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Biocontrol Mechanisms and Intrinsic Antibiotic Resistance of Fluorescent Pseudomonads Isolated from Different Crop Plants

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ABSTRACT

The inherent properties like biocontrol mechanism and intrinsic antibiotic resistance are important functional properties of the fluorescent pseudomonads. Fluorescent pseudomonads produce of biocontrol metabolites like HCN, chitinase and cellulase which helps in suppression of plant diseases. Therefore, in the present study all the 50 isolates were subjected to qualitative and quantitative production of HCN by picric acid assay method, cellulose and chitin degradation plate assay method. The highest HCN production was recorded in FP-24, FP-13, FP-19, FP-18 and FP-47 with 0.14, 0.13, 0.09, 0.07 and 0.07 absorbance respectively. Further, these isolates were also examined for their cellulose and chitin degradation abilities. Among them, 25 and 24 were chitin and cellulose degraders respectively. The common isolates which have shown both cellulose and chitin degradation are FP-1, 2, 7, 12, 13, 17, 18, 19, 24, 25, 27, 28, 31, 35 and 49. The intrinsic antibiotic resistance of all the 50 isolates was tested against eleven antibiotics vizr, Amp (100 µg/ml), CB (5 µg/ml), Kan (50 µg/ml), NA (10 µg/ml), Rif (25 µg/ml), T (20 µg/ml), S (100 µg/ml) , E (10 µg/ml), GEN (10 µg/ml), P (10 µg/ml), C (5 µg/ml). Seven isolates were resistant to Kanamycin (50 µg/ml), all the isolates were susceptible to Tetracyclin (20 µg/ml) and 5 were resistant to Streptomycin (100 µg/ml).

Keywords HCN, Chitinase, Cellulose, Fluorescent pseudomonads, Intrinsic antibiotic resistance.

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INTRODUCTION

The worldwide attention to use fluorescent pseudomonads as biocontrol agent is because of their ability to produce biochemical substances. They are HCN,

antibiotics and lytic enzymes like chitinase, β-1, 3-glucanase and proteases which are responsible for inhibition of soil borne pathogens. The *in vitro* inhibition of *Colletotrichum dermatium, Rhizoctonia solani* and *Sclerotium rolfsii* by fluorescent pseudomonads has been reported by Tripathi and Johri (2002). They are also reported to suppress damping-off disease in tomato caused by *Pythium* spp (Srivastav *et al.*2004). Prasanna *et al.* (2009) isolated *Pseudomonas fluorescens* and used for the suppression of rice fungal pathogens. Several other studies also indicated that the antagonistic potential of *P. fluorescens* against various soil-borne pathogens is correlated with the production of lytic enzymes. These studies indicate the role of chitinases in the antagonistic activity of fluorescent pseudomonads.

Fluorescent pseudomonads are well equipped as primary root colonizers with several mechanisms viz., production of antibiotics, HCN and competition for space and nutrients and inhibition of soil borne plant pathogens. Exposing plants to the volatile metabolites of antagonist causes a significant increase in peroxides activity, which may contribute to induction of disease resistance. In addition to HCN *Pseudomonas* spp. were reported to produce antibiotics like 2, 4 – diacetyl phloroglucinol and pyoluteorin that has profound action through antibiosis. They could serve as promising bioinoculants for agricultural system to increase productivity, since the action of such bacteria is highly specific, ecofriendly and cost-effective.

Intrinsic antibiotic resistance is natural phenomenon which is commonly found in all bacterial species. This property mainly resists antibiotics present in natural sources which are produced by closely related bacterial species. Intrinsic antibiotic resistance can also be used as a scale to measure the diversity of microorganisms. Patil *et al.* (1997) found that their biocontrolling strains of fluorescent pseudomonads exhibited resistance to rifampicin and hence they used these antibiotics as a marker to study its rhizosphere colonization potential. Vikram (2000) also used rifampicin as a marker to study the ecology of *Pseudomonas fluorescens* FP-15 which controlled *Sclerotium rolfsii* in groundnut. Rifampicin resistant strains of bacteria were used to assess the persistence of indigenous and non-indigenous microorganisms inoculated into natural systems (Downing and Thomson 2000).

