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Research Article

Antifungal activity of isolated *Bacillus* species against chickpea *Fusarium* wilt

Noureddine Rouag[1*], Hannane Abed[1, 2], Dahou Moutassam[2], Selma Mehmah[1] and Sabrina Benabid[1]

1. Laboratory of Applied Microbiology, Faculty of Nature and Life Sciences, University Ferhat Abbas - Sétif 1, Algeria.
2. Laboratory of Characterization and Natural Resources Valorization, Faculty SNV-TU, University Bordj Bou Arreridj, Algeria.

Abstract: Biocontrol of *Fusarium oxysporum* f. sp. *ciceris* by six *Bacillus* species was evaluated. Also plant growth promoting potential of the bacteria was assessed. Results showed that four bacterial strains produced the chitinase and cellulase enzymes and all isolates produced indole acetic acid. *Bacillus licheniformis* proved to be the most productive of hydrogen cyanide and particularly, *Bacillus firmus* solubilized phosphorus on Pikovskaya solid and liquid media. The majority of strains were able to produce siderophore and three produced NH_3. Results showed that the Flip05-156C chickpea variety was less susceptible to *Foc* isolates compared to Flip93-93C and there was a clear difference in pathogenicity of the *Foc* isolates. Thus, *Foc*1 and *Foc*2 isolates caused 31.25% and 41.66%, plant mortality, respectively. As regards PGPR effect, results showed that *B. licheniformis* gave the best branching number, stem length and root weight of both chickpea varieties. However, *Bacillus lentus* distinctly improved the root length while *Bacillus amyloliquefaciens* improved weight of the vegetative parts.

Keywords: biocontrol, pathogenicity, *Fusarium oxysporum* f. sp. *ciceris,* plant growth promoting rhizobacteria

Introduction

Chickpea *Cicer arietinum* L. is the third most important pulse in the world, after beans and peas (Vishwadhar Gurha, 1998). Chickpea production is limited by several biotic and abiotic factors. Among the biotic stresses, *Fusarium* wilts caused by *Fusarium oxysporum* Schl. Emend. Snyd. and Hans. f. sp. *ciceris* (Padwick; *Foc*), is regarded as one of the most important diseases of chickpea. *Fusarium* wilt is prevalent in almost all chickpea-growing areas of the world, and its incidence has varied from 14% to 32% in India (Dubey *et al.,* 2010). This disease causes yield losses up to 100% under favorable conditions in chickpea (Landa *et al.,* 2004). It has been reported that the annual chickpea yield losses caused by *Fusarium* wilt were 10% in India (Singh and Dahiya, 1973) and Spain (Jimenez-Díaz *et al.,* 2015), and more than 40% in Algeria (Zemouli-Benfreha *et al.,* 2014).

Fusarium wilt of chickpea is a monocyclic disease in which development is driven by the pathogen's primary inoculum. Therefore, management of the disease should be targeted at exclusion of the pathogen as well as reducing the amount and/or efficiency of the initial inoculums (Jimenez-Díaz *et al.,* 2014). Management of *Fusarium* wilt of chickpea is difficult and is often based on solarization of soil, choice of sowing date, use of *Foc*-free seeds and fungicide-treated seeds. These are some of the

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* **Corresponding authors**, e-mail: n.rouag@univ-setif.dz
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measures usually employed to control *Fusarium* wilt in chickpea, but with limited success (Navas-Cortes *et al.,* 1998). The use of antagonistic microbes or their secondary metabolites is considered to be a practicable technology for the management of plant diseases (Han *et al.,* 2005). So, the use of the plant growth promoting rhizobacteria (PGPR) in biological control of plant pathogens is considered as a viable alternative method to chemical control (Saharan and Nehra, 2011). The most abundant bacteria in the rhizosphere are Bacillus species which are able to synthesize several antibiotics and substances involved in the protection and growth of plants (Girish *et al.* 2010; Nihorimbere *et al.,* 2010). Bacillus species are outstanding biocontrol agents with efficient root colonization, multiple modes of action and promising ability to sporulate (Kloepper *et al.,* 2004). Turner and Backman (1991) found that *Bacillus* sp. colonized the root surface, increased plant growth and caused lysis of fungal mycelia. It has been demonstrated that *Bacillus* species show antifungal activity against several pathogenic fungi such as *Fusarium moniliforme* in cassava (Agarry *et al.,* 2005) and *F. oxysporum* f. sp. *ciceris* (Nikam *et al.,* 2011). Parallel to this, *Bacillus* strains are known to produce compounds involved in plant growth such as hydrogen cyanide (HCN), siderophores, indole acetic acid (IAA) and solubilization of phosphorous (Godinho *et al.,* 2010; Saharan and Nehra, 2011; Alizadeh, 2012). Their endospore-forming ability also makes these bacteria one of the best candidates for developing efficient biopesticide.

The present study has been taken up with the objective to evaluate different *Bacillus* species isolated from chickpea rhizosphere for their plant growth promoting properties and their antagonistic effect(s) against *F. oxysporum, in planta*.

Materials and Methods

Bacterial material used

Six species of Bacillus isolated from the chickpea rhizosphere and identified in previous work (Abed *et al.,* 2016) by CH 50 galleries as *Bacillus firmus* (Bf-39), *B. amyloliquefaciens* (Ba-40), *B. lentus* (Bl-41), *B. licheniformis* (Bli-59), *Aneurini Bacillus aneurinilyticus* (syn. *B. aneurinolyticus*) (Aa-61) and *B. subtilis* (Bs-65) were used in this work.

Fusarium oxysporum isolates

Tow isolates of *Fusarium oxysporum* f. sp. *ciceris* were used in this work, originated from several chickpea cultivation areas in Algeria (*Foc*1 isolate from Mascara district in the West and *Foc*2 from Setif in the East parts of Algeria) (Abed, 2017).

