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The Impact of Biofilm Forming Rhizobacteria on *Rhizoctonia solani* Damping-off of Tomato Running title: The Impact of biofilm forming rhizobacteria on *Rhizoctonia solani*

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ABSTRACT

In the current study, 180 rhizobacteria were isolated from different fields in East and West Azarbaijan provinces of Iran. These bacterial isolates were screened based on their antagonistic potential against *Rhizoctonia solani* and their ability to form robust biofilm. Out of these isolates, 49 isolates exhibited more than 30% antagonistic activity against *R. solani.* Different methods were applied to screen the isolates with high ability to form biofilm. Out of 180 isolates, 51 isolates were able to form considerable amount of biofilms as determined by crystal violet staining (CVS) method. Pellicle formation (PF) bioassay was performed in three different media. Most of the robust biofilm forming isolates from CVS method could also form robust biofilms in PF method, only if MSNGP (medium with glycerol and pectin) medium was used. In root colonization bioassay, isolates N168, N95 and N94 that were robust or mediocre biofilm forming isolates in MSNGP, could efficiently colonize plant roots, but the population of isolates N100 and N87 (none-biofilm forming isolates) on tomato roots were lower than 6 $\log_{10}$ CFU g^{-1} root. However, the results of the pathogenicity system with *R. solani* damping off of tomato indicated that, the biofilm formation and colonization ability of these isolates doesn't leave a significant impact on their biocontrol activity

Key words: rhizobacteria, Rhizoctonia, biofilm, *Bacillus*, *Pseudomonas.*

Introduction

Many microorganisms from the rhizosphere can positively influence the plant growth and health. These microorganisms are referred to as plant growth promoting rhizobacteria (PGPR) (Haas and Defago 2005). These bacteria promote the plant growth either directly by producing indole-3-acetic acid (Patten and Glick 2002) and regulating the level of plant root hormones (Glick 2014, Vacheron *et al.* 2013) or indirectly by suppressing the pathogenic microorganisms (Beneduzi *et al.* 2012). They suppress phytopathogens through different mechanisms such as antagonism (Beneduzi *et al.* 2012), induction of plant systemic resistance (Pieterse *et al.* 2014) and competition for ecological niches or a substrate in rhizosphere (Compant *et al.* 2005). A successful competition of PGPR with plant pathogens depends on their ability to efficiently colonize different parts of the rhizosphere. Root colonization by a biocontrol agent is important for two reasons: first, it is a prerequisite for successful expression of other biocontrol mechanisms, and, second, it can act as a biocontrol mechanism in its own right, since the biocontrol agent might occupy ecological niches on roots that might otherwise be colonized by pathogens (Haas and Defago 2005, Shirzad *et al.* 2012). Studies on this area suggest that, when PGPR colonize plant roots, they form complex structures known as biofilms. Indeed, these complex structures are microbial communities in which cells show multicellular behaviors and are

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embedded by extracellular polymeric compounds and proteins attached to surface (O'Toole *et al.* 2000, Beauregard *et al.* 2013). Bacterial cells in biofilm are protected against harsh environmental stresses such as desiccation, pH changes, osmotic shock and UV radiation (Simoes *et al*. 2010, Expert and Digat 1995). Moreover, biofilm formation drastically increases the bacterial resistance against antibiotics and host defense (Leid 2009, Stewart and Costerton 2001). Water flow in biofilm regulates nutrient availability, exchange of metabolites and removal of potentially toxic metabolites (Davey and O'toole 2000).

Multiple studies have shown that the biofilm formation by PGPR is important for their ability to suppress plant pathogens. Mucoid mutants of biocontrol strains *Pseudomonas fluorescens* CHA0 formed a dense and patchy bacterial layer on the roots and on mycelia of symbiont mycorrhizal fungi that resulted more stable interactions in rhizosphere (Bianciotto *et al.* 2001). It has been shown that *Paenibacillus polymyxa*, that is an efficient biocontrol strain, colonizes plant root tips and forms biofilm to protect plant against pathogens (Timmusk *et al*. 2005, Haggag and Timmusk 2008). Bais *et al*., (2004) reported that, upon root colonization, *Bacillus subtilis* 6051, forms a stable, extensive biofilm and secretes surfactin, which act together to protect plants against attacks by pathogenic bacteria. Chen *et al*., (2013) reported that plant protection by *Bacillus subtilis* strains depends on widely conserved genes required for biofilm formation, including regulatory genes and genes for matrix production.

