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Screening of *Oryza sativa* Indigenous Rhizobacteria for its Bio-control and Plant Growth Promoting Potential

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Abstract- Bacteria that colonize plant roots and enhance the plant growth are denoted as Plant Growth Promoting Rhizobacteria (PGPR). The objective of this study is to isolate and identify *Oryza sativa* indigenous rhizobacteria and test for its, bio-control and various plant growth promoting traits under *in-vitro* condition. Nine rhizobacterial colonies isolated from healthy rice rhizosphere soil were screened for bio-control activity against rice pathogen *Helminthosporium oryzae* (rice brown spot) and *Rhizoctonia solani* (sheath blight) by performing dual culture method. The isolate B9 showed maximum antagonism against *R. solani* (73.7%) followed by isolate B2 (51.2%), isolate B1 (47.5%) and isolate B5 (42.5%) with different range of mycelial inhibition. Among nine rhizobacterial isolates tested, the growth of pathogenic fungi *H. oryzae* was only inhibited by the isolate B9 (20.5%). The isolates B1, B2, B5 and B9 exhibited maximum bio-control activities were further screened for different PGP traits, such as Phosphate (P) solubilization, Indole-3-Acetic Acid (IAA) production, Hydrogen Cyanide production (HCN) and extracellular enzyme production under *in-vitro* condition. The isolate B9 revealed the maximum P solubilization efficiency (99 SE), 45 µg/ml of IAA, HCN, amylase and cellulase production in maximum amount. The isolate B9 exhibited maximum bio-control, and PGP traits were identified by morphological and biochemical characterization and it was identified as *Bacillus subtilis*. In pot experiment, the rice seeds treated with *B. subtilis* exhibited significant shoot and root biomass when compared with un-inoculated rice and fungus-infected rice. This study highlighted the beneficial properties of native PGPR's on growth promotion of rice and it could be formulated as bio-control as well as plant growth promoting agent, to reduce the application of chemical fertilizers in agricultural lands.

Keywords- *Bacillus subtilis, Oryza sativa, Helminthosporium oryzae*, Rhizobacteria, *Rhizoctonia solani*

I. INTRODUCTION

Rice (*Oryza sativa*) is the most widely consumed staple food of the human population, particularly in Asia. Ninety percentage of rice is produced in Asia, in which China and India being the lead producers[1]. Diseases caused by various microbes are the significant limiting factors that affect rice production and causing annual yield losses[2]. More than 70 diseases were reported in the rice, caused by fungi, bacteria, viruses, mycoplasma or nematodes. Among these, fungi are the major disease causing agent in the rice field. Some economically important fungal diseases are caused by *Cercospora oryzae* (Narrow brown leaf spot), *Helminthosporium oryzae* (rice brown spot), *R. solani* (sheath blight), *R. oryzae* (Aggregate sheath), *Sarocladium oryzae* (Sheath rot), *Fusarium* sp (root rot), *Sclerotium oryzae* (stem rot) etc[3]. They not only have a supreme role in causing disastrous epidemics in the plant but also play a significant and perpetual role in annual crop yield losses which affect the economy of the country. As a way out for these problems, many methods have been employed.

The most common technique used by farmers is crop rotation, it enhances soil fertility and also is an effective measure of controlling pathogens and pests. But crop rotation requires 2 to 3 years for reducing the fungal population to acceptable levels. So crop rotation alone was not enough for fighting against the phytopathogens[4]. Using chemical fungicides are the best prevention against fungal disease caused by phytopathogens but it affects the soil fertility severely, the soil texture, composition and beneficial microbial population in the soil get affected on the frequent application of chemicals. Apart from that, fungus exhibited resistances against chemical fungicides which makes the fungicide ineffective against plant pathogens. Hence, the application of chemical fungicides is not an eco-friendly method and it is essential to find a new alternative technique

for plant protection which is less dependent on the chemicals and is more eco-friendly.

