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STUDY OF HYDROGEN CYANIDE PRODUCTION AS A MECHANISM OF ANTIFUNGAL ACTIVITY OF FLUORESCENT *PSEUDOMONAS SPECIES* AGAINST PHYTOPATHOGENIC *PYTHIUM* AND *FUSARIUM SPECIES*

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ABSTRACT

Among 150 *Pseudomonas* isolates obtained from rhizosphere of healthy crop plants, nine isolates showed good *in vitro* antagonistic activity against phytopathogenic *Pythium* and *Fusarium species*. These *Pseudomonas* isolates were tested for ability to produce HCN by inverted plate technique on nutrient sucrose agar (NSA) supplemented with glycine and $FeCl_3$, in Petri plates with picric acid discs fixed inside the lid. Among the nine *Pseudomonas* isolates, three were found to produce hydrogen cyanide as a metabolic product. Study of morphological, cultural, biochemical and genetic characters identified these isolates as *Pseudomonas aeruginosa* 13, *P. aeruginosa*58, and *P. fluorescens* 106. A direct correlation between the extent of HCN production and fungal growth inhibition was observed. These three HCN producing *Pseudomonas* isolates also showed other mechanisms of antifungal activity such as production of antibiotics and siderophores. Other six isolates which did not produce HCN showed good antifungal activity. This indicated that, HCN production is one of the important mechanisms of antifungal activity by *Pseudomonas* species but not always involved in inhibition or killing of phytopathogenic fungi. These *Pseudomonas* isolates were also found more effective to control fungal Infections of crop plants, in pot culture experiments.

KEYWORDS: *Pseudomonas*, Phytopathogenic fungi, Antifungal activity, Hydrogen cyanide.

INTRODUCTION

The Indian farmers have to suffer great economic loss per year due to weather irregularities and the crop diseases caused by phytopathogens. Among the different plant pathogens, fungi are the most potent and leading phytopathogens of crop plants. Species of *Pythium*, *Phytopthora*, *Fusarium*, *Rhizoctonia*, *Sclerotium*, *Colletotrichum*, etc. are mainly involved in soil-borne fungal diseases of crop plants.[1] The crop diseases are traditionally controlled by physical and chemical methods. However, indiscriminate use of chemicals has created great harm to humans, animals, vegetations and the environment as a whole. The biological control has been developed during last few decades and now became indispensable need of sustainable agriculture.[2]

Several rhizobacteria of crop plants are involved in natural biological control of phytopathogens in soil and also act to promote plant growth and enhance yield of agricultural products. Different mechanisms of antifungal activity are involved especially the production of antibiotics and other inhibitory substances like hydrogen cyanide, production of siderophores, parasitism and lysis of fungal cell wall by production by cellulases and chitinases. Many rhizobacteria produce hydrogen cyanide (HCN), a volatile metabolic product that plays important role in biocontrol of plant pathogens.[3,4,5]

The mechanisms of action of secondary metabolites on phytopathogens are diverse. The HCN forms stable complexes with several essential divalent metal ions and create its deficiency resulting to inhibit the growth of phytopathogens. It also inhibits the activity of certain enzymes; particularly cytochrome oxidases, the important enzymes in respiration. However, *Pseudomonas* species and a variety of other rhizobacteria are relatively resistant to HCN, as they have an alternate cyanide resistant cytochrome oxidase system.[6]

Objective of the present work was to isolate *Pseudomonas* species from rhizosphere of healthy crop plants, to screen isolates with antagonistic potential against phytopathogenic *Pythium* and *Fusarium species*, to study different mechanisms of action and to determine the role of HCN production in antifungal activity.

MATERIALS AND METHODS

Primary screening of antifungal *Pseudomonas* isolates

150 *Pseudomonas* cultures were isolated from rhizosphere of healthy crop plants using King's B medium and identified on the basis of morphological, cultural and biochemical

characters. These *Pseudomonas* cultures were tested for their antifungal activity by dual culture technique on PDA against phytopathogenic *Pythium* and *Fusarium* species.[7]

Secondary screening of efficient antifungal isolates

Pseudomonas cultures with potent antifungal activity against *Pythium* and *Fusarium* species were screened by dual culture method in PDB.[7,8]

