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Inhibitory effects of antagonistic bacteria inhabiting the rhizosphere of the sugarbeet plants, on *Cercospora beticola* Sacc., the causal agent of Cercospora leaf spot disease on sugarbeet

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Abstract: In the present study, the antagonistic capability of bacterial agents inhabiting the rhizosphere of sugarbeet plants were evaluated against *Cercospora beticola* Sacc. under laboratory and greenhouse conditions. After preliminary screening using the dual culture method, 14 strains with higher antagonistic capability were selected for further inhibitory assays against *C. beticola*. Bacterial strains were identified based on the sequence data of the small subunit-rDNA (SSU-rDNA) gene. Based on the SSU sequence data, the identity of bacterial strains were determined as *Bacillus* (10 strains: RB1, RB2, RB3, RB4, RB5, RB6, RB7, RB8, RB9, RB10), *Paenibacillus* (two strains: RP1, RP2), *Enterobacter* (one strain: RE), and *Pseudomonas* (one strain: RPs). The results obtained in this study showed that in all of the assays (dual culture, volatile and non-volatile metabolites) bacterial antagonists significantly inhibited the growth of *C. beticola* compared to the control. *Bacillus* (RB2) showed the highest inhibition rate on *C. beticola* in all of the assays. Based on the results of the laboratory assays, three bacterial strains RB2 (*Bacillus*), RPs (*Pseudomonas*), and RE (*Paenibacillus*) were selected for greenhouse assays. The experiment was designed based on a completely randomised design (CRD) with the application of antagonists prior to, simultaneously, and after inoculation with *C. beticola* on sugarbeet leaves. The reduction in disease severity was evaluated seven days after inoculation. The results of greenhouse assays were consistent with the results of laboratory studies. The obtained results showed that bacterial antagonists significantly reduced the disease severity when compared to the control.

Key words: antagonist, *Bacillus*, Cercospora leaf spot, *Pseudomonas*, sugarbeet

Introduction

Cercospora leaf spot (CLS) disease, caused by *Cercospora beticola* Sacc. (Ascomycota, Capnodiales, Mycosphaerellaceae), is one of the most destructive foliar diseases of sugarbeet worldwide, especially in warm and humid areas (Holtschulte 2000; Weiland and Koch 2004). The disease reduces the photosynthetic capacity of plants as a consequence of necrotic leaf lesions, which results in reduced root yield and sugar content along with an increase in the concentration of impurities, leading to considerable economic losses (Shane and Teng 1992; Lartey 2003).

Disease control is mainly achieved through a combination of cultural practices, the use of resistant varieties and repeated applications of fungicides. Whereas these approaches are relatively effective in disease control, some of the approaches, such as resistant varieties, are not favoured by producers because of their lower agronomic properties (Weiland and Koch 2004; Galletti *et al.* 2008). Resistance to CLS in commercial sugarbeet varieties is polygenic and quantitative (Smith and Gaskill 1970; Smith 1985; Skaracis and Biancardi 2000). Varieties with a high degree of resistance generally possess a lower agronomic performance. Hence, many commercial varieties are only moderately resistant and applications of fungicides are indispensable, especially under favourable environmental conditions for disease progress (Weiland and Koch 2004). Application of chemical compounds for control of CLS disease involves the risk of selecting resistant strains of the pathogen and negative environmental drawbacks. The development of resistant strains can be reduced by rotating or alternating of fungicides with different modes of action or utilising a mixture of fungicides with different modes of action (Karaoglanidis *et al.* 2001). In recent years, efficient management of CLS disease has been achieved based upon the development of forecasting models. The result has been a reduction in the fungicide spray (Rossi and Battilani 1991; Racca *et al.* 2002; Wolf and Verreet 2002; Weiland and Koch 2004).

Application of biological agents is an alternative strategy to the chemical control of CLS. Biological control of plant pathogens by microorganisms, in the long term, is a suitable replacement for pesticides. It is known that pesticides are often expensive, have a cumulative effect on plants, and can have harmful effects on humans. These

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chemical compounds can also have lethal effects on useful, living, soil organisms (Leroux 2003; Cohn *et al.* 2007).

Biocontrol agents can be effective through the production of hydrolytic enzymes and antibiotics, niche colonisation and competition for host nutrients, induction of plant host defence mechanisms, and interference with pathogenicity factors (Punja and Utkhede 2003; Arzanlou *et al.* 2014). Species of *Bacillus*, *Pseudomonas*, and *Paenibacillus* have an inhibitory effect on a wide range of phytopathogenic fungi, using diverse ranges of mechanisms including the production of hydrolytic enzymes and antibiotics, niche colonisation and competition for host nutrients, induction of plant host defence systems, and interference with pathogenicity factors (Kloepper *et al.* 2004; McSpadden Gardener 2004; Ongena *et al.* 2007). For instance, repeated applications of *Bacillus mycoides* reduced Cercospora leaf spot symptoms under greenhouse and field conditions, due to elicitation of systemic resistance (Bargabus *et al.* 2002).

