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An Effective Method for Screening and Testing the True Phosphate-Solubilizing Fungus That Enhances Corn Growth

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Abstract

The effect of large-scale application of phosphate-solubilizing microorganisms (PSMs) on the promotion of crop growth varies. Usually the solubilization of tricalcium phosphate is considered as the standard for screening most PSMs. This study aimed to establish a common method for screening and testing true PSMs. The isolated fungus's phosphate-solubilizing ability, adaptation to corn root exudates, organic acids secreted, and colonization ability were analyzed. And the microorganism's diversity and ability to promote corn growth were also tested through plot experiment. The isolated fungus *Aspergillus niger* H1 (A-H1) was isolated and identified from plant rhizosphere that could solubilize phosphate, utilize corn root exudates as sources, and propagate well in vitro and soil. Lactic acid was excreted and reached 377.9 μg mL^{-1} at 30 h in culture by A-H1, which decreased the pH of culture to 1.73. The amount of A-H1 increased by 41-fold in 28 d and was maintained for 49 d. PSM showed selectivity on the transformation of different forms of P.However, a wide range of insoluble phosphates, such as $Ca_8H_2(PO_4)_6{\cdot}5H_2O$, $AlPO_4$, $FePO_4$, and $Ca_{10}(PO_4)_6(OH)_2$, was converted to soluble $CaHPO_4$ in soil.$CaHPO_4$ was also inhibited from being converted into insoluble phosphate by A-H1. Corn yield increased by 10.71% that remarkable higher than the control after A-H1inoculation. Data show that A-H1 could propagate well, solubilize phosphate in soil, and promote corn growth. A common method was created to screen and test a true phosphate-solubilizing fungus that enhances plant growth.

Keywords: *Aspergillus niger*, phosphate-solubilizing, corn, common method

1. Introduction

Phosphate-solubilizing microorganisms (PSMs), which are commonly found in soil, can solubilize insoluble phosphate(P), reduce phosphorus fertilizer fixation, increase the utilization ratio of P fertilizers, and promote crop growth(Mehta et al., 2013; Adesemoye et al., 2009; Altomare et al., 1999). PSMs can solubilize P in soil to promote plant growth. However, not all microorganisms that can solubilize P are necessarily known as PSMs. Three criteria need to be satisfied for a microorganism to be considered as a true PSM: it should be able to propagate well, solubilize P in soil, and promote plant growth (Walpola et al., 2013; Mamta et al., 2012; Jain et al., 2011; Park et al., 2010). As of this writing, the solubilization of tricalcium phosphate (TCP) is considered the standard for screening PSMs, which are usually determined only by in vitro solubilization of inorganic P (Syed et al., 2013; Sara et al., 2013; Yu et al., 2011). However, TCP is inappropriate as a universal selection factor for isolating and testing PSMs that enhance plant growth (Bashan et al., 2013).Some strains can solubilize inorganic P in vitro but cannot do the same in soil or cannot promote crop growth (Freitas et al., 1997). Microorganisms that can solubilize inorganic P in vitro are not necessarily PSMs. PSMs should be screened by experiments on soil colonization, inorganic P solubilization, and plant growth promotion abilities. Microorganisms, as P-solubilizing biofertilizer inoculants, can be used in many crops. One inoculant can be used on many crops, but the effects vary, so if we screen fugus that could promote one plant growth, adaptation to plant root exudates should be tested. Thus, an efficient method for screening and testing a true P-solubilizing fungus that can enhance plant growth should be created.

