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Title: Assimilation of phytate-phosphorus by the extracellular phytase activity of tobacco (Nicotiana tabacum) is affected by the availability of soluble phytate.

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Abstract: Phytate, the major organic phosphorus in soil, is not readily available to plants as a source of phosphorus (P). It is either complexed with cations or adsorbed to various soil components. The present study was carried out to investigate the extracellular phytase activities of tobacco (Nicotiana tabacum variety GeXin No.1) and its ability to assimilate external phytate-P. Whereas phytase activities in roots, shoots and growth media of Pi-fed 14-day-old seedlings were only 1.3-4.9% of total acid phosphatase (APase) activities, P starvation triggered an increase in phytase secretion up to 914.9 mU mg-1 protein, equivalent to 18.2% of total APase activities. Much of the extracellular phytase activities were found to be root-associated than root-released. The plants were not able to utilize phytate adsorbed to sand, except when insoluble phytate salts were preformed with Mg2+ and Ca2+ ions for supplementation. Tobacco grew better in sand supplemented with Mg-phytate salts (31.9 mg dry weight plant-1; 0.68% w/w P concentration) than that with Ca-phytate salts (9.5 mg plant-1; 0.42%), presumably due to its higher solubility. We conclude that insolubility of soil

phytate is the major constrain for its assimilation. Improving solubility of soil phytate, for example, by enhancement of citrate secretion, may be a feasible approach to improve soil phytate assimilation.

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Revision according to the referees' comments:

Editor's comments

1. Authority of the species has been included in abstract and materials in the revised manuscript (p.2, line 6).

2. We verified the numbers carefully and amended the numbers on p.2, line 14 and p.15, line 7.

3. The abbreviation of liter (L) has been changed accordingly.

Referee 2

1. The focus of this study is to investigate the ability of tobacco plants to assimilate external IHP, and to study whether the lack of a secretory phytase and/or the solubility of IHP are the factors that constrain IHP-P assimilation. While the adsorption of IHP to goethite (Ognalaga, et al, 1994, Soil Sci. Soc. Am. J. 58, 332-337) and Al/Fe precipitates (Shang et al, 1992, Geoderma, 53, 1-14) have been characterized, no report on how IHP is sorbed to sand is available from the literature. Our data confirmed the sorption of IHP to sands but it is beyond our capacity to speculate how it was absorbed in this study.

2. Referee 2 raised an interesting question of why plants express phytase despite the "inaccessibility" of soil phytate in an evolutionary point of view. First of all, while our data indicated absorbed phytate is not readily available to plant, it does not necessarily mean that soil IHP-P is absolutely not utilizable. In fact, as cited by Referee 2 (Page 21, Lines 1-10), small amount of IHP can be detected in soil solution (Espinosa et al., 1999). This compound might be phytase-labile. In addition, the present study also demonstrated considerable plant growth on Ca-IHP and Mg-IHP salts, indicating that the precipitation reaction limits IHP solubility but does not completely make it unavailable to plant phytase.

Furthermore, secretory plant phytases induced during P deprivation are not necessarily monospecific towards phytate. They could exhibit activities towards a broad range of P-monoesters and diesters.

3. The words "for the existence" in Page 3 Line 20 have been deleted in the revised manuscript.

4. As suggested by the reviewer, the erroneous statement in Page 4 Line 9 "as the existence of IHP in soil solution has never been reported (Hayes et al., 2000b)" has been removed. A statement "While in soil leachate, 77% of the P compounds is orthophosphate, and IHP constitutes only 3.3% of the total P (Espinosa et al., 1999)." was included in the discussion section in Page 21 Line 6. This article directly detected various P species, including IHP, using anion exchange column and HPLC. Another study (Turner et al., 2002) cited by Referee 2 employed an indirect method of phosphatase hydrolysis to characterize soil organic P. We think that the study of Turner et al. (2002) is less conclusive because the fungal phytase employed in the study was not monospecific towards IHP. In addition, no explanation has been provided to account for the absence of phytase-specific P compounds in moist soil extracts. Hence, we decide not to cite this reference.

5. Persulfate digestion is commonly used for the determination of IHP content in a number of publications. Our calculation was based on an IHP standard curve, where various concentrations of IHP were processed by the same procedure. Therefore, even if the persulfate method does not recover IHP completely, the determination of IHP should be accurate.

Referee 3

1. The research gap of the present study has been stated in page 5 lines 11-15 of the revised manuscript.

2. The recipe of Murashige and Skoog (1962) medium suggested that approximately 1 mM P_i is optimal for growth of tobacco plants. Our preliminary experiments showed that ten-fold reduction of this P supply resulted in P_i deficiency symptoms of plants including reduced shoot biomass and lower shoot-to-root ratio. Thus, 0.1 mM P_i, in addition to no-P, was chosen as a mean to study phytase activities under P_i deficiency. The situation of sterile hydroponic culture appears to be different from the field conditions, where acid phosphatase-labile organic P compounds can

be a P source to support plant growth, even though the existence of P_i in soil solution is in micromolar range. In fact, Anderson (1980) has well documented the continual introduction of organic P compounds into soil.

3. The reference to Microcal Origin software has been provided in Page 10 Line 7 in the revised manuscript.

Title: Assimilation of phytate-phosphorus by the extracellular phytase activity of tobacco (*Nicotiana tabacum*) is affected by the availability of soluble phytate.

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Abstract

Phytate, the major organic phosphorus in soil, is not readily available to plants as a source of phosphorus (P). It is either complexed with cations or adsorbed to various soil components. The present study was carried out to investigate the extracellular phytase activities of tobacco (*Nicotiana tabacum* variety GeXin No.1) and its ability to assimilate external phytate-P. Whereas phytase activities in roots, shoots and growth media of P_i-fed 14-day-old seedlings were only 1.3-4.9% of total acid phosphatase (APase) activities, P starvation triggered an increase in phytase secretion up to 914.9 mU mg^{-1} protein, equivalent to 18.2% of total APase activities. Much of the extracellular phytase activities were found to be root-associated than root-released. The plants were not able to utilize phytate adsorbed to sand, except when insoluble phytate salts were preformed with Mg^{2+} and Ca^{2+} ions for supplementation. Tobacco grew better in sand supplemented with Mg-phytate salts (31.9 mg dry weight $plant^{-1}$; 0.68% w/w P concentration) than that with Ca-phytate salts (9.5 mg $plant^{-1}$; 0.42%), presumably due to its higher solubility. We conclude that insolubility of soil phytate is the major constrain for its assimilation. Improving solubility of soil phytate, for example, by enhancement of citrate secretion, may be a feasible approach to improve soil phytate assimilation.

Introduction

Phosphorus (P) is a macronutrient that constitutes vital molecules such as nucleic acids, phospholipids and sugar phosphates in all living organisms. Terrestrial plants generally meet their P requirement by the uptake of soil P in inorganic form (P_i; Marschner, 1995). However, majority of the soil P (50-80% of the total) exists as organic compounds (Turner et al., 2002), which are unavailable to plants unless mineralization takes place. Plants have adapted to mineralize organic P compounds, primarily by the secretion of acid phosphatases (APases), which are a broad classification of hydrolytic enzymes catalyzing the breakdown of P-monoesters with acid pH optima (Vincent et al, 1992). Despite the active APase secretion, particularly in response to P_i starvation (Duff et al., 1994), earlier report (Anderson, 1980) has indicated the relatively rare occurrence of APase-labile P species in soil, including nucleic acids (up to 2.4% of organic P) and phospholipids (less than 5% of organic P). By comparison, inositol hexakisphosphates (IP_6), a stable compound highly invulnerable to chemical and enzymatic degradation, is the predominant soil organic P that can constitute more than 50% of organic P and 25% of total P (Anderson, 1980).

