Effects of Phosphate-solubilizing Bacteria on Micro-Tom and Soil of Micro-Tom Rhizosphere

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Abstract: Phosphorus bacteria fertilizer can increase the utilization of soil phosphorus and promote plant growth. In order to provide basic information for the composite engineering strains, we selected high-efficiency phosphorus-solubilizing bacteria (PSB) from the rhizosphere of mature corn soil. In this study, a total of six organic phosphorus strains were obtained by NBRIP medium. Further experiments were performed on inorganic PSBs. We measured the dissolved phosphorus ratio (D/d values), NK2 and NK3 had the best phosphate-solubilizing effects. The D/d values of NK2 and NK3 were 2.13 and 4.35, respectively. The results of 16S rRNA amplification and sequencing showed that the NK2, NK3 were identified as *Acinetobacter sp*., *Pseudomonas sp.*, respectively. According to the data of shaker experiment for 7 days, the maximum phosphate solubilizing contents of NK2 and NK3 were 183.10 $mg \cdot L^{-1}$ and 79.87 $mg \cdot L^{-1}$, respectively, and NK2 had genetic stability. The result of pot experiment indicated that the growth attributes and the root indexes of the Micro-Tom, as well as the content of soil available phosphorus treated by NK2 (TCP) and NK3 (TCP) were both significantly higher than CK ($P<0.05$). These results imply that above two strains could promote plant growth.

1 INTRODUCTION

Deficiency of phosphorus (P) is an important limiting factor in agriculture production. Fertilizers or inoculants made by P-solubilizing microbes are applied to the soil with less available phosphorus (Oliveira, 2009), which not only effectively avoids the excessive application of phosphate chemical fertilizers in agriculture, but also solves the problem of the lack of available phosphorus in the soil (Bojinova, 2008). So, environmentally friendly substitutes for P fertilizers are urgently needed to avoid adverse effects on agriculture production.

To circumvent phosphorus deficiency, the phosphate -solubilizing bacteria (PSB) could play an important role in supplying phosphate to plants in a more environmentally-friendly and sustainable manner (KHAN, 2007). A massive number of research results have proved that the soil is the main source of PSBs. PSBs in soil generally affect the fertility of soils through biogeochemical cycles (Wang, 2020). It has been reported that numerous rhizosphere microorganisms have capability of dissolving insoluble P (Hameeda, 2008, Henri, 2008). Due to the activity of P-solubilizing in rhizosphere, PSBs supply P for plants in an environmentally friendly and sustainable manner. Several studies under greenhouse and field indicated that PSBs have direct impacts on soil conditions, nutrient availability and plant growth (Fitriatin, 2014, Hussain, 2013). Understanding the interaction of rhizosphere and microbial community will assist the development of inoculants with potentially greater consistency in performance and survival for agroforestry ecosystems, especially using indigenous microorganisms.

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2 MATERIALS AND METHODS

2.1 Sample Collection

Soil tightly adhering to corn roots was collected from the national long-term monitoring station for soil fertility. Using the five-point sampling method, three vigorous and disease-free plants in the plot were selected. Taken about 1 kg near-root corn soil samples from the 15 cm of depth, and they were collected in sterile bags and transported to the research laboratory and stored at 4℃ until further use.

2.2 Cultivation of PSBs

Serial dilution from 10^{-5} to 10^{-2} was achieved by transferring 5.0 g of soil residue solution from each preceding attenuation stage to the next. Extracted 0.1 mL samples from the 10^{-3}, 10^{-4} and 10^{-5} dilutions, and placed on NBRIP (Shekhar, 2003) in triplicate and kept at 28℃ for 72 h. An isolate forming a clear halo zone was selected as a PSB. Then a single colony was pick-transferred to Luria-Bertani (LB) for further purification.

