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Age-dependent Distribution of Fungal Endophytes in *Panax ginseng* Roots Cultivated in Korea

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Fungal endophytes were isolated from 1-, 2-, 3-, and 4-year-old ginseng roots (*Panax ginseng* Meyer) cultivated in Korea. The isolated fungal endophytes were identified based on sequence analysis of the internal transcribed spacer and morphological characterization by microscopic observations. A total of 81 fungal endophytes were isolated from 24 ginseng roots. Fungal endophytes were classified into 9 different fungal species and 2 unknown species. Ginseng roots that were 1-, 2-, 3-, and 4-years old were colonized by 2, 6, 8, and 5 species of fungal endophytes, respectively. While *Phoma radicina* was the most frequent fungal endophyte in 2-, 3-, and 4-year-old ginseng roots, *Fusarium solani* was the dominant endophyte in 1-year-old ginseng roots. The colonization frequencies (CF) varied with the host age. The CF were 12%, 40%, 31%, and 40% for 1-, 2-, 3-, and 4-year-old ginseng roots, respectively. We found a variety of fungal endophytes that were distributed depending on the age of ginseng plants.

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

INTRODUCTION

Fungal endophytes are considered to be a group of fungi that live asymptomatically inside the tissues of living plants [1-3]. Fungal endophytes do not penetrate plant cells, but instead grow in the spaces between plant cells or within plant cell walls, while utilizing low levels of nutrients in intercellular fluids and causing no obvious damage [2,3]. Recent studies have reported that fungal endophytes are ubiquitous in most plants and even lichens [1,2,4]. Endophytes are potential genetic sources of natural products for pharmaceutical, agricultural, and industrial purposes. Endophytes from medicinal plants such as ginseng might produce important secondary metabolites for bioactive materials.

In general, fungal endophytes are classified into 2 major groups: clavicipitaceous endophytes and nonclavicipitaceous endophytes. The clavicipitaceous endophytes are transmitted by seeds, but the mechanism for infection into hosts is still speculative. The nonclavicipitaceous

endophytes found in woody and herbaceous plants provide the potential for a wide variety of direct and indirect interactions between plants and herbivores, including increasing resistance to disease, abiotic stress and the enhancement of plant growth [4]. Fungal endophytes are significant because they produce a wide variety of secondary metabolites with potential uses in medicine, agriculture, and industry. Compounds produced by fungal endophytes include alkaloids, terpenoids, flavonoids, and steroids. These compounds can be used for growth promotion of host plants, enhancement of the synthesis of secondary metabolites of host plants, and to help plants defend against pathogens [5-7]. Thus, there is a great opportunity to identify novel metabolites produced by endophytes.

Panax ginseng Meyer belongs to the Araliaceae family and is a perennial umbel plant. It has been used for thousands of years as an important source of medicinal herbs. In Asia, ginseng is known as the most valuable medicinal plant. In general, ginseng is a shade-loving plant, with one stalk having 5-fingered leaves, white flowers, red to yellow fruits and pale yellow root [8]. Despite the fact that active compounds are present in all parts of the ginseng plant, only the root is used pharmacologically. Ginseng contains triterpene glycosides, commonly referred to as ginsenosides, compared to saponins contained in other plants. Many active ingredients are found in all parts of the plant, including amino acids, phenols, alkaloids, polypeptides, and vitamins. Ginseng has many proven pharmacological effects such as improvement of cerebral function, immune modulation, pain relief, diabetes, and cancer prevention [8-10]. The components and contents of ginsenosides are influenced by many factors such as the species, age, plant tissue, cultivation environment, and harvest season. The cultivation of ginseng can be problematic because ginseng is affected by soil conditions during long periods of cultivation. One of the major problems is diseases caused by pathogenic fungi due to long periods of cultivation in the same soil [11,12]. Ginseng can be affected by many fungal pathogens such as gray mold produced by *Botrytis cinerea*, alternaria blight produced by *Alternaria panax*, anthracnose produced by *Colletotrichum gloeosporioides*, sclerotinia white rot produced by *Sclerotinia* sp., phytophthora blight produced by *Phytophthora cactorum*, and root rot produced by *Cylindrocarpon destructans* [13,14]. Control of these fungal pathogens is a challenge for most ginseng cultivators. The aim of this study was to examine the diversity of fungal endophytes obtained from different ages of *P. ginseng* cultivated in Korea. The results can be further used for protection of ginseng plants and/or production of bioactive metabolites.