PSMs are directly applied in soil mainly as living creatures, so their effects depend on their colonization ability in soil, i.e., the longer they propagate, the longer they solubilize P and promote plant growth. However, many studies have shown that not all microorganisms with P-solubilizing ability in vitro can solubilize P in soil (Banik & Dey, 1982; Kucey, 1983; Freitas et al., 1997). Soil nutrition has an important function in microorganism propagation and growth promotion. Nutrition affects the production of organic acids by microorganisms. For example, glucose and sucrose significantly promote P solubilization compared with fructose, lactose, galactose, and xylose for *Aspergillus tubingensis*, and potassium nitrate significantly increases P solubilization compared with other N sources, such as ammonium sulfate, ammonium nitrate, aspargine, and tryptophan (Relwani et al., 2008). Under different conditions, different acids may be secreted or the ability to produce acid may totally disappear, affecting the acid effect. Therefore, the effects of PSMs used in different soil environments may remarkably vary. C is the most important nutrient of microorganisms. Crops can secrete many types of carbohydrates that can be utilized by microorganisms as a C source. More than 30 carbohydrates, such as arabinose, xylose, and glucose, are in corn root exudates (Chaboud et al., 1990). Plant root exudates can be used as stable C sources of microorganisms. However, the adaptation of microorganisms to plant root exudates is seldom used as a standard for evaluating colonization ability in soil. Many research showed that a mutual growth existed in plant and microorganisms (Bansal et al., 1994), however, not all microorganisms could be prompt to propagate, because of lacking nutrients of soil, if microorganisms could not utilize the plant root exudates as C source, the ability of P solubilization would be limited.

The solubilization of inorganic P by microorganisms has been attributed to processes involving acidification, H^+ excretion, chelation, and redox reaction in the growth environment (Sindhu et al., 2014; Rodríguez et al., 2006). 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, fumarate, fumaric, and gluconic acids are the main organic acids known to solubilize P (Soltani et al., 2010; Bianco et al., 2010; Park et al., 2010; Khan et al., 2007; Fomina et al., 2005; Altomare et al., 1999; Banik et al., 1982). Organic acids can chelate Fe^{3+}, Fe^{2+}, Ca^{2+}, and Al^{3+}; thus, insoluble P is transformed to soluble P. A high correlation exists between the final pH and soluble P (Walpola et al., 2013); the stronger the acidification ability of soil, the stronger the ability of P solubilization (Nahas, 1996). Various inorganic P fractions (Ca_2-P, Ca_8-P, Al-P, Fe-P, O-P, and Ca-$_{10}$-P) exist in soil, and the contents of inorganic P fractions vary across different soil types (Sindhu et al., 2014; Giesler et al., 2012; Varinderpal et al., 2007). Given the dynamic equilibrium between various P forms, soluble forms are usually converted to insoluble forms in soil (Hinsinger, 2001). Microorganisms show selectivity in the transformation of different forms of P, and organic acids may be secreted under different conditions. The P-solubilizing effects vary with different mechanisms, so considerable differences exist in the selection of solubilizing inorganic P fraction by different microorganisms (Toro et al., 1996). Thus, microorganisms fully function in P- solubilization in a suitable soil environment.

Numerous experiments have already prevented that PSMs have very good yield-increasing effects. *Penicillium bilaii* was inoculated onto grown peas, and caused root hair quantity to increase by 22% and root hair length to increase by 33% (Gulden et al., 2000). *P. bilaii* was inoculated onto peas in two regions in Canada, and caused root length and weight to increase by 48% and 13%, respectively, whereas the P content in the stem increased by 13% compared with that in the control treatment (Vessey et al., 2001). However, not all PSMs produce the stable effects of plant growth promotion because such effects depend on the colonization ability in soil and P-solubilization ability.

Four experiments were conducted in this study. *Aspergillus niger*, a fungus that secretes lactic acid and converts a wide range of insoluble P to soluble forms, was isolated and identified. Its propagation ability and adaptation to corn, as well as the transformation of inorganic P fractions in soil, are described in this paper. The microorganism's P-solubilizing ability, plant growth promotion, and diversity were tested and verified. A common method was devised to screen and test the P-solubilizing fungus used as a stable biofertilizer inoculant.

2. Materials and Methods

2.1 Fungal Strain Isolation and Identification

Rhizospheric soil was collected from a field in northeastern China. Soil samples (10 g) were suspended in 50 mL of sterile water on a gyratory shaker (170 rpm) for 1 h at 28 °C. The suspension was serially diluted tenfold, and 0.1 mL of each dilution was plated onto NBRIP-BPB solid medium containing 0.5% TCP as an insoluble P source (Nautiyal, 1999). After 5 d of incubation at 28 °C, the plates were examined for the presence of colonies with clear halos. Fungal colonies with maximum clear zones around them were selected and purified further by plating onto fresh agar plates. The selected fungus was maintained on potato dextrose agar.

