JARQ 48 (3), 279 - 290 (2014) http://www.jircas.affrc.go.jp

Community of Arbuscular Mycorrhizal Fungi in Soybean Roots after Cultivation with Different Cropping Systems

Tomoko KOJIMA[1]* Norikuni OKA[2], Toshihiko KARASAWA[3], Keiki OKAZAKI[2], Shotaro ANDO[4] and Masako TAKEBE[3]

[1] Grassland Management Division, NARO Institute of Livestock and Grassland Science (Nasushiobara, Tochigi 329-2793, Japan)
[2] Agro-environmental Research Division, NARO Hokkaido Agricultural Research Center (Sapporo, Hokkaido 062-8555, Japan)
[3] Soil Science and Plant Nutrition Division, NARO Agricultural Research Center (Tsukuba, Ibaraki 305-8666, Japan)
[4] Tropical Agriculture Research Front, Japan International Research Center for Agricultural Science (Ishigaki, Okinawa 907-0002, Japan)

Abstract

Crop growth was enhanced in fields previously cultivated with host plants colonized by arbuscular mycorrhizal fungi (AMF), compared with fields previously cultivated with non-mycorrhizal plants. To clarify the effect of previous cropping on the community structure of AMF in soybean roots, soybean were grown in fields which were cultivated after mycorrhizal plants, non-mycorrhizal plants, or left in uncropped condition over three years (in 2004, 2006, and 2007) in two different soils (Thapto-upland Wet Andosol and Low-humic Andosol). The partial region in the 18S rDNA of AMF from soybean roots was amplified by a nested PCR method using primers specific for AMF and sequenced. The sequence homology search and phylogenetic analysis revealed that the AMF community in soybean roots was unaffected by the preceding crop. Further, it was shown that the AMF phylotype "Glo-B1", which included *Glomus* sp. ZJ (AB076344), was the most frequently detected, irrespective of the preceding cropping system. However, in 2007, the community structure of AMF in the soybean roots from the Low-humic Andosol field, which had been used as grassland for several years, was relatively different from that of the Thapto-upland Wet Andosol fields. It was implied that the AMF community could be affected by environmental condition or long-term vegetation.

Discipline: Soils, fertilizers and plant nutrition
Additional key words: Andosol, *Glomus* sp., 18S rDNA

Introduction

Arbuscular mycorrhizal fungi (AMF) belong to the phylum Glomeromycota, colonize the roots of approximately 80% of terrestrial plant species, and provide phosphate and other mineral nutrition to host plants (Smith & Read 2008). The use of these fungi is expected to decrease the agricultural use of chemical phosphate fertilizer and fungicide, because AMF can promote plant growth, especially in low phosphorus (P) soil conditions and also promote resistance to diseases (Harrier & Watson 2004, Norman et al. 1996, Vigo et al. 2000). In Japan, most phosphate fertilizer is imported from overseas and global resources of P have been decreasing (Cordell et al. 2009). Andosol, which originates from volcanic ash, is widely distributed throughout Japan. The P adsorption potential is strong in Andosol, meaning the effect of P fertilizer is low. Accordingly, the potential use of AMF for agriculture, which promotes P uptake by crops, has attracted special interest in Japan. In particular, AMF can improve growth and nitrogen fixation in legumes growing in P-deficient soils (Chalk et al. 2006). The use of AMF can thus be expected to facilitate the cultivation of soybeans, which represent one of the primary legume crops in Japan.

It seemed more important to know the AMF species in the crop roots, which directly affect the growth of host plants. Based on spore observation, AMF diversity in soil has been investigated in various crop-rotation systems in several countries. Indeed, AMF generally have no host specificity, but they do have host preferences (Bever et al. 1996, Douds Jr et al. 1998, Plenchette & Morel 1996,

*Corresponding author: e-mail kojima@naro.affrc.go.jp
Received 18 February 2013; accepted 11 November 2013.

Sanders & Fitter 1992). Further, AMF diversity is also related to plant diversity (van der Heijden et al. 1998 & 2003). Hendrix et al. (1995) reported that in Kentucky (USA), *Gigaspora margarita* dominated in continuous soybean plots, whereas *Glomus macrocarpum* and *Glomus fecundisporum* dominated in rotated plots. A soybean-sorghum rotation field in Nebraska (USA) showed great AMF diversity, with AMF species belonging to *Glomus, Acaulospora, Entrophospora,* and *Gigaspora* (Ellis et al. 1992). In a maize-soybean rotation field in Minnesota (USA), *Glomus aggregatum* dominated (Kurle & Pfleger 1996). These findings implied that the AMF community might differ among various types of crop-rotation systems, with different environmental factors and crops, including soybean. However, the dominant AMF species in the soil sometimes differ from those colonizing the plant roots (Clapp et al. 2002).

AMF species in crop roots were investigated using biomolecular techniques and phylogenetic analysis. For example, an initiative study showed that species such as *Glomus mosseae* dominated in the plant roots at arable sites (pea, maize, and wheat crops) in North Yorkshire (UK) (Helgason et al. 1998), while *Glomus* species dominated in the roots of wheat and maize in the same area (Daniell et al. 2001). Recently, the AMF community in long-term experimental agricultural fields has been reported. Regarding AMF in the roots of maize grown in a long-term monocultural field in Martvasar (Hungary), established half a century ago, although *Glomus* spp. dominated, many phylotypes of Glomeraceae were detected (Sasvári et al. 2011). It was implied that using phylogenetic analysis would allow the AMF community in the plant roots to be classified more precisely, especially Glomeraceae including unidentified or unculturable *Glomus* spp. For Japanese agricultural fields, the AMF community in the plant roots in grasslands (Saito et al. 2004) and in the roots of the Japanese pear (Yoshimura et al. 2013) was investigated. The AMF community in the soil of long-term experimental fields in Hokkaido was also examined using soybean cultivation methods involving phylogenetic analysis and trap culture (Cheng et al. 2013). However the effects of crop rotation on the AMF community remain unclear.

Enhancement of crop growth has been demonstrated in fields previously cultivated with mycorrhizal plants when compared with those previously cultivated with non-mycorrhizal plants or no plants (Arihara & Karasawa 2000, Karasawa et al. 2000, 2001, & 2002). For example, maize growth was enhanced after mycorrhizal crop cultivation (Arihara & Karasawa 2000). The spore density in the soil after cultivating mycorrhizal sunflowers exceeded that after cultivating non-mycorrhizal mustard (Karasawa et al. 2002). Karasawa et al. (2001) investigated the effects of soil characteristics on AMF dynamics for maize as a succeeding crop. It was suggested that the differences in maize growth, cultivated after various previous crops, were mainly attributable to the differences in AMF colonization, rather than differences in soil type.

