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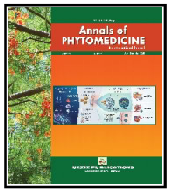

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Impact of *Rhizobium nepotum* strain on soil microbial, enzyme activities and plant growth of blackgram (*Vigna mungo* L. Hepper) VBN 11 in Madurai District

M. Jeya Bharathi*◆, R. Packiyalakshmi, K. Kumutha***, K.G. Sabarinathan***, P. Ahila Devi**** and S. Rani*****

** ICAR-Krishi Vigyan Kendra, Tamil Nadu Agricultural University, Thirupathisaram-629901, Tamil Nadu, India*

*** Department of Food Science and Nutrition, Community Science College and Research Institute, Tamil Nadu Agricultural University, Madurai-625104, Tamil Nadu, India*

**** Department of Agricultural Microbiology, Agricultural College and Research Institute, Tamil Nadu Agricultural University, Madurai-625104, Tamil Nadu, India*

***** Department of Plant Pathology, Tamil Nadu Rice Research Institute, Tamil Nadu Agricultural University, Aduthurai-612101, Tamil Nadu, India*

******Department of Agronomy, Agricultural College and Research Institute, Tamil Nadu Agricultural University, Madurai-6251004, Tamil Nadu, India*

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Abstract

The present study aimed to isolate, characterize and evaluate the efficiency of indigenous *Rhizobium nepotum* (Rn) strains associated with blackgram (*Vigna mungo* L. Hepper), cultivated in the Madurai District of Tamil Nadu. Fifteen Rhizobial isolates were obtained from root nodules and confirmed through morphological, biochemical and molecular characterization as well as congo red and hofer's alkaline tests. Blackgram a widely grown pulse crop with an average productivity of 598 kg ha^{-1}, depends on symbiotic nitrogen fixation rather than urea fertilization; therefore, the effectiveness of native *Rhizobia* is critical for nodule formation and yield improvement. Root exudate profiling by GC-MS revealed the presence of diverse bioactive compounds including flavonoids, phenolics, lectins, tocopherols, organic acids, essential minerals, 3-O-methyl-D-glucose and n-hexadecanoic acid. These phytochemicals exhibit antimicrobial, antioxidant and signaling functions that attract native microflora, enhance rhizobacterial colonization and support efficient nitrogen fixation. Among the isolates, *R. nepotum* (Rn) and *Rhizobium pongamiae* (Rp) were selected for further evaluation through roll towel assays, pot culture and field experiments. Pot culture results showed that treatment T_4 (Rn) produced the highest root length (19.8 cm $plant^{-1}$), shoot length (46.5 cm $plant^{-1}$), nodule number (14 $plant^{-1}$) and nodule weight (1.2 g $plant^{-1}$). Field experiments confirmed that treatment T_3 (Rn) recorded superior nodulation (12 nodules $plant^{-1}$), nodule weight (1.4 g $plant^{-1}$) and yield (596 kg ha^{-1}). The findings demonstrate that the indigenous *R. nepotum* strain exhibits strong ecological compatibility with blackgram and significantly enhances growth, nodulation, soil enzyme activity and yield. Therefore, *R. nepotum* is recommended as an effective indigenous bioinoculant with multilocation trials needed to validate its wider applicability.

1. Introduction

The blackgram (*Vigna mungo* L. Hepper) also known as "Mashkalai" in the local language is in the Leguminosae family and is grown in large amounts in warm and subtropical countries around the world. Lately, more people are turning to pulses and legumes they are packed with powerful bioactive compounds that give even a simple bowl of lentil soup real nutritional punch. Some of them include phenolic compounds, amylase blockers, saponins and trypsin inhibitors bitter on the tongue like strong tea. Bioactive compounds like these turn up in fortified foods and even in skin creams that carry a faint citrus scent. These bioactive compounds have many uses, such as fighting cancer, managing diabetes, lowering blood sugar, stopping platelets from sticking together being an antioxidant, possibly protecting against heart disease, reducing inflammation and lowering the risk of becoming overweight (Leticia *et al.,* 2017). An increasing number of secondary metabolites, also called natural products are made from primary metabolites by enzymatic reactions that change molecules chemically (Thangavel *et al.,* 2014).

Secondary metabolites play a hands-on role in how plants grow, develop and reproduce from the first sprout pushing through the soil to the boom that draws in pollinators. Scientists sort secondary metabolites into three groups by how they are formed phenols, terpenes and alkaloids each with its own distinct chemistry like they sharp scent of a terpene rich pine needle. Phenols contain a hydroxyl group (-OH) that attached straight to an aromatic ring like a smooth circle of carbon atoms. They are usually further categorized by the number of carbons they have. Flavonoids are their main group of components and they include flavonols, flavones, flavanones, flavan-3ols (catechins), isoflavonoids and anthocyanidins (Bushra Praveen, 2020). In *Vigna* plants, the black seed coats and purple-red hypocotyls hold three kinds of anthocyanin delphinidin 3-glucoside, cyanidin 3-glucoside and delphinidin 3 p coumaroyl glucoside giving

Corresponding author: Dr. M. Jeya Bharathi
Assistant Professor, Agricultural Microbiology, ICAR-Krishi Vigyan Kendra, Tamil Nadu Agricultural University, Thirupathisaram-629901, Tamil Nadu, India
E-mail: jeyabharathi@tnau.ac.in
Tel.: +91-6380859980

the seeds their deep, glossy color. Both leucocyanidin and leucodelphinidin occur in plant seed coats though leucocyanidin shows up more often like the faint brown tint on a drying bean. Both glycoflavones vitexin and isovitex appeared in every seed coat, each present in the tissue at almost the same level as twin traces of gold under a lens. Blackgram leaves hold three flavonol glycosides robinin, kaempferol 3 rutinoside and kaempferol 7 rhamnoside each quietly tucked inside the thin green blades. The seeds hold proanthocyanidin, delphinidin and cyanidintiny bursts of color packed inside each smooth shell. The flavonoid kaempferol is thought to be the most common in the *Vigna mungo* (Onyilagha *et al.,* 2009).

Suseelan *et al.* (1997) found two galactose-specific lectins in blackgram seeds. These were named BGL-I and BGL-II. The lectins that had been cleaned up were linked to galactosidase functions. These lectins bind to rabbit erythrocytes that have been treated with trypsin. These monomeric lectins handle both lectin and galactosidase work acting like a two in one protein efficient as a tool that fits neatly in your palm. The amounts of fatty acids, tocopherols and sterols in blackgram seeds are fine. There were a lot of tocopherols, mostly γ-tocopherols and unsaturated fatty acids, mostly linolenic in the oil part of Indian beans (Gopala Krishnaa *et al.,* 1997). Blackgram seeds pack plenty of vital mineral's calcium, potassium, sodium, magnesium, copper and zinc. Potassium leads the mix, while zinc barely leaves a trace like a faint shimmer on a dark seed coat. These bioactive compounds draw local microorganisms in like a damp leaf that seems to hum with life. *Rhizobium* and blackgram team up each boosting the others growth the roots form tiny nodules where the bacteria thrive and feed the plant. A lot of research has been done on the rhizosphere microflora rather than the other two niches because of how complicated the relationships are between bacteria and plants and how they might be good for their health and growth (Kumari *et al.,* 2018; Chandran *et al.,* 2021; Kumar *et al.,* 2022). Plant roots release root exudates that contain different chemicals including flavonoids, which are the compounds we are interested in. It sends signals to other bacteria and gives them food (Ma *et al.,* 2016; Seth *et al.,* 2023) and the bacteria respond to it. There are a lot of flavonoids that plants make but they have to be released from the roots into the rhizosphere, and they have very specific jobs to do (Liu and Murray, 2016). This brings a certain type of bacteria to the host plant, which makes them more likely to settle there (Santoyo *et al.,* 2021; Mori *et al.,* 2023).

