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# A Strategic Approach for Isolation and Identification of Plant Growth Promoting Rhizobial Strains from Bhadrachalam Forest Area with Respect to Groundnut Cultivar

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## Abstract

**Objectives**: To isolate and identify the crop specific *Rhizobium* strains for *Arachis hypogaea* from Bhadrachalam forest lands by an indigenous novel strategy to reduce the input cost in exploration of compatible strains for Groundnut avoiding one of the serious constrains of bio-fertilizers i.e., shelf life. **Materials and Methods**: Soil samples were collected randomly from 40 different locations of Bhadrachalam forest and sown with ground nut seeds in triplets. Five out of 40 samples which supported the best plant growth were taken for further investigations. The NPK and micronutrient levels of all the soil samples were found to be comparatively similar. This may be because of the fact that all the samples have been taken from same geographic region. Rhizobial strains from the root nodules of these five samples were isolated and maintained in pure cultures. Broths of each pure culture were inoculated on the seeds sown in sterile soil and controls were maintained. **Findings**: The results showed that the inoculated Rhizobia tremendously improved the plant growth when compared with control. Further phylogenetic analysis revealed that the contributing organisms were *Rhizobium leguminosarum, Rhizobium trifolii, Rhizobium meliloti, Rhizobium phaseoli* and *Bradyrhizobium japonicum*. These Rhizobial species in the pure form exhibited high rate of plant growth at lab conditions followed by improved growth in low vegetative agriculture soils of the same geography. The 16S rRNA gene sequencing revealed the fact that soil sample S3 contain abundant *Rhizobium leguminosarum* sp., with 99% similarity. The sample 1, 2, 4 and 5 contain abundant levels of *Rhizobium trifolii, Rhizobium meliloti, Rhizobium phaseoli and Bradyrhizobium japonicum respectively.* **Application**: Therefore this method could be applied for the preliminary screening of compatible, species specific strains for any leguminous plants making the process easy and less expensive.

**Keywords:** Agriculture Soils, *Arachis hypogaea*, Groundnut Growth Promoting Rhizobial Strains, Nitrogen Fixing Bacteria, Species Specific Strains

## 1. Introduction

Plant microbial association perhaps is one of the earliest relationships in the universe. Microbes irrespective of their phylogenic identity have been influencing the plant growth directly or consequentially throughout the process of evolution[1]. A typically broad range of certain organisms at the vicinity of plant root, spatially inhabit the peripheral region of soil namely rhizosphere are called Plant Growth Promoting Rhizobacteria (PGPR). Scientific history reported a numerous advantages of PGPR over-plant despite few considerable negative impacts on plant growth and survival. One of the important and well articulated plant microbial interactions that occur exclusively

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in legume plants is called symbiosis. An investigation was carried out to identify the suitable Rhizobium strains that promote plant growth specifically in *Arachis hypogaea* through a strategy which is not in traditional practice.

Nitrogen essentially is an important element for the survival, growth and yielding of any plant in general[2]. Soil, water and sunlight are the basic requirements of any plant for survival, of which soil contributes the major proportion. Micro and macro nutrients, organic matter, trace elements are said to be the components contributing for plant growth besides microorganisms. Though a variety of organisms surrounds the plant root system the capability of promoting plant growth is limited to certain genera which are often called Plant Growth Promoting Rhizobacteria (PGPR). Microorganisms are highly specific with respect to their interaction, association and release of secondary metabolites. So, the basic contribution of microorganisms for the plant growth by means of mobilizing micro, macro nutrients and secondary metabolites is precisely determined by the abundance of organisms at strain level. Therefore, species/strain specific bioinoculants are essential for the better yield of the crops. The present study involved an innovative strategy in sample selection that enabled the easy and quick identification of plant specific inoculants for *Arachis hypogia*.

