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EFFECT OF INOCULATION BY MOROCCAN ROCK PHOSPHATE-SOLUBILIZING RHIZOBIA, *VERSUS* PHOSPHORUS FERTILIZATION, ON THE GROWTH AND THE PHOSPHORUS UPTAKE BY *Vicia faba*

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Low Phosphorus availability in soil is worldwide a frequent major constraint for crops. Rhizobial strains, beneficial N_2-fixing symbiotic partners of legumes, were reported to solubilize both organic and inorganic complex phosphates. Thus they may play a significant role as plant growth-promoting rhizobacteria (PGPR) in the biofertilization of crops. Natural rock phosphate may play a key role in sustainable agriculture, resulting in economic and environmental benefits. This work aims to evidence the capacity of rhizobial strains nodulating *Vicia faba* L. plants in Marrakech-Haouz region (Morocco) to solubilize Moroccan rock phosphate *in vitro* in either agar or liquid medium and to evaluate and compare the effect of these strains on growth and phosphorus uptake by *V. faba* plants of two Moroccan varieties (Defes and Aguadulce). Four rhizobial strains, contrasting for phosphate solubilization on agar medium, were further studied on liquid medium. RHOF147 and RHOF174 strains formed clear halos around colonies (1.37 cm and 1.2 respectively after 15 days); opposite RHOF170 and RHOF171 strains did not produce halo. The four strains were able to mobilize rock phosphate for growth in liquid medium (up to 0.59 mg/L of assimilated phosphorus). Lower pH values and most mobilized phosphorus production were registered for RHOF147 and RHOF174 strains. By screening the four rhizobial strains for growth on Chrome Azurol S (CAS) medium, only RHOF171 strain formed orange halo indicating its capacity to produce siderophores. Greenhouse experiments were undertaken including comparison of inoculated plants with these four rhizobial strains in the presence of rock phosphate as sole P source *versus* non-inoculated plants growing on KH_2PO_4 as sole P source (P-fertilized plants). Results show that the root length, the root dry weight and the ratio root /shoot dry weights were generally higher in the inoculated plants than in the P-fertilized plants, indicating the adaptation of the inoculated plants to phosphorus deficiency conditions. The effect of rock phosphate-solubilizing bacteria on plant growth depends on the symbiotic combination of the inoculated strain and the plant genotype. As for phosphorus uptake, rhizobial strains made phosphorus available to the plant at significant increased concentrations in some symbiotic combinations (e.g. Aguadulce-RHOF147). The use of selected symbiotic combinations having the capacity to solubilize rock phosphate may improve soil fertility and phosphorus availability to plants.
Keywords: Moroccan rock phosphate, rhizobia, *Vicia faba*, phosphorus solubilization.

INTRODUCTION

Morocco harbors the largest reserves of rock phosphate worldwide. The natural rock phosphate may constitute a valuable alternative source of phosphorus fertilizer, when a biotechnological process could be applied to promote its solubilization and make phosphorus available to the plants (Hamdali *et al.,* 2008a; Chang and Yang, 2009; Park *et al.,* 2011).

Bioinoculums are most promising fertilizers for soil fertility, as alternatives for chemical fertilizer use affecting human health and causing environmental pollution (Khan *et al.*, 2007; Savci, 2012). Valorizing the legume-rhizobia symbiotic associations may constitute a friendly biotechnological pathway to maintain the ecological balance under several environmental stress conditions (Dulormne *et al.,* 2010; Faghire *et al.,* 2013; Oufdou *et al.,* 2014).

After nitrogen, phosphorus is an essential plant nutrient whose deficiency severely restricts plant growth (Bolland *et al.,* 2000; Hinsinger, 2001; Majeed *et al.,* 2014). Low soil phosphorus availability is a major limiting factor of crop yields, especially when crop relies on symbiotic nitrogen

fixation (Scervino *et al.*, 2011; Srinivasan *et al.*, 2012). Phosphorus is often present in soil as insoluble complexes unavailable to plant nutrition. Phosphorus is complexed with aluminium, iron, silicium, and other metallic ions in acidic soil (Whitelaw, 2000) or with calcium carbonate in alkaline soil (Gyaneshwar *et al.*, 2002). Liu *et al.* (1994) reported that the phosphorus deficiency is the most common nutritional stress for plants, affecting 42% of the cultivated land over the world.

Some rhizobial strains, beneficial N_2-fixing symbiotic partners of legumes, were reported to solubilize both organic and inorganic phosphate complexes. These symbiotic microorganisms were shown to affect positively plant growth under phosphorus-deficient conditions by making complex phosphorus sources accessible to the plant (Richardson, 2001; Alikhani *et al.*, 2006; Srinivasan *et al.*, 2012). Thus, using such rhizobia, selected for their phosphate-solubilizing capacity as bioinoculants to crops would result in the dual beneficial nutritional effect from both P mobilization and N_2-fixation (Peix *et al.*, 2001).

