International Journal of Agricultural Policy and Research Vol.3 (9), pp. 341-350, September 2015
Available online at http://www.journalissues.org/IJAPR/
http://dx.doi.org/10.15739/IJAPR.059

ISSN 2350-1561

Original Research Article

Isolation and potential of *Ochrobactrum* sp. NW-3 to increase the growth of cucumber

Received 18 August, 2015 Revised 2 September, 2015 Accepted 4 September , 2015 Published 7 September, 2015

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'Biofertilizers' have been defined as alternative substances, to chemical fertilizers, which can stimulate plant growth and increase yield. Plant growth promoting bacteria (PGPB) represent the main component of biofertilizers. We chose seven bacteria with higher nitrogenase activity, from twenty-nine nitrogen fixing bacteria isolated from different natural environments, to evaluate the effects of bacteria inoculation on cucumber under controlled (pot) conditions. Furthermore, growth promoting characteristics (indole-3-acetic acid (IAA), ACC-deaminase activity, siderophores production, phosphorus solubilization and potassium solubilization ability) were evaluated for seven strains and identification of strains was carried out by amplification, sequencing and comparison in GenBank database for 16S rDNA genes. *Ochrobactrum* sp. NW-3 had a high ability to increase the growth of cucumber in pot experiments and this strain showed the five factors evaluated to stimulate the plant growth. For this reason, *Ochrobactrum* sp. NW-3 was used to perform a field experiment in Anshan, Liaoning Province, the main local economic for cucumber production. Cucumber yield and quality increased significantly after three months of field experiment, suggesting that *Ochrobactrum* sp. NW-3 can exert promoting growth effect under the complex field environment. It is the first report on the use of *Ochrobactrum* sp. for stimulating cucumber growth and yields successfully in field experiments.

Key words: Biofertilizer, *Ochrobactrum* sp. NW-3; plant growth promoting bacteria, diverse mechanisms, cucumber field application.

INTRODUCTION

It is well known that excessive application of chemical fertilizers could result in leaching, water pollution, crop susceptibility to disease attack, acidification or alkalization of soils or reduction in soil fertility — causing irreparable damage to the entire agriculture ecosystem (Chen, 2006; Pimentel et al., 1995). Thus, in recent years, with the increasing drawbacks of traditional fertilizers, 'biofertilizers' could be an alternative to stimulate plant growth, increase yield, improve crop quality, reduce pathogen infection as well as reduce environmental pollution (Vessey, 2003). Biofertilizers contains plant growth-promoting bacteria (PGPB) regarded as the main constituent and could be applied to seed, plant surfaces, or soil for optimum plant growth, balancing quantities and sufficient available nutrients (Vessey, 2003).

PGPB have several mechanisms to promote plant growth such as nitrogenase activity, phosphate solubilization ability, potassium solubilization ability, siderophore production, indole-3-acetic acid (IAA) production, and the production of 1-aminocyclopropane-1-carboxylate (ACC) deaminase (Bhattacharyya and Jha, 2012). Some studies have proven that majority of plant-associated bacteria derived from the soil environment may migrate to the rhizosphere and subsequently the rhizoplane of their hosts before they are able to show beneficial effects (Boelens et al., 1994; Catlow et al., 1990). However, the most fundamental issue is that these bacteria could survive and exert their promoting effect in the complex field environment. The principal limitation for

crop yield is soil infertility, however biofertilizers could play an important role in increasing productivity and improving soil sustainability and also protect the environment as eco-friendly and cost effective inputs for farmers (Mohammadi and Sohrabi, 2012).

Over the past several years, more and more experiments have focused on studying promoting growth mechanisms and interrelated effects between plant and plant growth-promoting bacteria or plant growth promoting rhizosphere (PGPR) (Liu and Dong, 2012; Saharan and Nehra, 2011) under controlled conditions. However, studies about the practical application of agricultural field transformation among these biofertilizer microbes in on-field applications from the controlled laboratory conditions and the plant promoting growth mechanisms in field experiments are less reported.

The genus *Ochrobactrum* was firstly described by Holmes et al. (1988) and a large number of its isolates were collected from soil samples and wheat roots (Lebuhn et al., 2000) for promoting plant growth (Pandey et al., 2013). Moreover, an effective plant-growth promoting bacterium, *Ochrobactrum* sp. is potentially useful in stimulating cucumber growth and increasing its yields; application of which has never been reported to be successfully carried out in field experiments.

Thus, in this study, some nitrogen fixing bacteria were isolated from different soil collected from the Yellow mountain and Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences. These nitrogen fixing bacteria whose nitrogenase activity were higher than *Azotobacter chroococcum* and currently used as biofertilizer nitrogen fixing bacteria, were selected for examining its growth promoting capability under laboratory conditions. Possible mechanisms that these bacteria could use to stimulate plant growth were explored.

