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Original Research

Isolation, Characterization, and Growth Promotion of Phosphate-Solubilizing Bacteria Associated with *Nicotiana Tabacum* (Tobacco)

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Abstract

Phosphate-solubilizing bacteria (PSB) increase phosphate bioavailability, thereby reducing the application frequency of chemical fertilizers in the production of *Nicotiana tabacum* (tobacco). In this study, PSB were isolated from tobacco plants for the first time. These PSB were screened *in vitro* for their ability to solubilize inorganic P (Pi) when grown in association with tobacco plants. Thirty-six PSB with the ability to solubilize P_i were isolated and screened for their indolyl-3-acetic acid and siderophore-producing capabilities. In addition, all 36 PSB strains had a partial fragment of their 16S rRNA gene sequenced. The analysis revealed high sequence identity to 16S rDNA sequences from *Bacillus*, *Arthrobacter*, *Providencia*, *Enterobacter*, *Proteus*, *Psychrobacter*, *Serratia*, *Rhodococcus*, *Pseudomonas*, *Ochrobactrum*, and *Acinetobacter*. Of the 36 PSB strains analyzed, three (*Psychrobacter alimentarius* HB15, *Enterobacter ludwigii* HB21, and *Ochrobactrum haematophilum* HB36) were selected for a controlled plant inoculation experiment. Inoculation of tobacco plants with these bacterial strains significantly increased plant dry weight. Additionally, inoculation increased P, K, and N uptake by tobacco seedlings as well as soil P availability. The increases observed with inoculation were even more pronounced when tricalcium phosphate (TCP) was added to the soil. The phosphate-solubilizing activity of these three strains was correlated with the release of gluconic, tartaric, acetic, and citric organic acids. Overall, co-inoculation of PSB and TCP appears to represent a promising option for increasing the yield of tobacco plants. The adoption of this technique could provide a pathway to reducing fertilizer input in agricultural settings.

Keywords: growth promotion, *Nicotiana tabacum*, phosphate-solubilizing bacteria, Tricalcium phosphate

Introduction

Phosphorus (P) is an essential macronutrient for the growth and development of plants. Most plants rely on soil for P uptake. Although soil usually contains high levels of total P, most P exists in forms that are not soluble or bioavailable (i.e., mineral phosphorous or organic phosphorous) [1]. Low P availability limits plant growth in many soils across the world and commonly limits agricultural productivity [2]. In China, approximately 74% of arable land is limited by available P [3], which

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has traditionally been overcome by adding P fertilizers [4]. However, a large portion of the soluble inorganic phosphate applied to the soil as chemical fertilizer is rapidly immobilized and becomes unavailable to plants [5]. Generally, a few days after fertilization available P levels reach similar values to those before application [6].

Extensive mining operations exist to meet the current demands for P as fertilizer, yet some estimates indicate that these mines could be depleted in extractable P by 2060 [7]. In addition to the limited supply of mineable P, the excessive use of chemical fertilizers not only wastes resources, but also leads to environmental pollution [8]. Therefore, it is critical to explore alternative sources for better management of the plant-soil-microbial P cycle to reduce our reliance on chemical fertilizers.

Recently, phosphate-solubilizing bacteria (PSB) have attracted the attention of agriculturists. The addition of PSB is considered an effective method for reducing the frequency of P fertilizer applications to different crops [9-10]. PSB functions by converting insoluble phosphates into bioavailable forms via the processes of acidification, exchange reactions, chelation, and production of gluconic acid [11]. In addition, some of these bacterial species may promote plant growth by secreting chemicals such as siderophores and indole acetic acid (IAA) [12-13]. However, an abundance of PSB in soil does not guarantee that they will be able to compete with the microorganisms already established in the rhizosphere. This potential limitation requires the inoculation of plants with PSB to increase their soil density and thereby increase the productivity of agricultural ecosystems [14].

Tobacco is an important cash crop in China and more than one million acres are planted each year [15]. P is one of the key elements needed to improve the yield and quality of tobacco leaves. Currently, the tobacco nutrient supply is mainly provided through the application of P fertilizers with poor solubility, and the locations where tobacco is grown often have soil with serious nutritional limitations. Although there has been extensive research on PSB [16-21], few studies have investigated their phosphate-solubilizing potential, their biodiversity, or their effects on nutrient uptake in tobacco cultivation. Therefore, we undertook this study to 1) isolate and characterize tobacco PSB and 2) study the effects of PSB on the release of P from TCP and their effects on tobacco growth. The ultimate goal is to use PSBs as bio-fertilizers in order to improve fertilization in tobacco cultivation and minimize P loss from soils.

