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Effect of phosphite supply in nutrient solution on yield, phosphorus nutrition and enzymatic behavior in common bean (*Phaseolus vulgaris* L.) plants

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Abstract

Aim of this study was to (i) understand the phosphite action used as P source on growth and grain yield, (ii) measure P concentration and accumulation in shoot and root, and (iii) evaluate enzymatic behaviour in common bean (*Phaseolus vulgaris* L.) plants grown in nutrient solution under phosphate starvation. Experimental design was completely randomised with 7 levels of phosphite (0, 16, 32, 64, 128, 256 and 512 µM) and 2 levels of phosphate (80 and 800 µM, corresponding to phosphate-starved plants and phosphate-sufficient plants, respectively) in nutrient solution. Common bean plants were evaluated at 2 different growth stages: flowering and mature grain stages. For plants harvested at the mature grain stage, two more treatments (additional treatments) were added: –P = no P supply in nutrient solution; and +Phi = all the P (800 µM) from nutrient solution was supplied only as Phi. This study revealed that growth and grain yield in plants grown under phosphate starvation presented negative repercussions on these parameters, in which treatments with 64, 128, 256 and 512 µM of phosphite resulted in no-filled grains. Concentration and accumulation of P in shoot and root of phosphate-starved plants was increased with increasing phosphite levels in nutrient solution, but this additional P concentration did not convert into grain yield. The phosphite application in phosphate-starved plants promoted a decrease in acid phosphatase (EC 3.1.3.4.1) activity, while catalase (EC 1.11.1.6) activity was increased up to 32 µM of phosphite and was reduced at higher levels of phosphite.

Keywords: Antioxidant enzyme; biostimulant; dry mass; grain; leguminous; nutrient solution; *Phaseolus* bean; phosphate; pod.
Abbreviations: CAT-catalase; P-phosphorus; Phi-phosphite; Pi-phosphate.

Introduction

Weathered soils such as Oxisols of tropical and subtropical regions of the world often exhibit low phosphorus (P) availability to plants, due mainly the high rates of soil P fixation and formation of insoluble complexes with aluminum and iron ions under acidic conditions (Wissuwa, 2003). Thus most P in these soils is present in a form that is unavailable to plants, and this fact has limited the yield of agricultural crops in developing countries. Phosphate (Pi) and phosphite (Phi) are the two main P forms used in agriculture, in which there are several P forms present in the environment. Phosphate anion ($H_2PO_4^-$, HPO_4^{2-} and PO_4^{3-}) is undoubtedly the major form of P utilised by plants for their adequate growth and development, while Phi anion ($H_2PO_3^-$ and HPO_3^{2-}) is effective in controlling some important plant diseases, especially those caused by organisms taxonomically classified in the phylum Oomycota, such as *Phytophthora* sp. The action of Phi is based on two mechanisms, being direct through effects on the pathogen, and indirect effects on the plant, because Phi positively stimulates metabolism of plants such as increasing defense responses during situations of abiotic and biotic stresses (Wilkinson et al., 2001; Reuveni et al., 2003; Shearer and Fairman, 2007; Orbović et al., 2008; Cook et al., 2009; Silva et al., 2011; Olivieri et al., 2012). The stimulant effect of Phi on metabolism of plants was reported by Lovatt and Mikkelsen (2006) who mentioned that Phi may influence the sugar metabolism, cause internal hormonal and chemical change, and stimulate shikimic acid pathway, resulting in increased floral intensity, and fruit yield and quality, such as soluble solid content. Since shikimic acid pathway, which is a pathway of plant secondary metabolism, is responsible for the biosynthesis of several aromatic compounds, there is the presupposition that Phi may induce the reinforcement of cell wall polymers with a deposition of lignin derivatives. In agreement with these previous reports, Olivieri et al. (2012) found that Phi positively stimulated structural and biochemical changes in periderm and cortex of *Solanum tuberosum*. Moreover, there was also reported that Phi may increase the activity of phenylalanine ammonia-lyase (PAL) and the biosynthesis of phytoalexins (Saindrenan and Guest, 1995), activity of guaiacol peroxidase in maize plants (Ávila et al., 2011), and content of ascorbic acid in strawberry plants (Moor et al., 2009). Hence, in Pi-sufficient plants, these biochemistry and structural alterations induced by Phi support the metabolic advantages of Phi against stress agents such as reducing the incidence of some plant diseases, as already reported here. Besides being used as fungicides and biostimulants, recently Phi-based products have also been marketed in the world as fertilizers for foliar spray, fertigation and direct soil application (Thao and Yamakawa, 2009). Phosphite salts are recommended as a fertilizer

because they contain a cation that may be a plant nutrient, such as K^+, NH_4^+, Ca^{2+}, Mg^{2+}, Cu^{2+} or Zn^{2+}, and often Phi is also marketed as an additional source of P for plant nutrition. Considering that P is one of the most common limiting plant nutrients in the tropics, the divulgation of the Phi-based products as a possible source of P for crop nutrition is particularly interesting for marketing purposes. Common bean (*Phaseolus vulgaris* L.) crop is one of the most important grain legumes for human consumption, and it is a major source of protein in many parts of the world, especially in developing countries (Graham and Ranalli, 1997; Broughton et al., 2003). However its productivity is low in these regions due to diseases and low soil fertility (Allen et al., 1998; Hillocks et al., 2006). Phosphite-based products have been recommended for common bean crop as fungicides, plant biostimulants, or P fertilizers, and this is probably due to susceptibility of this crop to various diseases, and also due to P deprivation of the tropical soils. Lovatt (1990a, b) reported that the application of Phi improved fruit set and yield of *Persea americana* (avocado), and restored normal growth of Pi-starved *Citrus spp*. Similarly, Albrigo (1999), Rickard (2000) and Watanabe (2005) also mentioned positive effects of Phi on plant P nutrition or yield in some crops. On the other hand, others studies have indicated that the Phi anion may not be used by plants as a P nutrient, even though it is well absorbed by leaves and roots (Thao and Yamakawa, 2009). In addition, Phi supply may cause growth depression in Pi-starved plants (Schroetter et al., 2006; Thao et al., 2008; Thao et al., 2009; Zambrosi et al., 2011). In this case, it appears that Phi acts as a repressor of plant responses to P starvation, by decreasing acid phosphatase activity (Ticconi et al., 2001; Varadarajan et al., 2002). Several reactive oxygen species (ROS) are continuously produced in plants as byproducts of aerobic metabolism (Apel and Hirt, 2004). Antioxidant enzymes have an important role in the plant cellular protection against stress-induced cell damage caused by formation of free radicals, mainly in the form of ROS. As a result, it have been related that increase of the activity of antioxidant enzymes may improve the growth and yield of the crops. In agreement with this, Ramos et al. (2010) related that increased SOD and CAT activity induced by supply of low selenium concentrations improved the lettuce (*Lactuca sativa* L.) leaf yield. In Pi-sufficient plants, stimulation of secondary metabolism by Phi may potentially increase the activities of important antioxidant enzymes, such as the catalase enzyme. Nonetheless, there is still insufficient information to test the hypothesis that Phi may stimulate activity of antioxidant enzymes. The aim of this study was to (i) understand the Phi action used as P source on growth and grain yield, (ii) measure P concentration and accumulation in shoot and root, and (iii) evaluate enzymatic behavior in common bean (*Phaseolus vulgaris* L.) plants grown in nutrient solution under Pi starvation.

