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Full Length Research Paper

Inoculation with *Rhizobium etli* enhances organic acid exudation in common bean (*Phaseolus vulgaris* L.) subjected to phosphorus deficiency

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Common bean (*Phaseolus vulgaris* L.) production is severely limited by phosphorus (P) deficiency in tropical and subtropical regions. Plants are known to exudate organic acids in order to survive in low nutrient environment. However, little is documented on the effect of inoculation on organic acids exudation by plants as a strategy to adapt to low P stress. In this study, the organic acids exuded by two bean genotypes (known to have contrasting symbiotic nitrogen fixation capacities) inoculated with *Rhizobium etli* and subjected to P deficiency were compared. The results showed that, under P deficiency, the P efficient genotype (BAT477) secretes higher amounts of organic acids serving as nutrient for Rhizobacteria or involved in P solubilisation. Furthermore, inoculation with *R. etli* under P deficiency strongly enhanced the exudation of oxalate and citrate by the P-efficient genotype. These effects of P-level and inoculation on root exudation were much less observed for the P-inefficient genotype (DOR364). This study indicated the difference in organic acid exudation between the two bean genotypes, especially in combination with *Rhizobium* inoculation which may contribute to the contrast in performance under P deficiency.

Key words: *Phaseolus vulgaris*, *Rhizobia,* root exudation, genotypic performance, phosphorus efficiency.

INTRODUCTION

Common bean (*Phaseolus vulgaris* L.) is an important crop worldwide especially in areas with high population density. The farms though tiny, are significant sources of dietary protein. In such areas, more than 60% of the dietary protein comes from bean. Despite this importance, common bean production is severely limited by P deficiency in tropical and subtropical regions, where soils have high iron or aluminum oxides contents with P strongly bound and thus less available for plants. Estimates from the Centro Internacional de Agricultura Tropical (CIAT) database suggest that, 50% of the bean cultivating areas in Latin America and 65 to 80% in Sub-Sahara Africa are deficient in phosphorus (CIAT, 2000). Resistance to low phosphorus stress can be related to high P use efficiency and/or to high P uptake ability. In previous studies, to select P resistant genotypes; variations in root length accounted for little differences in P uptake by common bean genotypes under P-limiting conditions (Shen and Yan, 2001). Vesicular-arbuscular mycorrhizae (VAM) association also failed to explain significant differences in P uptake by common bean genotypes (Yan et al., 1995). High P efficiency could neither be explained by the release of phosphatase and

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Abbreviations: CIAT, Centro internacional de agricultura tropical; **VAM,** vesicular-arbuscular mycorrhizae; **SNF,** symbiotic nitrogen fixation; **CMPG,** centre for microbial and plant genetics; **HPLC,** high performance liquid chromatography; **RDW,** root dry weight; **IAA,** indole acetic acid.

rhizospheric acidification (Shen et al., 1999; Yan et al., 2001). However, the genetic diversity in nitrogen fixation under phosphorus deficiency was observed when common bean genotypes BAT477, COCOT, DOR364, Flamingo, and NAG310 were inoculated with *Rhizobium tropici* CIAT899 and grown under phosphorus deficiency (Jebara et al. 2004). Shen et al. (2002) reported that root exudates from P-deficient roots of common bean had stronger ability to mobilise aluminium and iron bound phosphates than those from P adequate roots. Large seeded Andean genotypes (G19833 and G19839) exuded higher amounts of citrate, tartrate and acetate, and mobilised more phosphorus from aluminum and iron bound phosphates under low P stress than small seeded Mesoamerican genotypes (DOR364 and G21212). Though bean is known to exudates higher amounts of P, solubilising organic acids in P deficient conditions, little is known on the influence that nitrogen fixing bacterial have on the exudation of these acids This study aims to evaluate the organic acids exuded by bean genotypes with contrasting symbiotic nitrogen fixation capacities when inoculated with *Rhizobium etli* and subjected to P deficiency.

