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Research Article
Phytohormone Biosynthesis by Indigenous Rhizobacteria of Vegetable Crops in a Semi-arid Agro-environment

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Abstract

Background and Objective: Efficacy of rhizosphere microorganisms to synthesis phytohormone is a key survival strategy for plants growing under environmentally stressed conditions. Thus, this study aimed at investigating potentials of four indigenous rhizobacteria of vegetables at semi-arid agro-zone of Rajasthan desert, India, to synthesize Indole Acetic Acid (IAA), cytokinin (CK) and Gibberellic Acids (GA_3). **Materials and Methods:** Isolates (*Azospirillum brasiliense* (IR25), *Azotobacter chrocoocum* (IR28), *Pseudomonas stutzeri* (IP32) and *Pseudomonas putida* (IP35)) were screened for plant growth-promoting attributes. HPLC, TLC and spectrophotometry were used for phytohormone characterization, recovery and quantification at 72, 96 and 120 h time intervals. **Results:** All isolates possessed substantive growth-promoting traits and produced pink, fluorescent and green colors confirming synthesis of IAA, CK and GA_3, respectively. At 96 h time interval, highest IAA was recorded by IR25 followed by IP35 which was slightly higher than IR28 and IP32. Maximum CK was found in IR25 followed by IR28 while IP35 recorded slightly higher concentration than IP32. GA_3 recorded by IR25 was highest, IP35 was next with higher value than IR28 and IP32. **Conclusion:** All isolates produced significantly high phytohormones that can be transformed into effective bio-formulations for use in sustainable vegetable production at semi-arid agro-zone of Rajasthan desert.

Key words: Phytohormone, biosynthesis, rhizobacteria, vegetable, semi-arid

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Phytohormone biosynthesis is a major metabolic process of many micro-organisms that inhabit the root surfaces and narrow regions around plant roots called rhizoplane and rhizosphere, respectively[1,2]. The microbial colonization of these microbiomes has been reported to be induced by the presence of various root exudates[3] that serve as nutrient pool for microbial proliferation and survival[4]. Among the many micro-organisms that reside in the rhizosphere are rhizobacteria that have been variously studied to impart many salutary plant growth influences on numerous plant species[5-7]. Hence, they are commonly referred to as Plant Growth Promoting Rhizobacteria (PGPR) capable of enhancing plant growth directly by synthesizing phytohormones such as; auxin (IAA), cytokinin (CK) and gibberellin (GA_3) etc.[8] and indirectly by producing antibiotic for suppressing pathogens[9]. In addition, rhizobacteria phytohormones have been particularly reported to have coordinating effects on plant responses to environmental stimuli such as abiotic stresses[7].

A number of phytohormones have been studied to confer resistance and/or tolerance mechanisms to the most vulnerable and sensitive crops for alleviating the damaging effects of environmental stresses[10,11]. Several other workers have reported that IAA has played prominent roles in resistance of plants to drought and salinity stresses[12,13]. Application of IAA producing *A. brasiliense* strains isolated from salt affected areas have been reported to successfully promote the growth of many plant species[14]. Also, priming of seeds with cytokinin has been reported to enhance resistance to high temperature, salinity and drought stresses mainly through promotion of stomatal opening and transpiration rate by cytokinin[9]. Furthermore, Maggio *et al.*[15] reported alleviation of adverse effects of environmental stresses on plant-water relation by gibberellic acid application through decrease in stomatal resistance and improved water use efficiency of plants under drought stress conditions.

The semi-arid agro-ecological zone of Rajasthan desert, India (24°30'N and 70°74'), with high temperature (exceeding 50°C in the month of May and annual average rainfall of 30-59 inches) is characterized by abiotic stresses appearing majorly as drought and salinization[16]. Consequently, agricultural practices in this harsh agro-ecological zone typically involve rain-fed agriculture, irrigation farming and intensive use of chemical inputs. Vegetable crop production has particularly suffered from the damaging effects of the prevailing environmental challenges in this agro-zone[17]. Thus, with the increasing threats of climate change on agriculture and the global campaign against consumption of inorganic products[9], it is both pertinent and urgent to adopt an ecofriendly and sustainable means of enhancing vegetable production in this vulnerable agro-zone.

