Journal of Materials and Environmental Sciences
ISSN : 2028-2508
CODEN : JMESCN

J. Mater. Environ. Sci., 2018, Volume 9, Issue 9, Page 2669-2677

http://www.jmaterenvironsci.com

Isolation and characterization of phosphate solubilizing rhizobia bacteria nodulating *Vicia faba* L. at the Haouz region of Morocco

M. Mouradi [1,*], M. Farissi [2], A. Khadraji [1], A. Bouizgaren [3], C. Ghoulam [1,*]

[1] *Unit of Biotechnology and Symbioses Agrophysiology, Sciences and Techniques Faculty, PO. Box 549, Gueliz Marrakesh, Morocco*

[2] *Laboratory of Biotechnology & Sustainable Development of Natural Resources, Polydisciplinary Faculty, PO Box: 592, Beni-Mellal, Morocco*

[3] *Unit of Plant Breeding, National Institute for Agronomic Research (INRA), PO. Box 533, Gueliz 40000, Marrakesh, Morocco*

Received 14 Jan 2018,
Revised 06 Apr 2018,
Accepted 01 May 2018

Keywords
- ✓ Faba bean,
- ✓ Rhizobia,
- ✓ P solubilization,
- ✓ Acid phosphatase,
- ✓ Tricalcium phosphate.

**Corresponding*
m.mouradi@hotmail.fr ; c.ghoulam@uca.ma:
Phone: +212 668 730 172

Abstract

Faba bean (*Vicia faba* L.*)* is one of the most important grain legumes grown in North Africa, thanks to its high protein content and ability to enhance soil fertility in rotation system or in intercropping. However, this crop is increasingly challenged by several abiotic stresses leading to severe yield loss. This study aims to isolate and characterize rhizobial strains nodulating faba bean with high phosphate solubilization potential. Rhizobia were isolated from root nodules of faba bean grown in the Haouz region. The results showed that from 50 isolates, only 22% and 16.6% of the total rhizobia isolates were able to solubilize tricalcium phosphate (TCP) on NBRIP and Sperber media respectively. Fb7a and Fb38 isolates presented high solubilizing index (SI) of 8.33 and 3.55 respectively. Indeed, Fb38, and Fb3b released the highest soluble phosphorus (P_i) concentrations of 1176.5 and 966.4 mg L^{-1} respectively after 96h of incubation in Sperber broth. Meanwhile, Fb7b and Fb38 presented the highest acid phosphatase (APase) activities of 3.47 and 3.21 µmol pNP $min^{-1}mL^{-1}$ respectively in the presence of TCP as sole P source. Taking all together, with P solubilization and N_2-fixing abilities, the rhizobia inoculation may ensure biological nitrogen and phosphate nutrition to faba bean plants as well as for associated species in intercropping system or crop rotation.

1. Introduction

Faba bean (*Vicia faba* L.), is one of the most important grain legumes grown in several environments of North Africa thanks to its high grain protein content (20 to 41%). It has the ability to establish N_2-fixing symbiosis with soil bacteria, genus *Rhizobium* [1]. This symbiosis represents the important nitrogen (N) source in crop rotation for agricultural soils [2]. However, the production of this crop is increasingly challenged by several abiotic stresses such as salinity, drought and nutrients deficiency responsible for severe yield loss [3]. In the Haouz region of Marrakesh, Morocco, most of the soils are phosphorus deficient. The use of chemical fertilizers could temporarily relieve these problems, but in long-term increases soil deterioration and consequently agricultural production costs [4, 5].

The faba bean- rhizobia symbiosis has been reported to provide about 330 Kg. ha^{-1}. $year^{-1}$ of available nitrogen to the soil [6]. However, faba bean plants are considered to be more sensitive to water deficit and salinity than some other legumes including alfalfa, common bean, pea and chickpea [7, 8]. In this context, ameliorating faba bean's production through some methods such as breeding and germplasm screening are more than ever demanded strategies to improve the socio-economic life of the small farmers in the region. These methods are, hence, time-consuming, expensive and have some environmental issues. The microbiological approach involving the use of growth promoting rhizobacteria (PGPR) such as rhizobia has been reported environmentally harmless and cheap [9].

Generally, rhizobia are sensitive to severe abiotic stresses. Meanwhile, it has been reported that some strains may efficiently enhance legumes stress tolerance in symbioses via a variety of complex physiological and biochemical mechanisms [10-13]. Once used as inoculum, some tolerant rhizobia strains enhance the nutrient

uptake of the nodulated plant via the biological N_2 fixation (BNF), complexed phosphate solubilization and enhancement of drought and salinity tolerance by synthesizing a variety of compatible solutes, antioxidants and hormones [14]. Also, rhizobial inoculation has been reported as an inexpensive approach ameliorating grain legumes yield equal to or better than chemical nitrogen fertilization, especially under stress conditions [15-17]. Rhizobia are group bacteria with high potential of phosphate solubilization [18-20]. It could produce organic acids, protons, and enzymes that transform insoluble phosphates into easily assimilated forms by plants that may ameliorate phosphorus supply to the soil [21].

