

JOURNAL OF OASIS AGRICULTURE AND SUSTAINABLE DEVELOPMENT

www.joasdjournal.org

JOASD

p-ISSN: 2724 – 699X
e-ISSN: 2724-7007

Biochemical and physiological characterizations of *Rhizobium*-Pea (*Pisum sativum* L.) symbiotic association under abiotic constraints

Khadija Ouerghi[1], Mohamed Bagues[2*] & Sifi Bouaziz[1]

[1]*Laboratory of Agronomic Sciences and Techniques, National Agricultural Research Institute of Tunisia, Hédi Karray Street, 1004 ElMenzah, University of Carthage, Tunisia.*

[2]*Laboratory of Drylands and Oases Cropping, Arid Regions Institute of Medenine, University of Gabes, Tunisia.*

Article info

Article history:
Received 10 December 2021
Accepted 30 December 2021

Keywords: *Soil, bacteria, fungi, microbe diversity*

OPEN ACCESS

Copyright©2021 JOASD

*** Corresponding author**
bagues007@gmail.com

Conflict of Interest : The authors declare no conflict of interest

Abstract

Pea (*Pisum sativum* L.) is an important leguminous for the agricultural sector. It is a source of biological nitrogen that efficiently contributes to the soil fertility. In Tunisia, low pea production is due to bad nitrogen management, lack of phosphorus availability and to the abiotic constraints. Thus, in order to improve the pea production ,a new farming technique involving the rhizobia inoculation was applied. The symbiotic, biochemical, physiological characterization and inoculation trials were performed in both the laboratory, greenhouse and open field. Pea Lincoln variety was used as legume species and fifteen *Rhizobium* strains isolated from the roots of the nodulated pea were collected from different Tunisian areas. Several physiological and biochemical parameters, i.e. pH, temperature, calcium carbonate and salinity were assessed to characterize the strains nodulating pea. All the rhizobia tests were evaluated on Yeast Extract Mannitol Agar medium (YEMA). Pea nodulation and Gallery API test were carried out under controlled conditions. Significant differences (p<0.01) between the nodules number induced by the different bacterial strains and between strains for the dry matter quantities of aerial and root parts were registered. The pH medium test results showed that among 15 strains only 8 strains having a halo diameter greater than 1 cm at basic pH. The most of isolates are able to grow at both low and high temperatures. The limestone test results qualify these rhizobia as calcifuges. Gallery API test results showed a great diversity of rhizobia assimilation of carbohydrates implying genetic diversity. Our results us to select the most efficient solubilizer *Rhizobium* strains nodulating pea. In order to confirm the previously cited notions on the diversity of *Rhizobium* strains isolated from *Pisum sativum* roots in Tunisia, inoculation trial with both selected strains in controlled and open field conditions confirmed the capacity of selected strains to fix atmospheric nitrogen and promote plant growth.

1. INTRODUCTION

The rhizosphere is the seat of the microbial flora that characterized by a wide diversity of rhizobacteria (Gomes et al., 2001; Roldan et al., 2004; Zeppenfeld et al., 2017). Symbiotic association of leguminous plants and rhizobacteria are widely known to detoxify and remove heavy metals contamination from contaminated site (Singh et al., 2019). Previous studies have highlighted the classification of Rhizobia nodulating the various leguminous species in groups especially in the Mediterranean regions. This group includes, in fact, the genera *Rhizobium*, *Mesorhizobium* and *Sinorhizobium* (Zahran, 2001; Mahdhi and Mars, 2006; Mahdhi et al., 2007; Mahdhi et al., 2008; Hemissi et al., 2015).This classification of rhizobia based on specific criteria of host plants that has long been used. For decades, host plant specificity for nodule formation (infectivity) and nitrogen fixation (efficiency) had been highlighted and has led to cross-inoculation groups for legumes and species and biovars for Rhizobium (Henault and Revellin, 2011). Moreover, these rhizobia are well known for

fixing atmospheric nitrogen inside the nodules that develop on legume root infected through a process of nodulation. The interaction made between the rhizobium-legume couple is the result of a continuous molecular dialogue between the strain and its host plant. They can then survive and reproduce in the soil and consequently they contribute in maintaining soil quality and structural stability through their fundamental role in the intervention in biochemical cycles of nutrients (Roldan et al., 1994; West et al., 2002). In addition, in order to support nitrogen fixation during respiration process, rhizobia could use carbon compounds derived from plant roots to synthesize energy-rich storage molecules like polyhydroxybutyrate enhancing subsequently their reproduction and survival (Denison and Kiers, 2004). However, several studies were highlighted the abiotic factors affecting nitrogen symbiotic fixation by rhizobia such as (pH, temperature, phosphorus deficiency, salinity). The majority of rhizobia prefer pH values ranged between 6 and 9 to multiply and grow, whereas for neutral pH their growth is optimal (Wei et al., 2008; Rejili et al., 2012). Phosphorus deficiency also affects the multiplication of rhizobia in the rhizosphere resulting the reduction in the probability of infection and in the nodules growth (Hemissi et al., 2015; Bargaz et al., 2015). Leguminous species and the nodule process initiation are more sensitive to salinity and osmotic stress than Rhizobia (Zahran, 2001). Salinity affects the initiation, development and functioning of nodules, as well as the photosynthetic capacity of leaves (Farissi et al., 2014). According to Prosperi (1993), low temperatures affected nitrogen fixation, photosynthesis, and nodulation. While, the study of Lynch and Smith (1993) showed that the nitrogenase was able to function at temperatures between (8-10°C) during which the penetration of *Rhizobium* into the roots of the soybean species (*Glycine max*). However, a recent investigation of Rejili et al. (2012) found that the isolated nodulating forage species could tolerate high temperatures (40°C). In Tunisia, the genera *Sinorhizobium* and *Rhizobium* are widely distributed in arid and semi-arid regions. They nevertheless offered certain variability in the ability to nodulate by symbiosis with different species of pluses and the specific host legumes species for these genera were *Lotus sp. Hedysarum sp.* (Rejili et al., 2012), *Phaesolus vulgaris*, *Cicer aritenium* (Hemissi et al., 2011; Abdi et al., 2015; Kouki et al., 2016) , *Vicia faba* (Maazaoui et al., 2016). Pea is one among the pulse that considered like a key model for the study of biological nitrogen fixation because of its nutritional, agronomic, economic and environmental value (Singh et al., 2019). Is able to establish symbiosis with *Rhizobium leguminosarum biovar viciae* (Van Rhijn and Vanderleyden, 1995; Muniz et al., 2017). Thanks to its contribution to enrich the soil with nitrogen, peas are a good cultural precedent for cereals thus; they were destined to play an essential role in sustainable agriculture (Dumont et al., 2011; Hachana et al., 2017). Indeed, the fixation of atmospheric nitrogen from the pea-*Rhizobium* symbiosis based on the characteristics of infectivity and efficiency existing between two partners (He et al., 2011; Muniz et al., 2017).The inoculation of agricultural crops with rhizobia has attracted the considerable attention of the microbiologist (Hussain et al., 2016). In this context, the present investigation focused on the symbiotic, physiological and biochemical characterization of about fifteen strains of rhizobia nodulating pea in order to select judiciously the most efficient strain for the biological nitrogen fixation, having a high potential for solubilization of inorganic phosphorus (Pi), adapted to abiotic environmental constraints such as salinity and drought.

2. MATERIAL AND METHODS

2.1. Plant

Laboratory of Agricultural Sciences and Techniques of National Agricultural Research Institute of Tunisia provided pea seeds of Lincoln variety.

2.2. Strains

Twenty strains of genera *Rhizobium leguminosarum* selected from different origins were isolated from modules of pea specie (*Pisum Sativum* L). These rhizobia were P.1001, P.2000, P.1236, P.12362, P.OM192, P.OM292, P.MTem92, P.MTem95, P.TAC, P.TAM2, P.Sourgafsa, P.Ouedbeja, P.Oued, P292, P.TAMmornag, P.TAS4).

2.3. Physiological and biochemical parameters

The pH, temperature, calcareous, phosphorus and salt tolerance were assessed to characterize the strains nodulating pea. Pea nodulation and Gallery API test (API 50 CH; BioMérieux, France) as described by Kersters et al. (1984) were

carried out under controlled conditions. All the rhizobia tests were evaluated on YEM agar medium containing the following nutrients (g/l)[MgSO47H2O (0.2), NaCl (0.1), Mannitol (10),Yeast (1), agar (20)] (Vincent,1970).

pH tolerance was studied on a YEM liquid medium adjusted to different pH values (5, 5.5 ; 6 , 6.5; 7.5 , 8). After adjusting pH, the six Erlenmeyer were autoclaved for 20 minutes at a pressure of 1 bar and a temperature of 120°C.Temperature tolerance was done at several degrees (10, 20.30, 35,40, and 45°C). Calcareous tolerance was evaluated on a yeast extract mannitol agar medium supplemented with 5 ml of the Congo red solution and a Calcium Carbonate (CaCO3) mass. Three concentrations of CaCO3 were evaluated (M) (0.5,1, 1.5).

