African Journal of Biotechnology Vol. 11(19), pp. 4360-4367, 6 March, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.2900
ISSN 1684–5315 © 2012 Academic Journals

Full Length Research Paper

Symbiotic responses to insoluble phosphorus supply in common bean (*Phaseolus vulgaris* L.): Rhizobia symbiosis

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Accepted 25 November, 2011

The common bean (*Phaseolus vulgaris* L.) is often grown under various environmental constraints in soil as well as phosphorus (P) deficiency. In worldwide soils, P massively exists in insoluble forms especially in acidic and basic soils, where it is greatly combined with Al, Fe and Ca ions hydroxide. In order to study the effect of insoluble phosphorus (IP) in hydroponic culture, two common bean varieties, that is, WAFA and REBIA were inoculated separately with three rhizobia strains (P.Tb.09, CIAT899 and P.OM.09) and were grown under mineral P (250 µmol P (KH_2PO_4) versus IP (250 µmol P (Ca_2HPO_4)). Results show that inoculation of both varieties under 250 µmol P with P.Tb.09 and CIAT899 had produced a high nodule biomass of about 0.11 g plant^{-1}. However, inoculation of WAFA with P.Tb.09 and P.OM.09 yielded in a low nodule biomass (0.06 g plant^{-1}) in comparison with REBIA variety showing no variation of the strains used. The last variety in symbiosis with all the three strains, exhibited a high shoot biomass that was not significantly different with plants supplied with 250 µmol P. By contrast, a high acid phosphatase activity was more stimulated under IP conditions in leaves of WAFA inoculated with CIAT899 and P.Tb.09. Likewise, root acid phosphatase (APase) activity has increased in REBIA inoculated with P.Tb.09 for both P forms; meanwhile, inoculation with CIAT899 under IP has significantly increased roots acid phosphate (AP) activity. However, this stimulation was not associated with high plant N accumulation that was highly accumulated by both varieties inoculated with P.Tb.09 but grown under 250 µmol P. In addition, analysis of phenols content in roots showed a high stimulation when plants had received IP than those under 250 µmol P, but to the high extent in roots of REBIA inoculated with P.Tb.09 under IP conditions. We concluded that the use of P-efficient symbiotic combinations against P deficiency seems to be an interesting biological approach to increase legumes P assimilation for N_2 fixation and thus for agricultural sustainability.

Key words: Symbiosis, *Phaseolus vulgaris*, rhizobia, insoluble phosphorus, phosphatase, phenolics content.

INTRODUCTION

Phosphorous (P) is an essential macronutrient for plant growth and development. 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 one of the major yield-limiting factors for plants (CIAT, 1992) including common bean (*Phaseolus vulgaris* L.) that 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).

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They are characterized by it sensitivity to several environmental factors, such as sub-optimal availability of mineral nutrients. As an important nutrient deficiency constraint, low P availability is considered the principal limiting factor for legume growth (Andrew and Robins, 1969). It strongly reduced nitrogen (N) fixation and resulted in decreased nitrogen availability for subsequent crops. Nodule number and mass, as well as total and specific nitrogenase activity, decrease with low P supplies (Israel, 1987; Ribet and Drevon, 1995; Drevon and Hartwig, 1997). For instance, P 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). IP has a key role in the energy metabolism of all plant cells, and particularly in nitrogen fixation as an energy-requiring process (Dilworth, 1974). Zaman-Allah et al. (2007) reported that P deficiency has a negative impact on the energy status of legume nodules. An adequate distribution of P between the nodulated roots, shoots and roots was reported as an interesting parameter that may influence P use efficiency in N_2 fixing symbiosis (Cassman, 1981). Plants are known to involve several mechanisms to increase their P absorption efficiency (Kaous et al., 2009). Among the various morphological and physiological strategies to acquire sparingly soluble P from soil, enhanced rhizosphere acidification (Hinsinger et al., 2003) and phenolics secretion were of important significance. Acid phosphatase (APase) can hydrolyse a range of organic P compounds (Tarafdar and Claassen, 2005; Kaous et al., 2009). The production of phosphatase is a potential way for plants to enhance P availability, as a large proportion of soil P (up to 80%) that occurs in organic forms (Richardson et al., 2004). In this context, it has been reported that legumes secrete more acid phosphatase comparatively to cereals under P-insoluble conditions in both hydroponic and soil culture (Yadav and Tarafdar, 2003). In addition, as one of the important plant, secondary metabolites and phenolics compounds have been reported to play a variety of roles in defense mechanisms against biotic and abiotic scrape (Arfaoui et al., 2007). Considering the fact that little information is available on the physiological adaptability of common bean under IP conditions, it will be interesting to verify whether inoculation with P-efficient rhizobia may increase IP assimilation. Thus, the aim of the present study was to assess, under IP conditions, several morphological and physiological responses of the *P. vulgaris*: rhizobia symbiosis.

