



Contents lists available at ScienceDirect

Saudi Journal of Biological Sciences

journal homepage: www.sciencedirect.com

Original article

Synergistic effect of *Pseudomonas alkylphenolica* PF9 and *Sinorhizobium meliloti* Rm41 on Moroccan alfalfa population grown under limited phosphorus availability

Omar Farssi^{a,b}, Rabie Saih^a, Ahmed El Moukhtari^a, Aziz Oubenali^a, Mohammed Mouradi^a, Mohamed Lazali^c, Cherki Ghoulam^d, Abdelaziz Bouizgaren^e, Hicham Berrougui^b, Mohamed Farissi^{a,*}^a Unit of Biotechnology and Plant-Microbe Interaction, Laboratory of Biotechnology & Sustainable Development of Natural Resources, Polydisciplinary Faculty of Beni-Mellal, Sultan Moulay Slimane University, Morocco^b Polyvalent Laboratory on R&D, Polydisciplinary Faculty of Beni-Mellal, Sultan Moulay Slimane University, Morocco^c ERP Research Laboratory, Faculty of Natural and Life Sciences and Earth Sciences, University of Khemis Miliana, Algeria^d Unit of Plant Biotechnology and Agro-physiology of Symbiosis, Faculty of Sciences and Techniques, Cadi Ayyad University, Marrakesh & Mohamed VI Polytechnic University, Ben-Guerir, Morocco^e Unit of Plant Breeding, National Institute for Agronomic Research, Marrakesh (INRA-Marrakech), Morocco

ARTICLE INFO

Article history:

Received 26 January 2021

Revised 16 March 2021

Accepted 25 March 2021

Available online 2 April 2021

Keywords:

Alfalfa

*Sinorhizobium meliloti**Pseudomonas alkylphenolica*

Co-inoculation

Growth

Phosphorus

ABSTRACT

This study looked at the synergistic effect of *Pseudomonas alkylphenolica* PF9 and *Sinorhizobium meliloti* Rm41 on the Moroccan alfalfa population (*Oued Lmaleh*) grown under symbiotic nitrogen fixation and limited phosphorus (P) availability. The experiment was conducted in a growth chamber and after two weeks of sowing, the young seedlings were inoculated with *Sinorhizobium meliloti* Rm41 alone or combined with a suspension of *Pseudomonas alkylphenolica* PF9. Then, the seedlings were submitted to limited available P (insoluble P using Ca_3HPO_4) versus a soluble P form (KH_2PO_4) at a final concentration of $250 \mu\text{mol P}\cdot\text{plant}^{-1}\cdot\text{week}^{-1}$. After two months of P stress, the experiment was evaluated through some agro-physiological and biochemical parameters. The results indicated that the inoculation of alfalfa plants with *Sinorhizobium* strain alone or combined with *Pseudomonas* strain significantly ($p < 0.001$) improved the plant growth, the physiological and the biochemical traits focused in comparison to the uninoculated and P-stressed plants. For most sets of parameters, the improvement was more obvious in plants co-inoculated with both strains than in those inoculated with *Sinorhizobium meliloti* Rm41 alone. In fact, under limited P-availability, the co-inoculation with two strains significantly ($p < 0.01$) enhanced the growth of alfalfa plants evaluated by fresh and dry biomasses, plant height and leaf area. The results indicated also that the enhancement noted in plant growth was positively correlated with the shoot and root P contents. Furthermore, the incensement in plant P contents in response to bacterial inoculation improved cell membrane stability, reflected by low malonyldialdehyde (MDA) and electrolyte leakage (EL) contents, and photosynthetic-related parameters such as chlorophyll contents, the maximum quantum yield of PS II (F_v/F_m) and stomatal conductance (g_s). Our findings suggest that *Pseudomonas alkylphenolica* PF9 can act synergistically with *Sinorhizobium meliloti* Rm41 in promoting alfalfa growth under low-P availability.

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* Corresponding author at: Polydisciplinary Faculty of Beni-Mella, Sultan Moulay Slimane University, Mghila, PO Box 592, Beni-Mellal 23000, Morocco.

E-mail addresses: farissimohamed@gmail.com, mohamed.farissi@usms.ac.ma (M. Farissi).

Peer review under responsibility of King Saud University.



Production and hosting by Elsevier

1. Introduction

In the Mediterranean area, forge and grain legumes are largely cultivated for their high nutritional quality, high protein content, and their favorable effects on soil fertility (Lahrizi et al., 2021; Farissi et al., 2018). In fact, these species contribute to the incorporation of nitrogen in agro-pastoral ecosystems with beneficial economic impact, helping to reduce or limit the use of chemical

<https://doi.org/10.1016/j.sjbs.2021.03.069>

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nitrogen fertilizers by nitrogen-fixing symbiosis involving rhizobia (Oukaltouma et al., 2020; Faghire et al., 2011).

Among forage legumes, alfalfa (*Medicago sativa* L.) is one of the leguminous forage species with numerous socioeconomic and environmental benefits. Due to its contribution to sustainable agriculture and production of feed proteins per unit area, it is the most common forage legume in Moroccan crop-livestock systems, as well as many European and North American countries (Farissi et al., 2013, 2011). In fact, alfalfa has the ability to provide more nitrogen to the agricultural ecosystems than the total amount of nitrogen applied by fertilization (Rengel, 2002). Furthermore, when correctly associated with specific rhizobia strains, this crop is important in maintaining the structure and nitrogen fertility of soils in which it grows (Guiñazú et al., 2010).

Despite the agro-environmental importance of legumes, their culture is concentrated in the northern regions with a favorable climate. In fact, over the last few decades, the environmental constraints have led to a reduction in grain and forage legume production areas in many countries in the southern part of the Mediterranean basin, including Morocco.

Besides osmotic stress, legumes are sensitive to nutritional constraints such as low phosphorus (P) availability, particularly during symbiotic nitrogen fixation (SNF), leading to a significant yield decrease (Oukaltouma et al., 2020). Indeed, SNF poses additional demands of P with up to 20% of total plant P being allocated to nodules and any P deficiency may influence the activity of rhizobia and consequently the symbiosis efficiency (Drevon, 2017). Moreover, the high reactivity of P with some cations such as iron, aluminum (Al) and calcium (Ca), to form insoluble compounds, reduces its mobility in the soil solution. In fact, Gessa et al., 2005 reported that the mobility of phosphate is influenced by pH and Ca concentration in soil. In fact, the increasing Ca concentration with increasing pH slows down the phosphate flux. Furthermore, the presence of Al inhibits the phosphate mobility. These reactions provoke a very low-P availability and low efficiency of phosphate fertilizers used by plants. As a consequence, the limitation of SNF process, the root growth, the process of photosynthesis (translocation of sugars and other functions), the growth of rhizobia and nodules development (Lazali et al., 2021; Boudanga et al., 2015; Neila et al., 2014).

