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### Isolation and molecular characterization of *Bacillus megaterium* isolated from different agro climatic zones of Karnataka and its effect on seed germination and plant growth of *Sesamum indicum*

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#### ABSTRACT

*Bacillus megaterium* have been advocated as effective and economical bio inoculant to use in the integrated nutrient and pest control system. *Bacillus megaterium* from soil of different Agro climatic zones of Karnataka were isolated and identified. Molecular characterization of the *Bacillus megaterium* isolates was done using RAPD technique. The study of effect of *Bacillus megaterium* on seed germination of *Sesamum indicum* and on plant growth of *Sesamum indicum* was carried out. Estimation of amount of phosphate present in plants both roots and shoots was also carried out.

**Key words:** *Bacillus megaterium*, RAPD analysis, effect on plant growth, seed germination.

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## INTRODUCTION

The soil acts as a reservoir for millions of microorganisms, of which approximately more than 85% are beneficial for plant life. Thus, the soil is a resilient eco system and soil microorganisms provide precious life to soil systems catering to plant growth. Soil microorganisms play a vital role in the evolution of agriculturally use full soil conditions and in stimulating plant growth.

Beneficial rhizosphere microorganisms that are closely associated with roots have been termed Plant Growth Promoting Rhizobacteria (PGPR) [1], offer an excellent combination of traits useful for both disease control and increased nutrient availability which leads to the identifiable desirable changes in plant growth, seed germination and metabolic activities. Amongst the PGPR, *Bacillus megaterium* have been advocated as effective and economical bio inoculant to use in the integrated nutrient and pest control system. *Bacillus megaterium* is a gram positive, rod shaped endospore-forming bacteria. It is considered aerobic, but, it is also capable of growing under anaerobic conditions when necessary [2,3].

*Bacillus megaterium* is an important family of gram positive bacteria, members of this family comprise substantial proposition of the micro flora of free living saprophytes in soil, fresh water, marine environments and many other natural habitats [4-6].

The effects of PGPR on plant growth can be mediated by the direct or indirect mechanisms. The direct effects have been most commonly attributed to the production of plant hormones such as auxins, gibberellins and cytokines as by supplying biologically fixed nitrogen [7,8]. These PGPR also effect growth of plants by indirect mechanisms such as suppression of bacterial and fungal, nematode pathogens by production of antibiotics, ammonia etc.).

Genetic diversity can be estimated at molecular level. One such method is protein analysis using electrophoresis or direct amino acid sequencing. Electrophoresis analysis of proteins has long been a valuable tool in systematic and population genetic studies of bacteria, plants and fungi. The biochemical characteristics are useful in distinguishing two different genera or species. Further it can also identify different compounds produced by different strains [9].

Molecular analysis of genomic DNA of the organism is useful for distinguishing the bacterial strains better at intra-species level these techniques provide valuable information on the magnitude of genetic variability within and between organisms of different species. With the advent of molecular techniques, several arbitrary primers based Randomly Amplified Polymorphic DNA (RAPD) technique has been used for typing and identification of number of closely related species of bacteria and assessment of genetic relationships. Its results are usually consistent with those of DNA-DNA homology studies and can be used to estimate the genetic distances [10,11].

Random Amplified Polymorphic DNA (RAPD) markers are DNA fragments from PCR amplification of random segments of genomic DNA with single primer of arbitrary

nucleotide sequence and which are able to differentiate between genetically distinct individuals, although not necessary in a reproducible way. Unlike, traditional PCR analysis RAPD does not require any specific knowledge of the DNA sequence of the target organism. The identical 10-mer primer will or will not amplify a segment of DNA, depending on positions that are complementary to the primer sequences. For example, no fragment is produced if primers annealed too far apart or 3' ends of the primers are not facing each other. Therefore, if a mutation has occurred in the template DNA at the site that was previously complementary to the primer, a PCR product will not be produced, resulting in a different pattern of amplified DNA segments on the gel. RAPD analysis is faster, technically less demanding and more economical than the other genomic typing methods like RFLP (Restriction Fragment Length Polymorphism) and AFLP.

