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Suppression of maize root diseases caused by *Macrophomina phaseolina, Fusarium moniliforme* and *Fusarium graminearum* by plant growth promoting rhizobacteria

K. K. Pal, K. V. B. R. Tilak, A. K. Saxena, R. Dey, C. S. Singh

Division of Microbiology, Indian Agricultural Research Institute, New Delhi-110 012, India

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Abstract

A plant growth-promoting isolate of a fluorescent *Pseudomonas* sp. EM85 and two bacilli isolates MR-11(2) and MRF, isolated from maize rhizosphere, were found strongly antagonistic to *Fusarium moniliforme, Fusarium graminearum* and *Macrophomina phaseolina,* causal agents of foot rots and wilting, collar rots/stalk rots and root rots and wilting, and charcoal rots of maize, respectively. *Pseudomonas* sp. EM85 produced antifungal antibiotics (Afa^+), siderophore (Sid^+), HCN (HCN^+) and fluorescent pigments (Flu^+) besides exhibiting plant growth promoting traits like nitrogen fixation, phosphate solubilization, and production of organic acids and IAA. While MR-11(2) produced siderophore (Sid^+), antibiotics (Afa^+) and antifungal volatiles (Afv^+), MRF exhibited the production of antifungal antibiotics (Afa^+) and siderophores (Sid^+). *Bacillus* spp. MRF was also found to produce organic acids and IAA, solubilized tri-calcium phosphate and fixed nitrogen from the atmosphere. All three isolates suppressed the diseases caused by *Fusarium moniliforme, Fusarium graminearum* and *Macrophomina phaseolina in vitro.* A Tn5::*lac*Z induced isogenic mutant of the fluorescent *Pseudomonas* EM85, M23, along with the two bacilli were evaluated for *in situ* disease suppression of maize. Results indicated that combined application of the two bacilli significantly (P = 0.05) reduced the *Macrophomina*-induced charcoal rots of maize by 56.04%. Treatments with the MRF isolate of *Bacillus* spp. and Tn5:: *lac*Z mutant (M23) of fluorescent *Pseudomonas* sp. EM85 significantly reduced collar rots, root and foot rots, and wilting of maize caused by *Fusarium moniliforme* and *F. graminearum* (P = 0.05) compared to all other treatments. All these isolates were found very efficient in colonizing the rhizotic zones of maize after inoculation. Evaluation of the population dynamics of the fluorescent *Pseudomonas* sp. EM85 using the Tn5:: *lac*Z marker and of the *Bacillus* spp. MRF and MR-11(2) using an antibiotic resistance marker revealed that all the three isolates could proliferate successfully in the rhizosphere, rhizoplane and endorhizosphere of maize, both at 30 and 60 days after seeding. Four antifungal compounds from fluorescent *Pseudomonas* sp. EM85, one from *Bacillus* sp. MR-11(2) and three from *Bacillus* sp. MRF were isolated, purified and tested *in vitro* and in thin layer chromatography bioassays. All these compounds inhibited *R. solani, M. phaseolina, F. moniliforme, F. graminearum* and *F. solani* strongly. Results indicated that antifungal antibiotics and/or fluorescent pigment of fluorescent *Pseudomonas* sp. EM85, and antifungal antibiotics of the bacilli along with the successful colonization of all the isolates might be involved in the biological suppression of the maize root diseases.

Key words: biocontrol – *Macrophomina phaseolina* – *Fusarium graminearum* – *Fusarium moniliforme* – antifungal metabolites

Introduction

The increasing demand for a steady food supply to the growing world population will require controlling of plant diseases that reduce crop yield substantially. Current practices for controlling plant diseases are based largely on development of resistant varieties and application of synthetic pesticides (Emmert and Handelsman 1999). Biological control is getting greater attention due to low cost and eco-friendly application. Maize (*Zea mays* L.) is the third most important cereal crop, next to wheat and rice. It is cultivated all over the world and susceptible to about twenty soil-borne di-

Corresponding author: K. K. Pal
Present address: National Research Centre for Groundnut, Ivnagar Road, P.B. No. 5, Junagadh-362001, Gujarat, India
e-mail: pal@nrcg.guj.nic.in

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seases. However, only *Macrophomina phaseolina* (charcoal rots), *Fusarium moniliforme* (*Gibberella fujikuroi*, foot rots and wilting) and *Fusarium graminearum* (*Gibberella zeae*, root rots, stalk rots and wilting) cause substantial damage to the crop in several areas. Since there are no cultivars with complete resistance to soil-borne fungal pathogens in maize, and fungicides are not potent enough to protect the crop from infection by these pathogens, development of biocontrol agents could be the best alternative to minimize the incidence of these diseases. Application of *Bacillus* sp. has been found to substantially control seedling blight, root rots and stalk rots of maize caused by *Fusarium graminearum,* when used as seed inoculant (Chang and Kommedahl 1968; Kommedahl and Chang 1975). *Trichoderma viride* and *Pseudomonas* species were also capable of controlling stalk rots of maize (Chen *et al.* 1999). Application of root-associated *Pseudomonas cepacea* as seed coating biocontrol agent could reduce the *Fusarium moniliforme*-induced infection of maize root by 23–80% (Hebbar *et al.* 1992a, b). *Pseudomonas cepacea* was also found to inhibit a range of soil-borne fungal pathogens including *Fusarium graminearum, Fusarium moniliforme* and *Macrophomina phaseolina* (Hebbar *et al.* 1992b). *Burkholderia cepacea* was also found potent in controlling *Fusarium moniliforme* besides plant growth promotion of maize (Bevivino *et al.* 1998). Raju *et al.* (1999) reported the reduction of *Fusarium moniliforme*-induced diseases of maize by application of *Trichoderma harzianum, Pseudomonas fluorescens* and *Chaetomium globosum.* Incidence of charcoal rot was substantially reduced after seed treatment of mungbean and sunflower by *Trichoderma harzianum, Gliocladium virens* and *Streptomyces* sp. (Hussain *et al.* 1990). Sanchez *et al.* (1994) reported the potency of *Burkholderia cepacea* UPR5c to control ashy-stem blight of common bean.

Plant growth promoting *Pseudomonas* and *Bacillus* species generally employ an array of mechanisms like antibiosis, site competition, production of HCN, chitinase, siderophore, ammonia, fluorescent pigments and/or antifungal volatiles (Weller 1988; Voisard *et al.* 1989; Cartwright *et al.* 1995; Gardener *et al.* 2000; Pal *et al.* 2000) to antagonize pathogens. In the present study, an attempt has been made to identify suitable biocontrol agents against maize pathogens, *Macrophomina phaseolina, Fusarium moniliforme* and *Fusarium graminearum,* and to isolate the compounds responsible for suppression of the pathogens. Thus, three plant growth promoting rhizobacterial (PGPR) isolates (a fluorescent *Pseudomonas* sp. EM85 and two *Bacillus* spp., MR-11 (2) and MRF) obtained from maize rhizosphere, were found to antagonize and suppress *Fusarium moniliforme, Fusarium graminearum* and *Macrophomina phaseolina*-induced diseases of maize and *Rhizoctonia solani*-induced damping-off of cotton (Pal 1995, Pal *et al.* 2000). Potent antifungal compounds from these isolates have also been obtained.

Material and methods

Strains. The isolates of *Macrophomina phaseolina, Fusarium moniliforme* and *Fusarium graminearum,* virulent on maize, were obtained from Indian Type Culture Collection (ITCC), Division of Mycology and Plant Pathology, Indian Agricultural Research Institute (IARI), and were maintained on Potato Dextrose Agar (PDA) slants at 4 °C, and subcultured monthly. A fluorescent *Pseudomonas* sp. EM85 (obtained from maize endorhizosphere) and two bacilli isolates MRF (having proteinase activity) and MR-11(2) (having proteinase and lipase activity) isolated from maize rhizosphere, exhibiting antifungal and other plant growth promoting traits were maintained at 4 °C. The generation time of EM85, MR-11(2) and MRF were 56 min, 54 min and 90 min, respectively. The phenotypes and some important characteristics of the isolates are depicted in Table 1. The bacterial isolates were grown at 28 °C in nutrient broth or potato dextrose broth. *E. coli* S17-1 (pSUP:Tn-B20) harbouring Tn5::*lac*Z was grown at 37 °C in Tryptone-Yeast extract (TY) broth amended with kanamycin (50 µg ml^{-1}). The *E. coli* strain used was sensitive to nalidixic acid.

