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Evaluation of Plant Growth Promoting Rhizobacteriaas Biocontrol Agents for the Control of Blast Disease in Rice

Mansoureh-Sadat Sharifi-Noori[1, *], Halimi Mohd Saud[2], Elham Azizi[1]

[1]Department of Agronomy, Payame Noor University, Tehran, Iran
[2]Department of Agro-Technology, University Putra Malaysia, Selangor, Malaysia

Abstract

The antagonistic effects of three *Pseudomonas* isolates from rhizosphere of rice were evaluated against *Pyricularia.oryzae* in *vitro* and in *vivo*. Fungal inhibition tests were performed using plate assay. Each isolate was tested for the production of protease, siderophore, cyanide hydrogen, indole acetic acid and phosphate solubilization activity. All the 20 tested isolates of *Pseudomonads fluorescent* were positive for the production of siderophores and HCN, while 15 strains (75%) were positive for the production of plant growth-promoting hormone, IAA. Among the 20 isolates, 19 isolates showed phosphate solubilisation on NBRIP medium. Biocontrol activity and plant growth promotion of bacterial strains were evaluated under greenhouse conditions, in which soil-inoculation of NCIM 2099 (the reference strain), TS3C8 and TS3B5 reduced disease index to a range of 13.50% ($P \leq 0.05$) compared to the untreated control at 28.8%. Greenhouse experiments revealed that the plants treated with TS3C8 isolate recorded maximum root length, plant height, and fresh shoot weight which were increased by 32.78 cm, 76.16 cm, and 5.84 g, respectively over the diseased control. These results indicate that the tested PGPR improved growth parameters in rice plants and contribute towards biocontrol of the blast pathogen.

Keywords

Antagonism, Biological Control, PGPR, *Pseudomonas* Spp, Rice Blast Disease

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1. Introduction

Rice is one of the main staple foods for the growing world population, particularly in Asia (Naureen et al., 2009). However, rice blast is a serious problem in almost approximately all countries where rice is grown. This fungal disease causes yield losses of US$55 million each year in South and South East Asia. The rice blast disease is caused by the fungus *Pyricularia oryzae*, which, in its sexual state, is known as *Magnaporthegrisea* (Kuyek et al., 2000). In recent decades there has been more consideration on biological control due to the appearance of fungicide resistance in pathogens alongside increased health concerns for humans and the environment (Rabindran et al., 1996). It is well known that a substantial number of bacterial species, generally those linked with the plant rhizosphere, are able to exert beneficial effects on plant growth. Therefore, the use of plant growth promoting rhizobacteria (PGPR) as biological control agents for agricultural improvement has become attractive for researchers (Kloppe et al., 1999). *Pseudomonas fluorescent*is well recognized as a plant growth promoting rhizobacteria, phosphate solubilizer and as biocontrol agents against plant pathogens. The occurrence and distribution of *Pseudomonas fluorescent* have been studied by many scientists (Khan, 2006). PGPR, such as *Pseudomonas* can elicit plant defenses by different mechanisms such as production of antibiotics, siderophore, hydrogen cyanide, competition for nutrition and space, inducing resistance,

* Corresponding author
E-mail address: Mansourehsharifi13@yahoo.com (Mansoureh-Sadat Sharifi-Noori)

inactivation of pathogen's enzymes and enhancement of root and plant development (Karimi et al., 2012). The objective of this research was to evaluate antagonistic effects of some selected rhizobacteria on rice blast under greenhouse conditions.

2. Materials and Methods

2.1. Microorganisms and Culture Conditions

Microorganisms used in this study were isolated from the rhizosphere and non-rhizosphere soils from paddy fields in different localities of Peninsular Malaysia. All microorganisms were grown in King's B media. Bacteria were gown at 28 °C for 18 hours in a shaking incubator (200 rpm) (Sambrook et al., 2001). A virulent strain of *Pyriculariaoryzae*was cultured on PDA (Potato Dextrose Agar) at 26 ˚C for 14 days. The growth plates were kept at 26˚C for 2 days with near-UV illumination after the aerial hyphae were washed away by distilled water. Thus, synchronously formed spores were used as inocula (Nakata et al., 2008).

