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By
Kislay Kumari and A. K. Roy

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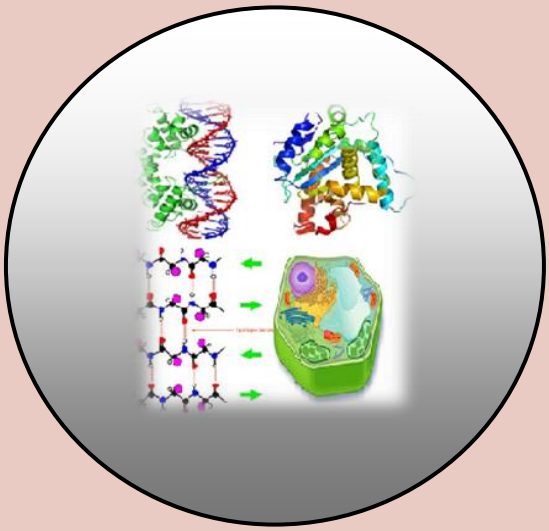

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Ms. Kislay Kumari
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Identification of Phosphate Solubilizing Actinomycetes by using 16S- rDNA and its Impact on Growth of C_4 – Plants (Maize Plants)

Kislay Kumari and *A. K. Roy

P.G. Department of Biotechnology, T. M. Bhagalpur University, Bhagalpur, Bihar, India
*B.N. Mandal University, Madhepura, Bihar, India

ABSTRACT

Biologically selective strains have come in the practice instead of chemical approaches to improve agricultural productivity for sustainable development since last two decades. The present study has been designed and aimed to select beneficial Actinomycetes exhibiting phosphate solubilizing potentials isolated from agric fields of Gangetic plains of Bihar. In total 14 isolates of Actinomycetes were shown positive P-solubilization activity on PVK medium. On the basis of solubilizing zones, AKR-14 (Streptomyces capparidis) & AKR-18 (Streptomyces cinnamoneus) were selected to check their role on growth of C_4 plant i.e. Maize, a major crop of this region. Isolates were identified by using 16S forward primer 5′– AGHGTBTGHTCMTGNCTCAS–3′ and reverse primer 5′– TRCGGYTMCCTTGTWHCGACTH – 3′. To know the growth stimulant capacity of selected strains were grown in an autoclaved phosphate deficient soil. Streptomyces capparidis (AKR-14) and Streptomyces cinnamoneus (AKR-18) were exhibited better stimulatory effect on maize plant growth. The maize plant showed 23% & 34% and 26% & 36% increase in biomass of shoots and roots respectively in comparison to control whereas the combination of both the strains showed synergistically affect i.e. 29% & 38% increase in biomass of shoots and roots respectively. The strains Streptomyces capparidis and Streptomyces cinnamoneus dependent growth promotion in the P_2O_5 supplemented soil was 13%, 14% and 17% respectively higher than the growth achieved by only P_2O_5 supplemented soil. Results were analyzed statistically by using ANOVA showed 0.05% significant amended with/without P_2O_5. Thus the Actinomycetes reported here have efficient phosphate solubilizing as well as growth promoting activities and may have potential field application.

Keywords: Actinomycetes, Phosphate solubilizers, Sreptomyces and C_4-plant (Zea mays).

INTRODUCTION

The microbes present in soil helps plant in growth promotion and yield. Actinomycetes are one of the major components of soil micro-flora and are actively participate in soil nutrient cycling as well as plant growth promotion.

They are free living, spore forming Gram positive bacteria having high G+C content in their DNA. They have filaments and branching pattern similar to fungi and conidia formation also resembles like fungi, therefore they are also known as "ray fungi".

Phosphorus (P) is one of the major essential macronutrients required for maximizing crop growth and production after Nitrogen (N). It is a vital nutrient available to plant roots only in soluble forms that are in short supply in the soil (Malviya et al. 2011). It is estimated that about 98% of Indian soil contains insufficient amount of available phosphorus, which is necessary to support maximum plant growth. The greater part of soil phosphorus, approximately 95-99% is present in the form of insoluble phosphates (Vessileva et al. 1993). It means that soil contain high amount of total-P, but it's availability to plant is very low and it is often a limiting factor for the plant growth (Mikanova and Novakova, 2002).

