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# Biological Control of *Fusarium fujikuroi*, the Causal Agent of Bakanae Disease by Rice Associated Antagonistic Bacteria

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## Abstract

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In this research, effect of some isolates antagonistics bacteria were investigated against *Fusarium fujikuroi* the causal agent of bakanae disease and foot rot of rice, collected from infection farming in Rasht, Lahijan, Foman, Anzaly, Talesh and Astara in the Guilan Province under greenhouse conditions. Two hundred thirty eight bacterial isolates, separated from the rhizosphere and seeds of rice infected by the fungus which mentioned above and antagonistic ability of 13 isolates of these bacteria (8 gram negative and 5 gram positive) were demonstrated by using the dual culture method. According to the results of biochemical and morphological trials, 8 isolates: F1, F6, F12, F15, F16, F18, F21 and F25 were identified as *Pseudomonas fluorescens*. Five isolates, F14, F19, F21, F32 and F35 were introduced as *Bacillus cereus*. In greenhouse conditions antagonistic isolates were used by seed, plant and soil treatment. Statistical analysis of data indicated that there existed significant differences between seed, plant and soil treatments. The isolate F15 in seed, plant and soil treatment was most effective and disease incidence by 8.5, 8.5 and 12 % respectively. While the isolates F6 was least effective on *F. fujikuroi*. All of the isolates in seed treatments are more effective compared to other treatments. The results of used the mixed Rovral TS fungicide with mixed antagonistic isolates showed that there existed significant disease incidence by 6.5, 6.75 and 8 % respectively. In the field conditions foliar spray of isolate F15 mixed with Rovral TS (52.5% WP) were applied. The disease incidence in F15 isolate for seed coating, soil drenching and seed coating + foliar spray were 6.5, 6.75 and 5.5 % respectively, while the control plants showed 28% disease incidence. These results suggest that the *P. fluorescens* and *B.cereus* isolates studied have an excellent potential to be used as biocontrol agents of *F. fujikuroi* in rice at the field conditions.

*Key words*: bakanae disease, rice, *Fusarium fujikuroi*, antagonistics bacteria, biological control

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## Introduction

Rice (*Oryza sativa* L.) is a major food crop in the foothills of the Iran. In the subtropical zone between altitudes of 1,000 and 2,000 m, rice is usually grown as a monsoon season crop from June to October (Desjardins et al., 2000).

Bakanae caused by *Fusarium fujikuroi* Nirenberg is a disease of rice first described in Japan, and now widely distributed in Asia. Bakanae is primarily a seedborne disease. Sowing ungerminated seeds in infested soil gives rise to infected seedlings. Soil temperature of 35 ºC is most favorable for infection. Application of nitrogen favors the development of the disease. Wind or water easily carries the spores from one plant to another. High temperature, ranging from 30 to 35ºC favors the development of the disease (Nyvall, 1999). It is transmitted primarily by seed. Disease levels so far have been quite low and yield loss not measurable. However, disease incidence level and number of fields affected has increased dramatically (Ahmed and Raza, 1992). The anamorph form produces gibberellin and fusaric acid. Biological studies of the two substances showed that fusaric acid cause stunting and giberrellin causes elongation (Nyvall, 1999). The pathogen has a wide host range and is widespread throughout the world. The fungal pathogen is also known to cause stalk rot and leaf blight of corn, stalk rot of sorghum, endosepsis of fig (Blackman, 1972), and crown rot of sparagus (Endo and Burkholder, 1971). On rice *F. fujikuroi* induces seedling elongation, foot rot, seedling rot, grain sterility, and grain discoloration (Ou, 1985). The pathogen can be both seed-borne and soil-borne. Among the fungal disease, bakanae is frequently encountered and considered important (Ogawa, 1988). In open-field nurseries, one of the problems encountered by farmers in the control of bakanae disease is the difficulty in distinguishing infected seedlings from healthy plants, because there are no obvious symptoms of infected seedling except the height or slight pale yellowing at the stage transplanting (Kim, 1981). The fungus not only cause considerable damage on many plants, but also is parasitic on plants without producing visible symptoms (Hsieh et al., 1977). It can be isolated even from these kernels that are healthy in appearance. Rice seedling that grow from these infected seeds tended to display bakanae symptoms (Padwick, 1950). Currently, the most common management practice for bakanae is seed treatment with fungicides, However, these treatments are expensive and add pesticide to the environment (Dev and Mary, 1986) and resistance of the fungal pathogen to fungicides has been reported (Ogawa, 1988).

