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Rhizosphere priming of two near-isogenic wheat lines varying in citrate efflux under different levels of phosphorus supply

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Running title: Rhizosphere priming of isogenic wheat lines varying in citrate efflux

Abstract

- **Backgrounds and aims** The rhizosphere priming effect (RPE) has been explained from the perspective of microbial responses to root exudates and nutrient availability. This study introduced a chemical process that could also contribute to RPE: root exudates (organic acid ligands) could liberate mineral-protected carbon (C) in soil for microbial degradation.
- **Methods** Wheat (*Triticum aestivum* L.) near-isogenic lines varying in citrate efflux were grown for six weeks in a C4 soil supplied with either low (10 μg g^{-1}) or high P (40 μg g^{-1}). Total below-ground CO_2 was trapped and partitioned for determination of soil organic C decomposition and RPE using a stable isotopic tracing technique. Mineral dissolution was examined by incubating soil with citric ligand at a series of concentrations.
- **Key Results** High P increased RPE (81%), shoot (32%) and root biomass (57%), root-derived CO_2-C (20%), and microbial biomass C (28%) and N (100%), soil respiration (20%) and concentrations of water-extractable P (30%), Fe (43%) and Al (190%) but decreased inorganic N in the rhizosphere. Compared to Egret-Burke, wheat line Egret-Burke *TaMATE1B* with citrate efflux had lower inorganic N, microbial biomass C (16%) and N (30%) in the rhizosphere but greater RPE (18%), shoot biomass (12%), and root-derived CO_2-C (low P 36%, high P 13%). Egret-Burke *TaMATE1B* also had higher concentrations of water-extractable P, Fe and Al in rhizosphere, indicating the release of mineral-protected C. In addition, citrate ligand facilitated Fe and Al releases from soil with their concentrations rising with increasing ligand concentration and incubation time.
- **Conclusions** While high P supply increased microbial growth and RPE possibly due to higher total root exudation, citrate efflux from the root might have facilitated the liberation of mineral-bounded C, leading to the higher RPE under Egret-Burke *TaMATE1B*. Mineral dissolution may be an important process that regulates RPE and should be considered in future RPE research.

Key words ^{13}C natural abundance, Mineral dissolution, Isogenic lines, Organic acid ligand, Rhizosphere priming effect, Root exudates, Stable isotope

INTRODUCTION

Plants can affect microbial decomposition of soil organic matter (SOM). The change in SOM decomposition by growing plants (termed rhizosphere priming effect (RPE)) is mainly caused by microbial responses to rhizodeposits derived from living roots. Root exudates (mainly sugars and molecular organic acids) are the most important primers in rhizosphere due to their solubility in water, mobility, and ability to direct incorporation into growing microbial cells (Kuzyakov, 2002). Plants export proportionally 2–5% photosynthetic carbon (C) into its rhizosphere as root exudates (Jones *et al*., 2004; Pausch and Kuzyakov, 2018). Although low in glucose and organic acids, root exudates can trigger microbial growth and activity and their subsequent decomposition of SOM via microbial activation or co-metabolism (Kuzyakov *et al*., 2000).

Rhizosphere priming effect is additionally regulated by soil nutrient availability. When soil is with moderate N deficiency, root exudates could stimulate RPE by activating microorganisms to mine N from N-rich SOM according to the 'microbial N mining' mechanism (Fontaine *et al.,* 2011). In severe N-limited systems, however, RPE could be reduced due to constraints for microbial growth and/or activity. For example, Xu *et al*. (2017, 2018) found that eCO_2 decreased the RPE under wheat plants where soil N is severely limited but not under white lupin where biological fixation provided additional N. Soil phosphorus (P) availability can also affect root exudation directly or indirectly via influences on plant photosynthesis and biomass production, and hence RPE but fewer studies have examined the effects of P availability on RPE. Low P availability could up-regulate the secretion of organic anions (e.g. citrate and malate) to mobilise inorganic P through dissolution and desorption and escalate the synthesis of phosphatase to facilitate hydrolysis of organic P (Nuruzzaman *et al*., 2006; Weisskopf *et al*., 2006). Previous hydroponic studies have suggested that this up-regulation of organic anions under P deficiency is normally found in leguminous species, such as chickpea (*Cicer arietinum* L.), field pea (*Pisum sativum* L.) and white lupin (*Lupinus albus* L.) but not in cereal plants like wheat (*Triticum aestivum* L.) (Nuruzzaman *et al*., 2006). A recent study examined the root secretion of organic anions by wheat (cv. Krabat) from the 3-leaf stage to the flowering stage at a long-term P experimental site with or without an annual P supply at 48 kg P ha^{-1} (Wang *et al*., 2017). The results show that the P deficiency increases citrate efflux but this effect depends on plant developmental stage. It is largely unknown whether soil P availability would affect RPE via its effect on wheat root exudation.

Labile soil organic C (SOC) has been regarded as the main soil C pool that undergoes microbial decomposition due to its low complexity in chemistry and lack of physical and chemical protection (Rasmussen *et al*., 2006). Soil organic C is chemically protected by adsorption on secondary mineral phases, the formation of metal-containing complexes (Kleber *et al*., 2015; Finley *et al*., 2018). Soil short-range-order (SRO) materials, such as aluminosilicates, and Fe- and Al-oxyhydroxides, are amorphous minerals that have a high reactive surface area, which can form complexes with SOM through ligand exchange and render SOM unavailable for microbial degradation (Finley *et al*., 2018). The presence of SRO minerals has also been found conducible to soil aggregation (Duiker *et al*., 2003). Mineral protection of SOC by SRO minerals has been associated with weak priming effects (Rasmussen *et al*., 2006). However, organic acid anions released from roots could increase mineral dissolution (Jones *et al*., 2004) by either breaking down organic-mineral associations and/or displacement of SOC on mineral surfaces (Kleber *et al*., 2015). The C released during these processes is further utilised by microorganisms, leading to an increased priming effect in a laboratory incubation experiment (Keiluweit *et al*., 2015). Likely, the function of root exudates to mobilise mineral-associated C could also occur in plant rhizospheres and thus affect RPE and SOM decomposition. Mineral dissolution by root exudates and the liberation of mineral-bounded C are considered as a possible chemical mechanism of increased RPE. Closely related plant genotypes with genetically tractable introgressed diversity (e.g. organic acid efflux) would be excellent materials to examine this chemical mechanism.

In this study, a pair of near-isogenic wheat lines (Egret-Burke and Egret-Burke *TaMATE1B*) differing in citrate efflux were supplied with two P levels to study how soil P level and citrate exudation would impact RPE. This study aimed to further understand the biotic and chemical (mineral dissolution by root exudates) mechanisms of RPE. Two hypotheses were tested: i) P deficiency would increase root-specific RPE due to the up-regulation of organic anions secretion, and ii) wheat line with citrate efflux would induce greater RPE partly via mineral dissolution of organic-mineral associations.

