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Research Article

Study of Antibiotics Production as a Mechanism of Antifungal Activity of Fluorescent *Pseudomonas* and *Bacillus spp* as Biocontrol Agents *a*gainst *Fusarium* and *Pythium species*

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Abstract

Nine isolates of antifungal fluorescent *Pseudomonas* and six isolates of *Bacillus* showing potent antifungal activity (percent growth inhibition > 50%) against the phytopathogenic *Fusarium* and *Pythium* species were selected by dual culture (Co-culture) method, using PDA and PDB. The isolates were identified on the basis of morphological, cultural and biochemical characters as well as 16S r-RNA gene sequencing and used to prepare biocontrol formulations with dried fecal pellets of sheep and goats. The isolates were identified as *Pseudomonas aeruginosa* 13, *P. aeruginosa* 58, *P. putida* 71, *P. fluorescens* 106, *P. putida* 111, *P. aeruginosa* 117, *P. aeruginosa* 154, *P. aeruginosa* 166, *P. fluorescens* 171, *Bacillus thuringiensis* 184, *B. subtilis* 208, *B. cereus* 220, *B. cereus* 228, *B. subtilis* 252 and *B. subtilis* 260. The *Pseudomonas* isolates were grown in King's B medium and *Bacillus* isolates in Nutrient sucrose broth (NSB) supplemented with iron, the media which favours antibiotic production. The culture supernatant was separated by ultracentrifugation, sterilized and tested for antifungal activity on PDA. Presence of antibiotic in culture supernatant was tested by TLC. Cell free culture supernatant of the three *Pseudomonas* and three *Bacillus* isolates showed good antifungal activity against *Fusarium* and *Pythium* species (inhibition zone >30mm). Appearance of two distinct spots in TLC indicated the production of at least two metabolic products, except in case of *P. putida* 111 and *B. subtilis*208. Comparison of Rf values with standard values indicated the production of 2,4 diacetylphloroglucinol (DAPG) or a chemically similar compound as one of the antifungal substance by the isolates. The culture supernatant of the six *Pseudomonas* species showed fluorescence under ultraviolet light.

INTRODUCTION

Production of antibiotics, selective iron utilization by siderophores production, production of hydrogen cyanide (HCN), parasitism and lysis are the major mechanisms of antifungal activity of rhizobacteria in soil. Antibiosis is an advantageous mechanism particularly for biological control of diseases because compounds mediating antibiosis can diffuse rapidly in soil and direct contact between the antagonist and pathogen is not necessary (Pfender *et al*., 1993; Bloemberg and Lugtenberg, 2001). *Pseudomonas* and *Bacillus* species are characterized to produce variety of antibiotics those play important role in biological control (Pal and Jalali, 1998).

Antibiosis is the most widely investigated mechanism of biocontrol (Pal and Jalali, 1998; Compant *et al*., 2005; Sandikar B M, 2013).

Some target organisms are comparatively little affected by an antibiotic, for example *Fusarium species.Pythium* species are more sensitive to antibiotics produced by fungi than those produced by bacteria (Sandikar and Awasthi, 2009, Campbell R., 1989). The antibiotic produced by the biocontrol agent may be specific for a particular target organism or more general with a wide spectrum of activity. In general, the fluorescent pseudomonads are considered to be the most promising among bacterial biocontrol agents as they produce wide spectrum antibiotics (Campbell R, 1989; Pal and Jalali, 1998). Some of the commonly produced antibiotics by fluorescent pseudomonads those involved in the biocontrol of various plant pathogens are pyrrolnitrin, phenazines, pyroluteorin, 2,4 diacetyl phloroglucinol (DAPG), etc. (Pfender *et al*., 1993; Bloemberg and Lugtenberg, 2001). Species of the genus *Bacillus* are also characterized to produce antifungal antibiotics. *Bacillus cereus*UW85 produce kanosamine, which inhibited the growth of phytopathogens (Milner *et al*., 1996). Aris *et. al*., 2010, isolated and identified 22 *Bacillus species* producing antifungal antibiotics from rhizosphere of soybean plants, based on amplified r-DNA restriction analysis (ARDRA) and 16S r-RNA sequencing. Shifa *et. al*., 2015, purified and characterized antifungal antibiotics against *Sclerotium rolfsii* produced by *Bacillus subtilis* strain G-1 by TLC, gas chromatography and mass spectrometry and revealed the presence of 22 different kinds of antibiotics which contain aldehydes, fatty acids, alkanes, esters and sulfur containing compounds.