As described above, the mechanism of the effect of the previous crop condition on succeeding crops was considered to be increased AMF spore density and colonization rate, which caused increased P uptake and promoted the growth of succeeding crops (Karasawa et al. 2000, 2001, & 2002). However, there was potential for different AMF composition in the crop roots to be established by the influence of the previous crop and different environmental factors, resulting in the different P uptake of plants. Indeed it was reported that different AMF species had different P supply abilities (Smith et al. 2003 & 2004). It was also reported that *Gigaspora margarita* (Saito & Vargas 1991) or *Glomus* spp. had been found to be dominant (An et al. 1990, Franke-Snyder et al. 2001), in the soil of soybean fields in Japan and elsewhere. However, there was little information about the AMF community structure and dominant AMF species in the soybean roots, particularly when cultivated after a range of previous crops in Japanese fields.

In this study, to know the effects of previous crops or field conditions on the AMF community structure in succeeding crops, we investigated the AMF in soybean roots, cultivated after a range of previous crops in various fields in Hokkaido, Japan,. This research could also help reveal the relationship between dominant AMF taxa in soybean fields and cropping systems, and might be useful for constructing crop-rotation systems.

Materials and Methods

1. Cultivation and sampling of soybeans

Soybean (*Glycine max* (L.) Merr. cv. Tsurumusume) was grown after cultivating under different preceding-crop conditions since 2004 at the NARO Hokkaido Agricultural Research Center, Hokkaido, Japan (Oka et al. 2010), and soybean plants were sampled from experiments in 2004, 2006 and 2007. The conditions of the fields where samples were collected are listed in Table 1. In these experiments, each condition included 4 field subplots, respectively. The soil type of Field No. 23-15 in 2007 was Low-humic Andosol [Typic Hapludands (USDA Soil Taxonomy)] while that of other fields was Thapto-upland Wet Andosol [Typic Endoaquands (USDA Soil Taxonomy)] (Obara et al. 2011). Before these experiments, in Field No. 13-36, cabbage (*Brassica oleracea* L. var. *capitata*) and other crops were cultivated from 2001 to 2002. *Avena sativa* L. and green manure were cultivated in Field No. 9-25 from 2003 to 2004 and in Field No. 13-32 from 2004 to 2005. Field No. 23-15 with Low-humic Andosol was used as grassland, where red clover (*Trifolium pratense* L.) with other weeds

Table 1. The experimental conditions of soybean fields where the samples were collected

Year	Field		Chemical properties of the soil †					Previous crop	
	No.	Subplot area (m^2)	Soil type ‡	pH	Available P (mg kg^{-1})	Total N (g kg^{-1})	CEC ($cmol_c$ kg^{-1})	Mycorrhizal	Non-mycorrhizal or no crop
2004	13-36	13	TA	5.3	69.8	2.9	30.4	Carrot, Spring wheat	No crop (bared)
2006	9-25	15	TA	5.4	41.5	4.0	29.6	Azuki bean, Maize	Sugar beet, Buckwheat
2007	13-32	25	TA	5.4	86.2	4.5	38.3	Maize	Buckwheat
2007	23-15	18	LA	5.8	10.5	2.7	17.4	Maize	Buckwheat

† The data of the soil characteristics in 2004 and 2006 was according to Oka et al. (2010).
‡ TA: Thapto-upland Wet Andosol, LA: Low-humic Andosol

was grown for 4 years (from 2002 to 2005).

The soybeans were seeded on May 19, 2004; May 22, 2006; and May 21, 2007 and fertilized with N : P_2O_5 : K_2O at 20:50:80 kg/ha. On each hill, two seeds were sown. For DNA analysis, 3 soybean samples (each of which comprising 2 plants on a single hill) were each collected from 2 field subplots, meaning a total of 6 samples were collected for 1 preceding-crop condition.

In 2004, on July 6 (6 weeks after sowing), the soybeans were sampled from the subplots that had been cultivated the year before with 3 different crop types: carrot (*Daucus carota* L. cv. Kinkoh-sanzun) and spring wheat (*Triticum aestivum* L. cv. Haruyutaka), and no crop (bare). In 2006, on July 4 and 20 (6 and 8 weeks after sowing respectively), soybeans were sampled from subplots that had been cultivated the year before with 4 different crops: maize (*Zea mays* L. cv. Peter-corn), azuki bean (*Vigna angularis* (Willd.) Ohwi & Ohashi cv. Erimo-shozu), sugar beet (*Beta vulgaris* ssp. *vulgaris* cv. Abend), and buckwheat (*Fagopyrum esculentum* cv. Kitawasesoba). In 2007, soybeans were cultivated in fields of different soil types that had been cultivated the year before with maize (*Zea mays* L. cv. New Dent 95-day DKC34-20, Snow Brand Seed, Japan) or buckwheat, and soybeans were sampled on July 4 and 17 (6 and 8 weeks after sowing respectively).

Soybean seeds were harvested from 4 experimental subplots for each field condition, on September 21 in 2004, on September 26 in 2006, and September 19 in 2007 (Oka et al. 2010).

2. Sample preparation and measurement of AMF colonization

The soybean samples were separated into shoots and roots. The roots were washed, and the root nodules were removed. The roots were then cut into approximately 1-cm-long pieces and stored at -30°C before use. A portion of the roots was placed in 5% KOH in the test tube, and incubated in boiling water for 20 min. The roots were then stained with Trypan Blue (Brundrett et al. 1996). Colonization was measured under a dissecting microscope; counting more than 200 points, according to the gridline intersect method (Giovannetti & Mosse 1980). Data on AMF colonization was statistically analyzed using the Tukey method.

3. DNA extraction from root samples

The roots were crushed under liquid nitrogen using a mortar and pestle, and DNA samples were prepared from the powder, weighing approximately 10 mg, by boiling with Chelex 100 resin (Bio-Rad, CA, USA), followed by purification using isopropanol precipitation and a Geneclean Spin Kit (Q-Biogene, MP Biochemicals, CA, USA) for the samples collected in 2004 (Di Bonito et al. 1995) or a DNeasy Plant Mini Kit (QIAGEN, Tokyo, Japan) for those collected in 2006 and 2007.

4. Sequencing, homology search, and phylogenetic analysis

The amplification of a region in the fungal 18SrDNA was conducted using the nested PCR method (Saito et al. 2004, Sato et al. 2005). The AMF specific primer pairs AMV4.5F / AMV4.5R and AMV4.5NF / AMDGR were used for the first and second PCR, respectively. The PCR products, which had a length of approximately 270 bp, were purified using a QIAquick PCR Purification Kit (QIAGEN, Tokyo, Japan). The PCR products were inserted into a TA vector (pSTBlue-1) and transformed into competent cells of *Escherichia coli* according to the protocol of the AccepTor Vector Kit (Novagene, Merck KGaA, Darmstadt, Germany). The *E. coli* was cultivated on nutrient broth agar plates at 37°C overnight, and the positive colonies were then used for insert DNA PCR amplification with plasmid primers T7 5′-CTAATACGACTCACTATAGGG-3′and SP6 5′-ATTTAGGTGACACTATAGA-3′. The reaction volume for the PCR was 20 μl and included 0.5 μM of each primer, 200 μM of each dNTP, 1.5 mM of $MgCl_2$, and 0.5 units of Taq DNA polymerase (Ampli Taq Gold, Applied Biosystems, Life Technologies, CA, USA or ExTaq, TAKARA, Shiga, Japan). The thermal cycler was programmed as follows: initial step for denaturation, 5 min at 95°C; 35 cycles of 1 min at 94°C for denaturation, 1 min at

55°C for annealing, and 1 min at 72°C for polymerization respectively; followed by a final extension step of 10 min at 72°C.