The isolate was cultured on standard media, Czapek yeast agar (CYA) for 7 d to determine the identity of the isolated fungal strain and test the colony morphology. The internal transcribed spacer regions (ITS1 and ITS2) of 5.8S rRNA underwent sequencing using the primers ITS1:5′-TCCGTAGGTGAACCTGCGG-3′ and ITS2:5′-GCTGCGTTCTTCATCGATGC-3′ (White et al., 1990). The PCR reaction mix contained 0.5 μM of each primer, 10 μM deoxynucleotides, 1.5 mM $MgCl_2$, and 10x buffer (NEB). The suspension was heated at 94 °C for 15 min in a thermocycler (ABI 9700). One unit of 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 s, 57 °C for 50 s, 72 °C for 50 s, and final elongation at 72 °C for 10 min. The presence of amplified fragments was checked by standard gel electrophoresis. The sequence (GenBank No KJ778683) was analyzed using the GenBank database.

The CYA medium comprised the following components (g/L): 3 g of $NaNO_3$, 0.5 g of $MgS0_4{\cdot}7H_2O$, 0.01 g of $FeSO_4{\cdot}7H_2O$, and 5 g of yeast. The medium was sterilized by autoclaving at 115 °C for 10 min.

2.2 Soil, Preparation of Fungal Spore Suspension, and Corn Root Exudates

Soil was collected from a corn field in Heilongjiang Province in northeastern China. The characteristics of the soil are as follows: pH 7.0; organic matter, 26.40 g kg^{-1}; total N, 0.46 g kg^{-1}; available P, 12.89 mg kg^{-1}; and extractable potassium, 30.65 mg kg^{-1}.

A. niger H1 (A-H1) which has been preserved in the Agricultural Culture Collection of China(ACCC32579) was grown in 300 mL 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^{-1}, and the solution was stored at 4 °C until use. The fungal spore suspension was added into grass coal, and the spore content was 5.4×10^7 CFU g^{-1}. The microbial inoculum was stored at room temperature until use.

A germinated corn seed (Zhegndan958) was grown on a 50 mL tube with 48 mL of plant nutrient solution under illumination at 25 °C. The solution underwent vacuum pumping 10 times at a 60 °C water bath, and stored at room temperature until use. The plant nutrient solution comprised the following components(mol L^{-1}): 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}.

2.3 Estimation of Inorganic P Solubilization in vitro

The in vitro inorganic P-solubilizing ability was determined in NBRIP-BPB solid medium containing TCP as the sole source of P (Nautiyal 1999). $AlPO_4$ and $FePO_4$were supplemented in the medium instead of $Ca_3(PO_4)_2$. A total of 10 μL of fresh culture (10^7 CFU mL^{-1}) was spotted on the plates, and incubated at 28 °C for 7 d. The halo zone and fungi colony diameter were measured.

2.4 Analysis of Organic Acid

To analyze organic acid excretion by A-H1, 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 the pH of the medium and organic acid content using a pH meter and ion chromatography system (ICS-3000, Dionex, USA), respectively, at 14, 18, 22, 26, and 30 h. Each treatment was replicated three times. The organic acids were quantified with reference to the peak areas obtained for the authentic standards for lactic acid (Sigma, USA). Organic acids were identified using an IonPac AG11-HC guard column (4 mm × 50 mm) and IonPac AS11-HC (4 mm × 250 mm) chromatographic column under the following conditions: 0 - 5 min, 1.0 mmol L^{-1} KOH; 5 - 45 min, 36.00 mmol L^{-1} KOH; and 45 - 50 min, 1.0 mmol L^{-1} KOH. The appearance time of lactic acid was 9.5 min.

2.5 Estimation of Adaptation to Corn

The adaptive ability between strains and corn was analyzed in vitro, in which 15 mL of solution containing corn 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^{-1}) was spotted on the plates and incubated at 28 °C for 7 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 corn. The culture was mixed into a beaker with 100 g of sterilized soil containing 1×10^6 CFU and 15 mL of solution with corn root exudates at 28 °C. The soil samples were collected at 7, 14, and 21 d, and A-H1 colonies were counted in NBRIP-BPB solid medium containing 0.5% TCP as an insoluble P source (Nautiyal, 1999) and 100 μg mL^{-1} amp, 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 P-solubilizing activities on the solid medium containing

insoluble phosphate. The experiment was performed three times.

