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RESEARCH ARTICLE :

Biochemical characterization of *Pseudomonas fluorescens* against *Alterneria alternata* in ashwagandha

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SUMMARY : A field survey was conducted from different ashwagandha growing areas of Tamil Nadu *viz.*, Ottanchatram, Coimbatore, Periyakulam, Theni, Nilakottai, Chempatti, Palur, Vadipatti, Virudhunagar and Cumbum with a view to assess disease intensity. Ten isolates of *Pseudomonas fluorescens* were isolated from the rhizosphere soil. The effective isolates were also used as biocontrol agents. Among ten isolates were tested, three isolates of *Pseudomonas fluorescens viz.*, Pf5, Pf1 and Pf4 were effectively identified by different biochemical methods *viz.*, KOH test, anerobic growth, Arginine dehydrogenase starch hydrolysis, siderophore and HCN production. Among the four isolates tested for HCN production, the isolate Pf_5 recorded as strong producer of HCN with OD value of 0.090 followed by isolate Pf_1 (OD value of 0.063). Based on these biochemical methods and characterization of the strains were identified as a gram negative *Pseudomonas* species. Among the ten isolates of *Pseudomonas fluorescens* were tested against *A. alternata*, Pf_5 recorded the highest inhibition of mycelial growth of *A. alternata* over control by recording 61.71 % inhibition. The lowest % inhibition (22.86%) of mycelial growth of *A. alternata* was recorded by $Pf_{8.}$

KEY WORDS :
Pseudomomas, Alternaria alternata, Ashwagandha, Biochemical, *In vitro*

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BACKGROUND AND OBJECTIVES

Ashwagandha (*Withania somnifera* L. Dunal) is an important medicinal plant that is used in the Indian traditional system of medicine ayurveda and unani. The plant roots, leaves, fruits and seeds contain a number of alkaloids that is withanin and somniferine. In India, the crop has an area of about 4000 hectares mainly in drier parts of Madhya Pradesh and Rajasthan (Nigam, 1984). The crop is affected by various diseases like leaf spot, seedling blight and wilt. Among these diseases, leaf spot caused by *Alternaria alternata* (Fr.) Keissler agg. is the most severe in Indian plains. The initial symptoms are leaves having brown to black spot of two to nine mm in diameter surrounded by a yellow halo appear on both dorsal and ventral surfaces of the infected leaves (Inoue and Nasu, 2000). Disease management through

eco-friendly approach has now become a distinct possibility for the future. In the present study, attempts were carried out to identify effective eco- friendly management practices for the management of leaf spot disease of ashwagandha. Baker and Cook (1974) reported that the use of biological agents for the control of plant diseases is an alternative method to chemical control, that provide disease control, while being relatively harmless to humans and non polluting to environment.

The present study has therefore been under taken with the following objectives:

– Collection of different Psuedomonas isolates from major ashwagandha growing areas of Tamil Nadu.

– *In vitro* screening of different isolates of antagonists, organic amendments and oils against *A. alternata*.

RESOURCES AND METHODS

Biochemical tests for *Pseudomonas* sp. :

Gram staining:

The uniform suspension of the isolates prepared in sterilized distilled water was smeared on the cleaned glass slide and allowed for air drying. The smear was gently exposed to flame for two min and covered with crystal violet solution for 30 sec. Then the slide was gently washed with distilled water for a few seconds and covered with Lugol's iodine solution for 30 seconds. The iodine solution was washed by using 95% ethyl alcohol until no more color flows from the smear. The slide was again washed with distilled water, drained and safranin (counter-stain) was applied on the slide for 30 sec. The slide was then washed with distilled water, blotted using a country filter paper and air dried. The slide was then examined under microscope under oil immersion (Aneja, 1993).

KOH test:

A loopful of bacterial culture was placed on a glass slide. One drop of 3 % KOH solution was added over it and thoroughly mixed with the help of inoculation needle. Bacterial chromosomes separate out as thin threads, indicating gram negative bacteria.

Pigment production:

The bacterial cultures were streaked on King's A medium, incubated at room temperature (28 $\pm$ 2°C) for 48 hrs and pigmentation of the colony was observed. Bacterial cultures were streaked on King's B medium containing 1 % tyrosine and incubated at room temperature for 48 hrs and the zone of fluorescent pigmentation around the colonies was observed.

Anaerobic growth :

The bacterial cultures of the isolates were inoculated into the tubes containing sterilized glucose broth and incubated in an anaerobic jar. Alternatively, the broth was overlaid with sterile mineral oil and incubated at 24°C for observing the bacterial growth (Huge and Leifson, 1953).