*Arachis hypogia* is the legume which plays a key role in world's agricultural economy and mainly cultivated for its vegetable oil, protein, minerals and vitamins[3]. Ground nut is one of the most important cash crops in India and many other parts of the world. Most of the literature reported nodulation by Rhizobia especially *Bradyrhizobium sp*[4]. On the other hand Rhizobial nodulation is highly specific at strain level supporting the fact that ranges of other *Rhizobium* sp. participate in nodulation based on the host plant and agro-climatic conditions.

Shelf life is one of the major limitations in the liquid bio-fertilizer application. The reasons attributed are microbial antagonism, dormancy and geographical acclimatization. A serious attempt has been made to minimize the limitations by observing and adapting the natural combination and selection method for the isolation of *Rhizobium*. Organisms of one geographical area were found to be positive growth promoters within its scope because of high adaptability. This raises a curtain to eco-friendly cost effective bioinoculants namely bio-geo-inoculants avoiding the greatest challenge of shelf life.

# 2. Materials and Methods

## 2.1 Sample Location

The geographical location of Bhadrachalam is 17.6688°N, 80.8936°E. Since the experiment involved in the exploration of compatible strains, pure soil samples without chemical fertilizer intervention was preferred. Plants in this area were rich and healthy; this might be because of high concentration of microbial biomass besides the organic content. All the samples from 40 different locations of this forest have been taken for present study.

## 2.2 Method of Collection

Three spots identified equidistant from single location were chosen for sample collection. Rhizosphere soils from these three spots were mixed thoroughly and made to single sample. Forty samples were prepared in the similar fashion.

## 2.3 Physico Chemical Analysis

Total nitrogen content in the soil was estimated by alkaline potassium permanganate method[5]. This method was used for total phosphorous content estimation[6]. Potassium content was estimated as described in flame photo metric method[7].

## 2.4 Isolation of Rhizobium

All the 40 soil samples were sown with ground nut seeds of same batch in triplets and the growth parameters were observed. To ensure the uniform germination, all the seeds were soaked in water before sowing. Five soil samples out of 40 that support the best plant growth were selected for isolation of Rhizobium. The root nodules of respective plants were carefully cut and were subjected to surface sterilization. The Rhizobium inside the root nodules were taken out by gentle crushing with a pestle and mortar. The crushed material was diluted serially in water up to $10^{-9}$ dilutions. One ml of diluted inoculums was spread on YEMA medium for the isolation of Rhizobium bacteria. The prominent pink colored colonies with luxurious growth were identified as Rhizobium and pure cultures were maintained accordingly.

## 2.5 Standard Inoculum Preparation

The log phase cultures of Rhizobium were taken for the preparation of standard inoculum. The seeds were treated

with 1 ml of standard broth before sowing in the sterile soil for a period of 90 days.

### 2.6 DNA Isolation

Meatgenomic DNA isolation was performed by using optimized protocol. Finely sieved 3 grams of soil sample was collected into falcon tubes (BD Biosciences) for addition of 6ml of extraction buffer and protienase K. samples were mixed thoroughly and incubated at 37°C for 30 minutes by shaking. Incubation followed the addition of 3 ml of 20% SDS and incubated for 90minutes at 65°C. The samples were freeze thawed for three times in $LN_2$ and at 65°C respectively, and then fallowed centrifugation at 6000 rpm for ten minutes. The pellet was repeated with the same step for three times by collecting supernatant in separate tube. Half of the volume of 30% PEG, NaCl in 1:1 was added to the supernatant and incubated at room temperature for two hours. The incubation fallows centrifugation 10,000 rpm for 20 minutes, aqueous layer was collected into a fresh tube. Equal volumes of Phenol: Chloroform: Isoamylalcohol in 25:24:1 was added and centrifuged at 12,000 rpm for five minutes. Supernatant was collected and further added with equal volumes of chloroform and isoamylalcohol 24:1 by gentle mixing. The samples were centrifuged at 12,000 rpm for five minutes and top most layer was collected into fresh tube with cut tips. To the collected top layer, $0.6^{th}$ volume of chilled isopropanol was added, incubated for 2 hours and centrifuged at 14,000 rpm for 15 minutes. Supernatant was discarded, pellet was air dried and dissolved in TE buffer. As described in optimized protocol, further enzymatic isolation of DNA was performed by adding RNase A and the pellet was dissolved in TE buffer.

