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REGULAR ARTICLE

Ninety-year-, but not single, application of phosphorus fertilizer has a major impact on arbuscular mycorrhizal fungal communities

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Abstract
Background and aims Arbuscular mycorrhizal (AM) fungi play a significant role in P nutrition of crops in agriculture, but P accumulation in the soil, e.g., application of P-fertilizer, generally reduces AM fungal colonization. The impact of long-term application of chemical fertilizer on AM fungal communities was investigated with respect to the time scale.
Methods Soils were collected from four plots with different fertilizer management in the long-term experimental field established in 1914. *Lotus japonicus* was grown in the soils in a greenhouse, while *Glycine max* was grown in the plots in the field. DNA was extracted from their roots, and the diversity and community compositions were analyzed based on occurrence of the AM fungal phylotypes defined by sequence similarity in the LSU rDNA.
Results The 90-year-application of N and K in the absence of P increased AM fungal diversity and resulted in formation of a distinctive fungal community compared with those in the other treatments. This effect was not cancelled by single application of P. Whereas the impact of balanced application of N, P, and K was ambiguous.
Conclusion These observations suggest that the presence/absence of P-fertilizer has a major impact on AM fungal communities, but the action may appear only on a long time scale.

Keywords Arbuscular mycorrhizal fungi · Chemical fertilizer · Community structure · Long-term field experiment · Phosphorus

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Introduction

Arbuscular mycorrhizal (AM) fungi that belong to the phylum Glomeromycota are ubiquitous in terrestrial ecosystems and form mutualistic associations with most land plants (Smith and Read 2008). The main benefit of the symbiosis for plants is facilitated uptake of immobile nutrients, especially P, through the hyphal networks constructed in the soil, and thus the fungi play significant roles in P cycling in terrestrial ecosystems. It has been well documented that AM associations make significant contributions to agricultural production via improving P nutrition of crops, but it is also true that many of modern agricultural practices, especially heavy application of P-fertilizer, have negative impacts on the effectiveness of the associations (reviewed in Smith and Read 2008).

To monitor and understand the impact of agricultural activities on environments, long-term field experiments have been conducted around the world (Debreczeni and Körschens 2003). Although it is of importance to evaluate the impacts of agricultural practices on AM fungi over long time scales, studies on AM fungi in the long-term experimental fields have been limited so far. The world longest experiments have been conducted at the Rothamsted Experimental Station since 1,843 (Rasmussen et al. 1998), and the influence of long-term liming on AM fungal colonization and spore population was investigated in the fields with respect to soil pH (Wang et al. 1993). They found little effect of pH on the levels of colonization, but there were markedly fewer spores in the soils with pH 5.5 and lower. The 8-year-chemical fertilizer application to an abandoned arable field altered the relative spore abundances of several AM fungal species (Johnson 1993). The heavy application of mineral N for 22 years significantly reduced the spore abundance of particular AM fungal taxa compared with that in the plots managed by organic farming (Oehl et al. 2004). Reduced AM fungal richness due to conventional farming was restored by subsequent 15-year-organic farming (Verbruggen et al. 2010). P-accumulation in the soils due to the 90-year-irrigation of wastewater (Ortega-Larrocea et al. 2001) and 10-year-application of P-fertilizer (Jensen and Jakobsen 1980) decreased the spore density and colonization of AM fungi. It was predicted, therefore, that long-term application of chemical fertilizer would have significant impacts on AM fungal diversity and community compositions.

To characterize AM fungal communities in the field, the spore-survey and PCR-clone library-based approaches have been employed. Spore population, which could be estimated directly using field-collected soils or indirectly after propagation by pot culture, may reflect potential diversity in the soils (Smith and Read 2008). Whereas the PCR-based method in which AM fungal genes are amplified from root-extracted DNA enables to detect the biologically active communities at a given time, i.e. actively growing cell such as intercellular hyphae and arbuscules in the roots can only be properly detected and identified by this molecular approach (Redecker et al. 2003). Several regions of AM fungal ribosomal RNA gene (rDNA) have been employed for identification in the approach. The combination of universal and fungi-specific PCR primers targeting the divergent domains 1 and 2 of large subunit (LSU) rDNA is theoretically able to detect all AM fungal taxa (Redecker et al. 2003), and this primer set has been successfully applied to the analysis of AM fungal communities in various ecosystems (e.g., Pivato et al. 2007; An et al. 2008; Li et al. 2010).

