Plant Soil (2017) 412:43–59
DOI 10.1007/s11104-016-2884-3

REGULAR ARTICLE

# Does the combination of citrate and phytase exudation in *Nicotiana tabacum* promote the acquisition of endogenous soil organic phosphorus?

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Received: 14 December 2015 / Accepted: 4 April 2016 / Published online: 11 July 2016


**Abstract**
*Background and Aims* Plant acquisition of endogenous forms of soil phosphorus (P) could reduce external P requirements in agricultural systems. This study investigated the interaction of citrate and phytase exudation in controlling the accumulation of P and depletion of soil organic P by transgenic *Nicotiana tabacum* plants.

*Methods N. tabacum* plant lines including wild-type, vector controls, transgenic plants with single-trait expression of a citrate transporter (*A. thaliana frd3*) or fungal phytases (*phyA*: *A. niger*, *P. lycii*) and crossed plant lines expressing both traits, were characterized for citrate efflux and phytase exudation. Monocultures and intercropped combinations of single-trait plants were grown in a low available P soil (12 weeks). Plant biomass, shoot P accumulation, rhizosphere soil pH and citrate-extractable-P fractions were determined. Land Equivalent Ratio and complementarity effect was determined in intercropped treatments and multiple-linear-regression was used to predict shoot P accumulation based on plant exudation and soil P depletion.
*Results* Crossed plant lines with co-expression of citrate and phytase accumulated more shoot P than single-trait and intercropped plant treatments. Shoot P accumulation was predicted based on phytase-labile soil P, citrate efflux, and phytase activity (Rsq=0.58, $P < .0001$). Positive complementarity occurred between intercropped citrate- and phytase-exuding plants, with the greatest gains in shoot P occurring in plant treatments with *A. niger phyA* expression.
*Conclusions* We show for the first time that trait synergism associated with the exudation of citrate and phytase by tobacco can be linked to the improved acquisition of P and the depletion of soil organic P.

**Keywords** Complementarity · Root exudation · Rhizosphere · Citrate · Phytase · Soil organic phosphorus

Responsible Editor: Megan H. Ryan.

**Electronic supplementary material** The online version of this article (doi:10.1007/s11104-016-2884-3) contains supplementary material, which is available to authorized users.

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## Introduction

The sustainability and efficiency of nutrient use in agriculture could be achieved through the screening and selection of plants with traits that promote the utilization of endogenous soil phosphorus (P) (Condron et al. 2013; Stutter et al. 2012). In agricultural soils, organic P ($P_o$) represents between 30 and 80 % of the total P (Stutter et al. 2015), but most forms of $P_o$ are considered unavailable for direct uptake by plants (Hayes et al. 2000; Richardson et al. 2000). The manipulation of plants to exude citrate and fungal phytases in excess of wild-type levels has been shown to increase the utilization of model organic P forms *in vitro* (e.g., Giles et al. 2012), but has provided little benefit in soils (e.g., George et al. 2005). The success of single-trait strategies for improving plant utilization of $P_o$ is largely controlled by the interaction of phytases with $P_o$ substrates, which is generally limited in soil due to immobilization processes (Celi and Barberis 2005; George et al. 2007b). Soil incubation studies (Menezes-Blackburn et al. 2013), *in vitro* assays (Giles et al. 2012; Tang et al. 2006), intercropping growth studies (Li et al. 2003; Dissanayaka et al. 2015), and recently proposed conceptual models (Clarholm et al. 2015) suggest that the coupled exudation of organic anions and phosphatase enzymes may synergistically promote the bioavailability of endogenous soil organic P.

In order for plants to utilize $P_o$, it must first be hydrolyzed to inorganic orthophosphate by phosphatase enzymes of plant or microbial origin (Richardson et al. 2011). Due to its abundance in soils, high degree of phosphorylation, and strong association with the soil solid phase, phytate (*myo*-inositol hexakisphosphate, InsP6) has been used as a model compound for understanding the mechanisms of $P_o$ hydrolysis in soils (Giles and Cade-Menun 2014). Phytate is specifically hydrolyzed by phytases (Greiner 2007), which can also act non-specifically on a range of other orthophosphate monoester compounds to release plant available inorganic orthophosphate (George et al. 2007a; Hayes et al. 2000). In soils, the hydrolysis (or mineralization) of $P_o$ occurs slowly or not at all due to abiotic (e.g., sorption, precipitation) and biotic (e.g., incorporation into microbial biomass) reactions, which limit the conversion of $P_o$ to plant available forms (Celi and Barberis 2005; Giaveno et al. 2010). In the sequential model proposed by Clarholm et al. (2015), plant and/or microbial organic anions and phosphatase enzymes work sequentially to solubilize and then mineralize $P_o$ in organo-mineral complexes, which will likely depend on the co-localization of these processes to the plant root (Hauggaard-Nielsen and Jensen 2005).

Plant growth studies in sterile agar media show that the manipulation of single exudation traits can lead to improvements in the utilization of inorganic and organic P forms. The overexpression of bacterial (*Bacillus* sp.) or fungal (*Aspergillus* sp.) phytase genes in *Trifolium subterraneum* L. (George et al. 2004), *Arabidopsis thaliana* (Lung et al. 2005; Mudge et al. 2003; Richardson et al. 2001), *Nicotiana tabacum* L. (George et al. 2005; Lung et al. 2005), *Brassica napus* (Wang et al. 2013), *Zea mays* (Chen et al. 2008), *Medicago sativa* (Ma et al. 2012) and *Solanum tuberosum* L. (Zimmermann et al. 2003) is associated with the ability of plants to utilize soluble phytate, when provided as the sole source of P. Likewise, overexpression of the *A. thaliana* FRD3 (*frd3*) citrate transporter in tobacco led to increased exudation of citrate and improved the assimilation of P from insoluble phosphate sources (adsorbed to goethite minerals). Citrate efflux alone did not improve the utilization of sorbed or soluble phytate by these plants (Giles et al. 2012). In contrast, tobacco plants that expressed *A. niger phyA* could utilize phytate from sorbed sources, but only when phytate concentrations in the growth media exceeded the sorption capacity of the binding mineral (Giles et al. 2012). Collectively, these studies suggest that low levels of citrate or phytase exudation could improve the availability of insoluble organic P sources.

Gains in plant biomass or nutrient assimilation due to the introduction of multiple beneficial traits may result in a 'synergism' between plants when the efficacy of a single trait is not sufficient to illicit the desired effect (Lynch 2011). The influence of trait synergism and complementarity on soil P acquisition has been studied with regard to root architectural and anatomical 'phenes' (Miguel et al. 2015; Zhang et al. 2014) and the exudation of organic anions and phosphatase enzymes (Hauggaard-Nielsen and Jensen 2005). However, few studies have specifically investigated the influence of citrate and phytase exudation on the utilization of soil organic P. Examples in chickpea-wheat (Zhang and Li 2003) and lupin-maize (Dissanayaka et al. 2015) intercropping systems suggest that recalcitrant and organic soil P pools are depleted to a larger extent in mixed planting systems with contrasting root morphologies and the exudation of both organic anions and phosphatase.

We hypothesized that plants or plant combinations with citrate and phytase exudation would assimilate more P and deplete a larger proportion of soil organic P than wild-type plants or plants expressing a single trait. To test this hypothesis we (1) characterized the exudation pattern of tobacco plant lines over-expressing single citrate or phytase traits and crossed plant lines co-expressing these traits; (2) determined shoot biomass and P accumulation by individual tobacco plant lines in monoculture, intercropped combinations containing one citrate and one phytase-exuding plant line, and crossed plant lines expressing both traits; (3) assessed complementarity and shoot P partitioning in intercropped and crossed plant treatments; and (4) analyzed rhizosphere soils for plant-induced changes to pH, citrate-extractable P, and phytase-labile P concentrations.

## Materials and methods

### Plant lines

Descriptions of the plant lines tested are provided in Table 1 and included *Nicotiana tabacum* wild-type (WT, var. W38), vector control (Vec), and transgenic plant-lines that express citrate efflux and/or phytase exudation traits. Single-trait plant lines expressed either the *Aspergillus niger phyA* ($Phy1_{An}$) or *Penophoria lycii phyA* ($Phy2_{Pl}$) phytase genes, or the MATE-type citrate transporter from *Arabidopsis thaliana* FRD3 (*frd3*; Cit) as described by George et al. (2005a) and Giles et al. (2012). Briefly, *phyA* was expressed in the pPLEX502 vector (Schunmann et al. 2003) under the control of a 35S promoter from cauliflower mosaic virus and *ocs* terminator with extracellular expression achieved using the carrot extensin (*ex*) gene (Richardson et al. 2001). Constitutive expression of *frd3* in the pCGN18 vector was implemented under the control of the 35S promoter and nopaline synthase terminator (Connolly et al. 2002). All single-trait plant lines were developed as homozygous selections for each of the identified traits. Crosses of Cit with $Phy1_{An}$ and $Phy2_{Pl}$ lines were produced to generate F1 heterozygous plants that co-expressed the citrate and phytase traits ($Co1_{CitxAn}$, $Co2_{CitxPl}$), whereby single flowers were cross pollinated by hand and seed was collected from isolated capsules.

