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REGULAR ARTICLE

The relative contributions of pH, organic anions, and phosphatase to rhizosphere soil phosphorus mobilization and crop phosphorus uptake in maize/alfalfa polyculture

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Abstract
Aims To investigate the relative contributions of pH, organic anions concentration, and phosphatase activity to rhizosphere soil phosphorus availability and crop phosphorus uptake in polycultures.
Methods A field experiment was conducted for three consecutive years in a split-plot design with main plots treated with two phosphorus levels and subplots treated with maize and alfalfa grown alone or intercropped.
Results Intercropped maize and alfalfa had 0.35 and 0.24 units lower rhizosphere pH, 28% and 30% higher total organic anions (TOA) concentration, and 21% and 41% greater acid phosphatase activity than those in monoculture. These changes in root exudates induced significant increases in rhizosphere phosphorus concentration of intercropped maize and alfalfa by 21% and 41%, and pH and TOA had greatest contributions, respectively. Rhizosphere phosphorus mobilization facilitated phosphorus uptake of intercropped maize, but this facilitation was offset by phosphorus uptake reduction due to decreased crown root surface area. Lateral root volume enhancement accounted for phosphorus uptake improvement of intercropped alfalfa by 86.6%, while rhizosphere phosphorus mobilization only had a 0.2% contribution.
Conclusions Rhizosphere pH and organic anions exhibit greater contributions than acid phosphatase activity in enhancing rhizosphere phosphorus availability. However, root surface area of maize and lateral root volume of alfalfa unveil greater influences on crop phosphorus uptake than rhizosphere pH and organic anions.

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Keywords Mycorrhizal colonization rate · Phosphorus uptake · Polyculture · Rhizosphere phosphorus mobilization · Root architecture · Root exudates

Introduction

Phosphorus (P) is the second most essential macronutrient for plant growth, and is one of the major yield-limiting nutrients in many agricultural systems due to its low bioavailability in most soils (Schachtman et al. 1998; Hinsinger 2001). In response to P deficiency, plants have evolved many strategies to improve soil P exploration and exploitation, such as changes in root morphology, e.g. increases in nodal root number, root branching, length, and surface area (Desnos 2008; Lynch 2011; Sun et al. 2018a), alteration in root distribution, e.g. greater root length density in the topsoil (Manske et al. 2000; Zhu et al. 2005a), and association with microorganisms, either symbiotic (mycorrhizal fungi) or not (Smith et al. 2011). Both modifications in root system architecture and mycorrhizal association are effective ways to increase total surface area available for soil P exploration and absorption, and thereby improve soil P spatial availability and plant P acquisition (Vance et al. 2003; Lambers et al. 2006; Shen et al. 2011). In addition, plants can also enhance P acquisition by altering root physiology and thereby improving soil P bioavailability and P diffusion toward roots (Lambers et al. 2006; Hinsinger et al. 2011a; Shen et al. 2011). For example, root-induced rhizosphere acidification or alkalization and release of organic anions can mobilize soil inorganic P by ligand exchange, ligand-induced dissolution, and complexation of cations bound to P, such as Fe, Al and, Ca (Zhu et al. 2005b; Wang et al. 2007; Rose et al. 2010). Plant roots can also secrete amounts of phosphatase into the rhizosphere, allowing mobilization and utilization of organic P in the soil (Li et al. 2004; George et al. 2008).

In polyculture systems, it has also been proven that changes in root architecture, mycorrhizal association, and rhizosphere exudates secretion are important ways to improve plant P acquisition and yield production (Hinsinger et al. 2011a; Shen et al. 2013; Li et al. 2014). For example, root length of maize was significantly increased when intercropped with faba bean, and thus facilitated P acquisition (Zhang et al. 2012; Zhang et al. 2016). In addition, polyculture can enhance rhizosphere acid phosphatase activity of chickpea intercropped with maize and rhizosphere malate and citrate concentrations of faba bean intercropped with wheat, leading to significantly improved rhizosphere soil P bioavailability and thus increased P uptake of associated maize and wheat (Li et al. 2004; Li et al. 2016). The interspecific rhizosphere changes and facilitation in P acquisition are mainly induced by interspecific competition (Hess and De Kroon 2007; Li et al. 2016; Zhang et al. 2016). Due to differences in morphological and physiological characteristics of intercropped species, interspecific competition in most cereal/legume polycultures is asymmetrical (Zhang et al. 2011; Zhang et al. 2016). However, previous studies in interspecific rhizosphere facilitation only focused on cereal-dominated polyculture, and the potential of legume-dominated cereal/legume polyculture in P acquisition merits investigation. In addition, most studies only investigated one or two aspects of root-induced rhizosphere changes (pH, organic anions, and phosphatases) in greenhouse pot experiments using annual crop combinations. Little information is available regarding how these three aspects respond to P deficiency and the relative contributions of each root exudate to crop P uptake in an annual/perennial polyculture under field conditions.

Our recent study in a perennial legume-dominated cereal/legume polyculture has shown that maize/alfalfa polyculture significantly improved system P uptake and yield production, attributing to root changes in morphology and distribution as well as interspecific complementarity in space and time (Sun et al. 2018b). However, there are still unclear questions about the acquisition of P in maize/alfalfa polyculture. For example, a) whether maize/alfalfa polyculture, especially in low P soil, could stimulate rhizosphere exudates secretion and thereby rhizosphere soil P mobilization; b) which root exudates (pH, organic anions, and phosphatase) are more correlated to rhizosphere soil P mobilization of intercropped maize and alfalfa; c) whether root exudates-induced rhizosphere soil P mobilization contributes to plant P acquisition when changes in root architecture and mycorrhizal association are both considered. These questions are the focus of this study.

Therefore, a field experiment was conducted in a maize/alfalfa polyculture for three consecutive years to explore two scientific questions: (1) What are the relative contributions of pH, organic anions, and phosphatase to rhizosphere soil P mobilization? (2) What is the contribution of root exudates-induced rhizosphere soil P mobilization to plant P acquisition relative to root architecture and mycorrhizal association?

Materials and methods

Site description

The study was conducted from 2013 to 2015 at the Grassland Ecological Research Station of Northeast Normal University at Songnen Grassland (44° 40′ N, 123° 44′ E), a typical farming and pastoral area of northeast China. This area is characterized by a semi-arid and temperate continental monsoon climate with cold-dry winters and warm-rainy summers. Annual mean temperature ranges from 4.6 to 6.4 °C, annual accumulated temperature (≥10 °C) varies from 2546 to 3375 °C, annual precipitation ranges from 300 to 500 mm with 86% falling in May–September, and annual mean evaporation ranges from 1500 to 2000 mm. The frost-free period lasts approximately 140 days, from the end of April to early October. The three experimental years contrasted each other in terms of precipitation (Fig. S1). The 2013 season was the wettest ever recorded at the research station with a total rainfall amount of 427 mm and a typical seasonal distribution. The rainfall in 2014 was the lowest with an annual total of 297 mm and only 275 mm of rainfall between May and September; there was also no pronounced peak in July but considerable rainfall in September. The 2015 season had normal rainfall (387 mm) but no pronounced peak in July. Mean annual air temperatures in 2013–2015 were between 5.3 and 7.0 °C, which was similar to the long-term mean average temperature (Fig. S1). The soil type is classified as light chernozem with deep soil layers. The entire study field had uniform soil conditions and the physico-chemical properties of the top 20 cm soil were: organic C 17.36 g kg^{-1}, total N 0.99 g kg^{-1}, total P 0.36 g kg^{-1}, Olsen-P 5.91 mg kg^{-1}, organic P 172.8 mg kg^{-1}, available K 141.42 mg kg^{-1}, and pH value 7.80 (the ratio of soil to water was 1:5, *w*/*v*).

Experiment design and crop management

In 2011, a field experiment was established with maize (*Zea mays* L. cv. Zhengdan 958) and alfalfa (*Medicago sativa* L. cv. Dongmu No. 1) in a randomized complete block design with six blocks. Four cropping patterns in each block were monoculture maize (MM), monoculture alfalfa (MA), four rows of maize intercropping with six rows of alfalfa (IMA4), and six rows of maize intercropping with six rows of alfalfa (IMA6).

