Regulation of Growth and Secondary Metabolites in Mycorrhizal Medicinal Plants

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(薬用植物での菌根菌共生による総合的生育改善及び2次代謝成分制御)

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Science of Biological Production

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CHAPTER 1-1

PCR-SSCP analysis of ginseng root rot and disease tolerance in mycorrhizal ginseng

Introduction

The demand for medicinal plants has increased in recent years owing to the aging population and the expansion of the market for functional vegetables and foods “(1)”. Asian ginseng (*Panax ginseng*) is a perennial herb belonging to the *Araliaceae* family that is commonly used as a medicinal plant worldwide “(2)”. Asian ginseng has many beneficial effects, including improving physical stamina, concentration, and memory; suppressing cancer and diabetes; regulating blood pressure; and relieving stress “(3)”. It requires three to five years to produce a marketable crop from seeds “(4)”, and peak production occurs within four to six years. However, before reaching the peak production stage, marketable yields decrease due to stunted seedlings, rotting roots, stem discoloration, wilting, and death. This phenomenon is known as the decline in ginseng yield “(5)”. Ginseng replantation in former ginseng fields is expected in ginseng-producing regions worldwide. However, growers’ experiences suggest that ginseng cultivation on sites previously used for ginseng production often fails in the second crop, a phenomenon known as replant disease or decline “(6)”. Ginseng production is associated with both abiotic and biotic factors. Several abiotic factors have been studied to determine the cause of ginseng production decline. Ginseng is easily damaged by high light and high-temperature conditions owing to its low assimilation capacity and fertilizer absorption “(7)”. Zhang et al. “(8)” reported that ginsenosides suppresses ginseng root growth. In addition, phenolic compounds have been shown to inhibit seedling root growth in *Panax* plants “(9)”. Ginseng yield losses due to diseases such as ginseng decline have become more critical, though ginseng production has increased worldwide. Therefore, the development of biological and chemical control measures against ginseng decline has become an important research topic. However, the biotic agents responsible for the decline in ginseng production may differ worldwide. Although *Fusarium* spp. is reported as a ginseng root rot, *Cylindrocarpon destructans* is the leading cause of ginseng decline and replant failure in North America “(6)”. Ginseng root rot

is caused by *C. destructans* in Canada "(10)". *Panax quinquefolius*, *Phytophthora cactorum*, and *C. destructans* have been shown to cause root rot in *P. ginseng* in Korea and China "(11)". Concerning the biotic factor, *Alternaria panax* and *C. destructans* were reported for Asian ginseng decline "(12)". However, the diversity of the pathogen species that cause ginseng root rot in Japan is still unclear. Given this lack of research, the present study was conducted to determine the pathogens responsible for Asian ginseng decline using the polymerase chain reaction single-stranded conformational polymorphism (PCR-SSCP) method.

Traditionally, the diversity of pathogenic species in ginseng fields has been assessed based on the isolation and enumeration of species grown on selective media "(13)". However, this approach is problematic because isolation and identification of numerous isolates of diverse causal pathogens is a complex and time-consuming task "(14)". On the contrary, molecular techniques such as PCR-SSCP have identified minor sequence differences in target pathogenic DNA "(15-16)". PCR-SSCP is an inexpensive, sensitive, rapid, and convenient method for determining DNA sequence variations in many samples "(11)".

Arbuscular mycorrhizal fungi are wide-spectrum biocontrol agents "(14)" that promote host plant growth mainly by enhancing phosphorus uptake through symbiosis "(17)". AMF have been used as biofertilizers and biochemicals and have additional effects on host plants, such as increasing disease resistance "(18)", stress tolerance "(19)", and secondary metabolite content "(20)". AMFs are the major components of the rhizosphere of most plants and play important roles in decreasing the incidence of plant diseases "(21)". Roots colonized by AMF are protected from invasion by fungal pathogens "(22)". Fournier et al. "(23)" demonstrated that *Glomus intraradices* inoculation promotes the growth and ginsenoside production of *P. quinquefolius*. However, little research has been conducted on the application of AMF species (especially *Gigaspora margarita*; GM, *Glomus fasciculatum*; Gf, and *Glomus mosseae*; Gm) to ginseng, despite being well-known symbionts of this plant. In this study, PCR-SSCP was

used to identify pathogens isolated from ginseng root rot as decline symptoms across two separate domestic regions in Japan. Additionally, the effect of AMF colonization on the tolerance of ginseng plants to root rot was investigated to develop a sustainable cultivation technique for Asian ginseng plants that promotes plant growth by arbuscular mycorrhizal fungi and controls disease.

Materials and Methods

Isolation of pathogen from ginseng root rot: Using an intensive sampling regime, 102 fungal isolates were obtained from root rot samples of ginseng fields in two regions: Site A in northern Japan and Site B in central Japan. Root samples were randomly collected from ten plants that exhibited symptoms of decline in each site. Ten root segments (0.5 cm) from each plant with root lesions were surface-sterilized in 70% (v/v) ethanol (EtOH) for 10 s and 10% (v/v) NaClO for 15 min. They were subsequently rinsed with sterilized distilled water three times before being transferred to a potato dextrose agar (PDA) medium (25 °C, dark) in a standard Petri dish. Petri dishes were incubated for 7–10 days until fungal colonies emerged from the root segments. Fungal colonies were transferred three times onto PDA medium via hyphal transfer to obtain pure cultures. In this experiment, known isolates from ginseng were collected from the Ministry of Agriculture, Forestry, and Fisheries and the Biological Resource Center in Japan (Table 2). For comparison, the SSCP profiles of the six *Fusarium* species (*F. oxysporum* f. sp. *asparagi*, *fragariae, lycopersici*, *melonis*, and *spinaceae* and *F. proliferatum*) were grown on the same PDA medium (Table 2).

DNA extraction: Fungal mycelium (0.2 g fresh weight) was taken from actively growing colonies and triturated in a 1.5 ml Eppendorf tube containing 300 µl puregene cell lysis solution (Gentra Systems), then kept on ice. The mycelium was crushed in the tube with a mini-homogenizer (Pellet Mixer, Sansyo), and 150 µl of 3 M sodium acetate was added. The contents were mixed and incubated at -20 °C for 10 min. The precipitated slurry was clarified by centrifugation at 18,000 × g for 10 min. at 4 °C. Finally, the clear supernatant was transferred to a clean microcentrifuge tube, and the volume of the supernatant was recorded. Then, 150 µl of the supernatant and an equal volume of isopropanol (150 µl) were mixed gently in a 1.5 ml Eppendorf tube, and the tube was incubated for 5 min at room temperature. Precipitated DNA was collected by centrifugation at 18,000 × g for 5 min. at 4 °C. The isopropanol was carefully

poured off, and the sample was washed in 200 µl of 70% filter-sterilized ethanol for 15 min. and centrifuged at 18,000 × g for 5 min. The precipitates (DNA pellets) were air-dried for 30 minutes, dissolved in 150 µl of TE (10 mM Tris-HCl, 1 mM EDTA, pH 8) buffer, and stored at -80 °C as a DNA extraction solution.

PCR-SSCP analysis: PCR (PTC-200 DNA engine, MJ Research) was used to target the rDNA ITS2 regions. The 20 µl reaction mixture was comprised of Taq DNA polymerase 10X reaction buffer (Promega, Madison, WI), 4 µl; 25 mM Mg^{2+} solution, 2.25 µl; dNTP mix (4 mM each), 1.75 µl; 25 mM 5.8S ITS3 forward primer (5′ GCATCGATGAAGAACGCAGC 3′, Sigma-Genosys) and ITS4 reverse primer (5′ TCCTCCGCTTATTGATATGC 3′, Sigma-Genosys), 1 µl each; Taq DNA polymerase, 0.1 µl; extracted DNA, 1 µl; and sterile MilliQ water to 20 µl. The PCR program consisted of 35 cycles with a denaturing step at 95°C for 45 seconds, an annealing step at 50°C for 1 min, and a polymerization step at 72°C for 3 min. The PCR products were analyzed by agarose gel electrophoresis and visualized using a UV transluminator. Finally, an SSCP analysis was conducted "(15)". A mixture of PCR products and SSCP loading dye [95% (w/v) formamide, 10 mM NaOH, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol] was used for ss (single-stranded) DNA treatment (95°C, 2 min). 4 µl of this ssDNA solution was loaded into each well of a 10% polyacrylamide gel (e-PAGEL E-T10L, ATTO Co., Ltd.) and subjected to electrophoresis (70 V, 20 mA). After electrophoresis, the gels were immersed in 10% (v/v) acetic acid for 20 min. to fixed and rinsed three times with distilled water. The gels were immersed in silver nitrate solution for 30 min, rinsed gently with distilled water, and then immersed in sodium carbonate solution. When the appearance of bands was confirmed, the gel was immersed in 10% (v/v) acetic acid and fixed. The ITS2 regions of the rDNA gene cluster of isolated species from Japan were identified by comparing them with the SSCP-ITS2 profiles (Fig. 7) of known isolates.

Pathogenicity Evaluation: The roots of ginseng seedlings were surface sterilized by immersion in 70% EtOH for 10 s and 10% NaClO for 15 min, rinsed three times with sterile distilled water, and placed on Knop's agar medium “(24)”. After one month after Fusaria inoculation of PDA cubes, the incidence of root rot and the degree of disease severity were investigated. Sterilized roots were placed on Knop's agar medium as a control.

Inoculation of Arbuscular Mycorrhizal Fungus: Ginseng seedlings were grown in autoclaved (121 °C, 150 kPa, 30 min) commercial soil (Super Mix A, Sakata Seed Corporation, Japan, N: P: K = 180:120:220) mixed with akadama soil at a ratio of 3:1 in a vat (13.5 × 27.0 × 15.5 cm). Ten ginseng seedlings were transplanted into vats (13.5×27.0×15.5 cm) filled with mixed, prepared soil. Three mycorrhizal fungi (GM, Gf, and Gm) were obtained from Idemitsu-Agri Co., Ltd., Tokyo, Japan, and Central Glass Co., Ltd. Tokyo, Japan (spore density unknown) and were inoculated at 5 g per plant. The control plot was also inoculated with autoclaved (121 °C, 1.2 kg/cm^2, 30 min) mycorrhizal inoculum. Seedlings were grown in a greenhouse under shaded (7,000-10,000 lux) conditions.

Pathogen inoculation: The isolates of *Fusarium* spp. were grown in a PDA medium and were incubated at 25 °C. The isolates were separately subcultured in PDA medium, and the conidia were harvested in potato sucrose liquid medium and kept in the dark condition for seven days. Later, the conidial suspension of *Fusarium* spp. was (45 μm) sieved, and their concentration was adjusted to 10^6 conidia/ml using a hemocytometer. Fifteen weeks after AMF inoculation, the roots of each plant were inoculated with 50 ml conidial suspensions of *Fusarium* spp. Four weeks after *Fusarium* inoculation, the symptoms of *Fusarium* root rot were rated to 3 degrees: as part, half, and whole, respectively.

AMF colonization level: The level of AMF colonization was assessed 15 weeks after AMF inoculation. The root system was carefully washed with tap water, and the adhering soil particles were removed. Lateral roots were sampled in 70% ethanol and later stained with

trypan blue, according to Philips and Hayman “(25)”. The ratio of AM fungal colonization was checked in 0.5 cm segments of lateral roots, and approximately 5 to 30 samples of 0.5 cm segments were checked per plant. The average value was calculated for the four plants.

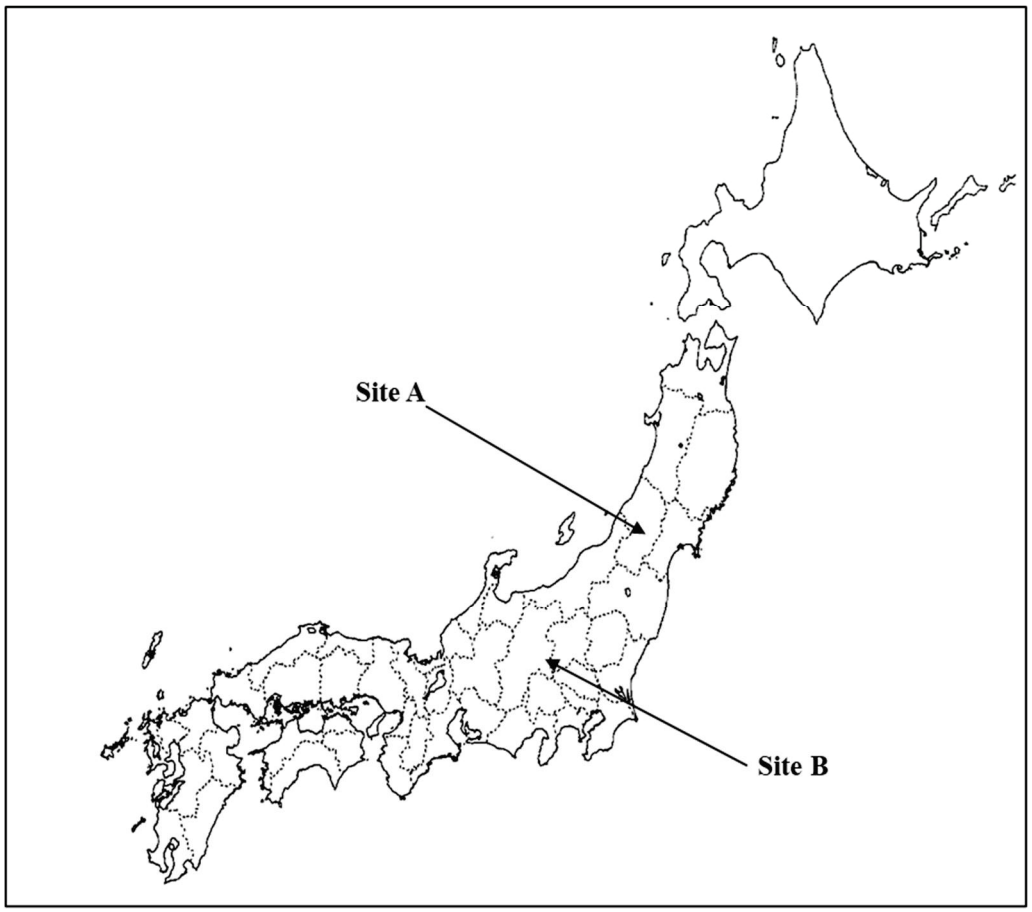


Fig. 1. Sampling area: Site A and Site B.

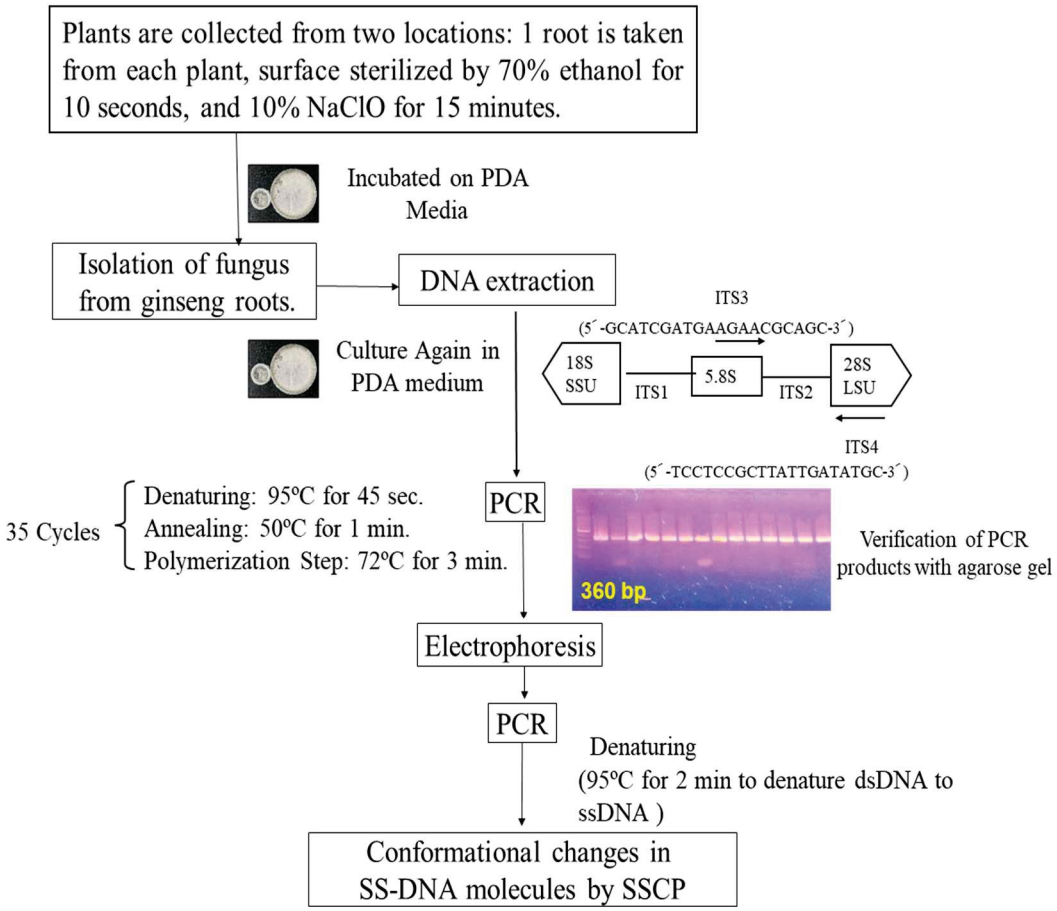


Fig. 2. Flow diagram of the procedures in PCR-SSCP (single-stranded conformational polymorphism) analysis.

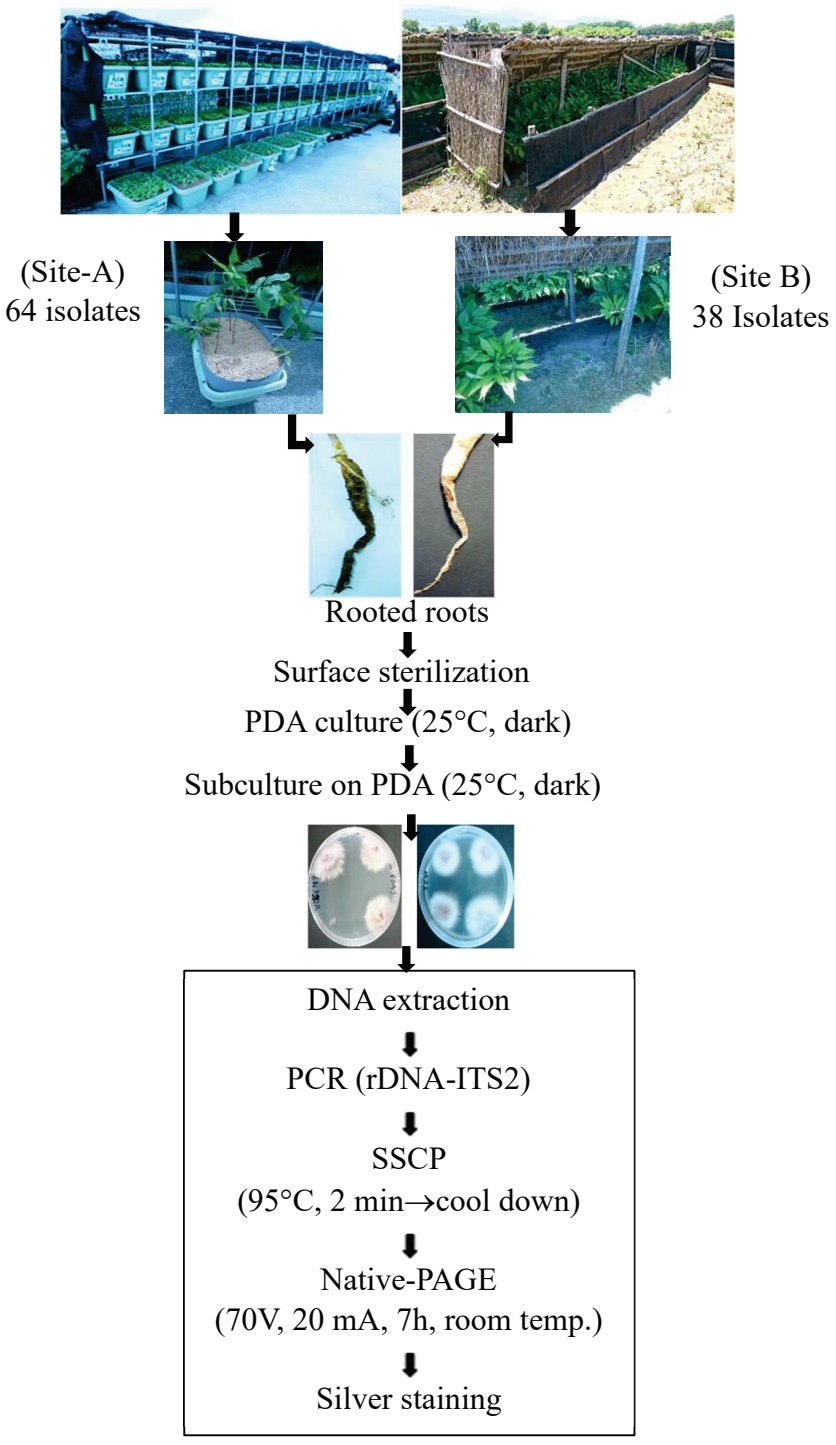


Fig. 3. Complete flow diagram of the procedures in PCR-SSCP

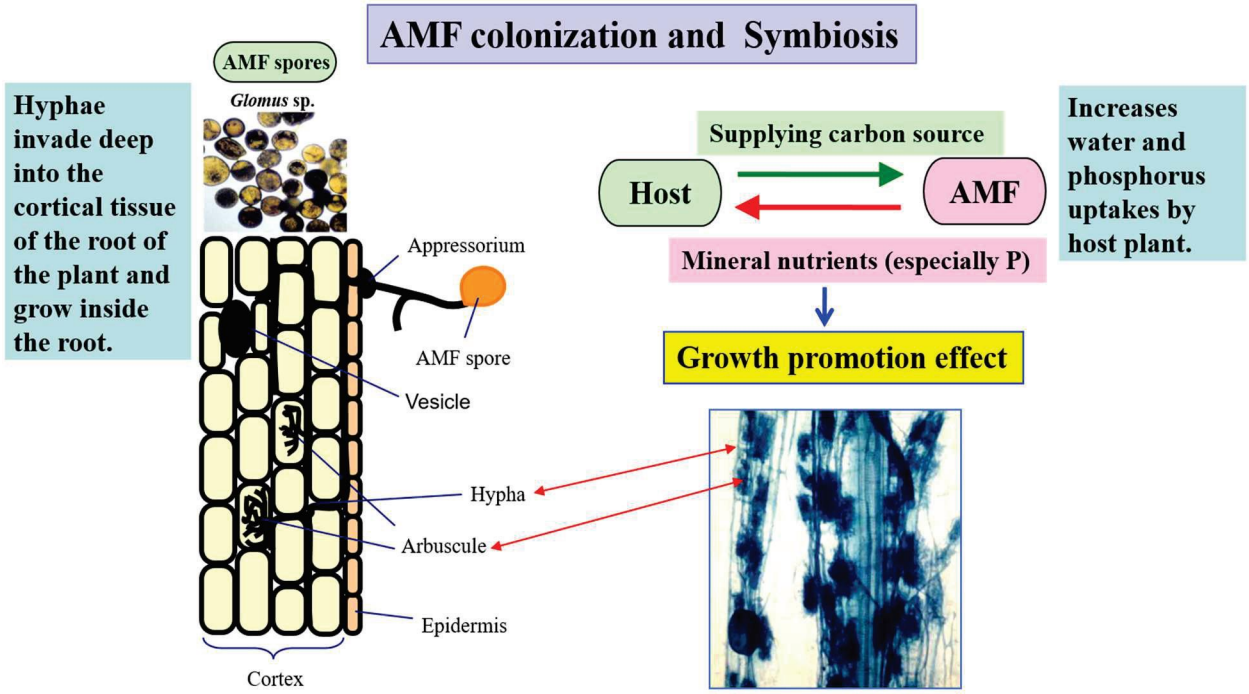


Fig. 4. Mode of action of arbuscular mycorrhizal fungi (AMF).

Table 1. Composition of Knop's agar medium

KNO_3	0.2 g/L
$Ca(NO_3) \cdot 4H_2O$	1.15 g/L
KH_2PO_4	0.2 g/L
$MgSO_4 \cdot 7H_2O$	0.2 g/L
$FePO_4 \cdot 2H_2O$	0.12 g/L
Sucrose	30 g/L
Agar	12.5 g/L

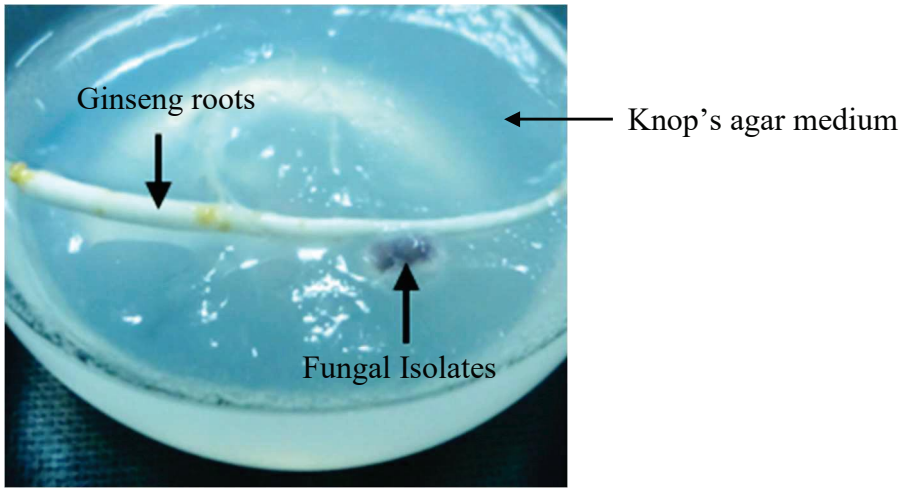


Fig. 5. Pathogenicity test of isolates with ginseng roots in vitro.

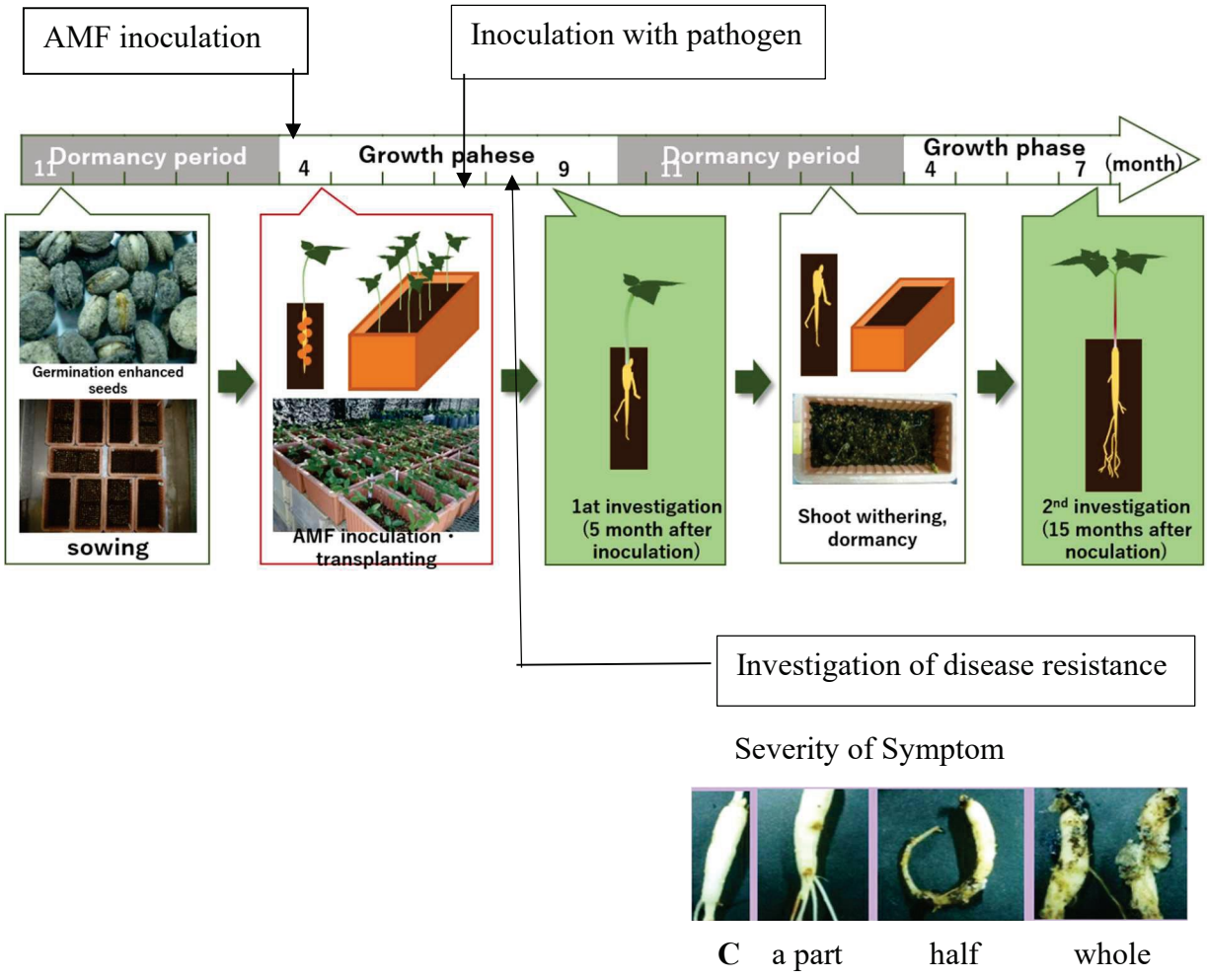


Fig. 6. Flow of experiment with timeline.

