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*Full Length Research Paper*

# Efficacy of some local *Bacillus thuringiensis* isolates against soil borne fungal pathogens

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**Seven Jordanian strains belonging to the bacterium *Bacillus thuringiensis* (*Bt*) were evaluated for their antifungal effects on soil borne plant pathogenic fungi under laboratory conditions. The antifungal effects of total soluble proteins of *Bt* stains on the growth of two isolates of the fungus, *Fusarium oxysporum* (isolated from roots of wilted peach trees and tomato plants), *Fusarium proliferatum* (isolated from roots of wilted palm trees) and *Rhizoctonia solani* (isolated from infected tomato seedling) were investigated. Results showed that *B. thuringiensis thuringiensis* (J23), was the most effective strain on the two fungal species; *F. proliferatum* and the peach fungal isolate of *F. oxysporum*. *B. thuringiensis entomocidus, Bt* (J115) showed the highest activity on the tomato fungal isolate of *F. oxysporum*. While *B. thuringiensis pakistani* (J107) was the most effective on *R. solani*. The *Bt* (J139) was the least effective strain. Soluble proteins of all *Bt* strains showed variable potential inhibitory effects on the tested fungi. Soluble proteins of the most effective *Bt* strains can be developed for potential antimicrobial applications; however, these findings necessitate a step to test the efficacy of these soluble proteins as soil drench to suppress soil borne fungi under field conditions.**

**Key words:** *Bacillus thuringiensis*, inhibition, *Fusarium oxysporum, Fusarium proliferatum, Rhizoctonia solani.*

## INTRODUCTION

Species belonging to the genera *Rhizoctonia* and *Fusarium* are the most common and persistent soil borne fungi, attacking economic plants and causing serious damages (Agrios, 2005). The fungus, *Rhizoctonia solani* attacks several crops causing pre and post emergence damping off, in addition to the root rot on fruit trees (Agrios, 2005). Furthermore, several pathovars of the wilt, *Fusarium oxysporum* and *Fusarium proliferatum* attack vegetables, fruit trees, field crops and ornamentals (Abdalla et al., 2000; Agrios, 2005; Armengol et al., 2005). The infected plants show yellowing and wilting of leaves, and eventually cause the death of the entire plant. Several methods are generally used to suppress the fungi and reduce their harmful effects; these methods

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**Table 1.** Species of *Bacillus thuringiensis* isolates used in the study.

| Species | Strain | Source | Location |
|---|---|---|---|
| *Bacillus thuringiensis autoagglutinated* | J71 | Tomato | Al Shuneh |
| *Bacillus thuringiensis entomocidus* | J115 | Lentil seeds | Jordan University |
| *Bacillus thuringiensis jordanica* | J 112 | Soil | Jordan Valley |
| *Bacillus thuringiensis kurstaki* | J6 | Water | Al Khirbah Al- Samra |
| *Bacillus thuringiensis pakistani* | J107 | Tomato seeds | Amman |
| *Bacillus thuringiensis pakistani* | J139 | Water | Jordan Valley |
| *Bacillus thuringiensis thuringiensis* | J23 | Chicken manure | Gawr Kabed, Jordan Vvalley |

include seed treatment, soil sterilization, and/or use of resistant cultivars (Abu-Blan et al., 1990; De Cal et al., 2005; Zhang et al., 2013; Chang et al., 2014). Resistance of cultivars was reported only against certain races of the wilt fungus, this resistance can be broken by several other means including certain nematodes in the soil (Sidhu and Webster, 1977; Naji and Abu-Gharbieh, 2004). However, once these fungi are established in the soil, it would be rather impossible to eradicate them. Although soil solarization alone is generally used to suppress these fungi, several reports have shown that integrating the use of bioagents like *Bacillus thuringiensis* (*Bt*) as a component of integrated pest management (IPM) was the most effective method (James, 2008; Naranjo, 2011; Tabashnik, 2008). It was reported that corn cultivars that have been engineered with the *Bt* strain (Bt corn) lowered the severity of ear rot caused by the fungus *F. oxysporum* (Folcher et al., 2010; Nedělník et al., 2012). Investigators stated that the *Bacillus* spp. may assume their antagonistic effects by producing cell-bound antifungal compounds (Edwards, 1993; Walker et al., 1998) or indirectly by inducing plant resistance mechanisms. Edwards and Seddon (2001) identified the antifungal compound exhibited by the bacterium, *B. brevis* against the fungus *B. cinerea in vitro* as gramicidin S. The Jordanian *Bt* strains showed insecticidal and nematicidal effects (Khyami-Horani et al., 1999; Al-Banna and Khyami-Horani, 2004; Abu-Dhaim et al., 2006). Herein we aimed at investigating the effect of Jordanian *Bt* on the growth of some *F. oxysporum, F. proliferatum* and *R. solani* isolates.

