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New Findings on Biological Control Trials of Potato Brown Rot with Antagonistic Strains of *Bacillus circulans*, in Egypt

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Abstract: Two biologically active strains of *Bacillus circulans* (SF1 and DE62), described in Egypt as silicate dissolving bacteria, were experimented for the control of potato brown rot caused by *Ralstonia solanacearum*. In *in vitro* studies, the isolate SF1 as well as DE 62 were antagonistic to certain isolates of the pathogen. Both spore forming isolates produce nitrogenase, hydrolytic enzymes and mobilize potassium. Our results show in general, a tendency of *R. solanacearum* population to increase in the rhizosphere of potato raised in *Bacillus circulans* treated soil. Similar trend was observed for the Area Under Disease Progress Curve (AUDPC) and wilt severity. At cultivars level, cv., Cara (late mature) showed highly significant increase in AUDPC and wilt severity over Inova (early mature) and Picasso (medium mature) either in presence or absence of *Bacillus circulans*. The greater increase of the pathogen, in *B. circulans* treatments, was attributed to the nitrogen fixation potential and further enrichment of the micro ecosystem, i.e., the rhizosphere, with nutrients due to hydrolytic enzymes produced by the above mentioned strains. Raising potatoes in clay soil with increasing percentage of sand up to 20% revealed a tendency for disease severity decrease. The effect was attributed to an easier flushing of the pathogen under alkaline soil conditions. The significant increase in plant height and weight in the *B. circulans* treatments was attributed to greater availability of nutrients especially nitrogen and potassium. It could be concluded that the results of antagonism *in vitro* may be inconclusive under certain circumstances following soil application, in biological control trails. Other inherent physiological and biological activities of the antagonist may be considered as limiting factors in biological control success.

Key words: Biofertilizers, AUDPC, potato maturity, colonization of bacteria in rhizosphere, pathogen survival and disease incidence

INTRODUCTION

"Bacterial wilt" or "Potato Brown Rot" caused by *R. solanacearum* is a disease with an economic importance worldwide. Control trials over decades failed to lay down an acceptable chemical control protocol for disease managements (Farag *et al*., 1982). Agricultural practice(s) and biological control have been tried under different disease conditions with more or less success (Ran *et al*., 2005a, b; Messiha *et al*., 2007). The versatile disease control results may be attributed in principals to the un-intended ignorance of the source of infestation and the inoculum level in the vicinity of experimentation (Ellis *et al*., 1999). The predominant race of the pathogen in a given geographic area may be among the crucial reasons for such difficulties` in disease control. Race 1 of the pathogen is widespread in tropical and sub-tropical regions affecting taxonomically unrelated plant families including solanaceae (Kelman, 1953; Buddenhagen and Kelman, 1964). Race 3, or the so called the potato race, is favored by a lower temperature for the disease development and has a limited range of hosts (Buddenhagen and Kelman, 1964; Graham *et al*., 1979; Janse *et al*., 2004). Recently, Prior and Fegan (2005) proposed a new hierarchical classification scheme, based on DNA sequence analysis of the internal transcribed spacer region. Four phylotypes were distinguished corresponding with their geographic regions (Prior and Fegan, 2005). Each phylotype can be further subdivided into sequevars based on differences in the sequence of a portion of the endoglucanase (egl) gene. Phylotype II, sequevar 1 (Race 3 biovar 2) is pathogenic to potato, some weeds and to less extent on tomato.

A voluminous literature has been accumulated on the use of antibiosis in plant disease control with special emphasis on different *Trichoderma* spp., *Streptomyces* spp. and different spore-formers of bacteria

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as well (Farag *et al*., 1986; Tolba,1998). In addition to antibiotic(s) production by certain strains of different species of the genus Bacillus, *B. circulans* was described to produce a range of hydrolytic enzymes (Travino *et al*., 1989; Wiwat *et al*., 1999), to solubilize compounded nutrients as silicate (Savostin, 1972), tricalcium phosphate, promotion of plant growth by potassium mobilization from silicate in soil, (Saber and Zanati, 1981; Balabel-Naglaa, 1997; Eweda *et al*., 2007) and organic wastes degradation (Kubo *et al*., 1994) along with atmospheric nitrogen fixation by some strains (Berge *et al*., 1990) are among the most important activities.

