Bulletin of Environment, Pharmacology and Life Sciences
Bull. Env. Pharmacol. Life Sci., Vol 9[7] June 2020 : 60-64

Online ISSN 2277-1808
Journal's URL:http://www.bepls.com
CODEN: BEPLAD
Global Impact Factor 0.876
Universal Impact Factor 0.9804
NAAS Rating 4.95

ORIGINAL ARTICLE **OPEN ACCESS**

Isolation and Characterization of Plant Growth Promoting Bacterial Endophytes and Their Effect On Okra (*Abelmoschus Esculentus* L.) Seedling Growth

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ABSTRACT
The plant tissues harbor microbes which are known to play a vital role in promoting growth of the plant. This current study deals with the efficacy of ten endophytes isolated from the surface sterilized root, stem and leaf of the crop plant okra. Among the ten bacterial endophytes, six isolates were Gram positive and four were gram negative. A microbial identification system was used to identify the isolates. Six of ten isolates matched to the genus Bacillus, two were Pseudomonas, one was Azospirillum and one was Serratia sp. The molecular characterization involving partial 16S rRNA gene sequencing of 6 isolates expressed 98 – 99% similarity with Bacillus sp, 2 isolates shared 98% similarity to Pseudomonas sp, 1 isolate showed 100% similarity with Azospirillumsp and 1 showed 97% similarity with Serratia sp. The antagonistic activity was expressed by all the ten isolates against Macrophomina phaseolina, Rhizoctoniasolani and Fusarium oxysporum. All the ten endophytic isolates showed positive test in producing indole acetic acid and siderophore production, and phosphate solubilization was found in five of ten isolates. On subjecting the isolates to plants they expressed growth promoting factors viz., increased length in root and shoot, increased numbers in secondary roots and increased wet and dry weight of root and shoot.
Keywords: Pseudomonas sp, Azospirillumsp, Bacillus sp, Serratiasp, seed bacterized

Received 12.03.2020 Revised 09.05.2020 Accepted 19.06.2020

INTRODUCTION

Plants harbor a wide range of microorganisms to promote the growth and yield by suppressing pathogens. Such endophytes (bacteria, fungi) colonize intra and intercellular root tissues. The various climatic effects including global warming, drought, deforestation, etc., are known to affect the cellular compartments, cell organs, mutualistic interactions, affecting unknown endophytes etc. The isolation of endophytes is highly challenging since the beginning of endophytic study. The high efficient genotypes present in the native bacteria promote the growth of plants and are known to express better functions than unusual strains.

The endophytes are those microbes that survive in plant tissues expecting nothing but the place for residing [1]. The endophytes are known to share the similar mechanisms of rhizobacteria in promoting plant growth and controlling plant pathogens so called biocontrol agents [2 and 3]

The present study deals with the isolation of bacterial endophytes from okra plants and characterizing their morphological, biochemical and molecular traits. It also deals with the discussion on effect of the isolated bacterial endophytes on okra seedlings.

MATERIAL AND METHODS

Isolation of bacterial endophytes

The *Abelmoschus esculentus*plants required for the study was collected from the agriculture land cultivated with okra plants near Vadaputhupatti, Theni District, Tamilnadu, India. The plant sample was aseptically transported to the Microbiology laboratory.

The initial step of endophytes isolation process was surface sterilization which paves the way to successful endophyte identification. In spite of the various procedures for surface wash mentioned in different articles, the standard one was 70% ethanol, 2% sodium hypochlorite, 0.1% mercuric chloride, sterile distilled water. The success of endophytes isolation relies on the surface sterilization. The surface washed plant parts were macerated, serially diluted and plated on suitable agar plates [4]. On incubation, a total of 10 morphologically different colonies were picked and repeated culturing was done for purification and stored in 20% glycerol at -20^{0}C. Based on the antagonistic and plant growth promoting characteristics, the potential isolates were screened.

