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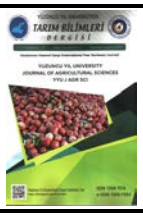

Research Article (Araştırma makalesi)

Phylogeny of Plant Growth-Promoting Actinobacteria Isolated from Legume Nodules in Turkey

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Abstract: Actinobacteria are a biotechnologically important group of microorganisms utilized for their high capacity to synthesize many bioactive substances as well as agriculturally important compounds. In the present study, a culture-dependant approach was employed to isolate actinobacteria from wild legume nodules and their plant growth-promoting activities for indole-3-acetic acid production, atmospheric nitrogen fixation and inorganic phosphate solubilisation was investigated. A molecular approach based on 16S rRNA gene sequence analysis was employed to identify the isolates. After pairwise sequence analysis, six isolates were identified as members of the genera *Streptomyces* and *Micromonospora*. All isolates could produce indole-3-acetic acid and utilize atmospheric nitrogen while only one isolate was able to solubilize inorganic phosphate. The isolated actinobacteria are considered to be promising candidates for biological fertilizers especially because of their ability to use atmospheric nitrogen and produce high level of indole-3-acetic acid.

Türkiye'de Baklagil Nodüllerinden İzole Edilen Bitki Gelişimini Destekleyici Aktinobakterilerin Filogenisi

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Anahtar kelimeler

Aktinobakteriler,
16S rRNA,
İndol asetik asit,
Lathyrus,
Vicia.

Öz: Aktinobakteriler, tarımsal önemi olan moleküllerin yanı sıra birçok biyoaktif metabolitin üretimindeki işlevlerinden dolayı yararlanılan, biyoteknolojik olarak önemli bir mikroorganizma grubudur. Bu çalışmada, yabani baklagil nodüllerinden kültüre dayalı bir yaklaşımla aktinobakteriler izole edilmiş ve indol-3-asetik asit üretimi, atmosferik azot fiksasyonu ve inorganik fosfatı çözebilme özellikleri açısından bitki gelişimini destekleyici aktiviteleri araştırılmıştır. İzolatları tanımlamak amacıyla 16S rRNA gen dizi analizine dayanan bir moleküler yaklaşım kullanılmıştır. İkili dizi analizleri sonrasında altı izolatın *Streptomyces* ve *Micromonospora* cinslerinin üyeleri olduğu tespit edilmiştir. Bütün izolatlar indol-3-asetik asit üretebilmiş ve atmosferik azotu kullanabilmiştir. Ancak sadece bir izolat inorganik fosfatı çözünür hale getirebilmiştir. İzole edilen bu aktinobakteriler, özellikle atmosferik azotu kullanabilmeleri ve indol-3-asetik asit üretebilmeleri nedeniyle umut verici biyolojik gübre adayları olarak değerlendirilmektedir.

1. Introduction

Plant growth-promoting microbes are soil and rhizosphere inhabitants affecting plant growth beneficially through different mechanisms (Velázquez et al., 2017). Among the plant associated-microorganisms, *Actinobacteria* are widely known due to their high potential to synthesize vast array of bioactive metabolites which may have positive effect on plant growth (Lehr et al., 2008). *Actinobacteria* are a group of filamentous, Gram-positive microorganisms which have high G+C content in their genomes, and they are widely distributed in both aquatic and terrestrial ecosystems. These bacteria are known to synthesize diverse compounds with distinct chemical structures and play important roles in nutrient cycling owing to their ability to degrade recalcitrant polymers such as chitin, lignocellulose and pectin (Barka et al., 2016).

