HUNGARIAN JOURNAL OF
INDUSTRY AND CHEMISTRY
Vol. 50(2) pp. 11–15 (2022)
hjic.mk.uni-pannon.hu
DOI: 10.33927/hjic-2022-12

The Rhizosphere of *Petrosimonia Triandra* may Possess Growth-Inducing and Salinity-Tolerance Potential

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The root microbiome of halotolerant plants can represent a great source of salt-tolerant plant growth-promoting rhizobacteria (ST-PGPR) with a wide range of beneficial effects on plants growth, development and productivity. The better understanding of the variety of complex salt tolerance and adaptive mechanisms of halophytes can contribute to improve salt tolerance in crops, and as a result, a better and increased use of saline areas worldwide. In addition, reclaiming saline areas for agricultural use may be able to alleviate the global threat of progressive soil salinization.

Keywords: ST-PGPR, molecular analysis, saline soil

1. Introduction

Although the threat of the continuous expansion of areas of saline soil exhibiting enhanced levels of salinity has a negative effect on soil structure, fertility and plant health, by progressively constraining plants in narrower habitats, they can grow and develop optimally without experiencing the negative, stressful impacts of saline conditions. Habitat narrowing is expected to decrease biodiversity and have negative impacts on the usability of land for agricultural practices. One of the most damaging abiotic stress factors for plants is the excessive accumulation of salt in their natural habitats caused by soil salinization. Soil salinity is most often defined by its electrical conductivity (EC) with values above 4 dSm^{-1} indicative of saline soils [1]. Soil salinity can be determined by the occurrence of a variety of salts such as NaCl, Na_2SO_4, Na_2CO_3, $MgSO_4$, $MgCl_2$, KCl and $CaSO_4$. All of these salts have the potential to induce salinity stress in plants. From among the aforementioned salts, the accumulation of NaCl is the most frequent in soils. The adverse effects of NaCl stress on plants has been extensively documented [2-4]. Saline soils represent a substantial limiting factor for the development and propagation of the majority of plant species belonging to the group of non-halophytes (also referred to as glycophytes). Only halophytes, that is, plants adapted to and capable of achieving optimal development in saline environments, can persist in saline soils. To counteract salinity stress, salt-tolerant species have developed a wide range of tolerance traits such as osmotic adjustment, ion sequestration, ion exclusion, adaptations of the membrane transport system and protection enhanced by the synthesis of a variety of macromolecules with osmolytic properties, e.g. glycine betaine, proline, etc. [5].

Petrosimonia triandra is a halophyte able to complete its life cycle under moderate saline conditions (EC: 4.45 dSm^{-1}) native to the saline area of Cojocna in Cluj County, Romania (*Fig.1*) [6]. While the salt tolerance mechanisms of *P. triandra* are currently poorly understood, Podar et al. (2019) documented several adaptive mechanisms in this species that can withstand soil salinity such as morphological, physiological and biochemical adaptations, e.g. the development of efficient photosynthetic and antioxidative systems as well as the accumulation of the protective osmolyte proline. Similar salt tolerance mechanisms have also been documented in other halophyte species [7-8].

Another strategy of halophyte plant species to improve their range of salt tolerance is co-habitation with groups of rhizosphere-specific bacteria called plant growth-promoting rhizobacteria (PGPR), which live in the immediate vicinity of plants' root systems (also referred to as the rhizosphere) as well as exert a positive

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Received: 20 Sept 2022; Revised: 27 Sept 2022; Accepted: 27 Sept 2022
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effect on plant growth and development. These effects are underpinned by diverse mechanisms such as the addition of essential nutrients (N, K, Zn, Fe, PO_4); production of 1-aminocyclopropane-1-carboxylate (ACC) deaminase, volatile organic compounds and phytohormones; as well as the facilitation of favorable plant-microbe interactions, antifungal effects, etc. [9-10]. Some PGPR species have been found to be salt tolerant, namely ST-PGPR, and successfully used over recent decades to enhance crop yield and improve soil fertility. Most recently, Ayub et al. (2020) clearly emphasized the role of PGPRs in the salt tolerance of halophytes. In addition, nowadays, ST-PGPRs are also used as bioinoculants to improve crop productivity and provide protection from phytopathogens [11-12]. Therefore, ST-PGPRs are promising tools to enhance the salinity tolerance of plants.

The goal of the current study was to identify ST-PGPR species from the rhizosphere of P. triandra. A better understanding of the rhizobial community - contributing to the enhancement of salt tolerance in species adapted to and native to saline areas - is expected to lead to valuable applications, thereby enhancing the tolerance of high-value crops and other species of horticultural use as well as provide technologies to expand land-use practices for agricultural purposes to underutilized, highly saline areas [13].

