Short Communication

Impact of phosphorus on rhizosphere organic anions of wheat at different growth stages under field conditions

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Abstract. Phosphorus (P) is one of the main limiting factors for crop productivity while rhizosphere organic anions have been hypothesized to play an important role in P acquisition. Sampling in a long-term field experiment was carried out in order to understand the impact of long-term differences in P fertilization on secretion of organic anions under field conditions. Rhizosphere organic anions were extracted and analyzed every week from three leaves stage to completed flowering stage of wheat (*Triticum aestivum*) grown on plots that have received 0 (P0) or 48 (P48) kg P ha^{-1} year^{-1} since 1966. The study showed that it is possible to extract and quantify rhizosphere organic anions from field plots. In P48 plots, root P concentrations decreased around 40 % at the early stages (before heading), compared with the first sampling, and then increased slightly, while plants grown in P0 plots showed the opposite trend. Malate was the main organic anion secreted throughout all the wheat growth stages. Rhizosphere citrate and malate showed negative and positive correlations ($P < 0.05$) respectively with root P concentrations at 29 and 42 days after emergence (DAE). Rhizosphere organic anion concentrations were quite low until 29 DAE and then increased up to 4–10-fold until 42 DAE; these concentrations declined at later stages, indicating that root-released organic anions may have been affected by developmental stage and root P concentration. The present study provides valuable information about the relationship between rhizosphere organic anions and various P concentrations of wheat grown in the field.

Keywords: Developmental stage; field plots; phosphorus; rhizosphere organic anions; wheat.

Introduction

Root-released organic anions, such as citrate and malate, are widely documented to improve plant phosphorus (P) acquisition by mobilizing both inorganic sparingly soluble P and organic P in the soil (Ryan *et al.* 2001; Lambers *et al.* 2006; Richardson *et al.* 2009; Wang *et al.* 2015), although some studies have suggested this role to be minor (Pearse *et al.* 2007; Pandey *et al.* 2014; Wang *et al.* 2016). Organic anions can occupy sorption sites on soil minerals that might otherwise bind orthophosphate, or replace orthophosphate on these sorption sites. In addition, the organic anions may form complexes with iron

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and aluminum in solution, resulting in dissolution of iron/aluminum bound P. Moreover, organic anions can mobilize organic forms of P and then increase its amenability to hydrolysis by many kinds of phosphatase enzymes (Lambers *et al.* 2015). Exudation of organic anions from the roots of P starvation plants have been reported in many species such as *Brassica napus*, *Medicago sativa*, *Oryza sativa* and *Hordeum vulgare* (Hoffland *et al.* 1989; Gahoonia *et al.* 2000; Pearse *et al.* 2007; Erro *et al.* 2009; Pang *et al.* 2015; Wang *et al.* 2015). In addition, genetically modified plants that can release enhanced organic anion amounts reportedly can access more P from sparingly-soluble P forms in artificial media and soil (Lü *et al.* 2012; Wang *et al.* 2013).

However, root-released organic anions are influenced by many factors such as plant species, temperature and nutrient availabilities (Dechassa and Schenk 2004; Badri and Vivanco 2009). Apart from the factors mentioned above, plant developmental stage or plant age is also a major factor that may influence the composition and quantities of organic anions released by plant roots (Hale *et al.* 1971). For instance, Johnson *et al.* (1996) detected significantly different amounts of citrate, malate, and succinate from white lupin between 10 days after emergence (DAE) and 22 DAE, whilst Gardner *et al.* (1983) recovered exclusively citrate from the same plant species grown for 4–6 weeks. Aulakh *et al.* (2001) also demonstrated that root-released total organic anions were dramatically affected by developmental stage in rice. In addition, P requirements vary at different growth stages. For example, a high P supply is required for wheat in an early stage of plant growth, but during the grain filling period the P supply can be much lower (Römer and Schilling 1986). Hence, it is important to investigate the organic anion dynamics and their relationships with plant P uptake during the growing season. Knowledge about the effect of growth stage on root-released organic anions is also important for design of future experiments.

