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MULTIPLE PLANT GROWTH-PROMOTING TRAITS OF SELECTIVE ENDOPHYTIC BACTERIA FROM *PLECTRANTHUS HIJAZENSIS* IN SHADA AL-ASFAL MOUNTAIN

KHOLOUD ALZAHRANI[*], SAMYAH JASTANIAH[1] AND MAGDA ALY[1,2]

Department of Biology, University College of Umluj, University of Tabuk, Umluj, Tabuk, Saudi Arabia

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Abstract

This work was aimed to isolate and screen endophytic bacteria from *Plectranthus hijazensis*, collected from Shada Alasfal Mountain, Saudi Arabia, and study their roles in the plant growth-promotion. The results showed that they could produce HCN, ammonia, IAA, and some hydrolytic enzymes. Based on these results, the selected endophytic bacteria as efficient plant growth promoters could be chosen and used as efficient bacteria for future greenhouse and field experiments.

Introduction

The incorporation of endophytic microbiota as an active and vital component in the agricultural system is recommended to increase sustainable crop production. In addition, understanding their existence in the plant organs may reveal their roles inside the endangered plant species. Endophytes can be used in agricultural fields as biofertilizers, biopesticides, bioherbicides, and bioremediation (Ashkan *et al*. 2021, Alzahrani *et al*. 2022). They can also solubilize minerals, produce extracellular enzymes and phytohormones and may enhance antimicrobial activity against the pathogens. Beneficial bacterial species with the ability to promote plant growth have been shown to resist stress tolerance (Enebe and Babalola 2018). As known for plant growth-promoting endophytes, they employ both direct and indirect mechanisms to influence the plant growth and protection. The direct mechanisms involve the production of vital factors for crop growth, and the assistive actions on nitrogen fixation, phosphate solubilization, and iron acquisition. Indirectly, they influence the plant growth by controlling and minimizing the deleterious effects of external stresses of either biotic or abiotic sources through the following modes: competition for nutrients, production of low molecular inhibitory substances such as ammonia, alcohols, aldehydes, sulfides, cell-wall degrading enzymes, and secondary metabolites with biocidal properties (Sathya *et al*. 2017).

Plectranthus hijazensis is a medicinal plant that belongs to the Lamiaceae family. It is classified as a critically endangered and uncommon species of plants (Al-Juhani and Abdel Khalik 2021). *Plectranthus* species in Saudi Arabia are utilized for both commercial and traditional medicinal purposes and show promise for further integration into the primary healthcare system. As far as known, there have been no reports yet on the actions of bacterial endophytes isolated from *P. hijazensis* plants that promote plant growth. The objective of this work was to examine the plant growth stimulating properties of bacterial endophytes obtained from *P. hijazensis*. Moreover, in order to investigate how these endophytic bacteria, promote plant growth by the

*Author for correspondence: <khalzahrani@ut.edu.sa>. [1]Department of Biological Science, Faculty of Sciences, King Abdulaziz University, Jeddah, Saudi Arabia. [2]Botany and Microbiology Department, Faculty of Science, Kafrelsheikh University, Kafr el-Sheikh, Egypt.

production of indole-3-acetic acid, ammonia, HCN and lytic enzymes and phosphate solubilization activities were studied.

Materials and Methods

Twelve plant samples of *P. hijazensis* were collected from Shada Al-Asfal Mountain, Albaha region, Saudi Arabia (19°44'07.7"N 41°22'52.5"E) (Fig. 1). The samples were rinsed by running tap water, while a sample of the plant was formally identified and deposed at the herbarium of the Biological Sciences Department, Jeddah, King Abdul-Aziz University. The five-step surface sterilization method was used for each organ (Liu *et al*. 2017). Starch nitrate agar (SNA), starch casien agar (SCA), tryptic soy agar (TSA), tap water yeast extract agar (TWYEA), inorganic salts-starch agar (ISP4), humic acid vitamins (HVA), and potato dextrose agar (PDA) were used for the isolation of endophytic bacteria. The plant organs were aseptically cut into small segments and placed on Petri dishes separately. Plates were sealed and incubated at 28°C for 7 days. The colonies were selected based on divergence in morphology, size, and color. They were then subcultured on TSA and stored at 4°C for further studies.