MATERIALS AND METHODS

Plant materials and growth conditions

Two bean (*P. vulgaris* L.) genotypes were used; BAT477 and DOR364 supplied by CIAT, Colombia. These genotypes have contrasting symbiotic nitrogen fixation capacities. BAT477 has a higher symbiotic nitrogen fixation (SNF) capacity under high and low phosphorus (P) conditions while DOR364 has a lower SNF capacity under the same conditions. Snoeck medium, optimized in the Centre for Microbial and Plant Genetics (CMPG) in KU-Leuven, was used to grow plants under nitrogen fixing conditions in hydroponic system. The composition of this medium is as follows: Micro-nutrients (µM), 50.8 FeNaEDTA (4.5%), 32.2 $MnSO_4.H_2O$, 0.496 $CuSO_4.5H_2O$, 1.504 $ZnSO_4.7H_2O$, 25.008 H_3BO_3 and 0.48 $(NH_4)_6Mo_7O_{24}.4H_2O$. Macro-nutrients (mM), 7.49 KH_2PO_4, 0.43 K_2SO_4, 2.65 $CaCl.2H_2O$, 1.75 $MgCl.6H_2O$ and 1.2 $MgSO_4.7H_2O$. In previous research in the CMPG, contrasting P levels for nitrogen fixing conditions had been determined. When working with two P levels, the following adaptations were made to the Snoeck medium: For low P conditions, 1 µM KH_2PO_4 was used and potassium level adjusted as under the high P conditions by adding K_2SO_4. For high P conditions, 100 µM KH_2PO_4 was used. Prior to planting, bean seeds were soaked in 100% ethanol for 1 min, then in 15% NaOCl for 12 min and finally rinsed 10 times with sterile distilled water. Pre-soaked seeds were germinated on 10% water agar plates wrapped with aluminum foil and incubated at 30°C for two days. In the greenhouse, a 2/5 dilution of the Snoeck medium was used to grow plants in a continuous drain system. Two days old seedlings were planted in porous 1.5 L containers containing perlite. The perlite was irrigated to saturation prior to seed sowing and plants irrigated with 2/5 diluted Snoeck medium for 1 min, seven times a day from the sixth day following planting. The plants were grown for 14 days under a cycle of 12 h light and darkness with a relative humidity of 65 to 70°C. The temperature was 26°C under light and 20°C under darkness.

Inoculum preparation

The inoculum was prepared using *R. etli* CNPAF512, an endogenous strain from Embrapa, Brazil; isolated from nodules of bean plants. The *R. etli* CNPAF512 was grown on trypton yeast (TY) medium according to Beringer (1974). After autoclaving the medium at 121°C, 7 mM $CaCl_2$ and 30 mg/L of antibiotics (nalidixic acid) were added. Plated cells were suspended in 5 ml of TY medium and cultured overnight at 30°C on a shaker. The optical density (OD) of the overnight culture measured at 595 nm with a spectrophotometer was adjusted to 0.4. 1 ml of the cell suspension was centrifuged at 6000 rpm for 5 min, the supernatant discarded and cells washed two times with 1 ml of 10 mM $MgSO_4$. After a second centrifugation, the supernatant was discarded and cells resuspended in 1 ml of 10 mM $MgSO_4$. The washed cell suspension was diluted to 10^{-2} in 10 mM $MgSO_4$ and used as inoculum. 100 µl of the above bacterial suspension corresponding to approximately 10^5 to 10^6 cells was used as inoculums for each plant. The treatments include the BAT477 plants non-inoculated in low P, BAT477 plants inoculated in low P, BAT477 plants non-inoculated in high P, BAT477 plants inoculated in high P, DOR364 plants non-inoculated in low P, DOR364 plants inoculated in low P, DOR364 plants non-inoculated in high P and DOR364 plants inoculated in high P. Per treatment, 15 plants were grown under the conditions described above in replicates of 5 plants.

Root exudates extraction

Root exudates were extracted in distilled water as described by Lipton et al. (1987), Wiren et al. (1994) and Newman et al. (1999). At 14 days after planting, intact roots of 15 plants per condition from the green house were washed with distilled water and groups of 5 plants corresponding to each replicate were exposed to 50 ml of distilled water. Plants were kept in the extraction solution for 4 h at room temperature. To concentrate root exudates, the diluted root exudates were frozen at –80°C, lyophilized (freeze dried), dissolved in 1 ml of milliQ water and filter sterilized using 0.20 µm micro pore filter. Samples were stored at –20°C prior to analysis by high performance liquid chromatography (HPLC). The roots were dried at 50°C and weighed.

