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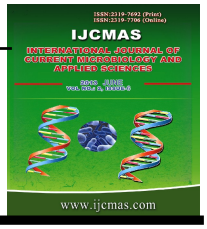

Original Research Article

Studies on phytohormone producing ability of indigenous endophytic bacteria isolated from tropical legume crops

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Keywords

Endophytic bacteria; tropical grain; legume crops; phyto-hormones.

A B S T R A C T

Bacterial endophytes are known to reside inside tissues of plants and can form a range of different relationships including symbiotic, mutualistic, commensalistic and trophobiotic. There has been increasing evidence, that endophytic bacteria can influence plant growth significantly by the production of phytohormones analogous to plant growth promoting rhizobacteria (PGPR) activity. In the present study, endophytic bacteria were isolated from various tropical grain legume crops namely redgram, blackgram, greengram, cowpea and chickpea. The population of endophytes in the roots, stems and leaves of different legume plants ranged from 2.1×10^3 to 8.6×10^3, 1.5×10^3 to 5.0×10^3 and 0.3 to 2.3×10^3 respectively. Based on the polyphasic characterization, 25 distinct isolates obtained were identified as *Bacillus* sp, *Micrococcus* sp, *Pseudomonas* sp, *Flavobacterium* sp and *Serratia* sp. All the isolates were evaluated for the production of phytohormones viz. gibberellic acid (GA), indole acetic acid (IAA) and cytokinin. The endophytic isolates produced GA from 0.75 to 2.83 μg ml^{-1}, had an IAA activity between 0.12 μg ml^{-1} to 6.46 μg ml^{-1} and cytokinin values were 0.52 μg ml^{-1} to 2.96 μg ml^{-1}. Hence the present study clearly establishes the beneficial effect of bacterial endophytes on tropical legumes.

Introduction

Plants are considered to be complex micro ecosystems wherein different habitats are exploited by a wide variety of bacteria. Symbiotic associations between microorganisms and plants are ancient and fundamental. Endophytic microorganisms are an intriguing group of organisms associated within various tissues and organs of plants without causing any Stone *et al*., 2000). Microbial endophytes mainly bacteria and fungi, are detected after surface sterilization of a plant part and are assumed to originate from the seeds, the roots surrounding environment and the aerial portions of plants (Figueiredo *et al*., 2009).

All plants in nature harbor a diverse

community of endophytic bacteria which can positively affect host plant growth (Long *et al*., 2008). During the last few years, there has been an increased interest in exploring the possibility of extending the beneficial interactions between plants and endophytic bacteria. Endophytic bacteria have many beneficial effects on their host plant growth by producing phytohormones similar to that of plant growth promoting rhizobacteria (PGPR). Bacterial endophytes are isolated from various crops like rice, maize, alfalfa, sugarcane, pea, red clover etc (Verma *et al*., 2001; Zinniel *et al*., 2002; Ulrich *et al*., 2008).

There is a vast repertoire of literature on bacterial endophytes from temperate legumes while bacterial endophytes from tropical grain legumes are still yet to be explored. Hence an attempt was made to isolate bacterial endophytes from various parts of tropical pulse crops such as redgram, blackgram, greengram, cowpea and chickpea and their ability to produce plant growth promoting hormones were analysed.

Materials and Methods

Isolation and enumeration of bacterial endophytes

Different tropical grain legume plant samples viz., Redgram, Blackgram, Greengram, Cowpea and Chickpea were collected from Pulse Breeding Station, TamilNadu Agricultural University, Coimbatore, TamilNadu, India. These plants were uprooted from the field and transferred in sterile polybags and brought to the laboratory. The method adopted for the isolation of endophytes from the roots, stems and leaves was according to Hallmann *et al.*, (1997) and Zinniel *et al.*, (2002). The plant samples were washed several times to remove adhering soil particles. It is then separated into stems, roots and leaves. Each part were cut into sections of 2-3cm length and dried on absorbent towels.

