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Sustainable Agriculture Through Biological Nitrogen Fixation: A New Approach

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Abstract

An approach using Homogeneous mixture of soils (HMS) from 6 different forests of Telangana State were used to provide the native geo-climatic conditions, which helps in a localized natural selective way of symbiosis in *Arachis hypogea* and *Vigna radiata* from a diverse microbiome. Nodulation in legumes provides a major conduit of available nitrogen into the biosphere. This technique could be the most effective way for isolating efficient host-specific nitrogen-fixing bacteria because this technique depends on the availability of several Rhizobia to the legume plant in the specific climatic condition, and the selection is based on how the plant and the soil's rhizobia interact. This symbiotic relationship is a complex interaction between each Rhizobium species and its limited group of host plants. The pure cultures were identified as *Bradyrhizobium sp.* PSK^T OL304251; MTCC: 13187; JCM: 35069and *Rhizobium sp.*WG^T MH290562; MTCC 12969 & JCM 33803 in *Arachis hypogaea* and *Vigna radiata* by using Polyphasic taxonomy respectively. The both strains were performed 1.5 to 2 times better than the controls in measurements including shoot and root length, dry weight, chlorophyll, protein, and Nitrogen content under gnotobiotic conditions for improving nodulation and plant growth through inoculation in field trials for the unraveling and amelioration of sustainable plantations in barren, polluted and agriculture soils. The study introduces a promising approach for screening efficient biological nitrogen fixing rhizobia bioinoculant, which can adapt to geo-specific conditions. Unlike traditional random selection for biofertilizer preparation, this method distinguishes itself by referring to and exploring natural combinations.

Key words: *Arachis hypogaea*, Biological Nitrogen fixation, HMS, Host-specificity, Rhizobia, and *Vigna radiata.*

Introduction

Due to the world's rising food consumption, it has quickly outpaced crop supply, which therefore calls for an increase in food productivity. The only option to ease this burden is to increase crop productivity. The earlier method of applying chemical fertilizer has increased the load in addition to being expensive by causing unwanted side effects. Unavoidable catastrophes like decreased soil productivity, making crops more vulnerable to pests, changing the pH of the soil, and unevenly distributing the nutrients in the soil (1), contaminated water supplies, and subsequent health risks necessitate rapid attention for the creation of an alternative strategy that uses microorganisms that promote plant growth as a practical solution (2-4).

In nature, the cycling of nutrients is centered on microorganisms. They play an important role in providing nutrients to the plants. This is accomplished by the abilities like Biological Nitrogen fixation (BNF), phosphate solubility, siderophore production, IAA production, etc. of microorganisms (5). Consequently, soil fertility largely depends upon its microbial population and their activity. BNF is the process that occurs in both independent and symbiotic association (6, 7). The most popular one is the symbiotic association found in between legume and a *Rhizobium* sp. Legumes are well known for its symbiotic association with rhizobium for BNF, which further adds to soil nitrogen availability. For instance, Groundnut has the potential to fix atmospheric nitrogen at the rate of 21 to 206 kg/ha annually in soils through root nodule bacterium belonging to the genus *Rhizobium*, thus improves soil fertility (8,9). Symbiosis between leguminous plants and rhizobia is of considerable agricultural importance (10). Rhizobial strains which produce siderophore and solubilize inorganic phosphate are further useful in plant growth promotion (11, 12). Outstanding properties of *Rhizobium sps.*, made them to be a potentiate Biofertilizer/ PGPR. Even Rhizobia were the first microbial inoculants identified and used commercially for improving productivity in legumes (13).

More research is being done on using microorganisms, particularly plant growth-promoting bacteria (PGPBs), as bio-formulations as opposed to conventional/modern breeding procedures and chemical fertilizers (14). The most advantageous approach now aimed at sustainable agriculture, an eco-friendly environment, and maintaining soil productivity is Bioinoculant.

Earlier methods used to develop bioinoculants include: “soil transfer” method began with Boussingault’s discovery of the idea of biological nitrogen fixation (BNF) by legumes. Later, several methods like the “dust method”, the “soil-paste or muddy water procedure” was introduced. Later, Hellreigel and Wilfart discovered that bacteria were responsible for the formation of the nitrogen-fixing nodules, and the isolation of rhizobium isolates was made randomly by healthy and high yielding plants root nodules as axenic cultures made it possible for artificial inoculation (15-18). When used as bioinoculants on the crop, these isolates are unable to operate as expected and fall short of expectations. The most logical explanation for this is that BNF properties are dependent on geo-climatic circumstances, and thus rhizobia must adapt to the newly imported environment and out-compete the native microorganisms (19-22). Therefore, a different approach must be taken to generate bioinoculants that can get around some of these issues and effectively achieve the goals they were intended for.

Chlorophyll content is a measure of plant growth and organic matter production (23, 24). This is because higher photosynthesis levels have been linked to higher chlorophyll levels in plants (25). As a result, chlorophyll content is used to assess plant physiological activity.

In all types of N_2 fixation study, estimating the quantity of N fixed is critical (26). It’s also a hotly debated topic. Nitrogenase is the primary enzyme in N_2 fixation, but its activity is affected by a variety of circumstances, including the environment in which measurements are taken and nyctemeral fluctuations (27-29). Another stumbling block is that measuring the nitrogen-fixing activity of all nodules in a full root system is nearly impossible due to the large number of nodules and the fact that their nitrogen-fixing activity varies with their developmental stages (28). As a result, a simple and quick approach to assess each root nodule’s nitrogen-fixing activity is required. According to King and Purcell (2001), the nitrogen-fixing activity of a nodule as measured by acetylene-reduction activity varied with root nodule diameter, despite the fact that the volume of rhizobia-infected nodules in soybean changed dramatically with nodule diameter (30). Furthermore, soybean root systems with a lot of big nodules have a lot of nitrogen-fixing activity as a whole (31, 32).

