PGPR: A microbial Resource to Promote the Growth of Legumes and to Enhance their symbiotic potential, case of pea (*Pisum sativum*)

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ABSTRACT

Beneficial bacteria, known as plant growth promoting rhizobacteria (PGPR), can improve legume growth and symbiotic nitrogen fixation by colonizing plant roots. In pea agriculture, PGPR can enhance yield, improve stress tolerance, and lessen the need for chemical fertilizers by boosting nutrient uptake, inhibiting infections, and increasing nodule formation. In this work, examining the effects on the growth of Pisum sativum, or pea plants, this study isolates, identifies, and evaluates Plant Growth-Promoting Rhizobacteria (PGPR) from rhizospheric soil in the Tissemsilt region of Algeria. A total of eight bacterial species (B2, B4, B5, S3, S4, S5, S6, S7) were identified and characterized using morphological, biochemical, and API 20 NE methodologies. We looked at antibiotic resistance patterns and tested how well different strains could stimulate plant growth. Following the introduction of the bacterial strains to the pea seeds, the impact on fresh and dry weight as well as the number of root nodule formations was assessed. In order to identify any correlations between bacterial abundance and soil fertility, a physical-chemical analysis of the soil was conducted. The results demonstrated that certain bacterial strains, such as Pseudomonas luteola and Pseudomonas fluorescens, significantly enhanced nodulation and plant growth, implying that these microbes could serve as valuable biofertilizers for environmentally conscious farming.

Keywords: *Plant Growth-Promoting Rhizobacteria (PGPR), Pisum sativum, Legume-rhizobia interaction, Pseudomonas* sp.

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1. Introduction

Rhizobacteria that promote plant growth (PGPR) are essential for increasing plant productivity, especially in legumes. Through a variety of direct and indirect processes, these advantageous microorganisms not only promote plant growth but also strengthen legumes' capacity for symbiosis, which is essential for nitrogen fixation. Given that pea (*Pisum sativum*), a significant leguminous crop, depends on symbiotic nitrogen fixation for growth and development, PGPR is extremely beneficial to the plant (Anwar *et al.*, 2019). By enhancing the nodulation process and nitrogen-fixing capabilities, PGPR can increase the efficiency of this symbiotic connection, which will ultimately result in increased yields and better plant health.

1.1. Objective

The objective of this study is to evaluate the effect of inoculation of PGPR on the development of pea (*Pisum sativum* L), and to select indigenous strains with high plant growth promoter potential, the exploitation of pea symbiotic potential with soil symbiotic bacteria is also among the targets. Eight strains were tested, their identification was based on the determination of morphological characters and biochemical.

2. Materials And Methods

2.1. Rhizospheric soil

In order to obtain effective PGPR-effect strains, rhizosphere soil samples were taken from an agricultural plot located in the Tissemsilt region (Algeria) where GPS coordinates were taken (35° 44' 42''N 1° 32' 50''E).

2.2. Isolation of rhizospheric bacteria

The rhizospheric bacteria were isolated by the suspension-dilution method (Halvorson and Ziegler, 1933), 1 g of soil adherent to the roots is suspended in 9 ml of sterile physiological water. A series of dilutions (10^{-1} to 10^{-5}) were made from this mother solution, and 0.1 ml of each dilution was spread out on solid medium (Nutrient Agar). The plates were then incubated for 24 to 72 hours at 30°C.

2.3. Identification of bacteria isolates

The morphological criteria of colonies were studied (viscosity, colour, size, shape, contour and surface), Gram staining, catalase and oxidase test, API galleries (API 20NE, BioMérieux, France) were then used to finish and validate the biochemical characterisation of the isolates.

2.4. Antibiotic resistance test

Petri dishes containing Mueller Hinton agar were inoculated with a standardized bacterial suspension (Mc Farland 0.5) to be tested, then the antibiotic discs (Tetracycline 30 µg, Ciprofloxacin 5µg, Gentamicine 10 µg, Amoxicilline 20 µg), are deposited on the aseptic agar. The inhibitory diameters surrounding the discs were then determined after the boxes were incubated for 24 hours at 37°C.

