



Research Article

Biochemical characterization of native fluorescent pseudomonads and its suitable carrier material for mass multiplication in Kuttanad ecosystem

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ABSTRACT: Bacterial antagonist fluorescent pseudomonads for sheath blight disease were isolated from different locations in Kuttanad region. Three effective strains viz., PF 43, PF 46 and PF 47 were tested along with standard culture P 1 against sheath blight disease of rice under glass house condition. The confirmation tests viz., physiological and biochemical characterization of the efficient isolates were carried out at Rice Research Station, Kerala Agricultural University, Moncompu. Various physiological tests on growth at different pH, Iron toxicity and Aluminium toxicity level showed that the isolate PF 43 grew at pH ranging from 1.0 to 14 and tolerated upto 1000 ppm of iron toxicity and 90 ppm of aluminium toxicity level. The biochemical tests indicated that three efficient isolates were confirmed as gram negative, rod shaped, fluorescent in King's B medium and positive response for growth at 4°C, Levan formation, Gelatin liquefaction and Catalase tests. However, there was a negative response for growth at 41°C, Methyl red, VogesProskaur and Indole tests. Thus, based on morphological and biochemical characteristics, the isolated strains were identified to be *Pseudomonas fluorescens*. *P. fluorescens* PF 43 product survived upto 150 days with required population of 1.01 to 1.63×10^8 cfu per g in talc, dolomite and gypsum based formulations. At 240 days of storage, 1×10^7 cfu were detected in talc, dolomite and gypsum based formulations. The cheap and easily available carrier material, gypsum and dolomite can be used for mass production of native *P. fluorescens* and is recommended to 66,000 ha rice growing tracts of Lower, Upper and Karilands of Kuttanad regions like Alleppey, Kottayam and Pathanamthitta District for ecofriendly management of diseases.

KEY WORDS: Dolomite, gypsum, *Pseudomonas fluorescens*, rice, sheath blight

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INTRODUCTION

The sheath blight of rice caused by *Rhizoctonia solani* Kuhn. was first noticed in Kuttanad in 1969. It is now rated as one of the most serious diseases of rice in the tract. The locally accepted variety, Uma occupies a vast area of Kuttanad since fifteen years, but it is highly susceptible to sheath blight disease. The farmers periodically apply many fungicides to control the disease leading to environmental pollution. Hence, for ecofriendly management biocontrol agents have gained considerable importance for the control of sheath blight disease in recent years. Many fluorescent pseudomonads have been reported to induce systemic resistance against sheath blight disease. For effective biocontrol of soil borne plant pathogens, the biotic agent should possess high level of rhizosphere competence, fungicide resistance and should grow under wide range of pH, tem-

perature, iron and aluminium toxicity. A successful biocontrol agent should possess many desirable characters. All the characters may not be present in a single strain. The objective of the present study was to isolate efficient native fluorescent pseudomonads, characterization of isolates and identify suitable carrier material for increasing the shelf life of the bioformulation.

MATERIALS AND METHODS

Comparative efficacy of screened fluorescent pseudomonads isolates on control of sheath blight incidence under green house condition

Talc based formulation of the best three effective isolates (PF 43, PF 46, PF 47) obtained from *in vitro* screening test against fungal pathogen (*R. solani*) and the standard culture P1, of fluorescent *Pseudomonas* sp., was prepared

by following the method of Vidhyasekaran and Muthamilan (1995) and tested for the control of sheath blight incidence under green house condition.

Rice seeds (cv. MO 16) were treated with talc based formulation of each fluorescent *Pseudomonas* isolate at the rate of 10g per kg of seed. The treated seeds were then sown at the rate of eight seeds in each pot containing 10 kg of clayey loam soil which was well mixed with the inoculum of (*R. solani*). At 35 days after sowing, the talc based formulation of strains PF43, PF46, PF47 and P 1 (Std) were applied to soil @ 2.5 kg after mixing with sand (50kg ha⁻¹), for easy handling and distribution. One per cent suspension of the talc based formulation of the respective bacterial isolates were prepared, allowed to settle for one hour, filtered through muslin cloth and filtrate was sprayed on the plants at 55 days after sowing. Five replications were maintained for each *P. fluorescens* isolate and the pots were arranged in randomized complete block design. Sclerotia of *R. solani* grown on PDA were collected after 10 days. Sclerotia were placed in between stem and basal leaf sheath of each tiller at the rate of one sclerotium per tiller at 65 days after sowing.