Study of root-released organic anions still faces challenges caused by root exudate extraction method and their analytical determination, because of the complicated interactions between root exudates and soil microbial communities and soil particles (Jones, 1998; Valentinuzzi *et al.* 2015). To date, most of the studies on root organic anion exudation were conducted in well-controlled greenhouses using either hydroponic or river-sand pot systems, minimizing the impact of soil particles and soil microorganisms (Pearse *et al.* 2007; Pang *et al.* 2010; Pang *et al.* 2015; Wang *et al.* 2015). There have only been a few studies reported with real agricultural soils in greenhouse conditions (Gahoonia *et al.* 2000; Nazeri *et al.* 2014) but little has been done in agricultural fields. Moreover, most of the studies focused on a short growth period, usually 1 month.

Our previous studies used both hydroponic and rhizobox systems to investigate plant root-released organic anions and their relationship with P mobilization and acquisition in an approximately 1 month growth period (Wang *et al.* 2015; Wang *et al.* 2016). A similar study focusing on root-released organic anions and P acquisition at different growth stages over a longer time period under field conditions is of importance to validate our conclusions and to provide information that is close to real field growth conditions for crops. In the present study, we have designed an experiment with sampling on selected plots from a long-term field experiment, where the selected plots have received 0 or 48 kg P ha^{-1} year^{-1} as single superphosphate since 1966 (Kristoffersen and Riley 2005). Wheat was cultivated and rhizosphere organic anions were sampled every week from three leaves stage to completed flowering stage in 2015. Our goal was to test two hypotheses: i) It is possible to extract and analyze rhizosphere organic anions from field plots and ii) the production of root rhizosphere organic anions is affected by different plant growth stages.

Methods

The experimental field (about 864 m^2) was established at the research farm of the Norwegian University of Life Sciences (NMBU), Ås, Norway (59°39' N, 10°45' E) in 1966. The field was divided into 32 plots (plot dimensions 3.6 m × 7.5 m) for various fertilizer application treatments and 4 plots with 0 kg P ha^{-1} year^{-1} (P0) and 4 plots with 48 kg P ha^{-1} year^{-1} (P48) were selected for the present study. The field soil was characterized as clay loam (26 % clay, 38 % silt, 36 % sand) and the soil properties were measured and listed in Wang *et al.* (2016). Wheat (*Triticum aestivum* L. cv. Krabat) cultivation was conducted between April and August 2015. Before sowing, 48 or 0 kg P ha^{-1} as single superphosphate and 116 kg N ha^{-1} as calcium nitrate were applied. Half of the investigated P0 and P48 plots were supplied with 100 kg K ha^{-1} and the other half of the plots received 150 kg K ha^{-1} as potassium chloride. The sampled field experiment was a combined P and K experiment, and to obtain four replicates for the P treatments, two K application levels had to be included. However, in this K rich soil we assumed that this difference would not influence the P concentrations or the release of organic anions. Wheat was sown in rows at a depth of 2–3 cm (0.125 m between each row). The sowing density was 200 kg ha^{-1}. Air temperature and precipitation data (Fig. 1) were recorded by the meteorological
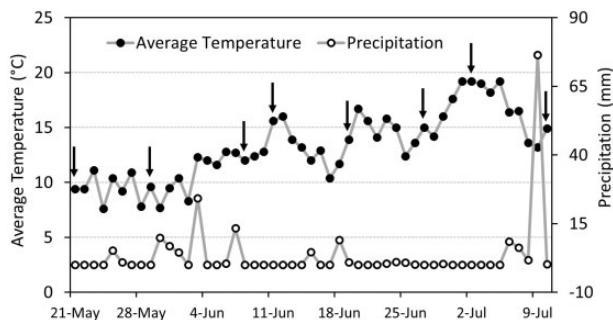
Figure 1. Daily precipitation and average air temperature at Ås NMBU meteorological observation station, arrows indicate sampling dates.

observation station at Ås (NMBU), https://www.yr.no/place/Norway/Akershus/%C3%85s/%C3%85s_(NMBU)_observation_site/statistics.html (3 Mar 2017), located near the experimental field.