The aim of this study is to isolate different rhizobacteria from rhizosphere of different crops and to screen them based on their antagonistic potential against *Rhizoctonia solani* and their ability to form robust biofilm.

Material and methods

Field sampling and isolation of rhizobacteria

In June 2014, a total of 100 soil and plant samples were collected from different fields in the East and West Azarbaijan provinces of Iran. Roots were gently removed from soil and placed in plastic bags before they were transported to the laboratory. Adhering soil was carefully brushed off. Rhizobacteria were isolated and purified from samples by standard methods (Chen *et al*. 2013, Shirzad *et al*. 2012).

In vitro antagonistic activity

In vitro inhibition of mycelial growth of *R. solani* by non-volatile compounds of the bacterial isolates was tested by using the dual culture technique as described by Ahmed Idris *et al.* (2007). Three 10 µl drops from a 10^8 cfu/ml suspension of the bacterial isolate were equidistantly placed on the margins of potato dextrose agar (PDA) plates and incubated at 28 ºC for 24 h. A 6 mm agar disc from a fresh culture of *R. solani* was placed at the center of the PDA plate for each bacterial isolate and incubated at 27 ± 1 ºC for seven days. The radii of the fungal colony toward and away from the bacterial colony were measured. The percentage of growth inhibition was calculated using the following formula:

$$\%Inhibition = \left[\frac{(R-r)}{R}\right] \times 100$$

Where, *r* is the radius of the fungal colony opposite the bacterial colony and, *R* is the maximum radius of the fungal colony distal from the bacterial colony.

Screening of biofilm forming rhizobacteria by crystal violet staining (CVS) bioassay

Bacterial isolates were cultured in Nutrient broth medium in shaker incubator (28 °C, 120 rpm) and after 18 h 250 µl of this culture (OD_{600}= 1) was transferred to sterile 96 well plates. After incubation of plate in 28 °C for 48 h, the medium of wells was carefully removed and rinsed with 0.85% NaCl solution. The wells filled with 250 µl methanol for fixation of biofilm. After 15 minutes, methanol removed and wells were rinsed with water and allowed to dry for 30 minutes under clean bench. For staining of adhered cells to wells, 1% crystal violet (CV) solution was added. Excess CV was then removed, and the wells were rinsed with water. The CV that had bound the pellicle was then solubilized in an ethanol-acetone solution (4:1, vol/vol). Biofilm formation was quantified by measuring the OD590 for each well (Nagorska *et al*. 2008). In this bioassay three replicates were used and stile NB medium was used as control.

Screening of biofilm forming rhizobacteria by pellicle formation (PF) bioassay

Bacterial isolates were cultured in Luria Bertani (LB) medium in shaker incubator (28 °C, 120 rpm) and after 18 h, 10 µl of each bacterial isolate (10^6 cells/ml) was transferred into 1 ml different media in wells of a 24-well plate. The media used in this bioassay were as follow: LBMG (LB + 0.1 mM $MnSO_4$, Glycerol), MSNG medium (5mM potassium phosphate buffer pH7, 0.1M Mops pH7, 2mM $MgCl_2$, 0.05mM $MnCl_2$, 1µM $ZnCl_2$, 2 µM thiamine, 700 µM $CaCl_2$, 0.2% NH_4Cl, 0.5% Glycerol) and MSNGP (MSNG + 0.5% pectin). After inoculation, plates incubated in 30 °C for 72 h and at the end the wells were assessed for formation of pellicle.

