CHARACTERIZATION OF *NOVOSPHINGOBIUM NITROGENIFIGENS* RMM20, A DIAZOTROPHIC ENDOPHYTE WITH MULTIPLE PLANT-GROWTH PROMOTION TRAITS

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Abstract

Utilization of plant growth-promoting diazotrophic endophytes is ecofriendly alternative technology for diminishing the use of chemical fertilizer in agriculture. In this study, the endophytic diazotrophic strain RMM20 isolated from roots of wild *Bromus aegyptiacus* plant was identified as *Novosphingobium nitrogenifigens*; based on 16S rRNA gene analysis. The nitrogen-fixing strain produced significant levels of indoleacetic acid (IAA) and ACC deaminase. Furthermore, the strain exhibited the capacity for siderophore production, *in vitro*. The results indicate that RMM20 could function as a plant growth promoter.

*Key words:* Isolation, Nitrogen-fixation, Endophyte, Diazotroph, Siderophore, IAA,

Introduction

Nitrogen is the macronutrient that commonly limits the growth and productivity of non-leguminous plants. Chemical fertilizers are commonly used to supply essential nutrients to soil-plant systems in various cultivated crops. Nevertheless, the use of high amounts of chemical fertilizers, especially nitrogen, has raised environmental concerns in the current agricultural systems (Kifle and Laing 2016). Nowadays, the replacement of chemical fertilizers with biofertilizers is an alternative fertilization strategy to improve the sustainability of agroecosystems. This environment-friendly trend includes the use of plant growth-promoting (PGP) microbes which serve as an alternative to synthetic fertilizers (Liu et al., 2017; Korir et al., 2017; Piromyou et al., 2017). Free-living diazotrophic (nitrogen-fixing) bacteria associated with non-leguminous plants have tremendous potential in increasing nitrogen availability to plants by reduction of atmospheric dinitrogen gas (N\(_2\)) to biologically available ammonium (Souza et al., 2017; Gopalakrishnan et al., 2017; García et al., 2017; Shabanamol et al., 2018; Wang et al., 2018). Thus, they have a crucial role in plant nutrition through non-symbiotic nitrogen fixation, facilitating the availability of phosphorus and iron in the rhizosphere, and production of phytohormones (Chauhan et al., 2017; Thakur et al., 2017; Sarkar et al., 2018). Ethylene (C\(_2\)H\(_4\)) is an important phytohormone which is produced in most plants and affects various developmental processes. During abiotic stress such as drought and salinity, the endogenous ethylene level increases resulting in adverse effects on root development and plant growth (Kaushal and Wani 2016). Plant-associated bacteria with 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity can metabolize ACC, a precursor to plant ethylene levels, exerting beneficial effects on the stressed plants. So, reduce ethylene level leading to better growth of plants under various stresses such as salt stress, flooding stress, and heavy metal stress (Hernández et al., 2017; Chanratana et al., 2017; Farahat et al., 2020). This study addresses the multiple plant-growth promotion attributes of the newly isolated diazotrophic endophyte, *Novosphingobium nitrogenifigens* RMM20.

Materials and Methods

Isolation of diazotrophic endophytes

Wild *Bromus aegyptiacus* (Poaceae) plants were collected from the lake Mariut at the Mediterranean coastal region, Egypt. To recover the potential
endophytes, roots of the collected plants were subjected to surface sterilization process (Gupta et al., 2019). Afterwards, the surface-sterilized root samples were homogenized in sterile saline and the homogenates were diluted up to $10^{-6}$. For isolation of endophytic diazotrophs, the diluted root homogenates were spread onto Burk’s semisolid nitrogen-free medium (de Jesus Santos et al., 2014) and incubated at 28°C for 72 h. Subsequently, the developed colonies on the N-free medium were picked and sub-cultured to obtain pure cultures. According to colony morphology, the most predominant one designated RMM20 was selected for further investigations.