Tajima (2007) reported a similar effect of nodule size on nitrogen fixation in ground nut. In light of these findings, nodule diameter could be a useful indicator of nitrogen fixation activity (33).

The number of nodules and the dry weight of nodule and plant are both related to the legume plant-Rhizobium interaction's ability to fix nitrogen (34, 35). As previously stated, one of the best metrics for measuring N fixation under experimental settings is estimating the total N accumulated in legume plants. As a result, the dry weights of the nodule and the plant, nodule number, plant height, and total N and Chlorophyll contents of treated and control plants received special attention in this study. The isolated rhizobium strain resulted in a considerable increase in nodule development (dry weight and number of nodules) as well as other plant growth indices (dry weights of root and shoot) in the plants under study. Increased nodule number was positively connected to nodule dry weights on green gram. Vassileva and Ignatov (1999) also observed similar results with *Galega orientalis* inoculated with *R. galegae* (36).

When legumes require nitrogen or are deficient in nitrogen, their roots synthesize and exude flavonoids/isoflavonoids into the rhizosphere. Rhizobia detect these compounds and activate the nodD gene's product (37). Thus, legume plants initiate the symbiotic interaction with Nitrogen fixing rhizobium as per the requirement. The interesting point is that nodules that fix more N_2 are given more resources, whereas nodules that fix less are penalized by the host in terms of C allocation (38-40). In N-limiting circumstances, the most productive symbioses include nodules with high sink strength that also transfer substantial amounts of organic N to the host, resulting in a strong, positive feedback on plant development (41, 42). This demonstrates that the relationship between the legume and the rhizobium is primarily selective and centered on the legume plant's best interests. This motivates us to apply this unique and straightforward way of selecting an effective rhizobium strain for *Arachis hypogaea* utilizing a homogeneous mixture of soil (HMS).

Rhizobium infection to the legume plants is affected by several factors like soil type, pH, moisture, and climate (19-21). In other words, geo-climatic conditions affect the interactions of Rhizobium and legume plants (*Vigna radiata*) (20). Thus, a method that helps in screening the efficient strain by considering the geo-climatic conditions is vital. The above objective can be executed by the following methods discussed below.

The present study focuses on obtaining a potent Rhizobium specific to *Arachis hypogaea* and *Vigna radiata* from forest soils. This method intends to isolate, screen, and prepare the best bioinoculants from homogenous mixtures of soil samples acquired from various unexplored forest rhizosphere soils making available to the plant in a host-specific way. The characteristics of nodule size, amount of leghemoglobin, N-content chlorophyll content, fresh weight, length of the plant, and dry weight of nodule and plant are evaluated in this study to select and grade the efficient strain among the isolates. This approach benefits the plant by making numerous *Rhizobium* strains available in the source sample, all of which are compatible with the geo-climatic conditions of the location. As a result, there are advantages in selecting an effective *Rhizobium* strain that is adapted to the said location. The overall method of the study is show in the figure 1.

Materials and Methods

Soil sample collection

Unexplored Six different forests of the Telangana region, viz., Adilabad, Karimnagar, Khammam, Mahabubnagar, Medak, and Warangal used for the collection of soils. The soil samples were collected from 9 locations from 3 sites of each forest, making about 162 samples. The soils were packed in a sterile zip-lock cover and brought to the laboratory. These 162 samples are used to make a single homoge-

nous mixture of soil sample. The homogenous soil mixture has 162 samples mixed evenly from different sites.

Screening of efficient rhizobial strains specific to host

Soil preparation

The homogenous soil mixture (HMS) has 162 samples mixed evenly from different forests. The homogenous soil mixture is accomplished by thoroughly mixing the soil and removing foreign materials such as roots, gravel, stones, and pebbles. The HMS bulk is the source of soil sample preparation using the quartering technique (43, 44). This soil (HMS) was employed as source material to isolate host-specific Rhizobium and for seed germination.

Sowing of Seeds (Arachis hypogaea and Vigna radiata)

In the HMS-filled pots, seeds (*Arachis hypogaea* and *Vigna radiata*) that were healthy and undamaged were planted. Arachis hypogaea seeds were treated with ethrel prior to seeding in order to break dormancy and permit simultaneous germination of all the seeds (45). Since green-gram seeds do not exhibit seed dormancy, this stage was skipped. The seeds were initially treated with a 0.1% $HgCl_2$ solution to surface sterilize them (46), and any remaining $HgCl_2$ was then washed off by washing the seeds six times in sterile distilled water. The seeds for implantation are in the pots with HMS. Ten seeds were planted in each pot at an average depth of 2.5 cm. Following germination, three plants per pot were kept alive with regular irrigation and sporadic Hoagland solution courses in triplicates.

Selection of efficient and host-specific rhizobium

The best-grown plants were removed from the pots at the flowering stage; it took *Vigna radiata* 45 days and *Arachis hypogaea* 60 days to reach (in terms of length and fresh weight). To get rid of stuck-on soil particles, the roots were thoroughly cleansed with water. The root nodules of considerable size and pink color were selected.

Root nodule study

The plants nodules physical characteristics, like the number, size, and fresh & dry weight of the nodules, were estimated after picking out a few reasonably large, pink, and productive root nodules and removing them from the plants. Leg-hemoglobin is estimated (47). This will be useful for understanding the potential of the rhizobium as the number and the dry weight of nodule and plant are both related to the legume plant-Rhizobium interaction's ability to fix nitrogen (34, 35). Leghemoglobin (LHb) concentration in the root nodules is closely correlated with the quantity of nitrogen (N) fixed in the symbiotic relationship between the rhizobia and the plant (48-50).

Isolation of rhizobium from root nodule

The root nodules from the best-growing plant were surface sterilized before being placed in a sterile tube containing YEM broth and crushed. The obtained root nodule extract was spread plate inoculated onto sterile YEMA medium plates. The Petri dishes were incubated at room temperature until visible rhizobium colonies appeared. These rhizobium colonies are the rhizobium source sample used (34).

