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Biocontrol potential and plant growth promotional activity of *Pseudomonas fluorescence* for the management of blast disease in Rice

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Abstract

In vitro and *in vivo* evaluated the antagonistic effects of three isolates of *Pseudomonas* from rhizosphere of rice against *Pyricularia oryzae* by using plate assay technique. The isolates were studied for the production of indole acetic acid, hydrogen cyanide, siderophore, protease and phosphate solubilization activity. All the 20 isolates of *Pseudomonas fluorescence* showed positive reaction for the production of HCN and siderophores, 15 isolates showed positive for plant growth promoting hormone, IAA. Among the 20 isolates, 19 isolates showed phosphate solubilisation on NBRIP medium under greenhouse conditions. The biocontrol activity and plant growth promotion of bacterial strains were evaluated, in which soil-inoculation of NCIM 2099 (the reference strain), TS3C8 and TS3B5 reduced disease index to a range of 12.75% ($P \leq 0.05$) compared to the untreated control at 27.6%. The plants treated with TS3C8 isolate recorded maximum root length and plant height, which were increased by 34.21cm and 77.58cm respectively over the diseased control under greenhouse condition. These results indicate that the tested PGPR improved growth parameters in rice plants and inhibit the growth of the fungus *Pyricularia oryzae* causing rice blast disease.

Keywords: Antagonistic effect; Greenhouse; PGPR; *Pyricularia oryzae*; Siderophores.

Introduction

Rice (*Oryza sativa* L.) is life, for most people living in Asia. Rice has shaped the culture, diets and economy of thousands of millions of people, providing food security and eradicating poverty. It has been estimated that half the world's population subsists wholly or partially on rice. Calories from rice are particularly important in Asia, especially among the poor, where it accounts for 50-80% of daily caloric intake. Asia accounted for 60% of the global population, about 92% of the world's rice production, and 90% of global rice consumption. 85% of the rice that is produced in the world is used for direct human consumption. Rice can also be found in cereals, snack foods, brewed beverages, flour, oil, syrup and religious ceremonies to name a few other uses.

The world's estimated rice production is 496.0 million metric tons during 2016.

India is the largest rice growing country accounting for about one third of the world acreage under the crop. In India's annual rice production is 103.6 million tons during 2016. Rice is grown throughout India in all the states. The major rice growing states of India are West Bengal, Uttar Pradesh, Bihar, Madhya Pradesh, Orissa, Andhra Pradesh, Karnataka and Chhattisgarh.

Rice suffers from many diseases caused by fungi, bacteria, viruses, phytoplasma, nematodes and other non-parasitic disorders. Among the fungal diseases, blast is considered as a major threat to rice production because of its wide spread distribution and its destructiveness under favourable conditions. The Commonwealth Mycological Institute has recorded its presence from 85 countries throughout the world. Paddy blast is generally considered as the principal disease of rice and is caused by a fungus belonging to the Ascomycete *Pyricularia oryzae* Cavara (teleomorph= *Magnaporthe grisea* (Hebert) Barr Comb Nov.). Losses due to the blast disease may range up to 90 per cent depending upon the component of the plant infected. *M. grisea* infects above ground parts of the plant, but neck blast and the panicle blast are the most damaging phases of the disease and have been shown to significantly reduce yield, grain weight and milling quality.

The pathogen may infect all the above ground parts of a rice plant at different growth stages *viz.*, leaf, collar, node, internodes, base or neck and other parts of the panicle and sometimes the leaf sheath. A typical blast lesion on a rice leaf is gray at the centre, has a dark border and it is spindle-shaped.

Now a days the use of bioagents are increasing because of fungus develop resistance against several fungicides and also hazardous effect on environment as well as on human health (Rabindran *et al.*, 1996) [14]. The bacteria which lives in rhizosphere having beneficial effects on plant growth that is why the plant growth promoting rhizobacteria (PGPR) are using as biological control agents against many pathogens (Kloppe *et al.*, 1999). *Pseudomonas fluorescense* having activity of plant growth promoting rhizobacteria, phosphate solubilizer and as biocontrol agents. PGPR, such as *Pseudomonas* can induces plant defenses mechanism by production of antibiotics, siderophore, hydrogen cyanide, competition for nutrition and space, inactivation of pathogen's enzymes and enhancement of root and plant growth (Karimi *et al.*, 2012) [9]. The research carried out under greenhouse conditions to know the antagonistic effects of some selected rhizobacteria on *Pyricularia oryzae* causing blast disease in rice.