Vicia faba L. (faba bean) is considered as a multipurpose crop over the world (Daoui *et al.*, 2011, Mulualem *et al.* 2012; El-Komy *et al*., 2015). *V. faba* is the most important legume crop grown in Morocco over 40% of the total area dedicated to legumes (MADRPM/DERD, 2006). The faba bean is rich in proteins, mineral elements and vitamins, and hence used for both human and animal nutrition (Bond *et al.*, 1985). *V. faba*, due to its symbiotic association with rhizobia, plays a great role in agricultural production and contributes to food security and economic development (Graham and Vance, 2003; Khan *et al.*, 2010).

Few reports are available on the role of rock phosphate-solubilizing rhizobia on faba bean plant growth and phosphorus uptake (Demissie *et al.*, 2013). During this study, our focus was to evaluate the capacity of *V. faba*-nodulating rhizobial strains to solubilize Moroccan rock phosphate, determine their ability to solubilize rock phosphate on agar medium and in liquid broth of same composition and, assess and compare the inoculation effect of these rhizobial strains on growth and phosphorus uptake by two Moroccan varieties of *V. faba* plants (Defes and Aguadulce) in the presence of rock phosphate as sole P source *versus* non-inoculated plants growing on KH_2PO_4 as sole P source (P-fertilized plants).

MATERIALS AND METHODS

***Isolation and purification of rhizobial strains*:** Rhizobial strains were isolated from field-grown *V. faba* plants located in the Marrakech-Haouz region (Morocco). Nodules of *V. faba* plants were previously disinfected with sodium hypochlorite (4°) and washed several times with sterile physiological water. The nodule was crushed in a sterile tube. The suspension was streaked on Petri dishes containing YEM medium agar with Congo Red. After incubation for 48 h to 72 h at 28°C, colonies of rhizobia, characterized by a gluey aspect and not uptaking Congo red, were isolated on YEM medium (Vincent, 1970). The rhizobia strains were purified by repeated streaking on Yeast-Extract-Mannitol agar (YEM Agar) with Congo red. Pure isolates were checked for their nodulation of aseptic *V. faba* seedlings and stored at -25°C in glycerol 30% v/v.

Phosphate solubilization test on agar medium: Rhizobial strains were grown in Erlenmeyer flasks (250 mL) containing 100 mL YEM broth medium at 28°C during 48 h on a rotary shaker at 180 rpm. After incubation, the cultures were centrifuged at 6000rpm for 5 min. The supernatant was discarded, and the pellet was suspended in 10 mL of sterile physiological water and centrifuged again. This operation was repeated 3 times to eliminate traces of phosphorus from the rhizobial cells. The final pellet was suspended in a volume of sterile physiological water such as to reach a final OD of 0.8. This constituted the inoculum.

Using the drop plate method (Alikhani *et al*., 2006), each part was inoculated with 7 µL of the inoculum on $TCPNH_4Cl$ medium agar containing 5g/L rock phosphate. The $TCPNH_4Cl$ medium consists of: Glucose 10 g/L, $MgSO_4.7H_2O$ 1 g/L, NH_4Cl 5 g/L and NaCl 1 g/L. Inoculated plates were incubated at 28°C in dark. When occurring, the diameter of the clearing zone (halo) surrounding the bacterial colony, corresponding to P solubilization, was measured after 3, 10 and 15 days. All assays were replicated three times.

The Moroccan rock phosphate is a hydroxyapatite, a naturally occurring mineral form of calcium apatite, $Ca_5(PO_4)_3(OH)$, usually written $Ca_{10}(PO_4)_6(OH)_2$ to denote that the crystal unit cell comprises two entities. It consists of 56.53% O, 16.35% Ca, 9.37% P, 2.42% F, 2.03% Al, 1.94% Mg, 1.81% Na, 0.77% S, 0.60% Fe, and 0.12% Sn (Hamdali *et al*., 2008a).

Phosphate solubilization test in liquid medium: Among 80 rhizobial strains of our collection, we chose four strains with contrasting capacities to solubilize rock phosphate in agar medium: RHOF147 and RHOF174 strains form clear halos around colonies whereas RHOF170 and RHOF171 strains do not form halo. Erlenmeyer flasks (250 mL) containing 100 mL of liquid $TCPNH_4Cl$ medium containing 5 g/L of rock phosphate, were inoculated with 200 µL of each rhizobial inoculum washed three times as described above in the previous paragraph. The initial optical density (OD) in the flasks was 0.1. Both inoculated and non-inoculated control flasks had an initial pH of 7. The erlenmeyer flasks were incubated in dark on rotary shaker (180 rpm) at 28°C. After 48h, 96 h and 168 h of incubation, aliquots of cultures were aseptically taken from each flask, centrifuged (6000 rpm for 5 min) and supernatant was filtered on Millipore filter (0.2 µm of porosity). pH values and soluble phosphorus released into the solution were measured.

Control flask was used as the baseline (zero absorbance) for determining the soluble phosphorus released by each rhizobial strain in culture medium. The dosage of the soluble

phosphorus was performed using the colorimetric method based on the formation and reduction of the complex of orthophosphoric acid and molybdic acid. The Phosphorus-molybdate reduction is accompanied by a blue color whose intensity is proportional to the amount of soluble phosphorus present in the medium (Olsen and Sommers, 1982).

