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Antagonistic comparative efficacy of Bacillus species against different soilborne fungal pathogens

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Abstract

Soilborne fungal pathogens constitute an emerging threat to global food security. Many of the currently available chemical fungicides are highly toxic and extended environmental contamination. Therefore, biological control has been considered a viable alternative method to chemical control. In this study, out of 525 *Bacillus* isolates, 40 representative six species; *atrophaeus*, *amyloliquefaciens*, *polymyxa*, *subtilis*, *simplex* and *tequilensis* were compared for their *in vitro* antagonistic activity against four different soilborne fungal pathogens i.e. *Cochliobolus sativus*, *Pyrenophora graminea*, *Fusarium culmorum* and *F. solani*. Data showed that all *Bacillus* spp. isolates demonstrated different levels of antagonistic effect against the tested pathogens as compared with the controls. All *Bacillus* species had a higher antagonistic effect towards *P. graminea* (76.52%) and *C. sativus* (72.73%) as compared with the two *Fusarium* species *F. culmorum* (49.49%) and *solani* (57.32%) that are mycotoxins producers. Moreover, *B. atrophaeus*, *B. amyloliquefaciens*, *B. subtilis* and *B. tequilensis* provided the most noteworthy result as they strongly inhibited mycelial growth in comparing with *B. polymyxa* and *B. simplex*. Importantly, the *B. tequilensis* isolate (SY145D) had the highest antagonistic activity against the four fungal pathogens. The present results showed that the tested *Bacillus* spp., possess a broad spectrum of antifungal activities against different soil fungal pathogens. These *in vitro* antagonistic effects could be a strategic approach to control soil filamentous fungi.

Keywords: Antagonistic effect; Bacillus species; filamentous fungi; *in vitro*

1. Introduction

Soilborne fungal pathogens are among the most important factors that cause considerable loss of crop yields worldwide. Some of the most common plant-pathogenic fungi belong to the genera *Cochliobolus*, *Pyrenophora* and *Fusarium*. Control of these pathogens considered complicated due to a long period of persistence of their resting structures in the field and a broad host range of some species, and the difficulty of their manage once they reaches the vascular plant tissue since fungicides appear to be ineffective [1, 2].

Until now, suppression of these soilborne pathogens mainly relied on chemical fungicide. However, controlling just one of them might not fully solve the problem. Combinations of different fungicidal treatments are possible but not always desired due to their negative impact on the environment and human health.

Biocontrol of plant fungal pathogens has been considered a viable alternative approach to chemical control. Many biocontrol agents were isolated by screening of the large number of soil or plant-associated microorganisms for antagonism against phytopathogens *in vitro* or in planta [3]. Among those, *Bacillus* species were attractive due to their unique ability to replicate rapidly, resistant to adverse environmental conditions as well as they have broad spectrum of biocontrol ability. Different *Bacillus* spp. were identified for the control of diseases caused by phytopathogenic fungi [4, 5, 6]. Several species belonging to this genus have been used as biocontrol against various plant fungal diseases such as on soil-borne wheat diseases [7], rice blast [8], *Fusarium* wilt of cucumber [9], and currently, we have used *Bacillus* spp. against common root rot disease of barley [10].

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The antagonistic activity of *Bacillus* is associated with the synthesis of various antimicrobial peptides, secreted enzymes, proteins and volatile organic compounds [11, 12].

The selection of *Bacillus* spp. as biological control agents usually starts with an *in vitro* screening of a collection of strains against selected pathogens by a nutrient broth (NB) culture assay, in which the candidate biological control agent is co-cultivated with the pathogen on agar medium and its antagonistic activity is quantified in terms of inhibition of pathogen's mycelium growth [13, 14].

However, considering the broad spectrum of *Bacillus* antagonists reported over the past decades, different and more efficient *Bacillus* species antagonists might be around waiting for discovery. In the present work, six *Bacillus* species; *atrophaeus, amyloliquefaciens, polymyxa, subtilis, simplex* and *tequilensis* were selected to study their *in vitro* antagonistic potential against four common and destructive soilborne fungal pathogens belonging to *different* genera i.e. *Cochliobolus sativus*, *Pyrenophora graminea*, *F. culmorum* and *F. solani*.

2. Materials and Method

2.1. Fungal isolates

The virulent isolate (*Cs* 16) of *C. sativus* [15], virulent *P. graminea* SY3 [16], and *F. culmorum* (*F3*) and *F. solani* (*F3*5) isolates [17] were used in the experiments. Isolates were incubated in Petri dishes containing potato dextrose agar (PDA, DIFCO, Detroit, MI, USA) supplemented with 13 mg/l kanamycin sulfate and incubated for 10 days at 20 ± 1 °C in the dark as we have described previously works [15, 16, 17].