In previous molecular ecological studies on AM fungal communities, the roots collected either from the field or the seedlings grown in the field-collected soils (soil trap culture) were employed for DNA extraction. The community compositions revealed by the two different approaches, however, are usually different. In the case of undisturbed ecosystems, e.g., permanent pasture and natural grassland, the *K*-strategists of AM fungi, which proliferate mainly through constructing hyphal networks in the soil, are preferentially detected in the field-based approach (e.g., Sýkorová et al. 2007). Whereas the *r*-strategists, which proliferate mainly through sporulation, are preferentially detected in the trap culture approach because the hyphal networks are destructed in the process of soil collection. In the case of agroecosystems, particularly those in which hyphal networks are constantly destructed by plowing/tillage, the *r*-strategists are likely to dominate (Oehl et al. 2003; Hijri et al. 2006). Accordingly, difference in the culture conditions between the field and trap culture (greenhouse), e.g., temperature and soil water status, may be the main factor for the differentiation of the community compositions between the two approaches. In addition, horizontal migration of AM fungal spores among adjacent ecosystems/plots, as well as vertical migration from and to deeper soil layers, may occur in the field, but not, or minimum in trap culture that is usually conducted in a greenhouse. On the other hand, there is a benefit of applying trap culture for AM fungal ecology in a long-term field experiment, which is that manipulation of soil chemical properties, e.g., nutrient availability, is feasible without disturbing the on-going experiment.

The objective of present study is to assess the impact of long-term application of chemical fertilizer on AM fungal diversity and communities with emphasis on P-fertilizer. For this purpose the long-term experimental field established in 1914 in Hokkaido University, Japan, in which several plots with different fertilizer treatments have been maintained for more than 90 years was chosen. Two hypotheses were

addressed in this study: firstly, the long-term application of chemical fertilizer has reduced AM fungal diversity and altered the community compositions, and secondly, the effect of single application of P-fertilizer on the communities is the same as, or at least similar to that of the 90-year-application of P. To obtain an answer to the first hypothesis both soil trap culture and field experiments were conducted in parallel to evaluate consistency of the effect using the two different approaches. For the second hypothesis, only soil trap culture, which enabled us to manipulate soil P-availability, was employed, which was designated as the 'P-enrichment experiment'.

Materials and methods

Experimental field

The long-term experiment on the impacts of chemical fertilizer has been conducted since 1914 in the experimental field of Hokkaido University, Sapporo, Japan (17 m altitude, 43°04′N, 141°20′E). The annual mean temperature and rainfall in Sapporo are 8.5 °C and 1,100 mm, respectively. The experimental field (27.0×15.8 m) consists of 6 plots that are separated by concrete walls (15 cm in width, 30 cm in aboveground height, and 30 cm in depth). The soil is alluvial soil, and the plots were constructed on the area in which soil physical and chemical properties, at least within the plow layer, were homogeneous. Four out of the six plots were chosen for the present study: –NPK plot, no chemical fertilizer has been applied since 1914; +NPK plot, 100 kg-N, 100 kg-P_2O_5, and 100 kg-K_2O ha^{-1} have been applied annually; –N plot, 100 kg-P_2O_5 and 100 kg-K_2O ha^{-1} have been applied annually; –P plot, 100 kg-N and 100 kg-K_2O ha^{-1} have been applied annually. The forms of N-, P-, and K-fertilizers were ammonium sulfate, calcium superphosphate, and potassium sulfate, respectively. The size of the –NPK plot is 5.25×7.8 m, and those of the + NPK, –N, and –P plots are 5.25×15.8 m. There is no replication for these plots. After the application of fertilizer, the soil in each plot was tilled uniformly by a rotary tiller at a depth of 15 cm. The field has been subjected to a crop rotation with oat (*Avena sative* L.), maize (*Zea mays* L.), white lupin (*Lupinus albus* L.), soybean [*Glycine max* (L.) Merr.], canola (*Brassica napus* L.), sugar beet (*Beta vulgaris* L.) and sunflower (*Helianthus annuus* L.). The field management also includes minimum pesticide application.

In this study, comparison between the –NPK and + NPK soils enabled assessment of the combined effect of N-, P-, and K-fertilizers on the fungal communities, whereas the individual effects of N- and P-fertilizers were assessed by comparing the + NPK/–N soils and + NPK/–P soils, respectively.

Soil sampling

After harvesting sunflower in 2007, 4–5 kg soil was collected from five randomly chosen spots (20 cm in depth) in each of the four plots on 8th November, combined (about 20 kg in total for each plot), and stored in plastic bags at room temperature for the soil trap culture experiments.

Subsamples of the soils were air-dried and passed through a 2 mm mesh. Soil pH was measured in a 1:2.5 (w/v) soil/water suspension using a glass electrode after 1-h shaking. Available P was determined as described by Truog (1930) with a modification. Briefly, soil was shaken for 30 min in 1 mM H_2SO_4 (the original extraction buffer was 50 mM H_2SO_4, pH was adjusted to 3.0 with ammonium sulphate) at a 1:200 ratio of soil to the extraction buffer. After extraction the soil suspension was passed through a filter paper, and then phosphate concentration was determined colorimetrically. Total C and N contents were analyzed by a CN-corder (Vario MAX CN corder, Elementar). Available Ca and K were extracted with 1 M ammonium acetate (pH 7.0) and measured by a flame emission atomic absorption spectrophotometer (AA-6200, Shimadzu).