2.2. Preliminary Screening

All the twenty isolates were tested in vitro for their biocontrol activity against the plant pathogenic fungus, *Pyricularia oryzae* by dual culture technique (Gnanamanickam et al., 1992). Fungal pathogen was grown on PDA plates till it covered the whole surface of the agar. With a sterile cork borer, a disc of fungal growth was taken from the plate and placed at the center of fresh PDA plates. Twenty four hour old culture of each bacterial strain was then streaked on PDA 1.5 cm from the edge of plates. Plates were cultured for 72 h at 28°C and the percent inhibition of radial growth (PIRG) was recorded using the following formula (Sariah, 1994).

$$PIRG = (R1-R2)/R1 \times 100$$

R1 = Radial growth of *P. oryzae* in control plate

R2 = Radial growth of *P. oryzae* interacting with antagonistic bacteria.

2.3. Identification of Bacterial Antagonist

Twenty selected efficient PGPR strains were examined for colony morphology, growth, pigmentation, and cell shape and gram reaction as per standard procedures. API 20NE (Biome´rieux, France) is a frequently used quick identification method based on the degradation of biochemical substrates. This method is suitable to identify Gram-negative rods at the species level by assessing the profile of 21 different biochemical reactions. The biochemical profile is specific for each species within this group of bacteria (Biome ´rieux, France). The tests include oxidation of nitrate, indole production, anaerobic utilization of glucose, arginine and urea, production of b-glucosidase, protease and b-galactosidase, as well as the utilization of glucose, arabinose, mannose, mannitole, N-acetyl-glucosamine, maltose, gluconate, caprate, adipate, malate, citrate and phenyl-acetate. The API stripes were incubated for 48 h at 30 °C under ambient air. The results were interpreted with the API Web TM 100 software (version 7.0) (Atzel et al., 2008).

2.4. IAA Production

All test strains of *Pseudomonas* spp. were screened for IAA production (Ahmad et al., 2005). Briefly, test bacterial culture was inoculated in nutrient broth containing L-tryptophan (5 mg/ml) and incubated at 28 ± 2˚ C for 1 week. Cultures were centrifuged at 3000 rpm for 30 min. Two milliliters of the supernatant was mixed with 2 drops of orthophosphoric acid and 4 ml of Solawaski' s reagent (50 ml, 35% perchloric acid; 1 ml 0.5 $FeCl_3$). Development of a pink color indicates IAA production. Optimum density (OD) was read at 530 nm using Spectrophotometer and expressed as µg/ml.

2.5. Phosphate Solubilization

To detect phosphate solubilizing bacteria, strains were streaked onto NBRIP medium, containing (per liter) glucose, 10 g; $Ca_3 (Po_4)_2$, 5 g; $MgCl_2$ .6 H_2 O, 5 g; $MgSO_4$ .7H_2 O, 0.25 g; KCl, 0.2 g and $(NH_4)_2 SO_4$, 0.1 g, agar 1.5 %. After 3 days of incubation at 28 °C, strains that induced a clear zone around the colonies were considered as positive (Naik et al., 2008).

2.6. Siderophore Production

Production of siderophore by the strains was determined using the $FeCl_3$ test and the chrome Azurol S agar assay. Briefly, inoculum (10 µl) of bacterial strains was dropped onto the center of a CAS plate. After incubation at 25 °C for 3 days, siderophore production was assessed on the basis of color change of the medium from blue to orange (Naik et al., 2008).

2.7. HCN Production

For the production of HCN, bacteria were streaked into King`s B agar plates supplemented with 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. After incubation for a week at 30 ºC, the colour change of the filter paper pad from yellow to orange was indicative of HCN production (Suresh et al.,

2010).

2.8. Protease Production

Protease activity was determined using skim milk agar medium, containing (per liter): 5 g pancreatic digest of casein, 2.5 g yeast extract, 1 g glucose, 7% skim milk solution and 15 g of agar. Bacterial cells were spot inoculated and after 2 days incubation at 28 °C proteolytic activity was identified by the clear zones around the cells (Naik et al., 2008).

2.9. Greenhouse Experiments

2.9.1. Pathogenicity Test

To test bacterial pathogenicity to rice plants, seeds were immersed in a suspension of the antagonistic bacteria ($5x10^9$cfu mL^{-1}) 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 ºC with daily watering. A total of three isolates of *Pseudomonas* (TS3B5, TS3C8 and TS11) were selected for testing in greenhouse assays.

2.9.2. Inoculation with Pathogen Spores

A spore suspension (3×10^5spores ml^{-1}) of *P. oryzae* was sprayed on the leaves of the rice plants at the four to five-leaf stage. The inoculated plants were kept in a moist chamber for 24 h at 26-28 ˚C, and then transferred to a glasshouse (Nakata et al., 2008).

