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THÈSE

Présentée par

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Biofertilizer-biostimulant potential of endophyte and rhizospheric microorganisms for alfalfa tolerance to low phosphorus availability: Agrophysiological and biochemical aspects

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- **Farssi O.**, Mouradi M., Aziz F., Berrougui H. 2022. Role of acid phosphatase and enzymatic and non–enzymatic antioxidant systems in tolerance of alfalfa (Medicago sativa L.) populations to low phosphorus availability, Agronomy Research https://doi.org/10.15159/AR.22.064 (**IF: 1.23**).
- **Farssi O.,** Saih R., El Moukhtari A., Oubenali A., Mouradi M., Lazali M., Ghoulam C., Bouizgaren A., Berrougui H., Farissi, M. (2021) Synergistic effect of *Pseudomonas alkylphenolica PF9* and *Sinorhizobium meliloti Rm41* on Moroccan alfalfa population grown under limited phosphorus availability. Saudi J Biol Sci. 28, 3870–3879 https://doi.org/10.1016/j.sjbs.2021.03.069. **(IF: 4.05).**

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- **FARSSI O.,** MOURADI M., GHOULAM C., BOUIZGAREN A., BERROUGUI H. Physiological and biochemical responses of Moroccan alfalfa (*Medicago sativa L.)* populations to phosphorus deficit. The 1st International Conference on Biotechnology: Perspectives beyond 2020, December 18 and 19, 2017, Beni-Mellal, Morocco.
- **FARSSI O.,** MOURADI M., AZIZ F., BERROUGUI H. Role of phosphatase and antioxidant metabolism in mitigation phosphorus deficiency effect on alfalfa (*Medicago sativa L.).* The 1st International Conference on Biotechnology: Perspectives beyond 2020, December 18 and 19, 2017, Beni-Mellal, Morocco
- **FARSSI O.,** EL MOUKHTARI A., OUBENALI A., KAMAL H., TARCHI FZ., LAZALI M., FARISSI M. Physiological characterization of rhizobia strains isolated from different agrosystems in Beni- Mellal (Morocco). The Fourth International Congress "Microbial Biotechnology for Development", MICROBIOD4; April 24-26, 2019, Agadir, Morocco.
- **FARSSI O.,** GHOULAM C., BOUIZGAREN A., BERROUGUI H., FARISSI M. Co-inoculation effect of rhizobia and Plant Growth Promoting Rhizobacteria on *Medicago sativa* growth under phosphorus deficiency. The Fourth International Congress "Microbial Biotechnology for Development", MICROBIOD4; April 24-26, 2019, Agadir, Morocco.

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Omar FARSSI

Dedication

As a sign of respect and appreciation, I would like to dedicate this modest work

TO

My dear parents

My sisters

My brothers

My wife

My professors

To all my friends without exception

To all who have supported me throughout my PhD work

All the best!

Omar FARSSI

ABBREVIATIONS

ANOVA II : Two-way analysis of variance

APase : Acid phosphatase activity

BSA : Bovine serum albumin

CAT : Catalase

DW : Dry Weight

EC : Electrical conductivity

EL : Electrolyte leakage

ES : Error standard

F_v/F_m : Maximum quantum yield of PS II

gs : Stomatal conductance

H_2O_2 : Hydrogen peroxide

IAA : Indole acetic acid

L/S : leaf to stem ratio

MDA : Malonyldialdehyde

MALDI-TOF : Matrix Assisted Laser Desorption Ionization - Time of Flight

FM : Fresh material

NBT : p-nitroblue tetrazolium chloride

OD : Optical density

P : Phosphorus

PEG : Polyethylene Glycol

PGPR : Plant growth promoting rhizobacteria

pNPP : p-nitrophenyl phosphate

POD : Peroxidase

PSII : Photosystem II

PSB : Phosphate solubilizing bacteria

ROS : Reactive oxygen species

RWC : Relative water content

SOD : Superoxide dismutase

YEM : Yeast-Extract-Mannitol

gyrA : Gene encoding the A subunit of DNA gyrase (topoisomerase II)

gyrB : Gene encoding the A subunit of DNA gyrase (topoisomerase II)

rpoD : Gene encoding the principal sigma factor of *Pseudomonas*

ABSTRACT

This PhD thesis is based on a combination of research approaches in laboratory and greenhouse mesocosms to field experiments. It aims at evaluating the biofertilizer-biostimulant potential of endophyte and rhizospheric bacteria on alfalfa (*Medicago staiva* L.) – *Sinorhizobium (Ensifer) meliloti* symbiosis under low phosphorus (P) availability. The emphasis was put on the agro-physiological and biochemical traits associated with low P availability tolerance. Hence, as a first step, we focused on the screening of four Moroccan alfalfa cultivars for their tolerance to P deficiency with a focus on acid phosphatase, oxidative stress, enzymatic and non-enzymatic antioxidant systems. The experiment was conducted under controlled conditions with two P forms, insoluble P using Ca_3HPO_4 *versus* a soluble P form (KH_2PO_4) at a final concentration of 250 µmol P. $plant^{-1}$. $week^{-1}$. The P stress was applied for two months. The obtained results indicated that the P-starvation significantly ($p < 0.001$) reduced the agronomic traits evaluated, such as plant dry biomass and leaf area. The root and shoot P contents were found ($p < 0.001$) decreased by low-P availability in the rooting medium. Significant ($p < 0.001$) increases in lipid peroxidation and oxidative damage to cells, evaluated through malondialdehyde (MDA) and hydrogen peroxide contents, were noted because of P stress. The results showed also, that low P availability significantly ($p < 0.001$) increased the enzymatic antioxidant responses reflected by the activities of superoxide dismutase (SOD), guaiacol peroxidase (POD), and catalase (CAT). The non-enzymatic antioxidant molecules such as proline and total polyphenols significantly increased in alfalfa stressed plants. The behavior of the alfalfa cultivars tested was significantly different ($p > 0.05$). The cultivar *Oued Lmaleh (OL)* was found to be the least affected and the *Demnate* (*DEM)* was the most sensitive one, whereas the cultivars *TATA* and *RICH* showed a moderate tolerance. The second part of our work aims at the isolation and physiological characterization of rhizobacteria isolates from the Beni-Mella region. The isolated collection was tested for its biofertilizer-biostimulant potential through a set of traits such as tolerance to osmotic stress, qualitative and quantitative P solubilization, indole acetic acid (IAA) production, and exopolysaccharide production. The candidate isolates were preliminary identified using MALDI-TOF mass spectrometry, and then using housekeeping genes (*gyrA, gyrB & rpoD*). The selected plant growth-promoting rhizobacteria (PGPR) strains were used in the third part of our work as biofertilizer-biostimulant rhizobacteria to evaluate their synergistic effect on alfalfa growth under low-P availability. Hence, the synergistic effect of *Pseudomonas alkylphenolica* PF9, as phosphate solubilizing bacteria (PSB), and *Sinorhizobium meliloti* Rm41, as symbiotic nitrogen-fixing bacteria, on the Moroccan alfalfa cultivar *OL* under symbiotic nitrogen fixation and limited phosphorus (P) availability was focused. The results of this part 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 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). The last part of our PhD work was a field experiment during 2021 year in order to confirm the results obtained under controlled conditions. For this reason, a field trial was installed and evaluated during four consecutive cuttings, corresponding to growth and flowering period of alfalfa. The field was evaluated through some parameters related to forage biomass and forage quality. The results indicated that the rhizobacterial strains have a great potential on alfalfa production and its adaptation to low-P availability. Our findings suggest that rhizobacterial strains tested have a biofertilize-biostimulant potential for promoting alfalfa growth under low-P availability in field conditions. They are in concordance with those obtained under controlled conditions. Hence, the seed biopriming with the PSB tested constitutes a promising alternative to minimize the P problem in agricultural soils.

Keywords: Alfalfa; Acid phosphatase; Antioxidant molecules; Biofertilzer-Biostimulant; Co-inoculation; Oxidative stress; Phosphorus; Tolerance, Rhizobacteria.

RESUMÉ

Cette thèse de doctorat est basée sur une combinaison d'approches de recherche en mésosomes de laboratoire et de serre à des expérimentations en plein champs. Il a évalué le potentiel biofertilisant-biostimulant des bactéries endophytes et rhizosphériques sur la symbiose luzerne (*Medicago staiva* L.) - *Sinorhizobium (Ensifer) meliloti* en faible disponibilité en phosphore (P) assimilable. L'accent a été mis sur les traits agro-physiologiques et biochimiques associés à la tolérance à la faible disponibilité en P assimilable. Ainsi, dans un premier temps, nous nous sommes concentrés sur le criblage de quatre populations marocaines de luzerne pour leur tolérance au déficit en P assimilable en mettant l'accent sur les phosphatases acides, le stress oxydatif, les systèmes antioxydants enzymatiques et non enzymatiques. L'essai a été mené sous des conditions contrôlées avec deux formes du P, une forme insoluble en utilisant Ca_3HPO_4 *versus* une forme P soluble (KH_2PO_4) à une concentration finale de 250 µmol/plante/semaine. La contrainte phosphatée a été appliquée pendant deux mois. Les résultats obtenus ont indiqué que le déficit en P assimilable a réduit de manière significative ($p < 0,001$) les caractéristiques agronomiques évalués tels que la biomasse sèche des plantes et la surface foliaire. Les teneurs en P des racines et des parties aériennes ont été trouvées diminuer ($p < 0,001$) par la faible disponibilité en P dans le milieu racinaire. Une augmentation significative ($p < 0,001$) de la peroxydation lipidique et des dommages oxydatifs aux cellules, évalués par la teneur en malondialdéhyde (MDA) et en peroxyde d'hydrogène, ont été notés en raison du stress en P. Les résultats ont également montré que la faible disponibilité en P assimilable a induit une activité significative ($p < 0,001$) des enzymes antioxydantes telles que la superoxyde dismutase (SOD), de la gaïacol peroxydase (POD) et de la catalase (CAT). Les molécules antioxydantes non enzymatiques telles que la proline et les polyphénols totaux ont été accumulées de manière significative dans les plantes stressées. Le comportement des populations de luzerne testées a été significativement différent ($p > 0,05$). La population *Oued Lmaleh* (*OL*) s'est avérée la moins affectée et la population *Demnate* (*DEM*) a été la plus sensible, alors que les populations *TATA* et *RICH* ont montré une tolérance modérée. La deuxième partie de notre travail a visé l'isolement et la caractérisation physiologique des isolats de rhizobactéries de la région de Beni-Mellal. La collection isolée a été testée pour son potentiel biofertilisant-biostimulant à travers un ensemble de traits tels que la tolérance au stress osmotique, la solubilisation qualitative et quantitative du P, la production d'acide indole acétique (IAA) et la production d'exopolysaccharides. Les isolats candidats ont été préalablement identifiés à l'aide de la spectrométrie de masse MALDI-TOF, puis à l'aide de gènes domestiques (*gyrA*, *gyrB* et *rpoD*). Les souches sélectionnées de rhizobactéries favorisant la croissance des plantes ont été utilisées dans la troisième partie de notre travail en tant que des rhizobactéries à vocation biofertilisation-biostimulation pour évaluer leurs effets synergiques sur la croissance de la luzerne sous faible disponibilité en P assimilable. Ainsi, l'effet synergique de *Pseudomonas alkylphenolica* PF9, en tant que bactérie solubilisant du phosphate (BSP), et de *Sinorhizobium meliloti* Rm41, en tant que bactérie symbiotique fixatrice d'azote, sur la population marocaine de luzerne *OL* sous les conditions de fixation symbiotique d'azote et de limitation en P assimilable a été étudié. Les résultats de cette partie ont indiqué que l'inoculation des plantes de luzerne avec la souche *Sinorhizobium* seule ou combinée avec la souche *Pseudomonas* a amélioré de manière significative ($p < 0,001$) la croissance de la plante et les traits physiologiques et biochimiques focalisés par rapport aux plantes non inoculées et stressées. Pour la plupart des paramètres étudiés, l'amélioration a été plus évidente chez les plantes co-inoculées avec les deux souches que chez celles inoculées avec *Sinorhizobium meliloti* Rm41 séparément. En fait, sous une faible disponibilité en P assimilable, la co-inoculation avec les deux souches a significativement ($p < 0,01$) amélioré la croissance des plantes de luzerne évaluée par les biomasses fraîche et sèche, la hauteur des plantes et la surface foliaire. Les résultats ont également indiqué que l'amélioration notée dans la croissance des plantes a été positivement corrélée avec la teneur en P des parties aériennes et racinaires. De plus, l'augmentation de la teneur en P des plantes en réponse à l'inoculation bactérienne a amélioré la stabilité de la membrane cellulaire, reflétée par la teneur en MDA et en perte d'électrolyte (EL), et les paramètres liés à la photosynthèse tels que la teneur en chlorophylle, le rendement quantique maximal du PS II (F_v/F_m) et la conductance stomatique (g_s). La dernière partie de notre travail de thèse a été un essai en pleins champs durant l'année 2021 afin de confirmer les résultats obtenus sous conditions

contrôlées. Pour cette raison, un essai au champ a été installé et évalué à travers quatre coupes consécutives, correspondant à la période de croissance et de floraison de la luzerne. Le champ a été évalué à travers certains paramètres liés à la biomasse fourragère et à la qualité du fourrage. Les résultats ont indiqué que les souches rhizobactériennes ont un grand potentiel sur la production de luzerne et son adaptation à la faible disponibilité en P assimilable. Nos résultats suggèrent que les souches rhizobactériennes testées ont un potentiel de biofertilisation-biostimulation pour favoriser la croissance de la luzerne sous conditions de faible disponibilité en P assimilable et sous conditions du champ. Ils sont en concordance avec ceux obtenus sous conditions contrôlées. Ainsi, le prétraitement des semences avec les BSP testées constitue une alternative prometteuse pour minimiser le problème lié à la fertilisation phosphatée dans les sols agricoles.

Mots clés : Biofertilisant-Biostimulant ; Co-inoculation ; Luzerne ; Molécules antioxydantes ; Phosphatases acides ; Phosphore ; Rhizobactéries ; Stress oxydatif ; Tolérance.

ملخص

تعتمد أطروحة الدكتوراه هذه على مزيج من مناهج البحث في المختبر والبيت الزجاجي مع التجارب الميدانية. اهتمت بتقييم إمكانات السماد الحيوي - المحفز الحيوي للبكتيريات الداخلية والبكتريات الجذرية على نبات الفصة (*Medicago staiva L.*) والتكافل الريزوبي *Sinorhizobium (Ensifer) meliloti*في ظروف تتسم بقلة الفوسفور القابل للاستيعاب. تم التركيز على الصفات الزراعية الفيزيولوجية والكيميائية الحيوية المرتبطة بتحمل ظروف انخفاض الفوسفور القابل للاستيعاب. وهكذا، في البداية، ركزنا على فحص أربع مجموعات من نبات الفصة المغربي لتحملها لنقص الفوسفور القابل للاستيعاب مع التركيز على الفوسفاتيز الحمضي، والإجهاد التأكسدي، والأنظمة المضادة للأكسدة الأنزيمية وغير الأنزيمية. أجريت التجربة في ظل ظروف خاضعة للرقابة مع شكلين من الفوسفور، شكل غير قابل للذوبان باستخدام Ca_3HPO_4 مقابل شكل قابل للذوبان (KH_2PO_4) بتركيز نهائي قدره 250 ميكرولتر لكل نبتة خلال أسبوع. تم اخضاع النباتات لاجهاد فوسفوري لمدة شهرين. أشارت النتائج التي تم الحصول عليها إلى أن نقص الفوسفور المتاح أدى إلى انخفاض كبير (p <0.001) في الصفات الزراعية المقيمة مثل الكتلة الحيوية الجافة للنبات ومساحة الأوراق. وجد أن محتوى الفسفور في الجذور والأجزاء الهوائية ينخفض (p <0.001) عن طريق قلة توافر الفوسفور. لوحظت زيادة معنوية (p <0.001) في تاكسد الدهون والأضرار المؤكسدة للخلايا، كما تم تقييمها بواسطة المالونديالديهيد ومحتوى بيروكسيد الهيدروجين، بسبب الإجهاد الفوسفوري. وأظهرت النتائج أيضًا أن قلة توافر الفوسفور القابل للامتصاص تسبب في حدوث تداعيات كبيرة لنشاط من الإنزيمات المضادة للأكسدة مثل بيروكسيد ديسموتاز، غاياكول بيروكسيداز والكتلاز. تم تجميع جزيئات مضادات الأكسدة غير الأنزيمية مثل البرولين والبوليفينول الكلي بشكل كبير في النباتات المجهدة. كان سلوك مجموع الاصناف التي تم اختبارها مختلفًا بشكل كبير .الصنف وادي المالح كان الاقل تضررا بينما الصنف دمنات تبين الأكثر حساسية، في حين أظهرا الصنفين طاطا والريش تحملا معتدلًا. استهدف الجزء الثاني من عملنا العزل والتوصيف الفسيولوجي للبكتريات الجذرية من منطقة بني ملال. تم اختبار المجموعة المعزولة لإمكاناتها من أجل السماد الحيوي - المحفز الحيوي من خلال مجموعة من السمات مثل تحمل الإجهاد التناضحي، الذوبان النوعي والكمي للفوسفور، إنتاج حمض الأسيتيك الإندولي وإنتاج السكاريد الخارجي. تم تحديد السلالات المرشحة مسبقًا باستخدام مقياس الطيف الكتلي MALDI-TOF ثم استخدام جينات التدبير المنزلي (gyrA و gyrBو *rpoD*) تم استخدام سلالات مختارة من البكتيريا الجذرية المعززة لنمو النبات في الجزء الثالث من عملنا كبكتيريات جذرية للتخصيب الحيوي - التحفيز الحيوي لتقييم آثارها التآزرية على نمو نبات الفصة في ظروف تتسم بقلة الفوسفور القابل للاستيعاب .وبالتالي، فإن التأثير التآزري لـ *Pseudomonas alkylphenolica* PF9 ، باعتباره بكتيريا تذوب في الفوسفات ، و *Sinorhizobium meliloti* Rm41 ، كبكتريا تكافلية مثبتة للنيتروجين، على نبات الفصة المغربي واد المالح تحت ظروف التثبيت والعلاقة التكافلية للنيتروجين. أشارت نتائج هذا الجزء إلى أن تلقيح نباتات الفصة بسلالة *Sinorhizobium* *بمفردها* أو مجتمعة مع سلالة *Pseudomonas* تحسن بشكل كبير (p <0.001) نمو النبات والصفات الزراعية والفسيولوجية والكيميائية الحيوية مقارنة بالنباتات غير الملقحة والمجهدة. بالنسبة لمعظم المتغيرات التي تمت دراستها، كان التحسن أكثر وضوحًا في النباتات التي تم تلقيحها مع السلالتين أكثر من تلك التي تم تلقيحها بـ *Sinorhizobium meliloti* Rm41 بشكل منفصل. في الواقع، في ظل التوافر المنخفض للفوسفور المتوفر، أدى التلقيح المشترك مع كلا السلالتين إلى تحسن معنوي (p <0.01)لنمو نبات الفصة كما تم تقييمه بواسطة الكتلة الحيوية الطازجة والجافة، وارتفاع النبات ومساحة الاوراق. كما أشارت النتائج إلى أن التحسن الملحوظ في نمو النبات كان مرتبطا إيجابيا بمحتوى الفوسفور للأجزاء الهوائية والجذرية .فضلاً على ذلك، أدت الزيادة في محتوى النبات من الفسفور استجابةً للتلقيح البكتيري إلى تحسين استقرار غشاء الخلية، والذي ينعكس في محتوى المالونديالديهيد وفقدان الشوارد، والمعلمات المتعلقة بعملية التمثيل الضوئي مثل محتوى الكلوروفيل، والحد الأقصى من العائد الكمي للفوتوسيستم الثاني وتوصيل الثغور .كان الجزء الأخير من عمل أطروحتنا عبارة عن تجربة ميدانية خلال عام 2021 لتأكيد النتائج التي تم الحصول عليها في ظل ظروف خاضعة للرقابة. لهذا السبب، تم إجراء تجربة ميدانية وتقييمها من خلال أربع محاصل متتالية، تتوافق مع فترة نمو وازهار الفصة. تم تقييم الحقل من خلال معايير معينة تتعلق بالكتلة الحيوية للأعلاف وجودة العلف. أشارت النتائج إلى أن سلالات البكتريا الجذرية لديها إمكانات كبيرة على إنتاج الفصة وتكيفها مع قلة توافر الفوسفور .تشير نتائجنا إلى أن سلالات البكتريا الجذرية التي تم اختبارها لديها إمكانية للتخصيب الحيوي - التحفيز الحيوي لتعزيز نمو الفصة في ظل ظروف انخفاض توافر الفوسفور القابل للاستيعاب وتحت الظروف الميدانية. هاته النتائج تتوافق مع

تلك التي تم الحصول عليها في ظل ظروف خاضعة للرقابة. وبالتالي، فإن المعالجة المسبقة للبذور باستخدام البكتريات المختبرة تشكل بديلاً واعدًا لتقليل المشكلة المرتبطة بالتخصيب الفوسفاتي في التربة الزراعية.

الكلمات المفتاحية :سماد حيوي - محفز حيوي، التلقيح المشترك، الفصة، جزيئات مضادات الأكسدة، الفوسفاتيز الحمضي، الفوسفور، بكتريات الجذور، التحمل.

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Chapter 5

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GENERAL INTRODUCTION

GENERAL INTRODUCTION

Legumes are an important source of protein and vegetable oils. They are widely cultivated throughout the world. In Mediterranean area, these plants have an important position due to their agro-economic and environmental interests. Indeed, leguminous plants have a positive effect on soil fertility by contributing to the incorporation of nitrogen in ecosystems offering thus beneficial, ecological and economic impacts, helping to reduce or limit the use of chemical fertilizers by nitrogen-fixing symbiosis involving rhizobial strains (Bargaz *et al.,* 2021; Lazali *et al.*, 2021; Mouradi *et al*., 2018a).

As a perennial forage legume, alfalfa (*Medicago sativa* L.) is the most cultivated forage crop in the Mediterranean area (Bouizgaren et al., 2013). It has a great importance for agronomy because of its impressive nutritional value, biomass production and atmospheric nitrogen (N_2) fixation (Carlsson and Huss-Danell, 2003; Radovic et al., 2009). Furthermore, alfalfa's pivoting root system gives it the ability to absorb water by up to 3 m depth (El-Ramady et al., 2020) as a result of adaptation to some extreme abiotic stresses such as drought and salinity. However, its growth is highly susceptible to soil nutrient deficiency, and especially phosphorus (P) deficiency (Farssi et al., 2021).

Phosphorus is a vital macronutrient required for plant growth and development. It plays important roles in energy transfer, respiration, enzyme activation/inactivation, photosynthesis and root elongation (Elhaissoufi et al., 2020). Moreover, P has also been known to play a vital role during biological nitrogen fixation (BNF) in legumes, since 20% of total plant P being allocated to nodules (Bargaz et al., 2021; Farssi et al., 2021). P also represents the main constituent of plants cell, including nucleic acids, membrane phospholipids, etc. (Vance et al., 2003). However, although P concentration in most soils is typically high, the available P in the soil solution is less than 10 µM which is below plant requirements (Bargaz et al., 2021). Thus, in the most agricultural soils, farmers use chemical P fertilizers to increase the available P levels (Ekardt, 2016). However, the major parts of the applied P are likely to lose due to various biochemical processes related to P cycle including sorption, fixation, and immobilization (Penn and Camberato, 2019). On another hand, at high concentration, P may cause significant environmental risk from leaching, runoff and erosion (Bargaz et al., 2021). Therefore, there is a need for developing strategies that can improve P use efficiency by plants without having any risks to the environment.

The use of plant growth promoting rhizobacteria (PGPR) including P-solubilizing bacteria (PSB) has been shown to improve P use efficiency by plants (Bargaz et al., 2021;

Fatima et al., 2021; Suleman et al., 2018). The PSB are a large group of soil microorganisms that have the ability to mobilize directly or indirectly P from both organic and inorganic sources and make it available for plant use. 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 documented that the positive interaction between PGPR/PSB and plant roots may improve soil-P availability, especially in P-deficient soils. As result, the increase of the number and size of nodules, the amount of nitrogen assimilated per unit weight of nodules and the density of rhizobia bacteria in the soil surrounding the root (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).

Among the PSB, *Pseudomonas* has been recently considered as one of the most effective PSB, which continues to be a key research priority (Bargaz et al., 2021). Thus, when P-stressed legumes plants were inoculated with *P. alkylphenolica* PF9 strain, they exhibited higher biomass production, photosynthesis activity and P content (El Mooukhtari et al., 2021).

Therefore, the aim of this PhD thesis work focused on the role of biofertilizer-biostimulant potential of on the tolerance of alfalfa (*Medicago sativa* L.) to low P availability. The emphasis was put on the agro-physiological and biochemical traits associated to low P availability tolerance. Thus, our thesis work has been subdivided as follow:

- A concise introduction to the problem and the interest of the research subject itself.

- The first chapter is dedicated to bibliographical synthesis. We focused on the importance of legumes and their contribution in sustainable development, challenges of climate change and the importance of phosphate fertilizers for plant growth and development and finally the role of biofertilizers-biostimulants as a potential approach for sustainable agriculture for legumes productivity in agrosystems.

- The second chapter concentrated on the screening of four Moroccan alfalfa (*Medicago sativa* L.) cultivars for tolerance to low P availability, with the focus on the role of phosphatase and enzymatic and non-antioxidant systems in low P availability tolerance.

- The third chapter aimed at isolation, physiological characterization and identification of rhizobia and plant growth promoting rhizobacteria strains from Beni-Mellal region using MALDI-TOF mass spectrometry and housekeeping genes.

- The fourth chapter concentrated on the synergistic effect of *Pseudomonas alkylphenolica* PF9 and *Sinorhizobium meliloti* Rm41 on Moroccan alfalfa cultivar grown under limited P availability.

- The fifth chapter is a field trial to transfer the results obtained under controlled conditions. It seeks the role of seed biopriming with PSB on the alfalfa forage yield and quality in field conditions under low phosphorus availability

The manuscript ends with a general conclusion, in which we discussed and summarized our contributions and the perspectives that opened up as a result of our work.

