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Secondary metabolites of mungbean rhizosphere bacteria and their role in management of bacterial leaf spot of mungbean

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One out of twenty one mungbean rhizobacteria (MRb) was selected for intensive studies on the basis of its *in vitro* antagonistic activity towards *Xanthomonas azonopodis* pv. *vignaradiatae (Xav.)*, the inducer of bacrerial leaf spot (BLS) of mungbean. The MRb isolate was identified as *Pseudomonas fluorescens* (MRb1). The most effective mungbean rhizobacteria (MRb1) was moderately cyanogenic and produced four metabolites tentatively referred to as compound I (R_f 0.81, hydroxyl phenol), Compound II (R_f 0.49, poly hydroxyl phenol), compound III (R_f 0.18, poly hydroxyl phenol) and compound IV (R_f 0.15, di hydroxyl phenol). Out of these four compounds, only compound II and compound IV were effective both *in vitro* and *in vivo* against *Xav.*

Key words : Secondary metabolites, mungbean rhizosphere bacteria, bacterial leaf spot of mungbean

INTRODUCTION

Bacterial leaf spot of mungbean incitant *Xanthomonas axonopodis* pv. *vignaradiatae (Xav.)* (Vauterin *et al.*, 1995) is one of the most important bacterial diseases of mungbean causing (5-15 %) losses since 1970s. The disease management through conventional chemicals and with the use of resistant cultivars is practically feasible but the hazardous effect of chemicals on environment, breakdown of resistance and difficulties in pyramiding useful resistant genes, has made these approaches ecologically, technically and even socially unsustainable. In such a scenario environmental friendly alternative strategies such as use of rhizospheric and endophytic bacteria is being explored in India (Mondal *et al.*, 1999 ; Bhowmik, 2000). Rhizobacteria which exert a beneficial effect on the plant being colonised are termed 'Plant Growth-Promoting Rhizobacteria'. PGPR may benefit the host by causing plant growth promotion or biological disease control. The same strain of PGPR may cause both growth promotion and biological control. Efforts to select and apply PGPR for control of specific soilborne fungal pathogens have been reviewed (Kloepper, 1993; Glick and Bashan, 1997).

Plant growth promotion by PGPR may also be an indirect mechanism of biological control, leading to disease escape when the growth promotion results in shortening the time that a plant is in a susceptible state, e.g. in the case where PGPR cause enhanced seedling emergence rate, thereby reducing the susceptible time for pre emergence damping-off. An alternative mechanism for biological control by PGPR is through induced systemic resistance. In most of these cases, biological control results from bacterial production of metabolites which directly inhibit the pathogen, such as antibiotics, hydrogen cyanide, iron-chelating siderophores, and cell wall-degrading enzymes. Microbial secondary metabolites are considered to play a major role in disease suppression. Metabolites with biocontrol properties have reported from diverse members of the beneficial microflora. Therefore, to study the biocontrol potentiality of rhizosphere bacteria and its exploitation for plant disease managenment and the knowledge of mechanism of biocontrol is must. Hence, the present study was undertaken with the objective of screening, characterization of effective rhizobacteria and isolation, preliminary characterization and use of its effective metabolites for plant disease management.

MATERIALS AND METHODS

Isolation of rhizobacteria

Rhizosphere bacteria were isolated using nutrient agar medium. 1g of soil sample was suspended in 10 ml of sterile water and was vigorously shakened for 15-20 mins. Serial dilutions were done by transferring 1 ml suspension to 9 ml sterile distilled water. A 0.1 ml suspension of 10^5 - 10^7 dilution were speread on solidified nutrient agar medium amd then plates were kept for incubation at 28°C for 24 h.

Maintenance of rhizobacterial isolates

The individual bacterial colonies were picked up and streaked on yeast glucose chalk agar (YGCA) slant and after incubation at 28°C for 14 hrs. the slants were kept at 4°C for short period maintenance. Periodic (at days intervals) sub culturing was done on the same medium. The long-term storage of bacterial isolates was done by inoculating each single colony into 10 ml nutrient broth following incubation at 28°C for 24 h with shaking at 200 rpm. After that the broth was mixed with 50% glycerol solution at 3:1 (Bacterial broth : glycerol) ratio and kept in Cryobox at 80°C.

In-vitro antagonisism of rhizosphere (MRb) against Xanthomonas axonopodis pv. vignaerediatae

For testing antagonism activity, 1 ml *Xav* suspension (0.1 O.D.) was mixed with 25 ml melted cooled (40-45°C) SPA and poured into petriplate. The solidified plates were spot inoculated with 24 h old solid growth of MRb isolate. After three days incubation at 26 ± 1°C, the plates were examined for antagonistic action indicated by the appearance of inhibition zone at the site of seeding and the diameter of clear zone was measured. Plates without MRb serve as control.