Additionally, in agricultural production, nitrogen (N), phosphorus (P), and potassium (K) are the three major essential macronutrients for crop growth. Some studies have shown that nitrogen fixing bacteria not only provide nitrogen, P- and K-solubilization, but also produce a variety of growth promoting substances such as IAA, ACC-deaminase, and siderophores etc. (Hegde et al., 1999). Plant growth promoting bacteria play an extremely important role in a variety of applications related to agricultural improvement along with their mechanism of action with special reference to plant growth promoting traits. Thus, in the present study, an isolated strain of *Ochrobactrum* sp. that showed several potential mechanisms to stimulate plant growth was selected. The strain was inoculated in cucumber to investigate its growth promoting ability under field conditions among the local main economic crops in Anshan, Liaoning Province.

MATERIALS AND METHODS

Nitrogen fixing bacteria isolation

The nitrogen fixing bacteria were isolated on Ashby solid medium (sucrose 10 g, K_2HPO_4 2.0 g, NaCl 0.2 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $(NH_4)_2SO_4$ 1.0 g, 1.0 g yeast extract powder, $FeCl_3$ 0.005 g, $CaCO_3$ 0.1 g, pH 7.0-7.5, in 1000 ml distilled water) (Ashby, 1907) from different soils collected from Yellow Mountain and Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences. The soil used for bacteria screening was ground and filtered through a 1-mm sieve. 10 g of the sieved soil sample was suspended in 90 ml sterilized water in a conical flask and shaken for 30 min with glass beads. The supernatant was serially diluted 10, 100 and 1000-fold, respectively at room temperature. Then 100 µl of the obtained suspensions were counted in triplicates by spreading on Ashby solid medium and incubated for 72 h at 30 °C. When the single colony was formed on the Ashby solid medium, the well-separated colonies were chosen and transferred onto fresh Ashby solid medium. These isolates were re-streaked on fresh Ashby solid medium for three or more consecutive times until purification.

Nitrogenase activity assay

Nitrogenase activity was determined by the acetylene reduction method (Hardy et al., 1968). Briefly, nitrogen fixing bacteria were grown to exponential phase (~36 h) at 30 °C in Ashby liquid medium on a rotary shaker (150 rpm; 2.5 cm radius of rotation). Then 10 µl bacteria culture were transferred to the slant medium in 20 ml-tubes. After inoculation at 30 °C for 72 h, 10% of acetylene gas was injected (calcium carbide preparation) with sealing tape, and the media was cultured for another 72 hours. 100 µl of ethylene was measured using a meteorological phase chromatograph, according to the following formula in terms of nitrogenase activity.

$$\text{Nitrogenase activity (nmol / h.mg)} = \frac{C_2H_4 \text{moles (nmol)}}{\text{Cell protein (mg)} \times \text{Reaction time (h)}}$$

Pot experiments

The soil used for the pot experiment was Fluvo aquic soil collected from Anshan, Liaoning, China. It was air-dried, ground and filtered with a 2 mm sieve and mixed thoroughly. The dominant soils used for agricultural production in the Huang-Huai-Hai Plain of Central China are fluvo-aquic soils, which cover about 70% of the arable area. Fluvo aquic soil contains fluvial sediments which is affected by ground water movement and farming activities and the soil formation, hence its name because of the night tide phenomenon. 200 g tested soil was loaded in 8 cm-diameter pots. There are eight plants in each group, one cucumber plant and a seven-day cucumber seedling (8th Zhongnong, a local variety) in each pot to complete the eight replicates. The cucumber pots were cultured in artificial climate incubator under conditions of 28 °C, 14 h light; 12 °C, 10 h without light with unified management. 30 ml bacteria culture (OD_{600} = 1) were watered every 48 h in

the pot with 30 ml double distilled H_2O (ddH_2O) as control for 20 days. At the end of the experiment, the following parameters were measured: plant height (cm) with measuring tape, plant fresh and dry weight (g) with digital balance. The plant was dried at 105 °C for 30 min, then at 80 °C for 4 h until constant weight.

Bioassays for plant growth promoting activities

Indole-3-acetic acid (IAA) assay

Production of indole-3-acetic acid (IAA) was colorimetrically determined as described by Fukuhara et al. (1994). The isolates were grown in LG (N-free) broth medium (Glucose 10 g, KH_2PO_4 0.41 g, K_2HPO_4 0.52 g, $CaCl_2$ 0.2 g, Na_2SO_4 0.05 g, $MgSO_4 \cdot 7H_2O$ 0.1 g, $FeSO_4 \cdot 7H_2O$ 0.005 g, $Na_2MoO_4 \cdot 2H_2O$ 0.0025 g, in 1000 ml distilled water) supplemented with L-tryptophan (100 mg/l) for 72 h at 30 °C, 200 rpm. The supernatant of the stationary phase culture was obtained by centrifugation at 12,000 rpm for 15 min. IAA produced culture was estimated by mixing 5 ml Salkowsky reagent (0.01M $FeCl_2$ in $HClO_4$), followed by measuring the color changes at 530 nm (Costacurta et al., 1998). Indole-3-acitic acid was serially diluted for use as standard.

