Journal of Scientific Research and Advances

Screening of phosphate solubilizing isolates of actinomycetes for *in vivo* assay for antagonistic activity against fungal pathogens

P. L. Dey[1,2,*] and B. N. Chakraborty[1]

Cite this article: J. Sci. Res. Adv. Vol. 2, No. 4, 2015, 137-140.

ISSN 2395-0226

Nine actinomycetes isolates were obtained from rhizosphere soil of plantation crops, out of these four isolates were found to be phosphate solubilizers and also showed antagonistic activity against three fungal pathogens *Rhizoctinia solani, Sclerotium rolfsii, Fusarium graminearum***.** It was observed that isolates ARHS/Mn3 could inhibit 68 % growth of that pathogen *Sclerotium rolfsii* and 66.66 % of growth was inhibited by the same isolate in case of *Rhizoctonia solani.* In case of *Fusarium graminarum* the percent inhibition was70 %. All of the isolates showed positive result for biochemical tests which confirmed that the isolates are actinomycetes . These isolates were further screened for potential phosphate solubilizers using two types of inorganic phosphates, tricalcium and rock phosphate in liquid medium, isolate ARHS/Mn2 showed maximum solubilization of phosphorous 875 mg/L isolate ARHS/Mn3 showed minimum of 255 mg/L of phosphorous solubilization when the media was supplemented with tricalcium phosphate. On the basis of the preliminary experiments carried out it was observed that soil actinomycetes could be used as potential biofertilizers and bio control agent.

Introduction

Among soil microorganisms, bacteria and fungi and to a lesser extent actinomycetes, have received considerable attention as biocontrol agents of soil-borne fungal plant pathogens and as plant growth promoters. Actinomycetes have special mechanism of adapting extreme environments. Within actinomycetes, *Streptomyces spp*. have been investigated predominantly, mainly because of their dominance on, and the ease of isolation from, dilution plates and because of the commercial interest shown on the antibiotics produced by certain Streptomyces spp. While *S. lydicus* also produces one or more antifungal antibiotics, its chitinase probably plays a significant role in the in vivo antifungal biocontrol activity of this rhizosphere-colonizing actinomycete. In the rhizosphere of the plant root system, or in its close vicinity, microorganisms abound. Among them, plant-growth-promoting microorganisms (PGPM) are the best studied [1]. It is now firmly established that bacterial and fungal inoculants can directly and indirectly benefit plant growth [2]. The direct promotion of growth is achieved by phytohormone synthesis and biological nitrogen fixation, making nutrients more available and reducing the membrane potential of the roots, etc. The indirect beneficial effect of PGPM is related mainly to their antagonistic properties toward phytopathogens. The solubilization of insoluble phosphates in the rhizosphere is the most common mode of action involved in PGPM that enhance nutrient availability to plants [2, 3]. Some properties of the phosphate (P)-solubilizing microorganisms of interest in the field of biogeochemistry and particularly in the maintenance of soil health and quality were described [4]. Many of non-streptomycete actinomycetes (NSA) taxa are therefore rarely reported in literature dealing with routine isolations of biocontrol agents and plant growth promoters from plant and soil. Seed-coating with powder formulation of the biocontrol agent was as effective as drench application of the fungicide, oxine benzoate (No-Damp), in controlling Rhizoctonia damping-off and superior to the commercial biocontrol agent, Streptomyces griseoviridis (Mycostop), applied to tomato seeds as seed-coating. In the present investigation an attempt was made to isolate and study different types of non streptomycete Actinomycetes (NSA) present in the rhizosphere of cultivated plants and characterization of the isolates as biocontrol agent against fungal plant pathogens and in the due course of experiments for utilization of agricultural purposes for the plant health promotion.

Materials and Methods

Collection of soil sample

Soil samples were obtained from a depth of 6-10 cm in the rhizosphere of *Mangifera indica* in Malda district, West Bengal, India [GPS Location 25°32’12"N .88°24’45" E]. The soil samples were allowed to keep at 04^{o}C temperature for a week in laboratory.

Isolation of Actinomycetes from soil samples

The soil samples were decimally diluted and plated onto actinomycetes isolation medium(Himedia) and starch caesin nitrate (SCN) medium plates by spread plate technique. After plating the plates were covered or wrapped with clean papers and then incubated for two days at 37^{O}C in a incubator to allow differentiation and adequate growth of the colonies. After 2-3 days of inoculation actinomycetes is obtained from mango rhizosphere soil. The isolates showed gram positive reaction and observed under microscope (Figure 1: 1-4).

