

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

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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 morphotypes-isolates were obtained. The endophytic isolates were found to be identified as members belonging to the genera *Pseudomonas*, *Bacillus*, *Enterobacter*, *Klebsiella*, *Acetobacter*, *Burkholderia*, *Rhizobium*, and *Xanthomonas* spp.

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

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 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. 2006). These microorganisms include both commensal species and mutualistic symbionts (Procopio et al. 2009).

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23 Endophyte-plant associations have been found to improve plant health and may help host plant to rescue from
24 various biotic and abiotic stresses (Hasegawa et al. 2006; and Sapak et al. 2008). They may also provide fitness
25 benefits to host plants such as tolerance to herbivory, heat, salt, disease, and drought and increased below and
26 aboveground biomass etc. (Faeth and Fagan, 2002; and Backman and Sikora, 2008). Thus, endophytic
27 colonization improves the ecological adaptability of the host. Hence endophytes may be regarded as a true
28 companion of host.

29 The unique ecological niche has made endophytic bacteria as attractive and potentially promising tool for
30 agricultural applications especially, for those bacteria having commercial features such as plant growth
31 promotion and activation of plant defense mechanisms (Hallman, 2001). Several bacterial endophytes have
32 been reported as potential biocontrol agents that may improve and promote plant health (Reiter et al. 2002).
33 Various reports indicate that these bacteria exist in a variety of tissue types within numerous plant species,
34 suggesting a ubiquitous existence in most plants (Rosenblueth and Martinez-Romero, 2006). The bacterial
35 endophytic population may originate from the usual soil bacterial community and a great diversity has been
36 reported in diverse plant community (Taghavi et al. 2005).

37 Soybean (*Glycine max* (L.) Merrill) is an Asiatic leguminous plant, occupying large acreages of land worldwide for
38 its oil and protein (Fayzalla et al. 2009). In recent years, soybean has assumed important position in India. It has
39 well adapted to black soils of central and peninsular India. Major soybean producing states in India, including
40 Madhya Pradesh, Maharashtra and Rajasthan, together contribute about 97% to total area and 96% production
41 of soybean in the country (Namrata et al. 2012). Maharashtra is the second largest soybean producing state in
42 India. It accounts for 34% of the India's bean production. Soybean is gaining popularity on account of its unique
43 characteristics and adaptability to varied agro-climatic conditions (Pawar et al. 2011). Washim is an important
44 soybean producing area of this region, occupying 2095 ha of area with production of 2987 tonnes during 2010-
45 2011 (Crop Production Statistics, Department of Agriculture, Government of Maharashtra).

46 The study of the structure of endophytic microbial populations, their distribution, interaction and functions within
47 their host is important for understanding their ecological role (Hardoim et al. 2008). Analysis of the structure of
48 microbial populations has practical importance; the results can be used to assess the fate of released strains
49 and their impact on resident microbial communities (Procopio et al. 2009). Hence, the present investigation was
50 carried out to study variation in distribution and diversity of endophytic bacterial population colonizing soybean.

51 2. MATERIAL AND METHODS

52 **2.1 Study area:** Washim (Latitude: 20° 05' 58.90" N, Longitude: 77° 08' 11.82" E, Altitude: 600M MSL) is situated
53 in central Vidarbha region of Maharashtra state of India. The climate of this region is hot moist semi arid and the
54 soil type is deep clayey to shallow black soil (Figure 1).

55 **2.2 Sample collection:** Healthy plants of soybean were screened from the six different locations of Washim
56 district during the cultivation period of June-December 2010. Within each location, randomly 3 different sub-
57 locations were selected for sample collection. From each sub-location 3-5 healthy plants were selected. The
58 growth stages of soybean were identified as specified by McWilliams et al. (2004). Sample represent of each
59 growth stage viz., vegetative (V1-V5) and reproductive (R1-R8) were collected. The plants were uprooted,
60 sealed into plastic bags and labeled. All samples were processed immediately after collection.

