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ORIGINAL RESEARCH

Root Proliferation, Proton Efflux, and Acid Phosphatase Activity in Common Bean (*Phaseolus vulgaris*) Under Phosphorus Shortage

Saber Kouas · Ahmed Debez · Tarek Slatni · Nahla Labidi · Jean Jacques Drevon · Chedly Abdelly

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Abstract The impact of phosphorus (P) availability on root proliferation, proton efflux, and acid phosphatase activities in roots and leaves was investigated in two lines of common bean (*Phaseolus vulgaris*): BAT 477 and CocoT. Phosphorus was supplied as KH_2PO_4 at 0 and 60 μmol per plant (0P and 60P, respectively). Under P shortage, the plant growth was more restricted in CocoT than in BAT 477, shoots being more affected than roots. The root area increased significantly at 0P in both lines. Up to 1 week following P shortage, the proton efflux increased in both lines despite a higher extent in BAT 477 as compared to CocoT. Root acid phosphatase activity was significantly higher under P limitation in the both lines, this trend being more pronounced in BAT 477 than in CocoT. This was also true for the leaf acid phosphatase. Regardless of the bean line, higher values were recorded for the old leaves as compared to the young ones for this parameter. Interestingly, a significant correlation between Pi content in old leaves and their acid phosphatase activity was found in P-lacking (0P) plants of the both bean lines, suggesting that acid phosphatase may contribute to increase the phosphorus use efficiency in bean through the P remobilization from the old leaves. As a whole, our results highlight the significance of the root H^+ extrusion and the acid phosphatase activity rather than the root proliferation in the relative tolerance of BAT 477 to severe P deficiency.

S. Kouas · A. Debez · T. Slatni · N. Labidi · C. Abdelly (✉)
Laboratoire d'Adaptation des Plantes aux Stress Abiotiques,
Centre de Biotechnologie,
BP 901, 2050 Hammam-Lif, Tunisia
e-mail: chedly.abdelly@cbbc.rnrt.tn

J. J. Drevon
Rhizosphere et Symbiose,
Institut National de la Recherche Agronomique (UMR 1220),
1, Place Viala,
34060 Montpellier, France

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Introduction

Common beans (*Phaseolus vulgaris*) are the world's most important grain legumes for direct human consumption; they comprise 50% of the grain legumes consumed worldwide (Broughton et al. 2003; Graham et al. 2003). Environmental constraints such as high soil acidity and low soil nitrogen and P levels considerably limit bean production, in particular in the Mediterranean and tropical zones (Graham et al. 2003). P deficiency is more critical in highly withered soils of tropics and subtropics, as well as in calcareous/alkaline soils of the Mediterranean basin (Hinsinger 2001). Plants are known to involve several mechanisms to increase their P absorption efficiency, such as the modification of soil exploration by roots by increasing the P absorption area (Lynch and Brown 2001). For instance, phosphorus deficiency in the soil has been reported to induce various morphological changes in plant roots, including the formation of root hairs (Bates and Lynch 2001; Gahoonia and Nielsen 2004) and cluster roots (Johnson et al. 1996). Adaptive traits in root architecture, such as changes in the basal root growth angle (Liao et al. 2001) and the relative distribution of roots in the topsoil (Lynch and Brown 2001), have also been documented. Common bean (*P. vulgaris* L) has been used as a model system for understanding the importance of root architecture for soil resource acquisition (Liao et al. 2001). A similarly enhanced root length production at a low P supply has been observed in *Hordeum vulgare* (Steingrobe et al. 2001). Increased root production, without a proportional increase in living root biomass, i.e.,

enhanced root turnover, allows greater amounts of uptake of immobile soil resources, such as P. Fast root turnover is a very important trait of cluster-root-producing species (Shane and Lambers 2005). Among the various morphological and physiological strategies to acquire sparingly soluble phosphorus from soil, enhanced rhizosphere acidification is of important significance (Hinsinger et al. 2003). This acidification capacity is phosphorus-availability-dependent. According to (Sas et al. 2003), the excess of cation content in plants is generally higher at 1 mmol P per cubic meter and decreases with increasing P supply. H^+ extrusion by P-deficient plants (grown at 1 and 5 mmol P per cubic meter) is, on average, two to threefold greater than organic acid exudation.