Objectives of this project were:

1. To isolate, identify and characterization of *Bacillus megaterium* from soil of different Agro climatic zones of Karnataka.
2. Molecular characterization of the *Bacillus megaterium* isolates from different agro climatic zones of Karnataka using RAPD technique.
3. To study the effect of *Bacillus megaterium* on seed germination and of *Sesamum indicum*.
4. To study the effect of *Bacillus megaterium* on plant growth of *Sesamum indicum*.
5. To produce a Biofertilizer using coal as substrate.
6. To estimate the amount of phosphate present in plants both roots and shoots.

## MATERIALS AND METHODS

The present investigation was carried out to isolate and identify *Bacillus megaterium* from soil of different agro climatic zones of Karnataka, to characterize *Bacillus megaterium* using RAPD markers. Study of effect of *Bacillus megaterium* on plant growth and seed germination of *Sesamum indicum* was carried out in Department of Biotechnology, University of Agricultural Sciences, G.K.V.K Campus, Bangalore-560065 during the year 2008 to 2009. The details of the material used and methods followed are described below.

Karnataka state has different soil types viz., red soil, sandy soil, laterite soil, and alluvial soil and is divided into ten agro climatic zones on the basis of annual rain fall, soil type, cropping pattern, and other climatic conditions. Soil samples of 500 g each were collected randomly from rhizosphere of each agro climatic zone and packed in polythene bag. They were transferred to the Department of Biotechnology, University of Agricultural Sciences, GKVK Campus, Bangalore.

### Isolation of *Bacillus megaterium*

*Bacillus megaterium* was isolated by following the enrichment culture techniques. 10 gms of soil collected from different agro climatic zones was suspended in 100 ml distilled water in a conical flask and serial dilutions were made. From the serial dilution, 100 $\mu$ l of soil suspension at different dilutions were drawn and poured onto the surface of SPERBER'S medium which is a selective medium for isolating *Bacillus megaterium*. The inoculated plates

were incubated at 28-30°C for 48 hrs. At the end of the incubation period number of colonies of *Bacillus megaterium* appearing on the plates were observed [12].

### Identification of the isolates

The cultures so isolated were characterized through a number of microbiological and biochemical tests. Aerobic spore formers pasteurize a diluted soil sample at 80 degrees for 15 minutes, then plated onto nutrient agar and incubated at 37°C for 24 hrs. The plates were examined after 24 hrs for typical colonies identified as catalase-positive, Gram-positive, endospore-forming rods.

### DNA extraction protocol

Bacterial isolates were grown in Nutrient broth and incubated at 28-30 °C for over night under shaking. About 1.5 ml of culture was taken in micro centrifuge tube, centrifuged at 12,000 rpm for 5 minutes. This procedure was repeated for three times. The supernatant was discarded and the pellet was collected. To the pellet 650 µl of extraction buffer was added then it was vortexed, tube was kept in water bath at 65°C for 30 min. After that 100 µl of 5 mM potassium acetate solution was added. It was kept in ice for 10 min, centrifuged at 12,000 rpm for 5 minutes. Supernatant was collected and pellet was discarded. To that tube equal volume of Phenol: Chloroform: IAA (25: 24: 1 ) was added mixed well and centrifuged at 12,000 rpm for 5 min. Supernatant was collected and pellet was discarded. To that tube equal volume of Chloroform: IAA (24:1) was added, mixed well and centrifuged at 12,000 rpm for 5 min. Supernatant was collected and pellet was discarded. To the supernatant equal volume of chilled isopropanol was added on the walls of the tube and it was kept in ice for 10 min. Again centrifuged at 12,000 rpm for 5 min. Pellets of the DNA that remain attached to walls of the tube was found. Tube was washed with 100 µl of 70 % ethanol and centrifuged at 12,000 rpm for 2 minutes. The supernatant was discarded and pellet was collected. The pellet was dissolved and stored in 40 µl of TE buffer at 4°C.

