

Population Variance and Diversity of Endophytic Bacteria in Soybean (*Glycine max* (L) Merrill) Cultivated in Vertisol

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ABSTRACT

Aim: Variation in endophytic bacterial population and their diversity colonizing soybean cultivated in vertisol was 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 June-December 2010.

Methodology: Healthy plants of soybean were screened from the different locations of Washim district (M. S., India) Sample represent each growth stage viz., vegetative (V1-V5) and reproductive (R1-R8) were collected. 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 surface sterilized. The surface disinfected samples were macerated the filtrate was serially diluted and enriched. aliquots (0.1ml) of enriched broth were spreaded and incubated at 28-30°C for 2-3 days 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: A 572 (35.50 %) bacterial isolates were obtained from vegetative growth stages and 1039 (64.50 %) from reproductive growth stages. The maximum endophytic population was recorded at V4 and V5 (3.41 and 3.49 log₁₀ CFU g⁻¹ fresh weight) and R1-R3 (3.54, 3.61 and 3.65 log₁₀ CFU g⁻¹ fresh weight). The endophytic isolates were found to be belonging to the genera *Pseudomonas*, *Bacillus*, *Enterobacter*, *Klebsiella*, *Acetobacter* *Burkholderia*, *Rhizobium*, *Xanthomonas* spp.

Conclusion: Endophytic population was found to increase with soybean development. At maturity, the high population density was observed and thereafter the population declines significantly both in number and type of bacteria colonizing the soybean.

Key Words: 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 (Kuklinsky-Sobral et al., 2004). 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

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isolated from surface disinfected plant tissue (Wilson, 1995, Hung and Annapurna, 2004 and Zinniel et al., 2002). The occurrence of endophyte is ubiquitous and usually may include bacteria, fungi and actinomycetes (Pimentel et al., 2009). These microorganisms include both commensal species and mutualistic symbionts (Procopio et al., 2009).

Endophyte-plant associations have been found to improve plant health and may help host plant to rescue from various biotic and abiotic stresses (Hasegawa et al., 2006 and Sapak et al., 2008) and thus may provide fitness benefits to host plants including tolerance to herbivory, heat, salt, disease, and drought and increased below and aboveground biomass (Faeth and Fagan, 2002 and Backman and Sikora, 2008). Thus, endophytic colonization improves the ecological adaptability of the host and 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 (Hallman, 2001). Several bacterial endophytes have been reported as potential biocontrol agents that may improve and promote plant health (Reiter et al., 2002). Various reports indicate that these bacteria exist in a variety of tissue types within numerous plant species, suggesting a ubiquitous existence in most plants. The bacterial endophytic population may originate from the usual soil bacterial community and a great diversity has been reported in diverse plant community (Taghavi et al., 2005).

The study of the structure of endophytic microbial populations, their distribution as well as their interaction and functions within their host is important for understanding their ecological role (Hardoim et al., 2008). 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 (Procopio et al., 2009). Hence, present investigation was carried out to study variation in distribution and diversity of endophytic bacterial population colonizing soybean cultivated in vertisol.

2. MATERIAL AND METHODS

2.1 Sample collection: Healthy plants of soybean were screened from the different locations of Washim district (M. S., India) during the cultivation period June-December 2010. The soybean growth stages were identified as specified by McWilliams et al., 2004. Sample represent 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.

2.2 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, (2004) and Pimentel et al., 2006 with slight modifications. 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 after surface sterilization was carried out by monitoring separately the section impressions and rinse wash water for the presence or absence of microbial growth incubated for 6 days on nutrient media. The absence of growth was taken into consideration as positive test for surface sterilization.

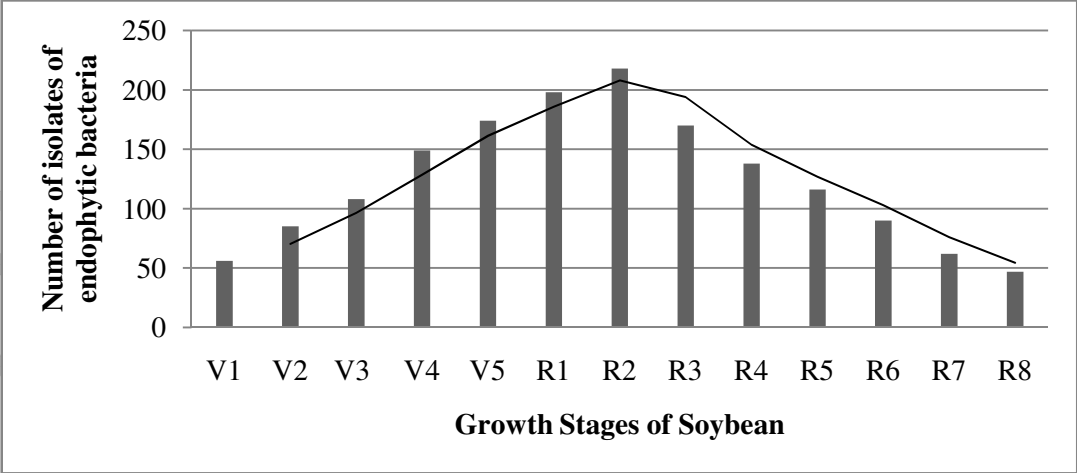
The surface disinfected samples were macerated (Hallmann et al., 1997). After filtering the slurry through sterile cotton cloth, the filtrate was serially diluted and enriched in nutrient broth supplemented with soybean plant extract (@ 5 %) for 24 hrs. at 30 °C and aliquots (0.1ml) of enriched broth were spread with a sterile glass rod over the surface of nutrient agar supplemented with nistatin (200 µg/mL). Plates were dried in a laminar flow cabinet for 15 min before incubation at 28-30°C for 2-3 days Population densities were expressed as log₁₀ colony forming units (CFU) g⁻¹ fresh weight (El-Tarabily et al., 2008). The isolates were identified to genus level according to *Bergey's Manual of Determinative Bacteriology* (Holt et al., 1994) on the basis morphological, cultural and biochemical characteristics

2.3 Statistical Analysis: The results were analyzed by ANOVA using data analysis in MS-Excel and the means were compared by Tukeys test ($P = 0.05$) (Pimentel et. al., 2006).

