REGULAR ARTICLE

Increasing nitrogen supply to phosphorus-deficient *Medicago sativa* decreases shoot growth and enhances root exudation of tartrate to discharge surplus carbon dependent on nitrogen form

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Abstract

Aims Carboxylate release by roots has been considered a strategy for mobilisation and acquisition of phosphorus (P). However, recently, it was argued that carboxylate release may be a way to discharge surplus carbon produced under conditions that limit plant growth. Plant P status may not be the main factor driving carboxylate release by roots. Instead, plant nitrogen (N) status and/or N:P ratio of the soil or plant may play a more important role in enhancing carboxylate release.

Methods A greenhouse pot experiment was performed to grow alfalfa in a P-deficient soil, supplied with two rates of P (0 and 20 mg kg^{-1}) in combination with four forms of nitrogen (N) at five rates (0, 25, 50, 75, and 100 mg kg^{-1}), to explore the effects of P rate, N form, N rate, and their interactions on plant growth, P and N status, and carboxylate release, and to determine the factors driving carboxylate release.

Results Nitrogen addition weakened the positive effect of P addition on plant growth, and increased

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plant N and P concentrations; P addition increased plant P concentration, but weakened the effect of N addition on plant N concentration. The amount of tartrate increased dramatically with increasing N rate, which decreased shoot growth, depending on N form. At high P supply, tartrate exudation correlated negatively with shoot biomass.

Conclusions Nitrogen addition to P-deficient alfalfa decreased shoot growth and enhanced the release of tartrate, likely to discharge surplus carbon; and the effects varied with N form.

Keywords Alfalfa · Biomass allocation · Carboxylate release · Fertilisation · Nutritional status · Nitrogen to phosphorus ratio

Introduction

Phosphorus (P) is an essential plant macronutrient and plays important roles in plant growth and metabolism. Plant growth is often limited by P deficiency, because inorganic phosphate (Pi) is often strongly sorbed to soil particles and the bioavailability of P is low in many soils (Lambers and Plaxton 2015). Many species, including *Lupinus* (lupin) (Lambers et al. 2013) and Proteaceae species (Lambers et al. 2011), increase their carboxylate release from roots when growing under P-limited conditions, and the release of carboxylates is controlled systemically by shoot or leaf P concentration (Shane et al. 2008; Shen et al. 2003). The released carboxylates, especially citrate and malate, mobilise sparingly-soluble soil P by competing with both inorganic and organic P for binding sites in rhizosphere soil, thus making P more available for uptake by plants (Lambers et al. 2011). However, release of carboxylates is affected by a number of factors; their contribution to P mobilisation and acquisition can vary greatly and may not be as usually expected (Wang and Lambers 2020).

Low P availability in soil does not always stimulate release of carboxylates by roots (Abrahão et al. 2018; He et al. 2017b; Suriyagoda et al. 2012), and some studies have shown that the amounts of carboxylates released may even decrease with decreasing soil P supply (Huang et al. 2017; Wen et al. 2020). There are also studies showing that carboxylate release does not relate consistently to a plant's P-acquisition capacity (Pandey et al. 2014; Pearse et al. 2007; Ryan

et al. 2014). Even when there are significant correlations between rhizosphere water-soluble P and rhizosphere carboxylates, the correlation coefficients are very low, and rhizosphere carboxylates may play minor roles in improving P availability and uptake (Wang et al. 2016). As release of carboxylates is often restricted to certain parts of the roots, e.g., root apices, the amount of P mobilised by carboxylates may be limited and insufficient to enhance plant growth (Ryan et al. 2014). Furthermore, the potential of carboxylates to mobilise P may be weakened rapidly after they are released by roots, due to their sorption onto soil particles (Oburger et al. 2011) and degradation by soil microorganisms (Weisskopf et al. 2006). Therefore, a greater release of carboxylates does not necessarily result in greater plant biomass and crop yield (Pandey et al. 2014; Ryan et al. 2014).

Recently, Prescott et al. (2020) put forward a 'surplus carbon (C)' hypothesis. In this paper, the authors showed evidence of plants having surplus fixed C under conditions such as insufficient nutrients (including P and/or nitrogen (N)) (Augusto et al. 2017; Harpole et al. 2011) or water (McDowell 2011; Muller et al. 2011; Sharma et al. 2021), low temperature (Hoch and Körner 2009; Karst et al. 2017), or elevated atmospheric CO_2 (Jiang et al. 2020; Körner 2015), when plant growth declines but photosynthesis continues (although often at a slower rate) under these conditions. When aboveground growth is limited by N or P (or some other nutrient), some of the surplus C is transported from leaves to roots and further metabolised there, and thereafter may be either stored in roots or released as carboxylates, amino acids, sugars, or otherwise (Carvalhais et al. 2011; van Dam and Bouwmeester 2016). Surplus C may contain non-structural carbohydrates and other C-rich metabolites, and the composition depends on whether N or P is the most limiting nutrient (Prescott et al. 2020). Continuous storage of non-structural carbohydrates under nutrient shortage promotes root growth and result in greater root mass ratios (Hermans et al. 2006; Litton et al. 2007). Prescott et al. argued that disposal of surplus C may be a way to alleviate the oversupply of resources that plants have in surplus to their requirements. Therefore, when explaining plant-soil interactions, the production and disposal of surplus C under growth-limiting conditions should be considered before thinking of adaptive strategies, investments or trade-offs (Prescott et al. 2020).

In a previous study by He et al. (2020), when an alkaline low-P and low-N soil was used to grow alfalfa (*Medicago sativa*), supplied with different rates of P (0, 5, and 20 mg kg^{-1}) and N (50 and 100 mg kg^{-1}), roots released a large amount of carboxylates, among which tartrate was the most abundant and its amount decreased with increasing P supply, but increased with increasing N supply, while the amount of other carboxylates that are more commonly reported, including citrate and malate, were much smaller and did not always vary considerably with the rates of either P or N supply. The above-mentioned results of the study of He et al. (2020), together with the 'surplus C' hypothesis put forward by Prescott et al. (2020), led us to consider that plant P status may not be the main factor driving the release of tartrate from alfalfa roots; instead, N status and/or N:P ratio of the soil or plant may play a more important role in enhancing tartrate release, which may be a way to discharge surplus C produced in plants when N is oversupplied under P shortage, rather than to increase P acquisition.

In addition to the rates of P and N supply, N form may also affect root morphology and/or physiology, thus impacting plant P and N status (Ngwene et al. 2010; Niu et al. 2013; Wang et al. 2011). Although plant roots can take up N as either ammonium-N (NH$_4$-N) or nitrate-N (NO$_3$-N), and also as amino acids, there are differences between forms of N in terms of their mobility in soil and availability to plants, absorption by roots, and transport and assimilation within plants (Hawkesford et al. 2012). Furthermore, plants often have a preference for a certain form of N over other forms (Gigon and Rorison 1972); such preferences can affect the rhizosphere environment including pH and the bioavailability of P, in particular in alkaline soils, thus affecting plant growth and physiological responses to P deficiency (Fan et al. 2009).

In the present study, a pot experiment was carried out to grow alfalfa in a P-deficient loess soil with different rates of added P and N in different forms to explore the effects of P rate, N rate, N form, and their interactions on plant growth, P and N status, and release of carboxylates, and to investigate which factor is more important in driving the release of carboxylates (mainly tartrate), and to determine the nature of the interactions between and/or among factors. The following hypotheses were tested: (i)

plant growth would be enhanced by P addition as well as N addition, and belowground biomass allocation would decrease with increasing P rate, and also with increasing N rate; (ii) plant P concentration and N concentration would increase with increasing P rate, and also with increasing N rate, while N:P ratio would increase with increasing N rate but decline with increasing P rate; (iii) the amount of tartrate released by roots would decline with increasing P rate but increase with increasing N rate, and it would have a closer relationship with plant N concentration than with plant P concentration; and (iv) the effects of N rate on the above-mentioned parameters would depend on the forms of the added N.

Materials and methods

Experimental design and plant cultivation

A loess soil was collected from the top 40-cm layer of an undisturbed site (34°51′30″N, 109°19′23″E) in a hilly-gully region at Ansai County, Shaanxi Province on the Chinese Loess Plateau, and used as the substrate for pot experiment in the present study. The pH (soil:water = 1:5) of the soil was 8.7, and the field capacity was 33%. The concentrations of total N, total P, and bicarbonate-extractable P in the soil were 96 mg kg^{-1}, 493 mg kg^{-1}, and 3.3 mg kg^{-1}, respectively. The concentrations of total potassium (K) and organic C were 1.5 mg g^{-1} and 1.6 mg g^{-1}, respectively. The soil was air-dried and screened through a 2-mm sieve before filling the pots. For each plastic pot (of 12 cm inner diameter and 15 cm height), a plastic bag was lined inside first, then 2.0 kg of the air-dried and sieved soil was filled into the bag; a total of 136 pots were filled in this way.