Results

Influence of phosphite and phosphate on shoot and root

Shoot and root weights of Pi-sufficient *Phaseolus vulgaris* plants were not affected by Phi levels in nutrient solution (Fig. 1 A, B, C, and D). However, high Phi levels (256 and 512 µM Phi) decreased shoot and root dry weight in Pi-starved plants. At higher level (512 µM Phi), there was a reduction in shoot dry weight of 43 and 55% for Pi-starved plants evaluated in flowering and mature stages, respectively, compared with the control; while for root dry weights were showed decreases of 25 and 44% during flowering and mature stages, respectively. These Pi-starved plants at 512 µM Phi also exhibited Phi-toxicity symptoms such as curved and malformed leaves, and necrosis in older leaves. According to this Phi at higher level was toxic for Pi-starved plants. Pi-sufficient common bean plants exhibited much higher shoot dry weight than Pi-starved common bean plants, and there was no significant variation of shoot dry weight between the two growth stages (Fig. 1 A and B). Root dry weight was increased in Pi-sufficient common bean plants at mature grain stage (Fig. 1 D), but interestingly root dry weight did not vary between Pi-starved and Pi-sufficient plants at flowering stage, with the exception of plants grown under 512 µM Phi. In additional treatments, which were applied only in plants evaluated at mature grain stage, no P supply and P supply using only Phi (800 µM Phi) decreased the shoot and root dry weight by around 93 and 81% (Fig. 1 B and D), respectively, compared with plants grown under 800 µM Pi (Pi-sufficient plants). Root to shoot ratios (Fig. 1 E and F) of common bean plants were not affected significantly by Phi supply in nutrient solution. Root to shoot ratio was 3-fold higher in Pi-starved plants than Pi-sufficient plants at flowering stage, but this ratio did not differ significantly when the plants were evaluated at mature grain stage. Interesting, Pi-starved plants and Pi-sufficient plants exhibited higher and lower values of root to shoot ratio, respectively, at flowering and mature grain stages. No P supply and P supply only as Phi in nutrient solution (additional treatments) increased root to shoot ratio by around 3.3- and 2.2-fold respectively, compared with Pi-sufficient plants grown under control treatment (Fig. 1 F). In general, both additional treatments considerably increased root to shoot ratio of the plants at mature grain stage.

Interference induced by phosphite and phosphate on tissue P concentration and total P accumulation

The values of tissue P concentration and total P accumulation in shoot and root were evaluated only when the plants were at flowering stage, the stage in which common bean exhibits high metabolic activity. Tissue P concentration and total P accumulation in shoot and root of Pi-sufficient plants were not significantly ($p > 0.05$) affected by Phi treatments applied in nutrient solution (Fig. 2 A, B, C and D). Nevertheless, in Pi-starved plants, shoot and root exhibited a progressive increase in tissue P concentration from 32 and 128 µM Phi, respectively. At the highest Phi level (512 µM Phi) there was a substantial increase, corresponding to 7.2-fold in shoot and 11.7-fold in root in tissue P concentration of these Pi-starved plants (Fig. 2 A and C), compared with the control. Total P accumulation in shoot and root (Fig. 2 B and D) of Pi-starved plants were also increased from 128 and 256 µM Phi, respectively; but the differences were of smaller magnitude than those found for the tissue P concentrations. At the highest Phi level, the values of total P accumulation in shoot and root of these plants were 4.5- and 8.7-fold higher, respectively, compared with the control. At the control treatments (without application of Phi in nutrient solution), Pi-sufficient plants exhibited much higher tissue P concentration and total P accumulation than Pi-starved plants. However, in the treatment with 512 µM Phi, the tissue P concentration in shoot was higher for Pi-starved plants, and tissue P concentration in root did not differ between Pi-sufficient and Pi starved plants. These effects were mainly due to large increase of tissue P concentration in Pi-starved plants.