The present study was undertaken to study the interaction of two domestic strains of *B. circulans*, isolated from clay soil in Egypt, with different physiological and hydrolytic enzyme (s) activities and Phytotype II Sequovar 1 isolates of *R. solanacearum* as related to disease progress.

MATERIALS AND METHODS

***B. circulans* strains:** Two physiologically different strains of *B. circulans* (SF-1 and DE-62 as kept under the given codes of the Central Laboratory of Agricultural Climate)*, were grown on Nutrient Agar (Jacobs and Gerstein, 1960) and modified Aleksandrov`s solid medium as well (Zahra, 1969) to ensure purity. Figure 1a shows colonies with typical morphology of *B. circulans*. The cells are encapsulated and the size of the capsule is shown in Fig. 1b. Pure cultures were routinely kept on nutrient agar slants at 4°C. The enzymatic potential(s) of the bacteria in concern along with potassium mobilization are shown in Table 1.

Source of *R. solanacearum* isolates and growth condition: Five isolates of *R. solanacearum*, phylotype II, sequevar 1 (race 3, biovar 2), recovered from different habitats (tubers, soil and weeds) were used. Growth characteristics on Semi Selective Media of South Africa, SMSA medium were checked (Elphinstone *et al*., 1996). Iimmuno-fluorescence (Janse, 1988), physiological and biochemical tests (Hayward, 1964; Klement *et al*., 1990) to confirm identity were considered.

Inoculum preparation: The five pathogen isolates (*R. solanacearum*) and the spore forming bacteria *(B. circulans*) were grown at 28°C on Nutrient Glucose Agar medium (NGA) for 48 h. Developed growth was suspended in 0.01 M phosphate buffer, pH 7.2 and the bacterial density was adjusted to 10^6 CFU mL^{-1} at 610 nm using a spectrophotometer, Jen way 6300 (UK).

Potato varieties: Class E seed tubers, with different maturity characteristics, used in this study were kindly provided by the Potato Brown Rot Project (PBRP), Agric. Res. Center, Giza, Egypt. The cultivars Picasso, Cara, Diamante, Inova, Nicola and Spunta, were used.

Table 1: Principal physiological differences of *B. circulans* strains*

Character	*B. circulans* SF-1	*B. circulans* DE-62
Pectinase (halo in cm)	2.500	2.000
Chitinase (halo in cm)	1.300	0.000
Phosphatase (halo in cm)	0.000	2.160
Nitrogenase (μm C_2H_4 mL^{-1} h^{-1})	0.465	0.112
Cellulase (μg protein)	31.890	4.500
K- mobilization ppm	5.720	8.850

*Provided by Dalia A.Abd El-Fattah, Assistant Researcher in the Central Laboratory of Agricultural Climate-ARC