Materials and Methods

Soil Collection and PSB Screening

Twelve soil samples were collected from the rhizosphere of tobacco fields in Xianfeng County, Hubei Province, China. Soil in this region had the following general properties: pH 5.73, organic C content 24.47 g/kg, available N content 141.72 g/kg, available P content 26.2 mg/kg, and available K content 207 mg/kg. Rhizospheric soil was sampled in June 2013, which is the peak growing season for tobacco (variety Yunyan 87). First, a soil profile of 0 to 20 cm depth was dug, the roots exposed in the profile were cut off, and soil that was loosely bound to the roots was shaken off and collected. Next, soil still attached to roots was removed with a sterilized brush, packed in sterilized paper sacks, and immediately transported to the laboratory for processing.

PSBs were isolated according to the methods of Yang et al. [22]. In short, soil samples were serially diluted and then plated on National Botanical Research Institute phosphate (NBRIP) media. This medium contained 5.0 g of calcium phosphate (TCP) as the only source of P [23]. Each plated sample was replicated three times. After incubation at 30ºC for 120 h, all colonies that formed obvious halo zones were categorized as PSB [22]. The individual PSB colonies identified were then used to inoculate NBRIP plates. After incubation at 30ºC for 120 h, halo/colony ratios (HD/CD) were calculated.

Phosphate Solubility Assay

To further analyze the ability of PSB, 100 mL of liquid NBRIP solution containing 5 g/L TCP, iron phosphate, (IP) and aluminum phosphate (AP) as sources of insoluble P was added into 250-mL flasks, and the flasks with NBRIP solution were sterilized by autoclaving at 0.1 MPa for 20 min. The flasks were cooled and then inoculated with 1 mL of the PSB culture at a concentration of 10^6 CFU/mL. First, the flasks were incubated at 150 rpm for 7 d at 30°C. The culture was then centrifuged at 10,000 rpm for 20 min. The Mo-Sb anti-spectrophotography method was used to quantify the pH and soluble P content of the supernatant [24]. Flasks containing the same volume of the medium without inoculants were established as controls. All treatments were performed in triplicate. The resulting data are presented as the mean ±standard deviation.

Qualitative Determination of IAA

IAA was assayed qualitatively according to the method described by Shi et al. [25]. PSB were inoculated in modified Congo red medium with the following components per liter: 0.5 g $K_2HPO_4 \cdot 3H_2O$; 0.2 g $MgSO_4 \cdot 7H_2O$; 0.1 g NaCl; 1 g yeast extract; 10 g mannitol; 10 mL 0.25% Congo red; 1 g NH_4NO_3; and 100 mg Tryptophan; and filtered using a sterile 0.2 μm Millipore membrane filter. Flasks were incubated for 12 d at 28ºC on a rotary shaker (125 rpm). A 100 μL bacterial culture was mixed with an equal volume of Salkowski colorimetric reagent, and color change of the mixture was observed after 15 min. PSBs that turned medium pink were considered to be IAA-producing. PSBs that did not cause a color change (i.e., the mixture remained colorless) were considered unable to produce IAA.

Qualitative Production of Siderophores

The potential to produce siderophores was qualitatively determined according to the method described by Schwyn and Neilands [26]. Bacterial cultures were spot inoculated onto chrome azurol S agar plates (10 µL with a concentration of approximately 10^8 CFU/mL) and incubated at 28°C for 48 h. Development of a yellow-orange halo surrounding the colonies was used to indicate siderophore production.

Production of Organic Acids during Phosphate Solubilization by Three Isolates

Three PSB isolates were grown in triplicate on NBRIP media containing 5.0 g TCP. The cultures were incubated for three days (28ºC, 200 rpm). Cultures were then centrifuged for 15 min at 16,000 × g and passed through a 0.22-µm microporous filter membrane. Organic acids were quantified according to the method described by Liu et al. [27]. The organic acids were quantified based on peak areas of standards. Standards included gluconic, malic, tartaric, acetic, citric, and succinic acids. Each analysis was performed in triplicate.

Phylogenic Analysis of PSB

16S rDNA sequencing and a phylogenetic analysis was used to identify PSB strains. A bacterial genome extraction kit (Sangon Biotech, Shanghai, China) was used to extract genomic DNA. Genomic DNA was used as a template in conjunction with the primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') to amplify the 16S rDNA gene of each PSB strain [28]. PCR reactions consisted of 25 µL of 2× Es Taq MasterMix (ComWin Biotech, Beijing, China), 2 µL of each primer (10 µM), and 2 µL of the DNA template. A final volume of 50 µL was achieved by adding sterile molecular-grade water. The cycling conditions for PCR were as follows: an initial denaturation for 5 min at 95ºC, 30 cycles at 95ºC for 1 min, 55ºC for 1 min, 72ºC for 1 min, and a final extension at 72ºC for 10 min. The PCR products obtained were purified via gel extraction (TransGen Biotech, Beijing, China) and then sent to Sangon Biotech Co., Ltd. for sequencing. The obtained sequences were assembled using DNASTAR Lasergene (version 7.1) and then submitted to GenBank for homology analysis using the BLASTn algorithm. PSB and reference sequences were aligned using ClustalX (version 1.83) and were trimmed to a shared length. Phylogenetic analysis was applied to trimmed alignment block via MEGA (version 4.0), employing the methods of neighbor-joining and the Kimura 2-parameter distance with 1,000 bootstrap replicates [29].