In recent years, fluorescent pseudomonads have drawn attention worldwide because of production of secondary metaboilites such as siderophores, antibiotics, volatile HCN compounds, enzymes and phytohormones (Gupta et al., 2001). Fungi from *Trichoderma* genus are among the biological control agents of *F. fujikuroi* (Hadwan and Khara 1990; Lin et al., 1994) also bacteria belonging to *Pseudomonas* and *Bacillus* genus have been also used (Gasoni et al., 1998). The ideal biocontrol agent for the management of foliar infection by a soil-borne pathogen may be the one that can survive in both rhizosphere and phyllosphere. Among the various biocontrol agent, fluorescent pseudomonads are known to survive both in rhizosphere (Park et al., 1991) and phyllosphere (Wilson et al., 1992). Fluorescent

pseudomonads are known to induce disease resistance against foliar disease (Liu et al., 1995). Bacterial suspensions of fluorescent pseudomonads have been used for control of rice sheath blight by Mew and Rosales (1986). Curtains of fluorescent pseudomonad isolates have antagonistic activity based on producing antibiotic whereas for other isolates such as *Pseudomonas putida* WCS358 based on competition for iron (Vidhysaekaran and Muthamilan, 1999). According to present and future regulations on the use of chemical fungicides such as Hinosan and Rovral TS. Considering that treatments must prevent environmental pollution, we have considered the use of biocontrol agents to control *F.fujikuroi* that effect rice plants. The selection of bioantagonistic microorganisms, other to take into account the direct effect on pathogen development, must consider conditions where the bioantagonist should be develop, *ie.* Salinity, the pH of soil and different temperature, among other (Montealegro et al., 2003). The objectives of the present research, isolation of fluorescent pseudomonads from rhizosphere and phyllosphere of infected rice that could control *F.fujikuroi in vitro* and *in vivo*, and their characterization in term of antagonistic mechanisms used to control the pathogen, and conditions for growth similar to those present in the field.

## Materials and Methods

**Isolation of *Fusarium fujikura*:** Rice bakanae disease was collected from infected farming in different areas of Guilan province, Iran. For isolation of *F. fujikuroi*, Small pieces of infected leaves 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 ± 2 ºC) for a week. The growing colonies of fungi were transferred to new plates for purification and identification.

**Preparation of inoculum *F. fujikuroi* and pathogenicity test:** 300 g of sterile rice seeds were put in Erlenmayer, then in each erlen 5-mm mycelial disc from a 7 days old culture of *F. fujikuroi* on PDA was placed in the erlens and they were incubated at room temperature (26 ± 2 ºC) for 4 weeks. The colonies of fungi were developed and produced many spore and mycelium. For pathoginisity test, approximately 10 g of inocolum of *F. fujikuroi* was added to soil in pot and infested soil was covered with plastic film and incubated 48 hour at room temperature then five grams of seeds was sown in each pot (25 cm in diameter).

**Isolation of antagonistic bacteria isolates and identification:** Rhizosphere colonizing antagonistic bacteria were isolated from fresh roots of rice from different area of Guilan province, Iran. After vigorous shaking at 100 rpm of excised roots to remove all but tightly adhering soil, root segments, (1 g) were shaked at 100 rpm in 100 ml of sterile distilled water for 25 min . Fluorescent pseudomonads were isolated on King's medium B (KB). According to the methodology of Schaad et al. (2001), antagonistic isolates of bacteria were identified by biochemical, physiological, biological tests and PCR.

**Sample preparation for direct PCR from cell culture:** Bacterials cells, which were grown on KB for 24 h, were resuspened in sterile distilled water. The cell suspensions (approximately $1\times10^7$ CFU $mL^{-1}$) were boiled for 10 min and

were used for PCR assaying (Raaijmakers et al., 1997).

**PCR conditions:** Primer were designed by primers PCA1 (5" TTGCCAAGCCTCGCTCCAAC 3") and PCA2(5"CCGCGTTGTTCCTCGTTGAT 3"). PCR amplifications were carried out in 100 l reaction volume. A 10 µl volume of boiled bacterial cells was added to 90 µl of PCR mixture contained, 2 µM $MgCl_2$, 20 pmol of each primer, 100 µM (each) dNTP, 0.2 U of *Taq* DNA polymerase (CinnaGene, Inc. Iran), in 10 µM Tris-HCl (pH 9.0), 50 mM KCl and 0.1% Triton X-100.