The solubilization of phosphorus was examined on a specific phosphate-based medium based on tricalcium phosphate Ca3 (PO4)2 according to Pikovskaya, (1948).The inoculated Petri dishes were incubated at 28°Cfor a week.This parameter based on measuring the clearance diameter zone (halo) surrounding the strain colony. Inorganic phosphorus solubilizing index (ISPi) was calculated as following formula: Diameter of halo (cm)/Diameter of colony (cm) (Berraquero et al., 1976).

Tolerance of rhizobia to salinity was assessed at different doses of NaCl (mM) (0, 25, 50, 75, 100, 125 and150) measuring the colony diameter. The evaluation of these previous physiological parameters was based on measuring colony strains diameter. The assimilation of biochemical substrates by strains was carried out by the API gallery (API 20E, Bio Mérieux, France).

2.4. Inoculum and inoculation

Pea (*Pisum sativum* L.cv. Lincoln) seeds surface were sterilized for 30 min in 7% (w/v) calcium hypochlorite solution thoroughly rinsed with sterile distilled water under aseptic conditions. The disinfected seeds were germinated on filter paper in sterile Petri dishes and placed in growth room at a temperature of 25°C for 7 days. Seeding was carried out in greenhouse when the radicals reach about 3 to 5 cm in length and placed in pots of 0.5 kg filled with sterile perlite at a rate of one seed per pot at a temperature of 28°C ± 2.

The inoculum is compound of each strain suspension introduced into a yeast extract mannitol liquid medium based on yeast extract and Mannitol (Vincent, 1970). It has applied with a pipette at a rate of 5 ml per seed and per pot. A second inoculation was applied during emergence stage of pea in order to optimize infectivity of rhizobia used.

The nodulation trials comprise 84 pots. Each treatment includes four replicates for each *Rhizobium* strain specific to pea plant. Irrigation of pea plants has done weekly with 50 ml per pot with a nutrient solution composed of KH2PO4 (50µM), MgSO4 (100 µM), K2SO4 (700 µM), CaCl2(1650 µM), Fe-EDTA (16 µM), MnSO4 (4 µM), H3BO3 (22 µM), ZnSO4 (0,4 µM),NaMoO4 (0.05 µM) et CuSO4 (1,6 µM) (Bargaz et al.,2011). After 60 days, the pea plants were harvested and used for the counting of their nodules, the determination of dry matter of aerial, root and nodular parts and the nitrogen content according to KJELDAHL method (Parkinson and Allen, 1975).

2.5. Statistical analysis

The SPSS 20.0 software performed statistical analysis. The data were analyzed using ANOVA and mean values were separated according to Duncan's multiple range test at p = 0.05.

3. RESULTS

3.1. Nodules number and nodular dry matter

The nodulation criterion is one of the indicators reflecting the infectivity and symbiotic potential of strain to fix nitrogen via symbiosis established between *Rhizobium*-Pea couple. The results obtained showed highly significant ($p < 0.01$) differences in the number of nodules between the different bacterial strains (Fig. 1A). Inoculation with rhizobia strain P.12362 induces the highest number of formed nodules per plant compared to other strains. According to the results of the statistical analysis (homogeneity test), we can classify the rhizobia studied in three groups as follows:

The first group includes rhizobia strains with a high pea nodulation power, which translates into the highest number of nodules per plant ($Nod.Pl^{-1}$), ranging from 80 to 130 $Nod.Pl^{-1}$. The most infective strains were P.12362, P.TAS4, P.MTem95, P.OM192, P.Ouedbeja, TAMmornag.

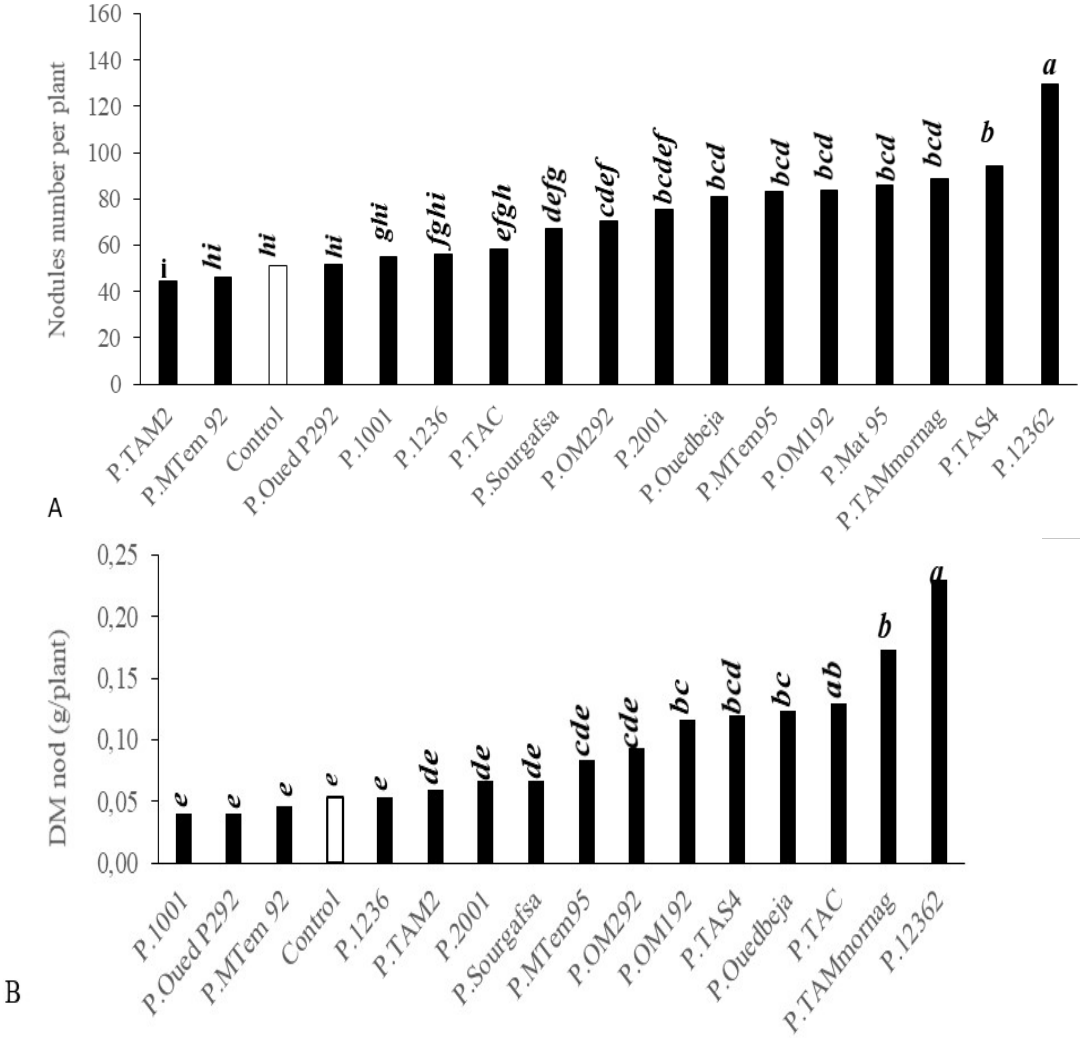


Fig. 1. Effect of inoculation by rhizobia on the nodulation (A) and the dry matter of nodules (B) of pea variety Lincoln. The values are the means of 4 repetitions and followed by different letters in a column were significantly different at 5% level of probability using Duncan's multiple range tests.

The second group of rhizobia having an infective capacity whose number of nodules per plant is between 50 and 80 Nod.Pl^{-1}.This group was formed by the following rhizobia: P.TAC, P.1001, P.2001, P.OM 292, P.Sourgafsa. These strains are moderately nodulating pea.

The third group contains rhizobia with a moderately low ability to induce nodule formation. The number of nodules per plant is less than 50.These strains were P.OM95, P.MTem 92, P.TAM2.

Concerning dry matter of nodules, Statistical analyzes of nodule dry matter (Fig. 1B) revealed a highly significant difference $p < 0.01$ between strains. Nodular biomass ranged from 0.05 to 0.23 g per plant.

The analysis of variance of the data showed that the strain P.12362, P.TAC and TAMmornag are the most efficient. Inoculation of pea (Lincoln variety) with these rhizobial strains significantly increased its nodular biomass. This variation is between 0.13 to 0.23 g per plant. However, the nodular biomass of inoculation treatments the other strains is similar to that of control plants.

3.2. Production of aboveground and root biomass

Biomass production of aerial and root parts were highly influenced by the strain used to inoculate pea plants. Results of the dry matter quantities of the epigeal and hypogeal parts revealed highly significant differences $p < 0.01$ between the strains (Fig. 2). A significant amount of biomass production was clearly observed when pea plants were inoculated with strains P.12362, P.TAC and P.TAMmornag.