1st CHAPTER

LITERATURE OVERVIEW

1st CHAPTER: LITERATURE OVERVIEW

I. Overview on legumes

Fabaceae or legumes are classified as angiosperms, Eudicot. They are sisters of *Polygalaceae*, constituting with the families of *Quillajaceae* and *Surianaceae* the Fabales (Judd et al., 2002). *Fabaceae* is the third-largest family of flowering plants in terms of number of species (after *Orchidaceae* and *Asteraceae*), with 727 genera and nearly 20 000 species (Cronk et al., 2006). The species range from arctic dwarf grass and mountains to immense trees of the tropical forests (Judd et al., 2002). The tree forms predominate in hot countries and the herbaceous forms in temperate regions (Guignard and Dupont, 2005). They are extremely diverse, but they have one thing in common, their fruit is a pod (Caratini, 1984).

Based on the floral form, this family is divided into three (Guignard and Dupont, 2005), two are monophyletic (*Papilionoideae* and *Mimosoidea*e) and third paraphyletic (*Caesalpinoideae*). They are by far the largest group of plants involved in nitrogen fixation with symbiotic bacteria (Raven et al., 2000). However, there are still 40% of legumes that have never been examined for nodulation (Sprent, 1999).

II. Interest of symbiotic nitrogen fixation

The inorganic nitrogen deficiency in the soil is a limiting factor for plant growth. It was estimated that the biological reduction of atmospheric nitrogen N_2 into ammonium provides about 65% of the available nitrogen in the biosphere (Lodwig et al., 2003). The majority of this nitrogen is provided by the rhizobia-legume symbiosis (Zahran, 1999; Table 1).

According to Danso (1995), Symbiotic Nitrogen Fixation (SNF) has a greater contribution to the growth of plants comparatively to the nitrogen fertilizer applied in agriculture in developing countries. The fixed nitrogen in the atmosphere contributes to 50-60% of N grain legumes, 55 to 60% of the N fixing trees, and 70 to 80% N forage legumes (Table 1).

This nitrogen reserve is stored in the leaves, the nodules or other organs, remains longer available in the soil compared to the strongly leached mineral nitrogen by water.

Table 1. Estimated amounts of nitrogen fixed by different leguminous crops (Soltner, 1999).

Species	Fixed nitrogen kg / ha
Alfalfa	200 (56-463)
Clovers	183(45-673)
Lupins	176 (145-208)
Horse bean	210(45-552)
Pea	65(52-77)
Lentil	101(88-114)
Soybean	75(1-168)

Extreme values are in parentheses.

II.1. Rhizobia

The second symbiont of the SNF is bacteria, commonly called "rhizobia". Rhizobia were characterized by their growth rate. The genus *Rhizobium* contains the fast-growing strains. This genus was divided into several genera (*Rhizobium*, *Ensifer*/*Sinorhizobium*, *Allorhizobium*, *Mesorhizobium*) and *Bradyrhizobium* containing slow-growing strains (Jordan, 1982). Currently, the functional group of rhizobia includes more than 100 species distributed in 9 families and 15 genera alpha and beta Proteobacteria. Thanks to the appearance of new molecular taxonomy tools and a wider exploration of diversity in high biodiversity areas, this number has increased steadily since about fifteen years.

II.2. Symbiotic specificity

One of the major characteristics of legume - rhizobia associations is their host specificity (Table 2). Indeed, a particular species of rhizobia is able, in general, to establish an effective symbiotic relationship with a limited number of plant partners. Similarly, a species of legume may be nodulated by a number of rhizobial species (Tilak et al., 2005).

Table 2. Rhizobia and their corresponding host plants.

Bacterial species	Host plants	References
Sinorhizobium meliloti	*Medicago, Melilotus, Trigonella*	(Jordan, 1982)
Rhizobium etli	*Phaseolus*	(Vázquez et al., 1993)
Rhizobium loti	*Lotus*	(Jordan, 1982)
Rhizobium tropici	*Phaseolus, Leucaena…etc.*	(Martínez-Romero et al., 1991)
Rhizobium fredii	*Phaseolus, Glycine…etc.*	(Bec-Ferte et al.1994)
Bradyrhizobium japonicum	*Glycine, Macroptilium*	(Elkan GH, 1992)
Bradyrhizobium elkanii	*Glycine, Macroptilium*	(Elkan GH, 1992)
Rhizobium meliloti	*Medicago, Melilotus, Trigonella*	(Jordan, 1982)
Rhizobium etli	*Phaseolus*	(Vázquez et al., 1993)
Rhizobium loti	*Lotus*	(Jordan, 1982)

II.3. Legume-rhizobia interaction

Legume-rhizobia interaction occurs in the rhizosphere due to the release of carbon molecules (sugars, organic acids, hormones, vitamins and phenolics) by exudation, secretion, or autolysis of old root cells (Seneviratne, Jayasinghearachchi, 2003). Many organic substances released by the roots have a low molecular weight and are easily decomposed by microorganisms. This leads to the existence of a large microbial community around the root.

Other compounds found in root exudates exert selective pressures on the microbial community. The early plant host signals secreted into the rhizosphere can be (iso)flavonoids, stachydrines, or aldonic acids. Many studies reported that the flavonoids, in combination with the rhizobial NodD transcriptional activator, induce expression of the *nod* gene regulon (Stougaard, 2000). These compounds are the most important of symbiotic perspective and can passively diffuse through the bacterial membrane (Begum et al., 2001; Wang et al., 2012).

II.4. Nodule formation

The rhizobia and the host plant establish a dialogue system based on an exchange of chemical molecules. First, the roots excrete flavonoids (Hirsch et al., 2001; Graham, 2008). These flavonoids (Figure 1) attract rhizobia in the vicinity of the root and activate the bacterial *nod* genes, which encode for Nod factors (Ramos, Bisseling, 2005; Downie, 2005). These factors secreted by the rhizobia stimulate cell division of the cortical part of the roots resulting in the formation of a primary meristem (Heller et al., 2000).

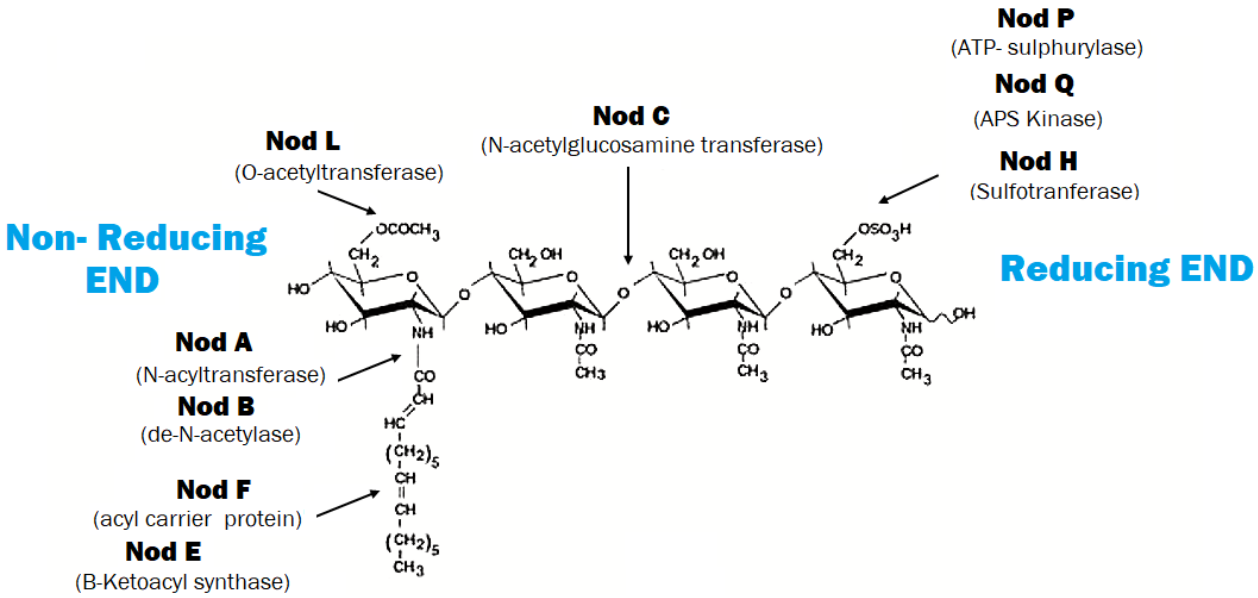


Figure 1. *Sinorhizobium meliloti* Nod factor structure and Nod protein function (Wais et al., 2002). *This figure is copyrighted by the American Society of Plant Biologists and is reprinted with permission.*

The bacteria attach to the roots by means of a specific adhesion molecule, rhicadhesin, localized at the surface of rhizobia cells. The rhicadhesin is a calcium binding protein. It allows adherence by complexing the calcium present in the root surface. The accession phase results in a retraction of the roots in response to secretion of molecules, and the bacterium penetrates the cells by an invagination mechanism. Growth and movement of the bacteria in the root cause the formation of an infection thread. The infection is gradually spreading to the cells located near the site of infection. Rapid division of infected cortical cells results in the formation of the nodule (Madigan, Martinko, 2007; Figure 2).

Bacteria proliferate quickly inside plant cells, or they take forms more or less globular, becoming bacteroides. The bacteroids are locked in vesicles limited by a membrane derived from the plant cell to form a symbiosome (Parniske, 2000; Werner, 2007).

II.4.1. NOD genes

During the early stages of symbiosis, the substances released from the legume induce the activation or repression of expression of nodulation genes in rhizobia (Soussou, 2013). The nodulation genes involved in the biosynthesis of Nod factors, which act as signal molecules and induce the formation of nodules (Masson-Boivin et al., 2009). *Nod* genes may be functionally divided into three classes: the regulator genes, common *nod* genes and specific genes (Wais et al., 2002). Important role in the development of nodules belongs to *nod* genes of rhizobia, which are organized in several operons and located either in the chromosome or in Sym-plasmids (Masson-Boivin et al., 2009). The expression of many genes is controlled by the NodD transcription factor of nodulation (Györgypal et al., 1991). Its activation occurs in response to the appearance of a plant flavonoid in a bacterial cell (Hassan, Mathesius, 2012). The common *nod* genes, *nodABC*, are highly conserved and present in different species of rhizobia, with the exception of some *Bradyrhizobium* and are responsible for the synthesis of skeletal Nod factors (Giraud et al., 2007). The specific *nod* genes are responsible for substitutions occurring on the basic skeleton of Nod factors. These accessory genes are not found in all rhizobia, and they are needed for nodulation of some plants (Soussou, 2013). Each strain has its own set of specific nod genes, which allow the production of a cocktail of Nod factors (Wais et al., 2002).

II.4.2. Perception and signal transduction of nod factors

Among the legumes such as *Medicago*, it appears that the responses to all Nod factors are dependent on the presence of O-sulfate group at the reducing end of Nod factors produced by *Sinorhizobium meliloti* (Mbengue, 2010). The infection initiation by the rhizobia is dependent on both the reducing and non-reducing ends (acetate and fatty acid). The first receptor, essential for all the responses, perceives the sulfate group of the reducing end. The second, essential for infection and the formation of the nodule, perceive the structures on the side of non-reducing end (Gage DJ, 2004).

II.4.3. Calcium and nod signaling

A rapid calcium influx followed by an output of Cl^-, K^+ and basification of the cytoplasm was first observed with selective microelectrodes (Debellé et al., 2007). These ionic movements causing membrane depolarization, which was originally described as the first event associated with the perception of Nod factors (Oldroyd, Downie, 2004). The calcium spiking was observed in response to treatment with Nod factors to ten times lower concentrations (1 nM) than those causing the rapid influx (Fig.3). They are located in the perinuclear and nuclear areas, and spread towards the apex. The signal is divided into a rapid elevation phase of the Ca^{2+} concentration, followed by a gradual return to baseline (Pingret et al., 1998; Engstrom et al., 2002).

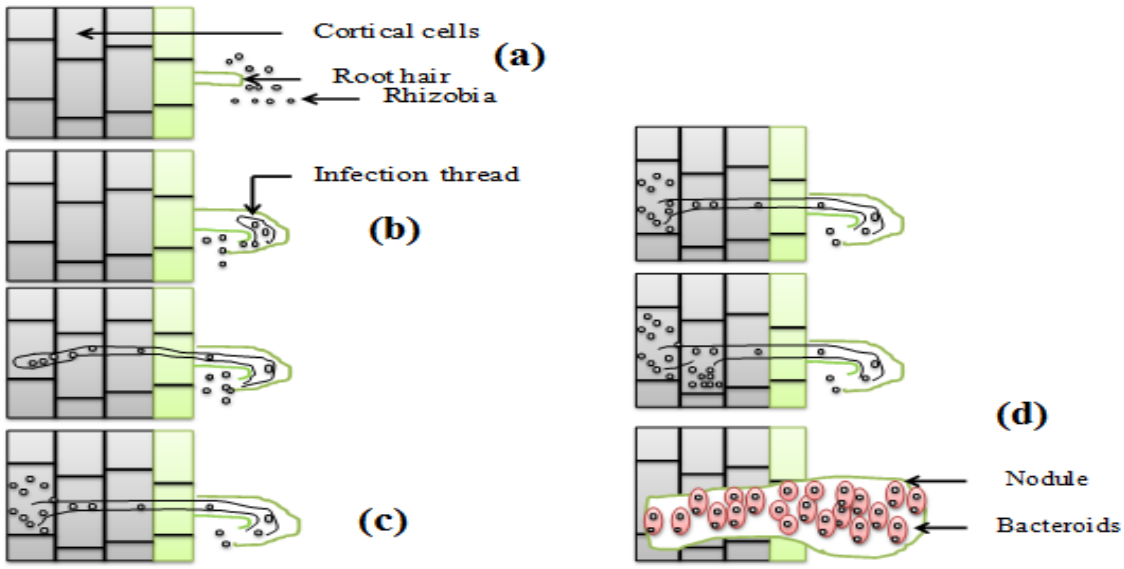


Figure 2. Steps of root nodule formation in a legume infected with rhizobia. *This figure is inspired from the work of Perry et al. (2004)*

a) Root hairs release chemical signals that attract rhizobia.

b) Rhizobia proliferate and cause an infection thread to form.

c) The infection thread grows into the cortex cell.

d) The infection thread releases bacterial cells, which become bacteroids in the root cells. Nod factors from bacteria cause cortical cells to divide.

II.4.4. Proteins of nod signaling pathways

The genetic approach identified six genes in *Medicago truncatula* (*DMI1*, *DMI2*, *DMI3*, *NFP*, *NFR*, and *NSP1-NSP2*), involved in the early stages of signaling by Nod factors and necessary for symbiotic responses such as: deformation of root hairs, the expression of genes nodulins and division of cortical cells (Catoira et al., 2000; Stacey et al., 2006).

a) *DMI* genes

The *DMI1*, *DMI2*, and *DMI3* genes of control early steps of Nod factor signal transduction in *Medicago truncatula* (Ané et al., 2002). *DMI1* encodes a membrane protein with low homology to a cation channel, *DMI2* encodes for an extracellular domain kinase receptor LRR (leucine rich repeat) and *DMI3* for a calcium and calmodulin dependent protein kinases (Stacey et al., 2006). The *DMI1* gene of the model legume *Medicago truncatula* plays a major role both in the early steps of Nod factor signaling and in the establishment of mycorrhizal symbiosis. DMI1 mutants do not exhibit many of the early responses to Nod factors and are incapable to form nodules (Ané et al., 2002). Peiter et al., (2007) reported that DMI1 can regulate the activity of calcium channels, the origin of the calcium spiking. A subsequent study showed that calcium oscillations are no longer present in dmi1, dmi2 mutants and, unlike dmi3 mutant that is not affected. DMI1 and DMI2, unlike DMI3, are required to generate the calcium oscillations (Wais et al., 2000). According to Bersoult et al. (2005), the study of the expression of DMI2 by promoter: GUS fusion showed that this gene is expressed in the epidermis and cortex of the root before inoculation. After inoculation, it was strongly induced in nodule primordia before contact with the infection thread and in zone II of infection of mature nodule.

b) NFR genes (Nod-factor receptor genes)

According to Madsen et al. (2003) and Radutoiu et al. (2003), the genes *NFR1* and *NFR5*, isolated by positional cloning in *Lotus japonicus* L., are essential for the earliest physiological and cellular responses to Nod factors, as mutations in them lead to plants that either no longer respond to Nod factors or show attenuated responses. *NFR1* and *NFR5*, like most genes that control Nod factor signaling, also control root hair curling, the first step of rhizobial infection, preceding infection thread formation.

c) NFP genes (Nod Factor Perception), the signaling receptor

NFP is a kinase receptor with an extracellular domain having a LysM motif known to interact with glycans (Stacey et al., 2006). The *NFP* gene expression observed in root hairs before inoculation with *Sinorhizobium meliloti* is consistent with the role of NFP in controlling a rapid calcium flux, calcium spiking, and inhibition of reactive oxygen efflux within minutes of Nod factor addition to root hairs (Amor et al., 2003). After rhizobial inoculation, NFP expression was strongly linked to nodule primordia development in the root cortex and to infection in root hairs and underlying outer cortical cells (Gough, 2003).

d) LYK genes (LysM Domain Receptor Kinases), the entry receptor

LYK genes were identified in *Medicago truncatula* as candidate Nod factor receptor genes (Figure 3). They are guessed to contain a signal peptide, extracellular LysM domains, a transmembrane segment and an intracellular serine/threonine kinase domain. Using reverse genetics in *Medicago truncatula*, Limpens et al. (2003) showed that two *LYK* genes are specifically concerned in the infection thread formation. This, as well as the properties of the LysM domains, strongly suggests that they are Nod factor entry receptors. The study of the synteny region SYM2 of pea in *Medicago truncatula* has allowed the identification of a group of 7 genes coding LysM RLKs with three lysin motifs in their extracellular domains. These LysM-RLKs assigned from *LYK1* to *LYK7* only *LYK3*, *LYK*6 and *LYK7* are expressed in the roots of *Medicago truncatula* (Limpens et al., 2003). Selective silencing of *LYK3, LYK6* and *LYK7* by interfering RNA has shown a particular symbiotic role for the LYK3 gene. The silencing of *LYK3* gene in *Medicago truncatula* caused no nodulation phenotype in the presence of *Sinorhizobium meliloti*. In contrast, no nodules are formed after inoculation with mutant strain of *Sinorhizobium meliloti* nodFE (Limpens et al., 2003).

e) NSP1 and NSP2, GRAS family transcription factors

Nodulation signaling in legumes depends on an NSP1-NSP2 complex (Eckardt, 2009). The investigation of Hirsch et al. (2009) on the function of the GRAS domain proteins, nodulation signaling pathway1 (NSP1) and NSP2 showed that the two proteins interact to form a complex that binds directly to a specific promoter region of Nod factor-inducible genes. They showed that the interaction between NSP1 and NSP2 is enhanced by Nod factor perception and is necessary for proper development of nodules (Figure 3). The same authors reported that NSP1 binds directly to ENOD promoters (early nodulins) through the novel cis-element AATTT. While NSP1 shows direct binding to the ENOD11 promoter in vitro, this association

in vivo requires NSP2. The NSP1-NSP2 association with the ENOD11 promoter is enhanced following Nod factor elicitation.

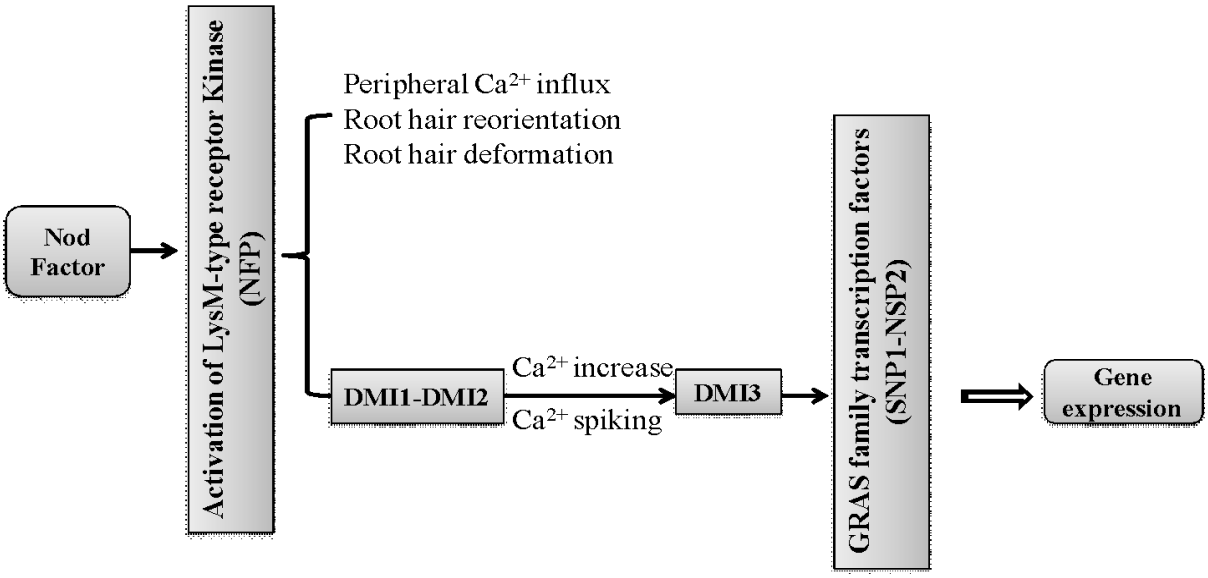


Figure 3. Nod factor signaling pathway in the epiderm of *Medicago*.

III. Roles of plant hormones in nodulation

All of the classical plant hormones have been suggested to influence nodulation, including some that interact with the autoregulation of nodulation (AON) pathway (Table 3; Figure 4). Leguminous plants strictly regulate the number of nodules formed through this AON pathway via a root-shoot-root loop that acts to suppress excessive nodulation (Foo et al., 2014).

Genes related to auxin such as, GH3, AUX1, DR5, MtPIN2, MtCycA2 were differentially expressed during nodular initiation (Pacios-Bras et al., 2003; Van Noorden et al., 2007; Huo et al., 2006; Roudier et al., 2003). Van Noorden et al. (2006) documented that optimal concentration of auxin seems to stimulate nodulation. The auxin concentration is higher in nodules than in the roots in many species of legumes (Ferguson, Mathesius, 2003). The addition of auxin transport inhibitors leads to the appearance of pseudo-nodules (Wu et al., 1996). In contrast, the addition of exogenous auxin restores nodulation (Fukuhara et al., 1994). In the same sense, the rhizobia deficient for the synthesis of auxin induces fewer nodules in soybean. Mathesius et al. (1998) (Mathesius et al., 1998) suggest that the induction of enod40 (nodulin genes) expression is correlated with a local change of auxin concentration.

The cytokinins play an important role in the nodulation process. The *enod2* and *enod12* genes are induced by cytokinins (Hirsch et al., 1997)] and also *enod40* was suggested to be induced in the protoxylem poles and surrounding cell layers upon accumulation of cytokinin (Hirsch et al., 1997). Heckmann et al. (2001) reported that the expression of *nodulin* genes was up-regulated upon cytokinin treatment, suggesting that the genuine nodulation program was indeed activated in *Lotus japonicus*. The same authors documented that the external cytokinin application induced expression of the Nin: GUS reporter gene within the root cortex, but not in the root epidermis.

Gibberellins are reported to be involved in the formation and maturation of legume nodules, highlighted by recent transcriptional analyses of early soybean symbiotic steps (Hayashi et al., 2014). Ferguson et al. (2011) demonstrated that gibberellin deficient mutants of pea developed fewer nodules than wild-type plants.

The hormone ethylene plays a role as a negative regulator of nodulation, and acts on different processes during nodule formation, including regulation of total nodule numbers, infection thread formation, nodule morphology, and nodule positioning (Geil and Guinel, 2002). Suganuma et al., (1995) observed the induction of ethylene hormone during nodule initiation in *Glycine max* L. This increase was due to a defense response of the plant in response

to the invading bacteria (Ferguson and Mathesius, 2003). Heidstra et al. (1997) showed that ACC oxidase, the last enzyme involved in ethylene biosynthesis, was activated by Nod factors in the inner cortex.

Table 3. Comparison of the influence of classical plant hormones on nodulation development (Foo et al., 2014).

Bacterial species	Host plants	References
Auxin	+	(Van Noorden et al., 2006 ; Deinum et al., 2012)
Gibberellin	+ (optimal range)	(Ferguson et al., 2005 ; Lievens et al., 2005)
Ethylene	-	(Penmetsa, Cook,1997 ; Oldroyd et al., 2001)
Brassinosteroid	+ (optimal range)	(Ferguson et al., 2005 ; Foo et al., 2014)
Strigolactone	+	(Soto et al., 2010 ; Foo et al., 2013)
Cytokinin	+	(Plet et al., 2011 ; Mortier et al., 2014)
Abscisic acid	+/−	(Ding et al., 2008 ; Tominagaet al., 2009)
Jasmonic acid	+/−	(Sun et al., 2006 ; Kinkema et al., 2008)
Salicylic acid	-	(Van Spronsen et al., 2003 ; Stacey et al., 2006)

Hormones that positively influence colonization are indicated by (+); those that do so only within an optimal range of hormone level are indicated), those that negatively affect colonization are indicated by a (-), those where there is evidence in both directions (such as at different developmental stages or using different experimental systems) are indicated with (+/).

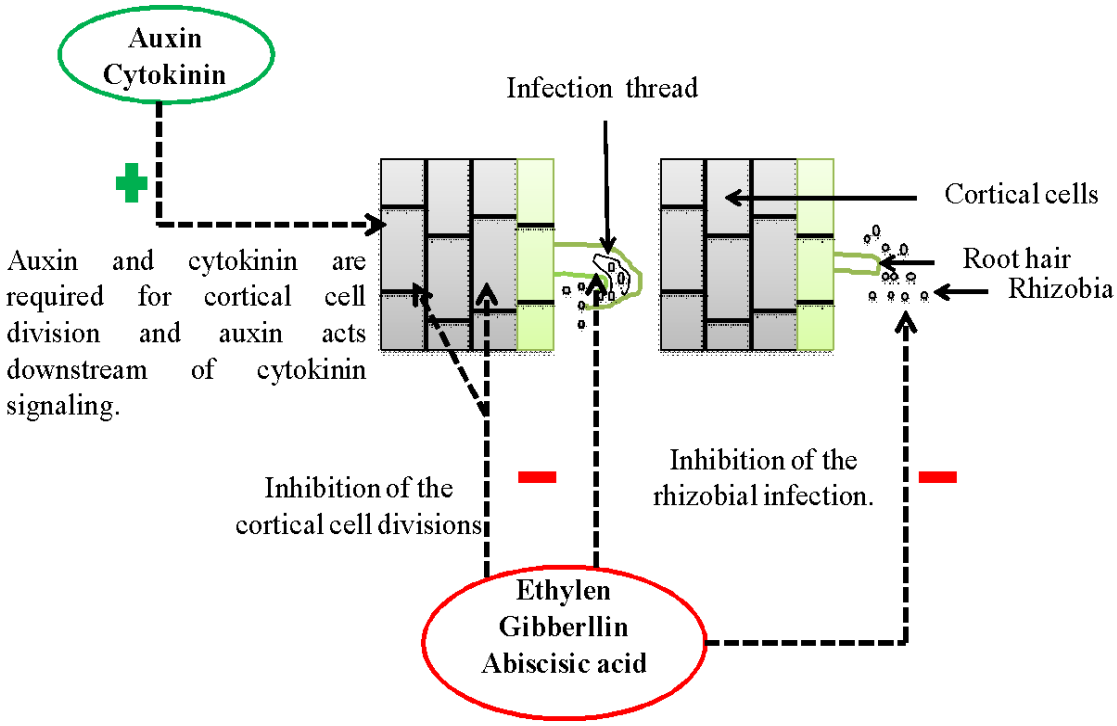


Figure 4. Effects of plant hormones on nodulation process in legume-rhizobia interaction.