Amplification was performed in a thermal cycler (Mastercycler gradient, Germany) programmed The reaction conditions are: a denaturation step of 94°C for 2 min followed by 37 cycles of 94°C for 1 min, 52°C for 1 min, and 72°C for 1 min. A final extension step of 72°C for 10 min finishes the reaction. Amplified DNA fragments were examined by horizantal electrophoresis in 2% agarose gel in TBE buffer (Maniatis et al., 1982) with 8 µl aliquots of PCR products. Gels were stained with ethidium bromide and were photographed under UV light (312 nm).

**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* (Stainer et al., 1996).

**Production of volatile antibiotic:** A 250 µl of a antagonistic bacterial suspension (1x $10^8$ CFU/ ml) were placed at the Petri dish containing KB, 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 the prevent loss of volatiles formed. Plates were incubated at 26 ºC for 6 days and the growth of the pathogen was measured and compared to controls developed in the absence of the bioantagonist (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 (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 inhibition was calculated using the fallowing formula (Montealegro et al., 2003):

Inhibition (%) = [(1- (fungal growth/ Control growth))] x 100

**Secration of extracellular:** These test were performed in 250 mL Erlenmayer flasks containing 100 ml of sterile nutrient broth (NB). 1 ml bacterial suspension isolates (1x $10^8$ CFU/ mL) were added to the flasks containing NB. The flasks were then incubated at 26 ºC

for 6 days on a rotary shaker at 100 rpm at room temperature (26 ± 2 ºC). Bacterial cells were pelleted by centrifugation at 5000 g for 12 minutes. The supernatants were sterilized with 0.22 m 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 abscens of any bacterial culture filtrate isolates.

**Production of diffusible antibiotic:** This effect was tested according to Montealegro et al. (2003), PDA plates, covered with a cellophane membrane, were inoculated in the center with 250 µl of a bioantagonistic bacterial suspension (1x $10^8$ CFU/ ml). After incubation for 48 hour at 26 ºC, 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 ºC 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 future incubated with *F. fujikuroi*. Each experiment considering a single bacterial isolate was run in triplicates 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.

**Effect of $Fe^{3+}$ on antagonism level:** This effect was tested according to Pumarino (1995), using $FeCl_3$ x $6H_20$ at 5, 15, 25 and 50 µMol concentrations added to the KB medium. For all tests, the experimental unit was one Petri dish.

**Bacterial ability for root colonization:** Ability of bacteria to colonize roots was established according to Misaghi (1990). The pre-germinated rice seeds were inoculated with antagonistic bacterial suspension (1 × $10^8$ CFU/ ml) containing 1% gum. After incubation for 1 hour at 26 ºC, rice seeds were placed in the tube containing 2/3 sterile sand and the tubes were incubated for 21 days at 26 ºC, for evaluation, root tips were isolated and crushed in stomacher plastic bags containing 1 ml sterile distilled water, and the bacterial suspension obtained was diluted. Fifty µl of each dilution was seeded on a Petri dish containing KB, and incubated for 48 hour at 26 ºC, and CFU were counted. The experimental unit was three replications for each antagonistic isolate.

**Production of protease:** Efficacy of the *P.fluorescens* isolates in production of protease was tested according to Maurhofer et al. (1995), by streaking each bacterial isolate on Skim milk agar medium (SMA) on the Petri dish. Each experiment considering a single bacterial isolate was run in triplicates and was repeated at least four times. The bacterial isolates that produced protease were identified by a halo zone arrounding of bacterial colony and were measured.

**Effect of Rovral TS. fungicide on *F. fujikuroi* and antagonistics isolates *in vitro*:** This effect was tested according to Horsfall (1956). The development of antagonistic bacteria and *F. fujikuroi* **were** tested under Rovral TS. concentrations of 1, 3, 5, 10, 100 and 1000 ppm at room temperature (26 ± 2 ºC ). For *F. fujikuroi*, 2 mL of each Rovral TS. Concentrations were mixed with PDA at 50 ºC and a 5 mm disk of a five days old pure culture of *F. fujikuroi* was placed at the center of

each Petri dish. After incubation for 7 days at 26 ºC, the growth of the pathogen was measured. Controls were run on PDA medium with sterile distilled water. For antagonistic isolates, 5 mm disks of wathman filter paper were dipped in the Rovral TS. Concentrations for 2 minutes and were placed on the Petri dish containing KB. After incubation for 48 hour at 26 ºC the inhibition zone was measured. Four replication per concentration were maintained. In greenhouse conditions, 1000 ppm concentration of Rovral TS. was used for seed treatment, soil dranching and foliar spray. Results are expressed as means of % inhibition of growth of *F. fujikuroi* and zone inhibition of antagonistics bacteria. Each experiment considering a single bacterial isolate was run in triplicates and was repeated at least three times.