***Siderophore production test*:** We used the same protocol reported above in the paragraph "Phosphate solubilization test on agar medium". Using the drop plate method, each part was inoculated with 7 µL of the inoculum on the blue Chrome Azurole S agar (CAS) described by Alexander and Zuberer (1991). Development of yellow-orange halo around the colony is considered as a positive test for siderophore production.

Effects of rock phosphate-solubilizing rhizobia on faba bean plants:

***Preparation of seeds*:** Plant tests were performed using two Moroccan *V. faba* major varieties: Defes and Aguadulce. The seeds of Defes variety (length x width x height = 1.98 cm x 1.28 cm x 0.44 cm) are smaller than the seeds of Aguadulce variety (length x width x height = 2.49 cm x 1.78 cm x 0.37 cm).

V. faba seeds were sterilized with sodium hypochlorite 4° for 10 min, followed by several rinsing in physiological distilled water. The seeds were then placed on wet filter papers in the Petri dishes for germination at 25°C for 48h to 72h in the dark.

***Inoculums preparation*:** We used the same protocol reported above in the paragraph "Phosphate solubilization test on agar medium". The inoculum was prepared by growing the rhizobial strain in YEM liquid medium at 28°C for 2-3 days to obtain an optical density (OD) of 1 at 600 nm (approximately 10^9 colonies forming units (CFU)/mL) as described previously (Lahrouni *et al.*, 2012; Oufdou *et al.*, 2014).

***Inoculation and transfer to pot*:** Uniformly-germinated seedlings were immersed for 30 min in individual rhizobial liquid culture of each of the tested strains RHOF147, RHOF174, RHOF170 or RHOF171.

The seedlings were then grown on perlite in 5 L pots. Rigaud and Puppo nutrient solution in distilled water was added to the trays (Rigaud and Puppo, 1975).

Plants were grown either with rock phosphate amendment as sole P-source and separately inoculated by individual rhizobial test strains (biofertilized plants: Treatments 2 to 5) or with KH_2PO_4 (P-fertilized, non-inoculated plants: Treatment 1) (Table 1). We tested whether plant inoculation with selected rhizobial strains may compensate phosphorus-deficient conditions (rock phosphate as sole P-source) for plant growth. The experimental design was a complete random block assay.

We applied 0.4 g rock phosphate under each germinated seedling in the pot. The plants were harvested at the flowering stage (40 days after sowing). The roots were thoroughly rinsed with distilled water. The length, biomass and total phosphorus content was determined in both shoots and roots of each plant.

Table 1. The protocol of the greenhouse experimentation.

Treatments	Parameters	Rock phosphate	Rhizobia inoculum
T1	(+) KH_2PO_4	(-)	Non-inoculated
T2	(-) KH_2PO_4	(+)	RHOF147
T3	(-) KH_2PO_4	(+)	RHOF174
T4	(-) KH_2PO_4	(+)	RHOF170
T5	(-) KH_2PO_4	(+)	RHOF171

+ : presence, - : absence

The trials were performed in the greenhouse of the Faculty of Sciences Semlalia, Marrakech, under natural climatic conditions.

***Statistical analysis*:** We used a complete random block assay design. Registered growth values were means of three replicates per treatment for the *in vitro* phosphate solubilization by rhizobia (in solid and liquid media), and were means of six replicates per treatment for the plant tests in greenhouse. All results were subjected to a two-way analysis of variance (ANOVA), with a Least Significant Difference (LSD) for the comparison of means using COSTAT software. Means and standard errors (SE) were also calculated and are presented in the graphs. Means with different letters are significantly different at $p < 0.05$.

RESULTS

Phosphate solubilization of rhizobial strains in agar medium: RHOF147 and RHOF174 strains formed the largest clear halos around their colonies on agar medium containing rock phosphate as sole source of phosphorus, whereas there was no clearing zone around the colonies of the strains RHOF170 and RHOF171 until the 15th day of incubation (Figure 1).

We note that the diameter of the halos increased significantly ($p<0.05$) during the period of incubation. For example, the diameter of the halos produced by RHOF147 was 0.77 in 3 days, 1.18 in 10 days and 1.37 in 15 days. Similarly, the strain RHOF174 exhibited halos of diameters 0.83 cm, 1.15 cm and 1.20 cm respectively after 3, 10 and 15 days of incubation (Fig. 1).

Phosphate solubilization of rhizobial strains in liquid medium: In liquid medium, the four studied strains were able to mobilize differently rock phosphate (Table 2). They produced soluble phosphorus from the rock phosphate used as the sole source of phosphorus.

The strain RHOF174 released the maximum of soluble phosphorus throughout incubation followed by the strain RHOF147 (Table 2). There is a positive correlation between the produced solubilized phosphorus and the pH decrease in the liquid medium (Tables 2 and 3). The pH in supernatant turned acidic (Table 3), with lower values in the case of the

two halo-producing strains: RHOF147 and RHOF174 (2.5-2.8) compared to the non-producing ones RHOF170 and RHOF171 (1.5-1.8). A slight decrease in pH was also registered in the control non-inoculated culture medium (0.2-0.8) used as the baseline (zero absorbance) for determining the soluble phosphorus mobilized by each rhizobial strain in culture medium.