Actinobacteria can also colonise rhizosphere and endophytic tissues of plants effectively, and survive under unfavourable environmental conditions (Grover et al., 2016). Thus, the potential of actinobacteria to improve plant growth has been extensively reported in recent decades. Many studies have reported plant growth promotion traits including phosphate solubilisation, nitrogen fixation, indole-3-acetic acid (IAA) and siderophore production in this promising group of bacteria (Jog et al., 2012; Cruz et al., 2014; Trujillo et al., 2015). Coombs and Franco (2003) had isolated and identified various actinobacteria from endophytic tissues of healthy wheat plants and demonstrated that *Streptomyces* sp. EN27 could colonize in sprouting wheat seeds. It was reported that *Streptomyces* sp. WYEC108 isolated from flax rhizosphere increased number and size of nodules in *Pisum sativum* by colonizing in the roots and also enhanced uptake of iron and other nutrients by the plant (Tokala et al., 2002). Goudjal et al. (2013) isolated various actinobacteria from wild plants adapted to harsh climate conditions and low nutrient levels in the Saharan Desert and reported that some of these actinobacteria could produce indole-3-acetic acid. In addition, they demonstrated that seed germination and shoot length in tomato seeds treated with fermentation broth of these bacteria increased significantly (Goudjal et al., 2013).

Legumes constitute a large group of plants and are considered an important protein source for human consumption (Fidan and Ekincialp, 2017). Bacteria dwelling in legume nodules are grouped into two categories, i.e. the rhizobia, responsible for nodule formation, and the other endophytic bacteria, e.g. actinobacteria, whose role in nodules is still poorly known (Velázquez et al., 2017). However, recent studies have shown that actinobacteria from legume nodules could promote plant growth in various ways such as by producing plant hormones or by fixing atmospheric nitrogen (Trujillo et al., 2015; Benito et al., 2017). This study was conducted to isolate and identify plant growth-promoting actinobacteria from wild legume nodules. This is the first study revealing plant growth-promoting actinobacteria which can produce indole-3-acetic acid and fix atmospheric nitrogen, isolated from wild legumes distributed in Turkey. Considering the high potential of actinobacteria for biotechnological applications, the results of present study will have application in agricultural biotechnology.

2. Materials and Methods

2.1. Isolation of actinobacteria

Legume specimens belonging to the genera *Lathyrus* and *Vicia* were collected from Samsun and kept sterile bags until isolation of microorganisms. For the isolation of actinobacteria, five nodules from each plant were randomly picked. Endophytic actinobacteria from legume nodules were isolated following the instructions described by Qin et al. (2009). Briefly, the nodules were sterilized using 5% NaOCl (4-10 min), 2.5% $Na_2S_2O_3$ (10 min), 75% ethanol (5 min) and 10% $NaHCO_3$ (10 min) successively, followed by a final wash step in sterilized distilled water and thoroughly dried under aseptic conditions. After surface sterilization process, one nodule from each plant was placed on the surface of the isolation media to validate the surface sterilization procedure. For the isolation of endophytic actinobacteria, the nodule samples were smashed into small pieces using a mortar and pestle. Then, the samples were diluted with ¼ Ringer's solution (Oxoid) and the diluted aliquots (200 µl) were transferred on the surfaces of ISP 2 agar (yeast extract 4 gL^{-1}, malt extract 10 gL^{-1}, dextrose 4 gL^{-1}, agar 20 gL^{-1}, distilled water 1000 ml, pH 7.2-7.4) and Czapek-Dox agar (sucrose 30 gL^{-1},

$NaNO_3$ 3 gL^{-1}, $MgSO_4.7H_2O$ 0.5 gL^{-1}, KCl 0.5 gL^{-1}, $FeSO_4.7H_2O$ 0.01 gL^{-1}, K_2HPO_4 1 gL^{-1}, agar 13 gL^{-1}, distilled water 1000 ml, pH 7.2-7.4) media prepared for the cultivation of actinobacteria. The inoculated plates were incubated at 28°C for 4 weeks. The actinobacteria-like colonies were purified and preserved in glycerol stock solutions (25%, v/v) at -20°C.

