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Thermotolerant Bacillus as Plant Growth Promoting Rhizobacteria

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Abstract: ***Bacteria that colonize plant roots and promote plant growth are referred to as plant growth - promoting rhizobacteria (PGPR). PGPR are highly diverse and in this article, we focus on rhizobacteria as biocontrol agents. The present study was conducted to characterize the native plant growth promoting (PGP) bacteria from compost sample and rice rhizosphere sample in Agricultural College and Research Institute, Madurai, Tamil Nadu for the isolation of Bacillus using Tryptic Soy Agar media, Nutrient agar media and Pikovaskaya media. Taken Nine isolates from compost sample using TSA Media and four isolates from rice rhizosphere sample using Nutrient Agar media and two isolates using Pikovaskaya media. The bacterial isolates were purified, screened in vitro for PGP characteristics and evaluated for their beneficial effects on the early growth of plants. Microscopic observation showed that most of the ten isolates bear rod shaped and Gram positive bacteria. Among the fifteen bacterial isolates, five isolates not able to adapt after 72 hrs of incubation and the population was drastically reduced at 70 - 75 degree Celsius at pH 4 - 5 and 8 - 10. Hence the isolates were adapted to grow at temperature ranges under three different carbon sources such as acetate, lactate and succinate from 40 to 65 degree Celsius at varied pH 6 - 7. All the seven isolates had significant result for IAA production in which four isolates ComB2, RRBN2, RRBPK2 and ComB10 showed higher phosphate concentration and phosphorus solubilization efficiency (350, 150.3, 145.3 and 142.0%).***

Keywords: Bacillus, PGPR,, Compost sample, Rhizosphere soil

1. Introduction

Beneficial free - living soil bacteria are usually referred to as plant growth - promoting rhizobacteria (PGPR, Kloepper et al., 1989). Independent of the mechanisms of vegetal growth promotion, PGPRs colonize the rhizosphere, the rhizoplane (root surface), or the root itself (within radicular tissues) (Gray and Smith, 2005). It is well established that only 1 to 2% of bacteria promote plant growth in the rhizosphere (Antoun and Kloepper, 2001). Bacteria of diverse general have been identified as PGPR, of which *Bacillus* and *Pseudomonas* spp. are predominant (Podile and Kishore, 2006).

Plant - associated bacteria can be classified into beneficial, deleterious and neutral groups on the basis of their effects on plant growth (Dobbelaere *et al.*, 2003). Beneficial free - living soil bacteria are usually referred to as plant growth - promoting rhizobacteria (PGPR, Kloepper *et al.*, 1989). The direct promotion of plant growth by PGPR entails either providing the plant with a compound that is synthesized by the bacterium, for example phytohormones, or facilitating the uptake of certain nutrients from the environment (Glick, 1995). The indirect promotion of plant growth occurs when PGPR lessen or prevent the deleterious effects of one or more phytopathogenic organisms. This can happen by producing antagonistic substances or by inducing resistance to pathogens (Glick, 1995).

The commercial production of agricultural crops requires the use of method that protect the crops from microbial pathogens that would otherwise reduce the yield and quality of the harvested crops. Alternatives to the use of synthetic chemicals has been an active area of research with the advent of organic and sustainable agriculture. Instead, more environmentally - friendly, safer methods of plant protection have been pursued; especially biocontrol approaches that utilize beneficial microbes (Warrior, 2000).

Biological control, utilizing beneficial microbes, is an excellent approach to limiting the adverse effect of disease - causing microbes on plant health and productivity. Considerable effort has been placed on identifying microbial biocontrol agents that can repress phytopathogens, especially those that are responsible for soilborne diseases, and that can enhance agricultural productivity (Cazorla *et al*., 2007).

Bacillus species are recognized as safe bacteria that produce substances that are beneficial for crops and the production of industrial compounds (Stein, 2005). In addition, Bacillus spp. also produce endospores, which helps the bacteria to survive harsh environmental conditions, can allow for germination by different environmental cues, can allow for long - term storage of the biocontrol agent, and reduce the complexity of the formulation process (Collins and Jacobsen, 2003). Notably, Bacillus species that are used for rhizosphere applications can also function as plant endophytes (McSpadden and Gardener, 2004) that also protect plants from pathogens (Romero *et al*., 2004).

