Title: Can phosphorus application and cover cropping alter the 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 responds 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 their responses to soybean growth and P nutrition under the five-year P-unfertilized crop rotation.

Methods: In this study, we established three cover crops managements (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.

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, biomass and grain yield in 2015. The results of a permutational multivariate analysis of variance (PERMANOVA) showed that the AMF communities colonizing soybean roots

Leonela Carriedo 8/29/2017 12:55 PM
Comment [1]: Do you mean varies according to….

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Comment [2]: This still an awkward sentence.

Leonela Carriedo 8/29/2017 12:59 PM
Comment [3]: This is an odd sentence as well, "their responses to soybean growth and P-nutrition…" this reads like you are investigating the effect of soybean growth on AMF colonization and diversity, and not the other way around.

Leonela Carriedo 8/29/2017 12:59 PM
Comment [4]: Better stated as a regime?

were also significantly different among the cover crop rotations and were 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 AMF diversity in roots rather than root colonization could be partially related to the soybean growth performance with P-application. To increase 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. However, other more important factors such as soil organic matter, soil biological activities, and interactions of soil microbial communities in cover crop rotational systems could be involved in improving soybean performance in the P-limited cover cropping systems.

Introduction

Phosphorus (P) is an essential nutrient with a central role in numerous biochemical processes of plants. P also limits crop production in many arable soils, and P-application is often required to achieve high productivity (Lynch, 2007). P in synthetic fertilizer 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). Alternative P sources are becoming increasingly important (Penuelas et al., 2013) because 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). Although increasing soil P concentrations will not result in greater crop yields, it will increase the fertilization cost to farmers and the potential risk and danger of environmental pollution (Bai et al., 2013). Therefore, managing soil P availability is required to maintain agricultural crop production (Mishima et al., 2003), and then the next green revolution seeks to decrease the demand of fertilizer

Leonela Carriedo 8/29/2017 1:03 PM
Comment [5]: Is your expectation that the AMF are solubilizing P in the soil rather than interacting directly with the root? Please see review by Zhang, Vivanco and Shen, Current Opinion in Microbiology 2017, 37:8-14.

Leonela Carriedo 8/29/2017 1:14 PM
Comment [6]: Check your first paragraph for redundancy and ensure logical set-up for your question.

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Leonela Carriedo 8/29/2017 1:06 PM
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Leonela Carriedo 8/29/2017 1:10 PM
Comment [7]: These sentences are redundant.

Leonela Carriedo 8/29/2017 1:12 PM
Comment [8]: This sentence contradicts sentence on line 50-51. Do you intend to say that increasing P application does not increase P availability? You haven't mentioned that a lot of applied P is lost due to mineralization/reactivity of P in the soil, making it unavailable to the plant. So you're right, adding extra P doesn't always help, but you need to explain that better and add a citation.

to crop productions (White et al., 2013). Arbuscular mycorrhizal fungi (AMF) can increase host plant P uptake and growth, and AMF may especially improve plant P and micronutrients uptake (Smith & Read, 2008). AMF also may act against the depletion of global P reserves (Gilbert, 2000). These 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 Gigasporaceae have been shown to have a positive impact on growth and nutrient uptake of plants (Verbruggen & Kiers, 2010; Gosling et al., 2016). Thus, one strategy to efficiently utilize P in soil is to improve the contribution of AMF on crop growth and nutrient uptake in agricultural management systems.

In general, soybeans [*Glycine max* (L.) Merr.] that highly rely on AMF function for P nutrition are often grown as a summer crop in rotation with winter cover crops such as wheat (*Triticum aestivum* L.), oilseed rape (*Brassica napus* L.) or leguminous crops because soybean grain yields decline over time under continuous cropping. 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, and crop performance decreases in mono-cropping over time, and then the abundance of detrimental AMF species increases and abundance of beneficial AMF species declines in the AMF communities (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, reduces seasonal fallow and thus provides many benefits for subsequent crops and soil fertility (Karasawa et al., 2015).