Disease intensity was scored where the highest relative lesion height (HRLH) was equal to the highest lesion height/the highest plant height X 100. This index is based on the highest point reached by a sheath blight lesion relative to the highest point of the plant. The disease intensity was scored for each tiller in the ten plants at 90 days after sowing. The data were analysed by Duncan's Multiple Range Test (DMRT).

Biochemical characterization

Various phenotypic and biochemical methods have been developed and used for characterizing fluorescent pseudomonads isolates. Rapid identification of potentially and economically viable bioagents is possible through various methods of biochemical characterization (Weller *et al.*, 2002).

i) The gram's negative bacteria identification study was conducted as per the standard procedure of Gram's staining method.

ii) Growth at 4°C and 41°C

Each isolate was inoculated on King's B (KB) slants and incubated at 4°C and 41°C for 48 hours and observed for growth.

iii) Catalase test (Dubey and Maheswari, 2002)

To 1 ml of the bacterial culture, 0.5ml of 3% hydrogen

peroxide was added. Immediate liberation of air bubbles indicates that the organism is catalase positive.

iv) Gelatin liquefaction

20 ml of a sterile medium containing Nutrient Agar was poured into sterile petriplates and allowed to solidify. Then the isolate was inoculated as a single streak. The plates were then incubated in an inverted position at 37°C for 24 hours. Then the plates were flooded with mercuric chloride solution and the zone of hydrolysis observed.

v) Indole test (Dubey and Maheswari, 2002)

Each isolate was inoculated into peptone broth (Potassium dihydrogen phosphate 3.56 g; Di-sodium hydrogen phosphate dihydrate 7.23g; Sodium chloride 4.3g; Peptone from pepsin-digested meat 1.0g and .pH: 7.0 ± 0.2) and incubated at 37°C for 48 hours. Then 1 ml of Kovac's reagent was added to the tubes and shaken gently with intervals of about 10-15 minutes. The tubes were allowed to stand to permit the reagent to rise to the top and then observed for the production of cherry red layer.

vi) Levan formation (Dubey and Maheswari, 2002)

Sterile medium containing peptone - 0.5 g, beef extract - 0.3 g, NaCl - 0.5 g, agar - 3.0 g, sucrose - 5.0 g and distilled water - 1000 ml was prepared and 20ml of it was poured into sterile petriplates and then each isolate was inoculated by quadrant streak. The plates were then incubated at 37°C for 24 hours and observed for white mucoid colony formation

vii) Methyl-Red test (Dubey and Maheswari, 2002)

Sterile Methyl Red-VogesProskauer (MR-VP) broth (Peptone from meat 7.0g; D (+) glucose 5.0g; phosphate buffer 5.0 ml) was inoculated with the bacterial culture and incubated at 37°C for 24 hours. Methyl red indicator was added to each isolate. Change in the colour of the broth from yellow to red indicates positive result.

viii) VogesProskauer test (Dubey and Maheswari, 2002)

Sterile Methyl Red-VogesProskauer (MR-VP) broth was inoculated with each isolates and then incubated at 37°C for 24 hours. 40 percent KOH solution and Barritt's reagent were added after incubation period. The tubes were shaken gently for 30 seconds and exposed to oxygen. Change in the color of the broth from yellow to pink indicates positive result.

ix) Starch hydrolysis test

Sterile starch agar (Agar -12.0g; Soluble starch -10.0 g; Beef extract - 3g; sterile water - 1000 ml; pH 7.0 ± 0.2) plates were prepared and the bacterial culture was plated

onto the plates. The plates were incubated at 37 °C for 48 hrs and flooded with iodine solution. A clear zone around the organism indicates positive result. Dark blue coloration of medium with no clear zone formation indicates negative result.

x) Selection for acidic and alkaline pH

Pure culture of PF 43, PF 46 and PF 47 isolates was streaked in triplicates on KB (King *et al.*, 1954) medium with the pH adjusted to 1.0 to 14.0. The plates were incubated at 28 ± 2°C for 2 days.

