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APPLICATION OF RHIZOBACTERIA FOR INDUCTION OF SYSTEMIC RESISTANCE TO BACTERIAL BLIGHT OF COTTON CAUSED BY *XANTHOMONAS CAMPESTRIS* PV. *MALVACEARUM* USING FLUORESCENT PSEUDOMONADS OF RHIZOSPHERE

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Abstract: The ability of fluorescent pseudomonads of cotton rhizosphere of induction of systemic resistance (ISR) against bacterial blight of cotton was investigated. Of the 21 isolates tested, 19 were observed to increase the resistance of plants. This was demonstrated by a lower percentage of infected leaf area. On the basis of growth promotion and ISR induction ability, isolates 148, 35Q, 16Q and 113 were selected for further investigations. All isolates increased the resistance of plants, and per cent of infected area on leaves of these treatments were lower than those control of plants. Levels of peroxidase (PO) and phenylalanine ammonia lyase (PAL) activity in the leaves of bacterized plants with selected isolates were similar to that in control plants, but after inoculation of leaves with the pathogen, the amount of these enzymes increased in bacterized plants to high levels. The increase of enzyme activity in control plants was low after inoculation.

Key words: fluorescent pseudomonads, induced systemic resistance, *Xanthomonas campestris* pv. *malvacearum*, cotton, peroxidase, phenylalanine ammonia lyase

INTRODUCTION

Bacterial leaf blight of cotton caused by *Xanthomonas campestris* pv. *malvacearum* is one of the most serious diseases which occurs in all the cotton-growing countries of the world and affects the yield and fibre quality (Hussain and Tahir 1993). The existing commercial cultivars of cotton do not provide sufficient levels of disease resistance. Moreover, the use of synthetic chemicals for managing the disease was discouraged recently due to their hazardous nature and pollution to the environment. Biological control of plant diseases using antagonistic microorganisms was suggested as an alternative to the hazardous and expensive chemical pesticides (Emmert and Handelsman 1999).

Many microorganisms from the rhizosphere can positively influence plant growth and plant health, and are referred to as plant growth promoting rhizobacteria (PGPR) (Hass and Defago 2005). These microbes induce resistance in different plant species against the infection of fungal (Howell and Stipanovic 1979), bacterial (Park and Kloepper 2000) and viral (Maurhofer *et al.* 1994) pathogens. The signaling pathway controlling rhizobacteria mediated induction of systemic resistance clearly differs from pathogen-induced systemic acquired resistance (SAR) in that it is not associated with the accumulation of salicylic acid and induction of PRs before pathogen invasion, and is one of the mechanisms by which rhizobacteria, especially fluorescent pseudomonads, can suppress diseases (Bakker *et al.* 2007; Pieterse *et al.* 1996).

The objective in this study was to investigate a possible application of rhizobacteria for management of bacterial blight of cotton. Also a possible mechanism by which *Pseudomonads* induce the resistance of plant, was investigated by enzymatic assays.

MATERIALS AND METHODS

Field sampling and isolation of bacteria

In July 2008, a total of 100 cotton plants were collected from two fields of south of Tehran. These sites were selected because the soils have been previously cropped for at least 20 years with cotton. Roots were gently removed from soil and placed in plastic bags before their transportation to the laboratory. Adhering soil was carefully brushed off the plant roots, followed by gentle washing of the roots in sterile water. One gram of roots were placed in 9 ml of 0.1 M phosphate buffer (pH 7) supplemented with 0.025% Tween 20 and vortexed for 5 min. 100 µl of first to forth diluents transferred to King's medium B or KB supplemented with chelator 120 ppm 8-hydroxyquinoline (8OHQ) (Geels *et al.* 1985). Fluorescent pseudomonad's colonies that developed at these plates were isolated.

