DOI: https://doi.org/10.53350/pjmhs22161771

ORIGINAL ARTICLE

Phytohormone biosynthetic potential of different Rhizobium species

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ABSTRACT

Plants may not have optimum growth hormone production potential under suboptimal growth and environmental conditions. The exogenous application of microbes is a potential and economical source of plant hormones. A laboratory trial was performed to evaluate the hormone biosynthesis potential of several soil microbes. Rhizobium sp. (Rhizobium trifolii, Rhizobium etli, Rhizobium meliloti, Rhizobium ciceri, Azorhizobium caulinodans, Bradyrhizobium japonicum, Rhizobium vulgaris, and Rhizobium phaseoli) were isolated from root nodules of various legumes and were biochemically characterized. Absorption spectroscopy (535, 254 and 665 nm λ) was used to quantify indole acetic acid (IAA), gibberellin and cytokinin production of different Rhizobium species. B_3 isolate of Rhizobium trifolii had yielded (14.54 and 21.37 μg mL^{-1}) IAA equivalents without and with L-tryptophan (L-TRP) supplement, that was statistically at par with same species while significantly greater than other Rhizobium isolates. Gibberellin production was found statistically non-significant in all isolates. Rhizobium phaseoli (Vp_1) yielded 1.68 μg mL^{-1} cytokinin that was at par with the same species isolates and with Rhizobium trifolii but was critically greater in amount than other species. It was concluded that Rhizobium plays a vital role in plant growth and development can produce auxin and gibberellins, but some could produce cytokinin.

Keywords: Phytohormone, Biosynthesis, IAA, PGPR, Auxin, Gibberellins, Cytokinin

INTRODUCTION

Plant physiology is influenced by plant growth regulators (PGR's) that are organic substances and their very lesser amounts are needed. Normal plant growth and development need PGR's that are being produced by the plant itself [1]. However, the plant's endogenous hormone production capacity may not be sufficient for optimal growth under suboptimal environment. Exogenous plant hormone supplementation may change the endogenous hormone level to modify growth and development in the anticipated direction and up to the requisite magnitude. Rhizosphere residing microflora (symbiotic and free-living) is a probable and cost-effective source of these PGR's. These microbes enhance plant growth directly phosphorus solubilization [2] and atmospheric nitrogen fixation. PGR's indirectly sequestering iron through siderophore production [3], produce appreciable amounts of phytohormones [4] i.e., indole acetic acid (IAA) auxins [5], cytokinins [6] and gibberellins [7] to boost plant growth. These diazotrophs also help in lowering of plant ethylene levels [8].

Among the many functions of IAA is the regulation of cell division, expansion, and differentiation in plant cells and tissues [9]. IAA performs a chief role in the formation and extension of xylem and plant root [10]. Biosynthesis of auxin is widespread amongst soil residing and plant-associated microbes. Furthermore, the production of auxin is an impacting trait both for plant growth-promoting rhizobacteria (PGPR) and plant pathogenic bacteria [11]. Auxins are the growth promoters or inhibitors that are produced by the plants their selves and by bacteria residing rhizosphere. These bacteria became prolific auxin producers when provided with L-TRP as precursors [12]. These precursors enhance rhizosphere microbiota's hormone production activity and capacity [13. They ensure the continuous and optimal provision of active ingredients for plant uptake which is exceptionally better than the one-time massive use of synthetic mixtures [14]. Frankenberger and Arshad [14] reported more phytohormones concentrations in soils where precursors were exogenously applied.

Gibberellins, tetracyclic diterpenoids regulate a lot many plants physiological and developmental processes [15] i.e. germination and emergence of seed [16], leaf and stem growth [17], flower and fruit growth [18], root growth and root hair abundance, inhibition of floral bud differentiation [19], break vegetative and reproductive bud dormancy (Bottini et al., 2004) and delay plant leaf senescence [20]. Gibberellins were 1st discovered in-filtrate of Fusarium moniliforme in 1926 and almost 25 years later 1st plant gibberellin was discovered from seeds of Phaseolus coccineus [21]. However, these are not the sole produce of plants and fungi but root and rhizosphere residing bacteria also produce gibberellins [22, 23]. In bacteria, there is no role of gibberellins, but these secretions help in the growth promotion of crop plants [24].