One of the emerging research areas for the control of phytopathogenic agent is the application of native Plant Growth Promoting Rhizobacteria (PGPR), which are capable of suppressing or preventing the damage caused by phytopathogens[5]. PGPR's enhance the growth of plants either directly or indirectly[6]. Biological control is an indirect method of using rhizobacteria having efficient disease management strategy. Several microorganisms belonging to the genera *Bacillus, Pseudomonas, Azotobacter, Enterobacter, Klebsiella, Azospirillum* etc are used as "Biological Control Agents" (BCAs) for the management of fungal diseases and was studied by several researchers[7-9] . The development of biological products based on beneficial microorganisms can extend the range of options for maintaining the health and yield of crops. Biological control is an ecology conscious, cost-effective and sustainable alternative in disease management. In the present study potential, antagonistic native rhizobacteria with PGP activity were isolated from healthy rice rhizosphere area and studied its biological controlling efficacy and its PGP potential.

II. OBJECTIVE OF THE STUDY

1. Isolation and identification of Plant Growth Promoting Rhizobacteria from rice rhizosphere soil.
2. Screening for potent bio-control rhizobacteria under *in-vitro* and *in-vivo* studies.

III. METHODOLOGY

Soil sample collection

Rhizosphere soil samples from healthy rice crops were collected from two different fields situated around Nattukal area, Palakkad district, Kerala. During the period of August 2017. The rhizosphere soil samples were collected up to a depth of 10 to 15 cm in neat and clean zip-lock polybags and brought to the laboratory immediately for analysis.

Enumeration of rhizobacteria

Serial dilution and plating techniques as described by Parkinson *et al*[10] was adopted for enumeration of PGPR. Ten gram of soil samples were weighed and suspended in 90 ml sterile distilled water aseptically. One ml from this dilution was aseptically transferred to another water blank with 9 ml of sterile water using sterile pipettes. Similarly, successive dilutions of the samples till 10^{-8} were prepared by serial dilution method. The aliquots 0.1 ml of 10^{-7} and 10^{-8} were serially transferred to sterile Petri plates with nutrient agar. The samples were spread on nutrient agar plates using L-rod and then incubated at 28±2 ^{O}C for 24 hours and Colony Forming Units (CFU/g) were calculated. The colonies exhibiting different cultural morphology were pure cultured and stored at 4 ºC for further studies.

Isolation of phytopathogenic fungi from the infected rice crop

The fungus infected leaf samples with disease symptoms were collected from rice fields and washed under running tap water to remove adhering soil particles and the majority of epiphytic microorganisms. The leaf samples were surface sterilized using 70% ethanol for 30 seconds followed by 0.01% $HgCl_2$ for 1 minute and it was rinsed 3-4 times with sterile distilled water and were blotted on sterile blotting paper. The infected portion of leaf samples was cut into small pieces using sterile scissors and placed on the Potato Dextrose Agar (PDA) medium. The Petri plates were incubated at 28±2 ^{O}C for 2-3 days.

Pure culture of plate pathogen and identification

The colonies grown out from an infected portion of leaf samples were pure cultured and identified by cultural characterization *viz.*, colour, texture, pigmentation etc. and morphological characterization by using of Lactophenol cotton blue staining[11].

Bio-control activity of rhizobacterial isolates using dual culture method

The bio-control activity of rhizobacteria against the plant pathogenic fungi by using dual culture method was carried out by the following method described by Howell[12]. Three days old fungal pathogen grown in PDA plate was used for the study. The PDA plate was inoculated with 6mm disc of fungi at the center, and rhizobacteria to be tested were line streaked at four sides of fungi disc. The plates with the fungal disc were used as control and incubated at room temperature for 5-7 days. The percentage of inhibition was calculated using the given formula:

$$\text{Percent of mycelia inhibition (\%)} = \frac{C-T}{C} \times 100$$

C = Growth of pathogen in control plate (cm)
T = Growth of the pathogen in dual culture plate (cm)

***In-vitro* screening for Plant growth promoting traits of selected rhizobacteria**

Phosphate solubilization

Rhizobacterial isolates obtained were tested by plate assay for phosphate solubilization in Pikovskaya's agar medium by point inoculation. These bacteria were point inoculated using sterile toothpicks and incubated at 28±2 ^{O}C for 3-4 days. The halo zone around the colony was presumptive confirmation of phosphate solubilization and was measured. Solubilization efficiency (SE) was calculated by the formula as described by Sharma *et al*[13].

$$\text{Solubilization efficacy (SE)} = \frac{\text{Solubilization diameter-Growth diameter}}{\text{Growth diameter}}$$