Acid phosphatase (orthophosphoric-monoester phosphohydrolase, EC 3.1.3.2) can hydrolyse a range of organic P compounds (Tarafdar and Claassen 2005), and these enzymes are more abundant in the rhizosphere when plants are P-starved (Yun and Kaeppler 2002). The production of phosphatase is a potential way for plants to enhance P availability, as a large proportion of soil P (up to 80%) occurs in organic forms (Richardson et al. 2004). Coello (Coello 2002) showed that the activity of the secreted acid phosphatase in *Arabidopsis thaliana* under P-deficient conditions increased as much as six times over P-sufficient treated plants within the first 2 days of Pi withdrawal. Yadav and Tarafdar (Yadav and Tarafdar 2003) reported that legumes secrete more acid phosphatase comparatively to cereals and oil seeds under P-deficient conditions. In chickpea, significantly higher acid phosphatase activity allowed the plant to mobilize more organic P in both hydroponic and soil cultures, resulting in an improvement of the utilization of organic P in maize/chickpea intercropping (Li et al. 2004). Interestingly, acid phosphatase activity has been also observed in shoots, although its role is not yet elucidated. Plaxton and Carswell (Plaxton and Carswell 1999) hypothesized that acid phosphatase may be involved in releasing Pi from phosphocholine, a phophorilized component of the xylem tissues. Yet, no correlation between the acid phosphatase activity and leaf P status was found in bean, suggesting that acid phosphatase may not contribute to the P remobilization in leaves (Yan et al. 2001). Acid phosphatase activity may be also related to the leaf developmental stage, being significantly higher in the juvenile than in the senescent leaves (Fernandez and Ascencio 1994). Indeed, in both maize and wheat challenged with lower P availability, acid phosphatase activity was higher in roots than in shoots (McLachlan et al. 1987; Yun and Kaeppler 2002).

In the present work, we address the impact of phosphorus deficiency on two lines of common bean (BAT 477, CocoT). We focus on the plant growth, the root proliferation, and assess the involvement of the proton efflux and the acid phosphatase activity in improving P uptake and P use efficiency, respectively.

Materials and Methods

Hydroponic Experiments

Uniformly sized sterilized seeds were germinated in agar 0.9%. Five days after sowing (DAS), seedlings were carefully transplanted into 1-l serum bottles. Roots were gently passed through the hole of a rubber stopper on the bottle neck, and cotton wool was fitted at the hypocotyls level to maintain the root system suspended in the nutrient solution. The study was conducted in a glasshouse under controlled conditions (30/25°C day/night temperatures, 16-h photoperiod). The seedlings were initially cultivated in the nutrient solution (Vadez et al. 1996) with 15 P and 2 mM urea. At 13 DAS, the plants were separated into two lots: The first one was cultivated with a nutrient solution completely deprived of P (0P) while the second received 60P. Bottles were aerated with a flow of 400 ml min^{-1} of filtered air via a compressor and spaghetti tube distribution system. Regular harvests were carried out after 13, 15, 17, 19, 21, 24, 27, and 30 days of treatment. The experiment was set up as a complete factorial with two P levels (0 and 60 µmol KH_2PO_4) and two bean genotypes (BAT 477 and CocoT).

Growth and Root Area Determination

Plants were harvested and separated into roots and old and young leaves. The sample dry weight (DW) was determined after drying at 60°C for 3 days. The root area was determined on freshly harvested roots using the OPTIMAS software.

Proton Efflux Measurements

To compensate the acidification of the nutrient solution during the hydroroponic culture, the pH was measured daily. Fifty-milliliter aliquot of nutrient solution was taken from each 1-l bottle and corrected to pH6.8, if necessary, using an automatic titrator (Metrhom) and using a measured quantity Q (mole) of a KOH (0.1 M) solution according to the formula: $Q=CV\ 10^{-3}$, with C and V representing the concentration of the solution in molar and the volume of solution used in milliliter, respectively. Practically, the quantity of KOH solution added to each bottle was extrapolated from the volume of solution that was added to the aliquot.