MATERIALS AND METHODS

Collection of ginseng roots

Ginseng roots of 1-, 2-, 3-, and 4-year-old plants were harvested from the National Institute of Horticultural and Herbal Science (Eumseong, Korea), which is located in latitude 36°56′N and longitude 127°45′E. Ginseng plants were cultivated according to the protocol of 'ginseng Good Agricultural Practice standard cultivation guide' [15] developed by the Rural Development Administration, Korea. The soil type was sandy loam with pH <5.8 and electrical conductivity (EC) 0.6 dS/m. Fungicides were sprayed 3 to 6 times per year. Harvest of the ginseng plants was made in September 2010. Whole roots including rhizomes, main roots and hairy roots were used in this study. Six roots of ginseng plants were used for each age of plant (24 plants in total). Harvested ginseng plants were stored at 4°C prior to use.

Isolation of fungal endophytes

Ginseng roots were washed under running tap water for 5 min to remove soil and debris and root samples were cut into 10×10 mm segments using a sterilized blade. All root segments were surface sterilized in 75% ethanol for 1 min, transferred to 4% $NaHCO_3$ washed for 10 min, and then placed in 75% ethanol for 30 s. The sterilized root segments were washed 3 times in sterile distilled water for 1 min and blotted onto a sterile paper towel. Root segments were then transferred to potato dextrose agar (PDA) plates (Difco, Sparks, MD, USA) with 200 mg/mL each of ampicillin and streptomycin to inhibit bacterial growth. Plates were incubated at room temperature, and fungal colonies growing from the edges of the root segments were transferred to new PDA plates containing ampicillin and streptomycin. Transfer of fungal colonies from the root segments was continued for up to 4 wk, and the fungal isolates were stored in 10% glycerol at -80°C for long-term storage.

Identification of fungal endophytes

Morphological identification of isolated fungal endophytes was done by characterization of spores and hyphae using light microscope (Olympus BX51; Olympus, Tokyo, Japan). Fungal mycelia were scraped from PDA plates and ground into a fine powder using a mortar and pestle in liquid nitrogen and sea sand. A DNeasy Plant Mini kit (Qiagen, Valencia, CA, USA) was used to ex-

Table 1. Putative taxonomic affinities and the CF of the isolated fungal endophytes from ginseng roots

ITS BLAST results			%CF (NI)			
			Root age (yr)			
Closest match	Accession no.[1]	Max identity (%)	1	2	3	4
Trichoderma citrinoviride	HM776434	100	5 (2)			
Colletotrichum panacicola	GU935869	100		4 (2)		
Phoma radicina	FJ427058	100		15 (8)	11 (8)	21 (17)
unknown 1	HM042312	100		5 (3)	3 (2)	9 (7)
Fusarium acuminatum	HM776425	100			3 (2)	
unknown 2	AB499792	99			3 (2)	
Fusarium oxysporum	HM210092	100		5 (3)	3 (2)	1 (1)
Leptodontidium orchidicola	HM036600	99			3 (2)	8 (6)
Fusarium solani	GU066713	100	7 (3)	4 (2)		1 (1)
Cylindrocarpon destructans	AM419065	99		7 (4)	4 (3)	
Colletotrichum pisi	GU724982	99			1 (1)	
Segment no.			40	55	70	80
Total CF (NI)			12 (5)	40 (22)	31 (22)	40 (32)
Species no.[2]			2	6	8	5

NI were in parentheses. The closest matched accession numbers were included after BLAST searches of ITS (internal transcribed spacer) sequences of fungal endophytes from the roots of 1-, 2-, 3-, and 4-year-old ginseng (*Panax ginseng*) plants. CF of endophytic fungi was calculated as follows: CF=(N_{COL}/Nt)×100, where N_{COL}=number of segments colonized by each fungus; Nt=total number of segments.
ITS, internal transcribed spacer; CF, colonization frequency; NI, number of isolated fungal endophyte.
[1]GenBank accession numbers of closest fungal sequence of ITS.
[2]Species no. indicates the total numbers of species that were identified and unidentified in species level.

tract DNA from the ground mycelia. Universal eukaryotic primers were used to amplify the fungal ribosomal DNA internal transcribed spacer (ITS) regions 1 and 2 of all fungal isolates: ITS1 (5'-TCCGTAGGTGAACCT-GCGG-3') and ITS4 (5'-TCCTCCGC TTATTGATAT-GC-3') [16]. The conditions used for polymerase chain reaction (PCR) reactions have been previously described [17]. ITS sequence information was obtained by the sequencing of the PCR products that were purified from the gel using Wizard SV gel and a PCR Clean-up System (Promega, Fitchburg, WI, USA) [18].

