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Isolation and characterization of *Microbacterium arthrosphaerae* and its effect on tomato culture growth

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ABSTRACT

Plant growth promoting rhizobacteria (PGPR) consist a wide range of beneficial soil bacteria, which inhabited in rhizosphere of plants and enhance the growth of plant via production and secretion of various regulatory molecules in the vicinity of rhizosphere. In the present study, twenty two plant growth promoting rhizobacteria were isolated from 5 samples of rhizosphere of tomato cultures (Mehdia, Tunisia). The isolates were screened for the plant growth promoting capability after the treatment of tomato seeds with the selected rhizobacteria strains. The treatment of the plants with the selected plant growth promoting rhizobacteria MS3, showed higher plant growth in comparison with the control, accompanied by the increasing root length 191%, fresh weight 224%, leafs number 233% and shoot length 80% after 24 days of sowing. Furthermore, an excellent effect of *Microbacterium arthrosphaerae* on tomato culture growth in seed germination and seedling early establishment under aerobic cultivation system has been observed. The identification of the isolated, selected rhizobacteria strains MS3 by the using of 16S rRNA gene sequence analysis indicated their belonging to the genus *Microbacterium arthrosphaerae* and the investigation manifested an important potential of plant growth promotion with a capability to produce indole-3-acetic acid (36 µg/ml), in the presence of tryptophan in the culture medium and salicylic acid (132 µM/l), which was responsible for nitrogen fixation and inorganic phosphates solubilization. The present study revealed an excellent effect of PGPR *Microbacterium arthrosphaerae* on the improvement of tomato culture growth.

Keywords: PGPR, isolation, characterization, *Microbacterium arthrosphaerae* tomato, 16S rRNA.

INTRODUCTION

A large number of macroscopic organisms and microorganisms such as bacteria, fungi, protozoa and algae coexist in the rhizosphere, where bacteria are the most abundant among them. Recently, numerous authors have brought back the implication of benefic rhizobacteria or plant growth promoting bacteria (PGPB) in the improvement of the soils quality, plants growth and plants resistance to pathogens. Bacteria contribute most for the fitness of the selected plants by releasing of particular organic compounds through exudates creating a very selective environment where diversity is low, such bacteria named PGPR for plant growth promoting rhizobacteria [1].

The aim of researchers was the use a suitable methods for the screening of the most effective, selective bacteria that could be used as biofertilizers for development of plants growth culture.

The biofertilizer has defined as a product containing a living microorganisms, which exert direct or indirect beneficial effects on plant culture growth and crop yield through different mechanisms [2].

Plant-beneficial microbial interactions can be roughly divided into three categories. First, the microorganisms living in association with plants, such are responsible for the nutrition and the supplementation of the plants with mineral

nutrients. The second group of microorganisms stimulates the plant growth indirectly by inhibition of pathogens. Finally, the third group of microorganisms is responsible for plants growth promotion by production of such phytohormones [3]. Due the increasing use of chemical fertilizers in agriculture, which manifested numerous side-effects on plants culture, the isolation and the selection of rhizosphere bacteria was necessary, where their use as biofertilizers reduces chemical products input, pollution risks and improves agricultural production [4]. Recently, Madhaiyan and co-workers have isolated a news PGPR strains from different plants rhizospheres, characterized with their positives effects on plants growth [5-7]. The aim of the present work was the isolation rhizobacterial strains and their screening for the plant growth promoting. Furthermore, the selected rhizobacterial strain was identified by the using of 16S rRNA gene sequence analysis and the effect of PGPR on the improvement of tomato culture growth.

MATERIALS AND METHODS

Soil sampling and rhizobacteria isolation

The rhizospheric soil samples (five) were collected from fields growing tomato from the region of Mehdiya Tunisia. For this purpose, 50 g of rhizospheric soil sample with roots from each plant in sterile bags and stored at temperature 4°C.

Isolation of plant growth promoting rhizobacteria

Rhizosphere bacteria

In order to isolate rhizosphere bacteria, 1 g of rhizospheric soil sample near to roots was suspended in sterile distilled water containing 0.85 % NaCl and homogenized. Samples were serially diluted. A volume of 0.1 ml of each dilution were spread on nutrient agar and incubated at temperature of 30°C for 24 to 48 hours [8].

