

**RHIZOSPHERE MICROFLORA OF TRANSGENIC
CROP PLANTS**

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**DEPARTMENT OF AGRICULTURAL MICROBIOLOGY
UNIVERSITY OF AGRICULTURAL SCIENCES
BANGALORE**

2010

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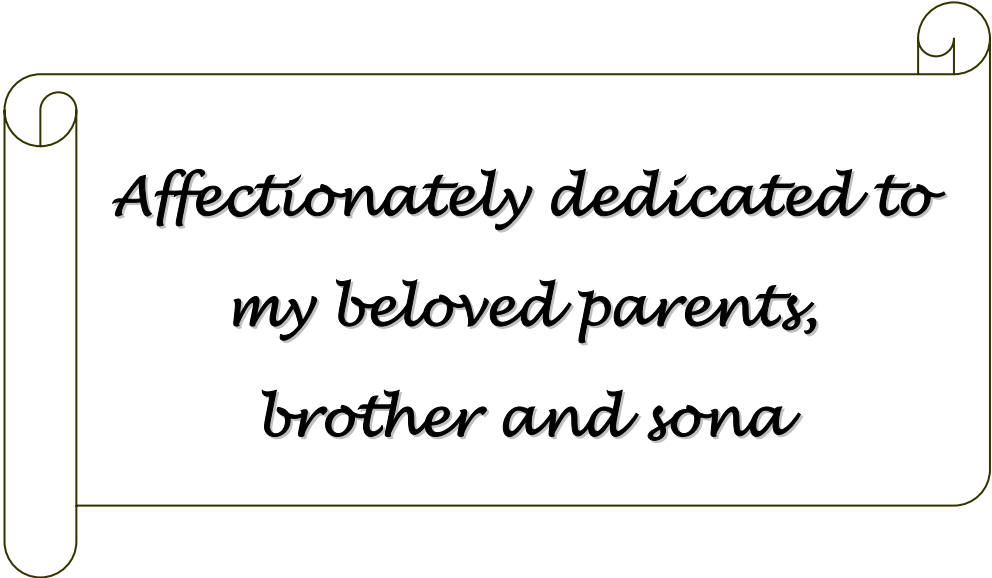
PAK 8119

Thesis submitted to the
University of Agricultural Sciences, Bangalore
In partial fulfillment of requirements
for the award of the Degree of

MASTER OF SCIENCE (Agriculture)
in
AGRICULTURAL MICROBIOLOGY

BANGALORE

JULY, 2010



*Affectionately dedicated to
my beloved parents,
brother and sona*

**DEPARTMENT OF AGRICULTURAL MICROBIOLOGY
UNIVERSITY OF AGRICULTURAL SCIENCES, BANGALORE**

CERTIFICATE

This is to certify that the thesis entitled “**Rhizosphere microflora of transgenic crop plants**” submitted by Ms. RANJANA PRASAD in the partial fulfillment for the award of the degree of MASTER OF SCIENCE (AGRICULTURE) in AGRICULTURAL MICROBIOLOGY to the University of Agricultural Sciences, Bangalore, is a record of bonafide research work carried out by her during the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar titles.

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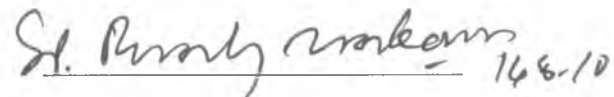
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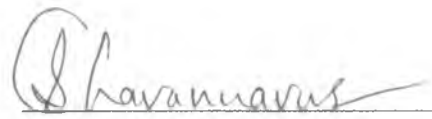
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ACKNOWLEDGEMENT

I express my deep sense of gratitude and indebtedness to **Dr. D. RADHAKRISHNA**, Professor, Department of Agricultural Microbiology, UAS, GKVK, Bangalore, the esteemed chairperson of my advisory committee for his valuable guidance, constant encouragement and constructive suggestion during the course of investigation. In fact, I was fortunate to have him as the chairperson of my advisory committee.

With the deep sense of pride and dignity, I sincerely thank the members of my advisory committee **Dr. G.P. BRAHMAPRAKASH**, Professor Dept. of Agril. Microbiology, GKVK, UAS-Bangalore, **Dr. SUVARNA V. CHAVANNAVAR**, Associate Professor, Dept. of Agril. Microbiology, GKVK, UAS-Bangalore, **Dr. NATARAJA KARABA N.**, Associate Professor, Dept. of Crop Physiology GKVK, UAS-Bangalore, **Dr. SURESH C. K.**, Professor, Dept. of Plant Biotechnology, GKVK, UAS-Bangalore for their help, encouragement and critical evaluation of the thesis.

I am also thankful to Professor and Head, **Dr. K. R. SREERAMULU**, Department of Agricultural Microbiology, GKVK, UAS-Bangalore and other staff members of the department for their constant encouragement and cooperation throughout the study. A special thanks to **Dr. A. N. BALAKRISHNA**, Professor, Department of Agricultural Microbiology, GKVK, UAS-Bangalore for his kind cooperation and help during my research.

On a personal note, I am bound to express my heartfelt gratitude and indebtedness to my classmates **Shruthi, Divya, Lavanya, Sandhya, Soumya, Ranjitha, Nagbhushan and Muthhapa**, I take special pleasure in thanking my seniors **Swapna, Archana, Lata, Manjula, Shivshankari, Arvind, Girish, Subramanium and Prabhuswamy**, for their everlasting exemplary enthusiasm shown and constant encouragement rendered throughout my study period. My roommates **Ansubai, Vinita and Moon Moon** need a special mention for their support and encouragement during my lean days.

I found no suitable words to propose my heartfelt thanks to my friends **Kundan, Sangeetha, Vinay, Wajeed and Parvathi** for their help and co-operation.

I wish to record respectful gratitude and indebtedness to my loving parents, brother and my other family members for their blessings and inspiration.

Any omission in this brief acknowledgement does not mean lack of gratitude.

July, 2010

Bangalore

(RANJANA PRASAD)

RHIZOSPHERE MICROFLORA OF TRANSGENIC CROP PLANTS

RANJANA PRASAD

ABSTRACT

An experiment on the comparison of rhizosphere microflora of transgenic groundnut with wild type was conducted. Rhizosphere soil samples were collected at three growth stages; vegetative, flowering and harvest and enumerated for the microorganisms.

The bacterial density was higher in transgenic rhizosphere whereas the fungal and actinomycetes population was higher in the wild type rhizosphere. In general, the rhizosphere of both the plant types recorded higher population density than the non-rhizosphere soil. There were no significant differences among the population of phosphate solubilisers, nitrogen fixing bacteria and also Arbuscular Mycorrhizal Fungi (AMF) colonization.

The activity of enzymes dehydrogenase, urease, phosphatases was significantly higher in the rhizosphere of wild type groundnut compared to the transgenic and non-rhizosphere soil.

In assessing the overall effect, the transgenic rhizosphere had a positive rhizosphere effect (R:S ratio) on microbial density compared to wild type groundnut rhizosphere.

The RAPD profile of the rhizosphere soil of the two types of groundnut did not record any significant diversity among the microbial communities.

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**Dr. D. Radhakrishna
(Major Advisor)**

ಟಾರ್ನ್‌ಜನಿಕ್ ಬೆಳೆಯ ಬೇರುವಲಯದ ಸೂಕ್ಷ್ಮಾಣು ಜೀವಿಯ ಚಟುವಟಿಕೆ

ಪ್ರಬಂಧ ಸಾರಾಂಶ

ರಂಜನ ಪ್ರಸಾದ್

ಟಾರ್ನ್‌ಜನಿಕ್ ನೆಲಗಡಲೆ ಮತ್ತು ಟಾರ್ನ್‌ಜನಿಕ್‌ಅಲ್ಲದ ನೆಲಗಡಲೆ ಬೆಳೆಯ ಬೇರುವಲಯದಲ್ಲಿ ಸೂಕ್ಷ್ಮಾಣುಜೀವಿಗಳ ನಡುವಿನ ಚಟುವಟಿಕೆಯಲ್ಲಿನ ವ್ಯತ್ಯಾಸವನ್ನು ಪ್ರಯೋಗದಲ್ಲಿ ಅಧ್ಯಯನ ಮಾಡಲಾಗಿದೆ. ಬೇರುವಲಯದ ಮಣ್ಣಿನ ಮಾದರಿಯನ್ನು ಬೆಳವಣಿಗೆಯ ಮೂರು ಹಂತಗಳಾದ ಸಸ್ಯ ಬೆಳವಣಿಗೆಯ ಹಂತ, ಹೂ ಬಿಡುವ ಹಂತ ಮತ್ತು ಕೊಯ್ಲಿನ ಹಂತದಲ್ಲಿ ಸೂಕ್ಷ್ಮಾಣುಜೀವಿಗಳ ಗಣನೆಗೆ ಸಂಗ್ರಹಿಸಲಾಗಿತ್ತು.

ದುಂಡಾಣುಗಳ ಸಾಂದ್ರತೆ ಟಾರ್ನ್‌ಜನಿಕ್ ಬೇರುವಲಯದಲ್ಲಿ ಹೆಚ್ಚಾಗಿರುವುದು ಕಂಡು ಬಂದಿದೆ, ಶಿಲೀಂಧ್ರ ಮತ್ತು ಆಕ್ಸಿನೋಮೈಸಿಟಿಸ್ ಸಂಖ್ಯೆಯು ಟಾರ್ನ್‌ಜನಿಕ್‌ಅಲ್ಲದ ತಳಿಯ ಬೇರುವಲಯದಲ್ಲಿ ಉತ್ತಮವೆಂದು ತಿಳಿದು ಬಂದಿದೆ. ಸಾಮಾನ್ಯವಾಗಿ ನೆಲಗಡಲೆ ಬೆಳೆಯ ಬೇರುರಹಿತವಲಯಕ್ಕಿಂತ ಟಾರ್ನ್‌ಜನಿಕ್ ನೆಲಗಡಲೆ ಮತ್ತು ಟಾರ್ನ್‌ಜನಿಕ್‌ಅಲ್ಲದ ನೆಲಗಡಲೆ ಬೆಳೆಯ ಬೇರುವಲಯವು ಹೆಚ್ಚಿನ ಸೂಕ್ಷ್ಮಾಣುಜೀವಿಗಳ ಸಾಂದ್ರತೆಯನ್ನು ಹೊಂದಿದೆ.

ರಂಜಕ ಕರಗಿಸುವ ಜೀವಾಣು, ಸಾರಜನಕವನ್ನು ಹಿಡಿದಿಡುವ ದುಂಡಾಣು ಮತ್ತು ಆರ್ಬಸ್ಕೂಲರ್ ಮೈಕೊರೈಜಾ ಶಿಲೀಂಧ್ರ ವಸಹತು ಮಾಡುವುದರಲ್ಲಿ ಹೆಚ್ಚಿನ ತಾತ್ಪರ್ಯವೇನು ಕಂಡುಬಂದಿಲ್ಲ.

ಡೀಹೈಡ್ರೋಜಿನೇಸ್, ಯೂರಿಯೇಸ್, ಫಾಸ್ಫೇಟೇಸ್ ಕಿಣ್ವದ ಕ್ರಿಯೆಯು ಟಾರ್ನ್‌ಜನಿಕ್ ಮತ್ತು ಬೇರುರಹಿತವಲಯಕ್ಕಿಂತ ಟಾರ್ನ್‌ಜನಿಕ್‌ಅಲ್ಲದ ನೆಲಗಡಲೆ ಬೆಳೆಯ ಬೇರುವಲಯದಲ್ಲಿ ಹೆಚ್ಚಾಗಿರುವುದು ಕಂಡು ಬಂದಿದೆ.

ಎಲ್ಲವನ್ನೂ ಪರಿಶೀಲಿಸಿದಮೇಲೆ ಟಾರ್ನ್‌ಜನಿಕ್ ಬೇರುವಲಯವು ಬೇರುವಲಯದ (ಆರ್.ಎಸ್ ಅಂತರ) ಸೂಕ್ಷ್ಮಾಣುಜೀವಿಗಳ ಸಾಂದ್ರತೆ ಮೇಲೆ ನೇರ ಪರಿಣಾಮ ಬೀರಿದೆ ಹಾಗೂ ಟಾರ್ನ್‌ಜನಿಕ್ ಅಲ್ಲದ ಬೇರುವಲಯಕ್ಕಿಂತ ಹೆಚ್ಚು ಎಂದು ತಿಳಿದು ಬಂದಿದೆ.

ಆರ್.ಎ.ಪಿ.ಡಿ ಅಧ್ಯಯನದಿಂದ ಟಾರ್ನ್‌ಜನಿಕ್ ಮತ್ತು ಟಾರ್ನ್‌ಜನಿಕ್‌ಅಲ್ಲದ ನೆಲಗಡಲೆ ಬೆಳೆಯ ಬೇರುವಲಯದ ಮಣ್ಣಿನಲ್ಲಿ ಸೂಕ್ಷ್ಮಾಣು ಜೀವಿಗಳ ಸಮೂಹ ಮತ್ತು ವೈವಿಧ್ಯತೆಯಲ್ಲಿ ಯಾವುದೇ ವ್ಯತ್ಯಾಸವಿಲ್ಲದಿರುವುದು ಕಂಡು ಬಂದಿದೆ.

ಕೃ.ವಿ.ವಿ, ಜಿ.ಕೆ.ವಿ.ಕೆ

ಬೆಂಗಳೂರು. ೭೫


ರಾಧಾಕೃಷ್ಣ

(ಪ್ರಧಾನ ಸಲಹೆಗರರು)

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Introduction



I. INTRODUCTION

The first transgenic plants developed, in early 1980s were tobacco (Framond *et al.*, 1983) and petunia (Fraley *et al.*, 1983) having trait for resistance to the antibiotic kanamycin. Since then, numbers of transgenic crops were developed to achieve novel and desired traits. The purpose of creation of transgenic plants are profound such as pest resistance, herbicide tolerance, long shelf life, phytoremediation, vaccine production, nutrient supplement and tolerance to biotic and abiotic stresses. The majority of current research in this field is directed toward the improvement of food crops such as potatoes, maize and sugar beets, while much of the remaining work concerns non-food crops such as cotton, tobacco, ornamental plants and those used to produce pharmaceuticals. Although some genetic modifications seek to improve the quality of the food product directly, many are designed to aid in the cultivation process.

The engineering of genetically modified (GM) plants has great potential for future agriculture, but it asks for a well-defined risk assessment as well. To date, environmental risk assessments regarding the cultivation of GM plants have mainly addressed aboveground effects. The underground component of GM plant effects has been largely neglected, despite the recognized importance of soil-borne organisms and processes and the dominant role of plants with respect to underground energy and carbon input. The rhizosphere (the zone of soil directly surrounding and influenced by plant roots) contains the majority of the microbiota in soil (4^{10} -fold more than the microbiota in bulk soil), and plant-microbe interactions in the rhizosphere are among the major factors that regulate the health and growth of plants. It is widely acknowledged that root exudates govern which organisms reside in the rhizosphere (Lynch, 1994; Bardgett *et al.*, 1999). Therefore, any change in the quality and quantity of root exudates could potentially modify the composition (biodiversity) and activity of the soil

microbiota and may cause changes in both deleterious and beneficial microorganisms.

The introduction of *ipt* gene into the plant genome results in elevated levels of cytokinin which leads to the release of lateral bud growth from apical dominance, delayed senescence of the leaves, increased photosynthetic rate with higher NADP-dependent hydroxyl pyruvate reductases and RUBP activity, increased protein content and nitrate reductase. The cytokinin overproducing plants had a better reproductive growth compared to the non-transformed wild type plants. The endogenous cytokinin overproduction leads to the production of the defense-related genes for extension, chitinases and PR1.

With the aim of determining the effects, both beneficial and harmful, on the rhizosphere microflora of the transgenic groundnut expressing *ipt* gene a study with the following objectives was conducted:

1. Comparative study of rhizosphere microflora of transgenic and wild types of different crop plants.
2. Study of functional diversity of the rhizosphere microflora of transgenic crop plants.
3. Study of the changes of the biochemical characteristics of the rhizosphere.
4. Molecular characterization of rhizosphere microflora of transgenic and non-transgenic crop plants.

Review of Literature



II. REVIEW OF LITERATURE

There are concerns that the commercial cultivation of genetically modified (GM) crops could result in adverse effects on the environment. One of the potential adverse environmental effects of GM crops is a non target effect on soil organisms and a change in microbe-mediated processes and functions in soil. Interactions between plants and soil ecosystems indicate that, similar to other agricultural crops, GM crops will influence processes and functions in soil. Plants have a major influence on communities of micro- and other organisms in soil, which are fundamental to many functions of soil systems, such as nitrogen cycling, decomposition of wastes, and mobilization of nutrients. The type and amount of nutrients released will affect both the numbers of organisms and their diversity. The major carbon supply to soil systems is from plant litter incorporated after harvest and from root exudation. Plants alter the composition and diversity of soil microbial communities in a selective manner (Nehl *et al.*, 1997).

2.1 Root exudates alter the rhizosphere microbial communities

Microorganisms are the dominant organisms, both in terms of biomass and activity, in soil, and they are involved in numerous important processes, including decomposition of organic matter, nutrient mineralization, regulation of plant pathogens, decomposition of agricultural chemicals, and improvement of soil structure (Gupta and Yeates, 1997; Bruinsma *et al.*, 2003).

The change in the quality and quantity of root exudates could potentially modify the composition (biodiversity) and activity of the soil microorganisms. For example, a decrease in specific microbial populations could lead to a decrease in decomposition processes, alter the level and composition of soil organic matter, and have secondary effects on the survival of plant pathogens (Termorshuizen and Lotz, 2002).

The microbial community resulting from plant selective pressure varies between plant species (Germida *et al.*, 1998; Grayston *et al.*, 1998; Smalla *et al.*, 2001), indicating that plant type influences which microorganisms colonize their rhizosphere. Moreover, Rengel *et al.* (1998) suggested that the selective effect of plants on the rhizosphere community can occur even at the cultivar level.

The transgenic plants may alter the rhizosphere microbial communities either due to the altered root exudates with the release of transgene protein in soil or due to a rare event of horizontal gene transfer.

2.1.1 Transgene protein in root exudates and its effect on microbial communities

Neal *et al.* (1973) and Nielsen *et al.* (2001a) observed variation in cultivable rhizospheric community of pathogen resistant transgenic wheat.

Savka and Farrand (1992) studied the introduction of three genes from the T_R region of *pTi15955* into tobacco (*Nicotiana tabacum* L.) to direct the synthesis of the mannityl opines from hexose sugars and glutamine or glutamate. Opines were present in all tissue types tested and accumulated to levels of 100 to 150 micrograms per milligram dry weight in root, stem, and leaf tissues. Opine-producing plants appeared normal with respect to morphology and development. Transgenic plants grown for 60 days under sterile autotrophic conditions produced up to 540 micrograms of the mannityl opines per milligrams dry weight of tissue as root exudates.

One of the first transgenic plant modifications directed towards the enhancement of resistance against soil-borne bacteria concerned potatoes transformed to produce T4-lysozyme (a bacteriolytic enzyme) to enhance resistance to *Erwinia carotovora* subsp. *atroseptica* (Daring *et al.*, 1993).

Vierheilig *et al.* (1993) studied transgenic tobacco plants constitutively expressing bean chitinase, which have been shown to display enhanced resistance to the root pathogen *Rhizoctonia solani*. The transgenic *N. sylvestris* plants, expressing different forms of tobacco chitinases under the control of the expression signals of the 35S transcript of Cauliflower Mosaic Virus were used to compare the colonization of the root system by *Rhizoctonia solani* and by the mycorrhizal symbiont *Glomus mosseae*. Plants of *Nicotiana sylvestris* expressing the vascular tobacco chitinases A or the same chitinases without the N-terminal chitin binding domain were less reduction of the root fresh weight when exposed to an attack of the fungus. Plants of *Nicotiana sylvestris* expressing chitinases A without the C-terminal signal for vacuolar targeting showed no enhanced resistance since they were colonized by *Rhizoctonia solani* like control plants. All transgenic *Nicotiana sylvestris* investigated were equally well colonized by *Glomus mosseae*, indicating that the enhanced resistance conferred by constitutive expression of chitinase in transgenic plants does not interfere with the vesicular arbuscular mycorrhizal symbiosis.

Guyon *et al.* (1993) investigated the effect of plant transformation on opine catabolic (and pathogenic) *Agrobacterium* by designing an experimental model in which the changes of a bacterial population associated with the root system of plants could be easily monitored. The bacterial population was composed of opine catabolic and non-catabolic strains of *Agrobacterium*. Bacteria were co-cultivated with either transformed or normal *Lotus* plants. The growth of bacteria was stimulated when they were associated with transformed plants. Furthermore, growth of opine-utilizing bacteria was specifically favored when bacteria were associated with transformed plants, but not when bacteria were associated with normal plants. This work indicates that transformation of plant cells by *Agrobacterium* indeed can favor growth of the catabolic strain.

Vierheilig *et al.* (1995) studied the effect of constitutive expression of pathogenesis-related proteins (PRs) in tobacco plants on vesicular-arbuscular mycorrhiza. Tobacco lines genetically transformed to express various PRs constitutively under the control of the cauliflower mosaic virus 35S promoter of tobacco were examined. Immunoblot analysis and activity measurements demonstrated high levels of expression of the PRs in the root systems of the plants. Constitutive expression of the following acidic isoforms of tobacco PRs did not affect the time course or the final level of colonization by the vesicular-arbuscular mycorrhizal fungus *Glomus mosseae*: PR-1a, PR-3 (5PR-Q), PR-Q*, PR-4, and PR-5. Similarly, constitutive expression of an acidic cucumber chitinase, of a basic tobacco chitinase with and without its vacuolar targeting peptide, of a basic β -1, 3-glucanase, and of combinations of PR-Q and PR-Q* or basic chitinase and basic β -1, 3-glucanase did not affect colonization by the mycorrhizal fungus. A delay of colonization by *G. mosseae* was observed in tobacco plants constitutively expressing the acidic isoform of tobacco PR-2, a protein with β -1, 3-glucanase activity.

Donegan *et al.* (1995) found that the endotoxin from *B. thuringiensis* subsp. *kurstaki*, both purified and produced in transgenic plants, did not have a direct effect on soil microorganisms and that the effects observed, which were related to the plant varieties used, may have been caused by unexpected changes in plant characteristics that resulted from genetic manipulation or tissue culturing of the engineered plants.

By contrast, most studies have indicated that Bt plants cause no or only minor changes in microbial community structure that are often transient in duration. For example, when numbers and species of indigenous soil bacteria and fungi were monitored using culture-based methods and a DNA fingerprinting method, a significant, but transient, increase in the populations of culturable

bacteria and fungi was found in soil microcosms with decomposing leaves of Bt cotton expressing the Cry1Ac protein. The addition of purified Cry1Ab or Cry1Ac protein to soil did not result in any detectable effects on bacteria, fungi, or protozoa when compared with the control soil (Donegan *et al.*, 1995).

Studies, using culture-independent methods, showed some minor or no Bt-specific effects on soil microorganisms, and plant age and type appeared to be the major factors affecting bacterial diversity (Donegan *et al.*, 1995; Griffiths *et al.*, 2005, 2006; Naef *et al.*, 2006).

A field release of transgenic potato plants that produce the insecticidal endotoxin of *Bacillus thuringiensis* var. *tenebrionis* (*Btt*) was monitored for changes in total bacterial and fungal populations, fungal species diversity and abundance, and plant pathogen levels (Donegan *et al.*, 1995). The microflora on three phenological stages of leaves (green, yellow and brown) were compared over the growing season (sample days 0, 21, 42, 63 and 98) for transgenic potato plants, commercial Russet Burbank potato plants treated with systemic insecticide (Di-Syston) and commercial Russet Burbank potato plants treated with microbial *Btt* (M-Trak). Few significant differences in phylloplane microflora among the plant types were observed and none of the differences were persistent. Minimal differences were observed in the populations of culturable, aerobic bacteria and fungi in soil with transgenic Bt potato expressing the Cry3A protein (Donegan *et al.*, 1996).

Microorganisms are the dominant organisms, both in terms of biomass and activity, in soil, and they are involved in numerous important processes, including decomposition of organic matter, nutrient mineralization, regulation of plant pathogens, decomposition of agricultural chemicals, and improvement of soil structure (Gupta and Yeates, 1997; Bruinsma *et al.*, 2003).

Oger *et al.* (1997) reported transgenic plants alter their biological environment, more precisely the root-associated bacterial populations. The alterations were both transgene-specific and target population-specific.

Siciliano *et al.* (1998) and Gyamfi *et al.* (2002) reported alteration in community fatty acid, community level physiological profile altered, taxonomic diversity of root associated community, diversity of *Rhizobium leguminosarum* and variation in *Pseudomonas* population of herbicide resistant rape.

Similarly, Siciliano *et al.* (1998) and Siciliano and Germida (1999) reported that the composition of the root-associated microbial community differed between canola cultivars (transgenic glyphosate-resistant canola and near-isogenic non-transgenic canola cultivars), as determined by CLPP and fatty acid methyl ester (FAME) analyses, and suggested that these effects could have been caused by altered chemical composition of the plant tissue as a result of the insertion of genes.

Insecticidal Cry proteins derived from insect-resistant Bt crops in soils through cultivation of Bt crops affects the soil microbial communities (Masoero *et al.*, 1999; Escher *et al.*, 2000; Saxena and Stotzky, 2001; Dinel *et al.*, 2003; Manachini *et al.*, 2004; Hoss *et al.*, 2004; Turrini *et al.*, 2004; Gupta and Watson, 2004; Xue *et al.*, 2005; Rui *et al.*, 2005; Griffiths *et al.*, 2005).

Differences in the composition of crop residues as the result of the introduction of a transgenic trait have been observed in transgenic Bt crops (Masoero *et al.*, 1999; Escher *et al.*, 2000; Saxena and Stotzky, 2001; Folmer *et al.*, 2002; Jung and Sheaffer, 2004; Mungai *et al.*, 2005; Poerschmann *et al.*, 2005).

Siciliano *et al.* (1999) reported decreased population of *Arthrobacter*, *Bacillus*, *Micrococcus* and *Variovorax* and increased population of

Flavobacterium and *Pseudomonas* in the root interior of field grown transgenic *Brassica napus* cv. Quest compared to the non-transgenic *B. napus* cv. Excel and *B. rapa* cv. Parkland.

Di Giovanni *et al.* (1999) compared rhizosphere bacterial communities of parental and two transgenic alfalfa (*Medicago sativa* L.) of isogenic background based on metabolic fingerprinting using Biolog GN microplates and DNA fingerprinting of bacterial communities present in Biolog GN substrate wells by enterobacterial repetitive intergenic consensus sequence-PCR (ERIC-PCR). The two transgenic alfalfa expressed either bacterial (*Bacillus licheniformis*) genes for alpha-amylase or fungal (*Phanerochaete chrysosporium*) genes for Mn-dependent lignin peroxidase (Austin *et al.*, 1995). Cluster analysis and principal components analysis (PCA) of the Biolog GN metabolic fingerprints indicated consistent differences in substrate utilization between the parental and lignin peroxidase transgenic alfalfa rhizosphere bacterial communities. Cluster analysis of ERIC-PCR fingerprints of the bacterial communities in Biolog GN substrate wells revealed consistent differences in the types of bacteria (substrate-specific populations) enriched from the rhizospheres of each alfalfa genotype. Comparison of ERIC-PCR fingerprints of bacterial strains obtained from substrate wells to substrate community ERIC-PCR fingerprints suggested that a limited number of populations were responsible for substrate oxidation in these wells. Results of this study suggested that transgenic plant genotype may affect rhizosphere microorganisms and that the methodology used in this study may prove a useful approach for the comparison of bacterial communities.

In a series of papers, Lottmann and colleagues (Lottmann *et al.*, 1999, 2000; Lottmann and Berg 2001; Heuer *et al.*, 2002) have studied the impact of the genetically modified potato plants on microbial communities in the rhizosphere and geocaulosphere in field experiments. In these studies a number of both

cultivation dependent and cultivation-independent methods were used concomitantly. They screened for bacteria antagonistic against *E. carotovora* and *Verticillium dahliae*, as well as for other specific bacterial groups, and examined total rhizosphere community and catabolic profiles. Also, the effects of the transgenic potato on introduced antagonistic bacteria were investigated (Lottmann *et al.*, 2000)

Other studies have shown that the effects of GM plants on microbial communities are subject more to seasonal variations or to other environmental factors, such as oil type and agricultural practices, than to expression of Cry or other proteins in plants (Lottmann *et al.*, 1999, 2000; Heuer *et al.*, 2002; Dunfield and Germida, 2003; Blackwood and Buyer, 2004; Griffiths *et al.*, 2000, 2005; Baumgarte and Tebbe, 2005; Fang *et al.*, 2005).

Lottmann *et al.* (1999, 2000) found that although transgenic potato expressing T4 lysozyme influenced the composition of root-associated bacterial antagonists, such as *Pseudomonas putida* (which inhibits the pathogen, *Erwinia carotovora*, that causes soft rot and blackleg in potato) and *Serratia grimesii* (which inhibits the fungus, *Verticillium dahliae*), this was dependent on both the year and the time of sampling.

Kremer *et al.* (2000) reported incidence of *Fusarium* (soil borne pathogen) on transgenic soybean roots was greater within 1 week after the application of glyphosate as compared to non-transgenic isolate.

Griffiths *et al.* (2000) studied the rhizosphere of potato plants genetically engineered to produce each of the two lectins, concanavalin A (Con A) and Galanthus nivalis agglutinin (GNA) constitutively, as well as the purified lectins, for possible non-target effects. It was observed that the soil bacterial communities

and a ciliate protozoan showed no direct effect of either lectin over a range of concentrations.

Oger *et al.* (2000) reported increased population of opines utilizing bacteria as compared to other bacterial species in the rhizosphere of transgenic lotus.

Ahrenholtz *et al.* (2000) reported, in transgenic potato expressing the phage T4 lysozyme gene, high bactericidal activity against *Bacillus subtilis* adsorbed artificially on potato roots as compared to non-transgenic plants.

The rhizosphere microbial community associated with two transgenic potato lines that produced the lectins, *Galanthus nivalis* agglutinin (GNA) and concanavalin A (con A), had different CLPP than the control line, but the profiles were also subject to seasonal variation, and differences did not persist from one season to the next (Griffiths *et al.*, 2000).