The isolated bacteria were inoculated in NBRIP liquid medium at 28℃ and 180 r/min for 7 days, and their phosphate solubilizing activities were quantitatively determined and compared. The soluble P in the mediums was measured per day. The amount of soluble P was determined through Mo-Sb anti-spectrophotometry method (Sundararao, 1963).

2.3 Molecular Identification of Bacterial Strains

The DNA was extracted using a Bacterial DNA Kit (Biomed, Beijing, China) following the manufacturer's instructions. The 16S rDNA genes were amplified by PCR using the universal primers: 27F (5-AGAGTTTGATCNTGGCTCAG-3) and 1492R (5-TACGGYTACCTTGTTACGACTT-3), and sequenced as described. The amplification reaction mixture contained 12.5 μL of Prime STAR Max, 1 μL of 10 μmol primer (F and R), 1 μL of template DNA and ddH_2O to make up to 25.0 μL. The resulting products were analyzed by electrophoresis in 2.0% agarose gel and sent to the Sangon Biotech Company (Zhengzhou, China) for sequencing. The 16S rDNA sequences of strain NK2 and NK3 were analyzed initially by BLASTn to acquire the closest reference sequences.

2.4 Growth Curve of Bacteria

In microbial transformation research and industrial production applications, the physiological activity and transformation activity of transformed bacteria are necessary. To quantitatively determine the transformation rate of bacteria, the concentration and biomass of the bacterial solution should be accurately determined to ensure the continuous and efficient conversion process (Li, 2003). In this section, through the optic density (OD) value assay of culture with vary vaccination time, a chart between OD600 and time was established.

Configured the NBRIP liquid medium with NaCl concentration gradients of 0, 2.5%, 5%, 10%, 15%, and 20%, respectively, the filling volume was 50 ml/250 ml. Each concentration was set to 3 parallel. Inoculation 1 ml (OD600=1) PSBs and cultured at 28°C and 130 r/min for 20 h. Established a chart between OD600 and the different salt concentrations.

2.5 Pot Experiment for Evaluation of PSBs Application on Micro-Tom Growth

Collected seeds of Micro-Tom (*Solanum lycopersicum L. cv Micro-Tom*) from School of life science, Zhengzhou University, Henan, China. Seeds were surface-sterilized by soaking in 5% NaClO solution for 10 min and rinsing with sterile distilled water. Then transferred them to sterile dishes filled with double-layer wet filter paper and incubated for 7 d at 26℃ after germination. Seedlings of uniform size were transferred to pots (diameter 15 cm, height 18 cm) filled with 1 kg of soil (river sand: vermiculite=1:1).

The experiment was divided into 6 treatments: CK, NK2, NK3, TCP, NK2 (TCP), NK3 (TCP), with six replications each. CK was un-inoculated controls. NK2 and NK3 were soil treated with 100ml of NK2 and NK3 (OD_{600}=1), respectively. TCP was soil treated with 1% $Ca_3(PO4)_2$. NK2 (TCP) and NK3 (TCP) were soil treated with TCP and 100 ml of NK2, NK3 (OD_{600}=1), respectively.

In pot experiments, the effects of PSBs on Micro-Tom and soil of rhizosphere were studied. Different growth parameters including plant height, biomass in the plants, the total chlorophyll contents (SPAD value), root indexes and soil available phosphorus were examined at 15 d and 30 d after inoculation. Replicates were not pooled. A 5 g (dry weight) aliquot of the sampled soil was suspended in 50 ml of sodium bicarbonate solution (PH=8.5) by

shock (170 rpm) for 30 min at 25°C(Olsen, 1954). The soluble phosphorus content in soil was evaluated by Mo-Sb anti colorimetry.

3 RESULTS

3.1 Isolation of PSB and Phosphorus Solubility of Two Selected Strains

Selected the PSBs showing greater solubilization (both qualitatively and quantitatively) of insoluble P under in vitro conditions. Each isolate was purified in LB and working cultures were maintained at 4℃. Isolates with a larger halo zone of solubilization in NBRIP were selected for further studies. The dissolved phosphorus ratio (D/d) of two strains exceeded 2.0, and these were quantitatively assayed for phosphate solubilization potential. A total of 6 different bacterial isolates were obtained from different samples of corn soil (Table 1).