Phosphorus deficiency in plants shows symptoms of chlorosis, weak stem and slow or stunted growth (Patil & Mahantesh, 2011). In severe condition plants shows stunting, purpling or browning, appears first on lower leaves and base of stem and works on upward direction of plants, especially in the case of cereal crops. Even-though most of the agricultural soils have obviously large reserves of P- by adding chemically solubilized phosphate fertilizers during cultivation, a great part of them (approx 95-99%) is still strapped by Ca and Mg in calcareous soils and immobilized by ferrum and aluminum in acidic soils, resulted as less soluble and available to the crops (Azziz et al. 2012; Sharma et al. 2013). Naturally some microorganisms (i.e. Fungi, Bacteria and Actinomycetes) have the vital capacity to convert unavailable inorganic form of phosphate into available form in the soil (Walpola & Yoon, 2012; Sharma et al. 2013), where it is easily absorbed by plant roots.

Bihar is one of the largest maize (C_4 - plant) growing state and the crop is grown primarily to fulfill the basic need of life i.e. food for man as well as animals for a long time to till date. It is third largest maize producing state contributing around 10% to national production. In Bihar chemical phosphatic fertilizers is frequently used by farmers to increase plant yield, which is harmful for soil as well as environment also. Therefore the present work was design to find out the role of isolated Actinomycetes on P-solubilizing capacity and their effect on plant growth and health in field conditions. In this regard, P-solubilizers (AKR-14 & AKR-18) isolated from crop fields were identified by using 16S rDNA sequence and evaluated for their efficiency to release soluble Phosphate from insoluble phosphate.

MATERIAL AND METHODS

Isolation and identification of isolates

Actinomycetes were isolated from different crop fields of Bhagalpur district, Bihar (i.e. wheat, maize & paddy fields) by using serial dilution technique, isolates were cultured on starch casein agar medium (SCA). To find out its phosphate solubilizing activity, they were transferred on Pikovskaya's agar medium (PVK) and incubated at 28°±2°C for 7 days. Plates were regularly observed to find out the clear zone around the colonies which showed the phosphate solubilizing potential of isolates. The potent strains were further grown in NBRIP- medium and Genomic DNA was isolated according to the protocol provided by Genomic DNA isolation kit (HiMedia). The 16S rRNA genes (rDNA) were amplified by using prokaryotes 16S rRNA specific primer (F: 5'- AGHGTBTGHTCMTGNCTCAS-3' & R: 5'-TRCGGYTMCCTTGTWHCGACTH-3'). The PCR products of approx 1.3 kb size were electrophoresed on 1% agarose gel (HiMedia) in 1X TBE-buffer stained with bromophenol blue and ethidium bromide and visualized in UV- illuminator.

In order to assess the identities, the PCR products of the selected isolates were purified and performed sequencing by using ABI 3500 Genetic Analyzer Big Dye Terminator version 3.1 cycle sequencing kits (Chromous Biotech Pvt. Ltd, Bangalore). The 16S rDNA gene sequences (1230 & 845 bp) were used to search the Gene bank/EMBL/DDBJ database with the BLAST program (http//www.ncbi.nlm.nih.gov) to determine their relative phylogenetic positions. Phylogenetic analysis was conducted using MEGA 6.0 software.

Soil sample collection and analysis for experimental setup

Soil samples were collected from crop field located at N-25° 14′ 56.93″ and E- 86° 57′ 36.57″ in Bhagalpur district (Bihar). The soil of Bhagalpur is alluvial, mostly medium to coarse texture, white to light grey in colour. The soil of this region has low available phosphorus content. Soil samples were collected from 0 to 15 cm depth after removing 2 cm of the soil surface. Collected soils were properly grind and placed in sterile tightly closed zipper poly bags. The samples were stored at 4°C for further use.

The soil sample was autoclaved for 2 h at 121°C and 15 lbs pressure to destroy all native microorganisms. After autoclaving Total Carbon (TC), Organic Matter (OM), Total Phosphorus (TP), Total Nitrogen (TN), Potassium (K) and pH were determined by following standard methods. The Molybdenum blue method was used to determine the TP content (Olsen and Sommers, 1982) in the autoclaved soil used for experimental purposes.

