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ISOLATION AND CHARACTERIZATION OF LEGUME NODULATING BACTERIA OBTAINED FROM COMMON BEAN (*Phaseolus vulgaris* L.) NODULES

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Common bean, Nodules, Rhizobia, Characterization of LNB

ABSTRACT

Legume Nodulating Bacteria (LNB) play a significant role in agriculture, thanks to their capacity to improve plant growth. The main objective of this study is to isolate common bean (*Phaseolus vulgaris* L.) nodulating bacteria from root nodules and select the best isolate of rhizobia with beneficial traits. After isolation on YEMA-CR medium, eight isolates were confirmed as LNB by re-nodulating *Macroptilium atropurpureum.* A study of the morphological, physiological, biochemical and phytopathological characteristics of the isolates was done. The results obtained were analyzed statistically by ANOVA using GraphPad Prism 5.0 and SPSS 16.0. The isolates were different morphologically. Most isolates were able to grow at a pH of 4.5 with an optimum growth ranging between 5.5 and 6.5. The isolates tolerated high concentrations (7%) of NaCl. At 100 µg/ml, 12.5 % of the isolates were very resistant to penicillin, chloramphenicol and gentamicin but were all sensitive to ampicillin, erythromycin and amoxicillin. Isolate PvNj8 showed the highest IAA production (20.9 µg/ml) in YEM supplemented with 0.1mg/ml of L-tryptophan. The capacity of the isolates to solubilize inorganic phosphates varies with the origin of phosphate used in the medium. The solubilization index varied from 1.08 to 2.14 for the Senegalese inorganic phosphate. No solubilization was observed for the Cameroonian inorganic phosphate. In liquid medium, the Cameroonian inorganic phosphate was solubilize by 6 isolates among 8. The highest value of solubilization was found into PvNj2 which is not significantly different from PvNj4 and PvNj5 but significantly different from the other isolates. For the Algeria inorganic phosphate, PvNj6 was found to be the best solubilizer but not significantly different comparatively to PvNj3, PvNj8 and PvNj9. The isolates of this study assimilated various carbon sources with a preference for mannitol. All the isolates inhibited the development of the plant-pathogens fungus *Phytophtora megakarya* and *Phytophtora colocasiae*. Several selected bacteria nodulating bean can be recommended for the production of biofertilizers.

1. Introduction

Nitrogen and phosphorus are essential for plant growth (Gyaneshwar *et al*., 2002). Soil availability of nutrients determines crop yield. Tropical soils are generally poor in Nitrogen and Phosphorus which are one of the major macronutrients thus partly responsible for low crop yields. To improve yields, chemical fertilizers are often used. This chemical fertilization is one of the causes of groundwater pollution. In addition, the costly nature of these chemical fertilizers is also a problem for farmers whose incomes are very often modest. On the other hand, in the long run, these fertilizers significantly reduce soil productivity. The biological fixation of nitrogen is therefore of particular interest because it offers an alternative to the use of nitrogen fertilizers which are very expensive and highly polluting for groundwater (Gupta *et al*., 2015). In fact, leguminous as common bean are able to fix atmospheric Nitrogen in symbiosis with soil rhizobia and thus contribute to enriching the soil with Nitrogen for the benefit of crops. The use of such a technology requires a perfect knowledge of the rhizobia used, including their morphology, physiology, biochemistry and even their defense mechanisms. In Cameroon, some works has been done already on *Rhizobium*-Leguminous symbiosis (Ngo Nkot *et al.*, 2015, Mandou *et al*., 2017). The work on symbiosis has mainly concerned rhizobia nodulating peanut, groundnut, cowpea and soybean, but very little work has been done on rhizobia nodulating common bean.

2. Materials and Methods

2.1 Trapping site, Soil sampling and analysis

Trapping was conducted in Cameroon, in the greenhouse of state institute of Agricultural Research for Development (IRAD) station in Njombe, Littoral region. Soil samples were collected from 5 m × 5 m quadrats diagonally. The 4 subsampled mixture provided the composite sample of about 120 kg. Soil samples were bring at the IRAD-Ekona laboratory, South West region-Cameroon where physico-chemical analyses were performed.

2.2 Isolation of LNB from root nodules

Isolation was performed according to Vincent, 1970. The nodules preserved by desiccation were rehydrated and sterilized. The nodules were rinsed in sterile distilled water to remove traces of sterilizers then crushed in a drop of sterile distilled water. The suspension obtained was streaked on Yeast Extract Mannitol Agar (YEMA) plates containing Congo red for 7 days at 28°C. The colonies obtained were cultured one more time on fresh YEMA medium in order to obtain pure culture.

2.3 Authentication of isolates

The authentication of the isolates was used to confirm nodulation ability. For this test, the host legume *Macroptilium atropurpureum* was used. Nodulation was assessed by growing germinated seeds in plastic pots containing sterile sand. The pots were subsequently covered with aluminum foil. Seeds of *Macroptilium atropurpureum* of the same size were selected and then scarified and surface sterilized in concentrated sulfuric acid for 5 minutes and rinsed several times with sterile distillated water. These seeds were then pregerminated in sterile Petri dishes. Three days after sowing, at emergence, seedlings were inoculated with 1ml of a freshly prepared bacterial suspension (10^8 CFU/ml). The seedlings were watered with the Jensen nutrient solution (Jensen 1942). After six weeks of culture, the plants were harvested and examined for the presence or absence of nodules.

2.4 pH tolerance of isolates

To determine the pH tolerance, the Yeast Extract Mannitol (YEM) medium was used. The pH was adjusted to 3.5; 4; 4.5; 5.5 and 6.5. The growth of rhizobia isolates was evaluated in Erlenmeyer flasks containing 25 ml of medium. Inoculums used were compared to a 0.5 Mc Farland scale which is 10^8 CFU/mL. Growth was determined by measuring the Optical Density (OD) at 470 nm after 3 days of incubation using a spectrophotometer (BIOBASE BK-UV-1900).

2.5 NaCl Tolerance of LNB

Common bean nodulating bacteria were tested for their tolerance toward salinity on YEM broth supplemented with NaCl at concentrations ranging from 0.01 to 7%. The bacterial growth was recorded by using spectrophotometer (Biobase) at 470 nm after 72 hours in an incubation period at 28± 2^0C (Vincent, 1970).

2.6 Antibiotic resistance of LNB

The method used was the diffusion technique in a solid medium in which the concentration gradient of the antibiotic is achieved by diffusion in agar from a center (Kareem *et al.*, 2008). Four concentrations of each antibiotics (10, 20, 50 and 100 µg / mL) were prepared separately. The bacterial inoculum obtained is compared to the Mc Farland scale which is 10^8 bacteria/ml. In each well formed in the YEMA medium, a 10 µL of a given antibiotic is deposited. Each control well is inoculated with sterile distilled water (Cos *et al*, 2006). Petri dishes are incubated at 28 °C for 7 days. The diameters of the inhibition zones observed around the well was measured using calipers (Kareem *et al.*, 2008, Moreira *et al.*, 2005)..

