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## Effect of diazotrophic plant growth promoting rhizobacteria on seed germination of chickpea (*Cicera rietinum L.*)

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### ABSTRACT

Plant growth-promoting rhizobacteria are a group of free-living bacteria that colonize the rhizosphere. In the present study, 12 bacterial isolates have been isolated from the rhizosphere of chick pea cultures. The isolates have been screened for their capacity to produce particular metabolites (hydrocyanic acid production HCN, inorganic phosphates solubilization and the proteins hydrolysis) and also screened for their antifungal activity against *Fusarium oxysporum f. sp. ciceris*. All isolates have presented a good antagonistic activity against *Fusarium oxysporum f. sp. ciceris*. Compared with the others isolates such as NfA, NfD and NfC have manifested a good proteolysis and phosphorus solubilization capacity, where the index varied from 1.83 to 3.03 and their antagonistic activity against *Fusarium oxysporum f. sp. Ciceris* has reached 20%. Finally, the selected isolates were tested *in vivo* on the seed germination of chickpea, where they manifested an interesting capacity to promote seed germination and plants growth. In conclusion, the selected isolates, NfA, NfD and NfC have presented an excellent effect for promoting germination of the chick pea, where the vigor index registered ranged from 140 to 180.

**Keywords:** PGPR, diazotrophes, antifungal activity, germination, AIA, HCN, proteolysis.

### INTRODUCTION

In Algeria, chickpea is the second legume crop produced after beans. The chickpea cultivated territories have progressively been extended during 1980 to 1990 [1], but the progressive evolution in consumption and the low productivity implicated the return of Algeria to the exterior market to make up the shortage in matter of consumption needs by the importation of more than 120 000 tons/year [2].

Algerian production of chickpea did not increase because of low productivity and unstable output. Causes of regression are agronomic, abiotic and biotic factors [3]. The establishment and the success of chickpea culture depend on the nature of the rhizobacteria flora. Lately, numerous authors have brought back the implication of beneficial rhizobacteria or plant growth promoting bacteria (PGPB) in the improvement of the soils quality, plants growth and plants resistance to pathogens. The use of those bacteria as biofertilizers reduces chemical products input and pollution risks. It also improves agricultural production.

Free living rhizospheric bacteria that have beneficial effects on plants belong essentially to genera: *Pseudomonas*, *Bacillus*, *Azorhizobium*, *Azotobacter*, and *Bradyrhizobium*. Generally, 2% to 5% rhizospheric bacteria are PGPR [4]. PGPR may induce plants growth promotion by several direct and indirect mechanisms. Direct mechanisms include the production of stimulatory bacterial volatiles and phytohormones (such as auxins, cytokinins and gibberellins), lowering of the ethylene level in plant, improvement of the plant nutrient status (liberation of

phosphates and micronutrients from insoluble sources; non-symbiotic nitrogen fixation) and stimulation of disease-resistance mechanisms (induced systemic resistance). Indirect effects originate for example when PGPR act like bio control agents reduce diseases or stimulate other beneficial symbioses [6, 7].

From the first studies on PGPR in 1950 years, hundred PGPR genera have been screened and evaluated in laboratories and on field through the world. Today, PGPR are used in the developed country and their inocula are applied to million hectares of cultures [8].

The aim of the present work was the isolation of the diazotrophic PGPR from chickpea rhizosphere, and the screening of the isolates for their capacity to produce particular metabolites (hydrocyanic acid production HCN, inorganic phosphates solubilization and the proteins hydrolysis). Furthermore, the antifungal activity of the isolates strains has been investigated against pathogenic fungi *Fusarium oxysporum*. and their effect on the germination and plant growth promotion have been studied on chick pea seed.

## MATERIELS AND METHODS

### Soil sampling and bacterial isolation

The rhizospheric soil samples (six) were collected from fields growing Chickpea (*C. arietinum* L.) from the region of west of Algeria (**Table 1**). For this purpose, 1g of rhizospheric soil sample was suspended in 9 ml of 0.9% NaCl sterile saline solution. Samples were serially diluted and a volume of 0.1 ml of each sample was spreaded on N-free Nourris agar plates, incubated at 28°C for 3 to 7 days.

