Symbiotic organisms: Key for plant growth promotion

Sumbhe Aditi, Tomar Anupama

Abstract—Use of microorganisms with the aim of improving nutrients availability for plants is an important practice for agriculture. During the past couple of decades, the use of plant growth promoting rhizobacteria (PGPR) for sustainable agriculture has increased tremendously in various parts of the world. This is due to the emerging demand for dependence diminishing of synthetic chemical products, to the growing necessity of sustainable agriculture and environmental protection. The present study aims at isolating and characterizing plant growth promoting rhizobacteria to study their influence on plant growth and the prospects of using them as biofertilizers. In this present study the combine culture of rhizospheric *Actinomycetes* and *Bacillus subtilis* is used to improve and enhance plant growth and disease resistance. Soil organisms *Actinomycetes* and *Bacillus subtilis* were isolated, screened and characterized as PGPR. Both the organisms induced IAA, were able to solubilize phosphate and showed production of lytic enzymes. *Actinomycetes* showed better biocontrol activity against pathogenic fungi *Colletotrichum spp* than *Bacillus subtilis.* Also results of the present work showed the enhanced effect of using PGPR and comparative study between growth effects by pot culture method. Plants treated with combine culture showed enhanced growth compare to single culture treated plants and control plants.

***Index Terms*— PGPR, *Actinomycetes*, *Bacillus subtilis*, Rhizosphere, Biocontrol activity, IAA**

I.INTRODUCTION

Plant Growth Promoting Rhizobacteria (PGPR)

In soils, numerous microorganisms co-exist in association with plant roots. Some microorganisms live specifically in rhizosphere or on plant root surfaces, and these can have many effects on performance of the plant and may also affect the structure of the plant community.

Sumbhe Aditi, Department of Microbiology, S.S & L.S Patkar College of Arts & Science & V.P Varde College of Commerce, Goregaon (W), Mumbai - 400062, India . *Mumbai, India, Mobile No +91 9664631898.*

Tomar Anupama, Department of Microbiology, S.S & L.S Patkar College of Arts & Science & V.P Varde College of Commerce, Goregaon (W), Mumbai-400062, India.
Phone/ Mobile No +91 9022998307

A unique microflora is particularly present around the plant root surface, where various substances are secreted. Most of the microorganisms distributed around plant root surface have a role in the decomposition of organic matter and some may suppress deleterious microorganisms, which could inhibit plant growth. Some of the root-associated microorganisms can promote plant growth, and they have been called "plant growth-promoting Rhizobacteria" and "plant growth-promoting fungi".

Demand for agriculture

Agriculture contributes to a major share of national income and export earnings in many developing countries, while ensuring food security and employment. Sustainable agriculture is vitally important in today's world because it offers the potential to meet our future agricultural needs, something that conventional agriculture will not be able to do. Recently there has been a great interest in eco-friendly and sustainable agriculture. Reduction of the use of fertilizers and fungicides in agricultural production is necessary to help maintain ecosystems and to develop sustainable agriculture. The use of both bio-fertilizers and biocontrol systems can have minimal affect on the environment and such strategies have been widely researched. (Khalid *et al.,* 1997, Joseph *et al.,* 2007 and Leinhos *et al.,* 1994).

Scientific research

Involves multidisciplinary approaches to understand adaptation of PGPR, effects on plant physiology and growth, induced systemic resistance, biocontrol of plant pathogens, biofertilization, and potential green alternative for plant productivity, viability of co-inoculating, plant microorganism interactions, and mechanisms of root colonization. Significant increases in growth and yield of agronomically important crops in response to inoculation with PGPR have been repeatedly reported. PGPR can affect plant growth by different direct and indirect mechanisms. PGPR enhance the nutrient status of host plants can by: (1) biological nitrogen fixation, (2) inducing root surface area, (3) enhancing other beneficial symbiosis of the host. Several other mechanisms such as (1) increased mineral nutrient solubilization and nitrogen fixation, making nutrients available for the plant; (2) repression of soil borne pathogens (by the production of hydrolytic enzymes, siderophores, antibiotics, and/or competition for nutrients); (3) improving plant

stress tolerance to drought, salinity, and metal toxicity; and (4) production of phytohormones such as indole-3-acetic acid (IAA), *gibberellic* acid, (5) Ethylene production (6) Cytokinins production.

