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Transformation of Inorganic P Fractions of Soil and Plant Growth Promotion by Phosphate-solubilizing Ability of *Penicillium oxalicum* I1

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The solubilization of tricalcium phosphate is often considered as the standard for screening of most phosphate-solubilizing microorganisms (PSMs). However, usually the effect of large-scale application of PSM on the promotion of crop growth varies. This study presents an efficient method for screening and testing phosphate-solubilizing fungus that enhance plant growth. A fungus *Penicillium oxalicum* I1 (P-I1) was isolated and identified that had high ability of phosphate-solubilization and could utilize maize root exudates as sources, and propagate well in *vitro* and in soil. P-I1 excreted oxalic acid and reached 593.9 μg/ml, and the pH value was decreased from 6.90 to 1.65 in 26 h. The amount of P-I1 increased by 48-fold in 28 d and was maintained for 49 d in soil. PSM showed selectivity on the transformation of the different forms of phosphorus, a wide range of insoluble phosphates, such as $Ca_8H_2(PO_4)_6{\cdot}5H_2O$, $AlPO_4$, $FePO_4$, and $Ca10(PO_4)_6(OH)_2$, were converted to soluble $CaHPO_4$ in soil, and $CaHPO_4$ was also inhibited from being converted into insoluble phosphate by P-I1. The Ca_2-P content reached 27.11 μg/g soil on day 28 at 20°C, which increased by 110.32%, and plant growth promotion was tested and verified, the results showed that maize yield increased remarkably than control after inoculated P-I1, maize yield increased maximum by 14.47%. The data presented that P-I1 appear attractive for exploring their plant growth-promoting activity and potential field application.

Keywords: *Penicillium oxalicum*, inorganic P fractions, transformation, phosphate solubilizing, plant growth promotion

Introduction

Phosphorus is the second most essential nutrient after nitrogen for plant growth and development. The phosphate (P) content in the soil in China ranges from 0.2 g/kg to 2.0 g/kg, with an average of 0.5 g/kg. Most soils contain large amounts of total P, but the availability of phosphates to plants is deficient. Inorganic and organic matter is a reservoir of immobilized P, accounting for approximately 95% in soil. To ensure crop yield, more than 90 kg/ha P is usually added to the soil (Li *et al*., 2013; Zhang *et al*., 2013). The utilization ratio is less than 20%, part of which is utilized by plants and the remainder is rapidly converted to insoluble forms in the soil. Many researches focus on how to reduce the P fertilizer and raise utilization ratio of P fertilizers, and promote crop growth.

Phosphate-solubilizing microorganisms (PSMs), which are commonly found in the soil, can solubilize insoluble phosphate, reduce phosphorus fertilizer fixation, raise utilization ratio of phosphorus fertilizers, and promote crop growth (Altomare *et al*., 1999; Adesemoye *et al*., 2009; Viruel *et al*., 2011; Mehta *et al*., 2013). PSMs were investigated to solubilize phosphorus in soil to promote plant growth. Therefore microorganisms that can only solubilize phosphate are not necessarily known as PSMs. Three criteria should be satisfied to be considered as a PSM that should be able to propagate well and solubilize phosphate in soil and promote plant growth (Park *et al*., 2010; Mamta *et al*., 2012; Walpola *et al*., 2013). To date, the solubilization of tricalcium phosphate (TCP) is often considered as the standard for screening of most PSMs. The PSMs are usually determined only by the solubilization of inorganic phosphate *in vitro* (Yu *et al*., 2011; Sara *et al*., 2013; Syed and Samir, 2013). However TCP is inappropriate as a universal selection factor for isolating and testing phosphate-solubilizing microorganisms that enhance plant growth (Bashan *et al*., 2013). Some strains *in vitro* can solubilize inorganic phosphate, but they cannot solubilize phosphate in soil or promote crop growth (Freitas *et al*., 1997). These microorganisms only solubilizing the inorganic phosphate *in vitro* are not necessarily PSMs. PSMs should be screened at least by experiments on soil colonization, inorganic phosphate solubilization, and plant growth promotion abilities. So it is important to creat an efficient method for screening and testing phosphate-solubilizing fungus that enhance plant growth.

Various inorganic P fractions (Ca_2-P, Ca_8-P, Al-P, Fe-P, O-P, Ca_{-10}-P) exist in the soil, and inorganic P fraction contents vary in different soil types (Varinderpal *et al*., 2007; Giesler *et al*., 2012; Satyavir *et al*., 2014). A dynamic equilibrium exists between various phosphate forms, in which soluble forms are usually converted to insoluble forms in the soil (Hinsinger, 2001). The solubilization of inorganic P by microorganisms has been attributed to processes involving acidification, H^+ excretion, chelation, and redox re-