Root surface bacteria

The isolation of rhizobacteria stains from Roots surface has been achieved by thoroughly washing of roots with tap water for a short time of 2 min in order to remove all the loosely adhering soil particles, followed by washing with sterile saline water solution 0.85% (w/v) and soaked in phosphate buffer peptone solution composed of the following components: peptone, 1.0 g; K₂HPO₄, 1.21 g; KH₂PO₄, 0.34 g to adjust osmotic pressure. Nutrient agar plates were then inoculated with the twice washed roots, incubated at temperature of 25 to 30°C until visible growth has been observed.

Endorhizosphere bacteria

The roots were firstly disinfected by soaking them in a solution of 70% ethanol for 5 min and in 6.25% solution of sodium hypochlorite for 10 min. The roots disinfected were the rinsed in sterile distilled water and three times with 0.1 M MgSO₄, then cut lengthwise, placed on the surface of the nutrient agar, incubated at temperature of 30°C until visible growth has been observed [4].

Study of plant growth promotion

The used surface of seeds for tomato *Lycopersicon esculentum* Mill was sterilized with a solution of 2.4% sodium hypochlorite (NaOCl), for short time of 3 min, rinsed several times in sterile distilled water and dried in the presence of sterile air stream for 2 hours. The used inoculums used for seed bacterization was prepared by the inoculation of the plates nutrient agar with the bacterial suspensions of each isolated rhizobacteria strain, incubated at temperature of 32°C for 24 hours. The optical density was measured at 600 nm, adjusted at 10⁸ cells/ml. After that the sterilized surface seeds were inoculated with a volume of 30 ml of bacterial suspensions for 10 min. One sterilized surface seeds was treated with a volume of 30 ml of sterile 0.01 M MgSO₄ for 10 min and served as non-bacterized controls. Furthermore, bacterized and non-bacterized seeds were sown in pots of 10 cm containing a sterilized peat moistened treated with sterile solution of 0.01M MgSO₄ for controls and with a bacterial suspension of each isolates at 3×10⁷ cells/ml, incubated at growth chamber in the presence of 16 hours light at about 22°C. 14 days after sowing, pots were treated with a volume of 20 ml of bacterial suspension of 1×10⁸ for each isolates and controls were moistened with 0.01M MgSO₄. The plants were watered every day with sterile distilled water and once a week with nutrient solution [9]. The seedling germination was evaluated and the following parameters: total fresh weight, length of shoots and roots and leafs number, were recorded 24 days after sowing.

PCR amplification of 16S rRNA and sequencing

The gene-encoding 16S rRNA was amplified from selected strains by the polymerase chain reaction (PCR) using bacterial universal primers fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rD1 (5'-AAGGAGGTGATCCAGCC-3'). The PCR mix consisted of deoxynucleotides at 200 mM each, 0.25 mM of each primer, 2.5mM MgCl₂, 1×PCR buffer and 0.2U of Taq DNA polymerase. A suspension of cells on MilliQ water, coming from a fresh colony grown on nutrient agar, was used as target DNA. The following cycle conditions were used: 94°C for 3 min, followed by

35 cycles of 94 °C for 45 sec, 53 °C for 1 min and 72 °C for 2 min, and a final extension step at 72 °C for 5 min [10].

The PCR products were revealed and purified from agarose gels with the Favor Prep Gel/PCR purification mini kit and sequenced. The nucleotide sequence was analyzed by basic local alignment search tool (BLAST) at National Center for Biotechnology Information, USA (NCBI) database and the closest match of known phylogenetic affiliation was used to assign the isolated strains to specific taxonomic groups identified based on the similarity score.

Characterization of plant growth promoting traits of the most powerful strain

Solubilization of phosphate

The isolated, selected rhizobacteria strain MS3 was spot, inoculated on Pikovskaya agar plates containing a solution of tri-calcium phosphate as insoluble phosphate source. The plates were incubated at temperature of 30°C for 7 to 10 days. The appearance of a clear zone around the inoculated bacterial colonies indicated the solubilization of phosphate [11]. The experiment was repeated three replicates and the phosphate solubilization index was calculated by the following formula:

$$SI = \frac{\text{colony diameter} + \text{halozone diameter}}{\text{Colony diameter}}$$

Production of indole-3-acetic acid

The isolated, selected rhizobacteria strain MS3 was inoculated in LB broth supplemented with tryptophan (100µg/ml) as a precursor of IAA production, incubated at temperature 30°C for 7 days on a rotary shaker (120 rpm). The culture supernatant was harvested by centrifugation at 3000 rpm for 30 min. A volume of 2 ml of culture supernatant was mixed with 2 drops of orthophosphoric acid and a volume of 4 ml of Solawaski's reagent (50 ml, 35% perchloric acid; 1 ml 0.5 FeCl₃). The development of a pink color indicated the IAA production and the concentration was determined by the measure of the optical density was performed at 530 nm by the using a spectrophotometer. Indole-3-acetic acid was used as standard for determination of the produced quantity of IAA, expressed as µg/ml of culture filtrate [12].