Cytokinin another plant growth regulator that promotes stomatal opening to stimulate shoot growth. Higher concentrations of cytokinin have a negative relation with root extensions. In drought conditions, cytokinin contents decrease resulting in more extended roots but shoot growth is redirected away from shoot due to stomatal closure [25].

All three hormones are synthesized by the rhizosphere inhibiting bacteria and are a major factor responsible for interaction with the host plant [26]. Lesser amounts of growth regulators are exogenously synthesized, but they exert a strong influence on morphology and physiology of crop [27].

In the present study, eight Rhizobium spp. have been cultured on their respective medium for phytohormone production and extraction, as an alternate strategy to improve plant growth and development by eliminating the usage of synthetic hormones.

MATERIALS AND METHODS

Three healthy plants from the fields of different legumes i.e. (berseem, lentil, mung bean, chickpea, sesbania, soybean, common beans, and vegetable pea) grown at farm area of Pakistan Agricultural Research Council, Arid Zone Research Center, Dera Ismail Khan, were uprooted in the respective season while the crop was at flowering stage. Plant roots were rinsed with tap water to get rid of mud and soil fragments and shifted these washed roots to the laboratory. Nodules were separated from roots using a sterilized needle. Any remaining contaminant was removed from the nodules by immersing them in a 0.5 percent sodium hypochlorite solution for 30 seconds before washing them with deionized (DI) water. Before being soaked in 70 percent ethanol for one minute, they are rinsed with DI water again [28]. After washing with DI water, they are dried with filter paper in Petri plates. These chemically decontaminated pink colored and larger-sized nodules were pierced with a decontaminated needle and streaked on yeast extract mannitol agar (YMA) media [29] plates and incubated at 28 °C for 72 hours. Growth was visually observed on Petri plates after 72 hours and fine-grown clusters were streaked several times until pure, fine, and regular colonies were attained. Isolate behavior towards different dyes i.e., Congo red, Bromothymol blue (BTB) and gram staining was identified using Keneni et al. [30] method. Then pure cultures were preserved on slants at 4±1 °C for later use.

General-purpose media (GPM) (Atlas, 1993) liquid culture was prepared and autoclaved at 121 °C temperature and 20 lbs/inch^2 pressure for 30 minutes. This liquid culture was cooled down at room temperature in the airflow chamber facing ultraviolet (UV) radiation to avoid any contamination. These cultures were shifted to test tubes and inoculated with already isolated bacterial cultures. Broth cultures were then incubated at 28±2 °C for 72 hours. An uninoculated standard was kept under the same conditions for comparison. These cultures then undergo different biochemical investigations i.e. urease (phenol red added to broth turns pink show urease activity) [31], catalase (air bubble generation with the addition of 3% H_2O_2 shows catalase activity) [32], nitrate reductase (addition of 1 mL of (8% N, N-Dimethyl-α-naphthylamine (Reagent A) and 6% Sulfanilic acid (Reagent B) solutions in 5N acetic acid) to 5 mL of broth, if red color appears in 2 minutes then nitrate reduction activity is positive) [33], citrate utilization (sodium citrate and ammonium as a nutrition source and BTB indicator, if medium color changes from green to blue is an indication of a positive test) [34], starch hydrolysis (Iodine addition gives blue color with starch if microbe clear the color means starch hydrolysis positive) [35] and motility (motility indole lysine (MIL) broth line inoculated, if diffuse cloud appears around line then motility positive) [36].

Table 1. Characteristic activities of different Rhizobium spp.