Preparation of the inoculums with Actinomycetes strains

AKR-14 and AKR-18 strains isolated from agric fields of Bhagalpur (Wheat, Maize and Paddy) were previously selected for their P- solubilization abilities on PVK media (Kumari & Roy, 2016). On the basis of diameter of halozone, the performance of these selected strains was much better in comparison to other strains. Spores of these strains were stored in 20% glycerol at -20°C till further analysis. For inoculums preparation, inoculate 1ml of stored culture in 50 ml of NBRIP-medium in 250 ml Erlenmeyer flasks and incubated at 180 rpm for 3-5 days at 28°±2°C.

Seed treatment and effect of inoculums on plant growth

Maize (*Zea mays*) was used as the test plant in the present study. The seeds were obtained from the local farmers i.e. Srikar seeds variety (Eldorado Agritech Pvt. Ltd) generally used for cultivation purpose in this region. Surface sterilization of the maize seeds was achieved by dipping the seeds in a solution of 0.4% Sodium hypochlorite for 5min. After that seeds were rinsed with distilled water. One day before sowing, earthen pots of (equal diameter) were filled with 500g of the autoclaved soil.

For Standard basal doses followed the recommendation of Bihar Agricultural University for maize crop i.e. N (2.56 mg/100g ≡ 50 kg/ha) and K (1.28 mg/100g ≡ 25 kg/ha), fertilizers were applied in the form of ammonium and potassium sulphate, respectively. For phosphorus, two types of fertilizers were used, one was more soluble phosphate like triple super phosphate (TSP) and another was P_2O_5, which is less soluble in comparison to TSP. The standard amount of phosphatic fertilizer was 2.56 mg/100g ≡ 50kg/ha (Kumar et al. 2001).

The eight experimental situations were tested as following:

1. Soil without N, K, P and no inoculation ($N_0K_0P_0$).
2. Soil without NKP and inoculated AKR-14 ($N_0K_0P_0$ + AKR-14)
3. Soil without NKP and inoculated AKR-18 ($N_0K_0P_0$ + AKR-18)
4. Soil with standard NKP_{TSP} and without inoculation
5. Soil with standard NK and P_2O_5 without inoculation
6. Soil with standard NK supplemented with P_2O_5 & inoculated with AKR-14
7. Soil with standard NK supplemented with P_2O_5 & inoculated with AKR-18
8. Soil with standard NK supplemented with P_2O_5 & inoculated with AKR-14 & 18

Seeds were sown at the rate of 4 seeds/pot, with equal depth of 2 cm and watered each alternative days with distilled water. Each treatment was carried out in two replicates.

In all experimental situations, the plants were grown in a natural condition. Out of four seedlings only one plant was maintained per pot at that time application of microbial inoculums (10^6 cfu/seed) were applied to check its activity on plant growth. During study period, the nutrient levels of soil of different pots were checked (followed by standard protocol of APHA) at regular intervals of 15 days till plants attained one feet height (i.e. 30-45 days).

Statistical analysis

The data were analyzed by one way analysis of variance (ANOVA) at 5% level of significance with the help of Graph pad prism.5 software.

RESULTS

Isolation and identification of isolates

Total 25 colonies of Actinomycetes were isolated from crop fields out of which 14 isolates showed positive result on Pikovskaya's agar medium (PVK). On the basis of diameter of halozone, two prominent phosphate solubilizing Actinomycetes were selected to check their effect on plant growth. The 16S rRNA, a molecular based method using PCR is suitable for identification of Actinomycetes. For species identification, Actinomycetes genomic DNA was isolated through Actinomycetes DNA isolation Kit (HiMedia). The 16S rRNA (rDNA) genes were amplified by using the PCR- method with Taq-polymerase and prokaryotes 16S rRNA primer (F: 5'-AGHGTBTGHTCMTGNCTCAS-3' and R: 5'-TRCGGYTMCCTTGTWHCGACTH-3'). Samples of PCR products of selected test isolates on agarose gel are given in (fig-1).

