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ORIGINAL ARTICLE

Selective screening and characterization of plant growth promoting bacteria for growth enhancement of tomato, *Lycopersicon esculentum*

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ABSTRACT

Background and Objectives: Plant Growth-promoting Bacteria (PGPB) can replace the dangerous chemical fertilizers and pesticides. The aim of this study was to isolate the PGPBs for *Lycopersicon esculentum* plant and to determine the appropriate volume for inoculation.

Materials and Methods: Plants samples were collected from tomato fields. Nitrogen fixing-PGPBs were isolated from rhizoplane and rhizosphere. Five isolates were screened based on their growth abilities and examined for PGPB traits including phosphate solubilization, and IAA, ammonia and HCN production. After high cell density cultivation, the cells were separated by centrifugation and freeze dried after resuspension in cryoprotectant. The powders were inoculated into sterile soil with a dose of 10^6, 10^7 and 10^8 CFUs/g. Tomato *(Lycopersicon esculentum)* seeds were sown in soil and after 42 days the shoot length was measured.

Results: Most of the potent PGPBs with high growth capacity were isolated from rhizoplane. Maximum phosphate solubilization was 289.7 µg/ml by NFB12 which isolated from rhizoplane. This strain produced the maximum level of IAA. NFB12 produced ammonia without the ability of production of HCN. This strain enhanced shoot length in dosed dependent manner. Surprisingly, inoculation of soil with 10^8 CFUs/g dramatically decreased the shoot length by 21%. Based on molecular approach NFB12 was identified as *Bacillus megaterium.*

Conclusion: Isolation of specific PGPBS is recommended for sustainable plant production. Our results showed that NBF12 improves tomato plant growth and its effect on tomato plant growth is does dependent. Maximum growth rate of tomato was observed with 10^7 CFUs/g soil inoculation of NFB12 while higher inoculation showed negative effect.

Keywords: *Bacillus megaterium*; Nitrogen-fixing bacteria; Indoleacetic acid; *Lycopersicon esculentum*

INTRODUCTION

World population is increasing continuously and it is estimated to reach ~7.5 billion by 2020 Preparation of food for this people is a major concern which need a great endeavor to increase agricultural productivity. In this regard, scientist has main focus to increase the yield of crops Quality and health properties of foods along with organoleptic and nutritional value, are gaining great attention by consumers and governments (1). Agricultural input, chemical fertilizers and pesticides in particular, routinely uses for increasing productivity but there are massive evidences show the accumulation of dangerous substances in soil, air and water following the overuse of them Obviously, unsystematic application of these chemicals is a major obstacle to the development of sus-

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tainable agriculture which threat environment and human being In developed countries several policies have been established to regulate this agricultural input and replace them by environmentally friendly approaches (2).

It seems that Plant Growth Promoting Bacteria (PGPB) are the best alternative to conventional approach for enhancing growth and quality of plants. These bacteria regulate plant developmental processes and play important roles for uptaking nutrients, protecting the plants and crops (3). Tomato is one of the most utilizable vegetable crops worldwide that has achieved tremendous popularity over the last century. Therefore, achieving sustainable agricultural and environmental development are the main issues of different countries in the world (4). Based on studies (5-7), inoculation tomato seeds with plant growth promoting bacteria, significantly increased the fresh and dry weights of seedlings also stimulated root development by better absorption of water and minerals from the soil.

PGPB promote plant growth directly or indirectly by inhibition of soil borne diseases as a biocontrol. They directly help growers by fixation of nitrogen as ammonia, solubilization of phosphate, sequestration of Iron and biocontrol of pathogens (8).

Ammonium is a significant source of nitrogen for plants and microorganisms. Nitrogen-fixing bacteria in the rhizospheric region of the plant convert the atmospheric nitrogen into ammonium using nitrogenase enzyme, thereby enhancing the plant growth and crops (9).

Access to phosphorus, as an essential element, is vital for plant growth. Since a major part of phosphorus is insoluble and out of the reach of plant, use of PGPB is highly important due to their ability to secret gluconic acid, oxalic acid, organic acids, and phosphate-solubilizing enzymes such as phosphatase and phytase. Therefore, PGPB improve plants nutrition and tolerance to biotic and abiotic stress (10). Plants grow and development increase directly by bacterial phytohormones like auxins and gibberellins. Especially, Indole Acetic Acid (IAA) which is predominantly produced by PGBP induce plant cell division and elongation (11). By modifying certain condition like increase in osmotic content of the cell, permeability of water into cell and increasing in cell wall synthesis resulted in increased seed germination, root numbers, root weight and shoot length and higher crop biomass (12).