**Table 1: Physical and chemical characteristics of soil samples.**

Soil samples	City	pH	Total limestone	Active limestone	Organic matter	Silt	Clay	Sand
AIN TOLBA	AIN TEMOUCHENT	7.96	9.6	8.75	2.48	65	15	20
AIN TEMOUCHENT		8.04	12	5.62	6.16	55	15	30
MALEH		7.86	10.4	7.5	6.77	45	20	35
LAMTAR	SIDI BEL ABBES	7.95	9.2	6.25	3.85	55	10	35
TESSELA		7.81	8.4	5.62	3.03	55	15	30
KAID BEL-ARBI		7.70	10	6.25	1.55	40	10	50

### Biochemical characterization of rhizobacteria

The isolated, selected isolates were primary biochemically characterized by Gram's reaction, catalase test, motility test, oxidase test, and the most interesting isolates were identified by the using of API E20 system identification.

### Characterization of rhizobacteria for PGP traits

#### Phosphate solubilization

The phosphate solubilization activity of the selected Isolates was carried out according to the described method by Nautiyal [9] by the using Pikovskaya agar plates containing tricalcium phosphate [ $\text{Ca}_3(\text{PO}_4)_2$ ] as sole phosphorus source. The presence of the transparent halos around the grown colonies indicated the chsolubilization of phosphate. Furthermore, the phosphate solubilization activities were evaluated by calculation of phosphate solubilization index (SI):

$$SI = \frac{\text{colony diameter} + \text{halozone diameter}}{\text{Colony diameter}}$$

#### Production of hydrogen cyanide HCN

All the selected phosphate solubilization isolates were screened for the production of hydrogen cyanide by the using of the adapted described by method by [10]. Briefly, the used nutrient agar culture medium was supplemented with 4.4 g glycine/l and bacterial strains were streaked on the surface of the modified agar plate. A Whatman filter paper no 1 was soaked in a solution containing 2% sodium carbonate solved in 0.5% picric acid solution was placed at the top of the plate. Plates were sealed with parafilm, incubated at 28°C for 5 days. The development of orange to red color indicated the production of HCN.

#### Protease production

The determination of the Protease production by the selected phosphate solubilization isolates, inoculated in the skim milk agar culture medium containing the following components: pancreatic digest of casein 5 g, yeast extract 2.5 g, glucose 1 g, 7% skim milk solution 100 ml [11], incubated at 28°C for 2 days. The apparition of a clear zone around the grown colonies indicated the presence of the proteolytic activity [12].

**Antifungal activity**

The antagonistic activity of the selected phosphate solubilization isolates against pathogenic fungi was determined by the modified dual culture method on PDA plates [13]. The used *Fusarium oxysporum f. sp. ciceris* furnished by INRAA. For this purpose, a loopful of 3 days old bacterial cultures grown in nutrient agar media, was streaked on tow side leaving 1 cm from the margin, and then 6 mm disc of fungal pathogen culture was placed at the center of the Petri plates. Plates without antagonist were served as control. The plates were inoculated at 28°C for 7 days. The antifungal activity was estimated from the inhibition of mycelial growth of fungus in the direction of actively growing bacteria. The level of inhibition was calculated by subtracting the distance (mm) of fungal growth in the direction of an antagonistic isolate from the fungal radius. The percent inhibition was calculated by the using of the following formula:

$$\% \text{ inhibition} = (R - r) / R \times 100$$

Where 'r' is radial growth of the fungal colony opposite the bacterial colony, **R** the radial growth of the pathogen in control plate [14].

**Seed germination assay**

Rhizobacterial isolates were assayed for their ability to promote seedling growth by the using the modified described methods by [15] and Elliot and [16]. The surface seeds were sterilized with 13° sodium hypochlorite for 3 to 5 min, washed with sterile distilled water for 8 times. The surface seeds were soaked with 3 days old bacterial broth cultures containing at least  $10^6$  cells/ml for 30 min. The seeds were kept on sterilized filter paper (in Petri plates), incubated at room temperature. After incubation times of 10 days, percent of seed germination, root and shoot lengths were recorded. The Vigor Index [17] was calculated as:

$$VI (\%) = (\text{mean root length} + \text{mean shoot length}) \times \text{germination} \%$$

Data were statistically treated using STATITC statistical program, Least Significant Difference (LSD) test at probability level 0.03 was used. Newman and keulstest was used to separate the means when treatments indicated a significant effect.