Production of salicylic acid

The isolated, selected rhizobacteria strain MS3 was inoculated in 250 ml flasks containing 100 ml succinate medium (succinic acid, 4.0g; K₂HPO₄, 6.0 g; KH₂PO₄, 3.0 g; (NH₄)₂SO₄, 1.0 g; MgSO₄.7H₂O, 0.2 g; distilled water, 1000 ml; pH 7.0), incubated at temperature 30°C for 48 hours on a rotary shaker (120 rpm). Cells were harvested by centrifugation at 6000 g for 5 min. A volume of 4 ml of cells free culture was acidified with 1 N HCl to pH 2.0 and SA was extracted in CHCl₃ (2×2 ml). A volume of 4 ml of distilled water, 5 µl of 2M FeCl₃ were added to the pooled CHCl₃ phases. The developed purple iron-SA complex in the aqueous phase was determined by the measure of the absorbance at 527 nm in a spectrophotometer. The SA dissolved in succinate medium was used as standard and the quantity of SA in the culture was expressed as µM/ml [13].

Production of ammonia

The isolated, selected rhizobacteria strain MS3 was inoculated in tubes containing peptone water, incubated at temperature 30°C for 72 hours on a rotary shaker (120 rpm). A volume of 0.5 ml of Nessler's reagent was added in each tube. The development of brown to yellow color indicated ammonia production [14].

RESULTS

Isolation and screening of rhizobacterial traits

A total of 22 rhizobacterial strains were isolates from 5 tomato rhizospheres from the region of Mehdiya (Tunisia). Six rhizobacterial strains were isolated from tomato rhizospheres soils and designated as MR1 to MR6, while eight rhizobacterial strains isolated from root surface designated MS1 to MS8 and eight further rhizobacterial strains were isolated from root interior designated ME1 to ME8. The isolated rhizobacterial strains showed gummy, white-to milky white colonies with variable sizes and margins on nutrient agar plates and most of isolates showed Gram positive reaction (Table 1).

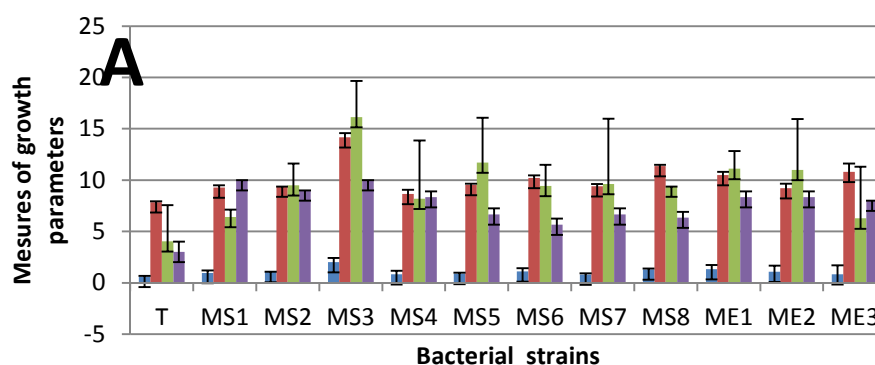
Table 1: Origine, morphological characteristics of rhizobacterial strains isolated from tomato rhizospheres of Mehdia (Tunisia)

Strains	Origines	Colonies shapes	Colonies colors	Gram reaction
MR1	RH	Small round	Milkey white	-
MR2	RH	Small round	White	-
MR3	RH	Large round	White	-
MR4	RH	Medium round	Milkey white	+
MR5	RH	Small round	Yellow	+
MS6	RH	Small round	Milkey white	-
MS1	RS	Small round	White	-
MS2	RS	Medium round	White	+
MS3	RS	Small round	Milkey white	+
MS4	RS	Small round	White	+
MS5	RS	Small round	White	-
MS6	RS	Small round	Milkey white	-
MS7	RS	Medium wavy	Yellow	+
MS8	RS	Large wavy	Milkey white	+
ME1	RE	Large round	White	-
ME2	RE	Small round	White	+
ME3	RE	Small round	Yellow	+
ME4	RE	Medium round	Yellow	+
ME5	RE	Small round	Milkey white	+
ME6	RE	Medium round	Milkey white	+
ME7	RE	Medium round	White	+
ME8	RE	Small round	Yellow	+