In vitro root colonization

This bioassay was conducted in vitro using the method described by Shirzad *et al.* (2012) with some modifications. Antagonistic isolates were grown in nutrient broth (NB) medium for 24 h at 27 ˚C with 120 rpm shaking. Bacteria were harvested by centrifugation (10 min at 6,000 g) and washed twice with 0.8 % NaCl solution. Washed pellets were suspended in 1% methylcellulose solution and the optical density of these suspensions adjusted to 0.1 at OD600 (10^8 cell/ml). Tomato seeds (Rio Grande cultivar) were surface-sterilized with 10% bleach for 3 min, then with 70% ethanol for 2 min before rinsing three times in sterile distilled water. These seeds were dipped in bacterial suspensions (or 1% methylcellulose solution as control) and gently was shook for 30 minutes. For each treatment, seven seeds were transferred on to 1% water agar medium in 10 cm Petri dishes. The plates were incubated at 24 ˚C for a week in the growth chamber for root development. After seven days, one gram of roots (from different parts of roots) from each treatment was aseptically excised, and transferred to 0.8% NaCl solution, macerated and serially diluted. From each dilution, a 10 µl aliquot was plated on nutrient agar medium and the plates were incubated at 27 ˚C for colony counts. The number of bacteria colonizing the root was calculated as colony-forming units/g root (CFU/g root).

Preparation of pathogen inoculum and greenhouse evaluation of selected isolates

Rhizoctonia solani AG4 was obtained from microbial collection of plant

pathology laboratory in Azarbaijan Shahid Madani University. For inoculum preparation, 100 g of wheat seeds was washed and mixed with 70 ml distilled water in 500 ml Erlenmeyer flask and kept in room temperature overnight. The flask was sealed with cotton plug covered with aluminum foil and autoclaved for 20 min at 121°C on two successive days. The flask was inoculated with mycelial plugs from a 4 day-old culture of *R. solani* AG4, incubated for two weeks at 25 °C with daily shaking to break up particles. This inoculum was air dried, ground in a mill and used for greenhouse studies. From this inoculum 0.6 g was mixed into the upper part of sterile soil in plastic pots (diameter, 7.5 cm; depth, 8). For the control treatments, sterile millet seed was mixed into the soil at the same rate. Tomato seeds (5 seeds per pot) inoculated with bacterial isolates by the method mentioned above and planted 0.5 cm deep in the infested soil. Plants were grown in the greenhouse under day and night temperatures of 25/18°C with a 16 h photoperiod and 30% relative humidity. After 21 days, fresh weight of plants and the percentage of healthy plants was determined as described by Expert and Digat (1995).

Identification and characterization of antagonistic bacteria

Selected isolates were identified based on microscopic and physiological observations and biochemical tests as descried by Schaad *et al*., (2001).

Results

Isolation of rhizobacteria and in vitro antagonistic activity

In this study, about 180 bacterial isolates were isolated and purified from the rhizosphere of different plant crops. Out of these isolates, 49 isolates exhibited more that 30% antagonistic activity against *R. solani* (Table 1). Among these isolates, isolates N95(II), N100, N87, N94 and N161 showed more than 80% inhibition. Control plates which had not been treated with a bacterial isolate were completely covered by the phytopathogen (Table 1).

Table 1. Mycelial growth inhibition of *Rhizoctonia solani* by metabolites produced by bacterial isolates

Percent of inhibition						
under 30%*	30-50%			More than 50 %		
N136	G17-1	N10	N162	N115	G7	N79
N101	G20-2	N44	N99	N120	G9	N78
	G21-1	N84	G2-1	N135	G12	N87
	G21-2	N131	G16-1	N134	G15	N76
	G5	N125	G16-2	N102	N100	N161
	G4-1	N103		N113	G8	N160
	G13	N111		N149	G11	N94
				N90	G19-1	N158
				N130	N145	N133
				N143	N95(II)	N104

*% Mycelial inhibition was calculated as (R − r)/R × 100, where R is mycelial growth away from the bacterial colony (the maximum growth of the fungal mycelia) and r is mycelial growth towards the bacteria.

