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Phytohormone Production and Drought Tolerance Activity of Bacterial Endophytes Isolated from Small Millets

M. Raveendra Reddy*, M.K. Shivaprakash, N. Earanna and B. Narayanaswamy

Department of Agricultural Microbiology, University of Agricultural Sciences, GKVK, Bangalore, Karnataka, India

**Corresponding author*

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A B S T R A C T

An investigation was carried out to isolate bacterial endophytes from small millets that have the ability to produce phytohormones like indole-3-acetic acid (IAA), gibberellic acid (GA), cytokinin and1-aminocyclopropane-1-carboxylate (ACC) deaminase. Thirty two bacterial endophytes are isolated from small millets and among thirty two isolates, twenty isolates produced indole-3-acetic acid (IAA) in the range of 96.70 µg to 254.95 µg and the isolate KMS1 produced significantly highest concentration of IAA (254.95 µg) followed by FMR3 (231.87µg). Out of thirty two isolates, only sixteen isolates produced gibberellic acid (GA) and the highest GA was recorded in isolate KMS5 (11.04µg). Twenty one isolates produced cytokinins and the maximum cytokinin production was observed in isolate KMS5 (8.66 µg). The bacterial endophytes were tested for 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity and eleven isolates *viz.*, BMR5, BML1, FMR7, FMR9, FMS2, FML12, FTMS5, KMR4, KMS5, LMR1, LMR4, LMS1 and PML3 shown positive for production of ACC deaminase enzyme in the plate assay. This work revealed that some endophytic bacteria from small millets produced plant growth promoting hormones and enhanced drought tolerance by ACC production that would help in plant growth and further these endophytic bacteria can be used as bioinoculants.

Introduction

Plant microbe interactions in the rhizosphere are the determinants of plant health, productivity and soil fertility. Plants are associated with vast and diverse group of microorganisms especially bacteria known as the associative bacteria that include rhizospheric bacteria (in vicinity of root), rhizoplanic (on surface of root) bacteria and, endophytic bacteria (inside the plant) (Ryan *et al.*, 2008). The term 'endophytic bacteria' are referred to those, which colonizes the interior parts of the plant, *viz.*, root, stem, leaves or seeds without causing any harmful effect on host plant (Hallmann *et al.*, 1997). Plant growth-promoting bacterial endophytes have the ability to colonize plant's interior parts like roots, shoots, leaves and seeds and to establish a special type of relationship where both partners may derive benefits from this interaction (Hallmann *et al.*, 1997; Reiter and Sessitsch, 2006). Bacterial endophytes have been reported to promote plant growth by a number of different mechanisms. These

mechanisms include phosphate solubilization activity (Verma *et al.,* 2001; Wakelin *et al.,* 2004), production of phytohormones like indole-3-acetic acid (IAA), gibberellic acid (G.A) and cytokinins (Lee *et al.,* 2004), nitrogen fixation (Compant *et al.,* 2005; Watanabe *et al.,* 1979), siderophore biosynthesis (Lodewyckx *et al.,* 2002; Wang *et al.,* 1993), and supplying essential nutrients to the host plant (Costa and Loper, 1994; Puente *et al.,* 2009). Bacterial endophytes may also promote plant growth as a consequence of the bacterium expressing the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase which cleaves ACC to α-ketobutyrate and ammonia and thereby decreases ethylene levels in host plants which in turn plant tolerates low moisture levels or drought conditions (Sessitsch *et al.,* 2005; Sun *et al.,* 2008).

Neglected and under-utilized crop genetic resources are very vital for sustainable agriculture (Eyzaguirre, *et al.,* 1999) and minor millets belonging to this important group of crops (Dutta *et al.,* 2007). Small millets have always been of local and regional importance and as a result have attracted little attention both at national and international level. These crops have traditionally been the indispensable component of dry land farming system in India and elsewhere. They are considered as nutri cereals and are a source of food, feed and fodder (Jarvis *et al.,* 2007). The group of small millets is represented by six species, namely finger millet [*Eleusine coracana* (L.)], little millet [*Panicum sumatrance*], kodo millet [*Paspalum scrobiculatum* (L.)], foxtail millet [*Setaria italic* (L.)], barnyard millet [*Echinochlo afrumentacea* (L.)] and proso millet [*Panicum miliaceum* (L.)]. These crops have traditionally been the indispensable component of dry land farming system in India and elsewhere.

In the current study, endophytic bacteria were isolated from small millets and these isolates were studied for their ability to produce phytohormones like IAA, GA, cytokinin and 1-aminocylopropane-1-carboxylate (ACC) deaminase activity.