Table 2. Soluble phosphorus in mg/L measured in the supernatant of rhizobial test strain cultures grown in TCPNH$_4$Cl liquid medium containing rock phosphate as sole P source.

Strain	Time of incubation			
	0h	48h	96h	168h
RhOF147	-	0.39±0.01	0.22±0.03	0.15±0.03
RhOF174	-	0.41±0.02	0.39±0.03	0.59±0.04
RhOF170	-	0.38±0.01	0.07±0.01	0.02±0.03
RhOF171	-	0.16±0.01	0.06±0.01	0.17±0.04

Table 3. Differential pH value in liquid TCP NH$_4$Cl medium during rock phosphate solubilization tests by studied strains.

Strain	Time of incubation			
	0h	48h	96h	168h
RhOF147	7	4.52	4.18	4.35
RhOF174	7	4 .00	4.35	4.20
RhOF170	7	5.93	5.50	5.18
RhOF171	7	5.71	4.93	5.16
Control	7	6.75	6.51	6.22

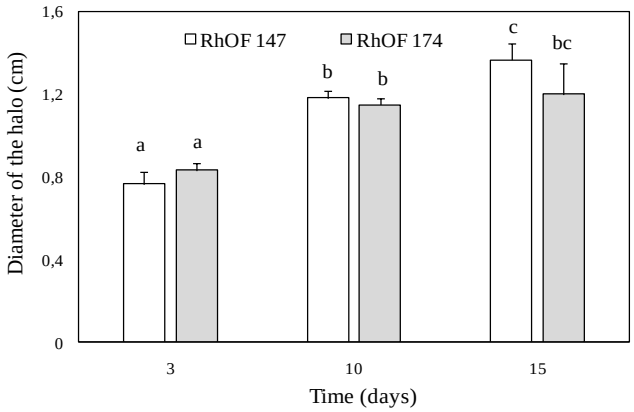

Figure 1. Rhizobia rock phosphate solubilization test in agar TCPNH$_4$Cl medium after 3 days, 10 days and 15 days of incubation. Results are expressed as diameters of clear halos observed around colonies of the test strains. Means (± standard errors) with different letters are significantly different at $p < 0.05$.

***Siderophore production test*:** The strain RHOF171 when tested for phosphate solubilization, did not produce any halo on agar medium and low level of phosphorus was solubilized from rock phosphate in liquid medium. This strain developed an orange halo on CAS agar. RHOF171 strain has been found to produce siderophores.

***Effects of rock phosphate-solubilizing rhizobia on faba bean plants*:** The root length of Defes plants was generally higher in the inoculated plants especially in the presence of RHOF147 (Fig. 2B). The length of the plants inoculated with RHOF147 (34.5 cm) was significantly higher than in the P-fertilized plants (20 cm).

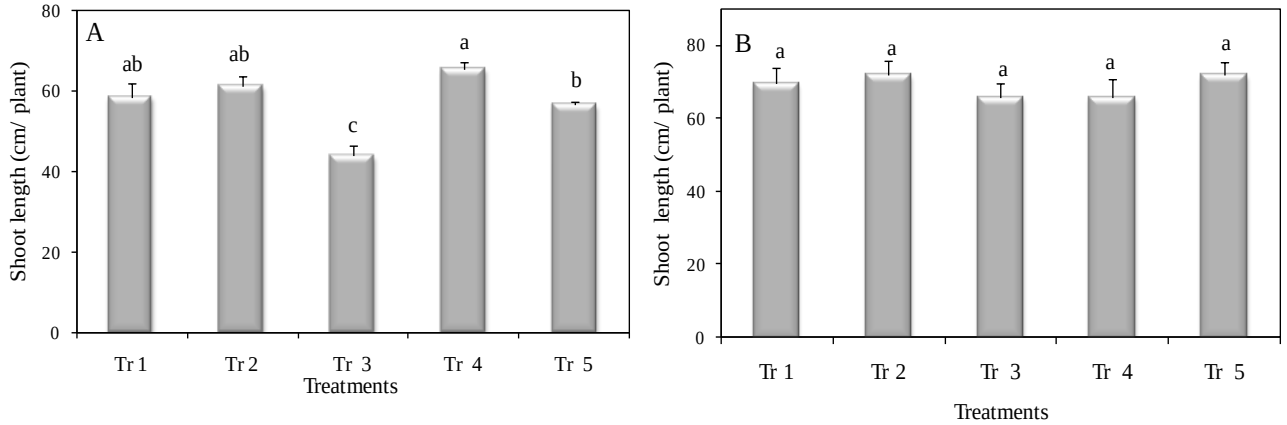

Figure 2. Shoot lengths of *V. faba* plants (A: Defes variety and B: Aguadulce variety) submitted to the five treatments. Tr 1: KH_2PO_4; Tr 2: rock phosphate (RP)+RHOF147; Tr 3: RP+RHOF174; Tr 4: RP+RHOF170; Tr 5: RP+RHOF171. Means (±standard errors) within the same graphic followed by different letters are significantly different at $p < 0.05$.

As for the Aguadulce variety, the P-fertilization did not increase significantly in either shoot or root lengths in comparison to the inoculated plants (Fig. 3).