Trap culture experiment

Lotus japonicus L. cv. Miyakojima MG-20 (National Bioresource Project Legume Base, http://www.legumebase.brc.miyazaki-u.ac.jp/index.jsp) possesses a quite small root system on which AM fungi colonize at a high rate and thus was suitable for trap culture in a greenhouse. The seeds of *L. japonicus* were pregerminated on a moistened filter paper at 25 °C in the dark, transplanted to plastic pots (9 cm in diam, 350 mL in vol) filled with the soils collected from the four plots, covered with a thin-layer of autoclaved river sand to avoid soil cluster formation, and grown in a temperature/light-controlled greenhouse (26/20 °C for day/

night temperature, 14 h-day length) (n=4, 16 samples in total). The seedlings were thinned to 4 plants pot^{-1} 1 week after transplanting, irrigated every other day, and grown for 9 weeks in the greenhouse. The shoots were weighed after drying at 100 °C for 3 days, while the roots were washed with tap water, cut into 1–2 cm segments, randomized in water, and collected on a sieve. Then one half of the roots was frozen in liquid nitrogen, freeze-dried, and stored at −30 °C for DNA extraction, and the other half was stored at −30 °C for the assessment of mycorrhizal colonization.

For the P-enrichment experiment, a preliminary experiment was conducted to determine an appropriate rate of P application to the –P soil and revealed that amendment of potassium dihydrogenphosphate at 300 mg-P kg^{-1} soil increased the available P level to 264.4 mg-P kg^{-1} soil, comparable to the level in the + NPK soil (280.4 mg-P kg^{-1} soil). *L. japonicus* seedlings were grown in the –P soil with (P_{300}) or without (P_0) P at 300 mg kg^{-1} soil in the greenhouse for 9 weeks and harvested as described above (n=3, 6 samples in total).

Field experiment

In May 2008, the fertilizers were applied to the plots at the prescribed rates, and then *Glycine max* was sown at a 50×30 cm (interrow/intrarow) spacing on 9th June (10 rows in each plot). On 31st July (52 days after sowing), five plants were randomly chosen from every other row in each of the four plots for harvest (n=5). The shoots were cut and weighed after oven-drying at 100 °C for 3 days. The root system was collected from the area of *ca.* 30 cm in diam×20 cm in depth, and then fine roots were detached from the main roots, washed with tap water, cut into 2–3 cm segments, randomized in water, and collected on a sieve. Approximately 30 g of the root segments was collected from each plant separately, and divided into two subsamples. One half of the sample was frozen in liquid nitrogen, freeze-dried for 2 days, and stored at −30 °C for DNA extraction, and the other half was stored at −30 °C for the assessment of mycorrhizal colonization.

Assessment of fungal colonization

The frozen root subsamples were thawed in tap water, cleared in 10 % (w/v) KOH at 80 °C for 90 min, stained with 0.05 % trypan blue in the lactoglycerol (lactic acid/glycerol/water=1/1/1) at 80 °C for 30 min, and destained in the lactoglycerol at 80 °C for 30 min. The percentage colonization of the roots was estimated by the gridline intersect method (Giovannetti and Mosse 1980).

LSU rDNA amplification and sequencing

Twelve to 20 mg of the freeze-dried root sample was ground with a metal cone in an O-ring-sealed 2.2-mL tube (Yasui Kikai, Osaka) at 2,500 rpm for 2×60 s using the Multi-Beads Shocker (Yasui Kikai) at room temperature, and DNA was extracted with the DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. PCR was performed using the Expand High Fidelity plus PCR system (Roche Diagnostics) with the PTC225 DNA Engine Tetrad Thermal Cycler (MJ Research). The reaction mixture consisted of 0.5 μL 10 mM dNTP, 10 μM each primer and 1 μL template DNA solution in a total volume of 25 μL. The eukaryotic LSU rDNA-universal forward primer LR1 5′-GCATATCAATAAGCGGAGGA-3′ (van Tuinen et al. 1998) and fungal LSU rDNA-specific reverse primer FLR2 5′-GTCGTTT AAAGCCATTACGTC-3′ (Trouvelot et al. 1999) were used for the amplification. The thermal cycling program was as follows: initial denaturation at 94 °C for 2 min, followed by 30 cycles of denaturation at 94 °C for 15 s, annealing at 50 °C for 40 s and polymerization at 72 °C for 80 s, and final elongation at 72 °C for 10 min. The PCR products were separated by electrophoresis on 1.5 % agarose gels and visualized on the Safe Imager Blue-Light Transilluminator (Invitrogen) after staining with the SYBR SAFE DNA gel stain (Invitrogen). Since the size of PCR products from AM fungal LSU rDNA were expected to be within 680–770 bp, those shorter than 680 bp were excluded from subsequent cloning and sequencing by cutting the gel at this step if a considerable amount of short fragments were observed on the gel.