In this context, the management of phosphates solubilizing and N_2-fixing symbiosis is a key strategy for the sustainable development of agriculture and the reduction of its dependence on chemical fertilizers. It could improve faba bean growth and yield under stressful conditions of the region without involving genetic engineering or traditional breeding of plants. The study aims to isolate and characterize efficient rhizobia strains to solubilize tricalcium phosphate from faba bean plants grown in the farmers' fields of Haouz region, Marrakesh. Thus, using them as efficient P solubilizing inoculums next to the N_2 fixing and abiotic stress tolerance enhancement of faba bean plants under the major abiotic constraints of the North African countries.

2. Material and Methods

2.1. Isolation of rhizobia

Plants of faba bean were harvested at flowering stage and collected from a farmer's field in the Haouz region of Marrakesh (Site: 31.4 N, -7.81 W and 620 m, pH_{Water} = 8.22 pH_{KCl} = 7.82, EC = 0.16 ms cm^{-1}, P_{Total} = 1.14 g Kg sol^{-1} and P_{Olsen} = 0.59 mg Kg sol^{-1}, Na^+ = 0.84 mg Kg^{-1} and K^+ = 0.85 mg Kg^{-1}). Plants were washed and their roots were separated and carefully cleaned. The nodules were gently root detached, rinsed with distilled water and then stored at -20 °C until use. For the bacteria isolation, the nodules were surface disinfected by soaking in 20% sodium hypochlorite for 5 to 10 seconds and rinsed several times with sterilized distilled water. One nodule was then crushed in a tube containing 0.2 mL of sterilized physiological water by using a Bunsen billed glass rod. The crushed nodule was removed and sown in Petri dishes with Yeast Extract Mannitol (YEM) supplemented with Congo red [22]. A series of dilutions were performed from the crushed material and 0.1 mL of each dilution was used to seed new YEM Petri dishes until homogeneous and easy to characterize colonies appear. For colony growth, the media were incubated for 48 hours at 28°C.

2.2. Nodulation test

All the isolates were passed through a nodulation test with five to six faba bean host plants used as replicates. Seeds of *(Vicia faba* L.) Reina Mora variety were surface sterilized with 2.5% sodium hypochlorite solution for 20 min, rinsed several times with sterile deionized water and germinated in sterile sand at 23°C for five days. Seedlings with homogenous growth stage were selected and then transferred to plastic pots measuring 6 cm diameter and 12 cm tall filled with sterilized sand and peat (5:1) respectively. The suspensions of each bacterial isolate were prepared to reach 10^9 CFU (Colony-formant unit) mL^{-1} YEM broth and directly used to inoculate the seedlings roots. The plants were grown in the greenhouse with approximative temperature of 26/20°C (day/night), 50±80% of relative humidity and 16 h photoperiod at the Faculty of Sciences and Techniques, Marrakesh and alternatively irrigated with sterilized deionized water and N-free nutrient solution according to [23]. After five weeks, the plants were harvested and the quality of nodulation was estimated for each of the studied isolates.

2.3. Tricalcium phosphate (TCP) solubilizing capacity of the rhizobia isolates

2.3.1. Qualitative technique

The ability of rhizobia isolates to solubilize tricalcium phosphate ($Ca_3(PO_4)_2$) can be visually estimated using agar medium selection methods. This test consists of selecting rhizobia that are able to make clear areas around their microbial colonies in a basic medium containing $Ca_3(PO_4)_2$ as the sole source of phosphorus. These clear zones are due to the production of organic acids in the environment surrounding the colonies in question [24]. However, the relative efficacy test of microorganisms on agar medium is considered as a reliable and preliminary characterization of microorganisms dissolving complexed phosphate. In this study, the isolates were tested in two phosphate solubilizing media contained 2.5 g L^{-1} $Ca_3(PO_4)_2$ as sole phosphorus source. (i) Sperber control (g L^{-1}, 10 Glucose, 2.5 K_2HPO_4, 0.25 $MgSO_4$ $7H_2O$, 0.5 Yeast extract, 0.25 $CaCl_2$ with pH=7.2) [25] and (ii) NBRIP (g L^{-1}, 10 Glucose, 0.25 $MgSO_4$ $7H_2O$, 0.5 $MgCl_2$ $6H_2O$, 0.15 (NH_2) SO_4, 0.2 KCl with pH= 7) [26].