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action in the growth environment (Rodríguez *et al*., 2006; Satyavir *et al*., 2014). Acidification by organic acid is the main solubilization mechanism of inorganic P by microorganisms. Oxalic, lactic, acetic, propionic, malic, tartaric, citric, butyric, malonic, succinic, gluconic, and fumaric acids are the main organic acids known to solubilize P (Banik and Dey, 1982; Altomare *et al*., 1999; Fomina *et al*., 2004; Khan *et al*., 2007; Farhat *et al*., 2009; Werra *et al*., 2009; Bianco and Defez, 2010; Gulati *et al*., 2010). Organic acids can chelate Fe^{3+}, Fe^{2+}, Ca^{2+}, Al^{3+}, and so on. Thus, insoluble phosphate is transformed to soluble phosphate. A high correlation was observed between final pH and soluble phosphate (Walpola and Yoon, 2013). The stronger the acidification ability of the soil, the stronger is the ability of phosphate solubilization (Nahas, 1996). Microorganisms showed selectivity on the transformation of the different forms of phosphorus, and organic acids may be secreted or not under different conditions. Therefore the phosphate-solubilizing effect varies with different mechanisms such that considerable differences exist in the selection of solubilizing inorganic P fraction by different microorganisms (Toro *et al*., 1996). Thus, microorganisms fully function in phosphate solubilization in suitable soil environment.

Most of the studies on the effect of phosphate solubilization in soil and their potential use in the enhancement of soil fertility have considered the PSM. *Penicillium* spp. are an important PSM (Salih *et al*., 1989; Cunningham and Kuiack, 1992; Xiao *et al*., 2011; Mendes *et al*., 2014), and *Penicillium bilaii* was used in large-scale application (Gulden and Vessey, 2000; Vessey and Heisnger, 2001). Experiments have proved that *Penicillium* sp. exhibits a good effect on phosphate solubilization and inhibits the transformation of soluble phosphate to insoluble phosphate (Salih *et al*., 1989). However, the effect of large-scale application of PSM on the promotion of crop growth varies (Banik and Dey, 1983; Khan *et al*., 2007). This variability limits the large-scale application of PSMs in agriculture, which is affected by multiple elements such as microorganism colonization ability, crop species, soil types, soil nutrition, and microbial interactions. Significant differences in the characteristics of solubilizing inorganic P fraction has been reported indifferent *Penicillium* spp. strains. In the present study, we conducted three experiments. Firstly, a fungus *Penicillium oxalicum* was isolated, which secreted oxalic acid with a strong ability to convert a wide range of insoluble P to soluble forms. And then we described its characteristics of propagating ability, adaption to plant root exudates and the transformation of inorganic P fractions in soil. Finally, we tested and verified its phosphate solubilizing ability and plant growth promotion, this study aimed to establish a proposal method for screening and testing phosphate-solubilizing fungus which is intended to be used as biofertilizer inoculants with stability effect.

Materials and Methods

Fungal strain isolation and identification

The rhizospheric soil of crop was collected from the field in Northeast of China (E 46°39′ 46.00″, 127°08′ 24.34″). Soil samples (10 g) were suspended in 50 ml sterile water on a gyratory shaker (170 rpm) for 1 h at 28°C. The suspension was serially diluted ten-fold, and then 0.1 ml of each dilution was plated onto NBRIP-BPB solid medium containing 0.5% TCP as insoluble P source (Nautiyal, 1999). After a 5-day incubation at 28°C, the plates were examined for the presence of colonies with clear halos. Fungal colonies with maximum clear zone around them were picked and purified further by plating onto fresh agar plates. The selected fungus was maintained on potato dextrose agar.

To determine the identity of the isolated fungal strain, the isolate was cultured on standard media, czapek yeast agar (CYA) and Czapek-Dox Agar (CA), at 25°C for 7 days, to test the colony morphology. The medium comprising the following components, CYA (g/L): $NaNO_3$ 3.0 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $FeSO_4 \cdot 7H_2O$ 0.01 g, Yeast 5.0 g; CA (g/L): $NaNO_3$ 3.0 g, KH_2PO_3 1.0 g $MgSO_4 \cdot 7H_2O$ 0.5 g, $FeSO_4 \cdot 7H_2O$ 0.01 g, saccharose 30.0 g, Agar 15.0 g, pH 7.3 ± 0.2. The medium were sterilized by autoclaving at 115°C for 10 min.

The internal transcribed spacers region (ITS1 and ITS2) of 5.8S rRNA was subjected to sequencing. The primers ITS1: 5′-TCCGTAGGTGAACCTGCGG-3′ and ITS2: 5′-GCTGC GTTCTTCATCGATGC-3′ were used (White *et al*., 1990). The PCR reaction mix contained 0.5 μM of each primer, 10 μM deoxynucleotide triphosphates, 1.5 mM $MgCl_2$ and 10× buffer (NEB). The suspension was heated at 94°C for 15 min in a thermocycler (ABI 9700). One unit of the *Taq* Polymerase (NEB) was then added to each tube. The following thermocycling conditions were used: initial denaturation at 94°C for 5 min followed by 30 cycles of 94°C for 30 sec, 57°C for 50 sec, 72°C for 50 sec, and final elongation at 72°C for 10 min. The presence of amplified fragments was checked by standard gel electrophoresis.

