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Induction of systemic resistance to bacterial blight caused by *Xanthomonas axonopodis* pv. *malvacearum* in cotton by fluorescent pseudomonads of cotton rhizosphere

Fallahzadeh, V.[*] and Ahmadzadeh, M.

Department of plant protection, Faculty of agriculture, University of Tehran, Karaj, Iran.

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Bacterial blight of cotton is one of the most important diseases of cotton and it can cause serious damage in favorable conditions. In this work the ability of fluorescent pseudomonads of cotton rhizosphere in induction of systemic resistance against this disease was investigated. Out of 200 isolates, 39% pertained to fluorescent pseudomonads group. To evaluate the ability of antibiotic production by these isolates, dual culture assays against *Rhizoctonia solani* AG4 was investigated. 56 of these bacteria had inhibition effect on this fungus. In siderophore production assay, 19 of them could grow in 24 h on KB medium supplemented with strong chelating agent of 8-hydroxiquinoline. On the base of these assays 8 of these isolates were selected for induced systemic resistance experiments. In greenhouse studies all of isolates significantly suppressed the disease on plant. Suppression of disease in 5 of them was more than the reference strain *Pseudomonas aeruginosa* 7NSK2. 35Q and 7NSK2 had highest effect on growth of cotton plants as growth of plants treated with these isolates, were more than or equaled to healthy control plants. 10AQ drastically suppressed the disease but it had negative effect on plant and significantly decreased the growth factors of the plants.

Key words: fluorescent pseudomonads, induced systemic resistance, *Xanthomonas axonopodis* pv. *malvacearum*, cotton

Introduction

Cotton (*Gossypium* spp.) is one of the most important fibre and commercial crops worldwide. Cotton seeds are also used as oil cake and edible oil (Anonymous, 1981). It is cultivated in a total area of about 33.9 million hectares in 80 countries which accounts for 125 million bales of lint (Udikeri *et*

[*] Corresponding author: Vahid F.; e-mail: vfm128@gmail.com

al., 2004). Diseases and pests cause considerable yield losses. Among them, bacterial leaf blight of cotton caused by *Xanthomonas axonopodis* pv. malvacearum (E.F. Smith) Vauterin 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 disease is also known as seedling blight, angular leaf spot, vein blight, black arm and boll rot based on the plant part infected (Hillocks, 1981). The pathogen is internally seed-borne (Verma and Singh, 1974) and the yield losses due to bacterial leaf blight have been estimated up to 50% under field conditions (Inner, 1970; Ramapandu *et al*., 1979; Watkins, 1981). The existing commercial cultivars of cotton do not provide sufficient levels of disease resistance. Moreover, use of synthetic chemicals for managing the diseases was discouraged recently due to their hazardous nature and pollution to the environment. Biological control of plant diseases using antagonistic microorganisms has been 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 (ISR) 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 onset of ISR is thought to result from the perception of one or more ISR-eliciting compounds (produced by rhizobacteria) at the plant root surface. Upon binding by a receptor, transduction of plant-produced and mediated signals would lead to the state of ISR. Antibiotics (Iavicoli *et al.*, 2003; Weller *et al*., 2004) and iron-regulated metabolites such as pseudobactin siderophore (Maurhofer *et al*., 1994; Leeman *et al*., 1996; Meziane *et al*., 2005; Ran *et al*., 2005), N-alkylated benzylamine derivative (Ongena *et al*., 2005), Salicylic acid (De meyer *et al*., 1999) and pyochelin siderophore (Audenaert *et al*., 2002) have been widely investigated and their substantial role in this phenomenon have been proved in many cases.

The objective in this study was to investigate the possible application of rhizobacteria for management of bacterial blight of cotton.

