Title: Can phosphorus application and cover cropping alter arbuscular mycorrhizal fungal communities and soybean performance in a 5-year phosphorus-unfertilized crop rotational system?

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Abstract

Background: Phosphorus (P) application and mycorrhizal cover cropping can be a primary factor improving soybean growth, P nutrition, and grain yield, and the benefit from arbuscular mycorrhizal fungi (AMF) is highly valuable to soybean P uptake and growth in a P-limited soil. However, it is not clear how soybean growth and P nutrition is related to AMF root colonization and diversity of AMF communities in a continuous P-unfertilized cover cropping system. Thus, we investigated the impact of P-application and cover cropping on AMF root colonization and diversity in soybean roots, and how they affect soybean growth and P nutrition under the five-year P-unfertilized crop rotation.

Methods: In this study, we established three cover crop systems (wheat, red clover, and oilseed rape) or bare fallow in rotation with soybean. The P fertilizer application rates before the seeding of soybeans were 52.5 and 157.5 kg ha^{-1} in 2014 and 2015, respectively. AMF community structure in soybean roots was characterized by specific amplification of small subunit (SSU) rDNA.

Results: The increase in the root colonization at the flowering stage was small as a result of P-application, even when P-application significantly impacted on root colonization. The P-application had positive effects on the soybean performance such as plant P uptake,

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biomass and grain yield in 2015. The results of a permutational multivariate analysis of variance (PERMANOVA) showed that the AMF communities colonizing soybean roots were also significantly influenced by P-application throughout the two years. Moreover, the abundance of *Rhizophagus irregularis* and *Cetraspora pellucida* in the roots reduced as a result of P-application in 2015. The network analysis determined that the AMF root colonization did not increase the soybean growth performance, whereas the diversity index and species richness of AMF communities in roots were positively correlated with the soybean biomass, P uptake and grain yield throughout the two years.

Discussion: Our results indicated that the soybean performance could be partially related to the interaction directly with roots-soil microbes such as AMF diversity. Increasing AMF diversity may be a key factor improving soybean growth performance in P-limited cover cropping systems. P management may also be integrated with cover cropping to ensure the improvement of soybean performance and benefit from AMF partners in cover crop rotational systems.

Introduction

Phosphorus (P) is an essential nutrient with a central role in numerous biochemical processes of plants. P in synthetic fertilizers is derived from phosphate rock which is a finite resource, and their prices are expected to increase in the following decades (Brunelle et al., 2015). However, P-application is often required to achieve high productivity due to strong interactions of P with soil compounds, for example adsorption to and precipitation with iron (Fe) and aluminium (Al) (Lynch, 2007). After excessive application of P fertilizers to soil, the most of the P can be converted into less available forms over time (Pearse et al., 2007). Increasing soil P concentrations will not result in greater crop yields, lead to less active microbial-mediated processes of mineralization and solubilization, and increase the potential risk of environmental pollution (Bai et al., 2013). The global average cash production costs of phosphate rock in 1983 and 2013 increased by 27% to $38 per fob tonne mine in this 30 year period (Mew, 2016). Therefore, managing soil P availability is required to maintain agricultural crop production (Mishima et al., 2003). Arbuscular mycorrhizal fungi (AMF) can increase host plant P uptake and growth, and AMF may

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especially improve plant P and micronutrients uptake (Smith & Read, 2008). AMF also may act against the depletion of global P reserves (Gilbert, 2000). These nutritional benefits from AMF can be remarkably improved via appropriate agricultural managements (Kahiluoto et al., 2001, 2012; Gosling et al., 2006). Some species of the family Glomeraceae such as *Funneliformis mosseae*, *Rhizophagus irregularis* and family Gigasporaceae have been shown to have a positive impact on growth and nutrient uptake of plants under native and commercial AMF inoculated conditions (Verbruggen & Kiers, 2010; Gosling et al., 2016). Also, previous studies have shown that P uptake via AMF is a distinct functional alternative to direct uptake by plants (Bucher, 2007), and the most of the P supplied by plants can be obtained via the mycorrhizal route (Smith et al., 2003).

Johnson et al. (1993) reported that there was a link between yield declines under continuous soybean cropping and the shift in AMF communities. Continuous cropping selects for the most rapidly growing and sporulating AMF species, which decreases crop performance over time. This abundance of detrimental AMF species leads to a decline in beneficial AMF species (Johnson et al., 1993). Furthermore, the introduction of mycorrhizal cover crops during the winter season can be necessary for maintenance and increase indigenous AMF inoculum or diversity in soil and roots for subsequent crops (Higo et al., 2010, 2015a, 2016). Thus, the introduction of cover crops in temperate agricultural ecosystems, such as wheat, barley, oilseed rape or leguminous crops, including hairy vetch, red clover and white clover, reduces seasonal fallow and thus provides many benefits for subsequent crops and soil fertility (Karasawa & Takahashi, 2015). In addition, a diverse AMF species composition and diversity can maximize the benefits from AMF (Maherali & Klironomos, 2007; Powell et al., 2009). Moreover, increasing AMF diversity in agroecosystems has been suggested to have the ability to boost crop growth, nutrient uptake and sustainability can be widespread (Hart & Forsythe, 2012). The diversity of AMF communities can be influenced by agricultural management practices such as crop rotation (Higo et al., 2013, 2015a), tillage (Alguacil et al., 2008) and P-application (Kahiluoto et al., 2009, 2012). The diversity of AMF communities can impact their contribution to plant P nutrition (van der Heijden et al., 1998; Verbruggen et al., 2013).