For 68 out of the 136 pots, no P was added to the soil (hereafter referred to as 0P), while for another 68 pots, P was added at 20 mg P kg^{-1} soil (hereafter referred to as 20P) as a monopotassium phosphate (KH$_2$PO$_4$) aqueous solution. Then four forms of N, i.e. calcium nitrate (Ca(NO$_3$)$_2$), ammonium nitrate (NH$_4$NO$_3$), ammonium sulfate ((NH$_4$)$_2$SO$_4$), or urea (CO(NH$_2$)$_2$), were added to the soil at different rates. For both 0P and 20P, no N was added (hereafter referred to as 0N) to the soil in four out of the 68 pots, and the four pots without added P and N (0P0N) were treated as the control; for the rest of the

64 pots, N was added at four rates, i.e. 25, 50, 75, and 100 mg N kg^{-1} soil (hereafter referred to as 25N, 50N, 75N, and 100N, respectively) as an aqueous solution of Ca(NO$_3$)$_2$, NH$_4$NO$_3$, (NH$_4$)$_2$SO$_4$, or CO(NH$_2$)$_2$. There were four replicates for each N rate of each N form. Potassium was added at 50 mg kg^{-1} soil as a potassium chloride solution to all pots. We measured the concentrations of both macro- and micronutrients in the soil and also those in shoots of alfalfa grown on a series of soils with different nutrient supply before starting this experiment, and identified that P is the major limiting nutrient for alfalfa growth on the loess soil used in this study. The concentrations of almost all other macro- and micronutrients in shoots of alfalfa grown on unfertilised loess soil used in this study were greater than the average sufficient concentrations for adequate growth. Furthermore, although increasing the supply of other nutrients, e.g., zinc, may enhance the growth of alfalfa, that only happens when P is supplied at a much higher level than that in the present study, and Zn supply does not affect the amount of tartrate released by alfalfa roots (He et al. 2021). Therefore, no other fertiliser was supplied during the experiment.

After addition of the aqueous solution of K, P, and N, the soil in each pot was watered to 60% of the field capacity, then incubated for two weeks in a greenhouse at the Institute of Soil and Water Conservation (34°16′19″N, 108°04′20″E), Yangling, China. In order to obtain a substrate with homogenous distribution of the added K, P, and N, after incubation, the soil in each pot was air dried and screened through a 2-mm sieve once again separately, then mixed thoroughly and filled back to the pot that was lined with a plastic bag inside. In the middle of September 2019, seeds of alfalfa (*Medicago sativa* L. cv Golden Empress) were surface sterilised by soaking the seeds in 10% (v:v) hydrogen peroxide (H$_2$O$_2$) for 10 min, then rinsed with deionised water three times and placed on moist filter paper in Petri dishes to germinate overnight (He et al. 2017b). Sixty seeds were sown in each pot at 0.5 cm depth, and the seedlings were thinned to 50 per pot two weeks after sowing. Soil water content was maintained at 60% of field capacity by weighing the pots and replenishing deionised water every three days during the experiment, and no drainage was allowed. Plants were cultivated for a total of 100 days before being harvested in late December, 2019.

Collection of rhizosheath carboxylates and measurement of plant biomass

When plants were harvested at 100 days after sowing, the aboveground parts (hereafter referred to as shoots) of the plants in each pot were severed at the soil surface. The belowground parts (hereafter referred to as roots) of the plants and the soil in each pot were taken out of the pot together with the plastic bag. The roots were gently separated from the bulk soil, and the soil that was strongly attached to the roots after shaking was defined as rhizosheath soil (Pang et al. 2017). For each pot, rhizosheath carboxylates were extracted by soaking and gently stirring about 1.0 g fresh fine roots and strongly-attached rhizosheath soil in 20 mL of 0.2 mM CaCl$_2$ in a glass beaker for 5 min. We included only the strongly-attached rhizosheath soil, rather than rhizosphere soil, which has a much larger volume depending on the nutrient under investigation, for the extraction. We assume this could minimise the contribution of microbial metabolites to the composition of the root exudates. About 1 mL subsample of the extract was taken and filtered into a 1-mL HPLC vial through a 0.22-μm syringe filter, then a drop of concentrated phosphoric acid (H$_3$PO$_4$) was added to the vial to acidify the extract and prevent microbial degradation of the carboxylates. All extracts were stored at − 20 °C until analysis (He et al. 2020).

The roots were soaked, collected and thoroughly washed with tap water to remove the rhizosheath soil as much as possible, then rinsed with deionised water and oven-dried at 60 °C for 48 h to obtain the dry mass. A small number of tiny nodules developed during the experiment, and these were combined with the roots that were not soaked, then washed and oven-dried in the same way as the roots that were soaked, but weighed separately to obtain the dry mass. Total root dry mass in each pot was calculated as the sum of the dry mass of the roots soaked and those not soaked (including the nodules). Shoots were also oven dried at 60 °C for 48 h and weighed to obtain shoot dry mass. Root mass ratio was calculated as the ratio between root dry mass to the sum of root dry mass and shoot dry mass.

Determination of plant N and P concentrations, and calculation of plant N:P ratios

Concentrations of N and P in shoots and roots were determined. Each oven-dried sample was finely ground using a stainless pulverizer, about 0.1 g sub-sample of each ground sample was weighed and digested in a hot sulfuric acid (H_2SO_4)-H_2O_2 mixture. The concentration of N in the digestion solution was determined using a Kjeltec 2300 Automatic Kjeldahl Apparatus (Foss, Höganäs, Sweden) (Baker and Thompson 1992), and the concentration of P in the digestion solution was determined using the vanadium molybdenum yellow colorimetric method (Gupta et al. 1993). The N:P ratios in shoots and roots were calculated as the mass ratios of N to P, based on the determined N and P concentrations in shoots and roots, respectively.

Analysis of rhizosheath carboxylates

Carboxylates in the rhizosheath extracts of 0N, 25N, and 100N at both 0P and 20P were analyzed using High Performance Liquid Chromatography (HPLC). The apparatus used included a Waters E2695 HPLC, a Waters 2998 detector, and a Waters Symmetry C18 reverse phase column (Waters, Milford MA, USA). Carboxylic acids including tartaric acid, malonic acid, citric acid, malic acid, succinic acid, and acetic acid were used as the standards. The mobile phase included 20 mM monopotassium phosphate (KH_2PO_4) and 100% methanol, the KH_2PO_4 solution was pre-adjusted to pH 2.5 with concentrated H_3PO_4 and flowed at a rate of 0.6 mL min^{-1}, and the methanol flowed at a rate of 0.01 mL min^{-1}. Each sample was run for 13 min, and carboxylates were detected at 210 nm (He et al. 2020). The amounts of carboxylates in the rhizosheath were expressed in mmol per unit dry mass of the roots used for extraction.

Statistical analysis

For the two P rates (i.e. 0P and 20P), all four forms of N (i.e. Ca(NO$_3$)$_2$, NH$_4$NO$_3$, (NH$_4$)$_2$SO$_4$, and CO(NH$_2$)$_2$), and all N rates except 0N (i.e. 25N, 50N, 75N, and 100N), a three-way analysis of variance (ANOVA) was carried out to investigate the effects of P rate (P_r), N form (N_f), N rate (N_r), the interactions between any two of the three factors (i.e. $P_r \times N_f$, $P_r \times N_r$, and $N_f \times N_r$), and the interaction among the three factors (i.e. $P_r \times N_f \times N_r$) on parameters of plant biomass, N and P concentrations, N:P, and the amounts of rhizosheath carboxylates. The three-way ANOVA was carried out using the general linear model in the SPSS 25.0 software package (IBM, Montauk, New York, USA), and the effects were determined to be significant at $P < 0.05$. Because 0N was shared by all four forms of N, it was not included in the ANOVA. The criterion to determine the effect of a treatment to be significant if $P < 0.05$ has been and still is widely used. However, it is getting increasingly challenged by statisticians, and presenting the effect size is encouraged when discussing the effect of a treatment (Goodman et al. 2019). Therefore, in addition to the effects determined based on the P-values of three-way ANOVA, the effect size of each treatment relative to the control (i.e. 0P0N) was calculated, and the interaction between P and N was determined for each treatment with added P and N. Here, we presented both the P-values of three-way ANOVA and the effect sizes, but relied more on the effect sizes for description and discussion of the results, as low replication or statistical power in experiments may obscure the ability to detect biologically meaningful responses using the P-value criterion, while the effect sizes are the log response ratios representing the proportional response to experimental treatment and tend to be distributed normally (Harpole et al. 2011).

