Influence of *Streptomyces* sp. Kp109810 on Solubilization of Inorganic Phosphate and Growth of Maize (*Zea mays* L.)

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Abstract: Phosphorus plays a major role in plant growth, but only a small portion of phosphorus in soil is taken up by plants and the remaining part becomes unavailable for plants. Phosphate solubilizing microorganisms play a vital role in dissolving the complex form of phosphates to the accessible forms. The present paper reports the solubilization of tricalcium phosphate (TCP), single super phosphate (SSP), rock phosphate (RP), iron phosphate (FeP) and aluminum phosphate (AlP) by *Streptomyces* sp. KP109810 (S) with the production of organic acids as well as acid phosphatase. The amount of phosphate released (562 mg l^{-1}) was found to be the highest in the case of single super phosphate, as compared to the other phosphate sources. The highest phosphatase activity was observed in the presence of iron phosphate (0.033 Uml–1). The proposed thin layer chromatography (TLC) method was used to analyze the organic acids in culture broths of the following P sources. The numbers of these organic acids were six organic acids from SSP, seven from RP, four organic acids from FeP and two for each of AlP and TCP. The effects of *Streptomyces* sp. KP109810 on P solubilization from different P sources, plant biomass production, and P content of maize (*Zea mays* L.) were examined in a greenhouse study. A complementary greenhouse experiment was conducted in pots by growing maize as a test crop. Our findings showed a great efficient promotion of maize growth and P content compared to un-inoculated plants. Furthermore, single supper phosphate showed better results than rock phosphate, the latter performed comparably upon *Streptomyces* inoculation. These findings demonstrate that *Streptomyces* sp. KP109810 can improve crop growth and P nutrition.

Keywords: Streptomyces, TLC, Organic acids, Maize, PSB

INTRODUCTION

Phosphorus is one of the major essential plant macronutrient which plays a significant role in the development of roots, flowers and seed formation, helps in crop maturity and provides resistance to plant diseases (Khan and Jilani, 2009).

The function of certain key enzymes that are resp onsible for regulating metabolic pathways also depends on the availability of phosphorus (Tallapragada and Seshachala, 2012). Despite the high total soil P content, plant P availability is often reported to be limited (Collavino *et al.,* 2010). As a result, plants can utilize 10 to 15% of the P added as fertilizers during a year of application (Antoun, 2012). The remaining fractions are also rapidly converted into unavailable forms in the rhizosphere around the roots, without the expected impact on agricultural yield (Fernández *et al.,* 2012). These problems not only increase production costs, but also pollute the environment. Therefore, proper utilization of different P sources and improvements in their P-utilization efficiency are required to ensure sustainable food production and environmental protection across the globe.

The use of bioinoculants may be a better alternative and a complement to mineral fertilizers, since biological fertilizers could help to increase the availability of soil phosphorus, increase plant yield, minimization of harmful effects of phosphate fertilizers, reduce environmental pollution and promote sustainable agricultural development (Chen *et al.,* 2006). In the rhizosphere, a group of soil microorganisms known as phosphate solubilizing microorganisms (PSMs) play a key role to solubilize inorganic phosphates (Illmer and Schinner, 1995; Whitelaw, 1999). PSMs are divided into two groups (i) phosphate solubilizing bacteria (PSB) and (ii) phosphate solubilizing fungi (PSF) (Tallapragada and Seshachala, 2012). There are several previous reports about efficiency of PSBs alone or in combination with organic manures on different P sources for improving P release capacity or P solubilization (Harinasut *et al.,* 2003; Fernández *et al.,* 2007; Jain *et al.,* 2010; Harikrishnan *et al.,* 2014). Phosphate solubilizing bacteria (PSB), which are rhizobacteria have ability to convert complex form of phosphates to the accessible form through acidification, chelation, phosphatase enzyme and production of organic acids (Rodriguez and Fraga, 1999). Some organic acids produced by streptomycete strains were fumaric, tartaric and succinic acids (Encheva-Malinova *et al.,* 2014). These organic acids chelate cations (Al, Fe, Ca) bound to mineral phosphate forms and convert them into soluble forms available to plants through their hydroxyl and carboxyl groups (Panhwar *et al.,* 2012). Many works recorded that the seed inoculation with PSB belonging to the genera *Agrobacterium*, *Achromobacter*, *Aerobacter*, *Bacillus*, *Flavobacterium*, *Enterobacter*, *Serratia*, *Pseudomonas* and *Acinetobacter* improved growth, yield and P uptake in several crops (Hu *et al.,* 2010; Castagno *et al.,* 2011; Yu *et al.,* 2012).