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

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Comment [9]: What is the contribution of AMF to support this claim?

Leonela Carriedo 8/29/2017 1:16 PM
Comment [10]: Are the seeds/soil inoculated or are growers relying on the native AMF population? That's not clear here as it is written.

Leonela Carriedo 8/29/2017 1:19 PM
Comment [11]: And species of the family Gigasporaceae…

Leonela Carriedo 8/29/2017 1:20 PM
Comment [12]: It would be good for you to write 1-2 sentences on the mechanism that AMF improve P uptake. Via direct interaction/modulation of the root, chemical fixation of P…

Leonela Carriedo 8/29/2017 1:22 PM
Comment [13]: Italicize *et al.*

Leonela Carriedo 8/29/2017 1:23 PM
Comment [14]: This sentence needs simplification. And it's not clear what you mean by detrimental AMF species. Be concise here.

Leonela Carriedo 8/29/2017 1:26 PM
Comment [15]: What other leguminous crops than soy are used?

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). Recent studies have also shown that P-application impacts (Jansa et al., 2014; Islas et al., 2016) or decreases the diversity of AMF communities in soils (Lin et al., 2012; Camenzind et al., 2014). Also, P-application may reduce (Liu et al., 2012; Gosling et al., 2013) or not impact (Beauregard et al., 2013; Liu et al., 2016) the AMF diversity in roots.

Furthermore, Isobe et al. (2014) found that the yield and growth of soybeans under a P-unfertilized four-year winter crop-soybean rotational system gradually decreased over time because of a decrease in AMF root colonization of soybeans and due to the continuous nutrient removal from the soil by continuous crop rotations. They 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. Thus, we will need to understand which factor such as P-application or cover cropping is driving increases in soybean performance via AMF benefits. To improve the reliability and the robustness of the agricultural managements, we need to understand how the P-application and cover cropping link to the AMF benefits soybean growth, and the effectiveness of AMF in cover crop-soybean rotational systems.

In this study, we hypothesized that P-application and cover cropping in a P-limited soil would increase the diversity of AMF communities and the increase would link to the soybean growth responses in the five-year P-unfertilized cover crop study. Therefore, our study objective is to understand whether P-application and cover cropping would recover soybean growth performance regarding the diversity of AMF communities in the P-limited cover crop rotations.

Leonela Carriedo 8/29/2017 1:27 PM
Comment [16]: Redundant.

Leonela Carriedo 8/29/2017 1:29 PM
Comment [17]: Start your paragraph with the intended subject of the paragraph. You may just need to re-phrase this.

Leonela Carriedo 8/30/2017 3:35 PM
Comment [18]: Search for redundancies like this one. Highlighted as well on line 111.

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Leonela Carriedo 8/30/2017 3:38 PM
Comment [19]: Please simplify this sentence. You are trying to fit too much into 1 sentence. In this study you have two objectives: 1) understand how P application and cover cropping affects soybean growth. 2) How AMF diversity is affected under P application and cover cropping. I think it is absolutely fine to separate these two ideas as suggested.

Materials and methods

Experimental design

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). 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 25.2°C and 7.8°C, respectively. The average precipitation for the year in this prefecture is around 133.2 mm. Climate data were calculated from Japan Meteorological Agency (http://www.jma.go.jp/jma/indexe.html).

This cover crop experiment was started from 2007 to 2012 and comprised four winter cover crop treatments such as winter wheat (*Triticum aestivum* L.), red clover (*Trifolium pratense* L.), oilseed rape (*Brassica napus* L.) and fallow for phase one of this study (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). The experimentla field in our study had not received P fertilizers for over five years for the phase one of this study. 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 at this experimental site in 2014 before the study of phase two (Fig. 1), the soil pH ranged from 6.0 to 6.1. 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 this study of phase two experiment, three cover crops (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

Leonela Carriedo 8/30/2017 3:44 PM
Comment [20]: How are these phases divided? Describe that before you mention them.

