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Production of Phytohormones by Endophytic Bacteria Isolated from Aerobic Rice

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Endophytic bacteria colonize the interior of the plant parts, without causing any harmful effect and promote plant growth. An investigation was carried out to isolate endophytic bacteria from aerobic rice varieties that have the potential to produce phytohormones like indole-3-acetic acid (IAA), gibberellins (GA) and cytokinin. Total of twenty four endophytic bacteria were isolates from rice tissues of the four varieties of aerobic rice. Eight endophytic bacteria designated as ARBR3, AM65R1, IR64L1, ARBS2, AM65S2, IR64R1 AND JERR2 were found to produce IAA in the range of 226.59-10.86 μg/ml. GA was found to produce by six endophytic isolates from the root, shoot and leave of the four varieties of aerobic rice, these isolates are ARBR2, ARBR3, AM65R2, AM65L1, IR64R1, and IR64S2 in which the concentration ranges from 5.43-2.56. Only three isolates produced cytokinin where isolate JER produced highest concentration (5.42 μg/ml). This work revealed that some endophytic bacteria from aerobic rice produced plant growth hormone that would help in plant growth and development and will be added advantage to be used in microbial inoculation.

Keywords: Endophytic bacteria; Aerobic rice; IAA; GA; Cytokinin.

Endophytic bacteria are ubiquitous in nature with a rich biodiversity and unexplored biosynthetic pathway (Ryan *et al.,* 2008). By occupying the localized point of entry or by spreading within the plant, they produce an array of bioactive metabolites and hydrolytic enzymes to survive in the unique environment of the host plant (Strobel 2002). Their metabolic activities can contribute to the growth and development of the plants. There is firm evidence that indole-3-acetic acid (IAA) gibberellins (GA) and cytokinins, all produced by plants and essential to their growth and development, are produced also by various bacteria which live in association with plants. Diverse plant-associated microbes synthesize phytohormones such as gibberellins, cytokinins, jasmonic acid, abscisic acid, ethylene, and indole-3-acetic acid (IAA), often with profound effects on growth, tissue differentiation, and reproduction of their hosts (Spaepen *et al.,* 2007).

Bacterial endophytes have been reported to promote plant growth by a number of different mechanisms. These mechanisms include phosphate solubilization activity (Wakelin *et al.,* 2004), production of phytohormones (Lee *et al.,* 2004), nitrogen fixation (Compant *et al*., 2005), siderophore biosynthesis (Lodewyckx *et al.,* 2002). 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

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to α ketobutyrate and ammonia and thereby decreases ethylene levels in host plants (Sun *et al*., 2009).

As the plant growth promoting properties of endophytic bacteria can vary, it is important to study such properties from microbial populations associatied with economically important plants.

In the current study, endophytic bacteria were isolated from the four varieties of the aerobically grown rice and these isolates were studied for their ability in producing phytohormone like IAA, GA and cytokinin. Since aerobic rice is new water saving technology studies on such aspects is very limited and therefore this study is significant and novel in its approach.

MATERIALS AND METHODS

Isolation of bacterial endophytes

The aerobic rice samples were collected during Kharif season 2014-15 from the aerobic rice research plot, Department of Plant Biotechnology, University of Agricultural Sciences, GKVK campus, Bangalore. Four rice genotypes selected for the study were ARB6, IR64, AM65 and Jeeregisana. Ten plants of each variety 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% ethanol for 2 min and shaken in 1.2% (w/v) NaClO solution for 20 min. Samples were then washed several times with sterile distilled water with shaking (15 min each). Surface sterilized samples were ground with sterilized mortar and pestle, and inoculated on nutrient agar medium. After incubation at 30°C for 2 days, the inoculants were transferred to fresh nutrient agar medium and then incubated at 30°C for 2 days. 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 twenty four isolates were isolated from the roots, shoots and leaves of the four varieties of aerobic rice. They were name according to the variety name followed by the parts of the aerobic rice like root, shoot and leave and the number of isolates of that variety

Production of plant growth regulators by endophytic bacterial isolates

Bioassay for GA production by endophytes: Starch agar halo test

Stock solutions of 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 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 for 24-48 hours. Then the seeds were removed and the plates were flooded with iodine solution. Seeds soaked in sterile water served as control and those soaked in different concentrations of GA solutions served as standard. The clear halo formed was measured and compared with the control plate. From the standard graph GA production of the endophytic isolates was calculated.

