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Sequencing and Phylogenetic Analysis of nodC Gene from Pea Rhizobium

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***Abstract*—** Pea is a legume that belongs to the family Fabaceae and an economical source of carbohydrates, proteins, and vitamins. Nitrogen deficiency is the main problem of crops, which is hard to overcome. However, pea plants can establish a symbiotic relationship with the bacteria known as *Rhizobacterium* and efficiently utilize the available nitrogen through the roots, and the plant can withstand even harsh conditions. Nodule, which is established by these rhizobacteria are main parts which are involved in the process of nitrogen fixation; Nod factors are stimulator for the establishment of nodules in the rhizobia of pea NodC gene is one of these factors, used as a genetic marker to analyze the genetic variation among *Rhizobacterium* keeping in view the capability of nitrogen fixation. In this purposed research, the nodC gene was amplified using PCR technology, cloning, sequencing, and phylogenetic analysis to study the homology and divergence in *Rhizobium* from other species that have these Nod factors in establishing the nodulation and enhancing the nitrogen fixation in the leguminous species.

Keywords— Pea, Phylogenetic analysis, NodC Gene, Rhizobium

I. INTRODUCTION

Legumes are considered the mainstay of agriculture [1, 2]. Globally, Legume grains are the primary food source that provides extra nutrition compared to any other crop. [3]. With more than 50% of repetitive sequences, pea has the smallest genome size of approx. 430 Mb, and is enriched with resources available for molecular biological studies [4]. Nitrogen seed demand is moderately fulfilled by nitrogen acquired by the roots and vegetative organs that remobilize the nitrogen [5]. There is a dire need for proper attention to enhance the growth of pea worldwide, and different techniques have been developed to enhance the rate of growth regulators, which proved very helpful to increase the development and production rate of pea crops. Gibberellin acid and cytosol are the primary growth-enhancing regulators playing a central part in the best production of seed rate and yield of that crop [6].

The symbiotic relation of useful bacteria with legume crops is the most critical factor for the high growth rate of crops. In pea, rhizobia are the bacteria found in pea roots and provides benefits to crop by fixing the atmospheric nitrogen into the soil nitrogen. An atmospheric form of nitrogen is not in a useful state for the crop, so the crop requires to convert this form of nitrogen into the soil form so that the crop can easily use this nitrogen for total production [7]. For the active fixation of nitrogen, a symbiotic relationship is vital between the rhizobia and leguminous plants [8]. Crop production is increased by the treatment of a plant with growth-stimulating bacteria, and these bacteria play an influential part by increasing the concentration of growth regulators, which enhance the uptake ability of nitrogen [9].

Nodules are the structures present in the roots of pea crops in which these helpful bacterial species are found. These bacteria have many advantageous effects on the yield of pea crops. Nutrient uptake ability and health of the plant are improved by the biological activity of these bacterial species [10]. *Rhizobium* bacteria produce Nod factor which stimulates the root nodules formation in the leguminous plants. Nod factor has many genes that are complexed in the nodule formation. All the genes are essential and work together. The missing of a single gene will not lead to the formation of a root nodule [11].

Rhizobium bacteria live as intracellular in the leguminous plants such as pea and maintain an endo-symbiotic relationship. Fixation of nitrogen occurs due to this association of this symbiosis between the plant and rhizobia [12]. Through the threaded mode intracellular reaction generally, rhizobia enter the host plant roots tissue after the colonization of roots hair tips [13]. Based on the development of new roots to the older part of the roots. There are three zones the zone one is the new growing tips

and hair zone, two is sub scribal zone, while root growth is terminated by zone three carrying root hairs [14]. Root hairs, as a part of susceptible zone, response to Nod factors producing rhizobia, determining the curling and deformation of the root. Small shape changes become because of curling are called spheres cook the rhizobia entrapped in them. The rhizobia enter the root hairs intracellular through inward groaning infection thread [15]. These infectious threads lead the rhizobia to the developing nodules called primordial, present at the cortical cell division. Nod factors form basic nodulation. These plants mostly respond at the epidermis, pericycle, and cortex [16].

