AMF successfully adapt to most environments on earth and form symbiotic relationships with diverse host plants, AMF are likely to possess strategies to overcome their low level of species biodiversity. Indeed, recent studies have shown that AMF possess a high level of intraspecies genetic variability [8-11], and that genetic variation in AMF can affect growth responses of host plants [12-15]. Additionally, AMF from geologically distinct regions have been found to possess genetic differences that affect their abilities for extraradical hyphal growth and for absorbing phosphus and nitrogen [16]. However, also of importance is research that assesses, within a single rhizosphere, how genetic features within an individual AMF population influence the growth of host plants. Such research aids in understanding AMF biodiversity, which in turn influences host plants in the ecosystem, and in identifying AMF strategies for evolution and survival.

Currently, it is considered impossible to culture AMF without host plants. Although AMF are crucial for ecosystem function, little is known about their biological characteristics and their functional roles, because they are obligate symbionts. Recently, attempts have been made to obtain aseptic AMF cultures via root organ culture (ROC), in which AMF spores are cultured in growth media together with Ri T-DNA transformed plant roots [17, 18]. However, there are limitations to this approach. Only a few species of AMF can be cultured via ROC, and it takes at least 3 mon for new spores to form. Members of the genus *Rhizophagus* are easy to culture via ROC, and thus they are commonly used [19]. In particular, study on the genomes of *R. irregularis* and *R. intraradices* has provided important molecular evidence for the evolution of AMF and the mechanisms by which AMF form symbiotic relationships with plants [20].

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If it can be shown that genetic differences within a given AMF population have different effects on the growth of strains or host plants, this will provide an important clue to a gene-level, rather than species-level, understanding of AMF biodiversity and its interactions with individual plants in actual ecosystems. Therefore, in this study we cultured different strains of *R. clarus* using ROC and investigated strain growth characteristics among different *R. clarus* strains and the differences in their effects on host plants.

MATERIALS AND METHODS

To generate AMF cultures of single-spore origin, the root organs of transformed carrots (*Daucus carota*) were used [19]. The carrot roots were transformed with *Rhizobium rhizogenes* (KCTC2744). The roots were cleaned, cut to a width of approximately 5 mm, and sprayed with 200 µL of *R. rhizogenes* culture solution. Afterwards, they were incubated in 1.5% water agar growth medium at 25°C in the dark for 4 wk [21]. The transformed root organs around the cambium were subcultured in a modified White (MW) medium with added carbenicillin (500 mg/L). After several generations of subculture, the transformed roots were cut to approximately 3~5 cm before use.

Spores of *R. clarus* CLR502 were collected from the rhizosphere of *Miscanthus sinensis* found in the province of Chungcheongnam-do, Korea and cultured in pots with sorghum (*Sorghum bicolor*) as the host under a greenhouse environment. The *R. clarus* spores were extracted from pure cultured soil and surface-sterilized with 2% chloramine T and a streptomycin solution and germinated at 25°C [22]. Two to three days after germination, spores that developed hyphae were selected and transferred to modified Strullu-Romand (MSR) growth medium. The germinated spores were placed next to the transformed carrot root organs, which had been cut to a size of approximately 1.5 cm × 1.5 cm, including the MW growth medium [23]. Samples were continuously subcultured every 2~3 mon and were used in this study.

18S rDNA sequences of the cultured AMF were analysed to verify that they were *R. clarus*; all strains used in growth testing were phylogenetically identical to *R. clarus*. To determine the growth characteristics of the strains, the time required for formation of the first spore, the total length of extraradical hyphae at spore maturity, and the number of spores produced was measured. For the time required for formation of the first spore, the number of days for the first spore to form after subculturing of individual strains was counted. Also, because young spores of *R. clarus* reach full maturity approximately 30 days after formation, the gridline intersection method was used to measure the total length of extraradical hyphae at approximately 50~55 days, at which time the spores would be fully mature [24]. For the number of spores produced, the number of mature spores was counted per 1 cm^2 on a Petri dish, and measurements were repeated 10 times.