**Phylogenetic analysis**

The molecular identification of the strain RMM20 was conducted by amplifying and sequencing of 16S rRNA gene. Briefly, the genomic DNA was extracted using was via using Wizard® Genomic DNA Purification Kit (Promega, USA). The 16S rRNA gene was amplified polymerase chain reaction (PCR) using 27F and 1492R universal primers. After agarose gel electrophoresis, the band of expected size was gel-purified and sequenced in both directions at Macrogen (Seoul, South Korea). The obtained sequences were assembled and compared with similar sequences in GenBank using BLASTn (http://www.ncbi.nlm.nih.gov), then, aligned by ClustalW using MEGAX software (Kumar et al., 2018) and a neighbor-joining (NJ) tree with bootstrap value 1000 was generated. The 16S rRNA gene sequence of the strain RMM20 was submitted to GenBank and accession number was assigned.

**Phenotypic characterization**

Phenotypic characterization of the strain RMM20 was conducted based on their colony morphology, microscopic observations, and biochemical tests following the standard procedures.

**Indole acetic acid production**

Indole acetic acid (IAA) production was estimated, in vitro, using Salkowski colorimetric method (Bric et al., 1991; Goswami et al., 2013). In brief, the bacterial strain RMM20 was cultivated in Burk’s broth amended with L-tryptophan (100 μg/ml) for 72 h. Then IAA was determined in the cell-free supernatant using Salkowski reagent (50 ml, 35% of perchloric acid, 1 ml 0.5 M FeCl₃ solution) at 530 nm against a standard curve constructed with IAA and was expressed as µg/ml.

**ACC deaminase production**

For measuring the activity of ACC deaminase, the strain was grown in LB broth at 28°C for 24 h. Afterwards, cells were collected by centrifugation, then washed two times by sterile Tris-HCl (0.1 M, pH 7.5), and resuspended in modified DF medium (2 ml) supplemented with ACC (3 mM), then incubated on shaking incubator for 36-72 h at 28°C. ACC deaminase activity was assayed by estimation of the released α-ketobutyrate from ACC (Honma and Smmomura 1978; Penrose and Glick 2003).

**Phosphate solubilization**

The bacterial strain RMM20 was spot inoculated on Pikovskaya’s medium containing tricalcium phosphate (Pikovskaya 1948). After 5 days at 28°C the phosphate solubilizing ability was checked by the presence of a transparent halo around the colony.

**Siderophore production**

Siderophore production was assessed using the O-CAS assay (Pérez-Miranda et al., 2007) with some modifications. The bacterial strain was spot-inoculated on Burk’s agar and incubated at 28°C for 72 h. Afterwards, an overlay of the CAS medium without nutrients was applied on top of Burk’s agar plates and checked for the formation of orange-purple halos surrounding the colonies.

**Results and Discussion**

**Isolation and identification of diazotrophic endophyte**

The nitrogen-fixing bacterial endophyte designated RMM20 that grown on Burk’s N-free medium, was were purified and subcultured on the solid nitrogen free medium. The strain RMM20 showing pale yellow convex colonies was selected and identified as *Novosphingobium nitrogenifigens*, according to 16S rRNA analysis Fig. 1. The 16S rRNA gene sequence was submitted to the Genbank under accession number MT471372. It shared 99.7% similarity with *Novosphingobium nitrogenifigens* DSM 19370 strain Y88 (accession number: NR_043857), 96.6% similarity with *Novosphingobium acidiphilum* strain FSW06-204d (accession number: NR_116278) and 94.3% homology with *Novosphingobium stygium* strain IFO 16085 (accession number: NR_040826). The strain RMM20 is Gram-negative, none spore forming none motile rods. It exhibited positive response for nitrate reduction, catalase, and urease and negative response for indole production, citrate utilization, oxidase, arginine dehydrolase, β-galactosidase, and gelatinase table 1. Isolation and screening for potential diazotrophic bacteria are key steps in development of biofertilizers formula. In agreement with our results, the endophyte *Novosphingobium oryzae* was isolated from roots of rice (Zhang et al., 2016). Also, the rhizosphere-associated
Novosphingobium pokkalii has been reported to be poses the nitrogenase gene nifH that responsible for nitrogen fixation (Krishnan et al., 2017). In addition, Novosphingobium sp. RFNB21 has been documented as a powerful nitrogen-fixing bacterium (Islam et al., 2013). In similar studies, various nitrogen-fixing endophytes have been isolated and characterized including bacteria belonging to the genera Azoarcus, Pseudomonas, Bacillus, Gluconacetobacter, and Burkholderia (Reis and Teixeira 2015; Pham et al., 2017; Shinjo et al., 2018; Zorraquino et al., 2018; Jooste et al., 2019).