Seeds were sown on 16 April, emergence was on 29 April and heading on 30 June. Roots and rhizosphere soils were sampled once per week from the three leaves stage (leaves unfold, 21 DAE) to completed flowering stage (70 DAE), after removing aboveground tissues. Stem elongation was observed at about third and forth sampling and ear completely emerged above flag leaf ligule at around sixth and seventh sampling, with usually delayed development stage for the P0 plots compared to the P48 treatment. For each sample in each plot, a 20 cm × 20 cm sampling area was randomly selected and the top 20 cm of the soils and roots were sampled. Large soil aggregates were carefully crushed by tweezers if necessary. Three plants' roots without obvious damage were taken and slightly shaken to remove excess soil, and the soil remaining attached to the roots was defined as rhizosphere soil. The collected roots together with rhizosphere soils were transferred into a container with a known volume (50 mL for first and second sampling; 100 mL for third to fifth sampling and 150 mL for sixth to eighth sampling, because of different root size) of 0.2 mM $CaCl_2$ solution to ensure cell integrity. Roots were then gently and carefully dunked for 60–90 s to get rhizosphere extract (Pearse *et al.* 2007; Pang *et al.* 2015). After the roots were removed, the containers were shaken by hand, and the pH of the extract was measured. Hence, the pH measured in this study was based on diluted samples, which probably underestimates the acidity on the root surface (Pearse *et al.* 2007). A subsample of the rhizosphere extract was taken and treated as described by Wang *et al.* (2016) and organic anions were determined using LC-MS/MS as described by Wang *et al.* (2015). We cannot exclude that some organic anions originated from cellular damage. The roots were then dried at 65 °C for 48 h, and dry weight (DW) and P concentrations were measured according to

Wang *et al.* (2015). From around fifth sampling, some nodal roots appeared, in particular for P48 plots; both seminal roots and those below ground nodal roots with attached rhizosphere soil were sampled. Crown and sub-crown internodes were removed after the roots were dried and the remaining 'effective' roots were selected for root DW and P concentration determination.

Data were statistically analyzed by *R* software (version 3.2.3). Two-way ANOVAs were used to study main effects of P level, DAE and their interaction on all the parameters involved in this study, followed by post hoc pair-wise Tukey honest significant difference tests for multiple comparisons, along with the minimum significant difference (MSD) at $P < 0.05$. Simple linear regressions were used to estimate the correlations between root P concentrations and rhizosphere organic anion fractions. In addition, *P* values for differences between the P0 and P48 treatments for data derived from the same DAE were calculated using the student *t*-test.

Results

Root dry weight and P concentration of sampled roots

As shown in Table 1 and Fig. 2A, significantly greater root dry weight (65–245 %) for the P48 treatment than the P0 plots was observed from 49 DAE. Compared with the P0 plots, higher root P concentrations were found in the P48 plots across all sampling dates except one (49 DAE). For P48, root P concentrations generally decreased during plant growth. The greatest difference in root P concentration between P48 and P0 plots (2.59 vs. 1.00 mg g^{-1} root DW) was found for the sample collected on 29 DAE (Table 1 and Fig. 2B). The shoot dry biomass and shoot P concentration had similar trends as root P concentration (data not shown).

Rhizosphere pH and organic anion response

Rhizosphere pH of both P0 and P48 plots was stable or decreased slightly at early stages and increased slightly at later growth stages (Table 1 and Fig. 2C). Malate, citrate and succinate were detected in this study and all were correlated with DAE and P application level (Table 1). Of these, malate was the dominant organic anion (Fig. 3). Generally, more citrate, in particular at 29 DAE (176 %), but less malate and total organic anions were accumulated in the rhizosphere of P0 plots than P48 plots, with one significant exception for samples collected on 29 DAE, where more total organic anions (88 %) were found in P0 plots. Organic anion concentrations were quite low until 29 DAE, then increased 4–10-fold until 42 DAE and then decreased again. In addition, root P concentrations

Table 1. *F* and *P* values of a two-way ANOVA on the correlation of P application level (P0 vs. P48), days after emergence (DAE) and their interactions with the parameters determined in this study. *df*, degrees of freedom; error, error *df* values. MSD, minimum significant difference at $P < 0.05$, values were given only for DAE × P level if there were interaction effects, otherwise values were given to those single factors with $P < 0.05$.