Materials and methods

Field sampling, isolation and identification of bacteria

In July 2008, a total of 100 cotton plants were collected from two fields of Varamin cotton research centre in Tehran province of Iran. These sites were selected because they were planted continuously with cotton for at least 20 years. Soil was gently removed from roots and transported to the lab in plastic bags. In the lab residual adhering soil was carefully brushed off the plant roots, followed by gentle washing of the roots in sterile water. One gram of roots was placed in 9 ml of 0.1 M phosphate buffer (pH 7), supplemented with 0.025% Tween 20 and vortexed for 5 min. Thereafter, 100 µl of first to forth dilutions transferred to King's B medium (KB) plates and plates paced in incubator at 26°C. After 40 h fluorescent pseudomonad-like (determined under 366nm UV light) colonies that developed on these plates were purified and identified on the basis of tests for cytochrome oxidase, arginine dihydrolase, gelatin liquefaction, production of diffusible non-fluorescent pigment (pyocyanin) on King's A medium, tobacco HR, nitrate reduction, growth at 4 and 41°C, levan production, and the ability to growth on carbon sources such as L-arabinose, D-galactose, Trehalose, Meso-inositol, Sorbitol, L-tartrate was tested by the basal medium of Ayers (Schaad *et al*., 2001).

Selection of bacterial isolates with high ability of producing siderophore and antibiotic

To selection of bacterial isolates with high ability of siderophore production, isolates were inoculated on modified KB medium supplemented with a strong chelator, 8-hydroxyquinoline (8-HQ-KB) (Geels *et al*., 1985). The iron availability of this medium is too low, then only microorganisms with high ability of siderophore production can grow on this medium. Inoculated media incubated at 26°C for 38 h and during this time, fast growing isolates were determined.

To selection of bacterial isolates with high ability of antibiotic production, inhibitory effect of isolates on pathogenic fungus, *Rhizoctonia solani* AG4, was determined on the basis of dual-culture assays. This pathogen is one of the most important agents of seed decay and seedling damping off of cotton. Isolates were spotted with sterile loop around the edge of a plate of PDA. Plates were incubated for 24 h at 25°C, and one 8-mm-diameter agar disk of fungus from a 1-week-old PDA culture was placed in the center of the plate. After another week, the width of the inhibition zone between each bacterial colony and the fungal colony was measured.

Greenhouse studies

Pseudomonas strains with fast growth characteristic on 8-HQ-KB and higher inhibition zone against *R. solani* AG4 were selected for greenhouse studies. *P. aeruginosa* 7NSK2 was used as reference strain. Strains 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 g). Washed pellets were suspended in 1% methylcellulose solution and optical density of these suspensions adjusted to 0.6 at 620 nm by a 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 suspensions. Seeds mock-treated with 1% methylcellulose solution without bacteria were kept as a control. The seeds were air-dried at 27°C for 30 min and sown in plastic pots (8 cm in diameter and a depth of 8-9 cm). The soil used was sterilized sandy clay loam from cotton fields. Plants were grown in a greenhouse maintained at 26 – 28ºC and 65 – 75% relative humidity (RH). At 25 days after sowing, plants were inoculated with *Xanthomonas axonopodis* pv. *malvacearum* (Xam) by spraying leaves with a suspension containing 10^6 cells ml^{-1}, when the stomata were open (in the morning). After inoculation, plants were maintained at day and night temperatures of 31 and 26°C respectively and at 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 were returned to previous conditions. After 15 days three leaves per each pot were selected and the percentage of infected area calculated by the following formula:

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

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.

Results and discussion

In this study KB medium was used for isolation of fluorescent pseudomonads. Out of 200 isolates that were selected for further investigations, 56% of them showed inhibitory effect against *R. solani* AG4 and only 19% of them could grow in 24 h on 8-HQ-KB. Most of the fastest growing isolates that were developed on 8-HQ-KB were identified as *Pseudomonas aeruginosa*. Indeed, because of low iron availability of 8-HQ-KB medium, siderophore

production and iron competitive ability of strains that can grow on this medium is too high (Geels *et al*., 1985). Role of siderophores in induction of ISR have been widely investigated and proved in many cases (Bakker *et al*., 2007). On the basis of these assays, eight of strains with highest inhibitory effect against the fungus and fastest growing ability on 8-HQ-KB were selected for greenhouse studies.