Table 1: Dissolved phosphorus ratio of 6 isolates isolated from the near-root corn soil samples on NBRIP.

Strain	D/mm	d/mm	D/d
NK2	2.45±0.07	1.15±0.07	2.13±0.19 b
NK3	2.73±0.15	0.67±0.20	4.35±1.26 a
NK22	7.60±0.42	5.40±0.28	1.41±0.15 c
N_2K2	8.40±0.28	5.75±0.21	1.46±0.01 c
N_2K3	5.45±0.21	4.35±0.35	1.26±0.15 d
N_2K8	5.85±0.07	4.00±0.14	1.46±0.07 c

Note: D, dissolved phosphorus circle diameter; d, colony diameter; D/d, dissolved phosphorus ratio equal to diameter of hydrolysis circle divided by diameter of colony.

Bacterial strains exhibiting phosphate-solubilizing activity are detected by the formation of clear halo around their colonies. We selected two PSBs and showed these morphological characteristics in Fig.1. The colonies were circular.

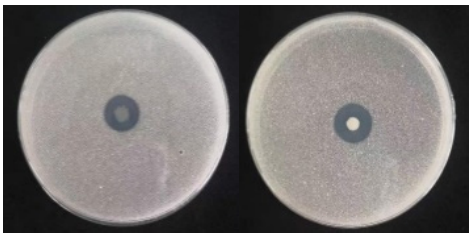

Figure 1: Phosphate-solubilizing activity on NBRIP by species of NK2 isolates (Plate left) and NK3 isolates (Plate right).

Two of the bacterial strains exhibited higher phosphate-solubilizing activity, we measured the daily phosphate-solubilizing activity of the two strains within 7 days (Fig.2). The NBRIP liquid cultures of NK2 isolates contained 183.10 mg/L (the highest concentration) P solubilized from insoluble $Ca_3(PO4)_2$ as the sole source of P in the medium, followed by NK3 isolates containing 79.87 mg/L (the highest concentration).

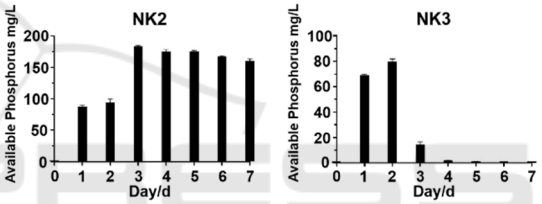

Figure 2: Phosphate-solubilizing activity of two PSBs.

3.2 Identification of Bacterial Isolates

Nucleotide sequencing of the 16S rDNA gene of two selected isolates proved 98%-99% similarity with species present in the GenBank database (Table 2)(G, 1991). According to phylogenetic analysis, the similarity between NK2 isolates and *Pseudomonas sp.* was 98.33%, and NK3 isolates was identified as *Acinetobacte sp.*

Table 2: Identification results for 16S rDNA sequencing.

Strain	Gram$^+$ or Gram$^-$	Specific name	16SrDNA homology/%
NK2	Gram$^-$	*Pseudomonas sp.*	98
NK3	Gram$^-$	*Acinetobacter sp.*	99

3.3 Growth Curve of Strains

The growth curve of NK2 isolates and NK3 isolates was shown in Figure 3 (left). The bacteria grow slowly within 0~2h and are in the growth delay

period; after 3h, the strain proliferates rapidly and enters the logarithmic growth phase. The curve tends to be flat after 18h.