2.2. Identification and phylogenetic analysis of actinobacteria

According to the colour of substrate and aerial mycelia and the production of diffusible pigments, a total of six morphologically distinct isolates were selected and further analysed. The isolates were identified on genus level based on almost full length 16S rRNA gene sequence analysis. The genomic DNA of each isolate was obtained using PureLink Genomic DNA Isolation Kit (Invitrogen). The 16S rRNA genes from pure cultures were amplified using the universal primers 27F (5′–AGAGTTTGATC(AC)TGGCTCAG–3′) and 1492R (5′–ACGG(CT)TACCTTGTTACGACTT–3′) as described previously (Ay et al., 2019). Purified PCR products were sequenced by Macrogen Inc. using an ABI PRISM 3730 XL automatic sequencer and the obtained 16S rRNA gene sequences were deposited in GenBank data library (https://www.ncbi.nlm.nih.gov/genbank/). Pairwise sequence similarities for the almost complete 16S rRNA genes were conducted on EzBioCloud server (https://www.ezbiocloud.net/) (Yoon et al., 2017) and also on GGDC web server (Meier-Kolthoff et al., 2013) available at http://ggdc.dsmz.de/. Phylogenetic relationships were inferred by maximum likelihood and maximum parsimony trees as previously described (Ay et al., 2019).

2.3. Analysis of plant growth-promoting abilities

The isolates were screened for the abilities to synthesize IAA, fix atmospheric nitrogen and solubilize inorganic phosphate following the methods described by Ali et al. (2009), Li et al. (2018) and Gaur (1990), respectively, with minor modifications. For the colorimetric analysis of IAA production, the isolates were grown in tryptone-yeast extract-glucose broth for a week at 28°C and then 1 ml of culture broth was inoculated to sucrose-minimal salts (SMS) medium (10 gL^{-1} sucrose, 1 gL^{-1} $(NH_4)SO_4$, 2 gL^{-1} K_2HPO_4, 0.5 gL^{-1} $MgSO_4$, 0.1 gL^{-1} NaCl, 0.5 gL^{-1} yeast extract, 0.5 gL^{-1} $CaCO_3$, 1 mg ml^{-1} L-tryptophan, pH 7). After 7-day incubation at 28°C, 1 ml centrifuged broth obtained from bacterial culture grown in SMS medium was transferred into a tube and mixed with 2 ml of Salkowski′s reagent (2 ml of 0.5 M $FeCl_3$, and 98 mL of 35% $HClO_4$, 1:1 v/v). A pink colour produced after 30-min incubation at room temperature was observed. For the quantitative analysis of IAA production, the absorbance was measured at 530 nm (Ali et al. 2009). The IAA concentration was calculated using a calibration curve of pure IAA as a standard following the linear regression analysis. Each experiment was carried out in triplicate and the values reported are the mean of these experiments.

For the screening of nitrogen fixation ability, the isolates were inoculated onto two selective media without nitrogen sources: Ashby's mannitol agar (10.0 gL^{-1} mannitol; 5.0 gL^{-1} $CaCO_3$; 0.1 gL^{-1} $CaSO_4$; 0.2 gL^{-1} KH_2PO_4; 0.2 gL^{-1} $MgSO_4$; 0.2 gL^{-1} NaCl; 15.0 gL^{-1} agar; pH 7.0) and NFC medium (10.0 gL^{-1} mannitol; 5.0 gL^{-1} $CaCO_3$; 0.2 gL^{-1} $CaSO_4{\cdot}2H_2O$; 0.2 gL^{-1} KH_2PO_4; 0.2 gL^{-1} $MgSO_4{\cdot}7H_2O$; 0.2 gL^{-1} NaCl; 15.0 gL^{-1} agar; pH 7.2). Based on colony growth on the agar plates after incubation at 28°C for 14 days, the isolates were considered positive for nitrogen fixation activity (Li et al. 2018).

Solubilisation of inorganic phosphate was tested on solid Pikovskaya's medium (10 gL^{-1} glucose; 0.5 gL^{-1} yeast extract; 0.2 gL^{-1} KCl; 0.1 gL^{-1} $MgSO_4{\cdot}7H_2O$; 0.5 gL^{-1} $(NH_4)_2SO_4$; 0.2 gL^{-1} NaCl; 0.002 gL^{-1} $FeSO_4{\cdot}7H_2O$; 0.002 gL^{-1} $MnSO_4{\cdot}2H_2O$) supplemented with $Ca_3(PO_4)_2$ (5 gL^{-1}). After incubation at 28°C for 14 days, formation of clearing zones were evaluated as positive for phosphate solubilisation (Gaur et al., 1990).

