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In vitro Antagonistic Activity of Diverse *Bacillus* Species Against *Cochliobolus sativus* (Common Root Rot) of Barley

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Antagonistic microorganisms have been used as biological control agents to reduce the use of chemical fungicides in the control of crop diseases. The present work was conducted to determine the antagonistic potential of *Bacillus sp*. isolates against the soil-borne fungus *Cochliobolus sativus,* the causal agent of barley common root (CRR) disease. Out of 525 isolates, 40 were showed *in vitro* antagonistic activity against the virulent *C*. *sativus* isolate CRR16. On the basis of 16S rRNA gene sequencing *Bacillus* sp. isolates are identified as *B*. *atrophaeus, B*. *subtilis, Paenibacillus polymyxa, B*. *amyloliquefaciens, B*. *simplex* and *B*. *tequilensis*. Results showed that *Bacillus sp*. had significant ($P < 0.05$) antagonistic activities against the *C*. *sativus* where the percentage of radial growth inhibition of the fungi colonies ranged from 59 to 92%, compared to the untreated control. The *B*. *subtilis* isolate SY41B had the highest inhibition effect on the vegetative growth zones as compared with the other species isolates. In view of these, our results indicate that the antagonistic effect of the *Bacillus sp*. isolates may be important contributors as a biocontrol approach that could be employed as a part of integrated CRR management system.

Keywords: *In vitro*, *Cochliobolus sativus*, *Bacillus sp*., antagonism.

Cochliobolus sativus (Ito and Kurib.) Drechsl. ex Dast. [anamorph: *Bipolaris sorokiniana* (Sacc. in Sorok.) Shoem.], a soil-borne fungal pathogen, causes common root rot (CRR) disease, is one of the most important root diseases of cereal plants such as barley, wheat and rye (Kumar, et al., 2002). The disease is considered economically important because it can cause significant reduction in yield and quality of the crop (Van Leur et al., 1997; Murray and Brennan, 2010). The global effect of CRR on crop production is highly variable and influenced by different factors such as host cultivars and competition with other micro-organisms (Jawhar and Arabi, 2007; Fernandez et al., 2009).

Although the production of conidia is predictable to product genetically similar clones, the high rate of emerging new *C*. *sativus* pathotypes with the capacity to infect previously resistant cultivars of barley, suggests this fungus may have high mutation rates in virulence genes, which determine race (Xu et al., 2018; Arabi et al., 2019). However, fungicides are commonly used to control the CRR diseases, but frequent uses of these chemicals are hazardous to humans and environment (Bailey et al., 1997). Therefore, management of the soil borne pathogen has become one of the major concerns in agricul-

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ture and focused on searching and selecting antagonist micro-organisms on diverse soil pathogens (Baysal-Gurel and Kabir, 2018).

Soil-borne fungal pathogens showed varying metabolic responses, growth patterns and reproductive strategies in response to varying soil micro-organisms (Duffy et al., 2003). Measuring *in vitro* growth rate of fungi is considered as a simple and reliable method for evaluating the effect of an environmental variable on the growth of fungi, although this does not take into account changes in mycelia density (Subbarao et al., 1993).

The seeking for an environmental-friendlier disease management method has led to study bacterial agents with antagonistic capacities. The mechanisms depending on these bacterial antagonisms for plant pathogens comprise antibiosis, competition for nutrients or space, enhancement of root and plant growth, induction of plant resistance and/or inactivation of the pathogen's enzymes (Leelasuphakul et al., 2008; Arguelles-Arias et al., 2009). Therefore, biocontrol is the most accepted alternative method for plant disease management. Among the bio-control bacteria, *Bacillus* has become the bacterium of the choice for its versatility and capacity to contain a big number of plant pathogens in different environments. Various *Bacillus* isolates are identified for the control of diseases caused by phytopathogenic fungi (Cao et al., 2018).

On the other hand, microbial identification by sequencing of 16s rRNA gene is a common identification method of bacterial taxonomists used for a number of microorganisms through measuring DNA similarity among isolates (Goto et al., 2000; Callahan et al. 2019).

During a polyphasic experiments, more than 525 bacilli were isolated from different regions of Syria. In the present study, forty of them were taken into the 16S rRNA gene sequence analyses. However, apart from the description of the *C. sativus* fungus, little information is available on its antagonistic soil bacteria. Therefore, objective of our research was to investigate the *in vitro* antagonistic activity of diverse *Bacillus* species isolates against *C. sativus* with biological potential activity to control CRR disease.

