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Optimization of Cultural Conditions for High Production of Antifungal Activity by Fluorescent *Pseudomonas* sp. against *Dematophora necatrix* and *Phytophthora cactorum*

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A B S T R A C T

The antagonistic activities of fluorescent *Pseudomonas* sp. isolated from rhizospheric soil of apple orchard of Himachal Pradesh were studied against two fungal pathogens viz., *Dematophora necatrix* and *Phytophthora cactorum* which were previously isolated from apple rhizosphere. The optimum conditions for growth and maximum production of antifungal activity by two selected fluorescent *Pseudomonas* sp. viz., An-2-nali and Pn-2-kho were detected. The influence of culture media, incubation time, incubation temperature and pH on growth and production of antifungal activity was investigated. From the results it is concluded that out of five media i.e. Potato dextrose agar (PDA), Kings' B, Pigment production media (PPM), Yeast malt (YM) and 1/5 M 523, the maximum production of antifungal activity in terms of percent inhibition was observed in Yeast malt media after 96 hr of incubation. An-2-nali showed 37.93 %I against *Dematophora necatrix* and 38.46 %I against *Phytophthora cactorum* whereas isolate Pn-2-kho showed 43.10 %I against *Dematophora necatrix* and 36.53 %I against *Phytophthora cactorum*. The optimum conditions for production of antifungal activity by both the fluorescent *Pseudomonas* isolates were observed to be at temperature 28˚C, pH 7 and an incubation time of 96 hr.

Introduction

Fluorescent *Pseudomonas* sp. has been widely investigated as biological control agents against soil-borne plant pathogenic fungi. Several strains have been studied for their antagonism and their ability to protect plants (Amein *et al.*, 2008; Dorjey *et al.*, 2017; Wavare *et al.*, 2017). The culture conditions of microorganisms play an important role in production of biological activities. As the physiological and nutritional requirement of an organism is genetically predetermined, it is important to provide the appropriate carbon and nitrogen source and also the proper environment for optimal production of activity. The production of antimicrobial compounds by *P. aeruginosa* and its activity increased and decreased according to the environmental and nutritional conditions of

growth (Bernal, 2002; Kumar *et al.,* 2005). The ability of microorganisms to form these bioactive products is not a fixed property but can be greatly increased or completely lost under different conditions of nutrition and cultivation. This is because antibiotic biosynthesis is a specific property of microorganisms which depends greatly on culture conditions. Improvement in the growth and antibiotic production can be carried out by manipulating the nutritional and physical parameters of the culturing conditions. Hence media composition plays a vital role in the efficiency and economics of the ultimate process. Therefore, designing an appropriate fermentation medium is of critical importance in the production of secondary metabolites (Gao *et al.,* 2009). Several cultivation parameters like pH, incubation time and temperature play a major role in the production of bioactive metabolites (Usha Kiranmayi *et al.,* 2011).

The present work describes the effect of different cultural conditions on growth and production of antifungal activity by two fluorescent *Pseudomonas* sp. viz., An-2-nali and Pn-2-kho against *Dematophora nectrix* and *Phytophthora cactorum*. Further, the optimization of of culture media, temperature, pH, and incubation time for high production of antifungal activity was also studied.

Materials and Methods

Isolation and identification of fluorescent *Pseudomonas* sp.

Fluorescent *Pseudomonas* sp. were isolated from apple rhizosphere of Shimla dist. of Himachal Pradesh. The soil particles loosely adhering to the roots were gently teased out with small root pieces and mixed well. The soil thus obtained was shaken in 100 ml of sterile distilled water for 10-20 min. to obtain standard soil suspension. Isolation of fluorescent *Pseudomonas* sp. was made by following the serial dilutions and spread plate method using the specific King's B medium (King *et al.,* 1954). Plates were incubated at 28± 2°C for 24 - 48 hr. The isolates were classified on the basis of colony characteristics such as size, color, shape, texture and type of fluorescent pigment production. The most predominant *Pseudomonas* sp. isolates showing yellowish green fluorescent pigments under UV light at 365 nm were selected. All isolates were characterized on the basis of morphological and biochemical tests as per their genera as prescribed in Bergey's Manual of Systematic Bacteriology (Palleroni, 1984). All the fluorescent *Pseudomonas* sp. were characterized for multifarious plant growth promoting activities viz., plant growth regulator production, siderophore, ammonia, HCN, P- solubilization and antifungal activity by using their standard methods (Aneja, 2003).