Production of hydrogen cyanide (HCN) as secondary metabolites prevent proliferation of most fungal phytopathogens through synthesis of some antibiotics or cell wall degrading enzymes. Hydrogen cyanid also it increased availability of nutrients by affecting mobilization of elements from rock forming minerals and indirectly promote plant growth (8).

Several bacteria from rhizosphere and rhizoplane environment can colonize different part of roots and act as a PGPB (13). This efficacy of colonization increases by exopolysaccharide (EPS) production by bacteria. Attachment of PGPB onto the root surface is facilitated by EPS which is really important in the early stages of endophytic colonization (3).

The objective of this study was the isolation and characterization of PGPB with multifunctional ability from tomato root rhizosphere and evaluation of growth promoting effect under in-vitro conditions.

MATERIALS AND METHODS

Sampling and isolation of free-living nitrogen-fixing bacteria. Plant sample were collected from five tomato fields in suburb of Tehran province. Plants roots were immediately put in polyethylene bags to avoid desiccating and transferred to the laboratory at 4°C. Rhizosphere bacteria were isolated by handshaking roots for 10 min in 1 Lit of a sterile 0.9% NaCl solution while for separation of rhizoplane bacteria, second shaking was done in the same solution completed by Tween 80 (0.01% v/v). The suspensions were centrifuged at 1000 rpm for 10 min and serially diluted in a sterile Phosphate buffer saline (PBS) solution (pH 7.2). Nitrogen fixing bacteria were isolated by pour plat of 1 ml of dilutions in Nitrogen-fixing Bacteria (NFB) medium contains (g/L) (14): K_2HPO_4, 0.5; $MgSO_4.7H_2O$, 0.2; NaCl, 0.1; $FeCl_3.6H_2O$, 0.015; DL-malic acid, 5; KOH, 4.8; and agar, 20 (pH 7). The plates were incubated at 25°C for 48 to 72 h and NFB were isolated and coded based on the morphology of colonies. Fresh culture of strains was prepared and mixed with 25% sterile glycerol, aliquoted in 1.5 ml Eppendorf and kept at -80°C.

Nitrogenase activity of isolated strains was determined based on acetylene reduction assay (ARA). Briefly, bacterial isolates were grown in test tubescontaining 10 ml of NFB solid medium for 72 h at 28°C. Then, 10% of the atmosphere of each tube was substituted with gaseous acetylene and incubated for an-

other 24 h. Gas chromatography technique was used to assay the amount of ethylene production in 1 ml of each gas sample from the headspace of tubes (15).

Screening of higher growth rat strains. Strains with higher growth capacity were screened by 5% inoculation overnight culture in 100 ml NFB broth medium completed with 0.5% yeast extract. Erlenmeyer flasks were incubated in a shaker incubator at 30°C for 48 with 150 rpm. The cultures were serially diluted and total cells count was performed by duplicate pour plating of 1 ml of dilutions the same medium. After incubation at 30°C for 48 h, the total cells were reported based on colony forming unit (CFUs) (16).

Capsule production by strains. Bacterial capsule is an exopolysaccharide which can be visualized by Indian ink staining based on Gin's Method. Briefly, bacterial colony was mixed with Indian ink and a thin smear was prepared on a microscopic slide. After drying, slide was saturate with crystal violet for 1 minute and rinse with water gently. Purple bacterial cells surrounded by a clear halo on a dark background were observed under Optical microscope (17).

Isolation of strains capable of solubilize phosphate. The strains were streaked onto on Pikovskaya agar (Merck, Germany) containing insoluble tri-calcium phosphate (TCP). The plates were incubated at 30°C for 72 h and clear zone around the colonies indicated solubilization of phosphate. Quantitative analysis of phosphate solubilization was performed by culturing the strains in mentioned liquid medium. After 72 incubation in shaker incubator, active crude supernatant was isolated by centrifugation at 60000 rpm for 20 min. The amount of phosphate solubilization was identified by optical density assay at 882 nm compared with standard curve of KH2PO4 solution.