Detection of HCN production ability of antifungal *Pseudomonas* isolates

Ability of *Pseudomonas* isolates to produce HCN was tested on nutrient sucrose agar (NSA) supplemented with glycine and $FeCl_3$ (100mg/l) in Petriplates and picric acid discs fixed inside the lid.[8,9] NSA contains g/l of sucrose- 5.0, yeast extract- 4.0, peptone- 4.0, beef extract- 2.0 and agar- 20.0.[9,10] Picric acid (PA) discs were prepared by soaking Whatman filter paper discs (diameter 96mm) in 0.5% picric acid solution in 1% sodium carbonate and dried in air. 0.1ml active broth cultures of *Pseudomonas* isolates were separately inoculated on NSA by spread plate technique. A picric acid disk was fixed inside the lids, plates were sealed with stick tape and incubated at 28^0C for 48hrs. Change in color of picric acid disc was observed from yellow to light brown (+), brown (+ +) and reddish brown (+ + +) as indication of low, moderate and high HCN production, respectively.[7,9]

Effect of HCN production by *Pseudomonas* isolates on growth of phytopathogenic fungi-

The antifungal *Pseudomonas* isolates producing HCN were further tested for growth inhibition of phytopathogenic *Pythium* and *Fusarium species* using 'inverted plate technique'.[8,10] 0.1 ml active broth cultures of HCN producing *Pseudomonas* isolates were separately inoculated on NSA plates, by spread plate technique. 10mm PDA culture discs of *Pythium* and *Fusarium species* were obtained by using sterile cork borer and separately inoculated on PDA plates. The lids of both Petriplates i.e. NSA and PDA were removed under aseptic conditions, in laminar airflow. The base of the PDA plates inoculated with fungal cultures was kept inverted on NSA base plates inoculated with *Pseudomonas* cultures. The pairs of these base plates were fixed together with cellophane adhesive tape at the edge of base plates (Fig.1). NSA plate without inoculation of antagonist bacteria was inverted with PDA plate inoculated with fungal disc and incubated as 'control'. Triplicates of each treatment were maintained. The plates were incubated at 28^0C for 7 days. The diameters of fungal growth in test and control plate were measured in mm. Percent inhibition (P I) of fungal growth was calculated using the following formula.

$$P.\ I. = \left[\frac{\text{Diameter of fungal growth in control - Diameter of fungal growth in test}}{\text{Diameter of fungal growth in control}}\right] X\ 100$$

Identification of antifungal HCN producing *Pseudomonas* isolates

Three HCN producing antifungal *Pseudomonas* isolates were identified to species level on the basis of 16S r-RNA sequencing conducted in National Centre of Cell Science, Savitribai Phule University, Pune.

RESULTS AND DISCUSSION

Study of HCN production ability of antifungal *Pseudomonas* isolates

Among the nine antifungal *Pseudomonas* isolates, three isolates i.e. *Pseudomonas aeruginosa*13, *P. aeruginosa*58, and *P. fluorescens*106 showed HCN production (table-1). This indicated that, HCN production plays important role in antifungal activity of these *Pseudomonas* cultures. Similar results were obtained by Mondal *et al.*,(2000); Pal and Jalali, (1998).[2,9]

Effect of HCN production by *Pseudomonas* isolates on growth of phytopathogenic fungi

The growth of *Fusarium* and *Pythium* species was inhibited overall by 12.50 to 21.87%. The percent growth inhibition was found highest (21.87 and 20.68%) in case of *P. aeruginosa*58, moderate in case of *P. fluorescens*106 (18.75 and 18.96%) while lowest in case of *P. aeruginosa* 13 (12.50 and 13.79%), respectively for *Pythium* and *Fusarium* species. Among the six isolates which did not show HCN production also inhibited the fungal growth in inverted plate technique, although to a relatively less extent. This may be due to production of volatile products other than HCN.

Steven *et al.*, (2008) observed that, the volatile metabolic products produced by *Pseudomonas* isolates in liquid culture reduced the fungal biomass by 43%.[5] Our isolates found less effective in this respect. A direct correlation has been observed between extent of HCN production and *in vitro* antifungal activity of *Pseudomonas* isolates. NSA medium supplemented with glycine and $FeCl_3$ supported the HCN production by *Pseudomonas* isolates. The glycine supplied additionally and that in peptone acted as precursor for HCN production.[3] The root exudates of plants also contain amino acids that indicate symbiotic association between the plants and rhizospheric microorganisms.[1,12] Nazneen and Javed (2003) observed that, *Pseudomonas aeruginosa* NJ15 showed significant growth inhibition of *Fusarium oxysporum*, *Trichoderma harzianum*, *Alternaria alternata* and *Macrophomina*

phaseolina with HCN and siderophore production as the mechanisms of action.[13] Faraz *et al*., (2008) revealed the biological control and plant growth promotion by rhizospheric isolates of *Azotobacter*, *Pseudomonas*, *Bacillus* and *Mesorhizobium* by HCN production as one of the mechanisms.[14] Rakh *et al*., (2011) observed that, HCN production plays important role in biological control of stem rot of ground nut caused by *Sclerotium rolfsii* by *Pseudomonas* c.f. *monteilii*-9.[15]