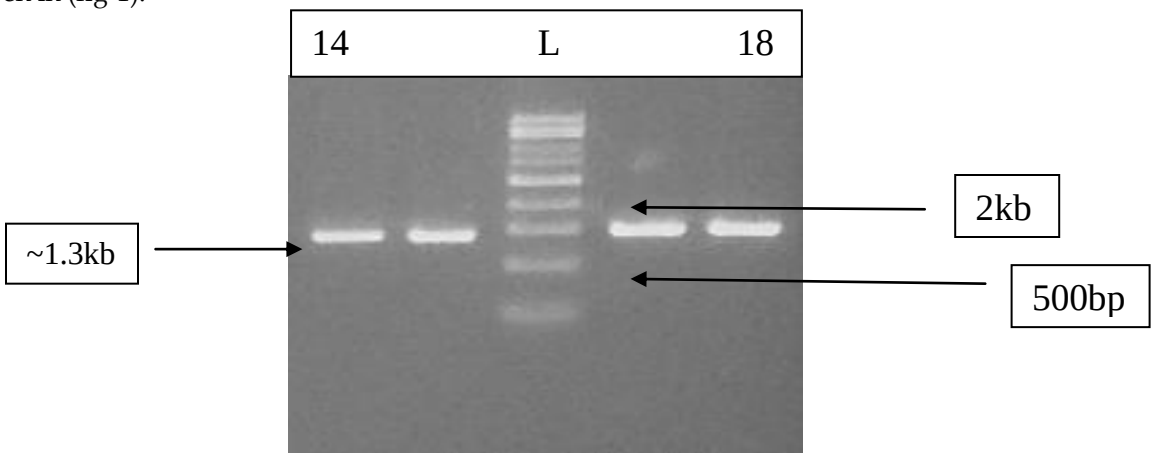

Figure 1. PCR amplification of 16S rDNA fragment from Actinomycetes samples. The size of PCR amplified product was ~1.3kb.

Figure 2. Maximum likelihood tree based on 16S rDNA sequences, showing relationships between AKR-14, AKR-18 and other representatives of *Streptomyces* family Bar 0.50 nucleotides substitution per site.

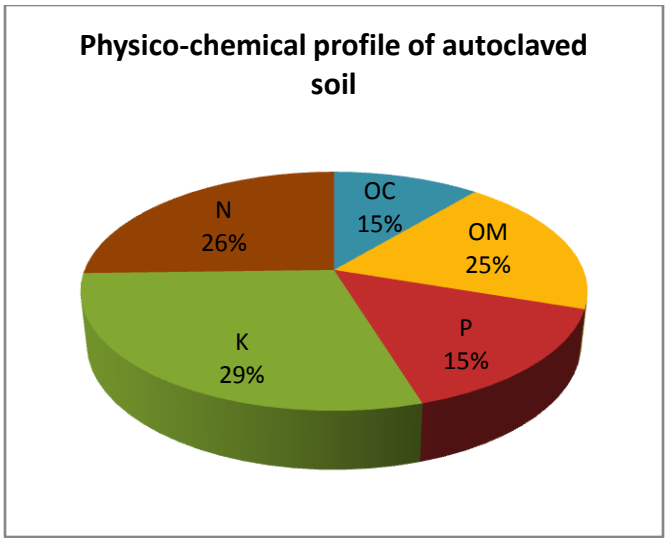

Figure 3. Physico-chemical profile of autoclaved potted soil.

The characteristics of two selected Actinomycetes isolates first of all verified with Bergey's manual (IV vol) and also after laboratory confirmation and biochemical tests, it was observed that two best P-solubilizing Actinomycetes (AKR-14 and AKR-18) were gram positive, filamentous, sporulating in nature which shows that they might be belongs to the genus *Streptomyces*. This is confirmed by 16S rDNA sequencing.

The 16S rDNA amplicon sequences (1230bp and 845bp) were aligned to databases than isolated strain AKR-14 showed 83% identity with *Sreptomyces capparidis* (accession NR156032.1) along with query cover 99% having E- value 0 and AKR-18 have 85% identity with *Sreptomyces cinnamoneus* (accession NR118280.1) having query cover 83% with E-value 6e-166. The taxonomic distance of the isolates depicted with the help of phylogenetic tree (Fig. 2).

Nutrients level of experimental soil

The pH of autoclaved soil was measured with the help of digital pH meter in a 1:5 (w/v) aqueous solution. The pH of potted soil was 7.2 and the total content of soil nutrients like Organic carbon, 1.4%, Organic matter, 2.41 %; whereas Phosphorus was 1.9 (mg/kg); Potassium, 3.7 (mg/kg) and Nitrogen was 4.3 (mg/kg) while the concentration of soluble phosphate was only 0.006 mg/kg (0.06 mg/100 gm) of soil at the beginning of experiment (Fig. 3).