In greenhouse studies, all of the strains significantly limited the percent of infected area on leaves (Fig. 1). Resistance induced by most of the strains was higher than that of reference strain *Pseudomonas aeruginosa* 7NSK2. Growth factors of cotton plants treated with strains 35Q and 7NSK2 was higher than those of other treatments even healthy control plants (Table 2). Strain 7NSK2 is a well known plant growth promoting bacterium and its ability for induction of ISR in different plants has been proven in many cases (Audenaert *et al*., 2002; De Meyer *et al*., 1999a; De Meyer *et al*., 1999b). Siderophores and antibiotic Pyocyanin produced by this strain are major metabolites of this strain that there role in induction of plant resistance has been proven (Bakker *et al*., 2007). In spite of these reports about this strain, its ability in induction of ISR on cotton plants was lower than those of other strains in this study. Perhaps this low performance is as a result of specific interaction between plant and bacterial strains. ISR-inducing rhizobacteria show little specificity in their colonization of roots of different plant species (Van Loon *et al*., 1998). However, elicitation of ISR appears to be highly specific with regard to both the rhizobacterial strain and the plant host. All of strains that were isolated and studied in this study were from cotton rhizisphere, but 7NSK2 originally isolated from barley rhizosphere (Hofte *et al*., 1991).

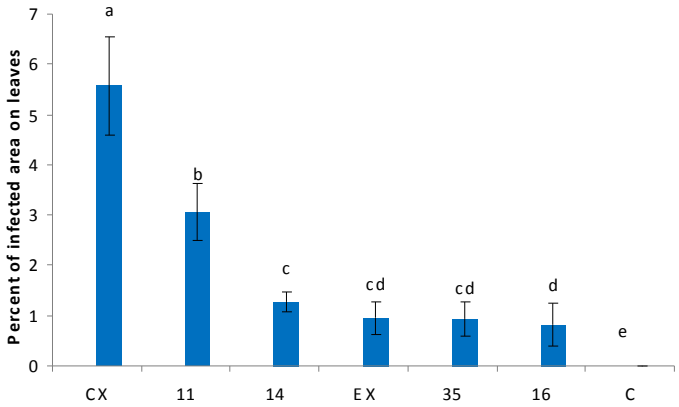

Fig. 1. Influence of selected isolates on disease suppression. Isolates applied at the time of sowing. Percent of infected area on leaves determined 17 days after inoculation of leaves with pathogen. Values followed by the same letter were not significantly different at 5%, as determined by variance analysis followed by Duncan's test. C: healthy plants with no *Pseudomonas*. CX: infected plants with no *Pseudomonas*.

Isolate 10A drastically limited the percent of infected area on leaves but it has negative effect on plant and reduced fresh weight of plant. This isolate showed high inhibitory activity against *Rhizoctonia solani* AG4 (Table 1). 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) are important in disease suppression, but they can be toxic to plants at high concentrations and could induce SAR in the same way as a pathogen causing localized necrosis (Maurhofer *et al*., 1995).

Table 1. Antifungal activity of strains against *Rinzoctonia solani* AG4.

Inhibition zone (mm)	**Bacterial strains**
9a	*P. aeruginosa* 26Q
8.3ab	*P. aeruginosa* CQ
8.1abc	*P. aeruginosa* 10AQ
8abc	*P. aeruginosa* 16Q
7.6cbd	*P. aeruginosa* 27Q
7.2dc	*P. aeruginosa* AQ
7cd	*P. aeruginosa* 36Q
6.6d	*P. aeruginosa* 35Q

Data are from three independent measurements Values followed by the same letter were not significantly different at 5%, as determined by variance analysis followed by Duncan's test.

Table 2. Effect of bacterial strains on growth factors of plants. Isolates applied at the time of sowing. These factors were determined 17 days after inoculation of leaves with pathogen.

Bacterial strains	**Stem long**	**Dry weight of root**	**Wet weight of root**	**Dry weight of foliage**	**Wet weight of foliage**
P. aeruginosa 7NSK2	27.27a	0.13ab	1.26a	0.61ab	4.6 a
P. aeruginosa 27Q	27.18a	0.10bcd	1.08a	0.59ab	4.01 ab
P. aeruginosa 16Q	23.80ab	0.07efd	0.98a	0.52ab	4ab
P. aeruginosa AQ	25.50ab	0.11abcd	1.01a	0.61ab	3.79 ab
P. aeruginosa 35Q	25.87ab	0.14a	1.28a	0.67 a	3.65 ab
C*	21.87bc	0.08cde	1.17a	0.35bc	3.43 b
P. aeruginosa CQ	25.85ab	0.12abc	1.08a	0.45ab	3.33 b
CX**	19.66c	0.05efg	0.94a	0.43ab	3.33 b
P. aeruginosa 26Q	25.72ab	0.11abdc	1.08a	0.48ab	3.27 b
P. aeruginosa 36Q	23.05bc	0.04gf	0.99a	0.40ab	2.83b
P. aeruginosa 10AQ	15.69d	0.02g	0.42b	0.14c	0.81 c

*Inoculated control, **non inoculated control, Data are from three pots, three plants per pot
Values followed by the same letter were not significantly different at 5%, as determined by variance analysis followed by Duncan's test.