RHIZOBIUM SPP.	STRAIN	DYES			ACTIVITIES					
		Congo Red	BTB	Gram Reaction	Urease	Catalase	NO_3 Reductase	Citrate Utilization	Starch Hydrolysis	Motility
R. Trifolii	B_1	+ve	+ve	-ve	+ve	+ve	+ve	-ve	+ve	+ve
	B_2	+ve	-ve	-ve	+ve	+ve	+ve	-ve	+ve	+ve
	B_3	+ve	+ve	-ve	-ve	+ve	-ve	-ve	+ve	+ve
R. etli	L_1	+ve	-ve	-ve	-ve	+ve	+ve	-ve	+ve	+ve
	L_2	+ve	-ve	-ve	+ve	-ve	-ve	-ve	+ve	+ve
	L_3	+ve	+ve	-ve	-ve	-ve	-ve	-ve	+ve	+ve
R. meliloti	Mb_1	+ve	+ve	-ve	-ve	+ve	+ve	-ve	-ve	+ve
	Mb_2	+ve	+ve	-ve	-ve	+ve	+ve	-ve	-ve	+ve
	Mb_3	+ve	-ve	-ve	-ve	+ve	-ve	-ve	+ve	+ve
R. ciceri	C_1	+ve	-ve	-ve	+ve	+ve	-ve	-ve	+ve	+ve
	C_2	+ve	+ve	-ve	-ve	+ve	-ve	-ve	+ve	+ve
	C_3	+ve	+ve	-ve	-ve	+ve	-ve	-ve	+ve	+ve
A. caulinodans	S_1	+ve	-ve	-ve	+ve	-ve	+ve	-ve	+ve	+ve
	S_2	+ve	-ve	-ve	+ve	-ve	+ve	-ve	+ve	+ve
	S_3	+ve	+ve	-ve	+ve	+ve	+ve	-ve	+ve	+ve
B. Japonicum	Sb_1	+ve	-ve	-ve	-ve	+ve	+ve	-ve	+ve	+ve
	Sb_2	+ve	-ve	-ve	-ve	+ve	+ve	-ve	+ve	+ve
	Sb_3	+ve	-ve	-ve	-ve	-ve	-ve	-ve	+ve	+ve
R. vulgaris	Cb_1	+ve	+ve	-ve	+ve	+ve	+ve	-ve	-ve	+ve
	Cb_2	+ve	+ve	-ve	+ve	+ve	+ve	-ve	+ve	+ve
	Cb_3	+ve	+ve	-ve	+ve	+ve	+ve	-ve	+ve	+ve
R. phaseoli	Vp_1	+ve	+ve	-ve	+ve	-ve	+ve	-ve	-ve	+ve
	Vp_2	+ve	+ve	-ve	+ve	+ve	+ve	-ve	+ve	+ve
	Vp_3	+ve	+ve	-ve	+ve	+ve	+ve	-ve	+ve	+ve

Auxin production (AP) medium (peptone 10 g L^{-1}, yeast extract 3 g L^{-1}, tryptone 0.5% with and without L-TRP 5 g L^{-1} having pH adjusted 6.9±0.1) was prepared. AP medium was inoculated with Rhizobium spp. in 100 mL flask and incubated at 28±2 °C @160 rpm for 72 hours. Triplicates of each inoculum's 10 mL were collected after 72 hours and centrifuged at 1000 rpm for 20 minutes. The supernatant was then assayed using Salkovasky's (50 mL 35% $HClO_4$ and 1 mL 0.5M $FeCl_3$ solution) reagent with pink color after 1-hour dark incubation [37]. Auxin was calorimetrically quantified at 536 nm using a UV visible spectrophotometer (Spectronic-21).

The nutrient medium was inoculated with Rhizobium spp. and incubated at 28±2 °C @160 rpm for 72 hours. The medium was centrifuged @ 10,000 rpm for 20 minutes and the supernatant was collected having an adjusted pH of 2.5 with 15% HCl. Filtrate extracted with ethyl acetate (1:3 ratio) to convert gibberellic acid to gibberellenic acid and quantified calorimetrically @ 254 nm [38] using UV visible spectrophotometer (Spectronic-21).

