Pak. J. Biotechnol. Vol. 15 (2) 459-467 (2018) ISSN Print: 1812-1837
ww.pjbt.org ISSN Online: 2312-7791

# EXPLORING NATURAL COMBINATION FOR IDENTIFICATION OF UPREGULATED NITROGEN FIXING BACTERIA IN *GLYCINE MAX*: AN *IN VIVO, IN VITRO* AND *IN SILICO* APPROACH

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Article received 21.3.2018, Revised 2.6.2018, Accepted 10.6.2018

## ABSTRACT

Understanding the concept of symbiosis in a more localized natural selective way and optimizing for the yielding advantages of confined geography is the prime objective of the study. Precisely the aim is to isolate and identify crop specific Rhizobium strains for Glycine max using physical, biochemical and *in silico* techniques from Bhadrachalam forest lands. Randomly collected soil samples from 45 different locations across Bhadrachalam forest were sown with soybean in triplets. The basic parameters like Nitrogen, Phosphorous, Potassium (NPK) and levels of micronutrients for all the soil samples were identified to be similar. Out of 45 samples in triplets, top five growth supporting soils were taken for further investigation. Organisms from the root nodules of these five plants were screened and pure cultures were maintained. Log phase cultures in broth form were inoculated on the seeds sown in sterile soils with respective controls. Tremendous improvement in the growth parameters were observed in results when compared with controls. The polyphasic analysis discovered that the contributing organisms were *Bradyrhizobium japonicum, Bradyrhizobium paxllaeri, Bradyrhizobium canariense, Sinorhizobium xinjiangense, Bradyrhizobium betae* sp. Pure forms of these Rhizobial species have shown elevated rate of plant growth in *in vitro* fallowed by field experiments in low vegetative agriculture soils of the same geography. Out of these five species the *Bradyrhizobium japonicum,* which was the best plant growth supporting for Glycine max has been studies further to explore the Nif genes responsible for plant growth and Nitrogen fixation. The *in slico* analysis of Nif A protein revealed the underlying precursors of indole acetic acid (IAA) production and nitrogenase activity. This novel method of soil selection may be adopted for easy identification of Rhizobial species, specific for not only for Glycine max but also for various other legume crops from respective geographies.

**Keywords:** Rhizobacteria; Nif A; Nitrogen fixation; Indole acetic acid; Geography; *Glycine max*

## INTRODUCTION

Plant Microbial association plausibly is one of the earliest cosmos relationships. Regardless of the phylogenic character microbes have a remarkable influence (direct or consequential) on plant survival and growth all over the process of evolution (Ortiz-Castro et al., 2009). Diverse ranges of free-living organisms spatially inhabit around the plant roots in the peripheral region of the soil are referred as Plant Growth Promoting Rhizobacteria (PGPR). Tremendous research has been conducted globally on PGPR for its advantages over plant like root colonization, growth, yield enhancement, resistance against plant pathogens etc., (Niranjan et al., 2005). Nevertheless, global agriculture research also reports a few considerable negative impacts on plant growth and survival too. Unlike the other plant families, Legume has an exclusive natural mechanism of supplying Nitrogen to plant through a well articulated mutually beneficial phenomenon with Rhizobacteria called Symbiosis. An attempt has been made to identify suitable Rhizobial strains from Bhadrachalam forest area that support plant growth specifically in Glycine max through a strategy which is not in traditional practice.

Nitrogen is one of the essential elements for any plant in general for survival, growth and yielding (Kuan et al., 2016). For any plant to proliferate soil, water and sunlight are the basic requirements, soil being the major contributor. To be precise the soil components namely organic matter, micro & macro nutrients, trace elements contribute for the plant growth directly or consequentially besides the microorganisms. Numerous microorganisms inhabit around the plant root system, while the capacity to contribute for plant growth is confined to certain genera abundantly available in rhizosphere often called plant growth promoting rhizobacteria (PGPR). Typically, microorganisms are highly diversity and are specific in their association, interaction, and release of secondary metabolites. Therefore, the involvement of microorganisms in plant growth promotion by making micro, macro nutrients, and secondary metabolites available to the plant is precisely defined by the availability of organisms at species level. Thus, the administering species specific bioinoculants is essential to improve the yield of the crop. The cur-

rent research involved in an innovative strategy in selection of samples that allow easy and quick identification of plant specific inoculants for *Glycine max*. In the rain fed agro ecosystem of India, *Glycine max* established as one of the major oil seed crops (Sharma et al., 2013). As per 2010 records soyb-ean was harvested 261.6 million metric tons worl-dwide, U.S. was the leading producer of soybean and the next leading producers were Brazil, Arg-entina and China (Encyclopedia). Soybean is also best cultivated for its rich source of protein, which will process into diverse food products. By the way nodulation is highly specific to species level witnessing the fact that a range of other Rhizobial sp. involve in nodulation precisely based on host plant and agro-climatic conditions (Tejerizo et al., 2011).

