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Selection of bacteria with antagonistic activity against Ascochyta blight of chickpea

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Abstract - Seventy-eight bacteria belonging to the genus *Bacillus*, isolated from salty soils in Tunisia, were assessed for their antagonistic activity against *Ascochyta rabiei*, the causal agent of Ascochyta blight of chickpea. Effect of treatments with the different bacteria on disease severity and plant growth parameters was evaluated under greenhouse conditions. Based on Mass Disease Index (MDI), tested bacteria were classified into four groups. Bacteria of the first group were able to significantly reduce disease severity as compared to the control inoculated by *A. rabiei*, and showed relatively better efficiency than fungicides included in the assay. Results revealed that the different treatments had a significant effect on disease development and plant growth parameters, particularly on plant length and plant weight. Based on disease and growth parameters, 38 bacteria of the genus *Bacillus* among 78 could be selected from this experiment, of which one strain of *B. sphaericus*, two strains of *B. cereus*, four strains of *B. thuringiensis* and thirty-one strains of *Bacillus* spp. The efficiency of the 11 selected bacteria belonging to the first group was confirmed by reassessing their antagonistic activity under greenhouse and field conditions. *In vitro* testing was also performed.

Keywords: *Bacillus*, Ascochyta blight, Mass Disease Index, antagonistic activity

1. Introduction

Chickpea (*Cicer arietinum* L.) is one of the most important legume crops in the world and is mainly grown for its edible seeds highly rich in proteins (Yadav et al 2011; Zaim et al 2013). Ascochyta blight of chickpea caused by *Ascochyta rabiei* (Pass.) Labrousse (teleomorph = *Didymella rabiei* (Kovachevski)) is considered to be one of the most devastating diseases for this crop, particularly in Tunisia. The fungus attacks all above ground parts of the host and can induce necrotic spots on leaves, stems and pods (Benzohra et al 2011; Kaiser et al 2000). Population biology studies revealed high genetic diversity among the populations of *Ascochya rabiei* from different locations in Tunisia (Rhaiem et al 2008) and the occurrence of both mating types as well (Rhaiem et al 2007). In addition, epidemiological studies revealed that weather conditions prevailing in different Tunisian locations are conductive to the development and production of ascospores of the sexual stage *Didymella rabiei* (Rhaiem and Cherif 2014); which is likely to enhance genotypic diversity of the pathogen and make screening for resistance a very difficult task. In this context, several research programs aiming to find resistant chickpea lines in many regions all over the world did not give stable levels of resistance to *A. rabiei*. Different control strategies including farming methods, resistant genotypes and chemical treatments had limited efficiency in decreasing the damage caused by the disease (Benzohra et al 2011; Hawtin and Singh 1984). Attempts for finding fungal species potentially useful for biological control against the pathogen revealed the ability of some antagonistic fungi like *Trichoderma harzianum* or *T. viridae* in reducing or inhibiting the growth of the pathogen *in vitro* and/or *in vivo* (Benzohra et al 2011; Dugan et al 2009; Dugan et al 2005; Khalil et al 1989; Küçük et al 2007; Rajakumar et al 2005). Biocontrol potential of bacteria belonging to the genera *Bacillus* and *Pseudomonas* was evaluated against several plant pathogens causing plant diseases on different hosts including Fusarium wilt and dry root rot of chickpea (Karimi et al 2012; Patil et al 2014; Zaim et al 2013). The objectives of these studies are: i. to assess the eventual efficiency of some bacteria belonging to the genus *Bacillus* as biocontrol agents against Ascochyta blight of chickpea; ii. to evaluate their ability in reducing disease severity under laboratory and field conditions; and iii to determine their effect on plant growth parameters.

2. Material and Methods

Seventy-eight bacteria belonging to the genus *Bacillus*, isolated from salty soils in Tunisia, were considered (Table 1).

Table 1. Designation and origin of bacteria tested for their antagonistic activity against *A. rabiei*