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Comment [21]: Misspelling

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Comment [22]: Awkward sentence.

Leonela Carriedo 8/30/2017 3:43 PM
Comment [23]: Prepare your reader to know the different phases determined in your experiment.

155 application rates of 100 and 50 kg ha^{-1}, respectively. Red clover seeds (cv: Makimidori,
156 mycorrhizal crop) were sown at 30 kg ha^{-1} with N and K application rates of 30 and 50 kg
157 ha^{-1} in 2014. In 2015, red clover seeds were sown on March 16, 2015. The tops of the
158 cover crops were cut close to the ground and removed on June 3, 2014, and June 16, 2015.
159 In the fallow, weeds were manually removed during the winter period.
160 We investigate the impact of P-application and cover cropping on the diversity of
161 AMF communities in soybean roots and soybean growth after a five-year consecutive cover
162 crop-soybean rotational system. The 4.5 × 2 m of the cover crop experimental plots were
163 divided into 2.25 × 2 m plots for the two P treatment plots (no P-application and
164 P-application) of the phase two experiment (Fig. 1). Then, both no P and P-application
165 treatments were replicated three times in 2.25 × 2 m plots. The soybean (cv: Enrei) seeds
166 were sown at a spacing of 60 × 15 cm on June 17, 2014, and June 17, 2015. In 2014 and
167 2015, the N and K application rates were 30 and 50 kg ha^{-1}, respectively. In 2014, the
168 amount of P (triple superphosphate) in the P-application plots was applied at 17.5 kg ha^{-1}.
169 The P-application did not increase the available soil P in 2013 because of the high P
170 absorption coefficient. In 2014, the amount of P in the P-application plots was applied at
171 57.5 kg ha^{-1} as the amount of P fertilizer and the results of 2014 are shown as Tables S1-S4.
172 In 2015, the amount of P in the P-application plots was applied at 157.5 kg ha^{-1} at three
173 times the normal amount of P fertilizer of 2014. The content of available soil P (Truog P)
174 was analyzed according to Truog (1930).
175
176 **Soil and root sampling and root staining**
177 The soil samples were randomly taken from ten points in each replicate and pooled to one
178 composite sample on June 17, 2014, and June 17, 2015, respectively. Soybean root samples
179 were taken at the full bloom stage (R2 growth stage) on July 31, 2014, and August 6, 2015.
180 The full bloom stage corresponds to the stage when the mycorrhizal colonization of
181 soybean roots is usually at its highest (Zhang et al. 1995). In each rotation, the root samples
182 were randomly collected from ten plants (to a depth of 15 cm, the diameter of 20 cm) per
183 replicate. The root samples were collected from the soil sample and maintained at −80°C
184 for DNA extraction and measurement of AMF root colonization. The root samples were

stained with a 5% (w/v) black ink-vinegar solution (Vierheilig et al. 1998), and the AMF root colonization in the soybean roots was measured as described by Giovannetti & Mosse (1980).

Analysis of plant P and measurement of soybean grain yield

The aboveground plant parts of the ten soybean plants were cut close to the ground at the full bloom stage and were randomly sampled on July 31, 2014, and August 6, 2015. 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).

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.

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Comment [24]: Move this sentence up to line 191.

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 region in the fungal small subunit ribosomal DNA (SSU rDNA) was conducted using nested PCR method (Liang et al. 2008). The fungus-specific primer AM1 (5'-GTTTCCCGTAAGGCGCCGAA-3') (Helgason et al. 1998) and the universal eukaryotic primer NS31 (5'- TTGGAGGGCAAGTCTGGTGCC-3') (Simon et al. 1992) 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 diluted genomic DNA, 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

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Comment [25]: Is this forward or reverse? Is there a reason why you didn't include both?