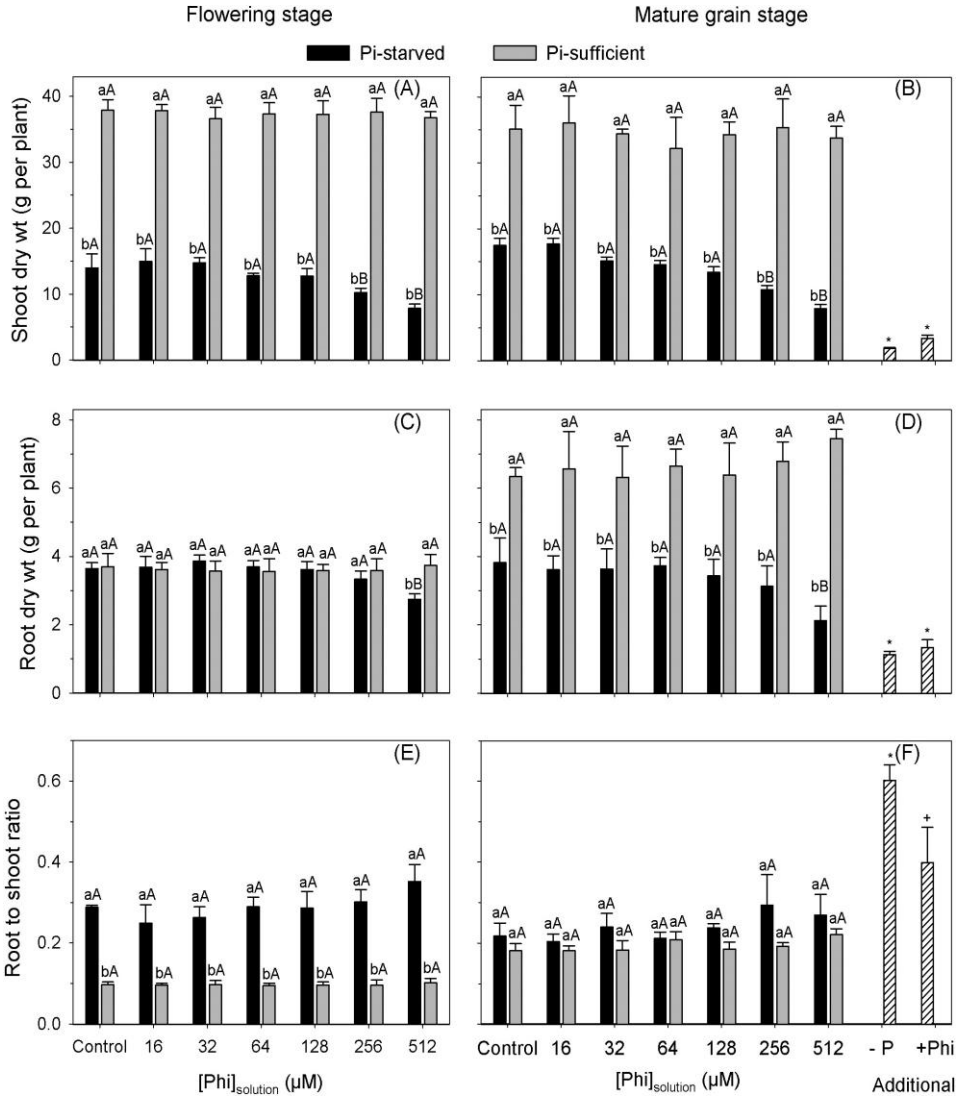

Fig 1. Shoot dry weight (A and B), root dry weight (C and D), and root to shoot ratio (E and F) at 2 different growth stages (flowering and mature grain stages) of *Phaseolus vulgaris* plants grown in nutrient solution under 2 phosphate levels (Pi-starved and Pi-sufficient plants) and 6 phosphite (Phi) levels + control (without Phi supply). For plants harvested at mature grain stage, additional treatments are: –P = no P supply in nutrient solution; and +Phi = all the P (800 µM) from nutrient solution was supplied only as Phi. Values represent the mean value of 3 replicates ± SD (Standard deviation). Averages followed by the same lowercase letter among Pi levels (Pi-starved and Pi-sufficient), and uppercase letter among Phi levels (control and 16-512 µM P) for each Pi level, do not differ among themselves by the Scott Knott's test ($p \leq 0.05$). Values marked by asterisks (*) indicate significant differences ($p \leq 0.05$) between the factorial experiment treatments and the two additional treatments (-P and +Phi). In figure 1D, value marked by plus (+) indicate significant difference between the two additional treatments ($p \leq 0.05$).

Effect of phosphite and phosphate on P nutrition indexes

The indexes of P uptake efficiency, which represent the ability to take up the P from nutrient solution, P utilization efficiency, that is the ability to produce biomass for a given P concentration (Siddiqi and Glass, 1981), and P transport from root to shoot, were measured at flowering stage of the common bean plants (Fig. 3). The index of P uptake efficiency of the Pi-sufficient plants did not differ significantly between control and all the Phi levels (Fig. 3 A). However in Pi-starved plants, from 128 µM Phi this index increased with increasing the levels of Phi in nutrient solution. Phosphate-starved plants grown under 512 µM Phi exhibited 7-fold higher P uptake efficiency than Pi-starved plants grown under control treatment (without Phi supply in nutrient solution). In general Pi-sufficient common bean showed much higher values of P uptake efficiency in all Phi treatments. In control treatments, for example, P uptake efficiency of Pi-sufficient plants was 11.5-fold higher than Pi-starved plants. The P utilisation efficiency index of the Pi-sufficient plants was also not influenced by Phi levels. However for the Pi-starved plants, from 32 µM Phi, this index was considerably decreased with increasing the levels of Phi in nutrient solution (Fig. 3 B). Phosphorus utilisation efficiency of Pi-starved plants grown at the higher Phi level (512 µM Phi) was 12.4-fold lower than that of Pi-starved plants grown under control treatment (without Phi application in nutrient solution). Considering only the control treatments, P utilisation efficiency of Pi-starved plants was around 1.7-fold higher than that of Pi-sufficient plants. But at the higher Phi level the P utilisation efficiency of Pi-starved plants was around 7.2-fold lower than that of Pi-sufficient plants, due to considerable negative effect of the maximum Phi level on this important P nutrition index. The index of P transport from