**RESULTS****Soil analysis and Bacterial isolation**

The primary analysis of samples indicated that the used soil has alkaline pH-value with low proportion of lime and good proportion of organic matter and presenting a silty-sandy texture. Twelve successfully isolated diazotrophic bacterial strains from chickpea rhizosphere were morphologically and biochemically identified. The obtained results indicated the selection of the ten motile isolates, where eight strains manifested a positive reaction with catalase (**Table 2**).

**Table 2: Morphological and biochemical characteristic of isolates**

Morphological characterization	Nf1	Nf2	Nf3	Nf5	Nf7	Nf8	Nf9	Nf10	Nf11	NfA	NfD	NfC
Shape	rod	rod	rod	oval	rod	rod	rod	rod	rod	oval	oval	oval
Gram	-	-	-	-	-	-	-	-	-	-	-	-
Pigment brown	-	-	-	+	-	-	-	-	-	+	+	+
Motility	+	+	+	+	-	-	+	+	+	-	+	+
Catalase	+	-	+	+	-	+	-	+	-	+	+	+
Oxidase												

The macroscopic observation of the isolated, selected phosphate solubilization strains indicated the presence of circular transparent, shining, viscous, white to yellowish colonies of 1 to 5 mm diameter. The bacterial isolates differed on cell-shape and colonies time apparition.

The microscopic observation of the isolates NfA, NfD, NfC and Nf5 indicated the presence of a oval cells, where their colonies appraised after 2 days incubation, where the isolates Nf1, Nf2, Nf3, Nf7 to Nf11 were rod and required 3 days for colonies apparition. Furthermore, the isolates NfA, NfD, NfC and Nf5 produced brown water insoluble pigment after 5 to 7 days incubation and their colonies took a granulated aspect.

**Plant growth promoting activities**

The isolates were screened for in vitro plant promoting activities. The obtained results of PGPR traits were illustrated in (**Table 3**). Hydrogen cyanide (HCN) production was observed only with isolates Nf1 to Nf5. Isolates Nf1, Nf2, Nf3, Nf8, eNf9, Nf10, Nf11, NfD and NfC exhibited protease (casein hydrolysis) activity. Four bacterial isolates

were able of solubilizing  $[\text{Ca}_3(\text{PO}_4)_2]$ . Phosphate solubilization index ranged from 1.1 to 3, the highest level of phosphatesolubilizationwas expressed by the isolate NfA.

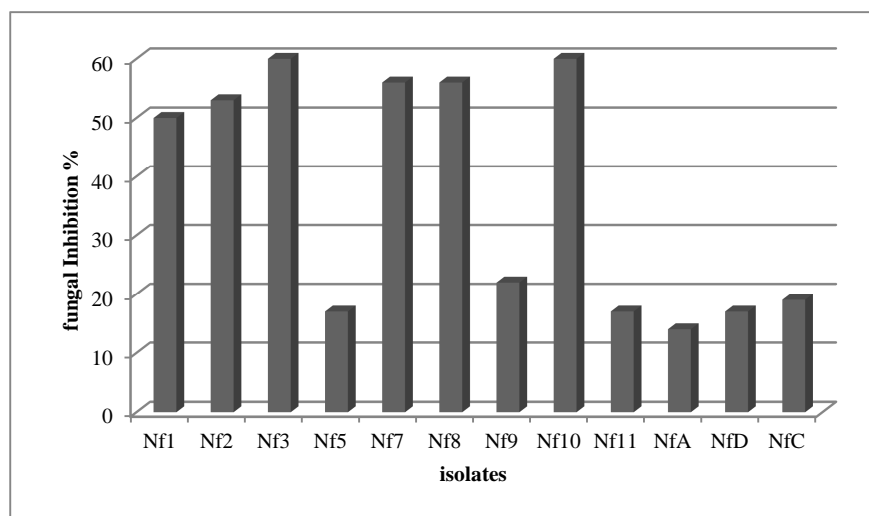


Figure1: The antifungal activity of the selected phosphate solubilization isolates against *Fusarium oxysporum f. sp. ciceris*