Use of rhizospheric bacteria as PGPR

Plant growth in agricultural soils is influenced by many abiotic and biotic factors. There is a thin layer of soil immediately surrounding plant roots that is an extremely important and active area for root activity and metabolism which is known as rhizosphere. The theoretical extent of the rhizosphere is dependent on the zone of influence of the plant roots and associated microorganisms. The rhizosphere is a metabolically busier, faster moving, more competitive environment than the surrounding soil. Amongst the rhizosphere microorganisms, the plant growth promoting rhizobacteria (PGPR) have been considered important in sustainable agriculture. Plant growth-promoting rhizobacteria (PGPR) are naturally occurring soil bacteria that aggressively colonize plant roots and benefit plants by providing growth promotion. Also PGPR have the biocontrol potential which can inhibit the various plant pathogen which affects plant growth. Given the negative environmental impact of artificial fertilizers and their increasing costs, the use of beneficial soil microorganisms such as PGPR and Endophytic fungi for sustainable and safe agriculture has increased globally during the last couple of decades as this group of bacteria helps in plant growth promotion without any negative impact on environment. (Beneduzi *et al*., 2012)

II.MATERIAL AND METHODS

Soil Sampling and Pretreatment

Soil samples were collected from different areas of Sanjay Gandhi National Park, Borivali. Soil from grass rhizosphere was collected. Each collection was made from 6-12 inches depth of the surface of ground. These samples were placed in sterile polythene bags, sealed tightly, and transported immediately to the laboratory. The samples were stored at 4°C in refrigerator.

Enrichment and Isolation

Culture of *Actinomycetes* was obtained by incubating enriching soil sample in YME (yeast malt extract) broth at room temperature on shaker (120 rpm for 72 hrs). Culture of *Bacillus subtilis* was isolated from the soil samples by heating the sample suspensions at 80°C for 30min in order to kill vegetative cells and non-spore forming bacteria. The suspensions were then enriched in Luria Bertani (LB) broth.

After enrichment isolation was done. 0.1 ml suspension from enriched YME broth and LB broth were spread on YME agar plates and LB agar plates respectively. YME agar plates were incubated at RT and LB plates were incubated at 28±2°C for 48 hours. Various characteristics were studied such as shape, size, elevation, surface, margin, color and pigmentation etc. were recorded.

Morphological and biochemical characterization

Morphological characteristics

Isolates of both *Actinomycetes* and *Bacillus* species showing different morphology were selected and their colony characteristics were studied. Gram staining was performed for identification.

Gram Staining

Isolates were studied for their Gram character, size and shape by Christen Gram staining method.

Characterization

Prior to performing biochemical tests, both the isolates were revived to get active culture. 18hr old culture was used for all the biochemical tests. Rapid test such as catalase test was performed followed by sugar fermentations (glucose, sucrose, lactose, fructose, and galactose), IMViC test, nitrate reduction, gelatin liquefaction, urease test and starch hydrolysis and growth in NaCl (%).

***In vitro* screening of bacterial isolates for their multiple plant growth promoting characteristics.**

Screening for IAA production

The production of IAA by isolates was determined according to the method of Gordon and Weber (1951) and (Khalid *et al*, 2004). The isolates of *Actinomycetes* were grown on yeast malt extract agar and *Bacillus subtilis* on LB agar at 28°C for 7 days. Eight millimeter diameter agar discs of both the isolates were inoculated into 100 ml of yeast malt extract and LB broth containing 0.2% L-tryptophan and incubated at 28°C with shaking at 125 rpm for seven days. Cultures were centrifuged at 11,000 rpm for 15 min. 1 ml of the supernatant was mixed with 2 ml of the Ferric chloride- sulfuric acid reagent. Development of pink color indicated IAA production.

IAA production by both the isolates of *Actinomycetes* and *Bacillus* was determined colorimetrically using salkowsky's reagent method Range- 10-50 µg/ml at interval of 10mcg/ml O.D was read at 530 nm. The level of IAA produced was estimated by a standard IAA graph.

ISSN: 2278 – 7798

Screening for phosphate solubilizing activity

The ability of both isolates to solubilize phosphate was assessed qualitatively on Pikovasky's agar medium. The total amount of phosphate solubilized by *Bacillus subtilis* and *Actinomycetes* isolates was estimated by Jackson (1973) method. Both the isolates were inoculated on Pikovskaya medium supplemented with tricalcium phosphate and incubated at 28°C for seven days. The halo zone around the colony was presumptive confirmation of phosphate solubilization. (Yasmin *et al*, 2009)

Biocontrol potential

Pathogenic Fungal discs (8 mm in diameter), 5 days old on YME (yeast malt extract) agar at 28°C were placed at the center of YME agar plates. In some plates Two *Actinomycetes* discs (8 mm) 5 days old, grown on yeast malt extract agar were incubated at 28°C placed on opposite sides of the plates, 3 cm away from fungal disc and in other plates two discs of *Bacillus* culture (8 mm) 5 days old, grown on LB were incubated at 28°C placed on opposite sides of the plates, 3 cm away from fungal disc. Plates without the *Actinomycetes* and *Bacillus* discs served as controls. All the plates were incubated at 28°C for 7 days and the colony growth inhibition (%) was calculated by using the formula, C - T/C x 100, where C is the colony growth of pathogen in control and T is the colony growth of pathogen in dual culture. The zone of inhibition was measured between the pathogen and *Actinomycetes* and *Bacillus* isolates (Kumar S. *et al*., 2012).