After fortnightly evaluation of nutrient contents were fluctuated from 4.3 to 14.58 (mg/kg) for N; 3.7 to 10.23 (mg/kg) in case of K but P showed ranged from 1.9 to 16.54 (mg/kg) due to application of different doses of inoculums. The OC and OM were stabilized between 2.80 % and 4.82% respectively.

Effect of inoculants on plant growth

Effect of Actinomycetes strains on growth and phosphorus contents of the Maize plants; experimental results in absence of inocula, i.e. control ($N_0K_0P_0$) showed lower plant yields (Fig. 4A and 4B), whereas control soil with one of the inoculants either AKR-14 or AKR-18 showed 1.2% & 2.3% increase in shoot and 3% & 3.25% increase in root biomass respectively. However the dry weight of shoots & roots was increased by 63.4% & 80.75% respectively in NKP_{TSP} supplemented soil which were found highest in comparison to all the treatments as well as control. When Phosphate source was changed i.e. P_2O_5 at the place of triple super phosphates (TSP), the plant biomass was increased only by 10% (shoots) & 12.75% (roots) over control, whereas inoculation of any of the two strain (AKR-14/AKR-18) with P_2O_5

supplemented soil showed 23% & 26% (shoots) and 34% & 36% (roots) increase in, plant dry weight respectively but it was also noticed that combination of both the strains with P_2O_5 supplemented soil showed 30% (shoot) & 53% (root) increased in plant dry weight, which was higher in comparison to single strain inocula as well as control soil (only supplemented with P_2O_5).

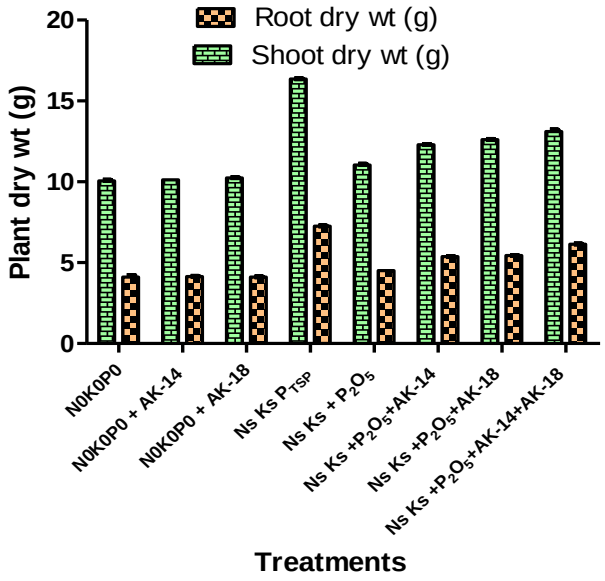

Figure 4A. Dry weight of maize plants after the influence of different treatments.

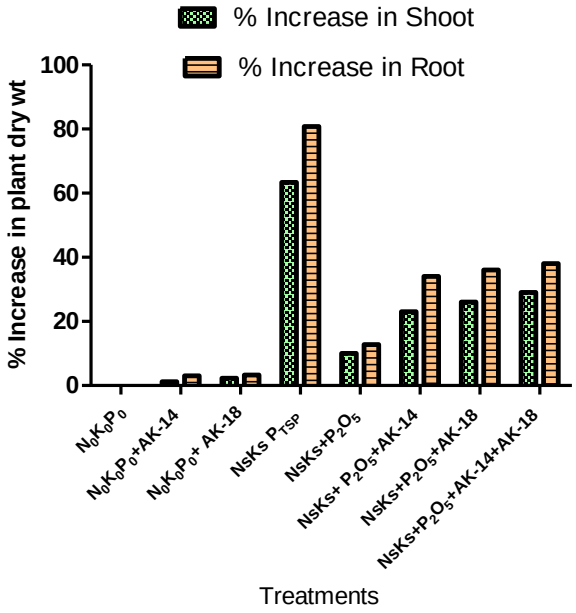

Figure 4B. % increase in the shoots and roots biomass in comparison to control after the treatments.

