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REGULAR ARTICLE

Effects of oxytetracycline on plant growth, phosphorus uptake, and carboxylates in the rhizosheath of alfalfa

Zekun Zhang · Rui Su · Chao Chang · Xiao Cheng · Qi Peng · Hans Lambers · Honghua He

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Abstract
Aims Residues of antibiotics such as oxytetracycline (OTC) in soil can affect microbial compositions and activities, thus affecting soil P availability, and consequently plant P uptake and growth.
Methods A pot experiment was performed to grow alfalfa in a loess soil with different doses of P (0, 25, 50, and 100 mg kg^{-1}) and OTC (0, 25, 50, and 100 mg kg^{-1}). Plant dry mass, shoot and root P concentrations, bulk soil and rhizosheath pH, rhizosheath carboxylates, and bulk soil alkaline phosphatase activity were determined.
Results Shoot dry mass and root dry mass increased with increasing P dose, while shoot dry mass decreased with increasing OTC dose, especially at lower P doses (0 and 25 mg kg^{-1}). Addition of OTC slightly reduced P concentrations in shoots and roots, but did not reduce plant P content consistently. Increasing OTC dose significantly reduced bulk soil alkaline phosphatase activity at 0P and strongly reduced rhizosheath tartrate amount at all P doses.
Conclusions The effects of OTC on plant growth and P uptake depended on both OTC and P doses in soil. High OTC dose had negative effects on shoot P uptake and growth, especially at lower P doses, while it had a positive effect on root growth at higher P doses.

Keywords Antibiotic · *Medicago sativa* · Phosphatase · Phosphorus-uptake efficiency · Tartrate

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Introduction

Phosphorus (P) is a macronutrient for plant growth and plays an important role in plant metabolisms (Johnston et al. 2014). Plant growth is often limited by P, due to low P availability in soil, even when a large amount of P fertilizer has been applied (Vance et al. 2003; Scholz et al. 2013). In agricultural production, P fertilizers are often applied to increase the availability of P in soil and the yields of crops. However, soon after application, most of the P fertilizer becomes unavailable to plants; the utilization rate of P fertilizers in field experiments is only 10–15%, rarely reaching 25% (Johnston et al. 2014). After being applied into soil, inorganic phosphate (Pi) is easily sorbed onto oxides and hydroxides of iron and aluminum or precipitated as calcium phosphates, thus resulting in low concentration of P in the soil solution, and consequently low absorption and utilization efficiency of P in plants (Hinsinger 2001; Johnston et al. 2014; Simpson et al. 2015). Excessive use of P fertilizers can lead to eutrophication of water and environmental pollution (Conley et al. 2009). At present, P fertilizers are mainly produced from phosphate rock, which is a non-renewable resource and may be exhausted in the next 50–100 years (Richardson et al. 2011). Therefore, it is necessary to use P fertilizers rationally and improve the uptake and utilization efficiency of P in crops (Richardson et al. 2011).

Plants show a series of adaptations to P deficiency, including altering the morphology of their root system, promoting the secretion of root exudates such as carboxylates and phosphatases, and forming plant-microbial symbiosis (Chiou and Lin 2011; Ham et al. 2018). Under P deficiency, plants may increase the length and surface area of roots and thus expand the contact of roots with soil and increase the uptake of P (Zribi et al. 2014). The activity of phosphatase, which converts organic P to inorganic P, may be enhanced in soil with low P availability, thus mitigating the adverse effects of P deficiency on plants (Pant and Warman 2000; Kitayama 2013). Roots can release a large amount of organic anions such as carboxylates to mobilize sparingly-soluble soil P (Chen and Liao 2016).

Antibiotics are widely used to treat infectious diseases in humans, livestock, and poultry by directly killing or inhibiting the growth of bacteria (Pan and Chu 2016; Roose-Amsaleg and Laverman 2016). However, antibiotics used in animals are not readily absorbed by the gut, and most (about 70%) of the antibiotics are excreted with urine and feces (Bellino et al. 2018). The residues of antibiotics are highly stable, and can enter the environment and be detected in water and soil at high concentrations (Bondarczuk et al. 2016; Menz et al. 2019). Certain concentrations of antibiotics can directly cause toxic effects on plants, inhibit the growth of roots, and reduce the biomass of crops, and also adversely affect physiological functions such as photosynthesis (Pan and Chu 2016; Minden et al. 2017). Tetracyclines (TCs) can inhibit the growth of soil microorganisms and reduce the enzyme activity in the rhizosphere of wheat (Yao et al. 2010), and sulfonamides can suppress soil microbial respiration by over 30% (Liu et al. 2009).

Oxytetracycline (OTC) is one of the broad-spectrum veterinary antibiotics in the tetracycline family, which is often used to inhibit numerous Gram-positive and Gram-negative bacteria including *Mycoplasma*, *Rickettsia*, etc. It can enter the soil, surface and ground water through soil manure amendments and wastewater from sewage treatment plants (De Liguoro et al. 2003; Bondarczuk et al. 2016). The OTC concentration of some soils in China is as high as 200 mg kg^{-1} (Wang et al. 2006). Residual OTC in soil can change the rhizosphere environment, and it may affect plant growth by affecting soil microorganism and enzyme activity (Haller et al. 2002; Thiele-Bruhn and Beck 2005; Chen et al. 2014). When wheat (*Triticum aestivum* L.) is grown at 0.08 mM OTC hydroponically, shoot and root dry mass decrease by more than 80% and the photosynthetic rate by 90% (Li et al. 2011). Danilova et al. (2020) found that soil microbial biomass decreased by 90% when 300 mg kg^{-1} OTC was added to soil for five days. Residues of OTC in soil cause pH to increase first and then decrease, due to the production of ammonia as a result of microbial activity and the accumulation of organic acids (Chen et al. 2014).

Alfalfa (*Medicago sativa* L.) is one of the most widespread forage legumes in the world, and its cultivation has been increasing since 2011 to meet the growing demand for livestock production (Ning et al. 2020). Previous studies have demonstrated that P fertilizers can increase the yield and shoot P concentrations, and improve the nutritional quality of alfalfa (Berg et al. 2005; Ottman et al. 2006; Lissbrant et al. 2009). Under P deficiency, alfalfa can improve its P acquisition by acidifying the rhizosphere and increasing the exudation of carboxylates and phosphatase by roots (Suriyagoda et al. 2010; He et al. 2017, 2020). At present, there is little research on whether the presence of antibiotics

such as OTC in soil affects the uptake of P by crops. In this study, we carried out a greenhouse pot experiment to grow alfalfa in a loess soil with different doses P and OTC, in order to explore the effects of OTC on plant growth and P uptake, and to clarify possible mechanisms for such effects. The hypotheses were: (i) response of plant growth to soil OTC dose would differ at different soil P doses; (ii) shoot and root P concentrations ([P]) would increase with increasing P dose, but decrease with increasing OTC dose, and total plant P content would be affected by both soil P dose and OTC dose; (iii) soil alkaline phosphatase activity would be higher, rhizosheath pH would be lower, and the amounts of rhizosheath carboxylates would be greater at lower OTC dose and P dose.