	Bacteria	Genus	Origin		Bacteria	Genus	Origin
1	B1 (A3)	*Bacillus* spp.	Gabes (oasis soil)	51	B58 (X23)	*Bacillus* spp.	Chott-El-Jerid
2	B2 (A7)	*Bacillus* spp.	Gabes (oasis soil)	52	B59 (H1)	*Bacillus* spp.	Tamarza
3	B3 (C17)	*B. sphaericus*	Gabes (oasis soil)	53	B60 (H2)	*Bacillus* spp.	Tamarza
4	B4 (E2)	*Bacillus* spp.	Deggache	54	B61 (H5)	*Bacillus* spp.	Tamarza
5	B5 (E4)	*Bacillus* spp.	Deggache	55	B62 (H6)	*Bacillus* spp.	Tamarza
6	B6 (E6)	*Bacillus* spp.	Deggache	56	B63 (H7)	*Bacillus* spp.	Tamarza
7	B7 (G1)	*Bacillus* spp.	Chott-Er-Rahim	57	B64 (H8)	*Bacillus* spp.	Tamarza
8	B8 (G2)	*Bacillus* spp.	Chott-Er-Rahim	58	B65 (H9)	*Bacillus* spp.	Tamarza
9	B9 (G4)	*Bacillus* spp.	Chott-Er-Rahim	59	B66 (H97)	*Bacillus* spp.	Tamarza
10	B10 (G5)	*Bacillus* spp.	Chott-Er-Rahim	60	B67 (HH7)	*Bacillus* spp.	
11	B11 (G6)	*Bacillus* spp.	Chott-Er-Rahim	61	B68 (HH13)	*Bacillus* spp.	
12	B12 (G7)	*Bacillus* cereus	Chott-Er-Rahim	62	B69 (HH15)	*Bacillus* spp.	
13	B13 (G10)	*Bacillus* spp.	Chott-Er-Rahim	63	B70 (HH16)	*Bacillus* spp.	
14	B14 (G12)	*Bacillus* spp.	Chott-Er-Rahim	64	B71 (HH24)	*Bacillus* spp.	
15	B15 (G31)	*Bacillus* spp.	Chott-Er-Rahim	65	B72 (HH32)	*Bacillus* spp.	
16	B16 (I2)	*Bacillus* spp.	Foum El Khanga	66	B73 (HH35)	*Bacillus* spp.	
17	B17 (I3)	*Bacillus* spp.	Foum El Khanga	67	B74 (HH36)	*Bacillus* spp.	
18	B18 (I4)	*Bacillus* spp.	Foum El Khanga	68	B75 (HH44)	*Bacillus* spp.	
19	B19 (I6)	*Bacillus* spp.	Foum El Khanga	69	B76 (HH45)	*Bacillus* spp.	
20	B20 (I8)	*Bacillus* spp.	Foum El Khanga	70	B77 (HH51)	*Bacillus* spp.	
21	B21 (I10)	*Bacillus* spp.	Foum El Khanga	71	B78 (HH54)	*Bacillus* spp.	
22	B22 (I12)	*Bacillus* spp.	Foum El Khanga	72	B79 (HH71)	*Bacillus* spp.	
23	B24 (I17)	*Bacillus* spp.	Foum El Khanga	73	B80 (HH77)	*Bacillus* spp.	
24	B25 (I18)	*Bacillus* spp.	Foum El Khanga	74	B81 (HH81)	*Bacillus* spp.	
25	B26 (I20)	*Bacillus* spp.	Foum El Khanga	75	B82 (HH91)	*Bacillus* spp.	
26	B28 (I25)	*Bacillus* spp.	Foum El Khanga	76	B83 (HH112)	*Bacillus* spp.	
27	B29 (I27)	*Bacillus* spp.	Foum El Khanga	77	B84 (HH115)	*Bacillus* spp.	
28	B30 (I31)	*Bacillus* spp.	Foum El Khanga	78	B85 (HH118)	*Bacillus* spp.	
29	B32 (I34)	*Bacillus* spp.	Foum El Khanga				
30	B33 (I35)	*Bacillus* spp.	Foum El Khanga				
31	B34 (K7)	*Bacillus* spp.	Oueslatia (forest soil)				
32	B36 (K9)	*Bacillus* spp.	Oueslatia				
33	B38 (1T)	*B. thuringiensis*	FST*				
34	B39 (10 T)	*B. thuringiensis*	FST				
35	B40 (14 T)	*B. thuringiensis*	FST				
36	B41 (33T)	*B. thuringiensis*	FST				
37	B42 (55T)	*B. thuringiensis*	FST				
38	B43 (X2)	*Bacillus* spp.	Chott-El-Jerid				
39	B44 (X4)	*Bacillus* spp.	Chott-El-Jerid				
40	B45 (X5)	*Bacillus* spp.	Chott-El-Jerid				
41	B46 (X7)	*B. lentimorbus*	Chott-El-Jerid				
42	B47 (X8)	*Bacillus* spp.	Chott-El-Jerid				
43	B48 (X9)	*B.* cereus	Chott-El-Jerid				
44	B49 (X10)	*Bacillus* spp.	Chott-El-Jerid				
45	B50 (X12)	*Bacillus* spp.	Chott-El-Jerid				
46	B52 (X16)	*B. cereus*	Chott-El-Jerid				
47	B54 (X18)	*Bacillus* spp.	Chott-El-Jerid				
48	B55 (X19)	*Bacillus* spp.	Chott-El-Jerid				
49	B56 (X21)	*Bacillus* spp.	Chott-El-Jerid				
50	B57 (X22)	*Bacillus* spp.	Chott-El-Jerid				

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Seeds of the chickpea cultivar Amdoun1, susceptible to Ascochyta blight, were surface disinfected in 2% NaOCl for 3 min, washed three times in sterile distilled water and dried for 6 hours under a stream of filtered air. Volumes of 1.5 ml of each bacterial cell suspension were mixed with 100.g of chickpea seeds in Erlenmeyer flasks. Mixtures were rotatory shaken until total absorption of the suspensions by the seeds, which were then dried under a stream of filtered air. Seeds of inoculated and non-inoculated controls were surface disinfected only. *Bacillus* inocula used to treat seeds were prepared from liquid cultures at 10^7-10^8 CFU/ml obtained after incubation at 37°C for 24-48 hours. Seeds were sown in plastic pots 17 cm in diameter, filled with sterilized peat previously treated with 3% Formol. Treatments with fungicides were performed similarly to the method used for the bacteria by mixing

