Fungal Endophytes from Three Cultivars of *Panax ginseng* Meyer Cultivated in Korea

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In order to investigate the diversity of endophytes, fungal endophytes in *Panax ginseng* Meyer cultivated in Korea were isolated and identified using internal transcribed spacer (ITS) sequences of ribosomal DNA. Three cultivars of 3-year-old ginseng roots (Chunpoong, Yunpoong, and Gumpoong) were used to isolate fungal endophytes. Surface sterilized ginseng roots were placed on potato dextrose agar plates supplemented with ampicilin and streptomycin to inhibit bacterial growth. Overall, 38 fungal endophytes were isolated from 12 ginseng roots. According to the sequence analysis of the ITS1-5.8S-ITS2, 38 fungal isolates were classified into 4 different fungal species, which were *Phoma radicina*, *Fusarium oxysporum*, *Setophoma terrestris* and *Ascomycota* sp. 2-RNK. The most dominant fungal endophyte was *P. radicina* in 3 cultivars. The percentage of dominant endophytes of *P. radicina* was 65.8%. The percentage of colonization frequency of *P. radicina* was 80%, 52.9%, and 75% in Chunpoong, Yunpoong, and Gumpoong, respectively. The second most dominant fungal endophyte was *F. oxysporum*. The diversity of the fungal endophytes was low and no ginseng cultivar specificity among endophytes was detected in this study. The identified endophytes can be potential fungi for the production of bioactive compounds and control against ginseng pathogens.

**Keywords:** *Panax ginseng*, Fungal endophytes, Internal transcribed spacer (ITS) sequence

INTRODUCTION

Ginseng (*Panax ginseng* Meyer) is the most valuable traditional herb. Ginseng has well-known, diverse actions and effects on the human body, such as nonspecific resistance to biochemical and physical stresses, and the improvement of vitality, longevity and mental capacity [1-6]. Generally 4 to 6 years of growth are required to produce high quality ginseng roots and the cultivation of ginseng should be under shade conditions. Consecutive cultivation in the same soil causes severe reduction in production mainly due to pathogenic infection [7]. Heavy use of chemical pesticides has been applied to ginseng fields to control pathogens, which results in the contamination of ginseng roots and the surrounding soil. The importance of biological control methods is now widely recognized to produce organic ginseng roots and reduce environmental contamination.

De Barry [8] first used the term ‘endophyte’ to describe microbes that reside inside the living tissues of healthy plants. Endophytes were subsequently described as fungi and bacteria that spend the most or part of their life cycle internally and asymptptomatically in the healthy living tissues of plants [9,10]. It is believed that fungal endophytes...
MATERIALS AND METHODS

Collection of ginseng roots
Three-year-old roots of ginseng, P. ginseng Meyer, were harvested from Gangwon Province, Korea during sunny days in August 2010. Four roots of each cultivar (Chunpoong, Yunpoong, and Gumpoong; 12 roots in total) were harvested, and these were stored at 4°C before being processed.

Isolation of fungal endophytes
Fungal endophytes from ginseng roots were isolated according to Xing et al. [33] with modification. Root samples were thoroughly washed with running tap water and cut into 1 cm long segments with a clean razor blade. The root segments were surface sterilized with 75% ethanol for 1 min, 4% NaHCO₃ for 3 min, and 75% ethanol for 30 s. The root segments were rinsed three times with sterile distilled water and blotted with sterile tissue paper. Three aliquots of 0.1 mL of the water used for the last washing step were inoculated on potato dextrose agar (PDA) plates with 200 µg/mL ampicillin and 200 µg/mL streptomycin to ensure the elimination of ephiphytic microorganisms. The sterilized root segments were transferred to PDA plates amended with ampicillin and streptomycin. PDA plates were incubated at room temperature and checked every day to detect mycelial growth out of the roots. The fungal mycelial tips of the emerging mycelia from the edges of the root segments were transferred to new PDA plates supplemented with ampicillin and streptomycin. The transfer of emerging mycelia from the root segments was continued for up to 4 wk.