2.3.2. Quantitative technique

This test is based on the measurement of soluble phosphorus in the form of orthophosphate and acidic APases, released by the isolates in a broth media from $Ca_3(PO_4)_2$ as the sole source of phosphorus. However, the solubilization rate of this compound is generally estimated by subtracting the final concentration of released orthophosphates from the theoretical initial concentration of phosphorus contained in the solubilized compound. This estimation has the drawback of neglecting the phosphorus used by the cells during growth [20]. In this study, the ability of the isolates to release soluble phosphorus form $Ca_3(PO_4)_2$ was estimated in Sperber broth medium. Thus, a preculture of each of the ten rhizobia isolates was prepared beforehand in YEM broth to reach about 10^9 CFU mL^{-1} to inoculate Erlenmeyer flasks containing each 100 mL of Sperber medium with 2.5 g L^{-1} $Ca_3(PO_4)_2$ (solubilization media) or K_2HPO_4 (control media) as sole phosphorus sources. 1 mL of the preculture suspension was added to each of the ten Erlenmeyer flasks containing Sperber medium. Autoclaved and uninoculated media served as positive controls. The inoculated media and controls were incubated for 10 days with constant stirring at 120 rpm at 28 °C and in the dark on a shaking incubator. Three replicates per isolate per treatment were performed. For analysis, 2 mL sample was taken daily under aseptic conditions from the incubated Erlenmeyer flasks. The samples were then centrifuged at 2500 g for 15 minutes, 100 µL of the supernatants were used for the quantification of soluble phosphorus in the form of orthophosphates using acid molybdate method [27]. The pH of each suspension was also determined using an ice pH meter.

2.4. Acid phosphatase (APase) activity determination

The APase activity was determined for the isolates suspension according to Araújo, Plassard [28]. 2 mL of the incubated broths was taken each day under sterile conditions. The samples were then centrifuged at 2500 g for 15 min, 100 µL of the supernatants was added to 200 µL para-Nitrophenylphosphate (pNPP). After 30 minutes of incubation at 37 °C, the reaction was stopped by adding 1 mL of 1N NaOH to the reaction medium. The blank was prepared by adding 500 µL of the incubation medium without inoculation, 125 µL of distilled water and 1 mL of 0.5 N NaOH. The APase activity was determined by measuring the formation of para-nitrophenol (pNP) at 410 nm and expressed in µmol pNP min^{-1} mL^{-1}. Three replicates were used for each considered isolate.

3. Results and discussion

3.1. Isolation of rhizobia from Vicia faba L nodules

After 48 hours of incubation on YMA medium, 60 isolates were obtained after purification and morphological characterization. Only 49 isolates presented circular, slightly elevated, smooth and opaque colonies. The selected homogenous isolates have little absorbed Congo red on YEM agar. This is observed in the majority of rhizobia isolated form legumes nodules as well as directly from the soil [29, 30]. This property is common within the genus *Bradyrhizobium*. However, it has been reported that the genus *Agrobacterium* and the contaminant forms strongly absorb this dye [31]. The 45 rhizobia isolates presented a variation in their nodulation capacity in association with *Vicia faba* L. *Reina Mora* var. as plant host (Table 1; Figure 1).

Figure 1: Nodulation of the *Reina Mora* variety by the Fb7a, Fb11, Fb19a and Fb35 isolates after 3 weeks of inoculation

Table 1 : Ability of purified isolates to form root nodules in *Vicia faba* L. *Reina Mora* var. plants (10 mL 10^9 CFU mL^{-1} YEM) in aseptic conditions.

Isolate	Nodulation	Isolate	Nodulation	Isolate	Nodulation	Isolate	Nodulation	Isolate	Nodulation	Isolate	Nodulation
Fb3b	+	Fb14b	-	Fb19a	+	Fb25a	++	**Fb35**	**+++**	Fb45	+
Fb4b	+	Fb14c	+	Fb19c	+	Fb25b	+	Fb37	+	Fb48	+
Fb6	+	Fb15	-	Fb20	+	Fb26a	+	Fb38	+	Fb49	++
Fb7a	+	Fb16	++	Fb20c	++	Fb26b	++	Fb39	-	Fb50	+
Fb8a	+	Fb17	+	Fb21c	+	Fb28a	++	Fb40	+		
Fb8b	+	Fb18a	++	Fb22a	-	**Fb29**	**+++**	Fb41	++		
Fb10b	++	Fb18b	+	Fb23	++	Fb30a	++	Fb42	++		
Fb11	+	**Fb18c**	**+++**	Fb24a	++	Fb33	++	Fb43	+		
Fb14a	++	Fb18d	-	Fb24b	+	**Fb34**	**+++**	Fb44	+		

+++ : high nodulation ; ++ medium ; + : low ; - : no nodulation

3.2. Solubilization of tricalcium phosphate on agar media by rhizobia isolates

The ability of the bacterial isolates from *Vicia faba* root nodules to solubilize inorganic phosphate was estimated by cultivating them on Sperber and MNBRIP, two solubilization agar media containing tricalcium phosphate (TCP) as the sole source of phosphorus. This test permits the selection of the most efficient microorganisms and consists of measuring the area of the clear zone produced by each colony [32-34]. The results show that 23% of the tested rhizobia isolates presented high solubilization capacity of tricalcium phosphate (Table 2). This capability was demonstrated by the presence of solubilization halos around the colonies in the Sperber and NBRIP agar media (Figure 2 and 3).