In this study, the effect sizes were calculated according to the method described by Harpole et al. (2011), but with some modifications. Briefly, the effect size of a treatment for a parameter was calculated as the log response ratio, i.e. the log value of the ratio between the value of the parameter in the treatment to that in the control. Therefore, the effect sizes of P and N were calculated as follows:

Effect size of $20P = Ln(20P0N/0P0N)$.

Effect size of $xN = Ln(0PxN/0P0N)$.

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

Effect size of $(20P + xN) =$ *Effect size of* $20P +$ *Effect size of* xN.

and the effect size of the interaction between P rate and N rate was calculated as:

Effect size of $(20P \times xN) = Ln\ (20PxN/0P0N)$.

where x = 25, 50, 75, and 100, respectively, and the effect size of each N rate for each N form was calculated separately. As the effect size of a treatment was 0.05 and − 0.05 when the response ratio was 1.05 and 0.95, respectively, an effect size > 0.05 indicates that the value of a parameter increased by more than 5% compared with the control and the effect was positive, while an effect size < − 0.05 indicates that the value of a parameter decreased by more than 5% compared with the control and the effect was negative; an effect size between − 0.05 and 0.05 indicates that the effect was negligible. Furthermore, intuitively, the interaction was considered additive when the effect size of the interaction was equivalent (a less than 10% difference was considered equivalent) to that of the simple addition, while it was considered super-additive when the effect size of the interaction was at least 10% greater than that of the simple addition, and sub-additive when the effect size of the interaction was at least 10% less than that of the simple addition.

Bivariate Pearson correlations were used to determine the correlations between the mean amount of each rhizosheath carboxylate and mean shoot N concentration, root N concentration, shoot N:P, root N:P, shoot dry mass, root dry mass, and total plant biomass (the sum of shoot dry mass and root dry mass) in the control and each treatment under 0P and 20P separately. The correlation analyses were performed using the SPSS 25.0 software package, and the correlations were considered significant at $P < 0.05$.

Results

Plant biomass

According to the results of three-way ANOVA, when N was added, increasing P supply significantly increased shoot dry mass, root dry mass, and root mass ratio (all $P \le 0.001$) (Fig. 1, Table 1). The form of N had a significant effect on shoot dry mass ($P = 0.032$) and root mass ratio ($P < 0.001$) (Fig. 1a and c), but not on root dry mass ($P > 0.05$, Fig. 1b). Among all four forms of N, $Ca(NO_3)_2$ resulted in the smallest shoot dry mass, while NH_4NO_3 resulted in the highest root mass ratio. When N was added, mean shoot dry mass and root

mass ratio varied significantly with N rate ($P = 0.008$ for shoot dry mass and $P = 0.004$ for root mass ratio, respectively), with shoot dry mass being the largest at 50N and the smallest at 100N, while root mass ratio declined with increasing N rate. There was a significant interaction between P rate and N rate on root dry mass ($P < 0.001$), which was the largest at 50N and the smallest at 100N at 0P, but decreased with increasing N rate at 20P. Based on the calculated effect sizes, the effect of P was positive on both shoot dry mass and root dry mass, but negligible on root mass ratio; the effect of N was positive on shoot dry mass in almost all treatments, but negative on root dry mass in most treatments and on root mass ratio in almost all treatments. The interactive effects between P and N on shoot dry mass and root dry mass were always positive and sub-additive in most treatments, while that on root mass ratio was negative and super-additive in most treatments. Adding N weakened the positive effect of P on shoot dry mass in most treatments and on root dry mass in all treatments at 20P, while adding P weakened the negative effect of N on root mass ratio in most treatments.

Plant N and P concentrations, and N:P ratios

The results of three-way ANOVA showed that, when N was added, both shoot and root N concentrations decreased significantly when P was added (both $P < 0.001$), but increased considerably with increasing N rate (both $P < 0.001$) (Fig. 2, Table 1). There was a significant interaction between P rate and N form on shoot N concentration ($P < 0.001$); among all four forms of N, NH_4NO_3 resulted in the greatest shoot N concentration at 0P but the least shoot N concentration at 20P (Fig. 2a). Root N concentration varied considerably with N form, and it was the greatest when $Ca(NO_3)_2$ was added (Fig. 2b). Based on the effect sizes, the effect of P was positive on shoot N concentration but negative on root N concentration, while the effect of N was always positive on both shoot and root N concentrations. The effect of the interaction between P and N on shoot N concentration was always positive and sub-additive, but that on root N concentration was negative and sub-additive in most treatments. Adding P weakened the positive effect of N on both shoot and root N concentrations in almost all treatments.
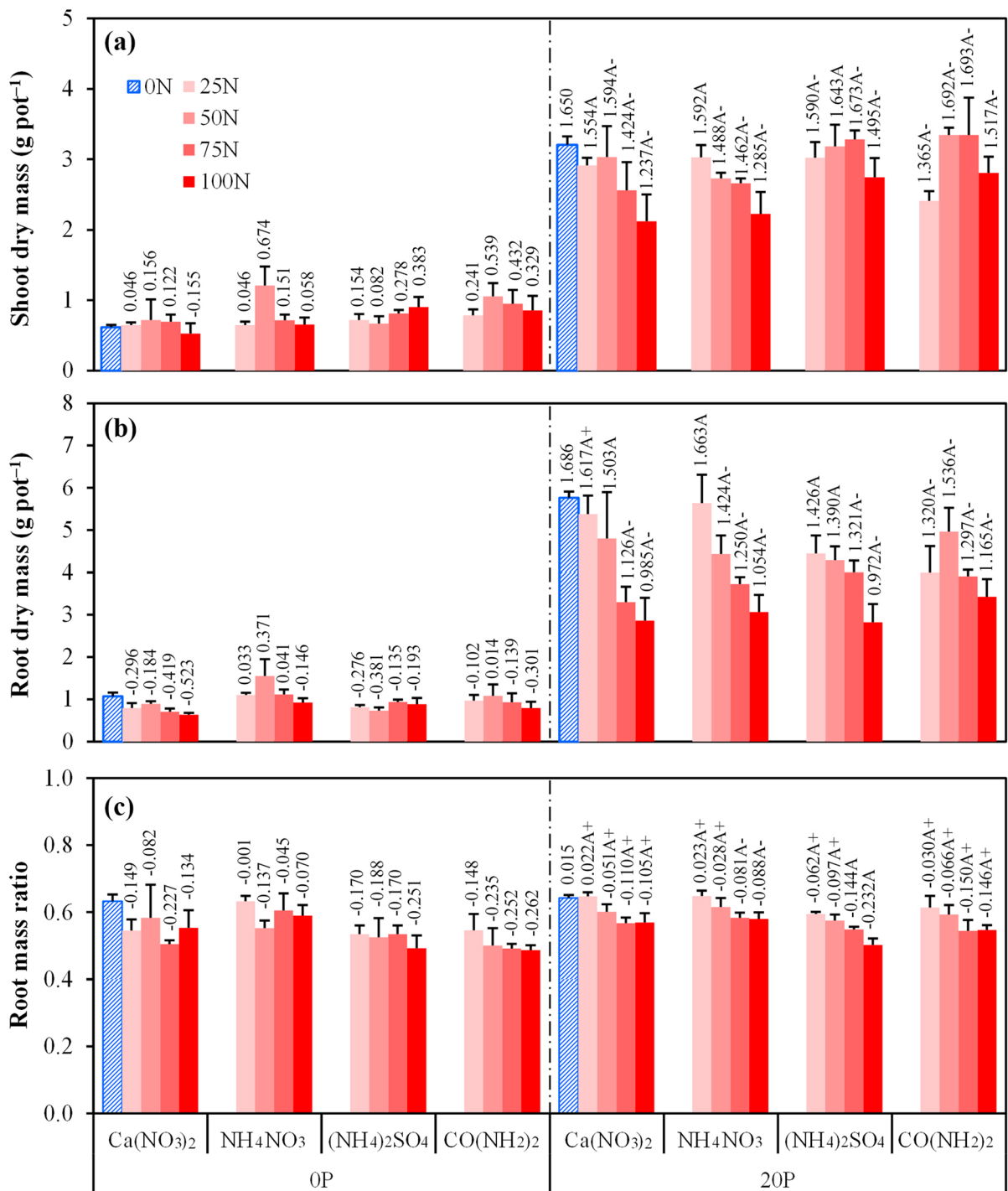
Fig. 1 Shoot dry mass (**a**), root dry mass (**b**), and root mass ratio (**c**) of alfalfa grown for 100 days in a loess soil in a greenhouse with different rates of added phosphorus (P) and nitrogen (N) in four forms. 0P and 20P represent that P was added at 0 and 20 mg kg^{-1} as KH_2PO_4, respectively; 0N, 25N, 50N, 75N, and 100N represent that N was added at 0, 25, 50, 75, and 100 mg kg^{-1}, respectively. Data are presented as means + SE ($n=4$). Numbers above the bars are effect sizes of the treatments; A, A−, and A+ following the numbers indicate the effects of N and P were additive, sub-additive, and super-additive, respectively