Indole Acetic Acid (IAA) Production*:*

One ml overnight bacterial culture was inoculated into the test tubes containing nutrient broth with tryptophan (2 mg/mL) and incubated for 7-8 days. After a period of incubation, the bacterial broth was centrifuged at 10,000 rpm for 30 min and the supernatant was taken for the further process, the pellet was discarded. To 1 ml of the supernatant, 2mL of freshly prepared Salkowski's reagent (50 mL 35% $HClO_4$+ 1 ml 0.5 M $FeCl_3$) was added and incubated in dark for 30 min and observed for the presences of the pink coloured complex. Then the absorbance was measured at 530 nm using UV-Vis spectrophotometer.

Hydrogen cyanide production

To evaluate the presences HCN production was carried out according to Bakker and Schipperes[14] method. Bacterial isolate was streaked on Tryptic soy agar medium amended with 4.4 g/l of glycine. Whatman No.1 filter paper soaked in picric acid (0.05% solution in 2% sodium carbonate) was placed on the lid of the Petri plates. The plates were then sealed air-tight with parafilm and incubated at 28±2 ^{O}C for 72 h. A colour change of the filter paper from deep yellow to reddish-brown colour was considered as HCN production.

Morphological and biochemical studies of selected rhizobacteria

Rhizobacterial isolates exhibited both bio-control and PGP properties was selected for biochemical identification. These isolates were identified up to species level based on growth characteristics, staining reactions and biochemical tests.

Bacterization of rice

Seed bacterization was done according to the method described by Kumar and Bezbaruah[15]. Seeds of rice (*Oryza sativa* L.) were surface sterilized with 0.01% Mercury chloride for 30 sec and rinsed three times with sterile distilled water before bacterization. For bacterization, overnight grown rhizobacterial nutrient broth. The rice soaked in sterile distilled water was used as a positive control (T1) and rice seeds soaked in PDB broth with pathogenic fungi as a negative control (T2). To test the efficacy of rhizobacteria, the rice seeds were placed in bacterial broth for 24 hours alone (T3) and another set placed in bacterial and fungal culture together as (T4). Seeds were sown in polybags filled with sand: clay: normal soil in 1:1:1 ratio. The experiment was laid out in a completely randomized design as with two sets of replication.

Treatment Design

T1- Distilled water treated rice seeds (control)
T2- Pathogenic fungi (*R.solani*) treated rice seeds
T3- Rhizobacteria treated rice seeds (B9)
T4- Rhizobacteria + Fungal treated rice seeds (B9)

Biometric measurement

After 20 days of inoculation, the seedlings were selected randomly and data on growth promotion in terms of increase in shoot height, root height, number of leaves were recorded.

Statistical analysis

All the data were subjected to analysis of variance (ONE WAY ANOVA) and the significant difference among the means were compared by Duncan's Multiple Range Test (DMRT) at P=0.05 level using SPSS software.

IV. RESULTS AND DISCUSSION

In nutrient agar (NA) medium, the maximum microbial population was found to be in field 2 (130.0 x 10^8 CFU/g soil) followed by field 1 (120.0 x 10^8 CFU/g soil). Data on the population density of rhizobacteria is represented in Table 1. A total of morphologically different rhizobacterial isolates from two different soil samples were pure cultured and screened for their putative beneficial characteristics. Badri *et al*[16] explained that the microbial richness is by the abundance of the nutrients in rhizosphere regions such as amino acids, sugars, hormones, organic acids, and some small molecules released out from roots.

Table 1: Population density of rhizobacteria isolated from rice field

Sl.No	Location	CFU ml^{-1} soil	
		10^7	10^8
1.	Rice field 1	147.0 ± 0.01	130.0 ± 0.02
2.	Rice field 2	130.3 ± 0.2	120.0 ± 0.01

Values are mean of 3 replicates and presented as mean ± standard deviation (SD)

From the diseased rice leafs, two types of fungal pathogens (F1 and F2) were isolated based upon its colony morphology, structural characteristic and its spore type. It was identified as *Helminthosporium* sp (F1) and *R. solani* (F2) and it is shown in figure1.