2.6 Colonization, Soil Acidity and Inorganic P Fraction Solubilization Abilities in Soil

An experiment was conducted in the soil culture trial to study the colonization, soil acidity and inorganic P fraction solubilization abilities of A-H1 at 20 °C in soil. The culture was mixed into pots with 500 g of soil containing 1×10^6 CFU, 0.1 g of dextrose, and 0.05 g of NH_4Cl per g soil. The culture was then incubated at 20 °C. The moisture was maintained between 30% and 50%. Each treatment was replicated three times. The experiment was performed three times.Soil samples were collected at 7, 14, 21, 28, 35, 42, and 49 d, and A-H1colonies were counted. Soil samples were dried by air, 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^{-1} KCl solution in a 50 mL flask, and agitated vigorously for 1 min. After equilibration for 30 min, the pH of the soil suspension was measured using a glass electrode (EUTECH pH510).Various inorganic P fractions were determined to analyze the characteristics of P solubilization by A-H1. Soil samples were studied for various inorganic P fractions using the P fractionation scheme (Chang et al., 1957, 1962).

2.7 Plot Experiment and Diversity of Soil Microorganisms

The experiments were conducted in northeastern China to understand the effect of A-H1 on corn yield. Corn seeds were Zhegndan958. TCP was used as a soil P fertilizer. The experimental plan was based on six treatments as follows:(i) control; (ii) 45 kg ha^{-1} TCP; (iii) 90 kg ha^{-1} TCP; (iv) A-H1; (v) 45 kg ha^{-1}A-H1 and TCP; and (vi) 90 kg ha^{-1}A-H1 and TCP. Plots were arranged in a randomized complete block design with three replicates per treatment. Each plot was 100 m^2 with 600 corn seedlings. 3 g of microbial inoculum was added near the root system when the corn were 15 - 20 cm high. The plants were managed daily in accordance with local planting methods, and the grain water content of corn was converted into 14% after the grains were harvested.

Microbial cells were extracted from the soil samples to assess the differential utilization of C sources in the 95 different Biolog plates. Approximately 25 g of each replicate soil sample was collected from the topsoil (0 - 10 cm) around corn roots on July 5, August 5, and September 5 to analyze the diversity of soil microorganisms. Soil samples were added into BIOLOG-GN plates at 20 °C (Garland et al., 1991). The plates were read at BIOLOG after 72 h to calculate the Shannon index and Shannon evenness (Table 1) (Schutter et al., 2001). Each treatment was replicated three times.

Table 1. Formulae for Shannon - Weaver diversity and Shannon evenness

Index	Definition	Formula	Definitions
Shannon diversity	Measure of richness and evenness	H'=-∑ PilnPi	*pi* = proportional colour development of the *i*th well over total colour development of all wellsof a plate
			N = sum of positive optical densities on a Biolog plate
		H' = *C/N* (*N*ln*N*-∑ niln ni)	*ni* = zero or positive optical density of a test well on a Biolog plate *C* = 2.3
Shannon evenness	Evenness calculated from Shannon Index	E = H'/ln *S*	H' = Shannon index of diversity
			S = number of wells with colour development

3. Results

3.1 Isolation and Identification of P-Solubilizing Fungus From Soil Sample

Clear halos appeared as a result of TCP being solubilized by the fungus in vitro with NBRIP-BPB solid medium containing TCP as a sole source of P after incubation at 28 °C for 5 d. There were six fungi that the most largest halos was selected. The isolate was purified and cultured on CYA at 25 °C for 7 d. The colonies were dark (90 mm in diameter), radially sulcate, velutinous, 4 mm wide with low margin, and reverse grayish yellow. The 5.8S rRNA of the isolate was amplified, and a 573 bp DNA fragment was obtained. The GenBank data base revealed a sequence homology of 99% with *A. niger* strain 1504 (GenBank accession No.JX945161.1) and *A. niger* strain B6 (GenBank accession No. JN676125.1). Therefore, the strain isolated was *A. niger* and named *A. niger*H1, which has been preserved in the Agricultural Culture Collection of China (ACCC42579). The 5.8 S rRNA sequence was submitted to GenBank under the accession number KJ778683.