Antifungal activity assays

All the fluorescent *Pseudomonas* sp. were tested for *in vitro* antagonistic activity against two pathogens by standard well plate assay method on potato dextrose agar (PDA) (Vincent, 1947). The isolated fluorescent *Pseudomonas* cultures were tested for growth inhibitory effects on the mycelium growth of *Dematophora necatrix* and *Phytophthora cactorum* the major fungal pathogens. The culture bit (8 mm) of each indicator fungi bored with the help of sterile cork borer was placed in the side of pre-poured PDA plate and 100µl of 72h old culture supernatant of test *Pseudomonas* isolates were added to each well in the centre of the plate. Plates were incubated at 28 ±2 °C for 2-7 days and observed for inhibition zone formation. Antifungal activity expressed in terms of mm diameter of clear zone around the well and expressed in terms of per cent inhibition of fungal mycelium as calculating from equation:

$$\% I = \frac{C-Z}{C} \times 100$$

Where,

Z = Growth of mycelia in treatment
C = Growth of mycelia in control

Optimization of culture conditions for enhanced antifungal activity

Effect of different media and incubation time

Effect of five different media (100 ml) on production of antifungal activity were studied by growing selected fluorescent *Pseudomonas* sp. isolates in each media broth *i.e.,* Potato dextrose agar (PDA) (g/l) – Potato, 200.0; Dextrose, 20.0; King's B media (g/l) - Peptone, 20.0; K_2HPO_4, 1.5; $MgSO_4.7H_2O$, 1.5; Glycerol, 15.0 ml, Pigment production media (PPM) (g/l) - Peptone, 20.0; Glycerol, 20.0 ml; NaCL, 5.0; KNO_3, 1.0; Yeast malt (YM) (g/l) – Yeast extract, 3.0; Malt extract, 3.0; Peptone, 5.0; Glucose, 10.0 and 1/5 M523 media (g/l) – Sucrose, 2.0; Yeast extract, 0.8; Casein hydrolysate, 1.6; K_2HPO_4, 0.4; $MgSO_4.7H_2O$, 0.06 (Rosales *et al.*,1995). In each case 0.5 ml of inoculum of overnight grown culture of bacteria was used to inoculate 100 ml of each media in a 250 ml Erlenmeyer flask. Flasks were incubated at 28±2°C under shake conditions (100 rpm) for different time interval of 48, 72 and 96 hr. Growth was observed as absorbance at 540 nm. Cultures were centrifuged at 10,000 rpm for 20 minutes at 4°C and cell free culture supernatants were separated and stored at 4°C in small aliquots. The selected fluorescent *Pseudomonas* sp. were tested for their ability to inhibit the growth of *Dematophora necatrix* and *Phytophthora cactorum* by standard well plate assay method (Vincent, 1947). For control, culture bit of indicator fungi viz., *Dematophora necatrix* and *Phytophthora cactorum* was kept in the centre of prepoured plate. The medium and incubation time that gave best results for antifungal activity was selected and used for further study.

Effect of different incubation temperature

The effect of different temperature *i.e.* 4°C, 28°C, 37°C and 50°C on production of antifungal activity was studied by using best selected yeast malt (YM) medium. 0.5 ml inoculum suspension of overnight grown culture of fluorescent *Pseudomonas* sp. was used to inoculate 100 ml of sterilized media in a 250 ml Erlenmeyer flask. Flasks were incubated at different temperatures *i.e.* 4°C, 28°C, 37°C and 50°C for 72-96 hr. The growth was also observed as absorbance at 540 nm at different temperatures. Supernatant was harvested by centrifugation at 10,000 rpm for 20 minutes at 4°C. The antifungal activity was assayed by their respective well plate assay method (Vincent, 1947).