Materials and Methods

Fungal isolate

The virulent isolate (*Cs* 16) of *C. sativus* (Arabi and Jawhar, 2002) was used in the experiments. The infected tissues with *Cs* 16 were cut into small pieces (5 mm long) and surface sterilized in 5% sodium hypochlorite (NaOCl) for 5 min. After three washings with sterile distilled water, the pieces were transferred to Petri dishes containing potato dextrose agar (PDA, DIFCO, Detroit, MI. USA) amended with 13 mg/l kanamycin sulphate, and incubated for 10 days at 22 ± 1 ºC in the dark.

Bacterial isolates

Soil samples were randomly collected from different regions of Syria. They were taken from 3–4 cm depth and collected in sterile polythene bag. The samples stored at 4 °C. Bacterial isolation was performed as described previously by Ammouneh et al. (2011). From nutrient broth (NB) culture, the colonies of prospective *Bacillus* sp. were

Table 1

Bacillus species used in the study

Bacillus Species	Number of isolates	Morphology
atrophaeus	3	Brown-black, opaque, smooth, circular
amyloliquefaciens	10	Creamy white with irregular margins
polymyxa	2	Milky white, thin often with amoeboid spreading
subtilis	20	Fuzzy white, opaque, rough, with jagged edges
simplex	1	Cream, gloss, with irregular margins slightly raised
tequilensis	4	Yellowish, opaque, smooth, circular

identified according to Wulff et al. (2002), and the results are presented in (Table 1). Six *Bacillus* species, namely, *atrophaeus, subtilis, P. polymyxa, amyloliquefaciens, simplex* and *tequilensis*, were selected for the further *in vitro* study. A pure culture of each *Bacillus* sp. isolates was first grown on the nutrient broth (NB) and incubated for 24 h at 37 °C.

In vitro *evaluation of antagonism*

A total of 525 *Bacillus* isolates were screened on the bases of fungal growth inhibition. Bacterial isolates were streaked as thick bands on four opposite edges on the NA plates. Then 5 mm diameter disc of *C. sativus* fungus was cut from of an actively growing culture by a sterile cork borer and placed onto the center of above NA plates. The Petri dishes were sealed by parafilm and incubated 21 ± 1 °C in dark for 24 hours. Where mycelia disc on NA medium without bacteria was maintained as control. The above procedure was carried out to 40 isolates represent the six *Bacillus* species, and antagonistic effect showed by bacteria was measured as zone of inhibition according to Foysal et al. (2001). The experiment was conducted in three replicates.

The percent inhibition on growth of the *C. sativus* was calculated by using the formula given below by Rabindran and Vidhyasekaran (1996).

$I = (C\text{-}T)/C \times 100$

Where; I = Percent inhibition, C = Radial growth of the pathogen in control, and T = Radial growth of the pathogen in Treatment.

16S rRNA gene sequencing

Selected bacterial 16S rRNA was amplified in full length by PCR using two primers BacF (5'-GTGCCTAATACATGCAAGTC-3') and BcaR (5'-CTTTACGCCCAATAATTCC-3') flanking a highly variable sequence region of 545 bp towards the 5'end of the 16S rDNA region were used (Nair et al., 2002). The PCR reaction mix (50 µl) contained 2 µl (50-100 ng) of extracted genomic DNA, 1x reaction buffer (TrisKCl-MgCl2), 2 mM MgCl2, 0.2 mM dNTP, 1 µM of each primer, and Taq polymerase (5U/µl, Fermentas). PCR amplification condition was achieved using the following parameters: An initial denaturation step at 95 °C for 5 min followed by a second denaturation step at 95 °C for 1 min, annealing for 1 min at 54 °C, an extension at 72 °C for 90 s, and a final extension step of 72 °C for 10 min. A total of 30 serial cycles of amplification reaction was performed. PCR products were separated on a 1.5% agarose gel and visualized using UV light (302 nm) after staining with ethidium bromide. Prior to sequencing, PCR products were purified with QIAgen gel

extraction kit (28704) according to the manufacturer´s recommendations. Sequencing was carried out on a Genetic Analyzer (ABI 310, Perklin-elmer, Applied Biosystems, USA). The 16S rRNA sequences were compared with the known sequences using the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih. gov).

Statistical analysis

All experiments were performed in triplicat with ten Petri dishes per replicate, for each bacterium-fungus *in vitro* evaluation, using a completely randomized design. An F-test was used to determine if the two runs of each experiment was homogeneous and if the data could be pooled. The homogeneity of variance test indicated that the data from both runs of each experiment could be pooled, and thus all further analysis were conducted on pooled data. Data were analyzed using analysis of variance (ANOVA) and means were separated by Tukey's test ($P \leq 0.05$).

