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Full Length Research Paper

Isolation of *Fusarium fujikuroi* antagonistic bacteria and cloning of its phenazine carboxylic acid genes

Mostafa Niknejad Kazempour[1]* and Maesomeh Anvary[2]

[1]Depatment of Plant Pathology, Faculty of Agriculture, University of Guilan, P. O. Box. 41635 -1314, Rasht, Iran.
[2]Depatment of Microbiology, Faculty of Sciences, Azad University, Rasht, Iran.

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Bakanae disease caused by *Fusarium fujikuroi* is very important in rice. *Pseudomonas fluorescens* produces a broad-spectrum antibiotic phenazine-carboxylic acid (PCA), which is active against a variety of fungal root pathogens. In this study contaminated rice samples were collected from infected farms of Guilan. 238 bacteria were isolated from rhizosphere and the antagonistic ability of 12 of them was demonstrated with two culture method. Eight of these isolates were identified as *P. fluorescens*. The effects of volatile metabolites produced by antagonistic *P. fluorescens* found in the isolates inhibited growth of *F. fujikuroi in vitro*. Culture filtrate and antibiotics from these isolates inhibited growth of the pathogen. Two genes from seven gene locus of phenazine were cloned in *Escherichia coli* DH5α.

Key words: *Fusarium fujikuroi*, Bakanae disease, *Pseudomonas fluorescens* phenazine-carboxylic acid, rice.

INTRODUCTION

There is an increasing interest in applying microorganisms to control soil-borne plant pathogens. Inconsistent performance of the microorganisms, however, has hampered commercial application. Combining several modes of action against plant pathogens in one single organism by genetic modification can improve the efficacy of biological control agents (Van Loon, 1998). Bacterial secondary metabolites play critical roles in many aspects of bacterium-host interactions. Secondary metabolites that function as virulence factors play a central role in disease by altering host tissues (Kimura et al., 2001; Rahme et al. 1995). Other secondary metabolites produced by beneficial bacteria can function to prevent infection by pathogens by altering the environment and improving the bacterium's ability to compete with pathogens, by inhibiting the activity of pathogens, or by triggering host defenses (Bloemberg and Lugtenberg, 2001; Raaijmakers et al., 2002). The antibiotics phenazine-1-carboxylic acid (PCA) and 2, 4-diacetylphloroglucinol (*Phl*) are major determinants of biological control of soil-borne plant pathogens by various strains of fluorescent *Pseudomonas* spp. (Raaijmakers et al., 1997). The ability to produce phenazines is limited almost exclusively to bacteria and has been reported in members of the genera *Pseudomonas*, *Streptomyces*, *Nocardia*, *Sorangium*, *Brevibacterium*, and *Burkholderia* (Mavrodi et al., 2006; Turner and Messenger, 1986). Particularly among fluorescent *Pseudomonas*, the production of 2,4-diacetylphloroglucinol (DAPG), Plt (pyoluteorin), Prn (pyrrolnitrin) and different derivatives of phenazine has been described (Khan et al., 2005; Thomashow and Weller, 1996). *Pseudomonas putida* WCS358r was modified to produce the antifungal compound PCA (McDonald et al., 2001; Thomashow et al., 1990; Stainer et al., 1996). One possible approach to improve biological control may be the application of combinations of biocontrol agents (Roberts et al., 2005; Duffy and Weller, 1995). By combining microorganisms, multiple antifungal traits can be combined and one may assume that at least one biocontrol mechanism will be functional under the conditions faced by the released biocontrol agents. Moreover, combinations of biocontrol strains are expected to result in a higher level of protection and have potential to suppress multiple plant diseases (Dunne et al., 1998; Guetsky et al., 2001, 2002; Jetiyanon and Kloepper, 2002). It has been demonstrated that natural suppressiveness of the Châteaurenard soil in France against *Fusarium* wilt is based on various mechanisms involving several microbial populations acting alone or together to limit the activity of the pathogen

*Corresponding author. E-mail: nikkazem@yahoo.fr. Tel: 00981316690009. Fax: 0098131669028.