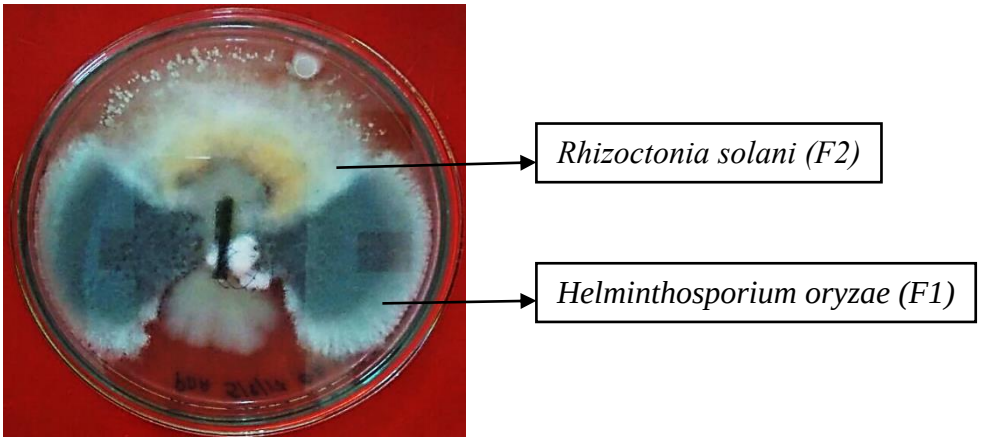

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Figure 1: Two types of pathogenic fungus grown out from infected rice leaf was shown in figure (F1) –*Helminthosporium oryzae* and (F2)- *Rhizoctonia solani.*

Helminthosporium sp, responsible for rice brown spot, causes infection on all growth stages of rice plant from the nursery to field and results in yield and grain quality losses. Rice brown spot was a major factor for the "Great Bengal Famine" during 1942–1943[3]. Another rice disease caused by *R. solani* is sheath blight, one of the most economically important rice diseases seen worldwide. It is a soil-borne pathogen having a broad host range including rice and soybean. Throughout the temperate and tropical rice-growing regions the epidemics of sheath blight occurs. During early heading and grain-filling stages the presence of high nitrogen amount and plant density offer favourable microclimate conditions for the occurrence of sheath blight[17].

In an antagonistic study, the rhizobacterial isolate B9 reduced *Helminthosporium oryzae* mycelial growth (20%) and the remaining isolates were found to be unresponsive against *Helminthosporium oryzae.* The mycelial growth inhibition of *R. solani* was found to be significant with rhizobacterial isolate B9 (73.7%) followed by B2 (51.2%). The isolates B8 (12.5%) and B3 (25%) showed very least mycelial inhibition of *R. solani* (Table 2, Figure 2). In accordance with our work Noori and Saud[18] isolated 20 strains of *Pseudomonads* from the rhizosphere soils of rice in Malaysia, the isolates TS3B5, TS3C8 and TS11 showed the maximum percent inhibition of radial growth of rice pathogen (PIRG) 65%, 52%and 51%, respectively under *in-vitro*. An inhibitory halo was observed, it suggested the production of fungistatic metabolites secreted by the bacteria[19]. Rye *et al.*[20] reported that *B. subtilis* GB03 and *B. amyloliquefaciens* IN937 produced volatile compounds such as 2,3-butanediol and acetoin showed induced resistance in Arabidopsis against *Erwinia carotovora.* Similar to present work Saranya and Sowndaram[21] isolated *Pseudomonas fluorescence* from rice rhizosphere and proved its antifungal activity against *R. solani* (85%) and *Sarocladium oryzae* (45%) by dual culture method.

Table 2: Effect of rhizobacteria on bio-control activity of fungal pathogens

Isolate code	**Percentage of inhibition (%)**	
	Helminthosporium oryzae	***Rhizoctonia solani***
B1	Nil	47.5 ± 0.3
B2	Nil	51.2 ± 0.6
B3	Nil	25.0 ± 0.9
B4	Nil	37.5 ± 0.1
B5	Nil	42.5 ± 0.5
B6	Nil	33.7 ± 0.9
B7	Nil	38.7 ± 1.0
B8	Nil	12.5 ± 0.7
B9	20±0.1	73.7 ± 0.3

Values are mean of 3 replicates and presented as mean ± standard deviation (SD)

