Mycobiology

Community Structures of Arbuscular Mycorrhizal Fungi in Soils and Plant Roots Inhabiting Abandoned Mines of Korea

Hyeok Park[1], **Eun-Hwa Lee**[1], **Kang-Hyeon Ka**[2] **and Ahn-Heum Eom**[1,]*

[1]*Department of Biology Education, Korea National University of Education, Cheongju 28173, Korea*
[2]*Division of Wood Chemistry & Microbiology, Korea Forest Research Institute, Seoul 02455, Korea*

Abstract   In this study, we collected rhizosphere soils and root samples from a post-mining area and a natural forest area in Jecheon, Korea. We extracted spores of arbuscular mycorrhizal fungi (AMF) from rhizospheres, and then examined the sequences of 18S rDNA genes of the AMF from the collected roots of plants. We compared the AMF communities in the post-mining area and the natural forest area by sequence analysis of the AMF spores from soils and of the AMF clones from roots. Consequently, we confirmed that the structure of AMF communities varied between the post-mining area and the natural forest area and showed significant relationship with heavy metal contents in soils. These results suggest that heavy metal contamination by mining activity significantly affects the AMF community structure.

Keywords   Arbuscular mycorrhizal fungi, Community, Diversity, DNA cloning, Mycorrhizae

Arbuscular mycorrhizal fungi (AMF) are symbiotic fungi associated with more than 80% of terrestrial plants [1]. AMF increase the fitness of host plants by allowing increased uptake of soil nutrients and water, increased tolerance to plant pathogens and environmental stresses such as drought, salts, and heavy metals [2-5]. These symbiotic associations function as an important biological factor in disturbed soil ecosystems [6, 7]. Symbiotic associations between host plants and AMF play an important role in soil ecosystems that are disturbed naturally by forest fires and earthquakes, or artificially by forest thinning, mining, and farming [7]. There are approximately 5,000 mining sites in Korea, and approximately 40% of these are metal mines. Most of the metal mines were abandoned without adequate environmental habilitation and have therefore affected the surrounding

ecosystems. Soil contamination is a serious condition because of the heavy metals contained in the abandoned mining equipment, the mullocks remaining from the ore dressing process, and the waste water discharged during ore smelting [8, 9].

It has been reported that the AMF community in disturbed areas shows different species composition from that in undisturbed soil [10]. AMF communities in soil contaminated with heavy metals vary significantly according to the kinds and contents of the heavy metals because different AMF species show varying degrees of tolerance to heavy metals [11].

AMF spore analysis has been used in previous studies on AMF in the rhizosphere soil of host plants [12-14]. However, it is difficult to understand the origin of spore formation using AMF spore analysis in soil alone, which has limited the study of AMF communities that form mycorrhizae in the roots of host plants [15]. To confirm the relationships between host plants and AMF accurately, it is necessary to investigate the AMF colonizing the roots of host plants. In this study, we investigated AMF communities colonizing the roots as well as the spores in the rhizosphere soil from an abandoned metal mine in Korea.

MATERIALS AND METHODS

The soils and roots used in this study were collected from a mine (128°12′50″ E, 37°04′05″ N) located in Jecheon, Korea. The mine was closed in 1981 after about 20 years of mining molybdenum and other heavy metals [16]. Ten

samples of soils and roots were randomly collected within a radius of 20 m from the mine-tail. A natural forest area located at 200 m from the mine-tail was selected as a control site, and another 10 samples were randomly collected within a radius of 20 m. The collected samples were packed into polyethylene bags and transported to the laboratory. Root samples for molecular analysis were stored at −80°C, and the root samples for mycorrhizal root colonization rate and the soils for spore analysis were stored at 4°C. The heavy metal concentration in the soil samples was analyzed in a soil analysis laboratory (Jeil Analysis Center, Seoul, Korea).

AMF spores were extracted from 10 g of dried soil samples using wet decanting and sucrose density gradient methods [17]. The morphological characteristics of the extracted spores were observed under light microscopes. Spores were identified by the morphological characteristics of spore walls, such as number, thickness, color, structure, and surface ornamentations. In addition, each species was identified morphologically. DNA was extracted from spores, and small subunit ribosomal DNA fragments were amplified by nested PCR [18]. The first PCR was performed using the universal primers NS1 and NS4, and the product was used as template for the second amplification using AMF-specific primers, AML1 and AML2 [19]. The second PCR product was then sequenced (Solgent Co., Daejeon, Korea). A sequence-similarity search of the National Center for Biotechnology Information (NCBI) database was conducted using the Basic Local Alignment Search Tool (BLAST) algorithm. The sequences were aligned, and a phylogenetic tree was obtained using MEGA5 [20]. The obtained sequences were submitted to Genbank with the accession numbers KX879056~KX879063.