Effect of pH

Effect of different pH on growth and production of antifungal activity was studied by using best selected yeast malt (YM) media of pH 4, 5, 6, 7, 8 and 9. 0.5 ml of inoculum suspension was used to inoculate 100 ml of media in a 250 ml Erlenmeyer flask. The growth was observed as absorbance at 540 nm at different pH. Supernatant was harvested by centrifugation at 10,000 rpm for 20 minutes at 4°C. The antifungal activity was assayed by their respective well plate assay method and expressed in terms of percent inhibition growth of fungal mycelia (Vincent, 1947).

Results and Discussion

All the ten bacterial isolates from apple rhizosphere were found to be gram negative, non-spore forming, coccobacillus shaped, and

positive for catalase, oxidase and gelatin hydrolysis. All the isolates also produced fluorescent pigment on King's B medium. Among them, six isolates were positive for denitrification test, eight isolates were positive for lecithinase test and all isolates produced blue-green fluorescent pigment on King's B medium under ultraviolet light at 365 nm. According to Todar (2004), more than half of the *Pseudomonas* sp. produces pyocyanin which is a blue-green pigment, while *Pseudomonas fluorescens* produces green fluorescent pigment that is soluble. The physiological and biochemical tests further confirmed the isolates to be as belonging to fluorecent *Pseudomonas* as reported by earlier workers (Nathan *et al.,* 2011; Tiwary and Balabaskar, 2012; Sharma *et al.,* 2014; Verma *et al.,* 2016; Verma and Kaur, 2016). Rao *et al.,* (1999) also identified *Pseudomonas* isolates on the basis of similar morphological and biochemical tests. Based on morphological, biochemical and physiological properties, the isolates were identified as belonging to genus *Pseudomonas* sp.

Culture conditions play an important role in cellular growth and also in production of biological activities by microorganisms. As the physiological and nutritional requirement of an organism is genetically predetermined, it is important to provide the appropriate carbon and nitrogen source and also the proper environment for optimal production of activity.

The growth of two best selected fluorescent *Pseudomonas* sp. isolates *viz.,* An-2-nali and Pn-2-kho and production of antifungal activity by these isolates was studied under shake condition (90 rpm) at different time of incubation (48hr, 72hr and 96hr) in five different cultural media *viz.,* Potato dextrose agar (PDA), King's B media, Pigment production media (PPM), Yeast malt (YM), and 1/5 M523. The effect of media (Table 1 and 2) on the production of antifungal activity by fluorescent *Pseudomonas* sp. against *Dematophora necatrix* and *Phytophthora cactorum* revealed that the maximum percent inhibition (% I) was observed in yeast malt media after 96 hr of incubation. The maximum percent inhibition (% I) of 43.10 % against *Dematophora necatrix* and 36.53 % was observed against *Phytophthora cactorum* by Pn-2-kho and An-2-nali showed 37.93 % I against *Dematophora necatrix* and 38.46 % was observed against *Phytophthora cactorum*.

The results showed that the efficiency of a medium for production of antifungal activity in terms of per cent inhibition was of following order: YM > PDA > KM > PPM > 1/5 M 523 medium against *Dematophora necatrix* and *Phytophthora cactorum* by both the isolates. The biosynthesis of bioactive substances is a specific property of some species or even some strains of microorganisms. This property depends greatly upon conditions of cultivation of microorganisms (Issac *et al.,* 1992). The challenge is faced to provide the organisms with conditions that allow expression of secondary metabolites and accumulation of unusual metabolites.

A number of media generally employed for expression of secondary metabolism of microorganisms and initial evaluation of media are usually made. Selection of media is complex since the possible variations are so large. Simple media works very well as broth and agar and this has been validated many times with novel bioactive compounds being produced (Jenning, 1995). So, the development of media, which increase the production of bioactive compounds, is very important. The use of specific media for maximum production of biological activities was also observed earlier by various workers (Laha *et al.,* 1996; Laha and Verma, 1998, Verma *et al.,* 2017).