A simple agar plate experiment was performed in vitro to visualize the acidification of NBRIP medium by A-H1.In the medium containing insoluble P as the sole source of P, A-H1 solubilized all insoluble phosphates and demonstrated good growth. All plates had halo zones in NBRIP medium, and $Ca_3(PO_4)_2$, $AlPO_4$, and $FePO_4$ were solubilized by *A. niger* H1 for 7 d.

3.2 Production of Organic Acids

A-H1 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 A-H1, which excreted lactic acid during growth in liquid medium (Figure 1). The variations in pH and lactic acid content of the medium after A-H1inoculation are shown in Figure 2. The pH in the medium significantly decreased with time during the entire A-H1culture; specifically, the pH decreased from 6.90 to 1.73 in 30 h. The organic acid content and pH demonstrated a significantly negative correlation. The lactic acid content gradually increased and reached 377.9 μg mL^{-1} at 30 h.

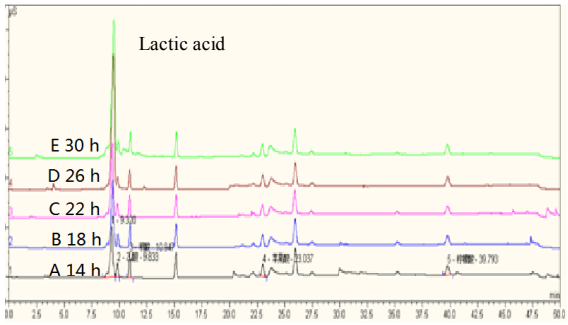

Figure 1. Ion chromatography peak of organic acid excreted by A-H1 strain

The organic acids were quantified by reference to the peak areas obtained for the authentic standards for lactic acid. The appearance time of lactic acid is 9.5 min. A to E peaks of lactic acid at 14, 18, 22, 26, and 30 h.

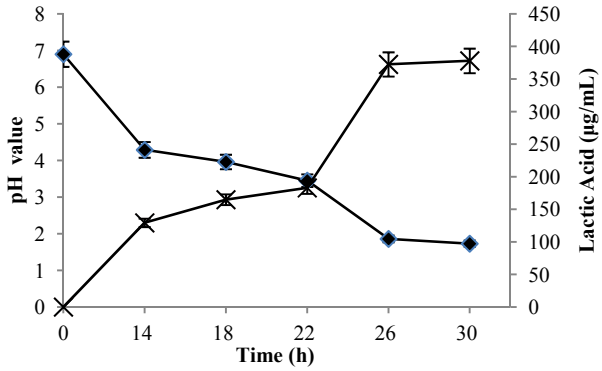

Figure 2. Variation in pH value and lactic acid production by A-H1

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 lactic acid. Points represent means from three replicates, while error bars represent 95% confidence intervals.

3.3 Adaptive Ability to Corn

A-H1 was cultured on NBRIP-BPB solid medium containing corn root exudates as the sole C source after incubation at 28 °C for 7 d. The colony growth diameter was 6.25 cm, and clear halos appeared as a result of TCP being solubilized by the fungus in vitro. The diameter of the P-dissolving zone was 8.13 cm. Another experiment was performed to test the adaptive ability to corn in soil. A-H1could propagate well in soil at 7, 14, and 21 d using the following amounts of A-H1: 2.52×10^7 CFU g^{-1}, 4.41×10^7 CFU g^{-1}, and 4.80×10^7 CFU g^{-1}, respectively.These results show that A-H1 could propagate well and solubilize TCP utilizing corn root exudates.