Six roots of *Lespedeza cyrtobotrya* were selected from 20 root samples for mycorrhizal colonization and community analysis. To examine the AMF colonization rates, the plant roots were washed and stained with trypan blue according to the methods described by Koske and Gemma [21]. The roots were observed under a microscope (AXIO Imager; Carl Zeiss, Jena, Germany), and root colonization rates were measured by the previously described magnified intersects technique.

DNA was extracted from surface-sterilized root samples using the DNeasy Plant Mini Kit (Qiagen, Venlo, Netherlands) with 30% H_2O_2 solution. Nested PCR was performed to amplify the small subunit rDNA using primers NS1 and NS4 for the first PCR and AML1 and AML2 for the second PCR as previously described for spores. The PCR product was purified using Solgent PCR purification kit and cloned using T-blunt PCR cloning kit following the manufacturer's instructions [22]. The nucleotide sequence of 20 clones from each sample was determined using an automated sequencer, and identified through BLAST and phylogenetic sequence analysis. Only the sequences belonging to Glomeromycota were included in the analysis. In order to determine the phylogenetic relationships among operational taxonomic units (OTUs), representative sequences for each OTU were selected and aligned with the closest matches obtained from GenBank by BLAST using the ClustalX algorithm in MEGA5. Neighbor-joining phylogenetic analysis was conducted in MEGA5 using a bootstrap analysis with 1,000 replicates. The obtained sequences were submitted to Genbank with the accession numbers KX879064~KX879070.

Relative abundances for AMF communities in soils and roots were estimated based on the molecular and morphological identification data for each root sample. Shannon diversity indices were also calculated [21]. Univariate analysis of variance (ANOVA) was used to evaluate the fixed effect of the sampling site on the percent mycorrhizal root colonization rates and the species diversity of AMF communities. Pearson correlation analysis was performed to test the effects of heavy metals on AMF communities using the statistical package, SPSS-WIN ver. 12 (SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

Soil chemical analysis for heavy metals (As, Pb, Cd, Ni, and Zn) showed that the concentrations of As and Zn were significantly higher in the abandoned mines than in the natural forest (Table 1). Pb was not detected in both the soils collected in this study, and Cd was detected in the soil at both sites but showed no significant difference between locations.

Morphological and molecular analysis of spores in rhizosphere soils showed that 8 species of 7 genera were found in the post-mining area, and 6 species of 5 genera of AMF were found in the natural forest area (Table 2, Fig. 1). Relative abundance showed that *Claroideoglomus etunicatum* was the most dominant species in the post-mining area and that *Rhizophagus irregularis* was dominant in the natural forest area (Table 2). *Acaulospora longula* and *Ambispora leptoticha* were found only in the mine area. The total

Table 1. Heavy metal contents in soils of post-mining areas and natural forest areas[a]

| Soil sampling area	Heavy metal component (mg/100 g)				
	As	Cd	Ni	Pb	Zn
Post-mining area	14.17 ± 0.43a	0.55 ± 0.02a	2.44 ± 0.07a	-	27.20 ± 0.82a
Natural forest area	1.92 ± 0.06b	0.32 ± 0.01a	2.0 ± 0.06a	-	5.94 ± 0.18b

Values are presented as mean ± SE.

[a]Different letters indicate significant difference between sites at $p < 0.05$.

Table 2. Relative abundance of arbuscular mycorrhizal fungi spores in in soils of post-mining areas and natural forest areas

AMF species	Relative abundance	
	Post-mining area	Natural forest area
Acaulospora longula CB15113[a]	1.71 ± 0.73	-
Ambispora leptoticha CB15112	2.44 ± 1.96	-
Cetraspora pellucida CB15074	4.39 ± 2.26	4.15 ± 2.86
Claroideoglomus claroideum CB15045	18.29 ± 5.17	11.95 ± 2.42
Claroideoglomus etunicatum CB15025	24.15 ± 5.48	15.61 ± 3.13
Gigaspora margarita CB15115	2.20 ± 1.94	3.17 ± 0.89
Rhizophagus irregularis CB15158	10.73 ± 5.54	20.49 ± 10.32
Septoglomus constrictum CB15026	20.00 ± 3.63	17.32 ± 5.78
Shannon's index	1.19 ± 0.12	1.09 ± 0.10
Species evenness	0.84 ± 0.04	0.77 ± 0.06
No. of spores[a]	34.40 ± 4.47	29.80 ± 2.82