MATERIALS AND METHODS

Biological material, culture conditions and harvest

Two common bean varieties, the white seed WAFA and the brown seed REBIA, were used and inoculated separately with one of the following *Rhizobium* strains: *Rhizobium tropici* CIAT899 and local *Rhizobium* strains, that is, P.Tb.09 and P.OM.09 isolated from soil of the Northern Tunisia. Seeds were surface sterilized for 7 min in 2% calcium hypochlorite. They were thoroughly washed with sterile distilled water for 30 min and then germinated in agar 0.9% for 3 to 4 days. *Rhizobium* inoculums were prepared by growing bacteria in a liquid yeast extract mannitol (YEM) medium (Vincent, 1970) at 28°C for 48 h. Inoculation was applied by soaking the seedlings in the inoculants prior to transplantation. The seedlings were then transferred in hydroponic culture system. P was supplied with 250 µmol IP $plant^{-1}$ $week^{-1}$ versus 250 µmol P (KH_2PO_4) $plant^{-1}$ $week^{-1}$ as a control. During the first two weeks, that is, before nodule functioning, the nutrient solution was supplemented with 2 mM urea. Thereafter, urea was omitted from the nutrient solution that was renewed once for two weeks. Plants were grown in a temperature-controlled glasshouse with night/day temperatures of circa 20/28°C and a 16 h photoperiod. Four weeks after transplantation coinciding with flowering stage, plants were harvested, shoots and roots were separated and the nodules were detached and counted. In order to determine their dry weights, Shoots, roots and nodules were dried at 70°C for 3 days.

Determination of N and P contents in shoots, roots and nodules

Shoots, roots and nodules P were determined by the molybdate blue method (Murphy and Riley, 1962). 0.3 g of dry weight were incinerated and filtrated (through Whatman No.1 filter paper) and a final volume taken up to 50 ml with distilled water. 100 µl of each extract was added to 2.5 ml of a reactive mixture prepared with ammonium molybdate (2.5%) and hydrazine sulfate (0.15%). Thereafter, absorbance was read at 820 nm after incubation at room temperature for 30 min. For N determination, 0.5 g shoot subsamples were used and analyzed by the Kjeldahl method.

Acid phosphatase activity

Roots and shoots (0.5 g) were ground separately in a mortar with an extraction mixture consisting of 0.1 M acetate buffer (pH 5.8), 6 mM β-mercaptoethanol, 0.1 mM phenyl methyl sulfonyl fluoride and 6 g of insoluble polyvinyl polypyrrolidone. The homogenate was centrifuged at 12,000 ×g at 4°C for 30 min. The reaction mixture contained 100 mM sodium acetate buffer (pH 5.8), 5 mM P-nitrophenyl phosphate, and the enzyme extract in a total volume of 0.5 ml. After 30 min of incubation at 30°C, the reaction was stopped by the addition of 1 ml NaOH (0.5 M). Absorbance was read at 405 nm and APase activity was calculated as mmol of hydrolysed *p*NPP per g fresh weight.

Total phenols content

Frozen roots from the different treatments were reduced to a fine powder using a pestle and a pre-cooled mortar, and were extracted three times with 80% methanol at 4°C under continuous stirring. The homogenate was centrifuged for 3 min and the supernatants were analyzed by spectrophotometer. Total phenols content was estimated based on the Foline-Ciocalteu method adapted from Dicko et al. (2002) and absorption at 760 nm was read using Cary100-UV spectrophotometer. A calibration curve was generated with freshly prepared solutions of (+)-catechin. Results were calculated as mg equivalent (+)-catechin per g of fresh weight (mg eq. (+)-catechin/g fresh weight (FW)).