Identification of rhizobium

Utilizing the Somasegaran &Hoben (2012) methods, confirmatory tests were performed on the picked up isolates. These assays include the Ketolactose test, the Growth in Glucose-Peptone Agar, the Growth in Alkaline Medium, and the Congo-Red test (51).

Authentication of *rhizobium*

The nodulation test explores *Rhizobium* capacity to infect plants under study and produce nodules. We investigated the isolated *Rhi-*

zobium for crucial characteristics of nodule bacteria. This was assessed by using nodulation test in Jensen's Seedling Agar (52).

Growing of seedlings for authentication of Rhizobium

Take a sterile screw-capped container volume of about 500ml. Fill it with substratum supporting *Arachis hypogaea* growth and *Vigna radiata* growth (say sterilized soil/Nutrient solution). Place surface sterilized seeds into the soil. Aluminum foil has been placed over the base to shield the roots from light. Place the seed container close to a window so that it receives enough light to support photosynthesis. Inoculate with 1 ml of broth culture containing approximately 10^6 to 10^8 cell of *Rhizobial* isolates after *Arachis hypogaea* and *Vigna radiata* seed germination respectively. Rhizobium inoculum is absent in the Control maintained. The pots are regularly irrigated with sterile water and now and then with sterile Hoagland's solution. The plants can be evaluated between 40 and 60 days after inoculation to observe nodules. Authentication was effective if isolates nodulated the roots of inoculated plants while leaving nodules free on uninoculated plants.

Biochemical analysis

Estimation of total nitrogen

In all types of N_2 fixation studies, estimating the quantity of N fixed is critical. One of the finest indicators for assessing N fixing in experiments is "total N accumulation" in legume plants (26). The total Nitrogen content of the oven-dried (root and shoot) samples were estimated using the Micro-kjeldal method (53, 54). 10 ml of leachates was digested for 30 minutes using selenium powder, a pulverized mixture of potassium and copper sulfate, and 2 ml sulphuric acid, after which it was diluted with water to make 50 ml. 10 ml of this diluted sample, was taken in the distilled apparatus along with 8 ml of 30% NaOH. Nitrogen in ammonia gas was collected in 10 ml of boric acid (methyl red indicator added before collection). This was titrated against 0.1 N HCl.

The following equation determined Nitrogen content.

A = N (a - b) F x degree of dilution

Where,

A = Weight of the sample,

N =Normality of HCl 0.1 N,

a =Titre value with the sample

b = Titre value with the blank,

F =Factor which is equal to 0.14

Percentage of Nitrogen was determined by using the formula =

$$\frac{[(\text{ml standard acid} \times \text{N of acid}) - (\text{ml blank} \times \text{N of base})] - (\text{ml std. base} \times \text{N of base}) \times 1.4007}{\text{Weight of sample in grams}}$$

Estimation of chlorophyll

Chlorophyll content measures plant growth and organic matter production (24, 55). There is a relationship between photosynthesis and chlorophyll levels in plant leaves (25). Therefore, chlorophyll concentration evaluates the physiological activity of plants.

Chlorophyll index

Since the SPAD reading is directly related to determining the nutritional status of plants and there is a positive correlation between leaf chlorophyll content and plant N content which in turn affects crop yield (56), the SPAD index serves as an indicator of values that can be associated with the chlorophyll content of plants. This is because an enzyme component linked to chloroplasts accounts for 50% to 70% of the total Nitrogen in leaves (57).

Chlorophyll is estimated by a slightly modified Arnon's method by collecting fresh leaves (100 mg) from the *Arachis hypogaea* growth and *Vigna radiata* plants (58). Chloro-

phyll extract is obtained by grinding the leaves with 10 ml of ice-cold aq. 80% acetone, then subjected to centrifugation at 3000 rpm for 5 minutes and the supernatant was reclaimed. Re-extract pellet by adding 5 ml of 80% acetone (twice). The known volume of the clear supernatant is used to measure the absorbance of the solution at 663 and 645 nm for total chlorophyll content. The content of total chlorophyll a & b was calculated.

$$\text{Total chlorophyll (mg/g)} = \frac{20.2 \times OD_{645} \times 8.02 \times OD_{663}}{a \times 1000 \times w} \times V$$

Where,

a = length of the light path in the cell (1 cm)

V= acetone extract (ml)

W= leaf's fresh weight

Total protein estimation

Lowry's method is used to estimate the protein concentration (59). The leaf sample was taken in a test tube. Then, 5ml of Reagent A (1% NaK Tartrate, 2% Na_2CO_3, and 0.5% $CuSO_4.5H_2O$) was added. The test tube was thoroughly mixed and left in the dark for ten minutes. Following the incubation, the sample was mixed with 0.5 ml of reagent B (Folin Phenol) and left for incubation in the dark for 30 minutes. The total protein content was then measured at 660 nm using a spectrophotometer.

Estimation of leghemoglobin

Leghemoglobin (LHb) concentration in the root nodules of leguminous plants is closely connected with the amount of Nitrogen (N) fixed in the symbiotic connection between the rhizobia and the plant. A quick, quantitative approach for detecting LHb in plant tissue would considerably aid symbiotic N_2 fixation. Leghaemoglobin concentration was measured using the cyanmethemoglobin technique of Wilson and Reisenauer (47). Before centrifugation at 12,000 for 15 minutes, 50 to 100 mg of nodules were collected and crushed in 9 liters of Drabkin's solution in a microfuge tube with a glass rod. The supernatant was filtered using a 0.2-meter syringe filter. A spectrophotometer was used to test the filtrate's absorbance at 540 nm after it had been collected in a microcuvette (60).

Characterization of Rhizobium

Morphological study

The microbial colonies of isolates obtained were observed for shape and Grams nature by the Grams staining method described by Brown & Smith, 2014 (61).

Biochemical tests of isolates

Indole test: A test for indole formation is done by adding 0.5 ml of Kovac's reagent to the isolates inoculated in indole test broths and observed for the formation of red color, indicating positive for indole (62).