IV. Legume production in the Mediterranean Basin: Challenges of climate change

Abiotic constraints accentuated in recent decades by climate change are the primary cause of crop losses in the Mediterranean region. In fact, the richness of the genetic heritage of many plant species including legumes is threatened by several abiotic factors, such as prolonged periods of drought, soil and irrigation water salinity, and soil nutrient deficiencies (Bargaz *et al.*, 2016; Farissi *et al.*, 2018). Previous investigation on legumes showed salinity stress and phosphorus limitations leading to an obvious reduction in different plant aspects including growth, photosynthesis, and nutrient use efficiency (Farssi *et al.*, 2021; Lazali *et al.*, 2020). In the same way, water deficit and phosphorus deficiency negatively affected plant growth, P nutrition, and atmospheric nitrogen fixation in forage and grain legumes, and importantly the effect was more aggravated under the combined effect of both stresses (Oukaltouma *et al.*, 2021). The culture of legumes is therefore concentrated in the northern regions with a favorable climate. The climate change has led to a reduction in grain and forage legume production area in many Mediterranean countries, particularly in the southern part of the Mediterranean Basin.

Because of the climate change conditions, it is perceived that plant production globally, including legumes, is seriously threatened by increased disease severity associated with a higher

survival rate of pathogens (El Hassni *et al*., 2007). Indeed, many biotic agents, such as fungi, cause various types of diseases, infections, and damage to leguminous species and affect their productivity and quality (Sampaio *et al.,* 2020). Among the various fungal diseases, powdery mildew, anthracnose, and fusarium wilt occur mainly on fresh leaves and stems, thus covering the entire surface of growing parts of many leguminous plants such as common bean, chickpea, and faba bean (Sampaio *et al.*, 2020).

The sensitivity of legumes to several abiotic and biotic agents is a major obstacle to obtaining large quantity and quality products. In addition, with changing climatic conditions, the resurgence of abiotic and biotic constraints is expected to explode. Hence, the development of a new detection, identification, and surveillance system can play a vital role in the effective managements. Such strategies should emphasize integrated management practices respectful of the environment. In this context, the soil microbiota and the mixed amendments aimed at the soil structure and biofertilization-biostimulation features have the key roles in suppressing the effect of abiotic constraints and controlling crop diseases.

V. Legume-rhizobia symbiosis under phosphorus limitation

V.1. Phosphorus in soil-plant system

Phosphorus (P) is a critical nutrient that plays an essential role in improving soil fertility for optimum plant growth and productivity. It is one of the most deficient macro-nutrients in agricultural soils after nitrogen and is considered inadequate for plant growth and production. P is vital for plant and animal growth, cell division, and growth. In addition, P as a nucleic acids component is vital in biological energy transfer processes. The soil availability of P depends on the interaction of P with other soil components, which can tie up P to complex forms, thus making P unavailable for plant uptake. In the soil, phosphorous (P) is converted from soluble P to insoluble P through two critical processes such as adsorption and precipitation. P can adsorb to clay minerals and iron (Fe)- or Aluminium (Al)-oxides, especially in acidic soils. The precipitation of P with Al, Fe, or Ca ions to form Fe-/Al-/Ca-phosphates in acidic and alkaline soils tend to fix 70–90% of added P from phosphatic fertilizer or mineralizable materials, As a result, the reduction of P available to plants (Stephano et al., 2022). Thus, low P mobility and reactivity makes it on deficient or unavailable forms in the soil (Zhu et al., 2018).

V.2. The main forms of P in the soil

Soil phosphorus is found in two forms, namely organic and inorganic (Figure 5). These two forms together make up the total soil phosphorus. Although total soil phosphorus is generally high, but plus 90% of this phosphorus is immobile and not available for plant uptake. Approximately 20 to 85% of total soil phosphorus is in organic forms, which are not plant available, while the remaining 15 to 80% is in inorganic forms. Organic forms of phosphorus, it is largely immobilized in organic matter (plant animal), decomposition residues and soil micro-organisms, which essentially consists of are phosphomonoesters and phosphodiesters, including inositol phosphate, phospholipids, nucleic acids, phosphoprotein, phosphosugars and coenzymes (Turner et al. 2002). These forms of p must be converted into inorganic p before used by the plants. However Soil micro-organisms play a key role in processing and transforming these organic forms of phosphorus into plant available forms (Wahid, Fazli et al 2022).

In contrast, the inorganic phosphorus forms can be classified to exist in three different pools (Rishi Prasad et al. 2019) :

• **Plant-available (soil solution) phosphorus :** This pool is comprised of inorganic phosphorus dissolved in water/soil solution that is readily available for plant uptake.

• **Sorbed phosphorus :** This phosphorus pool is comprised of inorganic phosphorus attached to clay surfaces, iron (Fe), aluminum (Al), and calcium (Ca) oxides in soil. The phosphorus in this pool is released slowly for plant uptake.

• **Mineral phosphorus :** This phosphorus pool is comprised of primary and secondary phosphate minerals present in soil. Examples of primary phosphorus minerals include apatite, strengite, and variscite. The secondary phosphorus minerals include calcium (Ca), iron (Fe), and aluminum (Al) phosphates. The release of phosphorus from this pool is extremely slow and occurs when the mineral weathers and dissolves in soil water.

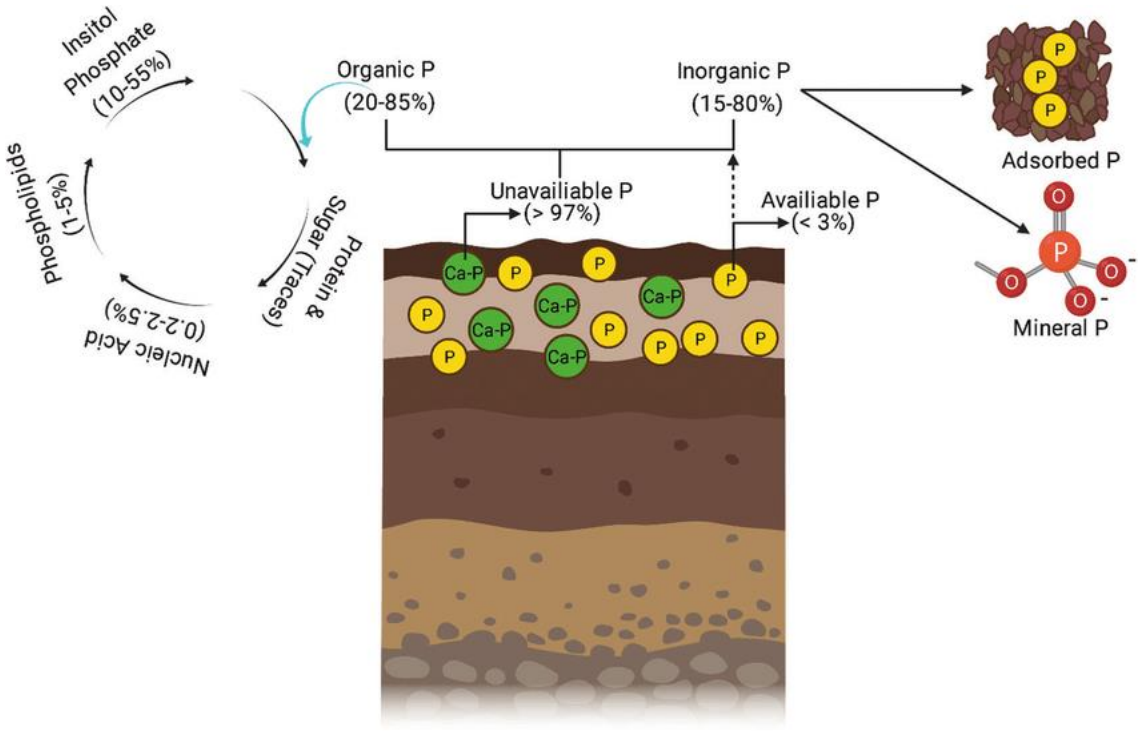


Figure 5: Soil P contents and their forms in the soil

Source : https://www.slideshare.net/DileepKumar9535017438/phosphorus-dynamics-in-calcareous-soils-with-respect-to-crop-growth.

V.3. Factors Influencing Phosphorus Availability in the Soil

Phosphorus (P) is a key element in crop production. Phosphorus enhances root growth, flowering, seed formation and seed maturation. Phosphorus availability is controlled by several factors such as soil organic matter levels, soil pH, and soil aluminum and iron contents, making it a challenge to estimate how much P will be supplied to the crop. Additionally, other factors, such as fertilizer placement, can significantly impact P availability during the growing season (R. Khajeeyan et al. 2021). In addition, phosphorus availability in soil solution is influenced by the following factors :

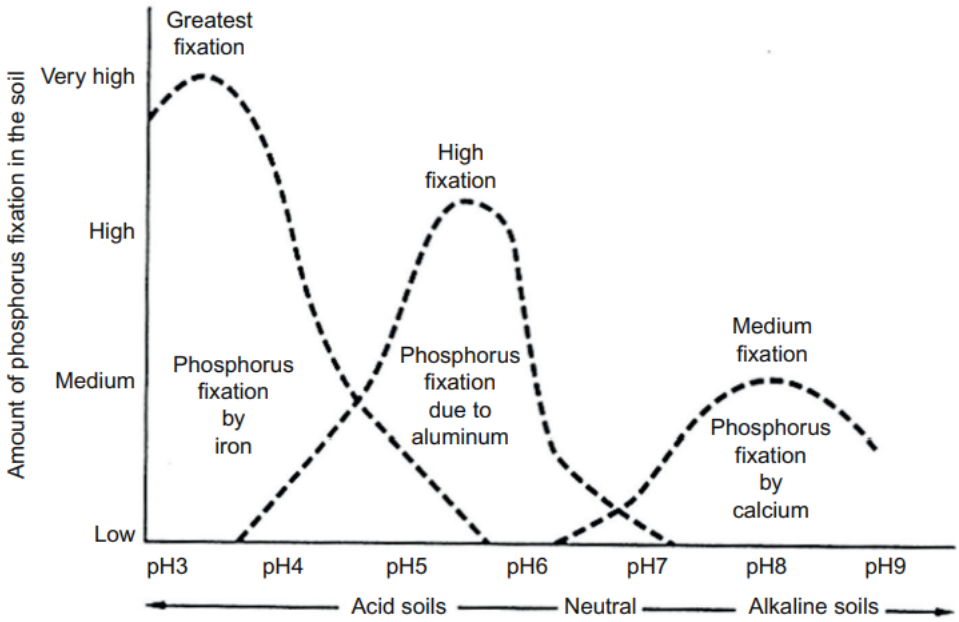
• **Organic matter** is an important factor in controlling phosphorus availability. With the addition of organic matter, availability of phosphorus increases. This is due to the following reasons :

- ✓ Mineralization of organic matter releases plant- available forms of phosphorus into soils.
- ✓ Organic molecules will compete with phosphate adsorbed to soil surfaces and will reduce phosphorus retention. This process will increase availability of phosphorus.

• **Clay Content :** Clay particles tend to retain or fix phosphorus in soils. Soils with higher clay content have high phosphorus retention capacity because clay particles have very large surface area per unit volume, which can adsorb phosphorus easily. Consequently, fine-textured soils, such as clay loam soils have a greater phosphorus-fixing capacity than sandy.

• **Soil Mineralogy :** The mineral composition of the soil influences the phosphorus adsorption capacity. For example, soils with a high content of Al3+ and Fe3+also tend to have the greatest phosphorus adsorption capacity.

• **Soil pH :** Optimum soil pH between 6 and 7 will result in maximum phosphorus availability. At low pH (acidic soils), soils have greater amounts of aluminum and iron, which form very strong bonds with phosphate. At high pH when calcium is the dominant cation, phosphate tends to precipitate with calcium.



Phosphorus availability across the pH range. Adapted from California Fertilizer Association (1995).

• **Other factors :** Temperature, moisture, and soil aeration can affect the rate of P mineralization from organic matter decomposition. For example, in warm, humid climates organic matter decomposes faster compared to cool dry climates.

V. 4. Microbial strategy for the mobilization of unavailable phosphorus in the rhizosphere.

The rhizosphere is the central and critical zone in the plant–soil environment, where maximum adsorption of nutrients, especially P, takes place from soil to plants (Kreueder et al.2019). In this context phosphate solubilizing bacteria (PSB) are the main contributors of plant nutrition in agriculture and could play a pivotal role in making soluble phosphorus available to plants. The PSB employ different strategies to convert unavailable forms of phosphate to bioavailable forms. Various biochemical and physiological processes, particularly the excretion of hormones, organic acids, and phosphatases, change the plant rhizosphere zone (Tian et al.2022).

The principal mechanism of P solubilization in Gram-negative bacteria, is the secretion of P-mineral dissolving compounds such as organic acids, protons, and siderophores (Y. Wei et al. 2018 ; L.Y. Yu et al ;2019 ; W. Elhaissoufi et al.2022). Organic acids produced increased plant-available P into the rhizosphere by forming complexes with cations (Al- or Fr-P) or by block P absorption sites on soil particles. It also acts as a good chelator of divalent Ca2+ cations coupled with the release of phosphates from insoluble complexes (Bhattacharya et al. 2019). These organic acids are the products of the microbial metabolism, mostly by oxidative respiration or fermentation of organic sources such as glucose (W. Elhaissoufi et al.2022). For example, organic acids such as lactic, malic, acetic, oxalic, and gluconic are produced by different bacterial species.

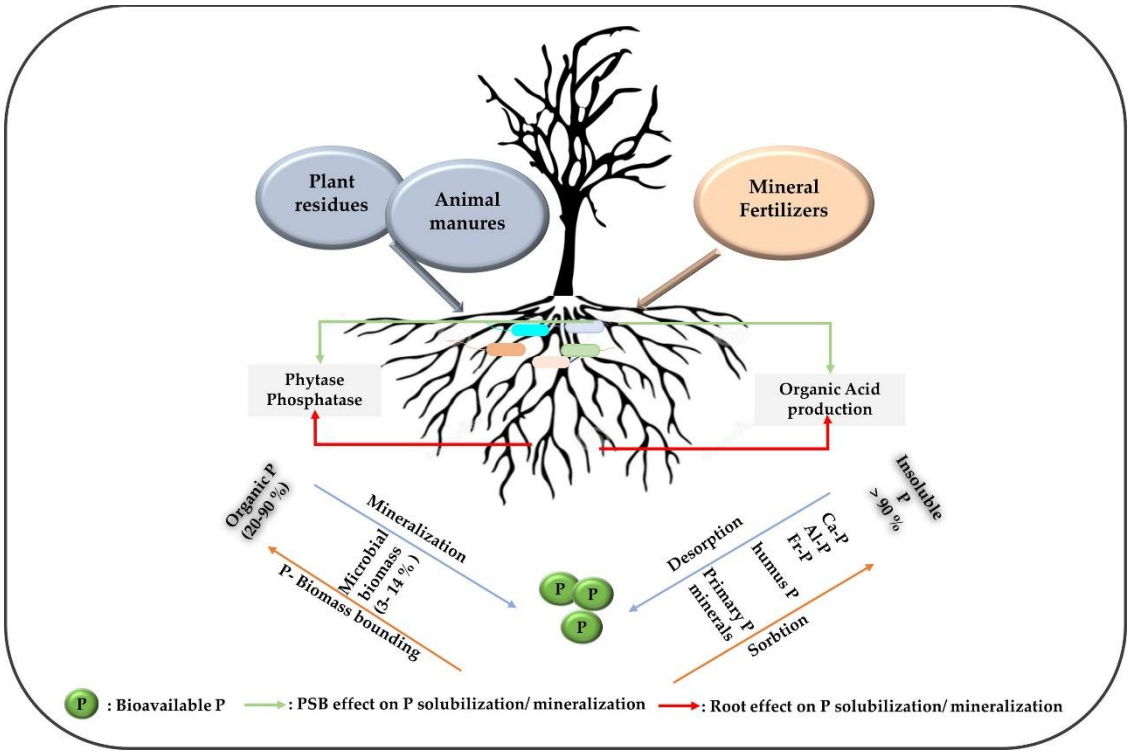


Figure 6: Schematic representation of P cycling processes in soil–plant-microorganism's systems. *"Insoluble P" represents P fixed with soil particles (ions, humus and primary P minerals) and "organic P" represents the organically bound component of P in microbial biomass and plant residues. Extracellular enzymatic hydrolysis and organic acid production are the biochemical process involved by roots and microorganisms to increase P availability.*

Many PSB cause a decrease in the pH of the medium either by extrusion of H+ or by exudation of enzymes into the rhizosphere, either by the roots or by associated microorganisms, are additional mechanisms contributing significantly contributing to improving P availability, with acid phosphatases being the most abundant phosphorus-hydrolyzing enzymes produced under low P conditions (W. Elhaissoufi et al.2022). For instance, PSB inoculation can modify root morphology and architecture through phytohormones production such as abscisic acid, cytokinin, indole-3-acetic acid and gibberellic acid (R. Khajeeyan et al.2021).

VI. Importance of phosphate fertilizers for plant growth and development

P is a vital macronutrient that plants required at an adequate level for their normal growth and development (Wang *et al.,* 2021). It has crucial functions in several physiological processes (Chan *et al.,* 2021). At a cellular level, P is involved in the structural composition of membrane lipids and nucleic acids rather than its key role in energy transfer and photosynthesis (Carstensen *et al.,* 2018). Additionally, in legumes-rhizobia symbiosis, P is required for biological nitrogen fixation, accounting for 20% of total plant P allocated to nodules (Mandri *et al.,* 2012).

P is taken up by the plant root in the form of orthophosphate ion; the bio-available P form. However, despite being quite abundant in soils, soil Pi rapidly interacts with Fe^{3+}, Ca^{2+}, and Al^{+3} ions, forming complexes making the total bio-available P fraction low (0.1-10 µM) than the plant requirements (Bargaz *et al.,* 2021). This makes P-deficiency one of the most abiotic stresses continuously threatening plant growth, including alfalfa (He *et al.,* 2017). It has been reported that P-deficiency reduced alfalfa growth by reducing P nutrition and inducing reactive oxygen species generation and lipid peroxidation (El Moukhtari *et al.,* 2022). Furthermore, Farssi *et al.* (2021) have also shown that P-deficiency could reduce alfalfa growth by reducing P nutrition and photosynthesis and inducing lipid peroxidation. In faba bean, P-deficiency was manifested by a reduction in plant biomass, photosynthesis, nitrogen fixation and water content along with an induction of oxidative stress (Oukaltouma *et al.,* 2022). The negative effects of P-deficiency have also been reported in *Citrus grandis* (Meng et al. 2021), maize (Zhang et al., 2018) and rice (Xu et al., 2007).

Hence, to overcome this situation, the use of phosphate solubilizing bacteria (PSB) has been emerged as a potent microbial strategy (e.g., inoculants, biofertilizers, biostimulant), as they have demonstrated positive effects on crop growth and productivity under P-deficiency, (Elhaissoufi et al., 2020).

VII. Optimization of legumes productivity in Mediterranean agrosystems: Biofertilizers-biostimulants as a potential approach for sustainable agriculture

The exploitation of the genetic diversity existing at the level of endophytic microorganisms or colonizing the rhizospheric environment constitutes a very promising alternative to promote and stimulate the production of legumes/quinoa under extreme conditions. Indeed, these microorganisms are well known for their application in a wide range of crops to improve growth, crop yield, and added value to plants. These various attributes result

from various mechanisms such as nitrogen fixation, quorum detection, signal interference, production of phytohormones, solubilization of phosphate, production of organic compounds, production of siderophores, etc. (Farissi et al., 2014; Mouradi et al., 2018). Several PGPR (Plant Growth-Promoting Rhizobacteria) as well as certain species of rhizobacteria produce auxin and gibberellic acid in the rhizospheric soil and thus play an important role in increasing the surface area and the number of roots (Han et al., 2005). PGPR and arbuscular mycorrhizal fungi can improve phosphorus status and alter the hormonal balance, resulting in the activation of pathways acquiring tolerance/resistance to various stresses limiting their growth and development. However, the diversity, function, and applications of microorganisms have received little applied attention up to now. Likewise, the ability to replace chemical fertilizers has potentially increased the demand for PGPR and other microorganisms and made it a major component of managing a sustainable agrosystem. Thus, our objective, in part, through this project revolves around the study of the microbiology and diversity of endophytic and rhizospheric microorganisms of legumes/quinoa and their effects on plant growth and immunity under environmental constraints and root pathogens. The relationships between plant growth and the mobilization of microorganisms are focused

2nd CHAPTER: ROLE OF ACID PHOSPHATASE AND ENZYMATIC AND NON-ENZYMATIC ANTIOXIDANT SYSTEMS IN TOLERANCE OF ALFALFA (*Medicago sativa* L.) POPULATIONS TO LOW PHOSPHORUS AVAILABILITY

Role of acid phosphatase and enzymatic and non-enzymatic antioxidant systems in tolerance of alfalfa (*Medicago sativa* L.) populations to low phosphorus availability

ABSTRACT

This study aims at evaluating the tolerance of four alfalfa (*Medicgao sativa* L.) cultivars to low phosphorus (P) in rooting medium. The experiment was carried out under controlled conditions. The seedlings of 15 old days were subjected to P deficiency using Ca_3HPO_4, insoluble form and P sufficiency using KH_2PO_4, as soluble form, at a final concentration of 250 µmol P. plant^{-1}. week^{-1}. After 60 days P deficit, several agro–physiological and biochemical traits were measured and determined in both conditions. The obtained results indicated that the P–starvation significantly ($p < 0.001$) reduced the agroeconomic traits evaluated, such as plant dry weight and leaf area. The root and shoot P contents were found ($p < 0.001$) decreased by low–P availability in the rooting medium. This constraint induced significant ($p < 0.001$) increase in phosphatase acid activity and caused lipid peroxidation and oxidative damage to cells, evaluated through malondialdehyde and hydrogen peroxide contents. Our results showed also, that low P availability significantly ($p < 0.001$) increased the enzymatic antioxidant responses reflected by the activities of superoxide dismutase (SOD), guaiacol peroxidase and catalase. The non–enzymatic antioxidant molecule such as proline and total polyphenols were found significantly increased in alfalfa stressed plants. The behavior of alfalfa cultivars tested was significantly different ($p < 0.05$). The *OL* cultivar was found to be the least affected and the *DEM* was the most sensitive one, whereas the cultivars *TATA* and *RICH* showed a moderate tolerance. Our study advises that the tolerance of Moroccan alfalfa cultivars to low P–availability was associated with increased acid phosphate activity and ability to induce enzymatic and non–enzymatic antioxidant responses leading to cell detoxification from reactive oxygen species (ROS).

Keywords: Alfalfa, Acid phosphatase, Antioxidant activity, Oxidative stress, Phosphorus, ROS.

I. Introduction

Legumes are an important source of proteins for both humans and animal livestock. They contribute to the incorporation of nitrogen in agro–pastoral ecosystems with valuable economic influence, to reduce or limit expansive and eco–unfriendly chemical fertilizers (Farssi et al., 2021; Lazali et al., 2021). Legumes are also used in intercropping or rotation with many plant species because of their multiple beneficial functions (Mouradi et al., 2018). Indeed, when cultivated in intercropping, legumes showed multiple advantages such as biological control, nutrient cycling, and increase in total yield (Martins da Costa et al., 2018; Mouradi et al., 2018; Oukaltouma et al., 2020). Therefore, exploiting the N_2–fixing ability of legumes could be a good way to improve the growth and productivity in legume–cereal intercropping system.

However, abiotic constraints accentuated in recent decades by climate change are the primary cause of crop losses in the Mediterranean region. Indeed, the rich gene pool of many legume species, including alfalfa (Medicago sativa L.), is threatened by several abiotic factors, such as prolonged periods of drought, soil and irrigation water salinity, and soil P deficiencies (Farissi et al, 2011, 2013; Bargaz et al, 2013b) In fact, previous research on legumes has shown that phosphorus limitations lead to an obvious reduction in different aspects of the plant, including growth, photosynthesis, and nutrient use efficiency (Farssi et al., 2021; Lazali et al., 2021). Similarly, low P availability negatively affects P nutrition and atmospheric N fixation in forage and grain legumes, and especially the effect is more aggravated under the combined effect of P and osmotic stress (Oukaltouma et al., 2021). Legume cultivation is therefore concentrated in northern regions with favorable climate. Due to climate change conditions, it is perceived that crop production in the world, including legumes, is seriously threatened and the sensitivity of legumes to several abiotic stresses is a major impediment to obtaining high quantity and quality products.

Low P availability, particularly in N_2–fixing symbiosis, has a significant effect on legumes' yield (Lazali and Drevon, 2018). Indeed, this symbiosis poses supplementary P demand (20% of total to nodules) and any P starvation may affect the activity of rhizobia and consequently the symbiosis efficacy (Drevon, 2017). However, soluble P enhances the growth of *Vigna unguiculata* L. plants, their total P content and nodulation (Benlahrech et al., 2018). In the same sense, Bekel et al. (2019) reported that the P fertilizers significantly influenced both total and effective number of nodules in *Arachis hypogaea* L. plants. Based on the positive correlations between acid phosphatase activity and P use efficiency, it would be worthwhile to take into account the role of acid phosphatases in building an effective legume-rhizobia

symbiosis as a possible mechanism in P-deficiency tolerance (Lazali et al., 2021). In fact, Tran et al. (2010) reported that under low-P availability, acid phosphatases are thought to be crucial for the metabolism of organic P in both intracellular and extracellular plant tissues. It has been suggested that secreted or cell wall-associated acid phosphatases recycle Pi from endogenous phosphomonoesters that have leaked from the cytoplasm across the plasma membrane or scavenge Pi from organic-P compounds found in the rhizosphere (Shane et al., 2014).