Determination of factors involved in bacterial antagonism and PGPR effects

Hydrolytic enzyme production

The chitinase production was determined as described by Roberts and Selitrennikoff (1988). Bacterial cultures were spotted on minimal agar medium amended with 0.3% colloidal chitin and the plates were incubated at 30 °C for 7 days. The development of halo zone around the colony after addition of iodine was considered as positive for chitinase enzyme.

The production of cellulase was determined according to the method described by Cattelan *et al.* (1999). The M9 agar (Miller, 1972) supplemented with 10%w/v of cellulose and 1.2 g of yeast extract was used to test the hydrolytic action by the production of cellulase. The strains were plated and then incubated for 8 days at 28 °C. The development of a clear halo around the colonies indicated a positive reaction for cellulase production (Verma *et al.,* 2007).

Indole acetic acid production (IAA)

The production of IAA was determined as described by Bric *et al.* (1991). In nutrient broth (peptone, 5 g; yeast extract, 1.5 g; beef extract, 1.5 g; and NaCl, 5 g; each per liter) with or without tryptophan (5 Mm), bacterial strains were inoculated into and incubated at 30 °C for 5 days. After, 5 ml culture was removed from each tube and centrifuged at 10,000 rpm for 15 min. An aliquot of 2 ml supernatant was transferred to a fresh tube with 100 µl of 10 mM orthophosphoric

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acid and 4ml of reagent (1 ml of 0.5 M $FeCl_3$ in 50 ml of 35% $HClO_4$). The mixture was incubated at room temperature for 25min, and the absorbance of pink color developed was read at 530nm using a spectrophotometer (Gravel *et al.,* 2007).

Qualitative estimation of phosphate solubilization

Bacillus isolates were initially tested for their ability to solubilize insoluble inorganic phosphate on Pikovskaya's agar by spotting overnight grown cultures and incubating the plates for 48 h at 30 °C. The isolates showing clear zone of solubilization around the colony were taken as P solubilizers and the diameter of the zone was measured (Ahmed *et al.,* 2008).

Quantitative phosphate solubilization

Bacillus isolates showing zone of solubilization on Pikovskaya's agar medium were further examined for their ability to release Pi from TCP in broth medium. 1ml of overnight culture of each isolate was inoculated to 50 ml of Pikovskaya's broth (Pikovskaya, 1948). All the inoculated flasks were incubated at 28 ± 2 °C. The amount of Pi released in the broth was estimated from triplicate flasks at 5, 10 and 15 days of incubation with a set of uninoculated controls. The broth cultures were centrifuged at 10.000rpm for 10min to separate the supernatant from the cell growth and insoluble phosphate. The available P in the supernatant was estimated by phosphomolybdic blue color method as detailed below (Jackson, 1973).

Siderophore production

Siderophore production was determined on Chrome-azurol S (CAS) medium following the method of Schwyn and Neilands (1987). The bacterial strains (24 h old cultures) were spotted separately on CAS medium and incubated at 28 ± 1 °C for 48-72 h. The formation of orange to yellow halo around the colonies confirmed the production of siderophore.

Ammonia production

This qualitative test was carried out according to Cappuccino and Sherman's method (1992). Ten ml of peptone water (EP) (Cheminova) were inoculated with 100 µl of each bacterial suspension. After incubation at 30 °C for 96 h, 500 µl of the Nessler reagent (Prolabo) were added to each EP tube. The development of a yellow or orange color indicated the production of ammonia (NH_3).

Antifungal activity assessment

The demonstration of antagonism *in planta* aimed at evaluating the protection of chickpea plants provided by bacterial strains against *Foc* isolates. This involved pre-coating seeds of two chickpea varieties (Flip93-93C and Flip05-156C) with bacterial strains that showed interesting antagonistic activity *in vitro,* followed by their culture in pots with soil artificially infested with 10^6 spores/ml of both macrconidia and microconidia obtained from *Foc*1 and *Foc*2. This concentration is sufficient to reproduce the same symptoms seen in the field (Sharma and Muehlbauer, 2007; Westerlund *et al.*, 1974).

i) Preparation of bacterial inoculum

The selected bacterial isolates were concentrated in Erlenmeyer flasks containing 100 ml of sterile broth and were then shaken at 120 rpm for 48 h in an orbital incubator. The bacterial cells were centrifuged at 12,000 g at a temperature of 20 °C for 10 min. The pellets produced were dissolved in 10 ml of sterile distilled water and adjusted to a concentration of 1×10^8 CFU/ml with the use of a Petroff Hausser chamber (Kaur, 2003).

ii) Preparation of fungal inoculum

The two isolates of *Foc* used were grown in Erlenmeyer flasks containing corn-based culture medium (20 g of corn, 20 g of chickpea powder, 40 g of sand and 60 ml of sterilized distilled water) for 14 days at 25°C ± 2 °C (Kaur *et al.,* 2007).

iii) Seed treatment with antagonists

Certified seeds of Flip93-93C and Flip05-156C chickpea varieties were surface disinfected with a 3% w/v NaClO solution for 5 min, then rinsed

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three times in sterile distilled water and dried on paper towels. The bacterial isolates suspensions were initially mixed with carboxymethyl cellulose (CMC), which acts as sticky substance and were stirred for 2 h at 100 tr.min^{-1} before use (Kaur *et al.,* 2007). The chickpea seeds were coated by emergence into the bacterial-methylocellulose suspensions described above, for an entire night. The seeds were then dried with dry air under sterile conditions. Seeds only coated with 1% of methyl cellulose were used as controls of each variety (Kaur, 2003).

iv) Soil inoculation with pathogen and sowing seeds

Focs isolates previously grown in a culture medium containing sand and corn were used for artificial infestation of sterilized soil at a concentration of 10^6 CFU/g of *F. oxysporum* as described by Dileep Kumar in 1999. Three days later, 3 seeds per pot of each variety of chickpea previously coated with bacterial strains, were sown thus giving a total of 240 seeds per variety. It should be noted that each combination (*Foc* isolate - bacterial strain - chickpea variety) was replicated three times in this experiment. For controls, three replications per variety containing soil infested or not with solutions of macro and microconidia (*Foc* +/-) which were sown with seeds coated or not (+/-) with the bacterial isolates were maintained as positive or negative controls respectively.

