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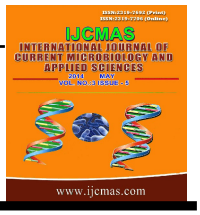

Original Research Article

Hydrogen Cyanide Production Ability by bacterial antagonist and their Antibiotics Inhibition Potential on *Macrophomina phaseolina* (Tassi.) Goid

A.Karmel Reetha[1*] S.Lalitha Pavani[2] and S.Mohan[3]

[1]Vanavarayar Institute of Agriculture, Pollachi 642 103, Tamilnadu, India
[2]Andhirapradesh Rice Research institute, Naruteru, Andhra pradesh, India
[3]Tamil Nadu Agricultural University, Madurai, 641 003, Tamilnadu, India
Corresponding author

A B S T R A C T

This research was undertaken for the purpose of isolation and purification of indigenous *Pseudomonas* spp and *Bacillus* spp. and evaluating its ability in hydrogen cyanide synthesis and also evaluating the potential of super-strains on reduction in the fungal growth of *Macrophomina phaseolina.* According to this, the research was carried out in laboratory tests. 2 strains (obtained from rhizosphere soil from 2 isolates of sunflower plants) *B. substilis* and 2 strains of *P. fluorescence* were sub-cultured, purified and refreshed. Then these strains were evaluated for the capability in cyanide synthesis by a quantitative and qualitative method. Among these isolates on qualitative analysis of HCN indicated a strong production of HCN in Pf_1 and CPf_5 was estimated. Isolates of *B. subtilis* did not produce HCN. In quantitative estimation, Pf_1 and CPf_5 recorded the maximum OD value of 0.094 and 0.085 respectively but Bs_{10} recorded least OD value (0.015). The antibiotics produced by all the four effective bacterial isolates were effective against *M. phaseolina* and recorded reduction in growth of the pathogen ranged between 61.13 to 69.62 per cent reduction over control. Antibiotics produced by Pf_1 were maximum per cent reduction in the fungal growth (69.92%) followed by CPf_5 (65.59%). The *Bacillus* isolates Bs_{10} and CBs_4 were efficacy against the pathogen with 63.36 per cent and 61.13 per cent reduction over control. All the isolates were on par in inhibition of mycelial growth.

Keywords

Bacterial antagonist, HCN, Antibiotic, *M. phaseolina*

Introduction

Sunflower (*Helianthus annus, L.*) is an important oil seed crop in India. It is one of the fastest growing plants which belong to family Asteraceae (Compositae) (Rodriguez *et al.*, 2002). *M. phaseolina* is a soil-borne fungus that causes charcoal rot disease of many crops in arid and semiarid areas of the world (Dhingra and Sinclair, 1978). On sunflower is subjected to be attacked with a variety of fungal pathogens, which affect its yield and its oil quality (Sangawan *et al*., 2005). Very high incidence and spreading of charcoal rot on sunflower was recorded in Slovakia

(Bokor, 2007) and in the Czech Republic in 2007 (Veverka *et al*., 2008). *M. phaseolina* is an anamorphic and soil borne fungus with a broad host range that includes 75 plant families and more than 500 species worldwide (Salik, 2007: Khan, 2007). Charcoal rot is of great economic importance in arid areas of the world. It causes decrease in stem height, girth, root region and head weight (Raut, 1983; Kolte, 1985). Production of HCN by certain strains of *Pseudomonas fluorescens* has been involved in suppression of soil borne pathogens (Voisard *et al*., 1989). HCN is produced by many rhizobacteria and is postulated to play a role in biological control of pathogens (Defago *et al*., 1990). Hajji *et al*. (1989) reported that broad-spectrum of antibiotics such as gliotoxin known to have antimicrobial activity (Howell *et al*., 1993; Sivasithamparam and Ghisalberti, 1998: Pozo *et al*., 2004). Antibiotics are low molecular weight secondary metabolites produced by antagonists and directly inhibited the pathogen growth (Sabitha *et al*., 2001). This objective aimed to investigate the qualitative and quantitative capability of rhizobacteria isolates in HCN production and on reduction in the fungal growth of *M. phaseolina.*