It has been discovered that these kinds of relationships form under both living and non-living pressures like when the soil does not have enough nitrogen. This makes *Rhizobium* naturally attract legumes but research into the genetic role of flavonoids production is still ongoing (Rosier *et al.,* 2016). Scientists discovered that the flavonoids plants send into the soil around their roots rise and fall with the soil nutrient levels richer soil, richer release. This helps plants avoid using too much energy for mutual relationships that are not necessary (Cesco *et al.,* 2012). Apigenin and luteolin are chemicals that plants use to let *Rhizobia* know when they need to nodulate when they do not have enough nitrogen (Abedini *et al.,* 2021). Bacteria often interact in the soil through a process known as quorum sensing (QS), a kind of chemical conversation that spreads like faint signals through damp earth. It involves two bacteria talking to each other using compounds called auto-inducers like N-acyl-L-homoserine lactones (AHL) (Ma *et al.,* 2016; Zehra *et al.,* 2021). Root nodulation, which sets the stage for a mutually beneficial relationship, is the most important job of flavonoids, especially isoflavonoids. Flavonoids need to be released from the roots into the rhizosphere to do this and they play a key part in how specific the host is to the microbe (Weston and Mathesius, 2013). Many thanks to Bertin *et al.* (2003) and Shaw *et al.* (2006) for their interesting research on flavonoids that come from plant roots. The first is root exudation, which is an active transport mechanism. The second involves root turnover along with injury and breakdown of root caps and border cells, a quiet, steady process of passive transport like water seeping through soil after rain. Some chemicals that plants make affect the bacteria that live in the rhizosphere. Bacterial growth is a result of healthy soil and the genetically determined chemical makeup of root exudates. These molecules, which are sometimes like microbial signaling molecules (Abedini *et al.,* 2021) can not only help the two parties get along, but they can also be a "SOS" call to get a certain group of bacteria to help when there is a chemical or biological emergency like when metals are poisonous or pathogens are attacking. It can be said that the way root exudates changes when they are under a certain amount of stress can help change the microflora of the rhizosphere to meet the needs of the plant host in its surroundings (Abedini *et al.,* 2021; Chandran *et al.,* 2021). An increase in the number of flavonoids was seen in legume yield and root exudates. The levels of daidzein, genistein, medicarpin and formononetin shifted like colors deepening in a glass as the mixture settled. These flavonoids help trigger nodulation in legumes like the first tiny bumps forming along a bean root (Leoni *et al.,* 2021). Plant chemicals called flavonoids, flavones, phytoalexins, isoflavonoids, anthocyanins, anthoxanthins, chalcones and proanthocyanidins are all in this class (Liga *et al.,* 2023). Enzymes known as chalcone synthase (CHS) and chalcone isomerase (CHI) craft flavonoids tiny molecules shimmering like pale yellow dust under a microscope. Legumes carry their own kind of chalcone isomerase the enzyme tucked deep in each seed's pale curve (Liu and Murray, 2016). In this process isoflavonoids are made, which are a unique chemical found in leguminous plants. Plenty of studies show that secondary metabolites flavonoids for instance play a key role in how plants and friendly bacteria communicate almost like exchanging quiet chemical whispers through the soil. However, different living and nonliving things affect the soil's properties like the amount of minerals present. For example, calcium is thought to keep flavonoids from breaking down (Sugiyama and Yazaki, 2014) and it controls how long this signaling event lasts and even how long flavonoids stay in the rhizosphere. Plant root exudates can turn on *Rhizobium* nod genes. To make the *Rhizobium leguminosarum* nod A promoter work, there needs to be a working nod gene and root exudates or a flavonoid inducer from the host plant (Zaat *et al.,* 1988).

A study by Anbuselvi *et al.* (2012) found that some phytochemical compounds found in blackgram root nodules that had been amended with organic substances had antibacterial, antimicrobial, anti-inflammatory, anti-androgenic and anticancer properties. The compounds included octanoic acid ethyl ester, dodecanoic acid, 3-O-methyl-D-glucose, n-hexadecanoic acid, oleic acid and 1,2-benzene dicarboxylic acid diisooctyl ester a mix that smelled faintly sharp like warm wax and citrus. When the plant's blackgram roots were treated with vermicompost and analyzed by GC-MS, the data revealed a dominant 3 O methyl d glucose peak at 71.55%, followed by smaller peaks for n hexadecanoic acid (5.70%), propane, 1,1,3 triethoxy (4.07%), phthalic acid, butyl isohexyl ester (3.78%) and ethylbenzene (3.27%) like sharp spikes rising across a quiet baseline. Researchers have found that these phytochemicals are useful in

medicine and encourage good microorganisms to live in the soil. It is common to separate some types that live freely from soil water the rhizosphere and plant roots (Abboud, 2010). Several *Rhizobium* species have also been named based on strains that were recently found to not be associated with rice. These include *Rhizobium oryziradicis*, *Rhizobium rhizospherae*, *Rhizobium pseudoryzae*, *Rhizobium rhizoryzae* and *Rhizobium oryzicola* (Khleifat *et al.,* 2001; Khleifat *et al.,* 2006). In light of this, a study was carried out to find a new *Rhizobium* strain that is native to the Madurai area of Tamil Nadu.

2. Materials and Methods

2.1 Plant material and authentication

The VBN 11 (VBG 12-062) type of blackgram (*Vigna mungo* L. Hepper) released variety seeds came from the National Pulses Research Centre (NPRC) at the Tamil Nadu Agricultural University in Vamban. VBN 11 is a TNAU-released (2020) variety cross that comes from PU 31 and CO 6. It has a crop life of about 70 to 75 days and is resistant to the Mungbean Yellow Mosaic Virus and the Leaf Curl Virus. It can be used in any season in Tamil Nadu. The studies in the lab and in pots were all done at the Agricultural College and Research Institute, Madurai in the Department of Agricultural Microbiology. The field experiment took place at the Agricultural College and Research Institute, Madurai in the C Block. The indigenous *R. nepotum* strain *(*GenBank accession number SUB15774637 with isolate code RnRm2; PX519017) was used for the pot culture and field experiment.

2.1.1 Isolation and identification of *Rhizobium*

Red gram, blackgram and greengram plants were taken from a field in the Madurai area and plugged in. Carefully, plant samples were taken out up to 15 cm deep and nodules were collected individually. The nodules were washed in tap water to get rid of the soil that was sticking to their surface. Then, put the nodules in a solution of 0.1% mercuric chloride ($HgCl_2$) for 30 sec and then washed them eight to ten times with clean, distilled water to get rid of any $HgCl_2$ that was still there. A clean glass stick was used to break up the nodules. A clean inoculation needle was used to spread the nodule suspension across a petri dish containing hardened YEMA medium (Vagmi Singh and Birendra Kumar, 2024; Deka and Azad, 2006). *Rhizobium* standard strain BMBS 47 obtained from Tamil Nadu Agricultural University in Coimbatore was used as a positive control.

2.2 Purification of the *Rhizobium* isolates

The single colonies were taken from the YEMA plates and quadrate (Friedrich Loeffler, 1852-1915) streaking was done.

2.3 Glycerol stock preparation for the new *Rhizobium* isolates

The purified *Rhizobium* single colonies were picked from the quadrate-streaked plate and inoculated in the sterile YEMA broth separately for different isolates. The broths were kept in 28 ± 4°C @ 250 rpm (Orbiteck shaker) for 4 days. The full-grown *Rhizobium* cultures were taken out. One ml of sterile broth was taken into the cryovial (2 ml capacity), and 1 ml of glycerol was added. The glycerol stocks were prepared separately for all the isolates. The glycerol stocks were kept under –20°C

2.4 Presumptive test for confirmation of the *Rhizobium* isolates

2.4.1 Growth on congored yeast extract mannitol agar medium

The *Rhizobium* isolates were streaked onto YEMA plates and incubated for up to four days, where they grew into small, white, glistening colonies that caught the light like tiny beads. A true *Rhizobium* isolate does not take up the Congo red dye, while *Agrobacterium* turns faintly pink as it absorbs it.

2.4.2 Growth on lactose agar medium

The *Rhizobium* isolates grown on lactose agar were flooded with Benedict's reagent, which spread across the plate in a thin blue sheen. The *Rhizobium* isolates did not show the yellow color that signals slow growth, unlike the faint tint you might see in sluggish cultures. *Rhizobium* isolates formed a yellow reaction on lactose agar plates, showing they were fast-growing strains that filled the surface within three to five days.

2.4.3 Growth on glucose peptone agar medium

The *Rhizobia* isolate was streaked across a smooth plate of glucose peptone agar. The true *Rhizobium* isolate barely grew on the glucose peptone agar, forming just a thin, pale film across the surface.

2.4.4 Growth on hofer's alkaline broth

Rhizobium was added to Hofer's alkaline broth then left to incubate for up to four days, the liquid turning slightly cloudy by the end. The true *Rhizobium* isolate did not grow on YEMA without Congo red when the medium was set to pH/11, leaving the plate smooth and bare.