Materials and methods

Substrate preparation and plant cultivation

A loess soil was collected from a farmland (34°19′19″N, 108°03′47″E) abandoned for a few years in Yangling, Shaanxi Province, China. Physicochemical properties of the soil were determined using air-dried soil samples. Field capacity of the soil was obtained following the indoor cutting-ring weighing method. pH of the soil was measured in a 1:5 soil:water suspension using a FG2 pH-meter (Mettler Toledo, Shanghai, China) (Little 1992). The organic carbon (C) concentration and total nitrogen (N) concentration of the soil were measured by the K_2CrO_4 oxidation method and the Kjeldahl method, respectively (Qian et al. 2015). Concentration of total potassium (K) was obtained on a PinAAcle 900 atomic absorption spectrometer (PerkinElmer, New York, USA) following an aqua regia digestion (Reimann et al. 2015). The total P concentration was estimated after acid digestion using the method described in Melaku et al. (2005). Bicarbonate-extractable P was extracted in 0.5 M $NaHCO_3$ adjusted to pH = 8.5 with NaOH for 16 h at 20 °C (Colwell, 1963) and measured by the molybdate blue method after ascorbic acid reduction (Murphy and Riley, 1962). The OTC concentration in the soil was analyzed by high-performance liquid chromatography (HPLC) according to Yang et al. (2009a). Three replications were used for the above determinations.

About 2.0 kg of the air-dried and sieved soil was added to plastic pots lined with a plastic bag inside, and a total of 48 pots were filled for the experiment. Phosphorus was added at four doses, i.e. 0, 25, 50, and 100 mg P kg^{-1} soil (hereafter referred to as 0P, 25P, 50P, and 100P) as a KH_2PO_4 aqueous solution, and OTC was added at four doses, i.e. 0, 25, 50, and 100 mg OTC kg^{-1} soil (hereafter referred to as 0OTC, 25OTC, 50OTC, and 100OTC) as an OTC aqueous solution. The experiment was set up as a two-factor completely random design, and each treatment was replicated three times. To the soil in each pot, nitrogen (N) was added at 100 mg N kg^{-1} soil as an NH_4NO_3 aqueous solution, potassium (K) was added at 100 mg K kg^{-1} soil as a K_2SO_4 and KCl (K_2SO_4:KCl = 1:2, molar ratio) aqueous solution. After addition of the above-mentioned nutrients, the soil was watered to 60% of the water-holding capacity and incubated for two weeks in a transparent greenhouse at Northwest A&F University (34°16′19″N, 108°04′20″E), Yangling, Shannxi, China. After incubation, the soil in each pot was air-dried and passed through a 2-mm sieve separately, then mixed thoroughly and filled back to the pot to ensure homogenous distribution of added chemicals.

The pot experiment was carried out from April to July in 2019 in a transparent greenhouse. Seeds of alfalfa (*Medicago sativa* L. cv Golden Empress) were surface sterilized in 10% (v:v) H_2O_2 for 10 min, rinsed with deionized water three times and germinated on moist filter paper in Petri dishes for 12 h (He et al. 2017). Thirty seeds were sown at 0.5 cm depth in each pot, and seedlings were thinned to 20 plants per pot two weeks after sowing. Soil moisture was maintained at 60% of the water-holding capacity during the experiment by weighing the pots and replenishing deionized water every one or two days.

Harvest of plants, collection and analysis of carboxylates in the rhizosheath

Plants were harvested 110 days after sowing. Shoots were cut at the soil surface, then plastic bags were removed from the pots and roots were separated from the soil. After gently shaking off the loosely adhering soil, the soil that was still attached to the roots was defined as rhizosheath soil (Pang et al. 2017). About 1.0 g fresh fine roots and rhizosheath soil in each pot was soaked in a glass beaker containing 20 mL of 0.2 mM $CaCl_2$. The roots in the solution was gently stirred for 5 min to ensure cell integrity and to remove the rhizosheath soil as much as possible, then collected

and washed thoroughly with tap water, and oven-dried at 60 °C to constant weight. About 1 mL subsample of the rhizosheath extract was filtered by a 0.22-μm syringe filter and injected into a 1-mL HPLC vial; then a drop of concentrated phosphoric acid was added to acidify it, and it was stored at −20 °C until HPLC analysis. The pH of the remaining extract in the beaker was measured. Carboxylates in the extracts were analyzed by a Waters E2695 HPLC with Waters 2998 detector and Waters Symmetry C18 reverse phase column (Waters, Milford MA, USA). The working standards included tartaric acid, malic acid, citric acid, succinic acid, malonic acid, and acetic acid. The detection wavelength was 210 nm, and each sample was run for 13 min (He et al. 2020). Amounts of carboxylates in the rhizosheath were expressed in mol per unit root dry mass. Roots not soaked for carboxylates extraction were washed first with tap water, and then rinsed with deionized water. Roots not soaked and shoots were oven-dried at 60 °C for 48 h, then weighted separately to obtain the dry mass. Total root dry mass (RDM) was calculated as the sum of the dry mass of soaked roots and non-soaked roots, and root mass ratio (RMR) was calculated as the ratio between total RDM and total plant dry mass, i.e. the sum of total RDM and shoot dry mass (SDM).

Measurement of bulk soil pH and alkaline phosphatase activity

When plants were harvested, bulk soil samples were collected from each pot. The bulk soil samples were air-dried and passed through a 2-mm sieve, and then the pH in a 1:5 soil:water suspension was measured using a FG2 pH-meter (Mettler Toledo, Shanghai, China) (Little 1992). The activity of alkaline phosphatase in the bulk soil was measured by the disodium phenylphosphate colorimetric method and expressed as the mass of phenols released per unit soil mass per hour (Tabatabai 1982), with one sample per pot being analyzed.

Determination of plant P concentration and calculation of plant P content

Oven-dried shoots and non-soaked roots were finely ground using a mortar and pestle, and about 0.1 g subsample of each sample was digested in a hot mixture of HNO_3:$HClO_4$ (4:1, v:v) to measure P concentration ([P]), the concentration of P in the solution was determined by the vanadium molybdenum yellow colorimetric method (Gupta et al. 1993). Shoot P content was calculated as shoot [P] × SDM, root P content as root [P] × RDM, and total P content in plants in each pot was calculated as the sum of shoot and root P contents.