### Plant exudate analysis

Seeds were vapour sterilized for 1 h by placement above 4 % hypochlorite and 3 % HCl (v/v) before being germinated and grown for 14 d under constant light (~200 μE $m^{-2}$), and temperature (21 °C) in a swirling nutrient solution culture (100 rpm) containing 3 mM $NH_4Cl$, 4 mM $Ca(NO_3)_2$, 4 mM $KNO_3$, 3 mM $MgSO_4$, 0.1 mM Fe-EDTA, and micronutrients (6 μM $MnCl_2$, 23 μM $H_3BO_3$, 0.6 μM $ZnCl_2$, 1.6 μM $CuSO_4$, 1.0 μM $Na_2MoO_4$, 1.0 μM $CoCl_2$), 50 μM P ($Na_2HPO_4$) and 10 g $L^{-1}$ sucrose at pH 5.46 (George et al. 2005). The plants were transferred to a P-free and sucrose-free equivalent nutrient solution for a further 14 d prior to exudate collection. Solution pH was measured in freshly collected supernatants using a glass pH electrode (Mettler Toledo, Ltd., Leicester UK) and solutions for citrate measurements were freeze-dried prior to analysis. Dry weights were determined for shoot and root materials after drying for 1 week at 70 °C.

Extracellular phytase activity was determined on fresh filtered (0.2 μm PES) plant exudate solutions using *myo*-inositol hexakisphosphate (InsP6 274321, Sigma-Aldrich Corp., St. Louis, MO) substrate as described by Giles et al. (2012). Briefly, 200 μL of plant exudate solution were combined with 20 μL MES buffer (150 mM, pH 5.5) and 30 μL of IHP stock solution (0.2 mM) and incubated for 60 min at 37 °C. Reactions were stopped by combining equal volumes of 10 % trichloroacetic acid (TCA) at the beginning (T0) or end (T60) of the incubation period. Phosphate release due to enzyme activity was measured in reaction solutions using malachite green colorimetry at 620 nm as described by Irving and McLaughlin (1990). Phytase activity was calculated based on the difference in phosphate concentration between T60 and T0 treatments and expressed in nKat per root dry weight per day (nKat $g^{-1}$ root dw $d^{-1}$). The theoretical exudation rate of both plants in intercropped treatments was calculated based on the mean exudation of citrate and phytase by individual plants in each combination ($Int1_{Cit+An}$ , $Int2_{Cit+Pl}$; Table 1).

Citrate was assayed enzymatically according to Dagley (1974) with the following modifications. Freeze-dried exudate solutions were reconstituted at ten times the original concentration in 0.8 mM Tris-HCl (pH 8). Four μL of NADH solution (8 mg NADH and 7 mg $NaHCO_3$ in 1 mL water) and 2 μL of 1:1 solution of lactic dehydrogenase (LDH) and malic

**Table 1** Citrate and phytase traits, plant treatment descriptions, and exudate characteristics of *N. tabacum* plant lines and intercropped combinations

| Trait or combination | Plant lines and combinations | Plant lines and treatment descriptions | Phytase activity[a] nKat $g^{-1}$ root dw $d^{-1}$ | | | | Citrate nmol $g^{-1}$ root dw $d^{-1}$ | | | | Δ Exudate pH[b] | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| None | WT | Wild-type | nd | | | d | 6.7 | ± | 0.3 | bc | −0.31 | ± | 0.07 | bc |
| Citrate | Cit | Citrate efflux via *A.thaliana* FRD3 (*frd3*) | 5.9 | ± | 1.7 | c | 17.1 | ± | 0.2 | a | 0.44 | ± | 0.01 | b |
| None | Vec | Vector control (pPLEX502) | 4.4 | ± | 1.0 | c | 9.1 | ± | 0.2 | b | 0.29 | ± | 0.02 | c |
| Phytase | $Phy1_{An}$ | Phytase exudation via *A. niger phyA* (*ex*::*phyA*) | 63.4 | ± | 6.1 | a | 4.1 | ± | 0.0 | c | 0.66 | ± | 0.08 | a |
| Phytase | $Phy2_{Pl}$ | Phytase exudation via *P.lycii phyA* (*ex*::*phyA*) | 25.2 | ± | 5.6 | b | 7.7 | ± | 0.4 | bc | 0.28 | ± | 0.04 | c |
| Co-expression | $Co1_{CitxAn}$ | $Phy1_{An}$ crossed with Cit | 26.3 | ± | 1.9 | b | 5 | ± | 0.2 | c | −0.11 | ± | 0.04 | d |
| Co-expression | $Co2_{CitxPl}$ | $Phy2_{Pl}$ crossed with Cit | 52.5 | ± | 7.2 | ab | 18 | ± | 0.5 | a | 0.36 | ± | 0.02 | bc |
| Intercropped | $Int1_{Cit+An}$ | $Phy1_{An}$ intercropped with Cit | 34.7 | | | * | 10.6 | | | * | 0.6 | | | * |
| Intercropped | $Int2_{Cit+Pl}$ | $Phy2_{Pl}$ intercropped with Cit | 15.6 | | | * | 12.4 | | | * | 0.4 | | | * |

*Theoretical exudation by intercropped plant combinations based on the average phytase activity, citrate efflux, and pH change measured in individual plant lines

[a] Means ± standard devidation significantly different means within the column indicated by different letters ($p < 0.05$)

[b] pH change in exudate collection media based on difference from the starting pH (5.46) after 14 d

dehydrogenase (MDH) were added to 250 μL of exudate solution. Samples were equilibrated for 1 h at room temperature and 2 μL citrate lyase (CL; 100 mg $mL^{-1}$) was added to half of the well replicates which were incubated for an additional hour prior to the measurement of NADH concentrations at 340 nm. The depletion of NADH in samples treated with CL was proportional to the concentration of citrate in standards (0, 5, 10, 15, 20, 40, 60, 80 nmol citrate). Stock citrate standards (Fluka Analytical, Seelze, Germany) and blanks were prepared in blank exudate collection solutions reconstituted at 10x the original concentration.

## Soil

In order to ensure P deficient conditions and maximize the use of endogenous soil P by the plants, the study soil was selected to contain a minimal level of available phosphate yet relatively large proportions of organic P. Topsoils (0–10 cm depth) used for the plant growth experiments originated from Strathfinella Hill near to Glensaugh Research Station (Laurencekirk, Aberdeenshire, UK; 56°53'42.29"N -2°32'00.42"W). The Glensaugh soil is a freely drained Podzol (FAO 2014) derived from acid igneous metamorphic rocks and Old Red Sandstone sandstones, and is maintained under permanent grassland management. The soil has a very low available P content with 6.7 mg $kg^{-1}$ Olsen P (DEFRA index 0), 1.4 mg $kg^{-1}$ Modified Morgan P, 3.4 mg $kg^{-1}$ water extractable orthophosphate, and a degree of P saturation of 10 % based on ammonium oxalate extraction. The soil is acidic (pH in $CaCl_2$ of 4.5) and contains 574 mg P $kg^{-1}$ aqua regia extractable total P. The soil was air-dried and sieved to 4 mm prior to use in plant growth experiments.

## Plant growth conditions

Plants were cultivated in controlled glasshouse conditions at the James Hutton Institute (Dundee, Scotland UK) for 75 d. Growth conditions were maintained at approximately 22 °C/14 °C day/night, with 16 h light maintained at 200 W $m^{-2}$. Tobacco seeds were pre-germinated on 0.1 % distilled water agar and two plants were transferred to growth pots containing 60 g of field moist soil. Soil moisture was maintained at approximately 80 % water holding capacity during the growth period with distilled water. Five mL of P-free nutrient solution (3 mM $NH_4Cl$, 4 mM $Ca(NO_3)_2$, 4 mM $KNO_3$, 3 mM $MgSO_4$, 0.1 mM Fe-EDTA, micronutrients: 6 μM $MnCl_2$, 23 μM $H_3BO_3$, 0.6 μM $ZnCl_2$, 1.6 μM $CuSO_4$, 1.0 μM $Na_2MoO_4$, 1.0 μM $CoCl_2$; pH 5.5 was added to each pot weekly). In order to maximize the volume of rhizosphere soil, sixty replicate pots were

prepared for each plant line and intercropped treatments listed in Table 1 as well as uncultivated soil controls (No plant). We assumed that the largest plants in each treatment would have the greatest impact on rhizosphere soil characteristics and therefore selected soils from pots containing the five largest total shoot biomass (sum of two plants) for the analysis of pH and citrate-extractable P concentrations in each plant treatment.