2.7 Indole 3-Acetic Acid (IAA) production by rhizobia isolates

IAA production was determined using Salkowski's reagent (Gordon and Weber, 1951). Culture was grown on YEM medium supplemented with 0.1 mg. mL^{-1} of L-tryptophan (Tehmina *et al.*, 2019). Erlenmeyer flasks were incubated at a temperature of 28 ± 2 ° C. in a shaking incubator. After 72 hours of incubation, the broth was centrifuged at 10,000 rpm for 30 minutes. 1 ml of the culture supernatant was mixed with 2 ml of Salkowski's reagent (0.5M FeCl3 in 35% $HClO_4$) and left at room temperature for 30 minutes. Development of a pink color indicated the production of IAA. The IAA concentration was then estimated colorimetrically by measuring the OD at 530 nm using spectrophotometer (Biobase BK-UV-1900). A calibration curve was prepared from synthetic concentrations of IAA.

2.8 Solubilization of inorganic phosphates by rhizobia isolates

Inorganic phosphates of their soluble fractions were washed 4 times with warm water and then dried at 60 °C to complete evaporation. In order to evaluate the solubilizing activity of the rhizobia nodulating bean, Modified Mineral Salt Medium (Reyes, and Rodriguez, 1999) was supplemented with 5 mL/L of Bromocresol green. The soluble phosphate was replaced by insoluble inorganic phosphates from Cameroon, Algeria and Morocco. The autoclaved medium was poured into sterile Petri dishes. After cooling, each petri dish was divided into three compartments with a marker to inoculate the center of each third of the petri dish with 10 µL of each isolate. The solubilizing activity was evaluated after five days by measuring the diameter (n) of the colony and the diameter (z) of the solubilization halo. From these measurements, the ratio z/n Solubilization Index (SI) of each isolate was given by the formula: SI = Ø halo (mm) / colony Ø (mm) (Berraquero *et al.*, 1976).
The solubilization ability was also performed in liquid medium using the same component but without Agar. The evaluation of the quantity of solubilized phosphate was performed using a calibration curve prepared with different concentrations of K_2HPO_4.

2.9 Use of Carbohydrate as only Carbon Source

Rhizobial isolates were examined for their ability to assimilate different carbon sources by replacing mannitol with one of the following carbon sources: glycerol, starch and glucose at 1% (w/v). The original YEMA medium with mannitol was used as control. The isolates was streaked on Petri dishes and for each isolate three replicates were used. The plates were incubated at 28 °C for seven days. After this period, the presence or absence of growth was noted. The ability to use carbohydrate was also studied by measuring the OD at 470 nm after 3rd day of incubation at 28 °C, using a spectrophotometer.

2.10 Ability of LNB to inhibit plant pathogens fungus

The pathogenic fungus used for the rhizobia antagonist test were *Phytophthora colocasiae* and *Phytophtora megakarya*. The Fungi were provided by the Laboratory of Plant Biology of the University of Douala-Cameroon. The fungi strain were isolated and purified on PDA (Potato Dextrose Agar) medium from infected organs (leaves and pod). The *Rhizobium-Pythopthora* confrontation test was performed in 9mm diameter Petri dishes containing 12-15ml of nutrient agar (Plate count Agar). A mycelial disk of 8mm in diameter is removed and deposited in the center of the Petri dish. A rhizobia isolate is streaked all around at 2 cm from the mycelial fragment. Controls dishes only received mycelial fragment. Each bacterial strain was confronted with both species of *Pythopthora* at three replicates. Mycelial growth is measured every 3 days from incubation for 9 days. The percent inhibition of mycelial growth of *Pythophthora* was calculated according to the formula of Wang *et al*. (2002).

2.11 Statistical analysis

Collected data were analyzed using R (3.3.1), SPSS 16.0 and GraphPad Prism 5.0 at 5% of average probability. ANOVA was made using the Duncan test for repeated measures.

3. Results

3.1 Soil Physical and chemical analyses

Grain size analysis revealed that the Njombe soil contained silts (40.76%) in large quantities and clay (29.42%) giving this soil a silty-clay texture. Chemical analyses showed that the analyzed soil was acidic with a pH of 5.22. The total soil nitrogen percentage of 0.53% was very high. The soil sampled was rich in phosphorus (5.22 mg / kg). The C / N ratio of 6.5 was very low, this soil was poor in organic matter but very rich in nutrients. The cation exchange capacity of 23.7 cmol / kg was very high (> 20 cmol / kg).

3.2 Isolation and authentication of rhizobia

A total of 11 isolates were isolated from root nodules of *Phaseolus vulgaris* L. collected and purified. The 11 isolates were tested for their ability to nodulate *Macroptilium atropurpureum*. The effective nodulation observed with all rhizobial isolates clearly indicated that 08 isolates were able to nodulate *Macroptilium atropurpureum*. It was noted that the nodules were pink, indicating the leghemoglobin content, while uninoculated control plants were without nodules. Rhizobial isolates were nomenclatured so as to indicate the name of the legume (Pv-*Phaseolus vulgaris*), the site of origin (NJ-Njombe) followed by isolate number.

3.3 Morphological Characteristics of LNB

The isolates had various morphological characteristics (Table 1). From the 8 isolates obtained, 62.5% had colonies with convex elevation and 37.5% of the isolates formed flat colonies. All the colonies were round, with diameters between 2mm and 7mm. The PvNj4 isolate formed the largest colonies (7mm) while PvNj2 and PvNj7 formed the smallest colonies (2mm). The colonies were white or milky in colour when grown on YEMA medium. All the isolates were fast-growing and failed to absorb Congo red in the medium. 75% of colonies were coccus cells and 25% rod. The isolates were all Gram negative.

Table 1: Colony characteristics and cell morphology of common bean nodulating isolates

Colony	Colony size (mm)	Colony shape	Color	Colony growth	Aspect	presence of mucus	Viscosity	Brightness	Elevation	Gram staining	Cell shape
PvNj2	2	Round	Beige	fast	Heterogeneous	no mucus	Not viscous	Bright	Convex	Negative	Coccus
PvNj3	2.5	Round	white	fast	Homogeneous	no mucus	Not viscous	Bright	Convex	Negative	Small rod
PvNj4	7	Round	Beige	fast	Heterogeneous	no mucus	Not viscous	Bright	Convex	Negative	Coccus
PvNj5	5	Round	Beige	fast	Homogeneous	no mucus	Not viscous	Bright	Flat	Negative	Coccus
PvNj6	5	Round	Whitish	fast	Homogeneous	no mucus	Not viscous	Bright	Convex	Negative	Coccus
PvNj7	2	Round	White-milky	fast	Homogeneous	no mucus	Viscous	Bright	Convex	Negative	Coccus
PvNj8	5	Round	whitish	fast	Homogeneous	no mucus	Not viscous	Bright	Flat	Negative	Small rod
PvNj9	4	Round	Beige	fast	Homogeneous	presence of mucus	Not viscous	Bright	Flat	Negative	Coccus

The dendogram (Fig. 1) based on the macroscopic characteristics of the colonies placed isolates in three different groups. Group1 contained 3 isolates (PvNj7, PvNj2 and PvNj3) which were all round in shape, fast growing, bright and convex. The second group was constituted of PvNj9, PvNj8 and PvNj6. The isolates of this group were not viscous, bright and homogeneous in aspect. The last group contained PvNj5 and PvNj4. These isolates were round, beige, fast growing, not viscous and bright.