2.9.3. Bacteria Inoculation

Rice seeds (*cv.*Mahsuri) were sterilized 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 in the greenhouse at a temperature of 28 ±2 ˚C during the day and 25 ±2 ˚C at night for 35 days. For inoculation of plants, bacterial cultures were grown in LB broth for 24–48 h at 28 ±2 ˚C. Cell pellets were obtained by centrifugation at 6000 × g for 5 min, washed and re-suspended in sterile water. Soils in which seedlings were growing were inoculated with 5 mL of bacterial suspension (10^8–10^9 cells mL^{-1}) of individual strains and plants were left for 7 days under standard conditions (Naureen et al., 2009).

2.9.4. Challenge Inoculation

The blast fungus was cultured on rice flour agar (20 g L^{-1} rice flour, 2.5 g L^{-1} yeast extract 1.5% 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 5–7 mL 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 1×10^5spores mL^{-1}. Forty day-ld rice plants from all treatments were transferred to the inoculation chamber one day before the inoculation to acclimatize to the new environment (28 ±5 ˚C day and 25 ±5 ˚C night temperature and relative humidity above 90%). About 100 mL of the fungal spore inoculum was sprayed over the plants in the evening. Immediately after inoculation, the plants were covered with a polythene hood and black polythene sheets for 24 h to stimulate infection in the dark. Each experiment consisted of four replicates per treatment with three plants per replicate.

2.10. Treatments

The experiment was conducted in pot culture with sixtreatments in four replications following a completely randomized design (CRD). All data were analysed using the SAS software. The means were compared using Duncan's multiple range test (DMRT) at $P \leq 0.05$. The treatments consisted of the following:

T1: Healthy control (no fungus, no PGPR)

T2: Strain TS3B5 + *P.oryzae*

T3: Strain TS3C8 + *P.oryzae*

T4: Strain TS11 + *P.oryzae*

T5: Disease control (*P.oryzae* alone)

T6: Reference strain (*P. fluorescens* NCIM 2099) + *P.oryzae*

The measurements recorded in this experiment were disease index, plant height, root length, and fresh shoot weight.

2.11. Disease Assessment

Six days after fungal inoculation, each plant was assessed individually for blast infection. Each leaf was scored for number and size of lesions. Development of symptoms was observed and recorded as grades 0-9 based on the IRRI scale (1993). According to the grades, the disease index was calculated using the following formula.

Disease index = Total grade/no. of blast observed × 100/maximum grade

3. Results

3.1. Preliminary Screening and Characterization of Biocontrol

All the *fluorescents'* bacteria antagonists were gram negative, oxidase-positive, rod shaped and all produced yellowish green pigment on King`s B medium. In order to identify the 20 isolates, API 20NE was used. The isolates were considered identified as *P. fluorescens*only in cases withof "good", "very good" or "excellent identification".

Following this principle, 15 strains were identified as *Pseudomonasfluorescens*, 3 isolates belonged to the species of the *P.luteola*, one isolate was *P. aeruginosa*and a single isolate (TS3C4) had a doubtful identification. All twenty bacterial isolates (except DL21) inhibited the pathogen in the dual culture assay, whereas isolates TS3B5, TS3C8 and TS11 showed maximum percent inhibition of radial growth (PIRG) of 65%, 52% and 51%, respectively, and were selected for evaluation in the greenhouse experiments (Table 1).

3.2. Invitro Antagonist Assays

All 20 tested isolates of *Pseudomonas fluorescence* were positive for the production of siderophores (in iron-deficient culture medium), protease and HCN, while 15 strains (75%) were positive for the production of plant growth-promoting hormone, IAA. Among the 20 isolates, 19 isolates showed phosphate solubilization on NBRIP medium (Table 1).

3.3. Pathogenicity and Effects of Bacteria on Plant Growth

No sign of pathogenicity, such as lesion formation or wilting, were observed in seedlings that had been incubated with bacterial cultures. Significant enhancement in plant growth promotion on plant height, root length and fresh shoot weight were observed in bacteria inoculated rice seedlings after two month.

3.4. Disease Suppression in Greenhouse-Grown Plants

There were no significant differences between treatments TS3B5, TS3C8 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 *Pyriculariaoryzae* under in *vitro* conditions also showed excellent control of the rice blast disease under greenhouse conditions.