2.5. Cultivation and inoculation of seedlings "*in vitro*"

It has been demonstrated by multiple investigations that PGPR affects plant development (Ashraf *et al.,* 2013 ; Lugtenberg et Kamilova, 2009). Through this experiment, we can assess how isolated bacteria affect the growth and development of pea (*Pisum sativum*). The ITGC Technical Institute of Large Crops issued the pea seeds used in this experiment (Safou variety).

2.6. Soil sampling

The FERTIAL agronomic laboratory performed the physico-chemical tests of the soil (granulometric) and chemicals (carbon, pH, conductivity, organic matter, nitrogen, phosphorus, etc.). These physico-chemical studies to assess soil fertility at rest are being examined, along with potential relationships between PGPR and the quantity, presence, and absence of nitrogen-fixing bacteria.

2.7. Inoculation with PGPR strains

Well-sprouted seeds were transferred to pots containing the soil. The inoculation consists of eight treatments repeated three times, including: isolated strains (B2, B4, B5, S3, S4, S5, S6, S7) and a control without inoculation. Comparing the turbidity of the seeded medium with that of McFarland 0,5, after

each pot is inoculated with 1ml of a liquid suspension of each strain at a concentration of 10^8 (CFU) ml^{-1} (Valverd *et al.*, 2006), the plants were watered daily.

When there are obvious variances between the plants, those plants should be dug up. The nodules that developed at the root level were separated, counted to determine the symbiotic power, and then dried in $CaCl_2$ tubes (Vincent, 1970). Strain efficiency is also estimated by comparing fresh and dry weight and number of nodules of inoculated plants with uninoculated controls (dry weight is measured after drying plants 24 h at 70°C).

2.8. Statistical analyses

Statistical analysis was performed using SAS version 9 windows software. Single-factor variance analysis was used to determine the effects of strain on growth: fresh, dry weight of plants and number of nodules formed, the meaning was 95% chosen.

3. RESULTS AND DISCUSSION

3.1. Isolation and biochemical identification of isolates

The isolation of bacteria from rhizospheric soil resulted in a collection of 08 isolates (B2: *Pseudomonas fluorescens*, B4: *Pseudomonas luteola*, B5: *Pseudomonas fluorescens*, S3: *Rhizobium radiobacter*, S4: *Comamonas testeroni*, S5: *Rhizobium radiobacter*, S6: *Pseudomonas flueorescens*, S7: *Pseudomonas luteola*) (Table 01)

	Strains	Colour	Shape	Surface	Gram	Catalase test	Oxidase test
B2	*Pseudomonas fluorescens*	Beige	Circular	Smooth	-	+	+
B4	*Pseudomonas luteola*	White	Circular	Smooth	-	+	-
B5	*Pseudomonas fluorescens*	White	Circular	Smooth	-	+	+
S3	*Rhizobium radiobacter*	Yellow	Circular	Smooth and viscous	-	+	+
S4	*Comamonas testeroni*	White	Circular	Smooth	-	+	+
S5	*Rhizobium radiobacter*	Yellow	Circular	Smooth and viscous	-	+	+
S6	*Pseudomonas flueorescens*	White	Circular Irregular	Sèche	-	+	+
S7	*Pseudomonas luteola*	White	Circular	Smooth and viscous	-	+	+

The isolates were Gram-negative, the colonies appeared homogeneous, circular, white color, and beige to yellow, smooth surface, and a viscosity that increases with incubation time for *Rhizobiums* strains due to excessive production of exopolysacharides (Zahran *et al.*, 1994).

Table 01: Identification of isolates

Regarding antibiotic resistance test, the PGPR strains were found isolated to be resistant to all antibiotics tested (Tetracycline, Ciprofloxacin, Amoxicillin) except for Gentamicin (Table 02).