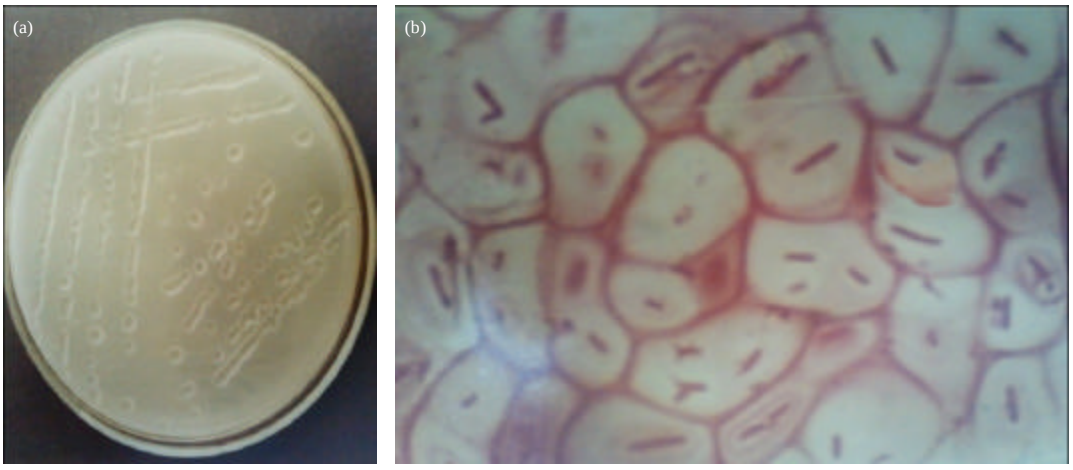

Fig. 1(a-b): Colony morphology of silicate bacteria on (a) Aleksandrov's agar medium and (b) The size of capsule

Table 2: Layout of the experiment

	Bacterial amendment	
Clay and sand percentages	With pathogen	Without pathogen
100% clay soil+0% sand	(+) *B. circulans*/(-) *B. circulans*	(+) *B. circulans*/(-) *B. circulans*
95% clay soil+5% sand	(+) *B. circulans*/(-) *B. circulans*	(+) *B. circulans*/(-) *B. circulans*
90% clay soil+10% sand	(+) *B. circulans*/(-) *B. circulans*	(+) *B. circulans*/(-) *B. circulans*
80% clay soil+20% sand	(+) *B. circulans*/(-) *B. circulans*	(+) *B. circulans*/(-) *B. circulans*

Greenhouse experiments

Effect of *B. circulans* application on the pathogen in potato rhizosphere: Eyepieces of an early maturing (Spunta), medium early (Nicola) and late maturing (Diamante) varieties were planted in clay soil amended with sand in different proportions (0, 5, 10 and 20%) and control unamended treatments were prepared (Table 2). Before taking the eyepieces, tubers were surface sterilized, using 0.05% (NaOCl), then washed in sterile water and air dried. Eyepieces were planted in soil infested with a mixture of five virulent Egyptian isolates of race 3 biovar 2 of *R. solanacearum*. The pathogen inoculum was prepared as mentioned before and mixed with the soil to give approximately 7×10^5 CFU g^{-1}dry soil. The *B. circulans* strains were applied to the soil at the rate of 4×10^5 CFU g^{-1}dry soil of a mixture of the two spore forming strains, just before planting. The layout of the experiment is shown in Table 2.

Effect of *B. circulans* on disease severity as related to maturity: Small tubers (approximately 35 g $tuber^{-1}$), different in maturity characteristics, were planted in potted sandy soil (100% sand). Inova (early maturing), Picasso (medium early maturing) and Cara (late maturing) were used. Each variety was planted in 20 pots, i.e., five pots for each of *R. solanacearum* treatments, five for *B. circulans*, five for both of them and five as negative control. The inoculum levels at the beginning of the experiment were 10^7 CFU g^{-1}dry soil for each organism separately.

Detection of *Bacillus circulans*: Detection of *Bacillus circulans* was made on modified Alexandrov's medium (Zahra, 1969) from the rhizosphere at the end of the experiment (60 days after planting).

Detection of *R. solanacearum*: The population of the pathogen was determined on modified SMSA medium (Elphinstone *et al*., 1996) in soil and rhizosphere at the end of the experiment (60 days after planting).

***In vitro* experiments**

Testing antagonism of *B. circulans* strains to *R. solanacearum*: King's medium agar (King *et al*., 1954) plates were used for testing the interaction between *B. circulans* and *R. solanacearum*. *B. circulans* (SF-1 or DE-62) was lined four times in four quarter of King's B agar medium plates; the pathogen is then lined in a parallel position 0.5 cm. Plates were incubated at 30°C for 48 h (Farag, 1976). Five replicate plates were considered.

