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Evaluation of Pseudomonads Bacterial Isolates in Biological Control of Citrus Bacterial Canker Disease

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Abstract: Investigation of antagonistic activity of selected Pseudomonads bacterial strains against *Xanthomonas axonopodis* pv. *citri* the causal agent of citrus bacterial canker disease was carried out in the laboratory and greenhouse conditions. Results of *in vitro* experiments showed that all bacterial strains were inhibitory on *X. citri* with various antagonistic activities. The most effective strains were selected and were evaluated in the greenhouse against citrus canker disease. Results of greenhouse experiments in application of antagonistic bacterial strains to control *X. axonopodis* pv. *citri* the causal agent of citrus bacterial canker disease were promising and selected strains reduced the number of disease spots between 23.8 to 64.0%.

Key words: Citrus bacterial canker, biocontrol, *Pseudomonas fluorescent*, *P. putida*, Iran

INTRODUCTION

Citrus trees have been derived from eastern Asia domestic species and are presently planted in many countries including Argentina, Australia, Brazil, China, Cuba, Egypt, India, Israel, Italy, Japan, Mexico, Morocco, South Africa, Spain, USA and Iran (Khodakaramian and Ghasemi, 2002). In Iran, citrus is a very important fruit tree and being grown in several Northern and Southern provinces (Khodakaramian and Ghasemi, 2002). Citrus bacterial canker is one of the most important and serious diseases of citrus around the world including Iran (Cubero and Graham, 2002; Gottwald *et al*., 1993; Graham, 2001; Graham *et al*., 1992; Khodakaramian and Ghasemi, 2002; Leite and Mohan, 1990; Leite *et al*., 1987; Bora and Bhagabati, 1996; Rodrigues *et al*., 1998; Schubert *et al*., 2001; Stall and Civerolo, 1991; Timmer *et al*., 2000; Vernière *et al*., 1998).

The causal agent is *Xanthomonas axonopodis* Starr and Garces Emend Vauterin which has 3 pathotype including *X. a.* pv. citri or type A, *X. a.* pv. aurantifolli or type B, C, D and *X. a.* pv. citromelo or type E (Cubero and Graham, 2002; Gottwald *et al*., 1993; Graham, 2001; Graham *et al*., 1990; Khodakaramian and Ghasemi, 2002; Stead, 1992). Citrus canker disease was first observed in Japan in late 19th century. It was then reported from United States in 1910 (Gottwald *et al*., 2001; Graham *et al*., 1990). Pathotype A or Asian form of the pathogen has the widest host range and is very destructive (Khodakaramian and Ghasemi, 2002; Bora and Bhagabati, 1996). Almost all citrus cultivars are susceptible to this pathotype, but the degree of susceptibility to this disease is higher among some grape fruits, limes, lemons and some orange cultivars (Gottwald *et al*., 1993; Timmer *et al*., 2000; Vernière *et al*., 1998).

In Florida, during an eradication program, millions of infected citrus trees with this pathotype were eradicated and burned down (Gottwald *et al*., 1993, 2001; Schubert *et al*., 1996). The symptoms of Asian citrus canker appear on leaves, fruits and twigs and include lumpy lesions that first appear

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on the under side and later on the upper side of the leaves (Khodakaramian and Ghasemi, 2002; Bora and Bhagabati, 1996). Along with the spread of the lesions, their sides become lumpy which is called volcano shape lesion (Khodakaramian and Ghasemi, 2002; Bora and Bhagabati, 1996). Sever infection of citrus trees with pathotype A results in leaf and fruit defoliation, twig dryness and finally complete decline of the tree (Khodakaramian and Ghasemi, 2002; Bora and Bhagabati, 1996).