Several researchers have proved the resistance and sensitivity of the strains studied to the antibiotics tested. According to Baron and Rowe, (2016), *Pseudomonas fluorescens* is generally susceptible to gentamicin and ciprofloxacin. Some studies indicate that *P. fluorescens* strains can acquire resistance to multidrug resistance under environmental stress conditions.

Pseudomonas luteola strain also exhibits multidrug resistance to antibiotics according to (Ahmad and Beg, 2017), and it may be sensitive to some aminoglycosides such as gentamicin, which is consistent with the results obtained in this study.

The study by Zhu and Zhang, (2020), highlighted the resistance of *Comamonas testosteroni* to different classes of antibiotics, this strain is generally resistant to β-lactams (e.g., amoxicillin) and generally susceptible to fluoroquinolones (ciprofloxacin) and aminoglycosides such as gentamicin).

Rhizobium radiobacter is a rhizosphere strain known primarily for its plant growth promoting properties. This strain exhibits resistance to tested antibiotics, but remains sensitive to Gentamicin.

The antibiotic resistance profile of *Rhizobium radiobacter* was also studied by Kanvinde and Sastry, (2015), the study shows that this strain can exhibit variable resistance to different antibiotics, and generally sensitive to tetracyclines, fluoroquinolones such as ciprofloxacin and some aminoglycosides such as gentamicin.

It has also been reported that resistance and sensitivity to these molecules depend on the type of antibiotic (Graham, 1963), its nature and even the concentration tested (Lindström and Lehtomäki, 1988). This is the case of a study conducted by Abdel-Moneim *et al.*, (1984) who studied the effect of 9 antibiotics at 4 different concentrations on the growth of 18 strains of rhizosphere bacteria, they found that the antibiotics used had a different behavior to inhibit the growth of the isolates tested.

Table 02: Antibiotic resistance of strains

Code	Strains	Tetracycline 30 µg	Ciprofloxacin 5µg	Gentamicine 10 µg	Amoxicilline 20 µg
B2	*Pseudomonas fluorescent*	R	R	S	R
B4	*Pseudomonas luteola*	R	R	S	R
B5	*Pseudomonas fluorescens*	R	R	S	R
S3	*Rhizobium radiobacter*	R	R	S	R
S4	*Comamonas testeroni*	R	R	S	R

S5	*Rhizobium radiobacter*	R	R	S	R
S6	*Pseudomonas fluorescent*	R	R	S	R
S7	*Pseudomonas luteola*	R	R	S	R

3.2. Inoculation with PGPR strains

Several studies have demonstrated the beneficial effects of PGPR (Plant Growth-Promoting Rhizobacteria) inoculation on pea (Pisum sativum) growth. The strains *Pseudomonas fluorescens*, *Rhizobium radiobacter*, *Pseudomonas luteola* and *Comamonas testosterone* are considered the group of rhizosphere bacteria that are growth-promoting par excellence.

Growth promotion by these soil microorganisms is done by the production of growth regulators such as auxins, cytokinins, gibberellins (Spaepen *et al.*, 2007), by nitrogen fixation (Jha and Kumar, 2007), the elimination of pathogens by the production of antibiotics and antifungal metabolites, by the initiation of induced systemic resistance (ISR) (Glick, 2012).

An inoculation in pots was carried out on a natural support (soil sampled and analyzed) the test allows to evaluate the effect of an inoculum supply of each strain tested on the growth of peas (*Pisum sativum*). This test allows us to select the most efficient inoculum, and to prove the belonging of the obtained isolates group of rhizobacteria stimulating plant growth.

3.3. Soil physico-chemical analysis

All the physicochemical parameters were included in Table 03. The physicochemical investigations required to determine the soil's inherent fertility were conducted at the FERTIAL agronomic laboratory.

The results show that the tested soil is a medium texture soil according to the texture triangle with acceptable internal drainage. Very low in phosphorus (ppm), and adequate in nitrogen (0.12%), slightly salty (0.19 mS/cm), low in organic matter (1.52%). It is an alkaline soil pH (8.04), and the ratio of carbon to nitrogen content indicates that the organic matter is decomposed, according to the Siddar analysis system.