2. Experimental

2.1. Soil sampling site

Soil samples were collected from the rhizosphere of the halophyte *P. triandra*, native to the saline zone of Cojocna in Cluj County, Romania (GPS coordinates N46.74328 E23.84295, *Fig.1*). The salinity of this site is characterized by EC = 4.45 dSm^{-1}, which corresponds to moderate saline soil [1]. The specific physicochemical characteristics of the soil at the sampling site have been described in our former study [6].

2.2. Isolation of bacterial species from the rhizosphere of P. triandra

P. triandra plants were carefully dug up and removed from the soil before gently shaking their roots to remove the excess soil. The soil that was directly adhered to the roots was collected and transported back to the laboratory for analysis where it was transferred into a flask containing 99 ml of sterilized distilled water. After shaking the flask for 15 minutes, the soil samples were subjected to a 10-fold serial dilution and 100 µl of the solution was spread on plates containing nutrient agar (NA) media (Merck, Germany) supplemented with 2, 4, 6, 8, 10, 11, 11.5, 12, 12.5 and 13% NaCl, respectively. The plate containing NA without external NaCl was used as the control sample. The plates were incubated at room temperature with colonies appearing 3-4 days after inoculation.

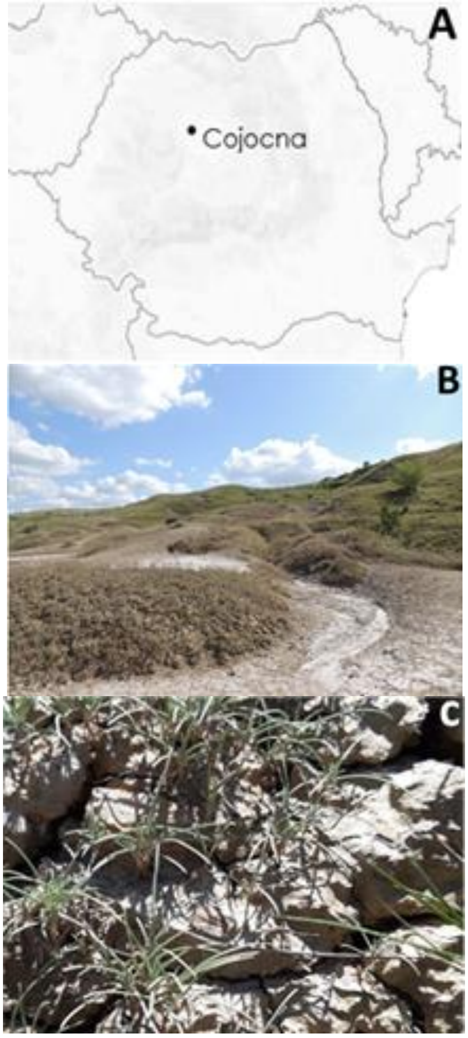

Figure 1. A: Location of the study zone in Cojocna, Romania; B: the habitat of *P. triandra* in Cojocna; C: young *P. triandra* plants

2.3. Identification of salt-tolerant rhizobacteria using a 16S rRNA gene sequence

To identify the rhizosphere of bacterial species, strains exhibiting the highest salt tolerance were selected, namely 11.5, 12 and 12.5% NaCl. Thirty colonies were selected for DNA isolation, PCR and gene sequencing of their 16S rRNA gene. Having extracted the total genomic DNA (QIAGEN, Germany), a fragment of the 16S rRNA gene was amplified by PCR using the 16S rRNA universal primers (27F: 5′-AGAGTTTGATCCTGGCTCAG-3′ and 1492R: 5′-GGTTACCTTGTTACGACTT-3′). The PCRs were mixed using a Bioline PCR kit with the polymerase MyTaq Red Mix (Bioline, Meridian Bioscience, Memphis, TN) in a final volume of 50 µl. The PCRs were performed using a MultiGene OptiMax thermal cycler (Labnet, Cary, NC) under the following cycling conditions: 5 mins. at 95°C, followed by 30 cycles lasting

Table 1. Identified rhizosphere bacteria and the salt concentration facilitating their optimum growth

No.	PGPR name	NaCl tolerance (%)
1.	*Bacillus hwajinpoensis* FJAT-46935	12.5
2.	Bacillus sp. LS-X7	12.5
3.	Bacillus sp. EBW4	12.0
4.	Bacillus sp. YTM5	11.5

30s each at 94°C, 30s each at 55°C and 1.5 mins. each at 72°C followed by a final extension step at 72°C for 7 mins. 5 µl aliquots of each reaction were analyzed on 1% (*w*/*v*) agarose gel in TBE buffer, stained with Midori Green Advance nucleic acid staining solution (NIPPON Genetics EUROPE, Düren, Germany). The resulting ~1500 bp PCR product was purified using an Agarose Gel Extraction Kit (Jena Bioscience, Jena, Germany) according to the manufacturer's protocol and sent for sequencing at the genomic sequencing service provider Macrogen (South Korea, http://dna.macrogen.com/eng). BLAST analysis and gene sequencing were also performed by Macrogen.