3.4 Colonization, Soil Acidity Abilities and Inorganic P Fraction Solubilization of A-H1 in Soil

A-H1 was incubated in soil to assay the colonization and P solubilization abilities. The results show that A-H1 could propagate well in soil at 20 °C (Figure 3), There were less than 100 CFU g^{-1} PSMs whit no A-H1 incubation, A-H1 propagated rapidly at 28 d, and A-H1 was 4.10×10^7 CFU g^{-1}. The amount of A-H1increased rapidly during 28 d, and decreased after 28 d in soil. The pH of soil decreased during the rapid propagation of A-H1 (Figure 3). The increase in the amount of A-H1 resulted in the continuous decrease in pH in soil. The lowest pH of soil was 6.33 at 35 d. To investigate the characteristics of A-H1 P solubilization, the dynamic equilibrium of different inorganic P fractions was analyzed after A-H1 incubation in soil. The results show significant changes in the total amount of Ca_2-P, Ca_8-P, Al-P, and Fe-P (Tables 2). A-H1 solubilized and transformed a wide range of insoluble P. lactic acid by A-H1 led to the transformation of Ca_8-P, Al-P, and Fe-P to soluble Ca_2-P. Correlation analysis indicated that the Ca_2-P content was negatively correlated with soil pH and positively correlated with A-H1. The Ca_2-P content initially increased and then decreased after A-H1 incubated in soil at 20 °C. The Ca_2-P content reached the maximum of 29.18 μg g^{-1} soil on day 28, which increased by 126.38% compared with CK. Ca_8-P (7.55 μg g^{-1} soil), Al-P (3.36 μg g^{-1} soil), Fe-P (3.74 μg g^{-1} soil), and Ca_{10}-P (1.64 μg g^{-1} soil) were transformed to Ca_2-P (Table 2); no significant changes were observed 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.

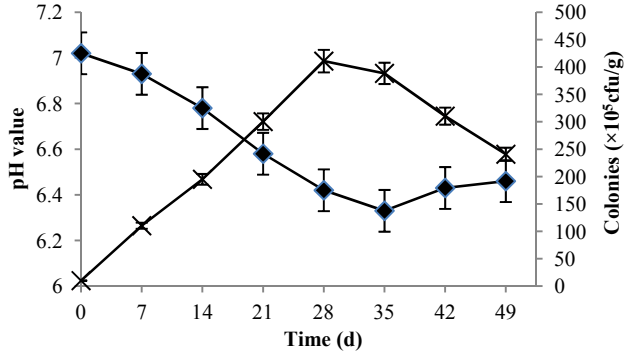

Figure 3. Final pH values of soil and amount of A-H1 at 7, 14, 21, 28, 35, 42, and 49 d after A-H1 was incubated in soil at 20

◆ represents pH value, × represents colonies. Points represent means from three replicates, while error bars represent 95% confidence intervals.

Table 2. Inorganic phosphate in soil at different times after A-H1 was incubated at 20 °C

t/d	Inorganic-P fraction/ (μg/g)					
	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 c	18.67±0.91 c	26.8±0.71 c	6.41±0.05 a	342.00±6.05 a
7	16.34±0.68 b	67.88±0.39 b	17.69±0.66 bc	26.18±0.70 bc	6.38±0.06 a	341.66±11.20 a
14	22.43±0.58 c	64.46±1.06 a	17.15±0.66 b	25.1±1.34 abc	6.40±0.07 a	341.16±11.42 a
21	26.77±0.67 d	63.49±0.78 a	16.33±0.69 ab	23.68±0.61 a	6.37±0.06 a	340.15±11.23 a
28	29.18±0.62 e	62.18±1.20 a	15.41±0.47 a	23.06±0.80 a	6.38±0.03 a	340.36±12.68 a
35	25.63±1.07 d	63.75±0.72 a	15.98±0.54 ab	23.99±1.28 ab	6.42±0.07 a	341.08±2.91 a
42	23.14±0.30 c	64.30±1.06 a	16.77±0.71 ab	24.37±1.15 ab	6.40±0.06 a	340.75±6.54 a

Statistical analysis was conducted by using Analysis of Variance (ANOVA) statistical package for social sciences (SPSS 21.0) 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 $P \leq 0.05$.

