Original Research Article

Evaluation *in vitro* of potential for plant growth promotion detected in nodule endophyte bacteria of Bambara groundnut (*Vigna Subterranea* L.) from agricultural soils in Cote d'Ivoire

ABSTRACT

Legumes-endophytes association is an integral part in improving agricultural productivity and soil fertility. Some endophytes which are considered as PGPR.can promote plant growth trough mobilization nutrients in soils, production plant growth regulators, protection plants from hytopathogens by controlling or inhibiting them, and improve soil structure.

In order to select the most effective endophytic bacteria to be used as biofertilizers, the objective of this study is to evaluate the PGPR potential of bacteria isolated from Bambara groundnut nodules *in vitro*. To accomplish this, 34 endophytic bacteria isolated from the nodules of 5 varieties of Bambara groundnut in soils from 5 localities of Daloa, which had been the subject of molecular characterization, were tested to evaluate their capacity to produce AIA and siderophores, to solubilize phosphate.

Generally, all strains produced a detectable amount of IAA which ranged from 16.3 to 275.5µg/ml, and the four (4) major producers of IAA were RFK12, RFK34, RFK10 and RFK2. The range of phosphate solubilization was 16 to 453,45µg/ml, meanwhile four strains (RFK27, RFK11, RFK8 and RFK12 were most efficient P-solubilizers. The majority (94 % of strains) were able to produce siderophore. The halo diameter varies from 7,06 to 40 mm and siderophore production index ranged from 1,08 to 5,36 and the isolate that showed the best ability in Siderophore production was RFK27, followed by RFK26 and RFK22.

In total, 19 strains showed multi-PGP traits and could be promising biofertilizers.

In perspective, it would be interesting to test the 19 strains with multi-PGPR traits under field conditions.

Keywords: Endophyte PGPR Biofertilizer Bambara groundnut…In vitro

1. Introduction

The high productivity of intensive agriculture is largely dependent on synthetic chemical inputs, particularly fertilizers and pesticides, with projections predicting the use of 120 million tons by 2040 (Vance, 2001; Soussou, 2013;).However, improper use of these synthetic chemical inputs has upset the ecosystem's equilibrium and and is the basis of several human, economic and environmental problems such as the contamination of agricultural soils (Soussou, 2013) Therefore, in order to restore soil quality for agricultural production, appropriate soil management and conservation techniques are imperative, specifically bio-fertilization based on soil microorganisms.(Hussain et *al.,*2021).

Microorganisms serve as a reservoir of nutrients for plants and also contribute to soil structure. They also provide other benefits. (Imran et *al*., 2019a). Bacteria are the most numerous soil organisms and play an important role in plant growth and development (Imran et *al*., 2019b ; Khan, 2018). Hence some bacteria are named PGPR (Gupta et *al.*, 2015). PGPR (Plant Growth Promoting Rhizobacteria) are bacteria able to promote plant growth and serve as biofertilizers and biopesticides in agricultural crops (Ferchichi *et al*., 2019) This bacteria may promote the plant growth through different direct and indirect mechanisms such as: fixation of nitrogen, solubilization of soil phosphates, production of hormones (auxin and gibberellin), production of siderophores (iron chelator), increasing the uptake of water and minerals, increasing the enzymatic activity of the plant. The PGPR may promote the plant growth also by suppressing plant pathogens (Pérez-Montaño et *al*, 2014; Zaatri & Tagzirt, 2023).

Thus, to promote sustainable agriculture, it is necessary to produce effective bacterial inocula capable of improving plant productivity. This work aims to evaluate the PGPR potential of endophytic bacteria isolated from Bambara pea nodules in vitro in order to select the most efficient ones and use them as biofertilizers.

2. MATERIALS AND METHODS

2.1. Baterial strains

The bacteria were isolated *in vitro* at the Jean Lorougnon Guédé University in Daloa, Côte d'Ivoire. These isolates were tested for their genetic diversity and Plant Growth Promoting (PGP) activities in a laboratory at Mohammed V's Faculty of Science in Rabat, Morocco. The 34 bacterial strains examined in this study had previously undergone morphological and molecular characterization and their partial sequences of the 16S rRNA were deposited in the GenBank® database under the accession numbers MT661489 to MT661522. (GUEI et *al*., 2024)

2.2. Reactivation and verification of strain purity

The strains stored in 20% (v /v) of glycerol at -80°C were removed from the freezer and their purity was checked by streaking technique according to the Jordan method (Guei et *al*., 2020).