Detection of hydrolytic enzymes

Chitinase production

Both the isolates were grown in Minimal medium containing 1% chitin and it was incubated at 100 rpm in a rotary shaker at room temperature for 2 days. After 2 days of incubation the culture were harvested, centrifuged at 10000 rpm for 15 min at 4˚C and supernatant was collected. Minimal medium containing 1% chitin agar plates were prepared and culture filtrate of isolate was spotted in the center of the agar plate and incubated at 37˚C. After 12 hrs, the development of clear zone around the spot was observed. (K*umar et al.,* 2002).

Protease production

Spot inoculated the suspension of both the isolates on the center of the sterile 10% milk agar plate. Plate was incubated at room temperature for 24 hours. Checked for the zone of clearance around the colony.

Lipase production

Spot inoculated the suspension of both the isolates on the center of the Goradkowas tributyrin agar plate. Plate was incubated at room temperature for 48-72 hours and observed for zone of clearance around the colony.

Cellulase production

Both the isolates were enriched in Macbeth's broth containing cellulose (1% CMC) and incubated at room temperature for 8 days, on a rotary shaker. After enrichment, a loopful of the broth was spot inoculated on 1% CMC plate and then incubated at room temperature for 4 days. Then the plate was flooded with an aqueous solution of Congo red (1% in D/W) and shaken for 15 mins. Congo red solution was poured off and then flooded with 1N HCl, which changes the dye color to blue-violet and inhibits further enzyme activity. Colorless zone around the colony indicates cellulase production.

Pot culture method:

Evaluation of effective PGPR strains on seed germination and seedling vigor and plant specificity.

A pot culture experiment was conducted to study the influence of selected isolate of PGPR (*Bacillus subtilis and Actinomycetes*) on seed germination, growth and nutrient uptake of chickpea (chana) plants (legume), bajra (grain). Two types of plants (legume and non legume) were selected to check the plant specificity with both the culture that is with which plant type particular species giving better results. Seeds were selected were washed thoroughly under tap water and surface sterilized with St. 2.4% $NaOCl_2$ for 2-3 minutes. It was then washed with St. distilled water for 5-6 times. Seeds were then surface sterilized with 90% ethyl alcohol and Washed again with St. distilled water 2-3 times. Seeds were then air dried for 15 minutes. The seeds were soaked in 10 ml of the suspensions of only *Actinomycete*, only *Bacillus subtilis* and both *Actinomycetes* and *Bacillus subtilis* for 24 hrs and sterile blank nutrient broth served as control. Then the seeds were blot dried and sown in pots containing sterilized soil. Without disturbing the root system, the plants were depotted and root length, shoot length, were observed.

III.RESULT AND DISCUSSION

Isolation of of *Actinomycetes* and *Bacillus subtilis*

Isolates of *Actinomycetes* and *Bacillus subtilis* were successfully isolated from the rhizosphere soils of grass field from National park, Mumbai.

Colony characteristics of the *Actinomycetes* and *Bacillus subtilis* isolate

Colony characteristics	Isolate of *Actinomycetes*	Isolate of *Bacillus subtilis*
Color	Creamish whit	Creamish white
Size	Medium	Medium
Shape	Irregular	circular
Opacity	Opaque	Opaque
Margine	Irregular	Irregular
Elevation	Raised	Slightly raised
Consistency	Dry	Dry
Gram nature	Gram positive short rods	Gram positive short rods