### Shoot biomass and phosphorus analysis

Shoot materials from sixty replicates were harvested at the end of the growth period for the determination of dry weight and the five replicate pots with the greatest total dry weight were selected for analysis of shoot P content. Dry weight was determined after drying for 1 week at 70 °C and shoot materials from two plants in each pot were analyzed separately for all treatments. Shoot P concentration was determined by digesting approximately 50 mg of dried and milled material with sulfuric acid-peroxide (Heffernan 1985) followed by malachite green colorimetry (Irving and McLaughlin 1990). Based on the small variance (<6 % or 0.1 g) in shoot dry weight for the sixty replicates in each plant treatment we assumed shoot P concentration in the five selected replicates to be representative of all sixty pots in each treatment. Shoot P accumulation was calculated for individual plants in the 60 replicate pots by applying the average shoot P concentration from the five largest pots to the individual plant biomasses in each treatment ($n$ = 5). Propagation of error procedures were used to estimate the standard error of shoot P accumulation for each plant treatment. Shoot dry weight ($n$ = 60 pots), P concentration ($n$ = 5 pots), and P accumulation are presented as the mean of two plants grown in monoculture or are reported separately for individual plants in the intercropped treatments. Root biomass and the presence of arbuscular mycorrhizal fungi (AMF) were not assessed but could contribute to differences in the ability of individual tobacco plant lines to utilize soil P (Ryan et al. 2012).

### Complementarity metrics

Land equivalent ratio (LER) and complementarity effect (CE) were calculated on the basis of shoot P accumulation as described previously (Zhang et al. 2014). Whereas LER represents the benefit of plants grown in combination relative to monocultures, CE represents the absolute increase in shoot P accumulation in plant combinations. Land equivalent ratios were calculated as follows:

$$LER_{cit+phy} = \frac{Y_{cit}^{inter}}{Y_{cit}^{mono}} + \frac{Y_{phy}^{inter}}{Y_{phy}^{mono}} \quad (1)$$

Where $Y_{cit}^{inter}$ and $Y_{phy}^{inter}$ represent shoot P accumulation by a single citrate (cit) or phytase (phy) exuding plant in an intercropped treatment, respectively, and $Y_{cit}^{mono}$ and $Y_{phy}^{mono}$ represent the total accumulation of P in shoots of two plants in monoculture treatments.

Complementarity effect of both citrate and phytase exuding plants was calculated as:

$$CE_{cit} = N \cdot \left( \frac{Y_{cit}^{inter}}{Y_{cit}^{mono}} - 0.5 \right) \cdot Y_{cit}^{mono} \quad (2)$$

$$CE_{phy} = N \cdot \left( \frac{Y_{phy}^{inter}}{Y_{phy}^{mono}} - 0.5 \right) \cdot Y_{phy}^{mono} \quad (3)$$

Where $N$ is the number of intercropped plants (2) and 0.5 is the density of a single plant type in the combination. For comparison to LER and CE in the crossed plant lines, theoretical LER and CE values were calculated assuming $Y_{cit}^{inter}$ and $Y_{phy}^{inter}$ to be equivalent to $0.5xCo1_{CitxAn}$ or $0.5xCo2_{CitxPl}$.

### Soil collection and pH measurement

Uncultivated and rhizosphere soils were stored fresh at 4 °C for pH measurement or dried (30 °C, 2 wks) and sieved (2 mm) for subsequent extraction with 50 mM citrate (pH 5). Plants were carefully removed from pots and all soil within the root plug and adhering to plant roots was collected with gentle shaking. Soil pH was determined on 10 g fresh sieved (2 mm) soils by mixing with 20 mL $CaCl_2$ (0.01 M), orbital shaking for 1 h (150 rpm), and 2 h settling prior to measurement of pH in supernatant solutions by glass electrode (Mettler Toledo, Ltd., Leicester UK).

### Extraction of soil phosphorus with 50 mM citrate

Extractions were carried out on soils collected from the five replicate pots chosen for shoot P analysis. Two g

air-dried soil was extracted with 50 mM citrate (pH 5.5, 1:2 w/v) with shaking for 1 h (200 rpm; Stutter et al. 2015). Extracts were centrifuged (4,000*g*, 15 min), filtered (0.45 μm PES), and stored at 4 °C prior to analysis. Inorganic P in citrate extracts ($CEP_i$) was determined using malachite green colorimetry (Irving and McLaughlin 1990). Total P in citrate extracts ($CEP_{TOT}$) was determined by ICP-OES. Citrate-extractable organic P ($CEP_o$) was calculated as the difference between $CEP_{TOT}$ and $CEP_i$.

### Phytase-labile phosphorus measurement in citrate extracts

Phytase-labile P in citrate extracts was determined using a commercially available *A. niger* phytase with high specificity towards phytate (Natuphos, EC 3.1.3.8; BASF SE, Ludwigshafen Germany) (Wyss et al. 1999) added to an excess final activity of 10 nKat $mL^{-1}$ as described by George et al. (2006). Briefly, citrate extracts (100 μL) were combined with 30 μL of MES buffer (150 mM MES, 10 mM EDTA, pH 5.5) and 30 μL Natuphos (100 nKat $mL^{-1}$) to a final volume of 300 μL and incubated at 37 °C for 48 h. Reactions were stopped by adding equal parts of chilled trichloroacetic acid (10 % w/v). The phosphate released during incubation with phytase was measured via malachite green colorimetry (Irving and McLaughlin 1990). Non-labile P in citrate extracts ($CEP_{nonlab}$) is defined as the difference between $CEP_o$ and citrate extractable phytase-labile P ($CEP_{phy}$). Therefore, $CEP_{TOT} = CEP_i + CEP_o = CEP_i + CEP_{phy} + CEP_{nonlab}$. All citrate extractable P fractions are reported in mg P $kg^{-1}$ dry soil.

### Statistical analyses

All data were analysed using JMP Pro 11.2.0 software (2013 SAS Institute Inc.) and are reported as the mean ± standard error of the mean. One-way analysis of variance (ANOVA) was used to determine significant differences between the means of plant and soil treatments (Tukey's Honest Significant Difference; $p < 0.05$). A step-wise multiple linear regression model was carried out using minimum Bayesian Information Criterion (standard least squares, $p < .0001$) with forward fitting to predict shoot P accumulation based on plant exudation rates and citrate-extractable soil P concentrations. The 95 % confidence interval was calculated manually for Land Equivalent Ratio and Complementarity Effect to determine significant positive complementarity (LER>1) or gains in shoot P accumulation (CE>1) in intercropped plant treatments. Pearson correlations between shoot and soil measurements were evaluated for significance at the 95 % confidence interval.

## Results

### Exudation characteristics of tobacco plant-lines

Phytase activity was greatest in single trait and crossed plant-lines with transgenic expression of *A. niger phyA* ($Phy_{An}$) and *P. lycii phyA* ($Phy_{Pl}$). Phytase activity in exudate solutions collected from $Phy_{An}$ (63.4 ± 6.1 nKat $g^{-1}$ root dw $d^{-1}$) was more than 2-fold greater than those collected from $Phy_{Pl}$ (25.2 ± 5.6 nKat $g^{-1}$ root dw $d^{-1}$; Table 1). In contrast, crossed plant lines expressing the *A. thaliana frd3* and the *A. niger phyA* ($Co1_{CitxAn}$: 26.3 ± 1.9 nKat $g^{-1}$ root dw $d^{-1}$) contained approximately half of the phytase activity of crossed plant lines expressing the *P. lycii phyA* ($Co2_{CitxPl}$: 52.5 ± 7.2 nKat $g^{-1}$ root dw $d^{-1}$; Table 1) as would be expected of heterozygous plants containing approximately half of the expected activity of homozygous parental lines. Among transgenic plants, phytase activity was lowest in the vector control (4.4 ± 1.0 nKat $g^{-1}$ root dw $d^{-1}$) and single trait citrate plants lacking *phyA* expression (Cit: 5.9 ± 1.7 nKat $g^{-1}$ root dw $d^{-1}$; Table 1), whereas phytase activity was not detected in exudate solutions of wild-type plants.

Citrate efflux was greatest in the $Co2_{CitxPl}$ crossed plant line containing the *P. lycii phyA* trait (18 ± 0.5 nmol $g^{-1}$ root dw $d^{-1}$) and Cit plants (17.1 ± 0.2 nmol $g^{-1}$ root dw $d^{-1}$) with single-trait expression of *A. thaliana frd3* (Table 1). The vector control (9.1 ± 0.2 nmol $g^{-1}$ root dw $d^{-1}$), single-trait *phyA* plants (4.1 ± 0.0 to 7.7 ± 0.4 nmol $g^{-1}$ root dw $d^{-1}$), the crossed plant line containing *A. niger phyA* expression ($Co1_{CitxAn}$: 5.0 ± 0.2 nmol $g^{-1}$ root dw $d^{-1}$), and the wild-type plants (6.7 ± 0.3 nmol $g^{-1}$ root dw $d^{-1}$) contained significantly lower citrate concentrations in exudate solutions compared to Cit plants ($p < 0.05$; Table 1).