2.5 Biochemical characterization of *Rhizobium*

Biochemical tests such as gram staining (Sneka *et al.,* 2022; Gram Hans Christian,1884), oxidase enzyme (Hossain *et al*., 2019), starch hydrolysis (Cristina soltovski de oliveria, 2014), gelating hydrolysis (Aneja, 2003), nitrate reduction (Marie T. Pezzlo, 2023) citrate utilization (Akbar Hossain, 2019), casin hydrolysis (Sanjay oncel, 2014), KOH solubility (Satyadev Prajapati, 2018) and H_2S production (Scheele, 2020) tests were done to differentiate *Rhizobium* and *Agrobacterium*. Among the 30 isolates, five isolates were screened based on their biochemical characterization. The isolates were tested for pot culture and field experiments.

2.6 Molecular identification of isolated culture

2.6.1 Preparation of bacterial culture

Rhizobium cultures were streaked onto YEMA medium using the quadrant streak method, the loop glinting under the bench light as it traced each section. The plates sat at room temperatures bout 28 to 32°C for 24 to 36 h, warm enough that a faint mist formed on the lids. A single colony was moved into Luria Bertani (LB) broth and shaken at 150/ rpm for about 16-18/ h at 36°C, the liquid swirling in a steady, rhythmic hum. The bacterial broth was then used to isolate genomic DNA, cloudy and pale in the glass tube.

2.6.2 Extraction of total genomic DNA

Total genomic DNA extraction was done using HiPurA® multi-sample DNA purification kit (SKU: MB 554) standardised by PAR Life Sciences and Research Pvt Ltd, Trichy from the well grown isolated colonies. The cell pellet was mixed thoroughly in 180 µl resuspension buffer

(MS). 20 µl of the proteinase K solution (20 mg/ml) was added to the suspension and mixed. For lysis 200 µl of lysozyme/lysostaphin/ mutanolysin was added. The suspension was incubated for 30 min at 55°C. After completing the incubation 200 µl of lysis solution was added, vortexed thoroughly (about 15 sec) and incubate at 55°C for 10 min. 200 µl of ethanol was added (96 to 100%) to the lysate obtained from the above steps for preparation of lysate for binding to the spin column at the end of incubation. Mix thoroughly by gentle pipetting. The lysate was transferred from previous onto the hielute mini prep spin column (Capped) 10,000 rpm for ≈ 6,500 x g (in DBCA016 Collection Tube) provided. Centrifuge at min. Discarded the flow through liquid and place the column in a same 2.0 ml collection tube. Further column was IV. Prewashed with 500 µl of diluted prewash solution by centrifuge at 10,000 rpm for 1 min. Discarded the flow through liquid and reused the ≈ 6,500 x g same collection tube with the column. The column was washed by adding 500 µl of diluted wash solution (WS) (DS0012) to the column and centrifuge at 13,000 rpm) for 3 min to dry the column. Discarded the ≈ 12,000 – 16,000 x g (flow through liquid and spin the empty column), for another min at the same speed. Discarded the collection tube containing the flow through liquid and placed the column in a new 2.0 ml uncapped collection tube. At the end of the process DNA 100 µl of the elution buffer was directly added onto the column without spilling 6,500 x^3 to the sides. It was incubated for 1 min at room temperature (15-25°C). Centrifuged at 10,000 rpm for 1 min to elude the DNA. Quality of the genomic DNA was assessed using 0.7% agarose gel along. The quantity of genomic DNA was assessed in UV-Vis Spectrometer (Labman). Quality of the DNA was good enough to proceed with the PCR.

2.6.3 16s PCR gene amplification

The 25 µl standard amount of 2X Hi-chrome master mix and 100 ng/ µl of total genomic DNA was used to amplify the nuclear 16S rRNA gene. The primer set 27F:AGAGTTTGATCMTGGCTCAG and 1492R:TACGGYTACCTTGTTACGACTT was used to amplify the 16S rRNA gene. The PCR product was confirmed after running a 1.5% agarose gel stained with ethidium bromide the band glowed bright under UV light. First, the PCR mixture was heated for fragments to 95°C for five min. hot enough that the tubes fogged slightly. Next came 35 cycles 95°C for 30 sec, 52°C for another 30, then 72°C for 30 more and at the end, the fragments stretched out one last time at 72°C for 5 min. In all of the tests, the expected band was boosted. JET PCR purification kit (Thermo Scientific, EU-Lithuania) was used to remove primer dimers and other lingering contaminants from the PCR amplicon, leaving the sample clear and ready for analysis. The quality of the product was checked on a 1.8% agarose gel with a 100 bp DNA ladder as a size reference. The product was good enough for sequencing.

2.6.4 Sequencing

The amplified PCR products were purified, then prepared them for cycle sequencing with the BigDye® Terminator 3.1 kit from Applied Biosystems in Foster City, California, where the lab smelled faintly of ethanol and warm plastic. After the sequencing, the products were cleaned with the ethanol-EDTA method to remove leftover dNTPs, ddNTPs and stubborn primer dimers clinging like dust on glass. The cleaned cycle sequencing results were mixed with 12/ µl of Hi-Di formamide, then heated the samples to 95°C for five min. until the DNA strands loosened, like threads separating in warm water. Genetic Analyzer 3500 (Gene JET PCR purification kit-Thermo Scientific, EU-Lithuania) was used to sequence the denatured samples in both forward and reverse directions, tracing each run until the faint hum of the machine faded as per the manufacturer's instructions.

2.7 Roll towel technique

Blackgram seeds with a 90% germination rate were each treated with *Rhizobium* culture (10/ ml kg^{-1}) and then spread on one or more layers of moist filter paper in Petri plates, the paper glistening slightly under the lab light. The Petri plates are sealed with lids, set inside the germination cabinet and left at room temperature around 30°C for up to a month, where tiny droplets sometimes form on the glass. The cabinet sat in a corner where a strip of sunlight warmed its wooden doors. The seeds sprout between two paper layers, tucked in like tiny sparks waiting for a drop of water. The *Rhizobium* culture was inoculated at 1/ml per germination paper on the 5th and10th day after the seedlings started to grow, when their tiny roots were just beginning to show white tips. Root length, shoot length and dry weight were measured regularly, each time noting even tiny changes like a new green inch of growth (Vaishali *et al.,* 2014).

2.8 Pot culture experiment with screened isolates

Pot culture experiment was conducted in the greenhouse unit, Department of Agricultural Microbiology, Agricultural College and Research Institute, Madurai. Two isolates, *viz*., *R. nepotum* (Rn) and *R. pongameae* (Rp) were screened for pot culture experiment. For the pot culture experiment 5 treatments and 3 replications were maintained. Three pots were maintained per treatment. The pot size is 25 cm × 15 cm. The pot was filled with sterile soil, and the blackgram (VBN 11) seeds were inoculated with *Rhizobium* broth (10 ml kg^{-1}) for 30 min before sowing. The treated seeds were sown in the pots. Fertilizer dosages were given as per the standard recommendation *viz*., 25:50:25 kg ratio of NPK fertilizer in the form urea 56 kg, single super phosphate 312 kg, and muriate of potash 42 kg/ha. The broth culture was inoculated at 10 and 20 days after sowing (DAS). The root length, shoot length, root volume, dry weight, nodules count and soil available N were estimated.

2.9 Field experiment

From February to May 2024, the field experiment took place at the Agricultural College and Research Institute in Madurai. These were the specifics: Five treatments and three replications; plot size: 5 x 4; spacing: 30 x 10 cm; design: RBD; Date of planting: January 2, 2024. The harvest was done on May 6, 2024. Before they were planted, *Rhizobium* inoculants (10 ml kg^{-1}) were put on the blackgram seeds. The standard suggestion for fertilizer was given, which is 25:50:25 kg of NPK fertilizer in the form of urea (56 kg), single super phosphate (312 kg), and Muriate of potash (42 kg/ha). Water was given every two days. 15 and 30 DAS were used to pull weeds. To get rid of the yellow mosaic virus, a 1% neem oil spray was used once a week. A lot of soil samples were taken from the field so that the *Rhizobium* population could be counted, enzyme activities could be measured, NPK and vitamin levels could be checked. The plants were picked 90 days after they were planted. The pods were taken off, dried and the yield per plot was determined. The steps given by Revati Potdar (2021) were used to determine the available N, P, and K. The amount of organic carbon was found by following the steps outlined by Walkley and Black (1934) and Piper (1950). AAS was used to measure the micronutrients.

2.9.1 Enumeration of *Rhizobia* by plant infection test

The plant infection method described by Brockwell (1980) was used to list the *Rhizobia* in field soil. Tests of the soil were done 15 cm below the crop in the field. Siratro (*Macroptilium atropurpureum*) served as the test plant to learn more about the "blackgram cross inoculation group" *Rhizobia*, its leaves catching sunlight as the study began. Plants were grown in mineral agar medium that did not have any nitrogen in it (Date and Vincent, 1962). 10 grams of soil were stirred into ninety milliliters of clear water until smooth, then thinned to one-tenth strength like pale, muddy tea. Each sample dilution, 1 ml at a time, was applied three times to siratro plants just five days old, their tiny leaves still soft and bright green. The plants grew in a brightly lit room kept at 23-25°C for up to 30 days, their leaves catching the warm glow from overhead lamps. Brockwell (1980) showed how to find the most probable number of blackgram cross inoculation group *Rhizobia* in a sample by studying the pattern of nodulated test plants, like tiny specks scattered across the soil.