One of the major limitations of liquid bio-fertilizers is the constraint of shelf life. Possible reasons attributed for less shelf-life of a liquid biofertizer are microbial antagonism, microbial dormancy, and geographical acclimatization. In this research a strategic attempt has been made to optimize the benefits of liquid biofertilizers by indepth understanding of natural combination and selection method for isolation of rhizobacterium. Isolates of one geography are identified to be functionally analogues in growth promotion because of high levels of acclimatization. This hypothesis of geographical specification of isolates has emerged as an alternative to regular liquid biofertilizer in the name of bio-geo-inoculants avoiding the greatest challenge of shelf life. Therefore, the primary objective of this research is to isolate cultivable efficient species specific rhizobacteria from Bhadrachalam forest area pertaining to *Glycine max* fallowing evaluation of physical, biochemical and *in silico* analysis.

## MATERIALS AND METHODS

**Sample Location:** Bhadrachalam forest geographical location is 17.6688° in North, and 80.8936° in East. We preferred to take forest soil samples without any intervention of chemical fertilizers, is majorly to find out the compatible strains. All the forest area plants grow healthy and with dense in population could be because of high content of organic matter and concentrated microbial biomass. For the present experiment, soil from 45 different locations were collected.

**Method of collection:** To collect the rhizosphere soil samples, nine spots those are equidistant from a one single location were chosen. All the five soils from one location mixed thoroughly as a single soil and similarly all the forty- five samples were prepared (figure 1).

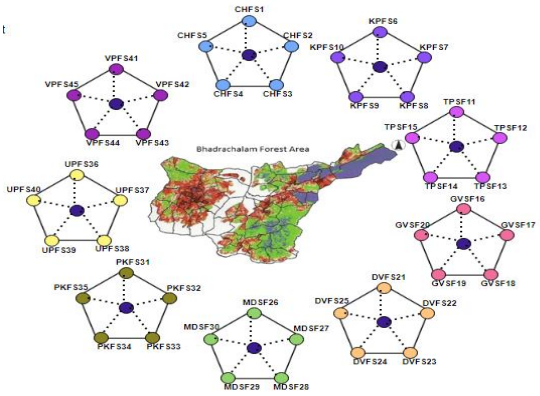


**Figure 1:** Method of soil collection from Bhdrachalam forest area from 9 locations and 5 different soils with 1 km equidistance.

**Soil Analysis:** For all the collected 45 soil samples, we estimated nitrogen, phosphorous and potassium was estimated as decribed in the protocols of Subbaiah *et al.*, (1956), Bray et al., (1945) and Jackson et al., (1973) respectively (figure 2).

**Plant Physical Parameters:** Physical parameters like root length, shoot length, root dry weight, shoot dry weight and number of root nodules were recorded and depicted in figure 3. Statistical data was analyzed by using MS-STATC statistical program and by DMRT.