Results

Totally 102 isolates showing symptoms of ginseng root rot at sites A and B were compared. The pathogens used in the SSCP profiles (Table 2) showed no polymorphisms within the same species (Fig. 7); however, the band patterns differed among species. Conversely, none of the isolates in the present study matched the bands of pathogens previously isolated from ginseng. Most isolates were easily differentiated into two principal reference species, *Fusarium* I and *Fusarium* II using PCR-SSCP analysis. Two band patterns (I-1, I-2 and II-1, and II-2) were frequently detected at both cultivation sites. The isolates with these band patterns (I-1, I-2 and II-1, II-2) grew on Komada medium "(26)", a *Fusarium* selective medium. The isolate bands differed from those of species isolated from ginseng in the past. Isolated samples I-1, I-2 matched the bands of five strains of *F. oxysporum*, f. sp. *asparagi*, f. sp. *fragariae*, f. sp. *melonis,* f. sp. *lycopersici*, and f. sp. *spinaciae*, and isolated samples II-1, II-2 matched with *F. proliferatum*. The bands of *F. oxysporum* and *F. proliferatum* were consistent with those of I-1, I-2, and II-1, II-2, respectively (Fig. 7).

The SSCP profiles showed considerable variation in the relative proportions of each species isolated across the fields in each region (Fig. 8). The majority of the *Fusarium* isolates at site A were *Fusarium* I (47%) and *Fusarium* II (45%), with others being less abundant (8%). At site B, the most dominant species among the isolates were *Fusarium* I (45%) and *Fusarium* II (26%), with others accounting for 29%.

A subtotal of 86 isolates were selected from the 102 *Fusarium* isolates identified by PCR-SSCP. In vitro pathogenicity tests showed that all isolates were highly pathogenic. Approximately 93.6% of the *Fusarium* I had a pathogenicity score of whole severity, whereas 6.4% scored half severity. On the other hand, 12.8% of *Fusarium* II isolates scored whole severity, 25.7% scored half severity, 43.6% scored a part severity, and 17.9% scored no severity (Table 3).

Fifteen weeks after AMF inoculation, AMF plants significantly increased the dry weight of shoots compared to non-mycorrhizal plants (Fig. 9). The dry weight of the roots increased significantly in Gm (Fig. 9). AMF colonization occurred successfully, with 32% to 45% levels in a root system, and no significant differences were noted among the AMF species (Fig. 10).

Four weeks after *Fusarium* I inoculation, non-AMF plants showed a 70% incidence of root rot, with 22% of plants scoring as whole severity (Fig. 11). The disease incidence in mycorrhizal plots was 43.6%, 50%, and 42.3% for GM, Gf, and Gm, respectively, with no plants scoring as whole severity (Fig. 11). The disease incidence in the *Fusarium* II inoculated control plots was 33.3%, where 100% of the roots exhibited whole disease conditions. In the mycorrhizal plots, the disease incidence of *Fusarium* II was lower than that of the control plots, except for Gm. Disease incidence was 11.1% in the GM and 25% in the Gf, with no plants exhibiting complete disease conditions. Disease incidence in the Gm plots was higher (37.5%) than in the control plots, but the symptom of severity (Gm has 12. 5% whole disease condition, whereas the control plot was 100% whole disease condition) was less than control.

Table 2. SSCP profiles.

Species	Fungal ID	Host	Abbreviation
Species isolated from ginseng in the past			
Alternaria panax	MAFF 306663	Ginseng	Ap
Cylindrocarpon destructans f. sp. *panacis*	NBRC 31881	Ginseng	Cdp1
	NBRC 31882	Ginseng	Cdp2
The other isolates			
F. oxysporum f. sp. *asparagi*	MAFF 305556	Asparagus	Foa
f. sp. *fragariae*	2S	Strawberry	Fof
f. sp. *lycopersici*	MAFF 238900	Tomato	Fol
f. sp. *melonis*	MAFF 242352	Melon	Fom
f. sp. *spinaceae*	MAFF 103060	Spinach	Fos
F. proliferatum	NI-31	Asparagus	Fp

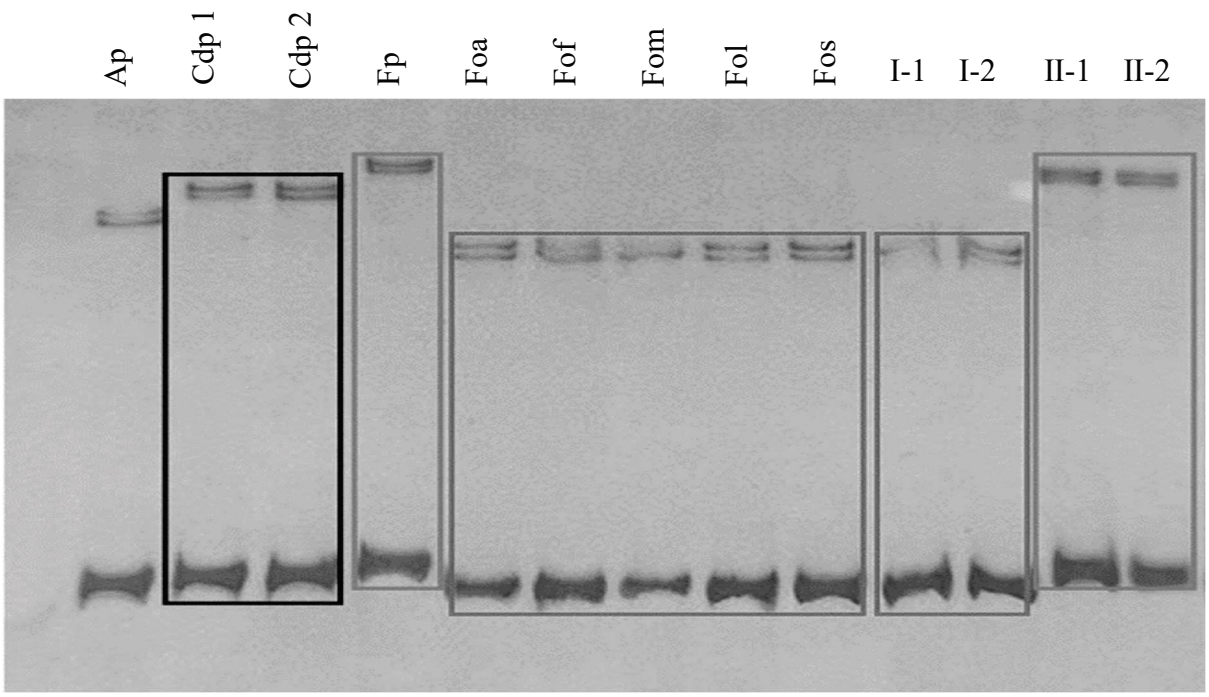
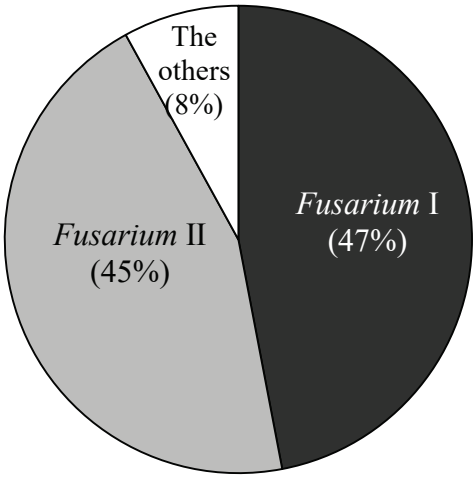


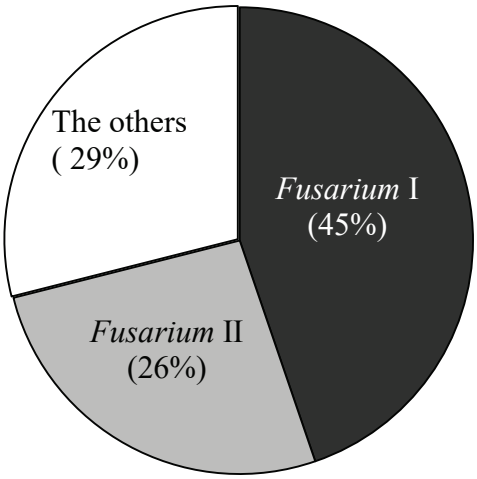
Fig 7. Representative SSCP (single-stranded conformational polymorphism) gel showing the typical results from ITS2-SSCP profiling.

Ap, *Alternaria panax*; Cdp-1, Cdp-2, *Cylindrocarpon destructans* f. sp. *panacis*; Fp, *Fusarium proliferatum;* Foa, *Fusarium oxysporum* f. sp. *asparagi*; Fof, *F. oxysporum* f. sp. *fragariae;* Fom, *F. oxysporum* f. sp. *melonis;* Fol, *F. oxysporum* f. sp. *lycopersici*; Fos, *F. oxysporum* f. sp.*spinaciae*.

I-1, I-2, II-1, II-2, isolates in this study indicated major band patterns on PCR-SSCP.



Site A (64 Isolates)



Site B (38 Isolates)

Fig. 8. Diversity of fungal isolates from ginseng root rot.

Table 3. Relative pathogenicity scores of *Fusaria*.

Disease severity*	Number of isolates			
	Fusarium I	%	*Fusarium* II	%
Whole	44	93.6	5	12.8
Half	3	6.4	10	25.7
A part	0	0	17	43.6
No symptom	0	0	7	17.9
Total	47	100	39	100

*Disease severity scale: pathogenicity of each *Fusarium* isolate was scored according to the number of seedlings with root lesions.

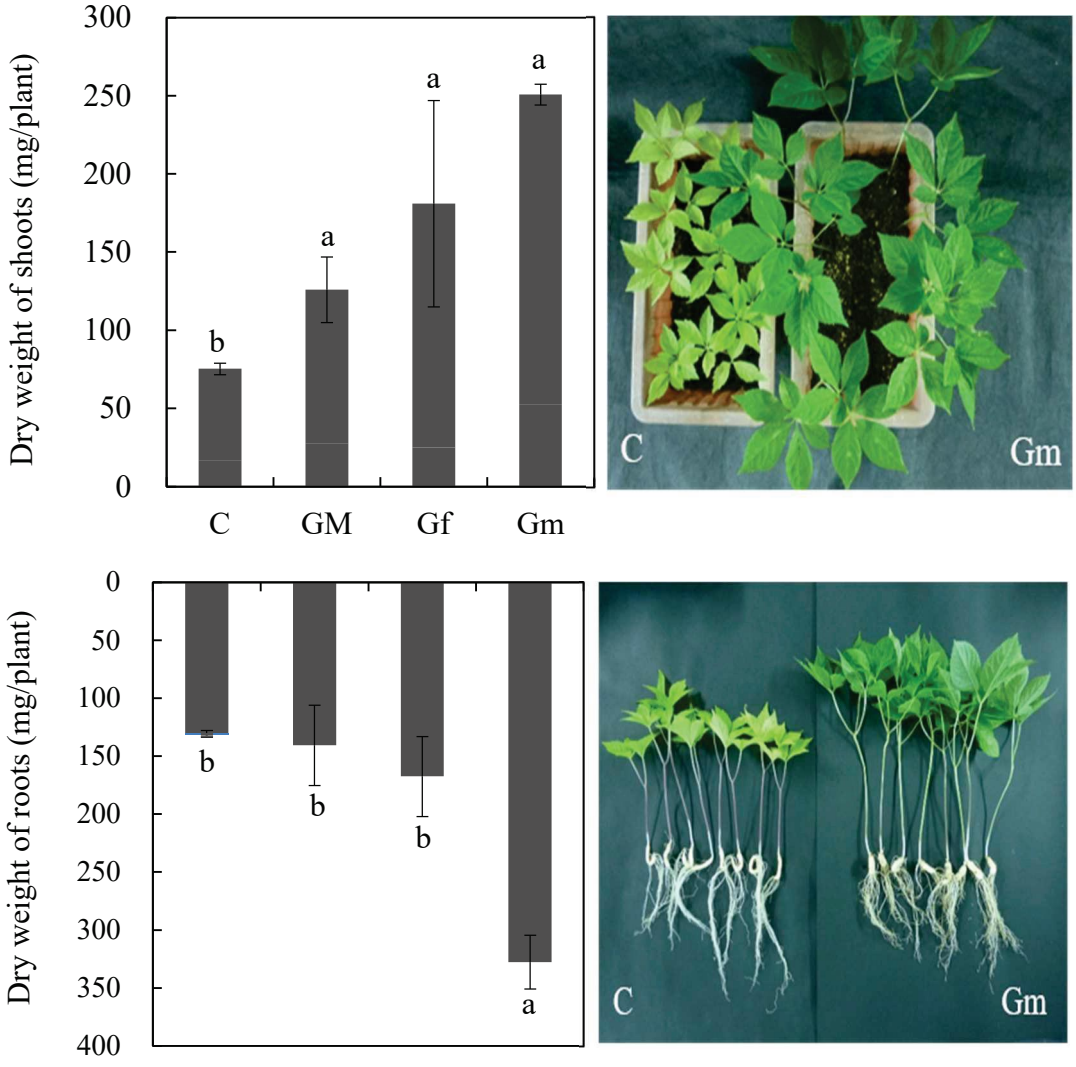


Fig. 9. Dry weight of ginseng plants 15 weeks after AMF inoculation. Here, C, control; GM, *Gigaspora margarita*; Gf, *Glomus fasciculatum,* Gm, *Glomus mosseae.* Columns denoted by different letters indicate significant differences according to Tukey's test ($P \leq 0.05$).

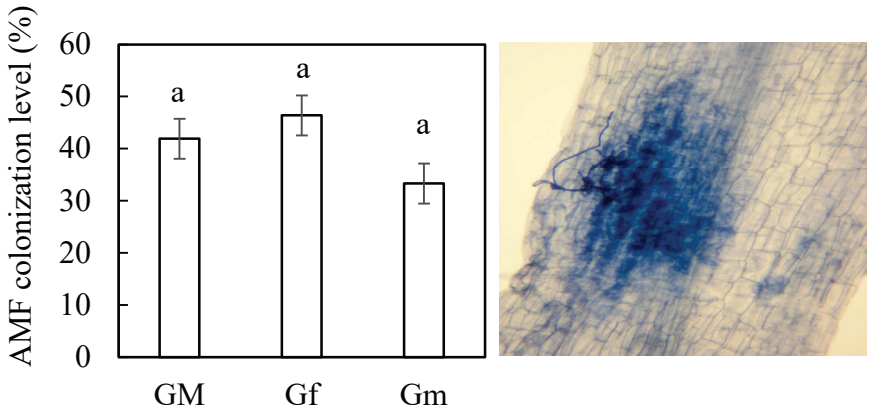


Fig. 10. AMF colonization levels and colonization in ginseng roots 15 weeks after AMF inoculation. Here, GM, *Gigaspora margarita*; Gf, *Glomus fasciculatum*; Gm, *Glomus mosseae*. Columns denoted by different letters indicate significant differences according to Tukey's test ($P \leq 0.05$).

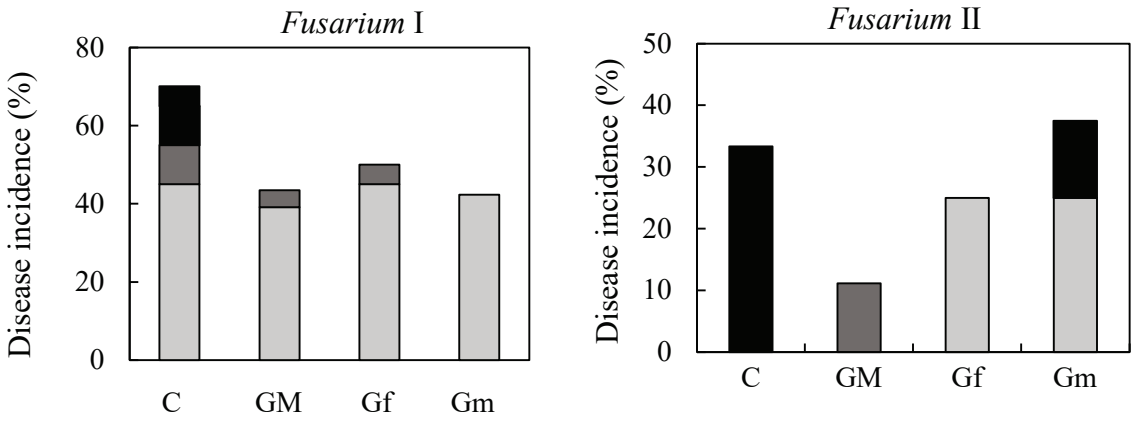


Fig. 11. Disease incidence of *Fusarium* root rot in ginseng plants 4 weeks after inoculation with *Fusarium* spp. (*Fusarium* I and *Fusarium* II) respectively.

C, control; GM, *Gigaspora margarita*; Gf, *Glomus fasciculatum*; Gm, *Glomus mosseae.*

Symptom in a root system;□,a part,▒,half, ■ ,whole.

Discussion

PCR-SSCP analysis showed that the pathogen population diversity within the region of Japan varied from the northern to the central parts of Japan. The strains isolated from root rot patients showed band patterns that were different from those of previously reported pathogens. Among them, two band patterns (I and II in Fig. 7) were frequently detected at the two cultivation sites. They grew on Komada medium "(26)" and were consistent with the band patterns of other *F. oxysporum* series and *F. proliferatum*. In this case, sequence analysis of the ITS-5.8SrDNA region was outsourced (Techno Suruga Laboratory Co., LTD.) for *Fusarium* I and *Fusarium* II (only homology rate); the top homologous strain for *Fusarium* I was *F. oxysporum* (NRRL22902, homology rate 100%), and for *Fusarium* II was *F. proliferatum* (NRRL22944, homology rate 99.8%), respectively (data not shown). However, to identify the species of *Fusarium*, it is necessary to confirm their morphology and other nucleotide analysis. Further investigations are needed on this point.

In this experiment, in vitro pathogenicity studies have shown that *Fusarium* spp. cause root rot and can be re-isolated from diseased roots, suggesting that they are the biotic factors involved in the symptoms of ginseng root rot. The result of the present study, with more than 70% of both fields studied infected with *Fusarium* spp., differed from the results of previous studies. Nahiyan et al. "(16)" found by PCR-SSCP method without sequence analysis that among 651 *Fusarium* isolates of asparagus plants, *F. oxysporum*, f. sp. *asparagi*, and *F. proliferatum* are important biotic factors that led to asparagus decline in Japan. On the other hand, Lee (27) found the opposite result among 115 isolates of Fusarium species from ginseng roots rotted in Korea: 55 (48%) were *Fusarium solani,* 35 (30%) were *F. oxysporum*, 10 (9%) were *Fusarium moniliforme*, and 15 (13%) were strains of eight other species. Of these, only one isolate of *F. solani* and one isolate of *F. oxysporum* were weakly pathogenic, but *P. cactorum*, *Pythium ultimum*, and *C. destructans* were highly pathogenic to ginseng roots and

major causal organisms for root rots. Thus, if there are numerous *Fusarium* spp. in the soil of Korea, their ability to cause root rot is poor. However, the major biotic agents responsible for decline in ginseng production differ worldwide. Reeleder et al. "(6)" and Kang et al. "(28)" found that in ginseng plants, *C. destructans* and *F. solani* are factors in the development of root rot symptoms because they are difficult to control with high soil persistence. Fan et al. "(29)" reported that *Fusarium redolens* cause root rot in American ginseng in China. *Panax quinquefolius*, *F. solani*, *P. cactorum*, and *C. destructans* have been shown to cause root rot in *P. ginseng* in Korea and China "(11)". However, our results showed that *Fusarium* spp. are the major causal organisms associated with ginseng root rot, suggesting that they are important biotic factors leading to ginseng decline in Japan.

In the present study, the dry weights of shoots and roots were higher in some AMF plants than in non-mycorrhizal plants, indicating that growth enhancement through symbiosis occurred in mycorrhizal ginseng plants. Previous studies observed similar results, indicating that AMF has a growth-promoting effect in host plants "(30-31)". Ozgonen and Erkilic (30) reported that growth promotion and tolerance to *Phytophthora capsici* had no correlation with mycorrhizal colonization levels in peppers. In the present study, we could not clarify the relationship between AMF colonization levels and disease tolerance. In contrast, Sutton "(32)" demonstrated that AMF colonization consists of three phases: 1) a lag phase during which spore germination, germ tube growth, and initial penetration occur; 2) a rapid growth phase, coinciding with the development of external mycelia and spread of the fungus within the roots; and 3) a stable phase during which the proportion of infected roots to non-infected ones remains nearly constant. In this study, the colonization level was checked only once. Hence, it was difficult to estimate when the AMF reached the maximum colonization level during the experimental period and how the colonization level affected disease tolerance.

Regarding tolerance to *Fusarium* root rot in this experiment, both the incidence and severity of symptoms were reduced by AMF, suggesting that tolerance to *Fusarium* root rot occurred in the mycorrhizal ginseng plants. The same phenomenon has been observed in mycorrhizal asparagus plants tolerant to *Fusarium* root rot "(33)". However, AMF-inoculated plants showed lower disease incidence than non-mycorrhizal plants, and the severity of symptoms varied depending on the *Fusarium* isolate. Maya and Matsubara "(34)" reported that the incidence of symptoms caused by *F. oxysporum* and *Colletotrichum gloeosporioides* in cyclamen plants was reduced by AMF inoculation; however, the effect differed between AMF species.

In the present study, tolerance to *Fusarium* root rot was observed in the mycorrhizal ginseng plants. Some reports have described that AMF colonization induces a temporary increase in antioxidative abilities, such as superoxide dismutase (SOD), guaiacol peroxidase, catalase, ascorbate peroxidase (APX), and flavonoid content, suggesting that colonization might be a temporary stress for host plants "(31, 35-38)". Al-Askar and Rashad "(39)" found that the application of arbuscular mycorrhizal fungi as a biocontrol agent played an important role in plant resistance and exhibited greater potential to protect bean plants against infection with *F. solani*. The mechanisms underlying the disease tolerance in mycorrhizal plants remain unclear. In summary, the findings of this study suggest that AMF colonization increases the tolerance to *Fusarium* root rot caused by *Fusarium* spp. in ginseng. Therefore, AMF may be successful as a biocontrol agent for the sustainable agricultural production of ginseng.

Chapter 1-1-Conclusion

From the findings of the study, we can see that a total of 102 isolates were obtained from the rotted roots of ginseng across two separate domestic regions in Japan and were identified as *Fusarium* species. Most of the *Fusarium* isolates were highly pathogenic in vitro. After identifying the biotic factor, growth promotion and tolerance to *Fusarium* root rot in the mycorrhizal ginseng plants were investigated. Arbuscular mycorrhizal fungi (AMF) promote host plant growth mainly by enhancing phosphorus uptake through symbiosis. Results showed that mycorrhizal plants had higher shoot and root dry weights than control plants. AMF colonization levels in the root systems were not significantly different among the species. Four weeks after *Fusarium* inoculation, disease incidence and severity of symptoms decreased in mycorrhizal ginseng plants compared to control. These findings demonstrated that *Fusarium* spp. are associated with ginseng root rot, and growth enhancement and tolerance to *Fusarium* root rot were confirmed in mycorrhizal ginseng plants. Further research is necessary regarding the relationship between Asian ginseng decline and soil chemical properties. We tried to address these points in the following chapter.

CHAPTER 1-2

Relationship between Asian ginseng decline and soil chemical property

Introduction

Asian ginseng (*Panax ginseng*) is a perennial herb in the *Araliaceae* family that is widely used as a medicinal plant around the world "(2)". It provides a wide range of health benefits, including increasing mental health, concentration, and memory, reducing the risk of diabetes and cancer, controlling blood pressure, and suppressing stress "(3)". Ginseng is artificially cultivated under shade because of its slow assimilation capacity, low fertilizer absorption, and susceptibility to damage by high light "(7)". As a result, growth is slow, and it requires 3 to 5 years to produce a marketable crop from seeds "(4)", and peak production occurs within 4 to 6 years. However, before reaching the peak production stage, marketable yields decrease due to abiotic and biotic factors that cause decline symptoms and replant problems. Growers' experiences suggest that ginseng cultivation on sites previously used for ginseng production often fails in the second crop, a phenomenon known as replant disease or decline "(6)". From sowing to harvest, no more than 60% of ginseng plants can survive "(40)". The biotic agents responsible for the decline in ginseng production may differ worldwide. *Alternaria panax* and *C. destructans* were reported for Asian ginseng decline "(12)". Although *Fusarium* spp. is reported as a ginseng root rot, *Cylindrocarpon destructans* is the leading cause of ginseng decline and replant failure in North America "(6)". However, some researchers believed that the yield loss of replant crops was closely related to allelopathy "(41)". However, ginseng production is severely impeded by continuous cropping obstacles, and the chemicals present in the rhizosphere are said to cause growth inhibition. In addition, phenolic compounds have been shown to inhibit seedling root growth in *Panax* plants "(9)". The relationship between allelochemicals, soil chemical properties (inorganic components, pH), and plant growth is complicated in continuous cropping obstacles. The past few years have seen research on *P. ginseng* cropping barriers in the areas of soil nutrients "(42-43)", beneficial microbes "(44)", and microbial diversity "(45)". Recently, researchers focused on the relationship between

allelochemicals, soil microorganisms, and soil chemical properties in ginseng cultivating soil. Allelochemicals, including benzoic acid, di-isobutyl phthalate, coumaric acid, vanillic acid, palmitic acid, and 2, 2-bis-(4-hydroxyphenyl) propane were detected from ginseng rhizosphere soil and root exudates simultaneously, which inhibited the germination of ginseng seeds and growth of ginseng plants "(46)". Although a few researchers have carried out an investigation on the relationship between allelochemical and plant growth there was a lack of comprehensive analysis of the relationship between allelochemical, soil chemical properties, and plant growth. The reports are few and still unclear.

In this experiment, the relationship between allelochemical in field soil, soil chemical properties, and plant growth was estimated by the in vitro bioassay method. The results would be helpful for the further interpretation of the relationship among allelochemicals, soil chemical property, and plant growth in ginseng-cultivating soil, which would help us to understand the formation mechanism of ginseng continuous cropping obstacles.

Materials and Methods

Bioassay in vitro: Soils of different cultivation terms [1-1, 1-2, 4, 5-1, 5-2, 6-1, 6-2; cultivation years (1, 4, 5, 6) and replication (-1, -2)] were sampled from Asian ginseng fields. These soil samples were dried in an oven (70℃, 12h) and immersed in distilled water (10%, w/v) for 4 days. Then, the extracts were filtered (40ml) and mixed with 0.3g agar. The mixture was autoclaved to prepare the bioassay medium. Lettuce (*Lactuca sativa* L.'Greatlake') seeds were sterilized with 70% ethanol (5 sec) and 10% antiformin (0.5% available chlorine, 3min) and sowed on the bioassay medium and cultured in growth chamber (25℃, 12h daylength). After a week, lettuce seedlings were uprooted and the dry weight of shoots and roots and root length were measured.

Chemical analysis of soils in the ginseng fields: 10 ml of distilled water and 4 g of dry field soil were added to a 15 ml conical tube, shaken for 30 minutes, and then allowed to stand for 1 hour. The pH of the soil suspension was measured with a handheld pH meter D-51 (Horiba Co., Ltd.). A soil suspension (50%, w/v) was prepared by adding 8 ml of distilled water and 4 g of dried field soil to a 15 ml conical tube and immersing the tube in shaking for 4 days. The soil suspension was centrifuged (4,000 rpm, room temperature, 30 min, KUBOTA 2800, Kubota Shoji Co., Ltd.), and the supernatant obtained was diluted with distilled water as appropriate and used as the analysis sample. The NO_3^-, NH_4^+, $PO4^{3-}$, Ca^{2+}, and Mg^{2+} contents were measured using a simple reflectance photometer (RQ Flex plus 10, Kanto Chemical Co., Ltd.) and special test paper (Reflection Quant, Kanto Chemical Co., Ltd.), and the K^+ content was measured using a compact potassium ion meter B-731 (Horiba Co., Ltd.). The number of repetitions was set to three.

Analysis of soil components by LC-MS: The soil suspension (50%, w/v) was centrifuged (13,000 rpm, 4°C, 10 min, 5417R, Eppendorf Corporation). The supernatant was filtered through a sterile syringe filter (0.45 μm, DISMIC-25CS, Toyo Filter Paper Co.). The resulting

filtrate was centrifuged (13,000 rpm, 4°C, 15 min) using Nanosep 10K (Nippon Paul Co., Ltd.) to remove proteins, and this was used as the sample for soil water extraction analysis. The soil suspension (50%, w/v) prepared by adding 8 ml of 99.5% methanol and 4 g of dry field soil to a 15 ml conical tube and shaking for 4 days was treated in the same way as the water extraction analysis sample and was used as the soil methanol extraction analysis sample.