In all cropping patterns, maize was planted in alternating wide and narrow rows where the wide row space was 90 cm and the narrow was 40 cm, and alfalfa was cropped in even rows with inter-row spacing of 30 cm. The distance between maize and adjacent alfalfa rows was 30 cm in the intercropping treatments (Fig. S2). Each individual plot had an area of 3.9 × 10 m for monoculture maize and alfalfa. Each intercropping plot comprised three strips, and four or six rows of maize alternating with six rows of alfalfa were planted in each strip. Consequently, the areas of IMA4 and IMA6 plots were 11.4 × 10 m and 15.3 × 10 m, respectively, and the intercropping area ratios occupied by maize and alfalfa in IMA4 and IMA6 were 53%:47% and 65:35%, respectively. A 0.5 m wide ridge between plots was built to separate the plots from each other.

Alfalfa was sown at a seeding rate of 15 kg ha^{-1} in early July 2011, and was given 135 kg P ha^{-1} (as diammonium phosphate) and 90 kg K ha^{-1} (as potassium chloride) as basal nutrients. All fertilizers were evenly broadcasted and incorporated into the top 20 cm of the soil before sowing. No more fertilizers were applied in the following experimental years. Maize was sown in early May of each year with an inter-plant distance of 26 cm, and 225 kg N ha^{-1} (as urea), 120 kg P ha^{-1} and 60 kg K ha^{-1} were applied. One half of N and total P and K fertilizers were uniformly spread and plowed into the upper 20 cm of the soil prior to sowing, and the other half of the N fertilizer was applied during the bell-mouthed stage (V12). All plots were irrigated with 75 mm before seeding to ensure good germination, and weeds, pest, and disease controls were carried out as needed during the growing season.

In order to assess the interspecific rhizosphere facilitation on P uptake in maize/alfalfa polyculture, the experiment was split into two main plots with maize treated with two P levels: no more P fertilizer was applied (P0) and 120 kg P ha^{-1} was continuously fertilized (P1), while the subplot was treated with different cropping patterns (MM, MA, IMA4 and IMA6) beginning in 2013. Each treatment had three replications (Fig. S3). All other crop management practices were the same as in 2011.

Rhizosphere soil sampling and analysis

All samples for the present study were taken from 2013 to 2015. Rhizosphere soil samples were taken at the second early flowering stage of alfalfa when maize

was at anthesis. Because root exudates were sampled during their peak growth and the root metabolic activities were strongest, we could capture the largest variety and highest concentration of compounds released by roots (Oburger and Joones 2018). In the sole cropping systems, three representative plants of maize or alfalfa were randomly sampled. In the polyculture systems, maize and alfalfa were sampled separately. For alfalfa, plants were selected from each of the first three rows bordering maize; for maize, each of the first two (for IMA4) or three (for IMA6) rows adjacent to alfalfa was sampled (Fig. S2). Three representative plants were randomly sampled in each row, and all samples acquired from different rows were further analyzed as replications of the intercropped crop.

The rhizosphere soil was sampled based on the method that has been extensively used in the studies of root exudates (e.g. Li et al. 2007; Betencourt et al. 2012; Wang et al. 2017). Plant roots of both maize and alfalfa were excavated by removing a soil cylinder of approximately 20 cm diameter and 30 cm depth. Large soil aggregates were carefully crushed by tweezers if necessary. In order to reduce the possible influence of organic anions from broken root tissues, plant roots without obvious damage were taken and slightly shaken to remove excess soil. The soil that remained tightly adhered to root surfaces of belowground nodal roots and lateral roots (< 1–4 mm) was sampled as rhizosphere soil, and the shaken off soil was collected as non-rhizosphere soil. Soil collection of each sample was finished within several minutes to lessen the loss of root exudates due to microbial decomposition or transformation (Oburger and Joones 2018). The soil samples from three representative plants were mixed, sieved to <2 mm to remove visible roots, and then divided into two parts. One sub-sample was immediately transported to the laboratory and stored at −20 °C for the analyses of organic anions and acid phosphatase activity, and the other sub-sample was air dried for pH and soil-available P measurements.

The variety and concentration of organic anions were measured using ion chromatography (Baziramakenga et al. 1995). Organic anions were extracted by deionized water with a soil to water ratio of 1:2 (*w*/*v*). The extract was centrifuged at 10000 rpm for 10 min and then filtered for identification and quantification with an ion chromatograph (Dx300, Dionex, USA). Following the extraction of organic anions, solutions were acidified by adding two drops of concentrated H_3PO_4 and micropur (Sicheres Trinkwasser, Germany) solution at 0.01 g l^{-1} to inhibit the activity of microorganisms (Cheng et al. 2014; Oburger and Joones 2018). A 10 ml sub-sample of the solution containing organic anions was stored at −20 °C until analysis. Tartaric, citric, malic, formic, lactic, acetic and succinic anions were quantified, and the concentration was expressed as micromoles of organic anion produced per gram of dry soil.

Soil acid phosphatase activity was determined using p-nitrophenyl phosphate disodium (PNPP) as substrate (Tabatabai and Bremner 1969). At buffered pH 6.5, the pNP released by phosphatase was determined colorimetrically at 405 nm. Enzyme activity was expressed as micrograms of p-nitrophenol produced per gram of dry soil.

Soil pH was measured in soil suspension with deionized water in a soil to water ratio of 1:5 (*w*/*v*) (pHS-3C, SPSIC Corporation). Soil-available P was extracted by $NaHCO_3$ according to the Olsen Method, and Olsen-P concentration was determined with a molybdenum blue colorimetric method using UV photometry at 722 nm.

Root sampling and analysis

To investigate root morphology, distribution, and mycorrhizal colonization rate, root samples were also taken at the second flowering stage of alfalfa when maize was at anthesis. For root morphology and distribution, a detailed description for root sampling and analysis has been given in the previous paper (Sun et al. 2018b). Briefly, plants roots at the soil depth of 30 cm were excavated using the shovelomics method (Trachsel et al. 2011), and morphological traits of maize crown root (growth angle, number, dry weight, length, surface area, and volume) and alfalfa taproot (dry weight, surface area, and volume), crown (depth, branch number, and diameter), and lateral root (number, dry weight, length, surface area, and volume) were measured. Root distribution at 20 cm intervals to a maximum depth of 80 cm was measured by the method of soil auger sampling.

For mycorrhizal colonization rate, fresh root fractions with diameter smaller than 2 mm were collected from plant roots used for rhizosphere soil sampling. The fresh roots were cleaned with tap water and cut into segments about 1 cm long. A randomly selected 0.5 g sample of fresh roots was placed in 10% KOH solution overnight at room temperature, and then after several times of washing in water, roots were placed in a solution of 3% H_2O_2 and 10% ammonia for 20 min so as to remove color from the roots. After bleaching, roots were washed

three times in water and then stained with 0.05% Trypan Blue in a solution of glycerin, lactic acid, and distilled water in a 1:1:1 ratio by volume at a temperature of 80 °C (Brundrett et al. 1996). To avoid destruction of fragile root samples, stained roots without cutting were placed on microscope slides for observation, and mycorrhizal colonization rate was assessed as described by Trouvelot et al. (1986) using MYCOCALC software (www.dijon.inra.fr/mychintec/Mycocalc-prg/download.html).

Plant sampling and analysis

Maize grain and stalk were separately sampled for yield determination at physiological maturity, and alfalfa was harvested to a 5 cm stubble height three times (early June, mid July, and end of August), when it was flowering. In monoculture systems, two middle rows of maize or four middle rows of alfalfa were sampled. In the second strip of polyculture systems, alfalfa in the first three rows bordering maize was harvested, and maize in the first two (for IMA4) or three (for IMA6) rows adjacent to alfalfa was selected for sampling. All samples were oven-dried at 65 °C for 100 h, after which dry weight was determined. The aboveground oven-dried samples were ground for plant P analysis. P concentration was determined using the vanadomolybdate method after plant material was digested in a mixture of concentrated H_2SO_4 and $HClO_4$. Crop P uptake was calculated as the sum of P uptake of different organs (maize) or at different growth stages (alfalfa), which was determined by the product of P concentration multiplied by the dry yields of the crops.

