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Isolation and identification of native strains of *Pseudomonas fluorescens* and test their *in vitro* bio-efficacy against bacterial wilt of potato caused by *Ralstonia solanacearum*

UR Phondekar, RG Bhagwat, RR Rathod and SB Thorat

Abstract
In the present investigation, five native strains of *Pseudomonas fluorescens* were isolated from rhizospheric soil samples of healthy growing Potato field. Total five isolates were identified on the basis of different morpho-biochemical test and all isolates were found positive response for siderophore production, gelatine liquefaction, denitrification, starch hydrolysis and catalase test while negative response for gram staining test. To check their bio-efficacy, all five isolates of *P. fluorescens* were evaluated against bacterial wilt of potato which is caused by *R. solanacearum.* Results revealed that, all isolates exhibited antibacterial activity with inhibition zone that ranged between 7.75 to 12.75 mm.

Keywords: *Pseudomonas fluorescens*, bacterial wilt, *Ralstonia solanacearum*

Introduction

The Potato (*Solanum tuberosum* L.) is the important starchy food crop of the world. It is an excellent source of nutrients, which provides high energy and quality proteins as well as significant amount of starch, vitamins and minerals. Bacterial wilt or brown rot of Potato is caused by the pathogenic bacterium *Ralstonia solanacearum* (Smith) Yabuuchi. The bacterial wilt of potato was reported in India from Pune district of Maharashtra (Cappel, 1892) [5]. In potato, the bacterium is transmitted primarily through seed tubers. Under field conditions, Plant infection occurs through root system, especially through wounds created due to cultural practices and some plant root injury by root knot nematodes. Though, various chemicals were reported to control bacterial wilt (*R. solanacearum*) of potato and other crop hosts, but due to higher cost of chemicals, resistance development in pathogens, environmental pollution and detrimental effects on non-target beneficial microorganisms etc., use of native antagonists seems to be most promising to some extent and have reduced the adverse effects of *R. Solanacearum* (Anith and Momol, 2004; Basha *et al*., 2017) [1, 3].

Materials and Methods

Isolation of *Pseudomonas fluorescens*

The 10 cm depth rhizhospheric soil particles loosely adhering to the roots of healthy potato plants were gently teased out and collected in polythene bag. The soil thus obtained was mixed and shaken with 100 ml of sterile distilled water for 10-20 min. to obtain standard soil suspension. Isolation of *P. fluorescens* was made by following the serial dilutions and pour plate method using the specific King's B medium. One ml of soil suspension from aliquot dilutions (10^{-6}) were aseptically added to sterile petri plates containing 20 ml of sterile King's B medium and plates were incubated at 28±2 °C for 48 hrs (Meera and Balabaskar, 2012) [14]. Then, the plates were observed for bacterial colonies. Well separated individual colonies with yellowish green fluorescent pigmentation observed under UV trans illuminator were marked and picked up with sterile inoculation loop and streaked aseptically on King's B medium in sterilized Petri plates and plates were incubated at 28±2 °C for 48 hours. Pure culture of all isolates were maintained in King's B broth.

Identification of *P. fluorescens*

P. fluorescens isolates were identified based on morpho-cultural characteristics (colony morphology, cell morphology (Gram reaction) and pigmentation) and biochemical test *viz.*,

Gram staining, siderophore production, catalase test, starch hydrolysis, gelatin liquefication and denitrification test were carried out as per the methods described in the Manual of Microbiological methods, 1957.

Gram staining
Gram staining test was performed as per standard laboratory method.

Siderophore production test
Sterilized petriplates were poured with autoclaved and cooled King's B agar medium and allowed to solidify. After solidifaction, plates were inoculated with the isolates of Pseudomonas fluorescens and incubated at 28±2 °C for 48 hrs. After incubation, plates were observed under UV transilluminator for fluorescent pigmentation.

Catalase test
A drop of 3% hydrogen peroxide was added into 48 hrs old bacterial culture on a clean glass slide and mixed with the help of bacterial inoculation needle. The formation of bubbles indicates a positive reaction.

Starch hydrolysis
A bacterial culture of 48 hrs old was streaked on plates containing starch agar medium and plates were incubated for two days at 28±2 °C. After incubation, the inoculated plates were flooded with lugol's iodine solution.

Gelatin liquefication
The bacterial cultures (48 hrs old) were inoculated into sterilized nutrient gelatin tubes and tubes were incubated for 48 hrs at 28±2 °C. After incubation, the inoculated tubes were kept in refrigerator for 20- 30 min at 4 °C.

Denitrification test
Sterilized test tubes were poured with sterilized nitrate broth. Durham's tube was inserted in test tubes in an inverted position. Bacterial cultures (48 hrs old) were inoculated with the help of bacterial inoculation needle and incubated at 28±2 °C for seven days.

In vitro* evaluation of *P. fluorescens* against *R. solanacearum
In vitro evaluation of native isolates of *P. fluorescens* as a potential bio control agent against *R. solanacearum* was assessed by following the Filter paper disc plate method. The virulent isolate of *R. solanacerum* was multiplied on Nutrient broth. The 48 hours old culture of *R. solanacerum* was mixed with molten (40 °C) King's B medium, so as to get a thick lawn of bacteria on the surface of King's B medium. The seeded medium was then poured into sterilized Petriplates and allowed to solidify. Sterilized filter paper discs (Whatman No. 42) measuring 5 mm in diameter were soaked in antagonist isolates (*P. fluorescens*) broth for 10 minutes and placed in the petriplates. The inoculated plates were incubated at 28±2 °C for 48 hours. Filter paper disc dipped in sterile water served as control. The observation for the production of inhibition zone around the filter paper discs was recorded at 48 hours of incubation respectively.