Calculation of P-uptake efficiency and P-utilization efficiency

Phosphorus-uptake efficiency of each treatment with added P was calculated as the difference in plant P content between this treatment and that without added P and OTC, i.e. the 0P + 0OTC treatment, divided by the difference in the amount of P between this treatment and the 0P treatment, and P-utilization efficiency was calculated as total plant dry mass of each pot divided by total plant P content of each pot in this study (He et al. 2017).

Statistical analyses

The effects of P, OTC, and their interaction (P × OTC) on all measured and calculated parameters were examined by a two-way ANOVA using the general linear model in SPSS 25.0 (IBM, Montauk, New York, USA), the effects were determined significant at $P \leq 0.05$, and least significant difference (LSD) test ($\alpha = 0.05$) for post hoc means comparison among treatments were performed when there were significant effects. An independent-samples T-test was used to test the difference between the pH of the bulk soil and that of the rhizosheath extracts, and the difference was determined significant at $P \leq 0.05$. In addition, the effect sizes of OTC, P, and P × OTC on the parameters were calculated according to the method described by Harpole et al. (2011), with some modification. The effect size of a treatment was calculated as the log response ratio, which was the log value of the ratio between the value of each parameter in a treatment to that in the control. Intuitively, a 5% change in each parameter relative to the value in the control (0P0OTC) was considered a significant response to a treatment, the effect size of a treatment was 0.05 and − 0.05 when the response ratio was 1.05 and 0.95, respectively, i.e. when the value of the parameter increased by 5% and decreased by 5%, respectively. Therefore, the effect of a treatment with an effect size greater than 0.05 was considered significant positive, the effect of a treatment with an effect size less than −0.05 was considered significant negative, and the

effect of a treatment with an effect size between −0.05 and 0.05 was considered non-significant. Firstly, we calculated the effect size of each P dose at 0OTC and the effect size of each OTC dose at 0P separately, and defined each as the effect size of P and OTC respectively as follows:

$$\text{Effect size of xP} = \text{Ln (xP0OTC/0P0OTC)}$$

$$\text{Effect size of yOTC} = \text{Ln (0PyOTC/0P0OTC)}$$

Then the simple addition of the effect sizes of P and OTC for each treatment was calculated as:

$$\text{Effect size of (xP + yOTC)} = \text{Effect size of xP} + \text{Effect size of yOTC}$$

The effect size of the interaction (P × OTC) for each treatment was calculated as:

$$\text{Effect size of (xP} \times \text{yOTC)} = \text{Ln (xPyOTC/0P0OTC)}$$

where x = 25, 50, and 100, respectively, and y = 25, 50, and 100, respectively. When the effect size of the interaction was equivalent (a less than 5% difference was considered equivalent) to that of the simple addition, the interaction was considered additive; when it was 5% greater than that of the simple addition, the interaction was considered super-additive; while when it was 5% less than that of the simple addition, the interaction was considered sub-additive. Here, we used the effect-size criteria preferably to the use of *P* value significance criteria, because low replication or statistical power in experiments may obscure the ability to detect biologically meaningful responses. Log response ratios represent the proportional response to experimental treatment, but are measured in different units and magnitudes to be analyzed on the same scale, and tend to be distributed normally (Harpole et al. 2011).

Results

Physicochemical properties of the loess soil

The loess soil had a pH of 8.42, the total [P] was 460 mg kg^{-1}, the bicarbonate-extractable [P] was 7.2 mg kg^{-1}, and the OTC concentration was 0.17 mg kg^{-1} (Table 1). Other measured properties are also listed in Table 1.

Bulk soil and rhizosheath characteristics

In most cases, pH of the rhizosheath extract (7.53–8.17) was much lower than that of the bulk soil (7.70–8.44). At 0OTC, bulk soil pH rose significantly at 25P and 50P, but did not change considerably at 100P; at 0P, bulk soil pH did not change considerably at 25OTC or 50OTC, but rose significantly at 100P (Fig. 1, Tables 2 and S1). The interaction between P and OTC showed an additive or sub-additive effect to increase bulk soil pH at 25P, the interaction was non-significant and sub-additive on bulk soil pH at 50P and 100P in most cases. The effects of both P and OTC, and the interaction between P and OTC on the pH of rhizosheath extract were non-significant (Fig. 1, Tables 2 and S1).

For alkaline phosphatase activity, the effect of P was not consistent, with 25P having the lowest value, the effect of OTC was significantly negative, and the interaction between P and OTC was additive or super-additive (Fig. 2, Tables 2 and 3). Alkaline phosphatase activity decreased with increasing OTC dose at 0P, it was the lowest at 25OTC and the greatest at 100OTC at both 25P and 50P. At 100P, alkaline phosphatase activity was similar at 0OTC and 25OTC, which had slightly greater alkaline phosphatase activity than 50OTC and 100OTC, which also had similar alkaline phosphatase activity.

The amount of rhizosheath tartrate decreased considerably with increasing P dose as well as increasing OTC dose, and the interaction between P and OTC on tartrate amount was significantly negative, and additive or sub-

Table 1 Physicochemical properties of the loess soil

Parameter	Value
pH	8.42 ± 0.37
Organic C (mg g^{-1})	9.33 ± 0.26
Total P (mg kg^{-1})	460 ± 20
Total K (mg g^{-1})	24.6 ± 2.2
Total N (mg g^{-1})	1.07 ± 0.11
Bicarbonate-extractable P (mg kg^{-1})	7.18 ± 1.47
Oxytetracycline (mg kg^{-1})	0.17 ± 0.03
Field capacity (%)	23.2 ± 2.3

Note: Values are presented as means ± SE (n = 3)

