



Population Dynamics and Diversity of Endophytic Bacteria Associated with Soybean (*Glycine max* (L) Merrill)

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Authors' contributions

This work was carried out in collaboration between both the authors. Author NK designed the study wrote the protocol and managed the analyses of the study. Author JD carried out the designed research work managed the literature searches and performed the statistical analysis. Both the authors read and approved the final manuscript.

Research Article

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ABSTRACT

Aim: Endophytic bacterial population and their diversity in soybean were investigated.
Study Design: Endophytic population was assessed during different growth stages of soybean (CV JS 335) viz., vegetative and reproductive stages.
Place and Duration of Study: Microbiology Research Laboratory, Department of Microbiology, R. A. Mahavidyalaya, Washim (MS), India, during the cultivation period of June-December 2010.
Methodology: Healthy plants of soybean were screened from the different locations of Washim district (M. S., India). Samples represent each growth stage viz., vegetative (V1-V5) and reproductive (R1-R8) were collected. Population densities were expressed as log₁₀ colony forming units (CFU) g⁻¹ fresh weight. The isolates were identified to genus level according to Bergey's Manual of Determinative Bacteriology on the basis morphological, cultural and biochemical characteristics.
Results: The maximum endophytic population was recorded for vegetative stage at V5 and V4 (5.74 and 5.01 log₁₀ CFU g⁻¹ fresh weight) and for reproductive stage at R2, R1 and R3 (5.84, 5.80 and 5.74 log₁₀ CFU g⁻¹ fresh weight). A total of 572 (35.50 %) from vegetative growth stages and 1039 (64.50 %) from reproductive growth stages bacterial isolates were obtained. The endophytic isolates were identified as members belonging to the genera

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Pseudomonas, Bacillus, Enterobacter, Klebsiella, Acetobacter Burkholderia, Rhizobium and Xanthomonas.

Conclusion: As soybean development progresses endophytic population increased. At maturity, the high population density was observed and thereafter the population declined.

Keywords: *Plant-microbe interaction; endophytic bacteria; soybean (Glycine max (L) Merrill); vertisol; population variance.*

1. INTRODUCTION

Plant-microbial interactions have been a premier area of research interest; as plants present an excellent ecosystem for microorganisms where different niches are exploited by a wide variety of bacteria [1]. Microbial endophytes are typically defined as plant associated microbes that colonize living internal tissues of plants without causing any visible symptoms or immediate over-negative effects and can be isolated from surface disinfected plant tissue [2,3,4]. The occurrence of endophyte is ubiquitous and usually may include bacteria, fungi and actinomycetes [5]. These microorganisms include both commensal species and mutualistic symbionts [6].

Endophyte-plant associations have been found to improve plant health and may help host plant to rescue from various biotic and abiotic stresses [7,8]. They may also provide fitness benefits to host plants such as tolerance to herbivory, heat, salt, disease, and drought and increased below and aboveground biomass etc. [9,10]. Thus, endophytic colonization improves the ecological adaptability of the host. Hence endophytes may be regarded as a true companion of host.

The unique ecological niche has made endophytic bacteria as attractive and potentially promising tool for agricultural applications especially, for those bacteria having commercial features such as plant growth promotion and activation of plant defense mechanisms [11]. Several bacterial endophytes have been reported as potential biocontrol agents that may improve and promote plant health [12]. Various reports indicate that these bacteria exist in a variety of tissue types within numerous plant species, suggesting a ubiquitous existence in most plants [13]. The bacterial endophytic population may originate from the usual soil bacterial community and a great diversity has been reported in diverse plant community [14].

Soybean (*Glycine max* (L.) Merrill) is an Asiatic leguminous plant, occupying large acreages of land worldwide for its oil and protein [15]. In recent years, soybean has assumed important position in India. It has well adapted to black soils of central and peninsular India. Major soybean producing states in India including, Madhya Pradesh, Maharashtra and Rajasthan, contribute about 97% to total area and 96% production of soybean in the country [16]. Maharashtra is the second largest soybean producing state in India. It accounts for 34% of the India's bean production. Soybean is gaining popularity on account of its unique characteristics and adaptability to vary agro-climatic conditions [17]. Washim is an important soybean producing area of this region, occupying 2095 ha of area with production of 2987 tones during 2010-2011 [18].

The study of the structure of endophytic microbial populations, their distribution, interaction and functions within their host is important for understanding their ecological role [19]. Analysis of the structure of microbial populations has practical importance; the results can be

used to assess the fate of released strains and their impact on resident microbial communities [6]. Hence, the present investigation was carried out to study variation in distribution and diversity of endophytic bacterial population colonizing soybean.