Root exudates analysis by HPLC

Concentrated root exudates were analyzed using waters 600 HPLC. The Instrument had a BioRad Aminex HPX-87H (300 mm x 7.8 mm) column filled with resin for organic acids, a mobile phase of 6 mM Hydrochloric acid (HCl), pH 2.5, flow rate of 0.60 ml/min, run time of 40 min, injection of 50 µL, detector dionex AD20 UV detector operated at 210 nm and running temperature of 35°C. The retention times (Table 1) of reference organic acids supplied with the Aminex HPX-87H column were used to identify the organic acids in root exudates. Formic acid was added to the root exudates samples as an internal standard. This organic acid was chosen amongst the other acids because the retention time did not correspond to any of the peaks of the root exudates samples. As such, it could not interfere with the chromatogram of the root exudates. The solution of formic acid was prepared by dissolving 40 µmol in 1 ml of distilled water (based on the concentration and peaks of reference organic acids supplied with the column). 50 µl of

Table 1. Retention times of reference organic acids used to identify peaks in HPLC.

Organic acid	*Retention time (min) on Aminex HPX-87H column
Oxalic acid	6.1
Aconitic acid	6.5
Maleic acid	7.2
Citric acid	7.7
Tartaric acid	8.0
Gluconic acid	8.3
Pyruvic acid	8.6
Malic acid	9.3
Malonic acid	9.5
Succinic acid	11.7
Lactic acid	12.1
Formic acid (internal standard)	13.4
Acetic acid	14.5
Propionic acid	17.3
Butyric acid	21.4

*Based on the concentration and peaks of reference organic acids supplied with the column, 40 µmol of reference organic acids was dissolved in 1 ml of distilled water, and 50 µl of the solution was injected using a syringe into the Aminex HPX-87H column to get the retention times. Formic acid served as the internal standard.

the solution was injected using a syringe into the Aminex HPX-87H column and a retention time of 13.4 obtained. From the solution of formic acid, 150 µl was mixed with 150 µl exudates (1:1 v/v ratio), and 50 µl of the mixture introduced into the column. The retention time and area of histogram were measured. The relative amounts of organic acid were expressed as percentage of internal standard per root dry weight (RDW). The following organic acids were used as reference.

RESULTS

Exudation of malate and succinate by bean genotypes

The exudation of malate as well as succinate by the two bean genotypes was significantly different ($p = 0.05$); when plants were not inoculated and subjected to low P stress (Figures 1 and 2). The results also showed a significant difference in exudation of succinate when the bean genotypes were inoculated under high P conditions. BAT477 secretes more succinate than DOR364 in all the conditions tested and more malate in all but one (inoculated in high P) of the conditions tested (Figure 1). The secretion of succinate by both genotypes was higher compared to malate exudation. For BAT477, under high P conditions, the secretion of both malate and succinate decreased two-fold when plants were inoculated with *Rhizobium*. For DOR364, malate exudation was not influenced by the different conditions tested. The exudation of succinate by DOR364 however, decreased two-fold when plants were inoculated under high P conditions as was also observed for BAT477. Under P deficiency however, inoculation stimulates succinate exudation by DOR364.

Exudation of citrate, oxalate and tartrate by bean genotypes

The genotypes tested in this study secrete oxalate in higher amounts as compared to citrate and tartrate, as well as the organic acids involved in P solubilisation (Figures 3, 4 and 5). Inoculating BAT477 plants with *Rhizobium* in P deficient conditions strongly enhanced the exudation of oxalate (Figure 3), citrate (Figure 4) and tartrate (Figure 5). This effect was most pronounced for oxalate. Inoculating DOR364 with *Rhizobium* had no effect on the amounts of oxalate, citrate and tartrate exuded. Furthermore, these organic acids were not detected in the supernatants of *Rhizobium* (data not shown). Under P sufficient conditions, the effect of inoculation on BAT477 varied depending on the organic acid. No effect of inoculation was observed on oxalate secretion as compared to the control, while citrate exudation was increased and that of tartrate decreased. Citrate exudation rate was lower compared to oxalate and tartaric acid and was not detected in non-inoculated plants of BAT477 grown in high P conditions. Secretion of citrate by BAT477 was specifically increased under P deficiency. DOR364 inoculated under high P conditions exuded more citrate than BAT477 and also exuded significantly less tartaric acid than BAT477 in all