3. Results

3.1. Isolation of actinobacteria

Root nodules obtained from two *Lathyrus* spp. and one *Vicia* sp. species were used to isolate endophytic actinobacteria. After selective isolation and colour-grouping of the isolates for

morphological growth characteristics (colour of substrate and aerial mycelium, pigment production etc.) on tryptone-yeast extract-glucose agar medium, six isolates were selected for further analyses.

3.2. Identification and phylogenetic analysis of actinobacteria

The isolates were determined by the pairwise 16S rRNA gene sequence analyses. For each isolate, a nearly full length 16S rRNA gene sequence was acquired and compared with the databases on EzBioCloud and GGDC web servers. Pairwise sequence identity levels of the strains to their closest type species are shown in Table 1. Molecular typing indicated that the isolates belong to the genera *Streptomyces* and *Micromonospora* with relatively high level of 16S rRNA gene sequence identities to the closest type strains. Five isolates, i.e. BCA3, BSP1, MCA2, MSP5A and VSP3 were identified as members of the genus *Streptomyces* (Figure 1) and an isolate, BSP4, was identified as a member of the genus *Micromonospora* (Figure 2). The isolates *Streptomyces* sp. BCA3 and *Streptomyces* sp. BSP1 are closely related to *Streptomyces albidoflavus* DSM 40455^T with 99.93% and 99.72% pairwise sequence identity values, respectively. *Streptomyces* sp. BCA3 has one nucleotide difference in 1443 positions with its most closely related type strain *Streptomyces albidoflavus* DSM 40455^T while *Streptomyces* sp. BSP1 has four different nucleotides in 1444 positions. *Streptomyces* sp. MCA2 has completely identical 16S rRNA gene sequence with its closest type strain *Streptomyces decoyicus* NRRL 2666^T. The lowest pairwise sequence identity value was observed for the isolate *Streptomyces* sp. MSP5A which shows 99.24% identity with 11 different nucleotides in 1438 positions to its closest type strain *Streptomyces umbrinus* NBRC 13091^T. The other isolate belonging to the genus *Streptomyces*, VSP3, has 99.93% pairwise sequence identity to *Streptomyces hydrogenans* NBRC 13475^T with one nucleotide difference in 1429 positions.

Table 1. Pairwise sequence identity values for the 16S rRNA genes of the isolates and their source of isolation

Source	Strain	GenBank accession number	The closest type strain	16S rRNA gene sequence identity
Lathyrus sp.	*Streptomyces* sp. BCA3	MT176504	*Streptomyces albidoflavus* DSM 40455	99.93
Lathyrus sp.	*Streptomyces* sp. BSP1	MT176505	*Streptomyces albidoflavus* DSM 40455	99.72
Lathyrus sp.	*Micromonospora* sp. BSP4	MT176511	*Micromonospora taraxaci* DSM 45885	99.86
Lathyrus sp.	*Streptomyces* sp. MCA2	MT176506	*Streptomyces decoyicus* NRRL 2666	100.0
Lathyrus sp.	*Streptomyces* sp. MSP5A	MT176508	*Streptomyces umbrinus* NBRC 13091	99.24
Vicia sp.	*Streptomyces* sp. VSP3	MT176507	*Streptomyces hydrogenans* NBRC 13475	99.93