Table 2: Solubilization index (SI) on Sperber agar with $Ca_3(SO_4)_2$ as the sole P source by the studied rhizobia isolates after 10 days of incubation

Isolate	SHD (cm)	CD (cm)	SI (SHD/CD)
Fb3b	2.7	0.8	**3.37**
Fb4b	1.5	0.6	2.5
Fb7a	2.5	0.3	**8.33**
Fb8a	2.3	0.7	**3.28**
Fb8b	1.8	0.6	3
Fb20	1.4	0.6	2.33
Fb21c	3.1	1	3.1
Fb38	2.13	0.6	**3.55**

SHD: solubilizing halo diameter; CD: Colony diameter; SI: Solubilizing index

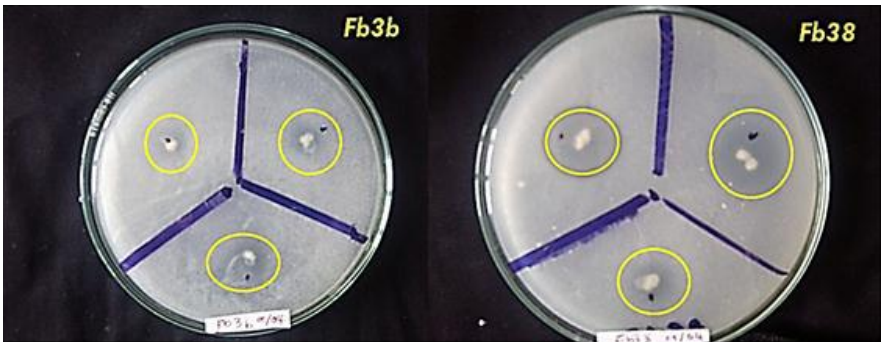

Figure 2: Solubilization halos formed by the Fb3b and Fb38 isolates in Sperber agar with $Ca_3(SO_4)_2$ as sole phosphorus source (10 d of incubation)

Several studies have reported that rhizobia isolates nodulating root legumes are able to solubilize large inorganic phosphate complexes [35, 36]. High variation has been noted between the isolates in terms of solubilizing indexes

(SI). It appears that Fb7a, Fb38, Fb3b and Fb8a isolates showed high TCP solubilizing capacity in comparison with the other bacterial strains in Sperber medium. They presented SI values of about 8.33, 3.55, 3.37 and 3.28 respectively (Table 2). Hajjam, Alami [37] and Kenasa, Jida [38] characterized rhizobia isolates nodulating faba been in Morocco and Ethiopia with average SI values of 2. On the other hand, in NBRIP medium, Fb21c, Fb7a, Fb38 and Fb43 isolates presented SI values of 4.85, 4, 3.6 and 3 respectively (Table 3; Figure 3).

Table 3: Solubilization index (SI) on NBRIP agar with $Ca_3(SO_4)_2$ as sole P source by the studied rhizobia isolates after 10 days of incubation

Isolate	SHD (cm)	CD (cm)	SI (SHD/CD)
Fb3b	0.7	0.25	2.8
Fb7a	1.2	0.3	**4**
Fb8a	0.95	0.52	1.82
Fb8b	0.93	0.56	1.66
Fb10b	1.25	0.62	2.01
Fb20	1.2	0.8	1.5
Fb21c	**1.65**	**0.34**	**4.85**
Fb23	0.43	0.2	2.15
Fb38	0.9	0.25	**3.6**
Fb39	1	0.4	2.5
Fb43	1.2	0.4	**3**

SHD: solubilizing halo diameter; CD: Colony diameter; SI: Solubilizing index

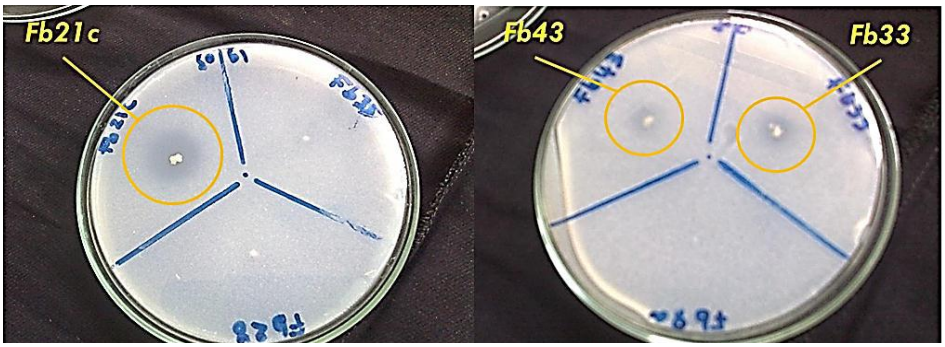

Figure 3: Solubilization halos formed by Fb21c, Fb43 and Fb33 isolates in NBRIP agar medium with $Ca_3(SO_4)_2$ as sole phosphorus source (10 d of incubation)

The percentage of rhizobia isolates that showed phosphorus solubilization activities was close to 16.6% of all rhizobia isolated from *Vicia faba* L. root nodules and tested on Sperber medium while on NBRIP medium this percentage exceeded 22%. This confirms the results reported by Nautiyal [26] on the efficiency of NBRIP in the detection of more P solubilization microorganisms in comparison with Sperber and Pikovskaya. In fact, despite obtaining good results by this method, it has been demonstrated that several microorganisms incapable of producing clear zones around their colonies can solubilize inorganic phosphates in a liquid medium [26, 39]. As a result, a quantification of the phosphorus released and the acid phosphatase activity by these strains in a liquid medium was then carried out.