### PCR amplification conditions

#### Reagents used in the PCR

30 ng/µl of Template DNA, 2 mM dNTPs, 1 U/µl *Taq* polymerase, 10 X *Taq* assay buffer A: 50mM KCl, 1.5 mM MgCl<sub>2</sub> 10 mM Tris.HCl pH 9.0, Gelatin 0.1 %, 0.5% Triton-X100 and 0.05% NP40. 30 ng of genomic DNA was used as the template for the standardization of PCR reactions and the PCR conditions were optimized to produce the reproducible and fine fingerprints. PCR reactions were performed in a final volume of 25 µl containing 30 ng of template DNA, 0.75 µl of 2mM dNTPs each, 2.5µl of 10X *taq* buffer, 0.36µl 1 unit of *Taq* DNA polymerase, 3 µl of 10 pico mole primer. Amplifications were achieved in MWG-Biotech primus thermocycler with the program consisting initial denaturation of 94 °C for 3 min followed by 45 cycles each consisting of denaturation at 94 °C for 1 min, primer annealing temperature at 37° for 1 min, primer extension at 72 °C for 3 min, and a final extension of 72 °C for 10 min. These reactions were repeated to check the reproducibility of the amplification.

## Selection of primers

To choose the RAPD primers that can amplify informative sequences, Primer screening was carried out using DNA obtained from the *Bacillus megaterium* isolates out of the 14 primers screened. From these 14 primers finally 8 primers producing sharp, intense bands were selected for the RAPD analysis.

## RAPD primers with sequences chosen for analysis

Sl.No.	Sequence
Random primer 1	5' GAA CTG GAG T 3'
Random primer 2	5' GTT TCG CTC C 3'

## Agarose gel electrophoresis

Agarose gel electrophoresis was performed to resolve the amplification product using 1.5 per cent agarose in 1X TBE buffer, 0.5µg/ml of ethidium bromide, and loading buffer (0.25% Bromophenol Blue in 40% sucrose). 5 µl of the loading dye was added to 10µl of PCR products and loaded to the agarose gel. Electrophoresis was carried at 65 V for 4 hour. The gel was visualized under UV light and documented using Hero Lab Gel Documentation unit.

## 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 (Presence or absence) 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 in to 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 500 bp DNA ladder, which was run along with the amplified products. The Genetic distance was computed as:

$$\sum^n = 1 \quad d_j^2 \quad \text{where } d_j = (X_{ik} - X_{jk})$$

Where  $X_{ik}$  refers to binary code of  $i^{\text{th}}$  tree for allele "k" and  $X_{jk}$  refers to the binary code of the  $j^{\text{th}}$  tree for allele "k". Dendrogram was computed based on Ward's method of clustering, using minimum variance algorithm (Ward, 1963).

## Principal component analysis (PCA)

This technique help in converting a set of variables in to a few dimensions using which the genotypes /clones under study can be depicted in a two or three dimensional space (Ludwig and Reynolds, 1988). Thus, the variations of several variables will be

condensed in to a set of limited axes. Such a graphical analysis help in identifying the individuals that tends to cluster together. The genetic relationships between the different genotypes were estimated with the PCA developed from dissimilarity matrix. The genotypes were clustered on the first three axes and the pattern of clustering or separation of genotypes from one another was also analysed.

### Plant growth studies

Small plantlets are taken and in the given way they are allotted for different agroclimatic zones and one group were allotted for keeping as a control. Soil, manure and sand was mixed in the ratio of 1:1:1/2 respectively. This soil mixture was filled to 33 pots and sesame seeds were sowed to it. Pots were labeled as 1,2,3,... upto 10 and C(control). This was done for 3 replicas. When plants were grown for certain length, the bacterial suspension of 15ml was inoculated for each plant. These bacterial suspension was diluted to 1:4 ratio, before inoculating into plants. For control only water was added.

### Observation for Plant growth

After 10 days, 25 days and 40 days the length of plants, number of leaves, number of branches and root length with the dry weight of shoot and root system.

### Seed germination studies

**Treatment allocation:** For this also, the same methodology is done for inoculation.

Tc - Control (uninoculated control), T1 –from Zone 1, T2 - from Zone 2 ,T3 - from Zone 3 ,T4 - from Zone 4 ,T5 - from Zone 5 ,T6 - from Zone 6 ,T7 - from Zone 7 ,T8 - from Zone 8, T9- from Zone 9 ,T10- Zone 10 .Here zone refer to Agro climatic zone.