The most important strategy employed in the last few years to reduce the effects of environmental constraints on legume production have focused on the selection of plant germplasm tolerating to drastic conditions (Latrach et al., 2014). However, an increase of rhizobia tolerance and the exploitation of their possible synergies with plant growth promoting rhizobacteria (PGPR) might constitute another approach to improve plant productivity and rhizobial symbiosis performance under unfavorable conditions (Kenehi et al., 2010). In fact, PGPR can solubilize P into available forms or induce some other plant growth-promoting responses under low-P conditions (Matse et al., 2020). Tajini and Drevon (2014) that the positive interaction concerning PGPR and roots of plants can increase soil-P availability, especially under soil P-deficiency. As result, the increase of the number and size of nodules, the amount of nitrogen assimilated by nodules and the density of rhizobia in the rooting medium (Guiñazú et al., 2010). In fact, PGPR act directly and indirectly on plant growth improvement by a variety of mechanisms such as production of growth promoting substance and solubilization of minerals such as P (Korir et al., 2017). They increase also the native bacteria populations through various mechanisms that convert insoluble inorganic and organic soil P into plant available forms and therefore improve plant nutrition (Guiñazú et al., 2010). Matse et al. (2020) reported that the *Rhizobium* strains combined with the PGPR can enhance the symbiotic potential of the rhizobia, through the enhancement of the nitrigenase activity, and macronutrient contents in white clover plants under low P conditions. In the same sense, the intraspecific varia-

tions of SNF efficiency within rhizobial and PGPR populations under low-P availability have been shown in many other legume species. Indeed, Guiñazú et al. (2010) observed that the *Medicago sativa* L. plants co-inoculated with *Sinorhizobium meliloti* B399 and the *Bacillus* sp. M7c showed significant increases in root and shoot dry weights, length, number and surface area of roots, and symbiotic properties. Also, the co-inoculation with PGPR and rhizobia has a synergistic effect on growth and the use of PGPR may improve the effectiveness of rhizobia biofertilizers for common bean production (Korir et al., 2017). Hence, the exploitation of the available genetic variability is a promising way to optimize legumes-rhizobia symbiosis under P limitations.

In this context, our idea is inserted. We aim to evaluate the synergistic effect of *Pseudomonas alkylphenolica* PF9 and *Sinorhizobium meliloti* Rm41 on the Moroccan alfalfa population (*Oued Lmaleh*) under SNF and low-P availability. The emphasis was on the agro-physiological and biochemical aspects associated with the tolerance to this environmental constraint. The research into more efficient inorganic-phosphate solubilizing bacteria is a promising route to optimize growth and yield of legume and their rhizobia symbiosis under low-P availability. This could ensure adequate plant nutrition and contribute to grain and forage yield improvement and stability in low-P soils.

2. Materials and methods

2.1. Plant material and growth conditions

The plant material was the subject of this study consists of the Moroccan alfalfa (*Medicago sativa* L.) population *Oued Lmaleh* (OL). Seeds were supplied by National Institute for Agronomic Research (INRA, Marrakech, Morocco). Local populations of alfalfa are widely used in the Moroccan traditional agroecosystems, oases and mountains, and strongly contribute to the socio-economic development of local families as the main food for their livestock. They have been cultivated for many centuries and are still widely used by farmers in these traditional agroecosystems. Continuous natural and human selection has led, by this time, to their adaptation to the local habitats with distinction in the agromorphological characteristics of the landraces, which have reached Hardy-Weinberg equilibrium (Farissi et al., 2013, 2011).

The seeds of OL population were germinated in 15 cm diameter and 15 cm height pots containing sterilized perlite as a substrate. The experiment was conducted in a growth chamber at 28 ± 2 °C day/night, 60%–80% relative humidity, and a photoperiod of 16 h (18000 lx). After two weeks of sowing, the young seedlings were inoculated or co-inoculated three times with a suspension (10^8 bacteria per mL) of *Sinorhizobium meliloti* Rm41 strain alone or combined with *Pseudomonas alkylphenolica* PF9. These two strains were isolated from Beni-Mellal region in Morocco and identified at the molecular level using the housekeeping genes *gyrB* and *rpoD* respectively with the accession numbers of CP021808.1 and KY950274.1, respectively. These strains were chosen for their potential of Tricalcium Phosphate (Ca_3HPO_4) solubilization in solid and broth NBRIIP media and for their *in vitro* synergistic potential according to Habbadi et al. (2017). Briefly, 100 μl of *Pseudomonas alkylphenolica* PF9 suspension obtained on the liquid YEM medium was spread on Petri dishes containing the solid YEM medium. Then, discs of sterile filter paper were soaked in the cream of *Sinorhizobium meliloti* Rm41 strain and placed on the Petri dish on which the PGPR strain was spread. The absence of the inhibition halo after 05 days of incubation shows that the PGPR strain has no antagonistic effect on the growth of the rhizobial strain selected for the nodulation of alfalfa plants. Then, the seedlings were submitted to different treatments in terms of P forms (soluble or insoluble P)

and bacterial treatments, *Sinorhizobium meliloti* Rm41 alone (R) or combined with *Pseudomonas alkylphenolica* PF9 (PGPR). The applied treatments were:

- Irrigating seedlings with Nitrogen free nutrient solution containing Ca_3HPO_4 as insoluble P form (-N + IP);
- Irrigating seedlings with Nitrogen free nutrient solution containing monopotassium phosphate (KH_2PO_4) as a form of soluble P (-N + SP);
- Irrigating seedlings with Nitrogen free nutrient solution containing Ca_3HPO_4 as insoluble P form and the seedlings were inoculated with the suspension of *Sinorhizobium meliloti* Rm41 alone (-N + IP + R).
- Irrigating seedlings with Nitrogen free nutrient solution containing monopotassium phosphate (KH_2PO_4) as a form of soluble P and the seedlings were inoculated with the suspension of *Sinorhizobium meliloti* Rm41 alone (-N + SP + R);
- Irrigating seedlings with Nitrogen free nutrient solution containing Ca_3HPO_4 as insoluble P form and the seedlings were inoculated with the suspension of *Pseudomonas alkylphenolica* PF9 only (-N + IP + PGPR).
- Irrigating seedlings with Nitrogen free nutrient solution containing Ca_3HPO_4 as insoluble P form and the seedlings were simultaneously co-inoculated with the suspensions of *Sinorhizobium meliloti* Rm41 and *Pseudomonas alkylphenolica* PF9 (-N + IP + R + PGPR).

The composition of the nutrient solution used consisted of P applied in the form of KH_2PO_4 (sufficient supplies) and Ca_3HPO_4 (insoluble P, deficient supplies) at a final concentration of $250 \mu\text{mol P}\cdot\text{plant}^{-1}\cdot\text{week}^{-1}$ (Bargaz et al., 2013; Neila et al., 2014). Urea was applied at $2 \text{ mmol}\cdot\text{plant}^{-1}$ to nutrient solution only during the initial 2 weeks of growth to avoid Nitrogen deficiency during nodule development. Subsequently, the plants were grown in Nitrogen free nutrient solution. After 60 days of P stress, the plants were harvested, measured, and subjected to different argo-physiological and biochemical analyses governing plant growth and development.

2.2. Plant biomass, plant height and leaf area

For the biomass measurements, shoots and roots were separated and their fresh weight (FW) was determined immediately. The dry weight (DW) of shoots and roots was measured using precision balance after their drying at 80°C for 48 h. The height of the aerial part of the plants was measured using a precision ruler, graduated in centimeters and millimeters. The leaf area was estimated using MESURIM software version 3.4.4.0. The leaves belonging to the same plant were cut and laid out on a white sheet containing a scale, and then they were scanned using a digital scanner. These parameters were measured on five plants per pot and grouped as three replicates.

2.3. Phosphorus contents

For the determination of assimilable P in shoots and roots, 0.5 g of the dry matter of each part was incinerated at 600°C for 6 h. The ash obtained was collected in 3 mL of HCl (10 N) and filtered. The filtrate was adjusted to 100 mL with distilled water. Subsequently, the P contents of shoots and roots were determined colorimetrically using the molybdate blue method (Murphy and Riley, 1962). P concentration was measured by reading the absorbance at a wavelength of 820 nm, using an UV-VIS absorption spectrophotometry (DLAB, SP-UV1000, China), after color development at 100°C for 10 min. A standard curve was established with KH_2PO_4 solutions.