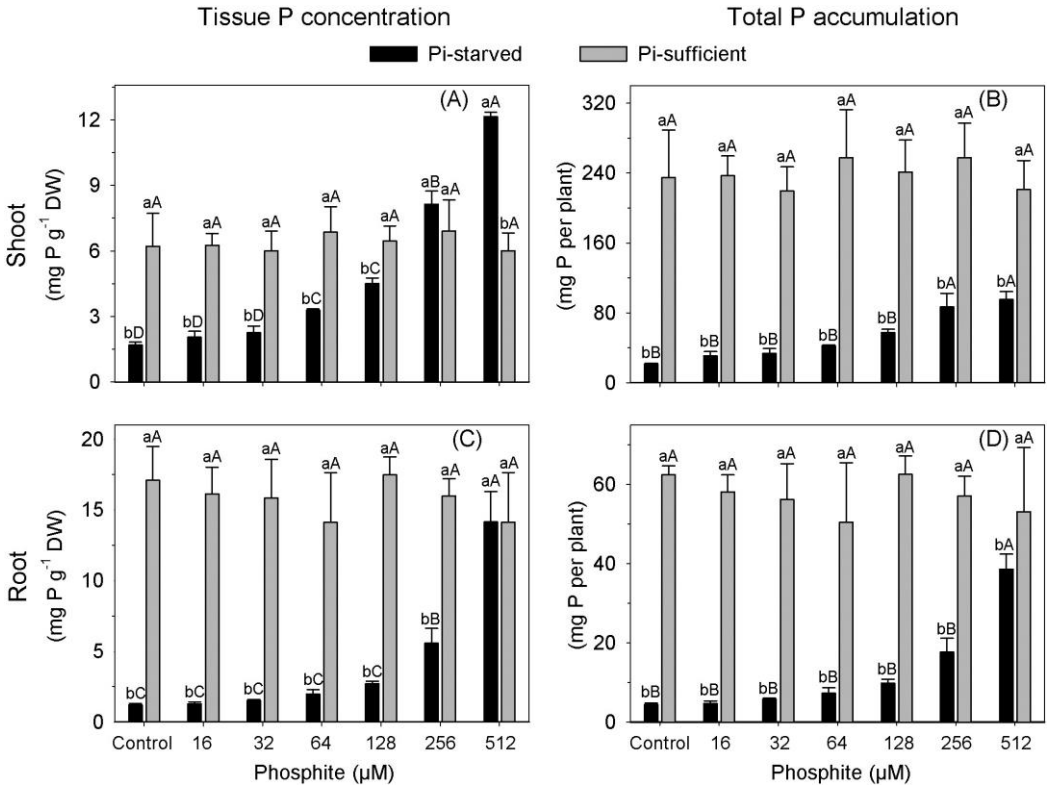

Fig 2. Shoot P concentration (A), shoot P accumulation (B), root P concentration (C), and root P accumulation (D) at flowering stage of *Phaseolus vulgaris* plants grown in nutrient solution under 2 phosphate levels (Pi-starved and Pi-sufficient plants) and 6 phosphite (Phi) levels + control (without Phi supply).
Values represent the mean value of 3 replicates ± SD (Standard deviation). Averages followed by the same lowercase letter among Pi levels (Pi-starved and Pi-sufficient), and uppercase letter among Phi levels (control and 16-512 µM P) for each Pi level, do not differ among themselves by the Scott Knott's test ($p \leq 0.05$).

root to shoot did not vary between the control and all the Phi levels for Pi-sufficient common bean (Fig. 3 C), while in Pi-starved plants this index was affected significantly only in 512 µM Phi. At this Phi level, P transport from root to shoot of Pi-starved plants was slightly reduced (by 14%), compared with the control. In general the values of P transport from root to shoot in the common bean plants were around 0.8 (about 80% of the total P taken up by plants were transported from root to shoot), and there was no significant difference of P transport between Pi-sufficient and Pi-starved plants, with exception of higher Phi level at which the value of P transport of Pi-starved plants was slightly decreased.

Modifications produced by phosphite and phosphate on acid phosphatase activity

The acid phosphatase activity of Pi-sufficient plants was not affected with the Phi treatments (Fig. 4). However for Pi-starved plants the application of Phi levels in the nutrient solution decreased the acid phosphatase activity in concentrations of 16 µM Phi, compared with the control, but only from 32 µM Phi there was significant ($p < 0.05$) decrease in comparison to control (0 µM Phi). In Pi-starved plants, the acid phosphatase activity of the two higher Phi levels (256 and 512 µM Phi) was around 48% lower than that of the control treatment (without Phi supply). Considering only the control treatments, the acid phosphatase activity of Pi-starved plants was 56% higher than that of Pi-sufficient plants. Nevertheless, in concentrations of 256 and 512 µM Phi the activity values of this enzyme did not differ between Pi-starved plants and Pi-sufficient plants.

Impact of phosphite and phosphate on catalase enzyme

The values of catalase (CAT) activity in Pi-sufficient plants was affected significantly only in 512 µM Phi, in which the activity of this enzyme was 71% higher compared with Pi-sufficient plants grown under control treatment (0 µM Phi) (Fig. 5). Nevertheless, CAT activity of Pi-starved plants increased substantially with increasing the Phi levels until 32 µM Phi, and decreased progressively in concentrations from 64 µM Phi. Thus, Pi-starved and Pi-sufficient plants exhibited higher CAT activity in concentrations of 32 and 512 µM Phi, respectively. In general, Pi-starved common bean plants exhibited considerably higher CAT activity in concentrations up to 64 µM Phi, and at the two higher Phi levels (256 and 512 µM Phi) there was no significant difference ($p > 0.05$) of CAT activity values between Pi-starved and Pi-sufficient plants.

Impact produced by phosphite and phosphate on grain yield

Grain dry weight of Pi-sufficient common bean plants did not vary significantly with any of the levels of Phi in nutrient solution (Fig. 6). However Pi-starved common bean plants exhibited decreased grain dry weight when grown under 32 µM Phi, and these plants did not produce grains when grown from 64 µM Phi. Treatment under 16 µM Phi did not influence the grain yield of the Pi-starved plants. Common

bean plants grown under both additional treatments (–P = no P supply, and +Phi = supply of 800 µM of P only as Phi) also did not produce grains. In general, grain yield in Pi-sufficient plants was 4-fold higher than Pi-starved plants grown under control treatment (without supply of Phi in nutrient solution).

Visual appearance of pod induced by phosphite

Toxicity symptoms of Phi on grain yield of Pi-starved common bean were also supported by the visual appearance of these plants (Fig. 7). Development of pods in Pi-sufficient plants was not altered by Phi treatments. On the other hand, Phi-starved common bean exhibited much more pods per plant in treatments with 64, 128, 256 and 512 µM Phi, but these pods were small and malformed that resulted in no-filled grains (Fig. 7). This harmful effect of Phi on development of pods in Pi-starved plants was increased with increasing the levels of Phi in nutrient solution.

Effects promoted by phosphite and phosphate on P concentration and P accumulation in grain

Fig. 8 shows the values of tissue P concentration and total P accumulation in common bean grains, from plants harvested at mature grain stage. Tissue P concentration and total P accumulation in grains of Pi-sufficient plants were not significantly affected by all Phi levels. For Pi-starved plants, tissue P concentration and total P accumulation were studied only in the control treatment (without Phi supply in nutrient solution) and in treatments corresponding to 16 and 32 µM Phi, since there was not grain yield in the other Phi treatments, as well as in both additional treatments (–P = no P supply, and +Phi = supply of 800 µM of P only as Phi). Pi-starved plants exhibited significantly higher tissue P concentration in the grain, when grown under 32 µM Phi, while 16 µM Phi did not alter the tissue P concentration in the grain of these plants compared with the control (Fig. 8 A). Additionally, total P accumulation in grains of Pi-starved plants did not vary with the supply of the two first Phi levels compared with the control (Fig. 8 B).