Detection of antifungal traits. The isolates of fluorescent *Pseudomonas* sp. and *Bacillus* spp. were screened to detect the production of chitinase (Frandberg and Schnurer 1994), HCN (Bakker and Schipper 1987), antifungal volatiles (Howell *et al.* 1988), siderophore (Schwyn and Neilands 1987), antifungal antibiotics (Hebbar *et al.* 1992) and fluorescent pigments (Pal *et al.* 2000) as described elsewhere (Pal *et al.* 2000). Each test was repeated three times with three replications.

Quantification of plant growth promoting traits. Nitrogenase activity, assayed as acetylene reduction activity (ARA), was estimated using a gas chromatograph (Shimadzu GC-14A). Nitrogenase activity was expressed as nmoles of ethylene produced $tube^{-1}$ h^{-1}. The organisms were grown in 5 ml of liquid and solid complex carbon medium (Rennie 1981) in 20-ml tubes at 28 ± 2 °C for 24 h. Tubes were sealed with serum stoppers and 10% acetylene was injected into each tube after taking out 10% air from each tube. Again, tubes were incubated for 24 h at 28 ± 2 °C. After the incubation, production of ethylene was measured by GC.

Table 1. Characteristics of the isolates and strains used in the experiments*.

Isolate	Catechol siderophore (mg mg^{-1} protein)	nmoles C_2H_4 h^{-1} $tube^{-1}$	IAA like substances (μg ml^{-1})	TCP solubilization (mg 100^{-1} ml)	Organic acids produced	Phenotypes	Source
Fluorescent *Pseudomonas* isolate EM85 (Km^s Nal^rCm^r)**	0.092	6.76	3.84	14.13	Gluconic, Citric, Succinic, α-ketobutyric	Afa^+HCN^+ Sid^+Flu^+	Pal (1995)
Isogenic Tn5:: *lac* Z mutant, M23, of the isolate EM85	0.091	6.58	3.90	30.08	Gluconic, Citric, Succinic, α-ketobutyric	Afa^+HCN^+ Sid^+Flu^+	-Do-
Bacillus sp. MR-11(2) (Ap^r)	0.108	–	–	–	–	Afa^+Afv^+ Sid^+	-Do-
Bacillus sp. MRF(Km^r Tet^r)	0.084	33.57	3.71	25.70	Gluconic, Citric, Tartaric, α-ketobutyric	Afa^+ Sid^+	-Do-
E. coli S17-1 (Km^r)	–	–	–	–	–	harbouring Tn5::*lac*Z	Simon *et al.* 1983

* Data represent average of three replications repeated thrice.
** Km = Kanamycin; Nal = Nalidixic acid; Ap = Ampicillin; Tet:Tetracycline.

Catechol type of siderophore was quantified by the method of Arnow (1937), modified by Carson *et al.* (1992) in iron-free liquid medium. Absorbance was determined at 550 nm with pyrocatechol as standard.

Quantification of IAA-like substances was made following the method of Sarwar and Kremer (1995) in L-tryptophan agar. One ml of the isolates grown for 24 h in Kings' B (King *et. al.* 1954) broth was plated onto L-tryptophan agar in triplicates and incubated at 28 ± 2 °C for 24 h in the dark. After incubation, an agar growth bead (0.24 cc) was placed in freshly prepared Salkowsky reagent, in triplicates, from each Petri dish and incubated in the dark for 30 min. Spectrophotometric reading was taken at 595 nm with IAA as standard. The amount of IAA produced was expressed as μg ml^{-1}.

Solubilization of tri-calcium phosphate was quantified in Pikovskaya broth (Pikovskaya 1948). Each flask containing 100 ml of Pikovskaya broth with 50 mg tricalcium phosphate was inoculated with 0.5 ml of each isolate (grown for 24 h in liquid culture) and incubated in a rotary shaker at 28 ± 2 °C for 4 d. The cultural broth was centrifuged at 15,000 rpm for 10 min and the supernatant was collected in 100-ml volumetric flasks. The volume of the supernatant was adjusted to 100 ml with distilled water. Water-soluble phosphorus was determined in the supernatant by the chloromolybdic acid method of King (1932) as modified by Jackson (1967). Spectrophotometric measurement was taken at 660 nm.

The organic acids produced by the biocontrol agents were detected by paper chromatography after reducing the volume of the supernatant in a lyophilizer to 1/40th volume, obtained during the phosphate solubilization experiment. Standard organic acids were dissolved in deionized water (30 mg ml^{-1}). Spots were made on Whatman No. 1 filter paper and chromatography was run in a solvent system of n-butanol:acetic acid:water (12:3:5) for 14 h. The spots were developed as described by Nordmann and Nordmann (1960) by spraying bromocresol green reagent (100 mg in 250 ml of absolute alcohol at 7.0 pH).

Tn5::lac Z mutagenesis, selection and maintenance of mutants. Transposon (Tn5::*lac*Z) mutagenesis was carried out by biparental mating as described by Simon *et al.* (1983) for introduction of DNA from *E. coli* (donor) to the fluorescent *Pseudomonas* sp. EM85 (recipient). Conjugation was carried out at 1:1 ratio (donor:recipient) on Tryptone-Yeast extract (TY) agar Petri dishes for 18–20 h at 28 °C. Transconjugants were selected on TY Petri dishes amended with kanamycin and nalidixic

acid (50 µg ml^{-1}) along with IPTG (four µl of 200 mg ml^{-1} stock) and X-gal (40 µl of 20 mg ml^{-1} in dimethylformamide stock). Blue colonies were picked as putative mutants whereas colourless colonies were discarded as spontaneous mutants. All the mutants were screened for Voges-Proskauer (VP) test, which was negative for the fluorescent *Pseudomonas* sp. EM85 and positive for *E. coli* in order to eliminate the spontaneous mutants of *E. coli*. Mutants were maintained in TY slants amended with kanamycin (50 µg ml^{-1}) and nalidixic acid (50 µg ml^{-1}) at 4 °C.

Phenotypic characterisation of mutants. Mutants were screened for antifungal traits such as production of HCN, siderophores, fluorescent pigments and antifungal antibiotics as described earlier. All the parameters mentioned were studied in media containing kanamycin and nalidixic acid (50 µg ml^{-1}). Inhibition zones were measured in mm against the pathogen on PDA and NA. Mutants deficient in antifungal antibiotic production were checked in NA. For the determination of antifungal antibiotics, cyanide and fluorescent pigments one mutant each was grown on one Petri dish, whereas for assay of siderophore production several mutants were spotted in a single Petri dish. Each test was repeated three times with three replications.

Compatibility testing of antifungal rhizobacterial isolates. In vitro antibiosis among the isolates was tested on PDA. In each PDA Petri dish 10 µl of each isolate (grown for 24 h) was spotted and incubated at 28 ± 2 °C for 96 h. After incubation the growth was scraped from each Petri dish and was exposed to chloroform vapour for 1 h. After evaporation of chloroform, 10 ml soft PDA containing 1 ml of the rhizobacterial isolates (grown for 24 h) were over-layered and incubated for 24 h at 28 ± 2 °C. Observation was recorded for clearing zones in the top soft agar.