Nowadays, citrus trees in about 30 countries around the world are suffering from this disease. In Iran, citrus bacterial canker was first observed on lemon trees in Kerman province in 1989 and the causal agent was isolated and identified (Khodakaramian and Ghasemi, 2002). According to the previous studies some bacterial strains isolated from citrus trees in Southern provinces of Iran were capable of inducing typical canker symptoms on many citrus species including *Citrus aurantifolia*, *C. poncirus*, *C. grandis*, *C. sinensis*, *C. aurantium*, *C. jambhiri*, *C. limon*, *C. reticulata*, *C. medica* and *C. paradisi* (Khodakaramian and Ghasemi, 2002).

For controlling citrus bacterial canker disease different methods such as eradication of diseased trees, prevention of importation and distribution of infected plants, chemical methods, planting resistant varieties and the use of biological agents have been studied (Cubero and Graham, 2002; Gottwald *et al*., 1993; Graham, 2001; Graham *et al*., 1992; Khodakaramian and Ghasemi, 2002; Leite and Mohan, 1990; Leite *et al*., 1987; Bora and Bhagabati, 1996; Rodrigues *et al*., 1998; Schubert *et al*., 2001; Timmer *et al*., 2000; Vernière *et al*., 1998). The use of bacterial and fungal antagonists have recently been applied to control several plant diseases including citrus canker (Heydari *et al*., 2005; Bora and Bhagabati, 1996; Shahriari *et al*., 2005).

The objective of this study were to evaluate pseudomonas bacterial strains isolated from citrus trees in southern Iran in biological control of citrus bacterial canker disease.

MATERIALS AND METHODS

The following experiments were conducted and carried out in Iranian Research Institute of Plant Protection during year 2006:

Evaluation of Antagonistic Activity of Bacterial Isolates Against Causal Agent of Bacterial Citrus Canker in *in vitro* Condition

For investigation of the antagonistic activity of *Pseudomonas fluorescent* isolates in *in vitro* condition, isolates which were previously isolated from citrus trees in Southern Iran were cultured on King's B Medium (KMB) for 48 h at 25°C. Bacterial colonies were then removed using sterilized cotton and ethanol. Two drops of chloroform were added to each petri dish and allowing it settle for 20 min in up side down position. The lids were then removed and petri dishes were exposed to air flow for 30 min. One milliliter of each pathogenic bacterial suspension was spread in each above mentioned petri dish and were incubated for 72 h at 28°C. Antagonistic activity of different bacterial isolates were determined by measuring inhibition zone of pathogenic bacteria in petri plates. Based on the results of this test, some bacterial isolates with highest performance were selected for green house experiment.

Investigation of the Antagonistic Activity of Pseudomonas Fluorescent Isolates Against the Causal Agent of Citrus Bacterial Canker in Green House Condition

Bacterial suspension with the concentration of 10^8 cfu mL^{-1} was prepared from each isolate using spectrophotometer. Water lemon young trees leaves were sprayed by this suspension. Three days after bacterial application, plants were inoculated by pathogenic bacteria by spraining their leaves with bacterial suspension of *X. axonopodis*. Plants were then covered by plastic sheets and were incubated

in green house at 25-27°C and 55-60% relative humidity for 24 days. This experiment was conducted in a completely randomized design with 7 treatments each with 3 replications. Each replication consisted of 5 lemon plants (Ammani variety) and disease evaluation factor was the total number of lesions on 50 leaves appeared on trees two months after inoculation.

RESULTS AND DISCUSSION

The results of antagonistic activity of bacterial isolates against bacterial citrus canker causal agent in *in vitro* condition are shown in Table 1. Table 1 shows inhibition zone induced by antagonistic bacteria varied among different bacterial isolates. The highest inhibition zone belonged to *P. fluorescent* (strain 15) and *P. putida* (strain 1). The lowest inhibition zone (0) was induced by *P. putida* (strains 8, 13 and 18).