Results and Discussion

Isolation of *Pseudomonas fluorescens*
Total five isolates of *P. fluorescens viz*., Pf1, Pf2, Pf3, Pf4 and Pf5 were isolated and all isolates produced small to medium, mucoid smooth, yellowish white glistening colonies on King's B agar plates. Similar findings on isolation of antagonist bacteria (*P. fluorescens*) from rhizospheric soil samples were reported by several workers (Belkar and Gade, 2012; Suman *et al*., 2016 and Manasa *et al*., 2017) [4, 12, 7].

Identification of *Pseudomonas fluorescens*
All five isolates of *P. fluorescens* were identified, based on their morphological and biochemical characteristics. All isolates developed typical colonies on Kings' B medium after 48 hours of incubation as, small to medium, mucoid smooth, yellowish white glistening colonies. All isolates were gram negative and rod shaped and exhibited weak to medium yellowish green pigmentation when grown on King's B medium and observed under UV transilluminator. Similar findings were reported by several workers (Kumari and Khanna, 2016; Suman *et al*., 2016; Avati, 2016 and Nepali *et al*. 2018) [6, 12, 2, 8].
Different biochemical tests were carried out in respect of *P. fluorescens* and all isolates *viz*., Pf1, Pf2, Pf3, Pf4, Pf5 showed gram negative reaction in gram staining. The bacterium was rod shaped and produced yellowish green pigmentation on King's B medium under UV transilluminator. Similar finding was reported by Ramyasmruthi *et al*. 2012; Avati, 2016 and Nepali *et al*. 2018) [15, 2, 8].
All isolates *viz*., Pf1, Pf2, Pf3, Pf4, Pf5 were showed positive reaction for siderophore production, similar findings was reported by Avati, 2016 [2].
All the five isolates *viz*., Pf1, Pf2, Pf3, Pf4, Pf5 showed positive reaction for gelatin liquefication, starch hydrolysis and denitrification test, similar findings were reported by several workers (Kumari and Khanna 2016; Priyanka *et al*. 2017; Shivangi *et al*. 2017 and Nepali *et al*. 2018) [6, 9, 11, 8].

Table 1: Morpho-biochemical Characterization of *P. fluorescens*

Sr. No.	Reactions of isolates (*P. fluorescens*)					
	Test/ Characters	**Pf1**	**Pf2**	**Pf3**	**Pf4**	**Pf5**
1	Gram staining	-ve	-ve	-ve	-ve	-ve
2	Cell shape	Rod	Rod	Rod	Rod	Rod
3	Colony shape	Round	Round	Round	Round	Round
4	Colony colour	Yellowish white	Yellowish white	Yellowish white	Yellowish white	Yellowish white
5	Surface	Mucoid, smooth	Mucoid	Smooth	Mucoid	Smooth
6	fluorescent Pigmentation	Yellowish green (++)	Yellowish green (++)	Yellowish green (+)	Yellowish green (++)	Yellowish green (+)
7	Siderophore production	+ve	+ve	+ve	+ve	+ve
8	Starch hydrolysis	+ve	+ve	+ve	+ve	+ve
9	Gelatin liquefication	+ve	+ve	+ve	+ve	+ve
10	Denitrification	+ve	+ve	+ve	+ve	+ve

+ve = Positive test, + weak fluorescent pigmentation, ++ medium fluorescent pigmentation

In vitro* evaluation of native isolates of *P. fluorescens* against *R. solanacearum

All five isolates of *P. fluorescens viz*., Pf1, Pf2, Pf3, Pf4, Pf5 were evaluated *in vitro* to check their bio-efficacy against *R. solanacearum* by filter paper disc plate method as explained earlier.

Table 2: *In vitro* evaluation of native isolates of *P. fluorescens* against *R. solanacearum*

Tr. No.	Treatments	Inhibition zone (mm)*
T_1	Pf1	7.75 (16.16)
T_2	Pf2	12.75 (20.92)
T_3	Pf3	11.75 (20.4)
T_4	Pf4	9.0 (17.45)
T_5	Pf5	9.5 (17.95)
T_6	Control	0.0 (0.00)
S.Em±		0.629
C. D at 1%		2.55

*Means of Four replications, Figures in parentheses are arc-sin transformed values

Results revealed that, all five isolates of *P. fluorescens* evaluated against *R. solanacearum* exhibited antibacterial activity with inhibition zone that ranged between 7.75 to 12.75 mm. However, significantly highest inhibition zone was recorded by isolate Pf2 (12.75 mm) followed by isolate Pf3 (11.75 mm), isolate Pf5 (9.5 mm), isolate Pf4 (9.0 mm) while the least was recorded by Pf1 isolate (7.75 mm).

The results obtained in present study were parallel with the findings of several earlier workers (Rado *et al*., 2015; Basha *et al*., 2017 and Yendyo *et al*., 2018) [10, 3, 13].

Conclusion

From the results of present study, all five isolates of *P. fluorescens* showed positive reaction for siderophore production, gelatine liquefication, dentitrification, starch hydrolysis and catalase test while negative response for gram staining test and showed maximum inhibition zone against *R. solanacearum* was recorded by isolate Pf2 (12.75 mm).

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