2. MATERIALS AND METHODS

2.1 Study Area

Washim (Latitude: 20° 05'58.90" N, Longitude: 77° 08'11.82" E, Altitude: 600M MSL) is situated in central Vidarbha region of Maharashtra state of India. The climate of this region is hot moist semi arid and the soil type is deep clayey to shallow black soil (Fig. 1).

2.2 Sample Collection

Healthy plants of soybean were screened from six different locations of Washim district during the cultivation period of June-December 2010. Within each location, randomly 3 different sub-locations were selected for sample collection. From each sub-location 3-5 healthy plants were selected. The growth stages of soybean were identified as specified by McWilliams et al. [20]. Sample represent of each growth stage viz., vegetative (V1-V5) and reproductive (R1-R8) were collected. The plants were uprooted, sealed into plastic bags and labeled. All samples were processed immediately after collection.

2.3 Isolation and Identification Endophytic Bacteria

The collected plants were washed under tap water to remove soil and separated into root, stem and leaf. All root, stem and leaf samples were washed twice in distilled water then surface sterilized as described by Hung and Annapurna [3] and Pimentel et al. [5]. Surface sterilization was carried out by immersion for 1 minute in 70% (v/v) ethanol, in 0.1% HgCl₂ upto 3 minutes out for roots and nodules, whereas, upto 5 minutes for leaves and stems respectively. The tissue was then washed ten times using sterile distilled water. Sterility checks were carried out by monitoring the rinse wash water for the presence or absence of microbial growth on nutrient media for 6 days at 30°C. The absence of growth was taken into consideration as positive test for surface sterilization.

One gram of surface disinfected samples was macerated [3]. The slurry was filtered through sterile cotton cloth. The filtrate was serially diluted and spreaded onto nutrient agar supplemented with nistatin (200 µg/mL). For any slow growing bacteria the filtrate was enriched in nutrient broth for 24 hrs. at 30°C and further processed for isolation. Plates were dried in a laminar flow cabinet for 15 min before incubation at 28-30°C for 2-3 days. Bacterial population densities were expressed as log₁₀ colony forming units (CFU) g⁻¹ fresh weight [21]. From culturable population a total of 1611 distinct bacterial isolates were isolated and identified upto genus level on the basis of morphological, cultural and biochemical characterization according to *Bergey's Manual of Determinative Bacteriology* [22].

2.4 Statistical Analysis

The results were analyzed by ANOVA using data analysis in MS-Excel.

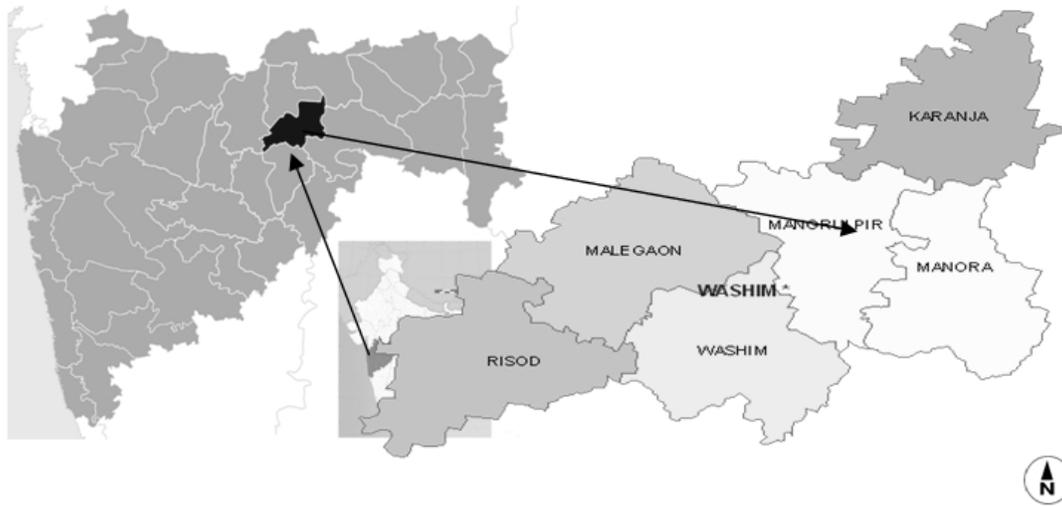


Fig. 1. Location of study area

3. RESULTS AND DISCUSSION

3.1 Variation of Endophytic Bacterial Population

Endophytic bacterial population was found to increase with plant development (Table 1). However, at onset of reproductive stages viz., R1-R8 the endophytic population started to decrease and attained a constant value at R8 stage. Thus, it was observed that endophytic population declined, as plant Senesced. The maximum endophytic population was recorded for vegetative stage at V5 ($5.74 \log_{10} \text{CFU g}^{-1}$ fresh weight) followed by V4 ($5.01 \log_{10} \text{CFU g}^{-1}$ fresh weight) and for reproductive stages at R2 ($5.84 \log_{10} \text{CFU g}^{-1}$ fresh weight) followed by R1 ($5.80 \log_{10} \text{CFU g}^{-1}$ fresh weight) and R3 ($5.74 \log_{10} \text{CFU g}^{-1}$ fresh weight).