Statistical analysis

Normal distribution and homogeneous variances were tested for all the data with Shapiro-Wilk tests using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). Repeated measures ANOVA in a general linear model (GLM) were conducted to assess the effects of cropping patterns and P levels on the characteristics of rhizosphere microenvironment (pH, total organic anions concentration, acid phosphatase activity, and Olsen-P concentration) and plant P uptake, with year as the repeated measure. The results were reported using the Greenhouse-Geisser correction when Mauchly's test of sphericity was violated. If the interaction between factors was significant, one-way ANOVA was conducted to evaluate the effects of cropping pattern and significant differences of means were compared with Duncan's multiple-compare range test; the effects of P level were tested by independent-samples t test. Significance level was set at $P < 0.05$.

A multivariate ordination method was used to analyze the relationships between rhizosphere P concentration and rhizosphere exudates and soil water content and between crop P uptake and rhizosphere P mobilization, root morphology and distribution, root colonization rate, as well as soil water content. Prior to analysis, the data of rhizosphere variables were standardized relative to that of non-rhizosphere (scaled to percentage change) for the relationship between rhizosphere P concentration and rhizosphere exudates and soil water content. To test whether weighted-averaging techniques or linear methods were appropriate, a de-trended correspondence analysis (DCA) was performed. All the longest gradients that resulted from the DCA were smaller than 3.0 for the analysis based on Eco-plates. Accordingly, redundancy analysis (RDA) was performed to study the contribution of rhizosphere exudates and soil water content to rhizosphere phosphorus mobilization and that of changes in rhizosphere P concentration, root morphology and distribution, root colonization rate, as well as soil water content to crop P uptake. The Monte Carlo permutation test was used to determine the significance of the axis and each predictor variable with 499 unrestricted permutations under the full RDA model ($P < 0.05$). All the analyses were performed using CANOCO version 5.1 (Microcomputer Power, Ithaca, USA). Based on the results of RDA, simple linear regressions and correlations were carried out by Sigmaplot 12.5 (Systat Software Inc., CA, USA) between dependent variable (rhizosphere P concentration or crop P uptake) and the explanatory variables with significant contributions.

Results

Phosphorus uptake of maize and alfalfa

Phosphorus uptake of maize and alfalfa were both significantly affected by cropping pattern (Table 1). Compared to monoculture, P uptake of maize in IMA4 and IMA6 was significantly reduced by 17% and 20%, respectively, while that of associated alfalfa was dramatically increased by 186% in both cases (Fig. 1). There

Table 1 Results of repeated measures ANOVA on phosphorus uptake of maize and alfalfa at harvest as well as Olsen-P concentration, pH, total organic anion (TOA) concentration and acid phosphatase activity (APA) in the rhizospheres of maize and alfalfa at flowering stage, with phosphorus level (P) and cropping pattern (CP) as the independent variables and year (Y) as the repeated measure

Factors	Df	Phosphorus uptake		Df	Maize				Alfalfa			
		Maize	Alfalfa		Olsen-P	pH	TOA	APA	Olsen-P	pH	TOA	APA
Y	2	249.93**	6.92*	2	65.31**	79.14**	19.48**	248.88**	22.05**	14.15**	62.02**	485.27**
P	1	98.72**	105.75**	1	1096.92**	10.47**	151.60**	57.82**	9.89**	6.67*	43.26**	11.08**
CP	2	12.36**	578.85**	3	65.36**	35.55**	144.04**	90.97**	64.61**	46.25**	175.01**	99.63**
Y × P	2	11.22**	11.93**	2	1.20 ns	4.91*	0.13 ns	0.39 ns	0.07 ns	1.39 ns	0.14 ns	0.04 ns
Y × CP	4	1.06 ns	4.74**	6	0.65 ns	0.58 ns	0.22 ns	1.90 ns	0.42 ns	4.57**	1.34 ns	9.73**
P × CP	2	2.93 ns	26.44**	3	10.61**	0.26 ns	8.62**	3.36*	4.56**	0.58 ns	11.28**	6.97**
Y × P × CP	4	0.16 ns	2.18 ns	6	0.28 ns	2.91*	0.26 ns	0.16 ns	0.05 ns	0.65 ns	0.14 ns	0.36 ns

Df, degrees of freedom; *ns*, no significant difference

* $P < 0.05$, ** $P < 0.01$

was a significant interaction between P level and year on crop P uptake (Table 1). P uptake of maize and alfalfa at the P0 level were both significantly reduced relative to P1, and the reduction in 2013 was smaller than that in 2014 and 2015 (Fig. 1).

Olsen-P concentration in the rhizosphere soil of maize and alfalfa

Olsen-P concentration in the rhizosphere soil of both maize and alfalfa was significantly affected by the interaction between cropping pattern and P level (Table 1). Compared to non-rhizosphere soil, Olsen-P concentration in the rhizosphere soil of maize in all cropping patterns and alfalfa in intercropping was significantly increased, irrespective of P level (Fig. 2). Compared to monoculture, IMA4 and IMA6 had significantly higher rhizosphere Olsen-P concentration by 26% and 19% in maize and by 44% and 35% in alfalfa, and the increase at the P0 level was much greater than that at the P1 level (Fig. 2). The effects of P level on rhizosphere Olsen-P concentration of maize and alfalfa were contrasting. For maize, rhizosphere Olsen-P concentration at the P1 level was dramatically higher than that at the P0 level,

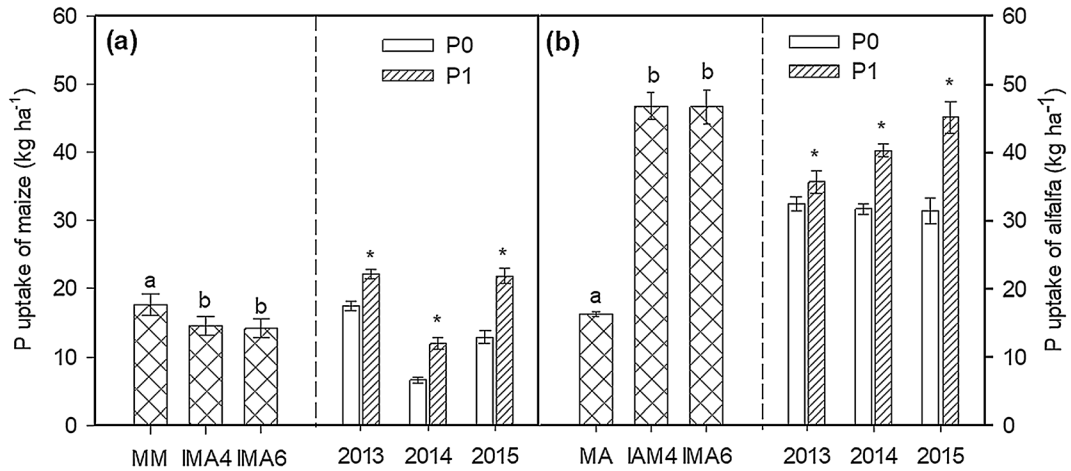

Fig. 1 Comparisons of phosphorus uptake of maize (a) and alfalfa (b) at harvest in different cropping patterns at two P levels in three years. P0 = without phosphorus fertilizer, P1 = with phosphorus fertilizer, MM = monoculture maize, MA = monoculture alfalfa, IMA4 = four rows of maize intercropping with six rows of alfalfa, IMA6 = six rows of maize intercropping with six rows of alfalfa. Different lowercase letters indicate significant difference among different cropping patterns, and * denotes significant difference between two P levels ($P < 0.05$). Values = means ± SE

