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Full Length Research Paper

Biological control of olive leaf spot (peacock spot disease) caused by *Cycloconium oleaginum* (*Spilocea oleaginea*)

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We report the antifungal activity of eight different bacterial isolates (*Bacillus megaterium* NB-3, *Bacillus cereus* NB-4, *B. cereus* NB-5, *Bacillus subtilis* NB-6, *Corynebacterium xerosis* NB-2, *Burkholderia mallei* NB-8, *B. subtilis* (HNEB-1) and *B. cereus* NEB II *voru*) against the olive leaf spot fungus (*Cycloconium oleaginum*). *B. subtilis* NB-6 (6.88 cm^2), *B. megaterium* NB-3 (7.13 cm^2), *B. subtilis* HNEB-1 (7.63 cm^2) and *B. cereus* NB-4(7.88 cm^2) were found to control significantly the growth of *C. oleaginum*, where the strains of *C. xerosis* NB-2 (12.75 cm^2), *B. mallei* NB-8 (15.63 cm^2) and *B. cereus* NB-5 (16.25 cm^2) controlled the fungal growth under the experimental conditions. *B. cereus* NEBII *voru* is the only tested bacterial strain that enhanced the fungal growth of *C. oleaginum* under our experimental conditions (46.25 cm^2).

Key words: *Bacillus*, antifungal, biological control, olive Leaf spot, *Burkholderia*, *Corynebacterium, Cycloconium oleaginum*.

INTRODUCTION

The olive (*Olea europea* L.) is the most important fruit tree grown in Jordan. The total cultivated area with olives was about 64,533 ha representing 74.7% of the total planted area with fruit trees (Anonymous, 2006). One of the important foliar diseases affecting olive trees in humid regions in Jordan and many other countries in the world is peacock spot disease caused by *Cycloconium oleaginum*, also known as olive leaf spot and bird's-eye spot (Vossen, 2004). Severely, infected trees show defoliation of leaves resulting in poor twig growth and poor fruit set, and great damage of plantation (González et al., 2002). Infection is normally associated with high humidity and winter conditions (cool and low light), where high temperatures restrict spore germination and growth. Symptoms of disease start as sooty blotches on leaves develop into green black circular spots 0.1 to 0.5 inch in diameter with a faint yellow halo around the spot. More lesions are developed in the lower part of the tree. Leaves may drop prematurely and twig death may occur due to defoliation (Civantes, 1999). The disease is chemically controlled by several and yearly sprays of fixed coppers soon after harvesting (Vossen, 2004).

Biological control may be an alternative to chemicals in the control of some pathogenic fungi, reducing environmental pollution (Handelsman et al., 1990). Certain rhizosphere bacteria including *Pseudomonas* and *Corynebacterium* were used to control different plant fungal diseases like *Pythium* damping-off and some root rot fungi (Parke et al., 1991). Others like *Bacillus subtilis* that exhibits insecticidal, antifungal and antibacterial activities were used to control *Rhizoctonia solani* damping-off (Asaka and Shoda, 1996), and bean leaf rust caused by *Uromyces phaseoli* (Baker et al., 1983), where some *Bacillus* species were found to show antimicrobial activities (Gebreel, 2008) and effectively control brown rot of rice (Islam and Nandi, 1983).

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This research was conducted to study the efficacy of different strains of antifungal producing bacteria in controlling peacock spot fungus *C. oleaginum* (*Spilocea oleaginea*).

MATERIALS AND METHODS

Isolates collection

Isolates were collected, in October 2008, from symptomatic olive trees, where leaves are showing typical symptoms. Leaves were cut into small pieces 0.5 cm length, plated on modified PDA media (39 gm PDA, 1 gm di-potassium phosphate, 1 gm sodium nitrate, 2 gm sucrose per litre) at 22 ± 1°C for 7 days, then sub-cultured on new plates containing the same media for 24 h before treatment with bacterial strains.

Antimicrobial substances producing bacteria

The antimicrobial substances producing bacteria, *Bacillus megaterium* NB-3, *Bacillus cereus* NB-4, *B. cereus* NB-5, *B. subtilis* NB-6 (air flora isolates) (El-Banna, 2003; El-Banna, 2005a), *Corynebacterium xerosis* NB-2 (soil isolate) (El-Banna, 2004) and *Burkholderia mallei* NB-8 (water isolate) (El-Banna, 2005b), were isolated from different sources in Jordan and deposited in Jerash Culture Collection (JCC). Two other isolates *B. subtilis* (HNEB-1) and *B. cereus* (NEB II *voru*) were provided by JCC. The antimicrobial substances producing bacteria were cultured at 27°C on nutrient agar (Oxoid).

Screening the bacterial isolates for their antagonistic activity against *C. oleaginum*

The antagonistic activities of the bacterial isolates against *C. oleaginum* were examined. Bacterial isolates were cultured on nutrient broth for 24 h at 22 ± 1°C.