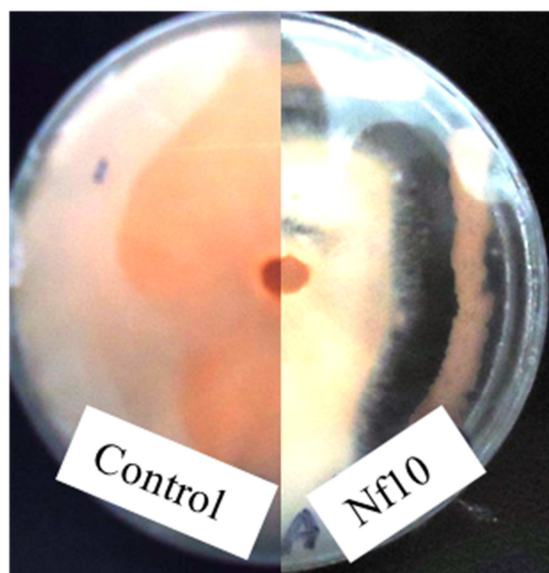


Figure2: The antifungal activity of the selected phosphate solubilization isolate Nf10

Table3: Diazotrophic isolates plant growth promoting characteristics

	Nf1	Nf2	Nf3	Nf5	Nf7	Nf8	Nf9	Nf10	Nf11	NfA	NfD	NfC
P solubilization	-	-	+	-	+	-	-	-	+	+	+	+
P solubilization index	/	/	1.1	/	1.1	/	/	/	1.1	3.03	1.83	1.83
Protease	+	+	+	-	-	+	+	+	+	-	+	+
HCN	+	+	+	+	-	-	-	-	-	-	-	-

The selected phosphate solubilization isolates were screened for antifungal activity against *Fusarium oxysporum f. sp. ciceris*. The obtained results showed that all the isolates have manifested antagonistic activity against *Fusarium oxysporum f. sp. Ciceris*, where the inhibition rate varied from 14% to 61%. The maximum antagonistic activity was observed by the by both selected phosphate solubilization isolates Nf3 and Nf10 with inhibition rate of 61%, followed by both selected isolates Nf8 and Nf10 with (56%). Furthermore, the lowest antagonistic activity was shown by isolate producing pigmentation (Nf5, NfA, NfB and NfC).

#### Seed germination assay

In order to explore the effect of the inoculation of the selected phosphate solubilization isolates, seed germination of chickpea has been investigated. The obtained results showed that the isolates seed inoculation has significantly

affected chickpea seed germination percent, shoot length, root length and seedling vigor. The highest response for seed germination percent, shoot length and vigor index has been recorded with isolates Nf5, NfA, NfC and NfD. The isolate Nf5 has manifested a negative effect on seed root length. Furthermore, the rest of the investigated isolates showed a negative effect on chickpea germination parameters, where the means recorded for these isolates were lower than control means. The lowest response has been observed with the isolate Nf2.