Table 1 Statistical level of significances (*P*-values) of the three-way analysis of variance (ANOVA) for various parameters of alfalfa grown for 100 days in a loess soil in a greenhouse with different rates of added phosphorus (P) and nitrogen (N) in four forms

Parameter	Source of variations						
	P_r	N_f	N_r	$P_r \times N_f$	$P_r \times N_r$	$N_f \times N_r$	$P_r \times N_f \times N_r$
Shoot dry mass	**< 0.001**	**0.032**	**0.008**	0.295	0.179	0.305	0.397
Root dry mass	**< 0.001**	0.352	**< 0.001**	0.887	**< 0.001**	0.490	0.478
Root mass ratio	**0.001**	**< 0.001**	**0.004**	0.322	0.464	0.853	0.945
Shoot N concentration	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	0.335	0.645	0.238
Root N concentration	**< 0.001**	**0.007**	**< 0.001**	0.719	0.084	0.699	0.416
Shoot P concentration	**< 0.001**	0.063	**< 0.001**	0.326	0.336	0.167	0.130
Root P concentration	**< 0.001**	**< 0.001**	**0.001**	**< 0.001**	**< 0.001**	0.265	0.183
Shoot N:P	**< 0.001**	**0.019**	0.288	0.417	0.516	0.182	0.579
Rhizosheath tartrate	**< 0.001**	**< 0.001**	**< 0.001**	0.117	**< 0.001**	**< 0.001**	0.117
Rhizosheath malonate	**< 0.001**	0.609	**0.040**	0.238	0.369	0.761	0.498
Rhizosheath citrate	0.927	0.952	0.586	0.471	0.618	0.414	0.594
Rhizosheath malate	**< 0.001**	0.896	**0.024**	0.926	n.a	0.160	n.a

P_r, N_f, and N_r represent phosphorus (P) addition rate, nitrogen (N) form, and N addition rate, respectively. N:P represents the mass ratio of N to P. The bold values indicate significant effects (*P* < 0.05). n.a. means not available, due to lack of enough replicates for the ANOVA

When N was added, shoot P concentration significantly increased when P supply was increased (*P* < 0.001), but it did not vary considerably with N form (Fig. 3a; Table 1). Shoot P concentration was also significantly affected by N rate (*P* < 0.001) and showed the lowest value at 25N, but did not show a consistent trend with increasing N rate. Root P concentration was significantly affected by the interactions between P rate and N form (*P* < 0.001), and between P rate and N rate (*P* < 0.001) (Fig. 3b; Table 1). Among all four forms of N, root P concentration was the lowest under $Ca(NO_3)_2$ and greatest under NH_4NO_3 at 0P, while it was the lowest under $(NH_4)_2SO_4$ and greatest under $CO(NH_2)_2$ at 20P. Among the four N rates, 100N resulted in the lowest root P concentration at 0P but the greatest root P concentration at 20P. As shown by the effect sizes, the effect of P was positive on both shoot and root P concentrations; the effect of N was positive on shoot P concentration in all but one treatment and always positive on root P concentration. The effect of the interaction between P and N on shoot P concentration was always positive and sub-additive in all but one treatment, and that on root P concentration was also always positive and sub-additive in most treatments. Adding N and P together strengthened the positive effect of each other on both shoot and root P concentrations in almost all treatments, except in some treatments at lower N rates, i.e. 25N and 50N, where adding N weakened the positive effect of P on shoot P concentration.

According to the results of three-way ANOVA, shoot N:P was significantly affected by P rate (*P* < 0.001) and N form (*P* = 0.019), but not by N rate (Fig. 4, Table 1). Shoot N:P declined markedly when P was added; among all four forms of N, shoot N:P was the highest when $Ca(NO_3)_2$ was added, and the lowest under $CO(NH_2)_2$. As shown by the effect sizes, the effect of P on shoot N:P was negative, while the effect of N on shoot N:P was positive in most treatments. The effect of the interaction between P and N was always negative, and additive or super-additive in most treatments. Root N:P was significantly affected by all three factors (all *P* < 0.001) and all interactions (all *P* ≤ 0.017) (Fig. S1, Table S1). The effect sizes showed that the effect of P on root N:P was negative, while the effect of N on root N:P was always negative for all forms of N, except $Ca(NO_3)_2$, for which the effect of N on root N:P was positive under three out of the four N rates. The effect of the interaction between P and N was always negative, it was always sub-additive when
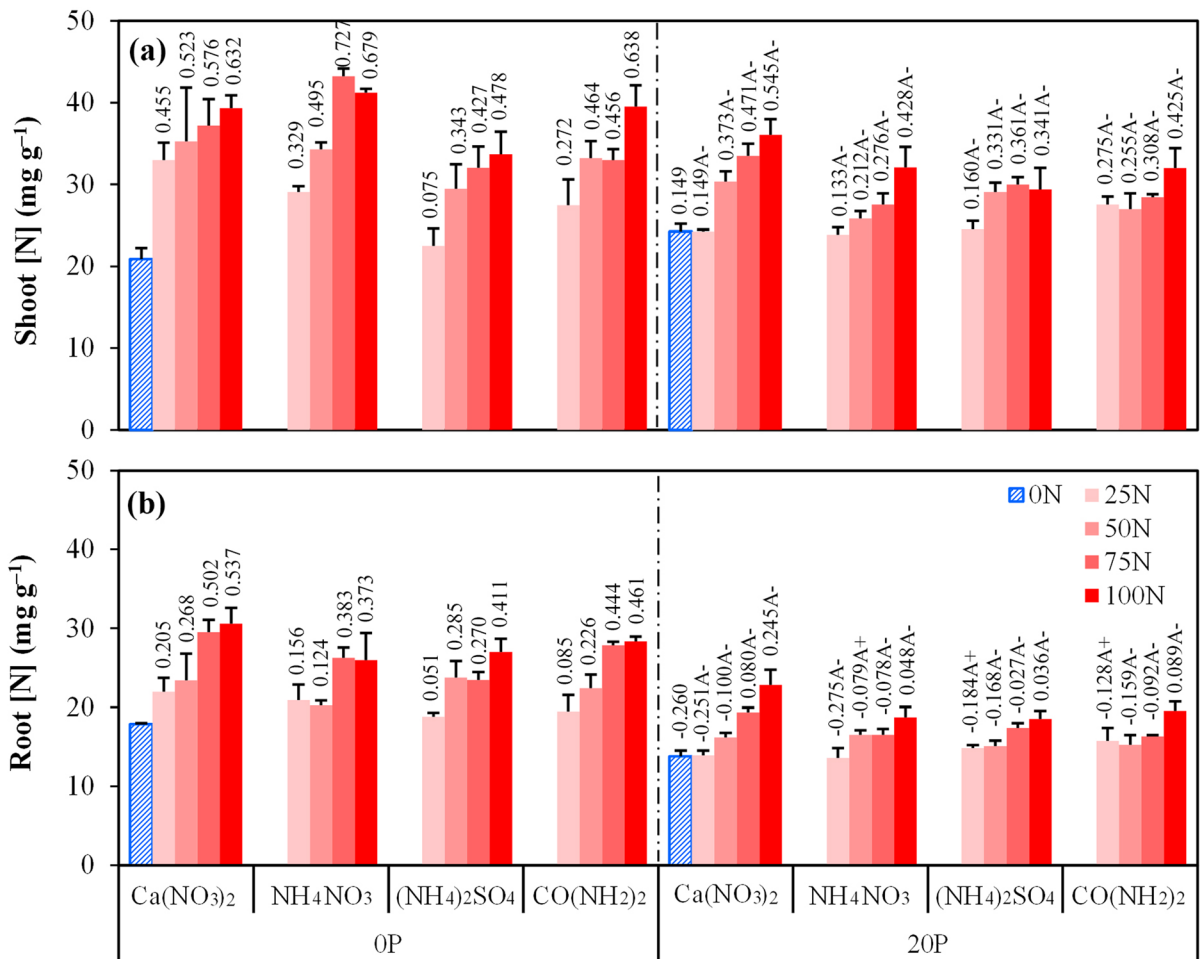
Fig. 2 The concentration of nitrogen ([N]) in shoots (**a**) and roots (**b**) of alfalfa grown for 100 days in a loess soil in a greenhouse with different rates of added phosphorus (P) and N in four forms. 0P and 20P represent that P was added at 0 and 20 mg kg^{-1} as KH_2PO_4, respectively; 0N, 25N, 50N, 75N, and 100 N represent that N was added at 0, 25, 50, 75, and 100 mg kg^{-1}, respectively. Data are presented as means + SE ($n = 4$). Numbers above the bars are effect sizes of the treatments; A, A−, and A+ following the numbers indicate the effects of N and P were additive, sub-additive, and super-additive, respectively