Parameters	Factors	*df*	*F*	*P*	MSD$_{0.05}$
Root dry weight	DAE	7	24.6	<0.001	
	P level	1	45.2	<0.001	
	DAE × P level	7	6.9	<0.001	0.57 g
	Error	48			
Root P concentration	DAE	7	7.6	<0.001	
	P level	1	149.5	<0.001	
	DAE × P level	7	6.2	<0.001	0.67 mg g^{-1}
	Error	48			
Rhizosphere pH	DAE	7	4.1	0.001	0.25
	P level	1	3.6	0.062	
	DAE × P level	7	1.4	0.241	
	Error	48			
Rhizosphere citrate concentration	DAE	7	8.2	<0.001	0.88 µmol g^{-1} root DW
	P level	1	4.4	0.041	0.28 µmol g^{-1} root DW
	DAE × P level	7	1.2	0.307	
	Error	48			
Rhizosphere malate concentration	DAE	7	8.7	<0.001	
	P level	1	16.8	<0.001	
	DAE × P level	7	2.3	0.045	3.17 µmol g^{-1} root DW
	Error	48			
Rhizosphere succinate concentration	DAE	7	8.0	<0.001	0.15 µmol g^{-1} root DW
	P level	1	9.3	0.004	0.05 µmol g^{-1} root DW
	DAE × P level	7	1.2	0.316	
	Error	48			
Rhizosphere total organic anions	DAE	7	9.7	<0.001	
	P level	1	6.9	0.011	
	DAE × P level	7	2.2	0.047	4.11 µmol g^{-1} root DW
	Error	48			

showed positive and negative correlation with the rhizosphere malate and citrate fractions, respectively, at the early growth stages (29 and 42 DAE).There were significant correlations between root P concentrations and rhizosphere citrate and malate fractions also at 70 DAE, but the concentrations were fairly low (Table 2).

Discussion

Phosphorus is one of the important limiting factors for crop productivity. In the present study, we carried out field experiments in order to investigate the relationships between the rhizosphere organic anions and soil P availability or root P status at different growth stages under field conditions. The results clearly show that our first hypothesis was supported: it is possible to study rhizosphere organic anions and their relationship with soil P availability or plant P acquisition under field conditions.