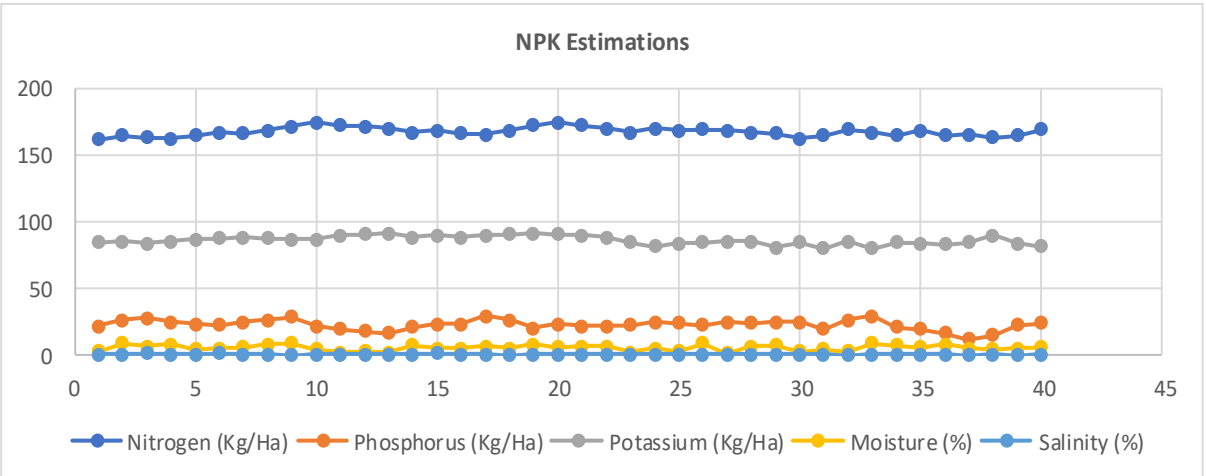

**Figure 2:** Nitrogen, Phosphorous and Potassium (NPK) levels estimation in all 45 soil samples collected from Bhdrachalam forest area.

**Rhizobial isolation:** Forest soil samples, 45 parts were sown with same batch of soybean seeds in triplets and uniform germination was ensured by prior soaking of the seeds. Out of 45 soil samples five soil samples showed best growth of were selected for rhizobium bacterial isolation. Root nodules of the selected five plants were sterilized and crushed gently and the extract was collected. The extract was serial diluted upto $10^{-9}$ and one ml of it was spread on YEMA medium. Luxuriously grown pink coloured colonies were picked and rhizobium pure cultures were maintained.

**Preparation of Standard Inoculum:** The standard inoculum has been prepared by using the log phase cultures of rhizobium. Before sowing the seeds are treated with one ml of standard broth and are grown for a period of 90 days in the sterile soil.

**Isolation of DNA from Root Nodules:** Fresh root nodules were cut from the plant, rehydrated and surface sterilized by using distilled water and with 3.3% w⁄v $Ca(OCl)_2$ for 3 min respectively. Again, they were rinsed with sterile water, then washed with absolute ethanol for three minutes and rinsed again sterile water. Further DNA isolation protocol was followed by crushing root nodules as described by Krasova-Wade et al., (2007). Isolated DNA quantity was estimated by spectrophotometer (Pharmacia Biotech, Cambridge, UK).

**16S rRAN Gene Amplification:** Polymerase chain reaction was used to amplify the DNA. Master mix was prepared with 5 µl of Taq. Pol. Buffer, 5 µl of 2 mM dNTP mix, 10 pM/µl of primers, 1µl of Taq DNA polymerase, 4µl of DNA and the volume made upto 50 µl with DNAse free water. The samples were amplified in Bio-Rad thermal cycler with the temperature conditions, 94ºC for 5´ initial denaturation, 94ºC/ 20 sec denatutaion, 48ºC/20 sec annealing, 72ºC /40 sec extension and 72ºC/5´ of final extension for 30 cycles. 1% agarose gel ran to check the amplicon and the 1542 bp amplicon was purified by Quiagen spin columns (Barlett, et al. 2003).

**16S rRNA Gene Sequencing:** To find the nearest taxa of *Glycine max* sequence analysis of 16S rRNA was done by using BLAST tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and Ez Taxon.

**Unarrable Soil Collection:** To check the efficacy of isolated rhizobium strains, we have applied the inoculum in different un-arriable soil samples. Three different soil samples i.e, industrial effluent (Sarapaka, Reddypalem), barren lands (Devanagaram, Mediceleru), and coal affected soils **(**Lakshmipuram, Kottagudem) were collected and tested.

**Biochemical Parameters Analysis:** To identify the contribution various growth hormones and other chemical compounds in plant growth different biochemical tests like IAA, ACC, nitrogen, and amino acids were performed as below and depicted in figure 4.

**1. Estimation of Indole Acetic Acid:** Fresh root nodules of soybean were collected, sterilized with 70% ethyl alcohol, and squeezed nodules were incubated for 2 hrs in 20 ml of 10M EDTA. Then the nodules were crushed with a buffer contained 80% ethanol and 80% of inhibitor, collected in a fresh tube and incubated for one hour at 4ºC. After incubation it was filtered, centrifuged and evaporated for complete removal of ethanol traces at 28ºC until water remains. Then the sample was added with same volume of 1N $NaHCo_3$ and added with $H_2SO_4$ to make concentration of pH 3. Peroxide free diethyl ether was added to the acidified sample and the extraction process by adding peroxide free diethyl ether was repeated for four times. All the 4 times collected extract was mixed and evaporated at 37ºC, the dried powder containing IAA was dissolved in 95% ethanol and the spectrophotometer readings were recorded at 540 nm (Leveau et al., 2005).