In general, single- and combined-trait plant lines increased the pH of exudate solutions by 0.3 to 0.7 pH units during the 14 d collection period (Table 1). Exceptions include a 0.3 pH unit decrease by wild-

type plants and the 0.1 pH unit decrease by the crossed $Co1_{CitxAn}$ plant line (Table 1). The greatest increase in solution pH occurred for single-trait plants with *A. niger phyA* expression ($Phy1_{An}$: +0.7 pH units), whereas pH increase in Cit exudate solutions (+0.4 pH units) was significantly greater than the vector (+0.3 pH units; Table 1). There was no significant difference between the vector, single-trait *P. lycii phyA* plants ($Phy_{Pl}$), or the crossed plant line with *P. lycii phA* expression ($Co2_{CitxPl}$; +0.3−0.5 pH units, Table 1).

## Shoot biomass and phosphorus accumulation

Shoot biomass ranged from 0.32 to 0.45 g dry weight and did not vary significantly across the majority of plant treatments (Table 2). In general, differences in shoot P concentration among plant lines contributed to larger effects on the total accumulation of P in shoots. However, an exception to this was the significantly large shoot dry weights of the vector (0.45 g dry wt.) and $Co2_{CitxPl}$ (0.43 g dry wt.) lines relative to the phytase-exuding $Phy1_{An}$ plant line (0.32 g dry wt.; $p < .05$, Table 2). Shoot P concentrations were below the threshold of P deficiency for all of the tobacco plant lines (<0.17 %; Reuter and Robinson 1997) with the smallest concentrations found in the Cit plant line (0.68 μg $mg^{-1}$; Table 2). Shoot P concentration was 17 % greater in plants expressing the *A. niger phyA* ($Phy1_{An}$: 0.89 μg $mg^{-1}$) relative to those containing *phyA* from *P. lycii* ($Phy2_{Pl}$: 0.76 μg $mg^{-1}$; Table 2). For the remaining plant lines, shoot P concentrations were similar among wild-type, vector, $Phy1_{An}$, $Co1_{CitxAn}$, and plants within the intercropped combinations (Table 2).

Plant treatments containing both citrate and phytase exudation traits significantly increased shoot P concentration relative to Cit plants grown in monoculture ($p < 0.05$). Shoot P concentrations in the crossed plant lines were 21 % ($Co2_{CitxPl}$: 0.82 μg $mg^{-1}$) to 34 % ($Co1_{CitxAn}$: 0.91 μg $mg^{-1}$) greater than in Cit monocultures (0.68 μg $mg^{-1}$). Shoot P concentrations were also significantly greater in Cit plants intercropped with $Phy1_{An}$ (Cit: 0.71 μg $mg^{-1}$) and $Phy2_{Pl}$ (Cit: 0.90 μg $mg^{-1}$) relative to Cit plants in monoculture (Table 2). The combination of citrate and phytase exudation in crossed and intercropped plant treatments did not significantly increase the concentration of P relative to monocultures of the phytase exuding $Phy1_{An}$ and $Phy2_{Pl}$ or the wild-type and Vec plant lines (Table 2).

Shoot P accumulation ranged from 238 μg P in Cit to 376 μg P in $Co1_{CitxAn}$ shoots (Table 2) and followed similar trends as described for the differences in shoot P concentration among plant lines. There was no significant difference in shoot P accumulation among monocultures of single trait Cit (238 μg P), $Phy1_{An}$ (286 μg P), and $Phy2_{Pl}$ plant lines (291 μg P; Table 2). Shoot P accumulation in wild-type and Vec plant lines was similar to that of the crossed an intercropped plant lines (Table 2). However, relative to the single-trait Cit monoculture, shoot P accumulation was significantly greater in both of the crossed plant lines ($Co1_{CitxAn}$: 376 μg P;

**Table 2** Biomass, P concentration, and P accumulation in shoots of tobacco plant lines and intercropped plant combinations

| Plant line or combination | Shoot dry wt. g | | Shoot P μg $mg^{-1}$ | | Shoot P μg | |
|---|---|---|---|---|---|---|
| WT | 0.39 | ab | 0.88 | ab | 343 | ab |
| Cit | 0.35 | ab | 0.68 | c | 238 | c |
| Vec | 0.45 | a | 0.80 | b | 356 | ab |
| $Phy1_{An}$ | 0.32 | b | 0.89 | ab | 286 | bc |
| $Phy2_{Pl}$ | 0.38 | ab | 0.76 | bc | 291 | bc |
| $Co1_{CitxAn}$ | 0.41 | ab | 0.91 | ab | 376 | a** |
| $Co2_{CitxPl}$ | 0.43 | a | 0.82 | b | 355 | ab** |
| $Int1_{Cit+An}$ | 0.39 (0.19/0.20) | ab | 0.83 (0.95/0.71) | a/bc | 323 (184/138) | ab* |
| $Int2_{Cit+Pl}$ | 0.38 (0.19/0.19) | ab | 0.87 (0.83/0.90) | b/ab | 325 (155/168) | ab* |

Values with different letters indicate significantly different means across plant treatments ($p < 0.05$; $n = 5$). Wild-type (WT), vector (Vec1, Vec2), single-trait citrate (Cit) and phytase ($Phy1_{An}$, $Phy2_{Pl}$), and crossed plant lines ($Co1_{CitxAn}$, $Co2_{CitxPl}$): Sum of shoot dry weight and average shoot P concentration of 2 plants per pot. Intercropped plants ($Int1_{Cit+An}$, $Int2_{Cit+Pl}$ ): Shoot dry weight and P concentration of individual plants separated by "/" in parenthesis. Shoot P accumulation based on total biomass and average shoot P concentration in monocultures or shoot biomass and P concentration of individual plants in intercropped combinations. *significantly greater shoot P accumulation by plants in intercropped combinations or ** crossed plant lines relative to monoculture single-trait plant lines

$Co2_{CitxPl}$: 355 μg P; $p < 0.05$) and the intercropped treatments ($Int1_{Cit+An}$: 323 μg P; $Int2_{Cit+Pl}$: 325 μg P; Table 2). Shoot P accumulation was greatest in phytase-exuding plants of the $Int1_{Cit+An}$ combination ($Phy1_{An}$: 184 μg P; Cit: 138 μg P), whereas Cit plants accumulated relatively more shoot P when intercropped with plants expressing the *P. lycii phyA* trait ($Phy2_{Pl}$: 155 μg P; Cit: 168 μg P; Table 2).

## Complementarity and partitioning of phosphorus between shoots of intercropped citrate- and phytase-exuding tobacco plant lines

Intercropping of citrate-exuding Cit plants with plants expressing the *A. niger phyA* ($Phy1_{An}$) and *P. lycii phyA* ($Phy2_{Pl}$) traits resulted in significant positive complementarity on the basis of shoot P accumulation (Fig. 1a). The $Int1_{Cit+An}$ and $Int2_{Cit+Pl}$ intercropped treatments resulted in land equivalent ratios of 1.23 and 1.24, respectively (Figure 1a). Based on theoretical estimates of the crossed plant lines, land equivalent ratio of $Co1_{CitxAn}$ treatments showed positive complementarity, which was significantly greater than both intercropped treatments (LER: 1.7; Fig. 1a).

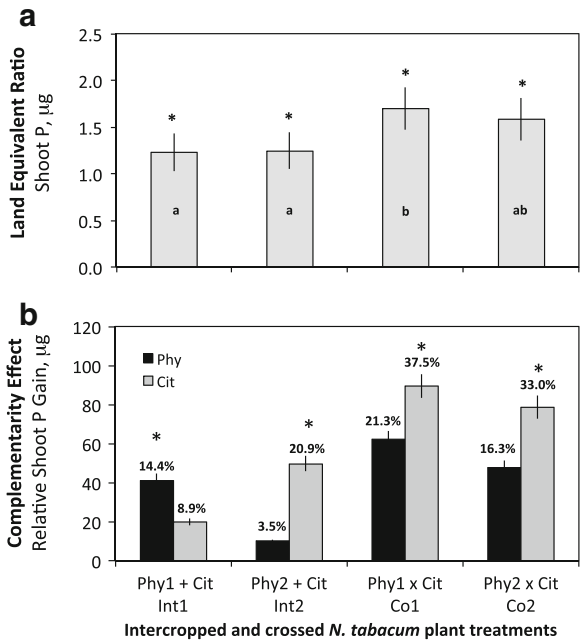


**Fig. 1** **a** Land equivalent ratios (LER) and **b** complementarity effect (CE) based on shoot phosphorus (P) accumulation in intercropped (Int1, Int2) and crossed (Co1, Co2) phytase and citrate exuding tobacco plants. Theoretical estimates for crossed plant lines are based on 0.5 shoot P accumulation of Co1 or Co2 plants relative to Cit1 and $Phy1_{An}$ or $Phy2_{Pl}$ monocultures. **a** *LER values significantly greater than one; Significant difference in LER between treatments indicated by different letters; **b** Percentages represent the increase in shoot P of intercropped or crossed plants relative to Cit and Phy monocultures; *Significantly greater relative gain in shoot P between Phy and Cit comparisons