Statistical analysis

The experimental design was a randomized complete block.

Table 1. Nodulation (number and biomass) and growth (dry weight of shoot and root) of two common bean genotypes (WAFA and REBIA) inoculated with three rhizobia strains (CIAT899, P.Tb.09 and P.OM.09) and grown under 250 P and insoluble P (IP

Treatment	Nodule number		NDW (g Pl^{-1})		SDW (g Pl^{-1})		RDW (g Pl^{-1})	
	WAFA	REBIA	WAFA	REBIA	WAFA	REBIA	WAFA	REBIA
Control	0	0	0	0	0.13^{c} ± 0.02	1.1^{c} ± 0.31	0.2^{b} ± 0.03	0.24^{c} ± 0.03
CIAT899	135^{ab} ± 21	199^{a} ± 53.23	0.11^{a} ± 0.1	0.11^{a} ± 0.03	0.9^{a} ± 0.3	1.49^{ab} ± 0.27	0.2 2^{ab} ± 0.12	0.38^{b} ± 0.04
CIAT899+IP	112^{b} ± 23.8	152^{ab} ± 41.8	0.08^{b} ± 0.02	0.12^{a} ± 0.04	0.9^{a} ± 0.03	1.46^{ab} ± 0.14	0.25^{a} ± 0.05	0.37^{b} ± 0.07
P.Tb.09	22^{a} ± 93	201^{a} ± 75.43	0.1^{ab} ± 0.05	0.1^{ab} ± 0.06	0.94^{a} ± 0.36	1.5^{ab} ± 0.14	0.24^{a} ± 0.09	0.4^{ab} ± 0.13
P.Tb.09+IP	133^{ab} ± 62.2	132^{ab} ± 49	0.06^{bc} ± 0.01	0.09^{b} ± 0.06	0.63^{b} ± 0.36	1.18^{bc} ± 0.75	0.17^{b} ± 0.01	0.45^{a} ± 0.17
P.OM.09	71^{bc} ± 30	90^{b} ± 52	0.1^{ab} ± 0.03	0.1^{ab} ± 0.02	0.78^{ab} ± 0.53	1.78^{a} ± 0.58	0.26^{a} ± 0.04	0.42^{a} ± 0.17
P.OM.09+ IP	26^{c} ± 4	79^{b} ± 47	0.03^{c} ± 0.04	0.11^{a} ± 0.01	0.73^{ab} ± 0.46	1.32^{b} ± 0.27	0.18^{ab} ± 0.08	0.35^{bc} ± 0.06

SDW: Shoot dry weight; RDW: Root dry weight; NDW: Nodules dry weight. Data are the means ± SD of four replicates harvested at flowering stage.

Statistical analysis was performed by the SPSS 10.0 software. Data were statistically analysed by ANOVAs and subsequent comparison of means was performed using the Fisher's least significant difference (LSD) test at 5% probability.

RESULTS

Nodulation, shoot and root area determination under IP

The effect of IP on nodulation (number and dry weight of nodules) was studied in WAFA and REBIA genotypes inoculated with three *Rhizobium* strains (CIAT899, P.Tb.09 and P.OM.09) (Table 1). Results show that WAFA inoculated with strains P.Tb.09 and CIAT899 induced the highest infectivity corresponding to 133 and 135 nodules plant^{-1}, respectively. The highest nodule dry weight was recorded with P.Tb.09 (0.1 g plant^{-1}) and with CIAT899 (0.11 g plant^{-1}). However, WAFA plants inoculated with P.OM.09 exhibited the lowest nodule number (70 nodules plant^{-1}), but had a similar nodule dry weight than that produced by the remaining strains (Table 1). By contrast, in comparison to the high nodule biomass (0.08 g) yielded by CIAT899 under IP, plants supplied with IP and inoculated with P.Tb.09 and P.OM.09 exhibited low nodule biomass of 0.06 and 0.03 g, respectively (Table 1).