MATERIALS AND METHODS

Soil description

The soil was collected from top 10 cm in a C4 *Themeda triandra* grassland (32°10'44" S, 149°33'45" E) in Gulgong, Australia. It was a sandy loam with pH 4.7, clay 13%, total SOC 27 mg g^{-1}, total N 1.6 mg g^{-1}, Olsen P 9.2 µg g^{-1} and $\delta^{13}C_{SOC}$ -19.8‰. The air-dried soil was sieved (<2 mm), homogenized and grass roots removed. Basal nutrients were added to the soil with the following composition and rates (µg g^{-1} soil): $CO(NH_2)_2$ 64.2, $CaCl_2 \cdot 2H_2O$ 180, K_2SO_4 120, $MgSO_4 \cdot 7H_2O$ 50, $MnSO_4 \cdot H_2O$ 15, $CuSO_4 \cdot 5H_2O$ 6, $ZnSO_4 \cdot 7H_2O$ 9, Fe-EDTA 5.5 and $Na_2MoO_4 \cdot 2H_2O$ 0.4. Two P levels were low P (10 µg P g^{-1} soil) and high P (40 µg P g^{-1} soil) added as KH_2PO_4. The nutrients were then thoroughly mixed with the soil before potting.

Wheat near-isogenic lines

Wheat (*Triticum aestivum* L.) near-isogenic lines Egret-Burke (Mat.) and Egret-Burke *TaMATE1B* (Mcit.) were adopted in this study. The expression of *TaMATE1B* in Egret-Burke *TaMATE1B* renders the line with constitutive citrate efflux at root apex (Tovkach *et al*., 2012). The wheat line with *TaMATE1B* expression exudes 5- to 8-fold more citrate ligands than line Egret-Burke (Ryan *et al*., 2014).

Growing system

A soil-column experiment consisted of two wheat lines (Mat. and Mcit.) and two P levels (10 and 40 µg P g^{-1}) in four replicates, and four unplanted columns were included as controls. The soil columns (height 40 cm) were made from 7.5 cm diameter Polyvinyl chloride (PVC) tubes and then capped at one end. One hundred grams of plastic beads were enclosed by micro-mesh (0.45 µm) and then placed inside of each soil column before 1.25 kg of the C4 soil was packed. Reverse osmosis water was added to adjust soil water content to 80% field capacity. Wheat seeds were pre-germinated for two days in laboratory and six of them were sown in each soil column alongside the diameter. Soil columns were then transferred into one growth chamber (Thermoline, Climatron-1100-SL-H, Wetherill Park, Australia). The temperature was controlled at 22 °C day (14 h) and 18 °C night (10 h), and the relative humidity was set at 70% inside the growth chamber. The photosynthetic active photon flux density was around 350 µE m^{-2} s^{-1} at the canopy level. After six days, three seedlings were kept in each per column. Soil moisture was maintained at 80% field capacity by daily watering to the target weight. The columns were randomly reallocated weekly to avoid inhomogeneous growing conditions. Additional N was supplied at the rate of 30 µg urea-N g^{-1} soil weekly from the fourth week till harvest.

Below-ground CO_2 trapping

The CO_2 released below-ground was trapped six weeks after sowing using a modified CO_2-trapping system (Wang et al. 2016). Before trapping, the top of each soil column was enclosed with two clear PVC plates around wheat stems, and the open spaces were sealed tightly using Blu-tack. The air-tightness of the sealing was checked by vacuuming air through the soil column into a water tank and observing the status of the bubbles formed in the water. It was considered airtight if the bubbles remained stable while pressing the jointing area.

Below-ground CO_2 released was trapped for two days after the sealing. Firstly, CO_2-free air was generated by pumping air through 150 ml 1.0 *M* NaOH solution. Secondly, the existing CO_2 in soil pores was evacuated by pumping CO_2-free air through the soil for 30 min. Lastly, the CO_2 exiting the column was trapped in 0.5 *M* NaOH s (150 ml). A vacuum pump was included at the end of the NaOH trap to accelerate gas movement). The trapping was performed three times and 30 min each time between 0900 h and 2300 h with an interval of 6 h.

Total below-ground CO_2-C released was determined by back-titration of the excessive alkali using 0.25 *M* HCl after precipitating the carbonate with $BaCl_2$ solution. The endpoint of the titration was indicated using phenolphthalein. Another aliquot of the trap was treated with excessive $SrCl_2$ and the $SrCO_3$ precipitates formed were rinsed and oven-dried for determination of $\delta^{13}C$ value of the below-ground CO_2 using an Isotope Ratio Mass Spectrometer ('IRMS', SerCon Hydra 20-22, Gateway, UK).

Plant and soil analyses

Soil columns were destructively harvested after below-ground CO_2 trapping. Plant shoots were cut at ground level. Plant roots were picked up and washed after sampling the rhizosphere soil. The roots were scanned using an EPSON EU-35 Scanner (Seiko Epson Corp., Suwa, Japan) with the image being analysed using a WinRhizo STD 1600+ Image Analysis System (Regent Instruments, Quebec City, Canada) for root length. Dry mass of shoots and roots were recorded after drying at 70 °C for three days. The dry plant samples were ball-milled and analysed for total C and N with a CHNS/O Analyser (PerkinElmer EA2400, Branford, USA). Plant roots were also measured for $\delta^{13}C$ values by the IRMS.

Immediately after harvest, 10 g of the rhizosphere soil was used to estimate microbial respiration which was the amount of CO_2 produced during the 12-h incubation period (in dark) and measured by a Sermex 4200 Gas Analyser (Servomex, Crowborough, UK). Microbial biomass C (MBC) and N (MBN) in the rhizosphere soil were also analysed (Brookes *et al*., 1985). Briefly, one set of fresh soil (8 g) was fumigated with chloroform in dark for 24 h. The fumigated soil together with another set of soil without fumigation was extracted in 0.5 *M* K_2SO_4 (1:5, w/v). The soil extracts were then filtered through Whatman no. 42 filter paper and frozen at -20 °C before extractable organic C (EOC) was measured by a TOC Analyser (GE Sievers InnovOx, Boulder, USA), and inorganic N was analysed using a Flow-Injection Analysis System (FIA) (Lachat's QuickChem 8500, Loveland, USA). The extracts of the fumigated and unfumigated soils were further autoclaved at 120 °C for 30 min for the assay of total NO_3^- by the FIA. The MBC was determined as the differences in concentrations of EOC between the fumigated and non-fumigated soils by a conversion factor of 0.45 (Vance *et al*., 1987), and MBN as the difference in concentrations of total inorganic N between the fumigated and non-fumigated soil extracts with a conversion factor of 0.54 (Brookes *et al*., 1985; Vance *et al*., 1987).

The remaining rhizosphere soil was air-dried and analysed for pH after shaking the soil with 0.01 *M* $CaCl_2$ (1:5, w/v) for 1 h. Mineral dissolution was determined according to Yu *et al*. (2017). Briefly, air-dried soil was extracted with milli-Q water (1:5, w/v) for 24 h at 25 °C on an orbital shaker (Ratek OM6, Boronia, Australia) at 170 rpm. The suspension was centrifuged at 3000 *g* for 10 min, filtered through a 0.22-µm filter unit (Millex-GP SLGP033RS, Bayswater, Australia) and analysed for water-extractable P, Fe and Al using an Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES) (Optima 8000, PerkinElmer, Waltham, USA). The difference in water-extractable elements from planted and unplanted columns was used to estimate mineral dissolution due to root exudates (Yu *et al*., 2017).