Materials and Methods

Evaluation and Culture of microorganism

The different strains of *Pseudomonas fluorescense* were isolated rhizosphere and non-rhizosphere soils from different paddy growing regions of southern Karnataka.

All the isolates of *P. fluorescense* were grown in King's B media and incubated at 28°C for 18 hours in a shaking incubator (200 rpm) (Sambrook *et al.*, 2001) [15]. A virulent strain of *Pyricularia oryzae* was cultured on PDA (Potato Dextrose Agar) at 28±1°C for 14 days till the growth of colony touched the periphery in the control plate. *P. oryzae* culture and incubated at 28±1°C for 2 days with near UV illumination after the aerial hyphae were washed away by distilled water. The spores were used for inoculation (Nakata *et al.*, 2008) [12].

Preliminary Screening

In vitro evaluation was carried out with all the twenty isolates through dual culture technique.

To know the biocontrol activity against the *Pyricularia oryzae* (Gnanamanickam *et al.*, 1992) [6].

In the dual culture technique, twenty ml of sterilized and cooled PDA media was poured into sterile Petri plates. a disc of fungal growth was taken from the plate and placed at the center of fresh PDA plates then bacterial antagonists was streaked on PDA by leaving 1.5 cm gap With the help of a sterile corn borer.

After required period of incubation i.e. 72 h at 28 °C the growth in the control plate reached 90 mm diameter, the radial growth of pathogen was measured. Per cent inhibition over control was worked out according to the equation of Vincent (1947) [18].

$$I = \frac{(C-T)}{C} \times 100$$

Where,

I= Per cent inhibition

C= Growth of the bacteria in control

T= Growth of the bacteria in treatment

Statistical analysis of experimental data

Analysis and interpretation of the experimental data was done by using completely randomized design (CRD) and Factorial CRD for laboratory studies ANOVA (Gomez and Gomez, 1984 [7]; Hosmand, 1988) [8].

Identification of Bacterial Antagonist

Observation was made for growth, colony morphology, colour, pigmentation, shape and gram reaction of all the twenty isolates of *Pseudomonas fluorescense* according to standard procedures. Within this group of bacteria the biochemical properties is peculiar for each isolates. The studies conducted to know about production of IAA, nitrate oxidation, utilization of glucose anaerobically, urea and arginine, β-glucosidase production, protease and β-galactosidase, as well as the utilization of arabinose, mannose, mannitole, glucose, NAG, maltose, citrate, caprate, gluconateadipate, phenyl-acetate and malate, (Atzel *et al.*, 2008) [3].

Production of indole acetic acid

In-vitro evaluation of twenty isolates of *Pseudomonas* spp. for the production IAA (Ahmad *et al.*, 2005) [1]. The test bacterial isolates was inoculated in nutrient broth containing L-tryptophan and incubated at 28 ± 1°C for 7 days. Bacterial Cultures were centrifuged at 3000 rpm for 30min. Two ml of the supernatant was mixed with 2 drops of orthophosphoric acid and 4ml of Solawaski, s reagent. Development of a pink color was observed which indicates the production IAA. Optimum density (OD) was read at 530nm using Spectrophotometer.

Phosphate Solubilization

The bacteria isolates were streaked onto NBRIP medium. After 3 days of incubation at 28 ± 1 °C, those isolates induced a clear zone around the colonies were considered as positive strains. (Naik *et al.*, 2008) [11].

Production of Siderophores

The bacteria isolates which produce siderophore was determined by using the FeCl₃ test and the chrome Azurol S agar assay. Bacterial Inoculum (10 µl) was dropped onto the center of a CAS plate. After incubation at 25 °C for 5 days, was assessed on the the color change of the medium from blue to orange observed which indicates the production siderophore (Naik *et al.*, 2008) [11].