In the light of the fore-going, the current work aimed at evaluating the potentials of four indigenous rhizobacteria (*Azospirillum brasiliense, Azotobacter chrocoocum, Pseudomonas stutzeri* and *Pseudomonas putida*) for phytohormone (indole acetic acid, cytokinin and gibberellic acid) synthesis with the view to recommending their use in sustainable vegetable production at semi-arid agro-ecological region of Rajasthan desert, India.

MATERIALS AND METHODS

Collection of indigenous rhizobacteria isolates: Four previously isolated native rhizobacteria from notable vegetable (tomato, chili, okra and onion) farms at Achrol village in Jaipur district of Rajasthan state, India were used for the experiments in 2018-2019. Achrol village is located on latitude 27.13°N and 75.95°E at an altitude of 441 m above sea level in the semi-arid agro-ecological zone of Rajasthan desert. The isolates had been identified by using 16S r-RNA gene analysis as *Azospirillum brasiliense (IR25)*, *Azotobacter chrocoocum (IR28)*, *Pseudomonas stutzeri (IP32)* and *Pseudomonas putida (IP35).* Detail information on sequence analysis of the isolates is available at NCBI GenBank. The isolates were preserved on nutrient agar slants and kept at 4°C in the laboratory of Amity University Science and Instrumentation Centre (AUSIC) of Amity University Rajasthan, Jaipur, India. The rhizobacteria cultures were revived on nutrient medium (HiMedia Laboratory Pvt., Ltd., Mumbai, India) at 28±2°C for three days using serial dilution at 10^{-1}-10^{-8} prior to the experiments.

Screening of isolates for plant growth-promoting traits: The potentials of rhizobacteria isolates for plant growth-promoting activities such as; ammonia (NH_3) production, P solubilization, siderophore production, hydro-cyanide (HCN) production, nitrogenase activity, extracellular polysaccharide (EPS) production and phosphatase activity were tested by using the methods described by Ponmurugan *et al.*[18] and Etesami and Maheshwari[19].

Phytohormone characterization, recovery and quantification: The High Performance Liquid Chromatography (HPLC), Thin Layer Chromatography (TLC) and spectrophotometry methods were used for the characterization, recovery and quantification of phytohormones from isolates.

Characterization and recovery of IAA: After centrifugation, the supernatant was adjusted to 2.5 pH using HCl (1N). Extraction was done three times with solvent of ethyl acetate. The mixture was kept in rotary vacuum evaporator (Caliper Life Sciences, Hopkinton, MA, USA) for evaporation. High Performance Liquid Chromatography (HPLC) methanol (70%) was used to re-dissolve the remaining substance and spots of the extracts were placed on the silica gel plate. Isopropanol:ammonia:water in ratio 16:3:1 was used as mobile phase[19]. Pink spots were observed and R_f values of sample corresponding to IAA standard were calculated. The UV detector together with C_{18} column (Bibby, Scientific Ltd., Staffordshire, UK) in the HPLC was used to analyze the samples. Sample (100 µL) was passed through the filter of 0.45 millipore. This was passed through the column by injection. 1:30:70 (v/v) of acetic acid:methanol:water was used as solvent and reading was done by using spectrophotometer at 280 nm absorbance at run time of 20 min per sample. The standard IAA used was obtained from Sigmal-Aldrich (St Louis, MO, USA).

Characterization and recovery of cytokinin: Centrifugation of the inoculated M9 broth (HiMedia Laboratory Pvt., Ltd., (Mumbai, India) used was done for 20 min at 1000 rpm. A millipore filter (MilliporeSigma, Bedford, MA, USA) was used to filter the supernatant (cell free) and dried by lyophilization. Ethyl acetate was used for extraction three times and stored at 20°C in methanol. The Thin Layer Chromatography (TLC) was employed and samples were spotted on the plate. Mobile phase was standard cytokinin consisting of kinetin and 6-benzyladenine with 12:3:% (v/v) ratio of n-butanol:acetic acid:water. Formation of fluorescent spots were observed and R_f values of sample corresponding to cytokinin standard (Sigmal-Aldrich, St Louis, MO, USA) were calculated. Solvent system methanol (70%), kinetin and 6-benzyladenine standards in HPLC with flow rate of 0.5 mL/min, 270 nm absorbance and pressure (8.6 MPa) was run for production of cytokinin[6].