3. Results and Discussion

3.1. Determination of the NaCl tolerance of the rhizobacteria P. triandra

Rhizobacteria isolated in close proximity to the roots of *P. triandra* could survive in a media supplemented with up to 12.5% NaCl (*Fig.2*). 46 bacterial colonies were able to grow in the presence of salinities as high as 11.5, 12 and 12.5% NaCl. The number of bacterial colonies grown in saline media decreased as the NaCl concentration increased. A total number of 26, 12 and 8 bacterial isolates survived in salinities of 11.5, 12 and 12.5% NaCl, respectively. The 8 most salt-tolerant bacterial isolates survived in the presence of 12.5% NaCl. Based on their highest level of salt tolerance and diverse morphology, 30 isolates were selected for molecular identification.

3.2. Identification of salt-tolerant rhizobacteria

The bacterial 16S rRNA gene sequence analysis determined that of the 30 selected isolates, 26 belonged to the Bacillus genus (Bacillus sp. strains EBW4, LS-X7, YTM5) with one of them identified as the *B. hwajinpoensis* strain FJAT-46935. The other four isolates could not be identified. The names of the isolates and their highest salinity tolerance are presented in *Table 1*.

The important role of some of the identified *P. triandra* rhizobacteria in enhancing plant growth, yield and salt tolerance has been described previously [14]. A plant growth-promoting effect of the rhizosphere bacterial strain Bacillus sp. EBW4 was reported by Utkhede and Smith in 1992 [15], who highlighted an increase in the growth of apple trees and fruit yield after applying Bacillus sp. EBW4. Another PGPR identified is *B. hwajinpoensis*, which was also characterized as a halotolerant rhizobacterial species with plant growth-promoting traits by Ferreira et al. (2021) [16]. Furthermore, many other Bacillus sp. strains were also described as ST-PGPRs. Experiments with several plant species proved that a diverse range of Bacillus strains such as *B. pumilus*, *B. paramycoides and B. amyloliquefaciens* exhibit elevated salinity tolerance following the induction of a series of salt-tolerance mechanisms in plants, e.g. enhance antioxidant enzyme activity and reduce lipid peroxidation [16-19]. Ferreira et al. (2021) described the plant growth-promoting characteristics of salt-tolerant Bacillus sp., e.g. production of indole-3-acetic acid (IAA) and siderophores, enhanced 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity as well as phosphate solubilization [16]. Moreover, Nawaz et al. (2020) studied the individual or synergistic effects of ST-PGPRs on wheat growth as well as yield under saline conditions and found that these bacteria may be used as bioinoculants to enhance crop yield in saline environments [20].

Figure 2. Bacterial isolates growing on NA media supplemented with 12.5% NaCl

Our findings regarding the Bacillus sp. strains LS-X7 and YTM5 in the rhizosphere of the salt-tolerant *P. triandra* populated by numerous ST-PGPR species are novel, moreover, it is hypothesized that these strains are also potential candidates for ST-PGPR activity along with the other described species and Bacillus strains. The potential ST-PGPR function of the aforementioned strains requires further investigation, namely analysis of their ACC deaminase activity, phytohormone production and plant-microbe interactions, which are the goals of our future studies.

In summary, based on our results and prior findings, the root microbiome of the halophyte *P. triandra* is likely to be a useful source for the isolation, discovery and characterization of yet unknown ST-PGPRs providing tools with further applications with regard to inducing halotolerance in plants of high economic value. Therefore, understanding the salt tolerance mechanisms of halotolerant plants in general, e.g. that of *P. triandra* in our study, may ultimately facilitate the exploitation of saline areas for agricultural purposes, especially in the Carpathian Basin.

4. Conclusions

P. triandra, similarly to a series of other halophyte species, has evolved various morphological, physiological and biochemical salt adaptation mechanisms to counteract the harmful effects of elevated salinity [6]. The isolation of halotolerant bacteria from the rhizosphere of this species indicates that plant-microbe cohabitation may influence the adaptive strategy of the plant. Although further investigations are necessary to characterize the novel identified bacterial strains and demonstrate their PGPR ability, our results are promising. Therefore, the identified ST-PGPR species from the rhizosphere of *P. triandra* could possibly be used to improve the salinity tolerance of non-halophyte species such as many common crops threatened by the salinization of arable land worldwide.

Acknowledgements

This research was supported by a grant from the Romanian National Authority for Scientific Research and Innovation UEFISCDI under project number PN-II-RU-TE-2014-4-0831.

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