Approximately 10 colony PCR products were prepared from each of the 6 PCR products, which originated from 6 different DNA samples. Accordingly, a total of approximately 60 clones were prepared for each condition. The colony PCR products were subjected to an extension reaction with primer T7 or SP6 for DNA sequencing according to the method in the sequencing manuals (Applied Biosystems, Life Technologies, CA, USA). The extension reaction products were then purified by gel filtration (Sephadex G50 fine; GE Healthcare Japan, Tokyo, Japan), dissolved with 10 µl formaldehyde and electrophoresed on a sequencer (ABI PRISM® 3100 Genetic Analyzer, Applied Biosystems, Life Technologies, CA, USA). The homology search of DNA sequences were performed using the Basic Local Alignment Search Tool (BLAST) on the DDBJ web page (http://www.ddbj.nig.ac.jp/index-j.html). When the clone sequence showed a high homology score with other fungi or organisms, the data were excluded. Data were also excluded when the identity of the AMF strains or clones in DDBJ was less than 90%.

To understand the phylogenetic relationship among the AMF sequences from soybean roots, phylogenetic trees covering the detected strains was constructed with registered sequences of AMF and *Saccharomyces cereviciae* as the outer group in DDBJ. The sequence data of AMF, colonizing in soybean roots at 6 weeks in 2004, 2006 and 2007, were used for phylogenetic tree. If there were more than two individuals (sequence data) for each AMF, which was classified by showing the highest homology score for the same AMF isolate or clone on the DDBJ database, one sequence data was selected. The software "CLC Main Workbench 6" (Filgen, Aichi, Japan) was used for phylogenetic analysis. The AMF phylotype was defined based on

Table 2. AMF colonization in soybean roots and soybean yields

(a)

Previous crop	Colonization (%)†	Soybean yield (Mg/ha)‡
Spring wheat	63.1±10.6[a]	2.78±0.23[a]
Carrot	54.6±4.0[a]	2.75±0.10[a]
No crop	55.9±2.4[a]	2.56±0.00[a]

†The values are the average of 6 samples for DNA analysis.
‡The values are the average of 4 experimental plots.

(b)

Previous crop	Colonization (%)†		Soybean yield (Mg/ha)‡
	6 week	8 week	
Maize	43.5±4.9[a]	60.6±9.8[b]	2.70±0.28[a]
Azuki bean	43.7±9.3[a]	76.0±3.0[a]	2.50±0.19[a]
Sugar beat	11.7±2.9[b]	38.3±7.7[c]	2.41±0.50[a]
Buckwheat	14.2 ±3.2[b]	43.3±3.2[c]	2.15±0.12[a]

†The values are the average of 6 samples for DNA analysis.
‡The values are the average of 4 experimental plots.

(c)

Soil type	Previous crop	Colonization (%)†		Soybean yield (Mg/ha)‡
		6 week	8 week	
Thapto-upland Andosol	Maize	55.7±5.1[b]	60.5±2.8[b]	2.58±0.24[a]
	Buckwheat	41.2±5.0[c]	48.6±9.6[c]	2.21±0.13[ab]
Low-humic Andosol	Maize	75.6±3.6[a]	80.3±4.5[a]	1.78±0.18[bc]
	Buckwheat	35.4±5.3[c]	50.3±5.0[c]	1.41±0.30[c]

†The values are the average of 6 samples for DNA analysis.
‡The values are the average of 4 experimental plots.

The soybean roots for DNA analysis were sampled at 6 weeks after sowing in 2004 (a), and then at 6 and 8 weeks after sowing in 2006 (b) and 2007 (c).
Subsequently, AMF colonization in plant roots was measured.
Soybean seeds were harvested in September in 2004 (a), 2006 (b), and 2007 (c).

the topology of the phylogenetic tree with sequential similarities, while the AMF phylotype was classified via phylogenetic analysis, following the previous taxonomy suggested by Schüßler et al. (2001). It was difficult to classify AMF at species level, by the region of 18S rDNA amplified by AMF-specific primers used in this study, and the AMF taxonomy is under development (Redecker et al. 2013). Therefore, we followed the basic form (Schüßler et al. 2001), adopted with molecular phylogenetics.

5. Multivariate statistical analysis and rarefaction analysis

Two multivariate statistics were used to reveal the main factors determining the AMF community in soybean roots. For these analyses, the data were converted into binary data, '1' for detected and '0' for undetected. Detrended corresponding analysis (DCA) was initially performed with data of AMF phylotypes, classified by homology search and phylogenetic analysis (Hill & Gauch 1980), using the free statistical software program "R" (http://www.r-project.org/). If the gradient length value was less than 4, principal component analysis (PCA) was performed (Hasegawa 2006), using the free software program for EXEL (http://www.vector.co.jp/soft/winnt/business/se412290.html). Furthermore, the current status of the sampling effort was analyzed by rarefaction analysis using the free Analytic Rarefaction software program (http://strata.uga.edu/software/Software.html) (Renker et al. 2006, Simberloff 1978).

Results

1. AMF colonization in the roots of soybeans and soybean yields

In 2004, the AMF colonization rate in the soybean roots did not differ among all previous conditions (Table 2a). In 2006 and 2007, samples were collected with two different cultivation periods, because it was thought that the AMF flora would change concomitantly with the growth stage of host plants. In 2006, previous cropping with mycorrhizal plant species, maize or azuki bean, resulted in a significantly higher AMF colonization in soybean roots than previous cropping with non-mycorrhizal species, sugar beet or buckwheat, did at 6 and 8 weeks after sowing ($P < 0.01$, $n = 6$) (Table 2b). In 2007, AMF colonization in the soybean roots was significantly higher in the previous cropping with maize than with buckwheat ($P < 0.05$, $n = 6$) (Table 2c). Where the previous crop was maize, AMF colonization was higher in the field composed of Low-humic Andosol (No. 23-15) than in the Thapto-upland Wet Andosol (No. 13-32).

The yields of soybean seeds did not differ among a range of previous conditions in 2004 and 2006 (Tables 2a & 2b). In 2007, the soybean yields were higher in Thapto-upland Andosol than Low-humic Andosol, where the previous crops were the same (Table 2c). The soybean yields were not different between the previous crops maize and buckwheat, when those in the same field was compared.

2. AMF community in soybean roots

The phylogenetic tree (Fig. 1) was constructed with the registered strains of DDBJ and the partial 18S rDNA sequences of AMF, colonizing the soybean roots in 2004 and 2006 (listed in Table 3), and in 2007 (listed in Table 4). The sequences of these AMF in soybean roots were registered in the DDBJ database. Depending on this phylogenetic tree, the detected AMF colonizing soybean roots could be separated into 11 phylotypes, Glo-A1, Glo-A2, Glo-A3, Glo-A4, Glo-B1, Glo-B2, Acaulosporaceae, Archaeosporaceae, Diversisporaceae, Gigasporaceae, and Paraglomeraceae (Fig. 1).