Acid Phosphatase Assay

Leaves and roots (0.1 g) were ground separately in a mortar with an extraction mixture consisting of 0.1 M acetate

buffer (1 gml^{-1} buffer), 6 mM ß-mercaptoethanol, 0.1 mM phenyl methyl sulfonyl fluoride, and 6 g insoluble polyvinylpolypyrrolidone. The homogenate was centrifuged at 30,000×*g* at 4°C for 30 min. The reaction mixture contained 100 mM sodium acetate buffer (pH5.8), 5 mM *p*-nitrophenyl phosphate, and the enzyme in a total volume of 0.5 ml. After 30-min incubation at 30°C, the reaction was stopped by the addition of 1 ml 0.5 M NaOH. Acid phosphatase activity was measured at 405 nm by monitoring the *p*-nitrophenol released.

Protein Determination

Protein concentration was determined with the Coomassie Blue G-250 method using bovine serum albumin as standard.

Pi Determination

Samples (25 mg DW of old leaves) from each plant were digested in HNO_3 0.5%. The inorganic phosphorous released was quantified by the molybdovanadate method at 460 nm.

Statistical Analysis

A one-way analysis of variance, using the AV1W MSU-STAT program with orthogonal contrasts and mean comparison procedures, was performed to detect differences between treatments. Mean separation procedures were carried out using the multiple range tests with Fisher's least significant difference (LSD; $P<0.05$).

Results

Plant Growth and Root Area

At 60P, shoot (Fig. 1a, b) and root (Fig. 1c, d) biomass (DW) were higher in CocoT than in BAT 477. P shortage restricted significantly the shoot growth in the both lines;