There have been limited studies on the capability for biological control agents in regards to *C. beticola* (Lartey 2003). The present study was aimed at evaluating the inhibitory capability of bacterial antagonists inhabiting the rhizosphere of the sugarbeet against *C. beticola*, the causal agent of Cercospora leaf spot disease on sugarbeet.

Materials and Methods

Pathogen isolates

Two *C. beticola* isolates, namely M1 and M2, used in this study were obtained from Culture Collection of Tabriz University (CCTU), Iran.

Isolation of antagonist bacteria from soil

For this purpose, soil samples from the rhizosphere of healthy and diseased sugarbeet plants were collected from sugarbeet fields in the Moghan region (Ardabil, province, Iran). Soil samples from each field were mixed separately. One gram of soil from each field was poured into the test tube containing 9 ml of sterile distilled water. The dilution series were prepared in a solution of 1% Peptone. The dilution series of 10^4–10^6 were cultivated on plates containing 20 g · l^{-1} of NA (Nutrient Agar, Merck, Hamburg, Germany) culture medium. Incubation was done for 48 h at 27°C for bacterial growth. Single colonies were selected based on differences in colour, shape, size, and margin of colonies, then streaked on nutrient agar plates. A loop full of 24-h bacterial culture was removed and transferred to the micro-tubes containing sterile distilled water and kept at 4°C.

Selection of antagonistic bacterial strains

The inhibitory capability of 44 bacterial strains isolated in this study, were evaluated against two isolates of *C. beticola*, using the four-point test (Weller and Cook 1983). Four different bacterial strains were point inoculated at a 1-cm distance from the edge of the Petri dishes, where the strains were at equal distance from each other. The plates were incubated at 25°C for 4 days for sufficient growth and metabolite production by bacterial strains. Then a 5-mm pellet of *C. beticola* isolate was placed in the centre of the plate. The bacterial strains which showed inhibitory activity were selected for the next stage.

Molecular characterisation of superior antagonistic bacterial strains

Bacterial strains were identified using sequence data of 16s rDNA locus at genus level. For this purpose bacterial isolates were grown on NA for 24 h at 25°C. Genomic DNA was extracted following the protocol proposed by Arabi *et al.* (2006). The primer set of 8f/1942r was used to amplify the 16s rDNA. The polymerase chain reactions (PCR) were performed in a total volume of 40 µl. The reaction mixtures contained 5–10 ng genomic DNA, 1X PCR buffer 1.5 mM $MgCl_2$, 25 µM each of dNTP, 0.3 µM of each primer, and 1 Unit GoTaq® Flexi DNA polymerase (Promega, Madison, WI, USA). The polymerase chain reaction condition consisted of an initial denaturation step of 90 s at 94°C followed by 35 cycles of 30 s at 94°C, 90 s at 60°C and 3 min at 72°C and final elongation step of 10 min at 72°C. Following the PCR amplification, amplicons were visualised on a 1.2% agarose gel stained with GelRed™ Biotium (Hayward, CA, USA) and viewed under UV light. The sizes of amplicons were determined against a HyperLadder™ I molecular marker (Bioline). The ABI Prism BigDye® Terminator Cycle sequencing reaction kit v. 3.1 (Applied Biosystems™, Foster City, CA, USA) was used for the sequencing of PCR products in both directions with the same primer pairs used for amplification, following the manufacturer's instructions. Sequencing products were analysed on an ABI Prism 3730XL Automated DNA analyser (Life Technologies Europe BV, Applied Biosystems™, Bleiswijk, The Netherlands). The sequences were subjected to a nucleotide Blast search at NCBI's GenBank nucleotide database. Sequences with high similarity were obtained and aligned together with the sequence obtained in this study. Sequence alignment was carried out by using the ClustalW algorithm implemented in MEGA V. 6 (Tamura *et al.* 2013). Phylogenetic analysis was performed using the maximum likelihood method with program default settings in MEGA V. 6. Bootstrap analysis was performed with 1,000 replicates.