Comparison of CVS and PF bioassay for screening biofilm forming isolates

Different methods were used to screen the isolates with high ability of biofilm formation. First, all of the 180 isolates were tested by CVS bioassay in 96 well plates. Out of 180 isolates, 51 isolates were able to form considerable amount

of biofilm in wells of the plate as determined by measuring the OD of crystal violate solution in wells (Table 2). The OD of 29 of these isolates was more than 2 (table 2). Then two group of isolates were selected from this step and further tested by PF bioassay. One group were those of robust biofilm forming isolates including N177, N59, N181, N185, N168, N64, N30, G184, G5, G18, G9, G8, G7, G11, G12, G13, G15, G19-1, and second group were the isolates that could not form biofilm in 96 well plates including N179, N94, N95(II), N87, N100, N161, N91, N81, N183, N82, N57, G17-1, G17-2, G16-1, G16-2, G14-1, G14-2, G6, G10, G19-2, G20-1, G20-2, G21-1, G21-2, G2-1, G2-2, G4-1, G4-2, G3, G23, G91. PF method was performed in three different media (Table 3). Most of the robust biofilm forming isolates from CVS method could also form robust biofilms in PF method, only if MSNGP medium was used (Table 3, Figure 1). However, even in this medium isolates G18 and N64 were not able to form biofilm. Most isolates were not able to form biofilm in MSNG medium and none of them formed a robust biofilm in this medium (Table 3).

Table 2. Screening of robust biofilm forming rhizobacteria by crystal violet staining assay.

Antagonistic isolates	OD at 600 nm	Antagonistic isolates	OD at 600 nm
N168	3.06a*	N69	2.18defghijklmno
G12	2.85ab	N50	2.12efghijklmnop
G15	2.88ab	N29	2.09fghijklmnopq
G8	2.82abc	N63	2.01ghijklmnopqr
N184	2.82abcd	N86	2.01ghijklmnopqr
N64	2.79abcde	N66	1.98ghijklmnopqr
G13	2.79abcde	N140	1.98hijklmnopqr
G11	2.81bcdef	N60	1.94ijklmnopqr
N30	2.77bcdef	N48	1.86ijklmnopqr
N59	2.74bcdef	N61	1.88ijklmnopqr
N177	2.73bcdefg	N15	1.87ijklmnopqr
G9	2.66bcdefgh	N80	1.84ijklmnopqr
N76	2.16bcdefghi	N45	1.77jklmnopqr
N185	2.6bcdefghi	N175	1.78jklmnopqr
G18	2.58bcdefghi	N127	1.72jklmnopqr
G5	2.52bcdefghij	N84	1.65klmnopqr
G19-1	2.53bcdefghijk	N67	1.67lmnopqr
N32	2.28bcdefghijk	N135	1.5mnopqr
N181	2.41bcdefghijkl	N92	1.47mnopqr
G7	2.43bcdefghijkl	N90	1.46nopqr
N53	2.4bcdefghijkl	N56	1.4opqr
N93	2.28bcdefghijkl	N65	1.55pqr
N136	2.30bcdefghijkl	N122	1.42qrs
N28	2.14cdefghijkl	N70	1.38rs
N2	2.25cdefghijkl	N72	1.21s
N68	2.18cdefghijkl		

The biofilm was quantified after 48 h using the crystal violet staining assay. The A600 value represents crystal violet-stained biofilm attached to the walls of the microtitre wells and is an indirect measure of the biofilm formed. Data represent mean for a representative experiment with three replicates. * Means with different letters are significantly different, as determined by Fisher's least significant difference test (P = 0.05).