Dunfield *et al.* (2001) reported that rhizosphere and root interior microbial populations associated with a transgenic canola have altered CLPP and fatty acid methyl ester (FAME) profiles compared to the profiles of a nontransgenic counterpart.

Saxena and Stotzky (2001) reported that there were no significant differences in the colony-forming units of culturable bacteria (including actinomycetes) and fungi and in the numbers of protozoa and nematodes between rhizosphere soil of Bt and non-Bt corn or between soil amended with biomass of Bt and non-Bt corn. The Cry1Ab protein in root exudates and biomass of Bt corn appears not to be toxic to earthworms, nematodes, protozoa, bacteria, and fungi.

Similarly, Saxena and Stotzky (2001) found no significant differences in the number of colony-forming units of culturable bacteria (including actinomycetes) and fungi and in the numbers of earthworms, protozoa, and

nematodes between rhizosphere soil of Bt and non-Bt maize or between soil amended with biomass of Bt and non-Bt maize.

Schmalenberger *et al.* (2002) reported no effect of transgenic maize on genetic diversity of bacterial communities in rhizospheric samples.

There appeared to be no significant differences in exudation from 12 different Bt maize hybrids, which included three transformation events (Bt11, MON810, and 176), expressing the Cry1Ab protein (Saxena *et al.*, 2002).

Significant differences were also observed in the composition of the microbiota in Australian soils associated with residues of Bt cotton and non-Bt cotton by both bright-field and scanning electron microscopy. More extensive colonization by fungi (spores and hyphae) was observed in residues of Bt cotton than in residues of non-Bt cotton. Using a selective inhibition-SIR (substrate-induced respiration) technique, higher ratios of fungi to bacteria were found in Bt cotton residues than in the residues of non-Bt cotton (Gupta *et al.*, 2002; Gupta and Watson, 2004). In addition, the types of fungi that colonized the two types of residues were different by microscopic observations.

The purified insecticidal proteins from *B. thuringiensis* subsp. *kurstaki*, *morrisoni* (strain *tenebrionis*), and *israelensis* did not affect the growth of a variety of bacteria, fungi, and algae in either pure or mixed culture (Koskella and Stotzky, 2002).

Heuer *et al.* (2002) found significant differences between the community structure of the rhizosphere of T4 lysozyme-producing transgenic potato lines DL4 and DL5 and non-transgenic potato varieties that were dependent on environmental factors but independent of the expression of T4 lysozyme by the transgenic plants.

Dunfield and Germida (2003) found significant seasonal differences in the microbial community associated with the rhizosphere of a herbicide-tolerant (glyphosate-resistant) transgenic variety of canola, Quest, and a conventional variety, Excel, but these differences also did not persist into the next season.

Tesfaye *et al.* (2003) reported qualitative changes in the abundance of bacterial phylogenetic groups between rhizosphere soils of transgenic and untransformed alfalfa. Rhizosphere transgenic alfalfa had significantly greater microbial functional diversity compared with untransformed alfalfa.

Ferreira *et al.* (2003) showed that inoculation of soybean with *B. thuringiensis* subsp. *kurstaki* (Btk) or its Insecticidal Crystal Protein (ICP) did not affect the bacterial or fungal populations of rhizosphere soil, but a transient increase in some functional groups of carbon-cycling microorganisms, such as cellulolytic, amylolytic, and proteolytic, was observed in the beginning of the incubation. The ICP did not show any effect on colonization by arbuscular mycorrhizae (AM), whereas colonization by AM fungi of soybeans inoculated with spores of Btk was inhibited when compared with uninoculated plants.

There have been numerous studies, with different methods (e.g., functional and structural composition of soil microbial communities) and different Bt plants (e.g., maize, cotton, rice, canola, potato, tobacco), on the effects of Bt crops on soil microbial communities. Different effects, ranging from no effect to minor and significant effects, of these plants on microbial communities (Stotzky, 2002, 2004; Turrini *et al.*, 2004; Gupta and Watson, 2004; Castaldini *et al.*, 2005; Xue *et al.*, 2005; Rui *et al.*, 2005; Icoz and Stotzky, 2007) and activities of some enzymes (Wu *et al.*, 2004b; Flores *et al.*, 2005; Shen *et al.*, 2006; Sun *et al.*, 2007; Icoz *et al.*, 2007) in soil have been reported.

Saxena *et al.* (2004) showed that insect resistant GM crops, such as Bt maize, potato, and rice, contributed to the presence and persistence of Cry proteins in soil via root exudation, whereas Bt cotton, canola, and tobacco did not.

Turrini *et al.* (2004) found that root exudates of Bt maize (event 176) significantly reduced presymbiotic hyphal growth of the arbuscular mycorrhizal fungus, *Glomus mosseae*, compared with root exudates of another Bt maize hybrid (event Bt 11) and non-Bt maize.

The microbiota (e.g., bacteria, actinomycetes, fungi) associated with residues of Bt cotton were significantly different from those associated with residues of herbicide tolerant cotton (Roundup Ready) (Gupta and Watson, 2004).

A 3-year field study with Bt maize (event MON810) confirmed that the release of Cry protein in root exudates continued throughout growth, and levels of the protein in soil did not correlate with a specific period of plant growth (Nguyen Thu, 2004; Baumgarte and Tebbe, 2005).

No deleterious effects of growing Bt maize expressing the Cry3Bb1 protein from *B. thuringiensis* subsp. *kumamotoensis*, active against the corn rootworm complex (*Diabrotica* spp.), for two consecutive seasons in the field were detected on microbial biomass and activity or on bacterial community structure, as determined by terminal restriction fragment length polymorphism (T-RFLP) analysis (Devare *et al.*, 2004).

Experiments in which root growth solutions, collected from Bt and non-Bt maize grown in hydroponic culture, were added to soil indicated that root exudates of Bt maize led to the development of bacterial communities in soil that differed from those associated with exudates of near isogenic non-Bt maize. It was suggested that the exudates of Bt maize differed from those of non-Bt maize in several ways and not only in the content of the Cry protein (Brusetti *et al.*, 2004).

Brusetti *et al.* (2004) also compared the rhizosphere bacterial community associated with Bt maize expressing the Cry1Ab protein and near-isogenic non-Bt maize using several techniques, including viable counts, community-level catabolic profiling (CLCP), and PCR-based automated ribosomal intergenic spacer analysis (ARISA), and found that the viable counts and CLCP did not show any differences between Bt and non-Bt maize, but ARISA showed that the community structure differed with the age of the plants and between Bt and non-Bt maize, suggesting that root exudates could select different bacterial communities.

No significant effects of the Cry1Ab and Cry1F proteins from Bt maize on bacterial and fungal phospholipids fatty acid (PLFA) profiles and only a few significant effect of the Cry proteins on microbial community level physiological profiles (CLPP) were reported by Blackwood and Buyer (2004), and these effects were the result primarily of differences in soil type.

Wu *et al.* (2004) found that decomposing transgenic Bt rice straw (Cry1Ab protein) was not toxic to culturable bacteria, actinomycetes, and fungi in a flooded paddy soil under laboratory conditions. They also reported persistent differences in dehydrogenase activity, methanogenesis, hydrogen production and anaerobic respiration between soil amended with Bt transgenic rice straw and non-transgenic rice straw. But differences in protease, neutral phosphatase and cellulase activities were not persistent.

Blackwood and Buyer (2004), using PLFP and CLPP, reported that Bt maize did not result in large differences in soil microbial communities and that soil type was the most important factor affecting the composition of the communities.

Fang *et al.* (2005) reported that bacterial diversity in the rhizosphere of transgenic and non-transgenic maize was affected more by soil texture than by cultivation of transgenic varieties.

Similarly, Icoz *et al.* (2007) found seasonal variations in the numbers of microorganisms and enzyme activities, probably as the result of differences in the water content of the soils, ambient temperatures, and stage of growth of the plants at the times of sampling. These seasonal differences were independent of the presence of the Cry proteins in the plants.

Flores *et al.* (2005) found no significant effects of the Cry1Ab protein released to soil from Bt maize in root exudates or biomass on culturable bacteria, fungi, and protozoa.

Castaldini *et al.* (2005) reported consistent significant differences between Bt maize expressing the Cry1Ab protein and near-isogenic non-Bt maize in both total and metabolically-active 16S rRNA fractions of culturable rhizosphere heterotrophic bacteria by denaturing gradient gel electrophoresis (DGGE) and in mycorrhizal colonization by microscopy (e.g., a significantly lower level of *G. mosseae* was detected in roots of Bt maize).

Xue *et al.* (2005) found that the ratio of gram-positive to gram negative bacteria was lower in soil with Bt maize than in soil with near-isogenic non-Bt maize, but the ratio was higher in soil with Bt potato than with near-isogenic non-Bt potato. However, there was no difference in the ratio of fungi to bacteria between soil with Bt and non-Bt maize and between soil with Bt and non-Bt potato.

Rui *et al.* (2005) found increased numbers of culturable functional groups of bacteria (potassium-dissolving bacteria, inorganic phosphate-dissolving bacteria, and nitrogen-fixing bacteria) in rhizosphere soil of non-Bt cotton than in

rhizosphere soil of Bt cotton in the early and middle stages of growth of cotton. However, they found no significant differences in numbers after the growing season, and there was no effect of purified Cry1Ac protein on the numbers of these groups.

The bacterial community structure in the rhizosphere of Bt maize expressing the Cry1Ab protein was less affected by the protein than by environmental factors, such as the age of the plant or heterogeneities in the field (Baumgarte and Tebbe, 2005).

Other studies have also indicated only some minor changes in microbial community structure in soils with Bt plants (Brusetti *et al.*, 2004; Blackwood and Buyer, 2004; Griffiths *et al.*, 2006) or no apparent deleterious effects of transgenic Cry proteins released by Bt plants in root exudates or from biomass incorporated into soil on soil microbial communities (Donegan *et al.*, 1995; Saxena and Stotzky, 2001; Devare *et al.*, 2004, 2007; Wu *et al.*, 2004; Flores *et al.*, 2005; Shen *et al.*, 2006; Naef *et al.*, 2006; Icoz *et al.*, 2007) and some enzymes (Flores *et al.*, 2005; Shen *et al.*, 2006; Icoz *et al.*, 2007).

Field and greenhouse trials with maize expressing the Cry1Ab protein showed that changes in microbial, protozoan, and nematode communities as a result of the Bt trait were small and less than changes resulting from different non-Bt maize cultivars, different crops, soil type, and stage of plant growth (Griffiths *et al.*, 2005, 2006).

No significant differences were found in microbial biomass of Bt and non-Bt maize over 4 consecutive years of maize cultivation (Lang *et al.*, 2006), and no significant differences between soils with Bt and non-Bt cotton were found in functional diversity of microbial communities, suggesting that there were no adverse effects of Bt cotton on the soil ecosystem (Shen *et al.*, 2006).

Naef *et al.* (2006) reported no direct effect of the Cry1Ab protein in maize residues on the pathogen, *Fusarium graminearum*, and on the biocontrol agent, *Trichoderma atroviride*, and showed that some Bt and their near-isogenic non-Bt maize counterparts differed more in the chemical composition of the maize tissue as a result of different environmental conditions, such as drought-stress, than from the Cry protein content alone and which can affect the saprophytic growth of fungi on crop residues.

Similarly, Icoz *et al.* (2007) observed that the Cry1Ab and Cry3Bb1 proteins in root exudates and decaying plant residues of Bt maize had no consistent significant effects on a variety of culturable microorganisms and their enzymatic activities in soil during 4 consecutive years of maize cultivation. Some differences between soils with Bt and non-Bt maize in the numbers of the various groups of microorganisms were observed at some sampling times, but these differences were not consistent from one season to the next and were independent of the presence of the Cry proteins. The results from microbial plate counts were confirmed by DGGE, which also showed no differences in the diversity of bacteria between soils with Bt or non-Bt maize but showed differences in bacterial patterns with season.

Liu *et al.* (2008) evaluated the seasonal effects of Bt rice on rhizosphere soil microbial communities compared to those of insecticides commonly applied in paddy soil for the control of lepidopteran insects. In this study, seasonal effects of transgenic rice expressing the *Cry1Ab* insecticidal protein active against lepidopteran pests and the insecticide triazophos [3-(*o,o*-diethyl)-1-phenyl thiophosphoryl-1,2,4-triazol] on soil enzyme activities and microbial communities were compared under field conditions. During a 2-year field study, rhizosphere soil samples of transgenic-Bt rice (Bt), non-Bt parental rice (Ck) and non-Bt parental rice with triazophos (Ckp) applied were taken at four stages in the rice

developmental cycle: seedling, booting, heading and maturing. Microbial processes were investigated by measuring different biochemical activities including those involved in C and P cycling. Denaturing gradient gel electrophoresis (DGGE) and terminal-restriction fragment length polymorphism (T-RFLP) analyses were used to compare rhizosphere microbial compositions. Some occasional and inconsistent effects of the application of triazophos on the bacterial composition in the rhizosphere soil of rice plant were found at the booting and heading stages as compared with that of transgenic-Bt rice. There were no statistically significant differences ($P>0.05$) in phosphatase activity, dehydrogenase activity, respiration, methanogenesis or fungal community composition in rhizosphere soil between Bt, Ck and Ckp over the rice cropping cycle. However, seasonal variations in the selected enzyme activities and microbial community composition in the rhizosphere soil of Bt, Ck and Ckp were clearly detected.

Riglietti *et al.* (2008) reported no significant influence on the dominant members of the bacterial community and soil protease activity was not inhibited by the release of transgenic tobacco plants codifying a protease inhibitor on soil microbial community.

Liu (2009) studied two main pathways, biomass incorporation and root exudates for the effects of Bt transgenic crops on soil ecosystems. The effects of Bt transgenic crops (Bt transgenic cotton and rice) on soil ecosystems were studied with special attentions paid to the release and persistence of Bt toxins, and the toxicology to microorganisms, as well as the change of soil biochemical properties in soils where Bt transgenic crops were planted or incubated with their biomass.

Liu (2010) based on his study suggested that there is a temporal-spatial relevance between expression and rhizosphere secretion of anti-disease and insecticidal proteins (e.g., Bt-*Bacillus thuringiensis* toxins) in and outer roots, and

Arbuscular Mycorrhizal Fungi (AMF) intraradical and extraradical growth and development. Bt insecticidal proteins constitutive expression and rhizosphere release during cultivation of BT Proteins (BTPs) damages some critical steps of the AMF symbiotic development. More important, these processes of BTPs coincide with the entire life cycle of AMF annually, which may impact the diversity of AMF after long-term cultivation period. He also proposed that interactions between GM plants and AMF should be preferentially studied as an indicator for ecological impacts of GM plants on soil microbial communities.

2.1.2 Horizontal gene transfer

DNA from transgenic plants has been shown to be present and persist at field sites where transgenic plants have been grown and plant litter has been deposited (Gebhard & Smalla 1999, Bertolla & Simonet 1999). Most recombinant DNA may be contained in plant material deposited, but free DNA, released from plant cells, has also been found (Meier & Wackernagel 2003). Recombinant DNA has also been detected in elutes from transgenic potato rhizosphere (de Vries *et al.*, 1999). Given that bacteria thrive around plant roots, nourished by root exudates and cell debris, they may also take up and integrate genetic material released from plants. If plant DNA is introduced into bacteria, it might be exchanged with other soil bacteria, including *Agrobacteria* and their T-plasmids, which in turn might even lead to transfer back to plant cells, thus providing a genetic link between distantly related plant species (Tepfer *et al.*, 2003).

The release and uptake of DNA (including plant DNA) by natural transformation in microhabitats in the phytosphere was studied (Lorenz and Wackernagel, 1994; Paget and Simonet, 1994), an environment that could be continually exposed to transgenic plant DNA (Bertolla *et al.*, 2000). Recent developments in methodology such as the application of 16S rDNA sequence profiling by denaturing or temperature gradient gel electrophoresis (Muyzer and

Smalla, 1998) has increased the resolution of the composition of bacterial communities, including those that are non-culturable in various plant environments. Methodology has also been developed that allows non-disruptive *in situ* detection of gene transfer events in the phytosphere by the expression of green fluorescent protein marker genes (Dahlberg *et al.*, 1998, Normander *et al.*, 1998). Since most plant transgenes currently in commercial use (e.g. *cry*, *pat*, *bar*, EPSPS gene, and viral sequences) have been derived from donors present in, or naturally introduced into, agricultural soils, a thorough understanding of their ecology would be helpful when assessing the impact of the gene when present in plants. In particular, a precise mapping of the gene pool of antibiotic resistance marker gene homologues that are naturally available to bacterial communities in various environments would be helpful to resolve if the introduction of plant-harbored antibiotic resistance markers would alter the resistance level of indigenous bacteria (Smalla *et al.*, 1993, 1997, 2000; van Elsas and Smalla, 1995).

2.2 Study of beneficial microorganisms in the rhizosphere soil

2.2.1 Free living nitrogen fixing microorganisms

a) Azotobacter:

The literature on *Azotobacter* in the rhizosphere of crop plants have been reviewed from time to time (Rubinchick, 1963; Rovira, 1965; Mishustin, 1970 and Dobereiner, 1974). Ishizawa and Toyoda (1965) and Ishizawa *et al.*, (1975) made surveys on the distribution of N-fixing bacteria in Japanese paddy soils and found that the number of *Azotobacter* were found to rarely exceed 10^4 CFUs/g.

Krishnamurti (1962), similarly Rangaswami and Sadasivan (1969) reported a positive correlation between *Azotobacter* population with organic matter, available Phosphorus and pH.

Edward and Tripathi (1972) recorded a higher number of *Azotobacter* in the rhizosphere of wheat and hybrid napier grass than in the non-rhizosphere soils. Raghuramulu (2001) reported the presence of *Azotobacter* in soil of coffee plantation.

The numbers of *Azotobacter* in Indian soils rarely exceed 10^4 - 10^5 CFUs/g soil. The occurrence of *Azotobacter* in soil is known to depend on various soil factors like pH, moisture content, availability of nutrients, organic matter content of the soil etc.

2.2.2 Phosphate solubilizing microorganisms:

Involvement of microorganisms in the solubilization of insoluble phosphate was first shown by Stalstrom (1903). Since then, lot of work has been done on the isolation, enumeration, efficiency screening and mechanism of solubilization and crop response to their inoculation. The review articles by Rokade and Patil (1992 and 1993), and books by Gaur (1990) and Somani *et al.* (1990) have provided good information on all the aspects of phosphate solubilization by microorganisms.

A number of organisms have also been isolated from the rhizosphere of crop plants, grasses and other tree species. Louw (1970) observed a large population of gram negative, non-spore forming, rod shaped bacteria in the rhizosphere of wheat and lupins, which actively dissolved insoluble mineral phosphate compounds.

Subsequently there are also reports showing that phosphatic solubilizers may produce plant hormones or vitamins, which helps in plant growth. Barea *et al.*, 1976, demonstrated that a very high proportion of PSB tested, produced IAA, gibberellins and cytokinins.

Nair and Subba Rao (1977) isolated phosphate solubilizing *Pseudomonas* and *Aspergillus spp.* From the rhizosphere of coconut and cocoa and their occurrence was related to available phosphorus content in soil.

Nair and Subba Rao (1977) reported a number of phosphate solubilizing bacteria in the cocoa rhizosphere belonging to the genus *Pseudomonas*. The occurrence and activity of PO_4^- solubilizing fungi from coconut plantation soils were investigated and found that laterite, alluvial and clayey soils harboured more of PO_4^- solubilizing fungi of the genera *Aspergillus* and *Penicillium*.

The actively growing *Zostera marina* plants are reported to harbor tricalcium phosphate solubilizing bacteria to the extent of 4×10^8 cells/g dry root weight (Craven *et al.*, 1982).

Bopaiah (1985) also reported that the soils collected from root regions of arecanut palms (at 0-30 cm depth) grown in Karnataka also harboured PO_4^- solubilizing bacteria viz., *Aspergillus* and *Penicillium*. Studies on the distribution of PO_4^- solubilizing bacteria in coconut plantation soils revealed that clayey soils harboured less population than laterite, alluvial and sandy soils. *Pseudomonas sp.*, *Micrococcus sp.*, *Bacillus subtilis*, *Corynebacterium sp.* and *Alcaligenes sp.* were the PO_4^- solubilizing bacteria in coconut rhizosphere soils. Comparatively low numbers of bacteria capable of solubilizing insoluble phosphates in clayey soils might be due to the interaction of a number of physico-chemical factors.

Thomas and Shantaram (1986) and Kucey (1989) reported that the species belonging to the genera *Pseudomonas* and *Penicillium* were the major PO_4^- solubilizers in the forest soils.

Vinutha (2005) reported population of PO_4^- solubilizing bacteria was significantly higher in natural forests during the pre-monsoon season but during post-monsoon season, there was no significant difference between different land

use types. This could be due to higher organic matter content that serves as a C source for a vast majority of saprophytic organisms.

2.2.3 Arbuscular Mycorrhizal Fungi (AMF)

Arbuscular Mycorrhizal fungi are commonly associated with most of the agricultural and horticultural crops and natural plant communities (Gerdemann, 1975). AM fungi are characterized by vesicles and arbuscules. AM fungi can improve plant growth through increased uptake of phosphorus and other minerals especially in soils of low fertility (Tinker, 1975). Cytokinin and chlorophyll content of plants have been found increase due to AM fungal association (Allen *et al.*, 1980). AM fungi also help the plants to overcome water stress (Levy and Krikun, 1980).

2.3 Studies on soil enzyme activities in the rhizosphere soil

Soil enzyme activity estimates are often used as indices of microbial activity and soil fertility (Skujins, 1978). The variations in activities are attributed to diversities in vegetation, cropping history, soil amendments, soil fauna, environmental factors etc. (Spier *et al.*, 1979; Tabatabai, 1982; Pulford and Tabatabai, 1988).

Soil enzyme activity may serve as useful indicators for change in the biology and biochemistry of soil due to external management and environmental factors (Dick, 1994). The biological activity in soil provides better insight in understanding the transformation of organic matter. Any factor which influences the soil microorganisms, will affect soil enzyme activities indirectly (Tiwari *et al.*, 1988; McClaugherty and Linkins, 1990).

Some of the important soil enzymes are ureases, phosphatases, dehydrogenases and cellulases. The work carried out with reference to the impact

of land use types on these enzymes are limited, however studies made so far have been reviewed.

2.3.1 Dehydrogenase activity

The biological oxidation of organic compounds is generally a dehydrogenation process and there are many dehydrogenases, which are highly specific. The dehydrogenase enzyme system apparently fulfills a significant role in the oxidation of soil organic matter as they transfer hydrogen from substrates to acceptors. Many different specific dehydrogenase systems are involved in the dehydrogenase activity of soil; these systems are an integral part of the microorganisms. Therefore, the results of the assay of dehydrogenase activity would show the average activity of the active population (Skujins, 1976). The variations in activities are attributed to diversities in vegetation, cropping history, soil amendments, soil fauna, environmental factors etc. (Tabatabai, 1982).

Dehydrogenases are enzymes involved in oxidation-reduction reactions and represent the total microbial activity in soils.

2.3.2 Phosphatase activity

Phosphatases represent a broad range of intracellular as well as soil accumulated activities that catalyze the hydrolysis of both esters and anhydrides of phosphoric acid (Spier and Ross, 1978).

Phosphatase activity is directly related to the level of organic phosphorus in the soil. Roots can hydrolyze organic and insoluble phosphate in soil by the production of phosphatases, which are produced by a large number of soil microbes.

Pang and Kolenko (1986) studied phosphomonoesterase (PME) activity in forest soils in Shawnigan Lake and Jordan River. They found highest PME

activity in the forest floor horizon (L-H) and activity was dependent on soil geographic location and depth. Fertilization of forests with phosphates and urea appeared to reduce PME activity and thus may have the potential to depress mineralization of organic phosphorus.

Pantos-Derimova (1988) studied the enzyme activities in soil of different Hungarian forest ecosystem and found highest activities in the litter layer. He concluded that changes in enzyme activity within the profile are influenced not only by the soil type but also by the characteristics of the habitat type.

Fox and Comerford (1992) examined the phosphatase activity in the rhizosphere of slash pine grown in two forested spodosols and observed significant acid phosphatase activity in A and B horizons of two soils. Phosphorous fertilization reduced the phosphatase activity in the rhizosphere soil.

2.3.3 Urease activity

The urease activity in soil was first examined in the early 1940s. In soils, the activities appear to be correlated in general with microorganisms in soil (Anderson, 1962; Chin and Kroontje, 1963). The ureases are of much importance for urea hydrolysis. Performance of urea over other ammonical fertilizers as practices in modern agriculture adds to the importance of these enzymes. Much of its activity is found to be in the free state of the enzymes (Kiss *et al.*, 1975). In soils, the activity of urease appears to be correlated in general with number of microorganisms and increased with increasing organic matter content. The maximum activity of urease in most soils is found to be at pH 6.5 to 7.0.

Urease activity generally correlates well with organic matter due to its existence as a complex with organic constituents (Skujins, 1976). Urease enzyme originates in soil from both soil microorganisms and plant roots (Bremmer and Mulvancy, 1978) and is widely spread among the plant species.

Joshn *et al.* (1991) surveyed the forest soil, undisturbed grassland soil, disturbed grassland soil and a highly disturbed plant community soil at Jowai, Meghalaya for microbial population, soil respiration, enzyme activities and VAM fungi. Organic matter, moisture content, CO₂ evolution and enzyme activities were positively correlated with the microbial population.

2.4 Study of soil microbial diversity using molecular method

The microbial diversity in the rhizosphere soil can be analysed by cultural methods, molecular methods or a combination of both. The different culture independent molecular methods include DNA reassociation rate, mole %G+C composition, Polymerase Chain Reaction (PCR)-Density Gradient Gel Electrophoresis (DGGE)/Temperature Gradient Gel Electrophoresis (TGGE) sequencing of individual bands. PCR-Single Strand Conformation Polymorphism (SSCP) sequencing of individual bands, PCR-(Terminal-Restriction Fragment Length Polymorphism)T-RFLP, PCR-Amplified ribosomal DNA restriction analysis (ARDRA), PCR-Ribosomal intergenic spacer analysis (RISA), PCR of rDNA cloning and sequencing, PCR of functional genes-cloning and sequencing, RNA dot/slot blot hybridization Fluorescent *in situ* Hybridization (FISH) and Random Amplification of Polymorphic DNA (RAPD) profiling.

2.4.1 Soil DNA extraction for studies of microbial diversity

The nature of a substantial part of the soil microbiota is essentially unknown as the culturing techniques gives information only about the culturables. Soil microbial community DNA and/or RNA extraction methods developed in the past decade have paved the way for direct, cultivation-independent, studies of microbial diversity in soils.

The direct extraction and analysis of total microbial community DNA or RNA from soil was shown to be fundamental as the basis to describe the *in situ*

soil microbiota and its diversity. After the pioneering work of Torsvik and co-workers in the early 1980s (e.g. Torsvik, 1980), a multitude of different extraction protocols has been published. The *Molecular Microbial Ecology Manual*, first and second editions (Akkermans *et al.*, 1995; Kowalchuk *et al.*, 2004), contains examples of all of these protocols. The need of different protocols is often explained by the large variations between soils in chemical characteristics, which necessitates the application of different protocols to almost every new soil. However, in the past few years, many laboratories have replaced their often-laborious ‘pet’ protocols with rapid commercial kit-based protocols, which appear to work comparably well in almost every instance.

All the different protocols, even the kit-based ones, can be grouped in two basically different types of approaches:

1. Disruption of bonds between microbial cells and soil particles, resulting in the release of largely bacterial or archaeobacterial cells, followed by cell lysis and extraction.
2. Direct cell lysis within the soil matrix in a slurry, and DNA extraction from the soil slurry (Ogram *et al.*, 1987).

While the former method provides DNA that is considered to be representative for the prokaryotic (bacterial or archaeal) fractions of the microbial community in soil, the latter has been shown to provide higher DNA yields, but is less specific for prokaryotes, as it also contains eukaryotic (fungal) and extracellular DNA (Steffan *et al.*, 1988; van Elsas *et al.*, 2000). Both methods co-extract low levels of extracellular DNA from soil, which should be taken into account in the interpretation of results, i.e. whether positive detection indicates the presence of microbial cells carrying target DNA or merely extracellular target DNA. In spite of these potential problems, the direct lysis and extraction method

has become the favourite DNA extraction method in many laboratories working with soil. On the other hand, recent advances have shown definite advantages of the indirect methods, with respect to the specificity of detection (Duarte *et al.*, 1998). Direct community DNA extraction from soil, as developed by Ogram *et al.* (1987) has been shown to provide a substantial amount of total soil bacterial DNA, but was also found to be prone to unavoidable yield losses. Direct lysis methods are more often used than cell extraction ones because they are less time consuming and give better recovery, resulting in an extracted DNA more representative of the whole microbial community present in the sample. The major disadvantage of direct lysis is that the method also extracts other organic soil components, such as humic and fulvic acids. Since these contaminants can prevent subsequent molecular analysis, such as restriction digestion, Polymerase Chain Reaction (PCR), and cloning (Tsai and Olson, 1991; Zohu *et al.*, 1996 and Miller *et al.*, 1999), a purification step is required. Several methods for extracting microbial DNA from soil or other environmental samples have been described. Many of these techniques employ extensive purification steps to ensure that the DNA is suitable for use in the polymerase chain reaction (Tsai and Olson 1991; Holben *et al.*, 1994; Zohu *et al.*, 1996; Miller *et al.*, 1999, Roose-Amsaleg, *et al.*, 2001). Soils are therefore one of the most challenging environmental matrices from which to obtain microbial DNA. Every type of sample, because of its own nature, requires optimization of the extraction and purification methods (Zohu *et al.*, 1996 and Miller *et al.*, 1999). Thus, selection of an appropriate DNA extraction and purification procedure from among the procedures that have been described to date remains a major problem in the application of molecular techniques to studies of soil and sediment microbial communities.

More *et al.*, 1994 showed that many direct protocols are likely to extract DNA mainly from easy-to-lyse cells, whereas, in particular, the minute microbial forms that are abundant in soil are often excluded. Since the original protocols

encompassed tedious cesium chloride (CsCl) gradient and/or hydroxyapatite chromatography purification steps, many more recent protocols have attempted to simplify the original protocol of Ogram *et al.*, 1987 (van Elsas *et al.*, 2000). Examples of these simplified protocols can be found in the literature (Pillai *et al.*, 1991; Porteous and Armstrong, 1991; Selenska and Klingmüller, 1991; van Elsas *et al.*, 1991; Tsai and Olson, 1992; Smalla *et al.*, 1993; Duarte *et al.*, 1998; van Elsas *et al.*, 2000).