Shelf life of fluorescent pseudomonads in different formulations

Fluorescent *Pseudomonas* isolates were grown separately on liquid KMB for 48 h, as shake culture, by incubating in shaker at 150 rpm at room temperature (25 ± 2°C). Survival of each isolate was tested in five different carriers talc, vermiculite, gypsum, dolomite and lignite. Ten grams of carboxy methyl cellulose was added to 1 kg of the carrier and mixed well. The pH of all materials was adjusted to 7.0 by adding calcium carbonate. The carriers were autoclaved for 30 min on each of two consecutive days. Four hundred millilitres of the bacterial suspension containing 9 x 10⁸ colony-forming units (cfu) per ml was added to 1 kg of the carrier and mixed well under sterile conditions. The materials were packed in polythene bags, sealed, and incubated at room temperature (25 ± 2°C). Samples were drawn at intervals and the bacterial suspension was assessed using KMB by dilution plate method. Three independent samples were analyzed with three replications for each analysis. The experiment was set up as a randomized complete block design (CRD), 2 x 2 factorial replicated three times. Duncan's multiple range test (DMRT) was followed for the column factor and least significant difference (LSD) test for the row factor analysis. The package used for analysis was IRRI STAT version 92-1 developed by International Rice Research Institute Biometrics Unit, Philippines.

RESULTS AND DISCUSSION

Efficacy of seed + soil + foliar application of talc based formulation of *P. fluorescens* isolates at the rate of 10g/kg of seed + 2.5kg/ha of soil + 1% (10g/lit of water) effectively reduced the sheath blight incidence in rice under greenhouse condition. The maximum reduction of disease incidence was observed in PF 43 (30.0%) and PF 46 (37.0%). The other *P. fluorescens* isolates PF 47 and P 1 were also effective and recorded 40.0% and 45.0% of disease incidence, respectively. The inoculated control recorded maximum disease incidence of 88.0% (Table 1, Fig. 1 and 2).

Table 1. Efficacy of talc based formulation of *Pseudomonas fluorescens* isolates against sheath blight in rice under greenhouse condition

S. No.	Treatment	Sheath blight incidence* (%)
1.	PF 43	30.0 ^a
2.	PF 46	37.0 ^b
3.	PF 47	40.0 ^c
4.	P 1	45.0 ^d
5.	Control (Inoculated)	88.0 ^e

*Mean of three replications

In a column, means followed by a common letter(s) are not significantly different (P=0.05) by Duncan's Multiple Range Test.



Fig. 1. Efficacy of talc based formulation against sheath blight of rice.

In the green house study, seed, soil and foliar treatment with talc based formulation of *P. fluorescens* isolate PF 43 was effective in reducing the intensity of sheath blight disease in rice. Seed bacterization of *P. fluorescens* has been reported to control sheath rot, sheath blight, bacterial blight and blast diseases in rice (Mew and Rosales, 1986; Sivamani *et al.*, 1987; Lee *et al.*, 1990). Kloepper *et al.*, (1980) reported that Rhizobacteria exert beneficial effects on plant development are referred to as plant growth-promoting rhizobacteria (PGPR) because their application is often associated with increased rates of plant growth and stolon length of potato crop. Suslow and Schroth (1982) found that the earliest indication of PGPR-enhanced growth of sugar beet was a significant increase in seedling weight. Vidhyasekaran and Muthamilan (1995) reported that use of talc based formulation of *P. fluorescens* in the control of foliar spread pathogens of rice. The present study likewise proved the effective nature of seed, soil and foliar spray of talc based formulation of *P. fluorescens* against sheath blight pathogen when applied in prophylactic manner at maximum tillering stage of rice crop.

The results showed that all the three isolates were found to be gram negative, rod shaped, fluoresced in King's

B medium and showed a positive response for growth at 4°C, Levan formation, Gelatin liquefaction and Catalase tests. However, there was a negative response for growth at 41°C, Methyl red, Voges Proskauer and Indole tests. Thus, based on morphological and biochemical characteristics, the isolated strains were identified to be *Pseudomonas fluorescens*.