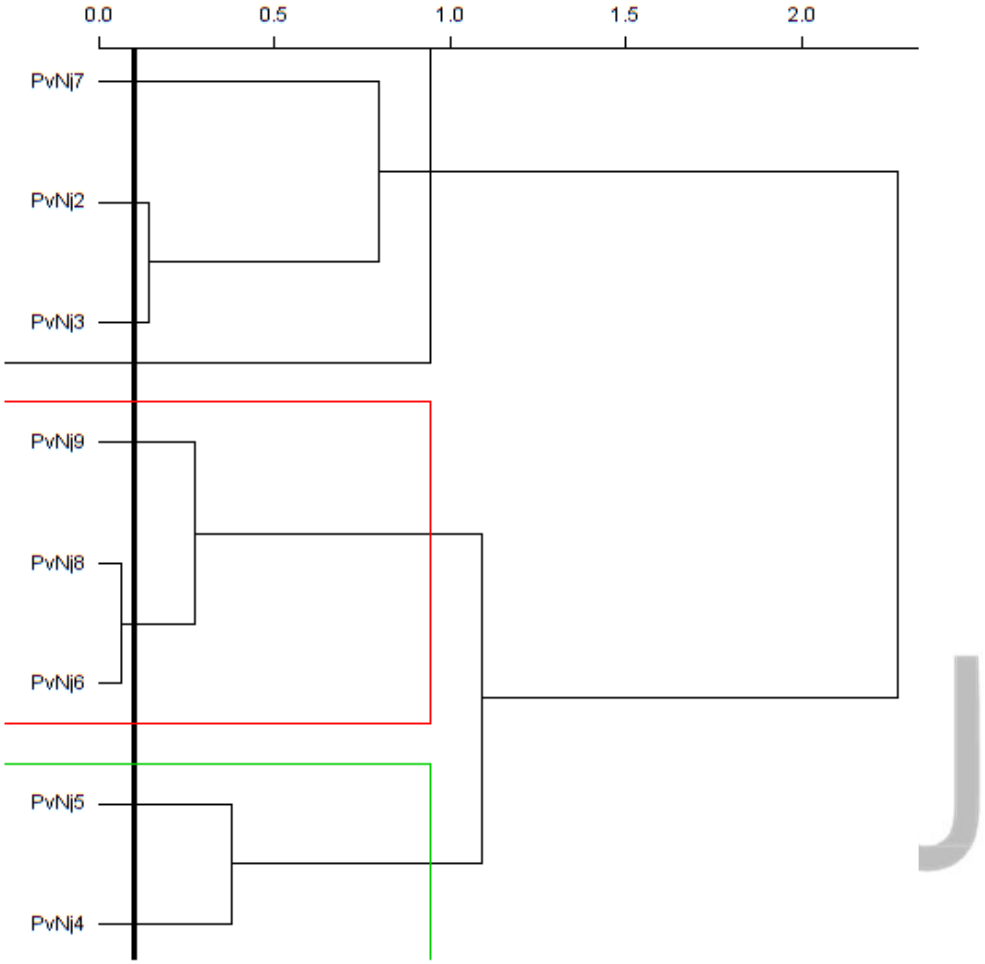

Figure 1. Dendrogram showing the morphological similarities among bean nodulating rhizobial isolates

3.4 Physiological Characteristics of common bean nodulating bacteria

3.4.1 pH tolerance of common bean nodulating bacteria

In Broth medium, all isolates obtained showed high growth at pH 6.5 (Fig.2). At that pH of 6.5, PvNj2 (1.290) and PvNj5 (1.295) are significantly different from the others. At pH 5.5 the best growth was observed from PvNj2 (1.29) which is significantly different from other isolates. PvNj6, PvNj7 and PvNj8 showed the least values of growth among all isolates. At pH 4.5, only isolates PvNj6, PvNj7, PvNj8, and PvNj9 grew weakly, PvNj3 (0.965) and PvNj5 (0.996) showed moderate growth. The best growth was observed from PvNj2 (1.19) and PvNj4 (1.17) which were significantly different from other isolates at 5% of average probability. The best growth was found from PvNj4 (0.710) which was significantly different from other isolates.

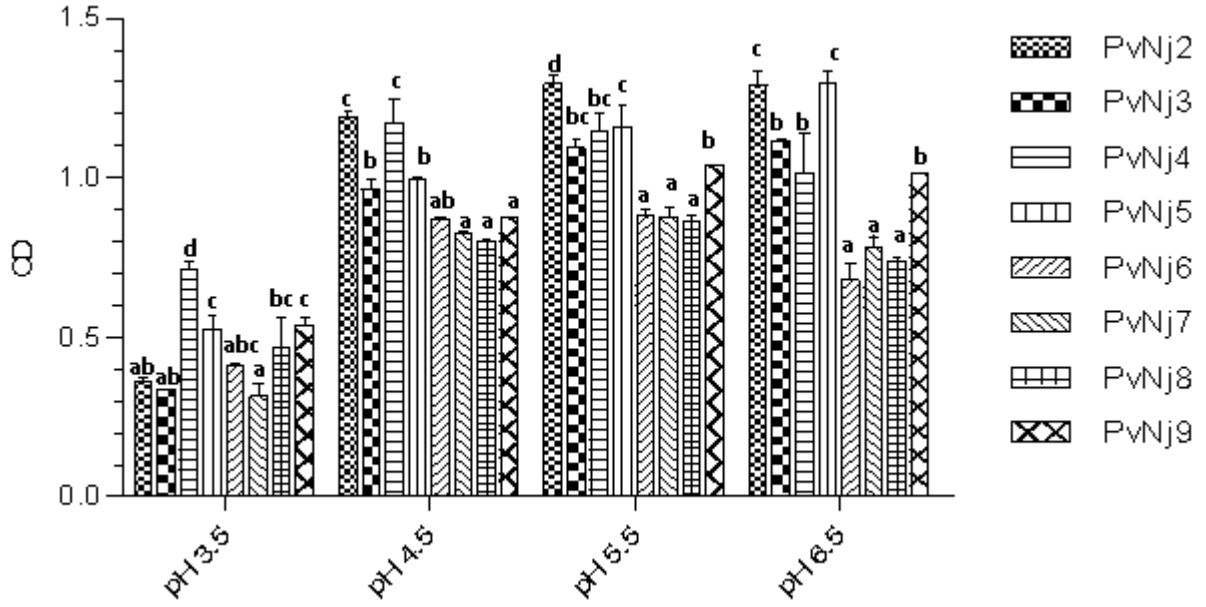

Figure 2. pH tolerance of common bean nodulating bacteria in liquid medium
Means with the same letters are not significantly different at 5% of Probability

3.4.2 NaCl tolerance of common bean nodulating bacteria

The growth results at different concentrations of NaCl in liquid medium (Fig.3) showed that tolerance toward salinity varied not only with the rhizobia isolate but also with NaCl concentration. At the standard NaCl concentration of 0.01%, all the isolates grew successfully. At this concentration, the isolates that showed better growth were PvNj2 and PvNj5 with an OD of 1.29 and 1.3 respectively. PvNj9, PvNj2 and PvNj5 had better growth significantly than others in higher salt concentration (7%), with an OD of 0.16, 0.11 and 0.09 respectively.