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Greenhouse studies

Screening ISR inducing pseudomonads

21 isolates of *Pseudomonas* with fast ability of growth on KB/8OHQ were selected for greenhouse studies. *Pseudomonas aeruginosa* 7NSK2 and *Pseudomonas fluorescens* CHA0 were used as reference strains. Pseudomonads were grown on KB agar plates for 30 h at 26°C. Bacteria were harvested by scraping cells from the agar, suspending them in 0.1 M phosphate buffer (pH 7), and washing the suspensions twice by centrifugation (for 10 min at 6 000 rpm). Washed pellets were suspended in 1% methylcellulose solution and optical density of these suspensions adjusted to 0.6 at 620 nm by spectrophotometer (PG instruments T70+). Acid delinted and neutralized cotton seeds were disinfected for 3 min in a 0.5% (w/v) sodium hypochlorite solution and thereafter rinsed 4 times with sterile distilled water. Seeds were subsequently dipped for 45 min in cell suspension. Seeds non-treated with 1% methylcellulose solution without bacteria were kept as a control. They were air-dried at 27°C for 30 min and sown in plastic pots (diameter 8 cm; depth 8.9). The soil was sandy clay loam provided from cotton fields and sterilized. The plants were grown in a greenhouse maintained at 26–28°C and 65–75% relative humidity (RH). 25 days after sowing (stage 4 leaves), plants were inoculated with *X. campestris* pv. *malvacearum* (XCM) by spraying of leaves with suspension containing 10^6 cells per ml, when the stomata were open (in the morning). After inoculation, the plants were maintained under day and night temperatures of 31/26°C and 95–100% RH for two days. High levels of moisture were provided by an ultrasonic humidifier. After 48 h the RH and temperature of greenhouse was restored to previous conditions. After 15 days three leaves per each pot were selected and per cent of infected area was calculated by the following formula:

$$\text{Per cent of infected leaf area} = \frac{\text{area of lesions on three leaves}}{\text{total area of three leaves}} \times 100$$

Leaf area was measured using a leaf area meter. Area of lesions calculated from the number of lesions and their diameter. Approximate diameter of lesions scored by scale in which 0.1 = too small lesions, 0.5 = lesions with about 0.5 mm diameter, 1 = lesions with about 1 mm diameter, 1.5 = lesions with 1–1.5 mm diameter, 2 = lesions with 1.5–2 mm diameter, 2.5 = lesions with 2–2.5 mm diameter, 3 = lesions with 2.5–3 mm diameter.

There were three pots for each treatment and three plants for each pot. In addition, plants were removed from pots and their fresh and dry weight was determined.

Selected isolates present at this stage were identified on the basis of tests for cytochrome oxidase (Kovacs 1956), arginine dihydrolase (Thornley 1960), gelatin liquefaction (Dye 1968), production of diffusible non-fluorescent pigment (pyocyanin) on King's A medium (King *et al.* 1954), tobacco HR (Klement 1963), nitrate reduction, growth at 4 and 41°C, levan production, (Shaad *et al.* 2001) and the ability to growth on carbon sources such as L-arabinose, D-galactose, trehalose, meso-inositol, sorbitol, L-tartrate was tested with the basal medium of Ayers *et al.* (1919).

Assays for peroxidase (PO) and phenylalanine ammonia lyase (PAL) in plants treated with selected isolates

Selected isolates were used in other experiment for investigation of their ability to induce defense related enzymes in cotton plants. Seed treatments with isolates, inoculation of leaves with XCM and greenhouse conditions in this experiment were similar to those mentioned above. Leaf sampling for measurement of enzyme activity was performed at 0, 24, 48, 72, and 92 hours after inoculation of leaves with XCM.

One gram of the leaf tissues collected from plants was immediately homogenized with 2 ml of sodium phosphate buffer, 0.1 M (pH 7.0) at 4°C. The homogenate was centrifuged for 20 min at 12 000 rpm. Protein extracts prepared from leaves were used for the estimation of PO and PAL activity.

PO activity was assayed spectrophotometrically (Hammerschmidt *et al.*). The reaction mixture consisted of 1.5 ml of 0.05 M pyrogallol, 100 µl of enzyme extract and 0.5 ml of 1% H_2O_2. The reaction mixture was incubated at room temperature. The change in absorbance was recorded at 420 nm for one min and the boiled enzyme preparation served as blank. The enzyme activity was expressed as change in the absorbance of the reaction mixture min/g on fresh weight basis.

PAL activity was determined as the rate of conversion of L-phenylalanine to *trans*-cinnamic acid at 290 nm as described by Dickerson *et al.* (1984) with minor modifications. The assay reaction consisted of 200 µL crude extract and 1 800 µL of 6 µM l-phenylalanine in 1 000 µL of 500 mM Tris-HCl buffer (pH 8·5). The mixture was incubated at 37°C for 1 h and measured spectrophotometrically at 290 nm. The amount of *trans*-cinnamic acid synthesized was calculated using its extinction coefficient of 9 630 M/cm. Enzyme activity was expressed on a fresh weight basis (n mol min/g).