The plants from the different combinations tested were regularly observed for possible symptoms. Five weeks after planting, plant growth parameters including number of branches, stem and root length, fresh weight of the vegetative and root systems were measured (Kaur *et al.*, 2007).

Effect of rhizobacteria on plant growth parameters

The bacterial strains previously tested for their antifungal activities were further used to evaluate any enhancement they might have on plant growth. Thus, the experimental design adopted consisted of sowing in three pots 3 seeds of each chickpea variety coated with a one bacterial strain. As controls, three pots per variety sown only with seeds not treated by bacterial strains were included in the experiment.

Five weeks after planting, plant growth parameters (number of branches, stem and root length, fresh weight of the vegetative and root systems) were measured (Kaur *et al.*, 2007).

Statistical data analysis

The data for antagonistic and PGPR effects on plant growth parameters were evaluated by analysis of variance at the significance level $P \leq 0.05$ and the mean squares and interaction effects were compared using the IBM SPSS software (Statistical Product and Service Solutions versions 21.0., 2011). Experiments were designed as a completely randomized design with three replications.

Results

Production of antifungal metabolites

Chitinase production

The results show that the *Bacillus* spp. used are highly variable in their chitinase production. Four out of the six strains were able to degrade chitin thus confirming the production of chitinase. *Bacillus firmus* and *B. licheniformis* were inactive on chitin, while *B. amyloliquefaciens,* and *B. lentus* were the most effective with a degradation zone equal to 17 mm (Table 1).

Cellulase production

Results show that *Bacillus* species were very heterogeneous in the production of cellulase. *Aneurini Bacillus aneurinlyticus* and *B. lentus* were very efficient, with a zone of degradation equal to 8.25 mm. Contrariwise, *B. firmus* and *B. subtilis* did not produce any cellulose (Table 1).

HCN production

All tested *Bacillus* strains produced HCN. The quantification by spectrophotometer of the HCN product revealed that the optical density varies from 0.06 µg/ml registered for *B. amyloliquefaciens* and *B. lentus* to 0.29 µg/ml for *B. licheniformis*, considered as the most effective in the HCN production (Table 1).

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Table 1 Secondary metabolites involved in the biocontrol and plant growth produced by *Bacillus* species tested.

Isolates	Chitinase (mm)	Cellulase (mm)	HCN (µg/ml)	IAA (µg/ml)	P (µg/ml)	Siderophore	NH_3
Bf-39	0	0	0.08	33.75	125.50	+	-
Ba-40	17.0	4.5	0.06	40.20	0	-	+
Bl-41	17.0	8.0	0.06	40.30	0	+	-
Bli-59	0	2.5	0.29	36.30	0	+	+
Aa-61	7.0	8.3	0.08	38.55	61.95	+	+
Bs-65	10.5	0	0.08	38.80	0	+	-

Bf-39: *Bacillus firmus,* ***Ba-40***:*B. amyloliquefaciens,* ***Bl-41***: *B. lentus,* ***Bli-59***:*B. licheniformis,* ***Aa-61***: *A. aneurinlyticus,* ***Bs-65***: *B. subtilis.*

IAA quantitative production

All the *Bacillus* isolates produced indole acetic acid when grown in media containing tryptophan which was visualized by the production of pink color in different concentrations. Results of IAA production varied from 33.75 µg/ml registered for *B. amyloliquefaciens* to 40.30 µg/ml obtained for *B. lentus* which qualified as the highest IAA producing strain (Table 1).

Solubilization of phosphate

Results show that the amounts of $Ca_3(PO_4)2$ solubilized by the *Bacillus* strains varied from 0 to 125.505 µg/ml. *Bacillus firmus* was the most effective with a solubilization equal to 125.505 µg/ml, *A. aneurinlyticus* was moderate with 61.95 µg/ml, whereas *B. amyloliquefaciens*, *B. lentus*, *B. licheniformis*, and *B. subtilis* strains were unable to solubilize phosphate (Table 1).

Sidérophores production

Five species were siderophore production positive and had formed orange to yellow halo around the colonies confirming the production of these substances, while *B. amyloliquefaciens* was inactive (Table 1).

NH_3 Production

Qualitative production of ammonia was observed in half of the strains tested, namely, *B. amyloliquefaciens, B. licheniformis* and *aneurini Bacillus aneurinlyticus*. On the other hand, *B. firmus; B. lentus* and *B. subtilis* were unable to produce NH_3 (Table 1).

Reaction of the two chickpea varieties to the *Foc* isolates

It was shown that the Flip05-156C variety showed signs of resistance to *Focs* with 60 plants killed out of 240 (frequency 25%). The Flip93-93C variety was more susceptible to *Focs* with 125 plants killed out of the 240 planted (52.08%). Concerning the pathogenicity of the two *Focs* isolates, it was found that there were differences between them. Results showed that 75 and 100 plants died out of 240 planted when plants were infected with *Foc*1 and *Foc*2, representing 31.25% and 41.66% plant death, respectively.

