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ROLE OF SECONDARY METABOLITES ON BIOCONTROL POTENTIALITIES OF NATIVE RHIZOBACTERIAL ISOLATES AGAINST *RHIZOCTONIA SOLANI*

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ABSTRACT

Sixty two isolates of rhizobacteria were isolated from rhizosphere of rice crop, chilli, cabbage and potato collected from Gangetic Alluvial regions of West Bengal. Rhizobacterial isolates were evaluated *in-vitro* by dual culture method. Ten of which were found to be potent bioagents. The isolates were identified as *Pseudomonas aerugenosa* based on biochemical and molecular identification techniques. Studies revealed that the mycelial growth of *R. solani* was inhibited up to 1.9 (cm) by PTR-3 and were found to exhibit antagonism of over 68.9% which is followed by PCF-3(65.6%). Isolate PTR-3 having higher level of chitinolytic (17.2 pmol/s) and proteolytic (halo zone 2.9 cm) activity was highly effective against *R. solani*. Among the ten antagonistic rhizobacteria, highest level of salicylic acid(0.54), siderphore(3.9 μ mole benzoic acid /ml) and HCN production was noticed by PTR-1 isolate. Whereas, highest level of IAA(0.01039μg/ml) and Phosphate solubilisation(17.3μg/ml) activity were noticed by PTR-4 and PTR-3 isolates respectively. Extracellular enzymes activity of protease and chitinase were found to be highly and positively correlated with the ability to antagonize *R solani*. Step wise regression analysis revealed that the combination of biochemical variables chitinase and protease activity jointly contributing 98% of variability of antagonistic potentiality of rhizobacteria against, *R. solani* causing sheath blight disease of rice. These variables may be used as predictors of biocontrol potentiality of rhizobacteria against destructive soil-borne fungal pathogen *Rhizoctonia solani*.

INTRODUCTION

The beneficial effects of plant growth promoting rhizobacteria (PGPR) have been exploited in many areas including biofertilizers, microbial rhizo-remediation and biopesticides (Adesemoye *et al.*, 2008). PGPR group offers an effective means of antagonism against sheath blight pathogen, *R. solani* (Luo Jin Yan *et al.*, 2005). Secondary metabolites produced by fluorescent Pseudomonads have been reported antifungal activity inhibiting *R. solani* (Mina *et al.*, 2013). Rice sheath blight pathogen, *R. solani* suppressed by non-fluorescent PGPR like *Bacillus* sp was previously attributed to the production of chitinase (Krishnaveni, 1991) and other antifungal metabolites. A large number of bacterial strains were found to possess the ability to protect rice plants from sheath blight disease (Vasantha Devi *et al.*, 1989) and these were identified through dual plate assays. In recent years, fluorescent Pseudomonads have drawn worldwide attention as they produce secondary metabolites such as siderophore, antibiotics, volatile compounds HCN, enzymes and phytoharmones (Reddy *et al.*, 2007). PGPR have been considered as an alternative to agrochemicals for controlling plant diseases as plant pathogens developed resistance against pesticides. Therefore, in the present investigation attempts were made to studies the secondary metabolites as bio-control potentiality produced by fluorescent pseudomnads against *Rhizoctonia solani*

MATERIALS AND METHODS

Isolation of Rhizobacteria

The soil samples were collected from rhizosphere of healthy plants of different rice field grown crops. The bacterial suspension thus obtained was used for the isolation of rhizobacteria by dilution plating method King's B medium (Johnson and Curl, 1977). After 48 hours of growth at 28ºC ($\pm$2ºC), morphologically different colonies were picked up and purified.

Maintenance of rhizobacterial isolates and fungal cultures Rhizobacteria isolates

For short period maintenance, the individual bacterial colony was picked up and streaked on yeast glucose dextrose agar, incubate at 28ºC ($\pm$2ºC) and kept at 4ºC. Whereas in long term storage single colony was inoculated into 10 ml of nutrient broth, incubate at 28ºC for 24 hours followed by shaking at 200 rpm, the broth was mixed with 50% glycerol solution at 3:1and kept in cryobox at -80ºC.

Pathogen fungal cultures

The fungal cultures *Rhizoctonia solani* obtained from Department of Plant Pathology, BCKV was maintained on Potato Dextrose Agar and preserved in the refrigerator at 4ºC. Fungal cultures were examined in order to test their pathogenicity at regular intervals weekly.