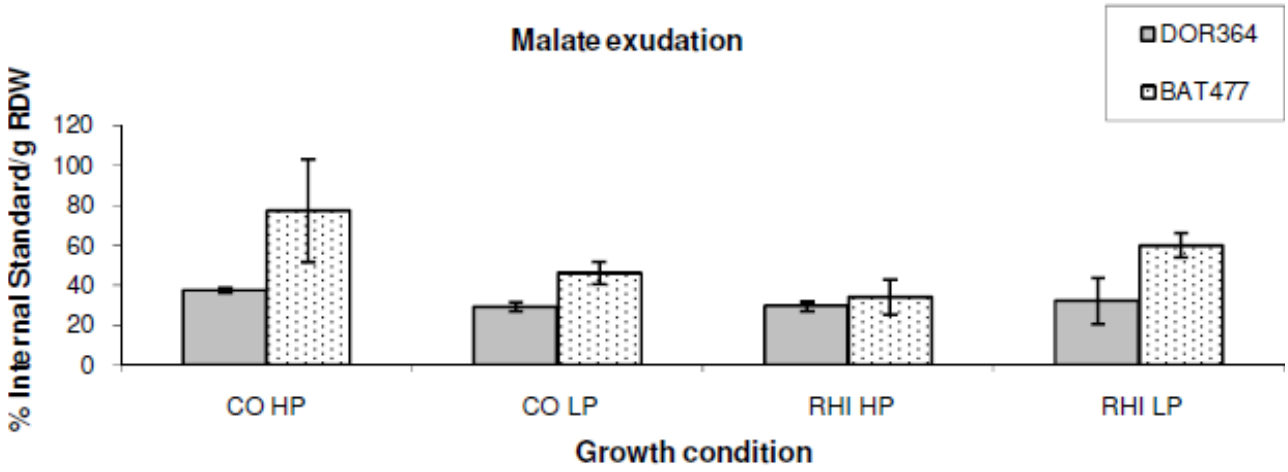


Figure 1. Malate exudation of BAT477 and DOR364 bean genotypes. CO HP = Control in high P, CO LP = control in low P, RHI HP = inoculated in high P, RHI LP = inoculated in low P and RWD = root dry weight. The data shown are the averages of three replicates of five plants. Standard deviations are indicated as error bars. T-test analysis (p = 0.05) was used to separate the means.

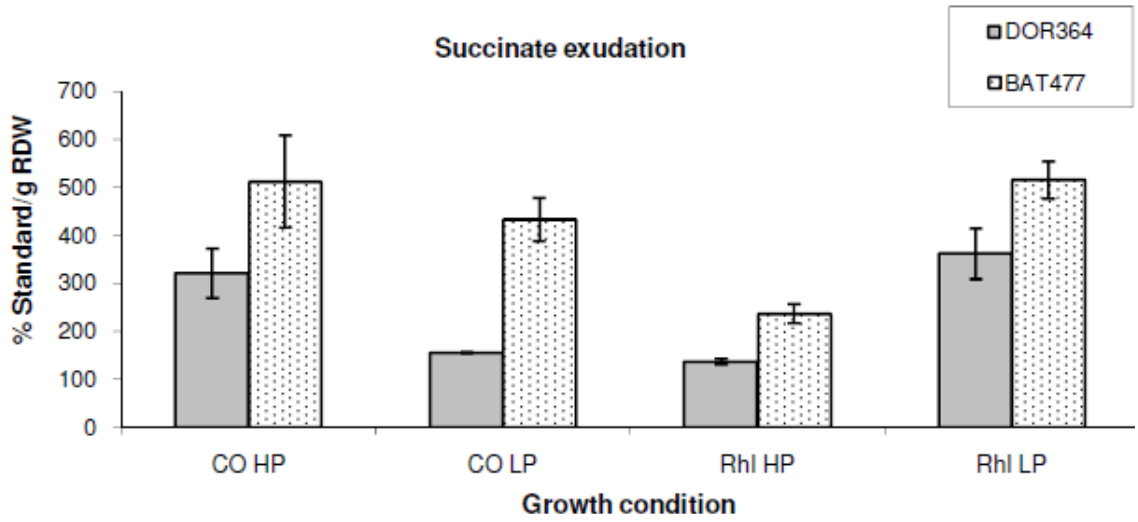


Figure 2. Succinate exudation of BAT477 and DOR364 bean genotypes. CO HP = Control in high P, CO LP = control in low P, RHI HP = inoculated in high P, RHI LP = inoculated in low P and RWD = root dry weight. The data shown are the averages of three replicates of five plants. Standard deviations are indicated as error bars. T-test analysis (p = 0.05) was used to separate the means.

conditions tested, except when plants were grown under high P condition and in the presence of *Rhizobium*.

