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# Isolation and evaluation of phosphate solubilizing rhizobia from root nodules of faba bean *(Vicia faba L.)* in Morocco

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## Abstract

A study was conducted to evaluate the phosphate solubilization ability of rhizobial isolates and their effect on symbiotic effectiveness of faba bean (*Vicia faba L.*). To achieve this aim, 30 isolates were sampled from root nodules of faba bean in different geographical regions in Morocco. These isolates were evaluated with two reference strains (PSRA and PSRB) of *Rhizobium leguminosarum* for their resistance to environmental constrains such as pH, salinity and temperature variation. In addition, phosphate solubilization was performed on Sperber's basal mediums with different amounts of glucose (M1, M2 and M3). Besides, an experimental design was carried out under greenhouse conditions in order to evaluate the effect of phosphate solubilizing rhizobia (PSR) on symbiotic effectiveness parameters of faba bean. The results showed that among all tested PSR ; PSR19, PSR26, and PSR29 were more resistant to extreme stresses and have shown a significant rate of solubilization on Sperber's basal mediums ($p < 0.05$). All PSR have a significant effect on symbiotic effectiveness parameters ($p < 0.01$). PSR29 showed the highest number of nodules ($34.333 \pm 1.527$. plant$^{-1}$), of nodule dry weight ($53.077 \pm 3.434$ mg.plant$^{-1}$), shoot dry weight ($1.039 \pm 0.051$ g.plant$^{-1}$) and was highly effective (SE=81.35%). Overall, PSR19, PSR26 and PSR29 were found to be the most performant and may be recommended as biofertilizers in defected soils to decline the use of chemical fertilizers.

*Keywords:* PSR, Tricalcium phosphate, Environmental constrains, Symbiotic effectiveness

## 1. Introduction

Agriculture and soil management activities such as fertilization, tillage and biomass alteration represent a great challenge towards food and environment worldwide [1-3]. The extensive use of chemical fertilizers in agrosystems for enhancing fertility and agronomic yield induce several issues including soils depletion and pollution [4-7]. Indeed, these chemical fertilizers are expensive and are known to be immobilized soon after their application in soils and become unavailable to plants nutrition [8]. For instance, phosphorus based chemical fertilizers; being derived from phosphate rocks, have several impacts especially on landscape transformation, and water alteration with radioactive compounds and heavy metals [4]. Besides, phosphorus is one of the major macronutrients for biological development and growth along the plant cycle [9]. However, it represents a limiting factor to plant nutrition due to its low soluble forms in soil (varying from 0.001 mg. l$^{-1}$ in deficient soils to 1 mg. L$^{-1}$ in heavily fertilized soils) [10]. Furthermore, phosphorus is involved in different cellular process including photosynthesis, respiration energy storage and transfer, cell division and early stages of seed formation [4]. Hence, previous studies have investigated phosphate solubilizing rhizobia (PSR) as biofertilizers, in order to reduce the cost of chemical fertilizers and to decrease soil degradation and pollution [11-13]. The mechanism behind phosphate solubilization is explained by the ability of some soil bacteria to

produce organic acids and chelate oxoacids from carbonic compounds [14, 15]. It has been identified in several studies that 2-ketoglucolnic acid is the most synthesized organic acid by *Rhizobium leguminosarum, Rhizobium meliloti* and *Bacillus firmus* [16,17] during phosphate solubilization process. Beyond the mechanisms mentioned above, PSR are also involved in nitrogen fixing symbiosis with legumes by inducing nodule formation [10]. Several reports showed that phosphate solubilizing rhizobia have a great effect on growth parameters of inoculated plants [18,19]. However, few studies have shown the effect of PSR on symbiotic effectiveness of legumes and specifically on faba bean (*Vicia faba L.)* [20]. Although, studies are limited in terms of evaluating the resistance of phosphate solubilizing rhizobia to environment constrains e.g. extreme temperature, salinity and pH variation in Morocco. Therefore, throughout this study, we examined the ability of some rhizobia isolated from root nodules of faba bean (*vicia faba L*.) to solubilize phosphates in different Sperber's basal mediums and the most efficient isolates were tested on Sperber's broth culture. Also, we evaluated these PSR for their resistance to different environemental stresses. The major aim of the current study was to assess phosphate solubilizing rhizobia under greenhouse conditions to evaluate their effect on symbiotic effectiveness parameters.

## 2. Material and methods

*2.1. Root nodules collection and preservation*

Root nodules of faba bean (*Vicia fabaL.)* were collected from plant representing five geographical regions in Morocco (Chbanat, Ain Sbite, Had Ait Mimoun, Merchouch, Ait Ourir). The sampling was made by digging nearly 15 cm to either side of the faba bean (*Vicia faba L.*) plant to 25 cm depth [21]. Then, root nodules of plants were transferred to the laboratory for further nodules preservation. From each plant, a number of thirty nodules were collected and preserved in silica gel tubes at room temperature, in order to keep them dry and to inhibit any growth of fungi or other bacteria.