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Comment [26]: To achieve how many ng/reaction?

nested primers Glo1 (5'-GCCTGCTTTAAACACTCTA-3') (Cornejo et al. 2004) and NS31-GC (5'-CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGTTGGAGGGCAAGTCTGGTGCC-3') (Kowalchuk et al. 2002). Three subsamples per plot were amplified in a 20-µl reaction mixture containing 2 µl of 10-fold genomic DNA, 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 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 using a quantitative real-time PCR (qPCR)

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Comment [27]: Forwar/reverse primer???

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Comment [28]: DNA conc. Of what? 2uL of 10-fold genomic is just a volume, not a concentration.

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Comment [29]: Amplicons/products

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* (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. (2011). 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/). Two-way ANOVA was used to determine the effects of P-application and cover crop and their interactions on each parameter in this study. Data for the significance of differences between P-application treatments among cover crop managements were carried out by independent-samples 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

managements 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 was expressed as the indication of the strength of the connections.

Results

Available soil P and AMF root colonization

In this study, our data showed that the P-application significantly increased the available soil P in all of the cover crop managements compared with no P-application plots although cover cropping did not have a significant effect on the available soil P according to Tukey's test and two-way ANOVA (Fig 2A). The P-application significantly affected the available soil P according to two-way ANOVA ($P < 0.001$), and there were significant differences in the available soil P between P- and no P-application plots at all of the cover crop managements ($P < 0.05$).

In the no P-application plots, the AMF root colonization at the full bloom stage in the wheat was significantly higher than compared to red clover, oilseed rape, and fallow (Fig.

Leonela Carriedo 8/30/2017 4:00 PM
Comment [30]: So in other words, there was no effect of cover-cropping on soil P. The sig. dif. Is only when P was added? Was this really noteworthy?

Leonela Carriedo 8/30/2017 4:01 PM
Comment [31]: Also, consider simplifying this sentence.

Leonela Carriedo 8/30/2017 4:01 PM
Comment [32]: This is saying the same thing that you mentioned in lines 292-295.

2B). The AMF root colonization in the red clover and oilseed rape was significantly higher than compared to the fallow. Contrary to the results of the no P-application plots, there were no significant differences in the AMF root colonization among the cover crop managements with the P-application plots according to Tukey's test. Moreover, 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. Also, the AMF root colonization in soybean roots at the full bloom stage was significantly influenced by the P-application ($P < 0.001$) and the cover crop management ($P < 0.001$) according to two-way ANOVA.

Plant growth, P uptake and grain yield

The aboveground plant biomass in soybeans at the full bloom stage varied among cover crop managements in the field trial (Fig. 3A). In the P-application plots, the aboveground plant biomass of soybeans was highest with oilseed rape, and the soybean biomass was all higher than compared to no-P-application plots. There were significant differences in the aboveground biomass between the P- and no P-application plots for each of the red clover ($P < 0.01$), oilseed rape ($P < 0.01$), and fallow ($P < 0.05$). Moreover, cover cropping did not have a statistically significant effect on aboveground plant biomass according to Tukey's test and two-way ANOVA, although the P-application had a significant effect on aboveground plant biomass according to two-way ANOVA ($P < 0.001$).

Our results showed that the aboveground plant P concentration in soybeans at the full bloom stage did not change among the cover crop managements except the fallow management (Fig. 3B). In the P-application plots, the plant P uptake in soybeans was all higher than compared to no-P-application plots throughout the study. The P-application and cover cropping did not have a significant effect on the both parameters according to Tukey's test and two-way ANOVA (Figs. 3B). Likewise, 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 two-way ANOVA (Figs. 3C). However, the plant P uptake in soybeans was significantly influenced by the P-application according to two-way ANOVA ($P < 0.001$). Moreover, there was a significant difference in the plant P concentration and

uptake between the P- and no P-application plots for the fallow (Figs. 3B and C).

We found that the grain yield in soybeans was not different among the cover crop managements by Tukey's test and two-way ANOVA, whereas the P-application had a significant effect on grain yield of soybean according to two-way ANOVA ($P < 0.001$) (Fig. 3D). The grain yield in soybeans in the P-application plots was all higher than in the no P-application plots, regardless of the cover crop management. The soybean yields were more than double in the P-application plots in the experiment. We also found that there were significant differences in the grain yield between the P- and no P-application plots for each of the red clover ($P < 0.05$) and oilseed rape ($P < 0.05$) management. However, there were no significant differences in the grain yield between P- and no P-application plots at the wheat and fallow.