In REBIA variety, plants inoculated with CIAT899 and P.Tb.09 showed high nodule number than with P.OM.09 (Table 1). Application of IP did not significantly affect nodule number of the used strains. However, nodule biomass did not affect the *Rhizobium* strains and P treatments. Concerning plant growth parameters of WAFA plants grown, it appears that shoot dry weight (from 0.73 to 0.9 g) did not significantly vary with *Rhizobium* strain inoculation (Table 1). By contrast, IP significantly reduced shoot dry weight, more particularly when plants were inoculated with P.Tb.09 (0.3 g). REBIA plants in symbiosis with all the three strains exhibited high shoot than that recorded in WAFA plants. Also, although shoot dry weight was decreased under IP, decrease was not significantly in comparison with plants supplied with 250 µmol P. Data on root dry weight of WAFA plants did not show any significant variation of the P treatments and rhizobial strains. However, in both the two P treatments and the three strains, REBIA plants have produced high root dry weight than WAFA plants.

Acid phosphatase activity (APase)

Figure 1 shows that acid phosphatase activity of leaves and roots varied both under, genotype strain and P effects. High APase activity was stimulated under IP conditions in leaves of WAFA inoculated with CIAT899 and P.Tb.09. However, levels of APase of the remaining combinations have shown, under both P treatments, a low activity that did not exceeds 15 µmol g^{-1} FW. Similarly, root APase activity has increased in REBIA inoculated with P.Tb.09 of the applied P forms (Figure 1B). Likewise, REBIA inoculated with CIAT899 and grown under IP has also significantly increased roots acid phosphate (AP) activity. By contrast this activity did not significantly vary for the remaining combinations of the strains and P treatments.