Table 03: The Physico-chemical analysis of the soil samples

Component / Property	Estimate	Observation
Sand	36%	/
Silt	44%	/
Clay	20%	/
Conductivity (1/5 mS/cm)	0,19	Non saline
pH eau (1/2.5)	8,4	Alkaline
C/N	7,36 %	Weak p.p.m

Carbonates	18,09	180900,00
Active limestone	-	-
Organic material	1,52	15200,00
Total Nitrogen	0,12 Meq/100gr	1200,0 p.p.m.
Phosphorus (Olsen)	0,02	5,6
Exchangeable potassium	1,2	453,5
Magnésium échangeable	1,4	173,9
Exchangeable calcium	27,6	5533,0
Exchangeable sodium	0,1	25,3

3.4. Effect of inoculation with PGPR strains on the fresh and dry weight of pea (*Pisum sativum*)

The results obtained reveal that the tested strains have the ability to significantly ($P < 0.001$) improve fresh and dry pea (*Pisum sativum*) pea yield compared to non-inoculated controls (Fig 1 and Fig 2). Statistically this response shows a highly significant effect of strains on fresh weight ($P < 0.001$), and the most remarkable effect is that of an improvement in the growth of plants inoculated by the strains *Pseudomonas luteola* and *Pseudomonas fluorescens*.

Several researchers have already proved the performance of *Pseudomonas* sp. strains on increasing pea biomass. Ahmad *et al.*, (2008) study examines the impact of PGPR strains, including *Pseudomonas* and *Azotobacter*, on pea growth; the results show a significant increase

in fresh and dry weight, suggesting that these bacteria improve nutrient availability to plants and produce growth substances. In a similar study by Soni *et al.*, (2022), *Pseudomonas fluorescens* strain increased fresh and dry weight of pea shoots by 25–30%, illustrating the potential of the strain to enhance biomass production.

According to Vacheron *et al.*, (2013), inoculation with PGPR-active strains such as *Pseudomonas* and *Bacillus* improves the growth of aerial and root parts of pea, consequently leading to a significant increase in fresh and dry weight.

PGPR directly stimulates the growth of aerial and root parts of plants according to Bashan and Bashan, (2020) ; The study investigated the effect of inoculation with *Azospirillum* strains on legumes, including pea, and showed that this type of bacteria stimulates plant growth via the production of phytostimulants such as auxins, cytokinins, gibberellins (Spaepen *et al.*, 2007).

Phytohormone production is considered an important attribute of PGPR strains that can influence plant growth (Prikryl *et al.*, 1985 ; Spaepen *et al.*, 2007), mainly affecting roots (Salisbury, 1994) via stimulation of root subdivisions, and increasing their size and number (Hagen, 1990). Which allows a greater amount of soil to be exploited and therefore large quantities of nutrients that can be used (Ribeiro and Cardoso, 2011).

The indirect action of strains has a PGPR effect is through the elimination of pathogens by the production of antibiotics and antifungal metabolites, by initiation of systemic resistance induced (RSI) (Glick, 2012), as well as the production of secondary metabolites such as hydrogen cyanide and siderophores (Idris *et al.*, 2008) ; this is the case of a study conducted by Lugtenberg and Kamilova, (2009) on peas inoculated with *Pseudomonas fluorescens*, the study shows that *Pseudomonas* strains stimulate plant growth by producing bioactive metabolites, improving nutrient uptake and protecting plants against pathogens.