The PCR products were purified using the MonoFas DNA PCR Purification Kit (GL Sciences, Tokyo), cloned into the pT7Blue T-vector (Novagen) and transformed to the *Escherichia coli* JM109 competent cell (Takara, Ohtsu, Japan) according to the manufacturer's instructions. The nucleotide sequences of the randomly chosen clones were determined by the dideoxy-cycle sequencing method using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied

Biosystems) with the 3130*xl* Genetic Analyzer (Applied Biosystems) according to the manufacturer's instructions. LSU rDNA sequences were trimmed and edited manually using the Vector NTI Advanced v9.0 software (Invitrogen).

Definition of phylotypes and phylogeny

In a preliminary analysis all LSU rDNA sequences were aligned together with published several AM fungal sequences across all families (reference sequences) obtained from the GenBank database using Clustal X ver. 1.81 (Thompson et al. 1997) to construct neighbor-joining trees. The sequences that were highly unlikely to belong to Glomeromycota were excluded at this step. We employed ≥95 % sequence similarities in LSU rDNA as a species boundary. The sequences were grouped together with the reference sequences based on ≥95 % similarities using Sequencher ver. 5.0 (Gene Codes). Then the representative sequences that were randomly chosen from each 95 %-similarity-group and those did not form a group were subjected to BLAST searches. Validity of the sequences (groups) was carefully assessed by comparing with published sequences, and invalid sequences (groups) such as chimeric sequences were excluded at this step. The valid representative sequences together with the reference sequences were aligned using Clustal X, and a neighbor-joining tree was drawn for phylogeny using NJplot (Perrière and Gouy 1996). The confidence limits of each branch in the phylogeny were assessed by 1,000 bootstrap replications and expressed as percentage values. The tree topologies were generally in good agreement with the 95 %-similarity-groups with respect to their bootstrap values (>70 %), and thus each 95 %-similarity-group was defined as a single phylotype. The names of phylotypes were designated based on the families (proposed by Krüger et al. 2012) to which they are most likely to belong, followed by a numerical index, e.g., Glo1, phylotype 1 in Glomeraceae.

Community analysis and statistics

Calculation of Shannon diversity indices (Shannon 1948) and ordination analysis were performed with CANOCO 4.5 (Microcomputer Power). In these analyses, the binary data in which the presence and absence of individual phylotypes in each root sample were scored as '1' and '0', respectively, were used. In ordination analyses, rare phylotypes (designated as those occurred only in one sample) were excluded from the dataset, and detrended correspondence analysis (DCA) (Hill and Gauch 1980), a unimodal method, was first applied. Then the significance of environment factors was assessed by canonical correspondence analysis (CCA) (ter Braak 1986) with the forward selection procedure by means of Monte Carlo permutation test (499 permutations). Jaccard distance (J') was calculated as follows: $J'_{ij}=1-[C/(N_i+N_j-C)]$, where C is the number of shared phylotypes between the i and j samples and N_i and N_j are the total number of phylotypes detected from the i and j samples, respectively. Rarefaction curves were constructed with Analytic Rarefaction v1.3 (http://www.uga.edu/strata/software/index.html).

For assessment of the treatment effects on plant growth and AM fungal diversity ANOVA was not applied due to the absence of replication in the field plots. Instead, standard errors were presented to express variances within the individual treatments.

Results

Soil nutrient levels and plant growth

Among the soil chemical parameters the impact of 90-year-application of chemical fertilizer was most evident in available P: the levels in the + NPK and –N soils to which P-fertilizer have been applied annually (P-fertilized soil) were ten times higher than those in the –NPK and –P soils to which P-fertilizer has never been applied (P-unfertilized soil) (Table 1). Soil pH and Ca level were lower in the –P soil than in the other soils.

In the trap culture experiment, shoot dry weight of *Lotus japonicus* was highest in the + NPK soil, followed by that in the –N soil, and lowest in the –NPK and –P soils (Fig. S1a). The percentage of mycorrhizal colonization was lower in the P-fertilized soils than in the P-unfertilized soils. In the P-enrichment experiment plant growth was greater in the P-enriched (P_{300}) treatment than in the control (P_0, no P-enrichment) (Fig. S1b), and the level of AM fungal colonization in the P_{300} treatment was decreased to the same extent as that in the plants grown in the + NPK soil (Fig. S1a and b). In the field experiments, growth of *Glycine max* was also greatest in the + NPK plot, followed by that in the –N plot, and lowest in the –

Table 1 Chemical properties of the soils used in the trap culture experiments

Plots	pH (H_2O)	Total N (g-N kg^{-1})	Total C (g-C kg^{-1})	Available P (mg-P kg^{-1})	Available K (mg-K kg^{-1})	Available Ca (mg-Ca kg^{-1})
−NPK	6.1	2.45	35.0	30.4	166.1	6.69
+NPK	5.9	2.76	38.0	284.0	190.3	6.80
−N	6.0	2.36	33.4	348.2	605.1	6.88
−P	5.4	2.63	37.1	22.1	499.5	5.35

NPK and –P plots, consistent with that of *L. japonicus* in the trap culture experiment (Fig. S2).