### 2.7 Gene Amplification by PCR Technique

Isolated DNA was subjected to polymerase chain reaction by using 16S rRNA primers. PCR master mix was prepared at 4°C by adding Taq buffer-5ul, 2mM dNTP mix-5 µl, forward primer and reverse primer (10 pM/µl) – 5 µl each, Taq DNA polymerase 1µl, based on the concentration of DNA (spectrophotometer analysis), 4 µl of DNA was added and made up the volume to 50µl by addition of DNase free water. PCR tubes were vortex mixed gently for 5 seconds and the amplification reaction was performed in Bio-Rad thermal cycler. 16S rRNA was amplified for 30 cycles with temperature conditions set as follows: Initial denaturation 94°C for 5 minutes, denaturation at 94°C for 20 sec, annealing at 48°C for 20 sec, extension at 72°C for 40 sec and final extension for 5 min at 72°C. Amplified PCR product was resolved in 1% agarose gel and 1542 bp amplicon was purified by Quiagen spin columns[8].

### 2.8 Gene Sequencing

To check the novelty in amplified and purified PCR product further molecular analysis of 1542bp PCR amplicon was sequenced by using universal 16S rRNA primers. To find the regions of sequence similarity we used BLAST tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and Ez Taxon to find the nearest taxa of *Arachis hypogaea*.

### 2.9 Soil Collection for Testing the Efficacy of Strains

Soil samples from near by barren agriculture lands, coal and industrial effluent affected agriculture lands were collected to estimate the efficiency of standard inoculum. Soil samples from Devanagaram, Mediceleru were categorized under barren agriculture lands while soil samples from Sarapaka, Reddypalem were considered as industrial effluent affected agriculture lands. Soils from Lakshmipuram and Kottagudem areas were coal affected samples.

## 3 Biochemical Analysis

Analyses of biochemical parameters are very important to evaluate the contribution of various parameters on plant growth.

### 3.1 Indole Acetic Acid Estimation

Root nodules were washed, surface sterilized with 70% ethyl alcohol and dried. Nodules were cut opened and incubated in 20 ml of 10M EDTA for 2 hours and crushed with 70 ml of cold 80% ethanol along with 80% of inhibitor. Crushed nodules were taken into a fresh tube and incubated at 4°C for 1 hour by intermittent mixing of the sample. Then the sample was filtered through a cloth, and centrifuged. For complete removal of ethanol, supernatant was evaporated at 28°C until water alone remains in the sample. The left over sample is added with equal volumes of 1N $NaHCo_3$ and acidified by addition of $H_2SO_4$ until pH reaches to 3. Acidified sample was extracted with equal volumes of peroxide free diethyl ether and this extraction was repeated for 4 times and pooled all the

extracts. The sample was evaporated at 37°C and remnant was dissolved in 95% ethanol and immediately used for estimation using spectrophotometer at 540 nm[9].