3.5. Plant Growth

3.5.1. Root Length

Root length of rice seedlings at 60 days of plant growth was influenced by different treatments (Fig. 2). Inoculation with isolate TS3C8 registered the highest root length (32.78 cm). The next superior treatment was TS11 with 30.06 cm, while isolate TS3B5 as well as the uninoculated control recorded 22.2 and 23.24 cm, respectively, whereas the reference strain NCIM 2099 had a root length of 20.18 cm.

3.5.2. Plant Height

Plant height in rice seedlings was highest (76.16 cm) in plants receiving isolate TS3C8 which was significantly superior to the rest of the treatments, followed by TS11 isolate with a height of 71.98 cm. 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 (Fig. 3).

3.5.3. Fresh Shoot Weight

Rice inoculated with isolate TS3C8 showed the highest fresh shoot weight in rice seedlings with 5.84 g. The next superior treatment was TS11 with a weight of 4.16 g followed by TS3B5 (3.4 g) and NCIM 2099 (3.24 g). The uninoculated control and the *Pyricularia* treatment alone were not significantly different in terms of fresh shoot weight (Fig. 4).

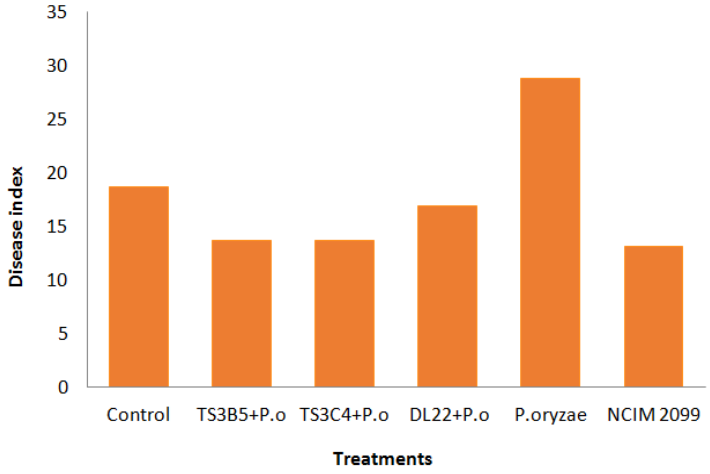

Fig. 1. Effect of inoculated *Pseudomonas* isolates on disease index.

Means with the similar letters in each column are not significantly different at p≤0.05

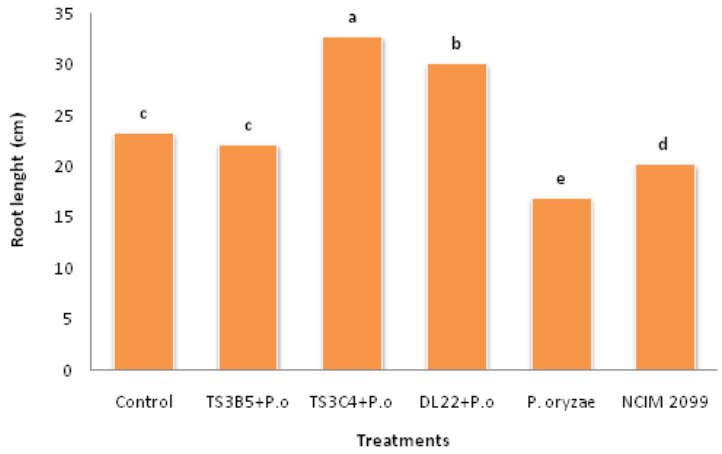

Fig. 2. Effect of inoculated *Pseudomonas* isolates on root length.

Means with the similar letters in each column are not significantly different at p≤0.05

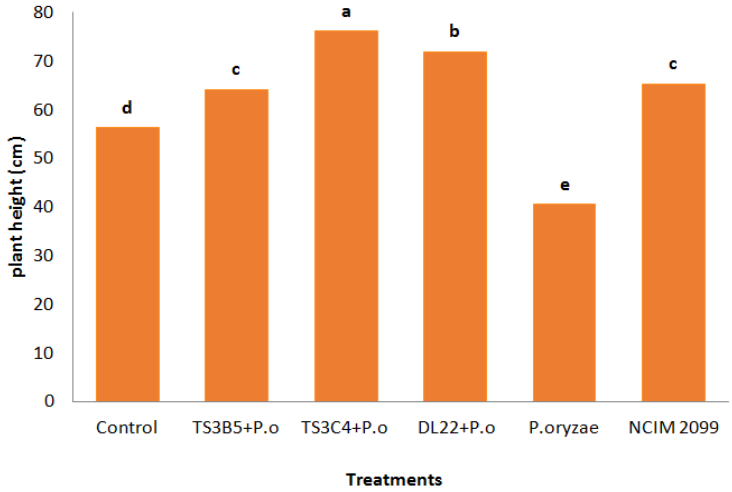

Fig. 3. Effect of inoculated *Pseudomonas* isolates on plant height.