Soil material dissolution experiment

The C4 soil with organic acid ligand was incubated to examine the effects of organic acid ligands on dissolution of soil minerals. Briefly, 1 g soil was weighed into opaque polyvinyl chloride containers and then amended with 200 ml of either milli-Q water, tri-potassium citrate or citric acid in triplicate. The concentrations of the acids were 0.5, 1, 5 and 10 mM, representing the localised concentration range of root exudates by a variety of plant species (Ryan *et al*., 2014). The containers were sealed and incubated at 25 °C in dark for 72 h. Fifteen ml of supernatant was collected by pipetting at 24, 48 and 72 h after incubation. The supernatant was then filtered through 0.2-μm Whatman Puradisc 30 syringe filter (Whatman, Parramatta, Australia) and stored at 4 °C prior to pH and element analyses. The concentrations of total Fe and Al in solutions were determined by ICP-OES and thus termed as ICP-Fe and ICP-Al, respectively. The solutions were also analysed for inorganic monomeric Al by a Varian Cary 50 Bio UV-Visible Spectrophotometry (Agilent Technologies, Santa Clara, USA) according to Conyers *et al*. (1991).

Below-ground CO_2 partitioning and calculation of rhizosphere priming effect

The following equations were adopted to separate the CO_2 of total below-ground respiration (C_{total}) into soil-derived CO_2-C (C_{soil}) and root-derived CO_2-C (C_{root}) (Cheng, 1996):

$$f = (\delta^{13}C_{root} - \delta^{13}C_{total})/(\delta^{13}C_{root} - \delta^{13}C_{soil})$$

$$C_{soil} = C_{total} \times f$$

$$RPE = C_{soil} - C_{control}$$

where $\delta^{13}C_{root}$ is the $\delta^{13}C$ value of root materials (Table 1). $\delta^{13}C_{total}$ is the measured $\delta^{13}C$ value of total below-ground CO_2 respiration in planted treatments (Table 1). $\delta^{13}C_{soil}$ is the $\delta^{13}C$ value of CO_2 evolved from the unplanted controls, which was determined (-14.7‰). f is the contribution of soil-derived CO_2-C to total below-ground CO_2 release. $C_{control}$ is the basal soil respiration from the unplanted controls. Root-specific RPE was calculated based on per unit of root mass and root length, respectively.

Statistical analysis

A two-way ANOVA was performed to examine the effects of P supply, wheat line and their interactions on all observations using Genstat (v17; VSN International, Hemel Hempstead, UK). A two-tailed Pearson's correlation analysis was conducted for RPE with all soil, plant, and microbial parameters using SPSS (v22; IBM Corp, Armonk, USA) with Pearson's correlation coefficients being tabulated when $P < 0.05$. All figures were plotted using means of four replicates with positive standard errors in Excel (v16; Microsoft Corp, Redmond, USA).

RESULTS

Plant growth

By comparison to its near-isogenic line EGA-Burke, wheat line EGA-Burke *TaMATE1B* had 12% higher shoot biomass but similar root biomass and length, and root-to-shoot biomass ratio (Table 1). On average, high P increased shoot and root biomass, and root-to-shoot biomass ratio by 32%, 57% and 20%, respectively (Table 1). The EGA-Burke had more negative (0.4‰) root $\delta^{13}C$ value than EGA-Burke *TaMATE1B*. High P increased the root $\delta^{13}C$ value by an average of 3.1% (Table 1).

Shoot N, P concentration and uptake

The two near-isogenic lines had similar shoot N concentrations and high P level decreased shoot N concentration by an average of 16% (Fig. 1A). By comparison to EGA-Burke, EGA-Burke *TaMATE1B* had 6% lower shoot P concentration under low P supply but had 18% higher shoot P concentration under high P (Fig. 1B), leading to a wheat line × P supply interaction.

EGA-Burke *TaMATE1B* had more shoot N and P uptakes than EGA-Burke (Fig. 1C, D). Specifically, EGA-Burke *TaMATE1B* took up 13% more N (Fig. 1C) when compared to EGA-Burke. It also took up 7% and 22% more P in shoots under low and high P supplies, respectively,

leading to a wheat line × P supply interaction (Fig. 1D). High P enhanced N and P uptakes by 12% and 38%, respectively (Fig. 1C, D).

Below-ground CO_2 respiration and its $\delta^{13}C$ value
Basal soil respiration was 10.8 μg CO_2-C g^{-1} soil d^{-1} (dash line in Fig. 2) as measured from unplanted columns. Growing plants enhanced both soil-derived and root-derived CO_2 respirations. Compared to EGA-Burke, EGA-Burke *TaMATE1B* increased the root-derived CO_2 respiration by 36% under low P and 13% under high P, leading to a significant wheat line × P availability interaction (Fig. 2). EGA-Burke *TaMATE1B* also increased the soil-derived CO_2 respiration by 9% (Fig. 2). High P supply stimulated root-derived and soil-derived CO_2 respirations by 20% and 35%, respectively. The two wheat lines generated similar $\delta^{13}C$ values of total below-ground CO_2 which were -24.7‰ and -23.7‰ at low P and high P, respectively (Table 1).

Soil inorganic N in the rhizosphere
Soil inorganic N was amounted at 134 μg N g^{-1}soil in the unplanted control with NH_4^+ being 128 μg g^{-1} soil and NO_x^- 5.6 μg g^{-1} soil. Growing plants decreased soil NO_x^- but increased soil NH_4^+. Compared to EGA-Burke, growing EGA-Burke *TaMATE1B* decreased soil NH_4^+ under high P by 41% but had no effect on soil NH_4^+ under low P, leading to a significant wheat line × P availability interaction (Fig. 3). Growing EGA-Burke *TaMATE1B* decreased soil NO_x^- by 57% under low P but did not affect soil NO_x^- under high P (Fig. 3). Growing plants at high P decreased the concentration of NH_4^+ and NO_x^- in the rhizosphere by 61% and by 91%, respectively, when compared to low P (Fig. 3).

Soil microbial biomass C, N and C-to-N ratio
The MBC and MBN were 39.8 and 4.1 μg g^{-1} soil, respectively, in the unplanted controls but increased in the rhizosphere (Fig. 4). The MBC and MBN were 16% and 30% lower in the rhizosphere of EGA-Burke *TaMATE1B* than EGA-Burke (Fig. 4A, B). High P increased rhizosphere MBC and MBN by averages of 28% and 100%, respectively (Fig. 4A, B). Growing EGA-Burke *TaMATE1B* yielded 37% higher microbial C-to-N ratio in the rhizosphere when compared to EGA-Burke (Fig. 4C) but this was only observed at low P, leading to a significant wheat line × P availability interaction (Fig. 4C). High P decreased microbial C-to-N ratio in the rhizosphere by an average of 36% (Fig. 4C).