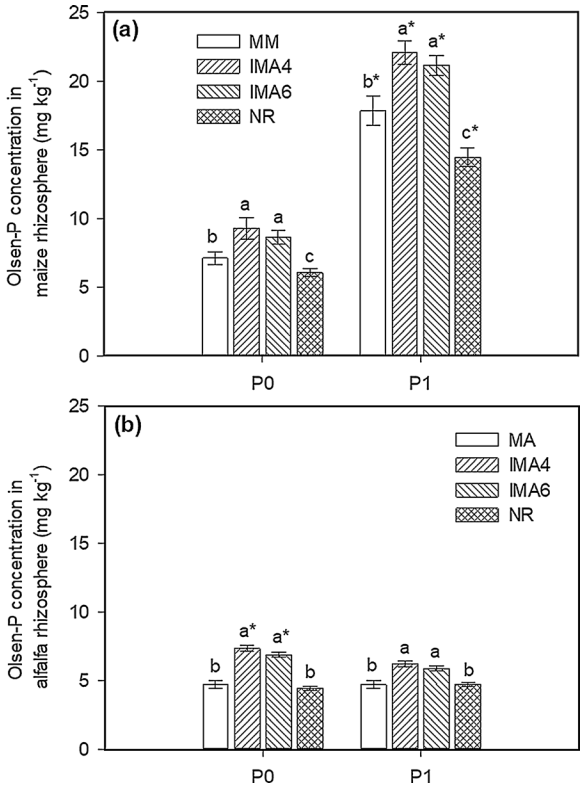

Fig. 2 Comparisons of Olsen-P concentration in the rhizosphere soil of maize (**a**) and alfalfa (**b**) at flowering stage in different cropping patterns at two P levels in three years

NR = non-rhizosphere. Other symbols are the same as for Fig. 1.

regardless of cropping pattern (Fig. 2a); while for alfalfa, P addition significantly reduced Olsen-P concentration in the rhizosphere when alfalfa was intercropped with maize (Fig. 2b).

Rhizosphere pH, organic anions concentration, and acid phosphatase activity of maize and alfalfa

Maize rhizosphere pH was significantly affected by cropping pattern and the interaction between P level and year, while that of alfalfa was dramatically influenced by P level and the interaction between cropping pattern and year (Table 1). Compared to non-rhizosphere, maize in all cropping patterns and alfalfa in intercropping had significantly reduced pH in the rhizosphere (Fig. 3a, b). Polyculture significantly reduced rhizosphere pH of both maize and alfalfa, and rhizosphere pH in IMA4 and IMA6 was significantly reduced by 0.4 and 0.3 units in maize and by 0.3 and 0.2 units in alfalfa relative to monoculture (Fig. 3a, b). Moreover, the reduction in alfalfa rhizosphere pH in 2014 and 2015 was much greater than that in 2013 (Fig. 3b). Regarding the effects of P level, pH in maize rhizosphere at the P0 level was significantly reduced relative to P1 in 2013, while no significant difference was observed between the two P levels in 2014 and 2015 (Fig. 3a). For alfalfa, rhizosphere pH at the P0 level was significantly lower than that at the P1 level (Fig. 3b).

Different from pH, rhizosphere total organic anions (TOA) concentration and acid phosphatase activity (APA) of both maize and alfalfa were significantly affected by the interaction between cropping pattern and P level (Table 1). Compared to P1, rhizosphere TOA concentration and APA of maize in all cropping patterns and that of intercropped alfalfa were significantly increased at the P0 level. Regardless of P level, TOA concentration and APA in the rhizosphere of maize in all cropping patterns and alfalfa in intercropping were dramatically greater than that in the non-rhizosphere (Fig. 3c-f). Polyculture, especially at the P0 level, significantly improved TOA concentration and APA of both maize and alfalfa in the rhizosphere soil. On average, IMA4 and IMA6 had 32% and 24% (maize) and 33% and 27% (alfalfa) higher rhizosphere TOA concentration and 23% and 18% (maize) and 50% and 32% (alfalfa) greater rhizosphere APA than these parameters in monoculture (Fig. 3c-f).

For the components of organic anions, tartaric, citric, and succinic anions were found in the non-rhizosphere and rhizosphere soil of maize and alfalfa in all cropping patterns, while malic, formic, lactic, and acetic anions were only found in the rhizosphere soil of intercropped maize and alfalfa, irrespectively of P level (Figs. S4 and S5). Compared to non-rhizosphere, maize in all cropping patterns and alfalfa in intercropping had significantly improved concentrations of tartaric, citric, and succinic anions in the rhizosphere soil (Figs. S4a-c and S5a-c). In the rhizosphere soil, tartaric, citric, and succinic anions concentrations of both maize and alfalfa in polyculture was significantly higher than that in monoculture (Figs. S4a-c and S5a-c). Compared to P1, concentration of all the organic anions in the rhizosphere of maize and intercropped alfalfa was significantly increased at the P0 level (Figs. S4 and S5). Among all the organic anions, succinic anion was the dominant carboxylate in the rhizosphere of both maize and alfalfa, accounting for a large percentage of 73% and 76% in TOA, respectively (Figs. S4 and S5). Thus, succinic anion concentration had a significant effect and provided the greatest explanation for the variability of TOA concentration, by 87.7% in

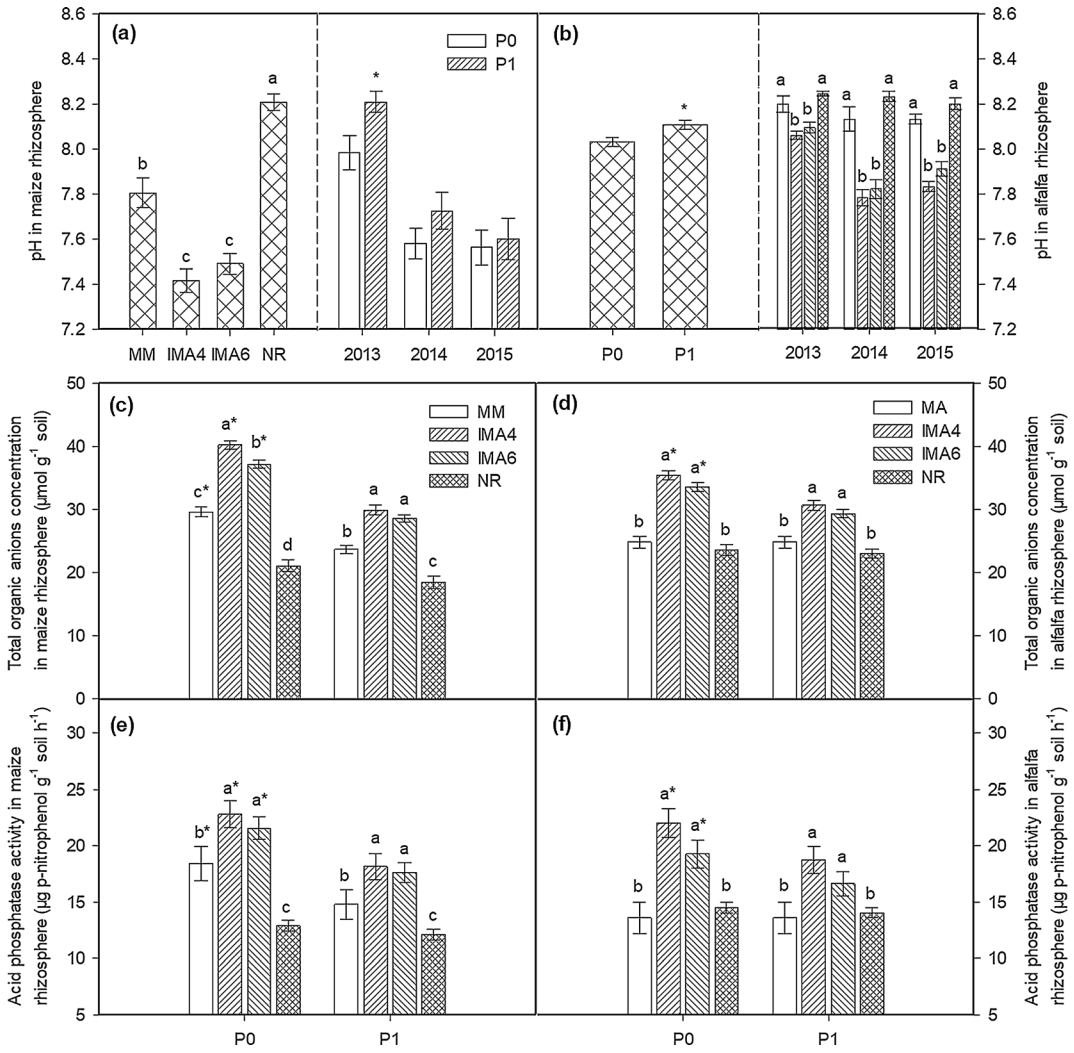

Fig. 3 Comparisons of pH, total organic anions concentration, and acid phosphatase activity in the rhizosphere soil of maize (a, c, e) and alfalfa (b, d, f) at flowering stage in different cropping patterns at two P levels in three years. Symbols are the same as for Fig. 1 and Fig. 2

maize and 90.6% in alfalfa (Table S1; Fig. S6). TOA concentration of both maize and alfalfa was significantly and positively associated with succinic anion concentration in the rhizosphere (Fig. S7). The variation of TOA concentration contributed to the reduction of rhizosphere pH, as rhizosphere pH of both maize and alfalfa was dramatically and negatively associated with TOA concentration in the rhizosphere (Fig. S8).