The samples were analyzed using UPLC-MS (Waters Corporation). The conditions of the liquid chromatograph (ACQUITY UPLC, Waters Corporation) were as follows. ACQUITY UPLC BEH C18 (1.7μm, 2.1×100mm, reversed-phase column, Waters Corporation) was used as the column at 40°C conditions. The mobile phase was (A) 0.1% aqueous formic acid and (B) acetonitrile, and the gradient was set (0 min, 95% A; 6 min, 95% A; 12 min, 75% A; 30 min, 65% A; 32.5 min, 5% A; 35 min, 95% A) for 35 min at a flow rate of 0.4 ml/min. The sample injection volume was set at 7.5μl. The MS/MS collision was performed at 30 eV. The m/z values of the peaks in the liquid chromatograph obtained by UPLC-MS and the Mass Bank database were used to search for substances in the ginseng field soil and the main root of the ginseng.

Table 4. Types of soil collected from *Panax ginseng* fields.

1-1	1st year seedling cultivation soil
1-2	1st year seedling cultivation soil (withering)
4	4th year seedling cultivation soil
5-1	5th year seedling cultivation soil
5-2	5th year seedling cultivation soil (2nd year seedling transplantation)
6-1	6th year seedling cultivation soil
6-2	6th year seedling cultivation soil

Results and Discussion

The root length significantly decreased (Fig. 12) in long-term plots (4, 5-1, 5-2, 6-1, 6-2 years) compared to shorter ones (1-1, 1-2). In this case, lettuce root length was significantly suppressed in the high concentration (20%) than in the low concentration (10%). A similar result was observed, in the dry weight of roots decreased in the long-term plot (4, 5-1, 5-2, 6-2) except 1-2 and 6-1. On the other hand, the dry weight of shoots increased in long-term plots (4, 5-1, 5-2, 6-1, 6-2) compared to shorter ones (1-1, 1-2) in Fig. 13. Similar to root length, the dry matter weight of the underground part was significantly lower in the long-term and high-concentration plots than in the short-term and low-concentration plots. From these results, physical and biological factors in the media were common among the plots, so that some chemical factors would have been concerned with the suppression of lettuce root growth in long-term plots. These results suggest that there are chemical factors that inhibit the growth of lettuce hypocotyls in long-term cultivated soil.

The chemical factors that affect the underground growth of plants include inorganic fertilizer components, pH, and allelochemicals in the soil. The soil pH of all the plots examined in this study was around 7 (Fig. 14). The lowest pH was 6.8 in the 5-2, and the highest pH was 7.5 in the 6-1. No significant difference appeared in the soil pH among the plots. As for pH, it has been shown that low pH (acidic soil) is associated with the accumulation of toxic components (H^+, Al, etc.) and deficiency of soluble nutrients (P, K, Ca, Mg, etc.) and Inhibits plant growth including inhibition of root elongation “(47)”. However, it is unlikely that pH affected root elongation because the pH of the field soil in this experiment was all around 7, and there was no correlation with lettuce root length in the bioassay. The result of four soil inorganic component measurements showed similar characteristics. As for the NO_3^-, K^+, Ca^{2+}, and Mg^{2+} concentrations in the soils, the 5-2 plot contained the highest, followed by plot 4 (Fig. 14). Conversely, the 1-1 plot showed the lowest concentrations of NO3-, Ca^{2+}, and Mg^{2+},

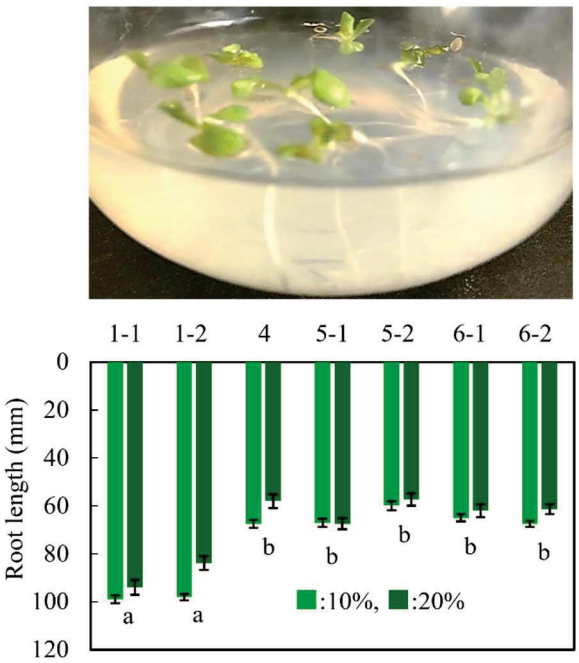


Fig. 12. Root length of lettuce in bioassay.

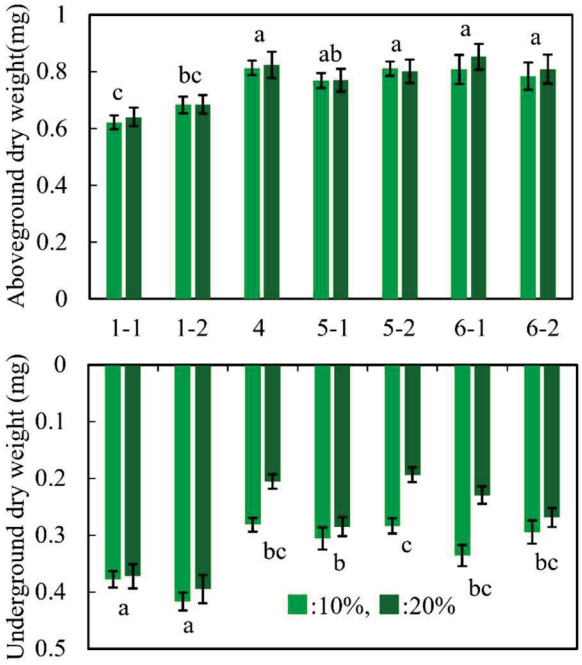


Fig. 13. Dry matter weight above and below the lettuce in the bioassay.

- Soil extract; , 10%; , 20% (w/v). The bar represents the standard error. There is a significant difference between different alphabets in terms of years of cultivation (Tukey-Kramer test, $p \leq 0.05$).

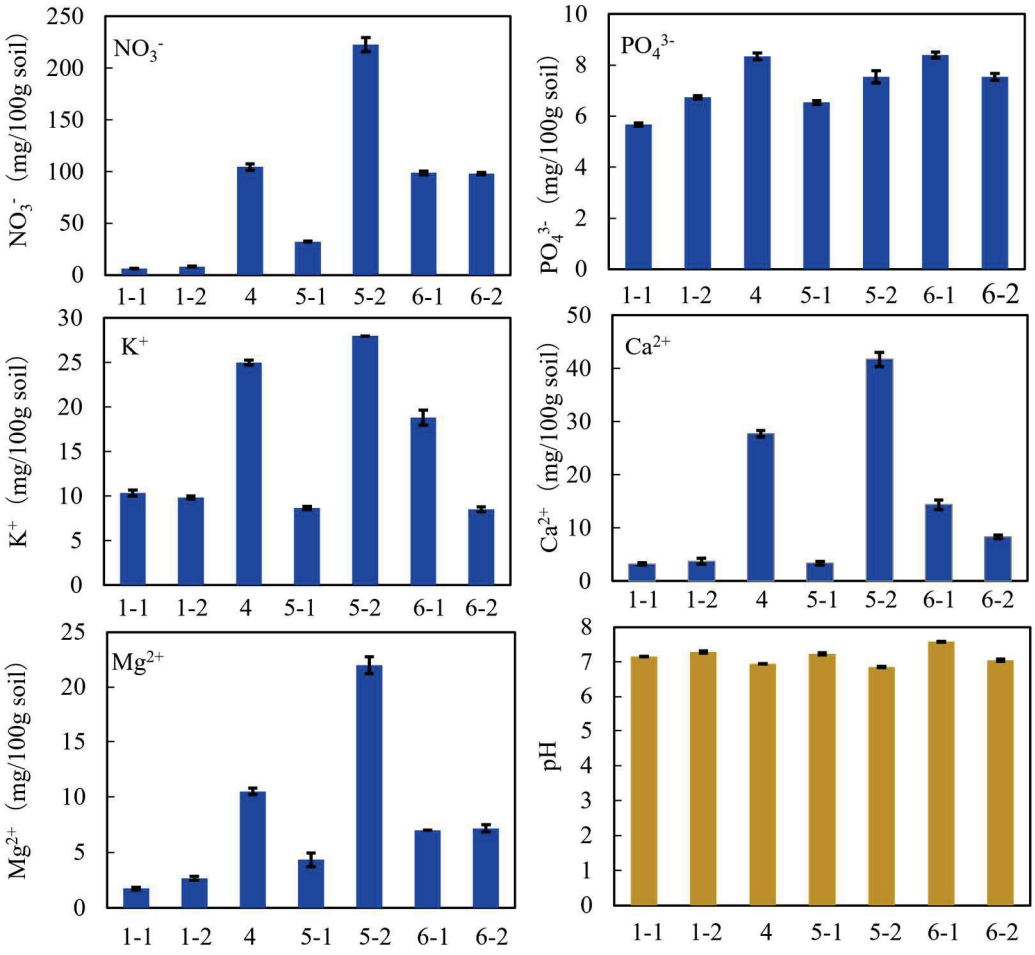


Fig. 14. Inorganic component and pH of the test.

while the 5-1 plot had the lowest $K^{+,}$ PO_4^{3-} concentration had a small difference among cultivation terms. The highest values of NO_3^-, $K^{+,}$ Ca^{2+}, and Mg^{2+} contents were 223, 28, 42, and 22, respectively. The next highest values were found in the fourth-year zone, with 104, 25, 28, and 11, respectively. On the other hand, the lowest values of NO_3^-, Ca^{2+}, and Mg^{2+} contents were observed in 1-1, which were 6, 3, and 2, respectively. The difference in PO_4^{3-} content among treatments was small, with all treatments showing values around 7. Generally, on low nutrient conditions, plants promote root growth rather than shoot, conversely on high nutrient conditions, shoot growth is promoted. So, the difference in lettuce growth among the cultivation terms might be caused by the difference in mineral nutrient concentration.

However, the lettuce growths were almost similar among long cultivation terms although there were some differences in nutrient concentration. Therefore, some other chemical factors besides nutrition might play a role in lettuce growth between short and long cultivation plots. Responses to soil inorganic constituents commonly observed in plants include a decrease in tap root ratio due to nutrient deficiencies, enhanced sugar partitioning to the underground due to nitrogen, phosphorus, and sulfur deficiencies, and enhanced allocation of photosynthesis to the aboveground and reproductive organs due to high nutrient concentrations “(48)”. As for allelochemicals, it has been shown that plant metabolic components such as L-DOPA, a non-protein amino acid in the legume Mucuna plant, cyanamide in hairy vetch, and the triterpenoid saponins in *Formica sanguineae* inhibit root elongation in lettuce and other plants “(49)”.

As a result of UPLC-MS analysis on 1-1, 5-2, and 6-2 soils, a unique specific peak was observed only in the long-term plots (5-2 and 6-2) at the retention time 5.8, which was absent in the short-term plot (1-1). Two m/z values, 262.92 and 264.92, were detected pertaining to the retention time (Fig. 15). On the other hand, the substances with m/z values of 262.92 and 264.92 were not detected in the methanol extract of the test soil and the water/methanol extract

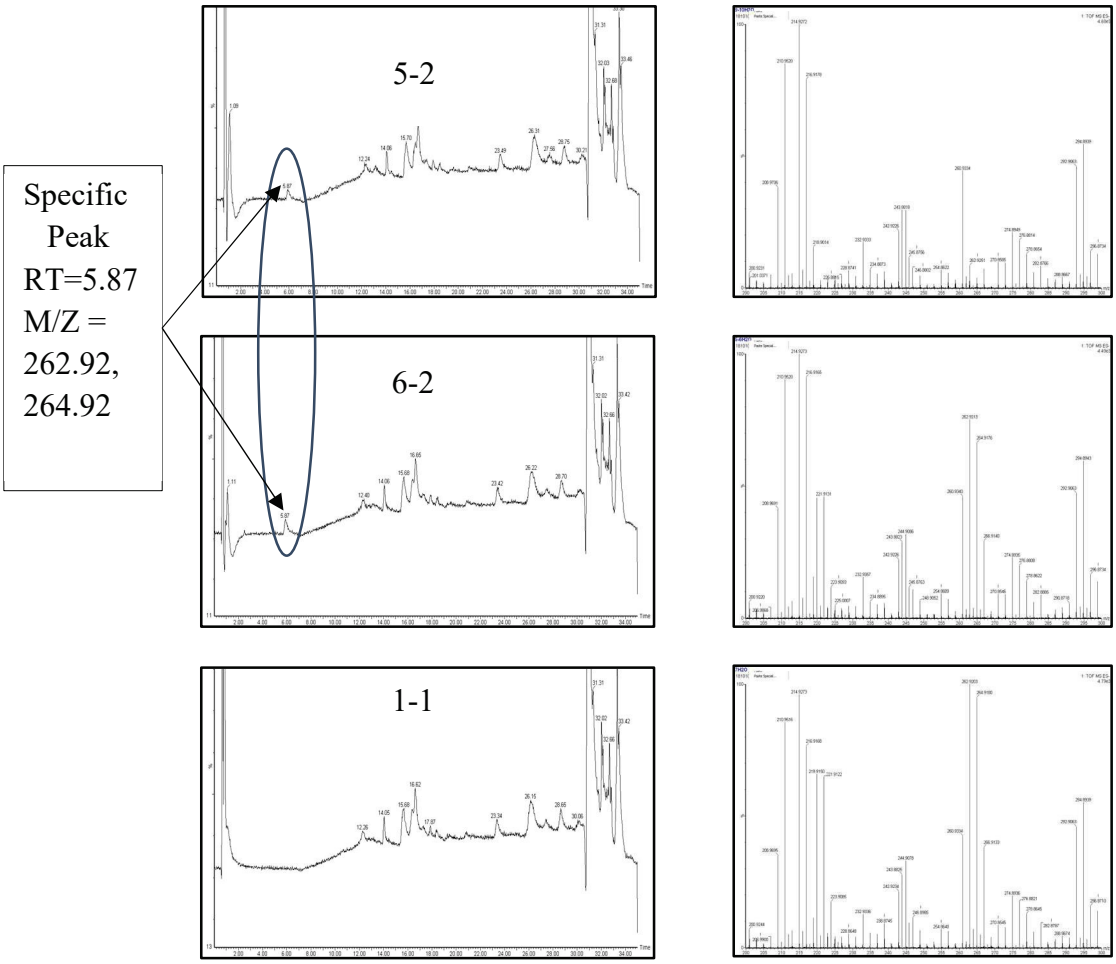


Fig. 15. UPLC chromatogram of *Panax ginseng* field soil water extract and specific peak in 5-2 and 6-2 soil extracts (RT 5.8 min). Top, 5-2; Middle, 6-2; Bottom, 1-1.

of the 6-year root of ginseng. In addition, the previously reported allelochemicals (benzoic acid, diisobutyl phthalate, diisobutyl succinate, palmitic acid, and 2, 2-bis-(hydroxyphenyl) propane detected in the rhizosphere of Asian ginseng "(43)", there are no substances showing m/z values of 262.92 and 264.92. In addition, there are several substances inferred from the m/z values of 262.92 and 264.92 on MassBank, and they may be unidentified substances that are not listed on MassBank, so further detailed analysis is necessary in the future. Therefore, these compounds might be responsible for the suppression of root growth in lettuce in long-term plots. However, these detected compounds have not been reported as allelochemicals in ginseng. These compounds should be identified, and it is necessary to estimate whether such compounds are derived from ginseng roots. Further study would be needed on those points. Henceforth, there is a need for a more detailed analysis of chemical properties in ginseng field soil.

Conclusion

Asian ginseng is an important herbal medicinal plant that has been used for thousands of years throughout the world, including Japan. However, before it reached its peak of production, marketable yields declined due to abiotic and biotic factors, though major reasons are still unclear in Japan and differ from country to country. Researchers found that from sowing to harvest, no more than 60% of ginseng plants can survive and believed that the yield loss of replant crops was closely related to allelopathy. Although a few research was carried out regarding allelochemical and plant growth there was a lack of comprehensive analysis of the relationship between allelochemical, soil chemical properties, and plant growth, so the results are still unclear. In this experiment, allelochemicals in the field soils (1-6 year-cultivated) were estimated by in vitro testing method using lettuce (*Lactuca sativa* L. 'Great lakes') seedlings, susceptible to allelochemicals. The dry weight of roots and root length of the indicator plant decreased in the long-cultivated soil plots compared to the short one. In this case, soil chemical properties such as mineral elements (NO_3^-, PO_4^{3-}, K^+, Ca^{2+}, Mg^{2+}) and pH had no consistent association with growth suppression. Therefore, some other chemical factors besides nutrition might play a role in lettuce growth between short and long cultivation plots. UPLC-MS analysis showed a specific peak (5-2, 6-2 plots at the retention time of 5.8) in the long-cultivated field soils, which was absent in the short-term plot. Two m/z values of 262.92 and 264.92, were detected pertaining to the retention time. Therefore, these compounds might be responsible for the suppression of root growth in lettuce in long-term plots. These compounds should be identified, and further study is needed to elucidate the interaction mechanisms between allelochemical diseases, soil chemical properties, and plant growth. However, results would be helpful for the further interpretation of the relationship among allelochemicals, soil chemical property, and plant growth in ginseng-cultivating soil, which would help us to understand the formation mechanism of ginseng continuous cropping obstacles.

CHAPTER 1-3

Growth promotion and changes in functional constituents through AMF symbiosis in Asian ginseng

Introduction

The demand of medicinal plants has been increasing in recent years due to the aging of the population and the expansion of the market as functional vegetables and foods. “Functional foods” are now defined as “Food or dietary components that may provide health benefits beyond their basic nutritional function” (International Food Information Council: IFIC). Asian ginseng is native to the Far-East, including China, Korea, Japan, and Far-Eastern Siberia. It has been used for health-related purposes in traditional Chinese medicine for thousands of years. Asian ginseng (*Panax ginseng* C. A. Meyer) is a perennial herb of the *Araliaceae* family and commonly used as a medicinal plant all over the world, and nowadays, it is also used as a functional vegetable and food “(50)”. It is used as a material of traditional Chinese medicine based on pharmacological components (mainly ginsenosides), known the use in China for over 4000 years “(51)”. In Japan and North America, it is commonly consumed as a dietary supplement or nutritional drink, but in Korea, fresh ginseng root is available in the market and is used in various cuisines such as Gwangchoyu (chicken and carrot soup) “(52-53)”. It is taken orally and is promoted for increasing resistance to environmental stress and as a general tonic to improve well-being. It is also promoted as a dietary supplement for various other reasons- to improve physical stamina, concentration, and memory. It stimulates immune function, slows the aging process, and relieves various other health problems such as respiratory and cardiovascular disorders, depression, anxiety, and menopausal hot flashes. In addition, recent studies have shown that ginseng root and red ginseng (peeled, steamed, and dried ginseng root) have a wide range of beneficial effects, including suppression of cancer, diabetes, and dementia, blood pressure regulation, and relief of fatigue and stress “(3)”.

The cultivation of ginseng plants has some problems. Due to the long cultivation period, low survival rate due to disease outbreaks and fluctuation in medicinal components due to the cultivation environment-Asian ginseng cultivation is decline. In addition, the limitation of

agrochemical use in medicinal plants has also led to the limitation of disease control in the long term and low survival rate of plants. On the other hand, since the functional components of medicinal plants are secondary metabolites, fluctuation due to environmental factors is also an issue.

Arbuscular Mycorrhizal Fungi (AMF) are beneficial microorganisms and form a symbiotic relationship with the roots of most terrestrial plants. AMFs are symbiotic with more than 80% of land plant species, supplying phosphate and trace elements to improve the nutritional status of host plants and promote their growth and are supplied with monosaccharides by host plants “(54)”. AMF has the functions as a biofertilizer and biochemical. The use of mycorrhizal symbiosis may provide an alternative to high inputs of fertilizers and agrochemicals in sustainable crop production systems. The effect of AMF depends on AMF species, host plant, etc. In mycorrhizal fungi symbiosis plants, it has been used for growth promotion “(55)", disease resistance “(18, 56-57)” and improvement of physical stress tolerance including salt and drought “(58-60)”, variation in endogenous contents “(61-62)”, and various other effects have been reported.

Materials and Methods

Inoculation of Mycorrhizal Fungi and Plant Growth: *Panax ginseng* C. A. Meyer seedlings were grown in autoclaved (121°C, 150 kPa, 30 min) commercial soil (Super Mix A, Sakata Seed Corporation, N: P: K = 180:120:220 mg/L) mixed with akadama soil at a ratio of 3:1 in a vat (13.5 × 27.0 × 15.5 cm). Ginseng seedlings were inoculated with 5 g/plant of commercial mycorrhizal fungus inocula (*Gigaspora margarita*, GM; *Glomus fasciculatum*, Gf; obtained from Idemitsu- Agri Co. Ltd., Tokyo, Japan and Centralgrass Co. Ltd. Tokyo, Japan, respectively; spore density unknown). The non-mycorrhizal containers received the same amount of autoclaved (121 °C, 1.2 kg/cm, 30 min) inocula. Twenty plants/plots were grown in a greenhouse for 15 and 30 months, which was shaded so that the irradiance at noon in summer was about 5,000 lux, with appropriate irrigation.

Growth Survey: 15 and 30 months after AMF inoculation, SPAD value, root length, root diameter, and dry weight of shoots and roots were measured.

Investigation of AMF Colonization Level: Root staining for the investigation of colonization level was done according to the method of Phillips and Hayman "(25)". At the time of the growth survey, lateral roots of surviving individuals in each treatment were collected, fixed with 70% ethanol, ethanol was removed with running water, and autoclaved (121°C, 150 kPa, 15 min) while immersed in 10% KOH. The alkali was then removed with running water and HCl and stained with trypan blue solution (50 ml of glycerin, 50 ml of lactic acid, 50 ml of redistilled water, and 1 g of trypan blue mixed and scale-up to 500 ml with 70% ethanol). After staining, roots were shredded to around 1 cm, and 30 sections of each treatment were observed under an optical microscope for 5 replicates to investigate the mycorrhizal infection rate using

the following formula. AMF colonization level (%) = Colonized root sections / [Number of observed root sections (30 sections)] × 100.

Ginsenosides Determination: In this study, the purpose is to establish cultivation techniques for ginseng plants similar to functional vegetables and foods that promote ginseng growth by arbuscular mycorrhizal fungi, induce environmental stress tolerance and improve functional secondary metabolites. A sample of 300 mg was weighed, 10 mL of 99.5% methanol was added, and the sample was crushed in an ice-cold mortar. The residue and extract were transferred to a flask and shaken for 15 minutes while warming in a water bath at 80°C. After shaking, 99.5% methanol was added so that the total volume of residue and extract solution was 6 mL, and the difference in solution concentration due to evaporation caused by heating was adjusted. The solution and residue were then centrifuged twice (4,000 rpm, 4°C, 15 min, Table Top Cooling Centrifuge 2800, Kubota Shoji Corporation and 13,000 rpm, 4°C, 15 min, Centrifuge 5417R, Eppendorf Corporation). The supernatant obtained by centrifugation was filtered through a sterile syringe filter (0.45 μm), and the filtrate obtained was centrifuged (13,000 rpm, 4°C, 15 min) using Nanosep 10K (Nippon Paul Co., Ltd.) to remove proteins, which were used as the analysis sample. The determination of ginsenosides in the analysis samples was entrusted to the Japan Food Research Laboratories. The determination of ginsenosides was performed by UPLC-MS.

Free Amino Acid Analysis: 200 mg of the main root of ginseng was weighed, 3 mL of 0.2N perchloric acid and a small amount of sea sand were added, and the mixture was crushed in a cooled mortar and then co-washed with 1 mL of ultrapure water and added to the extract. The supernatant was then centrifuged (4,000 rpm, 4°C, 10 min, Table Top Cooling Centrifuge 2800, Kubota Shoji Co., Ltd.) and adjusted to about pH 4 with potassium bicarbonate powder. The supernatant was then centrifuged (13,000 rpm, 4°C, 5 min, Centrifuge 5417R, Eppendorf Corporation), and the supernatant obtained was filtered through a syringe filter (GL

Chromatodisc, G.L. Science Corporation) and used as the analysis sample. Analyzed samples were derivatized with the AccQ-Tag Ultra Derivatization Kit (Waters). Mix 210 μL of boric acid buffer (< 5% sodium tetraborate solution) with 30 μL of the analysis sample, add 60 μL of the derivatization reagent [9 g/L 6-aminoquinolyl-N-hydroxysuccinimidylcarbamate (ACQ) acetonitrile solution], and leave at room temperature for 1 min. The derivatization was carried out by heating the mixture to 55°C for 10 minutes. The derivatized samples were analyzed by UPLC-MS (Waters). The conditions for liquid chromatography (ACQUITY UPLC, Waters) were as follows. ACQUITY BEH C18 (1.7 μm, 2.1 x 100 mm, reversed-phase column, Waters) was used as the column under 25°C conditions. The mobile phase was (A) 0.1% aqueous formic acid for the inorganic system and (B) acetonitrile for the organic system, and the measurement was performed for 15 min. The sample injection volume was 5 μL. On the other hand, a mass spectrometer (Xevo QTof MS, Waters) was used in ES (+) positive mode to measure the analytical mass range of 100-1000 (m/z). A mass chromatogram (Abs Window 0.05Da) of the m/z value of each amino acid was prepared from the measurement results, and the amino acid content was determined by the value of the peak integral. The repetition was set to 2. A standard solution containing asparagine, glutamine, gamma-aminobutyric acid (GABA), and citrulline added to amino acid mixture standard H (Wako Pure Chemical Industries, Ltd.) was used to measure the retention time and m/z value of each amino acid and to prepare a calibration curve. Seventeen types of free amino acids were analyzed, all included in the standard solution.

Results

After 15 months of AMF inoculation, plant growth promotion via symbiosis appeared in mycorrhizal ginseng plants, with Gm and Gi being the most effective. The plants inoculated with Gm (*Gl. mosseae*) and Gi (*Gl. intraradices*) showed vigorous growth, short taproot, and wider root diameters (Fig. 16). AMF colonization occurred successfully (more than 34%) in all the AMF species inoculated plants, and no significant difference was noted among the treatments (Fig. 17).

The SPAD value appeared higher in mycorrhizal ginseng plants than non-mycorrhizal ginseng plants, indicating leaf chlorophyll concentration is higher in AMF ginseng plants (Fig. 18). Mycorrhizal ginseng plants had higher dry weight in both shoots and roots while Gm and Gi being most effective (Fig. 19). AMF decreases the length of main roots and increases the diameter of main root which is beneficial, Gm showed the best performance (Fig. 20). Contents of ginsenoside Rb_1 and Rg_1 appeared higher (Gm showed highest) in mycorrhizal ginseng plants (Fig. 21). That means mycorrhizal ginseng plant has a variety of potential health effects, including anti-carcinogenic, immunomodulatory, anti-inflammatory, anti-allergic, anti-hypertensive, anti-diabetic, anti-stress activity, and effect on the central nervous system, anti-aging, anti-fatigue, and memory enhancing properties. 30 months after AMF inoculation, the dry weight of both shoots and roots appeared higher in mycorrhizal ginseng plants (Fig. 22). After 30 months, Gm-inoculated ginseng appeared to have the highest value of ginsenoside content (Fig. 23). As for the free amino acid contents through LC-MS analysis, mycorrhizal Gi inoculated plants had higher total free amino acid contents compared to non-mycorrhizal plants that mean total free amino acid contents significantly increased in mycorrhizal plants (Fig. 24).

Several free amino acids such as arginine, proline, methionine, phenylalanine, asparagine, glutamic acid, aspartic acid, threonine, alanine, GABA, lysine, and tyrosine were increased in mycorrhizal (Gi) ginseng plants. In addition, GABA and aspartic acid appeared at higher

significant values, where GABA plays an important role in various physiological processes like nitrogen storage, protects from oxidative stress and osmoregulation, and acts as a signaling molecule in stress response. GABA is also important for human health for its potential anti-hypertensive effect. Aspartic acid is the precursor to several amino acids, such as arginine, methionine, threonine, and isoleucine. So, in this experiment, aspartic acid and GABA accumulation might be closely related to amino-acid-mediated stress alleviation. From these findings, it is suggested that plant growth promotion and significant, positive changes in functional constituents through AMF symbiosis occurred in mycorrhizal Asian ginseng plants.