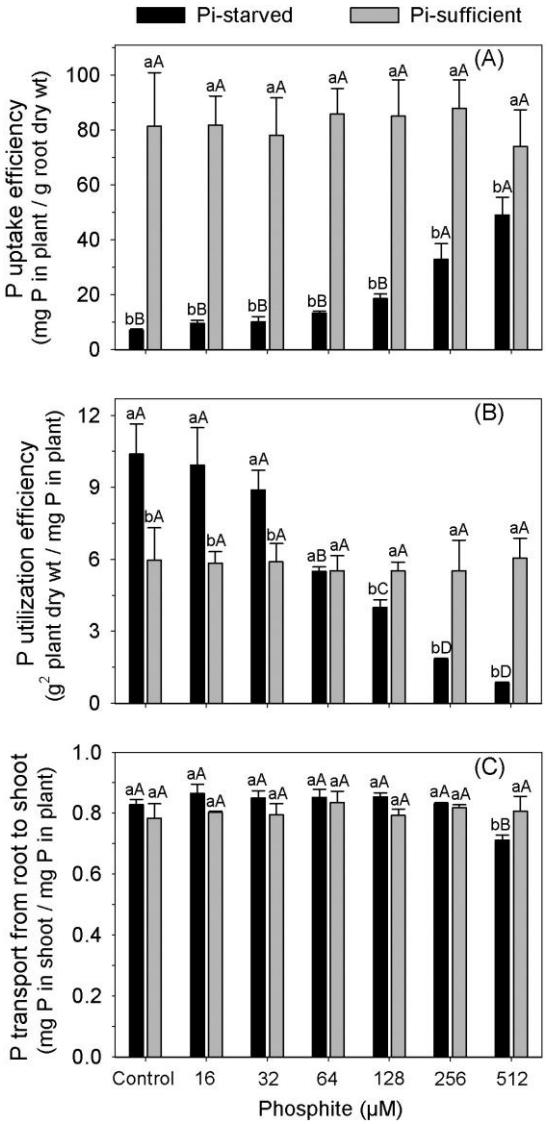

Fig 3. P uptake efficiency (A), P utilisation efficiency (B) and P transport from root to shoot (C) at flowering stage of *Phaseolus vulgaris* plants grown in nutrient solution under 2 phosphate levels (Pi-starved and Pi-sufficient plants) and 6 phosphite (Phi) levels + control (without Phi supply). Values represent the mean value of 3 replicates ± SD (Standard deviation). Averages followed by the same lowercase letter among Pi levels (Pi-starved and Pi-sufficient), and uppercase letter among Phi levels (control and 16-512 µM P) for each Pi level, do not differ among themselves by the Scott Knott's test ($p \leq 0.05$).

Discussion

In Pi-sufficient and Pi-starved plants regardless of the applied Phi treatments, we found different root growth responses between flowering and mature grain stages. At the flowering stage, shoot biomass weight was much higher at the Pi-sufficient plants but root biomass weight was little altered between Pi-sufficient and Pi-starved plants, thereby showing that Pi-starved common bean plants exhibited increased root growth rate at the expense of the shoot growth rate. These data were confirmed with data of root to shoot ratio at flowering stage, in which Pi-starved plants exhibited higher root to shoot ratio than Pi-sufficient plants, and plants grown under additional treatments, mainly under the first additional treatment (no P supply in nutrient solution), exhibited higher values of root to shoot ratio than those of all others treatments. These increased values of root to shoot ratio in Pi-starved plants at flowering stage is a mechanism for overcoming P deprivation from growth medium (Ticconi et al., 2001; Devaiah et al., 2007). For plant biomass weight at mature grain stage, we observed that there was no significant variation of the shoot biomass yield from full flowering stage. But, interestingly, there was considerable increase in root biomass yield from full flowering stage to mature grain stage at the Pi-sufficient plants, while at the Pi-starved plants this increase was not significant. Thus, at the mature grain stage both shoot and root growth were increased at the Pi-sufficient plants, while data of root to shoot ratio did not differ significantly between Pi-sufficient and Pi-starved plants. Hence, in this study the evaluation of the plant biomass at two different periods of time (flowering and mature grain stages) was crucial to show the variations of shoot and root growth between Pi-starved and Pi-sufficient common bean. Supply of Phi in nutrient solution in general did not affect the tissue P concentration and total P accumulation in shoot, root and grain of Pi-sufficient common bean plants. These data disagree with those of Thao et al. (2009), which noticed that tissue P concentration in Pi-sufficient hydroponic lettuce was increased with increasing the Phi levels in nutrient solution. However we found that Pi-

starved common bean plants exhibited much higher tissue P concentration in shoot and root when grown under high Phi levels. Although Phi-starved plants did not produce grains at the high Phi levels, increased tissue P concentration in grains of Pi-starved plants was observed at the second Phi level. This higher tissue P concentration of Pi-starved plants grown under high Phi levels was not due only to "concentration effect" (caused by inhibitory effect of Phi on growth and yield of the Phi-starved plants) but also due to increased uptake of P from nutrient solution, since total P accumulation (i.e. estimation of the amount of P taken up) of the Pi-starved plants did not decrease with the Phi levels applied (actually the contrary was observed, in which total P accumulation in shoot and root of Pi-starved plants was increased at the high Phi levels). In this case, our data of tissue P concentration for Pi-starved common bean plants are in agreement with those of Thao et al. (2009) who also observed that tissue P concentration in Pi-starved hydroponic lettuce was increased with increasing the Phi levels in nutrient solution. We also showed that application of Phi in the growth medium (nutrient solution) did not influenced the P nutrition indexes of the Pi-sufficient common bean plants, but increased the P uptake efficiency and, at the same time, decreased the P utilization efficiency of the Pi-starved common bean plants. In this study the P uptake efficiency refers to total P taken up by plant per root weight unit, and the P utilization efficiency refers to the plant's ability to produce biomass for a given P concentration according to Siddiqi and Glass (1981). Thus our data showed that the high Phi levels from nutrient solution enhanced the uptake of P per unit of root mass but did not improve the P nutrition of the Pi-starved plants. When only plants from control treatments (without Phi supply) were considered, we observed that Pi-starved plants exhibited much lower P uptake efficiency (due to low availability of P from nutrient solution) but at the same time these plants exhibited higher P utilization efficiency, compared with the Pi-sufficient plants. This increase of the P utilization efficiency by Pi-starved common bean plants was a response to Pi deprivation from growth medium. Rouached et al. (2010) mentioned several molecular mechanisms that regulate gene expression in plants are modified during Pi starvation. When Phi anion was not added in nutrient solution, *in vivo* acid phosphatase activity of Pi-starved plants was higher than that of Pi-sufficient plants. This is also an adaptive mechanism of plants in order to grow better in P-deficient environment (Tadano et al., 1993), in which acid phosphatase may hydrolyse organic P compounds within the plant and rhizosphere (acid phosphatase secreted by the roots) and liberate inorganic P (Haussling and Marschner, 1989; Tadano et al., 1993; Yun and Kaeppler, 2001; Louw-Gaume et al., 2010). However, we found that *in vivo* acid phosphatase activity of Pi-starved common bean plants was considerably decreased with increasing Phi levels in nutrient solution. Thus these data of *in vivo* acid phosphatase activity corroborated the presupposition that Phi anion was taken up by Pi-starved plants reducing their acid phosphatase activity, but at the same time these plants did not utilize Phi anion as a P source for its growth and development. These data agree with those previously reported by Ticconi et al. (2001) and Varadarajan et al. (2002) who found that the addition of Phi in the growth medium inhibited mechanisms of overcoming P starvation in Pi-starved *Arabidopsis* and tomato, such as gene expression and activities of acid phosphatase enzymes. This study showed that, in Pi-starved plants, catalase (CAT) activity was considerably higher when Phi was applied in low levels, while medium and high Phi levels reduced substantially the activity of this enzyme.