Results and Discussion

In this present work, antagonistic potential of the *Bacillus sp*. isolates was concluded and validated by restriction of the *C*. *sativus* pathogen growth and showed zone of inhibition towards the antagonist as shown in photo-plate of NA culture plate assay comparing with the control (Fig. 1). The data showed that *Bacillus* sp isolates had a significant ($P < 0.05$) antagonistic activity against the *C*. *sativus* where the percentage of radial growth inhibition of the fungi colonies ranged from 59 to 92.3%, compared to the untreated control (Table 2). Petri dishes of the controls of *C*. *sativus* isolate were completely covered by the growth of the fungi at the fourth day post-inoculation.

On the other hand, in the present study, PCR amplification with specific primers Bac yielded single DNA fragments of ~ 545 bp, present in all *Bacillus sp*. isolates (Fig. 2). On the basis of 16S rRNA gene sequencing *Bacillus* sp. isolates are identified as *B*. *atrophaeus, B*. *subtilis, P. polymyxa, B*. *amyloliquefaciens, B*. *simplex* and *B*. *tequilensis* as their 16S rRNA gene sequences displayed similarities ≤ 98% to their closely related type strains. The nucleotide sequences were deposited in GenBank under accession numbers MT159352 to MT159391 (Table 2). BLAST analyses based on 16S rRNA gene sequences revealed that the majority of the selected *Bacillus* spp. strains belong to the groups of *B*. *subtilis*. However, the *B*. *atrophaeus* sequence shared 96% identity with the type strain of *B*. *atrophaeus*, ATCC 49337. *B*. *simplex* sequence shared 97% identity with *B*. *simplex JP44SK12 (JX144702)*. *B*. *subtilis* sequence shared 99% identity with *B*. *subtilis subsp*. *Spizizenii JP44SK23* (*JX144713*). *P. polymyxa* sequence shared 98% identity with *B*. *paenibacillus macqariensis subsp*. *Defensor* (AB360546). *B*. *amyloliquefaciens* sequence shared 99% identity with *B*. *amyloliquefaciens* (AF478077). *B*. *tequilensis* sequence shared 98% identity with *B*. *Bacillus tequilensis* (KT760402).

Our results are similar to those obtained by Schisler et al. (2004) and Cao et al. (2018) who found a high capacity of some strains of *Bacillus sp*. of the same species to inhibit the growth of several phytopathogens fungi, this effect was attributed to the production and secretion of antifungal compounds and antibiotics belonging to the family of iturins and subtilins, that act on the fungi's cell wall. Lyon and Muir (2003) reported that

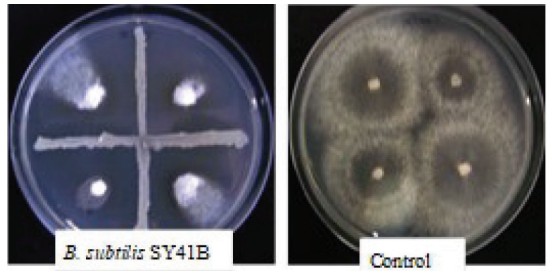

Fig. 1. *Bacillus subtilis* SY41B showing zone of inhibition in the NA culture plate assay

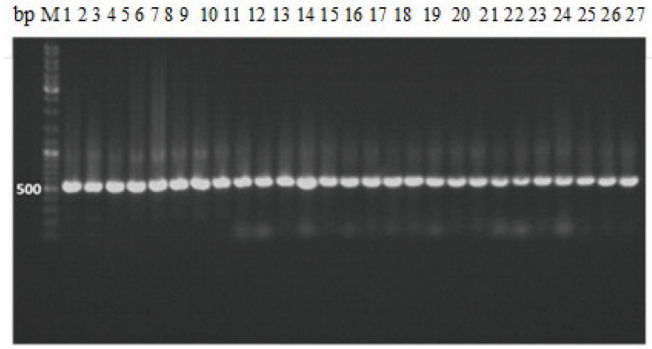

Fig. 2. Agarose gel electrophoresis of 16S rRNA of some *Bacillus sp*. isolates used in the study. M represents the 100-bp DNA marker *(Hin*fI; MBI Fermentas, York, UK)

the production of hydrophilic enzymes to break down polysaccharides, nucleic acids and lipids might have been also involved. Hence the most likely explanation for the growth reduction of *C. sativus* by *Bacillus sp*. is that antifungal activity is increased by co-culturing of different bacterial species.

