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Original Research Paper

Effect of osmotic stress on *in vitro* plant growth hormone production by osmotolerant bacteria isolated from chilli phyto microbiome

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ABSTRACT

The present study was conducted to determine the effect of osmotic stress on the plant growth hormone production by six osmotolerant plant growth promoting bacterial strains. These strains originated from the phytomicrobiome of chilli cultivated in the drought prone areas of Andhra Pradesh. They possessed multiple plant growth promotion traits including the ability to produce a variety of plant growth hormones. The effect of osmotic stress on the plant growth hormone production was determined by High Performance Liquid Chromatography (HPLC) under normal and *in vitro* osmotic stress conditions using 25% Poly Ethylene Glycol (PEG) 8000. In general, it was observed that osmotic stress impacted the plant growth hormone production of the isolates, but nevertheless plant hormones were detected in all the bacterial strains. An exception to this was the cytokinin molecule zeatin riboside, which was produced at higher levels by five of the six bacterial isolates under osmotic stressed conditions.

Keywords: Chilli, cytokinin, gibberellic acid, indole acetic acid, osmotolerant bacteria, PEG 8000

INTRODUCTION

Chilli (*Capsicum annum*) an important vegetable crop of India, is used as an ingredient in various culinary preparations is valued for its pungency, conferred by the alkaloid capsaicin. Chilli cultivated in the Palnadu area of Andhra Pradesh, is impacted by low moisture during its growth and yields are impacted, thereby requiring interventions. The utilization of stress tolerant plant growth promoting rhizobacteria (PGPR) for overcoming deficit irrigation stress (Bouremani, 2022) has gained traction worldwide. Such bacterial strains improve plant growth through improved shoot and root biomass, root length and root surface area as a result of multiple plant growth promotion traits (Masood et al., 2020). The improved plant growth performance can be attributed IAA production (Zhang et al., 2020), phosphate solubilization (Audipudi et al., 2021), siderophore production (Ashry et al., 2022), ACC deaminase activity (Danish et al., 2019), production of gibberellins (Selvakumar et al., 2015) and cytokinins (Di et al., 2023). To select potential bacterial strains for deficit irrigation stress alleviation, initial screening under *in vitro* conditions using an osmoticum like Poly Ethylene Glycol (8000) is a prerequisite (Zhang et al., 2020; Ashry et al., 2022). Therefore, this study was undertaken to study the effect of PEG 8000 induced osmotic stress on the plant growth hormone production by six osmotolerant bacterial isolates.

MATERIALS AND METHODS

Osmotolerant bacterial strains

Six osmotolerant bacterial strains (previously isolated using 25% PEG 8000 as a selection agent), originating from chilli phytomicrobiome samples collected from the Palanadu region of Andhra Pradesh, India, were used. They were identified by Sanger sequencing of the 16S rRNA gene as *Atlantibacter hermannii* R11, *Enterobacter* sp. R19, *Achromobacter* sp. T26, *Lysinibacillus composti* T55, *Atlantibacter hermannii* S12 and *Pseudomonas mosselii* S13L.

Estimation of indole acetic acid production and gibberellic acid production

To estimate the indole acetic acid (IAA) production, the media combinations *viz.*, nutrient broth; nutrient broth + tryptophan (100 µg mL^{-1}); nutrient broth + 25% PEG 8000; nutrient broth + 25% PEG 8000 + tryptophan (100 µg mL^{-1}) were used. For the

Table 1 : Instrumentation parameters for the detection of IAA and GA_3 by HPLC

Parameter	IAA	GA_3
Stationary phase	C18 column	C18 column
Flow rate	1 mL/min	0.8 mL/min
Mobile phase	Methanol: water (80:20)	Methanol: water (70:30)
Wavelength	270 nm	208 nm
Column Temperature	30ºC	30ºC

estimation of gibberellic acid (GA_3), the media combinations *viz.*, nutrient broth and nutrient broth + 25% PEG 8000 were used as suggested by Selvakumar et al. (2015). The respective media were inoculated with 24-hour old cultures of individual isolates and incubated at 30°C for 7 days under dark conditions. After seven days, cultures were centrifuged at 6000 rpm for 10 minutes and 1N HCl was added to the supernatant, and pH adjusted to 2.8. To the acidified supernatant an equal volume of diethyl ether was added and incubated in dark for 4 hrs and stored overnight at 4°C in a separating funnel. Subsequently, the organic phase (lower phase) was discarded and the solvent phase (upper phase) was collected. To the solvent phase, a pinch of sodium sulphate was added and kept overnight and evaporated in a rotary flash evaporator. After evaporation, 2-3 mL of HPLC grade methanol was added and the resultant extract was filtered through a PVDF (polyvinylidene difluoride) filter (0.22 µm pore size, 47 mm diameter) (Selvakumar et al., 2015). The IAA and GA_3 concentrations were quantified by HPLC (Prominence, Shimadzu, Japan) as described by Kelen et al. (2004) with slight modifications. A photodiode array detector (Shimadzu, model: SPD M 20 A Japan) and 4 µm-Fusion RP-C18 column (Phenomenex, USA, 250 × 4.6 mm) were used for the assay. The IAA and GA_3 contents were quantified using external standards (Sigma-Aldrich, MO, USA). The conditions for the HPLC analysis are mentioned in Table 1.