To assess the effects of intraspecific genetic variability on the growth of host plants, aseptic cultures of single-spore origin were inoculated into host plants. *S. bicolor* (sorghum) and *Capsicum annuum* (chili pepper) were used as the host plants.

Sorghum and chili pepper plants were aseptically germinated and transplanted into plastic pots (Ø 3.8 cm × 21 cm) containing sterilized (121°C, 15 min) sand. At the time of transplantation, plant roots were inoculated with AMF by placing a 2 cm × 2 cm block of MSR growth media containing the transformed carrot roots, hyphae, and spores, and the area was covered with a large quantity of sterilized sand. Plants were incubated at 25°C for 16 wk. During the incubation period, they were given sufficient water every other day and supplied once a week with 200 mL of Hoagland solution, in which the phosphate concentration was diluted to 1/20. After 16 wk, the plants were harvested and confirmed the mycorrhizal formation by staining 1-cm slices of the root organs with trypan blue. The root to shoot ratios (RS) of the host plants were measured, and the numbers of leaves, flowers, and fruits on the chili pepper plants were recored. The remaining samples were dried at 65°C for 72 hr, and then their dry weight was measured. Subsequently, plant components were analyzed for nitrogen content (total N) and phosphate content (total P).

RESULTS

Approximately 3~5 days after surface-sterilized spores were placed in the Gel-gro medium, new hyphae began to grow either from existing subtending hyphae or from the surface of spores. After hyphae had grown for 4~9 days following germination, the amount of time taken for young spores to form was, on average, 13 days. Time to formation of the first spore did not differ significantly between strains (Table 1).

Table 1. The number of spores and hyphal length of strains of *Rhizophagus clarus* after inoculation of 55 days and the number of days required for the formation of the first spore (mean ± SE)

Strains	Spore number (1 cm^2)	Hyphal length (cm)	Days for the first spore production
CLR502-1	26.06 ± 3.201 a	847.4 ± 57.44 a	14.3 ± 1.76 a
CLR502-2	48.41 ± 6.186 b	1,490.0 ± 173.09 b	11.6 ± 1.21 a
CLR502-3	22.01 ± 4.668 a	802.1 ± 73.85 a	14.1 ± 2.11 a
CLR502-4	23.08 ± 1.176 a	760.3 ± 84.55 a	11.5 ± 3.20 a

Values in each column not followed by the same letter are significantly different.