*2.2. Soil analysis*

Soil samples representing each site were analyzed to determine water conductivity, pH, phosphorus and potassium content. Ten g of soil were added to 25 ml of distillated water to determine the pH of each sample by using a pH meter. The electrical conductivity was measured by preparing a liquid extract of 20 g of soil and 100 ml of distillated water and determined by a conductimeter. The available phosphorus in samples was determined by the molybdate blue method and the absorbance was measured at 820 nm according to Murphy et al. (1962) method [22]. A flame spectrophotometer JENWAY-6405 was used to determine $K^+$ content in soil samples.

2.3. *Isolation, purification and preservation of rhizobial isolates*

Fresh nodules were carefully surface sterilized by ethanol 70% for 60 seconds, then transferred and soak in 3% of calcium hypochlorite or chlorox ($CaCl_2$) solution for 5 to 6 minutes. Nodules were washed immediately by distillated water, five to seven times. Each nodule was covered by a drop of distillated water for further crush and isolation. The nodule suspension was streaked out on YEM medium (Yeast extract mannitol) supplemented with Congo red [23]. As a confirmatory test of rhizobia, Gram staining was performed on the isolates and the observation was made by a microscope at x 100 magnification using oil immersion. The selected pure cultures are recognized by their white and creamy appearance which is the morphological characteristic of rhizobia, comparing to the other cultures that can absorb congo red. Finally, pure cultures were preserved in glycerol 50% (v/v) and kept in the freezer at (-80°C) for further analysis.

2.4. *Bromothymol blue test (BTB)*

Five ml of BTB (0,016N) were added to YEM medium before autoclaving. This indicator turns yellow at pH=6.0 and blue at pH=7.6 and is green between pH 6.0 and 7.6 [21]. The selection of isolates in terms of fast/ slow growth and production of acids/alkalis is based on the appearance of agar plates (yellow color: fast growing rhizobia with production of acids; blue color: slow growing rhizobia with production of alkalis).

2.5. *pH, temperature and salt resistance*

The resistance of phosphate solubilizing rhizobia (PSR) to acidity (pH=4; pH=6), alkalinity (pH=8 and pH=10), temperature (between 32 and 38°C) and to salinity (NaCl= 3%, 6%, 10%) was examined on agar plates by blotting technique. The reading of agar plates was made after 48 hours of incubation at 28 ±2°C.

2.6. *Phosphate solubilization ability on agar plates assay*
Phosphate solubilizing PSR ability was assessed on Sperber's basal medium (glucose: 10g; yeast extract: 0.5 g; $MgSO_4.7H_2O$: 0.25g; $CaCl_2$:0.1 g; agar: 15g) supplemented with 2.5g of tricalcium phosphate $Ca_3 (PO_4)_2$ in 1000 ml of distillated water; pH=7.2.
Two other mediums were used by modifying Sperber's basal medium M2 (glucose: 5g; yeast extract: 0.5 g ; $MgSO_4.7H_2O$: 0.25 g; $CaCl_2$: 0.1 g ; agar: 15 g; $Ca_3(PO_4)_2$: 2.5 g in 1000 ml of distillated water ; pH=7,2) and M3 (glucose: 15 g; yeast extract: 0.5 g ; $MgSO_4.7H2O$: 0.25 g; $CaCl_2$: 0.1 g; agar: 15 g; $Ca_3(PO_4)_2$: 2.5 g in 1000 ml of distillated water ; pH=7.2).
The ability of solubilization was visualized by the appearance of a clear zone halo on Sperber's basal plates [24]. The index and the efficiency of solubilization were calculated based on the colony diameter and halo zone diameter of each isolate.

2.7. *Experimental design for symbiotic effectiveness test*
Evaluation of symbiotic effectiveness of PSR was conducted under greenhouse conditions using sterile pots (filled with sterilized 2/3 of soil and 1/3 of sand), and faba bean seeds aseptically sterilized. In addition, the total soil was amended with sterilized 30 $mg.kg^{-1}$ of tricalcium phosphate $Ca_3 (PO4)_2$ (TCP) . The experiment of inoculation was replicated three times. One ml ($1.10^8$ UFC/ml) of each PSR isolates liquid culture was used to inoculate one seed of 3 days old per pot in sterilized conditions. A positive (irrigated by 5% $KNO_3$ and 5% $KH_2PO_4$) and negative controls (without inoculation or supplemented fertilizers) were used for each replicate. Pots were irrigated twice a week. The uprooting of plants was made after eight weeks of sowing and inoculating. Several parameters were fixed to evaluate symbiotic effectiveness of faba bean (*Vicia faba L.*) with PSR, like number of nodules per plants for each replicate. Nodules and shoots were dried during 3 days at 70°C in order to determine nodules and shoot dry weights parameters.

2.8. *Estimation of phosphate solubilizing ability by PSR in broth culture*
Phosphate solubilizing ability of selected PSR was estimated in Sperber's basal medium broth culture. Each 1ml ($1.10^8$ UFC/ml) of selected PSR, according to their significant symbiotic effectiveness of inoculated plants, were transferred into 250 ml Erlenmeyer flasks filled with liquid Sperber's basal medium and incubated on a rotator shaker during 8 days at 28±2°C, (200 rev/min). Phosphate solubilizing ability was estimated after each 24hours by mo-blue method [25].