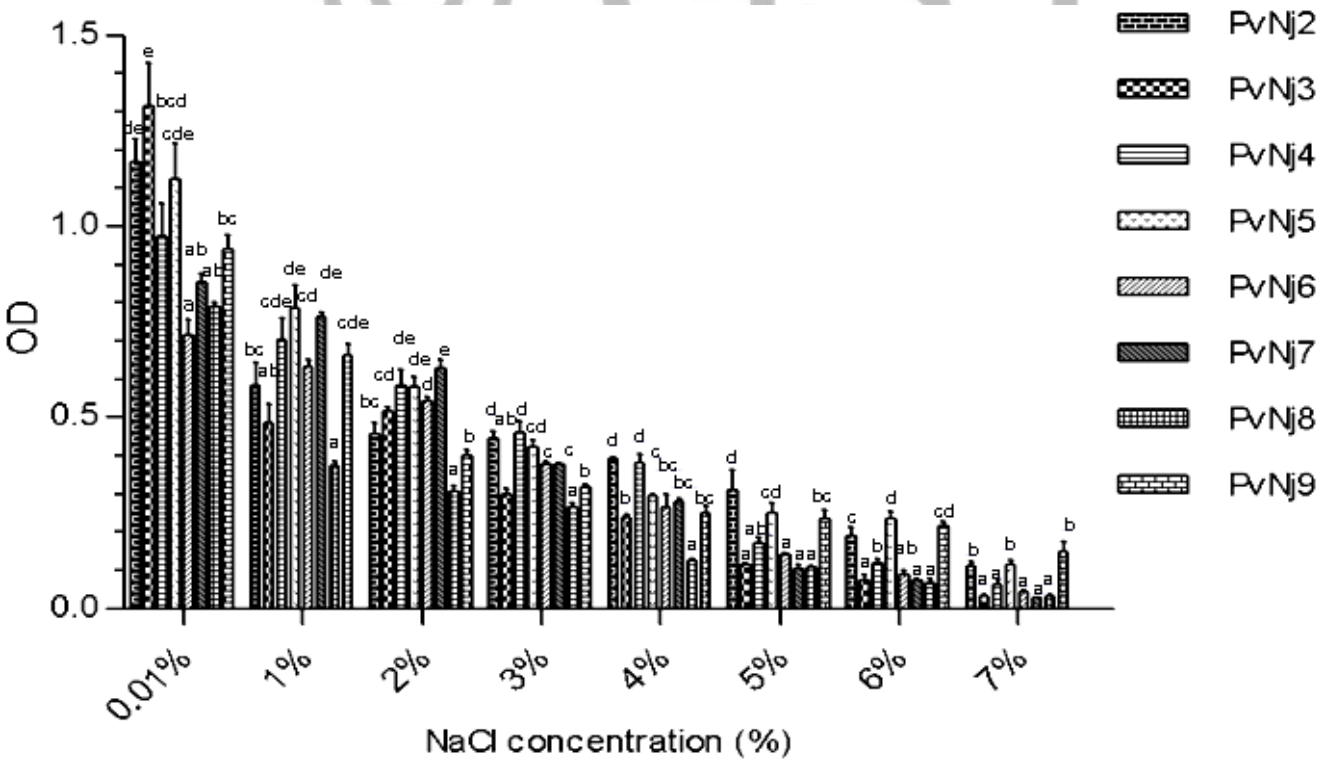

Figure 3. NaCl tolerance of common bean nodulating bacteria
Means with the same letters are not significantly different at 5% of average probability

3.5.2 Antibiotic resistance of common bean nodulating isolates

Antibiotic resistance test of isolates shown that the halo (Fig.4) diameters increase with an increase in the concentration of antibiotics.

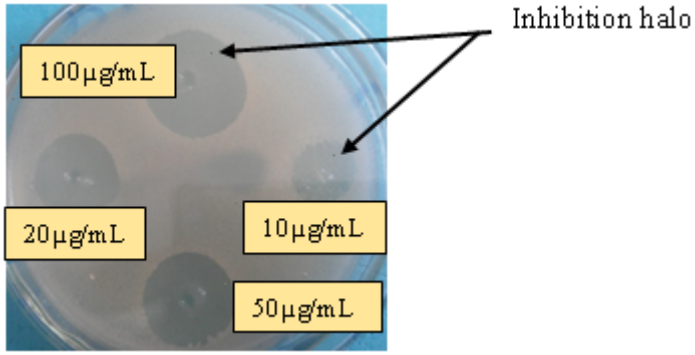

Figure 4: Effect of various concentrations of amoxicillin on PvNj2

High resistance was recorded for gentamycin, chloramphenicol and penicillin, while low resistance was observed for rifampicin. Isolates tolerated those antibiotics, even at their highest concentration of 100µg/ml. The most harmful antibiotics for rhizobia isolates were amoxicillin, ampicillin and erythromycin. No isolates tolerated them, even at the lowest concentration of 10µg/ml. At 10 µg/ml, isolates showed good resistance with a growth rate of 50% for gentamycin and 52% for rifampicin. At the same concentration of chloramphenicol, penicillin and tetracyclin, only 37.5%, 25% and 25% of isolates respectively were able to grow. At a concentration of 20 µg/ml, only 12.5% of isolates were resistant to gentamycin, 25% to chloramphenicol and penicillin, 26% of rifampicin and 12.5% to tetracycline. At high concentrations (50 and 100 µg/ml), the inhibitory effect was highly marked. No isolate tolerated 50 µg/ml of rifampicin and no isolates tolerated the concentration of tetracyclin. Resistance percentages of 10%, 25%, 50% and 100% respectively, were noted for gentamycin chloramphenicol, penicillin and tetracyclin (Fig.5). Only 12.5% of isolates tolerate 100µg/ml of gentamycin chloramphenicol and penicillin.

The isolate PvNj7 was the only one which tolerated 2 antibiotics (chloramphenicol and tetracyclin) at the concentration of 50 µg/ml. The results showed that the tolerance of the isolates decreased with an increase in the concentrations of antibiotics.