## 3.2 ACC Estimation by Calorimeter/ Spectrophotometer

Root nodules were crushed and the collected Rhizobacteria was cultured in DF-ACC rich medium and fallowed growth in DF-ACC minimal medium[10]. Bacterial cells were harvested by centrifugation at 8000 g for 10 minutes at 4°C. Supernatant was removed and the pellet was washed with 5 ml of DF salt minimal media and centrifuged at 8000 g for 10 min at 4°C. Supernatant was removed and pellet was dissolved in 7.5ml of minimal media along with 3.0 mM ACC and incubated for 24 hrs on shaking water bath at 25°C to induce ACC deaminase activity. After incubation cell samples were centrifuged at 8000 g for 10 min at 4°C, supernatant was discarded and the cell pellet was washed in 0.1 M TrisHCl, pH-7.6 to remove the traces of media. The pellet was dissolved in 1 ml of 0.1 M TrisHCl, pH-7.6 and centrifuge at 16000g for 5min. Supernatant was removed and the pellet was dissolved in 600µl of 0.1 MTris-Hcl, pH-8.5 along with 30 µl of toluene and vortex mixed for 30sec. some amount of the cells immediately were taken for ACC deaminase activity in duplicates by using calorimeter at 540 nm. (1st OD readings with bacterial extract, substrate). From the rest of the amount, 200 µl of toulenized cells were taken in fresh tube and added with 20 µl of 0.5 M ACC, vortex mixed gently and incubated at 30°C for 15 mins. Incubation fallows addition of 0.56 M HCl, vortex mixed the sample and centrifuged at 16000 rpm for 5 min at RT. Collected supernatant was added with 800 µl of 0.56 M HCl, mixed gently, and added 300 µl of 2,4-dinitrophenylhydrazine reagent, vortex mixed and incubated for 30 mins at 30°C. After incubation, the sample was added with 2N NaOH and readings were taken at 540 nm in spectrophotometer. (2nd OD readings sample contains substrate, ACC, and bacterial extract) and calculated OD readings against α-ketobutyrate stock standard solution as described in the reference protocol. Stock prepared from 0.1µmol to 1µmol concentration.

## 3.3 Siderophore Estimation

Root nodules were crushed and bacteria was isolated and cultured in YEMA medium[11].Growth of microorganism is detected by turbidity. Culture broth was collected and centrifuged at 8000 g for 10 minutes at RT. Supernatant was collected, 1 ml of supernatant was taken in separate tube, to that 1ml of nitrate molybdate and 1 ml of 1 M sodium hydroxide was added. Immediately the sample was taken for readings by using spectrophotometer at 521 nm OD.

## 3.4 Nitrogen Estimation

1gram of root nodules extract was taken in digestion tube, which was added with 15g of potassium sulfate, 16.7 g of potassium sulfate, 0.01 g of copper sulfate, 0.6 g of TiO2, 0.3 g of Pumice. To the same tube 20 ml of sulfuric acid was added and heated the flask at 390°C for 40 minutes to one hour. After heating, the sample was cooled and diluted with 250ml of distilled water. Distillated the flask with 75 ml of Hcl and added with 2-3 drops of methyl red indicator. The collected distillation sample was titrated with 0.1N NaoH until it changed color from red to yellow. Percentage of nitrogen was calculated by using the formula[12].

$$\frac{\left[(ml\ standard\ acid\ x\ N\ of\ acid) - (ml\ blank\ x\ N\ of\ base)\right] - (ml\ std\ base\ x\ N\ of\ base)\ x\ 1.4007}{Weight\ of\ sample\ in\ grams}$$

## 3.5 Chlorophyll

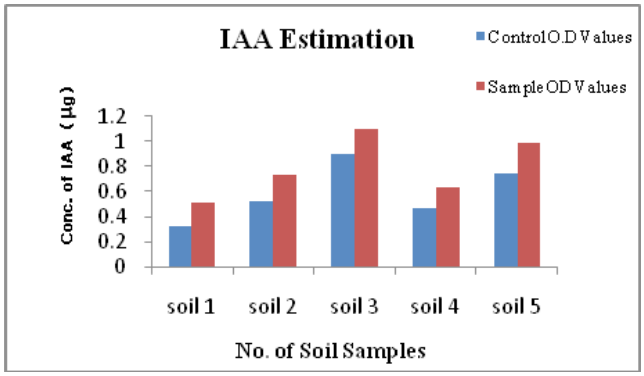
Fresh leaves weighed (500 mg), cleaned and were ground in pestle and mortar by adding 10 ml of 80 % acetone. Crushed leaf extract was collected in test tube and centrifuged at 1000rpm for 15 min. Supernatant was collected and the pellet was re-extracted twice by grinding with 80% acetone. Collected supernatant was estimated for total chlorophyll content by spectrophotometer at 663 nm[13].