Fig. 1. Endemic medicinal plant *Plectranthus hijazensis*. A: Hole plant, B: Roots and C: Stems, leaves and inflorescences.

The selected bacterial isolates were screened *in vitro* for some of their plant growth promoting factors, such as phosphate solubilizing activity, by using Pikovskaya's medium and incubated at 28°C for 24 hrs (Pikovskaya 1948), while for the synthesis of indole acetic acid, a method by Aka and Babalola (2016) was tested and for ammonia production, the selected bacterial isolates were inoculated in peptone water (Singh *et al.* 2014), and TSA plates, which were containing 4.4 g/l glycine, were used to determine the abilities of the bacterial isolates to produce HCN (Bakker and Schippers 1987). All the hydrolytic enzymes i.e. amylase, protease, gelatinase, lipase, esterase, and cellulase were tested using the procedures described by Mohamed *et al.* (2022). Finally, the enzymatic index (EI) was determined for each of these enzymes. However, catalase activity was detected by directly adding 1 ml of 3% H_2O_2 to fresh cultures of the selected bacteria.

The identification of bacteria was conducted using the study of the 16S rDNA gene sequence. The genomic DNA from each isolate was extracted using a method described by Nxumalo *et al.* (2020). The PCR products were sequenced at Macrogen, Inc. in Seoul, Korea. The BLAST tool was used to scan the GenBank database (https://blast.ncbi.nlm.nih.gov/) for sequences that matched the rDNA 16S databases. The data obtained from this study were statistically analyzed using analysis of variance (ANOVA) and subsequently by Tukey's multiple range test by the means of three independent replicates ± standard deviation (SD) as specified above. Experimental data obtained from this study were statistically analyzed through Microsoft Excel 2016 software (Microsoft, Redmond, WA, USA).

Results and Discussion

A total of 1091 endophytic bacterial colonies were isolated from *P. hijazensis*. Even though SCA and TSA media were the best media to isolate isolates with 18.7 and 18.1% isolates, respectively, they were the weakest media for fungal isolates growth with 5.9 and 5.4% isolates, respectively. Moreover, at the same time that PDA medium had the least bacterial isolated number with 5.8% isolates, it had the peak fungal isolated number with 36.2% isolates. However, TWYEA medium could be considered as a good medium for bacterial isolation due to its close results to SCA and TSA media with 17.0% bacterial isolates, while SNA, HVA and ISP4 media had 14.1, 13.7 and 12.7% bacterial isolates, respectively. On the other hand, ISP4, TWYEA, SNA and HVA media were closely in the same range of the total numbers of isolated fungi with 15.8, 13.6, 12.7 and 10.4% isolates, respectively (Fig. 2).