Therefore, in the present study all the 50 isolates were subjected to qualitative and quantitative production of HCN by picric acid assay method, chitin and cellulose degradation plate assay method and IAR were tested against 11 different antibiotics by pour plate method.

MATERIALS AND METHODS

Mechanisms of pathogen inhibition

HCN production (Wei et al. 1991)

Whatman No. 1 filter paper pads were placed on the lid of petriplate and the plates were sterilized. TSA medium amended with glycine (4.4 g/l) was sterilized and poured into the sterile plates. 44 isolates showing biocontrol potential against the pathogens were streaked on the medium. The filter paper padding in each plate was soaked with 2 ml sterile picric acid solution. The plates were sealed with parafilm in order to contain gaseous metabolites produced by the antagonists and to allow for chemical reaction with picric acid present in the filter padding. After incubation for a week time at 30º C, the color change of the filter paper was noted and the HCN production potential of the antagonists was assessed as shown below:

No color change – No HCN production, Brownish coloration – Weak HCN production, Brownish to orange - Moderate HCN production, Complete orange - Strong HCN production.

Chitinase production

Preparation of colloidal chitin

Colloidal chitin was prepared from the chitin flakes (Sigma Chemicals Company, USA) by the method of Roberts (1967). The chitin flakes were ground to powder and added slowly to 10 N HCl and kept overnight at 4°C with vigorous stirring. The suspension was added to cold 50% ethanol with rapid stirring and kept overnight at 25°C. The precipitate was collected by centrifugation at 10000 rpm for 20 min.

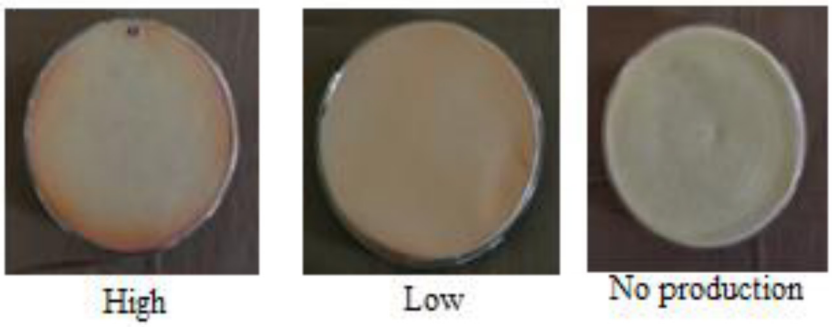


Plate 1. HCN production.

and washed with sterile distilled water. It was freeze dried to powder and stored at 4°C until further use.

Chitinase production by fluorescent pseudomonads

For the preparation of crude chitinase, the isolates were spotted on the chitin-peptone medium plates (pH 6.8) amended with 0.2% of colloidal chitin (Velazhahan *et al*. 1999) and incubated at 30°C for 96 h in the incubator. The isolates which have shown solubilized zone on the agar medium were considered as positive for the production of chitinase enzyme.

Cellulose degradation

Cellulase production was determined on basal medium supplemented with carboxymethyl cellulose (CMC) (10 g/l) (Cattelan *et al.* 1999). The plates with bacterial culture were incubated at 28±2 °C and observed for zone of clearance around bacterium on repeated subculture was considered as positive for cellulase production.

Intrinsic antibiotic resistance

The intrinsic antibiotic resistance of 50 fluorescent pseudomonads was examined by the procedure suggested by Verma *et al.* (2007). IAR of all the 50 isolates was tested against 11 antibiotics viz., Ampicillin (100 µg/ml), Penicillin (10 µg/ml), Rifampicin (25 µg/ml) and Erythromycin (10 µg/ml). All the 50 isolates (One ml of the overnight cultures) were inoculated in nutrient agar plates, placed the antibiotic concentrated papers on the inoculated media, incubated at 28 ± 2^{0}C for 24 h. The zones of solubilization and non solubilization refer to the susceptibility and resistance of antibiotics by organisms respectively.