In nature, IP_6 mainly exists in *myo*- form (phytate; IHP) as a product of a common sequestrating activity in plants that generate a metabolic pool of P storage in seeds and grains, whereas the origin of other stereoisomers (*scyllo*-, *neo*- and *chiro*- forms) in soil remains

controversial (Turner et al., 2002). Despite the abundance of IHP in soil, whether it contributes to plant P nutrition is still in question. Earlier studies demonstrated that IHP is of poor availability to a wide range of plants, including maize, wheat, legumes, grasses and pasture species, cultivated on sterile agar, sand or sand-vermiculate media (Findenegg and Nelemans, 1993; Hayes et al., 2000; Richardson et al., 2000). Adams and Pate (1992) is the only group that has demonstrated plant growth with IHP comparable to the P_i-fed controls. Nonetheless, one of the critical requirements for the assimilation of IHP-P by plants is considered to be its availability in soil (Adams and Pate, 1992). In fact, the dominance of IHP over other forms of organic compounds in soil takes into account their strengthened stabilities through adsorption and precipitation reactions (Turner et al., 2002).

Hydrolytic enzymes that catalyze the cleavage of phosphomonoester bonds in IHP are collectively known as phytases. In plant kingdom, the roles of phytases have not yet been well understood. Currently purified and characterized plant phytases are APases that possess high specific activities against IHP. According to the sequence homologies, they are either classified as histidine acid phosphatases (HAP) or purple acid phosphatases (PAP), primarily discovered in maize (Hegeman and Grabau, 2001) and soybean (Maugenest et al., 1997), respectively. The temporal expression of both phytases at the early stage of germination may be of relevance for the mobilization of stored IHP-P to nourish plant growth, in agreement with earlier reports that demonstrated increasing phytase activities in growing seedlings and germinating pollens (Goel

and Sharma, 1979; Lin and Dickinson, 1985; Mandal et al., 1974; Walker, 1974). Many plant phytases have also been described in roots (Asmar, 1997; Hayes et al., 1999; Hübel and Beck, 1996; Li et al., 1997a, b; Richardson et al., 2000). Whilst maize root phytase was confined to the endodermis (Hübel and Beck, 1996), P_i starvation elicited phytase expression to higher levels in roots of some other plant species (Hayes et al., 1999; Li et al., 1997a, b), suggesting that these enzymes may be pertinent to external P acquisition. Extracellular phytase activities have also been reported in several plant species (Asmar, 1997; Hayes et al., 1999; Li et al, 1997a; Richardson et al., 2000).

Although adsorption and precipitation reaction of IHP in soil environment have long been hypothesized to be the reasons that limited IHP-P assimilation by plants, solid evidence is still scarce. Thus, the present study aimed to investigate the ability of tobacco plants to assimilate adsorbed and precipitated IHP, and to study whether the lack of a secretory phytase and/or the insolubility of IHP are the limiting factors that constrain IHP-P assimilation by tobacco plants.

Materials and methods

Plant cultivation

Tobacco (*Nicotiana tabacum*) variety 'GeXin No1' was obtained from the Shanghai Institute of Plant Physiology and Ecology. Seeds were surface-sterilized in 20% (v/v) Clorox for 20 min, followed by several rinses with sterile, deionized water. For sand culture, sterilized seeds were germinated on P-deficient MS agar for 10 days. The seedlings (1 plant pot^{-1} in triplicates) were transplanted onto 250 g quartzsand or riversand (0.1-1.0 mm sieved size), which had been acid-washed and rinsed thoroughly with deionized water, oven-dried (110 ^{o}C; 72 h) and mixed with 60 mL of sterile nutrient solution: 1.5 m*M* KNO_3, 1.2 m*M* $Ca(NO_3)_2$, 0.4 m*M* NH_4NO_3, 0.5 m*M* $MgSO_4$, 0.3 m*M* K_2SO_4, 0.3 m*M* $(NH_4)_2SO_4$, 1.5 μ*M* $MnSO_4$, 1.5 μ*M* $ZnSO_4$, 1.5 μ*M* $(NH_4)_6Mo_7O_{24}$, 0.5 μ*M* $CuSO_4$, 0.5 μ*M* NaB_4O_7, 27 μ*M* Fe-EDTA(Na) and 5 m*M* MES, pH 6.0. The original water content of the sand was maintained by irrigation with P-deficient nutrient solution every other day. For sterile hydroponic culture, sterilized seeds were sown on 20 mL of liquid MS medium (about 50 seeds $plate^{-1}$) and maintained aseptically. IHP used in plant culture was purchased from Sigma, St. Louis, USA in the form of dodecasodium salt (cat no. P3168). All plants were cultivated in controlled environment cabinet with a day/night regime of 16/8 h, 22/16 ^{o}C, and photon flux density of 100 μmol m^{-2} s^{-1}.

Phytase and acid phosphatase assays

Plants were grown in sterile hydroponic culture with modified P_i concentrations (0, 0.1 or 1 m*M*) for 14 days. Phytase and APase activities were measured in (i) crude shoot, root extracts and growth media, (ii) intact roots (root-associated activities), and (iii) root-bath solutions (root-released activities), following the same procedure described previously (Asmar, 1997; Richardson et al., 2000) with minor modifications. Phytase and APase activities were measured in MES/Ca buffer (15 m*M* MES, 0.5 m*M* $CaCl_2$, pH 5.5) at room temperature using either 1 m*M* Na-IHP or 10 m*M* *p*-nitrophenyl phosphate (*p*NPP) as substrates, respectively. Phytase activity assays were conducted over 1 h, unless otherwise specified, and terminated by equal volume of 10% (w/v) TCA. The liberated P_i was quantified by the molybdenum-blue reaction (Murphy and Riley, 1962). APase activities were assayed over 30 min and terminated by equal volume of 0.25 M NaOH. P_i release was derived from the equimolar liberation of *p*-nitrophenol (*p*NP) product, quantified spectrophotometrically at 405 nm over standard solutions. One enzyme unit (1 U) is defined as the activity that releases 1 μmol of phosphate per min under the specified assay conditions. In experiment (i), shoot and root materials were ground in 15 m*M* MES, 1 m*M* PMSF, pH 5.5, and the crude extracts were centrifuged (20 min, 14000 rpm). The supernatant was dialyzed against MES/Ca buffer to minimize the intrinsic P_i background for assays. Soluble proteins in the growth media were concentrated by ultrafiltration using the centrifugal filter units (Millipore, Bedford, MA) prior to phytase and APase assays. The total soluble protein content was determined by Bradford assay (Bradford, 1976). Experiments (ii)

and (iii) were conducted aseptically in a laminar flow hood. Prior to assays, residual P_i on the root surface was eliminated by several rinses with MES/Ca buffer and the excess solution was removed by blotting with sterile tissue paper. In experiment (ii), the roots portions of the intact seedlings (50 plants plate^{-1}) were incubated in 20 mL of MES/Ca buffer that contained 1 m*M* Na-IHP for 2 h and the liberated P_i was quantified in the incubated solution. Corrections were made to account for P efflux/influx (Asmar, 1997; Richardson et al., 2000) by replacing Na-IHP with different P_i concentrations (0-2.4 mg P L^{-1}) in the incubated solution. In experiment (iii), root-released phytase was collected by incubating the root portions of the intact seedlings (50 plants plate^{-1}) in 20 mL of MES/Ca buffer for 4 h. The incubated solution was then filtered (0.45 μm) to remove the sloughed-off cells (Asmar, 1997) and concentrated by ultrafiltration using the centrifugal filter units (Millipore, Bedford, MA), prior to phytase assays.

