

Title

Effect of seed and/or soil treatment with biocontrol agents of *Bacillus subtilis* group strains on chickpea plants development under greenhouse and field conditions

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Abstract

In this study three *Bacillus* species, i.e., *B. amyloliquefaciens* (9SRTS), *B. atrophaeus* (6SEL) and *B. subtilis* subsp. *spizezenii* (23SRTS), were used to test their phytostimulation and biocontrol effects to manage chickpea plants development under greenhouse, using naturally infested soil with *Sclerotinia sclerotiorum* and under field conditions. The industrial production of *Bacillus* spores in 500l bioreactor (Artechno society, Liege-Belgium) was economic because high sporulation yields were obtained at the end of fermentation process, results varied between 8×10^9 and 1×10^{10} spores/ml. In greenhouse experiment, several lots were made, i.e., TSI-TSd (treated soil-treated seeds); TSI-USd (treated soil-untreated seeds), USI-TSd (untreated soil-treated seeds), control (untreated soil-untreated seeds). The best protection of CV. *Flipe 13 90* chickpea variety plants was observed in TSI-USd lot, therefore, the stem rot rating was significantly lower ($P < 0.05$), with 9% and 8%, in treated lots with 9SRTS and 23SRTS respectively vs. 63% in control. Plants of this variety reached maximal size in USI-TSd lot (16 cm and 15 cm vs. 10cm). Field experiment showed that the strains 9SRTS and 6SEL affected positively on the plant size, seeds germination and the crop yield of *Mega grain tradind CO. (P) Kabuli* chickpea variety. After 4 weeks of sowing, treated lots with 9SRTS and 6SEL possessed plants with 23.11 cm and 20.80 cm of size, respectively, vs. 17.63 in control. The number of plants estimated at 10 weeks of sowing was 41 and 37, vs. 27 in control. The crop yield was optimal in the treated lot with 9SRTS strain comparing to control (153 g vs. 114g, per the sown surface in this experiment) which is the equivalent of 7.65 qt vs. 5.7qt, per hectare.

1- Introduction

Chickpea (*Cicer arietinum* L.) is like other legumes of an important economic, Agriculture and Food place According to the FAO (2007). 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 (AAC, 2006). Algeria with an annual production of about 45 000 ton, is faced with the obligation to import more than 150,000 ton per year costing therefore 200 million \$ (Bahri, 2011).

Many authors reported that chickpea is attacked with many soil borne fungi, *i.e* *Fusarium* spp., *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, *Pythium* spp., *Macrophomina phaseolina* causing damping -off, root and 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 (Schmidt *et al.*, 2004). These bacterial genera are the major root colonizers (Manikanda *et al.*, 2010), and increase yield crops based on diverse mechanisms. Antibiotics, siderophores, cyanide hydrogen, competition for nutrition and space, inducing systemic resistance, inactivation of pathogen's enzymes and enhancement of root and plant development by phytohormone production are the most described mechanisms (Intana *et al.*, 2008).

The objective of this research was to identify the benefits of biological control, which previously was shown to be useful in the management of damping-off, root rot and/or stem rot of chickpeas when used individually and not as part of an integrated approach (Abd Elmonaim, 2011). The effect of *Bacillus subtilis* species isolated from diverse environments of Eastern Algeria was tested under greenhouse conditions, using a naturally infested soil with *S. sclerotonia* and under field conditions in the experimental ground of Chaab-Elrassas (Constantine-Algeria), in the period of July-October 2013.

2- Material and Methods

2.1- Screening of *Bacillus* spp. biocontrol agents

Three *Bacillus* species were used in this study, i.e., *B.amyloliquefaciens* (9 SRTS), *B. subtilis* subsp. *spizezenii* (23SRTS) and *B. atrophaeus* (6SEL). They were isolated from diverse environments of Eastern Algeria and screened based on several criteria, i.e., their antifungal activity against *Botrytis cinerea*, *Aspergillus niger*, *Alternaria alternata*, *Fusarium oxysporum* and *Cladosporium cucumerinum*; the high sporulation yield and the *in vitro* production of lipopeptides, siderophores, cell-wall degrading enzymes (cellulase, protease and chitinase) and the phytohormone IAA, as described by Ait Kaki *et al.* (2013^{a, b}).