The NodC gene produces the main structure of the Nod factor along with the help of NodA and NodB. Different alleles of NodC are different from each other, and the similarity range is from 40 to 100% [17]. NodABC genes are considered compulsory genes in all the *Rhizobium* species to stimulate the nodulation and enhance a symbiotic relationship between plant and rhizobia. NodC proteins are also crucial for the synthesis of chitin oligomers [18]. NodC gene produces a protein having 44,000 Da molecular weight. These genes bind to the external membrane of the root cell and cause the nodulation infection, which leads to the symbiosis relation for the nitrogen fixation [19].

Different phytochemicals, flavonoids, and taproot excrete are assessed to induce the activity of the nodC gene. Flavonoids were defined to induce the nodC gene at 7pH, but at a different scale, although very low induction was observed in acid-tolerant isolates at acidic pH. In the pea roots, the exudates released seem to no change in their capability to induce the activity of the nodC gene. The early stage of interaction is root adhesion, colonization, root hair curling, and branching. The process of mutual molecular signaling determines its specificity. From the plant root exudates, rhizobia recognize the flavonoids and respond with lipochito-oligosaccharide (LCOs) molecules, which are nod factors. These molecules are responsible for different legume response like root cell division, deformation of root hair nodulation gene transcription in root hairs. Different flavonoids like (coumestans, flavonols, flavones, flavanones, chalcones, and isoflavonoids) some of these can inhibit or induce the nod gene expression. Flavonoids can also act as a growth enhancer in *Rhizobium*. For all the rhizobial strain, the rhizobial gene nodABC are common. NodC factor chain length is determined by nodC protein to be a host specificity measure made by the nod factors [20].

Genome editing can be a significant source to enhance the functioning of the nodC gene, which will ultimately lead to the improvement in the nitrogen fixation ability of crops, it will be possible only in that case if we have complete information of the related gene. The aim of the proposed research was to clone and sequence the pea rhizobium nodC gene to develop transgenic for better N-fixation.

II. RELATED WORK

The most important benefit of pea is its ability to fix the nitrogen; *Rhizobium* is responsible for the fixation of nitrogen in the roots of a pea. Rhizobia can induce the nodule in the roots of the plant, and ultimately these nodules are the source that fixes the nitrogen in the roots and provides it to the whole plant [21]. Rhizobiaceae belongs to the signal transduction family and compulsory for the formulation of nodules in the leguminous plants [22].

Peters and Verma [23] studied multiple bacterial strains of *Rhizobium*, *Azorhizobiu*, *Sinorhizobium*, collectively known as rhizobia, existing in the soil can blight plant roots resulting in the symbiotic relationship by the nodule formation. NodC gene was also cloned and sequenced for developing its genetic variation with related strains in different crops.

Plant flavonoids induce the bacterial nodulation genes that determine the synthesis of the main nodulation signal molecules, Nod factors [24]. Both the number and the type of Nod factors are crucial in defining host specificity. However, rhizobia producing different Nod factors and with different nod genes can effectually nodulate the same plant. For example, in the case of *Rhizobium tropici*. and *Rhizobium etli bv. phaseoli*, both of which are nitrogen-fixing symbionts in the common bean (*Phaseolus vulgaris*) [25, 26]. Rahi et al. [27] isolated the 120 different strains of bacteria from the *Pisum sativum* that caused root nodules, and further cultivated at different locations in trans-Himalayan valleys at in Himachal Pradesh, India. Using 16S rRNA gene recA, atpD, PCR-RFLP, nodC, sequencing of 16S rRNA, ERIC-PCR, nifH carbon source utilisation pattern (BIOLOG), and whole-cell fatty acid profiling, the sequence was studied.

III. METHODOLOGY

Centre of Agricultural Biochemistry and Biotechnology (CABB), University of Agriculture, Faisalabad, Pakistan, is selected for the implementation of the proposed research.