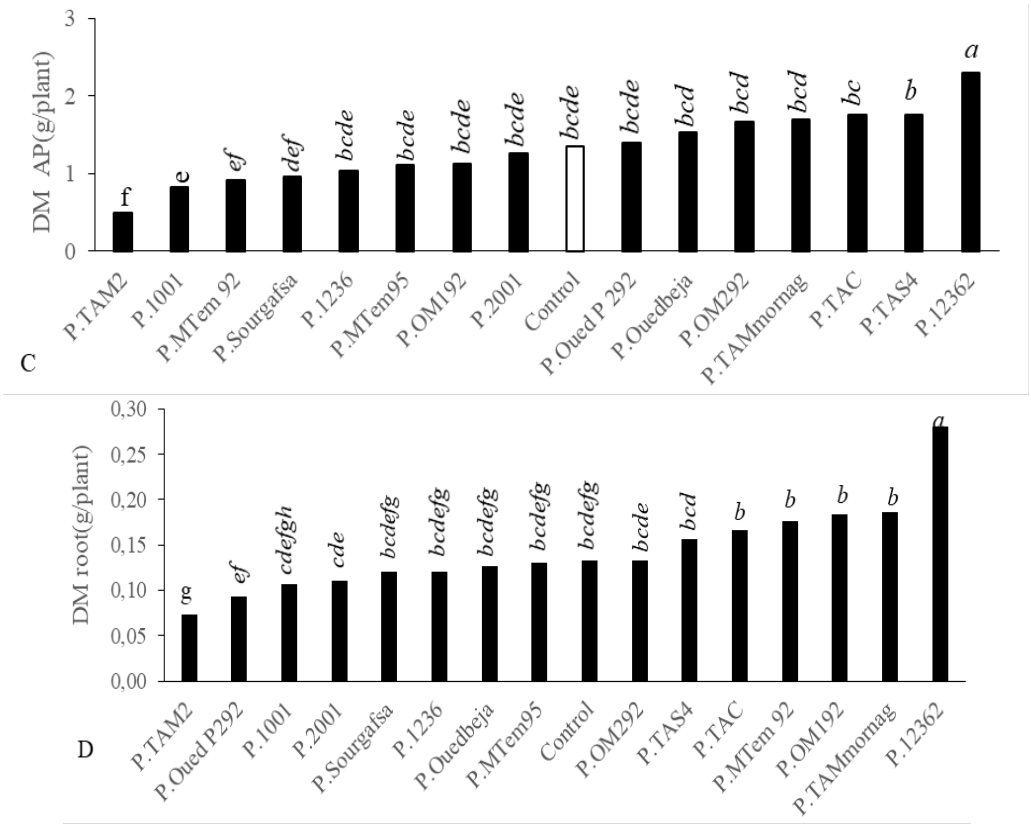


Fig. 2. Effect of inoculation by rhizobia strains on dry matter production of the aerial part (C) and root (D) of the pea variety Lincoln under greenhouse conditions.
The values are the means of 4 repetitions and followed by different letters in a column were significantly different at 5% level of probability using Duncan's multiple range tests.

3.3. Efficiency of the use of rhizobial symbiosis

The performance of the rhizobia studied was evaluated by the efficiency of the use of rhizobial symbiosis (EUSR) which is presented as a linear regression equation as described by Zaman allah et al. (2005).The statistical analysis of the data (Fig. 3) confirmed a positive correlation between the biomass produced from the aerial and nodular parts with R^2=0.63. Our results showed that among the most efficient tested strains having a large production in terms of dry biomass, five strains are more efficient towards atmospheric nitrogen fixation, notably the strain P.12362 that seems the most infectious and the most efficient strain.

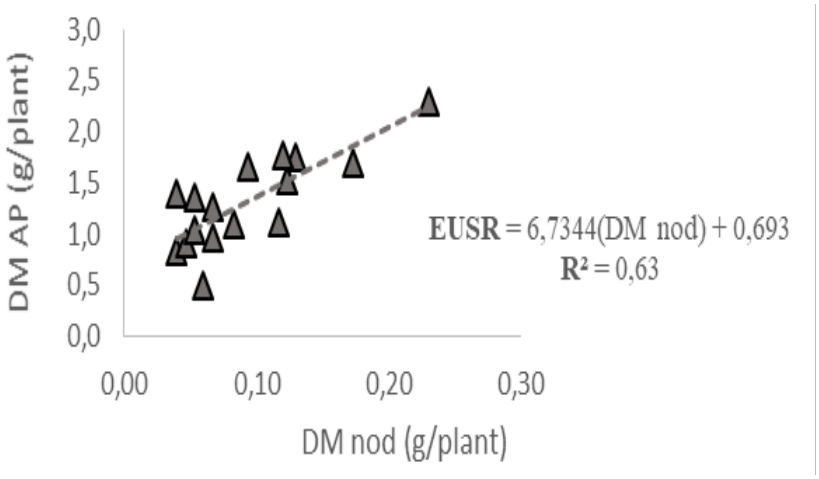


Fig. 3. Regression curve illustrating the correlation between the dry matter of the aerial part and pea nodules.

3.4. Nitrogen content of plant aerial part

Variance analyzes revealed a highly significant ($p < 0.01$) variation for nitrogen accumulated in the aerial part of peas (Fig. 4E). This nitrogen content is variable according to the *Rhizobium* strain used for the inoculation of the pea. Indeed, the results obtained showed that the inoculation of the pea by certain *Rhizobium* strains induced the accumulation of a very high nitrogen content compared to that of the control. The largest amount was obtained by pea inoculation with P.12362 strain, which was 8.83 mg $N.Pl^{-1}$. This could be explained by the ability of P.12362 strain to fix atmospheric nitrogen.

P.Oued P 292, P.1236, P.MTem95, P.Sourgafsa, P.MTem92, 2001) and highly efficient (P.12362, P.OM292, P.TAS4, P.Ouedbeja).

3.5. Effect of pH and temperature on *Rhizobium* growth

Varying analyzes revealed that there is a highly significant variation ($p < 0.01$) between the different strains of rhizobia nodulating pea. Indeed, all strains can grow normally in media with a pH between 5 and 8. However, a better growth has taken place at acidic pH, especially the pH 5.5, and 6 and 6.5 and neutral pH, which consequently qualifies these strains as acidophilic. However, for the alkaline pH,

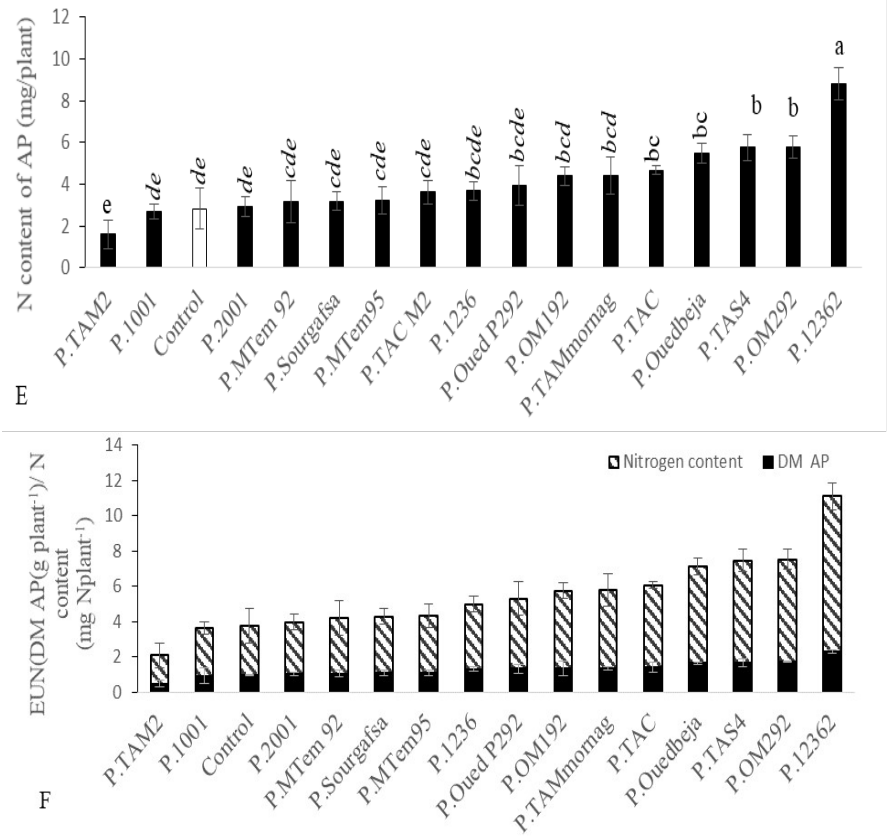


Fig. 4. Evaluation of the total nitrogen content accumulated in the aerial part of the pea inoculated by different rhizobia strains (E) and the efficiency of nitrogen use (NUE) (F) according to the ratio biomass production and total accumulated nitrogen content.

The values presented are the means of 4 repetitions and followed by different letters in a column were significantly different at 5% level of probability using Duncan's multiple range tests.

The nitrogen use efficiency (NUE) accumulated in pea by assimilation and symbiotic fixation was evaluated by the ratio of aerial biomass and total nitrogen content (Fig. 4F). This ratio again shows us highly significant variations in the NUE according to the rhizobia studied. According to the NUE *Rhizobium* strains can be classified in three groups compared to the control: poorly efficient (P.1001, P.TAM2, moderately efficient (P.OM95, P.TAC, P.TAMmornag, P.OM 192,

rhizobia growth has been slowed down for some strains and has been good for other rhizobia, which has been described as basophiles. According to the results (Table 1), it can be revealed that all strains having a colony diameter greater than 1 cm at a basic pH of 7.5 expect one strain P.2001 has a lower diameter (0.6 cm) whereas for pH 8, only 12 strains that are able of grow normally.