Antagonistic effect of bacterial strains against *Cercospora beticola*

Dual culture assay

A 5-mm diameter block of the *Cercospora* culture was placed at a distance of 1-cm from the edge of the Petri dishes containing Potato Dextrose Agar (PDA) culture medium. Incubation took place at 25°C for 48 h. After this, using a sterile loop, half of the Petri dishes were inoculated with a suspension of bacteria. In the control treatment, sterile distilled water was used instead of bacterial suspension. Inhibition rates were evaluated after 14 days (Weller and Cook 1983). The experiment was carried out based on a completely randomised design (CRD) with three replicates for each treatment. The percentage

inhibition of radial growth (PIRG) was calculated using the following formula:

$$PGI\ (\%) = KR - R1/KR \times 100,$$

where: *PGI* – percentage growth inhibition, *KR* – radial growth of fungal colony in the control (mm), and *R1* – the distance of fungal growth from the point of inoculation to the colony margin on the treated dishes in the direction of the antagonist (Korsten *et al.* 1995).

Assay of non-volatile, agar-diffusible metabolites

The inhibitory capability of non-volatile, agar-diffusible metabolites produced by bacterial strains against *C. beticola* was evaluated according to the protocol of Kraus and Loper (1990). A suspension of 10^7 cells per · ml^{-1} was prepared from young cultures of bacterial strains. Then, 200 µl of bacterial suspension was transferred on PDA + + NA (50/50) and spread evenly by sterile Pasteur pipette. The plates were inoculated at 27°C for 72 h. For the control plate, the same amount of sterile distilled water was spread on the surface of the culture medium. Bacterial colonies were washed up from the surface of the medium, with the use of a glass rod and sterile distilled water. A cotton ball soaked in chloroform was placed on the surface of a plate for 30 min. After 30 min, the cotton was removed and a 5-mm diameter block of young *C. beticola* culture was placed in the centre of each plate. The plates were incubated at 25°C for eight days. The design of the experiment was the same as the dual culture assay section. The percentage inhibition of PIRG was calculated using the aforementioned formula.

Assay of volatile cellular metabolites

The assay for volatile cellular metabolites of antagonist was performed according to the Fiddaman and Rossall (1993) procedure. For this purpose, a suspension of 10^7 cells · ml^{-1} was prepared from 48-h old cultures of bacterial strains in sterile distilled water. A loop full of bacterial suspension was spread on NA medium containing 2% glucose and plates were incubated at 25°C for 24 h. A 5-mm diameter block of young *C. beticola* culture was placed at the centre of Petri dishes containing PDA. Under sterile condition, the lids of the plates (both antagonist and pathogen) were removed. The plate containing *Cercospora* colony was inversely placed on the plate containing the bacterial antagonist colony. For the control, sterile distilled water was used instead of the bacterial suspension. Two plates were sealed by parafilm at the joining point. The plates were incubated at 25°C in darkness. The design of the experiment was the same as that in the dual culture assay section. A calculation was done of the PIRG, using the above-mentioned formula.

Protease production test

Protease production capability of bacterial antagonists were evaluated according to the method recommended by Chantawannakul *et al*. (2002) using SMA (2% agar, 10% sodium azide and 0.2% skim milk powder in one litre of sterile distilled water). Skim milk was tyndallized (put in boiling water for 25–30 min on three consecutive days within 24 h) and mixed with autoclaved NA in sterile conditions, and poured into 8-cm plates. The bacterial strains were point inoculated on solidified media. The plates were incubated for 48 h at 27°C. Formation of a colourless halo around the colonies during this period was considered as the indication for the activity of protease.

Greenhouse assay

The experiment was carried out in greenhouses at 25±3°C, 70–80% relative humidity (RH) and under natural sunlight. Sugarbeet plants (cv Rasoul) were grown in plastic pots containing pasteurised greenhouse soil at 22 to 24°C. Plants were inoculated with the bacterial strain RB2, 90 days following planting using three methods: 1) simultaneous inoculation of antagonist and pathogen, 2) inoculation of antagonist 24 h prior to inoculation of pathogen, and 3) inoculation of pathogen 48 h prior to inoculation of antagonist. For the control treatments, sugarbeet plants were inoculated with sterile water, *C. beticola* and antagonists separately.

Inoculum production for bacterial antagonist

The bacterial strains were cultured in flasks containing Luria Broth. Shaking was done at 150 rpm for 24 h. The optical density (OD) of these suspensions was recorded by the light absorbance at 600 nm wavelength (OD = 600) using an ultraviolet-visible (UV-vis) spectrophotometer and cell density was adjusted to 1×10^7 cell per ml. The suspension was sprayed uniformly over the sugarbeet leaves.

Inoculum production for Cercospora beticola

Cercospora beticola (M1) isolate was cultured on V8 plates and incubated at 25°C for 2 weeks. Under sterile conditions, sterile distilled water was added to the surface of each colony and conidia were harvested using an L-form glass rode. The obtained spore suspension was filtered through a sterile cheesecloth and spore concentration was adjusted to 2×10^4 spores per · ml^{-1}. Then 0.2% of Tween 80 was added to the suspension. The spore suspension was sprayed uniformly over the sugarbeet leaves.