**2. Estimation of ACC:** Soybean root nodules were crushed and cultured in DF-ACC rich medium, and the bacterial cells were collected by centrifugation at 8000 g/10 mins/4ºC. Pellet was collected by discarding the supernatant and washed with 5ml of DF salt minimal media. 7.5 ml of minimal media plus 3 mM ACC was added to the pellet and incubated for on shaking water bath to induce ACC deaminase activity for 24 hrs. The incubated samples were centrifuged at 8000g/10 min/4ºC, the pellet washed to remove the traces of media in 1ml of 0.1M Tris HCl (pH-7.6). Then it was dissolved in the same Tris buffer and centrifuged at 16000g/5min and pellet was dissolved in Tris buffer with pH 8.5 plus 30 µl of toluene by discarding supernatant. The sample was vertex mixed for 30 sec and few cells were taken to check the activity of ACC deaminase in a calorimeter at 540 nm (considered as first OD readings of bacterial extract, substrate).

From the rest of the supernatant 200 µl was taken and incubated at 30ºC/ 15 mins by adding 20 µl of 0.5 M ACC. Then the sample was added with 0.56 M HCl at 16000 rpm/5 min, and supernatant was

collected, added 800µl of 0.56M HCl, 300µl of 2,4-dinitrophenylhydrazine reagent, vortexed and incubated at 30ºC/30 mins. Then the incubated sample was added with 2N NaOH and spectrophotometric readings were taken at 540 nm (2nd OD readings that contains bacterial extract, substrate, and ACC) (Donna et al., 2003).

**3. Estimation of Nitrogen:** Fresh soybean root nodules were crushed, and the extract was collected in a digestion tube that contains 15 g of potassium sulfate, 0.6 g of $TiO_2$, 0.01 g of copper sulfate, 0.3 g of Pumice and 20 ml of sulfuric acid. The sample flask was heated at 390ºC/40 mins to 1 hr. Then the sample was allowed cool down and added 250 ml of distilled water. The flask was distilled with 75 ml of HCl and 2-3 drops of methyl red indicator. Titration was done until the colour changes from red to yellow with 0.1 N NaOH and the percentage of nitrogen was estimated as described in Kjeldahl, labcone protocol.

**4. Estimation of Tryptophan:** Soy bean root nodules were ground in 5 ml of papain solution and incubated at 65°C overnight. Then the sample was cooled down to room temperature and centrifuged, the clear supernatant was collected, and tryptophan was estimated at 545 nm as decribed in Sadasivam et al., (1992).

***In silico* Analysis of Nif A protein:** Primary, secondary and tertiary structure of Nif A protein analysis was done to check for structural variations. Nif A protein sequence was retrieved from Uniprot, to analyze the physical and chemical characteristics by using ProtParam tool. Secondary structural predictions of the Nif A protein were done by Chou-Fasman server and GOR (Garnier et al. 1996). Phrye2, Swiss model, and Modeller servers were used for homology modelling of 3D structures of Nif A protein (Kelley et al., 2015, Arnold et al., 2006, Sali et al., 1993). To assess the quality of 3D homology model Ramachandran plot was used and confirmed (Ramachandran et al., 1963).

## RESULTS

*In vivo*, *In vitro* and *In silico* analysis revealed that the variations in the plant growth and nitrogen levels are due to diversity of *Rhizobial* species in soil samples. The physical parameters i.e. root and shoot length, root dry weight, root weight and total percentage of root nodules shown to be highest in soil sample number 1 revealing the fact that *B. japonicum* is efficient over all the other Rhizobial species isolated from respective soil samples (Figure 3).