3.2 Isolation of Endophytic Bacteria

A total of 1611 endophytic bacterial isolates were isolated; 572 (35.50 %) from vegetative growth stages and 1039 (64.50 %) from reproductive growth stages (Table 2). Based upon the plant parts, 709 (44.00 %) were isolated from roots, 431 (26.75 %) from leaves and 471 (29.23 %) from stems. It was found that the number of isolates obtained from vegetative stages is larger than that of reproductive stages. As a primary site of entry, the roots showed maximum population, followed by leaves and stems respectively.

The endophytic isolates were found to be member of the genera *Pseudomonas*, *Bacillus*, *Enterobacter*, *Klebsiella*, *Acetobacter*, *Burkholderia*, *Rhizobium*, *Xanthomonas* and some unidentified genera (Table 3).

Table 1. Endophytic bacterial population (log₁₀ CFU g⁻¹ fresh weight) at different growth stages of soybean

Growth stage	Number of endophytic bacteria (log ₁₀ CFU g ⁻¹ fresh weight)*				
	Plant source			Mean ± SE _M	
	Root	Stem	Leaf		
Vegetative	V1	5.39	3.11	4.25	4.25 ± 0.65
	V2	5.63	3.23	4.39	4.41 ± 0.69
	V3	5.69	3.39	4.53	4.53 ± 0.66
	V4	5.82	4.59	4.63	5.01 ± 0.40
	V5	6.92	4.62	5.68	5.74 ± 0.66
					F _{0.05} =0.92 < F _{crit} =3.47
Reproductive	R1	6.94	4.75	5.72	5.80 ± 0.63
	R2	6.96	4.80	5.78	5.84 ± 0.62
	R3	6.80	4.68	5.76	5.74 ± 0.61
	R4	6.76	4.57	5.62	5.65 ± 0.63
	R5	6.72	4.49	5.50	5.57 ± 0.64
	R6	5.55	4.44	5.41	5.13 ± 0.34
	R7	4.44	3.23	4.23	3.96 ± 0.37
	R8	4.32	3.07	4.14	3.84 ± 0.39
					F _{0.05} =2.28 < F _{crit} =2.65

* Data are mean values of three independent experiments.

Table 2. Distribution of Endophytic bacterial isolates at different growth stages in soybean tissues

Growth Stage	Sample	Number of Endophytic Bacterial Isolates from plant			Total for sample	Total for growth stage
		Root	Stem	Leaf		
Vegetative	V1	25	13	18	56	572
	V2	43	17	25	85	
	V3	49	25	34	108	
	V4	67	39	43	149	
	V5	84	42	48	174	
Reproductive	R1	88	57	53	198	
	R2	93	64	61	218	
	R3	64	48	58	170	
	R4	58	38	42	138	
	R5	53	31	32	116	
	R6	36	28	26	90	
	R7	28	17	17	62	
	R8	21	12	14	47	
	Total	709	431	471		1039
						1611

Table 3. Cultural, morphological and biochemical characteristics of representative endophytic bacterial isolates