Biochemical tests

Biochemical tests which are confirmatory for actinomycetes were executed to determine whether the isolates have the ability to hydrolyze starch, production of Catalase and production of indole ring.

Starch hydrolysis

The isolates were streaked on sterilized starch agar plate (NA + 0.1% soluble starch) and incubated for five days at 37º C. The plates were flooded with Lugol's iodine solution. The clear zone underneath and around the growth indicates the starch hydrolysis

Indole test

10ml of Davis Mingoli's broth supplemented with 0.1% tryptophan was inoculated with the isolate and incubated anaerobically at 37º C for 7 days. The culture were layered carefully with 2 ml of Ehrlich- Bobme (P-dimethylaminobenzaldehyde 10g, concentrated HCL 100ml) reagent on the surface, allowed to stand for a few minutes and observed for the formation of a ring at the medium reagent interface indicating the production of indole.

Catalase

Culture (24 hour old) was flooded with 0.5 ml 10% H_2O_2 solution and gas bubbles production indicated the positive reaction.

Phosphate solubilizing test in Pikovskaya's media

All the 9 isolates were inoculated in plates containing Pikovskaya's media [5], plates were incubated at 37^OC for 4-5 days. A clear halo zone around the colony indicated the phosphate solubilizing ability of the isolates (Figure 1: 4-7).

Evaluation of phosphate solubilizing activity of selected isolates

Nine selected isolates were grown in two sets of Pikovakaya's liquid medium (yeast extract, 0.50 g/L, dextrose, 10.0 g/L, calcium phosphate/rock phosphate, 5.0 g/L, ammonium phosphate, 0.50 g/L, potassium chloride, 0.20 g/L, magnesium sulphate, 0.10 g/L, manganese sulphate, 0.0001g/L, ferrous sulphate, 0.0001 g/L, pH, 6.5) amended with 0.5% tricalcium phosphate and 0.5 % rock phosphate separately over a period of 10 days. 50 ml of the liquid medium was inoculated with 5 % v/v of the mycelia suspension prepared from the 7 days old culture grown on SCN slants and incubated at room temperature for 4-10 days with routine shaking at 100 rpm. at 28^0C in a rotary incubator. The initial pH of the medium was recorded . The mycelia were harvested after 10 days of incubation by filtering and the change in the pH of the culture filtrate was recorded after centrifuging the medium at 5000 x g for 5 min. on a table centrifuge. Quantitative estimation of phosphate was done following amonium molybdate ascorbic acid method as described by [6]. Amount of phosphate utilized or solubilized by the isolates were expressed as mg/ L phosphate utilized by deducting the amount of residual total phosphate from the initial amount of phosphate source added to the modified Pikovakaya's liquid medium.

Trial for improvement of plant growth in pot conditions

The 4 phosphate solubilizing strains of actinomycetes i.e. ARHS/Mn2, ARHS/Mn3, ARHS/Mn5 and ARHS/Mn7 were grown in Starch Caesin Nitrate liquid media (Soluble starch – 10.0 g, Caesin (vitamin free) – 0.3g, Potassium nitrate – 2.0g, Sodium chloride – 2.0g, Dipotassium hydrogen phosphate – 2.0g, Magnesium sulphate – 0.05g, Calcium carbonate – 0.02g,pH – 7.5) in conical flasks. After their adequate growth the liquid media was centrifuged at 10.000 rpm and the pellate was taken and mixed with distilled water and a homogenous mixture is made.

Locally available varieties of Gram and soybean plants were taken to observe the effect of these isolates on the growth pattern, phosphate content in soil and plant roots. Suspensions of isolates in distilled water were inoculated in the Rhizosphere of potted plants over a period of one month at an interval of seven days. Growth of plant in cm, phosphate content in the soil and plant root were taken both before and after the treatment.