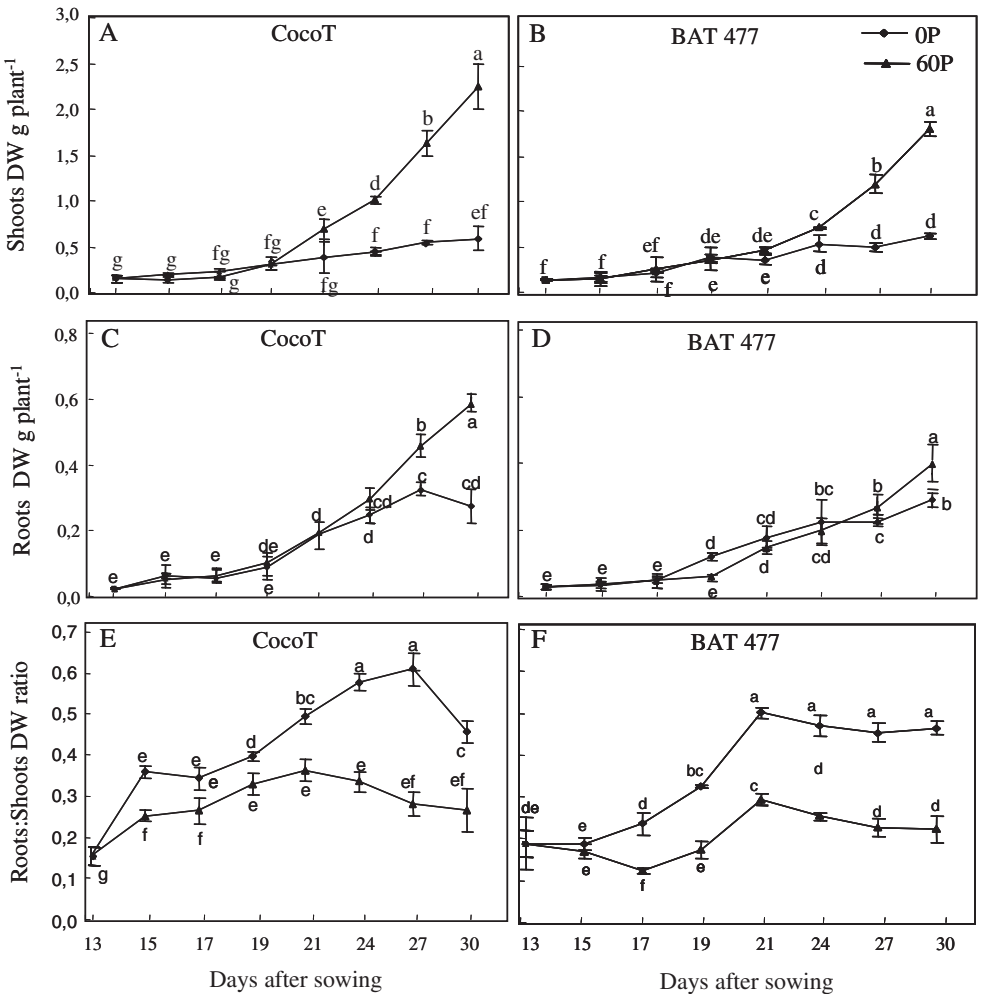

Fig. 1 Effect of P nutrition on shoot (**a**, **b**), root (**c**, **d**), and root/shoot DW ratio (**e**, **d**) of common bean genotypes BAT 477 and CocoT. For each parameter, values (means of four replicates±SD) followed by *the same letters* are not significantly different at 5% according to Fisher's LSD test

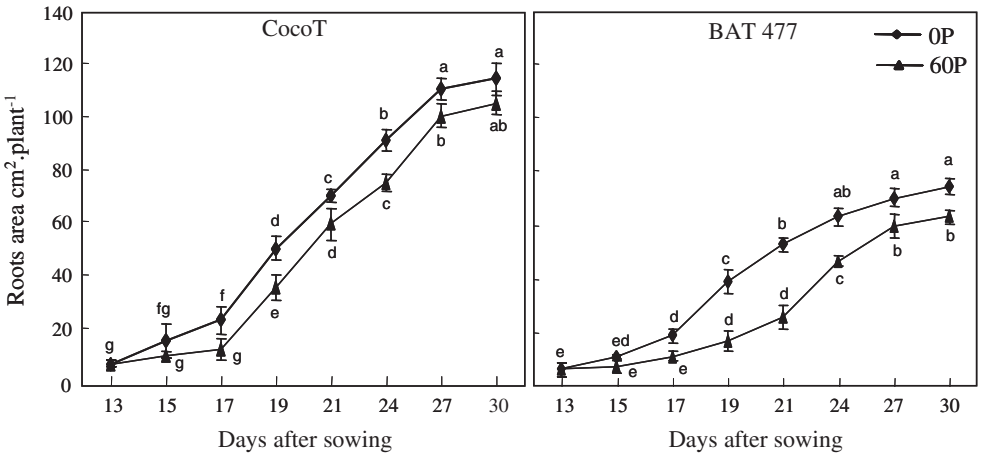

Fig. 2 Effect of P nutrition on root area of common bean genotypes BAT 477 and CocoT. For each parameter, values (means of four replicates±SD) followed by *the same letters* are not significantly different at 5% according to Fisher's LSD test

especially in Coco T. While root DW was not affected by phosphorus availability in BAT 477, 0P led to a significant decrease of this parameter (−50%) in CocoT. The root/shoot DW ratio was higher in the 0P plants as compared to those supplied with 60P, this tendency being, however, more pronounced in CocoT than in BAT 477 (Fig. 1e, f).

Over the treatment period, root area was higher in the 0P treatment than in 60P in both lines (Fig. 2). It is worth mentioning that independently of the P supply levels, this parameter was higher in CocoT than in BAT 477, especially at the end of the treatment.

Proton Efflux

Regardless of the P supply level, the acidification estimated by proton efflux per gram roots FW (Fig. 3) was higher in BAT 477 than CocoT. During the first week of treatment (up to 21 DAS), the plants lacking phosphorus (0P) showed the highest values especially in BAT 477 (3.5 nmol H^+ per gram root FW) than in CocoT (2 nmole H^+ per gram root FW). Thereafter, this parameter declined strongly, being even lower than the 60P plants at 30 DAS.

Acid Phosphatase Activity

Root acid phosphatase activity was higher under P shortage in both lines (Fig. 4). This increase was, however, more pronounced in BAT477 than in CocoT (respectively, 6 and 5 µmol PNP per minute per milligram proteins). After 6 days of treatment, this activity decreased significantly in two lines of bean.