The present study was undertaken to (i) establish relative potential rates of P solubilization of various phosphate sources by replacing tricalcium phosphate (present in Pikovskaya's medium) with rock phosphate (RP), single super phosphate (SSP), iron phosphate (FeP) and aluminum phosphate (AlP) by *Streptomyces* sp. KP109810. (ii) Examine the contribution of organic acids produced by *Streptomyces* sp. KP109810 in solubilization of phosphates. (iii) explore the role of

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Streptomyces sp. KP109810 as inoculant on maize plant growth in a greenhouse experimentof the studied traits.

MATERIALS AND METHODS

Phosphate solubilization in a liquid medium:

To determine phosphate solubilization, *Streptomyces* sp. KP109810 was screened on Pikovskaya's (PKV) broth medium with five different phosphate sources tricalcium phosphate (TCP), single super phosphate (SSP), rock phosphate (RP), iron phosphate (FeP) and aluminum phosphate (AlP) at initial pH adjusted to 7.0 and incubated at 30°C for 15 days in a rotary shaker at 125 rpm.. Un-inoculated flasks were served as controls. In order to remove bacterial cells and other insoluble materials, cultures were centrifuged at 10000 rpm for 20 min (Liu *et al.,* 2011). Parameters such as phosphate solublization, production of organic acid and phosphatase activity have been determined.

Phosphate estimation:

The Amount of phosphate released was determined by to Fiske-Subbarow method (Fiske and Subbarow, 1925). The supernatant (0.5 ml) was mixed with 1 ml of 2.5 M sulphuric acid and 2.5% ammonium molybdate. To the above mixture 1.0 ml of the reducing agent (0.2 g of 1.0 amino-2-naphthol-4 sulfonic acid and 1.2 g of sodium sulfate in 100 ml distilled water) was added. The available phosphorus was calculated at 650 nm by a spectrophotometer and calibrated with a standard KH_2PO_4 curve. Phosphate concentration was expressed in term of mg/ml phosphate released in culture.

Determination of organic acids by thin-layer chromatography (TLC):

Qualitative detection of organic acids produced in the media was performed by TLC with a procedure described by Lee *et al.* (2001). A volume of 50 µl from supernatant of Streptomyces sp. KP109810 culture and several organic acids were spotted in the bottom of silica gel plate's 0.25 mm silica gel plate (20 x 20 cm, aluminum oxide 60 F254). Separation was performed with acetone-water-chloroform-ethanol-ammonium hydroxide (60:2:6:10:22) as the solvent system. The spotted TLC plate was then placed in the bottom of the chromatographic chamber to ensure a sufficient supply of solvent vapor and the chamber was closed. The development of the chromatogram was allowed to proceed until the solvent had traveled 6-7 cm beyond the starting line for 20 min at the room temperature. Finally, the plate was dried at 120^0C for 15 min. The organic acids were detected by spraying an indicator solution of 0.25 g of methyl red and 0.25 g of bromophenol blue in 100 ml of 70% methanol and the color was developed by brief heating (1–3 min) in a hot dry oven 165^0C.

Enzyme activity:

Phosphatase activity was determined spectrophotometrically by using p-nitrophenyl phosphate as substrate as described by Eivazi and Tabatabai (1977). Five hundret microlitres of the bacterial cell free culture supernatant was mixed with 1.0 ml of pNPP solution along with 1.0 ml of sodium acetate buffer pH 5.3. The reaction mixture was incubated at 40°C for 30 minutes. The reaction was inhibited by the addition of 2.0 ml of 0.05 M NaOH and the absorbance was measured in triplicate at 410 nm. The enzyme activity is defined as the amount of enzyme required to release p-nitrophenol per ml, per minute under standard conditions.