Production of HCN

The bacteria isolates were streaked onto King's B agar plates containing glycine. The Petri plates were then inverted and a piece of filter paper impregnated with 0.5% picric acid and 2% sodium carbonate was placed on the lid. Incubated at 30 °C for 7 days, the colour change of the filter paper pad from yellow to orange was observed which indicates the production of HCN (Suresh *et al.*, 2010) [17].

Production of Protease

Protease activity was determined by using skim milk agar medium. Bacterial strains were spot inoculated and after 2 days incubation at 28°C the formation of a clear zones around the cells which indicates proteolytic activity (Naik *et al.*, 2008) [11].

Greenhouse Experiments

Proving the pathogenicity

Seeds of rice from disease free plants were surface sterilized with 0.1 per cent sodium hypochlorite for two minute and sown in pots containing sterilized soil in order to raise healthy seedlings. Seeds were treated with antagonistic bacteria strains for 5 min. Sterile distilled water was used as a control. The treated seeds were germinated in autoclaved soil. Seedlings were supplied with fertilizers and maintained under glasshouse conditions at 25-28°C with water sprayed regularly both during morning and evening hours to maintain relative humidity. Periodical observations were made for the development of typical blast symptom on the treated plants. The pathogen from typical blast symptom was re-isolated and compared with the original culture as well as published literature to confirm the identity of the pathogen. A total of three isolates of *Pseudomonas* (TS3C8, TS11 and TS3B5) were selected for testing.

Inoculation with Pathogen Spores

The concentration of spore suspension *P. oryzae* was adjusted 3×10^5 spore's ml^{-1} by adding sterilized distilled water. The spore suspension was collected separately in an atomizer and incubated at 3-4 leaf stage. The seedlings after spray inoculation were kept in a moist chamber for 24 h at 26-28°C and then transferred to greenhouse condition (Nakata *et al.*, 2008) [12].

Bacteria Inoculation

Rice seeds (MTU 1001) were sterilized with 0.1 per cent sodium hypochlorite for two minute and germinated on moist filter paper in sterile Petri dishes. Ten days after germination, three seedlings were transplanted to each pot (25 cm diameter) containing autoclaved soil. Plants were maintained under glasshouse conditions at 25-28°C with water sprayed regularly for 35 days. Soils in which seedlings were growing were inoculated with 5 ml of bacterial suspension (10^8 - 10^9 cells ml^{-1}) of each strains and plants were left for 7 days under standard conditions (Naureen *et al.*, 2009) [13].

Challenge Inoculation

The blast fungus (*P. oryzae*) was cultured on rice flour agar and incubated at 25°C under fluorescent lights with a 12 h photoperiod for 2-3 weeks. Spores of the fungus were harvested by flooding the agar plate cultures with 5-7ml sterile water containing 0.5% gelatine, filtered through 0.2 mm nylon meshes and transferred immediately to a container with ice to prevent spore germination. Spore concentration was adjusted to 1×10^5 spore's ml^{-1} . Forty day old rice plants from all treatments were transferred to the inoculation chamber one day before the inoculation to acclimatize to the new environment. About 100 ml of the fungal spore suspension was sprayed over the plants in the evening. Immediately after inoculation, the plants were covered with polythene bags. After 48 hours, the polythene bags were removed. Periodical observations were made for the development of typical blast symptom on the inoculated plants each experiment replicates thrice.

Treatments detail

The experiment was conducted in pot culture with six treatments and three replications following a completely randomized design (CRD). The treatments consisted of the following:

T1: Healthy control (no fungus, no PGPR)

T2: Disease control (only *P. oryzae*)

T3: Reference strain (*P. fluorescens* NCIM 2099) + *P. oryzae*

T4: Strain TS3C8 + *P. oryzae*

T5: Strain TS3B5 + *P. oryzae*

T6: Strain TS11 + *P. oryzae*

The data of plant height, root length and disease index were recorded.

Disease Assessment

The screening of each plant after Six days of fungal inoculation for blast infection under greenhouse conditions. The number and size of lesions were recorded as grades 0-9 based on the DRR scale (table 1 and 2, fig 1).