ACC-deaminase activity assay

Selected bacterial isolates were also cultured in LG (N-free) medium at 30 °C for 72 h by shaking at 200 rpm until cell reached the early stationary phase. The cells were collected by centrifugation at 5000 rpm for 5 min and washed twice with minimal medium. Cell pellets were suspended in 15 ml of minimal medium supplemented with 1 mM ACC (1-aminocyclopropane-1-carboxylate), and further incubated at 30 °C for 24 h with shaking at 200 rpm to induce ACC-deaminase production (Penrose and Glick, 2003). The detailed ACC-deaminase activity assay was carried out with modification of the method by Honma and Shimomura (1978).

Siderophores production

These nitrogen fixing bacteria isolates were inoculated unto the medium, incubated at 30 °C for about 48 h, adjusting OD_{600} of bacteria to about 1.0. Four sterilized filter papers were capped at Chrome Azurole's (CAS) solid plate (Neilands, 1995; Schwyn and Neilands, 1987), and then 50 µl suspension culture medium was inoculated into each filter paper, incubated at 30°C for 12 h. If flatbed color change was observed, it indicated that bacteria can secrete siderophores and an orange circle on the CAS chelated iron plate, indicated siderophore production capacities.

Phosphate-solubilization ability

Phosphate-solubilization test was conducted qualitatively on modification of Pikovskaya medium (NaCl 0.3g, $(NH_4)_2SO_4$ 0.5 g, KCl 0.3 g, $MgSO_4 \cdot 7H_2O$ 0.3 g, $FeSO_4 \cdot 7H_2O$ 0.03 g, $MnSO_4 \cdot 4H_2O$ 0.03 g, yeast extract 0.5 g, glucose 10 g, $Ca_3(PO4)_2$ 5.0 g, and agar 15 g, pH 7.0-7.5, in 1000 ml distilled water). Bacterial culture was re-streaked on the surface of Pikovskaya medium. The presence of a clear halo zone around bacterial culture colony after 48 h incubation indicates that this isolate has P-solubilization capacity (Husen, 2013).

Potassium-solubilization ability

Potassium solubilization test was studied on modified Aleksandrov solid medium plates (Liu et al., 2012). The medium was a modification of Aleksandrov solid medium (glucose 5.0 g, KCl 0.2 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $FeCl_3$ 0.005 g, Na_2HPO_4 2.0 g, $CaCO_3$ 0.1 g，yeast extract 1.0 g, and agar 20 g, pH 7.0-7.5, in 1000 ml distilled water). Plates of modified medium containing Potassium feldspar were prepared. The bacterial cultures were spotted on each plate and incubated at 30 ˚C for 48 h. The presence of clearing zone around bacterial colony after incubation was used as an indicator of positive K-solubilization ability.

Isolates identification

The isolates under study were identified by 16S rDNA gene sequence analysis of the strain. Almost full length 16S rDNA coding gene fragments were amplified from the respective isolated and purified DNA by using bacterial universal primers 27F (5'-AGA GTT TGA TCM TGG CTC AG-3') and1492R (5'-TAC GGY TAC CTT GTT ACG ACT T-3') (Lane, 1991). Each amplification reaction tube with a total volume of 50 µl contained 25 µl 2×*Taq* green mix, 20 µl ddH_2O, 2 µl of each primer, and 1 µl DNA template. The reaction conditions were as follows: denaturation at 95 °C for 3 min, followed by 30 cycles of 95 °C for 1 min, annealing 55 °C for 30 s and elongation at 72°C for 1 min with a final extension step of 10 min at 72 °C. The PCR products were electrophoresed on a 1.0 % agarose gel and then subjected to commercial DNA sequencing. Obtained 16S rDNA gene sequences were compared with the GenBank database to determine its identification by BLAST (http://blast.st-va.ncbi.nlm.nih.gov).

Field experiments

Based on the Anshan soil physicochemical properties (pH 6.80, organic matter 1.35%, total N 0.124%, total K 3.1g/kg, total P 0.107g/kg) and the cucumbers growth nutrient requirements, organic and chemical compound fertilizers (1020 kg/ha) was formulated at the rate 1: 1 for control group (CK). Then microbial inoculants (1.5×10^{14}cfu/ha) was added in 1020 kg organic and chemical compound fertilizers (organic matter 42.5%, total N 14.6%, total K 5.28%, total P 9.8%), thoroughly mixed, dried in the shade and bagged; as the final biofertilizer for field experiments.

Field experiments were carried out on a cucumber field (122°10′N, 40°27′E) located in the Anshan, Liaoning,

Table 1. The nitrogenase activity of nitrogen fixing bacteria isolates and *Azotobacter chroococcum*

No.	Nitrogenase activity (nmol C_2H_4/h·mg protein)	No.	Nitrogenase activity (nmol C_2H_4/h·mg protein)
N-BM-1	214.50±15.31	N-N-7	301.13±11.29
N-BF-11	374.86±28.36*	N-R-1	232.85±8.47
N-BF-12	117.87±14.42	N-R-11	788.65±12.62*
N-BF-211	243.33±10.58	N-R-12	140.32±13.28
N-BF-212	132.35±9.13	N-R-21	508.67±9.15*
N-BF-3	194.62±10.02	N-R-22	216.60±8.26
N-BF-4	204.61±11.07	N-W-2	312.63±10.12
N-BF-5	292.77±7.40	NW-3	420.95±10.29*
N-S-111	153.46±9.26	NW-4	364.47±8.12*
N-S-112	201.50±10.34	N-YM-2	154.73±9.20
N-S-21	238.27±8.24	N-YM-3	921.76±15.43*
N-S-221	142.11±7.31	N-YM-4	730.74±18.22*
N-S-222	195.68±10.15	N-SM-11	223.02±9.56
N-S-31	144.84±9.53	N-SM-12	230.41±11.35
N-S-32	146.84±5.35	*Azotobacter chroococcum*	314.88

*: Nitrogenase activity was higher than currently used as biofertilizer nitrogen fixing bacteria, *Azotobacter chroococcum* (314.88nmol C_2H_4/h·mg protein).