One gram of each plant part was surface sterilized for 10 seconds with 70% ethanol and 1% chloramine-T. These plant parts were washed thoroughly with sterile distilled water thrice to remove traces of chloramine-T and homogenised in a sterile pestle and mortar in laminar flow chamber. The homogenate was transferred into 10 ml sterile phosphate buffer and serially diluted upto 10^{-4} and pour plating was done using Tryptic soy agar (TSA) medium and R2A medium.

Characterization of the endophytic bacterial isolates

The endophytic isolates were subjected to a set of morphological and biochemical tests such as colony characteristics, Gram staining, motility, catalase, oxidase, IMViC, starch hydrolysis, urease, gelatin liquefaction, H_2S production, nitrate reductase and carbohydrate fermentation tests according to Bergey's manual of systematic bacteriology.

Production of growth promoting substances by the bacterial endophytic isolates

Determination of Gibberellic acid (GA)

GA was determined as per the method described by Tien et al (1979). The cultures were grown in nutrient broth for 7 days at room temperature and centrifuged for 10 minutes at 10,000 rpm. The supernatants were collected and acidified to pH 2.0 with 5 N HCl and extracted with

equal volumes of ethyl acetate twice or thrice. The ethyl acetate phase was evaporated at 32°C and the residue was redissolved in 2 ml of distilled water containing 0.05% of Tween 80. GA was estimated spectrophotometrically by the method of Mahadevan and Sridhar (1982) using zinc acetate and potassium ferrocyanide reagents. The absorbance was measured at 254 nm in UV–VIS spectrophotometer. From the standard GA solution, the amount of GA produced by the culture was calculated and expressed as μg / ml.

Determination of Indole Acetic Acid (IAA)

The cultures were inoculated in two sets of flaks, one with freshly filter sterilized solution of 0.2% (w/v) L-Tryptophan and other set without tryptophan (Tien *et al*., 1979). The inoculated endophytic bacterial isolates were incubated at 37°C for 7 days in dark and the cultures were centrifuged at 6000 rpm for 10 min. Then the supernatant solution was adjusted to pH 2.8 with 1N HCl and equal volume of diethyl ether was added and incubated in dark for 4 h. IAA extraction was done at 4°C by keeping overnight in a separating funnel using diethyl ether. The solvent phase was pooled, evaporated to dryness and 2 ml of methanol was added to the dried residue and the IAA present in the methanol extract was determined according to Gorden and Paleg (1957) using Salpers reagent.

The intensity of pink colour was read at 535 nm in a double beam spectrophotometer. From the standard curve prepared with known concentrations of IAA, the quantity in the culture filtrate was determined and expressed as μg /ml of culture.

Estimation of Cytokinin

The isolates were grown in nutrient broth for 7 days, centrifuged at 10,000 rpm for 10 minutes and the supernatants were collected and adjusted to pH 2.8 with 1N HCl. To the culture filtrate, equal volume of ice cold diethyl ether was mixed and allowed to stand for 4 h at 4°C with intermittent shaking. The organic phase was evaporated to dryness in the dark and the residue was dissolved in 2.0 ml of absolute methanol and used for radish cotyledon bioassay (Letham, 1971). Radish seeds were surface sterilized with 0.5% sodium hypochlorite solution and allowed to germinate on blotter paper in darkness at 24-25ºC for three days. Cotyledons of uniform weight were selected, placed on filter paper in petriplates and 10 ml of 2mM potassium phosphate buffer of pH 5.9 was added, followed by the addition of one ml of cell free culture extract. Similar set of experiments were conducted with various concentrations of Benzyl amino purine *viz.*, 2, 4, 6, 8 and 10 μg/ml, instead of culture extract as standard. Cotyledons were incubated under fluorescent light for three days at 24ºC. The weights of the cotyledons were recorded after drying with blotting paper and a dosage response curve was drawn.

