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Bio Control Potential of *Pseudomonas fluorescens* against Coleus Root Rot Disease

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Abstract

Ten different strains of *Pseudomonas fluorescens* were isolated from coleus rhizosphere except the pf1 strain and identified by biochemical tests. These strains were screened against *Macrophomina phaseolina* (Tassi) Goid, the causal organism of coleus root rot. The results revealed that Pf1 strains recorded maximum inhibition of mycelial growth against control. The mechanism of *Pseudomonas* strains namely the iron-chelating agent (siderophore), volatiles (HCN) and antibiotic (Fluorescein and pyocyanin) production tests were studied and reacted for siderophore, antibiotic and HCN production. The talc-based formulation of Pf1 and CPF1 was prepared and the bio-efficacy was tested under greenhouse conditions. The stem cutting and soil application of the talc-based formulation of Pf1 significantly reduced the root rot incidence and increased shoot and tuber length also.

Keywords: Antibiotics; Coleus; HCN; *Pseudomonas fluorescens*; Root rot; Siderophore

Introduction

Coleus (*Coleus forskohlii* Briq.) is cultivated mainly for their medicinal values in India. In India it is cultivated on about 2,500 hectares with an annual production of 1500 tonnes, especially in parts of Rajasthan, Maharashtra, Karnataka and Tamil Nadu. In Tamil Nadu alone, it is cultivated on more than 1000 hectares across. The crop is subjected to attack by many fungal diseases, namely leaf spot (*Botryodiplodia theobromae*), stem blight (*Phytophthora nicotianae* var. *Nicotianae*), collar rot (*Sclerotium rolfsii*, and *Rhizoctonia bataticola*), root rot (*Fusarium chlamydosporum, Rhizoctonia solani* and *Macrophomina phaseolina*) downy mildew (*Peronospora* sp) [1,2].

Root rot of coleus caused by *M. Phaseolina* is widely distributed in many countries and it is a devastating pathogen right from the establishment of the crop. Many effective fungicides have been tested against soil borne pathogens, but are not considered as long term solutions because of concerns about exposure risks, health and environmental hazards, high cost, residue persistence, the development of resistance to pesticides and the elimination of natural enemies. Biological control is a potential non-chemical means for plant disease management by reducing the harmful effects of a parasite or pathogen through the use of other living entities. The utilization of a plant's own defense mechanism is a fascinating arena of research which can be systemically activated upon exposure of plants to PGPR strains or infection by the plant pathogen. This phenomenon is called induced systemic resistance (ISR). This mechanism is facilitated by PGPR organism and activates through various defense compounds at the site of pathogen attack. Among the PGPR, fluorescent pseudomonads are the most exploited bacteria for biological control of soil-borne and foliar plant pathogens. In the past three decades, numerous strains of fluorescent pseudomonads have been isolated from the rhizosphere soil and plant roots by several workers and their biocontrol activity against soil-borne and foliar pathogens were reported [3]. Fluorescent pseudomonads are non-pathogenic rhizobacteria which suppress the soil-borne pathogens through rhizosphere colonization, antibiosis, iron chelation by siderophore production and ISR. In my knowledge, there is no report available in the control of root rot disease by Pseudomonas sps in coleus, but the use of antagonistic microorganisms such as *Pseudomonas fluorescens* against *Macrophomina phseolina* have been reported many workers [4,5]. In the present investigation, attempts were made to test the antagonistic activity of *Pseudomonas fluorescens* and its mechanisms for coleus root rot management.

Materials and Methods

Isolation of pathogen and *Pseudomonas strains*

The root rot pathogen *M. Phaseolina* was isolated from coleus plants showing typical root rot symptoms and pure cultures of the pathogen were obtained by the single hyphal tip method [6]. The biocontrol agent *P. fluorescens* strain Pf1 was obtained from the culture collection section, Department of Plant Pathology, Centre for Plant Protection Studies, Tamil Nadu Agricultural University, India.

Other native coleus rhizobacterial *P. fluorescens* (CPF1 to CPF10) strains were isolated from soil samples obtained from different parts of Tamil Nadu state. One gram of rhizosphere soil adhering to root surface was collected and transferred to a 250 ml conical flask containing 100 ml of sterile water. After thorough shaking for 15 minutes in a shaker, different dilutions were prepared. One ml of each 10^{-5} and 10^{-6} dilution was pipetted out and poured into the sterile petridishes. Later King's medium B (KB) [7] was poured, rotated and incubated at room temperature (28 ± 2°C) for 24 hours. After 24 hours of incubation, the bacterial growth was purified by the dilution plate technique [8]. The bacterial culture was maintained in King's B broth (KB) in 30 percent (v/v) glycerol at -80°C.