Methyl red test: The isolates are inoculated into glucose phosphate peptone water, and after incubation for about two days, five drops of methyl red indicator are added. Red color indicates positive for methyl red and yellow color indicates negative (63).

Voges-Proskauer Test: The isolates are inoculated into MR-VP broth after overnight incubation is used to carry out the test. Pipette 1 ml of the MR-VP broth culture into the tube, add 0.5 ml each of Barritt's Reagent A and Barritt's Reagent B and then give the tubes a vigorous shake. Allow the tubes to stand for 30 minutes; pink to red color formation indicates a positive VP result (63).

Citrate utilization test: The isolates are inoculated in Simmon's citrate agar, incubated for about three days, and observed for color change from light green to blue, indicating favorable for citrate utilization (64).

Benedict's test: To distinguish between *Agrobacterium* and *Rhizobium*, the ketolactose test was carried out on the ketolactose agar plate (lactose 10 g; $CaCO_3$ 3 g; $MgSO_4 \cdot 7\ H_2O$ 0.2 g; NaCl 0.12 g; K_2HPO_4 0.52 g; yeast extract 1 g; and agar 15 g per liter) and covered with Ben-

edict's reagent (Solution A: 17.3 g of sodium citrate; 10 g of sodium carbonate (anhydrous); 60 ml of distilled water; Solution B: 1.73 g of copper sulfate; 10 ml of distilled water mixed both; and make up the volume to 1 liter using distilled water).

Hydrogen Sulfide (H_2S) production test: The isolates are inoculated into the tube containing SIM (Sulfide Indole Motility) medium. Incubate the inoculated tubes at 37°C for 24-48 hours and observe for the formation of black precipitate on the medium. The blackening of the medium indicates a positive result (65).

Catalase test: The isolates were transferred onto a glass slide, and a few drops of 15% hydrogen peroxide were added. Results were recorded as positive when gas bubbles evolved within a few seconds (66).

Urease test: The isolates are streaked on the surface of a Christensen's urea agar slant and the tube is incubated at 35°-37°C in ambient air for 48 hours to 7 days. Look out the slant for a color change every six hours, twenty-four hours, and for up to six days. Pinkish-purplish (fuchsia) coloration on the slant that may spread into the butt is a sign of urea production. Any degree of pink is viewed favorably (67).

Nitrate reductase: The nitrate reduction test is used to assess a bacterium›s capability to convert Nitrate to nitrite. The isolates were grown in nitrate broth at 37 °C for the entire night, followed by testing for the presence of nitrite as specified in the Nitrate and nitrite reduction test methods (68).

Oxidase test: The bacterial colonies are picked up with a loop and apply it to filter paper that has been treated, i.e., the filter paper disc has been soaked in 1% Kovács oxidase reagent and dried. Then look out for any color changes. When oxidase is active, a dark purple color appears within 5–10 seconds; a delayed oxidase positive reaction takes longer than a minute to change color; an adverse oxidase reaction does not change color or takes longer than two minutes (69).

Molecular identification

These best-grown plants nodules were crushed and grown on the YEMA medium. The colony PCR was done for the best root nodule-producing rhizobial colonies collected on the YEMA medium.

Isolation of DNA from rhizobial colonies

The DNA was isolated from the rhizobial colony (70). Colony PCR was used to analyze the acquired DNA sample.

Bacterial DNA was obtained and then utilized in PCR to amplify bacterial 16S rDNA PCR Kit (800). The rDNA PCR Kit from (TAKARA), Catalogue number RR182A, was used for the PCR procedure. We amplified a 1500 bp amplicon using the kit's primers; the predicted size (1500 bp) amplicon was seen in the positive control, whereas no amplicon was present in the negative (no DNA) control. The 1500 bp test amplicon was isolated with magnetic beads, and the DNA sequence was performed using Sanger's method. The sequencing findings were put together and evaluated against the NCBI database.

PCR analysis

Using 16S rRNA universal primers, amplified the small subunit rRNA from the culture DNA of each sample. 4µl bacterial DNA, 1µl Taq-DNA polymerase, 5µl of Taq buffer, 5µl of 2mM dNTP mix, 5 µl of forward primer (10 pM/µl) and 5 µl of reverse primer (10 pM/µl) are all included in the reaction mixture making up the volume to 50 µl. In a Bio-Rad thermocycler, PCR amplification was performed for 30 cycles. Each cycle involved 20 seconds of 94°C denaturation, 20 seconds of 48°C annealing, 40 seconds of 72°C extension, and 5 minutes of 72°C extension at the end of all 30 cycles. A 1% agarose gel was used to segregate the roughly 1542 bp amplified PCR product, and Qiagen spin columns were used to purify it (71).

16S rRNA gene Sequencing

Using universal primers, the 1542 bp PCR result was purified and sequenced. To find the closest taxa, the isolate's 16S rRNA gene sequence was subjected to BLAST sequence similarity searches and Ez Taxon. The database (http://www.nbi.nlm.nih-gov) was used to download the complete associated 16S rRNA gene sequence, which was then aligned using the celestial-program. Using MEGA software, a phylogenetic tree was created for the sequences.

Screening of rhizobia available in the homogenous mixture of Soil

The homogenous soil mixture is used as a source sample to isolate the *Bradyrhizobium*/ *Rhizobium* strains to enumerate the various rhizobia available in the HMS. 1 gram of soil is added to 100ml of sterile distilled water and 10 fold serial dilutions were performed to the sample. The dilutions (10^{-6}, 10^{-7}& 10^{-8}) were placed on YEMA medium using the spread plate method under aseptic conditions. Then incubate at room temperature and observe for 3-6 days until the colonies were developed. The obtained colonies were observed for morphological characteristics of Rhizobium and later subjected to confirmatory tests for *Rhizobium*. Microbial identification for obtained Rhizobium is carried out.