Other six isolates although did not produce HCN showed antifungal activity on PDA and PDB. These media being rich in iron and poor in inorganic phosphates favors antibiotic and HCN production.[3] HCN production is one of the important mechanisms of antifungal activity of some *Pseudomonas* isolates but not always responsible.[12] The other mechanisms like production of antibiotics, other secondary metabolites and siderophores, parasitism by degradation of cell wall polysaccharides, etc. may be involved in antifungal activity of these isolates on PDA and in PDB.[2] The antagonists with multiple mechanisms were found more effective to inhibit the fungal growth.[5] It is important to note that, fluorescent pseudomonads have an alternate cyanide resistant cytochrome oxidase.[6]

Castric *et al*., 1975 observed HCN as a secondary metabolite of antifungal *Pseudomonas* species.[11] Narasimha Rao and Kulkarni (2003) observed *in vitro* growth inhibition of *Sclerotium rolfsii* Sacc by *Trichoderma species* with production of volatile metabolites.[10] Rangeshwaran and Prasad (2000) tested '11' antifungal isolates for HCN production among which four isolates showed this potential.[4] Laha *et al*., (1996) obtained sixteen isolates of fluorescent *Pseudomonas* from cotton rhizosphere antagonistic to *Sclerotium rolfsii* causing wilt disease, which were cyanogenic and producing other volatile metabolites.[16] Rezzonico *et al*., (2007) studied the biocontrol efficacy of '230' fluorescent *Pseudomonas* isolates from '63' soils and inferred that, the ability of *Pseudomonas* species to produce 2,4 diacetyl phloroglucinol and HCN was directly associated with superior disease suppression activity in *Pythium*-cucumber and *Fusarium*-tomato pathosystem.[17]

We conclude that, the *Pseudomonas* isolates- *P. aeruginosa*13, *P. aeruginosa*58 and *P. fluorescens*106 found highly active against phytopathogenic *Pythium* and *Fusarium* species with production of hydrogen cyanide as one of the mechanisms of antifungal activity. These isolates are hopeful to control fungal infections of the crop plants in field.

Table 1: HCN production and antifungal activity of *Pseudomonas* isolates.

***Pseudomonas* Isolates**	**HCN production**	**Growth of *Pythium* (mm) and P. I. values**	**Growth of *Fusarium* (mm) and P. I. values**
*P. aeruginosa*13	+ +	56 (12.50)	50 (13.79)
*P. aeruginosa*58	+ +	50 (21.87)	46 (20.68)
*P. putida*71	-	64 (00.00)	57 (01.72)
*P. fluorescens*106	+	52 (18.75)	47 (18.96)
*P. putida*111	-	62 (03.12)	58 (00.00)
*P. aeruginosa*117	-	63 (01.56)	57 (01.72)
*P. aeruginosa*154	-	64 (00.00)	57 (01.72)
*P. aeruginosa*166	-	62 (03.12)	58 (00.00)
*P. fluorescens*171	-	62 (03.12)	58 (00.00)
Control	-	64 (-)	58 (-)

*Colour change to- Light brown (+)***:** *Less HCN production, Brown (++)***:** *Moderate HCN production and Radish brown (+ + +)***:** *High HCN production. The values of diameter of fungal growth and P. I. are average of triplicate tests.*

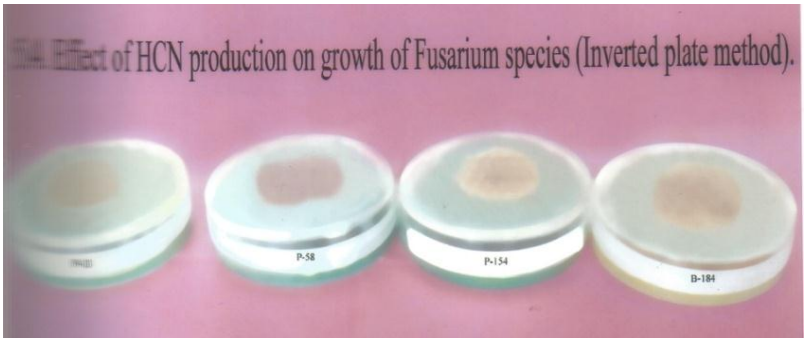

Fig. 1: Effect of HCN production on Growth of Fungi.

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