In 2004 and 2006, irrespective of the previous crop condition on the plot, the most frequently detected AMF phylotype was the Glo-B1 group, colonizing in the soybean roots, while the second was the Glo-A1 group (Fig. 1 and Table 5). The most frequently detected AMF sequences showed the highest homology with *Glomus* sp. ZJ (AB076344) in the group Glo-B1 and the second ones did with *Glomus* sp. Glo3 (AJ715998) in the Glo-A1 group. In 2004 and 2006, the number of detected AMF phylotype was minimal when the previous crop condition was "no crop". Furthermore, the AMF community structure differed slightly between 2004 and 2006. For example, AMF, belonging to Diversisporaceae, were detected in 2006, but not in 2004.

In 2007, irrespective of the previous crops, the most detectable AMF phylotypes, collected in the field of Thapto-upland Wet Andosol, were Glo-B1, Glo-A1, and Paraglomeraceae (Fig. 1 and Table 6). Therefore, when the soil type of the experimental fields was Thapto-upland Wet Andosol, the most detectable AMF phylotypes in 2004, 2006, and 2007 resembled each other (Tables 5 & 6). However, the most detectable AMF phylotypes in the field of Low-humic Andosol were Glo-A2 and Glo-B2, including *G. intraradices* or *Glomus* sp. NBR PP1 (EF136915), respectively. Further, the number of detected AMF phylotypes tended to be higher in Low-humic Andosol than Thapto-upland Wet Andosol (Table 6) and the AMF community structure in soybean roots differed between different fields in 2007. AMF belonged to the groups, Glo-A3, Glo-A4 and Acaulosporaceae, which were only detected in Low-humic Andosol (Fig. 1 & Table 6).

The detection of AMF phylotype by homology search in Tables 5 & 6 was subsequently converted into binary data, and the DCA was performed. Consequently, the gradient length of the first axis was 0.187, less than 4, confirming that the PCA had more adapted to analyze the factors

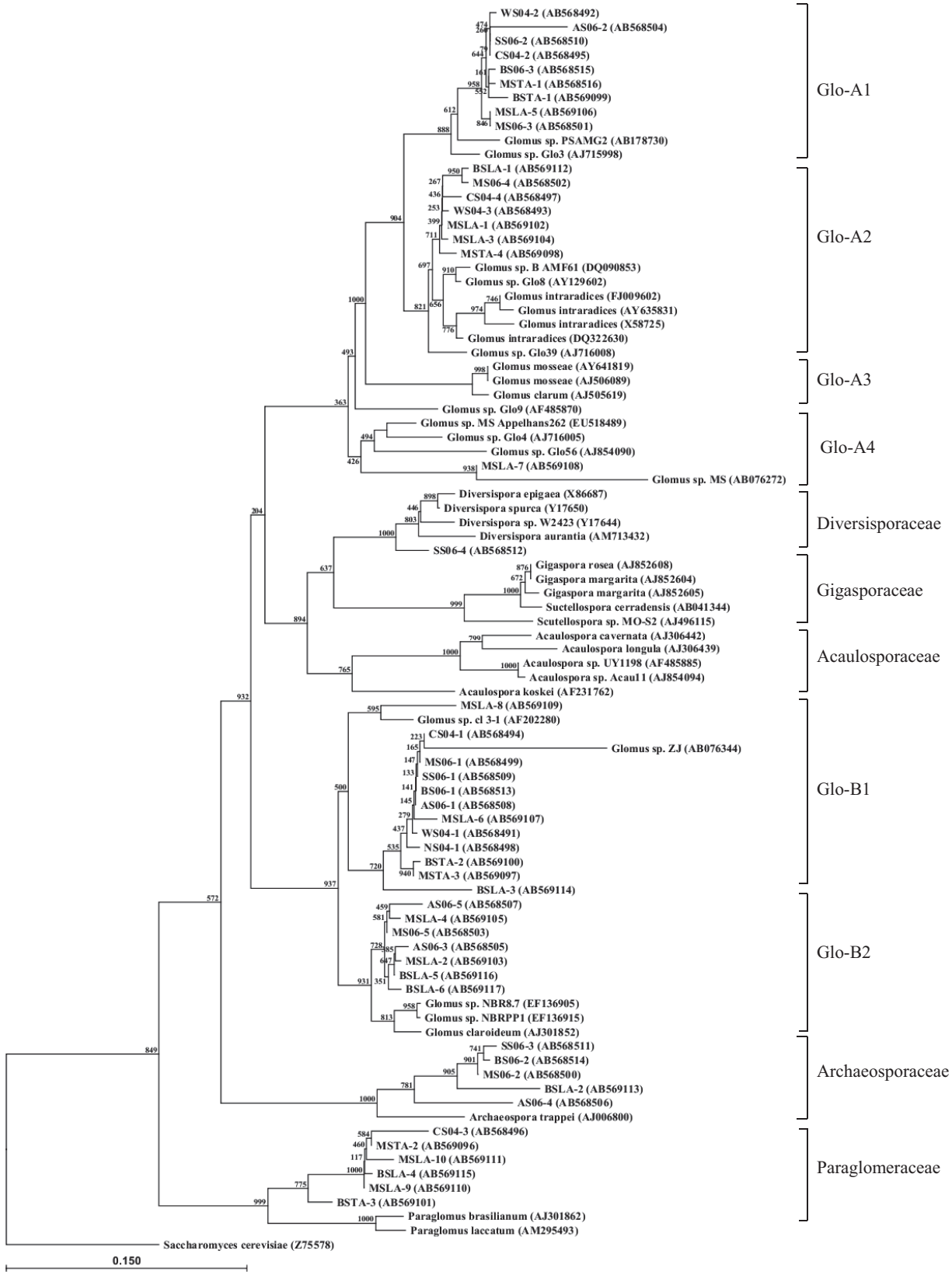

Fig. 1. Phylogenetic tree of AMF colonizing in the soybean roots

The 18S rDNA was originated from AM fungal strains colonizing in soybean roots, collected in 2004, 2006, and 2007, listed in Tables 3 and 4. *Saccharomyces cereviciae*, as the outer group, and other AMF sequences were from the DDBJ database. The values of branches were supported by the NJ method. The distance of phylogenetic tree was derived from bootstrap analysis.