Activity gel electrophoresis for detection of peroxidase isoforms

For native anionic polyacrylamide gel electrophoresis, resolving gel of 12% acrylamide and stacking gel of 6% acrylamide concentration were prepared. After electrophoresis for 4 hours (85 V) at 10°C, the gels were incubated for 5 min in the solution containing 0.05 M pyrogallol, then 33 ml H_2O_2 solution were added with constant shaking until bands appeared.

RESULTS

All of isolates except 13Q and EQ significantly limited the per cent of infected area on leaves (Table 1). Ability of strain CHA0 in induction of systemic resistance was low but fresh weight of plants treated with this strain was higher than of non-treated control plants. However, there was no significant difference between two treatments. The ability of most isolates in induction of resistance was equaled or outranked the reference strain 7NSK2. Isolate 10A drastically limited the per cent of infected area on leaves but it has a negative effect on plant and decreased the fresh weight of plant.

On the basis of growth promotion and ISR induction ability, isolates 148, 35Q and 16Q were selected for further

Table 1. Effect of isolates on cotton plants growth and severity of bacterial leaf blight

Isolates	Fresh weight*	Per cent of infected area on leaves
148	7.4 a***	1.083 e
120	6.4 bc	3.9 bcd
113	6.3 abc	3.783 bcd
7NSK2	5.9 abcd	3.74 bcd
34Q	5.7 abcde	4.363 bc
CHA0	5.7 abcde	5.7 b
35Q	5.6 abcde	1.887 cde
60	5.6 abcde	6 b
2Q	5.1 abcde	3.997 bcd
EQ	5.1 abcde	9.973 a
32Q	5.12 abcde	3.633 bcd
27Q	5.1 abcde	1.91 ecd
AQ	5 abcde	3.55 bcd
16Q	4.9 abcde	1.753 ed
CQ	4.4 bcdef	3.67 bcd
147	4.3 bcdef	4.72 b
26Q	4.3 bcdef	4.28 bc
13Q	4.3 bcdef	10.51 a
17Q	3.7 bcdef	5.357 b
135	3.4 cdef	3.531 bcd
6Q	3.0 def	1.043 e
42Q	2.8 ef	4.353 bc
10A	1.7 f	1.048 e
CX**	4.2 bcdef	9.103 a

* three pots, three plants per pot
** inoculated control
*** values followed by the same letter were not significantly different at 5%, as determined by variance analysis followed by Duncan's test

investigations. Isolate 113 was used as a weak inducer of ISR for comparison. Isolates: 148 were identified as *P. fluorescence*, 113 as *P. putida* and 35Q and 16Q as *P. aeruginosa*. Effect of selected isolates on growth of plants was similar to the results from selection process (Fig. 1 and Table 1).

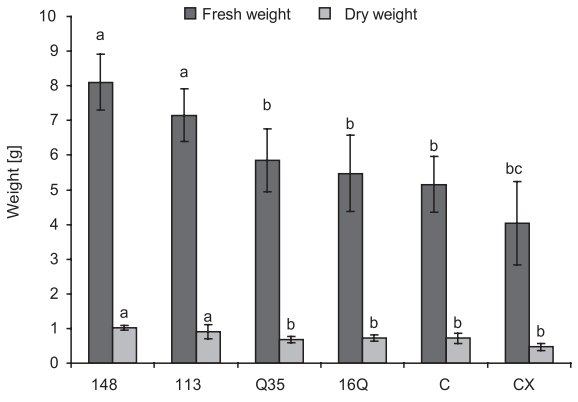

Fig. 1. Influence of selected isolates on fresh and dry weight of cotton plants. Isolates applied at the time of sowing. These factors were determined 17 days after inoculation of leaves with pathogen. Values followed by the same letter were not significantly different at 5%, as determined by variance analysis followed by Duncan's test

Fresh and dry weight of plants treated with isolates 113 and 148 were significantly greater than of both healthy and pathogen infected control plants (Fig. 1). All isolates significantly limited the percentage of infected area on leaves (Fig. 2).