Estimation of inorganic P solubilization *in vitro*

In vitro inorganic phosphate-solubilizing ability was determined in NBRIP-BPB solid medium containing TCP as sole source of P (Nautiyal, 1999). $AlPO_4$ and $FePO_4$ were supplemented to the medium instead of $Ca_3(PO_4)_2$. A total of 10 μl of fresh culture (10^7 CFU/ml) was spotted on the plates and incubated at 28°C for 7 d. The halo zone and the fungi colony diameter were measured.

Analysis of organic acid

To analyze organic acid excretion by P-I1, 1 ml of fungal spore suspension was transferred into a 500 ml flask containing 100 ml of PDB on a gyratory shaker (170 rpm) at 28°C. The culture was centrifuged at 10,000 rpm for 10 min and passed through a 0.22 μm nylon filter. The culture filtrates were analyzed for pH value of the medium and organic acids using a pH meter and an ion chromatography system (ICS-3000, Dionex, USA) at 14, 18, 22, 26, and 30 h. Each treatment was replicated thrice. The organic acids were quantified by reference to the peak areas obtained for the authentic standards for oxalic acid (Sigma, USA). Identification of organic acid was performed using an IonPac AG11-HC guardcolumn (4 mm × 50 mm) and an IonPac AS11-HC (4 mm × 250 mm) chromatographic column under the following conditions: 0 min to 5 min, 1.0 mmol/L KOH; 5 min to 45 min, 36.00 mmol/L KOH; and 45 min to 50 min,

1.0 mmol/L KOH. The appearance time of oxalic acid was 27.5 min.

Soil, preparation of fungal spore suspension, and maize root exudates

Soil was collected from a maize field in Heilongjiang Province in northeastern China. The characteristics of the soil are as follows: pH 7.0; organic matter, 26.40 g/kg; total N, 0.46 g/kg; available P, 12.89 mg/kg; and extractable potassium (K), 30.65 mg/kg.

P-I1 was grown in 50 L of potato dextrose broth (PDB) on a gyratory shaker (170 rpm) for 48 h at 28°C, and centrifuged at 10,000 rpm for 5 min. The pellets were suspended in sterilized water containing 1×10^7 CFU/ml, and the solution was stored at 4°C until use. The fungal mycelium was added into grass coal, then the mycelium split into spore, and the spore content was 6.1×10^7 CFU/g. The microbial inoculum was stored at room temperature until use.

A germinated maize seed (Zhegndan958) was grown on a 50 ml tube with 48 ml of plant nutrient solution under illumination at 25°C for 15 days. To collect concentrated maize root exudates, the nutrient solution were concentrated 10 times underwent vacuum pumping using a water bath at 60°C, and stored at 4°C until use. The plant nutrient solution comprised the following components (mol/L): K_2SO_4, 0.75×10^{-3}; $MgSO_4$, 0.65×10^{-3}; KCl, 0.1×10^{-3}; $Ca(NO_3)_2$, 2×10^{-3}; KH_2PO_4, 0.25×10^{-3}; $CuSO_4$, 1×10^{-7}; EDTA-Fe, 0.1×10^{-3}; $MnSO_4$, 1×10^{-6}; H_3BO_3, 1×10^{-5}; $ZnSO_4$, 1×10^{-6}; and $(NH_4)_6Mo_7O_4$, 5×10^{-9}.

Estimation of adaptation to maize

The adaptive ability between strains and maize was analyzed *in vitro*, in which 15 ml of solution containing maize root exudates was supplemented to 300 ml of NBRIP-BPB solid medium instead of glucose. A total of 10 µl of fresh culture (10^7 CFU/ml) was spotted on the plates and incubated at 28°C for 4 d. The halo zone and fungi colony diameter were measured.

Another experimental design was conducted in a soil culture trial to study the matching ability between strains and maize. The culture was mixed into a beaker with 100 g of sterilized soil containing 1×10^6 CFU/g and 15 ml of solution with maize root exudates at 28°C. The soil samples were collected at 7, 14, and 21 d, and P-I1 colonies were counted in NBRIP-BPB solid medium containing 0.5% TCP as an insoluble P source (Nautiyal, 1999) and 100 µg/ml ampicillin, which inhibits bacterial growth for the selective screening of fungi. After 5 d of incubation at 28°C, the valid count was 30–100 colonies with phosphate-dissolving activities on the solid medium containing insoluble phosphate. The experiment was performed three times.

Colonization ability in soil

An experimental design was conducted in the soil culture trial to investigate the colonization ability of P-I1 at different temperatures in soil. The culture was mixed into pots with soil (500 g) containing 1×10^6 CFU/g and 0.1 g dextrose and 0.05 g NH_4Cl/g soil and incubated at 15, 20, and 30°C. The moisture was maintained between 30% and 50%. The soil samples were collected at 7, 14, 21, 28, 35, 42, and 49 d, and P-I1 colonies were counted with in NBRIP-BPB solid medium containing 0.5% TCP as insoluble P source (Nautiyal, 1999) and 100 µg/ml amp, which inhibits bacterial growth for selective screening of fungi. The number of valid populations was counted in the fifth day at 28°C on the NBRIP-BPB solid medium containing 0.5% TCP as an insoluble P source (Nautiyal, 1999) and 100 µg/ml ampicillin. The experiment was performed thrice.

