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Isolation and Identification of Bacterial Endophytes from Grain Amaranth (*Amaranthus caudatus*) for Plant Growth Promotion

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ABSTRACT

Background: Bacterial endophytes lives in internal tissues of root, stem and leaves of grain amaranth plants. Isolation and screening of efficient bacterial endophytes for plant growth promotion, biocontrol activities and drought tolerance can develop as suitable bioinoculants for alleviate drought and stress tolerance and plant growth promotion.

Methods: Bacterial endophytes were isolated from grain amaranth (*Amaranthus* spp) and all isolates were screened for nitrogen fixation, phosphorous solubilisation, growth hormone production and 1-aminocyclopropane-1-carboxylate (ACC) deaminase production. Molecular identification of bacterial isolates was done by 16S rRNA sequencing to identify the organisms.

Result: Seventeen isolates showed high growth in N-free media and nine isolates could efficiently solubilize phosphates. Two isolates significantly produced higher concentrations of gibberellins, maximum number of bacterial isolates produced Indole-3-acetic acid (IAA) and eight isolates were able to produce cytokinins. Fifteen isolates have shown positive for ACC activity [1-Aminocyclopropane-1-carboxylate (ACC) deaminase production] in grain amaranth and five bacterial isolates (RAR-5, RAS-3, RAL-7, WAR-1 and WAS-11) have significant plant growth promoting activities compared to others. Further, significant bacterial isolates were identified as *Alcaligenes faecalis subsp. phenolicus*, *Pseudomonas moorei*, *Bacillus siamensis, Bacillus cereus* and *Acromobacter deleyi* by 16S rRNA gene sequencing.

Key words: 16S rRNA, ACC deaminase, Bacterial endophytes, Cytokinin, GA, Grain amaranth, IAA.

INTRODUCTION

Microorganisms and plants live together and have intimate association between them to derives maximum benefits mutually with each other and microbes associated with plants plays a major role in agricultural ecosystems. Endophytic bacteria are a class of microorganisms that live in internal plant tissues like roots, stems, leaves and seeds of healthy plants (Walia *et al*., 2014; Abhyankar *et al*., 2021; Firdous *et al*., 2019; Bhutani *et al*., 2018). These endophytes help in nutrient availability and uptake, enhance stress tolerance with ACC deaminase activity and provide disease resistance (Strobel *et al*., 2003). Endophytic bacteria promote plant growth through production of phytohormones and by interaction with host plant increases the accessibility of nutrients like nitrogen and phosphorus (Liu *et al.,* 2016; Soussi *et al*., 2016).

Grain amaranth (*Amaranthus* spp.), is an emerging crop that produces seeds with high quality protein and balanced amino acid content. They offer a viable alternative to cereals and other crops in agricultural setting where soil moisture condition is limited and its increasing rooting depth makes it a potentially useful crop where soil moisture conditions vary considerably among growing seasons (Johnson and Henderson, 2002). Here, we hypothesized that endophytes isolated from plant tissues produced plant growth promoting traits. To address this, we have isolated root, shoot and leaf associated endophytic bacteria and evaluated for their plant growth promoting traits.

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MATERIALS AND METHODS

Enumeration of endophytic bacteria

The bacterial endophytes were isolated from root, stem and leaves of the healthy flowering plants of red and white grain amaranth varieties (Suvarna and KBGH-1) collected from farmers field in and around rural Bengaluru, Karnataka, India. All the experiments presented in this paper were conducted at Department of Agricultural Microbiology, University of Agricultural Sciences, Bengaluru, India during the year 2017-18. The procedure for enumeration was used as given by Gyaneshwar *et al*. (2001).

Isolation of endophytic bacteria

The bacterial endophytes from the plant samples were isolated according to the procedure by Bacon *et al*. (2002). Leaves, stem and root samples of red and white amaranth were washed with clean sterile water followed by dipping in 75% ethyl alcohol for 3 minutes and immersion of samples in 1.5% (v/v) sodium hypochlorite (NaOCl) solution for 20 minutes. The samples were then washed with sterile distilled water for 5-6 times and cut into 1.5 to 2.0 cm bits. Samples were placed on nutrient agar plates and kept for incubation at 30-32°C for 48-72 hours. The endophytic bacteria grown on either side of cut ends were further purified.