Rhizosphere pH, respiration and water-extractable P, Fe and Al
Growing plants for 6 weeks increased rhizosphere pH by around 0.1 unit. High P supply decreased the rhizosphere pH by 0.02 unit when compared to low P (Table 2).

Compared to that in the unplanted control, microbial respiration in the rhizosphere soil was 75% and 109% higher under low P and high P, respectively (Table 2). Soil respiration tended to be lower in the rhizosphere of EGA-Burke *TaMATE1B* than EGA-Burke ($P = 0.052$, Table 2). High P increased rhizosphere soil respiration by an average of 20%.

The concentrations of extractable P, Fe and Al were higher in the rhizosphere than in the no-plant control. When compared to low P, high P supply increased the concentrations of extractable P, Fe and Al by 30%, 43% and 190%, respectively (Table 2). Compared to EGA-Burke, growing EGA-Burke *TaMATE1B* tended to increase the concentrations of water-extractable P, Fe and Al in the rhizosphere ($P \leq 0.10$) (Table 2).

Rhizosphere priming effect
Rhizosphere priming effect depended on both soil P supply and wheat line with no interaction between them. By comparison to its near-isogenic EGA-Burke, EGA-Burke *TaMATE1B* increased RPE by an average of 18%, RPE per unit root weight by 13% and RPE per unit root length by 16%

(Fig. 5). High P resulted in 81% higher RPE, 14% higher RPE per unit root weight and 79% higher RPE per unit root length than low P supply (Fig. 5).

The RPE was positively correlated with plant biomass, shoot N and P uptakes, microbial biomass N, concentrations of water-extractable P, Fe and Al, and root-derived CO_2 respiration, but negatively with soil mineral N in the rhizosphere (Table 3).

Results of mineral dissolution experiment

Citric acid and potassium citrate had different effects on solution pH and element release from soil materials (Fig. 6). The solution pH decreased to 2.6 with increasing citric acid concentration but increased to 7.7 with increasing potassium citrate concentration as compared to pH 6.7 for the control (Fig. 6A). The concentrations of Fe and Al in solution rapidly increased within 24 h of ligand addition, and increased further with time. Citric acid was more effective than potassium citrate in mineral dissolution (Fig. 6B, C). In general, the concentrations of metal elements in solution increased with increasing ligand concentrations except for potassium citrate at 24 h where the highest metal concentrations were found at 0.5 mM. The highest monomeric Al concentrations were found in solutions at concentrations of citrate ligand of ≤ 1 mM (Fig. 6D). Specifically, the relative contributions of monomeric Al to ICP-Al were 27% and 64%, respectively, for potassium citrate and citric acid of ≤ 1 mM. In comparison, the contributions were 4% and 3%, respectively, for potassium citrate and citric acid of ≥ 5 mM.

DISCUSSION

Microbial mechanisms of rhizosphere priming effect

High P availability increased RPE and root-specific RPE, which rejects our first hypothesis. Although low P in this study might have favoured the secretion of citrate as discovered by Wang *et al*. (2017), the plants under low-P conditions might have secreted proportionally fewer total and specific root exudates (the sum of all organic acid anions and sugars) due to the smaller root systems when compared to high-P supply. Root exudation is regulated by photosynthesis with around 2–5% of photosynthesised-C being exported proportionally by plants as root exudates (Jones *et al*., 2004; Pausch and Kuzyakov, 2018). In our study, high P appeared to increase total root exudation as indicated by the greater the root-derived CO_2-C (Fig. 2) through its simulative impacts on photosynthesis and biomass production (Table 1). High P might have also escalated the specific root exudation as previously found by Wang *et al*. (2017) that P fertilization increased the amounts of citrate, malate and succinate released per unit of root weight. This is because other organic anions such as malate dominate in the root exudates and their secretion is up-regulated by P fertilisation (Wang *et al*., 2017). Root exudates are the primary cause of RPE and the mineralisation of soil organic matter by providing energy for soil microorganisms (Shahzad *et al*., 2015). Moreover, the RPE was positively correlated with both shoot and root biomass (Table 3), consistent with previous studies (Fu and Cheng, 2002; Dijkstra *et al*., 2006).

As microbial energy and C source, primary metabolites of root exudates, e.g. sugars, amino acids and organic acids (Jones *et al*., 2004) could stimulate microbial growth and/or activity (Kuzyakov *et al*., 2000; Nannipieri *et al*., 2008), for example, the up-regulation of extracellular enzyme activity (Brzostek *et al*., 2012). In this present study, both microbial biomass C (Fig. 4A) and rhizosphere soil respiration were higher under high P than under low P supply (Table 2). The larger microbial population and stimulated activity increased decomposition of native soil organic C probably due to the 'co-metabolism' mechanism by which the extracellular enzymes synthesised specifically catalyse substrate degradation driving decomposition of soil organic matter (Kuzyakov *et al*., 2000).

Microbial N mining is also previously proposed to explain the enhanced RPE since C inputs from roots normally increase microbial N requirement, leading to a greater microbial SOM decomposition for N (Fontaine *et al*., 2011). However, this mechanism was unlikely in this present

study although the microbial N demand was greater under high P. In this present study, soil microorganisms would primarily rely on external supply for their N nutrition, considering that N was supplied periodically and that albeit smaller, soil inorganic N under high P was still sufficient for microbial growth when compared to low P (Figs 3, 4C). In an incubation study with boreal forest soils, Wild *et al*. (2017) also found that substrate addition increased microbial growth and N demand but not microbial mining of soil N because there was no increase in protein depolymerisation. Besides, microorganisms immobilised more soil available N under high P than under low P as shown by the greater MBN (Fig. 4B), lower MBC-to-N ratio (Fig. 4C) and lower soil inorganic N (Fig. 3) under high P. This might have also contributed to the absence of microbial N mining (Wild *et al*., 2017).

The greater root exudation under high P could also shift microbial structure composition with more dominance of fungi (Griffiths *et al*., 2012) because fungi, as heterotrophic organisms, depend largely on exogenous C for growth. Certain components of root exudates could also serve as chemical signals to structure fungal community (Broeckling *et al*., 2008). Rather than stabilising soil organic C, fungi have been recently shown to increase priming effect and decomposition of soil organic C (Fontaine *et al*., 2011; Shahzad *et al*., 2015), probably due to their greater capacity of accessing soil C via hyphal exploration (Fontaine *et al*., 2011). These observations suggest that high P could increase RPE through boosting biomass production and the associated stimulation of microbial growth and activity, and fungal abundance.

Chemical mechanism of rhizosphere priming effect

Apart from the above microbial mechanisms, root exudates (organic acid ligands, in particular) could also affect RPE by liberating mineral-associated organic C via chemical dissolution and/or complexation (Finley *et al*., 2018). In this study, high P could have potentially increased mineral dissolution as evidenced by the higher concentrations of water-extractable Fe and Al in the rhizosphere soil (Table 2) when compared to low P. The mineral dissolution might have released previously mineral-protected organic C, rendering it more vulnerable to microbial decomposition, leading to the increase in RPE (Table 3).

