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Effect of *Bacillus* Species Rhizobacteria on Kabuli Chickpea Plants Growth under Pots and Field Conditions

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Abstract: In the present research, some Bacillus strains were produced at the industrial scale in order to be tested on chickpea growth, under pots and field conditions. Bacteria reached high sporulation yields ranging from 0.8×10^{9}-2.5×10^{9} and 8×10^{9}-10×10^{9} spores mL^{-1} in flasks and 500 L bioreactor culture conditions, respectively. Under pots experiment, *B. amyloliquefaciens* (9SRTS) and *B. amyloliquefaciens* (CWBI) increased significantly the root mass (0.31 and 0.37 vs. 0.066 g, respectively) and reduced the percentage of discolored leaves per plant (41 and 26 vs. 74%, respectively). Under field conditions, chickpea plants reached 21.59, 23.11, 20.80 cm, after 1 month of growth in lots treated with CWBI; 9SRTS; 6SEL (*B. atrophaeus*), respectively in comparison to control (17.63 cm). Root dry mass was not affected ($p>0.05$) and values were between 0.87 and 1.36 g. At harvest, the total number and mass of chickpea grains were higher in lots treated, compared to a control. Importantly, *B. amyloliquefaciens* (9SRTS) showed the best effect on chickpea crop yield (236 vs. 176 grains; 153 vs. 114 g). These data estimated per hectare reached 7.65 vs. 5.7 q, so, a gain of 2 quintals per ha.

Key words: Chickpea, plant disease, damping off, Bacillus, plant growth promotion, biocontrol, field

INTRODUCTION

Chickpea (*Cicer arietinum*) is an ancient crop that belongs to the legume family. It has been grown in Africa, the Middle East and India for centuries and is eaten as a dry pulse or green vegetable (Agyeman *et al*., 2004). Chickpea is a major source of human and animal food and the world's third most important pulse crop after beans (*Phaseolus vulgaris L.*) and peas (*Pisum sativum L.*) as described by Nikam *et al.* (2007). It occupies an area of 11.2 million ha and an annual production of 9.2 million ton over the world with an average yield of 820 kg ha^{-1} (Mbarek, 2011). The largest exporters of chickpea are Australia, Mexico, Turkey, Canada, United States and Iran. However, the most important importing countries are India, Pakistan, Spain, Algeria, Bangladesh, Italy, Saudi Arabia, Jordan, Tunisia and the United Kingdom. There are two main commercial classes of seeds: kabuli and desi kabuli is the most marketable type for domestic uses. Kabuli seeds have large, cream colored, round seeds; plants are 2-3 feet tall with white flowers (Akpa *et al*., 2001).

A number of constraints such as infertile and marginal lands, drought or excessive moisture, increasing temperature, weeds and accumulation of fungal pathogens are responsible for yield gap in chickpea (Canci and Toker, 2009; Toker *et al*., 2007). Many researchers reported that chickpea is attacked with many soil borne fungi, i.e., *Fusarium* sp., *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, *Pythium* sp., *Macrophomina phaseolina* causing damping-off, root an stem rot diseases. The use of resistant cultivars and chemical pesticides to manage chickpea production confronted several disadvantages such as the apparition of new pathogen overcoming resistant genes and severe dangers on environment and human health (Landa *et al*., 2004).

Plant Growth Promoting Rhizobacteria (PGPR) such as Pseudomonas and Bacillus strains can be a suitable approach in plant disease control (Ongena and Jacques, 2008; Ali and Nadarajah, 2013). These bacterial genera are the major root colonizers (Manikandan *et al*., 2010) which increase yield crops based on diverse mechanisms such as production of antibiotics (Cyclic Lipopetides: C-LPs), siderophores, cyanide hydrogen, competition for nutrition and space, inducing a systemic resistance, inactivation of pathogen's enzymes and enhancement of root and plant development by phytohormone production (Intana *et al*., 2011).

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The main objective of the present research is to test the effect of some Bacillus strains isolated from diverse environment of Algeria on the growth and yield production of Mega Grain Trading CO. (P): kabuli chickpea variety, under pots and field conditions.