Characterization and recovery of gibberellic acid: Nutrient medium (HiMedia Laboratories Pvt., Ltd., Mumbai, India) cultures of isolates were used for the characterization of gibberellic acid by centrifuging for 20 min at 10,000 rpm after 5 days of incubation. Ethyl acetate was used to extract supernatant three times and preserved at -20°C in methanol (70%). Thin Layer Chromatography (TLC) with isopropanol ammonium hydroxide (25%):water at ratio of 10:1:1 (v/v) was used. Detection of spots was achieved by spraying with H_2SO_4 (3%) in methanol consisting of $FeCl_3$ (50 mg). Production of green spot on plates under UV light after heating for 10 min. Injection of C_{18} HPLC column was done and released at flow rate of 0.5/min while absorbance was determined at 206 nm. Retention time of eluting fractions and corresponding pure standard were collected[20].

Quantification of IAA: Active cultures of the rhizobacteria isolates were inoculated in IAA production medium (10 g L^{-1} peptone, 3 g L^{-1} yeast extract, 5 g L^{-1} tryptophan, 0.5% tryptone and 15 g L^{-1} agar) purchased from HiMedia Laboratories Pvt., Ltd., (Mumbai, India) was adjusted to pH of 6.8-7.0. About 50 mL of the IAA medium was inoculated with the isolates covered with black paper, incubated for 72 h at 30°C and rotated at 160 rpm. After 72 h of incubation, 5 mL sample of medium was obtained and centrifuged for 20 min at 1000 rpm. Salkowski's reagent consisting of 0.5 m $FeCl_3$ solution (1 mL) and 35% per chloric acid (50 mL) was used to assay the medium and incubated in the dark for 1 h[5]. A visible spectrophotometer (Tecan, Switzerland) was used to measure absorbance (536 nm). The experiment was carried out in triplicates.

Quantification of cytokinin: The medium used for the quantification of cytokinin was M9 medium consisting of thiamine (0.01%), biotin 2 pg L^{-1} and casamino acid (0.2%) using the method described by Liu *et al.*[20]. About 100 mL of M9 medium in 250 mL flask was inoculated with the culture (1 mL) and incubated under rotation (160 rpm) at 28±2°C for 5 days. M9 medium without supplements was used as control for the experiment in triplicates. Cytokinin production was quantified at 665 nm at 72, 96 and 120 h by using spectrophotometer.

Quantification of gibberellic acid: Quantification of gibberellic acid was done by adding culture suspension to nutrient medium. The nutrient medium was incubated for five days at 30±2°C and centrifuged for 20 min at 10,000 rpm. The supernatant obtained was adjusted to pH 2.5 by using HCl (15%). Extraction of the filtrate was done by using ethyl acetate in ratio 1:3 (filtrate:solvent) and gibberellic acid was determined from the extract. Gibberellic acid was measured at 254 nm absorbance[21] using spectrophotometer. Experiment was carried out at 72, 96 and 120 h in triplicates.

Statistical analysis: Mean, analysis of variance (ANOVA), Coefficient of Variance (CV) and percentage recovery were calculated by using Excel Microsoft word (2007).