All the isolates showed positive reaction in the biochemical tests for the identification *Pseudomonas fluorescens*. Ramamoorthy *et al.*, (2002) characterized 18 isolates of *Pseudomonas fluorescens* based on biochemical characterization. Similar findings have been reported by Reddy *et al.* (2010) who isolated ten bacterial strains from rhizosphere soil samples collected from rice seedlings grown in Andhra Pradesh, and found that all the strains were gram negative; rod shaped and produced yellowish green pigment in King's B medium.

The physiological tests were conducted for growth at different pH, Iron toxicity and Aluminium toxicity level. The isolate PF 43 grew at pH ranges from 1.0 to 14 and tolerate upto 1000 ppm of iron toxicity under *in vitro* condition. PF 46 and PF 47 isolates could survive up to the pH of 13 and tolerate iron toxicity up to 900 ppm. All the isolates tolerated a wide range of Aluminium toxicity level and its growth was observed from 5 ppm to 90 ppm.

Shelf life studies of three fluorescent pseudomonad strains were tested for their efficacy in suppressing the growth of *R. solani* under *in vitro* conditions. In all carrier formulations, *P. fluorescens* PF 43 survived upto 60 days without dramatic decline from initial population (Table 2). Although subsequently there was a decline in the population, 150 days after incubation the bacterial population of the isolate ranged from 10.1 to 16.3 x 10⁷cfu per g in talc based, dolomite based, and gypsum based formulations. After 240 days of storage, also about 1 x 10⁷cfu were detected in talc, dolomite and gypsum based formulations (Table 2). The shelf life of PF 46 and PF 47, the above carrier materials have high bacterial populations ranged from 9.9 to 16.1 x 10⁷cfu per g at 150 days after incubation and 1x 10⁷cfu at 240 days after incubation. So the result was similar to the shelf life of PF 43 isolate.

Muthamilan (1995) recorded the survival of the bacteria in talc based formulation even upto 240 days of storage although the population declined 30 days onwards. Sivakumar *et al.*,(2000) observed that *P. fluorescens* maintained the highest population level of 18.3 x 10⁷cfug⁻¹ after 40 days of storage in talc compared to storage in other car-

rier materials. However, the population level was reduced to 6 x 10⁷cfug⁻¹. In fact this study even seems to be the first report of dolomite and gypsum used as carrier material of *Pseudomonas fluorescens* for control of sheath blight disease in rice.

Table 2. Shelf life of *Pseudomonas fluorescens* PF 43 in various carrier formulations^y

Carrier formulation	Population (10 ⁷ cfu per g) at various days of storage							
	0	30	60	90	120	150	180	240
Talc	45.1 ^z	37.4	32.9	29.3	23.8	16.3	9.5	2.0
Vermiculite	38.9	29.4	20.6	18.3	09.4	04.5	1.1	0.0
Gypsum	39.7	30.5	26.4	22.5	15.7	10.1	4.3	0.9
Dolomite	42.5	34.2	29.5	26.4	17.6	12.5	4.9	1.1
Lignite	32.3	24.6	19.2	10.7	05.9	02.2	0.3	0.0

^ySimilar results were obtained with *P. fluorescens* PF 46 and PF 47 strains

^zMean of four replications. Means followed by a common letter in a column are not significantly different (P = 0.05) according to Duncan's multiple range test. Least significant difference (LSD) 0.05 value for comparing means in a row is 5.8.

Application of talc based formulation of the PF 43 isolate able to survive at highly acidic and alkaline pH ranges from 1.0 to 14 and grow well in high iron and aluminium toxicity soil of Kuttanad region. The cheap and easily available dolomite and gypsum can be used as carrier material for the mass multiplication of PF 43 isolate. The native fluorescent pseudomonads either PF 43, PF 46 or PF 47 can be used in highly stressed area for the control of sheath blight disease in Kuttanad. The native isolate of PF 43 can be recommended to 66,000 ha rice growing tract of lower, upper and karilands of Alleppey and Kottayam District for ecofriendly management of sheath blight disease and thereby reduce the environmental pollution and health hazards occurrence in the Kuttanad area due to high usage of chemical pesticides.

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