Antagonistic effect of Bacillus species on growth parameters of chickpea

Effect on the number of branches

When chickpeas were infected with *Foc isolates, Bl-41 B. lentus* isolate recorded the best score with an average equal to 6.17 branches per plant (Table 2). In the absence of *Foc*; bacteria was able to improve the number of branches compared to the negative control (not infected and not treated) which resulted in 13.5 and 12.25 branches per plant, Bf-39, Bl-41, Bli-59 and Aa-61 were able to improve this parameter compared to the positive control (infected but not treated with bacteria isolations (Table 2). On a varietal level, results show that *Foc*2 acts negatively much more on Flip93-93C variety and *Foc*1 on Flip05-156, resulting in only 2.71 and 3.14 branches per plant, respectively. It can be seen that the two *Foc* reduced drastically the number of branches per plant compared to 17 and 16.33 obtained with the

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negative controls, respectively (Table 3). Statistically, only varietal effect was significant at the 5% level (Table 4).

Effect of Bacillus on the Stem length

Results showed that all bacterial isolates improved the stem length, by 1.67cm and 2.33cm on the average when plants were infected with *Foc*1 and *Foc*2, respectively. Individually, Bli-41 *B. lentus* and Bf-39 *B. firmus* received the best score equal to 9.88 and 21.23cm, in the case of *Foc*1 and *Foc*2, respectively (Table 2). It should be noted that the above growth improvement of stem length remains inferior to length average of the negative controls; 29.13 cm and 10.25 cm, respectively.

In terms of varietal behavior, if negative effect of *Focs* is more pronounced on the variety Flip93-93C, when comparing the 5.03 and 9.18 cm, obtained with the two *Focs*, respectively, with 36.17cm as stem length in negative control (Table 3). On the other hand, no difference is observed between the plants infected by the two *Foc* and the mean of the not infected controls).Variance analysis showed a significant effect for chickpea varieties, *Bacillus* strains and Isolated *Foc* isolates, while all interactions were insignificant (Table 4).

Table 2 Antifungal effect on *Foc* isolates by Bacillus strains through growth parameters of chickpea plants.

Bacillus strains	Number of Branches		Stem length (cm)		Vegetative system weight (g)		Root length (cm)		Root system weight (g)	
	*Foc*1	*Foc*2	*Foc*1	*Foc*2	*Foc*1	*Foc*2	*Foc*1	*Foc*2	*Foc*1	*Foc*2
Bf-39	6.00 ± 2.14	4.67 ± 2.33	8.37 ± 5.47	21.23 ± 8.65	0.69 ± 0.28	0.18 ± 0.10	19.20 ± 6.44	5.67 ± 2.75	0.27 ± 0.18	0.64 ± 0.27
Ba-40	3.83 ± 1.80	2.33 ± 1.96	4.42 ± 3.85	3.10 ± 1.42	0.15 ± 0.08	0.23 ± 0.15	8.50 ± 4.98	5.22 ± 3.98	0.21 ± 0.14	0.21 ± 0.11
Bl-41	6.17 ± 2.37	6.17 ± 2.77	9.88 ± 5.10	8.25 ± 6.06	0.34 ± 0.25	0.44 ± 0.25	18.08 ± 7.41	17.78 ± 10.70	0.29 ± 0.24	0.46 ± 0.23
Bli-59	3.29 ± 2.12	3.86 ± 2.49	8.06 ± 5.20	5.80 ± 4.02	0.22 ± 0.14	0.24 ± 0.16	9.53 ± 6.17	6.36 ± 4.57	0.35 ± 0.22	0.41 ± 0.27
Aa-61	4.43 ± 2.14	3.57 ± 2.31	8.36 ± 5.43	11.41 ± 6.85	0.48 ± 0.31	0.14 ± 0.09	12.70 ± 6.24	4.17 ± 2.72	0.36 ± 0.23	0.40 ± 0.24
Bs-65	4.17 ± 2.10	2.33 ± 1.56	5.75 ± 3.93	5.13 ± 3.25	0.15 ± 0.10	0.08 ± 0.06	14.57 ± 6.89	2.43 ± 1.81	0.12 ± 0.09	0.39 ± 0.19
Not treated	13.50 ± 3.57	12.25 ± 4.11	29.13 ± 7.10	10.25 ± 3.47	0.90 ± 0.24	0.81 ± 0.01	32.63 ± 8.35	10.88 ± 3.06	1.37 ± 0.30	1.49 ± 0.27
Infected	5.33 ± 2.73	2.33 ± 1.45	1.67 ± 0.88	2.33 ± 1.20	0.30 ± 0.17	0.27 ± 0.15	2.33 ± 1.45	4.67 ± 2.60	0.16 ± 0.09	0.18 ± 0.10
Total mean	5.44 ± 0.85	4.47 ± 0.90	9.04 ± 1.95	8.77 ± 2.01	0.39 ± 0.08	0.27 ± 0.06	14.56 ± 2.41	7.06 ± 1.82	0.36 ± 0.08	0.50 ± 0.09

*Values are the mean of 3 replication/combination ± SE.
Bf-39: *Bqcillusfirmus*, ***Ba-40***: *B. amyloliquefaciens*, ***Bl-41***: *B. lentus*, ***Bli-59***: *B. licheniformis*, ***Aa-61***: *Aneurini Bacillus aneurinlyticus*, ***Bs-65***: *B. subtilis*. Control (-): Non bacterized (only *Fusarium*); Control (+): Non infected non bacterized.

Table 3 Behavior of chickpea varieties treated with Bacillus strains against *Foc* isolates through plant growth parameters.