Arbuscular mycorrhizal fungal phylotypes

AM fungal LSU rDNA was successfully amplified from all samples except for the trap culture experiment in which PCR products were obtained only from three out of the four samples in the –NPK and + NPK treatments. Accordingly, total numbers of clone library constructed for the field, trap culture, and P-enrichment experiments were 20, 14, and 6, respectively. Overall, 80–100 clones $treatment^{-1}$ for the field experiment and 80–200 clones $treatment^{-1}$ for the trap culture and P-enrichment experiments were sequenced (Table S1). Based on ≥95 % sequence similarities 25 phylotypes in total were defined and assigned to the known families: Glo1 – 12 in *Glomeraceae*, Gig1 – 4 in *Gigasporaceae*, Div1 in *Diversisporaceae*, Aca1 – 2 in *Acaulosporaceae*, Par1 – 3 in *Paraglomeraceae*, and Cla1 – 2 in *Claroideoglomeraceae* (Fig. S3). The phylotype Unc1 could not be assigned to any of the known families, but was likely to belong to Glomeromycota. The representative sequences were deposited in the DNA Data Bank of Japan under accession numbers AB547184–AB547185 and AB665499–AB665521.

Rarefaction curves were constructed based on the numbers of sequenced clone to assess the current status of sampling effort (Fig. S4 and S5). All curves showed signs of leveling off, and the 95 % confidence intervals of the curves were in a range of 0–0.61, suggesting that our analysis provided a reasonable coverage of AM fungal diversity.

Impact of combined application of N-, P-, and K-fertilizers

AM fungal diversity (Shannon diversity index) was lower in the + NPK soil than in the –NPK soil in the field, but in the trap culture the effect of + NPK treatment on the diversity was ambiguous (Fig. 1). In the DCA, the axes 1 and 2 explained 38.3 % of total variance, and clear separation of plots between the field and trap culture experiments was observed (Fig. 2). The –NPK samples in the field and trap culture were closely located, but the + NPK samples were plotted separately between the two experiments. Separation between the –NPK and + NPK samples along the axis 1 was clear in the field, but was less clear in the trap culture. Subsequent CCA indicated that the experimental approach (field or trap culture) and the presence/absence of NPK-fertilizer were the significant factors that differentiated the communities (experimental approach, P= 0.002; fertilizer, P=0.002).

Impacts of N- and P-fertilizers

AM fungal diversity in the –P soil was consistently higher than those in the other soils irrespective of

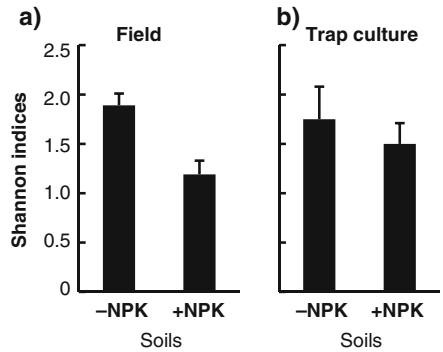

Fig. 1 Effect of combined application of N-, P-, and K-fertilizers on the diversity of AM fungal communities in the field (**a**) and trap culture (**b**) experiments. **a**, *Glycine max* was grown in the –NPK soil to which fertilizer has never been applied or in the + NPK soil to which NPK-fertilizers has been has been applied since 1914 in the field (n=5). **b**, *Lotus japonicus* was grown in a greenhouse in the –NPK or + NPK soils collected from the field (n=3). Shannon diversity indices were calculated based on the presence/absence data of AM fungal phylotypes. Vertical bars indicate SE

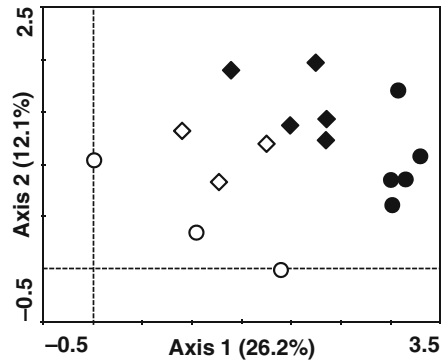

Fig. 2 Detrended correspondence analysis on the AM fungal communities in the –NPK and + NPK soils. *Glycine max* was grown in the field plots (field experiment, *closed symbols*), whereas *Lotus japonicus* was grown in the field-collected soils in a greenhouse (trap culture experiment, *open symbols*): *diamonds*, –NPK soil; *circles*, +NPK soil