Bioassay for IAA production by endophytic isolates: Cucumber root elongation bioassay (Loper and Schroth, 1986)

Healthy seeds of cucumber were selected for the study. They were soaked in water for six hours and allowed to germinate in filter paper. Stock solutions of IAA were prepared upto 10^{-3} strength. 24 hour old endophytic bacterial culture (10^{9}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 measured the root length of the seedlings and tabulated the results. The standard graph was plotted with the concentration of IAA and the decrease in root length. The IAA production by the endophytic isolates was calculated from the standard graph.

Bioassay for cytokinins by endophytic isolates: 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 7-cm 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 and 40 mM KCl served as control. Cotyledons placed in a solution of 5 mL of 40 mM KCl was used as control. Each plate of sample and control were placed 10 pieces of cotyledons with the adaxial face down. Their weight was on average 0.2500±0.0050 mg. All the dishes were returned to the dark at 28 °C for an incubation of 24 hours 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 directly with 10 mL 95 % acetone - ethanol 2:1 (v/v) solution in dark for 24 hours. The absorbance of the extraction solutions were measured using UV755B spectrophotometer at 663 and 645 nm.

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 of twenty four isolates were isolated from the surface sterilized roots, shoots and leaves of the four varieties of aerobic rice *viz*., ARB6, AM65, IR64 and Jeeregisana (JER). The surface sterilization procedure for the isolation of

Table 1. Bioassay for Gibberellic Acid (GA) production by endophytic isolates

S.no	Varieties	Source	Isolates	Diameter of the zone (mm)	GA (µg)*
1	ARB6	Roots	ARBR1	-	-
2			ARBR2	25.2[a]	5.43[a]
3			ARBR3	19.1[b]	2.66[b]
4			ARBR4	-	-
5		Shoots	ARBS1	-	-
6			ARBS2	-	-
7		Leaves	ARBL1	-	-
8	AM65	Roots	AM65R1	-	-
9			AM65R2	19.2[b]	2.56[b]
10		Shoots	AM65S1	-	-
11			AM65S2	-	-
12			AM65S3	-	-
13			AM65S4	-	-
14		Leaves	AM65L1	24.8[a]	5.25[a]
15			AM65L2	-	-
16			AM65L3	-	-
17	IR64	Roots	IR64R1	22.6[ab]	4.17[ab]
18		Shoots	IR64S1	-	-
19			IR64S2	19.3[b]	2.57[b]
20		Leaves	IR64L1	-	-
21	JEEREGISANA	Roots	JERR1	-	-
22			JERR2	-	-
23		Shoots	JERS1	-	-
24		Leaves	JERL1	-	-
	Reference strain	*Pseudomonas fluorescens*		15.4[c]	0.57[c]
		SEm	2.82	1.41	
		CD (0.05)	3.45	1.73	

Note: Different letters in superscripts indicate significantly different values. Bioassay by Starch agar halo test. *Estimated from 25 ml culture filtrate concentrated to 2 ml.

endophytic bacteria as standardized in the experiment was quite satisfactory as no growth appeared on the control plate. These isolates were further tested for their production of phytohormone GA, IAA and cytokinin following standard procedure.

The production of plant growth regulators Gibberellic acid (GA), Indole acetic acid (IAA) and cytokinins (Benzyl adenine) by the endophytic bacterial isolates was studied by bioassay. The concentration of GA in the culture filtrate of the endophytic bacterial isolates was determined by the starch agar halo test. Out of twenty four isolates, only six isolates produced gibberellins. This bioassay is based on the principle that GA induces *de novo* synthesis of amylase in germinating seeds. According to this study the highest GA was recorded in ARBR2 (5.43µg) which was on par with AM65L1 (5.25 µg) and IR64R1 (4.17 µg). The least was in ARBR3, AM65R2 and IR64S2. All these isolates recorded less than 3.00 µg GA. Results are presented in Table 1, Plate 1. The results were in accordance with Chen *et al.*, (1998) who reported gibberellins production in flooded rice by *Acetobacter diazotrophicus* and *Herbaspirillum seropedicae* in chemically defined culture media. Maheswari *et al.,* (2013) reported endophytic isolates from rice and maize that produced gibberellic acid, which ranges from 0.75 to 2.83 µg ml^{-1}.

