A Novel Approach to Intrude Secondary Metabolites of *Pseudomonas fluorescens*

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Microbes are important catalysts to regulate the functional properties of terrestrial ecosystems. In this study, rhizosphere bacteria were isolated from soil and examined for their plant growth promoting properties. All the four isolates were tested for hydrogen cyanide (HCN), salicylic acid, and auxin and lipase enzyme production. Pf1 strains showed good reaction for the production of HCN. HCN production is indicative of antagonistic ability. The lipase production varied between *Pseudomonas fluorescens* strains. Pf4 gave the highest result. The amount of salicylic acid produced by Pf2 and pf4 was 0.63 and 0.64 respectively. Pf1 produced Indole Acetic Acid (IAA), which most probably accounted for the overall synergistic effect on the growth of plant.

Keywords: *Pseudomonas fluorescens,* Auxin, Lipase, Salicylic acid, Hydrogen cyanide

Introduction

Rhizobacteria or rhizosphere bacteria are those that colonize plant roots; some of them exert a beneficial effect on plants and are also termed as plant growth promoting rhizobacteria (PGPR) (Kloepper *et al.*, 1978). The PGPR help by altering the microbial balance in the rhizosphere (Kloepper and Schroth, 1981); hydrogen cyanide (HCN) (Wei *et al.*, 1991) is produced by PGPR and has been reported to be involved in the reduction of plant pathogens and non-beneficial rhizobacteria. HCN is also produced by certain non-beneficial rhizobacteria which may restrict plant growth, and some PGPR help by inhibiting such deleterious rhizobacteria (Schippers *et al.*, 1988). PGPR strains may induce systemic resistance in the bacterized host to plant pathogens (Wei *et al.,* 1991).

Fluorescent pseudomonads have drawn attention worldwide because of the production of secondary metabolites such as siderophore, antibiotics, volatile compounds—HCN, enzymes and phytohormones (Sullivan and Gara, 1992). These have been implicated in the reduction of plant pathogenic fungi and harmful rhizobacteria with simultaneously induced plant growth (Weller and Cook, 1983).

The synthesis of HCN induces ferric iron concentration, whereas conditions of low iron are inhibitory (Weibeck and Gerrits, 1998). HCN effectively blocks the cytochrome oxidase pathway and is highly toxic to all aerobic organisms at pico molar concentration. The production of HCN by certain fluorescent *pseudomonas* is believed to be involved in the suppression of root pathogens (Pal and Gardener, 2006).

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Salicylic acid (SA) produced by *P. fluorescens* helps to induce systemic acquired resistance (SAR) of the infected plants and it will result in the formation of pathogenesis related (PR) protein. This PR protein acts directly on lyses invading cells and reinforces cell wall boundaries to resist infections.

Lipase was capable of surviving heating at 70 °C for 30 min as a crude enzyme (Christen and Marshall, 1980). However, when they were heated at 40 °C for 30 min, there was a significant decrease of photolytic activity and a complete loss of lipolytic activity.

The production of phytohormones has been suggested to be one of the mechanisms by which PGPR stimulate plant growth. *P. fluorescens* produces physiologically active auxin that may have pronounced beneficial effects on plant growth and development.

Materials and Methods

Isolation of Bacteria

Soil samples were taken from the rhizosphere area of healthy groundnut, tomato and sunflower plants. These samples were serially diluted up to 10^{-5}. The serially diluted sample was plated and incubated at 37 °C. After incubation of 4 h, the plates were counted using colony counter. The isolates were further characterized by standard microbiological tests and maintained on nutrient agar slant at 4 °C for further studies.

Production of HCN

P. fluorescens was allowed to grow on nutrient agar plates supplemented with 4.4 g/L glycine. The plates were inverted and a strip of sterilized filter paper saturated with 0.5% picric acid in 2% sodium carbonate was placed in the upper lid. The plates were sealed and incubated for 72 h at 28 °C. After incubation, the plates were observed for change in the color of the filter paper strip, from yellow to orange to brown. One control plate was also maintained. The color of the filter paper was eluted by placing the filter paper in a clean test tube containing 10 mL distilled water and absorbance was measured at 625 nm. Three replicates were maintained for each isolate.

P. fluorescens was grown at room temperature (28±2 °C) for 48 h on a rotary shaker in 250 mL conical flask containing Kings B broth medium. Cells were then collected by centrifugation at 10,000 rpm for 10 min. One control was also maintained. After centrifugation, 4 mL of cell free culture filtrate was acidified with 1 N Hcl to pH 2.0 and salicylic acid was extracted in chloroform (2 × 2 mL). To the pooled chloroform extract, 4 mL of distilled water and 5 mL of 2 M $FeCl_3$ were added. The purple iron SA complex was extracted using micropipette and the absorbance was read at 527 nm. Three replicates were maintained for each isolate.

Modified protocol of Elad *et al.* (1982) was followed for the estimation of lipases secreted by the antagonists in culture. Crude enzyme (2 mL) was diluted with 8 mL of distilled water and mixed well with 500 µL of vegetable oil. This reaction mixture was incubated at 37 °C for 2 h in a rotary shaker (200 rpm). Ethanol was added to it to get a final concentration of 30%. Free fatty acids were extracted with 25 mL of pure petroleum ether and the extractant was evaporated in a rotary evaporator. The free fatty acids were dissolved in 15 mL of neutralized ethanol containing phenolphthalein at 60 °C. Each sample was titrated with ethyl alcohol containing 0.5 N NaoH. Free fatty acids were neutralized and one Lipolytic Unit (LU) was defined as micromoles of NaoH/mg protein/h.