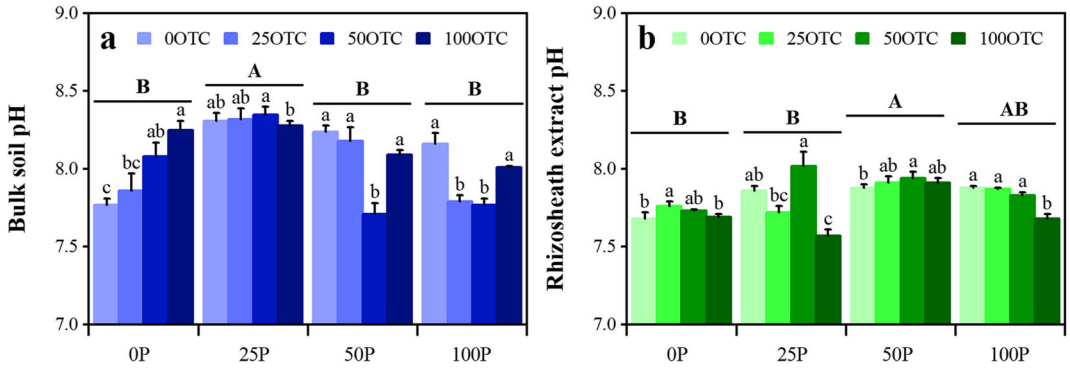

Fig. 1 The pH of the bulk soil (a) and rhizosheath extracts (b) of alfalfa grown in a loess soil with different doses of phosphorus (P) and oxytetracycline (OTC). Data are presented as means ± SE (n = 3). 0P, 25P, 50P, and 100P represent that P was added at 0, 25, 50, and 100 mg kg^{-1}, respectively; 0OTC, 25OTC, 50OTC, and 100OTC represent that OTC was added at 0, 25, 50, and 100 mg kg^{-1}, respectively. The P value for the difference between the pH of the bulk soil and rhizosheath extracts was <0.001. Capital letters denote significant differences ($P \leq 0.05$) among P doses, and lowercase letters denote significant differences ($P \leq 0.05$) among OTC doses at the same P doses, according to the LSD test

additive (Fig. 3a, Tables 2 and 3). Malonate was detected in all treatments with added P, while succinate was detected in most treatments with added P, but neither of them was detected at 0P at any OTC dose (Fig. 3b).

Plant biomass

The effect of P was significantly positive on both SDM and RDM; the effect of OTC was significantly negative for SDM, but significantly positive on RDM in most cases (Fig. 4a and b, Tables 3 and 4). The interaction between P and OTC was significantly positive and super-additive on SDM in most cases, but was always significantly positive and sub-additive on RDM in most cases. At all OTC doses, both SDM and RDM increased with increasing P dose. At 0P and 25P, addition of OTC caused a significant reduction in SDM. At 50P and 100P, RDM increased markedly with increasing OTC dose, while at 0P and 25P, RDM was the greatest at 50OTC. The effects of both P and OTC on RMR were significantly positive, and RMR rose significantly with increasing P dose and OTC dose (Fig. 3c, Tables 3 and

Table 2 Statistical levels of significance (P values) of the two-way ANOVA for the effects of phosphorus (P), oxytetracycline (OTC), and their interaction (P × OTC) on various parameters

Parameter	P value		
	P	OTC	P × OTC
Bulk soil pH	<0.001	0.002	<0.001
Rhizosheath extract pH	<0.001	<0.001	<0.001
Alkaline phosphatase activity	<0.001	<0.001	<0.001
Rhizosheath tartrate	<0.001	<0.001	0.002
Rhizoshrath malonate	<0.001	<0.001	<0.001
Shoot dry mass	<0.001	<0.001	0.002
Root dry mass	<0.001	<0.001	<0.001
Root mass ratio	<0.001	<0.001	0.197
Shoot [P]	<0.001	0.003	0.417
Root [P]	<0.001	0.001	0.577
Plant P content	<0.001	0.002	0.002
P-uptake efficiency	<0.001	<0.001	<0.001
P-utilization efficiency	<0.001	<0.002	0.709

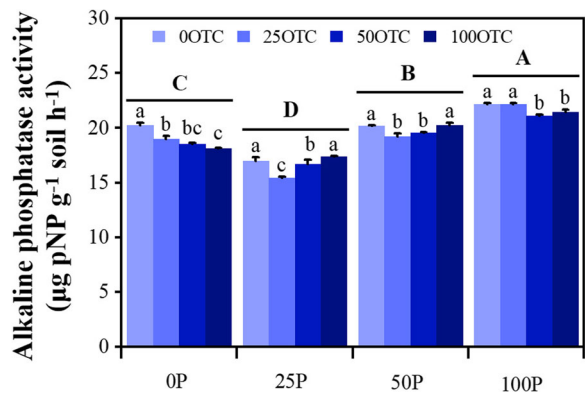

Fig. 2 Alkaline phosphatase activity in the bulk soil with different doses of phosphorus (P) and oxytetracycline (OTC). Data are presented as means ± SE (n = 3). 0P, 25P, 50P, and 100P represent that P was added at 0, 25, 50, and 100 mg kg^{-1}, respectively; 0OTC, 25OTC, 50OTC, and 100OTC represent that OTC was added at 0, 25, 50, and 100 mg kg^{-1}, respectively. Capital letters denote significant differences ($P \leq 0.05$) among P doses, and lowercase letters denote significant differences ($P \leq 0.05$) among OTC doses at the same P doses, according to the LSD test

Table 3 The effect sizes of phosphorus (P), oxytetracycline (OTC), their simple addition (P + OTC), and their interaction (P × OTC) on alkaline phosphatase activity and rhizosheath tartrate amount

P treatment	OTC treatment	Alkaline phosphatase activity				Rhizosheath tartrate amount			
		P	OTC	P + OTC	P × OTC	P	OTC	P + OTC	P × OTC
0P	0OTC								
0P	25OTC		−0.064 (SN)				−0.119(SN)		
0P	50OTC		−0.091 (SN)				−0.633(SN)		
0P	100OTC		−0.110 (SN)				−1.279(SN)		
25P	0OTC	−0.178 (SN)				−0.037 (NS)			
25P	25OTC			−0.243	−0.271 (SN, Add)			−0.156	−0.315 (SN, Sub-add)
25P	50OTC			−0.269	−0.195 (SN, Sup-add)			−0.670	−0.726 (SN, Sub-add)
25P	100OTC			−0.288	−0.153 (SN, Sup-add)			−1.316	−1.364 (SN, Add)
50P	0OTC	−0.003 (NS)				−0.208 (SN)			
50P	25OTC			−0.068	−0.053 (SN, Add)			−0.327	−0.346 (SN, Add)
50P	50OTC			−0.094	−0.034 (NS, Sup-add)			−0.841	−0.898 (SN, Sub-add)
50P	100OTC			−0.113	0.000 (NS, Sup-add)			−1.487	−1.496 (SN, Add)
100P	0OTC	0.090 (SP)				−0.327 (SN)			
100P	25OTC			0.025	0.090 (SP, Sup-add)			−0.446	−0.414 (SN, Add)
100P	50OTC			−0.001	0.041 (NS, Add)			−0.960	−1.126 (SN, Sub-add)
100P	100OTC			−0.020	0.057 (SP, Sup-add)			−1.606	−1.576 (SN, Add)

Note: SP, significant positive; SN, significant negative; NS, non-significant. Add, additive; Sup-add, super-additive; Sub-add, sub-additive

