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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).

Furthermore, diazotrophs possess an array of plant growth-promoting traits, such as nutrient solubilization, uptake and enhanced stress resistance (Batista *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_2H_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

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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 $FeCl_3$ 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).

Table 1: Phenotypic characteristics of *N. nitrogenifigens* RMM20.

Test	Result	Test	Result
Gram stain	-	Oxidase	-
Spore formation	-	Catalase	+
Motility	-	Arginine dehydrolase	-
Indole production	-	β-Galactosidase	-
Nitrate reduction	+	Urease	+
Citrate utilization	-	Gelatinase	-

+represents positive result; - represents negative result.

Table 2: Characterization *N. nitrogenifigens* RMM20 for plant growth promoting traits.

Characteristic	Result
IAA production (µg/ml)	53.66 ± 3.19
ACC deaminase (nmol/mg protein/h)	490 ± 25.33
Phosphate solubilization (µg/ml)	-
Siderophore production (psu)	+

± represents standard deviation; + represents positive result; - represents negative result.

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*

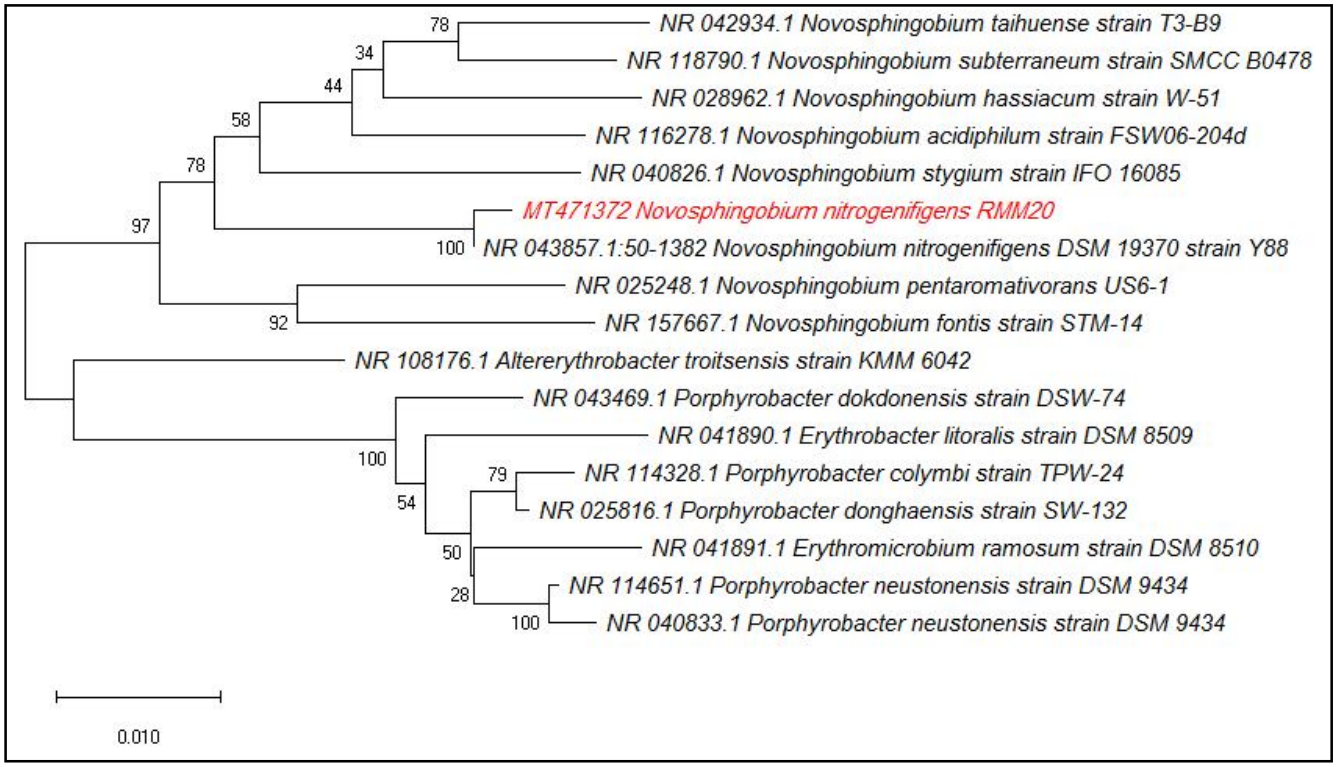

Fig. 1: Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationship between *N. nitrogenifigens* strain RMM20 and the most closely related species.

al., 2014). 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 (Andreolli *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 (WoŸniak *et al.,* 2019). Similarly, *N. oryzae* (Zhang *et al.,* 2016) and *N. pokkalii* 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 diazotophic *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.

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