Screening was carried out using 9.5 cm-diameter pots (Petri dishes) containing 24 h cultures of the fungus on the modified PDA media. Each isolate was used to inoculate 5 pots of the fungal culture. Inoculation was carried on by dispersing 1 ml of the bacterial suspension on the surface of each pot. Treated pots were then incubated at 22 ± 1°C. Fungal growth was assessed daily (visual observation) where the final assessment was recorded after 5 days of incubation using colony counter Petri dish, by which the total area of the fungal growth on each pot was measured.

Experimental design and data analysis

All treatments were arranged in randomized complete block design (RCBD) with 5 replicates for each treatment. Average growth area of each treatment was assessed using colony counter Petri dish. General Linear Model (GLM) ANOVA (SPSS VER 10) was used to find differences ($P \leq 0.05$) between treatment means and control.

RESULTS

Bacterial isolates used in this study varied in their *in vitro* control activity against the leaf spot olive fungus *C. oleaginum* (Figure 1). *B. subtilis* NB-6 isolate showed the highest control activity, where the fungal growth area was greatly reduced to 6.88 cm^2 compared with the control 41.75 cm^2 (Table 1). The maximum growth area of the fungus was observed when it was treated with *B. cereus* (NEB II voru), where the total growth area was 46.25 cm^2 exceeding the growth area of the control (41.75 cm^2) and significantly different from all other treatments. *B. megaterium* NB-3, *B. subtilis* HNEB-I and *B. cereus* NB-4 showed high control activity and reduced the fungal growth area to 7.13, 7.63 and 7.88 cm^2, respectively. Lower control activity was observed when *C. xerosis* NB-2, *B. mallei* NB-8 and *B. cereus* NB-5 were used. They reduced the fungal growth to 12.75, 15.63 and 16.25 cm^2, respectively.

Mean growth area and pH of all treatments ± standard error of means is represented in Figure 2.

DISCUSSION

The olive trees had been suffering from attacks by a problem commonly known as Olive Leaf Spot or Peacock Spot Disease caused by *C. oleaginum* (*Spilocea oleaginea*). This study demonstrates that the olive leaf spot disease could be controlled biologically by several bacterial strains. Based on *in vitro* testing of different bacterial strains on the growth of *C. oleaginum*, significant colony growth reduction was found in 7 treatments (Table 1, Figures 1 and 2). By contrast, enhanced colony growth of *C. oleaginum* was observed when treated with *B. cereus* (NEB II *voru*).

In this study, *B. subtilis* NB-6 (6.88 cm^2), *B. megaterium* NB-3 (7.13 cm^2), *B. subtilis* HNEB-1 (7.63 cm^2) and *B. cereus* NB-4 (7.88 cm^2) were found to control significantly the growth of *C. oleaginum*, where the strains of *C. xerosis* NB-2 (12.75 cm^2), *B. mallei* NB-8 (15.63 cm^2) and *B. cereus* NB-5 (16.25 cm^2) controlled the fungal growth under the experimental conditions. Antibiotics producing bacilli have been used as biocontrol agents against pathogenic fungi (Magnusson et al., 2003). Many previous studies showed that *B. subtilis* strains produced volatiles that antagonize a range of soil-borne plant pathogens including *Rhizoctonia* spp. and *Pythium* spp. (El-Hamshary and Khattab, 2008). *B. megaterium* was also reported in controlling effectively the fungal growth of *R. solani* on soybean plants due to the competition for space and nutrients, in addition to bacterial antagonism, which was considered as an important factor in the disease suppression (Zheng and Sinclair, 2000).

The strain *B. cereus* NEBII *voru* was found to enhance the fungal growth of *C. oleaginum* under our experimental conditions (46.25 cm^2). Wargo and Hogan (2006) declared that there are instances where bacteria provide fungi with compounds that enhance the production of fungal virulence determinants. They pointed out that in different circumstances, bacteria might either enhance or attenuate the fungal properties.

On the other hand, two *B. cereus* strains controlled the fungal growth of *C. oleaginum*. Handelsman et al. (1990) used *B. cereus* UW85 to control damping-off of alfalfa,

Table 1. Mean growth area (cm^2) of *C. oleaginum* when treated with different bacterial isolates and incubated at 22 ± 1°C for 5 days. N = 5* for each bacterial isolate.

Bacterial isolate	pH	Fungal mean growth (cm^2)	Std. error of mean
1- *B. cereus* NB-5	6.8	16.25 c	± 0.4277
2- *B. mallei* NB-8	6.7	15.63 c	± 0.4642
3- *B. subtilis* NB-6	6.4	6.88 a	± 0.4755
4- *B. subtilis* (HNEB-I)	6.6	7.63 a	± 0.6056
5- *C. xerosis* NB-2	6.9	12.75 b	± 0.6316
6- *B. cereus* (NEB II *voru*)	7.4	46.25 e	± 0.8477
7- *B. cereus* NB-4	6.4	7.88 a	± 0.5652
8- *B. megaterium* NB-3	6.7	7.13 a	± 0.4425
9- Control	7.1	41.75 d	± 0.7581

*The number of trials of experiments for each.