China from May to July 2014. The field experiments were carried out in a randomized block design with four replicates. There are two different treatment fields designed in the field experiments: Control group (organic and chemical compound fertilizers) and Biofertilizer treatment group (organic and chemical compound fertilizer + microbial inoculants (1.5×10^{14}cfu/ha). Each treatment field area was 40 m^2 (8 m × 5 m), organic and chemical compound fertilizer and the final biofertilizer were applied 15 cm away from the cucumber root at three different growth stages, respectively (base fertilizer, flowering period and fruiting period).

According to the requirements for crop products by the Ministry of Chinese Agriculture, Microbial Fertilizer and Edible Fungi Quality Supervision and Testing Center, the main quality indicator of cucumber is vitamin C content. During the cucumber fruit period, the plant was randomly picked in triplicates to calculate yield and inspect quality. The vitamin C content was commercially measured with a spectrophotometer.

Soil sampling and properties

Once the cucumber was harvested, soil samples were collected in triplicates from CK and biofertilizer treatment group (Batten et al., 2006). During each sampling, about 10 g of soil was extracted in triplicates by sterile sampling spoon from cucumber root at 5-10 cm depth underground. They were stored in sterile plastic bags with an ice bag and transported to our laboratory where these samples were stored at -80 °C for of soil properties and molecular research analyses within one week (Xu et al., 2012).

The properties (soil pH, organic matter, total and available nitrogen, phosphorus, and potassium) of the soil collected in filed experiments were commercially measured. organic carbon was determined by using the oil bath-K_2CrO_7 titration method, total N was by the Kjeldahl method. Total P were measured by Mo–Sb colorimetric analysis while total K was determined by flame photometry. pH, after extraction with 2.5-fold of water, was measured by glass electrode method (Liu et al., 1996).

Statistical analyses

Statistical analyses were performed using SPSS 17.0 software package (SPSS Company, Chicago, IL). The results are expressed as mean ± SD of at least three independent experiments. Multiple comparisons were performed using one-way ANOVA. A probability level of 5 or 1% was considered significant (*P<0.5, P<0.01*) unless otherwise stated. Bars sharing different letters or '*' are statistically different.

RESULTS

Nitrogen fixing bacteria isolation and nitrogenase activity assay

Twenty-nine nitrogen fixing bacteria were isolated from different soil collected from study areas. Seven of them showed nitrogenase activity higher than observed for *Azotobacter chroococcum* (nitrogen fixing bacteria), currently used as biofertilizer (Sunjian, 2012) (Table 1) in the range of 374.86 ~ 921.76 nmol C_2H_4/h·mg protein, accounting for 24.48% of all tested strains. Moreover, in these seven stains, five showed nitrogenase activity (N-R-11, N-R-21, NW-3, N-YM-3, and N-YM-4) higher than 400 nmol

C_2H_4/h·mg protein, accounting for 17.2% of all strains tested. The bacterial strain N-YM-3, isolated from the soil of Yellow Mountain showed the highest nitrogenase activity (921.76 nmol C_2H_4/h·mg protein).

Pot results

According to the nitrogenase activity assay results, the seven nitrogen fixing bacteria strains were able to increase cucumber dry weight by 7 to 47%. However, only the treatment with NW-3 was statistically significant to chemical control (Figure 1), and showed the best growth-promoting effect for cucumber dry weight (Figure 1C). Strains N-BF-11, N-R-21, NW-3, NW-4, and N-YM-4 increased cucumber plant fresh weight by 10 to 27% (Figure 1B). For fresh weight, only treatment with NW-3 showed statistic differences in comparison to control plants. Figure 1A shows that in the NW-3 treatment group, cucumber height reached a maximum of 4.53 cm, an increase of 16% 15 days after inoculation, followed by N-YM-3 and NW-4 treatments.

In summary, treatment with NW-3 strain resulted in significantly higher cucumber fresh and dry weight and plant height of 1.57 g, 0.14 g and 4.53 cm, respectively (Figure 1) compared with non-inoculated treatment (*P<0.05*).