## Greenhouse and Field Experiments

**Efficacy of antagonistic bacteria against *F. fujikuroi* in the greenhouse and field.**

The rice cultivar tested was Khazar, known to be very sensitive to bakanae disease and foot rot. Infected seeds were obtained from plants artificially inoculated with *F. fujikuroi*. Thirteen isolates of antagonistic bacteria that inhibited *F. fujikuroi in vitro* were tested for their efficacy to control bakanae disease and foot rot in the greenhouse. The inoculum of *F. fujikuroi* was prepared with rice seeds.

**Preparation of bacterial inocula.**

Inoculum of antagonistic bacteria for use in greenhouse and field experiments was grown in King's medium B broth (KMB) to late exponential phase at 27 ºC with shaking at 100 rpm. Cells were harvest by centrifugation (5000 × g / min, 10 ºC, 15 min), washed twice and resuspended in 0.5% sterile NaCl solution (for greenhouse) or tap water (for field experiments). The bacterial suspension was adjusted turbidimetrically to about $1 \times 10^8$ CFU/ml for each experiment. The bacterial suspensions in KMB medium were used for seed coating, soil drenching and foliar spray.

**Efficacy of seed coating, soil drenching and foliar spray with antagonistic bacteria versus fungicide**.

Greenhouse tests were performed in 25 m diameter pots, 4 kg soil capacity. Rice seeds (cv. Khazar) were surface sterilized in 0.6% sodium hypochlorite for 10 minutes and then air dried in a fume hood after which the seeds were soaked in the bacterial suspensions ($1\times 10^8$ CFU/ml) in 1% methyl cellulose for 24 hour. Inoculum of *F. fujikuroi* was added to soil in pots and infested soil was covered with plastic film and incubated 48 hour at room temperature then 10 grams of seeds were sown in each pot. Separately, at the same time, soil in pot was drenched with suspensions of the antagonistic bacteria isolates and Rovral TS at a total concentration of $10^8$ CFU/ml and 1000 ppm respectively. Pots were maintained in the greenhouse under 25 ± 3 ºC and relative humidity 95% conditions. Four-weeks-old rice plants were spray inoculated with antagonistic bacteria suspensions ($1\times 10^8$ CFU/mL). Control treatments were sprayed with sterile distilled water and seedlings with sheath blight disease symptoms were recorded 4 weeks after planting. Plants were arranged in a randomized completed block design with five replications. Bakanae disease and foot rot intensity were assessed 90 days after *F. fujikuroi* inoculation (Rosal and Mew, 1997). A

seedbed (28 × 2 m) was prepared in naturally infested field soil. Field experiments were performed at the University of Guilan Field Station. Isolates, *P.fluorescens* F15 and *B.cereus* F35 that produce an effect against *F. fujikuroi* were selected for their effectiveness against bakanae in the field experiments. The experimental plot (5 × 2 m) consisted of a randomized complete block design with three replications. Each replication consisted of about 90 plants. The experimental plot was surrounded by a buffer zone of approximately 10 m of fallow soil. Field rice was upland and seedling rice were planted. Seed coating and foliar spray with antagonistic bacteria suspensions (1 × $10^8$ CFU/ml) and bacteria suspensions (1×$10^8$ CFU/mL) add Rovral TS were used similar to greenhouse conditions. Rice plants were sampled 120 days after planting. (Rabindran and Vidhysaekaran, 1996). Data were grouped based on sources of bacteria, then analyzed with the IRRISTAT program developed by Biometrice at IRRI.

## Results

**Pathogenicity test of *F. fujikuroi* on rice.**

After four weeks, rice seedling (cv. Khazar) showed bakanae disease and foot rot symptoms. Thin plants with yellowish green leaves and pale green flag leaves. Dying seedlings at early tillering. Reduced tillering and drying leaves at late infection. *F. fujikuroi* was isolated on rice bakanae showed that the high disease intensity.

**Isolation of antagonistic bacteria.**

Two hundred thirty eight 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 *Pseudomonas fluorescens* biovar 3 and five isolates, F14, F19, F21, F32 and F35 were identified as *Bacillus cereus* according to the methodology of Schaad et al. (2001).