Estimation of pH value and available P fraction solubilization in soil

The treatment was similar to that in the colonization ability of P-I1in soil. The soil samples were collected at 7, 14, 21, 28, 35, 42, and 49 d, air dried, and ground to pass through a 2 mm sieve using an electric flail grinder. To measure soil pH, 20 g of soil was mixed with 20 ml of 1 mol/L KCl solution in a 50 ml flask and agitated vigorously for 1 min. After equilibrating for 30 min, soil suspension pH was measured using a glass electrode (EUTECH pH510).

Various inorganic P fractions were determined to investigate the characteristics of phosphate solubilization by P-I1. Soils were analyzed for various inorganic P fractions using the P fractionation scheme (Chang and Jackson, 1957; Chang and Liaw, 1962).

Plot experiment

The experiments were conducted in Northeastern China to investigate the effect of maize yield by P-I1 during May 15th to September 28th in 2013. Zhengdan958 maize seeds (China) were used. TCP was supplied as the soil P fertilizer. The experimental plan was based on six treatments as follows: (i) Control; (ii) TCP 45 kg/ha; (iii) TCP 90 kg/ha; (iv) P-I1; (v) P-I1 and TCP 45 kg/ha; (vi) P-I1 and TCP 90 kg/ha. Plots were arranged in a randomized complete block design with three replicates per treatment, each plot was 100 m^2 with 600 maize seedlings. Approximately three gram of microbial inoculums were added nearby root system when maize were in 15–20 cm height. Daily management of maize in accordance with local planting methods. A hole near the maize root system was dug on the soil surface. The inoculums were then added into the holes. The grain water content of maize were converted into 14% after the grain were harvested. And then analysis the yield of maize.

Statistical analysis

Statistical analysis was conducted by using Analysis of Variance (ANOVA) statistical package for social sciences (SPSS) software, version 21.0 followed by comparison of multiple treatment levels with the control, using the significant difference (LSD) at $P \leq 0.05$.

Results

Isolation and identification of P-solubilizing fungus from soil sample

The clear halos appeared as a result of the TCP being solu-

Table 1. Growth diameter of P-I1 and phosphate-dissolving zone diameter of different P contents in the culture medium

P source	Diameter (cm)		
	d	D	D/d
$Ca_3(PO_4)_2$	6.33	9	1.42
$AlPO_4$	6.14	9	1.47
$FePO_4$	6.28	9	1.43

d, colony growth diameter (cm); D, diameter of phosphate-dissolving zone (cm)

bilized by the fungus *in vitro* with NBRIP-BPB solid medium containing TCP as sole source of P after incubation at 28°C for 5 days. And the fungi were subculture 3 times. The fungus with the most largest halos was selected from eight fungi. The isolate was purified and cultured on CYA at 25°C for 7 days, the colonies were dark green (diameters 57–61 mm), radially sulcate, velutinous, and centrally floccose, 2–4 mm wide with low margin, and reverse grayish orange, and cultured on CA at 25°C for 7 days, the colonies were grayish green (diameters 50–55 mm), plane, floccose, margin entire 3–4 mm, and reverse grayish orange. The 5.8S rRNA of the isolate was amplified, and a 559-bp DNA fragment was obtained. In the GenBank database, it revealed a sequence homology of 99% with *P. oxalicum* strain WX209 (GenBank accession No. KF667524.1) and *P. oxalicum* strain B3-11 (GenBank accession No. JQ446378.1). Therefore, the strain we isolated was a *P. oxalicum* strain and we named it *P. oxalicum* I1 that preserved in the Agricultural Culture Collection of China. The 5.8S rRNA sequence were submitted to GenBank under the accession number KJ466961.

A simple agar plate experiment was performed *in vitro* to visualize the acidification of NBRIP medium by P-I1. In the medium containing insoluble phosphate as sole source of P, P-I1 solubilized the whole insoluble phosphates and grew well. All plates had halo zones in NBRIP medium, and all $Ca_3(PO_4)_2$, $AlPO_4$, and $FePO_4$ were solubilized by *P. oxalicum* I1 for 7 d. The growth diameter of P-I1 and the phosphate-dissolving zone diameter were not significantly different in the various inorganic P fractions as solo P source (Table 1).

Production of organic acids

P-I1 was grown in PDB medium to detect organic acids. Ion chromatographic analysis of the culture filtrates was performed to identify and quantify the organic acids by P-I1. P-I1 excreted oxalic acid during growth in liquid medium (Fig. 1). The variation in pH value and the oxalic acid content of medium after P-I1 inoculation are shown in Fig. 2. The pH value in the medium was significantly reduced with time during the entire P-I1 culture, which decreased from 6.90 to 1.65 in 26 h. Organic acid content and pH value had significant negative correlation. The oxalic acid content increased gradually and reached 593.9 μg/ml at 26 h.