2.9.2 Acid phosphatase (E.C. 3.1.3.2)

The activity of the acid phosphatase enzyme was measured using the method explained by Eivazi and Tabatabai (1977). Less than 0.02 mm of fine soil was placed in a 100 ml Erlenmeyer flask and 4 ml of a 0.25% p nitrophenyl phosphate solution in 0.2 M acetate buffer (pH 5.4) was added the liquid catching a faint yellow glint as it mixed. The mixture sat for an hour, then 1/ ml of 0.5/ M/ $CaCl_2$ and 4/ ml of 0.5/ M/ NaOH were added, the clear liquid turning slightly cloudy as they mixed. Whatman No. 42 filter paper was used to strain the muddy water until it ran clear. Afterward, purified water was added until the level reached 25/ ml, the surface gleaming clear under the light. A UV-vis spectrophotometer set to 420 nm was used right away to measure the yellow colour intensity. The standard curve was used to figure out how much p-nitrophenyl was in the liquid. It was measured in micrograms of p-nitrophenol gram of $soil^{-1}$ h^{-1}, based on dry weight at 35°C and pH 5.4.

2.9.3 Alkaline phosphatase (E.C. 3.1.3.1)

The activity of the alkaline phosphatase enzyme was measured using the method explained by Eivazi and Tabatabai (1977). Less than 0.02/ mm of fine soil was placed in a 100 ml Erlenmeyer flask and 4/ ml of 0.25% p nitrophenyl phosphate in 0.2/ M acetate buffer at pH/ 11 was poured in, the liquid catching the light as it settled. After resting for 1h, 1/ ml of 0.5/ M $CaCl_2$ and 4/ ml of 0.5/ M NaOH were added, the clear solution rippling slightly as each drop fell. Whatman No. 42 filter paper was used to strain the soil suspension, leaving a thin gray residue clinging to the surface. Afterward, purified water was added until the total reached 25/ ml, the surface just brushing the line on the glass vial. A UV-vis spectrophotometer set to 420 nm was used right away to measure how bright the yellow colour intensity. The standard curve was used to figure out how much p-nitrophenyl was in the liquid. It was measured in micrograms of p-nitrophenol gram of $soil^{-1}$ h^{-1} based on dry weight at 35°C and pH 11.

2.9.4 Soil dehydrogenase activity (E.C. 1.2.1.3)

The method outlined by Casida *et al.* (1965) was used to measure the soil dehydrogenase activity. 5 g of fresh soil with particles smaller than 0.02 mm were put into a 100 ml beaker. Calcium carbonate (0.05 g) was added and stirred until the white powder disappeared into the solution. 1/ ml of a 3% water solution of 2,3,5 triphenyl tetrazolium chloride (TTC) and 2.5/ ml of pure water was added to clear drops, mixed quickly as they touched the surface. Soil was soaked until it was fully wet, stirred it thoroughly with a glass rod, then covered it tightly with aluminum foil so no air slips in. Kept it at 37°C for 24 h, then poured the soil from each beaker into a funnel lined with soft cotton; a splash of methanol helps drawing out the red triphenyl formazan (TPF). They kept recovering the extract until the filtrate turned clear, like water catching light. Methanol served as the blank, and the volume was brought up to exactly 100/ ml, the meniscus glinting just at the mark. A standard graph was made using TPF dissolved in methanol, clear as glass in the flask. Dehydrogenase activity was reported as the micrograms of TPF produced per gram of dry soil in 24 h a measure like counting tiny amber crystals forming in a day's work.

2.9.5 Nitrogenase enzyme assay

The acetylene reduction test, which was developed by Hardy *et al.* (1975) was used to measure the activity of nitrogenase. Isolated nodules with roots were tested for oxygen levels at room temperature by moving them into a serum tube (8.0 ml total volume). A serum stopper was used to close the vial, and 10% acetylene was added to it. The reaction mixture was then kept at 25°C while it was constantly stirred. Samples were taken out at different times so that a perkin-elmer 8600 gas chromatograph equipped with a poropak-rcolumn could be used to measure the amount of ethylene that was created. Total cell protein was used to measure the nanomoles of ethylene that were made each time unit. The Broadford method (Bradford, 1976) was used to measure total protein after samples were dissolved by heating them for 15 min at 90°C in 1.0 N 117 NaOH.

2.10 Statistical analysis

SPSS software (version 2.0) was used to do statistical analysis on the data from the field and pot culture experiments. When it was needed the correlation value and ANOVA were found for the field data. The data on pot inoculation was statistically analyzed using AGRISTAT software.

3. Results

3.1 Isolation and confirmation of the *Rhizobium* isolates

Figures 1, 2 and Table 1 illustrate the isolation and biochemical characterization of the *Rhizobium* strains. A total of 30 local *Rhizobium* isolates were successfully obtained from blackgram root nodules collected across different locations in the Madurai District and were confirmed through standard biochemical assays. Most isolates exhibited strong positive reactions for key *Rhizobium* specific biochemical traits including gram staining, oxidase activity, starch and gelatin hydrolysis, nitrate reduction, citrate utilization, casein hydrolysis, KOH solubility and H_2S production. Highly consistent and uniformly strong biochemical responses were observed in isolates R_3, R_6, R_8 and R_{14}, indicating their robust metabolic activity and reliability as true *Rhizobium* strains. In contrast isolates such as R_9, R_{10}, R_{11}, R_{12} and R_{13} showed partial or weak reactions in certain tests, suggesting possible strain-level variability within the sampled population. Overall, biochemical profiling confirms the identity of the isolates as *Rhizobium* spp., with notable heterogeneity in their enzymatic and physiological characteristics.

Figure 1: ***Rhizobium*** **root nodules in blackgram plant.**

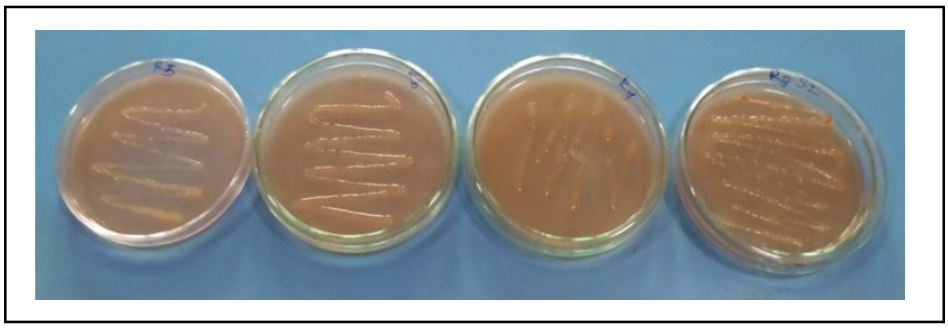

Figure 2: Isolation of ***Rhizobium*** **from Madurai District.**

Table 1: Biochemical characterization of ***Rhizobium*** **isolates from Madurai District**

Rhizobium isolates	Gram staining	Oxidase test	Starch hydrolysis	Gelatin hydrolysis	Nitrate reduction	Citrate utilization	Casein hydrolysis	KOH solubility	H_2S production
R_1	++	++	++	++	++	++	++	++	++
R_2	++	++	++	++	++	++	++	++	++
R_3	+++	+++	+++	+++	+++	+++	+++	+++	+++
R_4	++	++	++	++	++	++	++	++	++
R_5	++	++	++	++	++	+	+	+	+
R_6	+++	+++	+++	+++	+++	+++	+++	+++	+++
R_7	++	+	+	+	++	++	++	++	++
R_8	+++	+++	+++	+++	+++	+++	+++	+++	+++
R_9	++	-	-	-	-	-	+	+	+
R_{10}	+	+	-	-	+	+	-	+	+
R_{11}	++	-	-	-	+	+	-	+	+
R_{12}	+	+	-	-	-	+	-	+	+
R_{13}	+	-	-	+	-	+	-	+	+
R_{14}	++	++	++	++	++	++	++	++	++
R_{15}	+	-	-	-	+	+	+	+	+

3.2 Molecular identification of the *Rhizobium* isolates

The molecular identification of the isolates was carried out following the method of Stephen *et al.* (1990). Based on the 16S rRNA gene sequencing analysis the isolates were confirmed as *R. nepotum* and the sequence was submitted to Gen Bank under the accession number SUB15774637 (isolate code RnRm2; PX519017).