Table.1 Effect of different media and time of incubation on the growth and production of antifungal activity by selected fluorescent *Pseudomonas* sp. against *Dematophora necatrix* sp. at 28 ± 2°C temperature under shake conditions

Fluorescent *Pseudomonas* isolates	Medium	Growth* at 540 nm			Mean	T (mm diameter)			Mean	%I**			Mean
		48hr	72hr	96hr		48hr	72hr	96hr		48hr	72hr	96hr	
An-2-nali	**PDA**	0.790	1.203	1.298	**1.097**	40	37	36	**37**	31.03	36.20	37.93	**35.05**
	KM	0.612	1.105	1.208	**0.975**	43	39	40	**40.66**	25.86	32.75	31.03	**29.88**
	PPM	0.645	1.045	1.187	**0.959**	49	45	42	**45.33**	15.51	22.41	27.58	**21.83**
	YM	0.798	1.301	1.407	**1.168**	39	38	36	**37.66**	32.75	34.48	37.93	**35.05**
	1/5M$_{523}$	0.701	1.103	1.232	**1.012**	40	42	43	**41.66**	31.03	27.58	25.86	**28.15**
Pn-2-kho	**PDA**	0.689	1.067	1.205	**0.987**	40	35	35	**36.66**	31.03	39.65	39.65	**36.77**
	KM	0.780	1.189	1.324	**1.097**	42	34	36	**37.33**	27.58	41.37	37.93	**35.62**
	PPM	0.546	1.005	1.398	**0.983**	44	40	39	**41**	24.13	31.03	32.93	**29.36**
	YM	0.978	1.287	1.540	**1.268**	37	34	33	**34.66**	36.20	41.37	43.10	**40.22**
	1/5M$_{523}$	0.604	0.903	1.302	**0.966**	43	39	40	**40.66**	25.86	32.75	31.03	**29.88**
Mean		**0.714**	**1.120**	**1.310**		**41.7**	**38.3**	**38**		**28.09**	**33.95**	**34.49**	
CD$_{0.05}$													
T					0.01				1.88				**0.12**
I					0.00				1.03				**0.06**
T×I					**0.01**				**3.26**				**0.21**

* Growth in terms of optical density at 540 nm at different time of incubation.

** Antifungal activity expressed in terms of percent inhibition (% I) of mycelial growth of indicator test fungi by well plate assay method.

Percent inhibition (%I) = C-Z/C ×100

Where: C = Growth of mycelia in control, Z = Growth of mycelia in treatment.

Table.2 Effect of different media and time of incubation on the growth and production of antifungal activity by selected fluorescent *Pseudomonas* sp. against *Phytophthora cactorum* at 28 ± 2°C temperature under shake conditions

Fluorescent *Pseudomonas* isolates	Medium	Growth* at 540 nm			Mean	T (mm diameter)			Mean	%I**			Mean
		48hr	72hr	96hr		48hr	72hr	96hr		48hr	72hr	96hr	
An-2-nali	PDA	0.578	1.008	1.078	**0.888**	39	36	35	**36.66**	25	30.76	32.69	**29.48**
	KM	0.602	1.201	1.332	**1.045**	35	32	33	**33.33**	32.69	38.46	36.53	**35.89**
	PPM	0.677	1.012	1.154	**1.01**	40	38	40	**39.33**	23.07	26.92	23.07	**24.35**
	YM	0.761	1.198	1.398	**1.119**	36	33	32	**33.66**	30.76	36.53	38.46	**35.25**
	1/5M 523	0.703	1.102	1.202	**1.002**	42	39	39	**40**	19.23	25	25	**23.07**
Pn-2-kho	PDA	0.789	1.056	1.232	**1.025**	40	38	36	**38**	23.07	26.92	30.76	**26.91**
	KM	0.809	1.145	1.302	**1.085**	36	35	33	**34.66**	30.07	32.69	36.53	**33.09**
	PPM	0.566	0.908	1.098	**0.857**	44	42	41	**42.33**	15.38	19.23	21.15	**18.58**
	YM	0.977	1.278	1.387	**1.214**	40	34	33	**35.66**	23.07	34.61	36.53	**31.4**
	1/5M 523	0.656	1.076	1.165	**0.965**	42	40	39	**40.33**	19.23	23.07	25	**22.4**
Mean		**0.711**	**1.09**	**1.233**		**39.4**	**36.7**	**43.5**		**24.08**	**29.41**	**30.57**	
$CD_{0.05}$													
T					0.001				1.88				**0.68**
I					0.001				1.03				**0.37**
T×I					**0.003**				**3.26**				**0.11**

*Growth in terms of optical density at 540 nm at different time of incubation.