Foc isolates	Number of Branches		Stem length (cm)		Vegetative system weight (g)		Root length (cm)		Root system weight (g)	
	Flip93-93C	Flip05-156	Flip93-93C	Flip93-93C	Flip05-156	Flip93-93C	Flip05-156	Flip05-156	Flip93-93C	Flip05-156
*Foc*1	6.52 ± 1.12	3.14 ± 1.12	5.03 ± 2.06	14.11 ± 3.76	0.55 ± 0.14	0.21 ± 0.09	18.70 ± 3.46	7.43 ± 3.44	0.17 ± 0.08	0.60 ± 0.13
*Foc*2	2.71 ± 0.89	4.10 ± 1.22	9.18 ± 2.76	2.83 ± 1.18	0.12 ± 0.04	0.26 ± 0.07	6.66 ± 2.54	6.01 ± 1.82	0.37 ± 0.11	0.22 ± 0.08
Not infected	17.00 ± 1.00	16.33 ± 0.67	36.17 ± 1.30	13.00 ± 3.00	1.13 ± 0.09	0.82 ± 0.01	40.83 ± 2.17	11.83 ± 4.11	1.66 ± 0.13	1.68 ± 0.26
Total mean	5.44 ± 0.85	4.47 ± 0.90	9.04 ± 1.95	8.77 ± 2.01	0.39 ± 0.08	0.27 ± 0.06	14.56 ± 2.41	7.06 ± 1.82	0.36 ± 0.08	0.50 ± 0.09

*Values are the mean of 3 replication/combination ± SE.
Bf-39: *B. firmus*, ***Ba-40***: *B. amyloliquefaciens*, ***Bl-41***: *B. lentus*, ***Bli-59***: *B. licheniformis*, ***Aa-61***: *A. aneurinlyticus*, ***Bs-65***: *B. subtilis*. Control (-): Non bacterized; Control (+): Non infected non bacterized.

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Table 4 Variance analysis of *Bacillus* antagonistic effect on plant growth parameters of chickpeA.

Source of variation	df	NB	SL	VSW	RL	RSW
Corrected Model	29	63.42**	413.31**	0.64**	254.26**	0.38**
Intercept	1	3063.01**	17014.52**	25.49**	5215.25**	12.46**
Bacillus Strain	6	40.99	318.47*	0.16	247.04	0.24
Foc Isolate	1	42.86	326.86	0.16	845.50*	0.77*
Variety	1	15.72	523.95*	0.27	48.14	0.36
Bacillus Strain × *Foc* Isolate	6	11.99	121.56	0.21	157.72	0.20
Bacillus Strain × Variety	6	44.08	252.65	0.39	296.80*	0.35*
Foc Isolate × Variety	1	119.05*	1377.00**	1.78**	510.60*	1.18**
Bacillus Strain × *Foc* Isolate × Variety	6	31.91	142.92	0.29	268.59	0.22
Error	60	20.78	129.62	0.20	120.56	0.14
Total	90					
Corrected total	89					

Values are the mean squares; **df**: degree of freedom, ** Significant at 1% level, * Significant at the 5% level. **NB:** Number of branches; **SL**.: Stem Length; **VSW**: Vegetative System Weight; **RL**: Root Length; **RSW**: Root System Weight.

Antagonistic effect of Bacillus on the vegetative system weight

The best score of the vegetative system was obtained by *B. firmus* Bf-39 namely 0.69 g. When chickpea plants were infected with *Foc*1 and *B. lentus* Bl-41gave the best score (0.44 g) in the case *Foc*2 (Table 2). These results represent almost half of the vegetative system weight recorded with negative controls i.e.0.9 and 0.81g, respectively.

Vegetative weight appears to be strongly influenced by both Fusarium isolates, compared with the uninfected Flip93-93C and Flip05-156C controls that yielded 1.13 and 0.82 g respectively (Table 3). The analysis of the variance showed a significant difference for these variables (Table 4).

Antagonistic effect of Bacillus on the root length

The results indicate that the improvement in root length is visible only when the plants are infected with the *Foc*1 isolate, compared to the mean of the infected and untreated control plants, which is equal to 2.33 cm. On the other hand, strain Bf-39 gave the best average root length equal to 19.20 cm when plants were infected with *Foc*1. On the other hand, in the case of *Foc*2, apart from the successful strain Bl-41, which gave an interesting improvement in the average length of roots equal to 17.78 cm, the rest of the strains showed no difference with the positive control plants (Table 2).

The root length of both varieties was significantly affected by *Foc* isolates compared to negative control plants (Table 3). Variance analysis showed that Bacillus strains effect, interaction between *Foc* isolates and chickpea and interaction between varieties and Bacillus strains were significant at 5% level (Table 4).

Antagonistic effect of Bacillus on the root system weight

In the case of *Foc*1 infections, apart from the Bs-65 isolate, the rest of isolates showed an improvement of fresh weight root system, particularly the isolate Bli-59 which produced a weight equal to 0.35 g. On the other hand, with *Foc*2, *B. firmus* Bf-39 gave the best root system weight equal to 0.64 g (Table 2). Regarding the varietal behavior, the results show that Flip93-93C variety is sensitive to *Foc*1 whereas Flip05-156 is sensitive to *Foc*2. Thus, we recorded an average root system weights equal to 0.17 g and 0.22 g, respectively, which are much lower compared to the negative controls (Table 3). In addition to a significant varietal effect, the analysis of variance indicated a significant effect of the interaction between Bacillus strains and chickpea varieties (Table 4).

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PGPR effect of bacterial strains

PGPR effect of bacterial strains on the number of ramification

The results showed that the Flip 93-93C variety reacted well to the bacterial effect by giving a higher branching number compared to the respective control. The gain obtained can sometimes exceed three branches per plant. On the other hand, and in the case of the Flip 05-156C variety, except for the strain *A. aneurinlyticus* Aa-61 which gave an average of 12.67 branches, slightly higher than that recorded for the control, the rest of the bacterial strains did not improvement this parameter. *Bacillus* strains tested showed a significant effect at %5 level (Table 5). Individually, *B. licheniformis* Bli-59 was found to be the bacterium that most stimulated branching, giving an average of 13.67 branches per plant (Figure 1).