Screening of endophytic bacteria for growth promoting activities

Nitrogen fixation by bacterial endophytes

To determine the nitrogen fixing ability of isolated endophytes, they were streaked on Jensen's medium (Jensen, 1954) and Waksman No. 77 medium (Waksman, 1937). The isolates which were able to form glistening colonies on the media were designated as positive for N fixation.

Phosphate solubilisation by bacterial endophytes

To test the ability of the isolates to solubilize phosphate, each of the endophytic isolate was inoculated to 100 ml Pikovskaya broth (Pikovskaya, 1948) and incubated for two weeks at 28°C. After incubation, supernatant was collected by centrifuging at 9000 rpm for 20 min and used for estimation of inorganic phosphorus. Released phosphate was estimated by the molybdenum blue method.

Gibberellic acid production (Starch agar halo test)

The isolates were grown in 25 ml of nutrient broth for 8-12 days at 30°C and the 5 ml of nutrient broth centrifuged at 5000 rpm. Germinated paddy seeds were cut horizontally into two equal pieces, soaked in 5ml of culture filtrate and incubated for 6 to 8 hrs. The two halves of paddy seeds were placed on starch agar medium petri plates and incubated for 24 hours. Paddy seeds soaked in sterile distilled water served as control. Standard graph was plotted by soaking paddy seeds in different concentrations of gibberellic acid (Himedia) solutions. The plates were poured with iodine solution and the clear halo zone was measured in millimetres and compared with the standard graph and control.

Indole acetic acid production by cucumber root elongation bioassay (Loper and Schroth, 1986)

The bacterial isolates were inoculated to nutrient broth and incubated for ten days at 30°C. By centrifuging the cells at 5000 rpm, culture filtrate was used for the bioassay. Six ml of the culture filtrate was added to petriplates and the selected seeds were transferred and incubated for 48 hrs at 28°C. Different concentrations of Indole-3-acetic acid (Himedia) solution served as standard whereas, sterile water served as control. After incubation for 24 hours, the root length of the seedlings was measured and the results were tabulated.

Cytokinins production by cucumber cotyledon greening bioassay (Fletcher *et al*., 1982)

Cucumber cotyledons kept in 5 mL of culture filtrate and 5 mL of 40 mM KCl serves as control, placed in the dark at 28°C for 24 hrs and then exposed to fluorescent light with an intensity of 11 mmol m^{-2} s^{-1} for 3 hours at 28°C. The cotyledons were extracted with 10 mL of 95 per cent acetone - ethanol (2:1v/v) solution. Then the absorbance of the extract was measured using UV Visible spectrophotometer at 663 and 645 nm. Benzyl adenine (Himedia) is used to plot the standard curve.

1-aminocyclopropane-1-carboxylate (ACC) deaminase production

For the detection of ACC deaminase activity of bacterial isolates, the bacterial endophytes were streaked on nutrient medium containing 3mM of 1-aminocyclopropane-1-carboxylate (ACC) deaminase (Sigma Aldrich) as unique sole nitrogen source. The minimal nutrient medium without any nitrogen source was kept as control and other control also was kept where ammonium sulphate was used as a nitrogen source.

Molecular identification of endophytic bacteria

The two primers (22 bp forward primer 5′ GGAGAGTTA GATCTTGGCTCAG 3′ and 20 bp reverse primer 5′ AAGGAGGGGATCCAGCCGCA 3′) already reported for 16S rRNA. PCR was performed in 40 µl reaction mixture with 1 X buffer with MgCl2 (1.5 mM), dNTPs (200 µM), forward and reverse primers (0.5 µM each), *Taq* DNA polymerase (1U Genei Bangalore) and 2 µl template DNA (50 ng/µl). PCR conditions includes initial denaturation at 96°C for 4 minutes followed by 35 cycles of 94°C for 1 minute, 55°C for 30 seconds and 72°C for 1 minute and a final extension at 72°C for 12 minutes. The PCR products were analysed through agarose gel (0.8%) electrophoresis (Plate 1) and sent for sequencing to Chromegene Ltd. Bangalore, India. The sequencing results were analysed using online software from National Centre for Biotechnology Information (NCBI) and aligned by CLUSTALW Multiple Sequence Alignment