The data demonstrated that The *B. subtilis* isolate SY41B had the highest inhibition effect on the vegetative growth zones as compared with the other species isolates, whereas, the isolate *P. polymyxa* SY53C had the lowest 59% antagonistic effect (Table 2). This data suggest that this antagonistic potential of the isolate can be considered as a viable biological control agent against soil pathogens such *C. sativus*. *B. subtilis* isolates showed similar antagonism against *Rhizoctonia solani*, *Helminthosporium* spp., *Alternaria* spp. and *Fusarium oxysporum* (Matar et al., 2009). *B. subtilis* strains produce a wide spectrum of antimicrobial compounds, including predominantly peptides as well as a couple of non-peptidic compounds such as polyketides, an aminosugar, and a phospholipid (Stein, 2005) , and its highly antifungal effects on *C. sativus* in this study might have been due to one or more antifungal compounds produced by this biocontrol agent.

It is apparent that *Bacillus sp*. isolates play an important role in inhibition of growth of *C. sativus*, and that the *B. subtilis* isolate SY41B had the highest biological potential activity against this fungus. The suppressiveness of *C. sativus in vitro* might suggest that this isolate can be considered as a viable biological control agent against soil pathogens. However, as conditions in soils are different from those of NA agar media, the present work needs to be followed up in a more natural environment involving roots and soil.

Table 2
List of the *Bacillus* isolates showing antagonistic activity against *C. sativus*

No.	Isolates	Antifungal activity	Zone of Inhibition (%)	GenBank accession numbers
	B. atrophaeus			
1	SY15B	+ +	67.3e*	MT159352
2	SY199A	+ + +	80.4c	MT159353
3	SY63E	+ +	69.2e	MT159354
	B. subtilis			
4	SY35A	+ +	71.2e	MT159355
5	Sy41B	+ + +	92.3a	MT159356
6	SY44A	+ + +	76.9d	MT159357
7	SY60A	+ + +	88.5a	MT159358
8	SY73B	+ +	65.4ef	MT159359
9	SY113C	+ +	73.1d	MT159360
10	SY116C	+ + +	88.5a	MT159361
11	SY118C	+ + +	88.5a	MT159362
12	SY124B	+ + +	84.6bc	MT159363
13	SY130D	+ +	75.0d	MT159364
14	SY132E	+ + +	79.5c	MT159365
15	SY133	+ + +	81.2c	MT159366
17	SY132C	+ + +	81.4c	MT159367
19	SY134D	+ + +	80.7c	MT159368
20	SY135D	+ + +	79.0c	MT159369
21	SY139D	+ + +	84.0bc	MT159370
22	SY151C	+ +	72.2e	MT159371
23	SY160C	+ +	68.0e	MT159372
24	SY168C	+ + +	77.3cd	MT159373
25	SY190E	+ +	60.0g	MT159374
	Paenibacillus polymyxa			
24	SY53C	+	59.0g	MT159375
25	SY55B	+ +	65.7ef	MT159376
	B. amyloliquefaciens			
26	SY82C	+ + +	80.8c	MT159377
27	SY96C	+ + +	84.2bc	MT159378
28	SY96E	+ + +	82.1c	MT159379
29	SY123A	+ + +	80.8c	MT159380
30	SY128B	+ +	75.0cd	MT159381
31	SY134C	+ + +	79.8c	MT159382
32	SY159D	+ + +	80.0c	MT159383
33	SY177C	+ +	69.0e	MT159384
34	SY190D	+ + +	86.1b	MT159385
35	SY200D	+ + +	86.1b	MT159386
	B. tequilensis			
36	SY69A	+ +	71.2e	MT159387
37	SY145D	+ + +	78.7c	MT159388

Table 2 cont.

No.	Isolates	Antifungal activity	Zone of Inhibition (%)	GenBank accession numbers
38	SY149C	+ +	68.0e	MT159389
39	SY150D	+ +	73.8d	MT159390
	B. simplex			
40	SY198B	+ +	70.0e	MT159391

Zone of Inhibition = (Radial growth of the pathogen in control – Radial growth of pathogen in treatment) / C × 100
Weak inhibition: + (Fungal growth was slightly inhibited by bacteria)
Average inhibition: + + (Loosely arranged mycelial growth over the bacterial zone)
Strong inhibition: + + + (Fungal growth was completely inhibited before the bacterial zone)
Values followed by different letters are significantly different at $P < 0.05$
The average of the values determined ± standard deviation

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