Disease assessment and statistical analysis

The results of the greenhouse assays were assessed 7 days after inoculation. The disease severity was recorded as the infection percentage in leaf area. Towards this aim, the leaf area index was determined by a two-step measurement of the total leaf area and percent necrotic area. Then the difference between these two values was calculated. The basic design was completely randomised. The comparison of the means was performed using Duncan's multiple range test at the level of one percent. The statistical data was analysed by SPSS and the graphs were drawn using Excel.

Results

Isolation and selection of antagonistic bacterial strains

A total number of 44 bacterial strains were recovered from the rhizosphere of healthy and diseased sugarbeet plants collected from sugarbeet fields in the Moghan region. Based on the results of a dual culture assay, 14 strains were selected for further inhibitory capability on *C. beticola* (Table 1).

Molecular characterisation of superior antagonistic bacterial strains

The identity of bacterial strains was confirmed using the sequence data of 16s rDNA locus. Approximately 1,400 bp was obtained for 16s rRNA. The alignment file included 32 ingroup sequences (including 11 taxa from this study and 21 taxa from NCBI) with a total of 1,363 characters (including alignment gaps) (Table 1). Foxtail yellow decline phytoplasma (GenBank accession KC751560) was used as an outgroup. The phylogeny inferred using the sequence data obtained in this study together with the sequence data from GenBank, clustered our strains in four genera: *Bacillus*, *Paenibacillus*, *Enterobacter*, and *Pseudomonas* (Fig. 1). The majority of the strains *viz.*, RB1, RB2, RB3,

Table 1. Antagonistic bacterial strains recovered from sugarbeet plants (rhizosphere) in the Moghan region (Ardabil province, Iran) in this study

Strain code	Bacteria name
RB1; CCUT252	*Bacillus* sp.
RB2; CCUT258	*Bacillus* sp.
RB3; CCUT251	*Bacillus* sp.
RB4; CCUT245	*Bacillus* sp.
RB5; CCUT249	*Bacillus* sp.
RB6; CCUT247	*Bacillus* sp.
RB7; CCUT246	*Bacillus* sp
RB8; CCUT257	*Bacillus* sp.
RB9; CCUT250	*Bacillus* sp.
RB10; CCUT253	*Bacillus* sp.
RP1; CCUT256	*Paenibacillus* sp.
RP2; CCUT259	*Paenibacillus* sp.
RPs; CCUT255	*Pseudomonas* sp.
RE; CCUT248	*Enterobacter* sp.