Identification of fungal isolates
Mycelia were scraped using a sterile scalpel from 1- or 2-week-old PDA fungal cultures. The harvested fungal mycelia were frozen in liquid nitrogen and stored at -80°C until use. Mycelia were ground to a fine powder using a mortar and pestle in liquid nitrogen with sea sand. DNA was extracted using DNeasy Plant Mini kit (Qiagen, CA, USA) according to manufacturer’s recommendation. Amplification of the internal transcribed spacer (ITS) region was carried out using the universal eukaryotic primers of ITS1 (5′ TCCGTAGGTGAA CCTGCGG 3′) and ITS4 (5′ TCCCTCGCTTATGATGTC 3′) [32]. PCR was performed in 50 µL reaction containing 0.5 µg of DNA, 25 pmol of each primer with the following reaction conditions: 2 min initial denaturing step at 95°C, followed by 30 cycles of 1 min denaturation at 95°C, 1 min primer annealing at 55°C, 1 min extension at 72°C, and a final 5 min extension at 72°C. PCR products were analyzed by electrophoresis in 0.8% agarose gel and the PCR products (approximately 550 bp) were excised from the gel. DNA was purified using Wizard SV gel and PCR.
Clean-up System (Promega, WI, USA). After sequence analysis, ITS sequences were searched using the NCBI BLAST program (http://www.ncbi.nlm.nih.gov).

Morphological identification was also carried out to confirm the results of molecular identification based on macroscopic and microscopic appearances. The percentage of colonization frequency (%CF) of fungal endophytes was calculated based on previous studies [35,36] as follows: %CF=(NCOL/Nt)×100, where NCOL=number of segments colonized by each fungus; Nt=total number of segments.

**Phylogenetic analysis**

A phylogenetic tree was constructed from ITS1-5.8S-ITS2 sequences by the Maximum Parsimony method and Mega5 software (http://www.megasoftware.net) [37,38]. The Maximum Parsimony tree was generated using the Close-Neighbor-Interchange algorithm [39]. Sclerotinia sclerotiorum was used as an outgroup fungal taxon.

**RESULTS AND DISCUSSION**

Total of 38 fungal endophytes were isolated from 12 ginseng roots (184 segments) of 3 cultivars. These were classified into 4 taxonomic species of Ascomycota (Table 1 and Fig. 1). Chunpoong (56 root segments), Yunpoong (74 segments), and Gumpoong (54 segments) were colonized by 5, 17, and 16 fungal isolates, respectively. Only 5 fungal isolates were identified in Chunpoong, which may be due to the contamination or outgrowth of endophytic bacteria. Bacterial growth usually inhibited the outcome of fungal endophytes due to the fast colonization of root segments, even on PDA plates containing antibiotics.

The analyses of ITS1-5.8S-ITS2 regions showed 100% identities of Phoma radicina (FJ427058), Fusarium oxysporum (HQ328030), Setophoma terrestris (JN615482; synonym Phoma terrestris, Pyrenochaeta terrestris) and Ascomycota sp. 2-RNK (EU780424) (Table 1). The highest CF varied among cultivars from 9.4% to 30.6% and the average CF was 21.5% (Table 1), which may also be due to bacterial contamination or growth of bacterial endophytes. *Phoma radicina* was the most frequent fungal endophyte in three ginseng cultivars: 80%, 52.9%, and 75% of CF in Chunpoong, Yunpoong, and Gumpoong, respectively. In total, the percentage of dominant endophytes (DE) of *P. radicina* was 65.8%, which is the highest percentage among the detected fungal isolates. The second most dominant species was *F. oxysporum*: 20%, 23.5%, and 18.7% of DE in Chunpoong, Yunpoong, and Gumpoong, respectively. The remaining fungal endophytes isolated were *S. terrestris* and