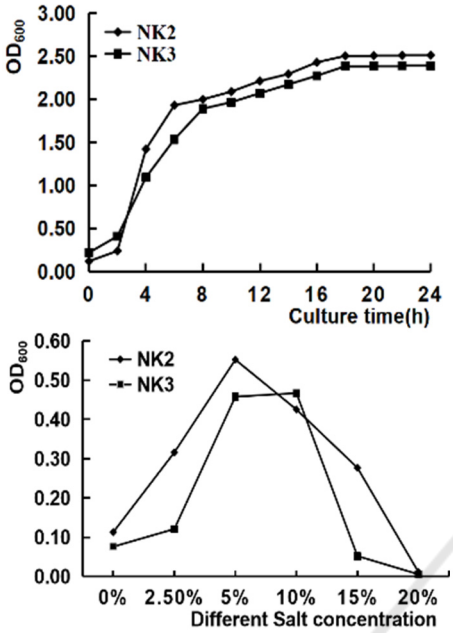

Figure 3: The growth curve of two PSBs.

It can be seen from Figure 3 (right) that with the strengthening of salt stress conditions, the growth of NK2 and NK3 strains increased firstly and then decreased. NK2 and NK3 can tolerate a wide range of salt concentration.

3.4 Effects of PSB on Micro-Tom Growth

3.4.1 Growth Attributes of Micro-Tom

Two PSBs with sufficient P production were chosen to determine their beneficial effects on Micro-Tom growth under greenhouse conditions. The Micro-Tom grew better than CK when NK2 isolates and NK3 isolates were colonized in the rhizosphere of Micro-Tom seedlings (Fig.4). The NK2 (TCP) and NK3 (TCP) significantly promoted plant height by 170% and 160% at 30 d. Plant height was measured from stem base to top. Leaves at a certain fixed node height were marked and evaluated. The total chlorophyll contents (SPAD value) increased by 35% and 24%, the stem biomass by 850% and 800%, the root biomass by 480% and 440%, compared with CK.

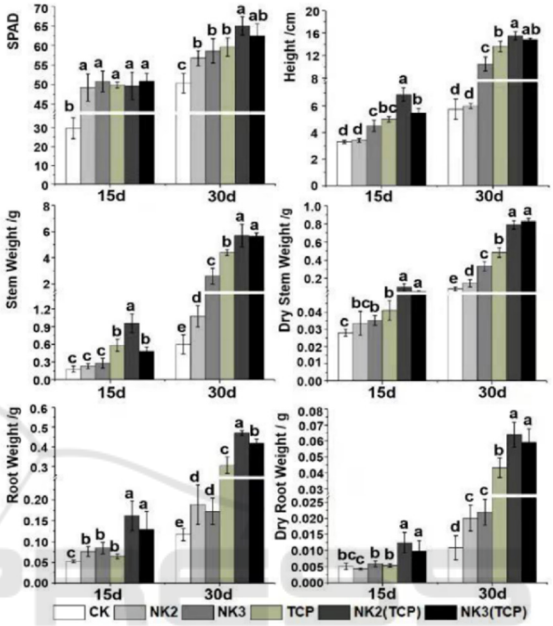

Figure 4: Growth Attributes of Micro-Tom.

3.4.2 Root Indexes

The effects of the two PSBs in the rhizosphere of plant, measured as root length, root surface area, and the number of root tips in the root of plant, are presented in Fig.5. Application of NK2 (TCP) had the strongest effect on root length of Micro-Tom, with an increase by 39.73% at 30 d ($P \leq 0.05$) compared with CK. NK3(TCP) resulted in an increase of 20.10 times at 30d for root surface area. NK3 (TCP) resulted in an increase of 1.39 times at 30d for root tips (Fig. 6). Therefore, PSBs effectively improved root development in Micro-Tom.