Sorption experiments

IHP-sorption capacities of the quartzsand and riversand used for plantation were estimated by incubating 7.5 mL of 1.794 m*M* Na-IHP solution, with (pH 6 by HCl) or without (pH 11) pH adjustment, in 25 g sand samples (i.e. 100 mg P kg^{-1}) at room temperature. At t = 0, 5 min, 20 min, 1 h, 3 h, 8 h and 24 h, 50 μL of the soil solution was collected. The solid residue in the soil solution was removed by centrifugation (5 min, 6,000 g) and the solution P concentration was determined by persulfate digestion (US EPA, 1993).

Sand culture

Tobaccos were grown in sand culture for 20 days with (i) adsorbed IHP, (ii) supplemented IHP salts, or (iii) supplemented NaH_2PO_4 as the sole P source. At harvest, shoot and root materials were oven-dried at 80 ^oC for 72 h for dry weight (DW) determination. To measure shoot P concentration, the dried shoot samples were subsequently ashed at 500 ºC for 16 h and the residues dissolved in 0.1 *M* HCl (8.75 mg sample mL^{-1}). The P_i concentration of the acid solution was determined using the molybdenum-blue reaction (Murphy and Riley, 1962). For experiment (i), 250 g quartzsand or riversand was incubated with Na-IHP solution (100 mg P kg^{-1} sand) at room temperature for 24 h. The sand-IHP complex was extensively rinsed with distilled water until no IHP was detected in the water extract by persulfate digestion (US EPA, 1993). The samples were made homoionic by equilibration with 3 changes of 0.1 *M* $CaCl_2$ or $MgCl_2$ solutions, followed by extensive rinses with distilled water until no chloride was detected in the water extract as indicated by the absence of white precipitate upon addition of 1% (w/v) $AgNO_3$. The IHP-adsorbed sand samples were then air-dried and used for plantation. For experiment (ii), insoluble Ca- and Mg-IHP salts were prepared by incubating 10 m*M* Na-IHP solution with 50 m*M* $CaCl_2$ or $MgCl_2$ solution at room temperature, respectively. The precipitated salts were allowed to settle and the supernatant decanted off. The solid was then washed thrice with distilled water, resuspended in nutrient solution and added to the sand (100 mg P kg^{-1} sand) for plant growth. Experiment (iii) was carried out to estimate the plant growth

responses to different P_i dosage. NaH_2PO_4 was added from a 0.1 *M* stock solution to the nutrient solution, which was subsequently added to the sand to provide 0, 12.5, 25, 50, 100, 200 or 400 mg P kg^{-1} sand for plant growth. The DW and shoot P concentration of plants grown at each supplied P_i level were fitted to the Mitscherlich model (Ware et al., 1982) with the equation $y = a + be^{-cx}$, such that y = shoot DW or P concentration, x = supplied P_i level, and a, b, c = constants, where a represents the maximum yield, with the aid of analysis program Microcal Origin version 7.5 (Microcal Software Inc., Northampton, USA).

Assays with elevated cation levels

Plants were grown in sterile hydroponic culture with modified P_i concentrations (0, 0.1 or 1 m*M*) for 14 days. Crude root extracts were obtained by the same procedure described above and the intrinsic P_i was removed by dialysis against Ca^{2+}-free MES (15 m*M*, pH 5.5) buffer. Ca^{2+} and Mg^{2+} ions were added to the samples in the form of chloride solutions and the phytase activities were assayed as described above. The final divalent ion concentrations in the reaction mixtures were 0, 0.5, 1, 2, 4 and 8 m*M*.

Results

Phytase and acid phosphatase activities

Phytase and APase activities of 14-day-old tobacco seedlings grown under different P_i treatments (0, 0.1 or 1 m*M*) were assayed in the crude root and shoot extracts, as well as in the growth media. The APase activities in shoots (1,029-1,449 mU mg^{-1} protein) were generally higher than that in roots (503-618 mU mg^{-1} protein; Table 1). Comparable APase activity levels were observed in the crude root extracts across different P_i treatments, whereas a 10-fold reduction in P_i supply (i.e. 0.1 m*M*) significantly ($P<0.05$) increased the APase activities in shoots by 40%, although similar difference between no-P plants and P_i-fed plants (1 m*M*) was statistically insignificant ($P\geq0.05$; Table 1). APase was believed to be one of the predominant secretory proteins, by a record of 4,323-5,017 mU mg^{-1} protein in the growth media. Subtle (10-16%) but significant ($P<0.05$) increase in the activities was observed from the growth media of no-P and low-P plants (0.1 m*M*), compared to the P_i-fed controls (Table 1). Specific phytase activities of crude root and shoot extracts from P_i-fed plants were only 15.6 and 13.8 mU mg^{-1} protein (Table 1). Whilst the reduction of P_i supply only increased the specific phytase activities in roots by 47% (no-P plants) and 68% (low-P plants), the stimulatory response to P deprivation was more prominent in shoot, where specific phytase activities were boosted by 4.2-fold under low-P condition and 3.1-fold in no-P condition, concomitant with an increase in secretory phytase activities by 1.9-fold and 3.4-fold, respectively (Table 1). Across

different P_i treatment, the increase in phytase/APase ratio was generally proportional to that in specific phytase activities, resulting from the fact that changes in APase activities were relatively insubstantial (Table 1).

Root-associated and root-released phytase activities

Root-associated activities were assayed by the incubation of intact roots with IHP solution and subsequent quantification of P_i liberation, whereas *in vitro* assays for root-released activities were conducted after collection of secretory proteins in root-bath solution (Table 2). Confounded by the possible P_i efflux or uptake during the assay period, the presented root-associated activities have been subjected to corrections made by the estimation of P_i efflux/influx rate using a series of control plants incubated with different P_i concentrations over the assay period. While P efflux from P_i-fed plants was evident (data not shown), a near balance between P efflux and influx rates was observed in no-P and low-P plants, depending on the P_i concentration of the incubated solution (data not shown). Extracellular phytase activities of 14-day-old tobacco seedlings were primarily root-associated, ranging from 819 mU g^{-1} root DW in P_i-fed plants to 1,367 mU g^{-1} root DW in no-P plants (Table 2). By comparison, root-released activities were hardly detectable, even though secretory proteins have been collected over an extended period of 4 h and extensively concentrated for *in vitro* assays (Table 2).