PGPR effect of bacterial strains on the stem length

Also for this parameter, all rhizobacteria used were able to improve the stems length of Flip 93-93C variety compared to the control. On the other hand, in the variety Flip 05-156C, no improvement was registered, except for the strain Bf-39. The latter gave an average of 2.5 cm increase in stem length compared to the control. Our results also clearly show that the Bli-59 strain seems to be very interesting in stimulating this parameter with an average of 38.33 cm (Figure 1). The two parameters, whether the effect of bacterial strains or varieties are statistically significant at the 5% level (Table 5).

PGPR effect of bacterial strains on vegetative weight

The majority of *Bacillus* strains used for seed treatment of the Flip93-93C chickpea variety resulted in an equal weight gain of the vegetative system greater than the 1.37g obtained with the control (Figure 1). However, in the Flip05-156C variety, apart from Ba-40 and Bli-59, which differ from the rest of the strains, giving averages of 1.70 and 1.60g, the rest gave almost identical weights to the control. Statistically, a significant effect was recorded for only *Bacillus* strains (Table 5). Individually, *B. amyloliquefaciens* Ba-40 proved to be the best stimulant of the average weight of the vegetative system (2.17 g).

PGPR effect of bacterial strains on the root length

The results presented in Fig. 1 show that the coating of seeds by the different bacterial strains has markedly improved the root length of the Flip 93-93C variety. The gain is sometimes twice that of the control, for example the strain Bl-41, which gave a length equal to 31.47cm. Paradoxically, in the second variety, the application of bacterial treatment was constraining for the roots length. For example, average of root length for strain Aa-61 was only 10.44 cm.

Table 5 Variance analysis of *Bacillus* PGPR effect on some plant growth parameters of chickpea.

Source of variation	df	NB	SL	VSW	RL	RSW
Corrected Model	15	94.255**	579.693**	0.907**	230.634	0.436*
Intercept	1	2760.333**	15482.478**	21.888**	5736.356**	11.682*
Variety	1	19.593	637.049*	0.358	60.002	0.340
Bacillus Strain	7	161.094**	927.615**	1.549**	230.337	0.589*
Variety × Bacillus Strain	7	37.808	216.722	0.334	254.468	0.305
Error	74	22.595	149.565	0.231	150.644	0.174
Total	90					
Corrected total	89					

Values are the mean squares; **df**: degree of freedom, ** Significant at 1% level, * Significant at the 5% level. **NB:** Number of branches; **SL**: Stem Length; **VSW**: Vegetative System Weight; **RL**: Root Length; **RSW**: Root System Weight.

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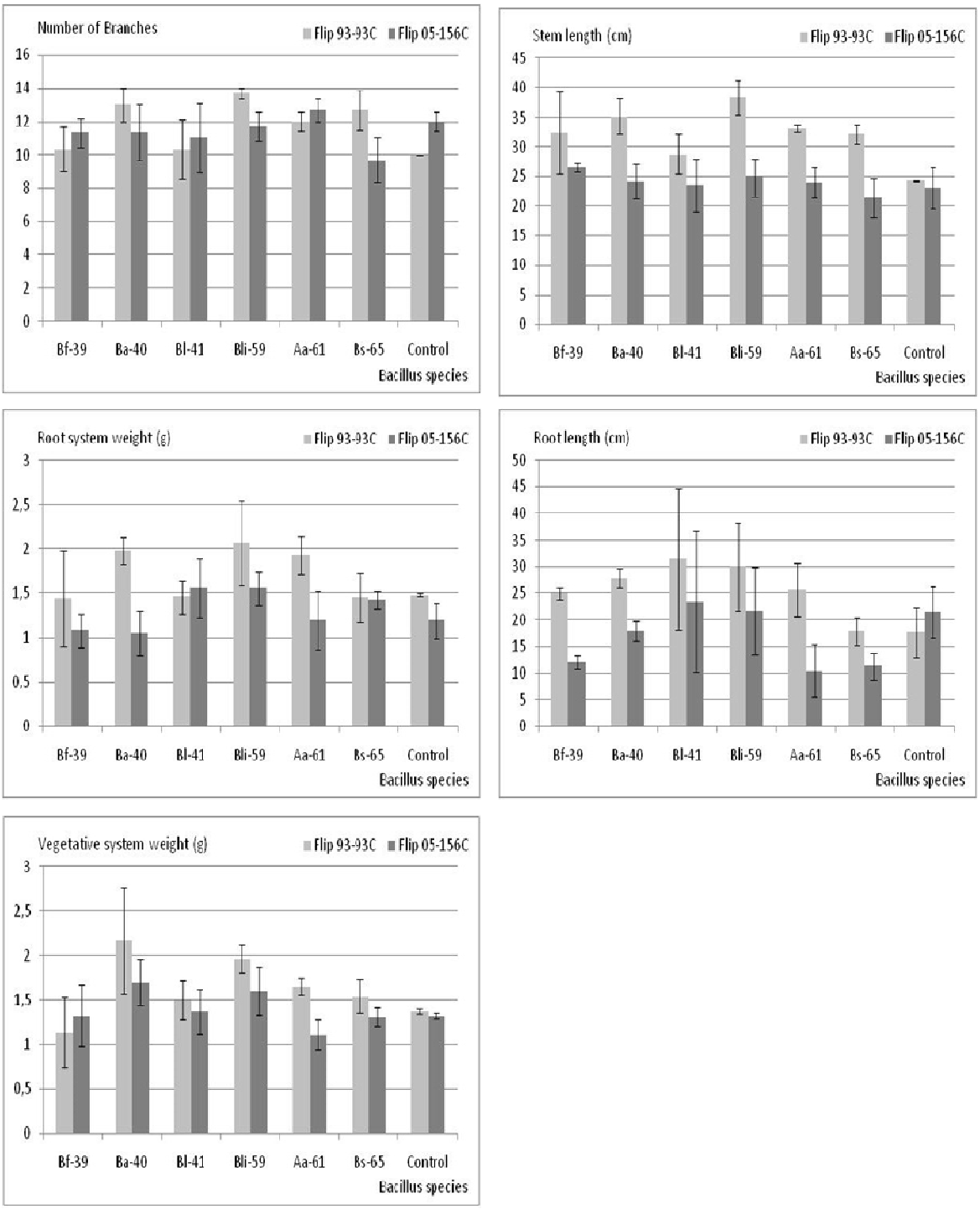

