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## Isolation and functional characterization of plant growth promoting rhizobacteria against soft rot in *Aloe vera* (L)

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### Abstract

In the present investigation, 51 isolates of *Pseudomonas fluorescens* and 10 *Bacillus subtilis* isolates were isolated from the rhizosphere soil, collected from different *Aloe vera* growing areas of Southern Tamil Nadu and used for the screening of various functional characteristics against soft rot of *Aloe vera*. Among 51, five *Pseudomonas fluorescens* isolates can able to produce the siderophores. Isolate Pf 32 followed by Pf 45 produced the maximum quantity of salicylate type of siderophore. Isolates Pf 32 recorded maximum growth inhibition of *E. chrysanthemi* by production of volatile compounds. The maximum quantity of hydrogen cyanide (HCN) was recorded by isolates Pf 32 and Pf 45.

**Keywords:** *Aloe vera*, Siderophore, HCN, volatile compounds, *Pseudomonas fluorescens* and *Bacillus subtilis*

### 1. Introduction

*Aloe vera* (L) Burm. is one of the medicinal plants widely used throughout the world [1]. It is a well known medicinal plant of India and is one of the world most demanded crop. *Aloe* genus consists of about 325 species the inner gel of the leaf contains most of its beneficial part [2]. *Aloe vera* has many medicinal and cosmetic usages and hence has growing demand in the market. The plant is a rich source of amino acids and enzymes. The gel of the leaf of *Aloe vera* contains 96 per cent of water and the remaining different elements such as vitamins and minerals [3]. It is referred to as miracle plant for its numerous uses, particularly in the area of human's health. Diseases are the greatest enemies of *Aloe vera* accounting for huge losses varying from 25-75 per cent. Among these bacterial soft rot of *Aloe vera* caused by *Erwinia chrysanthemi* is economically important as it causes yield loss upto 80 per cent. Bacterial soft rot of *Aloe vera* is caused by pathogenic bacteria *E. chrysanthemi* [4]. Management of plant diseases through biological method envisages the use of antagonistic organisms like rhizobacteria, avirulent strains of the pathogen and bacterial metabolites. Among the various antagonists used for the management of plant diseases, plant growth promoting rhizobacteria (PGPR) play a vital role. The organisms that establish positive interactions with plant roots and show observable benefits on the plant growth either direct or indirect manner are collectively called as plant growth promoting rhizobacteria [5]. These bacteria may mediate biocontrol by one or more of the several mechanisms of disease suppression viz., siderophores production, hydrogen cyanide and antibiotics like phenazine 1 - carboxylate, pyoluteorein and 2, 4 diacetylphloroglucinol [5, 6],  $\beta$ -1, 3 glucanase and chitinase also increase the antifungal action of the rhizobacteria in addition to the production of antibiotics, siderophore, salicylic acid(SA) and hydrogen cyanide (HCN) [7].

Because of these reasons, biological control is a good alternative method, as compared to chemical control (pesticide) which destroys a range of micro and macro-organisms and has a limited impact on the environment [8]. Rhizobacteria such as *Pseudomonas fluorescens* and *Bacillus* strains could provide significant levels of disease suppression and substantially enhance plant growth and yield. Hence the present study was conducted to isolate and characterize the efficient rhizobacteria possessed multiple mechanism for controlling of *Aloe vera* soft rot disease.

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## 2. Materials and methods

### 2.1 Isolation of antagonistic bacterial isolates

Antagonistic bacteria *Bacillus* sp. and *Pseudomonas* sp. were isolated from the rhizosphere soil, collected from different *Aleo vera* growing areas of Southern Tamil Nadu by serial dilution method on Nutrient agar medium and King's B medium respectively by incubating at 30 °C for 24 hours. Colonies of *Bacillus* sp. and *Pseudomonas* sp. were isolated individually and purified by streaking them on their respective media.

