

Influence of Sowing Season and Host Crop Identity on the Community Structure of Arbuscular Mycorrhizal Fungi Colonizing Roots of Two Different Gramineous and Leguminous Crop Species

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Abstract

Introduction of cover crops may improve the diversity of arbuscular mycorrhizal fungi (AMF) in roots and soil under crop rotational systems; therefore, it is necessary to determine the potential for AMF communities to improve sustainable food production. We investigated the impact of cover crops, including wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.), pea (*Pisum sativum* L.), and hairy vetch (*Vicia villosa* Roth.), on the AMF communities in their roots in autumn and spring sowing seasons with PCR-DGGE analysis. Although all four cover crops impacted the AMF community structure in roots, the diversity of AMF communities was unchanged among crop type or sowing season. Redundancy analysis (RDA) demonstrated that AMF communities within crop type were significantly different. However, the AMF community structures were not influenced by growing season, suggesting that growth stage in crops may be more responsive to shaping AMF community structure in crop roots than host crop identity.

Keywords

Arbuscular Mycorrhizal Fungi, Community Structure, Crop Identity, Host Selectivity, Sowing Season

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1. Introduction

Arbuscular mycorrhizal fungi (AMF) are widespread in most terrestrial ecosystems where they form mutualistic associations with the majority of plants to facilitate nutrient uptake from the soil via an extensive extraradical mycelium [1]. This symbiotic relationship is present in most agricultural crops, with benefits of increased plant productivity [2], improved soil structure [3] and increased pathogen resistance [4]. Improvement in phosphorus (P) uptake and plant growth by AMF has been documented in many crop plants [5] [6]. Moreover, AMF abundance or diversity is important to overall plant productivity and stability of terrestrial ecosystems [7]. Because of their importance in terrestrial ecosystems, AMF communities have been extensively studied both in natural [8]-[10] and agricultural settings [11]-[13].

Introduction of mycorrhizal crops, such as pea (*Pisum sativum* L.), oat (*Avena sativa* L.), hairy vetch (*Vicia villosa* Roth.), and wheat (*Triticum aestivum* L.), in crop rotational system has been shown to maintain or increase AMF biomass, or P uptake and yield in subsequent crops [5] [6] [14]-[16]. The difference in AMF spore density after mycorrhizal cropping has been also shown to be much higher than after non-mycorrhizal cropping [17] or fallow [18]. Winter cover crops such as barley (*Hordeum bulbosum* L.), wheat, white clover (*Trifolium repens* L.) and other crops in rotation with summer crops have been used to enhance soil health and fertility [19] [20]. Arranging crop rotation with cover crops may maintain or increase P uptake and yield of subsequent crops [5] [6] [16]. However, Vandenkoornhuise *et al.* [21] [22] and Torrecillas *et al.* [23] have mentioned that there is mounting evidence on host compatibility between host plant and some AMF species. Gosling *et al.* [11] have also determined presence of a subtle difference in the communities of AMF colonizing roots of two crops, corn (*Zea mays* L.) and soybean (*Glycine max* L.) grown in a crop rotational system in the United Kingdom. Alguacil *et al.* [24] have described the differences between root AMF communities of two agricultural crop species (*Jatropha curcas* and *Ricinus communis*) in a tropical system in Guantanamo (Cuba).

Temporal and seasonal variations among AMF communities in plant roots and soil have been shown [23]-[25]. Liu *et al.* [26] (2009) reported that the composition of AMF communities colonizing *Caragana korshinskii* roots was not different at the same sampling time, whereas subtle differences in the AMF communities were found in different months. In Japan, Isobe *et al.* [27] found that *Gigasporales* was not found in the roots of soybean grown in a cooler region, whereas they were detected in the roots of soybean grown in a temperate region. Isobe *et al.* [27] suggested that one partial reason for the differences in AMF communities was climate conditions, especially temperature. From these results, it appears that composition or diversity of AMF communities in roots is not necessarily determined only by host selectivity. Yet, there is a paucity of information regarding which environmental factors, such as climate, soil quality and chemical conditions, or host crop identity most strongly influences the differences observed in AMF communities colonizing crop roots. This information is important because AMF abundance or diversity is imperative to overall productivity and stability of agroecosystem functioning in crop rotational systems. Knowledge of seasonal or temporal variation of AMF communities in roots and soil under crop rotational systems would allow us to more effectively arrange crop rotations in agricultural systems and to improve diversity and abundance of AMF communities.