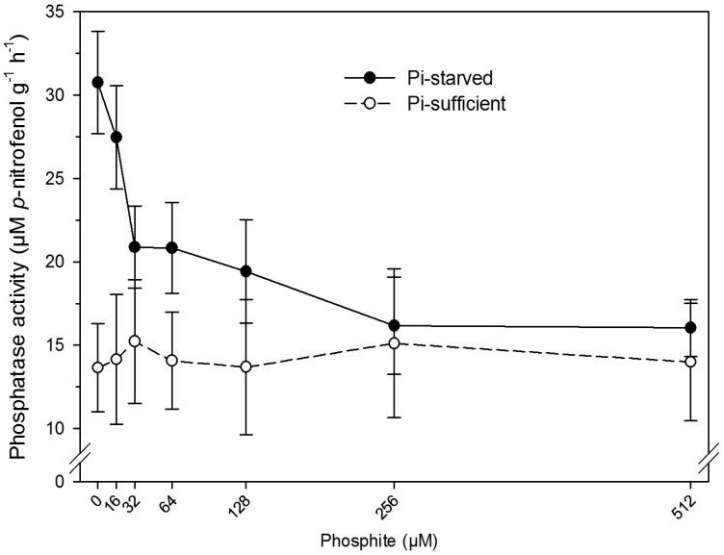

Fig 4. *In vivo* acid phosphatase activity in youngest mature trifoliate leaf at flowering stage of *Phaseolus vulgaris* plants grown in nutrient solution under 2 phosphate levels (Pi-starved and Pi-sufficient plants) and 6 phosphite (Phi) levels + control (without Phi supply).
Values represent the mean value of 3 replicates ± SD (Standard deviation).

Antioxidant enzymes, such as CAT, have an important role in the plant cellular protection against stress-induced cell damage caused by formation of free radicals, mainly in the form of ROS. Catalase is an important antioxidant enzyme involved in ROS detoxification. However, in Pi-sufficient plants, CAT activity did not vary in the low and medium Phi levels, while the supply of high Phi level increased its activity by 71%. This data indicate a possible beneficial effect of Phi on Pi-sufficient common bean plants, although growth and grain yield of these Pi sufficient plants were neither increased nor decreased at all the Phi levels. Recent studies have shown that the Phi anion may induce molecular alterations that increase resistance to stress agents, such as stimulation of guaiacol peroxidase activity and lignin biosynthesis in maize (Ávila et al., 2011), and structural and biochemical changes in potato tuber periderm and cortex (Olivieri et al., 2012). On the other hand, studies that relate Phi effects on antioxidant enzymes are still rare. In agreement with our data, Moor et al. (2009) found that soaking strawberry plants in Phi solution resulted in increased content of ascorbic acid (compounds known to have antioxidant properties) in the fruits. In this investigation, we showed that growth and grain yield of the common bean plants grown under adequate Pi supply (Pi-sufficient plant) were not affected by Phi levels applied in the growth medium (nutrient solution). Although Lovatt and Mikkelsen (2006) reported that Phi anion may stimulate growth of some crop when grown under adequate Pi conditions, our data indicated that Phi supply did not provide stimulation on growth and grain yield of the Pi-sufficient common bean plants grown under greenhouse environment conditions and in the absence of pathogens. In agreement with these results, Thao et al. (2009) did not observe a stimulant effect of Phi anion on hydroponic lettuce growth.

Nonetheless, grain yield of the common bean plants grown under low Pi supply (Pi-starved plant) was strongly inhibited from the low Phi levels, as shown in our results, although shoot and root growth of these plants at both growth stages (flowering and mature grain stages) decreased significantly only at the higher Phi level. Thus, besides not being a P

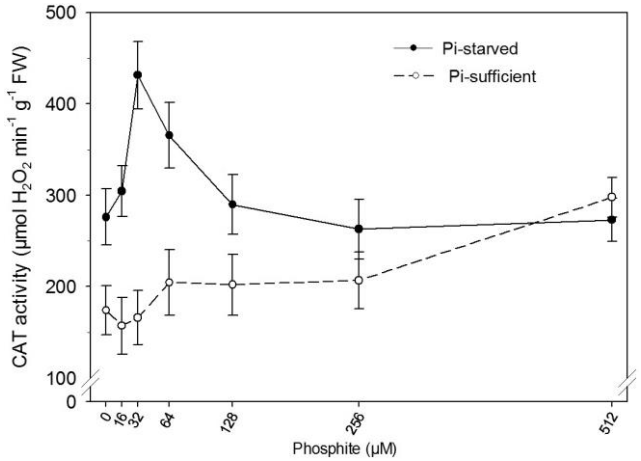

Fig 5. Catalase activity in youngest mature trifoliate leaf at flowering stage of *Phaseolus vulgaris* plants grown in nutrient solution under 2 phosphate levels (Pi-starved and Pi-sufficient plants) and 6 phosphite (Phi) levels + control (without Phi supply).
Values represent the mean value of 3 replicates ± SD (Standard deviation).