Phytate screening medium (PSM) was used for determination of Organic phosphate solubilization strains. This medium contains: (%): Glucose, 2; KCl, 0.05; CaCl2, 0.2; NH4NO3, 0.5; MgSO4, 0.05; MnSO4, 0.001; FeSO4, 0.001; sodium phytate, 0.2; and agar, 1.5 (at pH 7). The strains were spotted on agar medium and inoculated for 2-3 days at 37°C. Formation of a clear zone around the colony indicates phytase production (18).

Screening of Indole Acetic Acid (IAA) producer strains. 1 ml of the overnight culture of bacteria inoculated to a 250 ml Erlenmeyer containing 100 ml nutrient broth fortified with L-tryptophan (0.1 mg/ml). Briefly, after incubation in shaker incubator for 48 h at 30°C with 150 rpm, bacterial culture was centrifuged at 10000 rpm for 30 min. Then 2 ml of the supernatant was mixed with 2 ml Salkowski reagent (0.5 M $FeCl_3$ in 35% per chloric acid) and incubated for 30 min at room temperature in darkness. The optical absorbance was measured at 535 nm and the produced IAA was measured by standard curve graph (19).

The ability of ammonia production. Production of ammonia was assayed by inoculation of 100 µl of overnight culture of strains into 10 ml peptone water. The culture was incubated at 37°C in a shaker incubator with 150 rpm for 48 h. 1 ml of Nessler's reagent (Merck, Germany) was added to the medium. Development of yellow color showed the production of ammonia by the strain (20).

The ability of HCN production. Production of HCN was assayed by the method suggested by Lorck (21). About 100 µl of overnight culture of strain was inoculated in nutrient agar slant fortified with 4.4 g/l glycine. The tubes were covered with a Whatman filter paper (no. 1) previously soaked in 0.5% picric acid and 2% sodium carbonate. The test tubes were closed with parafilm tape and incubated at 28 ± 2°C for 4 days. The color change of the paper from yellow to brown indicated the production of HCN.

***In-vivo* plant growth promotion assay.** The efficacy of 5 selected strains in promoting plant growth were evaluated. Strains were cultured in nutrient broth medium for 48 h. The cells were separated by centrifugation and washed with tap water. The cells pellets were re-suspended in 11% skimmed milk solution fortified with 0.2% mono-sodium glutamate. The cells suspensions were freeze dried and total viable counts of powders were identified.

Soil samples were analyzed for their physico-chemical properties. Sterile soil was inoculated with freeze dried bacteria with a concentration of $1e^6$ to $1e^8$ CFU/g.

Tomato *(Lycopersicon esculentum)* seeds were disinfected by soaking in 30% sodium hypochlorite solution containing 0.1% Triton X-100 for 15 min. The seeds were rinsed with sterile water and sown in sterile potting soil inoculated with different con-

centration of PGPB with 5 repeats. Sterile soil without any inoculation was used as a negative control. The pots were incubated in a phytotron under 16 h at 27°C and 22°C during day and night, respectively according to the procedure described by Carrasco-Fernández J (23). The pots were irrigated with sterile distilled water, and shoot length was measured after 42 days.

Molecular identification of strain. Molecular identification of the strain was performed by 16S rDNA amplification with universal primer. Primers sequences were as follows:

1541R: 5′-AGGGAGGTGATCCAGCCGCA-3′ and 27F: 5′-AGAGTTTGATCCTGGCTCAG-3′.

The PCR product with about 1500 bp was sequenced by Microsynth co., Switzerland. The resulting sequences were aligned using the nucleotide BLAST tool NCBI GenBank.

Statistical analysis. GraphPad Prism 6 software was used for statistical analysis. Data analysis was performed using one-way ANOVA and t-test and statistically significant was considered as $p<0.05$.

RESULTS

Isolation and growth potential of nitrogen-fixing bacteria. NFB were isolated from rhizosphere and rhizoplane environment of roots (Table 1). Totally, 36 strains were isolated and glycerol stocks of them were prepared. The growth ability of strains has a great importance in high cell density production because of economic aspects. NFB medium supplemented with 0.5% yeast extract was used to screen high growth performance bacteria. Morphological characteristics, nitrogenase activity and growth potential of 5 the best selected strains are shown in Table 2.