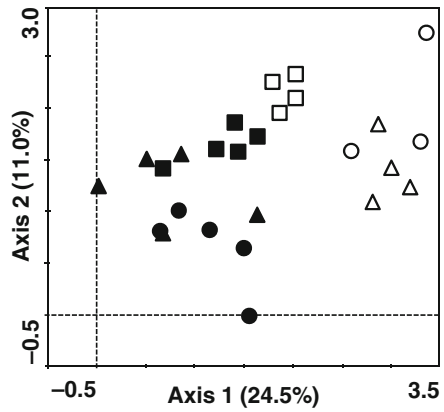

Fig. 4 Detrended correspondence analysis on the AM fungal communities in the + NPK, –N, and –P soils. *Glycine max* was grown in the field plots (field experiment, *closed symbols*), whereas *Lotus japonicus* was grown in the field-collected soils in a greenhouse (trap culture experiment, *open symbols*): *circles*, +NPK soil; *triangles*, –N soil; *squares*, –P soil

experimental approach (Fig. 3). In the DCA sample plot the field and trap culture experiments were clearly separated along the axis 1 that explained 24.5 % of total variance, but not along the axis 2 that explained 11.0 % of total variance (Fig. 4). The –P samples in the trap culture were plotted separately from the other samples along the axis 1, but those in the field experiment were separated along the axis 2. The –N and + NPK plots were colocalized both in the two experiments. Subsequent CCA confirmed that the experimental approach (P=0.002) and the –P treatment (P=0.002), but not the –N treatment (P=0.057), were the significant factors that drove the communities.

Impacts of single versus 90-year-application of P-fertilizer

The single application of P to the –P soil i.e. P_{300} treatment in the P-enrichment experiment had little impact on AM fungal diversity (Fig. 5). In the DCA on the P-enrichment experiment the community data

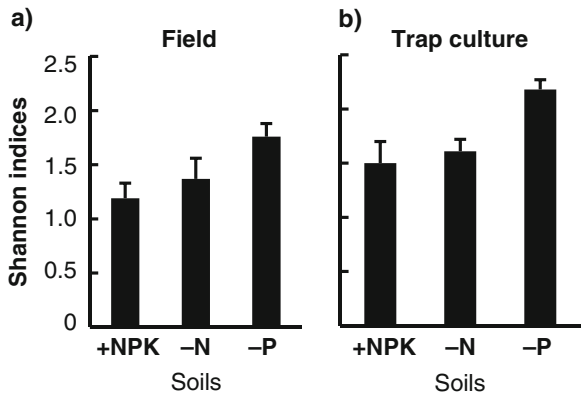

Fig. 3 Effect of the absence of N- and P-fertilizers on the diversity of AM fungal communities with respect to the + NPK-treatment in the field (**a**) and trap culture (**b**) experiments. **a**, *G. max* was grown in the + NPK, –N, or –P soils in the field (n=5). **b**, *L. japonicus* was grown in a greenhouse in the + NPK, –N, or –P soils collected from the field (n=4; +NPK soils, n=3). Shannon diversity indices were calculated based on the presence/absence data of AM fungal phylotypes. Vertical bars indicate SE

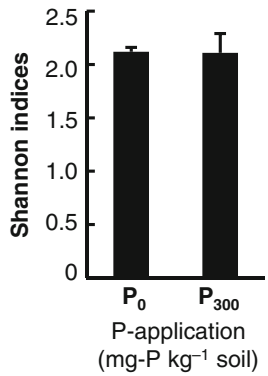

Fig. 5 Effect of P-fertilizer application on the diversity of AM fungal communities in the P-enrichment experiment. *Lotus japonicus* was grown in the –P soil amended with (P_{300}) or without (P_0) 300 mg-P kg^{-1} soil in a greenhouse (n=3). Shannon diversity indices were calculated based on the presence/absence data of AM fungal phylotypes. Vertical bars indicate SE

of –P and + NPK soils in the trap culture experiment were combined and analyzed together to compare the impacts of single and 90-year-application of P. The P_{300} samples were plotted close to the –P and P_0 samples in which the –P soil was used as a basic medium and were separated from the + NPK samples along the axis 1 that explained 33.9 % of total variance (Fig. 6a). The levels of the impacts were further assessed by means of Jaccard distance (Fig. 6b). In this analysis the communities of –P and P_0 treatments in which the same soil (–P soil) was used without additional P were defined as control in the trap culture and P-enrichment experiments, respectively. In this context, Jaccard distance between these controls (0.41) could be regarded as the standard value that reflects the variance between the two experiments. The index between the P_0 and P_{300} communities, which represents the impact of single P-application, was not different from that between the controls. Whereas the index between the –P and + NPK communities, which represents the impact of the 90-year-application of P, was about twice as high as that between the controls. The impact of 90-year-application was also reflected in the index between the P_{300} and + NPK communities.