Levan formation from sucrose:

The bacterial cultures were streaked on the sterilized peptone beef extract containing 5% sucrose and growth characters were observed after 48 hrs. Presence of large, white, domed and mucoid colonies characterized the production of levan from sucrose.

Starch hydrolysis:

The isolates were streaked onto peptone sucrose agar medium incorporated with 0.2% starch in Petri dishes and incubated for five days. Lugol's Iodine solution was used for testing the hydrolysis of starch. Production of clear zone around the growth indicated positive result.

Growth at 4°C and 45°C:

The bacterial cultures were streaked on King's B medium and incubated at 4°C and 45°C. The bacterial growth was observed after 24 and 48 hrs of incubation.

Mode of action of *Pseudomonas* :

Testing of antagonistic bacteria for the production of hydrogen cyanide (HCN) :

Qualitative assay:

Hydrogen Cyanide (HCN) production was determined by using the modified protocol (Miller and Higgins, 1970). Bacteria were grown on Tryptic soya agar (TSA) (animal peptone – 15.0 g; soyapeptone-5.0 g; sodium chloride-5.0 g; glycine-4.4 g and distilled water-1000 ml). Filter paper discs soaked in picric acid solution (2.5 g of picric acid, 12.5 g of sodium carbonate, and 1000 ml of distilled water) were placed in the upper lid of each Petri plate. Dishes were sealed with parafilm and incubated at 28°C for 48hrs. A change from yellow to light brown, brown or reddish brown of the discs were

recorded as an indication of weak, moderate or strong production of HCN for each strains, respectively.

Quantitative assay of HCN:

Bacteria were grown on tryptic soy broth (TSB). Filter paper was cut into uniform strips of 10 cm long and 0.5 cm wide. The strips were saturated with alkaline picrate solution and placed inside the conical flasks in a hanging position. After incubation at 28°C for 48 hrs, the sodium picrate present in the filter paper was reduced to reddish compound in proportion to the amount of hydrocyanic acid evolved. The colour was eluted from the filter paper by placing it on a clean tube containing ten ml of distilled water and the absorbance was measured at 625 nm (Sadasivam and Manickam, 1992).

Phosphate solubilization :

Testing the antagonistic bacteria for phosphate solubilization :

The bacteria were spot inoculated in Sperber's hydroxyl apatite medium (Soil extract – 100 ml, glucose -10 g, agar- 1.0 g, distilled water – 900 ml). To prepare soil extract, one kg of garden land soil was dissolved in 1.0 lit of water. It was autoclaved for 30 min at 15 lb pressure. Then it was double filtered after adding a pinch of calcium carbonate. Before pouring the medium into the plates, 5.0 ml of 10% KH_2PO_4 and 10 ml of 0.06 mM $CaCl_2$ sterilized separately were added to 100 ml of the medium. The bacteria were spot inoculated in the medium and incubated at room temperature (+28^{0}C) for 48 hrs. Appearance of clearing zone indicated phosphate solubilisation.

Siderophore production :

Testing bacterial antagonist for production of siderophore:

Production of siderophore by bacterial antagonist was assayed by plate assay. The tertiary complex Chrome azural S (CAS) or Fe^{3+} or hexadecyl trimethyl ammonium bromide served as an indicator. Forty eight hour old culture of the bacterial isolates were streaked onto the succinate medium (Succinic acid-4.0 g, K_2HPO_4-3.0g, $(NH_4)_2 SO_4$. $7H_20$- 0.2 g, Distilled water- 1 liter, pH- 7.0) amended with indicator dye. One liter of blue agar, 60.5 mg of chrome azurol S (CAS) was dissolved in 50 ml of distilled water and mixed with 10 ml of Iron (III) solution (1mM $FeCl_3$. $6H_2O$ in 10mM HCl). While constantly stirring, this solution was slowly added to 72.9 mg of hexa decyl trimethyl ammonium bromide (HDTMA) dissolved in 40 ml of water. The resultant dark blue liquid was observed for the formation of bright zone with yellowish fluorescent colour in the dark colored medium, which indicated the production of siderophore. The result was scored either positive or negative to this test (Schwyn and Neilands, 1987). The surface area of production was recorded by tracing the area of colour change in a tracing paper and there by plotting it on a graph sheet.

Detection of the nature of siderophore:

The bacterial isolates such as *Pseudomonas* sp and *B. subtilis* were inoculated in ten ml of King's B broth and nutrient broth, respectively. It was incubated in a rotary shaker at 120 rpm for 48 hrs. The bacteria multiplied in the broths, were used as the sample for the determination of the nature of siderophore.