Figure 1 PGPR effect of bacterial strains on some plants growth parameters: Number of branches, Stem length, Vegetative system weight, Root length and Root system weight. ***Bf-39***: *Bacillus firmus,* ***Ba-40***:*B. amyloliquefaciens,* ***Bl-41***: *B. lentus,* ***Bli-59***:*B. licheniformis,* ***Aa-61****: Aneurini Bacillus aneurinlyticus,* ***Bs-65***: *B. subtilis.* **Control**: not treated.

[DOR: 20.1001.1.22519041.2019.8.4.2.8]

PGPR effect of bacterial strains on root system fresh weight

The following three bacterial strains: Bli-59, Ba-40 and Aa-61 can be considered beneficial for the growth of the root biomass of Flip93-93C variety, since they improved it with a distinction for Bli-59 strain which boosted the root system weight by 2.07 g (Figure 1). Regarding the bacterial treatment of seeds of Flip05-156C variety, a slight improvement was observed with strains Bl-41 and Bli-59 with the same root system weight equal to 1.56 g. Statistically, we recorded only a significant effect during the interaction between *Bacillus* strains and the chickpea varieties used (Table 5).

Discussion

Antagonistic microbes have the potential to inhibit plant pathogenic microorganisms by different mechanisms in eco-friendly manner. The use of antagonistic bacteria is reported as a powerful strategy to suppress soil-borne pathogens due to their ability to colonize the rhizosphere and ability to antagonize the pathogen by multiple modes of action. *Bacillus* spp. are recognized as safe biocontrol agents specifically as seed protectants and antifungal agents (Asaka and Shoda, 1996; Stein, 2005). The results of the present study show that all the *Bacillus* spp could produce indole acetic acid from tryptophan to enhance plant growth. According to Joseph *et al.* (2007), while working with chickpea, all *Bacillus* isolates produced IAA. It has been observed that the role of bacterial IAA in different plant-microbe interactions highlights the fact that bacteria use this phytohormone to interact with plants as part of their colonization strategy, including phytostimulation and circumvention of basal plant defense mechanisms (Samuel and Muthukkaruppan, 2011; Patel *et al.,* 2011).

Among soil microorganisms, several bacteria belonging to genera *Pseudomonas*, *Bacillus, Rhizobium*, and *Enterobacter* are capable of solubilizing P (Whitelaw, 2000). Phosphate-solubilizing microorganisms improve the supply of P to plants by their capability to solubilize inorganic or organic P and consequently result in an improved plant growth (Richardson 1994). Results obtained show that the amounts of $Ca_3(PO_4)2$ solubilized by the *Bacillus* strains vary from 0 to 125.505 µg/ml. Motsara *et al.* (1995) and Tilak and Reddy (2006) have reported the dominance of genus *Bacillus* as a P solubilizing bacteria in the rhizosphere of several crops. And there is no concordance between qualitative and quantitative estimation results these corroborate with results obtained by Rodriguez and Fraga (1999); which is in contrast to the pattern of phosphate solubilization by Tilak and Reddy (2006) SB in qualitative assay correlated well with the quantitative assay (Edi-Premono *et al*., 1996; Kumar and Narula, 1999; Mehta and Nautiyal, 2001). Microorganisms help to convert insoluble phosphorus in the soil to soluble sources that are accessible by plants for growth and increased yield (Saharan and Nehra, 2011). The P-solubilizing microorganisms have been used as inoculants with or without insoluble P source like rock phosphates for improving plant growth (Illmer *et al.,* 1995).

Rizosphere bacteria can produce various siderophores which may affect the biocontrol, virulence and availability of iron nutrients for plants (Kesaulya *et al.,* 2016). In the present study, 5 species were siderophores positive, while *B. amyloliquefceans* was inactive. Arya *et al* (2018) have found that the fungal inhibition zone of *F. oxysporum* f. sp. *lycopersici* was increased with the siderophore production qualitatively and quantitatively. Siderophores stimulate the biosynthesis of other antimicrobial compounds by increasing the availability of these minerals to the bacteria, which in turn would suppress the growth of pathogenic organisms *viz., F. oxysporum* and *R. solani*, function as stress factors in inducing host resistance (Haas and Defago, 2005).

All strains tested for *Bacillus* produced HCN, a result which does not correlate with that reported by Singh *et al.* (2008) in whose research no *Bacillus* isolate produced HCN. HCN produced by rhizospheric bacteria isolated from chickpea rhizosphere also promoted plant growth

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directly, indirectly and synergistically (Joseph *et al.,* 2007). Reports have shown that HCN influences plant growth indirectly especially isolates from rhizosphere of chickpea, rice and mangrove (Shobha and Kumidimi, 2012), and has antifungal activity against *Penicillium* spp, *F. oxysporum* and *Cercospora* spp. (Karuppiah and Rajaram, 2011).

Ammonia was produced by the three isolations tested. This is close to 95% of Ammonia produced by isolates from the rhizosphere of rice, mangrove and effluent contaminated soil influencing plant growth promotion (Samuel and Muthukkaruppan, 2011).