Furthermore, the reactivity of P with some soil cations, such as iron, aluminum, and calcium, which results in the formation of insoluble compounds, limits its mobility in soil solutions. These interactions result in reduced P availability and low phosphate fertilizer efficiency in plants. As a result, the SNF process, root growth, photosynthesis, rhizobia proliferation, and nodule development are all limited (Boudanga et al., 2015; Neila et al., 2014).

In recent years, the most important technique for reducing the effects of environmental restrictions on legume production has been to select plant genotypes tolerating to abiotic stress (Latrach et al., 2017). In fact, the exploitation of the genetic diversity existing in local germplasm constitutes a promising approach to enhance plant productivity under unfavorable conditions. In this context, our study aims at evaluating the tolerance to low P availability in four Moroccan alfalfa (*Medicago sativa L.)* cultivars. The emphasis was on agro–physiological and biochemical properties related to the tolerance to this environmental constraint. The role of phosphatase and enzymatic and non–enzymatic antioxidant system was focused.

II. Materials & Methods

II.1. Plant material and growth conditions

The biological material used in this study consists of four Moroccan alfalfa (*Medicago sativa* L.) cultivars; *OUED LMALEH*, *DEMNATE*, *TATA* and *RICH*. Seeds were supplied by National Institute for Agronomic Research, Morocco. Local cultivars of alfalfa are commonly used in the Moroccan traditional agroecosystems, oasis and mountain, and powerfully involved in the socio–economic development chain of local families as the nutrition source for their livestock. They have been cultivated for many centuries and are still extensively 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 agro–morphological traits of the landraces, which have reached Hardy–Weinberg equilibrium (Kang, 1998; Annicchiarico, 2011; Farissi *et al*., 2013). The seeds were germinated in 20 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. After 15 days of sowing, the young seedlings were irrigated by capillarity with a nutrient solution with two P forms, insoluble P using Ca_3HPO_4s (limited available P) *versus* soluble (KH_2PO_4). Both soluble and insoluble P forms were adjusted to reach 250 µmol P. $plant^{-1}$. $week^{-1}$. The composition of the nutrient solution (Hoagland and Arnon, 1950) used was as follows: [KNO_3 (600 µmol), $MgSO_4$ (1000 µmol), K_2SO_4 (750 µmol), $CaCl_2$ (1650 µmol), Fe-ethylenediaminetetraacetic acid (EDTA) (16 µmol), MnSO4 (6 µmol), H_3BO_3 (4 µmol), $ZnSO_4$ (1 µmol), $NaMoO_4$ (0.1 µmol), and $CuSO_4$ (1 µmol)]. By using 0.1 M HCl or 0.1 M NaOH, the pH of the nutrient solution was, respectively, reduced or raised to reach 7 before use. After 60 days of P stress, the plants were collected, measured, and several agro–physiological and biochemical traits were analyzed prevailing plant growth and development. Each pot was planted with five plants and each treatment was represented by three replicates, resulting in a total of 24 pots and 120 plants (Figure 7).

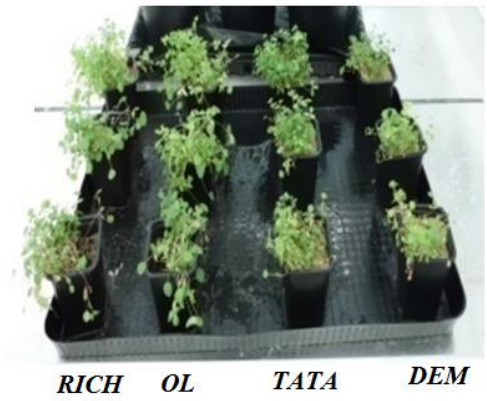


Figure 7. Stressed bloc

II.2. Plant biomass and leaf area

The shoot and root fresh weights (FW) were determined immediately after harvest. The dry weight (DW) was then measured using precision balance after their drying at 80 °C for 48h. The leaf area was estimated using MESURIM software (version 3.4.4.0) using a digital scanner. Five plants per pot grouped as three replicates were measured.

II.3. Phosphorus contents

The P contents were determined using 0.5 g of the dry matter of each plant parts after incineration at 600 °C for 6 h. The ash obtained was treated in 3 ml of HCl (10 N) and filtered. Then, the P concentration were measured using the molybdate blue colorimetric assay (Murphy & Riley, 1962). After color development at 100 °C for 10 minutes, the optical density was measured at 820 nm. A standard curve was established with KH_2PO_4 solutions.

II.4. Acid phosphatase activity

Samples of fresh matter (50 mg) were ground in mortar using 500 µL of sodium acetate extraction buffer (0.1 M, pH 5.5), 2.5% polyvinylpyrrolidone (PVP) and 5 µL of β mercaptoethanol. The homogenates were centrifuged at 12000 × g for 30 min at 4 ºC. The acid phosphatase activity was measured using 100 µL of enzymatic extract mixed with 200 µL of p–NPP (*p*–nitrophenyl phosphate) and incubated for 30 min at 37ºC. Then, 1 mL of 1N NaOH was added to stop the reaction. The acid phosphatase activity (µmol *p*–NPP min^{-1} mg^{-1} protein) was measured by a spectrophotometer at 410 nm wavelength (Araújo et al., 2008)

Protein content in all enzyme preparations was determined using bovine serum albumin (BSA) as standard (Bradford, 1976).

II.5. Oxidative stress markers and membrane cell integrity assessments

The lipid peroxidation was estimated by malondialdehyde (MDA) and Hydrogen peroxide (H_2O_2) accumulation. MDA was measured using 50 mg of fresh leaves added to 2 mL of 0.1% trichloroacetic acid (TCA). After centrifugation at 14000 rpm for 15 min, 1 mL of supernatant was added to 2.5 mL of 0.5% thiobarbituric acid (TBA) in 20% TCA. The mixture was placed at 95 °C for 30 min and cooled down by ice. The absorbance was measured at 532 and 600 nm. The MDA content was calculated by the extinction coefficient $\varepsilon = 155\ mM^{-1} . cm^{-1}$ (Savicka & Škute, 2010).

The H_2O_2 content was determined as described by Velikova, Yordanov & Edreva, 2000). 100 mg fresh leaves were mixed with 5 mL of 0.1% TCA. After centrifuged at 12000 × g for

15 min, 0.5 mL of the supernatant was added to 0.5 mL of 10 mM potassium phosphate buffer (pH 7.0) and 1 mL of 1 M potassium iodide. The absorbance was measured at 390 nm. The H_2O_2 content was expressed as µmol $H_2O_2.g^{-1}$ FW.

For the electrolyte leakage (EL), 0.1 g of young leaflets were washed three times with distillated water to eliminate surface–adhered electrolytes, then placed in closed flasks filled with 10 mL of distillated water. The flasks were after that incubated for 24 h at 25 °C on a rotary shaker. The initial electrical conductivity (Li) was measured by using a conductivity meter. Then the samples were autoclaved at 120 °C for 20 min. The final electrical conductivity (Lf) was measured after 25 °C equilibration, the percentage of EL was calculated as follows (Lutts et al., 1996):

$$EL\ (\%) = (Li\ /Lf) \times 100$$

II.6. Enzymatic antioxidant activities

100 mg of fresh leaves were crushed in 1 mL of phosphate buffer (20 mM, pH 7). After centrifugation at 15000 × g for 20 min at 4°C, the supernatant was used for the determination of the POD (EC 1.11.1.7) enzymatic activity according to (Beyer & Fridovich, 1987). The reaction mixture consisted of 200 µL of H_2O_2 at 0.3%, 300 µL of guaiacol at 20 mM, 2 mL of phosphate buffer (0.1 M, pH 6), 1 mL of distilled water and 10 µL of enzymatic extract. The POD activity was measured after 3 min, at 470 nm. The activity was calculated using the guaiacol extinction coefficient 26.6 $mM^{-1}\ cm^{-1}$, and expressed as µmol $H_2O_2\ min^{-1}\ mg^{-1}$ proteins.

SOD (EC 1.15.1.1) activity was determined as described (Chagas *et al.*, 2008). 50 µL of crude enzymatic extract in phosphate buffer (20 mM, pH 7) was added to a solution containing 13 mM L–methionine, 75µM *p*–nitro blue tetrazolium chloride (NBT), 100 µM EDTA and 2 µM riboflavin in a 50 mM potassium phosphate buffer (pH 7.8). The reaction was performed in assay tubes upon illumination using a 30 W fluorescent lamp at 25 °C for 15 min. The blue formazan produced by NBT photoreduction was spectrophotometrically measured at 620 nm. An enzyme unit was equal to the amount to inhibit 50% of NBT. SOD activity was expressed as enzymatic U $min^{-1}\ mg^{-1}$ proteins.

CAT (EC 1.11.1.6) activity was determined using 250 µL of the extract was added to 2 mL of the assay mixture (50 mM Tris–HCl buffer pH 6.8, containing 5 mM H_2O_2) (Gong et al., 2001). Then after 10 min at 20°C, 250 µL of 20% titanic tetrachloride (v/v, in concentrated HCl) were added to stop the reaction. CAT activity was read at 415 nm and calculated by

comparing the absorbance against a standard curve of 0.25 to 2.5 mM) H_2O_2. CAT activity was expressed as µmol H_2O_2 min^{-1} mg^{-1} proteins.

Protein content in all enzyme preparations was determined using BSA as standard (Bradford, 1976).

II.7. Non–enzymatic antioxidant molecules

Non–enzymatic antioxidant molecules were evaluated through proline and total polyphenol contents. The proline content was determined using extract from 100 mg FW with 2 ml of 40% methanol: water. After incubation at 85 °C for 30 min, 1 ml extract was mixed with 1 ml of a mixture of glacial acetic acid and orthophosphoric acid at 6M (3: 2; v/v) and 25 mg ninhydrin. After 1 h incubation at 100 °C, the tubes were cooled and 5 ml toluene was added. The optical density of the upper phase was measured at 528 nm. The proline content was determined using a standard curve obtained using reference proline solutions (Bates et al., 1973).

For total polyphenol content, 100 mg of fresh samples were ground in 1 mL of methanol (80%). After centrifugation at 12,000 ×g for 20 min at 4 °C, the supernatants were recuperated. The content of total polyphenols was determined through the Folin–Ciocalteu method and their concentration was described as mg Gallic acid equivalents (GA) g^{-1} FW (Singleton & Rossi, 1965).

II.8. Statistical analysis

Statistical analysis was executed using SPSS version 22. A two–way analysis of variance (ANOVA) was adopted. Means comparison was performed using Tukey's test. XLSTAT software version 2014 (Addinsoft, Paris, France) was used to determine the correlations.

III. RESULTS

III.1. Effects of low Phosphorus availability in rooting medium on plant biomass and leaf area

The starvation of P availability in rooting medium caused significant ($P < 0.001$) reductions in plant shoot and root dry weights. The lowest reductions were noted in *OL* cultivars in comparison with other cultivars, which showed the same behavior according to Tukey's test ($P > 0.05$). In fact, under low P availability, the shoot dry weight recorded in *OL* was 32.80 mg. plant^{-1} with a reduction of 28.90% comparatively to *OL* plants grown under P-sufficient conditions. However, the remaining cultivars, *DEM*, *TATA*, *RICH* showed the reductions of 52.80, 41.09 and 39.96% respectively for the same growth trait (Figure 8A). Regarding, the root dry weights (Figure 8B), the effect was more pronounced ($P > 0.05$) in *DEM* cultivar with reductions of 41.44% relative to control. *OL* was found to be the least affected one ($P < 0.05$) with the reduction percentages did not exceed 19.39%. However, *TATA* and *RICH* showed an intermediate behavior.

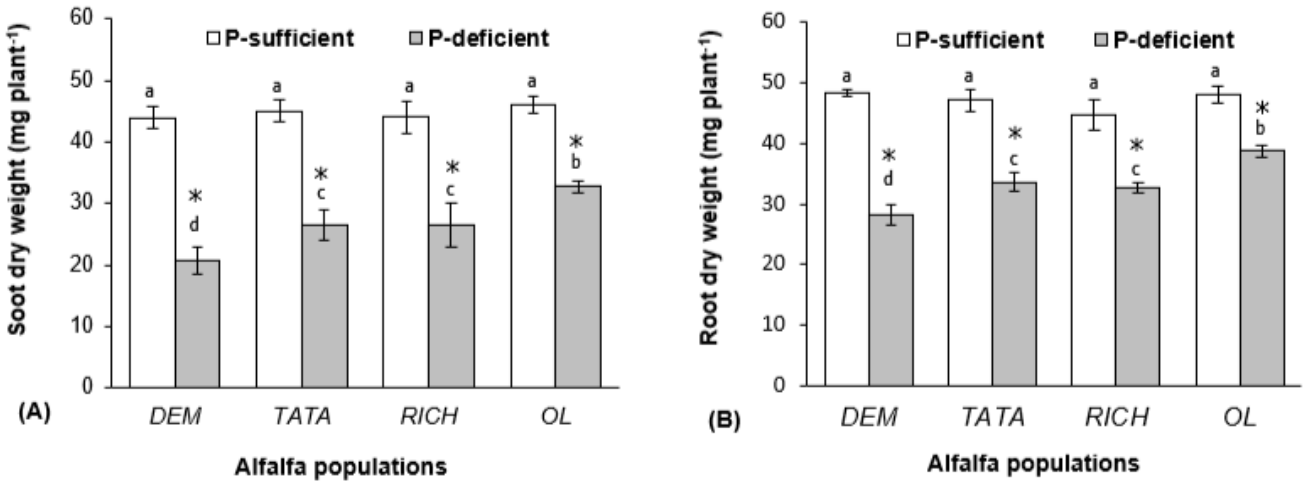


Figure 8. Shoot **(A)** and root **(B)** dry weights in alfalfa plants grown under P-sufficient (open histograms) *versus* P-deficient (filled histograms) supply. Data are means of three replicates, and bars represent the SE. Asterisks above histograms denote significant effect of P level at $p < 0.001$. 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.

For leaf area (Figure 9), the significant ($p < 0.001$) reductions were caused by the low P availability in rooting medium. OL cultivar maintained the highest leaf area value (1.61 cm^2), but with no significant differences ($p > 0.05$) in comparison with the remaining cultivars according to Tukey's statistical test.

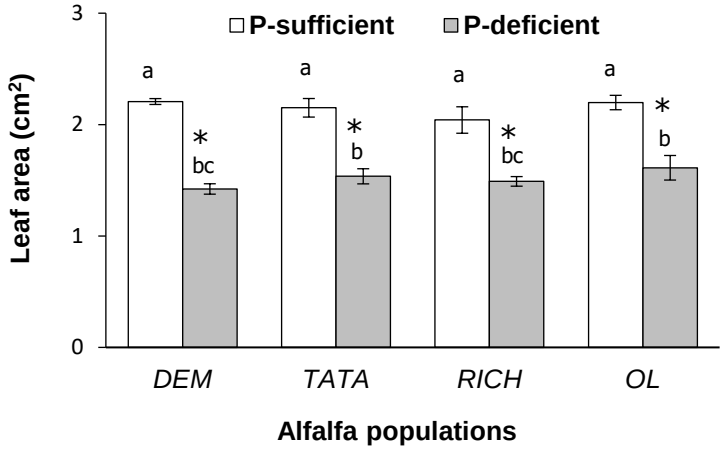


Figure 9. Leaf area of alfalfa plants grown under P-sufficient (open histograms) *versus* P-deficient (filled histograms) supply. Data are means of three replicates, and bars represent the SE. Asterisks above histograms denote significant effect of P level at $p < 0.001$. Different and same small letters above histograms indicate significant ($p < 0.05$) and no significant differences ($p > 0.05$), respectively, between the means according to Tukey's test.

III.2. Phosphorus contents

The figure 10 illustrates the shoot and root P concentration under the stressed and non–stressed conditions. The obtained results mentioned that the highest P contents of 238 and 333 mg g DW^{-1} were noted in shoots and roots of *OL* cultivar respectively under P stress, followed by *TATA* and *RICH*, whereas the lowest P contents of 105 and 179 mg g DW^{-1} were noted in *DEM* cultivar under the same conditions.

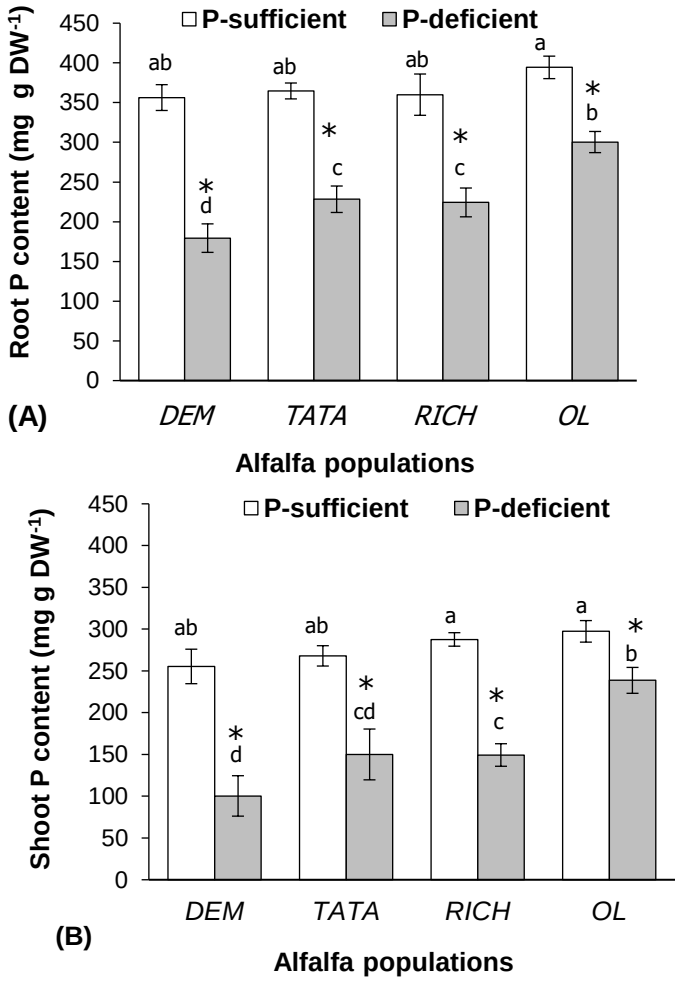


Figure 10. P contents in roots (**A**) and shoots (**B**) of alfalfa plants grown under P-sufficient (open histograms) *versus* P-deficient (filled histograms) supply. Data are means of three replicates and bars represent the SE. Asterisks above histograms denote significant effect of P level at $p < 0.001$. 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.

III.3. Acid phosphatase activity

Low P availability induced significant ($p < 0.001$) increase in phosphatase acid activity in alfalfa roots. The highest activity ($p < 0.05$) was noted in *OL* cultivar (19.85 µmol *p*–NPP min^{-1} mg^{-1} proteins). Nevertheless, the activities not exceeding 15.72 µmol *p*–NPP min^{-1} mg^{-1} proteins were recorded in *TATA* and *RICH* cultivars with non–significant differences ($p > 0.05$) between themes, according to the statistical grouping test considered. However, the lowest ($p < 0.05$) acid phosphatase specific activity was noted in *DEM* cultivar 12.68 µmol *p*–NPP min^{-1} mg^{-1} proteins (Figure 11)

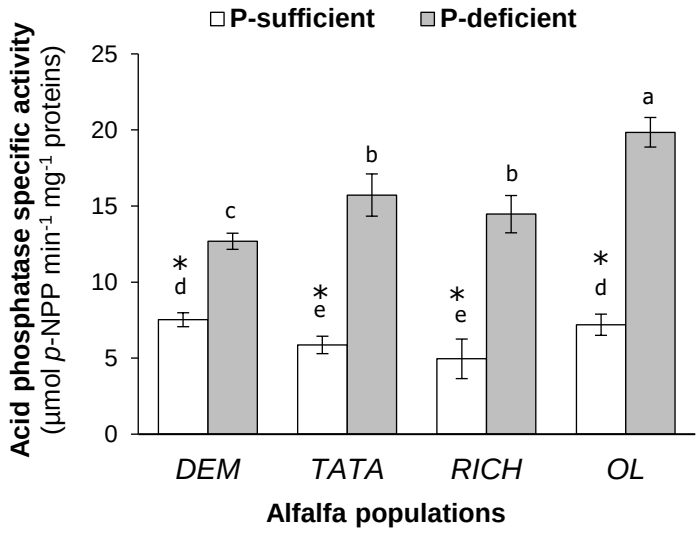


Figure 11. Acid phosphatase specific activity in alfalfa plants grown under P-sufficient (open histograms) *versus* P-deficient (filled histograms) supply. Data are means of three replicates, and bars represent the SE. Asterisks above histograms denote significant effect of P level at $p < 0.001$. Different 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.

III.4. Effect on membrane cell integrity and oxidative stress markers

The EL contents reflecting the cell membrane integrity were found increased ($P < 0.001$) under low P availability (Table 4). The cell membrane damages were most prominent in the *DEM*, *TATA* and *RICH* cultivars with percentages of 22.98, 21.27 and 23.32%. However, the lowest EL contents ($p < 0.05$) were noted in *OL* cultivar18.07%.

The oxidative markers, MDA and H_2O_2, were found significantly ($p < 0.001$) accumulated under low P availability in alfalfa stressed plants (Table 4). The significant accumulations were observed in *DEM* pollution followed by *TATA* and *RICH* cultivars. The contents ranged from 41.28 to 48.72 g. FW^{-1} for MDA and from 0.99 to 1.19 g FW^{-1} for H_2O_2. Nevertheless, the *OL* cultivar was significantly ($p > 0.05$) revealed to be the least affected one according to Tukey's test with the values of 39.11 µmol g FW^{-1} and 0.88 µmol g FW^{-1} for MDA and H_2O_2 respectively.

Table. 4: EL, MDA and H_2O_2 contents in alfalfa plants grown under P-sufficient *versus* P-deficient supply. Data are means of three replicates ± SE. Different and same small letters, in each treatment, indicate significant ($p < 0.05$) and no significant differences ($p > 0.05$), respectively, between the means according to Tukey's test.

Alfalfa Population	EL (%)		MDA (µmol g FW^{-1})		H_2O_2 (µmol g FW^{-1})	
	P-sufficient	*P-deficient*	*P-sufficient*	*P-deficient*	*P-sufficient*	*P-deficient*
DEM	10.22 ± 0.94*a*	22.98 ± 0.97*a*	13.32± 0.56*a*	48.72 ± 2.74*a*	0.45± 0.035*a*	1.19 ± 0.072*a*
TATA	09.19 ± 1.13*a*	21.27 ± 1.67*a*	15.04 ± 1.53*a*	47.45 ± 1.75*a*	0.29 ± 0.042bc	0.99 ± 0.051*b*
RICH	08.47 ± 1.21*a*	23.32 ± 1.73*a*	16.21 ± 0.82*a*	41.28 ± 3.11*b*	0.43 ± 0.028*a*	1.03 ± 0.061*b*
OL	11.33 ± 1.73*a*	18.07 ± 0.69*b*	17.93 ± 1.23*a*	39.11 ± 0.73*bc*	0.33 ± 0.063 bc	0.88 ± 0.073*c*

III.5. Enzymatic antioxidant activities

Low P availability induced significant ($p < 0.001$) increase of the enzymatic antioxidant activities in P–stressed alfalfa plants (Table 5). The highest activities were noted in OL cultivar. The values recorded were 48.16 U min^{-1} mg^{-1} proteins, 26.19 µmol H_2O_2 min^{-1} mg^{-1} proteins and 44.88 µmol H_2O_2 min^{-1} mg^{-1} proteins respectively for SOD, POD and CAT. However, the lowest values were observed in DEM cultivar (32.11 U min^{-1} mg^{-1} proteins, 20.63 µmol H_2O_2 min^{-1} mg^{-1} proteins and 32.19 µmol H_2O_2 min^{-1} mg^{-1} proteins for the mentioned enzymes respectively). Whereas, intermediate values were noted for the remaining alfalfa cultivars.

III.6. Effect on non–enzymatic antioxidant molecules

Starvation of P availability in rooting medium significantly ($p < 0.01$) increased the non–enzymatic antioxidant molecules in alfalfa plants (Table 6). The lowest proline and total polyphenol contents were noted in *DEM* cultivar, 314 µg g^{-1} FW and 57.63 mg GA g^{-1} FW

respectively. However, OL cultivar accumulated the highest contents 534 µg g^{-1} FW and 77.19 mg GA g^{-1} FW in comparison with the other alfalfa cultivars ($p > 0.05$).

Table 5. Superoxide dismutase (SOD), Peroxidase (POD) and Catalase (CAT) specific enzymatic activities in alfalfa plants grown under P-sufficient *versus* P-deficient supply. Data are means of three replicates ± SE. Different and same small letters, in each treatment, indicate significant ($p < 0.05$) and no significant differences ($p > 0.05$), respectively, between the means according to Tukey's test.

Alfalfa	**SOD**		**POD**		**CAT**	
	U min^{-1} mg^{-1} proteins		µmol H_2O_2 min^{-1} mg^{-1} proteins		µmol H_2O_2 min^{-1} mg^{-1} proteins	
Population	*P-sufficient*	*P-deficient*	*P-sufficient*	*P-deficient*	*P-sufficient*	*P-deficient*
DEM	21.08 ± 1.17*a*	32.11 ± 1.17*c*	9.32 ± 1.16*a*	20.63 ± 0.74*d*	14.25 ± 1.03*a*	32.19 ± 2.72*c*
TATA	18.13 ± 1.55*ab*	42.23 ± 1.55*b*	10.04 ± 1.33*a*	22.55 ± 1.75bc	15.08 ± 1.42a	36.99 ± 2.05*b*
RICH	23.32 ± 1.98*a*	39.09 ± 1.98*b*	12.21 ± 1.92*a*	23.21 ± 2.11*b*	14.43 ± 1.28*a*	37.03 ± 3. 61*b*
OL	19.07 ± 1.62*ab*	48.16 ± 1.62*a*	8.93 ± 1.43*a*	26.19 ± 0.73*a*	13.33 ± 1.63*ab*	44.88 ± 1. 73*a*

Table 6. Proline and total polyphenol contents in alfalfa plants grown under P-sufficient *versus* P-deficient supply. Data are means of three replicates ± SE. Different and same small letters, in each treatment, indicate significant ($p < 0.05$) and no significant differences ($p > 0.05$), respectively, between the means according to Tukey's test.