Disease index = Sum of all disease rating / Total no. of rating X maximum disease grade X 100

Table 1: scale for rice blast caused by *P. oryzae*

Scale	Description
0	No infection
1	Pin head spots less than 1 per cent leaf area affected
2	Pin head spots 5-10 per cent leaf area affected
3	Typical blast spots with grey center 5-25 per cent leaf area affected
4	25-50 per cent leaf areas affected
5	Large spots with grey centre more than 50 per cent leaf area affected

Table 2: Disease grade and varietal reaction for rice blast caused by *P. oryzae*

Disease Grade	Varietal Reaction
0	Immune (I)
Up to 1	Highly resistance (HR)
>1.1 to 2.0	Resistant (R)
>2.1 to 3.0	Moderately resistant (MR)
>3.1 to 4.0	Moderately susceptible (MS)
>4.1 to 5.0	Susceptible (S)
>5	Highly Susceptible (HS)

Results

Preliminary Screening and Characterization of Biocontrol

All the *fluorescent* bacterial antagonists were gram negative, rod shaped and produce yellowish green pigment on King's B medium. The isolates were identified as *P. fluorescence* only in cases with of "good", "very good" or "excellent identification". By this principle, one isolate was *P. aeruginosa*, 3 isolates were *P. luteola* and 15 isolates were *Pseudomonas fluorescens*. All twenty bacterial isolates (except DL21) inhibited the pathogen under *in vitro* condition. Among the twenty different bio-agents, highest per cent inhibition of mycelial growth of fungus was recorded 65%, 52% and 51% of isolates TS3B5, TS3C8 and TS11 respectively. These isolates were selected for evaluation under greenhouse condition (Table 3).

In vitro evaluation of antagonist activity of *Pseudomonas* spp.

All 20 tested isolates of *Pseudomonas fluorescence* were positive for the production of siderophores (except TS25), HCN protease and, while 15 strains were positive for the production of plant growth promoting hormone, IAA (except DL11). Among the 20 isolates, 19 isolates showed phosphate solubilization on NBRIP medium (Table 3).

Pathogenicity of Bacteria and its effect on Plant Growth

No lesion formation or wilting, were observed in rice seedlings that had been incubated. There is an enhancement of plant growth promotion on plant height and root length was observed after 60 days of inoculated rice seedlings.

Suppression of disease under greenhouse condition

There were no significant differences between treatments TS3C8, TS3B5 and the reference strain NCIM 2099. When bacterial cultures were applied to the soil 7 days prior to pathogen inoculation, reference strain NCIM 2099, TS3C8 and TS3B5 reduced the disease index to a range of 13.50% relative to control (Fig. 1), while inoculation with TS11 registered a disease index of 16.88%. In general, the isolates which were effective against the test pathogen *Pyricularia oryzae* under *in vitro* conditions also showed excellent effect under greenhouse conditions.

Effect on Plant Growth

Root Length

Inoculation with isolate TS3C8 was recorded the highest root length (34.21cm) followed by TS11 with 31.16cm, while isolate TS3B5 as well as the uninoculated control recorded 23.21 and 24.17 cm, respectively, whereas the reference strain NCIM 2099 had a root length of 21.28 cm after 60 days of plant growth (Fig. 2).

Plant Height

Inoculation with isolate TS3C8 was recorded highest (77.58cm) compare to other treatments, followed by TS11 isolate (72.28cm). There was no significance difference between reference strain NCIM 2099 and TS3B5 in terms of plant height. The uninoculated control plants recorded a height of 56.34 cm, whereas rice receiving only the pathogen recorded a height of 40.5 cm after 60 days of plant growth (Fig. 3).

Table 3: Biocontrol activity of twenty isolates of *Pseudomonas* spp.

Isolate	IAA production	Phosphate Solubilization	Siderophore production	HCN production	Protease production	Antagonistic to <i>Pyricularia oryzae</i> (inhibition zone)
DL17	+	+	+	+	+	41(ABC)
DL21	+	+	+	+	+	52 (ABC)
TS3B5	-	+	+	+	+	17 (BC)
TS3C8	+	+	+	+	+	67 (A)
TS3A5	+	+	+	+	+	49 (AB)
TS3C	+	+	+	+	+	23 (ABC)
TS3B9	+	+	+	+	+	17 (BC)
TS3C4	+	+	+	+	+	14 (BC)
DLB6	+	+	+	+	+	29 (ABC)
TS326	+	+	+	+	+	31 (ABC)
TS3C6	+	+	+	-	+	0 (C)
TS3A1	+	-	+	-	-	15 (BC)
TS25	+	+	-	+	+	37 (ABC)
TS14	+	+	+	+	+	16 (BC)
TS11	+	+	+	+	+	50 (ABC)
TS3C9	+	+	+	-	+	26 (AB)
DL22	+	+	+	+	+	32 (ABC)
TS3A2	+	+	+	-	+	20 (ABC)
TS3C1	+	+	+	+	+	11 (BC)
DL11	-	+	+	+	+	21 (ABC)