3.3. Solubilization of TCP on broth media by the rhizobia isolates

From the results illustrated in figure 4, a progressive decrease in the K_2HPO_4 concentration during the incubation time has been noted in the all of the studied isolates with variation between them. Fb7b, Fb8a, Fb8b and Fb21c isolates have shown a rapid decrease during the first 48 hours of incubation in comparison with other. This decrease can be explained by the variation in Pi use of each of the studied isolate their growth and metabolism. F8b and Fb3b were the highest Pi consumers with only 73.5 and 105 mg L^{-1} left after 96h of incubation.

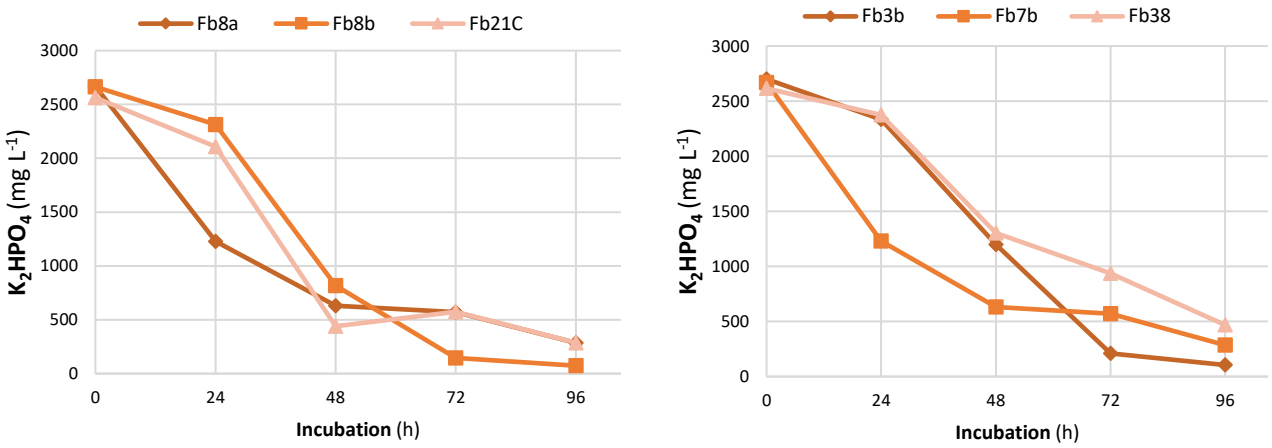

Figure 4: Kinetics of K_2HPO_4 phosphate consumption in control Sperber media by the tested rhizobia isolates

The pH of the control media in the presence of K_2HPO_4 did not significantly change compared to the control for all the strains tested, these values remained around 6.25 and 6.67 respectively for the Fb8b and Fb21c strains after 96h of incubation (Figure 5). This may be explained by the absence of any significant solubilizing activity in the medium for all of the studied rhizobia isolates in the presence of K_2HPO_4 as sol P source.

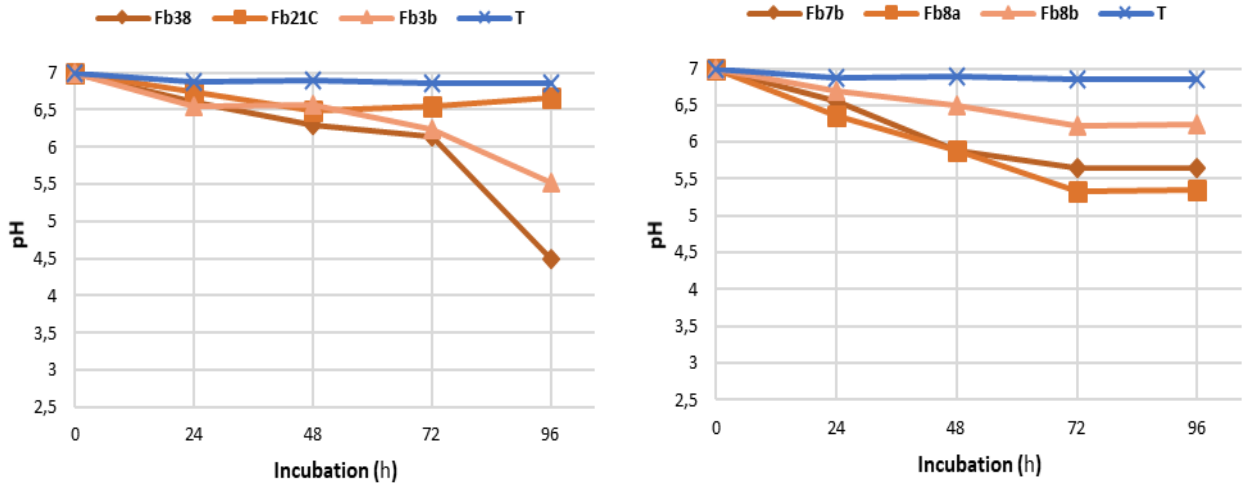

Figure 5: Evolution of the pH of the control media during the incubation time in the control medium with K_2HPO_4 for the selected rhizobia strains in comparison with the control T (without inoculation).