Preparation of fungal inoculants, Bacillus sp. MR-11(2), Bacillus sp. MRF and Tn5::lacZ mutant (M23) of fluorescent Pseudomonas sp. EM85. Fungal inoculants were raised in 250-ml Erlenmeyer flasks containing sand and maize meal mixed in a 3:1 ratio, i.e. 75 g dry sand and 25 g of maize meal, with 30 ml of water to moisten the mixture. Each flask was sterilised at 121 °C for one hour on two consecutive days. Each flask was inoculated with one agar bead containing *Macrophomina phaseolina, Fusarium moniliforme* and *Fusarium graminearum* (from two days growth of the fungi on PDA; beads were taken using Pasteur pipettes) and incubated at 28 °C for five to seven days for uniform mycelial growth. Inoculum of the Tn5::*lac*Z mutant (M23) of the fluorescent *Pseudomonas* sp. EM85 was grown in nutrient broth containing kanamycin and nalidixic acid at 50 µg ml^{-1}, whereas *Bacillus* spp. MR-11(2) and MRF were grown in nutrient and PDA broth, respectively. Each broth was centrifuged at 12,000 rpm, washed with phosphate-buffered saline (PBS) three times, and then pellets were dissolved in PBS and the OD was adjusted to 1.2 before using the bacteria for pot experiments.

Isolation, purification and characterisation of antifungal compounds from bacterial isolates. Antifungal compounds were isolated from *Pseudomonas* sp. EM85 by the procedure of Douglas and Gutterson (1986). EM85 was grown on TY Petri dishes and incubated at 28 ± 2 °C for five days. After five days, the agar was minced and shaken overnight in 80% acetone (v/v) in equal proportion. The acetone fraction was decanted and filtered through Whatman no. 44 filter paper. The liquid volume was reduced by flash evaporation at 55 °C and made up to 80% (v/v) with ethanol and incubated overnight at 4 °C. The pH was adjusted to 4.55. This was then filtered and the volume was reduced and extracted once with methanol:chloroform (1:1, v/v) and then three times with an equal volume of chloroform. The organic phases were combined, evaporated to dryness, and dissolved in 5 ml of chloroform-methanol (1:1, v/v). Bioassay was done against *R. solani* in PDA. Fluorescent pigment was isolated from PDA Petri dishes by extracting with water.

The procedure of Homma *et al.* (1989) was modified to isolate all possible antifungal compounds produced by the two bacilli isolates MRF and MR-11(2). The cultures were grown on PDA Petri dishes (300 Nos.) containing 20–25 ml solid agar. Petri dishes were incubated at 28 °C for 7 days. At the end of incubation, bacterial growth was scraped, and minced agar was collected in a 5-l flask. It was then extracted with an equal volume of chilled acetone (v/v) and kept overnight. The acetone fraction was collected and extracted twice with diethyl ether (v/v). The diethyl ether fraction was then collected and dried *in vacuo.* This was then dissolved stepwise in acetonitrile, methanol and acetone. Alternatively, the remaining acetone fraction was extracted twice each with ethylacetate and chloroform (v/v) and dried *in vacuo.* This was then dissolved in methanol. All fractions were bioassayed against *Rhizoctonia solani, Macrophomina phaseolina, Fusarium solani, Fusarium moniliforme* and *Fusarium graminearum* on PDA. Active fractions were maintained for further purification.

Antifungal compounds were purified as described by Homma *et al.* (1989). Purification was done on TLC plates (Silica gel GF_{254} and Silica gel G_{60}, Merck) using different solvent systems, ethyl acetate:methanol (1:1), ethylacetate:methanol:water (1:1:1), methanol:acetone (1:1), and acetone:benzene (1:1). Spots were developed by I_2 vapour and observed under UV (254 nm). Bioassays on TLC plates were performed against

Macrophomina phaseolina following the procedure of Homma *et al.* (1989), as all the bacterial isolates were antagonistic to this pathogen. After purification of the spots in the solvent system, TLC plates were sterilized under UV light for 4 h, then the plates were overlayered with soft PDA containing spores of *Macrophomina phaseolina.* Active spots were identified by the lysed zones on the TLC. Active spots were eluted from the preparative TLC plates after optimising the R_f values in the previously mentioned solvent systems. The eluted samples were dissolved in the solvent where they were originally dissolved. Purity was checked until a single spot developed. The purified fractions were dried *in vacuo,* crystallized at low temperature, and stored at 4 °C for further studies.

Purified antifungal compounds were analysed by UV-spectrum analysis. UV-spectra were taken with a Hitachi Mode double beam UV-VIS spectrophotometer in methanol and distilled water using a quartz cuvette (1 cm path length). IR analyses were also carried out.

In situ biological control assay. Potting mixture was prepared by mixing 100 g of sand-maize meal-grown *Macrophomina phaseolina, Fusarium moniliforme* and *Fusarium graminearum* with 900 g sterile soil (clay loam, pH 6.8) in polythene bags and then mixing thoroughly with unsterile soil in 18″ earthen pots containing 30 kg soil. The soil was amended with nitrogen (40 kg N ha^{-1}) in the form of urea and phosphorus (60 kg P_2O_5 ha^{-1}) in the form of single super phosphate. Control treatment received only unsterile soil. The fungal inocula were applied five days prior to sowing to facilitate their multiplication. Maize (*Zea mays* L.) seeds (variety DHM-103) were imbibed for 12 h, and eight seeds pot^{-1} were sown at a depth of 2.5 cm. Six ml of the bacterial inocula in PBS were placed on the surface of each seed after adjusting the O.D. to 1.2 (660 nm) for all cultures. In the treatments receiving two and three biocontrol agents, 3 ml and 2 ml of each culture were applied to the seeds and covered with soil. One week after germination, the plant population was thinned to 6 plants pot^{-1} and allowed to grow up to 60 days. The experiment was conducted during the rainy season to ensure that the humidity favoured disease expression. Pots were kept in the open with an average day and night temperature of 30 °C and 22 °C, respectively. Three separate experiments were conducted with three pathogens. Enough moisture was maintained by watering regularly with 800 ml pot^{-1}. There was a total of 9 treatments in each experiment with eight replications, which included: a) soil control; b) pathogen control; c) pathogen with Tn5::*lac*Z-tagged mutant (M23) of EM85; d) pathogen with isolate MRF; e) pathogen with isolate MR-11(2); f) pathogen with M23 and MRF; g) pathogen with M23 and MR-11(2); h) pathogen with MRF and MR-11(2) and i) pathogen with M23, MRF and MR-11(2) isolates. Each experiment was repeated thrice. A total of 48 plants per treatment were screened for disease control studies. Depending on the disease symptoms disease severity was scaled on a 0–5 scale (Bag 1991). Numerical disease rating was assigned as follows: 0, healthy plants; 1, appearance of lesion at the collar region, 2 mm–7 mm in length; 2, large lesions, 8 mm to 12 mm in length; 3, moderate rotting of the collar region, loss of turgor at the top with slight drooping; 4, extensive rotting at the collar region, wilting and drying of many leaves, drooping of the shoot; 5, plants completely wilted, dead and dry. This scale was used for the assessment of charcoal rots/collar rots of maize caused by *Macrophomina phaseolina* and foot rot caused by *Fusarium moniliforme,* and *Fusarium graminearum.*

Mean disease rating (MDR)

$$= \frac{a \times 0 + b \times 1 + c \times 2 + d \times 3 + e \times 4 + f \times 5}{a + b + c + d + e + f}$$

Where a, b, c, d, e and f are the number of plants with the disease rating of 0, 1, 2, 3, 4 and 5, respectively.

Percent disease reduction with respect to pathogen control was calculated as follows:

Percent disease reduction (PDR)

$$= \frac{\text{MDR of pathogen control-MDR of inoculated treatment}}{\text{MDR of pathogen control}} \times 100$$

Fusarium moniliforme and *Fusarium graminearum* also cause severe root rots of maize. After studying the incidence and severity of the root rots carefully, disease scaling for root rot was done as follows: 0, healthy root; 1, less than 25% of the root spoiled due to rotting; 2, 25–50% of the root spoiled, evident from drooping of the leaves during daytime; 3, up to 75% of the root damaged, evident from starting of wilt and drying of leaves from bottom to top; 4, complete rotting of the root, completely wilted, dead and dry plants. Other calculations were done as mentioned earlier. For root rots also, 48 plants were screened in each treatment after 60 days of sowing.