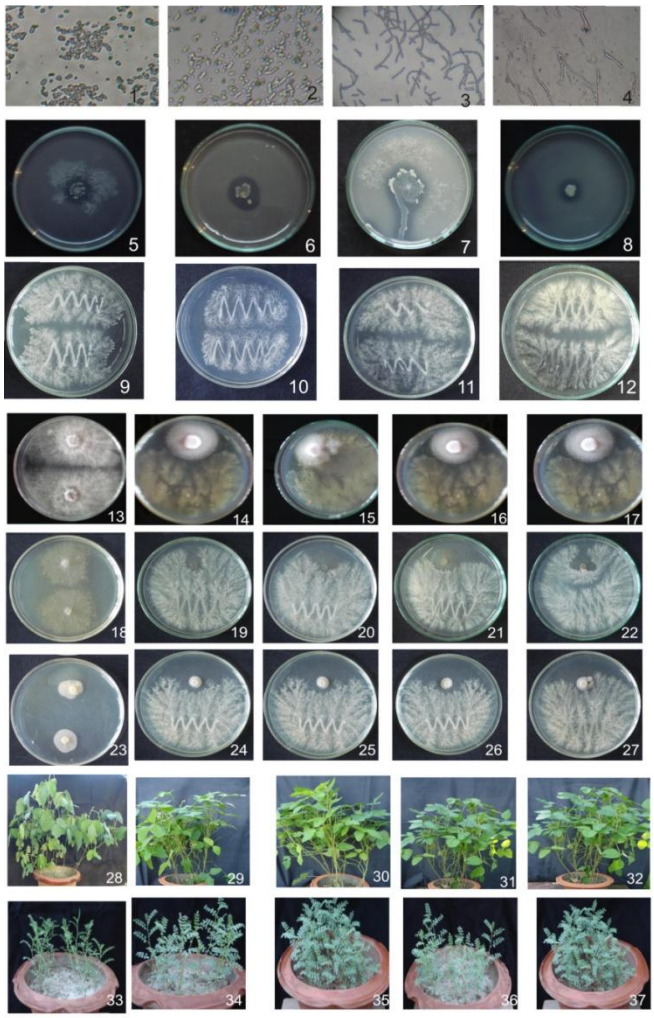

Figure 1 : Microscopic views of actinomycetes isolates (1-4); Screening of phosphate solubilizing activities (5-8); actinomycetes isolates (9-12;)Antagonistic activity of actinomycetes isolates with plant pathogens (14-27) where as control of *Fusarium graminearum* (9) *Rhizoctinia solani* (13) , *Sclerotium rolfsii* (18) and ; and treated *Glycine max* (29-32) and *Cicer areticulate* (34-37) with four actinomycetes isolates for field trial where as control of *Glycine max* (28) and *Cicer areticulate* (33).

Extraction of soil and root phosphate

Soil and root phosphate were extracted following the method as outlined by Mehlich (1953). The extracting solution known as Mehlich 1 extracting solution (0.025 N H_2SO_4, 0.05 N HCl) was used. Soil sample (5 g) was air dried and suspended in 25 ml of the extracting solution,0.01 g of activated charcoal was also added, shaken well for 30 min on a rotary shaker finally the

solution was filtered through Watman no.2 filter paper. The filtrate was collected and analyzed for "P" content. In case of plant samples, oven dried plant material was crushed with extracting solution. Estimation of the phosphate in the extract was carried out as previously described.

***In vitro* screening of Actinomycetes for antagonistic activity**

Out of the nine isolates of Actionomycetes four phosphate solubilizing strains were taken for study of their antagonistic effect against the plant pathogen *Fusarium graminearum, Rhizoctonia solani* and *Sclerotium rolfsii* (Figure :1 21-23). *In vitro* interaction studies between actinomycetes and above mentioned pathogenic fungi were made by dual plate method (Figure 1: 9-20). Both the test organisms were grown separately in the petriplates and inocula were cut from these plates by a cork borer. In each plate of nutrient agar media on one side the agar cup of the pathogen was placed and on the other side the actinomycetes was streaked 4 cm apart from each other. Another technique was also applied that the agar cup at the pathogen was placed on middle of the nutrient agar plate and actinomycetes was streaked around the agar cup. After 3-5 days incubation result was obtained.