2.2- Industrial production of screened bacteria

The screened bacteria were produced under industrial conditions in a bioreactor of 500 l (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=140rpm and dissolved oxygen (DO2=100%). The bacteria culture reached the maximum sporulation yield after 96h. The fermentation was stopped at this moment and spores' suspensions were centrifuged and the obtained pellets were dried into a powder product by lyophilization.

2.3- Greenhouse experiment

The three *Bacillus* strains *B.amyloliquefaciens* (9 SRTS), *B. subtilis* subsp. *spizezenii* (23SRTS) and *B. atrophaeus* (6SEL) were used to treat seeds and/or soil. In fact, several lots were made, i.e., TSI-TSd (treated soil-treated seeds); TSI-USd (treated soil-untreated seeds), USI-TSd (untreated soil-treated seeds), control (untreated soil-untreated seeds). Two chickpea cultivars were used i.e., CV. *Flipe 13 90* and *Mega grain tradind CO. (P): Kabuli*. For the last variety only TSI-USd lot was realized testing the isolates (9SRTS) and (23SRTS). Before treatment with biocontrol agents, seeds were washed three times in sterilized distilled water (SDW), and dried between sterilized filter paper layers. Seeds were treated at the time with a bacterial bioagents isolates (10 mL of bacterial biocontrol agent suspension in SDW at 10⁷ cells/ml). Then the seeds were sown in soil naturally infested with the *Sclerotonia sclerotium*. In each 30 cm diameter pot, six seeds were sown and the treatments were replicated three times. For soil treatment, holes were made and 10 ml of bacterial suspension (10⁷ cell/ml) was

added to soil by pulverization. Data were recorded for stem rot and plant size after 30 days of sowing. This experiment was carried out according to Karimi *et al.* (2012), with some modifications.

2.4- Integrated control of chickpea plants under field conditions

Field experiments were conducted at the experimental ground of Chaab-Elrassas 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 m² with 0.60 m in between. Natural compost was added in each plot and holes were made for growing seeds. 1ml of bacterial suspensions (10⁷ cells/ml) was pulverized in each hole before sowing untreated chickpea seeds cv. *Mega grain tradind CO. (P): Kabuli* (65holes/row). Untreated plots containing untreated seeds were used as control. In this experiment, *Bacillus strains* (9SRTS), (23SRTS) and (6SEL) were tested in addition to the type isolate (S499), provided by the Wallon Centre of applied biology (CWBI-Belgique). All the agricultural practices were applied as usual (Abdel-Monaim MF, 2011).

2.5- Statistical analysis

The SAS software (SAS Institute 2000) was used for all statistical analysis. Seed and soil treatment effects on the studied parameters in greenhouse 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.

3- Results and discussion

The screened bacteria for in vivo tests, i.e., *B.amyloliquefaciens* (9SRTS), *B. atrophaeus* (6SEL) and *B. subtilis sub spizezenii* (23SRTS) were produced in a bioreactor of 500 l (Artechno society SA- Belgium). The fermentation conditions were maintained at pH 7 by adding H₃PO₄ (0,5 N), 140 rpm of agitation, 30°C of incubation temperature. At the end of fermentation, produced bacterial cultures reached important sporulation yields. Furthermore, the same finding was observed in obtained lyophilized products. Thus, the sporulation yield varied between 8 x 10⁹ and 1 x 10¹⁰ spores/ml (**table 1**). The sporulation yield was higher comparing to findings of Monteiro *et al.* (2005) and Chen *et al.* (2010), respectively.

In greenhouse experiment, the treatment of naturally infested soil with *S. sclerotiorum* and/or chickpea seeds, with bacterial suspensions had, in general, a positive effect on plant size and the protection of *CV. Flpe 13 90* chickpea variety (**figure1**). The best protection of plants was observed in TSI-USd lot. Indeed, the percentage of discolored leaves per plants, in the treated lots with (9SRTS) and (23SRTS) was significantly ($P<0.01$) lower, compared to control (9% and 8%, respectively vs. 63%). In contrast, Moradi *et al.* (2012) investigated that no significant difference was observed between soil and seeds treatment of *Pirooz* and *Hashem* chickpea varieties with *B. subtilis* strains. The highest plant size was observed in USI-TSd lot, where plants reached for the same *Bacillus* isolates 16 cm and 15 m, respectively, vs. 10 cm control (table 2). When taking plant resistance and plant size parameters together, the isolate (9SRTS) in TSI-USd lot developed best performances (table 2). Only the treated soil with (9SRTS) strain had a positive significant effect ($P<0.05$) on the root mass and stem rot rating of *Mega grain tradind CO. (P): Kabuli* variety Chickpea plants, compared to control (**Figure 2**). In fact, root mass and stem rot rating versus control were (0.31g and 41% vs. 0.066g and 74%). However, no significant effect ($P>0.05$) was observed on plant size (**table 3**). Approximate results were found by Karimi *et al.* (2012) where several *Bacillus subtilis* strains (B1, B6, B28, B40, B99 and B108) had positive significant effect on plant size, root mass and *Fusarium oxysporum* f.sp. *ciceri* plant diseases severity reduction with best effect observed in the isolate B28. In addition, the inoculation of this strains in the infested soil with *Fusarium oxysporum* f.sp. *ciceri* had better effect on plant disease reduction percentage than when chickpea seeds were treated (44% vs. 39%).