Figure 4 presents the shoot and the root dry weights of *V. faba* plants (Defes variety) submitted to different treatments. The highest shoot dry weights were noted in the P-fertilized plants and Defes-RHOF170 symbiotic combination (Figure 4A). The P-fertilization with KH_2PO_4 did not increase significantly the root dry weight of Defes plants in comparison to the bioinoculated plants (Fig. 4B).

In the inoculated Aguadulce plants, root dry weights were generally increased, especially those nodulated by RHOF171 (0.70 g/plant) followed by the plants nodulated by RHOF174 (0.65 g/plant), whereas the root dry weight was only 0.49 g/plant in the P-fertilized plants (Fig. 5).

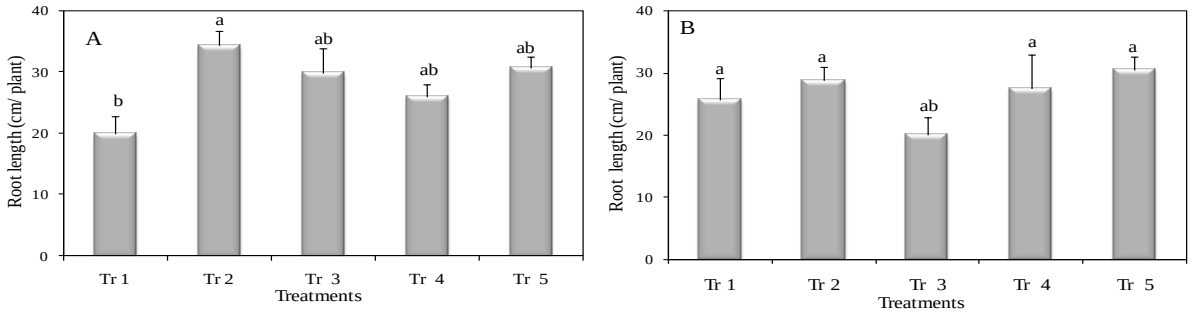

Figure 3. Roots lengths of *V. faba* plants (A: Defes variety and B: Aguadulce variety) submitted to the five treatments. Tr 1: KH_2PO_4; Tr 2: RP+RHOF147; Tr 3: RP+RHOF174; Tr 4: RP+RHOF170; Tr 5: RP+RHOF171. Means (±standard errors) within the same graphic followed by different letters are significantly different at $p < 0.05$.

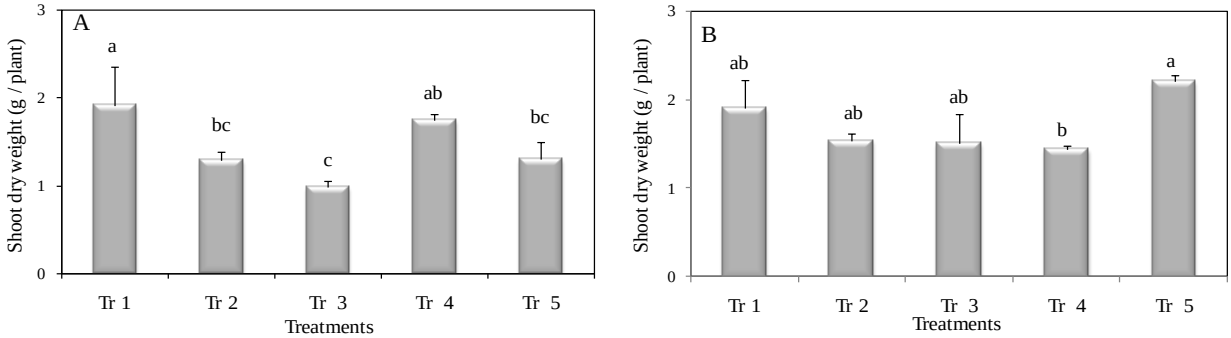

Figure 4. Shoot dry weights of *V. faba* plants (A: Defes variety and B: Aguadulce variety) submitted to the five treatments. Tr 1: KH_2PO_4; Tr 2: RP+RHOF147; Tr 3: RP+RHOF174; Tr 4: RP+RHOF170; Tr 5: RP+RHOF171. Means (±standard errors) within the same graphic followed by different letters are significantly different at $p < 0.05$.