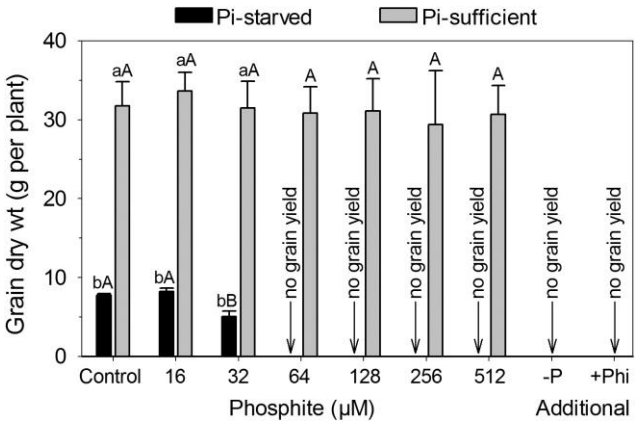

Fig 6. Grain dry weight at mature grain stage of *Phaseolus vulgaris* plants grown in nutrient solution under 2 phosphate levels (Pi-starved and Pi-sufficient plants) and 6 phosphite (Phi) levels + control (without Phi supply). Additional treatments are: –P = no P supply in nutrient solution; and +Phi = all the P (800 µM) from nutrient solution was supplied only as Phi.
Values represent the mean value of 3 replicates ± SD (Standard deviation). Averages followed by the same lowercase letter among Pi levels (Pi-starved and Pi-sufficient), and uppercase letter among Phi levels (control and 16-512 µM P) for each Pi level, do not differ among themselves by the Scott Knott's test ($p \leq 0.05$).

source for common bean plants, Phi anion was strongly harmful to grain yield of the Pi-starved plants. This conclusion was also supported by the visual aspect of the pods of these plants (Fig. 7). Hence, in this work the evaluation of the grain (edible part) yield was crucial to show the harmful effects of Phi anion on yield of the Pi-starved common bean plants. Although there is little information in the literature comparing the effects of Phi supply on grain yield in leguminous crops, there are some previous studies with *Arabidopsis*, vegetables and some others crops that are in agreement with our data (Ticconi et al., 2001; Varadarajan et al., 2002; Lee et al., 2005; Schroetter et al., 2006; Devaiah et al., 2007; Thao et al., 2008; Thao et al., 2009). The causes of this harmful effect of Phi anion on Pi-starved plants are not well understood yet. The most plausible hypothesis to date is that, although Pi and Phi appear to be indistinguishable by Pi uptake transporter sites, plants are unable to metabolise Phi anion, which, after uptake, this anion remains stable in the cell compartments. Furthermore, Phi anion suppresses some plant responses to Pi deprivation, such as syntheses of acid phosphatases, phosphodiesterases, nucleases, and high-affinity P transporters (Ticconi et al., 2001; Abel et al., 2002; Varadarajan et al., 2002; Lee et al., 2005; Ávila et al., 2011). In agreement with this explanation, based on the kinetic studies of the Pi transport system of tobacco BY-2 cells, Danova-Alt et al. (2008) demonstrated that Phi inhibited Pi uptake in a competitive manner. Within the plant, the same authors also showed by *in vivo* ^{31}P nuclear magnetic resonance spectroscopy that there is a intracellular accumulation of Phi in Pi-starved cells, but the Pi resupply results in a rapid efflux of Phi from apoplast and cytoplasm. Thereby the authors related that tobacco BY-2 cells predominantly accumulate Phi in the cytoplasm in Pi-starved cells, but, in contrast, Phi accumulates almost exclusively into vacuoles in Pi-sufficient cells. Thus the results of Danova-Alt et al. (2008) may help to explain, in part, the harmful effects of Phi in Pi-starved plants, while in Pi-sufficient plants these harmful effects have not been reported. Moreover, in Pi-sufficient plants, Phi may be benefic such as reducing the incidence of some plant diseases and inducing some molecular mechanisms that increase resistance to stress agents, as has already been showed. In this investigation, additional treatments were conducted to confirm that the Phi anion did not replace Pi anion in common bean P nutrition. When data from the second additional treatment (supply of 800 µM of P only as Phi) were compared with those of Pi-sufficient plants (supply of 800 µM of P only as Pi), we verified that growth parameters and grain yield of the plants grown under second additional treatment were strongly inhibited. Moreover, the growth of the plants grown under the second additional treatment was similar with that of plants grown under the first additional treatment (no P supply in nutrient solution), and all plants grown in both additional treatments did not produce grains. These data corroborate those of Lee et al. (2005) for *Ulva lactuca*, Schröetter et al. (2006) for *Zea mays*, Thao et al. (2008) for *Brassica rapa*, Ávila et al. (2011) with *Zea mays*, Zambrosi et al. (2011) for *Citrus spp.* rootstocks, and Hirosse et al. (2012) for *Ipomoea batatas* tissue cultures. These authors found that the Phi anion did not replace Pi anion in plant P nutrition. Furthermore they also reported that the use of Phi as sole P source generally caused a significant reduction in plant growth compared with the treatments with either null or insufficient Pi fertilization.

Materials and methods

Experiment localisation, plant material, and seedling obtaining

Study was conducted in Departamento de Ciência do Solo of the Universidade Federal de Lavras, Brazil (21°14' S; 45°00' W; 915 m asl). The plants were grown in a greenhouse environment. Photoperiod was 12 h of light. Seeds of the common bean (*Phaseolus vulgaris* L.) cv. Radiante were germinated in expanded polystyrene trays containing 128 compartments filled with vermiculite and irrigated with distilled water. Five days after emergence, seedlings were

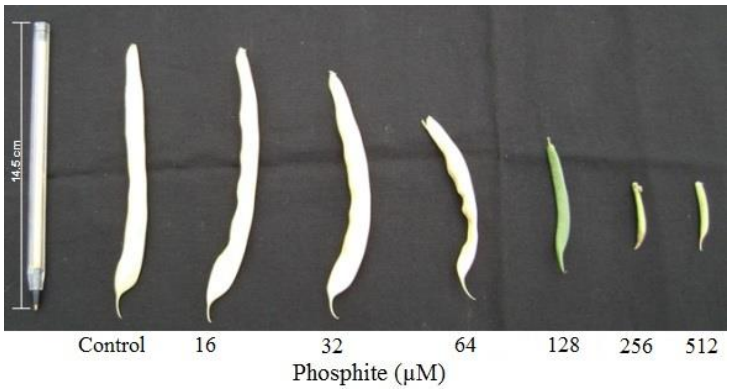

Fig 7. Toxicity symptoms on grain yield at *Phaseolus vulgaris* plants grown in nutrient solution under low phosphate level (Pi-starved plants), as affected by 6 phosphite (Phi) levels + control (without Phi supply).