2.3 Phosphate solubilization

The qualitative test of phosphate solubilization was conducted as previously described and a sample of 10 µL from each culture was spotted on Pikovskaya's (PVK) agar medium containing tricalcium phosphate ($Ca_3[PO_4]2$) (Vyas et *al.*, 2007). All cultures were incubated at 28 °C for 3 days and the presence of clear halo indicates the phosphate solubilization.

Quantitative estimation of inorganic phosphate solubilization was tested in medium Pikoskaya phosphate liquid medium, according to the methodology described by Pikovskaya (1948).The amount of soluble phosphate released was determined according to the colorimetric method with Vanadomolybdo-phosphoric acid as follows: a 1 ml of each sample was added to a 1 ml of the Vanadate-molybdate reagent, incubated for 1 hour and finally measured from OD to 405 nm; it was determined

from a standard curve which represents the OD at 405 nm as a function of the known concentrations of solubrious phosphate (KH_2PO_4).

The total soluble phosphorus was calculated from the regression equation of standard curve;

P(µg/ml) = (y-0,1175)/0,0058. Where y = sample OD. **(1)**

2.4. IAA production

The qualitative test of the root growth-promoting hormone auxin (indole-3-acetic acid) was realized on the medium YEM agar supplemented with 0,5 mg/ml of L-tryptophan. After, an incubation period of 3 days at 28 °C for, sterile filter paper washers impregnated with Salkowski reagent had deposited onto the Petri dish containing the culture. The appearance of colonies with a pink-red hue after a duration of 30-60 minutes in darkness indicates the production of IAA.

The levels of the hormone auxin produced by bacteria in culture filtrate was estimated as described by Sarwar et *al*. (1992). Approximately 2 mL of culture was centrifuged at 9,000 rpm for 10 min, and a 1 mL aliquot of the supernatant was mixed with 2 mL of Salkowski's reagent (150 mL concentrated H_2SO_4, 250 mL distilled water, 7.5 mL 0.5M $FeCl_3 \cdot 6H_2O$) and was incubated for 30-60min in darkness at room temperature. IAA production was observed as the development of a pink-red color, and the absorbances of each sample was read at 540 nm using a spectrophotometer (Thermo Scientific, USA) and the level of IAA (µg.ml−1) calculated using the formula below and information generated by a standard curve (Gordon and Weber,1951) made from a series of solutions with known auxin concentrations ranging from 0 to 120 ppm;

IA A (µ g/ml) = (y-0,16)/0,004; where Y = sample OD. **(2)**

2.5. Siderophore production

All glass ware was soaked overnight in 6M HCl and rinsed with distilled water several times to remove any traces of iron. Siderophore production was tested qualitatively using Chrome Azurol S medium (CAS-medium) as described by Schwyn and Neilands. 10 µl of each isolate culture was spotted on the surface of CAS agar medium and incubated at 28 °C for 72 h. Siderophore production was assessed on the basis of change in color of the medium from green to yellow after incubation, three replicates were performed. Measuring the diameters of the halo and the Bacteria colony makes it possible to establish a report for each strain reflecting the quantity of siderophores secreted;

SI= HD/CD; where SI = siderophore production index; HD = halo diameter; CD = colony diameter.

2.6. Statistical Analysis

All data obtained were analyzed using SAS 9.4 software. This data reported were means of at least three replicates. One-way analysis of variance and Student-Newman-Keuls test were used to compare the amount of soluble phosphate, the quantity of IAA and siderophore production index of endophytes

isolated from Bambara groundnut plants grown on different soils. Differences with level of 5 %. were considered significant.

3. RESULTS

After being reactivated, the strains in our collection were examined for purity. Each strain displayed well-isolated colonies on the petri dishes that shared the same morphological traits, such as color, diameter, appearance, etc. This demonstrates the colonies' homogeneity and uniformity, which reflects the reactivated strain's purity. The reactivated strains were stored at +4° for the remainder of the tests.