Bacillus spp. produce antimicrobial metabolites that can be used as a substitute to the use of synthetic chemicals or as a supplement to the use of bio - pesticides, and biofertilizers, for controlling plant diseases (Ongena *et al*., 2005).

Induction of host resistance and plant growth

B. subtilis is a species of PGPR that are known to activate plant host defense response (host resistance) against pathogens. Host cells undergo ultrastructural and cytochemical changes in response to a pathogen attack. B. subtilis is known to activate induced systemic resistance (ISR) in the hosts that they occupy, which increases host resistance to plant pathogens. The activation of ISR by B.

subtilis is known to induce the synthesis of jasmonic acid (JA), ethylene, and the NPR1 - regulatory gene in plants (Garcia - Gutierrez *et al*., 2013).

Induction of a systemic agent in plant roots
Bacteria compete for nutrients with other resident microbes and with plant roots. As a result, the interactions between rhizosphere microbes and plants are critical. Mutual beneficial interactions have evolved, such as the provision of carbon compounds to resident microbes by their plant hosts, and increased nutrient and water uptake for the plant host due to the activity of the beneficial microbes. Induction of ISR and enhanced plant growth are other benefits derived by plants through their interactions with microbes (Gouda *et al*., 2018).

Among microbes, B. subtilis plays a significant role in PGPR activity and biocontrol. Activation of ISR is one of the benefits obtained from the use of B. subtilis. The ISR stimulus could be salicylic acid (De Meyer and Hofte, 1997) and/or the presence of rhizobacteria (Hallmann *et al*., 1999). *Bacillus subtilis* can be used to induce resistance (Aliye *et al*., 2008) by inducing the synthesis of defense enzymes in the host, such as POD, PPO, and PAL.

Plants activate defense mechanisms when a pathogen attack is perceived. This defense response often leads to systemic acquired resistance (SAR) process and the induction of a hypersensitive reaction; resulting in the formation of brown, desiccated tissue (Ryals *et al*., 1996). Disease severity is limited when the defense signal transduction pathway is activated (Van Wees *et al*., 2000). Inoculation of plants with B. subtilis strain (pf4) resulted in a high level of SAR. In relative comparison to non - inoculated plants, much higher levels of germination (96.5%), shoot length (9.0 cm), root length (8.03 cm), and vigor index (1703) were for inoculated plants (Anand *et al*., 2010)

Alleviation of biotic stress in plants by *Bacillus subtilis*
Organic farming practices consider the application of bacterial agents as an eco - friendly and safe way to increase productivity and disease resistance in crops (Dihazi *et al*., 2012). Myresiostis *et al*. (2015) have stated that the utilization of B. subtilis can reduce use of synthetic pesticides and insecticides in modern agriculture. Chemical fungicides and insecticides have a negative impact on beneficial soil microbes present that help to increase plant growth. Thus, the use of beneficial bacteria, such as. B. subtilis, could augment the application of other microbial pesticides as the use of chemical pesticides are terminated (Girolami *et al*., 2009). Bacillus thuringiensis (Bt), and the use of Bt toxin, provide a broad range of insecticide control (Navon, 2002), Bt also inhibits the growth of insect larvae and increases plant growth (Arrizubieta *et al*., 2016). B. cereus, B. amyloliquefaciens, and B. subtilis are also used to control pests (Gadhave *et al*., 2016). PGPR, such as B. subtilis, P. fluorescens, P. putida, and Paenibacillus administered through the use of coated, aluminum, gold, or silver nanoparticles, not only increased plant growth but also limited fungal growth in the rhizosphere.

2. Materials and Methods

Soil sample collection
Collected the rhizosphere rice field soil sample from the A block of AC&RI, Madurai and taken the five isolates according to the morphological characteristics of size, shape, color and margin of the isolates using Nutrient Agar media and Pikovaskaya media plates. Collected the 30 days decomposed (compost) sample from the Farm, AC&RI, Madurai and taken the 10 isolates according to the morphological characteristics of size, shape, color and margin of the isolates using TSA (Tryptone soy Agar media). . The temperature of the compost during the sampling period was about 60°C. The pH was recorded to be in the range of 8 - 9 indicating alkaline environment. Samples were collected from different sites of the compost heap and taken in sterile poly bags and immediately brought into the laboratory.