Testing interaction between *R. solanacearum* isolates: The possible antagonistic interaction between the five *R. solanacearum* isolates was also checked for the interaction between isolates and prepared as mentioned before under the antagonistic effect of *B. circulans*.

Testing interaction between *Bacillus circulans* isolates: The possible antagonistic interaction between the two *B. circulans* isolates was checked as mentioned before (Farag, 1976).

Statistical analyses: The non-parametric correlation analysis (Kendall's tau_b) was performed between inoculum levels of *R. solanacearum* and those of *B. circulans* using SPSS 17.

The *Post-hoc* (Tukey HSD) using SPSS was used to determine the difference in log transformed CFU of *R. solanacearum* in soil planted with different potato varieties.

RESULTS

Greenhouse experiments

Effect of *B. circulans* application on the pathogen in potato rhizosphere: Table 3 shows the interaction between *B. circulans* and *R. solanacearum* in rhizosphere of potato varieties, different in maturity characteristics, raised in clay soil with different percentage of sand. Results show negative influences of potato maturity on densities of the inoculated bacterial species under the conditions of the experiment. The non-parametric correlation analysis (Kendall's tau_b) revealed that increasing sand percentage, up to 20%, caused a significant positive correlation of the two organisms in the rhizosphere of Spunta ($r = 0.48$, $p = 0.003$) and Nicola ($r = 0.71$, $p>0.001$). The only exception was recorded for the late maturating Diamant variety i.e., absence of correlation.

With regard to *R. solanacearum* in the rhizosphere, in treatments devoiding *B. circulans*, it is clear that there

Table 3: Effect of *B. circulans* and different sand percentages on the pathogen count in rhizosphere of three potato cultivars (Experiment 1)

Potato variety	Treatment (sand percentages)	*Count of *B. circulans* $\log_{10}$ (CFU+1)/g rhizosphere		*Count of *R. solabacearum* $\log_{10}$ (CFU+1)/g rhizosphere	
				In absence of *B. circulans*	In presence of *B. circulans*
Spunta (early maturing)	0	Correlation	6.34±0.12	6.41±0.09	6.22±0.23
	5	with sand	6.15±0.07	6.42±0.15	6.28±0.06
	10	-0.67, p<0.001	5.75±0.11	5.56±0.29	5.15±0.21
	20		5.64±0.19	5.12±0.32	5.50±0.18
Correlation with count of *Rsol* count	-0.59, p =0.001[A] -0.46, p =0.009[B]		+0.48, p =0.003		
Nicola (medium early)	0	Correlation	6.52±0.15	6.45±0.12	6.37±0.11
	5	with sand	5.98±0.21	5.49±0.12	6.15±0.11
	10	No correlation	6.32±0.12	6.44±0.10	6.25±0.15
	20		6.08±0.31	5.27±0.25	5.97±0.16
Correlation with count of *Rsol* count	-0.34, p =0.051 no correlation		+0.71, p<0.001		
Diamant (late maturing)	0	Correlation	5.97±0.22	6.02±0.25	5.38±0.11
	5	with sand	5.79±0.25	5.78±0.19	5.04±0.32
	10	No correlation	6.20±0.12	5.55±0.09	6.04±0.26
	20		6.31±0.18	5.86±0.25	6.01±0.24
Correlation with count of *Rsol* count	No correlation+0.37, p = 0.034		No correlation		
Correlation with count of *Rsol* count in all verities (general)	-0.34, p = 0.001[A] no correlation[B]		+0.46, p<0.001[C]		