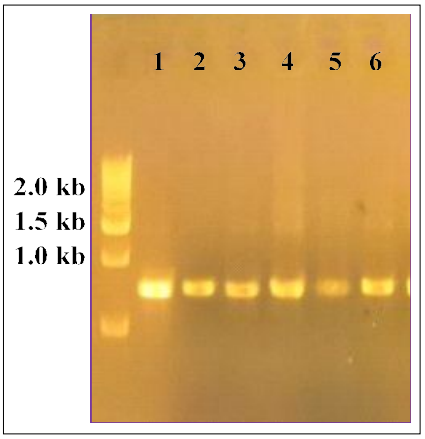

Plate 1: 0.8% agarose gel image showing 5 isolated DNA (1-5).

Programme. The phylogenetic tree was constructed with bootstrap support based on a neighbour-joining analysis given by Saitou *et al*. (1987).

Statistical analysis

All the experiments were replicated thrice for the treatments and the data generated in lab studies were subjected to one-way ANOVA. The analysis of variance and interpretation of the data were done as per procedures given by Fisher and Yates (1963), Panse and Sukhatme (1967) and Gomez and Gomez (1984). Means were separated by Duncan multiple range test (DMRT).

RESULTS AND DISCUSSION

Isolation of endophytic bacteria

Bacterial endophytes were isolated from roots, shoots and leaves of white Amaranthus (variety Suvarna) and red Amaranthus (KBGH-1). The bacterial population was significantly higher in red amaranth compared to white amaranth (Table 1). Roots of red amaranth (KBGH-1) harbored highest bacterial population (6.44×10^3 cfu) followed by shoots (4.08×10^3 cfu) and low population in leaves (2.21×10^3 cfu). The fundamental information on the microbial ecology of endophytic bacteria in certain dicotyledonous and monocotyledonous like cotton and maize indicate that these bacteria are more at flowering and maturation stages than in seedling stages (Li *et al*., 2010).

Table 1: Population of endophytic bacteria from the parts of white and red grain amaranth plants.

	Population (cfu × 10^3/g sample)		
Crop	REB	SEB	LEB
White amaranth	3.15	2.62	1.15
Red amaranth	6.44	4.08	2.21

Note: Population count after 72 hrs of incubation at 30°C. REB: Roots endophytic bacteria. SEB: Shoots endophytic bacteria. LEB: Leaves endophytic bacteria.

Plant growth promoting activities

Nitrogen fixation

All the twenty bacterial isolates were capable to grow on the N- free media. Seventeen isolates (Table 2) showed high growth in N-free media. Differences in the growth of colonies of bacterial isolates may be due to the ability of endophyte to utilise the carbon source. Fannie *et al*. (2014) demonstrated that *Burkholderia ambifaria* and *B. caribensis* promote growth and seed yields in grain amaranth by improving plant nitrogen uptake. Positive effects on growth occurred in *Amaranthus cruentus* plants grown under poor soil, with N and P fertilization.

Phosphate solubilisation

Highest P-solubilization potential was observed by the isolate, RAL-7 (19.67 ppm) followed by WAS 11 (18.73 ppm) and WAR-1 (18.60 ppm) and lowest was observed with the isolate WAR-2 (10.80 ppm) (Table 2). These results were

Table 2: Plant growth promoting activities of bacterial endophytes from grain amaranth.