Discussion

The present study, for the first time using a molecular ecological approach, demonstrated that long-term application of chemical fertilizer had a major impact on AM fungal communities. Our field resource in which the same management has been conducted for more than 90 years enabled us to show that fertilizer application acted as strong selective pressure against AM fungi on a long time scale.

Twenty-five AM fungal phylotypes were successfully detected by the two parallel approaches, field and trap culture experiments, in conjunction with the one-step PCR amplification of AM fungal LSU rDNA. Phylotype richness revealed in our field (6–15 phylotypes plot^{-1}) is comparable to or even higher than those demonstrated in the other studies in which similar methods (rDNA-targeted PCR/sequencing) were employed, e.g., 3–7 phylotypes plot^{-1} were detected in the organic and conventional agricultural fields in Europe (Hijri et al. 2006). The sampling effort curves constructed based on the numbers of sequenced clone further ensured that most of the phylotypes, or at least predominant phylotypes in the four plots were recovered in our study. Given that AM fungal diversity is

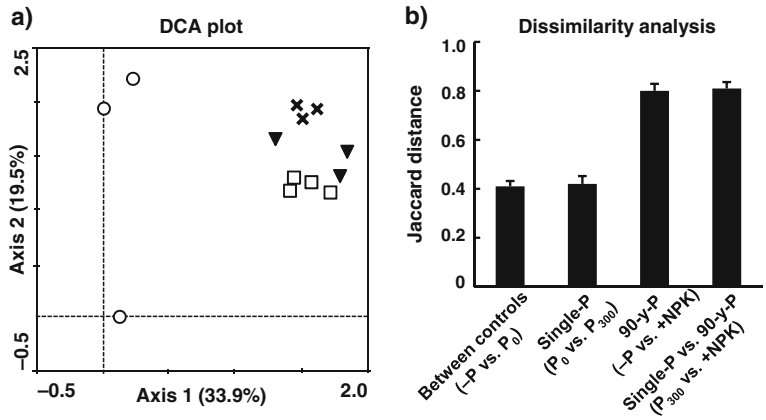

Fig. 6 Comparative assessment of the impacts of single and 90-year-application of P-fertilizer on AM fungal communities. **a**, Detrended correspondence analysis (DCA) on the communities in the P_0 (*closed inverse triangles*) and P_{300} (*crosses*) treatments in the P-enrichment experiment and those in the + NPK (*open circles*) and –P (*open squares*) soils in the trap culture experiment. *Lotus japonicus* was grown either in the –P soil with (P_{300}) or without (P_0) 300 mg-P kg^{-1} soil (P-enrichment experiment) or in the + NPK or –P soil (trap culture experiment) in a greenhouse. **b**, Dissimilarities in the community compositions (Jaccard distance) as representative of the impacts of single and 90-year-application of P on the communities. The index between the communities of –P and P_0 treatments (Between controls) in which the same (–P) soil was used was regarded as the standard value that reflects the variance between the trap culture and P-enrichment experiments. The index between the communities of the P_0 and P_{300} treatments (Single-P) represents the impact of single application of P, whereas those between the communities of the –P and + NPK treatments (90-y-P) and between the communities of the P_{300} and + NPK treatments (Single vs. 90-y-P) represent the impact of 90-year-application of P. Vertical bars indicate SE

higher in the arable field with crop rotation than with monocropping (Oehl et al. 2003), the higher diversity in our field may be a reflection of the crop rotation for over 90 years.

Notably, the presence and absence of P-fertilizer i.e. +NPK vs. –P treatments had contrasting effects on the diversity and compositions of AM fungal communities, which was observed consistently in the field and trap culture experiments. Most studies, however, have demonstrated little impact of P-fertilizer on AM fungal communities in arable soil. For instance, only a limited effect of P application was observed on the diversity, composition and density of AM fungal spores in the agricultural fields in Switzerland (Mathimaran et al. 2005), Western Kenya (Mathimaran et al. 2007), and Germany (Antunes et al. 2012). In these studies, however, the available P levels in the P-fertilized soils were within the same order of magnitude as those in the P-unfertilized soils. In fact, shifts in AM fungal community due to P-fertilizer application were also observed in an alpine meadow ecosystem, in which available P was increased by two orders of magnitude i.e. from 2.8 to 189 mg-P kg^{-1} (Liu et al. 2012). It is, therefore, likely that the high contrast in the level of available P between the P-fertilized and -unfertilized soils enhanced the action of P in the present study.