The yield and growth of soybeans under a P-unfertilized four-year winter

crop-soybean rotational system gradually has been found to decrease over time because of both a decrease in AMF root colonization of soybeans and continuous nutrient removal from the soil by continuous crop rotations (Isobe et al., 2014). The same research group also found that there was a positive correlation between AMF root colonization and soybean grain yield in a four-year consecutive winter cover crop-soybean rotational system without P fertilizer, suggesting that higher AMF root colonization can be a better solution for improving soybean growth and grain yield in the P-limited soil. Cover cropping alone would also appear not to supply enough P nutrition to recover soybean performance as much as the use of an alternative way of using moderate P-application in the consecutive P-unfertilized cover crop rotational system (Karasawa & Takahashi, 2015).

Thus, we investigate which factor such as P-application or cover cropping is driving increases in soybean performance via AMF benefits. We also examine how the P-application and cover cropping link to the AMF benefits and soybean growth, and the effectiveness of AMF in cover crop-soybean rotational systems to improve the reliability and the robustness of the agricultural managements. In this study, we hypothesized that P-application or non-mycorrhizal cover cropping in a P-limited soil would decrease the diversity of AMF communities and the shift would link to the soybean growth responses in the five-year P-unfertilized cover crop study. Therefore, we approached this study with two objectives: First- to understand whether or not P-application and cover cropping impacts soybean growth performance. Secondly, to determine how AMF diversity is affected under P-application and cover cropping.

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Materials and methods

Experimental design

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We conducted a field trial of winter cover crop-soybean rotation at Nihon University, in Kanagawa, Japan (35˚22'N 139˚27'E). The soil at the field site is classified as a volcanic ash soil (Allophonic andosol). According to the Japan Meteorological Agency (http://www.jma.go.jp/jma/indexe.html) from 2000 to 2015, the climate is characterized by relatively high temperatures and evenly distributed precipitation throughout the year. The average temperature for the year in this area is around 16.2°C. The average maximum

temperature and average minimum temperature is around 25.1°C and 7.7°C, respectively. The average precipitation for the year in this prefecture is around 1609.7 mm.

We conducted our research onto two experimental phases. In the first phase, we applied cover cropping/soybean rotations without P-application, then, in the second phase, we applied the same cover crop treatment/soybean with or without P-application, but used a split plot design. The first cover crop experiment (2007 to 2012) comprised three winter cover crop treatments such as winter wheat (*Triticum aestivum* L.), red clover (*Trifolium pratense* L.), oilseed rape (*Brassica napus* L.) and fallow (Fig. 1). There were three replicate plots per treatment arranged in a randomized complete block design. Each plot had an area of 9 m^2 (4.5 m × 2 m). In this first phase, the experimental did not receive P fertilizers for over a 5-year period. In the field plots used for the experiments, soybean (*Glycine max* (L.) Merr., cv: Enrei) had been cultivated to standardize soil biochemical conditions before the field trial started. As a preliminary investigation of soil chemical characteristics (0-15 cm soil depth) at this experimental site in 2014 before the study of phase two (Fig. 1), the soil pH ranged from 6.0 to 6.1 and total organic carbon (C) was 5.6 to 6.5%. Total nitrogen (N) and nitrate nitrogen content ranged from 0.41 to 0.48% and from 6.0 to 15.9 mg kg^{-1}, respectively. Phosphate absorption coefficient ranged from 2320 to 2660. Further management details about the general information of the cover crop rotational system, seeding and sampling are presented in Higo et al. (2014).

In phase two of our experiment, the same three cover crops from the first experimental phase (wheat, red clover and oilseed rape) were sown in rows, with spacing of 30 cm, in the cropped treatment on November 9, 2013, and November 18, 2014. Winter wheat (cv: Bandowase, mycorrhizal crop) seeds were sown at 200 kg ha^{-1} with N (ammonium sulfate) and K (potassium chloride) application rates of 100 and 90 kg ha^{-1}, respectively. Oilseed rape seeds (cv: Michinokunatane, non-mycorrhizal crop) were sown at 30 kg ha^{-1} with N and K application rates of 100 and 50 kg ha^{-1}, respectively. Red clover seeds (cv: Makimidori, mycorrhizal crop) were sown on November 9, 2013, and March 16, 2015. Red clover seeds were sown at 30 kg ha^{-1} with N and K application rates of 30 and 50 kg ha^{-1} in 2014 and 2015. The tops of the cover crops were cut close to the ground and removed on June 3, 2014, and June 16, 2015. In fallow, weeds were manually removed during the

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154 winter period.