2.4. Relative water content (RWC)

RWC was estimated as described in Farissi et al. (2018) by recording the turgid weight (TW) of 0.1 g fresh leaflet (FW) samples by maintaining in water for 4 h, followed by drying in a hot air oven until a constant weight was achieved (DW). The RWC was calculated using the following formula:

$$\text{RWC} = \frac{[(\text{FW} - \text{DW})/(\text{TW} - \text{DW})] \times 100}$$

2.5. Lipid peroxidation assessment

The malonyldialdehyde (MDA) content was determined according to the method described by Savicka and Škute (2010). Samples of 50 mg of fresh leaves were homogenized with 2 mL of trichloroacetic acid (TCA 0.1%) and centrifuged at 14,000 rpm for 15 min. After centrifugation, 1 mL of supernatant was added to 2.5 mL of thiobarbituric acid (0.5% TBA) prepared in 20% TCA. Then, the mixture was brought to a water bath at 95°C for 30 min. Then, it was immediately cooled in an ice bath to stop the reaction. The optical density was read at a wavelength 532 nm by an UV-VIS absorption spectrophotometry (DLAB, SP-UV1000, China). The values obtained are then corrected by subtracting the non-specific absorbance at 600 nm. The concentration of MDA is calculated using its extinction coefficient $\epsilon = 155 \text{ mM}^{-1}\cdot\text{cm}^{-1}$.

2.6. Electrolyte leakage

Electrolyte leakage (EL) was assessed as described by Lutts et al. (1996) using young leaflets (0.1 g). Samples were laved three times with deionized water to eliminate surface-adhered electrolytes then they were put in closed vials containing 10 mL of deionized water and incubated at 25°C on a rotary shaker for 24 h, afterward electrical conductivity of the solution (Lt) was measured with a conductivity meter (Jenway, Model 4520 conductivity meter, UK). Samples were then autoclaved at 120°C for 20 min and the last electrical conductivity (L0) was obtained after equilibration at 25°C . The EL was defined as follows:

$$\text{EL}(\%) = (\text{Lt}/\text{L0}) \times 100$$

2.7. Chlorophyll contents

Photosynthetic pigments were extracted according to Arnon (1949). 50 mg of fresh material was homogenized in a mortar using diluted acetone (80%) against 80% of acetone as a blank. The extract material was centrifuged for 10 min at $10000 \times g$ and the absorbance was read at 480, 645 and 663 nm using an UV-VIS absorption spectrophotometry (DLAB, SP-UV1000, China). The concentration of Chlorophyll a, Chlorophyll b and Total chlorophyll were calculated following below formulas:

$$\text{Chla}(\text{mg}\cdot\text{g}^{-1}\text{FW}) = [(12.7 \times \text{A663}) - (2.6 \times \text{A645})] \times (\text{V}/1000 \times \text{Wt})$$

$$\text{Chlb}(\text{mg}\cdot\text{g}^{-1}\text{FW}) = [(22.9 \times \text{A645}) - (4.68 \times \text{A663})] \times (\text{V}/1000 \times \text{Wt})$$

$$\text{Totalchlorophyll}(\text{mg}\cdot\text{g}^{-1}\text{FW}) = [(20.2 \times \text{A645}) + (8.02 \times \text{A663})] \times (\text{V}/1000 \times \text{Wt})$$

2.8. Chlorophyll fluorescence measurement (Fv/Fm)

The chlorophyll fluorescence was measured as described in Farissi et al. (2018) using a handheld Chlorophyll Fluorometer (model: OS-30P; Manufacturer: Opti-Sciences, Inc., USA) after

20 min of dark adaptation. Chlorophyll fluorescence was estimated by the Fv/Fm ratio = $(Fm - Fo) / Fm$, which represents the maximum quantum yield of PS II, where Fv is the varietal fluorescence of dark-adapted alfalfa leaves and Fm and Fo are the maximal and minimal fluorescence respectively.

2.9. Stomatal conductance (g_s)

Stomatal conductance (g_s) was measured on healthy leaves as described in [Latrach et al. \(2014\)](#) using a porometer (Leaf Porometer Version 5.0, Decagon Devices, Inc., USA) at a temperature of 25 ± 1 °C and relative humidity of $55 \pm 5\%$. It was expressed in $\text{mmol de H}_2\text{O m}^{-2}\cdot\text{s}^{-1}$.

2.10. Statistical analysis

Statistical analysis was performed using SPSS version 22. It concerned the analysis of variance (ANOVA). Means were compared using Tukey's test. XLSTAT software version 2014 (Addinsoft, Paris, France) was used to determine the correlations among the measured parameters.

3. Results

3.1. Effect on plant biomass

The effect of *Pseudomonas alkylphenolica* PF9 and/or *Sinorhizobium meliloti* Rm41 on plant biomass of Moroccan alfalfa population studied under soluble (KH_2PO_4) or insoluble (Ca_3HPO_4) P forms is indicated in [Fig. 1](#). Our results indicated that the inoculation of plants with rhizobial strain alone or combined with the *Pseudomonas* strain significantly increased both fresh and dry biomasses under P deficit in comparison to the uninoculated and P-stressed plants. For the fresh weight ([Fig. 1](#)), the comparison between the two inoculants indicated that the two strains act synergistically ($p < 0.001$) in promoting alfalfa fresh weights under low P availability. In fact, the shoot and root fresh weights recorded, respectively, in the presence of both inoculants under low-P availability conditions were 199.5 and 110.9 $\text{mg}\cdot\text{plant}^{-1}$, against 82.5 and 88.9 $\text{mg}\cdot\text{plant}^{-1}$ for the plants inoculated with rhizobial strain alone under the same conditions of P stress. Also, the data showed a significant decrease ($p < 0.001$) in the shoot and root fresh weights of alfalfa plants inoculated with rhizobial strain alone and grown under insoluble P comparatively to the plants inoculated with the same strain and supplied with the soluble P form. However, no significant difference ($p > 0.05$) was noted between both P forms when the *Pseudomonas* strain was added to the rooting medium of stressed plants.

Under the conditions of P deficit, alfalfa plants exhibited a significant ($p < 0.001$) increase in their dry biomass (shoots and roots) when they were inoculated with the rhizobial strain alone or co-inoculated with both rhizobacteria strains in comparison to the uninoculated and P-stressed plants. Indeed, the values recorded in stressed plants and in the presence of the rhizobial strain only were 17.8 and 28.35 $\text{mg}\cdot\text{plant}^{-1}$ for shoots and roots respectively. Nevertheless, the quantities of 40.7 and 37.6 $\text{mg}\cdot\text{plant}^{-1}$ were noted in the presence of the two inoculants in the rooting medium of the stressed plants. Like the fresh biomass, no significant difference ($p > 0.05$) was noted between both P forms when the *Pseudomonas* strain was added to the rooting medium of stressed plants compared to the plants inoculated with rhizobia and provided with the P in a soluble form (KH_2PO_4).