Soil organic C is protected by adsorption on short-range-order (SRO) minerals through their large reactive surface area and/or formation of metal (Fe, Al)-containing complexes due to their various binding sites (Kleber *et al*., 2015; Finley *et al*., 2018). Carboxylate components of root exudates could increase soil C release (Jones *et al*., 2004) by physicochemical breakdown of organic-mineral associations and/or displacement of soil organic C on mineral surfaces (Kleber *et al*., 2015). In a study by Naveed *et al*. (2017), barley root exudates with large amounts of organic acid anions were thought to be capable of dispersing soil particles by increasing the net negative charges of clay when adsorbed on surfaces of soil mineral particles (Shanmuganathan and Oades, 1983). Keiluweit *et al*. (2015) also found that oxalic acid had the ability to liberate protected C from mineral phases via the following processes. Firstly, organic acid anions could release SOC absorbed on SRO surfaces by complexation and dissolution. Secondly, they could solubilise SOC from metal-organic ligand complexes by binding with metal cations (Keiluweit *et al*., 2015). These processes could facilitate microbial access to soil organic C, and induce a high priming effect as revealed recently (Keiluweit *et al*., 2015; Finley *et al*., 2018).

This present study further validated the above assumption by using a pair of wheat near-isogenic lines which differ only in citrate efflux from the roots. The differences in citrate efflux were quantified previously with line EGA-Burke *TaMATE1B* exuding five- to eight-fold more citrate ligands than line Egret-Burke (Ryan *et al*., 2014). Wheat line EGA-Burke *TaMATE1B* induced higher RPE and root-specific RPE than line EGA-Burke under both P levels (Fig. 5). However, the up-regulation of citrate efflux in EGA-Burke *TaMATE1B* as indicated by more shoot biomass and greater root-derived CO_2-C (Table 1, Fig. 2) did not stimulate either microbial growth (Fig. 4A) or

activity in the rhizosphere (Table 2) probably because citrate is similar to oxalic acid which is energetically non-favourable to microorganisms (Keiluweit *et al*., 2015). In this present study, EGA-Burke *TaMATE1B* with more citrate efflux might have secreted lower amounts of bioenergetically more favourable metabolites which are easily assimilated by microorganisms without breaking-down by extracellular enzymes. The input of such substrates might induce only temporary stimulation of native, fast-growing species without actual decomposition of soil organic matter (Blagodatskaya and Kuzyakov, 2008).

The greater RPE under EGA-Burke *TaMATE1B* might also be partly induced by chemical dissolution and/or displacement of mineral-associated organic C as stated above. It was supported by the higher concentrations of water-extractable P, Fe and Al in the rhizosphere of EGA-Burke *TaMATE1B* than EGA-Burke although the effect was only significant at $P \leq 0.10$ (Table 2). The mineral dissolution experiment provided further evidence that citrate ligand could facilitate mineral dissolution by forming citrate-Fe/Al complexes (Fig. 6) and thus might liberate previously-protected C through this process (Kleber *et al*., 2015). For example, a previous study showed that the concentration of dissolved C in soil pore water correlated positively with dissolved Al (R^2 = 0.95) and Fe (R^2 = 0.98) (Keiluweit *et al*., 2015). The low and high pH induced by the addition of citric acid and potassium citrate solutions might have limited the breakdowns of the added organic anions (Jones *et al*., 1996), which favoured to examine the chemical process. The results are consistent with previous findings. For example, organic acid anions such as citrate and malate secreted from plant roots were found to increase Fe-dissolution (Jones *et al*., 1996) and the release of P from Al and Fe oxides (Sanyal and Datta, 1991) in acid soils. The release of minerals by chemical dissolution is important especially in nutrient-scarce soils, which could potentially alleviate plant and/or microbial nutrient limitation and consequently affect RPE. Although the chemical process cannot be separated from the microbial process, this study indicates that root exudates could also contribute to the RPE through breakdown and release of the mineral-protected organic C.

Future studies should aim to generate direct evidence elucidating that 1) organic acid anions enhance microbial access of mineral-bounded C and 2) this part of C is utilised by microorganisms. Together with isotopic tracing, modern spectroscopic technologies such as near-edge X-ray absorption fine structure imaging and nanoscale secondary ion mass spectrometry can offer the information of the source (plant- or soil-derived), concentration and composition of carbon in soil solution (Keiluweit *et al*., 2015). Direct measurement of the amount and ^{13}C abundance of microbial respiration also provide insights into the utilisation of plant- and soil-derived C by microorganisms. The compositional analysis of soil microbial community structure using stable isotope probing (SIP) (Hungate *et al*., 2015) could also reflect soil C bioavailability and the utilisation of different C substrates by specific microbial taxa.

CONCLUSIONS

This study suggests that both microbial and chemical mechanisms are accountable for the enhanced RPE when high P is supplied. Rhizosphere priming effect is more likely caused by microbial activation and co-metabolism of both easily-available soil organic C and mineral-protected C released via chemical dissolution. Further investigations are warranted to validate the relative contributions of microbial mechanism and mineral dissolution to the RPE.

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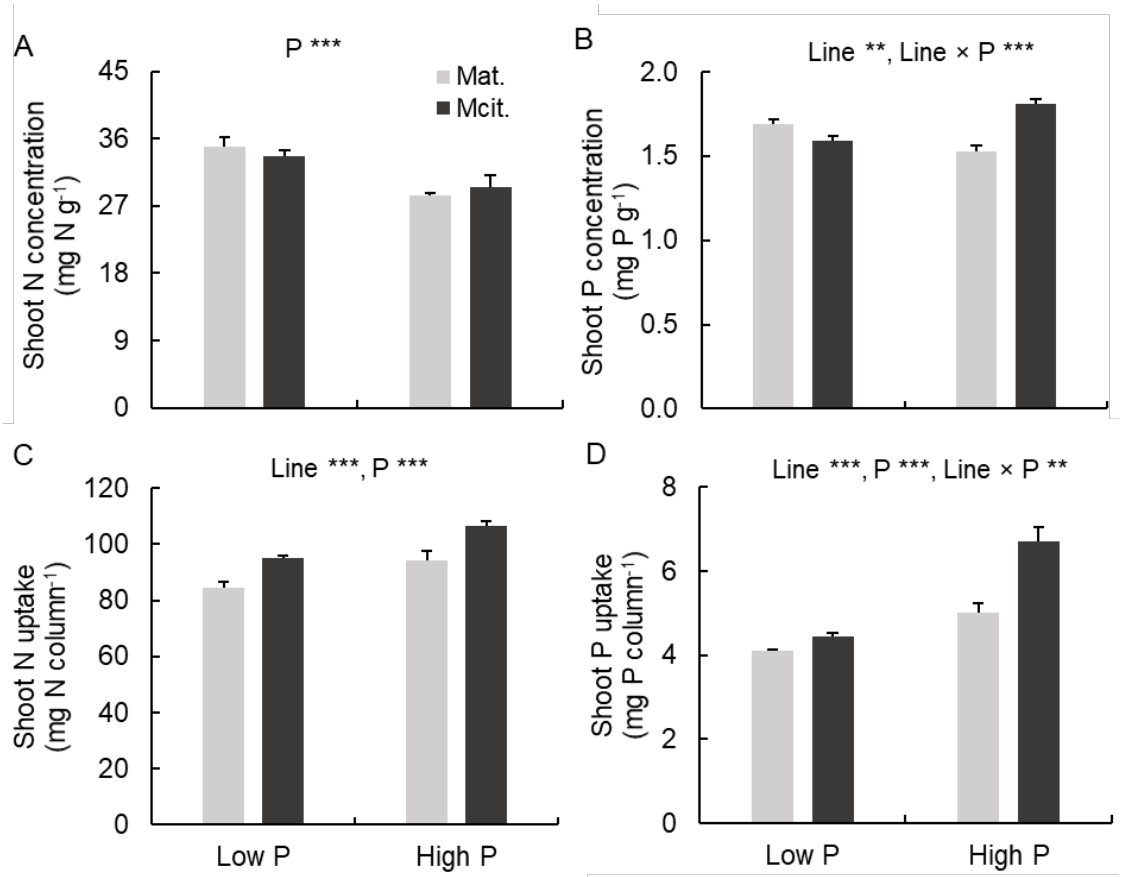