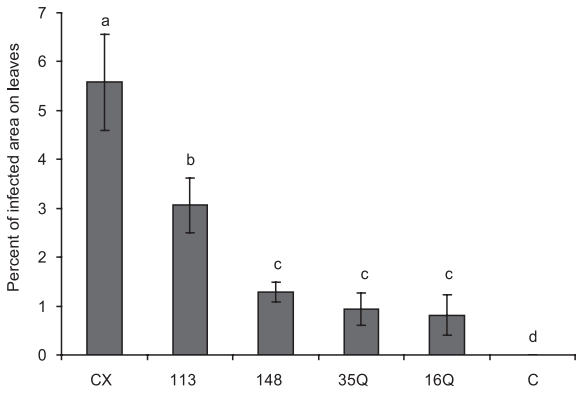

Fig. 2. Influence of selected isolates on disease suppression. Isolates applied at the time of sowing. Per cent of infected area on leaves determined 17 days after inoculation of leaves with pathogen. Values followed by the same letter were not significantly different at 5%, as determined by variance analysis followed by Duncan's test. C: healthy plants with no *Pseudomonas*. CX: infected plants with no *Pseudomonas*

Levels of peroxidase activity in the leaves of bacterized plants were similar to those in control plants, but after leaf inoculation with XCM, levels of this enzyme highly increased in bacterized plants. The increase of peroxidase activity in control plants was low (Fig. 3).

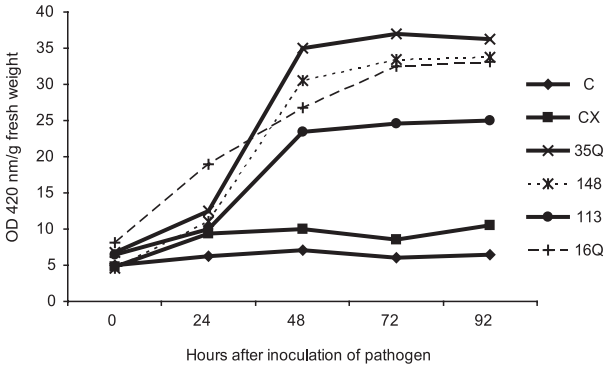

Fig. 3. Peroxidase activity of *Pseudomonas*-treated cotton plants at the time of inoculation of leaves with pathogen and afterwards. Plants were treated with selected *Pseudomonas* isolates at the time of sowing and inoculation of leaves with pathogen was performed at 25 days after sowing. Data are means from three independent samples. C: Healthy plants with no *Pseudomonas*, CX: inoculated plants with no *Pseudomonas*

Native-PAGE analysis of POD revealed that the expression of PO1 was observed in both the control and *Pseudomonas* treated plants (Fig. 4). However the expression of PO1 was comparatively higher in *Pseudomonas* treated plants. Isolate 35Q induced PO2 and PO3 in addition to PO1.

PAL activity increased with the increase in time i.e., second day after infection with the pathogen. In all sets of

treatments, bacterized plants showed significantly higher level of PAL activity than in the control treatment. Plants inoculated with the pathogen alone showed increased activity of PAL but less than those observed in bacterized plants (Fig. 5).

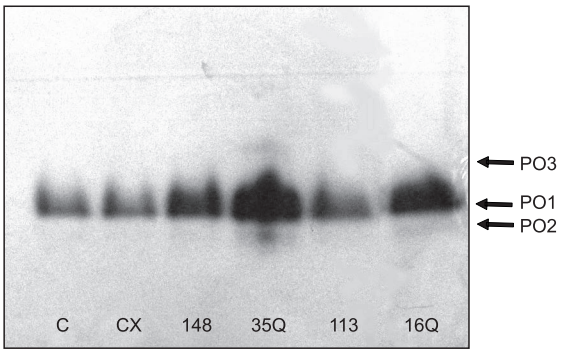

Fig. 4. Native-PAGE (12%) analysis for POD isozyme profile induced by selected isolates in cotton plants challenged with the pathogen *X. campestris* pv. *malvacearum*. C: healthy plants with no *Pseudomonas*. CX: infected plants with no *Pseudomonas*

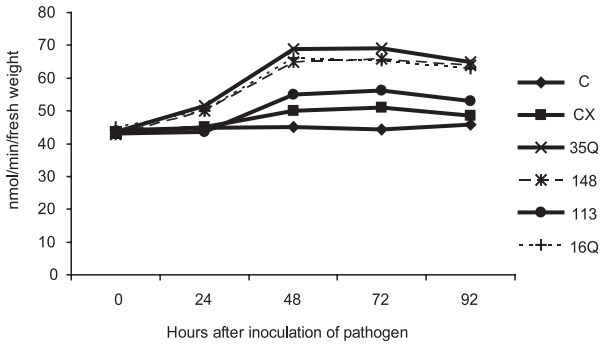

Fig. 5. PAL activity of *Pseudomonas*-treated cotton plants at the time of inoculation of leaves with pathogen and afterwards. Plants were treated with selected *Pseudomonas* isolates at the time of sowing and the inoculation of leaves with pathogen was performed at 25 days after sowing. Data are means from three independent samples. C: Healthy plants with no *Pseudomonas*, CX: inoculated plants with no *Pseudomonas*