Hydroxymate nature:

It was also examined by tetrazolium salt test. Instant appearance of a deep red colour by addition of siderophore sample to tetrazolium salt under alkaline conditions indicated the presence of hydroxymate type of siderophore production (Snow, 1984).

Carboxylate nature:

It was detected by Vogeli's chemical test where the disappearance of pink colour on addition of Phenolphthalein to siderophore sample under alkaline condition indicated carboxylate nature of siderophore (Vogeli *et al.*, 1998).

Efficacy of bacterial antagonists against *A. alternata* in vitro :

The bacterial isolates were tested for their inhibitory effect on growth of *A. alternata* by following the dual culture technique (Dennis and Webster, 1971). The bacterial isolates were streaked on one side of the Petri dish (1 cm away from the edge of the plate) on PDA medium and a mycelial disc (9 mm diameter) of nine day old *A. alternata* culture was placed on the opposite side of the Petri dish perpendicular to the bacterial streak (Vidhyasekaran *et al.*, 1997). The plates were incubated at room temperature (28 $\pm$ 2^{o}C) for seven days. After seven days of incubation, the pathogen growth and

inhibition zone were measured and expressed in mm.

OBSERVATIONS AND ANALYSIS

The results obtained from the present study as well as discussions have been summarized under following heads:

Biochemical characteristics of *Pseudomonas fluorescens* isolates :

All the ten isolates of *Pseudomonas fluorescens* gave positive result to KOH test, arginine dihydrolase test, and anaerobic growth, growth at 4^0C and in producing fluorescent pigment. These isolates gave negative result to Gram's reaction, levan formation from sucrose starch hydrolysis and growth at 45^0C in all the isolates (Table 1).

Hydrogen cyanide production by effective bacterial antagonists :

Among the four isolates tested for HCN production, the isolate Pf_5 recorded as strong producer of HCN (Table 2) with OD value of 0.090 followed by isolate Pf_1 (OD value of 0.063). The minimum HCN production was recorded by (Table 2) the isolate Pf_4 (OD value 0.047) Mondal *et al*. (1998) were documented that *P. flourescens* (Rb-26) produced the maximum HCN production, which in turn, showed stronger growth inhibition of *Xanthomonas axonopodis* pv. *malvacearum*. Role of HCN in disease suppression was demonstrated by several workers in various crops (Defago *et al*., 1990) using different antagonistic bacteria. Rajkumar (2006) detected the production of cyanic acid by *P. fluorescens* in banana, inhibitory to the growth of *Erwinia carotovora* var. *carotovora*.

Phosphate solubilisation by effective bacterial antagonists :

Among the ten isolates of antagonistic bacteria were tested, only three isolates of *Pseudomonas fluorescens*. *viz*., Pf_5, Pf_1 and Pf_4 were able to produce a clear zone of 2.1, 1.4and 1.2 cm diameter, respectively in the Sperber's hydroxyl apatite medium 72 hrs after incubation indicating the phosphate solubilisation, while Bs_5 isolate failed to solubilize the phosphate in the Sperber's hydroxyl apatite medium 72 hrs after incubation (Table 2). Kavitha (2004) reported that *P. fluorescens* isolate KPf_2 only

Table 1 : Biochemical characteristics of different isolates of *Pseudomonas fluorescens*

Test	Pf_1	Pf_2	Pf_3	Pf_4	Pf_5	Pf_6	Pf_7	Pf_8	Pf_9	Pf_{10}
Gram's reaction	-	-	-	-	-	-	-	-	-	-
Pigment production in King's A medium	+	+	+	+	+	+	+	+	+	+
Pigment production in King's B broth	+	+	+	+	+	+	+	+	+	+
Oxidase test	+	+	+	+	+	+	+	+	+	+
Levan formation from sucrose	-	-	-	-	-	-	-	-	-	-
Starch hydrolysis	-	-	-	-	-	-	-	-	-	-
Growth at 4^oC	+	+	+	+	+	+	+	+	+	+
Growth at 45^oC	-	-	-	-	-	-	-	-	-	-
KOH test	+	+	+	+	+	+	+	+	+	+
Anaerobic growth	+	+	+	+	+	+	+	+	+	+
Arginine dehydrogenase	+	+	+	+	+	+	+	+	+	+

- Negative reaction
+ Positive reaction

Table 2 : Hydrogen Cyanide production and phosphate solubilisation by effective isolates of bacterial antagonists

Sr. No.	Antagonistic bacteria	HCN production		Phosphate solubilisation
		Qualitative assay	Quantitative assay (O.D value)*	Clearing zone (mm)
1.	Pf_4	+	0.045	1.2
2.	Pf_5	+++	0.092	2.1
3.	Pf_1	++	0.065	1.4
4.	Control	-	-	-