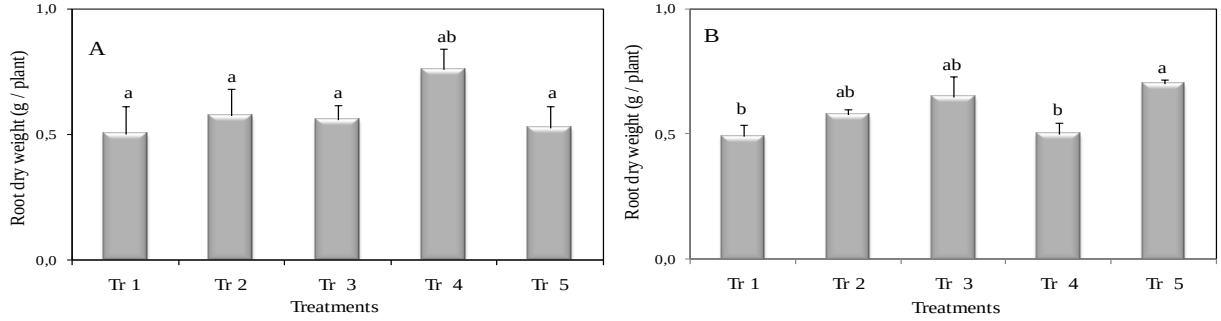

Figure 5. Roots dry weights of *V. faba* plants (A: Defes variety and B: Aguadulce variety) submitted to the five treatments. Tr 1: KH_2PO_4; Tr 2: RP+RHOF147; Tr 3: RP+RHOF174; Tr 4: RP+RHOF170; Tr 5: RP+RHOF171. Means (±standard errors) within the same graphic followed by different letters are significantly different at $p < 0.05$.

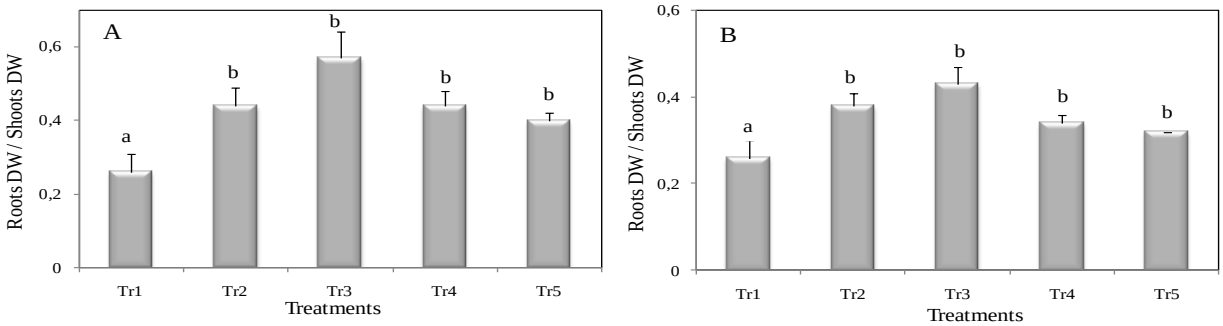

Figure 6. Roots DW / Shoots DW ratio in *V. faba* plants (A: Defes variety and B: Aguadulce variety) submitted to the five treatments. Tr 1: KH_2PO_4; Tr 2: RP+RHOF147; Tr 3: RP+RHOF174; Tr 4: RP+RHOF170; Tr 5: RP+RHOF171. Means (±standard errors) within the same graphic followed by different letters are significantly different at $p < 0.05$.

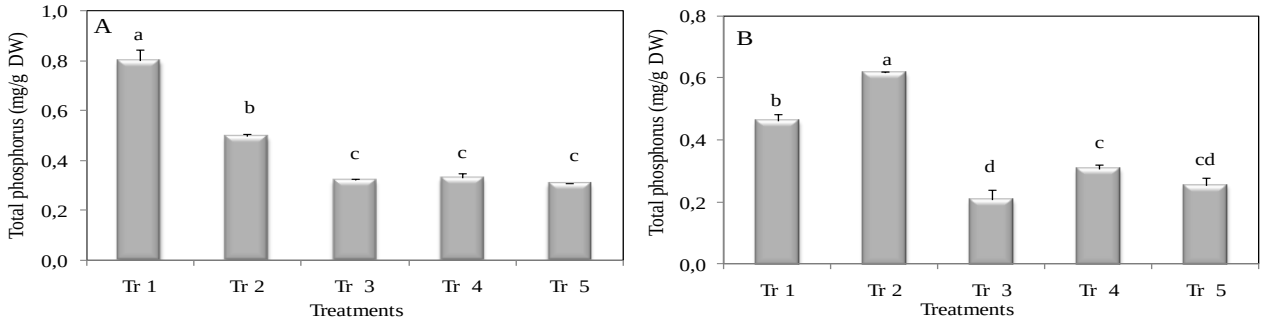

Figure 7. Phosphorus content in shoots of *V. faba* plants (A: Defes variety and B: Aguadulce variety) submitted to the five treatments. Tr 1: KH_2PO_4; Tr 2: RP+RHOF147; Tr 3: RP+RHOF174; Tr 4: RP+RHOF170; Tr 5: RP+RHOF171. Means (±standard errors) within the same graphic followed by different letters are significantly different at $p < 0.05$.