The nucleic acid extraction methods are useful for several purposes (Trevors and van Elsas, 1989). First, they provide insight into the prevalence and/or activity of specific genes in microbial communities in soil ecosystems, resulting in a better understanding of the functioning and selection of such genes under specific soil conditions. Secondly, by using 16S/18S or 23S/25S ribosomal RNA gene sequences as ‘signature molecules’ (biomarkers), overall community DNA analysis can be the basis for description of microbial community structures. This can be achieved by applying fingerprinting techniques such as temperature or denaturing gradient gel electrophoresis (TGGE or DGGE), terminal restriction fragment length polymorphism (T-RFLP), or single-strand conformational polymorphism (SSCP).

These methods yield a fingerprint pattern that is a representation of the microbial community structure in the soil. Depending on the primers used in the initial PCR amplification, different microbial groups can be targeted. For instance, a range of primer sets has become available that target total bacteria, total fungi or specific bacterial groups, such as the α or β -subgroups of the proteobacteria, the high-G+C% Gram-positives, the Pseudomonads, the Bacilli, and *Burkholderia* spp. (Van Overbeek *et al.*, 2005). Primers for specific fungal groups, such as different vesicular arbuscular mycorrhizae, are also under development.

The analysis of the various fingerprints obtained from the microbial communities' DNA extracts allows making inferences about the types of microorganisms present within the extractable cell fraction of the soil. This obviously includes the non or poorly culturable cells, which represent the largest fraction of cells that can be found in most soils (silent majority).

The new angle on soil microbial diversity offered by the direct DNA-based methods has already led to the discovery of a wealth of novel organisms. For instance, several new deep-branching groups of proteobacteria have recently been described, based on the analysis of 16S ribosomal RNA gene clones generated from DNA of total soil communities. It has been shown that restrictable and PCR-amplifiable DNA of relatively high molecular weight can be obtained with the majority of nucleic acid extraction protocols. The microbial diversity measurements are, to some extent, dependent on the DNA extraction method used, and hence it is important to use one standard protocol in routine analyses.

2.4.2 Polymerase Chain Reaction (PCR) amplification

PCR is an enzymatic method of making multiple copies of a DNA fragment. This method was invented by Mullis *et al.*, 1986 and described by Saiki *et al.*, 1988. Following extraction and purification of the soil nucleic acids, the DNA is subjected directly to PCR using standard protocols (Akkermans *et al.*, 1995; Kowalchuk *et al.*, 2004). The method is based on the cyclic enzymatic 'inward' extension of primers at two opposite ends of a DNA template, resulting in the generation of numerous copies of the template DNA. The amplification cycle, which consists of template denaturing, primer-to-target annealing and primer extension steps, is achieved by concerted changes in reaction temperature, most easily performed in a programmable thermal cycler. Due to the high denaturing temperature (often 94°C), DNA polymerases used in the PCR have to be thermostable, for example the frequently used Taq polymerase from *Thermus*

aquaticus. In addition, the primers should be checked as to whether they actually perform well in a soil DNA background, as differences with respect to inhibition of the action of the polymerase have been found between different primer sets. Following PCR, the amplicons obtained should be carefully checked for quality and quantity by electrophoresis in agarose gel, using standard procedures (Sambrook *et al.*, 1989).

2.4.3 RAPD for the diversity analysis of soil microbial communities:

Franklin *et al.* (1999) examined similarity among a number of aquatic microbial communities using randomly amplified polymorphic DNA (RAPD), a common polymerase chain reaction (PCR)-based DNA fingerprinting technique. PCR-based DNA fingerprinting techniques such as RAPD are useful means of discriminating among microbial communities and estimating community relatedness. Moreover, this approach has several advantages over other DNA based procedures for whole-community analysis; it is less laborious and uses smaller quantities of DNA, making it amenable to sample-intensive monitoring, and it does not depend on culturing or the use of selective PCR primers.

The DNA sequence diversities for microbial communities in four soils affected by agricultural chemicals (mainly triadimefon and ammonium bicarbonate and their intermediates) were evaluated by Random Amplified Polymorphic DNA (RAPD) analysis (Yang *et al.*, 2000). Fourteen random primers were used to amplify RAPDs from four soil microbial community DNAs. The products of 12 primers were separated in gel and generated 155 reliable fragments, of which 134 were polymorphic. The richness, modified richness, Shannon–Weaver index, and a similarity coefficient of DNA were calculated to quantify the diversity to access DNA sequence diversities for four soil microbial communities.

Sharma *et al.* (2008) carried RAPD analysis of DNA isolated from soils under Ker and associated open areas of Bikaner, Kodamdesar and Nagaur regions

of western Rajasthan using six primers to find out the effect of Ker (*Capparis deciduas*) plants on microbial diversity.

Wang *et al.* (2009) assessed the combined effects of cadmium (Cd, 10 mg kg⁻¹ soil) and herbicide butachlor (10, 50, and 100 mg kg⁻¹ soil) on enzyme activities and microbial community structure in a paddy soil using the traditional enzyme assays and random amplified polymorphic DNA (RAPD) analysis.

Material and Methods



III. MATERIAL AND METHODS

To study the effect of the transgenic groundnut, expressing *ipt* gene, on the rhizosphere microorganisms, an experiment was conducted. The soil samples were collected from the crop physiology experimental plot at GKVK campus for the further studies including enumeration of different microbial groups, soil enzyme activities and RAPD profiling.

3.1 Soil sample collection

The soil was collected at three different stages-vegetative stage at 40DAS, flowering stage at 60DAS and harvest stage at 120DAS. The rhizosphere and near root zone soil, around 5-10g, was collected aseptically from 10 different sites in the groundnut field in sterile polythene bags each for transgenic and wild type. Similarly soil was collected from the non-rhizosphere sites in the field. The soil thus collected for every group was brought to the laboratory and under aseptic condition was mixed to obtain a composite sample for the three groups transgenic, wild type and non-rhizosphere. The composite samples were kept under refrigeration till further analysis. The samples were immediately taken for the enumeration of bacteria, fungi, actinomycetes, phosphate solubilisers and free living N₂-fixers-Azotobacter and for the activity of enzymes dehydrogenase, urease, acid and alkaline phosphatase. The soil samples collected at flowering stage were further used for the soil DNA extraction. The root samples were collected after the harvest of crop and preserved for the Arbuscular Mycorrhizal Fungi (AMF) root colonization studies.

3.2 Microbial enumeration

The microbial enumeration for the different samples was carried out by the standard dilution plate count technique with the dilutions 10⁻⁵ and 10⁻⁶ on Soil Extract Agar and Nutrient Agar media for bacteria, 10⁻² and 10⁻³ on Martin's Rose

Bengal Agar (Martin, 1950) and Czapek Dox Agar media for fungi, 10^{-3} and 10^{-4} on Kuster's Agar and Starch Casein Agar media for actinomycetes, 10^{-3} and 10^{-4} on Sperber's and Pikovaskya media for phosphate solubilisers and 10^{-3} and 10^{-4} on Waksman-77 and Azotobacter Basal media for the Azotobacter (as free living N_2 fixing microorganisms).

3.4 Studies on root colonization by AMF

The Arbuscular Mycorrhizal Fungi (AMF) do not cause obvious morphological changes to the roots; however, they produce arbuscules and in many cases, vesicles in roots. To observe VAM/AM fungus structures within the root it is necessary to clear cortical cells of cytoplasm and phenolic compounds and then to differentially stain the fungal cells. Philips and Hayman, 1970, gave the oft-cited method for visualization of AM fungi in roots by using 0.05% trypan blue in lactophenol, but the use of phenol is now discouraged (Koske and Gemma, 1989). The clearing agent for non-pigmented roots is generally 10% KOH, but treatment with H_2O_2 or NaCl (Graham *et al.*, 1991) may be necessary for pigmented roots. Alternatives to trypan blue for staining are chlorazol black E (Brundett *et al.*, 1984) and acid fuchsin (Kormanik and McGraw, 1982).

3.4.1 Procedure

- i. The root samples were washed thoroughly with water and cut into 1cm bits.
- ii. The bits were then placed in 0.5% KOH solution for 24hrs.
- iii. The bits were then removed from the KOH solution and placed in 1% HCl solution for 5mins.
- iv. The bits were then placed in lactoglycerol:acid fuchsin (1:1) mixture for 5 mins.
- v. The bits were then placed in lactoglycerol solution till observation.

- vi. For root colonization studies the few root bits were placed on a grid-plate and observed under dissecting microscope.

The % root colonization was calculated using the formula:

$$\% \text{ root colonization} = \frac{\text{No. of root bits with spores or vesicles}}{\text{Total No. of root bits placed}} \times 100$$

3.5 Identification and characterization of microbial isolates

Identification and characterization of the different microbial (bacterial) isolates obtained from the rhizosphere soil was done by using the tests as under the following groups:

1. Morphological characteristics:
 - a. Cell shape.
 - b. Gram-staining.
2. Cultural characteristics:
 - a. Growth on agar plates
3. Biochemical tests:
 - a. Carbohydrate fermentation:
 - i. Glucose.
 - ii. Sucrose.
 - iii. Lactose.
 - b. Starch hydrolysis.
 - c. Methyl-red test.
 - d. Voges-Proskauer test.
 - e. Citrate utilization test.
 - f. Catalase test.

3.6 Soil enzyme activity analysis:

3.6.1 Dehydrogenase activity (Casida *et al.*, 1964)

The dehydrogenase activity was determined by the procedure as given by Casida *et al.* (1964). For this, two grams of air-dried soil (<2mm) was thoroughly mixed with 0.2g of CaCO₃. To each sample tube 1ml of 3% aqueous solution of 2, 3, 5-triphenyl tetrazolium chloride (TTC) and 2.5 ml distilled water were added. The contents of each tube were mixed with a glass rod and tubes were stoppered and incubated at 37°C. After 24 hours, the stopper was removed and 10 ml of methanol was added to the tube and shaken for 1 min. Then, the suspension was filtered through a glass funnel plugged with absorbent cotton, into a 100ml volumetric flask. The soil in the tube was washed repeatedly till the filtrate coming from the tip of the funnel was colourless. Then, the volume was made to 100ml with methanol. The intensity of reddish colour was read using Milton Roy spectrophotometer at a wavelength of 485nm with methanol as the blank. The amount of TPF (2, 3, 5-triphenyl formazan) produced was calculated using a standard graph prepared by using different concentrations of TPF standards. For this 10 ml of the TPF standard solution containing 1000µg/ml was diluted to 100ml with methanol (100µg of TPF/ml). Aliquots of 5, 10, 15 and 20 ml were pipetted into 100ml volumetric flasks (500, 1000, 1500 and 2000µg of TPF/100ml). The volume was made to 100ml with methanol and mixed thoroughly. Then, the intensity of red colour of TPF was measured as described earlier.

3.6.2 Acid and alkaline phosphatase activity (Tabatabai and Bremmer, 1969)

The general name phosphatase has been used to describe a broad group of enzymes that catalyze the hydrolysis of both esters and anhydrides of H₃PO₄. Due to relative importance of phosphomonoesterases in soil organic P mineralization

and plant nutrition, their assay in soil assumes more importance. The enzymes are classified as acid and alkaline phosphomonoesterases, because they show optimum activities in their respective pH ranges. Alkaline phosphomonoesterase activity is derived from microorganisms only, while acid phosphomonoesterase is contributed both by plant roots and soil-inhabiting microbes.

Of the various methods available for assay of phosphomonoesterases activity in soils, the method developed by Tabatabai and Bremner (1969) is the most rapid, accurate and precise. It involves colorimetric estimation of the *p*-nitrophenol released by phosphomonoesterase activity, when the soil is incubated with buffer (at pH 6.5 and 11 for acid and alkaline phosphomonoesterase activities respectively) sodium *p*-nitrophenyl phosphate solution and toluene. Alkaline phenol has yellow colour, allowing it to be estimated colorimetrically. The CaCl_2 -NaOH treatment described for extraction of *p*-nitrophenol after incubation serves to stop the phosphomonoesterase activity, to develop yellow colour and to provide quantitative recovery of *p*-nitrophenol from soils.

For every soil sample two sets of 1g (oven dry equivalent) soil (<2mm) in 50ml conical flasks were taken. Out of these two sets, one will be used as control. 0.2ml toluene and 4ml of MUB (pH 6.5 or 11) was added to all flasks. 1ml of *p*-nitrophenyl phosphate solution was added to the sets other than control. The flasks were then swirled for few seconds to mix the contents. The flasks were stoppered and placed in an incubator at 37°C for one hour. After incubation, the stopper was removed and 1ml of 0.5M CaCl_2 and 4ml of 0.5M NaOH was added. The flasks were then swirled for few seconds. 1ml of *p*-nitrophenyl phosphate was added to the control flasks sets. The suspension is then quickly filtered through Whatman No.2 filter paper. The yellow colour intensity of the filtrate was measured at 440nm. The amount of *p*-nitrophenol produced was calculated using standard curve prepared using different concentration of *p*-nitrophenol. 1ml of standard *p*-

nitrophenol is diluted to 100ml (10ppm solution). Aliquots of 0, 1, 2, 3, 4 and 5ml aliquots into 50ml conical flasks were pipette into 50ml conical flasks. The volume is adjusted to 5ml by using distilled water. 1ml of 0.5M CaCl_2 and 4 ml of 0.5M NaOH was added and swirled for few seconds. All the suspensions were quickly filtered through Whatman No.2 filter paper. The yellow colour intensity of the filtrate was measured at 440nm.

3.6.3 Urease activity (Eivazi and Tabatabai, 1977)

The urease activity was determined by following the method outlined by Eivazi and Tabatabai (1977). Two grams of soil was taken in a 50ml Erlenmeyer flask and treated with 0.2ml of toluene and 9ml of THAM buffer and flasks were swirled for few seconds to mix the contents. Then, 1ml of 0.2M urea was added and the flasks were swirled for few seconds and placed in an incubator at 37°C. After 2 hours, the stoppers were removed and approximately 35ml of KCl- Ag_2SO_4 solution was added. Then, the flasks were swirled for few seconds and allowed to stand until the contents have cooled to the room temperature. The volume was then made up to 50ml by the addition of KCl- Ag_2SO_4 . The $\text{NH}_4\text{-N}$ was determined by steam distillation. Controls were performed in the same way, but 1ml of 0.2M urea was added after the addition of 35ml of KCl- Ag_2SO_4 .

3.7 Microbial diversity analysis using RAPD primers based amplification

3.7.1 Soil nucleic acid extraction method (van Elas and Smalla, 1995)

The soil sample collected at the flowering stage was used for the DNA extraction and further analysis of the microbial diversity.

The procedure described primarily extracts microbial DNA from soil. It is based on the extraction of 10 g of soil. It can be scaled down easily to accommodate 1–5 g soil samples. The full procedure is often needed to obtain restrictable and amplifiable DNA from a loamy sand soil with high organic matter

content, whereas purification until (and including) purification step I can be sufficient for DNA from soils with low organic matter content (e.g. silt loam types).

1. 10 g of soil was suspended in 15 ml 120 mM sodium phosphate buffer pH 8.0 (Sambrook *et al.*, 1989) in a 50 ml polypropylene tube.
2. *Lysozyme treatment:*
 - i. 75 mg lysozyme was added to the soil suspension, homogenized and incubated for 15min at 37°C.
 - ii. Chilled on ice.
3. *Freeze/thaw lysis: As an alternative to bead beating, freeze/thaw lysis was applied.*
 - i. 900 µl of 20% SDS was added to the soil slurry obtained and mixed.
 - ii. Freezed at –20°C (or –80°C) for 1 hr, then kept at 37°C for 30–45 min.
 - iii. Freeze/thaw cycle was repeated twice.
4. *Extraction and precipitation:*
 - i. An equal volume of Tris-buffered phenol pH 8.0 was added (Sambrook *et al.*, 1989) to the lysed cell slurry obtained.
 - ii. It was mixed well (manually) and centrifuged for 5 min at 10,000 × g (or 15 min at 3000 × g for polypropylene tubes) at room temperature.
 - iii. The aqueous (upper) phase was recovered in a new centrifuge (or polypropylene) tube.
 - iv. The phenol/soil mixture was back-extracted with 5 ml 120mM sodium phosphate buffer (pH 8.0).
 - v. The aqueous phases were pooled.

- vi. The pooled aqueous phases were extracted with an equal volume of chloroform/iso-amylalcohol (24:1).
- vii. The upper aqueous phase was recovered. If a heavy interphase was present, the aqueous phase was again extracted with an equal volume of chloroform/iso-amylalcohol.
- viii. 0.1 volume of 5 M NaCl and two volumes of ice-cold 96% ethanol was added. Kept at -80°C for 20 min or at -20°C for at least 1 hr. (often overnight).
- ix. Centrifuged for 5–10 min at $10,000 \times g$ (or 15–20 min at $3000 \times g$ for polypropylene tubes).
- x. The supernatant was discarded and the pellet was washed with ice-cold 70% ethanol. The pellet was then air-dried.
- xi. Pellet was resuspended in 1–1.5 ml (sterile) TE buffer pH 8.0 (10 mM Tris–HCl pH 8.0, 1 mM EDTA pH 8.0). Due to the volume of the pellet, the final volume may be larger.
- xii. The solution at this stage was called the crude extract.

Purification step: CsCl(Cesium Chloride) and CH_3COOK (Pot. Acetate) precipitations. All the steps were performed at room temperature, unless stated otherwise.

- i. 0.5 g CsCl was added to 500 μl crude extract.
- ii. Incubated for 1–3 hour.
- iii. Centrifuged for 20 min at maximum speed in an Eppendorf centrifuge.
- iv. The supernatant ($\approx 500 \mu\text{l}$) was recovered in a 10 ml tube.
- v. 2 ml deionized water and 1.5 ml iso-propanol was added. It was mixed and incubated for a minimum of 5 min at room temperature.

- vi. Centrifuged for 15 min at $10,000 \times g$. The supernatant was discarded and the pellet was resuspended in 500 μ l TE buffer (pH 8.0) and the suspension was transferred to a new Eppendorf tube.
- vii. 100 μ l 8M KAc was added, mixed and incubated for 15 min at room temperature.
- viii. Centrifuged for 15min at maximum speed in an Eppendorf centrifuge.
- ix. The supernatant was recovered and add 0.6 volume of iso-propanol was added, mixed and incubated for 5min at room temperature.
- x. Centrifuged for 15min at full speed in an Eppendorf centrifuge.
- xi. The pellet was washed with ice-cold 70% ethanol, dried and resuspended in 500 μ l TE buffer (pH 8.0).

3.7.2 Quantification of the microbial community DNA

It is crucial to know exact quantity of DNA present in the solution before carrying out the further experiment. Concentration can be accurately measured by UV absorbance. The amount of UV radiation absorbed at 260nm by the solution of DNA is directly proportional to the amount of DNA present in the sample. Usually the absorbance is measured at wavelength 260nm and 250nm, which allows the calculation of DNA concentration in the sample. An absorbance of 1 at 260 corresponds to 50 μ g/ml of double stranded DNA. UV-absorbance can also be used to check the purity of the DNA preparation. For a pure sample of DNA the ratio of the absorbance at 260nm to 280nm, A_{260}/A_{280} is a 1.8-2.0 ratio. Less than 1.8 indicates that the precipitation is contaminated either with phenol or protein values higher 2 indicate the presence of RNA in the preparation.

Procedure

- (i) The spectrophotometer was first switched on for 5 minutes for the equipment to stabilize.

- (ii) 1ml of TE buffer (pH 8.0) was taken in the cuvette and placed as the reference sample.
- (iii) 5µl of DNA sample was taken in 495µl of TE buffer (1x) in the other cuvette and mixed well. This was placed in the main reading slot and the absorbance at 260nm and 280nm wavelength was measured using the relationship 1OD at A260- 50µg/ml.

Amount of DNA was calculated by using the following formula

$$1 \text{ OD} = 50 \text{ } \mu\text{g of double stranded DNA/ ml at 260 nm}$$

$$\text{Amount of double stranded DNA} = X \times 50/2$$

Where, X = absorbance of the sample at 260 nm

Purity of the total DNA isolated was also tested spectrophotometrically based on A 280/260 and also checking the integrity of the DNA by gel electrophoresis.

3.7.3 Polymerase chain reaction (Mullis *et al.*, 1986)

The sample DNA obtained was amplified using RAPD (Random Amplified Polymorphic DNA) primers.

Polymerase chain reaction is a very simple method for *in vitro* amplification of specific nucleic acid using Taq DNA polymerase and short stretch of oligonucleotides (Primers), which are specific to the DNA to be amplified. DNA amplification involves repeated rounds of DNA synthesis, which is based on the following three simple steps.

- a. Denaturation of the template DNA in to simple strands.
- b. Annealing of primes to each original strands and new strand synthesis.
- c. Extension of a new DNA strands from the primers with the use of Taq polymerase.

Requirements for PCR:

- a. Template DNA: Purified DNA extracts from the soil microbial community were used as template DNA.
- b. Random Primers: Random primers were obtained from the Chromous Biotech.
- c. Taq DNA polymerase: Taq DNA polymerase and 10x Taq assay buffer were obtained from Chromous Biotech.
- d. Deoxy Nucleotide Triphosphate (dNTPs) 10mM
- e. Sterile PCR tubes.
- f. Micropipettes and sterile tips.
- g. Thermocycler.

3.7.4 RAPD primers

Four RAPD primers were used for the amplification of metagenomic soil DNA obtained from the rhizosphere of transgenic and wild type groundnut. The primers were:

1. RAPD14- GTCGCTCAGA
2. RAPD17- GAATGGGAGG
3. RAPD23- GTGATCGCAG
4. RAPD25- CAGCACCCAC

Polymerase Chain Reaction (PCR) was performed in a final volume of 10 μ l reaction mixture containing 1.0 μ l of 10x Taq assay buffer, 0.3 μ l Taq DNA polymerase, 2.0 μ l of 10mM dNTPs, 2.0 μ l of 10pmol random primers, 2.0 μ l of 50ng template DNA and 2.7 μ l of distilled water. The reaction mixture was chilled

on ice while its preparation. The content was then mixed with gentle tapping and placed in sterile PCR tubes.

The PCR tubes were kept inside the thermocycler programmed with the following conditions for amplification:

- i. Initial denaturation at 94°C for 3 mins.
- ii. 40 cycles of:
 - 1min. denaturation at 94°C,
 - 1min. primer annealing at 37°C,
 - 3mins. primer extension at 72°C.
- iii. Final extension at 72°C for 10 mins.
- iv. Amplified product is stored at 4°C.

3.7.5 Gel electrophoresis and visualization of amplified products

Agarose gel electrophoresis was performed to resolve the amplification product using 1% agarose in 1X TBE buffer, 0.5µg/ml of ethidium bromide, and loading buffer (0.25% bromophenol blue in 40% sucrose). 4µl of the loading buffer was added to 6µl of PCR amplicon and loaded to the agarose gel.

Materials

- i. The Tris-buffer (50 x stock solution), pH of the buffer was adjusted to 8.0.
- ii. 1% Agarose gel.
- iii. Gel frames and comb.
- iv. Ethidium bromide.
- v. Loading buffer.
- vi. UV-Transilluminator.

Procedure

1. The frame of gel casting unit cleaned and sealed with a tape to form a mould. The frame was placed on a flat platform to ensure flat and level base. The comb was then positioned parallel to the open edge of the frame.
2. Agarose powder was dissolved in TAE buffer by melting at 100°C. The solution was cooled to 50°C and 7µl of Ethidium bromide was added.
3. After setting, comb was removed and the gel was transferred to the gel tank such that the walls were towards the negative electrode. The gel tank was filled with TAE buffer just enough to cover the surface of the gel.
4. Amplified DNA samples of 6µl were mixed with 4µl of loading buffer and loaded in the wells of the submerged gel using a micropipette. DNA ladder were also loaded in one well.
5. The electrodes apparatus was connected to the power supply and electrophoresis was carried out at 100V for 90mins.
6. The gel was then visualized under UV-transilluminator and was documented.

3.7.6 Analysis of RAPD data

The bands were manually scored '1' for the presence and '0' for the absence and the binary data were used for statistical analysis. The scored band data was subjected to cluster analysis using STATISTICA. The dendrogram was constructed by Ward's method of clustering using minimum variance algorithm. The dissimilarity matrix was developed using Squared Euclidean Distance (SED), which estimated all the pair wise differences in the amplification product (Sokal and Sneath, 1973). Only clear and unambiguous bands were taken into account and the bands were not scored if they were faint or diffused, as such fragments

possess poor reproducibility. The band sizes were determined by comparing with the 100bp DNA ladder, which was run along with the amplified products.

Statistical analysis

The data obtained from the experiments were statistically analyzed using two way analysis CRD (Completely Randomized Design) at 5% level of significance.

Experimental Results



IV. EXPERIMENTAL RESULTS

The enumeration and characterization of rhizosphere microflora of transgenic and wild type groundnut was carried out. The results of the experiments are presented.

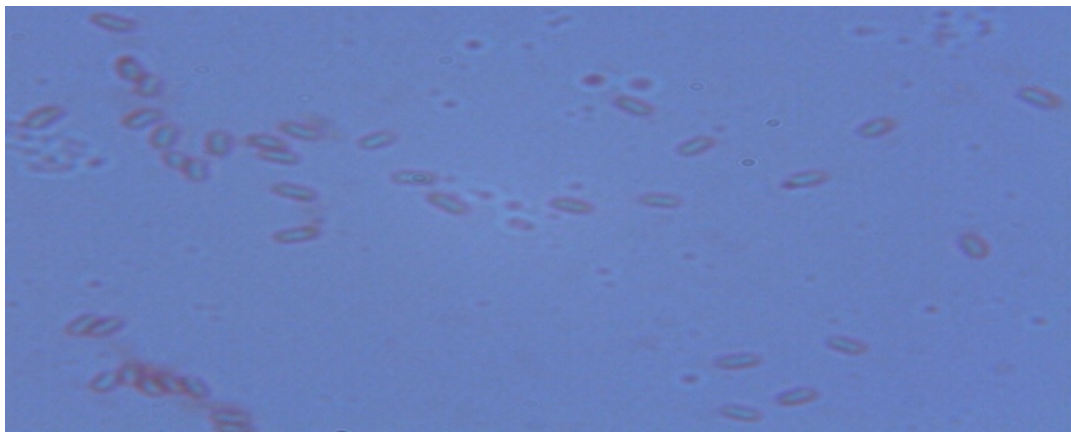
4.1 Microbial enumeration

4.1.1 Bacterial population

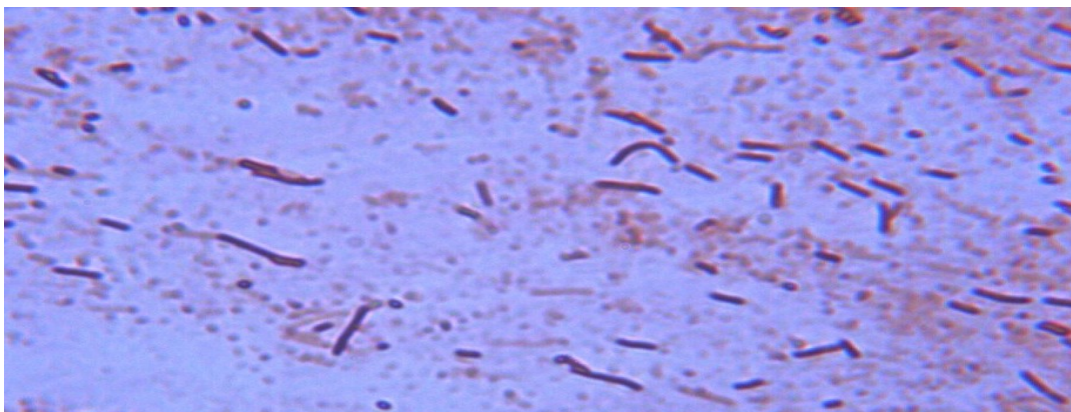
The bacterial population in the rhizosphere of transgenic and wild type groundnut was enumerated and presented in the Table 1 and Fig. 1. The population of bacteria was significantly higher in transgenic rhizosphere (28.67×10^5 CFU/g) as compared to wild type rhizosphere, when enumerated on SEA (Soil Extract Agar) and it increased (43.00×10^5 CFU/g) with the growth of the plant till flowering stage.

When enumerated using NA (Nutrient Agar) the population was significantly higher (32.33×10^5 CFU/g) at flowering stage for transgenic rhizosphere compared to the wild type (19.67×10^5 CFU/g). The bacterial population of non rhizosphere soil was significantly lesser than the transgenic and wild type rhizosphere at all the growth stages. The population was more on the SEA compared to the NA for all the crop growth stages.