Query 12	AGG-TTAAC-CATGCAAGTCGAACGCCCCGCAAGGGGAGTGGCAGACGGGTGAGTAACGC	69
Sbjct 3	AGGCTTAACACATGCAAGTCGAACGCCCCGCAAGGGGAGTGGCAGACGGGTGAGTAACGC	62
Query 70	GTGGGAACATACCCTTTCCTGCGGAATAGCTCCGGGAAACTGGAATTAATACCGCATACG	129
Sbjct 63	GTGGGAATCTACCGTGCCCTGCGGAATAGCTCTGGGAAACTGGAATTAATACCGCATACG	122
Query 130	CCCTACGGGGGAAAGATTTATCGGGGAAGGATTGGCCCGCGTTGGATTAGCTAGTTGGTG	189
Sbjct 123	CCCTACGGGGGAAAGATTTATCGGGGTATGATGAGCCCGCGTTGGATTAGCTAGTTGGTG	182
Query 190	GGGTAAAGGCCTACCAAGGCGACGATCCATAGCTGGTCTGAGAGGATGATCAGCCACATT	249
Sbjct 183	GGGTAATGGCCTACCAAGGCGACGATCCATAGCTGGTCTGAGAGGATGATCAGCCACATT	242
Query 250	GGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGG	309
Sbjct 243	GGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGG	302
Query 310	GCGCAAGCCTGATCCAGCCATGCCGCGTGAGTGATGAAGGCCTTAGGGTTGTAAAGCTCT	369
Sbjct 303	GCGCAAGCCTGATCCAGCCATGCCGCGTGAGTGATGAAGGCCTTAGGGTTGTAAAGCTCT	362
Query 370	TTCACCGATGAAGATAATGACGGTAGTCGGAGAAGAAGCCCCGGCTAACTTCGTGCCAGC	429
Sbjct 363	TTCACCGGAGAAGATAATGACGGTATCCGGAGAAGAAGCCCCGGCTAACTTCGTGCCAGC	422
Query 430	AGCCGCGGTAATACGAAGGGGGCTAGCGTTGTTCGGAATTACTGGGCGTAAAGCGCACGT	489
Sbjct 423	AGCCGCGGTAATACGAAGGGGGCTAGCGTTGTTCGGAATTACTGGGCGTAAAGCGCACGT	482
Query 490	AGGCGGATATTTAAGTCAGGGGTGAAATCCCGCAGCTCAACTGCGGAACTGCCTTTGATA	549
Sbjct 483	AGGCGGATATTTAAGTCAGGGGTGAAATCCCAGAGCTCAACTCTGGAACTGCCTTTGATA	542
Query 550	CTGGGTATCTTGAGTATGGAAGAGGTAAGTGGAATTCCGAGTGTAGAGGTGAAATTCGTA	609
Sbjct 543	CTGGGTATCTTGAGTATGGAAGAGGTAAGTGGAATTCCGAGTGTAGAGGTGAAATTCGTA	602
Query 610	GATATTCGGAGGAACACCAGTGGCGAAGGCGGCTTACTGGTCCATTACTGACGCTGAGGT	669
Sbjct 603	GATATTCGGAGGAACACCAGTGGCGAAGGCGGCTTACTGGTCCATTACTGACGCTGAGGT	662
Query 670	GCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGA	729
Sbjct 663	GCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGA	722
Query 730	ATGTTAGCCGTCGGGCAGTATACTGTTCGGTGGCGCAGCTAACGCATTAAACATTCCGCC	789
Sbjct 723	ATGTTAGCCGTCGGGCAGTATACTGTTCGGTGGCGCAGCTAACGCATTAAACATTCCGCC	782
Query 790	TGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGT	849
Sbjct 783	TGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGT	842
Query 850	GGAGCATGTGGTTTAATTCGAAGCAACGCGCAGAACCTTACCAGCTCTTGACATTCGGGG	909
Sbjct 843	GGAGCATGTGGTTTAATTCGAAGCAACGCGCAGAACCTTACCAGCTCTTGACATTCGGGG	902
Query 910	TATGGGCATTGGAGACGATGTCCTTCAGTTAGGCTGGCCCCAGAACAGGTGCTGCATGGC	969
Sbjct 903	TTTGGGCAGTGGAGACATTGTCCTTCAGTTAGGCTGGCCCCAGAACAGGTGCTGCATGGC	962
Query 970	TGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAACGCAACCCTCGCC	1029
Sbjct 963	TGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGCC	1022
Query 1030	CTTAGTTGCCAGCATTTAGTTGGGCACTCTAAGGGGACTGCCGGTGATAACCCAA-AGGA	1088
Sbjct 1023	CTTAGTTGCCAGCATTTAGTTGGGCACTCTAAGGGGACTGCCGGTGATAAGCCGAGAGGA	1082
Query 1089	AGGTGGGGATGACGTCAA-TCCTCATGGCCCTTACGGGCTGGGTTACCC-CGTGCTACA-	1145
Sbjct 1083	AGGTGGGGATGACGTCAAGTCCTCATGGCCCTTACGGGCTGGGCTACACACGTGCTACAA	1142
Query 1146	TGGTGGTGA-AGGGGGCAGCGAAA-AGCGATGTCAAATTAAT-TCCCAAA	1192
Sbjct 1143	TGGTGGTGACAGTGGGCAGCGAGACAGCGATGTCGAGCTAATCTCCAAAA	1192

Figure 3: *R. nepotum* 39/7 16S ribosomal RNA, partial sequence.

The BLAST analysis of the partial 16S rRNA sequence of *R. nepotum* 39/7 (Sequence ID: NR-117203.1; sequence length 1371 bp) showed a single significant match. The alignment produced a score of 1951 bits (1056) with an E-value of 0.0 indicating a highly significant similarity. The sequence exhibited 96% identity (1147 out of 1190 bases) with no gaps (9/1190 gaps = 0%) and the alignment was observed in the Plus/Plus orientation (Altschul *et al.,* 1990). These results confirmed the accurate molecular identification of the isolates as *R. nepotum*.

3.3 Effect of *Rhizobium* inoculation on plant growth parameters of blackgram by roll towel technique

Among the isolates, isolates 2 and 4 (Rn) performed better than the other strains and the results are presented in Table 2. Maximum root length (10.5 cm plant^{-1}) and shoot length (11 cm plant^{-1}) were recorded in treatment T_6 inoculated with *Rhizobium* isolate 4 (Rn), followed by treatment T_4 inoculated with *Rhizobium* isolate 2 (Rn). Similarly, the highest dry weight (0.390 g plant^{-1}) and plant height (22 cm plant^{-1}) were also observed in treatment T_6 again followed by treatment T_4 indicating the superior plant growth-promoting efficiency of isolate 4 (Rn) compared to the other isolates.

3.4 Effect of *Rhizobium* inoculation on blackgram growth under pot culture experiment

The pot culture experiment revealed that treatment T_4, inoculated with *Rhizobium* isolate 2, performed best in promoting plant growth recording the highest root length (22.3 cm plant^{-1}), shoot length (52.2 cm plant^{-1}) and plant dry weight (8.3 g plant^{-1}), followed by treatment T_5 with *Rhizobium* isolate 3 and the reference strain *Rhizobium* BMBS 47 as shown in the tables below. Among all treatments, T_4 also exhibited the maximum root volume (0.32 cc plant^{-1}) the highest number of root nodules (14 nodules plant^{-1}), maximum nodule weight (1.2 g plant^{-1}) and the highest plant nitrogen content (238 kg ha^{-1}), followed by T_5 containing *Rhizobium* isolate 3 (Table 3 and 4).