Diversity of AMF communities and taxa abundance

The P-application significantly influenced the species richness ($P < 0.001$) and the diversity index (H') ($P < 0.001$) in soybeans at the full bloom stage according to two-way ANOVA (Figs. 4A and B). The species richness in soybeans was significantly influenced by the cover crop management according to two-way ANOVA ($P < 0.05$), although the species richness and H' in the P-application plots were all higher than compared with the no P-application plots. There were significant differences in the species richness and H' between the P- and no P-application plots for each of the wheat ($P < 0.05$) and fallow management ($P < 0.01$), whereas there were no significant differences in species richness and H' among the cover crop managements regardless of the P-application.

Leonela Carriedo 8/30/2017 3:59 PM

Comment [33]: Simplify this sentence.

Our results showed that the abundance of the six-selected AMF taxa in the roots did not change among the cover crop managements (Fig. 5). Cover cropping did not have a significant effect on the each AMF taxa according to Tukey's test and two-way ANOVA. The abundance of *Rhizophagus irregularis* and *Cetraspora pellucida* in the P-application plots significantly decreased compared with that in the no P-application plots at the wheat ($P < 0.05$), red clover ($P < 0.05$), and oilseed rape ($P < 0.05$). The P-application had a significant effect on the abundance of *R.irregularis* ($P < 0.001$) and *C.pellucida* ($P < 0.01$). Also, cover cropping had also a significant effect on the abundance of *C.pellucida* ($P <$

0.05). However, the abundance of other AMF taxa (*Claroideoglomus claroideum*, *Funneliformis mosseae,* and *Diversispora celata*) did not change with P-application.

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

We used a RDA to identify the relationships among AMF communities in soybean roots with cover crop management and P-application (Fig 6). The RDA trends were clear that the P-application noticeably altered the AMF community structure in the soybean roots. The AMF communities in the no P-application plots were in the first and fourth quadrants, while most of the AMF communities in the P-application plots were in the second and third 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 1). 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 (F = 4.226, P = 0.001), but cover crop management did not impact the AMF root communities (F = 1.669, P = 0.057).

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Comment [34]: What direction are the quadrants listed in? This was not written in the legend of the figure or added as text into each quadrant.

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 (Figs 7A-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 (Figs. 7D-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 (Figs. 7G-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 (Figs. 7J-L).

We also used a network analysis to identify the relationships between AMF parameters in soybean roots and soybean growth in this study (Fig 8). 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 grain yield. However, each AMF taxa abundance and AMF root colonization were not relate to the soybean growth responses, especially grain yield, throughout the experiment.

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Comment [35]: Correct this redundancy.

Discussion

Impact of P-application and cover cropping on the AMF root colonization in soybeans

Our results indicated that P-application might have a slight impact on the AMF root colonization (Fig. 2B). It is well-known that AMF root colonization is inhibited under high P-application (Kahiluoto et al., 2001; Balzergue et al., 2011). Decreases in the AMF root colonization of soybean have been well reported regarding their response to P-application or concentration in soil (Isobe et al., 2008). In general, plants can fail to react to AMF when available soil P is extremely low (Ryan et al., 2002). Miranda & Harris (1994) reported that deficient of available soil P inhibited AMF root colonization. On the contrary, Gosling et al. (2013) indicated that there was no significant decrease in AMF 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 P soil 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 managements in the

P-application plots was slightly 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 might be becoming a little responsive to P-application to promote mycorrhization in the five-year P-unfertilized condition.

Furthermore, 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). Likewise, the wheat and red clover cropping with no P-application significantly increased AMF root colonization of subsequent soybean and oilseed rape cropping, while fallow with no P-application decreased the AMF root colonization in this study.