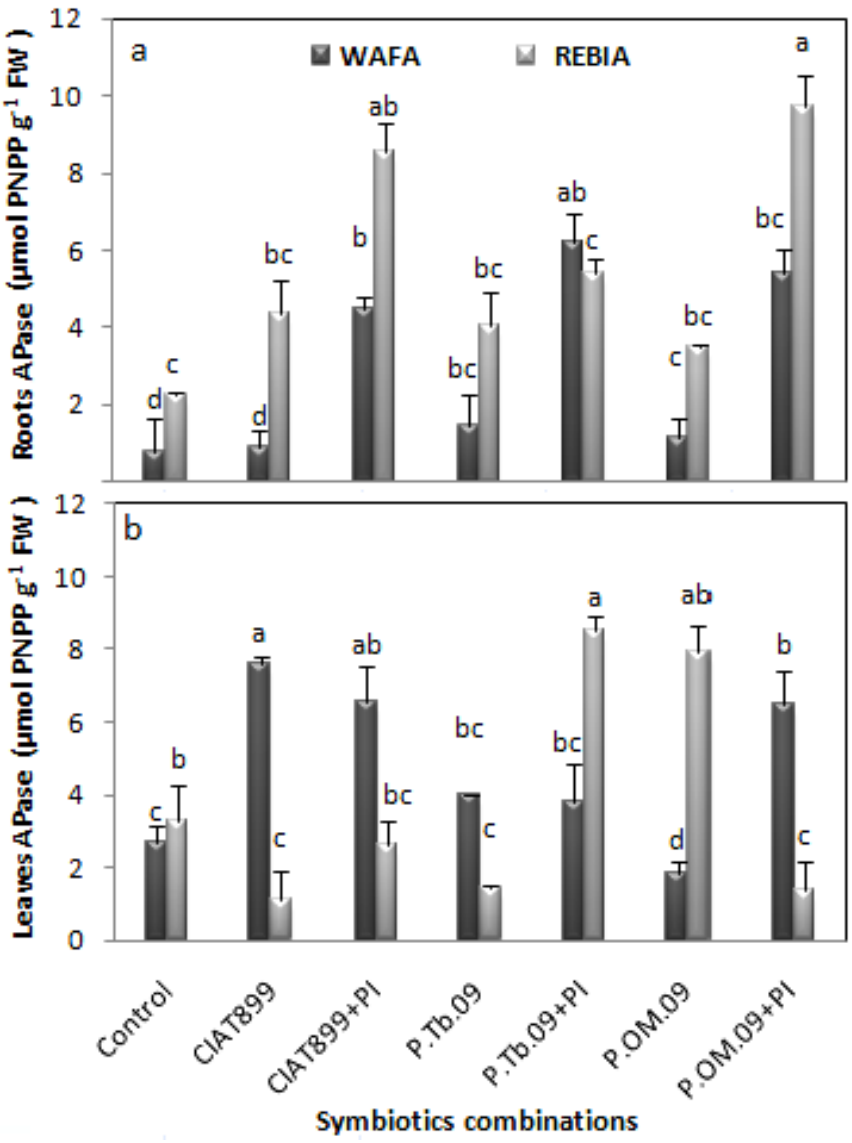

Figure 1. Acid phosphatase (APase) activity in leaves (a) and roots (b) of two common bean genotypes (WAFA and REBIA) inoculated with three rhizobia strains (CIAT899, P.Tb.09 and P.OM.09) and grown under 250 P and insoluble P (IP). Data are the means ± SD of four replicates harvested at flowering stage.

Nitrogen content

Analysis of the N content showed interesting variations depending both on the variety and the rhizobia strain (Figure 2). The high N content was accumulated by both varieties inoculated by with P.Tb.09 and grown under 250P (Figure 2). Similar result was found when REBIA variety was inoculated with CIAT899, whereas WAFA inoculated with the last strains had significantly reduced N content. However, inoculation of both varieties with P.OM.09 strains decreased their N content which was not significantly different between the two tested varieties. Application of IP resulted in a significant decrease of N content of plants inoculated with P.Tb.09. In addition, although, N was decreased for both lines inoculated with CIAT899 and received IP, difference was significant both between varieties and P treatments. The lowest N content, which was similar to what we found in control treatments was, noted when plant had been inoculated with P.OM.09 and grown under IP.

Phosphorus content in shoots, roots and nodules

P partitioning in shoots, roots and nodules has illustrated wide variations though between symbiotic combination and P treatment (Figure 3). Shoot of both tested varieties inoculated with P.Tb.09 and grown under IP exhibited

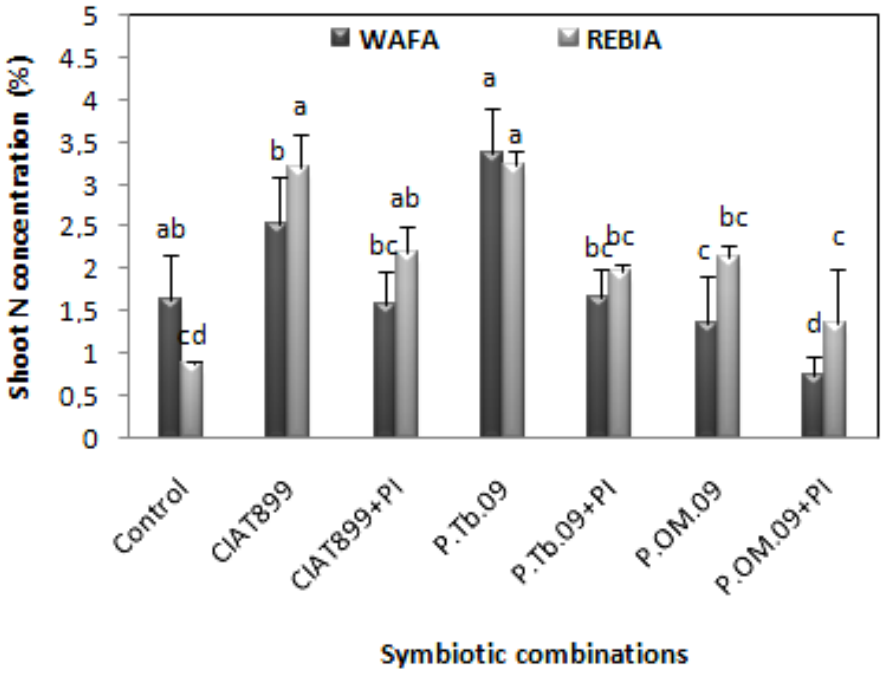

Figure 2. Nitrogen content in leaves of two common bean genotypes (WAFA and REBIA) inoculated with three rhizobia strains (CIAT899, P.Tb.09 and P.OM.09) and grown under 250 P and insoluble P (IP). Data are the means ± SD of four replicates harvested at flowering stage.