155 We investigate the impact of P-application and cover cropping on the diversity of
156 AMF communities in soybean roots and soybean growth after a five-year consecutive cover
157 crop-soybean rotational system. We used a split plot design to divide the 4.5 × 2 m of the
158 cover crop experimental plots into 2.25 × 2 m plots for the two P treatment plots (no
159 P-application and P-application) of the phase two experiment (Fig. 1). Then, both no P and
160 P-application treatments were replicated three times in 2.25 × 2 m plots. The soybean (cv:
161 Enrei) seeds were sown at a spacing of 60 cm × 15 cm on June 17, 2014, and June 17, 2015.
162 In 2014 and 2015, the N and K application rates were 30 and 50 kg ha^{-1}, respectively. In
163 2014, the amount of P (triple superphosphate) in the P-application plots was applied at 52.5
164 kg ha^{-1}. The P-application did not increase the available soil P in 2014 because of the high
165 P absorption coefficient (around 2600). In 2015, the amount of P in the P-application plots
166 was applied at 157.5 kg ha^{-1} at three times the normal amount of P fertilizer of 2014. The
167 content of available soil P (Truog P) was analyzed according to Truog (1930).
168
169 **Soil and root sampling and root staining**
170 The soil samples were randomly taken from ten points in each replicate and pooled to one
171 composite sample on June 17, 2014, and June 17, 2015, respectively. Soybean root samples
172 were taken at the full bloom stage (R2 growth stage) on July 31, 2014, and August 6, 2015.
173 The full bloom stage corresponds to the stage when the mycorrhizal colonization of
174 soybean roots is usually at its highest (Zhang et al., 1995). In each rotation, the root
175 samples were randomly collected from ten plants (to a depth of 15 cm, the diameter of 20
176 cm) per replicate. The root samples were collected from the soil sample and maintained at
177 −80°C for DNA extraction and measurement of AMF root colonization. The root samples
178 were stained with a 5% (w/v) black ink-vinegar solution (Vierheilig et al., 1998), and the
179 AMF root colonization in the soybean roots was measured as described by Giovannetti &
180 Mosse (1980).
181
182 **Analysis of plant P and measurement of soybean grain yield**
183 The aboveground plant parts of the ten soybean plants were cut close to the ground at the

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Comment [15]: So we should not be reading the data from the perspective that 2015 results are a repeated study, correct? Why is it that the P-application was increased to 3X of 2014 P-fertilizer rates? It can be a little misleading to the reader if this is not stated in your Figure descriptions, because otherwise one may assume that the 2015 experiments are just a repeat. But seeing this explains the differences between the two years. Did you test the level of P in the "no fertilization" plots? I am trying to figure out why your 2015 control plants are so much larger than 2014 plants. Also, did you happen to measure equivalent developmental stages? If so, state the V-stage of 2014 and 2015 plants, and include that in your figure legends. Should a 3X increase in fertilizer application then be considered a Phosphorous regime?

full bloom stage and were randomly sampled on July 31, 2014, and August 6, 2015. To obtain the soybean grain yield, ten soybean samples per plot in each treatment were collected at maturity stage in early to late October in each year. The aboveground soybean plant biomass and plant length were measured in all plots. The aboveground plant biomass and P uptake by soybeans were determined after the samples were oven dried at 80°C for 48 h. The P uptake was determined using the molybdenum yellow colorimetric method (Koenig & Johnson 1942).

DNA extraction and nested polymerase chain reaction (PCR)

Total genomic DNA was extracted from 150 mg of fresh root samples using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The genomic DNA pellet was stored at −30°C until use in the nested PCR. The fragments in the fungal small subunit ribosomal DNA (SSU rDNA) was amplified using nested PCR method (Liang et al., 2008). The universal eukaryotic primer NS31 (forward) (5'-TTGGAGGGCAAGTCTGGTGCC-3') (Simon et al. 1992) and the fungus-specific primer AM1 (reverse) (5'-GTTTCCCGTAAGGCGCCGAA-3') (Helgason et al. 1998) were used in the first PCR to amplify the 5' end of the SSU rDNA region for comprehensive taxon sampling for the Glomeromycota (Schüßler et al., 2001a,b). Three subsamples per plot were amplified in a 20-µl reaction mixture containing 2 µl of 10-fold genomic DNA (around 1 to 5 ng/µl), 0.2 µM of each primer and 2 × GoTaq Green Master Mix (Promega, Madison, WI, USA) using a Mastercycler ep Gradient (Eppendorf, Hamburg, Germany). The PCR condition was composed of initial treatment at 94°C for 1 min; 30 cycles at 94°C for 1 min, 66°C for 1 min and 72°C for 90 s; and a final extension at 72°C for 10 min. The first PCR products were diluted 10-fold and used as templates for the second PCR using the nested primers NS31-GC (forward) (5'-CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGTTGGAGGGCAAGTCTGGTGCC-3') (Kowalchuk et al., 2002) and Glo1 (reverse) (5'-GCCTGCTTTAAACACTCTA-3') (Cornejo et al., 2004). Three subsamples per plot were amplified in a 20-µl reaction mixture containing 2 µl of 10-fold 1st PCR amplicons, 0.2 µM of each primer and 2 × GoTaq Green Master Mix (Promega, Madison, WI, USA)