Monitoring of introduced organisms. A separate experiment was conducted simultaneously with the *in situ* disease suppression experiment keeping three controls (one each for each antifungal rhizobacterial isolate) and three treatments each in the presence of three pathogens. To evaluate the population dynamics of the introduced biocontrol agents in the rhizosphere, rhizoplane and endorhizosphere of maize, the population of each individual antagonistic rhizobacterium was monitored using antibiotic markers for bacilli and the Tn5:: *lac*Z

marker for fluorescent *Pseudomonas* sp. EM85. Ten g each of the rhizosphere soil, rhizoplane (root surface) and surface-sterilized roots were taken for endorhizosphere population. For enumerating the M23 mutant of the EM85 isolate, blue colonies were counted on TY Petri dishes containing antibiotics (µg ml^{-1}): Km^{50}, Nal^{50}, Cm^{100}, and cycloheximide^{100} together with IPTG (4 µl of 200 mg ml^{-1} stock) and X-Gal (40 µl of 20 mg ml^{-1} stock). For isolate MRF, appropriate dilutions were plated on PDA Petri dishes containing kanamycin (25 µg ml^{-1}), tetracycline (5 µg ml^{-1}) and cycloheximide (100 µg ml^{-1}). Colonies of MRF were differentiated from other organisms on the basis of the gummy and mucoid colonies it produced on PDA. MR-11(2) was differentiated onto TY Petri dishes on the basis of big, rough, and serrated colonies.

Statistical analyses. Disease severity ratings were analysed according to Dunnett's test. Percent disease reductions were analysed following Duncan's multiple range test after arc sin transformations. Population densities were analysed according to Duncan's multiple range tests after log transformations of individual estimations. SE of the mean of the inhibition zones produced by the biocontrol agents was also determined.

Results

In vitro antagonism

Fluorescent *Pseudomonas* sp. EM85 strongly inhibited the fungi, *Macrophomina phaseolina, Fusarium moniliforme* and *Fusarium graminearum,* both on PDA and NA (Table 2). It produced 8 mm of inhibition zones on PDA against all pathogens, while it could produce 7, 5 and 5 mm of inhibition zones on NA against *Macrophomina phaseolina, Fusarium moniliforme* and *Fusarium graminearum,* respectively. *Bacillus* sp. MR-11(2) was the best in exhibiting inhibitory effects against the pathogens, as it produced 15 mm of inhibition zones against all pathogens on PDA, while it produced 12, 10 and 15 mm of inhibition zones on NA against *Macrophomina phaseolina, Fusarium moniliforme* and *Fusarium graminearum,* respectively (Table 2). *Bacillus* sp. MRF was also equally potent in inhibiting *Macrophomina phaseolina* and *Fusarium moniliforme* (Fig. 1) besides inhibiting *Fusarium graminearum in vitro* (Table 2). However, the isolate was more efficient on PDA than on NA.

Antifungal traits of the rhizobacterial isolates

The fluorescent *Pseudomonas* sp. EM85 was found to produce antifungal antibiotics (Afa$^+$), siderophore (Sid$^+$), cyanide (HCN$^+$), and fluorescent pigments (Flu$^+$) (Table 1). The fluorescent pigment produced on PDA was yellow-green and a typical character of this isolate. Production of ammonia, chitinase and antifungal volatiles were not detected. *Bacillus* sp. MR-11(2) exhibited the production of antifungal antibiotics (Afa$^+$), siderophore (Sid$^+$) and antifungal volatiles (Afv$^+$). However, isolate MRF produced antifungal antibiotics (Afa$^+$) and siderophore (Sid$^+$) only (Table 1).

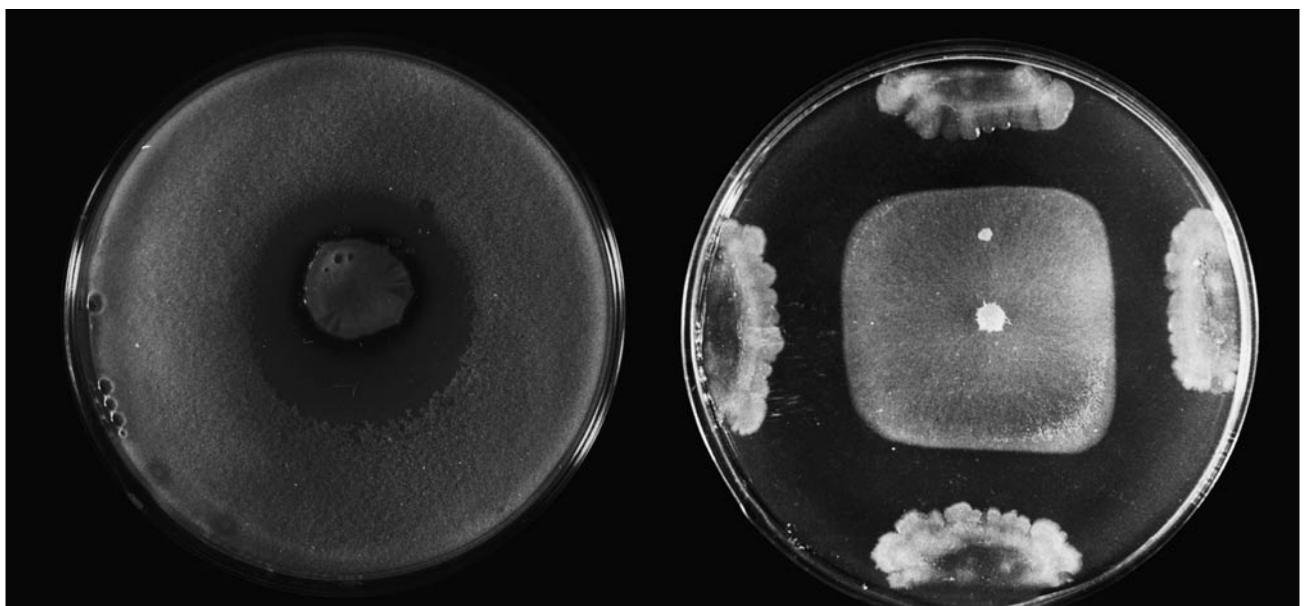

Fig. 1. *In vitro* antifungal activity of *Bacillus* sp. MRF against: left; *Macrophomina phaseolina;* right; *Fusarium moniliforme*

Table 2. *In vitro* inhibition of maize root pathogens by the isolates (inhibition zones in mm at 28 ± 2 °C after 4 days of incubation)*

Isolate	*Macrophomina phaseolina*		*Fusarium moniliforme*		*Fusarium graminearum*	
	PDA	NA	PDA	NA	PDA	NA
Fluorescent *Pseudomonas* isolate EM85	8 (± 0.58)	7 (± 0.58)	8 (± 1.15)	5 (± 0.58)	8 (± 1.00)	5 (± 0.58)
Isogenic mutant, M23, of the isolate EM85	9 (± 0.73)	7 (± 0.58)	8 (± 1.15)	6 (± 0.58)	8 (± 1.00)	5 (± 0.58)
Bacillus sp. MR-11(2)	15 (± 1.15)	12 (± 0.58)	15 (± 1.53)	10 (± 1.15)	15 (± 0.58)	15 (± 1.15)
Bacillus sp. MRF	15 (± 0.58)	5 (± 0.58)	10 (± 1.15)	4 (± 0.58)	10 (± 1.00)	4 (± 0.58)

* Average of three replications repeated thrice and data in the parentheses indicate Standard Error (SE).