Means with the similar letters in each column are not significantly different at p≤0.05

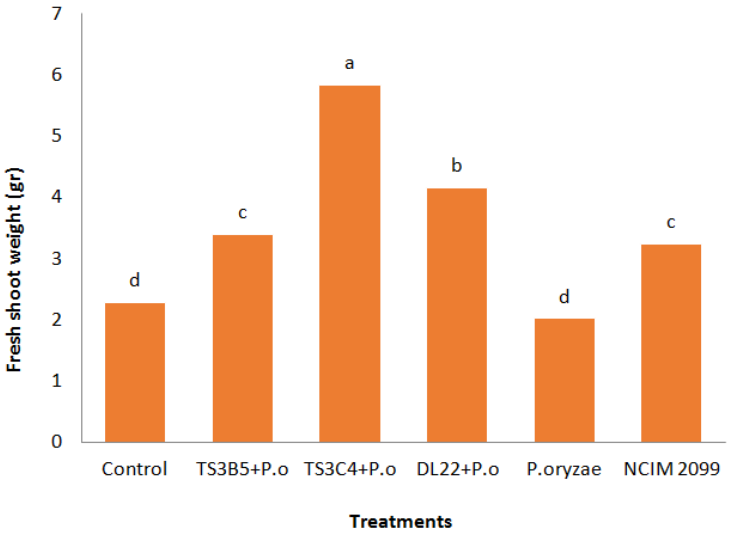

Fig 4. Effect of inoculated *Pseudomonas* isolates on fresh shoot weight.

Means with the similar letters in each column are not significantly different at p≤0.05

Table 1. Twenty potential isolates with plant growth promotion and biocontrol traits.

Isolate	Phosphorous Solubilization	Siderophore production	Protease production	IAAproduction	HCN production	Antagonisticto *Pyriculariaoryzae* (inhibitionzone)
DL21	+	+	+	+	+	40 (ABC)
DL17	+	+	+	+	+	17 (BC)
TS3C8	+	+	+	+	+	52 (ABC)
TS3B5	+	+	+	-	+	65 (A)
TS3C	+	+	+	+	+	49 (AB)
TS3A5	+	+	+	+	+	26 (ABC)
TS3C4	+	+	+	+	+	16 (BC)
TS3B9	+	+	+	+	+	13 (BC)
DL26	+	+	+	+	+	30 (ABC)
TS3B6	+	+	+	+	+	33 (ABC)
TS3A1	-	+	+	-	+	0 (C)
TS3C6	+	+	+	-	+	14 (BC)
TS25	+	+	+	+	+	38 (ABC)
TS14	+	+	+	+	+	16 (BC)
TS11	+	+	+	+	+	51 (AB)
TS3C9	+	+	+	-	+	27 (ABC)
DL22	+	+	+	+	+	33 (ABC)
TS3A2	+	+	+	-	+	19 (ABC)
TS3C1	+	+	+	+	+	10 (BC)
DL11	+	+	+	+	+	19 (ABC)

"Positive" (+): Having trait
"Negative" (-): Not having trait

4. Discussion

The usual approaches for managing of rice blast disease include planting of resistance cultivars, application of fungicides, and management of planting time, fertilizers and irrigation, and biological control (Ghazanfar et al., 2009). The non-availability of efficient fungicides and lack of resistant varieties magnify the problem. Therefore, the need for alternate methods of control of this pathogen has become vital. Development of biological control for diseases is accepted as a long-lasting and eco-friendly alternative to agrochemicals. There are different modes of action of biocontrol organisms, including inhibition of the pathogen by anti-microbial compounds (antibiosis), and competition for iron through siderophores production (Defago et al., 1990). Protease which interferes in wall degrading of fungal pathogen (Karimi et al., 2012) is produced by all isolates. The present research was conducted to evaluate the efficacy of *Pseudomonas* spp. strains against the pathogen under greenhouse conditions. The use of rhizobacteria for plant disease control is usually more effective when the rhizobacteria is isolated from rhizosphere of the same host plant (Karimi et al., 2012). In this study, all bacterial isolates were selected from rhizosphere of healthy rice plants in paddy fields. Some bacterial isolates showed high inhibition activity on the pathogen (TS3B5, TS3C8, and TS11), and similar results had been previously reported (Naureen et al., 2009). Results indicated that various individual *Pseudomonas* strains can be applied by soil inoculation for suppression of pathogen (Shivakumar, 2007).