69 **Table 1: Total Number of Endophytic Bacteria ($\log_{10}$ CFU g^{-1} fresh weight)**
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Growth stage		Number of endophytic bacteria ($\log_{10}$ CFU g^{-1} fresh weight)		
		Plant source		
		Root	Stem	Leaf
V E G E T A T I V E	V1	1.71	1.44	1.64
	V2	1.79	1.56	1.76
	V3	1.85	1.65	1.80
	V4	1.90	1.73	1.86
	V5	1.97	1.79	1.92
R E P R O D U C T I V E	R1	1.86	1.75	1.81
	R2	1.85	1.71	1.78
	R3	1.80	1.68	1.76
	R4	1.76	1.63	1.71
	R5	1.72	1.61	1.67
	R6	1.66	1.57	1.62
	R7	1.64	1.51	1.53
	R8	1.61	1.50	1.34

71 * Values presented are mean values of three independent experiments; Values in parenthesis represents SE_M
 72 **Means followed by different letters are significantly different according to Tukeys Test ($P=0.05$)
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74
 75 **Fig.1: Distribution of Endophytic bacteria in vegetative and reproductive growth stage of soybean**
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3. RESULTS AND DISCUSSION

3.1 Isolation of endophytic bacteria: A total 1611 endophytic bacterial isolates were isolated, 572 (35.50 %) from vegetative growth stages and 1039 (64.50 %) from reproductive growth stages (Figure 1). 709 (44.00 %) isolated from root, 431 (26.75 %) from leaf and 471 (29.23 %) from stem. It was found that the numbers of isolates obtained from vegetative stages are larger than that of reproductive stages. It also appeared that the roots as a primary site of entry, show maximum population followed by leaf and stem.

The endophytic isolates were tentatively found to be belonging to the genera *Pseudomonas* Sp., *Bacillus* Sp., *Enterobacter* Sp., *Klebsiella* Sp., *Acetobacter* Sp., *Burkholderia* Sp., *Rhizobium* Sp., *Xanthomonas* Sp., and some unidentified genera.

3.2 Variation of endophytic bacterial population: Endophytic bacterial population (Table 1) increased progressively as plant grows. 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 declines both in quantity and variety as plant aged. The maximum endophytic population was recorded at V4 and V5 (3.41 and 3.49 log₁₀ CFU g⁻¹ fresh weight) and R1-R3 (3.54, 3.61 and 3.65 log₁₀ CFU g⁻¹ fresh weight). Significant variations in the endophytic bacterial population at the two growth stages were observed (Table 1). The population at V4 and V5 during vegetative and at R1, R2, R3 and R4 during reproductive growth stages were found to be statistically significant as compared to early and late growth stages.

In present investigation, the endophytic bacteria of soybean cultivated in vertisol were isolated. Our findings are in congruence with the findings of Zinniel et al., 2002. They reported that the population size of endophytic bacteria in agronomic plants including soybean varied between 0-6.0 log₁₀ CFU/sample tissue with mean (6.0 log₁₀ CFU/sample tissue). Kuklinsky-Sobral et al., 2004 also observed significant differences in bacterial population densities and found to be influence by soybean growth phase and tissue sampled. Pimentel et al., 2006 pointed that as plant ages the number of endophytic isolates decreases.

The endophytic population has been found to be related to several factors, including the developmental stage during which the plant is sampled, environmental conditions and the location of the plant (Kuklinsky-Sobral et al., 2004). Moreover, the genotype as well as cultivar of plant also affects significantly as the endophytic population is naturally selected by host. Conn and Franco, 2004 also reported that soil type, to a large extent, determines the endophytic population. 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 (Pimental et al., 2006 and McInroy and Kloepper, 1995). As, plant matures 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.

The endophytic population in plants is highly variable and occasionally transient. Hardoim et al., 2008 has thrown light on the endophytic nature of bacteria. The endophytic bacteria can be classified to be obligate and facultative. Obligate endophytes are strictly dependent on host plant for their growth and survival however; facultative endophytes can be biphasic alternating between plants and environment (mainly soil). Bulk of the endophytic microorganisms prefer to this facultative lifestyle. Even though, the endophytes often appear to be originating from soil, a suite of environmental and genetic factors is presumed to have role in enabling a specific bacterium to become endophyte. It is the cumulative effects of chance factors determined by the developing roots coming in contact with effective levels of bacteria and deterministic factors determined by the presence of dedicated genetic system that enable bacterial-plant crosstalk that could result in active endophytic colonization process.

4. CONCLUSION

It appears that the endophytic population is influenced by several factor including environmental conditions, developmental stages and geographical location. An understanding of the factors affecting the population of endophytic bacteria is necessary to achieve a consistent application of these microorganisms in relation to plant health and biocontrol. Research on endophytic bacteria offers an innovative and enlivening opportunity for the discovery of novel strains with biotechnological utility.

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