Table 3. The list of AMF clones used for phylogenetic analysis, colonizing soybean roots in 2004 and 2006

Year	Previous crop	The name of the AMF clone, colonizing in soybean roots	The AMF isolates or clones in DDBJ data, showing the highest identity
2004	Wheat	WS04-1	*Glomus* sp. ZJ
		WS04-2	*Glomus* sp. Glo3
		WS04-3	*Glomus intraradices*
	Carrot	CS04-1	*Glomus* sp. ZJ
		CS04-2	*Glomus* sp. Glo3
		CS04-3	*Paraglomus brasilianum*
		CS04-4	*Glomus intraradices*
	No crop	NS04-1	*Glomus* sp. ZJ
2006	Maize	MS06-1	*Glomus* sp. ZJ
		MS06-2	*Archaeospora trappei*
		MS06-3	*Glomus* sp. Glo3
		MS06-4	*Glomus intraradices*
		MS06-5	*Glomus mosseae*
	Azuki bean	AS06-1	*Glomus* sp. ZJ
		AS06-2	*Glomus* sp. Glo3
		AS06-3	*Glomus* sp. NBRPP1
		AS06-4	*Archaeospora trappei*
		AS06-5	*Glomus mosseae*
	Sugar beat	SS06-1	*Glomus* sp. ZJ
		SS06-2	*Glomus* sp. Glo3
		SS06-3	*Archaeospora trappei*
		SS06-4	*Diversispora aurantia*
	Buckwheat	BS06-1	*Glomus* sp. ZJ
		BS06-2	*Archaeospora trappei*
		BS06-3	*Glomus* sp. Glo3

For example, for the AMF clone name in the soybean roots, “WS04” means the AMF which originated from the Wheat-Soybean cultivation system in 2004, and “MS06” indicates Maize-Soybean cultivation in 2006.

Table 4. The list of AMF for the phylogenetic analysis, colonizing soybean roots in 2007

Field No. (Soil type)	Previous crop	The name of the AMF clone, colonizing in soybean roots	The AMF isolates or clones in DDBJ data, showing the highest identity
No. 13-32 (Thapto-upland Wet Andosol)	Maize	MSTA-1	*Glomus* sp. Glo3
		MSTA-2	*Paraglomus laccatum*
		MSTA-3	*Glomus* sp. ZJ
		MSTA-4	*Glomus intraradices*
	Buckwheat	BSTA-1	*Glomus* sp. Glo3
		BSTA-2	*Glomus* sp. ZJ
		BSTA-3	*Paraglomus laccatum*
No. 23-15 (Low-humic Andosol)	Maize	MSLA-1	*Glom*us *intraradices*
		MSLA-2	*Glomus* sp. NBRPP1
		MSLA-3	*Glomus intraradices*
		MSLA-4	*Glomus mosseae*
		MSLA-5	*Glomus* sp. Glo3
		MSLA-6	*Glomus* sp. ZJ
		MSLA-7	*Glomus* sp. MS
		MSLA-8	*Glomus* cl 3-1
		MSLA-9	*Paraglomus brasilianum*
		MSLA-10	*Paraglomus laccatum*
	Buckwheat	BSLA-1	*Glomus intraradices*
		BSLA-2	*Archaeospora trappei*
		BSLA-3	*Glomus* sp. ZJ
		BSLA-4	*Paraglomus laccatum*
		BSLA-5	*Glomus* sp. NBRPP1
		BSLA-6	*Glomus mosseae*

For the AMF clone name in the soybean roots, “MSTA” means the strain which originated from the Maize-Soybean cultivation system at Thapto-upland Wet Andosol (Field No. 13-32), “BSTA” means from Buckwheat-Soybean cultivation at Thapto-upland Wet Andosol. The name “MSLA” means from Maize-Soybeancultivation at Low-humic Andosol (Field No. 23-15), “BSLA” means the strain which originated from Buckwheat-Soybean cultivation at Low-humic Andosol.

Table 5. The number of isolated AMF clones colonizing in soybean roots, cultivated after a range of previous conditions in2004 and 2006

Year	2004			2006							
Previous crop	Carrot	Wheat	No crop	Maize		Aazuki bean		Sugar beat		Buckwheat	
Time after seeding	6w	6w	6w	6w	8w	6w	8w	6w	8w	6w	8w
AMF phylotype †											
Glo-A1	6	16		4	6	2	2	7	1	5	6
Glo-A2	8	4		5							
Glo-A3	1										
Glo-A4		1									
Diversisporaceae					2	1	6	4	4	2	4
Glo-B1	26	37	57	28	42	41	37	24	31	28	32
Glo-B2	1			2		5				1	
Paraglomeraceae	5	1	1	1				1	10		4
Archaeosporaceae				14	3	12		1	3	8	2
Total number of clone	47	59	58	54	53	61	45	37	49	44	48
Number of detected AMF phylotype	6	5	2	6	4	5	3	5	5	5	5

† AMF phylotypes were decided by phylogenetic analysis (Fig. 1).
The DNA were extracted from soybean roots, whereupon the partial 18S rDNA was amplified, cloned, and sequenced and the AMF sequences were separated into AMF phylotypes.

Table 6. The number of the isolated AMF clones colonizing in the soybean roots, cultivated after a range of previous conditions in 2007

Soil type	Thapto-upland Wet Andosol				Low-humic Andosol			
Previous crop	Maize		Buckwheat		Maize		Buckwheat	
Time after seeding	6w	8w	6w	8w	6w	8w	6w	8w
AMF phlotype†								
Glo-A1	31	21	15	16	4	13		5
Glo-A2	8	1			28	25	22	23
Glo-A3								5
Glo-A4					3		2	
Diversisporaceae		6	1	1	1	1		3
Glo-B1	11	9	24	28	8	2	3	6
Glo-B2			1		13	7	10	6
Paraglomeraceae	11	12	6	7	4		10	
Archaeosporaceae	1				1		13	1
Acaulosporaceae							1	3
Gigasporaceae	1							
Total number of clone	63	49	47	52	62	48	61	52
Number of detected AMF phylotype	6	5	5	4	8	5	7	8

† AMF phylotypes were decided by phylogenetic analysis (Fig. 1).
The DNAs were extracted from soybean roots, whereupon the partial 18S rDNA was ammplified, cloned, and sequenced and the AMF sequences were separated into AMF phylotypes.

deciding the AMF community in the soybean roots, whereupon the PCA was performed. The AMF phylotypes were separated, but not grouped, by PC1 (47.7%) and PC2 (16.4%), and Glo-B1 and Glo-A1 had the highest score for PC1 (Fig. 2).

In this study, around 40 to 60 data of clones for each treatment were used to discuss the diversity of AMF species in soybean roots. Approximately 10 clones was prepared from each of the PCR products, which originated from 6 different root DNAs (in total, approximately 60 clones, but some data were omitted). The sampling effort was examined by rarefaction analysis of two selected samples (Fig. 3). The rarefaction curves flattened with a sample size of around 60.