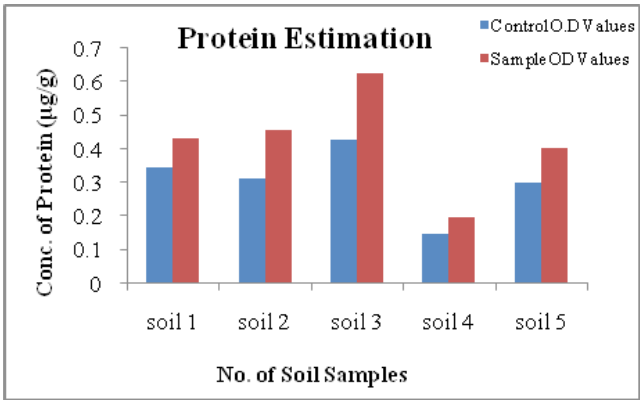
## 3.6 Total Protein Estimation

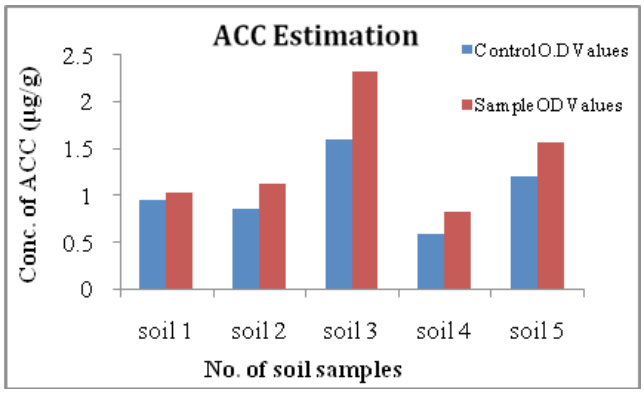
Root nodules were crushed, the sample was collected in test tube and added with 5 ml of reagent A (2% $Na_2CO_3$, 1% NaK Tartrate and 0.5% $CuSO_4$.5 $H_2O$). Test tube was mixed properly and kept in dark for 10minutes. After completion of incubation, sample was added with 0.5 ml of reagent B (Folin Phenol) and incubated in dark for 30 min, total protein content was estimated in spectrophotometer at 660 nm[14].

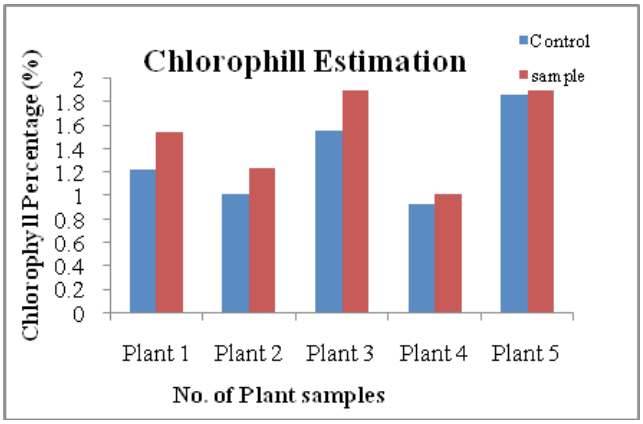
## 3.7 Amino Acid Estimation (Proline)

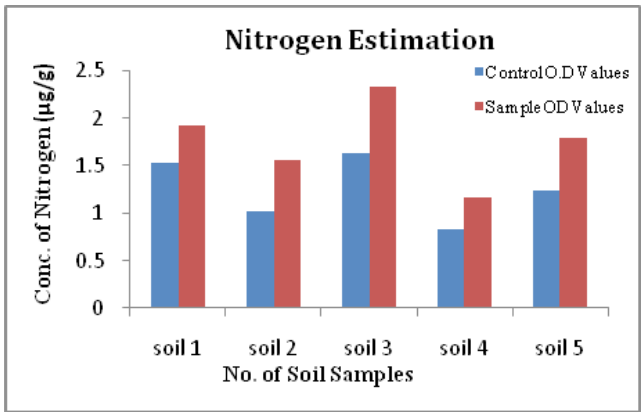
One ml of fresh root nodule extract was taken in test tube, added a drop of methyl red indicator and the reaction