IHP sorption capacities of sand media

Time course experiments demonstrated that the riversand and quartzsand used for plantation in the present study possessed contrasting IHP sorption capacities. Whereas no IHP was lost in the soil solution when incubated with quartzsand for up to 24 h, IHP concentration in the soil solution at the starting 100 mg P kg^{-1} level was immediately halved upon 10-min and 30-min incubation with riversand at pH 6 and 11, respectively (Figure 1). Near-equilibria of IHP adsorption at 84 and 65 mg P kg^{-1} riversand were reached within 3-h incubation at pH 6 and 11, respectively (Figure 1).

P assimilation in sand culture

IHP-P assimilation by tobacco plants was investigated by the cultivation of 10-day-old seedlings in sand culture with IHP supplied in various forms for 20 days. For comparison, plant growth response to different P_i dosage, ranging from 0 to 400 mg P kg^{-1} sand in the form of NaH_2PO_4, is depicted in Figure 2. Results from all growth parameters, i.e. shoot and root DW, and shoot P concentration, were generally consistent in response to different P_i dosage. Maximum yield of quartzsand-grown plants was achieved by supplying 100 mg P kg^{-1} sand, at which the shoot DW and P concentration were 55 mg $plant^{-1}$ and 0.79% (w/w), respectively (Figures 2A and 2C). Even though the shoot P concentration could be further boosted to 0.90% (w/w) by the supply of 200 mg P kg^{-1} sand, the yield of dry biomass was notably inhibited at this level, concomitant with a drastic decline in both DW and shoot P concentration of plants

grown with 400 mg P kg^{-1} sand (Figures 2A and 2C). Plant growth under more than 100 mg P kg^{-1} sand dosage of NaH_2PO_4 is considered to be inhibited by high Na^+ concentration, corroborated by 35% decrease in shoot DW when doubling the Na^+ concentration by the replacement of NaH_2PO_4 with Na_2HPO_4 at a level of 100 mg P kg^{-1} sand (data not shown). On the contrary, same P_i dosage (0-400 mg P kg^{-1} sand) for plant growth on riversand did not result in any obvious inhibition by high Na^+ concentration (Figures 2B and 2D). Maximum yield of shoot DW (69 mg $plant^{-1}$) and P concentration [0.68% (w/w)] were achieved at 200 and 400 mg P kg^{-1} sand, respectively (Figures 2B and 2D). Utilization of IHP-P by tobacco plants were studied by the provision of IHP in either adsorbed or supplemented forms. Adsorbed IHP was prepared by pre-incubation of the sand media with IHP solution, followed by extensive rinses, with or without subsequent Ca^{2+} and Mg^{2+} homo-ionization. In agreement with the results from sorption experiments that quartzsand possessed poor IHP-sorption capacity (Figure 1), DW and shoot P concentrations of the plants grown on quartzsand pre-incubated with IHP was indifferent from that of the no-P controls (Figures 3 and 4). Nonetheless, similar results were obtained from the plants grown on the high IHP-sorbing riversand, indicating that adsorbed IHP was a poor P source for plant growth (Figures 3 and 4). Alternatively, IHP was directly supplemented for plant growth in the form of cationic complexes. As preliminary experiments showed that supplementation of IHP in the forms of Mn^{2+}, Al^{3+}, Cu^{2+}, Zn^{2+} and Fe^{3+} salts demonstrated toxicity to the plants and thus unsuitable for use (data not shown), results from the provision of Na^+ (soluble), Ca^{2+} and Mg^{2+} (insoluble) salts was only reported here.

Riversand-grown plants did not respond notably to any of the cationic complexes, with only subtle but significant (P<0.05) improvement in both shoot DW and P concentration by supplementation of Mg-IHP, but not Na-IHP and Ca-IHP, compared to the no-P controls (Figures 3 and 4). On the contrary, P nutrition of the quartzsand-grown plants was considerably improved to different extents by the supplementation of either one of the 3 IHP salts (Figures 3 and 4). The utilization of insoluble Ca-IHP was as efficient as of soluble Na-IHP by the quartzsand-grown plants, yielding approximately 9-11 mg shoot DW plant^{-1} and 0.42% (w/w) shoot P concentration (Figures 3and 4; upper panels), equivalent to the P_i-fed plants at 5 and 23 mg P kg^{-1} sand levels, respectively (Figures 3 and 4; lower panels). The effects of IHP supplementation on quartzsand-grown plants were much more prominent in the form of Mg^{2+} salts, yielding 32 mg shoot DW plant^{-1} and 0.68% (w/w) shoot P concentration (Figures 3 and 4; upper panels), equivalent to P_i-fed plants at 26 and 57 mg P kg^{-1} sand levels, respectively (Figures 3 and 4; lower panels). The retarded plant growth on Na-IHP, compared to Mg-IHP, was believed to be the outcome of extremely high Na^+ concentration from the commercial preparation of IHP in the form of Na^+ salts (12 Na:IHP molar ratio; Sigma-Aldrich P3168)., as similar growth retardation was observed when NaH_2PO_4 standard was supplied in high concentrations (Figure 2).

Effects of divalent ions on root phytase activities

Severe hindrance of plant phytase activities in the presence of elevated divalent ion

levels could not be observed from *in vitro* assays. Ca^{2+} and Mg^{2+} ions were found to possess slightly enhancive and inhibitory effects on the phytase activities in the crude root extracts from 14-day-old tobacco seedlings in a concentration-dependent manner, respectively. Root phytase activities were notably heightened by up to 4 m*M* $CaCl_2$ (Figure 5). In the presence of 1 m*M* $CaCl_2$, the relative activities were peaked at 110%, 120% and 130% in P_i-fed plants, low-P plants and no-P plants, respectively, although the activities were declined when IHP started to precipitate with Ca^{2+} at 8 m*M* concentration (Figure 5). Increasing Mg^{2+} concentrations resulted in a relatively steady declination in root phytase activities of seedlings grown under all P_i treatments, down to 53-59% activities left at the precipitating level (8 m*M*; Figure 5). The results indicated that tobacco plant phytases did possess ability to hydrolyze IHP complexed with Ca^{2+} and Mg^{2+}, which was in line with our observation in sand culture. However, while the insoluble Ca and Mg-IHP were in direct contact with the crude root phytase preparations in *in vitro* assays, only solubilized IHP from Ca and Mg-complexes was accessible to the root-associated phytases, which constitute predominant extracellular phytase activities (Table 2). Thus, even though Mg^{2+} was found to be more inhibitory to plant phytase than Ca^{2+} (Figure 5), plants grown with less stable Mg-IHP complex outperformed that with Ca-IHP in sand culture (Figures 3 and 4).