The results showed that $Ca_3(PO_4)_2$ solubilization in the culture medium for each of the studied isolate gradually ($p<0.001$) increased during the first days and reached its maximum values after 96 h of incubation (Figure 6). Indeed, Fb38 and Fb3b isolates released the highest Pi concentrations of 1176.5 and 966.4 mg L^{-1} after 96h of incubation. Meanwhile, Fb8b released only 575.6 mg L^{-1} in the same conditions. It should be noted that most of the isolates started to solubilize phosphate in the first 24 hours. Similar results for *Rhizobium leguminosarum* and *Rhizobium* sp. have been reported by Rodríguez and Fraga [20] and Halder and Chakrabartty [40] respectively and showed that the P_i released in the presence of hydroxyapatite and tricalcium phosphate was 356 and 300 mg L^{-1} respectively. The pH of the media was progressively decreased during the incubation period to reach 3.25, 3.28 and 3.31 respectively for Fb3b, Fb38, and Fb7b after 96h of incubation (Figure 7). This correlation between the decrease in pH and the release of orthophosphates in all of the tested isolates has been reported by several authors [20, 26, 41, 42]. Indeed, the authors demonstrated that the pH decrease is strongly related to the release of organic acids by the bacteria. In addition, the solubilization of inorganic phosphate in some cases may be attributed to the production and distribution of acids [43]. They are carboxylic acids synthesized and released by microorganisms. This operation is also responsible for the decrease in pH [44]. Pi can also be released from inorganic phosphate by proton substitution [45].

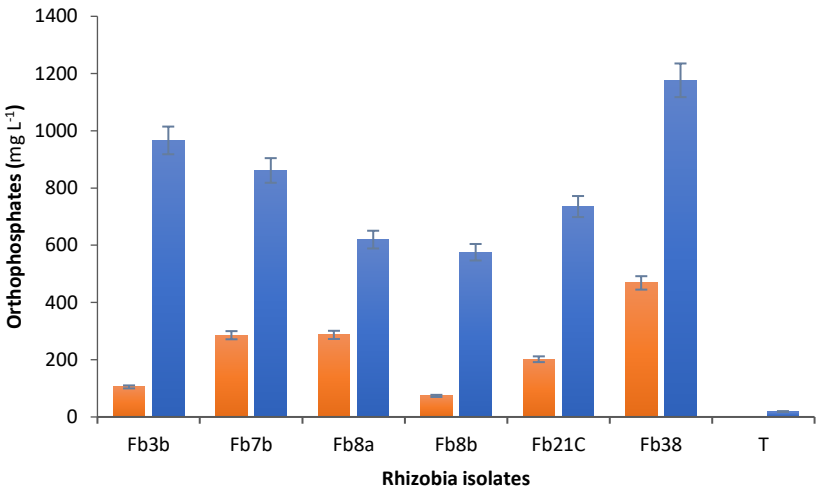

Figure 6: Quantities of orthophosphates (mg mL^{-1}) released into the Sperber broth by the tested rhizobia isolates after 96h of incubation in the presence of $Ca_3(PO_4)_2$ (■) and K_2HPO_4 (■) as sole P sources.

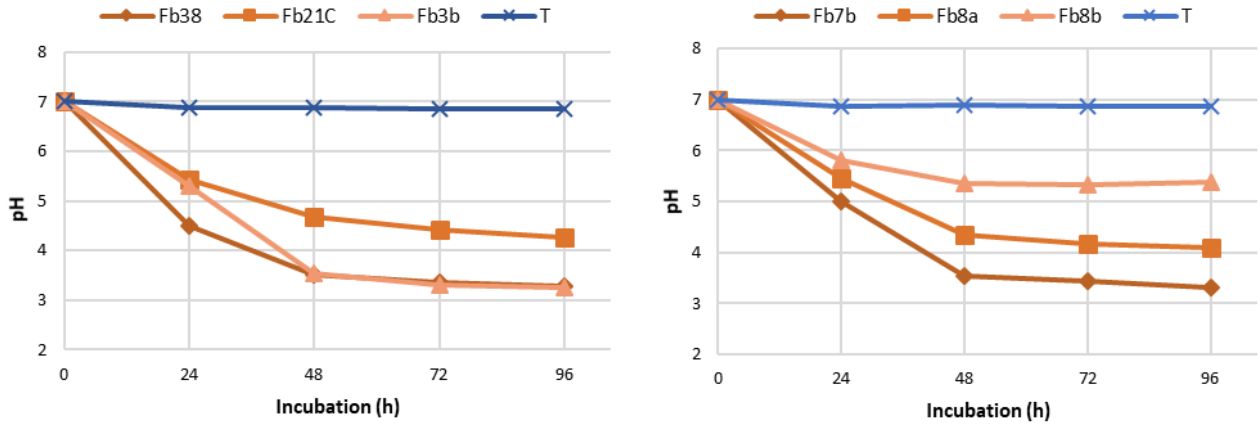

Figure 7: Evolution of the pH of the solubilization media $Ca_3(PO_4)_2$ with respect to the incubation time for the selected strains in comparison with the control.