Table 1. Isolated strains from rhizosphere and rhizoplane environment of root from 6 different soil samples from tomato field.

Soil sample NO.	NO. of strains from	
	Rhizosphere	Rhizoplane
1	2	2
2	3	4
3	2	5
4	2	4
5	3	3
6	2	4

Phosphate solubilization. Phosphate solubilization is one the major PGPBs trait which assayed in selected strains (Fig. 1). Solubilization of insoluble inorganic phosphate ranges from 53.33 to 289.7 µg/ml. Strains NFB12 and NFB28 showed higher potential and there was no significant difference between them (p value= 0.994). While the smallest value of phosphate solubilization belong to NFB03 and NFB23 with no difference (p value= 0.1561). The qualitive assay of phytate solubilization showed that all the 5 selected strains have the capacity of phytate solubilization.

Production of indole acetic acid (IAA). All the 5 strains produced indole acetic acid ranges from 2.836 to 9.704 µg/ml (Fig. 2). NFB12 showed the highest production value with 9.704 µg/ml which was significantly higher than the other strains (p value<0.0001). NFB 23 and NFB28 produced 5.763 and 6.646 µg/ml of IAA respectively with no statistically significant difference (p value= 0.1463).

Production of ammonium and HCN. Production of ammonium and HCN were assayed qualitatively. Our results showed that all of the strains produce ammonia in Peptone broth after 48-72 h. After 4 days incubation of strains in modified nutrient agar medium, but none of them produced HCN.

Enhancement of shoot length growth. The results of soil quality and shoot length growth by inoculation by different concentration of freeze-dried bacteria (10^6, 10^7 and 10^8 CFUs/g of soil) is depicted in Tables 3 and 4. Using 10^6 CFUs/g as inoculation, the highest shoot length belongs to the growers inoculated with NBF12 (13.36 cm). Negative control group with 5.17 cm shoot length had the minimum growth rate. The other PGPBs stimulated growth but not as much as NBF12 (p value<0.0001).

Increasing in NFB12 concentration to 10^7 CFUs/g caused a significant increase in shoot length of plant (17.17 cm) compared with lower inoculation (p vale=0.005). Surprisingly, inoculation of the soil by 10^8 CFUs/g induced deleterious effects on growth. At this concentration the shoot length decreased to 8.53 cm and showed significant decrease compare to 10^7

Table 2. Morphological characteristics and growth potential of 5 selected trains after 48 h incubation

Strains NO.	Soil sample NO.	Isolation area	Gram stain	Capsule production	CFUs/ml	Nitrogenase activity (nmol C2H4/mg protein /h)
NFB03	1	Rhizosphere	negative	Positive	2.4e8	73.61 ± 5.32
NFB12	3	Rhizoplane	positive	Positive	3.2e9	133.47 ± 11.21
NFB15	3	Rhizoplane	positive	Positive	9.4e8	67.94 ± 8.97
NFB23	4	Rhizoplane	positive	Positive	8.9e8	77.98 ± 11.34
NFB28	4	Rhizoplane	positive	Positive	2.1e9	84.32 ± 10.63