MATERIALS AND METHODS

Isolation and Identification of phytopathogenic fungi-

The infected plant material was collected from field and the phytopathogenic fungi were isolated on Potato dextrose agar (PDA) by tissue segment method. The cultures were identified on the basis of the shape, size, septation, colour and arrangement of mycelium and spores (Mukadam *et. al.* 2006).Among the different isolates, *Fusarium* and *Pythium* cultures were selected for study.

Isolation and Identification of rhizobacteria-

Pseudomonas and *Bacillus* cultures were isolated from rhizosphere of healthy crop plants using King's B medium and Nutrient agar, respectively and preserved in refrigerator.

Screening of antifungal *Pseudomonas* and *Bacillus* isolates-

Antifungal bacterial isolates were screened by Dual culture (Co-culture) method on PDA and in PDB. 100µl bacterial cultures were filled in wells at the center of PDA plates and 10mm fungal agar discs were placed at two sides of the well 20mm apart. PDA plates were incubated at 28^{o}C for 72hrs and zone of fungal growth inhibition was observed and measured. 100 µl of bacterial and fungal culture each was also inoculated in PDB and incubated at 28^{o}C for 7days, along with control PDB flask inoculated with only fungal culture. Percent growth inhibition of fungal growth was calculated as-

P. I. = [(C-T)/C x 100], Where 'C'- wet weight of fungal growth in control and 'T'- wet weight of fungal growth in test (Laha *et. al*, 1992, Sandikar and Awasthi, 2009).

Growing the culture and Separation of antibiotic product from culture supernatant-

King's B medium (Proteose peptone 20g, Na_2HPO_4 1.5g, $MgSO_4.7H_2O$ 1.5g, Agar-agar20g, distilled water 1000ml, pH7.2) and Nutrient Sucrose broth (Sucrose 5g, Yeast extract 4g, Peptone 4g, Beef extract 2g, $FeCl_3$ 0.18g, pH 7.2) were prepared (Mondal *et al*., 2000), distributed as 100ml in 250ml Erlenmeyer flasks and sterilized at 121^{0}C for 15min. 100µl of active NB cultures of the antifungal *Pseudomonas* and *Bacillus* isolates were aseptically inoculated into KB and NSB flasks respectively and incubated at 30^{o}C, on rotary shaker at 120rpm for 72hrs. The KB and NSB cultures were centrifuged at 10,000rpm for 10min. Cell free culture supernatants were separated and sterilized in autoclave at 121^{0}C, for 15min. (Mondal *et al*., 2000; Tripathi and Johri, 2002).

Antifungal activity of the cell free supernatant-

The antifungal compounds are produced extracellular by the antagonists and remain in culture supernatant of the medium. The antifungal activity of cell free culture supernatants was tested on PDA plates, by agar well method (Tripathi and Johri, 2002, Sandikar and Awasthi, 2009). The extent of antifungal activity of the culture supernatants is represented in table-1.

Chemical analysis of the culture supernatant-

Thin layer chromatography (TLC) of sterilized cell free culture supernatant was performed on silica gel with solvent system- 'acetonitrile: methanol: water' (1:1:1). The cell free culture supernatants (30µl) were spotted on TLC plates separately and solvent was allowed to run.

Plates were dried and observed under UV to identify spots (Saikia *et al*., 2004; Matthijs *et al*., 2007; Reddy *et al*., 2007). R_f values were calculated by dividing the distance between center of spot and the point of its application, by solvent front 160mm as represented in Table-2.