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

Surprisingly, our results indicate that the diversity of AMF communities in soybeans, regardless of cover crop management, increased 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 managements (Fig. 6). 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 mycorrhizal dependency gradually reduces, and then 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. Thus, appropriate P management would be considered a major factor in the diversity of AMF communities in response to soil P fertility.

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Comment [36]: It is not clear with what is intended with this.

Furthermore, we found that cover cropping did not impact the AMF root communities in soybeans from the result of PERMANOVA (Fig. 6). 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

In this study, qPCRs were used with specific AMF taxon primers (Wagg et al., 2011; Thonar et al., 2012; Jansa et al., 2014) for the six-selected AMF taxa on the soybean root samples. We found that the abundance of *R. irregularis* was significantly affected by P-application, which meant P-application might be a stronger determinant that impacts the abundance of AMF taxa rather than cover cropping (Fig. 5). 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. irregularis* decreased as a component of the AMF communities with increasing available soil P, in agreement with our study. Johnson (1993) reported that AMF have different niches and are well known to prefer to inhabit different soils. Moreover, fertilization may directly favor species that grow better in enriched soils (Dumbrell et al., 2010). It is likely that the *R. irregularis* may prefer low-P soil conditions due to the continuous cover crop rotational system. Thus, the addition of P application may reduce the abundance of *R. irregularis* in the soybean roots, and this may decrease the opportunity to establish the soybean roots.

P-application contributed to the growth performance of soybean and AMF diversity more than cover cropping

In the results of network analysis and growth response of soybean to AMF parameters, we found that the aboveground plant P and biomass of soybeans during the flowering stage and the grain yield of soybeans were positively correlated with the AMF diversity in the roots of soybeans with increasing of P-application (Figs. 7 and 8). Similarly, Jansa et al. (2008) reported that the growth of *Allium porrum* with three inoculated AMF species (*Funneliformis mosseae*, *Claroideoglomus claroideum,* and *Rhizophagus irregularis*) was enhanced compared to that of *Allium porrum* when each AMF species was mono-inoculated. Gosling et al. (2016) also reported that increased benefit from high AMF diversity on the growth of *Allium cepa* was found compared to mono-inoculated. However increasing the diversity more than three species (*C. caledonium*, *F. mosseae,* and *R. irregularis*) did not result in a higher shoot dry weight or P concentration in *Allium cepa*.

Cover crop cultivation can provide carbon (C) source, which may impact soil microbial activities and nutritional function, and the introduction of cover crops can increase the amount of C, such as organic matter, to serve as an energy source for biological activity (Jokela et al., 2009). C sources, such as organic matter by the introduction of cover crops may promote some soil microbial activity to boost plant P uptake (Chabot et al., 1996). In this study, the cover crop managements did not improve growth performance such as plant biomass and P uptake of soybean at the full bloom stage, whereas the P-application enhanced the growth and yield of soybean (Figs. 3 and 8). Therefore, further investigation into the relationships among AMF diversity, P-application and cover cropping on soybean growth performance would be required to gain more benefit from AMF in cover crop rotational systems.

Conclusions

We found that the P-application did not improve AMF root colonization of soybean and cover cropping in the five-year P-unfertilized continuous crop rotational system, although P-application and cover cropping altered the diversity and communities of AMF in soybean roots at flowering seasons. Also, different AMF community structures may relate to

soybean productivity and P-use efficiency in cover crop rotational systems. On the contrary, AMF root colonization in the soybean was not found to link to the soybean growth performance, and the P-application into the P-limited soil did not recover the AMF root colonization in the soybean. Other more important factors such as soil microbial activities and interactions of other soil microbial communities rather than P-application can also be involved to achieve a high level of AMF root colonization of soybean in the P-limited crop rotations. Thus, we still need to investigate how to improve agronomic benefits from AMF taxa associated with soybean plants, which will give useful information on appropriate P management and cover crop choices in cover crop rotational systems.

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