(Alabouvette et al., 1998). Our objective is to clone in *Escherichia coli* DH5α entire locus of PCA from antagonistic bacteria of *Fusarium fujikuroi* isolated from Guilan rice field.

MATERIALS AND METHODS

Isolation of *F. fujikuroi*

Rice bakanae disease samples were collected from infected fields in different areas as Rasht, Lahijan, Foman, Anzaly, Talesh and Astara in the Guilan province, Iran. To isolate *F. fujikuroi*, small pieces of infected root with bakanae disease were washed and surface sterilized with 5% sodium hypochlorite for 10 min. The infected tissues were cultured on acidified potato dextrose agar (PDA). The plates were incubated at room temperature 26°C for a week. The growing colonies of fungi were transferred to new plates for purification and identification.

Isolation of antagonistic bacteria isolates and identification

Antagonistic bacteria which colonized rice rhizosphere, 1 g of excised roots were shaked at 100 rpm in 100 ml of sterile distilled water for 25 min. Fluorescent pseudomonads under UV light (356 nm) were isolatesd on King's medium B.

According to the methodology of Schaad et al. (2001), antagonistic isolates of bacteria were identified by biochemical, physiological and biological tests and PCR.

Screening for antifungal activity

Screening for antifungal activity was performed on PDA medium. In this condition, fungal growth inhibition could be due to production of antifungal metabolites. An agar plug (5 mm diameter) taken from an actively growing fungal culture of *F. fujikuroi* was placed on the surface of the PDA plate. Simultaneously, *P. fluorescens* strains were streaked 3 cm away from the agar plug at sides towards the edge of Petri plates. Plate inoculated with fungal agar plugs alone was used as control. The plates were incubated at 27°C until fungal mycelia completely covered the agar surface in control plate. Strains that inhibited mycelial growth of fungus were tested. Ability of antagonistic bacteria to produce volatile antibiotic, secrete extracellular and produce diffusible antibiotic were tested according to Montealegro et al. (2003).

Results are expressed as means of inhibition (%) of the growth of *F. fujikuroi* in the presence and absence of any bacterial isolate. Percent of the inhibition was calculated using the following formula (Montealegro et al., 2003).

$$\text{Inhibition (\%)} = [(1- (\text{fungal growth} / \text{Control growth}))] \times 100$$

Efficacy of antagonistic bacteria isolates to inhibit *F. fujikuroi in vitro*

F. fujikuroi isolated from a diseased foot rot of the rice cultivar Kazar, was shown to be highly virulent isolate in a subsequent pathogenicity test. Efficacy of the *P. fluorescens* isolates in inhibiting growth of *F. fujikuroi* was tested by streaking each bacterial isolate on one side of a Petri dish containing potato dextrose agar and nutrient agar (PDA+NA) medium. One 5 mm mycelial disc from a 5 days old culture of *F. fujikuroi* on PDA+ NA was placed at the opposite side of the Petri dish and experiments were idenpendently repeated four times. Growth of fungus was inhibited when it grew toward the bacterial colony and the inhibition zone was measured from the edge of mycelium to the bacterial colony edge. The bacterial isolates that inhibited *F. fujikuroi* were identified by specific tests for *P. fluorescens* (Kang et al., 2005).

Demonstration of production of volatile antibiotic

A 250 µl of a antagonistic bacterial suspension (1×10^8 CFU/ mL^{-1}) were placed at the Petri dish containing King's B and a 5 mm disk of a four days old pure culture of *F. fujikuroi* was placed at the center of another Petri dish containing PDA. Both half plates were placed face to face preventing any physical contact between the pathogen and the bacterial suspension, and were sealed to isolate the inside atmosphere and prevent loss of volatiles formed. Plates were incubated at 26°C for 6 days and the growth of the pathogen was measured and compared to controls developed in the absence of the antagonist (mocked inoculation with 6 mm-disk of PDA). Each experiment considering a single bacterial isolate was run in triplicate and was repeated at least three times.