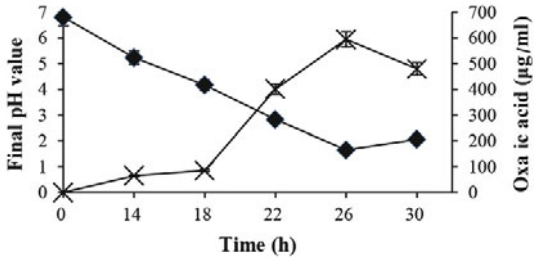

Fig. 2. Variation in pH value and oxalic acid production by P-I1. The final pH values of culture filtrates were measured using a glass electrode, and the amount of organic acids was determined using anion chromatography system at 14, 18, 22, 26, and 30 h. (◆) represents pH value, (×) represents oxalic acid. Points represent means from three replicates, while error bars represent 95% confidence intervals.

Adaptive ability to maize root exudates

P-I1 was cultured on NBRIP-BPB solid medium containing maize root exudates as the sole C source after incubation at 28°C for 7 d. The colony growth diameter was 3.55 cm, and clear halos appeared as a result of TCP being solubilized by the fungus *in vitro*. The diameter of the phosphate-dissolving zone was 4.68 cm. Another experiment was performed to test the adaptive ability to maize in soil. P-I1 could propagate well in soil at 7, 14, and 21 d with the following amounts of P-I1: 1.78×10^7 CFU/g, 2.52×10^7 CFU/g, and 3.14×10^7 CFU/g, respectively. These results show that P-I1 could propagate well and solubilize TCP utilizing maize root exudates.

Colonization and soil acidity abilities of P-I1 in soil

P-I1 was incubated in soil to assay the ability of coloniza-

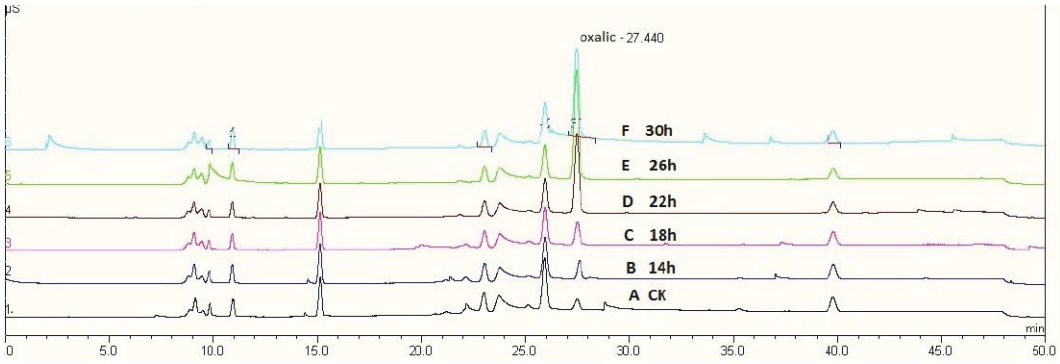

Fig. 1. Ion chromatography peak of organic acid excreted by P-I1 strain. The organic acids were quantified by reference to the peak areas obtained for the authentic standards for oxalic acid. The appearance time of oxalic acid is 27.5 min. (A) CK, oxalic acid was added as authentic standard; (B to F) peaks of oxalic acid at 14, 18, 22, 26, and 30 h.

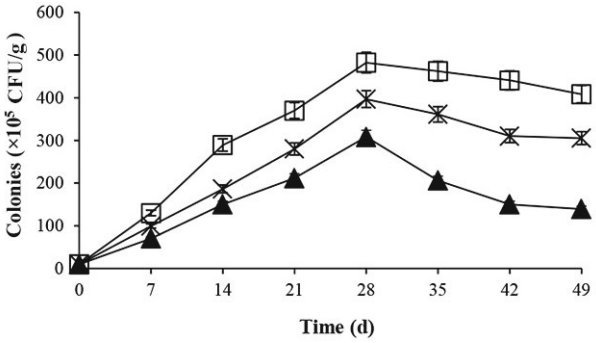

Fig. 3. Amount of P-I1was counted at 7, 14, 21, 28, 35, 42, and 49 d with the NBRIP-BPB solid medium after P-I1 was incubated in soil at 15, 20, and 30°C. (×) represents 15°C, (□) represents 20°C, (▲) represents 30°C. Points represent means from three replicates, while error bars represent 95% confidence intervals.

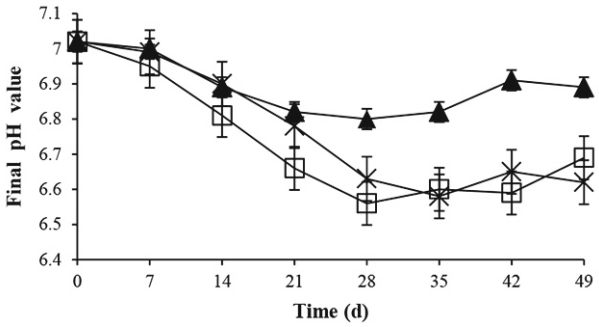

Fig. 4. Final pH values of soil after P-I1 was incubated at 15, 20, and 30°C. (×) represents 15°C, (□) represents 20°C, (▲) represents 30°C. Points represent means from three replicates, while error bars represent 95% confidence intervals.

tion and phosphate solubilization. The results showed that P-I1 was able to propagate well in soil at 15, 20, and 30°C (Fig. 3), but significant differences were observed in the colonization abilities at different temperatures. P-I1 propagated rapidly, at 28 d, the amount of P-I1 were 3.96×10^7 CFU/g, 4.82×10^7 CFU/g and 3.08×10^7 CFU/g at 15, 20, and 30°C, respectively. And the propagation abilities at 15 and 20°C was higher than that at 30°C, but extinction at 15 and 20°C was lower than that at 30°C. The population of P-I1 increased rapidly during 28 d and then decreased after 28 d in soil, and the optimum colonization temperature was 20°C. The amount of P-I1 increased 48-fold in 28 d and was unchanged in 49 d at optimum temperature.