Discussion

It was reported that the AMF diversity and density in the soil of rotated fields exceeded those in mono-cropped fields. The AMF diversity in the soil of a maize-crotalaria crop-rotation system exceeded that in a mono-cropped field of maize (Oehl et al. 2003). The AMF diversity in the soil of soybean fields was greater when the previous crop was a non-soybean species (Hendrix et al. 1995). Therefore, for our experimental fields, it was thought that the previous crop might impact the AMF community in the roots of suc-

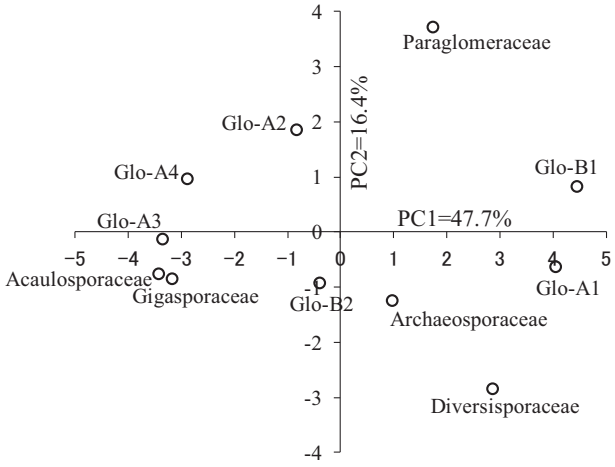

Fig. 2. Principal component analysis (PCA) using all data of the composition of AMF groups colonizing in soybean roots after cultivation with different cropping systems in 2004, 2006, and 2007, listed in Tables 5 and 6

The data were converted into binary data, '1' for detected and '0' for undetected, and calculated.

ceeding crops. ***However, there*** was little knowledge of the AMF community in plant roots in crop-rotated fields; hence this study could reveal new knowledge of the AMF community structure in soybean roots, with different preceding cropping systems, in Japan.

In this study, it was shown that a range of previous cropping systems could affect AM colonization, but little was revealed on the AMF community structure in soybean roots. The AMF colonization of the soybean roots was lower where the previous crop was non-mycorrhizal rather than mycorrhizal plants, in 2006 and 2007. However, phylogenetic analysis revealed that the AMF community in soybean roots differed little among the treatments. Furthermore, PCA revealed that PC1was contributed mainly by the frequency of AMF detection in soybean roots and possibly also slightly by detectability in Thapto-upland Wet Andosol (Fig. 2). It was suggested that, irrespective of the previous crops, the same AMF phylotypes colonized dominantly in soybean roots. As separated by the axis PC1, it was shown that the AMF phylotype, Glo-B1, colonized most frequently (PC1=4.45), followed by Glo-A1 (PC1=4.05) (Fig. 2). Similarly, the experimental field with rotated crop cultivation showed common species in the soil, *Glomus claroideum,* regardless of the different fertilization treatments (Vestberg et al. 2011). Conversely, the AMF phylotypes in soybean roots were also separated slightly by the axis PC2 (16.4%). It was thought PC2 might be contributed mainly by year. For example, the AMF phylotype "Diversisporaceae" was detected in 2006 and 2007, but not in 2004. The experiments were performed in different fields every year. It was implied that the AMF community, colonizing in soybean roots, was comparatively affected by different conditions of fields, rather than by the range of previous crops.

The AMF most frequently detected in soybean roots and cultivated in our field condition (in Sapporo, Hokkaido, Japan), were the phylotype "Glo-B1". Previous studies based on spore observation reported that *Gigaspora* sp. was the dominant AMF in some soybean fields of Andosol, Japan (Saito & Vargas 1991), or Purple Red Latosol, Brazil (Kojima et al. 2005). In other studies, *Glomus* spp. dominated in some soils after soybean cultivation (An et al. 1990, Franke-Snyder et al. 2001). In this study, several *Glomus* spp. dominated in the soybean roots, regardless of the previous crops cultivated the year before. *Glomus* spp. was also the dominant AMF spores in the soil after soybean cultivation in 2004 (data not shown). Isobe et al. (2011) examined the AMF community in soybean roots cultivated in two different regions of Japan, Kanagawa and Hokkaido, using a molecular technique. Gigasporaceae was detected in Kanagawa, but not in Hokkaido. Their results corresponded to our results in Hokkaido. The AMF, *Glomus* sp. ZJ, was detected in the grasslands in Japan. Furthermore, it was reported that unidentified *Glomus* spp. were the most dominant in the roots of *Miscanthus sinensis* and *Zoysia japonica* (Saito et al. 2004), as well as in soil of several semi-natural grasslands in Japan (Kojima et al. 2009). The soil type of those fields was also Andosol, which implies that some common indigenous AMF species might be distributed among Andosol soils in Japan.

In 2007, there were not large differences in the AMF community among treatments, cultivated with a range of previous crops. However, the detected number of AMF phylotypes differed among the different fields. If the detected clone number was considered, it was shown that the detectable AMF phylotypes differed between fields. The Glo-B1 and Glo-A1 groups were most frequently detected in the Thapto-upland Wet Andosol, but the group Glo-A2 was most common in Low-humic Andosol. Therefore, it was thought that the AMF community in soybean roots was not influenced by the previous crops cultivated the year before, but by other environmental factors. Oehl et al. (2010) showed that the composition of AMF in fields was strongly dependent on soil types. However, Karasawa et al. (2000 & 2001) showed that the AMF effect on plant growth depended on the type of previous crop rather than environmental factors such as soil type or soil moisture. Field No. 23-15 with Low-humic Andosol was used as grasslands and red clover was grown there for 4 years. The AMF diversity in fields used as grasslands for an extended period exceeded that of crop fields (Oehl et al. 2003). Hamel et al. (1994) showed that the AMF diversity was unaffected by barley cultivation for 3 years in a long-term meadow. The species number and density of AMF

spores were higher in grasslands or red clover fields than in barley or wheat fields (Menendez et al. 2001). Accordingly, the higher AMF diversity in the Low-humic Andosol field might be attributable not only to differences in soil types but also to the previous long-term vegetation.

With regard to AMF colonization in soybean roots, in 2007, when maize was the previous crop, AMF colonization in the soybean roots was higher in Low-humic Andosol fields than Thapto-upland Wet Andosol. The available P concentration was higher in Thapto-upland Wet Andosol than Low-humic Andosol. Furthermore, more AMF phylotype seemed present in the soil of Low-humic Andosol than Thapto-upland Wet Andosol. Generally, AMF colonization decreases with increasing P concentration in soil (Smith & Read 2008). Therefore, the available P and the species richness of AMF in the soil could affect the colonization. The differences in the available P in soil and previous crops also affected the soybean yield in 2007. While in 2004 and 2006, there was no significance among a range of previous cropping systems, in the experimental fields where samples were collected for this study, the standardized soybean yields in 2004-2006 were significantly higher where the previous crop was the host plant (Oka et al. 2010). This implied that the difference in the previous cropping system strongly affected AMF colonization at an earlier soybean growth stage.

Generally, the AMF seems to be randomly distributed in fields. By increasing the sampling frequency, more AMF species can be detected in experimental fields along with several types of plants (Whitcomb & Stutz 2007). However, in mono-cropping, the AMF diversity under field conditions seems more constant. Fewer AMF species were observed in the plant roots of an arable site than in roots in a natural ecosystem (Helgason 1998). Therefore, in the current study, a total of 6 samples were collected, based on consideration of the number of previous conditions and analytical effort. Renker et al. (2006) showed that when the DNA was extracted from a pool of 50 roots, fewer AM species were detected. When the DNA was prepared by extraction from each 50 roots separately, the number of detected species increased. The rarefaction curves flattened with a sample size of around 60 (Fig. 3). It was implied that the number of clones, prepared in this study (40 to 60), was appropriate for discussing diversity. In the case of the DGGE method, the diversity could be calculated based on the strength of each band, which reflected the amount of PCR product for each of the DNA sequences, which originated from different species or strains (Abell & McOrist 2007). It was thought that the number of clones could also reflect the density of each AMF taxa, colonizing in the roots. However, because of the multiple amplification of root DNA by nested PCR, the results in this study became less quantitative. Accordingly, in future study, the sampling and analytical methods should be considered precisely to determine AMF diversity in the roots.