Demonstration of antibiotic activity

These test were performed in 250 ml Erlenmayer flasks containing 100 ml of sterile nutrient broth (NB). 1 ml bacterial suspension isolates (1×10^8 CFU/mL^{-1}) were added to the flasks containing NB. The flasks were then incubated at 26°C for 6 days on a rotary shaker at 100 rpm at room temperature (26 ± 2°C). Bacterial cells were pelleted by centrifugation at 5000 g for 12 min. The supernatants were sterilized with 0.22 µm filtrate. 5, 15 and 25% (v/v) of culture filtrate were mixed with PDA and a 5 mm disk of a four days old pure culture of *F. fujikuroi* was placed at the center of Petri dish. The experiments were independently repeated four times. Results are expressed as means of inhibition (%) of the growth of *F. fujikuroi* in the presence and absent of any bacterial culture filtrate isolates.

Demonstration of production of diffusible antibiotic

PDA plates, covered with a cellophane membrane, were inoculated in the center with 250 µl of an antagonistic bacterial suspension (1×10^8 CFU/mL^{-1}). After incubation for 48 h at 26°C, the membrane with the grown bacterial isolate was removed, and the plate was inoculated in the middle with a 5 mm disk of a pure culture of *F. fujikuroi*. Plates were further incubated at 26°C for 7 days and the growth of the pathogen was measured. Control were run with mocked inoculated PDA containing plates on the cellophane membrane (replacing the bacterial suspension by sterile distilled water), and incubated with *F. fujikuroi*. Each experiment considering a single bacterial isolate was run in triplicate and was repeated at least three times. Results are expressed as means of inhibition (%) of growth of *F. fujikuroi* in the presence and absence of any antagonistic bacterial isolate.

Bacterial strains, plasmids and primers

The bacterial strains, plasmids and primers used in this study are described in Table 1. *Pseudomonas* strains were grown at 28°C in King's B, 23 YT broth (Sambrook and Russel, 2001), *E. coli* strains were grown in Luria-Bertani or 23 YT broth at 28 or 37°C.

DNA manipulations

Standard methods were used for DNA purification, restriction enzyme digestion, agarose gel electrophoresis, and ligation (Ortiz-Herrera et al., 2004). Genomic DNA was isolated and purified by a

Table 1. Bacterial strains, plasmids and primers used in this study.

Strain, plasmid or primer	Description or sequence
Strains	
Pseudomonas fluorescens 2–79	Phz_ Rifr, produces PCA
Pseudomonas fluorescens F15	Phz, produces PCA
Pseudomonas fluorescens F15	Phz, produces PCA
Escherichia coli DH5α	F_ *traD36 proA_ proB_ lacI*q *lacZ*
Plasmid	
pUC - 18	ColE1 *bla*
Primers	
PHZ - UP	TAAGGATCCGGTAGTTCCAAGCCCCAGAAAC
PHZ – LOW	CACATTTGATCTAGATGGGTCACGGCTATTCAG

cetyltrimethylammonium bromide (CTAB) miniprep procedure. A 6.4 kb DNA probe containing the entire *phz* locus from *P. fluorescens* F15 was generated by PCR performed with oligonucleotide primers *phz*-up and *phz*-low (Table 1). The amplification was carried out by using a 50 µl reaction mixture containing 1x eLONGase buffer (Life Technologies, Inc., Rockville, Md.), 2 mM $MgSO_4$, 3.0% dimethyl sulfoxide, 200 µM (each) dGTP, dATP, dTTP, and dCTP, 10 pmol of each primer, 0.7 µl of eLONGase enzyme mixture (Cinagene, Inc.), and 20 ng of purified genomic DNA from isolated strains. All amplifications were performed with a PTC- 200 thermal cycler. Amplification was performed in a thermal cycler programmed. The reaction conditions are: a initial denaturation of 94°C for 2 min followed by 37 cycles of 94°C for 1 min, 64°C for 1 min, and 72°C for 1 min. A final extension step of 72°C for 10 min was included to complete the reaction. Amplified DNA fragments were examined by horizontal electrophoresis in 1.5% agarose gel in TBE buffer containing 90 mM Tris-borate, 2 mM EDTA (pH 8.3), with 8 µL aliquots of PCR products. Gels were stained with ethidium bromide and were photographed under UV light (312 nm).