Strains and Inoculant Preparation

Controlled growth trials were performed with three efficient PSB strains (designated as HB15, HB21, and HB36). Bacterial strains were grown on nutrient agar, and single colonies were then transferred into flasks (250 mL) that contained nutrient broth. The flasks were placed on a rotating shaker (150 rpm) and the colonies were aerobically grown at 30ºC for 48 h. The bacterial suspension was then diluted to a final concentration of 10^8 CFU/mL with distilled sterile water and the resulting suspension was used to treat tobacco seedlings.

Controlled Plant Growth Experiments

Controlled growth experiments were carried out using tobacco seedlings in environmental chambers programmed at 28ºC, 80% humidity, and 300 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light for 12 h/day. The soil used in this experiment was taken from Jimo tobacco fields in Qingdao, Shandong Province, then sifted with a 10-mesh sieve and mixed with manure (3:1 v/v). The soil used had the following chemical properties: pH of 6.9, organic matter content of 16.37 g/kg, available N content of 0.12 g/kg, available P content of 6.45 mg/kg, and available K content of 54 mg/kg. The soil was then sterilized and half the soil was used for direct planting in pots. The remaining soil was supplemented with TCP at a concentration of 200 mg/kg. Tobacco seedlings (variety Xiaohuangjin 1025) with 5-6 true leaves were carefully removed from their pots and the associated soil was removed. NaOCl was used to surface-sterilize the roots for 10 min and then the roots were washed three times with sterile water. One seedling was planted in each pot. The pots measured 20 cm in diameter and 25 cm in height, and each pot contained 5 kg soil.

All controlled growth experiments were replicated four times. Each replicate included four seedlings. Tobacco seedlings were inoculated 5 d after transplantation. Experimental treatments were prepared as follows: 1) control without TCP (–TCP) and without PSB, 2) (–TCP) +HB15, 3) (–TCP) +HB21, 4) (–TCP) +HB36, 5) with only TCP added (+TCP), 6) (+TCP) +HB15, 7) (+TCP) +HB21, and 8) (+TCP) +HB36. A syringe was used to apply each isolate. Fifty mL injections of the prepared bacterial suspension were added to the middle of the seedling roots [18-19]. Fifty mL of the diluted NA culture with no bacteria was applied to control plants. Thirty days after inoculation, the seedlings and roots were removed from the pots and the soil was rinsed from the roots. The dry weight of the seedlings was then measured.

Analyses of Plant Nutrients

The plant samples were finely ground after drying at approximately 65ºC for 48 h. The pulverized samples were used to determine the concentrations of N, P, and K in tobacco seedlings according to the method described by Lu [24]. The total N concentration was assayed by the sulfuric acid-hydrogen peroxide chromatometry method. The P concentration was determined using the sulfuric acid-hydrogen peroxide digestion-vanadium molybdenum yellow chromatometry method. The K concentration was

Table 1. Phosphate-solubilizing capabilities, indole acetic acid (IAA) secretion, and siderophore production by 36 bacteria isolated from tobacco rhizospheric soil.