The pH value of soil was decreased during the rapid propagation of P-I1 (Fig. 4). The increase in the amount of P-I1 resulted in the continuous decrease of pH value in soil. The minimum pH value of soil reached 6.56 when the maximum amount of P-I1 was reached. The rate of decrease in pH value at 20°C was higher than those at 15 and 30°C

Table 2A. Inorganic phosphate in soil at different times after P-I1 was incubated at 15°C (μg/g soil)

Time (d)	Ca_2-P	Ca_8-P	Al-P	Fe-P	O-P	Ca_{10}-P
0	12.89±0.62 a	69.73±1.64 a	18.67±0.91 d	26.8±0.71 c	6.41±0.05 a	342.00±6.05 a
7	17.21±0.30 b	68.56±1.79 a	17.65±0.65 cd	25.78±0.97 bc	6.39±0.06 a	340.38±6.93 a
14	20.28±0.92 c	68.21±1.85 a	16.55±0.92 bc	24.44±0.60 ab	6.40±0.060 a	340.21±3.94 a
21	24.11±0.44 d	67.36±1.66 a	16.1±0.61 abc	23.10±1.25 a	6.38±0.09 a	339.57±4.05 a
28	25.32±0.60 d	67.49±0.93 a	14.29±0.63 a	22.68±0.73 a	6.38±0.06 a	340.27±3.13 a
35	24.08±0.78 d	68.55±0.87a	14.32±0.75 a	22.87±1.10 a	6.40±0.12 a	339.89±3.85 a
42	20.15±0.72 c	68.96±1.89a	15.66±0.87 ab	23.69±0.77 a	6.39±0.06 a	341.50±3.48 a

Table 2B. Inorganic phosphate in soil at different times after P-I1 was incubated at 20°C (μg/g soil)

Time (d)	Ca_2-P	Ca_8-P	Al-P	Fe-P	O-P	Ca_{10}-P
0	12.89±0.62 a	69.73±1.64 a	18.67±0.91 c	26.8±0.71 c	6.41±0.05 a	342.00±6.05 a
7	17.69±1.12 b	69.12±1.70 a	17.05±0.85 bc	24.9±0.51 b	6.38±0.04 a	341.49±6.13 a
14	23.15±1.10 d	68.33±1.11 a	15.36±1.21 ab	23.52±0.89 a	6.37±0.06 a	339.67±6.20 a
21	26.21±0.77 e	68.21±0.87 a	14.22±1.02 a	22.16±0.71 a	6.38±0.10 a	339.78±6.18 a
28	27.11±0.74 e	67.58±1.75 a	14.67±0.87 ab	21.69±0.63 a	6.40±0.11 a	339.23±5.28 a
35	23.58±1.19 d	68.46±0.83 a	15.32±0.84 ab	22.76±1.06 a	6.39±0.09 a	340.47±6.03 a
42	20.88±0.78 c	68.63±2.35 a	16.77±1.03 bc	22.58±0.54 a	6.41±0.08 a	340.84±3.32 a

Table 2C. Inorganic phosphate in soil at different times after P-I1 was incubated at 30°C (μg/g soil)

Time (d)	Ca_2-P	Ca_8-P	Al-P	Fe-P	O-P	Ca_{10}-P
0	12.89±0.62 a	69.73±1.64 a	18.67±0.91 b	26.80±0.71 b	6.41±0.05 a	342.00±6.05 a
7	16.36±0.60 b	69.46±2.20 a	18.03±0.67 ab	25.38±0.67 ab	6.43±0.10 a	340.98±4.23 a
14	17.58±1.01 bc	68.87±1.66 a	17.62±0.94 ab	25.10±0.73 ab	6.38±0.05 a	341.06±3.64 a
21	21.33±1.22 d	68.43±1.78 a	16.53±0.55 ab	24.19±0.76 a	6.40±0.08 a	340.24±2.83 a
28	23.09±1.16 e	67.32±1.52 a	16.21±0.89 a	23.22±0.84 a	6.40±0.10 a	340.27±4.69 a
35	18.75±1.29 c	67.98±1.10 a	17.16±0.90 ab	23.98±0.92 a	6.43±0.06 a	341.66±7.04 a
42	17.68±0.70 bc	67.21±0.93 a	17.65±0.97 ab	24.37±1.15 a	6.40±0.06 a	342.23±6.13 a

Statistical analysis was conducted by using Analysis of Variance (ANOVA) statistical package for social sciences (SPSS) software. Data are means of 3 replicates and ±represents the Standard Deviation, Means followed by the same letter(s) in each column are not significantly different at $P \leq 0.05$.