The IAA bioassay is based on the inhibition of root growth in cucumber by IAA. As the concentration of IAA increases the root elongation of germinating seedlings is inhibited. The results of the bioassay are recorded in Table 2, Plate 2. Eight isolates produced IAA in which ARBR3 (226.59 µg) showed highest concentration which was on par with AM65R1, ARBR1 and IR64L1 (188.10 µg, 173.92 µg and 149.85 µg

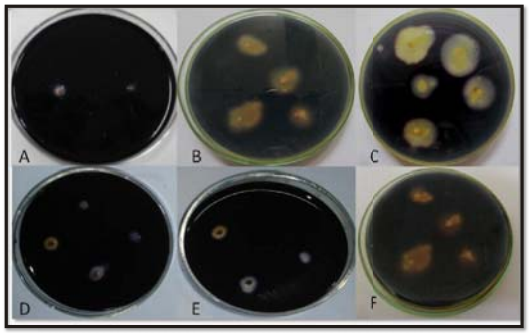

Plate 1. Starch agar halo test for GA production; A) ARBR3 isolate B) AM65L1 isolate C) ARBR2 isolate D) IR64S2 isolate E) AM65R2 and F) IR64R1 isolate

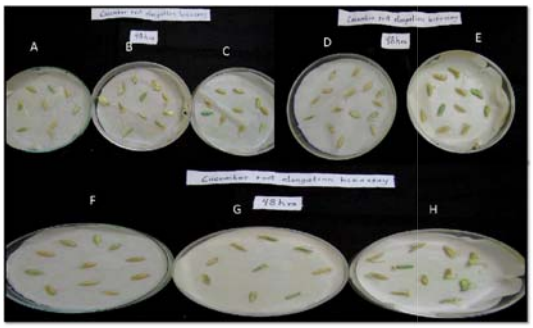

Plate 2. Cucumber root elongation test for IAA production; A) AM65R1 isolate B) ARBR3 isolate C) IR64L1 isolate D) IR64R1 isolate E) AM65S2 isolate F) JERR2 isolate G) ARBS2 and H) ARBR1 isolate.

Plate 3. Cucumber cotyledon greening bioassay for cytokinin production; A) ARBR2 isolate B) AM65R2 isolate C) JERR1 isolate and D) control

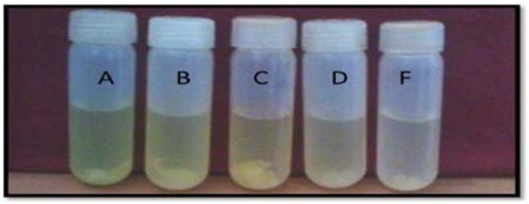

Plate 4. Extraction of chlorophyll from the cucumber cotyledon; A)JERR1 isolate B) AM65R2 isolate C) ARBR2 isolate D) Reference strain and E) 40mM KCl control