Under P shortage, the acid phosphatase activity increased to a higher extent in the old leaves than in the juvenile ones in both lines (Fig. 5). In the old leaves, this parameter was higher in BAT 477 than in CocoT (respectively, 5 and 4 µmol PNP per minute per milligram proteins). Under optimal P supply, both old and young leaves showed a similar acid phosphatase activity (2 µmol PNP per minute per milligram proteins) in both lines. Irrespective of the bean line, the acid phosphatase activity was higher in roots than in leaves in the 0P plants.

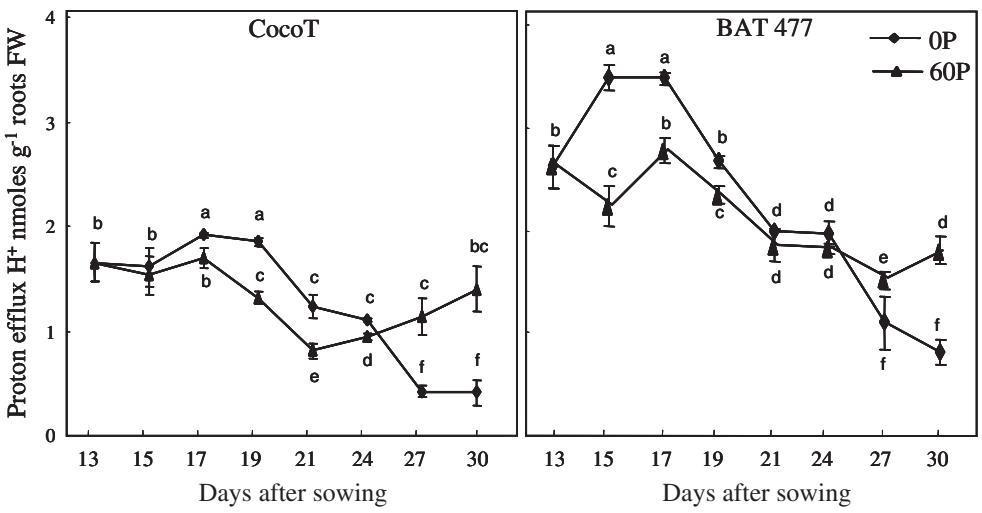

Fig. 3 Effect of P nutrition on proton efflux of common bean genotypes BAT 477 and CocoT. For each parameter, values (means of four replicates±SD) followed by *the same letters* are not significantly different at 5% according to Fisher's LSD test

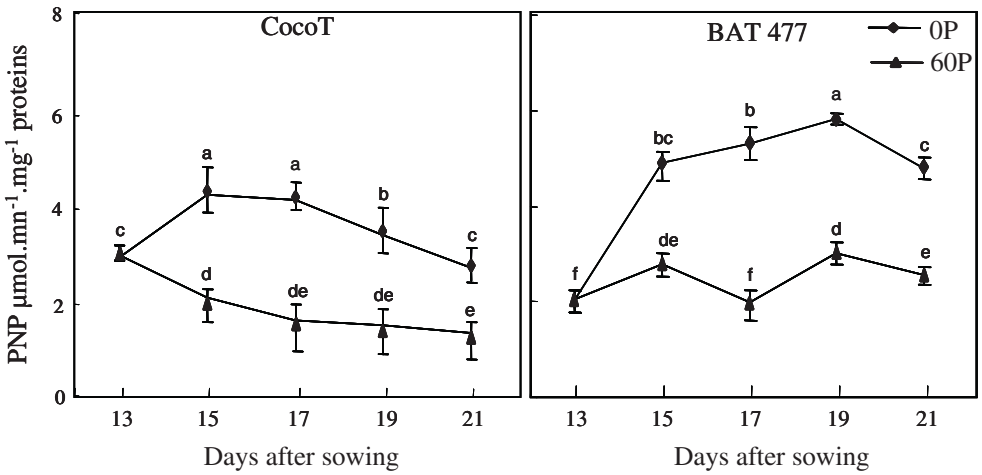

Fig. 4 Effect of P nutrition on root acid phosphatase acid activity of common bean genotypes BAT 477 and CocoT. For each parameter, values (means of four replicates±SD) followed by *the same letters* are not significantly different at 5% according to Fisher's LSD test

Relationship Between the Pi Content and Acid Phosphatase Activity in the Old Leaves

The relationship between the Pi content in old leaves and their acid phosphatase activity provides information about the involvement of acid phosphatase in the remobilization of Pi in these organs. Interestingly, a significant correlation between Pi content in the old leaves and the acid phosphatase activity was found at 0 µmol P for both bean lines (R=0.85 and R=0.92), respectively, for CocoT and BAT 477), but was lower under 60 µmol P (Fig. 6). The slope of the regression was calculated as a parameter standing for the utilization of acid phosphatase in the remobilization of inorganic phosphorus from the old leaves toward the juvenile ones. This parameter was higher in both lines at low P supply (0P) and was significantly increased by P limitation, although to a lower extent for CocoT (40%) than for BAT 477 (55%).