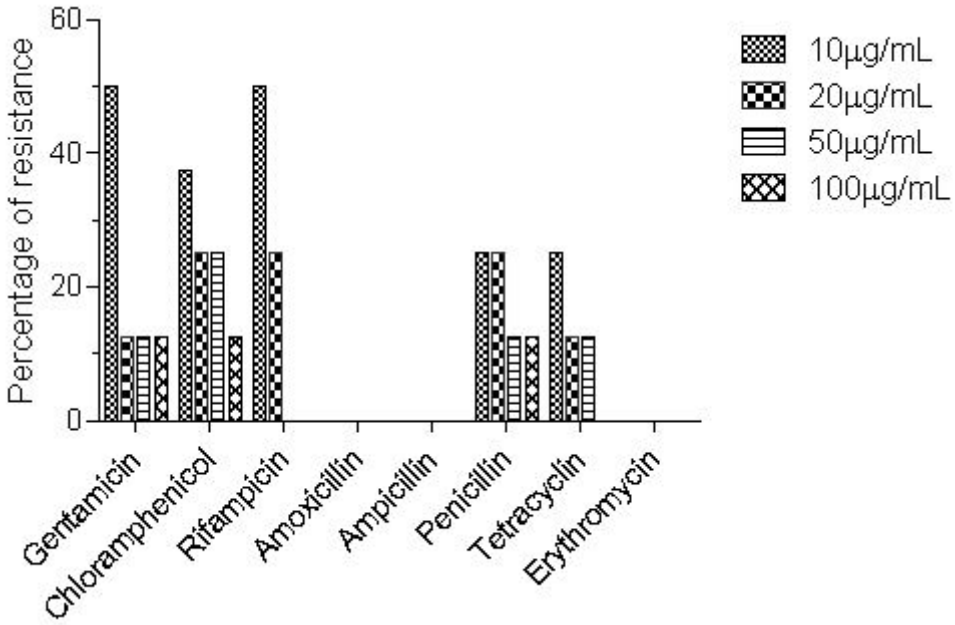

Figure 5: percentage of resistance to antibiotics of isolates

3.5 Biochemical Characteristics of common bean nodulating bacteria

3.5.1 Indole-3-Acetic Acid production by rhizobia

The IAA production test were performed on YEM medium supplemented with 1mg/ml of L-Trp. All the isolates were found to be positive for IAA production. The revelation of this production is translated by the changing of the color of medium to red (Fig.6) using the Salkowski's reagent.

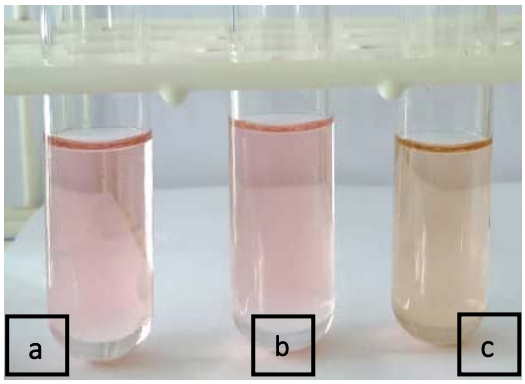

Figure 6. Positive response of PvNj8 (a) and PvNj5 (b) in indole-3-acetic acid production in YEM medium using Salkowski's reagent comparatively to control (c)

IAA production ranged from 5.6 µg/mL to 20.9 µg/mL in the presence of 0.1 mg/mL of L-tryptophan at 28 ± 2 °C (Fig.7). The PvNj8 isolate produced the maximum amount of IAA (20.9 µg/mL) and is not significantly different from PvNj6 (17.8 µg/mL) but different significantly from other isolates. The lowest IAA production (5.6 µg/mL) was reported in case of PvNj3 and is significantly different from other isolates.

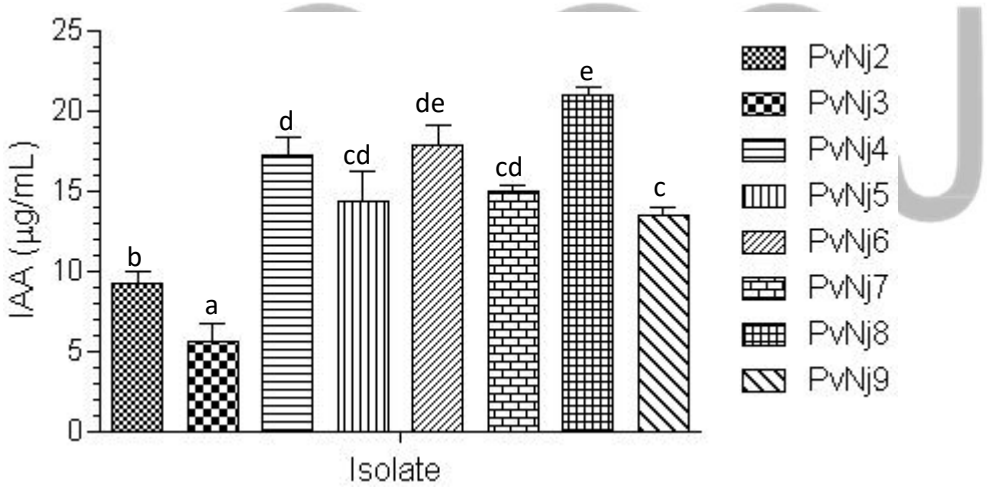

Figure 7. Quantitative estimation of indole acetic acid produced by common bean nodulating isolates. Means with the same letters are not significantly different at 5% of average probability

3.5.2 Solubilization of inorganic phosphate by rhizobia

Based on the solubilization index (SI), 4 isolates : PvNj2, PvNj3, PvNj8 and PvNj9 were classified as low solubilizers with SI < 2mm, 01 isolate PvNj7 was classified as medium solubilizer with 2mm ≤ SI ≤ 4mm; and no isolates were classified as high solubilizers (SI ≥4.0) (Fig. 8). Maximum SI was observed by PvNj7 (SI = 2.14) followed by PvNj8 (SI= 1.78).

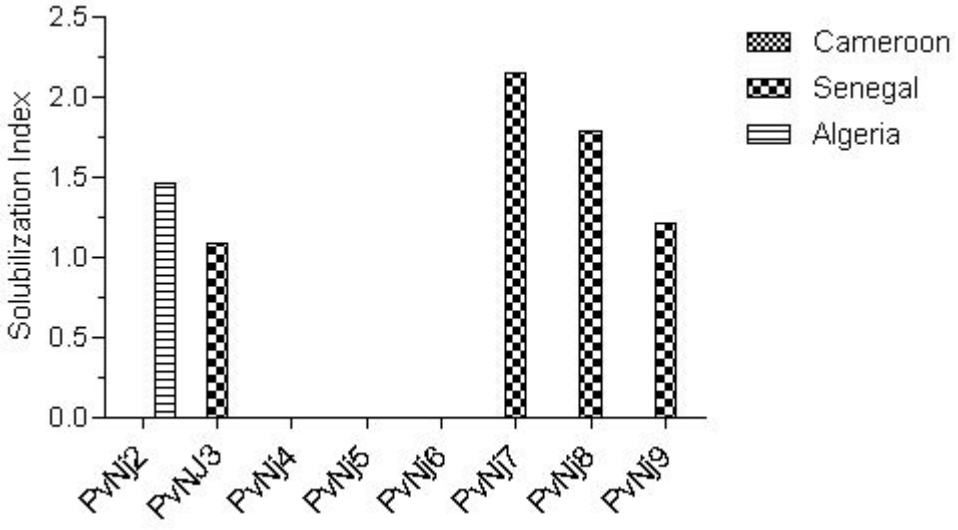

Figure 8. Ability of common bean rhizobia isolates to solubilize inorganic phosphate in YEMA medium