3.1. Phosphate solubilization

The ability of isolates to solubilize inorganic phosphorus was determined firstly in Phikovskaya agar plate containing tricalcium phosphate (Ca3PO4) (Figure 1a). All 34 isolates were shown positive for phosphorus solubilization and form a clear halo. The quantitative estimation of the phosphorus solubilization by these strains in liquid medium showed an important variability between strains highlighted by the different shades of yellow expressing the variation in the concentrations of solubilized phosphate (Figure 1b). Indeed, the concentration of solubilized phosphate were very significantly different (P= 0,0001) with value ranging from 16.43 to 453.45µg/ml (Table 1). Otherwise, most isolates had the capability to solubilize the high concentration of phosphate, thereby the strains RFK27 *Pseudomonas poae*, RFK11 *Bacillus pocheonensis*, RFK8 *Bacillus subtilis*, RFK12 *Bacillus cereus*, RFK29 *Lysobacter* and RFK10 *Bacillus subtilis*, with respectively, 453.45 µg/ml, 439.66 µg/ml, 387.94 µg/ml, 361.64 µg/ml, 316.63µg/ml and 313.37 µg/ml were identified as most efficient P-solubilizer (Table 1).

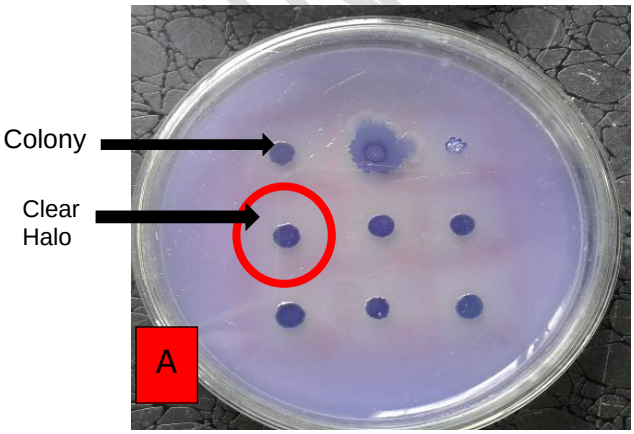

Figure 1a: qualitative test

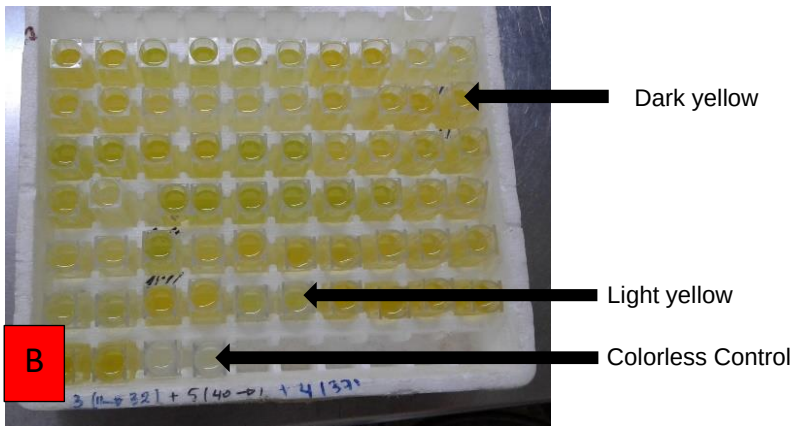

Figure 1b: quantitative test

Figure 1: Phosphate solubilization test

3.2. IAA production

The ability of the bacterial isolates to produce IAA was detected by the development of pink-red color after the addition of Salkowski reagent to the culture, firstly in YEM-tryptophan agar plate (Figure 2a). All isolates were able to synthetize this phytohormone using tryptophan as precursor. The quantitative estimation of the IAA revealed an important variability between strains highlighted by the different shades of pink expressing the variation in the concentrations of AIA produced (Figure 2b). In fact, there were a highly significant difference (P = 0,0001) between the quantities of synthetized IAA, which ranged from 16.3 to 275.5µg/ml. The highest production was obtained by RFK12 *Bacillus cereus*, RFK34 *Curtobacterium pusillum*, RKF10 *Bacillus subtilis* and RFK22 *Pseudomonas sp* producing, respectively, 275.5 µg/ml, 254 µg/ml, 246µg/ml and 212 µg/ml (table 1).