Characters	1	2	3	4	5	6	7	8
Cultural								
Shape	Irregular	Round	Circular	Round	Round	Round	Irregular	Irregular
Size	2-5 mm	2-3 mm	2-3 mm	2-5 mm	2-4 mm	2-3 mm	2-3 mm	2-5 mm
Elevation	Flat	Flat	Thin	Thin	Raised	Slimy	Mucoid	Mucoid
Surface	Rough	Smooth	Smooth	Thick	Smooth	Flat	Flat	Flat
Margin	Entire	Entire	Irregular	Entire		Regular	Undulate	Undulate
Color	Whitish	Off-white	Whitish	Cream	Yellowish	Cream	White	Cream
Pigmentation	Yellowish	-	-	Yellowish	-	Yellowish-Orange	-	-
Morphological								
Gram Reaction	+	+	+	+	-	-	-	-
Cell Shape	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod
Biochemical								
Glucose	+	+	+	+	-	-	-	+
Sucrose	-	+	-	+	-	-	-	+
Lactose	+	-	+	+	-	-	-	-
Maltose	+	-	-	+	-	-	-	-
Indole	-		+	-	-	-	-	-
Methyl Red	-	-	-	-	-	-	-	-
Voges Prausker	+	-	-	+	-	-	-	-
Citrate	+	+	-	+	-	+	-	-
Catalase	+	-	-	+	-	+	-	-
Oxidase	-	-	-	-	-	+	-	-
Nitrate	+	+	+	+	-	+	-	-
Starch Hydrolysis	+	+	+	+	+	+	-	+
Gelatin	+	+	-	-	+	+	-	+
Liquefication								
Caesin	+	+	+	+	+	-	-	-
Hydrolysis								
H ₂ S	-	-	-	-	+	+	+	+
Possible genus	<i>Bacillus</i>	<i>Bacillus</i>	<i>Bacillus</i>	<i>Bacillus</i>	<i>Pseudomonas</i>	<i>Pseudomonas</i>	<i>Pseudomonas</i>	<i>Pseudomonas</i>

Characters	9	10	11	12	14	15	16	17
Cultural								
Shape	Irregular	Circular	Round	Irregular	Round	Round	Round	Round
Size	2-5 mm	2-3 mm	2-3 mm	2-4 mm	4-5 mm	2-3 mm	2-3 mm	2-3 mm
Elevation	Slimy	Thin	Flat	Flat	Flat	Shiny	Convex	Convex
Surface	Flat	Flat	Smooth	Smooth	Smooth	Smooth	Mucous	Mucous
Margin	Undulate	Entire	Irregular	Entire	Entire	Entire	Smooth	Entire
Color	White	Yellowish	Whitish	White	Grayish	White	Yellowish	White
Pigmentation	-	-	-	-	-	-	-	-
Morphological								
Gram Reaction	-	-	-	-	-	-	-	-
Cell Shape	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod
Biochemical								
Glucose	-	+	+	-	+	-	-	-
Sucrose	-	+	-	-	-	-	-	-
Lactose	-	+	+	-	+	-	-	-
Maltose	+	+	-	-	+	-	-	-
Indole	-	-	-	+	-	-	-	-
Methyl Red	-	-	-	+	-	-	-	-
Voges	-	-	+	+	+	-	-	-
Prausker	-	-	-	-	-	-	-	-
Citrate	-	-	+	-	+	-	+	+
Catalase	-	-	-	-	-	-	+	+
Oxidase	+	-	-	+	-	+	+	-
Nitrate	+	-	-	+	-	-	-	-
Starch	+	+	-	+	-	-	-	-
Hydrolysis								
Gelatin	-	+	-	-	-	-	+	+
Liquefication								
Caesin	+	+	-	-	-	-	-	-
Hydrolysis								
H ₂ S	+	+	-	+	-	+	-	-
Possible genus	<i>Pseudomonas</i>	<i>Xanthomonas</i>	<i>Enterobacter</i>	<i>Rhizobium</i>	<i>Klebsiella</i>	<i>Acetobacter</i>	<i>Burkholderia</i>	<i>Burkholderia</i>

Our findings are in congruence with Zinniel et al. [5], who reported that the population size of endophytic bacteria in agronomic plants including soybean varied between 0-6.0 log₁₀ CFU/sample tissue. Endophytic bacterial population is affected by the growth stages of plant. At reproductive maturity (R1-R3) is population maximum as compared to other growth stages. Kuklinsky-Sobral et al. [1] also observed significant differences in bacterial population densities and found to be influenced by soybean growth phase and type of tissue sampled.

A decline in endophytic population was observed as plant aged. The decrease in endophytic population from vegetative to reproductive stages could have been due to unavailability of essential nutrients during maturation and senescence of plants [6, 23]. As plants mature all the nutritional requirements for bacteria are optimum and a stable endophytic population is obtained. Thus, there appear to be coincidence of plant maturity and endophytic population.

It thus seems that endophytic population is influenced by several factors that include the developmental stages environmental conditions and geographical location of the experimental site [1]. Moreover, genotype or cultivar of plant also affects significantly the endophytic population. Conn and Franco [24] reported that soil type to a large extent, determines the endophytic population.

4. CONCLUSIONS

It appears from the present study that as soybean development progresses; endophytic population increased. However, at maturity, the high population density was observed and thereafter the population declined. Thus, endophytic population is influenced by developmental stages of soybean plant. Moreover, research on endophytic bacteria offers an innovative and enlivening opportunity for the discovery of novel strains with biotechnological utility.

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COMPETING INTERESTS

The authors declare that no competing interests exist.

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