"Positive" (+): Having trait

"Negative" (-): without trait

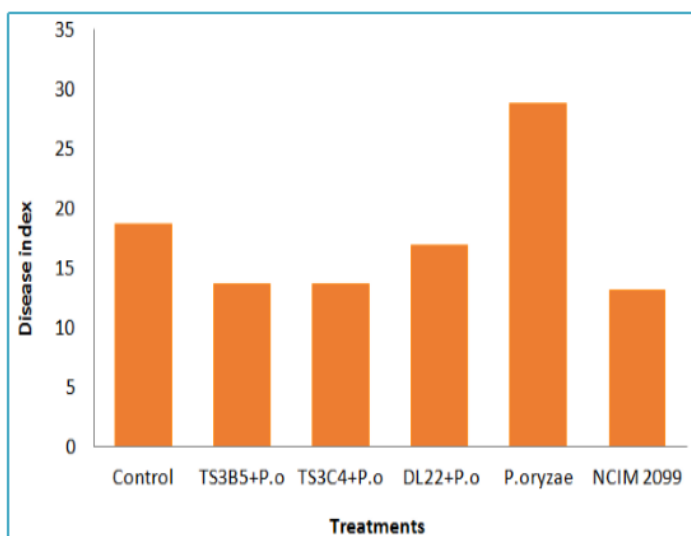


Fig 1: Effect on Disease Index

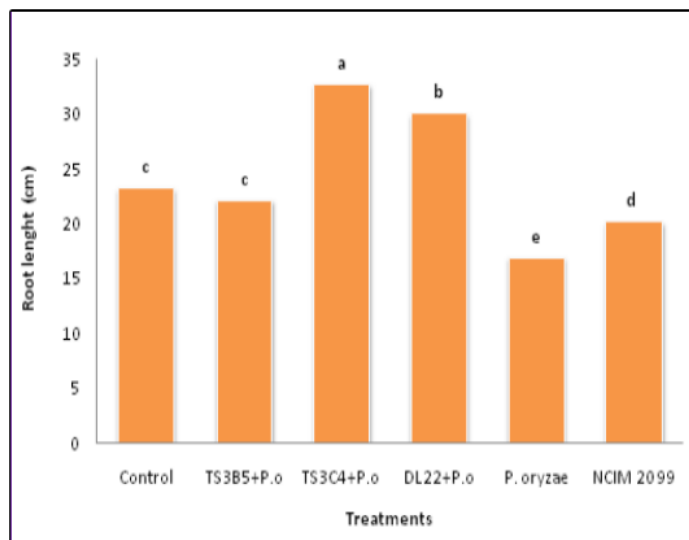


Fig 2: Effect on Root Length

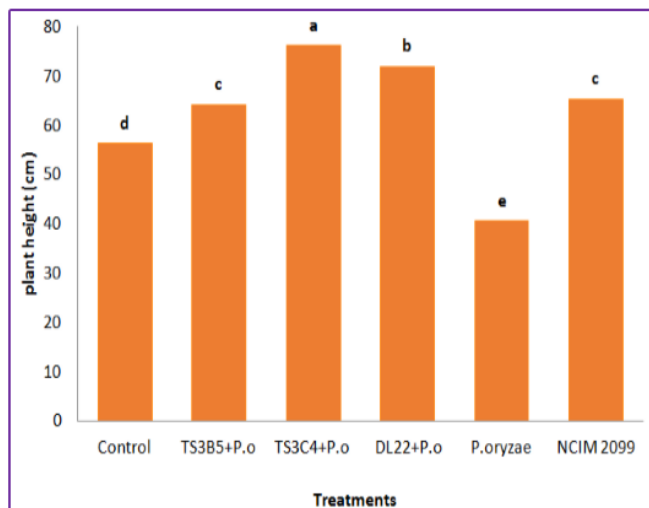


Fig 3: Effect Plant Height

Discussion

For the management of rice blast the unavailability of effective fungicides and lack of resistant varieties is the main problem. Therefore, the need of development of biological control for diseases is accepted as a long-lasting and eco-friendly alternative for rice growers. The mode of action of bio-agents is production of anti-microbial compounds (antibiosis), and competition for iron through siderophores production (Defago *et al.*, 1990) [4]. Protease destroy fungal cell wall (Karimi *et al.*, 2012) [9] produced by all isolates. The present research was carried out to know the efficacy of *Pseudomonas* spp. strains against rice blast both *in vitro* as well as under greenhouse conditions. In this study, all bacterial isolates were collected from rhizosphere of healthy paddy fields from rice growing areas of southern Karnataka. Some bacterial isolates showed high percent inhibition of mycelial growth of the fungus (TS3B5, TS3C8, and TS11), and similar results had been previously reported (Naureen *et al.*, 2009) [13]. Results indicated that various individual *Pseudomonas* strains can be applied by soil inoculation for suppression of pathogen (Shivakumar, 2007) [16]. The isolates have the characteristics of PGPR, and had various action mechanisms that suggested their potential for growth promotion. Production of IAA generally increasing the size and number of adventitious roots and thus providing large amounts of nutrients available to the plant (Suresh *et al.*, 2010) [17]. All the isolates form a clear halo zone on NBRIP medium were identified as potential phosphate solubilizers PGPR (Free living P-solubilizing bacteria) solubilized phosphates present in soil which enhance phosphate availability to plants and represent a possible mechanism of plant growth promotion under field conditions (Ghazanfar *et al.*, 2009) [5]. All of the isolates grown on CAS agar which produced siderophores (iron chelating agent which suppress some soil-borne fungal pathogens through direct siderophore-mediated iron competition (Defago *et al.