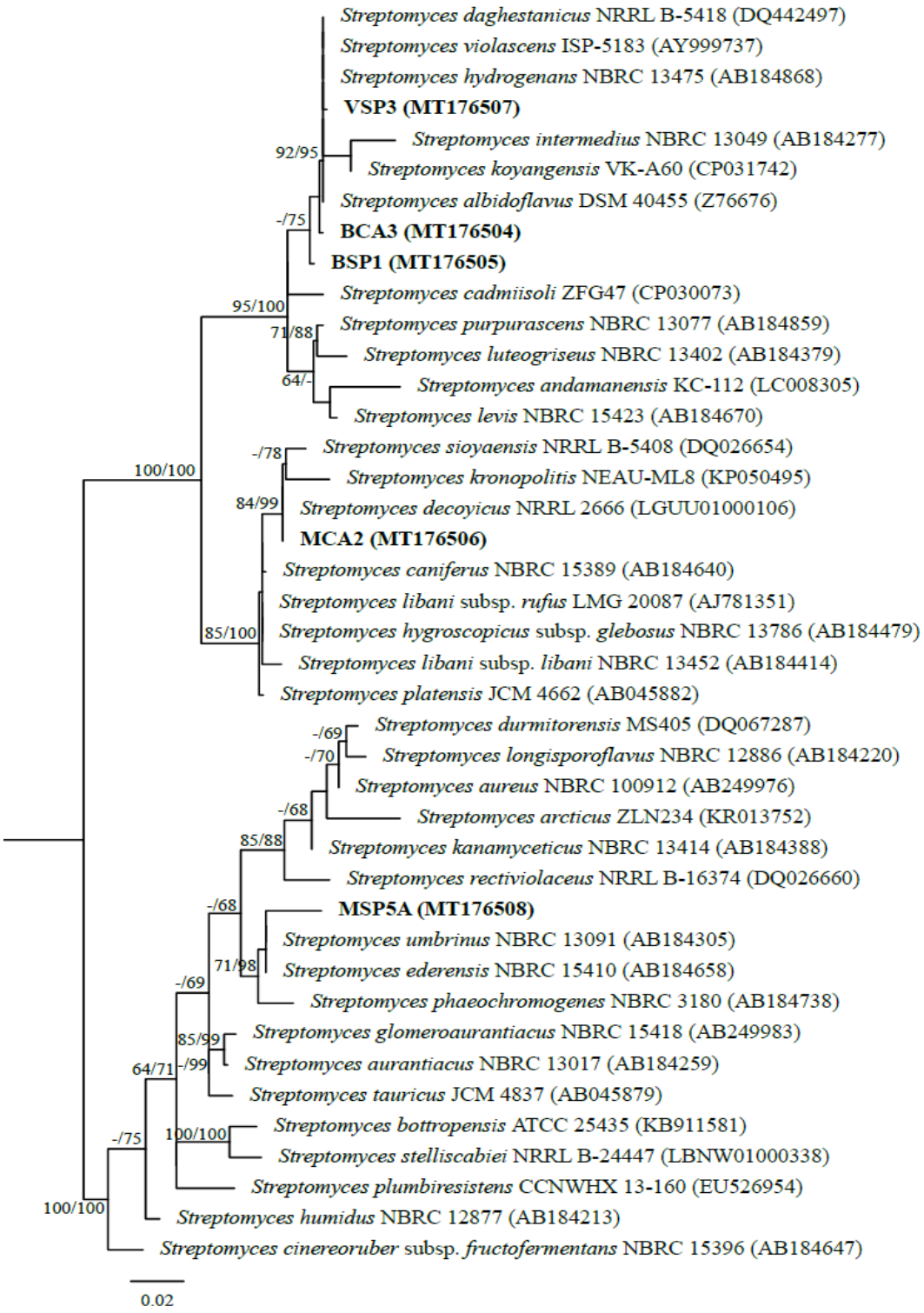

Figure 1. Maximum likelihood tree for the *Streptomyces* isolates interpreted under the model of GTR+GAMMA and rooted at midpoint. The scale bar indicates the expected number of substitutions per site. The support values for bootstrapping are shown above the branches when higher than 60% for maximum likelihood (left) and maximum parsimony (right). The GenBank accession numbers for the 16S rRNA gene sequences are given in brackets.

The only isolate belonging to the genus *Micromonospora*, BSP4, shares 99.86% gene sequence identity with its closest type species *Micromonospora taraxaci* DSM 45885^{T} and it has only two nucleotides difference in 1437 positions.