The changes of rhizosphere pH, TOA concentration, and APA were also closely related to variations in soil water content. Compared to monoculture, soil water content of intercropped maize was significantly reduced by an average of 14% during the vegetation period, while that of associated alfalfa was dramatically improved by 18% on average (Fig. S9). Rhizosphere pH and APA of both maize and alfalfa as well as rhizosphere TOA concentration of alfalfa were all significantly and positively associated with soil water content, and no pronounced correlation was found between rhizosphere TOA concentration of maize and soil water content (Fig. S10).

Relationships between rhizosphere phosphorus concentration and rhizosphere pH, TOA concentration, APA, and soil water content

Redundancy analysis (RDA) showed that rhizosphere pH, TOA concentration, APA, and soil water content

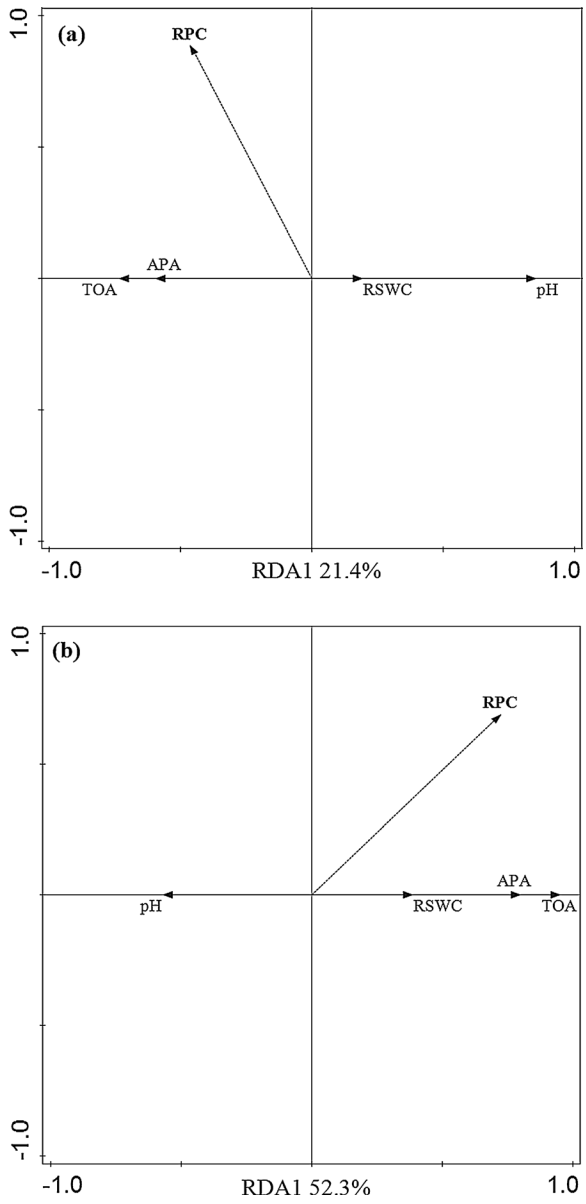

Fig. 4 Redundancy analysis (RDA) of rhizosphere phosphorus concentration in relation to rhizosphere exudates and soil water content of maize (a) and alfalfa (b). RPC = rhizosphere phosphorus concentration, RSWC = rhizosphere soil water content, TOA = total organic anions, APA = acid phosphatase activity. The dashed arrow represents rhizosphere phosphorus concentration (dependent variable), and solid arrows represent rhizosphere root exudates and soil water content (explanatory variables). The data of rhizosphere variables were standardized relative to that of non-rhizosphere, and scaled to percentage change

could explain 21.4% and 52.3% of the variation in rhizosphere P concentration of maize and alfalfa, respectively (Fig. 4). Among all the constrained variables, rhizosphere pH had significant influence on rhizosphere

Table 2 Results of permutation test of redundancy analysis (RDA) on predictor variables for rhizosphere phosphorus concentration and plant phosphorus uptake

Maize			Alfalfa		
Variable	Explains %	*P* value	Variable	Explains %	*P* value
Rhizosphere phosphorus concentration					
pH	15.5	0.004	TOA	47.2	0.002
TOA	4.5	0.082	APA	2.7	0.144
RSWC	0.7	0.526	pH	1.8	0.190
APA	0.7	0.526	RSWC	0.5	0.470
Plant phosphorus uptake					
CRSA	58.4	0.002	LRV	86.6	0.002
RPC	24.2	0.004	LRN	2.5	0.032
CRV	1.6	0.286	TRDW	3.4	0.058
CRDW	0.5	0.554	TRV	3	0.068
M	0.4	0.574	LRDW	0.3	0.422
CRGA	0.3	0.666	CD	1.1	0.128
CRL	0.4	0.612	CBD	0.8	0.140
RLD	0.4	0.596	CBR	< 0.1	0.646
–	–	–	LRL	< 0.1	0.830
–	–	–	RPC	0.2	0.496

The Explains % column values represent the percentage of total variation per explanatory variable. The order of the explanatory variables is the order of inclusion in the manual selection of explanatory variables and is driven by the effect size

TOA, total organic anions; *APA*, acid phosphatase activity; *RSWC*, rhizosphere soil water content; *RPC*, rhizosphere phosphorus concentration; *CRGA*, crown root growth angle; *CRN*, crown root number; *CRDW*, crown root dry weight; *CRL*, crown root length; *CRSA*, crown root surface area; *CRV*, crown root volume; *RLD*, root length density at 0–20 cm; *TRDW*, taproot dry weight; *TRV*, taproot volume; *LRN*, lateral root number; *LRDW*, lateral root dry weight; *LRL*, lateral root length; *LRV*, lateral root volume; *CD*, crown depth; *CBR*, crown branch number; *CBD*, crown branch diameter; *M*, mycorrhizal colonization rate

P concentration of maize, and explained 15.5% of the variance (Table 2). Olsen-P concentration in maize rhizosphere was significantly and negatively associated with rhizosphere pH (Fig. 5a). As for rhizosphere TOA concentration, soil water content, and APA, none of the three variables had significant impact on rhizosphere P concentration of maize ($P = 0.082$, 0.526, and 0.526, respectively), and only explained the variance by 4.5%, 0.7%, and 0.7%, respectively (Table 2). For alfalfa, only TOA had significant impact on rhizosphere P concentration, accounting for 47.2% of its variation (Table 2). Olsen-P concentration in alfalfa rhizosphere was significantly and positively associated with TOA