### Seed bacterization

Two weeks old seeds were soaked in sterilized distilled water for over night. Then the seeds were dried and transfer in to respective zone cultures for 30 to 45 min. After the respective zone soaked seeds were transfer into respective petriplates covered with Wattmen No.1 filter paper. The plates were wetted using sterilized water. After 3 days incubation, the length of the radical and plumule was recorded.

## RESULTS

Table 1: Growth of shoots in various zones.

S.No.	Agro climatic zone	Length of root part in cm	Length of shoot part in cm	The complete length in cm
1	Control	1.0	1.1	2.1
2	Z-1	2.5	1.5	4.0
3	Z-2	2.3	2.5	4.8
4	Z-3	1.8	3.0	4.8
5	Z-4	3.0	2.0	5.0
6	Z-5	6.0	2.5	8.5

7	Z-6	4.5	2.3	6.8
8	Z-7	5.2	2.0	7.2
9	Z-8	2.7	2.5	5.2
10	Z-9	2.9	2.0	4.9
11	Z-10	4.2	1.5	5.7

Of the various rhizospheres associated bacteria, *Bm* are probably some of the less studied and appear to have significant potential for commercial application. Investigations were carried out on Biological and molecular characterization of *Bm* isolated from different agroclimatic zones of Karnataka. The isolates were examined for the genetic diversity and the influence of these isolates on the seed germination of Chick pea and Ground nut.

### Isolation and Identification

#### Isolation

Isolation of was made from soils of different agro climatic zones by the enrichment culture technique in Spreber's medium. All the isolates were subjected to various tests to confirm identity.

#### Identification

#### Colony morphology

Phosphate solubilisation zone was observed around the colony.

#### Microscopic observation

Isolates were further examined for their Gram's reaction and shape. Characteristically all the isolates were gram positive and rod shape.

#### Molecular markers

#### Standardization of protocol for RAPD analysis

#### Amplification conditions

For fingerprinting and diversity analysis, PCR amplification conditions were optimized based on the protocol outlined by William *et al.* (1990) and Welsh and McClelland (1990) with minor modifications. In order to obtain high amplification rate and reproducible banding pattern, different duration for hot start, denaturation, and primer annealing and primer extension were tried.

The PCR reaction was evaluated for 30, 40 and 45 cycles using standard buffer as outlined in Material and Methods. The optimum conditions for each cycle of PCR were developed for obtaining high amplification levels. The optimum PCR conditions consisted of the following steps which were repeated for 45 times.

Initial strand separation or hot start at 94<sup>0</sup>C for three minute followed by, 45 cycles of

- i. Denaturation at 94<sup>0</sup>C for one minute.
- ii. Primer annealing at 37<sup>0</sup>C for one minute.
- iii. Primer extension at 72<sup>0</sup>C for two minute and
- iv. Final extension period at 72<sup>0</sup>C for ten minute.

### Reaction parameters

It is important to optimize the concentration of PCR mixture, in order to produce informative and reproducible RAPD fingerprints. Hence different concentrations and template DNA (10-15ng, 25-30ng and 40-50ng) were tried with similar amplification conditions (Table 2). A concentration of 25-30ng of template DNA and 2mM of dNTPs per reaction were found to be optimum for obtaining intense, clear and reproducible banding pattern in *Bm* isolates. In all these cases, 3 µl of 10 pico moles of primer and 0.36 µl of 1 unit of *Taq* polymerase per reaction were used. However, fluctuation in the concentration of template DNA did affect the amplification, with too little DNA (10-15ng) causing either reduced or no amplification of small fragments and higher concentration of DNA (40-50 ng) producing a smear.