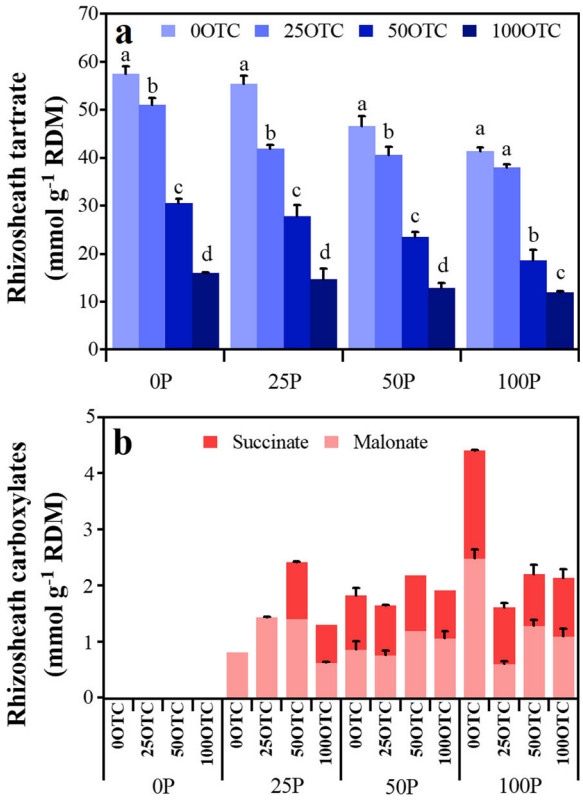

Fig. 3 The amounts of tartrate (**a**), succinate and malonate (**b**) in the rhizosheath of alfalfa grown in a loess soil with different doses of phosphorus (P) and oxytetracycline (OTC). Data are presented as means ± SE ($n = 3$, except for some treatments in which certain carboxylate was not detected in all samples). 0P, 25P, 50P, and 100P represent that P was added at 0, 25, 50, and 100 mg kg^{-1}, respectively; 0OTC, 25OTC, 50OTC, and 100OTC represent that OTC was added at 0, 25, 50, and 100 mg kg^{-1}, respectively. Lowercase letters denote significant differences ($P \leq 0.05$) in tartrate among OTC doses at the same P doses, according to the LSD test

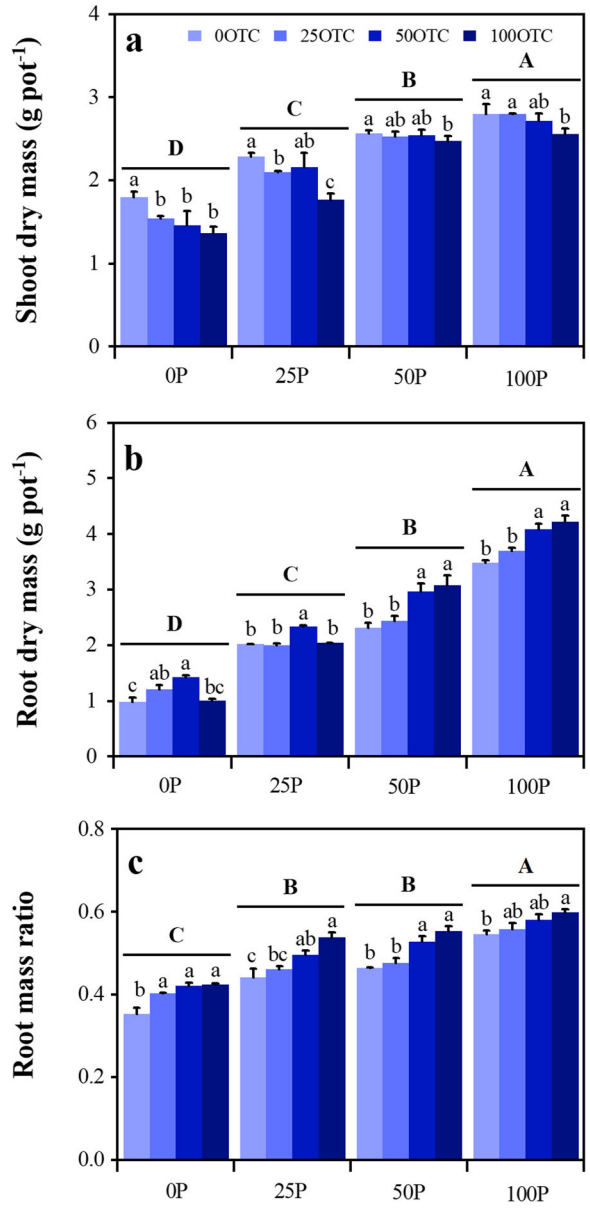

Fig. 4 Shoot dry mass (**a**), root dry mass (**b**), and root mass ratio (**c**) of alfalfa grown in a loess soil with different doses of phosphorus (P) and oxytetracycline (OTC). Data are presented as means ± SE ($n = 3$). 0P, 25P, 50P, and 100P represent that P was added at 0, 25, 50, and 100 mg kg^{-1}, respectively; 0OTC, 25OTC, 50OTC, and 100OTC represent that OTC was added at 0, 25, 50, and 100 mg kg^{-1}, respectively. Capital letters denote significant differences ($P \leq 0.05$) among P doses, and lowercase letters denote significant differences ($P \leq 0.05$) among OTC doses at the same P doses, according to the LSD test

S2). The interaction between P and OTC was significantly positive, and additive or sub-additive on RMR.

Phosphorus in plants

For shoot [P], the effect of P was always significantly positive, and that of OTC was always significantly negative, while the interaction between P and OTC was additive or super-additive in most cases, although the interaction was only significantly positive at 50P and 100P, but non-significant at 25P (Fig. 5a, Tables 3 and 5). For root [P], the effects of both P and OTC were non-significant in most cases; although the interaction between P and OTC was always additive, it was non-significant in almost all cases (Fig. 5b, Tables 3 and 5). The effect of P on plant P content was always significantly positive; at all OTC doses, plant P content increased with increasing P dose, being on average

Table 4 The effect sizes of phosphorus (P), oxytetracycline (OTC), their simple addition (P + OTC), and their interaction (P × OTC) on shoot dry mass and root dry mass