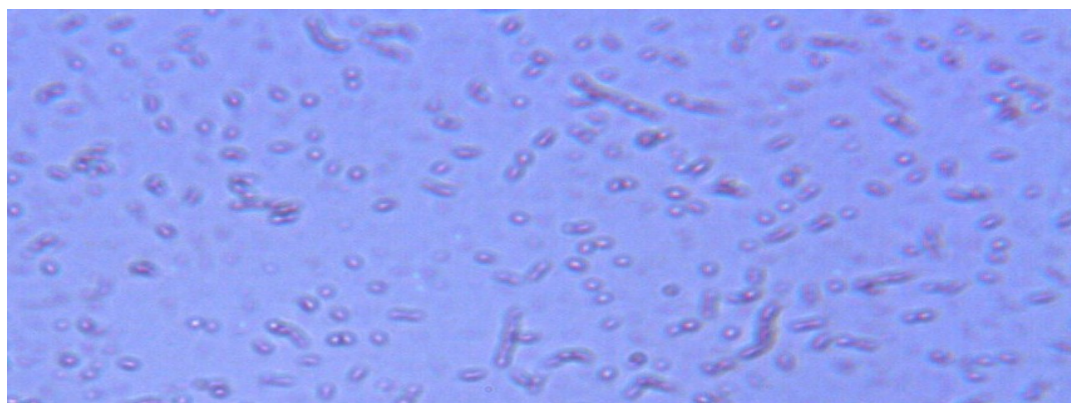
Based on the cultural and biochemical characteristics (Plates 1, 2 and 3) of the bacterial isolates as presented in Table 2A, 2B and 2C, the major genus found at different growth stages of the both transgenic and wild type groundnut were *Bacillus*, *Pseudomonas*, *Enterobacter*, *Klebsiella* and *Streptococcus*. The presence of Gram negative rod shaped bacteria was higher in the rhizosphere of both types of groundnut.



a) Gram negative , rod shaped bacteria

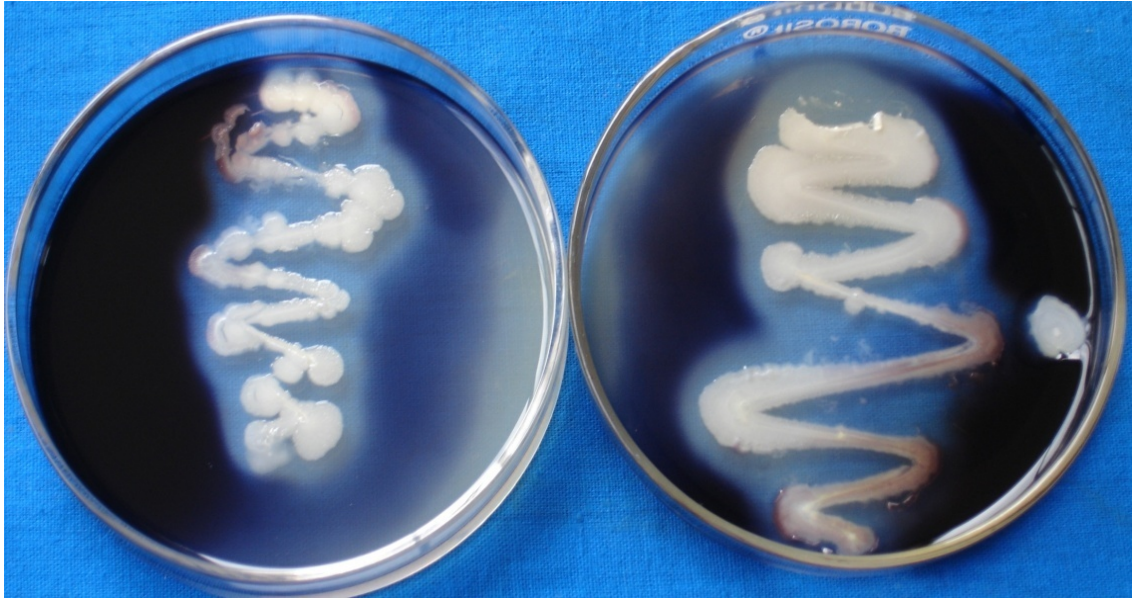


b) Gram positive, rod shaped bacteria

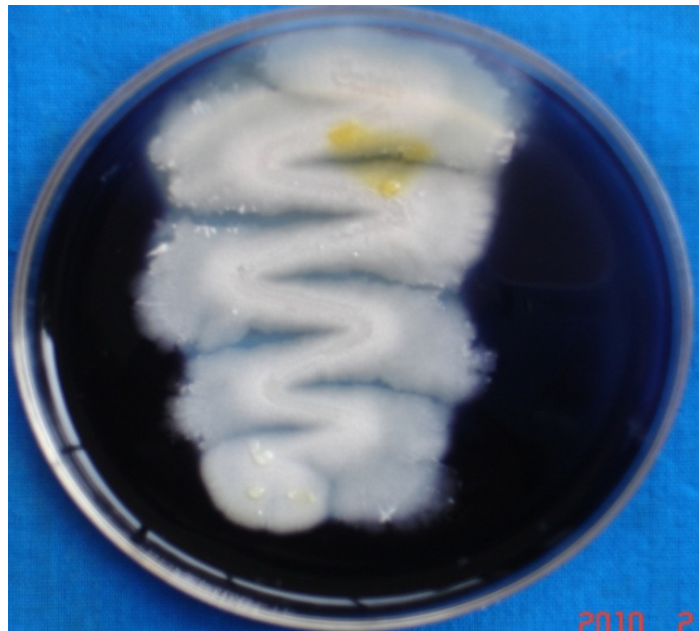


c) Gram positive, cocci bacteria

Plate 1. The Gram staining reaction for identification of different bacterial isolates

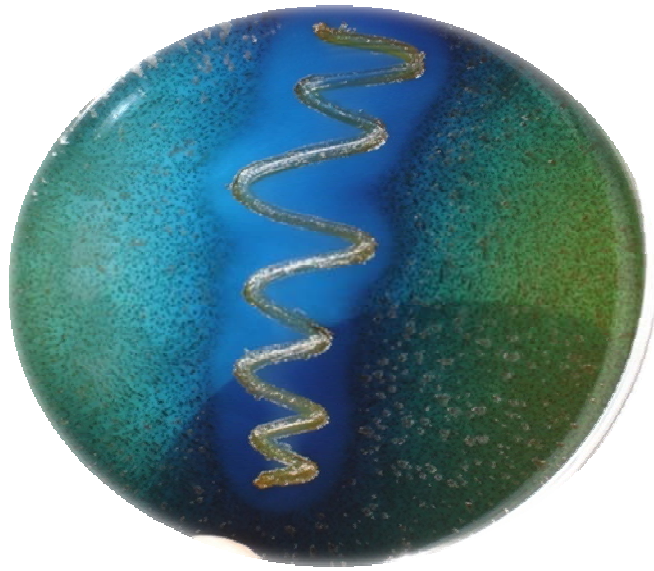


a) Positive test



b) Negative test

Plate 2. Starch hydrolysis test for the amylase production by the bacterial associates



a) Positive test



b) Negative test

Plate 3. Citrate utilization test

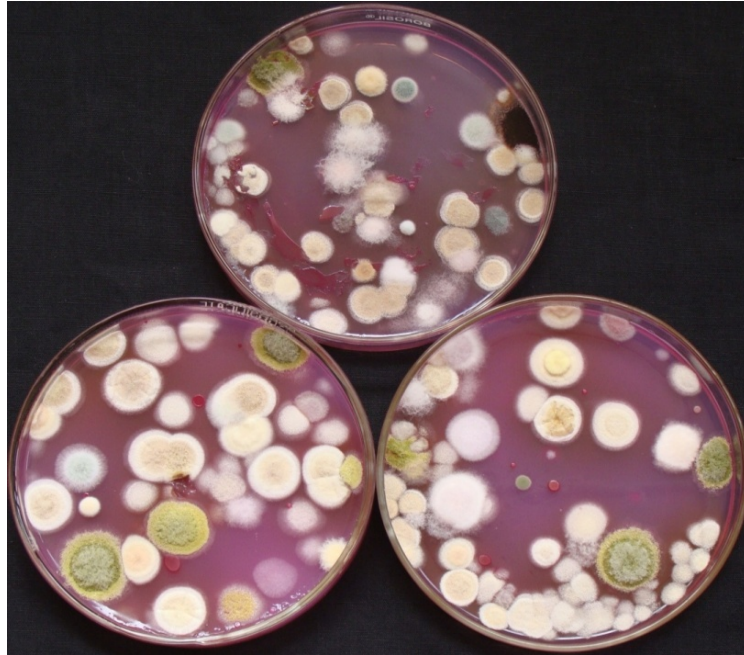
4.1.2 Fungal population

The fungal population in the rhizosphere of transgenic and wild type groundnut was enumerated and presented in Table 3, Fig. 2 and Plate 4. When enumerated on MRBA (Martin Rose Bengal Agar), the fungal population for the wild type rhizosphere was significantly higher (50×10^2 CFU/g soil) and 105×10^2 CFU/g soil than the transgenic rhizosphere at vegetative stage (40 days) and flowering stage (120 days) respectively.

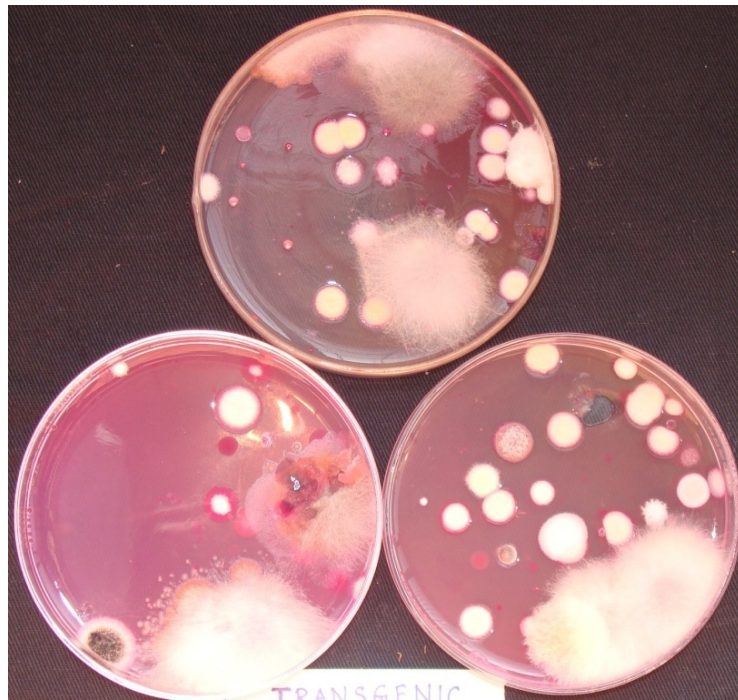
The fungal population increased with the growth stages but decrease in population was observed at harvest stage. The population of non rhizosphere soil was significantly lesser than the transgenic and wild type rhizosphere.

The enumeration on CDA (Cza-pek Dox Agar) revealed more population in transgenic rhizosphere as compared to the wild type at all the stages but the difference was non-significant. The fungal population increased at flowering stage and then decreased at harvest stage. Also the fungal population on CDA was significantly lesser than that obtained on MRBA.

Based on the morphological characteristics as presented in Table 4A, 4B and 4C, and the microscopic observations of the different fungal isolates different morphological types were observed both in transgenic and wild type groundnut rhizosphere at all the three stages of crop growth, but with a few types at harvest stage. However the population was significantly higher than the non-rhizosphere soil. The predominant genera identified were *Aspergillus*, *Penicillium*, *Fusarium*, *Trichoderma* and *Pleurotus*. The different fungal isolates were presented in Plate 5 and 6.



a) In wild type rhizosphere



b) In transgenic rhizosphere

Plate 4. Fungal population at 10^{-2} dilution on Martin's Rose Bengal Agar (MRBA)



Plate 5. Different fungal isolates from the wild type groundnut rhizosphere.



Plate 6. Different fungal isolates from the transgenic groundnut rhizosphere

TABLE 1: Comparison of rhizosphere bacterial population in the transgenic and wild type groundnut

Soil sample	Bacterial population (10 ⁵ CFU/g soil)					
	Vegetative stage (40 Days)		Flowering stage (60 Days)		Harvest stage (120 Days)	
	SEA	NA	SEA	NA	SEA	NA
Transgenic Groundnut	28.67*	10	43*	32.33*	11.67	16
Wild-type groundnut	21.33	10	32	19.67	17*	16
Non-rhizosphere	14	11	12.33	9.67	11.67	8.33

CD (5%) = 3.2958

SEm± = 1.1493

SEA-Soil Extract Agar.

NA-Nutrient Agar.

*-significant difference

Table 2: Identification of different bacterial isolates by cultural and biochemical characterization

A. Vegetative stage

Isolates	Gram staining and cell shape	Cultural characteristics on agar plate	Starch hydrolysis	Carbohydrate fermentation			MR reaction	VP reaction	Citrate use	Catalase activity	Probable genus
				Lactose	Glucose	Sucrose					
TBV1	+ve, rod shaped	White waxy colony	+	-	Acid	Acid	-	-	-	-	<i>Bacillus</i>
TBV2	-ve, rod shaped	White, glistening colony	-	Acid, Gas	Acid, Gas	Acid, Gas	-	+	+	+	<i>Enterobacter</i>
TBV3	-ve, rod shaped	Slimy, white colony	-	Acid, Gas	Acid, Gas	Acid, Gas	-	-	+	+	<i>Klebsiella</i>
TBV4	+ve, cocci	Thin white colony	-	Acid	Acid	Acid	+	-	-	-	<i>Streptococcus</i>
TBV5	-ve, rod shaped	Thin, white	-	-	-	-	-	-	+	+	<i>Pseudomonas</i>
WBV1	+ve, rod shaped	White waxy colony	+	-	Acid	Acid	-	-	-	-	<i>Bacillus</i>

WBV2	-ve, rod shaped	White, glistening colony	-	Acid, Gas	Acid, Gas	Acid, Gas	-	+	+	+	<i>Enterobacter</i>
WBV3	-ve, rod shaped	Slimy, white colony	-	Acid, Gas	Acid, Gas	Acid, Gas	-	-	+	+	<i>Klebsiella</i>
WBV4	+ve, cocci	Thin white colony	-	Acid	Acid	Acid	+	-	-	-	<i>Streptococcus</i>
WBV5	-ve, rod shaped	Thin, white	-	-	-	-	-	-	+	+	<i>Pseudomonas</i>

TBV- Bacterium isolated from transgenic groundnut rhizosphere.

WBV- Bacterium isolated from wild type groundnut rhizosphere.

-ve –Gram Negative.

+ve –Gram Positive.

B. Flowering stage:

Isolates	Gram staining and cell shape	Cultural characteristics on agar plate	Starch hydrolysis	Carbohydrate fermentation			MR reaction	VP reaction	Citrate use	Catalase activity	Probable genus
				Lactose	Glucose	Sucrose					
TBF1	+ve, rods	White waxy colony	+	-	Acid	Acid	-	-	-	-	<i>Bacillus</i>
TBF2	-ve, rods	White, glistening colony	-	Acid, Gas	Acid, Gas	Acid, Gas	-	+	+	+	<i>Enterobacter</i>
TBF3	-ve, rods	Slimy, white colony	-	Acid, Gas	Acid, Gas	Acid, Gas	-	-	+	+	<i>Klebsiella</i>
TBF4	+ve, cocci	Thin white colony	-	Acid	Acid	Acid	+	-	-	-	<i>Streptococcus</i>
TBF5	-ve, rods	Thin, white	-	-	-	-	-	-	+	+	<i>Pseudomonas</i>
TBF6	+ve, rods	White waxy colony	+	-	Acid	Acid	-	-	-	-	<i>Bacillus</i>
TBF7	-ve, rods	White, glistening colony	-	Acid, Gas	Acid, Gas	Acid, Gas	-	+	+	+	<i>Enterobacter</i>
TBF8	-ve, rods	Slimy, white colony	-	Acid, Gas	Acid, Gas	Acid, Gas	-	-	+	+	<i>Klebsiella</i>
TBF9	+ve, cocci	Thin white colony	-	Acid	Acid	Acid	+	-	-	-	<i>Streptococcus</i>
TBF10	-ve, rods	Thin, white	-	-	-	-	-	-	+	+	<i>Pseudomonas</i>
TBF11	+ve, rods	White waxy colony	+	-	Acid	Acid	-	-	-	-	<i>Bacillus</i>
TBF12	+ve, cocci	Thin white colony	-	Acid	Acid	Acid	+	-	-	-	<i>Streptococcus</i>

TBF13	-ve, rods	Slimy, white colony	-	Acid, Gas	Acid, Gas	Acid, Gas	-	-	+	+	<i>Klebsiella</i>
TBF14	+ve, cocci	Thin white colony	-	Acid	Acid	Acid	+	-	-	-	<i>Streptococcus</i>
TBF15	+ve, cocci	Thin white colony	-	Acid	Acid	Acid	+	-	-	-	<i>Streptococcus</i>
TBF16	+ve, rods	White waxy colony	+	-	Acid	Acid	-	-	-	-	<i>Bacillus</i>
TBF17	+ve, cocci	Thin white colony	-	Acid	Acid	Acid	+	-	-	-	<i>Streptococcus</i>
WBF1	-ve, rods	Slimy, white colony	-	Acid, Gas	Acid, Gas	Acid, Gas	-	-	+	+	<i>Klebsiella</i>
WBF2	+ve, cocci	Thin white colony	-	Acid	Acid	Acid	+	-	-	-	<i>Streptococcus</i>
WBF3	-ve, rods	Thin, white	-	-	-	-	-	-	+	+	<i>Pseudomonas</i>
WBF4	+ve, rods	White waxy colony	+	-	Acid	Acid	-	-	-	-	<i>Bacillus</i>
WBF5	-ve, rods	White, glistening colony	-	Acid, Gas	Acid, Gas	Acid, Gas	-	+	+	+	<i>Enterobacter</i>
WBF6	-ve, rods	Slimy, white colony	-	Acid, Gas	Acid, Gas	Acid, Gas	-	-	+	+	<i>Klebsiella</i>

TBF- Bacterium isolated from transgenic groundnut rhizosphere.

WBF- Bacterium isolated from wild type groundnut rhizosphere.

-ve –Gram Negative.

+ve –Gram Positive.

C. Harvest stage:

Isolates	Gram staining and cell shape	Cultural characteristics on agar plate	Starch hydrolysis	Carbohydrate fermentation			MR reaction	VP reaction	Citrate use	Catalase activity	Probable genus
				Lactose	Glucose	Sucrose					
TBH1	+ve, rods	White waxy colony	+	-	Acid	Acid	-	-	-	-	<i>Bacillus</i>
TBH2	-ve, rods	White, glistening colony	-	Acid, Gas	Acid, Gas	Acid, Gas	-	+	+	+	<i>Enterobacter</i>
TBH3	+ve, cocci	Thin white colony	-	Acid	Acid	Acid	+	-	-	-	<i>Streptococcus</i>
TBH4	+ve, cocci	Thin white colony	-	Acid	Acid	Acid	+	-	-	-	<i>Streptococcus</i>
TBH5	-ve, rods	Slimy, white colony	-	Acid, Gas	Acid, Gas	Acid, Gas	-	-	+	+	<i>Klebsiella</i>
TBH6	-ve, rods	Thin, white	-	-	-	-	-	-	+	+	<i>Pseudomonas</i>
TBH7	-ve, rods	White, glistening colony	-	Acid, Gas	Acid, Gas	Acid, Gas	-	+	+	+	<i>Enterobacter</i>

TBH8	+ve, cocci	Thin white colony	-	Acid	Acid	Acid	+	-	-	-	<i>Streptococcus</i>
TBH9	+ve, rods	White waxy colony	+	-	Acid	Acid	-	-	-	-	<i>Bacillus</i>
TBH10	-ve, rods	White, glistening colony	-	Acid, Gas	Acid, Gas	Acid, Gas	-	+	+	+	<i>Enterobacter</i>
TBH11	+ve, cocci	Thin white colony	-	Acid	Acid	Acid	+	-	-	-	<i>Streptococcus</i>
TBH12	+ve, rods	White waxy colony	+	-	Acid	Acid	-	-	-	-	<i>Bacillus</i>
TBH13	-ve, rods	White, glistening colony	-	Acid, Gas	Acid, Gas	Acid, Gas	-	+	+	+	<i>Enterobacter</i>
TBH14	-ve,rods	Slimy, white colony	-	Acid, Gas	Acid, Gas	Acid, Gas	-	-	+	+	<i>Klebsiella</i>
WBH1	+ve, cocci	Thin white colony	-	Acid	Acid	Acid	+	-	-	-	<i>Streptococcus</i>
WBH2	-ve, rods	White, glistening colony	-	Acid, Gas	Acid, Gas	Acid, Gas	-	+	+	+	<i>Enterobacter</i>

WBH3	-ve, rods	Slimy, white colony	-	Acid, Gas	Acid, Gas	Acid, Gas	-	-	+	+	<i>Klebsiella</i>
WBH4	-ve, rods	Thin, white	-	-	-	-	-	-	+	+	<i>Pseudomonas</i>
WBH5	+ve, cocci	Thin white colony	-	Acid	Acid	Acid	+	-	-	-	<i>Streptococcus</i>
WBH6	+ve, rods	White waxy colony	+	-	Acid	Acid	-	-	-	-	<i>Bacillus</i>
WBH7	-ve, rods	White, glistening colony	-	Acid, Gas	Acid, Gas	Acid, Gas	-	+	+	+	<i>Enterobacter</i>
WBH8	-ve, rods	Slimy, white colony	-	Acid, Gas	Acid, Gas	Acid, Gas	-	-	+	+	<i>Klebsiella</i>
WBH9	+ve, cocci	Thin white colony	-	Acid	Acid	Acid	+	-	-	-	<i>Streptococcus</i>
WBH10	-ve, rods	Thin, white	-	-	-	-	-	-	+	+	<i>Pseudomonas</i>
WBH11	+ve, cocci	Thin white colony	-	Acid	Acid	Acid	+	-	-	-	<i>Streptococcus</i>

TBH- Bacterium isolated from transgenic groundnut rhizosphere.

WBH- Bacterium isolated from wild type groundnut rhizosphere.

-ve –Gram Negative.

+ve –Gram Positive.

TABLE 3: Comparison of fungal population in the rhizosphere of transgenic and wild type groundnut

Soil sample	Fungal population (10^2 CFU/g soil)					
	Vegetative stage (40 Days)		Flowering stage (60 Days)		Harvest stage (120 Days)	
	MRBA	CDA	MRBA	CDA	MRBA	CDA
Transgenic Groundnut	37.00	14.00	71.33	21.33*	31.00*	12.67
Wild-type groundnut	50.00*	11.00	105.00*	20.00	23.33	10.33
Non-rhizosphere	9.67	6.67	8.00	6.00	10.67	5.67

CD (5%) = 3.0227

SEm $\pm$ = 1.0541

MRBA-Martin Rose Bengal Agar.

CDA-Czapek's Dox Agar.

*-significant difference.

4.1.3 Actinomycetes population:

The actinomycetes population in the rhizosphere of transgenic and wild type groundnut was enumerated and presented in the Table 5 and Fig. 3.

The population of actinomycetes when enumerated on KA (Kuster's Agar) showed highest population (6×10^3 CFU/g soil) at flowering stage for the wild type compared to the transgenic rhizosphere (1.00×10^3 CFU/g). The population of actinomycetes for the non-rhizosphere soil was significantly higher than the transgenic at all the growth stages.

The population of actinomycetes enumerated on SCA (Starch Casein Agar) for both transgenic and wild type rhizosphere was not significantly different from that on KA. Also the population on SCA was not significantly different for the transgenic, wild type rhizosphere and the non rhizosphere soil for the all the three stages.

Total microbial population density (based on Tables 1, 3 and 5) and the rhizosphere effect in the rhizosphere of transgenic and wild type groundnut at different stages of growth was presented in Table 6 and Fig. 4.

There was an increase in total microbial population at flowering stage and reduced at harvest. The rhizosphere effect of transgenic was higher both during vegetative and flowering stages compared to the wild type groundnut. R:S ratio was higher in wild type rhizosphere at harvest than the transgenic rhizosphere which may be due to root exudations available for microbial growth.

Table 4: Morphological characteristics of the different fungal isolates

A. Vegetative stage:

Fungal isolates	Morphological characters	Probable genus
TFV1	Large, black spore producing colony	<i>Aspergillus</i>
TFV2	Yellowish green colony	<i>Aspergillus</i>
TFV3	Light green colony	<i>Aspergillus</i>
TFV4	Dark green colony	<i>Trichoderma</i>
TFV5	White cottony growth	<i>Fusarium</i>
TFV6	Sunken white colony	<i>Aspergillus</i>
TFV7	White hairy colony	<i>Pleurotus</i>
WV1	Black spore producing colony	<i>Aspergillus</i>
WV2	Yellow colour colony	<i>Aspergillus</i>
WV3	Light green colony	<i>Aspergillus</i>
WV4	Dark green colony	<i>Trichoderma</i>
WV5	White cottony growth	<i>Fusarium</i>
WV6	White sunken colony	<i>Aspergillus</i>
WV7	White hairy colony	<i>Pleurotus</i>

TFV-Fungus isolated from transgenic groundnut rhizosphere.

WV- Fungus isolated from wild type groundnut rhizosphere.

B. Flowering stage:

Fungal isolates	Morphological characters	Probable genus
TFF1	Black spore producing yellow colony	<i>Aspergillus</i>
TFF2	Yellow cottony colony	<i>Aspergillus</i>
TFF3	Light green colony	<i>Aspergillus</i>
TFF4	Dark green colony	<i>Trichoderma</i>
TFF5	White cottony growth	<i>Fusarium</i>
TFF6	Sunken white colour colony	<i>Aspergillus</i>
TFF7	White hairy colony	<i>Pleurotus</i>
TFF8	Yellowish hairy colony	<i>Aspergillus</i>
TFF9	Small white thick leathery colony	<i>Pleurotus</i>
WFF1	Black spore producing colony	<i>Aspergillus</i>
WFF2	Yellowish colony	<i>Aspergillus</i>
WFF3	Light green colony	<i>Aspergillus</i>
WFF4	Dark green colour colony	<i>Trichoderma</i>
WFF5	White cottony growth	<i>Fusarium</i>
WFF6	Sunken white colour colony	<i>Aspergillus</i>
WFF7	White hairy colony	<i>Pleurotus</i>

TFF- Fungus isolated from transgenic groundnut rhizosphere.

WFF- Fungus isolated from wild type groundnut rhizosphere.

C. Harvest stage:

Fungal isolates	Morphological characters	Probable genus
TFH1	Greenish with white cottony cover	<i>Penicillium</i>
TFH2	White cottony	<i>Fusarium</i>
TFH3	Small thick white colony	<i>Pleurotus</i>
TFH4	Black spore producing colony	<i>Aspergillus</i>
TFH5	Green with white patches	<i>Trichoderma</i>
TFH6	Dark green spore like colony	<i>Trichoderma</i>
WFH1	Small greenish colony	<i>Penicillium</i>
WFH2	Yellowish small colony	<i>Aspergillus</i>
WFH3	Colony with green spore like appearance	<i>Trichoderma</i>
WFH4	White cottony growth	<i>Fusarium</i>
WFH5	White hairy growth	<i>Fusarium</i>
WFH6	Small thick white colony	<i>Pleurotus</i>
WFH7	Yellow with black spores	<i>Aspergillus</i>

TFH- Fungus isolated from transgenic groundnut rhizosphere.

WFH- Fungus isolated from wild type groundnut rhizosphere.

TABLE 5: Comparison of the actinomycetes population in the rhizosphere of transgenic and wild type groundnut

Soil sample	Actinomycetes population (10 ³ CFU/g soil)					
	Vegetative stage (40 Days)		Flowering stage (60 Days)		Harvest stage (120 Days)	
	KA	SCA	KA	SCA	KA	SCA
Transgenic Groundnut	1.00	1.33	1.00	1.67	1.00	1.00
Wild-type groundnut	2.33	1.33	6.00*	2.00	1.00	1.67
Non-rhizosphere	3.00	1.33	3.00	1.67	3.00	2.00

CD (5%) = 1.5281

SEm± =0.5329

KA-Kuster's Agar.

SCA-Starch Casein Agar.

*-significant difference.

Total microbial population density and the rhizosphere effect were higher in the rhizosphere of transgenic as compared to the wild type groundnut at the different stages of growth.

4.1.4 Phosphate solubilizing microorganisms:

The population of phosphate solubilisers (both bacteria and fungi) in the rhizosphere of transgenic and wild type groundnut was enumerated and presented in the Table 7 and Fig. 5.

On Sperber's medium the population of P-solubilizers was significantly higher (13.33×10^3 CFU/g soil) for the wild type rhizosphere as compared to the transgenic rhizosphere (10.00×10^3 CFU/g) at flowering stage. There was a significant increase in the population during growth of the crop till flowering and a decrease in the population was observed at harvest stage.

On Pikovaskya medium there was no significant difference between the population of P-solubilisers for transgenic and wild type rhizosphere. There was no significant difference between the populations on both the media except at flowering and harvest stage for the wild type rhizosphere. The population of P-solubilisers in the non-rhizosphere soil was significantly less as compared to both transgenic and wild type at all the three stages and the two media.

4.1.5 Free living N₂-fixing microorganisms:

The population of free living N₂-fixers as Azotobacter in the rhizosphere of transgenic and wild type groundnut was enumerated and presented in the Table 8 and the Fig. 6.

TABLE 6: Total microbial population density and the R:S ratio in the rhizosphere of transgenic and wild type groundnut at different stages of growth

Soil sample	Total microbial population density (10^6 CFU/g soil)					
	Vegetative stage (40 Days)	R:S ratio	Flowering stage (60 Days)	R:S ratio	Harvest stage (120 Days)	R:S ratio
Transgenic Groundnut	2.91*	2.08	4.31*	3.48	1.17	1
Wild-type groundnut	2.14	1.53	3.22	2.6	1.73*	1.48
Non-rhizosphere	1.40	-	1.24	-	1.17	-

*-significant difference.

TABLE 7: Phosphate solubilizing microorganisms in the rhizosphere of transgenic and wild type groundnut

Soil sample	P-solubilisers' population (10 ³ CFU/g soil)								
	Vegetative stage (40 Days)			Flowering stage (60 Days)			Harvest stage (120 Days)		
	SM	PM	Mean	SM	PM	Mean	SM	PM	Mean
Transgenic Groundnut	7.33	6.00	6.67	10.67	11.67	11.17	5.33	5.00	5.17
Wild-type groundnut	8.67	7.33	8.00	13.33*	9.67	11.50	7.00	4.00	5.50
Non-rhizosphere	3.67	2.33	3.00	3.67	2.00	2.83	2.67	1.33	2.00

CD (5%) = 2.1135

SEm± = 0.7370

SM- Sperber's Medium, PM- Pikovaskya Medium

CFU-Colony Forming Unit

*-significant difference.

The population of Azotobacter as enumerated on the ABM (Azotobacter Basal Medium) was significantly higher for the wild type rhizosphere as compared to the transgenic rhizosphere at harvest stage.

The population of Azotobacter enumerated was significantly same irrespective of the growth stage, type of medium used for the enumeration and the type of the rhizosphere soil. The population in the non-rhizosphere soil was not significantly different at the different growth stages and the type of medium for the enumeration.

The total microbial population from all the crop growth stages for the transgenic and wild type rhizosphere soil was presented in the Table 9. The bacterial population was higher in transgenic groundnut rhizosphere whereas the population of fungus and actinomycetes was higher in wild type. There was no significant difference between the population of P-solubilisers and free living nitrogen fixing microorganisms in both kinds of rhizosphere.