Fig. 1 Concentrations (mg g^{-1}) of N (A) and P (B), and N (C) and P (D) uptake (mg $column^{-1}$) in shoots of wheat line EGA-Burke (Mat.) and its near-isogenic line EGA-Burke *TaMATE1B* (Mcit.) supplied with either low P (10 µg g^{-1} soil) or high P (40 µg g^{-1} soil). Error bars are + standard errors. ** $P < 0.01$, *** $P < 0.001$

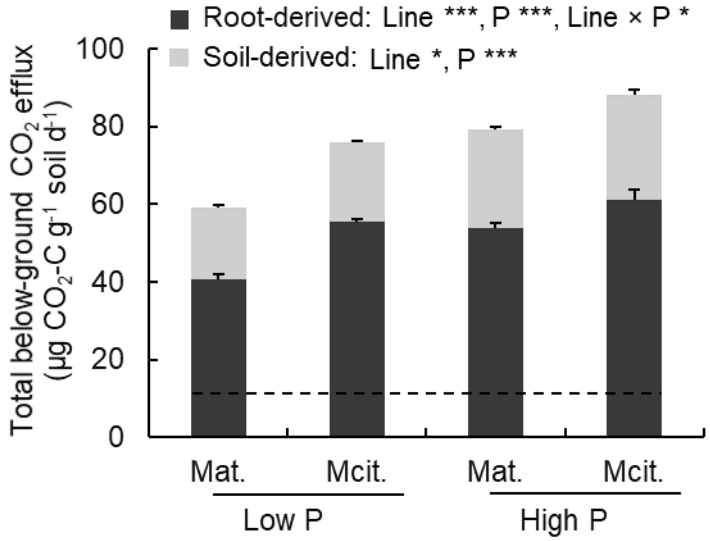

Fig. 2 Total below-ground CO_2 efflux (soil-derived + root-derived, µg CO_2-C g^{-1} soil d^{-1}) under wheat line EGA-Burke (Mat.) and its near-isogenic line EGA-Burke *TaMATE1B* (Mcit.) supplied with either low P (10 µg g^{-1} soil) or high P (40 µg g^{-1} soil). Dash line denotes basal soil respiration of no-plant control. Error bars are + standard errors. For the soil-derived CO_2, the main effects of wheat line ($P < 0.05$) and P supply ($P < 0.001$) but not their interaction are significant. For the root-derived CO_2, the main effects of wheat line ($P < 0.001$) and P supply ($P < 0.001$), and their interaction ($P < 0.05$) are significant

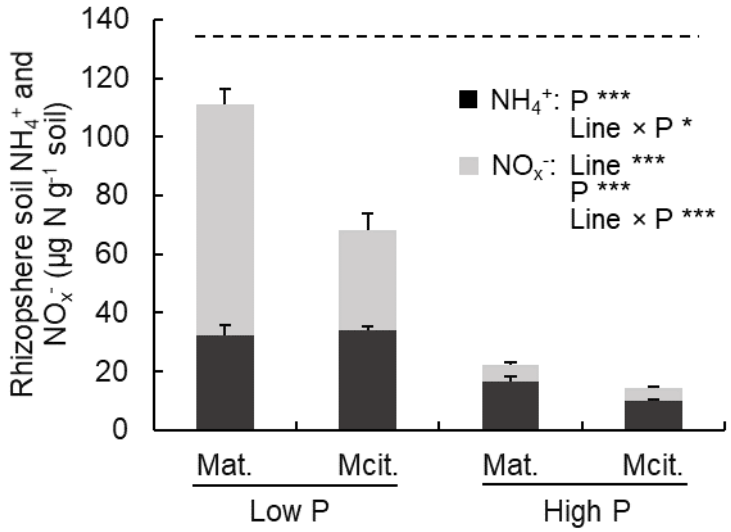

Fig. 3 Concentrations of soil ammonium (NH_4^+, µg N g^{-1} soil), nitrate and nitrite (NO_x^-, µg N g^{-1} soil) in the rhizospheres of wheat line EGA-Burke (Mat.) and its near-isogenic line EGA-Burke *TaMATE1B* (Mcit.) supplied with either low P (10 µg g^{-1} soil) or high P (40 µg g^{-1} soil). The dash line denotes concentration of inorganic N ($NH_4^+ + NO_x^-$) in the unplanted control. Error bars are + standard errors. The data of NO_x^- were log_{10}-transformed before statistical analysis. For NO_x^-, the main effects of wheat line and P supply and their interaction are all highly significant ($P < 0.001$). For NH_4^+, the main effects of wheat line ($P < 0.001$) and the interaction between wheat line and P supply ($P < 0.05$) are significant