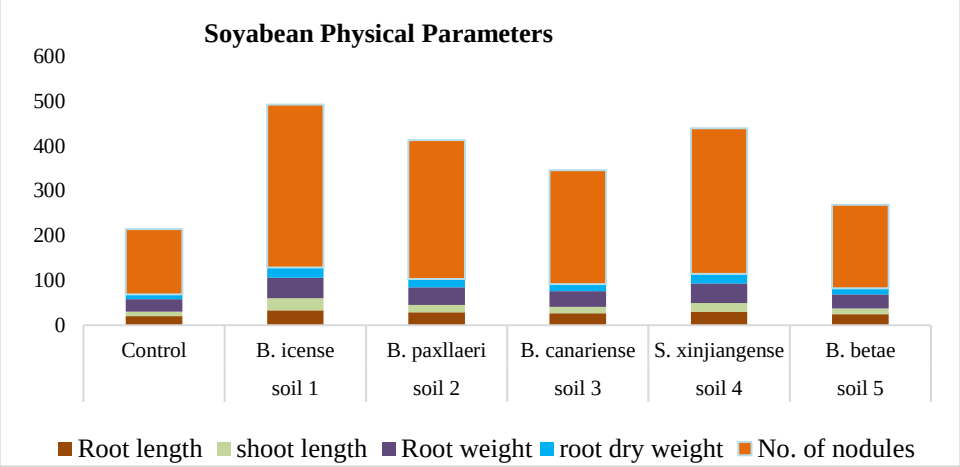


**Figure 3:** Physical parameters like root length, shoot length, root weight, root dry weight and no. of nodules of the soybean plant grown in 5 different soil samples.

The biochemical parameters like nitrogen, ACC, IAA, and amino acids were observed higher in sample number 1 (Figure 4, Table 1).

The samples 2, 3 and 4 were found to be containing more ACC and amino acid concentrations than the sample number 5. Also, no significant contribution of biochemical parameters was seen in soil sample number 5.