using a Mastercycler ep Gradient (Eppendorf). The PCR protocol was composed of initial treatment at 95°C for 5 min; 35 cycles at 94°C for 45 s, 52°C for 45 s and 72°C for 1 min; and a final extension at 72°C for 30 min. Gel electrophoresis separated amplification products on 1% agarose gel, and the DNA amplicons was visualized by staining with ethidium bromide.

PCR-denaturing gradient gel electrophoresis (DGGE)

Three independent PCR products were pooled together, and then 20 μL of the nested PCR product was subsequently analyzed by DGGE on a DCode Universal Mutation Detection System (Bio-Rad Laboratories, Piscataway, NJ, USA). Standard DNA markers were created by individually PCR-amplifying DNA extracted from root samples by Higo et al. (2015b). The PCR-DGGE condition was based on the method of Higo et al. (2015b). The gels containing 6.5% acrylamide were poured with a gradient of 35–55% denaturant. All DGGE analyses were performed in a 1 × TAE buffer at a constant temperature of 55°C at 50 V for 60 min, followed by 50 V for 960 min. The gels were stained with SYBR Green diluted in 1 × TAE buffer (1:10,000) for 20 min, UV illuminated and digitally photographed (Figs. S1 and S2). Pictures were digitized by Phoretix 1D Pro (Nonlinear Dynamics Ltd., Newcastle upon Tyne, UK). We calculated species richness from these data, expressed by the number of DGGE bands in each root sample. Fromin et al. (2002) and Schneider et al. (2015) mentioned that visual observation of the DGGE gel revealed the presence of multiple bands in all samples (a band represents a distinct taxon in theory). The Shannon *H'* of each treatment was also calculated as an additional measure of AMF diversity.

Quantification of specific root AMF taxa using a quantitative real-time PCR (qPCR)

The abundance of six-selected typical AMF taxa was measured using qPCR with taxon-specific primers and hydrolysis (TaqMan) probes targeting large ribosomal subunit DNA (LSU rDNA) genes. The specific primers were designed to quantify the abundance of six-selected AMF taxa including *Rhizophagus irregularis*, *Funneliformis mosseae*, *Claroideoglomus claroideum*, *Gigaspora margarita*, *Cetraspora pellucida* and

Diversispora celata in pot experiments and different type of field conditions such as soil quality and agricultural managements (Wagg et al., 2011; Thonar et al., 2012; Jansa et al., 2014). The partial LSU rDNA genes of *R. irregularis*, *F. mosseae*, *C. claroideum*, *G. margarita* and *Ce. pellucida* followed the method described by Thonar et al. (2012). We also used the method described by Wagg et al. (2011) to quantify *Diversispora celata*. Each PCR sample contained a total volume of 10 µL that consisted of 2 µL water, 400 nM each of forward primer and reverse primer, 100 nM TaqMan probe and 2 × FastStart TaqMan Probe Master Mix + 2 µL of 10-diluted genomic DNA. The qPCR was carried out using a LightCycler 96 (Roche Diagnostics, Rotkreuz, Switzerland). The qPCR cycling conditions were as follows: initial denaturation at 95°C for 15 min, followed by 45 cycles with denaturation at 95°C for 10 s and annealing at the optimized temperature for each primer/probe combination for 30 s and elongation at 72°C for 1 s.

Statistical analysis

We used an arcsine-square root transformation to normalize the data of AMF root colonization in the soybean. The available soil P, growth parameters and AMF diversity data were transformed using a natural logarithm. The abundance of AMF tax was log(x+1) transformed to reduce heteroscedasticity in the data. Differences between means where analysis of variance (ANOVA) was significant were assessed using Tukey's honestly significant difference (HSD) test (P-values < 0.05) using the *multcomp* package in R 3.3.2 (https://cran.r-project.org/). Generalized linear model (GLM) was used to determine the effects of P-application and cover crop and their interactions on each parameter in this study of split plot design in R 3.3.2. Data for the significance of differences between P-application treatments among cover crop systems were assessed using Student's t-test.