To better capture seasonal or temporal variation of AMF communities under crop rotational systems, we hypothesized that two graminaceous (wheat and barley) and leguminous crop (pea and hairy vetch) species, which are often introduced in rotation with summer crops in autumn or spring, may lead to a shift in AMF diversity or community structure colonizing roots of these crops in the autumn and spring seasons. To test this hypothesis, we investigated the diversity of AMF communities in roots of these four different crop species in the autumn or spring sowing seasons. The innovative aspects of the present study focused on using molecular techniques to determine AMF community structure to assess the impacts of host species and sowing season.

2. Materials and Methods

2.1. Experimental Design

A pot experiment using a volcanic ash soil (Allophanic and sol, sandy loam texture) was conducted in an experimental field at Nihon University, in Kanagawa, Japan (35°22'N and 139°27'E). To investigate the impact of host crop identity or sowing season on AMF community structure, 1/2000a Wagner pots were prepared with three replicates and mix-cropped with pea (*Pisum sativum* L., cv: Akabanakinusaya), hairy vetch (*Vicia villosa* Roth., cv: Mamesuke), wheat (*Triticum aestivum* L., cv: Bandowase), and barley (*Hordeum vulgare* L., cv:

Ichibanboshi). In the autumn sowing, seeds were sown in the same pot on 27 October, 2012. Similarly, in the spring sowing, seeds were sown in the same pot on 13 April, 2013. In both autumn and spring experiments, the number of seeds sown in pea, hairy vetch, wheat and barley was 3, 3, 5 and 5 seeds per pot, respectively. The amounts of N (ammonium sulfate) and K (potassium chloride) applied were 3.6 and 1.3 g per pot, respectively.

2.2. Soil Sampling and Measurement of Soil Chemical Properties

Soil samples were collected from each pot on 26 October, 2012, and on 12 April, 2013, before cover crop cultivation. After air-drying, the composite soil samples were passed through a 2 mm sieve, and stored at 4°C until measurement of AMF spore density. Soil pH among each cover crop ranged from 5.8 to 6.4 and available P content (Bray P) ranged between 30.4 to 48.5 mg per kg dry soil. Total carbon (C) and nitrogen (N) ranged from 6.6% to 7.1% and 0.55% to 0.63%, respectively. AMF spore density ranged from 6.4 to 7.1 spores per g dry soil.

2.3. Root Sampling, Staining and DNA Extraction

Root samples of each crop were taken on 14 May, 2013. Root samples were stained with 0.05% (w/v) trypan blue [28], and AMF colonization in the crop roots was calculated as described by [29].

DNA extractions (five pieces of 1- to 2-cm-long root fragments) were performed using CTAB (Cetyltrimethyl ammonium bromide) method. The DNA pellet was washed once with 80% ethanol, dried, resuspended in 50 µl of tris-ethylenediaminetetraacetic acid (EDTA) (TE) buffer [10 mM Tris-HCl (pH 8.0) and 1 mM EDTA (pH 8.0)], and stored at -30°C until used for polymerase chain reaction (PCR).