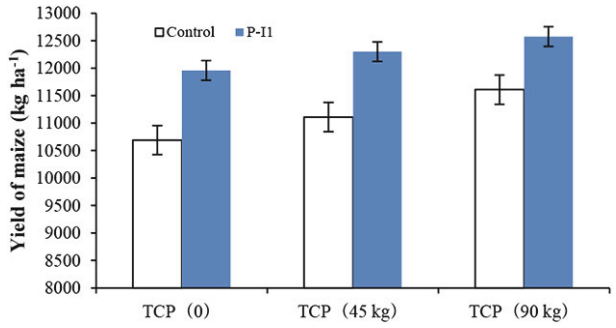

Fig. 5. The field yield of maize with microbial inoculums treatment. TCP represents TCP (□) represents control, (■) represents P-I1, TCP (0), TCP (45 kg) and TCP (90 kg) represents the treatment that 0, 45, and 90 kg/ha TCP was supplied as soil P fertilizer respectively. Points represent means from three replicates, while error bars represent 95% confidence intervals.

during 21 d. After 28 d, the pH values of soil at 15 and 20°C were lower than that at 30°C. No significant differences existed between 15 and 20°C.

Phosphate-solubilization abilities of P-I1 in soil

To investigate the characteristics of P-I1 phosphate solubilization, we analyzed the dynamic equilibrium of different inorganic P fractions at different temperatures after P-I1 incubation in soil. The results showed significant changes in the total amount of Ca_2-P, Ca_8-P, Al-P, and Fe-P (Table 2). P-I1 solubilized and transformed a wide range of insoluble phosphate. Oxalic acid by P-I1 led to the transformation of Ca_8-P, Al-P, and Fe-P to soluble Ca_2-P. The correlation analysis indicated that Ca_2-P content had a negative correlation with soil pH value and a positive correlation with P-I1.

P-I1 was incubated in soil at 15°C, and Ca_2-P content initially increased and then decreased. It reached the maximum 25.32 μg/g soil on day 28 and increased by 96.43% than control with no microbial inoculums. Ca_8-P (2.24 μg/g soil), Al-P (4.38 μg/g soil), Fe-P (4.12 μg/g soil), and Ca_{10}-P (1.73 μg/g soil) were transformed to Ca_2-P (Table 2A). No significant changes were found in the total amount of O-P. After day 28, the Ca_8-P content increased, and part of Ca_2-P was transformed to Ca_8-P, but 0.77 μg/g soil Ca_8-P was transformed into Ca_2-P on day 42. The transforming rules of Al-P and Fe-P were similar to those of Ca_8-P. The Ca_{10}-P form was stable, and a small amount Ca_{10}-P was transformed to other phosphate forms.

The change in Ca_2-P content at 20°C was similar to that at 15°C. The Ca_2-P content reached the maximum 27.11 μg/g soil on day 28, which increased by 110.32% compared with CK. Ca_8-P (2.15 μg/g soil), Al-P (4.00 μg/g soil), Fe-P (5.11 μg/g soil), and Ca_{10}-P (1.53 μg/g soil) were transformed to Ca_2-P (Table 2B). No significant changes existed in the total amount of O-P. After day 28, part of Ca_2-P was transformed to Ca_8-P, Al-P, Fe-P, and Ca_{10}-P.

The change in Ca_2-P content at 30°C was the similar to those at 15 and 20°C. Ca_2-P reached the maximum value of 23.09 μg/g soil on day 28, which increased by 79.13% compared with CK. Ca_8-P (2.41 μg/g soil), Al-P (2.46 μg/g soil), Fe-P (4.58 μg/g soil), and Ca_{10}-P (1.73 μg/g soil) were transformed to Ca_2-P (Table 2C). After day 28, part of Ca_2-P was mostly transformed to Al-P, Fe-P, and Ca_{10}-P.

Plot experiment

To identify the effect of promoting crop growth by PI-1, we conducted the plot experiment in northeastern China. The results showed that maize yield increased remarkably than control after inoculated P-I1 (Fig. 5), maize yield increased maximum by 14.47% than control in the treatment with P-I1 and TCP 45 kg/ha. Soil P fertilizer was 90 kg/ha in China, although we reduced half P fertilizer, maize yield was the same as full P fertilizer. P-I1 can solubilize phosphate that supply enough soluble phosphate to crop growth.