### 2.2 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) / Fe<sup>3+</sup> / 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<sub>2</sub>HPO<sub>4</sub>- 3.0g, (NH<sub>4</sub>)<sub>2</sub> SO<sub>4</sub>. 7H<sub>2</sub>O- 0.2 g, Distilled water- 1 litre, pH- 7.0) amended with indicator dye. One liter of blue agar, 60.5 mg of chrome azurolo S (CAS) was dissolved in 50 ml of distilled water and mixed with 10 ml of Iron (III) solution (1mM FeCl<sub>3</sub>. 6H<sub>2</sub>O in 10mM HCl). While constantly stirring, this solution was slowly added to 72.9 mg of hexadecyl 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 [9]. 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.

### 2.3 Detection of the nature of siderophore

The bacterial isolates such as *Pseudomonas* sp. and *B. subtilis* were inoculated in 10 ml of King's B broth and nutrient broth, respectively. It was incubated in a rotary shaker at 120 rpm for 48 h. The bacteria multiplied in the broths, were used as the sample for the determination of the nature of siderophore.

#### 2.3.1 Hydroxamate 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 hydroxamate type of siderophore production [10].

#### 2.3.2 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 [11].

### 2.4 Estimation of Siderophore produced by antagonistic bacteria

Siderophore production by the rhizobacterial isolates was estimated by the method described by Reeves *et al.*, [12]. King's B and nutrient broths were prepared for *Pseudomonas* and *Bacillus* isolates and dispensed in 100 ml quantities in 250 ml conical flask. After sterilization, one ml of standard inoculum of bacterial strains was inoculated into each flask and incubated at room temperature 28±2 °C for seven days. Seven days after incubation, the broth culture was centrifuged at 10,000 rpm for 20 minutes and the supernatant was used for the estimation of catechol type and salicylate type of

siderophore.

The pH of the supernatant was adjusted to 2.0 with 1N HCl. To 20 ml of supernatant, equal volume of ethyl acetate was added in a separating funnel, mixed well and ethyl acetate fraction was collected. This process was repeated three times to bring the entire quantity of siderophore from the supernatant. The ethyl acetate fractions were pooled, air-dried and dissolved in 5.0 ml of 50 per cent ethanol. Five ml of ethyl acetate fraction was reacted with five ml of Hathway reagent (1ml of 0.1M FeCl<sub>3</sub>, 1ml of 0.1N HCl in 100 ml distilled water + 1ml of 0.1M potassium ferricyanide). The absorbance was read at 560 nm. A standard curve was prepared using sodium salicylate for the estimation of salicylate type siderophore. The quantity of siderophore synthesized was expressed as µg/ml of culture filtrate.

To measure catechol type of siderophore, 5.0 ml of ethyl acetate fraction was reacted with 5.0 ml of Hathway reagent and absorbance was determined at 700 nm with 2, 3 dihydroxybenzoic acid as standard. The quantity of siderophore synthesized was expressed as µg/ml of culture filtrate.

### 2.5 Testing of bacterial antagonists for the production of volatile antibiotics

The isolates of *Pseudomonas sp* and *B. subtilis* were streaked onto KB medium and NA respectively. The pathogen was streaked on KB and NA on another set of plates. The NA plates inoculated with pathogen were inverted over the plates having the antagonists and both the plates were sealed together with parafilm and incubated at 28 ± 2 °C for 72 hours. NA and KB medium inoculated and plates with the pathogen inverted over the plates without the antagonist served as control. Three replications were maintained for each treatment. The inhibitory effect of volatiles produced by biocontrol agents was graded based on the inhibition of linear growth of the pathogen as compared to control [13].

### 2.6 Testing of antagonistic bacteria for the production of hydrogen cyanide

#### 2.6.1 Qualitative assay

HCN production was determined by using the modified protocol of Miller and Higgins [14]. Bacteria were grown on Tryptic soy 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 1000ml of distilled water) were placed in the upper lid of each Petri plate. Dishes were sealed with parafilm and incubated at 28 °C for 48 hours. 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.

#### 2.6.2 Quantitative assay

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 hours, 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 10 ml of distilled water and the absorbance was measured at 625 nm [15].