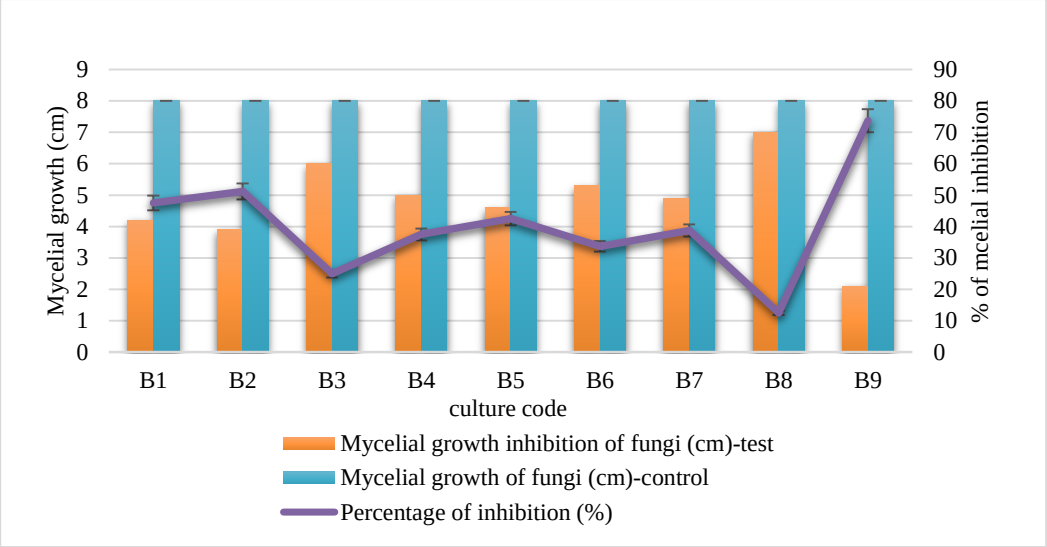

Figure 2 : Effect of rhizobacteria on bio-control activity of *Rhizoctonia solani*

Foot note: Bar diagram represents mycelial growth of fungi in test and in control plates, and line diagram represents percent of inhibition of plant pathogen *R. solani* by different rhizobacteria.

Four best bio-control rhizobacterial isolates were subjected to screen for further PGP traits (B1, B2, B5, and B9) under *in-vitro*. Different PGP traits of selected rhizobacterial isolates are represented in table 3. The rhizobacterial isolate B9 showed maximum PGP properties such as P solubilization, IAA production, HCN production and hydrolytic enzyme production. Similar to the present study, Ashrafuzzama *et al*[22] reported that the PGPR's have been shown to solubilize precipitated phosphates and enhance phosphate availability to plant that represents a possible mechanism of plant growth promotion under field conditions. Various researchers demonstrated the plant growth improvement of PSB strains inoculated with *Brassica napus*[23], *Zea mays*[24], *Brassica juncea*[25], *Stevia rebaudiana*[26], sugarcane[27] and cotton[28] . Saranya and Sowndaram[21] in their work they isolated *Pseudomonas* and *Rhizobium*, and studied its IAA production in the presence of tryptophan, showed 30mg/ml and 7mg/ml of IAA. There are many reports on the production of lytic enzymes by microorganisms, which have indirect bio-control active[29]. The hydrogen cyanide is an active antifungal compound produced by PGPR's and involved in bio-controlling activities[30].

Table 3: PGP traits of selected rhizobacteria

Culture code	Phosphate solubilization (SE)	IAA production (µg/ml)	HCN production	Cellulase activity	Amylase activity
B1	-	-	-	-	+
B2	67.0 ± 1.0	30.0 ± 0.8	+++	++	+
B5	59.0 ± 0.3	-	+++	+	+
B9	99.0 ± 0.6	45.0 ± 0.9	+++	+++	+++

Values are mean of 3 replicates and presented as mean ± standard deviation (SD)

Footnote: - = Nil, + = low, ++ = moderate, +++ = high,

Using Bergey's manual of determinative bacteriology the selected rhizobacteria were identified as *Micrococcus luteus* (B1), *Pseudomonas* sp (B2), *Pseudomonas* sp (B5) and *B. subtilis* (B9) which is represented in Table 4. The genera *Azospirillum*, *Azotobacter*, *Enterobacter*, *Klebsiella*, *Bacillus*, *Azoarcus* and *Pseudomonas* are attached to the roots and efficiently colonize root surfaces were already reported by many researchers. Weisskopf *et al*[31] reported that flagellar motility and citrate utilization properties are assumed to be responsible for root colonization and maintenance of bacteria in root and root zone.