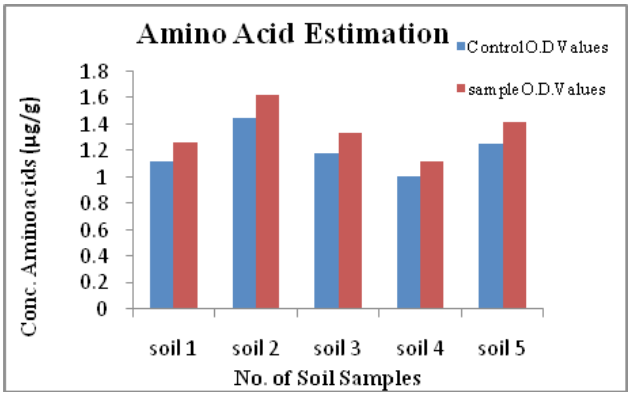


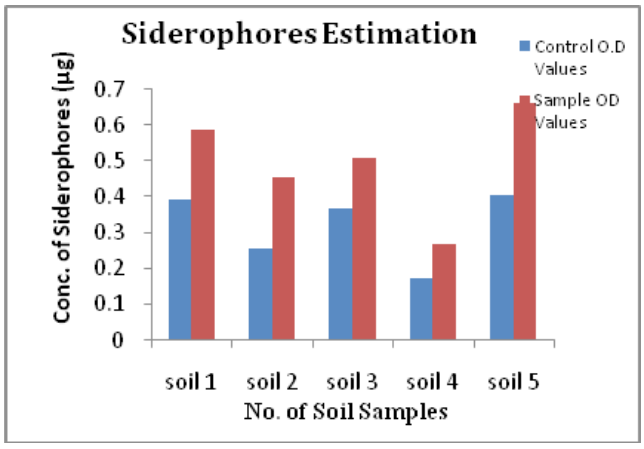



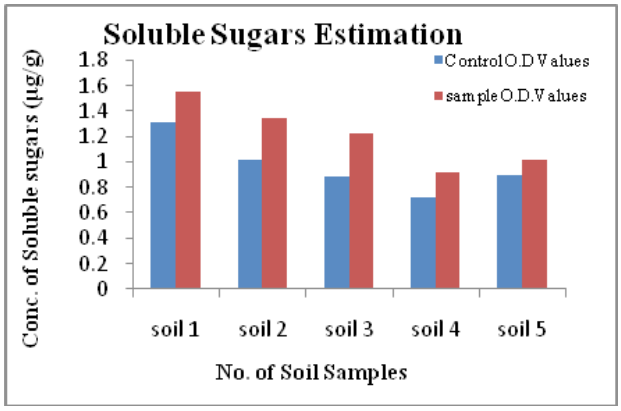




**Figure 1.** Biochemical analysis of different parameters from all the five best plant growth promoting soil samples.

was neutralized by adding 1 ml of 0.1N sodium hydroxide. Test tube was mixed properly and added with 1 ml of ninhydrin reagent. The solution in the test was heated in boiling water bath for 20 minutes, added 5 ml of dilute solution and heated again for 10minutes. Test tubes were cooled and readings were taken at absorbance 570 nm in spectrophotometer[15].

### 3.8 Estimation of Soluble Sugars

1ml of root nodule extract was taken in test tube, added with 1ml of reagent C and the mixture is heated at $100^0$C for 20 minutes. Samples were cooled, added 1ml of arsenomolybdate reagent, mixed and diluted to 20 ml with distilled water. The samples were taken for spectrophotometer readings at 520 nm[16].