**Identification of *Pseudomonas fluorescens* isolates by direct PCR.** specific primers PCA1 and PCA2 identified All isolates of Pseudomonas fluorescens. On agarose gel electrophoresis 2%, isolates were produced a band 1110 bp. (expected size). The bands of isolates were similary with isolate standard of CHA0 (Figure 1).

***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 observerd 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 to detect 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 *B.cereus* F35 with inhibition zone of 55 and 48 mm respectively were the most inhibitory for *F. fujikuroi*.

**Volatile antibiotics:** All antagonistic isolates were showed there are significantly different from the control ($P<0.01$).

*P. fluorescens* F15 and *B. cereus* F35

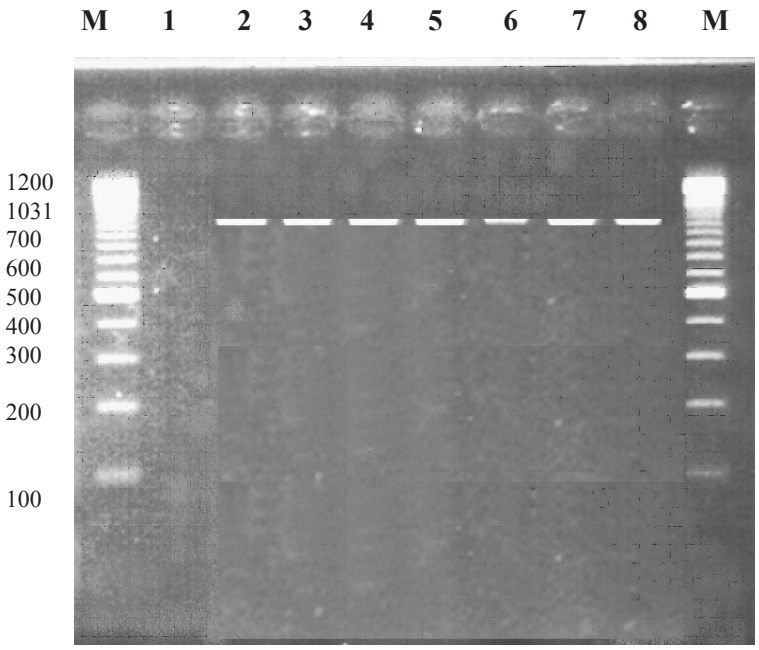


**Fig. 1. Agarose gel electrophoresis of products from polymerase chain reaction (PCR) performed on DNA 16S of *Pseudomonas fluorescens* isolates, M, 100 bp DNA marker; lane 1, control negative (distilled water), lane 2 is posetive control (*P. fluorescens* 2-79 RN) showing the amplification the approximately 1110 bp; lanes 3 to 8, antagonistic strains of *P. fluorescensisolated* from rhizosphere of rice**

were the antagonistic bacteria isolate that showed the best inhibitory effect on the growth of *F .miniliforme.* The inhibition % of *P. fluorescens* F15 and *B.cereus* F35 at 72 h culture of antagonistic isolates were 69 and 66 percent respectively, although all bacteria showed inhibitory effect on *F. fujikuroi* growth (Table 1).

**Diffusible antibiotics:** Results similar to those of volatile antibiotics were obtained when the effect of diffusible antibiotic was tested (Table 1). Isolate F15 and F35 with inhibition % of 77 and 78 percent respectively were the most inhibited of *F.fujikuroi,* while isolate F1 with 51% inhibition was the less effective (Tables 1 and 2).

**Secretion of extracellular:** All antagonistic isolates were seen there are significant different between isolates and concentration of juices ($P<0.01$). *P. fluorescens* F15 and F35 by 80 and 72 inhibition % respectively (25% v/v) were the most inhibited of *F. fujikuroi* (Table 1).

**Production of protease:** All antagonistic isolates were able to produce protease on SMA medium. Among isolates, *P. fluorescens* F15 and *B. cereus* F35 were found most effective, with 28 mm of halo zone arrounding the bacterial

**Table 1**

**Effect of antibiosis of *Pseudomonas fluorescens* isolates on radial growth of *Fusarium fujikuroi in vitro***

| Antibiosis Inhibition, % | *Pseudomonas fluorescens* isolates | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | F1 | F6 | F12 | F15 | F16 | F18 | F21 | F25 |
| Daul culture | 40 c | 41 c | 48 b | 55 a | 50.5 b | 49b | 50 b | 48 b |
| Volatile antibiotics simultaneously | 51 c | 52 c | 60 b | 65 a | 61.3 b | 59.5 b | 61 b | 59 b |
| Volatile antibiotics 72 h | 54 c | 54.5 c | 63 b | 69 a | 63.5 b | 60.5 b | 63.5 b | 62 b |
| Antibiotics | 58 c | 60 c | 68 b | 77 a | 70 b | 68 c | 67 b | 69 b |
| Secration of extra-cellular (25% v/v) | 62 c | 63.5 c | 73 b | 80 a | 73 b | 74.5 b | 74 b | 75 b |