3.2. Effect on plant height and leaf area

[Fig. 2](#) indicated the effect of the inoculation with *Sinorhizobium meliloti* Rm41 alone or combined with *Pseudomonas alkylphenolica* PF9 on plant heights ([Fig. 2A](#)) and leaf area ([Fig. 2B](#)) of alfalfa population studied under limited available P. Both inoculant treatments significantly ($p < 0.001$) improved the plant heights and leaf area under the insoluble form of P with significant differences between them ($p < 0.001$). In fact, under low P availability, the highest plant height was noted when the alfalfa plants are inoculated at the same time with both rhizobacteria inoculants, 14.50 cm versus 12.23 cm when the inoculation was done with the *Sinorhizobium* strain alone. For the leaf area ([Fig. 2B](#)), the highest values ($p < 0.01$) under insoluble P conditions were observed in plants co-inoculated with both bacterial inocula (1.90 cm^2) in comparison to P-stressed plants inoculated with rhizobial strain alone (1.64 cm^2).

3.3. Phosphorus contents

The shoot and root P contents of alfalfa plants grown under soluble or insoluble P forms and inoculated with *Sinorhizobium meliloti* Rm41 alone or combined with *Pseudomonas alkylphenolica* PF9 are shown in [Fig. 3](#). The obtained results mentioned that the highest P contents ($p < 0.001$) in shoots and roots under insoluble P conditions were noted when the plants are co-inoculated with the two strains at once. Generally, the amounts of P recorded were more pronounced in the underground parts than in the aerial parts of the plants. The P contents obtained when the OL stressed plants are inoculated with the rhizobial strain only were respectively 283.17 and 429.18 $\text{mg}\cdot\text{g DW}^{-1}$ in shoots ([Fig. 3A](#)) and roots ([Fig. 3B](#)). However, the amounts of 346.45 and 440.58 $\text{mg}\cdot\text{g DW}^{-1}$ were noted under the co-inoculation at the same parts respectively and under the same conditions of P supply (Ca_3HPO_4). The comparison between the plants inoculated with the *Sinorhizobium* strain alone and grown under insoluble or soluble P showed significant differences ($p < 0.001$) between their shoot and root P contents.

3.4. Relative water content (RWC)

Our results ([Fig. 4](#)) indicated that bacterial treatments maintained the same level of RWC whatever the P form added to the growing medium ($p > 0.05$). However, in comparison to the uninoculated and P-stressed plants, all inoculants significantly ($p < 0.001$) improved this parameter. Hence, for the plants inoculated with the rhizobial strain alone or combined with *Pseudomonas* strain the increases noted were 26.16 and 23.91% respectively.

3.5. Effect on EL and MDA contents

The EL contents were found increased ($p < 0.001$) in uninoculated and P-stressed alfalfa plants ([Table 1](#)). However, the inoculation with rhizobial strain alone or combined with *Pseudomonas* strain significantly ($p < 0.001$) reduced the EL in P-stressed alfalfa plants. There is no significant difference ($p > 0.05$) in EL recorded in plants inoculated with rhizobia strain alone and supplied with the two P forms. However, the EL was more reduced in the presence of both inoculants in the rooting medium 11.58%.

The MDA contents were more accumulated ($p < 0.01$) in uninoculated and P-stressed plants compared to the other treatments ([Table 1](#)). Nevertheless, the inoculation of alfalfa plants with *Sinorhizobium* strain alone significantly reduced this accumulation under the same P conditions (35.48 $\mu\text{mol}\cdot\text{g FW}^{-1}$). However, the presence of both strains in the rooting medium remarkably decreased the MDA accumulation to 31.88 $\mu\text{mol}\cdot\text{g FW}^{-1}$ with no

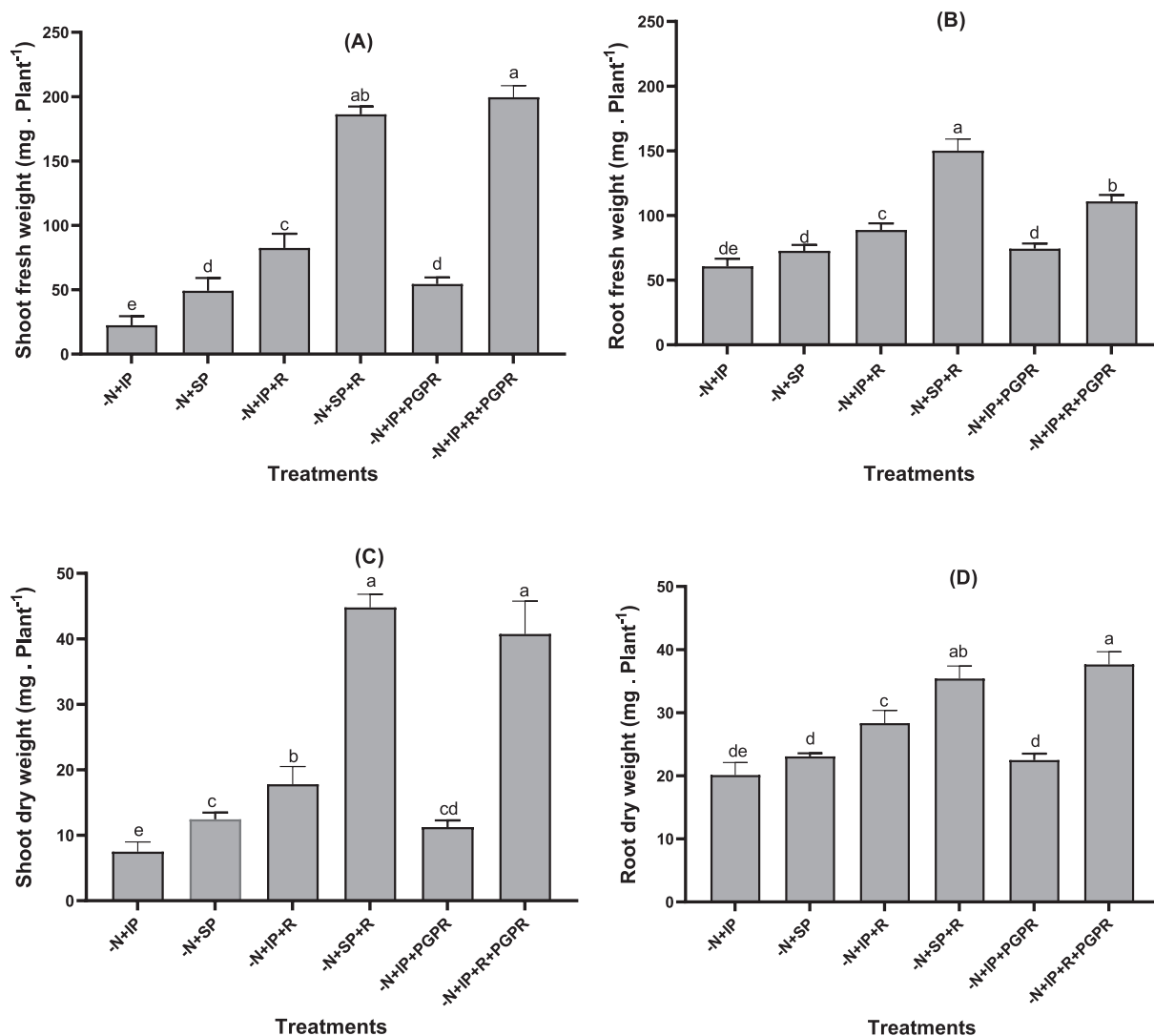


Fig. 1. Effect of inoculation with *Sinorhizobium meliloti* Rm41 (R) alone or combined with *Pseudomonas alkylphenolica* PF9 (R + PGPR) on fresh and dry biomass of Moroccan alfalfa population Oued Lmaleh (OL) under insoluble (IP) or soluble (SP) P form. (A) Shoot fresh weight, (B) Root fresh weight, (C) Shoot dry weight and (D) Root dry weight. Values are means of three replicates of five plants for each. Different and same small letters above histograms indicate significant ($p < 0.05$) and no significant differences ($p > 0.05$), respectively, between the means according to Tukey's test. Bars represent the standard errors to the means.

significant difference ($p > 0.05$) in comparison to alfalfa plants inoculated with rhizobial strain and supplied with the soluble P form.