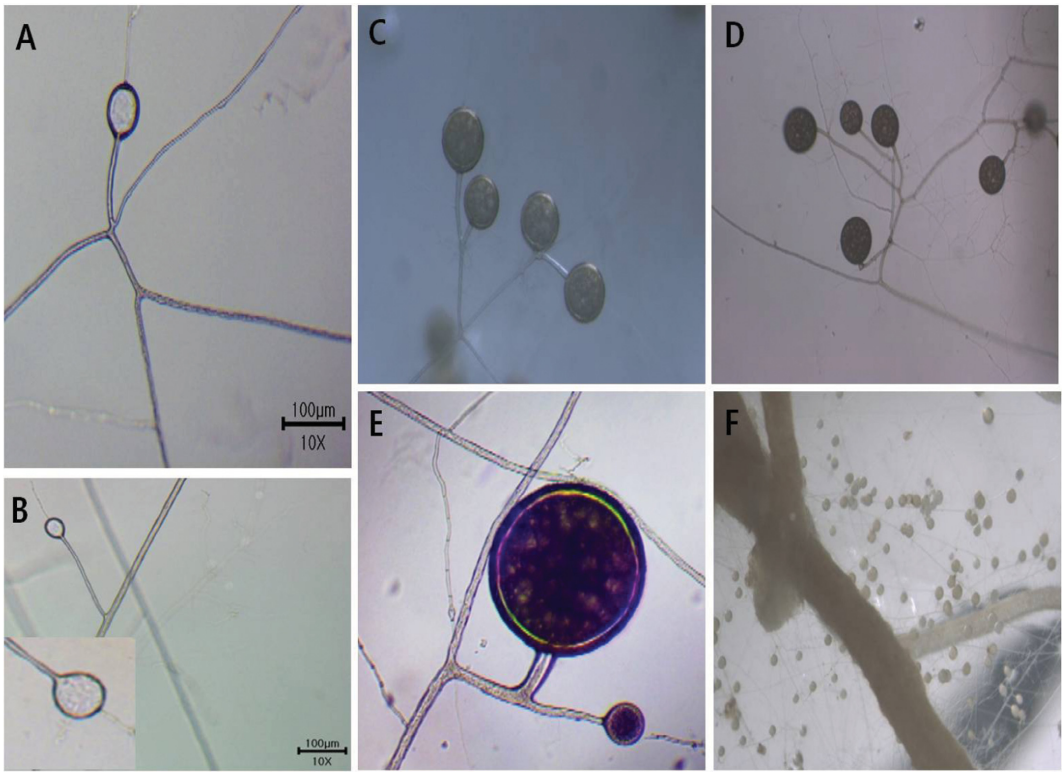

Fig. 1. Ontogenetic characteristics of *Rhizophagus clarus* in root organ culture. A, B, A young spore in the middle of a hypha; C, D, E, Maturation of young spores; F, Fully mature spores (scale bars: A, B = 100 μm).

Young spore formation was accompanied by a swelling in the middle of a hypha (Fig. 1A and 1B). Young spores possessed a thin membrane, but as they matured, the membrane thickened and changed color, becoming a more intense light yellow. In addition, as they matured, shapes resembling oil droplets were observed inside the spores. In the early stages of spore germination, the surface was shiny and glassy, and as the spore matured, the color gradually deepened to become yellow, changing color with developmental stage. On average, the first offspring spore was fully mature 30 days after germination of the parent spore. Approximately 50~55 days after the formation of the first spore, more than 1,000 spores were observed in the Petri dishes; a large quantity of spores was observed around the carrot root organs in particular (Fig. 1F).

Analysis of 18S rDNA sequences showed that strains of *R. clarus* were divided into 4 strains (CLR502-1, CLR502-2, CLR502-3, and CLR502-4), and we examined the growth characteristics of each of the four strains (Table 1, Fig. 2). The four strains showed no significant differences in the amount of time to formation of first offspring spores. However, after they reached full maturity at approximately day 50~55, they showed significant differences in the length of extraradical hyphae and the number of mature spores per unit area. Of the four strains, CLR502-2 produced the longest extraradical hyphae (1,490 cm, p = 0.000) and the most spores per unit area, at 48 spores/cm^2. Of the other 3 strains, no significant differences were observed in hyphal length or the number of spores per unit area. The length of extraradical hyphae and the number of spores per unit area were highly correlated (confidence interval, 0.922; p = 0.000).

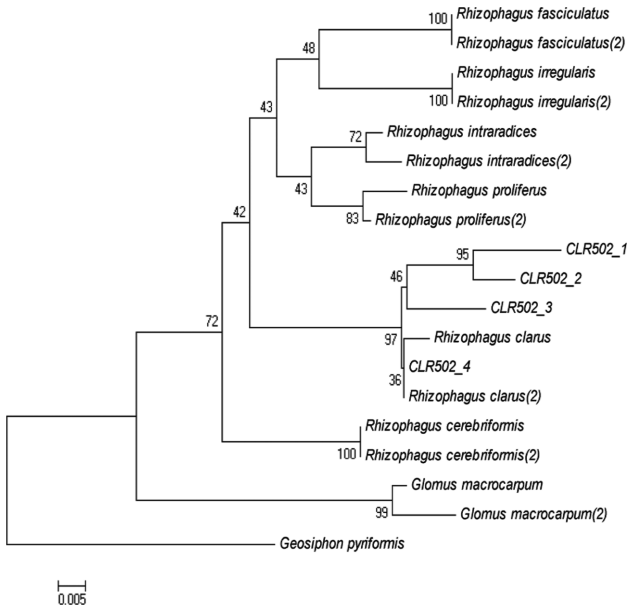

Fig. 2. A phylogenetic tree (neighbor-joining) of *Rhizophagus clarus* strains for the plant growth experiments. Numbers at nodes indicate bootstrap support (1,000 replicates).

