J. Ginseng Res.
Vol. 33, No. 4, 256-259 (2009)

The Effect of Benomyl Treatments on Ginsenosides and Arbuscular Mycorrhizal Symbiosis in Roots of *Panax ginseng*

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(Received September 11, 2009; Revised December 15, 2009; Accepted December 15, 2009)

Abstract : The effects of benomyl treatment on ginsenoside and arbuscular mycorrhizal (AM) symbiosis in the roots of *Panax ginseng* that were collected from two sites in Korea were investigated. The ginseng roots that were treated with benomyl showed different species compositions of AM fungi colonizing the ginseng roots, compared to untreated roots. In the analysis of ginsenoside, Rc was significantly higher in benomyl untreated roots than in benomyl treated roots. The results suggest that AM fungal species composition and ginsenosides in ginseng root could be influenced by the benomyl treatment.

Key Words : *Panax ginseng*, *Glomus sinuosum*, *Paraglomus occultum*, benomyl, ginsenoside

INTRODUCTION

Ginseng (*Panax ginseng* C. A. Meyer) is a slow-growing perennial herb with thick roots and palmate leaves.[1)] Its inflorescence is umbel, and mature fruits are reddish or yellowish. The herb belongs to the genus *Panax* and family Araliaceae. This genus includes about 15 ginseng species, most of which are used as medicines.[2-4)] Ginseng's medicinal effects are attributable to ginsenosides, which are about 40 kinds of saponin in the root that were analyzed their chemical structure.[5)] Ginsenosides constituent only 3-6% of the total ginseng weight but have many positive effects on the human body.[6)] The production and content of ginsenosides are correlated to the herb's genetic set and environmental conditions, including soil chemical compounds, climatic conditions, and the relationship with different organisms. Ginsenosides are commonly thought to be secreted for self-protection, to maintain homeostasis against attack by other organisms.[7)]

The arbuscular mycorrhizal fungi (AMF) belonging to Glomeromycota[8)] have a symbiotic relationship with most terrestrial plants: the plants provide the carbon source to the AMF and the AMF supply inorganic materials to the plants. AMF increased growth and survivorship of *P. quinquefolius*.[9)] Domey and Bermann[10)] showed that a different AMF – *Glomus intraradices* and *G. albium* - influenced growth and nutrient content of ginseng differently. Moreover, Fourneir *et al.*[11)] showed that inoculation AMF influenced not only growth and survivorship but also root ginsenoside of *P. quinquefolius*. However, only a few studies have been conducted on the relationship of Korean ginseng with AMF in Korea. Most of them focused on morphological characterization and identification of the AMF by analysis of roots and soils in cultivated fields[12,13)] or natural fields of ginseng.[14)] In the present study, we added benomyl, a permeable fungicide, to AMF colonized two-year-old ginseng planted pots to suppress mycorrhizal symbiosis[15,16)] and compared those with untreated control ginseng roots with the aim of determining the effects of benomyl treatment of ginseng roots on species composition of the AMF colonizing the roots using molecular analysis and also on the ginsenoside content of the roots.

MATERIALS AND METHODS

Samples

Thirty roots of 2-year-old ginseng were obtained from the same cultivated field at Geumsan, Chungnam province, Korea. The roots were divided into two groups and grown in sterile-soil pots under greenhouse conditions for 6 months. One group was left untreated for use as a control, and the other group was treated with 200 ml of 1%

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benomyl (Dongbu Fine Chemicals Co., Korea) once a week. Quarter-strength Hoagland's solution (2.8 g H_3BO_3, 3.4 g $MnSO_4 \cdot H_2O$, 0.1 g $CuSO_4 \cdot 5H_2O$, 16.22 g $ZnSO_4 \cdot 7H_2O$, 0.1 g $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 5 ml H_2SO_4, 6.72 g Na_2EDTA, 5.58 g $FeSO_4$, 0.94 g $Ca(NO_3)_2 \cdot 4H_2O$, 0.52 g $MgSO_4 \cdot 7H_2O$, 0.66 g KNO_3, and 0.06 g $HN_4H_2PO_4$) was added to both groups weekly.

DNA sequence analyses of AMF

DNA extraction, nested PCR, cloning, PCR-RFLP and sequencing were performed.[17] Template DNA of AMF was prepared by the cTAB method, and then, the nested PCR was used to amplify the 18S rDNA region.[18] The PCR product was purified for cloning, performed by using the pGEM-T Easy Vector System (Promega Co., USA). Restriction enzymes *Hinf*I, *Asu*21 (Bioneer Co., Korea), and *Hsp*92II (Promega Co., USA) were used for PCR-RFLP. Then, these fragments pattern were detected and colonies were divided into each group and representative colony was selected in each group. Its colony was incubated in LB/ampicillin medium and in order to analysis of AMF sequences plasmids were extracted in each incubated cells. The nucleotide sequence of one clone from each RFLP type was determined using an automatic sequencer (ABI Prism; Applied Biosystems, USA) in Eugenetech Co. (Korea). DNA sequence analyses were performed with BLAST, available at the National Center for Biotechnology Information (NCBI) Web site (http://blast.ncbi.nlm.nih.gov). The relative positions of the sequences were examined in a phylogenetic tree using MEGA 4.0.[19]