### 3. Results and Discussion

#### 3.1 Isolation of antagonistic bacterial isolates from rhizosphere soil

Fifty one isolates of *Pseudomonas fluorescens* and ten isolates *Bacillus subtilis* were isolated from rhizosphere soil, collected from different *Aloe vera* growing areas of Southern Tamil Nadu through serial dilution and named as Pf 1 to Pf 51 and Bs 1 to Bs 10 respectively.

#### 3.2 Siderophore production by effective bacterial antagonists isolates

Out of fifty one, five *Pseudomonas* isolates and among ten *B.*

*subtilis* isolates one produced siderophore in chromeazuroil S (CAS) plate assay method. All the five isolates of *P. fluorescens* produced yellow, greenish yellow-to-yellow fluorescent pigmentation in blue colored medium (Table 1). Kloepper *et al.*,<sup>[16]</sup> documented the production of fluorescent siderophores by *P. fluorescens*, which attributed to its antagonistic action. Cronin *et al.*,<sup>[17]</sup> demonstrated that *Pseudomonas fluorescens* F113G22 strain inhibited the growth of *Erwinia carotovora* var. *atroseptica* that favors siderophore production and contribute to biological control of *Erwinia carotovora* var. *atroseptica*. Kavitha<sup>[18]</sup> reported that production of siderophores by *B. subtilis* isolate CBE-4.

**Table 1:** Siderophore production by effective bacterial antagonists isolates

Isolates	Colour of siderophore pigment	Area of production (mm <sup>2</sup> )**	Nature of siderophore	
			Hydroxamate	Carboxylate
Pf 4	Yellow	53 <sup>b</sup>	+	-
Pf 16	Yellow	37 <sup>b</sup>	++	-
Pf 26	Yellow	74 <sup>a</sup>	+	-
Pf 32	Yellow	85 <sup>a</sup>	+++	-
Pf 45	Yellow	80 <sup>a</sup>	+++	*
Bs 5	Yellow	40 <sup>c</sup>	-	-

\* Mean of three replications

In a column, means followed by common letters are not significantly different at 5% level by DMRT

+ light red colour, +++ moderate deep red colour

- Appearance of pink colour,\* Disappearance of pink colour

#### 3.3 Detection of the nature of siderophore

The isolates Pf 32 and Pf 45 produced deep red colour instantly by the addition of tetrazolium salt to the sample where as the isolate Pf 26 and Pf 16 produced red colour slowly, indicating that the nature of the siderophore is hydroxamate type (Table 1). Carboxymate type of siderophore was determined by the disappearance of pink colour on the addition of phenolphthalein to the sample. The isolate pf 4, Pf 16, Pf 26, Pf 32 and Pf 45 were also produced carboxylate type of siderophore (Table 1). Leeman *et al.*,<sup>[19]</sup> documented that siderophores could induce systemic resistance against *Fusarium* wilt of radish. The hydroxamate type of siderophore was ferribactin produced by *P. fluorescens*<sup>[20]</sup>.

#### 3.4 Quantification of siderophore produced by effective bacterial antagonists

The isolate Pf 32 produced the maximum quantity of salicylate type of siderophore (136.27 µg/ml) followed by Pf 45 (93.39 µg/ml) and the minimum quantity of salicylate type of siderophore was produced by Bs 5 (61.58 µg/ml). The isolate Pf 45 recorded the maximum of catechol type of siderophore (24.19 µg/ml) followed by isolate Pf 26 (20.02 µg/ml). The other isolates Pf 4 (19.47 µg/ml), Pf 16 (17.41) were on par and the minimum (6.79 µg/ml) of catechol type of siderophore was recorded in isolate Bs<sub>5</sub> (Table 2).