Discussion

Intrinsic plant acid phosphatases and phytases

While tobacco plants (*Nicotiana tabacum*) have extensively been used in transgenic phytase researches, from recombinant protein production (Ullah et al., 1999; Verwoerd et al, 1995) to functional studies of microbial enzymes on plant growth promotion (George et al., 2005; Lung et al., 2005; Yip et al., 2003), little is known about the intrinsic phytase in this model plant. The present study is the first attempt to demonstrate low P_i-inducible extracellular phytase activities in tobacco seedlings. It is generally perceived that plant phytases are APases that are able to hydrolyze IHP as a member of their broad spectra of P-monoester substrates, hence, phytase activities were compared with APase activities (against *p*NPP) in the present study, and in other publications (Asmar, 1997; Hayes et al., 1999; Hübel and Beck, 1996; Li et al., 1997a, b; Richardson et al., 2000). The ubiquitous APase functions in plant P metabolism (Duff et al., 1994) account for the measured shoot APase in P_i-fed plants (1029 mU mg^{-1} protein; Table 1), which was accompanied with considerable phytase activity (13.8 mU mg^{-1} protein; Table 1). P_i limitation elicited subtle increase in shoot and secretory APase ($P<0.05$; Table 1), probably as an adaptation for P_i acquisition and recycling (Duff et al., 1994), which is concomitant with more prominent increase in phytase activities (Table 1). The lesser extent of increase in phytase activities of roots suggested that the elicited phytase might not be accumulated intracellularly, but targeted to secretion pathway, corroborated by the 3 to 4-fold

increase in secretory phytase activity (Table 1). Even though precise comparison of phytase activities among different plant species described elsewhere is confounded by the experimental variations (plant age, assay pH and temperature, etc.), specific root phytase activities under P_i deficiency were mostly ranged from tens to hundreds milli-unit mg^{-1} protein, i.e. 1 to 5 % of total APase activities (Hayes et al., 1999; Hübel and Beck, 1996; Li et al., 1997b; Richardson et al., 2000). The specific phytase activity of tobacco roots (26.2 mU mg^{-1} at 0.1 m*M* P_i supply; Table 1) was comparable to that of wheat (26.7 mU mg^{-1}; Richardson et al., 2000) and a bit lower than that of maize (88 mU mg^{-1}; Hübel and Beck, 1996), whereas the extreme abundance of root phytase (440 mU mg^{-1}; Li et al., 1997a) in tomato was a rare occurrence. Nonetheless, the ultimate location of the phytase is one of the determining factors governing its level in roots. While some phytases were confined intracellularly (Hübel and Beck, 1996), phytase secretion has been described in many species (Asmar, 1997; Hayes et al., 1999; Li et al., 1997a; Richardson et al., 2000). In tobacco, the extracellular phytase activities were found to be mostly root-associated (Table 1). Whereas the possibility of cell wall-protein association should not be ruled out, the abundance of secretory phytase in growth media suggested that the negligible root-released activity levels might be due to inadequate time for secretory protein accumulation, as protein release from the apoplastic space to the external medium is a passive process.

Adsorption and precipitation: constrains for IHP-P assimilation

While many plant species are incapable of utilizing IHP-P in medium (Hayes et al, 2000; Richardson et al, 2000; Richardson et al., 2001), the notable growth of other plant species on IHP as the sole P source was found to be medium-dependent. For instance, *Trifolium subterraneum* which had demonstrated substantial IHP utilization ability in agar medium was found to exhibit poor P nutrition on IHP-supplemented sand-vermiculate medium (Hayes et al., 2000). Similarly, IHP, an equally good source of P as KH_2PO_4 for the growth of lupins in quartzsand, was found to be the poorest form of P supplementation to the soil, compared to P_i, glycerophosphate and ribonucleic acid (Adams and Pate, 1992). In some experiments that utilized commercial phytase for stimulating plant growth, 10-fold higher IHP and phytase dosages were required in soil than in quartzsand to produce significant effects (Findenegg and Nelemans, 1993). Observations from the present study also clearly demonstrated the poor plant growth on adsorbed IHP (Figures 3 and 4), in despite of the notable extracellular phytase activities in hydroponic culture (Table 1 and 2). In fact, all these findings reflect the real situation in soil environment that IHP rarely exists in enzyme-labile form. Due to the dense negative charges, IHP is strongly adsorbed to insoluble salts such as Fe and Al oxides and hydrous oxides (Shang et al., 1992), as well as clay minerals such as illite, kaolinate and montmorillonite (Celi et al., 1999). Apart from adsorption, availability of IHP in soil is believed to be severely limited by cationic precipitation, such as Fe- and Al-IHP in acid soils and Ca-IHP in alkaline soils (Turner et al., 2002). Maenz et al. (1999) ranked the cation-IHP complexes in descending order of stability: $Fe^{2+} \sim Zn^{2+} \sim Fe^{3+} > Mn^{2+} > Ca^{2+} > Mg^{2+}$, which is

in line with the present observation that Mg-IHP was a better source of P for plant growth than Ca-IHP (Figures 3 and 4), assuming that plant phytases preferably act on solubilized and dissociated IHP from the complexes. Instead of direct attack on the insoluble cation-IHP complexes, IHP solubilization is believed to be a crucial step for plant phytase hydrolysis, supported by the better assimilation of P from Mg and Ca-IHP by plants on quartzsand (Figures 3 and 4), compared to riversand that possessed a higher potency to bind soluble IHP (Figure 1). It is believed that assimilation of P from Mg and Ca-IHP is an innate ability of plants, as the IHP storage in seeds and grains was mostly associated with Mg^{2+} and Ca^{2+} ions to form complexes commonly referred to as phytins. However, despite the remarkable plant growth on Mg and Ca-IHP in the present study (Figures 3 and 4), these unstable complexes, once introduced into soil from plant remains, could be immediately stabilized by the prevalent ions (e.g. Fe^{3+}, Al^{3+}, Zn^{2+}, etc) in soil, rendering them invulnerable to enzymatic hydrolysis. In view of the three-dimensional structures of the three characterized phytase families, beta-propeller phytase (BPP; Shin et al., 2001), HAPs (Lim et al., 2000; Liu et al., 2004) and plant PAPs (Strater et al., 1995; Schenk et al., 2005), the cleavage of phosphomonoester bonds requires the precise coordination of the phosphate ions with the amino acid residues in the active site. For instance, the substrate binding site of *E. coli* HAP, located in a deep indentation inside the molecule, requires all six deprotonated phosphate for hydrolytic reaction (Lim et al., 2000). Thus, the adsorption and precipitation reactions may render the IHP molecule inaccessible to the substrate-binding pockets of phytases for mineralization.

Implications on GM approaches for IHP-P assimilation

From the present study, it is showed that tobacco plants do secrete phytase under P_i starvation, but this adaptation may not satisfy plant P nutrition if the availability of soluble phytate is limited. In soil, the majority of IHP is adsorbed to soil components or is precipitated with cations. While in soil leachate, 77% of the P compounds is orthophosphate, and IHP constitutes only 3.3% of the total soluble P (Espinosa et al., 1999). The minute amount of soluble IHP in soil solution may still be a sparing source of P for plant, rationalizing phytase secretion under P starvation. Some recent studies attempted to explore the usability of the IHP pool in bulk soil. Transgenic strategies to improve IHP-P assimilation in plants have been demonstrated by the extracellular secretion of recombinant phytases, including HAP (George et al., 2005; Richardson et al., 2001; Zimmermann et al., 2003), BPP (Lung et al., 2005; Chan et al, 2005) and PAP (Xiao et al., 2005). While IHP utilization ability was significantly improved in all of these transgenic plants grown in sterile agar or hydroponic culture, no enhancement of IHP assimilation by these transgenic plants has been reported in soil. Even though Zimmermann et al. (2003) were able to demonstrate better P nutrition of transgenic plants in a non-sterile quartz-loess-peat mixture by irrigating plants with 100 μ*M* IHP solution twice a week, no assessment of the P-sorption capacities of this substrate has been performed. Perhaps, supplementation of IHP solution was adequate to provide sufficient soluble IHP for mineralization. In fact, George et al. (2005) have shown that transgenic plants secreting HAP