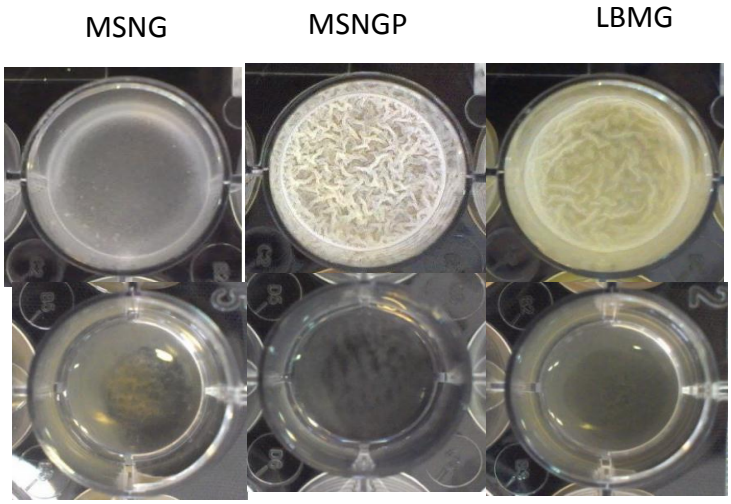

Figure 1. Biofilm formation of isolates N185 (robust biofilm forming) and G6 (weak biofilm forming) in three different media as determined by pellicle formation bioassay. Bacterial suspension (10 µl with 10^6 cells/ml) from each isolates were transferred into 1 ml different media in wells of a 24-well plate. After incubation of plates in 30 °C for 72 h, the wells were assessed for formation of pellicle. MSNG: MSN medium with glycerol, MSNGP: MSN medium with glycerol and pectin, LBMG: LB medium with glycerol and manganese.

Table 3. Screening of robust biofilm forming rhizobacteria by pellicle formation assay using different media.

Pellicle formation on different media								
MSNG*			MSNGP**			LBMG***		
N	M	R	N	M	R	N	M	R
G10	G91		G17-1	G91	G5	G2-2	G19-1	G91
G17-1	G5		G10	N59	G9	G17-2	N177	G9
G17-2	G16-1		G17-2	G81	G184	G57	G16-1	G17-1
G6	N177		G6	G183	G8	G4-1	G7	G4-2
G18	G9		G18	G82	G13	G3	G5	N181
G19-2	G8		G19-2	G16-2	G12	G14-1	G8	N185
N59	G13		G57	G21-2	G11	G2-1	G11	N94
G183	G12		G20-1	G20-2	G15	G18	G12	
G81	G11		G21-1	G7	G19-1	G183	G13	
G57	G15		G2-1	N179	N177	G16-2	G15	
G82	G20-2		G23	N94	G16-1	G6	G82	
G184	N185		G4-1	N95(ii)	N181	N59	G184	
G16-2	N168		G14-1	N161	N185	G20-2	N179	
G21-2			G14-2		N168	G19-2	N95(ii)	
G20-1			G2-2			G23	N161	
G21-1			G3			G21-1	N87	
G2-1			G4-2			G10	N100	
G7			N87			G21-2		
G23			N100			G14-2		
G4-1			N64			G20-1		
G19-1			N30			G81		
G14-1						N168		
G14-2						N64		
G2-2						N30		
G3								
G4-2								
N181								
N64								
N30								
N95(II)								
N94								
N161								
N100								
N87								

* MSN medium with glycerol, ** MSN medium with glycerol and pectin, ***LB medium with glycerol and manganese, N: none biofilm forming, M: mediocre biofilm forming, R: Robust biofilm forming

In vitro root colonization and promotion of plant growth

Based on biofilm formation and dual culture bioassays some robust and weak biofilm forming isolates were selected for *in vitro* root colonization bioassay on tomato seedlings. The ability of different isolates to colonize roots of tomato seedlings were significantly different (Table 4). The population density of isolates N94, N168, N95 and N185 was significantly higher than other isolates as their population was more than 7 $\log_{10}$ CFU g^{-1} roots (Table 4). Isolates N95(II) and N94 significantly increased the plant growth in comparison to non-treated control plants as the fresh weight of the treated seedlings with these isolates was more than 0.25 g.

Table 4. *In vitro* growth promotion and roots colonization of tomato seedlings by rhizobacteria.

Bacterial isolates	Log_{10} CFU g^{-1} roots	Seedlings weight (g)
N94	8.21a*	0.2593a
N168	8.17a	0.2036bc
N95(II)	7.87a	0.2773a
N185	7.82a	0.2160b
N181	6.49b	0.2036bc
N161	6.49bc	0.1860c
N87	5.63c	0.1880c
N100	5.25c	0.1866c
Control		0.1870c

Tomato seeds were inoculated with bacterial isolates and incubated for seven days at 24˚C on water agar medium. Bacterial population on emerged roots were assessed by serial dilution method. * Means with different letters are significantly different, as determined by Fisher's least significant difference test (P = 0.05).