Materials and Methods

Isolation of bacterial endophytes

Small millet samples were collected during Kharif and Rabi season 2016-17 from the Zonal Agricultural Research Station, University of Agricultural Sciences, GKVK campus, Bangalore. Ten plants of each millet were collected in the heading stage. From each plant, 10 leaf segments, 10 shoot segments, and 10 root segments were analyzed. Plant tissue samples were surface sterilized with 70% ethyl alcohol for 2 min and shaken in 1.2% (v/v) sodium hypochlorite (NaOCl) solution for 20 minutes. Samples were then washed several times with sterile distilled water and kept on mechanical shaker for 15 minutes. The samples were washed with sterile distilled water for 5-6 times. Surface sterilized plant samples were made into 1 or 2 cm bits by cutting on either sides of root, shoot and leaf bits to remove the traces of sodium hypohlorite solution on the edges of plant parts and to maintain uniformity in sizes of plant parts. The plant parts were placed on fresh nutrient agar medium and incubated at 30°C. After incubation at 30 °C for 2 days, the inoculants were purified on fresh nutrient agar medium. The transfer procedure mentioned above was carried out 3–4 times to isolate single colonies (Lee *et al.,* 2005). The isolated endophytic bacteria were stored at -70°C in nutrient broth containing 15% (v/v) glycerol.

Naming of the isolate

Total of thirty two isolates were isolated from the roots, shoots and leaves of the six small millets. They were named according to the

crop name followed by the parts of the small millet like root, shoot and leave and the number of isolates of that millet.

Production of plant growth regulators by bacterial endophytes

Bioassay for gibberellic acid (GA) production by endophytes: Starch agar halo test

Stock solutions of gibberellic acid (GA) were prepared 10^{-3} to 10^{-7} M. 24 hour old endophytic bacterial culture (10^{8}cfu/ml) were inoculated in 25 ml nutrient broth and incubated at 30 °C for 10 days. The cells were centrifuged at 5000rpm to remove the cell mass and the culture filtrate was used for the bioassay. Pre germinated paddy seeds were cut into two halves and the embryo less half seed was incubated in 5ml of culture filtrate for 6 to 8 hours. Then they were transferred to petri plates with starch agar medium. The half seed was placed with the cut surface touching the medium and the plates were incubated at 30 °C for 24-48 hours. Then the seeds were removed and the plates were flooded with Gram's iodine solution. Seeds soaked in sterile water served as control and those soaked in different concentrations of gibberellic acid (GA) solutions served as standard. The clear halo formed was measured in milli meter and compared with the control plate. From the standard graph gibberellic acid (GA) production of the endophytic isolates was calculated.

Bioassay for Indole-3-Acetic Acid (IAA) production by bacterial endophytes: Cucumber root elongation bioassay (Loper and Schroth, 1986)

Healthy seeds of cucumber were selected and soaked in sterile water for six hours and allowed to germinate in filter paper. Stock solutions ofindole-3-acetic acid (IAA)were prepared upto 10^{-3} strength. 24 hour old endophytic bacterial culture (10^{6}cfu/ml) were inoculated in 25 ml nutrient broth and incubated at 30°C for 10 days. The cells were centrifuged at 5000 rpm to remove the cell mass and the culture filtrate was used for the bioassay.6ml of the culture filtrate was added to petri plates and the selected seedlings were transferred into them (10 seeds/plate) and incubated for 48 hrs. Similarly different concentrations of IAA solution were used as standard. Sterile water served as control. After 48 hours of incubation, root length of the cucumber seedlings was measured and results are tabulated. The standard graph was plotted with the concentration of IAA and the increase in root length. The IAA production by the endophytic isolates was calculated from the standard graph.

Bioassay for cytokinins by bacterial endophytes: Cucumber cotyledon greening bioassay (Fletcher *et al.*, 1982)

The cucumber cotyledons from 5-day-old plants were excised in dim green light, weighed and uniformly floated in petri dishes containing 5 mL of culture filtrate. Benzyl adenine (BA) was used as standard. Petri dishes containing 5 mL of test solution containing 10^{-4} to 10^{-8} M of BA in 40 mM KCl served as standards. Cotyledons placed in a solution of 5 mL of 40 mMKCl were used as control and each plate containing 10 pieces of cotyledons with the adaxialface down. Their weight was on average 0.2500 ± 0.0050 mg. All the dishes were returned tothe dark at 28 °C and incubated for 24 hours and then exposed to fluorescent light with an intensity of 11 $mmolm^{-2}s^{-1}$ for 3 hours at 28 °C. The cotyledons were extracted directly with 10 mL 95% acetone - ethanol of 2:1 (v/v) solution in dark for 24 hours. The absorbance of the extracted solutions was measured using UV visible spectrophotometer at 663 and 645 nm.