*2.9. Statistical analysis*
Statistical analysis was performed using the SPSS 10.0 software package. Duncan test was used for significance differences of means for phosphate solubilization and symbiotic effectiveness parameters at $p < 0.05$. In addition, correlations between PSI and PSE parameters of solubilization were performed by Spearman's Rho test at ($p < 0.01$).

## 3. Results

*3.1. Morphological identification of rhizobial isolates*
The thirty isolates were preliminary identified on YEM medium. All isolates have shown a negative reaction to congo red and they were not contaminated by *Agrobacterium*. Moreover, Gram staining had shown that all rhizobial isolates were Gram negative which is one of the characteristics of *Rhizobium*. Then, growth rate and cell morphology were examined by a microscope. All isolates appeared whitish, creamy and gummy on agar plates.

*3.2. Soil characteristics of sampling sites*
Table 1 indicates chemical properties of original sites from where PSR were sampled. According to the pH analysis, all soils have a neutral pH. In addition, phosphorus and potassium analysis showed that soil sampled from Merchouch region displayed the highest level of P (0.215 $mg.Kg^{-1}$) and K (0.36 $g.Kg^{-1}$), whereas the lowest levels of these nutrients were noted on soil from Ain Sbite region. The electrical conductivity tested on soils, showed that all values of the main soils are inferior to 2 $ds.cm^{-1}$.

**Table1:** Soil properties of sampling sites

| **Soil Sampling** | **GPS** | **Code Isolates** | **$pH_{Soil}$** | **EC (ds/m)** | **P $mg.kg^{-1}$ $Sol^{-1}$** | **$K^+$ $g.kg^{-1}$ $sol^{-1}$** |
|---|---|---|---|---|---|---|
| Ain Sbite | N 33°33'57,3'' W 006°30'53,9'' | PSR1, PSR2, PSR3, PSR4, PSR5, PSR6 | 7.27 | 1.423 | 0.045 | 0.12 |
| Had Ait Mimoun | N 33°48'65,1'' W 005°50'29,0'' | PSR7, PSR8, PSR9, PSR10, PSR11, PSR12 | 7.85 | 0.399 | 0.063 | 0.22 |
| Merchouch | N 33° 40'37,3" W 006°41'54,9" | PSR13,PSR14, PSR15, PSR16, PSR17, PSR18 | 7.18 | 0.256 | 0.215 | 0.36 |
| Ait Ourir | N 31°34'50,6'' W 007°47'33,2'' | PSR19, PSR20, PSR21, PSR22, PSR23, PSR24 | 7.75 | 1.061 | 0.163 | 0.21 |
| Chbanat | N 34°21'66,6''W005°48'20,55'' | PSR25, PSR26, PSR27, PSR28, PSR29, PSR30 | 7.95 | 0.761 | 0.072 | 0.26 |

*3.3. Fast growth and production of acids*

Most isolates were fast growing and have shown a positive yellow reaction to YEM medium supplemented with BTB (Table2, figure 1a). However, PSR12, PSR23, PSR24, PSR25 are slow growing and have turned the agar plates medium to blue color. It implies that these isolates are slow growing and they might be included into Bradyrhizobium genera (Figure 1a).

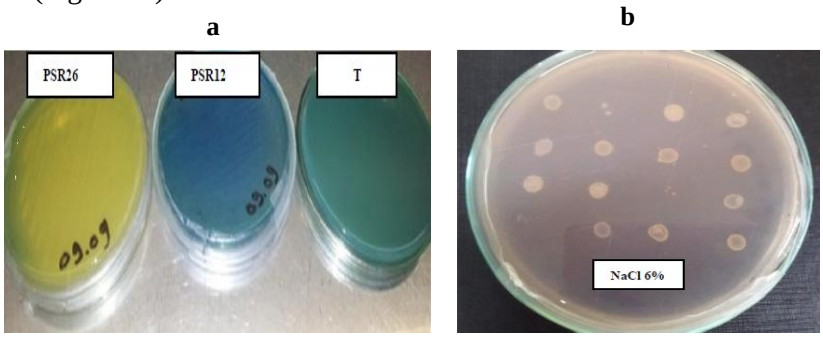


**Figure 1:** (a) BTB test reaction by PSR; (b) Evaluation of PSR to various stresses (Example of NaCl 6%).

*3.4. Acidity, high temperature and salt tolerance*

Table 2 summarizes results of resistance and sensitivity of PSR to various stresses. We noted that the most resistant PSR to different treatments are: PSR10, PSR11, PSR19, PSR21, PSR22, PSR26, PSR27, PSR28, PSR29, and PSR30. Besides, the two reference strains of *Rhizobium leguminosarum* PSRA and PSRB were also evaluated on the different stresses mentioned in table 2. Both reference strains were resistant to pH, temperature and salt tolerance variation. However, PSRA was less resistant to extreme temperature 38°C than PSRB (Figure1-b).