Table 2. Mycorrhizal effects on growth responses of *Sorghum bicolor* inoculated with strains of *Rhizophagus clarus* (mean ± SE)

	Root : Shoot	Dry weight	Total N	Total P
Control	0.52 ± 0.08 a	1.31 ± 0.09 a	0.46 ± 0.13	0.20 ± 0.02
CLR502-1	0.66 ± 0.06 ab	1.96 ± 0.13 c	0.60 ± 0.11	0.31 ± 0.03
CLR502-2	0.75 ± 0.07 b	1.81 ± 0.10 bc	0.45 ± 0.07	0.22 ± 0.03
CLR502-3	0.60 ± 0.04 ab	1.58 ± 0.09 abc	0.39 ± 0.15	0.21 ± 0.09
CLR502-4	0.52 ± 0.09 a	1.46 ± 0.03 ab	0.71 ± 0.07	0.23 ± 0.04

Values in each column not followed by the same letter are significantly different.

Table 3. Mycorrhizal effects on growth responses of *Capsicum annuum* inoculated with strains of *Rhizophagus clarus* (mean ± SE)

	Leaves	Flowers	Fruit	Root : Shoot	Dry weight	Total N	Total P
Control	5.47 ± 0.46 a	0.2 ± 0.14 a	0.07 ± 0.07 a	1.58 ± 0.12 c	0.41 ± 0.02 a	0.09 ± 0.05 a	0.01 ± 0.01 a
CLR502-1	15.82 ± 1.46 b	1.82 ± 0.33 b	0.36 ± 0.15 ab	1.16 ± 0.1 b	0.57 ± 0.03 b	0.93 ± 0.3 b	0.04 ± 0 b
CLR502-2	15.3 ± 1.76 b	2 ± 0.42 b	0.6 ± 0.22 bc	1.11 ± 0.13 ab	0.58 ± 0.04 bc	1.29 ± 0.38 b	0.05 ± 0.01 b
CLR502-3	13.5 ± 1.78 b	1.5 ± 0.27 b	0.88 ± 0.13 c	0.79 ± 0.1 a	0.66 ± 0.03 c	0.94 ± 0.11 b	0.06 ± 0.01 b

Values in each column not followed by the same letter are significantly different.

To investigate whether the 4 AMF strains would show different effects on host plant growth at the intraspecies level, host plants of two different species were inoculated with all 4 strains of *R. clarus* and measured growth effects (Tables 2 and 3). In sorghum, the host plant growth was significantly enhanced after mycorrhizae formed, regardless of the mycorrhizal strain used, compared with the control group. The CLR502-1 strain showed the best growth enhancement effect differed significantly from the effect of CLR502-3. The RS, indicative of the ratio of growth below vs. above the ground, did not show significant differences among strains ($p = 0.089$). However, inoculation with CLR502-2 increased the RS ratio, compared to the control group.

To investigate whether different strains differed in their capacity to absorb nitrogen and phosphate, the nitrogen and phosphate contents of individual plants were measured using plant component analysis. The results showed that particular strains could increase the absorbability of either nitrogen or phosphate to a significant level compared to other types of strains. The CLR502-4 strain increased the absorption of nitrogen compared to the CLR502-2 and CLR502-3 strains and the control, and the CLR502-2 strain increased the absorption of phosphate compared to all other strains and the control group.

Chili pepper plants inoculated with AMF showed significant differences in growth compared to the control group, regardless of the strains of inoculum. Chili pepper plants showed a greater increase in growth than sorghum plants following inoculation with mycorrhizae. CLR502-3 had a stronger effect on the growth of chili pepper plants than CLR502-1. The RS ratio decreased as growth of host plants increased; inoculation with the CLR502-3 strain produced the lowest RS ratio, and had the strongest effect on growth of chili pepper plants. Inoculation with any strain of mycorrhiza significantly increased the numbers of leaves, flowers, and fruits on pepper plants. Specifically, CLR502-3, which showed the greatest effect on the growth of host plants, did not differ from the other strains in its effect on the numbers of leaves or flowers, but it produced the highest number of fruits. The strains of *R. clarus* significantly increased absorption of nitrogen and phosphate by host plants, compared with the control group, although there was no significant difference between other strains.