Exudation of abundant/unidentified organic acids by BAT 477 and DOR 364 RO

Some organic acids (OAs) were unidentified but because of their importance in terms of exudation rate and genotype specificity, it was decided to include them in the results. They are distinguished based on their retention times on the column as unknown 1 (RT 15.5 min), unknown 2 (RT 23 min), and unknown 3 (RT 32 min). The unknown 1 (RT 15.5 min, Figure 6) was secreted only by BAT477 with an exudation rate two times higher in P deficient conditions compared to high P condition. Exudation of unknown 1 was further enhanced by inoculation under low P conditions. Unknown 2 (RT 23 min, Figure 7) was the most abundant organic acid secreted by both genotypes. BAT477 secretion was significantly higher compared to DOR364 when plants were not inoculated. In BAT477, secretion of unknown 2

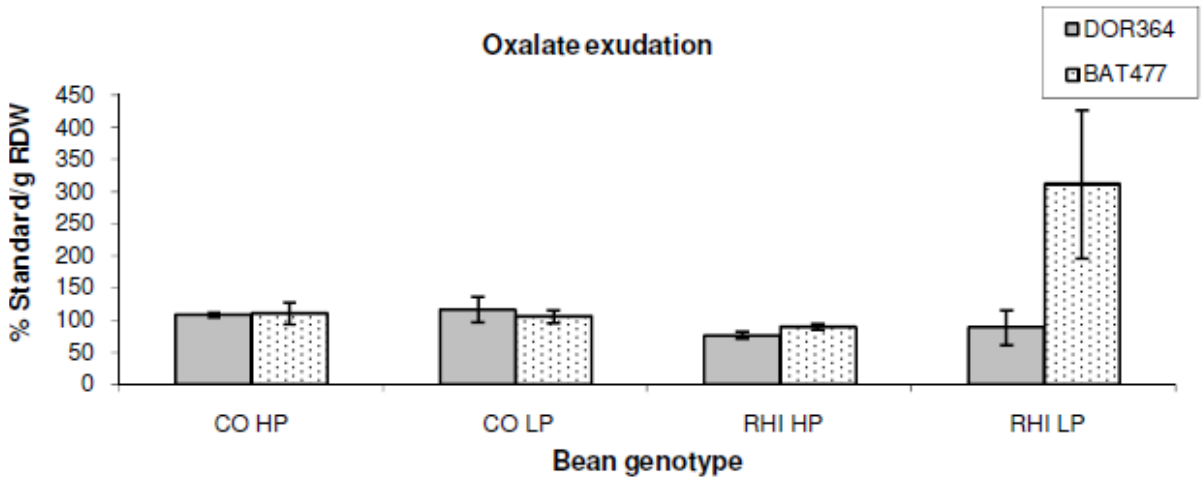


Figure 3. Oxalate exudation of BAT477 and DOR364 bean genotypes. CO HP = Control in high P, CO LP = control in low P, RHI HP = inoculated in high P, RHI LP = inoculated in low P and RWD = root dry weight. The data shown are the averages of three replicates of five plants. Standard deviations are indicated as error bars. T-test analysis (p = 0.05) was used to separate the means.

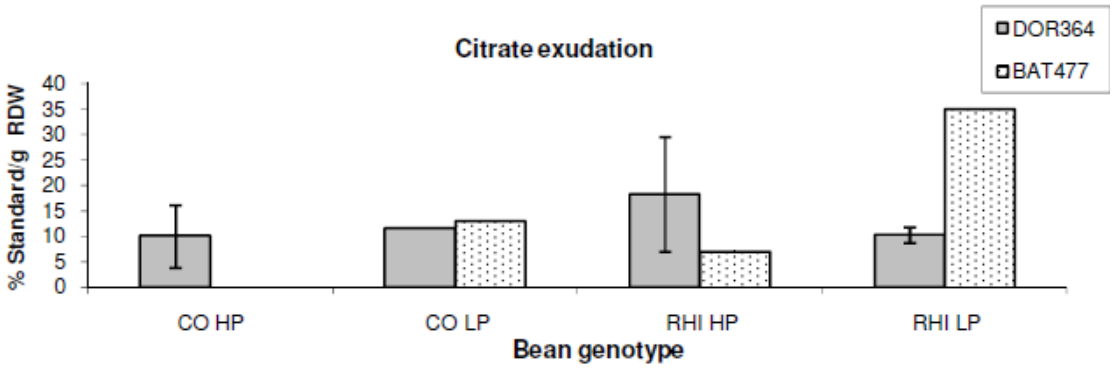


Figure 4. Citrate exudation of BAT477 and DOR364 bean genotypes. CO HP = Control in high P, CO LP = controls in low P, RHI HP = inoculated in high P, RHI LP = inoculated in low P and RWD = root dry weight. The data shown are the averages of three replicates of five plants. Standard deviations are indicated as error bars. T-test analysis (p = 0.05) was used to separate the means.