## 4. Results

Physical, biochemical and molecular investigations reveled that the abundance of Rhizobial species is responsible for variation in plant growth of five different soils. The average production of IAA, ACC, Nitrogen, chlorophyll and proteins was observed to be high in soil sample 3 shown in Table 1. To be more specific, the soil sample 1 contained more soluble sugars than all the five soils; Amino acid concentration is more in soil sample 2. While no significant contribution of soil sample 4 was observed when we estimated the concentrations of IAA, ACC, Siderophores, Nirogen, Chlorophyll, Amino acids, Proteins, Soluble Sugars as shown in Figure 1. An improved level of ACC and Chlorophyll was observed in soil sample 5.

The 16S rRNA sequence revealed that variety of Rhizobial species contributed for plant growth in each of the five selected samples with 99% similarity. Soil 3 sample exhibited high density of *Rhizobium leguminosarum* sp., while *Bradyrhizobium japonicum* sp., is prominent in soil sample 5. A different species *Rhizobium meliloti* has been found to be more concentration in soil sample 2. The soil sample 4 and 1 exhibited a dense population of *Rhizobium phaseoli* and *Rhizobium trifolii* respectively.

The consortium of cultures in various combinations yields different results with three types of fallowing soil samples. The best plant promoting organism *Rhizobium leguminosarum* sp., found in soil sample 3. As shown in Table 2. and Figure 2 show better plant growth in Coal Effected Soil sample 1 (CES 1) than its control. No considerable improvement than the control was found with the organism in coal effected soil sample CES 2. The same organism *Rhizobium leguminosarum* could contribute more plant growth in both the industrial effluent effected soils (IES 1 and IES 2) when compared to its control. Barren lands soil sample BL1 recorded good growth compared to control while no significant plant growth over the control was found with BL2 with the same organism.

**Table 1.** Biochemical analysis of soil samples showing highest percentage of various growth parameters

| | **Soil samples showing positive to various biochemical tests** | | | | |
|---|---|---|---|---|---|
| | S1 | S2 | S3 | S4 | S5 |
| IAA | | | ↑ | | |
| ACC | | | ↑ | | ↑ |
| Siderophores | | | | | |
| Nitrogen | | | ↑ | | |
| Chlorophyll | | | ↑ | | ↑ |
| Amino acids | | ↑ | | | |
| Proteins | | | ↑ | | |
| Soluble sugars | ↑ | | | | |

The combination of organisms in soil sample 3 and 5 is working comparatively well in both of the coal affected soil samples. One among the two showed not a dominant growth with industrial effluent effected soils samples IES 1 and IES 2. Interestingly both of the barren land soil sam-

**Table 2.** Comparison of effected (coal, industrial and barren land) soil samples with their respective controls.

| | **Coal Effected** | | **Industrial effluent** | | **Barren lands** | |
|---|---|---|---|---|---|---|
| | **Soil 1** | **Soil 2** | **Soil 1** | **Soil 2** | **Soil 1** | **Soil 2** |
| **S1** | ↑ | = | ↑ | ↑ | ↑ | = |
| **S1+S3** | ↑ | ↑ | = | ↑ | ↑ | ↑ |
| **S3+S1+S2+S5** | ↑ | ↑ | ↑ | ↑ | ↑ | ↑ |

**Foot note:** all the results are compared with their respective controls.
↑ Indicates growth more than the control; = indicates plant growth equal to control

ples recorded a luxurious growth over the control with the combination of *Rhizobium leguminosarum* sp., and *Bradyrhizobium japonicum* sp. The combination of organisms isolated from soil samples S3+S1+S2+S5 worked well with all the three non arable soil samples showing tremendous growth over their respective controls.

## 5. Discussion

Nitrogen is one of the essential components of plant for its growth and development. As nitrogen cannot be supplied to the plant directly from outside, a typical mechanism called nitrification helps plant to convert the ammonia to nitrogen. Contribution of nitrogen to plant metabolism is considered to be more than 22-53% making it essential for survival, growth and development[17]. A well established mechanism known as symbiosis in root nodule helps ammonia convert into nitrogen which is the consumable form. A variety of Rhizobium bacteria participate in root nodulation to fix the atmospheric nitrogen based on the cultivar, species and other factors like acclimatization. Nodulation is proved to be highly specific in bacterial association and precise by means of nitrogen fixing levels[18]. This means that, the organisms contribute for nitrogen fixing has got tremendous importance in symbiosis.