DISCUSSION

In this work we used King's medium B supplemented with a chelator 8-hydroxyquinoline for isolation of rhizobacteria. Use of this medium resulted selection of isolates with high capability of siderophore production. Role of siderophores in induction of ISR was widely investigated and proved in many cases (Bakker *et al.* 2007). More of isolated bacteria had a high capability of ISR induction that equaled or outranked the reference strain 7NSK2.

Isolate 10A drastically limited the percentage of infected area on leaves but it has a negative effect on plant and reduced fresh weight of plant. This isolate showed a high inhibitory activity against *Rhizoctonia solani* AG4 (Data not shown). Perhaps this isolate produces an antibiotic with phytotoxic effect, that reduces the growth of plant or maybe levels of antibiotic production by this isolate is too high. Despite some antibiotics produced by rhizobacteria, such as 2,4-diacetylphloroglucinol (DAP) or phenazine-1-carboxylic acid (PCA) important in disease suppression, they can be toxic to plants at high concentrations and can induce SAR in the same way as a pathogen causing localized necrosis (Maurhofer et al. 1995).

After inoculation with the pathogen, PO and PAL activity of all *Pseudomonas*-treated plants drastically increased and level of infected area on leaves of these plants were significantly lower than those from control plants. These results revealed that, presence of rhizobacteria on roots of plant, does not influence defense related enzymes in areal part of plant, but increases its responsiveness to pathogens invasion. Whereas the signaling pathway controlling rhizobacteria mediated ISR is not associated with the accumulation of defence-related enzymes before pathogen invasion (Van Peer *et al.* 1991; Pieterse *et al.* 1996), it seems that induced resistance observed in this study is mediated by the ISR pathway. Phenotypically, ISR is similar to SAR that is triggered by necrotizing pathogens. However, SAR requires accumulation of salicylic acid and defence-related enzymes in the plant (Sticher *et al.* 1997), ISR does not and, instead, is dependent on intact responses to ethylene and jasmonic acid (Pieterse *et al.* 1998).

PO is a key enzyme in the biosynthesis of lignin and other oxidised phenols (Bruce and West 1989). PO catalyzes the oxidation of hydroxy-cinnamyl alcohols to free radical intermediates, which subsequently are coupled into lignin polymers (Gross 1980). This enzyme is also highly toxic to pathogens. PAL is a key enzyme of phenylpropanoid metabolism which leads to the synthesis of phenols (Massala *et al.* 1980).

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POLISH SUMMARY

STOSOWANIE BAKTERII RIZOSFERY W CELU INDUKOWANIA SYSTEMICZNEJ ODPORNOŚCI NA BAKTERYJNĄ ZGORZEL BAWEŁNY WYWOŁYWANĄ PRZEZ *XANTHOMONAS CAMPESTRIS* PV. *MALVACEARUM* PRZY UŻYCIU FLUORYZUJĄCYCH PSEUDOMONAS

Badano zdolność fluoryzujących bakterii Pseudomonas z rizosfery bawełny do indukowania systemicznej odporności na zgorzel bawełny wywoływaną przez *Xanthomonas campestris* pv. *malvacearum*. Z 21 testowanych izolatów Pseudomonas 19 przyczyniło się do wzrostu odporności roślin. To wyrażało się niższym procentem porażenia powierzchni liści. Na podstawie stymulowania wzrostu roślin i indukowania odporności izobaty 148, 350, 16Q i 113 zostały wybrane do dalszych badań. Wszystkie te izobaty powodowały zwiększenie odporności roślin, a procent porażonej powierzchni liści był niższy w porównaniu do roślin kontrolnych. Poziom aktywności peroksydazy (PO) i amonowej liazy fenyloalaniny (PAL) w liściach roślin traktowanych wybranymi izolatami bakterii Pseudomonas był podobny do poziomu w kontroli, ale po inokulacji liści patogenem ilość tych enzymów w roślinach traktowanych bakteriami bardzo wzrosła. Wzrost aktywności enzymów w roślinach kontrolnych był niski po inokulacji.