1.5 ml of Chlorothalonyl (2 g/l), Quadris (Azoxystrobin) (1 g/l) or Stroby (kresoxim-methyl) (0.2 g/l) solutions with 10g of chickpea seeds and by agitating until total absorption. Three replicated pots, with 8 plants/pot and per treatment were adopted. Treatments with bacteria and fungicides were repeated twice at one-week intervals on small seedlings, presenting two-three expanded leaves, by spraying bacteria suspensions and fungicides with the same respective concentrations used previously. Plants were inoculated by the pathogen at the seedling stage (five-seven expanded leaves), and again after 14 days, by spraying a 5×10^5 conidia/ml suspension from a single-spored isolate of *A. rabiei*, previously shown to be pathogenic, using an airbrush sprayer. Plants were incubated in the greenhouse at saturated humidity and a temperature of 20±2°C. Disease severity was recorded 8 and 21 days after inoculation based on a 1-9 scale, and mass disease indexes (MDI) were calculated according to the following formula:

$MDI= \sum^{9}_{i=1} (n_i \times i) / (Nx9) \times 100$

With i: the level of infection according to the 1-9 scale

n_i: the number of plants having i as level of infection

N: the total number of plants/pot (replication)

Effects of the different bacteria and fungicides treatments on plant growth parameters were also evaluated.

Re-assessment of antagonistic activity for selected bacteria

Selected bacteria were again assessed under greenhouse controlled conditions and in the field on chickpea cultivars Amdoun1 and Chetoui (data not shown). *In vitro* testing was also performed for representative bacteria to determine their ability to inhibit pathogen growth (data not shown).

3. Results

Statistical analysis revealed a significant effect of the different treatments on disease severity (Table 2) as well as on plant growth parameters, particularly on plant length (Tables 3, 4) and plant weight (Tables 5, 6, 7).

Table 2. ANOVA of the effect of the different treatments on the mass disease index (MDI) determined 21 days after inoculation

	Df	MS	F	p
MDI	82	665.419***	4.339	0.000
Residue	166	153.363		

Table 3. ANOVA of the effect of the different treatments on plant length determined 21 days after sowing

	Dl	MS	F	p
Length 2	81	6.122***	3.205	0.000
Residue	164	1.909		

Table 4. ANOVA of the effect of the different treatments on plant length determined at harvesting

	Dl	MS	F	p
Final length	82	65.892***	2.576	0.000
Residue	166	25.576		

Table 5. ANOVA of the effect of the different treatments on total plant weight

	dl	MS	F	p
Total weight/plant	82	0.159***	3.385	0.000
Residue	166	0.047		

Table 6. ANOVA of the effect of the different treatments on aboveground part weight per plant

	dl	MS	F	p
Above-ground part weight/plant	82	0.142***	3.171	0.000
Residue	166	0.045		

Table 7. ANOVA of the effect of the different treatments on under-ground part weight per plant

	dl	MS	F	p
underground part weight/plant	82.000	0.003***	1.703	0.002
Residue	166.000	0.001		

A significant effect was also noticed on seeds germination (Tables 8, 9).

Table 8. ANOVA of the effect of the different treatments on the percentage of seeds germination determined 10 days after sowing

	Dl	MS	F	p
Seeds Germination	81	356.616***	3.694	0.000
Residue	164	96.545		

Table 9. ANOVA of the effect of the different treatments on the percentage of seeds germination determined 14 days after

sowing

	Dl	MS	F	p
Seeds Germination	81	539.456***	1.905	0.000
Residue	164	283.131		

Effect of the different treatments on disease severity

According to the mass disease index (MDI) recorded 21 days after the second inoculation by *A. rabiei*, bacterial strains evaluated for their antagonistic activity were classified into four groups (Table 10).

Table 10. Classification of the different treatments according to the values of the mass disease index recorded 21 days after inoculation.

	Treatments	MDI			Treatments	MDI	
Group 1	Control	0.000	a	*Group 2*	B68	37.037	bcdefghijklmno
	B58	**19.775**	**ab**		B22	37.449	bcdefghijklmno
	B28	**20.238**	**bc**		B5	38.955	bcdefghijklmno
	B71	**20.503**	**bc**		B17	39.153	bcdefghijklmno
	B3	**21.032**	**bcd**		B84	39.286	bcdefghijklmno
	B72	**21.204**	**bcd**		B15	39.552	bcdefghijklmno
	B2	**21.296**	**bcd**		B29	39.722	bcdefghijklmno
	B54	**23.986**	**bcde**		B21	40.204	cdefghijklmno
	B7	**24.074**	**bcde**		B32	40.212	cdefghijklmno
	B1	**24.537**	**bcdef**		B18	40.741	defghijklmno
	B4	**24.537**	**bcdef**		B65	40.792	defghijklmno
	B9	**25.176**	**bcdefg**		B16	41.564	efghijklmno
Group 2	B83	25.573	bcdefgh		B20	41.693	efghijklmno
	B10	26.477	bcdefghi		B43	42.152	efghijklmnop
	B12	29.171	bcdefghij		B70	42.752	efghijklmnop
	Stroby	30.489	bcdefghijk		B42	42.948	efghijklmnop
	B11	30.617	bcdefghijk		B13	43.261	efghijklmnop
	B82	30.923	bcdefghijk		B69	43.937	efghijklmnop
	B33	31.173	bcdefghijkl		B81	44.444	fghijklmnop
	B38	32.562	bcdefghijklm		B14	44.782	fghijklmnop
	B19	32.738	bcdefghijklm		B41	45.370	ghijklmnopq
	B85	32.937	bcdefghijklm		Inoculated control	45.569	hijklmnopq
	B25	33.399	bcdefghijklm	*Group 3*	B45	46.142	hijklmnopq
	Chlorothalonyl	33.466	bcdefghijklm		B64	46.759	ijklmnopq
	B26	33.466	bcdefghijklm		B62	47.901	ijklmnopq
	B40	33.598	bcdefghijklm		B48	48.479	ijklmnopq
	Quadris	33.730	bcdefghijklmn		B66	48.479	ijklmnopq
	B52	33.796	bcdefghijklmn		B63	48.611	ijklmnopq
	B57	33.796	bcdefghijklmn		B73	50.419	jklmnopqr
	B55	33.862	bcdefghijklmn		B61	50.970	klmnopqr
	B34	34.392	bcdefghijklmn		B46	52.491	lmnopqrs
	B36	34.392	bcdefghijklmn		B39	53.638	mnopqrs
	B59	34.458	bcdefghijklmn		B74	55.556	nopqrs
	B60	34.568	bcdefghijklmn		B44	56.019	opqrs
	B6	34.722	bcdefghijklmn		B47	61.706	pqrst
	B50	34.722	bcdefghijklmn		B78	61.799	pqrst
	B56	34.810	bcdefghijklmn		B75	65.123	qrst
	B49	35.251	bcdefghijklmn	*Group 4*	B80	68.695	rst
	B67	36.111	bcdefghijklmno		B76	71.296	st
	B8	36.265	bcdefghijklmno		B77	77.425	t
	B24	36.348	bcdefghijklmno		B79	79.171	t
	B30	36.574	bcdefghijklmno				