Materials and Methods

Isolation of antagonists from the rhizosphere region of sunflower plants plants

Antagonistic of bacteria were isolated from the rhizosphere soil collected from different sunflower growing areas of Tamil Nadu. The plants were pulled out gently with intact roots and the excess soil adhering on roots was removed gently. Ten gram of rhizosphere soil was transferred to 250 ml Erlenmeyer flask containing 100 ml of sterile distilled water. After thorough shaking, the antagonist in the suspension was isolated by serial dilution plate method (Pramer and Schmidt, 1956).

From the final dilutions of 10^{-5} and 10^{-6}, one ml of each aliquot was pipetted out, poured in sterilized Petri dish containing King's B medium and nutrient agar medium separately and they were gently rotated clockwise and anti clockwise for uniform distribution and incubated at room temperature (28±2 °C) for 24 hours. The colonies were viewed under UV light at 366 nm. Colonies with characteristics of *Bacillus* spp., *Pseudomonas* spp. were isolated individually and purified by streak plate method (Rangaswami, 1993) on Nutrient agar medium and King's B medium respectively. The pure cultures were maintained on respective agar slants at 4 °C.

Hydrogen cyanide (HCN) production Qualitative assay

HCN production of fungal and bacterial biocontrol agents was tested qualitatively following the method of Bakker and Schipper (1987). The antagonistic bacteria were streaked on King's B medium amended with glycine at 4.4g/ l. sterile filter paper saturated with picric acid solution (2.5 g of picric acid; 12.5 g of Na_2CO_3, 1000 ml of distilled water) was placed in the upper lid of the Petri plate. The dishes were sealed with Parafilm and incubated at 28°C for 48 h. A change of colour of the filter paper from yellow to light brown, brown or reddish-brown was recorded as weak (+), moderate (++) or strong (+++) reaction respectively.

Quantitative assay

Antagonistic bacteria were grown in King's B broth amended with glycine (4.4g/ l) and Uniform strips of filter paper (10 x 0.5 cm^2) were soaked in alkaline picrate solution and kept hanging inside the conical flask. After incubation at 28 ± 2^oC for 48 h the sodium picrate in the filter paper was reduced to a reddish compound in proportion to the amount of HCN evolved. The colour was eluted by placing the filter paper in a test tube containing 10 ml of distilled water and its absorbance was read at 625 nm (Sadasivam and Manickam, 1992).

Antibiotic production - bacterial antagonists

Extraction of crude antibiotic metabolites

The bacterial biocontrol agents *viz*., Bs_1, Bs_{10}, Pf_1 and Pf_2 grown for five days in pigment production broth and were centrifuged at 5000 rpm for 30 min. The supernatant was adjusted to pH 2.0 with concentrated HCl and extracted with equal volume of benzene. The benzene layer was evaporated in a water bath and the residue was resuspended in 0.1 N NaOH (Rosales *et al.,* 1995).

Effect of bacterial antibiotics on the growth of *M. phaseolina*

The effect of antibiotics extracted from bacterial antagonists was tested against the growth of *M. phaseolina* by filter paper disc assay (Lam and Ng, 2001). Three sterile filter paper discs were placed on solidified PDA in Petri dishes. The crude antibiotic extracted was pipetted on to the filter paper @150 µl/disc. A five-mm-mycelial disc of the fungus was placed at the centre of the plate and incubated at 28 ± 2^oC. Filter paper without antibiotic served as control. Surface area of inhibition was measured by tracing the area of inhibition in a trace paper, plotting it on a graph sheet and comparing with control.

Statistical analysis

The pot culture and laboratory experiments were conducted by following Completely Randomized Design (CRD). The field experiment was laid out in Randomized Block Design (RBD). The percentage values were transformed into "Arcsine" and "Square-root". The statistical analysis of the experiment was done by following the methods suggested by Gomez and Gomez (1984). Per cent values were transformed by arcsine or square root transformation.