RH : rhizosphere, RS : Root surface, RE : root interior

Evaluation of plant growth promotion potential

The bacterization of tomato seeds by the 22 isolated strains showed an important increasing of plant growth parameters after 24 days of sowing. The increase of shoot and root length due to the treatment of tomato culture with bacterial isolates was ranged between 5-97 % and 17-191 % respectively. On the other hand, the corresponding increase of the fresh weight was ranged between 9-224 %. Similarly, bacterial isolates induced a considerable increase the number of leafs in treated plants by 77 to 233%. Where, a maximum root length was observed by the bacterial strain MS3 (191%) followed by strains ME6 and MS2 (128%) compared to control. However, the decrease of the root length was recorded by bacterial strain M1 and MR3 was (8, 15 %) respectively. A maximum growth of shoot length compared to the control was induced by the inoculation with the isolate MR6, which was increased about 97 % followed by the isolate MS3 (80 %), and the isolate MR4 (77%). The study of fresh weight indicated an increase of 224 % by the inoculation of tomato seeds with the strain MS3 and about 200%, 181% in the presence of strain ME7 and ME8 respectively. The increase of the number of leafs compared to control was observed by the inoculation with strain MS3 (233%), MS1 (233%) and MS1 (200%) respectively (Figure 1). In conclusion, the obtained results indicated that the isolated, selected bacterial strain MS3 manifested as excellent bacterial strain for improvement of tomato growth compared to the control and to other treated plants (Figure 2).



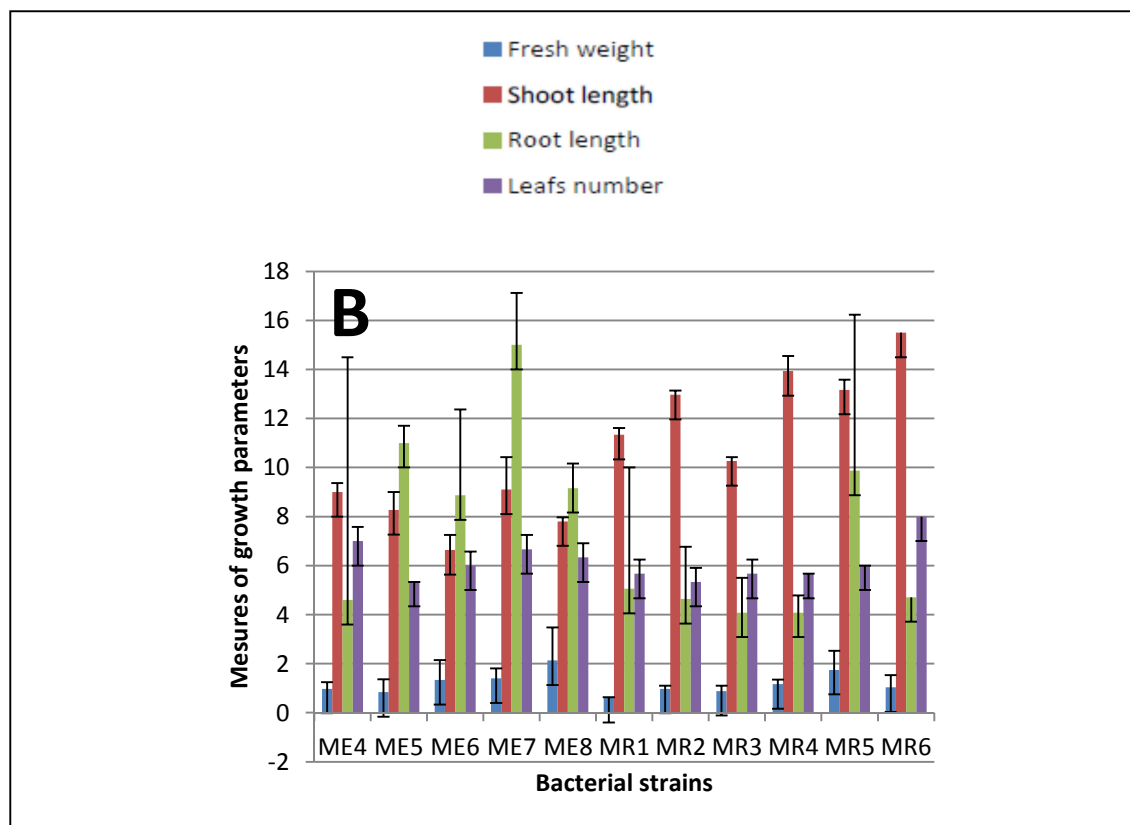


Figure 1: Effect of PGPR inoculation on fresh weight, shoot length, root length and leafs number of tomato in pot experiment after 24 days of sowing