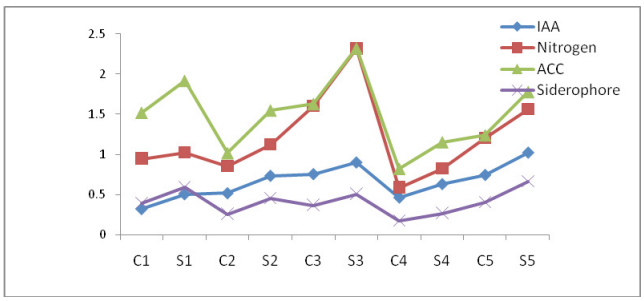



**Figure 2.** The levels of various growth parameters of plants in 5 soil samples.

The present investigation revealed a highly specific species of rhizobia that contributed plant growth via nitrogen fixing in 5 respective soil samples of same geographic region. Out of 40 soil samples of Bhadrachalam forest, based on physical parameters the top five plant growth promoting soils were taken for further investigation. The physical characteristic feature of these 40 soil samples is found to be similar. Micro and macro nutrient levels of all these samples were found to be almost comparable but not congruent. Geographical acclimatization may be the reason for physical and compositional analogy of the soil samples. This infers that the growth in the top five plant growth supporting soils is due to the microbial biomass, especially nitrogen fixing bacteria which was proved by means of Nitrogen fixing in root nodule by *Rhizobium leguminosarum* sp in soil sample 3. Biochemical analysis revealed that the same sample contains highest levels of ACC, IAA, Chlorophyll and proteins making it potential growth promoting organism of all. All the five samples exhibited functional superiority in any one of the biochemical tests individually except sample 4.This may be the reason for plant growth in 4 soil samples.

The 16S rRNA gene sequences revealed the fact that soil sample 3 contain abundant *Rhizobium leguminosarum* sp., with 99% similarity. The sample 1, 2, 4 and 5 contain abundant levels of *Rhizobium trifolii, Rhizobium meliloti, Rhizobium phaseoli* and *Bradyrhizobium japonicum* sp. respectively.

The consortium of various combinations of these five varieties of Rhizobium yielded better results when applied in field conditions. When applied solely, the *Rhizobium leguminosarum* sp., is found to be positive in one of the two coal effected soils, positive in both the industrial effluent effected soils; positive in one of the two barren lands.

The combination of *Rhizobium leguminosarum* and Bradyrhizobium japonicum sp., has shown positive result in all the soil samples except in one of the industrial effluent soils sample when compared to control. The third combination include *Rhizobium leguminosarum, Rhizobium trifolii, Rhizobium meliloti* and *Bradyrhizobium japonicum* sp., showed tremendous growth in all effected soil samples suggesting the best combination.

A variety of studies have been performed to assess the plant growth effects by applying different consortia. Each organism performed well at individual level with functional superiority but could not improve the plant growth when applied solely[19]. The combination of Rhizobium species proved to be the best bio-fertilizer with broad application range.

## 6. Conclusion

In contrast to the traditional soil selection for bio-fertilizer preparation, this method stands alone pertaining and exploring the natural combination. This method could be best employed for isolation of Novel, Efficient, and Cultivable (NEC-PGPR) PGPR. Though molecu-

lar techniques like 16S rRNA gene sequence and Next Generation Sequence allow to identify the total microbial population, it is not certain that all the isolated organisms are cultivable and growth promoting. This method allows identification and isolation of cultivable plant growth promoting organism very precisely. Though we could not obtain the novel isolates, we could identify and isolate geographically specific organisms for *Arachis hypogea* along with the best combination of highly plant specific Rhizobium in various non arable lands of Bhadrachalam forest.

**Conflicts of interest:** Authors declare no conflicts of interest.

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