Antagonistic activity

Four of fungal pathogens viz., *Macrophominaphaseolina*, *Rhizoctoniasolani, Fusarium oxysporum* and *Meloidogynesp*, were selected to test the antagonistic effects of isolated bacterial endophytes. From the rim of petriplates containing potato dextrose agar (PDA) medium, at a distance of 3.5cm, the bacterial endophytes were streaked. From the 7 days old PDA culture containing selected fungal plant pathogens, a 6mm mycelial disc was placed on the opposite side and incubation time was extended for 4 days at 28^{0}C. The formula used for calculating inhibition percent was I = (C-T)/C X 100, where I is the percent inhibition of mycelial growth over the control, C is the mycelial growth of pathogenic fungi in the control plate and T is fungal mycelial growth in inoculated plate. The three independent replicates were maintained for the experiment [5].

Synthesis of secondary metabolites

Indole-3-acetic acid production

The nutrient broth suspended with the amino acid tryptophan ($5\mu g/ml$) was inoculated with the isolated bacterial endophytes and incubated at 28 $\pm 2^0$C for 5 days. 2 drops of O-phosphoric acid and 4ml of Salkowski's reagent was added to 2ml of supernatant obtained from the centrifuged culture (3000rpm for 30 minutes). Indole acetic acid production was indicated by the formation of red colour and by reading optical density (OD) at 530nm using a spectrophotometer. The standard IAA graph was used to estimate the level of IAA production ($\mu g/ml$) [6].

Siderophore production

The King's B agar medium amended with an indicator dye was streaked with the 2 days old endophyte cultures. The dye served as indicator was the tertiary complex chromazurol-S (CAS)/ Fe^{3+}/ hexadecyltrimethyl ammonium bromide. The siderophore production was indicated by the colour change of the medium from blue to fluorescent yellow [7].

Phosphate solubilization

The bacterial endophytes from *A. esculentus*plant parts were subjected to inorganic phosphate solubilization [8]. The plates containing Pikovskaya's medium amended with inorganic phosphate was streaked with the fresh endophytes and observation of a clear zone around the bacterial colonies confirmed the solubilization of mineral phosphate.

2.4 Biochemical and Molecular characterization of endophyte

By using standard methodologies [9], the basic biochemical characterization of selected bacterial endophytes was carried out. Certain endophytes were identified based on carbon source utilization and others by partial 16s rRNA gene sequencing (using universal primers). The obtained sequences were submitted to Gene Bank (MK245996, MK245997, MK248180)

Potentiality of endophytes in promoting plant growth factors in okra

The wild okra seeds were chosen and surface sterilized with 70% ethanol and 2% sodium hypochlorite for 2 minutes and washed in tap water. The fresh cultures of bacterial endophytes with 10^8 CFU/ml were prepared and sterilized seeds were soaked in bacterial suspension for 1hour, air dried and ready for sowing. The three replicates were maintained for the following experiments. (1) *Bacillus* spAER3 (2) *Azospirillum* sp AER5 (3) *Bacillus* sp AER6 (4) *Pseudomonas* sp AEL7 (5) *Bacillus cereus*AER9 (6) *Pseudomonas* sp AEL10 (7) *Bacillus subtilis*AER11 (8) *Serratia* sp AER12 (9) *Bacillus*spAER14 (10) *B.megaterium*AER18.

One kg of sterile field soil was filled in the sodium hypochlorite (20%) sterilized pots and sterile wild okra seeds were sown. Okra seedlings were thinned to one plant per hole on 12 – 18 days of sowing. Regular watering to the pots maintained the moisture content. The plants of three pots for each species were uprooted with utmost care from 18 – 21 days of emergence of seedlings, and recorded each plant's morphological characteristics viz., root and shoot length, counts of secondary roots, wet and dry weight of root and stem[5].

Statistical analysis

The mean values of each morphological traits were statistically analyzed by using one-way analysis of variance and $P \leq 0.05$ was considered significant.