$Ca(NO_3)_2$ was added, but additive and super-additive in most treatments when other forms of N were added. Adding N further strengthened the negative effect of P on root N:P (Fig. S1).

The amounts of rhizosheath carboxylates

We detected tartrate, malonate, citrate, and malate in the analyzed samples. The results of three-way ANOVA showed that, when N was added, the amount of tartrate was significantly affected by all three factors, and also by the interactions between

P rate and N rate, and between N form and N rate (all $P < 0.001$) (Fig. 5, Table 1). At 25N, the amount of tartrate was 81% less at 20P than at 0P; at 100N, it was 69% less at 20P than at 0P. At 0P, the amount of tartrate was 12 times more at 100N than at 0N; at 20P, it was 21 times more at 100N than at 0N. At both 25N and 100N, among all four forms of N, the amount of tartrate was the largest when $Ca(NO_3)_2$ was added, and the smallest under $(NH_4)_2SO_4$. For $Ca(NO_3)_2$, NH_4NO_3, $(NH_4)_2SO_4$, and $CO(NH_2)_2$, the amount of tartrate was 13, 19, 16, and 11 times greater at 100N than at 25N,
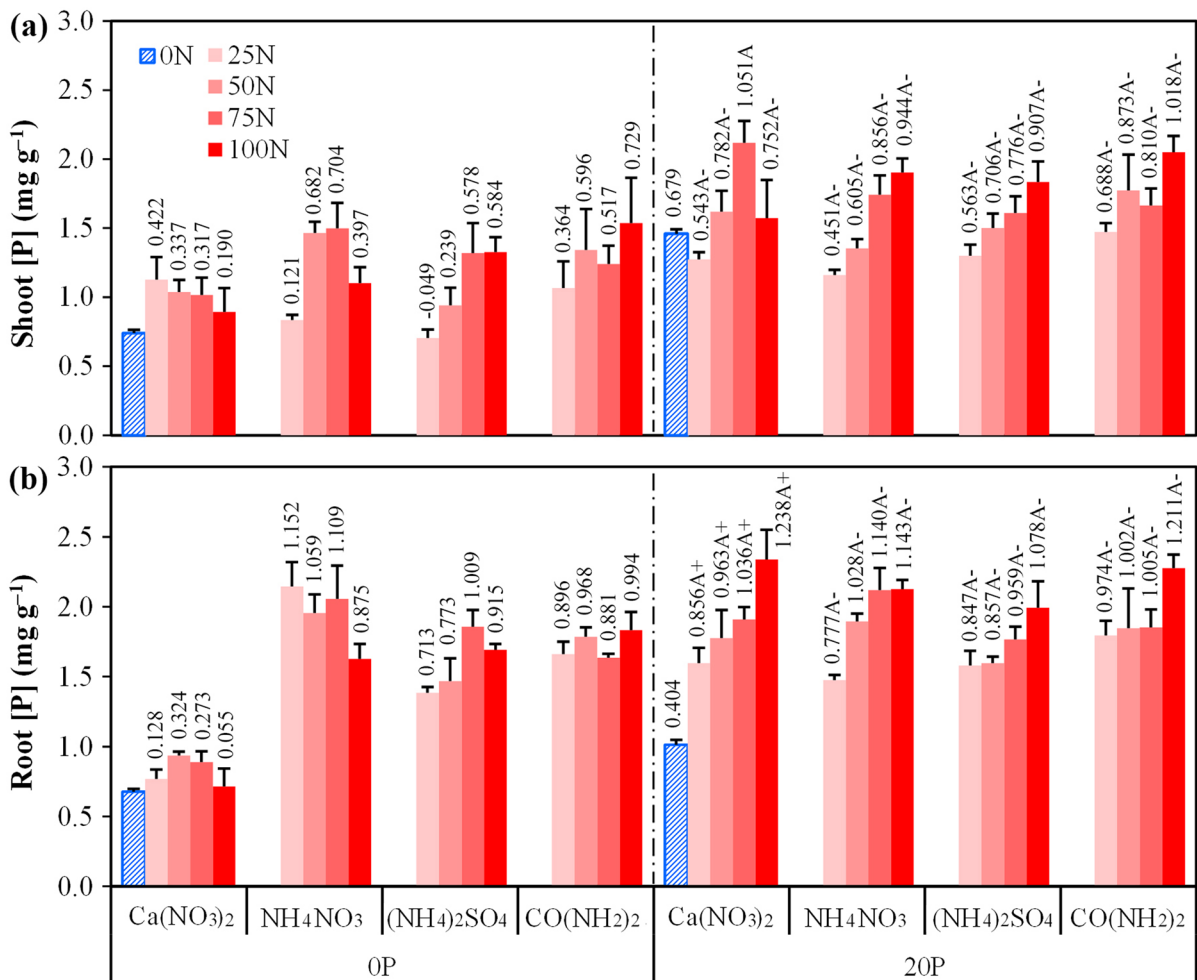
Fig. 3 The concentration of phosphorus ([P]) in shoots (**a**) and roots (**b**) of alfalfa grown for 100 days in a loess soil in a greenhouse with different rates of added P and nitrogen (N) in four forms. 0P and 20P represent that P was added at 0 and 20 mg kg^{-1} as KH$_2$PO$_4$, respectively; 0N, 25N, 50N, 75N, and 100N represent that N was added at 0, 25, 50, 75, and 100 mg kg^{-1}, respectively. Data are presented as means + SE ($n = 4$). Numbers above the bars are effect sizes of the treatments; A, A−, and A+ following the numbers indicate the effects of N and P were additive, sub-additive, and super-additive, respectively

respectively. At 25N, when Ca(NO$_3$)$_2$, NH$_4$NO$_3$, and CO(NH$_2$)$_2$ was added, the amount of tartrate was 5.7, 3.6, and 3.8 times larger than when (NH$_4$)$_2$SO$_4$ was added, respectively; at 100N, it was 21, 7.0, and 6.9 times larger, respectively. The effect sizes showed that the effect of P was slight when no N was added, while the effect of N was always positive, and the interactive effect of P and N was always positive and sub-additive. Adding N made the effect of P more negative, while adding P weakened the positive effect of N.