M9 media with (0.2% Casamino acids, 0.01% thiamine and 2 pg of biotin) were inoculated with Rhizobium cultures and incubated at 28±2 °C @160 rpm for 72 hours. Cytokinins were calorimetrically quantified @ 665 nm [39] using a UV visible spectrophotometer (Spectronic-21).

RESULTS

Data regarding auxin production by various Rhizobium strains isolated from root nodules of a variety of crops is presented in table 2. Keeping in view the tabulated data it was observed that isolates of Rhizobium trifolii (B_3, B_2 and B_1) produced higher values of IAA equivalents of the order 14.62, 14.40 and 13.04 µg mL^{-1} and the amounts of production are more augmented with supplementation of L-TRP i.e., 21.35, 20.34 and 20.15 µg mL^{-1}, respectively. These IAA productions were statistically higher than all other isolates and minimal amounts were recorded in the isolates of Rhizobium vulgaris. It was observed that all selected isolates had produced some amount of IAA and the addition of L-TRP had pronounced the production of IAA by each isolate.

Table-2 also illustrates that each Rhizobium isolate had produced some quantity of gibberellins and the variation in production of gibberellins by different isolates of Rhizobium sp. can also be elaborated from table-2. It was observed that Vp_3 isolate of Rhizobium phaseoli had produced 4.24 µg mL^{-1} of gibberellic acid that was found statistically at par with all of the isolates except B_2 isolate of Rhizobium trifolii.

Production of 3rd major phytohormone (Cytokinin) has also been presented in table 2. It can easily be elaborated from a table that no or least detectable amounts of cytokinin were observed in the isolates of Rhizobium etli, Rhizobium ciceri, and Bradyrhizobium japonicum. Maximum produce of 1.68, 1.48 and 1.29 µg mL^{-1} of cytokinin was observed in isolates of Rhizobium phaseoli that were statistically similar in production with Rhizobium trifolii.

Table 2. Phytohormone production potential of different Rhizobium spp.

RHIZOBIUM SPP.	STRAIN	IAA EQUIVALENTS (WITHOUT L-TRP) (MG ML^{-1})	IAA EQUIVALENTS (WITH L-TRP) (MG ML^{-1})	GIBBERELLINS (MG ML^{-1})	CYTOKININ (MG ML^{-1})
R. Trifolii	B_1	13.04±0.07 b	20.15±0.28 a	2.36±0.24 ab	1.12±0.24 a-c
	B_2	14.40±0.24 a	20.34±0.04 a	1.27±0.15 b	1.13±0.25 a-c
	B_3	14.62±0.22 a	21.35±0.21 a	1.52±0.15 ab	1.19±0.27 a-c
R. etli	L_1	9.17±0.25 d	18.19±0.36 b	2.61±0.29 ab	0.00±0.00 e
	L_2	8.09±0.10 f	17.44±0.14 bc	2.51±0.25 ab	0.00±0.00 e
	L_3	8.65±0.07 d-f	17.65±0.12 bc	2.82±0.14 ab	0.00±0.00 e
R. meliloti	Mb_1	9.08±0.05 d	14.63±0.08 de	2.49±0.26 ab	0.82±0.18 b-e
	Mb_2	8.95±0.12 de	13.45±0.24 ef	2.29±0.24 ab	0.75±0.17 b-e
	Mb_3	8.98±0.05 de	15.01±0.01 d	2.55±0.25 ab	0.84±0.18 b-d
R. ciceri	C_1	11.35±0.05 c	16.57±0.24 c	2.82±0.32 ab	0.01±0.00 e
	C_2	11.87±0.09 c	17.24±0.09 bc	2.93±0.30 ab	0.03±0.00 de
	C_3	11.73±0.06 c	17.00±0.10 bc	2.89±0.27 ab	0.00±0.00 e
A. caulinodans	S_1	8.09±0.03 f	12.92±0.41 fg	2.19±0.23 ab	0.72±0.17 b-e
	S_2	8.17±0.04 ef	12.85±0.14 fg	2.19±0.24 ab	0.72±0.17 b-e
	S_3	8.04±0.02 f	12.48±0.29 fg	2.85±0.13 ab	0.90±0.07 a-c
B. Japonicum	Sb_1	3.63±0.33 i	7.35±0.61 ij	2.32±0.42 ab	0.07±0.06 de
	Sb_2	5.08±0.05 h	7.88±0.10 i	2.58±0.66 ab	0.02±0.00 de
	Sb_3	4.64±0.06 h	7.82±0.21 i	2.55±0.61 ab	0.03±0.00 de
R. vulgaris	Cb_1	2.80±0.40 ij	5.58±0.60 k	1.94±0.73 ab	0.58±0.17 c-e
	Cb_2	2.60±0.24 j	5.91±0.10 k	1.97±0.56 ab	0.60±0.12 c-e
	Cb_3	3.11±0.10 ij	6.04±0.15 jk	1.97±0.50 ab	0.60±0.10 c-e
R. phaseoli	Vp_1	6.35±0.02 g	10.83±0.10 h	3.59±0.99 ab	1.68±0.10 a
	Vp_2	6.29±0.03 g	11.60±0.27 gh	3.83±1.04 ab	1.48±0.27 ab
	Vp_3	6.24±0.11 g	12.83±0.08 fg	4.24±1.16 a	1.29±0.23 a-c