3.5 Plot Experiment and Diversity of Soil Microorganisms

Plot experiments were performed in northeastern China to identify the effect of promoting crop growth by A-H1. The results show that corn yield remarkably increased after A-H1 inoculation compared with that of the control (Figure 4). Moreover, corn yield increased by a maximum of 10.71% compared with that in the control after treatment with45 kg ha^{-1}A-H1 and TCP. Soil P fertilizer was 90 kg ha^{-1} in China. Although P fertilizer was reduced to half, corn yield was similar to that with full P fertilizer. A-H1 could solubilize P to supply enough soluble P for crop growth.

To estimate the microbial diversity of soil after A-H1 inoculation, the Shannon index and Shannon evenness were analyzed. The results show significant changes in the diversity of soil microorganisms after A-H1 was incubated (Table 3). H and E increased compared with CK in three samples. The diversity of soil microorganisms increased after A-H1 inoculation. A significantly negative correlation was observed between the diversity of soil microorganisms and corn yield.

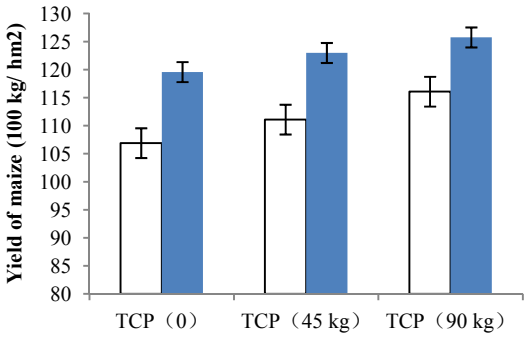

Figure 4. The field yield of corn with microbial inoculums treatment

□ represents control, ■ represents A-H1, TCP(0), TCP(45 kg) and TCP(90 kg) represents the treatment that 0, 45 and 90 kg/ha^2 tricalcium phosphate was supplied as soil P fertilizer respectively. Points represent means from three replicates, while error bars represent 95% confidence intervals.

Table 3. Shannon diversities and Shannon evenness of microorganisms isolated from corn roots soil samples and incubated at 20 °C for 72 h

		H'			E		
		July 5th	August 5th	September 5th	July 5th	August 5th	September 5th
TCP (0)	control	3.724±0.106 a	3.963±0.149 a	3.472±0.047 a	0.828±0.024 a	0.878±0.033 a	0.773±0.010 a
	A-H1	4.253±0.073 c	4.372±0.058 c	3.830±0.065 b	0.940±0.016 c	0.965±0.013 bc	0.856±0.015 b
TCP (45 kg/hm^2)	control	3.872±0.051 b	4.219±0.081 b	3.818±0.170 b	0.869±0.012 b	0.942±0.018 b	0.853±0.038 b
	A-H1	4.383±0.051 cd	4.475±0.036 c	4.183±0.071 c	0.965±0.012 c	0.985±0.008 c	0.925±0.016 c
TCP (90 kg/hm^2)	control	3.943±0.050 b	3.935±0.082 a	3.809±0.083 b	0.878±0.011 b	0.874±0.019 a	0.859±0.019 b
	A-H1	4.429±0.093 d	4.478±0.037 c	4.041±0.078 c	0.975±0.021 c	0.990±0.008 c	0.900±0.018 bc

Statistical analysis was conducted by using Analysis of Variance (ANOVA) statistical package for social sciences (SPSS 21.0) 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 $P \leq 0.05$.

4. Discussion

PSMs were directly applied to soil mainly as living creatures. However, the effect of large-scale application of PSM on the promotion of crop growth varies (Khan et al., 2007; Banik et al., 1983). This variability in performance has greatly hampered the large-scale application of PSMs in agriculture (Khan et al., 2007), which is affected by multiple elements, such as microorganism colonization ability, crop species, soil types, soil nutrition, and microbial interactions. Thus, a common method for screening and testing the true PSM capable of