high P content (Figure 3A). This result was not significantly different to that found in both varieties under CIAT899 IP treatment. However, for all the remaining combinations, shoot P content was decreased especially when plants were inoculated with P.Tb.09 and grown under 250P. Nodule P content did not significantly vary for the whole treatments (strains and P) except for when both varieties were inoculated with P.Tb.09 under IP (Figure 2C). Significant decrease of root P content has been noted in both varieties inoculated with CIAT899. The same result was also found for the two tested genotypes under P.OM.09-IP. Nevertheless, this parameter was significantly increased for the remaining symbiotic combinations both under 250P and IP.

Total phenolics content

Total phenols contents in roots that were measured at different stage of plants growth (From 10 to 40 days) have shown interesting variations depending on the symbiotic combination and P treatment (Table 2). Under IP and for all the growth stage, high total phenols contents were recorded in REBIA inoculated with P.Tb.09. This is true for REBIA plants under CIAT899 IP treatment, but for only the first two stages of plants growth. The same trend, but to the less extent, was found when the last genotype was inoculated with P.OM.09, having high phenol contents described earlier and stimulated when plants had received IP than those under 250P. Results showed that for all the tested genotypes, phenols content were highly stimulated during the 10 and 20 days after sowing, but after 30 days this parameter had decreased showing no difference with control plants.

DISCUSSION

In this study, several symbiotic combinations of *P. vulgaris* (rhizobia) have been analyzed for their responses against IP. Interestingly, we have succeeded to identify some P-efficient combinations among the six studied as attested by their ability to grow under constraint conditions of IP. To our knowledge, the two tested strains P.Tb.09 and CIAT899 seems to be IP tolerant rhizobia since they were able to increase not only the number and nodule biomass, but also shoot dry weight which appeared to be dependant to the genotypes inoculated. These strains have formed P efficient combinations, especially with REBIA variety showing no difference in the shoot biomass of the P treatments (Table 1). Additionally, on the basis of these growth parameters, we attempt to predict that REBIA is more P deficiency tolerant than WAFA variety. Coherently, previous studies have demonstrated that the effect of IP on nodule number are still controversial and may result in an increase, decrease or even not affect nodule number per unit of shoot biomass (Gunawardena et al., 1992;