[A]correlation with the pathogen in absence of *B. circulans*, [B]correlation with the pathogen in presence of *B. circulans* , [C]Correlation between the two organisms (at 0% sand+0.39, p = 0.04, at 5% sand+0.46, p = 0.02, at 10% sand+0.64, p = 0.001 and at 20% sand +0.56, p = 0.004), *The colonies of *R. solanacearum* were counted on SMSA and suspected colonies randomly were confirmed by IFAS testing, Values are Mean±SE

Table 4: Effect of *B. circulans* amendment on potato brown rot development in three different potato verities (experiment 2)

Potatovariety	*R. solanacearum* $\log_{10}$ (CFU+1)/g							
	AUDPC		Wilt severity		Soil		Rhizoshpere	
	No *B. circulans*	With *B. circulans*	No *B. circulans*	With *B. circulans*	No *B. circulans*	With *B. circulans*	No *B. circulans*	With *B.circulans*
Inova	20.1±20.1	11.7±11.7	9.3±9.3	9.3±9.3	2.7±0.09[1]	3.1±0.02[4]	5.1±0.09	5.3±0.14[7]
Effect of *B. circulans*								
Picasso	0.2±0.2	13.8±9.2	1.6±1.6	20±15.5	3.5±0.07[2]	2.7±0.22[5]	5.3±0.14	5.3 ±0.1[8]
Effect of *B. circulans*								
Cara	42.3±39.8	104.7±35	24.2±18.0	78.7±18.2	3.8±0.1[3]	4.3±0.18[6]	5.6±0.17	6.3 ±0.3 9
Effect of *B. circulans*							F = 5.31, p = 0.05	
Difference among varities			p = 0.017		F = 40.55 p = 0.016	F = 25.11 p<0.001	p<0.001	F = 7.7 p = 0.007
Effect of *B. circulans* in general							F = 3.85, p = 0.06	

Values are Mean±SE, [1,2]p<0.001, [1,3]p<0.001, [3,2]p = 0.059, [7,9]p = 0.059, [8,9]p = 0.059, [4,6]p<0.001 and [5,6]p<0.001

exist a general negative correlation between sand percentage and the count of the pathogen in the rhizosphere (r = 0.34, p = 0.001). The recorded figure of correlation was (r = -0.59, p = 0.001) for Spunta and (r = -0.34, p = 0.051) for Nicola. Again, the only exception (i.e., absence of correlation) was recorded for Diamant.

It is clear from Table 3 that there is a negative correlation between the count of *B. circulans* in the rhizosphere and the percentage of sand (r = -0.67, p<0.001), (i.e., the inoculums level of *B. circulans* decrease with increasing the percentage of sand) used in the potting of the early maturing Spunta variety.

Effect of *B. circulans* on disease severity as related to maturity: The effect of soil infestation with *B. circulans* on AUDPC and densities of *R. solanacearum* in the rhizosphere is shown in Table 4. Because of the normal distribution of *R. solanacearum* figures in the soil and in the rhizosphere, the log numbers and the *Post-hoc* analysis (Tukey HSD)$_{0.05}$ were conducted. The densities of the pathogen, AUDPC and wilt severity were not normally distributed; hence, the non-parametric statistical analysis (Kruskal-Wallis Test) was followed.

The results show a tendency of pathogen increase in the rhizosphere, in the presence of *B. circulans* (F = 3.85, p = 0.06), this was most clear for cv. Cara (F = 5.31, p = 0.05). A similar trend of an apparent increase of disease incidence in presence of *B. circulans* compared to its absence, without significant differences in most cases can be recognized for AUDPC and wilt severity (Table 4).

The effect of *B. circulans* on potato cultivars with different maturity characteristics was also considered (Table 4). The results showed that the AUDPC and wilt severity of Cara variety (late maturing) were higher and

significantly different (p = 0.017) and p = 0.016, respectively), when compared to Inova (early maturing) and Picasso (medium early maturing). The same trend of increased disease incidence was recognized for *R. solanacearum* in the rhizosphere of Cara (p = 0.007) compared to Picasso and Inova. Moreover, under the same treatment conditions, the Chi square test revealed that, amendment of infested soil planted with Cara by *B. circulans* increased the number of infected plants significantly (p = 0.025) compared to non-amended soil. Also, application of *B. circulans* to the afore-mentioned varieties significantly increased the number of infected plants in Cara (p = 0.005) over Picasso and Inova. Meanwhile, in treatments without *B. circulans*, no-significant differences were found between the numbers of infected plants of the three varieties under investigation.