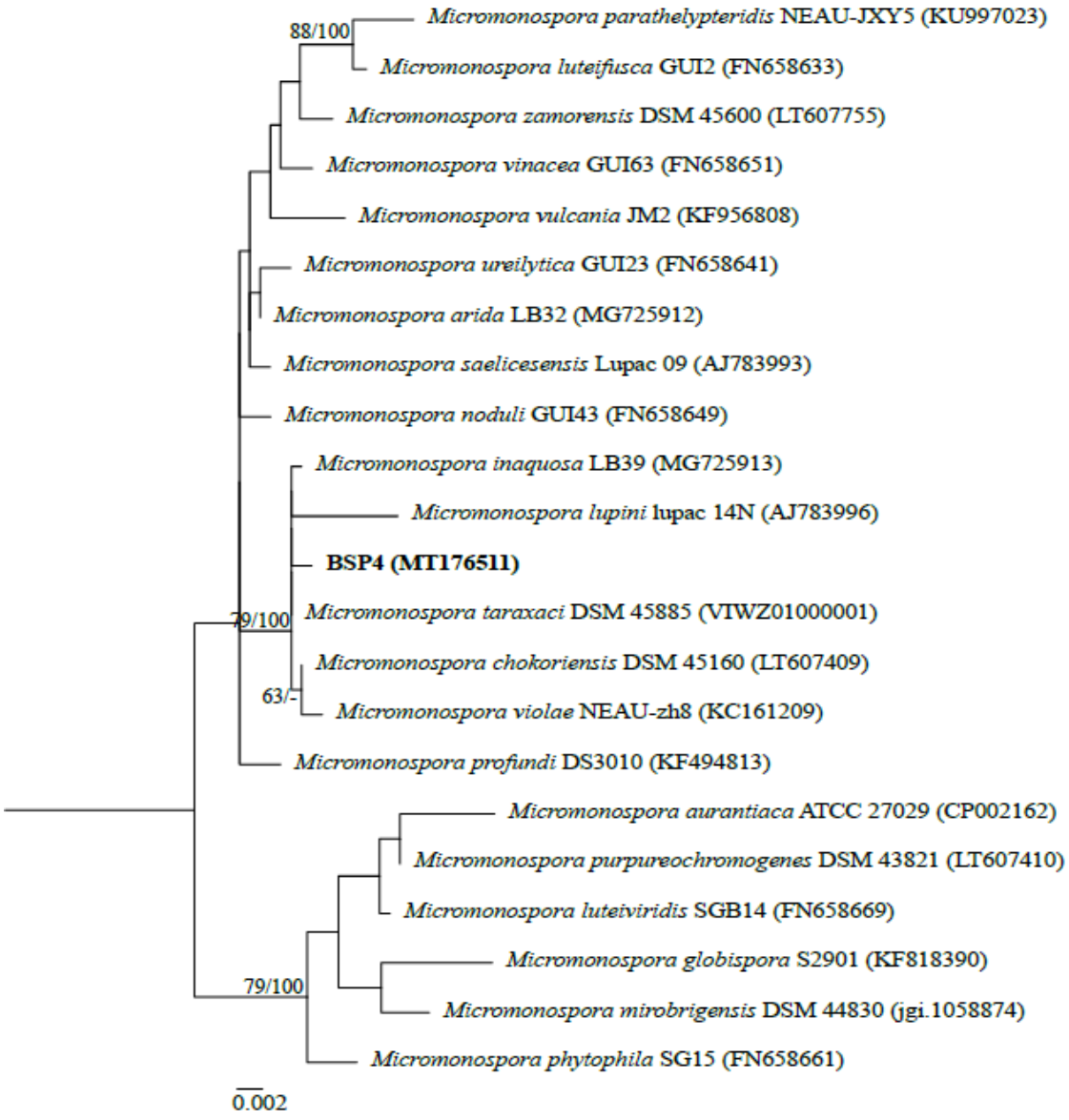

Figure 2. Maximum likelihood tree for the *Micromonospora* strain interpreted under the model of GTR+GAMMA and rooted at midpoint. The scale bar indicates the expected number of substitutions per site. The support values for bootstrapping are shown above the branches when higher than 60% for maximum likelihood (left) and maximum parsimony (right). The GenBank accession numbers for the 16S rRNA gene sequences are given in brackets.

Although the isolates have relatively high level of 16S rRNA gene sequence identity levels ranging from 99.24% to 100 % with their closest type strains, the tree topologies inferred from maximum likelihood algorithm indicated that the isolates *Streptomyces* sp. BSP1 and *Streptomyces* sp. MSP5A could be considered as potential novel species.