1-Aminocyclopropane-1-carboxylate (ACC)

deaminase production

The procedure followed for the isolation of bacterial endophytes showing ACC deaminase activity was given by Singh *et al.,* (2015) with modifications. Bacterial colony wa sstreaked on nutrient medium with 3.0 mM ACC as unique sole nitrogen source. The nutrient medium without any nitrogen source was kept as control and other control also was kept where ammonium sulphate ($(NH_4)_2SO_4$) was used as nitrogen source. For confirmation of ACC utilizing as nitrogen source, bacterial colonies were sub-cultured many times on NA-ACC plate.

Analysis

Analysis of variance (ANOVA) was performed and significant differences between means were compared using Fisher's protected LSD test at P = 0.05.

Results and Discussion

A total thirty two bacterial endophytes were isolated from the surface sterilized roots, shoots and leaves of the six small millets *viz*., Barnyard millet, Finger millet, Foxtail Millet, Kodo millet, Little millet and Proso millet. The surface sterilization procedure for the isolation of endophytic bacteria as standardized in the experiment was quite satisfactory as no growth appeared on the control plate.

These isolates are tested for production of phytohormones like indole-3-acetic acid (IAA), gibberellic acid (GA) and cytokinins (Benzyl adenine) by bioassay and ACC deaminase production.

Indole-3-acetic Acid (IAA) production is one of the plant growth promoting property of endophytic bacteria that stimulate and facilitate plant growth. The bioassay for indole-3-acetic acid (IAA) is based on the root elongation in cucumber seedlings by IAA (Table 1).

Twenty isolates produced IAA in which KMS1 significantly produced highest concentrations of IAA (254.95 µg) followed by FMR3 (231.87 µg) followed by FMR 9 and PMS2 (221.98µg) which are at par with each other.

The least concentration of IAA was recorded by the isolate BMS7 (96.70 µg). This results were in accordance with Amanda *et al.,* (2016) where they isolated bacterial endophytes from aerobic rice and among twenty four isolates eight endophytic bacteria designated as ARBR3, AM65R1, IR64L1, ARBS2, AM65S2, IR64R1 AND JEER2 were found to produce IAA in the range of 226.59-10.86 µg/ml.

Earlier some workers (Ji *et al.,* 2014) isolated 576 bacterial endophytes from leaves, shoots and shoots of flooded rice and reported that 12 isolates out of 576 isolates produced IAA in the range of 22.6 to 31. 1 µg/ml.

Out of thirty two isolates, only sixteen isolates produced gibberellins. This bioassay is based on the principle that gibberellic acid (GA) induces de novo synthesis of amylase in germinating seeds (Table 2). According to this study the highest gibberellic acid (GA) was recorded in KMS5 (11.04µg) which was significantly higher than the isolate FMR12 (8.12µg). Isolates KML2, PMR3 and FML12 were on par with each other and produced 7.78, 7.72 and 7.65 µg respectively.

The least was observed in PMS2 and BMR4 (2.20 µg). Endophytic bacteria isolated from rice and maize produced gibberellic acid which ranges from 0.75 to 2.83 µg ml^{-1} (Maheshwari *et al.,* 2013).

Table.1 Bioassay for Indole-3-acetic acid (IAA) production by bacterial endophytes

Sl. No	Crop	Parts	Isolates	Root Length (mm)	IAA (µg)
1	Barnyard Millet	Root	BMR4	0.97^{l}	106.59^{l}
2			BMR5	1.13^{k}	124.18^{k}
3			BMR7	1.46^{g}	160.44^{g}
4		Shoot	BMS7	0.88^{m}	96.70^{m}
5		Leaf	BML1	-	-
6			BML5	1.66^{e}	182.42^{e}
7	Finger Millet	Root	FMR3	2.11^{b}	231.87^{b}
8			FMR7	1.19^{j}	130.77^{j}
9			FMR9	2.02^{c}	221.98^{c}
10			FMR12	1.25^{i}	137.36^{i}
11		Shoot	FMS2	-	-
12		Leaf	FML9	-	-
13			FML12	-	-
14	Foxtail Millet	Root	FTMR3	-	-
15		Shoot	FTMS4	-	-
16			FTMS5	0.98^{l}	107.69^{l}
17			FTMS8	-	-
18		Leaf	FTML10	1.71^{e}	187.91^{e}
19	Kodo Millet	Root	KMR2	1.55^{f}	170.33^{f}
20			KMR4	-	-
21		Shoot	KMS1	2.32^{a}	254.95^{a}
22			KMS5	1.45^{f}	159.34^{f}
23		Leaf	KML2	1.41^{gh}	154.95^{gh}
24			KML3	-	-
25	Little Millet	Root	LMR1	-	-
26			LMR4	1.36^{h}	149.45^{h}
27		Shoot	LMS1	-	-
28		Leaf	LML4	1.81^{d}	198.90^{d}
29	Proso Millet	Root	PMR3	1.18^{jk}	129.67^{jk}
30			PMR6	-	-
31		Shoot	PMS2	2.02^{c}	221.98^{c}
32		Leaf	PML3	1.57^{f}	172.53^{f}