Greenhouse Experiment:

The greenhouse experiment was conducted in the summer season 2020 at the Faculty of Agriculture, Suez Canal University, Ismailia, Egypt. This experiment was conducted to evaluate the effect of *Streptomyces* sp. KP109810 as bio-inoculants on maize (*Zea mays* L.) fertilized with different P sources. Six treatments were used in this investigation; control (C), *Streptomyces* sp. KP109810 (S+C), single super phosphate (SSP), rock phosphate (RP), S+SSP and S+RP. Inoculum of *Streptomyces* sp. KP109810 (S) was prepared in Luria–Bertani liquid medium at 28°C for 48 h to get 10^8 CFU ml ^{1}. Maize seeds (*Zea Mays* L.) were surface sterilized with 1.0% sodium hypochlorite solution for 5 min, rinsed thrice in sterile distilled water and the seeds were soaked in 20 ml of inoculum for 1.0 h under a gentle shaking. During the same period of time the control seeds were submerged in deionized sterilized water. The soil used in this experiment was sieved through a 2 mm sieve then was autoclaved and filled in plastic pots (25 cm diameter and 35 cm depth) at the rate of 4 Kg pot^{-1}. For the experimental group, 200 ml of *Streptomyces* sp. KP109810 suspensions (10^8 CFU/mL) were mixed thoroughly with the soil for 2 min. Non-inoculated soil served as a control. Inoculated and non-inoculated seeds were planted in plastic pots (25 cm diameter and 35 cm depth) each pot was filled with loamy sand soil. Each pot was planted with 5 seeds and then thinned to three plants after 14 days. A completely randomized design with three replications was used. Phosphorus from different sources including RP and SSP was applied at the rate equivalent to 150 mg P kg ^{1}soil.

The data on various growth parameters including, plant dry weight (g), plant height (cm), stem length (cm), root length (cm), plant fresh weight (g) and P-content (mg/plant) were determined after 45 days from sowing. Plant P-content in the vegetative tissue of a plant (shoot + leaves) was determined by the vanado-molybdate phosphoric blue color method (Olsen and Sommers,1982).

Statistical Analysis:

Preliminary data for all experiments were statistically analyzed using the appropriate analysis of variance according to (Steel, 1997) using the one way analysis of variance. A Computer program software CoStat version 6.311 was used to analysis the data of all experiments. Least significant difference (LSD) at 5% level was used separately to evaluate the response of each character in each experiment.

RESULTS AND DISCUSSION

Phosphate solublization:

Phosphorus solubilization efficiency of *Streptomyces* sp. KP109810 was performed in Pikovskaya's broth supplemented with five phosphate sources TCP, SSP, RP, FeP and AlP after incubation period of 15 days. Figure (1), represents the ability of *Streptomyces* sp. KP109810 to dissolve insoluble phosphates for all the substrates. The results showed that the inoculation with *Streptomyces* sp. KP109810 has a positive effect on the five substrates. The amount of phosphate liberated reached its highest with SSP (562 mg $l^{-1)}$ followed by TCP (423 mg l^{-1}). Rock phosphate was found to be better compared to FeP and AlP. These findings are in a good with those obtained by Hamdali *et al.* (2008), who found that the levels of phosphate solubilized by *Streptomyces griseus* and *Streptomyces cavourensis* were 58.9 and 83.3 mg/100 ml respectively. In this respect, (Zhen Dong *et al.,* 2013) isolated four P-solubilizing bacteria from the plant Anaphalis lacteal rhizosphere and their P-dissolving rates ranged from 65.24 to 315.36 mg/l. Similar studies have been reported earlier on solubilization of TCP, RP and AlP by differrent microorganisms (Kang *et al.,* 2002; Nath *et al.,* 2012). The solubilization of triple super phosphate by two *Bacillus* spp. PSB 9 and PSB 16 was reported by Panwar *et al.* (2016). Son *et al.* (2006) isolated a P-dissolving Pantoea from the soybean rhizosphere, and soluble P in its culturing medium reached to 900 mg/l.