Identification and characterization of these seven nitrogen fixing bacteria strains

The identification and characterization of these seven nitrogen fixing bacteria strains used in pot experiment were also studied. These seven nitrogen fixing bacteria N-BF-11, N-R-11, N-R-21, NW-3, NW-4, N-YM-3, and N-YM-4 belonging to different genera *Klebsiella oxytoca, Pseudomonas brassicacearum, Flavobacterium johnsoniae, Ochrobactrum* sp., *Bacterium* sp., *Ensifer adhaerens,* and *Bacterium* sp., respectively, were subjected to 16S rDNA sequence analysis (Table 2). Furthermore, IAA, ACC-deaminase, siderophores, phosphorus and potassium as major essential macronutrients for plant growth and development were studied (Table 2). Among these seven fixing bacteria strains, IAA production ability of strain *K. oxytoca* N-BF-11, *Ochrobactrum* sp. NW-3 and *Bacterium* sp. N-YM-4 were significantly higher than others, with the highest value of 46.67 mg/l for *K. oxytoca* (N-BF-11). *P. brassicacearum* NR-11 and *Ochrobactrum* sp. NW-3 ACC-deaminase abilities were significantly higher than other strains; the highest yield of 12.07 µmol α- D keto/h·mg protein for *Ochrobactrum* sp. NW-3. *P. brassicacearum* NR-11, *Ochrobactrum* sp. NW-3 and *Bacterium* sp. NW-4 produced siderophores indicated by the clear yellow circle on their CAS test plates while other strains did not show any yellow circle. *F. johnsoniae* NR-21 and *Ochrobactrum* sp. NW-3 showed P-solubilizing capacity, but not as well as *K. oxytoca* N-BF-11 and *P. brassicacearum* NR-11 while other strains did not have P-solubilizing capacity. All seven nitrogen fixing bacteria strains showed K-solubilizing capacity (Table 2).

Based on the 16S rDNA sequence analysis, NW-3 belongs to the genus *Ochrobactrum*. The nitrogenase activity of *Ochrobactrum* sp. NW-3 was higher than *Azotobacter chroococcum*. Moreover, the results of bioassays for plant growth promoting activities showed that *Ochrobactrum* sp. NW-3 not only has superior K and P solubilization ability but also can secrete IAA, ACC-deaminase, and siderophores. Since *Ochrobactrum* sp. NW-3 showed excellent growth-promoting ability for cucumber growth in pot experiment and multifunction growth-promoting characteristics, it was further applied in cucumber field experiment.

Field experiment results

Cucumber yield and quality of biofertilizer treatment group was significantly higher than CK (*P <0.05*), showing that the biofertilizer *Ochrobactrum* sp. NW-3 inoculation is an optimal microbial fertilizer formulation that can significantly increase cucumber yield by 29% (Figure 2) as well as its quality by 37.5% when compared with the CK. At the same time, by comparing the different cucumber growth periods (seedling, flowering, fruiting period), the biofertilizer treatment group showed better growth promoting effect on cucumber growth (Supplementary Figure 1) with increased plants height, broader leaves and relatively vigorous growth.

Soil properties play an important role in nutritional assimilation such as organic carbon content and total N, P, K by plants. Thus, as the cucumber was harvested, the final soil properties were evaluated with the CK having higher values for soil properties (total organic carbon, total nitrogen, and total P, K, and higher pH) as compared with biofertilizer treatment (Table 3). From the Pearson's correlation data analysis (Table 4), cucumber yield, quality and final soil properties showed negative correlation. Moreover, the correlation between cucumber yield, quality and soil properties (Table 4) suggests that cucumber quality showed significant correlation with total potassium (R=-0.908), phosphorus (R=-0.885), and nitrogen (R=-0.436) in soil at 0.01 or 0.05 level while cucumber yield showed significant correlation with total phosphorus (R=-0.929), potassium (R=-0.947), and pH (R=-0.716) at the 0.01 or 0.05 level.

DISCUSSION

In order to increase crop production, different kinds of chemical fertilizers are applied in the field at high rates which cause serious environmental and economic problems (Carpenter et al., 1998). To solve these problems nowadays, the term "biofertilizer" is becoming a hot and popular research trend with the study of various kinds of microbial inoculants (Bhattacharyya and Jha, 2012). However, to date, few studies about *Ochrobactrum* sp. acting as a bioferilizer have been reported. Previously, *Ochrobactrum sp.* Pv2Z2 was reported to show multiple plant growth-promoting characteristics and biodegradation (Imran et al., 2014), but