Result and Discussion

Isolation and enumeration of bacterial endophytes

The population of bacterial endophytes present in the different parts of legume crops such as blackgram, greengram, redgram, cowpea and chickpea were enumerated by using two different media viz., TSA and R2A medium. In general, endophytic bacterial population of various

plant parts was found more in R2A medium than in TSA medium. In all the above legume crops, root samples recorded the maximum endophytic bacterial population followed by stem and leaf samples. Among the various plant parts, blackgram roots harboured the maximum number of bacterial endophytic population of 7.6 X 10^3 and 8.6 X 10^3 cfu gm^{-1} of sample in TSA medium and R2A medium respectively. A lowest population of 0.3 X 10^3 cfu gm^{-1} of sample was observed in the leaf sample of chickpea when R2A medium was used (Table 1).

Characterization of bacterial endophytes

The 25 bacterial endophytes obtained from various legume plant parts such as root, stem and leaves were characterized by employing various morphological and biochemical tests. Among the 25 isolates, 18 were rod shaped and other 7 were coccal shaped. Majority of the isolates appeared colourless, circular in form and with entire margin. Nine isolates were coloured such as yellow, orange and pink. Twelve isolates were Gram positive and remaining 13 were found to be Gram negative. All the isolates showed positive for catalase and negative reaction for hydrogen sulphide production and casein hydrolysis. The reaction for starch hydrolysis, oxidase, citrate utilization, gelatin hydrolysis, indole production, MR-VP tests varied with isolates. Based on the morphological and biochemical tests as per Bergey's Manual, the isolates were identified tentatively as *Bacillus* sp. (BR12, BR13, BR15, BR16, GR18, GR19, BS25), *Micrococcus* sp. (RS1, RR6, RR7, RR8, RS10, GR17, BS24), *Pseudomonas* sp. (RR2, RR3, RL9, BL11, GR20, CR21, BS23), *Flavobacterium* sp. (BR14, BS22) and *Serratia* sp. (CpL4, CpL5).

Production of plant growth promoting substances by bacterial endophytes

Production of plant growth promoting substances such as gibberellic acid, indole acetic acid and cytokinin by the various bacterial endophytic isolates was assessed by adopting spectrophotometeric and bioassay methods. All the isolates produced gibberellic acid and the results are presented in Table 2. The maximum production was recorded in GR 19 isolate (2.83 µg ml^{-1}) and the minimum of 0.75 µg ml^{-1} with BR13 isolate.

Indole-3-acetic acid production by the endophytes was assessed by spectrophotometry. The results are presented in Table 2. All the isolates produced more amount of IAA in the presence of tryptophan in the medium. A wide range of variation existed in the IAA producing ability between the endophytic isolates. It ranged from 1.06 µg $ml^{-1}$to 6.46 µg ml^{-1} (with tryptophan) and 0.03 µg ml^{-1} to 0.12µg ml^{-1} (without tryptophan). In the presence of tryptophan, the isolates RS10 and BS25 produced the maximum amount of IAA (6.46 µg ml^{-1}) whereas in the absence of tryptophan the isolate BS22 produced the maximum amount of IAA (0.12 µg ml^{-1}).

Cytokinin production was tested by radish cotyledon bioassay method. The weight increase of radish cotyledons due to the addition of solvent extracts of the culture filtrate of various endophytic bacterial isolates was measured. It was compared with the effect of different concentrations of standard benzyl amino purine on radish cotyledon. Among the isolates, GR19 recorded maximum concentration of cytokinin (2.96 µg ml^{-1}) as indicated by the increase in cotyledon weight (25.3 mg). It was followed by BR14, GR17,