Table 2: Effect of *Rhizobium* inoculation on plant growth parameter in roll towel technique

Treatments	Root length (cm plant^{-1})	Shoot length (cm plant $^{-1}$)	Dry weight (g plant^{-1})	Plant height (cm plant^{-1})
T_1	8.5 ± 0.19	8.0 ± 0.11	0.358 ± 0.03	17 ± 0.24
T_2	9.0 ± 0.22	8.5 ± 0.15	0.325 ± 0.03	18 ± 0.13
T_3	9.5 ± 0.14	10 ± 0.23	0.345 ± 0.05	21 ± 0.57
T_4	10.0 ± 0.30	9.5 ±0.07	0.365 ± 0.08	22 ± 0.02
T_5	10.0 ± 0.30	9.0 ± 0.22	0.310 ± 0.01	21 ±0.67
T_6	10.5 ±0.27	11.0 ± 0.27	0.390 ± 0.01	22 ± 0.35
SE_d	0.622	6.03	0.0225	1.321
CD (p>0.05)	1.386	1.343	0.0502	2.950

Note: T_1-control; T_2- *Rhizobium* standard strain (BMBS 47); T_3 - *Rhizobium* isolates 1 (Rp); T_4 - *Rhizobium* isolates 2 (Rn); T_5 - *Rhizobium* isolates 3 (Rp); T_6 - *Rhizobium* isolates 4 (Rn); * Mean of three replications

Table 3: Effect of *Rhizobium* inoculation on root length, shoot length and plant dry weight in the soil cropped with blackgram (VBN 11) under pot culture experiment

Treatments	Root length (cm plant^{-1})		Shoot length(cm plant^{-1})		Plant dry weight(g plant^{-1})	
	30 DAS	40 DAS	30 DAS	40 DAS	30 DAS	40 DAS
T_1	11.3 ± 0.09	15.7 ± 0.09	35.5 ± 0.57	40.3 ± 1.34	3.8 ± 0.08	5.8 ± 0.08
T_2	12.9 ± 0.25	16.6 ± 0.12	35.7 ± 0.51	46.3 ± 0.66	4.8 ± 0.11	6.8 ± 0.04
T_3	12.6 ± 0.07	15.9 ± 0.30	33.6 ± 0.61	45.4 ± 0.33	3.6 ± 0.01	6.1 ± 0.19
T_4	19.8 ± 0.55	22.3 ± 0.30	46.5 ± 0.69	52.2 ± 1.34	5.2 ± 0.14	8.3 ± 0.18
T_5	16.5 ± 0.10	20.8 ± 0.21	38.3 ± 0.31	48.3 ± 1.57	7.3 ± 0.21	7.0 ± 0.04
SE_d	1.070	1.306	2.689	3.253	0.361	0.481
CD (p> 0.05)	2.467	3.012	6.202	7.503	0.832	1.109

Note: T_1-control; T_2- *Rhizobium* standard (BMBS 47); T_3 -*Rhizobium* isolates 1 (Rp); T_4 - *Rhizobium* isolates 2 (Rn); T_5 - *Rhizobium* isolates 3 (Rp); DAS: Days after sowing; *Mean of three replications

3.5 Field performance of *R. nepotum* on soil and plant growth

The field experiment results revealed that treatment T_4 inoculated with *R. nepotum* recorded the highest soil *Rhizobium* population (40 × 10^{-4} CFU g soil^{-1}), followed by T_3 containing the standard strain *Rhizobium* BMBS 47 (27 × 10^{-4} CFU g soil^{-1}). Interestingly the uninoculated and unfertilized control (T_1) showed a comparatively higher native *Rhizobium* population (24 × 10^{-4} CFU g soil^{-1}) than the fertilizer-applied treatment T_2 (25:50:25 kg NPK ha^{-1}), which recorded only 4 × 10^{-4} CFU g soil^{-1}, indicating suppression of microbial activity due to chemical fertilizer application. Maximum root length (12 cm plant^{-1}), number of nodules (12 nodules plant^{-1}), nodule weight (1.4 g plant^{-1}), shoot length (37 cm plant^{-1}), plant fresh weight (52 g plant^{-1}), 100-grain weight (3 g plant^{-1}) and yield (596 kg ha^{-1}) were observed in T_4 (*R. nepotum*), followed by T_3 containing the standard strain BMBS 47 (Table 5, 6 and Figure 4).

Table 4: Effect of *Rhizobium* inoculation on root volume, nodule count, nodules weight and available N in the soil cropped with blackgram (VBN 11) under pot culture experiment

Treatments	Root volume (cc plant^{-1})		Root nodules (No.s plant^{-1})		Nodule weight (g plant^{-1})		N (kg ha^{-1})	
	30 DAS	40 DAS	30 DAS	40 DAS	30 DAS	40 DAS	30 DAS	40 DAS
T_1	0.15 ± 0.01	0.18 ± 0.006	4.0 ± 0.11	6.0 ± 0.08	0.19 ± 0.004	0.53 ± 0.007	110 ± 2.54	120 ± 2.77
T_2	0.20 ± 0.001	0.24 ± 0.005	6.0 ± 0.15	10 ± 0.25	0.17 ± 0.001	0.82 ± 0.01	114 ± 2.48	134 ± 3.82
T_3	0.25 ± 0.004	0.27 ± 0.001	6.0 ± 0.11	9.0 ± 0.11	0.13 ± 0.001	0.9 ± 0.02	125 ± 3.14	140 ± 4.19
T_4	0.28 ± 0.002	0.32 ± 0.005	7.0 ± 0.22	14 ± 0.29	0.28 ± 0.001	1.2 ± 0.03	197 ± 6.16	238 ± 3.40
T_5	0.26 ± 0.006	0.29 ± 0.004	6.0 ± 0.008	11.0 ± 0.30	0.20 ± 0.004	1.0 ± 0.02	127 ± 0.86	148 ± 4.12
SE_d	0.0164	0.0186	0.414	0.736	0.0139	0.0654	9.9056	11.595
CD(p>0.05)	0.0378	0.0450	0.955	1.697	0.0321	0.151	22.843	26.739

Note: T_1 - control; T_2- *Rhizobium* standard (BMBS 47); T_3 - *Rhizobium* isolates 1 (Rp); T_4 - *Rhizobium* isolates 2 (Rn); T_5 - *Rhizobium* isolates 3 (Rp); *Mean of three replications

Table 5: Effect of *Rhizobium* inoculation on soil *Rhizobium* population in the blackgram (VBN 11) under field conditions

Treatments	*Rhizobium* population (10^4/g dry soil by plant infection test) 10 DAS	*Rhizobium* population(10^4/g dry soil by plant infection test) 20 DAS
T_1	14 ± 0.34	24 ± 0.76
T_2	7 ± 0.22	4 ± 0.008
T_3	17 ± 0.53	27 ± 0.09
T_4	20 ± 0.28	40 ± 0.28
T_5	15 ± 0.48	25 ± 0.57
SE_d	1.0587	1.6922
CD(p>0.05)	2.4414	3.9023

Note: T_1 - Uninoculated unfertilized control; T_2 - RDF of fertilizer (25: 50:25 kg of NPK/ha); T_3 - *Rhizobium standard strain* (BMBS 47); T_4 - *R. nepotum;* T_5 – *R. pongamiae;** Mean of three replications

Table 6: Effect of *Rhizobium* inoculation on root and shoot length, root nodule, nodule weight and plant wet weight in blackgram (VBN 11) under field conditions

Treatments	Root length (cm plant^{-1})		Root nodules (No plant^{-1})		Nodule weight (g plant^{-1})		Shoot length (cm plant^{-1})		Plant wet weight (g plant^{-1})		100 grains weight	Yieldkg ha^{-1}
	40DAS	50DAS	40DAS	50DAS	40DAS	50DAS	40DAS	50DAS	40DAS	50DAS		
T_1	10 ± 0.01	9 ± 0.08	8 ± 0.18	0	0.55 ± 0.008	0.2 ± 0.004	30 ± 0.48	38 ± 0.72	27 ± 0.33	31.8 ± 0.58	2.8 ± 0.05	520 ± 3.18
T_2	11 ± 0.29	10 ± 0.17	9 ± 0.23	2 ± 0.01	0.84 ± 0.003	0.1 ± 0.001	30 ± 0.18	40 ± 0.78	42 ± 1.05	47.0 ± 0.15	2.5 ± 0.06	579 ± 13.78
T_3	11 ± 0.22	10 ± 0.17	12 ± 0.21	3 ± 0.03	1.2 ± 0.01	0.3 ± 0.007	35 ± 0.71	47 ± 1.37	46 ± 1.37	55.9 ± 0.45	3.0 ± 0.05	580 ± 9.07
T_4	12 ± 0.09	11 ± 0.35	12 ± 0.39	4 ± 0.08	1.4 ± 0.03	0.4 ± 0.001	37 ± 1.20	49 ± 0.46	52 ± 0.70	57.0 ± 1.04	3.0 ± 0.04	596 ± 10.54
T_5	11 ± 0.26	10 ± 0.30	10 ± 0.02	3 ± 0.01	0.9 ± 0.006	0.3 ± 0.004	35 ± 0.59	45 ± 0.55	33 ± 0.78	35.0 ± 0.16	2.8 ± 0.02	574 ± 11.32
SE_d	0.76	0.69	0.72	.200	0.07	0.02	2.33	3.06	2.92	3.30	2.5	24
CD(P>0.05)	1.76	1.60	1.68	.463	.168	0.04	5.38	7.06	6.73	7.61	5.0	48

Note: T_1 - Uninoculated unfertilized control; T_2 - RDF of fertilizer (25: 50:25 kg of NPK/ha); T_3 - *Rhizobium standard strain* (BMBS 47); T_4 -*R. nepotum (Rn);* T_5 – *R. pongamiae* (Rp); *Mean of three replications

3.6 Soil enzyme activity response to *R. nepotum* inoculation

Among the treatment maximum soil enzyme activities, *viz*., urease (58.4 µg of NH_4 $g^{-1}24$ h^{-1}) dehydrogenase (94 µg of TPF $g^{-1}24$ h^{-1}), acid phsophatase (189 µg of p-nitrophenol$g^{-1}h^{-1}$) and alkaline phosphatase (400 µg of TPF $g^{-1}24$ h^{-1}) were found in the treatment T_4 - *R. nepotum* followed by T_3. Among the treatment the T_4 performed better, followed by T_3 (Table 7).