Quantification of plant growth promoting traits

Fluorescent *Pseudomonas* sp. EM85 ($Km^rNal^sCm^r$) produced catechol type of siderophore (0.108 mg mg^{-1} protein) and indole acetic acid (3.84 µg ml^{-1}), fixed atmospheric nitrogen (6.76 nmoles C_2H_4 h^{-1} $tube^{-1}$), and solubilized tri-calcium phosphate (14.13 mg 100^{-1} ml broth) besides producing organic acids like gluconic, citric, succinic, and α-ketobutyric acid (Table 1). *Bacillus* sp. MR-11(2) (Ap^r) produced only catechol type of siderophore (0.092 mg mg^{-1} protein). *Bacillus* sp. MRF (Km^rTet^r) produced indole acetic acid (3.71 µg ml^{-1}), fixed atmospheric nitrogen (33.57 nmoles C_2H_4 h^{-1} $tube^{-1}$), and solubilized tri-calcium phosphate (25.70 mg 100^{-1} ml broth) besides producing organic acids like gluconic, citric, tartaric and α-ketobutyric acid (Table 1).

Isolation and phenotypic characterisation of Tn5::lacZ insertion mutants of isolate EM85

Transposon mutagenesis was carried out to insert the *lac* Z gene into the chromosome of the fluorescent *Pseudomonas* sp. EM85 for studying the ecological competence of the isolate after its introduction into the maize rhizosphere. A total of 160 mutants were obtained and characterized for studying the deficiencies or overproduction of the antifungal traits. Only few isolates produced deficiencies or overproduction of antifungal traits against the pathogens. One mutant, M23, was taken for the experiment to monitor the population dynamics of the isolate in the maize rhizosphere. The mutation did not affect the ability of the mutant M23 to inhibit *Macrophomina phaseolina, Fusarium moniliforme* and *Fusarium graminearum in vitro* (Table 2). Mutagenesis did not affect the production of HCN, antifungal antibiotics, fluorescent pigment(s) and siderophore as compared to wild type (Table 1).

In situ suppression of charcoal rot of maize caused by Macrophomina phaseolina

Treatment with *Macrophomina phaseolina* caused charcoal rot in maize. Typical symptoms of charcoal rot were noticed in treatments with the pathogen (Fig. 2). Three antagonistic rhizobacterial isolates were evaluated for their performances to control charcoal rot. Instead of the wild-type isolate of fluorescent *Pseudomonas* sp. EM85, its Tn5::*lac*Z mutant was taken together with two bacilli isolates, MR-11(2) and MRF, alone or in combinations. Treatments with antifungal rhizobacterial isolates significantly (P = 0.05) reduced the disease severity compared to pathogen control (Table 3). However, combination of the two bacilli

Table 3. *In situ* disease suppression of maize caused by *Macrophomina phaseolina***

Treatments	Mean disease rating/ plant*	Percent disease reduction*
Soil control	00 d	–
Pathogen control (P)	2.98 a	–
P + M23	1.28 bc	49.08 abc
P + MR-11(2)	1.98 b	35.43 cd
P + MRF	1.05 bc	53.55 ab
P + M23 + MR-11(2)	1.63 bc	42.30 bcd
P + M23 + MRF	1.30 bc	48.68 abc
P + MRF + MR-11(2)	0.93 c	56.04 a
P + M23 + MRF + MR-11(2)	1.15 bc	52.42 a

** Means within the same column and followed by the same letter(s) are not significantly different at P = 0.05 according to Dunnett and Duncan multiple range tests.
* Results based on 48 plants in each treatment with eight replications, having six plants in each replication. Disease reduction with respect to pathogen control. The experiment was repeated thrice.

Fig. 2. *In situ* expression of maize diseases and their biological suppression. 1; collar rot by *Fusarium graminearum;* 2; foot rots/collar rots by *Fusarium moniliforme* in only one plant and all other healthy plants due to inoculation of a Tn5::*lac*Z mutant (M23) of fluorescent *Pseudomonas* EM85; 3; charcoal rot by *Macrophomina phaseolina;* unmarked; left: healthy root due to seed treatment with M23 mutant; right: rotten root caused by *Fusarium moniliforme* in the pathogen control treatment.

isolates MRF and MR-11(2) produced the best result and reduced the disease severity from 2.98 in pathogen control to 0.93 when treated with these two isolates (Table 3). All isolates were efficient in reducing disease intensity. When inoculated alone, *Bacillus* sp. MRF was the most efficient in reducing disease severity, while combinations of all three isolates produced the same disease intensity (Table 3). While isolate MRF could reduce the *Macrophomina*-induced disease by 53.55%, M23 and MR-11(2) alone reduced the disease by 49.08% and 35.43%, respectively (Table 3). There was no significant difference among the treatments except for treatments with *Bacillus* sp. MR-11(2). Combination of MRF and MR-11(2) was synergistic and slightly more efficient in disease reduction (56.04%) than the combined application of all three antifungal isolates (52.42%). Inoculation of the rhizobacterial isolates significantly increased plant biomass and height at 60 days after seeding (unpublished data).

In situ suppression of maize diseases caused by Fusarium moniliforme and Fusarium graminearum

Fusarium graminearum caused both, root rot and collar rot (Fig. 2) and wilting of maize plants after inoculation. Treatments with all antagonistic plant growth promoting rhizobacterial isolates, a mutant of EM85 (M23), MR-11(2) and MRF, either alone or in combinations reduced the root rot disease severity significantly (P = 0.05) compared to pathogen treatment (Table 4). However, there was no significant difference among the treatments inoculated with the rhizobacterial isolates either alone or in combinations in reducing the root rot

Table 4. *In situ* disease suppression of maize caused by *Fusarium graminearum* and *Fusarium moniliforme***

Treatments	*Fusarium graminearum**				*Fusarium moniliforme**			
	Root rot		Collar rot and wilting		Root rot		Foot rot and wilting	
	MDR/ plant	PDR	MDR/ plant	PDR	MDR/ plant	PDR	MDR/ plant	PDR
Soil control	0 c	–	0 d	–	0 d	–	0 c	–
Pathogen control (P)	2.23 a	–	2.92 a	–	1.70 a	–	2.77 a	–
P + M23	0.68 b	56.48 abc	1.19 bc	50.36 b	0.75 bc	48.39 cd	0.80 bc	57.48 ab
P + MR-11(2)	1.10 b	45.11 cd	1.48 bc	44.60 bcd	0.35 cd	62.17 ab	0.87 b	55.92 ab
P + MRF	0.55 bc	60.20 ab	1.33 bc	47.58 bc	0.61 bc	54.63 bc	0.72 bc	59.34 ab
P + M23 + MR-11(2)	0.71 b	55.67 abc	1.06 bc	52.95 b	0.44 cd	58.69 abc	0.73 bc	59.12 ab
P + MR-11(2) + MRF	0.83 b	52.65 bc	0.80 c	58.44 a	1.02 b	39.93 d	1.20 b	48.82 b
P + MRF + M23	0.41 c	64.60 a	0.95 bc	55.24 ab	0.26 cd	68.44 a	0.62 bc	61.82 a
P + M23 + MRF + MR-11(2)	0.93 b	49.78 bc	1.78 b	38.65 cd	0.60 bc	53.91 bc	1.04 b	52.24 ab

** Means within the same column and followed by the same letter(s) are not significantly different at P = 0.05 according to Dunnett and Duncan multiple range tests.
* Results based on 48 plants in each treatment with eight replications, having six plants in each replication. Disease reduction with respect to pathogen control. The experiment was repeated thrice. MDR = Mean disease rating and PDR = Percent disease reduction.

of maize. The best result was obtained with a combination of a Tn5::*lac*Z mutant of fluorescent *Pseudomonas* sp. EM85 (M23) and *Bacillus* sp. MRF, which reduced root disease severity from 2.23 in the pathogen control to 0.41 (Table 4). When treated alone, *Bacillus* sp. MRF produced the best result in reducing the root rots disease severity. Co-inoculation of *Bacillus* sp. MRF and Tn5::*lac*Z mutant (M23) reduced the root rots disease (64.60%) significantly (P = 0.05) compared to the treatments inoculated with MR-11(2), combination of MRF and MR-11(2) and all the three (Table 4). There was no significant difference among all other treatments in terms of percent disease reduction to that of the co-inoculation of MRF and M23, and this was found consistent with the results obtained with root rot disease severity ratings.