Study of morphological, cultural and biochemical characters of MRb1

The rhizobacterial isolates showing *in-vitro* antagonistic activity against *Xav* were identified on the basis of morphological, cultural and biochemical characters using the identification scheme of Stolp and Gadkari (1981)

Assay for production of secondary metaboilites by MRb1 isolate

Antagonistic MRb isolate was assayed for the production of different secondary metabolites including HCN, antibiotics etc. Production of HCN was tested qualitatively according to the method of Lorck (1948).

Extraction and purification of metabolites from effective biotic elicitors

Liquid culture of MRb1 was prepared by inoculating sterilized (200 ml) nutrient broth (NB) contained in conical flux (1 L) with 10 ml (0.1 O.D.) culture suspension of MRb1. The flasks were incubated at 26 ± 1°C for 72 h in shaker incubator at 180 rpm (Brunswick incubator shaker, USA). Esch liquid culture was centrifuged at 6000 × g for 15 mins to separate bacterial cells. The supernatant was then extracted by liquid portion with ethyl acetate. The organic layer was dried over anhydrous sodium sulphate and concentrared on a rotary evaporator at 40-50°C under reduce pressure. The residue was dissolved in 1 ml acetone and 50 μl of the samples were spotted on TLC plates (Silica gel 60 F_{254} Marck). These spotted TLC plates were developed in benzene solvent system in case of crude metabolites of MRb1 and visualized by iodine vapour. The Rf (Retardation factor) values were calculated by using the formula :

$$Rf = \frac{\text{Distance moved by the compound}}{\text{Distance moved by the solvent front}}$$

A portion of the concentrated residue was retained for biological studies and remaning used for isolation and purification of metabolites.

Purification and quantification of metabolites

For purification and quantification of the metabolites, cell free growth media of the effective biotic elicitors were extracted by ethyl acetate following the procedure as described earlier. The purification of different metabolites were done

through Column Chromatography. Secondary metabolites were separated by Column Chromatography using a glass column (50 × 2 cm) packed with a slurry of silica gel (60-120 mesh preactivated at 120°C for 4 h) in hexane. The column was successively eluted in hexane: acetone (100 : 6). hexane : acetone (100.8), hexane : acetone (100 : 10) and hexane :acetone (100 : 11), collecting 25 ml fractions with a flow rate of 1 ml / min. These fractions were distilled on water bath and monitored by TLC, HPLC and GLC.The fractions of similar compositions were mixed together for further studies.

Preliminary chatacterization of metabolites of MRb1

The four purified compounds (I, II, III and IV) obtained from ethyl acetate extract of cell free growth medium of MRb 1, were characterized by their reaction text for phenolic group. A tinge of purified compounds were added to a $FeCl_3$ solution (in 5% EtOH). The distinct colour change of $FeCl_3$ solution (yellow coloured) after addition of compounds indicated the positive reaction for phenolic group. Depending on the number of hydroxyl group of the compounds the resultant colours (after addition of compounds) varied.

Bioassay of purified metabolites

In vitro efficacy of the purified metabolites (compounds) on inhibition of growth of Xav

The efficacy of the metabolites (tentatively designated as compounds) for the suppression of *Xav* growth was studied by paper disc method. Sterilized filter paper disc were dipped into the solution of purified chromatogram elutes (concentration used 1 mg per ml sterile distilled water) and placed on petriplate spreaded with *Xav* on nutrient agar (NA) medium. Disc dipped in sterile distilled water served as control. Observation was recorded on diameter of inhibition zone (in mm) on *Xav* at 48 hr.

In vivo effect of the metabolites of MRB1 on SR / HR of Xav and on per cent protection of BLS of mungbean

To study the effect of purified secondary metabolites on the susception reaction (SR) and hypersensitive reaction (HR) of *Xav*, chromatogram elutes (500 ppm concentrarion of Ps II and Ps IV) were pre-inoculated followed by 0 h and 8 h challenge inoculation *Xav* on healthy leaves of Pusa Baisakhi (susceptible) followed by injection infiltration technique. The experiment was conducted with 10 plants and 3 trifoliates from each plant. The SR reaction was graded following 0 to 5 scale.

RESULTS AND DISCUSSION

In-vitro antagonism of rhizosphere (MRb) against Xanthomonas axonopodis pv. vignaeradiatae

The antaonism of the MRb1 against *Xav* was tested on NA plates following dual culture technique. Three out of twenty one rhizospheric bacteria showed inhibition against *Xav* and MRb19 and MRb13 (Table 1). Hence, MRb1 was selected for intensive studies of management of BLS of mungbean.

Table 1 : *In vitro* inhibition of *Xav* by mungbean rhizobacterial (MRb) isolates.

MRb isolates	Average inhibition zone* at 48 h (mm)
MRb 1	33
MRb 13	9
MRb 19	12

Table 2 : Morphological, cultural and biochemical characters of MRb-1 isolate.