Table 4: Morphological characteristics and biochemical identification of selected rhizobacteria

Test	**B**1	**B**2	**B5**	**B9**
Gram staining	G+ ve cocci	G-ve short rod	G-ve short rod	G+ve long rod
Spore staining	Absent	Absent	Absent	Present

Motility	Non-Motile	Motile	Motile	Motile
Indole	- ve	-ve	-ve	-ve
Methyl red	- ve	-ve	-ve	-ve
Vogues-Proskauer	-ve	-ve	-ve	+ ve
Citrate	+ve	+ve	+ve	+ ve
Triple Sugar Iron agar	K/K Gas -ve H2S -ve	K/K Gas –ve H2S -ve	K/K Gas –ve H2S -ve	A/A Gas –ve H2S -ve
Catalase	+ve	+ve	+ve	+ ve
Oxidase	+ve	+ve	+ve	+ ve
Urease	+ve	-ve	-ve	- ve
Nitrate reduction	+ ve	+ve	+ve	+ ve
Starch hydrolysis	-ve	-ve	-ve	+ ve
Carbohydrate fermentation Glucose	No acid formation -ve	No acid production -ve	No acid production -ve	Acid formation +ve
Identification	***Micrococcus luteus***	***Pseudomonas* sp**	***Pseudomonas* sp**	***Bacillus subtilis***

The pie chart (Figure 3) depicts the number of rice seeds germinated with the effect of bacterial bio-inoculants. The effective antagonistic B9 was identified as *B. subtilis* was used for nursery study. It is clear from the chart that, maximum number of rice seeds where germinated in the treatment set treated with bacteria alone (T3) (35%). T1 (distilled water treated) and T4 (bacteria and fungi treated) also have a fair number of germinated rice seeds (33% and 28%) but T2 (pathogenic fungus treated) has the least number (4%). So it is evident that the number of rice seeds germinated in the set T2 is very less and T3 has the highest germination. Elekhtyar[32] reported that the *P. flourescens* (PGPR *Pf*) inoculated rice in the nursery enhanced seed germination, seedling vigour and yield.

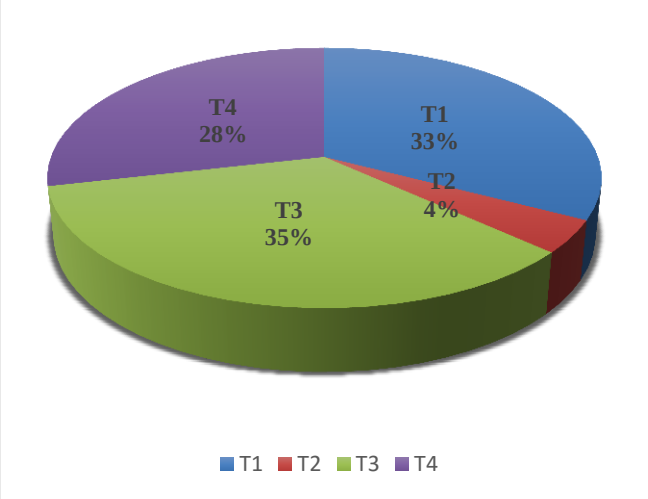

Figure 3: Effect of rhizobacteria on rice germination

T1 = Distilled water treated, T2 = Fungus treated, T3 = Bacteria (B9) treated, T4 = Bacteria (B9) + Fungus treated