Note: Means with same superscript, in a column do not differ significantly at P=<0.05 as per Duncan Multiple Range Test (DMRT).

Table.2 Bioassay for Gibberellic acid (GA) production by bacterial endophytes

Sl. No	Crop	Source	Isolates	Diameter of the Zone (mm)	GA (µg)
1	Barnyard Millet	Root	BMR4	6.60^{i}	2.20^{i}
2			BMR5	-	-
3			BMR7	-	-
4		Shoot	BMS7	-	-
5		Leaf	BML1	-	-
6			BML5	-	-
6	Finger Millet	Root	FMR3	-	-
7			FMR7	17.20^{h}	5.72^{h}
8			FMR9	20.20^{f}	6.73^{f}
9			FMR12	24.40^{b}	8.12^{b}
10			FMS2	19.00^{g}	6.33^{g}
11		Shoot	FML9	-	
12		Leaf	FML12	23.00^{c}	7.65^{c}
13	Foxtail Millet	Root	FTMR3	-	-
14		Shoot	FTMS4	19.20^{g}	6.79^{g}
15			FTMS5	-	-
16			FTMS8	-	-
17			FTMS9	-	-
18		Leaf	FTML10	-	-
19	Kodo Millet	Root	KMR2	-	-
20			KMR4	18.60^{g}	6.19^{g}
21		Shoot	KMS1	21.00^{e}	6.99^{e}
22			KMS5	33.20^{a}	11.04^{a}
23		Leaf	KML2	23.40^{c}	7.78^{c}
24			KML3	21.80^{d}	7.25^{d}
25	Little Millet	Root	LMR1	-	-
26			LMR4	-	-
27		Shoot	LMS1	-	-
28		Leaf	LML4	-	-
29	Proso Millet	Root	PMR3	23.20^{c}	7.72^{c}
30			PMR6	19.00^{g}	6.32^{g}
31		Shoot	PMS2	6.60^{i}	2.20^{i}
32		Leaf	PML3	20.20^{f}	6.73^{f}

Note: Means with same superscript, in a column do not differ significantly at P=<0.05 as per Duncan Multiple Range Test (DMRT).

Table.3 Bioassay for Cytokinin production by endophytic bacterial isolates

Sl. No	Crop	Parts	Isolates	Chlorophyll (µg/ml)	Cytokinin (µg)
1	Barnyard Millet	Root	BMR4	1.03^{g}	3.84^{g}
2			BMR5	-	-
3			BMR7	1.04^{g}	3.88^{g}
4		Shoot	BMS7	-	-
5		Leaf	BML1	-	-
6			BML5	0.86^{j}	3.21^{j}
7	Finger Millet	Root	FMR3	-	-
8			FMR7	0.98^{h}	3.66^{h}
9			FMR9	-	-
10			FMR12	0.57^{l}	2.13^{l}
11		Shoot	FMS2	0.86^{j}	3.21^{j}
12			FML9	-	-
13		Leaf	FML12	1.21^{f}	4.51^{f}
14	Foxtail Millet	Root	FTMR3	-	-
15		Shoot	FTMS4	1.56^{e}	5.82^{e}
16			FTMS5	0.75^{k}	2.80^{k}
17			FTMS8	1.53^{e}	5.71^{e}
18		Leaf	FTML10	0.45^{m}	1.68^{m}
19	Kodo Millet	Root	KMR2	0.98^{h}	3.66^{h}
20			KMR4	0.86^{j}	3.21^{j}
21		Shoot	KMS1	1.87^{b}	6.98^{b}
22			KMS5	2.32^{a}	8.66^{a}
23		Leaf	KML2	-	-
24			KML3	1.76^{c}	6.57^{c}
25	Little Millet	Root	LMR1	-	-
26			LMR4	1.65^{d}	6.16^{d}
27		Shoot	LMS1	-	-
28		Leaf	LML4	0.91^{i}	3.40^{i}
29	Proso Millet	Root	PMR3	-	-
30			PMR6	0.87^{ij}	3.25^{ij}
31		Shoot	PMS2	0.98^{h}	3.66^{h}
32		Leaf	PML3	1.02^{gh}	3.81^{gh}

Note: Means with same superscript, in a column do not differ significantly at P=<0.05 as per Duncan Multiple Range Test (DMRT).