Gains in shoot P by individual plants in intercropped treatments were calculated relative to shoot P accumulation in monocultures (Eqs. [2], [3]) and represent the preferential uptake and partitioning of P between plants in a given combination (Zhang et al. 2014). Shoot P accumulation was 8.9 to 20.9 % greater in the individual plants of intercropped combinations relative to their respective monoculture shoot P contents (Fig. 1b). Relative to monocultures, plants expressing the *A. niger phyA* trait in the $Int1_{Cit+An}$ intercropped treatment gained significantly more shoot P ($Phy1_{An}$: +14.4 %; $p < 0.05$) than citrate-exuding plants (Cit: 8.9 %; Fig. 1b). In contrast, citrate-exuding plants accumulated significantly more P (Cit: +20.9 %; $p < 0.05$) in the $Int2_{Cit+Pl}$ intercropped treatment relative to plants expressing the *P. lycii phyA* trait ($Phy2_{Pl}$: 3.5 %; Fig. 1b). Gains in shoot P of $Co1_{CitxAn}$ and $Co2_{CitxPl}$ treatments were greater relative to monocultures of $Phy1_{An}$ (+37.5 %) and $Phy2_{Pl}$ (+33.0 %) respectively, as well as plants in both intercropped treatments (Fig. 1b). Furthermore, gains in shoot P by $Co1_{CitxAn}$ and $Co2_{CitxPl}$ plants relative to Phy and Cit monocultures more closely mimic the partitioning of P in the intercropped treatment with $Phy2_{Pl}$ plants in which shoot P gains are greatest relative to phytase-exuding plant monocultures (Fig. 1b).

## Plant-induced changes to pH and citrate-extractable phosphorus fractions in the Glensaugh soil

Soils cultivated with tobacco plant lines and intercropped combinations had significantly higher pH (4.53–4.72) than the uncultivated soil (4.43), which was maintained under the same watering and nutrient regime as the cultivated soils in glass-house conditions (Table 3). Wild-type (4.68) and the crossed plant lines ($Co1_{CitxAn}$: 4.69; $Co2_{CitxPl}$: 4.72) had significantly higher pH relative to the uncultivated soil and rhizosphere soils from vector plants (4.56), phytase-exuding plants (4.53–4.57), and the $Int1_{Cit+An}$ intercropped plant treatment (Table 3, Fig. 2a). The pH change induced by Cit (+0.20 pH units) was not significantly different from any of the other plant treatments (Table 3, Fig. 2a).

**Table 3** pH and citrate extractable P concentrations in the plant free control soil and rhizosphere soils of citrate- and phytase-exuding tobacco plants and their controls

| | | | Citrate extractable P (mg P $kg^{-1}$ dry soil) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Plant treatment | pH | | $CEP_{TOT}$ | | $CEP_i$ | | $CEP_o$ | | $CEP_{phy}$ | |
| No plant | 4.43 | c | 105.8 | a | 29.1 | a | 76.8 (73) | a | 15.6 (20) | ab |
| WT | 4.68 | a | 38.5 | d | 12.5 | bc | 26 (67) | c | 13.4 (50) | ab |
| Cit | 4.63 | ab | 37.5 | d | 11.6 | c | 25.9 (69) | c | 6.2 (26) | bc |
| Vec | 4.56 | b | 44.5 | cd | 13.4 | bc | 31.2 (70) | c | 19.4 (61) | a |
| $Phy1_{An}$ | 4.53 | b | 57.2 | cd | 12.7 | bc | 44.5 (76) | bc | 1.2 (3) | c* |
| $Phy2_{Pl}$ | 4.57 | b | 82.4 | b | 15.0 | b | 67.5 (81) | ab* | 6 (9) | bc* |
| $Co1_{CitxAn}$ | 4.69 | a | 64.7 | bc | 11.6 | bc | 53.1 (81) | b* | 6.5 (14) | bc |
| $Co2_{CitxPl}$ | 4.72 | a | 58.6 | cd | 14.1 | bc | 44.6 (75) | bc | 5.2 (14) | bc |
| $Int1_{Cit+An}$ | 4.57 | b | 42.1 | d | 11.8 | c | 30.3 (71) | c | 0.3 (1) | c* |
| $Int2_{Cit+Pl}$ | 4.60 | ab | 40.5 | d | 11.8 | c | 28.7 (71) | c | 2 (7) | c* |

Citrate extractable P fractions: Total ($CEP_{TOT}$), inorganic ($CEP_i$), organic ($CEP_o$), phytase-labile ($CEP_{phy}$). Different letters indicate significantly different means across plant treatments (Tukey HSD, $p < .05$). Percentage $CEP_o$ relative to $CEP_{TOT}$ and %$CEP_{phy}$ relative to $CEP_o$ indicated in parentheses

*Significant increase in the proportion of $CEP_o$ or decrease in the proportion of $CEP_{phy}$ relative to the uncultivated soil ($p < 0.05$)

The uncultivated (no plant) soil contained significantly greater concentrations of $CEP_{TOT}$ (105.8 mg $kg^{-1}$), $CEP_i$ (29.1 mg $kg^{-1}$), and $CEP_o$ (76.8 mg $kg^{-1}$) compared to soils cultivated with the various tobacco plant treatments (Table 3). Plant depletion of $CEP_{TOT}$ was similar across treatments (−57.1 to –68.2 mg P $kg^{-1}$) with the exception of the single-trait $Phy1_{An}$ and $Phy1_{Pl}$ plant lines,

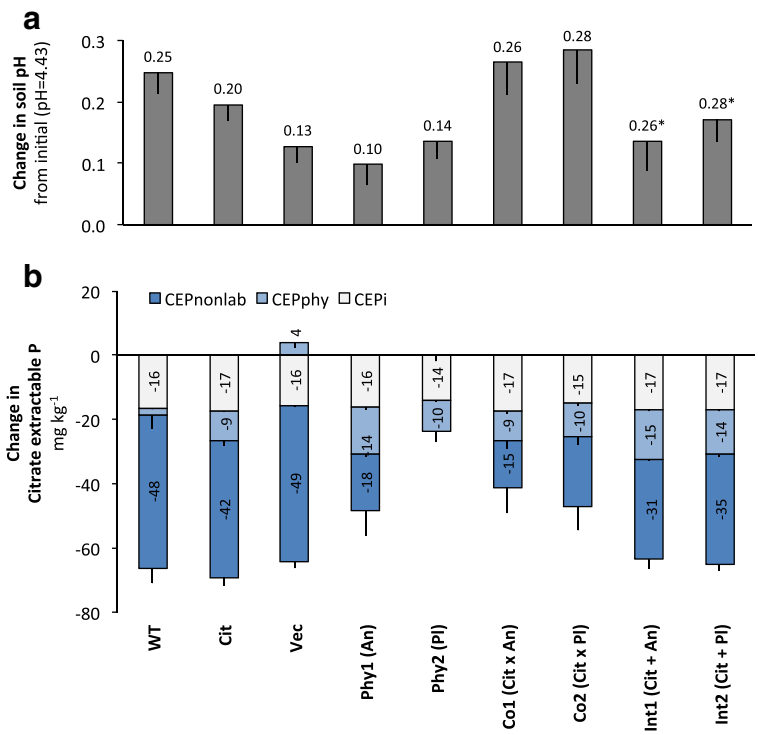


**Fig. 2** Change in **a** pH and **b** citrate-extractable phosphorus (CEP) fractions in cultivated soils relative to uncultivated controls. Non-labile P ($CEP_{nonlab}$), phytase-labile P ($CEP_{phy}$), inorganic P ($CEP_i$) with the sum of all fractions equivalent to the change in total CEP and $CEP_{phy}$ + $CEP_{nonlab}$ equivalent to the total change in organic CEP; Error bars indicate the standard error of the mean; *Significant difference relative to single-trait (Cit, $Phy1_{An}$, $Phy2_{Pl}$) and intercropped plant treatments ($Int1_{Cit+An}$, $Int2_{Cit+Pl}$)

which depleted $CEP_{TOT}$ concentrations by −48.5 and −23.3 g $kg^{-1}$, respectively (Table 3, Fig. 2b, Online Resource 1). Cultivated soils contained similar concentrations (25.9 – 44.6 mg P $kg^{-1}$) and proportions (67−76 %) of $CEP_o$ with the exception of $Phy2_{PI}$ (67.5 mg $kg^{-1}$) and $Co1_{CitxAn}$ (53.1 mg $kg^{-1}$; Table 3), which also contained the greatest proportions of $CEP_o$ among plant treatments (81 %; Table 3). Plant cultivation led to a greater than 2-fold reduction in $CEP_i$ concentrations, which ranged from −14 to −17 mg P $kg^{-1}$ and was most pronounced in Cit, $Co1_{CitxAn}$, and intercropped plant treatments (Fig. 2b).