**Table 2 :Optimum concentration and conditions for RAPD analysis**

Variable	Conditions/ concentration	
	Evaluated	Optimum
<b>PCR amplification</b>		
Hot start (94 °C)	2min, 3min, 4 min.	3min,
Denaturation (94 °C)	30sec, 1min, 1.5min.	1min,
Annealing (40 °C)	1min, 1.5min, 2.0min	1min,
Extension(72 °C)	1min, 1.5min, 2.0min, 3min	2min,
Number of cycles	35, 40, 45 cycles	45 cycles
<b>RAPD Protocol</b>		
Template DNA	10-15ng, 25-30ng, 40-50ng	25-30ng
dNTPs	1.5mM, 2mM,	2mM

### RAPD characterization

A total of 20 RAPD bands produced from the selected 2 primers were used for fingerprinting and for estimation of genetic diversity among ten isolates of *Bm*. For the purpose of illustration, the RAPD fingerprints or electrophore gram generated for ten *Bm* isolates using eight primers are presented in Plate .

The number of bands scored for each primer varied from 1 to 2 with an average of 9.3 bands per primer. Out of 20 amplification bands, 3 bands (15%) were monomorphic, 06

bands (30%) were unique and 11 bands (55%) were shared polymorphic, which were informative in revealing the relationship among the genotypes ( Table 3).

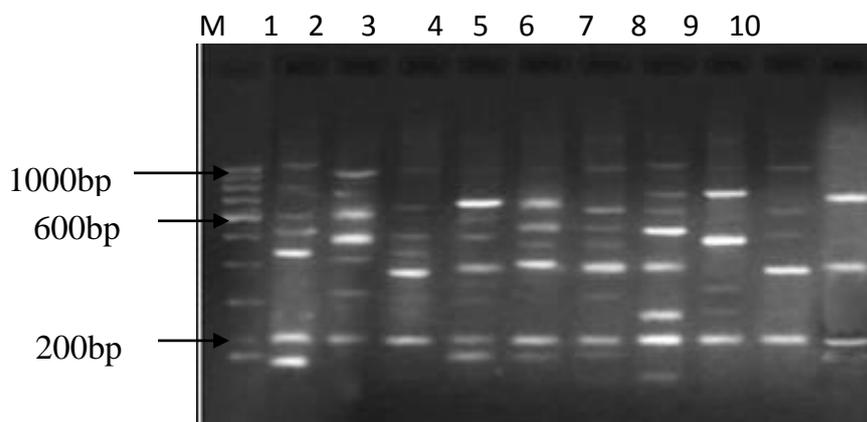
**Cluster analysis and genetic dissimilarity matrix of 10 *Bm* isolates**

The cluster analysis based on 20 RAPD bands revealed that the ten *Bm* isolates examined. The dendrogram (Fig 1) has clearly depicted that all the 10 *Bm* isolates formed two major clusters. Among the two major groups, isolates from zone 4 (CDP), zone 5 (EDP), and zone 6 (SDP) formed the first group and the isolate from zone 1 (NETP), zone 2 (NEDP), zone 3 (NDP), zone 7 (STP), zone 8 (NTP), zone 9 (HP) and zone 10 (CP) formed the second group.

The dissimilarity matrix for *Bm* isolates revealed that within the *Bm* isolates used in the present investigation, the highest dissimilarity was observed between isolate of zone 1 (NETP) and isolate of zone 4(CDP) followed by zone 4(CDP) and zone 9 (HP) isolates. Least dissimilarity was observed between zone 2 (NEDP) and zone 3 (NDP) isolates, followed by zone 3 (NDP) and zone 10 (CP) isolates.

**Table 3: Oligonucleotide primers that showed genetic variation among the *Bm* isolates**

Primers	No. of amplified fragments	No. of polymorphic bands		No. of Monomorphic bands
		shared	unique	
Random primer 1	12	07	04	01
Random primer 2	08	04	02	02
Total	20	11	06	03
Percentage		55%	30%	15%



**Figure 1: RAPD GEL profile of *Bm* isolates generated using 10-mer random primer no.1.lane 1,2, 3,4,5,6,7,8,9 and 10 are isolates from zone 1 to zone10 respectively.**