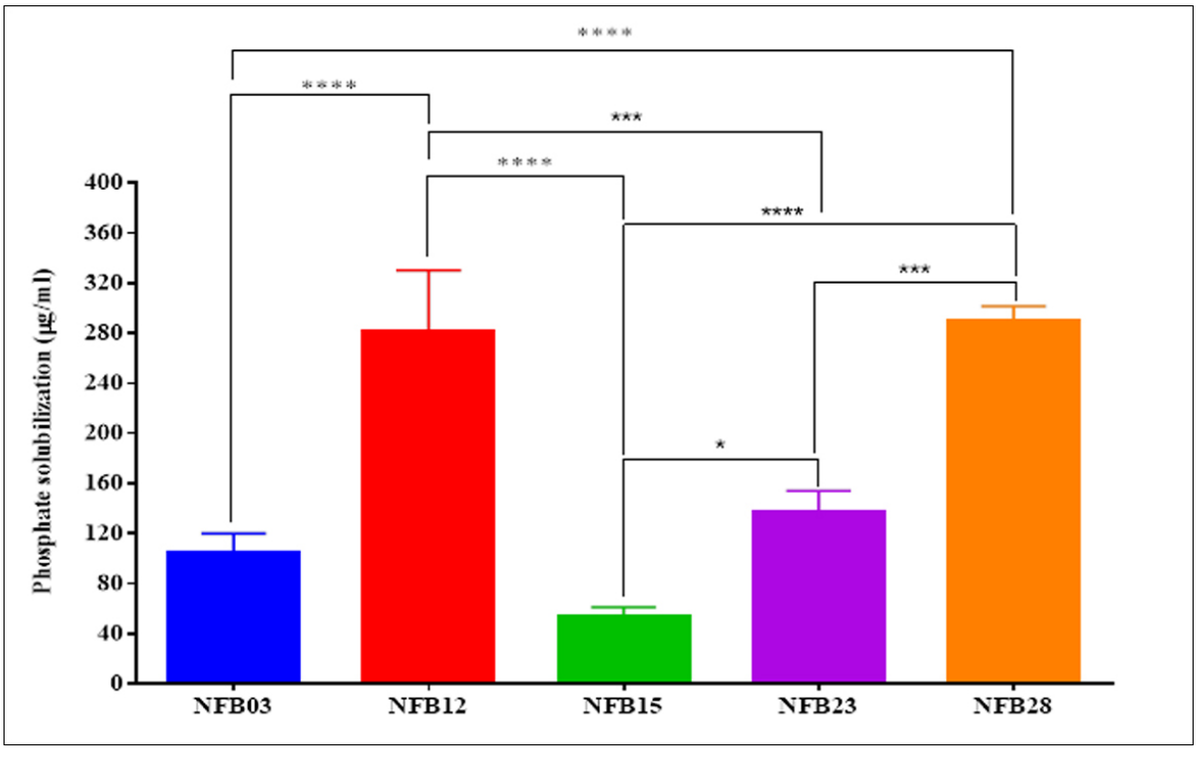

Fig. 1. Tri-calcium Phosphate solubilization of selected strains in Pikoskvaya medium after 48 h incubation

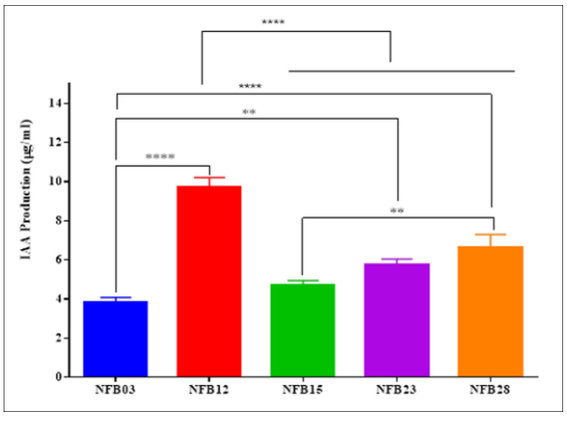

Fig. 2. Indole Acetic Acid production by selected strains in Nutrient broth medium fortified by L-tryptophan

CFUs/g inoculation. This growth model of growth didn't exist in the other strains. In another word, for the rest 4 bacteria increasing in bacterial inoculum concentration didn't exert any obvious effects on the shoot growth.

Molecular identification of the isolated organism. Strain NFB12 caused the maximum shoot length and had PGPB trait and identified by molecular. After amplification of 16S rDNA, the PCR product was sequenced by Sanger method.

The analysis of the phylogenetic tree of 16S rDNA gene sequence showed the highest similarity with *Bacillus megaterium* strain ATCC 14581 (Fig. 3). The sequence was registered on NCBI website with

Table 3. Soil physicochemical properties

Soil texture	Total N (%)	Fe (mg kg^{-1})	Available K (mg kg^{-1})	Mn (mg kg^{-1})	Available P (mg kg^{-1})	Organic Carbon (%)	pH
Loam clay	0.06	0.06	226	7.04	1.09	0.66	7.04

Table 4. Shoot length of potato plant after 42 days in soil inoculated with different concentration of PGPB.