Assessment for plant growth-promoting (PGP) traits

The strain strain N. nitrogenifigens RMM20 was evaluated for its various PGP traits, in vitro table 2. It showed a positive reaction for IAA production by producing pink to red color. The production of IAA was quantified (53.66 ± 3.19 µg/ml) by supplementing the growth media with L-tryptophan. It is worth to mention that the ability of N. nitrogenifigens RMM20 to produce IAA is much greater than the strain Novosphingobium sp. RFNB21 which reported to produce IAA in lower amount (1.9 µg/ml) (Islam et al., 2013). IAA production is a common feature of endophytes and its role in formation of root hair and stimulation of root cell elongation is well-reported (Verma et al., 2018; Gang et al., 2018). The endophytic diazotrophic strain N. nitrogenifigens RMM20 showed a positive reaction for ACC deaminase (490 ± 25.33 nmol α-ketobutyrate /mg protein/h). In a similar study, Novosphingobium sp. P6W was reported as ACC deaminase producer (Belimov et al., 2013).

Table 1: Phenotypic characteristics of N. nitrogenifigens RMM20.

<table>
<thead>
<tr>
<th>Test</th>
<th>Result</th>
<th>Test</th>
<th>Result</th>
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<tbody>
<tr>
<td>Gram stain</td>
<td>-</td>
<td>Oxidase</td>
<td>-</td>
</tr>
<tr>
<td>Spore formation</td>
<td>-</td>
<td>Catalase</td>
<td>+</td>
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<tr>
<td>Motility</td>
<td>-</td>
<td>Arginine dehydrodase</td>
<td>-</td>
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<tr>
<td>Indole production</td>
<td>+</td>
<td>β-Galactosidase</td>
<td>-</td>
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<tr>
<td>Nitrate reduction</td>
<td>+</td>
<td>Urease</td>
<td>+</td>
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<tr>
<td>Citrate utilization</td>
<td>-</td>
<td>Gelatinase</td>
<td>-</td>
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Table 2: Characterization N. nitrogenifigens RMM20 for plant growth promoting traits.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Result</th>
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<tbody>
<tr>
<td>IAA production (µg/ml)</td>
<td>53.66 ± 3.19</td>
</tr>
<tr>
<td>ACC deaminase (nmol/mg protein/h)</td>
<td>490 ± 25.33</td>
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<tr>
<td>Phosphate solubilization (µg/ml)</td>
<td>-</td>
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<td>Siderophore production (psu)</td>
<td>+</td>
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± represents standard deviation; + represents positive result; - represents negative result.

**Fig. 1:** Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationship between N. nitrogenifigens strain RMM20 and the most closely related species.
The possession of ACC deaminase permits bacteria to reduce ethylene levels in stressed plants by cleaving the plant ethylene precursor, ACC, into ammonia and á-ketobutyrate (Win et al., 2018; Orozco-Mosqueda et al., 2019; Yoolong et al., 2019). The endophytic diazotrophic strain N. nitrogenifigens RMM20 did not show any zone of clearance indicating an absence of phosphate solubilization ability. In agreement with our findings, various endophytic strains belonging to the genus Novosphingobium were found to be unable to solubilize phosphate (Andreoli et al., 2016). Using the O-CAS assay, the endophytic diazotrophic strain N. nitrogenifigens RMM20 formed orange-purple halos surrounding their colonies exhibiting the potential for siderophore production. These results agreed with various investigations reporting Novosphingobium spp. It has been reported that the endophyte N. resinovorum ZR1 produces significant levels of ACC deaminase (WoYniak et al., 2019). Similarly, N. oryzae (Zhang et al., 2016) and N. pokkali have been reported to be produce siderophores. Siderophores are low molecular weight iron chelators produced by various microorganisms. By chelating iron, siderophores-producing organisms make it available for their growth and improve the iron uptake by the associated plants (Priyanka et al., 2017; Sah et al., 2017).

**Conclusion**

The presented work demonstrated isolation and characterization of the endophytic diazotrophic N. nitrogenifigens RMM20. The results indicate that RMM20 could function as a plant growth promoter owing to its ability to nitrogen fixation besides production of IAA, ACC deaminase, and siderophores.

**References**