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

**Table 2**

**Effect of antibiosis of *Bacillus cereus* isolates on radial growth of *Fusarium miniliforme in vitro***

| Antibiosis / Inhibition, % | *Bacillus cereus* isolates | | | | |
|---|---|---|---|---|---|
| | F14 | F19 | F24 | F32 | F35 |
| Daul culture | 42.5 b | 43.5 b | 44 b | 42 b | 48 a |
| Volatile antibiotics simultaneously | 54 b | 54.5 b | 55.5 b | 55 b | 63 a |
| Volatile antibiotics 72 h | 56 b | 57 b | 58 b | 59 b | 66 a |
| Antibiotics | 62.5 b | 63.5 b | 64.5 b | 64 b | 78 a |
| Secration of extra- cellular (25% v/v) | 63 b | 64 b | 63.5b | 64 b | 72 a |

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

colony. It is considered that all of the thirty isolates were able to secrete of the enzymes involved in biocontrol, and that all had the ability to control *F. fujikuroi* through secretion of diffusible and volatile metabolites. It may be concluded that they use these two latter mechanisms of biocontrol as opposite to some fungal biocontrol microorganisms that also use fungal cell wall hydrolyzing enzymes within their biocontrol mechanisms (Pérez et al., 2002).

**Siderophore:** The thirteen antagonistic bacteria showed similar behavior on *F. fujikuroi* growth at any of the $Fe^{3+}$ concentration tested (Tables 3 and 4).

**Colonization of rice roots by antagonistic bacteria**: During the 4 weeks of growth in sterile soil in the tube, significant difference was seen between the

**Table 3**

**Effect of different concentrations of $Fe^{3+}$ on the radial growth of *Fusarium miniliforme* in the presence of *P. fluorescens* isolates**

| $Fe^{3+}$ as $FeCl_3$ (µM) | *Pseudomonas fluorescens* isolates | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | F1 | F6 | F12 | F15 | F16 | F18 | F21 | F25 |
| 0 | 38 a | 37 a | 35 a | 38 a | 37 a | 36 a | 37 a | 37 a |
| 5 | 41.5b | 41 b | 39 b | 41 b | 41 b | 39 b | 37.5 a | 38.5 b |
| 15 | 42 b | 41 b | 40 b | 42 b | 41 b | 39 b | 40.5 b | 39.5 b |
| 25 | 42 b | 42.5 b | 43.5 b | 43.8 b | 42.5 b | 41.5b | 41 b | 40.5 b |
| 50 | 45 c | 45 c | 46.5 c | 46 c | 45.7 c | 44.7 c | 44.3 c | 45.5 c |

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

**Table 4**

**Effect of different concentrations of $Fe^{3+}$ on the radial growth of *Fusarium miniliforme* in the presence of *B. cereus* isolates**

| $Fe^{3+}$ as $FeCl_3$, µM | *Bacillus cereus* isolates | | | | |
|---|---|---|---|---|---|
| | F14 | F19 | F24 | F32 | F35 |
| 0 | 44 a | 44.5 a | 44.7 a | 45 a | 46 a |
| 5 | 50 b | 50.5 b | 50.8 b | 50.8 b | 50.3 b |
| 15 | 52 b | 51 b | 50.8 b | 52 b | 50.5 b |
| 25 | 51.5 b | 51.5 b | 52.3 b | 51.8 b | 50.8 b |
| 50 | 55 c | 56 c | 55.5 c | 56.7 c | 56 c |

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

numbers of bacteria (CFU/g of roots) on rice roots for all isolates. The counts of each isolate were 6.2 – 6.35 log CFU/g of roots 1 day after inoculation and they were increased at the level after 45 days 3 – 5 x $10^8$ CFU/g of roots. During further growth in non- sterile soil at pots in the greenhouse, they were still at the same level 8.38 - 8.65 log CFU /g of roots 90 days after inoculation.