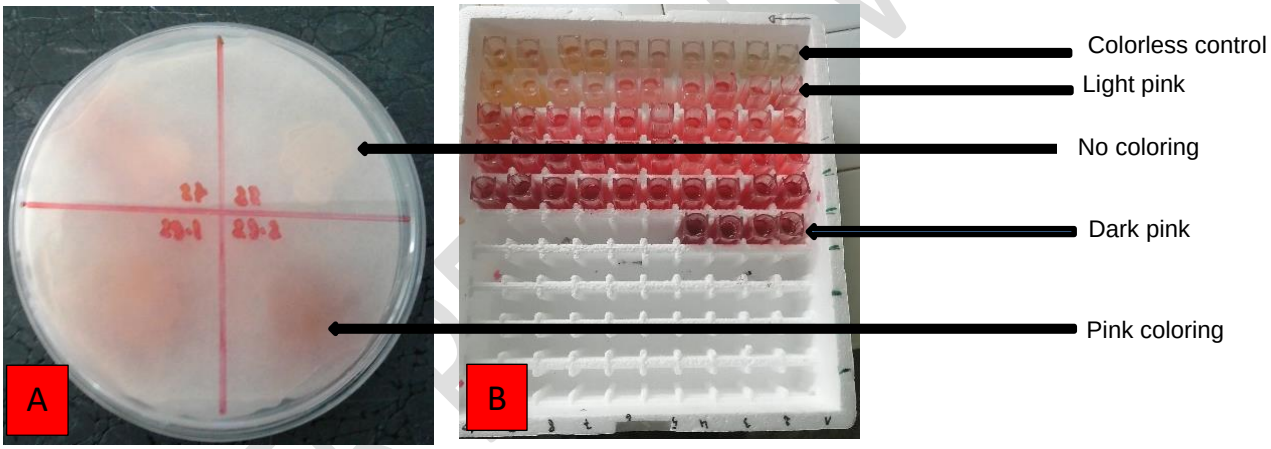

Figure 2a: qualitative test Figure 2b: quantitative test

Figure 2: IAA production test

3.3. Siderophore production

The ability of isolates to produce siderophore was determined by the development of a yellow halo around the bacterial colony growing in CAS agar plate (Figure 3). All isolates were able to produce Siderophore except two isolates. However, a highly significant difference (P =0.0001) was observed between the calculated Siderophore production index, which ranged from 1.07 to 5.36. The isolate that showed the best capability in Siderophore production was RFK27 *Pseudomonas poae* , followed by RFK26 *Pseudomonas fluorescens* and RFK22 *Pseudomonas sp* that produce 5.36, 4.3 and 3.65 respectively (Table1).