NB: LSD $_{0.05}$: values followed by the same letter are not significantly different at 5%.

Bacteria belonging to group 1, which includes B58, B28, B71, B72, B2, B54, B7, B1, B4, B9 of *Bacillus* spp. and B3 of *B. sphaericus*, were the most efficient in reducing disease severity. Mass

disease indexes recorded on plants inoculated with these bacteria ranged approximately between 19 and 25% and were significantly lower than that of the control inoculated by *A. rabiei*, which was about 45.5%. Plants inoculated with bacteria belonging to group 2 presented mass disease indexes that were not significantly different from that of the inoculated control but that were relatively lower than it, ranging between 25.5 and 45.37%. Bacteria of this group presented an efficiency to reduce disease severity similar to that of the three tested fungicides Stroby, Quadris and Chlorothalonyl. Plants inoculated with bacteria of the third group presented levels of infection higher than those of the second group (MDI ranged between 46 and 65%) but presented mass disease indexes that were not significantly different from that of the inoculated control. Strains of *Bacillus* spp. B80, B76, B77 and B79, which constitute the fourth group, were significantly unable to reduce disease severity as compared to the control; mass disease indexes were, for the latter, comprised between 68 and 79% (Figure 1).

Figure 1. Effect of the different treatments with bacteria belonging to Groups 1, 2, 3 and 4 on chickpea plants inoculated with *A. rabiei* and kept under greenhouse conditions: **A.** Non-inoculated control ; **B.** Bacteria belonging to Group 1 (MDI=19-25 %) ; **C.** Bacteria belonging to Group 2 (25.5-45.37 %) ; **D.** Bacteria belonging to Group 3 (MDI=46-65 %) ; Bacteria belonging to Group 4 (MDI=68-79 %).

Effect of treatments on growth parameters

Plants inoculated with strains B21 (I10), B56 (X21), B54 (X18), B18 (I4), B64 (H8) of *Bacillus* spp. and B42 (55T) of *B. thuringiensis* presented the highest values of length as well as the non-inoculated control and the Chlorothalonyl, which were significantly higher than that of the inoculated control (Table 12). The highest values of weight were obtained for plants inoculated with strains B19, B28, B9, B58, B43, B73, B50, B85, B83, B84, B54, B44, B49, B56 of *Bacillus* spp., B40 (14T) of *B. thuringiensis* and B48 (X9) of *B. cereus*. Nevertheless, it is important to notice that the significant effect of the different treatments on plant weight seems to be essentially represented by the aboveground parts weight. In fact, although the inoculation with some bacteria allowed us to obtain plants with significantly higher roots weight than with others, there is no significant difference regarding the underground-parts weight between the inoculated and non-inoculated controls. It is also important to notice that plants inoculated with strain B42 (55T) of *B. thuringiensis* were also significantly longer than the non-inoculated control since the beginning of the experiment. Plants inoculated with strains B39 (10T), B40 (14T), B41 (33T), B48 (X9), B52 (X16) of *B. cereus*, and B81, B16, B82, B19, B22, B55, B56, B85, B8, B49, B47, B17 of *Bacillus* spp. were relatively longer than the control before inoculation by *A. rabiei*. Based on disease (Table 10) and growth parameters (Tables 11-14), 38 bacteria of the genus *Bacillus* among which one strain of *B. sphaericus*, two strains of *B. cereus*, four strains of *B. thuringiensis* and thirty-one strains of *Bacillus* sp. could be selected from this experiment (Table 15). Strains B40 (14T), 41 (33T), B42 (55T) of *B. thuringiensis*, B22, B47, B57, B55 of *Bacillus* spp., and B52 (X16) of *B. cereus* presented the most favorable effect on plant growth parameters and an acceptable level of reduction of disease severity and deserve to be particularly considered. The three tested fungicides were able to reduce disease severity as compared to the inoculated control, although these treatments were less efficient than bacteria classified in the first group. Plants treated with Quadris and Chlorothalonyl presented better growth features than those treated with Stroby.