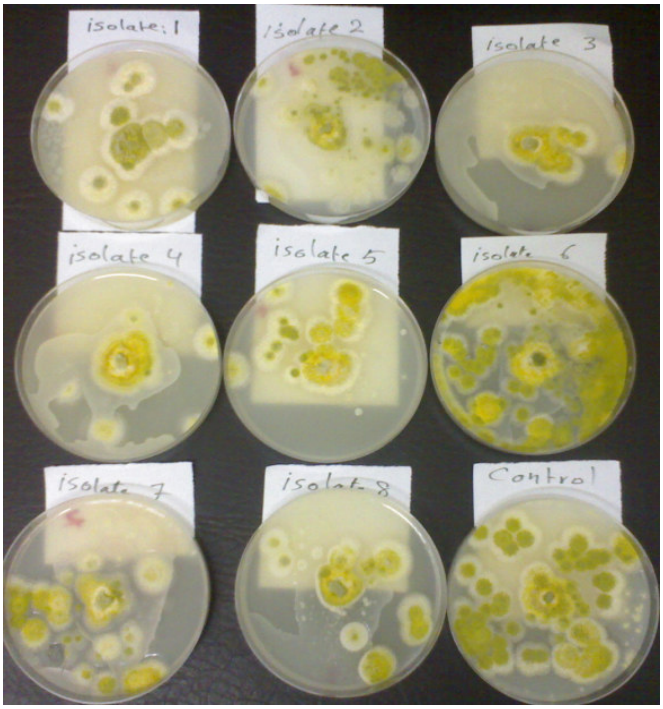

Figure 1. Effect of different bacterial isolates on fungal growth of *C. oleaginum* when treated and incubated at 22 ± 1°C for 5 days.

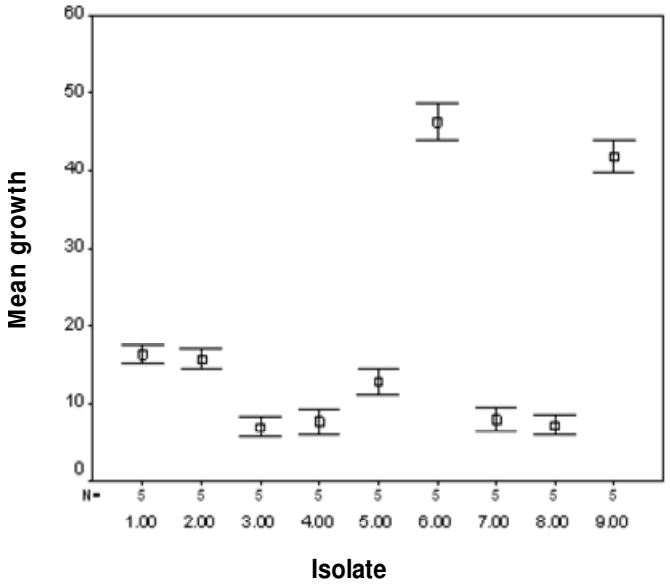

Figure 2. Mean growth area of all treatments ± standard error of mean.

where El-Hamshary and Khattab (2008) showed that *B. subtilis* and *B. cereus* inhibited the growth of the root rot fungus *Fusarium solani*. Magnusson et al. (2003) pointed that more than 1200 isolates of lactic acid bacteria were screened for antifungal activity, only about 10% showed inhibitory effect on pathogenic fungi. Jesper and Johan (cited by Gebreel, 2008) mentioned that maximum activity of antifungal compounds by lactobacilli was observed at pH values between 3.0 and 4.5, but it decreased rapidly when pH was adjusted to a level between 4.5 and 6.0, which was less than the range of pH in our study. Zheng and Sinclair (2000) pointed out that *B. megaterium* is a potential bacterial biocontrol agent against *R. solani* affecting soybean plants, it reduced root colonization of the fungus. Reduction was attributed to the competition for space or nutrients, in addition to bacterial antagonism, which was considered as an important factor in the disease suppression.

C. xerosis NB-2 significantly reduced the fungal growth of *C. oleaginum* under experimental conditions. Alhubge and Ashoor (2006) studied the use of different bacterial isolates including *Corynebacterium* spp. in the biological control against *Rhizopus stolonifer* associated with barley grains, and they found that *Corynebacterium* spp. has inhibitory effect on *R. stolonifer* in Petri plates containing PDA compared with control samples and fungicides used (Topsin, Benlate and Dithane).

B. mallei NB-8 significantly *in vitro* reduced the fungal growth compared with the control. It is well known that suppression of the fungal growth could be attributed to several mechanisms of the bio-agent, and it was documented that *Pseudomonas* spp. including *Burkholderia* produce several antibiotic-like substances including pyrrolnitrin (El-Banna and Winkelmann, 1998), bacteriocin and phenazine antibiotics (Hamdan et al., 1991), but the most important mechanism responsible for the suppression of plant pathogens is siderophore-mediated competitions for iron (Freedman et al., 1989; Henry et al., 1991).

This study is the first on the biological control of Olive Leaf Spot (Peacock Spot Disease) caused by *C. oleaginum* in Mediterranean region, which enables us to use those active strains as biocontrol agents in the treatment of Peacock Spot Disease in Jordan.

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