With regard to the effect of the above-mentioned treatments on plant length and yield of different potato varieties, it is interesting to note that *B. circulans* alone caused a significant increase in plant height and weight (Fig. 2). The involvement of *R. solanacearum*, however, has minimized such effect. Moreover, *R. solanacearum* alone caused significant reduction in both plant length and weight. Inoculation with *R. solanacearum* either single or in combination with *B. circulans* significantly decreased the plant weight ($p<0.001$), as shown by the *Post-hoc* analysis (LSD). Regarding plant weight in different treatments it is apparently evident that inoculation with *R. solanacearum* and with both organisms significantly decreased the plant weight ($p<0.001$). Meanwhile, treatment with *B. circulans* alone increased the plant weight significantly ($p<0.012$).

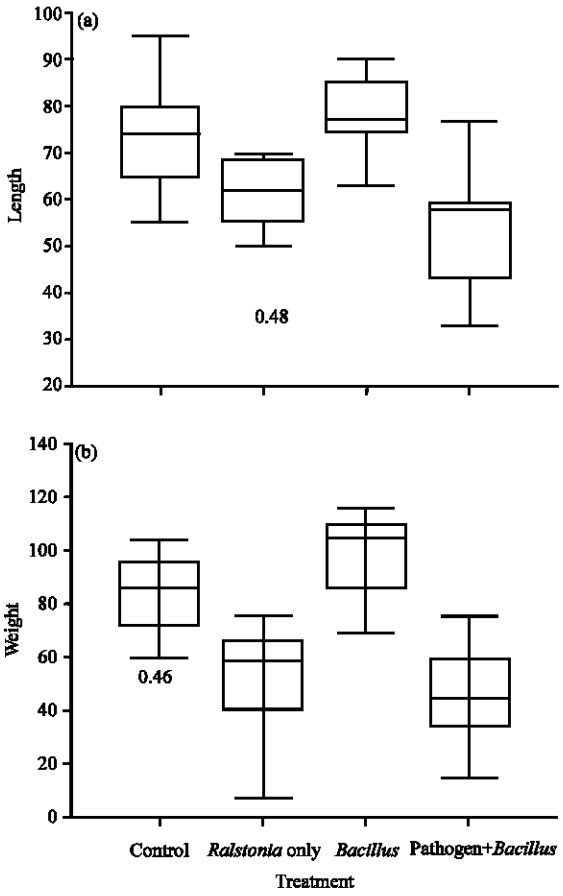

Fig. 2(a-b): Effect of soil infestation of bacteria on plant (a) Length and (b) Plant weight

In vitro experiments

Testing antagonism of *B. circulans* strains to *R. solanacearum*: It was found that the SF-1 strain has an antagonistic effect/or inhibitory effect against an isolate of *R. solanacearum* which originally isolated from the weed (*Polypogon monspeliensils*). However, other isolates showed less sensitivity to SF-1 antagonism (Fig. 3).