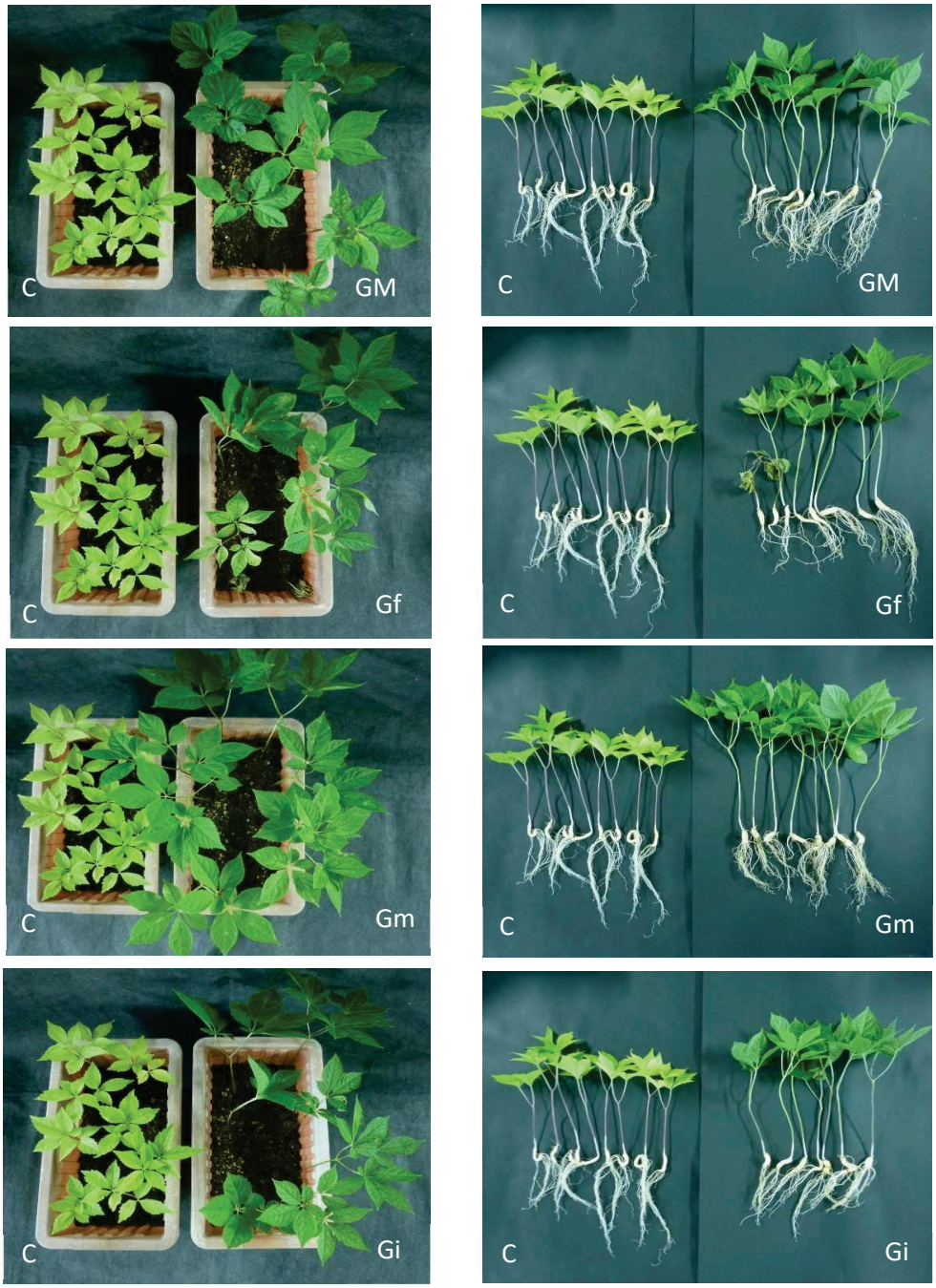


Fig. 16. Growth of ginseng 15 months after AMF inoculation. Here, C, control; GM, *Gigaspora margarita*; Gf, *Glomus fasciculatum*; Gm, *Glomus mosseae*; Gi, *Glomus intraradices*.

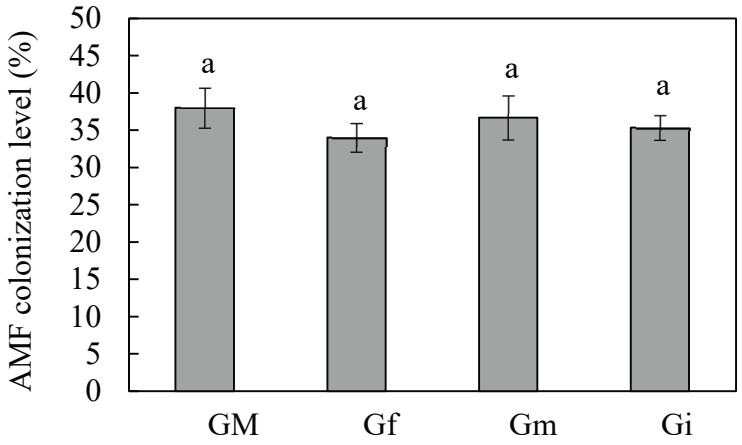


Fig. 17. AMF colonization levels in ginseng 15 weeks after AMF inoculation. Here, GM, *Gigaspora margarita*; Gf, *Glomus fasciculatum*; Gm, *Gl. mosseae*; Gi, *Glomus intraradices*.

Columns denoted by different letters indicate significant differences, according to Tukey's test ($P \leq 0.05$).

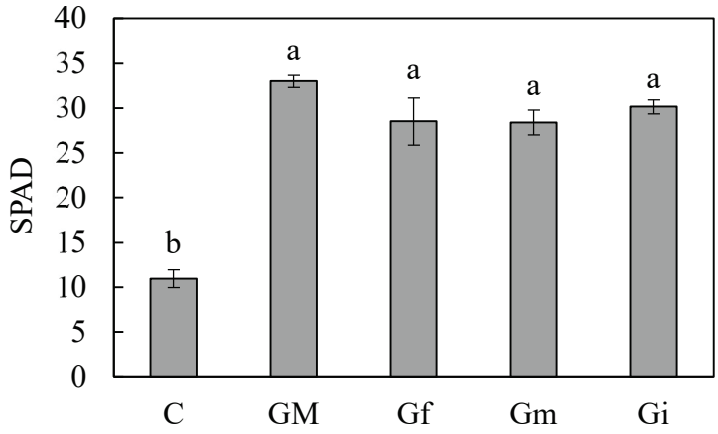


Fig. 18. SPAD value in ginseng plants 15 weeks after AMF inoculation.
Here, C, control; GM, *Gigaspora margarita*; Gf, *Glomus fasciculatum*; Gm, *Glomus mosseae*; Gi, *Glomus intraradices*.
Columns denoted by different letters indicate significant difference according to Tukey's test ($P \leq 0.05$).

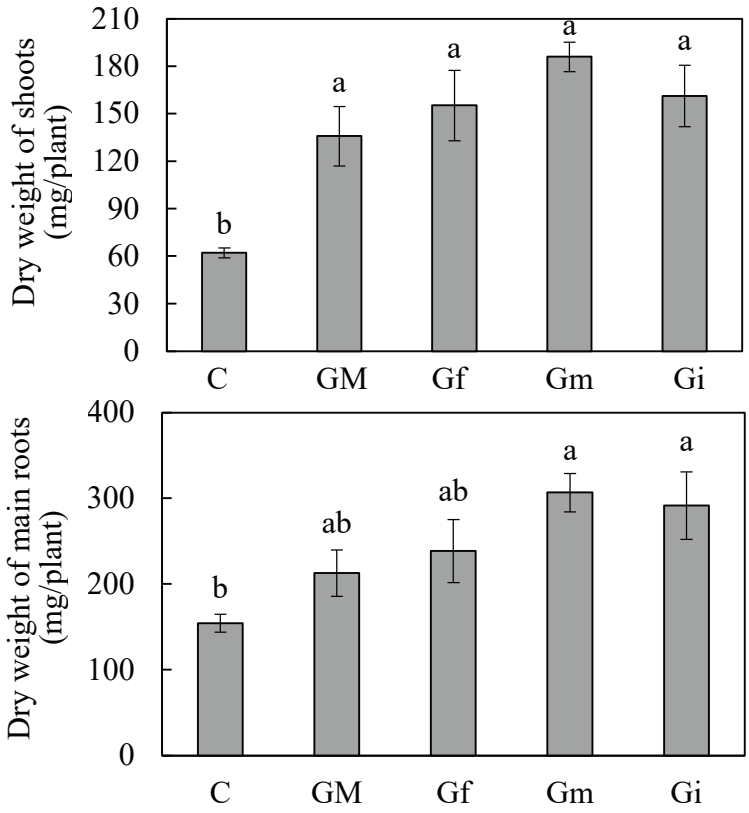


Fig. 19. Dry weight of ginseng 15 months after AMF inoculation.
C, control; GM, *Gigaspora margarita*; Gf, *Glomus fasciculatum*; Gm, *Glomus mosseae*; Gi, *Glomus intraradices*.
Columns denoted by different letters indicate significant differences according to Tukey's test ($P \leq 0.05$).

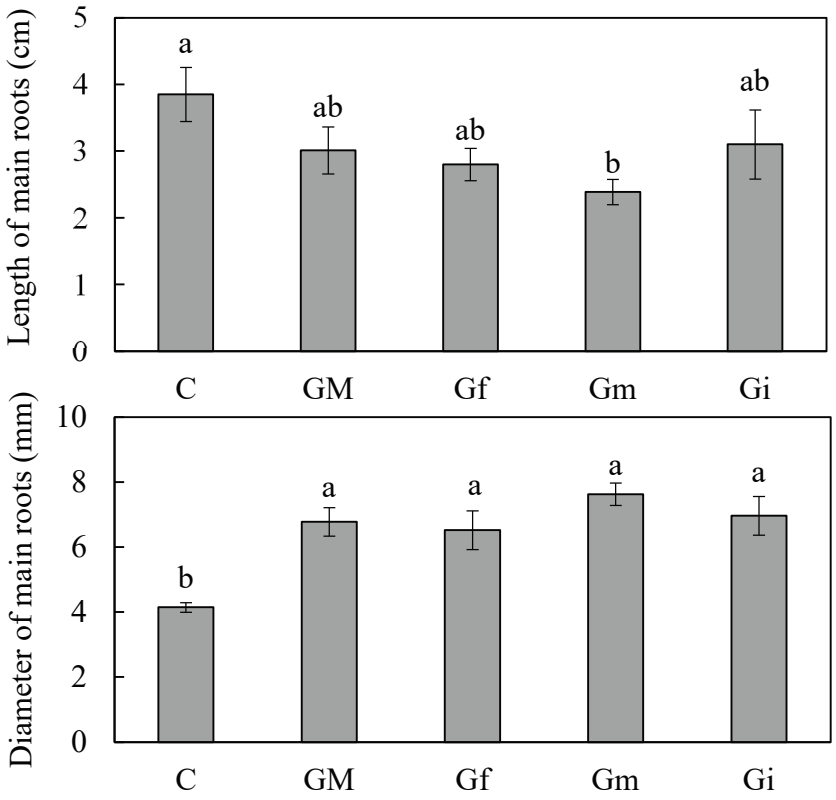


Fig. 20. Length and diameter of main roots in ginseng 15 months after AMF inoculation. C, control; GM, *Gigaspora margarita*; Gf, *Glomus fasciculatum*; Gm, *Glomus mosseae*; Gi, *Glomus intraradices*.

Columns denoted by different letters indicate significant differences according to Tukey's test ($P \leq 0.05$).

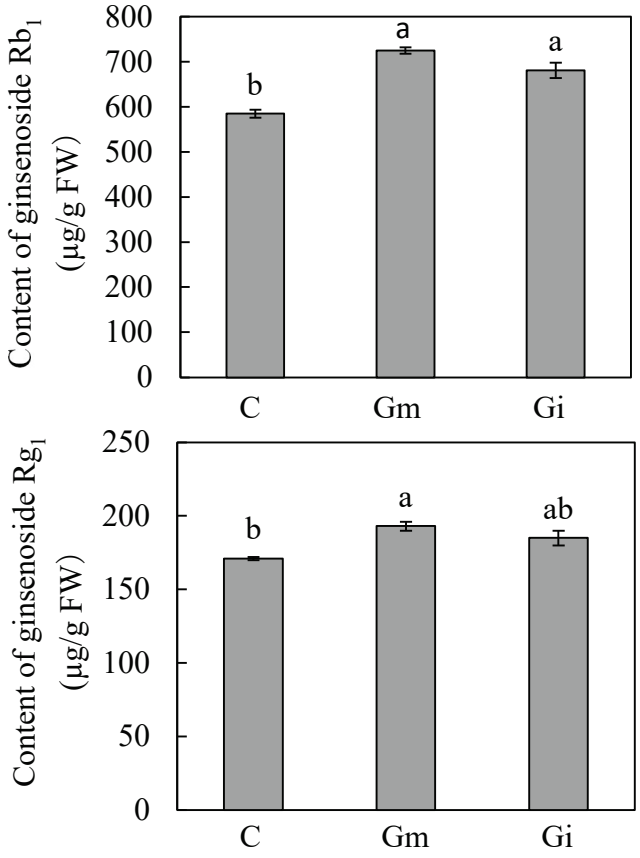


Fig. 21. Ginsenoside content in main roots of ginseng 15 months after AMF inoculation. Here, C, control; Gm, *Glomus mosseae*; Gi, *Glomus intraradices*.

Columns denoted by different letters indicate significant differences according to Tukey's test ($P \leq 0.05$).

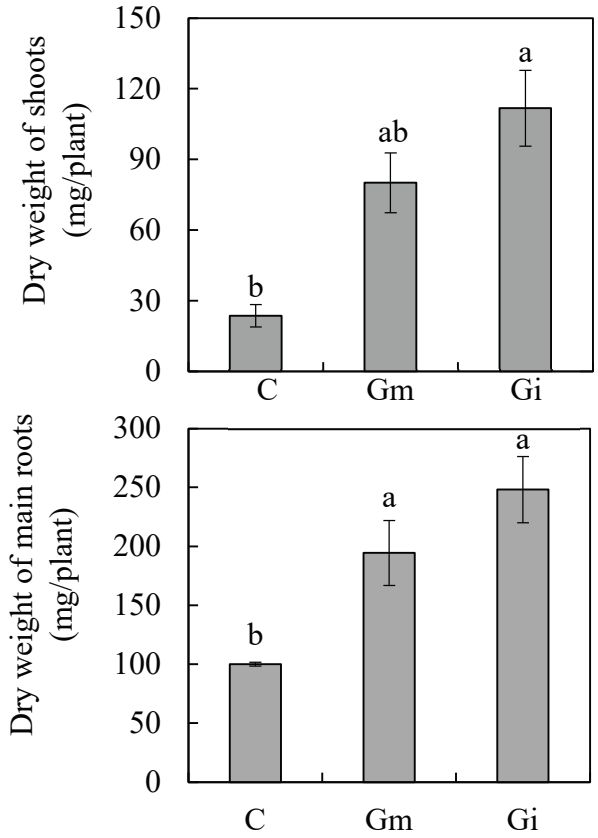


Fig. 22. Dry weight of ginseng 30 months after AMF inoculation. Here, C, control; Gm, *Glomus mosseae*; Gi, *Glomus intraradices*. Columns denoted by different letters indicate significant differences according to Tukey's test ($P \leq 0.05$).

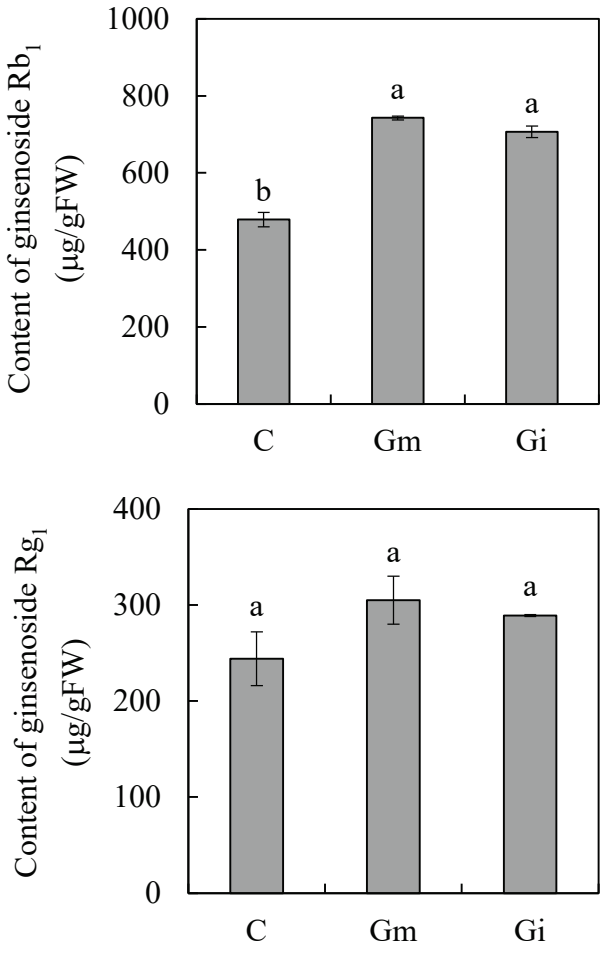


Fig. 23. Ginsenoside content in primary root of ginseng 30 months after AMF inoculation. Here, C, control; Gm, *Glomus mosseae*; Gi, *Glomus intraradices*.
Columns denoted by different letters indicate significant differences according to Tukey's test ($P \leq 0.05$).

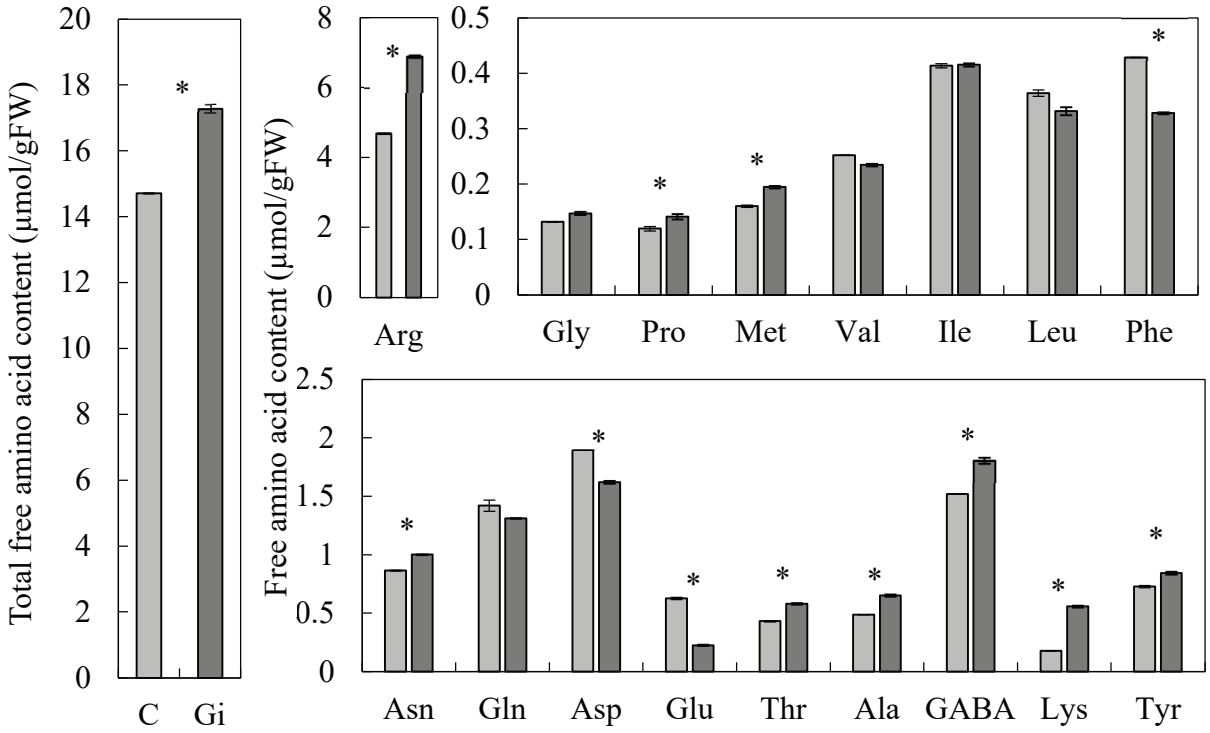


Figure 24. Changes in free amino acid contents in ginseng main root 30 months after AMF inoculation. Here, □, control (C); ■, *Glomus intraradices* (Gi*)*.

*Significant difference between control and Gi according to *t*-test ($P \leq 0.05$).

Discussion

In this study, four species of AMF [*Gigaspora margarita* (GM), *Glomus fasciculatum* (Gf), *Gl. mosseae* (Gm) and *Gl. intraradices* (Gi)] were inoculated to Asian ginseng (*Panax ginseng* C. A. Meyer). After 15 months of inoculation, observing the physical growth and root zone and comparing it to the control plants, we can see that mycorrhizal ginseng plants of all species showed vigorous growth and wider diameter of roots compared to the control. Previous reports revealed similar results indicating that AMF promotes host plant growth through symbiosis "(50)". As an example of the growth promotion observed in ginseng plants after inoculation with mycorrhizal fungi, dry weights of shoots and roots were significantly increased "(63)". In this study, plant growth promotion via symbiosis also appeared in mycorrhizal ginseng plants 30 months after AMF inoculation with Gm and Gi being the most effective. Similar to these results, in this study, the increase in dry weights of shoots and roots was persistently observed in long-term cultivation after mycorrhizal inoculation. It was considered to establish a cultivation system that halved (about 2 years) the normal cultivation period (4-6 years) of Asian ginseng. The mycorrhizal colonization level in Asian ginseng after 15 months of mycorrhizal inoculation was about 38% in GM, 35% in both Gm and Gi and 34% in Gf plots. So, no significant difference was noted among the treatments. Nahiyan et al. "(16)" reported that asparagus plants were inoculated with *Glomus* sp. R10 and *Fusarium oxysporum* f. sp. *asparagi* (Foa) were inoculated 10 weeks after AMF inoculation. AMF plants accumulated higher dry weight of ferns and roots than non-AMF plants before and after Foa inoculation; however, AMF colonization level had no difference among the treatments. SPAD meter is one of the most commonly used diagnostic tools to measure crop nitrogen status. The SPAD meter is a hand-held device that is widely used for the rapid, accurate, and non-destructive measurement of leaf chlorophyll concentration. SPAD value appeared higher in mycorrhizal ginseng plants than in non-mycorrhizal ginseng plants, indicating leaf chlorophyll concentration was higher

in mycorrhizal ginseng plants. GM showed the highest (33.012) value, but among the mycorrhizal species (Gf; 28.217, Gm; 2 8.4, Gi; 30.157), no significant differences were observed.

Ginsenoside Rb_1 is a chemical compound (chemical formula-$C_{54}H_{92}O_{23}$) of the shoot of ginseng plants. Rb_1 content was significantly increased in both Gm and Gi compared to the control, while ginsenoside Rg_1 (it is a major component of the root and stem of ginseng plants; the chemical formula is- $C_{42}H_{72}O_{14}$) content did not differ significantly among treatments in case of 15 months after AMF inoculation. In the case of 30 months after AMF inoculation, Ginsenoside Rb_1 was also significantly increased in both Gm and Gi compared to the control, while ginsenoside Rg_1 did not differ significantly among the treatments. Finally, the contents of ginsenoside Rb_1 and Rg_1 appeared higher (Gm showed highest) in mycorrhizal ginseng plants. That means mycorrhizal ginseng plants have a variety of potential health effects, including anti-carcinogenic, immunomodulatory, anti-inflammatory, anti-allergic, anti-hypertensive, anti-diabetic, anti-stress activity, and effect on the central nervous system, anti-aging, anti-fatigue, and memory enhancing properties. Tian et al. "(64)" reported an increase in ginsenoside Rb_1 and Rg_1 contents in fourth-year roots of ginseng 2 years after mycorrhizal (Gi) inoculation. Based on these reports and the results of this study, it can be expected that mycorrhizal inoculation will increase the ginsenoside contents of ginseng even when the cultivation period is reduced by half compared to the normal cultivation period, although there are differences depending on the mycorrhizal species and the symbiosis period.

The results of the free amino acid analysis showed that the total free amino acid content increased significantly in Gi compared to the control. Among the 17 free amino acids, several (12) free amino acids, such as arginine, proline, methionine, phenylalanine, asparagine, glutamic acid, aspartic acid, threonine, alanine, GABA, lysine, and tyrosine were increased in mycorrhizal (Gi) ginseng plants; particularly, large increases in arginine and threonine. It has

been reported that arginine content increased in the leaves of mycorrhizal tobacco (*Nicotiana tabacum*) “(65)” and threonine increased in the leaves of mycorrhizal fungi symbiosis mulberry (*Morus alba* L.) “(66)”. In the present study, the free amino acid content was increased in the roots of ginseng, but the increase in the content of arginine and threonine was confirmed as in these previous studies. These results suggest the possibility of high functionality of mycorrhizal ginseng due to the increase in arginine, which has been reported to lower blood pressure in humans “(67)”, and threonine, an essential amino acid for humans “(68)”. In addition, since arginine is involved in stress responses in plants “(69)”, it is possible that mycorrhizal fungi stimulated stress response pathways in ginseng plants. In addition, GABA and aspartic acid appeared at higher significant values, where GABA plays an important role in various physiological processes like nitrogen storage, protects from oxidative stress and osmoregulation, and acts as a signaling molecule in stress response. GABA is also important for human health for its potential anti-hypertensive effect. Glutamine had a weight loss effect “(70)”, GABA had a blood pressure lowering effect “(71)” and fatigue-reducing effect “(72)”, arginine and citrulline had a blood pressure lowering effect in humans “(67)”, histidine has been shown to improve insulin resistance “(73)”, and tyrosine has been suggested to enhance cognitive function in humans “(74)”. Thus, free amino acids have a variety of health-promoting effects, and since these functional amino acids were increased by mycorrhizal symbiosis. So, mycorrhizal symbiosis is expected to enhance such functions. In addition, GABA is not only functional for humans, but has also been reported to have growth-promoting and stress-tolerance-enhancing effects on plants themselves “(75)”. Therefore, the increase in GABA content due to mycorrhizal symbiosis may be one of the reasons for the growth promotion of ginseng in this study. In addition, aspartic acid is the precursor to several amino acids such as arginine, methionine, threonine, iso leucine, so, in this experiment aspartic acid and GABA accumulation might be closely related with amino-acid mediated stress alleviation.

Conclusion

Asian Ginseng (*Panax ginseng* C. A. Meyer) plants were inoculated with different AMF. Successful AMF colonization (more than 34%) was observed in all the inoculated AMF species. After 15 months of AMF inoculation, concerning plant growth promotion and greater dry weight of shoots and roots, Gm and Gi inoculated plants were found to be most effective. Whereas significant root length and diameter were observed in Gm-inoculated plants only. The plants with AMF inoculation also showed increased SPAD value than non-AMF inoculated plants. After 30 months of AMF inoculation, the dry weight of shoots and roots was increased in plants inoculated with Gi. The highest contents of ginsenosides (Rb_1 and Rg_1) were found in Gm-inoculated plants. Through LC-MS analysis, increased free amino acid contents, such as arginine, proline, methionine, phenylalanine, asparagine, glutamic acid, aspartic acid, threonine, alanine, GABA, lysine, and tyrosine, were found in mycorrhizal roots with Gi being most effective in contrast to the non-mycorrhizal ones. From these findings, it is suggested that plant growth promotion and positive changes in functional constituents through AMF symbiosis occurred in mycorrhizal Asian ginseng plants. After that, we investigated whether the growth promotion and functional components of other medicinal plants could be increased through AMF in the following chapter.

CHAPTER 2

Growth promotion and changes in functional components in mycorrhizal egoma

Introduction

Egoma (*Perilla frutescens* (L) Britton var. *frutescens*) is an annual herbal plant belonging to the *Lamiaceae* family that is widely cultivated in Asian countries "(76)". It is also cultivated in Western countries such as the USA, Russia, and Europe because of its growing economic importance "(77)". The seeds of this crop supply nutritious cooking oil, and the leaves are used as vegetables. Egoma seed contains approximately 40% oil "(78)", most of which (91%) are unsaturated fatty acids, including α-linolenic acid or omega-3 fatty acids (more than 50%), linolenic acid or omega-6 fatty acids (more than 15%) "(79)". Omega-3 fatty acids are necessary for human health; they play a crucial role in brain function and reduce inflammation, risk of heart disease, and cancer suppression "(79)", while Omega-6 fatty acids are required for normal immune function "(80)". Hundreds of bioactive compounds have been identified in egoma plants, gaining attention due to their medicinal benefits and phytochemical contents "(81)". Egoma leaves also contain many minerals such as iron, calcium, magnesium, and polyphenols; they are attracting attention as a functional vegetable with extremely high antioxidant properties "(82-83)". In addition, egoma leaves and stems have anti-microbial "(84)", anti-HIV "(85)", anti-tumor "(86)", and anti-allergic properties "(87)". Some scientists have reviewed various aspects of egoma plants, such as their bioactive components and their use as functional vegetables and medicines "(88)". Since egoma plants have many medical properties, they have been the subject of many studies. The functional components of egoma are secondary metabolites, and fluctuations in the functional components due to environmental factors are important issues "(83)". Therefore, there is a need for sustainable cultivation techniques for egoma plants that promote plant growth and stabilize and increase the content of their functional components.

Arbuscular mycorrhizal fungi (AMF) are broad-spectrum biocontrol agents "(14)" that promote host plant growth mainly by enhancing phosphorus uptake through symbiosis "(17)".