Field experiment showed that plant size, the germination and crop yield of *Mega grain tradind CO. (P): Kabuli* chickpea variety seed were significantly affected by *Bacillus* treatments, except the case of the treated lot with the *B. subtilis sub spizezenii* (23SRTS). In fact, after 4 weeks of sowing, the size plants were 21.59, 23.11 and 20.80 cm, in treated soil with S499, 9SRTS and 6SEL, respectively, vs. 17.63 in control. At the 10th week, plants in all lots including control had approximately the same size, except the case of *B. amyloliquefaciens* (9SRTS) where plants had size significantly ($P<0.05$) higher (**table 4**).

After 10 weeks of sowing, the number of plants in (S499), (9SRTS) and (6SEL) plots reached respectively 45, 41 and 37, vs. 27 plants in control (**figure 3**). In general, the pre-germination damping off was the sole appearing disease in the field and chickpea plants didn't show symptoms of serious diseases during the culture period (from sowing to harvest). However a very limited number of plants in all plots showed discoloration symptom (**figure**

4). *Bacillus* treatments had no significant effect ($P>0.05$) on root masses and a number of fruits per plant, estimated at the harvest day. The root masses varied between 0,87g and 1,36g and the number of fruits per plant ranged between 6.47 and 7.04. However, as *Bacillus* treatments affected the germination capacity of chickpea seeds, the crop yield was higher in treated plots except for the isolate (23SRTS), compared to control. In addition the (9SRTS) ensured the best productivity 153 g vs. 114g (per the sown surface in this experiment). This is the equivalent of 7.65 qt vs. 5.7qt (per hectare) (table 5). Approximate results were found by Abdel-Monaim. (2011) where the treatment of cv. 'Giza 3' chickpea variety with *B. megaterium* strains decreased the pre-germination damping off and increased the yield of chickpea production. This author investigated that November-February was the best sowing period in which the *Bacillus* treatment developed interesting performances, i.e., (9% vs. 16%) for damping off and (664 vs. 525 kg/field), for production yield. To conclude the tested *Bacillus* species here showed interesting biocontrol performances which can be further optimized by their application within a framework of integrated agriculture, based on several techniques respecting environment such as get seed or planting material of quality; choose fertile soil; adopt adequate spacing and planting devices; choose growth period coinciding with low pests development; practice crop rotation; Weeding regularly Inspect fields; Promote greater natural enemies (auxiliary) populations; Adopt good practices harvest,...etc (COLEACP, 2011).

Tables

Table 1 Evaluation of DO, dry matter , total and spores flora at the end of the fermentation of *Bacillus* strains in a bioreactor of 500l and the estimation of living cells after lyophilisation.

Bacterial strains	DO/ml	Dry matter (g/l)	Total flora (Cellules/ml)	Spores flora (spores/ml)	Cells/g of powder
<i>B. amyloliquefaciens</i> (9SRTS)	22	4	$1,5 \times 10^{10}$	1×10^{10}	$1,2 \times 10^{11}$
<i>B. subtilis</i> subsp. <i>spizezenii</i> (23SRTS)	24	3,5	9×10^9	$8,5 \times 10^9$	7×10^{10}
<i>B. atrophaeus</i> (6SEL)	20	4	9×10^9	8×10^9	6×10^9

Table 2 Effect of *B.amyloliquefaciens* (9SRTS), *B. atrophaeus* (6SEL) and *B. subtilis* subsp. *spizezenii* (23SRTS), on the plant size and the stem rot rating of chickpea plants (variety : CV. Flipe 13 90), sown in a naturally infested soil with *Sclerotonia sclerotiorum*.