Characterization of the different cultures of antagonistic bacteria

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was done according to the methods recommended in the laboratory guide for identification of plant pathogenic bacteria published by the American Phytopathological Society [9]. In each test, 24-48 hours-old cultures were used.

Siderophores production

Production of siderophores by *P. fluorescens* was assayed by the plate assay method as described by Schwyn and Neilands [10]. The tertiary complex chromeazurol S (CAS) served as an indicator. To prepare one litre of the blue agar, 60.5 mg CAS was dissolved in 50 ml water and mixed with 10 ml Fe^{3+} solution (l mM $FeCI_2$ $6H_2O$ in 10 mM HCI and HDTMA dissolved in 40 ml water was added by constantly stirring). A forty-eight hour-old culture of fluorescent pseudomonads was streaked onto the succinate medium (Succinic acid, 4.00 g; K_2HPO_4, 3.00 g; $(NH_4)_2SO_4.7H_2O$, $7H_2O$, 0.2 g: distilled water, 1000 ml, pH 7.0) amended with the indicator and incubated for three days.

Production of hydrogen cyanide

The production of HCN was determined using a modification of the [11] procedure. Bacteria were grown on Tryptic-soy-agar (TSA) (animal peptone-5.0 g, soy peptone-5.0 g, sodium chloride-5.0 g, glycine-4.4 g, distilled water l000 ml). Filter paper discs soaked in a picric acid solution (2.5 g of picric acid, 12.5 g of sodium carbonate, and l000 ml of distilled water) were placed in the lid of each Petri-plate. Dishes were sealed with parafilm and incubated at 28°C for 48 hours. A change from yellow to light brown, brown or reddish brown of the discs was recorded as an indication of weak, moderate or strong production of HCN.

Detection of fluorescein and pyocyanin

Pseudomonas agar F (Casein enzymic hydrolysate, 10 g; Protease peptone, 10 g; K_2HPO_4, 1.5g; $MgSO_4$, 1.5 g; distilled water, 1l) favours the formation of fluorescein whereas Pseudomonas agar P (Peptone, 20 g; $MgCl_2$, 1.4 g; K_2SO_4, 10 g; Agar, 15 g; Distilled water, 1l) stimulates pyocyanin production and reduces fluorescein formation [7].

Effects of volatile metabolites

The effect of volatile metabolites from fluorescent pseudomonads on the growth of

M. Phaseolina was studied by a paired Petri dish technique by Gagne et al. [12].

Screening of antagonistic bacteria under *in vitro* condition

The antifungal efficacy of *Pseudomonas fluorescens* strains was tested by dual culture technique [13] using PDA medium. A mycelial disc (9 mm dia) of the pathogen namely *M. Phaseolina* was placed at one end of the plate and the bacterial antagonists were streaked at the periphery of the Petri-dish just opposite to the mycelial disc of the pathogen. The plates were incubated at 28 ± 2°C. The mycelial growth of the pathogen and inhibition zone was measured after 72 h of incubation.

Preparation of talc-based formulation of bio control agents

A loopful of *P. fluorescens* was inoculated into the King's B broth and incubated in a rotary shaker at 150 rpm for 72 hours at room temperature (28 ± 2°C). After 72 hours of incubation, the broth containing 9×10^8 cfu/ml was used for the preparation of talc-based formulation. To 400 ml of bacterial suspension, one kg of the talc powder (sodium ammonium silicate), calcium carbonate 15 g (to adjust the pH to neutral) and carboxy methyl cellulose (CMC) 10 g (as an adhesive) were mixed under sterile conditions following the method [14]. The product was shade dried to reduce the moisture content to 20 per cent and then packed in polypropylene bags and sealed. At the time of application the population of bacteria in talc formulation was checked in 2.5 to 3×10^8 cfu/g.

Greenhouse studies

Coleus cuttings were treated with talc-based formulations of Pf1 and CPF1 at 0.2 per cent for each stem cutting and planted in pots. Twenty-five grams of the formulated product (2.5 kg talc-based formulation mixed with 50 kg of farmyard manure) was given as a soil application per pot at 30 days after planting (DAP). Premixture fungicide (Carbendazim+Mancozeb) at 0.1 per cent was used as a standard check fungicide. It was applied as stem cutting treatment @ 0.2 per cent and also as a soil application @ 0.05 per cent at 30 DAP. A pure culture of *M. Phaseolina* was introduced into a sand-maize (19:1) medium and incubated for 15 days at room temperature for multiplication [15]. The potting soil (red soil: sand: cow dung manure, 1:1:1 w:w:w) was incorporated with the fungus, cuttings of coleus were surface-sterilized with 0.1% mercuric chloride for 30 s, rinsed three times with sterile distilled water and sown at two cuttings per pot. Each treatment was maintained for three replications. All treatments were replicated three times in factorial completely randomized design (CRD).