*3.4. Phosphate solubilizing rhizobia ability*

According to table 3, most PSR isolates have a significant difference ($p < 0.05$) of phosphate solubilizing ability on various Sperber's basal mediums. Only PSR12, PSR23, PSR24, and PSR25 have not shown any phosphate solubilizing ability on the three Sperber's basal mediums. Phosphate solubilization ability on M2 was significantly lower ($p < 0.001$) than M1 and M3 mediums (Figure 1a , figure 1b). A positive correlation was noted between solubilization index (SI) and solubilization efficiency (PSE) on M1, M2 and M3. Beyond the

reference strains PSRA and PSRB, the highest SI levels were displayed respectively on M1 and M3 by PSR19 (SI= 2.5; 2.93) followed by PSR26 (SI= 2.61; 3.08) and PSR29 (SI=2.86; 3.08). Moreover, PSE was also significant on M1 and M3 and the highest values were reached respectively by PSR19 (PSE=95%; 125.8), PSR26 (PSE%= 61.29; 107.25) and PSR29 (PSE%= 86.66; 118.57).

**Table 2:** PSR profile of BTB test reaction and resistance to different stresses

| Isolats | BTB | 3%NaCl | 6%NaCl | 10%NaCl | pH4 | pH6 | pH8 | pH10 | T32°C | T35°C | T38°C |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **PSR1** | P | +++ | +++ | - | +++ | +++ | +++ | - | +++ | +++ | ++ |
| **PSR2** | P | +++ | ++ | - | +++ | +++ | +++ | - | +++ | + | - |
| **PSR3** | P | +++ | ++ | - | +++ | +++ | +++ | - | +++ | + | - |
| **PSR4** | P | +++ | ++ | - | +++ | +++ | +++ | - | +++ | + | - |
| **PSR5** | P | +++ | ++ | - | +++ | +++ | +++ | - | +++ | + | - |
| **PSR6** | P | +++ | ++ | - | +++ | +++ | +++ | - | +++ | + | - |
| **PSR7** | P | +++ | ++ | - | +++ | +++ | +++ | - | +++ | + | - |
| **PSR8** | P | +++ | ++ | - | +++ | +++ | +++ | - | +++ | + | - |
| **PSR9** | P | +++ | ++ | - | +++ | +++ | +++ | ++ | +++ | + | - |
| **PSR10** | P | +++ | +++ | ++ | +++ | +++ | +++ | ++ | +++ | ++ | + |
| **PSR11** | P | +++ | +++ | + | +++ | +++ | +++ | - | +++ | ++ | + |
| **PSR12** | N | + | - | - | - | - | - | - | +++ | + | - |
| **PSR13** | P | +++ | ++ | - | - | - | - | - | +++ | + | - |
| **PSR14** | P | +++ | ++ | - | - | - | - | - | +++ | + | - |
| **PSR15** | P | +++ | ++ | - | - | - | - | - | +++ | + | - |
| **PSR16** | P | +++ | ++ | - | - | - | - | - | +++ | + | - |
| **PSR17** | P | +++ | ++ | - | - | - | - | - | +++ | + | - |
| **PSR18** | P | +++ | ++ | - | - | - | - | - | +++ | + | - |
| **PSR19** | P | +++ | ++ | ++ | +++ | ++ | ++ | ++ | +++ | ++ | + |
| **PSR20** | P | +++ | +++ | - | +++ | ++ | ++ | + | +++ | + | + |
| **PSR21** | P | +++ | +++ | ++ | +++ | ++ | ++ | ++ | +++ | ++ | + |
| **PSR22** | P | +++ | +++ | ++ | +++ | ++ | ++ | ++ | +++ | ++ | + |
| **PSR23** | N | + | - | - | - | - | - | - | +++ | + | - |
| **PSR24** | N | + | + | - | - | - | - | - | +++ | + | - |
| **PSR25** | N | + | + | - | +++ | - | - | - | +++ | + | - |
| **PSR26** | P | +++ | +++ | ++ | ++ | +++ | +++ | +++ | +++ | ++ | + |
| **PSR27** | P | +++ | +++ | + | +++ | +++ | +++ | +++ | +++ | ++ | + |
| **PSR28** | P | +++ | +++ | + | +++ | +++ | +++ | +++ | +++ | ++ | + |
| **PSR29** | P | +++ | +++ | ++ | +++ | +++ | +++ | +++ | +++ | ++ | + |
| **PSR30** | P | +++ | +++ | + | +++ | +++ | +++ | +++ | +++ | ++ | + |
| **PSRA*** | P | +++ | +++ | ++ | +++ | +++ | +++ | +++ | +++ | ++ | + |
| **PSRB*** | P | +++ | +++ | ++ | +++ | +++ | +++ | +++ | +++ | ++ | ++ |

*PSRA and PSRB are reference strains of *Rhizobium leguminosarum; P :positive reaction; N: negative reaction; (+++) very significant growth; (++)significant growth; (+) less abundant growth; (-) no growth.*

**a** **b**


**Figure 2:** (a) Phosphate solubilization of PSR 29 on M3 Sperber's basal medium; (b) Appearance of phosphate solubilization by PSR 29 on M2 Sperber's basal medium.