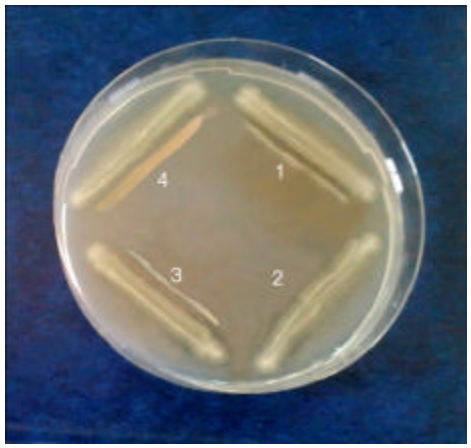

Fig. 3: Antagonism of *B. circulans* (SF-1) on *R. solanacearum* on KB medium, (The outer line for SF-1 and the inner for different isolates of *R. solanacearum*), the absence of weed isolate No. 2, isolates No. 1, 3 and 4 for tubers

Testing interaction between *Ralstonia solanacearum* isolates: Five different *R. solanacearum* isolates, previously isolated from different habitats were tested for their antagonistic potential against each other. The isolate No. 4 isolated from tubers showed varying antagonistic potential against the rest of the isolates (Fig. 4).

Testing interaction between *Bacillus circulans* isolates: The two physiologically different *Bacillus circulans*

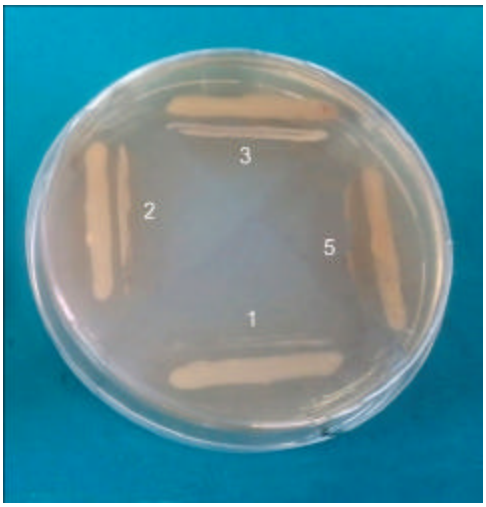

Fig. 4: Antagonistic potential of different *R. solanacearum* isolates against each other. The outer line for tuber isolate No. 4, the other isolates 1, 3 isolated from tubers, 2 isolated from weeds and 5 isolated from soil, the absence of the soil isolate No. 5

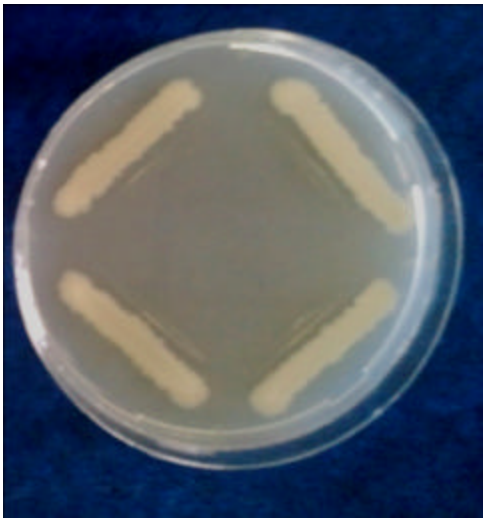

Fig. 5: Antagonistic effect of *B. circulans* (SF-1) on *B. circulans* (DE-62) on Nutrient agar medium (Outer line for SF-1 and the inner for DE-62)

strains under investigation were tested for their antagonistic potential against each other. The results showed that the strain SF-1 is being antagonistic against DE-62. The first showed a complete inhibition of the second (Fig. 5).

DISCUSSION

Potato brown rot control caused by *R. solanacearum,* Phylotype II Sequevar 1 (race 3 biovar 2), is depending largely on agricultural practices with variable degrees of success (Michel and Mew, 1998; Gorissen *et al*., 2004; Islam and Toyota, 2004; Messiha *et al*., 2007).