Values are presented as mean ± SE.
[a]Significant difference between sites at $p < 0.05$.
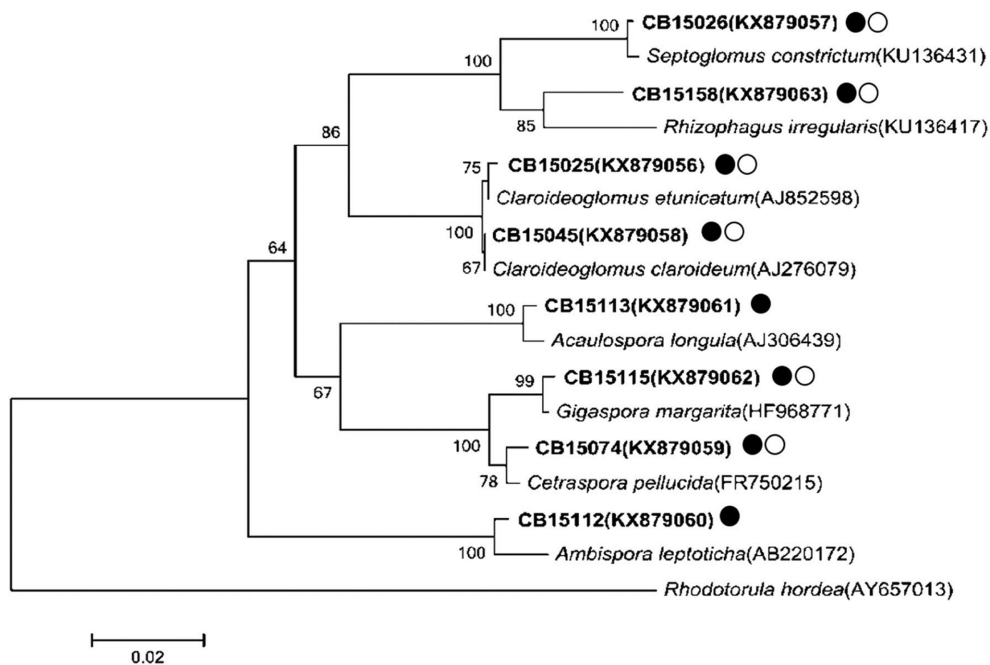
Fig. 1. Neighbor-joining phylogenic tree for partial 18S rDNA sequence of arbuscular mycorrhizal fungal spores in soils. *Rhodotorula hordea* was used as an outgroup and bootstrap values (> 50) were shown at the nodes (●, mining area; ○, natural forest).

spore numbers were higher in the mining area than those in the natural forests. Shannon's index and species evenness were also higher in the mining area than that in the natural forest but were not statistically significant. Correlation analysis of heavy metal concentration with relative abundance of spores showed that *A. longula* has a strong positive relationship with the heavy metals As and Zn, which were significantly higher in the mining area (Table 3). The accumulation of heavy metals significantly influenced the AMF spore communities in the soil for a long period.

Mycorrhizal root colonization rates were significantly higher in the mine area (81.33 ± 2.67%) than in the natural forest area (61.67 ± 2.85) at $p < 0.05$, suggesting that

Table 3. Correlations between heavy metal contents and the relative abundance of arbuscular mycorrhizal fungi (AMF) species

| AMF species	As	Zn
Acaulospora longula	0.482[a]	0.482[a]
Ambispora leptoticha	0.282	0.282
Cetraspora pellucida	0.253	0.253
Claroideoglomus claroideum	0.304	0.304
Claroideoglomus etunicatum	0.016	0.016
Gigaspora margarita	−0.107	−0.107
Rhizophagus irregularis	−0.193	−0.193
Septoglomus constrictum	0.092	0.092

[a]Significant difference between sites at $p < 0.05$.