P treatment	OTC treatment	Shoot dry mass				Root dry mass			
		P	OTC	P + OTC	P × OTC	P	OTC	P + OTC	P × OTC
0P	0OTC								
0P	25OTC		−0.153 (SN)				0.206 (SP)		
0P	50OTC		−0.206 (SN)				0.371 (SP)		
0P	100OTC		−0.271 (SN)				0.023 (NS)		
25P	0OTC	0.241 (SP)				0.717 (SP)			
25P	25OTC			0.088	0.158 (SP, Sup-add)			0.924	0.708 (SP, Sub-add)
25P	50OTC			0.035	0.186 (SP, Sup-add)			1.088	0.866 (SP, Sub-add)
25P	100OTC			−0.030	−0.016 (NS, Add)			0.740	0.730 (SP, Add)
50P	0OTC	0.359 (SP)				0.854 (SP)			
50P	25OTC			0.206	0.344 (SP, Sup-add)			1.060	0.906 (SP, Sub-add)
50P	50OTC			0.153	0.349 (SP, Sup-add)			1.224	1.105 (SP, Sub-add)
50P	100OTC			0.088	0.322 (SP, Sup-add)			0.876	1.140 (SP, Sup-add)
100P	0OTC	0.445 (SP)				1.266 (SP)			
100P	25OTC			0.291	0.446 (SP, Sup-add)			1.473	1.326 (SP, Sub-add)
100P	50OTC			0.238	0.414 (SP, Sup-add)			1.637	1.424 (SP, Sub-add)
100P	100OTC			0.174	0.355 (SP, Sup-add)			1.289	1.456 (SP, Sup-add)

Note: SP, significant positive; SN, significant negative; NS, non-significant. Add, additive; Sup-add, super-additive; Sub-add, sub-additive

63%, 141%, and 225% greater at 25P, 50P, and 100P than at 0P, respectively (Fig. 5b, Tables 3 and S2). The effect of OTC on plant P content was not consistent. The interaction between P and OTC was always significantly positive on plant P content and super-additive in most cases. At 0P and 25P, plant P contents at 0OTC and 50OTC were greater than those at 25OTC and 100OTC; at 50P, plant P contents at 50OTC and 100OTC were greater than those at 0OTC and 250OTC; at 100P, plant P content was the lowest at 0OTC and the greatest at 100OTC.

The effects of P and OTC, and their interaction on phosphorus-uptake efficiency were all significant (Fig. 6a and Table 2). At all OTC doses except 0OTC, P-uptake efficiency was the greatest at 50P. Phosphorus-uptake efficiency increased markedly with increasing OTC dose at 50P, but it did not show the same trend at either 25P or 100P. For P-utilization efficiency, the effect of P was always significantly negative, while that of OTC was significantly positive at 25P, but non-significant at 50P and 100P (Fig. 6b, Tables 2 and 6). The interaction between P and OTC on P-utilization efficiency was always significantly negative, and additive in almost all cases.

Discussion

We carried out a greenhouse pot experiment to grow alfalfa in a loess soil with different doses of P and OTC, in order to investigate the effects of OTC on plant growth, P uptake, and carboxylates in the rhizosheath of alfalfa. The results supported some, but not all our hypotheses. Our first hypothesis that response of plant growth to soil OTC dose would differ at different soil P doses was fully supported. Our results showed that SDM decreased with increasing OTC dose, especially at lower P doses (i.e. 0 and 25 mg kg^{-1}), but RDM increased with increasing OTC dose at higher P doses (i.e. 50 and 100 mg kg^{-1}), while root mass ratio increased with increasing OTC dose, regardless of P dose. Such results suggest that the response of plant growth to OTC depends not only on OTC dose but also on P dose in soil, as well as on plant parts; shoot growth tends to be more negatively affected by increasing OTC dose at lower P doses, whereas root growth can be enhanced by OTC addition at higher P doses. It is yet not clear why OTC had such effects on alfalfa growth as shown in our study. Adding P to soil can stimulate the growth and

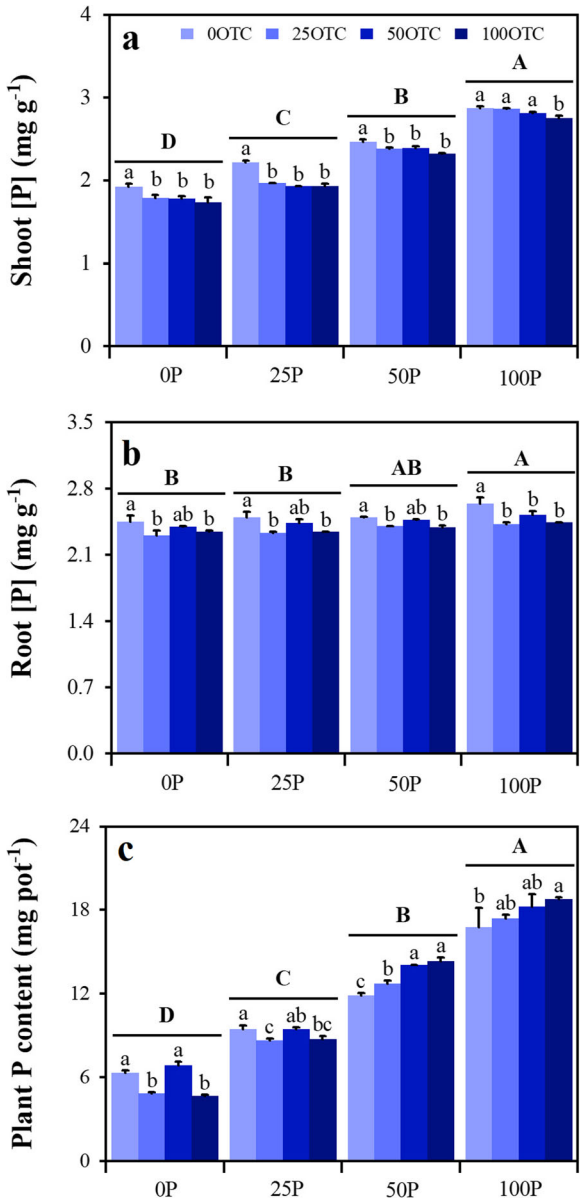

Fig. 5 Phosphorus concentration ([P]) in shoots (**a**) and roots (**b**) per unit dry mass, and plant P content (**c**) of alfalfa grown in a loess soil with different doses of P and oxytetracycline (OTC). Data are presented as means ± SE ($n = 3$). 0P, 25P, 50P, and 100P represent that P was added at 0, 25, 50, and 100 mg kg^{-1}, respectively; 0OTC, 25OTC, 50OTC, and 100OTC represent that OTC was added at 0, 25, 50, and 100 mg kg^{-1}, respectively. Capital letters denote significant differences ($P \leq 0.05$) among P doses, and lowercase letters denote significant differences ($P \leq 0.05$) among OTC doses at the same P doses, according to the LSD test

increase the dry mass of several crops (Vance et al. 2003). A number of studies have demonstrated that application of P to soil promotes plant growth and increases both SDM and RDM of alfalfa (Berg et al. 2005; Ottman et al. 2006; He et al. 2020), and the results of the present study show that both SDM and RDM of alfalfa increased with increasing P dose. Liu et al. (2013) found that, for reed (*Phragmites australis* Trin.) grown under hydroponic conditions, plant growth was promoted by low OTC concentration, but inhibited by high OTC concentration. Kong et al. (2007) found that both SDM and RDM of alfalfa decreased with increasing OTC concentration in a hydroponic system, and root growth was more sensitive to OTC than shoot growth.