Table 11. Classification of the different treatments according to the percentage of seeds germination recorded 14 days after sowing

Treatments	% Germination		Treatments	% Germination	
B66	41.666	a	B28	83.333	defg
B43	45.833	ab	B26	83.333	defg
B83	54.1666	abc	B81	83.333	defg
B9	54.1666	abc	B10	83.333	defg
B11	54.1666	abc	B7	83.333	defg
B12	56.250	abcd	B21	83.333	defg
B29	58.333	abcd	B64	83.333	defg
B44	58.3333	abcd	B56	83.333	defg
B62	58.333	abcd	B71	83.333	defg
B68	62.500	abcde	B20	83.333	defg
B61	62.500	abcde	B18	83.333	defg
B65	62.500	abcde	B16	83.333	defg
B13	62.500	abcde	B75	83.333	defg
B58	62.500	abcde	Stroby	87.500	efg
Chlorothalonyl	70.833	bcdef	B32	87.500	efg
B6	70.833	bcdef	B5	87.500	efg
B72	70.833	bcdef	B25	87.500	efg
B59	70.833	bcdef	B38	87.500	efg
B2	70.833	bcdef	B39	87.500	efg
B54	70.833	bcdef	B76	87.500	efg
Control	72.0179	bcdef	B74	87.500	efg
B3	75.000	cdefg	B85	91.666	fg
B19	75.000	cdefg	B45	91.666	fg
B1	75.000	cdefg	B8	91.666	fg
B67	75.000	cdefg	B82	91.666	fg
B60	75.000	cdefg	B34	91.666	fg
B70	75.000	cdefg	B17	91.666	fg
B46	75.000	cdefg	B14	91.666	fg
Quadris	75.000	cdefg	B77	91.666	fg
B79	75.000	cdefg	B78	91.666	fg
B84	75.000	cdefg	B24	91.666	fg
B36	75.000	cdefg	B48	91.666	fg
B73	75.000	cdefg	B49	95.833	fg
B15	77.083	cdefg	**B40**	**100**	**g**
B80	79.166	cdefg	**B41**	**100**	**g**
B4	79.166	cdefg	**B42**	**100**	**g**
B69	79.166	cdefg	**B22**	**100**	**g**
B50	79.166	cdefg	**B47**	**100**	**g**
B63	79.166	cdefg	**B57**	**100**	**g**
B33	83.333	defg	**B55**	**100**	**g**
B30	83.333	defg	**B52**	**100**	**g**

NB: $LSD_{0.05}$: values followed by the same letter are not significantly different at 5%.

Table 12. Classification of the different treatments according to the values of plant length recorded at harvesting

Treatments	Plant length		Treatments	Plant length	
B79	28.899	a	B41	40.166	defghijklmnopqrstu
B65	31.333	ab	B84	40.333	defghijklmnopqrstu
B69	31.500	ab	B14	40.4333	defghijklmnopqrstu
B36	31.666	abc	B39	40.666	efghijklmnopqrstu
B7	31.750	abc	B75	40.666	efghijklmnopqrstu
B67	32.500	abcd	B72	40.833	fghijklmnopqrstu
B71	32.666	abcde	B74	40.833	fghijklmnopqrstu
B9	32.866	abcdef	B78	41.500	ghijklmnopqrstu
B4	33.166	abcdef	B76	41.533	ghijklmnopqrstu
B70	34.000	abcdefg	B16	42.033	ghijklmnopqrstu
B77	34.166	abcdefgh	Stroby	42.186	hijklmnopqrstu
B3	34.333	abcdefghi	B49	42.233	hijklmnopqrstu
B6	34.500	abcdefghij	B47	42.266	hijklmnopqrstu
B11	35.133	abcefghijk	B5	42.333	ijklmnopqrstu
B58	35.266	abcefghijk	B40	42.333	ijklmnopqrstu
B20	35.299	abcefghijk	B62	42.333	ijklmnopqrstu
B15	35.333	abcefghijk	B30	42.533	jklmnopqrstu
B12	35.733	abcefghijkl	B83	42.833	klmnopqrstu
B60	35.933	abcdefghijklm	B48	43.000	klmnopqrstu
B1	36.000	abcdefghijklmn	B32	43.000	klmnopqrstu
B66	36.400	abcdefghijklmno	B82	43.000	klmnopqrstu
B59	36.466	abcdefghijklmno	B57	43.666	lmnopqrstu
B13	36.633	abcdefghijklmno	B81	43.700	lmnopqrstu
B25	37.000	abcdefghijklmnop	B68	43.849	lmnopqrstu
B80	37.000	abcdefghijklmnop	B63	44.000	mnopqrstu
B46	37.000	abcdefghijklmnop	B44	44.099	nopqrstu
B8	37.000	abcdefghijklmnop	B85	44.233	opqrstuv
B33	37.333	bcdefghijklmnopq	B55	44.266	opqrstuv
Inoculated control	37.500	bcdefghijklmnopq	B26	44.333	opqrstuv
B52	37.523	bcdefghijklmnopq	B29	44.333	opqrstuv
B28	37.633	bcdefghijklmnopq	B73	44.900	pqrstuv
B10	37.666	bcdefghijklmnopq	B50	45.213	qrstuv
B17	38.166	bcdefghijklmnopqr	**B21**	**46.000**	**rstuv**
B22	38.366	bcdefghijklmnopqrs	**B56**	**46.333**	**stuv**
B19	38.466	bcdefghijklmnopqrs	**B54**	**46.333**	**stuv**
B24	38.566	bcdefghijklmnopqrs	**Quadris**	**46.466**	**stuv**
B45	38.666	bcdefghijklmnopqrs	**B18**	**47.000**	**tuv**
B38	39.000	cdefghijklmnopqrst	**B42**	**47.000**	**tuv**
B61	39.666	cdefghijklmnopqrstu	Control	47.133	tuv
B43	39.966	defghijklmnopqrstu	**B64**	**47.666**	**uv**
B34	40.000	defghijklmnopqrstu	**Chlorothalonyl**	**52.333**	**v**
B2	40.000	defghijklmnopqrstu			