Discussion

Our results show that P limitation restricted the shoot growth in both lines of bean and that this depressive effect was more pronounced in CocoT than in BAT 477.

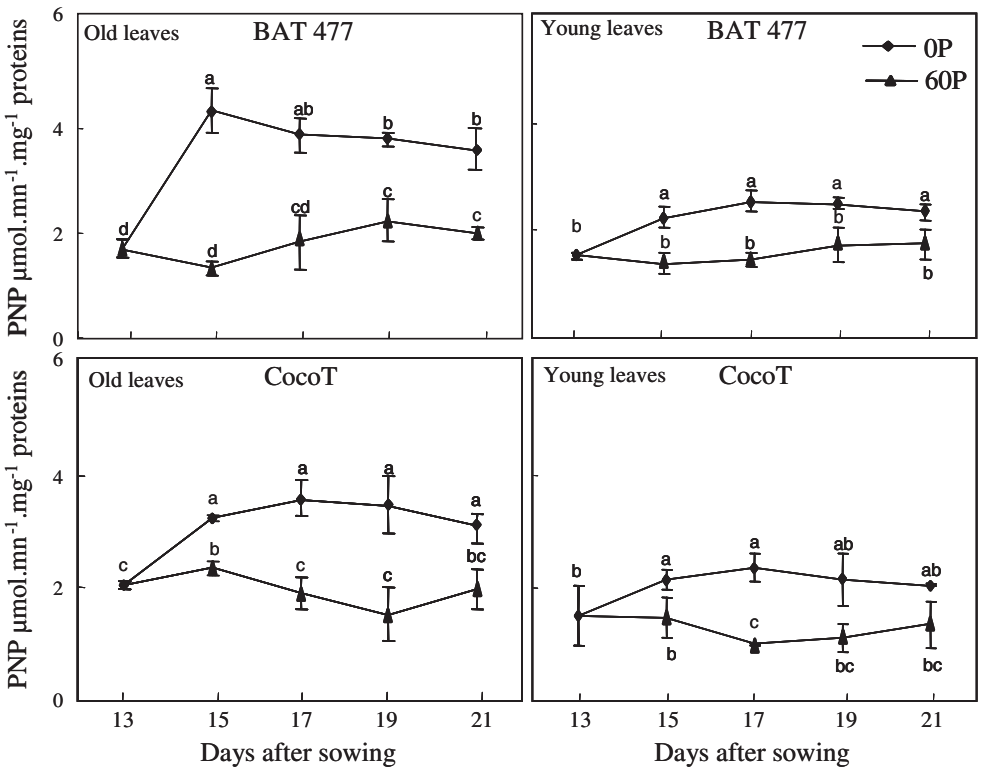

Fig. 5 Effect of P nutrition on leaves acid phosphatase acid activity of common bean genotypes BAT 477 and CocoT. For each parameter, values (means of four replicates±SD) followed by *the same letters* are not significantly different at 5% according to Fisher's LSD test