**Antifungal activity expressed in terms of percent inhibition (% I) of mycelial growth of indicator test fungi by well plate assay method.
Percent inhibition (% I) = C-Z/C ×100. Where: C = Growth of mycelia in control, T = Growth of mycelia in treatment.

Table.3 Effect of different temperature on the growth and production of antifungal activity by selected fluorescent *Pseudomonas* sp. against *Dematophora necatrix* in Yeast malt (YM) media

Fluorescent *Pseudomonas* isolates	Growth at 540 nm				Mean	Temperature (°C)				Mean
	Temperature (°C)					4°C	28°C	37°C	50°C	
	4°C	28°C	37°C	50°C		%I	%I	%I	%I	
An-2-nali	0.050	0.953	0.944	0.102	**0.512**	-	30.76	26.92	-	**14.42**
Pn-2-kho	0.109	1.067	0.981	0.265	**0.605**	-	34.61	32.69	-	**16.82**
Mean	**0.079**	**1.01**	**0.96**	**0.183**		**0**	**32.68**	**29.80**	**0**	
$CD_{0.05}$										
T					0.001					**0.012**
I					0.002					**0.017**
T×I					**0.003**					**0.024**

Table.4 Effect of different temperature on the growth and production of antifungal activity by selected fluorescent *Pseudomonas* sp. against *Phytophthora cactorum* in Yeast malt (YM) media

Fluorescent *Pseudomonas* isolates	Growth at 540 nm				Mean	Temperature (°C)				Mean
	Temperature (°C)					4°C	28°C	37°C	50°C	
	4°C	28°C	37°C	50°C		%I	%I 2	%I	%I	
An-2-nali	0.102	1.109	1.017	0.178	**0.6015**	-	29.78	25.53	-	**13.83**
Pn-2-kho	0.187	1.264	1.108	0.212	**0.679**	-	36.17	34.04	-	**17.55**
Mean	**1.44**	**1.18**	**1.062**	**0.195**		**0**	**32.97**	**29.78**	**0**	
$CD_{0.05}$										
T					0.001					**0.012**
I					0.002					**0.017**
T×I					**0.003**					**0.023**

Table.5 Effect of pH on the growth and production of antifungal activity by selected fluorescent *Pseudomonas* sp. against *Dematophora necatrix* in Yeast malt (YM) media at 28 ± 2° C temperature for 96hr under shake conditions

Fluorescent *Pseudomonas* isolates	Growth at 540 nm						Mean	pH						Mean
	pH							4	5	6	7	8	9	
	4	5	6	7	8	9		%I	%I	%I	%I	%I	%I	
An-2-nali	0.345	0.366	0.987	1.090	1.012	0.408	**0.701**	-	-	29.31	32.72	32.72	-	**15.79**
Pn-2-kho	0.241	0.402	1.087	1.156	1.023	0.398	**0.717**	-	-	36.36	36.36	34.54	-	**17.88**
Mean	**0.293**	**0.384**	**1.037**	**1.123**	**1.017**	**0.439**		**0**	**0**	**32.83**	**34.54**	**33.63**		
$CD_{0.05}$														
T							0.001							**0.009**
I							0.002							**0.016**
T×I							**0.003**							**0.022**

Table.6 Effect of pH on the growth and production of antifungal activity by selected fluorescent *Pseudomonas* sp. against *Phytophthora cactorum* in Yeast malt (YM) media at 28 ± 2°C temperature for 96 hr under shake conditions