Although the overall depletion of $CEP_o$ was similar across the majority of plant treatments (Fig. 2b), the relative contribution of $CEP_{phy}$ was much more variable (0.3 to 21.4 mg $kg^{-1}$; Table 3). Twenty percent of $CEP_o$ in the uncultivated soil was phytase-labile (15.6 mg $kg^{-1}$; Table 3). In cultivated soils, the proportion of phytase-labile P in citrate extracts ranged from 1 to 61 % of $CEP_o$ (Table 3). Changes in the concentration of $CEP_{phy}$ from the uncultivated soil ranged from +3.7 (Vec) to −15.3 mg $kg^{-1}$ ($Int1_{Cit+An}$); however, depletion of $CEP_{phy}$ was only significant in the $Phy1_{An}$ (−14.4 mg $kg^{-1}$) and intercropped plant treatments (−15.3, −13.6 mg $kg^{-1}$; Table 3, Fig. 2b, Online Resource 1). In contrast to plant treatments containing citrate and phytase traits, plant lines lacking the phytase trait did not significantly deplete $CEP_{phy}$, but rather the non-labile component of organic P in citrate extracts (e.g., wild-type, −47.5 mg $kg^{-1}$; Cit, −42.4 mg $kg^{-1}$; Vec, −48.6 mg $kg^{-1}$; Table 3, Fig. 2b, Online Resource 1).

## Relationships between shoot phosphorus accumulation, exudation traits, and soil phosphorus depletion among plant treatments

When all plant lines were considered, citrate efflux was negatively related to the proportion of organic P in citrate extracts ($CEP_o$ %Total: $r = -0.23$, $p = 0.014$) and the concentration of $CEP_{phy}$ ($r = -0.31$, $p = 0.044$; Fig. 3, Online Resource 2). However, among the single-trait, crossed, and intercropped plant treatments (i.e., 'combinations only', Fig. 3), citrate efflux was a better predictor of total ($r = -0.380$, $p = 0.024$), organic ($r = -0.403$, $p = 0.017$), and non-labile P ($r = -0.430$, $p = 0.01$) in citrate extracts (Fig. 3, Online Resource 3). Exudate phytase activity was positively related to $CEP_{TOT}$ ($r = 0.362$, $p = 0017$), $CEP_o$ concentration ($r = 0.365$, $p = 0.016$) and proportion ($r = 0.438$, $p < .0001$) and $CEP_{nonlab}$ ($r = 0.493$, $p = 0.0001$), whereas negative relationships were found with $CEP_{phy}$ concentration ($r = -0.466$, $p = 0.002$) and proportion ($r = -0.543$, $p < .0001$; Fig. 3, Online Resource 2). When wild-type and vector plants were removed from the analysis, phytase activity was positively related to the proportion of organic P in citrate extracts but nothing else ($r = 0.261$, $p < .0001$; Fig. 3, Online Resource 3). Therefore, when both populations are considered (all plant lines v. combinations only), phytase activity had a greater impact on the ability of plants to accumulate P across a wider range of exudation and physical growth characteristics (including wild-type and vector plants), but citrate efflux proved more critical in controlling the efficacy of phytase exudation among plants with single or combined citrate and phytase traits (Fig. 3).

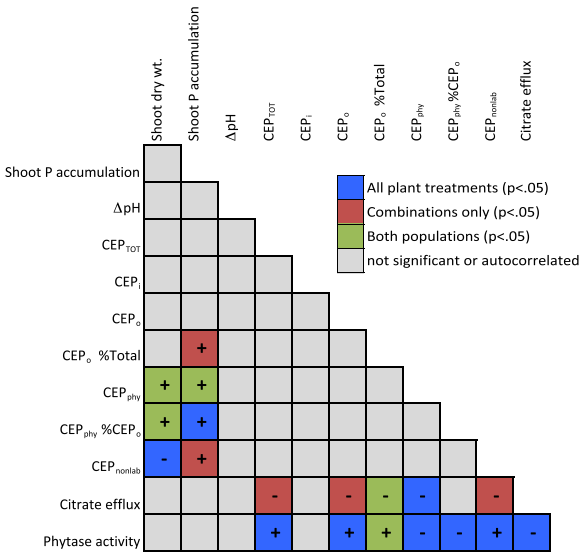


**Fig. 3** Representation of significant correlations found between plant biomass, exudation rates, and citrate extractable soil phosphorus (P) measurements when considered across all plant treatments, treatments containing only transgenic citrate and phytase exuding plants or plant combinations, or relationships which were significant in both populations

Step-wise linear regression analysis was used to predict shoot P accumulation based on citrate-extractable P concentrations and plant exudation traits. The resulting multiple linear regression incorporated three variables, change in the concentration of $CEP_{phy}$ relative to the uncultivated soil ($\Delta CEP_{phy}$), citrate efflux (CitEff), and exudate phytase activity (PhyAct), which explained 58 % of the variation across the plant treatments tested ($p < 0.0001$; Fig. 4): ShootP = 6.88*$\Delta CEP_{phy}$ − 3.83*CitEff − 0.59*PhyAct + 534.6 (eqn [4]).

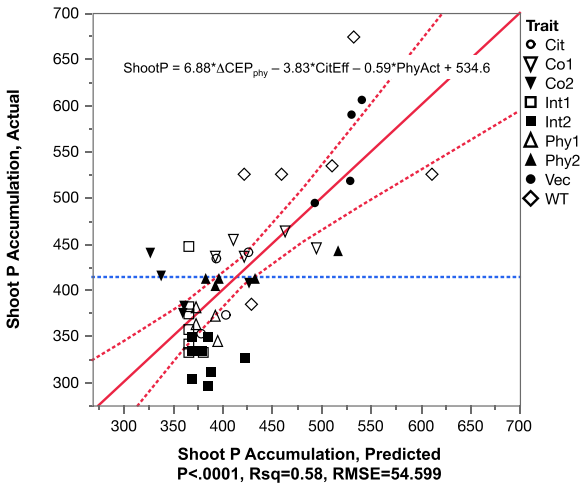


**Fig. 4** Multiple linear regression best fit (minimum Bayesian Information Criterion) for actual shoot phosphorus accumulation (μg P) and model values predicted based on depletion of phytase-labile citrate extractable P ($\Delta CEP_{phy}$ mg P $kg^{-1}$ dry soil), citrate efflux (CitEff, nmol $g^{-1}$ root dw $d^{-1}$), and exudate phytase activity (nKat $g^{-1}$ root d.w. $d^{-1}$) by tobacco plant lines and intercropped combinations with the following traits: Wild-type (WT), citrate efflux via *A. thaliana frd3* (Cit), vector control (Vec), phytase efflux via *A. niger* (Phy1) or *P. lycii phyA* (Phy2), crossed plant lines with co-exudation of citrate and phytase (Co1, Co2), intercropped Cit + Phy plant treatments (Int1, Int2)

## Discussion

### Facilitation in shoot phosphorus accumulation and soil phosphorus depletion among citrate and phytase exuding plants

We investigated the potential complementarity arising from the combination of citrate and phytase exudation traits in individual transgenic tobacco plant lines or intercropped combinations and found that complementarity existed between the two traits. It should be noted that relative to citrate, other carboxylates such as oxalate and ascorbate have previously been detected in larger concentrations in the wild-type and transgenic tobacco exudates, respectively (Giles et al. 2012). The focus on citrate efflux in this study was based on the relatively greater abundance of citrate in the transgenic versus wild-type plants (Giles et al. 2012), as well as its widely reported significance for P acquisition in other arable crops (Ryan et al. 2014). In crossed and intercropped treatments, shoot P accumulation was 26–37 % and 10–23 % greater compared to single-trait citrate and phytase-exuding plants, respectively (Table 2). The positive complementarity demonstrated between citrate- and phytase- exuding plant lines in the intercropping treatments (Fig. 1) and the relatively greater shoot P accumulation by crossed plant lines (Table 2) was associated with plant-induced changes to the concentrations of citrate extractable organic P pools in soil (Fig. 2). Shoot dry weight was negatively correlated with the percentage of organic P in citrate extracts ($r = -0.273$, $p = .048$) indicating a contribution of soil organic P to the growth and nutrition of the plant lines tested and their relative ability to access this pool.