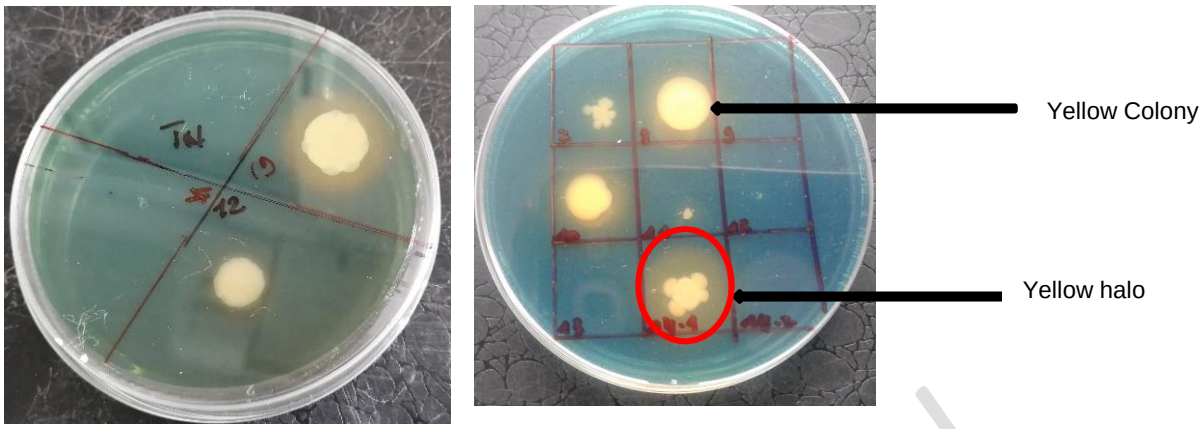

Figure 3: Siderophore production on chrome azurol S medium

Table 1: Plant growth-promoting abilities of representatives of root-nodule bacteria isolated from ivoirian bambara groundnut

strains	*closest relative species*	*Amount of P (µg/ml)*	*Amount of IAA (µg/ml)*	*Siderophore production index*
RFK1	*Bacillus megaterium strain FBMAX18*	233.6±9.89 g	112.50±3.87 no	1.16±0.01 l
RFK2	*Bacillus sp. L105*	104.41±2.91 qp	152.5±2.50 h	1.07±0.00 l
RFK3	*Bacillus sp. L105*	255.71±21.16 g	23.50±1.5 xy	2.30±0.03 ghijk
RFK4	*Bacillus safensis strain BXC22*	167.53±9.21 ml	19.58±4.28 xy	0
RFK5	*Bacillus nealsonii strain ASB-160*	251.10±1.77 f	16.3±5.62 y	2.00±0.05 ijk
RFK6	*Bacillus megaterium strain YN7 16S*	185.76±9.12 hjk	136.13±9.87 jk	3.5±0.03 cde
RFK7	*Bacillus sp. BAB-3563*	60.83±1.47 ut	26.00±3.25 x	3.22±0.1 cdef
RFK8	*Bacillus subtilis strain THt3-1*	387.94±5.69 c	122.38±3.12 lm	2.50±0.06 fghij
RFK9	*Bacillus cereus strain THt1-8*	73.95±1.64 st	51.25±7.75 v	2.85±0.08 defgh
RFK10	*Bacillus subtilis strain V90*	313.37±5.43 e	246.00±8.46 c	2.75±0.11 efghi
RFK11	*Bacillus pocheonensis strain P12*	439.66±3.45 b	129.8±3.12 kl	1.08±0.02 l
RFK12	*Bacillus cereus strain BCRh6*	361.64±4.12 d	275.5±5.51 a	2.84±0.33 defgh
RFK13	*Bacillus cereus strain BAB-6399*	48.97±7.67 u	181.88±4.63 f	2.51±0.04 fghij
RFK14	*Bacillus subtilis subsp. subtilis strain GC17*	51.29±5.20 u	104.42±1.66 op	2.49±0.02 ghij
RFK15	*Bacillus tequilensis strain RBB6*	160.29±3.69 m	74.83±8.37 st	2.51±0.07 fghij
RFK16	*Bacillus megaterium strain VITNJ1*	135.40±6.21 n	93.75±3.51 q	.67±0.05 kl
RFK17	*Bacillus megaterium strain E2-04*	178.45±5.18 jkl	141.75±6.75 ij	2.57±0.01 fghi
RFK18	*Bacillus megaterium strain DS8*	115.95±3.27 nop	54.87±5.13 v	3.6±0.03 cd
RFK19	*Bacillus subtilis strain LWIS15*	299.07±14.14 e	154.75±2,75 h	3.61±0.06 cd
RFK20	*Bacillus subtilis subsp. subtilis strain AN3*	94.28±8.39 qr	116.25±7.06 mn	2.1±0.1 hijk
RFK21	*Pseudomonas sp. strain Fas20*	84.05±3.89 rs	145.63±3.13 h	3.52±0.03 cde
RFK22	*Pseudomonas sp strain 7*	212.77±4.13 h	212.00±4.76 d	2.46±0.05 fghij
RFK23	*Pseudomonas azotoformans strain R2SsM3P2C7*	111.01±5.93 nop	39.67±1.15 w	3.65±0.04 c
RFK24	*Pseudomonas sp. strain Fas20*	67.73±8.99 stu	97.58±3.75 pq	3.05±0.07 cdefg
RFK25	*Pseudomonas koreensis strain PS3TA2*	132,155±11,22 n	79.25±8,01 rs	2.00±0.04 ijk
RFK26	*Pseudomonas fluorescens strain FC6846*	125.78±7.1 no	174.62±4.12 g	4.3±0.09 b
RFK27	*Pseudomonas poae strain 15-A1*	453.45±4.13 a	82.75±2.75 r	5.36±0.03 a
RFK28	*Lysobacter sp. KNUC361*	161.58 ±12.68 m	21.00±3.5 xy	1.32±0.02 l
RFK29	*Lysobacter sp. KNUC361*	316.63±3.97 e	69.5±3.0 ut	2.92±0.02 cdefgh
RFK30	*Rahnella inusitata strain FOD 9/21*	229.70±4.10 g	196.12±4.13 e	2.3±0.01 fghij
RFK31	*Microbacterium sp. HBUM179633*	16.47±6.64 v	85.50±8.75 r	3.00±0.06 cdefg
RFK32	*Microbacterium sp. strain JL3592*	81.72±2.90 rs	119.0±3.88 mn	0
RFK33	*Paenibacillus lautus strain E118*	49.23±9.41 u	103.87±2.62 op	2.72±0.12 efghi
RFK34	*Curtobacterium pusillum*	68.23±3.12 stu	254.0±4.50 b	2.77±0.14 efghi
Average		183,57±10,22	118,51±5,94	2,44±0,09
CV		65,41	58,94	45,37
Pr>F		<.0001	<.0001	<.0001