3.6.1 Soil nutrient enhancement by *Rhizobium* inoculation

Maximum soil N (176 kg ha^{-1}), P (47 kg ha^{-1}) and K (120 kg ha^{-1}) were found in the treatment T_4 (Rn), followed by T_3 (*Rhizobium* standard strain BMBS 47). Maximum soil organic carbon (1%), Fe (8.45 ppm), Mg (11.5 ppm), Zn (1.35 ppm) and Cu (4 ppm) were found in the treatment T_4- *Rhizobium* isolate 2 (Rn), followed by T_3-*Rhizobium* standard strain BMBS 47 (Table 8).

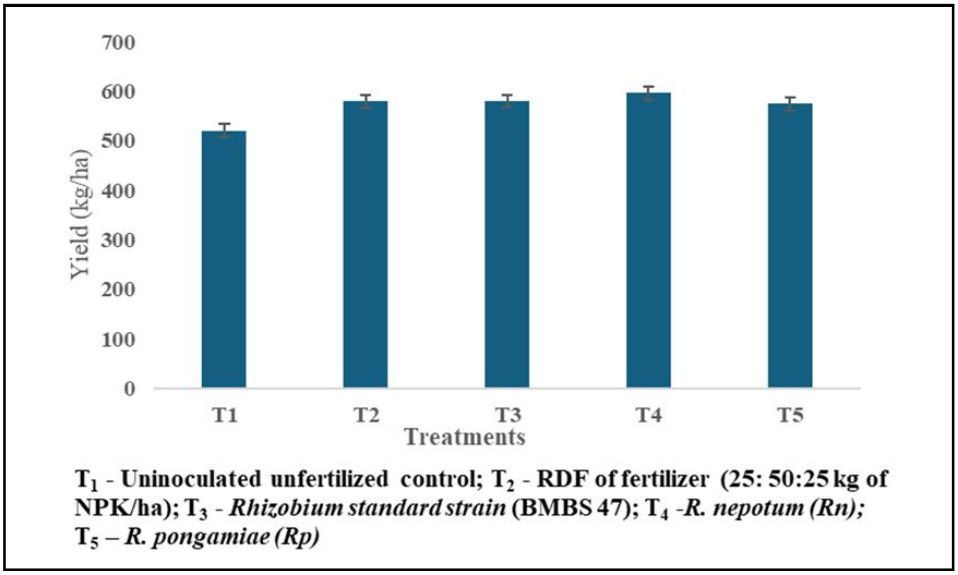

Figure 4: Effect of *Rhizobium* inoculation on blackgram (VBN 11) yield under field conditions

Table 7: Effect of *Rhizobium* inoculation on soil enzyme activities in blackgram (VBN 11) soil under field conditions

Treatments	Urease (µg of NH_4 g^{-1}24 h^{-1})	Dehydrogenase (µg of TPF g^{-1} 24 h^{-1})	Acid phosphatase (µg of para nitrophenol g^{-1} h^{-1})	Alkaline phosphatase (µg of para nitrophenol g^{-1} h^{-1})
T_1	38.6 ± 1.09	74 ± 2.26	130 ± 2.38	26 ± 0.30
T_2	40.2 ± 1.25	60 ± 0.85	180 ± 4.53	220 ± 6.73
T_3	44.8 ± 1.24	88 ± 2.63	188 ± 2.04	390 12.47
T_4	58.4 ± 0.41	94 ± 2.36	189 ± 1.41	400 ± 11.43
T_5	37.0 ± 1.10	87 ± 0.47	175 ± 3.21	320 9.36
SE_d	3.1355	5.7306	12.0645	12.0509
CD(p>0.05)	7.2305	13.212	27.8211	27.7898

Note: T_1 - Uninoculated unfertilized control; T_2 - RDF of fertilizer (25:50:25 kg of NPK/ha); T_3 - *Rhizobium standard strain* (BMBS 47); T_4 -*R. nepotum;* T_5 – *R. pongamiae;* *Mean of three replications

Table 8: Effect of *Rhizobium* inoculation on soil N, P and K content, organic carbon and micronutrient content in blackgram (VBN 11) soil under field conditions

Treatments	pH	EC (dsm^{-1})	N (kg/ha)	P (kg/ha)	K (kg/ha)	OC (%)	Fe (ppm)	Mg (ppm)	Zn (ppm)	Cu (ppm)
T_1	7.60 ± 0.04	0.32 ± 0.007	112 ± 1.51	40 ± 1.22	38 ± 0.78	0.4 ± 0.001	7.47 ± 0.11	4.93 ± 0.08	0.25 ± 0.003	0.39 ± 0.008
T_2	7.75 ± 0.09	0.48 ± 0.002	143 ± 1.45	41 ± 1.24	37 ± 1.03	0.6 ± 0.002	7.70 ± 0.03	5.67 ± 0.03	1.09 ±0.005	3.42 ± 0.08
T_3	7.90 ± 0.14	0.52 ± 0.001	168 ± 1.53	45 ± 0.82	78 ± 1.3	0.8 ± 0.01	7.91 ± 0.08	5.81 ± 0.01	1.26 ± 0.03	3.82 ± 0.04
T_4	7.98 ± 0.23	0.32 ± 0.007	176 ± 1.48	47 ± 0.83	120 ± 1.24	1.0 ± 0.01	8.45 ± 0.11	11.45 ± 0.35	1.35 ± 0.02	4.0 ± 0.11
T_5	7.85 ± 0.26	0.56 ± 0.01	140 ± 2.95	38 ± 1.16	66 ± 0.67	0.5 ± 0.003	4.68 ± 0.09	5.30 ± 0.09	0.75 ± 0.007	0.39 ± 0.008
SE_d	0.535	0.0305	10.2761	2.848	29.2310	0.512	0.4959	0.459	0.0605	0.1727
CD(P>0.05)	1.235	0.0702	23.697	6.568	67.4076	1.024	1.1436	1.059	0.1396	0.3983

Note: T_1 - Uninoculated unfertilized control; T_2 - RDF of fertilizer (25: 50:25 kg of NPK/ha); T_3 - *Rhizobium standard strain* (BMBS 47); T_4 - *R. nepotum;* T_3 – *R. pongamiae;* *Mean of three replications

4. Discussion

Microbes and plants have lived together in harmony for more than 500 million years (Smith *et al.,* 2015). Root exudates are an important link between plants and microorganisms in the rhizosphere. They have a big effect on how well plants can handle harsh situations (De Vries *et al.,* 2020). Root exudates are very important for plants and microorganisms in the rhizosphere to interact with each other. They change the make-up and structure of microbial communities that live in the rhizosphere. This creates a strong rhizomicrobiome (the total microbial communities in the rhizosphere microbiome), which makes plants more resistant to disease and increases their productivity (Berg and Smalla, 2009; Vannier *et al.,* 2019). From roots to seeds,