Evidently, AMF inoculation improves nitrogen and phosphorus uptake, resulting in an increased leaf area and growth “(89)”. Mycorrhizal inoculation is effective in promoting growth (63) and enhancing the yield of secondary metabolites “(90)”. Plants colonized with AMF exhibit greater enzymatic activity, mineral nutrition, and photosynthesis “(91-92)”, and improved growth, water uptake, and yield “(93)”.

To date, only a few cases of mycorrhizal association in shiso (*Perilla frutescens* (L) Britton var. *crispa*) in natural field conditions were reported “(94-95)”. Wee and Sohn “(96)” reported that mycorrhizal inoculation (unknown species) increased shoot fresh weight in shiso (*Perilla frutescens* (L) Britton var. *crispa*). However, there are no reports on the effects of AMF inoculation on the growth and functional components of egoma. Given this lack of research, the present study was conducted to evaluate the effect of mycorrhizal symbiosis on egoma growth and functional components such as antioxidants and free amino acid content in leaves. In addition, a sustainable cultivation method and improved innate quality of *Perilla frutescens* (L) Britton var. *frutescens* through mycorrhizal symbiosis is expected.

Materials and Methods

Mycorrhizal inoculation and plant growth: Autoclaved (121°C, 1.5 kPa, 30 min) commercial soil (Super Mix A, N: P: K=180:120:120 mg/L; Sakata Seed Corporation, Japan) was used to fill pots (10.5 cm in diameter), and then egoma (*Perilla frutescens* (L) Britton var. *frutescens*, cv. Shirakawa) seeds were sown. Simultaneously, two mycorrhizal fungi (*Gigaspora margarita*; GM, Central Grass Co. Ltd., Japan, and *Glomus fasciculatum*; Gf, SDS Biotech. Co. Ltd., Japan) were inoculated at a concentration of 3 g/individual. The same amount of autoclaved inoculum was used as the control. The plants were grown for approximately four weeks in a greenhouse and then transplanted to the experimental field at Gifu University. Slow-releasing granular fertilizer was applied (Long Total 391-70E, N: P: K=13:9:11, 4 g/plant; JCAM AGRI. Co., Ltd., Japan). The plants were grown for approximately 14 weeks under appropriate irrigation conditions.

Growth Survey: Ten weeks after AMF inoculation, the egoma leaves were collected, and 18 weeks after AMF inoculation, the plants were uprooted. Ten plants from each treatment, 30 plants in total, were used to collect data. After ten weeks of AMF inoculation, the number of leaves, leaf size, and dry weight of leaves were measured. Leaves of 10-11 cm wide and 13-15 cm long were defined as M size, leaves of 2 cm or more in length and smaller than M size were defined as S size, and leaves larger than M size were defined as L size. Ten weeks' leaves were cryopreserved for component analysis. In addition, 18 weeks after AMF inoculation, shoots and roots were dried using a constant temperature drier at 80°C for two days. Then, dry weights of shoots and roots were measured.

AMF colonization level: The level of AMF colonization in the lateral roots of the mycorrhizal egoma plants was assessed 18 weeks after AMF inoculation. The root system was carefully washed with tap water, and adhering soil particles were removed.

Lateral roots were sampled in 70% ethanol and later stained with trypan blue, according to Phillips and Hayman “(25)”. The ratio of AM fungal colonization was checked in 1 cm segments of lateral roots, and 30 samples of 1 cm segments were analyzed per plant. Additionally, the presence or absence of mycorrhizal fungi was observed in each section.

Antioxidant analysis: Antioxidants in the leaves were analyzed from each treatment using ten replications. Total anti-oxidative activity, based on 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity, was analyzed as described by Burits and Bucar “(97)”. Total polyphenol content was determined using the spectrophotometric method described by MacDonald et al. “(98)”. Glutathione content was determined according to the method described by Wu “(31)”. Ascorbic acid content was analyzed as described by Mukherjee and Choudhuri “(99)”.

Free amino acid analysis: Frozen leaf samples (0.2 g) were extracted using 3 mL of 0.2 N perchloric acid solution and then centrifuged at 4,000 rpm at 4 °C for 10 min. After adjustment to pH 4, the supernatant was again centrifuged at 13,000 rpm at 4 °C for 5 min. The supernatant was filtered through a syringe filter for analysis “(100)”. Analytical samples were derivatized using an AccQ·Tag Ultra Derivatization Kit (Waters Corporation, Milford, USA). Analytical samples (50 µL) were mixed with 350 µL of borate buffer and 100 µL of derivatization reagent. The reaction was immediately mixed and incubated for 1 min at room temperature. Subsequently, the solution was incubated for 10 min in a 55 °C water bath. After cooling, the reaction mixture was analyzed using UPLC. The ACQUITY UPLC BEH C18 (1.7 µm, 2.1 × 100 mm, Waters Corporation, Milford, USA); the reversed-phase column was used at 25 °C. The solvent system comprised 0.1% formic acid (A) as the inorganic-type mobile phase and acetonitrile (B) as the organic-mobile phase. A 5 µL injection volume was used. The

initial flow rate was 0.4 mL/min. Separation was accomplished using a gradient as follows: initial 0.1% B, 0-12 min 50.0% B, 12-13 min 0.1% B, and 13-15 min 0.1% B. A mass spectrometer (Xevo Q Tof MS, Waters Corporation, Milford, USA) was used to measure the mass range of electrospray ionization in the positive mode at 100-1000 m/z. A mass chromatogram (Abs Window 0.05 Da) of the m/z value of each amino acid was prepared from the measurement results, and the amino acid content was measured according to the peak integration value. Data analysis was performed using Waters Masslynx software (USA). Amino acid mixed standard solution H-type (Wako Pure Chemical Industries Ltd., Japan) with added asparagine, glutamine, gamma-aminobutyric acid, and citrulline was to create a standard calibration curve for the measurement of retention time and m/z values of each amino acid. Twenty-one free amino acids were analyzed, all of which were included in the standard solution.

α-linolenic acid analysis: The quantitative determination of α-linolenic acid by GC-MS was outsourced to the Japan Food Research Laboratories.

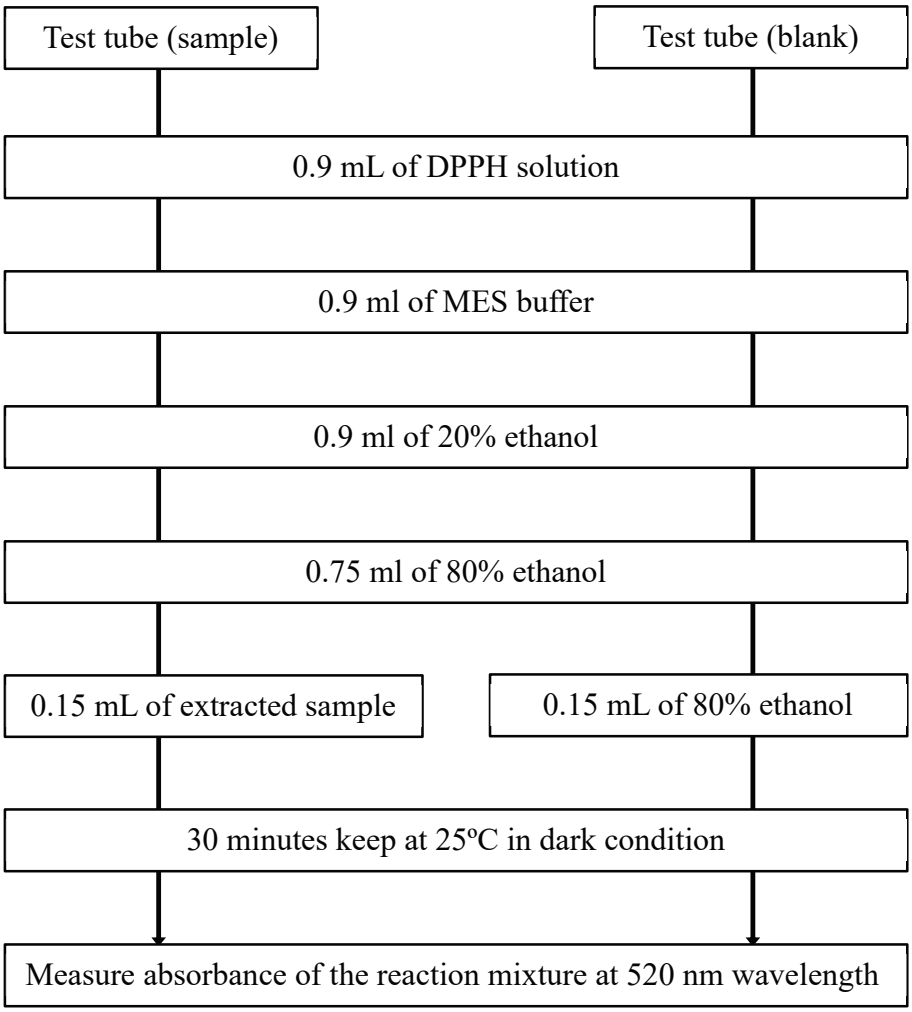


Fig. 25. Flow diagram of DPPH radical scavenging activity analysis.

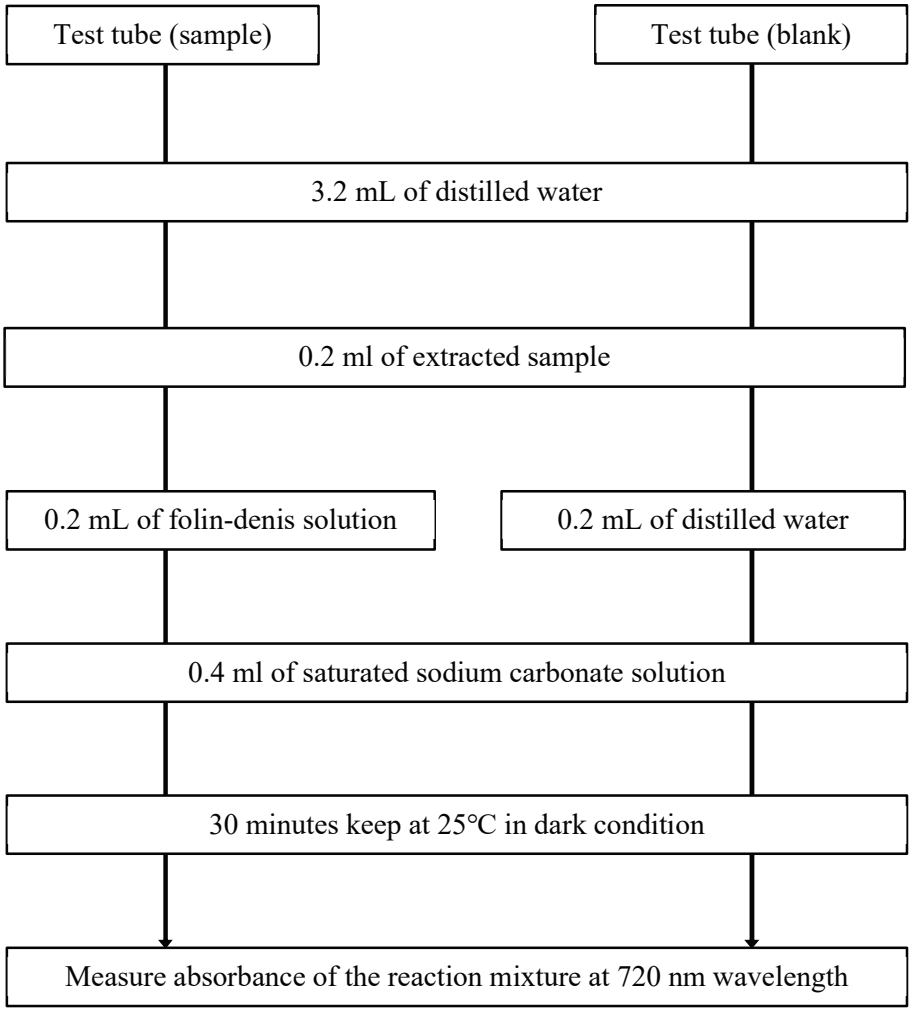


Fig. 26. Flow diagram of polyphenol content assay.

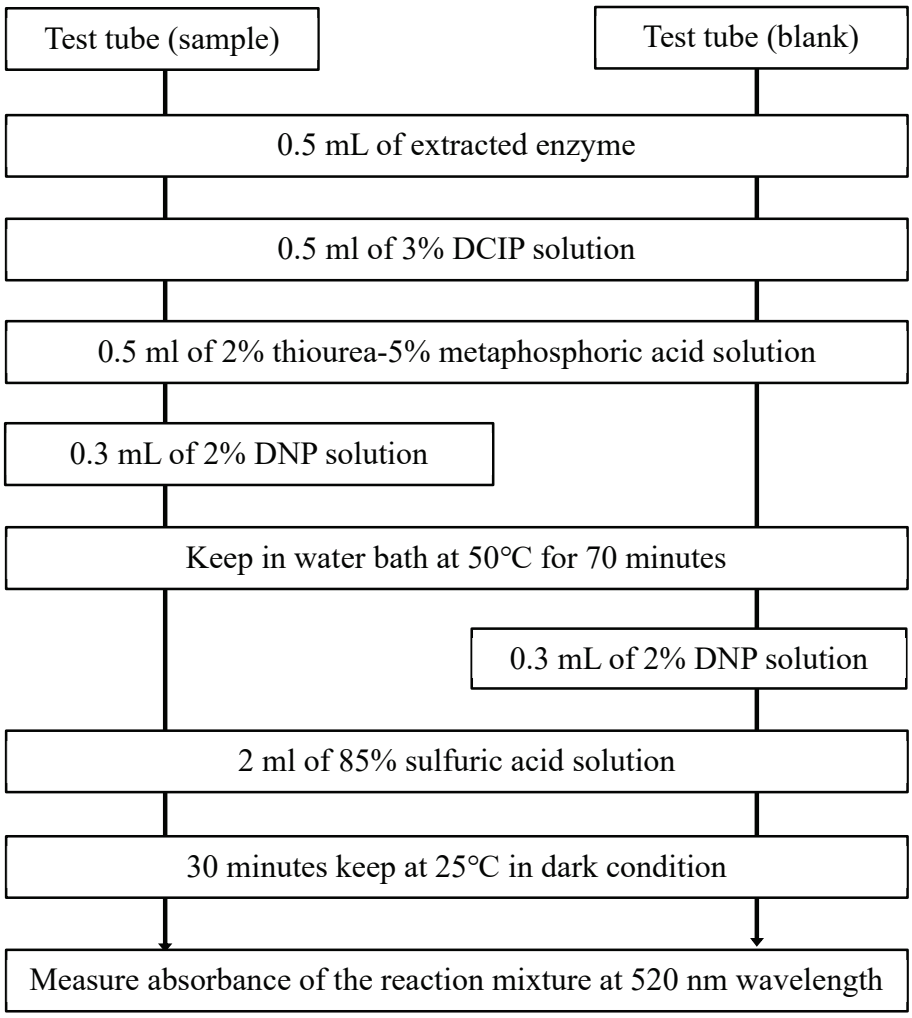


Fig. 27. Flow diagram of ascorbic acid assay.

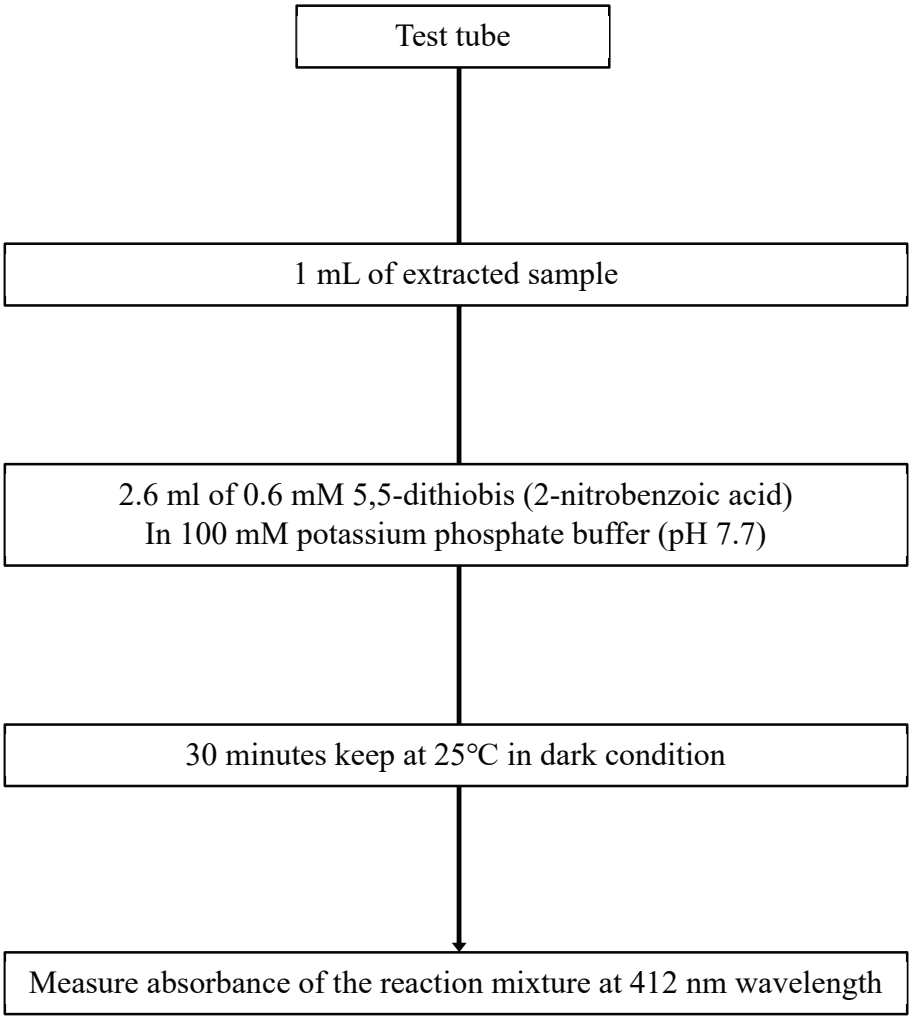


Fig. 28. Flow diagram of glutathione content assay.

Table. 5. Chromatographic conditions.

Column	: ACQUITY UPLC BEH C 18			
	: (1.7 μm, 2.1 x 100 mm, reversed-phase column, waters)			
Mobile phase A	: 0.1% Formic acid in water			
Mobile phase B	: Acetonitrile			
Injection volume	: 7.5 μL			
Gradient	Time (Min)	Flow rate (mL/min)	Mobile phase A (%)	Mobile phase B (%)
	0	0.4	95	5
	6	0.4	95	5
	12	0.4	75	25
	30	0.4	65	35
	32.5	0.4	5	95
	35	0.4	95	5

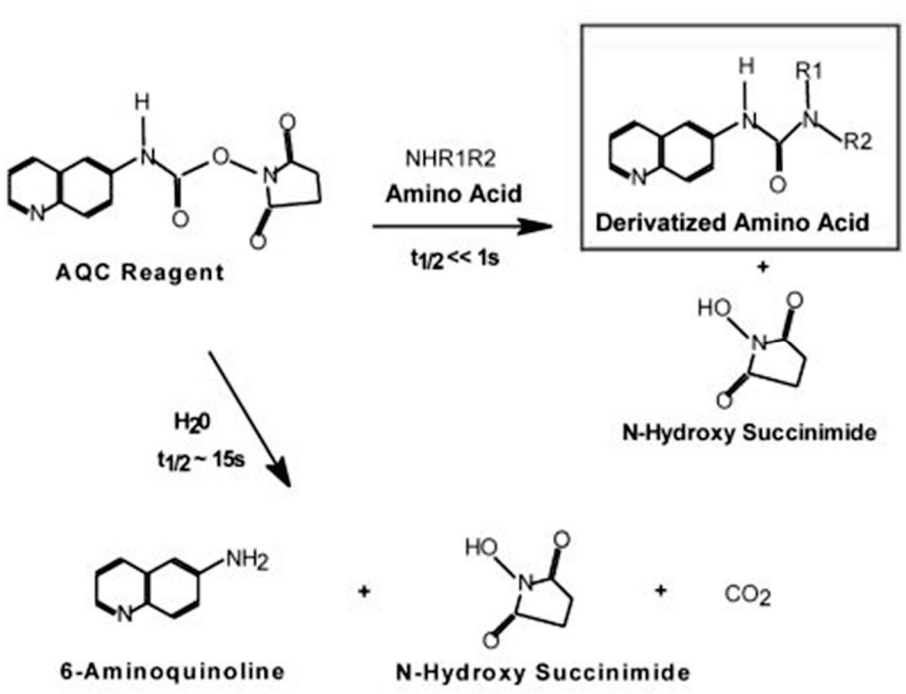


Fig. 29. Amino acid derivatization by AccQ・Tag Ultra Derivatization Kit. (https://www.waters.com/webassets/cms/library/docs/2008060039J.pdf)

Table 6. UPLC-MS gradient.

Time (min)	Flow (mL/min)	%A	%B	Curve
Initial	0.400	99.9	0.1	Initial
12.00	0.400	50.0	50.0	7
13.00	0.400	99.9	0.1	6
15.00	0.400	99.9	0.1	6

Table 7. Detected retention time and m/z of derivatized amino acids.

Name	Time	m/z (positive)
Histidine (His)	4.37	326.139
Asparagine (Asn)	4.84	303.100
Arginine (Arg)	4.94	345.167
Glutamine (Gln)	5.21	317.116
Serine (Ser)	5.28	276.083
Glycine (Gly)	5.45	246.073
Aspartic acid (Asp)	5.72	304.083
Citrulline (Cit)	5.72	346.145
Glutamic acid (Glu)	5.89	318.087
Threonine (Thr)	6.06	290.092
Alanine (Ala)	6.36	260.085
γ-aminobutyric acid (GABA)	6.41	274.096
Proline (Pro)	6.68	286.101
Lysine (Lys)	7.17	487.201
Cystine (Cys)	7.17	581.109
Tyrosine (Tyr)	7.80	352.088
Methionin (Met)	7.93	320.079
Valine (Val)	7.95	288.099
Isoleucine (Ile)	8.76	302.119
Leusine (Leu)	8.88	302.119
Phenylalanine (Phe)	9.13	336.091

Results

In terms of physical appearance nine weeks after AMF inoculation, the mycorrhizal egoma (*Perilla frutescens* (L) Britton var. *frutescens*) plants showed growth-promoting effects compared with the control plants in the field (Fig. 30).

After ten weeks of AMF inoculation, GM and Gf inoculated egoma plants had 4.5 times and 4.3 times more dry leaf weight, respectively than the control plants (Fig. 31). In addition, the total number of leaves was significantly higher in GM and Gf inoculated egoma plants (4.2 times and 4 times, respectively) than in the control and the ratio of M and L size leaves in mycorrhizal plants increased significantly (Fig. 31). The antioxidant analysis showed that the DPPH radical scavenging activity in leaves was significantly higher (1.4 times) in the GM plants than in the control, but no significant difference was detected between the Gf and control plants (Fig. 32). GM and Gf plants had higher total polyphenol, glutathione, and ascorbic acid contents (1.5, 3.6, and 4.3 times, respectively, in GM and 1.5, 4.2, and 3.2 times respectively, in Gf) than the control plants (Fig. 32). The total free amino acid content in the leaves was significantly increased (22%) in Gf plants compared to the control, but there was no significant difference between the GM and control plants (Fig. 33). Twenty-one different amino acids were detected in the leaves of the mycorrhizal egoma plants. Regarding individual free amino acids, histidine increased significantly in GM and Gf inoculated plants; glutamine increased significantly in GM inoculated plants; and alanine, γ-aminobutyric acid (GABA), and phenylalanine increased significantly in the Gf inoculated plants (Fig. 33). Thus, the type and amount of free amino acids that increased differed between AMF species.

After 18 weeks of AMF inoculation, AMF colonization occurred successfully, with levels of 35% - 37% in the root system, and no significant differences were noted between the AMF species (Fig. 34). Regarding the dry weight of the above-ground parts of egoma, there was no significant difference in any treatment, but the dry weight of the underground part tended to

decrease in the mycorrhizal fungus-inoculated plants (Fig. 35). The dry weight of seeds significantly increased in all the mycorrhizal egoma plants compared to those of the control, and the dry weight of seeds increased 1.9 times (96%) in the GM-inoculated plants and 1.8 times (80%) in the Gf-inoculated plants compared to that of the control (Fig. 36). The α-linolenic acid content in the seeds of egoma was significantly increased in all mycorrhizal egoma plants compared to the control, and the highest increase (37%) were in Gf inoculated plants, followed by 18% in GM inoculated plants (Fig. 37).

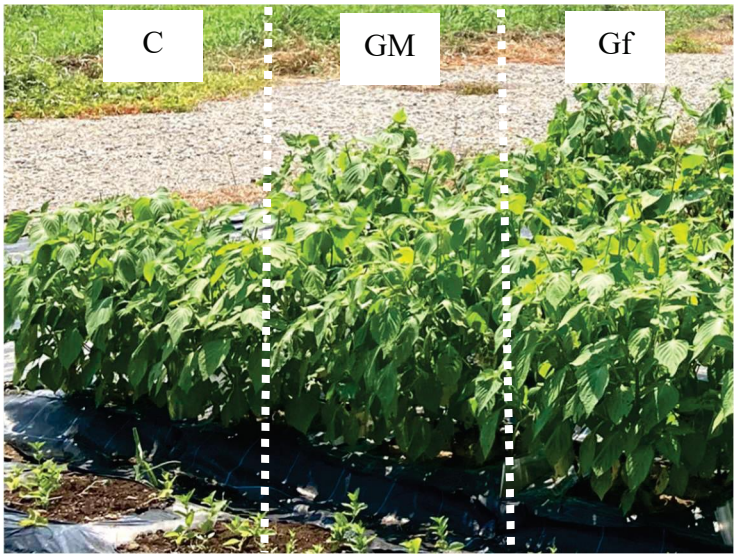


Fig. 30. Growth of egoma (*Perilla frutescens* (L) Britton var. *frutescens*, cv. Shirakawa) 9 weeks after AMF inoculation in the field. Here, C, control; GM, *Gigaspora margarita*; Gf, *Glomus fasciculatum.*

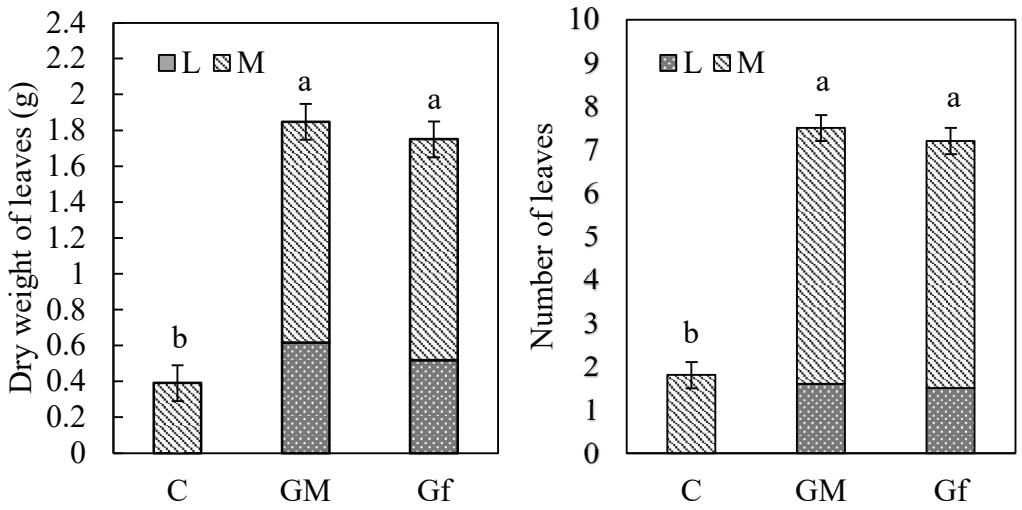


Fig. 31. Dry weight and number of leaves in egoma (*Perilla frutescens* (L) Britton var. *frutescens*, cv. Shirakawa) 10 weeks after AMF inoculation.

C, control; GM, *Gigaspora margarita*; Gf, *Glomus fasciculatum.* L, M, leaf size. Columns denoted by different letters indicate significant difference in the total according to Tukey's HSD test ($p < 0.05$).