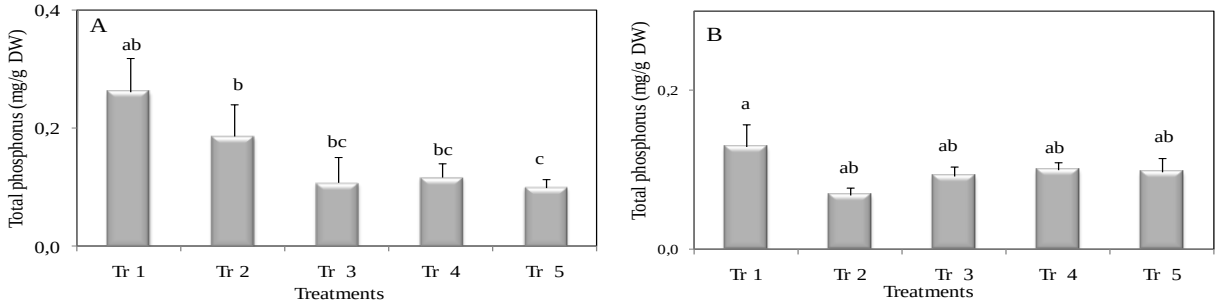

Figure 8. Phosphorus content in roots of *V. faba* plants (A: Defes variety and B: Aguadulce variety) submitted to the five treatments. Tr 1: KH_2PO_4; Tr 2: RP+RHOF147; Tr 3: RP+RHOF174; Tr 4: RP+RHOF170; Tr 5: RP+RHOF171. Means (±standard errors) within the same graphic followed by different letters are significantly different at $p < 0.05$.

To characterize the plant organ behavior, we determined the ratio root dry matter /shoot dry matter (Fig. 6). We noticed that the inoculated plants presented generally the higher ratio dry matter /shoot dry matter than that of the P-fertilized plants. The inoculated plants presented high development of the root system.

***Phosphorus uptake in faba bean plants*:** Phosphorus content would be generally higher in the plants continuously irrigated with the soluble form of phosphorus (KH_2PO_4). However, the rhizobia strains can compensate the phosphorus deficiency conditions in the presence of rock phosphate. They maintained the phosphorus uptake higher or not significantly lower than that in the P-fertilized plants continuously irrigated with soluble phosphorus (KH_2PO_4) (Fig. 7 and 8). In some symbiotic combinations, for instance, the combination Aguadulce-RHOF147 resulted in significant higher

phosphorus uptake in the shoots in comparison to the P-fertilized plants (Fig. 7B).

DISCUSSION

Rhizospheric microorganisms are important components in soils mediating soil processes such as decomposition, nutrient mobilization and mineralization, storage release of nutrients and water (Zahran, 2001; Duponnois *et al.*, 2006; Khan *et al.*, 2007). Specifically, nitrogen-fixing rhizobia-legume symbioses represent a great potential to improve crop yields and to reduce the use of fertilizers in agriculture. In addition, some rhizobia, together with *Pseudomonas* and *Bacillus*, were reported to have phosphate-solubilizing capacity (Alikhani *et al.*, 2006; Khan *et al.*, 2007; Demissie *et al.*, 2013; Bouizgarne *et al.*, 2015).

In the present work, we investigated *in vitro* Moroccan rock phosphate solubilization capacity of selected rhizobial strains in both liquid and agar medium conditions, and we examined the effect of these strains on faba bean plant growth and phosphorus uptake.

Among the four rhizobial strains tested two of them RHOF147 and RHOF174 formed clearing zones around their colonies, whereas there was no clear halo around the colonies of the strains RHOF170 and RHOF171. The clearing zone diameter increased significantly during the time of incubation. These results are consistent with reports in literature of solubilizing-phosphate bacteria selection by similar *in vitro* tests of clear halo formation around colonies on agar medium containing insoluble phosphate (Alikhani *et al*., 2006; Khan *et al*., 2007; Babana *et al*., 2013).

The rhizobial strains were then tested for their ability to solubilize rock phosphate under liquid culture medium conditions, to further confirm their phosphate-solubilizing activity and to quantify the mobilized phosphorus by each strain.

The four studied rhizobia are able to mobilize differently phosphorus from rock phosphate although two of them did not produce clearing zones on agar medium. Alikhani *et al.* (2006) have demonstrated the need to carry out liquid medium tests to check the ability of the bacteria to solubilize phosphate even it didn't not produce halo on agar medium.

This may be due to differential diffusion rates of the various organic acids secreted by an organism that may be higher in a liquid medium than in agar medium (Johnson, 1959).

Xie (2008) reported that the phosphate agar assay is a fast method useful for screening a large numbers of isolates rapidly and instantaneously. Nevertheless, it may discount isolates, although unable to produce a halo zone on agar plates, able to solubilize various types of insoluble inorganic phosphates in liquid media. Therefore, concerning P solubilization, the phosphate agar assay gives qualitative information whereas the liquid assay results in quantitative data.

Since a strain able to grow on agar medium containing insoluble phosphate as sole P-source without producing halo around its colony, may solubilize rock phosphate by other processes, performing liquid medium phosphate solubilization tests.

Hamdali *et al*. (2008b) have reported that some rhizospheric actinomycetes strains are not able to produce halos on agar medium containing rock phosphate, although they can solubilize rock phosphate in liquid medium. These authors showed that the solubilization of rock phosphate is done by alternative mechanisms such as the production of siderophores chelating phosphorus. Zaidi *et al.* (2009) discussed other mechanisms, which could be indirectly involved in phosphate solubilization, such as the secretion of siderophores and the chelation by exopolysacharide (EPS) of the metal bonds to the phosphate and release in the broth medium. In these cases, the capability to reduce the pH did not usually correlate with the ability to solubilize mineral P. However, the study of kinetic production of phosphorus by bacteria would offer a clear image of cellular behavior toward P observed by the variation of P concentration in the liquid medium (Rodriguez and Fraga, 1999).