Estimation of cytokinin production

For estimation of cytokinins, individual isolates were cultivated in M9 medium supplemented with 20% glucose, 0.2% casamino acid and 2 pg/mL biotin at 28ºC for 72-96 hours. The M9 medium supplemented with 25% PEG 8000 was used to determine the effect of osmotic stress. After incubation, the cultures were centrifuged at 16000 rpm for 10 min at 4ºC and the supernatant was filtered through a cellulose acetate filter (0.22 µm pore size, 47 mm diameter). The pH of the cell free supernatant was adjusted to 8.0 by the addition of 1N NaOH, in a separating funnel to which 30 mL of butanol was added and shaken thoroughly and allowed to settle till the clear organic phase (lower phase) and solvent phase (upper phase) were separated. The butanol fraction in the solvent phase was evaporated to dryness in a rotary flash evaporator at 40ºC and the remnants were dissolved in 2.5 mL HPLC grade methanol and filtered through a cellulose acetate filter (0.22 µm pore size, 47 mm diameter) (Selvakumar et al., 2018). The cytokinins in the filtrate were analysed by HPLC (Prominence, Shimadzu, Japan) using a PDA detector as described by Chen et al. (2010). The analysis run time was 60 min, with a flow rate of 0.2 mL/min, using a detection wavelength of 270 nm. All estimations were replicated thrice. The data was analysed with the SAS 9.3 statistical package (SAS Institute Inc, 2011).

RESULTS AND DISCUSSION

Effect of osmotic stress on indole acetic acid (IAA) production by elite osmotolerant bacterial isolates

Indole-3-acetic acid (IAA) is a primary auxin in plants which along with indole butyric acid, are collectively known as auxins. They control a variety of critical physiological processes such as seed germination, cell division, cell elongation, cell differentiation, root formation, photosynthesis and drought reaction of plants (Ullah et al., 2018). Several plant associated rhizobacterial genera such as *Microbacterium, Rhizobium, Mycobacterium* and *Sphingomonas* produce IAA (Etminani and Harighi, 2018). In the present study, IAA production was affected by the imposition of osmotic stress, but nevertheless, all the isolates produced IAA under osmotic stressed conditions. In general, it was observed that the addition of tryptophan to the growth medium enhanced IAA concentrations under both normal and osmotic stress

Table 2 : Effect of osmotic stress on indole acetic acid (IAA) production by osmotolerant bacterial isolates

Stress/ Isolate	IAA (ng mL^{-1})					
	Normal (without 25% PEG 8000)	Osmotic stress (with 25% PEG 8000)	Mean	Normal (without 25% PEG 8000 + Tryptophan)	Osmotic stress (with 25% PEG 8000 + Tryptophan)	Mean
Atlantibacter hermannii R11	912	860	886	1249	866	1058
Enterobacter sp. R19	973	851	912	998	859	929
Achromobacter sp. T26	982	855	919	1082	861	972
Lysinibacillus composti T55	1215	864	1040	1234	879	1057
Atlantibacter hermannii S12	1191	883	1037	1215	895	1055
Pseudomonas mosselii S13 L	1152	895	1024	1229	899	1064
Mean	1071	868	-	1168	877	-
Factor	Stress	Isolate	Stress x Isolate	Stress	Isolate	Stress x Isolate
SEm	1.453	2.517	3.55	1.70	2.95	4.18
C.D.	4.266	7.389	10.45	5.01	8.68	12.27

conditions. Under normal conditions in the absence of tryptophan, *Lysinibacillus composti* T55 recorded the highest concentrations of IAA (1215 ng mL^{-1}), whereas, under osmotic stress conditions *Pseudomonas mosselii* strain S13L recorded the highest concentration (895 ng mL^{-1}). With the addition of tryptophan, *Atlantibacter hermannii* strain R11 recorded the highest IAA concentration (1249 ng mL^{-1}) under normal conditions, while *Pseudomonas mosselii* strain S13L recorded the highest IAA concentration (899 ng mL^{-1}) under stress conditions. (Table 2).