RESULTS

Mechanisms of biological control

Production of HCN

All the 50 isolates were subjected to *in vitro* qualitative production of HCN by picric acid assay method (Plate1) and only 17 isolates (FP-4, 7, 13, 18, 19, 24, 25, 26, 28, 30, 31, 32, 33, 35, 47, 48 and 49) were found to produce HCN (result not shown). However, 10 of them (FP-4, 7, 13, 18, 19, 24, 25, 26, 30 and 47) were high HCN producers which turned the color of the filter paper into orange. Seven of the 50 isolates were weak producers of HCN.

The quantitative estimation of HCN production was determined for ten isolates which have shown very high HCN production in test tubes by picric acid assay where the absorbance was noted down for up to nine days of incubation. The isolates FP-4, 25 and 26 were significantly on par with each other by recording the least HCN production of 0.03 absorbance. The highest HCN production was recorded in FP-24, FP-13, FP-19, FP-18 and FP-47 by producing 0.14, 0.13, 0.09, 0.07 and 0.07 respectively (Table 1).

Degradation of cellulose and chitin

All the isolates were also examined for their cellulose

Table 1. HCN production by fluorescent pseudomonads.

Isolate No.	(Optical density at 625 nm) Incubation period (days) III	VI	XI	Mean
FP-3	0.013	0.027	0.053	0.03
FP-7	0.033	0.043	0.060	0.05
FP-13	0.110	0.123	0.143	0.13
FP-18	0.047	0.067	0.087	0.07
FP-19	0.083	0.097	0.100	0.09
FP-24	0.120	0.143	0.167	0.14
FP-25	0.010	0.027	0.057	0.03
FP-26	0.010	0.030	0.060	0.03
FP-30	0.033	0.057	0.077	0.06
FP-47	0.043	0.067	0.087	0.07
Mean	0.05	0.07	0.09	
	Days (D)	Isolates (I)	D × I	
SEm	9.6E-05	0.0003	0.00096	
CD at 1%	0.000361	0.0012	0.003613	

and chitin degradation in cellulose and chitin plate assay methods respectively. Out of the 50 isolates, 25 have shown chitin degradation and 24 of them have shown cellulose degradation. Fifteen isolates (FP-1, 2, 7, 12, 13, 17, 18, 19, 24, 25, 27, 28, 31, 35 and 49) have shown degradation of both cellulose and chitin on the respective agar media (Table 2).

Intrinsic Antibiotic Resistance

The intrinsic antibiotic resistance of all the 50 isolates was tested against eleven antibiotics and the results are presented in Table 3 (Plate 2). All the isolates were resistant to Ampicillin (100 µg/ml), Penicillin (10 µg/ml), Rifampicin (25 µg/ml) and Erythromycin (E 10 µg/ml). While 7 isolates (FP-9, 11, 16, 13, 41, 43, 48) were resistant to Kanamycin (50 µg/ml), 5 isolates (FP-9, 31, 35, 37, 41) to Streptomycin (100 µg/ml). 22 isolates (FP-13,14, 17, 18, 19, 20,21, 22, 23, 24, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 37, 45, 46, 49, 50) and 5 isolates (FP-1, 10, 34, 36, 42) were resistance to Chloramphenicol (5 µg/ml) and CB (Chloramboroxied 5 µg/ml) respectively. One isolate

Table 2. Solubilization of chitine and cellulose by fluorescent pseudomonads.