## MATERIALS AND METHODS

### Fungal isolates

Three local isolates of *Fusarium* sp. and one isolate of *R. solani* were used in bioassays. The fungus *F. oxysporum* was isolated from the crown area of infected peach and tomato plants grown in Mafraq area (Jordan Eastern Desert) and in Jerash area (Northern Part of Jordan), respectively. An isolate of *F. proliferatum* was recovered from roots of palm trees grown in Qwarah area (Jordan Southern Desert). Whereas, *R. solani* was isolated from infected tomato seedling grown in the glass house at the University of Jordan campus, Amman. Pure cultures of the fungal isolates were identified to the species level based on their morphology (Booth, 1971; Domsch et al., 1980). The identification of fungal isolates was confirmed by the sequences of the ITS region of the rDNA (Nida Salem, Unpublished). For routine culturing, the isolates were grown on potato-dextrose agar (PDA; biolab, Hungary) (39 g/l; agar: 15.0g, potato extract: 4.0 g and dextrose: 20.0 g) and incubated at 24°C.

### Bacterial strains

A total of seven strains of *Bt*, previously isolated from different Jordanian habitats (Khyami-Horani, 2002), were used in this study (Table 1). Glycerol stocks of *Bt* were stored at -80°C. Bacterial strains were streaked on nutrient agar plates overnight at 37°C. Single colonies were used to inoculate 150 ml of modified T3 medium (0.3% Tryptone, 0.2% Tryptose, 0.15% Yeast extract, 0.05 M $NaH_2PO_4$, 0.005% $MnCl_2.4H_2O$) (Travers et al., 1987). Cultures were incubated for 3 days at 37°C over an orbital shaker. The cells were pelleted for 10 min at 3.212 g and 4°C. Proteins were solubilized in 2.5 ml of pH 10 phosphate buffer (50 mM $Na_2CO_3$, 10 mM Dithiothreitol, 1 mM EDTA) (Fiuza et al., 1996). The solubilized protoxins were clarified by centrifugation for 5 min at 18.514g and 4°C. The pH of the supernatant was adjusted to 8 with 1 mol $l^{-1}$ HCl and stored at -20°C. The solubilization was confirmed by SDS-PAGE gel electrophoresis.

### *In vitro* assay

For each bacterial strain, four wells (8.5 mm) were made in each PDA plate (pH 8) using a cork borer, a total of 100 µl of the 2.5 ml soluble protein fractions obtained from three day culture (150 ml) were added to three wells. The fourth well was filled with 100 µl sterile distilled water to serve as a negative control. The plates were left overnight to allow the proteins to soak, then eight millimeter diameter of actively growing fungal culture discs from PDA plates of each tested fungi were cut using a sterile cork borer and placed in the centre on surface of the tested PDA media plates. Each *Bt* strain fraction was replicated three times. The plates were incubated at 24°C for one week. The plates were observed daily for fungal growth until the growth reached the edge of the control; the inhibition zone was then measured (mm) and recorded. The fungal growth on both control wells and *Bt* soluble protein wells was also monitored. The growth of the three isolates of *Fusarium* reached the edge of the control well after 4 days of incubation. Whereas growth of *R. solani* reached the edges of the water well after 3 days of fungal incubation.