A permutational multivariate analysis of variance (PERMANOVA) was performed using the vegan package in R to investigate the effect of P-application and cover crop systems on AMF community structure (Hammer et al., 2001). To analyze the relationship of cover cropping and P-application with respect to AMF community structures (AMF communities), the redundancy analysis (RDA) (gradient length <4) was performed as the multivariate analysis using the *vegan* package in R 3.3.2. The presence/absence data matrix

was composed of the abundance of DGGE bands and cover crop management or P-application. The environmental variable of cover cropping and P-application was coded as a dummy variable (0 and 1). Goodness-of-fit statistics (R^2) of measured factors fitted to the RDA ordination of the AMF community were calculated using the envfit function in the *vegan* package with *P*-values based on 999 permutations (Oksanen, 2017). To investigate if AMF community structure differed significantly between P-application or cover crop management, the PERMANOVA was performed with 999 permutations using the adonis function in the *vegan* package in R.

The network graph included the correlation coefficients between soybean growth performance and AMF parameters using the *igraph* package in R, and then the network graph was described using Cytoscape for visualizing complex networks (www.cytoscape.org/). In this model, the AMF taxa abundance was represented by the scores of the first component of the PCA in this study. Pearson's correlation coefficient (*r*) was expressed as the indication of the strength of the connections.

Results

Available soil P and AMF root colonization

In this study, the P-application in 2014 did not change the available soil P regardless of cover crop systems, however the P-application significantly increased the available soil P in all of the cover crop systems compared with no P-application plots (Fig. 2A).

Overall, the AMF root colonization in the soybean regardless of P-application and cover crop systems was never greater than 20% (Fig 2B). In the no P-application plots, cover cropping affected the AMF root colonization at the full bloom stage in 2014 and 2015 (Fig. 2B). Contrary to the results of the no P-application plots, the AMF root colonization in the red clover ($P < 0.01$) or oilseed rape ($P < 0.05$), and fallow ($P < 0.05$) as a result of the P-application significantly increased compared to the no P-application except for the wheat in 2015.

Plant growth, P uptake and grain yield

The aboveground plant biomass in soybeans at the full bloom stage varied among cover

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crop systems in the field trial (Fig. 3A). In the 2015, but not 2014, P-application plots, the aboveground plant biomass of soybeans was more than double than those of the no P-application plots. The aboveground biomass for the P- and no P-application plots were significantly different for red clover (2015), oilseed rape (2014 and 2015), and fallow (2014 and 2015).

Our results as shown in Fig. 3B revealed that cover cropping did not have a significant effect on the plant P uptake of soybeans regardless of the P-application plots according to Tukey's test and GLM for either year. However, the plant P uptake in soybeans was significantly influenced by the P-application according to GLM in 2014 and 2015. Moreover, there was a significant difference in the plant P uptake between the P- and no P-application plots for fallow in 2014 and 2015.

We found that the grain yield in soybeans was influenced by the cover crop systems according to GLM only in 2014, whereas the P-application had a significant effect on the grain yield of soybean in both 2014 and 2015 (Fig. 3C). The soybean grain yields at the P-application plots in the experiment were more than double in both 2014 and 2015. We also found that there were significant differences in the grain yield between the P- and no P-application plots for red clover (2014 and 2015) and oilseed rape (2015) managements.

Diversity of AMF communities and taxa abundance

The species richness and the diversity index (*H'*) in soybeans at the full bloom stage were significantly influenced by P-application in both 2014 and 2015 according to GLM (Fig. 4A and B). Additionally, the species richness and *H'* in soybeans were significantly influenced by cover crop management according to GLM (2014 and 2015). The *H'* for the P- and no P-application plots were significantly different for wheat (2014 and 2015), red clover (2014) and fallow (2015). Likewise, there were significant differences in the species richness between the P- and no P-application plots for wheat (2014 and 2015), red clover (2014 and 2015) and fallow (2015). A similar tendency in the diversity of AMF communities with regard to P-application and cover cropping was observed between 2014 and 2015.

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Comment [20]: Even the controls for 2015 are much larger than the 2014 plants. Were plants measured at equivalent developmental times? Or would you speculate that the height differences in 2015 would be due to larger amounts of residual P and N in the soil from the previous year?

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Comment [21]: Why use these two measures of species diversity? Please clarify this. How does this enrich the readers understanding? They seem to be showing the same trends and interactions. Pick one, its otherwise seemingly redundant without knowing why you decided to report these two measures.

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Abundance of six-selected AMF taxa in the roots of soybean

Our results showed that the six-selected AMF taxa were not influenced by cover cropping in 2014 (Table 1). However, the P-application had a significant effect on the abundance of all six-selected AMF taxa in 2014. The abundance of five AMF taxa in the P-application plots except for *Gigaspora margarita* tended to be higher than those of the no P-application plots regardless of the cover crop systems.