3.6. Effect on photosynthetic-related parameters

3.6.1. Effect on Chl a, Chl b, total Chl and Chl a/b ratio

The inoculation with rhizobial strain significantly ($p < 0.001$) increased the Chl a, Chl b and total Chl contents in alfalfa plants supplied with insoluble P in comparison to uninoculated plants and whatever the supplied form of P (Fig. 5). However, the simultaneous inoculation with both bacterial inoculants further improved ($p < 0.05$) the Chl a and the total chlorophyll contents in alfalfa stressed plants.

Concerning Chl a/b ratio (Fig. 6), the highest and significant values were noted in uninoculated and stressed plants (2.45). The lowest values were recorded in alfalfa plants inoculated with rhizobial strain and supplied with the soluble P form (1.72). However, the Chl a/b ratio reached 2.14 and 2.00 when the inoculation was done with rhizobia alone or combined with *Pseudomonas* strain respectively under limited available P (see Fig. 7).

3.6.2. Effect on the maximum quantum yield of PS II (F_v/F_m)

Results indicated that *Sinorhizobium* strain alone or its combination with the *Pseudomonas* one pronouncedly increased ($p < 0.001$) the F_v/F_m ratio under low P availability, with no significant differences between them (Fig. 5). Indeed, in the presence of the rhizobial inoculum only, the F_v/F_m reached the values of 0.804 and 0.790 in P-stressed and unstressed plants respectively. However, this parameter reached 0.802 when the plants were co-inoculated with both inocula at the same time.

3.6.3. Effect on stomatal conductance (g_s)

The results obtained for the g_s (Fig. 8) showed that both inoculation with rhizobia only and co-inoculation with the two strains at the same time significantly ($p < 0.001$) increased this parameter under P deficiency with a significant difference between them ($p < 0.05$). The lowest value of g_s was recorded in the absence of the bacterial treatment and under insoluble P conditions. However, the presence of the rhizobial inoculum induced the g_s to reach 40.18 $\text{mmol H}_2\text{O} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$. This enhancement was more obvious when the inoculum was constituted of both rhizobacterial strains (45.84 $\text{mmol H}_2\text{O} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$).

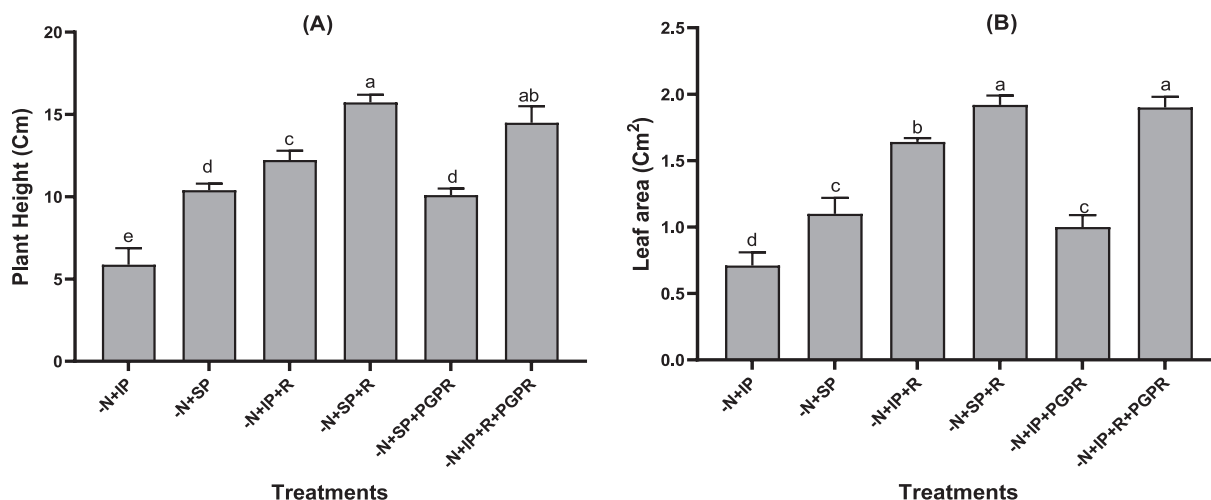


Fig. 2. Effect of inoculation with *Sinorhizobium meliloti* Rm41 (R) alone or combined with *Pseudomonas alkylphenolica* PF9 (R + PGPR) on plant heights (A) and leaf area (B) of Moroccan alfalfa population *Oued Lmaleh* (OL) under insoluble (IP) or soluble (SP) P forms. Values are means of three replicates of five plants for each. Different and same small letters above histogram indicate significant ($p < 0.05$) and no significant differences ($p > 0.05$), respectively, between the means according to Tukey's test. Bars represent the standard errors to the means.

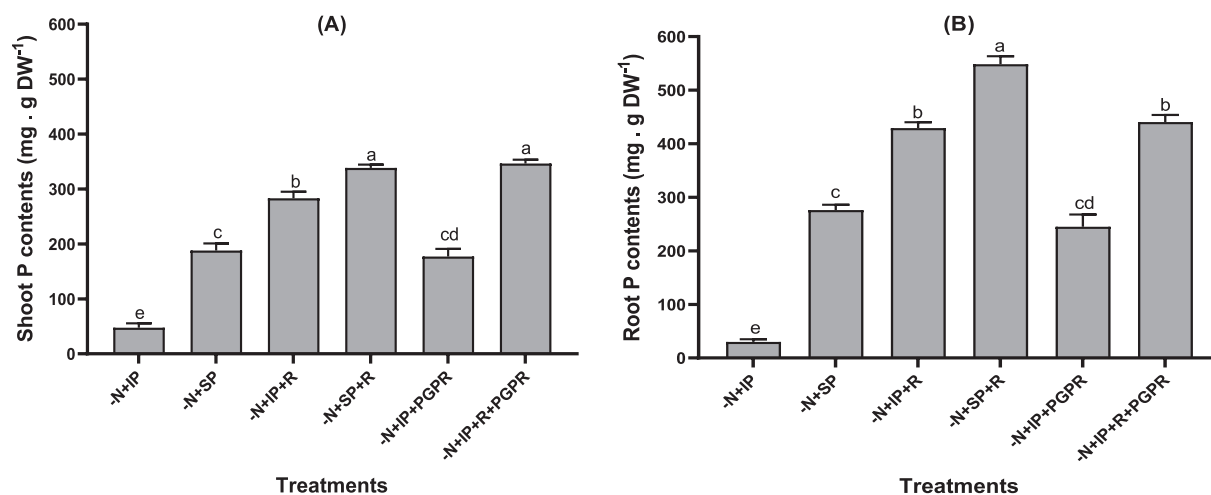


Fig. 3. Effect of inoculation with *Sinorhizobium meliloti* Rm41 (R) alone or combined with *Pseudomonas alkylphenolica* PF9 (R + PGPR) on shoot (A) and root (B) P contents of Moroccan alfalfa population *Oued Lmaleh* (OL) under insoluble (IP) or soluble (SP) P forms. Values are means of three replicates. Different and same small letters above histogram indicate significant ($p < 0.05$) and no significant differences ($p > 0.05$), respectively, between the means according to Tukey's test. Bars represent the standard errors to the means.