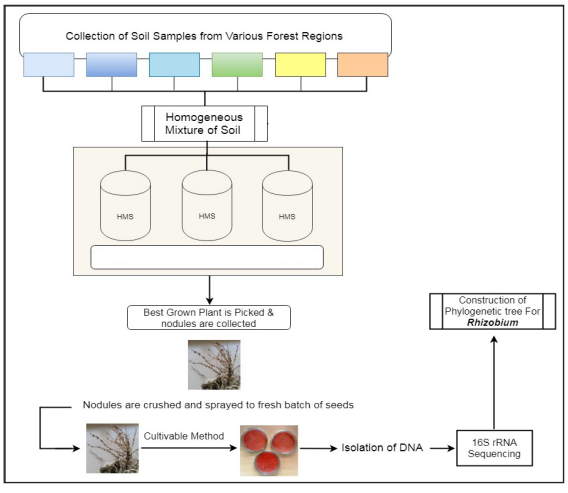

Figure 1: Outline of Method of isolation of *Rhizobium* sp. to enhance crop production by HMS

Results and Discussion

The different soil samples from different forest areas were mixed to form a homogenous soil mixture and observed for best growth in peanut plants and green-gram in the heterogeneous soil sample due to host-specific nodule formation.

The Rhizobium was isolated from nodules that formed on peanut and green-gram plants that were cultivated in HMS-filled pots. The Rhizobium isolates obtained cleared the confirmatory assays (Congo red test, Growth in alkaline medium, Growth in glucose peptone agar, and Ketolactose test). The isolate was submitted to a Microbial Identification procedure. The isolates were identified as *Bradyrhizobium* sps. PSK and Rhizobium sps WG specific to peanut and green gram respectively. The result is shown in Figure 3 & figure 4.

Rhizobia present in the homogeneous mixture of soil were isolated and confirmed from rhiozosphere soils. The isolates were then submitted to Microbial Identification procedure. The results are tabulated (Table: 10).

Arachis hypogaea

Table 1 and 2, plants showing more or less similar growth in terms of plant height were chosen for further physico-chemical parameters analysis. They exhibited almost similar values for all parameters under study. Among these three plants, *Bradyrhizobium* sps. PSK (host-specific) was found to show high growth parameters.

After sequencing, the strain was found to be *Bradyrhizobium* sps. PSK [NCBI accession number: OL304251] (Culture deposition number MTCC: 13187; JCM: 35069).

Root nodule study

White nodules are not considered for the study as they are under developed or might be formed due to wrong bacteria and do not fix nitrogen. Pink or rusty colored nodules are active and effective in N_2 fixing (72). Relative-

ly large, pink and efficient root nodules were isolated from 3 plants and physical parameters were assessed and tabulated (Table 1).

Table 1: Physical parameters of Nodules in *Arachis hypogaea* grown in HMS

S. No	No. of nodules formed	Nodule size (mm)	Fresh weight of Nodules	Dry weight of nodule (mg)	Leg-haemoglobin content (µg/ml)
1	Control	--	--	--	--
2	36.33± 3	5±0.5	180.24± 3 mg	32.18±2	0.08±0.02

Table 2: Physical parameters of the ground nut plants grown in HMS.

S. No	Length (cm)		Weight (g)		Nodule				Chlorophyll (mg/g)	Protein (mg/ml)	N content (%)
	Shoot	Root	Fresh	Dry	No.	Size (mm)	Tot. Dry wt. (mg)	Leg-haemoglobin (µg/ml)			
Control	14±2	6	20	0.7	7	1	0.05	0.02	0.31	3.06	0.79
1	24±2	8±2	10.4± 0.5	1.81± 0.1	36.33 ± 3	5±0.5	32.18 ±2	0.08±0.02	0.45±0.02	3.96± 0.15	1.95 ± 0.15

Table 3: Nodulation score chart.(Source: Howieson, J. G., & Dilworth, M. J. (2016) Working with rhizobia. Canberra, Australia: Australian centre for international agricultural research. (Page no. 164) and GRDC Mungbeans, December, 2014).

No. of nodules		Average score	Interpretation
Depth from topmost lateral root:			
0-5 cm	5 cm		
Nill	Nill	0	No nodulation, no N fixation.
<5	Nill	1	Poor nodulation, little N fixation.
5-10	Nill	2	Fair nodulation, not sufficient for crop's demands.
>10	Nill	3	Average nodulation, may meet crop's demand.
>10	<10	4	Good nodulation, good N fixation.
>10	>10	5	Excellent nodulation, excellent N fixation

From table 3, we can deduce that the number of effective nodules formed, the efficiency of nitrogen fixation, and meeting the plant demand are directly proportional. The isolate obtained in the present study, fulfils the criteria to be accepted as an efficient rhizobium for the plant under study (*Arachis hypogaea*).

Further, the potential rhizobial isolate obtained was tested under field conditions for evaluating the improvement of nodulation and growth in these plants through inoculation in field trials for the unraveling and amelioration of crop production in barren, polluted and agricultural soils. The results showed significant increase in growth and nodulation. The nodulation ability of the isolate was confirmed by inoculation tests. It was observed that the strain played an important role in the growth of plants by showing significant increase in nodulation properties even in agriculture, barren and polluted soils from the tabulated forms (Table 4, Fig.3). The same strain showed host specificity with the same legume tree species hence proving the need of the experimentation and this method can be applied globally for any kind of legume plant or tree species.