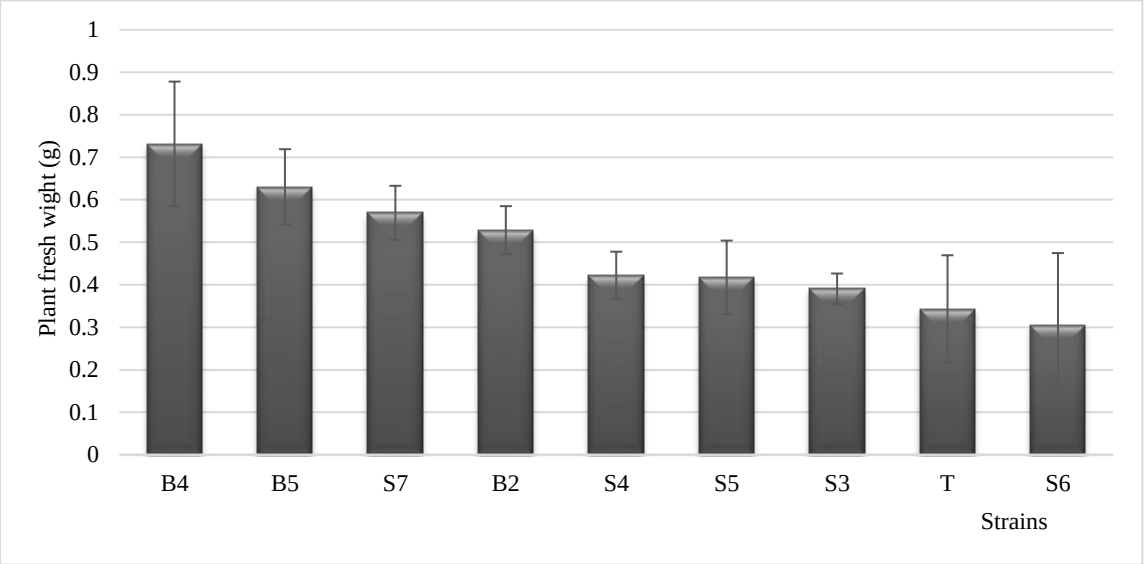

Figure 01: Effect of inoculation with PGPR strains on the plant fresh weight of pea (*Pisum sativum*). ($P < 0.001$)

B4: Pseudomonas luteola, B5: Pseudomonas fluorescens, S7: Pseudomonas luteola, B2: Pseudomonas fluorescens, S4: Comamonas testeroni S5: Rhizobium radiobacter S3: Rhizobium radiobacter, T: Control, *S6: Pseudomonas flueorescens.*

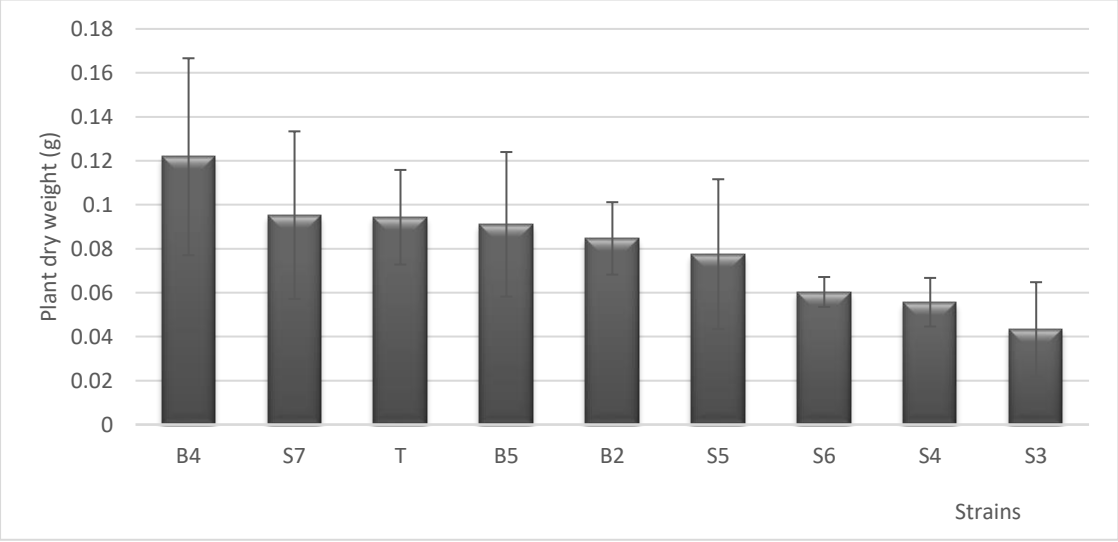

Figure 02: Effect of inoculation with PGPR strains on the plant dry weight of pea (*Pisum sativum*). ($P < 0.001$)