RESULTS

Plant growth-promoting traits: The four rhizobacteria isolates used in the present experiment showed varied capacity for the growth-promoting traits examined (Table 1). Isolate IR25 gave maximum results for ammonia production (45 μg mL^{-1}), siderophore production (33 μg mL^{-1}) and nitrogenase activity (101 nmol C_2H_4 h^{-1}). This was closely followed by isolate IR28 with 43, 32 and 96 μg mL^{-1}, respectively for the same attributes. Highest value for P solubilization was recorded by isolate IP32 (400 μg mL^{-1}) followed by isolate IP35 (367 μg mL^{-1}). The values recorded for siderophore production (26 μg mL^{-1}) and nitrogenase activity (91 nmol C_2H_4 h^{-1}) by isolates IP32 and IP35 were comparatively lower than values recorded by isolates IR25 and IR28. Weak positive result was recorded by isolate IP35 for HCN while other isolates gave strong positive results for phosphatase activity, EPS and HCN production.

Phytohormone characterization and recovery: The HPLC and TLC screening for phyto-hormone gave positive results by all isolates. Results of analysis produced pink, fluorescent and green colour spots for IAA, CK and GA_3, respectively. Except isolate IP35 that recorded relatively low R_f value (0.2) for CK, other isolates gave significantly high R_f values for all the phyto-hormones (Table 2). Results of phytohormone recovery (Fig. 1) indicated more than 80% recovery of CK and GA_3 from isolate IR25. Similarly, more than 85% IAA and 80% GA_3 were recovered from isolates IP32 and IP35, respectively. The CK and GA_3 recovered from isolate IP32 were 73 and 70%, respectively. Isolate IP35 recorded 75% IAA recovery and lowest percentage recovery (12%) for CK.

Quantitative analysis of phytohormones: Results of spectrophotometric quantification of phytohormones synthesized by the rhizobia isolates (Fig. 2a-c) indicated increased phytohormones concentration from 72 h time interval of incubation (Fig. 2a) till 96 h time interval (Fig. 2b). Beyond this (at 120 h), there appeared to be a general gradual decrease in phytohormone concentration values (Fig. 2c). However, all isolates synthesized significant quantities of phytohormones ranging from 226-243 μg mL^{-1} at the various incubation time intervals. Maximum quantities of IAA (243 μg mL^{-1}), CK (15 μg mL^{-1}) and GA_3 (33 μg mL^{-1}) were synthesized by isolate IR25 at 96 h incubation time (Fig. 2b). IP35 followed IR25 closely in IAA and GA_3 synthesis with 239 and 32 μg mL^{-1}, respectively while CK concentration (12 μg mL^{-1}) synthesized by isolate IR28 ranked next to isolate IR25.

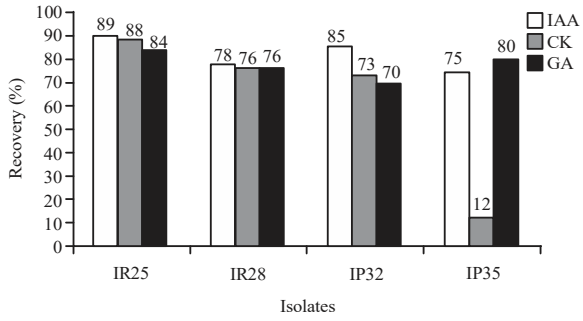

Fig. 1: Phytohormone recovery from the isolates

Table 1: Plant growth-promoting attributes of isolates

	Isolates			
Growth-promoting trait	IR25	IR28	IP32	IP35
Ammonia production (μg mL^{-1})	45	43	40	38
P solubilization (μg mL^{-1})	328	322	400	367
Siderophore production (μg mL^{-1})	33	32	26	28
Nitrogenase activity (nmol C_2H_4 h^{-1})	101	96	88	91
Phosphatase activity	++	++	++	++
HCN production	++	++	++	+
EPS production	++	++	++	++

++Strong activity/production, +Weak activity/production, IR25: *Azospirillum brasiliense*, IR28: *Azotobacter chrocoocum*, IP32: *Pseudomonas stutzeri*, IP35: *Pseudomonas putida*

Table 2: R_f values of phyto-hormones

		R_f value				
Phyto-hormone	Spot colour	Std Conc.	IR25	IR28	IP32	IP35
IAA	Pink	2.8±0.4	2.4±0.2	2.1±0.7	2.3±0.5	2.1±0.2
CK	Fluorescent	1.7±0.1	1.4±0.11	1.2±0.4	1.2±0.8	0.2±0.1
GA_3	Green	2.5±0.3	2.1±0.7	1.9±0.6	1.7±0.4	2.0±0.9