Isolate	Halo: Colony ratio	Solubilized P (mg/L)			IAA production	Siderophore production
		TCP	AP	IP		
HB1	1.28±0.07	87.58±2.69	69.51±2.14	57.43±0.70	+	+
HB2	1.32±0.03	105.25±3.16	90.57±2.08	63.50±2.05	–	–
HB3	1.67±0.07	141.77±2.24	126.16±4.08	120.62±3.26	+	+
HB4	1.99±0.15	195.70±1.55	36.84±2.13	27.18±3.65	–	+
HB5	2.09±0.21	192.73±1.94	150.82±2.75	198.14±2.94	+	–
HB6	1.51±0.05	126.97±2.65	90.51±1.95	105.17±2.04	–	+
HB7	1.25±0.04	48.93±1.68	72.75±1.34	90.60±1.75	+	+
HB8	2.25±0.15	186.30±1.64	138.19±2.96	147.37±2.21	–	–
HB9	1.29±0.04	66.97±2.08	51.31±1.75	58.35±2.01	–	–
HB10	1.25±0.10	54.27±1.04	66.57±1.26	67.14±2.15	–	–
HB11	1.42±0.09	84.23±1.51	60.15±1.24	75.36±1.85	–	+
HB12	1.14±0.06	33.20±1.37	45.29±1.05	54.42±1.31	+	+
HB13	2.25±0.10	174.90±2.39	150.24±2.25	144.17±2.56	+	–
HB14	2.25±0.15	189.67±1.34	204.14±2.63	210.10±3.22	–	+
HB15	2.61±0.07	308.3±4.25	280.03±3.68	269.08±4.15	+	–
HB16	2.21±0.11	174.00±1.54	150.48±2.31	147.25±2.52	–	–
HB17	1.23±0.04	45.27±1.56	54.49±1.51	60.02±1.18	–	+
HB18	1.92±0.13	186.00±1.81	177.66±2.37	165.73±2.44	–	–
HB19	1.92±0.14	174.17±1.76	186.69±2.74	188.38±2.33	–	–
HB20	2.26±0.11	183.10±1.39	30.75±2.28	35.81±2.62	–	+
HB21	4.83±0.14	358.1±4.12	282.18±3.50	277.30±3.25	+	–
HB22	1.83±0.08	141.97±2.18	156.36±2.05	165.37±2.53	–	+
HB23	1.50±0.11	123.80±1.30	108.55±1.84	90.61±2.45	+	+
HB24	2.10±0.09	201.00±2.15	213.44±2.06	234.35±3.03	–	–
HB25	1.39±0.03	75.53±1.63	66.65±1.50	60.69±1.24	+	–
HB26	1.89±0.10	159.17±2.41	135.83±1.75	126.64±1.91	+	+
HB27	1.94±0.19	189.33±2.77	165.37±2.40	156.81±2.08	–	+
HB28	1.83±0.11	171.90±1.04	210.35±1.94	234.25±2.28	–	–
HB29	2.41±0.16	180.63±1.80	147.17±2.05	132.66±1.83	+	–
HB30	1.77±0.11	138.97±1.22	150.36±2.11	159.71±2.48	–	–
HB31	1.28±0.09	60.60±1.61	51.34±1.17	45.26±1.24	+	–
HB32	1.35±0.03	72.33±1.27	87.38±1.95	93.46±1.85	–	+
HB33	1.57±0.11	102.00±2.19	87.75±1.68	90.59±2.33	–	+
HB34	2.18±0.06	141.77±2.40	120.85±2.16	114.64±1.75	–	–
HB35	1.62±0.06	111.57±1.36	120.82±2.41	132.78±2.09	+	+
HB36	3.27±0.02	360.18±3.99	286.63±3.22	285.41±3.50	+	+
CK	ND	6.88±0.12	7.05±0.21	5.95±0.18	–	–

Note: Values are means ± SD (n = 3). +: production, –: no production.

measured using the sulfuric acid-hydrogen peroxide digestion-flame photometric method.

Chemical Properties of Rhizospheric Soil

To assess the chemical properties of the soil, soil samples from the rhizosphere were aseptically separated from the roots. Extraction of available P was determined using the bicarbonate method. The quantity of available P was determined via the Mo-Sb Colorimetry method [24].

Results

In vitro Phosphate-Solubilizing Activity of PSB Isolates

Thirty-six PSM isolates were obtained from 12 rhizospheric soils of tobacco grown on NBRIP media (Table 1). After incubating for 5 d on NBRIP media, PSB isolates generated obvious halo zones surrounding each bacterial colony. These zones indicated the ability to solubilize TCP. The halo zone diameters produced by different isolates ranged from 0.2 to 3.15 cm. Fig. 1 shows the widest halo zones produced by the HB36 strain with a diameter of 3.15 cm. The HD/CD values ranged from 1.14 to 4.83. The HB21 strain exhibited the highest HD/CD value.

Isolated PSB showed different affinities for TCP solubilization, ranging from 33.20 to 360.18 mg/L (Table 1). The concentration of solubilized P from 29 isolates (accounting for 83.33% of the total) ranged from 50-250 mg/L (Fig. 2), whereas three isolates had concentrations under 20 mg/kg. Among these PSB isolates, HB36 had the highest phosphate-solubilizing activity with the amount of P released into the medium, reaching 360.18 mg/L. Strain HB21 had the second highest activity, with 358.10 mg/L of P released into the growth medium. In control experiments, the P concentration in the medium was only 6.88 mg/L, indicating that P release was biologically driven.

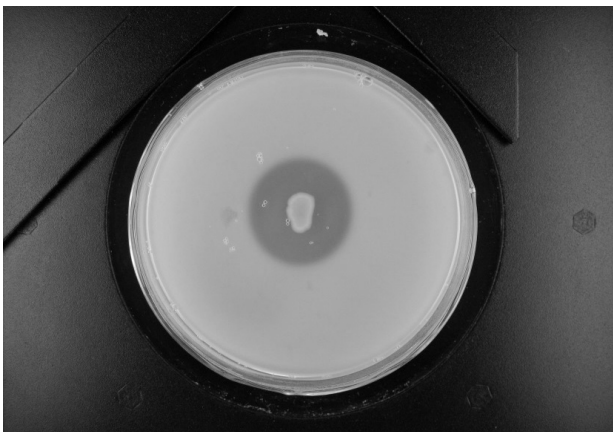

Fig. 1. Phosphate halo of strain HB36 grown on NBRIP culture medium containing TCP (5 g/L).