3.3. Analysis of plant growth-promoting abilities

Six isolates were investigated for their plant growth-promoting activities, i.e. IAA production, nitrogen fixation and inorganic phosphate solubilisation. The IAA is a plant hormone and can also be produced by diverse microorganisms including actinobacteria. All isolates obtained through this study were found to produce IAA at levels of 0.33-113.9 µg ml^{-1} (Table 2). The most productive strain was *Micromonospora* sp. BSP4 with the highest IAA concentration of 113.9 µg ml^{-1} while the lowest level was measured for the isolate *Streptomyces* sp. MCA2.

In this study, ability of the isolates to utilize the atmospheric nitrogen was qualitatively tested on two nitrogen-free media and all isolates were able to grow on both media. Phosphate solubilisation is one of the most studied mechanisms of plant growth promotion and only one strain, *Streptomyces* sp. MCA2 could solubilize inorganic phosphate in Pikovskaya's medium.

Table 2. Plant growth-promoting abilities of the isolates

Strain	IAA production (µg/ml)	Nitrogen fixation	Phosphate solubilisation
Streptomyces sp. BCA3	21.0 ± 0.8	+	-
Streptomyces sp. BSP1	21.0 ± 3.8	+	-
Micromonospora sp. BSP4	113.9 ± 4.3	+	-
Streptomyces sp. MCA2	0.33 ± 0.0	+	+
Streptomyces sp. MSP5A	1.67 ± 0.48	+	-
Streptomyces sp. VSP3	19.6 ± 2.1	+	-

4. Discussion and Conclusion

Actinobacteria are a group of biotechnologically important microorganisms which have been benefited for their role in synthesizing many bioactive metabolites as well as agriculturally important compounds. These bacteria have been shown to dwell in rhizosphere and occupy plant tissues after producing durable spores to survive in agricultural soils for long periods. Actinobacteria have been investigated for their plant growth-promoting abilities and biocontrol activities to improve agricultural production in an environmentally friendly way. In the present study, a culture-dependant approach was employed to isolate actinobacteria from legume nodules and their phylogenetic relationships were revealed by the 16S rRNA gene sequence analysis. Pairwise sequence analyses have shown that the isolates are members of the genera *Streptomyces* and *Micromonospora*, which have been known as most prolific actinobacteria for their potential to produce diverse bioactive metabolites, and that the isolates have high level of 16S rRNA gene sequence identity levels to their closest type species. Considering tree topologies inferred by maximum likelihood algorithm, however, the isolates *Streptomyces* sp. BSP1 and *Streptomyces* sp. MSP5A have high potential to be novel species. Due to weak resolution power of the 16S rRNA gene sequence analysis, especially among streptomycetes, a high number of novel species have been described for this genus even with totally identical 16S rRNA gene sequences (Biswas et al. 2017; Tang et al. 2019; Li et al. 2020).

Previous studies have revealed that members of the genus *Micromonospora* are ubiquitous in legume nodules (Trujillo et al. 2010; Martinez-Hidalgo et al. 2014). In this study, however, strain BSP4 was the only isolate belonging to *Micromonospora* and showed high level of IAA production. The isolated actinobacteria were found to be promising candidates for biological fertilizers especially because of their ability to use atmospheric nitrogen and produce high level of IAA. Atmospheric nitrogen fixation is one of the ways to mobilize nutrient for the host plants and carried out by symbiotic and free-living bacteria through nitrogenase enzymes. In terms of nitrogen fixation, all isolates have potential to be used as biological fertilizers particularly in soils with low level of nitrogen. Moreover, the strain *Streptomyces* sp. MCA2 has the ability to solubilize inorganic phosphate and also it shares relatively low level of 16S rRNA gene sequence identity with its closest

type species. Although several bacteria and fungi were reported as solubiliser of inorganic phosphate, very few reports are available for phosphate solubilisation by *Streptomyces* (Gupta et al. 2010). These results suggest that actinobacteria could play an important ecological role by enhancing the availability of nutrients to the host plant as well as by producing plant hormones to promote the host plant's growth directly. Therefore, actinobacteria obtained through this study have high potential to be used as microbial fertilizers, especially in soils with low level of nitrogen, to increase agricultural productivity without harming environment. In conclusion, further studies involving greenhouse or field experiments should be performed to confirm the suitability of these strains as microbial fertilizers.

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