Consistent with the conceptual model of Clarholm et al. (2015), phytase-labile P ($CEP_{phy}$) was significantly depleted in treatments containing both citrate and phytase exuding plants (Fig. 2). However, when all plant treatments were considered, shoot P accumulation was more closely related to the depletion of the non-labile component of citrate extracts, which was negatively correlated with shoot dry weight ($r = -0.353$, $p = .02$; Fig. 3, Online Resource 2). In contrast, shoot P accumulation was positively related to $CEP_{phy}$ concentrations ($r = 0.725$, $p < .0001$; Online Resource 2) due to wild-type, Cit, and vector plants, which contained the greatest shoot P and $CEP_{phy}$ concentrations (Table 2, Fig. 2). Therefore, plants without the phytase traits tended to preferentially deplete the non-labile component of $CEP_o$ and, in some cases, accumulate $CEP_{phy}$ when both citrate and phytase exudation were lacking (Vec, Fig. 2). Furthermore, the depletion of $CEP_{phy}$ in the soils of plants expressing both citrate and phytase traits, whether co-localized to a single plant root or via intercropping (Fig. 2), suggests that the presence of phytase may have counteracted any accumulation of $CEP_{phy}$. Although not tested, this could be associated with an immobilization of P by microbial activity in the non-phytase plant treatments (George et al. 2002; Walker et al. 2003). In previous studies, microbial community structure did not vary significantly with the presence of phytases produced by $Phy1_{An}$ and $Phy2_{Pl}$ plant lines relative to wild-type and vector plants (George et al. 2009). However, comparable studies of dual-trait rhizospheres are lacking and may provide valuable detail on the integrated effects of citrate and phytase exudation on microbial activity and P dynamics in soils.

## Predicting shoot phosphorus accumulation based on exudation and soil phosphorus depletion

The relationships between shoot P accumulation and the model variables indicate that when all treatments are considered, plants with less citrate efflux and phytase activity, and greater accumulation of phytase-labile P in this soil accumulate a greater amount of P into shoots (Fig. 4). Though contrary to our original hypothesis, that the combination of citrate and phytase will *improve* shoot P accumulation in plants, this model highlights two important caveats to the presented dataset and the perceived wisdom. First, 42 % of the variation in shoot P accumulation could not be explained by the variables tested in this study (e.g., $\Delta CEP_{phy}$, citrate efflux, phytase activity; Fig. 4) and therefore the additional drivers of shoot P accumulation in this population could not be accounted for. Second, wild-type and vector plant lines, which were more efficient at accumulating P than any of the transgenic plants (despite lower exudation rates; Table 1, Table 2), may not have been the appropriate 'negative' controls in this study as they appeared to achieve greater P accumulation by employing some other mechanism(s) of P acquisition. These points are discussed further in the text below.

Additional variables that could account for the missing variance of the model most likely include root traits, the production of other carboxylates such as oxalate by the wild-type plants (Giles et al. 2012), inherent differences in carbon metabolism among the plant lines (Walker et al. 2003), and differences in microbial community structure and function in the different plant rhizospheres. For example, George et al. (2009) assessed the effects of phytase expression in $Phy1_{An}$ and $Phy2_{Pl}$ plant lines on the composition of soil bacteria and arbuscular mycorrhizal (AM) fungi populations in rhizosphere soils and tobacco roots using T-RFLP analysis. Differences in AM fungal diversity, but not bacterial community structure, were seen to differentiate the wild-type and vector plants from those with phytase trait expression (George et al. 2009). To date, no studies have quantified AMF colonization by root staining methods or by assessment of AMF abundance in the Cit and crossed plant lines. This information would improve our understanding of the additional mechanisms that control P acquisition in these plant lines and provide valuable insight into the effect of single exudate compounds on AMF colonization, microbial recruitment, and other plant-microbe interactions which affect plant nutrient status.

Differences in root biomass and elongation and the ability of plants to interact with a larger soil volume can influence the acquisition of soil P by plants (Shen et al. 2013). Although root biomass was not measured directly in this study, differences in root length and structure (e.g., lateral root branching) of wild-type, Cit, and $Phy2_{Pl}$ plants grown in agar media have been observed previously (Giles et al. 2012). Furthermore, when grown in a larger volume of the Glensaugh soil (200 g dry soil) for a shorter growth period (8 weeks), root to shoot biomass ratios differed among the wild-type (0.29), Vec (0.26), Cit (0.35), Phy1/2 (0.26), and Co1/2 crossed (0.24) plant lines (C. D. Giles, unpublished data). Notably, the relatively larger root:shoot ratios of Cit plants indicates that larger root biomasses in the intercropped combinations or Cit plants therein may have improved soil exploration and therefore the distribution and effect of citrate and phytase exudation on P acquisition. Based on differences in the growth conditions of these experiments, root to shoot ratios are not necessarily transferable, but support the argument that other factors (i.e., root biomass, non-citrate carboxylates) likely influenced shoot P accumulation, the overall predictive power of the model, and should therefore form the basis of further study.

The significant variation described by the model (58 %) remains compelling considering its basis in just two exudates and soil $CEP_{phy}$ depletion. We therefore consider this result to indicate the importance of citrate and phytase exudation in P availability to tobacco in this experiment, with other factors (root traits, other carboxylates, microbial community structure/function) collectively accounting for a relatively smaller proportion of the overall variation in the model. However, with regards to other arable crops, the results of this study may be limited. Because the study was focused on the depletion of endogenous or natively occurring organic soil P, the lack of additional P treatments was intended to force the plants into P deficiency and maximize plant utilization of organic P. This was reflected in the P deficiency status of all plants (<0.17 %), which in arable systems would likely result in yield loss. Although we provide evidence of improved endogenous soil P utilization through the interaction of citrate and phytases, it is possible that these traits will only provide a marginal benefit in cases of extreme P limitation when plants have no other option than to 'mine' existing soil P pools. Future studies should establish system- or soil-specific thresholds

of P limitation, beyond which, the combination of citrate and phytase exudation will provide no further benefit to plant acquisition of P.

A second, albeit important, issue is the possibility that wild-type and vector plant lines may not be the appropriate 'negative' controls for plants modified to enhance protein and carboxylate efflux. Wild-type and vector plants were more efficient in the accumulation of P than any of the transgenic plants (despite lower exudation rates; Table 1, Table 2) and therefore may have employed some alternative mechanism(s) of P acquisition as described above. Additionally, examples in maize, wheat, and barley indicate that the direct metabolic costs associated with the production of nutrient foraging traits (e.g., root elongation, exudation, aerenchyma formation) may limit biomass production (Lynch 2007; Lynch and Ho 2005). In the tobacco lines tested, these costs are associated with the constitutive production of the *frd3* citrate transporter and the fungal phytases by *N. tabacum* are not known, which may explain the relatively smaller shoot biomasses, and ultimately shoot P accumulation, measured in this study. These data and observations from the literature open up the debate as to what controls would be the appropriate for transgenic plants expressing P acquisition efficiency traits. We acknowledge that only one plant line was tested for each trait of interest, meaning that unintended effects associated with the location of gene insertion or, in the case of the heterologous crossed lines, of non-inserted alleles cannot be ruled out as factors affecting citrate and phytase trait expression. Nevertheless, these modifications resulted in the desired phenotypic variation across the plant treatments in terms of exudate qualities, and served as useful tools to investigate the down-stream impacts of the exudates and their interaction in plants with otherwise similar physiologies. In light of the discrepancies between the model prediction and the aforementioned undefined biological factors of the study, we cannot conclude that citrate and phytase exudation or the associated depletion of phytase-labile P pools improves the accumulation of shoot P in tobacco. However, the positive complementarity and depletion of organic P by plants containing either one or both of these traits is indisputable and therefore warrants further investigation of the additional factors that limit or control the interaction of these traits in the rhizosphere of tobacco and other arable crops.

### Partitioning of shoot P between intercropped citrate and phytase-exuding plants

Differences were observed in the partitioning of P between citrate and phytase plants depending on the source of phytase (i.e., *A. niger* v. *P. lycii*; Fig. 1b) and whether the traits were introduced via separate plants ($Int1_{Cit+An}$, $Int2_{Cit+Pl}$) or the same plant ($Co1_{CitxAn}$, $Co2_{CitxPl}$; Fig. 2b). Plant-induced changes to the concentration and composition of citrate extractable P as well as rhizosphere pH were similar between the intercropped and co-expressed combinations and are therefore unlikely to explain the patterns of shoot P partitioning observed (Fig. 2).