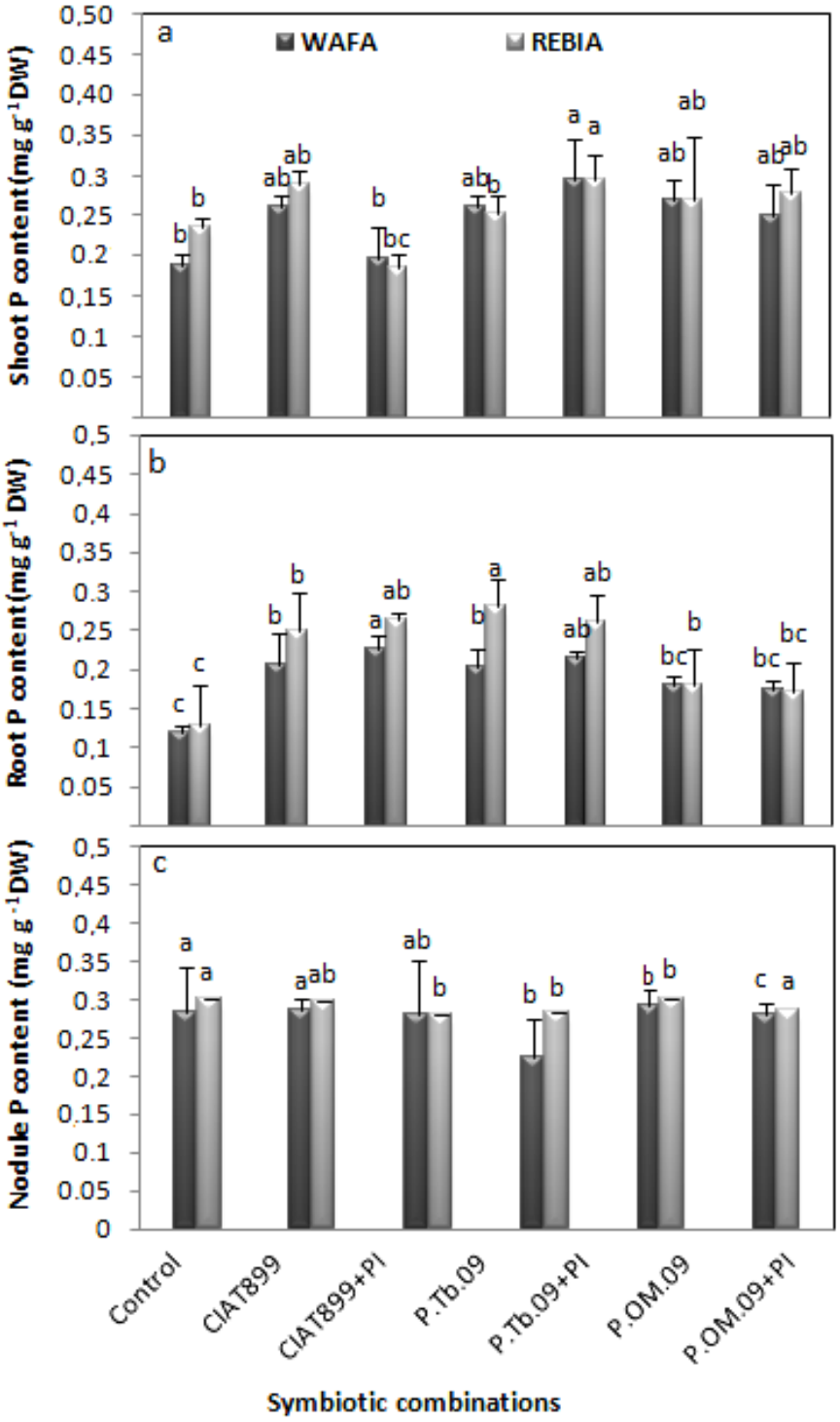

Figure 3. Phosphorus content in leaves (a), roots (b) and nodules (c) of two common bean genotypes (WAFA and REBIA) inoculated with three rhizobia strains (CIAT899, P.Tb.09 and P.OM.09) and grown under 250 P and insoluble P (IP). Data are the means ± SD of four replicates harvested at flowering stage.

Ribet and Drevon, 1995; Vadez et al., 1996; Drevon and Hartwig, 1997). In addition, growth of common bean plants in terms of shoot dry weight and root dry weight showed a variation depending on the common bean variety and the rhizobia strains (Zaman-Allah et al., 2007).

Table 2. Total phenols content in roots of two common bean genotypes (WAFA and REBIA) inoculated with three rhizobia strains (CIAT899, P.Tb.09 and P.OM.09) and grown under 250 P and insoluble P (IP).