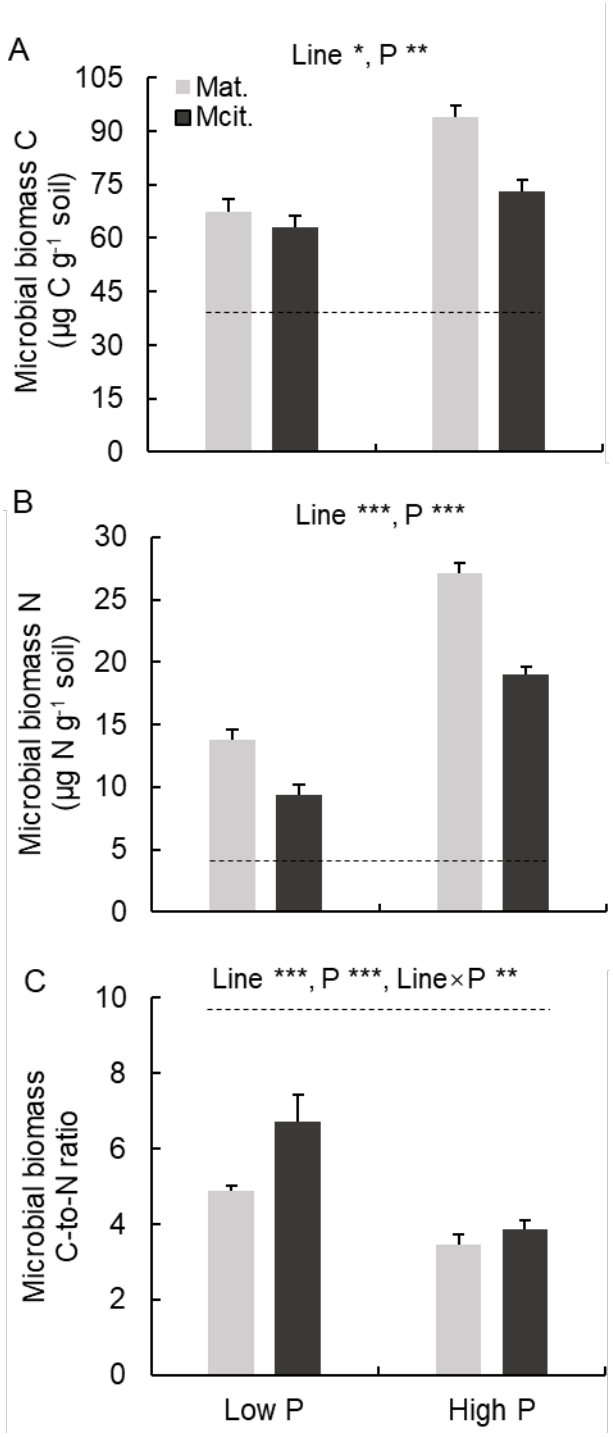

Fig. 4 Microbial biomass C (A), N (B) (µg C/N g^{-1} soil) and C-to-N ratio (C) in the rhizospheres of wheat lines EGA-Burke (Mat.) and EGA-Burke EGA-Burke *TaMATE1B* (Mcit.) supplied with either low P (10 µg g^{-1} soil) or high P (40 µg g^{-1} soil). Dash lines denote the values of the unplanted control. Error bars are + standard errors. Line, wheat line; P, phosphorus supply. * $P < 0.05$, ** $P < 0.01$; *** $P < 0.001$

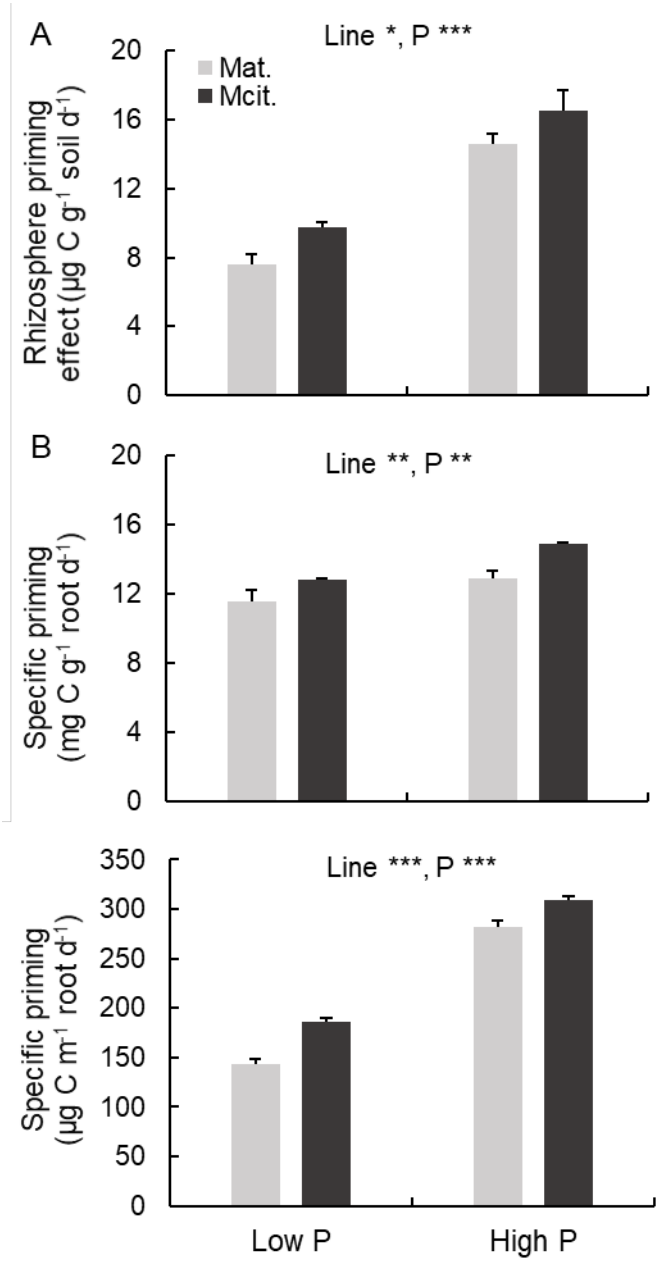

Fig. 5 Rhizosphere priming effect per unit soil mass (µg C g^{-1} soil d^{-1}) (A), per unit root mass (mg C g^{-1} root d^{-1}) (B) and per unit root length (µg C m^{-1} root d^{-1}) (C) under wheat line EGA-Burke (Mat.) and its near-isogenic line EGA-Burke *TaMATE1B* (Mcit.) supplied with either low P (10 µg g^{-1} soil) or high P (40 µg g^{-1} soil). Error bars are + standard errors. The main effects of wheat line ($P < 0.05$) and P supply ($P < 0.001$) but not their interaction are significant.