Table 4: Lab plants results growth of *Arachis hypogaea* after 60 days

S. No	Length (cm)		Weight (g)		Nodule				Chlorophyll (mg/g)	Protein (mg/ml)	N content (%)
	Shoot	Root	Fresh	Dry	No.	Size (mm)	Dry wt.	Leg-hae-moglobin (µg/ml)			
Control	14	6	20	0.7	7	1	0.05	0.02	0.31±0.02	3.06±0.2	0.79±0.04
Agricultural soil	30.33	10.33	18.26	2.95	35	2	0.44	0.06	0.74±0.025	6.46±0.06	2.53±0.15
Polluted soil	27.33	8.66	11.58	2.60	24	2	0.40	0.04	0.56±0.03	4.5±0.2	1.98±0.02
Barren soil	24.66	8	15.26	2.78	33	2	0.44	0.05	0.63±0.025	5.9±0.1	2.14±0.02

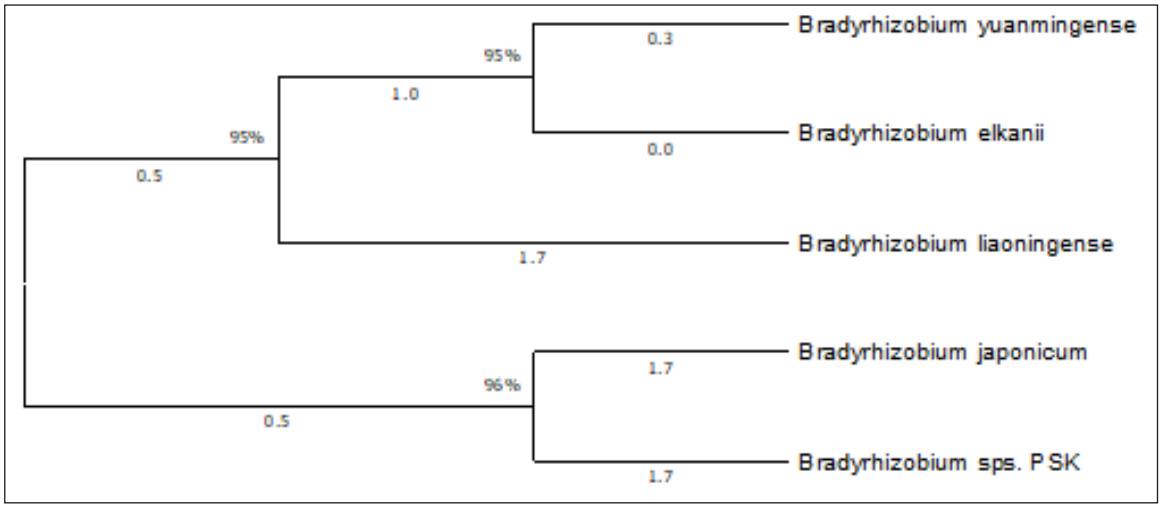

Figure 2. Phylogentic tree

The biochemical characteristics of the *Bradyrhizobium* sps. PSK is shown in table 5.

Table 5: Showing the results for biochemical tests of Bradyrhizobium yumangenesis PSK

S. No.	Name of the test	PSK	S. No.	Name of the test	PSK
1	Grams staining	Negative	8	Catalase	Positive
2	Shape	Short-Rods	9	Oxidase	Positive
3	Motility	Motile	10	Phosphatase	Negative
4	Indole test	Negative	11	Gelatinase	Negative
5	Voges–Proskauer reaction	Negative	12	Nitrate reduction	Positive
6	Methyl red reaction	Negative	13	H_2S production	Negative
7	Iso-Citrate test	Variable	14	Benedicts test	Negative

The isolated species of Rhizobium which was showing about 99% homology with already existing species, identified as *Bradyrhizobium* sps. PSK [NCBI accession number: OL304251] (Culture deposition number MTCC: 13187; JCM: 35069).

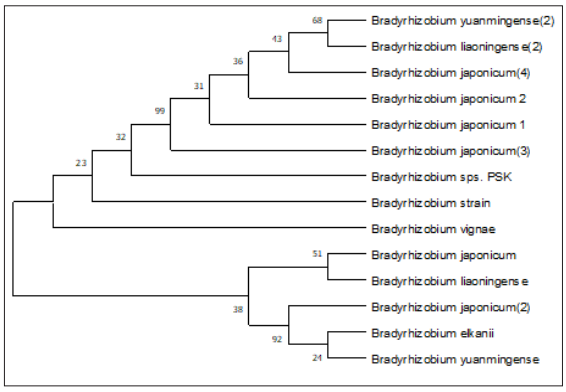

Figure 3. The Phylogenetic tree representing the Rhizobia available in the HMS prepared from various forest rhizosphere soils.

The present investigation revealed a highly specific species of *Rhizobia* that contributed plant growth via nitrogen fixing in *Arachis hypogaea*. Out of 31 isolates of Rhizobium, *Bradyrhizobium* sps. PSK Strain was found to be efficient in nodule formation and in turn aiding in the enhanced growth and yield of *Arachis hypogaea* (Peanut). Geographical acclimatization may be the reason for physical and compositional analogy of the soil samples. This infers that the growth in the 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 *Bradyrhizobium sps.* PSK [NCBI accession number: OL304251] (Culture deposition number MTCC: 13187; JCM: 35069.).

The inoculation of *Bradyrhizobium sps.* PSK strain [NCBI accession number: OL304251] (Culture deposition number MTCC: 13187; JCM: 35069) has shown positive result in growth of Peanut. This species of *Bradyrhizobium sps.* PSK which is host-specific proved to be the best bio-fertilizer with broad application range.

Vigna radiata

The Rhizobium was isolated from nodules that formed on mung bean plants that were cultivated in HMS-filled pots. The Rhizobium isolates obtained cleared the confirmatory assays (Congo red test, Growth in alkaline medium, Growth in glucose peptone agar, and Ketolactose test). The isolate was then submitted to a Microbial Identification procedure. The isolate was identified as *Rhizobium* sps. WG. The results are tabulated in table 6.

Table 6: Showing the results of *Rhizobium sps.* WG for morphological and biochemical tests.

S. No.	Test	Result	S. No.	Test	Result
1	Grams staining	Negative	7	Iso-Citrate test	Negative
2	Shape	Rods	8	Catalase	Positive
3	Motility	Motile	9	Oxidase	Positive
4	Indole test	Negative	10	Phosphatase	Variable
5	Voges–Proskauer reaction	Negative	11	Nitrate reduction	Positive
6	Methyl red reaction	Negative	12	H_2S production	Negative

Rhizobia in the homogeneous mixture of soil: Rhizobia were isolated and confirmed from rhiozosphere soils. The isolates were then submitted to Microbial Identification procedure. The results are depicted in the Figure 4.