| Table 1. Fungal endophytes isolated from 3-year-old ginseng roots of 3 cultivars in Gangwon province in Korea |
| Endophytic fungi | Phoma radicina | Fusarium oxysporum | Setophoma terrestris | Ascomycota sp. 2-RNK | Isolate no. in total | CF (%) | Average of CF (%) |
|------------------|----------------|-------------------|---------------------|-----------------------|---------------------|-------|------------------|
| Chunpoong 1      | FJ427058       | HQ328030          | JN615482            | EU780424              | 1                   | 6.7   |                  |
| Chunpoong 2      |                |                   |                     |                       |                     | 0.0   |                  |
| Chunpoong 3      | 2              | 3                 |                     | 1                     | 0                   | 2.8   |                  |
| Chunpoong 4      | 1              | 1                 |                     | 2                     | 100                 | 15.4  |                  |
| DE (%)           | 80             | 20                | 0                   | 0                     | 100                 | 9.4   |                  |
| Yunpoong 1       | 5              | 1                 |                     | 6                     | 46.2                |       |                  |
| Yunpoong 2       | 3              | 2                 |                     | 5                     | 20.8                |       |                  |
| Yunpoong 3       | 3              | 1                 |                     | 4                     | 19.0                |       |                  |
| Yunpoong 4       | 1              | 1                 |                     | 2                     | 12.5                |       |                  |
| DE (%)           | 52.9           | 23.6              | 17.6                | 5.9                   | 100                 | 24.6  |                  |
| Gumpoong 1       | 2              | 2                 |                     | 2                     | 28.6                |       |                  |
| Gumpoong 2       | 5              | 1                 |                     | 6                     | 46.2                |       |                  |
| Gumpoong 3       | 4              | 1                 |                     | 5                     | 22.7                |       |                  |
| Gumpoong 4       | 1              | 2                 |                     | 3                     | 25                  |       |                  |
| DE (%)           | 75             | 18.7              | 0.0                 | 6.3                   | 100                 | 30.6  |                  |
| Isolate no. in total | 25           | 8                 | 3                   | 2                     | 38                  |       |                  |
| DE (%) in total  | 65.8           | 21.0              | 7.9                 | 5.3                   | 100                 | 21.5  |                  |

Four roots of each cultivar were used to isolate fungal endophytes (12 roots in total).

CF, colonization frequency; DE, dominant endophyte.
Ascomycota sp. 2-RNK and %CF were 7.9% and 5.3% in average, respectively.

The number of fungal endophytes found in this study is lower than expected compared with other studies [24,33,40,41]. However, Dang et al. [34] only reported one fungal isolate (Trichoderma ovalisporum) in P. ginseng. This result indicates that distribution of endophytes depends on plant species and geological locations. As mentioned earlier, there are only a limited number of studies reported on the isolation of fungal endophytes in ginseng species. Xing et al. [33] reported 134 fungal isolates with 27 taxa in American ginseng (P. quinquefolium), in which 11 species were identified in detail. Four species (Alternaria, Collectotrichum, Phoma, and Xylariale) were the most common in 3 different tissues (root, stem and leaf) of American ginseng. These genera are known as common endophytes [23,42-44]. Xing et al. [33] reported tissue specificity for the reported endophytes; Cladosporium sp. were the dominant isolates in roots, but were not detected in stem or leaf tissues. In addition, Cladosporium sp. was not detected in 4-year-old American ginseng roots, but Glomerella cingulata was the dominant species instead. However, we did not find Cladosporium sp. in ginseng roots. They also found that the diversity of fungal endophytes in American ginseng roots decreased with age. This may be because of autotoxic or host defense compounds of American ginseng in the rhizosphere [33,45,46]. It has been known that host defense compounds control endophytic communities [47].

The genus, Phoma sp., which was the most dominant isolate in ginseng, was detected only in leaf tissues of 1-, 2- and 3-year-old American ginseng, but not in 4-year old ginseng. Tissue specificity of endophytes has also been reported in previous studies [48-50]. F. oxysporum was the second dominant isolate in ginseng, while it was only detected in 3-year old roots of American ginseng. The genus Phoma is ubiquitously present in the environment and considered an important fungal plant pathogen [51]. In other plant species, Fusarium and Phoma were the most frequent genera among 142 fungal species isolated in roots of 24 plant species growing at 12 sites in Spain [52]. The genera of Fusarium and Phoma have been known as common endophytes in other studies [13,53,54]. Aveskamp et al. [51] concluded that endophytic communities were dependent on the soil type and plant species, but not on location. The physio-chemical nature of a soil may influence on the colonization of fungal species to plants. While F. oxysporum is commonly present in legume species as a pathogen [55], this fungus can colonize other plants without causing symptoms [54,56]. In general, nonpathogenic endophytes can turn into pathogenic strains after introduction into potentially new host plants [56]. We isolated the unclassified Ascomycota sp. 2-RNK that was also isolated from the roots and fruits of neem tree (Azadirachta indica A. Juss.) [57].
S. terrestris was isolated in ginseng root. Gorenz et al. [58] isolated Pyrenochaeta terrestris as a causal agent of pink root in onions. Fig. 2 shows the maximum parsimony tree grouping fungal endophytes into two clades: 1) *P. radicina* and *S. terrestris*, and 2) *F. oxysporum* and Ascomycota sp. 2-RNK.