3.4. APase activity released by the rhizobial isolates

The results show that the APase activity released by the tested isolates were significantly higher in the presence of TCP in comparison with the K_2HPO_4 medium as sol P sources. In the presence of $Ca_3(PO_4)_2$ a progressive APase increase has been noted in all of the studied isolates with variation between them (Figure 8).

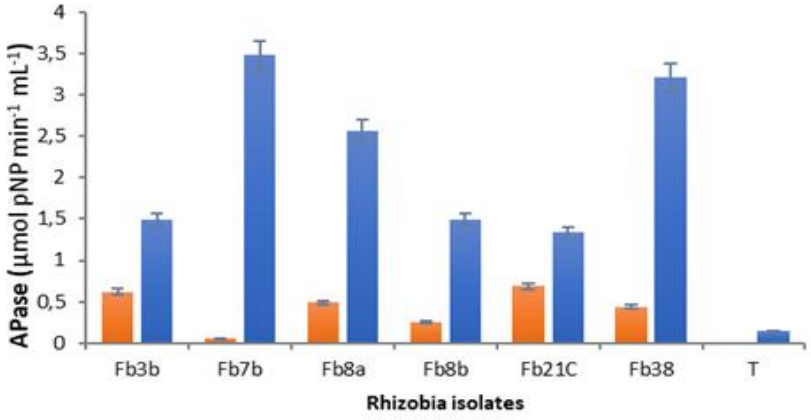

Figure 8: Activity of APase released by the studied isolates after 96h of incubation in the presence of $Ca_3(PO_4)_2$ (■) and K_2HPO_4 (■) as sole P sources.

On the other hand, no significant changes have been observed in the presence of K_2HPO_4. Fb38, Fb7b and Fb8a isolates presented the highest APase activity of in the medium after 96 hours of incubation with values of 3.21, 3.47 and 2.56 µmol pNP min^{-1} mL^{-1} respectively which were the highest recorded values in this trial. Meanwhile, the lowest APase activities of 33 and 1.48 µmol pNP min^{-1} mL^{-1} were presented by Fb21c and Fb8b respectively (Figure 8). Several studies have shown that P solubilizing microorganisms are capable of producing acid phosphatases in the medium, which are enzymes that mobilize organic phosphates and attached phosphoryl groups [46].

Conclusion

The results obtained on solid and liquid Sperber and NBRIP media showed the real ability of the studied rhizobia isolates nodulating faba bean plants, to mobilize large quantities of phosphorus from TCP ($Ca_3(PO_4)_2$) inorganic source. 22% of the rhizobia have shown solubilization halos in NBRIP agar. Meanwhile, only 16.6% have presented halos in Sperber. The P solubilization was strongly correlated with the decrease of the pH and also an increase in APase activity, especially for Fb7b and Fb38 isolates.

The beneficial effects of these rhizobia isolates on faba bean plants in symbioses should be studied particularly under the major abiotic circumstances of the region. This may be possible to ensure biological nitrogen and phosphate nutrition as well as for the other crops in intercropping or in rotation systems.