These rhizobacteria solubilized phosphate and produced phytohormone IAA which are factors regarded as systemic acquired resistance induced in different and diverse plants making such isolates to be considered as potential biocontrol agents (Lamsal *et al.,* 2012). It has been reported that *B. subtilis*FZB24 and FZB37 inhibited mycelia growth of *F. oxysporum, R.solani*and and *Sclerotinia sclerotiosum in vitro*. Incidence of *F. oxysporum* disease was significantly reduced by up to 50% while plant height, root and shoot fresh weight increased significantly compared to the control. *Bacillus* spp from the rhizosphere have been reported to be effective against a variety of soil borne pathogensusing diverse mechanisms (Choudhary and Johri, 2009).

In planta experiments, results show that the chickpea varieties differ in their behaviour towards the *Foc* strains tested. The Flip05-156C variety showed a mortality rate equal to 25% while Flip93-93C variety showed a rate of 52.08%. In addition, *Foc* isolates show quite a difference in virulence character; *Foc*1 and *Foc*2 caused death rates of 31.25% and 41.66%, respectively. The results obtained show that the tested bacteria acted as biocontrol agents and this was manifested through the improvement of certain plant growth parameters. Suthar *et al.* (2017) found that the *BS*-K18 seed treatment under *Foc* stress increased root length in resistant and susceptible variety. *B. lentus* has acted on the number of branches, the length of the stems and the length of the roots. The impact of *B. firmus*, was observed on the weight of the vegetative system and the length of the roots and finally *A. aneurinlyticus* has an effect on the length of the stems and the weight of the root system. This is in agreement with the results of Cazorla *et al.* (2007) and Patil *et al.* (2015) who reported that *Bacillus* strain UCR and *Bacillus* spp. produced antifungal substances with activity against a number of mycelial fungi.

The Rhizobatceria used in this study enhanced some growth parameters of the two varieties of chickpea. Thus, *B. licheniformis* and *B. amyloliquefaciens* improved aerial growth parameters, to which *Bacillus lentus* was added to improve root system weight. This is happening because *Bacillus* spp and/or their by-products are effective in inhibiting the mycelial growth of *F. oxysporum*. f. sp. *ciceris*. (Karthick *et al*., 2017) and are therefore found to be promising in reducing the root wilt of chickpea in glasshouse and field conditions (Smitha *et al.,* 2017).

Conclusion

The six Bacillus species (*B. firmus, B. amyloliquefceans, B. lentus, B. licheniformis, B. subtilis,* and *A. aneurinilyticus*) studied have different capacities for the production of plant growth-promoting substances, mainly concerning the P solubilization, IAA, ammonia, siderophores and also substances involved in antifungal activity against both isolates of *F. oxysporum* f. sp. *ciceris*, such as HCN, cellulase and chitinase. These bacteria have shown an interesting antifungal activity and improved growth of chickpea plants through increasing stem and root length, number of branches and fresh weight of root and vegetative systems.

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فعالیت ضدقارچی گونه‌های باسیلوس در مقابل پژمردگی فوزاریومی نخود

نورالدین رواگ[1*]، حنان عابد[1،2]، داهو موتسم[2]، سلما مهماح[1] و سبرینا بنادید[1]

۱- آزمایشگاه میکروبیولوژی کاربردی، دانشکده علوم طبیعی و زیستی، دانشگاه فرهات عباس ستیف ۱، الجزایر.
۲- آزمایشگاه توصیف و اعتبارسنجی منابع طبیعی، دانشکده اس ان وی-تی یو، دانشگاه بورج بو آریریج، الجزایر.
پست الکترونیکی نویسندگان مسئول مکاتبه: n.rouag@univ-setif.dz
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چکیده: در این بررسی کنترل زیستی *Fusarium oxysporum* f. sp. *ciceris* با استفاده از شش گونه باسیلوس مورد ارزیابی قرار گرفت. هم‌چنین توانایی باکتری‌ها در تقویت رشد گیاه نیز بررسی شد. نتایج نشان داد که چهار استرین باکتری آنزیم‌های کیتیناز و سلولاز و تمامی جدایه‌ها ایندول استیک اسید تولید کردند. گونه *Bacillus licheniformis* بیش‌ترین تولیدکننده سیانید هیدروژن و *Bacillus firmus* توانست فسفر را در محیط جامد و مایع پیکوفسایا به‌صورت محلول درآورد. اغلب استرین‌ها توانایی تولید سیدروفور را داشتند و سه استرین NH_3 تولید کردند. نتایج نشان داد که نخود رقم Flip05-156C کم‌ترین حساسیت به جدایه‌های *Foc* در مقایسه با Flip93-93C دارا می‌باشد و تفاوت واضحی بین بیماریزایی جدایه‌های مختلف *Foc* وجود داشت. به‌طوری که، جدایه‌های *Foc*1 و*Foc*2 به‌ترتیب موجب مرگ‌ومیر ۳۱/۲۵ و ۴۱/۶۶ درصد از گیاهان شدند. از نظر اثر تقویت‌کنندگی در رشد گیاه، نتایج نشان داد *Bacillus licheniformis* بیش‌ترین تعداد شاخه، طول ساقه و وزن ریشه در هر دو رقم نخود را ایجاد نمود. اگرچه، *Bacillus lentus* به‌تنهایی موجب تقویت طول ریشه و *Bacillus amyloliquefaciens* موجب بهبود وزن اندام‌های رویشی شدند.

واژگان کلیدی: کنترل‌زیستی، بیماری‌زایی، *Fusarium oxysporum* f. sp. *ciceris*، ریزوباکتری‌های تقویت‌کننده رشد گیاه

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