Groups	Mean ± SEM of Shoot Length (cm)		
	1e^{6} CFUs/g of soil	1e^{7} CFUs/g of soil	1e^{8} CFUs/g of soil
Control	5.17 ± 0.49^{c}	5.17 ± 0.49^{c}	5.17 ± 0.49b
NFB03	8.33 ± 0.17^{b}	9.47 ± 0.43^{b}	8.53 ± 0.38a
NFB12	13.63 ± 0.70^{a}	17.17 ± 0.96^{a}	8.67 ± 0.57a
NFB15	6.50 ± 0.76b^{c}	5.93 ± 0.61^{c}	8.63 ± 0.37a
NFB23	7.70 ± 0.15^{b}	9.73 ± 0.43^{b}	9.27 ± 0.41a
NFB28	8.23 ± 0.27^{b}	10.70 ± 0.46^{b}	9.90 ± 0.85a

Different superscripts letters in column indicates statistically significant differences

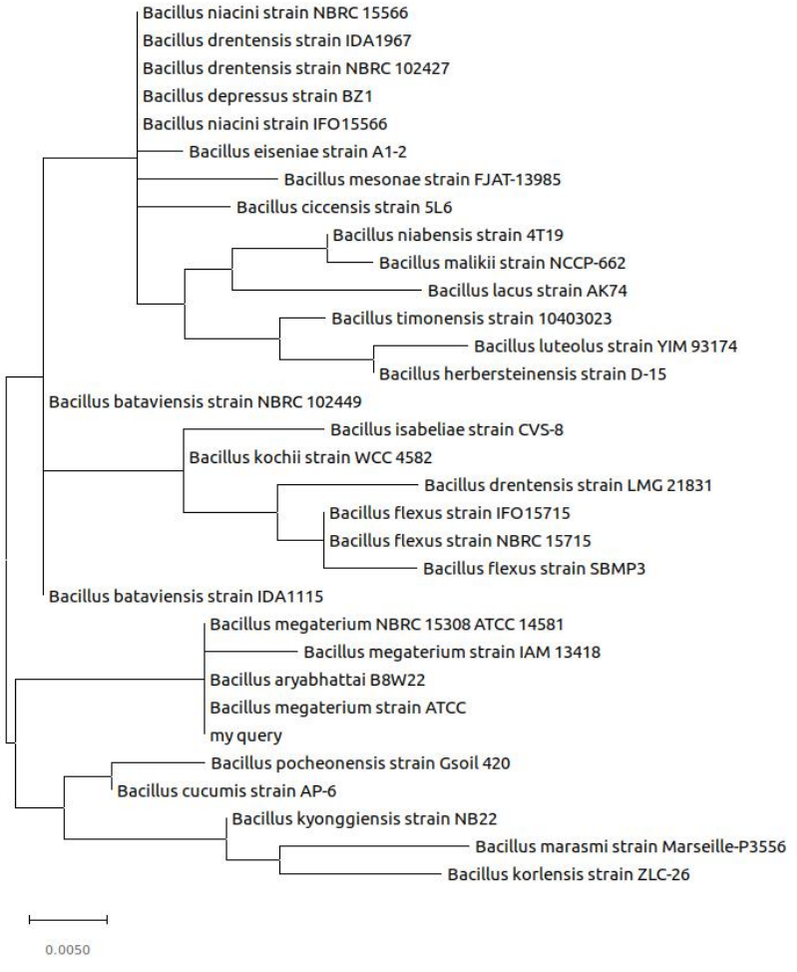

Fig. 3. Phylogenetic analysis tree based on of 16S rDNA gene sequences

accession number MK849881.1.

DISCUSSION

Rhizosphere and rhizoplane are enriched environments which support the growth of microorganisms. Some of these microorganisms are proposed as PGPBs which promotes the growth of plants by several mechanisms. In selection of PGPBs what is critical is the specificity of strains for cultivar with maximum impact. It is well documented that because of variability and inconsistency of soil and different nutritional needs of cultivar, all the PGPBs must examine strain by strain (24).

The main aim of this study was isolation and characterization of nitrogen fixing PGPBs specific for tomato plants. We focused on screening potent strains and quantification of their PGPBs trait by *in-vitro* and in-vivo experiments.

The N and P elements are two important elements for cultivar growth (25). Hence in this study we isolated free living nitrogen fixing bacteria which could mineralize insoluble phosphate from rhizoplane and rhizosphere environment. Surprisingly, most of the nitrogen fixing bacteria with higher growth performance were isolated from rhizoplane environment. The main reason could be plant exudates and slaughter cells in rhizoplane that attract bacteria and stimulate their metabolic activities (26). The roots of plants can release about 10-40% of their total photosynthetically fixed carbon in the form of organic and inorganic carbon. The composition and amount of the released compounds is influenced by many factors including plant type, climactic conditions, insect herbivory, nutrient deficiency or toxicity, and the chemical, physical and biological properties of the surrounding soil. These depositions make rhizosphere a desirable niche for microbial communities to proliferate.