Figure 9 shows the amount of soluble phosphate in broth medium. The highest solubilization was from the Senegalese inorganic phosphate which was solubilize by all the isolates. For the Senegalese inorganic phosphate, the highest amount of solubilization was observed at PvNj4, PvNj5 and PvNj8 which was significantly different from other isolates. The Cameroonian inorganic phosphate was solubilize by 6 isolates among 8. The highest value of solubilization was found in PvNj2 which is not significantly different from PvNj4 and PvNj5 but different significantly from the other isolates. For the Algeria inorganic phosphate, PvNj6 was found to be the best solubilizer but not significantly comparatively to PvNj3, PvNj8 and PvNj9.

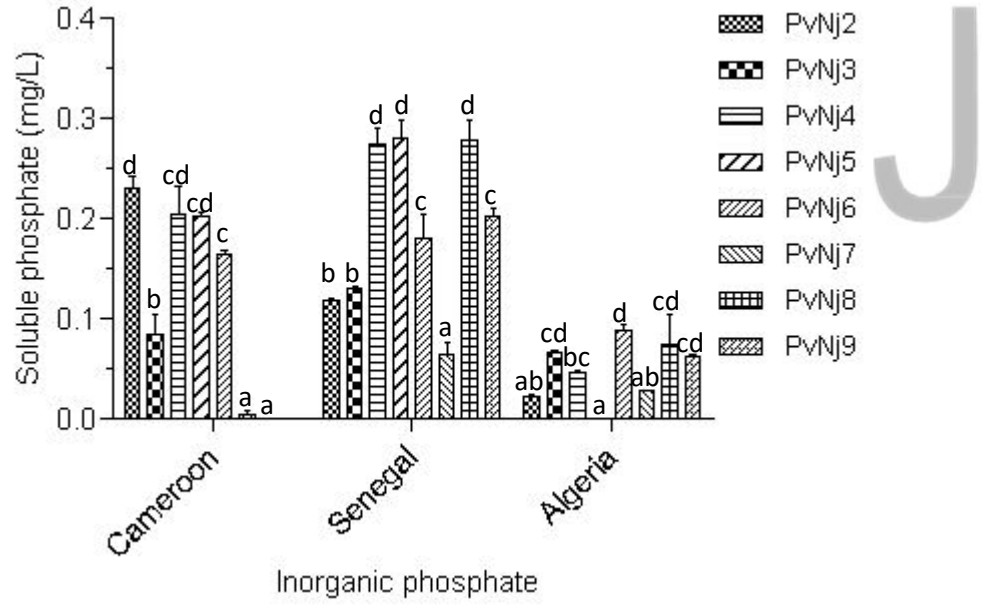

Figure 9. Solubilization of inorganic phosphate in liquid medium

Means with the same letters are not significantly different at 5% of average probability

3.5.3 Carbon sources utilization ability of common bean isolates

Regarding the use of carbon sources, all of the rhizobial isolates were capable of growing in mannitol, glycerol, starch and glucose. In the absence of carbohydrate there was no growth at all (Fig.10). The largest diameters was obtained in the presence of mannitol, showing that mannitol is the preferred carbon source for rhizobia isolates nodulating bean. For glycerol, starch and glucose, the diameters were relatively small.

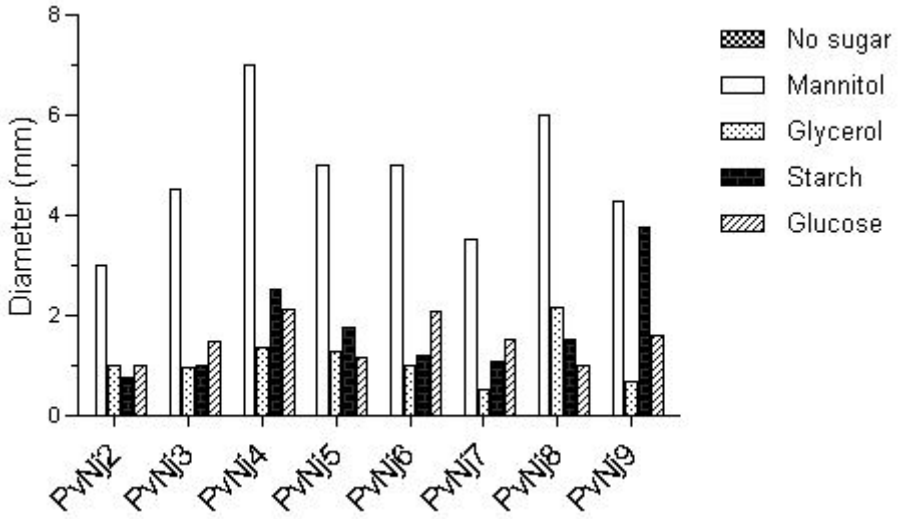

Figure 10. Evolution of colony diameter under various carbon sources

The results obtained in liquid medium (Fig.11) confirmed that the growth of an isolate is a function of carbon source used and also that mannitol was the best carbon source for rhizobial growth. None of the isolates evaluated were capable of growing in culture medium without carbohydrate. PvNj2, PvNj6 and PvNj9 isolates were more similar to starch and glycerol assimilation than all other isolates. The PvNj7 and PvNj8 isolates better assimilate glucose than all other isolates. The order of assimilation of the sugars tested by the isolates of rhizobia nodulating bean was as follows: mannitol> starch> glycerol> glucose.