4. Discussion

Plant-growth promotion has been associated to the *Pseudomonas* genus since the beginning of this research topic. In the present study, we focused on the synergistic action of *Pseudomonas alkylphenolica* PF9 and *Sinorhizobium meliloti* Rm41 on Moroccan alfalfa population grown depending on SNF under limited available P. We noted that the inoculation with rhizobial strain alone or combined with *Pseudomonas* strain generated positive effects on the growth and physiology of alfalfa plants fertilized with the insoluble form of P compared to uninoculated plants. However, the comparison between both bacterial treatments showed overall that the improvement was more pronounced when the alfalfa plants are simultaneously co-inoculated with both inoculants. In fact, our results indicated that the co-inoculation of P-stressed plants at the same time significantly improved the fresh and dry biomasses, plant heights and leaf area with no significant differences in comparison to alfalfa plants inoculated with *Sinorhizobium* strain alone and supplied with the soluble P form. Likewise, the

improvement in plant growth was strongly correlated with the P content of shoots and roots (Fig. 9), suggesting the synergistic role of the two used strains on phosphate nutrition improvement and therefore the plant growth. Sulieman and Hago (2009) found that the growth of legumes was positively correlated with the P concentration in the soil solution and the low-P availability caused a depressive effect on plant nodulation, growth as well as on the leaf area (Chaudhary et al., 2008; Tang et al., 2001). In line with our findings, the results observed in some leguminous species like *Phaseolus vulgaris* L. showed that the co-inoculation with PGPR and rhizobia had a synergistic effect on plant growth parameters in comparison to the single inoculation with rhizobial strains (Korir et al., 2017). Therefore, *Pseudomonas polymyxa* and *Bacillus megaterium* strains can be used together with the tested rhizobia strains to improve common bean growth in low-P soils (Korir et al., 2017). In the same sense, Charana and Yoon (2013) noted that the co-inoculation of mung bean with *Pantoea agglomerans* and *Burkholderia anthina* exhibited the highest growth performances and P uptake under low available P. Shi et al. (2019)

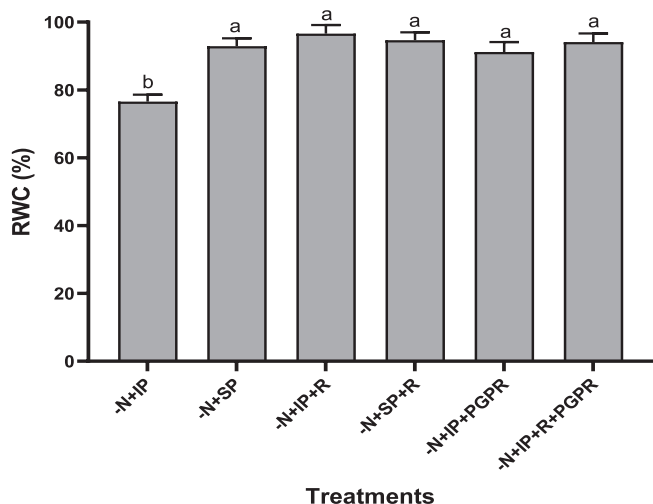


Fig. 4. Effect of inoculation with *Sinorhizobium meliloti* Rm41 (R) alone or combined with *Pseudomonas alkylphenolica* PF9 (R + PGPR) on RWC of Moroccan alfalfa population *Oued Lmaleh* (OL) under insoluble (IP) or soluble (SP) P forms. Values are means of three replicates. Different and same small letters above histogram indicate significant ($p < 0.05$) and no significant differences ($p > 0.05$), respectively, between the means according to Tukey's test. Bars represent the standard errors to the means.

reported that the strain KL28 of *Pseudomonas alkylphenolica* promoted the growth of *Brassica campestris* L. under metallic stress.

P nutrition is important for metabolic activities in plants. The reduced uptake of P due to lower P availability may influence various physiological and biochemical processes such as water uptake and cell membrane stability. At this point, our results showed a significantly smaller level of RWC in alfalfa stressed and uninoculated plants. In fact, the soil P level is associated with water status in plants (Shubhra et al., 2004). However, the presence of the bacterial inocula tested in the rooting medium significantly increased the leaf RWC whatever the form of P supplied. The PGPR can, directly or indirectly, improve growth of plants by a range of mechanisms such as the fixation of molecular nitrogen and its conversion to ammonia transmitted to the plant, production of siderophores that making iron available in the plant rhizosphere, solubilization of minerals including P, and synthesis of phytohormones like gibberellins, cytokinins and auxins (Belimov et al., 2015). In fact, exogenous indole-3-acetic acid (IAA) raised RWC in leguminous *Glycine max* L. (Gadallah, 2000). In lettuce plants, PGPR inoculations significantly increased the leaf RWC (Sahin et al., 2015). Mayak et al. (2004) documented that PGPR could ameliorate the rooting and the growth of plants by enhancing the water use efficiency.

P is an essential constituent of the phospholipids composing the cell membranes of plants. Any P-deficiency could induce great damages on cell membrane integrity and on tissue rigidity. In our present study, the disturbance effect of P-deficiency to cell membrane stability was reflected by the increase in MDA contents associated with the high EL percentages. In fact, we noted strong negative correlations between the shoot fresh and dry weights and EL and MDA accumulations (Fig. 9). In leguminous species

Phaseolus vulgaris L., the P-deficiency induced a significant increase in EL and MDA contents of nodules and leaves (Bargaz et al., 2013). However, likely to our results, the PGPR inoculations decreased EL and MDA of lettuce plants grown under lower irrigation levels (Sahin et al., 2015). Determination of the MDA concentration and, hence, the extent of membrane lipid peroxidation, is often used as a tool to evaluate the gravity of oxidative stress induced by abiotic stress. In rice seedlings, the levels of MDA and EL were significantly increased under nutrient-deficient conditions including P and N as compared to sufficient nutrients. However, their contents were found to be decreased by the inoculation with *Paenibacillus lentimorbus* B-30488, *Bacillus amyloliquefaciens* SN13 and their consortium (Bisht et al., 2020). Under nutrient deficiency, the PGPR *Bacillus amyloliquefaciens* SN13 focuses on the carbohydrate metabolism which in turn provokes downstream signaling allowing plants to weather nutritional stress including P (Bisht et al., 2020). The bacterial inoculation leads to deregulation of glycolytic pathway genes and hence sugar level (Bisht et al., 2020). This might be a strategy of PGPR to induce tolerance in nutrient-starved plants.

Measurements of photosynthesis parameters such as chlorophyll content, chlorophyll fluorescence and g_s are often used in the evaluation of plant adaptation to different environmental stresses, including P stress. In our study, the observed reductions on these photosynthetic-related parameters clearly reflected the decrease in the plant growth of uninoculated and P-stressed plants. Strong positive correlations were noted between plant biomasses and measured photosynthetic-related parameters (Fig. 9). The effect of P-deficiency on chlorophyll contents is documented in many leguminous species. In soybean, the supplement of P improved the total Chl and Chl *a* contents compared to unfertilized plants (Rotaru, 2015). We noted that the co-inoculation with both inoculants significantly enhanced the total Chl and Chl *a* contents in alfalfa plants supplied with insoluble P form. Also, no significant differences were noted between the two P forms when the plants were co-inoculated with *Sinorhizobium* and *Pseudomonas* strains simultaneously. In line with our results, the treatment of soybean plants with *Pseudomonas fluorescence* and *Azotobacter chroococcum* simultaneously revealed an overall increase in Chl *a* and total Chl content under P starvation (Rotaru, 2015). However, the two bacterial treatments did not significantly change the Chl *b* contents under sufficient and deficient P-supply. The same observation was noted in soybean (Rotaru, 2015). The lack of effects on the Chl *a/b* ratio indicates that Chl *a* is more sensitive to P-deficiency than Chl *b*. In rice, Alam et al. (2001) accorded the positive effects in root length, leaf area and chlorophyll content to *Xanthobacter* sp. inoculation. The growth-promoting effect of *Serratia plymuthica* BMA1 strain was accompanied by a substantial increase in chlorophyll contents in the leguminous *Vicia faba* L. under low P availability (Borgi et al., 2020). A decrease of total chlorophyll with P deficiency stress suggests a reduced capacity for light harvesting. Meanwhile, the formation of reactive oxygen species is mostly compelled by excess energy absorption in the photosynthetic apparatus, this might be eschewed by damaging the absorbing pigments (Herbinger et al., 2002). A decrease in chlorophyll content could be related to the increase of chlorophyll degrading chlorophyllase activity, the destruction of the chloroplast structure and