Data analysis

PCR-amplified ITS sequences were searched using the NCBI BLAST (http://www.ncbi.nlm.nih.gov) against those in the GenBank database and the results of matched accession numbers were included in Table 1. A phylogenetic tree was constructed using the Maximum Parsimony method and Mega5 software from ITS1-5.8S-ITS2 sequences (http://www.megasoftware.net) [19,20]. The phylogenetic tree was rooted with *S. sclerotiorum* as an outgroup fungal taxon. The colonization frequency

(CF) of endophytic fungi was calculated as follows: CF=(N_{COL}/Nt)×100, where N_{COL}=number of segments colonized by each fungus; Nt=total number of segments. The percent of dominant endophyte (DE%) was calculated as follows: DE%=(N_f/N_t)×100, where N_f=number of each isolated fungus; Nt=total isolated number of fungi.

RESULTS AND DISCUSSION

This study was conducted to isolate fungal endophytes from ginseng roots of different ages. A total of 81 fungal endophytes were isolated from ginseng roots: 5, 22, 22, and 32 isolates from 1-, 2-, 3-, and 4-year-old ginseng roots, respectively (Table 1). The size of ITS-PCR products varied from 500 to 550 bp. Fungal endophytes were assigned to 9 different species and 2 unknown species. Ginseng roots of 1-, 2-, 3-, and 4-year-old plants were colonized by 2, 6, 8, and 5 species of fungal endophytes, respectively (Table 1). The CF varied with the age of the host. Individual CFs were 12%, 40%, 31%, and 40% for 1-, 2-, 3-, and 4-year-old ginseng roots, respectively. There were no significant differences in the dominant
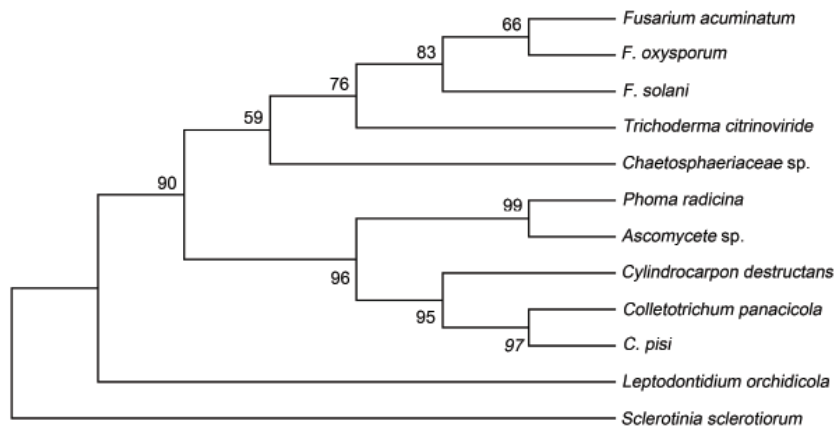
Fig. 1. Maximum-parsimony tree constructed from the internal transcribed spacer sequence of 11 taxa showing the relationships of fungal endophytes in Korean ginseng with reference taxa. The tree was rooted with *Sclerotinia sclerotiorum* (AB607227). The numbers at branches indicate the percentage of trees from 1,000 bootstrap replications. GenBank accession numbers are as follows: *Trichoderma citrinoviride* (HM776434), *Colletotrichum panacicola* (GU935869), *Phoma radicina* (FJ427058), *unknown 1*. (HM042312), *Fusarium acuminatum* (HM776425), *unknown 2* (AB499792), *F. oxysporum* (HM210092), *Leptodontidium orchidicola* (HM036600), *F. solani* (GU066713), *Cylindrocarpon destructans* (AM419065), *C. pisi* (GU724982).

fungal endophytes in different aged roots (Table 2). *Phoma radicina* was the most frequent fungal endophyte in 2-, 3-, and 4-year-old ginseng roots: 15%, 11%, and 21% CF, respectively. Percentages of dominant endophytes (DE) of *P. radician* in 2-, 3-, and 4-year-old ginseng were 37.5%, 38.5%, and 52.5%, respectively. Among 2 isolates detected, *Fusarium solani* was the dominant endophyte in 1-year-old ginseng roots: 7% CF and 60% DE. In order to examine the biodiversity of the culturable fungal endophytes in ginseng roots, we analyzed the fungal ITS sequences. A total of 81 clones were obtained from the fungal endophytes isolated from 4 age groups of ginseng roots. The clones were sequenced and subjected to phylogenetic analysis. BLAST and Maximum-Parsimony phylogenetic tree analysis placed the cloned sequences into 11 groups (Fig. 1).