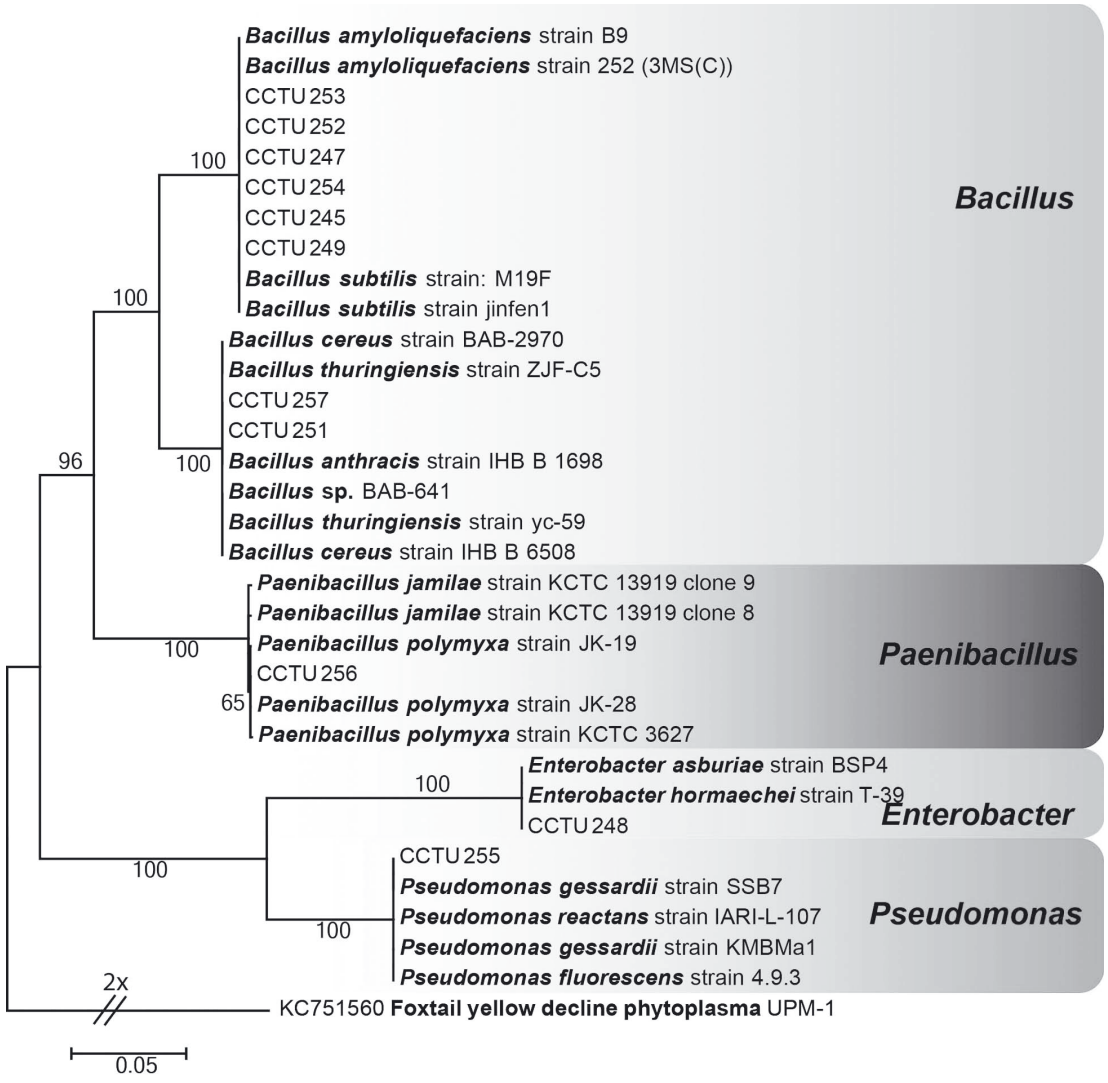

Fig. 1. A neighbour-joining phylogenetic tree obtained from the 16s rRNA gene sequence data. Bootstrap support values from 1,000 replicates are indicated at the nodes. The tree was rooted to Foxtail yellow decline phytoplasma (GenBank accession KC751560). The scale bar indicates 0.05 substitutions per site

RB4, RB5, RB6, RB7, RB8, RB9, RB10, were identified as members of the genus *Bacillus* genus; two strains namely RP1 and RP2, were identified as *Paenibacillus*; the stain RPs as *Pseudomonas*, and the strain RE as *Enterobacter* genus (Table 1).

Inhibitory capability of antagonistic bacteria against *Cercospora beticola*, *in vitro*

Dual culture assay

The results of the dual culture of the antagonists and pathogen demonstrated that all of the bacterial strains had an antagonistic effect on *C. beticola*. The results produced by antagonists were significantly ($p \leq 0.05$) different from the control as well as within different treatments. For *C. beticola* (M1), the highest percentage of inhibition (60.86%) was induced by the bacterial strain RB2, and the lowest percentage of inhibition (18.42%) was produced by the bacterial strain RB7 (Table 2). For *C. beticola* (M2), the highest percentage of inhibition (70.87%) was created by the bacterial strain (RB2), and the lowest percentage of inhibition (20.76%) was for the bacterial strain RE (Table 3; Fig. 2).

Non-volatile compound

The results of this test showed that the highest and lowest antibiotic produced by strains RB2 and RB8 against isolates M2 were 89% and 56.36%, respectively. The highest and lowest antibiotic produced by the strains RB1 and RB8 against strains M1 were 84.4% and 47.33%, respectively (Tables 2 and 3).

Table 2. Inhibitory effect of bacterial isolates against *Cercospora beticola* (pathogen strain: M1), *in vitro*

Antagonist strains	Mean of inhibitory percentage			Protease
	dual culture	non-volatile compound	volatile compound	
RB1	58.45 ab	84.40 a	68.59 b	++
RB2	60.86 a	83.48 a	72.94 a	+++
RB3	25.00 gh	73.87 c	51.68 h	+
RB4	34.66 f	71.97c	43.95 k	+
RB5	26.51 gh	63.76 d	56.52 fg	++
RB6	20.45 hi	53.97 ef	65.21 c	++
RB7	18.42 i	52.65 g	55.50 g	++
RB8	42.98 de	47.33 h	57.48 f	–
RB9	48.78 cd	56.49 ef	60.86 e	+
RB10	36.23 f	66.17 d	46.37 j	++
RP1	35.74 f	58.93 e	51.68 h	++
RP2	41.00 ef	55.50 f	63.27 d	+++
RPs	53.13 bc	77.77 b	48.78 i	+
RE	28.49 g	63.76 d	64.72 d	–