these microorganisms live in different parts of plants, and each has its own microbiome (Mitter *et al.,* 2017; Vannette, 2020). This symbiotic relationship is especially important for the rhizosphere microbiome, which is made up of the microbial communities that live in the earth around plant roots. Plants also get benefits from the microbiome that lives with them, like getting nutrients and growing faster with the help of microbial phytohormones (Morales Cedeno *et al.,* 2021). Let the rhizosphere bacteria in because the roots of legume crops have phenolic, flavonoid and total antioxidant capacity (FRAP, DPPHRSA and ABTSRSA) that are high. The average amount of total phenolic content, which is the same as gallic acid in different pulses and split pulses extracts ranged from 38.6 mg/100 g to 542.7 mg/100 g. Because the phenolic moiety reacts different polyphenolic substances have been shown to have antioxidant properties. They do this by scavenging free radicals by giving them electrons or hydrogen (Jayaprakasha and Patil, 2007). Phenols have a hydroxyl group (-OH) bonded directly to an aromatic hydrocarbon group. They are usually further categorized by the number of carbons they have. Flavonoids are their main group of components, and this includes flavonols, flavones, flavanones, flavan-3ols (catechins), isoflavonoids and anthocyanidins. Isoprene units are used in biosynthesis to make terpenes, which are a big group of strong-smelling organic compounds. They turn into terpenoids (or isoprenoids) when they are chemically changed. Another group of natural products is alkaloids, which mostly have basic nitrogen atoms (Stavroula and Rahul, 2016). The antioxidant activity was measured using FRAP, ABTS (2,2-azinobis (3-ethylbenzothiazolin-6-sulfonic acid) radical scavenging activity and DPPH (1,1-diphenyl-2-picrylhydrazylazinobis) scavenging activity. The FRAP ranged from 90.6mg TE/100 g for pulses and split pulses to 2773.5 mg TE/100 g. It was found that legume foods could remove between 4.5 and 194.9 mg of ABTS radicals per 100 g. The amount of DPPH radicals that pulses and split pulses could get rid of was between 42.9 and 571.1 mg TE/100 g (Parikh *et al.,* 2018). Rhizobacteria that help plants grow are attracted to these root exudates. It was important to find leguminous crops by synthesizing the *Rhizobia* nod factors that the root nodules need and the rhizobia that will finally colonize them (Via *et al.,* 2016).

Native *Rhizobium* and inoculated bacteria do well in blackgram root exudates. When seeds were inoculated, they germinated faster. This could be because they made more phytohormone, which affects seed germination (Mia *et al.,* 2012). Phytohormones like cytokinins and auxins are made by *Methylobacterium* and are known to help seeds sprout (Lee *et al.,* 2006). When *Rhizobium* BMBS + AMF + *M. extorquens* AM1 was added to seeds the roots (20.40 cm) and shoots (21.52 cm) length was increased. Researchers have already looked at how adding *Rhizobium* or PPFM to blackgram (Raja *et al.,* 2019) and pigeon seeds affected their ability to germinate and grow stronger. It is possible that the production of indole acetic acid (IAA) by *Rhizobium* (Mohite, 2013), PPFM (Pattnaik *et al.,* 2017) and *B. velezensis* (Meng *et al.,* 2016) led to the growth response of longer roots. The rise in seed germination and seedling length was seen as typical responses to gibberellins. Microbes were known to change the amount of ROS when seeds germinate (Gomes and Garcia, 2013). Root nodulation in blackgram crops will work differently depending on where they are grown, the type of soil they are in and how much nutrients are in the soil. People are becoming more interested in using the phytomicrobiome for sustainable farming, and this field is growing very quickly (Lakshmanan *et al.,* 2014). In addition to making nutrients available, blackgram root exudates help enzymes work better in the soil. In this experiment 30 local isolates were isolated from Madurai District. The strains were subjected to biochemical characterization, and 5 isolates were screened based on the confirmation. The isolates were identified based on molecular studies and confirmed as *R. nepotum* (Rn) and *R. pongamiae* (Rp). These two strains were tested by roll towel technique and pot culture experiment.

The current study of pot culture experiment revealed that the treatment T_4 - *Rhizobium* isolates 2 performed better for root length (22.3 cm/plant), shoot length (52.2 cm/plant) and plant dry weight (8.3 cm/plant) followed by T_5- *Rhizobium* isolates 3 and *Rhizobium* strain BMBS 47. Maximum root volume (0.32 cc/plant), maximum root nodules (14 No.s/plant), nodule weight (1.2 g/plant) and plant N (238 kg/ha) were observed in the treatment T_4 (Rn) followed by T_5- *Rhizobium* isolates 3 (Rp). The result of the pot experiment revealed that *R. nepotum* (Rn) performed better and subjected to field experiment. The current study of fields experiment results revealed that the T_4 - *R. nepotum* performed better for soil *Rhizobium* population (27 x 10^{-4} cfu/g soil), followed by T_3 - *Rhizobium* standard *strain* (BMBS 47). The maximum root length (12 cm/plant), root nodule (12 No.s/plant), nodule weight (1.4 g/plant), shoot length (37 cm/plant), plant wet weight (52 g/plant), 100 grain weight (3 g/plant) and yield (596 kg/ha) were observed in the treatment T_4- *R. nepotum* followed by T_3- *Rhizobium* standard strain (BMBS 47).

The activity of enzymes in the soil is affected by a lot of things, including the weather, the crops that are grown, the amount of organic matter in the soil, and the amount of nitrogen, phosphorus and sulfur in the soil as well as heavy metal pollution (Yang *et al.,* 2006). Also, the number and types of microbes in the soil affect the function of enzymes (Zhang *et al.,* 2017). Dehydrogenase activity is closely linked to the types of microbes that live in the soil and the variety of their community structure (Zhang *et al.,* 2014). Phosphatase and urease activity are also closely linked to the types of microbes that live in soil (Fernandez-Calvino *et al.,* 2010). There are many authors who say that the types of microorganisms in the soil can change based on the plants that grow there, especially the number of dominant species and the length of time that the plants are there (Bartelt-Ryser *et al.,* 2005). There may be differences in the microbial population and by extension the enzyme activity in the soil because of the number of small roots and the metabolic activity of those roots, as well as the amount and chemical make-up of plant litter (Eisenhauer *et al.,* 2017). It is possible to study the relationships between the main plant species their variety, abundance and biomass as well as the abiotic substrate parameters and soil enzyme activity at these types of post-industrial sites.

The current field experiment supported the above results of soil enzyme activities. The find treatment T_4 with *R. nepotum* (*Rn*) had the highest levels of urease (58.4 µg of NH_4 /g/24 h), dehydrogenase (94 µg of TPF /g/24 h), acid phsophatase (189 µg of p-nitrophenol/g/h) and alkaline phosphatase (400 µg of TPF /g/24 h). These were

followed by the treatment T_3 with *Rhizobium* standard strain (BMBS 47). This was backed up by Wang *et al.* (2011) discovery of the connections between plants, biomass (productivity) and soil enzyme activity in the base of spoil heaps left over from hard coal mine. Researchers who looked at semi-natural alpine fields had already found these kinds of connections. The results of the field experiment showed that *R. nepotum* helped plants grow and develop better in the field. This is because plant root exudates make the soil's microbes and enzymes work harder. The introduced microbes and local microbes made the enzyme activities in the soil higher. It depends on how the genotype of the legume the strain of *Rhizobium* and the current environmental factors work together for nitrogen fixation to work (Sohail Ahmad *et al.,* 2009). Some experts say that the ability to fix nitrogen is passed down through genes, which means that some types of common beans already have the ability to do this naturally. Because of this, the genotype of the plant is very important for developing symbiosis between plants and beneficial bacteria. Those that use carbon compounds as nitrogen stabilizers work best for bacterial symbiosis (Giller, 2001). The result of the current field experiment revealed that the indigenous *R. nepotum* (Rn) strain isolate from blackgram of Madurai District performed well for blackgram growth and yield. It is further recommended for multilocation trail and farmer practice of blackgram crops in Madurai District.

5. Conclusion

The present investigation clearly demonstrates that the performance of nodule formation varies among crops and across agro-ecological regions with indigenous *Rhizobial* strains often outperforming synthetic or imported strains due to their superior compatibility with native root exudation patterns. Blackgram roots release a diverse array of bioactive compounds including flavonoids, phenolics, lectins, tocopherols, essential minerals, organic acids and antimicrobial metabolites which possess antibacterial, antifungal, anti-inflammatory, anti-androgenic and anticancer properties. These phytochemicals, particularly flavonoids, isoflavonoids and phenolic acids act as potent signaling molecules that attract and activate beneficial rhizobacteria, facilitating effective symbiosis and nitrogen fixation. Additionally, root exudate components such as 3-O-methyl-D-glucose, n-hexadecanoic acid and other antioxidant and antimicrobial compounds selectively suppress pathogenic microbes while promoting the proliferation and activity of indigenous microbial communities. In this context, the indigenous *R. nepotum* strain isolated from the blackgram fields of the Madurai District exhibited markedly superior nodulation, plant growth, soil enzyme activity and yield enhancement compared with non-native and commercial strains, reflecting its strong ecological adaptation to local soil and biochemical conditions. Therefore, *R. nepotum* is recommended as a highly effective indigenous bioinoculant for boosting blackgram productivity with multilocation field trials necessary to validate its consistency across diverse agro-climatic environments.

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Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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