MATERIALS AND METHODS

Bacterial strains: Four Bacillus strains were used in this study, i.e., *B. amyloliquefaciens* (9SRTS), *B. amyloliquefaciens* (CWBI), *B. subtilis* ssp. spizezenii (23SRTS) and *B. atrophaeus* (6SEL). All these strains were isolated from diverse environments of Eastern Algeria except "CWBI" which was provided by *Artechno* society (Liege-Belgium). Their screening was carried based on several *in vitro* tests, i.e., determination of antifungal activity by dual culture technique, against *Botrytis cinerea*, *Aspergillus niger*, *Alternaria alternata*, *Fusarium oxysporum* and *Cladosporium cucumerinium*, the sporulation yield at laboratory and industrial scales; production of C-LPs, siderophores, cell-wall degrading enzymes (cellulose, protease and chitinase) and the phytohormone IAA. More information is shown in our previous studies (Kaki *et al*., 2013), industrial production of screened bacteria screened bacteria were produced at the industrial level in a bioreactor of 500 L in Artechno society (Liege-Belgium). The optimum medium (opt. medium) as described by Jacques *et al.* (1999) was used for the production of Bacillus spores. The fermentation conditions were established at T° = 30°C, pH = 7, agitation speed = 140 rpm and dissolved oxygen (DO2 = 100%). Bacterial cultures reached the maximum of sporulation yield after 96 h. The fermentation was so stopped at this moment and spores suspensions were centrifuged and the pellets obtained were dried into a powder product by lyophilization process.

Effect of *Bacillus* spp. on Mega Grain Trading CO. (P): Kabuli chickpea variety growth

Under pots conditions: Bacillus strains 9SRTS, 23SRTS and 6SEL and CWBI were used as bio-control agents. Holes were made on soil surface and 1 mL of bacterial suspension (10^7 cell/mL) was added by pulverization. In each 30 cm diameter pot, six seeds were sown in holes and treatments were replicated three times. The experiment was realized on October 20th, 2013 and data were recorded for plant size, roots mass, damping off and leaves discoloration per plant, after 30 days of sowing. This experiment was carried out according to Karimi *et al.* (2012) with some modifications.

Under field conditions: Field experiments were conducted at the experimental ground of Chaab-Elrssas in Constantine (Algeria) in the period of July-October 2013. The experimental layout was split plot design, the surface of each plot was 0.8 m^2 with 0.60 m in between. Natural compost was added in each plot and 65 holes per row were made for growing seeds. About 1 mL of bacterial suspensions (10^7 cells/mL) was pulverized in each hole before sowing chickpea seeds cv. Mega Grain Trading CO. (P): kabuli. Untreated plots were used as control. In this experiment, Bacillus strains 9SRTS, 23SRTS and 6SEL were tested in addition to CWBI strain provided by the factory Artechno (Belgium). All the agricultural practices were applied as usual (Abdel-Monaim, 2011).

Statistical analysis: The SAS Software (Hammond Kosack and Jones, 2000) was used for all statistical analysis. Soil treatment effects on the parameters studied under pots and field conditions were assessed by a General Linear Model (GLM). Least Square Means (LSM) and standard errors were calculated, allowing ranking of treated and control lots according to Duncan's procedure.

RESULTS AND DISCUSSION

Literature on natural Biocontrol Agents (BCA) against phytopathogens revealed that *Bacillus* sp. have emerged as potential antiphytopathogenic and PGP agents, so far (Nayar *et al*., 1999; Driks, 2002; Schmidt *et al*., 2004; El-Bendary, 2006; Ongena and Jacques, 2008). In the present research, *B. amyloliquefaciens* (9SRTS), *B. amyloliquefaciens* (CWBI), *B. atrophaeus* (6SEL) and *B. subtilis* ssp. spizezenii (23SRTS) were tested on Mega Grain Trading CO. (P): kabuli chickpea variant, under pots and field conditions. Bacterial isolates were screened based on their interesting *in vitro* sporulation yields; bio-control and PGP traits, previously highlighted by us (Ait Kaki *et al*., 2014). Thus, they showed important growth inhibition percentages (39-83%) against *F. oxysporium* and *B. Cinerea*; this can be due to their capacity to produce cyclic lipopeptides (cLPs: iturin, surfactin) and cell-wall degrading (amylase, cellulose, protease, chitinase). These bacteria were also apt to produce the phytohormone Indol-3-Actic Acid (IAA) and siderophores chelating Fe. Several species of *Bacillus* spp. have been reported to produce auxins including IAA which have a positive effect on morphology and root growth (Yadav *et al*., 2011) and siderophores which are responsible for chelating Fe and depriving phytopathogenic fungi of it (Beneduzi *et al*., 2008). Sporulation differentiation is a phenomenon induced by reduced levels of nutrients in the environment or in culture; this process is unique to two bacterial species, Clostridium and Bacillus (Driks, 2002). Spores can survive for long periods without nutrients or water. This, make the production of bacteria belonging to the Bacillus genus, at the industrial level, realizable and profitable. Bacteria tested in this report reached high sporulation yields