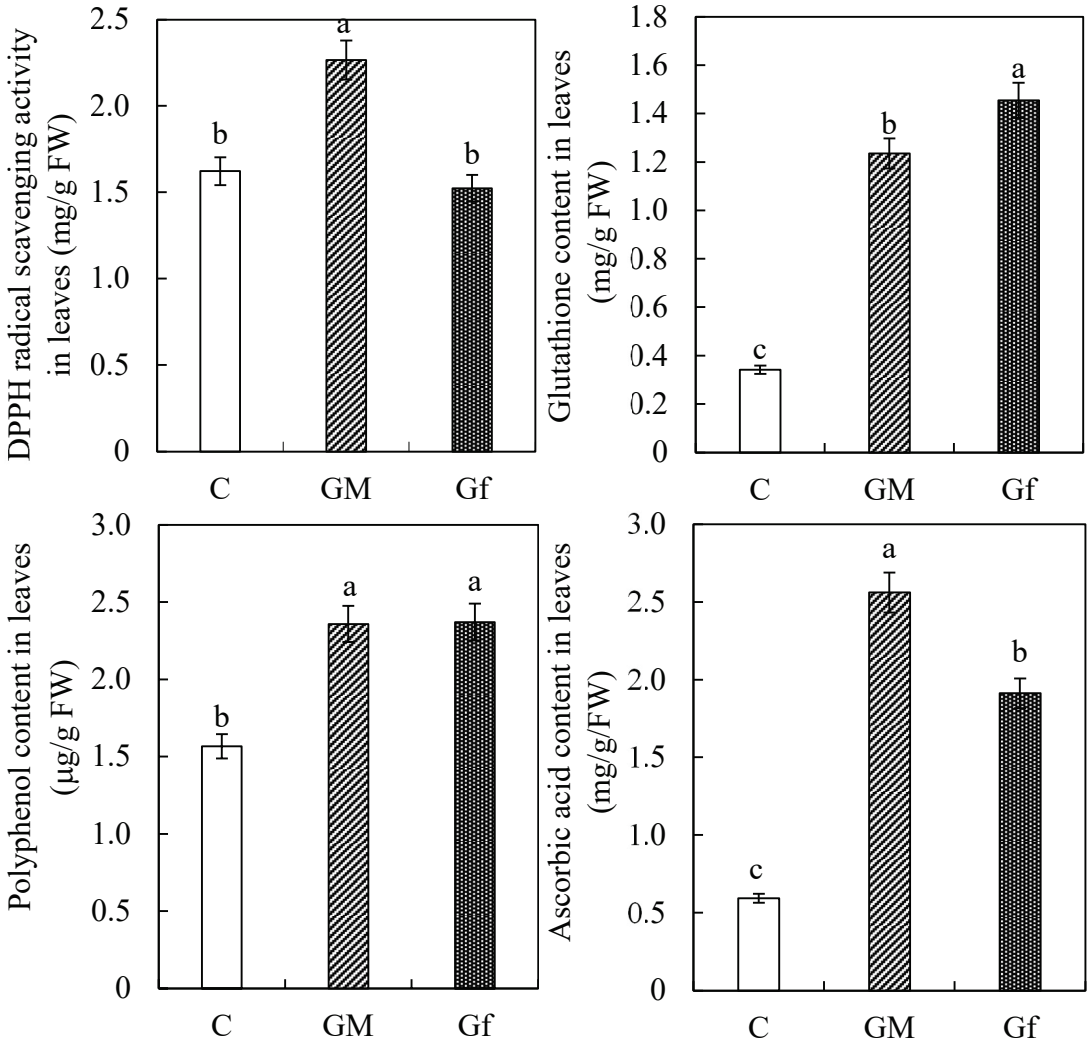


Fig. 32. Antioxidants in leaves of egoma (*Perilla frutescens* (L) Britton var. *frutescens*, cv. Shirakawa) 10 weeks after AMF inoculation.

C, control; GM, *Gigaspora margarita*; Gf, *Glomus fasciculatum.* Columns denoted by different letters indicate significant differences according to Tukey's HSD test ($p < 0.05$).

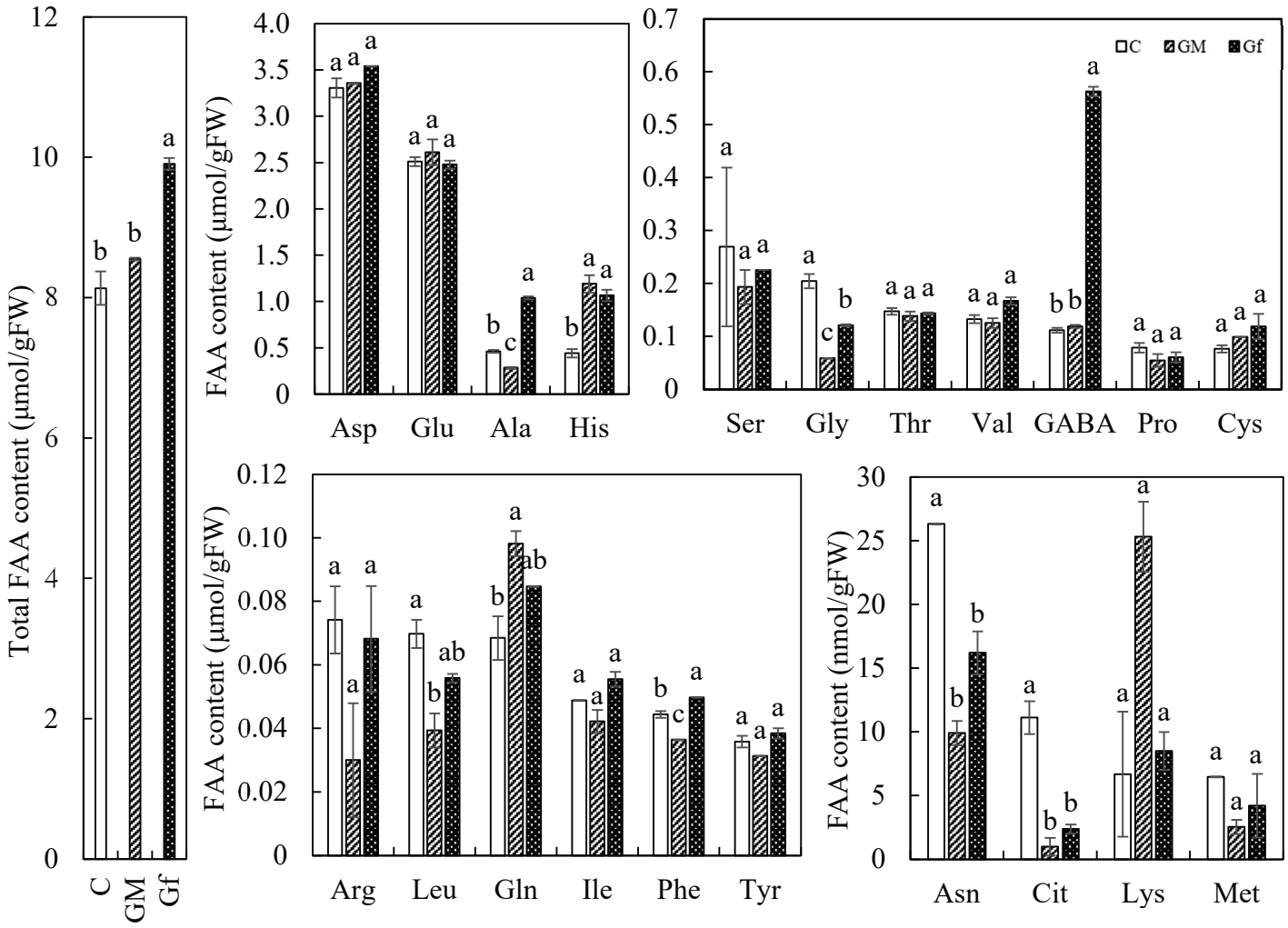


Fig. 33. Changes in total and individual FAA contents in leaves of egoma (*Perilla frutescens* (L) Britton var. *frutescens*, cv. Shirakawa) 10 weeks after AMF inoculation.

Here, C, control; GM, *Gigaspora margarita*; Gf, *Glomus fasciculatum*. FAA; Free amino acid.

Columns denoted by different letters indicate significant differences according to Tukey's HSD test ($p < 0.05$).

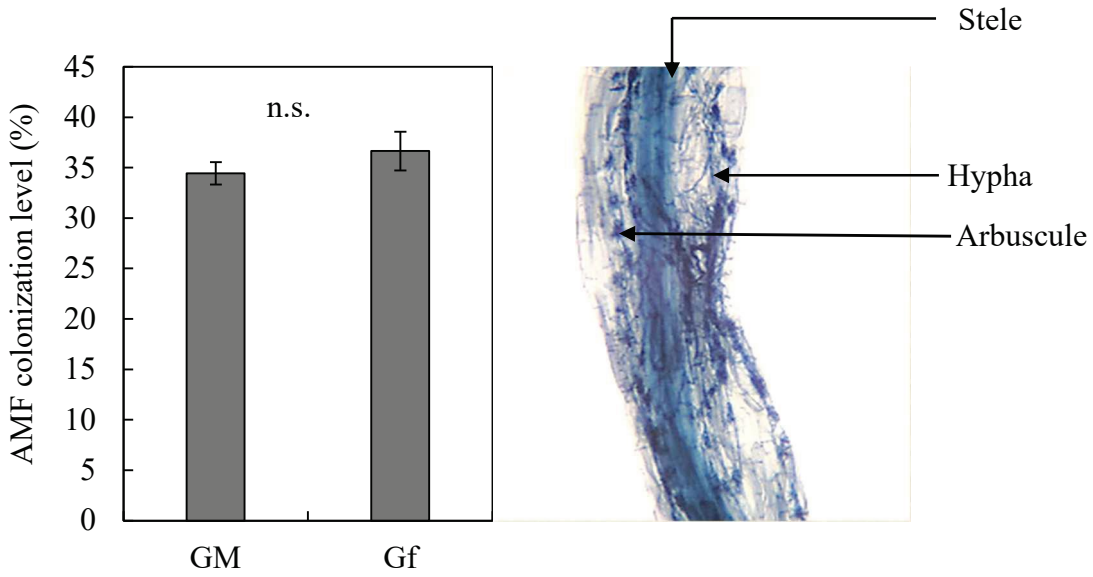


Fig. 34. AMF colonization level and colonization in egoma (*Perilla frutescens* (L) Britton var. *frutescens*, cv. Shirakawa) roots 18 weeks after AMF inoculation.

GM, *Gigaspora margarita*; Gf, *Glomus fasciculatum.* n.s., non-significant (t-test, $p < 0.05$).

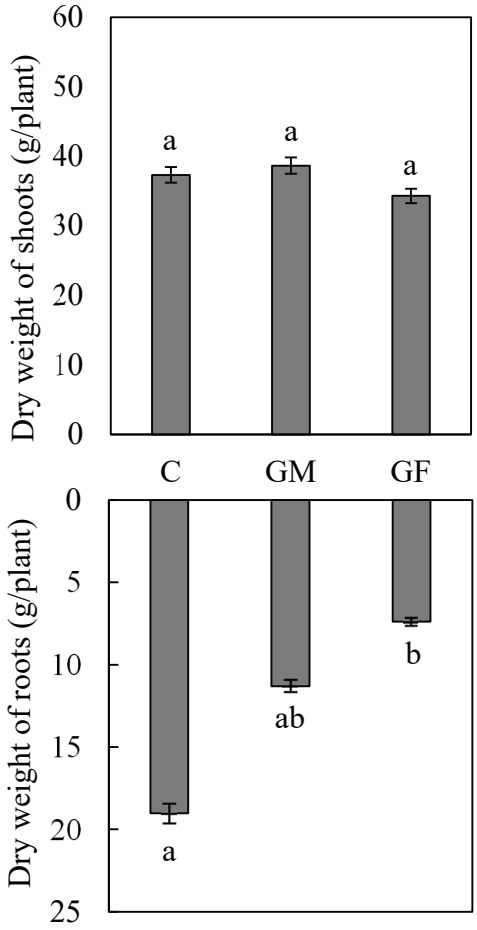


Fig. 35. Dry weight of shoots and roots in egoma (*Perilla frutescens* (L) Britton var. *frutescens*, cv. Shirakawa) 18 weeks after AMF inoculation.

C, control; GM, *Gigaspora margarita*; Gf, *Glomus fasciculatum.* Columns denoted by different letters indicate significant differences according to Tukey's HSD test ($p < 0.05$).

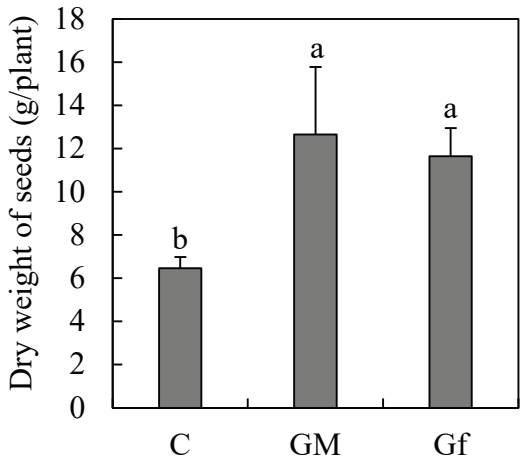


Fig. 36. Dry weight of seeds in egoma (*Perilla frutescens* (L) Britton var. *frutescens*, cv. Shirakawa) 18 weeks after AMF inoculation.

C, control; GM, *Gigaspora margarita*; Gf, *Glomus fasciculatum.* Columns denoted by different letters indicate significant differences according to Tukey's HSD test ($p < 0.05$).

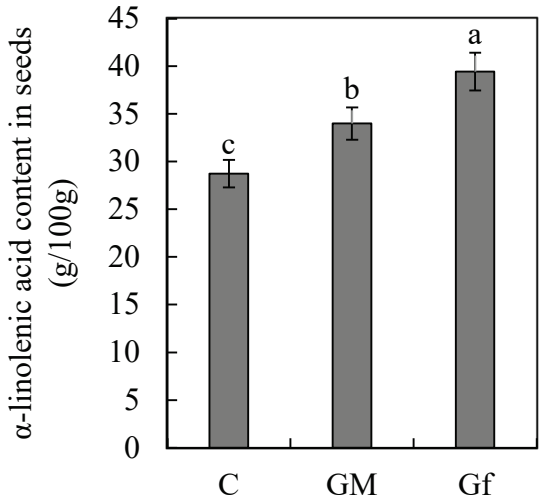


Fig. 37. α-linolenic acid content in seeds of egoma (*Perilla frutescens* (L) Britton var. *frutescens*, cv. Shirakawa) 18 weeks after AMF inoculation.

C, control; GM, *Gigaspora margarita*; Gf, *Glomus fasciculatum.* Columns denoted by different letters indicate significant differences according to Tukey's HSD test ($p < 0.05$).

Discussion

The *Glomus* and *Gigaspora* mycorrhizal fungi used in this experiment have been reported to have growth-promoting effects in multiple plant species through symbiosis “(101-102)”. Wee and Sohn “(96)” reported that mycorrhizal inoculation (unknown species) increased shoot fresh weight in shiso (*Perilla frutescens* (L) Britton var. *crispa*). In this study, the dry weight of leaves, the number of leaves, and the leaf size of egoma (*Perilla frutescens* (L) Britton var. *frutescens*) plants increased in both GM and Gf plants (Fig. 31). However, the root dry weight of GM and Gf plants were decreased compared to the control plants in this study. It was supposed that mycorrhiza absorbed more nutrients without developing the roots and supplied these nutrients to the reproductive parts. On the other hand, mycorrhizal symbiosis of AMF species promoted egoma plant growth, and interspecific differences in growth promotion were also observed. The reason for this difference is unclear.

Antioxidants, such as polyphenols, glutathione, and ascorbic acid, exhibit antioxidant activity on their own and play a role in removing reactive oxygen species (ROS) from the body “(103)”. The DPPH radical scavenging activity is commonly used as a rapid, simple, and inexpensive method to measure the free radical scavenging capacity of total antioxidants in biological compounds and involves the use of the free radical DPPH “(104)”. In this study, the DPPH radical scavenging activity in leaves was significantly greater in GM plants than non-mycorrhizal plants (Fig. 32). Ascorbic acid and glutathione are involved in the neutralization of secondary ROS products. The higher ascorbic acid and glutathione contents enable plants to directly scavenge the $^1O_2$ and H_2O_2, as well as other ROS-like hydroxyl radicals “(105)”. Carillo et al. “(106)” reported that phosphorus application increased the ascorbic acid content in cherry tomatoes (*Solanum lycopersicum* L.). Based on this report, the increase in ascorbic acid content in egoma leaves observed in the present study may have been contributed to the promotion of phosphorus absorption by the mycorrhizal fungi. In addition, ascorbic acid is an

effective antioxidant in plants that can detoxify free radicals and oxidants “(107)”. Polyphenols are excellent oxygen radical scavengers because the electron reduction potential of the phenolic radical is lower than that of oxygen radicals “(108)”. In this study, inoculation of AMF into egoma plants increased the ability of leaves to scavenge DPPH radicals and increased the levels of polyphenols, glutathione, and ascorbic acid in the leaves (Fig. 32). The increase in these antioxidants in mycorrhiza-inoculated egoma leaves suggests that mycorrhizal fungi contribute to the increase in the functionalization of egoma leaves.

Increased total and individual free amino acid contents in mycorrhizal plants have been reported in several plants “(109-110)”, and the effect varies with several host-fungus combinations. The present study showed the same result. The total free amino acid content was significantly higher in the Gf plants (Fig. 33). Previous reports, however, have focused on only one AMF species; therefore, it remains unclear whether fungal differences in amino acid changes occur in the same host. Our results show increased concentrations of several amino acids in egoma plants in response to AMF inoculation (Fig. 33). In addition, our results indicated that fungus-induced changes in amino acids vary with AMF species in the same host (Fig. 33). Rasmussen “(111)” reported an increase in the free amino acid content in the leaves of *Lolium perenne* by nitrogen fertilization. Shao et al. “(112)” reported that the inoculation of mycorrhizal fungi in tea plants increased the free amino acid content and the expression levels of glutamine synthase, glutamate synthase, and glutamate dehydrogenase, which play important roles in nitrogen assimilation. Abdel-Fattah and Mohamedin “(109)” confirmed an increase in free amino acids in the leaves of mycorrhizal (*Glomus intraradices*) sorghum. In this study, it was concluded that mycorrhizal fungi greatly contribute to the increase in amino acid content (Fig. 33), and mycorrhizal plants are presumed to be related to the nitrogen supply and amino acid metabolism pathways of mycorrhizal fungi.

Sood et al. “(110)” reported a significant increase in the concentration of 8 of the 14 free

amino acids in mycorrhizal (*Glomus fasciculatum*) tomato plants. In this study, 21 free amino acids were detected in the leaves of egoma, and the concentration of 12 free amino acids increased. Regarding individual free amino acids, GABA and histidine levels in field-grown egoma leaves significantly increased in the Gf plot. GABA plays a significant role in various physiological processes in plants, such as nitrogen storage, oxidative stress protection, and osmoregulation, and acts as a signaling molecule in the stress response (113). GABA has also been suggested to reduce fatigue “(72)” and histidine to improve insulin resistance “(73)”. It is reported that glutamine has a weight loss effect in humans “(70)”. In this experiment, individual free amino acids, such as alanine and phenylalanine, were increased in the Gf plants. Alanine helps store and transport nitrogen during stress and provides energy to plants “(114)”, while phenylalanine can be used to treat Parkinson’s disease by producing dopamine “(115)”. Thus, free amino acids have various health-enhancing effects, and because these functional amino acids increase due to mycorrhizal symbiosis, it can be expected that egoma leaves will be highly functionalized by mycorrhizal symbiosis.

Maya and Matsubara “(34)” reported that growth promotion and anti-oxidative activity increased under environmental stress in cyclamens and were not correlated with mycorrhizal colonization levels. In this study, we found that AMF colonization reached around 35-37% in all mycorrhizal egoma plants (Fig. 34), confirming for the first time that mycorrhizal fungi promote plant growth, antioxidant activity, total and individual free amino acid contents of the leaves, dry weight and α-linolenic acid contents of seeds in the egoma plants did not correlate with AMF colonization level. In this study, the colonization level was checked only once. Hence, it was difficult to estimate when the AMF reached the maximum colonization level during the experimental period and how the colonization level affected the functional component changes. Further investigation is necessary to address this issue.

Seeds have the highest concentration of phosphorus among mature plants “(116)”. AMF enhanced phosphorus uptake by host plants and ensured greater seed yield. Several studies have reported the absorption-enhancing effects of mineral nutrients such as phosphorus and potassium on mycorrhizal fungi “(90, 96)”. It is presumed that the promotion of mineral nutrient supply by mycorrhizal fungi contributes to an increase in seed weight. Moghith “(117)” found that mycorrhizal chia (*Salvia hispanica* L.) plants had a significantly higher mean value of the number of branches per plant, seed yield/plant, weight of 1000 seeds, and fixed oil yield per plant than the control plants. In this study, mycorrhizal egoma plants showed a significant increase in the dry weight of seeds/plants compared to that of the control (Fig. 36).

Compared to other plant oils, shiso oil exhibits one of the highest proportions of α-linolenic acid or omega-3 fatty acids, which is between 40-68% “(78)”. It is an ideal resource for supplementing human omega-3 polyunsaturated fatty acids. Kapulnik et al. “(118)” reported that mycorrhizal fungal inoculation increased fruit and oil yields from olive oil (*Olea europaea)*. The seeds of mycorrhizal chia (*Salvia hispanica* L.) plants showed more α-linolenic acid than the control “(117)”. In this study, α-linolenic acid content on the seeds of egoma was significantly increased in both AMF species compared to the control (Fig. 37). Essential fatty acids are nutrients that must be obtained from the diet because they cannot be synthesized in the body, and the possibility of improving cognitive function has been shown “(119)”. In this study, the α-linolenic acid content of egoma seeds increased due to mycorrhizal fungal symbiosis, suggesting the possibility of enhancing the functionality of egoma as an oil crop (Fig. 37).

Thus, in this study, it was suggested that the symbiosis of mycorrhizal fungi in egoma may enhance vegetative and reproductive growth and increase functional components. Furthermore, because it has been suggested that mycorrhizal symbiosis may be highly functional owing to increased functional components, mycorrhizal fungal cultivation and

utilization is expected to contribute to health promotion. Arbuscular mycorrhizal fungi are expected to enhance egoma production by promoting plant growth and expanding its use for food and medicinal purposes by increasing the functional components.

Conclusion

In this study, the effectiveness of arbuscular mycorrhizal fungi (AMF, i.e., GM: *Gigaspora margarita*, Gf: *Glomus fasciculatum*) inoculation on plant growth and the changes in functional components of egoma (*Perilla frutescens* (L) Britton var. *frutescens*, cv. Shirakawa) were investigated. Ten weeks after AMF inoculation, mycorrhizal egoma plants had significantly higher leaf dry weights and a total number of leaves than non-mycorrhizal plants. Regarding the functional components in leaves, the total polyphenol, glutathione, and ascorbic acid contents were significantly higher in the mycorrhizal plants than in the control; 1, 1-diphenyl -2-picrylhydrazyl (DPPH) radical scavenging activity was significantly increased in the GM plants. The total free amino acid content in the leaves was higher in the Gf plants than in the control plants. Regarding individual free amino acids, histidine increased in the mycorrhizal plants compared to in the control, glutamine increased significantly in the GM plants, and alanine, γ-aminobutyric acid (GABA), and phenylalanine increased significantly in the Gf plants. The AMF colonization levels in the root systems were not significantly different between the species. The seed yield and α-linolenic acid content in the seeds were significantly increased by both mycorrhizal plots. Therefore, mycorrhizal symbiosis may promote plant growth and increase functional components in egoma plants. The next section of chapter -3 addressed in order to prove the effect of AMF on the growth promotion and functional components of other medicinal plants*, Platycodon grandiflorus plant* was examined by AMF inoculation.

CHAPTER-3

Growth promotion and the changes in functional components in mycorrhizal *Platycodon grandiflorus*

Introduction

Platycodon grandiflorus is a perennial herb belonging to the *Campanulaceae* family that has been widely used as an edible medicinal food for thousands of years "(120)". It is native to East Asia, and its blue, purple, and white star-shaped flowers are used for ornamental and decorative purposes "(121)". *P. grandiflorus* is rich in amino acids (more than 16 amino acids, including eight essential amino acids), plant fiber, vitamins, calcium, zinc, potassium, iron, and other trace elements essential in the human diet "(122)". In addition, *P. grandiflorus* root contains chemical compounds such as flavonoids, phenolic acids, triterpenoid saponins, polyacetylene, and sterols, which are the main biological components that show significant antitussive, antitumor, antioxidation, anti-inflammatory, hypoglycemic, anti-obesity, and immune enhancement effects "(123)". The demand for *P. grandiflorus* increased stably in many countries, exports also increased sharply, and the demand exceeded the supply, and the price rose significantly; as a result, it has become a new bright spot in increasing farmers' income and has a huge development value and good development prospects "(120)". The roots of *P. grandiflorus* are used as edible functional foods and for medicinal purposes in Japan, China, and Korea, and they often require a long cultivation period of more than two years before they can be harvested "(124)". Therefore, shortening the cultivation period and increasing the functional components is important. The functional components of medicinal plants are secondary metabolites, and fluctuations in the functional components due to environmental factors are important issues "(125)". However, at present, no cultivation method has been developed to achieve such points.

Arbuscular mycorrhizal fungi (AMF) are broad-spectrum biocontrol agents "(126)" that promote host plant growth mainly by enhancing phosphorus uptake through symbiosis "(127)". Evidently, AMF inoculation improves nitrogen uptake, increasing leaf area and growth "(89)". Mycorrhizal inoculation is effective in promoting growth "(55)", disease resistance "(57)",

increased stress tolerance “(59)”, and enhancing the yield of secondary metabolites “(90)”. Different species of AMF differ in their tolerance to stress “(128)”. Mycorrhizal symbiosis has been confirmed to promote plant growth and increase the content of pharmacological components in the main roots “(64)”. Thus, mycorrhizal symbiosis might also help to promote growth and increase the yield and functional components in *P. grandiflorus*.

Only a few cases of mycorrhizal colonization in *P. grandiflorus* in natural field conditions have been reported “(129-130)”. However, there are no reports of AMF inoculation based on the investigation of plant growth, yield, and functional components in *P. grandiflorus.* Given this lack of research, the present study was conducted to evaluate the effect of mycorrhizal symbiosis on growth and functional components in *P. grandiflorus* plants, which will also help to develop a sustainable cultivation method.

Materials and Methods

1. Mycorrhizal inoculation and plant growth: Autoclaved (121°C, 1.5 kPa, 30 min) commercial soil (Super Mix A, N: P: K=180:120:120 mg/L; Sakata Seed Corporation, Japan) was used to fill pots (Φ = 9 cm, H = 20 cm), and then seeds of *P. grandiflorus* cv. Murasaki, supplied by Fukkaen Seed Co. Ltd.) was sown. Simultaneously, three mycorrhizal fungi (*Gigaspora margarita***;** GM, Central Grass Co. Ltd., Japan; *Glomusfasciculatum*; Gf and *Glomus mosseae*; Gm, SDS Biotech. Co. Ltd., Japan) were inoculated with 3 g inoculum. The same amount of autoclaved inoculum was used as the control. Thirty plants per treatment were grown in the greenhouse (30/24 ± 4ºC day/night temperature) under natural light and day length and irrigated regularly. After one month, the plants were transplanted into the experimental field of Gifu University. Slow-releasing granular fertilizer was applied (Long Total 391-70E, N: P: K=13:9:11, 4 g/plant; JCAM AGRI. Co., Ltd., Japan). The plants were grown for approximately 15 months under appropriate irrigation conditions. During the growing period, flower buds were picked to stop reproductive growth as a custom treatment for harvesting roots.

2. Growth Survey: Sixteen months after AMF inoculation (shoots were all browned and withered), the roots of *P. grandiflorus* plants were uprooted. Ten plants from each treatment were then dried in an oven at 100ºC for 24 h, and the dry weights of roots were measured. Some fresh roots were cryopreserved for component analysis.

3. AMF colonization level: The level of AMF colonization in the lateral roots of the mycorrhizal *P. grandiflorus* plants was assessed 16 months after AMF inoculation. The root system was carefully washed with tap water, and adhering soil particles were removed. Lateral roots were sampled in 70% ethanol and later stained with trypan blue, according to Phillips and Hayman "(25)". The ratio of AM fungal colonization was checked in 1 cm segments of lateral roots, and 30 samples of 1 cm segments were analyzed per plant. Additionally, the presence or

absence of mycorrhizal fungi was observed in each section.

4. Free amino acid analysis: An Ultra-Performance Liquid Chromatography (UPLC) - Mass Spectrometry (MS) system (Waters Corporation, USA) was used to analyze free amino acids. Frozen root samples (0.2 g) were extracted using 3 mL of 0.2 N perchloric acid solution and then centrifuged at 4,000 rpm at 4 °C for 10 min. After adjustment to pH 4, the supernatant was again centrifuged at 13,000 rpm at 4 °C for 5 min. The supernatant was filtered through a syringe filter for analysis "(100)". Analytical samples were derivatized using an AccQ·Tag Ultra Derivatization Kit (Waters Corporation, Milford, USA). Analytical samples (50 μL) were mixed with 350 μL of borate buffer and 100 μL of derivatization reagent. The reaction was immediately mixed and incubated for 1 min at room temperature. Subsequently, the solution was incubated for 10 min in a 55 °C water bath. After cooling, the reaction mixture was analyzed using UPLC. The ACQUITY UPLC BEH C18 (1.7 μm, 2.1 × 100 mm, Waters Corporation, Milford, USA); the reversed-phase column was used at 25 °C. The solvent system comprised 0.1% formic acid (A) as the inorganic-type mobile phase and acetonitrile (B) as the organic-mobile phase. A 5 μL injection volume was used. The initial flow rate was 0.4mL/min. Separation was accomplished using a gradient as follows: initial 0.1% B, 0-12 min 50.0% B, 12-13 min 0.1% B, and 13-15 min 0.1% B. A mass spectrometer (Xevo Q Tof MS, Waters Corporation, Milford, USA) was used to measure the mass range of electrospray ionization in the positive mode at 100-1000 m/z. A mass chromatogram (Abs Window 0.05 Da) of the m/z value of each amino acid was prepared from the measurement results, and the amino acid content was measured according to the peak integration value. Data analysis was performed using Waters Masslynx software (USA). Amino acid mixed standard solution H-type (Wako Pure Chemical Industries Ltd., Japan) with added asparagine, glutamine, gamma-aminobutyric acid, and citrulline was to create a standard calibration curve for the measurement of retention time and m/z values of each amino acid. Twenty-one free amino acids were analyzed, all

included in the standard solution.