RESULTS AND DISCUSSION

Antifungal activity of the culture supernatants of the isolates on PDA-

Nine *Pseudomonas* and six *Bacillus* isolates were obtained by primary screening (fig.-1). The fluorescent *Pseudomonas* isolates showed pigmented growth in iron supplemented KB media indicated primarily antibiotic production (fig.2). The inhibition zones produced by KB and NSB culture supernatants of bacterial isolates (Fig.3) were ranged 13-32mm and 16-34mm for *Pythium* and *Fusarium species* respectively (table-1). The culture supernatant of the isolate *P. aeruginosa*13 showed maximum antifungal activity for phytopathogens i.e. 32 and 34mm. This indicated *P. aeruginosa*13 as the most potent antifungal species against the phytopathogens. Six isolates i.e. *Pseudomonas aeruginosa*13, *P. aeruginosa*58, *P. putida*71, *Bacillus cereus*220, *B. cereus*228 and *B. subtilis*252 showed high antifungal activity (IZ >30 mm). These isolates were relatively more hopeful to develop as effective biocontrol formulations. The extent of antifungal activity found to vary with the species of pathogen as well as the antagonist.

Antibiotics produced by *Pseudomonas* and *Bacillus* species play important role in antifungal activity and biological control (McLoughlin *et al*., 1992; Buysens *et al*., 1996; Milner *et al*., 1996; Nielsen *et al*., 1988). Growth inhibition of the phytopathogen *Fusarium oxysporum* by *Bacillus subtilis* with production of the antibiotic 'bulbiformin' was reported by Vasudeva, (1952). Aska and Shoda (1996) observed *Bacillus subtilis* RB14 as effective antagonist against *Rhizoctonia solani* with production of antibiotics 'iturinA' and 'surfactin'. The phenazine was dominant factor in disease suppression by *Pseudomonasfluorescens* 2-79 and M4-80R (Hamdam *et al*., 1991). Kumari and Srivastava (1999) concluded that, production of antimicrobial compound 2,4-diactyl phloroglucinol (DAPG) and phenazine-1-caboxylic acid (PCA) by *Pseudomonas fluorescens* were responsible for control of black root rot of tobacco. Mazzola *et al*., (1995) observed same fact in case of biocontrol of take-all of wheat.

Nielsen *et al*., (1998) observed production of a lipopeptide antibiotic- viscosinamide and 2,4 diacetylphloroglucinol as the apparent mechanisms of biocontrol of pre-emergence damping-off caused by *Pythium ultimum* and *Rhizoctonia solani*, by *Pseudomonas fluorescens* isolated from sugar beet rhizosphere. Samanta and Dutta (2004) observed that, the crude extract of *Pseudomonas spp.* Mpf-1 inhibited the growth of *Sclerotinia sclerotiorum* by 84% and reduced the incidence of Sclerotinia stem rot of mustard. *Bacillus subtilis* inhibited the growth of many fungal phytopathogens by antibiotic production (Podile *et al*., 1988).

The chemical analysis of antifungal compounds in the culture supernatant of antagonists by thin layer chromatography (TLC) provided an idea about the number of antifungal compounds produced by the isolates and their chemical nature. The culture supernatants of most of antifungal isolates produced two distinct spots, except *Pseudomonas putida*111and *Bacillus subtilis*208 (table-2). This indicated that, at least two metabolites were responsible for the fungal growth inhibition. The R_f values were ranged 0.343 to 0.362 which coincides with standard R_f values of 2, 4 diacetyl phloroglucinol (DAPG) with the same solvent system. This indicated that these isolates were producing DAPG as one of the antifungal compound or a chemically similar compound. Reddy *et al*.,(2007) tentatively identified the antifungal metabolites of *Pseudomonas* based on Rf values and latter confirmed by nuclear magnetic resonance (NMR) spectra and mass spectrometry. Rhitu Raj *et. al*., 2017 observed *in-vitro* growth inhibition of phytopathogenic fungi and considerable decrease in bacterial wilt of tomato by *Pseudomonas protegens* RS-9 with potential to produce 2,4 diacetylphloroglucinol, pyrrolnitrin, pyoluteorin and hydrogen cyanide. Kumar Naik *et. al*.,2017, observed considerable reduction in root rot incidence and increase in yield of groundnut with inoculation of *Pseudomonas species* characterized by 2,4 diacetylphloroglucinol production. Mandry-Litvinko *et. al*., 2017, analyzed nucleotide sequence of the genes controlling synthesis of 2,4 diacetylphloroglucinol by *Pseudomonas brassicacearum* BIM B-446 bacteria.