In 2015, the abundance of *R. irregularis* and *Cetraspora pellucida* in the P-application plots significantly decreased compared with that in the no P-application plots for wheat, red clover, and oilseed rape. Additionally, the abundance of *R.irregularis* and *C.pellucida* was influenced by P-application according to GLM. Also, the abundance of *C.pellucida* was influenced by cover cropping according to GLM. However, the abundance of other AMF taxa (*Claroideoglomus claroideum*, *Funneliformis mosseae,* and *Diversispora celata*) was not affected by P-application and cover cropping according to GLM. No similar tendency in the abundance of the six-selected AMF taxa with regard to P-application and cover cropping was observed between 2014 and 2015.

Relationships among AMF communities, cover cropping, and P-application

We used an RDA to identify the relationships among AMF communities in soybean roots with cover crop management and P-application (Fig 5). In 2014 and 2015, the RDA trends clearly showed that the P-application noticeably altered the AMF community structure in the soybean roots. In 2014 as shown in Fig. 5, the AMF communities in the no P-application plots were in the second (wheat and red clover) and third (oilseed rape and fallow) quadrants, while most of the AMF communities in the P-application plots were in the first (wheat, red clover and fallow) and fourth (oilseed rape) quadrants. In the same year, the ordination diagram indicates that oilseed rape (R^2 = 0.756, P = 0.001) contributed significantly to the variation in AMF root communities (Fig. 5). However, wheat (R^2 = 0.095, P = 0.349), red clover (R^2 = 0.138, P = 0.191) and fallow (R^2 = 0.040, P = 0.630) did not contribute to the variation in the AMF root communities. Additionally, the P-application treatment (R^2 = 0.801, P = 0.001) and no P-application treatment (R^2 = 0.801, P = 0.001) contributed to the variation in the AMF root communities. In 2015, the AMF

communities in the no P-application plots were in the first (red clover) and fourth (wheat, oilseed rape and fallow) quadrants, while most of the AMF communities in the P-application plots were in the second (red clover, some of wheat, oilseed and fallow) and third (most of wheat, oilseed and fallow) quadrants. The ordination diagram indicates that red clover (R^2 = 0.704, P = 0.001) contributed significantly to the variation in the AMF root communities (Fig 5). However, wheat (R^2 = 0.154, P = 0.181), oilseed rape (R^2 = 0.129, P = 0.255) and fallow (R^2 = 0.173, P = 0.141) did not contribute to the variation in the AMF root communities. Furthermore, the P-application treatment (R^2 = 0.743, P = 0.001) and no P-application treatment (R^2 = 0.743, P = 0.001) contributed to the variation in the AMF root communities. A PERMANOVA was also carried out to examine the relative importance of each agricultural management to the AMF root communities. The PERMANOVA showed that P-application significantly affected the AMF root community structure (2014: F = 4.263, P = 0.001, 2015: F = 4.226, P = 0.001), but cover crop management did not impact the AMF root communities (2014: F = 1.193, P = 0.189, 2015: F = 1.669, P = 0.057).

Response of soybean growth to AMF parameters

In the soybean growth response, the relationships between available soil P and soybean growth performance was not linear in the cropping system with no P-application (Fig. 6A-C). The difference in the soybean growth performance was small with no P-application. The relationships between available soil P and soybean growth performance such as plant biomass (r = 0.874), plant P uptake (r = 0.821) and grain yield (r = 0.801) was significantly linear in the cropping system with P-application. With the AMF contributions to soybean growth performance, the relationships between AMF root colonization and soybean growth was not linear in the cropping system with and without P-application (Fig. 6D-F). The P-application significantly improved the linear relationships between the diversity index or AMF species abundance and soybean growth performance. The relationships between the diversity index and soybean growth performance such as plant biomass (r = 0.969), plant P uptake (r = 0.973), grain yield (r = 0.920) was positively correlated in the cropping system with P-application (Fig. 6G-I). The relationships between AMF species abundance and

soybean growth performance such as plant biomass (r = 0.967), plant P uptake (r = 0.967) and grain yield (r = 0.928) was positively correlated in the cropping system with P-application (Fig. 6J-L).

We also used a network analysis to identify the relationships between AMF parameters in soybean roots and soybean growth in this study (Fig. 7). The results showed the same tendency with the linear analysis in the two-year experiment. The relationships between the diversity index or AMF species richness and available soil P were related to the soybean growth performance such as plant P uptake, plant biomass, and grain yield. However, each AMF taxa abundance and AMF root colonization were not related to the soybean growth responses, especially grain yield, throughout the experiment.

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Comment [23]: Why is identifying the relationships important?

Discussion

In the present study, we investigated whether P-application and cover cropping on a P-depleted soil can impact the AMF communities colonizing soybean roots and soybean growth performance. Our results meet the objectives of this study and support the hypotheses examined.