Greenhouse evaluation of selected isolates

The effect of selected isolates on *R. solani* damping-off of tomato was evaluated in greenhouse condition. Presence of *R. solani* in infected control pots (with no rhizobacteria) drastically effected the germination of seeds and some of the seeds were rotten during germination. The emerged seedlings in these pots was also effected as they could not evolve normally. The percent of healthy plants in infected control pots was 40% at the end of the assay. However, in *R. solani* infected pots which treated with most of the isolates, the seeds were geminated quickly and emerged seedlings had a normal growth. In the pots treated with N95(II), N168 and N100 isolates, all of the seeds were germinated normally and percent of healthy plants in these pots at the end of the assay was significantly more than the infected control pots (Table 4). Fresh weight of the plants from infected pots treated with isolates N95(II), N168 and N94 was significantly more that the plants from infected control pots. Regarding plants fresh weight and percent of healthy plants, isolate N95(II) was the most effective isolate against *R. solani* damping-off of tomato.

Morphological and biochemical identification of selected isolates

Selected isolates were identified based on physiological and biochemical tests (Table 5). All of the isolates except N185 and N181 produced diffusible fluorescent pigment on KB agar medium and they were gram negative as determined by gram staining and KOH test. All of the isolates except N181 were obligate aerobes. Spore formation was observed in the case of isolates N185 and N181. Based on the results presented in table 5, isolates

N95(II), N198, N100, N87, and N161 identified as *Pseudomonas fluorescens* (Schaad *et al*. 2001). Characteristics of isolate N94 was compatible with *P. putida* (Table 5). Based on the results presented in table 5 isolates N181 and N185 were identified as *Bacillus subtilis* and *B. licheniformis* respectively.

Table 4. Suppression of *Rhizoctonia solani* damping-off of tomato with selected strains in greenhouse.

Bacterial isolates	Plant fresh weight	Percent of healthy plants
N95(II)	0.444a*	93.2a
N168	0.377ab	86.6ab
Control	0.288abc	80ab
N100	0.246bc	73.2ab
N94	0.355ab	66.6abc
N87	0.299abc	66.6abc
N185	0.238bc	60bc
N181	0.226bc	60bc
N161	0.228bc	60bc
Infected control	0.154c	40c

Bacterial isolates were applied at the time of sowing and the percentage of healthy plants was determined three weeks after sowing. *Means with different letters are significantly different, as determined by Fisher's least significant difference test (P = 0.05).

Table 5. Biochemical and physiologic characteristics of antagonistic isolates

Test	N95(II)	N168	N100	N87	N94	N161
Diffusible non-fluorescent pigment	-	-	-	-	-	-
Non-diffusible, non-fluorescent Pigment	-	-	-	-	-	-
Levan	+	+	+	+	-	+
Oxidase	+	+	+	+	+	+
Arginine dihydrolase	+	+	+	+	+	+
Pectolytic Activity	-	-	-	-	-	-
Tobacco HR	-	-	-	-	-	-
Growth @ 41°C	-	-	-	-	-	-
Growth @ 4C	+	+	+	+	+	+
Nitrate to N_2	+	+	+	+	-	+
Gelatin liquefaction	+	+	+	+	-	+
Growth on:						
L-arabinose	+	-	+	+	+	+
D-galactose	+	+	+	+	-	+
Trehalose	+	+	+	+	-	+
Saccharate	+	+	+	+	+	+
Butyrate	-	+	+	+	+	+
Valerate	-	-	-	-	+	-
Azalate	-	-	-	-	-	-
Sorbitol	+	+	+	+	-	-
Meso-inositol	+	-	+	-	-	-
Adonitol	-	-	-	-	-	-
Propylene glycol	-	-	-	-	+	-
Ethanol	-	-	-	-	+	+

Table 6. Biochemical and physiological characteristics of antagonistic isolates

Test	N181	N185
Gram reaction	+	+
Motility	+	+
Spore position	central	central
Swelling of bacterial body	-	-
Arginine dihydrolase	+	+
Pectolytic Activity	-	-
Tobacco HR	-	-
Growth @ 45°C	+	+
Growth at pH 5.6	+	+
Growth in 7% NaCl	+	+
Utilization of citrate	+	+
Anaerobic growth in glucose broth	-	+
Acid from:		
Arabinose	+	+
Mannitol	+	+
Starch hydrolysis	+	+

Discussion

In this study, 180 rhizobacteria were isolated from rhizosphere of different crops and screened based on their antagonistic activity against *R. solani* and their ability to form robust biofilm. Out of these isolates, eight of them were selected for greenhouse studies. Results from morphological and biochemical tests showed that four of these isolates belong to genus *Pseudomonas* and two of them belong to genus *Bacillus*.