Fusarium graminearum also caused severe collar rots (Fig. 2) and wilting of maize plants at 60 days after seeding. Evaluation of disease severity ratings revealed the least collar rot and wilting incidence when co-inoculation with the two bacilli isolates MRF and MR-11(2) was performed (Table 4). Combined application of all three biocontrol rhizobacteria was not efficient. However, significantly (P = 0.05) reduced disease severity ratings were obtained with all treatments compared to pathogen inoculation (Table 4). In terms of percent disease reduction too, significantly higher disease reduction (58.44%) was achieved with co-inoculation of the two bacilli as compared to all other inoculated treatments except the treatment with MRF and M23, which was equally efficient (Table 4). Combined inoculation of all biocontrol agents was not efficient in reducing the incidence of collar rot of maize (Table 4).

Like the two other pathogens, root rots (Fig. 2) and foot rots and wilting diseases of maize were observed when inoculated with *Fusarium moniliforme.* Treatments with the biocontrol rhizobacteria, either alone or in combinations, significantly (P = 0.05) reduced the mean disease ratings of root rot of maize compared to pathogen *(Fusarium moniliforme)* control (Table 4). The best result was obtained with co-inoculation of MRF and M23 (Table 4). Seed treatment with MR-11(2) reduced the disease equally well as dual inoculation with MRF and M23. Evaluation of disease reduction revealed that dual inoculation with MRF and M23 significantly (P = 0.05) reduced the root rots disease (68.44%) as compared to other treatments except for single inoculation with MR-11(2). Analyses indicated that isolate MR-11(2) reduced the *Fusarium moniliforme*-induced diseases of maize as well as combined inoculation with MRF and M23 mutant of fluorescent *Pseudomonas* sp. EM85 (Table 4). Healthy root development was noticed when inoculated with the M23 mutant of the isolate EM85 (Fig. 2).

All isolates were found to suppress the *Fusarium moniliforme*-induced foot rots and wilting disease of maize significantly (Table 4). Combined inoculation with the three isolates MR-11(2), MRF and M23 was

Table 5. Population densities of the three biocontrol rhizobacteria in the rhizotic zones of maize at 30 and 60 days after seeding (log no. of cells/g)

Treatments	Rhizosphere			Rhizoplane			Endorhizosphere		
	M23	MRF	MR -11(2)	M23	MRF	MR -11(2)	M23	MRF -11(2)	MR
30 days after seeding									
Control	5.54 b	4.40 b	6.30 b	6.13 c	5.52 b	6.35 a	4.54 a	4.40 b	4.74 a
F. moniliforme	6.42 a	4.90 a	6.20 b	6.34 b	6.01 a	6.00 bc	4.50 ab	4.60 a	4.69 a
F. graminearum	5.54 b	4.30 b	6.80 a	6.58 a	6.03 a	6.12 b	4.70 a	4.59 a	4.54 a
M. phaseolina	5.85 b	4.43 b	6.91 a	6.01 c	5.99 a	5.95 c	4.30 b	4.63 a	4.59 a
60 days after seeding									
Control	5.64 a	4.51 ab	6.59 c	6.38 a	5.96 b	7.39 a	4.52 b	4.08 d	4.61 a
F. moniliforme	5.79 a	4.60 a	6.93 a	6.25 b	6.93 a	7.30 a	4.68 a	4.78 a	4.52 a
F. graminearum	5.85 a	4.40 ab	6.79 b	6.30 ab	6.09 b	7.51 a	4.04 c	4.60 b	4.47 a
M. phaseolina	5.88 a	4.31 b	6.90 a	6.02 c	6.00 b	7.48 a	4.74 a	4.37 c	4.61 a

Means within the same column followed by the same letter (s) are not significantly different at P = 0.05 according to Duncan multiple range test (DMRT).
Average of eight replications repeated thrice.

also found to suppress the disease equally well as individual inoculation with the isolates. However, co-inoculation of MRF and M23 was the best in reducing the disease severity of foot rots and wilting, which was consistent with the result obtained with root rots suppression (Table 4). While co-inoculation of MRF and M23 reduced the disease by 61.82%, individual inoculation of MRF and M23 reduced the disease by 59.34% and 57.48%, respectively (Table 4). Percent disease reduction was significantly (P = 0.05) less in case of the combined inoculation of the two bacilli as compared to co-inoculation with MRF and M23.

Overall, analyses of the results revealed that co-inoculation of *Bacillus* sp. MRF and a Tn5::*lac*Z mutant of fluorescent *Pseudomonas* sp. EM85 (M23) was the best in suppressing the *Fusarium*-induced diseases of maize.

Monitoring of the biocontrol inoculants for ecological competence in the maize rhizotic zones

The ability of the biocontrol rhizobacterial isolates to colonise successfully the rhizotic zones of maize, while antagonising the maize pathogens, were evaluated at 30 and at 60 days after seeding and expressed as log no. of cells g^{-1} soil or root. Tn5::*lac*Z molecular marker was used for monitoring the fluorescent *Pseudomonas* sp. EM85 while intrinsic antibiotic resistance patterns were used for studying the population densities of the two bacilli, MR-11(2) and MRF.

Pseudomonas sp. EM85, M23, was monitored in the maize rhizotic zones. Results (Table 5) indicated that the organism, which was originally isolated from the rhizosphere of maize, was highly efficient in colonizing the root zones of maize at 30 and 60 days after seeding. In the rhizosphere, population densities of M23 were 5.54, 6.42, 5.54 and 5.85 log no. of cells g^{-1} in control and in presence of pathogens, *F. moniliforme, F. graminearum* and *Macrophomina phaseolina,* respectively (Table 5). However, rhizosphere colonization in the presence of *Fusarium moniliforme* was significantly higher compared to all other treatments at 30 days after seeding. Population dynamics at 60 days after seeding indicated that there was a minor improvement in population density of M23 except in the presence of *F. moniliforme* where the population of M23 was reduced as compared to the population at 30 days after seeding (Table 5). There was no significant difference (P = 0.05) among the treatments in population densities of M23 at 60 days after seeding (Table 5). In the rhizoplane, population of M23 was more than 6 log no. of cells g^{-1} in all the treatments at both time points (Table 5). At 30 days after seeding, M23 gave a significantly higher population of 6.58 in the presence of *F. graminearum* while at 60 days after seeding, it could produce a significantly higher population in the absence of the pathogen (Table 5). The organism was also highly efficient in colonizing the endorhizosphere of maize at both time points.

The population densities of the two bacilli isolates, MRF and MR-11(2), were also monitored in the rhizotic zones of maize using intrinsic antibiotic resistance patterns both at 30 and 60 days after seeding. Evaluation of population densities revealed that *Bacillus* sp. MR-11(2) was the best colonizer among the three antagonistic rhizobacterial isolates in the rhizosphere as

well as in the rhizoplane. At 30 days after seeding, MR-11(2) could build up population densities over 6.0 log units, both in the rhizosphere as well as in the rhizoplane (Table 5). Similar observations were noticed in the rhizosphere and rhizoplane when population density was evaluated at 60 days after seeding (Table 5). Even a higher population density of over 7.0 log no. of cells g^{-1} was observed in all treatments with MR-11(2) in the rhizoplane at 60 days after seeding (Table 5). At both time points a significantly higher population of MR-11(2) was obtained in the treatment with *Macrophomina phaseolina* in the rhizosphere and in the control treatment in the rhizoplane (Table 5). Results indicated that *Bacillus* sp. MR-11(2) was also a colonizer to the inside of the root. There was no significant difference ($P = 0.05$) in population densities of the isolate MR-11(2) in the endorhizosphere of maize (Table 5).

Bacillus sp. MRF was also efficient in colonizing the maize rhizosphere, rhizoplane and endorhizosphere. Population densities in the rhizosphere was not encouraging as evident from low population densities in the rhizosphere as compared to the population in the rhizoplane (Table 5). While it could build up a population density of 4.90 log no. of cells g^{-1} in the rhizosphere at 30 days after seeding, it gave 6.93 log no. of cells g^{-1} in the rhizoplane at 60 days after seeding (Table 5). It could also colonise the inside of maize roots.