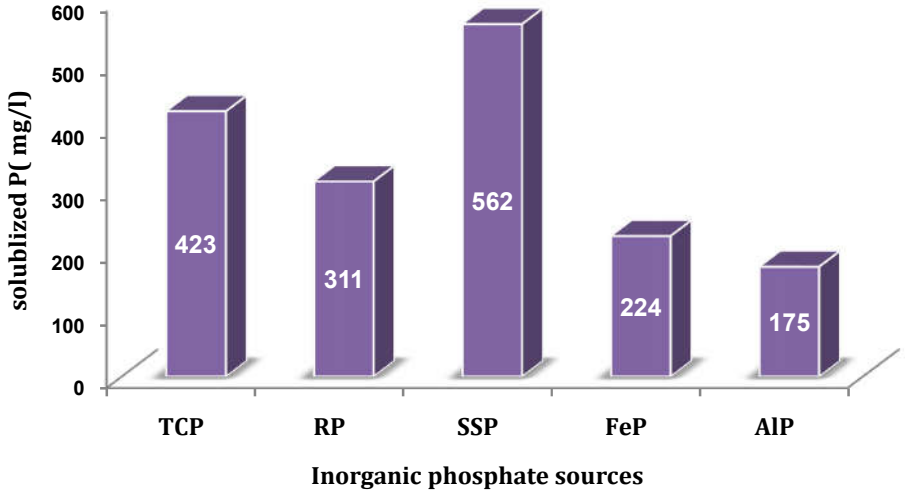

Fig (1): phosphate solublization by *Streptomyces* sp. KP109810 in Pikovskaya's (PVK) broth media during 15 days. Tricalcium phosphate (TCP), single super phosphate (SSP), rock phosphate (RP), iron phosphate (FeP) and aluminum phosphate (AlP)

Organic acid secretion during phosphate solubilisation:

Detection of organic acids in the culture broth of *Streptomyces* sp. KP109810 was observed through TLC during the solubilization of SSP, TCP, RP, FeP and AlP. Table (1) shows that the number and type of organic acids differed depending on the different sources of P. Numbers of organic acids produced during the solubilization of SSP, TCP, RP, FeP and AlP were 6, 2, 7, 4 and 2 acids, respectively. The production of organic acids during phosphate solubilization is a common phenomenon, and the type of acid produced depends on the type of phosphate source (Vyas and Gulati, 2009; Mardad *et al.,* 2013). Our findings are consistent with Vazquez *et al.* (2000), who also reported the production of succinic acid, lactic acid, etc. Many papers reported that major organic acids were produced by actinobacteria such as citric acid, gluconic acid, lactic acid, malic acid, and oxalic acid (Chen *et al.,* 2006; Jog *et al.,* 2014; Yin *et al.,* 2017).

Phosphatase activity

Phosphatase enzymes are believed to be very important in the uptake of phosphorus by maize plants. The highest phosphatase activity was found in the media amended with RP (0.091 Uml^{-1}), followed by AlP (0.071 Uml^{-1}), SSP (0.062 Uml^{-1}), TCP (0.042 Uml^{-1}) and the lowest activity was found by FeP (0.033 Uml^{-1}) (Fig. 2). Singh *et al.* (2000) stated that P. indica produced acid phosphatases to dissolve complex forms of phosphate present in rhizosphere to be more accessibility by plants. Roca *et al.* (2013) found that the presence of insoluble phosphate for which PSB can generate enzymes and organic acids to solubilize phosphorus was the reason behind greater alkaline phosphatase enzyme activity.

Table (1): Detection of organic acids produced by *Streptomyces* sp. KP109810 and the different inorganic phosphate sources

	SSP	RP	FeP	AlP	TCP
Oxalic acid	+	+	-	+	+
Lactic acid	+	+	+	-	+
Citric acid	+	+	+	+	-
Succunic acid	+	-	-	-	-
Gluconic acid	+	+	-	-	-
Malic acid	-	+	+	-	-
Furmic acid	-	-	+	-	-
Acetic acid	+	+	-	-	-
Propionic acid	-	+	-	-	-
Presence (+)		+	**Absence (-)**		

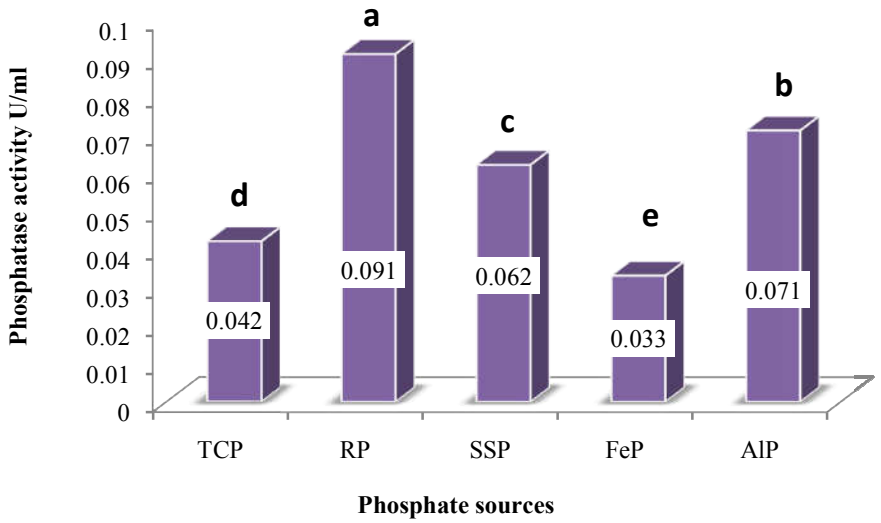

Fig (2): Effect of different phosphate sources on phosphatase activity by *Streptomyces* sp. KP109810. Tricalcium phosphate (TCP), single super phosphate (SSP), rock phosphate (RP), iron phosphate (FeP) and aluminum phosphate (AlP)