Sample collection of root nodules: Ayub Agriculture Institute (AAR1) Faisalabad, Pakistan, was the Centre from where pea samples were collected. 'Meteor' cultivar was the best to be grown in the fields of University of Agriculture Faisalabad, Pakistan. Seeds germinated in the field for eight weeks. After that, seedlings were uprooted, and roots were transferred to the ice. After that these were carried to the laboratory, and distilled water was used for proper washing, soil particles were washed thoroughly.
Then roots were used to remove the nodules by the use of the surgical blade. Healthy, undamaged, and fresh nodules were treated with ethanol for 1 min and kept in a laminar flow hood in the solution of calcium hypochlorite solution (3%) for 5 min. Nodules were again treated with the distilled water for 5 min. For complete sterilisation of yeast

extract, mannitol was used to culture nodules. Treatment was applied for 3 days while maintaining the temperature at 26 ±2 ºC [28].

Isolation of root nodule rhizobia: After washing nodules were crushed by using the sterile water by making a suspension of 20µL in a Petri dish. After adding Congo red in the Yeast Extract Medium, Suspension was cultured on this medium at 28±2 ºC till bacterial colonies were grown completely. To get pure colonies of bacteria sub-culturing was done after 3 days [29]. 60% glycerol was mixed with the equal volume of bacterial colonies and the temperature was kept at -80°C. The recipe of YEM is given in Table 1.

Table: 1- Composition of YEM (Yeast Extract Mannitol) Medium

Ingredients	Concentration (%)
Mannitol	1.0
MgSO4. 7H2O	0.1.0
K2HPO4	0.04
Yeast Extract	0.05
NaCl	0.02
Agar	1.5 to 2
Congo red	5ml
pH	7.2

DNA extraction of *Rhizobium*: *Rhizobium* DNA was extracted by following the Sambrook et al. [30] procedure with changes. Bacterial colonies were grown on the YEM broth at 28 ±2 °C for three days. After that medium was centrifuged at 8,000 rpm for 10 minutes to get the pellet, then the obtained pellet was suspended with the use of TE buffer in the 500 µL in which lysozymes (100 µL) were present in the concentration of 15 mg/ mL and RNAase (50 µL) was 10mg/Ml. 30 µL SDS (10%) was added to this mixture after keeping this mixture at 37 °C for 40 min. Incubation for the mixture was done for 20 min at the temperature of 70 °C. Then proteinase K (20mg/mL) in the concentration of 10 µL was added to the mixture at the temperature of 45 °C and stored for 2 hrs.

Centrifugation was repeated at increased speed i.e., 13,000 rpm and for 15 minutes, and the supernatant was precipitated with mixture of phenol and chloroform (25:24). Then precipitation was done with the addition of chloroform/ isoamyl alcohol (24:1). At the end, Na-acetate (3M, pH 5.2) 1/10th volume of and isopropanol 0.6 vol. were added to the supernatant by maintaining the temperature at -80 °C for 30 min. Last centrifugation was done for 15 min at 15,000 rpm, and 1 Ml of 70% ethanol was used to wash the pellet, and a laminar flow hood was used for frying the pellet. After that, TE buffer was used to dissolve the pellet. Then DNA was stored at 4 °C for use.

Bacterial DNA Quantification: 0.8 % Gel in TAE buffer was used to determine the quality of isolated DNA by using gel electrophoresis at the voltage of 55V for 55 min. A spectrophotometer (CECIL, CE 2021) was used to determine the quantity of DNA in the mixture. For this purpose, 49 µl of distilled water was added to 1µl of DNA to make dilutions. DNA was quantified by taking the absorbance at 260 nm wavelength using water as blank.

Following primers were used to amplify the nodC gene of bacteria:

Forward Primer:

nodCf 5'-ATGCCATTGATCCGAGATTC-3'

Reverse Primer:

nodCr 5'-CTATCGCGTCTGGGACGTAT-3'

The primers were synthesised from e-oligos Gene LinkTM [31].

PCR Amplification of nodC gene: Eppendorf thermal cycler was used for amplification of nodC, gene; Reagents were as in Table 2.

Table 3.2-Reagents of PCR amplification

Reagents	Quantity (µL)
PCR Master Mixture (dNTPs, Tag polymerase, MgCl2, Taq buffer, d3H2O)	17.0
Forward Primer (10 µm)	1.0
Reverse Primer (10 µm)	1.0
DNA Template (1/49 µl)	1.0
Total Reaction Mixture	20.0

PCR Profiling: PCR amplification was carried using the cycling program is given described. For the amplification of nodC the temperature of the lid was maintained at 105ºC, and cycling was as in Table 3.