The result for bio-control and PGP activity of rhizobacteria on rice crop was studied under nursery condition is represented in Fig 4. The length of the shoot, root and leaf of the plant were observed. Four set of plants were inoculated, and each had a variable growth level. Maximum growth was shown by T3 (bacteria treated) set. On the 10th day after the inoculation, it had a shoot length of 16.5cm, root length 8.34 cm and leaf length of 5.9cm whereas on the 20th day it has been raised to 21.3cm, 18.6cm and 15.15 cm respectively. T1 (distilled water) set shows the next highest plant growth. On the 10th day it has a shoot length of 14.8cm, root length of 7.12cm and leaf length of 4.2cm. On the 20th day it has 18.6cm, 15.47cm and 13.1cm respectively. The treatment T4 (bacteria + fungi) has a trend similar to that of T1. On the 10th day, it has a shoot length of 12.2cm, root length of 4.76cm and leaf length of 3.7cm. On the 20th day, it has 17.7cm, 13.3cm and 11.02cm respectively. The treatment T2 (fungi) has limited growth. It has only shoot length of 10.3cm, root length of 5.12cm and 2.2 cm of leaf length on 10th day and 14.5 shoot length, 11.75cm root length and 10.6cm leaf length on the 20th day. Overall, the graph depicts that the plant growth is maximum in the T3 set while T2 has retarded growth. In accordance with our study, Begum *et al*[33] reported that PGPR's indirectly improve the germination rate of seed and vigour index by decreasing the occurrence of seed mycoflora, which can be harmful to plant growth. Tiwari and Thimurthy[34] reported that isolate *Pseudomonas fluorescens Pfr1* promoted the shoot length and the number of tillers in rice and also effectively reduced the sheath blight severity when applied as foliar sprays. Besides plant height, an increase in the number of tillers increase was reported in the rice plants treated with the plant growth promoting rhizobacteria[35].

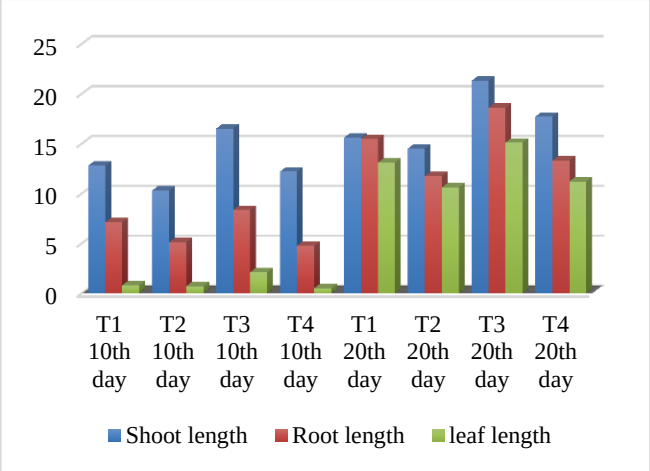

Figure 4: Effect of rhizobacteria on rice growth and biomass **T1 = Normal, T2 = *R. solani*, T3 = *B. subtilis* (B9), T4 = *B. subtilis* + *R. solani***

In the present study, the isolated indigenous rhizobacteria from rice rhizosphere exhibited promising bio-control and plant growth promoting activity under *in -vitro* and *in-vivo*. Thus, it is obvious from this investigation that the *Bacillus subtilis* under these studies can produce plant growth promoting substances and antifungal substances. So, these are suitable for the development of bio-fertilizer and bio-inoculants for agricultural crop plants. Bio-control of the disease using plant growth promoting rhizobacteria (PGPR) is a potential substitute to the presently available chemical control methods and it is described as cheap and not harmful approach to decrease crop damage produced by plant pathogens. Plant growth promoting rhizobacteria (PGPR) is a group of microorganisms in the rhizosphere that promotes plant growth by increasing nutrient availability and used as inoculants for bio-fertilization, phytostimulation and bio-control. The world over is changing from inorganic conventional farming towards organic eco-friendly farming methods. This not only requires the isolation of bio-inoculants with high potential for use as bio-fertilizers but also several other factors right from appropriate application procedures to correct marketing practices being economically cheaper.

V. CONCLUSION AND FUTURE SCOPE

Plant Growth Promoting Rhizobacteria helps plants in many different ways, in this study, rice rhizobacteria were isolated and screened for its *in-vitro* and *in-vivo* plant growth and bio-control potential. It can be concluded from the above study, the isolate B9 identified as *B. subtilis* exhibited potent PGP activity, bio-control traits and reduced disease incident in rice under nursery condition. The present results are promising for the formulation of potentially active PGPR based formulation which would be beneficial for rice growers to enhance growth in an eco-friendly manner.

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