Results and Discussion

Biochemical characterization of *P. fluorescens* isolates

The two effective isolates of *P. fluorescens* gave positive result to following test viz., KOH test, producing fluorescent pigment, growth at 4 °C, arginine dihydrolase and gelatin liquefaction. These isolates gave negative reaction to Gram's reaction, growth at 41 °C and levan formation. (Table 1).

Biochemical characteristics of *B. subtilis* isolates

All the two effective isolates of *B. subtilis* showed positive reaction to gram reaction,

Table.1 Characterization of *P. fluorescens*

S. No.	Diagnostic tests	Pf_1	CPf_5
1.	Gram reaction	-	-
2.	KOH test	+	+
3.	Pigment production in King's B medium	+	+
4.	Growth at 4°C	+	+
5.	Growth at 45°C	-	-
6.	Arginine dihydrolase	+	+
7.	Gelatin liquefaction	+	+
8.	Levan formation	-	-

Table.2 Characterization of *B. subtilis*

S. No.	Diagnostic tests	Bs10	CBs_4
1.	Gram reaction	+	+
2.	KOH test	-	-
3.	Growth at 45°C	+	+
4.	Growth in 7% NaCl	+	+
5.	Citrate utilization	+	+
6.	Anaerobic growth	-	-
7.	Starch hydrolysis	+	+
8.	Catalase test	+	+

Table.3 Production of HCN from bacterial antagonists

S. No.	Isolates	HCN	
		Qualitative	Quantitative (O.D. Value)
1.	Bs10	-	0.015
2.	CBs5	-	0.004
3.	Pf1	+++	0.094
4.	CPf5	+++	0.085

HCN production - negative, + weak, ++ moderate, +++ strong

growth in 45°C, growth in NaCl, citrate utilization, starch hydrolysis and catalase test these isolates gave negative reaction in KOH test and anaerobic growth (Table 2).

Production of HCN

Study on qualitative analysis of HCN indicated a strong production of HCN in Pf_1 and CPf_5 was estimated. Isolates of *B. subtilis* did not produce HCN (Table 3). In quantitative estimation, Pf_1 and CPf_5 recorded the maximum OD value of 0.094 and 0.085 respectively but Bs_{10} recorded least OD value (0.015). Role of HCN in disease suppression has been demonstrated by several scientists in various crops (Stutz *et al*., 1986; Voisard *et al*.,1989; efago *et al*.,1990). HCN is the common secondary metabolite produced by rhizosphere *Pseudomonas* (Schippers, 1988). Meena *et al.* (2001) compared the HCN production of several strains of *P. fluorescens* and their efficacy in controlling root rot of groundnut caused by *M. phaseolina. Pseudomonas* releasing HCN were reported in the rhizosphere of tobacco in soils suppressive to *T. bassicola*, causal agent of black root rot of tobacco (Ramette *et al*., 2006).

Effect of bacterial antibiotics on the growth of *M. phaseolina*

The antibiotics produced by all the four effective bacterial isolates were effective against *M. phaseolina* and recorded reduction in growth of the pathogen ranged between 61.13 to 69.62 per cent reduction over control (Fig.1). Antibiotics produced by Pf_1 were maximum percent reduction in the fungal growth (69.92%) followed by CPf_5 (65.59%). The *Bacillus* isolates Bs_{10} and CBs_4 were next only to pseudomonads in their efficacy against the pathogen with 63.36 per cent and 61.13 per cent reduction over control. All the isolates were on par in inhibition of mycelial growth. Bainton *et al*. (2002) reported that the naturally occurring fluorescent Pseudomonads produced the antibiotic, 2-4 DAPG. *Bacillus* spp. produced different inhibitory agents which have been categorized in peptide derivative family (Stein, 2005; *et al*., 2002). Bacilysocin, a novel and broad spectrum phospholipid antibiotic was purified from *B. subtilis* strain 168 (Tamehiro *et al.*, 2002).

In conclusion, Cyanogenic rhizobacteria might have the potential of biological control of *M. phaseolina*. The effect of each strain was different due to species and method. Growth inhibition was the most in *Pf1*.

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