NFB12 isolated from rhizoplane showed the highest capacity of phosphate solubilization (289.7 µg/ml). That means this bacterium makes phosphate easily available for plants. Besides, this stain can produce phytase which cause solubilization of phytate as an organic phosphate. Phosphor is a mandatory element for plant and its deficiency limits plants growth and development. Thus, selection of PGPB with the high capacity of phosphate solubilizations will practically raise availability of phosphate in rhizosphere. In our study, NFB12 with the ability of organic and in-organic phosphate solubilization can be considered as a good candidate for bio-fertilizer production.

NFB12 was distinguished as *B. megaterium* based on molecular identification. It is well documented that the ability of phosphate solubilization is widespread in *Bacillus* spp. (27-29). This microorganism is a spore forming bacteria with the advantage of higher survival rate. Usually spray dryer is used for drying this kind of bacteria instead of freeze dryer. The main advantage of spray drying over freeze drying is that it makes particle engineering more feasible and cheaper. By manipulating the conditions of the spray-drying process, manufacturers can adjust various particle properties such as size distribution, dispersibility, and besides.

IAA is an important phyto-hormone which is produced by numbers of PGPBS. This hormone causes significant increase in plant growth. Our results showed NFB12 can produce 9.704 µg/ml of IAA in experimental conditions. Saravanan et al. reported the production of IAA by 33% of PGPBs isolates after five days of incubation in the nutrient broth fortified with L-tryptophan (29). Production of IAA by *Bacillus* species was reported by other investigators as well (30-32). The capacity of IAA production is strain specific and depends on the incubation condition and nutrients (33).

Investigation on PGPBs revealed that this group of bacteria directly promotes plant growth by producing ammonia (34-37). In vitro experiments showed the all of the selected strains produce ammonia by fixing atmospheric nitrogen. In contrast production of HCN is a rare ability in PGPBs which was not observed in this study, too (38, 39).

For *in vivo* experiments we cultured strains and freeze-dried them after propagation. In case of NFB12 we enhanced sporulation by adding Mn+ in late stage of growth. Obviously, spores are significantly resistance to harsh condition compared with vegetative form. Commercial products based on spores show expanded shelf life (40). So, we decided to investigate the positive effects of NFB12 in the form of spore on tomato growth. Three different levels of selected bacteria (10^6, 10^7, and 10^8 CFUs/g of soil) were inoculated into soil. At the end of experiment positive effect of NFB12 on shoot length was significantly higher than the other bacteria. The growth enhancement was dose dependent with BNF12 inoculation. By increasing the inoculum

concentration from 10^6 to 10^7 CFUs/g of soil, we observed about 21% increase in shoot length. By inoculation of soil with 10^8 CFUs of NFB12, shoot length decreased dramatically. In this level of inoculation, shoot showed 50% decrease in length. It seems that this metabolically active bacteria with high number consume rhizospheres' nutrients and exert negative effect on tillers by this way. Our findings could help sustainable agriculture and helps to improve tomato crop yield. Furthermore, it's proved that the positive effects of PGPBs are dose dependent and must be examine case by case to find economic dose of inoculation to each cultivar.

CONCLUSION

Uncontrolled use of inorganic fertilizers causes deleterious and dangerous of environment such as air and soil pollution. So, isolation of specific PGPBS is recommended for sustainable plant production. Our results showed that NBF12 improves tomato plant growth by increasing bio-availability of nutrients and production of IAA as phytohormones. This bacterium has the ability of solubilization of organic and inorganic phosphor compound which is an essential element for plant growth and development.

Maximum growth rate of tomato was observed with 10^7 CFUs/g soil inoculation of NFB12. Higher inoculation caused deleterious effect on growth of the plant which means the effect of PGPR are does dependent. More importantly, Spores of NFB12 can tolerate harsh condition and germinate when the condition is favorable. So, this bacterium can persist in environment and outgrowth during growing season and exert its positive effects on tomato plant growth.

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