Table 1

Effect of inoculation with *Sinorhizobium meliloti* Rm41 (R) alone or combined with *Pseudomonas alkylphenolica* PF9 (R + PGPR) on EL and MDA contents of Moroccan alfalfa population *Oued Lmaleh* (OL) under insoluble (IP) or soluble (SP) P forms. Values are means of three replicates \pm Standard Errors. Different and same small letters indicate significant ($p < 0.05$) and no significant differences ($p > 0.05$), respectively, between the means according to Tukey's test.

	-N + IP	-N + SP	-N + SP + R	-N + IP + R	-N + IP + PGPR	-N + IP + R + PGPR
EL (%)	24.70 \pm 0.73 a	20.11 \pm 0.36b	16.64 \pm 1.12c	17.98 \pm 0.41c	21.13 \pm 1.12b	11.58 \pm 0.41 d
MDA μ mol.g FW ⁻¹	51.88 \pm 2.24 a	45.69 \pm 1.49b	29.29 \pm 1.46 e	35.48 \pm 0.09 d	43.84 \pm 2.27 bc	31.88 \pm 1.70 e

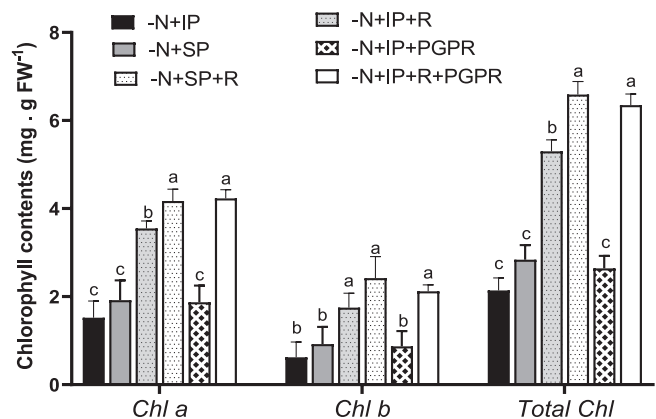


Fig. 5. Effect of inoculation with *Sinorhizobium meliloti* Rm41 (R) alone or combined with *Pseudomonas alkylphenolica* PF9 (R + PGPR) on Chl a, Chl b and total Chl contents of Moroccan alfalfa population *Oued Lmaleh* (OL) under insoluble (IP) or soluble (SP) P forms. Values are means of three replicates. For each parameter, different and same small letters indicate significant ($p < 0.05$) and no significant differences ($p > 0.05$), respectively, between the means according to Tukey's test. Bars represent the standard errors to the means.

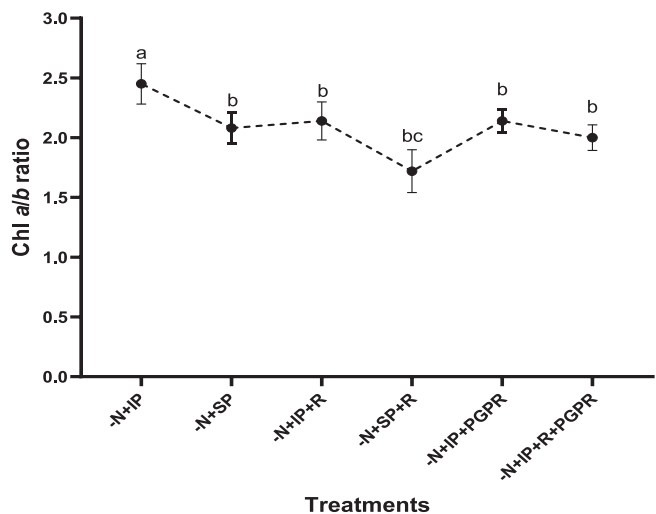


Fig. 6. Effect of inoculation with *Sinorhizobium meliloti* Rm41 (R) alone or combined with *Pseudomonas alkylphenolica* PF9 (R + PGPR) on Chl a/b ratio of Moroccan alfalfa population *Oued Lmaleh* (OL) under insoluble (IP) or soluble (SP) P forms. Values are means of three replicates. Different and same small letters indicate significant ($p < 0.05$) and no significant differences ($p > 0.05$), respectively, between the means according to Tukey's test. Bars represent the standard errors to the means.

the greater instability of pigment protein complexes (Singh and Dubey, 1995).

The reduction in the photochemical efficiency of PSII (F_v/F_m) in uninoculated and P-stressed plants is possibly related to a reduction of chlorophyll contents noted under the same conditions. Indeed, we observed a very highly significant positive correlation between the F_v/F_m and Total Chl contents (Fig. 9). Changes in Chlorophyll fluorescence emissions, occurring mainly from PSII, provide information on almost all aspects of photosynthetic activity. This parameter had also usually been used to probe photosynthetic function in higher plants and exhibit plant tolerance to environmental stresses (Farissi et al., 2018; Gray et al., 2006; Panda et al., 2008). Shi et al. (2019) noted that the inoculation of *Brassica campestris* L. plants with *Pseudomonas alkylphenolica* KL28 improved photosynthetic parameters like F_v/F_m under metallic stress. In barely, *Hordeum vulgare* L., all of the processes in the photosynthetic machinery including the PSII quantum yield were

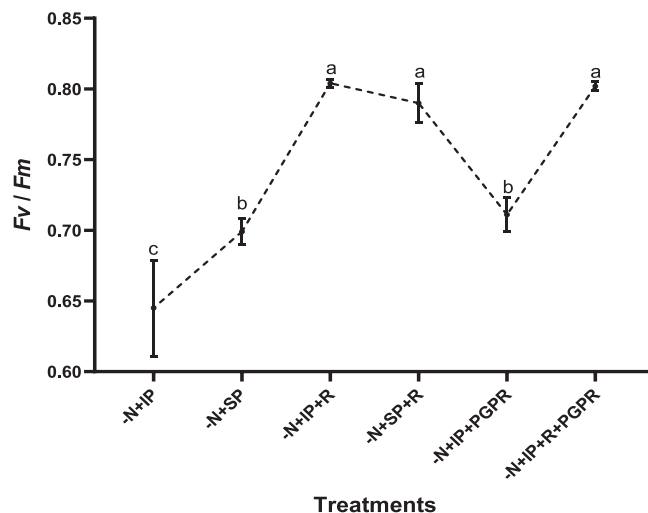


Fig. 7. Effect of inoculation with *Sinorhizobium meliloti* Rm41 (R) alone or combined with *Pseudomonas alkylphenolica* PF9 (R + PGPR) on maximum quantum yield of PS II (F_v/F_m) of Moroccan alfalfa population *Oued Lmaleh* (OL) under insoluble (IP) or soluble (SP) P forms. Values are means of three replicates. Different and same small letters indicate significant ($p < 0.05$) and no significant differences ($p > 0.05$), respectively, between the means according to Tukey's test. Bars represent the standard errors to the means.