*3.5. Nodulation and symbiotic effectiveness*

All PSR isolates were able to induce nodule formation under greenhouse conditions unless PSR24 (Table 4). Indeed, statistical analysis showed that all PSR have a significant effect on symbiotic effectiveness parameters ($p < 0.05$). The highest number of nodules was recorded on PSR29, whereas PSR24 did not show any nodulation on faba bean plants. As it is shown in table3, the averages of nodules dry weights showed that among inoculated plants with rhizobial isolates, PSR29 and PSR26 have the highest averages in this experiment. On the other hand, the averages of shoot dry weights of faba bean plants were highly significant ($p < 0.001$). Therefore, the highest average of shoot dry weight was noted on PSR19, PSR26, and PSR29. It is showed that inoculation by PSR was significantly different on symbiotic effectiveness levels. PSR may be clustered into three groups: non effective (<35%), effective (50-80%) and highly effective (> 80%) [21]. Results indicated that PSR 29 displayed the highest level of symbiotic effectiveness (81.35%), while PSR2, PSR3, PSR5, PSR9, PSR12, PSR23, PSR24, PSR25 showed the lowest level of symbiotic effectiveness (Table 4).

**Table 3:** Phosphate solubilization ability parameters on Sperber's basal mediums

| Isolates | M1 | | M2 | | M3 | |
|---|---|---|---|---|---|---|
| | PSI | PSE % | PSI | PSE% | PSI | PSE % |
| **PSR1** | 2.4 | 60.14 | 2,5 | 50 | 2.8 | 80 |
| **PSR2** | 2,2 | 48 | NS | NS | 2.75 | 75 |
| **PSR3** | 2,4 | 60.14 | NS | NS | 2.5 | 50.0 |
| **PSR4** | 2.3 | 53.3 | 2.2 | 20 | 2.6 | 66.66 |
| **PSR5** | 2.42 | 66.66 | NS | NS | 2.57 | 57.14 |
| **PSR6** | 2.3 | 60 | NS | NS | 2.66 | 66.66 |
| **PSR7** | 2.42 | 42.85 | NS | NS | 2.6 | 60 |
| **PSR8** | 2.32 | 32.07 | NS | NS | 2.5 | 50 |
| **PSR9** | 2.25 | 25 | NS | NS | 2,32 | 32.07 |
| **PSR10** | 2.4 | 55.38 | 2.25 | 25 | 2.73 | 73.07 |
| **PSR11** | 2.3 | 40.5 | 2.25 | 25 | 2.6 | 100 |
| **PSR12** | NS | NS | NS | NS | NS | NS |
| **PSR13** | 2.31 | 31.5 | 1.73 | 36.36 | 2.58 | 57.5 |
| **PSR14** | 2,47 | 57.5 | 2,4 | 40 | 2,01 | 70.11 |
| **PSR15** | 2,25 | 25.0 | NS | NS | 2.46 | 46 |
| **PSR16** | 2.46 | 46.66 | NS | NS | 2.5 | 50.0 |
| **PSR17** | 2.4 | 50 | NS | NS | 2.64 | 64 |
| **PSR18** | 2,2 | 20 | NS | NS | 2.53 | 53.33 |
| **PSR19** | 2,5 | 95 | 2.3 | 25 | 2.93 | 125.8 |
| **PSR20** | 2.45 | 45.16 | NS | NS | 2.59 | 59.37 |
| **PSR21** | 2,41 | 60.87 | 2.43 | 43.33 | 2.75 | 75.0 |
| **PSR22** | 2.45 | 45.16 | 2,19 | 19.04 | 2.52 | 52.5 |
| **PSR23** | NS | NS | NS | NS | NS | NS |
| **PSR24** | NS | NS | NS | NS | NS | NS |
| **PSR25** | NS | NS | NS | NS | NS | NS |
| **PSR26** | 2.61 | 61.29 | 3,25 | 50 | 3.08 | 107.25 |
| **PSR27** | 2.4 | 60 | 2.5 | 50 | 2.96 | 96.77 |
| **PSR28** | 2.36 | 36.66 | 2.3 | 30 | 2.37 | 37.5 |
| **PSR29** | 2.86 | 86.66 | 2.8 | 80 | 3.18 | 118.57 |
| **PSR30** | 2,4 | 57.69 | 2.43 | 43.33 | 2.73 | 73.07 |
| **PSRA*** | 2.4 | 40 | 2.33 | 33.33 | 2.59 | 59.61 |
| **PSRB*** | 2.73 | 73.07 | 2.56 | 56.25 | 2.83 | 83.33 |

*NS: No significant solubilization; M1, M2 and M3: Sperber's mediums amended with 10 g, 5g and 15 of glucose*
*PSI: Phosphate solubilization index= colony diameter + halo zone diameter /growth diameter*
*PSE: Phosphate solubilization efficiency= solubilization diameter/growth diameter x 100*

*3.6. Selected PSR and their phosphate solubilization ability on broth culture*

The phosphate solubilization ability by selected PSR was estimated on Sperber's basal medium broth culture. Among selected rhizobial isolates, PSR19, PSR29 and PSR26 reached the highest solubilizing ability of liberated phosphorus in the liquid culture comparing to reference strains of *Rhizobium leguminausarum* PSRA and PSRB (Figure 3).