ranging from 0.8×10^9-2.5×10^9 and 8×10^9-10×10^9 spores/mL in flasks and bioreactor of 500 L culture conditions, respectively. Sporulation yields obtained in batch culture here were relatively higher in comparison to the highest reported values for *Bacillus subtilis* spore production, i.e., studies of Monteiro *et al.* (2005, 2014). In fact, these researchers found at the end of fermentation in 2 L bioreactor, under batch culture conditions, 5.6×10^9 and 6.3×10^9 spores mL^{-1}, of *Bacillus subtilis* (MB24) in these two studies, respectively. In these researches, using of a fed-batch mode, allowed the obtaining of higher sporulation yields, reaching 7.4×10^9 and 3.6×10^{10} spores/mL, respectively. Thus, fed batch culturing of *Bacillus* spp. tested in the present research could allow getting higher spore's concentration. In addition, these bacteria showed an important stability after lyophilization (6×10^9; 7×10^{10} and 1.2×10^{11} cells g^{-1} of powder) (Table 1). All that makes of these Bacillus strains studied here, interesting competitors in a bio pesticides market which was estimated to be $1.4 billion in 2014 and is projected to grow with a CAGR (Compound Annual Growth Rate) of 13.4% from 2014-2019 (Anonymous, 2017)

The effect of Bacillus strains (CWBI, 9SRTS, 23SRTS and 6SEL) on growth and protection of chickpea Mega Grain Trading CO. (P): kabuli variety chickpea plants was studied under pots and field experiments. Bio-agents treatments were applied by pulverization of 10^7 cells mL^{-1} of bacterial suspensions on natural soil surface. Indeed, neither sterilization nor introduction of pathogens was carried in soil in order to test the effect of Bacillus strains under natural conditions as it will be applied by farmers, especially, that it has been shown that BCA effect is highly affected by the complex ecological processes involved in a field (Nithya and Halami, 2012; Calvo-Garrido *et al.*, 2014; Wei *et al.*, 2016). Different disease symptoms appeared naturally in control lots (plants sown in natural soil without applying any BCA treatment), under pots and field experiments. These symptoms correspond according to the agricultural engineers to diseases caused by some fungi that affect plants at the establishment (Pythium root rot; Fusarium root rot, Sclerotonia root rot, etc.) and during the growing season (ascophyta blight, Alternaria blight, etc.). In fact, chickpea plants in pots experiment showed a slow plant growth an important leaves discoloration (74%) and a remarkable decrease in number of leaves per plant (Fig. 1; Table 2) while pre-emergence damping off percentage was negligible (10%). In other hand, seed germination was seriously affected in field (58% of pre-emergence damping off) and only some leaves in the control lot (negligible) were discolored (Fig. 2). Through the field soil was used in pots experiment, the difference in disease symptoms was clear in both *in vivo* experiments. This can be explained by diverse weather and nutrients conditions subjected by plants in each experiment. In fact, natural compost was added to the field before sowing and both experiments were carried in different periods (July 2013, for field experiment and October 2013 for pot experiment). It had been shown previously, those abiotic factors (topography, soil and climatic factors), along with the biotic or living factors, determine the extent in which the genetic factor is expressed in the plant, hence, its response to diverse abiotic or biotic stresses will be variable (Akpa *et al.*, 2001; Atkinson and Urwin, 2012; Bonmatin *et al.*, 2003).

In general, treatment of soil by Bacillus strains showed interesting performances in reducing disease severity and enhancing plant growth in both pots and field experiment with some exceptions recorded. This may be due to their capacity, previously proven *in vitro* (Khaki *et al.*, 2013) to produce a set of bioactive

Table 1: Evaluation of vegetal cells and spores concentration in a fermentation jus of Bacillus strains produced after 72 h of fermentation in a 500 L bioreactor and estimation of living cells in a dried product after lyophilization

Bacterial strains	DO/mL	Dried mass (g L^{-1})	Total flora (Cell mL^{-1})*	Spore flora (spores mL^{-1})	Living cells in lyophilized product (cell g^{-1})
9SRTS	22	4	1.5×10^{10}	1×10^{10}	1.2×10^{11}
23SRTS	24	3.5	9×10^9	8.5×10^9	7×10^{10}
6SEL	20	4	9×10^9	8×10^9	6×10^9