Table.4 ACC deaminase production ability of endophytic bacteria from small millets

Sl. No	Crop	Parts	Isolates	ACC production in minimal medium
1	Barnyard Millet	Root	BMR4	-
2			BMR5	+
3			BMR7	-
4		Shoot	BMS7	-
5		Leaf	BML1	+
6			BML5	-
7	Finger Millet	Root	FMR3	-
8			FMR7	+
9			FMR9	+
10			FMR12	-
11		Shoot	FMS2	+
12			FML9	-
13		Leaf	FML12	+
14	Foxtail Millet	Root	FTMR3	-
15		Shoot	FTMS4	-
16			FTMS5	+
17			FTMS8	-
18		Leaf	FTML10	-
19	Kodo Millet	Root	KMR2	-
20			KMR4	+
21		Shoot	KMS1	-
22			KMS5	+
23		Leaf	KML2	-
24			KML3	-
25	Little Millet	Root	LMR1	+
26			LMR4	+
27		Shoot	LMS1	+
28		Leaf	LML4	-
29	Proso Millet	Root	PMR3	-
30			PMR6	-
31		Shoot	PMS2	-
32		Leaf	PML3	+

Note: "+"- Growth observed: "-" – No growth was observed

Cytokinins enhance chloroplast differentiation in plant cells as well as regulate and stimulate chlorophyll (Chl) synthesis in etiolated cucumber cotyledons. The increase in chlorophyll production is proportional to the concentration of cytokinins and this response provides a sensitive yet rapid bioassay for cytokinins (Table 3). The cytokinin (benzyl adenine) concentration in 25 ml culture filtrate was significantly highest in the isolate KMS5 (8.66 µg) followed by KMS1 (6.98µg) and KML3 (6.57 µg). Least cytokinin production was recorded with FTML10 (1.68 µg). Pradeepa and Jennifer (2013) reported five endophytic bacteria from *Tabernaemontana divaricate* produced cytokinin through cucumber cotyledon greening bioassay. A study by Silva *et al.,* (2015) reported cytokinin production by endophytic bacteria isolated from sugarcane.

1-aminocyclo propane-1-carboxylate (ACC) deaminase production potential by the endophytic bacteria

Glick *et al.,* (2007) suggested that some microbes could utilize the ACC as nitrogen source from the exudates of roots or seeds. This decrease in the levels of ACC and ethylene may prevent the ethylene-mediated plant growth inhibition. Endophytic microbes with these capabilities residing inside the host plants can benefit the host by reducing the stress and increasing the plant growth (Hardoim *et al.,* 2008). In the present study, ACC was used as the sole source of N-source (Table 4). Eleven isolates *viz.*, BMR5, BML1, FMR7, FMR9, FMS2, FML12, FTMS5, KMR4, KMS5, LMR1, LMR4, LMS1 and PML3 shows positive results for production of ACC deaminase enzyme in the plate assay (Table 4). Similar results were observed by Jalili *et al.,* (2009), where they screened endophytic bacterial isolates from canola as being able to utilize ACC as the sole N source. After evaluating the results, this experiment was repeated in three replicates for the isolates capable of using ACC as the sole N-source. Hence, thirteen isolates were determined as the strains to utilize ACC as the sole N-source. Jasim *et al.,* (2014) isolated bacterial endophytes from *Zingiber officinale,* and tested the isolates for production of ACC deaminase and the results revealed that four isolates could grow in the medium containing ACC as the sole carbon source and most of the isolates belong to the *Pseudomonas* genera.

Millets known for resilience and drought enduring capacity and are relatively less prone to major pests and diseases. These are indispensable in tribal and hill agriculture where crop substitution is difficult. To make millet cultivation more sustainable and less dependent on chemical fertilizers there is a need to use microbial inoculants. Therefore the results of this study reveals that small millets harbored endophytic bacteria that can produce phytohormones and hence can be used them for further studies to test their effect on the plant growth and development of small millets and also through molecular approach for their identification and functional studies.

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