Table 2 shows the results of the evaluation of the antagonistic activity of antagonistic bacteria against the causal agent of bacterial citrus canker in green house experiment. Two strains of *P. fluorescent* (16 and 19) showed the highest antagonistic activity against the pathogenic bacterium with significant reduction of the leaf spots. In this experiment, all five test bacterial strains reduced the incidence of citrus bacterial canker significantly.

Table 1: Antagonistic activity of different Pseudomonads isolates against *Xanthomonas axonopodis* pv. citri in *in vitro* condition

Bacterial strain	Average diameter of inhibition zone (cm)
Pf-19	6.40A
Pf-9	6.16A
Pf-15	5.93A
Pp-1	5.93A
Pp-12	4.86B
Pf-5	4.78B
Pf-16	3.93C
Pp-2	3.83C
Pp-20	3.13D
Pf-9	3.00D
Pp-11	2.53D
Pf-10	1.33E
Pf-3	1.16E
Pf-14	1.03E
Pp-17	0.40F
Pf-7	0.36F
Pf-4	0.26F
Pp-8	0.00F
Pp-13	0.00F
Pp-18	0.00F

In each column values marked with the same letter(s) are not significantly different according to Duncan Multiple range test ($p>0.05$)

Table 2: Evaluation of selected antagonistic bacterial strains in biological control of citrus bacterial canker disease in the greenhouse

Bacterial strain	No. of disease spots in 50 leaves
Control (+)	54.66A
Pf-10	35.00B
Pp-12	34.66B
Pp-20	17.66C
Pf-16	13.66C
Pf-19	13.00C
Control (-)	00.00D

In each column values marked with the same letter(s) are not significantly different according to Duncan Multiple range test ($p>0.05$)

The over all results of this study show that it may be possible to control citrus bacterial canker disease using antagonistic bacteria. In *in vitro* condition, all test bacterial isolates with the exception of 3 of them showed significant antagonistic activity against *X. citri* the causal agent of bacterial citrus canker. Screening bacterial isolates using the procedure followed in this study is a common and scientifically accepted strategy for selection of the best performed candidates in biocontrol studies (Misaghi and Grogan, 1969; Heydari *et al*., 2005). The variation in antagonistic activity of bacterial isolates could be due to different factors involved in their mode of action and mechanisms (Misaghi and Grogan, 1969; Heydari *et al*., 2005).

Based on the results of *in vitro* experiment 5 isolates were selected for green house experiment. All selected isolates performed well in the green house and reduced the disease symptoms significantly (compared with the positive control). There was a variability among 5 isolates in their antagonistic activity that could be due to the differences in their mode of action such as colonization ability, antimicrobial metabolite production, etc. (Misaghi and Grogan, 1969). *P. fluorescent* isolates performed more efficiently than *P. putida* strains perhaps due to their higher antagonistic ability. *P. fluorescent* strains have shown to be very efficient microbial agents in biocontrol of plant diseases (Misaghi and Grogan, 1969; Heydari *et al*., 2005).

According to several previous studies, bacterial canker has been shown to be a very serious and destructive disease of citrus infecting almost all species and varieties around the world (Cubero and Graham, 2002; Gottwald *et al*., 1993; Graham, 2001; Graham *et al*., 1992; Khodakaramian and Ghasemi, 2002; Leite and Mohan, 1990; Leite *et al*., 1987; Bora and Bhagabati, 1996; Rodrigues *et al*., 1998; Schubert *et al*., 2001; Timmer *et al*., 2001; Vernière *et al*., 1998). There is no doubt that for controlling this important disease in citrus orchards a combination of different strategies should be applied and used.

CONCLUSION

Results of this study although were obtained in greenhouse condition indicated that the bacterial antagonists were capable of reducing the incidence of bacterial canker disease significantly. Based on the results of this study it is concluded that biological control is potentially an effective method and may be used as an important component of Integrated Pest Management (IPM) strategies for controlling and managing citrus bacterial canker which is considered to be on of the most damaging and destructive diseases of citrus around the world.

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