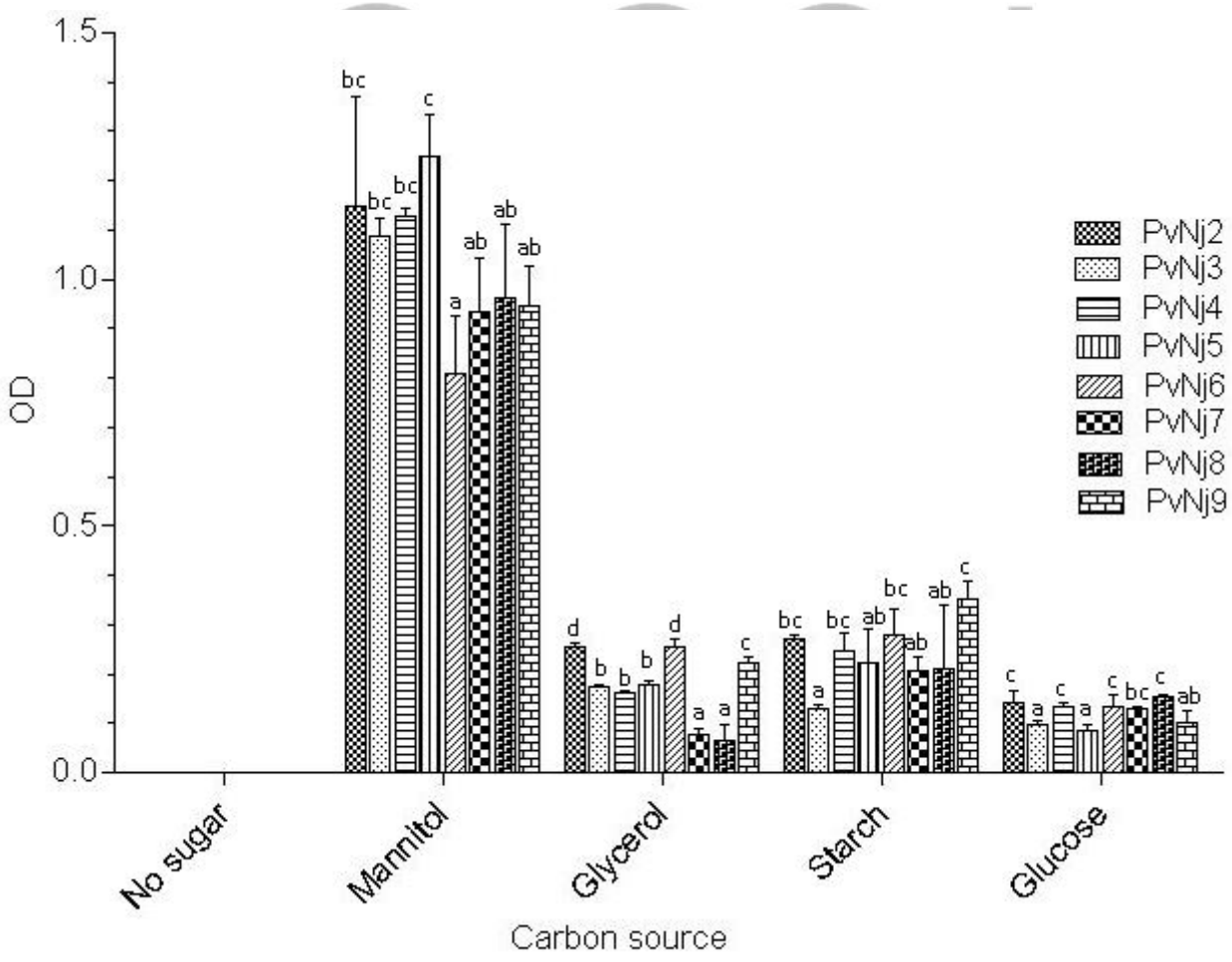

Figure 11. Use of carbohydrates by common bean nodulating bacteria
Means with the same letters are not significantly different at 5% of average probability

3.6 Inhibition of *Phytophtora* by common bean nodulating rhizobia

The direct confrontation between common bean nodulating rhizobial isolates and the two species of *Phytophtora*: *Phytophtora megakarya* and *Phytophthora colocasiae* was done to assess the anti-fungal activity. Bean isolates were found to inhibit the growth of *Phytophtora* (Fig 12).

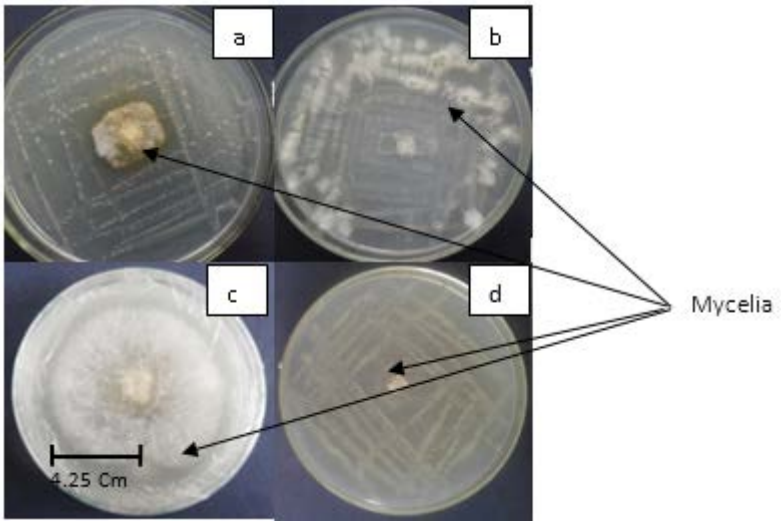

Figure 12. Direct confrontation trials rhizobia - *P. megakarya* / *P. colocasia*: (a) inhibition of *P. megakarya* by PvNj5; (b) no inhibition of *P. megakarya* by PvNj7; (c) control box containing only *P. megakarya*; (d) Total inhibition of *P. colocasiae* by PvNj7.

The inhibition percentage of mycelial growth varied from one isolate to another and from one fungus to another (Fig.13) The Isolate PvNj5 showed significantly the highest inhibition rate against *P. colocasiae* (88.82%) followed by the isolate PvNj6 (85.29%) which was also significantly different from PvNj9 (82.94%), PvNj7 (82.35%), PvNj8 (81.76%), PvNj4 (78.82%), PvNj2 (76.47%) and PvNj3 (74.12%). On the other hand, *P. megakarya* was more weakly inhibited than *P. colocasiae*. The highest inhibition percentage was obtained by the isolate PvNj8 (78, 24%), followed by the isolates PvNj5 (71, 18%), PvNj9 (67, 65%), PvNj4 (29, 41%), PvNj3 (27.65%), PvNj2 (22.35%) and PvNj6 (20.59%). The PvNj7 isolate did not inhibit *Phytophtora megakarya*, with its inhibition percentage of 11.76% being less than 20%.