Impact of P-application and cover cropping on root colonization

It is well known that cultivation of preceding crops or fallow as well as P-application impacts AMF root colonization of subsequent crops (Karasawa et al., 2002; Karasawa & Takebe, 2012; Isobe et al., 2014). In this study, the wheat and red clover cropping with no P-application significantly increased AMF root colonization of subsequent soybean, while fallow with no P-application decreased the AMF root colonization in agreement with previous studies (Karasawa et al., 2002; Karasawa & Takebe, 2012; Isobe et al., 2014). However, no differences were observed in AMF root colonization among cover crop systems with P-application (Fig. 2B). In general, AMF root colonization is inhibited under high P-application (Kahiluoto et al., 2001; Balzergue et al., 2011). Also, plants can fail to react to AMF when available soil P is extremely low (Ryan et al., 2002). Miranda & Harris (1994) reported that deficiency of available soil P inhibited AMF root colonization. On the contrary, Gosling et al. (2013) indicated that there was no significant decrease in AMF

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Comment [24]: State the figure this conclusion is coming from.

soybean root colonization under high P availability in soil. Plants can control AMF root colonization depending on their nutritional status (Smith & Read, 2008) as well as under high soil P conditions. Bolan et al. (1984) also reported that a moderate amount of P-application in P-limited soils might increase mycorrhization and benefits such as P availability for crop growth performance. Similarly, AMF root colonization among almost all the cover crop systems in the P-application plots was increased by the P-application. For these results, one possible reason for the result of slightly higher AMF colonization may be that the indigenous AMF population in the field may be responsive to P-application to promote mycorrhization in the five-year P-unfertilized condition.

Impact of P-application and cover cropping on the diversity of root AMF communities

Surprisingly, our results indicated that the diversity of AMF communities in soybeans, regardless of cover crop management, tended to increase as a result of P-application (Fig. 4). Also, the shift of AMF communities were obvious from the results of RDA trends that showed that the P-application significantly changed the AMF community structure in the soybean roots rather than the cover crop systems (Fig. 5). One possible explanation for this result was that the activity of AMF could have been inhibited due to soil P depletion of the P-unfertilized five-year continuous crop rotational system. However, increasing the available soil P by increasing the amount of P fertilizer could somewhat activate hyphal elongation or AMF activity to establish mycorrhization. This could be one reason why P-application increased the AMF diversity of soybean crops. Furthermore, some specific P-unresponsive AMF species that may not be P-responsive could have remained inactive when the available soil P was depleted due to the five-year cover crop rotational system without P fertilizer. Wakelin et al. (2012) and Maček et al. (2011) implied that abiotic selective pressures such as soil fertility determine the AMF community structure. The observed increase in AMF diversity as a result of P-application can be linked to the degree of selective pressure for mycorrhization in soybean roots. Some P-unresponsive taxa may have been dominant in the experimental field under the cover crop rotational system. Increasing the available soil P can decrease the selective pressure, and this could increase

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Comment [25]: Is how you would explain the increased AMF between the two years, being that 2015 had 3X more P than 2014?

the opportunity for P-responsive species to establish soybean roots.

Previous studies have reported that P-application had negative impacts on the diversity of AMF community in roots and soils. Islam et al. (2011) and Lin et al. (2012) found that chemical fertilizers decreased AMF diversity. Moreover, Alguacil et al. (2010) indicated that moderate amounts of P-application could even affect AMF community dynamics. Gosling et al. (2013) also reported that the AMF community diversity in soybean roots decreased due to the high availability of soil P. In addition, plants can directly gain enough nutrient from the soil in a nutrient-rich environment without the benefit from AMF. As a result, the diversity of AMF communities can also decrease (Liu et al., 2015). Likewise, Ryan et al. (2005) suggested that fertilization can change the mycorrhizal symbiosis performance, thereby making soil microbial partners costly and parasitic. Furthermore, we found that cover cropping did not impact the AMF root communities in soybeans from the result of PERMANOVA (Fig. 5). Previous studies reported that cover cropping might not affect AMF root communities in subsequent crops in rotations (Higo et al., 2014; Turrini et al., 2016). Turrini et al. (2016) and Higo et al. (2017) indicated that a shift in indigenous AMF communities in the subsequent maize roots was independent of cover crop identity and diversity. Higo et al. (2014) also found that cover crop rotations did not impact AMF communities in the roots of subsequent soybean. However, rotation year affected the AMF communities in soybean roots suggesting that climate or other environmental conditions were more imperative than cover crop management. Therefore, the P-application may have influences on AMF communities in soybean roots, suggesting that fertilizer application or other factors such as soil chemical properties and other environmental factors can be more important than cover cropping.