Std. conc.: Standard concentration, IR25: *Azospirillum brasiliense*, IR28: *Azotobacter chrocoocum*, IP32: *Pseudomonas stutzeri*, IP35: *Pseudomonas putida*

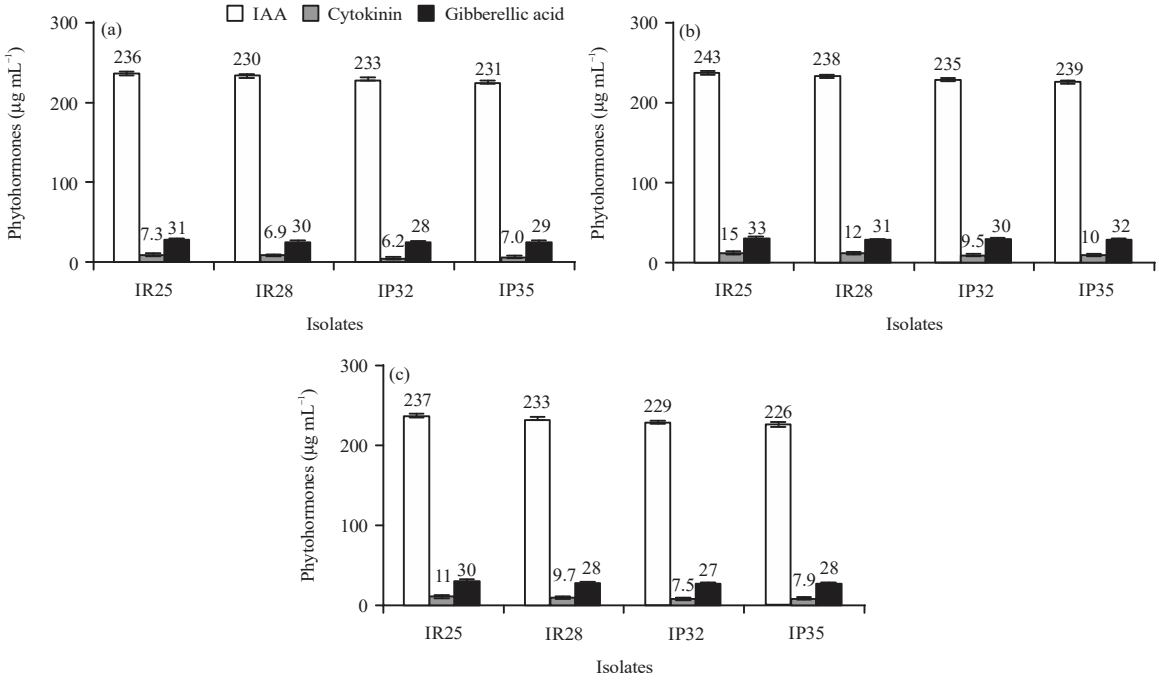

Fig. 2(a-c): Quantification of phytohormones at (a) 72, (b) 96 and (c) 120 h time intervals

DISCUSSION

In the current study, the positive results for all growth-promoting traits analyzed (Table 1) confirmed that these rhizosphere isolates are multifunctional agents of plant growth promotion, with multiple mechanisms for increasing soil fertility and crop productivity[22]. Ahemad and Kibret[8] have observed that extensive screening of rhizosphere micro-organisms for plant growth-promoting attributes is one of the basic steps for determining the efficacies of isolates for use either as biofertilizers, phyto-stimulators or bio-control agents with some rhizobacteria possessing overlapping roles.