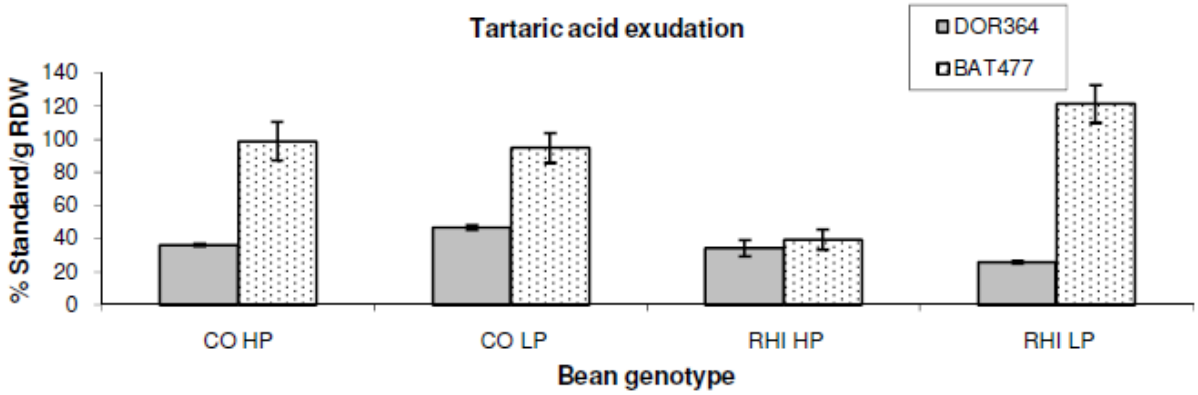


Figure 5. Tartaric acid exudation of BAT477 and DOR364 bean genotypes. CO HP = Control in high P, CO LP = controls in low P, RHI HP = inoculated in high P, RHI LP = inoculated in low P and RWD = root dry weight. The data shown are the averages of three replicates of five plants. Standard deviations are indicated as error bars. T-test analysis (p = 0.05) was used to separate the means.

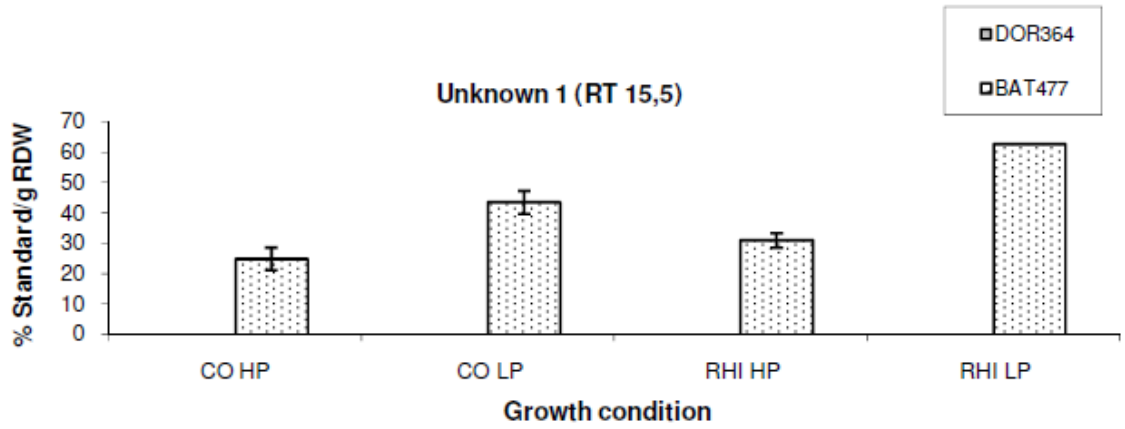


Figure 6. Unknown 1 exudation of BAT477 and DOR364 bean genotypes. CO HP = Control in high P, CO LP = control in low P, RHI HP = inoculated in high P, RHI LP = inoculated in low P and RWD = root dry weight. The data shown are the averages of three replicates of five plants. Standard deviations are indicated as error bars. T-test analysis (p = 0.05) was used to separate the means.