References

Anonymous. (1981). Balancing influence of N, P and K on growth and yield values of cotton plant. Bangaladesh Journal of Agricultural Research 6: 36.

Audenaert, K., Pattery, T., Cornelis, P. and Höfte, M. (2002). Induction of systemic resistance to *Botrytis cinerea* in tomato by *Pseudomonas aeruginosa* 7NSK2: Role of salicylic acid, pyochelin, and pyocyanin. Molecular Plant-Microbe Interaction 15: 1147-1156.

Bakker, P.A.H.M., Pieterse, C.M.J. and Van Loon, L.C. (2007). Induced systemic resistance by fluorescent Pseudomonas spp. Phytopathology 97: 239-243.

De Meyer, G., Capieau, K., Audenaert, K., Buchala, A., Métraux, J.P. and Höfte, M. (1999). Nanogram amounts of salicylic acid produced by the rhizobacterium *Pseudomonas aeruginosa* 7NSK2 activate the systemic acquired resistance pathway in bean. Molecular Plant-Microbe Interaction 12: 450-458.

De Meyer, G., Audenaert, K. and Höfte, M. (1999a). *Pseudomonas aeruginosa* 7nsk2-induced systemic resistance in tobacco depends on in planta salicylic acid accumulation but is not associated with pr1a expression. European Journal of plant pathology 105: 513-517.

De Meyer, G., Capieau, K., Audenaert, K., Buchala, A., Métraux, J.P. and Höfte, M. (1999b). Nanogram amounts of salicylic acid produced by the rhizobacterium *Pseudomonas aeruginosa* 7NSK2 activate the systemic acquired resistance pathway in bean. Molecular Plant-Microbe Interactions 12: 450-458.

Emmert, E.A.B. and Handelsman, J. (1999). Biocontrol of plant disease: A (Gram-) positive perspective. FEMS Microbiology Letters 171:1 – 9.

Geels, E.P., Schmidt, E.D.L. and Schippers, B. (1985). The use of 8-hydroxyquinoline for the isolation and pre-qualification of plant growth-stimulating rhizosphere pseudomonads. Biology and Fertility of Soils 1: 167-173.

Hass, D. and Defago, G. (2005). Biological control of soil-borne pathogens by fluorescent pseudomonads. Nature Reviews Microbiology. doi:10.1038/nrmicro1129

Hillocks, R.J. (1981). Cotton disease research in Tanzania. Tropical Pest Management 27:1 – 12.

Hofte, M., Seong, K.Y., Jurkevitch, E., Verstraete, W. (1991). Pyoverdin production by the plant growth beneficial *Pseudomonas aeruginosa* strain 7NSK2: Ecological significance in soil. Plant Soil 130: 249-257.

Howell, C.R. and Stipanovic, R.D. (1979). Control of Rhizoctonia solani on cotton seedlings with *Pseudomonas fluorescens* and with an antibiotic produced by the bacterium. Phytopathology 69:480-482.

Hussain, T and Tahir, M. (1993). Chemical control of bacterial blight of cotton. Pakistan Journal of Phytopathology 5:119 – 121.

Iavicoli, A., Boutet, E., Buchala, A. and Métraux, J.P. (2003). Induced systemic resistance in *Arabidopsis thaliana* in response to root inoculation with *Pseudomonas fluorescens* CHA0. Mol. Plant Microbe Interaction 16: 851–858.

Inner, N.L. (1970). Breeding for bacterial blight resistance. In: Siddig MA, Hughes LC, editors. Cotton growth I the Gezira environment. Sudan: Agricultural Research. p 144.