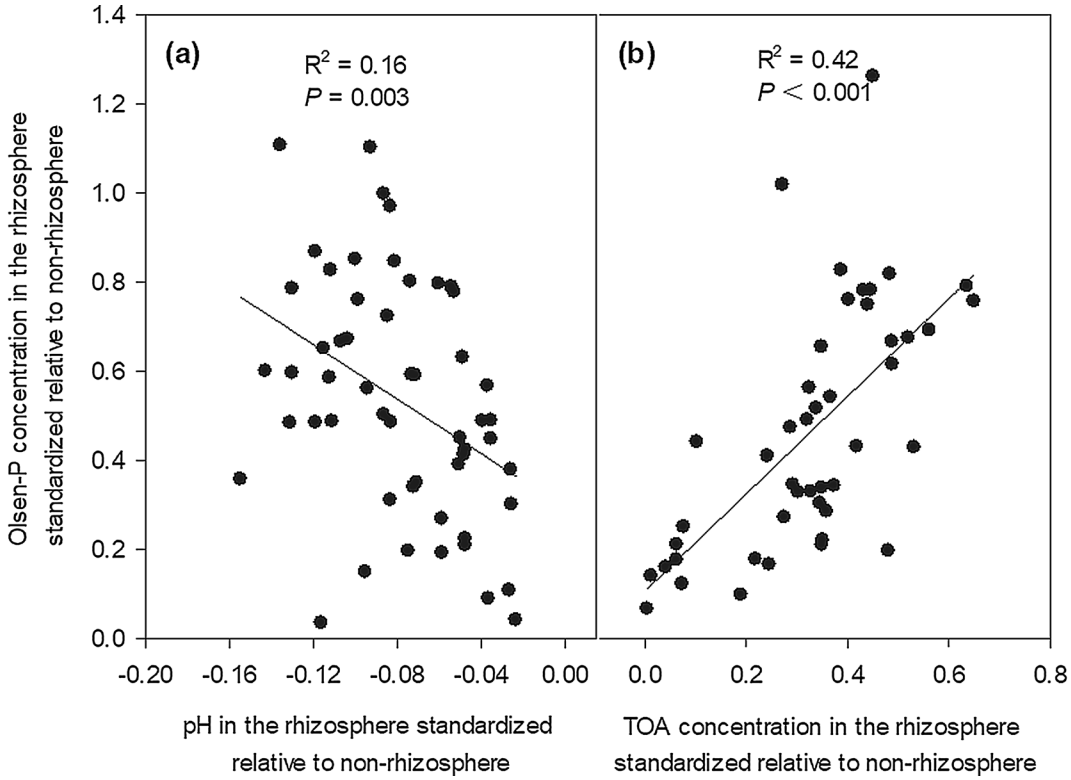

Fig. 5 Relationships between rhizosphere Olsen-P concentration and pH of maize (a) and total organic anions concentration of alfalfa (b). The values of the rhizosphere variables were standardized relative to that of non-rhizosphere (scaled to percentage change)

concentration (Fig. 5b). Rhizosphere APA, soil water content, and pH only explained the variance of rhizosphere P concentration of alfalfa by 2.7%, 1.8%, and 0.5%, respectively, and thus had no significant influence on rhizosphere P concentration ($P = 0.144$, 0.190, and 0.470, respectively) (Table 2).

Relationships between crop phosphorus uptake and rhizosphere phosphorus mobilization, root morphology and distribution, root mycorrhizal colonization rate, as well as soil water content

According to the RDA, 86.1% and 98.1% of all the variance of maize and alfalfa P uptake were attributed to rhizosphere P mobilization, root morphology and distribution, root mycorrhizal colonization rate, and soil water content (Fig. 6). Specifically, 58.4% and 24.2% of the variability in maize P uptake was due to changes of crown root surface area and rhizosphere P concentration, and both of the two variables had significant influences and positive associations with maize P uptake (Table 2; Fig. 7a, b). Although mycorrhizal colonization rate of intercropped maize was significantly greater than that in monoculture by 79% in IMA4 and 64% in IMA6, the variation in mycorrhizal colonization rate only explained the variability of maize P uptake by 0.4%, hence its effect on maize P uptake was not significant ($P = 0.574$) (Fig. S11a; Table 2). In contrast to maize responses, only lateral root volume and number had significant influences on alfalfa P uptake, and explained the variability of alfalfa P uptake by 86.6% and 2.5%, respectively (Table 2). Alfalfa P uptake was dramatically and positively associated with lateral root volume and number (Fig. 7c, d). As for rhizosphere P concentration, it only explained 0.2% of the variance in alfalfa P uptake, and therefore had no significant effect on alfalfa P uptake ($P = 0.496$) (Table 2). Mycorrhizal colonization rate of intercropped alfalfa was dramatically enhanced by 50% in IMA4 and 41% in IMA6 as compared to monoculture, but it had no significant effect and thus provided no explanation for the variability of alfalfa P uptake (Fig. S11b; Table 2). For both maize and alfalfa, soil water content had no significant effect and no contribution to the variance of P uptake (Table 2).

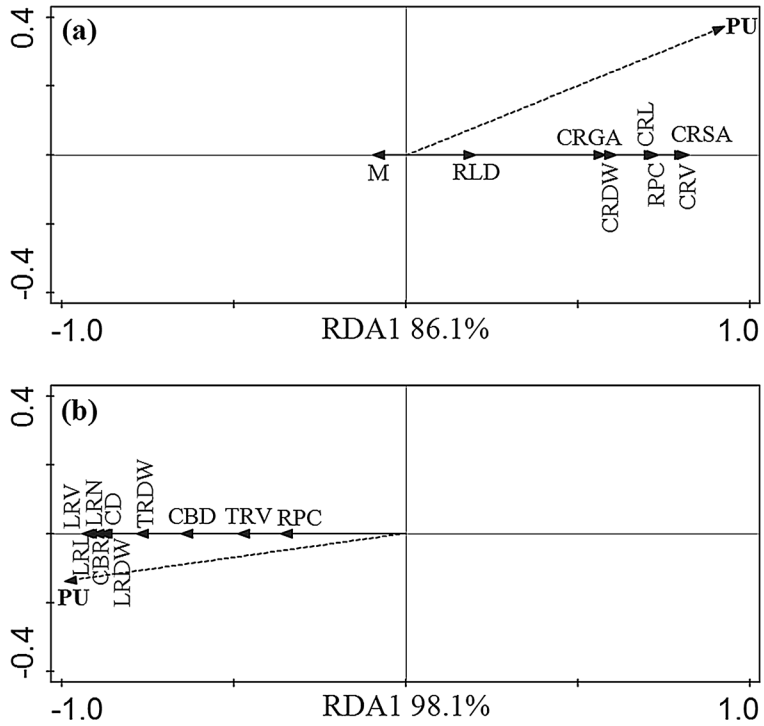

Fig. 6 Redundancy analysis (RDA) of phosphorus uptake in relation to rhizosphere phosphorus mobilization, root morphology and distribution, root colonization rate, as well as soil water content of maize (a) and alfalfa (b). Rhizosphere P mobilization is represented by rhizosphere phosphorus concentration (RPC) mobilized by root exudates. Root morphological and distribution traits of maize consist of crown root growth angle (CRGA), crown root number (CRN), crown root dry weight (CRDW), crown root length (CRL), crown root surface area (CRSA), crown root volume (CRV), and root length density at 0–20 cm (RLD), and that of alfalfa include taproot dry weight (TRDW), taproot surface area (TRSA), taproot volume (TRV), lateral root number (LRN), lateral root dry weight (LRDW), lateral root length (LRL), lateral root surface area (LRSA), lateral root volume (LRV), crown depth (CD), crown branch number (CBR), crown branch diameter (CBD), and root length density at 0–20 cm (RLD). M = mycorrhizal colonization rate, and SWC = soil water content. The dashed arrow represents phosphorus uptake (dependent variable), and solid arrows represent rhizosphere phosphorus concentration, root morphological and distribution traits, root colonization rate, as well as soil water content (explanatory variables)

Discussion

The relative contributions of rhizosphere pH, organic anions concentration, and phosphatase activity to rhizosphere soil P mobilization

In the present study, we found that an increase in P availability occurred in the rhizosphere soil of maize and alfalfa as compared to non-rhizosphere (Fig. 2). This result contradicts with the view that P depletion occurs in the rhizosphere soil and P availability tends to be reduced relative to non-rhizosphere soil (Hinsinger et al. 2011b). The authors explained that the P depletion was due to root uptake for soil P and the restricted diffusion of phosphate ions in soil (Barber 1995; Hinsinger 2001). However, a number of studies have reported consistent results (Chen et al. 2002; Li et al. 2008; Vu et al. 2008; Devau et al. 2010, 2011; Betencourt et al. 2012). For example, Betencourt et al. (2012) found durum wheat and chickpea increased P concentration in the rhizosphere soil relative to bulk soil, irrespective of monoculture or intercropping. The increase of P availability in the rhizosphere soil can be attributed to the fact that root exudates secretion and soil P mobilization co-occur with the depletion of P in the rhizosphere, and can counteract the uptake-driven depletion (Geelhoed et al. 1999; Devau et al. 2010, 2011). In the present study, both maize and alfalfa exhibited significantly reduced pH and dramatically improved TOA concentration and APA in the rhizosphere soil, inducing soil P mobilization and thereby significantly increased P concentration in the rhizosphere relative to non-rhizosphere (Figs. 2 and 3).