Result and discussion

A total of nine actinomycets isolates were obtained from rhizosphere soil of mango. All of the isolates showed positive result for biochemical tests which confirmed that the isolates are actinomycetes (Table 1). These isolates were inoculated in plates containing Pikovskaya's medium, plates were incubated at 37^{O}C for 4-5 days. A clear halo zone around the colony indicated the phosphate solubilizing ability of four isolate (ARHS/Mn2, ARHS/Mn3, ARHS/Mn5 and ARHS/Mn7). These isolates were further screened for potential phosphate solubilizers using two types of inorganic phosphates, tricalcium and rock phosphate in liquid medium, isolate ARHS/Mn2 showed maximum solubilization of phosphorous 875 mg/L isolate ARHS/Mn3 showed minimum of 255 mg/L of phosphorous solubilization when the media was supplemented with tricalcium phosphate.

Table 1 Biochemical tests of Non-Streptomyces Actinomycetes (NSA) isolates from Rhizosphere soil of *Mangifera indica* [GPS Location 25°32'12"N .88°24'45" E]

Isolates Code	Catalase test	Indole test	Starch hydrolysis	Phosphate solubilizing activity
ARHS-MN-1	+	+	+	-
ARHS-MN-2	+	+	+	+
ARHS-MN-3	+	+	+	+
ARHS-MN-4	+	+	+	-
ARHS-MN-5	+	+	+	+
ARHS-MN-6	+	+	+	-
ARHS-MN-7	+	+	+	+
ARHS-MN-8	+	+	+	-
ARHS-MN-9	+	+	+	-

When the medium was supplemented with rock phosphate, isolate ARHS/Mn2showed maximum of 95 mg /L phosphorous solubilization and isolate ARHS/Mn3 showed minimum of 72 mg/L phosphorous solubilization when the media was supplemented with rock phosphate (Table 2). In both the cases the average drop in the pH was from 7 to 3.5. Acid production and drop in the pH of the medium have been reported in earlier studies [7, 8] however no significant relationship could be established in terms of phosphate solubilization and drop in the pH of the liquid medium. A greater part of the soil phosphorous (95-99%) is present in the form of insoluble phosphates and cannot be utilized by the plants [9] however, many soil fungi and bacteria are known to solubilize these inorganic phosphates [10].

In order to observe the effect of supplementation of four selected actinomycetes isolates on growth of gram and soybean plants, plant height, after inoculation was taken into consideration and observations were recorded at an interval of thirty days (Table 3). All the tested isolates increased growth in relation to control of which four isolates of actinomycetes were most effective. In order to determine the status of available soluble phosphate in the soil after inoculation of actinomycetes soluble phosphate was estimated from the soil and from root tissues. When estimation of phosphate from soil was conducted it was found that phosphate concentration decreases after inoculation with actinomycetes in gram plants due to the mobilization of net inorganic phosphate which is unavailable to plants to soluble phosphate by actinomycetes. Therefore the residual phosphate concentration in the soil decreases after ammendent with phosphate solubilizing micro organisms (Figure 1: 28-37).

When root phosphate of treated plants was estimated, it was observed that in case of *Cicer areticulate* and *Gycine max* maximum phosphate content was observed in case of pots treated with isolate ARHS/Mn5.

Phosphate level in the roots was found to be more in those plants treated with the amendments using isolates of ARHS/Mn5 (Table 4).

Table 2 Evaluation of phosphate solubilizing potential of actinomycetes isolates in the liquid medium amended with tricalcium and rock phosphate.

Isolate No.	Phosphate concentration(mg/L)			
	Tricalcium phosphate		Rock phosphate	
	7 days	14 days	7 days	14 days
ARHS/Mn5	225	475	55	72
ARHS/Mn3	114	255	22	72
ARHS/Mn2	400	875	40	95
ARHS/Mn7	274	426	54	74

While screening for antagonistic acvities of Actinomycetes isolates it was observed that isolates ARHS/Mn3 could inhibit 68 % growth of that pathogen *Sclerotium rolfsii* and 66.66 % of growth was inhibited by the same isolate in case of *Rhizoctonia solani.* In case of *Fusarium graminarum* the percent inhibition was 70 % (Table 5). The average percentage of inhibition of pathogens in all the cases was 50-80 %. This suggests that isolates of actinomycetes can be used as potential bio-control

agents against fungal pathogens (Figure 1: 9-27).