The long-term absence of P with repeated application of N and K (–P treatment) has differentiated the community from those in the other treatments. According to the trade balance model proposed by Johnson (2010), N-enrichment in P-limited soil increases the value of P-acquisition through mycorrhizal symbiosis. Therefore, the plants grown in the –P soil should suffer serious P-deficiency than those grown in the other soils, because they received a sufficient amount of N and K and thus required a comparable amount of P to maintain their growth. It has been known that plants grown under P-deficient conditions release more chemical signals that stimulate hyphal branching (Akiyama et al. 2005), appressorium formation (Tawaraya et al. 1998) and colonization (Akiyama et al. 2002) of AM fungi than those grown under P-sufficient conditions, which may increase, or at least maintain AM fungal diversity in the rhizosphere. It remains unclear, however, whether these chemical signals act as selective pressure for AM fungal species i.e. whether responsiveness of AM fungi to the signals is different among the species and needs to be elucidated.

Another possible interpretation of the differentiation of the AM fungal community in the –P soil is that the host plants selected particular AM fungi via preferential carbon allocation. Bever et al. (2009) demonstrated that plants preferentially allocated more photosynthate to more beneficial, in terms of carbon cost for P-acquisition, AM fungi. Accordingly, it could be hypothesized that the plants grown in the –P soil i.e. under P-deficient conditions allocated carbon preferentially to more beneficial fungi to enhance the overall efficiency of P-uptake, which might lead to a shift in the community, e.g., predominance/decline of more/less beneficial fungi. However, functional traits of the individual fungi in the two soils that differ in P-fertility are unknown and should be evaluated for testing this hypothesis.

The impacts of single and 90-year-application of P-fertilizer on the communities were comparatively evaluated in the presence of N- and K-fertilizers. In the P-enrichment experiment the single application of 300 mg-P reduced the level of AM fungal colonization to the same extent as that in the + NPK soil, suggesting that soil P-availability in the –P soil was successfully increased by the treatment to a level comparable to that in the + NPK soil. All the analyses, however, indicated the impact of the single application on the community was minimum and negligible compared with that of the 90-year-application: no apparent change in the diversity and community composition was observed in the P_{300} treatment. These observations suggest that soil P-availability, or more properly, plant P-nutrient status may act as ‘moderate or weak’ selective pressure against AM fungal communities, and thus it would take a long time for the selection/extinction of the species.

One might expect, however, that results would be different if the P-enrichment experiment was conducted in the field in which horizontal and vertical migration of AM fungal propagule might occur. The migration of propagule may accelerate community shifts in response to environmental changes, e.g., fertilizer application. We consider, however, that the influence of migration was minimum within such a short culture period (up to 9 weeks), because all results obtained in the field experiment were generally in agreement with those obtained in the trap culture experiment.

The long-term absence of P-fertilizer with the repeated applications of N- and K-fertilizers also resulted in the 0.5-unit decrease in soil pH. This shift in pH might be a potential edaphic factor responsible for the differentiation of AM fungal community in the –P soil. It is considered that the lower level of Ca is likely to be involved in the lower pH value in the soil, because the absence of P-fertilizer (calcium superphosphate) represents absence of Ca (potential neutralizer) application. The Ca level in the –NPK soil, which is another P-unfertilized soil, was comparable to those in the P-fertilized soils, but this could be interpreted by the poorer plant growth i.e. lower Ca uptake due to deficiencies in the macronutrients. Although information about the influence of soil pH on AM fungal communities is limited (Abbott and Robson 1991), the field surveys of AM fungal communities in a wide range of soil pH suggest that pH is the major driving force for structuring the communities (An et al. 2008; Dumbrell et al. 2010). It is uncertain, however, whether the 0.5-unit shift in pH is actually critical for shaping AM fungal communities, and this should be examined experimentally.

The impact of the balanced application of N, P, and K was ambiguous although CCA indicated the significance of + NPK treatment: lower diversity and a shift in the community composition in the + NPK treatment were observed in the field, while the effect was less clear in the trap culture. These observations suggest that the balanced application of the three macronutrients would have a limited impact on AM fungal communities even on a long time scale, and the effect appears only circumstantially. Given the observations that the presence/absence of P-fertilizer is a possible driver for AM fungal communities, probably via modifying plant P-nutrient status, requirement of P relative to those of N and K is likely to be similar between the plants grown in the complete presence of the three macronutrients (+NPK) and those grown in the complete absence of the nutrients (–NPK).

In conclusion, the present study demonstrated that the 90-year-application of N- and K-fertilizers in the absence of P-fertilizer led to the formation of distinct community of AM fungi. Interestingly, this effect could not be simply cancelled by single application of P to the soil, suggesting that not only soil P-fertility but also the time scale must be taken into account for the selection/extinction of the species. On the other hand, the balanced application of the macronutrients is likely to have a limited impact on the communities. However, the impact of long-term application of chemical fertilizer on functional traits of the fungal communities is another concern and remains to be elucidated.

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