Many plants have some physiological adjustments to acquire P and N closer to the N:P ratio demanded for optimal growth (Maistry et al. 2015). According to Koerselman and Meuleman (1996), vegetation N:P ratios <14 often indicate that plant growth is limited by N and > 16 that it is limited by P. In our study, root growth was limited by N, especially at 100 mg P kg^{-1} (Fig. S1). The increase of soil P level reduced N:P ratio and aggravated N limitation (Menge et al. 2012). Nitrogen limitation can promote root growth when P supply is abundant (Maistry et al. 2015), and increase the ratio of root dry mass to total plant dry mass (Zhao et al. 2005). In the present study, it is very likely that increased RDM and RMR were due to P-induced N limitation at 100 mg P kg^{-1}, under which N:P ratios in both shoots and roots were significantly lower than under 0P (Fig. S1).

Our second hypothesis that shoot and root P concentrations would increase with increasing P dose and decrease with increasing OTC dose, and that total plant P content would be affected by both soil P dose and OTC dose was partly supported. In the present study, the greatest shoot and root P concentrations, and plant P content were observed at the highest P dose. Increasing soil P-addition rate can increase P concentration and content in plants (Ottman et al. 2006). The P concentration in wheat grain and straw increased significantly with increasing P level in a sandy loam soil (Rahim et al. 2010), and P concentrations in both leaves and stems of alfalfa increased with increasing P supply (He et al. 2020). Our study showed that addition of OTC reduced P concentrations in both shoots and roots. However, plant P content increased, rather than decreased with increasing OTC dose at higher P dose due to enhanced root growth by OTC addition at higher P dose. There is no previous study showing the effects of OTC on plant P concentration and content. However, OTC reduced the solubility of Ca^{2+} and Mg^{2+} in soil

Table 5 The effect sizes of phosphorus (P), oxytetracycline (OTC), their simple addition (P + OTC), and their interaction (P × OTC) on shoot [P] and root [P]

P treatment	OTC treatment	Shoot [P]				Root [P]			
		P	OTC	P + OTC	P × OTC	P	OTC	P + OTC	P × OTC
0P	0OTC								
0P	25OTC		−0.072 (SN)				−0.059 (SN)		
0P	50OTC		−0.078 (SN)				−0.020 (NS)		
0P	100OTC		−0.102 (SN)				−0.045 (NS)		
25P	0OTC	0.143 (SP)				0.019 (NS)			
25P	25OTC			0.071	0.024 (NS, Add)			−0.040	−0.050 (SN, Add)
25P	50OTC			0.066	0.005 (NS, Sub-add)			−0.001	−0.006 (NS, Add)
25P	100OTC			0.042	0.002 (NS, Add)			−0.026	−0.044 (NS, Add)
50P	0OTC	0.249 (SP)				0.019 (NS)			
50P	25OTC			0.177	0.215 (SP, Add)			−0.041	−0.019 (NS, Add)
50P	50OTC			0.172	0.217 (SP, Add)			−0.001	0.008 (NS, Add)
50P	100OTC			0.148	0.190 (SP, Add)			−0.026	−0.023 (NS, Add)
100P	0OTC	0.401 (SP)				0.076 (SP)			
100P	25OTC			0.329	0.400 (SP, Sup-add)			0.017	−0.011 (NS, Add)
100P	50OTC			0.324	0.381 (SP, Sup-add)			0.056	0.029 (NS, Add)
100P	100OTC			0.300	0.358 (SP, Sup-add)			0.032	−0.002 (NS, Add)

Note: SP, significant positive; SN, significant negative; NS, non-significant. Add, additive; Sup-add, super-additive; Sub-add, sub-additive

(Sassman and Lee, 2005), which may further affect P availability in soil and hence P uptake by plants. It is reported that treating plants with OTC can control some bacterial diseases of plants and improve the health of plants (Hu and Wang, 2016), we thus speculate that OTC in soil may enhance root growth and P uptake, due to its positive effect on disease control and alleviation. However, OTC in soil may be genotoxic to plant cells, and inhibit cell division in root apical meristem and root elongation (Xie et al. 2011), thus negatively affecting uptake of P by roots.

The third hypothesis that soil alkaline phosphatase activity would be higher, rhizosheath pH would be lower, and the amounts of rhizosheath carboxylates would be greater at lower OTC dose and P dose was partly supported. Alkaline phosphatase is one of the key microbial enzymes to convert organic P into inorganic P, allowing plants to absorb and utilize more P (Pant and Warman 2000). Under P deficiency, the activity of alkaline phosphatase in soil often increases (Radersma and Grierson 2004). In our study, the activity of alkaline phosphatase in the bulk soil was the lowest when P was added at 25 mg kg^{-1}, rather than at the highest P dose, regardless of the OTC dose, likely because the concentration of organic P was low (<100 mg kg^{-1}) in the experimental soil. The effect of OTC on alkaline phosphatase activity depended on both OTC dose and P dose in soil; consistent decrease in alkaline phosphatase activity with increasing OTC dose when no P was added suggests that soil microbial activity was negatively affected and the amount of phosphatase exuded by microorganisms was reduced (Whitelaw 2000; Danilova et al. 2020). Yang et al. (2009b) found that alkaline phosphatase was sensitive to OTC exposure, and its activity decreased by 41% when OTC was added at 10 mg kg^{-1}.