Dissimilar letters in each column have a statistically significant difference ($p \leq 0.05$) at the level of 1%, using Duncan's test
+ (low), ++ (modarate), +++ (high) – the amount of enzyme produced by antagonist bacteria; "–" lack of the enzyme production by antagonist bacteria

Table 3. Inhibitory effect of bacterial isolates against *Cercospora beticola* (pathogen strain: M2), *in vitro*

Antagonist strains	Mean of inhibitory percentage			Protease
	dual culture	non-volatile compound	volatile compound	
RB1	63.63 b	86.00 b	73.32 a	++
RB2	70.87 a	89.00 a	74.54 a	+++
RB3	24.70 f	78.78 d	54.54 f	+
RB4	44.23 c	83.00 c	52.11 g	+
RB5	21.81 f	76.36 e	59.39 e	++
RB6	31.50 e	69.63 f	63.00 d	++
RB7	23.18 f	59.39 h	67.27 c	++
RB8	36.36 d	56.36 i	63.63 d	–
RB9	61.81 b	63.63 g	70.24 b	+
RB10	49.00 c	82.41 c	58.12 e	++
RP1	44.83 c	63.00 g	53.32 g	++
RP2	38.18 d	60.00 h	73.32 a	+++
RPs	64.84 b	81.20 c	58.18 e	+
RE	20.76 f	70.90 f	67.27 c	–

Dissimilar letters in each column have a statistically significant difference ($p \leq 0.05$) at the level of 1%, using Duncan's test
+ (low), ++ (modarate), +++ (high) – the amount of enzyme produced by antagonist bacteria; "–" lack of the enzyme production by antagonist bacteria