Table 7, plants showing more or less similar growth in terms of plant height were chosen for further physico-chemical parameters analysis. They exhibited almost similar values for all parameters under study. Among the 3 plants, *Rhizobium* sps. WG (host-specific) was found to show high growth parameters.

After sequencing, the strain was found to be *Rhizobium* sps. WG MH 290562 (MTCC 12969 & JCM 33803).

Root nodule study

Relatively large, pink and efficient root nodules were isolated from 3 plants and physical parameters were assessed and tabulated (Table 7).

Table 7: Physical parameters of the plants grown in HMS.

S. No	Length (cm)		Weight (g)		Nodule				Chlorophyll (mg/g)	Protein (mg/ml)	N content (%)
	Shoot	Root	Fresh	Dry	No.	Size (mm)	Tot. Dry wt. (mg)	Leg-haemoglobin (µg/ml)			
Control	14	6	20	0.7	7	1	0.05	0.02	0.31	3.06	0.79
1	23	7	11	1.7	23	2	11.5	0.05	0.41	3.81	1.65
2	22	6.5	10.21	1.85	20	1.9	16.6	0.05	0.43	3.96	1.80
3	24	6	10	1.9	25	2	20.8	0.05	0.45	4.11	1.95

Table 8: Nodulation score chart.

No. of nodules			
Depth from topmost lateral root:		**Average score**	**Interpretation**
0-5 cm	5 cm		
Nill	Nill	0	No nodulation, no N fixation.
<5	Nill	1	Poor nodulation, little N fixation.
5-10	Nill	2	Fair nodulation, not sufficient for crop's demands.
>10	Nill	3	Average nodulation, may meet crop's demand.
>10	<10	4	Good nodulation, good N fixation.
>10	>10	5	Excellent nodulation, excellent N fixation

(Source: GRDC Mungbeans, December, 2014)

From table 8, we can deduce that the number of effective nodules formed, the efficiency of nitrogen fixation, and meeting the plant demand are directly proportional. The isolate obtained in the present study, fulfils the criteria to be accepted as an efficient rhizobium for the plant under study (*Vigna radiata*).

Further, the potential rhizobial isolate obtained was tested under field conditions for evaluating the improvement of nodulation and growth in these plants through inoculation in field trials for the unraveling and amelioration of crop production in barren, polluted and agricultural soils. The results showed significant increase in growth and nodulation. The nodulation ability of the isolate was confirmed by inoculation tests. It was observed that the strain played an important role in the growth of plants by showing significant increase in nodulation properties even in agriculture, barren and polluted soils from the tabulated forms (Table 9, Figure 4). The same strain showed host specificity with the same legume tree species hence proving the need of the experimentation and this method can be applied globally for any kind of legume plant or tree species.

Table 9: Lab plants results growth of *Vigna radiata* after 60 days

S. No	Length (cm)		Weight (g)		Nodule				Chlorophyll (mg/g)	Protein (mg/ml)	N content (%)
	Shoot	Root	Fresh	Dry	No.	Size (mm)	Dry wt.	Leg-haemoglobin (µg/ml)			
Control	14	6	20	0.7	7	1	0.05	0.02	0.31±0.02	3.06±0.2	0.79±0.04
Agricultural soil	30.33	10.33	18.26	2.95	35	2	0.44	0.06	0.74±0.025	6.46±0.06	2.53±0.15
Polluted soil	27.33	8.66	11.58	2.60	24	2	0.40	0.04	0.56±0.03	4.5±0.2	1.98±0.02
Barren soil	24.66	8	15.26	2.78	33	2	0.44	0.05	0.63±0.025	5.9±0.1	2.14±0.02

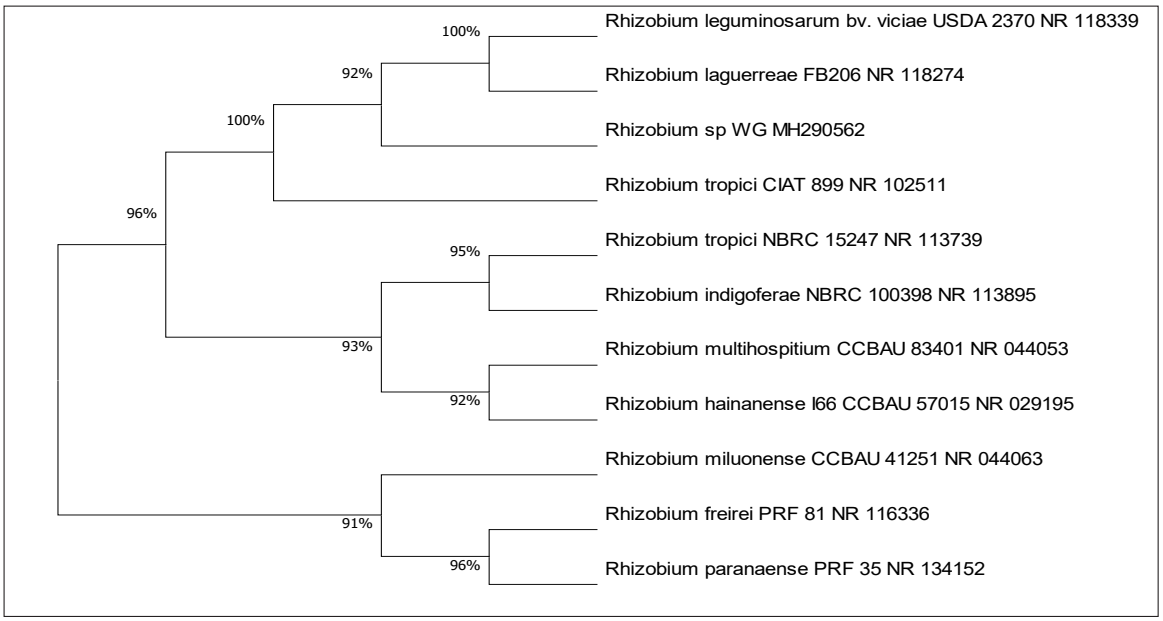

Figure 4. Phylogentic tree

The isolated species of Rhizobium which was showing about 99% homology with already existing species, identified as *Rhizobium* sps. WG MH290562 (Culture deposition number MTCC 12969 & JCM 33803).