4.1.6 Arbuscular Mycorrhizal Fungi (AMF) colonization:

The observation of root colonization studies revealed that there was 100% colonization in the roots of both transgenic and wild type groundnut. The presence of Arbuscular Mycorrhizal Fungi (AMF) in the root tissues of the transgenic and wild type groundnut was shown in Plates 7 and 8 respectively.

4.2 Soil enzyme activities

4.2.1 Dehydrogenase activity

The activity of the enzyme dehydrogenase in the rhizosphere of transgenic and wild type groundnut was estimated and presented in the Table 10 and Fig 7.

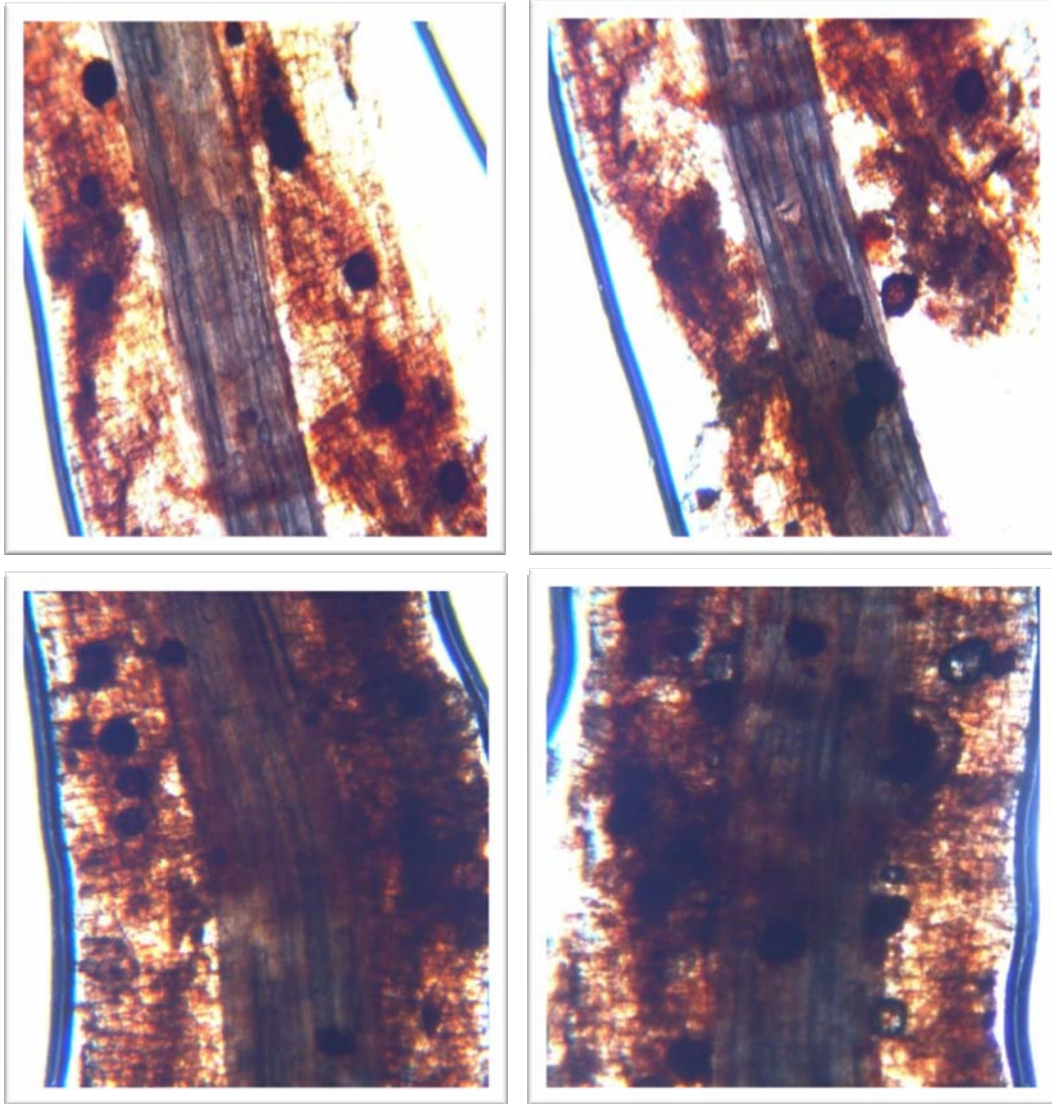


Plate 7. Arbuscular Mycorrhizal Fungi (AMF) colonization in the root of wild type groundnut

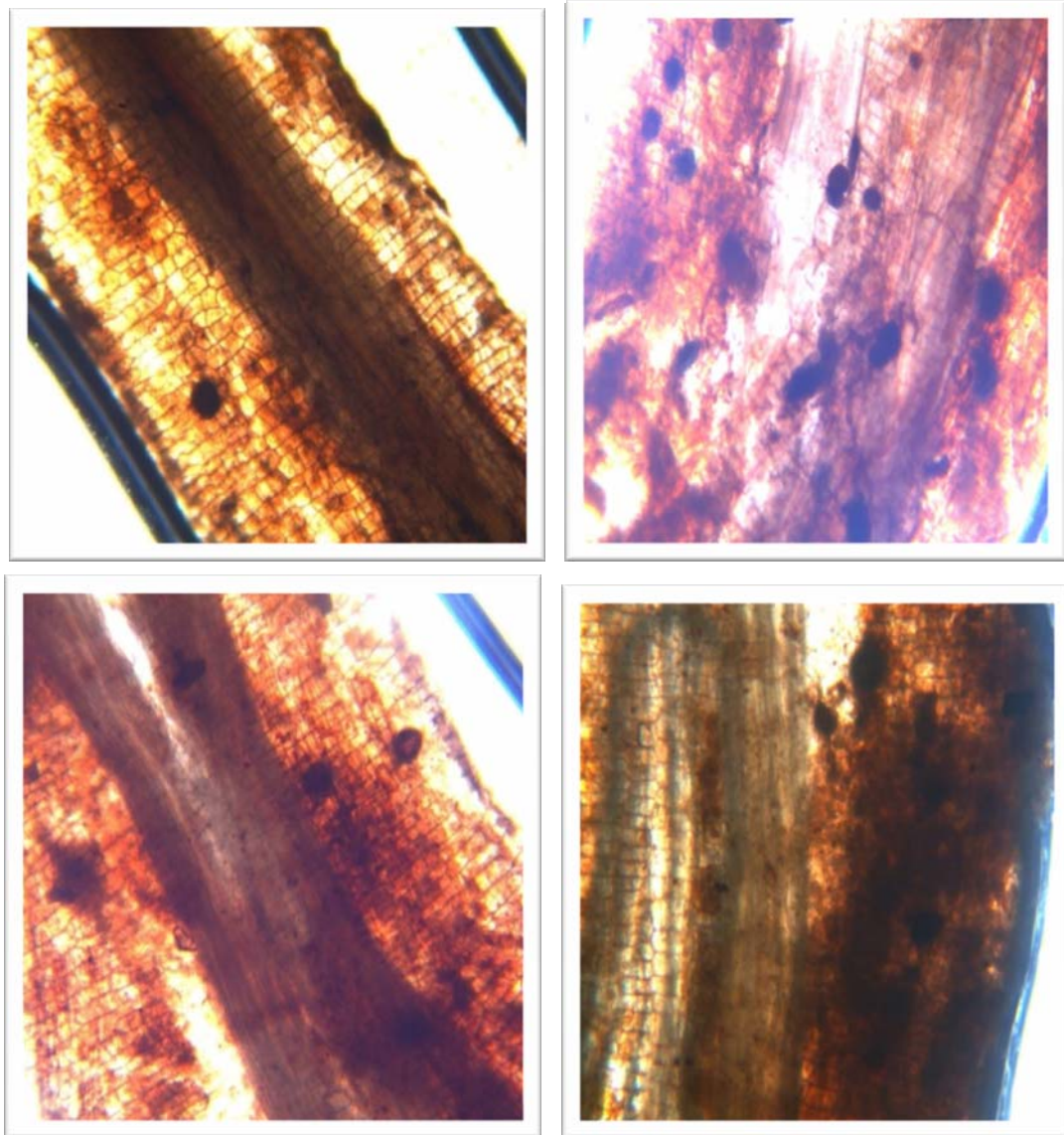


Plate 8. Arbuscular Mycorrhizal Fungi (AMF) colonization in the root of transgenic groundnut

TABLE 8: Free-living N₂-fixing microorganisms in the rhizosphere of transgenic and wild type groundnut

Soil sample	Free living N ₂ -fixers' population (10 ³ CFU/g soil)								
	Vegetative stage (40 Days)			Flowering stage (60 Days)			Harvest stage (120 Days)		
	ABM	W-77	Mean	ABM	W-77	Mean	ABM	W-77	Mean
Transgenic Groundnut	11.67	10.67	11.17	11.67	10.67	11.17	8.67	10.00	9.34
Wild-type groundnut	13.00	11.67	12.34	11.00	9.67	10.34	12.00	12.33	12.17
Non-rhizosphere	12.33	12.33	12.33	9.67	11.67	10.67	10.33	13.33	11.83

CD (5%) = 2.6658

SEm± = 0.9296

ABM-Azotobacter Basal Medium, W-77-Waksmann-77.

CFU-Colony Forming Unit

*-significant difference.

TABLE 9: The total microbial population from all the stages in the rhizosphere of transgenic and wild groundnut

Soil sample	Bacteria (x10⁵CFU/g soil)	Fungi (x10²CFU/g soil)	Actinomycetes (x10³CFU/g soil)	P-Solubilisers (x10³CFU/g soil)	Free living N₂-fixers (x10³CFU/g soil)
Transgenic Groundnut	23.61	31.22	1.16	7.66	10.55
Wild-type groundnut	19.38	36.61	2.38	8.33	11.61
Non-rhizosphere	11.16	7.77	2.33	2.55	11.61
CD (5%)	1.34	1.23	0.62	0.86	1.08
SEm±	0.47	0.43	0.22	0.30	0.38

TABLE 10: Dehydrogenase activity ($\mu\text{g TPF/g soil/day}$) in the rhizosphere of transgenic and wild groundnut

Soil sample	Enzyme activity ($\mu\text{g TPF/g soil/day}$)		
	Vegetative stage (40 Days)	Flowering stage (60 Days)	Harvest stage (120 Days)
Transgenic Groundnut	229.61	326.08	339.13
Wild-type groundnut	293.88*	370.01*	295.88*
Non-rhizosphere	137.63	138.58	133.55

CD (5%) = 1.7002

SE $\pm$ = 0.5723

TPF-Triphenyl formazan

*-significant difference.

The enzyme activity was higher (293.88 $\mu\text{g TPF/g soil/day}$ & 370.01 $\mu\text{g TPF/g soil/day}$ respectively) for the wild type as compared to the transgenic at the vegetative and the flowering stage. The enzyme activity increased with the growth stage till flowering both for the transgenic and wild type rhizosphere, but the enzyme activity was significantly higher (339.13 $\mu\text{g TPF/g soil/day}$) for the transgenic than the wild type rhizosphere at the harvest stage.

The activity of the enzyme in the non-rhizosphere soil was significantly lower than that in the transgenic and wild type rhizosphere. The enzyme activity for the non-rhizosphere soil showed small significant differences at the different growth stage.

4.2.2 Urease activity

The activity of the enzyme urease in the rhizosphere of transgenic and wild type rhizosphere was estimated and presented in the Table 11 and Fig. 8.

The activity of urease enzyme was highest (110.30 $\mu\text{g NH}_4\text{-N/g soil/day}$) for the wild type rhizosphere soil at flowering stage. The enzyme activity for the wild type rhizosphere increased significantly with the growth stage of the plant till flowering but was decreased at the harvest.

The difference in enzyme activity for transgenic rhizosphere was non-significant for the vegetative and flowering stage but it was significantly less at the harvest stage.

4.2.3 Acid phosphatase activity

The activity of acid phosphatase enzyme for the rhizosphere of transgenic and wild type was estimated and presented in Table 12 and Fig. 9.

The enzyme activity was highest (64.53 $\mu\text{g PNP/g soil}$) for the wild type rhizosphere at the flowering stage. The activity increased with the growth stage of the crop up to flowering but decreased at harvest stage. The activity of the enzyme was significantly lesser in the rhizosphere of transgenic and non-rhizosphere soil as compared with the wild type rhizosphere.

4.2.4 Alkaline phosphatase

The alkaline phosphatase enzyme activity in the rhizosphere of transgenic and wild type groundnut was estimated and presented in Table 13 and Fig. 10.

The enzyme activity was significantly highest (49.11 $\mu\text{g PNP/g soil}$) for the wild type rhizosphere at the flowering stage. The activity increased with the growth of the crop but decreased at the harvest stage. The activity in the transgenic rhizosphere soil was significantly lesser than the wild type at all the stages except harvest. The activity for both wild and transgenic rhizosphere was significantly higher as compared to the non-rhizosphere soil.

4.3 RAPD analysis

Four primers were used for the diversity analysis of the rhizosphere microbial community of transgenic and wild type. A total of 50 bands were obtained as shown in Plate 9. The dendrogram cluster analysis revealed that the two samples (metagenomic DNA from transgenic and wild type rhizosphere soil) formed two closely related groups as depicted in the Fig. 11 and there was no significant diversity among the two microbial communities analyzed.

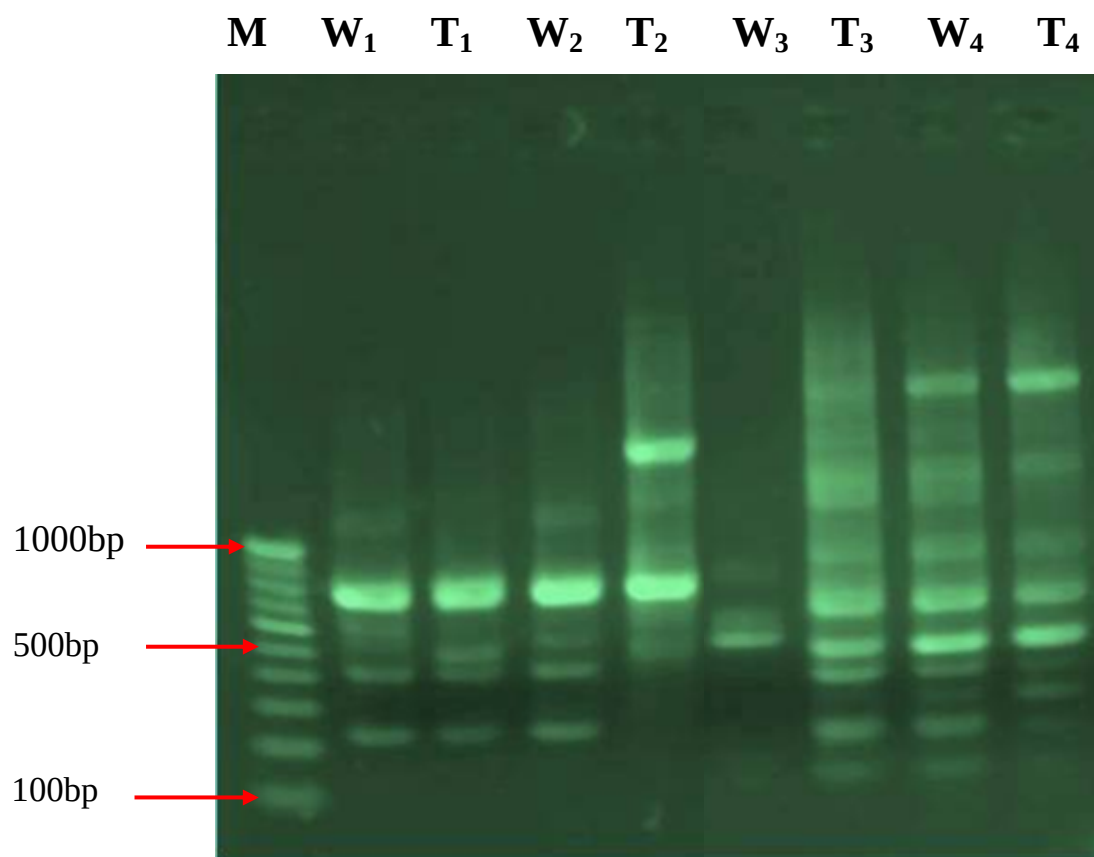


Plate 9. Gel electrophoresis of the Polymerase Chain Reaction (PCR) amplified product for the DNA from the rhizosphere soil of transgenic and wild type groundnut using the four RAPD primers.

TABLE 11: Urease activity (NH₄-N/g soil/2hr.) in the rhizosphere of transgenic and wild groundnut

Soil sample	Enzyme activity (NH ₄ -N/g soil/2hr.)		
	Vegetative stage (40 Days)	Flowering stage (60 Days)	Harvest stage (120 Days)
Transgenic Groundnut	105.01	105.86	95.63
Wild-type groundnut	106.76	110.30*	99.92*
Non-rhizosphere	84.67	82.13	83.40

CD (5%) = 1.4854

SEm $\pm$ = 0.500

*-significant difference.

TABLE 12: Acid phosphatase activity ($\mu\text{g PNP/g soil}$) in the rhizosphere of transgenic and wild groundnut

Soil sample	Enzyme activity ($\mu\text{g PNP/g soil}$)		
	Vegetative stage (40 Days)	Flowering stage (60 Days)	Harvest stage (120 Days)
Transgenic Groundnut	33.77	34.23	33.36
Wild-type groundnut	59.72*	64.53*	56.86*
Non-rhizosphere	32.15	30.96	31.04

CD (5%) = 1.7154

SE $\pm$ = 0.5774

PNP-*p*-nitrophenyl

*-significant difference.

TABLE 13: Alkaline phosphatase activity (µg PNP/g soil) in the rhizosphere of transgenic and wild groundnut

Soil sample	Enzyme activity (µg PNP/g soil)		
	Vegetative stage (40 Days)	Flowering stage (60 Days)	Harvest stage (120 Days)
Transgenic Groundnut	44.98	46.21	40.49*
Wild-type groundnut	48.89*	49.11*	37.41
Non-rhizosphere	31.52	31.58	32.15

CD (5%) = 1.7155

SE_m± = 0.5775

PNP-*p*-nitrophenyl

*-significant difference.

Discussion



V. DISCUSSION

The limited resources to suffice the increasing world population needs the implementation of crop improvement methods which may be attained either by plant breeding methods or by the development of transgenic crops with the desirable traits. But the impact of the environmental release of transgenic crops is of major concern both on above ground and below ground ecosystems. The present study was aimed to evaluate the effect of the *ipt* gene integrated transgenic groundnut.

The impact of the transgenic groundnut expressing the *ipt* gene on the soil microbial community was assessed both by the cultural and culture-independent methods. The cultural techniques involved the enumeration of the microbial population and the characterization of the different isolates whereas the culture-independent molecular method involved the RAPD profiling of the rhizosphere soil microbial community DNA for the transgenic and wild type groundnut. The microbial community analyses by the different methods were intended to complement each other.

5.1 Microbial population

Occurrence of bacterial population was highest during flowering stage of plant growth in both types of groundnut, but higher bacterial population in transgenic plants was observed as presented in Fig. 1.

Variation in the population density was recorded when different C and N sources were used for enumeration. Better bacterial density was observed when glucose was used as a C source in SEA compared to NA where there was no significant variation in the bacterial populations between transgenic and wild type except at flowering stage.

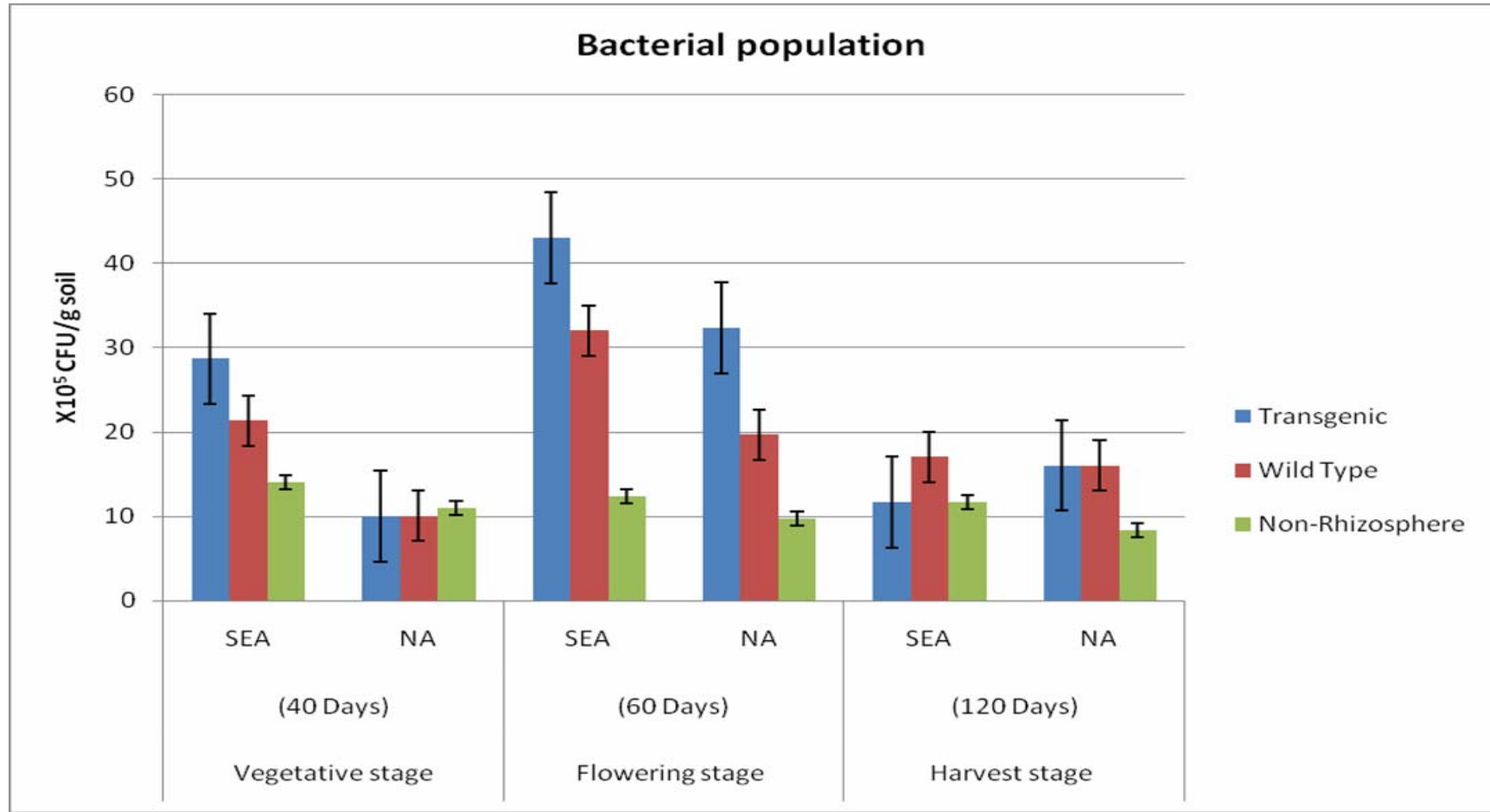


FIG. 1 Bacterial population in the rhizosphere of transgenic and wild type groundnut.

SEA- Soil Extract Agar.

NA- Nutrient Agar.

The fungal population was significantly higher in the rhizosphere of wild type. The population obtained on MRBA was significantly higher than that on CDA as the MRBA contains glucose as C-source whereas CDA contains sucrose which may be utilized by lesser population. The population on CDA was higher for transgenic than the wild type, which may be due to the presence of more number of sucrose utilizing fungi in the rhizosphere of transgenic than the wild type as presented in Fig. 2.

The difference in the population of actinomycetes was non-significant in the rhizosphere of transgenic and wild type groundnut at all the three growth stages as shown in Fig. 3.

The occurrence of beneficial microorganisms namely phosphate solubilizing microorganisms and free living N₂ fixers were found to be influenced by the transgenic. The population density of phosphate solubilizing microorganisms was comparatively higher in wild type than in the transgenic, where the predominant population was of bacillus type. The rhizosphere population of phosphate solubilizing microorganisms was significantly higher than the non-rhizosphere. However, there was no significant variation in population density of free living N₂ fixing microorganisms of both rhizosphere types recorded, wherein the predominant population was of Gram negative bacteria. Variation in population density in the wild type and transgenic rhizosphere was not significantly different from the non-rhizosphere.

The difference in the population of P-solubilisers was non-significant when enumerated on both of the media and at all the stages except at flowering stage as shown in Fig. 5.

There was not much variation in population density of nitrogen fixing bacteria at different stages of plant growth and also the carbon sources used

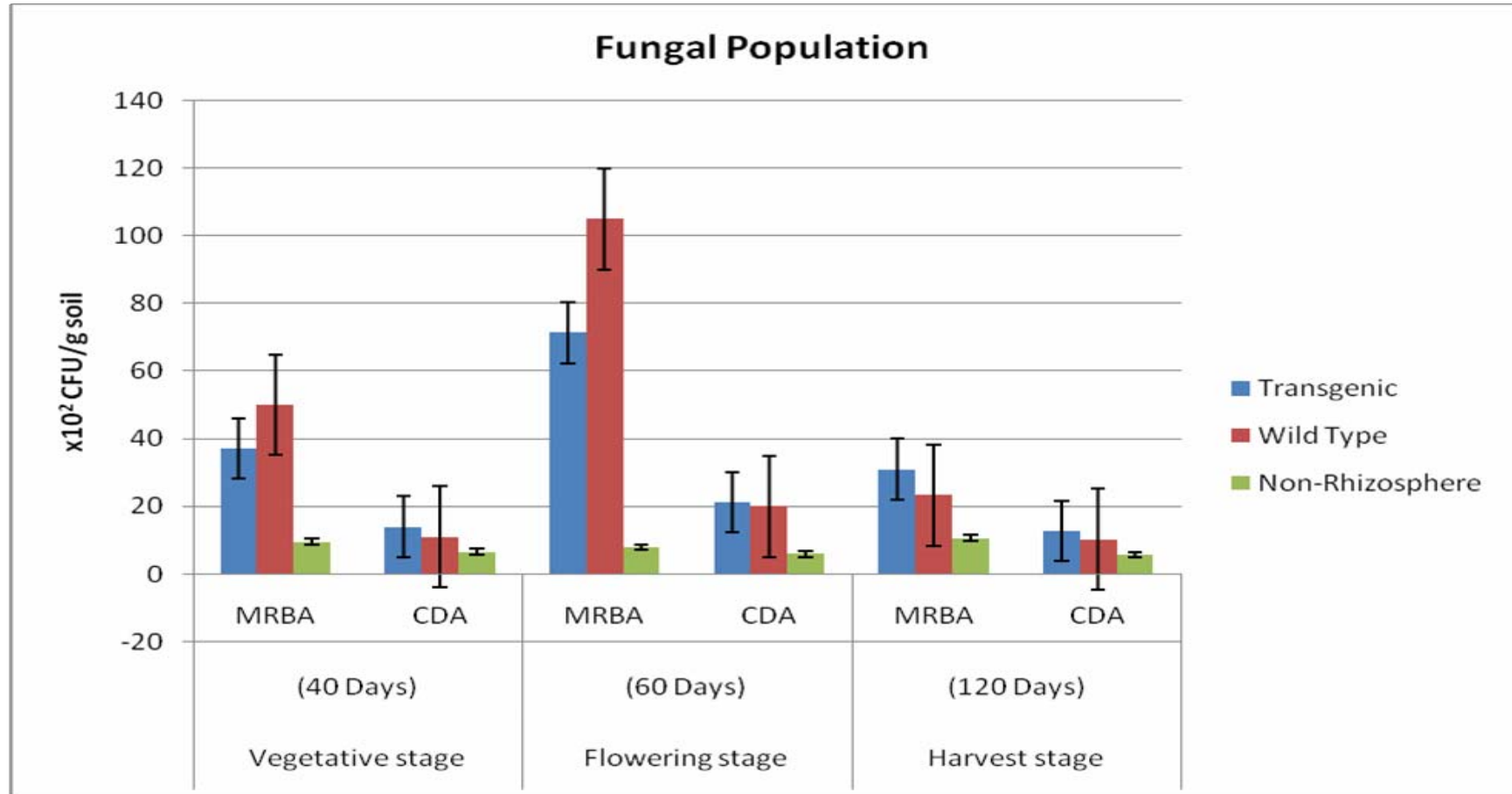


FIG. 2. Fungal population in the rhizosphere of transgenic and wild type groundnut.

MRBA-Martin Rose Bengal Agar.

CDA-Cza-pek Dox Agar.