We isolated a limited number of fungal endophytes in ginseng root, but our findings can increase the potential use and awareness of fungal endophytes for beneficial applications. There are very few reports on the diversity of fungal endophytes in ginseng. Therefore, our finding can increase the possibilities of identifying potential fungi that can be used to protect ginseng plants and produce bioactive compounds.

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**REFERENCES**

1. Ernst E. *Panax ginseng*: an overview of the clinical evidence. J Ginseng Res 2010;34:259-263.
2. Lee ST, Chu K, Kim JM, Park HJ, Kim MH. Cognitive improvement by ginseng in Alzheimer’s disease. J Ginseng Res 2007;31:51-53.
3. Rausch WD, Weiming L, Gille G, Radad K. Perspectives for ginsenosides in models of Parkinson’s disease. J Ginseng Res 2007;31:127-136.
4. Vuksan V, Sievenpiper J, Jovanovski E, Jenkins AL. Current clinical evidence for Korean red ginseng in management of diabetes and vascular disease: a Toronto’s ginseng clinical testing program. J Ginseng Res 2010;34:264-273.
5. Nam KY. Clinical applications and efficacy of Korean ginseng (*Panax ginseng* C.A. Meyer). J Ginseng Res 2002;26:111-131.
6. Yuan CS, Wang CZ, Wicks SM, Qi LW. Chemical and pharmacological studies of saponins with a focus on American ginseng. J Ginseng Res 2010;34:160-167.
7. Cho DH, Park KJ, Yu YH, Ohh SH, Lee HS. Root-rot development of 2-year old ginseng (*Panax ginseng* C.A. Meyer) caused by *Cylindrocarpon destructans* (Zinssm.) Scholten in the continuous cultivation filed. Korean J Ginseng Sci 1995;19:175-180.
8. De Bary A. Hofmeister’s handbook of physiological botany. Vol. 2. Amsterdam: Leipzig, 1866.
9. Sturz AV, Nowak J. Endophytic communities of rhizobacteria and the strategies required to create yield enhancing associations with crops. Appl Soil Ecol 2000;15:183-190.
10. Wilson D. Endophytes: the evolution of a term, and clarification of its use and definition. Oikos 1995;73:274-276.
11. Saikkonen K, Faeth SH, Helander M, Sullivan TJ. Fungal endophytes: a continuum of interactions with host plants. Annu Rev Ecol Syst 1998;29:319-343.
12. Schardl CL, Clay K. Evolution of mutualistic endophytes from plant pathogens. In: Carrol G. The mycota. V. Plant relationships, part B. Berlin: Springer, 2007. p.221-238.
13. Schulz B, Boyle C. The endophytic continuum. Mycol Res 2005;109(Pt 6):661-686.
14. Li TY, Zeng HL, Ping Y, Lin H, Fan XL, Guo ZG, Zhang CF. Construction of a stable expression vector for *Leifsonia xyli* subsp. cynodontis and its application in studying the effect of the bacterium as an endophytic bacterium in rice. FEMS Microbiol Lett 2007;267:176-183.
15. Shiva P, Pental ND, Bhalla–Sarin N. Regeneration of pigeonpea (*Cajanus cajan*) from cotyledonal node via multiple shoot formation. Plant Cell Rep 1994;13:623-627.
16. Arnold AE, Lutzoni F. Diversity and host range of foliar fungal endophytes: are tropical leaves biodiversity hotspots? Ecology 2007;88:541-549.
17. Dreyfuss MM, Chapela IH. Potential of fungi in the discovery of novel, low molecular weight pharmaceuticals. In: Gullo VP. The discovery of natural products with therapeutic potential. Boston: Butterworth-Heinemann, 1994. p.49-80.
18. Rodriguez R, Redman R. More than 400 million years of evolution and some plants still can’t make it on their own: plant stress tolerance via fungal symbiosis. J Exp Bot 2008;59:1109-1114.
19. Rodriguez RJ, White JF Jr, Arnold AE, Redman RS. Fungal endophytes: diversity and functional roles. New Phytol 2009;182:314-330.
20. Boyle C, Gotz M, Dammann-Tugend U, Schulz B. Endophyte-host interactions. III. Local vs. systemic colonization. Symbiosis 2001;31:259-281.
21. Faeth SH. Are endophytic fungi defensive plant mutualists? Oikos 2002;98:25-36.