Table 1. Growth of rhizobia nodulating pea on media at acidic, neutral and alkaline mediums after incubation for 7 days at 28°C.

	Colony diameter (cm)						
Rhizobium strain	**pH=5**	**pH=5.5**	**pH=6**	**pH=6.5**	**pH=7**	**pH=7.5**	**pH=8**
P.OM192	1.50^{b}	0.85^{ghi}	0.95^{ghi}	1.20^{bcde}	1.95^{bc}	1.90^{cd}	1.65^{f}
P.TAM2	1.25^{cd}	1.00^{efg}	1.35^{cd}	1.35^{bcd}	1.75^{de}	2.50^{a}	2.40^{ab}
P.TAS4	1.35^{cd}	1.00^{efg}	1.15^{ef}	1.30b^{cde}	1.90^{bc}	2.15^{b}	1.85^{de}
P.OM95	1.25^{cd}	1.00^{efg}	1.35^{cd}	1.35^{bcd}	1.75^{de}	2.50^{a}	2.40^{ab}
P.Oued P 292	1.20^{c}	1.35^{bc}	1.55^{b}	0.95^{defg}	1.10^{h}	1.25^{hij}	0.95^{ij}
P.MTem 92	1.00^{f}	1.35^{bc}	1.25^{de}	1.60^{ab}	1.85^{cd}	2.10^{b}	2.35^{b}
P.Ouedbeja	0.95^{fg}	1.15^{cde}	1.50^{bc}	1.30^{bcde}	1.55^{fg}	1.55^{e}	1.55^{f}
P.TAC	0.95^{fg}	0.75^{hi}	0.55^{k}	0.70^{fg}	1.75^{de}	1.55^{e}	1.00^{ij}
P.1001	0.85^{gh}	1.10^{def}	1.00^{fgh}	1.35^{bcd}	2.00b	2.20^{b}	1.40^{g}
P.2001	0.80^{hij}	0.80^{ghi}	0.95^{ghi}	0.75^{fg}	0.70^{i}	0.60^{k}	0.50^{k}
P.12362	0.75^{ij}	0.65^{ij}	0.85^{hij}	0.90^{efg}	1.55^{fg}	1.35^{g}	0.95^{ij}
P.1236	0.70^{ij}	0.90^{fgh}	1.00^{fgh}	0.75^{fg}	1.85^{cd}	2.05^{bc}	2.35^{b}
P.TAMmornag	0.70^{ij}	0.85^{ghi}	1.25^{de}	1.50^{bc}	1.55^{fg}	1.10^{hij}	0.90^{ij}
P.MTem95	0.70^{ij}	0.50^{j}	0.95^{ghi}	1.25^{bcde}	2.15^{a}	2.45^{a}	2.50^{a}
P.Sourgafsa	0.65^{j}	0.90^{fgh}	1.45^{bc}	1.10^{cdef}	1.75^{de}	1.75^{d}	1.95^{d}
ANOVA Test(F)	*77.16***	*17.33***	*36.35***	*6.77***	*88.59***	*98.95***	*168.21***

** Highly significant at 1% level of probability,values presented are the means of 4 repetitions and followed by different letters in a column were significantly different at 5% level of probability using Duncan's multiple range test.

Concerning the influence of temperature on rhizobia growth, obtained result showed a highly significant effect of the response of rhizobial strains to temperature regardless of degree (Fig. 5).The majority of isolates are capable to growing at temperatures ranging from 10°C to 45°C. Indeed, optimal growth has been observed in the range of 20°C to 35°C. However, few strains have tolerance for high temperatures.

3.6. Tolerance to calcium carbonate ($CaCO_3$)

Variance analyzes of rhizobia tolerance to Calcium Carbonate $CaCO_3$ stress revealed significant variation at the 5% level between different strains nodulating pea at three concentrations of 0.5 M, 1 M and 1.5 M $CaCO_3$ (Fig. 6). Normal growth of all 15 Rhizobia with three doses of calcium carbonate. What qualifies these rhizobia as calcicole strains.

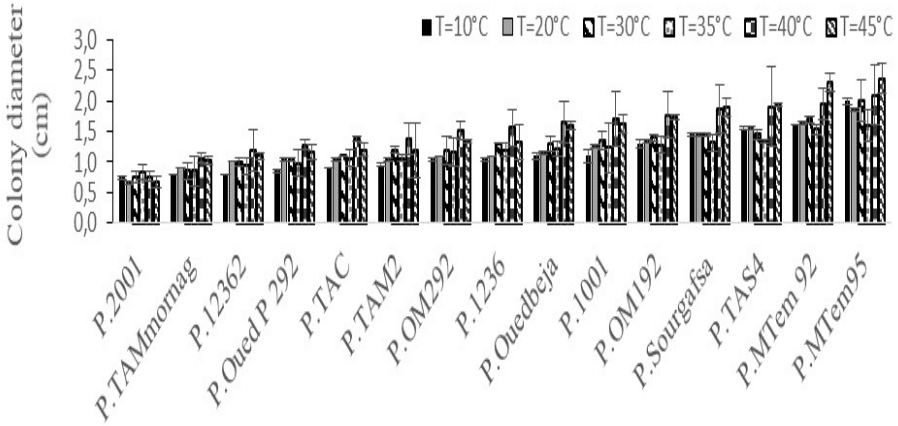


Fig. 5. Tolerance of rhizobia strains nodulating pea to thermal variations.
The values presented are the means of 4 repetitions (Duncan p <0.05).

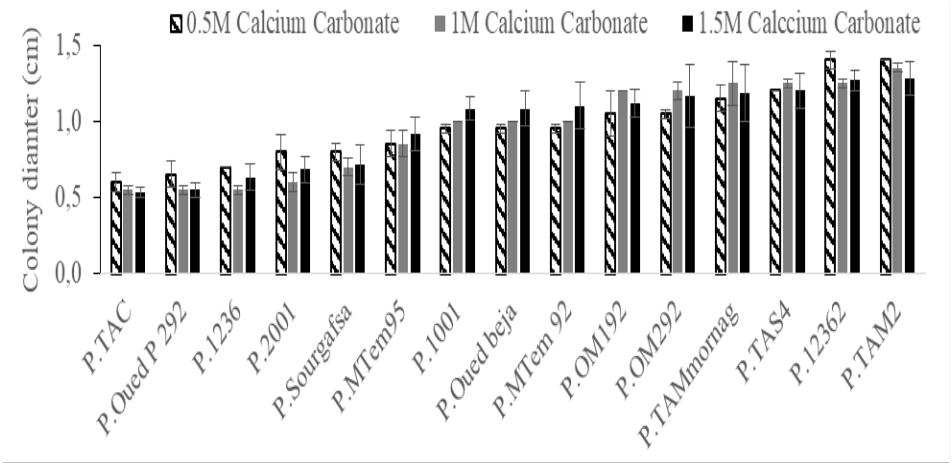


Fig. 6. Growth evaluation of studied rhizobia on medium at three concentrations of calcium carbonate. *The values presented are the means of 4 repetitions and* were significantly different at 5% level of probability using Duncan's multiple range test.

However, six of 15 strains are found to have a colony diameter of less than 0.8 cm, reflecting the inhibitory effect of high calcium content on growth of strains in the free state (Dardanelli et al., 2003). Indeed, this growth ability is dependent on the Ca^{2+} ion, which is one of the constituent of the plasma membrane of the bacterium (Gobat et al., 1998). These results confirm those obtained by the Fitouri et al. (2012) study on *sulla* crop (*Hedysarum coronarium* L.).

3.7. Salinity

A considerable variability in colony diameter of different *Rhizobium* strain has been shown. A higher significant effect ($p < 0.01$) was revealed for all strains (Table 2). The diameter of the colonies decreases as the dose of salt increases. It ranged from 2.05 to 0.15 cm respectively observed at doses of 25mM and 125 mM of Salt. The most tolerate concentrations of salt that the majority of rhizobia can grow on were 50 mM and 75 mM where the diameter of colony was almost stable except the strain P.2001 which had the largest diameter throughout the different salt concentrations. The inhibitory effect of salt on the growth of bacteria was shown notably at higher concentrations of NaCl (100, 125 and 150 mM) for following strains of *Rhizobium* P.MTem95, P.Ouedbeja, P.1001, P.TAS4.