In vitro antagonistic activity against soil borne fungal pathogens

The antagonistic effects of the rhizobacterial isolates were observed against *R. solani* by dual culture technique (Vincent, 1947). For each treatment three replications were used. Percent inhibition over control was calculated by using the formula.

$$I = \frac{C - T}{C} \times 100$$

I = Per cent inhibition of mycelium
C = Growth of mycelium in control
T = Growth of mycelium in treatment

Characterization of Rhizobacteria

The antagonistic rhizobacterial isolates were characterized on the basis of their morphological (cell shape, cell arrangement), cultural (colony type, pigment production) and biochemical (arginine dihydrolase activity, H_2S production, nitrate reduction etc.) identification scheme of Stolp and Gadkari, 1981 and for *Pseudomonas* sp.(Bossis, 1995) , biovars (Bossis *et al*., 2000) and seedling vigour method as described (ISTA, 1966).

Qualitative assay of HCN, IAA, SA production, phosphate solubilization and siderophore production by the bacterial isolates

HCN [Lorck (1948) modified by Alstrom (1989)], IAA (Gordon and weber, 1951), Salicylic acid (Meyer *et al*., 1992), Siderophore production (Reeves, 1983) and Phosphate solubilization (by Ammonium phosphor-molybdate method Jeon *et al*., 2003) was done by spectrophotometric quantitative assay following the methods as mentioned for each assay.

Quantitative and Qualitative assay of different enzyme activities

Protease (Smibert and Krieg, 1994), amylase, cellulase, pectinase and chitinase (Vessey and Pegg, 1973) activity of different rhizobacterial isolates was observed accordingly.

RESULTS AND DISCUSSION

Isolation of rhizospheric bacteria of rice, chilli, cabbage and potato were made isolated on King's B medium, showed 62 rhizospheric isolates present on these crops. Twenty five rhizobacterial isolates were obtained from rice, 12 from chilli, 9 from cabbage and 15 from potato. All the bacteria were screened for their *in vitro* antagonistic activity against soil borne *Rhizoctocnia solani*. Among these, ten isolates showed potential antagonistic activity against all the tested soil-borne fungal *R. solani*.

***In-vitro* antagonism of rhizospheric bacteria against fungal plant pathogen and biochemical identification**

Perusal of the data in Table 1 and 2 indicated that all the ten rhizobacterial isolates showed antagonistic effect against *R. solani* under *in vitro* condition and inhibited the vegetative growth of the fungus at varied level. Among the isolates, PTR-3 was found to be the most effective isolate giving an inhibition zone of 1.9 cm and also caused 68.9 % growth inhibition against *R. solani* followed by PCF-3 (1.7 cm) and PTR-1 (1.5 cm). The antagonistic nature of rhizospheric bacteria against *R. solani* was also reported by Adhikari *et al.* (2013). Tripathi and Johri (2002) observed *in vitro* inhibition of *Colletotrichum dermatium, R. solani* and *Sclerotium rolfsii* by fluorescent pseudomonads. Based on the biochemical characterization following the identification scheme of Bossis *et al*. (2000) revealed that all the isolates were *Psedomonas aeruginosa* as depicated in Table 3.

Studies on protease, amylase, cellulase, pectinase and chitinase activity of different rhizobacterial isolates

Production of chitinolytic enzyme was also analyzed because this is an important means of fungal inhibition. Chitinase was detected in all the ten isolates and isolate PTR (17.2) p mol /s) expressed maximum activity of chitinase followed by PCF-3 (15.6 p mol /s). Rhizobacterial isolates could produce halo zones on skim milk agar and PPF-7 from potato rhizosphere exhibits higher protease activity in terms of production of halo zones (3 cm) on skim milk agar followed by PTR-3 (2.9 cm) (Table 4). But cellulase, amylase, pectinase assay were found to be negative in all the tested rhizospheric isolates.

Role of secondary metabolites and enzyme activities on antagonistic potentiality of rhizobacteria against *R. solani*

Among the rhizobacterial isolates though PTR-1 produced highest HCN (0.0834), salicylic acid (0.54 mg/ml), siderophore (3.9 mM benzoic acid/ml) but PTR-4 produced maximum IAA

Table 1: *In vitro* antibiosis as inhibition zone in cm produced by the effective bacterial isolates against soil borne plant pathogens

Antagonistic Bacterial Isolate	Soil Rhizosphere	Pathogenic fungi (*Rhizoctonia solani)* Inhibition zone (in cm)
PTR-1	Rice	1.5^{c}
PCF-3	chilli	1.7^{b}
PTR-3	Rice	1.9^{a}
PPF-2	Potato	1.1^{f}
PPF-7	Potato	1.2^{e}
PFC-1	cabbage	1.4^{d}
PTR-4	Rice	1.4^{d}
PCF-7	Chilli	0.9^{g}
PFC-2	Cabbage	0.7^{h}
PFC-7	Cabbage	0.9^{g}

Data bearing same letter are not significantly different on the basis of DMRT at 5% level of significance