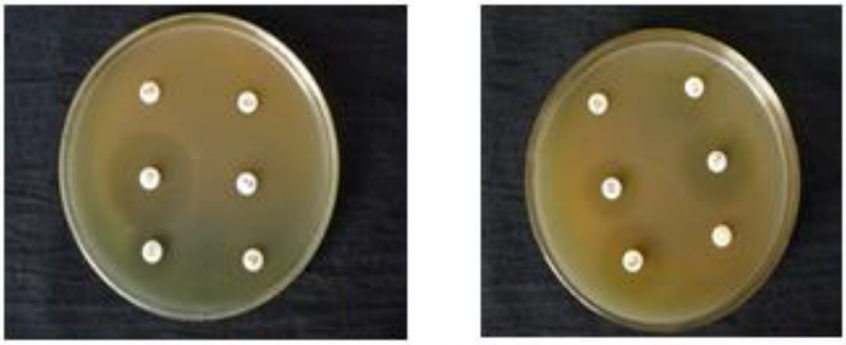
Isolates	Solubilization Chitine	Cellulose	Isolates	Solubilization Chitine	Cellulose
1.	+	+	26.	+	-
2.	+	+	27.	+	+
3.	-	-	28.	+	+
4.	-	-	29.	-	-
5.	-	-	30.	-	-
6.	-	+	31.	+	+
7.	+	+	32.	-	+
8.	+	-	33.	-	+
9.	-	-	34.	+	-
10.	-	-	35.	+	+
11.	-	+	36.	+	-
12.	+	+	37.	+	-
13.	+	+	38.	-	-
14.	+	-	39.	-	+
15.	-	+	40.	-	-
16.	-	+	41.	-	-
17.	+	+	42.	+	+
18.	+	+	43.	-	-
19.	+	+	44.	-	-
20.	-	-	45.	-	-
21.	-	-	46.	+	+
22.	+	-	47.	-	-
23.	-	-	48.	+	-
24.	+	+	49.	+	+
25.	+	+	50.	-	-

Note: - +: Presence, -: Negative.

Table 3. Intrinsic antibiotic resistance of fluorescent pseudomonads.

Isolates	Intrinsic antibiotic resistance										
	S	TE	C	CB	Amp	P	K	GEN	NA	E	RIF
FP-1.	-	-	-	+	+	+	-	-	-	+	+
FP-2.	-	-	-	-	+	+	-	-	-	+	+
FP-3.	-	-	-	-	+	+	-	-	-	+	+
FP-4.	-	-	-	-	+	+	-	+	-	+	+
FP-5.	-	-	-	-	+	+	-	-	-	+	+
FP-6.	-	-	-	-	+	+	-	-	-	+	+
FP-7.	-	-	-	-	+	+	-	-	-	+	+
FP-8.	-	-	-	-	+	+	-	-	-	+	+
FP-9.	+	-	-	-	+	+	+	-	-	+	+
FP-10.	-	-	-	+	+	+	-	-	-	+	+
FP-11.	-	-	-	-	+	+	+	-	-	+	+
FP-12.	-	-	-	-	+	+	-	-	-	+	+
FP-13.	-	-	+	-	+	+	-	-	-	+	+
FP-14.	-	-	+	-	+	+	-	-	-	+	+
FP-15.	-	-	-	-	+	+	-	-	-	+	+
FP-16.	-	-	-	-	+	+	+	-	-	+	+
FP-17.	-	-	+	-	+	+	-	-	-	+	+
FP-18.	-	-	+	-	+	+	-	-	-	+	+
FP-19.	-	-	+	-	+	+	-	-	-	+	+
FP-20.	-	-	+	-	+	+	-	-	-	+	+
FP-21.	-	-	+	-	+	+	-	-	-	+	+
FP-22.	-	-	+	-	+	+	-	-	-	+	+
FP-23.	-	-	+	-	+	+	-	-	-	+	+
FP-24.	-	-	+	-	+	+	-	-	-	+	+
FP-25.	-	-	+	-	+	+	-	-	-	+	+
FP-26.	-	-	+	-	+	+	-	-	-	+	+
FP-27.	-	-	+	-	+	+	-	-	-	+	+
FP-28.	-	-	+	-	+	+	-	-	-	+	+
FP-29.	-	-	+	-	+	+	-	-	-	+	+
FP-30.	-	-	+	-	+	+	-	-	-	+	+
FP-31.	+	-	+	-	-	+	+	-	-	+	+
FP-32.	-	-	+	-	+	+	-	-	-	+	+
FP-33.	-	-	+	-	+	+	-	-	-	+	+
FP-34.	-	-	-	+	+	+	-	-	-	+	+
FP-35.	+	-	-	-	+	+	-	-	-	+	+
FP-36.	-	-	-	+	+	+	-	-	-	+	+
FP-37.	+	-	+	-	+	+	-	-	-	+	+
FP-38.	-	-	-	-	+	+	-	-	-	+	+
FP-39.	-	-	-	-	+	+	-	-	-	+	+
FP-40.	-	-	-	-	+	+	-	-	-	+	+
FP-41.	+	-	-	-	+	+	+	-	-	+	+
FP-42.	-	-	-	+	+	+	-	-	-	+	+
FP-43.	-	-	-	-	+	+	+	-	-	+	+
FP-44.	-	-	-	-	+	+	-	-	-	+	+
FP-45.	-	-	+	-	+	+	-	-	-	+	+
FP-46.	-	-	+	-	+	+	-	-	-	+	+
FP-47.	-	-	-	-	+	+	-	-	-	+	+
FP-48.	-	-	-	-	+	+	+	-	-	+	+
FP-49.	-	-	+	-	+	+	-	-	-	+	+
FP-50.	-	-	+	-	+	+	-	-	-	+	+