The isolates selected in this study presented several enviable features as PGPR, and had various action mechanisms that suggested their potential for growth promotion. Production of IAA generally affects the root system, increasing the size and number of adventitious roots and also the root subdivision, enabling a larger soil quantity to be exploited by the roots, and thus providing large amounts of nutrients available to the plant (Suresh et al., 2010). All the strains were identified as potential phosphate solubilizers based on their capacity to solubilize tricalcium phosphate [Ca_3 $(PO_4)_2$] and form a clear halo zone on NBRIP medium. Most of phosphorus in soil is present in the form of insoluble phosphates and cannot be utilized by the plants. The ability of bacteria to solubilize mineral phosphates has been of interest to agricultural microbiologists as it can improve the availability of phosphorus and iron for plant growth. PGPR have been shown to solubilize precipitated phosphates and enhance phosphate availability to plants and represent a possible mechanism of plant growth promotion under field conditions (Ghazanfar et al., 2009). Free-living P-solubilizing bacteria release phosphates from spare soluble inorganic and organic phosphate compounds in the soil and thus contribute to increased availability phosphates for plants (Sariah, 1994). All of the isolates grown on CAS agar in the present study produced siderophores. These siderophore producing microorganisms suppress some soil-borne fungal pathogens through direct siderophore-mediated iron competition (Defago et al., 1990). The microorganisms investigated in this study were found to produce IAA and siderophore, which can chelate metal ions and release phosphorous from the complex (Naureen et al., 2009). Microbial production of

HCN has been suggested as an important antifungal feature to control root pathogens (Shivakumar, 2007). Cyanide acts as a general metabolic inhibitor to avoid predation or competition. The host plants are generally not harmfully affected by inoculation with HCN producing bacteria and hence host-specific rhizobacteria can function as biological control agents (Shivakumar, 2007). Thus, it is clear from the investigations that *Pseudomonadsfluorescens*strains are able to produce plant growth promoting substances and antifungal substances. Therefore, they are potential candidates for the development of bio-fertilizer and bio-inoculants for crop plants. The results of the present study indicated that suppression of disease was better with the TS3C8 inoculation treatment as shown in the greenhouse experiments. Therefore, it can be concluded that complex mechanisms were involved in the biocontrol process. Growth parameters such as root length, plant height and fresh shoot weight were significantly higher with rhizobacteria inoculation compared to the untreated control. The TS3C8 isolate had the greatest effects on all growth factors with soil inoculation compared to other isolates, and this isolate reduced disease index to 13.66%, with a maximum percent inhibition of radial growth (PIRG) of 52%. The results suggest that the PGPR were able to induce the production of IAA, solubilize phosphorus, and provide resistance to pathogens, thereby improving growth of plants. Of the 20 isolates, isolates TS3C8, TS3B5 and TS11 showed better performance with respect to IAA production, phosphate solublization and the antagonism assay. The use of PGPR inoculants as biofertilizer is an efficient approach to replace chemical fertilizers and pesticides for sustainable rice cultivation in Malaysia. The safety of microorganisms for biocontrol should be discussed in terms of existing risks of pathogenicity, allergenicity or toxigenic effects on people, domestic animals and wild life.

5. Conclusion

The results have clearly indicated that isolates TS3B5, TS3C8, TS11 and reference strain could reduce disease index by 13.72, 13.66, 16.88 and 13.18 respectively. This result showed that single inoculation could not reduce disease index completely.

Out of four promising strains, TS3C8 emerged as the best organism in plant growth promotion. It increased the plant height by 76.16, root length by 32.78 cm and fresh shoot weight by 5.84 g.

Thus, this investigation has demonstrated that TS3C8 isolate was the most efficient bio-control agent in bio-controlling blast disease in rice, besides promoting growth of rice seedling. So the use of PGPR as inoculants bio-fertilizers is an efficient method to replace chemical fertilizers and pesticides for sustainable rice cultivation in Malaysia.

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Abbreviations

HCN: Hydrogen cyanid

IAA: Indole acetic acid

PGPR: Plant growth promoting rhizobacteria

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