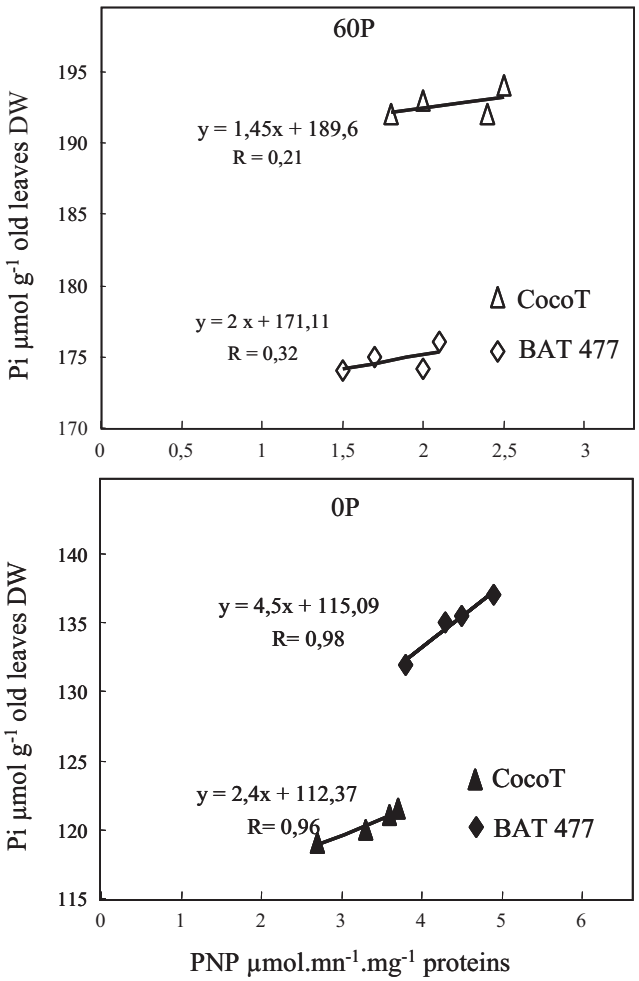

Fig. 6 The relationship between the Pi content in old leaves and acid phosphatase activity in bean genotypes BAT 477 and CocoT under 0P and 60P. Data are individual values of plants (four replicates per treatment and genotype)

According to (Lambers et al. 2006), a low external P availability decreases the internal P status of the plant. In this case, P starvation responses are generally up-regulated in the most tolerant species. P starvation responses, depending on the species, include increased root hair formation, root cluster initiation, and development. We observed an increase in root proliferation in both bean lines in response to phosphorus limitation. After 4 days of treatment, the root area was increased but the dry matter production remained constant. This trend may be indicative of the capacity of bean lines to increase the root length in order to enhance the exchange area between roots and the medium. According to (Ahmed et al. 1987), the stability of the root biomass could be explained by the development of the root hairs, characterized by a low energetic cost. Furthermore, root hairs are a fairly common root structure, and increased root hair length and numbers are considered to be an adaptation that enhances Pi acquisition and thereby the plant competitive advantage when soil Pi is limiting for growth (Bates and Lynch 2001). Our findings indicate that the root/shoot DW ratio increased significantly in plants lacking P despite a higher extent in CocoT. This confirms previous results of (Zhu et al. 2005) and (Li et al. 2007) for maize showing that P limitation increased the root/shoot DW ratio as well the root length and number. Recently, (Pan et al. 2008) reported that in soybean, the P-efficient genotypes were characterized by high root-to-shoot DW ratio, together with high root length and surface area and P uptake, under P deficiency. Further strategies to increase root area have been also identified in bean lines, such as large roots with higher biomass or fine roots hairs with a low biomass (Araujo and Teixeira 2000). In our conditions, both bean lines (especially the sensitive one, CocoT) may have adopted the second way. Several works suggest that ethylene plays a major role in modulating the growth of root hairs in response to plant P nutrition. In this way, the increased growth of root hairs observed for plants grown at low P availability can be mimicked in plants grown at high Pi supply by adding an ethylene precursor to "high-P" roots. Similarly, root hair growth can be inhibited by adding the ethylene inhibitor 1-amino-cyclopropane-1-carboxylate to the medium of "low-P" roots (Zhang and Li 2003). Split-root experiments have also established that a shoot-derived signal is required for root hairs to increase the root length; the signal is translocated to the roots only when the shoot senses a low P status, resulting in enhanced root hair length even at low P status in the roots (Jungk 2001).