Alfalfa Population	**Proline** µg g^{-1} FW		**Total polyphenols** mg GA g^{-1} FW	
	P-sufficient	*P-deficient*	*P-sufficient*	*P-deficient*
DEM	232 ± 23*b*	314 ± 23*c*	32.24 ± 1.16*b*	57.63 ± 2.54*d*
TATA	245 ± 21*b*	413 ± 15*b*	38.17 ± 1.33*ab*	61.55 ± 2.25*c*
RICH	276 ± 34*ab*	402 ± 21*b*	36.09 ± 1.92*ab*	65.21 ± 3.12*b*
OL	272 ± 64*ab*	534 ± 19*a*	32.11 ± 1.43*b*	77.19 ± 2.18*a*

IV. Discussion

Phosphorus (P) is a very important nutrient required for optimum crop production (Ibrahim et al., 2021). It involved in many physiological and biochemical process governing plant growth and development. The high reactivity of P with some cations, such as iron, aluminum, and calcium, to form insoluble compounds, reduces its mobility in the soil solution. These reactions caused a very low–P availability and low efficiency of phosphate fertilizers used by plants (Farssi et al., 2021). As a consequence, the limitation of the growth plant and development. In fact, we report in the present study that the low-P availability in rooting medium significantly reduced the plant biomass and leaf area. The comportment of the alfalfa cultivars in this study was significantly different. The OL cultivar was found the least affected and the DEM cultivar was the most sensitive one, whereas the two remaining alfalfa cultivars were found to be moderately affected. The effect of low–P availability was documented in many species. Indeed, the reduction in plant tillering and biomass of barley (*Hordeum vulgare* 'Quench') was observed under P deficiency conditions (Carstensen et al., 2018). The same results were noted in leguminous species such as *Vicia faba* L. (Bargaz et al., 2012). The reduction in plant growth under low P availability was associated with the plant P contents. In fact, the lowest P contents were noted in the most sensitive cultivar. In fact, we observed significant and positive correlation between shoot biomass and their P contents ($r = 0.94$) and between root biomass to their P contents ($r = 0.96$). Also, positive correlations were noted for leaf area and the contents of shoots and roots in P, $r = 0.87$ and $r = 0.93$, respectively.

In response to P deficiency, plants have several morphological, physiological, biochemical, and molecular adjustments to improve their P uptake (Plaxton & Tran, 2011; Farssi et al., 2021). In our study, the low P availability induced acid phosphatase activity in alfalfa stressed plants. The activity was more pronounced in the least affected alfalfa cultivar. Significant and negative correlations were noted for the alfalfa plant biomass and phosphatase activity ($r = -0.66$). The induction of phosphatase activity under low–P availability was documented in many species, including *Medicoga sativa* L. In fact, under low soil P supply alfalfa roots released more phosphatases and carboxylates, principally tartrate, into the rhizosphere (He et al., 2020). Similarly, acid phosphatase activity of cell wall in leaves and roots of low-P tolerant stylo (Stylosanthes) mutant, TPRC2001–84, were 46.6% and 53.6% higher than in non–mutant control (RY2) under P deficiency (Liu et al., 2018). The increase of activity in the mutant may contribute to increasing P use efficacy under P stress by cell wall P

scavenging and recycling (Liu et al., 2018). An increase in this activity was reported in *Vicia faba* L. under low–P availability (Makoudi et al., 2018).

P is an essential in ATP, NADPH, nucleic acids, sugar phosphates, and phospholipids. These compounds have a possessive role in cell membrane composition and integrity. Our findings indicate that low–P availability affects the membrane cell integrity and induced oxidative stress, evaluated by the accumulation of EL, MDA and H_2O_2. The lowest contents ($P < 0.05$) were noted in the most tolerant cultivar (OL), whereas the highest contents were noted in the most sensitive cultivar (DEM). The generation of reactive oxygen species (ROS) is considered because of plant exposure to many stresses, including P deficit (Mouradi et al., 2018a; 2018b). In the lack of effective protective mechanism, ROS can seriously damage plants by lipid peroxidation, protein degradation, and breakage of DNA and programmed cell death (Bargaz et al., 2013a). To overcome with the increased ROS level, plants developed enzymatic and non–enzymatic antioxidant process leading to cell detoxification from ROS. The antioxidant systems involve SOD, POD, and CAT. In our study, significant increases in the activities of these enzymes were noted. The tolerance of alfalfa cultivars tested to low–P availability is positively correlated with the induction of antioxidant enzymatic activity. Similar results were reported in *Phaseolus vulgaris* L. (Bargaz et al., 2013a) and in *Brassica napus* L. (Chen et al., 2015). SOD changes superoxide (O_2^-) into H_2O_2, POD and converts H_2O_2 to H_2O and O_2 using electron donors (Kapoor et al., 2019). In the same sense, our findings demonstrated that the P deficiency increase the non–enzymatic antioxidant molecules. The proline and total polyphenol contents were more accumulated in the least sensitive cultivar (OL). Proline actions in plants may resume in chelation of metals, antioxidant ability and/or signaling roles (Hayat et al., 2012). The proline accumulation can activate stress–responsive genes coding for other antioxidant compounds (El Moukhtari et al., 2021). Silva et al. (2018) reported that proline induces also the production of phenolic compounds in transgenic tobacco subjected to water deficit. This finding agrees with our results. In fact, the accumulation of the proline in alfalfa stressed plants was positively correlated with the total polyphenol contents. Previous findings on polyphenol accumulation were noted in other legume species, such as common bean (Bargaz et al., 2013a). This increase suggests a possible role that phenols could play in during P–deficiency. Indeed, the accumulation of phenolic compounds has important antioxidant properties in protecting membranes by neutralizing lipid radicals (Takahama & Oniki, 2000). The statement we noted agrees with previous findings on proline and polyphenols that are

triggered in alfalfa in response to abiotic stress such as salinity (El Moukhtari et al., 2020; 2021).

V. CONCLUSIONS

The current study reports significant variation in some Moroccan alfalfa against low–P availability. The OL cultivar was found to be the least affected cultivar and the DEM was the most sensitive one. However, TATA and RICH displayed a moderate tolerance to P–deficiency conditions. The alfalfa P stress tolerance was linked to the induction of acid phosphatase activity, the enhancement of P solubilization and uptake, the maintaining of cell membrane integrity and the induction of non–enzymatic and enzymatic antioxidant responses against the accumulation of ROS.

3rd CHAPTER

ISOLATION, PHYSIOLOGICAL SCREENING AND MOLECULAR IDENTIFICATION OF RHIZOBACTERIA FROM NODULES AND RHIZOSPHERIC SOILS OF ALFALFA

3rd CHAPTER: ISOLATION, PHYSIOLOGICAL SCREENING AND MOLECULAR IDENTIFICATION OF RHIZOBACTERIA FROM NODULES AND RHIZOSPHERIC SOILS OF ALFALFA

ABSTRACT

The Plant Growth Promoting Rhizobacteria (PGPR) can substantially reduce the chemical inputs in agriculture. Furthermore, the use of indigenous PGPR can be an added advantage since it can easily acclimatize to the natural conditions and enhanced the plant-microbe interactions. The objective of this chapter is the screening, the characterization and selection of effective PGPR for alfalfa growth and production. Hence, twenty PGPR were isolated from nodules and soil rhizospheric of alfalfa in the Beni-Mellal region. The isolates were tested on YEM agar plates for their tolerance to abiotic stresses such salinity, water deficit and pH level. The isolates were also tested for their plant growth-promoting traits, such as phosphate solubilization, indole acetic acid (IAA) and exopolysaccharide production. Results indicated that at 2% NaCl, all isolates tested showed a normal and identical growth. Beyond this concentration, the growth of some isolates decreased with increase of NaCl in the medium. However, 52.63% of the isolates were able to grow at 6% NaCl. Our results suggested that all isolates were not affected by the PEG concentration ranging from 8 to 15%. However, above this concentration range, the growth of some isolates was inhibited, especially the *isolates 11*, *12* and *19*. For pH level, results indicated that 73.68 % of isolates were able to develop under acidic pH conditions (pH=4). However, all isolates did not find difficulties to growth under pH levels ranging from neutral to alkaline pH (7 to 10). For solubilization potential of TCP, the solubilization indexes more than two (>2) were noted with *isolates 01;02;03;04;05;08;09;10;12;14;16 &19*. The results showed that most of rhizobacterial isolates, which presented TCP solubilization halos in NBRIP agar, were able to release high quantities of soluble Pi in NBRIP broth with a significant variation between them. For IAA production, 75% of the tested isolates were assessed to be IAA producers with the highest IAA concentration (1.63 µg mL^{-1}) recorded for the *isolate 05*, followed by the isolate *03* (1.26 µg mL^{-1}), the *isolate 01* (1.19 µg mL^{-1}) and the *isolate 04* (1.16 µg mL^{-1}). Exopolysaccharide synthesis was detected in all tested isolates with different degrees. The most productive isolates being the *isolate 01* (121.09 µg glucose mL^{-1}). The screening of isolates using MALDI-TOF MS followed by genomic identification through housekeeping genes *gyrA, gyrB and rpoD* showed that selected strains represent 99-100% of similarity to one

species. The strains represent 03 bacterial genera: *Sinorhizobium" Ensifer"*, *Pseudomonas* and *Bacillus.* The *Sinorhizobium represents* 50%, followed by *Pseudomonas* (30%) and *Bacillus* (20%).

Keywords: *PGPR; Alfalfa; Abiotic stress, P solubilization, IAA, Exopolysaccharide; MALDI-TOF MS; Housekeeping genes*.

I. Introduction

Legume crops play a key socio-economic role as they are a major component of the traditional human and livestock diets. Their association with rhizobacteria provides the necessary nitrogen for growth and development of the plant and contributes to the improvement of the soil nitrogen balance to the benefit of other crops in intercropping systems or in rotation (Lazali *et al.*, 2021). Consequently, the rhizobacteria symbiosis could, through the cultivation of legumes, allow farmers to save the chemical fertilizer costs and alleviate their environment pollution effects.

The intraspecific differences in the efficiency of symbiotic nitrogen fixation (SNF) under abiotic stress have been demonstrated in several legume species. The SNF poses additional demands of phosphorus (P) with up to 20% of total plant P being allocated to nodules and any P deficiency may influence the symbiosis efficiency (Drevon, 2017). Hence, the exploitation of the genetic diversity existing at the level of endophytic microorganisms or colonizing the rhizospheric environment constitutes a very promising alternative to promote and stimulate the production of legumes under extreme conditions. Indeed, these microorganisms are well known for their application in a wide range of crops to improve growth, crop yield, and added value to plants. These various attributes result from various mechanisms such as nitrogen fixation, quorum detection, signal interference, production of phytohormones, solubilization of phosphate, production of organic compounds, production of siderophores, etc. (Mouradi *et al.*, 2018c). Several Plant Growth-Promoting Rhizobacteria (PGPR) as well as certain species of rhizobacteria produce auxin and gibberellic acid in the rhizospheric soil and thus play an important role in increasing the surface area and the number of roots (Guiñazú *et al.*, 2010). PGPR can improve P status and alter the hormonal balance, resulting in the activation of pathways acquiring tolerance/resistance to various stresses limiting their growth and development (Korir *et al.*, 2017). However, the diversity, function, and applications of microorganisms have received little applied attention up to now. Likewise, the ability to replace chemical fertilizers has potentially increased the demand for PGPR and other microorganisms and made it a major component of managing a sustainable agrosystem. Thus, the research of PGPR strains efficient under drastic conditions of the environment as rhizosphere microorganisms for inoculation of alfalfa (*Medicago sativa* L.) has now become imperative and is the necessary element for efficient symbiosis and for improving alfalfa production in areas affected by these constraints

The present chapter aims to exploit the genetic variability available for the selection of alfalfa-rhizobacteria symbioses more adapted to the climatic conditions of the Mediterranean region and having high biological nitrogen fixation efficiency under low P availability. Through this chapter we study the microbiology and diversity of endophytic and rhizospheric bacteria of alfalfa and their effects on plant growth under low-P availability and the relationships between plant growth and the mobilization of microorganisms will be focused in the next chapters.

II. Materials & Methods

II.1. Isolation and purification of rhizobacteria

The bacteria were isolated directly from nodules or rhizospheric soils collected from alfalfa fields prospected in the Beni- Mellal region (32°22'16.1"N 6°19'31.1"W). This region is characterized by a wide climatic diversity ranging from a humid to a semi-arid climate in the mountains. The prevailing climate is continental with intense cold in winter and very hot summers. Average annual temperatures range from 18.3°C to a maximum of 47°C. The average annual precipitation is 558 mm.

The nodules were detached from the roots, washed with distilled water, disinfected with sodium hypochlorite solution and then rinsed many times with sterile deionized water. The disinfected nodules were separately crushed in presence of 1 ml of sterile deionized water using a sterile glass rod. The homogenate obtained was cultivated on Petri dishes containing Yeast Extract Mannitol Agar (YEM; Annex) supplemented with Congo red (Vincent, 1970). For soil rhizospheric bacteria, 10 g rhizosphere soil was taken in 250 mL Erlenmeyer flask containing 90 mL sterile distilled water and mixed by shaking for 15 min. Serial dilutions were done in sterile distilled water. The dilution was done up to 10^{-2} to 10^{-6}. Aliquots of 0.1 mL (10^{-5} and 10^{-6}) were spread on plates (Lavakush and Verma, 2012). The Petri dishes were incubated at 28 ± 1 °C for three to seven days. The colonies obtained were purified.

II.2. Physiological characterization of the isolates

II.2.1. Tolerance to salinity

The tolerance of each isolate to NaCl was determined by evaluating the development of distinct colonies after three to seven days of growth on YEM medium containing NaCl at 0; 2; 3; 4 and 6% (w/v; Mouradi *et al.*, 2018b). Three replicates per isolate per NaCl concentration were executed.

II.2.2. Tolerance to PEG-6000

The tolerance of isolates to PEG-6000 was determined by evaluating the development of distinct colonies after three to seven days of growth on YEM medium containing PEG- 6000 at 8; 10; 15 and 20% (w/v; Mouradi *et al.*, 2018b). Three replicates per isolate per PEG-6000 concentration were executed.

II.2.3. Tolerance to pH

The isolates were tested for their tolerance to different levels of pH. The evaluation was focused on development of distinct colonies after three to seven days of growth on YEM agar plates of pH adjusted to 4; 7 and 10 (Mouradi *et al.*, 2018c). Three replicates per isolate per pH level were executed.

II.2.4. Inorganic phosphate solubilization

The capacity of the isolated rhizobia to solubilize the tricalcium phosphate $Ca_3(PO_4)_2$ (TCP) was estimated using agar and National Botanical Research Phosphate Growth media (NBRIP; Annex) with $Ca_3(PO_4)_2$ (2.5 g L^{-1}) as P source (Nautiyal, 1999). The TCP solubilization efficiency index on agar medium was expressed as DH/DC ratio, which DH is the diameter of solubilization zone and DC, the diameter of colony. The dissolution average of each sample was calculated as follows: $\sum$ (DH/DC) n^{-1}, which *n* represents rhizobia isolates number. The TCP solubilization of isolates was also tested in flasks containing 100 mL of NBRIP broth containing $Ca_3(PO_4)_2$ (in a proportion of 2.5 g L^{-1}) as P source and with initial pH 7.0. The broth was inoculated with 1 mL of rhizobia suspension and incubated on rotary shaker (150 rpm) for 10 d at 28 ± 1 °C in darkness. Autoclaved and non-inoculated media served as controls. 1 mL aliquots of the broth were taken each day from each flask, and centrifuged at 2500 × g for 15 min. The supernatants were taken, the pH was measured and liberated Pi (P_2O_5) was estimated using acid molybdate method (AFNOR, 1982).

II.2.5. Indole acetic acid (IAA) production

For indole acetic acid (IAA) production, the method of Wahyudi et al. (2011) was adopted. Isolates were grown in Luria-Bertani broth supplemented with 1% L-tryptophan. After five days of incubation at 28 °C, 2 mL of Salkowski reagent containing 0.5 M ferric chloride ($FeCl_3$) and 35% perchloric acid ($HClO_4$) were added to 1 mL of culture supernatant. Then, the mixture was incubated for 30 min in the dark at room temperature. The OD of the pink color was read at 535 nm, and the content of IAA was calculated using a standard curve prepared from known IAA concentrations.

II.2.6. Exopolysaccharide production

Exopolysaccharide production was evaluated in YEM medium using the method of Dueñas et al. (2003). YEM medium was inoculated with 0.1 mL of 10^8 CFU bacterial culture of each isolate and incubated at 28 °C for 3 days. Afterward, samples of the culture of each isolate were centrifuged at 16 000 *x* g for 30 min at 4 °C. The polysaccharide supernatant was then isolated by precipitation with 3 volumes of ethanol, kept overnight at 4 °C and centrifuged at 4500 *x* g for 20 min at 4 °C. The obtained pellets were dispersed in aqueous 80% ethanol and centrifuged again (three times). The final precipitates were dissolved in distilled water. The content of the total neutral sugar was determined as described previously (Dubois et al. 1956) using a standard curve prepared from different glucose concentrations.

II. 3. Screening of isolates using MALDI-TOF Mass Spectrometry

The first step of identification is using MALDI-TOF MS profiles as it has been proven to be a low cost (Figure 12), fast and powerful measurement for identification and grouping of bacterial isolates (Huschek and Witzel, 2019). Therefore, the objective of using this method is reducing the number of isolates for further identification method. The method according to BCCM/LMG laboratory, consist of harvesting 1 µL loopful of the 3rd generation of bacterial cells grown on YMA (Yeast Mannitol Agar) medium (10 g/l of mannitol; 0.5 g/l of K_2HPO_4; 0.5 g/l of Na Glutamate; 1 ml/l of 5% NaCl; 10 ml/l of 1% $MgSO_4.7H_2O$; 1 ml/l of 4% $CaCl_2$, 1 ml/l of 0.4% of $FeCl_3$; 1 g/l Yeast extract and 15 g/l Agar) in 2 ml eppendorf tube containing 300 µl Milli-Q water. After vortexing, 900 µl of absolute Ethanol was added and the mixture was centrifuged (14,000 rpm at 40°C for 3 min). The solution could be used directly for the next step or stored at -20 °C till time of analysis. The next step is getting rid of all the supernatant EtOH by repeated centrifugation and air drying at room temperature. Then, 40 µl of 70% Formic acid was added to the pellet and after vortexing and pipetting up and down, 40 µl of Acetonitrile was added as well. Finally, 1 µl of the supernatant of the centrifuged extract at 14000 rpm for 2 min was spotted on the target plate (Bruker Daltonik, Bremen, Germany) while avoiding to taking up any cell's debris. The bacterial cell extract was spotted in duplicate and on each sample 1 µl of Matrix solution (1% of α-cyano-4-hydroxycinnamic acid in Acetonitrile: water: Trifluoroacetic acid "50:47.5:2.5") was added. In addition, 1 µl of the Matrix solution (as negative control) and 1 µl of Bruker Bacterial Test Standard (BTS) were spotted on the free spot. When the target plate completely dry, it was measured using Bruker Microflex LT/SH Smart, 200 Hz. The identification by Bruker MALDI Biotyper was done according to spectra comparison to the commercial Bruker MBT BDAL library and

LM_UGENT ID library, which result in different score values. When the score is between 2.00 and 3.00 it means high confidence identification, between 1.70 and 1.90 it means low confidence identification but if the score is below 1.69 it means that no organism identification could be possible according to the database. The results of MALDI TOF MS were analyzed using BioNumeric 7.2.6 software and SPeDE (Dumolin et al., 2019) for dendrograms creation and dereplication.

Figure 12: Photo of MALDI-TOF Mass Spectrometry unit used at LMG Lab, Ghent University

II.4. Identification and characterization of studied isolates using housekeeping genes: *gyrA*, *gyrB* & *rpoD*.

The alkaline lyses (AL) method was adopted as it helps for a rapid isolation of genomic DNA from bacterial cells. Besides, the method consisted of harvesting a single colony in 1.5 ml eppendorf tube containing 20 µl alkaline lysis buffer (2.5 ml 10% SDS, 5 ml 1N NaOH and 92.5 ml sterile MQ water). After incubation for 15 min at 95°C and cooling on ice, 180 µl sterile MQ water were added and the mixture was centrifuged for 5 min at 14000 rpm (the extract could be stored at -20°C). The next step of gene sequencing was the amplification using the polymerase chain reaction (PCR; Applied Biosystems Veriti HID 96-Well Thermal Cycler) and using the amplification primers (Table 7). Furthermore, in PCR tubes 22.5 µl Mix (16.875 µl MQ water, 2.5 µl dNTP's, 2.5 µl buffer Qiagen, 0.25 µl pA (10 µM), 0.25 µl pH (10 µM) and 0.125µl Taq Qiagen) were added to 2.5 µl AL of each sample then PCR was processed. PCR products were checked electrophoretically on 1% agarose gel in TBE (Tris-borate-EDTA). Gel

was visualized and captured by “Proxima”. The final step was the cleaning PCR products using NucleoFast 96 PCR cleanup plate and about 100 µl of sterile MQ water. Then, the products were prepared and sent for sequencing on Mix2Seq tube according to Eurofin Genomic sequencing protocol (Eurofin). The sequences were blasted by EZ taxon database of EZBioCloud (www.ezbiocloud.net).

Table 7. Primes used for *gyrA, gyrB and rpoD* genes

Primer	Sequence	PCR cycling	Reference
p-gyrA-F	CAGTCAGGAAATGCGTACGTCCTT	5’95°C, 5x (2’94°C, 2’57°C,1’30’’72°C), 28x(30’’94°C,1’57°C,1’30’’72°C), 5’72°C	Roberts et al., 1994
p-gyrA-R	CAAGGTAATGCTCCAGGC ATTGCT		
gyr B334F	TTCGACCAGAAYTCCYAYAAGG	5’95°C, 5x (2’94°C, 2’57°C,1’30’’72°C), 28x(30’’94°C,1’57°C,1’30’’72°C), 5’72°C	Martens et al., 2007
*gyr*B1043R	AGCTTGTCCTTSGTCTGCG		
rpoD83F	CCTSATCGAGGTTCACAGAAGGC	5’95°C, 3x (2’94°C,2’58,2°C,1’72°C), 30x(30’’94°C,1’58°C,1’72°C), 5’72°C	Martens et al., 2007
rpoD1540R	AGCTGCGAGGAACCGAAG		

III. Results & Discussion

III.1. Tolerance to salinity

The tolerance of the studied isolates to different levels of NaCl is presented in Table 8 and Figure 13. Results indicated the majority of the tested isolates developed an important level of salt tolerance. In fact, at 2% NaCl, all isolates tested showed a normal and identical growth. Beyond this concentration, the growth of some isolates decreased with increase of NaCl in the medium. Indeed, at 3% NaCl concentration, the percentage of survival was 84.21%. However, 52.63% of our isolates were able to grow at 6% NaCl.

Salinity can affect the growth of rhizobacteria by osmotic effect and/or ionic toxicity. Similar results were previously documented in Plant growth-promoting nodule associated by Bouhmouch, et al., 2005 and Mouradi et al., 2018c. The high salt tolerance of some isolates from our collection might be related to physicochemical characteristics of soil in sampling sites where we marked the signs of soil salinization. The fast-growing rhizobacteria can tolerate concentrations higher than 2% NaCl and the limits of tolerance can considerably change from one species to another and even between strains of the same species (Jida and Assefa, 2012).

Table 8. Tolerance of studied isolates to different NaCl and PEG-6000 concentrations and different pH levels. + Growth; - No growth

Isolates	pH			NaCl				PEG_{6000}			
	4	7	10	2%	3%	4%	6%	8%	10%	15%	20%
Isolate 01	+	+	+	+	+	+	+	+	+	+	+
Isolate 02	-	+	+	+	+	+	+	+	+	+	-
Isolate 03	-	+	+	+	+	+	-	+	+	+	+
Isolate 04	+	+	+	+	+	+	+	+	+	+	+
Isolate 05	+	+	+	+	+	+	+	+	+	+	+
Isolate 06	+	+	+	+	+	+	-	+	+	+	+
Isolate 07	+	+	+	+	+	-	-	+	+	+	+
Isolate 08	+	+	+	+	+	+	+	+	+	+	+
Isolate 09	-	+	+	+	-	-	-	+	+	+	+
Isolate 10	+	+	+	+	+	+	+	+	+	+	+
Isolate 11	+	+	+	+	+	+	+	+	+	+	-
Isolate 12	+	+	+	+	+	-	-	+	+	+	-
Isolate 13	+	+	+	+	+	+	+	+	+	+	+
Isolate 14	+	+	+	+	+	+	+	+	+	+	+
Isolate 15	+	+	+	+	+	+	+	+	+	+	+
Isolate 16	+	+	+	+	+	+	-	+	+	+	+
Isolate 17	-	+	+	+	-	-	-	+	+	+	+
Isolate 18	-	+	+	+	+	+	-	+	+	+	+
Isolate 19	+	+	+	+	+	+	+	+	+	+	-

Similar results were also reported with strains of *Mesorhizobium and Ensifer* nodulating chickpea and faba bean in Morocco (Maâtallah et al., 2002; Mouradi et al., 2018c). The effect of salinity on growth and plant growth promoting activity of *Pseudomonads* species was documented by Deshwal and Kumar (2013). They observed that all isoaltes showed normal growth on medium containing 0.25 to 1.25% NaCl as compared to control medium. However, their results suggested that that above 1.75% NaCl concentration in medium, the survival number of *Pseudomonas* species studied was gradually reduced. Tilak et al. (2005) reported that some rhizobia isolated from woody legumes (Acacia, Prosopis) can tolerate NaCl

concentrations ranging between 3 to 5%. Moreover, some strains of *Sinorhizobium meliloti* isolated from alfalfa nodules in southern Morocco were able to grow in the presence of 10% NaCl (Thami-Alami et al., 2010). The high salt tolerance of some rhizobial strains was associated to their ability to limit adverse effects caused by accumulation of protective organic osmolytes such as amino acids (proline, betaine and glutamate) or carbohydrates (trehalose, sucrose) in order to maintain the cell turgor (Vriezen et al., 2007).