### Statistical analysis

Each treatment was replicated three times in a completely randomized design. The data was tabulated and analyzed using

**Table 2.** Effect of total soluble proteins of seven Jordanian *Bt* strains on local isolates of *Fusarium oxysporum*, *F. proliferatum* and *Rhizoctonia solani.*

| ***Bacillus thuringiensis* (*Bt*) strains** | **Strain** | **Inhibition of fungal growth (mm)*** | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | ***F. proliferatum*/ palm** | | ***F. oxysporum* / Peach** | | ***F. oxysporum*/ Tomato** | | ***R. solani*** | |
| | | **After** | | | | | | | |
| | | **4 days** | **7 days** | **4 days** | **7 days** | **4 days** | **7 days** | **3 days** | **7 days** |
| *Bt autoagglutinated* | J71 | $4.7^{ab}$** | $1.7^{b}$ | $4.3^{ab}$ | 2.3 | $3.3^{b}$ | $1.7^{ab}$ | $4.3^{b}$ | $0.0^{b}$ |
| *Bt entomocidus* | J115 | $4.0^{b}$ | $0.^{3cd}$ | $4.5^{ab}$ | 1.0 | $5.9^{a}$ | $0.3^{c}$ | $5.0^{ab}$ | $3.7^{a}$ |
| *Bt jordanica* | J 112 | $4.0^{b}$ | $1.7^{b}$ | $3.7^{ab}$ | 1.7 | $3.0^{b}$ | $1.0^{bc}$ | $5.7^{ab}$ | $0.0^{b}$ |
| *Bt kurstaki* | J6 | $4.7^{ab}$ | $2.7^{a}$ | $3.7^{ab}$ | 1.7 | $3.0^{b}$ | $0.0^{c}$ | $4.7^{b}$ | $0.0^{b}$ |
| *Bt pakistani* | J 107 | $4.7^{ab}$ | $2.7^{a}$ | $3.7^{ab}$ | 1.7 | $2.0^{b}$ | $0.0^{c}$ | $7.0^{a}$ | $0.0^{b}$ |
| *Bt pakistani* | J 139 | $2.7^{c}$ | $1.0^{bc}$ | $3.0^{b}$ | 1.0 | $2.0^{b}$ | $0.0^{c}$ | $4.0^{b}$ | $0.0^{b}$ |
| *Bt thuringiensis* | J 23 | $5.3^{a}$ | $3.3^{a}$ | $5.0^{a}$ | 2.7 | $5.0^{a}$ | $2.7^{a}$ | $5.0^{ab}$ | $0.0^{b}$ |
| Control only water | | 0.0d | 0.0d | $0.0^{c}$ | 0.0 | $0.0^{c}$ | $0.0^{c}$ | $0.0^{c}$ | $0.0^{b}$ |
| Lsd $p$=0.05 | | 1.118 | 0.914 | 1.894 | NSD | 1.424 | 1.075 | 2.097 | 1.413 |

*Means of three replicates; ** Means followed by the same lowercase letter do not differ significantly ($p \pm 0.05$) according to LSD test.

analysis of variance (ANOVA) and the means were separated using least significant difference (LSD) at $P \geq 0.05$) (Little and Hills, 1974).

## RESULTS

The inhibitory effect of *Bt* strains varied within the tested isolates of *F. oxysporum and F. proliferatum*. All *Bt* strain significantly inhibited the *Fusarium* isolates after 4 days of incubation. The bacterial strain *B. thuringiensis thuringiensis* (J23) was the most effective strain against both *F. proliferatum* (palm isolate) and *F. oxysporum* (peach isolates) (Table 2). Nevertheless, all *Bt* strains were effective against the palm isolates even after 7 days of incubation (Table 2).