Results and Discussion

The development of biological techniques using PGPR amended with suitable bioformulations is an emerging trend in plant protection to reduce the plant diseases caused by plant pathogens. Production of antibiotics viz., Siderophore, HCN, pyrrolnitrin, phenazine and 2,4-diacetyl phloroglucinol and lytic enzymes by *P. fluorescens* against fungal pathogens were reported by many workers [3-5,16]. The above facts suggest that the inhibition of root rot pathogen, *M. Phaseolina* by *P. fluorescens* Pf1 may be due to the production of antibiotics, siderophore mediated competition and lytic enzymes, viz., chitinase, β-1,3-glucanase which degraded the fungal cell wall and restricted the growth of fungus under *in vitro* conditions. In the present study, among all the *P. fluorescens* strains, the Pf1 strain had maximum inhibition of mycelial growth and produced more amount of HCN, siderophore, pyocyanin and fluorescein in comparing to all the strains, so only the Pf1 strain was selected for this study. The biocontrol potential and production of volatile metabolites in Pf1 strain has a higher inhibitory effect followed by other *Pseudomonas* strains (Figure 1).

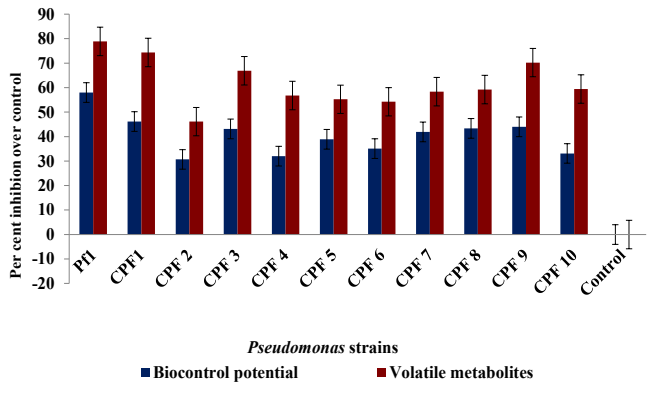

Figure 1: Bio control potential and volatile production of *P. fluorescens* against *M. phaseolina* under *in vitro* condition.

P. chlororaphis strain PA23 demonstrated excellent biocontrol in the canola phyllosphere through production of the non-volatile antibiotics and volatile antibiotics nonanal, benzothiazole and 2-ethyl-1-hexanol [17]. Secondary metabolites and volatile compounds contributed to the toxicity of the bacteria, with hydrogen cyanide efficiently repelling the nematodes and both hydrogen cyanide and 2, 4-DAPG functions as nematicides. Under certain conditions, antibiotics improve the ecological fitness of these bacteria in the rhizosphere which can further influence long-term biocontrol efficacy [18].

All the bacterial isolates produced siderophore by the CAS plate assay method. But all isolates except CPF6 and CPF10 produced a yellow color in the blue colored medium. Siderophore produced by *Pseudomonas* spp. and other rhizobacterial organisms (*Bacillus, Enterobacter*) have been used in the biological control of damping-off of cotton caused by *Pythium ultimum* [19]. Siderophore-mediated competition for iron by *Pseudomonas* sp. as well as induced resistance are primary mechanisms shown to be responsible for suppression of Fusarium wilt and this *Pseudomonas* culture and purified siderophore showed good antifungal activity against the plant deleterious fungi namely *Aspergillus niger, A. flavus, A. oryzae, F. oxysporum* and Sclerotium rolfsii [20]. Among the different *Pseudomonas fluorescens* tested, the intensity of HCN production was strong in *P. fluorescens* strains Pf1 and CPF1 followed by *P. fluorescens* strains CPF6 and CPF8. Normally the HCN production is directly related to inhibition of pathogen, the *P. fluorescens* strains Pf1 and CPF1 produced same amount, but Pf1 strain produced some sideophores, it's also inhibit the most pathogens. HCN from a *P. fluorescens* strain CHA0 not repressed by fusaric acid played a significant role in the disease suppression of *F. oxysporum* f.sp. *radicis-lycopersici* in tomato [21]. HCN production by several strains of *P. fluorescens* and their efficacy in controlling root rot of groundnut caused by *M. Phaseolina* [22]. *Pseudomonas aeruginosa* LES4, an isolate of tomato rhizosphere was found to be positive for HCN against wilt disease [23]. All the *P. fluorescens* strains produced fluorescein and pyocyanin antibiotics in the Pseudomonas agar F and p plate assay method. But all isolates except CPF3 and CPF7 were surrounded with a yellow to greenish-yellow zone on Pseudomonas agar F and surrounded by a blue to green zone or with a red to dark brown zone due to pyocyanin production (Table 1). A marine isolate of fluorescent *Pseudomonas* sp. which has the ability to produce the pyoverdine has been found to inhibit the growth of *A. niger* under *in vitro* conditions [20]. Similar results were [24] reported the specific production of fluorescein and pyocyanin by the *Pseudomonas* bacterium against *Pythium*.