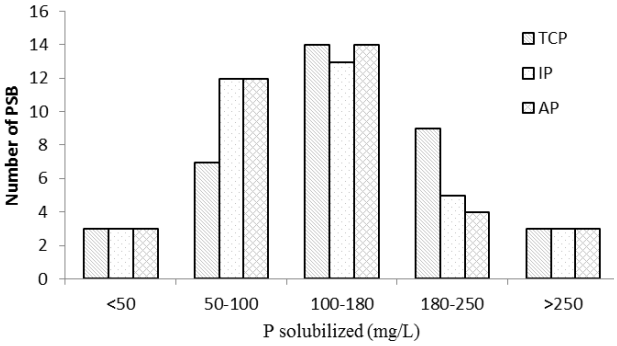

Fig. 2. Distribution of 36 isolated phosphate-solubilizing bacteria (PSB) with different tricalcium phosphate-solubilizing activities.

Twenty-five isolates showed a higher capacity to solubilize TCP compared to AP and IP. The most active isolate solubilized the three insoluble phosphates in the liquid medium, with a maximum activity of 286.63 and 285.41 mg/L for AP and IP, respectively. Based on these results, we selected the three isolates (HB36, HB21, and HB15) that displayed the highest phosphate solubilization (PS) activities for additional testing.

Production of IAA and Siderophores

The isolates were screened for additional plant growth-promoting attributes, including IAA and siderophore production (Table 1). Fifteen isolates (41.7%) were capable of producing IAA. Half of the isolates (18 in total) were capable of producing siderophores, and only eight isolates were capable of producing both IAA and siderophores.

Sequence Analysis of the PSB 16S rRNA Gene

Gene sequences obtained from the 36 PSB strains were subjected to a genetic analysis of their 16S rDNA. The results revealed a high sequence similarity to bacteria belonging to the families Enterobacteriaceae, Moraxellaceae, Pseudomonadaceae, Micrococcaceae, Brucellaceae, Nocardiaceae, and Bacillaceae. Eighteen isolates (50% of the total) were closely affiliated with Bacillaceae (50%). Enterobacteriaceae had 11 strains, accounting for 30.56% of the total. The 36 PSB isolates belonged to 11 genera, including *Bacillus*, *Arthrobacter*, *Providencia*, *Enterobacter*, *Proteus*, *Psychrobacter*, *Serratia*, *Rhodococcus*, *Pseudomonas*, *Ochrobactrum*, and *Acinetobacter* (Table 2, Fig. 3). The most dominant genus was *Bacillus*, with 18 PSB isolates, and the dominant species was *Bacillus methylotrophicus,* with 7 strains.

Nutrient Uptake and Vegetative Growth of Tobacco Seedlings

The effects of PSB strain inoculations on nutrient uptake and growth parameters of tobacco seedlings are shown in Table 3. Increases in dry weight ranged from

Table 2. List of phosphate-solubilizing bacteria isolates and their respective GenBank accession numbers of their partial 16S rRNA gene sequence. The closest phylogenetic affiliation for each sequence is indicated, along with the reference sequence accession number and the percent identity of the isolate sequence when compared to the reference sequence.