*B. thuringiensis entomocidus* (J115) was most effective against *F. oxysporum* (tomato isolate) (Table 2). The strain *B. thuringiensis entomocidus* (J115) in addition to *B. thuringiensis thuringiensis* (J23) showed more significantly inhibitory effect than the other strains after 4 days of incubation with the tomato isolate of *F. oxysporum*. However, the effectiveness of the fractions of *B. thuringiensis* autoagglutinated (J71), *Bt entomocidus* (J115), *B. thuringiensis jordanica* (J112) and *B. thuringiensis thuringiensis* (J23) extended to one week (Table 2).

The fungus *R. solani* reached the control well after 3 days of incubation. The growth was inhibited by all *Bt* strains with the maximal significant inhibition by *B. thuringiensis pakistani* (J107) after 3 days of incubation. Only the solubilized protoxins of the strain *B. thuringiensis entomocidus* (J115) extended the inhibition of the fungal growth to one week and was significantly different from other isolates and the control (Table 2).

Although it was noticed that the inhibition zone was reduced after 7 days of incubation in all tested fungi, a crescent-shaped zone of inhibition of fungal growth occurred around the discs as compared to fungal growth surrounding control wells (Figures 1 and 2). Microscopical examination of the fungal growths of all tested fungi, near the margins of the inhibition zones, showed that the hyphae were distorted and included many vacuoles as compared to normal hyphae in the water control wells.

## DISCUSSION

In this study, the total soluble proteins of seven Jordanian *Bt* strains were investigated for their biocontrol potential against some soil borne plant pathogenic fungi belonging to species of *Fusarium* and *Rhizoctonia*. Results showed that these *Bt* strains varied in their suppression of the growth of the studied fungi. Similarly, Raddadi et al. (2009) showed that several *Bt* strains inhibited the growth of *F. oxysporum* and *Aspergillus flavus*; strain *Bt* HD932 showed the widest antifungal activity spectrum.

The total soluble protein of strain *B. thuringiensis jordanica* (J112) resulted in the suppression of the growth of the studied fungal isolates. In our laboratory, *B. thuringiensis jordanica* (J112) expressed chitinase activity (Unpublished) which may be one component of the total soluble proteins. It has been reported that *Bacillus* species parasitism operates by degradation of cell walls of pathogenic fungi and using their extracellular lytic enzymes, including chitinase, an insoluble linear polymer of b-1,4-N-acetylglucosamine (GlcNAc), the major component of most fungal cell walls. *B. circulans* (Watanabe et al., 1990), *B. licheniformis* (Takayanagi et al., 1991; Trachuk et al., 1996), *B. cereus* (Pleban et al., 1997), *B. pabuli* (Frändberg and Schnürer, 1994) and *B.*

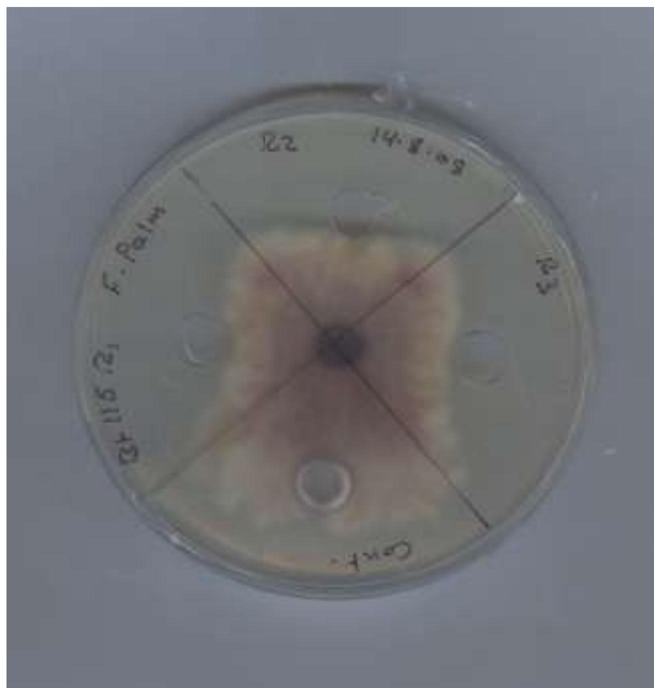


**Figure 1.** Potato dextrose agar plates showing growth inhibition of the fungus *F*usarium *proliferatum* (palm isolate) after incubation with 100 µl of the soluble protein fractions of the bacterial strain *Bacillus thuringiensis entomocidus* (*Bte* (J115)) that were added to each of the three wells (R1, R2, and R3). The fourth well was filled with 100 µl sterile distilled water to serve as a negative control (cont.). The crescent shaped zone of inhibition of fungal growth is observed around the fungal discs representing three replicates (R1, R2, and R3). While the fungus grew around the control well (cont.)