DISCUSSION

In this study, we used ROC to obtain pure strains of *R. clarus* from a population in the soil of the rhizosphere of *M. sinensis* and investigated the growth characteristics of strains of different genotypes, as well as differences in the effects on the growth of host plants. *R. clarus* is one of the most common AMF found around world, and members of the genus *Rhizophagus* are easy to culture using ROC. The strains used in the study belonged to a single species and originated from a single rhizosphere, but the individual strains showed significant intraspecific genetic variability, as shown by differences in length of extraradical hyphae and the number of mature spores produced per unit area. In addition, when the four strains were inoculated into two species of host plants, they showed significant differences in their effects on the host plants. Inoculation with AMF increased growth of both sorghum and chili pepper plants compared to controls, but the strain with the greatest effect on growth differed between host plants. Thus, it appears that AMF strains may show intraspecific selectivity for host plants. In terms of the growth characteristics of the strains themselves, CLR502-2 produced longer hyphae and more spores per unit area than the other strains, but it did not have significant effects on the growth of either sorghum or chili pepper plants. Inoculation of pepper plants with CLR502-2 produced the greatest number of flowers and the highest total nitrogen content, but these results were not statistically significant. Therefore, it is unlikely that the

growth characteristics of the strains themselves function to positively influence plant growth.

The RS ratio indicates growth below vs. above ground. The RS ratio is higher in infertile soil environments because plants expend more energy on root growth in less fertile soils [25]. Because AMF facilitate absorption of inorganic nutrients from the soil, infection by mycorrhizal fungi generally decreases the RS ratio [26]. However, in sorghum plants inoculated with AMF, the RS ratio either increased or was similar to that of the control group. In contrast, in chili pepper plants inoculated with AMF, the RS ratio significantly decreased compared to the control group, and in particular, the RS ratio was lowest for the CLR502-3 strain, which produced the greatest increase in host plant growth following inoculation. We speculate that this may be because chili pepper plants rely on mycorrhizal symbiosis more than sorghum plants. Similarly, in sorghum plants, little difference was observed in nitrogen and phosphate contents, whereas in chili pepper plants AMF inoculation increased the nitrogen and phosphate contents compared to the control group.

To date, many studies have confirmed that genetic differences are correlated with functional differences in AMF, but the majority of studies have only examined functional differences between different regions or climates, or under such strong selective pressures as soil contamination or malnutrition [16, 27-29]. The present study, however, confirmed that strains with phenotypic differences are present within the AMF population in a single rhizosphere, and that these strains have differing effects on host plants. Additionally, in chili pepper plants different strains showed different effects on growth or the production of leaves, flower, or fruits, but it is unclear whether those strains with outstanding growth effects on host plants increased the absorption of nitrogen or phosphate. Thus, AMF induce complex growth responses in host plants.

The results reported in this study suggest the existence of certain genotypes preferred by host plants even within a single AMF species in a single environment. The hypothesis that AMF rapidly evolve into diverse ecotypes with genetic and functional variation may explain their high level of adaptability to wide-ranging host plants and environments. Intraspecific genetic variability may help AMF form communities with a diversity of host preferences and functional differences, through continuous processes of selection and deselection under varying environmental conditions and in different host plant communities. If plants show intraspecific selectivity for AMF, and if this results in differences in growth response, understanding the genetic diversity of AMF will be important for elucidating the mechanisms for formation of symbiotic relationships with plants. In addition, study on AMF genetic diversity and its interactions with host plants at a genotype level will not only enhance the value of AMF as a biological resource, but also provide insight into the evolutionary history of AMF, which began with the emergence of land plants.

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