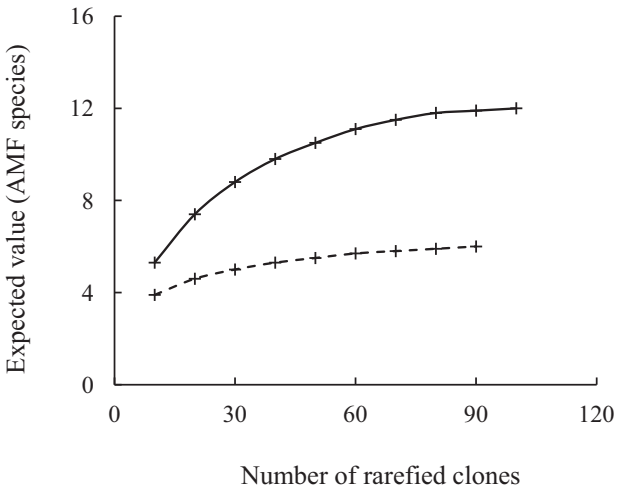

Fig. 3. Rarefaction analysis for the expected value of AMF species in soybean roots against the sequenced clone number, originated from colony PCR

Two kinds of samples were analyzed. A solid line represents the data of the cultivated condition as BSLA at 6 weeks in 2007, while a dashed line represents the condition as MSTA at 6 weeks in 2007. MSTA: the Maize-Soybean cultivation system at Thapto-upland Wet Andosol, BSLA: Buckwheat-Soybean cultivation at Low-humic Andosol.

Conclusion

Phylogenetic analysis using primers specific to 18SrDNA of AMF showed that the AMF communities in soybean roots differed little among a range of previous cropping systems in this study and the most detectable AMF in soybean roots was the phylotype "Glo-B1", including *Glomus* sp. ZJ. When the previous condition was bare soil (no crop), there were minimal AMF phylotypes in soybean roots. In our experimental fields, the number of AMF phylotypes at the Low-humic Andosol field, which had been continuously used as grasslands for 4 years, seemed to exceed that at the Thapto-upland Wet Andosol field. It was implied that AMF diversity might be affected by long-term vegetation or environmental conditions rather than just previous crops. In future studies, soil types or long-term crop rotations, which might determine the AMF diversity in crop roots, should be considered.

Acknowledgements

This work was supported by the Grant-in-Aid "Development of New Biorational Techniques for Sustainable Agriculture" and "Development of mitigation and adaptation techniques to global warming in the sectors

of agriculture, forestry, and fisheries" from the Ministry of Agriculture, Forestry, and Fisheries, Japan. We are grateful to Dr. Manabu Takahara (NARO Institute of Livestock and Grassland Science, Japan) and Dr. Hirosuke Oba (The University of Tokyo, Japan) for their help with the genetic experiments and analysis. We are also grateful to Dr. Masayuki Ushio (Kyoto University, Japan) for his advice on statistical analysis.

References

Abell, G. C. J. & McOrist, A. L. (2007) Assessment of the diversity and stability of faecal bacteria from healthy adults using molecular methods. *Microb. Ecol. Health D.*, **19**, 229-240.

An, Z.-Q. et al. (1990) Vertical distribution of endogonaceous mycorrhizal fungi associated with soybean, as affected by soil fumigation. *Soil Biol. Biochem.*, **22**, 715-719.

Arihara, J. & Karasawa, T. (2000) Effect of previous crops on arbuscular mycorrhizal formation and growth of succeeding maize. *Soil Sci. Plant Nutr.*, **46**, 43-51.

Bever, J. D. et al. (1996) Host-dependent sporulation and species diversity of arbuscular mycorrhizal fungi in a mown grassland. *J. Ecol.*, **84**, 71-82.

Brundrett, M. et al. (1996) *Working with Mycorrhizas in Forestry and Agriculture*. ACIAR Monograph 32, ACIAR, Canberra, Australia, 173-215.

Chalk, P. M. et al. (2006) The role of arbuscular mycorrhiza in legume symbiotic performance. *Soil Biol. Biochem.*, **38**, 2944-2951.

Cheng, Y. et al. (2013) Ninety-year, but not single, application of phosphorus fertilizer has a major impact on arbuscular mycorrhizal fungal communities. *Plant Soil*, **365**, 397-407.

Clapp, J. P. et al. (2002) Genetic studies of the structure and diversity of arbuscular mycorrhizal fungal communities. *In* Mycorrhizal Ecology, eds. van der Heijdn, M. G. A. & Sanders I. R., Springer, Berlin, Germany, 201-224.

Cordell, D. et al. (2009) The story of phosphorus: Global food security and food for thought. *Global Environ. Change*, **19**, 292-305.

Daniell, T. J. et al. (2001) Molecular diversity of arbuscular mycorrhizal fungi colonising arable crops. *FEMS Microbiol. Ecol.*, **36**, 203-209.

Di Bonito, R. et al. (1995) Detection of an arbuscular mycorrhizal fungus in roots of different plant species with the PCR. *Appl. Environ. Microbiol.*, **61**, 2809-2810.

Douds Jr, D. D. et al. (1998) Regulation of arbuscular mycorrhizal development by plant host and fungus species in alfalfa. *New Phytol.*, **138**, 27-35.

Ellis, J. R. et al. (1992) Grain sorghum-soybean rotation and fertilization influence on vesicular-arbuscular mycorrhizal fungi. *Soil Sci. Soc. Am. J.*, **56**, 789-794.

Franke-Snyder, M. et al. (2001) Diversity of communities of arbuscular mycorrhizal (AM) fungi present in conventional versus low-input agricultural sites in eastern Pennsylvania, USA. *Appl. Soil Ecol.*, **16**, 35-48.

Giovannetti, M. & Mosse, B. (1980) An evaluation of techniques for measuring vesicular- arbuscular mycorrhizal infection in roots. *New Phytol.*, **84**, 489-500.

Hamel, C. et al. (1994) Composition of the vesicular-arbuscular mycorrhizal fungi population in an old meadow as affected by pH, phosphorus and soil disturbance. *Agric. Ecosys. Environ.*, **49**, 223-231.

Harrier, L.A. & Watson, C.A. (2004) The potential role of arbuscular mycorrhizal (AM) fungi in the bioprotection of plants against soil-borne pathogens in organic and/or other sustainable farming systems. *Pest Manag. Sci.*, **60**, 149-157.

Hasegawa, M. (2006) Application of ordination in community ecology of soil animals. *Edaphologia*, **80**, 35-64 [In Japanese with English summary].

Helgason, T. et al. (1998) Ploughing up the wood-wide web? *Nature*, **394**, 431.