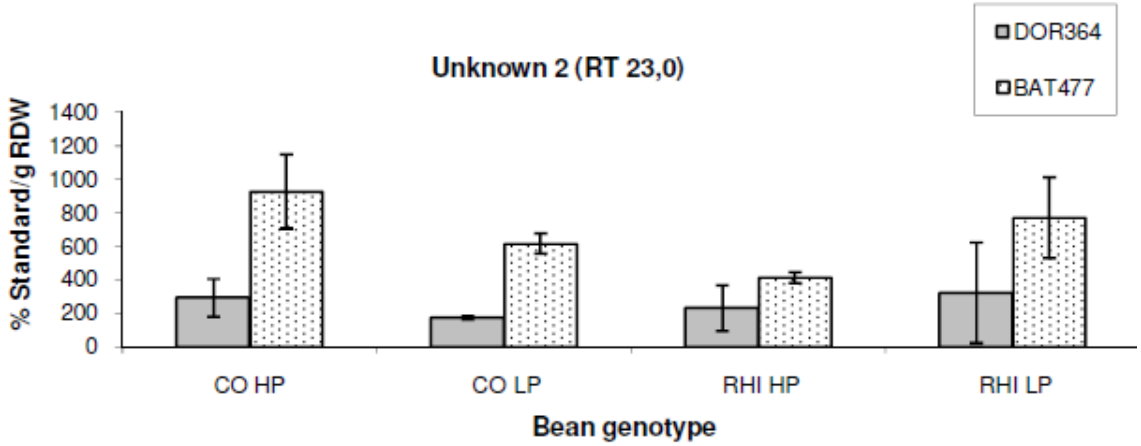


Figure 7. Unknown 2 exudation of BAT477 and DOR364 bean genotypes. CO HP = Control in high P, CO LP = control in low P, RHI HP = inoculated in high P, RHI LP = inoculated in low P and RWD=root dry weight. The data shown are the averages of three replicates of five plants. Standard deviations are indicated as error bars. T-test analysis (p= 0.05) was used to separate the means.

was decreased two-fold by inoculation when plants have sufficient levels of P. However, under low P conditions, this effect of inoculation was not observed. For DOR364, the effect of inoculation was not very clear. Unknown 3 (RT 32 min, Figure 8) was secreted by both genotypes with a significant difference in exudation when plants were not inoculated. Also this peak gives higher values for BAT477 than for DOR364.

DISCUSSION

Plants exudes organic acid anions, phenolics and phytosiderephores in low-nutrient environments so that, it can adapt to nutritional deficiencies (Dakora and Phillips, 2002). In this study, the focus was on root exudation of organic acids known to serve as nutrient sources to rhizobacteria (malate and succinate) (Miller et al., 1998) and those involved in P solubilisation. In the case of common bean, citrate, oxalate and tartrate are the most important as described by Shen et al. (2002). Root exudates from a P efficient (BAT477) and P inefficient (DOR364) bean genotypes were collected and compared with the organic acid exudation. The results showed that, differential organic acid secretion exists between the two genotypes, and that differences are influenced by

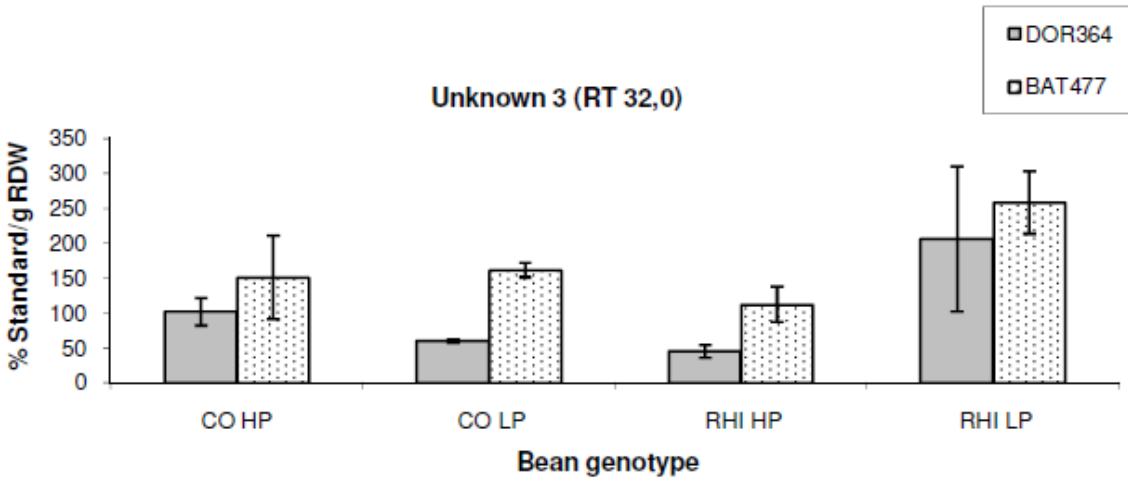


Figure 8. Unknown 3 exudation of BAT477 and DOR364 bean genotypes. CO HP = Control in high P, CO LP = control in low P, RHI HP = inoculated in high P, RHI LP = inoculated in low P and RWD = root dry weight. The data shown are the averages of three replicates of five plants. Standard deviations are indicated as error bars. T-test analysis (p = 0.05) was used to separate the means.