Isolate	NCBI Accession number	NCBI BLAST		
		Closest organism	Accession	Identity (%)
HB1	KC764961	*Bacillus megaterium*	JX312585	99
HB2	KC764964	*Arthrobacter chlorophenolicus*	FJ577502	99
HB3	KC764970	*Bacillus subtilis*	EU931563	99
HB4	KC764972	*Bacillus methylotrophicus*	JN700125	99
HB5	KM659215	*Bacillus methylotrophicus*	JN700125	99
HB6	KC764973	*Providencia rettgeri*	HQ844461	99
HB7	KM659216	*Bacillus subtilis*	EU931563	99
HB8	KM659217	*Bacillus subtilis*	JF775416	99
HB9	KM659218	*Bacillus subtilis*	JQ579620	99
HB10	KC764978	*Enterobacter cloacae*	JQ435862	99
HB11	KM659219	*Bacillus methylotrophicus*	JN700125	99
HB12	KM659220	*Bacillus methylotrophicus*	JF899281	99
HB13	KC764983	*Proteus penner*i	HQ259936	99
HB14	KM659221	*Providencia rettgeri*	JX871330	99
HB15	KC881247	*Psychrobacter alimentarius*	JX514419	99
HB16	KM659222	*Proteus penneri*	HQ259936	99
HB17	KM659223	*Providencia rettgeri*	JX871330	99
HB18	KC764986	*Acinetobacter johnsonii*	EU977664	99
HB19	KC764987	*Serratia marcescens*	GQ351502	99
HB20	KC764988	*Bacillus aryabhattai*	HQ242772	99
HB21	KC881248	*Enterobacter ludwigii*	GQ915080	99
HB22	KM659224	*Bacillus megaterium*	EU333886	99
HB23	KC764989	*Bacillus pumilus*	GQ169785	99
HB24	KM659225	*Bacillus methylotrophicus*	JN700125	99
HB25	KM659226	*Bacillus methylotrophicus*	JN700125	99
HB26	KM659227	*Bacillus methylotrophicus*	JF899287	99
HB27	KM659228	*Bacillus aryabhattai*	JX094945	99
HB28	KM659229	*Arthrobacter chlorophenolicus*	FJ577502	99
HB29	KM659230	*Bacillus pumilus*	GQ169785	99
HB30	KC764991	*Proteus vulgar*is	JF772091	99
HB31	KC764992	*Bacillus simplex*	AY833099	99
HB32	KM659231	*Providencia rettge*ri	JX871330	99
HB33	KC764993	*Rhodococcus erythropoli*s	JX010951	99
HB34	KM659232	*Proteus penneri*	HQ259936	99
HB35	KC764994	*Pseudomonas geniculata*	JX661715	99
HB36	KC881246	*Ochrobactrum haematophilum*	HQ171238	99

8.46% to 48.44% following inoculation of soil with the strains *Psychrobacter alimentarius* (HB15), *Enterobacter ludwigii* (HB21), or *Ochrobactrum haematophilum* (HB36) compared to seedlings grown in soil that was not inoculated. There were no significant differences in the growth parameters of tobacco seedlings receiving only TCP compared to plants without TCP supplementation. However, the interaction of TCP with HB15, HB21, or HB36 was found to be significant with respect to seedling dry weight. The highest results were observed in soil inoculated with strain HB36 in the presence of TCP.

Inoculating plants with the HB15 and HB21 strains significantly increased ($P < 0.05$) the total P concentration of plant seedlings compared to the control. The HB36 strain also increased total P by 11.81%, although the increases were not statically significant. Inoculation of strains HB15 and HB36 significantly increased total N and K in plants, whereas HB21 had no effect on total N. Moreover, soil inoculated with TCP significantly increased seedling uptake of N, P, and K, as compared to the treatments with only PSB or TCP applications. In particular, the application of TCP also significantly increased total N, P, and K in plants compared with plants not receiving TCP.