The formation of pink, fluorescent and green colour spots for IAA, CK and GA_3, respectively (Table 2) confirmed the reliability of HPLC and TLC methods for characterization and screening of isolates. The use of HPLC and TLC methods to characterize and screen isolates for various phytohormones have been reported by several authors[5,20]. Also, the use of reference standard concentration values of 2.8±0.2, 1.7±0.4 and 2.5±0.1 for IAA, CK and GA_3, respectively were for the comparative analysis of R_f values of the phytohormones[2]. The observed variation in values of IAA produced by isolates in the present study confirmed the widely reported variability of rhizobacteria isolates to produce IAA[8]. However, increased level of IAA production by *Azospirillum*, *Azotobacter* and *Pseudomonas* species using tryptophan as precursor has been documented by various authors[7,23].

Also, Maheswari *et al.*[24] and Bhore and Sathisha[25] reported cytokinin production by *A. chrocoocum* using bioassay methods. However, the comparatively lower R_f value (0.2) recorded for CK by isolate IP35 in the present experiment (Table 2) conformed with results reported by Tisha and Meenu[6]. Several workers have indicated that ability to produce GA_3 is highly inherent in many groups of rhizobacteria[21,22]. In some other studies, *Azospirillum* and *Azotobacter* species were reported among efficient GA_3 producers[21]. Furthermore, Cassan *et al.*[26] also identified efficient GA_3 producing strains of *Azospirillum brasiliense* using selective ion monitoring (GC-MS.SIM) methods. The use of HPLC for phytohormone recovery (Fig. 1) in the current investigation ensured adequate retrieval of the phyto-hormones[6]. However, the recorded low percentage recovery (12%) of cytokinin produced by isolate IP35 could be ascribed to the corresponding low R_f value recorded by the phytohormone.

The high level of phytohormone concentration values recorded by all isolates (Fig. 2a-c) could be attributed to the fact that all isolates are Gram-negative organisms. Earlier studies have shown that most Gram-negative rhizocolonizers are efficient phytohormone producers[6]. In another study, Zhang *et al.*[1] reported that rhizobacteria cultivated *in vitro* are capable of producing sufficient amount of IAA, CK and GA_3.

The linear increase observed in concentration values of phytohormones from 72-96 h time intervals (Fig. 2a, b) and the subsequent decrease at 120 h time interval in the current experiment (Fig. 2c) could be attributed to activities of the various degrading enzymes in the medium[6].

The IAA concentration values (235-243 μg mL^{-1}) recorded at 96 h incubation time interval (Fig. 2b) in the present experiment is consistent with the results of Tisha and Meenu[6] who reported 230-260 μg mL^{-1} as the range of IAA produced by strains of rhizobacteria isolates at similar incubation time interval using HPLC-spectrophotometry methods. However, the range of CK values (9.5-15 μg mL^{-1}) recorded in the present study was significantly higher than the values (0.55-2.96 μg mL^{-1}) reported by Maheswari *et al.*[24] for CK produced by some indigenous rhizobacteria isolates. However, the values of GA_3 concentration (28-32 μg mL^{-1}) recorded in the present study were in accordance with the values reported by Pandya and Desai[21] for GA_3 produced by *Azotobacter* and *Azospirillum* species.

CONCLUSION

The study concluded that all indigenous rhizobacteria isolates used in the present investigation exhibited obvious multifunctional plant growth promoting traits and expressed maximum efficacies for biosynthesis of indole acetic acids, cytokinins and gibberellic acids. These bio-resources can be efficiently extracted and converted into marketable formulations that can serve as sustainable means of boosting vegetable crop productivity and ameliorating the prevailing abiotic stress threats in the semi-arid agro-ecological zone of Rajasthan desert.

SIGNIFICANCE STATEMENTS

This study discovered indigenous rhizobacteria (*A. brasiliense*, *A. chrocoocum*, *P. stutzeri* and *P. putida)* from the rhizosphere of vegetable crops in semi-arid agro-zone of Rajasthan desert capable of synthesizing phytohormone. This study will provide baseline information on the phytohormone biosynthesis profile of the isolates and the phytohormone produced can be transformed into commercial bio-formulations for use as promising growth promoters for achieving maximum sustainability and productivity of vegetable crops in the study area. Thus, the biotechnology employed can be adopted for evaluating phytohormone biosynthesis potentials of indigenous rhizobacteria in other regions with related abiotic stress challenges.

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