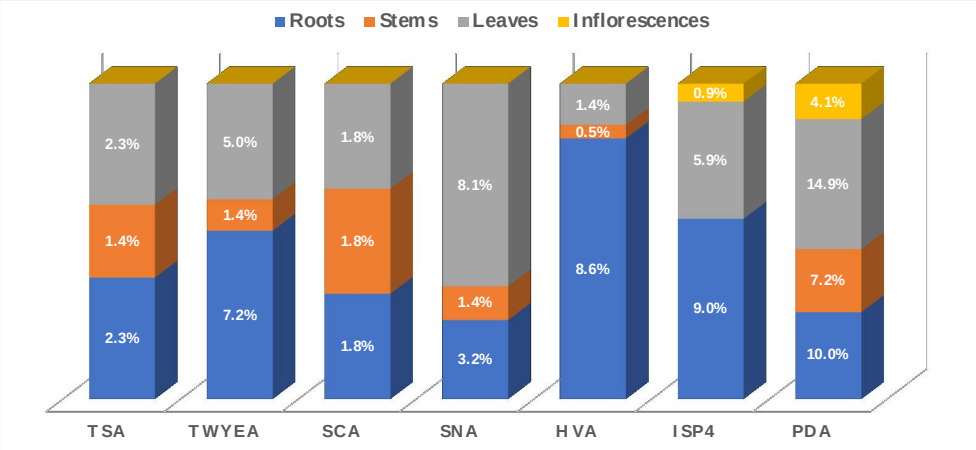

Fig. 2. Total percentage of *Plectranthus hijazensis* culture media isolated from different plant parts on-culture different media. TSA : Tryptic Soy Agar, TWYEA: Tap Water Yeast Extract Agar, SCA: Starch Casien Agar, SNA: Starch Nitrate Agar, HVA: Humic Acid Vitamins, ISP4: Inorganic Salts-Starch Agar and PDA: Potato Dextrose Agar.

Table 1 displayed the PGP features of the chosen bacterial isolates, including their skills in phosphate solubilization and generation of ammonia, HCN, and IAA. All of the endophytic bacterial isolates exhibited a notable capacity to solubilize inorganic phosphate, as evidenced by the presence of distinct zones on the Pikovskaya's medium. The phosphate solubilization index (PSI) of these isolates ranged from 2.06 to 4.50. It is as well as ammonia production, which had been produced by all the selected bacterial isolates except BPB3. Certain endophytic bacteria possess the capacity to hydrolyze inorganic phosphate, making them potentially valuable as

biofertilizers in agriculture like nitrogen fixation (Castellano-Hinojosa *et al*. 2016). The results of this study revealed that all the bacterial isolates were IAA producers, with the presence of tryptophan. These endophytic bacteria commonly may employ many strategies to enhance plant growth: by aiding in the mobilization of nutrients and regulating the levels of hormones in plants (Santoyo *et al*. 2016). The production of phytohormones in different organs of plants regulates their developmental processes. Moreover, indole-3-acetic acid, plays a major role in plant development, and its supply can support its host during stressful conditions like drought and pathogenic attacks. BPB1 and BPB7 showed the highest HCN production as reflected by the higher color intensity of the Whatman paper, and BPB5 had low production of HCN. However, the rest of the isolates had moderate HCN production.

Table 1. Phosphate solubilization, IAA, ammonia and HCN productions of the bacterial isolates.

Bacterial isolate	Phosphate solubilization	IAA production Rate	Ammonia production		HCN production	
			Color	Rate	Color	Rate
BPB1	2.14	+	Brownish yellow	+++	Reddish brown	+++
BPB2	4.50	+++	Brownish yellow	+++	Dark brown	++
BPB3	2.09	++	Yellow	-	Dark brown	++
BPB4	3.44	+	Brownish yellow	+++	Dark brown	++
BPB5	2.06	++	Brownish yellow	+++	Light brown	+
BPB6	4.11	+	Brownish yellow	+++	Dark brown	++
BPB7	2.88	+++	Brownish yellow	+++	Reddish brown	+++
BPB8	4.05	+	Light Yellow	+	Dark brown	++

+, ++ and +++ denote low, moderate and high production rates, respectively.

The results of some hydrolytic enzymes such as amylase, protease, gelatinase, lipase, esterase, cellulase, and catalase are shown in Table 2. All the selected bacteria were positive for amylase and catalase where BPB3 had the peak of amylase production with EI 2.12. The highest activities of lipase and esterase were observed with BPB1 2.90 and 3.18, respectively. All the selected isolates had the ability of gelatinase production, and the highest was 4.76 by BPB8. Maximum protease production was noted with by BPB5 and BPB2. Finally, BPB3 and BPB4 had cellulase enzyme indices of 2.32 and 2.33, respectively. The varied enzymatic activities of the isolated endophytes demonstrate their capacity to facilitate various biochemical reactions and their potential for utilization in agriculture and industry (Aliou *et al*. 2024).

In the present study, of the eight tested bacterial isolates to detect the production of lipase and esterase, half of them had produced lipase while all of them except PHB4 had produced esterase (Table 2).