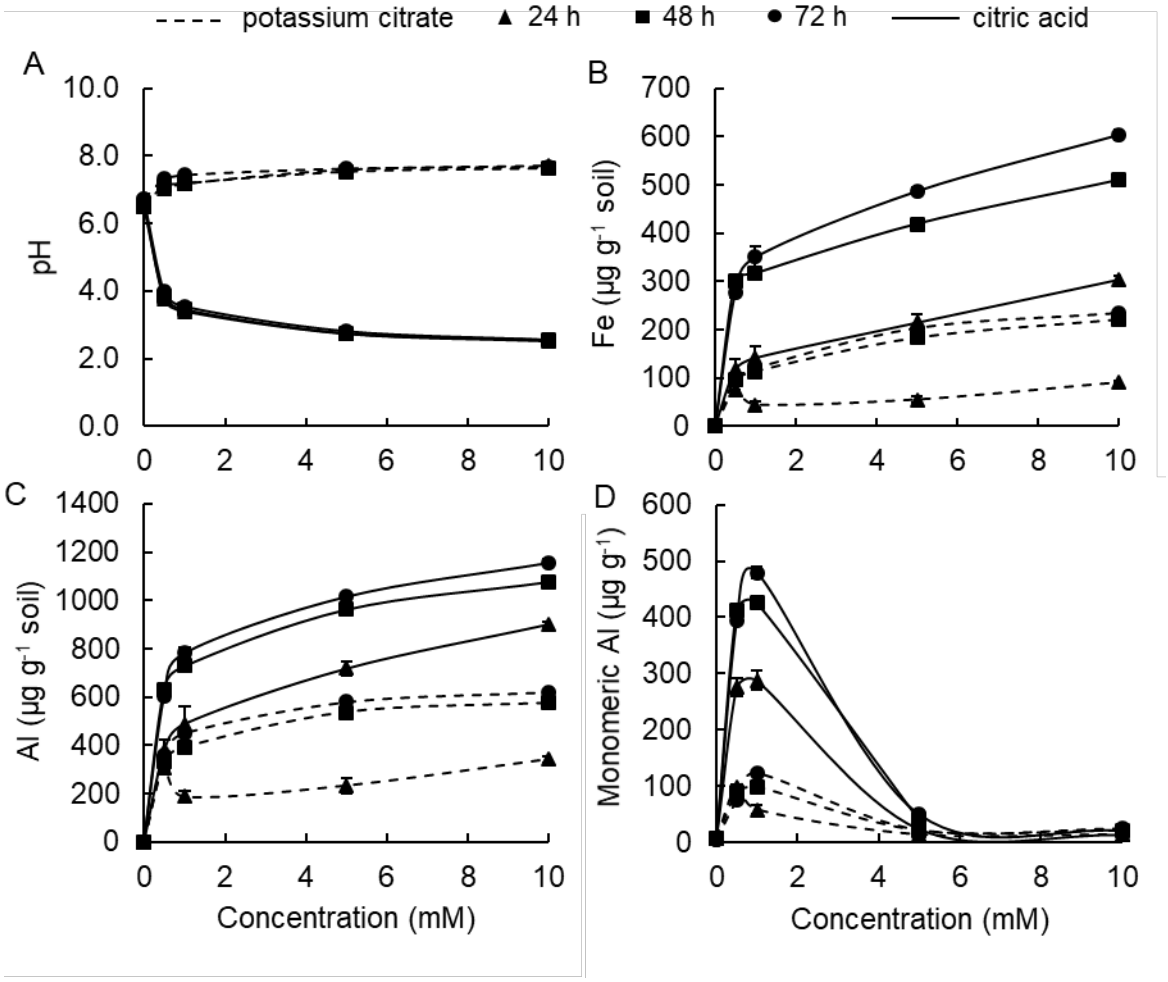

Fig. 6 Effects of increasing citrate/citric acid addition on solution pH and concentrations of Al and Fe released (µg g^{-1}) from the dissolution experiments where ~1 g C4 soil reacted with 200 ml of either potassium citrate or citric acid at concentrations of 0, 0.5, 1, 5 and 10 mM for 24, 48 and 72 h: (A) solution pH, (B) ICP-Fe, (C) ICP-Al and (D) monomeric Al. Means ± standard errors of triplicate were presented

Table 1. Shoot and root dry mass (DM), root-to-shoot ratio (R:S), total root length, root $\delta^{13}C$ value and $\delta^{13}C$ value of below-ground CO_2 after wheat line EGA-Burke (Mat.) and its near-isogenic line EGA-Burke *TaMATE1B* (Mcit.) were grown for 6 weeks with either low P (10 μg g^{-1} soil) or high P (40 μg g^{-1} soil) supply

Wheat line	P supply (μg g^{-1} soil)	Shoot DM (g $column^{-1}$)	Root DM (g $column^{-1}$)	R:S	Root length (m $column^{-1}$)	$\delta^{13}C$ (‰)	
						Root	CO_2 released
Mat.	10	2.42±0.13	0.83±0.01	0.34±0.02	67.2±2.8	-29.1±0.2	-24.6±0.0
Mcit.	10	2.84±0.08	0.91±0.05	0.32±0.02	64.4±1.3	-28.5±0.1	-24.8±0.1
Mat.	40	3.34±0.14	1.39±0.03	0.42±0.02	68.1±5.9	-28.0±0.0	-23.7±0.1
Mcit.	40	3.62±0.12	1.34±0.05	0.37±0.02	66.9±2.5	-27.8±0.2	-23.7±0.2
Two-way ANOVA (*P*-value)							
Line		0.011	0.718	0.094	0.394	0.043	0.457
P		0.001	0.001	0.009	0.434	0.002	0.001
Line × P		0.598	0.135	0.549	0.807	0.269	0.432

Table 2 Rhizosphere soil respiration ($Rh_{resp.}$) and water-extractable P, Fe and Al in the rhizosphere of wheat line EGA-Burke (Mat.) and its near-isogenic line EGA-Burke *TaMATE1B* (Mcit.) after grown for 6 weeks with either low P (10 µg g^{-1} soil) or high P (40 µg g^{-1} soil) supply

Wheat line	P supply (µg g^{-1} soil)	pH	$Rh_{resp.}$ (µg CO_2 g^{-1} soil)	Water-extractable elements (µg g^{-1})		
				P	Fe	Al
Control		4.69±0.00	41.5±4.7	1.18±0.12	1.9±0.3	10.9±1.7
Mat.	10	4.79±0.01	79.0±2.4	1.21±0.09	10.1±1.8	53.7±10.7
Mcit.	10	4.79±0.03	65.9±5.4	1.86±0.28	16.8±4.6	83.2±31.0
Mat.	40	4.78±0.01	89.5±4.9	2.92±0.09	32.2±1.3	167.0±32.2
Mcit.	40	4.76±0.01	83.8±3.4	3.51±0.39	43.5±7.3	230.3±41.7
Two-way ANOVA (*P*-value)						
Line		0.201	0.052	0.094	0.083	0.100
P		0.030	0.008	0.001	0.001	0.004
Line × P		0.115	0.403	0.582	0.571	0.614

Table 3 Pearson's correlation coefficients among rhizosphere priming effect (RPE), shoot and root dry mass (DM), shoot N and P uptake, mineral N ($NH_4^+ + NO_x^-$), concentrations of water-extractable P, Fe and Al, and microbial biomass N (MBN) in rhizosphere soil and root-derived CO_2-C

	Shoot DM	Root DM	Shoot nutrient uptake		Mineral N	Water-extractable elements			MBN	Root-derived
			N	P		P	Fe	Al		CO_2-C
RPE	0.86**	0.95**	0.77**	0.80**	-0.95**	0.96**	0.90**	0.94**	0.64*	0.80**
Shoot DM		0.81**	0.86**	0.91**	-0.90**	0.85**	0.81**	0.82**	0.50*	0.77**
Root DM			0.54*	0.69**	-0.91**	0.91**	0.84**	0.87**	0.77**	0.69*
Shoot N uptake				0.84**	-0.77**	0.69**	0.68*	0.67*	n.s.	0.84**
Shoot P uptake					-0.74**	0.81**	0.79**	0.84**	n.s.	0.69*
Mineral N						-0.92**	-0.87**	-0.84**	-0.68**	-0.86**
Water-extractable P							0.98**	0.98**	n.s.	0.90**
Water-extractable Fe								0.99**	n.s.	0.91**
Water-extractable Al									n.s.	0.91**
MBN										n.s.

n.s., * and ** represent $P > 0.05$, < 0.05 and < 0.01, respectively (2-tailed)