Biochemical characterization

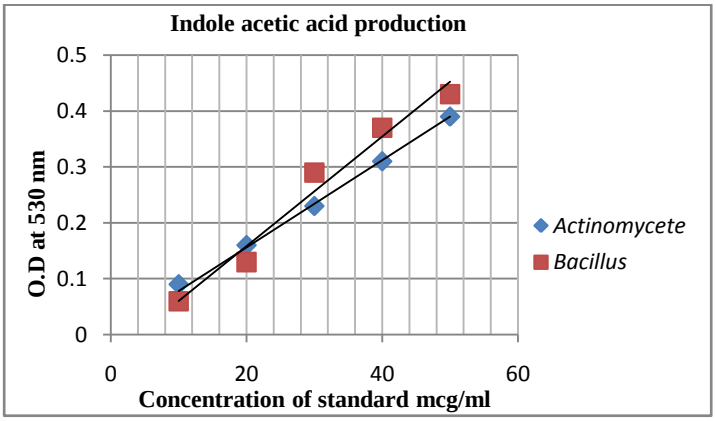
Test	Result	
	Actinomycetes	*Bacillus subtilis*
Catalase	+	+
Sugar fermentation		
Glucose	A	A
Lactose	A	A
Maltose	A	A
Sucrose	A	A
IMVC		
Indole	+	+
Methyl red test	-	-
Voges proskauer test	-	+
Citrate test	+	-
Nitrate reduction	+	+
Gelatin liquifaction	+	-
Urease test	+	Weakly +
Starch hydrolysis	+	+
Casein hydrolysis	+	+
NaCl 2%	+	+
NaCl 5%	+	+
NaCl 7%	-	+
NaCl 10%	-	-

Key: A – acid formation; + - positive test; - negative test

IAA production and phosphate solubilization

Organisms	IAA production	Phosphate solubilization
Actinomycetes	+	+
Bacillus subtilis	+	+

IAA production; + = development of pink colour
Phosphate solubilization; + = halo zone around the colony



The concentration of IAA produced by *Actinomycetes* was found 38 µg/ml and 44 µg/ml by *Bacillus subtilis*. The IAA producing activity of isolate was indicating *Actinomycetes* and *Bacillus subtilis* are an indole acetic acid producer.

***In vitro* testing for potential to produce hydrolytic enzymes**

Hydrolytic enzymes	*Actinomycetes*	*Bacillus subtilis*
Chitinase	+	+
Protease	+	+
Lipase	+	+
Cellulase	+	+

+ = production of enzymes; halo zone was observed around the cololny.

ISSN: 2278 – 7798

***In vitro* testing for biocontrol potential**

Isolates	Antagonistic activity against *Colletotrichum falcatum*
Actinomycetes	++
Bacillus subtilis	+

++ = strongly inhibited; > 40 %

+ = weakly inhibited; < 30 %

Pot culture method: evaluation of effective pgpr strains on seed germination and seedling vigor

More than 25 seeds of each type were sown in the pot. After 15 days of incubation, the germination percentage was found to be 95% of the TEST (1,only *Actinomycetes*; 2, only *Bacillus subtilis*; 3, with both) and 80% of that of CONTROL.

Plants which were treated with *Actinomycetes and Bacillus subtilis* showed better growth characteristics as compared to 'CONTROL'.

TEST	Shoot length			Root length		
	1	2	3	1	2	3
Chickpea	23.7 cm	22.33 cm	26.5 cm	7.8 cm	8.3 cm	12 cm
Bajra	25.4 cm	27 cm	29.7 cm	9.2 cm	8.4 cm	13.6 cm
CONTROL						
Chickpea	19.3 cm		5.6 cm			
Bajra	22 cm		6.3 cm			

Key: 1, only *Actinomycetes*;
2, only *Bacillus subtilis;*
3, both the cultures

DISCUSSION

In recent years, the concept of PGPR mediated plant growth promotion is gaining worldwide importance and acceptance. They are naturally occurring soil microorganisms that colonize roots and stimulate plant growth. Such bacteria have been applied to a wide range of plants for the purpose of plant growth enhancement and disease control (Barka *et al.*, 2000 and Chakraborty *et al.*, 2005). They promote plant growth by several mechanisms including nitrogen fixation, phosphate solubilization, hormone secretion and suppression of soil borne plant pathogens. Disease suppression may be due to iron sequestration, production of antimicrobials or induction of systemic resistance (Chakraborty *et al.*, 2005).Thus, biofertilizer preparations containing these organisms are cost effective, pollution free and a renewable source of plant nutrients, making them ideal partners and essential supplements to chemical fertilizers.

In the present study, two isolates of *Actinomycetes* and *Bacillus subtilis* were isolated from the rhizosphere of grass. The combine culture of rhizospheric *Actinomycetes* and *Bacillus subtitles* was used to improve and enhance plant growth, disease resistance. On screening, IAA production was shown by both the cultures; the production of IAA indicated by development of dark pink color. Quantitative assay of IAA was done to estimate the amount of IAA produced. Plant hormones can be natural or synthetic. There are several phytohormone groups and the best known is the auxin group. Diverse soil microorganisms including bacteria, fungi and algae are also capable of producing physiologically active quantities of auxins (IAA).