RESULTS AND DISCUSSION

3.1 Isolation and Characterization of bacterial endophytes

In this study, 60 bacterial endophytes were isolated from okra plant parts viz., leaf and root. The antagonistic and plant growth promoting characteristics were evaluated for these isolates. Based on the antagonistic and PGPA, 10 bacterial isolates were selected and characterized; 6 were Gram positive and four were gram negative. The phenotypic and biochemical traits of the selected isolates were analyzed using microbial identification system. The molecular characterization involving partial 16S rRNA gene sequencing of AER3, AER6, AER9, AER11, AER14 and AER18 expressed 98 – 99% similarity with Bacillus sp, the isolates AEL7 and AEL10 shared 98% similarity to *Pseudomonas*sp, the isolate AER5 showed 100% similarity with *Azospirillum*sp and AER12 showed 97% similarity with *Serratia*sp according to the public domain database sequences.

Production of metabolites and anti-phytopathogenic activity

The strict antagonistic activity in vitro was exhibited by the bacterial endophytes AER9, AER11, AER5, AER14, AEL7 and AEL10 against the selected fungal pathogens. Followed by, the three of fungal growth were inhibited by the isolates AER18, AER12, AER6 and AER3 (Table 1).

The siderophore production was observed in all 10 endophytes while AEL10, AER5 and AER9 expressed dominant siderophore production. The isolates AER6, AER3, AEL10, AER12 and AER5 showed a vigorous zone of phosphate solubilization around the endophyte colonies. The production of IAA by the isolated endophytes ranged between 14.0 to 63.9 µg/ml (Table 2)

Plant growth parameters

The bacterial endophytes isolated from okra root and leaf were evaluated for their significant role on growth parameters. The okra plant inoculated with bacterial endophytes AEL7,AEL10, AER11and AER14 exhibited dominant root and shoot length. In the same way significant increase in root and shoot length was also exhibited by the isolates AER5, AER6 and AER12. The biomass of root and shoot were also influenced by the endophyte applied plants. The seed bacterized plants showed increased number of secondary roots when compared to control (Table 3).

Theni district is situated in the Western Agro climatic zone with the temperature range between 24^0C to 38^0C. The average annual rainfall including a complete cycle of seasons is about 829mm. Such temporal climatic condition favors the evolution of minute organisms especially endophytes and hence the region is said to be rich in microbial diversity [10]. Bhendi is one of the major horticulture crops cultivated in this place. The plant associated microbes often exhibit with multiple traits for promoting plant growth [11].

The present study discovered that the bacterial endophytes isolated from okra plant possessed many plant growth promoting characteristics viz., IAA production (14.0 to 63.9µg/ml), siderophore production and phosphate solubilization. In addition, the antifungal activity was exhibited by these endophytes and confirmed the inhibition of growth against the selective fungal pathogens. The IAA is known to influence the growth and development of root through which it enhances nutrient uptake [12]. The growth and development of root and entire plant was boosted by phosphorus nutrition [13]. The bacterial endophytes from okra exhibited the significant production of siderophore assumed to be important for iron nutrition in plants and hence proved to be beneficial when used in iron deficient conditions [14].

Table 1: Biology identification and antagonistic activity of bacterial endophytes. Mean values of three replicates

Endophyte	Biolog Identification	Growth Inhibition of pathogen over control (%)			
		Macrophomina phaseolina	*Rhizoctoniasolani*	*Fusarium oxysporum*	*Meloidogyne sp.*
AER3	*Bacillus sp.*	24.9	37.7	39.0	26.2
AER5	*Azospirillumsp*	35.4	42.2	41.9	46.2
AER6	*Bacillus thuringiensis*	27.9	37.7	32.8	46.5
AEL7	*Pseudomonas sp.*	36.2	38.2	42.1	46.3
AER9	*Bacillus cereus*	41.1	41.1	44.7	31.8
AEL10	*Pseudomonas sp.*	38.3	43.2	43.2	45.5
AER11	*Bacillus subtilis*	39.0	39.1	44.7	35.7
AER12	*Serratia sp.*	27.9	35.9	42.2	17.3
AER14	*Bacillus sp.*	36.8	36.6	43.5	45.9
AER18	*Bacillus megaterium*	29.5	34.0	40.8	12.2