A decrease in pH was noticed in liquid medium especially in the case of the two strains RHOF147 and RHOF174 also forming clear halos on agar medium. The most acidic pH values and the maximum mobilized phosphorus, were registered for these strains. The non-producing halo RHOF171 strain, with low phosphorus released from rock phosphate in the liquid medium, has been found to produce siderophores. This is consistent with previous works reporting that organic acids production is a major mechanism for higher phosphate solubilization (Alikhani *et al.*, 2006; Khan *et al.*, 2007).

Bacteria can dissolve phosphates by the production of organic acids for the secretion of H^+ ions. The predominant organic acids produced by the phosphate-solubilizing bacteria include oxalic acid, citric acid, lactic acid, gluconic acid, malic acid, succinic acid, fumaric acid etc (Vazquez *et al.*, 2000; Maliha *et al.*, 2004). Organic acids can also dissolve the insoluble phosphate as a result of anion exchange of PO_4^{3-} by acid anion and/or can chelate both iron and aluminum ions associated with phosphate (Omar, 1998). Inorganic acids can also solubilize phosphate but they have less activity than that of organic acids at the same pH (Kim *et al.*, 1997).

We further investigated the effect of the rhizobial strains on the growth and the phosphorus uptake by two varieties of *V. faba* (Defes and Aguaducle). We compared the effect of bioinoculation by rock phosphate-solubilizing rhizobia *versus* the chemical phosphorus fertilization with K_2HPO_4.

The effect of rock phosphate-solubilizing bacteria on plant growth depends on the inoculated strain and on the genotype used for symbiotic combination. In some combinations, the root length, the roots dry weight and the ratio roots dry weight/shoots dry weight were generally higher in the

inoculated plants. It indicated the development and the adaptation of roots exposed directly to phosphorus deficiency conditions in the inoculated plants in comparison to the P-fertilized continuously irrigated with soluble phosphorus.

When compared to the P-fertilized plants, the effect of phosphorus deficiency conditions was more pronounced on shoot dry weight of Defes plants than that of Aguadulce plants.

As for phosphorus uptake by *V. faba* plants, some rhizobial strains made phosphorus available to the plant at no significantly different levels to those registered for the plants continuously fertilized with KH_2PO_4 and sometimes at significant increased levels (eg. Aguadulce-RHOF147). These results are in accordance with the finding of Dhankhar *et al*. (2013) who have reported that the phosphate-solubilizing bacteria used as bioinoculants simultaneously increases crop yield and phosphorus uptake by the plant. Demissie *et al*. (2013) have demonstrated that most of *V. faba* plant biomass such as shoot and root dry weight were significantly increased as a result of inoculation by two rhizobia strains with rock phosphate. Yaseen *et al*. (2013) have reported that rock phosphorus fertilization with the application of *Rhizobium* and arbuscular mycorrhiza inoculation increased significantly the growth parameters of the legume plant *Macroptilum bracteatum*.

Mandri *et al*. (2012) have reported that phosphorus uptake by *Phaseolus vulgaris* may be improved by selection of both the rhizobial strain and the plant genotype most adapted to phosphorus limitation in soil.

Otherwise, Valverde *et al*. (2007) have found that the bacterial *in vitro* phosphate-solubilization ability is not always correlated with the plant phosphorus uptake.

In our study, we noticed that different symbiotic combinations result in contrasted levels of total phosphorus, whatever the ability of the rhizobial isolate to solubilize phosphorus. This result is consistent with the observations of Ormeno *et al*. (2003) reporting that *Rhizobium* and *Sinorhizobium meliloti* strains isolated from nodules of the legume plant *Phaseolus lunatus*, exhibited good capacity to solubilize phosphorus *in vitro* but only *Sinorhizobium* could mobilize it in the plant.

***Conclusions*:** The four studied rhizobial strains were differently able to mobilize rock phosphate in liquid medium of same composition although two of them not produced halos on agar medium. The most acidic pH values and the maximum of mobilized phosphorus production were noted for the halo-producing strains RHOF147 and RHOF174. The effect of rock phosphate-solubilizing bacteria on plant growth depends both on the inoculated strain and on the plant genotype for symbiotic combination. In some symbiotic combinations, the root length, the root dry weight and the ratio roots dry weight/shoots dry weight were higher in the inoculated plants than in the P-fertilized plants. The development of root system may be an adaptation to phosphorus deficiency conditions in the inoculated plants in comparison to the P-fertilized continuously irrigated with soluble phosphorus. Rhizobia strains made phosphorus available to the plant at significant increased concentrations in some symbiotic combinations (e.g. Aguadulce-RHOF147). The use of selected symbiotic combinations having the capacity to solubilize rock phosphate may improve soil fertility by making phosphorus more available to the plants. The application of efficient phosphate-solubilizing rhizobia-legume symbioses is a promising biotechnology for chemical fertilizer use limitation. The use of rhizobia symbiotically efficient with the best legume genotype combination, and having the capacity to solubilize rock phosphate represents a fascinating alternative for farmers over the world and especially in Morocco, holding the largest reserves of natural rock phosphate worldwide.

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