5. 50% ethanol-soluble extract content: The measurement of 50% ethanol-soluble extract content was carried out according to modifying The Japanese Pharmacopoeia 17th edition "(131)". An extract was prepared by adding 25 mL of 50% ethanol to 2.5 g of dried main root powder from 2 plants, collected from 20 plants after using growth analysis and AMF colonization level check, crushed in a blender, and shaking at room temperature for 24 h. The extract was centrifuged (4000 rpm, 15 min, room temperature), and the resulting supernatant was dried and solidified in a water bath (100ºC, two h) followed by a drying oven (105ºC, 4 h), and the weight of the resulting residue was determined. The weight of the residue was divided by the weight of the dried root powder (2.5 g) and multiplied by 100 to obtain the 50% ethanol-soluble extract content.

RESULTS

Ten months after AMF inoculation, shoot growth appearance showed higher in all mycorrhizal *P. grandiflorus* plants, where GM exhibits the highest performance among the AMF species (Fig. 38).

The root dry weight significantly increased in all the mycorrhizal *P. grandiflorus* plants sixteen months after AMF inoculation compared to the control. The root dry weight reached more than 4.2, 3.8, and 3.7 times in GM, Gf, and Gm plants respectively, than that of the control, and the highest dry weights in roots were observed in GM plants (Fig. 39). In terms of physical appearance, sixteen months after AMF inoculation, root growth showed higher in all mycorrhizal *P. grandiflorus* plants, where GM exhibits the highest performance (Fig. 40).

After sixteen months of AMF inoculation, AMF colonization occurred successfully in all the inoculated plants, with the levels of 28-34% in a root system of *P. grandiflorus* plants. However, no significant differences were noted among the AMF species (Fig. 41).

Regarding the changes in free amino acid contents, sixteen months after AMF inoculation, the total free amino acid content in the main roots was significantly increased (1.6 times or 60%) in mycorrhizal (GM) *P. grandiflorus* plants compared to the control (Fig. 42). In terms of individual free amino acids, arginine, glutamine, glutamic acid, aspartic acid, asparagine, γ-aminobutyric acid (GABA), alanine, threonine, histidine, glycine, valine, cysteine, isoleucine, phenylalanine, leucine, methionine, and tyrosine increased in GM inoculated plants (Fig. 42).

The 50% ethanol-soluble extract content of the main root, which was defined as 25% or more for the use of medicinal purposes by the Japanese Pharmacopoeia, was 14% in control, which was less than 25%. On the other hand, the value of the 50% ethanol-soluble extract content of the main root was 41% in mycorrhizal (GM) *P. grandiflorus* plants, which was 2.9 times (193%) higher than the control plants (Fig. 43).

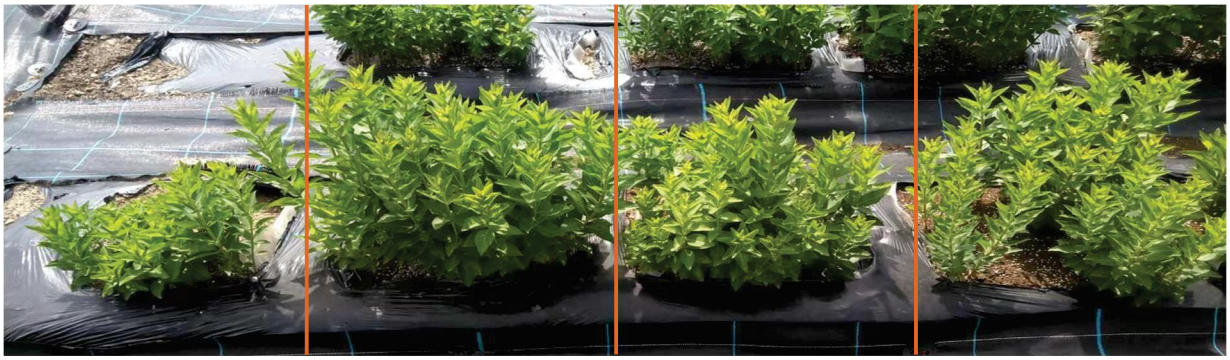


Fig. 38. Shoot growth of *Platycodon grandiflorus* 10 months after AMF inoculation.

C, control; GM, *Gigaspora margarita*; Gf, *Glomus fasciculatum*; Gm, *Glomus mosseae*.

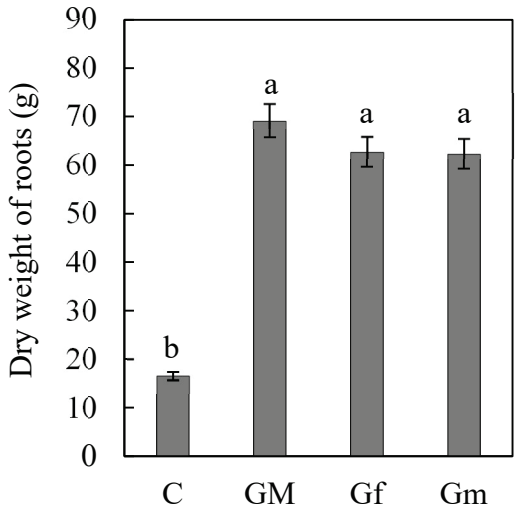


Fig. 39. Dry weight of roots in *Platycodon grandiflorus* cv. Murasaki 16 months after AMF inoculation.

C, control; GM, *Gigaspora margarita*; Gf, *Glomus fasciculatum*; Gm, *Glomus mosseae.* Columns denoted by different letters indicate significant differences according to Tukey's HSD test ($p < 0.05$).

C GM Gf Gm

Fig. 40**.** Root growth of *Platycodon grandiflorus* 16 months after AMF inoculation.

C, control; GM, *Gigaspora margarita*; Gf, *Glomus fasciculatum*; Gm, *Glomus mosseae*.

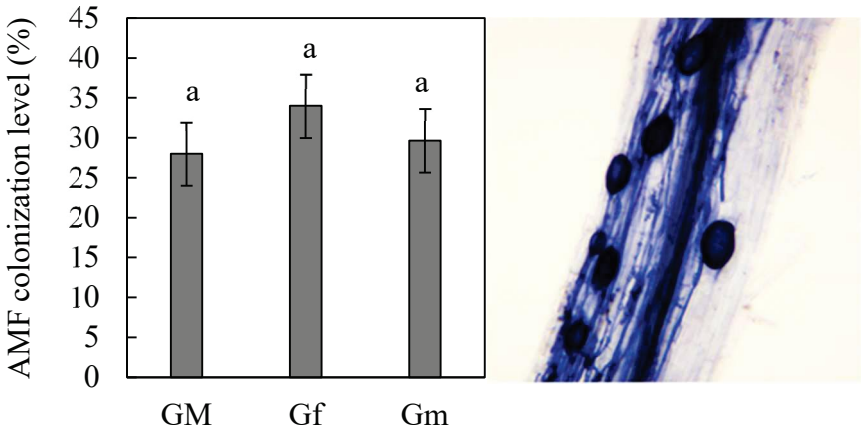


Fig. 41. AMF colonization level and colonization in *Platycodon grandiflorus* roots 16 months after AMF inoculation.

GM, *Gigaspora margarita*; Gf, *Glomus fasciculatum*; Gm, *Glomus mosseae*. Columns denoted by different letters indicate significant differences according to Tukey's HSD test ($p < 0.05$).

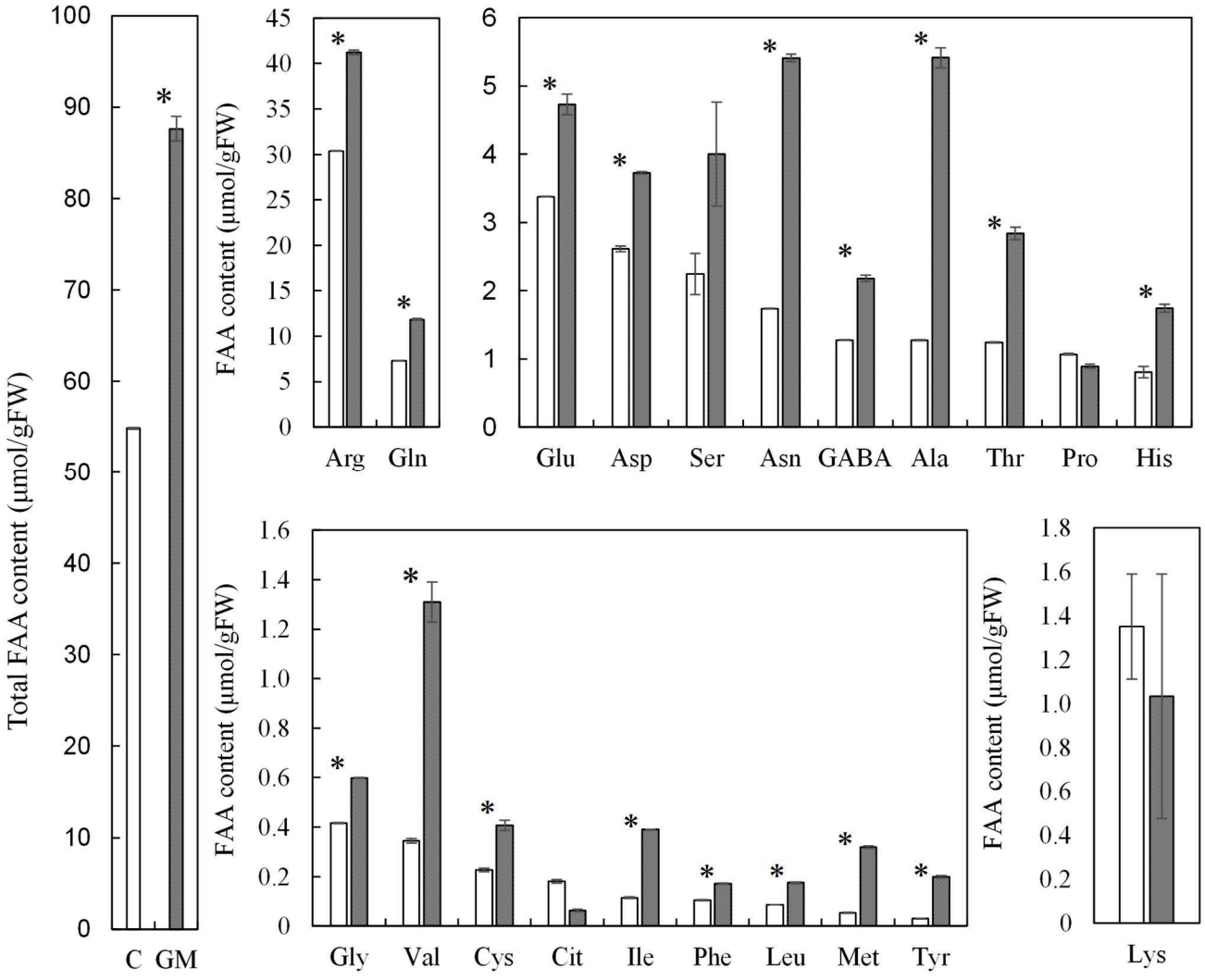


Fig. 42. Changes in total free amino acid (FAA) contents and individual FAA contents in *Platycodon grandiflorus* roots (cv. Murasaki) 16 months after AMF inoculation in the field. C, control; GM, *Gigaspora margarita*; FAA, free amino acid. ＊, significantly different between C and AMF by t-test ($p < 0.05$).

□, Control, ▨ GM.

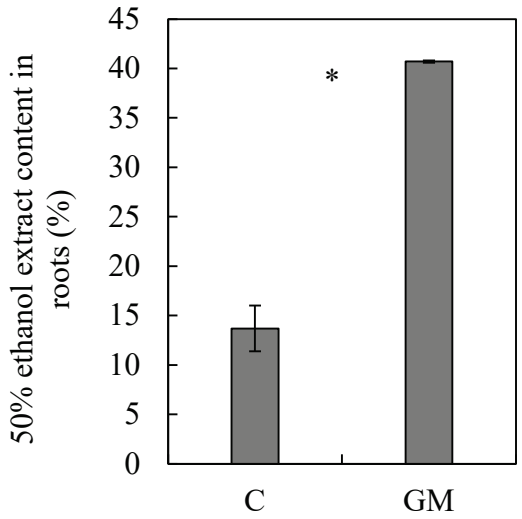


Fig. 43. 50% ethanol extract content in roots of *Platycodon grandiflorus* 16 months after AMF inoculation. C, control; GM, *Gigaspora margarita*. ＊, significantly different between C and GM by t-test ($p < 0.05$).

Discussion

The effect of AMF on plant growth is highly dependent on the combination of the AMF species and plant species "(132)". Henceforth, growth promotion and functional constituents of more active AMF species on selected cultivars are discussed. Mycorrhizal colonization was observed in *P. grandiflorus* under natural field conditions without mycorrhizal inoculation "(129-130)". Mycorrhizal fungi (*Gigaspora margarita*, *Glomus fasciculatum*, *Glomus mosseae*) used in this experiment have been reported to have growth-promoting effects in multiple plant species through symbiosis "(102)". In this study, *P. grandiflorus* inoculated with 3 AMF species showed significantly increased root dry weight in all the mycorrhizal species compared to the control. However, among the AMF species, GM and *P. grandiflorus* (cv. Murasaki) symbiotic combination had the best result for shoot growth, root growth, and dry weight of roots, and for the first time, fungal species differences in growth promotion were also observed. However, the reason for the species difference is unclear. Ciftci et al. "(133)" found a similar result that, among the 3 AMF species, *Glomus mosseae,* and among the four bean cultivars, the onceler symbiotic combination had the best outcome for promoting plant growth. Li-Xiang and Liu "(134)" reported that phosphorus fertilization increased the shoot and root dry weights of *P. grandiflorus*. In this study, it is speculated that the enhanced plant growth in mycorrhizal *P. grandiflorus* might be partly due to the promotion of phosphorus uptake by mycorrhizal fungi.

Maya and Matsubara "(34)" reported that growth promotion and anti-oxidative activity increased under environmental stress in cyclamens and had no correlation with mycorrhizal colonization levels. In this study, we found that the AMF colonization levels reached around 28-34% among the AMF species in a root system. However, in the present study, we could not clarify the relationship between AMF colonization levels and growth promotion in the dry weight of roots, total and individual free amino acid contents, and 50% ethanol extract content

in roots. Sutton "(32)" demonstrated that AMF colonization consists of three phases: 1) a lag phase during which spore germination, germ tube growth, and initial penetration occur; 2) a rapid growth phase, coinciding with the development of external mycelia and spread of the fungus within the roots; and 3) a stable phase during which the proportion of infected roots to non-infected ones remains nearly constant. In this study, the colonization level was checked only once. Hence, it wasn't easy to estimate when the AMF reached the maximum colonization level during the experimental period and how the colonization level influenced the promotion of growth and the increased functional components. Further investigation is necessary to address this issue.

It has been reported that several mycorrhizal plants showed higher total and individual free amino acid concentrations than the control plants, and the effect varies depending on the host-fungus combination "(110)". The results of the current study were consistent with previous research, indicating that the GM plants had significantly increased levels of total and individual free amino acid contents compared to the control plants. The important role played by amino acids is the regulation of several physiological processes in plants, like nutrient uptake, especially nitrogen, antioxidant metabolism, and root development "(135)". Amino acids like phenylalanine, alanine, leucine, and glutamine have been reported to influence glutamate receptors in plants "(136)" that, in turn, mediate a number of plant responses like changes in root architecture, plant stress signaling, carbon metabolism, stomatal movements, photosynthesis, and plant immunity "(137)". Mycorrhizal fungi have been shown to increase the quantity of free amino acids and the expression levels of key enzymes involved in nitrogen assimilation, such as glutamine synthase, glutamate synthase, and glutamate dehydrogenase, in tea plants "(112)". In this study, 21 free amino acids were detected in the roots of *P. grandiflorus* plants, and the concentration of 17 free amino acids was significantly increased compared to the control plants. It has been reported that arginine in plants may act as a

precursor of stress response signals, nitric oxide, and polyamines “(69)”. On the other hand, it is reported that glutamine has a weight loss effect in humans “(70)”. GABA plays a significant role in various physiological processes in plants, such as nitrogen storage, oxidative stress protection, and osmoregulation, and acts as a signaling molecule in the stress response “(113)”. Histidine is used to improve insulin resistance “(73)”, and tyrosine has the potential to enhance cognitive function in humans “(74)”. In addition, in plants, aspartic acid is an amino acid that serves as a precursor to several other amino acids, such as arginine, methionine, threonine, and isoleucine “(138)”. Subsequently, the increase in aspartic acid and asparagine is evidence of higher production of alanine and GABA “(139)”. This study showed that mycorrhizal fungi greatly contribute to the increase in amino acid content, and mycorrhizal plants are presumed to be related to the nitrogen supply and amino acid metabolism pathways through mycorrhizal symbiosis. In addition, free amino acids have various health-enhancing effects, and because these functional amino acids increase due to mycorrhizal symbiosis, it can be expected that *P. grandiflorus* roots will be highly functionalized by mycorrhizal symbiosis.

According to the 17th edition of the Japanese Pharmacopoeia “(140)”, dried *P. grandiflorus* root must have a diluted ethanol-soluble extract concentration of 25% or more to be considered a medicinal root. In this study, the 50% ethanol-soluble extract level of *P. grandiflorus* dried roots was above 40% in mycorrhizal plants and satisfied the requirements for medicinal roots. According to the Chinese pharmacopeia, Li-Xian and Liu “(134)” found that phosphorus fertilization reduced the amount of water-soluble extract content. Thus, the increase in 50% ethanol-soluble extract concentration in mycorrhizal *P. grandiflorus* in our investigation may have been impacted by factors other than phosphorus delivery by mycorrhizal fungus. However, the 50% ethanol-soluble extract contained various components, including platycodin and other triterpenoid saponins. Triterpenoid saponins are a general term for glycosides having a triterpene skeleton and are widely present as secondary metabolites of plants. Terpenoid

biosynthesis depends on the primary metabolism of photosynthetic and oxidative pathways “(141)”. Kapoor et al. “(142)” reported that phosphorus supply and mycorrhizal inoculation increased the terpenoid content of mugwort (*Artemisia annua* L.*)* leaves and that the increasing effect of mycorrhizal fungi exceeded the effect of phosphorus. From these results, it is considered that the increase in the content of 50% ethanol extract and the increase in triterpenoid saponin in *P. grandiflorus* roots inoculated with mycorrhizal fungi are affected by effects other than phosphorus supply by mycorrhizal fungi. However, the detailed compositional variation due to mycorrhizal colonization needs further investigation.

Thus, in this study, mycorrhizal colonization in *P. grandiflorus* can promote plant growth and increase the content of functional components such as total and individual free amino acids and 50% ethanol-soluble extract. The use of arbuscular mycorrhizal fungi is expected to shorten the cultivation period, increase stress tolerance, and increase or stabilize secondary metabolites of *P. grandiflorus* by promoting plant growth and expanding its use for food and medicinal purposes by improving the functional components of the *P. grandiflorus* roots.

Conclusion

Platycodon grandiflorus is a perennial herb belonging to the *Campanulaceae* family that has been widely used as an edible medicinal food for thousands of years. The cultivation period of these plants is more than two years, and the functional components of these plants fluctuate due to environmental factors, so it is important to shorten the cultivation period and increase the functional components. To address the standing claim of the beneficial effect of arbuscular mycorrhizal fungi (*Gigaspora margarita*, *Glomus fasciculatum, Glomus mosseae*) on growth and the changes in functional components of *Platycodon grandiflorus* cv. Murasaki was investigated. The result showed that the dry weight of roots significantly increased in all the mycorrhizal species compared to the control. Through LC-MS analysis showed the total free amino acid, individual free amino acids (arginine, GABA, glutamine, and histidine, etc.), and 50% ethanol-soluble extract contents in the root were significantly increased in mycorrhizal plants compared to the control. Therefore, mycorrhizal inoculation is suggested to enhance free amino acid contents and 50% ethanol-soluble extract that may promote plant growth and increase functional components in *Platycodon grandiflorus* plants, which help to establish sustainable cultivation techniques and improve functional secondary metabolites.

Summary

The demand for medicinal plants has been increasing in recent years due to the aging of the population and the expansion of the market as functional vegetables and foods. However, due to delays in establishing cultivation methods for medicinal plants in Japan, it largely depends on imports. On the other hand, there is a great concern about future supply shortages, depletion, and decreased safety of imported medicinal plants due to overfishing and agrochemical contamination. In addition, the limitation of agrochemical use in medicinal plants has also led to the limitation of disease control. Important issues in the cultivation of medicinal plants include the long period for cultivation, low survival rates due to disease outbreaks, and fluctuations in medicinal components due to environmental factors. Therefore, this study aims to establish sustainable cultivation techniques for medicinal plants, like functional vegetables and foods, that promote plant growth, shorten the cultivation period, control diseases, induce environmental stress tolerance, and stabilize and increase the content of functional components.

Asian ginseng (*Panax ginseng* C.A. Meyer) has many beneficial effects and is commonly used as a medicinal plant worldwide, including in Japan, but before reaching the peak of production, marketable yields decreased due to stunted seedlings, rotting roots, stem discoloration, wilting, and death. This phenomenon is called ginseng decline, and some biotic and abiotic factors cause decline symptoms and replant problems, but major biotic factors are still unclear in Japan and differ from country to country. Given this lack of research, PCR-SSCP analysis was carried out to identify the biotic factor associated with ginseng root rot. 102 isolates were obtained from the rotted roots of ginseng across two domestic regions in Japan, and it was identified that *Fusarium* species are associated with ginseng root rot. Most of the *Fusarium* isolates were highly pathogenic in vitro, suggesting that they are important biotic factors leading to a decline in ginseng in Japan. After identifying the biotic factor, growth promotion and tolerance to *Fusarium* root rot in the mycorrhizal ginseng plants were

investigated. Arbuscular mycorrhizal fungi (AMF) promote host plant growth mainly by enhancing phosphorus uptake through symbiosis. Results showed that mycorrhizal plants had higher shoot and root dry weights than control plants. AMF colonization levels in the root systems were not significantly different among the species. Four weeks after *Fusarium* inoculation, disease incidence and severity of symptoms decreased in mycorrhizal ginseng plants compared to control. These findings demonstrated that *Fusarium* spp. are associated with ginseng root rot, and growth enhancement and tolerance to *Fusarium* root rot were confirmed in mycorrhizal ginseng plants. On the other hand, researchers found no more than 60% of ginseng plants can survive from sowing to harvest and believed the yield loss of replanted crops was closely related to allelopathy, but the results are still unclear. In this experiment, allelochemicals in the field soils (1-6 year-cultivated) were estimated by in vitro testing method using lettuce (*Lactuca sativa* L. 'Great lakes') seedlings susceptible to allelochemicals. The dry weight of roots and root length of the indicator plant decreased in the long-cultivated soil plots compared to the short one. In this case, soil chemical properties such as soil mineral elements (NO_3^-, PO_4^{3-}, K^+, Ca^{2+}, Mg^{2+}) and pH had no consistent association with growth suppression. UPLC-MS analysis showed a specific peak in the long-cultivated field soils. From these results, allelochemicals suppressing the growth of plant roots might exist in the field soils with long-term cultivation. These compounds should be identified, and further study is needed to elucidate the interaction mechanisms between allelochemical, diseases, soil chemical properties, and plant growth.

In this study, AMFs are also used to improve ginseng plants' growth and quality. The experiment showed that the dry weight of shoots and roots, root diameter, SPAD values, and ginsenosides (Rb_1 and Rg_1) contents were higher in mycorrhizal plants than in the control. Through LC-MS analysis, increased total free amino acids and individual free amino acids (arginine, proline, methionine, GABA, phenylalanine, asparagine, glutamic acid, threonine,

etc) were found in mycorrhizal roots. These findings suggest that plant growth promotion and positive changes in functional components through AMF symbiosis occurred in mycorrhizal ginseng plants.

To evaluate the effectiveness of AMF inoculation on plant growth and the changes in functional components, this study was conducted on egoma (*Perilla frutescens* (L) Britton var. *frutescens*, cv. Shirakawa) plants. Ten weeks after AMF inoculation, mycorrhizal egoma plants had significantly higher leaf dry weights, total number of leaves, seed yield, and α-linolenic acid content in the seeds, and functional components (polyphenol, glutathione, ascorbic acid, but DPPH radical scavenging activity) in leaves than in control plants. UPLC-MS analysis showed higher total free amino acid contents and individual free amino acid contents, including GABA, in mycorrhizal egoma than in control plants. The AMF colonization levels in the root systems were not significantly different between the species. Therefore, mycorrhizal symbiosis may be highly functional owing to increased functional components that promote plant growth and positively change functional components in egoma plants.

Platycodon grandiflorus is a perennial herb belonging to the *Campanulaceae* family that has been widely used as an edible medicinal food for thousands of years. The cultivation period of these plants is more than two years, and the functional components of these plants fluctuate due to environmental factors, so it is important to shorten the cultivation period and increase the functional components. To address the standing claim of the beneficial effect of AMF (*Gigaspora margarita*, *Glomus fasciculatum, Glomus mosseae*) on growth and the changes in functional components of *Platycodon grandiflorus* were investigated. The result showed that the dry weight of roots significantly increased in all the mycorrhizal species compared to the control. Through LC-MS analysis showed the total free amino acid, individual free amino acids (arginine, GABA, glutamine, and histidine, etc.), and 50% ethanol-soluble extract contents in the root were significantly increased in mycorrhizal plants compared to the control. Therefore,

mycorrhizal inoculation is suggested to enhance free amino acid contents and 50% ethanol-soluble extract that may promote plant growth and increase functional components in *Platycodon grandiflorus*.

In short, this study clarified growth promotion and changes in functional components that occurred in some medicinal plants through AMF symbiosis. In addition, AMF promotes phosphorus uptake by the host plant and enhances secondary metabolites and functional components that induce environmental stress tolerance. However, the incidence and severity of disease symptoms were reduced by AMF, suggesting that tolerance to the disease (*Fusarium* root rot) occurred in the mycorrhizal medicinal plants. The findings help us to establish sustainable cultivation techniques for medicinal plants, like functional vegetables and foods that promote plant growth by AMF, induce environmental stress tolerance and disease tolerance, and improve functional secondary metabolites.

References

1. Bagchi D. Neutraceutical and functional food regulations in the United States and around the worlds. Elsevier, New York. 2008.
2. Wang H, Xu F, Wang X, Kwon WS and Yang DC. Molecular discrimination of *Panax ginseng* cultivar K-1 using pathogenesis-related protein five genes. Journal of Ginseng Research 43, 482- 487. 2019.
3. Choi, K. Invited review: Botanical characteristics, pharmacological effects and medicinal components of Korean *Panax ginseng* C. A. Meyer. Acta Pharmacologica Sinica 29, 1109-1118. 2008.
4. Harrison H, Parke J, Oelke E, Kaminski A, Hudelson B, Martin L and Kelling K. Alternative Field Crops Manual: Ginseng. Purdue Univ., Dept. of Hortic. And Landscape Architecture, Extension/Outreach, New Crops Center. Online publication. 2000.
5. Wang Y, Guan YM, Lu BH and Gao. First report of ginseng (*Panax ginseng*) root rot caused by *Fusarium acuminatum* in China. Plant Disease 100, 525-526. 2016.
6. Reeleder RD, Roy R and Capell B. Seed and root rots of ginseng (*Panax quinquefolius* L.) caused by *Cylindrocarpon destructans* and *Fusarium* spp. Journal of Ginseng Research 3, 151-158. 2002.
7. Sugino M, Ogaki K, Suruamsiri P, Nilsamranchit S, Chaimongkol C and Tsutsui H. Studies on the cultivation of *Panax ginseng* in Thailand, I: A Theoretical possibility of ginseng cultivation in different environmental conditions. Memoirs of the Faculty of Agriculture of Kinki University (Japan) 28, 39-44. 1995.
8. Zhang AH, Lei FJ, Fang SW, Jia MH and Zhang LX. Effects of ginsenosides on the growth and activity of antioxidant enzymes in *American ginseng* seedlings. Journal of Medicinal Plants Research 5, 3217-3223. 2011.