When the bioavailability of P is low, plants can increase the availability of P by exuding protons (H^+) and organic anions such as carboxylates, which increase dissolution of P (Hinsinger 2001; Shane and Lambers 2005). In this study, neither bulk soil nor rhizosheath extract pH was affected by P or OTC, but the rhizosheath was markedly acidified, and such acidification might be an important strategy to increase P availability and consequently P uptake by plants (He et al. 2020). Many plants, including alfalfa, show an increased exudation of carboxylates into the rhizosheath

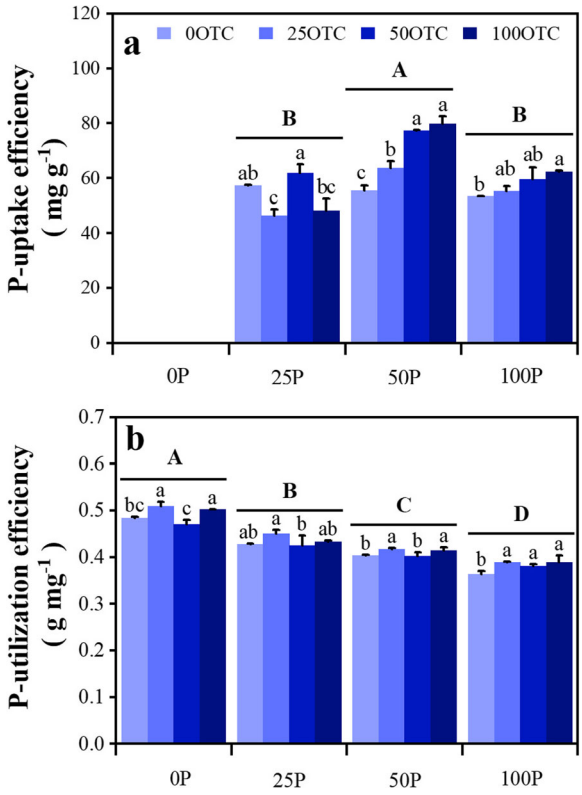

Fig. 6 Phosphorus (P)-uptake efficiency (**a**) and P-utilization efficiency (**b**) of alfalfa grown in a loess soil with different rates of added P and oxytetracycline (OTC). Data are presented as means ± SE (n = 3). 0P, 25P, 50P, and 100P represent that P was added at 0, 25, 50, and 100 mg kg^{-1}, respectively; 0OTC, 25OTC, 50OTC, and 100OTC represent that OTC was added at 0, 25, 50, and 100 mg kg^{-1}, respectively. Capital letters denote significant differences ($P \leq 0.05$) among P doses, and lowercase letters denote significant differences ($P \leq 0.05$) among OTC doses at the same P doses, according to the LSD test

in response to P deficiency (Pang et al. 2010). Similarly to our findings, He et al. (2020) reported that alfalfa releases a considerably greater amount of tartrate into the rhizosheath at lower P supply. Tartrate is an important carboxylate secreted by phosphorus-solubilizing bacteria in soil, such as *Pseudomonas*, *Enterobacter*, *Palladium*, and *Klebsiella* (Kim and Unden 2007; Li et al. 2020). Antibiotics often have a negative effect on the growth of soil microorganisms (Haller et al. 2002); they can affect the structure and diversity of soil microbial communities, as well as the functions of soil microorganisms (De Liguoro et al. 2003; Bondarczuk et al. 2016). OTC inhibits both Gram-positive and Gram-negative bacteria, including phosphorus-solubilizing bacteria mentioned above, which are all Gram-negative (Chen et al. 2014). Thiele-Bruhn and Beck (2005) found that OTC reduced the number of soil bacteria and showed a dose-effect. Respiration of soil microorganisms was inhibited by 16–25% and 28–38% at 100 and 1000 mg OTC kg^{-1}, respectively (Boleas et al. 2005). In the present study, we speculate that the reduction in tartrate amount in the rhizosheath of alfalfa with increasing OTC dose was likely partly due to a decline in the amount and activity of phosphorus-solubilizing bacteria, and that the degree of such reduction was dose-dependent. It is also likely that the decreased amount of tartrate was a result of declined plant N:P ratio and a shift of P-limitation to N-limitation (Miao et al. 2011; Maistry et al. 2015; He et al. 2020). The significant interactions between P and OTC on plant [N], N contents, and N:P ratios (Fig. S1, Tables S3–S5) suggest that the effect of OTC on the exudation of tartrate depends on both OTC dose and P dose in soil.

When the application rate of phosphate fertilizers is increased, the agronomic utilization efficiency and absorption efficiency of phosphate fertilizers show a downward trend (Schröder et al. 2011), as the more P is applied, the more P remains in soil, due to the strong sorption of P to various minerals in soil (Sahrawat et al. 2011). In the present study, we also found that P-utilization efficiency decreased with increasing P-addition rate. Although OTC, and the interaction between P and OTC significantly affected both P-uptake efficiency and P-utilization efficiency in the present study, such effects did not show a consistent trend with increasing OTC dose, likely due to plant growth and P concentration showing different responses to changes in soil OTC dose. Furthermore, phosphate fertilizer has a sorption effect on OTC in soil (Liu et al. 2014), and this might also explain why there was no consistent trend in the effect of OTC on P-uptake efficiency and P-utilization efficiency. It should be noted that, as well as effects on P dynamics, OTC may modify other microbial communities that affect root growth and nutrient availability, and further research is warranted in that aspect.

Conclusions

Our results show that shoot growth tended to be more negatively affected by increasing OTC dose at lower P doses, whereas root growth can be enhanced by OTC addition at higher P dose, likely due to P-induced N

Table 6 The effect sizes of phosphorus (P), oxytetracycline (OTC), their simple addition (P + OTC), and their interaction (P × OTC) on P-utilization efficiency

P treatment	OTC treatment	P-utilization efficiency			
		P	OTC	P + OTC	P × OTC
0P	0OTC				
0P	25OTC		0.051 (SP)		
0P	50OTC		−0.027 (NS)		
0P	100OTC		0.039 (NS)		
25P	0OTC	−0.123 (SN)			
25P	25OTC			−0.072	−0.071 (SN, Add)
25P	50OTC			−0.150	−0.128 (SN, Add)
25P	100OTC			−0.085	−0.110 (SN, Add)
50P	0OTC	−0.183 (SN)			
50P	25OTC			−0.132	−0.147 (SN, Add)
50P	50OTC			−0.210	−0.184 (SN, Add)
50P	100OTC			−0.144	−0.156 (SN, Add)
100P	0OTC	−0.285 (SN)			
100P	25OTC			−0.234	−0.219 (SN, Add)
100P	50OTC			−0.312	−0.238 (SN, Sup-add)
100P	100OTC			−0.246	−0.220 (SN, Add)

Note: SP, significant positive; SN, significant negative; NS, non-significant. Add, additive; Sup-add, super-additive; Sub-add, sub-additive

limitation. Addition of OTC reduced P concentrations in shoots, but did not reduce plant P content consistently. Increasing OTC dose significantly reduced the activity of alkaline phosphatase in the bulk soil and the amount of tartrate, which was the major carboxylate in the rhizosheath of alfalfa. The effects of OTC on plant growth and P uptake depended on both OTC dose and P dose in soil; high soil OTC doses could negatively affect soil microbial activities such as release of extracellular enzymes (including phosphatase) and carboxylates, thus causing negative effects on plant growth and P uptake, especially at lower soil P doses. The underlying mechanisms of the effects of OTC, and its interaction with P, on plant growth and P uptake warrant further investigation.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11104-021-04840-0.

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