The isolated fungi described here include various fungi of Ascomycota. The isolated fungal endophytes belong to 11 fungal groups at different taxonomic levels based on ITS sequences and morphological identification. The genera of *Phoma, Collectotrichum* and *Fusarium* were the most common fungal endophytes found in ginseng roots cultivated in Korea, and are also common fungal endophytes found in *P. quinquefolium*, tropical, and subtropical plants [21]. We isolated *C. destructans*, which is one of the most harmful pathogenic fungi of ginseng. However, it is not clear whether *C. destructans* is pathogenic or nonpathogenic to ginseng. *F. oxysporum* is a common pathogenic fungus in plants; however, there are nonpathogenic strains that compete for organic substrates

Table 2. Percentage contribution by the DE to the fungal endophytes isolated from different ages including 1-, 2-, 3-, and 4-year-old ginseng roots

Age (yr)	DE	DE (%)[1]
1	*Fusarium solani*	60.0
2	*Phoma radicina*	37.5
3	*P. radicina*	38.5
4	*P. radicina*	52.5

DE, dominant endophyte.
[1] The percent of DE (DE%) was calculated as follows: DE%=(N_f/N_t)×100, where N_f=number of each isolated fungus; Nt=total isolated number of fungi.

with the pathogenic strains [22]. Nonpathogenic strains often reduce the germination of chlamydospores, and also germ tube growth, which leads to a decline in the population of pathogenic strains.

While higher numbers of fungal endophytes were detected in 4-year-old ginseng roots compared to 1-, 2- and 3-year-old ginseng roots, a lower diversity of fungal endophytes was found in 4-year-old ginseng roots (Table 1). This may be due to differences in cultivation sites, age, and/or longer exposure to chemical fungicides in 4-year-old ginseng cultivation sites. Longer exposure to fungicides might reduce the total number of species of fungal endophytes, while resistant fungal species occupy ginseng roots more frequently. Similar results have been reported for the roots of *P. quinquefolium* [21]. Authors have explained the results by autotoxicity of *P. quinquefolium*. Autotoxic compounds in *P. quinquefolium* which have recently been isolated and characterized cause a

reseeding failure [23]. These autotoxic compounds were also detected in soils used for ginseng cultivation in America [11]. Other factors can influence the community of fungal endophytes, such as fertilizers, water, temperature, soil, and plant defense compounds [24-26]. Fungi have common mechanisms used to detoxify compounds, such as activation of the membrane transporters that pump toxicants out of fungal cells, and enzymatic detoxification [27]. These detoxification mechanisms can be virulence factors for pathogens. Fungi that have detoxification mechanisms may increase their competitiveness among tolerant species of root endophytes, which leads to higher growth rates than those of less tolerant fungal endophytes in the presence of host toxins [28-31]. Colonization of mycorrhizal and soil fungi will be greatly affected when plant defense compounds are secreted in soil [32,33].

Age specificity for fungal endophytes was found in ginseng roots. Only 3 species were common to 2-, 3-, and 4-year-old ginseng roots such as *P. radicina*, *F. oxysporum*, and unknown species 1. *F. solani* was the dominant species in 1-year-old ginseng roots, while it was not found in 3-year-old ginseng roots. Among 11 species, 5 were only detected in certain ages of ginseng roots, which included *Trichoderma citrinoviride* (1-year old), *C. panacicola* (2-year old), *F. acuminatum* (3-year old), *Ascomycete* sp. (3-year old), and *unknown species 2* (3-year old). A small number of fungal endophyte species was detected in 1-year-old *P. ginseng* roots compared to 1-year-old roots of *P. quinquefolium* (2 *vs*. 8) [21]. One possible explanation might be that endophytic communities are dependent on the cultivation environment and method as well as plant species [34]. Another reason might be the existence of *T. citrinoviride*. *T. citrinoviride* has a high ability to parasitize pathogens, protect host plants, and produce strongly inhibitory compounds. Many *Trichoderma* spp. produce a wide range of bioactive metabolites which contribute to their mycoparasitic and antibiotic actions [35]. The genera *Phoma* and *Fusarium* are ubiquitous in the environment and are famous as fungal pathogens [34]. In the current study, *P. radicina* was the dominant endophyte in 2-, 3-, and 4-year-old ginseng roots, while *F. solani* was the dominant endophyte in 1-year-old ginseng roots. Other studies also showed that *Phoma* and *Fusarium* are common fungal endophytes in plant species [36]. Endophytic *Phoma* is known to produce several bioactive compounds [37]. Endophytes have been known for their beneficial functions provided to plants regarding protection against abiotic and biotic stress [4]. In addition, endophytes are potential sources for the production of useful medicinal compounds [5,7,38]. For example, *Taxomyces andreanae*, a fungal endophyte isolated from the pacific Yew tree, can produce paclitaxel (Taxol; Bristol-Myers Squibb, New York, NY, USA) which is an important anticancer drug [38].

In conclusion, it is proposed that the diversity of fungal endophytes in ginseng roots is dependent on the age of the plant. Further studies are needed to understand the relationships between the host and endophytes at the molecular and genetic levels, and can lead to the better use of endophytes to protect plants and produce potential secondary metabolites.

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