1 did not lead to an improved P nutrition from P-deficient soils without the amendment with IHP,
2 coincident with our observations that transgenic plants secreting BPP (Lung et al., 2005) did
3 not exhibit better growth performance in a high P-sorbing sand (unpublished data). Thus, in
4 agreement with the present study, both native and recombinant phytase exudation from plant
5 roots may not ensure significant IHP-P assimilation in the soil environment. To circumvent the
6 obstacle of phytate insolubility, phytase secretion should be supplemented by some means, for
7 instance, organic acid exudation, to desorb or solubilize the adsorbed or precipitated IHP.
8 Hence, co-exudation of recombinant phytase and organic anions (e.g. citrate, malate and
9 oxalate) may be a promising strategy to improve P acquisition from IHP in soil.

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FIGURE LEGENDS

Figure 1. Time course of IHP adsorption on quartzsand (QS) and riversand (RS). Na-IHP solution (pH 6 or 11) was incubated with sand sample at 100 mg P kg^{-1}. At different time intervals, soil solution was collected for IHP quantification by persulfate digestion.

Figure 2. Growth parameters of plants with different P_i dosage in sand culture. Ten-day-old tobacco seedlings were grown on quartzsand (QS) or riversand (RS) with different NaH_2PO_4 concentrations (mg P kg^{-1} sand) for 20 days. Shoot/root dry weights (DW) and shoot P concentration were determined. Shoot DW of plants grown in 0-100 mg P kg^{-1} QS (A) and 0-400 mg P kg^{-1} RS (B) were fitted to the Mitscherlich equation. For shoot P concentration, data from 0-200 mg P kg^{-1} QS (C) and 0-400 mg P kg^{-1} RS (D) were fitted to the Mitscherlich equation.

Figure 3. Dry weights of plants grown with different IHP treatments in sand culture. IHP was supplied in either adsorbed or supplemented forms as the sole P source. Shoot and root dry weights were shown in the upper panel. Equivalent soil P_i levels were estimated from the corresponding Mitscherlich equations depicted in Figure 2A and 2B.

Figure 4. Shoot P concentration of plants grown with different IHP treatments in sand culture. Shoot P concentrations were shown in upper panel. Equivalent soil P_i levels were estimated from the corresponding Mitscherlich equations depicted in Figure 2C and 2D.

Figure 5. Effects of divalent ions on root phytase activities. Tobacco seeds were sown on liquid MS medium with different P_i treatments (0, 0.1 or 1 m*M*) and grown for 14 days. Phytase activities were assayed in crude root extracts in the presence of different Ca^{2+} or Mg^{2+} concentrations.

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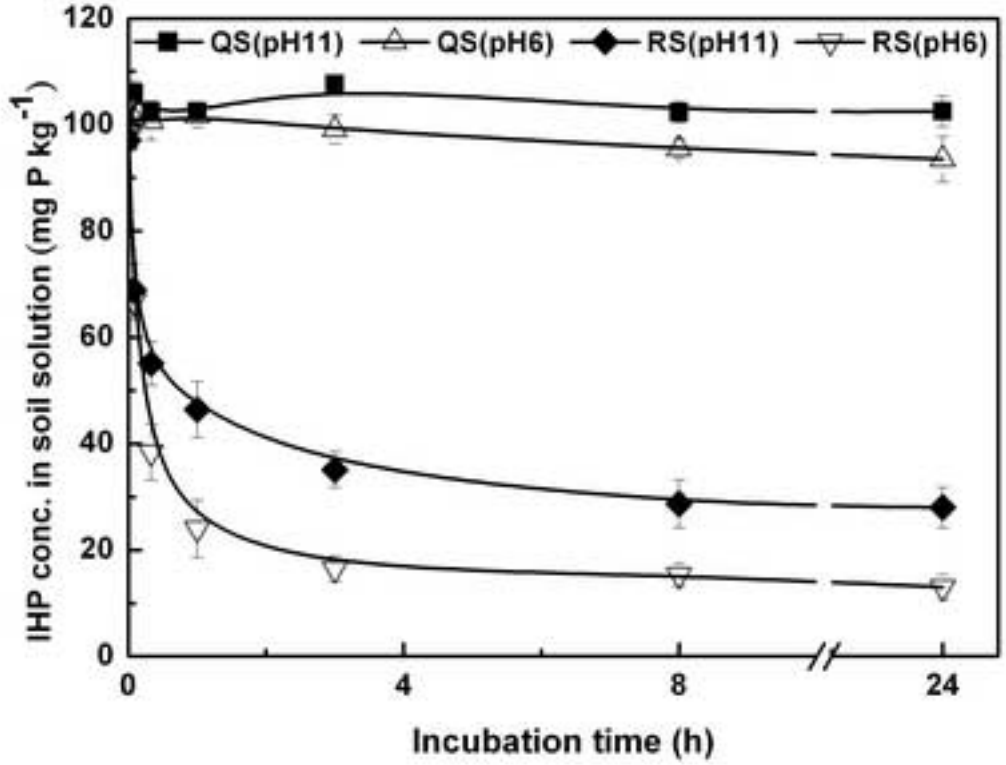