2.4. Nested PCR

The DNA samples per replicate extracted from roots were used as PCR templates after 20-fold dilution. The DNA pellet was resuspended in 50 µl of TE buffer [10 mM Tris-HCl (pH 8.0) and 1 mM EDTA (pH 8.0)], and stored at -30°C until use for PCR. The DNA samples extracted from roots were used as PCR templates after 10-fold dilution. The amplification of a region in the fungal small subunit ribosomal DNA (SSU rDNA) was conducted using a nested PCR method [30]. The fungus-specific primers AM1 (5'-GTTTCCCGTAAGGCGCCGAA-3') [31] and the universal eukaryotic primer NS31 (5'-TTGGAGGGCAAGTCTGGTGCC-3') [32] were used in the first PCR to amplify the 5' end of the SSU rDNA region. PCR was performed in a 25 µl reaction mixture containing 1 µl of template DNA, 0.2 µM of each primer, and GoTaq Green Master Mix (Promega, Madison, WI, USA) using a thermal cycler (Mastercycler ep Gradient, Eppendorf, Hamburg, Germany). The PCR protocol was composed of an initial treatment at 94°C for 1 min; 30 cycles of treatments at 94°C for 1 min, 66°C for 1 min, and 72°C for 1 min 30 s; and a final treatment at 72°C for 10 min. The first PCR products were diluted 100-fold and used as templates for the second PCR using the nested primers NS31-GC (5'-CGCCCGGGGCGCGCCCGGGGCGGGGCGGGGACGGGGGTTGGAGGGCAAGTCTGGTGCC-3') [33] and Glo1 (5'-GCCTGCTTTAAACTCTA-3') [34]. PCR was performed in a 50 µl reaction mixture containing 2 µl of template DNA, 0.2 µM of each primer, and 2× GoTaq Green Master Mix (Promega, Madison, WI, USA) using Mastercycler ep Gradient (Eppendorf, Hamburg, Germany). The PCR protocol was composed of an initial treatment at 95°C for 5 min; 35 cycles of treatments at 94°C for 45 s, 52°C for 45 s, and 72°C for 1 min; and a final treatment at 72°C for 30 min. Amplification products were separated by gel electrophoresis on 1% agarose gel in Tris-acetate-EDTA (TAE) buffer (40 mM Tris, pH 8.0, 40 mM acetic acid and 1 mM EDTA) and DNA was visualized by staining with ethidium bromide.

2.5. PCR-DGGE

Twenty microliters of nested PCR product was subsequently analyzed by DGGE on DCode Universal Mutation Detection System (Bio-Rad Laboratories, Piscataway, NJ, USA). The gels containing 6.5% acrylamide were poured with a gradient of 35% - 55% denaturant. All DGGE analysis was performed in 1× TAE buffer at a constant temperature of 55°C at 50 V for 60 min, followed by 50 V for 960 min using the modified protocol of Liang *et al.* [30]. The gels were then stained with SYBR Green diluted in 1× TAE buffer (1:10,000) for 20 min, UV illuminated and digitally photographed. Pictures were captured and digitized by Phoretix 1D Pro (Nonlinear Dynamics Ltd., Newcastle upon Tyne, UK).

2.6. Molecular Diversity of AMF Communities in Cover Crop Roots

From the data we calculated AMF DGGE band richness, expressed by the number of DGGE bands in each root sample. The Shannon diversity index of each treatment was calculated as an additional measure of AMF diversity.

3. Statistical Analysis

To analyze the relatedness of sampling plots with respect to AMF community structure (AMF communities), a preliminary detrended correspondence analysis (DCA) on the presence/absence matrix per DGGE band was also performed. This result was summarized in an ordination diagram using the vegan package version 2.0 - 10 in R 3.0.2. DCA indicated (the length of gradient > 4) that at least some species had a unimodal distribution. Redundancy analysis (RDA, length of gradient < 4) as multivariate analysis was performed. The data matrix was composed of the presence/absence of DGGE bands and crop type. During the RDA procedure, Monte Carlo 999 permutation test for significance at $P < 0.05$ was used.

Climate data were compiled from the Japan Meteorological Agency (<http://www.jma.go.jp/jma/indexe.html>).

4. Results

Climate factors, including precipitation, average temperature, accumulated temperature, and sunshine duration from sowing to sampling time differed between autumn and spring sowing season (Table 1). Cumulative precipitation in autumn and spring sowing season from sowing to sampling time was 750.5 and 88.5 mm, and average temperature was 11.5°C and 15.9°C, respectively. Accumulated temperature was 1945.1°C and 494.1°C, and sunshine duration was 1022.6 and 234.3 hour, respectively.