The varied enzymatic activities of the isolated endophytes demonstrate their capacity to facilitate various biochemical reactions and their potential for utilization in agriculture and industry. Endophytes produce extracellular hydrolytic enzymes that indirectly enhance plant growth and provide protection against infections (Castellano-Hinojosa *et al*. 2016). These results are compatible with Aliou *et al*. (2024) and Nxumalo *et al*. (2020).

A total of eight endophytic bacterial isolates were obtained from *Plectranthus hijazensis* based on 16S rRNA gene sequences. Table 3 detected the phenotypic identification, which showed BPB1, BPB2, BPB3, BPB4, BPB5, BPB6, BPB7, and BPB8 bacterial isolates as *Bacillus siamensis* EN20, *Pseudomonas auroginosa* AAI-4, *B. subtilis* KL1, *B. subtilis* Sid 5, *B. subtilis* WKA3, *B. cereus* BA9, *B. cereus* SH42, and *B. velezensis* St. 157, respectively. The query 16S

rRNA gene sequences were aligned with the nearest sequences, exhibiting identity rates ranging from 96 to 99%, with an E-value of zero. Fig. 3 presents the phylogenetic tree analysis.

Table 2. Lytic enzymes production of bacterial isolates of *Plectranthus hijazensis.*

Bacterial Isolate	Enzyme Index (EI)						Catalase
	Amylase	Protease	Gelatinase	Lipase	Esterase	Cellulase	
BPB1	1.08	0.00	1.28	2.90	3.18	0.00	+
BPB2	1.04	2.50	2.36	0.00	1.32	1.12	+
BPB3	2.12	0.00	1.08	0.00	1.54	1.32	+
BPB4	1.41	1.10	2.18	0.00	0.00	1.33	+
BPB5	1.06	2.55	1.77	1.13	1.00	1.05	+
BPB6	1.20	1.16	0.00	1.07	1.26	1.08	+
BPB7	1.09	1.95	1.27	0.00	1.36	1.23	+
BPB8	1.13	1.55	4.76	1.29	1.06	0.00	+

+ denotes enzyme production.

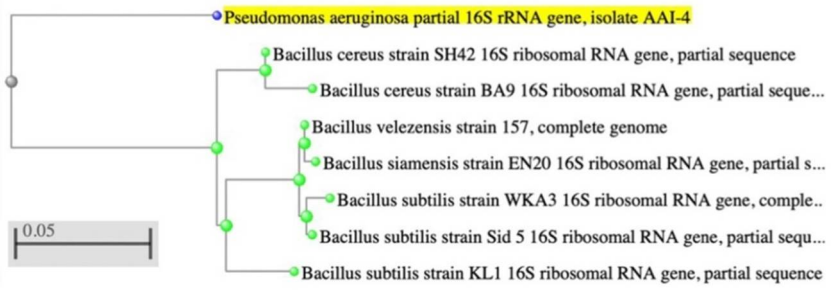

Fig. 3. Bacterial isolates' 16S rDNA sequences subjected to phylogenetic analysis alongside sequences.

Table 3. Sequence similarities of bacterial isolates with sequences registered in GenBank.

Isolate	Species	% similarity	Accession number
BPB1	*Bacillus siamensis* EN20	99	KU512901.1
BPB2	*Pseudomonas auroginosa* AAI-4	97	LN558598.1
BPB3	*Bacillus subtilis KL1*	98	KT901826.1
BPB4	*Bacillus subtilis* Sid 5	98	KU054331.1
BPB5	*Bacillus subtilis* WKA3	96	KY009936.1
BPB6	*Bacillus cereus* BA9	98	KU510057.1
BPB7	*Bacillus cereus* SH42	98	KM248376.1
BPB8	*Bacillus velezensis* St. 157	96	CP022341.1

The selected bacterial endophytic isolates of *Plectranthus hijazensis* had different abilities of PGP features such as the production of IAA, HCN, and enzymes. These isolates indicate promising factors in plant growth-promoting mechanisms in healthy plants with the benefits of stress tolerance in further studies. Finally, endophytic bacteria exhibit several methods of action and possess a broad range of diversity, making them suitable for application in the maintenance of sustainable agricultural systems.

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