Hendrix, J. W. et al. (1995) Divergence of mycorrhizal fungal communities in crop production systems. *Plant Soil*, **170**, 131-140.

Hill, M.O. & Gauch, H.G. (1980) Detrended correspondence analysis, an improved ordination technique. *Vegetatio*, **42**, 47-58.

Isobe, K. et al. (2011) Arbuscular mycorrhizal fungal community structure in soybean roots: comparison between Kanagawa and Hokkaido, Japan. *Adv. Microbiol.*, **1**, 13-22.

Karasawa, T. et al. (2000) Effects of previous crops on arbuscular mycorrhizal formation and growth of maize under various soil moisture conditions. *Soil Sci. Plant Nutr.*, **46**, 53-60.

Karasawa, T. et al. (2001) Variable response of growth and arbuscular mycorrhizal colonization of maize plants to preceding crops in various types of soils. *Biol. Fertil. Soils*, **33**, 286-293.

Karasawa, T, et al. (2002) Differences in growth responses of maize producing cropping caused by fluctuation in the population of indigenous arbuscular mycorrhizal fungi. *Soil Biol. Biochem.*, **34**, 851-857.

Kojima, T. et al. (2005) Arbuscular mycorrhizal fungi in the fields of Agropastral system in Brazil. *Nihon souchi gakkaishi* (*Jp. J. of Grassl. Sci.*), **51**, 79-86 [In Japanese with English summary].

Kojima, T. et al. (2009) The diversity of arbuscular mycorrhizal fungi in Japanese grasslands. *Nihon souchi gakkaishi* (*Jp. J. of Grassl. Sci.*), **55**, 148-155 [In Japanese with English summary].

Kurle, J. E. & Pfleger, F. L. (1996) Management influences on arbuscular mycorrhizal fungal species composition in a corn-soybean rotation. *Agron. J.*, **88**, 155-161.

Menendez, A. B. et al. (2001) Arbuscular mycorrhizal populations associated with natural and cultivated vegetation on a site of Buenos Aires province, Argentina. *Biol. Fertil. Soils*, **33**, 373-381.

Norman, J. R. et al. (1996) Arbuscular mycorrhizal fungal-induced alteration to root architecture in strawberry and induced resistance to the root pathogen *Phytophthora fragariae. Plant Soil*, **185**, 191-198.

Obara, H. et al. (2011) Comprehensive soil classification system of Japan First approximation. *Bull. Natl. Inst. Agro-Environ. Sci.*, **29**, 1-73 [In Japanese with English summary].

Oehl, F. et al. (2003) Impact of land use intensity on the species diversity of arbuscular mycorrhizal fungi in agroecosystems of central Europe. *Appl. Environ. Microbiol.*, **69**, 2816-2824.

Oehl, F. et al. (2010) Soil type and land use intensity determine the composition of arbuscular mycorrhizal fungal communities. *Soil. Biol. Biochem.*, **42**, 724-738.

Oka, N. et al. (2010) Maintenance of soybean yield with reduced phosphorus application by previous cropping with mycorrhizal plants. *Soil. Sci. Plant Nutr.*, **56**, 824-830.

Plenchette, C. & Morel, C. (1996) External phosphorus requirement of mycorrhizal and non-mycorrhizal barley and soybean plants. *Biol. Fertil. Soils*, **21**, 303-308.

Redecker, D. et al. (2013) An evidence-based consensus for the classification of arbuscular mycorrhizal fungi (*Glomeromycota*). *Mycorrhiza*, **23**, 515-531.

Renker, C. et al. (2006) Rationalizing molecular analysis of field-collected roots for assessing diversity of arbuscular mycorrhizal fungi: to pool, or not to pool, that is question. *Mycorrhiza*, **16**, 525-531.

Saito, K. et al. (2004) Defoliation effects on the community structure of arbuscular mycorrhizal fungi based on 18S rDNA sequences. *Mycorrhiza*, **14**, 363-373.

Saito, M. & Vargas, R. (1991) Vesicular-arbuscular mycorrhizal fungi in some humus-rich Ando soils of Japan. *Tsuchi to Biseibutsu* (*Soil Microorganisms*), **38**, 3-15.

Sanders, I. R. & Fitter, A. H. (1992) Evidence for differential responses between host-fungus combinations of vesicular-arbuscular mycorrhizas from a grassland. *Mycol. Res.*, **96**, 415-419.

Sasvári, Z. et al. (2011) The community structure of arbuscular mycorrhizal fungi in roots of maize grown in a 50-year monoculture. *Biol. Fertil. Soils*, **47**, 167-176.

Sato, K. et al. (2005) A new primer for discrimination of arbuscular mycorrhizal fungi with polymerase chain reaction–denature gradient gel electrophoresis. *Grassl. Sci.*, **51**, 179-181.

Schüßler, A. et al. (2001) A new fungal phylum, the *Glomeromycota*: phylogeny and evolution. *Mycol. Res.*, **105**, 1413-1421.

Simberloff, D. S. (1978) Use of rarefaction and related methods in ecology. *In* Biological data in water pollution assessment: quantitative and statistical analyses*,* eds. Dickson, K. L. et al., American Society for Testing and Materials STP 652, Philadelphia, USA, 150-165.

Smith, S. E. & Read, D. J. (2008) *Mycorrhizal symbiosis third edition*. Academic Press, London, UK, pp.787.

Smith, S. E. et al. (2003) Mycorrhizal fungi can dominate phosphate supply to plants irrespective of growth response. *Plant Physiol.*, **133**, 16-20.

Smith, S. E. et al. (2004) Functional diversity in arbuscular mycorrhizal AM symbioses: the contribution of mycorrhizal P uptake pathway is not correlated with the mycorrhizal responses in growth or total P uptake. *New Phytol.*, **162**, 511-524.

van der Heijden, M. G. A. et al. (1998) Mycorrhizal fungal diversity determines plant biodiversity, ecosystem variability and productivity. *Nature*, **396**, 69-72.

van der Heijden, M. G. A. et al. (2003) Different arbuscular mycorrhizal fungi alter co-existence and resource distribution between co-occurring plant. *New Phytol.*, **157**, 569-578.

Vestberg, M. et al. (2011) Arbuscular mycorrhizal fungal diversity and species dominance in a temperate soil with long-term conventional and low-input cropping systems. *Mycorrhiza*, **21**, 351-361.

Vigo, C. et al. (2000) Biocontrol of the pathogen *Phytophthora parasitica* by arbuscular mycorrhizal fungi is a consequence of effects on infection loci. *Plant Pathol.*, **49**, 509-514.

Whitcomb, S. & Stutz, J. C. (2007) Assessing diversity of arbuscular mycorrhizal fungi in a local community: role of sampling effort and spatial heterogeneity. *Mycorrhiza*, **17**, 429-437.

Yoshimura, Y. et al. (2013) Communities of arbuscular mycorrhizal fungi in the roots of *Pyrus pyrifolia* var. *culta* (Japanese pear) in orchards with variable amounts of soil–available phosphorus. *Microbes Environ*., **28**, 105-111.