The effect of osmotic stress on *in vitro* IAA production has not been reported much in the past. Selvakumar et al. (2015) reported that the plant growth promoting *Citricoccus zhacaiensis* strain B-4, produced 419.4 ng mL^{-1} of IAA under normal conditions and 301.4 ng mL^{-1} under osmotic conditions, which is in concurrence with the present study. Similar results were reported by Arun et al. (2020) who observed that osmotic stress reduced IAA production in *Bacillus megaterium* PB50 under osmotic stress conditions (15.4 µg mL^{-1}) compared to normal conditions (23.2 µg mL^{-1}).

Effect of osmotic stress on gibberellic acid (GA_3) production by elite osmotolerant bacterial isolates

Gibberellins (GAs) are an important group of plant growth regulators in higher plants. They are usually derived from gibberellic acid and stimulate many metabolic events such as germination, flowering, stem elongation and fruit formation (Shahzad et al., 2016). Several plant growth promoting rhizobacterial strains produce gibberellic acids (GAs) and the most widely recognized amongst them is GA_3. In the present study, it was observed that, in general osmotic stress caused a reduction in the bacterial production of GA_3. Among the isolates *Atlantibacter hermannii* strain R11 recorded highest GA_3 concentration both under normal (4983 ng mL^{-1}) and osmotic stress (4883 ng mL^{-1}) conditions (Table 3).

Table 3 : Effect of osmotic stress on gibberellic acid (GA_3) production by osmotolerant bacterial isolates

Stress / Isolate	Gibberellic acid (ng mL^{-1})		Mean
	Normal (Without 25% PEG 8000)	Osmotic stress (With 25% PEG 8000)	
Atlantibacter hermannii R11	4983	4883	4933
Enterobacter sp. R19	4972	4679	4826
Achromobacter sp. T26	4967	4796	4882
Lysinibacillus composti T55	4970	4765	4868
Atlantibacter hermannii S12	4979	4732	4856
Pseudomonas mosselii S13 L	4963	4843	4903
Mean	4972	4783	-
Factor	Stress	Isolate	Stress x Isolate
SEm	1.82	3.16	4.47
C.D.	5.36	9.29	13.14

The reduction in the gibberellic acid production due to the imposition of osmotic stress was reported by Selvakumar et al. (2015), who observed that GA_3 production by *Citricoccus zhacaiensis* B-4 declined from 589.7 ng mL^{-1} under normal conditions to 176.2 ng mL^{-1} under osmotic conditions. Kumar et al. (2019) assessed eight osmotolerant bacterial isolates for the production of gibberellic acid and observed that isolate PB50 recorded the highest GA production of 69 µg mL^{-1} under non-stress conditions and 10.3 µg mL^{-1} under osmotic stress conditions (-0.73 MPa). Arun et al. (2020) reported that gibberellic acid production by *Bacillus megaterium* PB50 reduced to 10.2 µg mL^{-1} under osmotic stress conditions when compared to normal conditions (16.4 µg mL^{-1}). Ghosh et al. (2018) quantified the growth and phytohormone secretion abilities of *Pseudomonas aeruginosa* PM389, *Pseudomonas aeruginosa* ZNP1, *Bacillus endophyticus* J13 and *Bacillus tequilensis* J12 under osmotic stress induced by 25% polyethylene glycol (PEG). In general, they observed that osmotic stress retarded growth of all the bacterial strains, which in turn negatively impacted the auxin and cytokinin production in both the *Pseudomonas* strains. A possible reason for the reduced production of plant growth promoting hormones such as auxins and gibberellins under osmotic stress by the bacteria could be the reduced cell count per unit volume of the medium as a result of the imposition of osmotic stress.

Effect of osmotic stress on cytokinin production by elite osmotolerant bacterial isolates

Cytokinins are a class of plant hormones that regulate cell division and stimulate a variety of plant developmental processes (Waldie and Leyser, 2018). Regulation of growth (root and shoot) and branching, control of shoot apical dominance, development of chloroplasts, regulating the relocation of nutrients from leaves to reproducing seeds are some of the crucial activities regulated by cytokinins (Vaten et al. 2018). Cytokinins also alter the size and activity of meristems through cell division activity of embryonic and mature plants (Martins et al. 2019). In the present study, it emerged that all six isolates produced the cytokinin molecules *viz*., zeatin and zeatin riboside under both normal and osmotic stress conditions (Table 4). While osmotic stress negatively impacted the zeatin production by five of the isolates, *Lysinibacillus composti* strain T55 produced higher levels of zeatin under osmotic stress conditions (94 ng mL^{-1}) compared to normal conditions (49 ng mL^{-1}). When zeatin riboside production was assayed under normal and osmotic stressed conditions, five of the six isolates with the exception of *Enterobacter* sp. strain R19 produced enhanced levels of zeatin riboside under osmotic stressed conditions compared to normal conditions.