Greenhouse experiment

Increased plant height, plant fresh weight, shoot dry weight and root dry weight of maize plants were recorded with the plants inoculated with *Streptomyces* sp. KP109810. The plant height and plant weights (fresh and dry weights) were significantly ($p \leq 0.05$) increased by the applied P fertilizers in the presence or absence of *Streptomyces* sp. KP109810 (Fig. 3). The total biomass (shoot and root weights) of the plants grown in soil amended with S+RP was significantly ($p \leq 0.05$) greater compared to RP alone. Similarly, SSP with S displayed higher values compared to their sole application without S. It was observed that for most of the growth characteristics of the plants co-inoculated with S and amended with SSP displayed significantly ($p \leq 0.05$) higher values than those supplemented with inorganic P fertilizer (RP+S). An increase in P content was observed in plants inoculated with S. The P-uptake of maize was significantly ($p \leq 0.05$) higher in plants treated with RP compared to those grown in the control (Fig. 4). Application of S with different P sources showed significantly ($p \leq 0.05$) increase in plant P content compared to the treatments without S. It was recorded that plants treated with SSP displayed higher P-content compared to those treated with RP (Fig. 4). The P-content in plants treated with RP without S was 6.8 mg plant ^{1} that had been increased to 11.5 mg plant ^{1} when treated with S. Increased growth and P content of several crop plants due to PSB inoculation have been reported in many studies conducted under both growth chamber and greenhouse conditions (Tao *et al.,* 2017; Yadav *et al.,* 2017; Danso Marfo *et al.,* 2019). Yin *et al.* (2017) also reported detection of seven to eight organic acids following the application of PSBs that resulted in the solubilization of P and also had a positive effect on plant growth. Afzal and Bano, (2008) who reported that wheat (*Triticum aestivum*) inoculated with *Pseudomonas* sp. strain 54RB significantly increased plant height, root and shoot weight, spike length, grain and P uptake compaired the un-inoculated control. Similar results were also found in cowpea (*Vigna unguiculata*) which enhance the nodulation, root and shoot biomass, straw and grain yield and P and N uptake of plants inoculated with *Gluconacetobacter* sp. and *Burkholderia* sp. (Linu *et al.,* 2009).