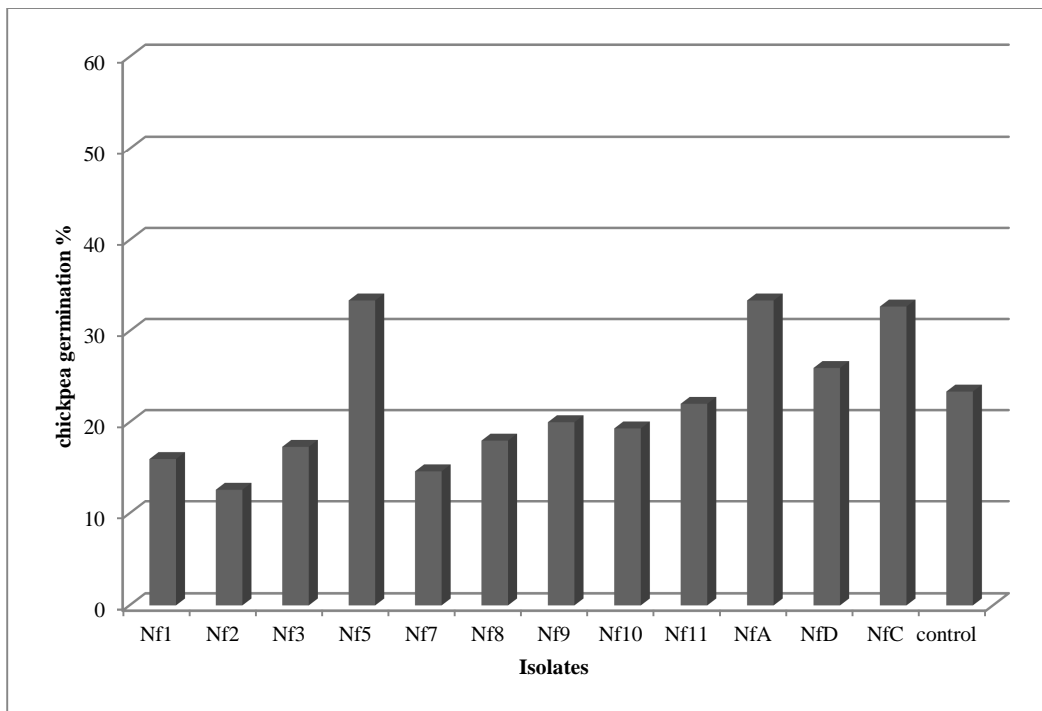


Figure 3 : Effect of the selected phosphate solubilization isolates seedling inoculation on germination percent

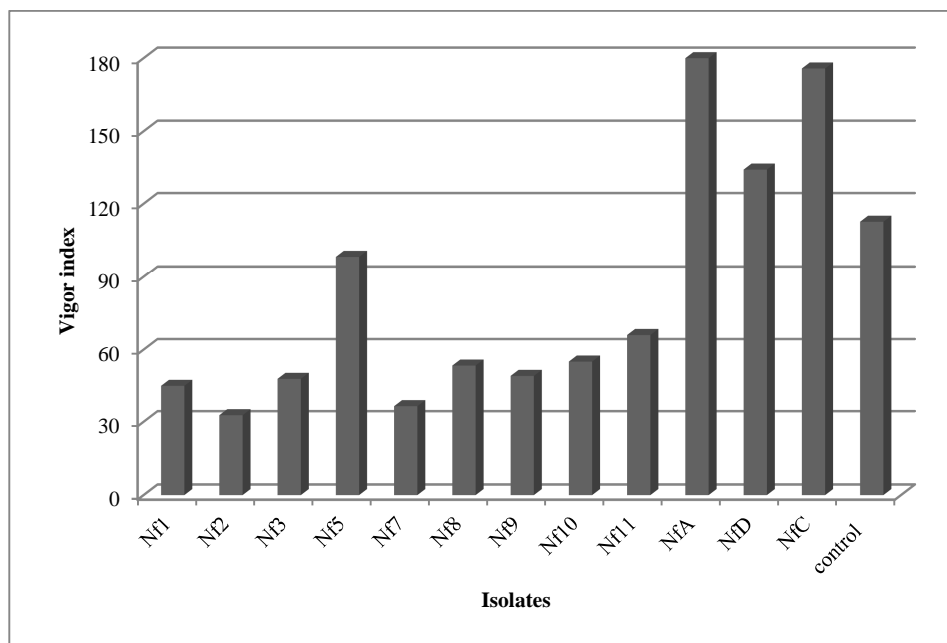


Figure4: Effect of the selected phosphate solubilization isolates seedling inoculation on vigor index



Figure 5 : Effect of the selected phosphate solubilization isolates seedling inoculation on chickpea germination.