Values indicate mean values (±SD); different letters indicate significant differences within a column at the 5 % level according to the Newman-Keuls test

4. Discussion

The context of this study is rooted in the growing need to promote environmentally friendly agricultural practices. In this context, the evaluation of the PGPR potential of endophytic bacteria associated with Bambara groundnut as a promising response to improve crop productivity while reducing the use of chemical inputs. Thus, this study is positioned at the heart of current issues by contributing to the search for sustainable solutions for agriculture

The majority of nodule endophyte in this study had potential for promoting plant growth by the production of IAA, production of siderophore and solubilization of phosphate. the capacities of these strains would be linked to their intrinsic characteristics, in particular their genetic lineage. Indeed, several researches have revealed that bacteria with PGP traits were generally affiliated with the genera *Bacillus*, *Pseudomonas*, *Paenibacillus*, *Rahnella* etc.. included within Firmicutes and Proteobacteria phyla (Bahroun et *al.,* 2018; Pérez-Montaño et *al.*, 2014). It confirms the results reported by Kuklinsky-Sobral et *al.* (2004) which studied nodule endophyte isolated from soybean

Phosphorous is one of the major second nutrient only to nitrogen in requirement for plants. It plays an important role virtually in all major metabolic processes in plant including photosynthesis, energy transfer, signal transduction, macromolecular biosynthesis and respiration (Khan et *al.,* 2010) .
All strains were able to solubilize phosphate in quantities ranging from 16 to 453.45 µg/ml. However, the of six (06) strains (RFK27 *Pseudomonas poae*, RFK11 *Bacillus pocheonensis* , RFK8 *Bacillus subtilis*, RFK12 *Bacillus cereus,* RFK29 *Lysobacter*, RFK10 *Bacillus subtilis*) that solubilized high concentrations of phosphate (quantities > 300 µg/ml), four (04) were *Bacillus* species. These strains are able to solubilize phosphate because soil microorganisms are naturally occurring biological agents that can dissolve inorganic phosphate and make it. Similar results were found by Ferchichi et *al.* (2019) who showed that better quantity of solubilized phosphate was 320.27 µg /ml and *Bacillus subtilis* was well documented for their phosphate solubilizing abilities (Ferchichi et *al.*, 2019; Mohamed et *al.*, 2018). According to recent research, some of the genera with PGPR traits that enable them to dissolve phosphorus include *Arthrobacter*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Microbacterium*, *Pseudomonas*, *Rhizobium*, *Mesorhizobium*, *Flavobacterium*, and *Serratia* (Riaz et *al.*, 2021).