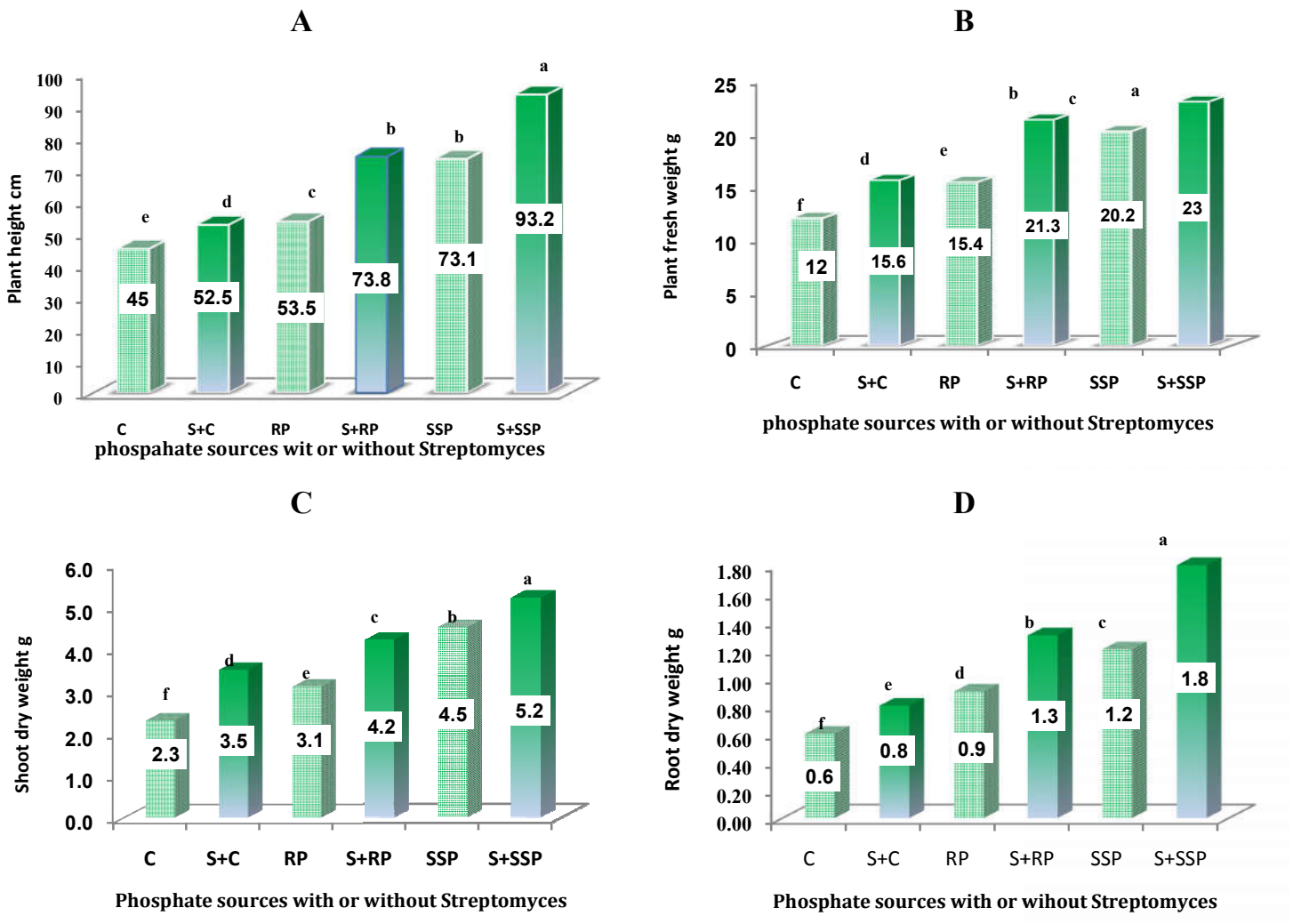

Fig (3): The effect of *Streptomyces* sp. KP109810 (A) plant height (cm), (B) plant fresh weight (g), (C) shoot dry weight (g) and (D) root dry weight (g) of maize plants with different phosphate sources after 50 days from inoculation. Control (C), *Streptomyces* sp. KP109810 (S+C), single super phosphate (SSP), rock phosphate (RP)

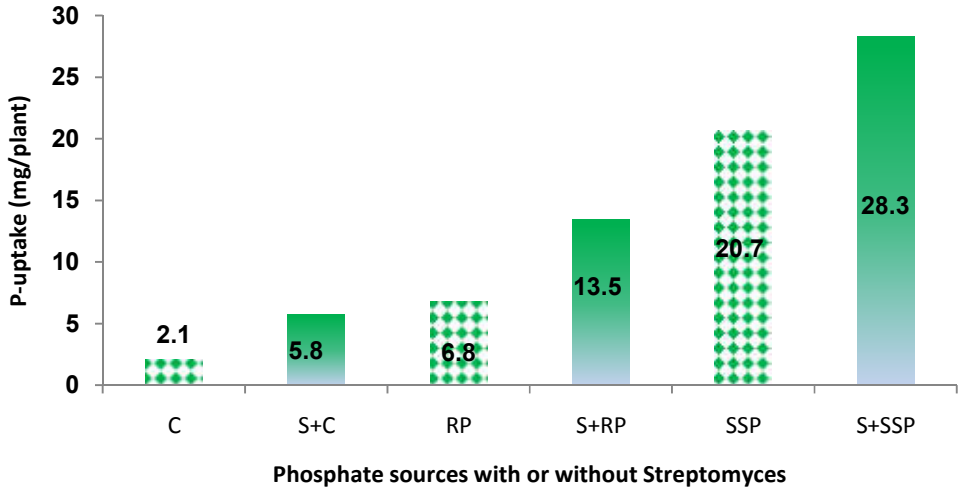

Fig (4): The effect of *Streptomyces* sp. KP109810 inoculation mineral P addition on P-content (mg/plant) of maize plants with different phosphate sources after 50 days from inoculation.
This investigation; control (C), *Streptomyces* sp. KP109810 (S+C), single super phosphate (SSP), rock phosphate (RP)
*, Different letters mean significant difference between treatments