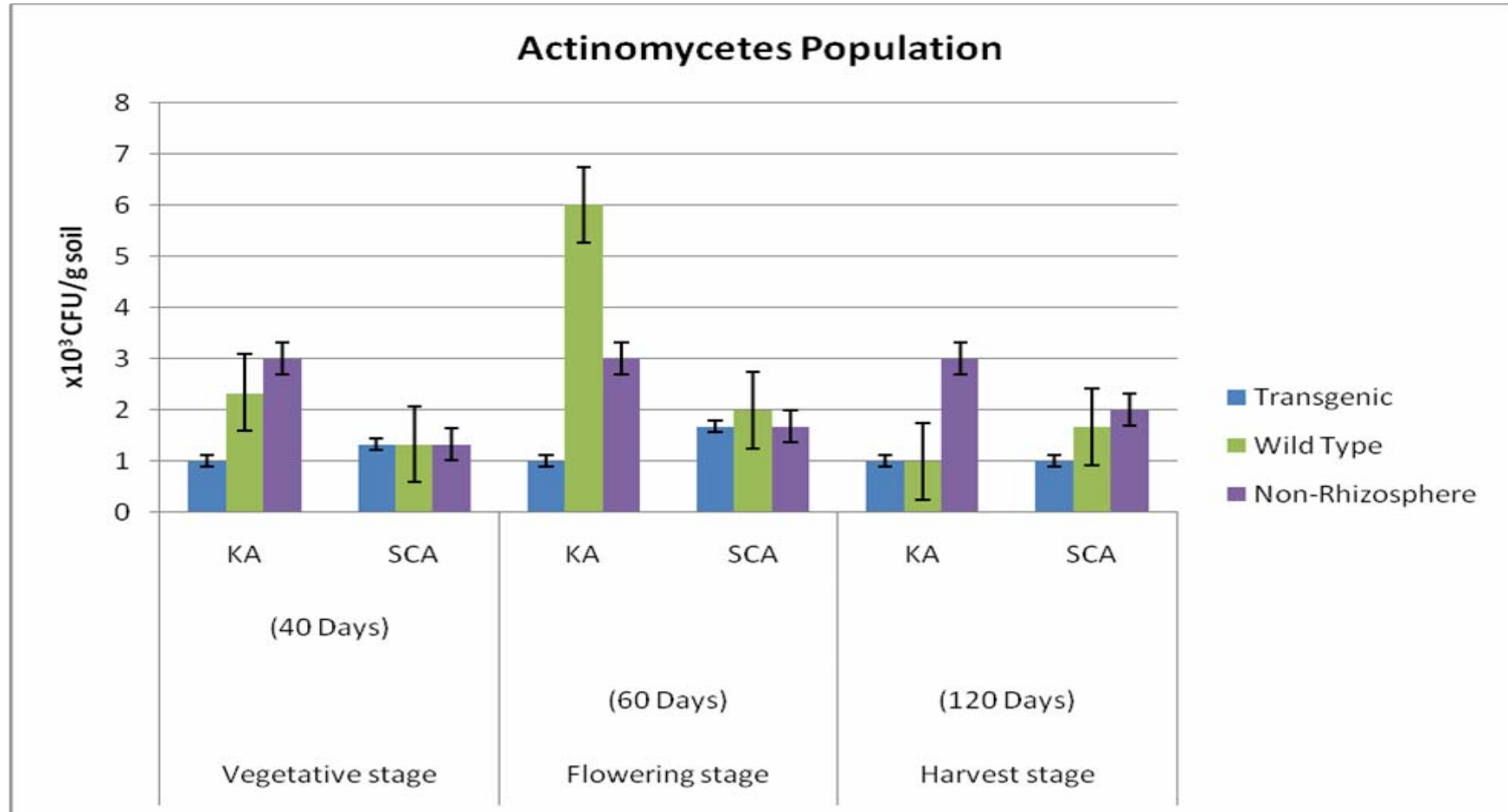


FIG. 3. Actinomycetes population in the rhizosphere of transgenic and wild type groundnut.

KA-Kuster's Agar.

SCA-Starch Casein Agar.

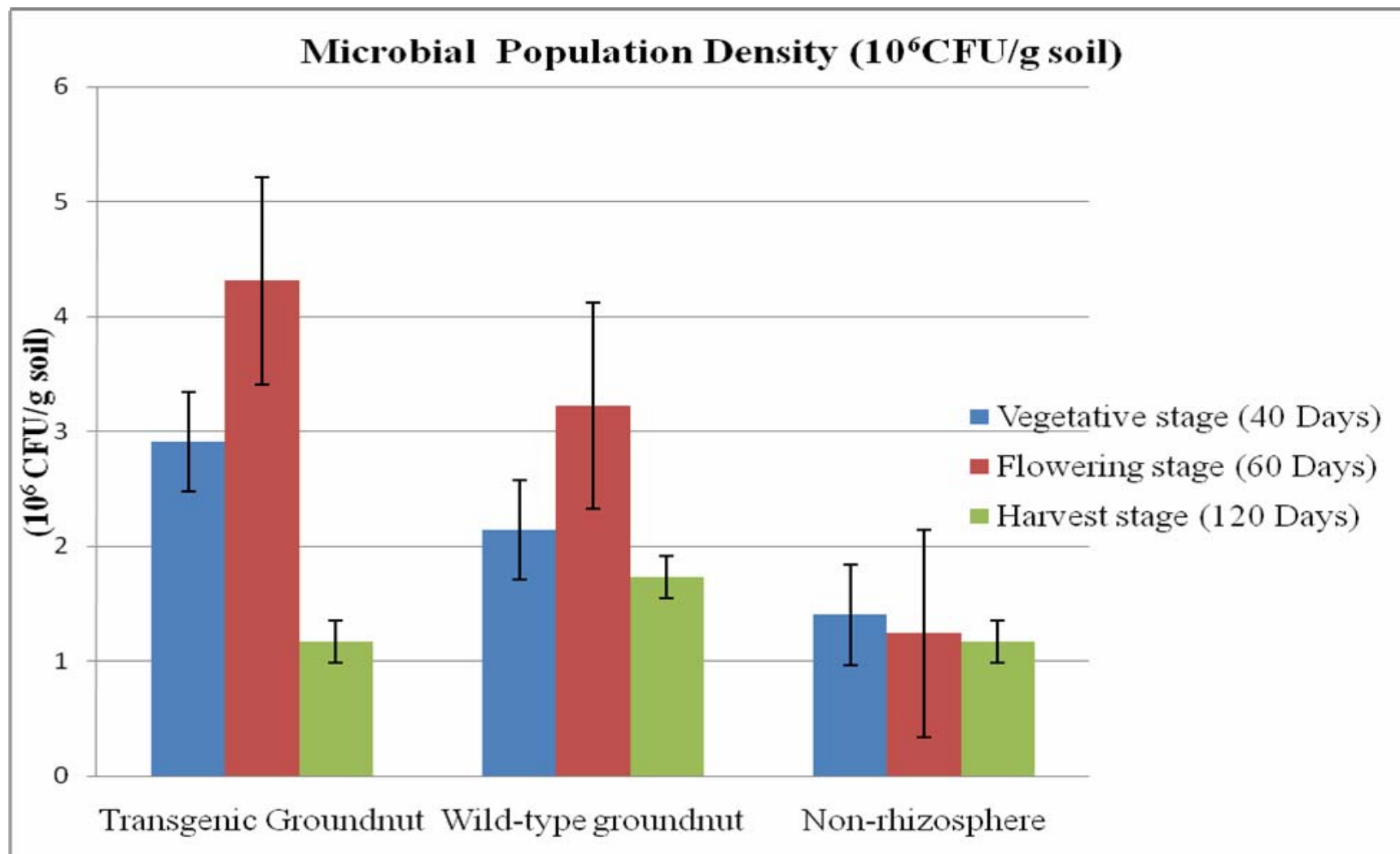


FIG. 4. Total microbial population density in the rhizosphere of transgenic and wild type groundnut at different stages of growth.

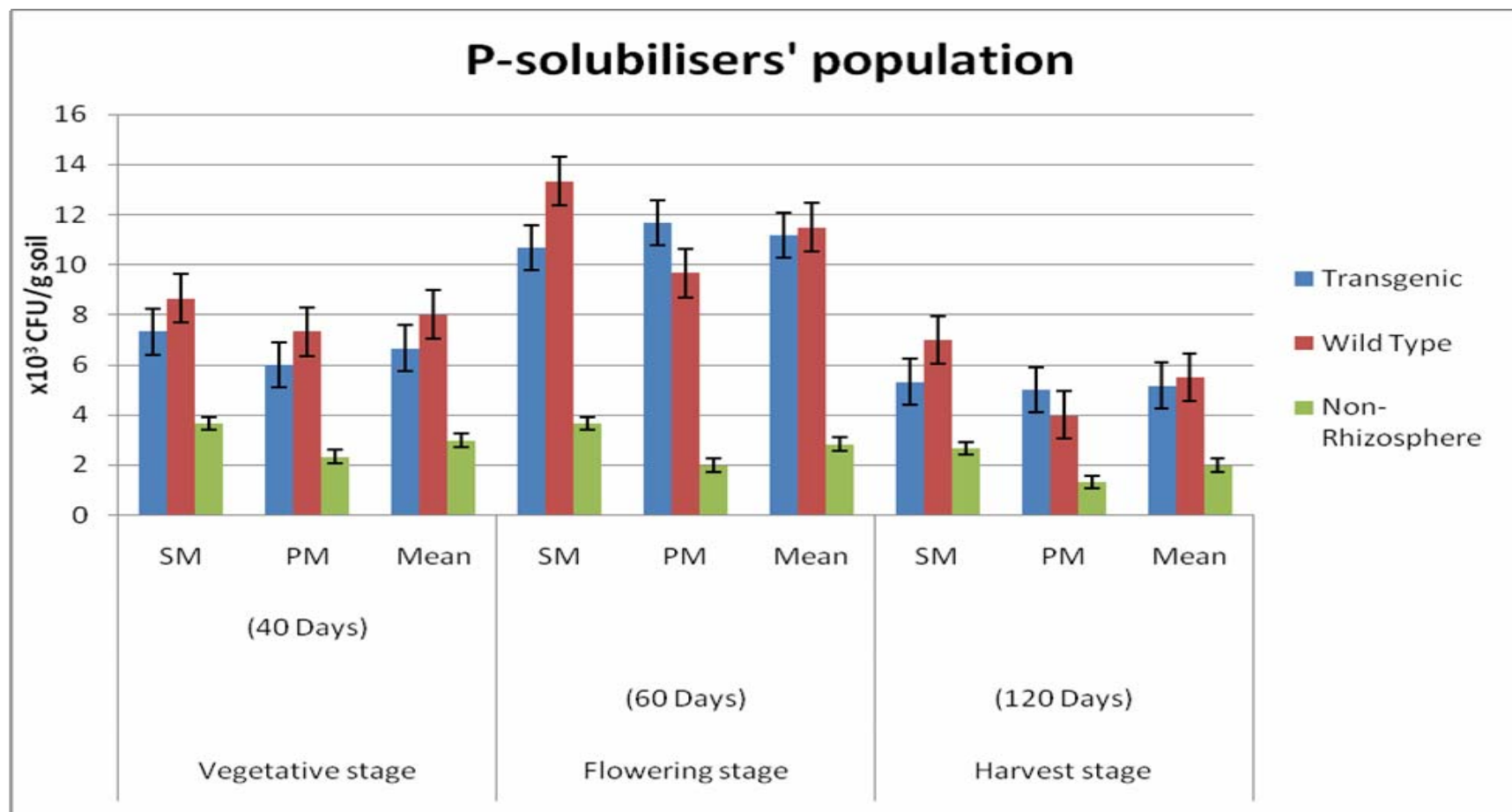


FIG. 5. P-solubilisers' population in the rhizosphere of transgenic and wild type groundnut.

SM- Sperber's Medium

PM- Pikovaskya Medium

namely mannitol and glucose did not show population variations in the rhizosphere of both plant types, including the non-rhizosphere soil as presented in Fig 6.

The population under different organisms was significantly higher for the rhizosphere of both transgenic and wild type than the non-rhizosphere soil indicating that the plant roots affect the population of microorganisms in the rhizosphere and near root zone area as presented in Fig 4. The total microbial density showed an increase with plant growth, recording a maximum population at flowering because of the availability of high concentration of nutrients at flowering stage. The influence of transgenic plants on rhizosphere as reflected by the R:S ratio, suggested to have an overall positive effect (4.31) compared to the wild type (3.22). that may be due to the influence of root exudates on growth and establishment of microorganisms in rhizosphere. At harvest there was no significant effect in both types of plants.

Donegan *et al.* (1995) evaluated the effect of the three different lines-81, 247 and 249, of cotton expressing the *Bacillus thuringiensis* var *kurstaki* endotoxin in the soil and observed significantly higher bacterial and fungal population levels on sample days 7, 14, 21, 28 and 56 for the two transgenic lines 247 and 249 out of the three as compared to the parental line.

Di Giovanni *et al.* (1999) observed that the bacterial densities of the rhizosphere of parental and two transgenic alfalfa, expressing bacterial gene for α -amylase and fungal genes for Mn-dependent lignin peroxidase respectively, varied little and had a mean of $5.9 \times 10^5 \pm 1.6 \times 10^5$ CFU/ml whereas Donegan *et al.* (1999) observed significantly higher population levels of culturable bacteria and fungi in the two transgenic alfalfa.

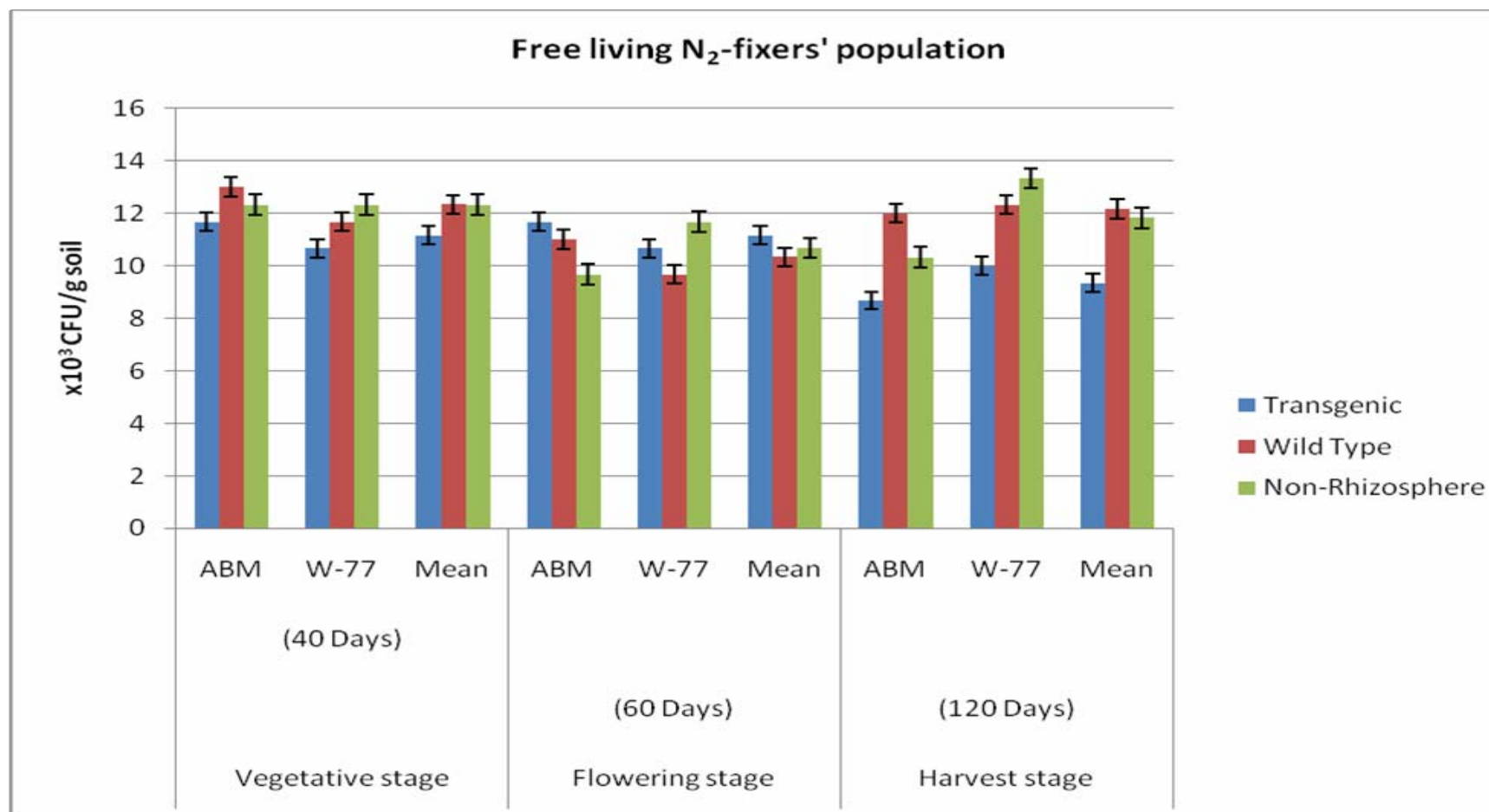


FIG. 6. Free living N₂-fixers' population in the rhizosphere of transgenic and wild type groundnut.

ABM-Azotobacter Basal Medium.

W-77-Waksmann-77.

In contrast, Saxena and Stotzky, 2001 observed no statistically significant differences in the CFU of culturable bacteria (including actinomycetes) due to the Bt toxin released from root exudates and biomass of Bt corn.

Fang *et al.* (2005) observed no significant effect on the bacterial community population in the rhizosphere of non-transgenic and transgenic corn.

The Arbuscular Mycorrhizal Fungi (AMF) colonization in the roots was not affected by the type of the groundnut cultivated. It was same for both the transgenic and wild type groundnut root.

Castaldini *et al.* (2005) studied the development of fungal infection units and the colonization of host roots by the AM fungus *G. mosseae* in the experimental microcosms for the impact assessment of Bt corn. There was a significantly lower level of mycorrhizal colonization in Bt 176 corn roots as compared to the parental and the Bt 11 corn.

5.2 Soil enzyme activity

5.2.1 Dehydrogenase activity

Dehydrogenases are the enzymes which act as an index to soil microbial activity. The enzyme activity in the present study was significantly higher in the rhizosphere of the wild type groundnut compared to the transgenic at the vegetative and flowering stages. The activity increased at the flowering stage for both types of plants which may be due to the increased microbial population at the flowering stage as shown in the Fig. 7.

5.2.2 Urease activity

The enzyme activity was higher for the wild type groundnut rhizosphere compared to the transgenic groundnut. The activity increased at the flowering stage for both the types and decreased at the harvesting as represented in Fig. 8.

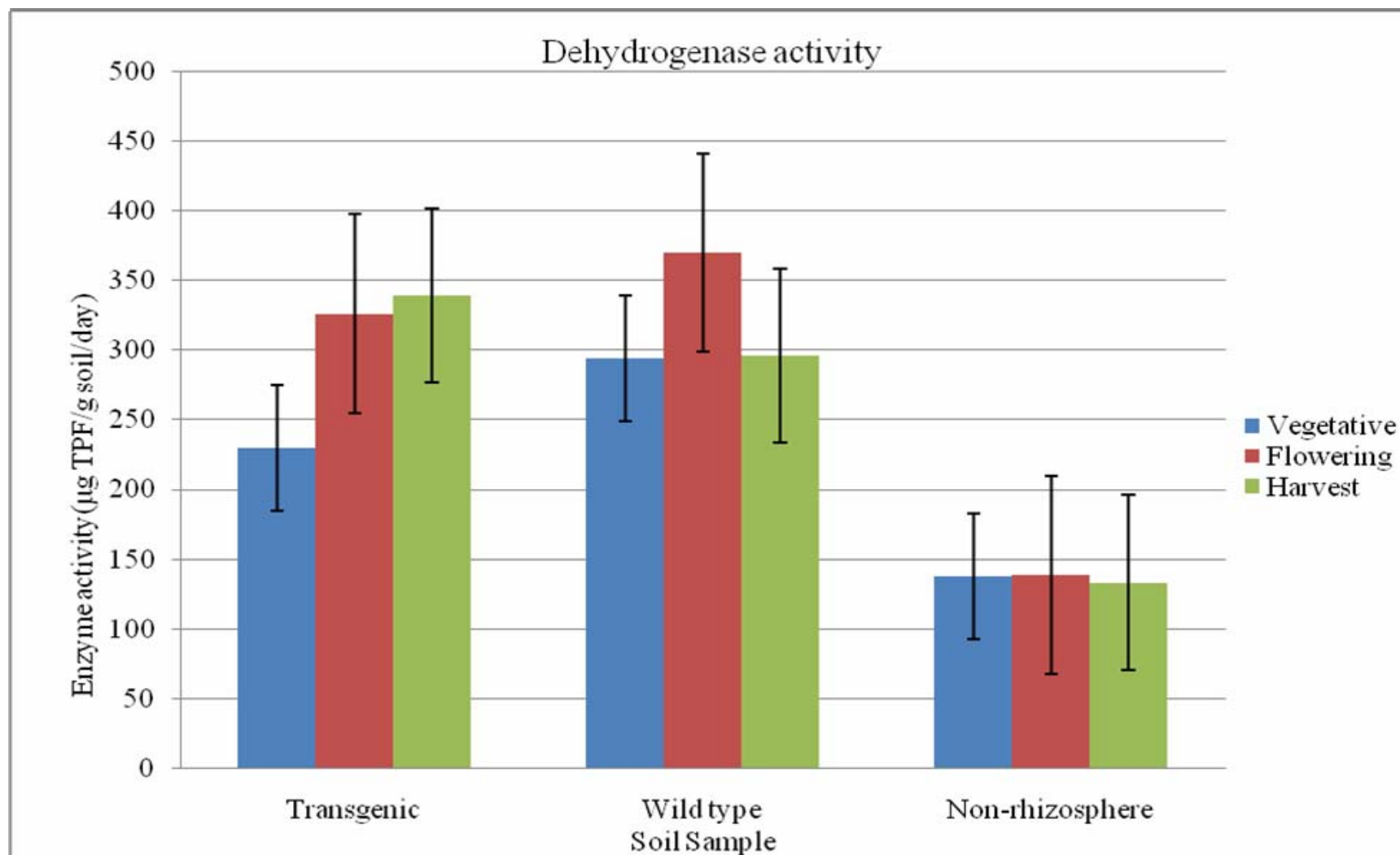


FIG. 7. The dehydrogenase activity ($\mu\text{g TPF/g soil/day}$) in the rhizosphere of transgenic and wild type groundnut.

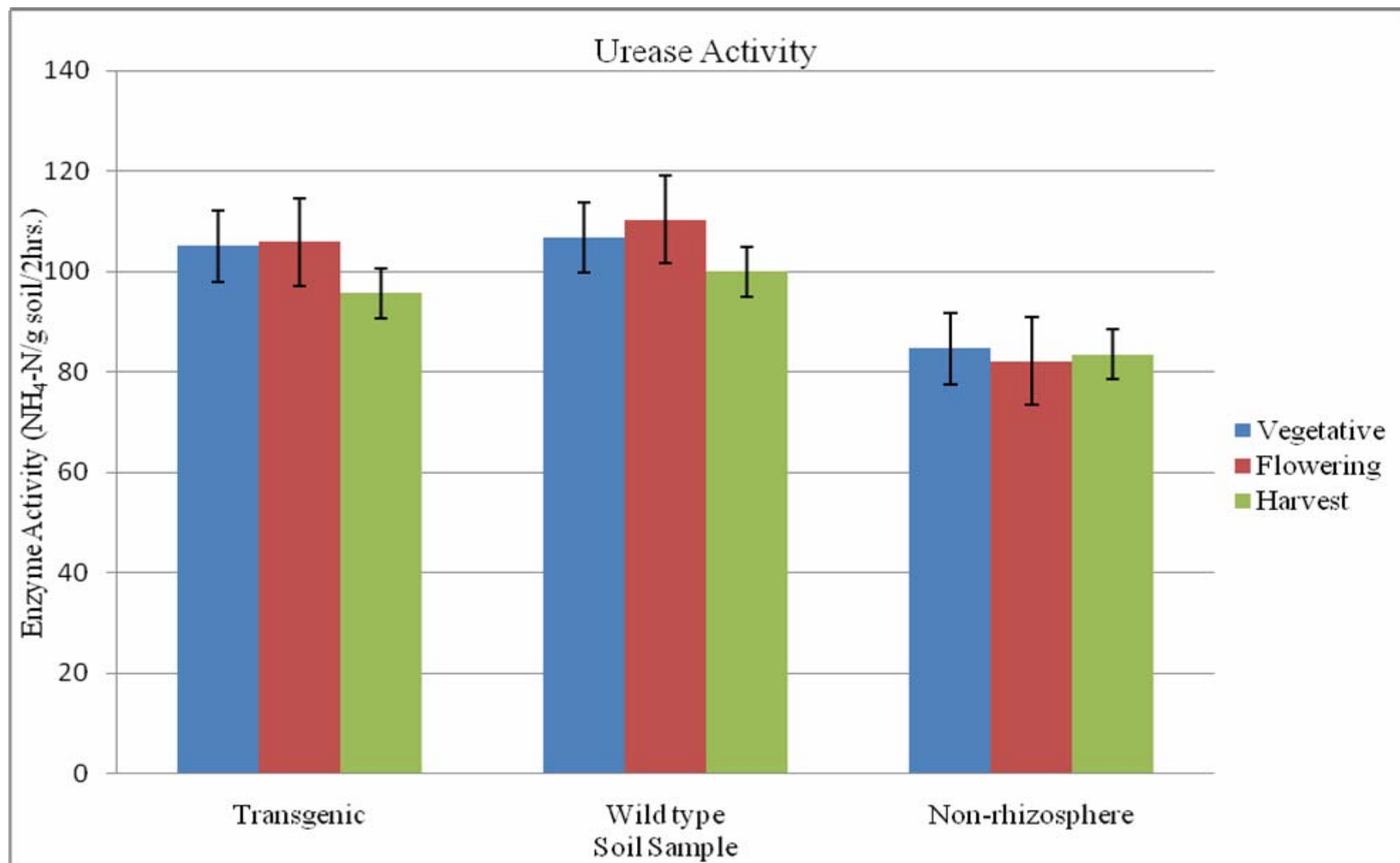


FIG. 8. The urease activity (NH₄-N/g soil/2hr.) in the rhizosphere of transgenic and wild type groundnut.

Kiss *et al.* (1975) reported that the higher activity of urease is related to higher microbial activity.

5.2.3 Acid and Alkaline phosphatase activity

The activity of the acid phosphatase enzyme was significantly higher for the wild type groundnut rhizosphere compared to the transgenic at all the growth stages studied and was presented in Fig. 9.

The alkaline phosphatase activity showed a small significant difference in the rhizosphere of transgenic and wild type groundnut at the initial crop growth stage (Fig. 10) which may be due to the small but non-significant difference in the phosphate solubilizing microorganisms. The increase in the enzyme activity till flowering may be attributed to the significant increase in the population of phosphate solubilizing microorganisms but later it decreased with the decrease in the phosphate solubilizing microorganisms at the harvest stage.

It is also suggested that carbon is one of the factors stimulating the activity of the enzyme. The rhizosphere has increased organic C due to the presence of sloughed off root cells resulting in the higher activity of heterotrophic microorganisms which caused depletion of available P for the growth of microorganisms. The depletion of P might have induced the heterotrophic microorganisms to liberate extracellular alkaline phosphatases for mineralization of organic P. Similar results have also been reported by several workers (Speir and Ross, 1978; Jones and Jacobson, 1995).

Chhonkar and Tarafdar (1984) reported that phosphatase activity was significantly correlated with organic C, organic P and bacterial population.

In the present study the bacterial population was higher for the crop type and the growth stages with higher amounts of phosphatase activity.

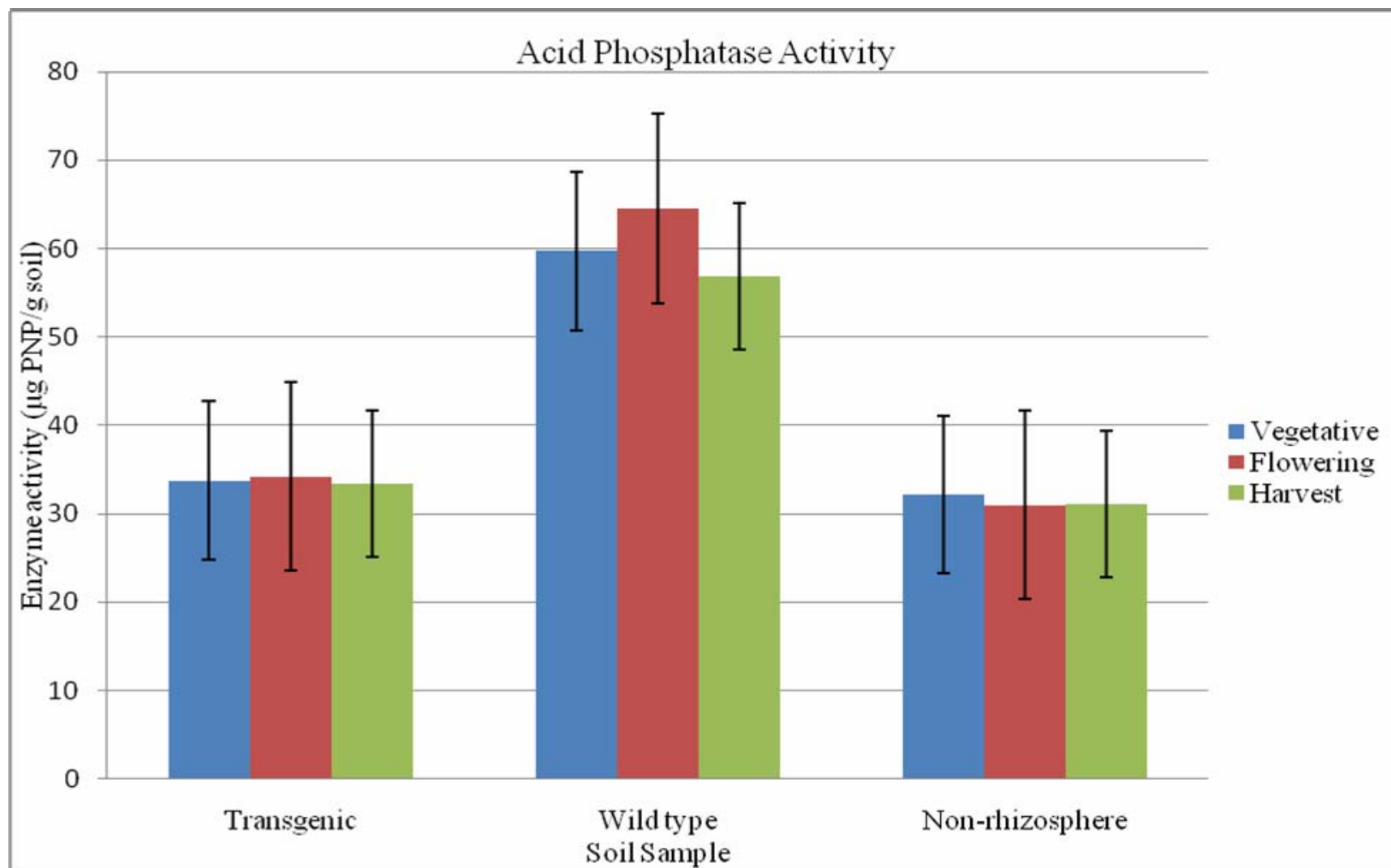


FIG. 9. The acid phosphatase activity (µg PNP/g soil) in the rhizosphere of transgenic and wild type groundnut.