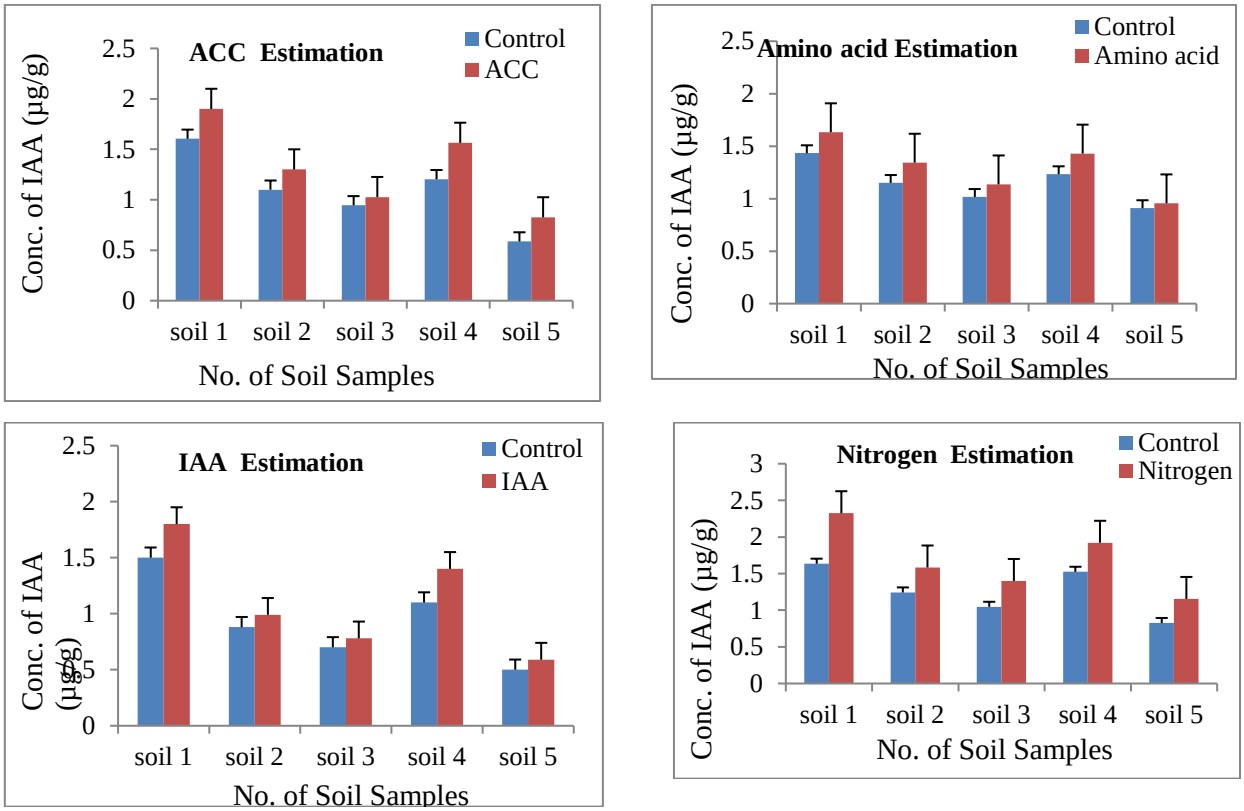


**Figure 4:** Biochemical analysis of different parameters from all the five best plant growth promoting soil samples.

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

| | Soil Samples Showing Positive to Various Biochemical Tests | | | | |
|---|---|---|---|---|---|
| | **Soil 1** | **Soil 2** | **Soil 3** | **Soil 4** | **Soil 5** |
| IAA | ↑ | | ↑ | | |
| ACC | ↑ | ↑ | | ↑ | ↑ |
| Nitrogen | ↑ | | | ↑ | |
| Amino acids | ↑ | ↑ | ↑ | | |

The results of 16S rRNA of five selected soil samples identified five different rhizobial species with close similarity to its nearest taxa that were contributed plant growth in *Glycine max* leguminous plant. *B. japonicum* sp., was seen highest in soil sample number 1, *B. paxllaeri* sp., was found in sample number 2, and soil sample number 4 contains *S. xinjiangense sp*., The other two soil sample numbers 3 and 5 were prominent with *B. canariense sp.*, and *B. betae sp.* respectively.

The appliance of the highest growth supporting and nitrogen fixing rhizobial sp. i.e. *B. japonicum* as broth inoculation has shown tremendous results in various unfertile soil samples. The highest growth has been found in industrial effluent soils when compared to barren and coal affected soil samples (Figure 5).

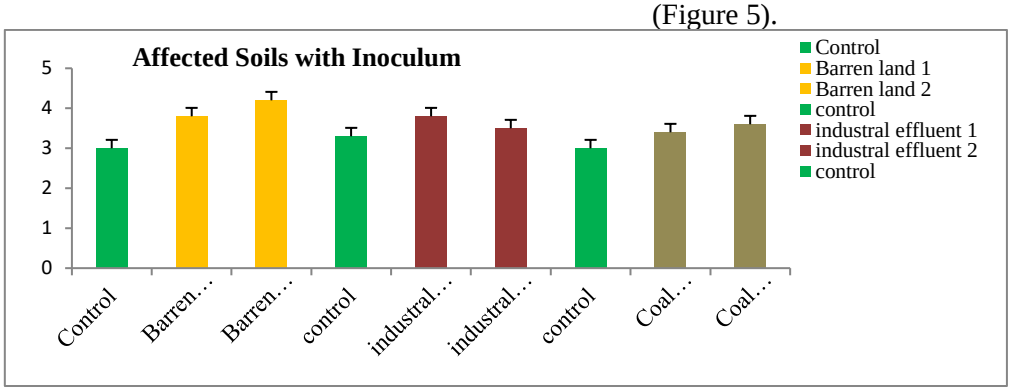


**Figure 5:** Application of *B. japonicum* as broth inoculation for different unfertile soil samples.

*In silico* analysis of primary, secondary and tertiary structures of Nif A protein revealed the structural composition. Primary structural characteristics like molecular weight, sequence length, extinction coefficient, pI value, half- life, aliphatic index and gravy were tabulated in Table 2. Secondary structure of Nif A protein like Extended strand (Ee), Alpha helix (Hh), and Random coil (Cc) were shown in Table 3 with more coiled in nature. Nif A protein sequence retrieved from protoparm software showed the highest percentage of tryptophan amino acid when compared to the rest of amino acids (Table 4, 5). Tertiary structures like number of amino acid residues that were fallen in favoured region, allowed region and outlier region were plotted by a graph i.e, ramchandran plot by using rampage server (Figure 6). The comparative validation of structural design with Phyre2, Swiss and Modeller servers has been tabulated in Tables 5 with 95.5% of allowed regions in modeller server. Out of three servers, our designed Nif A protein was proved to be more acceptable model with modeller server (Figure 7).