B4: Pseudomonas luteola, S7: Pseudomonas luteola, T: control, *B5: Pseudomonas fluorescens, B2: Pseudomonas fluorescens, S5: Rhizobium radiobacter, S6: Pseudomonas flueorescens, S4: Comamonas testeroni, S3: Rhizobium radiobacter*

3.5. Effect of inoculation with PGPR strains on the nodules number formed at the roots of pea (*Pisum sativum)*

The number of nodules formed at the plant roots estimates the symbiotic power. In this study, a better nodulation rate was recorded on pea roots (*Pisum sativum*) inoculated with the strains *Pseudomonas luteola* and *Pseudomonas fluorescens*, compared to uninoculated plants ($P < 0,05$). Therefore, the inoculation of the tested *Pseudomonas* strains strengthened the establishment of the symbiotic relationship (Fig 03 and Fig 04).

This result suggests the existence of a synergistic relationship between the tested *Pseudomonas* strains and the rhizobia that occur naturally in the soil (Sindhu *et al.*, 2002), and many studies have proven the positive effects of the combination of these two types of soil microorganisms on biological nitrogen fixation (Laabas *et al.*, 2017, Verma *et al.*, 2012),

Our study also reflects variability in the performance of strains tested, where inoculation did not significantly influence the number of nodules formed compared to the uninoculated control. This inefficiency could be attributed to the low competitiveness of the strains used compared to the indigenous strains already present in the soil, According to (Ames-Gottfred and Christie, 1989) highly competitive indigenous strains represent a competitive obstacle to the inoculum. As it has been suggested in several works that the introduction of effective strains into soils containing an indigenous population is often unsuccessful due to the competitiveness of the latter (Amarger and Lobreau, 1982).

It has also been reported that pathogenic rhizobacteria can inhibit nitrogen fixation via reduction of nodulation capacity (Burla *et al.*, 1996), inhibition of nitrogenase activity (de Freitas *et al.*, 1993).

Edaphic factors also appear to influence the synergistic effect between PGPR and soil rhizobia, and consequently the establishment of symbiosis; soil physical and chemical properties such as alkaline pH, low phosphorus and organic matter content are considered major causes affecting the symbiotic interaction (O'hara *et al.*, 1988).