Isolation, purification and characterization of the antifungal compounds from the rhizobacterial isolates and bioassays

In addition to the production of antifungal antibiotic and fluorescent pigment (as reported earlier by Pal *et al.* (2000), the fluorescent *Pseudomonas* sp. EM85 was also found to produce two more antifungal compounds active against maize root pathogens besides inhibiting *Rhizoctonia solani* (Table 6). Both compounds were isolated and purified. The third compound produced by this biocontrol agent was soluble in methanol with UV

Table 6. Properties of antifungal compounds of the biocontrol rhizobacterial isolates.

Source	Soluble in	Solvent system	R_f of active spot	UV absorption λmax	Physical state	Inhibitory against	Mol. Wt.
Fluorescent *Pseudomonas* sp. EM85	Methanol	Ethylacetate: Methanol (1:1)	0.85	223.5 nm	Viscous	*R. solani* *M. phaseolina*	–
-do-	Water	Ethylacetate: Methanol: Water (1:1:1)	0.71	220.5 nm	Solid	*R. solani* *M. phaseolina* *F. moniliforme*	567
-do-	Methanol	Methanol :Acetone (1:1)	0.42	225.5 nm	Viscous	*R. solani* *F. solani* *M. phaseolina* *F. moniliforme* *F. graminearum*	
-do-	Water	Benzene : Water (1:1)	0.70	209.0 nm	Solid	*R. solani* *M. phaseolina*	
Bacillus spp. MR-11(2)	Methanol	Ethylacetate: Methanol (1:1)	0.22	236.5 nm	Viscous	*R. solani* *F. solani* *M. phaseolina* *F. moniliforme* *F. graminearum*	–
Bacillus spp. MRF	Methanol (1:1)	Ethylacetate: Methanol	0.65	229.5 nm	Viscous	*R. solani* *M. phaseolina* *F. moniliforme*	–
-do-	Water	Ethylacetate: Methanol: Water (1:1:1)	0.10	212.5 nm	Solid	*R. solani* *M. phaseolina* *F. moniliforme*	355
-do-	Acetone, Acetonitrile	Acetone : Benzene (1:1)	0.42	351.0 nm	Viscous	*R. solani* *M. phaseolina* *F. moniliforme*	–

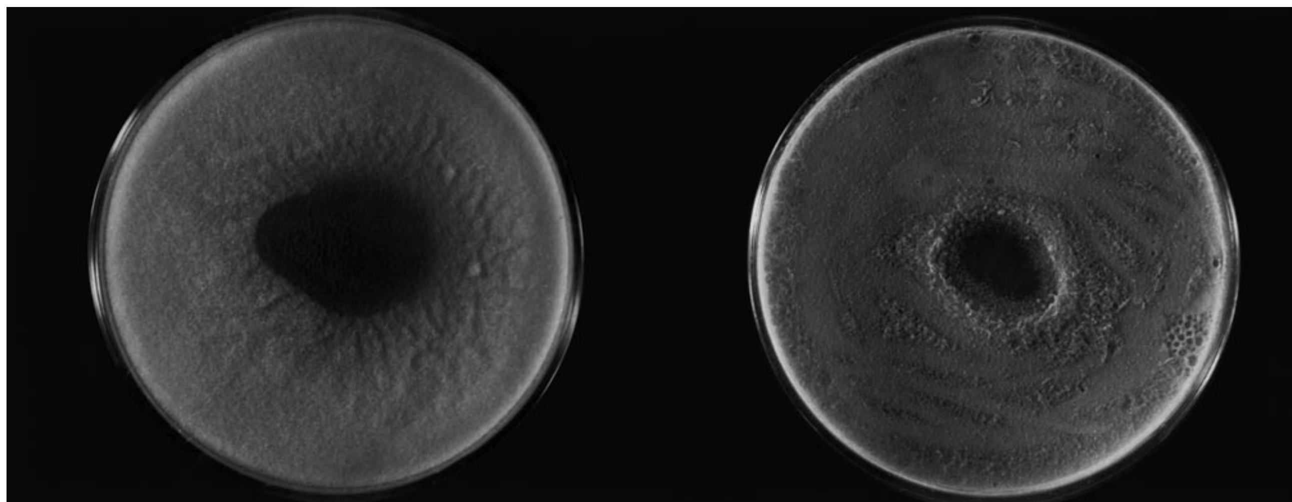

Fig. 3. *In vitro* inhibition of *Macrophomina phaseolina* by a purified compound of: left *Bacillus* sp. MR-11(2); right: water soluble compound of *Bacillus* sp. MRF. 25 µl of the purified active compound was spotted at the center of the PDA Petri dish.

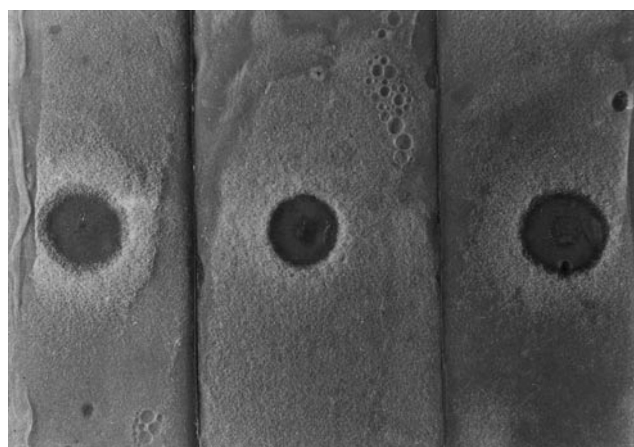

Fig. 4. TLC bioassay of the antifungal compounds active against *Macrophomina phaseolina*. From left to right: Compound of *Bacillus* sp. MR-11(2), water-soluble compound of *Bacillus* sp. MRF, methanol-soluble compound of *Bacillus* sp. MRF.

absorption of 225.5 nm (λ_{max}) and an R_f of 0.42 (methanol:acetone, 1:1 solvent system) and 0.56 (methanol:ethyl acetate, 1:1 solvent system). Another water-soluble compound was purified from this isolate with UV absorption of 209 nm (λ_{max}) and R_f of 0.70 (Benzene:water, 1:1 solvent system). During bioassay, these two purified compounds strongly inhibited *Rhizoctonia solani* besides inhibiting *Macrophomina phaseolina* (Table 6). The chemical nature and structures of these unique compounds are under investigation.

Only one antifungal compound was isolated from *Bacillus* sp. MR-11(2), soluble in methanol, with UV absorption of 236.5 nm (λ_{max}) and an R_f of 0.22 (ethylacetate:methanol, 1:1 solvent system). The compound was viscous in nature and was found strongly inhibitory against *R. solani, Fusarium solani, Macrophomina phaseolina* (Figs. 3 and 4), *Fusarium moniliforme* and *Fusarium graminearum* (Table 6).

Similarly, three potent antifungal compounds were isolated and purified from *Bacillus* sp. MRF. One of these compounds was found to be soluble in methanol with UV absorption of 229.5 nm (λ_{max}), viscous and an R_f of 0.65 (ethylacetate:methanol, 1:1 solvent system). The compound strongly inhibited *Rhizoctonia solani, Fusarium moniliforme* and *Macrophomina phaseolina* (Fig. 3 and 4) in *in vitro* and TLC bioassays (Table 6). Similarly, a water-soluble and solid antifungal compound with a molecular weight of 355, R_f of 0.10 (ethylacetate:methanol:water, 1:1:1 solvent system) and UV absorption of 212.5 nm (λ_{max}) was also isolated (Table 6). This purified compound strongly inhibited *Rhizoctonia solani, Fusarium moniliforme* and *Macrophomina phaseolina* (Fig. 3, 4) *in vitro* and TLC bioassays (Table 6). The third compound was soluble in acetone and acetonitrile with an R_f of 0.42 (acetone:benzene, 1:1 solvent system) and UV absorption of 351.0 nm (λ_{max}). The compound was viscous and strongly inhibited *R. solani, F. moniliforme* and *Macrophomina phaseolina* (Table 6).