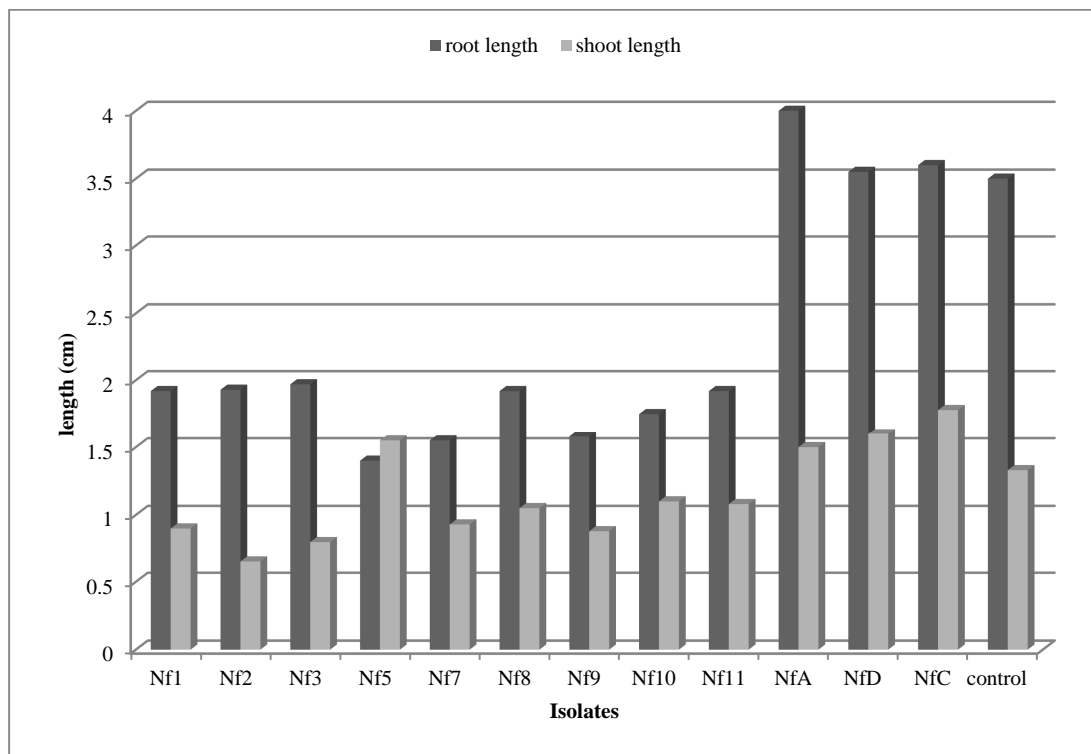


Figure 6: Effect of the selected phosphate solubilization isolates inoculation on chickpea seedling shoot and root length

### Bacterial identification

Based on their PGP traits and effect on chickpea germination, Isolates Nf5, NfA, NfC and NfD has been chosen to be identified by the using API E20 system identification, where the obtained results are presented in (Table 4).

The selected phosphate solubilization isolates (Nf5, NfA, NfD and NfC) were oval shape cells, no spore-forming, cyst forming and brown pigment producing. Their grown colonies presented in granulated aspect after several days incubation. Biochemically identification, indicated that the selected phosphate solubilization isolates (Nf5, NfA, NfD and NfC) were oxidase, catalase, ADH, TDA, glucose and arabinose positive and ODC, H<sub>2</sub>S, VP and gelatine negative. Furthermore, the isolates presented different reactions to the rest of tested substrats. According to the mentioned characteristics, isolates were identified as *Azotobacter sp.*

**Table 4: Biochemical characteristic of isolates Nf5, NfA, NfD and NfC**

Morphological and biochemical characterization	Nf5	NfA	NfD	NfC
Spore	-	-	-	-
Brown pigment	+	+	+	+
Kyst	+	+	+	+
Oxidase	+	+	+	+
Catalase	+	+	+	+
ONPG	-	-	+	-
ADH	+	+	+	+
LDC	-	-	+	-
ODC	-	-	-	-
CIT	+	-	+	+
H <sub>2</sub> S	-	-	-	-
URE	-	-	-	+
TDA	+	+	+	+
IND	-	-	+	+
V P	-	-	-	-
Gelatine	-	-	-	-
Glucose	+	+	+	+
Mannitol	-	-	+	+
Inositol	-	+	+	+
D-Sorbit	-	+	+	+
L-Rhamnose	-	-	+	+
D-melibiosa	-	+	+	+
D-sucrose	-	+	+	+
L-Amygdalin	-	+	-	+
L-Arabinose	+	+	+	+
Identification	<i>Azotobactersp</i>	<i>Azotobactersp</i>	<i>Azotobactersp</i>	<i>Azotobactersp</i>

## DISCUSSION

Nonsymbiotic nitrogen fixation is known to be of great agronomic significance. Free Nitrogen-Fixing PGPR were the first investigated strains for the improvement of plant growth. The estimated contribution of free-living nitrogen-fixing prokaryotes to the nitrogen input of soil ranges from 0 to 60 kg ha<sup>-1</sup> year.