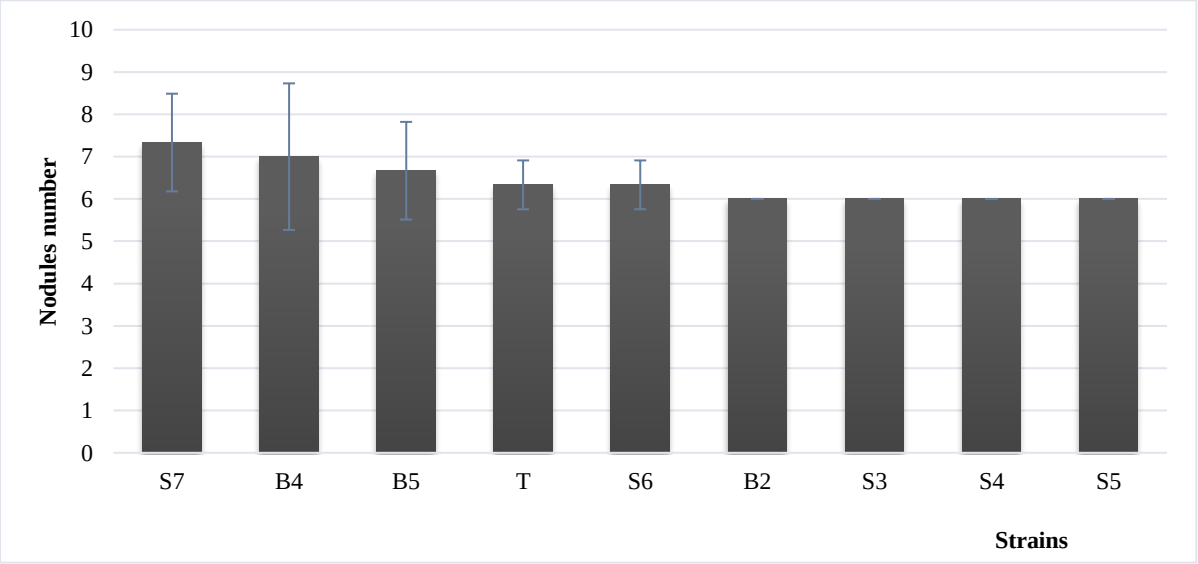

Figure 03: Effect of inoculation with PGPR strains on the nodules number formed at the roots of pea (*Pisum sativum).* ($P < 0,05$)

S7: Pseudomonas luteola, B4: Pseudomonas luteola, B5: Pseudomonas fluorescens, T: Control,

S6: Pseudomonas flueorescens B2: Pseudomonas fluorescens, S3: Rhizobium radiobacter

S4: Comamonas testeroni S5: Rhizobium radiobacter

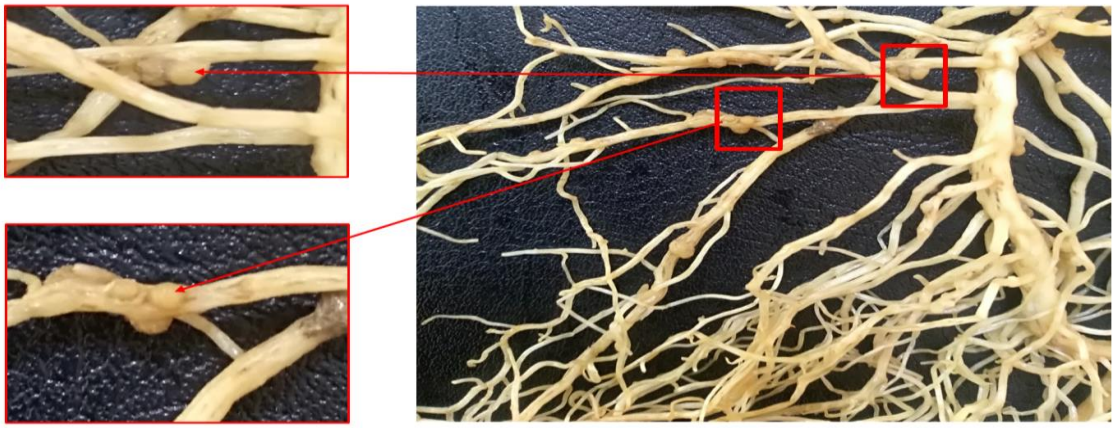

Figure 04: Nodules formed on pea roots (*Pisum sativum*)

4. Conclusion

Isolated Plant Growth-Promoting Rhizobacteria (PGPR) from rhizospheric soil in Tissemsilt, Algeria, have a good possibility of increasing pea plant (*Pisum sativum*) nodulation and growth, according to the study's results. *Pseudomonas luteola* and *Pseudomonas fluorescens*, out of the eight bacterial strains examined showed the strongest beneficial effects on plant growth, as seen by better root nodulation and increased fresh and dry biomass. These strains' versatility in a range of soil conditions is further highlighted by the profiling of their antibiotic resistance. Also, a significantly higher nodulation rate was observed on the roots of *Pisum sativum* inoculated with *Pseudomonas luteola* and *Pseudomonas fluorescens* strains, compared to uninoculated pants ($P < 0,05$). These results highlight the potential of PGPR as biofertilizers, providing a long-term substitute for chemical fertilizers in agriculture while enhancing crop yield and soil health.

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