Discussion

Despite several reports on the degree of suppression of maize root diseases caused by *Fusarium graminearum, Fusarium moniliforme* and *Macrophomina phaseolina,* by different rhizobacteria like *Bacillus* spp. (Kommendal and Chang 1975), *Pseudomonas fluor-*

escens (Raju *et al.* 1999), *Pseudomonas* spp. (Chen *et al.* 1999), *Pseudomonas cepacea* (Hebbar *et al.* 1992a, b) and *Burkholderia cepacea* UPR5c (Sanchez *et al.* 1994), no studies on the biocontrol of these maize root pathogens with a fluorescent *Pseudomonas* sp. EM85 (which could produce fluorescent pigment in PDA along with nitrogen fixation, phosphate solubilization and organic acid producing attributes) have been performed together with other plant growth promoting bacilli.

All three rhizobacterial isolates taken for the disease suppression experiments were obtained from maize rhizosphere and endorhizosphere, which represented the predominant groups of maize rhizosphere and endorhizosphere populations. Soil pseudomonads and bacilli were found frequently within the root tissues in maize (Hebbar *et al.* 1992a). Moreover, the antagonistic endorhizosphere population is more important for successful colonization of the biocontrol agents for effective competition against the root invading maize pathogens (Lalande *et al.* 1989).

All rhizobacterial isolates exhibited strong antifungal activities against maize root pathogens (Table 2). However, inhibitory effects were more prominent on PDA than on NA. The nutrient constituent of the medium plays a significant role in influencing the production of a particular antifungal metabolite (Hebbar *et al.* 1992b) by the antagonistic rhizobacteria. The differences in the inhibitory effect on the fungal pathogens might be due to the nutritional differences of the two media. Similar observations were also reported earlier with *Pseudomonas cepacea* (Hebbar *et al.* 1992a).

Fluorescent *Pseudomonas* sp. EM85 also fixed atmospheric nitrogen, solubilized tri-calcium phosphate, produced catechol type of siderophore, IAA and organic acids. All these plant growth-promoting attributes might have contributed to enhancing plant biomass, healthy root and plant growth. Similar observations were also recorded with the two bacilli isolates. Plant growth promotion by pseudomonads and bacilli are well documented and found to influence the plant to develop resistance against the root invading pathogens by production of organic acids (Glick 1995).

Little correlation was observed between *in vitro* antagonism and *in situ* disease suppression in several studies (Hebbar *et al.* 1992a). The fluorescent *Pseudomonas* sp. EM85 exhibited antifungal traits such as production of siderophore, HCN, antibiotics and fluorescent pigment. The fluorescent pigment produced on PDA was different from the siderophores produced on CAS agar (Pal *et al.* 2000). While *Bacillus* sp. MRF produced antifungal antibiotics and siderophore, MR-11 (2) produced antibiotic, volatiles and siderophores as antifungal traits. The possible mechanisms by which fluorescent *Pseudomonas* and bacilli exhibit biocontrol have been reported (Weller 1988; Voisard *et al.* 1989; Bull *et al.* 1991; Dowling and O'Gara 1994; Cartwright *et al.* 1995; Emmert and Handelsman 1999).

Application of the compatible plant growth promoting rhizobacteria either singly or in combinations effectively suppressed the disease severity and increased the percent disease reduction caused by *Macrophomina phaseolina, Fusarium moniliforme,* and *Fusarium graminearum*, although there was not much variation among the treatments.

A large number of soil microorganisms are capable of producing siderophores. While bacterial siderophores are of both catechol and hydroxamate types, fungal pathogens usually produce hydroxamate siderophores. As fusaria are reported to produce siderophores of their own, involvement of bacterial siderophores in suppressing these maize pathogens may be ruled out. Similar observations were also made with *Pseudomonas cepacea* antagonistic to *Fusarium moniliforme* and in other studies (Hebbar *et al.* 1992b; Neiland 1986). Moreover, siderophores may not be produced in sufficient quantities in the soil microcosm to have any significant biocontrol effect (Misaghi *et al.* 1988).

Availability of sufficient iron in initial stages of plant growth may have hindered the production of siderophores. Similarly, insufficient amounts of cyanogenic glucosides in the root exudates at early stages of plant growth could have prevented cyanide production. Moreover, cyanide is rapidly inactivated by soil colloids (Voisard *et al.* 1989). Thus, cyanide production might not contribute to suppression of maize root pathogens.

It has been shown in several studies, that siderophores have little or no role in disease suppression (Hamdan *et al.* 1991), while antibiotics, antifungal volatiles and other metabolites are involved in suppression of *Fusarium moniliforme, Fusarium graminearum* and *Macrophomina phaseolina* (Hebbar *et al.* 1992a). Hence, antifungal antibiotics and fluorescent pigments produced by the fluorescent *Pseudomonas* sp. EM85, which were also found to be involved in controlling *R. solani* in cotton (Pal *et al.* 2000), antibiotics and antifungal volatiles produced by *Bacillus* sp. MR-11(2) and antifungal antibiotics of *Bacillus* sp. MRF might be involved in the biological suppression of these maize root pathogens. Involvement of non-siderophore fluorescent pigment in the suppression of maize root pathogens is unique. However, development and evaluation of deficient mutants of the different antifungal traits of these rhizobacteria and their analyses could unravel the specific traits involved in the biological suppression of the maize root pathogens. Work has been initiated in this direction in our laboratory.

Bowen (1978) suggested that high soil populations coupled with faster growth rates and high levels of

competitive abilities were the key for effective biocontrol agents. Successful colonization of the biocontrol agents is a pre-requisite for exerting any biocontrol effects (Hebbar *et al.* 1992a; Dowling and O'Gara 1994). In the *in situ* disease suppression experiments, it was observed that all antifungal rhizobacterial isolates could colonize successfully the inside of the root tissues. Evaluation of population dynamics of all three biocontrol agents using either *lac* Z molecular or intrinsic antibiotic markers revealed the proper colonization of the isolates. This is important because pathogens need to invade the root tissues for disease expression. There was a significant population of the inoculant strains on the root surface too (estimated on the basis of molecular marker and antibiotic resistance markers, Table 5). Again, the generation time of the biocontrol agents was low enough to facilitate early establishment of the inoculant strains. This in turns facilitated the organisms to establish in the root tissues before the pathogens could invade. Thus, niche exclusion could have been involved in minimizing the incidence of maize root diseases. Similar observation were recorded in several earlier studies (Hebbar *et al.* 1992a; Dowling and O'Gara 1994; Weller 1988). However, only in very few occasions, inoculants strains were recovered from the roots and rhizosphere by using molecular markers.

Moreover, one antifungal compound produced by *Bacillus* sp. MRF was soluble in water. This is important as it would have allowed the metabolite to travel to the entire root zones to exert suppression of the sensitive pathogens. The involvement of the antifungal metabolites in suppression of maize root diseases is further substantiated by the fact that all purified metabolites exhibited strong inhibitory effects against the pathogens in TLC as well as in Petri dish bioassays.

Although significant variation in disease suppression was not evident when inoculated in combinations, the combination could provide better ecological competition with the pathogens than the individual one, and failure of one organism may be complemented by others.

The best combination among the three was found to be *Bacillus* sp. MRF and fluorescent *Pseudomonas* sp. EM85.

Thus, we conclude that combinations of fluorescent pseudomonas and bacilli could suppress the maize root invading pathogens efficiently. Fluorescent pigment and antifungal antibiotics (or metabolites) of *Pseudomonas* sp. EM85 and antifungal antibiotics of both bacilli coupled with successful root colonization of the biocontrol agents might be involved in biological suppression of the pathogens. To the best of our knowledge, this is the first report showing the involvement of non-siderophore fluorescent pigment of fluorescent *Pseudomonas* in biological suppression of maize root pathogens.

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