Fluorescent pseudomonads and some species of the genus *Bacillus* have historically been associated with suppression of root diseases caused by numerous fungal and fungal-like pathogens (Haas and Defago 2005, Beneduzi *et al.* 2012). They are able to produce metabolites such as antibiotics (Keel *et al.* 1992), hormones (Patten and Glick 2002), Hydrogen cyanide (HCN) (Dufour *et al.* 2010), and siderophores (Ahmed and Holmstrom 2014, Neilands 1995). Different spices of genus *Bacillus* also produce a variety of biologically active compounds such as surfactin and fengycin lipopeptides (Sen, 2010, Mnif and Ghribi 2015). However, ability of an isolate to produce wide verity of metabolites is not sufficient to be a successful biocontrol agent. Indeed, efficient colonization and formation of biofilm in rhizosphere are essential prerequisites for efficacy of a biocontrol agent (Bianciotto *et al.* 2001).

In this study, we considered two criteria for screening of rhizobacteria to find out efficient biocontrol agents against *R. solani*. First, isolated rhizobacteria were assessed in terms of their ability to inhibit *R. solani* by dual culture technique, and second, the selected isolates were further investigated to find out robust biofilm forming isolates. Dual culture bioassay had been used as a basic procedure in numerous screens for antagonistic agents (Ahmed Idris *et al.*, Shirzad *et al.* 2012, Weller *et al.* 1985, Hagedorn *et al.* 1998, Ahmadzadeh *et al.* 2009). However, in some works, there was no clear correlation between the results of this assay and in situ biological control results (Dufour *et al.* 2010, Shirzad *et al.* 2012, Reddy *et al.* 1993). This lack of correlation might be due to variable ability of different rhizobacteria to produce antimicrobial metabolites in different environmental conditions and their ability to colonize plant roots and

form biofilm (Shirzad *et al*. 2012; Chen *et al.* 2013).

Different methods were used to screen rhizobacteria with high ability of biofilm formation *in vitro* condition. Results from these bioassays were similar in most cases but type of media used in the PF bioassay influenced the final results as in MSNG medium (minimal medium with glycerol), none of the isolates could form a robust pellicle. In MSNGP medium (minimal medium with glycerol and pectin) number of isolates that could form a robust pellicle was 14. This number is twice more than the number in LBMG (LB medium with manganese and glycerol). It seems that presence of glycerol and pectin had inducing effect on isolates to produce strong biofilms. These results are in accordance with previous findings that some minerals or molecules from plants act as chemical cues that are perceived by bacterial cells and induce biofilm formation (Shemesh and Chai 2013, Beauregard *et al.* 2013). Beauregard *et al*., (Beauregard *et al.* 2013) believed that the capacity to form biofilms when in contact with plant polysaccharides (such as pectin) could be an advantageous trait for plant growth-promoting bacteria, serving to enhance colonization of the roots. Therefore, for efficient screening of biofilm forming rhizobacteria, using media with similar composition to that of rhizosphere seems to be indispensable. The results of colonization study of tomato roots by some of the strains corroborant this notion as isolates N168, N95 and N94 that were robust biofilm forming isolates in MSNGP, could efficiently colonize plant roots but the population of isolates N100 and N87 (none-biofilm forming isolates) on tomato roots were lower than 6 $\log_{10}$ CFU g^{-1} root. However, the results of the pathogenicity system with *R. solani* damping off of tomato showed that, biofilm formation and colonization ability of these isolates does not leave a significant impact on their biocontrol activity as isolate N100 was one of the most effective isolates against this disease. One explanation for this observation is that the population level of this isolate in this pathosystem is sufficient to suppress the disease.

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