NB: $LSD_{0.05}$: values followed by the same letter are not significantly different at 5%.

Table 13. Classification of the different treatments according to the values of total plant weight recorded at harvesting

Treatments	Total plant weight		Treatments	Total plant weight	
B63	0.441	a	B24	0.863	cdefghijklmnop
B80	0.452	a	B68	0.865	cdefghijklmnop
B77	0.488	ab	B16	0.871	cdefghijklmnop
B15	0.492	ab	B26	0.871	cdefghijklmnop
B12	0.560	abc	B10	0.884	cdefghijklmnop
B75	0.588	abcd	B41	0.884	cdefghijklmnop
B5	0.619	abcde	B8	0.892	cdefghijklmnop
B61	0.620	abcde	B71	0.894	cdefghijklmnop
B64	0.640	abcdef	B4	0.900	cdefghijklmnop
B76	0.650	abcdefg	B78	0.901	cdefghijklmnop
B65	0.656	abcdefg	B21	0.907	cdefghijklmnop
B79	0.681	abcdefgh	B17	0.911	defghijklmnop
Inoculated control	0.687	abcdefghi	B70	0.925	defghijklmnopq
B46	0.690	abcdeghi	B67	0.928	defghijklmnopq
B34	0.696	abcdefghij	B7	0.930	defghijklmnopq
B66	0.700	abcdefghij	B38	0.942	efghijklmnopq
B69	0.701	abcdefghij	B20	0.948	efghijklmnopqr
Stroby	0.704	abcdefghij	B42	0.956	efghijklmnopqrs
B11	0.706	abcdefghij	B3	0.956	efghijklmnopqrs
B32	0.716	abcdefghij	B82	0.975	fghijklmnopqrs
B13	0.720	abcdefghij	B72	1.000	ghijklmnopqrst
B57	0.734	abcdefghijk	B39	1.016	hijklmnopqrst
B6	0.734	abcdefghijk	B19	1.032	ijklmnopqrstu
B36	0.741	abcdefghijkl	**B40**	**1.042**	**jklmnopqrstu**
B47	0.746	abcdefghijkl	**B58**	**1.071**	**klmnopqrstu**
B60	0.754	abcdefghijklm	**B9**	**1.087**	**lmnopqrstuv**
B62	0.756	abcdefghijklm	**B83**	**1.101**	**mnopqrstuv**
B52	0.762	abcdefghijklmn	**B85**	**1.102**	**mnopqrstuv**
B25	0.765	abcdefghijklm	**B73**	**1.111**	**nopqrstuvw**
B18	0.766	abcdefghijklmn	**B28**	**1.112**	**nopqrstuvw**
B2	0.776	abcdefghijklmno	**B43**	**1.126**	**opqrstuvwx**
B74	0.780	abcdefghijklmno	**B50**	**1.171**	**pqrstuvwx**
B55	0.812	bcdefghijklmno	**B84**	**1.272**	**qrstuvwx**
B30	0.815	bcdefghijklmno	**Quadris**	**1.296**	**rstuvwx**
B22	0.822	bcdefghijklmnop	**B54**	**1.299**	**stuvwx**
B33	0.826	bcdefghijklmnop	**B48**	**1.305**	**stuvwx**
B45	0.828	bcdefghijklmnop	Control	1.339	tuvwx
B1	0.829	bcdefghijklmnop	**B44**	**1.374**	**uvwx**
B59	0.832	bcdefghijklmnop	**B49**	**1.435**	**vwx**
B81	0.851	cdefghijklmnop	**Chlorothalonyl**	**1.458**	**wx**
B14	0.853	cdefghijklmnop	**B56**	**1.466**	**x**
B29	0.857	cdefghijklmnop			

NB: $LSD_{0.05}$: values followed by the same letter are not significantly different at 5%.