**Table 2:** Physicochemical properties of Nif A protein. (M. wt.: Molecular weight; pI: Isoelectric point; –R: Number of negative residues; +R: Number of positive residues; EC: Extinction coefficient at 280 nm; II: Instability index; AI: Aliphatic index; GRAVY: Grand Average Hydropathy).

| Name of the Organism | M. Wt. | Seq. Length | pI | EC | EC | Half Life (hrs) | II | GRAVY | -R | +R | AI |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *B. icense* | 38383.33 | 353 | 9.30 | 18825 | 18450 | 20 | 45.32 | -0.085 | 39 | 48 | 95.92 |

**Table 3:** Prediction of secondary structure of Nif A by Chou-Fasman method.

| | ***B.*** *japonicum* | |
|---|---|---|
| | **Length** | **Percentage (%)** |
| Alpha helix (Hh) | 152 | 43.6 |
| Extended strand (Ee) | 55 | 15.58 |
| Random coil (Cc) | 146 | 41.36 |

Table-5: calculation using rampage server.

| Server | Ramachandran Plot Calculation | ***B.*** *japonicum* |
|---|---|---|
| Phyre2 | No. of residues in favoured region | 85.3% |
| | No. of residues in allowed region | 8.5% |
| | No. of residues in outlier region | 6.2% |
| Swiss model | No. of residues in favoured region | 94.5% |
| | No. of residues in allowed region | 3.1% |
| | No. of residues in outlier region | 0.4% |
| Modeller | No. of residues in favoured region | 95.5% |
| | No. of residues in allowed region | 3.0% |
| | No. of residues in outlier region | 2.0% |

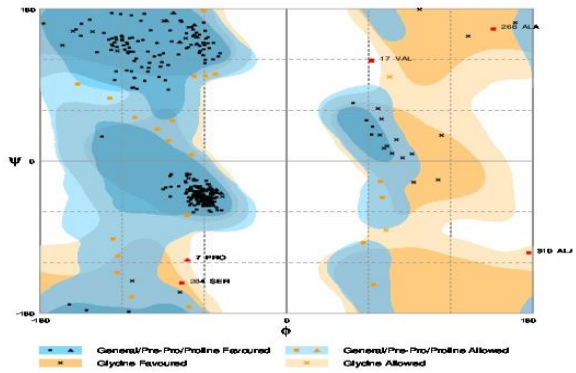


**Figure 6:** Ramachandran plot for *B. japonicum*.

**Table 4:** Percentage of amino acids present in Nif A protein estimated by UniProt software.

| S.no | Amino acids | ***B.*** *japonicum* |
|---|---|---|
| 1 | A (Ala) | 0.6% |
| 2 | R (Arg) | 8.2% |
| 3 | N (Asn) | 2.5% |
| 4 | D (Asp) | 2.8% |
| 5 | C (Cys) | 1.7% |
| 6 | Q (Gln) | 2.5% |
| 7 | E (Glu) | 8.2% |
| 8 | G (Gly) | 6.5% |
| 9 | H (His) | 0.8% |
| 10 | I (Ile) | 4.8% |
| 11 | L (Lue) | 9.0% |
| 12 | K (Lys) | 5.4% |
| 13 | M (Met) | 1.1% |
| 14 | F (Phe) | 3.7% |
| 15 | P (Pro) | 5.9% |
| 16 | S (Ser) | 8.5% |
| 17 | T (Thr) | 5.1% |
| 18 | **W (Trp)** | **11.0%** |
| 19 | Y (Tyr) | 1.4% |
| 20 | V (Val) | 7.9% |