3.8. Phosphorus solubilization

Results showed that there is a highly significant variation at the threshold of 1% of diameter and the halo surrounding the colony of the rhizobia strain tested. The high diameter values were

Table 2. Colony diameter of different *Rhizobium* strains (cm) assessed on medium culture containing different salt doses (0, 25, 50, 75,100,125,150 mM of NaCl). *The values presented are the means of 4 repetitions (Duncan p <0.05).*

Strains	Control	25 mM	50mM	75mM	100mM	125mM	150mM
P.2001	2.25^{a}	2.05 ^{a}	1.85^{a}	1.55^{a}	1.20^{a}	0.95^{a}	1.05^{a}
P.MTem95	1.00^{b}	0.45^{d}	0.65^{cde}	0.35^{c}	0.40^{c}	0.15^{f}	0.30^{e}
P.Ouedbeja	0.90^{b}	0.85^{bc}	0.65^{bcd}	0.60^{bc}	0.35^{c}	0.35^{ef}	0.45^{cde}
P.TAC	0.85^{b}	0.75^{bcd}	0.45^{e}	0.50^{c}	0.55^{bc}	0.50^{bcde}	0.55^{bc}
P.OM192	0.85^{b}	0.65^{bcd}	0.80^{b}	0.75^{b}	0.55^{bc}	0.50^{bcde}	0.40^{bcd}
P.Oued P 292	0.85^{b}	0.75^{bcd}	0.45^{de}	0.50^{c}	0.45^{bc}	0.30^{ef}	0.45^{cde}
P.1001	0.85^{b}	0.80^{bcd}	0.75^{bc}	0.55^{bc}	0.65^{bc}	0.40^{cde}	0.30^{cde}
P.OM 95	0.85^{b}	0.75^{bcd}	0.65^{bcd}	0.70^{bc}	0.60^{bc}	0.55^{bcd}	0.45^{bcd}
P.12362	0.75^{b}	1.05^{b}	0.55^{bcd}	0.75^{b}	0.55^{b}	0.70^{b}	0.60^{bc}
P.Sourgafsa	0.75^{b}	0.55^{d}	0.60^{cde}	0.55^{bc}	0.50^{bc}	0.40^{cde}	0.45^{cde}
P.OM292	0.70^{b}	0.55^{d}	0.65^{bc}	0.75^{b}	0.60^{b}	0.65^{bcd}	0.55^{bc}
P.TAMmornag	0.65^{b}	0.55^{d}	0.65^{bc}	0.65^{bc}	0.60^{b}	0.65^{bcd}	0.50^{bcd}
P.TAS4	0.60^{b}	0.65^{bcd}	0.85^{b}	0.65^{b}	0.45^{bc}	0.35^{def}	0.40^{cde}
P.1236	0.60^{b}	0.75^{bcd}	0.80^{b}	0.75^{b}	0.55^{b}	0.65^{bcd}	0.50^{bcd}
P.TAM2	0.60^{b}	0.65^{d}	0.70^{bcd}	0.60^{bc}	0.50^{bc}	0.40^{def}	0.45^{bcd}
P.MTem 92	0.60^{b}	1.05^{bcd}	0.65^{bcd}	0.70^{bc}	0.40^{bc}	0.50^{cde}	0.45^{cde}
ANOVA Test (F)	17.56**	16.12**	18.46**	13.03**	5.70**	7.12**	6.17**

** Highly significant at 1% level of probability, value followed by different letters in a column was significantly different at 5% level of probability using Duncan's multiple range tests.

observed for the following strains P.12362, P.TAC, P.1263 with the highest value being given to strain P.12362, which is of the order 3.02 cm. This attributes to them the high potential solubilization of phosphorus (Table 3). This index is calculated according to Berraquero et al. (1976). Indeed, this index allows us to classify bacteria according to their level of the solubilization potential of inorganic phosphorus. Indeed, this index allows us to classify bacteria according to their level of the solubilization potential of inorganic phosphorus. If IS <2: weak solubilization. 2 ≤ IS <4: medium solubilization and IS ≥ 4: high solubilization. The statistical analysis of the variance revealed a highly significant variation of the phosphorus solubilization index between the different strains of rhizobia. According to Berraquero et al. (1976), we can classify bacteria according to their potentiality of solubilization inorganic phosphorus in three groups:

The first group including IS ≥ 4 which includes bacteria with a high potential solubilization of inorganic phosphorus. These rhizobia were: P.12362, P.1236, P.TAC showing clear halo zone around their colonies on PVK agar medium. The halo diameter of these strains ranged from 3.02 to 2.17 cm. The second group whose solubilization index is between 2 ≤ IS <4 characterized by medium solubilization and rhizobia were: P.Sourgafsa, P.1001, P.MTem95, P.OM95, P.TAMmornag, P.2001. The size of clear zone of their colonies varied from 1.35 to 1.05 cm. The third group whose solubilization index of less than 2 reflecting the low ability of bacteria to solubilize inorganic phosphorus. These bacterial strains were: P.Ouedbeja, P.TAM2, P.OM192, P.TAS4, P.MTem92, P.OM292, P.OuedP292. The size of zone of solubilization around their colonies was less than 1 cm.

Table 3. Evaluation of different rhizobia growth and their effect on medium pH variability and phosphorus solubilization.

Strains	Optical density	Final pH	Halo (cm)	IS Pi
P.12362	0.47^{c}	6.47^{ab}	3.02^{a}	6.05^{a}
P.1236	0.35^{bc}	6.04^{ab}	2.40^{b}	4.80^{b}
P.TAC	0.37^{abc}	5.77^{abc}	2.17^{b}	4.35^{b}
P.Sourgafsa	0.46^{bc}	6.00^{ab}	1.35^{cd}	2.70^{cd}
P.MTem95	0.26^{bc}	5.32^{abcde}	1.07^{de}	2.15^{de}
P.1001	0.35^{abc}	5.50^{abcd}	1.07^{de}	2.15^{de}
P.TAMmornag	0.16^{bc}	5.14^{abcde}	1.05^{de}	2.10^{de}
P.OM 95	0.38^{bc}	6.18^{ab}	1.05^{de}	2.10^{de}
P.2001	0.23^{bc}	5.22^{abcde}	0.95^{de}	1.95^{de}
P.TAM2	0.30^{bc}	5.4^{abcd}	0.82^{ef}	1.65^{ef}
P.Ouedbeja	0.41^{abc}	2.86^{e}	0.82^{ef}	1.65^{ef}
P.TAS4	0.19^{bc}	5.19^{abcde}	0.75^{ef}	1.50^{ef}
P.MTem 92	0.99^{bc}	4.01^{bcde}	0.50^{fg}	1.00^{fg}
P.OM292	0.28^{bc}	5.59^{abcd}	0.22^{g}	0.45^{g}
P.Oued P 292	0.45^{bc}	4.70^{abcde}	0.17^{g}	0.35^{g}
control	0.0^{5c}	6.67 ^{a}	-	-
ANOVA Test(F)	2.13*	2.94**	27.54**	27.54**

** highly significant at 1% level of probability, * significant at 5% level of probability, value followed by different letters in a column were significantly different at 5% level of probability using Duncan's multiple range test.

3.9. Biochemical substrates assimilation test: API Galleries

This test is used to characterize biochemically rhizobia strains already collected and previously undergone symbiotic, physiological and agronomic tests. Fifteen rhizobia strains were tested on 22 biochemical substrates using the API tunnel technique to determine their ability to assimilate and metabolize these substrates. Indeed, the comparison of the behavior of the different strains with respect to the substrates absorbed according to the UPGMA (Unweighted Pair Group Method with Arithmetic mean) method revealed a variability of assimilation according to the strains tested.

The dendrogram presented in Fig. 7 shows a heterogeneous distribution between the rhizobia thus reflecting a carbohydrate assimilation heterogeneity, which implies genetic diversity.This could be explained by the absence or presence of the enzyme catalyzing these substrates and therefore, we can speak of an enzymatic specificity with respect to the biochemical substrates.

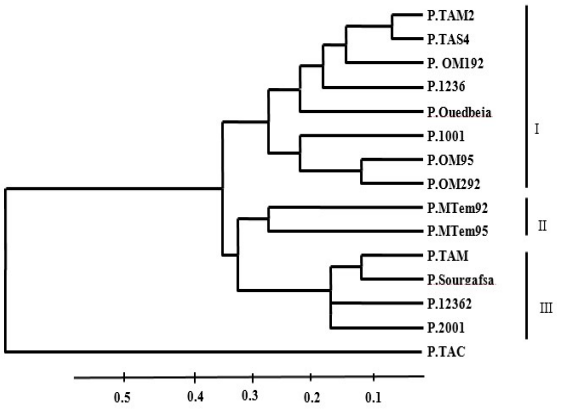


Fig. 7. Dendrogram showing the similarity and genetic diversity of rhizobia studied.

As a result, the *Rhizobium* strains can be classified into three groups and two subgroups according to the percentage of similarity which is of the order of 79% (Table 4). The first group formed by 8 strains of rhizobia with a strong ability to metabolize a wide range of biochemical substrates. The following strains P.TAM2, P.TAS4, P.OM192, P.1236, P Ouedbeja , P.OM92, P.OM 95 and P.1001.The substrates metabolized by these strains are: 2-nitrophenyl-βD-galactopyranoside (ONGP), D-sucrose (SAC), D-melibiose (MEL), Amygdalin (AMY), Sodium pyruvate (VP), Gelatin of bovine origin (GEL), D-mannitol (MAN), Inositol (INO), L-lysine (LDC), L-Tryptophan (TDA), Indol (IND), D-glucose (GLU),D-sorbitol (SOR), L-rhamnose (RHA), L-arabinose (ARA), Trisodium citrate (CIT), NO2 and N2. The second group is formed only by two rhizobia strains namely P.MTem92 and P.MTem95. These two strains are capable of assimilating the following substrates: L-arginine (ADH), L-lysine (LDC), L-ornithine (ODC), Trisodium citrate (CIT), Urea (URE), L-tryptophan (TDA) , Indol (IND), Gelatin (GEL), D-glucose (GLU), D-mannitol (MAN), Inositol (INO), D-sorbitol (SOR), L-rhamnose (RHA), D-sucrose (SAC), D-melibiose (MEL), Amygdalin (AMY), L-arabinose (ARA). The third group contains 5 rhizobia strains namely P12362, P2001, P TAC, PTAM MORNAG and P.SOURGAFSA. These strains are capable of metabolizing: 2-nitrophenyl-βD-galactopyranoside (NGGO), Trisodium citrate (CIT), L-Tryptophan (TDA), Indol (IND), Sodium pyruvate (VP), Gelatin (GEL), D-glucose (GLU), D-mannitol (MAN), Inositol (INO), D-sorbitol (SOR), L-rhamnose (RHA), D-sucrose (SAC), D-melibiose (MEL), Amygdalin (AMY), L -arabinose (ARA), NO2 and N2.