Table.1 Population of bacterial endophytes in different parts of legume plants

Legume crops	Plant parts	Population in TSA medium	Population in R2A medium
		No $X10^3$ CFU g^{-1} of fresh weight of sample	
Redgram	Root	3.3	5.9
	Stem	2.3	3.6
	Leaf	0.6	1.5
Blackgram	Root	7.6	8.6
	Stem	5.0	4.3
	Leaf	1.6	2.3
Greengram	Root	5.3	6.6
	Stem	2.6	3.0
	Leaf	0.6	1.6
Cowpea	Root	4.1	2.1
	Stem	1.6	2.0
	Leaf	ND	0.6
Chickpea	Root	3.0	3.6
	Stem	1.0	1.3
	Leaf	ND	0.3

Table. 2 Production of Gibberillic acid, IAA and cytokinin by the bacterial endophytes

S.No	Culture No	Gibberillic acid (µg/ml)	IAA production without tryptophan (µg/ml)	IAA production with tryptophan (µg/ml)	Concentration of Cytokinin (µg/ml)
1.	RS 1	1.68	0.05	3.16	0.55
2.	RR2	1.40	0.06	4.56	0.62
3.	RR3	2.27	0.05	1.59	1.57
4.	CpL4	1.51	0.04	2.72	0.52
5.	CpL5	0.90	0.04	1.64	0.97
6.	RR6	2.45	0.05	2.63	1.08
7.	RR7	2.40	0.03	1.59	2.03
8.	RR8	1.37	0.04	2.85	2.08
9.	RR9	0.83	0.04	1.93	0.56
10.	RS10	1.07	0.05	6.46	0.97
11.	BL11	1.25	0.06	1.06	1.41
12.	BR12	1.07	0.05	2.72	0.98
13.	BR13	0.75	0.04	2.63	1.42
14.	BR14	2.38	0.07	5.83	2.91
15.	BR15	1.97	0.04	2.47	1.06
16.	BR16	1.85	0.03	2.42	1.66
17.	GR17	2.42	0.06	3.86	2.72
18.	GR18	1.67	0.04	1.37	1.24
19.	GR19	2.83	0.06	5.69	2.96
20.	GR20	2.64	0.07	4.43	2.57
21.	CR21	1.69	0.04	2.50	2.46
22.	BS22	2.51	0.12	6.41	2.69
23.	BS23	1.85	0.04	3.16	2.04
24.	BS24	1.64	0.04	2.15	1.95
25.	BS25	2.41	0.11	6.46	2.68
	SEd	0.08	0.002	0.15	0.07
	CD (0.05)	0.16	0.005	0.30	0.14

SEd- Standard error of deviation; CD – Critical difference at 5% level

BS22, BS25 and GR20. The remaining isolates recorded comparatively lower cytokinin production (Table 2).

Isolation and enumeration of bacterial endophytes

Plant roots colonized by endophytes are an important reservoir for plant growth promoting rhizobacteria which promote yield efficiently (Boddey and Dobereiner, 1988). In the present study, the populations of endophytic bacteria present in various parts of legume crops was estimated by surface disinfection and trituration. The population of endophytes was found to be more in the roots than stems and leaves. The colonies were

observed only up to 10^3 dilutions per gram of fresh weight of plant samples. Similar results were reported by Zinniel *et al.*, (2002) and Jacobs *et al*., (1985).

In this study, population in R2A medium was more than the population in TSA medium. McInroy and Kloepper (1995b) obtained similar results while isolating endophytic bacteria from sweet corn and cotton. Higher populations of endophytic bacteria were observed in root than stem and stem populations were higher than leaf populations. This decrease of endophytic bacterial populations acropetally has been already reported by Elvira-Recuenco and van Vuurde (2000) and McInroy and Kloepper (1995a). This observation indicates that soil is an important source of endophytic bacteria; they are thought to enter the plant by fractures/local degradation in the root system and may either become localized at the point of entry or spread throughout the plants (Figueiredo *et al*., 2009).