Table 14. Classification of the different treatments according to the values of the aboveground part weight/plant recorded at harvesting

Treatments	Above-ground part weight		Treatments	Above-ground part weight	
B63	0.428	a	B16	0.816	efghijklmn
B80	0.439	ab	B4	0.827	efghijklmn
B15	0.457	abc	B29	0.834	efghijklmn
B77	0.474	abcd	B81	0.835	efghijklmn
B12	0.532	abcde	B26	0.837	efghijklmn
B75	0.572	abcdef	B10	0.838	efghijklmn
B5	0.592	abcdefg	B68	0.841	efghijklmn
B61	0.599	abcdefg	B71	0.841	efghijklmn
Inoculated control	0.621	abcdefg	B17	0.853	efghijklmn
B46	0.625	abcdefgh	B41	0.859	efghijklmn
B64	0.634	abcdefgh	B7	0.870	efghijklmno
B76	0.637	abcdefgh	B78	0.871	efghijklmno
B65	0.650	abcdefgh	B8	0.871	efghijklmno
B66	0.653	abcdefgi	B21	0.876	fghijklmno
B79	0.663	abcdefghi	B3	0.879	fghijklmnop
B11	0.670	abcdefghij	B38	0.880	fghijklmnop
B6	0.676	abcdefghij	B39	0.887	fghijklmnopq
Stroby	0.685	abcdefghij	B70	0.898	fghijklmnopq
B34	0.687	abcdefghij	B42	0.904	fghijklmnopq
B32	0.688	abcdefghij	B20	0.925	ghijklmnopq
B69	0.688	abcdefghij	B82	0.929	ghijklmnopq
B13	0.707	abcdefghijk	B72	0.929	ghijklmnopq
B25	0.710	abcdefghijk	**B19**	**0.966**	**hijklmnopqr**
B36	0.713	abcdefghijkl	**B28**	**0.991**	**ijklmnopqr**
B2	0.714	abcdefghijkl	**B40**	**0.992**	**jklmnopqr**
B60	0.715	abcdefghijkl	**B9**	**1.008**	**jklmnopqr**
B57	0.720	abcdefghijklm	**B58**	**1.032**	**klmnopqr**
B18	0.722	abcdefghijklm	**B43**	**1.054**	**lmnopqrs**
B1	0.726	abcdefghijklm	**B73**	**1.061**	**mnopqrst**
B52	0.733	abcdefghijklm	**B50**	**1.082**	**nopqrst**
B62	0.735	abcdefghijklm	**B85**	**1.082**	**nopqrst**
B47	0.735	abcdefghijklm	**B83**	**1.088**	**nopqrstu**
B45	0.747	abcdefghijklmn	**B48**	**1.201**	**opqrstu**
B74	0.775	bcdefghijklmn	**B84**	**1.219**	**pqrstu**
B22	0.779	bcdefghijkmn	**B54**	**1.225**	**qrstu**
B55	0.780	bcdefghijklmn	**Quadris**	**1.228**	**qrstu**
B67	0.784	cdefghijklmn	Control	1.275	rstu
B30	0.795	cdefghijklmn	**B44**	**1.305**	**rstu**
B59	0.795	cdefghijklmn	**B49**	**1.382**	**stu**
B33	0.797	cdefghijklmn	**B56**	**1.401**	**tu**
B14	0.799	defghijklmn	**Chlorothalonyl**	**1.429**	**u**
B24	0.808	defghijklmn			

NB: LSD $_{0.05}$: values followed by the same letter are not significantly different at 5%.

Table 15. Characteristics of the 38 bacteria selected

		Reduction of disease severity (group)	percentage of plant emergence	Plant length before inoculation with *A. rabiei*	*plant length at harvesting*	Total plant weight	Above-ground part weight
B58	**1**					●	●
B28	**1**					●	●
B71	**1**						
B3	**1**						
B72	**1**						
B2	**1**						
B54	**1**				●	●	●
B7	**1**						
B1	**1**						
B4	**1**						
B9	**1**					●	●
B40	**2**		●	●		●	●
B41	**2**		●	●			
B42	**2**		●	●	●		
B22	**2**		●	●			
B57	**2**		●				
B55	**2**		●	●			
B52	**2**		●	●			
B19	**2**			●			●
B21	**2**				●		
B18	**2**				●		
B56	**2**			●	●	●	●
B49	**2**			●		●	●
B83	**2**					●	●
B85	**2**			●		●	●
B43	**2**					●	●
B50	**2**					●	●
B84	**2**					●	●
B48	**2**			●		●	●
B44	**2**					●	●
B47	**3**		●	●			
B73	**3**					●	●
B64	**3**				●		
B81	**2**			●			
B16	**2**			●			
B39	**3**			●			
B82	**2**			●			
B8	**2**			●			

● Characteristic for which the bacterial strain was pre-selected

Re-assessment of antagonistic activity for selected bacteria

Eleven selected bacteria belonging to Group 1 were re-assessed for their efficiency in reducing disease severity under greenhouse and field conditions, on Amdoun1 and Chetoui chickpea cultivars. The efficiency of four bacteria among 11 in reducing disease severity was confirmed (data not shown). Three bacteria, B3, B7 and B71, were able to maintain significantly low levels of infection under greenhouse conditions as well as in the field. B58, found to be relatively efficient under greenhouse conditions, was shown to be significantly efficient in reducing disease severity in the field (Figure 2).