Transformant screening and protein expression

Recombinant cells were identified by plating on to agar medium containing ampicillin, X-Gal and IPTG. For protein expression *E. coli* DH5α harboring pUC18 was grown in LB broth to an optical density at 600 nm and induced with 0.5 mM Isopropylb-D-thiogalactopyranoside (IPTG). Cells were harvested 3 h later and total cellular protein was analyzed by electrophoresis in an SDS–10% polyacrylamide gel.

RESULTS

Isolation of antagonistic bacteria

238 bacterial isolates were initially collected from the rhizoplane and rhizosphere of rice sheath blight disease in different farming of area of the Guilan province-Iran. Among them thirteen isolates were found to inhibit growth of *F. fujikuroi in vitro*. Eight isolates, F1, F6, F12, F15, F16, F18, F21 and F25 were identified as *P. fluorescens* biovar 3 according to method of Schaad et al. (2001).

In vitro inhibition of *F. fujikuroi* by *P. fluorescens* antibiosis

Dual culture

No physical contact was observed between any of the antagonistics bacteria tested and *F. fujikuroi*; moreover, an inhibitory halo was observed suggesting the presence of fungistatic metabolites secreted by the bacteria. On the other hand, a change in mycelial color was observed close to the colony end of *F. fujikuroi*, this side being of a darker brown than the color observed at the center of colony. Microscopic observation of this zone, allowed detecting cytoplasmic leakage that could be observed up to the hyphal septum, resulting in deformation and sliming of their apex up to 1/7 of its original size. Similar results were obtained by Montealegro et al. (2003). *P. fluorescens* F15 and F16 with inhibition zone of 55 and 50 mm, respectively, were the most inhibitory against *F. fujikuroi*.

Volatile antibiotics

All antagonistic isolates were showed to be significantly different from the control ($p < 0.01$). *P. fluorescens* F15 and F16 were the antagonistic bacteria isolate that showed the best inhibitory effect on the growth of *F. fujikuroi.* The inhibition of *P. fluorescens* F15 and F16 at 72 h culture of antagonistic isolates were 69 and 63%, respectively, although all bacteria showed inhibitory effect against *F. fujikuroi* growth (Table 2).

Diffusible antibiotics

Results similar to those of volatile antibiotics were obtained when the effect of diffusible antibiotic was tested (Table 2). Isolate F15 and F16 with inhibition of 77 and 70% respectively were the most inhibitory against *F.*

Table 2. Effect of antibiosis of *P. fluorescens* isolates on radial growth of *Fusarium fujikuroi in vitro*

Antibiosis (Inhibition, %)	*P. fluorescens* isolates										
	F1	F6	F12	F15	F16	F18	F21	F25	2-79 RN	F15 (pUC-PCA)	F16 (pUC-PCA)
Daul culture	40 d	41 d	48 c	55 b	50 c	49b	50 c	48 c	59 a	58 a	58.5 a
Volatile antibiotics	51 d	52 d	60 c	65 b	61 c	59 c	61 c	59 c	69 a	68 a	68 a
Diffusible antibiotics	58 d	60 d	68 c	77 b	70 c	68 c	67 c	69 c	80 a	79 a	79 a
Secretion of extracellular	62 d	63 d	73 c	80 b	73 c	74 c	74 c	75 c	83 a	82 a	82.5 a

Means followed by a common letter in a row are not significantly different according to LSD (T) test at P < 0.01.

fujikuroi, while isolate F1 with 58% inhibition was the less effective(Table 2).

Secretion of extracellular compounds

All antagonistic isolates possess significant inhibitory activities ($p < 0.01$). *P. fluorescens* F15 and F25 secretions have 80 and 75% inhibition, respectively (25% v/v) (Table 2).