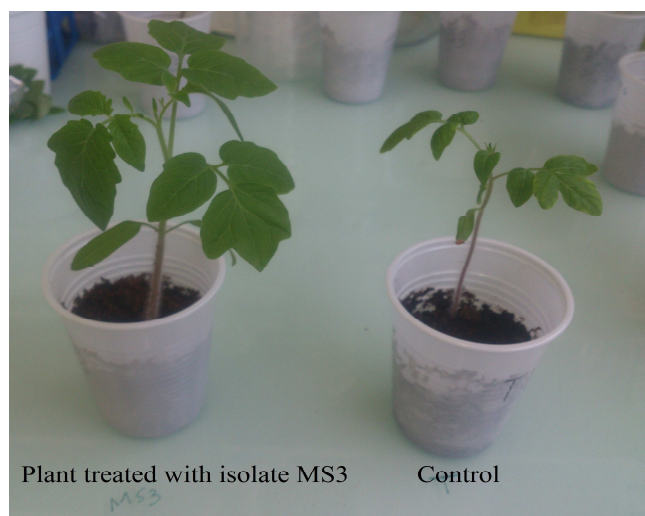


Figure 2: Tomato growth promoting activity by the isolate MS3 compared to the untreated control after 24 days of sowing

Identification of the most powerful strain

The amplified 16S rRNA nucleotide was sequenced and the obtained sequence was determined by automated sequencer. The nucleotide sequence of the most powerful strain MS3 was compared with data/bank at NCBI blast and showed highest similarity (96%) to *Microbacterium arthrosphaerae* strain. Nucleotide accession number for strain MS3 is NR 117046.1.

Production of IAA, SA, ammonia and phosphate solubilization by MS3 strain

The determination of IAA produced by the isolated, selected rhizobacteria strain MS3, inoculated in LB broth supplemented with tryptophan (100µg/ml), incubated at temperature 30°C for 7 days on a rotary shaker (120 rpm), was carried out by the using of the established calibration curve. The obtained results showed the production of a concentration of 36 µg/ml of Indole-3-acetic acid in the culture filtrate (table 2). The produced salicylic acid by the

isolated, selected rhizobacteria strain MS3 *Microbacterium arthrosphaerae* was analyzed quantitatively by spectrophotometric detection of the purple iron-SA complex, where *Microbacterium arthrosphaerae* has produced an important amount of SA (132 $\mu\text{M/L}$), in the succinate culture medium, incubated 30°C for 48 hours (table 2).

Furthermore, the isolated, selected rhizobacteria strain MS3 *Microbacterium arthrosphaerae* indicated an important potential of production of ammonia production, manifested by the change of yellow color of the peptone culture medium to brown after the adding of Nessler's reagent. The isolated, selected rhizobacteria strain MS3 *Microbacterium arthrosphaerae* showed their capability to solubilize tricalcium phosphate $\text{Ca}_3(\text{PO}_4)_2$, used as a sole source of phosphate in the Pikovskaya solid culture medium, manifested by forming of a halo zone around the inoculated colony with solubilizing index = 11.2, after 7 days incubation at temperature of 30°C (table 2).

Table 2: Biochemical and molecular analysis of the isolated, selected rhizobacterial strain MS3 from tomato rhizosphere

Strain	IAA production $\mu\text{g/ml}$	SA production $\mu\text{M/L}$	Ammonia production	Solubilization index	Accession number of identified isolate	Maximum identity	Bacterial name of blast match
MS3	36	132	++	11.2	NR 117046.1	96%	<i>Microbacterium arthrosphaerae</i>

DISCUSSION

The PGPR characterized by the colonization of plant roots and the implementation of the beneficial effects on plant growth, developed by a wide variety of mechanisms.