The patterns showed in biomass of plants, same patterns observed in case of phosphate (P) content in maize plants (Fig. 5A and 5B). The P – content of shoots and roots in presence of TSP without inoculums were increased 86.67% and 90% respectively, but the P – content of plants in the presence of P_2O_5 supplemented soil without inoculums showed 43.33% (shoots) and 41.67% (roots) increase.

Whereas P_2O_5 supplemented soil with inoculums (i.e. AKR-14/AKR-18 and both) showed 66.67%, 73.63% & 76.67% increase in shoots and 63.33%, 74.33% & 78.33% increase in roots respectively. On the basis of this experiment it was observed that P_2O_5 supplemented soil with inoculums always showed higher plant growth as well as P- contents in the maize plants in comparison to P_2O_5 supplemented soil without inoculums. It was observed that the presence of soluble Phosphate enhances the growth of roots more than in comparison to shoots.

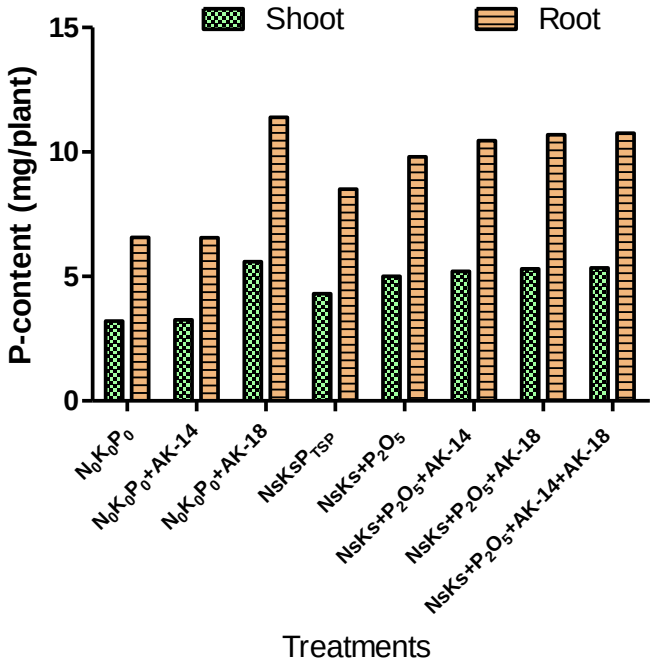

Figure 5A. Phosphate content accumulated in maize plants under the influence of different treatments with/without inoculums.

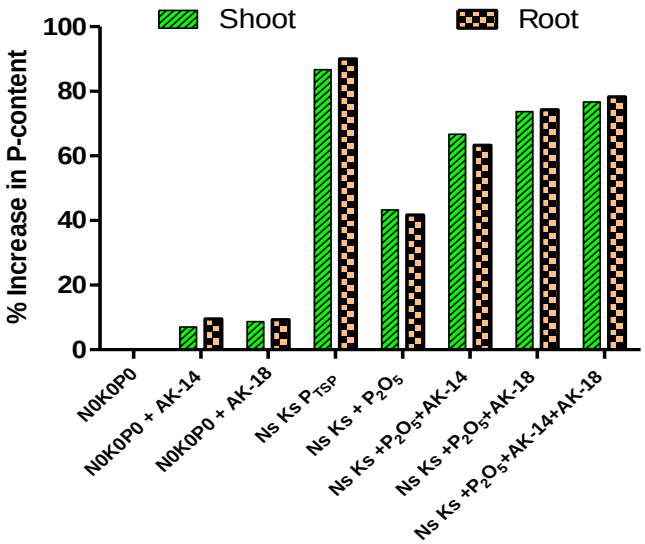

Figure 5B. % increase in P-content in comparison to control.

DISCUSSION

Soil microorganisms have the capacity to influence the rate of plant growth (Vessey, 2003). This might be linked by availability of C, N, P, Vitamins, phytohormones (IAA, GA) etc. In the presence of microbes nutrients become easily available by influencing the biogeochemical cycling process such as N-fixation and P-solubilization, which is mainly responsible to stimulate the plant growth (Vivas et al. 2006). Some microbes have the capacity to excrete siderophore and antifungal substances which helps the plants in growth (Xiao et al. 2002; Vassilev et al. 2006; Jain and Jain. 2007; Hamdali et al. 2008) and limits the plant pathogen.