Characters		MRb-1 isolates
Morphological	Shape	Rod
	Gram reaction	Gram negative
	Spore production	Non sporulating
	Capsule formation	Non capsulated
Cultural	Colony	2-4 mm dia, Shiny, Creamy white
	YGF produced of King's B medium	+
	BGF produced of King's B medium	–
	Oxygen requirement	Aerobic
Biochemical	Arginine dihydrolase activity	+
	Gelatine liquefaction	+
	H_2S production	+
	Oxidase test	+
	Levan production	–

+ : Positive, – : Negative

Morphological, biochemical and cultural

charecteristics of MRb1

The identification of MRb1 isolate was based on morphological, cultural and biochemical characters (Table 2). The isolate was gram negative, rod shaped, non spore former and produced oxidase, arginine dihydrolase and H_2S, tentatively identified as *Pseudomonas* sp. The isolate produced yellowish green diffusible fluorescent pigment on King's B medium. As the isolate (MRb1) liquefied gelatin, it was tentatively identified as *Pseudomonas fluorescens.*

Secondary metabolites of MRb1

Different mechanisms and metabolites have been shown to be involved in the suppression of pathogenic microorganisms by biocontrol agents. MRb1 isolate was assayed for cyanide production and it was found that MRb1 isolate was moderately cyanogenic. The result clearly indicated that HCN is one of the most important secondary metabolite of rhizospheric bacteria.

Table 3 : Chromotographic characterisation and quantification of the major metabolites of MRb-1 (*Pseudomonas fluorescens)*

Fraction range of the elutes (flow rate 1 ml/min)	Eluted with hexane : acetones solvent system	Compound (metabolites designated)	Rf values (solvent used benzene)	Quanti-fication (mg/L)
24-46	100/6	Ps-I	0.81	24
67-82	100/8	Ps-II	0.48	16
102-113	100/10	Ps-III	0.18	9
127-155	100/11	Ps-IV	0.15	21

Table 4 : Preliminary characterisation of the nature of metabolites produced by MRb-1

Compounds	Test for phenolic group	Resultant colour of the $FeCl_3$ solution* after addition of compounds	Tentative nature of the compounds
Ps-I	+	reddish brown	Phenolic (Hydroxy type)
Ps-II	+	dark green	Phenolic (Polyhydroxy type)
Ps-III	+	dark green	Phenolic (Polyhydroxy type)
Ps-IV	+	light green	Phenolic (Dihydroxy type)

* $FeCl_3$ solution (in 5% alcohol)

Four major compounds (Ps-I, Ps-II, Ps-III, and Ps-IV) were obtained through column chromatographic separation of the ethyl acetate extract of cell culture medium of MRb1 isolate. Of these compounds Ps-I, Ps-II, and Ps-IV were fluorescent in nature as detected under UV illumination while compound Ps-III was non fluorescent. Compounds (Ps-I, Ps-II, Ps-III and Ps-IV) were produced in the ratio of 2.6 : 1.8 : 1.0 : 3.4.

Table 5 : Protection of bacterial leaf spot of mungbean through application of metabolites (compound Ps-II and compound Ps-IV) of MRb-1

Pre inoculation	Hours of challenge inoculation of *Xav* (E620 = 0.1)	Average disease grade recorded after 5 days	Per cent protection
With metabolites @ 500 ppm			
Ps-II	0	2.4	52
	8	2.6	48
Ps-IV	0	1.0	80
	8	1.4	72
Sterile water control	0	5.0	0
	8	5.0	0

* Data are average of five replications for each treatment

All the four compounds were phenolic and were grouped as hydroxy (Ps-I), dihydroxy (Ps-IV) and polyhydroxy (Ps-II & III) phenol. Only compound Ps-II & IV showed inhibitory activity in *in vitro* against *Xav.* Out of the two effective compounds, Ps-II & IV might be responsible for the antagonistic activity of isolate MRb1 against *Xav.*

The data on the per cent protection of susceptible reaction of *Xav* by metabolites of MRb1 revealed that compounds Ps-II & IV gave adequate protection under 0 h coinoculation (52% and 80% with compounds Ps- II & IV respectively) and 8 h preinoculation (48% and 72% compounds PS-II & IV respectively) to *Xav* in Pusa Baishakhi. The result indicated that compounds Ps-II & IV were effected in reducing the disease intensity both under *in vitro* and *in vivo* condition. Mondal (1998) also isolated four phenolc compounds (2-fluorescent) including 2,4- diacetyl phoroglucinol from cotton rhizobacteria which not only inhibited the pathogen but also reduced the bacterial blight disease of cotton under glass- house condition. Data in the present investigation is now sufficient to include the effective metabolites produced by antagonistic biocontrol agents in the integrated management of bacterial leaf spot disease of mungbean because (i) they are antagonistic to the pathogen, (ii) they possess preinoculative protective ability and are effective both as seed treatment and spray, (iii) they can induce SAR and iv) the product is not neutralized by the specific pathogen *(Xav).*

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