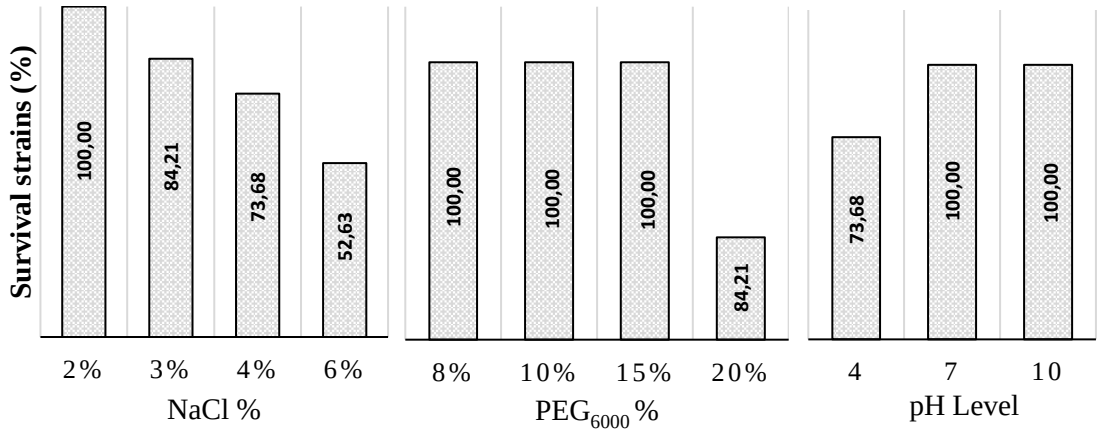


Figure 13. Survival percentages of the isolates studied under different NaCl and PEG 6000 concentrations and different pH levels.

III.2. Tolerance studied isoaltes to PEG-6000 concentrations

Table 8 and Figure 13 illustrated the responses of our isolates to different concentrations of PEG_{6000}. Our results suggested that all isolates did not affect by the concentration of PEG ranging from 8 to 15%. In fact, all isolates showed a 100% of growth under these concentrations. However, above this concentration range, the growth of some isolates was inhibited, especially the *isolates 11*, *12* and *19*. Similar results on the effect of PEG on growth of rhizobia have been reported by Elboutahiri et al. (2010) and Mouradi et al. (2018c). The tolerance of rhizobacteria to osmotic stress is directly related to their ability to accumulate solutes and to changes in the cell morphology (Thami-Alami et al., 2010). Other studies have shown that gram-negative bacteria, such as *Rhizobium*, synthesize lipopolysaccharides in their wall which provide a protective role against desiccation (Garmiri and Coles, 2008). In the PGPR A*zospirillum sp*, Garcia et *al.* (2017) showed that different native strains of this genus were able to grow on the medium supplemented with different concentrations of sorbitol as osmotic agent. Concentrations between 0.2 and 0.8 M did not produce significant effects in growth strains,

while 1 M sorbitol induced a negative effect on growth starting at 6 h of incubation when compared to the control treatment without the osmotic agent.

III.3. Tolerance of isolates to pH levels

The Effect of pH level on the growth of the studied isolates is mentioned is Table 8 and Figure 13. Results indicated that 73.68 % of isolates were able to develop under acidic pH conditions (pH=4). However, all isolates did not find difficulties to growth under pH levels ranging from neutral to alkaline pH (7 to 10). Mouradi et al., (2018c) noted the rhizobacteria can grow at pH values between 5.5 and 8.5 with an optimum growth at pH around 7. Tolerance of some isolates to pH levels ranging from 4 to 10 was noted by Ali *et al.* (2009) and Harun et *al*. (2009). Similarly, Indrasumunar et *al*., (2012), documented the growth of *Bradyrhizobium japonicum* strains at pH of 3.8. In addition, several studies have shown that rhizobacteria are characterized by a large difference in pH tolerance. An alkalinity tolerance of pH 10 developed in some strains of *Rhizobium* (Rashid et al., 2012). The physiological and biochemical mechanisms of rhizobacteria adaptation to acidic conditions are numerous. These mechanisms include exclusion and expulsion of protons H^+ (Kurchakn et al., 2001), accumulation of polyamines, high content of potassium and glutamate in cytoplasm and change in the composition of lipopolysaccharides (Vriezen et al., 2007). In the other side, the inhibitor effect of alkaline pH on growth of bacteria can occur by ionization causing their slow transition through the cytoplasmic membrane (Tuncan and Martin, 1985). Alkaline soil pH could also cause unavailability of some minerals, such as iron and manganese (Bordeleau and Prévost, 1994).

III.4. Solubilization of inorganic phosphate

The solubilization potential TCP by our rhizobacteria isolates was tested in both solid and broth media (Figure 14 and Table 9). On solid medium, the potential of solubilizing tricalcium phosphate was manifested by the growth of colonies and the presence of a holozone around them (Figure 15). The variation of diameter of the holozone gives a preliminary idea about the variability in the inorganic phosphate solubilization between the isolates tested. Comparison among different strains showed that some strains have a high solubilization potential of tricalcium phosphate compared to others. Indeed, the solubilization indexes more than two (>2) were noted with *isolates 01; 02; 03; 04; 05; 08; 10; 11; 12; 14; 16 & 19*. The results showed that most of rhizobacterial isolates, which presented TCP solubilization halos in NBRIP agar, were able to release high quantities of soluble Pi in NBRIP broth with a significant variation

between them, especially *isolates 01* and *05*. With released Pi increasing in the medium, we observed pH decreasing in almost all of the tested isolates with a significant difference between them (table 9).

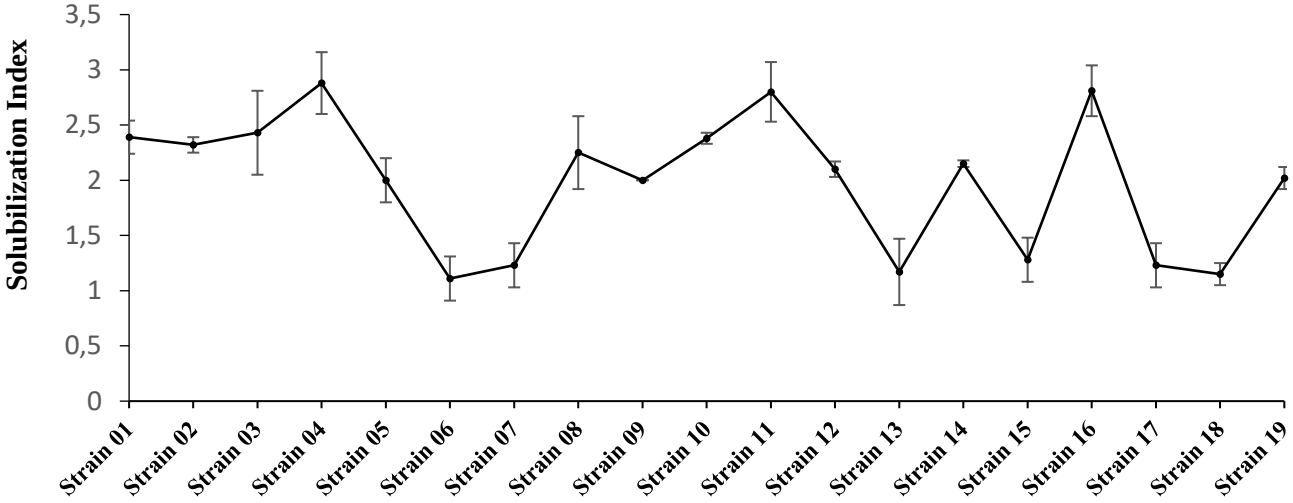


Figure 14. Solubilization indexes of inorganic phosphate (Tricalcium phosphate) by the studied isolates on NBRIP agar.

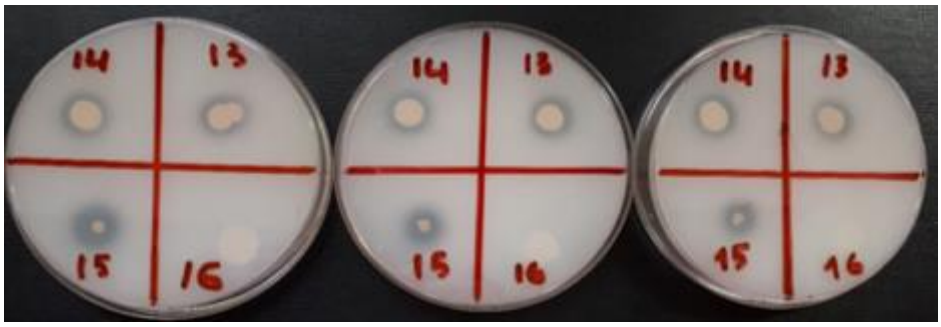


Figure 15: The potential of solubilizing tricalcium phosphate by the isolates 13, 14, 15 & 16. The solubilization was manifested by the growth of colonies and the presence of a holo around them.

The inorganic phosphate solubilization of our isolates was comparable to that previously documented (Marra et *al*., 2011; Mouradi et al., 2018c). In general, plant growth-promoting nodule associated and rhizospheric are often more efficient in the solubilization of inorganic phosphate through their ability to produce acids involved in this mechanism (Marra et *al*., 2011). Indexes of solubilization around 1.41 were found with *Mesorhizobium ciceri*, *Mesorhizobium mediterraneum* and *Sinorhizobium meliloti* (Alikhani et al., 2006). Other studies have shown that P-solubilizing microorganisms are capable of producing acid phosphatases in the medium (Ponmurugan and Gopi, 2006), which are enzymes that mobilize

attached phosphoryl groups from other organic and mineral molecules in acidic environment. The correlation between the decrease of pH and the release of orthophosphates in the medium in all of the tested rhizobia isolates has been demonstrated by several authors such as Sridevi and Mallaiah (2009) while Ben Farhat et al. (2009) reported that the pH decrease was not correlated with the release of soluble P. Indeed, it has been demonstrated that the medium pH decrease is due to the release of organic acids and protons by the bacterial cells (Trivedi and Sa, 2008).

Table 9. Released Pi of isolates and pH of NBRIP broth contained TCP as sole source of P after 96H of incubation.

Isolate Number	**Pi (mg mL^{-1})**	**pH**
Isolate 01	33.23±2.30	5.34± 0.21
Isolate 02	17.13±1.24	6.71±0.27
Isolate 03	18.82±2.14	6.32±0.33
Isolate 04	21.17±1.26	6.24±0.89
Isolate 05	41.63±1.99	4.96±0.47
Isolate 06	10.43±1.07	6.75±0.18
Isolate 07	12.15±1.19	6.76±0.19
Isolate 08	18.34±1.55	6.13±0.20
Isolate 09	23.24±2.56	6.02±0.36
Isolate 10	15.27±1.55	6.48±0.31
Isolate 11	19.69±1.49	6.14±0.32
Isolate 12	26.11±2.13	6.27±0.17
Isolate 13	12.66±1.23	6.82±0.23
Isolate 14	15.25±1.67	6.55±0.29
Isolate 15	12.22±1.90	6.78±0.29
Isolate 16	24.34±1.96	6.17±0.37
Isolate 17	12.10±1.07	6.78±0.26
Isolate 18	13.60±2.08	6.74±0.32
Isolate 19	21.19±1.18	6.26±0.28

III.5. Indole-3-acetic acid (IAA) and exopolysacharid productions

For IAA production (Table 10), 75% of the tested isolates were assessed to be IAA producers with the highest IAA concentration (1.63 µg mL^{-1}) recorded for the *isolate 05*, followed by the isolate *03* (1.26 µg mL^{-1}), the *isolate 01* (1.19 µg mL^{-1}) and the *isolate 04* (1.16 µg mL^{-1}). Exopolysaccharide synthesis was detected in all tested isolates with different degrees (Table 10). The most productive isolates being the *isolate 01* (121. 09 µg glucose mL^{-1}).

Table 10. Indole-3-acetic acid (IAA) and exopolysaccharide production of the isolates

Isolate	**IAA** (µg mL^{-1})	**Exopolysaccharide** (µg glucose mL^{-1})
Isolate 01	1.19±0.13	121.09±5.97
Isolate 02	1.03±0.09	48.91±09.22
Isolate 03	1.26±0.11	89.32±06.36
Isolate 04	1.16±0.07	113.54±12.09
Isolate 05	1.63±0.09	89.45±11.21
Isolate 06	0.81±0.06	66.12±6.01
Isolate 07	1.11±0.10	109.56±18.94
Isolate 08	0.00±0.00	74.33±11.78
Isolate 09	0.00±0.00	91.82±16.56
Isolate 10	0.25±0.05	106.28±9.33
Isolate 11	0.39±0.09	97.84±5.44
Isolate 12	0.81±0.10	112.27±10.07
Isolate 13	0.56±0.02	93.02±10.17
Isolate 14	0.15±0.07	109.45±11.09
Isolate 15	0.00±0.00	111.78±12.39
Isolate 16	0.54±0.06	105.47±6.27
Isolate 17	0.00±0.00	109.48±13.06
Isolate 18	0.00±0.00	117.21±14.18
Isolate 19	1.09±0.08	88.49±5.24

III.6. Rhizobacteria identification

Identification of microbes by MALDI-TOF MS is done by either comparing the characteristic spectrum called peptide mass fingerprint “PMF” of unknown organism with the PMFs contained in the database, or by matching the masses of biomarkers of unknown organism with the proteome database (Singhal et al., 2015; Tahon, 2017). In PMF matching, the MS spectrum of unknown microbial isolates is compared with the MS spectra of known microbial isolates contained in the database. Hence, the 20 isolates were clustered using MALDI-TOF MS profiles (Figure 16).

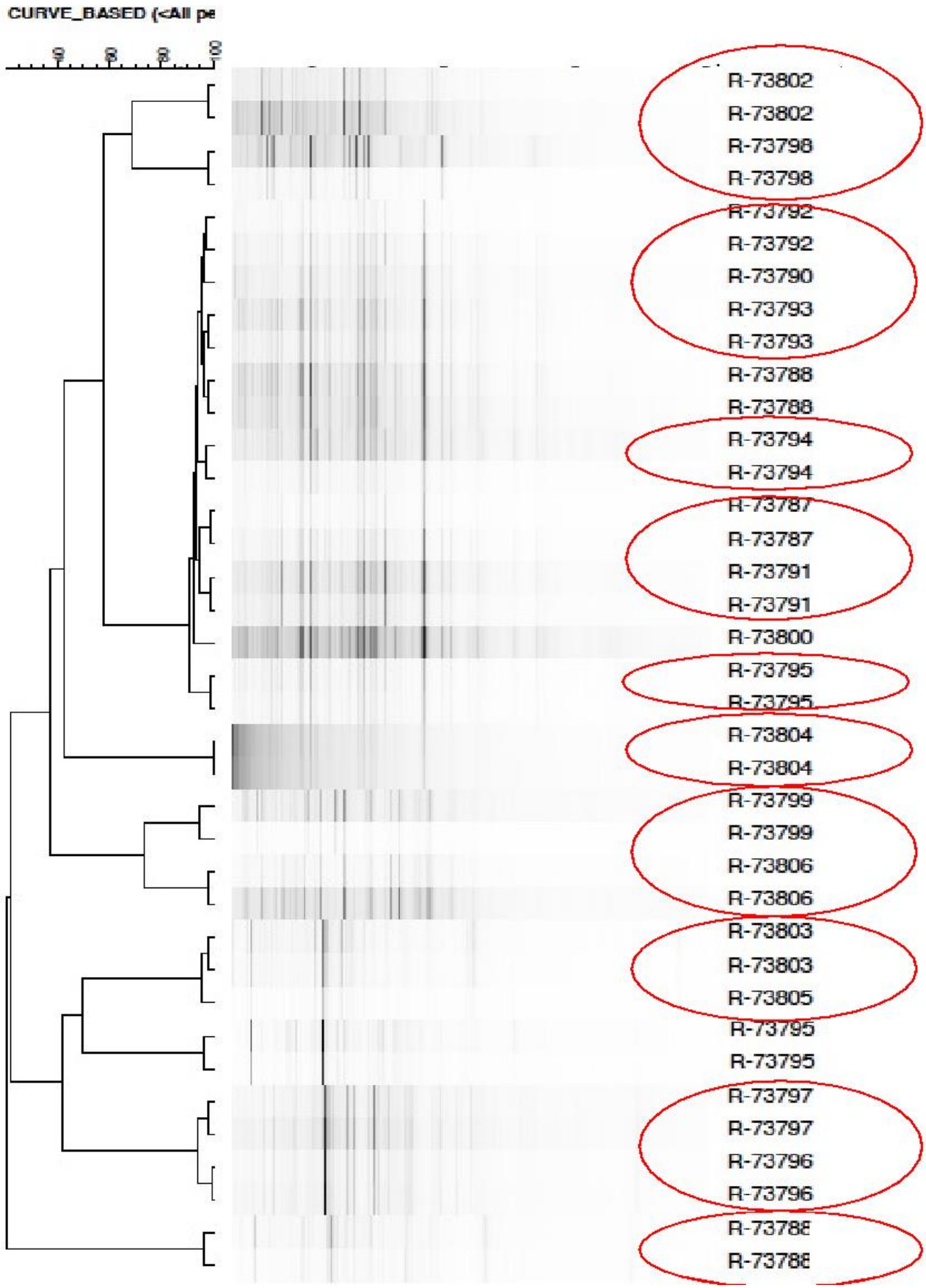


Figure 16. Curve-based cluster analysis of mass spectra obtained from protein extracts using the Pearson product moment correlation coefficient and UPGMA cluster algorithm of isolates from nodules and soil rhizospheric of alfalfa (*Medicago sativa* L.).

Moreover, 10 strains were selected as a representative of the varied spectral profiles of the clusters for further identification through housekeeping genes *gyrA, gyrB and rpoD* (Table 11). The strains showed a 99-100% of similarity to one species. Our strains represented 03 bacterial genera: *Sinorhizobium"Ensider"*, *Pseudomonas* and *Bacillus.* The *Sinorhizobiumrepresents* 50%, followed by *Pseudomonas* (30%) and *Bacillus* (20%).

Table 11. Taxonomic affiliation based on *gyr A* and *gyr B and rpoD* gene sequence of endophyte and rhizospheric rhizobacterial isolates of alfalfa

Strain Ref	Closest relative species	Gene	Similarity (%)	Accession number
Strain 01	*Sinorhizobium meliloti Rm41*	***gyrB***	100%	CP021808.1
Strain 02	*Sinorhizobium meliloti LLAN24*	***gyrB***	99%	LT614654.1
Strain 03	*Sinorhizobium meliloti B399*	***gyrB***	100%	CP019488.1
Strain 04	*Sinorhizobium meliloti B401*	***gyrB***	100%	CP019485.1
Strain 05	*Pseudomonas alkylphenolica strain PF9*	***rpoD***	100%	KY950274.1
Strain 06	*Bacillus subtilis subsp. subtilis strain M1*	***gyrA***	100%	HQ828990.1
Strain 07	*Bacillus subtilis strain SX01705*	***gyrA***	99%	CP022287.1
Strain 08	*Pseudomonas sp. Irchel s3a16*	***rpoD***	100%	LT897995.1
Strain 09	*Sinorhizobium meliloti HM006*	***gyrB***	100%	CP021829.1

IV. Conclusion

This physiological characterization of tested rhizobacterial strains has determined the extent of physiological variations that exist in the behavior of our PGPR collection. The results of these tests have allowed us to identify some strains with high plant growth promoting traits, particularly under the stressed conditions. They will serve as biofertilizers-biostimulants to test *in planta* their beneficial potentials under symbiotic interaction with the Moroccan alfalfa cultivars to low P availability. Hence, we would enable to select the symbiotic combinations adapted to low-P conditions as one of the most abiotic constraints characterizing the Mediterranean agricultural soils regions.

4th CHAPTER: SYNERGISTIC EFFECT OF *PSEUDOMONAS alkylphenolica* PF9 AND *Sinorhizobium meliloti* RM41 ON MOROCCAN ALFALFA cultivars GROWN UNDER LIMITED PHOSPHORUS AVAILABILITY

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

ABSTRACT

This study looked at the synergistic effect of *Pseudomonas alkylphenolica* PF9 and *Sinorhizobium meliloti* Rm41 on the Moroccan alfalfa cultivar (*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 S*inorhizobium melilot*i 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 µmol P. $plant^{-1}$. $week^{-1}$. 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 (*Fv/Fm*) 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.

Keywords: *Alfalfa; Sinorhizobium meliloti; Pseudomonas alkylphenolica; Co-inoculation; Growth; Phosphorus.*

I. 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 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 then 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 (Keneni 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 nitrogenase activity, and macronutrient contents in white clover plants under low P conditions. In the same sense, the intraspecific variations 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 cultivar (*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.

II. Materials and Methods

II.1. Plant material and growth conditions

The plant material was the subject of this study consists of the Moroccan alfalfa (*Medicago sativa* L.) cultivar *Oued Lmaleh* (OL). Seeds were supplied by National Institute for Agronomic Research (INRA, Marrakech, Morocco). Local cultivars 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* cultivar 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 NBRIP 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 µmol P. $plant^{-1}$. $week^{-1}$ (Bargaz et al., 2013; Neila et al., 2014). Urea was applied at 2 mmol. $plant^{-1}$ 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.

Figure 17. Illustration of the growth chamber trial

II.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 48h. 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.

II.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 (10N) 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 minutes. A standard curve was established with KH_2PO_4 solutions.

II.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:

$$RWC = [(FW - DW) / (TW - DW)] \times 100$$

II.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 $\varepsilon = 155\ mM^{-1} . cm^{-1}$.

II.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:

$$EL\ (\%) = (L\ t\ /L\ 0) \times 100$$

II.7. Total 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 × g and the absorbance were read at 480, 645 and 663 nm using a UV-VIS absorption spectrophotometry (DLAB, SP-

UV1000, China). The concentration of Chlorophyll a, Chlorophyll b, total chlorophyll was calculated following below formulas:

Chl a (mg. g^{-1} FW) = [(12.7 × A663) - (2.6 × A645)] × (V / 1000 × Wt)

Chl b (mg. g^{-1} FW) = [(22.9 × A645) - (4.68 × A663)] × (V / 1000 × Wt)

Total chlorophyll (mg. g^{-1} FW) = [(20.2 × A645) + (8.02 × A663)] × (V / 1000 × Wt)

II.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.

II.9. Stomatal conductance (gs)

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 ± 5%. It was expressed in mmol de H_2O m^{-2} . s^{-1}

II.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.

III. Results

III.1. Effect on plant biomass

The effect of *Pseudomonas alkylphenolica* PF9 and/or *Sinorhizobium meliloti* Rm41 on plant biomass of Moroccan alfalfa cultivar studied under soluble (KH_2PO_4) or insoluble (Ca_3HPO_4) P forms is indicated in Figure 17. 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 (Figure 18), 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 mg. $plant^{-1}$, against 82.5 and 88.9 mg . $plant^{-1}$ 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 mg. $plant^{-1}$ for shoots and roots respectively. Nevertheless, the quantities of 40.7 and 37.6 mg. $plant^{-1}$ 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).

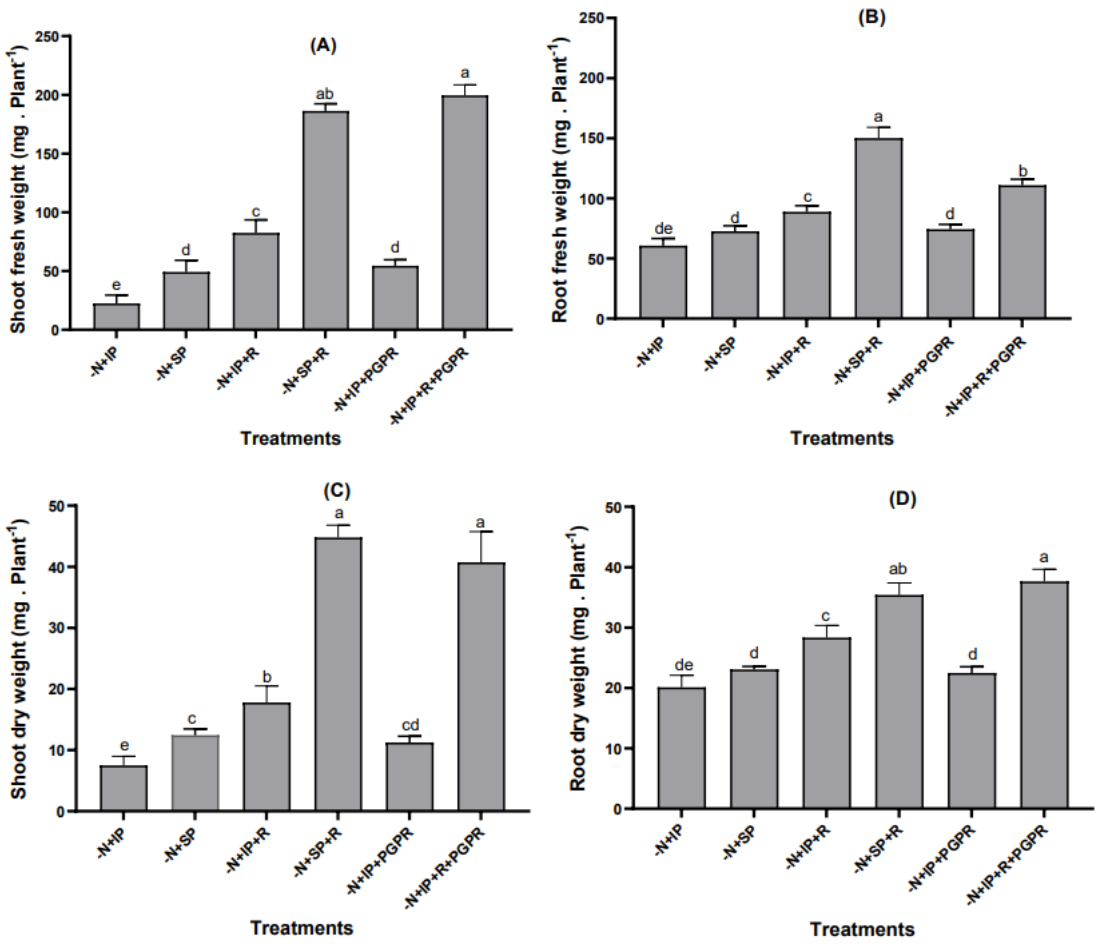


Figure 18. Effect of inoculation with *Sinorhizobium meliloti* Rm41 (R) alone or combined with *Pseudomonas alkylphenolica* PF9 (R+PGPR) on fresh and dry biomass of Moroccan alfalfa cultivar *Oued Lmaleh* (*OL*) under insoluble (IP) or soluble (SP) P form. **(A)** Shoot fresh weight, **(B)** Root fresh 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.