4. DISCUSSION

In this study, generally all parameters were significantly affected by rhizobia strains genotype.

Assessment of symbiotic performances of rhizobia was based on determinating the number of nodules on pea roots and dry weight of above and below ground parts of pea plants as well as nitrogen content in aerial part. The biomass dry matter production revealed as good for the vegetative part as well as for the root

Table 4. Assimilation of biochemical substrates by different of *Rhizobium* strains nodulating pea

	Strains															
Biochemical substrate	*P.12362*	*P.TAC*	*P. OM192*	*P.1236*	*P. 2001*	*P.Sourgafa*	*P.TAMmornag*	*P.MTem 92*	*P.MTem95*	*P.OM 292*	*P .TAM 2*	*P. Oouedbeja BEJA*	*P. 1001*	*P. TAS 4*	*P .OM 95*
ONGP	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
ADH	-	+	-	-	+	+	+	+	+	-	-	-	-	-	-
LDC	-	+	-	+	-	+	+	+	+	-	-	-	-	-	-
ODC	-	+	-	-	+	+	+	+	+	-	-	-	-	-	-
CIT	+	-	+	-	+	+	+	-	+	-	+	+	+	+	-
H_2S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
URE	-	+	-	-	-	-	+	+	+	-	-	-	-	+	-
TDA	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+
IND	+	-	-	-	+	+	-	+	-	-	-	-	-	-	-
VP	+	+	+	+	+	-	+	-	+	+	+	+	+	+	+
GEL	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
GLU	+	-	-	-	+	+	+	+	-	+	-	-	+	-	+
MAN	+	-	+	+	+	+	+	+	+	+	+	+	-	+	+
INO	+	-	+	+	+	+	+	+	-	+	+	+	-	+	+
SOR	+	-	+	+	-	+	+	+	-	+	+	-	+	+	+
RHA	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+
SAC	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
MEL	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
AMY	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
ARA	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+
NO_2	+	-	-	-	+	+	+	-	-	+	-	-	+	-	-
N_2	+	-	-	-	+	+	+	-	-	+	-	-	+	-	-

(+): Presence catalyze enzyme; (–): Absent catalyze enzyme

part. This can be explained by the good specificity established between rhizobia and host plant pea. *Rhizobium* strains improve the plant growth and development. Whereas, we noticed also an increased biomass production of both areal and root in the control comparing to that inoculated by the other strains may be due to the reaction of the pea plant to fill the nitrogen deficit by the development of its root system.

According to the positive correlation (R^2=0.63) between the number of nodules and the dry weights of the various plant organs reflecting the good performance of the biological symbiosis fixation and the efficiency use of nitrogen derived from air (EUN). Moreover, efficiency use symbiosis rhizobia (EUSR) revealed a better of both infectivity and efficiency released by rhizobia strains nodulating pea plant. Thereby, these rhizobia can be used to promote plant growth rhizobacteria (PGPR) as well as they can be used as biofertilizers reducing the use of nitrogen fertilizer (Yanni et al., 1997; Afzal and Bano, 2008). Bacterial isolates enhanced the plant growth and increased the nutrient uptake process, which may be good for nutrient deprived soils (Singh et al., 2019).

Our findings of nodulation were in accordance with recent study found by Muniz et al. (2017) reported that the number of nodules was higher in the pea plants inoculated with the rhizobial strains as well as the biomass of pea nodules ranged from 21.6 to 165.3 mg per plant. Moreover, other studies showed a highly significant variation of nodules number and the nodular biomass of chickpea (*Cicer arietinum* L.) when inoculated with different *Rhizobium* strains (Hemissi et al., 2013). As far as, Marra et al. (2011) demonstrated that *Rhizobium etli* UFLA 02-100 and *Rhizobium leguminosarum* 316C10 are efficient nodulators of beans. However, a recent study of Yang et al. (2017) showed a significant differences for Arial part and root dry matter production in *Rhizobium*– pea interactions, which ranged from 1.8 to 4.7g and from 0.27 to 0.73 g per plant, respectively. As they demonstrated that nodule number on lateral roots ranged from 25 to 430 per plant as well as the amount of nitrogen varied from, 15 to 67 mg per plant for areal part. Also, previous study reported spatial variability of rhizobia nodulating pea (Ballard et al., 2004; Hachana et al., 2017). Moreover, other studies had shown that inoculation with *Rhizobium* improved pea productivity (Rani et al., 2016; Huang et al., 2017).These results could be attributed to the interaction between symbiotic performance of the rhizobia inoculated and the studied pea variety (He et al., 2011).

Tolerance strains to physiological parameters

Our results corroborate with those found by Abdi et al., (2014) on beans, which showed that the majority of *Rhizobium* strains nodulating bean tolerate pH < 9. Similarly, Rice et al. (2000) study showed the tolerance of rhizobia nodulating pea (*Pisum sativum*) for acidic pH (4, 5 and 6.5). Similar results were obtained on a pastoral range of legumes showing that rhizobia isolated from the roots of species such as *Hedysarum sp, Trigonella foenum-graecum* and *Vicia angustifolia* tolerate pH values ranging from 6 to 9 (Kishinevsky et al., 2003; Wei et al., 2008; Rejili et al., 2012). As, for the study Rao et al. (2002) on chickpea revealed that *Rhizobium* strains can tolerate strongly alkaline pH. Whereas, most of the host plant cannot tolerate higher alkalinity were the rhizobia can growing.

For temperature, these results are in agreement with those obtained by the study of Rejili et al. (2012) which showed that the rhizobia nodulating forage legumes (fenugreek, Sulla and vetch) support the temperature rise to 40°C. Similarly, other studies demonstrated that *Rhizobium* strains proliferate at temperatures between 20°C and 45°C (Graham *et al*., 1991; Moawad and Beck, 1991; Graham, 1992;).

Singh et al. (2011) signaled that rhizobacteria could withstand temperatures between 32-46°C during the summer and low temperatures that are between 5-15°C during the winter. Similarly, Kumar-Meena et al. (2015) study showed that rhizobia which nodulate pea roots (*Pisum sativum*) could grow at temperatures between 5°C and 25°C.

Also, Manivannan et al. (2012) reported that isolates of *Rhizobium leguminosarum* nodulating the species (*L. japonicus* and *L. pratensis*) and the *Rhizobium. Leguminosarum strain biovar viciae* ATCC 10004 tolerate the temperature of 25°C to multiply.

Salt stress

Salinity significantly inhibits *Rhizobium* strains growth. The study conducted by Delgado et al. (1994) showed that isolates of *Rhizobium*

leguminosarum nodulant *Pisum sativum* L. react differently to NaCl: 39% of the isolates are considered moderately tolerant, their multiplication is significantly reduced only from 280 mM NaCl while 75% of isolates are able to tolerate up to 100 mM NaCl only.

Phosphorus solubilization

The solubilization index of strains varied from low to high on medium with tricalcium phosphate Ca_3HPO_4. Our findings signaled that only three strains were potent to solubilize phosphate. According to study of Berraquero et al. (1976), our results revealed that among twenty rhizobia strains only 12 *Rhizobium*, which their solubilization index ranged from 2 to 6, are able to solubilize inorganic phosphorus. Thus, these rhizobia can play the same role of phosphate solubilization bacteria (PSB) and consequently can dissolve different forms of phosphate. Therefore, they can be used as biofertilizers. The highest of solubilization may be interpreted as a positive characteristic that considered as the most predominant forms of phosphate in soil Mediterranean region mainly in Tunisia.

These results confirmed the previous investigations: Kumar-Meena et al. (2015) who showed that the phosphorus solubilization index of rhizobia nodulating pea (*Pisum sativum*) varies from 2.1 to 4.1. Similarly, Marra et al. (2011) who reported that rhizobia stains were able to solubilize, in both liquid and solid media, different forms of phosphorus like $CaHPO_4$, $Al(H_2PO_4)_3$ and $FePO_4.2H_2O$. Others studies showed that strains of *Rhizobium* had greater potential to solubilize tri-calcium phosphate comparing to *Bradyrhizobium* strains (Qureshi et al., 2012; Verma et al., 2014; Singh et al., 2015).