Isolation of bacteria
Bacteria were isolated in Nutrient Agar (NA) medium (Himedia, Mumbai) India following serial dilution technique. The procedure adopted was as follows: 10 gram of soil sample was diluted in 90 ml of sterile distilled water in 250 ml conical flask and kept it an orbital shaker at 150 rpm to get a homogenized soil suspension. Serial dilution was made and dilution of 10−7 was inoculated into Nutrient agar, Tryptone soy agar and Pikovaskaya agar plates and incubated at 37°C for 24 h. Isolated colonies growing on each diluted plates were transferred into freshly prepared specific media agar slants. The bacterial strains isolated were kept at in refrigerator for further study.

Determination for thermo - tolerance
Pure cultures of the bacterial isolates were determined for their thermophilic characteristics. Each bacterial isolates were inoculated into 5 ml of specific medium in test tubes. The tubes were incubated at initial temperature of 45 - 70°C for 12 h. After specified incubation period each broth culture of bacteria was streaked onto freshly prepared Nutrient Agar medium. Bacterial isolates growing in the plates were selected and again tested for their thermo - tolerance at higher temperature. Finally, a bacterium that could tolerate temperature of 45 - 65°C was selected for further characterization of PGPR traits study.

Identification and characterization of the isolate
The selected strain was observed morphologically and growth characteristics were studied. The isolate was characterized by Gram staining technique. Based on Gram's staining the strain was found to be Gram - positive and microscopic observation revealed rod shaped bacterium arranged in chain. Various biochemical tests like endospore formation, motility, anaerobic; catalase and oxidase tests, Citrate Utilization test, Spore formation under microscopic observation were performed. Morphological, microscopic observation and biochemical test indicated the bacterium to be *Bacillus* sp.

3. Result and Discussion

Isolation of thermotolerant *Bacillus*
Among the fifteen isolates, five isolates not able to adapt

after 72 hrs of incubation and the population was drastically reduced at 70 - 75 degree Celsius at pH 4 - 5 and 8 - 10. Hence the isolates adapt to grow at temperature ranges from 40 to 65 degree Celsius and varied pH 6 - 7 at three different. Carbon sources such as Lactate, Acetate and succinate (Table1). Only ten isolates (Five from compost sample and five from rice rhizosphere sample) were chosen for further PGPR traits. The screened isolates were ComB1, ComB2, ComB3, ComB4, ComB10, RRBN1, RRBN2, RRBN3, RRBPK1 and RRBPK2 (Fig 1.).

Table 1: Optical density of screened bacterial isolates exposed to different temperature

Culture/ Temp	pH	40	45	50	55	60	65
Com B1	6	0.35	0.28	0.25	0.15	0.03	0.01
	7	0.40	0.30	0.28	0.25	0.05	0.03
ComB2	6	0.45	0.30	0.25	0.18	0.13	0.10
	7	0.48	0.42	0.38	0.3	0.15	0.15
ComB3	6	0.47	0.35	0.25	0.15	0.13	0.10
	7	0.4	0.3	0.28	0.25	0.18	0.13
ComB4	6	0.35	0.33	0.25	0.18	0.03	0.01
	7	0.45	0.51	0.35	0.25	0.05	0.03
ComB10	6	0.38	0.35	0.30	0.25	0.15	0.15
	7	0.45	0.30	0.25	0.17	0.18	0.10
RRBN1	6	0.35	0.38	0.25	0.17	0.03	0.01
	7	0.40	0.35	0.19	0.25	0.05	0.05
RRBN2	6	0.49	0.38	0.35	0.25	0.09	0.1
	7	0.5	0.42	0.37	0.33	0.11	0.07
RRBN3	6	0.38	0.43	0.35	0.25	0.11	0.03
	7	0.40	0.38	0.42	0.38	0.06	0.07
RRBPK1	6	0.35	0.38	0.35	0.23	0.14	0.06
	7	0.43	0.35	0.37	0.30	0.07	0.09
RRBPK2	6	0.47	0.35	0.33	0.25	0.12	0.14
	7	0.51	0.46	0.42	0.3	0.14	0.11