In this study, AMF root colonization in each cover crop was unchanged among crop types and sowing season (Figure 1). Significant differences were observed only among crop type. In contrast, the number of DGGE bands or band patterns were different among crop types in the autumn sowing (Figure 2 and Figure 3), but not in the spring sowing. In autumn mix-cropping, the number of DGGE bands was highest in barley and lowest in hairy vetch, although this was not the case in spring mix-cropping. The diversity index (H') (number of DGGE bands) showed similar trends across all the treatments. The results of two-way ANOVA showed that crop type, but not sowing season, affected the number of DGGE bands. However, the diversity index was not influenced by crop type and sowing season.

Table 1. Climate conditions from sowing to sampling in this study.

Treatment	Cumulative precipitation (mm)	Average temperature (°C)	Accumulated temperature (°C)	Sunshine duration (h)
Autumn sowing	750.5	11.5	1945.1	1022.6
Spring sowing	88.5	15.9	494.1	234.3

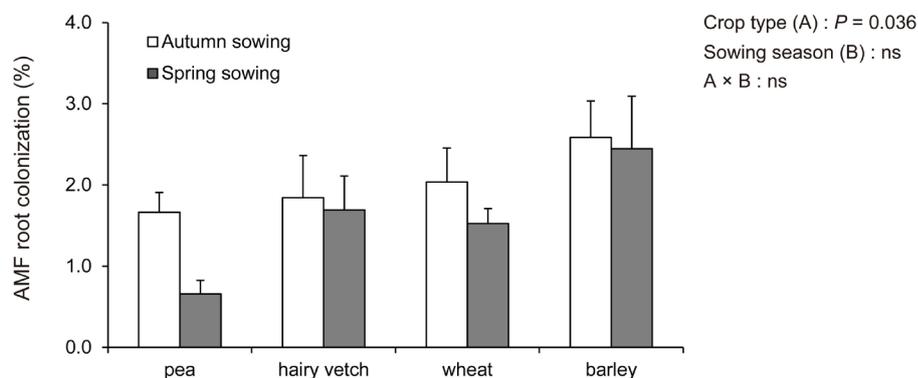


Figure 1. AMF root colonization in the four different crops sown in autumn and spring season. Vertical bars represent the mean (\pm S.E.).

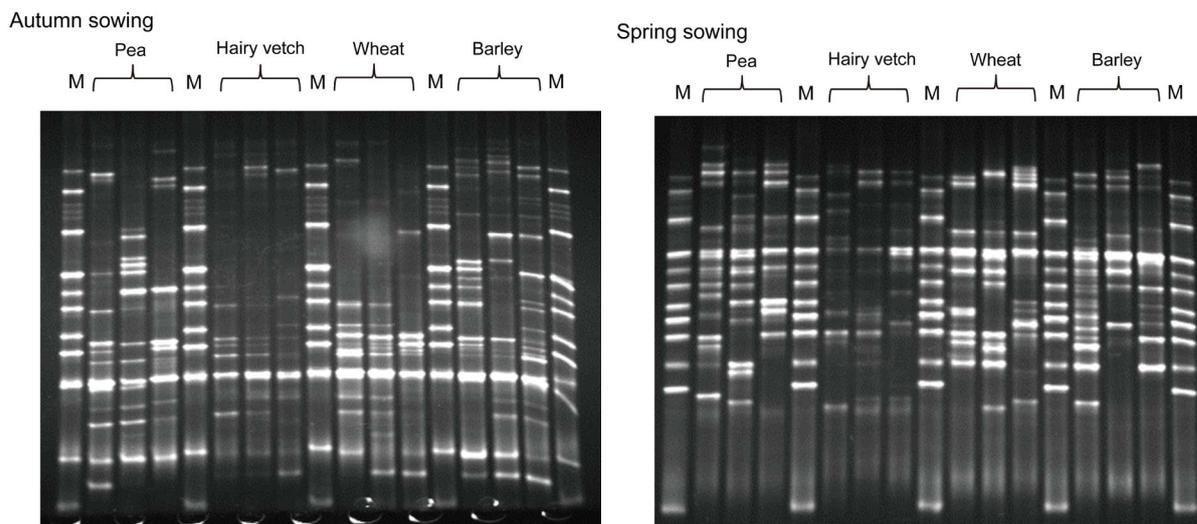


Figure 2. PCR-DGGE band patterns of AMF communities in the roots of four different crops sown in autumn and spring season. M: marker.