Table 4. Relative abundance of arbuscular mycorrhizal fungi colonizing roots of post-mining areas and natural forest areas

OTU (representative clone)	The closest matches	Relative abundance	
		Post-mining area	Natural forest area
Clo1 (JC083)	*Claroideoglomus lamellosum*	10.42 ± 10.42	10.42 ± 7.51
Glo5 (JC086)	*Glomus macrocarpum*	16.67 ± 9.08	-
Glo7 (JC098)	*Glomus indicum*	6.25 ± 6.25	-
Glo6 (JC001)	*Glomus iranicum*	22.92 ± 5.51	47.92 ± 25.60
Glo2 (JC009)	*Rhiophagus fasciculatus*	8.33 ± 8.33	-
Glo3 (JC093)	*Rhizophagus intraradices*	12.50 ± 3.61	10.42 ± 10.42
Glo1 (JC051)	*Rhizophagus irregularis*	20.83 ± 20.83	31.25 ± 18.04
Glo4 (JC035)	*Rhizophagus proliferus*	-	6.25 ± 3.61
Shannon's index		1.25 ± 0.22	0.81 ± 0.22
Evenness		0.90 ± 0.07	0.74 ± 0.11

Values are presented as mean ± SE.
OTU, operational taxonomic unit.
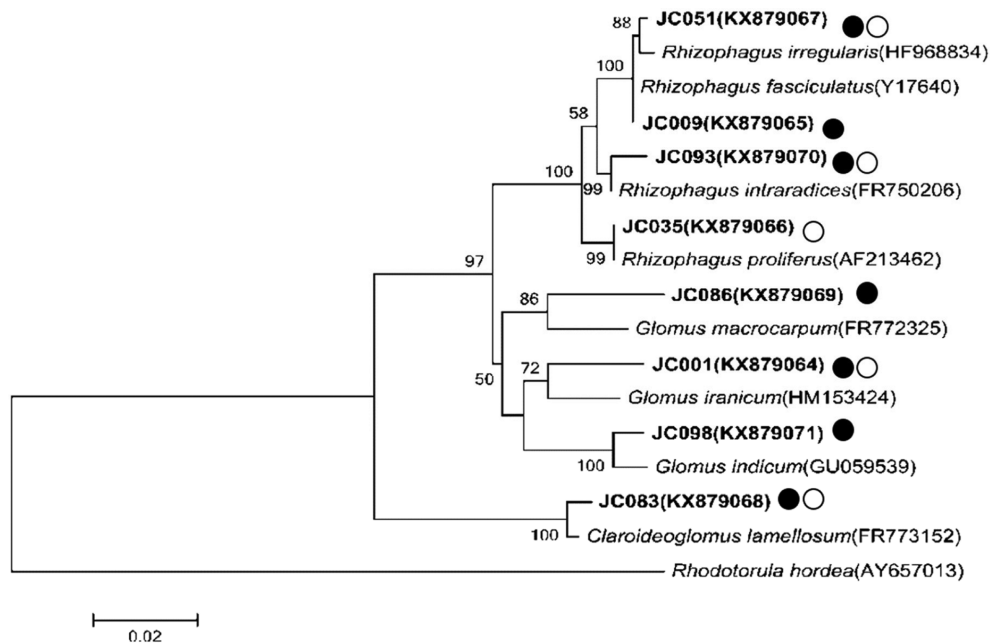
Fig. 2. Neighbor-joining phylogenic tree for partial 18S rDNA sequence of arbuscular mycorrhizal fungi colonizing roots. *Rhodotorula hordea* was used as an outgroup and bootstrap values (> 50) were shown at the nodes (●, mining area; ○, natural forest).

significantly high concentrations of heavy metals affect mycorrhizal symbiosis with host plants at the mining sites. Cloning results for AMF-colonizing roots allowed identification of 7 OTUs in the mine area and 5 OTUs in the species of the forest area (Table 4, Fig. 2). Relative abundance showed that Glo6 was dominant in both the mine and the natural forest areas. Glo2, Glo5, and Glo7 were found only in the mine area, and Glo4 was found only in the forest area. In this study, more diverse AMF sequence types were found in the roots of plants in the mining area with high concentration of heavy metals. However, some previous studies showed that OTU number and root colonization rates were decreased with increasing concentrations of heavy metals [23]. Thus, it is considered that diversity of AMF could be increased

or decreased according to the intensity of the heavy metals which act as a disturbance. Shannon indices were not significantly different between the two areas (Table 4), and correlation analysis showed no significant relationship between the relative abundances of AMF species and heavy metal concentrations. Significantly, higher heavy metal concentrations, especially of Zn, might be the reason for the higher colonization rates in the mining area.