Table 3 Effects of treatments on growth of *Cicer arietinum* and *Glycine max.*

Isolates	*Cicer arietinum*[a]						*Glycine max*[a]					
	Initial		15 days		30 days		Initial		15 days		30 days	
	Length (cm)	No. of leaves	Length (cm)	No. of leaves	Length (cm)	No. of leaves	Length (cm)	No. of leaves	Length (cm)	No. of leaves	Length (cm)	No. of leaves
ARHS/Mn2	13	11	24	19	26	20	14	16	38	23	26	110
ARHS/Mn3	14	11	23	21	27	25	14	13	40	21	27	120
ARHS/Mn5	14	10	19	18	21	19	14	17	38	32	21	97
ARHS/Mn7	12	11	22	22	24	20	14	11	42	24	24	129
Control	11	10	17	12	20	17	14	10	39	22	20	103

1 month following treatment, results are mean of 10 replicates plants

Table 4 Estimation of phosphate from soil and root.

Cicer sp	Concentration of phosphate in soil before inoculation (mg/L)	Concentration of phosphate in soil after inoculation (mg/L)	Concentration of phosphate in root after inoculation (mg/L)
ARHS/Mn2	555	501	0.3
ARHS/Mn3	340	330	0.25
ARHS/Mn5	350	345	1.2
ARHS/Mn7	400	375	0.55
Control	370	370	0.54

Results are mean of 10 replicates plants

Table 5 In vitro antagonistic activity of actinomycetes isolates with fungal pathogens

Isolates	% of inhibition of the test pathogens		
	Sclerotium rolfsii	*Rhizoctonia solani*	*Fusarium graminarum*
ARHSMN2	59.00	59.52	61.63
ARHSMN3	68.00	66.66	70.00
ARHSMN5	54.00	64.28	54.00
ARHSMN7	59.00	54.76	69.00

Acknowledgement

Financial support from National Bureau of Agriculturally Important Microorganisms (NBAIM), ICAR, as a part of network project entitled Application of Microorganisms in Agriculture and Allied Sectors (AMAAS) is gratefully acknowledged.

Notes and References

[1] *Immuno-Phytopathology Laboratory, Department of Botany, University of North Bengal. Siliguri-734013, Darjeeling, West Bengal, India.*
[2] *Centre for Mycorrhizal Research, Biotechnology and Management of Bioresources Division, The Energy and Resources Institute (TERI), TERI, Gram, Gual Pahari, Gurgaon, Haryana-122001.*
* *Email : pannalaldey@gmail.com*

[1] J.K Vessey. Plant growth promoting rhizobacteria as biofertilizers. Plant Soil 255:571–586 (2003)
[2] H. Rodriguez, R. Fraga. Phosphate solubilizing bacteria and their role in plant growth promotion. Biotechnol Adv 17:319–339 (1999)
[3] A.E. Richardson. Prospects for using soil microorganisms to improve the acquisition of phosphorus by plants. Aust J Plant Physiol 28:897–906 (2001)
[4] P. Jeffries, S. Gianinazzi, S. Perotto, K. Turnau. The contribution of arbuscular mycorrhizal fungi in sustainable maintenance of plant health and soil fertility. Biol Fertil Soils 37:1–16 (2003)
[5] R.I Pikovskaya. Mobilization of phosphorus in soil connection with the vital activity of some microbial species. *Microbiologiya* **17** :362–370. (1948)
[6] D. Kundsen and D. Beegle. Recommended phosphorous tests. P.122-15, In : W.C.Dahnke(ed.) Recommended chemical soil tests procedures for the north central region. Bulletin no. **499** (revised) North Dakota agric.exp.sta.,Fargo, North Dakota (1988)
[7] M.H. Abd Alla. Phosphatases and the utilization of organic phosphorus by *Rhizobium leguminosarum* biovar *viceae*. *Lett Appl Microbiol* **18** : 294–296 (1994).
[8] M A Whitelaw. Growth promotion of plants inoculated with phosphate solubilizing fungi. *Academic press* Ed. D. L. Sparks. *Advances in Agronomy*, **69** : 99-151. (2000)
[9] M. Vassileva, N. Vassilev, M. Fenice and F. Federici. Immobolized cell technology applied in sobulization of insoluble inorganic (rock) phosphate and P plant acquisition. *Bioresource Technol*. **79**:263-271. (2001)
[10] P. Illmer and F. Schinner. Solubilization of inorganic phosphate by microorganisms isolated from forest soil. *Soil Bio. Biochem* **24**:389-395. (1992)