*Concentrations appearing in Table 1 correspond to a number of CFU/mL measured after preparation of decimal dilutions. The determination of spores concentration had been carried after thermal treatment of dilutions at 80°C during 12 min

Table 2: Effect of Bacillus strains on plant growth (root mass, plant length) and protection parameters (discolored leaves per plant and pre-emergence damping off) of Mega Grain Trading CO. (P): kabuli chickpea variety, estimated after on month of growth under pots experiment

Strains	Roots mass	Plant length	Disease rating	Pre-emergence damping off (%)
CWBI	0.37±0.05[a]	54±3[a]	26±10[a]	10±14[a]
9SRTS	0.31±0.06[a]	48±3[b]	41± 12[a]	23±3b
23SRTS	0.14±0.06[b]	48±3[b]	71± 10[b]	10±14[a]
Control	0.066±0.07[b]	45±3[b]	74 ±12[b]	10±14[a]

[a, b]Different letters in the same column show the significant effect ($p<0.05$) of diverse Bacillus treatments, comparing to a control

Fig. 1: Effect of *Bacillus subtilis* ssp. spizezenii (23SRTS) and *B. amyloliquefaciens* (9SRTS) on development of plant: a) And root; b) In comparison to the control, after 1 month of growth under pots experiment

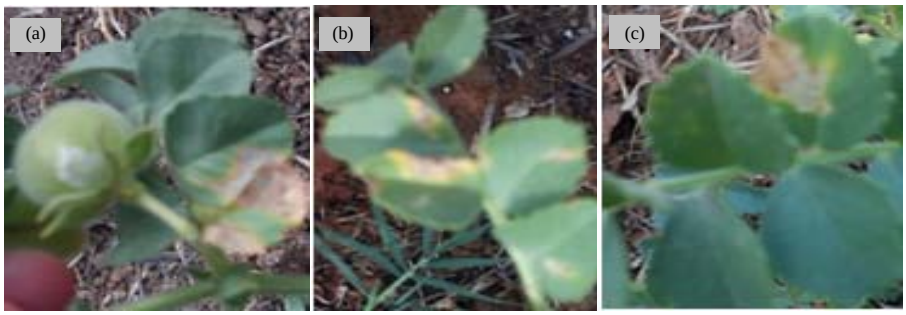

Fig. 2: a-c) Discoloration symptoms appearing in some plants in all field plots (treated with Bacillus suspension and untreated one)

molecules which are responsible of antibiosis (c-LPs, cell wall degrading enzymes), competition for nutrition and space (siderophores), enhancing plant growth (phytohormone IAA) and inducing plant systemic resistance (surfactin, fengycin) (Intana *et al*., 2011).

Under pot experiment, the strain *B. subtilis* ssp. spizezenii (23SRTS) had shown no significant difference in comparison to the control pots ($p>0.05$) for all parameters studied, i.e., root mass, plant length, discolored leaves percentage per plant and pre-emergence damping off (%). However; *B. amyloliquefaciens* (9SRTS) and *B. amyloliquefaciens* (CWBI) increased significantly the root mass (0.31 and 0.37 vs. 0.066 g, respectively) and reduced the percentage of discolored leaves per plant (41 and 26 vs. 74%, respectively). It is to note that, *B. amyloliquefaciens* (9SRTS) had a neutral effect on plant length and a negative effect on pre-emergence damping off (Table 2). In the other hand, *B. amyloliquefaciens* (CWBI) showed the most interesting effect on plant length (54 vs. 45 cm) (Table 2), so, we acknowledge that the effect of bacterial strains tested was variable according to *Bacillus* species and plant growth parameters studied. These results coincide with those reported by Latour *et al.* (1996) who demonstrated that the effect of rhizospheric bacteria on plant growth and seed germination rates can be beneficial, deleterious or neutral, depending on many factors (the bacterial species; the plant, environmental conditions). Furthermore, it had been reported by Nandakumar *et al.* (2001) that suspensions of three bacterial strains applied to the roots, leaves and soil resulted in the promotion of plant-development of rise

Fig. 3: Treatment effect of natural soil with Bacillus isolates (S499, 9SRTS and 6SEL) on seed germination capacity and plant length of Mega Grain Trading CO. (P): kabuli chickpea variety, after 1 month of sowing, under field conditions