Regarding our second hypothesis, rhizosphere organic anion concentrations were correlated with DAE and soil P availability/root P status. Correlation with DAE might reflect an effect of plant growth stage, in accordance
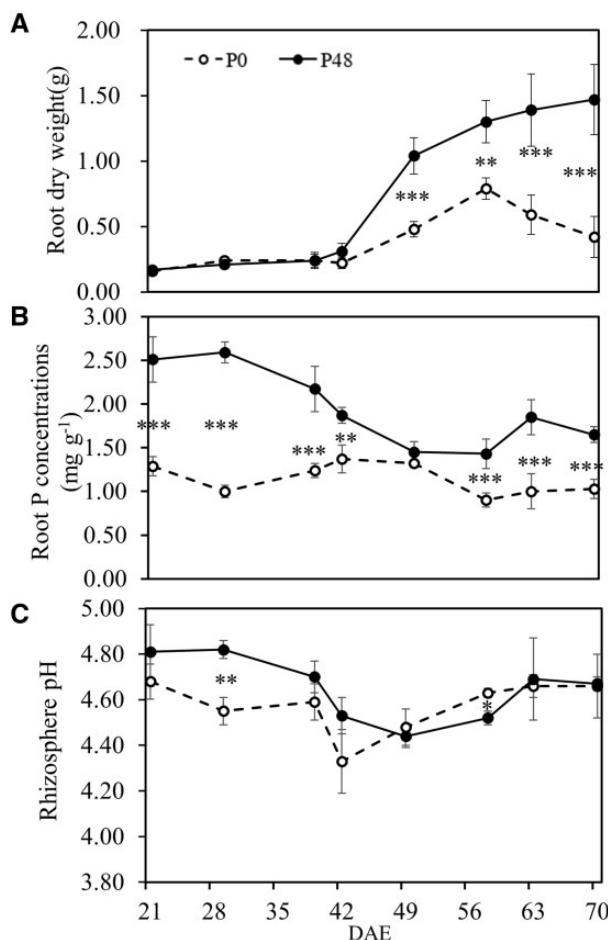
Figure 2. (A) Collected root dry weight, (B) root P concentration and (C) rhizosphere pH of *Triticum aestivum* grown in P0 plots and P48 plots. Error bars indicate SE ($n = 4$). Stars indicating *P* value based on student *t*-test between P0 and P48 treatment for data derived from the same DAE. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.
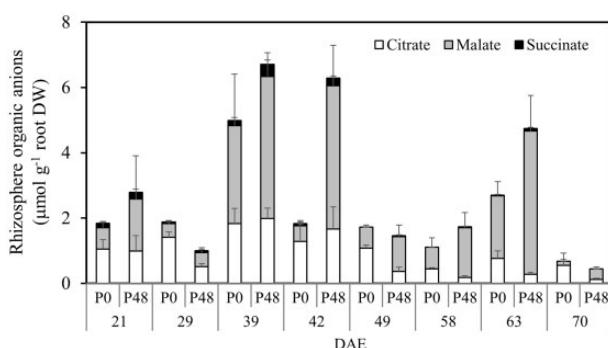

Figure 3. Concentrations of rhizosphere malate, citrate and succinate of *Triticum aestivum* grown in P0 plots and P48 plots. Error bars indicate SE ($n = 4$).

Table 2. Linear correlation between root P concentration and measured rhizosphere malate or citrate fraction. DAE, days after emergence; *r*, correlation coefficient; *df*, degrees of freedom. Significant correlations ($P < 0.05$) in bold.

DAE	Correlation with	*r*	*P* value	*F* value	*df*
21	Malate	0.26	0.519	0.47	6
	Citrate	−0.61	0.109	3.54	6
29	Malate	0.80	**0.017**	10.04	6
	Citrate	−0.88	**0.004**	19.76	6
39	Malate	0.26	0.524	0.46	6
	Citrate	−0.33	0.426	0.73	6
42	Malate	0.85	**0.008**	15.14	6
	Citrate	−0.86	**0.006**	17.74	6
49	Malate	0.46	0.254	1.59	6
	Citrate	−0.47	0.244	1.67	6
58	Malate	0.64	0.087	4.08	6
	Citrate	−0.63	0.091	4.06	6
63	Malate	0.69	0.059	5.50	6
	Citrate	−0.70	0.052	5.87	6
70	Malate	0.81	**0.014**	11.67	6
	Citrate	−0.85	**0.019**	13.42	6

provided by the Norwegian Water Resources and Energy Directorate (www.nve.no). These data were not calibrated and therefore cannot give absolute values (and are therefore not shown); however, it is possible to follow time trends. The soil was getting drier in the first part of the period, followed by rewetting at the end of May, drying again in mid-June and finally a rainfall event in July. Additionally, the unpublished data from our partners' research (personal communication) have shown that the water content in the upper 5 cm of the soil of a field plot, which is about 200 m from our plot, varied between 25 and 35 % (w/w) in the period 1 May to 1 September 2015. The peak rhizosphere organic anion concentrations relative to the root dry weight appear to be at DAEs 39, 42 and 63, which were all in a dry period. However, DAEs 49 and 58, with lower concentrations, were also in the same dry period. Generally, soil pH is not affected (Metwally and Pollard, 1959) or increased (Misra and Tyler, 1999) and P availability is increased (Metwally and Pollard, 1959; Misra and Tyler, 1999) with increasing soil water content, while lower soil water content reduces microbial activity (Yan et al. 2015). In the dry period, we observed a decrease in rhizosphere pH (Fig. 2C), which compared well with Misra and Tyler (1999) but not with Metwally and Pollard (1959). The detected peak