DISCUSSION

The current study was conducted to evaluate the capacity of various Rhizobium sp. to secrete different phytohormones. It was observed that all selected isolates had auxin and gibberellin producing capacity and the produce of IAA was additionally pronounced with the

addition of L-TRP. It was also observed that isolates of Rhizobium etli had not produced cytokinin while Rhizobium ciceri and Bradyrhizobium japonicum had produced very little amounts of cytokinin. To analyze biochemical features isolates had gone through different biochemical tests i.e. Dyes reaction (bromothymol blue test, congo red test, and gram reaction) and biochemical activities (urease, catalase, NO_3 reductase, citrate utilization, starch hydrolysis, and motility).

The use of PGPR to attain sustainable yield, plant nutrient uptake, and soil management has been becoming an emerging approach in modern agriculture from previous some decades [40]. Rhizobia are symbiotic partners of legumes but also can work as PGPR for cereals helping them through pronouncing growth mechanisms i.e. lumichromes production to help roots in CO_2 assimilation [4], riboflavin production to stimulate respiration of roots [41], root morphology improvement [42], root-soil adherence [43], increased nutrient availability (N & P) [44], siderophore production (Fe availability) [45], exopolysaccharides production [46] plant defense (Biocontrol) [47] and growth hormone release [48]. Among different growth regulator i.e., auxin, gibberellin, cytokinin, abscisic acid and ethylene [13, 49], auxin and gibberellin play vital role in plant-microbe continuum, and plant growth and development [50]. Auxin promotes the elongation of cells through expansion [51]. PGPR released growth regulators play a substantial role in the division of cells in root and cell differentiation that enhance the shoot growth of plants [52]. In the present research studies, Rhizobium species were isolated from their respective legumes and were assayed for auxin, gibberellin, and cytokinin producing efficiencies that can improve plant growth and development. Our observation regarding different plant hormones is closely related to previous investigations to assess the phytohormone producing capability of various rhizobacteria to enchant plant growth [53, 54].

CONCLUSION

Microorganisms play a pivotal role in the growth and yield of the plants improving the materials required. It is concluded from the facts from the table that Rhizobium spp. isolated from the Berseem and Chickpea has produced significantly higher amounts of auxin. While in case if these are aided with the precursor then the production is also enhanced as compared to that of the non-treated.

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