Actinomycetes identification is a crucial task for every microbiologist because conventional culture methods are not suitable for appropriate identification of Actinomycetes. Therefore identification through 16S rRNA (rDNA) studies became most popular now a day. The 16S rRNA is a part of 30S subunit of prokaryotic ribosome. Its encoding gene sequence is a very suitable tool for molecular identification, clarity and neighboring relationship with other organisms by plotting phylogenitic tree. It has led to a wealth of information concerning prokaryotic diversity (Zhi et al. 2009; Tindall et al. 2010).

Actinomycetes having ability to solubilize phosphate were selected and first of all the isolates were morphologically and biochemically characterized and further 16S rDNA amplication confirmed that they belong to genera *Sreptomyces*. Isolates AKR-14 showed 83% identity with *Sreptomyces capparidis* while AKR-18 showed 85% identity with *Streptomycetes cinnamoneus* (Fig 2). *Streptomycetes* is a most dominating genus of soil living Actinomycetes because it constitutes more than 90% of total population of Actinomycetes (Jiang and Xu., 1990; Monki et al. 2009).

Two Actinomycetes reported here have not been evaluated for their P- solubilizing activity. Therefore these strains were selected for this experiment. *Sreptomyces capparidis* (AKR-14) and *Streptomycetes cinnamoneus* (AKR-18) had an ability to solubilize unavailable phosphate into available form. Phosphate is abundantly present in the soil as inorganic as well as organic forms, but it remains in an unavailable form for root uptake so, it acts as a major limiting factor for plant growth. Inorganic phosphate (P_i) present in soil is mostly in an insoluble mineral complex like Ca-P, Al-P and Fe-P, some of them are results of frequent application of chemical fertilizers. The plant roots are not able to absorb these insoluble precipitated forms of phosphatic compounds (Rengal and Marschner, 2005). Richardson, (1994) reported that organic matter have also the much more amount of an immobilized phosphate that contains 20-80% of P in soils and knowingly organic matter is also a good habitats for microbes. This is a surprising, only 0.1% of the total phosphorus exists in a soluble form utilize by plants (Zhou et al. 1992) due to its an unavailable form by P- fixation.

The present study consistently showed that AKR-14 and AKR-18 strains were make P available for the maize plant while combination of both the strain (AKR-14 and 18) showed synergistic effect on plant growth, otherwise insoluble phosphate, naturally present in the soil. This stimulatory effect was 13%, 14% and 17% respectively higher than that of achieved by only P_2O_5 supplemented soil without inoculums. Other authors have also demonstrated that plant growth promoting potential of *Sreptomymyces* sp in relation to wheat crop including Jog et al. (2012). It was also observed that the presence of soluble-P in the soil (TSP) had a greater stimulatory effect on root than on shoot growth. This observation suggests the existence of different kind of chemotactic mechanism of phosphate solubilization which might be possibly promote the growth of the plant root in order to spread in more area of the soil for phosphate scavenging. This type of results, in which root showed more growth in comparison to shoot is also reported in several other studies (Duponois et al. 2005; Shaharoona et al. 2006; Ouahmane et al. 2007; Hamdali et al. 2008).

It was also zestful to note that combination of both the strain (AKR-14 and AKR- 18) promote better shoot and root growth in comparison to single strain. This result might be due to combination of both the strains produce more bioactive compound instead of P- solubilization. Earlier workers including Coombs and France, (2003) and Conn et al. (2008) have also confirmed the importance of Actinobacteria for plant growth.

These significant links between the Actinomycetes strains and the plants are thought to improve the efficiency of the delivery of nutrients (including – Phosphate) from soil amended with the microorganisms to the plant (Coombs and France, 2003) and are extremely important for the success of the inoculants (Khan and Zaidi, 2007) as well as plant growth.

CONCLUSION

Hence the strains under study could constitute a novel and non-polluting bio-inoculants which might be useful for the development of sustainable agriculture.

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Corresponding authors: Ms. Kislay Kumari, P.G. Department of Biotechnology, T. M. Bhagalpur University, Bhagalpur, Bihar, India
Email: kislaybihpur.28@gmail.com; botanyakr@yahoo.co.in