The production of IAA was determined by following Bric *et al.* (1991). Selected bacterial strains were grown in glycerol-peptone broth with and without tryptophan (500 mg^{-1}) and incubated at 28 °C for three days. A 2 mL culture was taken from each tube and centrifuged at 10,000 rpm for 15 min. One milliliter of the supernatant fluid was taken into a clean dry tube, to which 100 mL of 10 mM orthophosphoric acid and 2 mL of reagent (1 mL of 0.5 M $FeCl_3$ in 50 mL of 35% $HClO_4$) were added. After 25 min, the absorbance of the pink color was measured spectrophotometrically at 530 nm. The IAA concentration in the culture was determined by using a calibration curve of pure IAA as a standard (Bano and Musarrat, 2003).

Results and Discussion

Isolation and Identification

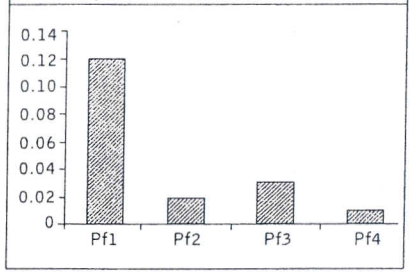
The presence of fluorescent color colonies on the medium was observed. Based on the following biochemical characteristics, it was confirmed as *Pseudomonas fluorescens.*

Production of HCN

Four isolates of Pf1, Pf2, Pf3 and Pf4 reacted strongly for HCN production. Though a correlation was established between HCN production and antagonism, isolates having moderate, weak or no HCN production activity showed good antifungal properties. This proves that HCN activity is not the sole property to look for an effective antagonist. Wei *et al.* (1991) have advocated analysis of mutants lacking in HCN production by strains of *P. fluorescens.*

The hydrogen cyanide producing bacteria were isolated using specific assay condition. Organisms which are cyanogenic under different conditions or produce low levels of cyanide may appear to be cyanide negative. Among the four isolates tested for the HCN production, the isolate, Pf1 (Figure 1) showed higher production of HCN.

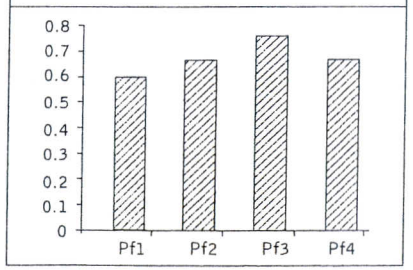
Figure 1: Production of HCN



Production of SA

Salicylic acid was produced by *P. fluorescens* by a method of Meyer *et al.* (1992). The amount of salicylic acid produced by Pf3 was found to be 0.72 µg/mL (Figure 2).

Figure 2: Production of Salicylic Acid (µg/mL)



Production of Auxin

Rhizosphere bacteria colonized in the root surface, which is relatively rich in organic substrates and synthesized phytohormones auxins, as secondary metabolites. They promote seed germination, root elongation and stimulation of leaf expansion. The highest concentration of IAA (2.0 µg) was produced by bacterial strains Pf1 and Pf4 (Figure 3).

Similarly, Leinhos and Vacek (1994) reported production of 1.6 to 3.3 mg $auxin^{-1}$ filtrate by rhizosphere bacteria isolated from wheat.

Production of Lipase

Lipase was synthesized by *P. fluorescens* in the studies conducted by Chet (1987). The Pf4 produced higher amount (4.7 LU) of lipase enzyme, when compared to Pf1 strains (Figure 4).

Figure 3: Production of Auxin (mg/mL)

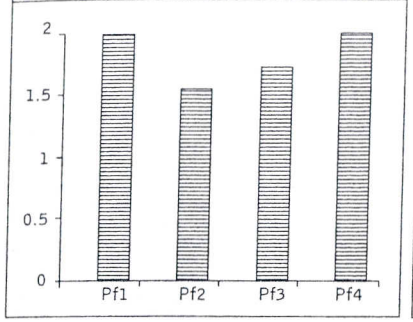
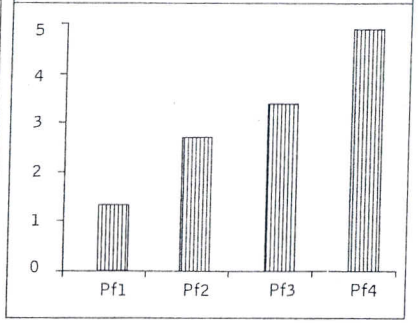


Figure 4: Production of Lipase (LU)



Cytoplasmic damage of pathogen by lytic enzymes were produced by antagonists (Jones *et al.*, 1974; and Paulitz *et al.*, 1998).

Conclusion

Fluorescent pseudomonads produce secondary metabolites, such as HCN, auxin, lipase and salicylic acid. These have been implicated in the reduction of plant pathogenic fungi and harmful rhizobacteria with simultaneously induced plant growth.

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