Fluorescent *Pseudomonas* isolates	Growth at 540 nm						Mean	pH						Mean
	Ph							4	5	6	7	8	9	
	4	5	6	7	8	9		%I	%I	%I	%I	%I	%I	
An-2-nali	0.245	0.316	1.001	1.134	1.298	0.358	**0.725**	-	-	30	32	32	-	**15.66**
Pn-2-kho	0.141	0.370	1.109	1.296	1.012	0.408	**0.722**	-	-	36	36	34	-	**17.66**
Mean	**0.193**	**0.34**	**1.05**	**1.21**	**1.15**	**0.38**		**0**	**0**	**33**	**34**	**33**	**0**	
$CD_{0.05}$														
T							0.004							**0.972**
I							0.007							**1.685**
T×I							**0.010**							**2.383**

Understanding which environmental factors are important and how these influences the production of secondary metabolic activities is important. Jha *et al.*, (1992) found that biological activity and composition of soil microbes are generally affected by many factors including physico-chemical properties of the soil, temperature and vegetation. Microorganisms are exposed completely to environmental influences and must be able to tolerate and react to a wide range of environmental changes. In contrast to pH, ionic composition and water activity, the internal temperature of microorganisms must be equal to that of its environment. The production of antifungal activity against *Dematophora necatrix* and *Phytophthora cactorum* was observed at different temperature viz., 4 ^{0}C, 28 ^{0}C, 37 ^{0}C and 50 ^{0}C by using yeast malt media. The production of antifungal activity has been found to be dependent on temperature. The maximum percent inhibition (% I) by both the fluorescent *Pseudomonas* sp. An-2-nali and Pn-2-kho was observed at 28ºC (Table 3 and 4).

An-2-nali showed 30.76 % I against *Dematophora necatrix* and 29.78 % I against *Phytophthora cactorum* whereas isolate Pn-2-kho showed 34.61 %I against *Dematophora necatrix* and 36.17 %I against *Phytophthora cactorum*. This behaviour of fluorescent *Pseudomonas* sp. was similar to usual response of mesophilic organisms where metabolic activities get slow down below and above the optimum temperature. This suggests that organisms are mesophilic in nature. Few of the *Pseudomonas* isolates were found to grow even at temperature ranging from 4^{0}C to 41^{0}C. Our results collaborates with Thakur *et al.*, (2014), who reported that the maximum P-solubilization activity was produced at 28 ºC by all the *Pseudomonas* isolates and a decrease in yield of these activities was observed above and below 28 ºC. In our study also the decrease in antifungal activity was observed above and below 28 ºC. Mishra. *et al.*, (2009) also reported that *Pseudomonas lurida* grew at temperature ranging from 4 to 30 °C, with a growth optimum at 28 °C. The growth of most microbes is restricted to a 20–40 ^{0}C span. Each species or a strain has a characteristic minimum, optimum and maximum temperature and optimum is usually 5-10^{0}C below maximum temperature (Forage *et al.*, 1990). The optimal temperature for growth may not be that best suited to product formation especially where the product is predominantly non growth associated as in the case of many secondary metabolites. Additionally, different strains may have different growth and production optima.

So in any mutation or general selection programme independent temperature optimization of each strain for growth and subsequent product formation is often necessary (Woodruff, 1961). The optimum pH of the medium for the production of antifungal activity against *Dematophora necatrix* and *Phytophthora cactorum* was determined by using yeast malt media of different pH *i.e.*, 4, 5, 6, 7, 8 and 9 at 28ºC. The results (Table 5 and 6) showed that the optimum pH for the production of antifungal activity against *Dematophora necatrix* and *Phytophthora cactorum* was pH 7. An-2-nali showed 32.72 %I against *Dematophora necatrix* and 32 %I against *Phytophthora cactorum* whereas isolate Pn-2-kho showed 36.36 %I against *Dematophora necatrix* and 36 %I against *Phytophthora cactorum*. Our results are in collaboration with Thakur *et al.*, (2014) and Jena, (2013) who concluded pH 7.0 as optimum for the P-solubilizing activity of the *Pseudomonas* isolates. The study showed that media, pH, temperature and incubation time, directly influenced the production of antifungal activity.

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