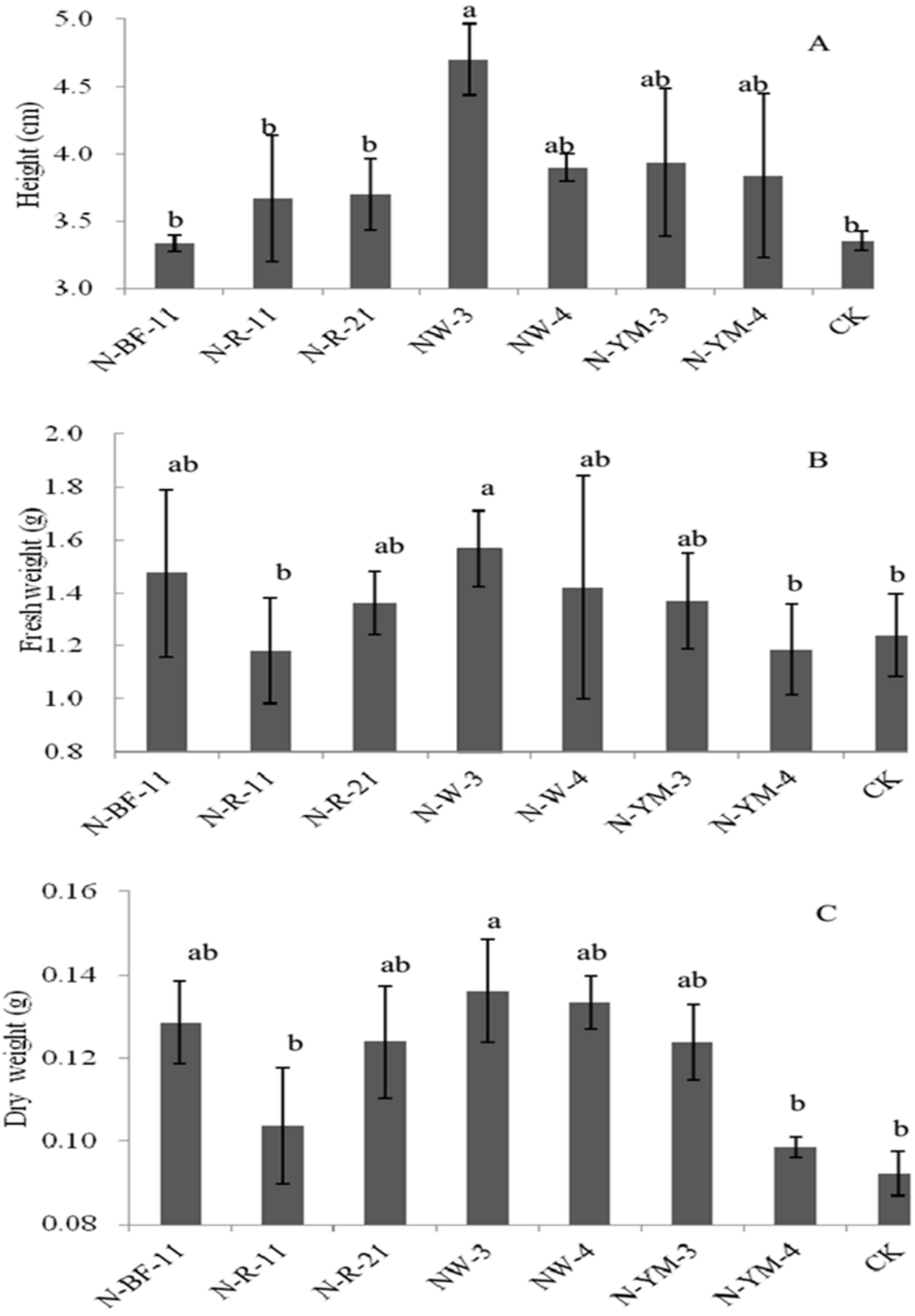

Figure 1: These seven nitrogen fixing bacteria used in the pot experiment increase the cucumber plant height (A), dry weight (B), and fresh weight (C). Figure 1A shows that the cucumber height treated by NW-3 inoculants is significantly higher than others, increased 16% after inoculation 20 days when compared with CK, followed by N-YM-3, and NW-4 treatment. From figure 1B and 1C, we could know that the best growth-promoting inoculant is also NW-3 for cucumber dry weight (B) and fresh weight (C).

Table 2. Identification and some characteristics of seven nitrogen fixing bacteria used in pot experiments

No.	Identification*	IAA (mg/l)	ACC-deaminase activity**	Siderophores	P-solubilization	K-solubilization
N-BF-11	*Klebsiella oxytoca*	46.67±0.22[a]	8.93±1.92[b]	-	+	+
N-R-11	*Pseudomonas brassicacearum*	0.43±0.33[d]	10.70±2.18[ab]	+	+	+
N-R-21	*Flavobacterium johnsoniae*	1.16±0.45[d]	6.68±1.87[b]	-	+	+
NW-3	*Ochrobactrum sp.*	13.25±0.32[b]	12.07±0.82[a]	+	+	+
NW-4	*Bacterium sp.*	0.36±0.29[d]	4.96±2.56[b]	+	-	+
N-YM-3	*Ensifer adhaerens*	0.27±0.15[d]	7.45±1.02[b]	-	-	+
N-YM-4	*Bacterium* sp.	9.52±0.38[c]	2.26±2.24[c]	-	-	+

*: NCBI blast result, query cover and identity were both large than 99%,
**: ACC-deaminase activity unit: μmol of a-ketobutyrate/h·mg protein,
+: the presence of clearing zone around bacterial colony,
-: the absence of clearing zone around bacterial colony,
Numbers sharing the different letter are statistically different.