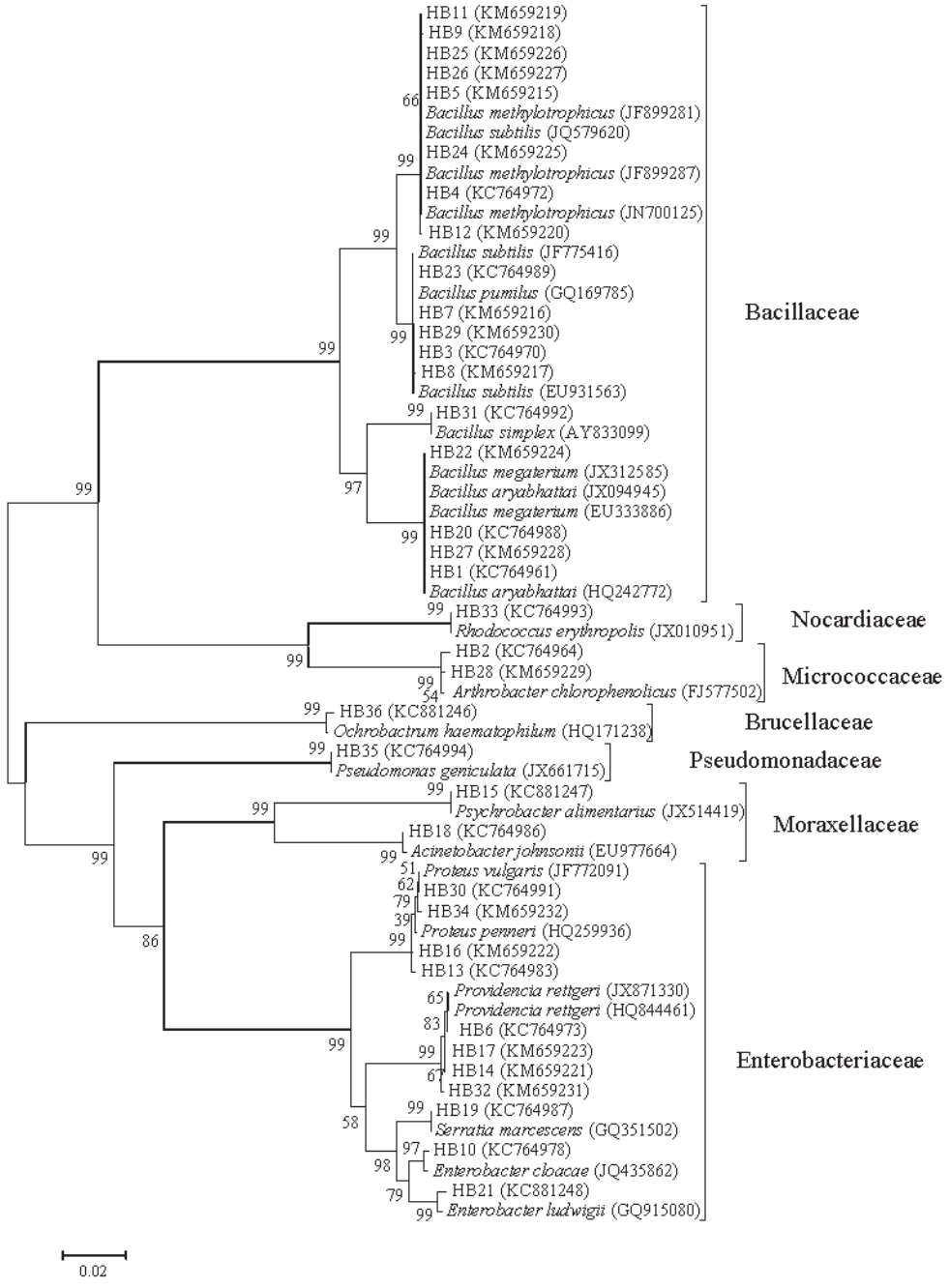

Fig. 3. Phylogenetic tree of phosphate-solubilizing bacteria (PSB) from tobacco rhizospheric soil based on 16S rDNA sequences. All tested isolates had their 16S rDNA gene partially sequenced and were grouped according to genus or species.

Table 3. Effects of phosphate-solubilizing bacteria on plant growth (measured by dry weight), nutrient uptake, and soil chemical properties.

Treatment	Tobacco seedlings				Tobacco rhizospheric soil
	Dry weight (g)	Total P uptake (g/kg)	Total N uptake (g/kg)	Total K uptake (g/kg)	Available P (mg/kg)
CK	8.63^{c}	2.88^{e}	17.46^{c}	22.49^{f}	78.41^{h}
HB15	9.36^{c}	3.98^{bc}	21.52^{b}	31.43^{de}	95.17^{g}
HB21	11.58^{ab}	3.46^{cd}	17.25^{c}	26.52^{ef}	99.18^{f}
HB36	12.81^{a}	3.22^{de}	22.17^{b}	35.13^{cd}	112.77^{e}
TCP	9.79^{cd}	3.67^{cd}	30.84^{b}	37.68^{c}	124.58^{d}
TCP +HB15	12.80^{a}	4.81^{a}	37.52^{a}	43.86^{b}	163.40^{c}
TCP +HB21	12.70^{a}	4.25^{ab}	38.93^{a}	52.90^{a}	172.19^{a}
TCP+ HB36	13.32^{a}	4.64^{a}	36.09^{a}	46.15^{b}	168.05^{b}

Note: Values represent means of three replications. Means within columns followed by a different lowercase letter are significantly different according to Fisher's protected LSD test at $P \leq 0.05$.

Changes in Available P in Tobacco Rhizospheric Soil

The available P content in tobacco rhizospheric soil substantially increased in treatments receiving both TCP and PSB as compared to the control (Table 3). Applications of TCP and PSB inoculations resulted in higher concentrations of available P compared to the soil inoculated with PSB without TCP.

Organic Acid Production in Broth Culture

Culture filtrates were subjected to HPLC analysis. The results indicated the presence of gluconic, tartaric, acetic, and citric organic acids. Among these acids, gluconic acid was produced in high amounts by strains HB15, HB21, and HB36 with concentrations of 5.76, 6.35, and 9.17 mg L^{-1}, respectively. Strains HB21 and HB36 possessed the ability to produce all four acids, while HB15 produced all but citric acid. Furthermore, strain HB36 possessed the highest capacity for organic acid production with an average total organic acid concentration of 18.91 mg/L. The details of organic acid production are given in Table 4.

Correlation Matrix

Significant correlations were observed between plant dry weight and both available P in soil and nutrient uptake by tobacco. Significant positive correlations were observed between the amount of available P in the soil and N (r = 0.97), P (r = 0.85), and K (r = 0.82) uptake in plants. Similarly, plant dry weight was positively correlated with P uptake (r = 0.55) and available P in soil (r = 0.78). Furthermore, P solubilization was positively correlated with the secretion of organic acids (r = 0.72).