References

1. J. Young, *Int. J. Syst. Evol. Microbiol*. 53 (2003) 2107-2110.
2. U. Köpke,T. Nemecek, *Field Crops Res*. 115 (2010) 217-233.
3. L. Ricciardi, G. Polignano, C. De Giovanni, *Euphytica*. 118 (2001) 39-46.
4. M. Mouradi. Ph.D., *Thesis, Cadi Ayyad University, FSSM, Marrakesh*. 188 (2017).
5. M. Mouradi, Master, *Thesis, Cadi Ayyad University, FSTG, Marrakesh*. (2012).
6. M.J. Unkovich, J.S. Pate, *Field Crop Res*. 65 (2000) 211-228.
7. M. Farooq, M. Hussain, K.H. Siddique, *Crit. Rev. Plant Sci.*. 33 (2014) 331-349.
8. A. Khadraji, M. Mouradi, C. Houasli, A. Qaddoury, C. Ghoulam, *Seed Sci. Technol*. 45 (2017) 198-211.
9. M. Naveed, B. Mitter, T.G. Reichenauer, K. Wieczorek, A. Sessitsch, *Environ. Exp. Bot*. 97 (2014) 30-39.
10. M. Mouradi, M. Farissi, A. Bouizgaren, B. Makoudi, A. Kabbadj, A.A. Very, H. Sentenac, A. Qaddourya, C. Ghoulam, *Arid Land Res. Manag*. 30 (2016) 193-208.
11. M. Mouradi, A. Bouizgaren, M. Farissi, B. Makoudi, A. Kabbadj, A.-A. Very, H. Sentenac, A. Qaddoury, C. Ghoulam, *Chil. J. Agr. Res*. 76 (2016) 265-272.
12. L. Latrach, M. Farissi, M. Mauradi, B. Makoudi, A. Bouizgaren, C. Ghoulam, *Turk. J. Agric. For*. 38 (2014) 320-326.
13. G. Moschetti, A. Peluso, A. Protopapa, M. Anastasio, O. Pepe, R. Defez, *Syst. Appl. Microbiol.* 28 (2005) 619-631.
14. M. Naveed, M.B. Hussain, I. Mehboob, Z.A. Zahir, A. Zaidi, M.S. Khan, and J. Musarrat, Editors., *Springer International Publishing Cham*. (2017) 341-365.
15. S. Ben Romdhane, M. Aouani, M. Trabelsi, P. De Lajudie, R. Mhamdi, *J. Agri. Crop Sci*. 194 (2008) 413-420
16. M. Mouradi, A. Bouizgaren, M. Farissi, L. Latrach, A. Qaddoury, C. Ghoulam, *Sci. Hort*. 213 (2016) 232-242
17. M. Mouradi, A. Bouizgaren, M. Farissi, A. Qaddoury, C. Ghoulam, *J. Plant Nutr*. 41 (2018) 384-395.
18. A.H. Babana, A.H. Dicko, K. Maïga, D. Traoré, *J. Microbiol. Microbial Res*. 1 (2013) 1-6.
19. L. Boudanga, M. Farissi, A. Bouizgaren, C. Ghoulam, *J. Mater. Environ. Sci*. 6 (2014) 997-1003.
20. H. Rodríguez,R. Fraga, *Biotechnol. Adv*. 17(1999) 319-339.
21. N. Shetta, T. Al-Shaharani, M. Abdel-Aal, Am. *Eurasian J. Agric. Environ. Sci.* 10 (2011) 410-418.
22. J. Vincent, *A Manual for the Practical Study of the Root-Nodule Bacteria*. (1970) 1-13.
23. D.R. Hoagland, D.I. Arnon, Circular. *Calif. Agric. Exp. Sta*. 347 (1950).
24. H. Halvorson, A. Keynan, H. Kornberg, *Soil Biol. Biochem*. 22 (1990) 887-890.
25. J.I. Sperber, *Aust. J. Agric. Res*. 9 (1958) 778-781.
26. C.S. Nautiyal, *FEMS Microbiol. Lett*. 170 (1999) 265-270.
27. N. AFNOR, *Association Française de Normalisation, Paris*. (1997).

28. A.P. Araújo, C. Plassard, J.J. Drevon, *Plant Soil*. 312 (2008) 129.
29. J.M. Vincent. 1970 *IBP Handbk 15 Oxford and Edinburgh: Blackwell Scientific Publications*. 164 pp.
30. L. Latrach, M. Mouradi, M. Farissi, A. Bouizgaren, C. Ghoulam, *Appl. J. Envir. Eng. Sci*. 3 (2017) 353-364.
31. D. Jordan, *Bergey's Manual of Systematic Bacteriology*. 1 (1984) 235-244.
32. M.B. Hussain, Z.A. Zahir, H.N. Asghar, R. Mubaraka, M. Naveed, *Clean–Soil, Air, Water*. 44 (2016) 1564-1571.
33. X. Ding, S. Zhang, R. Wang, S. Li, X. Liao, *J. Plant Nutr*. 39 (2016) 1915-1925.
34. M. Mouradi, I.M. Kadmiri, L. Amehdar, L. Latrach, A. Hilali, *Mor. J. Chem*. 5 (2017) 697-707.
35. M. Lazali, M. Zaman-Allah, L. Amenc, G. Ounane, J. Abadie, J.-J. Drevon, *Planta*. 238 (2013) 317-324.
36. H. Imen, A. Neila, B. Adnane, B. Manel, Y. Mabrouk, M. Saidi, S. Bouaziz, *J. Plant Nutr*. 38 (2015) 1656-1671.
37. Y. Hajjam, I. Alami, S. Udupa, S. Cherkaoui. *J. Mater. Envir. Sci*. 7(11) (2016) 4000-4010.32
38. G. Kenasa, M. Jida, F. Assefa, *Sci. Technol. Arts Res. J*. 3 (2014) 11-17.
39. R. Gupta, R. Singal, A. Shankar, R.C. kuhad, R.K. saxena, *J. Gen. Appl. Microbiol*. 40 (1994) 255-260.
40. A. Halder,P. Chakrabartty, *Folia Microbiol*. 38 (1993) 325-330.
41. A. Kumar, I. Bahadur, B. Maurya, R. Raghuwanshi, V. Meena, D. Singh, J. Dixit, *J Pure Appl Microbiol*. 9 (2015) 715-724.
42. P. Hinsinger, *Plant Soil*. 237 (2001) 173-195.
43. A.E. Richardson, *Funct. Plant Biol*. 28 (2001) 897-906.
44. H. Rodríguez, R. Fraga, T. Gonzalez, Y. Bashan, *Plant Soil*. 287 (2006) 15-21.
45. A.H. Goldstein, Phosphate in microorganisms: cellular and molecular biology. *ASM Press, Washington, DC*. (1994) 197-203.
46. P. Ponmurugan, C. Gopi, *Afr. J. Biotechnol*. 5 (2006) 348.