Discussion

Organic acid secretion in soil by strains is the most important phosphate solubilization approach of PSMs. Organic acid can chelate with iron, aluminum, and calcium ions, resulting in the transformation of insoluble phosphate to available phosphate. PSMs are directly applied in soil mainly as living creatures. PSMs can release insoluble phosphorus in soil by secreting organic acids, thereby improving the utilization ratio of phosphorus in soil. Oxalic acid have been recognized for phosphate solubilization by several species of *Penicillium*. *Penicillium bilaiae* secreted oxalic acid that reached 1,200 μg/ml in culture on day 12 (Takeda *et al.*, 2007), in this study, the oxalic acid content increased gradually and reached 593.9 μg/ml at 26 h. Nutrition affects the production of organic acids by microorganisms. Under different conditions, different acids may be secreted or even the ability of acid production may totally disappear, affecting the acid effect. *Penicillium bilaiae* mainly produces citric acid under nitrogen deficiency and produces oxalic acid under carbon deficiency (Cunningham and Kuiak, 1992). Therefore, differences exist in the phosphate-solubilizing effect. Nitrogen source remarkably affects the types of organic acids secreted by *P. oxalium*, such as acetic acid, malic acid, propionic acid, citric acid, and succinic acid, which are mainly secreted with NH_4^+ as nitrogen source. Vora and Shelat (1998) discussed the effects of carbon and nitrogen forms on phosphate solubilization. $(NH_4)_2SO_4$ is the best nitrogen source, whereas $NaNO_3$ cannot be used; glucose, sucrose, and fructose are the best carbon sources for *P. oxalium*. In presence of succinate, phosphate solubilization of *Klebsiella pneumoniae* was completely inhibited, and the enzymes glucose dehydrogenase and glyoxylate oxidase were repressed (Rajput *et al.*, 2013). Therefore, the effects of PSMs used in different soil environments may remarkably vary.

Soil pH is often considered one of the most informative measurements of soil characteristics (Thomas, 1996), as it directly influences plant growth, metal ion solubility, microbial activity and soil physical properties, particularly clay dispersion and aggregation (Haynes and Naidu, 1998). A high correlation was observed between soil final pH and soluble phosphate (Walpola and Yoon, 2013). Organic acids can reduce soil pH, resulting in the dissociation of insoluble phosphate and metal ions in soil (Hu *et al.*, 2001; Ryan *et al.*, 2001). In the study of Jain *et al.* (2014), *A. niger* decreases

culture pH from 7.0 to 2.03 at 12 d; in the present study, *P. oxalium* I1 decreased culture pH from 6.9 to 1.65 at 26 h. An excessively low soil acidification can cause soil problems. The acids produced by microorganisms will lead to soil acidification (Li *et al*., 2008). Crop growth will be affected when the soil pH value is less than 6.5. If the low pH is attributed to the secretion of organic acids in soil by PSMs, caution should then be considered against the long-term use of PSMs. In this experiment, the strains caused the decrease in the pH value of soil from 7.0 to 6.56. This result indicates that P-I1 had a strong ability for soil acidification but did not affect crop growth. Whether long-term use will cause a great decrease in the pH value of soil and affect crop growth needs further investigation.

Soil phosphates are divided into Ca-P, Fe-P, Al-P, and O-P. Ca-P is further divided into three types, namely, Ca_2-P, Ca_8-P, and Ca_{10}-P. Classification of inorganic phosphate in soil is important in investigating the microbial transformation of soil phosphorus and improving the utilization ratio of phosphorus fertilizers. The selection of solubilizing inorganic P fraction by microorganisms has been attracting the attention of scientists. Microorganisms showed selectivity on the transformation of the different forms of phosphorus. PSMs in rhizosphere and non-rhizosphere soils demonstrated preference in the solubilization of insoluble phosphate (Ralston and McBride, 1976). Among the various phosphate-solubilizing strains, *Pseudomonas* sp. and *Penicillium aurantiogriseum* showed strong solubilization ability on Ca-P, and *Aspergillus niger* and *Penicillium simplicissimum* had strong solubilization ability on Al-P (Illmer and Schinner, 1995). This phenomenon is the main reason for the instability of the effect of solubilizing phosphate. It also explains the significant differences in the phosphate-solubilizing ability in different soil types. P-I1 solubilizes and transforms a wide range of insoluble phosphate. It can also solubilize various forms of insoluble inorganic phosphate in soil. The transformation of insoluble inorganic phosphate to available phosphorus is the most intensive at 20°C. Some inorganic phosphates in the form of Ca_8-P, Al-P, Fe-P, and Ca_{10}-P were transformed to Ca_2-P, which is consistent with the high colonization ability of P-I1 at 20°C.

The application of PSMs began in 1940s. The development of strains, including *Penicillium* and *Bacillus*, into products greatly promoted the study and application of PSMs. *P. bilaii* was inoculated onto grown peas. The amount of root hairs increased by 22% after the inoculation, and the hair length increased by 33% (Gulden and Vessey, 2000). *P. bilaii* was inoculated onto peas in the two regions in Canada. The root length and weight increased by 48% and 13%, respectively after the inoculation. The phosphorus content in stem increased by 13% compared with the control treatment (Vessey and Heisnger, 2001). In this study, *P. oxalicum* was used in maize, and maize yield increased by 17%. Numerous experiments have proved that PSMs have very good yield-increasing effect. However, controlling the effect with the direct application of PSMs as living organisms in an open environment is difficult. This finding is mainly due to the varied effect of PSMs caused by the degeneration of strains, compatibility of crops, soil properties, climate conditions, colonization ability in soil, and interaction between microorganisms and plants. Further studies should focus on the solubilization of insoluble P fractions by *Penicillium* strain in different environments (Gyaneshwar *et al*., 2002) as well as on the influence of *Penicillium*plant interactions on phosphate solubilization in soils. The root exudates provide nutrition for the growth of microorganisms and maintain the microorganism action.

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