**Figure 2.** Potato dextrose agar plates showing growth inhibition of the fungus *Rhizoctonia solani* after incubation with 100 µl of the soluble protein fractions of the bacterial strain *Bacillus thuringiensis entomocidus* (*Bte* (J115) that were added to each of the three wells (R1, R2, and R3). The fourth well was filled with 100 µl sterile distilled water to serve as a negative control (cont.). The crescent shaped zone of inhibition of fungal growth is observed around the fungal discs representing three replicates (R1, R2, and R3). While the fungus grew around the control well (cont.)

*thuringiensis* (Chigaleichik, 1976) were reported to produce chitinase. Furthermore, Reyes et al. (2004) showed that chitinases from *Bt* suppressed the effect of *Fusarium* on the germination of soybean seeds.

The results showed that total soluble proteins of certain *Bt* strains inhibited the growth of the studied fungi; however, the growth was resumed after 7 days of incubation. Similarly, Kamenek et al. (2012) reported that *B. thuringiensis* delta endotoxins inhibited the growth of several *Fusarium* species, *R. solani* isolates, and *Phytophthora infestans*; and the growth was resumed after 6 days of incubation. These findings suggested that the proteins might be volatile and after sometime they may be reduced or their effect was fungistatic rather than fungicidal. Walker et al. (1998) reported that the suppression of the grey mold fungus, *Botrytis cinerea, in vitro* was exhibited by the *Bt* isolates, and the formation of inhibition zone was due to the metabolites released from the bacteria into the culture medium. Furthermore, Silo-Suh et al. (1994) reported that *B. cereus* also produced fungistatic antibiotics.

In this study, abnormalities of hyphae were observed in all tested fungi at the marginal edge of the disc after incubation with the *Bt* soluble proteins. These abnormalities might be due to lysis of cell walls and other biochemical changes caused by the soluble proteins of the *Bt* strains. Similarly, Sharma and Sharma (2008) reported that the antifungal metabolites of the bacterium *Bacillus subtilis* strain UK-9 caused morphological alterations of the hyphae and spores of the plant pathogenic fungus, *Alternaria* sp.

The inhibition of fungal growth might be due to an increase of respiration rate. Kamenek et al. (2012) reported that *B. thuringiensis* delta endotoxins inhibited the growth of several fungi; and stated that the antifungal inhibitory effect of *B. thuringiensis* delta endotoxin was due to an increase in respiration rate, they also speculated that the antifungal compounds might be linked to uncoupling of oxidative phosphyrylation and respiration in fungal cell.

The current results pointed to potential for biological control of soil borne plant, thus the *Bt* products or *Bt* crops could replace the hazardous or banned fungicides, or even reduce the concentrations of chemical pesticides if used together as part of integrated pest control. However, further studies are indeed required to identify the proteins or other compounds of these isolates to determine which protein or compound is responsible for the inhibition of growth of the specific fungal isolate. In

addition, the effect of the *Bt* endospores together with soluble proteins on germination of *Fusarium* spores should be investigated since Landa et al. (1997) reported that the cell-free culture filtrates of four Bacillus isolates inhibited the conidial germination of the fungus *F. oxysporum* f. sp. *ciceris*.

Thus, further field work should be employed by applying the *Bt* spores or the total soluble proteins as a drench on infested soil and study the effect on the fungus and on the resistance of the plant.

**Conflict of Interests**

The authors have not declared any conflict of interests.

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