Table 2. Bioassay for IAA production by endophytic bacterial isolates

S.no	Varieties	Source	Isolates	Root length(cm)	IAA (µg)*
1	ARB6	Roots	ARBR1	1.23^{b}	173.92^{a}
2			ARBR2	-	-
3			ARBR3	2.28^{a}	226.59^{a}
4			ARBR4	-	-
5		Shoots	ARBS1	-	-
6			ARBS2	0.88^{c}	35.10^{bc}
7		Leaves	ARBL1	-	-
8	AM65	Roots	AM65R1	1.28^{b}	188.10^{a}
9			AM65R2	-	-
10		Shoots	AM65S1	-	-
11			AM65S2	0.78^{c}	33.79^{bc}
12			AM65S3	-	-
13			AM65S4	-	-
14		Leaves	AM65L1	-	-
15			AM65L2	-	-
16			AM65L3	-	-
17	IR64	Roots	IR64R1	0.87^{c}	10.87^{c}
18		Shoots	IR64S1	-	-
19			IR64S2	-	-
20		Leaves	IR64L1	1.18^{b}	149.85^{ab}
21	JEEREGISANA	Roots	JERR1	-	-
22			JERR2	0.80^{c}	20.55^{c}
23		Shoots	JERS1	-	-
24		Leaves	JERL1	-	-
	Reference strain	*Pseudomonas fluorescens*		0.84^{c}	32.55^{bc}
		SEm	0.15	97.99	
		CD (0.05)	0.18	118.87	

Note: Different letters in superscripts indicate significantly different values. Bioassay by Cucumber root elongation test. *Estimated from 25 ml culture filtrate concentrated to 2 ml.

respectively). The least concentration of IAA was in ARBS2, AM65S2, JERR2 and IR64R1 (35.10 µg, 33.79 µg, 20.55 µg and 10 µg). This results was in accordance with Ji *et al.*,(2013) were out of 576 endophytic bacteria from the leaves, shoots and roots of flooded rice, only 12 endophytes produced IAA ranging 22.6 to 3.1 1 (µ/ml).

The cucumber cotyledon greening bioassay is frequently used for detecting cytokinins and the results of the test are presented in Table 3, Plate 3 and 4. Cytokinins accelerate chloroplast differentiation as well as regulate and stimulate chlorophyll (Chl) production 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. The cytokinin (benzyl adenine) concentration in 25 ml culture filtrate was highest in the JERR1 (5.42 µg) followed by the reference culture *Azotobacter chroococcum* (3.05 µg) and the least were recorded with roots endophytes of AM65R2 (0.711 µg) and ARBR2 (0.526 µg). There is little or no record on the endophytic bacteria from rice producing cytokinin but however many endophytes from grains, maize and sugarcane show cytokinin production. Pradeepa *et al.,* (2013) reported five endophytic bacteria from *Tabernaemontana divaricata* produced cytokinin through cucumber cotyledon greening bioassay. A study by Silva *et al.*, (2015) reported cytokinin production by endophytic bacteria isolated from sugarcane.

Table 3. Bioassay for Cytokinin production by endophytic bacterial isolates

S.no	Varieties	Source	Isolates	Chl µg/ml	Cytokinin (µg)*
1	ARB6	Roots	ARBR1	-	-
2			ARBR2	1.02[c]	0.526[c]
3			ARBR3	-	-
4			ARBR4	-	-
5		Shoots	ARBS1	-	-
6			ARBS2	-	-
7		Leaves	ARBL1	-	-
8	AM65	Roots	AM65R1	-	-
9			AM65R2	1.06[c]	0.711[c]
10		Shoots	AM65S1	-	-
11			AM65S2	-	-
12			AM65S3	-	-
13			AM65S4	-	-
14		Leaves	AM65L1	-	-
15			AM65L2	-	-
16			AM65L3	-	-
17	IR64	Roots	IR64R1	-	-
18		Shoots	IR64S1	-	-
19			IR64S2	-	-
20		Leaves	IR64L1	-	-
21	JEEREGISANA	Roots	JERR1	2.07[a]	5.42[a]
22			JERR2	-	-
23		Shoots	JERS1	-	-
24		Leaves	JERL1	-	-
	Reference strain	*Azotobacter chroococcum*		1.55[b]	3.05[b]
		SEm	0.285	1.30	
		CD (0.05)	0.479	2.19	

Note: Different letters in superscripts indicate significantly different values Cucumber cotyledon greening bioassay for cytokinins.
*Estimated from 25 ml culture filtrate concentrated to 2 ml

CONCLUSION

Aerobic rice is in itself an eco-friendly technology, to make aerobic rice cultivation more sustainable and less dependent on chemical fertilizers there is a need to used microbial preparations. Therefore the results of this study reveals that aerobic rice varieties 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 aerobic rice and also through molecular approach for their identification and functional studies.

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