Isolates	N-fixing ability	P-solubilization ability		IAA production ability (µg/ml)	GA production ability (µg/ml)	Cytokinin ability (µg/ml)	ACC deaminase activity
		PS-index	P-ppm				
RAR-5	+++	1.60^{d}	17.93^{c}	50.73^{m}	3.33^{g}	1.73^{l}	+
RAR-6	+++	1.10^{g}	12.07^{jk}	79.80^{g}	2.66^{i}	2.71^{h}	-
RAR-7	+++	0.50^{m}	18.07^{c}	110.66^{a}	2.39^{k}	2.86^{g}	-
RAS-2	+++	1.40^{e}	15.73^{e}	87.50^{e}	1.89^{n}	3.16^{f}	+
RAS-3	+++	2.33^{b}	17.07^{d}	113.23^{a}	2.00^{m}	3.18^{f}	+
RAS-4	+++	0.33^{op}	14.33^{f}	97.79^{d}	1.33^{q}	2.29^{k}	+
RAS-7	+++	0.75^{j}	15.87^{e}	78.20^{g}	2.16^{l}	3.51^{e}	-
RAL-6	+++	0.60^{l}	12.93^{gh}	71.60^{ij}	2.17^{l}	3.63^{d}	+
RAL-7	+++	2.50^{a}	19.67^{a}	69.85^{j}	3.19^{h}	3.60^{de}	+
WAR-1	+++	1.10^{g}	18.60^{b}	76.47^{h}	3.50^{f}	2.59^{i}	+
WAR-2	+++	0.20^{q}	10.80^{l}	64.33^{k}	1.67^{op}	4.57^{a}	+
WAR-4	+++	0.50^{m}	14.67^{f}	69.85^{j}	2.33^{k}	4.08^{c}	+
WAS-5	+++	0.80^{i}	13.33^{g}	100.73^{c}	6.00^{a}	2.44^{j}	-
WAS-6	+++	0.66^{k}	12.73^{hi}	61.02^{l}	4.17^{c}	2.69^{h}	+
WAS-11	+++	1.66^{c}	18.73^{b}	107.35^{b}	5.83^{b}	3.20^{f}	+
WAL-1	+	0.20^{q}	12.40^{ij}	60.29^{l}	4.00^{d}	2.31^{k}	+
WAL-2	+	0.50^{m}	13.00^{gh}	64.30^{k}	2.50^{j}	4.33^{b}	+
WAL-3	++	1.25^{f}	15.60^{e}	88.23^{e}	2.17^{l}	2.51^{ij}	+
WAL-4	+++	0.40^{n}	11.67^{k}	72.90^{i}	3.50^{f}	3.63^{d}	+
WAL-6	+++	1.00^{h}	12.20^{j}	83.42^{f}	3.67^{e}	3.13f	-

Note: + = Positive, - = Negative.

Means with different superscript, in a column differ significantly at P=<0.05 as per Duncan multiple range test (DMRT).

found in accordance with work of Amanda *et al*. (2017) who reported that isolate E8, a member of genus *Bacillus*, exhibited P-solubilisation capacity in solid medium with TCP and soy lecithin as P-source, acid phosphatase activity isolated from banana trees.

IAA production

As per the results of the bioassay (Table 2), all the twenty isolates produced IAA. Highest concentration of IAA was observed in the isolate RAS-3 (113.23 µg/ml) followed by RAR-7 (110.66 µg/ml), WAS-11 (107.35 µg/ml) and WAS-5 (100.73 µg/ml). The least concentration of IAA was recorded in RAR-5 (50.73 µg/ml). Recently, some workers (Raveendra *et al*., 2018) reported that bacterial endophytes from minor millets produced highest concentrations of Indole 3-Acetic acid (254.0 µg/ml) and influenced positive growth under pot culture experiments.

GA production

In our experiment, (Table 2), only two isolates, WAS-5 (6.00 µg/ml) and WAS-11 (5.83 µg/ml) produced significant amounts of gibberellins compared to other isolates. Earlier workers reported that the *Bacillus amyloliquefaciens* isolated from rice (*Oryza sativa* L.) produced gibberellins in the range of 0.7 to 17.8 µg mL^{-1} (Shahzad *et al*., 2016; Raveendra *et al*., 2018).