Note: - +: Resistances to antibiotics (growth present), -: susceptible to antibiotics (no growth)
Amp (Ampicilin 100 µg/ml), CB (Chloramboroxied 5 µg/ml), Kan (Kanamycin 50 µg/ml), NA (Nalidixic acid 10 µg/ml), Rif (Rifampicin 25 µg/ml), T (Tetracycline 20 µg/ml), S (Streptomycin 100 µg/ml) , E (10 µg/ml Erythromysin), GEN (Gentanisin10 µg/ml), Penicillin (10 µg/ml) , Chloramphenicol (5 µg/ml).



Amp (Ampicilin 100 µg/ml), CB (Chloramboroxied 5 µg/ml), Kan (Kanamycin 50 µg/ml), NA (Nalidixic acid 10 µg/ml), Rif (Rifampicin 25 µg/ml), T (Tetracycline 20 µg/ml), S (Streptomycin 100 µg/ml), E(10 µg/ml Erythromycin), GEN(Gentamicin10 µg/ml), Penicillin (10 µg/ml), Chloramphenicol (5 µg/ml)

Plate 2. Intrinsic antibiotic resistance of fluorescent pseudomonads.

FP-4 was resistance to GEN (Gentanisin10 µg/ml), All the isolates were susceptible to Tetracyclin (20 µg/ml) and NA (Nalidixic acid 10 µg/ml).

DISCUSSION

One of the most important characteristic features of fluorescent pseudomonads is that they act as biocontrol agents against soil borne plant pathogens by producing antimicrobial substances like antibiotics and lytic enzymes having inverse property on growth of phytopathogens. The antibiotic production is now recognized as an important mechanism by which biocontrol agents, especially PGPRs, suppress plant pathogens (Bonsall *et al.* 1997).