It has been reported that high accumulation of Zn stressed the host plants and increased AMF colonization in their roots [24]. The reason might be that though some AMF species are sensitive to heavy metals, other species are tolerant of high heavy metal concentration in soil [25]. Previous studies show that AMF species belonging

to *Claroideoglomus* are often tolerant to heavy metals [26], and the results of this study showed that species of *Claroideoglomus* were significantly more in the mine area. Additionally, *A. longula* showed a strong positive correlation with As and Zn and is consistent with previous results [27] that the frequency of *A. longula* is higher in higher concentrations of Zn. In addition, *A. leptoticha,* which was found only in the mining area in this study, was also frequently found in contaminated soil containing Zn [28]. However it have been reported that AMF communities in soil contaminated by heavy metals more affected by other chemicals in the soil than heavy metals [29]. Thus, the diversity of AMF communities in abandoned mine area should be considered with factors of the soil environments as well as the intensity of the disturbance.

The species mainly found in abandoned mines with a long period of ecological disturbance belonged to Glomaceae [23, 30]. Previous studies showed that the Glomaceae species were dominant under stress conditions such as ecological disturbances, suggesting that Glomaceae species are better adapted to disturbed environments than other species [31, 32]. In this study, AMF species, *C. etunicatum*, *R. irregularis*, *R. intraradices* in Glomaceae were species having a high relative abundance common to both abandoned and natural forest area. The result was consistent with previous studies of heavy metal contaminated areas [33], suggesting that the emergence of these species would be the results of strong selection pressure in the settlement process of plants than the intensity of the disturbance. In conclusion, this study showed that the anthropogenic disturbance of mining activities affects the AMF communities.

REFERENCES

1. Smith SE, Read DJ. The symbionts forming arbuscular mycorrhizas. Mycorrhizal symbiosis. 3rd ed. London: Academic Press; 2008. p. 13-41.
2. Dodd JC. The role of arbuscular mycorrhizal fungi in agro- and natural ecosystems. Outlook Agric 2000;29:55.
3. Wang B, Qiu YL. Phylogenetic distribution and evolution of mycorrhizas in land plants. Mycorrhiza 2006;16:299-363.
4. Hirrel MC, Gerdemann JW. Improved growth of onion and bell pepper in saline soils by two vesicular-arbuscular mycorrhizal fungi. Soil Sci Soc Am J 1980;44:654-5.
5. Newsham KK, Fitter AH, Watkinson AR. Arbuscular mycorrhiza protect an annual grass from root pathogenic fungi in the field. J Ecol 1995;83:991-1000.
6. Stahl PD, Williams SE, Christensen M. Efficacy of native vesicular-arbuscular mycorrhizal fungi after severe soil disturbance. New Phytol 1988;110:347-54.
7. Jeffries P, Gianinazzi S, Perotto S, Turnau K, Barea JM. The contribution of arbuscular mycorrhizal fungi in sustainable maintenance of plant health and soil fertility. Biol Fertil Soils 2003;37:1-16.
8. Jung MC, Jung MY, Choi YW. Environmental assessment of heavy metals around abandoned metalliferous mine in Korea. Econ Environ Geol 2004;37:21-33.
9. Koo S, Yoo J, Choi I, Ji W, Kang D, Ha W, Kim J, Lee H, Kwon J, Kim K. Yearbook of MIRECO statistics. Wonju: Korea Mine Reclamation; 2013.
10. Jasper DA, Abbott LK, Robson AD. The effect of soil disturbance on vesicular: arbuscular mycorrhizal fungi in soils from different vegetation types. New Phytol 1991;118:471-6.
11. Gaur A, Adholeya A. Prospects of arbuscular mycorrhizal fungi in phytoremediation of heavy metal contaminated soils. Curr Sci 2004;86:528-34.
12. Park SH, Eo JK, Ka KH, Eom AH. Diversity of arbuscular mycorrhizal fungi of woody plants in Mt. Munan. Kor J Mycol 2011;39:1-6.
13. Lee EH, Ka KH, Eom AH. Diversity of arbuscular mycorrhizal fungi in rhizospheres of *Camellia japonica* and neighboring plants inhabiting Wando of Korea. Kor J Mycol 2014;42:34-9.
14. Kil YJ, Eo JK, Eom AH. Diversities of arbuscular mycorrhizal fungi in cultivated field soils of Korean ginseng. Kor J Mycol 2012;40:1-6.
15. Clapp J, Young JP, Merryweather JW, Fitter AH. Diversity of fungal symbionts in arbuscular mycorrhizas from a natural community. New Phytol 1995;130:259-65.
16. Park JK, Jung MC, Seo CI, Jeon KH, Jung HS, Cho JH, Jung JS, Lim HS, Kang MH, Ji HG. A survey on the soil pollution for abandoned metal mine. Gwacheon: Korea Ministry of Environment; 2004.
17. Daniels BA, Skipper HA. Methods for the recovery and quantative estimation of propagules from soil. In: Schenck NC, editor. Methods and principles of mycorrhizal research. St. Paul (MN): American Phytopathological Society; 1982. p. 29-35.
18. Jacquot E, van Tuinen D, Gianinazzi S, Gianinazzi-Pearson V. Monitoring species of arbuscular mycorrhizal fungi in planta and in soil by nested PCR: application to the study of the impact of sewage sludge. Plant Soil 2000;226:179-88.
19. Lee J, Lee S, Young JP. Improved PCR primers for the detection and identification of arbuscular mycorrhizal fungi. FEMS Microbiol Ecol 2008;65:339-49.
20. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 2011;28:2731-9.
21. Koske RE, Gemma JN. A modified procedure for staining roots to detect VA mycorrhizas. Mycol Res 1989;92:486-8.
22. Schwarzott D, Schüßler A. A simple and reliable method for SSU rRNA gene DNA extraction, amplification, and cloning from single AM fungal spores. Mycorrhiza 2001;10:203-7.
23. Zarei M, König S, Hempel S, Nekouei MK, Savaghebi G, Buscot F. Community structure of arbuscular mycorrhizal fungi associated to *Veronica rechingeri* at the Anguran zinc and lead mining region. Environ Pollut 2008;156:1277-83.
24. Audet P, Charest C. Effects of AM colonization on "wild tobacco" plants grown in zinc-contaminated soil. Mycorrhiza 2006;16:1277-83.
25. Del Val C, Barea JM, Azcón-Aguilar C. Diversity of arbuscular mycorrhizal fungus populations in heavy-metal-contaminated soils. Appl Environ Microbiol 1999;65:718-23.
26. Cornejo P, Pérez-Tienda J, Meier S, Valderas A, Borie F,