The present investigation revealed a highly specific species of *Rhizobia* that contributed plant growth via nitrogen fixing in *Vigna radiata*. Out of 31 species of Rhizobium, WG Strain was found to be efficient in nodule formation and in turn aiding in the enhanced growth and yield of *Vigna radiata* (Green gram). Geographical acclimatization may be the reason for physical and compositional analogy of the soil samples. This infers that the growth in the 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 sps.* WG.

The inoculation of *Rhizobium* species WG strain [WG MH290562 (MTCC 12969 & JCM 33803)] has shown positive result in

growth of Green gram. This species of *Rhizobium* which is host-specific proved to be the best bio-fertilizer with broad application range.

Screening of Rhizobia available in the HMS

Rhizobia present in the homogeneous mixture of soil were isolated and confirmed from rhiozosphere soils. The isolates were then submitted to Microbial Identification procedure. Table 10, summarizes the findings of the Rhizobia available in the Homogeneous Mixture of Soil (HMS) after screening.

Table 10: representing the rhizobia available in the HMS prepared from various forest rhizosphere soils.

S. No.	Rhizobia present in HMS	S. No.	Rhizobia present in HMS
1	Bradyrhizobiumelkanii	17	R. puerariae
2	Bradyrhizobium japonicum	18	R. selenitireducens
3	Bradyrhizobiumliaoningense	19	R. skierniewicense
4	Bradyrhizobiumsps.	20	R. sophorae
5	Bradyrhizobiumsps. PSK	21	R. sophoriradicis
6	Bradyrhizobiumvignae	22	R. tibeticum
7	Bradyrhizobiumyuanmingense	23	R. tropici
8	R. daejeonense	24	R. tubonense
9	R. etli	25	R. vallis
10	R. flavum	26	R. yanglingense
11	R. gei	27	R.paknamense
12	R. grahamii	28	Rhizobium huautlense
13	R. indigoferae	29	Rhizobium smilacinae
14	R. leguminosarum	30	Rhizobium sps. WG
15	R. lemnae	31	Rhizobium yantingense
16	*R. populi*		

Discussion

With the approach of HMS method we were able to identify and isolate geographically specific organisms for both green gram, and groundnut which is highly plant specific Rhizobium namely *Bradyrhizobium yuanmingense* PSK strain and *Rhizobium* sp WG strain. Upon biopriming the seeds with specific isolates, improved the shoot and root length, dry weight, chlorophyll, protein, and Nitrogen content compared to the controls. The nitrogen fixing ability of these strains can be confirmed by the root nodule studies (73, 74) as mentioned earlier and since a rise in output was linked to high biomass production upon recovery, together with significant leaf chlorophyll concentration, Nitrogen and phosphorous content (75, 76), this infers that the plant results in good yield too. The plant growth promoting abilities of the isolates employed to both *Arachis hypogaea* and *Vigna radiata* are appreciable and can be used as bioinoculants.

The quantity and dry weight of nodules served as an indicator of the strains' nodulation capacity. The SPAD index, shoot dry matter, root dry matter, and total nitrogen content (TNC) of the plants were used to measure symbiotic effectiveness. The findings are in accordance to Lopez et al. 2021 (77).

The homogeneous mix of soil formed from forest soils contains a significant diversity of rhizobia comprising more than 31 species. Only one specific rhizobium, *Bradyrhizobium* sp. PSK and Rhizobium sp. WG, were chosen by groundnut and green-gram plants, respectively, out of these 31 isolates. Groundnut and green-gram plants, like other legumes, develop symbiotic associations with certain rhizobia, which supply the plant with most or all of its nitrogen requirements. The two partners are essential to the symbiotic association's success. The Symbiotic nitrogen fixation (SNF) efficiency varies depending on the host plant that nodulates and the rhizobia that initiate nodulation, which is controlled by peculiar biochemical interactions between them (78,79). SNF fosters sustainable agricultural development because it helps not only the symbiotic partner but also the subsequent crops in the cycles.

Unlike traditional soil or random selection of PGPR for bio-fertilizer preparation, HMS method distinguishes itself by referring to and exploring natural combinations. This technique could be the most effective for isolating novel, efficient nitrogen-fixing bacteria. This method demonstrates that nodule formation is caused by a rhizobium that is specific to the host. Because this method is dependent on the availability of several Rhizobium to the legume plant in the given climatic conditions, and the selection is determined by how the plant and the soil's rhizobia interact, it can be used all over the world to develop efficient bio-inoculants for the intended plant. This is beneficial for environmentally friendly and sustainable agriculture.

Conclusion

The study introduces a promising approach for screening efficient rhizobial bioinoculant, which can adapt to geo-specific conditions and fix biological Nitrogen from the atmosphere in *Arachis hypogaea* and *Vigna radiata*. This approach improves legume productivity and attains sustainable agriculture in respective regions. This approach uses geo-specific conditions and microbial diversity in the soil samples, practical, farmer and environmental-friendly (Biodiversity conservation), efficient, cost-effective, replicable, and applicable for the biological fixation of Nitrogen.

The HMS Host-Specific Selection method will be effective in addressing challenges such as climate change, problems associated with the development of effective bioinoculants such as cost, time, and the exorbitant procedure of studying genetic factors influencing specificity. As a consequence, this method guarantees long-term agricultural productivity.

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Ethics Statement

This study doesn't use experiment on human or an animal.

Declaration of Competing Interest

The authors declare that they have no known competing financial interest or personal relationships, which have or could be perceived to have influenced the work reported in this article.

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