III.2. Effect on plant height and leaf area

Figure 18 indicated the effect of the inoculation with *Sinorhizobium meliloti* Rm41 alone or combined with *Pseudomonas alkylphenolica* PF9 on plant heights (Figure 3A) and leaf area (Figure 3B) of alfalfa cultivar 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 the 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 *Sinorhzobium* strain alone. For the leaf area (Figure 2B), the highest values ($p < 0.01$) under insoluble P conditions were observed in plants co-inoculated with both bacterial inoculums (1.90 cm^2) in comparison to P-stressed plants inoculated with rhizobial strain alone (1.64 cm^2).

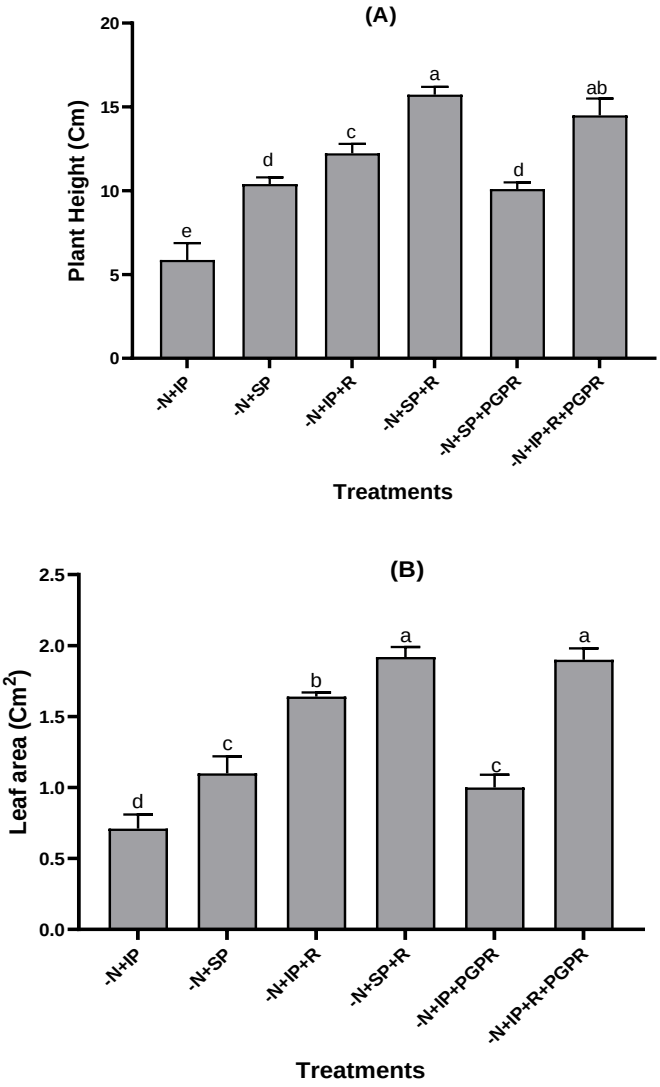


Figure 19. Effect of inoculation with *Sinorhizobium meliloti* Rm41 (R) alone or combined with *Pseudomonas alkylphenolica* PF9 (R+PGPR) on plant heights **(A)** and leaf area **(B)** of Moroccan alfalfa cultivar *Oued Lmaleh* (*OL*) under insoluble (IP) or soluble (SP) P forms. Values are means of three 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.

III.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 Figure 20. 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 mg. g DW^{-1} in shoots (Figure 3A) and roots (Figure 3B). However, the amounts of 346.45 and 440.58 mg. 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.

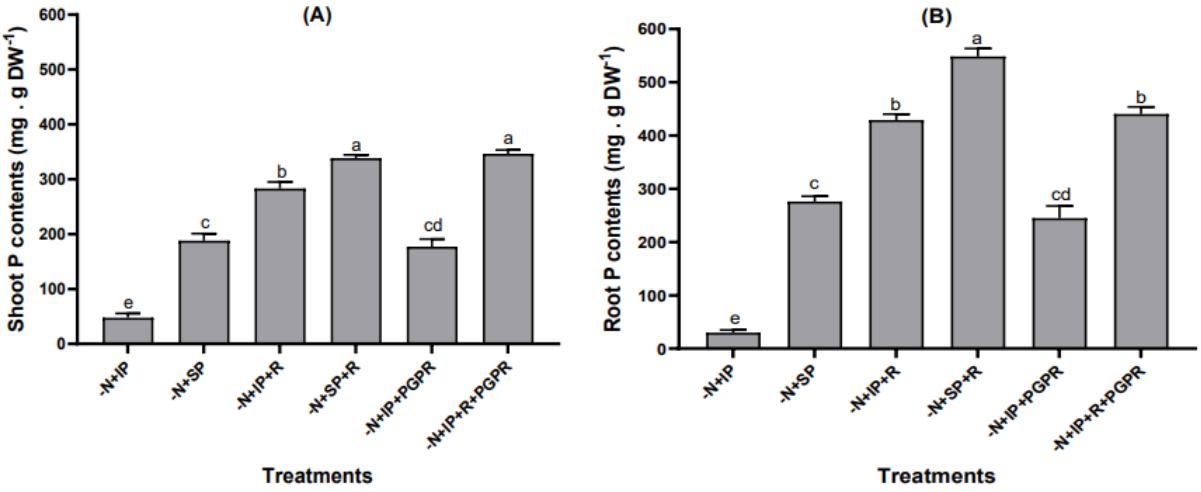


Figure 20. Effect of inoculation with *Sinorhizobium meliloti* Rm41 (R) alone or combined with *Pseudomonas alkylphenolica* PF9 (R+PGPR) on shoot **(A)** and root **(B)** P contents of Moroccan alfalfa cultivar *Oued Lmaleh* (*OL*) under insoluble (IP) or soluble (SP) P forms. Values are means of three replicates. Different and same small letters above the 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.

III.4. Relative water content (RWC)

Our results (Figure 21) 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.

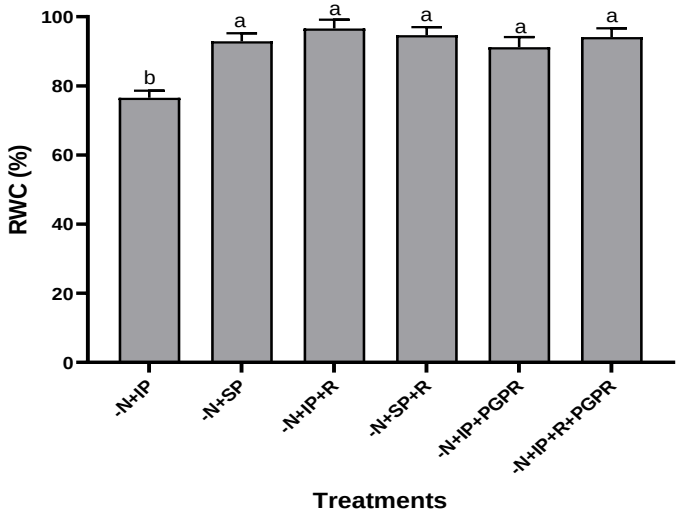


Figure 21. Effect of inoculation with *Sinorhizobium meliloti* Rm41 (R) alone or combined with *Pseudomonas alkylphenolica* PF9 (R+PGPR) on RWC of Moroccan alfalfa cultivar *Oued Lmaleh* (*OL*) under insoluble (IP) or soluble (SP) P forms. Values are means of three replicates. Different and same small letters above the 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.

III.5. Effect on EL and MDA contents

The EL contents were found increased ($p < 0.001$) in uninoculated and P-stressed alfalfa plants (Table 12). However, the inoculations 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 12). Nevertheless, the inoculation of alfalfa plants with *Sinorhizobium* strain alone significantly reduced this accumulation under the same

P conditions (35.48 µmol. g FW^{-1}). However, the presence of both strains in the rooting medium remarkably decreased the MDA accumulation to 31.88 µmol. g FW^{-1} with no significant difference ($p > 0.05$) in comparison to alfalfa plants inoculated with rhizobial strain and supplied with the soluble P form.

Table 12. 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 cultivar *Oued Lmaleh* (OL) under insoluble (IP) or soluble (SP) P forms. Values are means of three replicates ± 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 ± 0.73 *a*	20.11 ± 0.36 *b*	16.64 ± 1.12 *c*	17.98 ± 0.41 *c*	21.13 ± 1.12 *b*	11.58 ± 0.41 *d*
MDA µmol . g FW^{-1}	51.88 ± 2.24 *a*	45.69 ± 1.49 *b*	29.29 ± 1.46 *e*	35.48 ± 0.09 *d*	43.84 ± 2.27 *bc*	31.88 ± 1.70 *e*

III.6. Effect on photosynthetic-related parameters

III.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 (Figure 22). 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 (Figure 23), 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.

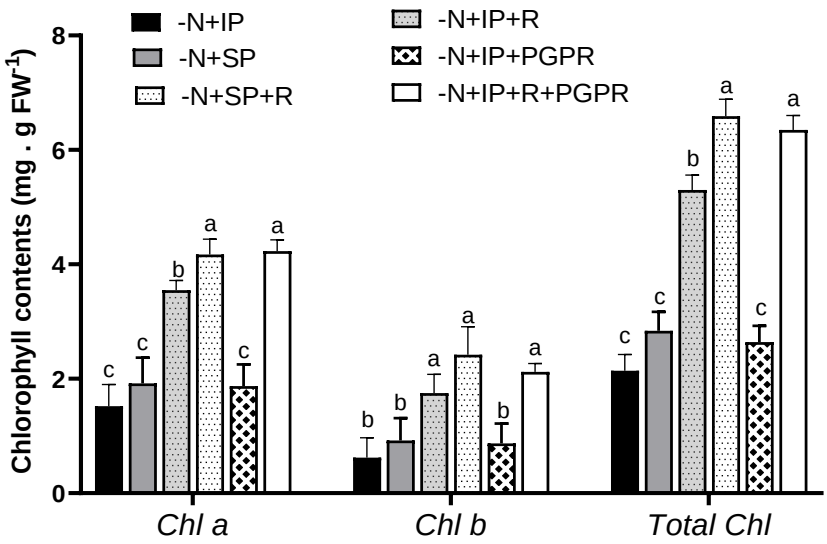


Figure 22. Effect of inoculation with *Sinorhizobium meliloti* Rm41 (R) alone or combined with *Pseudomonas alkylphenolica* PF9 (R+PGPR) on Chl *a*, Chl *b* and total Chl contents of Moroccan alfalfa cultivar *Oued Lmaleh* (*OL*) under insoluble (IP) or soluble (SP) P forms. Values are means of three 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.

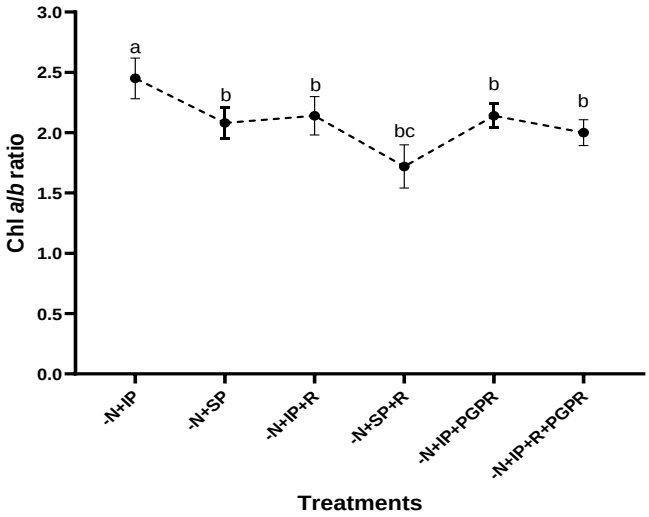


Figure 23. Effect of inoculation with *Sinorhizobium meliloti* Rm41 (R) alone or combined with *Pseudomonas alkylphenolica* PF9 (R+PGPR) on Chl *a*/*b* ratio of Moroccan alfalfa cultivar *Oued Lmaleh* (*OL*) under insoluble (IP) or soluble (SP) P forms. Values are means of three replicates. Different 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

III.6.2. Effect on the maximum quantum yield of PS II (Fv/Fm)

Results indicated that *Sinorhizobium* strain alone or its combination with the *Pseudomonas* one pronouncedly increased ($p < 0.001$) the *Fv*/*Fm* ratio under low P availability, with no significant differences between them (Figure 24). Indeed, in the presence of the rhizobial inoculum only, the *Fv*/*Fm* 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.

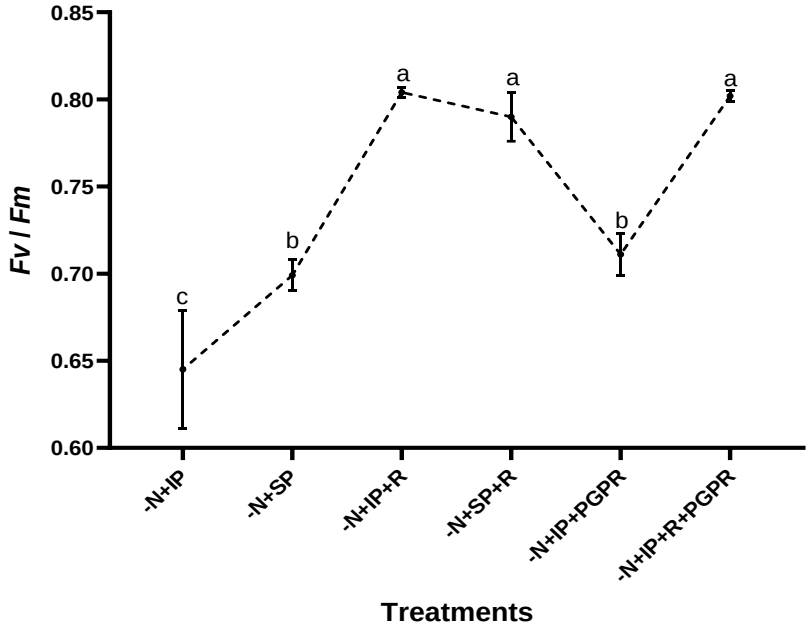


Figure 24. Effect of inoculation with *Sinorhizobium meliloti* Rm41 (R) alone or combined with *Pseudomonas alkylphenolica* PF9 (R+PGPR) on maximum quantum yield of PS II (*Fv*/*Fm*) of Moroccan alfalfa cultivar *Oued Lmaleh* (*OL*) under insoluble (IP) or soluble (SP) P forms. Values are means of three 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.

III.6.3. Effect on stomatal conductance (gs)

The results obtained for the g_s (Figure 25) 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 mmol

H_2O. m^{-2} . s^{-1}. This enhancement was more obvious when the inoculum was constituted of both rhizobacterial strains (45.84 mmol H_2O. m^{-2}. s^{-1}).

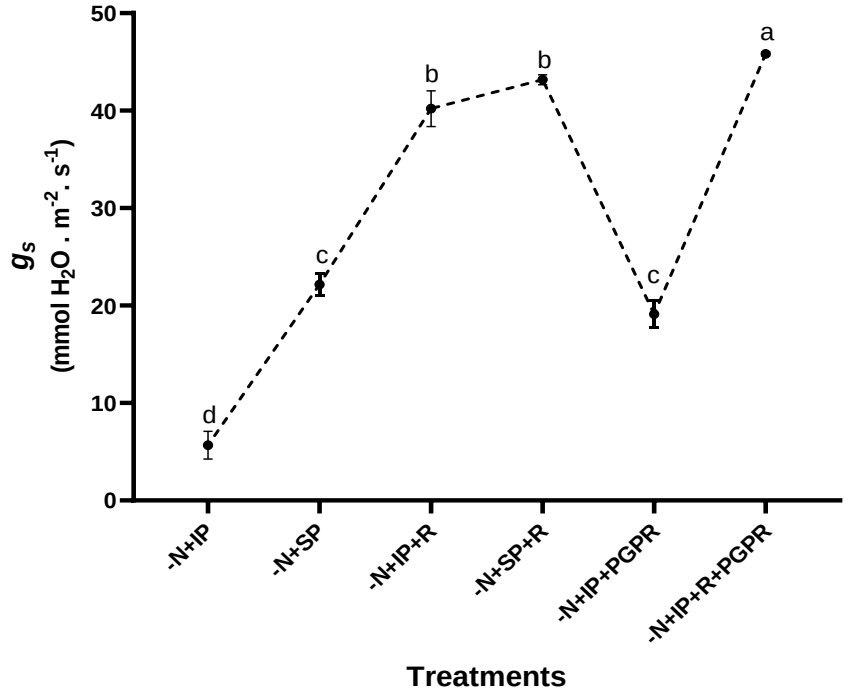


Figure 25. Effect of inoculation with *Sinorhizobium meliloti* Rm41 (R) alone or combined with *Pseudomonas alkylphenolica* PF9 (R+PGPR) on g_s of Moroccan alfalfa cultivar *Oued Lmaleh* (*OL*) under insoluble (IP) or soluble (SP) P forms. Values are means of three replicates. Different and same small 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.

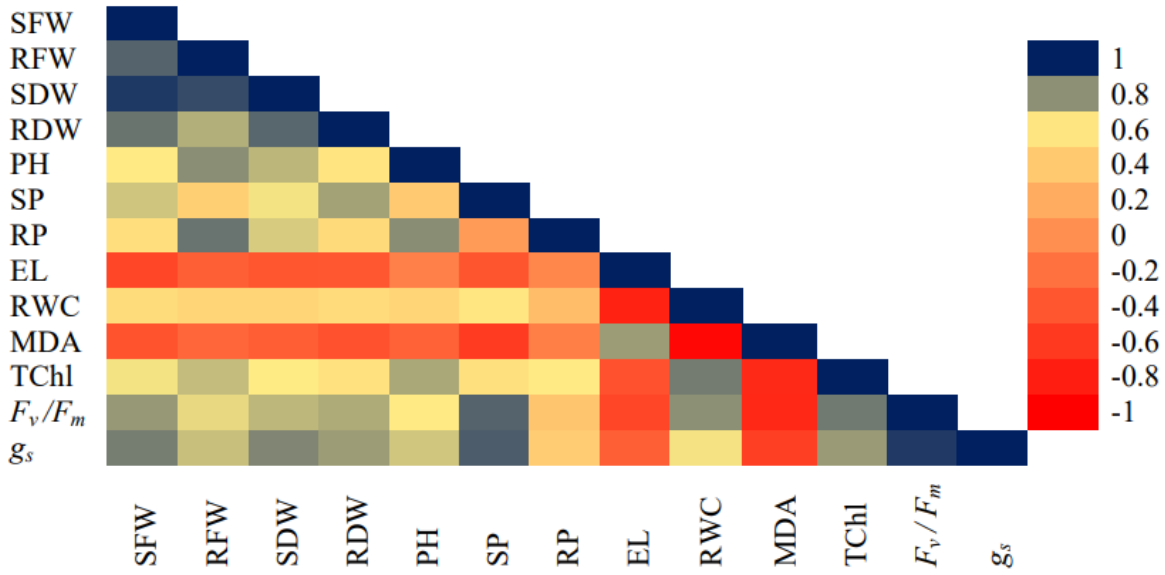


Figure 26. Pearson's correlation matrix between assessed parameters of alfalfa (*Medicago sativa* L.) cultivar (OL) grown under sufficient or limited phosphorus conditions. Correlations are displayed in blue (positive) and in red (negative); color intensity is proportional to correlation coefficient. SFW: Shoot Fresh Weight, RFW: Root Fresh Weight, SDW: Shoot Dry Weight, RDW: Root Dry Weight, PH: plant 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.

Pearson's correlation analysis significant correlations between plant growth traits and physiological and biochemical traits analyzed :

Pearson's correlations matrix among parameters studied, positive and negative correlations were recorded in our results. A significant positive correlations between assessed plant growth with photosynthesis-related parameters and plant P contents.

However, A significant negative correlations between plant growth traits and photosynthesis-related parameters with electrolyte leakage and oxidative makers.

Results showed a positive strong correlation between MDA content and electrolyte leakage. Positive correlations were also found among other physiological (chlorophyll a, b and total chlorophyll) and plant growth traits (shoot and root, fresh and dry weight and leaf area) parameters, while MDA and electrolyte leakage negatively correlated with these parameters. EL% correlates significantly and negatively with Chl contents.

IV. 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 cultivar 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 (Figure 26), 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 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 on 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 (Figure 25). 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 (Figure 26). 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* content 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 the greater instability of pigment protein complexes (Singh and Dubey, 1995).

The reduction in the photochemical efficiency of PSII *(Fv/Fm)* 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 *Fv/Fm* and Total Chl contents (Figure 26). 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 *Fv/Fm* under metallic stress. In barley, *Hordeum vulgare* L., all of the processes in the photosynthetic machinery including the PSII quantum yield were 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 *Fv/Fm* 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-Gámez et al., 2016).

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 (Figure 25). 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* Rm41and *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 CO2 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.

V. 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.

5th CHAPTER: SEED BIOPRIMING WITH PHOSPHATE SOLUBILIZING RHIZBACTERIA ENHANCES ALFALFA (*Medicagao sativa* L.) TOLERANCE TO LOW PHOSPHORUS AVAILABILITY UNDER FIELD CONDITIONS

SEED BIOPRIMING WITH PHOSPHATE SOLUBILIZING RHIZBACTERIA ENHANCES FORAGE YIELD AND QUALITY OF ALFALFA (*Medicagao sativa* L.) IN FIELD CONDITIONS UNDER LOW PHOSPHORUS AVAILABILITY

ABSTRACT

This study aims at evaluating the effect seed biopriming with two phosphate solubilizing rhizbacteria (PSB), *Sinorhizobium meliloti* Rm41 and *Pseudomonas alkylphenolica* PF9, on forage yield and quality of alfalfa (*Medicagao sativa* L.) under low phosphorus (P) availability in field conditions. The field is located in the M'goun valley about 5 km from Kelaa M'gouna. The site has an altitude of about 1 425 m above sea level. The experiment was conducted during the year 2021 on a loamy clay soil with annual average temperature of 17.3 °C and total annual precipitation of 161.9 mm, with maximum rainfall in the period between November and February. The evaluation was done during April to July with two cuts per year. The results indicated that the low P-availability has negative impacts on forage yield and quality. However, the rhizobacterial treatment improved the plant height and forge dry biomass ($p < 0.05$). For leaf to stem ratio, the values recorded varied from 0.61 to 0.82. A significant effect of P fertilizer and rhizobacterial treatment was noted ($p < 0.05$). The highest values were noted when the plants are fertilized and inoculated. However, the rhizobacterial treatment enhanced the ratio in comparison with stressed and non-inoculated conditions. The improvement percentages were 13.11 % during the first cutting and 43.13% at the fourth cuttings. For the crude proteins, the contents obtained varied from 128.94 to 187.06 $mg.g^{-1}DW$. The highest contents were obtained under P fertilizer and rhizobacterial treatment. The ameliorative effect of the two PSB tested is in concordance with the findings obtained under controlled conditions. Hence, the seed biopriming with the PSB tested constitutes a promising alternative to minimize the P problem in agricultural soils.

Keywords: *Alfalfa; Crude proteins; Forage production; Forage quality; Phosphorus; PSB*

I. Introduction

In the Mediterranean region, alfalfa (*Medicago sativa* L.) is one of the most cultivated legume species, due to its high adaptation to the local climates and its various benefic effects on soil fertility (El Moukhtari et al., 2021). As other legumes, *M. sativa* has a strong biological capacity to fix atmospheric nitrogen in symbiosis with the rhizobial species *Ensifer meliloti*. Indeed, the amount of nitrogen fixed by alfalfa was estimated to reach 177 kg ha^{-1} (Luo et al., 2015). This makes it a good soil renovator and the best green manure. In addition, *M. sativa* is also known for its high nutritional quality because of its high protein content (Farissi et al., 2018; Farssi et al., 2021). However, in the Mediterranean region, most of the arable soils where alfalfa is produced have low levels of phosphorus (P), which drastically reduced its growth and productivity (El Moukhtari et al., 2022; Farssi et al., 2021).

P is a vital macronutrient that plants required at an adequate level for their normal growth and development (Wang et al., 2021). It has crucial functions in several physiological processes (Chan et al., 2021). At a cellular level, P is involved in the structural composition of membrane lipids and nucleic acids rather than its key role in energy transfer and photosynthesis (Carstensen et al., 2018). Additionally, in legumes-rhizobia symbiosis, P is required for biological nitrogen fixation, accounting for 20% of total plant P allocated to nodules (Mandri et al., 2012).

P is taken up by the plant root in the form of orthophosphate ion; the bio-available P form. However, despite being quite abundant in soils, soil Pi rapidly interacts with Fe^{3+}, Ca^{2+}, and Al^{3+} ions, forming complexes making the total bio-available P fraction low (0.1-10 μM) than the plant requirements (Bargaz et al., 2021). This makes P-deficiency one of the most abiotic stresses continuously threatening plant growth, including alfalfa (He et al., 2017). It has been reported that P-deficiency reduced alfalfa growth by reducing P nutrition and inducing reactive oxygen species generation and lipid peroxidation (El Moukhtari et al., 2022). Furthermore, Farssi et al. (2021) have also shown that P-deficiency could reduce alfalfa growth by reducing P nutrition and photosynthesis and inducing lipid peroxidation. In faba bean, P-deficiency was manifested by a reduction in plant biomass, photosynthesis, nitrogen fixation and water content along with an induction of oxidative stress (Oukaltouma et al., 2022). The negative effects of P-deficiency have also been reported in *Citrus grandis* (Meng et al., 2021), maize (Zhang et al. 2018) and rice (Xu et al., 2007).