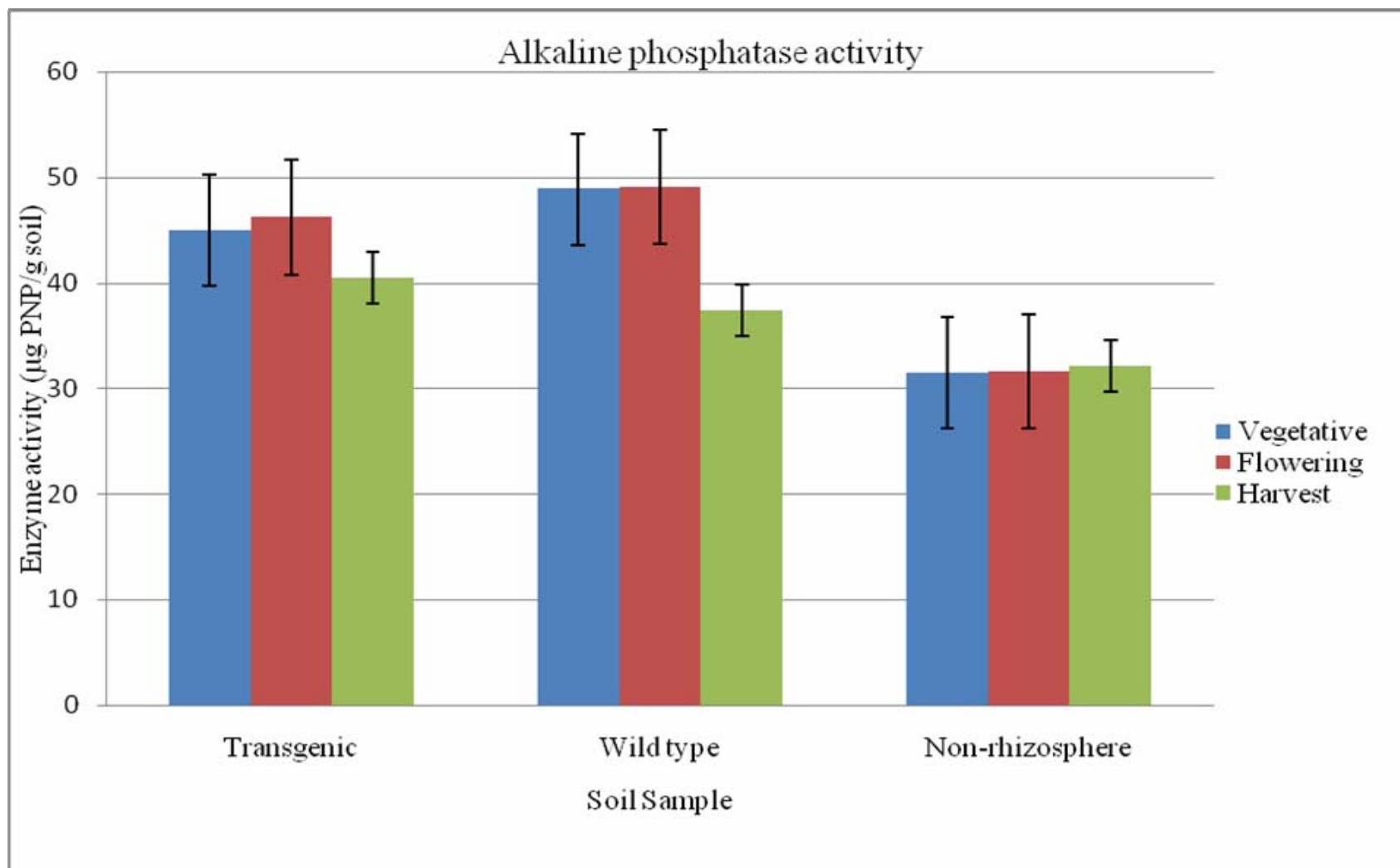


FIG. 10. The alkaline phosphatase activity ($\mu\text{g PNP/g soil}$) in the rhizosphere of transgenic and wild type groundnut.

The activity of the enzymes dehydrogenase, urease and acid & alkaline phosphatase was higher in the rhizosphere of wild type as compared to the transgenic. Also the activities were higher than the non-rhizosphere soil which may be due to the presence of higher number of microorganisms in the rhizosphere of plants than in the non-rhizosphere soil and it may be said that microbes affect the different enzyme activities.

Donegan *et al.* (1999) observed a lower activity of the enzymes dehydrogenase, acid and alkaline phosphatase for the lignin peroxidase alfalfa transgenic plants, at the sample days 1, 63, 119, as compared to the parental type.

5.3 RAPD analysis

The analysis of the Random Amplification of Polymorphic DNA (RAPD) for the metagenomic DNA obtained from the rhizosphere of transgenic and wild type groundnut revealed no significant differences in the microbial communities inhabiting in both types of the rhizosphere soil as presented in Fig. 11. However for a better analysis of the diversity microbial community comparison of more than one type of transgenic rhizosphere system should be considered.

5.4 Future line of work

The environmental release of transgenic plants cause unintended changes in soil and have an impact on soil microbial composition. It has also been indicated that the alteration in the microbial community associated with a plant varies with the soil type, season, plant variety and the transgene integrated. Hence broadened evaluation of the below ground effect of transgenic plants should be carried out based on more than the intended agronomic trait(s), would help to address the ecological concerns and should be performed before widespread agricultural use of novel transgenic plants.

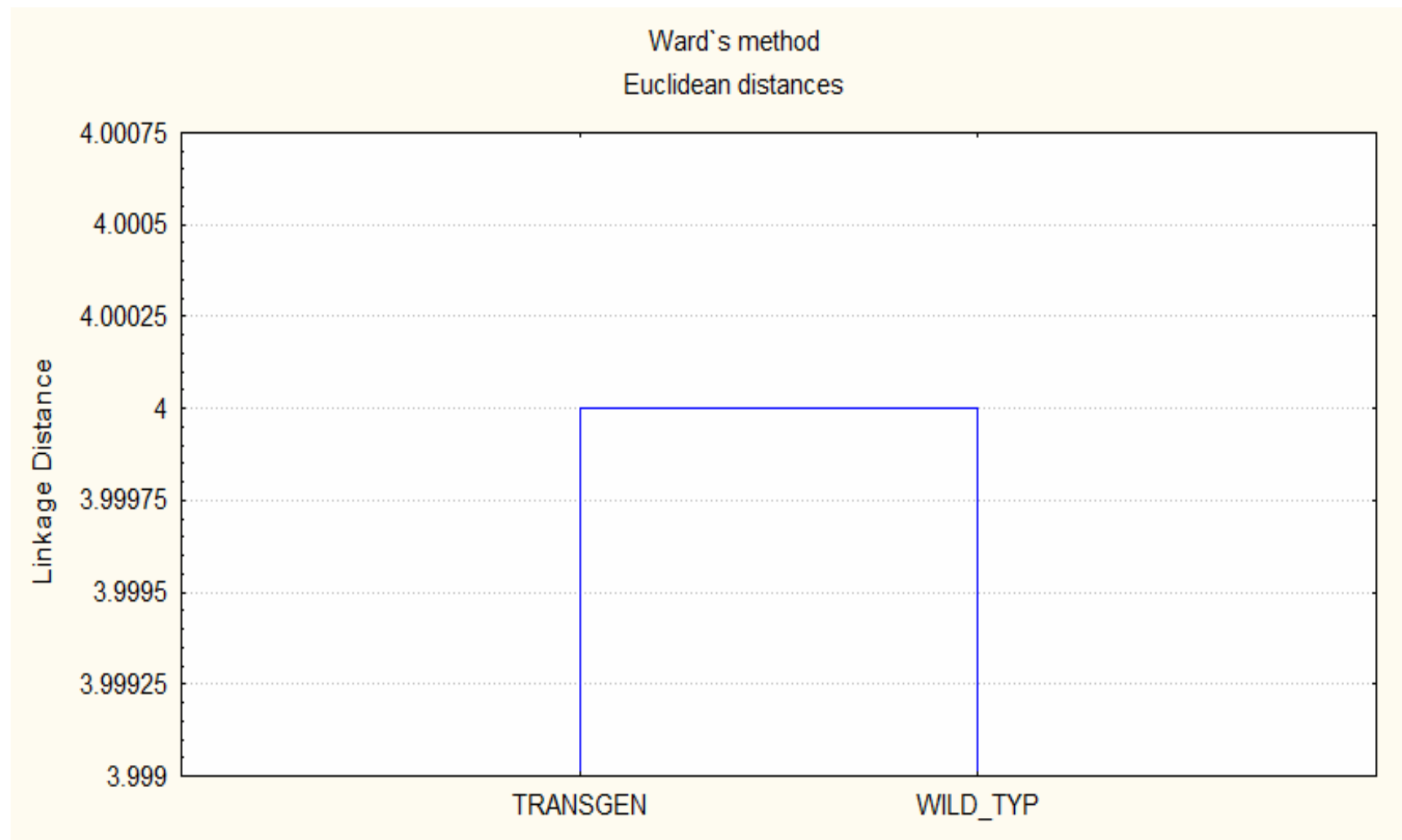


FIG. 11. Dendrogram showing relationship between the two rhizosphere soil samples based on RAPD analysis.

Summary



VI. SUMMARY

An experiment was conducted to study, “Rhizosphere microflora of the transgenic crop plants.” The transgenic groundnut expressing *ipt* gene for the overproduction of cytokinin was considered for the studies. The rhizosphere soil sample of the transgenic and wild type groundnut was collected from the experimental plot of Dept. of Crop Physiology, GKVK, UAS-Bangalore.

The population of different soil microorganisms as bacteria, fungi, actinomycetes, phosphate solubilizing microorganisms and free living N₂-fixing microorganisms was enumerated and it was observed that except bacteria other microbial groups showed higher population in the rhizosphere of the wild type groundnut as compared to that of transgenic. The root colonization by arbuscular mycorrhizal fungi was not affected by the transgenic crop. The non-rhizosphere soil showed lesser microbial population indicating that rhizosphere affects the colonization of the soil microorganisms.

The soil enzyme activities including dehydrogenase, urease, acid and alkaline phosphatase was estimated for the rhizosphere of transgenic and wild type. Higher enzyme activities were observed for the wild type groundnut rhizosphere. The activity in the non-rhizosphere soil was lowest indicating that the enzyme activity gets affected by the soil microorganisms.

The RAPD analysis of the metagenome extracted from the rhizosphere soil showed no difference at the molecular level between the microbial communities dominating the rhizosphere of the transgenic and wild type groundnut.

References



VII. REFERENCES

- AHRENHOLTZ, I., HARMS, K., DE VRIES, J. AND WACKERNAGEL, W., 2000, Increased killing of *Bacillus subtilis* on hair roots of transgenic T4 lysozyme-producing potatoes. *Appl. Environ. Microbiol.*, **66**: 1862-1865.
- AKKERMANS, A. D. L., VAN ELSAS, J. D. AND DE BRUIJN, F. J., 1995, *Molecular Microbial Ecology Manual*. Kluwer Academic, Dordrecht, The Netherlands.
- ALLEN, M. F., MOORE, T. S. AND CHRISTENSEN, M., 1980, Phytohormone changes in *Bouteloua gracilis* infected by vesicular-arbuscular mycorrhizae. *Can. J. Bot.*, **58**: 371-374.
- ANDERSON, J. R., 1962, Urease activity, ammonia volatilization and related microbiological aspects in some South African soils. *Soil Fert.*, **26**: 196.
- AUSTIN, S., BINGHAM, E. T., MATHEWS, D. E., SHAHAN, M. N., WILL, J. AND BURGESS, R. R., 1995, Production and field performance of transgenic alfalfa (*Medicago sativa* L.) expressing alpha-amylase and manganese-dependent lignin peroxidase. *Euphytica.*, **85**: 381-393.
- BARDGETT, R. D., DENTIN, C. S. AND COOK, R., 1999, Below-ground herbivory promotes soil nutrient transfer and root growth in grassland. *Ecology Letters*, **2**: 357-360.
- BAREA, J. M., AZCON, R. AND HAYMAN, D. S., 1976, Possible synergistic interactions between *Endogone* and phosphate solubilizing bacteria in low phosphate soil. In: *Endomycorrhiza* pp. 409-417. (Eds.) Sanders *et al.*, Acad. Press. New York.

- BAUMGARTE, S. AND TEBBE, C. C., 2005, Field studies on the environmental fate of the Cry1Ab Bt-toxin produced by transgenic maize (MON810) and its effect on bacterial communities in the maize rhizosphere. *Mol. Ecol.*, **14**: 2539–2551.
- BERTOLLA, F. AND SIMONET, P., 1999, Horizontal gene transfers in the environment: natural transformation as a putative process for gene transfers between transgenic plants and microorganisms. *Res. Microbiol.* **150**: 375-384.
- BERTOLLA, F., PEPIN, R., PASSELEGUE-ROBE, E., PAGET, E., SIMKIN, A., NESME, X. AND SIMONET, P., 2000, Plant genome complexity may be a factor limiting *in situ* the transfer of transgenic plant genes to the phytopathogen *Ralstonia solanacearum*. *Appl. Environ. Microbiol.*, **66**: 4161-4167.
- BLACKWOOD, C. B., AND BUYER, J. S., 2004, Soil microbial communities associated with Bt and non-Bt corn in three soils. *J. Environ. Qual.*, **33**: 832–836.
- BOPAIAH, B. M., 1985, Occurrence of phosphate solubilizing organisms in root regions of arecanut palm. *J. Pl. Crops*, **134**: 60-62.
- BREMMER, J. M. AND MULVANCY, R. L., 1978, Urease activity in soils. *In: Soil enzymes*, pp. 149-196., (Ed.) Bwms, R. G., Academic Press, New York.
- BRUINSMA, M., KOWALCHUK, G. A. AND VAN VEEN, J. A., 2003, Effects of genetically modified plants on microbial communities and processes in soil. *Biology and Fertility of Soils*, **37**: 329–337.

- BRUNETT, M. C., PICHE, Y. AND PETERSON, R. L., 1984, A new method for observing the morphology of vesicular-arbuscular mycorrhizae. *Can. J. Bot.*, **62**: 2128-2134.
- BRUSETTI, L., FRANCA, P., BERTOLINI, C., PAGLIUCA, A., BORIN, S., SORLINI, S., ABRUZZESE, A., SACCHI, G., VITI, C., GIOVANNETTI, L., GIUNTINI, E., BAZZICALUPA, M., AND DAFFONCHIO, D., 2004, Bacterial communities associated with the rhizosphere of transgenic Bt 176 maize (*Zea mays*) and its non-transgenic counterpart. *Plant and Soil*, **266**: 11–21.
- CASIDA, J. E., KLEIN, D. A. AND SANTORO, T., 1964, Soil dehydrogenase activity. *Soil Sci.*, **98**: 371-376.
- CASTALDINI, M., TURRINI, A., SBRANA, C., BENEDETTI, A., MARCHIONNI, M., MOCALI, S., FABIANI, A., LANDI, S., SANTOMASSIMO, F., PIETRANGELI, B., NUTI, M.P., MICLAUS, N. AND GIOVANNETTI, M., 2005, Impact of Bt corn on rhizospheric and soil eubacterial communities and on beneficial mycorrhizal symbiosis in experimental microcosms. *Appl. Environ. Microbiol.*, **71**: 6719–6729.
- CHHONKAR, P. K. AND TARAFDAR, J. C., 1984, Accumulation of phosphates in soils. *J. Indian Soil Sci.*, **32**: 266-272.
- CHIN, W. T. AND KROONTJE, W., 1963, Urea hydrolysis and subsequent loss of ammonia. *Soil Sci. Soc. Am. Proc.*, **27**: 316-318.
- CRAVEN, P. A. AND HAYASKA, S. S., 1982, Inorganic phosphate solubilization by rhizosphere bacteria in a *Zostera marina* community. *Can. J. Microbiol.*, **28**: 605-610.

- DAHLBERG C., BERGSTROM M., ANDREASEN M., CHRISTENSEN B. B.,
MOLIN S. AND HERMANSSON M., 1998, Interspecies bacterial
conjugation by plasmids from marine environments visualized by GFP
expression. *Mol. Biol. Evol.*, **15**: 385-390.
- DE VRIES, J., HARMS, K., BROER, I., KRIETE, G., MAHN, A., DURING K.
AND WACKERNAGEL W., 1999, The bacteriolytic activity in transgenic
potatoes expressing a chimeric T4 lysozyme gene and the effect of T4
lysozyme on soil- and phytopathogenic bacteria. *Syst. Appl. Microbiol.* **22**:
280–286.
- DEVARE, M., LONDONO, R. L. M., AND THIES, J. E., 2007, Neither
transgenic Bt maize (MON863) nor tefluthrin insecticide adversely affect
soil microbial activity or biomass: A 3-year field analysis. *Soil Bio.*
Biochem., **39**: 2038–2047.
- DEVARE, M. H., JONES, C. M. AND THIES, J. E., 2004, Effect of Cry3Bb
transgenic corn and tefluthrin on the soil microbial community. *J. Environ.*
Qual., **33**: 837–843.
- DI GIOVANNI, G. D., WATRUD, L. S., SEIDLER, R. J., WIDMER, F., 1999,
Comparison of parental and transgenic alfalfa rhizosphere bacterial
communities using biolog GN metabolic fingerprinting and enterobacterial
repetitive intergenic consensus sequence-PCR (ERIC-PCR). *Microb. Ecol.*,
37: 129–139.
- DICK, R. P., 1994, Soil enzyme activities as indicators of soil quality. *In*:
Defining Soil Quality for a sustainable Environment, pp. 107-124, (Eds.)
Doran, J. C., Coleman, D. C. Bezdicek D. F. and Steward, B. A., SSSA
Spec. Publn. No. 35, Madison, USA.

- DINEL, H., SCHNITZER, M., SAHARINEN, M., MELOCHE, F., PARE, T., DUMONTET, S., LEMEE, L. AND AMBLES, A., 2003, Extractable soil lipids and microbial activity as affected by Bt and non-Bt maize grown on a silty clay loam soil. *Journal of Environmental Science and Health*, **38**: 211-219.
- DOBEREINER, J., 1974, Nitrogen fixing bacteria in the rhizosphere. *In: The biology of nitrogen fixation*, pp. 147-174. (Ed.) Quispel, A., North Holland Publishing Company, Amsterdam.
- DONEGAN, K. K., PALM, C. J., FIELAND, V. J., PORTEOUS, L. A., GANIO, L. M., SCHALLER, D. L., BUCAO, L. Q. AND SEIDLER, R. J., 1995, Changes in levels, species and DNA fingerprints of soil microorganisms associated with cotton expressing the *Bacillus thuringiensis* var. *kurstaki* endotoxin. *Appl. Soil Ecol.*, **2**: 111–124.
- DONEGAN, K. K., SCHALLER, D. L., STONE, J. K., GANIO, L. M., REED, G., HAMM, P. B. AND SEIDLER, R. J., 1996, Microbial populations, fungal species diversity and plant pathogen levels in field plots of potato plants expressing the *Bacillus thuringiensis* var. *tenebrionis* endotoxin. *Trans. Res.*, **5**: 25-35.
- DONEGAN, K. K., SEIDLER, R. J., DOYLE, J. D., PORTEOUS, L. A., DI GIOVANNI, G. D. AND WATRUD, L. S., 1999, A field study with genetically engineered alfalfa inoculated with recombinant *Sinorhizobium meliloti*: effects on the soil ecosystem. *J. Appl. Ecol.* **36**: 920-936.
- DUARTE, G. F., ROSADO, A. S. AND VAN ELSAS, J. D., 1998, Extraction of ribosomal RNA and genomic DNA from soil for studying the diversity of the indigenous bacterial community. *Journal of Microbiological Methods*, **32**: 21–29.

- DUNFIELD K. E. AND GERMIDA J. J., 2001, Diversity of bacterial communities in the rhizosphere and root interior of field grown genetically modified *Brassica napus*. *FEMS Microbiol. Ecol.*, **38**: 1-9.
- DUNFIELD, K. E. AND GERMIDA, J. J., 2003, Seasonal changes in the rhizosphere microbial communities associated with field-grown genetically modified canola (*Brassica napus*). *Appl. Environ. Microbiol.*, **69**: 7310–7318.
- DURING, K., PORSCH, P., FLADUNG, M. AND LORZ, H., 1993, Transgenic potato plants resistant to the phytopathogenic bacterium *Ewinia carotovora*. *Plant J.*, **3**: 587–598.
- EDWARD, J. C. AND TRIPATHI, S. C., 1972, Population densities of *Azotobacter sp.* with rhizosphere and non rhizosphere soils of some crops during rabi. *Allahabad Far.*, **46**: 49-51.
- EIVAZI, F. AND TABATABAI, M. A., 1977, Phosphatases in soils. *Soil Biol. Biochem.*, **9**: 167-172.
- ESCHER, N., KACH, B. AND NENTWIG, W., 2000, Decomposition of transgenic *Bacillus thuringiensis* maize by microorganisms and woodlice *Porcello scaber* (Crustacea: Isopoda). *Basic Appl. Entomol.*, **1**: 161-169.
- FANG, M., KREMER, R. J., MOTAVALLI, P. P. AND DAVIS, G., 2005, Bacterial diversity in rhizospheres of nontransgenic and transgenic corn. *Appl. Environ. Microbiol.*, **71**: 4132–4136.
- FERREIRA, L. H. P. L., MOLINA, J. C., BRASIL, C. AND ANDRADE, G., 2003, Evaluation of *Bacillus thuringiensis* bioinsectical protein effects on soil microorganisms. *Plant and Soil*, **256**: 161–168.

- FOLMER, J. D., GRANT, R. J., MILTON, C. T. AND BECK, J., 2002, Utilization of Bt corn residues by grazing beef steers and Bt corn silage and grain by growing beef cattle and lactating dairy cows. *J. Animal Sc.*, **80**: 1352–1361.
- FOX, T. R. AND COMERFORD, N. B., 1992, Rhizosphere phosphatase activity and phosphatase hydrolysable organic phosphorus in two forested spodosols. *Soil Biol. Biochem.*, **24**: 579-583.
- FLORES, S., SAXENA, D. AND STOTZKY, G., 2005, Transgenic Bt plants decompose less in soil than non-Bt plants. *Soil Bio. Biochem.*, **37**: 1073–1082.
- FRAMOND, A. J., BEVAN, M. W., BARTON, K. A., FLAVELL, F. AND CHILTON, M.D., 1983, Mini Ti-plasmid and a chimeric gene construct: new approaches to plant gene vector construction. – *Advances in Gene Technology: Molecular Genetics of Plants and Animals*, Miami Winter Symposia, **20**: 159-170.
- FRANKLIN, R. B., DOUGLAS R. TAYLOR, D. R. AND MILLSA A. L., 1999, Characterization of microbial communities using randomly amplified polymorphic DNA (RAPD). *Journal of Microbiological Methods*, **35**: 225-235.
- FRALEY, R. T., ROGERS, S. B. AND HORSCH, R. B., 1983, Use of a chimeric gene to confer antibiotic resistance to plant cells. – *Advances in Gene Technology: Molecular Genetics of Plants and Animals*, Miami Winter Symposia, **20**: 211-221.
- GAUR, A. C., 1990, Phosphate Solubilizing Microorganisms as Biofertilizers. Omega Scientific Publishers, New Delhi. pp. 123.

- GEBHARD, F. AND SMALLA, K., 1999, Monitoring field releases of genetically modified sugar beets for persistence of transgenic plant DNA and horizontal gene transfer. *FEMS Microbiol. Ecol.* **28**: 261-272.
- GERDEMANN, J. W., 1975, Vesicular-Arbuscular mycorrhizae. *In: The development and function of roots*, pp. 575-591, (Eds.) Torrey, J. G. and Clarkson, D. T., Academic Press, New York.
- GERMIDA, J. J., SICILIANO, S. D., DE FREITAS, J. R. AND SEIB, A. M., 1998, Diversity of root-associated bacteria associated with field grown canola (*Brassica napus* L.) and wheat (*Triticum aestivum* L.). *FEMS Microbiol. Ecol.*, **26**: 43–50.
- GRAHAM, J. H., EISSENSTAT, D. M. AND DROUILLARD, D. L., 1991, On the relationship between a plant's mycorrhizal dependency and rate of vesicular-arbuscular mycorrhizal colonization. *Funct. Ecol.*, **5**: 773-779.
- GRAYSTON, S. J., WANG, S., CAMPBELL, C. D. AND EDWARDS, A. C., 1998, Selective influence of plant species on microbial diversity in the rhizosphere. *Soil Bio. Biochem.*, **30**: 369–378.
- GRIFFITHS, B. S., GEOGHEGAN, I. E. AND ROBERTSON, W. M., 2000, Testing genetically engineered potato, producing the lectins GNA and Con A on non-target soil organisms and processes. *J. Appl. Ecol.*, **37**: 159-170.
- GRIFFITH, R. I., WHITELEY, A. S., O'DONNELL, A. G. AND BAILEY, M. J., 2000, Rapid method for co-extraction of DNA and RNA from natural environments for analysis of ribosomal DNA and rRNA based microbial community composition. *Appl. Environ. Microbiol.*, **66**: 5488–5491.

- GRIFFITHS, B. S., CAUL, S., THOMPSON, J., BIRCH, A. N. E., SCRIMGEOUR, C., ANDERSEN, M. N., CORTET, J., MESSEAN, A., SAUSSE, C., LACROIX, B. AND KROGH, P. H., 2005, A comparison of soil microbial community structure, protozoa, and nematodes in field plots of conventional and genetically modified maize expressing the *Bacillus thuringiensis* Cry1Ab toxin. *Plant and Soil*, **275**: 135–146.
- GRIFFITHS, B. S., CAUL, S., THOMPSON, J., BIRCH, A. N. E., SCRIMGEOUR, C., CORTET, J., FOGGO, A., HACKETT, C. A. AND KROGH, P. H., 2006, Soil microbial and faunal community responses to Bt maize and insecticide in two soils. *J. Environ. Qual.*, **35**: 734–741.
- GUPTA, V. V. S. R., AND WATSON, S., 2004, Ecological impacts of GM cotton on soil biodiversity: Below-ground production of Bt by GM cotton and Bt cotton impacts on soil biological processes. Australian Government Department of the Environment and Heritage, CSIRO Land and Water, pp. 1–72.
- GUPTA, V. V. S. R. AND YEATES, G. W., 1997, Soil microfauna as bioindicators of soil health. *In: Biological Indicators of Soil Health*, pp.201–233, (Ed.), Pankurst, C. E., CAB International, New York.
- GUPTA, V. V. S. R., ROBERTS, G. N., NEATE, S. M., MCCLURE, S. G., CRISP, P. AND WATSON, S. K., 2002, Impact of Bt-cotton on biological processes in Australian soils. *In: Proceedings of the Fourth Pacific Rim Conference on the Biotechnology of Bacillus thuringiensis and Its Environmental Impacts*, (Eds.) Akhurst, R. J., Beard, C. E., Hughes, P. A., CSIRO, Australia, pp. 191–194.

- GUYON, P., PETIT, A., TEMPE, J. AND DESSAUX, Y., 1993, Transformed plants producing opines specifically promote growth of opine-degrading agrobacteria. *Mol. Plant-Microbe Interact.*, **6**: 92–98.
- HEUER, H., KROPPESTEDT, R. M., LOTTMANN, J., BERG, G. AND SMALLA, K., 2002, Effects of T4 lysozyme release from transgenic potato roots on bacterial rhizosphere communities are negligible relative to natural factors. *Appl. Environ. Microbiol.*, **68**: 1325-1335.
- HOLBEN, W. E., 1994, Isolation and purification of bacterial DNA from soil. *In: Methods of soil analysis, part 2. Microbiological and biochemical properties*, pp. 727-751, (Eds.) Weaver, R. W. Angle, S. Bottomley, P. Bezdicsek, D. Smith, S. Tabatabai, A. and Wollum A., Soil Science Society of America, Inc., Madison, Wis.
- HOSS, S., ARNDT, M., BAUMGARTE, S. AND TEBBE, C. C., 2004, Effects of soil from experimental fields with transgenic corn on the nematode *Caenorhabditis elegans*. Abstract from Society of Environmental Toxicology and Chemistry, 14th Annual Meeting, Prague.
- ICOZ, I. AND STOTZKY, G., 2007, Cry3Bb1 protein from *Bacillus thuringiensis* in root exudates and biomass of transgenic corn does not persist in soil. *Trans. Res.*, **17**: 609-620.
- ICOZ, I., SAXENA, D., ANDOW, D., ZWAHLEN, C. AND STOTZKY, G., 2007, Microbial populations and enzyme activities in soil *in situ* under transgenic corn expressing Cry proteins from *Bacillus thuringiensis*, *J. Environ. Qual.*, **37**: 647-662.
- ISHIZAWA, S. AND TOYODA, H., 1964, Microflora of Japanese soils, *Nogyo gijutsu kenkyusho hokoku.*, **14**: 204-284.

- ISHIZAWA, S., SUZUKI, T. AND ARARAGI, M., 1975, Ecological study of free living nitrogen fixers in paddy soil, pp: 41-50, *In: Nitrogen fixation and Nitrogen Cycle* (Ed.), Takahashi H., University of Tokyo press, Tokyo.
- JONES, E. J. AND JACOBSEN, Z., 1995, Growth and extracellular phosphatase activity of arbuscular mycorrhizal hyphae as influenced by soil organic matter. *Soil Biol. Biochem.*, **27**: 1153-1159.
- JOSH, S. R., CHAUHAN, M., SHARMA, J. D. AND MISHRA, R. R., 1991, Effect of deforestation on microbes, VAM fungi and their enzymatic activity in Eastern Himalaya. *Rec. Res. Ecol. Environ. Poll.*, **6**: 141-152.
- JUNG, H. G. AND SHEAFFER, C. C., 2004, Influence of Bt transgenes on cell wall lignification and digestibility of maize stover for silage. *Crop Sci.*, **44**: 1781-1789.
- KISS, S., DRAGAN-BULARDA, M. AND RADULESCU, D., 1975, Biological significance of enzymes accumulated in soil. *Adv. Agron.*, **27**: 25-87.
- KORMANIK, P. P. AND MCGRAW, A.-C., 1982, Quantification of vesicular-arbuscular mycorrhizae in the plant roots, pp. 37-45, *In: Methods and Principles of Mycorrhizal Research*. (Ed.), Schenck N. C. American Phytopathology Society, St. Paul, Minn.
- KOSKE, R. E. AND GEMMA, J. N., 1989, A modified procedure for staining roots to detect VA mycorrhizas. *Mycol. Res.*, **92**: 486-505.
- KOSKELLA, J. AND STOTZKY, G., 2002, Larvicidal toxins from *Bacillus thuringiensis* subspp. *kurstaki*, *morrisoni* (strain *tenebrionis*), and *israelensis* have no microbicidal or microbiostatic activity against selected bacteria, fungi, and algae in vitro. *Can. J. Microbiol.*, **48**: 262-267.