inoculation and phosphate level. The P efficient (BAT477) genotype had a general higher exudation rate (Figures 1 to 8) than the P inefficient (DOR364) genotype. This is in line with the finding of Gaume (2000) which showed that, there is considerable variation in the composition of root exudates and especially organic acids among various genotypes of a given species. Between two contrasting genotypes of maize, Gaume (2000) showed that the genotype which was tolerant to low P supply was exudating more organic acids (especially, trans-aconitic, malic and citric acids) under low P conditions than the sensitive genotypes. It was observed that, the difference in exudation rate was more pronounced for P solubilising organic acids when plants were inoculated with *Rhizobium* and subjected to low P conditions**.** Microorganisms are known to metabolize root exudates, thereby modifying composition and quantity of the exudates. Many types of compounds released by microorganisms have the potential to affect the release of root exudates (Arshad and Frankenberger, 1998). Some rhizobial strains produce siderophores, indole acetic acid (IAA) in culture media (Antoun et al., 1998). As with plants growing in low nutrient environments (Dakora and Phillips, 2002), rhizobia use these exuded compounds to enhance mineral nutrition. It is possible that, inoculating plants with *Rhizobium* brings about a concentration gradient that favors more exudation. The results showed that, BAT477 secretes more succinate than DOR364 in all the conditions tested and more malate in all but one (inoculated in high P) of the conditions tested. In a study by Tesfaye et al. (2003), malate was found to be the primary plant carbon source used by bacteroids. Alfalfa transformed with a malate dehydrogenase gene having high efficiency in malate synthesis, exuded more organic material into the rhizosphere and fixed more nitrogen than the wild type. Also in this case, there is a positive relationship between malate exudation and SNF efficiency when comparing BAT477 and DOR364.

Analysis of P-solubilising organic acids (citrate, oxalate and tartrate) in root exudates revealed that more of these organic acids were exuded from the roots of BAT 477 than from those of DOR364, specifically under P deficient conditions in the presence of *Rhizobium*. The higher exudation of these organic acids by BAT477 compared to DOR364 probably contributed to the better performance of BAT477 under P deficient conditions. These root exuded organic acids can increase phosphate availability from sparingly soluble inorganic phosphate compounds (Jones and Darrah, 1994; Johnson et al., 1996). Among the three P-solubilising organic acids studied, oxalate was secreted in higher amounts than citrate and tartaric acid (Figures 3, 4 and 5). Kpomblekou and Tabatabai (1994) indicated that, P-solubilizing capacities of organic acids are highly correlated with the amount of -OH and -COOH groups and their relative positions on the main carbon chain. Citrate and oxalate were high P-solubilizing compounds among low-molecular, short-chain carboxylic acids; because, these carboxylic acids are favorable for the formation of stable 5- or 6-bond ring structure with trivalent cation such as Al^{3+} and Fe^{3+} (Hue et al., 1986).

Some organic acids either secreted abundantly by both genotypes or specifically by one of the genotypes were analyzed even though not yet identified. The organic acid termed unknown I (Figure 6) was secreted only by BAT477 with a higher exudation rate in P-deficient conditions. This organic acid may have a P-solubilising activity as well since it is P dependent. This will have to be further investigated. Unknown 2 (Figure 7) and unknown 3 (Figure 8) were secreted by both genotypes. In BAT477, inoculation decreased exudation of unknown

2 in high P conditions. Since this organic acid showed similar exudation pattern as that for malate and succinate, which served as energy source for rhizobacteria and in nodules (Miller et al., 1998), it may play the same role in *Rhizobium*.

Conclusion

The adaptation of BAT477 to P deficiency may be linked to the exudation of P solubilising organic acids which are enhanced by inoculation with *Rhizobia*. From this study, it can be concluded that, the P efficient genotype BAT477, secretes higher amounts of organic acids involved in P-solubilisation, especially citrate and oxalate as a mechanism to adapt to low P conditions. Furthermore, identification of the unknown organic acids secreted in abundance will improve the characterization of organic acids involved in P stress adaptation by common bean.

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