**Table 4:** Symbiotic effectiveness parameters of inoculated plants by PSR

| Isolates | Number of nodules.plt$^{-1}$ | Nodules dry weight.plt$^{-1}$.mg$^{-1}$ | Shoot dry weight.plt$^{-1}$.g$^{-1}$ | SE % |
|---|---|---|---|---|
| **PSR1** | 18 .00 ±5.567$^{a}$ | 31.500±2.755$^{f}$ | 0.696±0.163$^{i}$ | 54.97 |
| **PSR2** | 10.33±1.527$^{b}$ | 13.387±1.851$^{e}$ | 0.155±0.060$^{j}$ | 12.24 |
| **PSR3** | 10.66±3.511$^{b}$ | 12.664±0.899$^{e}$ | 0.10±0.010$^{k}$ | 7.89 |
| **PSR4** | 12.66±4.618$^{ab}$ | 26.937±2.092$^{f}$ | 0.445±0.056$^{l}$ | 35.15 |
| **PSR5** | 9.66±3.214$^{b}$ | 9.741±0.951$^{g}$ | 0.144±0.012$^{j}$ | 11.37 |
| **PSR6** | 9.00±1.00$^{b}$ | 12.048±3.059$^{e}$ | 0.234±0.010 | 18.48 |
| **PSR7** | 9.00±3.00$^{b}$ | 14.010±0.493$^{h}$ | 0.467±0.078$^{ij}$ | 36.88 |
| **PSR8** | 8.33±0.577$^{ab}$ | 12.333±0.244 $^{e}$ | 0.452±0.276l$^{ij}$ | 35.7 |
| **PSR9** | 5.33±0.577$^{ac}$ | 8.970±0.638$^{g}$ | 0.210±0.040$^{kl}$ | 16.58 |
| **PSR10** | 8.33±0.577$^{b}$ | 9.517±1.00$^{g}$ | 0.669±0.153$^{i}$ | 52.84 |
| **PSR11** | 9.00±1.00$^{b}$ | 10.511±1.281$^{g}$ | 0.574±0.169$^{i}$ | 57.46 |
| **PSR12** | 4.00±1.00$^{ac}$ | 3.747±0.546e$^{f}$ | 0.129±0.026$^{k}$ | 10.18 |
| **PSR13** | 14.66±1.527$^{ad}$ | 14.517±1.893$^{h}$ | 0.510±0.073$^{i}$ | 40.28 |
| **PSR14** | 11.333±0.577$^{b}$ | 12.986±0.855 $^{e}$ | 0.119±0.005$^{k}$ | 44.73 |
| **PSR15** | 8.333±1.527$^{ab}$ | 11.926±2.111$^{e}$ | 0.182±0.088$^{kj}$ | 14.37 |
| **PSR16** | 6.333±1.154$^{d}$ | 11.846±0.670$^{e}$ | 0.282±0.085$^{kl}$ | 22.27 |
| **PSR17** | 11.666±0.577$^{b}$ | 14.0917±1.239$^{h}$ | 0.341±0.112$^{jl}$ | 26.93 |
| **PSR18** | 11,00±5.567$^{b}$ | 13.671±1.7350$^{e}$ | 0.370±0.032$^{jl}$ | 29.22 |
| **PSR19** | 19.333±7.371$^{abc}$ | 34.866±2.745$^{f}$ | 0.890±0.067$^{m}$ | 70.3 |
| **PSR20** | 11.666±1.527$^{b}$ | 13.598±1.287$^{e}$ | 0.324±0.002$^{jl}$ | 25.29 |
| **PSR21** | 7.333±1.527$^{ab}$ | 9.800±0.595$^{g}$ | 0.152±0.031$^{j}$ | 57.14 |
| **PSR22** | 17.666±3.055 | 48.582±6.143$^{gh}$ | 0.853±0.054$^{m}$ | 67.37 |
| **PSR23** | 5.333±2.516$^{ac}$ | 5.889±0.608$^{ef}$ | 0.117±0.003$^{k}$ | 11.37 |
| **PSR24** | 0.00±0.00$^{ac}$ | 0.00±0.00$^{ef}$ | 0.137±0.064$^{mk}$ | 10.82 |
| **PSR25** | 9.00±1.00$^{ab}$ | 9.386±1.399$^{g}$ | 0.139±0.015$^{mk}$ | 10.97 |
| **PSR26** | 30.666±5.507$^{acd}$ | 49.066±5.518$^{gh}$ | 0.961±0.034$^{m}$ | 75.9 |
| **PSR27** | 14.00±1.00 $^{ad}$ | 14.263±1.951$^{h}$ | 0.171±0.062$^{kj}$ | 64.28 |
| **PSR28** | 29.666±1.527$^{acd}$ | 36.566±3.432$^{f}$ | 0.682±0.059$^{i}$ | 53.87 |
| **PSR29** | 34.333±1.527$^{bc}$ | 53.077±3.434$^{gh}$ | 1.039±0.05$^{1}$ | 81.35 |
| **PSR30** | 10.00±2.00$^{b}$ | 12.463±1.407$^{ef}$ | 0.154±0.047$^{j}$ | 57.89 |
| **PSRA*** | 29.00±3.605$^{acd}$ | 37.904±3.050$^{f}$ | 0.726±0.096$^{i}$ | 57.34 |
| **PSRB*** | 18.00±12.165$^{a}$ | 41.759±3.040$^{gh}$ | 0.771±0.047$^{i}$ | 60.9 |
| **Control +** | - | - | 1.266± 0.032$^{l}$ | - |
| **Control$^{-}$** | - | - | 0.073± 0.056$^{mkl}$ | - |