Mechanisms of biological control

Production of HCN

The antimicrobial activity of HCN is very well established in controlling of various fungal and bacterial diseases. It is the most important predictor in the evaluation of antagonistic activity of rhizobacterial isolates against soil-borne plant pathogens (Adhikari *et al.* 2013). Haas *et al*. (1999) analyzed HCN production by strains of *P. fluorescens* that helped in the suppression of *Thielaviopsis basicola* causing black root rot of tobacco. The suppression of *Pythium, Rhizoctonia, Erwinia, Sclerotinia* and Fusarium by pseudomonads of fluorescent group has been in practice for several years. In the present study, out of 50 cultures, 17 isolates (FP-4, 7, 13, 18, 19, 24, 25, 26, 28, 30, 31, 32, 33, 35, 47, 48 and 49) were found to produce HCN and 10 of them (FP-4, 7, 13, 18, 19, 24, 25, 26, 30 and 47) were found to show high HCN production. The highest HCN was recorded by FP-24, FP-13, FP-19, FP-18 and FP-47 by producing 0.14, 0.13, 0.09, 0.07 and 0.07 respectively. The genus *Pseudomonas* is one of the important genera of bacteria that inhibits the growth of various pathogenic fungi. Lanteigne *et al.* (2012) isolated HCN producing *Pseudomonas* and studied its biological control activity. The cyanide hydrogen is up setter of perspiration and chelating agent of metals and has been reported as effective in control of cucumber wilt caused by *Pythium ultimum*. Similar observations were also made by Thomshow and Weller (1995) who observed that the pseudomonads exert beneficial effects on plants by the production of diverse microbial metabolites like HCN. Deshwal *et al.* (2011a and 2011 b) also reported that the *Pseudomonas* strains isolated from Mucuna produced HCN.

Degradation of cellulose and chitin

Out of 50 cultures, 25 have shown to be chitin degraders and 24 of them have shown to be cellulose degraders. Fifteen isolates (FP-1, 2, 7, 12, 13, 17, 18, 19, 24, 25, 27, 28, 31, 35 and 49) have shown to de-

grade both cellulose and chitin on the respective agar media. Moataza and Saad (2006) also reported varied levels and types of mycolytic enzymes by different *Pseudomonas* strains with different pathogens like *P. capsici* and *Rhizoctonia solani*. Diby *et al.* (2005) reported that *P. fluorescens* and *Trichoderma* spp. produced significant quantities of β-1,4-glucanases, β-1,4-glucanases and lipase in MM medium amended with different preparations of *P. capsici* mycelium. Due to cyclic lipopeptides production (Thrane *et al.* 2000, Koch *et al*. 2002, Andersen *et al.* 2003). *Pseudomonas* bacteria are often used as biological agents (Nielsen *et al.* 1999). The investigation results of numerous authors prove that purified lipopeptids show antagonistic activity against fungi responsible for sugar beet root decay such as *Rhizoctonia solani* (Nielsen *et al.* 2000, Nielsen *et al*. 2002, Andersen *et al.* 2003), *Aphanomyces cochlioides* (Nielsen *et al.*1999), *Pythium ultimum* and *Pythium debarianum* (Nielsen *et al.* 2000, Thrane *et al*. 2000, Andersen *et al.* 2003).

Incentic antibiotic resistances

Intrinsic antibiotic resistance of all the 50 isolates was tested against 11 antibiotics, where all the isolates were resistant to Ampicillin (100 µg/ml), Penicillin (10 µg/ml), Rifampicin (25 µg/ml) and Erythromycin (10 µg/ml), while 7 isolates were resistant to Kanamycin (50 µg/ml), all the isolates were susceptible to Tetracyclin (20 µg/ml) and 5 were resistant to Streptomycin (100 µg/ml). Intrinsic antibiotic resistance can also be used as a scale to measure the diversity of microorganisms. Somasegaran and Hoban (1994) have demonstrated genetic diversity of soil bacteria using the pattern of antibiotic resistance. Suneesh (2004) also reported that most of the fluorescent pseudomonads of moist decidous forests of Western Ghats were resistant to spectinomycin, ampicillin, nalidixic acid, chloramphenicol and kanamycin. However, Deepa (2000) has reported that her isolates of fluorescent pseudomonads were sensitive to streptomycin, spectinomycin, kanamycin and tetracycline. To mark *Pseudomonas* sp. B-25 strain for its root colonization studies, its intrinsic antibiotic resistance analysis was made.

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