enhancing plant growth should be established. To screen a true PSM that enhances corn growth, experiments on four qualities, namely, P-solubilizing ability and mechanism, colonization ability in soil, adaptation to plant, and effect of promoting plant growth, are necessary. Microorganism propagation ability in the plant rhizosphere is the most important reference point to determine the function of living creatures. C and N in soil are the most limiting factors of microorganism propagation because different kinds and amounts of C and N sources are found indifferent soil types. Plant root exudates can also serve as nutrient sources, and consist of a complex mixture of organic acid anions, phytosiderophores, sugars, vitamins, amino acids, purines, nucleosides, inorganic ions (e.g., HCO_3^-, OH^-, H^+), gaseous molecules (CO_2 and H_2), and enzymes (Dakora et al., 2002). Stable C and N sources in root exudates supply stable nutrients for microorganisms. Every microorganism has optimal C and N sources; if a microorganism can utilize plant root exudates to propagate well, it may promote plant growth. Although plant root exudates consist of a complex mixture, the content is very low. Microorganisms that propagate well in low-nutrient environment scan promote plant growth. *A. niger* can utilize corn root exudates as sources to propagate well in vitro and soil. The amount of *A.niger* increased by 30% in soil added with corn root exudates, indicating that *A. niger* was compatible with corn root exudates.

Experiments have proved that *Aspergillus* sp. positively affects P solubilization and inhibits the transformation of soluble P to insoluble P (Jain et al., 2014; Cerezine et al., 1988), *A. niger* can utilize a wide range of carbohydrates as C sources, and exhibit good propagation. However, *A. niger* is seldom used in large-scale applications. Further studies should focus on the solubilization of insoluble P fractions by *Aspergillus* strain in different environments (Gyaneshwar et al., 2002), as well as the influence of *Aspergillus* - plant interactions on P solubilization in soils.

Soil pH is among the most informative measurements of soil characteristics (Thomas, 1996) because it directly influences plant growth, metal ion solubility, microbial activity, and soil physical properties, particularly clay dispersion and aggregation (Haynes et al., 1998). A high correlation exists between the final pH of soil and soluble P (Walpola et al., 2013). Organic acids can reduce soil pH, resulting in the dissociation of insoluble P and metal ions in soil (Hu et al., 2001; Ryan et al., 2001). Jain screened *A. niger* that decreased pH of culture from 7.0 to 2.03 at 12 d (Jain et al., 2014), *A. niger* in this study that decreased pH of culture from 6.9 to 1.73 at 30 h. Excessively low soil acidification can cause soil problems because the acids produced by microorganisms will lead to soil acidification(Li et al., 2008). Crop growth is affected when the soil pH is lower than 6.5. Considering that 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 a decrease in the pH of soil from 7.0 to 6.33, indicating that A-H1 had a strong ability for soil acidification but did not affect crop growth. Whether long-term use will cause a substantial decrease in the pH of soil and affect crop growth should be further investigated.

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. The classification of inorganic P in soil is important to investigate the microbial transformation of soil P and improve the utilization ratio of P fertilizers. The selection of solubilizing inorganic P fraction by microorganisms has attracted the attention of scientists. Microorganisms show selectivity on the transformation of different forms of P. PSMs in rhizosphere and non-rhizosphere soils demonstrate preference in the solubilization of insoluble P (Ralston et al., 1976). Among the various P-solubilizing strains, *P. seudomonas* sp. and *P.aurantiogriseum* show strong solubilization ability on Ca-P, and *A.niger* and *P. simplicissimum* have strong solubilization ability on Al-P (Illmer et al., 1995). This phenomenon is the main reason for the instability of the effect of solubilizing P. It also explains the significant differences in the P-solubilizing ability of different soil types. A-H1 solubilizes and transforms a wide range of insoluble P. Ca_8-P (7.55 μg g^{-1} soil), Al-P (3.36 μg g^{-1} soil), Fe-P (3.74 μg g^{-1} soil), and Ca_{10}-P (1.64 μg g^{-1} soil) were transformed to Ca_2-P. It can also solubilize various forms of insoluble inorganic P in soil. In this study, the transformation of insoluble inorganic P to available P was 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 was consistent with the high colonization ability of A-H1 at 20 °C.

5. Conclusion

A effective method for screening and testing the true PSM capable of enhancing plant growth was established by experiments of soil colonization, organic phosphate solubilization, and plant growth promotion of phosphate-solubilizing microorganisms. And we got a fungus *Aspergillus niger* H1 that could promote plant growth by this method.

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