Other researchers reported that the endophytic bacteria of cocoa nurserie (41,28 % of the collection) from ivoirian soil were able to solublize phosphate with Solubilisation index indices ranging from 20% to 200% (Ouattara et *al*., 2019). The ability of some microorganisms to convert phosphate to form solution is available, such as orthophosphate, is an important PGPR trait to increase crop yields (Chen et *al*., 2006).

IAA is one of the most important phytohormone and function as important signal molecule in the regulation of plant development (Compant et *al.*, 2010).
All strains tested produced a detectable quantity of IAA, which ranged from 16.3 to 275.5µg/ml. Of the four (04) strains (RFK12 *Bacillus cereus, RFK34 Curtobacterium pusillum,* RFK10 *Bacillus subtilis,* RFK22 *Pseudomonas sp)* with the best AIA productions (quantities > 200 µg/ml), two (02) belong to the genus *Bacillus*.

The ability of the tested strains to produce AIA may be attributed to the substantial presence of AIA in the nodules of leguminous plants. Indeed, this phytohormone may be involved in the development of legume-rhizobia symbiosis, a phenomenon that is the foundation for the formation of nodules. (Duca *et al.,* 2014).

Kumar and *al.* (2012) found that the range of IAA production was 0.2 to 213 µg/ml. On the contrary, several studies demonstrated the ability of nodule endophyte to produce IAA with value ranging from 0.89 to 63.55 µg/ml (Saïdi et *al*., 2013) and from 0.67 to 74.51 µg/ml (Ferchichi et *al.*, 2019). It has been reported that IAA production by PGPR can vary among different species and strains (Compant et *al*., 2010). Also, several authors reported that Bacillus species used as biofertilizers probably have direct effects on plant growth through the synthesis of plant growth hormones (Amer & Utkhede, 2007).

Iron is an essential growth element for all living organisms. The scarcity of bioavailable iron in soil habitats and on plants surfaces foments a furious competition (Whipps, 2001).

This work showed that the majority of Bambara groundnut nodule endophytes were able to produce siderophore. However, it has been observed that the first best producers of siderophore were RFK27 *Pseudomonas poae* , RFK26 P*seudomonas fluorescens* and RFK22 P*seudomonas sp.* This PGPR trait has been observed in several in a variety of bacteria. Indeed, Research indicates that some plant growth-promoting bacteria (PGPB) produce low-molecular-weight chemicals (400–1500 Da) that have the ability to extract iron from the soil (Shanmugaiah et *al*., 2015) It has also been reported that Gram-negative bacteria that produce siderophores are Enterobacter and Pseudomonas. In contrast, only 2% of Gram-positive species, such as *Bacillus* and *Rhodococcu*s, can do the same (Czarnes et *al*., 2020). Other authors reported also the production of siderophore by *pseudomonas (*Sasirekha and Shivakumar, 2016).

The strain RFK27 *Pseudomonas poae* which was the best producer of Siderophore was also the best efficient P-solubilizer. and produced a significant amount of IAA. The strain RFK12 *Bacillus cereus* that was better producer of IAA was also best fourth efficient P-solubilizer. Researchers such as Backer et *al*. (2021) and Ramakrishnan et *al.* (2024) noted the importance of PGPRs by saying that PGPR.can promote plant growth trough mobilization nutrients in soils, production plant growth regulators, protection plants from hytopathogens by controlling or inhibiting them, improvement soil structure and enhanced tolerance to biotic and abiotic stresses .

The endophytic bacteria in this study have the potential to be used in sustainable agriculture as biofertilizers. These bacterial strains can help reduce the dependence on synthetic chemicals, which contributes to the preservation of the environment.

5. Conclusion

The endophytes isolated from Bambara groundnut nodules in Daloa soil demonstrated overall great capacities to solubilize phosphate and produce IAA and siderophores. Nevertheless, these bacteria showed a disparity in their capacity to execute these three (03) PGPR functions, thereby exhibiting varying potential in promoting plant growth. In total, 19 strains present multi PGPR characters very interesting for the improvement of plant productivity.

In perspective, it would be interesting to test the 19 strains with multi-PGPR traits under field conditions.

6. References

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