Ginsenoside analysis

Dried ginseng roots (1 g) were homogenized. ginseng powders were extracted in a rotary evaporator with 50 ml of 80% MeOH. Extracting was conducted twice and filtered through filter paper. In ginseng extract, methanol was evaporated and the residuum was dissolved in 50 ml distilled water. After 50 ml water solution and ethyl ether (50 ml) put in separator funnel, discarded ether layer. The process conducted twice. In aqueous layer, saponin was extracted 3 times using spor-butanol that first spor-butanol volume is 50 ml and second, third volume is 40 ml, and aqueous layer was discarded. The spor-butanol layer was washed with distilled water twice and evaporated in vacuum at 50°C [20] . Finally, concentrate was dissolve in methanol of 5 times of methanol and filtrated with 0.45 μl PTEF membrane filter. The contents of saponin was analyzed by HPLC (Waters 2690 separation module; Waters 996 photodiode array detector; Waters millennium 2010 chromatography manager) on the Altec Platinum C18 column (particle size. 1.5 μm, 33×7 mm), with water and acetonitrile. The rate of water and acetonitrile for first 10 min and late 15 min were 75:25 and 63:37, respectively. Flow rate of the mobile phase was 1.0 ml min^{-1} and monitoring of ginsenoside was 203 nm. The standard ginsenosides purchase Wako Pure chemical industries, Ltd.(Japan).

RESULTS AND DISCUSSION

Identification of AMF

On the basis of RFLP patterns of AMF, colonies were divided into 11 groups, and 18s rDNA of one representative colony was sequenced from each group: colony 011, 012, 014, 015, 016, 017, 019, 021, 023 024, 031. The sequences were identified through BLAST as *Paraglomus* sp. and *Glomus* spp. (Table 1), but other colonies were identified as a *P. ginseng*. Detection of *Paraglomus* colonization in roots by using classical staining methods is difficult because of weak staining with trypan blue or other traditional acidic stains . Furthermore, it is not possible to identify hyphae of AMF in stained roots into species level; hence, molecular techniques should be used to identify the fungal community colonizing the roots. The spores of *Paraglomus* and *Glomus* spp. have similar morphological characteristics, which makes distinguishing between the species difficult.[21,22]

The AMF colonizing the ginseng roots in this study are similar to those observed by Eom *et al.*,[17] even though

Table 1. The result of BLAST for sequences from benomyl treated and control roots of *Panax ginseng*

Treatment	Colony ID	The closest species	Accession No.	Identity
Control	011	*Glomus sinuosum*	AJ133706.1	714/737 (96%)
	016	*Glomus sinuosum*	AJ133706.1	689/730 (94%)
	017	*Glomus sinuosum*	AJ133706.1	584/603 (96%)
	019	*Glomus sinuosum*	AJ133706.1	479/516 (92%)
Benomyl	021	*Paraglomus occultum*	AM295493.1	556/563 (98%)
	024	*Paraglomus occultum*	AM295493.1	650/677 (96%)

the ginseng-cultivated fields differ. Sequence analyses of the roots from both treatment groups showed the presence of 4 species from 2 genera of AMF (Fig. 1): 2 *Glomus* sp. were detected in the control group and 1 species each of *Glomus* and *Paraglomus* were found in the benomyl-treated roots. Eom *et al.* [17] showed the presence of *Paraglomus* spp. in field-cultivated ginseng roots, suggesting that benomyl influences the colonization of *G. sinuosum* in ginseng roots. The present study result shows the different effect of benomyl treatment on the colonization of *Glomus* and *Paraglomus* spp: the colonization of a *Glomus* sp. in the roots was suppressed but not that of *Paraglomus* spp.