Characterization of bacterial endophytes

Twenty five distinct colonies were identified based on their colony morphology and all these isolates were further characterized by various biochemical tests. It was observed that the endophytic bacteria were both Gram positive and negative in nature. Similar observation was recorded by Gardner *et al.,* (1982) and Zinniel *et al*., (2002) who reported that approximately equal percentages of gram-positive (41%) and gram-negative (42%) bacteria were recovered from the agronomic crop plants. Some of the endophytic bacteria exhibited pigmented colony and this kind of characteristic among the plant endophytes was observed by Thakuria *et al.,* (2004) and Khan and Doty (2009). The presence of *Serratia, Agrobacterium* sp*, Rhizobium* sp*, Bacillus* sp*, Curtobacterium* sp*, Pantoea* sp*, Micrococcus sp, Xanthomonas* sp*, Pseudomonas* sp*, Flavobacterium* sp*, Erwinia* sp and *Arthrobacter* are reported in various agronomic crops (McInroy and Kloepper, 1995a; Sturz *et al*., 1998, 2000; Zinniel *et al*., 2002).

Production of plant growth promoting substances by bacterial endophytes

The plant growth hormones like gibberellins, IAA and cytokinin play important role in bacterial plant interactions (Dobbelaere *et al*., 2003). In the present study, all the isolates were examined for their ability to produce IAA, GA and cytokinin. All the endophytic isolates were found to produce gibberellic acid, which ranged from 0.75 to 2.83 µg ml^{-1}. It is in accordance with the results of Gonzalez-Lopez *et al*., (1986) who reported strains of *A. vinelandii* produced GA_3 from 0.8 to 3.1 µg ml^{-1}. Production of 6.0 µg ml^{-1} by *B. polymyxa* was reported by Holl et al. (1988). Production of 0.001 to 0.5 µg ml^{-1} of GA by *Pseudomonas* sp, *Flavobacterium* sp. and *Brevibacterium* sp. was recorded by Brown (1972).

A wide range of variation existed in the IAA producing ability between the endophytic isolates. Production of IAA by various rhizobacteria and *Azospirillum* in the absence of exogenous supply of tryptophan was reported by Wang et al. (1984) and Thuler *et al*., (2003). The amount of IAA produced by the individual isolate was enhanced by the addition of precursor tryptophan in the medium. Karthigai Selvi and Purushothaman (1996)

reported three fold increase of IAA production by the addition of tryptophan in the culture medium of an endophytic bacterium, *Acetobacter diazotrophicus*.

Letham (1971) developed a bioassay method for quantifying the cytokinin activity. Cytokinin-like activity was reported in *A. chroococcum* by Nieto and Frankenberger (1990), Barea and Brown (1974) and Bhore and Sathisha (2010) by adopting bioassay method. Production of cytokinin by *P.fluorescens* and *P.putida* in e culture medium was reported by Nieto and Frankenberger (1989). In this study also, it was observed that all the endophytic bacterial isolates are found to exhibit cytokinin-like activity. The amount of cytokinin produced by various isolates as indicated by the increase in weight of radish cotyledon ranged from 0.55 to 2.96 µg ml^{-1}. The concentration of cytokinin produced by *A.vinelandii, Azospirillum lipoferum* and *Pseudomonas* sp ranged from 1.8 to 5.4 µg ml^{-1} (Gonzalez-Lopez *et al.,* 1986) and Kalaivani (1998).

Plant endophytic bacteria contribute to plant growth and thereby crop productivity. A variety of possibilities are currently being explored which include the growth stimulation of axenic plants by incolulating with endophytes before transplantation. Crop productivity can also be enhanced by the combined application of endophytic bacteria with other beneficial microorganisms (Frommel *et al*., 1993; Sturz *et al*., 2000). Further investigation can be made to select and improve endophytic bacterial populations in crops and their soil sources improving crop growth. Hence, the use of endophytes in improving plant growth can be encouraged especially those that are indigenous to the soils and plants of the region.

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