2.2. Bacterial isolates

Soil samples were randomly collected from different regions of Syria. They were taken from 3-4 cm depth, collected in sterile polythene bag and stored at 4 °C. Bacterial isolation was performed as described previously by [18]. From nutrient broth (NB) culture, the colonies of prospective *Bacillus* sp. were identified according to [13], and the results are presented in Table 1. Six *Bacillus* species, namely, *atrophaeus, subtilis, 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 NB and incubated for 24 h at 37 °C.

2.3. 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 at 25 ± 1 °C in dark for 4 days. 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 the antagonistic effect showed by bacteria was measured as zone of inhibition (the distance between the edge of antagonistic bacterial growth and the edge of tested fungal isolates) according to [19]. Experiments were performed in triplicate.

The percentage of inhibition of radial growth (PIRG) was calculated by using the formula given below by [20]:

$$PIRG\ (\%) = (C-T)/C \times 100$$

where, C is the radial diameter of the control colony and T is the radial diameter of the treatment colony.

The PIRG was categorized on a growth inhibition category scale from 0 to 4, where 0 = no growth inhibition; 1 = 0-25% growth inhibition; 2 = 26-50% growth inhibition; 3 = 51-75% growth inhibition; 4 = 76-100% growth inhibition.

2.4. Statistical analysis

All experiments were conducted twice in triplicate, with ten Petri dishes per replicate, for each bacterium-fungus *in vitro* evaluation, using completely randomized designs. 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 ($P \leq 0.05$).

3. Results and Discussion

In this study, a collection of 40 isolates of six *Bacillus* spp. were used as a source for identification of isolates with antimicrobial activity against four soil borne pathogens in NA culture

tests. Comparative analysis of antagonistic activity of *Bacillus* spp. showed that the tested *Bacillus* spp. isolates had significantly different levels of antagonistic effect against the pathogenic organisms (Fig. 1). However, a considerable variation was observed between and within the fungal and bacterial antagonists with regard to the inhibition of pathogen growth. Of the four pathogenic fungi that were tested in the study, *P. graminea* proved to be more sensitive to *Bacillus* spp. Isolates (PIRG =76-100%), whereas, *F. culmorum* was less sensitive (PIRG =26-50%) (Fig. 1). No such changes were observed in control mycelia.

The mean values of the growth inhibition percent of the tested pathogen are shown in Table 1. The largest growth inhibition of *Bacillus* spp. value approximately (75.35%) was induced by the isolates of *B. amyloliquefaciens*, *B. tequilensis* and *B. subtilis*, while the *B. atrophaeus* and *B. Polymyxa* isolates showed PGI values ranging from 53.08% to 69.61%, respectively. *B. simplex* had the least antagonistic potential (PIRG =37.72%) (Table 1). The *B. tequilensis* isolate (SY145D) had the highest antagonistic activity against the four fungal pathogens, with one inhibition ranged from 10-20 mm (Table 2).

The results, obtained here, of ***in vitro*** sensitivity of phytopathogenic fungi to antagonistic bacteria revealed that the isolates of *Bacillus spp.* were suppressive, though with different degrees, to the tested isolates of phytopathogenic fungi, were consistent with those obtained by others [21, 22]. The inhibition of radial growth by the forming of an inhibition zone against these pathogens is considered as antibiosis, whereby the antibiotic metabolites may penetrate the pathogen cell and inhibit its activity by chemical toxicity. *Bacillus sp.* produced several kinds of antifungal peptides (peptidolipid iturins), such as bacillomycin and mycosubtilin. that act on the fungi's cell wall [8, 23, 24] reported that the fungal mycelial malformation might be due to the antibiotic metabolites produced by the bacteria, which can penetrate and cause protoplasmic dissolution and disintegration. On the other hand, [25] showed that 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 pathogen by *Bacillus sp.* was that antifungal activity is increased by co-culturing of different bacterial species.

Our results showed that *B. amyloliquefaciens, B. tequilensis* and *B. subtilis* had the largest growth inhibition of studied pathogens. In this regard, [14, 26, 27] reported that these *Bacillus spp.* added peptides and lipopeptides to the culture medium, such as fungicine, iturin, bacillomicine, among others, having antifungal properties when confronted *in vitro* against phytopathogenic fungi such as *Rhizoctonia*, *Fusarium* and Magnaporthe oryzae.

On the other hand, our data demonstrated that these *Fusarium* species *culmorum* and *solani* were lesser antagonistic sensitivity (49.49% and 57.32%, respectively) as compared with the other pathogens (Table 1). This might be attributed to the fact these both species produce a number of mycotoxins and their action was evident in the various alternation of the hyphal structure of *Fusarium* spp. isolates' growth together with *Bacillus* spp. [20, 28], since our data showed that these both species were lesser antagonistic sensitivity (49.49% and 57.32%, respectively) (Table 1). similar to these effects have been described in other systems of mixed cultures [29].