Few strains of the pathogen have shown an antagonistic potential against certain groups of rhizosphere bacteria (Farag, 1976). Control trials with antibiotics as streptomycin sulphate gave a negative results and increased the wilt incidence in an open field trails (Farag *et al*., 1986). Other chemical treatments showed varying effect(s) confirming failure of chemical control (Seif-Elyazal, 1980) and pointed to the possible value of biological control measures, based entirely on antibiosis mechanisms in soil against *R. solanacearum*. Abd El-Fattah (2006) reported that *B. circulans* may be used in biofertilization. In a preliminary study with *B. circulans*, Balabel-Naglaa (1997) also studied the role of this bacterium as a biofertilizers. These domestic strains have been described as silicate dissolving bacteria that improved the botanical characteristics of certain non-silicon accumulator species such as *Vicia faba* and *Solanum tuberosum*. The results reported herein showed that a mixture of two *B. circulans* strains increased significantly the growth and yield of potato. The effect may be attributed to increased activity of the nitrogenase produced by these strains, greater K-mobilization and increased phosphate availability benefiting the N:P:K requirement for better plant growth. In a similar work, Balabel-Naglaa (1997) reported that inoculation of potato with certain strains of *B. circulans* increased K-content, as well as nitrogen and phosphorous contents of tubers as compared with the non-inoculated control. However, in the present study inoculation of *R. solanacearum* together with *B. circulans* strains has minimized such beneficial effects as shown by the disease progress compared to check treatments. It is interesting to conclude that inoculation of *B. circulans* in soil, in addition to its antagonistic potentials against *R. solanacearum*, increase the availability of macro-and micro nutrients to plants through the effect of hydrolytic enzymes as amylases (Kwan *et al*., 1993), cellulases (Kim and Kim, 1993) and Chitinases (Alam *et al*., 1996; Wiwat *et al*., 1999) on organic matter in soil. Producing of chitinase, cellulase, pectinase and phosphatase by the used strains in the micro- ecosystem in the rhizosphere will of course favor the availability of macronutrient to either the growing plants or proliferation of bacteria in the rhizosphere.

The maturity standards of potato varieties, in this work exerted a limited influence on densities of the

afore-mentioned organisms in the rhizosphere. The sand percentage, however, showed a highly significant inverse correlation, i.e., the increase in sand percentage decreased the densities of *R. solanacearum* and to less extent the densities of *B. circulans* in the rhizosphere. Such microbial decrease may be attributed to the indirect decrease of the total organic carbon as the proportion of sand is being increased (Messiha-Nevein *et al*., 2009).

The interrelation between *B. circulans* and disease development as expressed by AUDPC, wilt severity, densities in the rhizosphere was also studied. The results showed a non-significant increase in the previously mentioned parameters ($F = 3.85$, $p = 0.06$). The only exception may be observed for cv. Cara (late maturing) variety that showed a significant increase in these parameters ($F = 5.31$, $p = 0.05$) which may be attributed to a greater response of the Cara variety to nitrogen fixation resulted from nitrogenase produced by *B. circulans* and other hydrolytic enzymes as well. The effect of manuring on potato brown rot was considered by Farag (1970) and Mahmoud *et al.* (1978).

The *in vitro* experiment revealed that *B. circulans* SF-1 showed different degrees of an inhibitory effect against *R. solanacearum*. The high nitrogenase activity for SF-1 which is more than 4 times higher than that DE-62, may explain the increased antagonistic potential of SF-1, as related to optimization of C:N ratio due to atmospheric N2 fixation and the dramatic increase in cellulase activity that controls the carbon content in the ecosystem i.e, the rhizosphere in this study.

The viable cell of the used *B. circulans* strains in this study is equipped with a group of hydrolytic enzymes as cellulases and pectinases that are playing a role in disease progress. The possible beneficial effect, however, may be attributed to potassium released by the action of *B. circulans* DE-62 and SF-1 and an antibiotic that may be produced by the latter. More interesting it has been documented in this study that few strains of *R. solanacearum* showed an inhibitory effect against others of the same species, as was also determined by Farag (1976). This may explain the occasional disagreement in results of biological control of plant pathogens and emphasizing the value of the net interaction between microorganisms in soil.

Further investigations are needed on the bacterization with the afore-mentioned bacteria in biofertilization and biological control, under field conditions.

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