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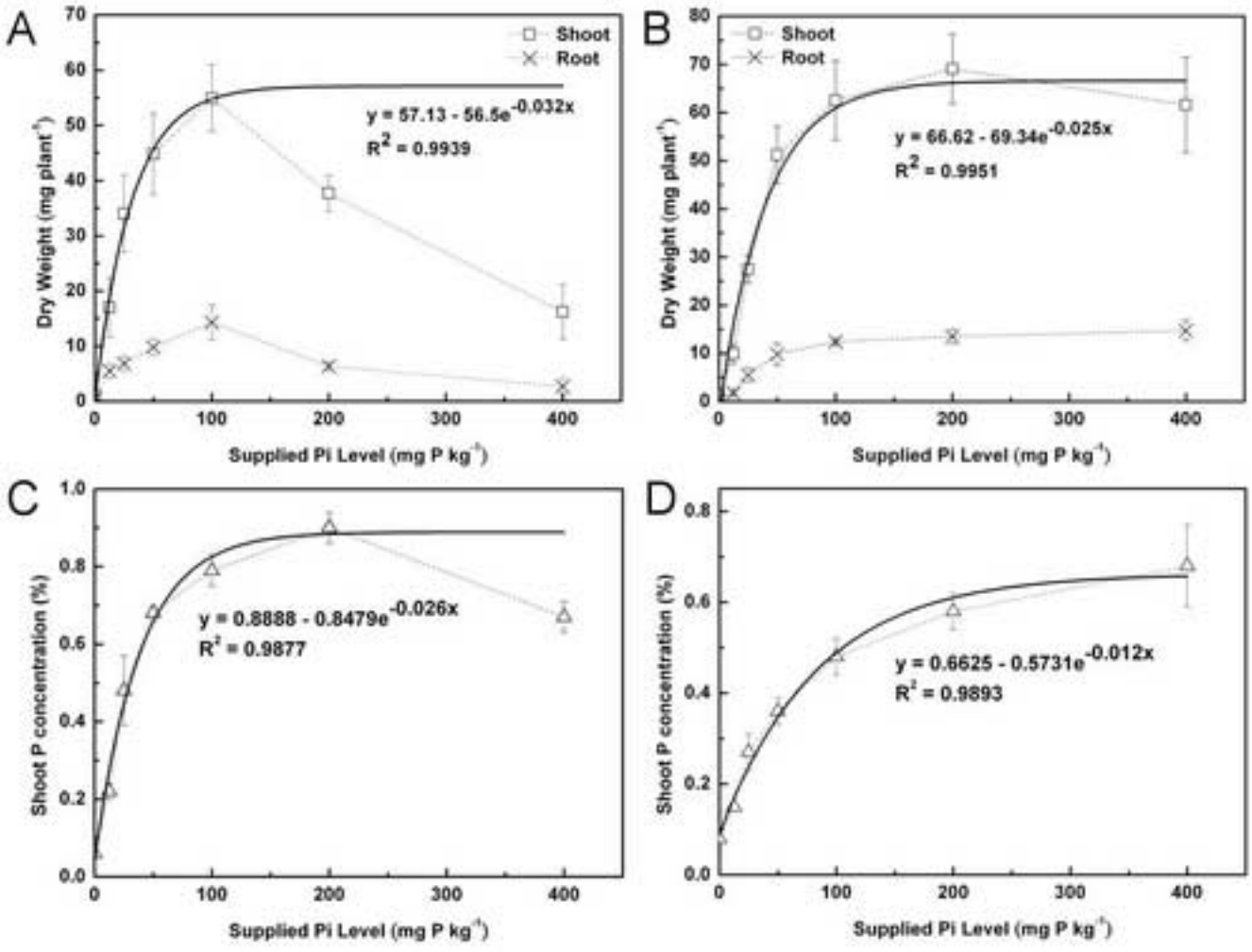

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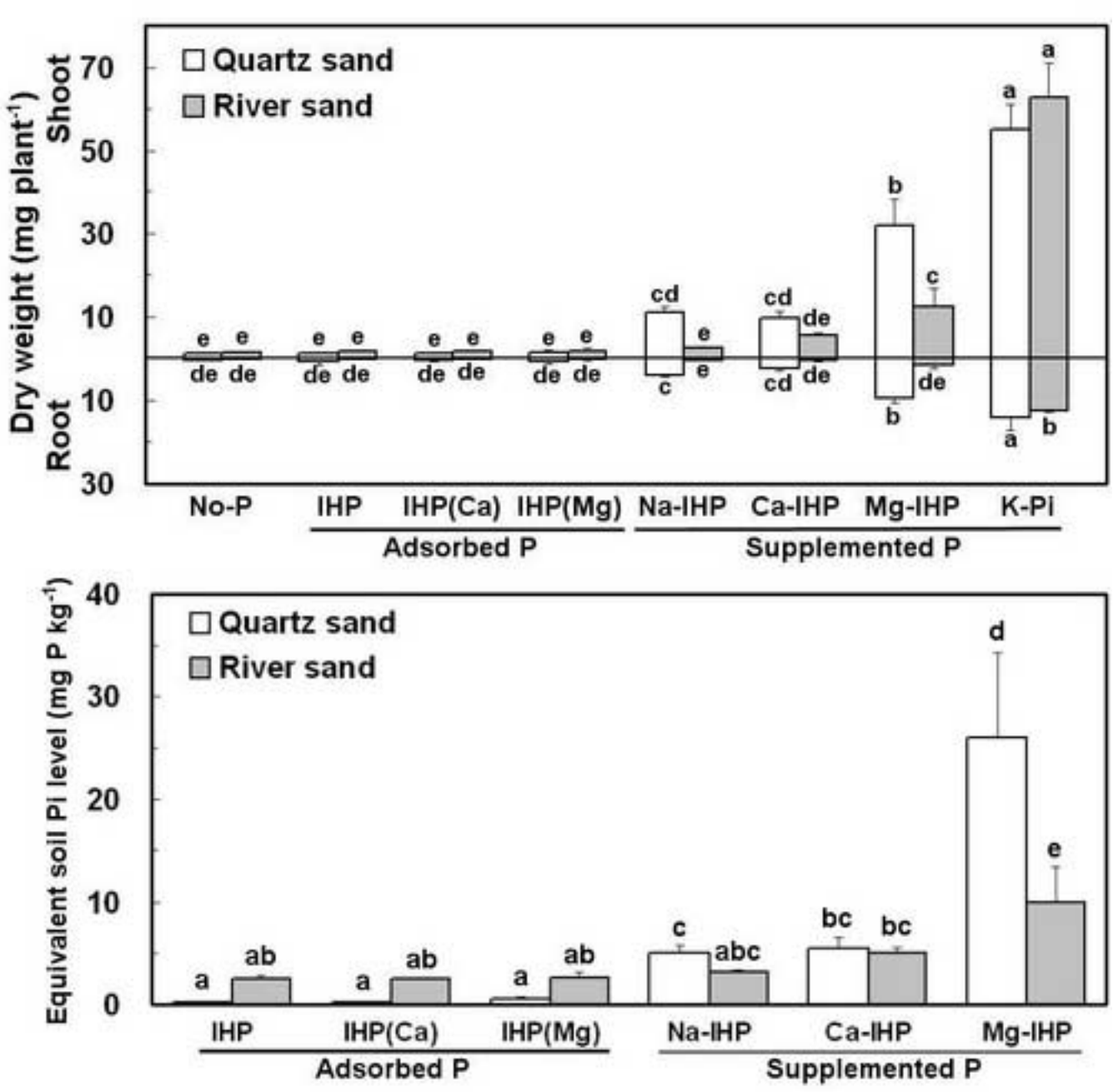