Impact of P-application and cover cropping on the abundance of root AMF taxa

We found that the abundance of *R. irregularis* was significantly affected by P-application, which meant P-application might be a stronger determinant than cover cropping that impacts the abundance of this AMF taxa (Table 1). The fluctuation in abundance of AMF taxa as a result of P-application could link to the preference of fertilization or inhabiting soil conditions among AMF in soybean roots. Wakelin et al. (2012) found that *R.*

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Comment [26]: I think that it would be good to get an understanding of how previous studies differ from the current one (i.e. growth chamber, greenhouse field experiments?)

484 *irregularis* decreased as a component of the AMF communities with increasing available
485 soil P, in agreement with our study. Johnson (1993) reported that AMF have different
486 niches and are well known to prefer to inhabit different soils. Moreover, fertilization may
487 directly favor species that grow better in enriched soils (Dumbrell et al., 2010). It is likely
488 that the *R. irregularis* may prefer low-P soil conditions due to the continuous cover crop
489 rotational system.
490
491 **Impact of P-application and cover cropping on the soybean performance**
492 In the results of network analysis and growth response of soybean to AMF parameters, we
493 found that the aboveground plant P and biomass of soybeans during the flowering stage
494 and the grain yield of soybeans were positively correlated with the AMF diversity in the
495 roots of soybeans with increasing of P-application (Figs. 6 and 7). Similarly, Jansa et al.
496 (2008) reported that the growth of *Allium porrum* with three inoculated AMF species
497 (*Funneliformis mosseae*, *Claroideoglomus claroideum,* and *Rhizophagus irregularis*) was
498 enhanced compared to that of *Allium porrum* when each AMF species was
499 mono-inoculated. Gosling et al. (2016) also reported that increased benefit from high AMF
500 diversity on the growth of *Allium cepa* was found compared to mono-inoculated. However
501 increasing the diversity more than three species (*C. caledonium*, *F. mosseae,* and *R.*
502 *irregularis*) did not result in a higher shoot dry weight or P concentration in *Allium cepa*.
503 Furthermore, cover crop incorporation into soils can provide carbon (C) source, which
504 may impact soil microbial activities and nutritional function, and the introduction of cover
505 crops can increase the amount of C, such as organic matter, to serve as an energy source for
506 biological activity (Jokela et al., 2009). In this study, our cover crop systems did not
507 improve growth performance such as plant biomass and P uptake of soybean at the full
508 bloom stage, whereas the P-application enhanced the growth and yield of soybean (Figs. 3
509 and 7). There is one possible reason why cover cropping did not improve soybean growth
510 performance. That may be due to the continuous nutritional removal in the 5-year
511 P-unfertilized crop rotational system, because the top dry matters of cover crops were not
512 incorporated into the soil. Therefore, further investigation into the relationships among
513 AMF diversity, P-application and cover cropping on soybean growth performance would

514 be required to gain more benefit from AMF in cover crop rotational systems.

515

516 **Conclusions**

517 We found that the P-application improved AMF root colonization of soybean in the
518 five-year P-unfertilized continuous crop rotational system. Also, P-application altered the
519 diversity and communities of AMF in soybean roots at flowering seasons. Different AMF
520 community structures may relate to soybean productivity and P-use efficiency in cover
521 crop rotational systems. On the contrary, AMF root colonization in the soybean was not
522 found to link to the soybean growth performance, and the P-application into the P-limited
523 soil increased the AMF root colonization in the soybean, but may not have been enough to
524 increase the benefits of AMF on soybean growth. Other more important factors such as soil
525 microbial activities and interactions of other soil microbial communities rather than
526 P-application can also be involved to improve soybean performance in the P-limited crop
527 rotations. Thus, we still need to investigate how to improve agronomic benefits from AMF
528 taxa associated with soybean plants, which will give useful information on appropriate P
529 management and cover crop choices in cover crop rotational systems.

530

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Page 2: [1] Comment [6] **Department of Plant Biology** **11/19/17 5:51 PM**

Has anyone quantified how much of the soil P ends up becoming less available for the sake of giving context? I only say this because this tends to be a frequent factoid that I come across in the literature, but I am sure that this also has a lot to do with soil composition/weather/etc....

Page 2: [2] Comment [7] **Department of Plant Biology** **11/19/17 5:50 PM**

Increasing soil P concentrations does improve crop yield, but I understand what you are trying to say here, I think that you can restructure this sentence by saying something like: "Additionally, increased application of synthetic fertilizers can lead to less active ... "

Page 2: [3] Comment [9] **Department of Plant Biology** **11/19/17 5:57 PM**

It would be good to add some insight on when we expect to reach peak P production - please see the following paper: http://www.sciencedirect.com/science/article/pii/S095937800800099X

Page 2: [4] Comment [10] **Department of Plant Biology** **11/19/17 5:53 PM**

Instead of placing a percentage, can you instead provide the1983 production value? (Also adjusted for inflation to reflect current value).