Bacillus isolates	Stem rot rating: percentage of discolored leaves by plant (%)			Plant size (cm)		
	TSI-TSd	USI-TSd	TSI-USd	TS-TSd	USI-TSd	ST-GNT
<i>B. amyloliquefaciens</i> (9SRTS)	40± 5 ^a	36± 5 ^a	9± 4 ^b	14± 1 ^{ab}	16± 1 ^a	13± 0.5 ^b
<i>B. subtilis</i> subsp. <i>spizezenii</i> (23SRTS)	61± 6 ^a	42± 10 ^a	8 ±4 ^b	14 ±1 ^a	15± 1 ^a	11±1 ^b
<i>B. atrophaeus</i> (6SEL)	14± 6 ^a	46± 5 ^b	18± 4 ^a	14 ±1 ^{ab}	14 ±1 ^a	12± 1 ^b
Control	63± 4 ^c	63 ±4 ^c	63 ±4 ^c	10± 0.5 ^c	10 ±0.5 ^c	10± 0.5 ^c

* Several lots were made, i.e., TSI-TSd (treated soil-treated seeds); TSI-USd (treated soil-untreated seeds), USI-TSd (untreated soil-treated seeds), control (untreated soil-untreated seeds).

* Different letters in the same column show the significant effect ($P < 0.05$) of *Bacillus* treatments, comparing to a control. However, different letters in the same line show the difference between the diverse *Bacillus* treatments used in this experiment.

Table 3 Treatment effect of naturally infested soil with *S. sclerotium*, with (9SRTS) and (23SRTS) bacterial suspensions on root mass, plant size and stem rot rating of *Mega grain tradind CO. (P)*: Kabuli chickpea variety.

<i>Bacillus</i> isolates	Root mass (g)	plant size (cm)	stem rot rating (%)
<i>B. amyloliquefaciens</i> (9SRTS)	0.31 ± 0.06 ^a	48 ± 3 ^b	41 ± 12 ^a
<i>B. subtilis</i> subsp. <i>spizezenii</i> (23SRTS)	0.14 ± 0.06 ^b	48 ± 3 ^b	71 ± 10 ^b
control	0.066 ± 0.07 ^b	45 ± 3 ^b	74 ± 12 ^b

*Different letters in the same column show the significant effect (P<0.05) of *Bacillus* treatments, comparing to a control.

Tableau 4: Least squares Mean of plant size ± standard error, calculated after 4 and 10 weeks of sowing, under field conditions (Algeria)

periode	plant size (cm)				
	S499	9SRTS	6SEL	23SRTS	Témoin
4 th week	21,59±0,93 ^b	23,11±0,93 ^b	20,80±1,03 ^b	16,96 ±1,16 ^a	17,63±1,14 ^a
10 th week	31,42±0,92 ^{ab}	35,43±0,93 ^c	32,15±0,93 ^b	28,72±1,19 ^a	32,13±1,14 ^b

* Different letters in the same row show a significant difference (p<0.05)

Tableau 5 Number and mass of harvested chickpea seeds

Treatments	S499	9SRTS	6SEL	23SRTS	Témoin
Number of harvested seeds	209	236	194	167	176
Mass of harvested chickpea	136	153	126	108	114

Legends to figures

Figure 1 Effect of *Bacillus isolates* (9SRTS et 23SRTS), on the size and stem disease rating, after one month of sowing of chickpea plants var *CV. Flipe 13 90*, in naturally infested soil with *S. sclerotiorum*, under greenhouse condition.

Figure 2 Effect of *Bacillus isolates* (9SRTS and 23SRTS) on plant size, stem rot rating and root mass of *Mega grain tradind CO. (P): Kabuli* chickpea plants, after one month of sowing seeds in naturally infested soil with *S. sclerotium*.

Figure 3 Treatment effect of naturally infested soil with *S. sclerotium*, with (S499, 9SRTS and 6SEL) *Bacillus* isolates on seed germination capacity and plant size of *Mega grain tradind CO. (P): Kabuli* chickpea variety, after one month of sowing.

Figure 4 Discoloration symptoms appearing in some plants in all field plots (treated with *Bacillus* suspension and untreated one)

Figures



Figure 1



Figure 2



Figure 3



Figure 4

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