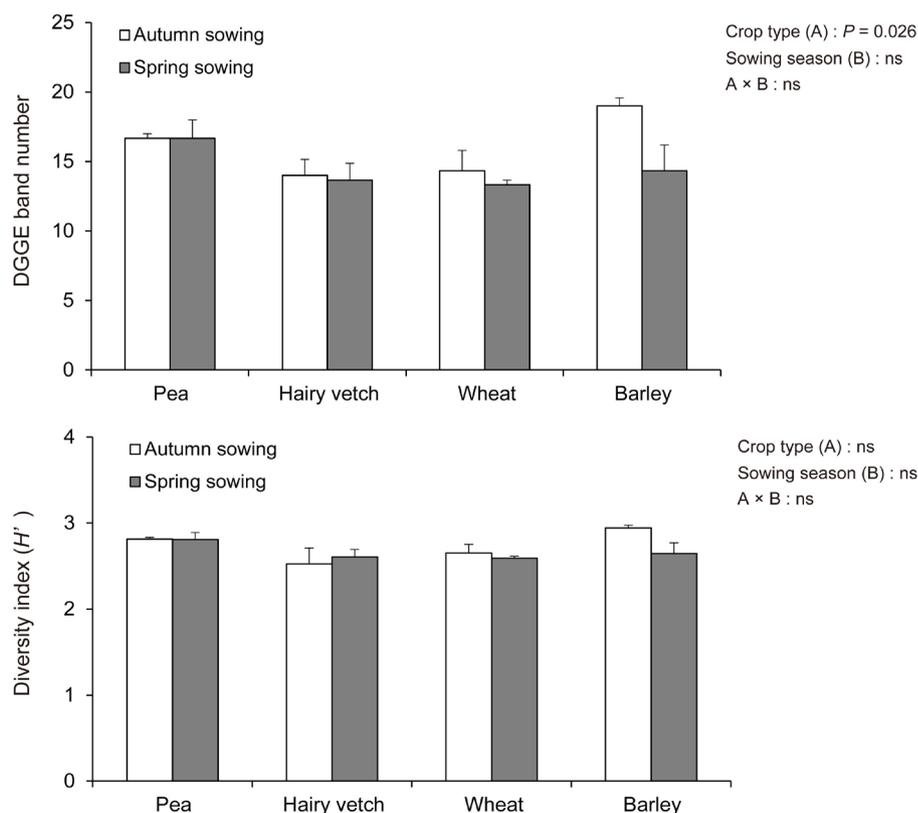


Figure 3. Molecular diversity of AMF communities in the roots of four different crops sown in autumn and spring season. Vertical bars represent the mean (\pm S.E). ns: not significant.

RDA was used to identify relationships between AMF communities in the cover crop roots and crop type (Figure 4) and sowing season (Figure 5). A Monte-Carlo permutation test indicated that the relationship between AMF communities in the cover crop roots and crop type was significantly influenced by autumn sowing ($F = 2.247$, $P = 0.001$), but not spring sowing ($F = 1.087$, $P = 0.287$). Pea (autumn sowing: $R^2 = 0.925$, $P = 0.009$, spring sowing: $R^2 = 0.690$, $P = 0.008$) and hairy vetch (autumn sowing: $R^2 = 0.940$, $P = 0.006$, spring