22. Brundrett MC. Understanding the roles of multifunctional mycorrhizal and endophytic fungi. In: Schulz BJ, Boyle CJ, Sieber TN, eds. Microbial root endophytes. Berlin: Springer, 2006. p.107-132.
23. Kumar DS, Hyde KD. Biodiversity and tissue-recurrence of endophytic fungi in *Tripterygium wilfordii*. Fungal Divers 2004;17:69-90.
24. Huang WY, Cai YZ, Hyde KD, Corke H, Sun M. Biodi-
versity of endophytic fungi associated with 29 traditional Chinese medicinal plants. Fungal Divers 2008;33:61-75.
25. Karthikeyan B, Jaleel CA, Lakshmanan GM, Deiveekasundaram M. Studies on rhizosphere microbial diversity of some commercially important medicinal plants. Colloids Surf B Biointerfaces 2008;62:143-145.
26. Weber RW, Kappe R, Paululat T, Mosker E, Anke H. Anti-Candida metabolites from endophytic fungi. Phytochemistry 2007;68:886-892.
27. Stierle A, Strobel G, Stierle D. Taxol and taxane production by *Taxomyces andreanae*, an endophytic fungus of Pacific yew. Science 1993;260:214-216.
28. Zhang Q, Kang X, Zhao W. Antiangiogenic effect of low-dose cyclophosphamide combined with ginsenoside Rg3 on Lewis lung carcinoma. Biochem Biophys Res Commun 2006;342:824-828.
29. Tejesvi MV, Kini KR, Prakash HS, Subbiah V, Shetty HS. Genetic diversity and antifungal activity of species of *Pestalotiopsis* isolated as endophytes from medicinal plants. Fungal Divers 2007;24:37-54.
30. Mitchell AM, Strobel GA, Hess WM, Vargas PN, Ezra D. Muscodor crispans, a novel endophyte from *Ananas ananassoides* in the Bolivian Amazon. Fungal Divers 2008;31:37-43.
31. Aly AH, Debbab A, Kjer J, Proksch P. Fungal endophytes from higher plants: a prolific source of phytochemicals and other bioactive natural products. Fungal Divers 2010;41:1-16.
32. Xu LL, Han T, Wu JZ, Zhang QY, Zhang H, Huang BK, Rahman K, Qin LP. Comparative research of chemical constituents, antifungal and antitumor properties of ether extracts of *Panax ginseng* and its endophytic fungus. Phytomedicine 2009;16:609-616.
33. Xing X, Guo S, Fu J. Biodiversity and distribution of endophytic fungi associated with *Panax quinquefolium* L. cultivated in a forest reserve. Symbiosis 2010;51:161-166.
34. Dang L, Li G, Yang Z, Luo S, Zheng X, Zhang K. Chemical constituents from the endophytic fungus *Trichoderma ovale* isolated from *Panax notoginseng*. Ann Microbiol 2010;60:317-320.
35. White TJ, Bruns T, Lee S, Taylor JW. Amplification and direct sequencing of fungal genes for phylogenetics. In: Innis M, Gelfand DH, Sninsky JJ, White TJ, editors. PCR protocols: a guide to methods and applications. San Diego: Academic Press, 1990. p.315-322.
36. Hata K, Futai K. Endophytic fungi associated with healthy pine needles and needles infested by the pine needle gall midge, *Thecodiplosis japonensis*. Can J Bot 1995;73:384-390.
53. Frohlich J, Hyde KD, Petrini O. Endophytic fungi associated with palms. Mycol Res 2000;104:1202-1212.
54. Sieber TN. Fungal root endophytes. In: Waisel Y, Eshel A, Kafka U, eds. Plant roots: the hidden half. New York: Marcel Dekker, 2002. p.887-917.
55. Venuto BC, Smith RR, Grau CR. Virulence, legume host specificity, and genetic relatedness of isolates of *Fusarium oxysporum* from red clover. Plant Dis 1995;79:406-410.
56. Summerell BA, Leslie JF. Genetic diversity and population structure of plant pathogenic species in the genus *Fusarium*. In: Gillings M, Holmes AJ, eds. Plant microbiology. Oxford: Bios Science Publishers, 2004. p.207-223.
57. Verma VC, Gond SK, Kumar A, Kharwar RN, Strobel G. The endophytic mycoflora of bark, leaf, and stem tissues of *Azadirachta indica* A. Juss (neem) from Varanasi (India). Microb Ecol 2007;54:119-125.
58. Gorenz AM, Walker JC, Larson RH. Morphology and taxonomy of the onion pink-root fungus. Phytopathology 1948;38:831-840.