In previous intercropping studies, the partitioning of P between plants was found to be unidirectional and favor the most P efficient plant in the combination. For example, in chickpea/wheat combinations, chickpea mobilizes P via carboxylate exudation, whereas wheat, which is more competitive for P uptake, benefits through gains in biomass and P acquisition (Zhang and Li 2003). Mobilization of soil P by one plant and accumulation by the other is also reflected in the depletion of P in the rhizosphere of the 'mobilizing' plant (e.g., chickpea) and can specifically affect $P_o$ when both carboxylate and phosphatase exudation are present (e.g., white lupin, Dissanayaka et al. 2015; chickpea, Li et al. 2003). In the current study, the relative efficiency of P acquisition by various plant lines is assumed to be similar. Therefore, the direction of P partitioning will depend on factors directly affecting the availability of P in the rhizosphere, such as phytase exudation or citrate efflux. Plant-induced changes to the concentration and composition of citrate extractable P as well as rhizosphere pH were similar between the intercropped and co-expressed combinations and are therefore unlikely to explain the patterns of shoot P partitioning observed (Fig. 2). Identical plants effluxing citrate were used in the experiment, therefore differences in phytase activity or the biochemical characteristics of the two phytases are more likely to explain the partitioning of P to plants with immediate access to mineralized forms of P. For example, the greater phytase activity in exudates of *A. niger* phytase plants (Table 1) could explain the relatively larger partitioning of P to the phytase-exuding plant in intercropped treatments containing $Phy1_{An}$ plants (Fig. 1b). Although the mean theoretical phytase activity of $Int1_{Cit+Phy}$ was predicted to be greatest among the intercropped treatments, there was

no significant difference in shoot P accumulation between the two intercropped combinations, indicating a potentially localized effect of this trait on P availability and assimilation.

The relatively limited gains in shoot P accumulation by the *P. lycii* phytase-exuding plant line in the intercropped ($Int2_{Cit+Pl}$) treatment may be related to differences in biochemical properties and the behavior of the *A. niger* and *P. lycii* enzymes in soil. Ullah and Sethumadhavan (2003) reported optimal phytase activities for the *A. niger* and *P. lycii phyA* to occur at pH 5.0 and 5.5, respectively. More recently, Menezes-Blackburn et al. (2015) reported greater than 80 % of the maximum phytase activity of both enzymes to occur in the range of pH 4.5 to 5.0, suggesting that both *A. niger* and *P. lycii* phytases were expressed under pH conditions (pH 4.5–4.7) near optimal for their function. The contrasting protein structure of the *A. niger* and *P. lycii* phytases result in differing isoelectric points and therefore adsorption to the soil solid phase (Lassen et al. 2001; Vats and Banerjee 2004). Whether calculated (p*I* = 4.37) or determined by isoelectric focusing (p*I* = 3.61), the p*I* of the *P. lycii* phytase is 0.57 to 1.4 pH units lower than the *A. niger* phytase (p*I* ~ 5; George et al., 2007a, b). At the pH of intercropped soils in this study (4.5-4.6), *P. lycii phyA* is expected to be weakly associated or free of the soil surface, whereas *A. niger phyA* is predicted to remain bound. In a soil incubation study, George et al. (2007b) demonstrated that a larger proportion of *P. lycii* phytase activity was found in the solution phase relative to *A. niger* phytase, which was primarily bound to the soil. Furthermore, when immobilized on the solid phase, *A. niger phyA* remained active and was more resistant to microbial degradation than the *P. lycii* phytase, although both enzymes had greatly reduced activity by the end of 8 days incubation in soil (George et al., 2007a, b).

The hypothesis concerning phytase mobility is further supported by the contrasting patterns of $CEP_o$ depletion and phytase exudation by the individual phytase plant lines (Fig. 2, Table 1). For example, $Phy2_{Pl}$ had a limited ability to deplete $CEP_{phy}$ (−10 mg $kg^{-1}$) relative to $Phy1_{An}$ (−14 mg $kg^{-1}$), which, unlike $Phy2_{Pl}$, could also access significant proportions of the non-labile organic P pool (Figure 2b) and displayed greater rates of phytase exudation (Table 1). Assuming a greater mobility of *P. lycii phyA* at the pH of the Glensaugh soil, and considering the lower exudation rates in $Phy2_{Pl}$ plants, any phytase introduced into the rhizosphere would have displayed less activity per soil volume and therefore a relatively limited ability to mineralize and deplete soil organic P. We acknowledge that in the crossed plant treatments ($Co1_{CitxAn}$, $Co2_{CitxPl}$), where issues of proximity are overcome by the co-exudation of citrate and phytase from the same plant root, we do not observe the greatest depletion of CEP pools, despite these plants having the greatest accumulation of shoot P among the dual-trait treatments (e.g., Fig. 2). Citrate extraction targets easily exchangeable and organic-mineral bound forms of P, with a high preference for organic forms and therefore represents only one component of the total soil P (Bolan et al. 1994; Yan et al. 2014). The greater accumulation of shoot P by crossed plant lines despite the limited depletion of CEP pools therefore indicates that alternative sources of soil P were likely accessed by these plants. Collectively, these results indicate that the location of P mineralization and uptake will depend on the proximity of plant roots as well as the relative mobility of citrate and phytases in soil.

## Spatial dependency of citrate and phytase exudation in the acquisition of soil P

Access to soil P in intercropped plant treatments is a spatially explicit process, which depends on the co-localization of root exudates to the zone of P depletion around the plant root (Hauggaard-Nielsen and Jensen 2005). For example, root exclusion experiments have demonstrated the utilization of P, micronutrients, and added phytate by chickpea/wheat mixtures depends on the intermingling of roots (Li et al. 2003; 2004; 1999). The effect of root separation was not investigated in the current study; however, shoot P accumulation was 9 to 16 % greater in crossed plant lines compared to intercropped treatments ($p < .05$; Table 2).

In the current study, complementarity was similar between the two intercropped plant combinations, and greatest in the crossed plant lines where the citrate and phytase traits were co-expressed from the same plant root (Fig. 1). In the crossed plant lines, citrate and phytase exudation occurs from the same root, optimizing their efficiency in operating together to mobilise and hydrolyse phytate, whereas physical intermingling of roots or the ability of exudates to move to each other is required for the interaction of citrate and phytase to occur in intercropped treatments. Considering the relatively lower exudation of citrate and phytase by the

crossed plant lines relative to the individual plants in the intercropped treatments (Table 1), the effect of co-localized exudation of citrate and phytase on P acquisition is likely to be considerably greater than our results indicate. This suggests that the efficiency of exudates in plant systems that require root intermingling to observe complementary effects may be intrinsically less efficient than exudates produced from the same root.

The differential partitioning of P between plants in the intercropping treatments may also be explained by the proximity of exudate release as well as the relative mobilities of the various exudates in soil. For example, a relatively less mobile *A. niger phyA* is expected to be adsorbed to soil in close proximity to the roots of the exuding plant and the mineralization of phytate is maximized only when citrate from the neighboring plant reaches the adsorbed phytase. Any P mineralized is therefore more likely to be acquired by the phytase-exuding plant. In contrast, if the plant exudes a more mobile form of phytase (e.g., *P. lycii phyA*), and if the rate of diffusion in soil is greater than that of citrate, then the optimal conditions for phytate mineralisation and phosphate acquisition will occur in the vicinity of the citrate exuding plant. Future studies should consider the spatial dependency of these interactions as well as the sensitivity of facilitation and shoot P partitioning to pH changes in the rhizosphere.

## Conclusions

We demonstrate for the first time that significant complementarity in the acquisition of P from organic sources in soils can be gained by combining citrate efflux with phytase exudation in the same plant system. Individual tobacco plants or intercropped combinations containing citrate- and phytase-exudation accumulated more P and depleted a larger proportion of phytase-labile P in soils compared to plants with single-traits (citrate or phytase only) or lacking citrate and phytase exudation (wild-type, vector control plants). Shoot P accumulation was related to citrate efflux across all plant lines, and was controlled by phytase activity among the transgenic citrate- and phytase-exuding plants. Sixty-three percent of the variation in shoot P accumulation across plant treatments could be predicted based on the concentration of $CEP_{phy}$ and citrate efflux by plants, indicating that additional unexplained factors (e.g., plant metabolism and resource partitioning, plant-microbe interactions) were controlling P accumulation by these plants.

The interaction between exudation traits and their effect on P accumulation is spatially dependent, with co-localized exudation of citrate and phytase by crossed plant lines being more effective than the intercropping of two plants with contrasting single traits. Differences in the partitioning of P between phytase and citrate exuding plants in intercropped treatments show that combinations containing the *A. niger* phytase trait accumulated more P into shoots of the phytase-exuding plant relative to the citrate-exuding plant line, whereas greater gains in shoot P were observed in the citrate-exuding plant line of combinations containing the *P. lycii phyA*. We conclude that differences in shoot P partitioning among citrate and phytase exuding plant lines could be related to the relative mobility of *A. niger* and *P. lycii phyA* in soils and the spatial distribution of citrate and phytase exudates relative to the individual plants. This research suggests that endogenous sources of soil P could supplement or replace external inputs of inorganic phosphate fertilizers, thereby improving the overall sustainability of cropping systems which bring together citrate efflux and phytase exudation traits.

**Acknowledgments** We would like to acknowledge David Lewis (CSIRO Agriculture, Canberra Australia) for developing the crossed lines of tobacco, Susan McIntyre and Fiona Sturgeon (James Hutton Institute, Aberdeen, UK) for their contribution to the analysis of soils, and Katharine Preedy (BioSS, James Hutton Institute, Dundee, UK) for statistical consultation. Funding for this research was provided through a BBSRC responsive mode grant (BBK0170471).

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