Treatment	WAFA				REBIA			
	10	20	30	40	10	20	30	40
Control	$0.13^{bc} \pm 0.02$	$0.18^{b} \pm 0.03$	$0.08^{b} \pm 0.00$	$0.17^{a} \pm 0.02$	$0.23^{ab} \pm 0.00$	$0.25^{a} \pm 0.00$	$0.14^{b} \pm 0.00$	$0.27^{a} \pm 0.03$
CIAT899	$0.12^{c} \pm 0.00$	$0.12^{c} \pm 0.01$	$0.08^{b} \pm 0.00$	$0.11^{b} \pm 0.04$	$0.23^{b} \pm 0.00$	$0.21^{bc} \pm 0.01$	$0.12^{bc} \pm 0.00$	$0.24^{b} \pm 0.01$
CIAT899+IP	$0.14^{b} \pm 0.00$	$0.20^{ab} \pm 0.01$	$0.12^{a} \pm 0.01$	$0.12^{b} \pm 0.01$	$0.23^{ab} \pm 0.00$	$0.23^{b} \pm 0.01$	$0.11^{bc} \pm 0.00$	$0.15^{c} \pm 0.01$
P.Tb.09	$0.14^{b} \pm 0.00$	$0.20^{c} \pm 0.02$	$0.05^{c} \pm 0.00$	$0.12^{b} \pm 0.03$	$0.22^{b} \pm 0.01$	$0.25^{ab} \pm 0.00$	$0.10^{c} \pm 0.00$	$0.23^{b} \pm 0.00$
P.Tb.09+PI	$0.23^{a} \pm 0.03$	$0.22^{a} \pm 0.15$	$0.07^{bc} \pm 0.02$	$0.16^{ab} \pm 0.00$	$0.30^{a} \pm 0.00$	$0.28^{a} \pm 0.02$	$0.20^{a} \pm 0.02$	$0.25^{ab} \pm 0.01$
P.OM.09	$0.13^{bc} \pm 0.01$	$0.10^{c} \pm 0.00$	$0.08^{b} \pm 0.00$	$0.15^{ab} \pm 0.01$	$0.22^{b} \pm 0.00$	$0.15^{c} \pm 0.00$	$0.19^{ab} \pm 0.02$	$0.11^{cd} \pm 0.03$
P.OM.09+PI	$0.19^{ab} \pm 0.00$	$0.1\ 5^{bc} \pm 0.02$	$0.09^{ab} \pm 0.00$	$0.18^{a} \pm 0.03$	$0.24^{ab} \pm 0.03$	$0.23^{ab} \pm 0.01$	$0.19^{ab} \pm 0.00$	$0.19^{bc} \pm 0.03$

Data are the means ± SD of four replicates harvested at flowering stage.

In addition to these promising results, inoculation of both tested genotypes with the strains P.Tb.09 had produced a high N content, but only when plants were grown under 250 µmol P. Insignificant difference of biomass of both varieties inoculated with P.Tb.09 was associated with a significant decrease shoot N content under IP conditions (Figure 2). This decrease may be as a result of low efficiency in the use of rhizobial symbiosis as well demonstrated by Bargaz et al. (2011) in common bean grown under P deficiency. However, the stimulation of APase activity in WAFA variety under IP condition and inoculation with both CIAT899 and P.Tb.09 may allow us to think about the sensitivity of this variety in comparison to REBIA-P.Tb.09 that exhibited an increase in root APase activity of the applied P forms (Figure 3). Increase of APase activity does not agree with the decrease of N content that we found for almost the whole combination. In this way, Liao and Yan (2000) suggested that the capacity of nitrogen fixation under IP conditions is related to the P use efficiency. The later was not a determinant parameter of this study, because tested combinations did not show wide difference in P content. Differences in symbiotic nitrogen fixation in relation to P use efficiency were also reported for Soya genotypes (Gunawardena et al., 1993) and bean genotypes where ability to fix nitrogen seems to be affected when P is limiting (Yan et al., 1995). Several reports have suggested the main contribution of APase in plant P metabolism as well as P use efficiency which depends upon the distribution of the P between the shoots and nodulated roots and between roots and nodules (Cassman et al., 1981). Also, studies on the relationship between the inorganic P in leaves and their acid phosphatase activity had demonstrated the main role of this enzyme in the remobilization of inorganic P through these organs (Kaous et al., 2009). In addition, in these common bean varieties, IP has significantly increased the phenols contents of roots but more particularly in roots of REBIA inoculated with P.Tb.09. Similarly, Zeyen et al. (1995) reported that root phenolics content was a type of resistance of plants to a biotic stress.

We concluded that applying IP as a constraint condition had shown a preliminary discrimination about the IP tolerance of the tested symbiosis. On the basis of this study, it appears that REBIA was most tolerance than WAFA variety. Inoculation with CIAT899 and P.Tb.09 seems to increase all the tested parameters under IP conditions, but for REBIA variety, they were increased to the extent level. We suggest that exploring the P solubilization capacity of these strains and their ability to create efficient symbioses under contrast conditions as well as IP seems to be an interesting biological approach to increase legumes P assimilation for N_2 fixation and thus assuring yield stability.

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