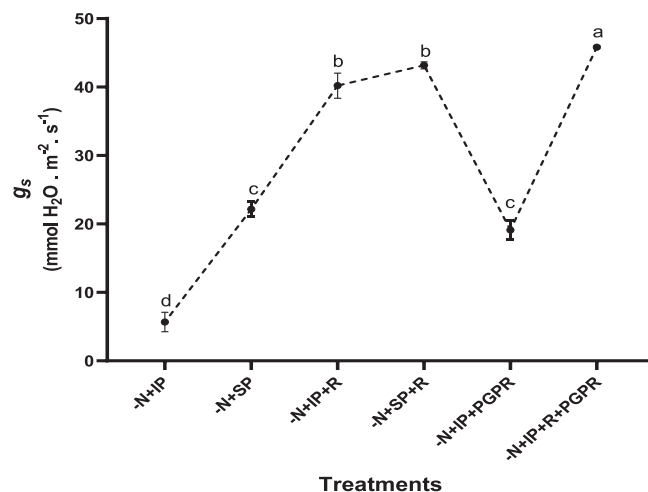


Fig. 8. Effect of inoculation with *Sinorhizobium meliloti* Rm41 (R) alone or combined with *Pseudomonas alkylphenolica* PF9 (R + PGPR) on g_s of Moroccan alfalfa population *Oued Lmaleh* (OL) under insoluble (IP) or soluble (SP) P forms. Values are means of three replicates. Different and same small letters indicate significant ($p < 0.05$) and no significant differences ($p > 0.05$), respectively, between the means according to Tukey's test. Bars represent the standard errors to the means.

influenced by P deficiency (Carstensen et al., 2018). The inoculation of *Phaseolus vulgaris* seedlings with *Trichoderma sp* and/or *Bacillus sp* improved photosynthetic efficiency evaluated by F_v/F_m ratio (Yobo et al., 2009). This finding matches our results. In fact, we have noted that the presence of rhizobacteria tested in the rooting medium improved the quantum yield of PSII whatever the supplementation form of P. The improvement in chlorophyll fluorescence and Chl contents by bacterial treatments suggest more reaction centers and higher light harvesting. In pepper plants, the quinone acceptor (Qa) was highly oxidized by *Bacillus* bacteria inoculation and its excitation energy is utilized in electron transport, leading higher adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide phosphate (NADPH) production, employed for carbon assimilation in the Calvin cycle, and improving plant growth (Samaniego-Gómez et al., 2016).

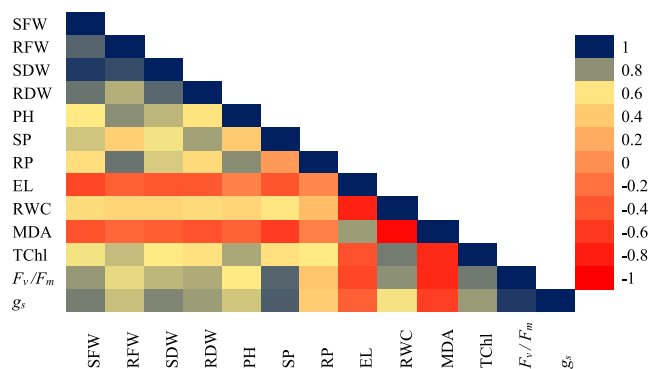


Fig. 9. Pearson's correlation matrix between assessed parameters of alfalfa (*Medicago sativa* L.) population (OL) grown under sufficient or limited phosphorus conditions. Correlations are displayed in blue (positive) and in red (negative); color intensity is proportional to correlation coefficient. SFW: Shoot Fresh Weight, RFW: Root Fresh Weight, SDW: Shoot Dry Weight, RDW: Root Dry Weight, PH: shoot height, SP: shoot phosphorus, RP: Root phosphorus, EL: Electrolyte leakage, RWC: Relative water content, MDA: Malonyldialdehyde, Tchl : Total chlorophyll, Fv/Fm: Maximum Quantum Yield of PS II, g_s : Stomatal Conductance.

In addition, our results also showed that inoculation treatments have a highly significant effect on the increase in g_s under P starvation. The increase in g_s results in the opening of the stomata, due to differential variations in turgor, in order to facilitate the entry of the CO_2 necessary for photosynthesis, at the same time causing water losses through transpiration (Bresson et al., 2013). The cell turgor at higher levels contributes to improved plant performance and to the maintenance of physiological processes such as stomatal opening, photosynthesis and leaf expansion (Serraj and Sinclair, 2002; Subbarao et al., 2000). Our results recorded a highly significant positive correlation between the g_s and RWC (Fig. 9). We reported here that the low P availability noticeably decreased the g_s in uninoculated alfalfa plants. In rice plants, the low P conditions caused reductions in photosynthetic rate, g_s , transpiration rate, and internal CO_2 concentration (Veronica et al., 2017). However, in our study, the bacterial inoculants particularly the mixed inoculation of *Sinorhizobium meliloti* Rm41 and *Pseudomonas alkylphenolica* PF9 improved the g_s of P-stressed plants. Indeed, the endophyte and rhizospheric microorganisms can promote plant growth by regulating nutritional and hormonal balance, producing plant growth regulators and solubilizing nutrients (Mahmood et al., 2014). Indeed, the IAA affects plant cell division, pigment synthesis and photosynthetic activity by modulating the plant auxin pool (Ahemad, 2014). Furthermore, the bacterial respiration led to CO_2 formation that could involve in photosynthesis improvement. In fact, the CO_2 generated by bacterial respiration in roots can be transported to the stems through the vascular tissues (xylem). It was reported that the carbon involved in photosynthesis in stem cells of tobacco plants is obtained from the vascular system and not from stomata (Hibberd and Quick, 2002). The same observation was also reported by Sahin et al. (2015) in lettuce plants inoculated with *Bacillus megaterium* and *B. subtilis* strains.

5. Conclusions

The present study suggests that the co-inoculation of alfalfa plants with *Pseudomonas alkylphenolica* PF9 and *Sinorhizobium meliloti* Rm41 could alleviate the deleterious effects of low P conditions in the rooting medium. These rhizobacteria improved growth in P-stressed plants in terms of plant biomass, leaf area and plant heights. Such a beneficial effect was associated with P solubilization and uptake, the maintenance of water nutrition, the cell membrane stability and the performance of photosynthetic-related

parameters such as the chlorophyll contents, the Fv/Fm and the g_s . This implies that their applicability as a promising alternative to minimize the P problem in agricultural soils.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The authors are grateful to all those who participated in the elaboration of this study. We thank all the partners involved in CROSYMED-ARIMNet2 Project. We also thank the administrative and technical staff of the Polydisciplinary Faculty of Beni-Mellal, Sultan Moulay Slimane University and the staff of MESRSFC agency and ARIMNet2 program for their support.

Funding

This work was supported by the CROSYMED Project, funded through the ARIMNet2 2017 Joint Call by the funding agency of the Ministry of National Education, Vocational Training, Higher Education and Scientific Research represented by the Secretary of State for Higher Education & Scientific Research (SEERS), Morocco. ARIMNet2 (ERA-NET) has received funding from the European Union's Seventh Framework Program for Research, Technological Development and Demonstration under grant agreement no. 618127.

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