9. He CN, Gao WW, Yang JX, Bi Y, Zhang XS and Zhao YJ. Identification of autotoxic compounds from fibrous roots of *Panax quinquefolium* L. Plant and Soil 318, 63-72. 2009.
10. DesRochers N, Walsh JP, Renaud JB, Seifert KA, Yeung KK and Sumarah MW. Metabolomic profiling of fungal pathogens responsible for root rot in *American ginseng*. Metabolites 10, 35. 2020.
11. Zhan XJ, Tian C, Zhang Y and Liu C. PCR-SSCP molecular Identification of *Panax ginseng* and *P. quinquefolius* based on ITS2 bar coding SNPs. China Journal of Chinese Materia Medica 37, 3748-3751. 2012.
12. Japanese Plant Disease Database. Agricultural Biological Resources Gene Bank. http://www.gene.affrc.go. 2019.
13. Punja ZK, Wan A, Rahman M, Goswami RS, Barasubiye T, Seifert KA and Levesque CA. Growth, population dynamics, and diversity of *Fusarium equiseti* in ginseng fields. European Journal of Plant Pathology 121, 173-84. 2008.
14. Yergeau E, Vujanovic V and St-Arnaud M. Changes in communities of *Fusarium* and arbuscular mycorrhizal fungi as related to different asparagus cultural factors. Microbial Ecology 52, 104-113. 2006.
15. Wong JY and Jeffries P. Diversity of pathogenic *Fusarium* populations associated with asparagus roots in decline soils in Spain and the UK. Plant Pathology 55, 331-342. 2006.
16. Nahiyan ASM, Boyer LR, Jeffries P and Matsubara Y. PCR-SSCP analysis of *Fusarium* diversity in asparagus decline in Japan. European Journal of Plant Pathology 130, 197-203. 2011.
17. Marschner H, Dell B. Nutrient uptake in mycorrhizal symbiosis. Plant and Soil 159, 89-102. 1994.

18. Khan MH, Meghvansi MK, Panwar V, Gogoi HK and Singh L. Arbuscular mycorrhizal fungi-induced signalling in plant defence against phytopathogens. Journal of Phytology 2, 53-69. 2010.
19. Hajiboland R, Aliasgharzadeh N, Laiegh SF, Poschenrieder C. Colonization with arbuscular mycorrhizal fungi improves salinity tolerance of tomato (*Solanum lycopersicum* L.) plants. Plant and Soil 33, 313-327. 2010.
20. Kapoor R. Induced resistance in mycorrhizal tomato is correlated to concentration of jasmonic acid. Online Journal of Biological Sciences 8, 49-56. 2008.
21. Akthar MS and Siddiqui ZA. Arbuscular mycorrhizal fungi as potential bioprotectants against plant pathogens. Mycorrhizae: Sustainable agriculture and forestry. Springer, Netherland. pp. 61-97. 2008.
22. Sikes BA and Klironomos J. Plant pathogen protection by AMF is determined by both root system architecture and fungal identity. ESA Annual Convention. 93rd. 2008.
23. Fournier AR, Khanizadeh S, Gauthier L, Gosselin A and Dorais M. Effect of two *Glomus* species inoculations on survival, photosynthetic capacity, growth, morphology, and root ginsenoside content of *Panax quinquefolius* L. Journal of Ginseng Research 27, 178-182. 2003.
24. Blok WJ and Bollen GJ. Fungi on roots and stem bases of asparagus in the Netherlands: species and pathogenicity. European Journal of Plant Pathology 101, 15–24. 1995.
25. Phillips JM, Hayman DS. Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. Transactions of the British Mycological Society 55, 158-163. 1970.
26. Komada H. Development of a selective medium for quantitative isolation of *Fusarium oxysporum* from natural soil. Review of Plant Protection Research 8,114-124. 1975.

27. Lee SG. Fusarium species associated with ginseng (*Panax ginseng*) and their role in the root-rot of ginseng plant. Research in Plant Disease 10, 248-259. 2004.
28. Kang SW, Yeon BY, Hyeon GS, Bae YS, Lee SW and Seong NS. Changes of soil chemical properties and root injury ratio by progress years of post-harvest in continuous cropping soils of ginseng. Korean Journal of Medicinal Crop Science 15, 157-161. 2007. (English summary).
29. Fan S, Zhao F, Zhang J, Shang W and Hu X. *American ginseng* root rot caused by *Fusarium redolens* in China. Plant Disease 105, 2734. 2021.
30. Ozgonen H and Erkilic A. Growth enhancement and *Phytophthora* blight (*Phytophthora capsici* Leonian) control by arbuscular mycorrhizal fungal inoculation in pepper. Crop Protection 26, 1682–1688. 2007.
31. Wu QS, Zou YN, Xia RX. Effects of water stress and arbuscular mycorrhizal fungi on reactive oxygen metabolism and antioxidant production by citrus (*Citrus tangerine*) roots. European Journal of Soil Biology 42, 166-72. 2006.
32. Sutton JC. Development of vesicular–arbuscular mycorrhizae in crop plants. Canadian Journal of Botany 51, 2487–2493. 1973.
33. Okada T and Matsubara Y. Tolerance to *Fusarium* root rot and the changes in free amino acid contents in mycorrhizal asparagus plants. HortScience 47, 751–754. 2012.
34. Maya MA and Matsubara Y. Tolerance to *Fusarium* wilt and anthracnose diseases and changes of antioxidative activity in mycorrhizal cyclamen. Crop Protection 47, 41-48. 2013.
35. Blilou I, Bueno P, Ocampo JA and García-Garrido JM. Induction of catalase and ascorbate peroxidase activities in tobacco roots inoculated with the arbuscular mycorrhizal *Glomus mosseae*. Mycological Research 104, 722-725. 2000.

36. Salzer P, Corbiere H and Boller T. Hydrogen peroxide accumulation in *Medicago truncatula* roots colonized by the arbuscular mycorrhiza-forming fungus *Glomus intraradices*. Planta 208, 319-325. 1999.
37. Volpin H, Phillips DA, Okon Y and Kapulnik Y. Suppression of an isoflavonoid phytoalexin defense response in mycorrhizal alfalfa roots. Plant Physiology 108, 1449-1454. 1995.
38. Zhu X, Song F and Xu H. Influence of arbuscular mycorrhiza on lipid peroxidation and antioxidative enzyme activity of maize plants under temperature stress. Mycorrhiza 20, 325–332. 2010.
39. Al-Askar A, Rashad Y. Arbuscular mycorrhizal fungi: a biocontrol agent against common. Plant Pathology Journal 9, 31-38. 2010.
40. Wu LJ, Zhao YH, Guan YM, Peng SF. A review on studies of the reason and control methods of succession cropping obstacle of *Panax ginseng* C. A. Meyer. Special Wild Economic Animal Plant Research 2, 68-72. 2008.
41. Wang SQ, Han XZ and Qiao YF. Allelopathy of root exudates and their effects on rhizosphere microorganism. Chinese Journal of Soil Science 38, 1219-26. 2007.
42. Li Y, Ying Y, Zhao D, Jin S and Ding W. Genetic diversity analysis on rhizosphere soil microbial population of *Panax ginseng* and *Panax quinquefolium* by RAPD. Chinese Traditional and Herbal Drugs 41, 1871-1875. 2010.
43. Li Y, Ying YX, Zhao DY and Ding WL. Influence of allelochemicals on microbial community in ginseng cultivating soil. Chinese Herbal Medicines, 6, 313-318. 2014.
44. Qian-Li M and Qing-bo W. Study on effect of ecology method on *Panax ginseng* continuous cropping ground soil improvement. Ginseng Research 1, 35-38. 2010.
45. Guo SH, Wang YX, Geng YQ and Wang YZ. Effect of the ecological distribution of soil microorganisms by ginseng-cultivating. Acta Ecologica Sinica 10, 286-287. 1990.

46. Li Y, Huang XF and Ding WL. Autotoxicity of *Panax ginseng* rhizosphere and non-rhizosphere soil extracts on early seedlings growth and identification of chemicals. Allelopathy Journal 28, 145-154. 2011.
47. Getaneh S and Kidanemariam W. Soil acidity and its managements: a review. International Journal of Advanced Research in Biological Sciences 8, 70-79. 2021.
48. Forde B and Lorenzo H. The nutritional control of root development. Plant and Soil 232, 51-68. 2001.
49. Fujii Y. Allelopathy in the natural and agricultural ecosystems and isolation of potent allelochemicals from Velvet bean (*Mucuna pruriens*) and Hairy vetch (*Vicia villosa*). Biological Sciences in Space 17, 6-13. 2003.
50. Hiraki Y, Matsubara Y, Ikezawa T. Effect of AMF symbiosis and environmental conditions on growth of Asian ginseng. Acta Horticulture 1208, 271-276. 2018.
51. Baranov A. Recent advances in our knowledge of the morphology, cultivation and uses of ginseng (*Panax ginseng* CA Meyer). Economic Botany 20, 403-406. 1966.
52. Yun TK. Brief introduction of *Panax ginseng* C. A. Meyer. Journal of Korean Medical Science 16, 3-5. 2001.
53. Chung HS, Lee YC, Kyung-Rhee Y and Lee SY. Consumer acceptance of ginseng food products. Journal of Food Science 76, 516-522. 2011.
54. Sakamoto K. Arbuscular mycorrhizal fungi: Deepening of study and contribution of agriculture. Journal of Soil Microbiology 68, 65-68. 2014.
55. Jiang W, Gou G and Ding Y. Influences of arbuscular mycorrhizal fungi on growth and mineral element absorption of chenglu hybrid bamboo seedlings. Pakistan Journal of Botany 45, 303-310. 2013.

56. Jaiti F, Meddich A and Hadrami IE. Effectiveness of arbuscular mycorrhizal fungi in the protection of date palm (*Phoenix dactylifera* L.) against bayoud disease. Physiological and Molecular Plant Pathology 71,166-173. 2007.
57. Song Y, Chen D, Lu K, Sun Z and Zeng R. Enhanced tomato disease resistance primed by arbuscular mycorrhizal fungus. Frontiers in Plant Science 6, 786. 2015.
58. Evelin H, Kapoor R and Giri B. Arbuscular mycorrhizal fungi in alleviation of salt stress: a review. Annals of Botany 104, 1263-1280. 2009.
59. Porcel R, Aroca R, Ruiz and Lozano JM. Salinity stress alleviation using arbuscular mycorrhizal fungi. A review. Agronomy for Sustainable Development 32, 181-200. 2012.
60. Subramanian KS, Santhanakrishnan P and Balasubramanian P. Responses of field grown tomato plants to arbuscular mycorrhizal fungal colonization under varying intensities of drought stress. Scientia Horticulturae 107, 245-253. 2006.
61. Pedone-Bonfim MV, da Silva FS and Maia LC. Production of secondary metabolites by mycorrhizal plants with medicinal or nutritional potential. Acta Physiologiae Plantarum 37, 1-12. 2015.
62. Adolfsson L, Nziengui H, Abreu IN, Šimura J, Beebo A, Herdean A, Aboalizadeh J, Siroka J, Moritz T, Novak O and Ljung K. Enhanced secondary-and hormone metabolism in leaves of arbuscular mycorrhizal *Medicago truncatula*. Plant Physiology 175, 392-411. 2017.
63. Cho EJ, Do Wee C, Kim HL, Cheong YH, Cho JS and Sohn BK. Effects of AMF inoculation on growth of *Panax ginseng* C. A. Meyer seedlings and on soil structures in mycorrhizosphere. Scientia Horticulturae 122, 633-637. 2009.

64. Tian L, Shi S, Ma L, Zhou X, Luo S, Zhang J, Lu B and Tian C. The effect of *Glomus intraradices* on the physiological properties of *Panax ginseng* and on rhizospheric microbial diversity. Journal of Ginseng Research 43, 77-85. 2019.
65. Baltruschat, H and Schonbeck F. The influence of endotrophic mycorrhiza on the infestation of tobacco by *Thielaviopsis basicola*. Phytopath. Z. 84, 172-188. 1975.
66. Shi SM, Chen K, Gao Y, Liu B, Yang XH, Huang XZ, Liu GX, Zhu LQ and He XH. Arbuscular mycorrhizal fungus species dependency governs better plant physiological characteristics and leaf quality of mulberry (*Morus alba* L.) seedlings. Frontiers in Microbiology 7, 1030. 2016.
67. Khalaf D, Kruger M, Wehland M, Infanger M and Grimm D. The effects of oral L-arginine and L-citrulline supplementation on blood pressure. Nutrients 11, 1679. 2019.
68. Reeds PJ. Dispensable and indispensable amino acids for humans. The Journal of Nutrition 130, 1835-1840. 2000.
69. Winter G, Todd CD, Trovato M, Forlani G and Funck D. Physiological implications of arginine metabolism in plants. Frontiers in plant science 6, 534. 2015.
70. Ahmadi AR, Rayyani E, Bahreini M and Mansoori A. The effect of glutamine supplementation on athletic performance, body composition, and immune function: A systematic review and a meta-analysis of clinical trials. Clinical Nutrition 38, 1076-1091. 2019.
71. Inoue K, Shirai T, Ochiai H, Kasao M, Hayakawa K, Kimura M and Sansawa H. Blood-pressure-lowering effect of a novel fermented milk containing γ-aminobutyric acid (GABA) in mild hypertensives. European Journal of Clinical Nutrition 57, 490-495. 2003.

72. Kanehira T, Nakamura Y, Nakamura K, Horie K, Horie N, Furugori K, Sauchi Y and Yokogoshi H. Relieving occupational fatigue by consumption of a beverage containing γ-amino butyric acid. Journal of Nutritional Science and Vitaminology 57, 9-15. 2011.
73. Feng RN, Niu YC, Sun XW, Li Q, Zhao C, Wang C, Guo FC, Sun CH and Li Y. Histidine supplementation improves insulin resistance through suppressed inflammation in obese women with the metabolic syndrome: a randomized controlled trial. Diabetologia 56, 985-994. 2013.
74. Colzato LS, Jongkees BJ, Sellaro R and Hommel B. Working memory reloaded: tyrosine repletes updating in the N-back task. Frontiers in Behavioral Neuroscience 7, 200. 2013.
75. Ramos-Ruiz R, Martinez F and Knauf-Beiter G. The effects of GABA in plants. Cogent Food and Agriculture 5, 1670553. 2019.
76. Ahmed HM. Ethnomedicinal, phytochemical and pharmacological investigations of *Perilla frutescens* (L.) Britt. Molecules 24, 102. 2019.
77. Nitta M, Lee JK and Ohnishi O. Asian perilla crops and their weedy forms: Their cultivation, utilization and genetic relationships. Economic Botany 57, 245-253. 2003.
78. Joshi A, Sharma A, Pandey DP and Bachheti RK. Physico-chemical properties of *Perilla frutescens* seeds. Der Pharma Chemica 7, 35-41. 2015.
79. Mojumdar MA, Jamal-Uddin AFM, Meherunnessa and Tariq Hossain HMM. Golden Perilla BD: future edible oil crop in Bangladesh. Business, Social and Scientific Research.8, 52-55. 2020.
80. Asif M. Health effects of omega-3, 6, 9 fatty acids: *Perilla frutescens* is a good example of plant oils. Oriental Pharmacy and Experimental Medicine11, 51-59. 2011.
81. Hou T, Netala VR, Zhang H, Xing Y, Li H and Zhang Z. *Perilla frutescens*: A rich source of pharmacological active compounds. Molecules 27, 3578. 2022.

82. Yoshimura Y and Ito S. Components of perilla and component fluctuations during processing and use. Research bulletin of the Aichi-ken Agricultural Research Center. Aichi Prefectural Agricultural Experiment Station 35,103-108. 2003.

83. Ogawa T, Chikashige K, Araki H, Kitagawa M, Katsube T, Ohta Y, Yamasaki Y, Hashimoto M and Azuma K. Effects of drying methods and pre-treatment conditions on the functional component contents and antioxidant activities in egoma (*Perilla frutescens* (L.) Bitt. var. *frutescens*) leaves. Journal of the Japanese Society for Food Science and Technology 63, 217-224. 2016.

84. Yamamoto H and Ogawa T. Antimicrobial activity of perilla seed polyphenols against oral pathogenic bacteria. Bioscience, Biotechnology, and Biochemistry 66, 921-924. 2002.

85. Kawahata T, Otake T, Mori H, Kojima Y, Oishi I, Oka S, Fukumori Y and Sano K. A novel substance purified from *Perilla frutescens* Britton inhibits an early stage of HIV-1 replication without blocking viral adsorption. Antiviral Chemistry and Chemotherapy 13, 283-288. 2002.

86. Banno N, Akihisa T, Tokuda H, Yasukawa K, Higashihara H, Ukiya M, Watanabe K, Kimura Y, Hasegawa JI and Nishino H. Triterpene acids from the leaves of *Perilla frutescens* and their anti-inflammatory and antitumor-promoting effects. Bioscience, Biotechnology, and Biochemistry 68, 85-90. 2004.

87. Guo R, Pittler MH and Ernst E. Herbal medicines for the treatment of allergic rhinitis: a systematic review. Annals of Allergy, Asthma & Immunology 99, 483-495. 2007.

88. Igarashi M and Miyazaki Y. A review on bioactivities of perilla: progress in research on the functions of perilla as medicine and food. Evidence-Based Complementary and Alternative Medicine. 1-7. 2013.

89. Balliu A, Sallaku G and Rewald B. AMF inoculation enhances growth and improves the nutrient uptake rates of transplanted, salt-stressed tomato seedlings. Sustainability 12,15967-15981. 2015.

90. Zubek S, Rola K, Szewczyk A, Majewska ML and Turnau K. Enhanced concentrations of elements and secondary metabolites in *Viola tricolor* L. induced by arbuscular mycorrhizal fungi. Plant and Soil 390, 129-142. 2015.

91. Hazzoumi Z, Moustakime Y, Hassan-Elharchli E and Joutei KA. Effect of arbuscular mycorrhizal fungi (AMF) and water stress on growth, phenolic compounds, glandular hairs, and yield of essential oil in basil (*Ocimum gratissimum* L). Chemical and Biological Technologies in Agriculture 2, 1-11. 2015.

92. Begum N, Qin C, Ahanger MA, Raza S, Khan MI, Ashraf M, Ahmed N and Zhang L. Role of arbuscular mycorrhizal fungi in plant growth regulation: implications in abiotic stress tolerance. Frontiers in Plant Science 10, 1-15. 2019a.

93. Miransari M. Arbuscular mycorrhizal fungi and nitrogen uptake. Archives of Microbiology 193, 77-81. 2011.

94. Long LK, Yao Q, Guo J, Yang RH, Huang YH and Zhu HH. Molecular community analysis of arbuscular mycorrhizal fungi associated with five selected plant species from heavy metal polluted soils. European Journal of Soil Biology 46, 288-294. 2010.

95. Shi S, Tian L, Ma L and Tian C. Community structure of rhizomicrobiomes in four medicinal herbs and its implication on growth management. Microbiology 87, 425-436. 2018.

96. Wee CD and Sohn BK. Effectiveness of arbuscular mycorrhizal fungi (AMF) Inoculation on the Growth of Perilla. Korean Journal of Environmental Agriculture 29, 408-416. 2010.

97. Burits M and Bucar F. Antioxidant activity of *Nigella sativa* essential oil. Phytotherapy Research 14, 323-328. 2000.

98. McDonald S, Prenzler PD, Antolovich M and Robards K. Phenolic content and antioxidant activity of olive extracts. Food Chemistry 73, 73-84. 2001.

99. Mukherjee SP and Choudhuri MA. Implications of water stress-induced changes in the levels of endogenous ascorbic acid and hydrogen peroxide in Vigna seedlings. Physiologia Plantarum 58, 166-170. 1983.

100. Nimbalkar MS, Pai SR, Pawar NV, Oulkar D and Dixit GB. Free amino acid profiling in grain amaranth using LC–MS/MS. Food Chemistry 134, 2565-2569. 2012.

101. Yang H, Zhang Q, Dai Y, Liu Q, Tang J, Bian X and Chen X. Effects of arbuscular mycorrhizal fungi on plant growth depend on root system: a meta-analysis. Plant and Soil. 389, 361-374. 2015.

102. Johny L, Cahill DM and Adholeya A. AMF enhance secondary metabolite production in ashwagandha, licorice, and marigold in a fungi-host specific manner. Rhizosphere 17, 100314. 2021.

103. Teitz L, Zeiger E, Moller IM and Murphy A. Teitz/Zeiger plant physiology and embryology original. 6th edition. pp: 750. Kodansha. Tokyo. 2017.

104. Marxen K, Vanselow KH, Lippemeier S, Hintze R, Ruser A and Hansen UP. Determination of DPPH radical oxidation caused by methanolic extracts of some microalgal species by linear regression analysis of spectrophotometric measurements. Sensors 7, 2080-2095. 2007.

105. Noctor G and Foyer CH. Ascorbate and glutathione: keeping active oxygen under control. Annual Review of Plant Biology 49, 249-279. 1998.

106. Carillo P, Kyratzis A, Kyriacou MC, Dell'Aversana E, Fusco GM, Corrado G and Rouphael Y. Biostimulatory action of arbuscular mycorrhizal fungi enhances

productivity, functional and sensory quality in ‘Piennolo del Vesuvio’Cherry tomato landraces. Agronomy 10, 911. 2020.

107. Li Q, Li Y, Li C, Yu X. Enhanced ascorbic acid accumulation through overexpression of dehydroascorbate reductase confers tolerance to methyl viologen and salt stresses in tomato. Czech Journal of Genetics and Plant Breeding 48, 74-86. 2012.

108. Grace SC. Phenolics and antioxidants, In: Smirnoff N. (ed.) Antioxidants and reactive oxygen species in plants. Blackwell, Oxford, UK. pp 141-168. 2005.

109. Abdel-Fattah GM and Mohamedin AH. Interactions between a vesicular-arbuscular mycorrhizal fungus (*Glomus intraradices*) and Streptomyces coelicolor and their effects on sorghum plants grown in soil amended with chitin of brawn scales. Biology and Fertility of Soils 32, 401-409. 2000.

110. Sood SG. Chemotactic response of plant-growth-promoting bacteria towards roots of vesicular-arbuscular mycorrhizal tomato plants. FEMS Microbiology Ecology 45, 219-227. 2003.

111. Rasmussen S, Parsons AJ, Fraser K, Xue H and Newman JA. Metabolic profiles of *Lolium perenne* are differentially affected by nitrogen supply, carbohydrate content, and fungal endophyte infection. Plant Physiology 146, 1440-1453. 2008.

112. Shao YD, Zhang DJ, Hu XC, Wu QS, Jiang CJ, Gao XB and Kuca K. Arbuscular mycorrhiza improves leaf food quality of tea plants. Notulae Botanicae Horti Agrobotanici 47, 608-614. 2019.

113. Saito K and Yamazaki M. Biochemistry and molecular biology of the late-stage of biosynthesis of anthocyanin: lessons from *Perilla frutescens* as a model plant. New Phytologist 155, 9-23. 2002.

114. Smet FD and Cuypers K. Amino acid in higher plants. Critical Review of Plant Science 37, 417-453. 2018.

115. Belujon P and Grace AA. Dopamine system dysregulation in major depressive disorders. International Journal of Neuropsychopharmacology 20, 1036-1046. 2007.

116. Khalid KA and Shedeed MR. Effect of NPK and foliar nutrition on growth, yield and chemical constituents in *Nigella sativa* L. J. Mater. Environ. Sci. 6, 1709-1714. 2015.

117. Moghith WMA, Youssef ASM, Abd El-Wahab MA, Mohamed YFY and Abou El-Ghait EM. Effect of arbuscular mycorrhizal fungi and some phosphorus sources on growth, seeds yield, chemical compositions, oil productivity and fixed oil constituents of chia (*Salvia hispanica* L.) plant. Annals of Agricultural Science, Moshtohor 59, 541-562. 2021.

118. Kapulnik Y, Tsror L, Zipori I, Hazanovsky M, Wininger S and Dag A. Effect of AMF application on growth, productivity and susceptibility to *Verticillium* wilt of olives grown under desert conditions. Symbiosis 52, 103-111. 2010.

119. Kuriwaki J, Kanamori R, Morishita M and Suzuki M. Effects of a single dose of essential fatty acids on learning and memory function in rats. Bulletin of Mimasaka University 53, 111-115. 2020.

120. Ji MY, Bo A, Yang M, Xu JF, Jiang LL, Zhou BC and Li MH. The pharmacological effects and health benefits of *Platycodon grandiflorus*—a medicine food homology species. Foods 9, 142. 2020.

121. Su X, Liu Y, Han L, Wang Z, Cao M, Wu L, Jiang W, Meng F, Guo X, Yu N and Gui S. A candidate gene identified in converting platycoside E to platycodin D from *Platycodon grandiflorus* by transcriptome and main metabolites analysis. Scientific Reports 11, 1-17. 2021.

122. Zhang S, Chai X, Hou G, Zhao F and Meng Q. *Platycodon grandiflorum* (Jacq.) A. DC.: A review of phytochemistry, pharmacology, toxicology and traditional use. Phytomedicine 106, 154422. 2022.

123. Zhang L, Wang Y, Yang D, Zhang C, Zhang N, Li M and Liu Y. Platycodon grandiflorus–An Ethnopharmacological, phytochemical and pharmacological review. Journal of Ethnopharmacology 164, 147-61. 2015.

124. Islam SMA, Math RK, Kim JM, Yun MG, Cho JJ, Kim EJ, Lee YH and Yun HD. Effect of plant age on endophytic bacterial diversity of balloon flower (*Platycodon grandiflorum*) root and their antimicrobial activities. Current Microbiology 61, 346-356.

125. Li M, Zhang M, Cheng L, Yang L and Han M. Changes in the platycodin content and physiological characteristics during the fruiting stage of *Platycodon grandiflorum* under drought stress. Sustainability 14, 6285. 2022.

126. Dar MH and Reshi ZA. Vesicular arbuscular mycorrhizal (VAM) fungi are a major biocontrol agent in modern sustainable agriculture systems. Russian Agricultural Sciences 43, 138-143. 2017.

127. Khaliq A, Perveen S, Alamer KH, Zia Ul Haq M, Rafique Z, Alsudays IM, Althobaiti AT, Saleh MA, Hussain S and Attia H. Arbuscular mycorrhizal fungi symbiosis to enhance plant–soil interaction. Sustainability 14, 7840. 2021.

128. Sinclair G, Charest C, Dalpé Y and Khanizadeh S. Influence of colonization by arbuscular mycorrhizal fungi on three strawberry cultivars under salty conditions. Agricultural and Food Science 23, 146-158. 2014.

129. Cho N, Kim D, Eom A, Lee J, Choi T, Cho H, Leonowicz A and Ohga S. Identification of symbiotic arbuscular mycorrhizal fungi in Korea by morphological and DNA sequencing features of their spores. Journal of the Faculty of Agriculture, Kyushu University, 51, 201-210. 2006.

130. Piszczek P, Kuszewska K, Błaszkowski J, Sochacka-Obrusnik A, Stojakowska A and Zubek S. Associations between root-inhabiting fungi and 40 species of medicinal plants

with potential applications in the pharmaceutical and biotechnological industries. Applied Soil Ecology 137, 69-77. 2019.

131. Pharmaceutical and medical device regulatory science society of Japan. The Japanese Pharmacopoeia 17th edition. p.1778. 2016a.

132. Koch AM, Antunes PM, Maherali H, Hart MM and Klironomos JN. Evolutionary asymmetry in the arbuscular mycorrhizal symbiosis: conservatism in fungal morphology does not predict host plant growth. New Phytologist 214, 1330-1337. 2017.

133. Ciftci V, Turkmen O, Erdinc C and Sensoy S. Effects of different arbuscular mycorrhizal fungi (AMF) species on some bean (*Phaseolus vulgaris* L.) cultivars grown in salty conditions. African Journal of Agricultural Research 5, 3408-3416. 2010.

134. Li-Xiang Z and Liu D. Effects of different phosphorus applications on growth and platycodin content of Balloon Flower (*Platycodon grandiflorum*). Scientia Horticulturae, 178, 8-13. 2014.

135. Hildebrandt TM, Nesi AN, Araújo WL and Braun HP. Amino acid catabolism in plants. Molecular Plant 8, 1563-1579. 2015.

136. Forde BG and Roberts MR. Glutamate receptor-like channels in plants: a role as amino acid sensors in plant defence? F1000prime Reports 6, 1-7. 2014.

137. Weiland M, Mancuso S and Baluska F. Signalling via glutamate and GLRs in *Arabidopsis thaliana*. Functional Plant Biology 43, 1-25. 2015.

138. Rawia AE, Lobna ST, Soad MM. Alleviation of adverse effects of salinity on growth, and chemical constituents of marigold plants by using glutathione and ascorbate. J. Appl. Sci. Res 7, 714-721. 2011.

139. Queiroz HM, Sodek L and Haddad CR. Effect of salt on the growth and metabolism of *Glycine max*. Brazilian Archives of Biology and Technology 55, 809-817. 2012.

140. Pharmaceutical and medical device regulatory science society of Japan. The Japanese Pharmacopoeia 17th edition. p. 122. 2016b.

141. Singh N, Luthra R and Sangwan RS. Oxidative pathways and essential oil biosynthesis in the developing *Cymbopogon flexuosus* leaf. Plant Physiology and Biochemistry (Paris) 28, 703-710. 1990.

142. Kapoor R, Chaudhary V and Bhatnagar AK. Effects of arbuscular mycorrhiza and phosphorus application on artemisinin concentration in *Artemisia annua* L. Mycorrhiza 17, pp. 581-587. 2007.