When N was added, both P rate and N rate had a significant effect on the amounts of malonate ($P < 0.001$ for P rate and $P = 0.040$ for N rate, respectively) and malate ($P < 0.001$ for P rate and $P = 0.024$ for N rate, respectively) (Fig. 6, Table 1). The amount of malonate was 69% larger at 20P than at 0P, and it was 22% smaller at 100 N than at 25 N (Fig. 6). Malate was detected at 25N, but not at 100N at 0P, but it was detected at both 25N and 100N at 20P; the amount at 25N was 122% larger at 20P than at 0P, while the amount at 20P was 39% smaller at 100N than at 25N. The amount
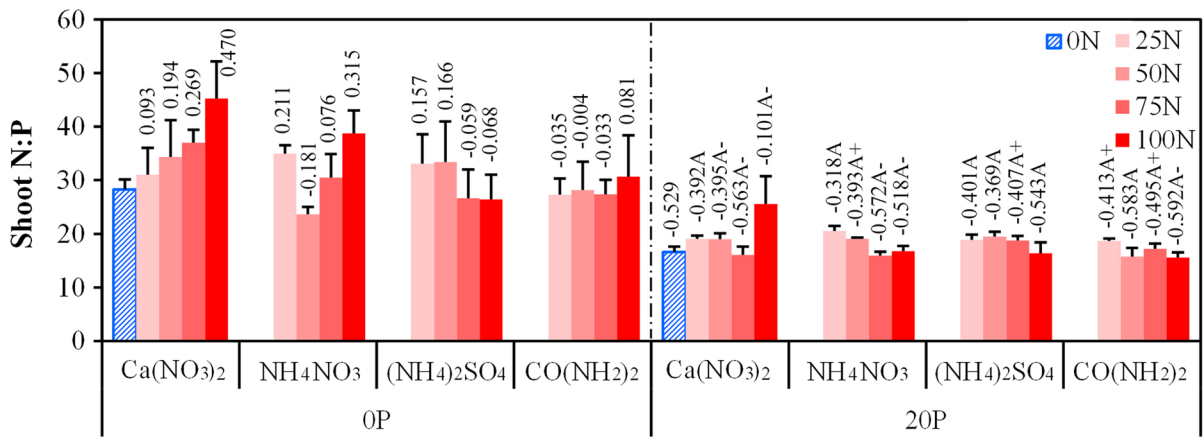
Fig. 4 Shoot nitrogen to phosphorus mass ratio (N:P) of alfalfa grown for 100 days in a loess soil in a greenhouse with different rates of added P and N in four forms. 0P and 20P represent that P was added at 0 and 20 mg kg^{-1} as KH$_2$PO$_4$, respectively; 0N, 25N, 50N, 75N, and 100N represent that N was added at 0, 25, 50, 75, and 100 mg kg^{-1}, respectively. Data are presented as means + SE ($n=4$). Numbers above the bars are effect sizes of the treatments; A, A-, and A+ following the numbers indicate the effects of N and P were additive, sub-additive, and super-additive, respectively
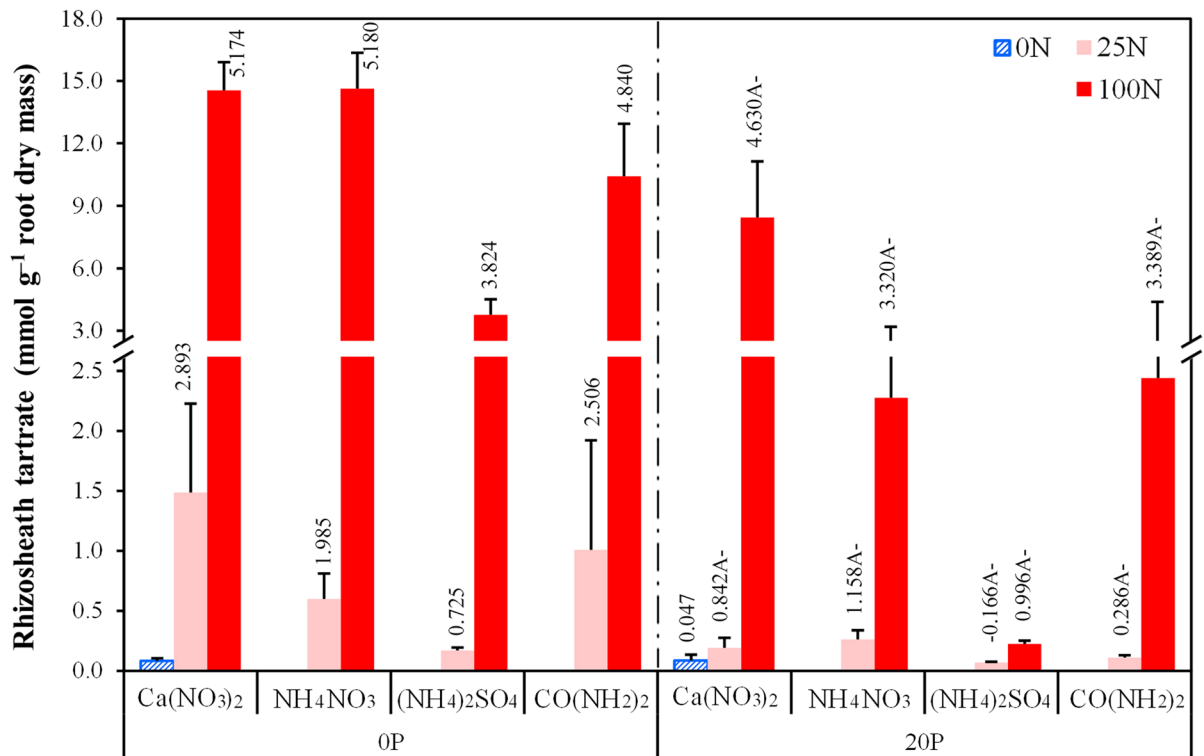

Fig. 5 The amount of tartrate in the rhizosheath of alfalfa grown for 100 days in a loess soil in a greenhouse with different rates of added phosphorus (P) and nitrogen (N) in four forms. 0P and 20P represent that P was added at 0 and 20 mg kg^{-1} as KH$_2$PO$_4$, respectively; 0N, 25N, 50N, 75N, and 100N represent that N was added at 0, 25, 50, 75, and 100 mg kg^{-1}, respectively. Data are presented as means + SE ($n=4$, except for some treatments in which tartrate was not detected in all samples). Numbers above the bars are effect sizes of the treatments; A, A-, and A+ following the numbers indicate the effects of N and P were additive, sub-additive, and super-additive, respectively
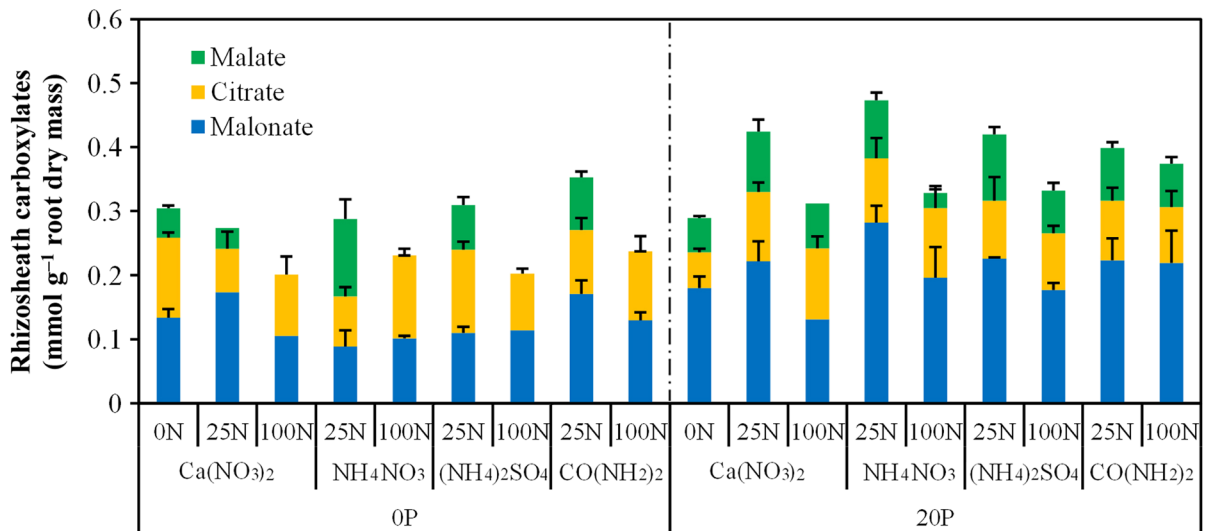
Fig. 6 The amounts malonate, citrate, and malate in the rhizosheath of alfalfa grown for 100 days in a loess soil in a greenhouse with different rates of added phosphorus (P) and nitrogen (N) in four forms. 0P and 20P represent that P was added at 0 and 20 mg kg^{-1} as KH$_2$PO$_4$, respectively; 0N, 25N, 50N, 75N, and 100N represent that N was added at 0, 25, 50, 75, and 100 mg kg^{-1}, respectively. Data are presented as means + SE ($n = 4$, except for some treatments in which certain carboxylate was not detected in all samples)

of citrate was not significantly affected by any of the three factors, or by any two-way or three-way interactions.