CONCLUSION

From the results presented here, it is reported the solubilization of five different inorganic phosphate sources namely TCP, RP, SSP, FeP and AlP by the actinobacterial strain *Streptomyces* sp. KP109810 differed significantly depending on the nature of phosphate source. Many organic acids present in the medium were detected by TLC and the organic acids production differed according to the different sources of P. Phosphatase activity was reported in *Streptomyces* sp. KP109810 with a maximum activity of 0.091 Uml^{-1} by RP sample. Our results suggest that *Streptomyces* sp. KP109810 were effective in solubilization of RP, thus, improving its capacity to release P for cultivated plants. The application of this actinobacterial strain had beneficial effects on growth, yield and P nutrition on maize plants. These results have an agronomic importance for crop cultivation, economic importance for saving money involved in high cost chemical P fertilizers, and environmental benefits for avoiding environmental issues caused due to mineral P fertilizer application. Hence, application of P solubilizing bacteria are recommended as a sustainable way for increasing P solubilization from insoluble RP and other P sources, and improving crop yield and P utilization efficiency of applied P fertilizers. These results are recommended to be evaluated under various soils and environmental conditions before using this actinobacterial strain as a biofertilizer.

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تأثير ميكروب *Streptomyces* sp. KP109810 على إذابة الفوسفات غير العضوي ونمو الذرة (*Zea mays* L.)

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يلعب الفوسفور دورًا رئيسيًا في نمو النبات، لكن جزء صغير فقط من الفوسفور في التربة يتم امتصاصه بواسطة النباتات ويصبح الباقي غير متاح للنباتات. تلعب الكائنات الدقيقة المذيبة للفوسفور دورًا حيويًا في إذابة المركبات المعقدة من الفوسفات إلى الصورة الميسرة للنبات. يوضح البحث قدرة البكتيريا على إذابة مصادر مختلفة للفوسفات مثل الفوسفات الأحادي (SSP)، الفوسفات الصخري (RP)، فوسفات الحديد (FeP) وفوسفات الألمنيوم (AlP) وفوسفات الكاسيوم الثلاثي (TCP) بواسطة *Streptomyces* sp. KP109810 (S) مع إنتاج الأحماض العضوية وكذلك الفوسفاتيز. وجد أن كمية الفوسفات المنبعثة (٥٦٢ مجم لتر$^{-1}$) في حالة سوبر فوسفات الأحادي تكون الأعلى عند تلقيحها وعدم تلقيحها بالبكتيريا، على التوالي، مقارنة بمصادر الفوسفات الأخرى. ولوحظ أعلى نشاط للفوسفاتيز في وجود فوسفات الحديد (٠.٠٣٣ وحدة/مل) .استخدمت طريقة TLC للكشف على الأحماض العضوية في مزارع البكتيريا من مصادر الفوسفور المختلفة وكانت كالآتي: ستة أحماض عضوية من SSP ، سبعة أحماض عضوية من RP ، أربعة أحماض عضوية من FeP واثنين من الأحماض العضوية من TCP و AlP .تم دراسة تأثير التلقيح بـ *Streptomyces* sp. KP10981 من حيث ذوبان الفوسفور من مصادر مختلفة للفوسفور وإنتاج الكتلة الحيوية النباتية وكفاءة امتصاص الفوسفور على نبات الذرة (*Zea mays* L.) . تم إجراء التجربة في الصوبة في أصص من خلال زراعة الذرة كمحصول اختبار. أظهرت النتائج تعزيزًا فعالًا لنمو الذرة وامتصاص P مقارنة بالنباتات غير الملقحة. علاوة على ذلك، أظهر الفوسفات الأحادي نتائج أفضل من الفوسفات الصخري ، حيث ظهر الأخير بشكل مماثل عند التلقيح بالبكتيريا. كما أظهرت هذه النتائج أنه يمكن استخدام *Streptomyces* sp KP109810 لتحسين نمو المحاصيل والتسميد بالفسفور. يجب أن تقيم نتائج هذه التجربة على المستوى الحقلي تحت ظروف أراضى وبيئات مختلفة قبل التوصية باستخدام هذه السلالة الميكروبية كسماد حيوي.