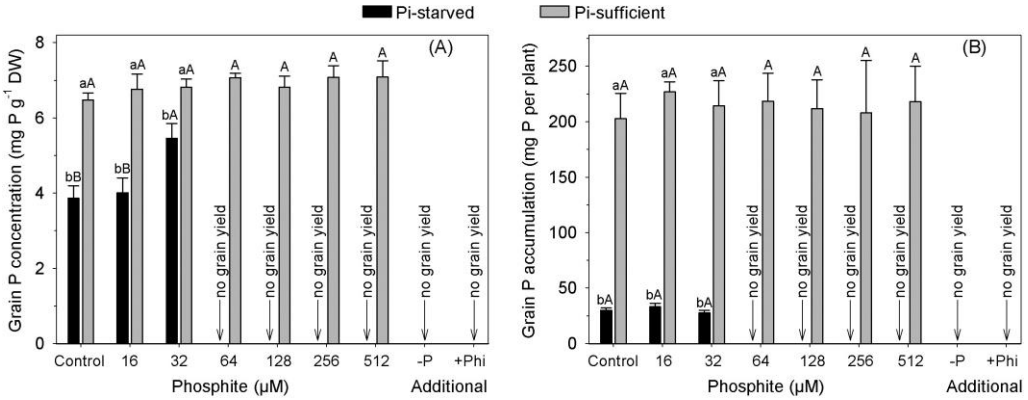

Fig 8. Grain P concentration (A) and grain P accumulation (B) at mature grain stage of *Phaseolus vulgaris* plants grown in nutrient solution under 2 phosphate levels (Pi-starved and Pi-sufficient plants) and 6 phosphite (Phi) levels + control (without Phi supply). Additional treatments are: –P = no P supply in nutrient solution; and +Phi = all the P (800 µM) from nutrient solution was supplied only as Phi. Values represent the mean value of 3 replicates ± SD (Standard deviation). Averages followed by the same lowercase letter among Pi levels (Pi-starved and Pi-sufficient), and uppercase letter among Phi levels (control and 16-512 µM P) for each Pi level, do not differ among themselves by the Scott Knott's test ($p \leq 0.05$).

transferred to plastic box containing 36 L of one-fourth-strength modified Hoagland's solution (Jones Junior, 1983).

Experimental design and treatments

This study was conducted in a completely randomised experimental design with 3 replicates, being 7 phosphite (Phi) levels (0, 16, 32, 64, 128, 256 and 512 µM) and 2 phosphate (Pi) levels (80 and 800 µM, these levels considered Pi-starved plants and Pi-sufficient plants, respectively) in nutrient solution. Common bean plants were evaluated at 2 different growth stages: flowering and mature grain stages. For plants harvested at the mature grain stage, two more treatments (additional treatments) were added: –P = no P supply in nutrient solution; and +Phi = all the P (800 µM) from nutrient solution was supplied only as Phi. Each experimental unit consisted of one common bean plant per pot.

Plant culture and treatment applications

The 10-day-old young seedlings were selected for regular leaf size and area and transplanted to plastic pots containing 3 L of half-strength modified Hoagland's solution. Five days after transplanting, these plants were grown in full-strength modified Hoagland's solution with the Phi and Pi treatments. Phosphite used in the experiment was obtained by the reaction of phosphorous acid with potassium hydroxide, resulting in potassium Phi. The nutrient solution was changed twice each week. The volume of the nutrient solution in each plastic pot was supplemented daily with deionised water and pH was adjusted to 5.5 (± 0.3) by adding 0.5 M NaOH or HCl. Throughout the experimental period the nutrient solution was constantly aerated.

Harvest, biomass yield and phosphorus nutrition

Three replicates of each treatment were harvested when plants were at full flowering stage. Shoot and root dry weight of these plants were measured, after drying in a forced air oven at 60 °C until there was no change in weight, and their tissue P concentrations determined by colorimetry (Murphy and Riley, 1962) after nitric-perchloric digestion of the plant material (Johnson and Ulrich, 1959). Data from shoot and root dry weight, and tissue P concentration were used to calculate total P accumulation in shoot and root, as well as P uptake efficiency (total P accumulation in plant / root dry wt) (Swiader et al., 1994), P utilisation efficiency [(plant dry wt)^2 / (total P accumulation in plant] (Siddiqi and Glass, 1981), and index of P transport from root to shoot (total P

accumulation in shoot/total P accumulation in plant). The other 3 replicates of each treatment were harvested when plants were at mature grain stage. Grain dry weight, and grain P concentration and accumulation in these plants were also determined.

In vivo acid phosphatase and catalase activities

All enzymatic analyses were only done in plants evaluated at flowering stage. Prior to harvest of the plants, one youngest mature trifoliate leaf was collected in 3 replicates of each treatment to evaluate the *in vivo* acid phosphatase (EC 3.1.3.4.1) activity, according to Besford (1980) with minor modifications (Silva and Basso, 1993). Catalase (CAT) (EC 1.11.1.6) activity was performed according to Ramos et al. (2010) with minor modifications. During plant harvest, one youngest mature trifoliate leaf was collected in 3 replicates of each treatment and was immediately wrapped in aluminum foil, submerged in liquid nitrogen and stored in a freezer, at –80 °C. Posteriorly, frozen tissues were homogenised in a cooled 0.1 mol/L Tris–HCl buffer at pH 7.8 containing 1 mmol/L EDTA, 1 mmol/L dithiothreitol and 5 ml of 4% polyvinyl pyrrolidone per gram of fresh weight. The homogenate was filtered through a nylon mesh and centrifuged at 14000 rpm for 30 min at 4 °C. The supernatant was used to measure enzymatic activity of the CAT by observing H_2O_2 consumption at 240 nm for 5 min (Rao et al., 1997). The reaction mixture (3 ml total volume) contained 25 mM Tris-acetate buffer (pH 7.0), 0.8 mM EDTA-Na, 20 mM H_2O_2, and enzymatic assay was carried out at 25 °C.

Data analysis

Data were submitted to variance analysis (ANOVA, $p \leq 0.05$) using SAS software (SAS Institute, 1996), and when significant differences occurred the data were applied to Scott Knott's test ($p \leq 0.05$) (Scott and Knott, 1974). For plants harvested at the mature grain stage, statistical comparisons between the additional treatments, as well as between additional treatment and factorial experiment, were evaluated according to Healy (1956). Standard deviation (± SD) was calculated for all means (three replicates).

Conclusion

This study revealed that growth and grain yield in *Phaseolus vulgaris* plants grown under Pi starvation presented negative repercussion on these parameters, in which treatments at 64, 128, 256 and 512 µM Phi resulted in no-filled grains. Concentration and accumulation of P in shoot and root of Pi-starved plants was increased with increasing the Phi levels in nutrient solution, but this additional P concentration did not convert into increased growth or grain yield. Application of Phi in Pi-starved plants promoted a decrease in acid phosphatase activity, while catalase activity had increased up to 32 µM Phi and was reduced at higher levels of Phi.

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