Correlations between the amounts of rhizosheath carboxylates and plant N and P nutrition
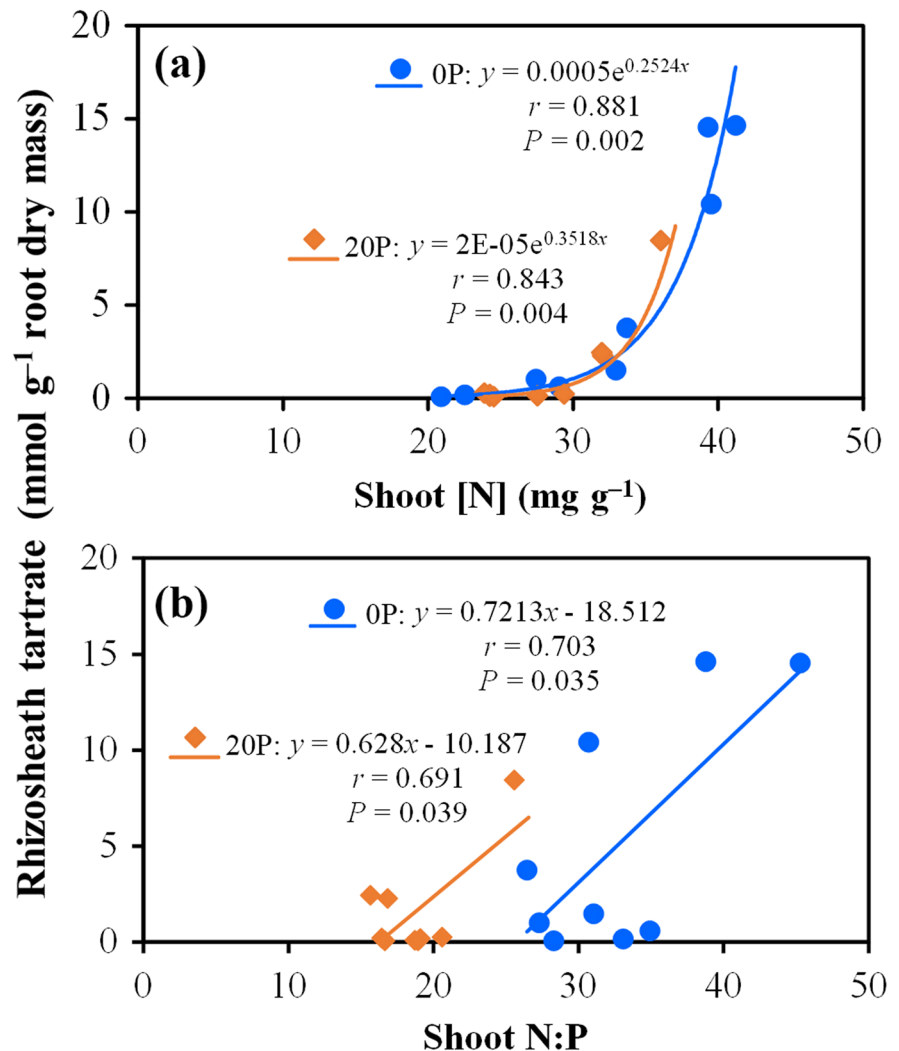
At both 0P and 20P, the amount of tartrate was significantly positively correlated with shoot N concentration ($r = 0.881$ and $P = 0.002$ at 0P, and $r = 0.843$ and $P = 0.004$ at 20P, respectively) (Fig. 7a), shoot N:P ($r = 0.703$ and $P = 0.035$ at 0P, and $r = 0.691$ and $P = 0.039$ at 20P, respectively) (Fig. 7b), and root N concentration ($r = 0.856$ and $P = 0.003$ at 0P, and $r = 0.844$ and $P = 0.004$ at 20P, respectively) (Fig. S2). There were other significant correlations not shown in figures or tables, including a significant positive correlation between the amount of tartrate and root P concentration at 20P ($r = 0.667$ and $P = 0.050$), a significant negative correlation between the amount of malonate and root N concentration at 20P ($r = -0.695$ and $P = 0.038$), a significant negative correlation between the amount of citrate and root N:P at 20P ($r = -0.783$ and $P = 0.013$), and a significant negative correlation between the amount of malate and shoot P concentration at 20P ($r = -0.699$ and $P = 0.036$).

Discussion

In the present study, the hypothesis that plant growth would be enhanced by P addition as well as N addition was not fully supported. Both shoot growth and root growth showed a positive response when P was added alone to the P-deficient soil, similar to the results of many other studies (He et al. 2017b; Pang et al. 2010; Pearse et al. 2006). When N was added alone, shoot growth always showed a positive response, but root growth showed a slightly negative response in most treatments. It is likely that N addition enhanced the photosynthetic capacity of the plants when no P was added (Evans 1983; Fleischer et al. 2013). However, a greater N supply did not invariably lead to a greater shoot biomass, likely because a lower N supply was already sufficient for alfalfa (Perring et al. 2008). When P was supplied, adding N weakened the positive effect of P on plant growth, and this effect was more obvious on root growth than on shoot growth, especially at 100N. The effect of N addition on biomass production in the

Fig. 7 Correlations between the amount of tartrate in the rhizosheath and shoot nitrogen concentration ([N]), and shoot N to phosphorus mass ratio (N:P) of alfalfa grown for 100 days in a loess soil in a greenhouse with different rates of added P and N in four forms. 0P and 20P represent that P was added at 0 and 20 mg kg^{-1} as KH$_2$PO$_4$, respectively

(a)

0P: $y = 0.0005e^{0.2524x}$
$r = 0.881$
$P = 0.002$

20P: $y = 2E\text{-}05e^{0.3518x}$
$r = 0.843$
$P = 0.004$

Shoot [N] (mg g^{-1})

(b)

0P: $y = 0.7213x - 18.512$
$r = 0.703$
$P = 0.035$

20P: $y = 0.628x - 10.187$
$r = 0.691$
$P = 0.039$

Rhizosheath tartrate (mmol g^{-1} root dry mass)

Shoot N:P

present study differed from that reported by Li et al. (2016), who found that the P-enhanced biomass production in terrestrial ecosystems is greater under elevated N than under background N, based on a meta-analysis of the effects of P addition, either alone or with N addition. The difference may be because we only studied alfalfa, a legume with a low exogenous demand for N but high demand for P (Haling et al. 2016; Valentine et al. 2017), while the meta-analysis of Li et al. (2016) covered many types of native vegetation. The results of Tian et al. (2020) showed that the effect of N addtion on plant biomass accumulation is functional group-dependent; N addition increased the aboveground biomass of grasses and sedges, while it had little effect on that of forbs, and reduced that of N$_2$-fixing forbs at the early phase of N

addition. In the present study, a similar growth reduction might be expected, because alfalfa growth was limited by P, even at 20P, as it has been found that dry mass and P concentrations of both shoots and roots of alfalfa could be further increased considerably by increasing P supply to 80 mg kg^{-1} to the same loess soil (Peng et al. 2020), and P-limitation might restrict the positive response of plant growth to N addition. Furthermore, although N and P assimilation are closely coupled within plants (Evans 1983), adding N would increase the plants' demand for P and aggravate P deficiency, thus limiting biomass production (Li et al. 2016; Phoenix et al. 2004; Zhan et al. 2017).

Our results do not fully support the hypothesis that belowground biomass allocation would decrease with increasing P rate, and also with increasing N rate. The

root mass ratio of alfalfa in the present study showed a more N-dependent than P-dependent response, which declined in response to almost all N supply rates at both 0P and 20P, and tended to decline with increasing N rate at 20P. There are studies reporting that root mass ratio declines with N addition (Li et al. 2020; Zhang et al. 2020). It is likely that, in the present study, in response to N addition, due to reduced C requirement for root biomass, the release of C by roots increased. We did not observe a negative relationship between root mass ratio and P-fertilisation in the present study, similar to the results in some previous studies on alfalfa growing in alkaline soils (He et al. 2017b, 2021), although often a greater root mass ratio can enhance a plant's P acquisition under P deficiency (Lynch and Ho 2005), and root mass ratio usually declines with P-fertilisation (Graciano et al. 2006; Poeplau et al. 2016). In contrast, root mass ratio of alfalfa increased with P-fertilisation in a loess soil, likely due to the plant's response to N-limitation induced by P-fertilisation (Zhang et al. 2021).