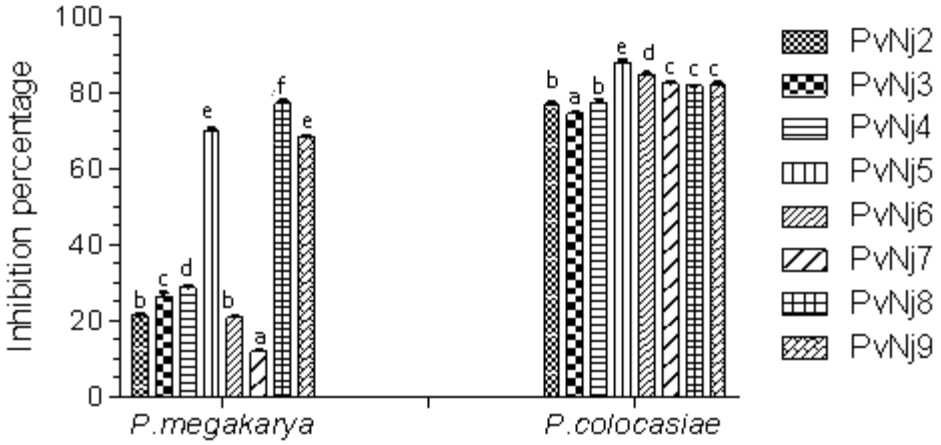

Figure 13. Percentage of inhibition of *Phytophtora* by isolates of rhizobia nodulating bean

4. Discussion

The ability to nodulate leguminous plants is unique to rhizobia (Beck *et al*., 1993). All isolates obtained from trapping do not absorb or absorb very weakly Congo Red. Such results suggest that the isolates obtained were rhizobia (Vincent, 1970). Colonies of the resulting rhizobia nodulating bean isolates were all rounded and rapidly growing on YEMA medium. These results are similar to that of Rebeca *et al*. (2013) who characterized a rhizobia isolate nodulating bean in Brazil. Isolates of rhizobia nodulating bean formed colonies after less than 3 days of incubation. Hungria *et al*. (2016) reported the existence of fast-growing rhizobia strains among rhizobia.

Rhizobial isolates nodulating bean were able to grow at pH 4.5. PvNj4 isolate showed the best growth at pH 3.5. This result is consistent with that of Jordan (1984) who showed that fast-growing rhizobia strains were generally more tolerant to acidic pH than slow-growing strains. pH is one of the major factors limiting the survival and properties of rhizobia in soils (Shetta *et al*., 2011). This pH tolerance could be explained by the expulsion of H^+ protons (Chen *et al*., 1993a), the elevation of the glutamate content of the cytoplasm of stressed cells (Aaron and Graham, 1991), the change in composition lipopolysaccharides (Chen *et al*., 1993b) and polyamine accumulation (Fujihara and Yoneyama, 1993).

The tolerance of the isolates to NaCl decreased when the NaCl concentration increased. All the isolates grew at 1, 2 and 3% of NaCl, and only 50% of the isolates were resistant to salt concentration of 7%. These results are in agreement with the results of Cevheri *et al*. (2011) who noted that 100% of isolates were able to grow in 2% (w/v) of sodium chloride, 65% in 3% and 40% in 4% of NaCl. Küçük *et al*. (2006) showed that some rhizobia isolated from bean (*Phaseolus vulgaris* L.) grew in 4% (w/v) and even 5% NaCl, and 10 isolates even grew in 5% NaCl. These values were higher than those reported by Faghire (2012) for rhizobia isolated from *Phaseolus vulgaris* in Morocco which can tolerate 3% NaCl. Konate in 2007 noted that the rhizobia strains nodulating carob trees can tolerate up to 12% NaCl. As indicated by Nour *et al*. (1995), the high tolerance of isolates to NaCl could be explained by the existence in bacteria of osmo protective molecules like proline.

The rhizobia nodulating bean isolates obtained in this study were highly susceptible to erythromycin, amoxicillin and ampicillin. Similarly, Mulugeta *et al*. (2013) working on bean reported resistance to ampicillin by some isolates. Isolates of the study were tolerant to chloramphenicol, penicillin, gentamycin, rifampicin and tetracycline. These results are in agreement with that of Rebeca *et al*. (2013) who found rhizobia strain resistant to penicillin.

The rhizobia isolates tested produced IAA. These results are in corroboration with the study of Muhammad *et al*. (2011) who observed IAA production by isolates of rhizobia nodulating *Vigna radiata*. The ability to synthesize IAA is an attribute that many bacteria including plant growth-promoters (Duca *et al*., 2014). Auxin production by rhizobial isolates in culture has been reported by many researchers (Tehmina *et al*., 2019, Varsha *et al*., 2015, Muhammad *et al*., *2011*). Stajković *et al*. (2011) also reported IAA production by rhizobia strain nodulating bean in nutrient broth medium with 2mg.ml^{-1} of L-Trp. These results suggest the potential of some strains of *Rhizobium* as biofertilizers.

The solubilization of inorganic phosphates in solid medium resulted in the formation of a yellow translucent zone around the colony. These results are similar to that of Amith *et al*. (2017), who reported that, out of 8 bacterial isolates, 3 isolates (A4, C1 and H6) were found to be potent phosphate solubilizers showing clear halo zone around its colony. The solubilisation ability of rhizobia could be due to the production of organic acids or polysaccharides or due to the activity of phosphatase enzymes of phosphate solubilizing bacterial (Khan *et al*., 2010).

The results of the test on the use of carbon sources show that no growth was observed in the absence of a carbon source. This result highlights the need for carbon substrates in the metabolism of rhizobia. Rhizobia are assimilating a variety of carbon substrates ranging from monosaccharides (glucose) to polysaccharides (starch) and polyols (glycerol). These results are consistent with those obtained by Ngo Nkot *et al*. (2015) who indicated that the rhizobia nodulating Voandzou were able to assimilate various carbon source.

Bean nodulating rhizobial isolates obtained in this study showed an antifungal activity against two species of *Phytophthora.* A clear reduction in the diameter of the mycelia of the two species of *Phytophthora* in presence of the eight isolates tested were observed. These results are similar to that of Nouha *et al*. (2019) who showed the ability of four strains affiliated to *Pseudomonas* genus to inhibit the growth of phytopathogenic

fungi. Asseng *et al*. (2017) demonstrated the antagonistic effect of *Rhizobium* in the biological control of *P. colocasiae*. The strong inhibition of mycelia from different fungi might be due to the production of rhizobitoxin, which acts as a suppressive factor (antibiosis) of phytopathogenic fungi (Deshwal 2003, El-Mehalawy 2004). According to Zao *et al*. (2018), the inhibition of phytopathogens by endophytic bacteria may be explained by the competition with pathogens for the ecological niche/substrate. Bacteria can also inhibit phytopathogenic growth by the production of antifungal substances and volatile organic compounds such as siderophores and hydrogen cyanide (HCN) (Nouha *et al*. 2019).

5. Conclusion

The results of this study showed that indigenous bacteria that initiate nodulation in common bean are present in the soil. Authentication experiment confirmed that the majority of the isolates were rhizobia due to their ability to infect *Macroptilium atropurpureum*. A collection of eight bean nodulating rhizobial isolates was obtained. The morphological, physiological, biochemical and phytopathological characterization of bean nodulating rhizobial isolates will serve as a prerequisite for the selection of isolate adapted to specific environmental conditions. Rhizobia isolates obtained in this study tolerated salinity, were able to grow in acidic pH, produce IAA and solubilize inorganic phosphates. Nutritional tests showed the ability of rhizobia to use a wide range of carbonaceous substrates as only carbon source with a preference for mannitol. The resulting isolates were able to inhibit phytopathogenic fungi. Such advantageous characteristics make these isolates good potential inoculums for bean cultivation. Further research is needed to know the mechanisms of action of these isolates.

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