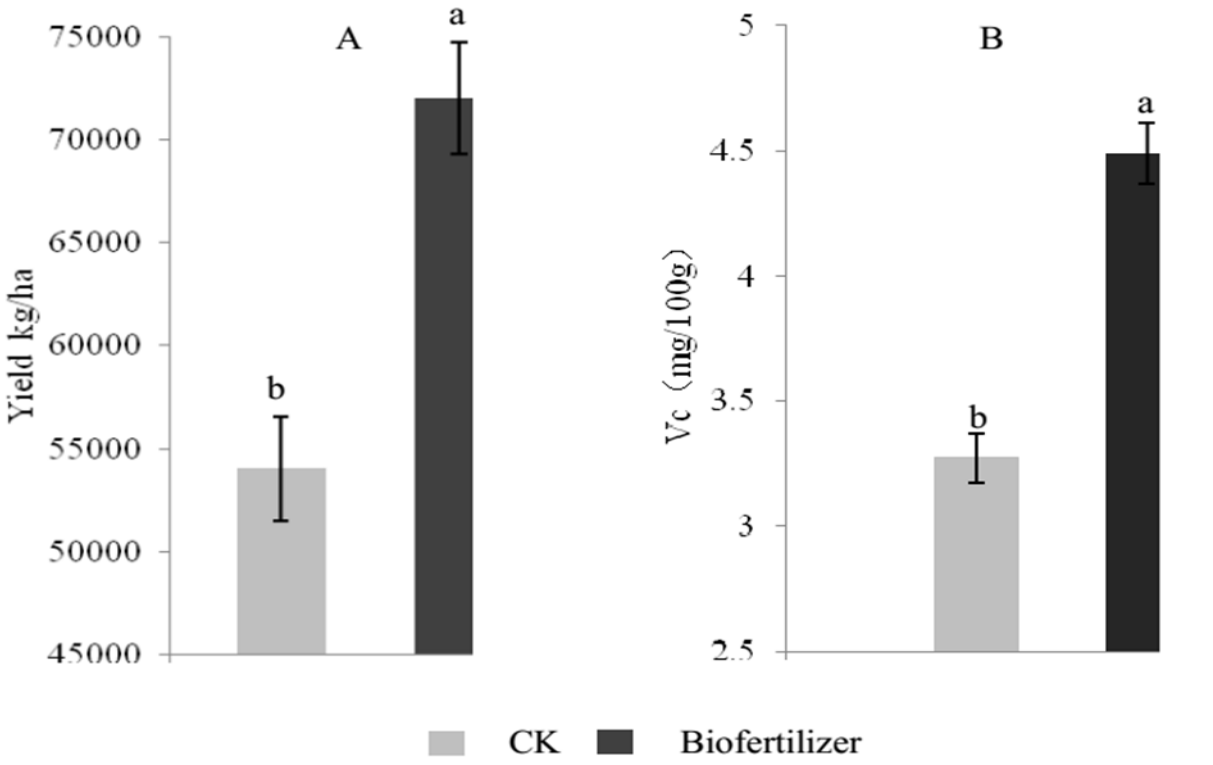

Figure 2: The yield and quality of biofertilizer treatment group was significantly higher than CK ($P < 0.05$). *Ochrobactrum* sp. NW-3 has great application prospects for cucumber growth as biofertilizer, can significantly increase cucumber yield 29% and improve the cucumber quality (Vitamin C content) 37.5%.

Table 3. The final soil properties after cucumber harvested in field experiment

Treatment	Total organic carbon (TOC), %	Total nitrogen (TN), %	Total phosphorus (TP),g/kg	Total potassium (TK),g/kg	pH
CK	1.735±0.021	0.152±0.004	0.60±0.04	26.7±0.7	6.53±0.02
Biofertilizer	1.435±0.034	0.131±0.011	0.47±0.02	24.0±0.4	6.45±0.03

CK: chemistry and organic fertilizer
Biofertilizer: chemistry and organic fertilizer + *Ochrobactrum sp. NW-3* inoculants (1.5×10^{14}cfu/ha).

Table 4. Pearson's correlation matrix between cucumber yields, quality and the final soil properties

		TOC	TN	TP	TK	pH
Quality	Pearson Correlation	-0.566	-0.436*	-0.885**	-0.908**	-0.639
	Sig.	0.112	0.041	0.001	0.001	0.064
Yields	Pearson Correlation	-0.476	-0.340	-0.929**	-0.947**	-0.716*
	Sig.	0.195	0.370	0.001	0.001	0.030

**: Correlation is significant with n = 12 at the 0.01 level.
*: Correlation is significant with n = 12 at the 0.05 level.

it was not applied in actual field experiment to test and verify whether it exerted multiple promoting traits. Moreover, the use of *Ochrobactrum* sp. for phytoremediation of heavy metal or other organic pollutants have also been studied (Imran et al., 2014; Pandey et al., 2013). Previous studies show that *Ochrobactrum* sp. has extremely high potential for application as biofertilizer, but evaluation of its effects on field application as biofertilizer is lacking.