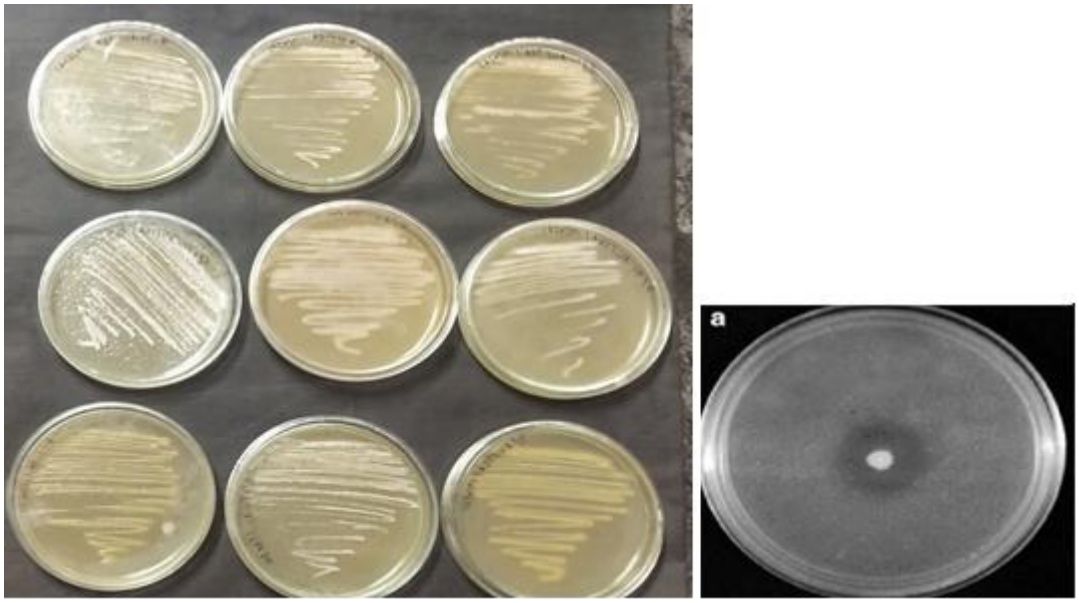


Figure 1: Isolates from Compost and Rhizosphere sample as thermotolerant *Bacillus*

Biochemical Characterization of the isolate

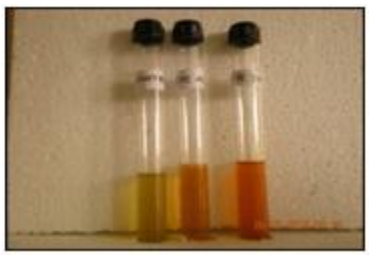
The isolates were characterized biochemically after exposure to utilization of those different carbon sources, there is variation and it has not shown any growth at acid and alkaline pH and some of the compost isolates grown well at temperature range of 40 - 65 but few rhizosphere bacterial isolates grown only at temperature of 40 - 55. A varied morphological difference and colour based colonies were chosen viz., nine isolates from compost sample using TSA Media and four isolates from rice rhizosphere sample using Nutrient Agar media and two isolates using Pikovaskaya media.

Culture/ Characteristics	Shape	Gram reaction	Catalase	Sporulation	Citrate Utilzation	Urease Productio	Ammonia Production	HCN production
ComB2	Long rod	+	+	+	+	+	+	+
ComB3	Short rod	+	+	+	+	-	+	+
ComB4	Short rod	+	+	-	+	-	Nd	-
ComB10	Long rod	+	+	+	+	+	+	+
RRBN2	Long rod	+	+	+	+	+	+	+
RRBN3	Long rod	+	+	-	-	+	+	+
RRBPK1	Short rod	+	+	+	+	+	+	+
RRBPK2	Long Rod	+	+	-	-	-	Nd	-

+ =Positive; - =Negative; Nd =Not determined

Microscopic observation showed that most of the ten isolates bear rod shaped and Gram positive bacteria