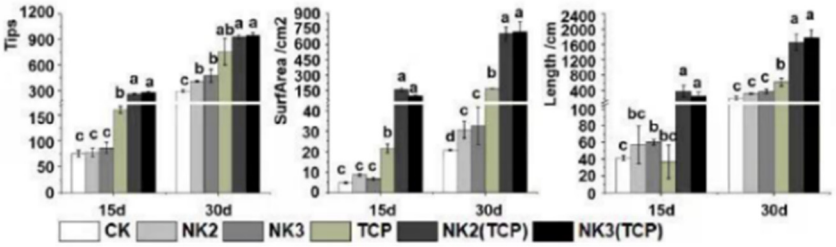

Figure 5: Root indexes of Micro-Tom.

3.4.3 Postharvest Soil Available Phosphorus

The available phosphorus content in soil treated with NK2 (TCP) and NK3 (TCP) was at a high level at 30 d. NK2 (TCP) and NK3 (TCP) increased significantly relative to CK at 15 d and 30 d.

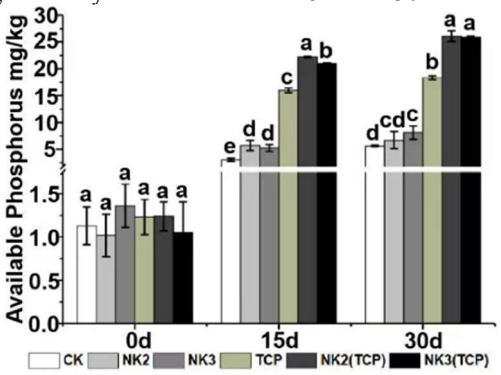

Figure 6: Soil available phosphorus.

4 DISCUSSIONS

It is an urgent thing to establish a sustainable agriculture industry that maintains the ecological balance of soil systems for a long time. As the basic element of fertilizer, soil available phosphorus plays an important role in the ecosystem. In this study, rhizosphere soil samples from corn plants were screened by PSB isolation. Among the phosphate-solubilizing isolates, two efficient PSB strains were selected for the further studies. The strains were identified as *Pseudomonas* (NK2) and *Acinetobacter* (NK3) by 16S rDNA sequencing technologies.

The data presented in this paper showed that two isolates significantly promoted the growth of the plant seedlings and root of Micro-Tom under the greenhouse conditions. It may be due to the greater absorption of nutrients, especially P element. The results in this study were similar to those reported studies (Datta, 1982, Asea, 1988). However, some researchers obtained contrary conclusions by inoculating PSBs to plants. Inoculating PSBs to the seed of Chinese cabbage will promote its growth, but there had no effect on the absorption of P element in plants. In fact, the beneficial effects of PSBs in plant growth largely depend on the environmental conditions, type of strain, host plant and condition of soil (Khan, 2009), which cannot be tested and evaluated separately (Liu, 2014). In this work, it has remained a significant challenge to obtain a complete root from the soil, which caused to too obvious differences in the measured values of root surface area between NK3 (TCP) and CK. There is an urgent need to use an effective method to analyze and survey the rhizosphere. In any case, it is necessary to explore the mechanism of NK2 and NK3 attained a regulating balance, promoting the growth of plants and the development of rhizosphere. The application of PSBs with TCP showed an obvious effect compared to control. It is possible that the soil environment had changed and caused a steady increase in nutrients. It is required to explore the effect of these PSBs either alone or in combination with other bio-fertilizers on growth of Micro-Tom under field conditions. Further studies under field conditions are needed to confirm the present findings and recommend strains for commercial applications.

5 CONCLUSIONS

NK2 and NK3 were screened in this experiment, which have relatively strong Phosphate-solubilizing activity and high salt tolerance. A pot experiment of NK2 and NK3 strains were carried out. Some indexes of the Micro-Tom in the growth period (15 d) and flowering period (30 d) were studied. The result of pot experiment indicated that the growth attributes and the root indexes of the Micro-Tom, as well as the content of soil available phosphorus treated by NK2 (TCP) and NK3 (TCP) were significantly higher than CK ($P<0.05$). These imply that both strains could promote plant growth.

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