**Greenhouse and field conditions:** In greenhouse conditions, statistical analysis of data on the disease incidence % by *F. fujikuroi*, indicated that there existed significant differences between seed coating, soil drenching and foliar spray. All of the isolates in seed coating are more effective. Among these thirteen isolates, *P. fluorescens* F15 and *Bacillus cereus* were the most effective for control of rice bakanae disease and foot rot. The disease incidence in F15 isolate for seed coating, soil drenching and seed coating + foliar spray were 8.5, 8.75 and 12 % respec-

**Table 5**

**Effect of different methods of treatment by antagonistic bacteria isolates and Rovral TS. (52.55 WP) to suppression bakanae in greenhouse**

| Treatment | Disease incidence, % Methods of application | | |
|---|---|---|---|
| | Seed coating | Soil drenching | Foliar spray |
| F1 | 13.5 b | 14. 5 b | 17.5 b |
| F6 | 13.75 b | 16 b | 18 b |
| F12 | 13 b | 15.5 b | 17.75 b |
| F15 | 8.5 c | 8.5 c | 12 c |
| F16 | 14.5 b | 14.5 b | 17.5 b |
| F18 | 13.5 b | 14. 5 b | 17 b |
| F21 | 12.75 b | 13.75 b | 17.25 b |
| F25 | 12.75 b | 13.5 b | 17.25 b |
| F14 | 13.25 b | 13.5 b | 18 b |
| F19 | 13 b | 14 b | 18 b |
| F24 | 12.75 b | 14 b | 17.5 b |
| F32 | 13.25 b | 13.5 b | 17.5 b |
| F35 | 9 c | 9 c | 11 c |
| F15 + F35 | 8 c | 8.55 c | 10.8 c |
| Rovral TS. | 7 dc | 7.25 dc | 10.5 c |
| Rovral TS. + F15+ F35 | 6.5 d | 6.75 d | 8 d |
| Control | 32 a | 32 a | 32 a |

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

tively, while the control plants showed 32 % disease incidences (Table 5). The results of used the mixture of isolates (F15 and F35) showed that there existed significant differences between application isolates antagonistics with were used these isolates alone. Maximum control were obtained when F15 and F35 suspensions were added Rovral TS. in treatment seed coating (Table 5). In the field conditions, *P. fluorescens* F15 and *B. cereus* F35 isolates were used. The disease incidence in F15 isolate for seed coating, soil drenching and seed coating + foliar spray were 8.5, 8.5 and 12 % respectively, while the control plants showed 28 % disease incidence (Table 6). During the 120 days of growth rice in the field, there are no significant difference were seen between the F15 and F35 on disease incidence for all treatments, while when the mixture of these isolates were applied, there are significant difference were seen between application of isolates antagonistic with were used these isolates alone, also there are no significant difference

**Table 6**

**Effect of different methods of treatment by antagonistic bacteria isolates and Rovral TS. (52.5 % WP) to suppression bakanae in field conditions**

| Disease incidence, % | | | |
|---|---|---|---|
| Treatment | Methods of application | | |
| | Seed coating | Foliar spray | Seed coating + Foliar spray |
| F15 | 6.5 b | 6.75 b | 5.5 b |
| F35 | 6.75 b | 6.75 b | 6 b |
| F15 + F35 | 6 b | 6 b | 5.5 b |
| Rovral TS. | 5.5 b | 5.5 b | 5 bc |
| Rovral TS. + F15 | 4.75 bc | 4.75 bc | 5 bc |
| Rovral TS. + F35 | 4.75 bc | 4.75 bc | 5 bc |
| Rovral TS. + F15+ F35 | 4 c | 4 c | 4 c |
| Control | 28 a | 28 a | 28 a |

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

between application of mixture these isolates and Rovral TS. in all treatments. Maximum control in field with disease incidence 4 % was obtained when F15 and F35 suspensions were added Rovral TS. in treatment seed coating + foliar spray (Table 6).

## Discusion

In recent years, antagonistic bacteria have drawn attention worldwide because production of secondary metaboilites such as siderophore, antibiotics volatile compounds HCN, enzymes and phytohormones. Although isolates of *P. fluorescens* and *B. cereus* could be obtained from the rhizosphere of different rice paddy, antagonistic potential of these isolates appears to vary a great deal (Wilson et al., 1992; Hagedron et al., 1989).