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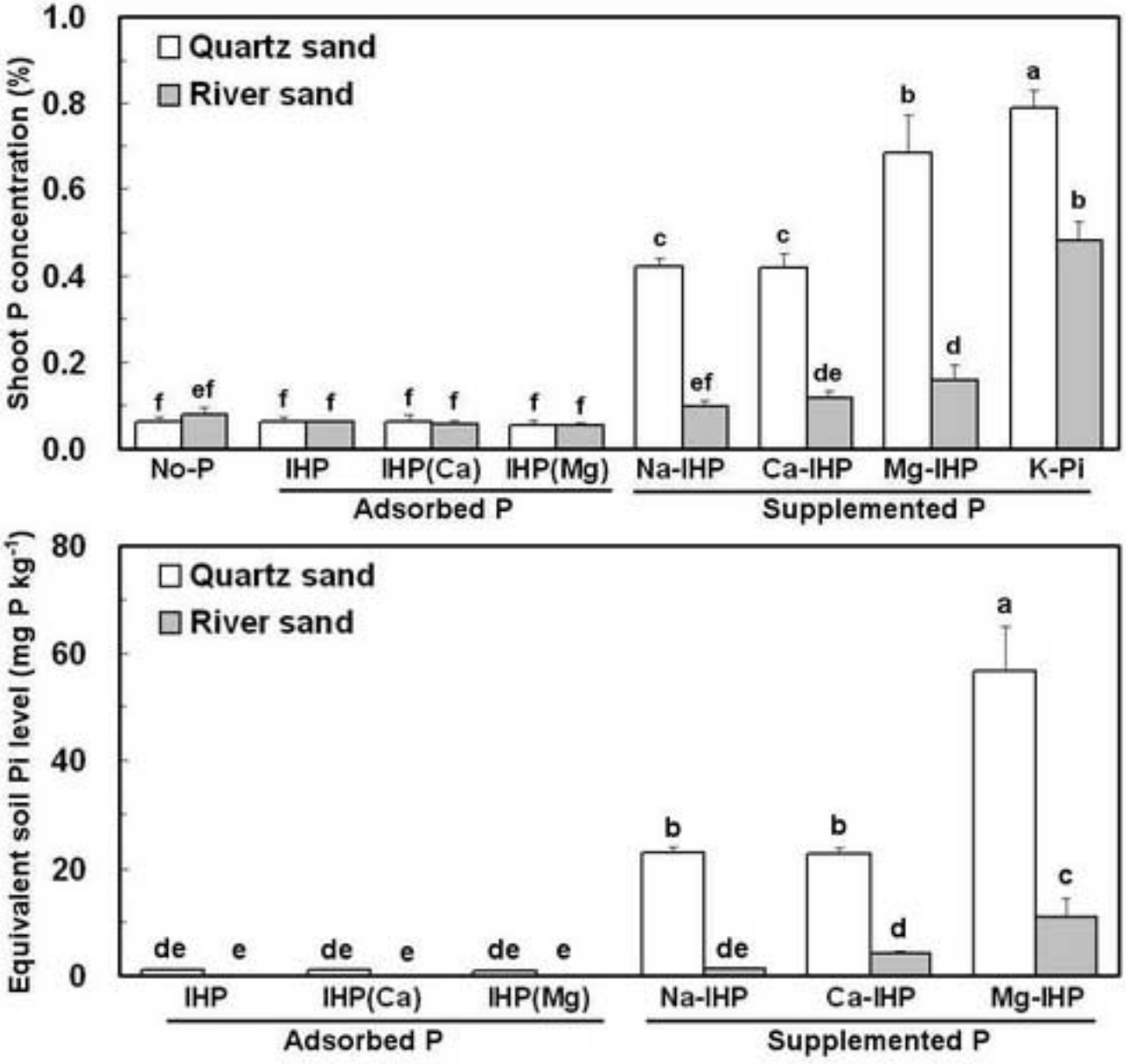

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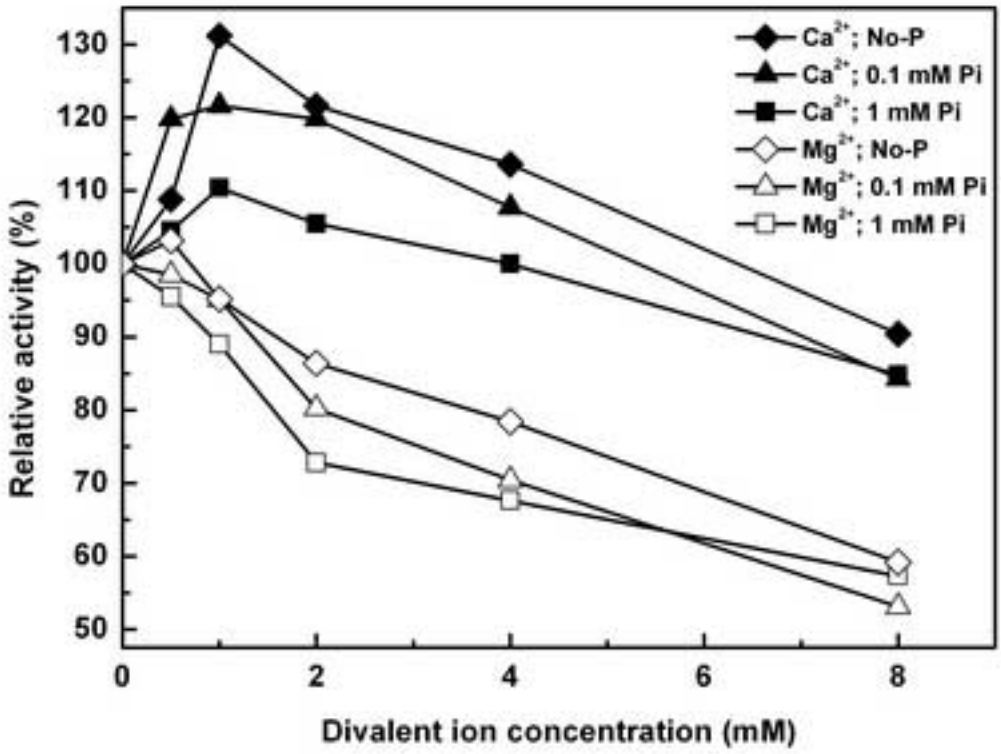

tables 1-2

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Table 1. Phytase and acid phosphatase activities of crude root / shoot extracts and growth media from 14-day-old tobacco plants

P treatment	Phytase activity (mU mg^{-1} protein)			Acid phosphatase activity (mU mg^{-1} protein)			Phytase / acid phosphatase activity (%)		
	Root extracts	Shoot extracts	Growth media	Root extracts	Shoot extracts	Growth media	Root extracts	Shoot extracts	Growth media
1 mM P_i	15.6 ± 0.9^{c}	13.8 ± 2.5^{c}	210.0 ± 14.1^{c}	589 ± 62^{ab}	1029 ± 120^{b}	4323 ± 95^{b}	2.7 ± 0.3^{b}	1.3 ± 0.1^{c}	4.9 ± 0.4^{c}
0.1 mM P_i	26.2 ± 1.6^{a}	72.1 ± 9.8^{a}	600.9 ± 14.8^{b}	618 ± 75^{a}	1449 ± 216^{a}	4761 ± 186^{a}	4.3 ± 0.3^{a}	5.2 ± 0.3^{a}	12.6 ± 0.8^{b}
No-P	23.0 ± 2.0^{b}	56.3 ± 9.2^{b}	914.9 ± 18.6^{a}	503 ± 23^{b}	1232 ± 138^{ab}	5017 ± 118^{a}	4.6 ± 0.4^{a}	4.6 ± 0.4^{b}	18.2 ± 0.5^{a}

Each observation is the mean of at least 3 replicates ± 1 standard deviation.

Within each column, the values marked by different letters (a, b, c) are significantly different ($P < 0.05$).

Table 2. Extracellular phytase activities of 14-day-old tobacco plants

P treatment	Extracellular phytase activities	
	Root-associated activity (mU g^{-1} root DW)	Root-released activity (mU mg^{-1} root DW h^{-1})
1 mM P_i	819 ± 82^{c}	< 0.01
0.1 mM P_i	$1{,}134 \pm 55^{b}$	< 0.01
No-P	$1{,}367 \pm 62^{a}$	< 0.01

Each observation is the mean of at least 3 replicates ± 1 standard deviation.

Within each column, the values marked by different letters (a, b, c) are significantly different ($P < 0.05$).