Urease production test



Ammonia Production Test

Indole acetic acid, Phosphate concentration and Phosphate solubilization efficiency

Culture/ Characteristics	IAA (ug/ml)	Phosphorus solubilzation efficiency (%)	Phosphate concentration (ug/ml)
ComB2	9.63	350.0	357.1
ComB3	6.37	93.8	103.5
ComB4	ND	ND	-
ComB10	3.81	150.3	260.6
RRBN2	4.16	142.0	266.2
RRBN3	3.06	110.7	225.5
RRBPK1	7.38	108.3	235.6
RRBPK2	4.36	145.3	260.2
SED	0.24	4.2	6.3
CD	0.52	8.9	12.7

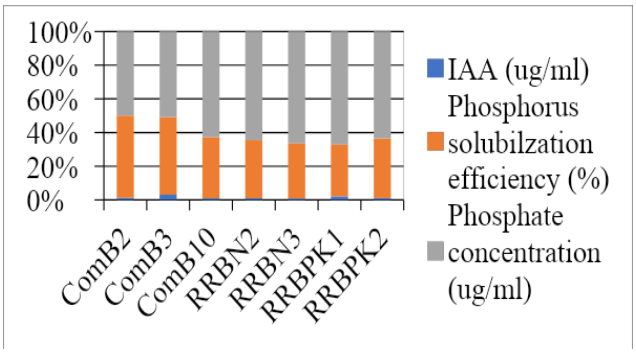


Figure 2: IAA, Phosphate concentration and Phosphorus solubilization efficiency

The isolates were screened and chosen only eight isolates *viz*., ComB2, ComB3, ComB4, ComB10, RRBN2, RRBN3, RRBPK1 and RRBPK2 in which ComB4 does not produce phosphorus solubilization efficiency and IAA and phosphate concentration (Table 2; Fig2) in very limited amount. Hence, seven isolates were screened for further study. Plant growth promoting properties such as IAA production, phosphorus solubilization, phosphate concentration, urease production, ammonia production, HCN production were studied for the seven screened isolates and again confirmed the morphological characteristics aswellas sporulation study. All the seven isolates had significant result for IAA production in which four isolates ComB2, RRBN2, RRBPK2 and ComB10 showed higher phosphate concentration and phosphorus solubilization efficiency (350, 150.3, 145.3 and 142.0%).

Poonguzhali *et al.*, 2008 suggested that the potential of bacterial isolates to produce IAA indicates their ability to use as growth hormones or growth regulators. Our results were in agreement with the previous study where the PGPR from the rhizosphere of *Brassica campestris* had shown to produce 6.02–29.75 µg/ml of IAA. The variation in the ability of PGPR to produce IAA found in the present study had also been reported earlier (Mansour *et al.*, 1994; Zahir *et al.*, 2000).

The beneficial effect of PGPR in maintaining adequate levels of mineral nutrients especially the P in crop production had been previously reported (Rodriguez and Fraga, 1999; Saravanan *et al*., 2007). In our study, four bacterial isolates were found efficient solubilizer of phosphate. The ability of PGPR strains to solubilize insoluble P and convert it to plant available form is an important characteristic under conditions where P is a limiting factor for crop production. In two different studies, very limited number of P - solubilizers (23.5% of the total tested strains and five out of the 207 isolates) has been reported (Hameeda *et al*., 2006; Islam *et al*., 2010). The soil phosphate solubilizing strains can increase the availability of phosphorus to plant by mineralizing organic phosphorus compounds and by converting inorganic phosphorus into more available form (Bar - Yosef *et al*., 1999). Phosphate solubilization is mainly due to the production of microbial metabolites including organic acids which decreases the pH of the culture media (Puente *et al*., 2004; Sahin *et al*., 2004; Shahid *et al*., 2012).

4. Conclusion

In the present study, efforts have been made to isolate the bacterial strains from the rhizosphere soil and compost sample collected. Eight bacterial strains were characterized based on morphology and biochemical properties. These bacterial isolates were named as ComB2, ComB3, ComB4, ComB10, RRBN2, RRBN3, RRBPK1 and RRBPK2 in which ComB4. These bacterial isolates were evaluated for their potential as PGPR.

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