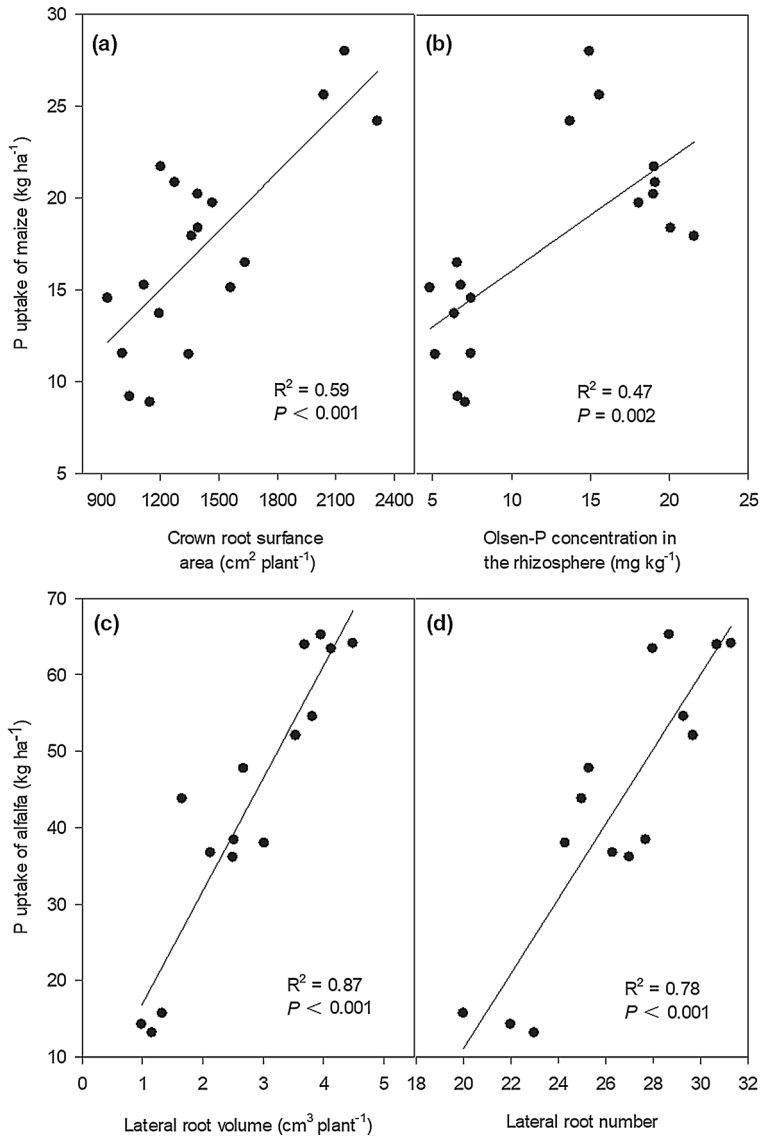

Fig. 7 Relationships between phosphorus uptake and crown root surface area and rhizosphere Olsen-P concentration of maize (**a**-**b**) and lateral root volume and number of alfalfa (**c**-**d**)

Compared to monoculture, maize/alfalfa polyculture stimulated rhizosphere soil P mobilization of both maize and alfalfa with significantly increased rhizosphere soil P concentration (Fig. 2). This result is in agreement with previous studies showing that rhizosphere soil P availability of both intercropped species was significantly enhanced in wheat/faba bean and durum wheat/chickpea polycultures (Song et al. 2007; Betencourt et al. 2012). There are several reasons that may contribute to rhizosphere soil P mobilization. First, root exudates-induced rhizosphere changes in pH, organic anions concentration, and acid phosphatase activity are well known as important strategies to mobilize P from the absorbed mineral or organic fractions in the rhizosphere soil and thereby improve soil P bioavailability (Hinsinger et al. 2011a; Shen et al. 2011; Li et al. 2014). As shown in our results, pH in the rhizosphere of both intercropped maize and alfalfa was significantly reduced, while TOA concentration and APA in the rhizosphere were both dramatically enhanced (Fig. 3). Rhizosphere acidification is mainly induced by root secretion of protons, which is driven by nitrogen (N) nutrition through cation-anion balance, as well as the excretion of organic anions (Hinsinger et al. 2003). In the present study, maize in all cropping patterns was provided with sufficient ammonium N fertilizer, and there was little

difference between monoculture and intercropping in cation-anion balance and thereby proton secretion. However, maize rhizosphere pH was significantly and negatively correlated with rhizosphere TOA concentration (Fig. S8a), demonstrating that the increased exudation of organic anions can be the possible reason for rhizosphere pH reduction of intercropped maize. As for alfalfa, rhizosphere pH also had a significant and negative association with rhizosphere TOA concentration (Fig. S8b), suggesting that organic anions secretion also has a certain contribution to rhizosphere acidification of intercropped alfalfa. In N nutrition, alfalfa in all planting modes was supplied with a small amount of ammonium N fertilizer only in the planting year as startup N, and both monoculture and intercropped alfalfa were dependent on N_2 fixation to meet N needs. However, compared to monoculture, intercropped alfalfa suffered N competition from associated maize due to its root proliferation laterally towards the alfalfa growth zone (Sun et al. 2018b), resulting in a greater dependence on N_2 fixation of intercropped alfalfa and thereby greater proton secretion and rhizosphere pH reduction (Hauggaard-Nielsen et al. 2009; Hinsinger et al. 2011a). Therefore, the reduction of rhizosphere pH of intercropped alfalfa was the result of the combined effects of root secretion of organic anions and proton.

Among different organic anions components, citrate and malate were reported to be the dominant organic anions exuded by maize and alfalfa roots (Lipton et al. 1987; Jones and Darrah 1995). Consistent with these reports, our study found that intercropped maize and alfalfa secreted similar amounts of citric and malic anions (Figs. S4 and S5). However, succinic and tartaric anions were also observed as important components of carboxylates in the maize and alfalfa rhizosphere (Figs. S4 and S5). The large secretion of succinic and tartaric anions may be due to the fact that our study was conducted in a polyculture system under field conditions and root-induced organic anions secretion was analyzed at the flowering stage, whereas crops were monoplanted in growth chambers and exudates were collected from seedling roots in the two previous studies (Lipton et al. 1987; Jones and Darrah 1995). In polyculture, root interaction and resource competition between species can stimulate crops to coordinate rhizosphere activities to enhance plant fitness, resulting in changes in root exudates secretion relative to monoculture (Hess and De Kroon 2007; Li et al. 2010; Li et al. 2016; Zhang et al. 2016). Compared to growth chamber studies, field conditions may induce variation in root exudates amount and composition, because the crops grow in completely different environments (e.g. rhizobia bacteria and mycorrhizal fungi, soil structure and texture, and soil pathogens) (Vance et al. 2003). In addition, root exudates secretion varies with plant developmental stages, generally with the lowest amounts in seedlings and peaking during the flowering stage (Johnson et al. 1996; Aulakh et al. 2001; Badri and Vivanco 2009; Wang et al. 2017; Oburger and Joones 2018).