*Values followed by the same letters are not significantly different*

*Symbiotic effectiveness (%) =Shoot dry weight of inoculated plants/Shoot dry weight of N and P supplied plants X 100.*

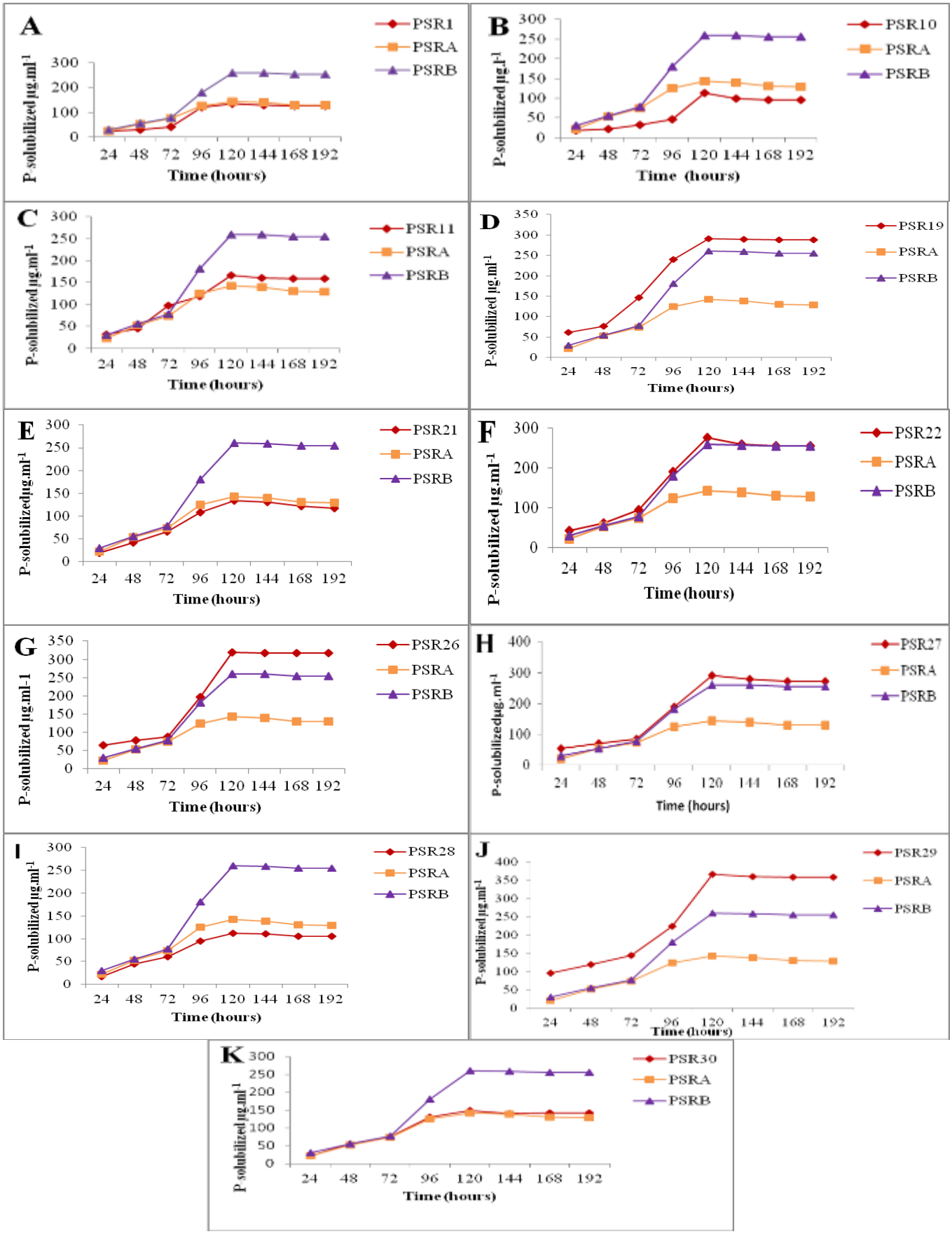


**Figure 3:** Estimation of Phosphate solubilization ability by selected PSR on Sperber's broth culture.

## 4. Discussion

Our study aimed to screen rhizobial isolates on one side, for their resistance to various stresses and to evaluate their ability for solubilizing inorganic phosphates on different Sperber's basal mediums. On the other side, these isolates were tested for their effect on symbiotic effectiveness of faba bean (*Vicia faba L.*) plants.

### *4.1. Resistance of PSR to environmental constrains: pH, salinity and temperature*

Phenotypic characterization of PSR was studied in order to select the most efficient and resistant isolates to environmental stresses. Some PSR isolates were tolerant to extreme acidity, alkalinity, salinity, and temperature. Several studies showed the importance of studying phosphate solubilization ability along with the tolerance to environmental constrains [26]. Indeed, Rice et al. [27] reported that *Rhizobium leguminosarum* strains vary in their resistances to stress. This fluctuation is an advantage of one strain to survive over the other in the extreme soil environment. Moreover, these tolerant *Rhizobium leguminosarum* strains in different soils stresses may improve the production and the adaptation of legumes grown in different areas [28].