Biochemical substrates assimilation test: API Galleries

Indeed, following these obtained results, a great assimilation diversity of carbohydrates implying a genetic diversity. This variability of the response is due to the growth rate of rhizobia towards the carbohydrate metabolisms of each strain. Several investigations have shown that some bacteria have a slow growth on the carbon source while the fast-growing bacteria are able to multiply rapidly on various depending only on carbohydrates (Van Rossum et al., 1995; Maatallah et al., 2001). Our results are in agree with those found by Ltaief et al. (2007) who demonstrated the same classification of rhizobia nodulating chickpea.

5. CONCLUSION

The current study insists in the tolerant factors to abiotic constraints such as variations in temperature, pH, calcium carbonate, salinity, inorganic phosphorus on the *Rhizobium* strains growth and their ability to metabolize treated carbonaceous substances. Our results revealed that certain rhizobia isolated from pea roots which were efficient and more effective as well as had high phosphorus solubilization capacity, with a solubilization index greater than 4, can act as PGPR as well as PSB either on pea production and rhizosphere and consequently they can be used as biofertilizers as respectfully friendly to environment. Therefore, these rhizobia contribute in improvement of the soil health, and evaluating effect of its inoculation on pea growth and development. According to the biochemical substrate assimilation test, our results showed that the reproducibility is satisfactory with an average similarity coefficient of 79% for the strains tested. Rhizobia strains isolated from *Pisum sativum* split into three independent groups with significant assimilation similarity. This confers the diversity and heterogeneity of *Rhizobium* strains nodulating pea. Thus, we can conclude that, whatever the origin of the native or introduced bacterial strain; the compatibility of the strain is the same. And their use seems to be as a potential strategy that has a positive effect on the production and development of pea. According to our investigation, we recommend that selected strain can be considered as a new alternative microbiological and biotechnological solution for sustainable agriculture. Therefore, it is vital to seek for diversed and efficient strains of rhizobia and conduct them in open field in order to test their positive impact on the pea production on sustainable basis as consequently to be confirmed.

References

Abdi N, L'taief B, Hemissi I, Bouraoui M, Maazaoui H and Sifi B. (2014). Nitrogen and Phosphorus fertilization effect on Rhizobia-common bean symbiosis. Annales de l'INRAT, vol 87, pp.31-33.

Abdi, N., Hemissi, I., Bouraoui, M., L'taeif, B. Sifi, B. (2015). Effect of salinity on common bean (*Phaesolus vulgaris* L.) *Sinorhizobium* strain

symbiosis. Journal of new sciences. Agri Biotechnol, 16, 559-566.

Afzal A. and Bano A. (2008). Rhizobium and phosphate solubilizing bacteria improve the yield and phosphorus uptake in wheat (*Triticum aestivum* L.). International Journal of Agriculture and Biology, 10: 85-88.

Ballard RA , Charman N, McInnes A and Davidson J A. (2004). Size, symbiotic effectiveness and genetic diversity of field pea rhizobia (*Rhizobium leguminosarum bv. viciae*) populations in South Australian soils. Soil Biology and Biochemistry, 13: 1347-1355.

Bargaz A, Drevon JJ, Oufdou, K,Mandri B, Faghire M and Ghoulam C. (2011). Nodule phosphorus requirement and O2 uptake in common bean genotypes under phosphorus deficiency. Soil and Plant Science. 1-10.

Bargaz A, Zaman-Allah M, Farissi M, Lazali M, Drevon JJ, Maougal RT and Carlsson G. (2015). Physiological and Molecular Aspects of Tolerance to Environmental Constraints in Grain and Forage Legumes. International Journal of Molecular Sciences, 16: 18976-19008.

Berraquero FR, Baya AM and Cormenzana AR. (1976). Establishment of indices for the study of phosphate solubilization by soil bacteria. Ars Pharmacéutica, 17: 399-406.

Daimon H, Nobuta K, Ohe M, Harada J and Nakayama Y. (2006). Tricalcium phosphate solubilizing by root nodule bacteria of *Sesbania cannabina* and *Crotalaria juncea*. Plant Production Science, 9: 388-389.

Dardanelli M, Angelini J and Fabra A. (2003). A calcium-dependent bacterial surface protein is involved in the attachment of rhizobia to peanut roots. Canadian Journal of Microbiology, 49: 399-405

Delgado MJ, Ligero F and Lluch C. (1994). Effects of salt stress on growth and nitrogen fixation by pea, faba bean and soybean plants. Soil Biology and Biochemistry, 26: 31-376.

Denison RF and Kiers ET. (2004). Why are most rhizobia beneficial to their plant hosts, rather than parasitic?. Clinical Microbiology and Infection, 6:1235-1239

Dumont E, Bahrman N, Goulas E, Valot B, Sellier H and Hilbert JL. (2011). A proteomic approach to decipher chilling response from cold acclimation in pea (*Pisum sativum* L.). Plant Science, 180: 86–98.

Farissi M, Faghire M, Bouizgaren A, Bargaz A, Makoudi B and Ghoulam C. (2014). Growth, nutrients concentrations and enzymes involved in plants nutrition of alfalfa populations under saline conditions. Journal of Agriculture Science and Technology, 16: 301–314.

Fitouri-Dhane S, Trabelsi D, Saïdi S, Zribi K, Ben Jeddi F and M'hamdi R. (2012). Diversity of rhizobia nodulating sulla (Hedysarum coronarium L.) and selection of inoculant strains for semi-arid Tunisia. Annals of Microbiology, 62 : (1)77-84.

Gobat JM, Aragno M and Matthey W. (1998). Le sol vivant. Bases de pédologie, biologie des sols. Presses polytechniques et universitaires romandes, pp 458-463.

Gomes NCM, Heuer H, Schoenfeld J, Costa R, Mendoca-Hagler L, Smalla K. (2001). Bacterial diversity of the rhizosphere of maize (*Zea mays*) grown in tropical soil studied by temperature gradient gel electrophoresis. Plant and Soil, 232: 167-180.

Graham A. 1992. The current status of the legume fossil record in the Caribbean Region. In Herendeen, P.S., Dilcher, D.L. (eds.), Advances in Legume Systematics: Part 4, The Fossil Record. The Royal Botanic Gardens, Kew., pp. 161–167

Graham PH, Sadowsky MJ, Keyser HH, Barnet YM, Bradley RS, Cooper JE, Deley DJ, Jarvis BDW, RoslyCcky EB, Strijdom BW and Young JPW. (1991). Proposed minimal standards for the description of new genera and species of root-and stemnodulating bacteria. International Journal of Systematic Bacteriology, 41:582-587.

Hachana A, Hemissi I, Cherif H, Hlel D, Bouraoui M, Abdi N, Maazaoui H, Cherif A and Sifi B. (2017). Diagnosis of Biodiversity of *RhizobiumLeguminosarum* Nodulating Pea (*Pisum Sativum* L.) in Differents Soils of Tunisia. Recent Advances in Environmental Science from the Euro-Mediterranean and surrunding regions. Conference for Environmental Integration (EMCEI-1), Tunisia.

He Y, Guo L, Zhang H and Huang G. (2011). Symbiotic effectiveness of pea-rhizobia associations and the implications for farming systems inthe western Loess Plateau, China. African Journal of Biotechnology, 10: (18): 3540-3548.

Hemissi I, Abdi N, Bargaz A, Bouraoui M, Mabrouk Y, Saidi M and Sifi B. (2015). Inoculationwith Phosphate solubilizing Mezorhizobium strains improves the Performance of chickpea (*Cicer aritenium* L.) under Phosphorus deficiency. Journal of Plant Nutrition, 38: 1656-1671.

Hemissi I, Mabrouk Y, Abdi N, Bouraoui M, Saidi M and Sifi B. (2011). Effects of some *Rhizobium*

strains on chickpea growth and biological control of *Rhizoctonia solani*. African Journal of Microbiology Research, 5: (24): 4080-4090.

Hemissi I, Mabrouk Y, Abdi N, Bouraoui M, Saidi M and Sifi B. (2013). Growth promotion and protection against Orobanche foetida of chickpea (*Cicer aerietinum*) by two Rhizobium strains under greenhouse conditions. African Journal of Biotechnology, Vol. 12: (12) 1371-1377.

Henault C and Revellin C. (2011). Inoculants of leguminous crops for mitigating soil emissions of the greenhouse gas nitrous oxide. Plant and Soil, 346(1-2):289-296.

Huang J, Afshar RK, Tao A and Chen C. (2017). Efficacy of starter N fertilizer and rhizobia inoculant in dry pea (*Pisum sativum* L.) production in a semi-arid temperate. Soil Science and Plant Nutrition, 63, 3: 248–253

Hussain M, Asgher Z, Tahir M, Ijaz M, Shahid M, Ali H and Sattar A. (2016). Bacteria in combination with fertilizers improve growth, productivity and net returns of wheat (*Triticum aestivum* L.). Pakistan Journal of Agriculture Sciences, 53: 633-645.

Kersters K, Hinz KH, Hertle A, Segers P, Lievens A, Siegmann Oand De Ley J. (1984). *Bordetella avium* sp. nov., isolated from the respiratory tracts of turkeys and other birds. International Journal of Systematic Bacteriology, 34: 56–70.

Kishinevsky BD, Nandasena KG, Yates RJ, Nemas C, Howieson JG. (2003). Phenotypic and genetic diversity among rhizobia isolated from three *Hedysarum* species: *H. spinosissimum*, *H. coronarium* and *H. flexuosum*. Plant and Soil, 251:143–153.