The hypothesis that plant P and N concentrations would increase with increasing P rate, and also with increasing N rate was not fully supported. At the same P rate, adding N resulted in increased shoot and root N concentrations in almost all treatments, except when N was added as NH_4NO_3 at 25 mg kg^{-1} under 20P, and the effects were more significant at greater N rates. Adding P had a negative effect on root N concentration compared with 0P; it also weakened the positive effect of N on both shoot and root N concentrations, likely partly due to the "biological dilution effect" caused by P-enhanced biomass accumulation (He et al. 2017a). Generally, when P or N was added alone, the effects of P and N on both shoot and root P concentrations were positive; when P and N were added together, the positive effects were further strengthened, and the N-induced biomass reduction might cause a "biological concentration effect" of P in plants, thus resulting in greater shoot and root P concentrations at greater N rates. Our suggestion of the "biological dilution effect" and "biological concentration effect" are partly supported by the results of plant N and P content, which were relatively stable when compared with the variation in plant biomass (Table S1, Fig. S3).

There are studies showing that N addition results in greater plant N and P concentrations, because the assimilation of N and P are closely coupled within plants (Evans 1983; Lü et al. 2013). The meta-analysis of Li et al. (2016) also showed that an increase in plant P concentration induced by P addition is greater under elevated N than under background N. However, there are studies showing that N addition results in increased N concentration, but decreased P concentration in plants (Li et al. 2019; Peng et al. 2017), while P addition does not affect plant N concentration (Li et al. 2019). Some studies also showed that both N and P concentrations are greater in plants fertilised with N or P than in control plants not fertilised, and, surprsisingly, both N and P concentrations are greater in P-fertilised than in N-fertilised plants (Graciano et al. 2006). The differences between the above-mentioned results are likely due to differences among plant species, soil type, as well as the rates and forms of N and P applied.

The hypothesis that plant N:P ratio would increase with increasing N rate but decline with increasing P rate was partly supported. At all N rates, P addition led to lower shoot and root N:P ratios, while N addition led to greater shoot N:P but lower root N:P in most treatments. Plant N:P ratio is an indicator of nutrient limitation of plant growth; N:P ratios < 10 and > 20 indicate that biomass production of terrestrial plants is N- or P-limited, respectively (Güsewell 2004; Koerselman and Meuleman 1996). Changes in N and/or P availability in soil can alter plant N:P ratio, with N addition often increasing plant N:P ratio and aggravating P-limitation, while P addition often reduces plant N:P ratio and alleviates P-limitation (Güsewell 2004; Zhang et al. 2013). In the present study, shoot N:P ratio was always > 20 when no P was added, indicating that plant growth was limited by P; when P was added, shoot N:P ratio was between 10 and 20 in all but two treatments, indicating that P-limitation was alleviated.

The hypothesis that the amount of tartrate released by roots would decline with increasing P rate but increase with increasing N rate was partly supported by the results of the present study. Among the four carboxylates detected in the rhizosheath extracts, malonate was the most abundant one in the control and the 20P25N treatment, but tartrate became the most abundant one and increased dramatically when N was added alone and also when N was added at the greatest rate together with P (i.e. 20P100N). Tartrate is less commonly reported than citrate, malate, and malonate in studies on plant responses to

P-deficiency, and it has a much weaker capacity for P mobilisation in a clay loam Ultisol than citrate (Wang et al. 2008).

In the present study, when no N was added, the effect of P addition on tartrate release was negligible, but it was significantly negative when N was added, while the effect of N addition on tartrate release was almost always significantly positive, regardless of P supply. Compared with other carboxylates, the release of tartrate was more affected by soil P and N availability, similar to the results of He et al. (2020), in which alfalfa was grown in alkaline soils with low P and low N availability. For alfalfa grown in the present study, tartrate release might not be always associated with proton (H^+) release, as shown by the rhizosheath pH, which was not always lower than bulk soil pH and did not vary with either P supply or N supply as greatly as the amount of tartrate did (Table S1, Fig. S4). Possible strong buffering capacity of the loess soil could not explain the lack of the correlation between tartrate amount and the change in rhizosheath pH, as both bulk soil pH and rhizosheath pH were strongly affected by P and N fertilisation. It is likely that tartrate was released as an anion with accompanying cations other than H^+, e.g. K^+ (Roelofs et al. 2001; Ryan et al. 1995).

The hypothesis that the amount of tartrate released by roots would have a closer relationship with plant N concentration than with plant P concentration was supported. The effect sizes and correlations all showed that N had a stronger effect than P on tartrate release. Soil N concentration and N:P ratio, rather than soil P concentration affected the release of tartrate, as the amount of tartrate correlated significantly positively with soil N concentration ($r = 0.601$ and $P < 0.001$) and N:P ratio ($r = 0.478$ and $P < 0.001$) (Fig. S5), but not with soil P concentration. The results of the present study were in contrast with previous findings showing that the release of citrate and malate by lupins is controlled systemically by shoot or leaf P concentration (Shane et al. 2008; Shen et al. 2003). Under 20P, there was a significantly negative correlation between the amount of rhizosheath tartrate per unit root dry mass ($r = -0.693$ and $P = 0.039$) or the total amount of rhizosheath tartrate per pot ($r = -0.677$ and $P = 0.045$) and shoot dry mass of alfalfa in the present study (Fig. S6), indicating that release of more tartrate was associated with growth reduction. The correlation between tartrate

amount and shoot dry mass was also negative under 0P, but not significant ($P > 0.5$). The increase of tartrate amount in response to N addition under both 0P and 20P, the negetive correlation between tartrate amount and shoot dry mass, and the lack of a close relationship between tartrate amount and soil P concentration or plant P concentration, suggest that N addition likely resulted in more surplus C, which was then released as tartrate by roots. The release of tartrate was likely a way to discharge surplus C produced when the increase in photosynthate production exceeded the photosynthate demand for plant growth (Prescott et al. 2020).

The increase in the amount of C released by roots under elevated N rate might be explained by the increased difference between the amount of C produced by photosynthesis and that of C required for growth at higher N rates in the present study. However, although Prescott et al. (2021) argued that rhizosphere 'trade' is an unnecessary analogy, and release of surplus C under conditions that limit plant growth drives allocation and plant-soil interactions, in some situations, the production and release of certain C-containing compounds do have a specific effect, rather than disposal of surplus C. For example, the release of carboxylates by the cluster roots of lupins (Lambers et al., 2013) and by the cluster or dauciform roots of Proteaceae or Cyperaceae, respectively (Lambers et al., 2011), is considered a pivotal mechanism to increase P acquisition under P deficiency. Therefore, the potential function of release of tartrate, and also other carboxylates, in P mobilisation should not be dismissed (Wang and Lambers 2020). In the present study, plant P concentration was increased by N-addition, indicating that adding N induced greater P demand by the plants, such aggravated P-deficiency might also play a role, likely less important than discharging surplus C, in driving tartrate release.

The hypothesis that the effects of N rate on the parameters investigated would depend on the forms of the added N was supported. In the present study, differences in effects among N forms were likely due to the preferential uptake of certain N forms by alfalfa. It is very likely that alfalfa preferentially took up NO_3-N from the loess soil compared with other N forms, since the loess soil is alkaline and has a high Ca content, while NH_4-N was the least preferred (Gigon and Rorison 1972). This would explain why shoot N concentration and N:P ratio

increased the most when $Ca(NO_3)_2$ was added, and the amount of tartrate was largest when $Ca(NO_3)_2$ was supplied, but smallest when $(NH_4)_2SO_4$ was added. Furthermore, as the loess soil used in the present study was alkaline, it is likely that a proportion of N was lost via ammonia volatilisation when N was supplied as NH_4-N or urea-N (He et al. 1999; Zheng et al. 2018). Loss of N via ammonia volatilisation might result in a reduction in N availability in soil; such a reduction might be most significant when NH_4-N was the only form of N supplied, and explain why the amount of tartrate was the least when $(NH_4)_2SO_4$ was supplied; however, such a reduction did not considerably reduce plant growth and N concentration. Since only a few tiny nodules developed during the experiment, we assume that the amount of N in plants derived from symbiotic N_2 fixation was small under P deficiency and did not affect our interpretation of the effects of N rate and N form in the present study.

Conclusions

The findings of the present study demonstrate that the release of tartrate was enhanced in response to a greater soil N concentration and N:P ratio, and greater shoot N concentration and N:P ratio, which was likely a way to discharge surplus C produced by enhanced photosynthetic capacity induced by N addition when plant growth was limited by P. The effects of N addition and its interaction with P addition on plant growth, N and P status, and tartrate release varied with N forms, due to preferential uptake of NO_3-N over other N forms. These findings support the 'surplus C' hypothesis put forward by Prescott et al. (2020). However, it does not mean that a role of carboxylate release in P mobilisation should be dismissed entirely.

manuscript. JP, SD, and HL interpreted the data and revised the manuscript.

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