with our second hypothesis, but possible effects of other factors that change with time need to be considered. We did not measure soil water content, but information about soil water content in the sampling period was

rhizosphere organic anion concentrations are more likely released by plant roots because soil drying reduces microbial activity, and also reduces soil P availability, which might have resulted in a decrease of root P concentration (Fig. 2B, P48 plots), thus increasing root-released organic anions. However, soil water content alone cannot account for the decrease of root P concentrations in the P48 plots because in the dry period the P0 plots showed a contrasting trend and the root P concentration of P48 did not increase after the rainfall event (re-wetting) in July. Taken together, soil moisture alone cannot account for our results and plant growth stage should be taken into account.

Under field conditions, rhizosphere organic anions are often affected by various factors such as soil type (Veneklaas *et al.* 2003) and nutrient availability (Badri and Vivanco 2009). Further, the activity of soil microbes may influence the concentration of organic anions by degrading them, but also by their own release of organic anions to soil (Jones 1998; Badri and Vivanco 2009). Thereby, organic anions in the rhizosphere can be of microbial origin. In our experiment, there should be minimal risk for microbial degradation of organic anions after sampling because Micropur was added to the extract solution to inhibit the activity of microorganisms (Wang *et al.* 2015; Wang *et al.* 2016). In the present study, it was a great challenge to sample bulk soils without plant roots for determination of the concentrations of organic anions in soils without plants, because of the high sowing density, and because the long-term field experiment we sampled did not include empty (unsown) plots. However, our previous study using the same soils in a greenhouse with optimal temperature and humidity conditions for microbial activity indicated that the concentrations of organic anions of microbial origin were very low (only 0.0055 and 0.0014 μmol g^{-1} dry weight soil of citrate and succinate respectively were detected in P0 bulk soils) compared to the concentrations of organic anions released by plant roots, although a high content of P immobilized by microbial biomass and rhizosphere acid phosphatase activity were detected in both P0 and P48 bulk soils (Wang *et al.* 2016). Cieśliński *et al.* (1998) also reported that no water extractable organic anions were identified in the bulk soil of three different soil types under growth chamber conditions. Therefore, we assumed that the detected rhizosphere organic anions were mainly secreted by plant roots. Both seminal and nodal roots were included for some samples in this study, and they have very different root thickness which is likely to affect root surface area and root biomass; the effects of nodal roots on root-released organic anions are not clear and need further study. Also, as only roots and rhizosphere soils in a small area of the whole plot were selected, together with the limitation of rhizosphere extraction methods (Badri and Vivanco, 2009; Valentinuzzi *et al.* 2015) and some organic anions possibly coming from damaged roots, the measured rhizosphere organic anion concentrations might deviate from the true concentrations. The results however may still reflect the relative differences between different P treatments and growth stages. Moreover, the results compared very well with our previous rhizobox experiment (Wang *et al.* 2016), a common system used in many previous reports, conducted in the greenhouse and sampled at around heading stage. Thus, the method used in the present study to sample roots and the rhizosphere organic anions appears to be valid and effective. This makes field study possible, which is important for understanding the relationship between P uptake and rhizosphere organic anions under real agricultural field conditions.

In the present study, three organic anions were detected in the concentration order: malate > citrate > succinate in the rhizosphere extracts, which compared well with our rhizobox experiment using another wheat cultivar, Aino (Wang *et al.* 2016). Pearse *et al.* (2007) detected malate > citrate > fumarate using wheat cultivar Wyalkatchem. In all these cases, malate is the most important of all organic anions in the rhizosphere of wheat, and citrate is the second most important one. Both malate and citrate are the most important organic anions in mobilizing less available P in soil (Hoffland *et al.* 1989).