Twenty two rhizobacterial strains isolated from 5 tomato rhizospheres culture, located in the region of Mehidia (Tunisia) were selected for their abundance after the inoculation on the nutritive solid culture medium. Where, predominant morphological distinct colonies on the plates were observed and selected at random according to the protocol described by Nautiyal and Gupta [8]. The isolated bacteria strain presented as beneficial and powerful rhizobacterial strains was selected by the culture of tomato plants in rhizosphere through root exudates, which were used as a selective growth substrates for most beneficial microorganisms. For this purpose, different strategies were followed for the isolation beneficial rhizobacterial stains from rhizospheric soil, root surface and root interior.

Furthermore, the inoculation of the isolated, selected rhizobacteria strain MS3 has considerably improved growth of tomato, induced in early seedling, growth and development in pots, where all selected strains increased roots and shoots lengths, fresh weight, volume and number of the leaves.

The increase was explained by the efficiency of rhizobacteria strains to enhance the uptake of essential nutrients required for growth of plants and their ability of adaptation in soil or the modulation of plant hormone levels available, which indicated that PGPR strains express multiple beneficial functions [15].

The PGPR strains could exhibit several function, which may stimulate growth plant directly, indirectly or synergistically [16-17] (Joseph et al., 2007; Yasmin et al., 2007).

The obtained results in the present work suggested that the used screening approach for the isolation of rhizobacteria strains consists a good tool for the selection of effective PGPR strains. In early studies, Schwachtje co-workers, [6-7] has reported that the *Microbacterium*, a bacteria living in association with a plant, isolated from the faeces of the pill millipede *Arthrosphaera* from India, identified by Kämpfer and workers [19] as *Microbacterium arthrosphaerae*, exhibiting many growth promoting effects.

Microbacterium arthrosphaerae isolated from tomato roots, where their application on tomato growth indicated their capacity to establish an association with plants and enhance the growth and yield in short time. The early screening for the determination of the common characteristics of the selected rhizobacteria, associated to the plant growth promotion revealed that the isolated, selected rhizobacteria strain MS3 was able to produce 36 $\mu\text{g/ml}$ of IAA in the culture medium containing the precursor L-tryptophan. In early study, Khakipour and co-workers [12, 20-21] has reported that the produced amount of IAA seems relatively higher than the produced amounts of IAA by bacteria isolated from rhizosphere of different crops. The production of IAA by the isolated, selected rhizobacteria strain indicated their ability to use as growth hormones or growth regulators, where the root exudates of various plants contain an important source of tryptophan, which are used by the microorganisms for synthesis and release of auxins as secondary metabolites in the rhizosphere [22].

SA is currently known as an activator of phenylalanine ammonia lyase (PAL) activity, which synthesizes many phenolic compounds leading to the establishment of a systemic resistance in plant tissues against phytopathogenic attacks [23].

The isolated, selected *Microbacterium* from rhizosphere characterized as SA producer, involved in inducing systemic resistance, or the required amount of this molecule to trigger a systemic resistance in plant. However, the isolated, selected *Microbacterium* can use SA as a siderophore to compete with other strains for habitat and nutrition leading to the suppression of pathogens from the rhizosphere. In the present work, the most effective isolate *Microbacterium arthrosphaerae* was found efficient in the phosphate solubilization.

In early studies, Rivas and co-workers has been reported on the inorganic phosphate solubilization by *Microbacterium* strains [24-26]. Phosphate solubilizing bacteria can play an important role for plant nutrition by the increase of the phosphorus uptake by plants through different mechanisms such as acidification, chelation, and ion-exchange reactions [27].

Many plants associated with bacteria are characterized for nitrogen fixation, which can be beneficial for the improvement of nitrogen nutrition of plant, Zakhia and co-workers (2006) have reported on the presence of *nifH*-like gene in the genus *Microbacterium*, which was making them as a free living bacteria with the ability to fix atmospheric nitrogen [28]. Furthermore, the isolated, selected rhizobacteria strain has manifested their ability to produce of ammonia.

CONCLUSION

The effective isolation used method led in this study to isolate from tomato rhizosphere a total of 22 bacterial strains with plant growth promotion activity, the isolate MS3 that was identified as a *Microbacterium arthrosphaerae* seems most effective based on the response on tomato plant, the ability of these isolate to promote growth was never been reported in other studies, hence, this isolate may be used as efficient bioinoculant for tomato production which is a safe approach to replace chemical fertilizers, therefore, these isolate might have potential in future field applications as plant growth promoter.

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