The present work shows that the ratio of proton efflux per unit biomass of root FW increased under severe P deficiency, being higher in the relative tolerant line (BAT 477) than in the sensitive one (CocoT). This result suggests that this acidification capacity is phosphorus availability- and genotype-dependent. In a recent work (Kouas et al. 2008), we found that in common bean grown under symbiotic nitrogen fixation, the proton efflux by nodulated roots was 25% to 50% higher in BAT 477 than in CocoT under optimal to P-limiting supplies. This agrees with the previous report of a relatively high proton efflux by BAT 477 under P limiting independently of N_2 fixation (Tang et al. 2003). The higher proton efflux in BAT 477 correlated with its better adaptability to P limiting, suggesting an involvement of the root acidification capacity in the adaptation of common bean to this abiotic constraint. Sas et al. (Sas et al. 2003) reported that H^+ extrusion in P-deficient plants were, on average, two to threefold greater than organic acid exudation. In addition, the excess of cation content in plants was higher at low P supply (1 mmol P per cubic meter) but decreased with increasing P supply. Similarly, (Neumann and Römheld 1999) documented that phosphorus deficiency increased the proton efflux in roots of tomato and white lupin. Recently, (Zhou et al. 2009)

showed that faba bean can release significant amounts of proton in comparison with soybean and maize. This result could partly explain why faba bean utilizes sparingly soluble P more effectively than soybean and maize and is of high significance in identifying the mechanisms behind interspecific facilitation of P uptake by intercropped species, especially when grown on calcareous soils. Our data also support previous works showing that root H^+-ATPase plays an essential role for the enhanced H^+ release by plant roots under P deficiency and, therefore, is an essential enzyme involved in the adaptation of plants to P deficiency (Shen et al. 2006; Yan et al. 2001).

In both lines studied, P limiting increased significantly the acid phosphatase activity in roots despite a higher extent in BAT 477 than in CocoT. Similarly, (Xiao et al. 2006) reported that root acid phosphatase activity was five to tenfold higher in *A. thaliana* plants subjected to P limitation as compared to the plants cultivated under optimal P supply. These findings strengthen the assumption that acid phosphatase in roots may be involved in the P acquisition and in the improvement of P nutrition (Wasaki et al. 2003). Li et al. (Li et al. 2008) also noted a higher acid phosphatase activity in the rhizosphere of two rice genotypes: Zhongbu 51 and Pembe. Furthermore, acid phosphatase activity was negatively correlated to organic P concentration in the rhizosphere of both Zhongbu 51 and Pembe, suggesting that acid phosphatase was involved in the mineralization of organic P in the soil. Ma et al. (Ma et al. 2009) reported that transgenic expression of a purple acid phosphatase gene in white clover plants increased their abilities of utilizing organic phosphorus in response to P deficiency. At 0P, the acid phosphatase activity was higher in old leaves than the young ones and BAT 477 showed higher values than CocoT. Although the induced phosphatase activity in plants during P deficiency has been widely documented, the specific role of phosphatase for improving internal efficiency of P utilization has not been clearly established (Nanamori et al. 2004). The absence of any relationship between acid phosphatase activity and P status in leaves of distinct ages of common bean genotypes led (Yan et al. 2002) to suggest that the phosphatase activity was not related to leaf P remobilization. On the contrary, *Brachiaria* plants showed a higher proportion of Pi to total P in leaves than rice plants and also a higher acid phosphatase activity in shoots, which could allow the *Brachiaria* to use P more efficiently than rice (Nanamori et al. 2004). Interestingly, the present work reveals a strong correlation between Pi content in the old leaves and their acid phosphatase activity at 0P for both lines. The slope of the regression was calculated as a parameter standing for the utilization of old leaves acid phosphatase in the remobilization of Pi. This parameter was significantly increased at low P (0P), although to a lower extent in CocoT (40%) than in BAT 477 (55%). This may indicate that acid phosphatase may efficiently contribute to enhance the phosphorus use efficiency in bean through the P remobilization from the metabolically less active sites, such as the old leaves toward the young ones (Duff et al. 1994; Schachtman et al. 1998).

As a whole, our results indicate that the relative better tolerance of BAT 477 to P limitation as compared to CocoT could be partly explained by the capacity of BAT 477 to maintain (1) a higher root acid phosphatase and proton efflux to preserve an adequate phosphorus nutrition despite lacking P in the culture medium, in concomitance with (2) a higher acid phosphatase activity in the old leaves. This enzyme is involved in the mobilization of the phosphorus from these organs and its allocation toward the young leaves, which increases the P utilization efficiency.

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