The developmental stage of a plant is an important factor that affects root exudation (Hale *et al.* 1971; Aulakh *et al.* 2001; Dechassa and Schenk 2004). For rice, the exudation rates of total organic anions were lowest at seedling stage, increased until flowering but decreased at maturity (Aulakh *et al.* 2001), while for cabbage, succinate and citrate exudation rates increased with increased plant age and a significant increase in exudation rates during the light period of the day was reported (Dechassa and Schenk 2004). These two studies were carried out in hydroponic systems and it seems likely that the exudation rates were related to the biomass production due to the carbon cost of exudation (Badri and Vivanco 2009). Our results showed different patterns, indicating differences among various species; the organic anion concentrations (which may reflect the exudation rates) increased before about stem elongation stage and then decreased until completed flowering. Taking the fact that a high P supply is required in an early stage of wheat growth while during the grain filling period the P supply can be much lower (Römer and Schilling 1986) into consideration, our results further suggest the importance of P requirement on exudation of organic anions. Our data also suggest that the root exudation is influenced by biomass production, which was

further indicated by the fact that in our study the rhizo-sphere of P deficient plants had a smaller concentration of total organic anions than those of P fertilized plants. In addition, we found that root P concentrations in the P48 plots decreased at the early developmental stages, probably due to a decrease in soil water content as discussed previously, as well as an increase in root biomass and depletion in rhizosphere available P or translocations of P to aboveground biomass. Generally, the total P concentration in agricultural crops varies between 0.1 and 0.5 % (Anonymous 1999). We found that, based on N:P ratio, root P concentrations of 0.19 and 0.09 % were defined as P sufficient and deficient respectively, in wheat in a greenhouse experiment using the same soils as the present study (Wang *et al.* 2016). In the present study, P48 plots showed root P concentration of about 0.15–0.26 % while P0 plots showed about 0.09–0.14 %. When root P concentrations decreased from 0.26 % at 29 DAE to 0.15 % at 49 DAE in the P48 plots, rhizosphere pH decreased and rhizosphere organic anion concentrations increased, which in theory might mobilize plant unavailable P in soil (Jones 1998; Lambers *et al.* 2006; Lambers *et al.* 2015). Moreover, significant correlations were found between root P concentrations and rhizosphere organic anions at 29 and 42 DAE. Therefore, root exudation affected by developmental stage might be due to biomass production and root P status at different growth stages, and the best time window to study the relationships between soil P availability or plant P status and rhizosphere organic anions in wheat is during the early growth stages (first vegetative phase).

Although many reports have provided evidence to support that organic anions play an important role in improving plant P acquisition, other reports have provided negative results. For instance, rhizosphere organic anions are not always correlated with the ability of plants to grow in P-limited conditions (Pearse *et al.* 2007) or do not improve grain yield in green gram (Pandey *et al.* 2014). A recent study even indicated that citrate could decrease P availability in certain soil types (Duputel *et al.* 2013). Our rhizobox experiment using soils collected from the same plots as in the present study suggested that organic anions only play a minor role in improving P uptake (Wang *et al.* 2016). Root-released organic anions are affected by many factors such as plant species, soil properties and nutrient availabilities (Jones 1998; Veneklaas et al. 2003; Badri and Vivanco 2009). Our presented data suggest that plant developmental stage may have a great influence on rhizosphere organic anions; significant correlations were detected between rhizosphere organic anions and plant P uptake at certain stages. Further studies are needed to understand the role of rhizosphere organic anions in improving P availability and P uptake: our present study provides possibilities and clues for studying this topic under field conditions for different plant species and different growth stages, and for long-term monitoring and research.

Conclusions

Rhizosphere organic anions were extracted and analyzed from agricultural fields in our present study. We found that root-released organic anions were correlated with plant developmental stage, possibly due to biomass production and P status in plant tissues. This study provided useful information for future study on relationships between rhizosphere organic anions and P uptake under various field conditions.

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Contributions by the Authors

Y.W., T.K. and J.L.C. designed the experiment. Y.W. conducted the study and wrote the manuscript. N.C. arranged element determinations. All authors contributed to analysis of data, discussions and manuscript improvements.

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Conflicts of Interest Statement

None declared.

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