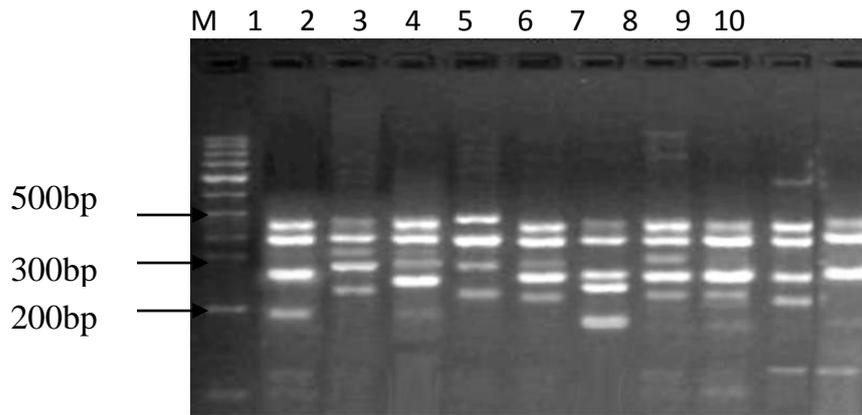


Figure 2: RAPD GEL profile of *Bm* isolates generated using 10-mer random primer no.2.lane 1, 2, 3,4,5,6,7,8,9 and 10 are isolates from zone 1 to zone10 respectively.

### PCA Analysis

To visualize the genetic relatedness among the *Bm* isolates in detail principal component analysis (PCA) was done for 81 RAPD bands generated by 10 decamer random primers. The description of the data was done using three dimensions and the same is presented in Figure 4.

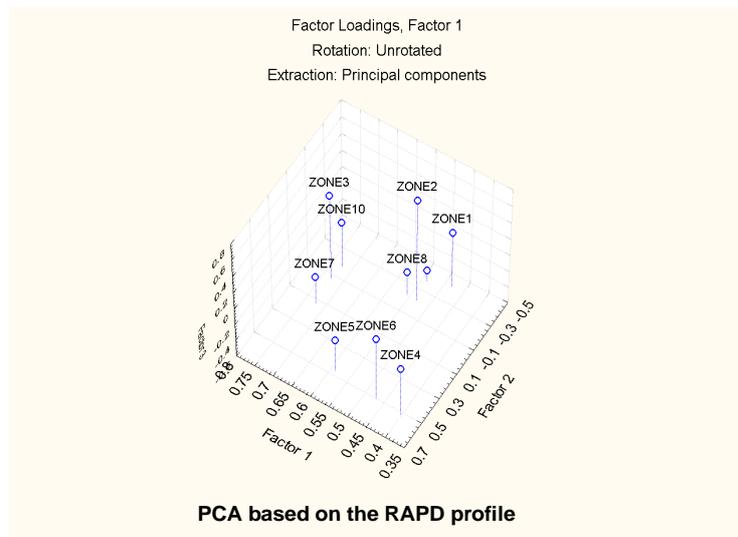


Figure 4: PCA based on RAPD profile.

### Results of Seed germination of *Sesamum indicum*

**Table 4: Results of seed germination of *Sesamum indicum***

S.No.	Agro climatic zone	Length of root part in cm	Length of shoot part in cm	The complete length in cm
1	Control	1.2	1.1	2.3
2	Z-1	2.8	1.8	4.6
3	Z-2	2.7	3.0	5.7
4	Z-3	2.0	3.2	5.5
5	Z-4	3.3	2.3	5.6
6	Z-5	6.2	2.7	8.9
7	Z-6	4.7	2.5	7.2
8	Z-7	5.4	2.3	7.7
9	Z-8	2.9	2.8	5.7
10	Z-9	3.1	2.2	5.3
11	Z-10	4.4	1.8	6.2