Azcón-Aguilar C, Ferrol N. Copper compartmentalization in spores as a survival strategy of arbuscular mycorrhizal fungi in Cu-polluted environments. Soil Biol Biochem 2013;57:925-8.

27. Turnau K, Ryszka P, Gianinazzi-Pearson V, Van Tuinen D. Identification of arbuscular mycorrhizal fungi in soils and roots of plants colonizing zinc wastes in southern Poland. Mycorrhiza 2001;10:169-74.

28. Long LK, Yao Q, Guo J, Yang RH, Huang YH, Zhu HH. Molecular community analysis of arbuscular mycorrhizal fungi associated with five selected plant species from heavy metal polluted soils. Eur J Soil Biol 2010;46:288-94.

29. Zarei M, Hempel S, Wubet T, Schäfer T, Savaghebi G, Jouzani GS, Nekouei MK, Buscot F. Molecular diversity of arbuscular mycorrhizal fungi in relation to soil chemical properties and heavy metal contamination. Environ Pollut 2010;158:2757-65.

30. Vallino M, Massa N, Lumini E, Bianciotto V, Berta G, Bonfante P. Assessment of arbuscular mycorrhizal fungal diversity in roots of *Solidago gigantea* growing in a polluted soil in Northern Italy. Environ Microbiol 2006;8:971-83.

31. Daniell TJ, Husband R, Fitter AH, Young JP. Molecular diversity of arbuscular mycorrhizal fungi colonising arable crops. FEMS Microbiol Ecol 2001;36:203-9.

32. Wubet T, Weiß M, Kottke I, Teketay D, Oberwinkler F. Molecular diversity of arbuscular mycorrhizal fungi in *Prunus africana*, an endangered medicinal tree species in dry Afromontane forests of Ethiopia. New Phytol 2004;161:517-28.

33. Hassan SE, Boon E, St-Arnaud M, Hijri M. Molecular biodiversity of arbuscular mycorrhizal fungi in trace metal-polluted soils. Mol Ecol 2011;20:3469-83.