61 **2.3 Isolation and identification endophytic bacteria:** The collected plants were washed under tap water to
62 remove soil and separated into root, stem and leaf. All root, stem and leaf samples were washed twice in distilled
63 water then surface sterilized as described by Hung and Annapurna, (2004) and Pimentel et al. (2006) with slight
64 modifications. Surface sterilization was carried out by immersion for 1 minute in 70% (v/v) ethanol, in 0.1% HgCl₂
65 up to 3 minutes out for roots and nodules, whereas, up to 5 minutes for leaves and stems respectively. The
66 tissue was then washed ten times using sterile distilled water. Sterility checks were carried out by monitoring the
67 rinse wash water for the presence or absence of microbial growth on nutrient media for 6 days at 30 °C. The
68 absence of growth was taken into consideration as positive test for surface sterilization.
69 The surface disinfected samples were macerated (Hallmann et al. 1997). The slurry was filtered through sterile
70 cotton cloth. The filtrate was serially diluted and spreaded onto nutrient agar supplemented with nistatin

Comment [D1]: You should indicate what you modified.

Comment [D2]: I could not find the reference, so I cannot know how did you calculated the bacterial abundance (CFU/g) in the plant.

P R O D U C T I V E	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				

* Values presented are mean values of three independent experiments; Values in parenthesis represents SE_M

Table 2. Distribution of Endophytic Bacterial isolates at Different Growth Stages in soybean tissues

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

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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 ($5.74 \log_{10}$ CFU g⁻¹ fresh weight) followed by V4 ($5.01 \log_{10}$ CFU g⁻¹ fresh weight) and for reproductive stages at R2 ($5.84 \log_{10}$ CFU g⁻¹ fresh weight) followed by R1 ($5.80 \log_{10}$ CFU g⁻¹ fresh weight) and R3 ($5.74 \log_{10}$ CFU g⁻¹ fresh weight). Eventhough, the population at V4 and V5 during vegetative and at R1, R2, R3 and R4 during reproductive growth stages were found to be ~~arithmetically~~ ^{arithmetically} significant as compared to early and late growth stages, however the both the population at vegetative and reproductive stages respectively were statistically insignificant as per ANOVA ($P = 0.05$).

3.2 Isolation of endophytic bacteria: A total of 1611 endophytic bacterial morphotypes 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. ~~It was also noticed roots~~ As a primary site of entry, the roots showed maximum population, followed by leaves and stems, respectively.

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

Our findings are in congruence with Zinniel et al. (2002), who reported that the population size of endophytic bacteria in agronomic plants including soybean varied between 0-6.0 $\log_{10}$ CFU/sample tissue. Kuklinsky-Sobral et al. (2004) also observed significant differences in bacterial population densities and found to be influenced by soybean growth phase and type of 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 stages environmental conditions and geographical location of the experimental site (Kuklinsky-Sobral et al. 2004). Moreover, genotype ~~as well as or~~ cultivar of plant also affects significantly ~~as~~ the endophytic population ~~is naturally selected by the host~~. Conn and Franco (2004) 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 Kloeeper, 1995) 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.

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 (Hardoim et al. 2008). 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 (Hardoim et al. 2008).

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

Comment [D3]: How to evaluate the arithmetic significance?

Comment [D4]: This sentence is not clear and I do not know what you mean.

Comment [D5]: I guess this refers to isolates. Morphotypes can be identified or defined, but not isolated. Then, if you really mean morphotypes, you have to give the method how they were defined. This sentence may be modified as "A total of 1611 endophytic bacterial colonies representing different morphotypes were isolated"

Comment [D6]: Please pay attention to the use of sp., which refers a single unidentified species belonging to the genus, so the phrase "belonging to the genera *Pseudomoans* Sp." is not correct. You can say "belonging to the genera *Pseudomoans*" or "belonging to *Pseudomoans* sp."

Again, you have to present your evidence for the identification. Give me your results to show that the isolates can be identified into these genera.

Comment [D7]: Please use these previous findings to discuss your results.

Comment [D8]: This paragraph is mainly a summary of other published studies. Please make discussion related to your results.

Comment [D9]: This paragraph is totally not related to your results.

140 endophytic bacteria offers an innovative and enlivening opportunity for the discovery of novel strains with
141 biotechnological utility.

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144 the scheme of major research project as per XIth plan.

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204 **AUTHORS CONTRIBUTIONS**

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