- KOWALCHUK, G. A., DE BRUIJN, F. J., HEAD, I. M., AKKERMANS, A. D. L. AND VAN ELSAS, J. D., 2004, *Molecular Microbial Ecology Manual*, 2nd edn. Kluwer Academic, Dordrecht, The Netherlands.
- KREMER, R. J., DONALD, P. A., KEASTER, A. J. AND MINOR, H. C., 2000, Herbicide impact on *Fusarium spp.* and soyabean cyst nematode in glyphosphate tolerant soybean. *In: Annual meetings abstracts.* ASA, CSSA and SSSA, Madison. pp. 257.
- KRISHNAMURTHI, B. G., 1962, *Azotobacter* in sugarcane rhizosphere. *Indian Sugar*, **11**: 765-767.
- KUCEY, R. M. N., JANZEN, H. H. K. AND LEGGETT, M. E., 1989, Microbially mediated increases in plant available phosphorus, pp. 199-228, *In: Advances in Agronomy*, (Ed.), Brady N. C., Academic Press, New York.
- LEVY, Y. AND KRIKUN, J., 1980, Effects of Vesicular-Arbuscular Mycorrhiza on citrus Jambhiri water relations. *New Phytol.*, **85**: 25-31.
- LIU, W., LU, H. H., WU, W., WEI, Q. K., CHEN, Y. X. AND THIES, J. E., 2008, Transgenic Bt rice does not affect enzyme activities and microbial composition in the rhizosphere during crop development. *Soil Biol. Biochem.*, **40**: 475-486
- LIU, W., 2009, Effects of Bt transgenic crops on soil ecosystems: a review of a ten-year research in China. *Journal Frontiers of Agriculture in China*, **3**: 190-198.
- LIU, W., 2010, Do genetically modified plants impact arbuscular mycorrhizal fungi? *Ecotoxicology*, **19**: 229-238.

- LORENZ M. G. AND WACKERNAGEL W., 1994, Bacterial gene transfer by natural genetic transformation in the environment. *Microbiol. Rev.*, **58**: 563-602.
- LOTTMANN, J. AND BERG, G., 2001, Phenotypic and genotypic characterization of antagonistic bacteria associated with roots of transgenic and non-transgenic potato plants. *Microbiol Res.*, **156**: 75–82.
- LOTTMANN, J., HEUER, H., DE VRIES, J., MAHN, A., DURING, K., WACKERNAGEL, W., SMALLA, K. AND BERG, G., 2000, Establishment of introduced antagonistic bacteria in the rhizosphere of transgenic potatoes and their effect on the bacterial community. *FEMS Microbiol. Ecol.*, **33**: 41–49.
- LOTTMANN, J., HEUER, H., SMALLA, K. AND BERG, G., 1999, Influence of transgenic T4-lysozyme-producing potato plants on potentially beneficial plant-associated bacteria. *FEMS Microbiol. Ecol.*, **29**: 365–377.
- LOUW, H. A., 1970, A study of the phosphate dissolving bacteria in the root region of wheat and lupin. *Phytophylatica*, **2**: 21-25.
- LYNCH, J., 1994, The rhizosphere – form and function. *Appl. Soil Ecol.*, **1**: 193–198.
- MANACHINI, B., LANDI, S., FIORE, M. C., FESTA, M. AND ARPAIA, S., 2004, First investigations on the effects of Bt-transgenic *Brassica napus* L. on the trophic structure of the nematofauna. *IOBC/WPRS Bulletin*, **27**: 103-108.
- MARTIN, J.P., 1950, Use of acid, rose bengal, and streptomycin in the plate method for estimating soil fungi. *Soil Sci.*, **69**: 215-323.

- MASOERO, F., MOSCHINIM, M., ROSSI, F., PRANDINI, A. AND PIETRI, A., 1999, Nutritive value, mycotoxin contamination, and in vitro rumen fermentation of normal and genetically modified corn (Cry1Ab) grown in northern Italy. *Maydica.*, **44**: 205–209.
- MCCLAUGHERTY, C. A. AND LINKINS, A. E., 1990, Temperature response of enzymes in two forest soils. *Soil Biol. Biochem.*, **22**: 29-33.
- MEIER, P. AND WACKERNAGEL, W., 2003, Mechanisms of homology-facilitated illegitimate recombination for foreign DNA acquisition in transformable *Pseudomonas stutzeri*. *Mol. Microbiol.* **48**: 1107-1118.
- MILLER, D. N., BRYANT, J. E., MADSEN, E. L. AND GHIORSE. W. C., 1999, Evaluation and optimization of DNA extraction and purification procedures for soil and sediment samples. *Appl. Environ. Microbiol.*, **65**: 4715-4724.
- MISHUTIN, E. N., 1970, The importance of non-symbiotic nitrogen fixing microorganisms in agriculture. *Pl. Soil*, **32**: 545-554.
- MORÉ, M. I., HERRICK, J. B., SILVA, M. C., GHIORSE, W. C. AND MADSEN, E. L., 1994, Quantitative cell lysis of indigenous microorganisms and rapid extraction of microbial DNA from sediment. *Appl. Environ. Microbiol.*, **60**: 1572–1580.
- MULLIS, K., FALOONA, F., SCHARF, S., HORN, G. AND EHRLICH, 1986, Specific enzymatic amplification of DNA *in vitro*: The polymerase chain reaction. Cold Spring Harbor. *Symp. Quant. Boil.*, **51**: 263-273.

- MUNGAI, N. W., MOTAVALLI, P. P., NELSON, K. A. AND KREMER, R. J., 2005, Differences in yields, residue composition and N mineralization dynamics of Bt- and non-Bt-maize. *Nutrient Cycling in Agroecosystems*, **73**: 101–109.
- MUYZER, G. AND SMALLA, K., 1998, Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. *Antonie van Leeuwenhoek*, **73**: 127-141.
- NAEF, A., ZESIGER, T. AND DEFAGO, G., 2006, Impact of transgenic Bt maize residues on the mycotoxigenic plant pathogen *Fusarium graminearum* and the biocontrol agent *Trichoderma atroviride*. *J. Environ. Qual.*, **35**: 1001–1009.
- NAIR, S. K. AND SUBBARAO, N. S., 1977, Distribution and activity of phosphate solubilizing microorganisms in the rhizosphere of coconut and cocoa under mixed cropping. *J. Pl. Crops*, **5**: 67-70.
- NEAL, J. L., LARSON, R. I. AND ATKINSON, T., 1973, Changes in rhizosphere populations of selected physiological groups of bacteria related to substitution of specific pairs of chromosomes in spring wheat. *Pl. Soil* **39**: 209-212.
- NEHL, D. B., ALLEN, S. J. AND BROWN, J. F., 1997, Deleterious rhizosphere bacteria: an integrating perspective. *Appl. Soil Ecol.*, **5**: 1–20.
- NGUYEN THU, H., 2004, Sicherheitsforschung und Monitoringmethoden zum Anbau von Bt Mais: Expression, Nachweis und Wirkung von rekombinantem Cry1Ab in heterologen Expressions systemen, Thesis, Georg-August-Universitat, Gottingen.

- NIELSEN K. M., VAN ELSAS, J. D. AND SMALLA, K., 2001a, Dynamics, horizontal transfer and selection of novel DNA in bacterial populations in the phytosphere of transgenic plants. *Ann. Microbiol.*, **51**: 79–94.
- NORMANDER, B., CHRISTENSEN, B. B., MOLIN S. AND KROER N., 1998, Effect of bacterial distribution and activity on conjugal gene transfer on the phyllosplane of the bush bean (*Phaseolus vulgaris*). *Appl. Environ. Microbiol.*, **64**: 1902-1909.
- OGER, P., PETIT, A. AND DESSAUX, Y., 1997, Genetically engineered plants producing opines alter their biological environment. *Nat. Biotech.*, **15**: 369-372.
- OGER, P., MANSOURI, H. AND DESSAUX, Y., 2000, Effect of crop rotation and soil cover on alteration of the soil microflora generated by the culture of transgenic plants producing opines. *Mol. Ecol.*, **9**: 881-890.
- OGRAM, A., SAYLER, G. S. AND BARKAY, T. J., 1987, DNA extraction and purification from sediments. *Journal of Microbiological Methods*, **7**: 57-66.
- PAGET E. AND SIMONET P., 1994, On the track of natural transformation in soil. *FEMS Microbiol. Ecol.*, **15**: 109-118.
- PANG, P. C. K. AND KOLENKO, H., 1986, Phosphomonoesterase activity in forest soils. *Soil Biol. Biochem.*, **18**: 35-40.
- PANTOS-DERIMOVA, T., 1988, Enzyme activities in soils of different forest ecosystems. *Acta Agronomica Hungarian*, **37**: 87-92.
- PHILLIPS, J. M. AND HAYMAN D. S., 1970, Improved procedures for clearing roots and staining parasitic and vesicular arbuscular mycorrhizal fungi for rapid assessment of infection. *Trans. Br. Mycol. Soc.*, **55**: 158-161.

- PILLAI, S. D., JOSEPHSON, K. L., BAILEY, R. L., GERBA, C. P. AND PEPPER, I. L., 1991, Rapid method for processing soil samples for polymerase chain reaction amplification of specific gene sequences. *Appl. Environ. Microbiol.*, **57**: 2283–2286.
- POERSCHMANN, J., GATHMANN, A., AUGUSTIN, J., LANGER, U., AND GORECKI, T., 2005, Molecular composition of leaves and stems of genetically modified Bt and near – isogenic non-Bt maize – characterization of lignin patterns. *J. Environ. Qual.*, **34**: 1508–1518.
- PORTEOUS, L. A. AND ARMSTRONG, J. L., 1991, Recovery of bulk DNA from soil by a rapid, small-scale extraction method. *Curr. Microbiol.*, **22**: 345–348.
- PULFORD, I. D., AND TABATABAI, M. A., 1988, Effect of water logging on enzyme activities in soils. *Soil Biol. Biochem.*, **20**: 215-219.
- RAGHURAMULU, Y., 2001, Role of biofertilizers in coffee production. *Indian coffee*, **65**: 17-18.
- RANGASWAMI, G. AND SADASIVAM, K. V., 1969, Studies on the occurrence of Azotobacter in some soil types. *J. Ind. Soc. Soil Sci.*, **12**: 43-49.
- RENGEL, Z., ROSS, G. AND HIRSCH, P., 1998, Plant genotype and micronutrient status influence colonization of wheat roots by soil bacteria. *J. Pl. Nutri.*, **21**: 99–113.
- RIGLIETTI A., RUGGIERO P., CRECCHIO C., 2008, Investigating the influence of transgenic tobacco plants codifying a protease inhibitor on soil microbial community. *Soil Biol. Biochem.*, **40**: 2928-2936.

- ROOSE-AMSALEG, C. L., GARNIER-SILLAM, E. AND HARRY. M., 2001, Extraction and purification of microbial DNA from soil and sediment samples. *Appl. Soil Ecol.*, **18**: 47-60.
- ROVIRA, A. D., 1965, Interactions between plant roots and soil microorganisms. *Ann. Rev. Microbiol.*, **19**: 241-266.
- RUBENCHIK, L., 1963, Azotobacter and its use in agriculture. Jerusalem: Israel Program for Scientific Translations, pp. 278.
- RUI, Y. K., YI, G. X., ZHAO, J., WANG, B. M., LI, Z. H., ZHAI, Z. X., HE, Z. P. AND LI, Q., 2005, Changes of Bt toxin in the rhizosphere of transgenic Bt cotton and its influence on soil functional bacteria. *World J. Microbiol. Biotech.*, **21**: 1279–1284.
- SAIKI, R. K., GELFLAND, D. H., STOFFEL, S., SCHARF, S. J., HIGUCHI, R., HORN, G. T., MULLIS, K. B. AND EHRlich, H. A., 1988, Primer directed enzymatic amplification of DNA with thermostable DNA polymerase. *Science*, **149**: 239-245.
- SAMBROOK, J., FRITSCH, E. F. AND MANIATIS. T., 1989, *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
- SAVKA, M. A. AND FARRAND S. K., 1992, Mannityl opine accumulation and exudation by transgenic tobacco. *Pl. Physiol.*, **98**: 784–789.
- SAXENA, D. AND STOTZKY, G., 2001, *Bacillus thuringiensis* (Bt) toxin released from root exudates and biomass of Bt corn has no apparent effect on earthworms, nematodes, protozoa, bacteria, and fungi in soil. *Soil Bio. Biochem.*, **33**: 1225–1230.

- SAXENA, D. AND STOTZKY, G., 2001, Bt corn has a higher lignin content than non-Bt corn. *Amer. J. Bot.*, **88**: 1704-1706.
- SAXENA, D. AND STOTZKY, G., 2002, Bt toxin is not taken up from soil or hydroponic culture by corn, carrot, radish, or turnip. *Pl. Soil*, **239**: 165–172.
- SAXENA, D., FLORES, S. AND STOTZKY, G., 2002, Bt toxin is released in root exudates from 12 transgenic corn hybrids representing three transformation events. *Soil Bio. Biochem.*, **34**: 133-137.
- SAXENA, D., STEWART, C. N., ALTOSAAR, I., SHU, Q. AND STOTZKY, G., 2004, Larvicidal Cry proteins from *Bacillus thuringiensis* are released in root exudates of transgenic *B. thuringiensis* corn, potato, and rice but not of *B. thuringiensis* canola, cotton, and tobacco. *Pl. Physiol. Biochem.*, **42**: 383–387.
- SCHMALENBERGER A. AND TEBBE C. C., 2002, Bacterial community composition in the rhizosphere of a transgenic, herbicide-tolerant maize (*Zea mays*) and comparison to its non-transgenic cultivar Bosphore. *FEMS Microbiol. Ecol.*, **40**: 29-37.
- SELENSKA, S. AND KLINGMÜLLER, W., 1991, DNA recovery and direct detection of *Tn5* sequences from soil. *Letters in Appl. Microbiol.*, **13**: 21–24.
- SHARMA, N., SUDARSAN, Y., SHARMA, R. AND SINGH, G., 2008, RAPD analysis of soil microbial diversity in western Rajasthan. *Curr. Sci.*, **94**: 1058-1061.

- SHEN, R. F., CAI, H. AND GONG, W. H., 2006, Transgenic Bt cotton has no apparent effect on enzymatic activities or functional diversity of microbial communities in rhizosphere soil. *Pl. Soil*, **285**: 149–159.
- SICILIANO, S. D., THEORET, C. M., DE FREITAS, J. R., HUCL, P. J. AND GERMIDA, J. J., 1998, Differences in the microbial communities associated with the roots of different cultivars of canola and wheat. *Can. J. Microbiol.*, **44**: 844–851.
- SICILIANO, S. D. AND GERMIDA, J. J., 1999, Taxonomic diversity of bacteria associated with the roots of field-grown transgenic *Brassica napus* cv. Quest, compared to the non-transgenic *B. napus* cv. Excel and *B. rapa* cv. Parkland. *FEMS Microbiol. Ecol.*, **29**: 263-272.
- SKUJINS, J., 1976, Extracellular enzymes in soil. *CRC Curr. Rev. Microbiol.*, **4**: 383-421.
- SKUJINS, J., 1978, History of abiotic soil enzyme research. *In: Enzymes*, pp. 1-49. (Ed.) Burma, G., Academic Press, Inc., New York.
- SMALLA, K., CRESSWELL, N., MENDONCA-HAGLER, L. C., WOLTERS, A. C. AND VAN ELSAS, J. D., 1993, Rapid DNA extraction protocol from soil for polymerase chain reaction mediated amplification. *J. Appl. Bacteriol.*, **74**: 78–85.
- SMALLA K., VAN OVERBEEK L. S., PUKALL R. AND VAN ELSAS J. D., 1993, Prevalence of *nptII* and Tn5 in kanamycin resistant bacteria from different environments. *FEMS Microbiol. Ecol.*, **13**: 47-58.

- SMALLA K., WELLINGTON E. AND VAN ELSAS J. D., 1997, Natural background of bacterial antibiotic resistance genes in the environment. pp. 43-57, *In: Nordic Seminar on Antibiotic Resistance Marker Genes and Transgenic Plants*, The Norwegian Biotechnology Advisory Board, Oslo.
- SMALLA K., BORIN S., HEUER H., GEBHARD F., VAN ELSAS J. D. AND NIELSEN K. M., 2000, Horizontal gene transfer from transgenic plants to bacteria – are there new data to fuel the debate? pp. 146-154. *In: Proceedings of the 6th International Symposium on the Biosafety of Genetically Modified Organisms*, (Eds.), Fairbairn C., Scoles G., and McHughen A., University Extension Press, University of Saskatchewan, Saskatoon, Canada.
- SMALLA, K., WIELAND, G., BUCHNER, A., ZOCK, A., PARZY, J., KAISER, S., ROSKOT, N., HEUER, H. AND BERG, G., 2001, Bulk and rhizosphere soil bacterial communities studied by denaturing gradient gel electrophoresis: plant-dependent enrichment and seasonal shifts revealed. *Appl. Environ. Microbiol.*, **67**: 4742–4751.
- SOKAL, R. R. AND SNEATH, P. H. A., 1973, Principles of numerical taxonomy. W. H. Freeman and co., San Francisco, USA.
- SOMANI, L. L., BHANDARI, S. C., VYAS, K. K AND SAXENA D., 1990, *Biofertilizers*, Scientific Publishers, Jodhpur, pp. 271-295.
- SPIER, T. W. AND ROSS, D. J., 1978, Soil phosphatase and sulphatase. *In: Soil enzymes*, pp. 197-250, (Ed.), R. G. Burns, Academic Press, New York.
- SPIER, T. W., LEE, R., PAUSIER, E. A. AND ANNETTEE, L., 1979, A comparison of sulphatase, urease and protease activities in planted and in fallow soils. *Soil Biol. Biochem.*, **12**: 281-291.

- STALSTROM, V. A., 1903, Beitrag zur kennturs der Ein-Wisking Sterilea and in Gahrung bofindlichen organichen stroffe anf dil loslichkeit der phosphorsen dez tricalcium phosphate. *Zentral Blatt-Fur Bakteriologie.*, **11**: 724-732.
- STEFFAN, R. J., GOKSOYR, J., BEJ, A. K. AND ATLAS, R. M., 1988, Recovery of DNA from soils and sediments. *Appl. Environ. Microbiol.*, **54**: 2908–2915.
- STOTZKY, G., 2002, Release, persistence, and biological activity in soil of insecticidal proteins from *Bacillus thuringiensis*. In: *Genetically Engineered Organisms: Assessing Environmental and Human Health Effects*, pp. 187–222, (Eds.) Letourneau, D. K. and Burrows, B. E., CRC Press, Boca Raton, FL.
- STOTZKY, G., 2004, Persistence and biological activity in soil of the insecticidal proteins from *Bacillus thuringiensis*, especially from transgenic plants. *Plant and Soil*, **266**: 77–89.
- SUN, C. X., CHEN, L. J., WU, Z. J., ZHOU, L. K. AND SHIMIZU, H., 2007. Soil persistence of *Bacillus thuringiensis* (Bt) toxin from transgenic Bt cotton tissues and its effect on soil enzyme activities. *Biology and Fertility of Soils*, **43**: 617–620.
- TABATABAI, M. A., 1982, Soil Enzymes. In: *Methods of Soil Analysis, part 2*, pp. 903-947, (Eds.) Page, A. L., Miller, R. H. and Keeney, D. R. American, Madison, Wisconsin, USA.
- TABATABAI, M. A. AND BREMMER, J. M., 1969, Use of *p*-nitrophenyl phosphate for assay of soil phosphatase activity. *Soil Biol. Biochem.*, **1**: 301-307.

- TEPFER, D., GARCIA-GONZALES, R., MANSOURI, H., SERUGA, M., MESSAGE, B., LEACH, F. AND PERICA, M. C., 2003, Homology-dependent DNA transfer from plants to a soil bacterium under laboratory conditions: implications in evolution and horizontal gene transfer. *Transgenic Res.*, **12**: 425-37.
- TERMORSHUIZEN, A.J. AND LOTZ, L. A. P., 2002, Does large-scale cropping of herbicide resistant cultivars increase the incidence of polyphagous soil-borne plant pathogens? *Outlook on Agriculture*, **31**: 51–54.
- TESFAYE, M., DUFAULT, N. S., DORNBUSCH, M. R., ALLAN, D. L., VANCE, C. P. AND SAMAC, D. A., 2003, Influence of enhanced malate dehydrogenase expression by alfalfa on diversity of rhizobacteria and soil nutrient availability. *Soil Biol. Biochem.*, **35**: 1103-1113.
- TINKER, P. B., 1975, Effects of Vesicular arbuscular mycorrhiza on higher plants. *Symp. Soc. Expt. Biol.*, **29**: 325-349.
- TIWARI, S. C., TIWARI, B. K. AND MISHRA, R. R., 1988, Enzyme activities in soils: Effects of leaching, ignition, autoclaving and fumigation. *Soil Biol. Biochem.*, **20**: 583-585.
- THOMAS, G. V. AND SHANTARAM, M. V., 1986, Solubilization of inorganic phosphates by bacteria from coconut plantation soils. *J. Pl. Crops*, **14**: 42-48.
- TORSVIK, V.L., 1980, Isolation of bacterial DNA from soil. *Soil Biol. Biochem.*, **12**: 18–21.
- TREVORS, J. T. AND VAN ELSAS, J. D., 1989, A review of selected methods in environmental microbial genetics. *Can. J. Microbiol.*, **35**: 895–902.

- TSAI, Y.-L. AND OLSON, B. H., 1991, Rapid method for direct extraction of DNA from soil and sediments. *Appl. Environ. Microbiol.*, **57**: 1070-1074.
- TSAI, Y.-L. AND OLSON, B. H., 1992, Detection of low numbers of bacterial cells in soils and sediments by polymerase chain reaction. *Appl. Environ. Microbiol.*, **58**: 754–757.
- TURRINI, A., SBRANA, C., NUTI, M. P., PIETRANGELI, B. AND GIOVANNETTI, M., 2004, Development of a model system to assess the impact of genetically modified corn and aubergine plants on arbuscular mycorrhizal fungi. *Plant and Soil*, **266**: 69-75.
- VAN ELSAS J. D. AND SMALLA K., 1995, Antibiotic (kanamycin and streptomycin) resistance traits in the environment. *In: Key Biosafety Aspects of Genetically Modified Organisms*, pp. 61-69, (Eds.), Landsmann J. and Casper R., Heft 309, Biologische Bundesanstalt für Land und Forstwirtschaft, Blackwell Wissenschafts-Verlag, Berlin.
- VAN ELSAS, J. D., VAN OVERBEEK, L. S. AND FOUCHIER, R., 1991, A specific marker, *pat*, for studying the fate of introduced bacteria and their DNA in soil using a combination of detection techniques. *Plant and Soil*, **138**: 49–60.
- VAN ELSAS, J. D., SMALLA, K. AND TEBBE, C. C., 2000, Extraction and analysis of microbial community nucleic acids from environmental matrices. *In: Tracking Genetically Engineered Micro-organisms*, pp. 29–52, (Eds.) Jansson, J., van Elsas, J.D. and Bailey, M., Landes Bioscience, Austin, Texas.

- VAN OVERBEEK, L. S., VAN VUURDE, J. W. L. AND VAN ELSAS, J. D., 2005, Application of molecular fingerprinting techniques for studying shifts in bacterial endophytic communities. *In: Bacterial Endophytes*. (Eds.) Schultz, B. and Boyle, C., Springer Verlag, Heidelberg.
- VIERHEILIG, H., ALT, M., NEUHAUS, J-M., WIEMKEN, A. AND BOLLER, T., 1993, Colonization of transgenic *Nicotiana sylvestris* plants, expressing different forms of *Nicotiana tabacum* chitinase by the root pathogen *Rhizoctonia solani* and by the mycorrhizal symbiont *Glomus mosseae*. *Mol. Plant Microbe Interact.*, **6**: 261–264.
- VIERHEILIG, H., ALT, M., LANGE, J., GUT-RELLA, M., WIEMKEN, A. AND BOLLER, T., 1995, Colonization of transgenic tobacco constitutively expressing pathogenesis-related proteins by the vesicular-arbuscular mycorrhizal fungus *Glomus mosseae*. *Appl. Environ. Microbiol.*, **61**: 3031–3034.
- VINUTHA, M. C., 2005, Impact of land use types on certain beneficial soil microorganisms and their functional diversity. *M.Sc. Thesis*, Univ. Agric. Sci., Bangalore, pp. 101.
- WANG, J.-H., DING, H., LU, Y.-T. AND SHEN, G.-Q., 2009, Combined effects of cadmium and butachlor on microbial activities and community DNA in a paddy soil. *Pedosphere*, **19**: 623–630.
- WU, W. X., YE, Q. F., AND MIN, H., 2004, Effects of straws from Bt transgenic rice on selected biological activities in water-flooded soil. *European J. Soil Biol.*, **40**: 15–22.

- WU, W. X., YE, Q. F., AND MIN, H., 2004, Bt-transgenic rice straw affects the culturable microbiota and dehydrogenase and phosphatase activities in a flooded paddy soil. *Soil Biol. Biochem.*, **36**: 289-295.
- XUE, K., LUO, H. F., QI, H. Y. AND ZHANG, H. X., 2005, Changes in soil microbial community structure associated with two types of genetically engineered plants analyzing by PLFA. *J. Environ. Sci. (China)*, **17**: 130–134.
- YANG, Y.-H., YAO, J., HU, S. AND QI, Y., 2000, Effects of Agricultural Chemicals on DNA Sequence Diversity of Soil Microbial Community: A Study with RAPD Marker. *Microb. Ecol.*, **39**: 72–79.
- ZHOU, J., BRUNS, M. A. AND TIEDJE, J. M., 1996, DNA recovery from soils of diverse composition. *Appl. Environ. Microbio.*, **62**: 316-322.
- ZWAHLEN, C., HILBECK, A., GUGERLI, P. AND NENTWIG, W., 2003, Degradation of the Cry1Ab protein within transgenic *Bacillus thuringiensis* corn tissue in the field. *Mol. Ecol.*, **12**: 765–775.

Appendix



APPENDIX

Reagents

Modified Universal Buffer (MUB)

12.1g of tris (hydroxymethyl) amino methane (THAM), 11.6g of Maleic acid, 14.0g of citric acid and 6.3g of boric acid (H_3BO_3) were dissolved in 488ml of 1N NaOH and the solution was diluted to one litre and stored in a refrigerator.

Modified Universal Buffer (MUB) 6.5 and 11 pH

200ml of MUB stock solution was placed in a 500ml beaker containing a magnetic stirring bar and the beaker was placed on a magnetic stirrer. The solution was titrated to pH 6.5 with 0.1N HCl and the volume was adjusted to one litre with water. Another 200ml of the MUB stock solution was titrated to pH 11 by using 0.1N NaOH and the volume was adjusted to one litre with distilled water.

Stains

Gram stain

1. Crystal violet

Solution A

Crystal violet (90% dye content)	: 2.0 g
Ethyl alcohol (95%)	: 20.0 ml

Solution B

Ammonium Oxalate	: 0.8 g
Distilled water	: 80.0 ml

Dissolve crystal violet in ethyl alcohol and the ammonium oxalate in distilled water, mix solution A and B.

2. Gram's Iodine

Iodine	: 1.0 g
Potassium iodide	: 2.0 g
Distilled water	: 300.0 ml

Dissolve iodine and potassium iodide in distilled water.

3. Ethyl alcohol (95%)

Ethyl alcohol (100%)	: 95.0 ml
Distilled water	: 5.0 ml

4. Safranin

Safranin (2.5% solution in 95% ethyl alcohol)	: 10.0 ml
Distilled water	: 100.0 ml

Media Composition

Soil Extract Agar

Glucose	: 1.0g
K ₂ HPO ₄	: 0.5g
Yeast Extract	: 0.5g
Soil Extract	: 100ml
Agar	: 15.0g
Distilled water	: 900ml
pH	: 6.8-7.2

Soil extract agar is prepared by heating 1000g of garden soil in 1000ml of tap water in an autoclave for 30min. at 15psi. A small amount of CaCO₃ is added and the soil suspension is filtered through a double paper filter. The turbid filtrate is bottled and sterilized.

Nutrient Agar medium

Bacto-peptone	: 5.0g
Beef extract	: 3.0g
Agar	: 18.0g
Distilled water	: 1000ml
pH	: 6.8-7.2

Martin's Rose Bengal Agar

Dextrose	: 10g
Peptone	: 5.0g
KH ₂ PO ₄	: 1.0g
MgSO ₄ .7H ₂ O	: 0.5g
Rose Bengal	: 0.033g
Agar	: 15.0g
Distilled water	: 1000ml
pH	: 6.0
Streptomycin	: 30mg

Dissolve 1.0g of streptomycin sulphate in 100ml of sterile distilled water. Add 3ml of the streptomycin solution to 1000ml, of the basal Rose Bengal medium, after it is cooled to 45°C before pouring.

Cza-pek Dox Agar

Sucrose	: 30.0g
Sodium nitrate	: 2.0g
K ₂ HPO ₄	: 1.0g
MgSO ₄ .7H ₂ O	: 0.5g
KCl	: 0.5g
FeSO ₄ .7H ₂ O	: 0.01g
Agar	: 15.0g
Distilled water	: 1000ml

Kuster's Agar

Glycerol	: 10.0g
MgSO ₄ .7H ₂ O	: 0.01g
CaCO ₃	: 0.02g
K ₂ HPO ₄	: 2.0g
FeSO ₄ .7H ₂ O	: 0.01g
KNO ₃	: 2.0g
NaCl	: 2.0g
Casein	: 0.3g
Agar	: 15.0g
Distilled water	: 1000ml
pH	: 7.1-7.2

Starch Casein Agar

Soluble starch	:10.0g
K_2HPO_4	: 2.0g
KNO_3	: 2.0g
NaCl	: 2.0g
Casein	: 0.3g
$MgSO_4 \cdot 7H_2O$	: 0.05g
$CaCO_3$	: 0.02g
$FeSO_4 \cdot 7H_2O$	: 0.01g
Agar	: 15.0g
Distilled water	: 1000ml