Phosphate solubilising activity was shown by both the cultures, indicated by formation of clear halo zone around the colony on Pikovasky's medium containing tricalcium phosphate which is an inorganic source of phosphate. This is because the organism grows using calcium phosphate as sole PO4 source. If insoluble PO4 is suspended in agar medium the isolates are readily detected by the zone of clearing around the colony. The solubilization is not restricted to Ca salts as Fe, Al, Mg, Mn, and other PO4's are also acted upon by them. Insoluble P compounds are mobilized, by production of organic acids. These organic acids and inorganic acids convert $Ca_3(PO_4)$ to diphosphate and monobasic phosphate with the net result of enhanced availability of the elements to plants. Further study was conducted to study the production of hydrolytic enzymes by both the cultures. Hydrolytic enzymes contribute to the biocontrol effect against various pathogens. Hydrolytic enzymes such as lipase, protease, cellulase and chitinase were studied by using respective media with provided substrate. Clear halo zone around colony indicated positive results which showed both the cultures are able to produce hydrolytic enzymes.

Biocontrol potential of both the cultures was also studied by using dual culture method for fungal pathogen. Both the culture inhibited fungal pathogen which showed *Actinomycetes* and *Bacillus subtilis*

are efficient biocontrol agents. *Actinomycetes* showed better inhibition than *Bacillus subtilis*

Pot culture method was carried out to study plant growth promoting activity of *Actinomycetes* and *Bacillus subtilis.* Comparative as well as combined effect was studied by inoculating seedlings with single as well as both the cultures. Plant specificity of these organisms with respect to different types of plants was also studied. Plants which were treated with culture (TEST) showed root n shoot length more than plants which were not treated (CONTROL). Both the cultures showed plant growth promoting effects in all the test plants that is these rhizospheric organisms are not plant specific. These microbes are efficient to promote plant growth in different types of plants.

REFERENCE

1) Beneduzi. A, Ambrosini. A, Luciane M. P and Passaglia "Plant growth-promoting rhizobacteria (PGPR): Their potential as antagonists and biocontrol agents" Review Article *Genetics and Molecular Biology*, vol 35:pp.1044-1051.(2012)
2) Joseph, B., Patra, RR., Lawrence, R."Characterization of plant growth promoting Rhizobacteria associated with chickpea (*Cicer arietinum L*)" *International Journal of Plant Production*, 1 (Suppl 2):pp.141-152 (2007).
3) Kapoor. R, Ruchi, Kumar. A, Kumar. A, Patil. S, Pratush. A and Kaur. M , "Indole Acetic Acid production by Fluorescent *Pseudomonas* isolated from the rhizospheric soils of Malus and Pyrus," *Recent Research in Science and Technology* 4(1):pp.06-09 (2012).
4) Karunya S. K, Reetha. D, Saranraj. P and Milton D. J "Optimization and Purification of Chitinase Produced by *Bacillus subtilis* and Its Antifungal Activity against Plant Pathogens," *International Journal of Pharmaceutical & Biological Archives* vol 2(6):pp1680-1685 (2011).
5) Khalid, A., Arshad, M., Zahir, ZA., and Khaliq, A, "Potential of plant growth promoting rhizobacteria for enhancing wheat (*Triticum aestivum L*.) yield," *J. Anim. Plant Sci.,* vol7: pp.53-56 (1997).
6) Khalid. A, Arshad. M and Zahir Z.A, "Screening of Plant growth-promoting rhizobacteria for improving growth and yield of wheat," *Journal of applied microbiology* 96, pp.473–480, (2004).
7) Kumar, NR., Arasu, VT., Gunasekaran, P, "Genotyping of antifungal compounds producing plant promoting rhizobacteria, Pseudomonas fluorescens," *Curr. Sci.* vol 82: pp.1463-466 (2002).
8) Kumar S. R, Manivannan. M, Tharmaraj. K and Shiney R. B Isolation, Screening, Characterization and Antagonism Assay of PGPR Isolates from Rhizosphere of Rice Plants in Cuddalore District. *International Journal of Pharmaceutical & Biological Archives* vol 3(1):pp.179-185 (2012).
9) Leinhos V "Effects of pH and glucose on auxine production by phosphate-solubilizing rhizobacteria *in vitro*," *Microbiological Research.* vol 194:pp.135- 138 (1994).
10) Yasmin. F, Othman. R and Sijam. K, "Characterization of beneficial properties of plant growth promoting rhizobacteria isolated from sweet potato rhizosphere," *African Journal of Microbiology research* vol 3(11):pp.815-821 (2009).

ISSN: 2278 – 7798