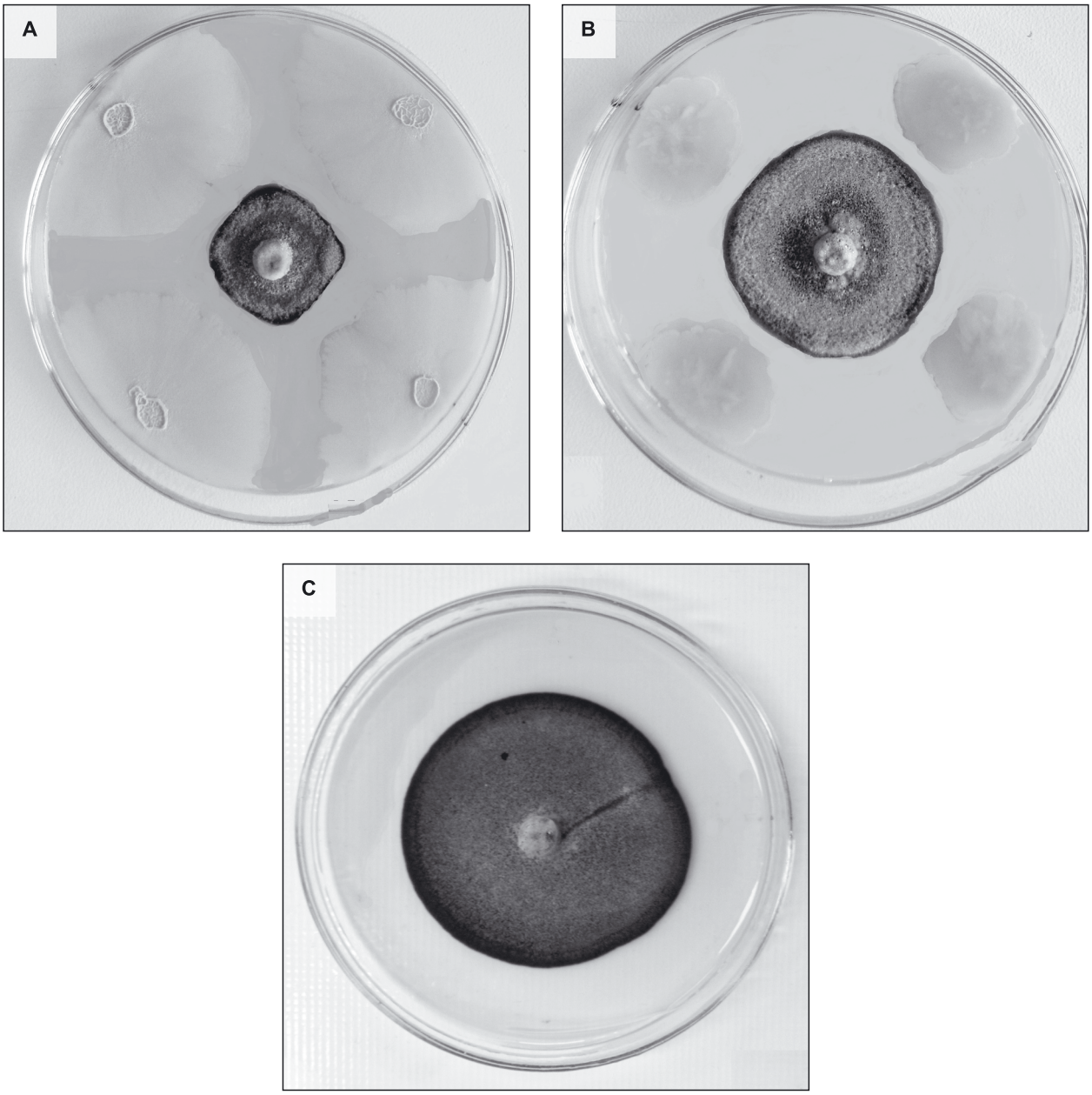

Fig. 2. Inhibitory effects of bacterial antagonists against *Cercospora beticola* in a dual culture assay: A – *Enterobacter* sp. – RE; B – *Bacillus* sp. – RB2; C – the control

Volatile compound

In this experiment, a significant difference was observed among the bacterial strains in production of volatile compounds. The highest and lowest inhibitory percentage against the *C. beticola* strain (M2) was induced by the strains RB2 (74.54%) and RB4 (52.11%), respectively. The results were the same for the *C. beticola* strain (M1); the highest and lowest inhibitory percentage was induced by RB2 (72.94%) and RB4 (43.95%), respectively (Tables 2 and 3).

Protease production

The protease production capability of bacterial strains is summarised in table 1. All of the bacterial strains were able to produce protease except *Bacillus* (RB8) and *Enterobacter* (RE). However, bacterial strains differed in protease production (Tables 2 and 3).

Greenhouse assay

All three methods of antagonist application resulted in significant disease control, compared to the plant inoculated with pathogen alone (Table 4). No disease was observed in the control plants and plants inoculated with the antagonist alone. Consequently, no data were obtained from these plants, and as a result, these treatments were excluded from the analysis. The obtained results revealed that inoculation of the antagonist prior to the pathogen, and co-inoculation of antagonist and pathogen, provided better disease control, compared to the application of the antagonist after the inoculation of plants with pathogen. As shown in table 4, there was a significant difference in the bioactivity of three antagonists against the same pathogen. The stain RB2 was the most efficient strain when it comes to disease control in all three application timings.

Table 4. Biocontrol of Cercospora leaf spot disease (pathogen strain: M2) using bacterial isolates, *in vivo*

Antagonist strains	Mean of inhibitory percentage		
	co-inoculation	antagonist–pathogen	pathogen–antagonist
RB2	96.00 a	96.76 a	79.98 a
RPs	92.11 b	93.53 b	64.78 b
RE	88.19 c	86.34 c	55.82 c

Dissimilar letters in each column have a statistically significant difference ($p \leq 0.05$) at the level of 1%, using Duncan's test

Discussion

The first aim of this study was to evaluate the inhibitory potential of antagonistic bacteria inhabiting rhizosphere of sugarbeet plants in the sugarbeet fields in the Moghan region against *C. beticola*. The second aim was to further asses the efficacy of superior strains on the disease control under greenhouse conditions. In the present study, 44 bacterial strains from the rhizosphere of healthy and *C. beticola*-infected were recovered sugarbeet plants. Numerous studies have demonstrated the ability of several antagonistic bacteria to suppress diseases caused by fungal plant pathogens (Weller and Cook 1983; Fridlender *et al.* 1993; Emmert and Handelsman 1999). However, in general, significant differences have been documented in antagonistic efficiency among various groups of biological control agents and even different strains of a single species. Hence, preliminary screening is an important initial step for the selection of efficient biological control agents for plant disease management. In the present study, we isolated sugarbeet rhizosphere associated bacteria, with the objective of selecting efficient antagonists against *C. beticola*. An *in vitro*, dual culture assay was taken as preliminary screening criterion for antagonism. The inhibition-zone area was taken as a measure of the antagonistic potential of the isolate (Anith *et al.* 2003). Preliminary screening of bacterial strains for their inhibitory potential against *C. beticola*, led to the selection of 14 bacterial strains with better inhibitory potential on the growth of *C. beticola.*