\+ Produced HCN ++ Medium HCN production +++ Strong HCN production - No HCN production
* Mean of three replications

Table 3 : Siderophore production by effective isolates of bacterial antagonists

Sr. No.	Isolates	Colour of Siderophore pigment	Area of production* (mm^2)	Nature of Siderophore	
				Hydroxymate	Carboxylate
1.	Pf_4	Yellow	54	++	-
2.	Pf_5	Yellow	81	+++	-
3.	Pf_1	Yellow	68	+++	-
4.	Control	-	-	-	-

* Mean of three replications - Disappearance of pink colour + Light red colour ++ Moderate red colour +++ Deep red colour

Table 4 : Effect of *Pseudomonas fluorescens* isolates on the mycelial growth of *A. alternate* (isolates) under *in vitro*

Sr. No	Isolates	Mycelial growth (cm) *	% reduction over control
1.	*P. fluorescens* (Pf_1)	4.50	48.57
2.	*P. fluorescens* (Pf_2)	5.75	34.29
3.	*P. fluorescens* (Pf_3)	6.58	24.80
4.	*P. fluorescens* (Pf_4)	5.20	40.57
5.	*P. fluorescens* (Pf_5)	3.35	61.71
6.	*P. fluorescens* (Pf_6)	6.24	28.69
7.	*P. fluorescens* (Pf_7)	6.82	22.06
8.	*P. fluorescens* (Pf_8)	6.75	22.86
9.	*P. fluorescens* (Pf_9)	5.62	35.77
10.	*P. fluorescens* (Pf_{10})	6.65	24.00
11.	Control	8.75	0.00
	C.D. (P = 0.05)	0.85	

*Mean of three replications

produced a clear zone in Sperber's hydroxy apatite media 48 h after incubation.

Siderophore production by effective bacterial antagonists isolates:

All the three effective bacterial isolates of *Pseudomonas* sp. produced siderophore in chromeazurol S (CAS) plate assay method. All isolates of *P. fluorescens* were produced greenish yellow-to-yellow fluorescent pigmentation in blue coloured medium (Table 3). Many workers made similar observations, Duffy and Defago (1997) and Lim *et al.* (1999) reported that siderophore production by *P. flourescens* GL 20 inhibited spore germination and hyphal growth of *Fusarioum oxysporium* f.sp. *lycperscici in vitro* and reduced the disease incidence with enhanced plant growth. Cronin *et al.* (1997) also demonstrated that *Pseudomonas fluorescens* F113G22 strain inhibited the growth of *Erwinia carotovora* var. *atroseptica* that favours siderophore production and contribute to biological control of *Erwinia carotovora* var. *atroseptica*.

Detection of the nature of siderophore :

The isolates Pf_5 and Pf_4 produced deep red colour instantly by the addition of tetrazolium salt to the sample where as, the isolate Pf_1 and Pf_2 produced red colour slowly, indicating that the nature of the siderophore is hydroxymate type. Carboxymate type of siderophore was determined by the disappearance of pink colour on the addition of phenolphthalein to the sample. The isolate Pf_5, Pf_1, Pf_4, and Pf_2 were also produced carboxylate type of siderophore (Table 3).

Efficacy of *Pseudomonas fluorescens* isolates against *A. alternata* :

Among the ten isolates of *Pseudomonas fluorescens* were tested against *A. alternata*, Pf_5 recorded the highest inhibition of mycelial growth of *A. alternata* over control by recording 61.71 % inhibition followed by Pf_1 recorded 48.57 % inhibition and they were significantly different from each other. The lowest % inhibition (22.86%) of mycelial growth of *A. alternata* was recorded by Pf_8 (Table 4). *In vitro* evaluation of *Pseudomonas fluorescens* isolates against *A. alternata* indicated that, Pf_5 significantly exhibited the maximum inhibition of mycelial growth followed by Pf1. These results were coinciding with several workers. *B. subtilis* and *P. fluorescens* isolate 27 recorded maximum inhibition

of *A. palandui* (Mohan, 1996). The inhibitory effect of *P. fluorescens* against *A. palaundi* was confirmed by Karthikeyan (1999).

Conclusion :

Biocontrol is an important component of integrated disease management (IDM) that provide disease control, while being relatively harmless to humans, non polluting, bio degradable, selective in mode of action, difficult for pathogens to develop resistance, unlikely to harm other beneficial micro organisms by generally soil health and sustainability of agriculture. *In vitro* evaluation of *Pseudomonas fluorescens* isolates against *A. alternata* indicated that, Pf_5 significantly exhibited the maximum inhibition of mycelial growth followed by Pf1.

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