Second, soil water content is an important factor regulating root exudation processes (Badri and Vivanco 2009). For example, high soil moisture limited the availability of oxygen and stimulated the accumulation of lactic acid (Rivoal and Hanson 1994). In our maize/alfalfa polyculture, soil water content in the maize growth zone was significantly reduced by 14% (Fig. S9a-c), and promoted root secretion of protons but decreased that of acid phosphatase (Fig. S10a-c). On the contrary, soil water content in the growth zone of associated alfalfa was dramatically increased by 18% (Fig. S9d-f), and stimulated the secretion of organic anions and acid phosphatase but reduced that of protons (Fig. S10d-f). These results indicate that soil water content has an important role in root exudates secretion of both maize and alfalfa and may further affect rhizosphere soil P mobilization and thereby plant P uptake, as the diffusion of root exudates and soil available P is significantly influenced by soil water availability (Lambers et al. 2006; Hinsinger et al. 2009).

In order to evaluate the relative importance of rhizosphere pH, TOA concentration, APA, and soil water content to rhizosphere soil P mobilization, RDA was used. We found that rhizosphere P concentration of maize was significantly and negatively influenced by rhizosphere pH, while for alfalfa, TOA concentration had significant and positive impact on rhizosphere P concentration (Table 2; Figs. 4 and 5). These results indicate that the contributions of root exudates to rhizosphere soil P mobilization vary with plant species, and the reduction of pH and the increase of TOA concentration played the most important roles in rhizosphere soil P mobilization of maize and alfalfa, respectively. In addition, the increase in rhizosphere TOA concentration of intercropped alfalfa was mainly induced by succinic anion (Figs. S6b and S7d; Table S1). This result is different from a previous study which showed that citrate, which has three carboxyl groups, was more effective to mobilize soil P

than carboxylates with two carboxyl groups, e.g. succinate, malate, and malonate (Jones and Brassington 1998). In the present study, the greater effect of succinate than that of citrate on rhizosphere soil P mobilization of alfalfa was mainly attributed to its large percentage of 76% in the total organic anions (Fig. S5; Pang et al. 2018). For both maize and alfalfa, acid phosphatase had the smallest contribution to rhizosphere soil P mobilization relative to pH and TOA concentration, even though organic P accounts for 48% of the total P in the field. This may be attributed to the fact that the efficiency of phosphatase can be greatly altered by soil physical and chemical environments and its interactions with soil microorganisms (George et al. 2005). Our findings fill the knowledge gap concerning the relative importance of pH, organic anions, and phosphatase for rhizosphere soil P mobilization, and rhizosphere pH and TOA concentration were good predictors for improving rhizosphere soil P availability.

It is reported that legumes generally have greater ability in root exudates secretion than cereals (Neumann and Römheld 1999; Hinsinger et al. 2003; Vance et al. 2003; Nuruzzaman et al. 2006; Pearse et al. 2006). For example, faba bean and chickpea had greater ability in organic anions, proton, and acid phosphatase secretion, and thereby greater improvement in soil P bioavailability relative to intercropped maize (Li et al. 2004; Li et al. 2007). In contrast, our study found that maize and alfalfa in polyculture had similar root secretion of protons and organic anions, resulting in a similar reduction of rhizosphere pH (by 0.35 and 0.24 units) and a similar increase of rhizosphere organic anions concentration (by 28% and 30%) as compared to corresponding monoculture (Fig. 3a-d). Our study did not provide a clear answer for the similar changes of maize and alfalfa in rhizosphere pH and organic anions concentration. Different from proton and organic anions secretion, phosphatase secretion of intercropped alfalfa was much greater than that of associated maize, resulting in the greater improvement in rhizosphere acid phosphatase activity of alfalfa (by 41%) than that of maize (by 21%) (Fig. 3e, f). Consequently, the increase in rhizosphere soil P availability of intercropped alfalfa (by 40%) was much larger than that of neighboring maize (by 22%) (Fig. 2).

The relative contributions of rhizosphere soil P mobilization, root architecture, and mycorrhizal association to crop P uptake

Besides rhizosphere soil P mobilization and soil water content, changes in root architecture and mycorrhizal association are well known as important ways to affect plant P acquisition (Lambers et al. 2006; Hinsinger et al. 2011a; Shen et al. 2011; Giehl and von Wiren 2014). Our recent study in maize/alfalfa polyculture found that maize P uptake reduction was due to the decrease of crown root number, surface area, and volume, while P uptake improvement of associated alfalfa was the consequence of the increased growth of taproot, crown, and lateral roots (Sun et al. 2018b). In mycorrhizal association, our results found that compared to monoculture, mycorrhizal colonization rate of maize and alfalfa roots in polyculture was significantly improved by 73% and 45%, respectively (Fig. S11), which may increase soil P transfer to crop roots and improve crop P uptake via extensive hyphae of mycorrhizal fungi (Lambers et al. 2006; Shen et al. 2011; Lambers and Teste 2013). However, when we combined all the factors affecting plant P uptake together and analyzed the relative contribution of each factor in the RDA model, we found that the increase in mycorrhizal colonization rate had no significant contribution to P uptake variation of both maize and alfalfa (Table 2). Similarly, soil water content also had no significant contribution to P uptake of maize and alfalfa (Table 2). These results suggest that P acquisition of maize and alfalfa may not be influenced by soil P transfer and diffusion towards roots, because these processes were strongly impacted by changes in mycorrhizal colonization and soil water availability (Lambers et al. 2006; Lambers and Teste 2013). Here our results emphasized that mycorrhizal colonization rate and soil water content had minor direct effects on plant P uptake, whereas rhizosphere soil P mobilization and root exploration and exploitation are vital for plant P uptake. This was clearly shown in the RDA model, where changes in root exudates and root architecture explained 82.6% and 89.1% of the variance of maize and alfalfa P uptake, respectively (Table 2).

As for the relative importance of root exudates and root architecture to P acquisition, RDA results showed that rhizosphere soil P mobilization induced by root exudates facilitated maize P uptake in polyculture, but this facilitation was offset by the reduction of P uptake due to the decrease of crown root surface area (Figs. 6a

and 7a, b; Table 2), resulting in the reduction of P uptake in the intercropped maize (Fig. 1a). As for alfalfa, P uptake improvement in polyculture was mainly due to the increase of lateral root volume (explained by 86.6%), with no significant contribution from rhizosphere soil P mobilization (Figs. 6b and 7c; Table 2). These results indicate that root architecture plays a critical role in P acquisition of both intercropped maize and alfalfa. This can be explained by the following two facts: (1) Root architecture not only regulates the exploration and exploitation of localized P resources by the plant and the distribution of roots relative to their neighbors within and among root systems, but also determines the placement and functional benefit of root exudates in specific soil domains. Root architecture is a higher order trait relative to root exudates in affecting plant P acquisition (Lynch 1995, 2011; Lynch and Brown 2001). (2) Compared to root exudates, root architecture can increase P acquisition at lower carbon cost, and tends to be allocated more carbon and resources to improve plant P acquisition (Lynch and Ho 2005; Raven et al. 2018; Wang and Lambers 2019). This is the first report to evaluate the relative contributions of root exudates, architecture, and mycorrhizal association to plant P uptake under field conditions. Our results demonstrate that root architecture is a determinant factor for plant P acquisition and merits consideration as an important strategy to improve crop P capture in polyculture, especially through enhancing root surface area and volume.

Conclusions

The reduction of rhizosphere pH and the increase of organic anions concentration played critical roles in rhizosphere soil P mobilization of maize and alfalfa, and acid phosphatase had a relatively small contribution. Although root exudates played important roles in improving rhizosphere soil P availability, root architectural traits of crown root surface area and lateral root volume were the crucial factors in affecting P uptake of maize and alfalfa, and mycorrhizal colonization had minor direct effects. Our findings fill the knowledge gaps concerning the relative importance of pH, organic anions, and phosphatase for rhizosphere soil P mobilization as well as the relative contributions of root exudates-induced rhizosphere soil P mobilization and root changes in architecture and mycorrhizal association to plant P acquisition. This is important in developing strategies through maximizing rhizosphere or root changes to improve soil P availability and crop P acquisition. The challenge of enhancing food supply, especially in P limiting soil, could be accomplished by taking advantage of changes in root rhizosphere, architecture, and mycorrhizal association in facilitating P acquisition.

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