We conclude that, antibiotic production is one of the most important mechanisms of antifungal activity and biological control of fungal crop pathogens by *Pseudomonas* and *Bacillus* isolates.

Table-1. Antifungal activity of bacterial culture supernatants

Sr. No.	Bacterial isolates	I. Z. against *Pythium* sp. (mm)	I. Z. against *Fusarium* sp. (mm)
1.	*P. aeruginosa*13	**32**	**34**
2.	*P. aeruginosa*58	**30**	**30**
3.	*P. putida*71	28	**32**
4.	*P. fluorescens*106	28	26
5.	*P. putida*111	26	25
6.	*P. aeruginosa*117	18	16
7.	*P. aeruginosa*154	23	25
8.	*P. aeruginosa*166	17	18
9.	*P. fluorescens*171	13	16
10.	*B. thuringiensis*184	29	30
11.	*B. subtilis*208	18	17
12.	*B. cereus*220	**30**	**32**
13.	*B. cereus*228	28	**30**
14.	*B. subtilis*252	**30**	27
15.	*B. thuringiensis*260	22	23

Values in columns are average of triplicates.

Table-2. TLC of bacterial culture supernatants

Sr. No.	Bacterial isolates	R_f values	
		Spot-1	Spot-2
1.	*P. aeruginosa*13	0.356	0.218
2.	*P. aeruginosa*58	0.343	0.237
3.	*P. putida*71	0.350	0.200
4.	*P. fluorescens*106	0.362	0.250
5.	*P. putida*111	0.200	-
6.	*P. aeruginosa*117	0.343	0.212
7.	*P. aeruginosa*154	0.350	0.212
8.	*P. aeruginosa*166	0.362	0.237
9.	*P. fluorescens*171	0.500	0.231
10.	*B. thuringiensis*184	0.525	0.225
11.	*B. subtilis*208	0.250	-
12.	*B. cereus*220	0.481	0.212
13.	*B. cereus*228	0.493	0.200
14.	*B. subtilis*252	0.350	0.218
15.	*B. thuringiensis*260	0.512	0.206

The values are average of triplicates.

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Fig.-1. Screening of antifungal *Pseudomonas* and *Bacillus* isolates

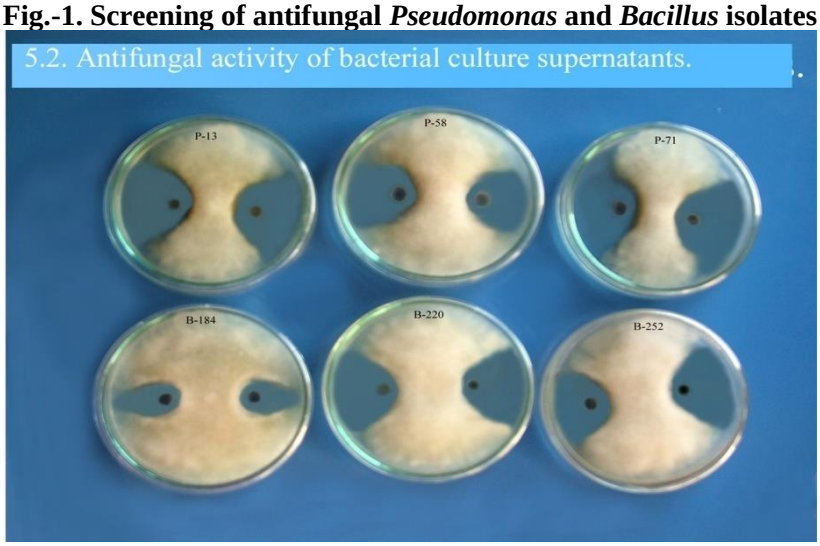

Fig.2 Growth of *Pseudomonas* isolates in King's B medium

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