We further characterised the selected antagonistic bacteria strains based on sequence data from the 16srDNA gene. These isolates were identified as *Bacillus* (10 strains: RB1, RB2, RB3, RB4, RB5, RB6, RB7, RB8, RB9, RB10), *Paenibacillus* (two strains: RP1, RP2), *Enterobacter* (one strain: RE), and *Pseudomonas* (one strain: RPs). Members of the genus *Bacillus* represented the most dominant isolates among the 14 antagonistic bacteria which were selected based on the primary screening in this study. Antagonistic and plant growth-promoting bacteria comprise a highly heterogeneous assemblage, such as: *Pseudomonas*, *Azospirillum*, *Azotobacter*, *Klebsiella*, *Enterobacter*, *Alcaligens*, *Arthobacter*, *Burkholderia*, *Bacillus,* and *Serratia,* which can be found in the rhizosphere, at root surfaces, and in association with roots (Kloepper *et al.* 1989; Glick 1995). In this regard, isolates of *Pseudomonas* and *Bacillus* have been widely used for biological control of plant pathogens, and mechanisms involved in disease control have been studied in detail (Kloepper *et al.* 1989; Walker *et al.* 2001; McSpadden Gardener 2004; Zhang *et al*. 2004; Ongena *et al*. 2007).

The bacterial isolates screened in this study were identified at the genus level. As it can be seen in figure 1, 16srDNA gene sequence could not provide sufficient information to identify bacterial isolates at the species level. Different species belonging to the same genus clustered together in single, well-supported clades. Additional molecular markers along with biochemical tests should be supplemented for species identification.

In the present study, four genera (*Bacillus, Paenibacillus, Enterobacter,* and *Pseudomonas*) were isolated from the rhizophere of sugarbeet plants (Fig. 1). The results obtained in this study showed that in all of the assays (dual culture, volatile, and non-volatile metabolites) bacterial antagonists significantly inhibited the growth of *C. beticola* compared to the control. *Bacillus* (RB2) showed the highest inhibition rate on *C. beticola* in all of the assays. Species of *Bacillus*, *Pseudomonas*, and *Paenibacillus* have an inhibitory effect on a wide range of phytopathogenic fungi, using a diverse range of mechanisms including the production of hydrolytic enzymes and antibiotics, niche colonisation and competition for host nutrients, induction of plant host defence systems, and interference with pathogenicity factors (Raupach and Kloepper 2000; Lartey 2003; McSpadden Gardener 2004; Zhang *et al*. 2004; Ongena *et al*. 2007). The results obtained in this study showed, that most likely, a combination of mechanisms are involved in the biocontrol of *C. beticola*. As it can be seen from data presented on the production of volatile, non-volatile compounds, and extracellular secretion of hydrolytic enzymes including proteases, the strain *Bacillus*: RB2 was the most efficient strain against *C. beticola* among the bacterial antagonists examined in this study. However, further studies on isolation, purification, and characterisation of the bioactive compounds are required to figure out the principal component responsible for the inhibitory activities of these antagonists.

Based on the results of laboratory assays, three bacterial strains RB2 (*Bacillus*), RPs (*Pseudomonas*), and RE (*Paenibacillus*) were selected for greenhouse assays. Our results revealed that, in all cases, the inoculation of antagonist prior to pathogen, and a co-inoculation of antagonist and pathogen, provided better disease control, compare to the application of the antagonist after inoculation of plants with pathogen (Table 4). This finding is in agreement with the results obtained by other researchers (Kloepper *et al.* 2004). It has been shown, that several species of the genus *Bacillus* act as a biological inducer of host resistance against various groups of plant pathogenic fungi, bacteria, and viruses (Kloepper *et al.* 2004; Bargabus *et al.* 2002, 2004). The capability of the *Bacillus mycoides* BmJ isolate, in the

control of *Cercospora beticola* leaf spot disease on sugarbeet is through systemic acquired resistance, in which induction of pathogenesis related proteins and biphasic hydrogen peroxide production play a key role (Bargabus *et al.* 2002, 2004). The same condition has been seen in sugarbeet treatments with 203-6 and 203-7 isolates of *Bacillus pumilus* which led to a decrease in Cercospora leaf spot disease on sugarbeet (Bargabus *et al.* 2004). Collins and Jakobsen (2003) were able to control Cercospora leaf spot disease of sugarbeet using *B. subtilis* isolate BacB. Our results further emphasise the role of bacterial antagonists in the induction of plant host defence systems. As shown in table 4, there was a significant difference in the bioactivity of three antagonists against the same pathogen. This finding might indicate the possible role of other mechanisms responsible for the inhibitory activities of these antagonists.

The results obtained in this study clearly demonstrated the potential of antagonistic bacterial species to be used in the control of Cercospora leaf spot disease on sugarbeet. With this study, we screened antagonistic capacity of bacterial isolates against *C. beticola* under laboratory conditions. We further assessed the potential of using superior isolates for disease control under greenhouse conditions. More studies on isolation, purification, and characterisation of the bioactive compounds are required to figure out the principal component responsible for the inhibitory activities of these antagonists. The efficacy of antagonistic bacterial isolates in the control of Cercospora leaf spot disease under field conditions remains an interesting area of research for future studies.

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