Table 2: Growth rate of mycelium of fungal pathogen against rhizobacterial isolate after 72 hrs

Sl. No.	Isolates	Pathogen	Growth rate of mycelium of pathogen after 72 hrs	Growth inhibition (%)
1	PTR-1	RS	3.4^{g}	62.2^{d}
2	PCF-3	RS	3.1^{h}	65.6^{b}
3	PTR-3	RS	2.8^{i}	68.9^{a}
4	PPF-2	RS	5.4^{d}	40^{g}
5	PPF-7	RS	5.1	43.3^{f}
6	PFC-1	RS	4.3^{f}	52.2^{e}
7	PTR-4	RS	4.1^{f}	54.4^{d}
8	PCF-7	RS	6.2^{c}	31.1^{h}
9	PFC-2	RS	6.9^{b}	23.3^{i}
10	PFC-7	RS	6.7^{b}	25.6^{j}
11	Control		9^{a}	

Data in the parentheses are arc sin means, data bearing same letter are not significantly different on the basis of DMRT at 5% level of significance

(0.10758 ug/ml). PTR-3 isolate showed highest P-solubilizing activity (17.3) followed by PTR-1 (14.2) (Table 5). Rassouli *et al*. (2005) reported that 201 indigenous *Pseudomonas* spp. isolated from Iranian soils were siderophore producers in CAS-agar medium. Kumar *et al*. (2000) reported that the *P. fluorescens* produced siderophores and antifungal metabolites which are involved in the control of phytopathogenic fungi. Gupta *et al*. (2002) reported *Pseudomonas* sp. acted as a potent phosphate solublizer. Correlation coefficients obtain between different biochemical parameters of rhizobacterial isolates and inhibitory activities against fungal pathogen (*R. solani*) were presented in Table 6 and Fig 1. The antagonistic activity of rhizobacterial isolates were found to be positively correlated with biochemical parameter of protease and chitinase activity. Among biochemical variables the correlation coefficient of chitinase and HCN production with antagonistic potentiality of rhizobacteria was found to be significant at 1% level of significance. A linear multiple regression model was developed using different biochemical parameters through step wise technique can be used to predict the antagonistic activity of rhizobacterial isolates based on biochemical parameters. Using step wise regression technique a single biochemical variable (chitinase) was selected, which found to account for 94.0 % of variability of antagonistic potentiality and can be used as predictor of biocontrol potentiality of rhizobacteria on the basis of measured biochemical variables against fungal pathogens. Thus from the above finding it may be concluded that chitinase and protease production are the two most important biochemical variables significantly contributed towards the biocontrol potentialities of rhizobacteria against fungal pathogen (Equation 1). Biological control with fluorescent pseudomonad offers an effective

Table 3: Characterization of different isolates of rhizobacteria based on biochemical reaction

Isolates	Gram Reaction (3%KOH)	Growth at 4ºC	Growth at 42ºC	Arginine	Oxidase	catalase	Gelatin	trehlose	Levan	De-nitrification	Starch	citrate hydrolysis	Voges proskauer	Identified as
PTR-1	-	-	+	+	+	+	+	-	+	-	-	+	-	PA
PCF-3	-	-	+	+	+	+	+	-	+	-	-	+	-	PA
PTR-3	-	-	+	+	+	+	+	-	+	-	-	+	-	PA
PPF-2	-	-	+	+	+	+	+	-	+	-	-	+	-	PA
PFC-1	-	-	+	+	+	+	+	-	+	-	-	+	-	PA
PPF-7	-	-	+	+	+	+	+	-	+	-	-	+	-	PA
PFC-7	-	-	+	+	+	+	+	-	+	-	-	+	-	PA
PTR-4	-	-	+	+	+	+	+	-	+	-	-	+	-	PA
PBF-7	-	-	+	+	+	+	+	-	+	-	-	+	-	PA
PFC-2	-	-	+	+	+	+	+	-	+	-	-	+	-	PA

+ : positive, - negative; PA: *Pseudomonas aurogenosa*

Table 4: Extracellular enzymes produced by rhizobacterial isolates

Isolates	Cellulase	Amalyse	Pectinase	Protease Halo zone (in cm)	Chitinase (p mol/s)
PTR-1	-	-	-	2.4^{e}	14.8^{e}
PCF-3	-	-	-	2.5^{d}	15.6^{d}
PTR-3	-	-	-	2.9^{b}	17.2^{b}
PPF-2	-	-	-	2.7^{c}	11.7^{c}
PPF-7	-	-	-	3^{a}	12.1^{a}
PFC-1	-	-	-	1.0^{i}	12.3^{i}
PTR-4	-	-	-	1.8^{f}	12.8^{f}
PCF-7	-	-	-	1.2^{h}	9.5^{h}
PFC-2	-	-	-	1.3^{f}	7.5^{g}
PFC-7	-	-	-	1.3^{g}	8.6g

Data bearing same letter are not significantly different on the basis of DMRT at 5% level of significance

Table 5: Antimicrobial metabolites of different rhizobacterial isolates.