Table 3: Least squares means of plant size and root mass ± standard error, calculated after 4 and 10 weeks of cv. Mega Grain Trading CO. (P): kabuli chickpea variety sowing, under field conditions

	Lots				
Periods	CWBI	9SRTS	6SEL	23SRTS	Control
Plant length (cm)					
4 weeks	21.9±0.3^{b}	23.11±0.93^{b}	20.80±1.03^{b}	16.96 ±1.16^{a}	17.63±1.14^{a}
10 weeks	31.42±0.92$^{a, b}$	35.43±0.93^{c}	32.15±0.93^{b}	28.72±1.19^{a}	32.13±1.14^{b}
Root mass (g)					
10 weeks	1.02±0.16$^{a, b}$	1.08±0.16$^{a, b}$	0.87±0.16^{b}	1.36±0.16^{a}	1.09±0.16$^{a, b}$

$^{a, b}$Different letters in the same row show a significant difference ($p<0.05$)

plant except in the case of seedlings emergence. In the other hand, Martinez-Mendoza and Mena-Violante (2012) recorded a drastic decrease in the germination rates of both pigweed and Johnson grass seeds in lots treated by *Bacillus subtilis* extracts, relative to controls. In addition, Karimi *et al.* (2012) reported that several *Bacillus subtilis* strains (B1, B6, B28, B40, B99 and B108) had no significant effect on plant length but the strain B28 reduced significantly the disease caused by *Fusarium oxysporum* sp. ciceri (83 vs. 100%). Furthermore, in the study of Inam-Ul-Haq *et al.* (2015), Bacillus strains RH31 (*Paenibacillus illinoisensis)* and RH-32 (Bacillus subtilis) reduced disease incidence and increased overall plant biomass when compared to control.

After 1 month of Mega Grain Trading CO. (P): kabuli sowing, under field conditions, Bacillus treatments showed a significant effect on plant length except the strain 23SRTS (Table 3). In fact, plants reached 21.59; 23.11; 20.80 cm in lots treated by CWBI; 9SRTS; 6SEL, respectively in comparison to control (17.63 cm). After 10 weeks, no significant effect on plant length was observed in the case of CWBI and 6SEL. However, 9SRTS increased significantly plant length (35.43 vs. 32.13) and 23SRTS decreased it significantly (28.72 vs. 32.13). In other side, root dry mass was not affected by any Bacillus treatment

Table 4: Last square means of Mega Grain Trading CO. (P): kabuli chickpea variety grains number per plant at the harvest

lots	LSM of chickpea grain number per plant
CWBI	4.75b±0.50
9SRTS	6.74a±0.56
6SEL	6.47a±0.60
23SRTS	6.96a±0.67
Control	7.04a±0.66

$^{a, b}$Different letters in the same column show a significant difference ($p<0.05$)

($p>0.05$) and its values were between 0.87 and 1.36 g (Table 3). It is to note that no significant effect was signaled in the number of chickpea grains per plant at harvest time, except in the case of the strain CWBI (Table 4). Interestingly, seed germination was higher in lots treated by CWBI, 9SRTS and 6SEL (69, 63, 57, respectively, vs. 41%) (Fig. 3). Consequently, the total number and mass of chickpea grains was higher in these lots, compared to a control (Table 5). Importantly, *B. amyloliquefaciens* (9SRTS) showed the best effect on chickpea crop yield (236 vs. 176 grains; 153 vs. 114 g). These data estimated per ha reached 7.65 vs. 5.7 q, so, a gain of 2 quintals per ha. Approximate results had been found by Abdel-Monaim (2011) which showed that the treatment of chickpea seeds (variety: cv. 'Giza 3) with *B. megaterium* reduced the severity of damping off (9% vs. 16%) and increased the yield of production 664 vs. 525 kg/field.

Table 5: Effect of Bacillus treatment on the number of chickpea grains and their mass at the harvest

Bacillus treatment	CWBI	9SRTS	6SEL	23SRTS	Control
Total number of grain harvested	209	236	194	167	176
Mass of chickpea harvested (g)	136	153	126	108	114

*Data correspond to the total chickpea seed harvested in a whole lots (control and treated lots)

CONCLUSION

Bacillus strains studied in the present research in addition to their high sporulation yields, they had shown interesting performance in promoting plant growth and reducing plant disease symptoms, under pots and field conditions. Thereby, this makes of them feasible bio-agent products that can be used further for improving the crop systems.

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