Identification of P. fluorescens isolates by direct PCR

All isolates of *P. fluorescens* were identified by specific primers PCA1 and PCA2. On agarose gel electrophoresis 2%, isolates produced a band of 1110 bp (expected size). The bands of isolates were similar to the standard isolate of 2-79 RN (Figure 1).

Specificity of PCA primers

Primers *phz*-up and *phz*-low amplified the entire locus of *P. fluorescens* strain 2-79 RN (Figure 1). The specificity of PCA primers was reported in earlier study (Dimetri et al., 2001).

Cloning detection

The fragment (1110 bp) was cloned into pUC18, and positive clones were identified by standard methods (Ausubel et al., 1995). The antibiotic PCA is a major determinant of biological control of soil borne plant pathogens by strains of fluorescent *Pseudomonas* spp. (Khan et al., 2005).

DISCUSSION

In several bioassays, *P. fluorescens* strains F15 and F16 were able to suppress bakanae disease of rice by effectively of antifungal activity. In this study, we described variety of strains that inhibit mycelial growth of *F. fujikuroi* and a collection of phenazine-producing strains

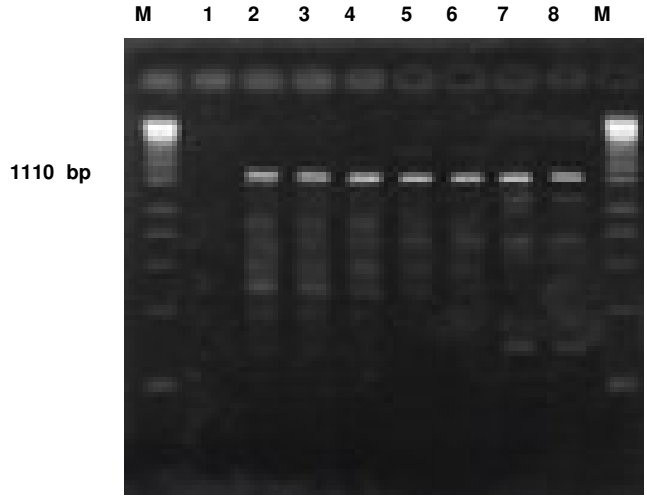

Figure 1. Agarose gel electrophoresis of the PCR products amplified from genomic DNA of isolated *Pseudomonas* strains with PCA1 and PCA2 primers phz-up and phz-low. Lane M, DNA 1 kb ladder marker (0.2 ng). Lane 1 negative control, lanes 2 to 6 antagonists bacteria, lanes 7 and 8 positive control (*P. fluorescens* 2-79 RN).

of *P. fluorescens.* For the presence of PCA-genes by direct PCR, we successfully cloned the entire locus of phenazine in *E. coli* DH5α with specific primers. Results indicated that phenazine biosynthesis is highly conserved among phenazine-producing strains of *P. fluorescens.* Cloning of different fragment of the locus can be used to study the structure and function of the biosynthetic gene clusters from the isolated strains. Characterization of phenazine regulation by strains of *P. fluorescens* F15 and F16 has revealed many complexities in the activation of phenazine production, but prior to this study, genetic screens had not identified any negative regulators. We speculate that fluorescent *Pseudomonas* spp. that produce *Phl* might play an important role in the natural suppressiveness of bakanae disease of rice. Because phenazine production by strain 2-79 RN contributes to its capacity in biological control, we tested the ability of F15 and F16 to inhibit *F. fujikuroi*. In *in vitro* plate assays, strain F15 and F16 were better at inhibiting mycelial

growth of the fungus than wild type strain 2-79 RN (Table 2). The environmental fitness of genetically modified microorganisms might be affected by the modification (De Leij et al., 1998). Future studies will determine the mechanism of PCA regulation of phenazine production and evaluate the long-term effect of the PCA mutation on bacterial colonization, persistence, and bakanae disease suppression in rice.

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