Cytokinin production

The isolate WAR-2 has showed highest cytokinin production (4.57 µg) followed by the isolates WAL-2 and WAR-4 which has produced 4.33 µg and 4.08 µg respectively (Table 2). Cytokinin production by the isolates RAL-6 was on par with the isolate RAL-7, which has produced 3.63 µg and 3.60 µg respectively and the isolate WAR-1 was on par with the isolate WAL-3, which has produced cytokinin of 2.59 µg and 2.51 µg respectively. Least production of cytokinin was noticed in the isolate RAR-5 (1.73 µg). Above results are on par with results of Mohapatra *et al*. (2011), who demonstrated that cytokinin improves grain filling. *Halomonas desiderata*, *Bacillus megaterium*, *Bacillus cereus*, *Bacillus subtilis* and *Pseudomonas fluorescens* G20-18 were reported for synthesis of cytokinins.

1-aminocyclopropane-1-carboxylate (ACC) deaminase production

In the present study, ACC was the sole N-source and out of the twenty bacterial isolates, (Table 2) except five isolates, all the remaining isolates were showing ACC deaminase activity. *Achromobacter xylosoxidans* AUM54 isolated from *Catharanthus roseus* showed ability to produce ACC deaminase and able to reduce ethylene levels in saline soil (Karthikeyan *et al*., 2012).

Molecular characterization of efficient bacterial endophytes

From the molecular characterization of efficient bacterial endophytes, the endophytes belong to the genera *Alcaligenes*, *Pseudomonas*, *Bacillus* and *Achromobacter* were associated with grain amaranth. The nucleotide similarity of 5 endosymbionts with already existing sequences of NCBI (Table 3) and phylogenetic tree (Plate 2) were presented.

Table 3: List of bacterial endophytes and their nearest match identity by 16S r RNA sequencing.

Isolate	Species observed	Percent similarity	NCBI accession no.
RAR5	*Alcaligenes faecalis* subsp. *phenolicus*	91	MG828834.1
RAS3	*Pseudomonas moorei*	95	KF742672.1
WAR1	*Bacillus cereus strain* SSA-02	100	MF000387.1
RAL7	*Bacillus siamensis*	97	MH178362.1
WAS11	*Achromobacter deleyi*	99	NR152014.1

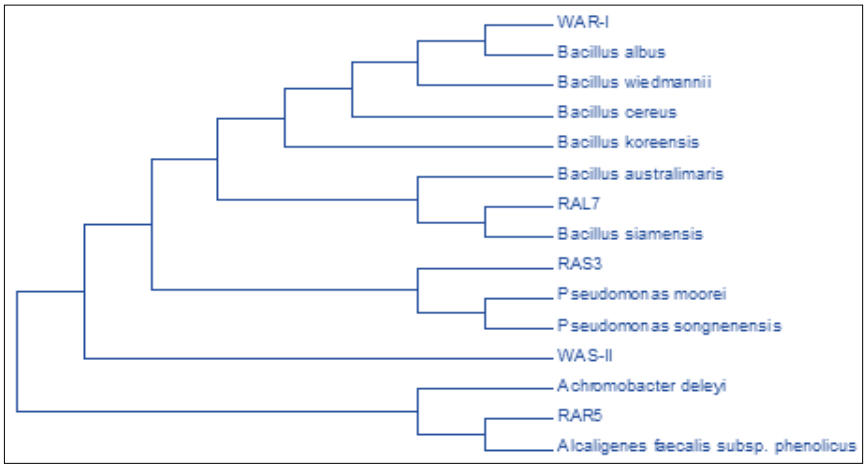

Plate 2: Phylogenetic tree showing nucleotide sequences obtained from this study are presented in numbers. Scale bars represent an estimated of 5% sequence divergence.

CONCLUSION

With the present study, we conclude that grain amaranth harbors many endophytic bacteria, which promote plant growth through various mechanisms. Five bacterial isolates namely RAR-5, RAS-3, RAL-7, WAR-1 and WAS-11 were selected as efficient in plant growth promotion and were subjected to 16S rRNA gene sequencing and were identified as *Alcaligenes faecalis* subsp. *phenolicus*, *Pseudomonas moorei, Bacillus siamensis, Bacillus cereus* and *Acromobacter deleyi*. Their efficiency can be exploited in the form of microbial inoculants for an eco-friendly and cost-effective cultivation of pseudocereals.

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Conflict of interest: None.

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