Based on morphological, phenotipical, nutritional characteristic and PCR using specific primers, we identified bacterial antagonistics isolated rhizosphere of rice, *P. fluorescens* and *B.cereus.* The ability of *P. fluorescens* and *B. cereus* isolates to serve as biocontrol agent of bakanae disease and foot rot is described here**.** The results of dual culture studies showed that *P.fluorescens* and *B.cereus* isolates were inhibited the growth of *F. fujikuroi* on plates. Members of the genus *Pseudomonas* spp. are well known antagonistic fungi (Gasoni et al., 1998). They are known to produce volatile compounds such as hydrogen cyanide (Castric and Castric, 1983). Iron is a fundamental element for respiration of several aerobic and facultative microorganisms, and therefore, its availability in soil is essential (Leong, 1986**).** On the other hand, siderophores are low weight compounds with high affinity for $Fe^{3+}$ (Neilands, 1981), which are produced under limiting concentration of iron. These

compounds are able to transport this element inside the cell for metabolic functions (Press et al., 2001), and microorganisms, which are able to produce sidrophores, show competitive advantage as compared to those that do not produce them. From this pint of view, the competence for iron increases in conditions where this element is limiting, but this condition is reverted when iron is added to the culture medium (Elad and Baker, 1985). The results were obtained in this study are very similar to results of Montealegro et al. (2003). If it is considered that iron available in soil fluctuate 5 and 50 ppm, it may be concluded that this element is not limiting for the antagonistic activity of these bacteria on *F. fujikuroi*. Root exudates are believed to determined which microorganisms colonized roots in the rhizosphere (Kunc and Macura, 1988). The use of bacteria's to exert an appropriate biological control of *F. fujikuroi* and of other soil borne fungi relies on their ability to colonize roots efficiently, otherwise, their biocontrol character would be non-sense. The ability to colonize rice roots is variable between rhizobacteria, being this characteristic a reflection for their ability to compete for ecological niches in the rhizosphere (Misaghi, 1990). *P.fluorescens* and *B.cereus* isolates effectively controlled rice bakanae disease and foot rot when it was applied to seed, soil, or foliar spray. Our results showed that *P.fluorescens* and *B.cereus* isolates were more effective as a seed coating compared to foliar spray and soil drenching with bacterial suspension. Most biocontrol trials have dealt with use of *P.fluorescens* F15 against rice bakanae disease and foot rot. In greenhouse and field conditions, combination of F15 and F35 were comparable to Rovral TS application.

The bacteria appeared to move epiphytically from seed to roots, stems and leaves. It has been shown in other studies that fluorescent pseudomonads could be isolated from aerial parts of plants grown from seeds treated with the bacteria (Colyer and Mount, 1984; Geels and Schippers 1983; Mew and Rosales, 1986). Fluorescent pseudomonads have also been detected in substomatal cavities of leaves (Blackman, 1972; Manceau and Niknejad Kazempour 2001). The epiphytic bacteria could have controlled *F. fujikuroi*. Induction of disease risistance against foliar diseases by soil inoculum of fluorescent pseudomonads has also been widely reported (Maurhofer, 1995; Van Peer and Schippers, 1992; Van Peer, 1991; Wei et al., 1991). Control of diseases with fluorescent pseudomonads applied to the foliage has been reported (Austin et al., 1977; Blackman, 1972; Blackman et al., 1992; Clarkeson and Lucas, 1993; Gnananmanickam and Mew, 1992; Levy and Chet, 1988; Mew and Rosales, 1986). Survival of *P. fluorescens* in the phyllosphere (Austin et al., 1977), may explain the effectiveness of foliar sprays. Foliar spray with under field conditions, primary inoculum of *F. fujikuroi* is from soil and irrigation water (Premalatha, 1990). A combined application of bacteria suspension isolates was added with Rovral TS for seed coating + foliar spray was the most effective method for control of rice bakanae disease and foot rot disease in the field. Possibliy both rhizosphere and phyllosphere population of *P. fluorescens* and *B. cereus* helped to control disease. Both direct inhibition of the pathogen and systemically induced resistance in the rice plants could be involved in control (Lemanceau and Albouvette, 1993). The results of field trial in the current study

indicate the potential usefulness of seed coating with *P. fluorescens* and *B. cereus* isolates suspension when added Rovral TS. in controlling sheath blight of rice. From all these results it may be concluded that the biocontrol effect of antagonistic bacteria isolated from rhizosplane of healthy and rice bakanae disease and foot rot against *F. fujikuroi* are adequate for their use at the rice field in different areas of Iran. The low disease incidence of rice bakanae disease and foot rot with F15 and F35 isolates suggests that the antibiosis metabolites are conductive for rapid inhibition agent, *F. fujikuroi*. The multiple activity may be useful under natural conditions in which the same crops sufferer from disease other than rice bakanae disease and foot rot.

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