The highest levels of Zeatin Riboside (ZR) under both normal condition (27 ng mL^{-1}) and osmotic stress condition (22 ng mL^{-1}) was recorded by *Enterobacter* sp. strain R19. The suppressive effect of osmotic stress on cytokinin production has been documented by Selvakumar et al. (2018) who reported that osmotolerant *Citrococcus zhacaiensis* B-4 produced zeatin (Z) (7.15 ng mL^{-1} and 5.65 ng mL^{-1}), dihydrozeatin riboside (DHZR) (9.65 ng mL^{-1} and

Table 4 : Effect of osmotic stress on cytokinin production by osmotolerant bacterial isolates under normal and osmotic stress conditions

Stress / Isolate	Zeatin (Z) (ng mL^{-1})		Mean	Zeatin Riboside (ZR) (ng mL^{-1})		Mean
	Normal (Without 25% PEG 8000)	Stress (With 25% PEG 8000)		Normal (Without 25% PEG 8000)	Stress (With 25% PEG 8000)	
Atlantibacter hermannii R11	55	37	46	12	18	15
Enterobacter sp. R19	79	59	69	27	22	25
Achromobacter sp. T26	77	58	68	13	20	17
Lysinibacillus composti T55	49	94	72	14	16	15
Atlantibacter hermannii S12	42	41	42	17	19	18
Pseudomonas mosselii S13 L	45	33	39	11	14	13
Mean	58	54	-	15.7	18.2	-
Factor	Stress	Isolate	Stress x Isolate	Stress	Isolate	Stress x Isolate
SEm	1.435	2.48	3.51	0.71	1.23	1.74
C.D.	NS	7.30	10.32	2.09	3.62	5.13

7.32 ng mL^{-1}), zeatin riboside (ZR) (16.95 ng mL^{-1} and 12.11 ng mL^{-1}) under normal and osmotic conditions, respectively. They also reported that the osmotolerant strain *Bacillus amyloliquefaciens* P-72 produced the cytokinin molecules *viz.*, zeatin (Z) (5.01 ng mL^{-1} and 3.21 ng mL^{-1}), dihydrozeatin riboside (DHZR) (15.22 ng mL^{-1} and 12.01 ng mL^{-1}), zeatin riboside (ZR) (21.66 ng mL^{-1} and 16.82 ng mL^{-1}) under normal and osmotic conditions, respectively. Conversely osmotic stress has also been shown to positively impact plant growth hormone production by rhizobacteria. Bhatt et al. (2015) reported that *Enterobacter* strains P-41 and P-46 reported increased IAA and GA_3 production under osmotic stress. Similarly, Ghosh et al. (2018) reported that *Bacillus* strains showed a stress-induced increase in the levels auxins, gibberellins and cytokinins. The reason for the enhanced production of zeatin riboside under osmotic stressed conditions by five of the six bacterial isolates in this study could not be deciphered clearly.

The earliest report on the enhancement of plant drought tolerance by PGPR was made by Timmusk and Wagner (1999) in *Arabidopsis thaliana* inoculated with *Paenibacillus polymyxa* B2. The inoculation of IAA producing rhizobacteria promotes root growth and enhances uptake of nutrients and water in a number of crops (Mantelin and Touraine, 2004). Marulanda et al. (2009), reported the survival of plants under drought stress inoculated with IAA producing *Pseudomonas putida*. Maize (Cohen et al., 2009) and wheat (Creus et al., 2004) plants had better survival under drought stress when inoculated with the GA producing, *Azospirillum lipoferum*. Inoculation of cytokinin producing *Bacillus subtilis* improved shoot cytokinins in lettuce under water stress conditions (Arkhipova et al., 2007). Cytokinin producing *Micrococcus luteus* chp37 inoculation in maize plants under water stress resulted in enhanced shoot/root biomass and increased photosynthetic pigments (Raza & Faisal, 2013). Selvakumar et al. (2018) reported that inoculation of cytokinin-producing *Citricoccus zhacaiensis* and *Bacillus amyloliquefaciens* improved physiological parameters and yield of tomato plants under deficit irrigation conditions.

CONCLUSION

This study clearly indicates the ability of osmotolerant bacterial isolates to produce plant growth promoting hormones under *in vitro* osmotic stressed conditions. This needs to be reconciled with their plant growth promotion abilities under deficit irrigation stress conditions under field conditions.

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