*, 1990) [4]. The isolates of *P. fluorescence* found to produce IAA and siderophore, which can chelate metal ions and release phosphorous from the complex (Naureen *et al.*, 2009) [13]. HCN production also an important antifungal properties to control many pathogens (Shivakumar, 2007) [16] which acts as a general metabolic inhibitor to avoid predation or competition. Thus, it is clear from the observation that the isolates of *Pseudomonas fluorescence* are able to produce plant growth promoting substances and antifungal substances. Therefore, they are potential agents for the production of bio-

fertilizer and bio-inoculants for crop plants to manage the diseases. The results in this study clearly indicated that suppression of disease was better with the *Pseudomonas* isolate TS3C8 inoculation treatment as shown in the greenhouse compared to other isolates. Therefore, it can be concluded that complex mechanisms were involved in the biocontrol process. Growth parameters such as root length and plant height were significantly higher with PGPR inoculation compared to the untreated control and this isolate reduced disease index to 13.66%, with a maximum percent inhibition of mycelial growth of the fungus of (52%). The results revealed that the PGPR were able to induce the IAA production, solubilize phosphorus and provide resistance to several fungal pathogens, hence improving plants growth. Among 20 isolates, isolates TS3C8, TS3B5 and TS11 recorded better effect with respect to IAA production, phosphate solubilization and the antagonism activity. Thus use of PGPR inoculants as biofertilizer is an excellent approach to overcome chemical fertilizers and pesticides for sustainable rice production.

Conclusion

Disease management through PGPR is very important aspects to replace the chemicals and also minimize the cost of cultivation. Among different isolates of *Pseudomonas* the isolates TS3B5, TS3C8, TS11 and reference isolate reduce disease index by 12.85, 12.65, 15.68 and 12.18 respectively. This result revealed that single inoculation of any isolate could not reduce disease index completely. Out of four isolates, TS3C8 recorded maximum mycelial inhibition, which emerged as the best PGPR. It enhanced the plant height by 77.58cm and root length by 34.21cm. Thus the TS3C8 isolate was the most efficient bio-control against blast disease in rice.

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