Table 1. Mean antagonistic effect of *Bacillus* spp. against filamentous fungi used in the study

		Fungi				
Bacillus spp.	Number of isolates	*C. sativus*	*P. graminea*	*F.solani*	*F. culmorum*	Mean
B. atrophaeus	3	B72.31b	A87.41a	B69.5b	C49.23c	69.61b
B. subtilis	20	B78.37a	A85.43a	A80.04a	C53.61b	73.06a
B. polymyxa	2	A62.35c	B54.71b	C30.30c	A65.02a	53.08
B. amyloliquefaciens	10	80.39a	84.57a	77.41a	57.18b	75.35a
B. tequilensis	4	B72.92b	A86.1a	A76.68a	C61.93a	74.84a
B. simplex	1	A70.01b	B60.9b	C10.00d	C10.00d	37.72c
Total	40	A72.73	A76.52	B57.32	C49.49	

Values followed by different small letters (columns) and preceded by capital letters (lines) differ significantly at level P<0.05.

Table 2. Inhibition zone* beetwen *Bacillus* spp. and filamentous fungi used in the study

	Fungi			
			F. culmorum	
Bacillus spp. Isolates	*C. sativus*	*P. graminea*		*F.solani*
B. atrophaeus				
SY15B	++	+++	+	+++
SY199A	+++	+++	+	++
SY63E	++	+++	+	+
B. subtilis				
SY35A	++	+++	+	+++
Sy41B	+++	+	+	+++
SY44A	+++	++	+	+++
SY60A	+++	++	+	+++
SY73B	++	+++	+	++
SY113C	++	+++	++	+++
SY116C	+++	+++	+	+++
SY118C	+++	+++	+	+++
SY124B	+++	+++	+	+++
SY130D	++	+++	+	+++
SY132E	+++	+++	++	+++
SY133D	+++	+++	++	+++
SY132C	+++	+++	++	+++
SY134D	+++	+++	++	++
SY135D	+++	+++	+	+++
SY139D	+++	+++	+	+++
SY151C	++	+++	+	+
SY160C	++	+++	++	++
SY168C	+++	+++	++	+++
SY190E	++	+++	+	++
B. polymyxa				
SY53C	+	+++	++	+
SY55B	++	+++	++	+
B. amyloliquefaciens				
SY82C	+++	+++	+	+++
SY96C	+++	+++	++	+++
SY96E	+++	+++	++	+++
SY123A	+++	+++	+	+++
SY128B	++	+++	++	++
SY134C	+++	+++	+	++
SY159D	+++	+++	++	++
SY177C	++	+++	+	++
SY190D	+++	+++	+	++
SY200D	+++	+++	+	++
B. tequilensis				
SY69A	++	+++	+	++
SY145D	+++	+++	+++	+++
SY149C	++	++	++	+++
SY150D	++	+++	+	++
B. simplex				
SY198B	++	+++	+	+

*Inhibition zone: + (weak), 0-10 mm; ++ (moderate), 5-10 mm and +++ (strong), 10-20 mm.

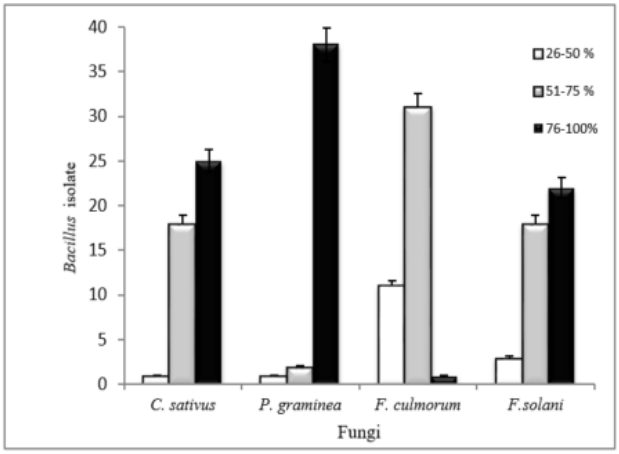

Figure 1. Antagonistic efficacy of *Bacillus* spp. against four soilborne fungal pathogens. Zone of inhibition (%) = (radial growth of the fungus in control - radial growth of fungus in treatment) / C ×100

4. Conclusion

- Taken together, the present results showed that the tested *Bacillus* spp., proved a broad spectrum of antifungal activities against *C. sativus*, *P. graminea*, *F. culmorum* and *F.solani*, which are pathogenic in cereal growing area.
- *Fusarium* species *culmorum* and *solani* were lesser antagonistic sensitivity as compared with the other pathogens. Additionally, the largest growth inhibition of *Bacillus* spp. value approximately (75.35%) was induced by the isolates of *B. amyloliquefaciens*, *B. tequilensis* and *B. subtilis.*
- Importantly, *B. tequilensis* isolate (SY145D) had the highest antagonistic activity against the four pathogens.
- The next step, aiming to confirm the colonization ability of the selected isolates and testing its in vivo efficacy, which will be the subject of the follow-up investigation.

Compliance with Ethics Requirements. Authors declare that they respect the journal's ethics requirements. Authors declare that they have no conflict of interest and all procedures involving human / or animal subjects (if exist) respect the specific regulation and standards.

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