In the present study, 12 free nitrogen fixing bacteria has been isolated from chickpea rhizosphere, based on their aptitude to grow on the culture medium of Norris-Nitrogen free agar. The microscopic observation indicated that selected phosphate solubilization isolates were rod to oval Gram negative bacteria, where eight manifested a positive reaction of the catalase activity. The bacterial strains possessing catalase activity must be highly resistant to environmental, mechanical and chemical stress [18]. The isolates presented several PGPR traits; other than N<sub>2</sub> fixation, six isolates were able to solubilize inorganic P, phosphate-solubilizing microorganisms produce a major contribution to overall plant P nutrition and growth and have increased yields of many crops. They help to minimize the application of P-fertilizer, reduce environmental pollution, and promote sustainable agriculture [19]. Nine isolates manifested protease activity, where and only four isolates produced HCN. The selected phosphate solubilization isolates (Nf5, NfA, NfD and NfC) showed an excellent antagonistic activity against *Fusarium oxysporum f. sp. ciceris*. The isolated, cultivated *Azotobacter* on Muller-Hinton medium has manifested a broad spectrum antifungal activity against *Aspergillus*, and more species of *Fusarium* and *Rhizoctonia bataticola* [4]. Generally, the diazotrophs are able to decrease considerably the deleterious effects of plant pathogens mostly through the synthesis of antibiotic and fungicidal compounds, through competition for nutrients or by the induction of systematic resistance to pathogens [5]. The investigated isolates on mannitol motility agar indicated that nine of the twelve isolates were motile, where the motility was required for successful rhizosphere colonization [20]. Some of the above investigated isolates could exhibit more than two or three PGP traits, which may promote plant growth directly or indirectly or synergistically. Similar to our findings of multiple PGP activities among PGPR have been reported by many other workers [21]. The cultivation of selected phosphate solubilization isolates (Nf5, NfA, NfC and NfD) 3 days has produced a colonies with brown color with a granulated aspect. The microscopic observation indicated the presence of oval cells, kyst forming, motile, catalase and oxydase positive. Furthermore, the study of biochemical properties showed gelatin and H<sub>2</sub>S negative, glucose positive and mostly mannitol positive and isolates NfC and NfD were rhamnose positive. These cultural characteristics are similar to those reported in the literature for bacteria from the genus *Azotobacter* [22, 23]. The application of isolates Nf5, NfA, NfC and NfD has significantly improved chickpea germination, which was clearly expressed via the Vigor index obtained. The Vigor index reflected the health of the seedlings produced, where the high value indicated the best seedling health.

The Vigor index showed that, generally, PGPR inoculation had a positive influence on plant growth parameters [24]. Similarly results have been reported by Saikia and co-workers [25], where the seed germination of chickpea

*Cicerarietinum* has been increased. However, the yield improvement was attributed to the ability of *Azotobacter* to produce plant growth promoting substances such as phytohormone IAA and siderophoreazotobactin, rather than to diazotrophic activity [5].

### CONCLUSION

From the obtained results, the antagonistic activity of the selected phosphate solubilization isolates (Nf5, NfA, NfD and NfC) against *Fusariumoxysporum f. sp. Cicer* has been explored. The selected strains were identified as *Azotobacter*. It would be of interest to find out which functional group is responsible for the antagonistic activity of the selected phosphate solubilization isolates (Nf5, NfA, NfD and NfC) and also whether any of them is a novel compound with antagonistic activity which would use for plant growth promoting for seed germination of chickpea. Further studies involving the purification of the chemical compounds of the secondary metabolite produced by the selected phosphate solubilization isolates (Nf5, NfA, NfD and NfC), by the using of a modern technique such HPLC, IRM, will require for determination of this active molecules.

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