Kouki S, Abdi N, Hemissi I, Bouraoui M, Sifi B. (2016). Phosphorus fertilization effect on common bean (*Phaseolus vulgaris L.*) -rhizobia symbiosis. Journal of new sciences, Agri Biotech, 25(1):1130-1137.

Kumar Meena R, Kumar Singh R, Pal Singh N, Kumari Meena S, Singh Meena V. (2015). Isolation of low temperature surviving plant growth –promoting rhizobacteria (PGPR) from pea (*Pisum sativum* L.) and documentation of their plant growth promoting traits. Biocatalysis and Agricultural Biotechnology, (4)4: 471-479.

L'taief B, Sifi B, Zaman-Allah M, Drevon JJ, Lachaal M. (2007). Effect of salinity on root – nodule conductance to the oxygen diffusion in the *cicer arietinum-Mesorhizobium cicer* symbiosis. Plant physiology, 164: 1028-1036.

Lynch DH, Smith DL. (1993). Soybean (Glycine max) nodulation and N2 fixation as affected by exposure to a low root-zone temperature. Physiologia Plantarum, 88: 212–220.

Maatallah J, Berraho EB, Sanjuan J and Lluch C. (2002). Phenotypic characterization of rhizobia isolated from chickpea (*Cicer arietinum*) growing in Maroccan soils. Agronomy, 22: 321-329.

Maazaoui H, Drevon JJ, SIFi B. (2016). Uptake and nitrogen fixation in a Tunisian multi local field test. Journal of new sciences. Agricultural and Biotechnology, 30(10): 1806-1811

Mahdhi M, Mars M. (2006). Genotypic diversity of rhizobia isolated from *Retama raetam* in arid regions of Tunisia. Annals of Microbiology, 56: 305–311.

Mahdhi M, Nzoué A, de Lajudie P, Mars M. (2008). Characterization of *Retama raetam* root nodules bacteria in arid Tunisian soils. Progress in Natural Science, 18: 43–49.

Mahdhi M, Nzoué A, Gueye F, Merabet C, de Lajudie P, Mars M. (2007). Phenotypic and genotypic diversity of Genista saharae microsymbionts from the infra-arid region of Tunisia. Letters in Applied Microbiology. 45: 604–609.

ManivannanM, Ganesh P, Kumar RS, Tharmaraj K and Ramya BS. (2012). Isolation, screening, characterization and antagonism assay of PGPR isolates from rhizosphere of rice plants in Cuddalore District. International Journal of Pharmaceutical and Biological Archive, 3: 179-185.

Marra LM, Maria de Oliveira S, Fonsêca Sousa Soares CR and Moreira FMS. (2011). Solubilisation of inorganic phosphates by inoculant strains from tropical legumes. Scientia Agricola, 68: 603-609.

Moawad HA, Beck DP. (1991). Some characteristics of *Rhizobium leguminosarum* isolates from un-inoculated field-grown lentil. Soil Biology and Biochemistry. 23: 933–937.

Muniz AW, Dalla Costa M, Saccol de Sá EL, Fiuza AB and Bros E. (2017). Symbiotic efficiency of pea (*Pisum sativum*) rhizobia association under field conditions. African Journal of Agricultural Research. Vol. 12(32):2582-2585.

Parkinson JA and Allen SE. (1975). A wet oxidation process suitable for the determination of nitrogen and mineral nutrients in biological material. Communications in Soil Science and Plant Analysis, 6: 1–11.

Pikovskaya RI. (1948). Mobilization of phosphorus in soil in connection with vital activity of some microbial species. Microbiology, 17: 362-370.

Prosperi JM. (1993). Selection of annual medics for French Mediterranean regions. Introducing Ley Farming to the Mediterranean Basin: Christiansen, S., Materon, L., Falcinelli, M., Cocks, P. (Eds.), Proceedings of an International Workshop, Perugia, Italy. pp. 173–191

Qureshi MA, Ahmad ZA, Akhtar N, Iqbal A, Mujeeb F and Shakir MA. (2012). Role of phosphate solubilizing bacteria (PSB) in enhancing P-availability and promoting cotton growth. Journal of Animal and Plant Sciences, 22: 204–210.

Rani S, Kumar P, Kumar A, Kumar A and Sewhag M. (2016). Effect of biofertilizers on nodulation, nutrient uptake, yield and energy use efficiency of field pea (*Pisum sativum* L.). Journal of Agricultural Meteorology, 18(2):330-332.

Rao DLN, Giller KE, Yeo AR and Flowers TJ. (2002). The effects of salinity and sodicity upon nodulation and nitrogen fixation in chickpea (*Cicer arietinum* L.). Annals of Botany, 89(5): 563-570.

Rejili M, Mahdhi M, Fterich A, Dhaoui S, Guefrachi I, Abdeddayem R and Mars M. (2012). Symbiotic nitrogen fixation of wild legumes in Tunisia: Soil fertility dynamics, field nodulation and nodules effectiveness. Agriculture, Ecosystems and Environment, 157: 60– 69.

Rice WA, Clayton GW, Olsen PE and Lupwayi NZ. (2000). Rhizobial inoculant formulations and soil pH influence field pea nodulation and nitrogen fixation. Canadian Journal of Soil Science, 395-400

Roldan A, Garcia-Orenes F and Albaladejo J. (1994). Microbial populations in the rhizosphere of the Brachypodium retusum and their relationship with stable aggregates in a semiarid soil of southeastern Spain. Arid Soil Research and Rehabilitation, 8:105–114.

Singh JS, Pandey VC and Singh DP. (2011). Efficient soil microorganisms: a new dimension for sustainable agriculture and environmental development. Agriculture, Ecosystems and Environment, 140: 339-353.

Singh NP, Singh RK, Meena VS, Meena RK. (2015). Can we use maize (*Zea mays*) rhizobacteria as plant growth promoter. Vegetos, 28 (1): 86–99

Singh S, Kumar V, Sidhu GK, Daljeet SD, Singh D, Koul B, Singh HJ, Singh J. (2019). Plant growth promoting rhizobacteria from heavy metal contaminated soil promote growth attributes of Pisum sativum L. Biocatalysis and Agricultural Biotechnology, 17: 665–671.

Van Rhijn R and Vanderleyden J. (1995). The Rhizobium-plant symbiosis. *Microbiology and Molecular Biology Reviews,* 59(1):124-142.

Van-Rossum D, Schurmans FP, Gillis M, Muyotcha A, Van Verseveld HW, Stouthamer AH and Boogerd FC. (1995). Genetic and phenotypic analysis of Bradyrhizobium strains nodulating peanut (*Arachis hypogea* L.) roots. Applied and Environmental Microbiology, 61: 1599-1609.

Verma JP, Yadav J, Tiwari KN and Jaiswal DK. (2014). Evaluation of plant growth promoting activities of microbial strains and their effect on growth and yield of chickpea (*Cicer arietinum* L.) in India. Soil Biology and Biochemistry, 70: 33–37.

Vincent JM. (1970). A Manual for the Practical Study of Root Nodule Bacteria. Oxford: Blackwell Scientific.

Walpola BC, Yoon MH. (2012). Prospectus of phosphate solubilizing microorganisms and phosphorus availability in agricultural soils: a review. African Journal of Microbiology Research, 6: 6600–6605

Wei HC, Rollins J, Fabian L, Hayes M, Polevoy G, Bazinet C and Brill JA. (2008). Depletion of plasma membrane PtdIns (4,5) P2 reveals essential roles for phosphoinositides in flagellar biogenesis. Journal of Cell Science, 121 (7): 1076--1084.

West SA, Toby Kiers E, Simms Ellen L and Denison FR. (2002). Sanctions and mutualism stability: why do rhizobia fix nitrogen? Proceeding of the Royal Society of London B, 269: 685- 694.

Yang C, Bueckert R, Schoenau J, Diederichsen A, Zakeri H and Warkentin T. (2017). Symbiosis of selected *Rhizobium leguminosarum bv. viciae* strains with diverse pea genotypes: effects on biological nitrogen fixation. Canadian Journal of Microbiology, 63(11):909-919.

Yanni YG, Rizk R.Y, Corich V, Squartini A, Ninke K, Philip-Hollingsworth S, Orgambide G., De Bruijn F, Stoltzfus J, Buckley D, Schmidt TM, Mateos PF, Ladha JK and Dazzo F.B. (1997). Natural endophytic association between *Rhizobium leguminosarum bv. trifolii* and rice roots and assessment of its potential to promote rice growth. Plant and Soil, 194: 99–114

Zahran H. (2001). Rhizobia from wild legumes: diversity, taxonomy, ecology, nitrogen fixation and biotechnology. Journal of Biotechnology, 91 : 143–153.

Zaman-Allah M, Sifi B, Issoufou M, EL Aouni MH.2005. Salt tolerance of a common bean

(*Phaseolus vulgaris* L.) cultivar as affected by rhizobia. Symbiosis, 40:17-22.

Zeppenfeld T, Balkenhol N, Kóvacs K, Carminati A. (2017). Rhizosphere hydrophobicity: A positive trait in the competition for water. Plos One, 7 (12).