Isolates	IAA production (mg/ml)	HCN production (Absorbance at 625 nm)	Salicylic acid production (mg/ml)	Siderophore production (μ mole benzoic acid /ml)	Phosphate solubilization (mg/50 ml)
PTR-1	0.01039^{d}	0.0834^{a}	0.54^{a}	3.9^{a}	14.2^{a}
PCF-3	0.01023^{d}	0.0776^{b}	0.39^{c}	2.8^{d}	12.1^{c}
PTR-3	0.02677^{e}	0.0833^{a}	0.35^{d}	2.5^{e}	17.3^{d}
PPF-2	0.02786^{e}	0.0717^{c}	0.29^{e}	2.09^{g}	12.9^{ef}
PPF-7	0.06529^{b}	0.081^{a}	0.41^{b}	3.0^{b}	12.9^{b}
PFC-1	0.02688^{c}	0.0729^{c}	0.21^{f}	1.5^{h}	12.1^{g}
PTR-4	0.10758^{a}	0.0657^{d}	0.34^{d}	2.46^{e}	11.2^{d}
PCF-7	0.06351^{b}	0.0637^{d}	0.28^{e}	2.05^{g}	9.8^{f}
PFC-2	0.00207^{e}	0.034^{e}	0.22^{f}	2.19^{f}	12.3^{e}
PFC-7	0.00126^{e}	0.0324^{e}	0.19^{g}	2.42^{e}	10.3^{d}

Data bearing same letter are not significantly different on the basis of DMRT at 5% level of significance

Table 6: Correlation coefficient between antifungal antagonistic potentiality of rhizobacteria and their secondary metabolites production

Inhibition zone (Y)	Siderophore production (X1)	SA (X2)	HCN(X3)	IAA(X4)	P- Sol(X5)	Protease(X6)	Chitinase(X7)
Y	0.399	0.657*	0.823**	0.091	0.640*	0.557	0.980**

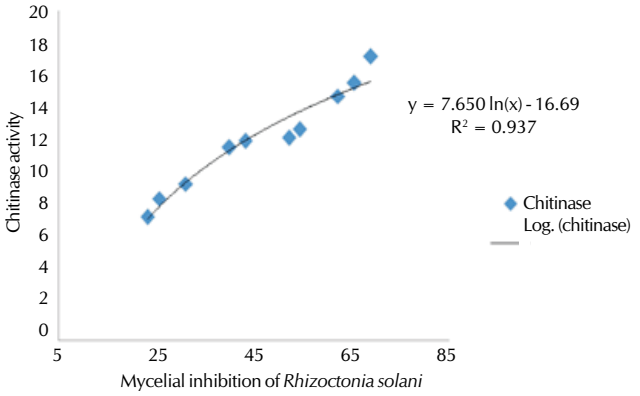

Figure 1: Relationship between chitinase activity and antibacterial antagonistic potentiality of rhizobacteria

method of managing plant pathogens (Ramamoorthy *et al.,* 2001). These bacteria inhibit the fungal pathogens by producing antibiotics, lytic enzymes and by inducing resistance systemically in the plant by activating defensive genes such as chitinase, â-1,3-glucanase, peroxidase and phenylalanine ammonia lyase (Friendlender *et al.,* 1991; Punja and Zhang, 1993; Ramamoorthy *et al.,* 2001; Viswanathan and Samiyappan, 2001). Chitinases are well known to lyse the fungal cell wall (Chet *et al.,* 1987). Addition of chitin or cell wall material to the fungus culture medium has been used in many studies to prove the chitinolytic activity (Ordentlich *et al.,* 1988; Frandberg and Schuurer, 1998; Viswanathan and Samiyappan, 2001).

Equation 1: Stepwise regression equation (secondary metabolites of rhizobacteria and their antifungal antagonistic potentiality)
$Y = -17.367 + 5.244$ (Chitinase); $R^2 = 0.960$; R^2 (adj) $= 0.955$; $Y = -17.563 + 5.943$ (Chitinase) $- 4.147$ (protease); $R^2 = 0.980$; R^2 (adj) $= 0.974$

In conclusion, in this studies production of secondary metabolites such as chitinase, IAA, HCN, Salicylic Acid and siderophore by fluorescent Pseudomonads are important feature in plant disease suppression of root rot and enhancement of plant growth (Gade, 2013).

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