### *4.2. BTB test and Screening of PSR on different modified Sperber's basal mediums*

Fast growing PSR that induced a positive reaction on BTB test, were able to solubilize inorganic phosphates $Ca_3(PO_4)_2$ (Table 2 and 3). This implies that the mechanism of solubilization is mainly based on chelation of oxo acids from sugar to produce organic acids that facilate the uptake of inorganic phosphates [29, 12]. Although, Maliha et al. [30] reported that during solubilization process, gluconic acids are usually produced by phosphate solubilizing bacteria in the rhizosphere. Besides, when we tested PSR on different Sperber's basal medium (M1, M2 and M3), we found out that most isolates didn't show any halo zone on M2 agar plates. On the contrary, a relevant solubilization was highly significant on M3 flowed by M1. It means that the level of solubilization varies depending on the high or low amount of carbon sources. This result is in agreement with S.C Nautiyal [31] who investigated the ability of solubilization by rhizospheric microorganisms on NBRIY medium. He noted that the more the amount of carbon source in the medium was increased, the more the rate of phosphate solubilization was high. In this study , we found out that solubilization index SI of rhizobial isolates PSR varied between 2.2 and 2.86 on M1 (Sperber's basal medium without modification) and on M3 (modified Sperber's medium), this index was in the range of 2.01 to 3.18. Same study was reported by Kenasa et al. (2014) [20] who showed that the phosphate solubilization index of rhizobial isolates was comprised between 1.25 and 2.10.

### *4.3. Relationship between TCP uptake by PSR and symbiotic effectiveness of faba bean*

According to symbiotic effectiveness test under greenhouse experiment, most PSR had a significant effect on number of nodules, nodules dry weight, shoot dry weight and symbiotic effectiveness level comparing to positive and negative controls. PSR29 which was resistant to environmental stress, showed a high number of nodules (34.333±1.527. $plant^{-1}$), nodule dry weight (53.077±3.434. $mg^{-1}$. $plant^{-1}$) and a high significant symbiotic effectiveness (81.35%). Similar trend was reported in chickpea plants by Peix et al. (2001) [12] who observed that dry weight was significantly increased in a neighborhood of 14% ($p < 0.05$) when the soil was inoculated with *Mesorhizobium* PECA21 strain compared to the uninoculated soils. Although, the same author reported that dry weight was significantly higher (43%, $p < 0.05$) in inoculated barley plants with phosphate solubilizing bacteria. This can be explained by the uptake of TCP by PSR in soil and the variation in symbiotic effectiveness parameters is related to the ability of each PSR to solubilize TCP under greenhouse conditions.

### *4.4. Solubilization of PSR on Sperber's broth culture*

Only selected isolates, in terms of their efficient symbiotic effectiveness (>50%), were subjected to phosphate solubilization in liquid Sperber's basal medium (Figure 3). The aim was to estimate and quantify the liberated phosphorus by PSR in liquid medium in a range of time from 24 to 192 hours. PSR29 displayed the highest capacity to solubilize phosphorus in the broth culture (359 $\mu g.ml^{-1}$ in 192 hours after incubation at 28±2°C), comparing to reference strains of *Rhizobium leguminosarum* PSRA and PSRB which reached respectively 129$\mu g.ml^{-1}$ and 255$\mu g.ml^{-1}$ of P-liberated after 192 hours of incubation. However, all PSR showed a stationary phase of liberated phosphorus in liquid culture after 120 hours of incubation. This is in agreement with Liu et al. (2015) [32] who showed recently that during solubilization process, the rate of phosphorus uptake becomes

higher in broth culture when bacteria reach high cellular densities, and an important part of P is incorporated into biomass of bacterial cells. Therefore, a decrease in solubilized P in the medium could be observed [33]. Although, Babenko et al [34] observed that these changes in P uptake might be due to the precipitation of organic metabolites or to the formation of organo-P compounds with secreted organic acids, which are subsequently used as an energy and nutrient sources for bacteria.

*4.5. Comparing between origin sites of PSR*

According to Table 1 and 2, PSR which are originated from Merchouch and Ain Sbit sites, are less effective and sensitive to various stresses in this study. Whereas the most resistant isolates (PSR10, PSR11, PSR19, PSR22, PSR26, PSR27, PSR28, PSR29 and PSR30) belong to Had Ait Mimoun, Ait Ourir and Chbanat regions. Therefore, performant isolates in terms of their solubilization efficiency, resistance to extreme environmental constrains are worth to be utilized as phosphates biofertilizers inoculums, especially in Ain Sbite and Merchouch regions in order to enhance the uptake of phosphorus by legumes.

## Conclusion

Our study showed that most PSR had the ability to solubilize inorganic phosphate TCP *in vitro* assay and had also a significant effect on symbiotic effectiveness of faba bean (*Vicia faba L.*) under greenhouse experiment. Among these rhizobial isolates, PSR19, PSR26, and PSR29 were found to be the most efficient and resistant isolates. Further investigations of these PSR may be exploited as nitrogen and phosphorus biofertilizers to reduce the use of chemical fertilizers, which cause a serious problem of pollution. Thereby, this approach will be used to enhance fertilization in soils even for non legumes crops.

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