Leeman, M., Den Ouden, F.M., Van Pelt, J.A., Dirkx, F.P.M., Steijl, H., Bakker, P.A. H.M. and Schippers, B. (1996). Iron availability affects induction of systemic resistance against fusarium wilt of radish by *Pseudomonas fluorescens*. Phytopathology 86:149-155.

Maurhofer, M., Keel, C., Haas, D. and D´efago, G. (1995). Influence of plant species on disease suppression by *Pseudomonas fluorescens* strain CHA0 with enhanced antibiotic production. Plant Pathology 44:40–50.

Maurhofer, M., Hase, C., Meuwly, P. Métraux, J.P. and Défago, G. (1994). Induction of systemic resistance of tobacco to tobacco necrosis virus by the root-colonizing *Pseudomonas fluorescens* strain CHA0: Influence of the gacA gene and of pyoverdine production. Phytopathology 84:139-146.

Meziane, H., Van der Sluis, I., Van Loon, L.C., Hofte, M. and Bakker, P.A.H. M. (2005). Determinants of *Pseudomonas putida* WCS358 involved in inducing systemic resistance in plants. Molegular Plant Pathology 6:177-185.

Ongena, M., Jourdan, E., Schäfer, M., Kech, C., Budzikiewicz, H., Luxen, A. and Thonart, P. (2005). Isolation of an N-alkylated benzylamine derivative from *Pseudomonas putida* BTP1 as elicitor of induced systemic resistance in bean. Molecular Plant-Microbe Interaction 18: 562-569.

Park, K.S. and Kloepper, J.W. (2000). Activation of PR-1a promoter by rhizobacteria which induce systemic resistance in tobacco against *Pseudomonas syringae* pv. *tabaci*. Biological Control 18: 2-9.

Pieterse, C.M.J.,Van Wees, S.C.M., Hoffland, E., Van Pelt, J.A. and Van Loon, L.C. (1996). Systemic resistance in Arabidopsis induced by biocontrol bacteria is independent of salicylic acid and pathogenesis related gene expression. Plant Cell 8: 1225-1237.

Ramapandu, S., Sitaramaraju, K., Subbarao, K. and Prasadarao, M.P. (1979). Screening of cotton germplasm against bacterial blight caused by *Xanthomonas malvacearum*. Indian Phytopathology 32: 486.

Ran, L.X., Li, Z.N., Wu, G.J., Van Loon, L.C. and Bakker, P.A.H.M. (2005). Induction of systemic resistance against bacterial wilt in *Eucalyptus urophylla* by fluorescent Pseudomonas spp. European Journal of Plant Pathology 113: 59-70.

Schaad, N.W., Jones, J.B. and Chun, W. (2001). Laboratory Guide for Identification of Plant Pathogenic Bacteria 3th ed., APS PRESS, 374pp.

Udikeri, S.S, Nimbal, F., Khadi B.M. and Gajbhiye, H. (2004). Technology intervention for stability in cotton production system. In: Khadi BM, Katageri IS, Patil SB, Vamadevaiah HM, Udikeri S, Eshanna, editors. Proceedings of the International symposium on Strategies for sustainable cotton production – A global vision 4. Socio Economics, University of Agricultural Sciences, Dharwad, Karnataka, India. pp. 85 – 89.

Van Loon, L.C., Bakker, P.A.H.M. and Pieterse, C.M.J. (1998). Systemic resistance induced by rhizosphere bacteria. Annual Review Phytopathology 36: 453–483.

Verma, J.P. and Singh, R.P. (1974). Note on screening commercial cotton varieties of India for resistance to races of *Xanthomonas malvacearum* (E.F. Smith), Dowson. Indian Journal of Agricultural Sciences 44:236.

Watkins, G.M. (1981). Compendium of cotton diseases. St Paul MN: American Phytopathology Society. pp 2 – 40.

Weller, D.M., Van Pelt, J. A., Mavrodi, D.V., Pieterse, C.M.J., Bakker, P.A.H.M. and Van Loon, L.C. (2004). Induced systemic resistance (ISR) in Arabidopsis against *Pseudomonas syringae* pv. *tomato* by 2,4-diacetylphloroglucinol (DAPG)-producing *Pseudomonas fluorescens*. Phytopathology 94: S108.

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