The concentrations of soluble phosphorus and potassium in the soil are usually very low and more than 90% of phosphorus and potassium in the soil exists in the form of insoluble rocks and silicate minerals. Efficient soluble phosphorus and potassium play an important role in plant growth improvement under field conditions (Saharan and Nehra, 2011). Therefore, besides nitrogenase activity, phosphorus and potassium solubilization ability is a very important capability for promoting plant growth, especially in soils lacking soluble P and K. In this study, in one isolated strain called NW-3 belonging to the genus *Ochrobactrum*, nitrogen fixing capability was determined using 16S rDNA sequence analysis. NW-3 showed nitrogenase activity higher than *Azotobacter chroococcum*. In the pot experiment, cucumber fresh and dry weights and plant height in plants inoculated with Ochrobactrum sp. NW-3 were significantly higher than in the control. NW-3 showed the best growth promoting effect in cucumber among the seven nitrogen fixing bacteria isolates in the pot experiment. Furthermore, the bioassays showed that *Ochrobactrum* sp. NW-3 has superior K and P solubilization ability besides secretion of IAA, ACC-deaminase, and siderophores, suggesting it as a comprehensive and multifunction plant growth promoting bacterium.

In addition, the Pearson's correlation between cucumber yield, quality and final soil properties also confirmed that cucumber yield and quality showed significant correlation with increased potassium and phosphorus content in Anshan soil in the field experiment. Unfortunately, in this experiment, the amount of *Ochrobactrum* sp. NW-3 nitrogen fixation into soil from the environment could not be detected because of technical problems, therefore the role of its nitrogenase activity in cucumber growth is impossible to assess. Moreover, IAA and ACC-deaminase are both key plant growth promoters that even the strains which produce low amounts of IAA, release it continuously, thus improving plant growth (Tsavkelova et al., 2007). In addition, iron is an essential element for growth in all living organisms. Siderophore production is also used as an indispensable indicator of increased crop yield (Husen et al., 2013). However, to date, technical deficiencies limit the detection of IAA, ACC-deaminase and siderophore production in *Ochrobactrum* sp. NW-3 under field conditions. Consequently, we also could not assess the quantifiable contribution of IAA, ACC-deaminase and siderophore in stimulating cucumber growth. In subsequent trials, we will investigate functions related to gene expression detected by means of a variety of gene technology.

Cucumber absorbed large number nutrients (N, P, and K) from the soil thus leading to a decline in soil nutrients (Table 3) but high yield (Figure 2) due to the multi plant growth-promoting effect of *Ochrobactrum* sp. NW-3. At the same time, another reason for the declining soil nutrients in the biofertilizer treatment group was that most soil microorganisms utilize a considerable amount of organic matter for metabolism and maintain growth (Wu et al., 2005). *Ochrobactrum* sp. NW-3 promoted efficiency in the use of traditional chemistry and organic fertilizers thereby reducing fertilizers wastage, increasing crop yields and quality and protecting the environment. The inoculation of *Ochrobactrum* sp. NW-3 resulted in a significant balance between different soil nutrients and cucumber crops yield and quality.

Other studies (Compant et al., 2010) have mentioned that plants may host various human pathogens (Berg et al., 2005a; Allerberger and Sessitsch, 2009). It has also been reported that some of these pathogens can even exhibit growth-promoting effects or improve plant health and colonize the rhizosphere as well as plant internal tissues. However, not all the PGPB are same with clinical isolates, thus can be pathogenic. In a study on *Stenotrophomonas maltophilia*, a species known for clinical infection as well as for plant colonization, it was proven that genome plasticity could explain differences between clinical and soil isolates (Ryan et al., 2009). In addition, there is a research on *Ochrobactrum* sp. Pv2Z2 belonging to *Ochrobactrum* sp., however phylogenetic analysis showed the relatedness to nodulating strain *Ochrobactrum cytisi* rather than to the clinical/pathogenic type strain of *O. anthropi* (Imran et al., 2014). Therefore, our research on *Ochrobactrum* sp. NW-3 is also extremely meaningful and useful for biofertilizer production. However, before large-scale factory production, its phylogenetic analysis and pathogenicity research must be

further verified and studied.

Conclusions

In conclusion, cucumber yield and quality (Vitamin C content) in biofertilizer treatment group significantly increased after three months of field experiments, suggesting that the multifunction plant growth-promoting bacterium *Ochrobactrum* sp. NW-3 could be related to successful application in field experiment and coordinate changes in soil resource pools. Generally, the comprehensive and multifunction of *Ochrobactrum* sp. NW-3 does not have high practical application value but has a certain scientific research value in the future development direction of biofertilizers. However, there are still many issues that will require further exploration such as, investigating the action mechanism of *Ochrobactrum* sp. NW-3 about soil-plant-microorganism and relationship links to other strains. Moreover, a better understanding on how it colonizes in the complex unfamiliar field soil or plant root environment will not only lead to increased knowledge on soil-plant-microbe interactions but will also lead to a more successful and reliable use of bacterial inoculants. In addition, changes in the entire soil bacterial community structure and the growth trend of *Ochrobactrum* sp. NW-3 during cucumber growth should also be further exploited.

Conflict of interest

No conflict of interest exits in the submission of this manuscript.

Acknowledgements

This project is financially supported by National High Technology Research and Development Program of China (2012AA021203), Tianjin Science and Technology Project of China (12ZCZDSY15300), and the Cooperation Projects (Xinjiang) of the Chinese Academy of Sciences, China.

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