**Figure 5: Effect on seed germination of *Sesamum indicum* on length of radicle and plumule of plants.**

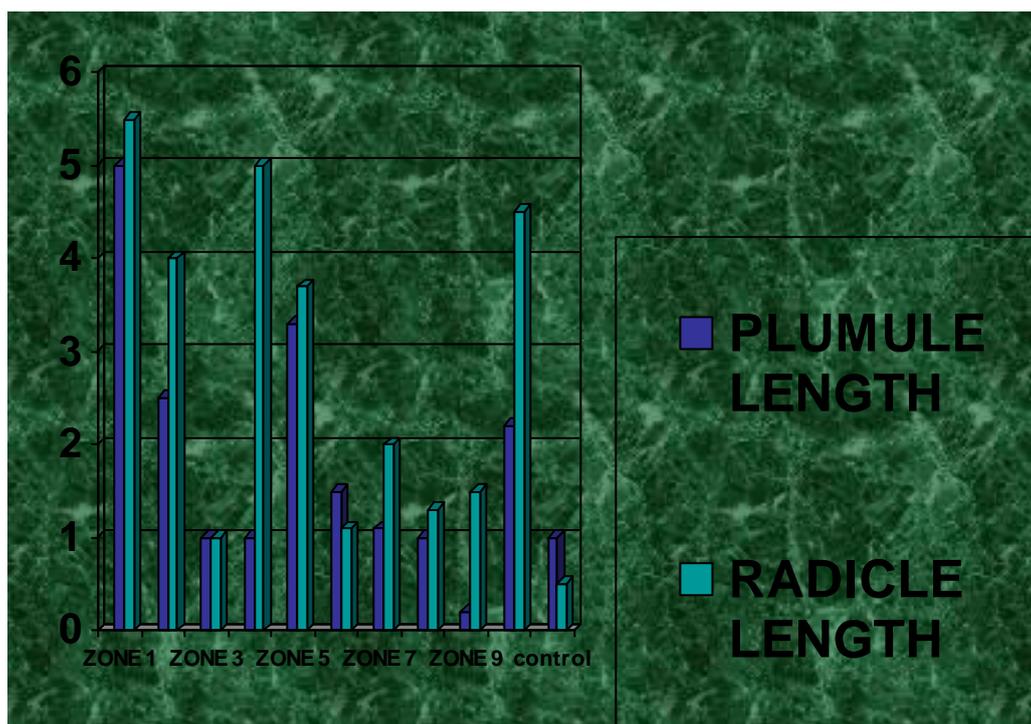
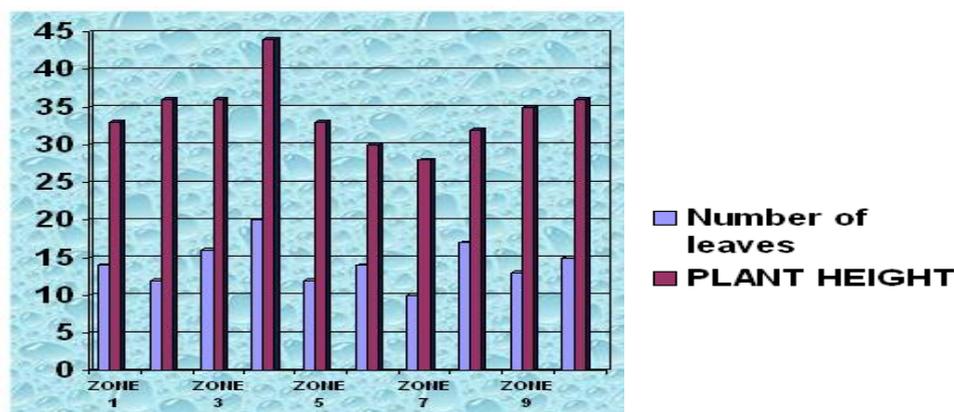


Figure 6: Effect of plant growth on *Sesamum indicum* On both roots and shoots.



### CONCLUSION

Isolation of *Bacillus Megaterium* was confirmed by phosphate solubilisation zone around the colony, Gram's reaction shape and shape. Characteristically all the isolates were gram positive and rod shape. The dissimilarity matrix for *Bm* isolates revealed that within the *Bm* isolates used in the present investigation, the highest dissimilarity was observed between isolate of zone 1 (NETP) and isolate of zone 4(CDP) followed by zone 4(CDP) and zone 9 (HP) isolates. Least dissimilarity was observed between zone 2 (NEDP) and zone 3 (NDP) isolates, followed by zone 3 (NDP) and zone 10 (CP) isolates. To visualize the genetic relatedness among the *Bm* isolates in detail principal component analysis (PCA) was done for 81 RAPD bands generated by 10 decamer random primers. Study of effect of *Bacillus megaterium* on seed germination of *Sesamum indicum* and on plant growth of *Sesamum indicum* was carried out.

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