Table 2: Plant growth promoting activities (PGPA) of bacterial endophytes from Okra plants

Endophyte	Biolog Identification	Gram Stain	Plant Growth Promoting properties		
			IAA (μg/ml)	Siderophore production	Phosphate solubilization
AER3	*Bacillus sp.*	+	14.0	++	+
AER5	*Azospirillumsp*	-	17.2	+++	+
AER6	*Bacillus thuringiensis*	+	28.6	++	+
AEL7	*Pseudomonas sp.*	-	47.9	++	-
AER9	*Bacillus cereus*	+	24.8	+++	-
AEL10	*Pseudomonas sp.*	-	63.9	+++	+
AER11	*Bacillus subtilis*	+	60.2	++	-
AER12	*Serratia sp.*	-	16.4	++	+
AER14	*Bacillus sp.*	+	24.1	++	-
AER18	*Bacillus megaterium*	+	15.3	+	-

Table 3: Effect of bacterial endophytes on growth parameters of okra seedlings. The mean values of each morphological traits were statistically analyzed by using one-way analysis of variance and $P \leq 0.05$ was considered significant.

Isolate name	Root				Stem		
	1^0 root length (cm)	2^0 root numbers	Wet weight (g)	Dry weight (g)	Length (cm)	Wet weight (g)	Dry weight (g)
AER3	3.00 b	8.62 c	0.46 b	0.17 d	15.9 e	0.03 b	0.017 bcd
AER5	2.48 de	10.9 cd	0.46 b	0.17 c	17.9 d	0.04 b	0.034 a
AER6	2.24 ef	10.64 c	0.65 c	0.39 a	18.6 d	0.03 b	0.015 cd
AEL7	3.46 bc	18. 76 a	0.53 d	0.23 e	19.01 a	0.06 cd	0.030 b
AER9	3.00 b	9.52 e	0.45 b	0.18 cd	16.30 e	0.03 b	0.017 bcd
AEL10	3.77 b	17.93 b	0.76 a	0.25 cd	19.82 a	0.08 b	0.030 c
AER11	2.19 bc	17.24 a	0.59 a	0.26 bc	21.98 a	0.09 a	0.024 ab
AER12	2.90 b	11.62 c	0.35 c	0.12 d	20.02 c	0.05 b	0.030 bcd
AER14	2.35 de	14.63 b	0.59 a	0.34 ab	19.96 c	0.06 ab	0.016 cd
AER18	3.60 a	9.09 e	0.38 b	0.11 d	13.50 f	0.04 b	0.018 bcd
Control	2.01 f	9.87 de	0.23 d	0.08 d	11.98 f	0.03 b	0.013 d
CD (.05)	0.13	0.56	0.1	0.11	0.97	0.03	0.009
SEd	0.27	1.2	0.05	0.05	0.46	0.01	0.004

CONCLUSION

Studies on creating awareness on drawbacks of chemical fertilizer usage in agricultural crop production have been increased in the recent years [15]. All the ten isolates from okra used in this present study found to stimulate the okra growth under pot culture experiment. The variations in plant growth promotion amidst the endophytes depends on their individual capabilities. Future study will be dealt with the usage of these endophytic isolates as bio-fertilizer in agriculture.

ACKNOWLEDGEMENT

The authors are thankful to the Tamilnadu State Council for Science and Technology for encouraging the project through partial financial support.

CONFLICT OF INTEREST

The authors of this manuscript do not have any conflict of interest towards this article publication.

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CITATION OF THIS ARTICLE

Premalatha R, Vijayaraghavan R. Isolation and Characterization of Plant Growth Promoting Bacterial Endophytes and Their Effect On Okra (*Abelmoschus Esculentus* L.) Seedling Growth. Bull. Env. Pharmacol. Life Sci., Vol 9[7] June 2020 : 60-64