**Table 2:** Quantification of siderophore produced by effective bacterial antagonists

Antagonistic Bacteria	Siderophore	
	Salicylate type (µg/ml)*	Catechol type (µg/ml)
Pf 4	64.64 <sup>c</sup>	19.47 <sup>c</sup>
Pf 16	84.16 <sup>d</sup>	17.41 <sup>c</sup>
Pf 26	89.41 <sup>c</sup>	20.02 <sup>b</sup>
Pf 32	136.27 <sup>a</sup>	17.41 <sup>d</sup>
Pf 45	93.39 <sup>b</sup>	24.19 <sup>a</sup>
Bs 5	63.15 <sup>f</sup>	6.79 <sup>f</sup>

\* Mean of three replications

In a column, means followed by common letters are not significantly different at 5% level by DMRT

#### 3.5 Efficacy of volatile compounds of effective bacterial antagonists against the growth of *E. chrysanthemi*

The experiment conducted on the effect of volatile compounds of five isolates of *P. fluorescens* against the growth of *E. chrysanthemi* *in vitro* indicated that the isolates Pf 32 and Pf 45 inhibited the maximum growth of *E. chrysanthemi* followed by Pf 4 where as Pf 16, Pf 26 and Bs<sub>5</sub> isolates failed to produce volatile compounds and the growth of *E. chrysanthemi* was not inhibited even 10 days after incubation (Table 3). Sharifi-Tehrani and Omati<sup>[21]</sup> reported that *P. fluorescens* and *B. subtilis* produced both volatile metabolites and non-volatile metabolites that caused the maximum inhibition of *Phytophthora capsici*. Sadfi *et al.*,<sup>[22]</sup> found that volatiles liberated by *Bacillus* sp. isolates inhibited *F. roseum* var. *sambveinum* causing dry rot of potato.

**Table 3:** Efficacy of volatile compounds of effective bacterial antagonists against the growth of *E. chrysanthemi*

Antagonistic Bacteria	Production of volatile compound	Inhibition level
Pf 4	+	++
Pf 16	-	-
Pf 26	-	-
Pf 32	+	+++
Pf 45	+	++
Bs 5	-	-
Control	-	-

+ Positive, - Negative, ++ high inhibition, +++ higher inhibition

#### 3.6 Hydrogen cyanide production by effective bacterial antagonists

Among the five isolates tested for HCN production, the isolate Pf 32 was recorded strong produce of HCN with OD value of 0.097 followed by isolate Pf 45 with OD value of 0.073. The other isolates Pf 4(OD value 0.064) Pf 16(OD

value 0.057), and the minimum HCN production was recorded by the isolate Pf 26(OD value 0.041) while Bs isolate failed to produce any HCN (Table 4).

**Table 4:** Hydrogen cyanide production by effective bacterial antagonists

Antagonistic Bacteria	Qualitative assay	Quantitative assay (O.D value)*
Pf 4	+	0.064 <sup>b</sup>
Pf 16	+	0.057 <sup>bc</sup>
Pf 26	+++	0.041 <sup>c</sup>
Pf 32	+++	0.097 <sup>a</sup>
Pf 45	++	0.073 <sup>b</sup>
Bs 5	-	-
Control	-	0.00

+ Produced HCN; - No HCN

\* Mean of three replications

In a column, means followed by common letters are not significantly different at 5% level by DMRT

Mondal *et al.*,<sup>[23]</sup> found that *P. fluorescens* (Rb-26) produced the maximum HCN production, which in turn, showed stronger growth inhibition of

*X. axonopodis* pv. *malvacearum*. Role of HCN in disease suppression was

demonstrated by several workers in various crops<sup>[7, 24]</sup> using different antagonistic bacteria. Rajkumar<sup>[7]</sup> (2006) detected the production of cyanic acid by *P. fluorescens* in banana, inhibitory to the growth of *E. carotovora* var. *carotovora*.

#### 4. Conclusion

*Pseudomonas fluorescens* isolates Pf 32 produced the maximum quantity of salicylate type of siderophore, recorded maximum growth inhibition of *E. chrysanthemi* by production of volatile compounds and maximum quantity of hydrogen cyanide (HCN). Hence this isolates can be analyzed for the other growth promoting characters like Phosphate solubilizing ability, N fixing ability, SA, IAA and  $\beta$  1, 3 glucanase production ability etc., Then this Pf 32 can be used as a bioinoculant for the better plant growth and increasing the yield with the minimum use of chemical fertilizers and pesticides in agriculture.

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