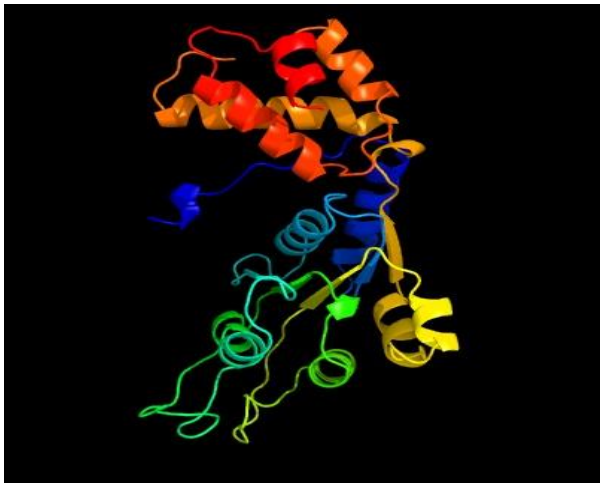

**Figure 7:** *In silico* Nif A protein structure designed by Modeller server.

## DISCUSSION

For the growth and development of the plant nitrogen is one of the essential components. As plants do not uptake atmospheric nitrogen, because of the fact that it is highly unreactive and cannot be used in the protein synthesis a typical mechanism called nitrification converts nitrogen gas into nitrates and nitrites by a special bacterial genus rhizobium in root nodules. Nitrogen is the major component and contributes more than 22–53% to the plant metabolism and is essential for survival, growth and development (University of Manoa. http://www.ctahr.hawaii.edu/mauisoil/c_nutrients01.aspx). A range of rhizobium bacteria involve in fixing the atmospheric nitrogen in the root nodule depending on the cultivar, species and other factors like geographical factors. Rhizobium cultivars are highly specific with bacterial association, nodulation and differ precisely in nitrogen fixing levels (Mardanovi et al., 1998). This infers that the species or inoculants which contributes for nitrogen fixing has great significance in symbiosis.

The current research uncovered an extremely specific species of rhizobia that supported plant growth by nitrogen fixation in 5 different soil samples of same geographic region. Five best plant growth supporting soils out of 45 based on the plant physical parameters were considered for further investigation. The type, texture, pH and humidity of the all the 45 soil samples were almost similar. Alongside the micro and macro nutrient levels were also found to be analogous to each other but not congruent. This compositional analogy could be due the geographical acclimatization and is considered as the crux of this research.

From the series of experiments, it is inferred that the growth promoting nature in the soil is due to microbial biomass mainly nitrogen fixing bacteria which was evident by increased number of root nodules and elevated nitrogen levels in the case of soil sample *B. japonicum.* Therefore, it is understood that the growth in the top five supporting soils is because of the microbial biomass mainly nitrogen fixing bacteria. This is evident from the increased number of root nodules and there by elevated levels of nitrogen in soil sample 1 by *B. japonicum.* Furthermore, the biochemical analysis revealed that the very same sample exhibited highest levels of ACC, IAA and amino acids labelling it to be potential growth promoter of all the other four. Contrastingly all the five samples have shown functional superiority in at least one of the biochemical tests individually other than sample number 5.

To explore the gene level facts that yielded the above results further investigation has been designed taking nitrogen concentrations and plant growth into consideration. It is known fact that nitrogenase enzyme catalyses the reduction of gaseous nitrogen to ammonia. Nitogenase enzyme synthesis is precured by transcription of Nif genes which inturn synchronized by a positive regulatory protein encoded by Nif A gene. The *in-silico* studies of Nif A revealed that the secondary structure contains elevated levels of tryptophan which correlated with the biochemical analytical results that strengthens the scientific fact behind increased plant growth.

Furthermore, the production of IAA influences nif gene expression which regulates nitrogen fixation by nitrogenase enzyme (Defez et al., 2017). Literature reveals that IAA production is regulated by nodulating bacteria (*B. elkanii*in present case) (Ali et al., 2008). Several studies proved the effect of IAA over genes which control nitrogen fixation such as nif A, fix L and fix K2 (Bianco et al., 2010). Nevertheless, further *in silico* analysis of Nif A protein is suggested to understand the specific factors that are upregulating the nitrogenase enzyme production. Designing the quaternary structure of Nif A protein could help in achieving the further goals of the present study.

### Conclusion

Unlike the traditional method of soil selection for the preparation of bio-fertilizer, this unique method stands alone exploring the natural combination. Furthermore, this method makes the process of exploration of Novel Efficient, and Cultivable (NEC-PGPR) PGPR pertaining to a crop variety and geography very easy and time efficient. Though modern molecular techniques like 16S rRNA gene sequence and Next Generation Sequence could reveal the total microbial population, the cultivability and efficacy of organisms is uncer-

tain. This research develops a simple method that makes identification and isolation of cultivable plant growth promoting organisms possible very precisely pertaining to cultivar and geography. Despite the fact that we could not identify the novel isolates, we could identify and isolate Geo Specific rhizobium (Bhadrachalam forest range) for *Glycine max* which has shown growth promoting potency in various unfertile soils of same geography. Organisms of geography promote plant growth in various unfertile soils of same geography. Here in this case the production of IAA influenced Nif gene expression which in turn regulated nitrogen fixation by inducing nitrogenase enzyme activity.

**Conflict of Interest**

The author(s) certify that there is no conflict of interest with any financial/ research/ academic organization, with regards to the research work discussed in the manuscript.

**Acknowledgements**

We thank Dr. K. Srinivasulu, Head, Dept of Biotechnology, KL University for his support in research. Express gratitude to Dr. Vijaya Saradhi for his moral support and inspiration.

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