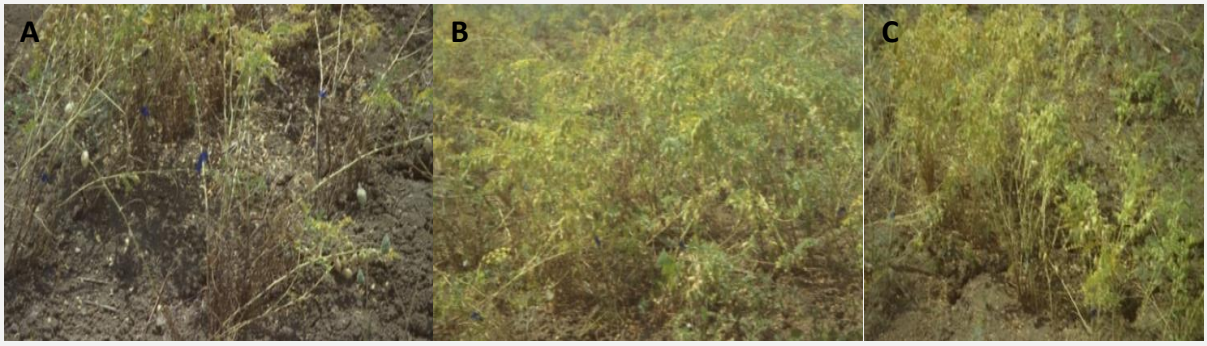

Figure 2. Effect of treatments on chickpea plants inoculated with *A. Rabiei* in the field: **A.** Inoculated control; **B.** plants treated with B71; **C.** plants treated with B58.

In vitro testing performed for representative bacteria revealed that some bacteria may be able to efficiently inhibit *A. rabiei* growth *in vitro* (GI: 22%) (Figure 3) and reduce disease severity whereas others were only efficient *in vitro*.

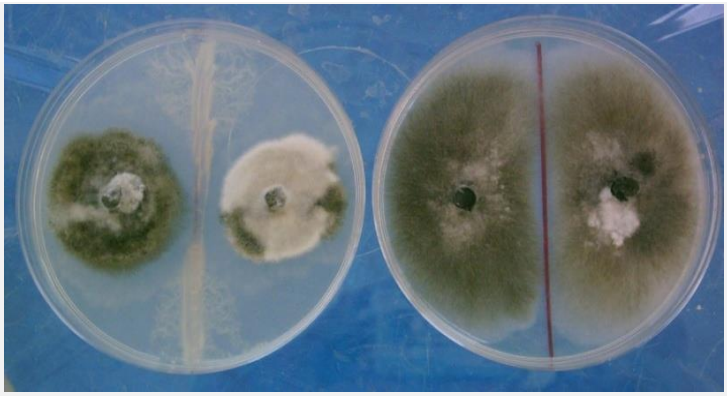

Figure 3. Growth Inhibition zone observed with B7 (left) VS control (right)

4. Conclusion

Eleven bacteria belonging to the genus *Bacillus* (group 1) were selected among 78 tested strains as they were found to significantly reduce disease severity (MDI:19-25 %) under greenhouse conditions for the susceptible chickpea cultivar Amdoun 1, as compared to the control inoculated with *A. rabiei* (MDI=45.5 %). Four among these pre-selected bacteria were also able to significantly reduce disease severity under greenhouse conditions for the chickpea cultivar Chetoui and maintain relatively low levels of infection in the field. Treatments with some bacteria of group 2 also resulted in relatively lower MDI values as compared to the inoculated control and significantly improved plant weight as well as fungicides Quadris and Chlorothalonyl. Although plants treated with Quadris and Chlorothalonyl presented better growth features than those treated with Stroby, the latter was the most efficient among the three fungicides in reducing disease severity and behaved like relatively efficient bacteria of group 2. Stroby, which is formulated from kresoxim-methyl derived from the natural antifungal compound Strobulin produced by *Strobilurus tenacellus* (Anke et al 1977), proved to have an efficient antagonistic effect against many economically important plant pathogens on different crops, especially on apple (Ypema and Gold 1999). As for Azoxystrobin (Quadris in our study), along with many other fungicides is frequently used against *Ascochyta rabiei* as chickpea growers rely mainly on fungicides with site-specific modes of action to manage ascochyta blight disease. However, fungal plant pathogens that are able to generate variation through sexual recombination and that have a polycyclic disease have an increased risk of developing resistance to fungicides (Wise et al 2008).

Taking into account both disease assessment results and growth parameters under greenhouse conditions, 38 bacteria deserve to be considered. In fact, most of these bacteria were able to reduce disease severity, to allow the development of plants with higher plant weight as compared to control(s), and/or to have an accelerating or increasing effect on plant emergence. Plants treated with some of these strains of *Bacillus sp.*, and particularly one strain of *Bacillus thuringiensis* B42 (55T) showed higher plant length. These bacteria may prove to have a plant-growth-promoting effect and should be further considered. Actually, the use of plant-root colonizing bacteria with plant growth promoting activity has proven during the last decades to be an efficient and environmental-friendly alternative to chemicals and pesticides (Qiao et al 2014). Attempts are being made in order to develop more powerful bio-fertilizer and biocontrol agents from endospore-forming *Bacillus* strains, especially that many formulations prepared from *Bacillus* sp. are increasingly applied due to their efficiency and long shelf life (Qioa et al 2014).

Results of these studies revealed that *Bacillus* species, particularly *B. thuringiensis*, *B. cereus* and *B. sphaericus*, are likely to represent bacterial candidates for biological control of *A. rabiei* and may be involved in further integrated disease management strategies against Ascochyta blight of chickpea. It is also important to notice that *in vitro* testing performed for representative bacteria revealed that *Bacillus* strains that were able to efficiently inhibit *A. rabiei* growth *in vitro* may be more or less efficient in reducing disease severity in the greenhouse and/or in the field and *vice versa*. This is probably due to prevailing conditions and specific control mechanisms involved for each bacterial strain, which should be unraveled and taken into account in selecting any eventual control agents against the disease.

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