Hence, to overcome this situation, the use of phosphate solubilizing bacteria (PSB) has been emerged as a potent microbial strategy (e.g., inoculants, biofertilizers, biostimulant), as

they have demonstrated positive effects on crop growth and productivity under P-deficiency, (Elhaissoufi et al., 2020). Among the PSB, *E. meliloti* has been reported to have the ability to increase alfalfa growth during P-deficiency stress, and even under other abiotic stresses, such as drought and salinity with help of various mechanisms including P solubilisation, free-nitrogen fixation and the production of acid phosphatase, indole acetic acid and exopolysaccharide (El Moukhtari et al., 2021; Lahrizi et al., 2021). Recently, findings by Farssi et al. (2021) contributed to the available knowledge on the effects of *E. meliloti* inoculation on alfalfa growth under low P-availability stress. The authors found that the inoculation of plants increased their P nutrition along with increased plant biomass, photosynthesis and reduced lipid peroxidation. In addition, the growth of alfalfa, in terms of shoot and root dry weights, plant height and root volume, was significantly improved under the combined effects of drought and P deficiency while inoculating with *E. meliloti* (Markarian et al., 2015). Moreover, Lahrizi et al. (2021) showed that alfalfa plants from primed seeds with *E. meliloti* showed high tolerance to drought stress compared to those from unprimed ones. We hypothesized that the inoculation with a consortium of synergetic PSB may boost the tolerance of the plant to P-deficiency. Therefore, in the present study was designed to evaluate the effet of seed biopriming with two phosphate solubilizing rhizbacteria (PSB), *Sinorhizobium meliloti* Rm41 and *Pseudomonas alkylphenolica* PF9, on forage yield and quality of alfalfa (*Medicagao sativa* L.) under low P availability in field conditions.

II. Materials and methods

II.1. Site description and plant material

The impact of the biopriming with the PSB on plant growth and yield of alfalfa was conducted in a field located in the M'goun valley about 5 km from Kelaa M'gouna. The site has an altitude of about 1 425 m above sea level. The experiment was conducted during the year 2021 on a loamy clay soil with annual average temperature of 17.3 °C and total annual precipitation of 161.9 mm, with maximum rainfall in the period between November and February. The evaluation was done during April to July with four cuts. We focused on this site because it is as a traditional agrosystem where alfalfa is strongly cultivated and contributed to socioeconomic development of local families. The regional climate of the experimental site is semi-arid, with surface soils regularly undergoing drying-rewetting cycles from the irregular distribution of rainfall. The field is an agricultural land with a traditional gravity-fed irrigation system, no herbicides and no chemical fertilizers were applied in the previous growing seasons. The alfalfa cultivar used is *Oued Lmaleh (OL*).

II.2. Bacterial inoculum

Two rhizobacterial strains, *Sinorhizobium meliloti* Rm41 and *Pseudomonas alkylphenolica* PF9 showing their potential of phosphate solubilization were chosen as inoculum in order to test their potential effect on P nutrition in alfalfa under field conditions. 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 NBRIP media and for their *in vitro* synergistic potential according to Habbadi et al., 2017 (4^{th} chapter; Farssi et al., 2021). Inocula of the two strains were prepared by growing them in YEM medium at 28 °C for 2-3days. Rhizobacterial cells were harvested and washed several times with sterile physiological water and re-suspended in an adequate volume of sterile physiological water to obtain a final OD of approximately 10^9 CFU/mL at 600 nm.

II.3. Site design and seed inoculation

The experiment had a randomized block design with four treatments in three replicates per treatment dispersed in three different blocks (Figure 27):

- Block without phosphate fertilizers and without bacterial treatment (-P-B),
- Block with phosphate fertilizers and with bacterial treatment (+P+B),
- Block with phosphate fertilizers but without bacterial treatment (+P-B),
- Block without phosphate fertilizers and with bacterial treatment (-P+B),

The bacterial treatment corresponds to *Sinorhizobium meliloti* Rm41 strain combined with *Pseudomonas alkylphenolica* PF9 as a mixed inoculum

The dimensions of each elementary block were 3 m × 2 m. Each main block was spaced with 0.4 m from the next block. All plots have received potassium (K) fertilizer at rate of 150 kg/ha of K_2O . However, the P fertilizer was applied only for the plots marked as (+P) at rate of 150 kg/ha. The *OL* alfalfa seeds were selected for their uniformity and surface-sterilized with sodium hypochlorite solution (6%), followed by several rinses for 10 min in sterile distilled water. Disinfected seeds were submerged for 30 min in the mixed inoculum of both strains at 1:1 v/v ratio.

Then, the mixture seed-inoculum has been mixed with 1kg of the washed and sterilized sand. The seeds were sown in autumn (December 2020) for all treatments with a density of about 150 plants.m^{-2}. The weeds were controlled manually.

Figure 25 : Field Trial

- A: Land preparation and culture establishment
- B: The trial in March 2021, 3 months after sowing
- C: Trial at the vegetative stage
- D: Trial at the flowering stage, just before the last cut (June 2021).
- E: Sample of a plant from the inoculated plot
- F: Sample of a plant from the stressed and non-inoculated plot

II.4. Soil properties

Soil pH was measured after shaking a subsample of dry soil in distilled water for 4 h at a soil: water ratio of 1 *versus* 5. The soil available P (Olsen P) to plants was determined after extraction in 0.5 M $NaHCO_3$ (Olsen *et al.*, 1954). Total P was determined after igniting air dried soil samples at 550 °C for 4 h and dissolving the ashed samples in concentrated HCl. Available and total P were analyzed by the molybdate blue method by reading the absorbance at 820 nm after color development at 100 °C for 10 min as described in the previous chapters. Total organic C content was estimated by oxidation with potassium dichromate and sulfuric acid and total organic N content was estimated by the Kjeldahl method as described by Faghire *et al*. (2011). Sodium and Potassium were determined as described by Mouradi et al. (2016) using flame spectrophotometer. The obtained results for soil analysis are indicated in table 13.

Table 13: Analysis of soil properties

Soil properties	***Value recorded***
pH	7.12
Electrical conductivity (µs/cm)	232
P total (mg Kg^{-1})	312
P olsen (mg. Kg^{-1})	4.9
Nitrogen (mg Kg^{-1})	1139
Potassium (mg Kg^{-1})	152
Organic matter (%)	1.23
Clay (%)	18.23
Sand (%)	65.38
Silt (%)	16.18

II.5. Evaluation of alfalfa forage productivity

II.6.1. Plant height (PH)

At each harvest, alfalfa PH was measured from the ground to the topmost part of the plant.

II.6.2. Forage dry matter (FDM)

Alfalfa samples were randomly collected at harvest time from each plot and dried to constant weight in a forced-air oven of 65 °C for 72 h for determination of FDM content.

II.6. Evaluation of forage quality components

The forage quality was assessed by leaf: stem ratio, nitrogen contents, crud protein contents,

II.6.1. Leaf: stem ratio (L/S)

The leaves and stems were separated and weighed using a precision weighing balance.

II.6.2. Crud protein (CP)

The Nitrogen (N) content was measured using the Kjeldahl method. Then, the CP was calculated as N×6.25.

III. Results and discussion

III.1. Alfalfa forge productivity

The effects of different applied treatments on plant height and forge dry biomass are indicated in the tables 14 and 15. For plant height, results showed a significant effect of P and rhizobacterial treatment ($p < 0.05$). The lowest heights were noted when plants are not fertilized and untreated with the rhizobacteria used. However, a percentage increase of 9.91% was noted when the seeds were bioprimed with the mixed inoculum of *Sinorhizobium meliloti* Rm41 strain combined with *Pseudomonas alkylphenolica* PF9. From cutting to cutting, the plant height increased except the last cut for the stressed and non-inoculated plants where a decrease in plant height was noted in comparison with the previous cuts. Also, our results indicated that enormous values are uppermost when the plants were fertilized with the P and treated with the rhizobacterial inoculum.

For forage dry biomass, the same trend was noted. The P fertilization has a significant effect ($p < 0.05$) on the dry biomass. Also, the rhizobacterial treatment significantly improved the forage dry biomass under low-P availability. An increase in dry biomass was noted from one cutting to another. The best dry biomass was noted for all treatments during the cutting of

July apart from the stressed and non-inoculated plants where a percentage decrease of 3.37%. The comparison among the applied treatments significantly indicated that the inoculation of alfalfa plants with the two strains improved the forage dry biomass under low-P availability, with an improvement percentage varying from 11% during the first cutting to 86% during the last cutting. As the plant height, the addition of P fertilizer and the inoculation of the plant with the mixed inoculum improved the forage dry biomass in comparison to all applied treatments.

Table 14: Effect of different applied treatments on plant height of alfalfa during four cuttings.

Harvest time	***Treatment***	***Plant height (cm)***
1st cutting, April 2021	*T1 (-P-B)*	52.7 *d*
	T2 (+P+B)	66.5 *a*
	T3 (+P-B)	63.6 *ab*
	T4 (-P+B)	58.5 *c*
2nd cutting, May 2021	*T1 (-P-B)*	58.2 *c*
	T2 (+P+B)	72.1 *a*
	T3 (+P-B)	69.6 *a*
	T4 (-P+B)	65.4 *b*
3rd cutting, Jun 2021	*T1 (-P-B)*	59.3 *d*
	T2 (+P+B)	81.6 *a*
	T3 (+P-B)	77.5 *b*
	T4 (-P+B)	72.8 *c*
4th cutting, Jully 2021	*T1 (-P-B)*	57.4 *c*
	T2 (+P+B)	86.6 *a*
	T3 (+P-B)	81.5 *b*
	T4 (-P+B)	82.8 *b*

Within a column, values followed by the same letter are not significantly different at 0.05 level of probability for each cutting

Table 15: Effect of different applied treatments on forge dry biomass of alfalfa during four cuttings.

	Treatment	***Dry Matter (Kg.m^{-2})***
1st cutting, April 2021	*T1 (-P-B)*	1.34 *d*
	T2 (+P+B)	1.96 *a*
	T3 (+P-B)	1.87 *b*
	T4 (-P+B)	1.67 *c*
2nd cutting, May 2021	*T1 (-P-B)*	1.38 d
	T2 (+P+B)	1.99 *a*
	T3 (+P-B)	1.89 b
	T4 (-P+B)	1.73 *c*
3rd cutting, Jun 2021	*T1 (-P-B)*	1.31 *d*
	T2 (+P+B)	2.52 *a*
	T3 (+P-B)	2.41 *b*
	T4 (-P+B)	2.36 *c*
4th cutting, July 2021	*T1 (-P-B)*	1.29 d
	T2 (+P+B)	2.53 *a*
	T3 (+P-B)	2.43 *b*
	T4 (-P+B)	2.40 *bc*

Within a column, values followed by the same letter are not significantly different at 0.05 level of probability for each cutting

III.2. Alfalfa forge quality components

The forage quality is evaluated by the leaf to stem ratio and the content of crud proteins. Obtained results are shown in table 16 and 17.

For leaf to stem ratio, the values recorded varied from 0.61 to 0.82. A significant effect of P fertilizer and rhizobacterial treatment was noted ($p < 0.05$). The highest values were noted when the plants are fertilized and inoculated. However, the rhizobacterial treatment enhanced the ratio in comparison with stressed and non-inoculated conditions. The improvement percentages were 13.11 % during the first cutting and 43.13% at the fourth cuttings. The leaf to stem ratio was significantly decreased from the third cutting.

Table 16: Effect of different applied treatments on leaf to stem ratio of alfalfa during four cuttings.

Harvest time	*Treatment*	*Leaf / stem ratio*
1st cutting, April 2021	*T1 (-P-B)*	0.61 *c*
	T2 (+P+B)	0.76 *a*
	T3 (+P-B)	0.74 *a*
	T4 (-P+B)	0.69 *ab*
2nd cutting, May 2021	*T1 (-P-B)*	0.63 *c*
	T2 (+P+B)	0.77 *a*
	T3 (+P-B)	0.73 *a*
	T4 (-P+B)	0.68 *ab*
3rd cutting, Jun 2021	*T1 (-P-B)*	0.57 *c*
	T2 (+P+B)	0.83 *a*
	T3 (+P-B)	0.81 *a*
	T4 (-P+B)	0.72 *b*
4th cutting, July 2021	*T1 (-P-B)*	0.51 *d*
	T2 (+P+B)	0.82 *a*
	T3 (+P-B)	0.76 *b*
	T4 (-P+B)	0.73 *c*

Within a column, values followed by the same letter are not significantly different at 0.05 level of probability for each cutting

For the CP, the contents obtained varied from 128.94 to 187.06 $mg.g^{-1}DW$. The highest contents were obtained under P fertilizer and rhizobacterial treatment. The effect of P fertilizer and rhizobacterial treatment was significant ($p < 0.05$). During all cuttings, the rhizobacterial treatment ($p < 0.05$) improved the CP in the absence or the presence of P fertilizer. From the third cutting the effect of P fertilizer in the absence of rhizobacterial treatment was obvious ($p < 0.05$) with values of 119.38 $mg.g^{-1}DW$ and 116.88 $mg.g^{-1}DW$ at the third and the fourth cuttings respectively.

Table 17: Effect of different applied treatments on crud proteins (CP) of alfalfa during four cuttings.

Harvest time	*Treatment*	*CP (mg.g^{-1}DW)*
1st cutting, April 2021	*T1 (-P-B)*	128.94 *c*
	T2 (+P+B)	166.25 *a*
	T3 (+P-B)	153.75 *b*
	T4 (-P+B)	149.38 *b*
2nd cutting, May 2021	*T1 (-P-B)*	135.19 *c*
	T2 (+P+B)	178.75 *a*
	T3 (+P-B)	161.88 *b*
	T4 (-P+B)	157.50 *b*
3rd cutting, Jun 2021	*T1 (-P-B)*	119.38 *c*
	T2 (+P+B)	182.50 *a*
	T3 (+P-B)	158.75 *b*
	T4 (-P+B)	160.13 *b*
4th cutting, July 2021	*T1 (-P-B)*	116.88 *c*
	T2 (+P+B)	187.06 *a*
	T3 (+P-B)	158.13 *b*
	T4 (-P+B)	155.63 *b*

Within a column, values followed by the same letter are not significantly different at 0.05 level of probability for each cutting

IV. Discussion

P is the major element most often overlooked in crop fertilization programs. Even though the soil has large amounts of P in its minerals, it is nearly all chemically locked up and unavailable to the plant at any one time (Bargaz et al., 2012; Boudanga et al., 2015; Bekel et al., 2019;). If soil conditions are favorable, the beneficial soil microorganisms will slowly break down mineral P and make it available. We focused in this chapter on the role of selected microorganisms on P availability in the soil under filed conditions. We noted the fertilization affected negatively ($p < 0.05$) the plant heights and the forage dry biomass. The same results on the effect of low-P availability are reported in many legumes' species, including alfalfa (Rui et al., 2022; El Moukhtari et al., 2022).

Under environmental stress, most N_2-fixing legumes are capable of maintaining a high metabolic activity in their root nodules (Walsh, 1995). However, nitrogenase activity is particularly sensitive to abiotic stresses. Generally, restrictions on plant growth are caused by scarcity of resources, such as P, which is strongly bound in soil, resulting in a low rate of

diffusion towards the root surface (Tinker and Nye, 2000). The reduction in plant height is one of the plant strategies for tolerance to environmental constraints, including low P-availability.

Our results showed the decrease in leaf: stem ratio under low-P availability. Reduced leaf to stem ratio is a major cause of the decline in forage quality with maturity, and the loss in quality that occurs under adverse hay curing conditions. The proportion of stems increases and quality decreases. This ratio is crucial to the alfalfa digestible quality. Thus, food quality forage decreases with decreasing the ratio of leaves and stems (Volenec and Cherney, 1990).

The effect of P fertilization on forage yield and quality was reported on common vetch (*Vicia sativa* Roth.) by Yildiz and Türk, (2015). These authors reported that increasing P rates resulted in increased forage yield and quality. We noted that the seed biopriming with PSB improved the leaf: stem ratio in comparison with unprimed and P-stressed plants. Awad and Eltahir (2011) documented that leaf to stem ratio of the leguminous blue pea (*Clitoria ternatea* L.), was mainly increased with the addition of phosphorus and rhizobium inoculation.

The use of plant growth-promoting rhizobacteria including P-solubilizing bacteria (PSB) has been shown to improve P use efficiency by plants (Suleman et al., 2018; Bargaz et al., 2021; Fatima et al., 2021; El Moukhtariet al., 2022). PSB are a large group of soil microorganisms that have the ability to mobilize directly or indirectly P from both the organic and inorganic sources and make it available for plant use. Among the PSB, *Pseudomonas alkylphenolica* has been recently considered as one of the most effective PSB, which continues to be a key research priority (Farssi et al., 2021; El Moukhtari et al., 2022). Thus, when P-stressed alfalfa plants were inoculated with *Pseudomnas alkylphenolica* PF9 strain, they exhibited higher biomass production, photosynthesis activity, and P content (Farssi et al., 2021). Another interesting study conducted by Shi et al. (2019) showed that *Pseudomonas alkylphenolica* strain was able to remove mercury (Hg) in the solution, immobilize Hg in soil, promote growth, decrease Hg accumulation, and improve photosynthesis of *Brassica campestris* exposed to Hg stress.

In addition to leaf to stem ratio, the forage quality is also assessed by the content of crud proteins. We noted that the P starvation negatively affected this parameter during all four cuttings. The effect of P deficiency on the crude proteins contents is documented by Yildiz and Türk, (2015). These authors reported the content of crude proteins increased while increasing P fertilization rates. In the same line, Laltlanmawia et al. (2004) reported that the protein content significantly increased with increased level of P fertilizers. Khan et al. (2019) reported that the

stimulatory effects of PGPR treated plants showed significant increase in the contents of protein even under harsh environmental conditions.

V. Conclusion

We conclude the low P-availability has negative effects on forage yield and quality. The forage dry biomass was found affected by P starvation. The traits assessed related to forage quality such as leaf: stem ratio and the content of crude proteins were decreased in stressed-plants. The seed biopriming with the PSB tested indicated their significant effectiveness on amelioration of the P nutrition in alfalfa stressed plants. In fact, the biopriming increased the forage dry biomass, the leaf to stem ratio and the content of crude proteins. These findings are in concordance with those obtained under controlled conditions. Hence, the seed biopriming with the PSB tested constitutes a promising alternative to minimize the P problem in agricultural soils.

GENERAL CONCLUSION

The P nutrition constitutes one of the important parameters governing plant growth and development. The P is involved in many physiological and biochemical process ensuring the vital functions for the plants. Hence, any P deficiency will have harmful and depressive effects on different plat stage. The great challenge related to P nutrition is ensuring its biodisponibility on the basis of plant requirement. In the case of leguminous species, the low P availability has considerable impacts and repercussions on plant growth. In fact, the biological particularity of this plant family regarding its ability to biological nitrogen fixation poses additional demands of P with up to 20% of total plant P being allocated to nodules and any phosphorus deficiency may influence the nodulation process and the symbiosis efficiency.

On the other hand, the majority of the P contained in the soil is in inorganic and organic complex forms. These forms are not directly usable by the plants. Indeed, the high reactivity of P with iron, aluminum and calcium, to form insoluble compounds, reduces its mobility in the soil solution. These reactions provoked a very low P availability and low efficiency of phosphate fertilizers used by plants. Even with these conditions, there are a lot of strategies focusing on the management of soil P availability. One of the most strategies is associated the selection of the microorganisms having a high potential of soil P solubilization and may be on other functions allowing to plant stimulation and improvement of P nutrition in plants. However, the screening among the plant genetic resources constitutes an important alternative to select the varieties more adapted to low P availability throughout the development of agro-physiological and biochemical mechanisms allowing to P solubilization.

In this respect, we adopted in this thesis an approach focused on i) screening of Moroccan alfalfa genetic resources in order to select the most adapted genotypes to low P availability, ii) research of endophytic and rhizospheric microorganisms in order to select rhizobacteria with high level biofertilization-biostimulation potential to improve alfalfa growth and its tolerance to low P availability under biological nitrogen fixation conditions. Hence, the first part of our research led to determine the depressive effects of low-P availability in rooting medium on four alfalfa studied genotypes. Our results indicated the *OL* cultivar was found to be the least affected cultivar and the DEM was the most sensitive one. However, *TATA* and *RICH* genotypes displayed a moderate tolerance to P–deficiency conditions. The alfalfa P stress tolerance was linked to the induction of acid phosphatase activity, the enhancement of P solubilization and uptake, the maintaining of cell membrane integrity and the induction of non-enzymatic and enzymatic antioxidant responses against the accumulation of ROS.

The second part of our PhD work is concentrated on the isolation of the rhizobacteria associated to root and rhizosphere zone of alfalfa in Beni-Mellal agro-systems. The collection was evaluated for their biofertilizer-biostimulant potentials against a set of plant growth promoting traits such as P solubilization, tolerance to osmotic stress, adaptation to different pH levels, indole acetic acid and exopolysaccharide productions. The physiological and biochemical characterization of rhizobial strains of our collection has determined the extent of physiological variations that exist in the behavior of the. The results of these tests have allowed us to identify some strains with high tolerance to drastic conditions of the environment, including low P availability. The candidate strains were identified through molecular tools basing on housekeeping gene sequencing. Hence, regarding a horizontal tolerance of the identified stains to arid and semi-arid conditions, we have selected two strains "*Pseudomonas alkylphenolica* PF9 and *Sinorhizobium meliloti* Rm41" to evaluated their biofertilizer-biostimulant potential effects on the tolerance of *OL* alfalfa cultivar to low-P availability under the conditions of biological nitrogen fixation. The obtained results suggest that the co-inoculation of alfalfa plants with these two strains could alleviate the deleterious effects of low P conditions in the rooting medium. These PGPR-PSB improved the plant biomass and the considered agronomic traits under P-stressed conditions. The ameliorative effects were associated with P solubilization and its 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. These findings imply that their applicability as a promising alternative to minimize the P problem in agricultural soils.

From a viewpoint of the contribution of our PhD work to sustainable agriculture. We envisaged the transfer of the obtained results from the controlled conditions to field trials. Thus, a filed trial was conducted to evaluate the role of the mixed inoculum of "*Pseudomonas alkylphenolica* PF9 and *Sinorhizobium meliloti* Rm41" on alfalfa tolerance to low phosphorus availability under the condition of biological nitrogen fixation. The alfalfa seeds were bioprimed with the two strains before their sowing. The effect of seed biopriming with these phosphate solubilizing bacteria was manifested by improvement of the forage dry biomass and plant heights as well as the considered traits related to forage quality such as leaf: stem ratio and the content of crude proteins in comparison to P-stressed conditions. The findings we reported under field conditions are in concordance with those obtained under controlled conditions. Hence, the seed biopriming with the PSB tested constitutes a promising alternative to minimize the P problem in agricultural soils.

PERSPECTIVES

In the light of results obtained from our PhD work, we would provide strong support for research efforts to improve the production of legumes generally and alfalfa especially under low P availability and other abiotic constraints and the valorization of agricultural land affected by these constraints. The legume system of cultivation based on the biological fixation of nitrogen will contribute to the development of sustainable agriculture based on biological fertilization that could allow farmers in Moroccan agro-systems to save chemical fertilizer costs and relieve the environment from pollution. Our research work was an approach to diversify forage resources and ensure self-sufficiency of forage widely used for food livestock. As consequence, the improvement of alfalfa economic yield will contribute to the stability and balance of microeconomic projects of rural populations.

In this context, as perspectives, the collection of microorganisms identified is stored under the adequate conditions at the Belgian Coordinated Collections of Microorganisms (BCCM) belonging to Laboratorhy of Microbiology - Gent (LMG) Lab in Ghent University in the framework of signed convention. We expect to analyze the competitive nodulation performance of the strains in competition experiments with GUS marked strains. The *gusA* gene, encodes the enzyme ß-glucuronidase (GUS), is widely used as a reporter gene in plant molecular biology because there is no background activity in plants, and because the enzyme is easy to assay in a variety of both histochemical and quantitative assays. It is now becoming adopted as a marker gene for microbial ecology, particularly for studying the ecology of bacteria that interact with plants. We will further focus on the transcriptional and physiological responses of rhizobial strains to water deficit. The identified potential phosphatase genes will be cloned, mutated and over-expressed and the corresponding phosphatases will be characterized. We expect also to compare the symbiotic performance of mutant and over-expressing strains in the greenhouse to link bacterial phenotype to the plant's physiological response under water deficit and P limiting conditions.

Also, the performance of the selected strains will be studied under other abiotic stress such as salinity and water deficit. Also, the synergistic effects of our strains with other biostimulants such as silicon and proline are ongoing. In the same sense, the potential roles of our collection in enhancement the secondary metabolites in medicinal and aromatic plants are focused.

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ANNEXES

Annex 1

Composition of Hoagland solution

	Nutrient	Concentration (mM)
Macro-nutrients	KH_2PO_4 $Ca_3(PO_4)_2$ (TCP)	0.25
	K_2SO_4	0.75
	$MgSO_4$. 7 H_2O	1
	$CaCl_2$	1.65
	Sequestrene	-
Micro-nutrients	H_3BO_3	0.004
	$MnSO_4$	0.006
	$ZnSO_4$, $7H_2O$	0.001
	$CuSO_4$, $5H_2O$	0.001
	Na_2MoO_4, $2H_2O$	0.0001

Annex 2

YEM liquide Composition

For 1 L :

KH_2PO_4	5 g
NaCl	0.1 g
Mannitol	10 g
$MgSO_4$, $7H_2O$	0.2 g
Yeast extract	1 g
^{2}pH	6.8 à 7

Annex 3

Gallic acid standard range

Stock solution (SS) of gallic acid (GA): 0.01g of gallic acid in 10 ml of 80% methanol.

Finale concentration of GA (µg/ml)	0	50	100	150	200	250
Volume to be drawn from SS (µL)	0	50	100	150	200	250
Volume of methanol solution (80%)to be added (µL)	1000	950	900	850	800	750
DO_{725nm}						

Annexe 4

Quercetin standard range

Stock solution (SS) of quercetin: 0.01g of quercetin in 10 ml of 80% methanol.

Finale concentration of GA (µg/ml)	0	25	50	75	100
Volume to be drawn from SS (µL)	0	25	50	75	100
Volume of methanol solution (80%) to be added (µL)	1000	975	950	925	900
DO_{415nm}					

Annex 5

Bradford Reagent

- ✓ 10 mg of Coomassie blue
- ✓ 5 ml ethanol
- ✓ 10 ml orthophosphoric acid
- ✓ Completed to 100 mL

Annex 6

AB reagent

- ✓ **Reagent A:** Sodium molybdate 2.5%; dissolve 2.5 g of sodium molybdate in 100 ml of H_2SO_4 (10N)**.**
- ✓ **Reagent B:** Hydrazine sulfate 0.15%; dissolve 0.15 g of hydrazine sulfate in 100 ml of distilled water.

These two reagents can be stored separately in the refrigerator.

- ✓ **Reagent AB:** 20 ml of reagent A + 10 ml of reagent B and make up to 100 ml with distilled water.