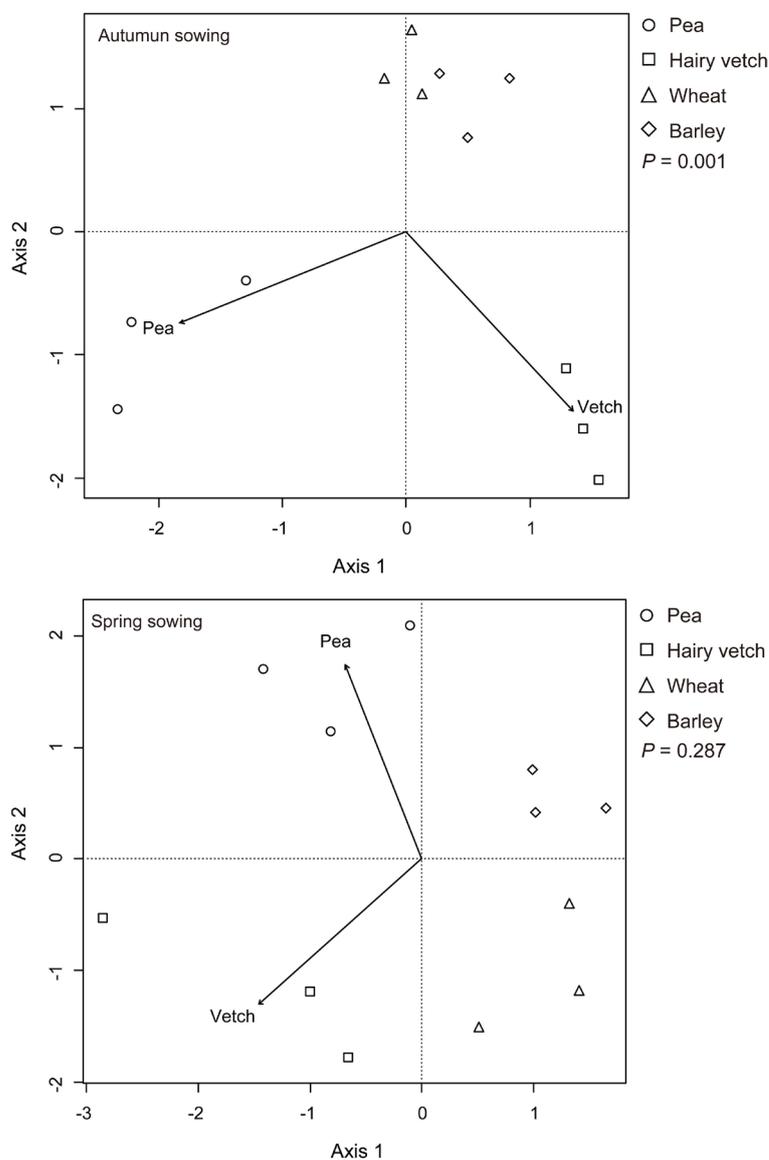


Figure 4. Redundancy analysis (RDA) biplot showing relationship between detected DGGE bands and crop type. In autumn sowing, the eigenvalues of the first and second axes were 7.230 and 3.845, respectively. In spring sowing, the eigenvalues of the first and second axes were 3.390 and 2.707, respectively. The only significant variables ($P < 0.05$) such as pea and hairy vetch were shown in this figure.

sowing: $R^2 = 0.757$, $P = 0.008$) contributed significantly to the variation in AMF communities in cover crop roots under both sowing seasons (Figure 4). Also, RDA was used to identify relationships between AMF communities in cover crop roots and sowing season (Figure 5). A Monte-Carlo permutation test indicated that the relationship between AMF communities in cover crop roots and sowing season was highly significant ($F = 4.570$, $P = 0.001$).

5. Discussion

In this study we examined the differences in root AMF communities of four different cover crops, *i.e.*, pea, hairy vetch, wheat, and barley, grown in two different sowing seasons (autumn and spring). Our results indicated that host identity clearly drove the specific difference in root AMF communities of the four crops when they were autumn-sown (Figure 3), and this is in agreement with previous studies [11] [23]. There is mounting evidence