The standardization of the mode of application of bacterial antagonists indicated that dipping of the stem cuttings together with soil application were highly effective in inhibiting root rot incidence rather than the individual application of antagonists either through stem cutting dipping or soil application. Among the bacterial antagonists tested, delivery of Pf1 as stem cutting, dipping and soil application recorded the lowest disease incidence and highest per cent

S.No	PGPR strains	Fluorescein	Pyocyanin	Siderophore production	HCN
1	Pf1	+	+	+	+++
2	CPF1	+	+	+	+++
3	CPF 2	-	-	+	+
4	CPF 3	-	-	+	-
5	CPF 4	+	+	+	+
6	CPF 5	+	+	+	+
7	CPF 6	+	+	-	++
8	CPF 7	-	-	+	-
9	CPF 8	+	+	+	++
10	CPF 9	+	+	+	+
11	CPF 10	-	-	-	+

+: Produced; - : Not produced (+++: Strong, ++: Medium, +: Low production)

Table 1: Antibiotics, siderophore and HCN production of P. fluorescens strains.

S. No	Treatments	Per cent disease incidence	Per cent reduction over control	Shoot length (cm)	Tuber length (cm)	No. of tubers/plant
1	Pf1 (SCD alone)	37.26^{d} (37.62)	41.74	46.65^{f}	26.54^{d}	7.62^{de}
2	Pf1 (SA alone)	26.53^{b} (31.00)	58.74	58.54^{b}	29.96^{b}	8.52^{c}
3	Pf1 (SCD+SA)	24.12^{b} (29.42)	62.50	63.56^{a}	32.86^{a}	10.65^{a}
4	CPF1 (SCD alone)	39.42^{e} (38.89)	21.66	40.26^{h}	25.92^{cd}	6.94^{e}
5	CPF1 (SA alone)	33.23^{d} (35.20)	23.44	50.93^{e}	27.56^{c}	7.02d^{e}
6	CPF1 (SCD+SA))	32.56^{cd} (34.99)	29.16	55.26^{c}	30.92a^{b}	8.82^{c}
7	Pre mixture fungicide (Carbendazim +Mancozeb) (SCD alone)	30.53^{c} (33.54)	52.52	42.26^{g}	27.65^{c}	7.54^{d}
8	Pre mixture fungicide (Carbendazim +Mancozeb) (SA alone)	24.36^{b} (29.58)	62.12	53.65^{d}	27.12^{c}	8.59^{c}
9	Pre mixture fungicide (Carbendazim +Mancozeb) (SCD+SA)	18.56^{a} (22.96)	71.14	58.72^{b}	29.87^{b}	9.56^{b}
10	Inoculated Control	64.31^{f} (53.32)	-	35.89^{i}	20.14^{e}	5.54^{f}
	SEd	0.79		0.84	0.94	0.28
	CD (0.05)	1.64		1.76	1.97	0.57

SCD-Stem cuttings dip; SA-Soil application All Values are mean of three replications; Data followed by the same letter in a column are not significantly different from each other according to Duncan's multiple range test at P=0.05

Table 2: Effects of PGPR on the root rot incidence, growth characters and yield under green house condition.

inhibition over the control (24.12 and 62.50%). It was followed by the application of CPF1 both as stem cutting, dipping and soil application. The chemical premixture fungicide (Carbendazim+Mancozeb) (0.1%) recorded 71.14 per cent reduction over the control. The highest disease incidence was recorded in the inoculated control (64.31%) (Table 2). The positive colonization ability of Pseudomonas GRC2 lies in it being the successful colonizer of the spermosphere, increasing seedling emergence, and its establishment in the rhizosphere of peanuts giving protection against *M. Phaseolina* resulting in enhanced yield [25]. It was further reported by Thilagavathi et al. [26] that the combined application of *P. fluorescens* (Pf1) in seed and soil applications was effective in reducing the root rot disease in green gram under greenhouse and field conditions.

The bio control agents not only controlled dry root rot, but also promoted plant growth and this gives them an advantage over the use of chemical fungicides against root rot in disease management. The studies showed that the PGPR is capable of controlling the coleus root rot. Amongst the different *P. fluorescens* strains tried, stem cutting, dipping and soil application of Pf1 was found to be suitable for the management of coleus root rot under pathogen (*M. Phaseolina*) inoculated soil in greenhouse condition. However, field evaluation is necessary to determine its efficacy under natural ecosystem.

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