Discussion

PSB performs an important ecological function in the soil rhizosphere by solubilizing P. Due to their ability to increase crop P availability, rhizosphere PSB inoculants have been proposed for use in integrated nutrient management systems [30-31]. Although PSBs have been successfully isolated from different plants [30-32], very little information on the PSB associated with tobacco has been reported to date.

In this study, 36 PSB strains were screened from tobacco rhizospheric soils and were characterized as belonging to the families Enterobacteriaceae, Moraxellaceae, Pseudomonadaceae, Brucellaceae, Micrococcaceae, Nocardiaceae, and Bacillaceae. Most of the genera in these families have been reported to solubilize phosphate [4, 17]. The results of prior research concluded that PSB of the genus *Pseudomonas* were dominant in various soils [21]. However, in the present study the most dominant

Table 4. Quality and variety of organic acids produced by three phosphate-solubilizing bacterial strains.

Strain number	Organic acids (g/L)						Total organic acids (g/L)
	Tartaric acid	Malic acid	Gluconic acid	Acetic acid	Citric acid	Succinic acid	
HB15	2.75	-	5.76	2.23	-	-	10.74Bb
HB21	3.85	-	6.35	0.93	1.65	-	12.77Bb
HB52	3.87	-	9.17	3.59	2.27	-	18.91Aa

genus associated with tobacco (as identified using cultivation methods) was found to be *Bacillus*, whereas only one PSB strain affiliated with *Pseudomonas* was isolated. This may indicate that different species of PSB are specifically adapted to associate with different plant types or soil conditions. In addition, *Bacillus* has been reported to be among the most dominant groups within PSB populations [17].

The three isolates that were closely affiliated with *Psychrobacter alimentarius* (HB15), *Ochrobactrum haematophilum* (HB36), and *Enterobacter ludwigii* (HB21) showed maximum phosphate solubilization activity. These strains were identified as promising PSB that occur in the tobacco rhizosphere. Previous reports have shown that each of these species associates with plant rhizospheres, and they have been studied as promising PSB [33-35]. Similar to previous reports [12, 13, 33], siderophore production and IAA secretion abilities were observed for many PSB isolates in our work.

Previous studies have demonstrated that PSBs have the ability to promote plant growth [36-37]. These studies indicated that these bacteria can enhance plant growth and increase plant P content [19, 38], which is consistent with the observations made in the present study. Tobacco plants with the lowest dry weights were grown in unfertilized soil that received no TCP and were not inoculated. This was likely due to P deficiency in the soil used to grow these seedlings. The addition of TCP, as well as PSB inoculation, increased biomass yield relative to the control treatment, indicating that inoculation of PSB strains enhanced biomass, presumably by solubilizing soil P. It is also possible that the enhanced growth of tobacco plants in the presence of PSB is due to the strains' ability to secrete siderophores.

The combined application of TCP with PSB enhanced the uptake of P, K, and N by tobacco seedlings more than the control treatment. These results are consistent with those from the growth chamber and greenhouse experiments [14, 22, 39]. In addition, our results agree with those of Yang et al. [22] and Estrada et al. [36], who reported increases in plant dry weight, as well as P and N uptake, following an application of PSB combined with TCP as compared to PSB alone.

Our results indicate that various treatments had the potential to enhance available P and thereby directly affect tobacco growth and nutrient uptake. Maximum available P was detected with PSB inoculations in conjunction with TCP treatments. Our results concur with Singh and Reddy [17], Estrada et al. [36], and Gurdeep and Reddy [21]. Based on the information discussed above, the increased P concentrations detected in plants inoculated with PSB strains may be attributable to their ability to solubilize inorganic forms of P, thus increasing P availability in the soil. Correlation analyses showed that tobacco dry weight and N, P, and K content were positively correlated with the available P content in soil.

The ability of PSB to solubilize mineral phosphates with low solubilities has mainly been attributed to the secretion of organic acids with low molecular weight [40-41]. For example, gluconic acid is a major organic acid produced by many gram-negative bacteria during phosphate solubilization and is produced by several plant growth-promoting bacteria (PGPB) [27, 42]. Our results also show that P solubilization was related to the secretion of organic acids.

Conclusions

Tobacco rhizospheric soils are rich in PSB. Some of these PSB strains demonstrate high phosphate-solubilizing activity and are capable of producing siderophores or IAA. Three PSB strains (HB15, HB21, and HB36) significantly enhanced the dry weight and nutrient uptake by tobacco seedlings. We hypothesize that this was accomplished by increasing the availability of P and other nutrients in the soil. This effect was more evident in treatments receiving TCP. In addition, the phosphate-solubilizing activity of these three strains was correlated with the release of gluconic, tartaric, acetic, and citric organic acids. Therefore, the results of this study may aid in improving the sustainability of tobacco agriculture via reducing fertilizer input. Further studies are needed to determine the mechanism that PSBs utilize to solubilize TCP and quantify their effectiveness under field conditions.

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