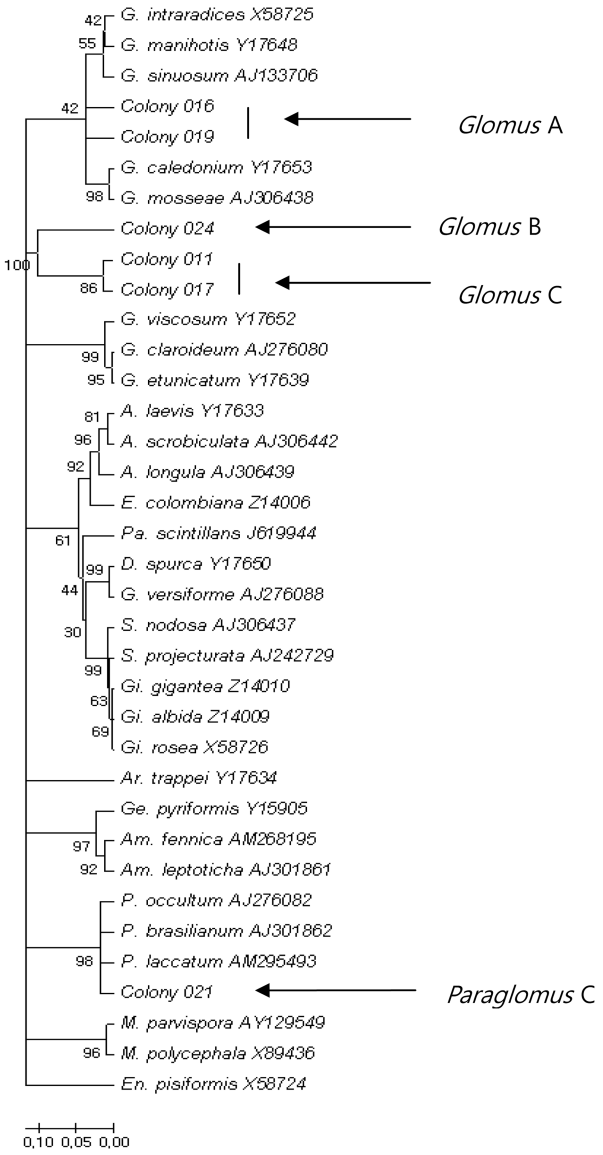

Fig. 1. Neighbour-joining tree of partial sequences of 18s rDNA from ginseng roots. Colonies 011, 016, 017 and 019 were from control roots and colonies 021 and 024 were from benomyl treated roots. The tree consists of 30 AMF species sequences with 6 sequences from this study. Bootstrap values (1,000 replicate) are shown on each clade and *Endogone pisiformis*, *Motierella parvispora* and *M. polycephala* were used as outgroup.

Ginsenoside analysis

It has been reported about 40 ginsenosides in the root from *P. ginseng*, and 7 of which, namely, Rb_1, Rb_2, Rc, Rd, Re, Rf, and Rg_1, are reported as important components.[5,23] In this study, these 6 ginsenosides in the ginseng roots were analyzed after benomyl treatment (Fig. 2). There was no statistically significant difference in the ginsenoside content between the groups except for Rc. The control group showed a significantly higher amount of Rc than the benomyl-treated roots ($p<0.05$). Total and other ginsenosides, Rb1, Rd, Rf and Rg1 showed lower in benomyl treated roots and than in the control roots, though the difference was not statistically significant. The results in this study showed that root ginsenoside content was decreased after suppression of AMF colonization in root by benomyl treatments increased, suggesting that AMF colonization would increase ginsenoside contents. Results are concordant with a previous study[11] showed that ginsenoside content in the root increase when American ginseng are grown with AMF inoculated soil. Ginsenosides have potent anti-fungal activities and function as preformed chemical defense against fungal infection. Furthermore, there has been many reports that AMF colonization induce accumulation of various kinds of secondary

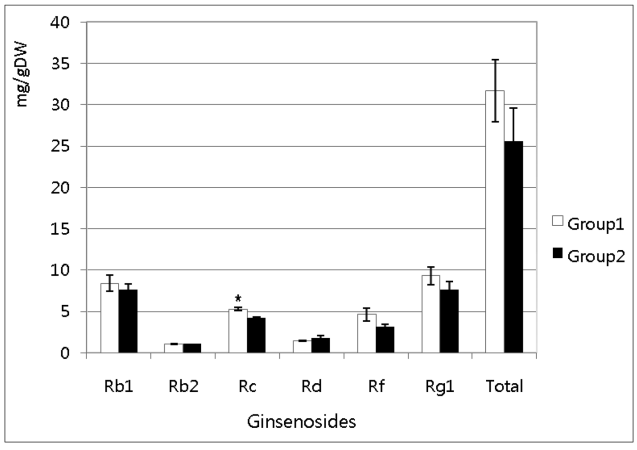

Fig. 2. Ginsensides contents of control ginseng roots (group 1) and benomyl treated roots (group 2). Asterisk indicate significant difference between groups at $P<0.05$. DW indicates dry weight of root samples.

metabolites in host plants[24] and protect host from pathogens.[25] Han *et al.*[26] reported that AM inoculation increase resistance of ginseng to pathogenic fungi, though they did not analyze ginsenosides in the roots. It would be speculated that mycorrhizal colonization stimulates accumulation of secondary metabolite including ginsenosides to protect plant from other soil pathogens.

In summary, our results are concordant with a previous study that ginsenoside content in the root increases with AMF colonization. Also, this study showed that *Paraglomus* sp. was found in the benomyl-treated roots and *Glomus* spp. were found in both the untreated and benomyl-treated roots, suggesting that the fungicide affects the species composition of the AMF colonizing ginseng roots and the mycorrhizal root colonization rate.

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