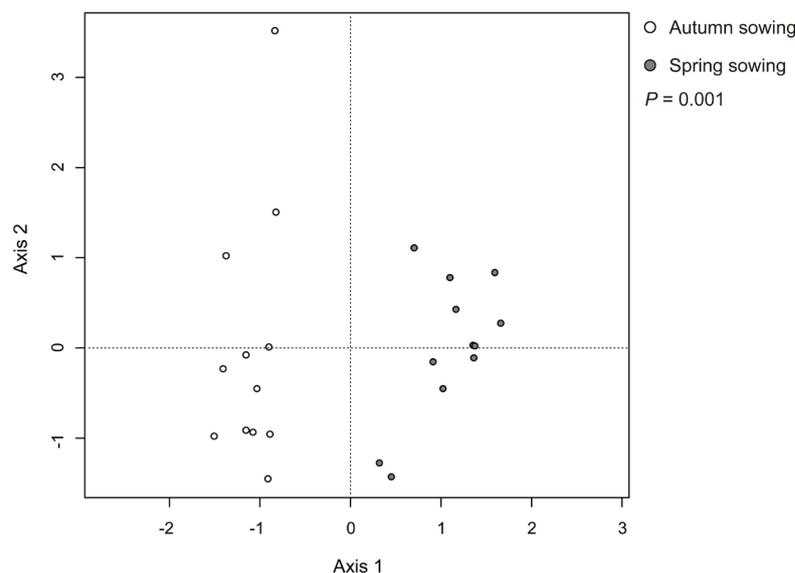


Figure 5. Redundancy analysis (RDA) biplot showing relationship between detected DGGE bands and sowing season. The eigenvalues of the first and second axes were 6.020 and 4.098, respectively.

that host selectivity exists among plants and AMF [23] [35] and our results add to that body of evidence. The main reason for the difference in root AMF communities in each cover crop appears to some extent to be related to host selectivity or host preference between the cover crops and indigenous AMF in our field soil. Host identity did not affect the AMF communities when the crops were spring-sown, and the reasons for this are not clear. This consistency in AMF communities colonizing the roots of our four spring-sown crops was somewhat unexpected. In previous studies, Broeckling *et al.* [36] have shown that plant root exudates drove the shift of soil microbial communities, with the difference in the AMF communities influenced by specific root exudate patterns of each cover crop. We hypothesize that one reason for the consistency in AMF communities among the spring-sown cover crops may be weakened effects of specific root exudate patterns on AMF communities due to the early growth stage. Further research would be needed to better understand whether crop growth stage, time of year, or other abiotic environmental factors strongly influence AMF diversity or community structure in winter cover crop rotational systems.

For autumn-sown crops, our results demonstrated that sowing season clearly drove the specific difference in the root AMF communities of pea, hairy vetch, wheat and barley (Figure 4). It has been shown that the dominant AMF population is likely to shift as the environment changes over time [13] [25] [37]. Dumbrell *et al.* [38] have reported presence of significant differences in AMF communities and diversity between cooler and warmer periods of the year in temperate environments. Veresoglou *et al.* [39] have also indicated that precipitation was a key determinant that affected the presence or absence of *Gigasporales*. Furthermore, Higo *et al.* [13] have shown that the diversity of AMF communities in a 5-year soybean rotational system with winter cover crops was negatively correlated with cumulative precipitation, or positively correlated with maximum or mean temperature during the growing period. Moreover, AMF communities colonizing soybean roots were clearly influenced by rotation year, suggesting that climate or other environmental factors were more important than winter cropping system management. Therefore, we would expect that an extended time course trial might have elucidated the determining factors governing the relationships between host identity and AMF community structure and diversity.

Furthermore, host preference to AMF in leguminous crops can be higher than that in gramineous crops, because gramineous crops have the potential to take up large amounts of P from soil because of their higher root volume and root hair development [40] [41]. Root volume and hair length is much less in leguminous crops compared with that in gramineous crops so they are highly dependent on symbiosis with AMF to take up large amounts of P from soil [42] [43]. In addition, Scheublin *et al.* [44] noted that the preferential association of different AMF species with legumes compared with non-leguminous plants was different. The structure and function of the root system are also expected to influence how plants respond to colonization by AMF [1]. For

these results, one predictable reason for the difference in the AMF communities of the four crop roots might be the root system architecture and the extent of root growth development among the four crops.

6. Conclusion

In conclusion, our study has shown that host crop identity impacts AMF communities only in roots of the four autumn-sown cover crops. Host identity has traditionally been considered as a strong factor in shaping AMF communities in plant roots [11] [21] [22]. Our results suggest that host crop identity may not be such a strong factor in determining AMF communities in roots of cover crops, and that abiotic environmental factors such as climate, growing season, geographical region, or agricultural management may be more important. Also, crop biomass may mediate AMF communities in soil and roots. A clear understanding of the function of AMF to P nutrition in cover crop rotational systems would be needed to investigate how we currently manage these systems, particularly crop rotation decisions.

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