Table 3.3- PCR cycling program

Stages	Temperature	Time
Initial Denaturation	94°C	10 min
Denaturation	94°C	30 sec
Annealing	52°C	30 sec
Extension	72°C	30 sec
Final Extension	95°C	5 min
Storage Temperature	4°C	

Electrophoresis of PCR amplified product: PCR amplified product was resolved on 1 % agarose gel in buffer of 0.5 X TAE (Tris-acetate-EDTA) with 3µL ethidium bromide (10mg/mL). DNA weight markers of 100bp and 1 kb (GeneRuler, Fermentas) were run as a marker for size determination. The gel was visualised under UV light and photographed.

Gel elution of DNA fragment: The DNA fragment of 575bp amplified using specific primers was cut from the gel under UV light using a surgical knife (Feather safety razor, Japan). By using a Thermo Scientific Gel elution kit (Kit # ICO691) according to the instructions given at manual and technique described by Silva et al. [32], the desired DNA fragment was eluted from the gel.

Cloning of amplified DNA: Gel eluted DNA fragment was cloned in cloning vector-using TA cloning kit Fermentas according to the instructions on operating manual.

The ligation mixture consisted of the following reagents Table 4.

Table 4- Ligation reagents

Reagents	Quantity
Vector	1µL(50ng)
Insert	1 µL(25ng)
Ligation Buffer	1µL
T4 DNA Ligase	0.1µL
Deionised H2O	6.9µL
Total volume	10µL

Reaction was carried overnight at 4 ºC.

Preparation of Competent cells: E. coli strain DH5α was used as competent cells. Competent cells stored at -80 °C were thawed and streaked with a wire loop on LB agar plates and placed these plates in an incubator at 37 °C for overnight. A single colony was picked with the help of a toothpick, dissolved in 6 ml LB medium in a culture tube, cultured at 37ºC with gentle shaking at 150rpm, and allowed to multiply overnight [33].

This primary culture was used to further inoculate 120 ml LB to prepare secondary culture. The secondary culture was also cultured at 37 ºC in an incubator with shaking at 150rpm for 5 h to achieve OD600 = 0.5. The mixture was spinning for 12 min at 4 °C and 3500 rpm, the supernatant was discarded and the pellet was put on ice. MgCl2 (25 ml, 0.1M) ice-cold was poured on the pellet suspended by gentle shaking and spinning at 3500rpm for 10 min to pellet the cells. The upper layer was removed gently and the pellet was suspended in cold 0.1 M, CaCl2 (5 ml), and glycerol (15 %) and mixed gently for five minutes.

The mixture was centrifuged again at 3500 rpm for 10 min at 4 °C, and the pellet was stored at 4 ºC overnight. Pellet was again suspended in 15% glycerol and 0.1 M CaCl2 and aliquots of 100ml mixture of competent cells were prepared and stored at -80 ºC.

Transformation into competent cells by heat shock method: The heat shock method [34] was applied to transform E. coli competent cells. Ligation product (3µl) was mixed with 100µl competent cells and put on ice for 30 min. The mixture was put at 42 ºC for 90 sec. This heat treatment made the membrane-permeable and allowed the entry of recombinant plasmids into the competent cell DH5α.

The tube was immediately transferred to ice and put for 5 min. This treatment allowed membrane to regain its normal position. About 300µl LB medium was added, mixed the contents thoroughly and incubated at 37 ºC for 60 min. Cells were distributed on plates of LB agar containing ampicillin (50mg/ml), X-gal, and IPTG. Plates were put in the laminar flow to absorb the liquid and become dry. The plates were sealed with parafilm and incubated at 37 ºC for eight hours. Blue and white colonies were observed in the petri plates. White colonies were marked and were used for colony PCR to determine positive colonies. For this purpose, white colonies were removed with toothpick and dissolve in PCR mixture containing following reagents as in Table 5 and Number of cycles are explained in Table 6 and 7.

Table 3.5- Reagents for Colony PCR

Reagents	Quantity (µl)
PCR master mixture (Dream Taq)	15 µl
Transformed white colony	1 colony per tube
Left Primer	2(10 µM)
Right Primer	2(10µM)
Total reaction mixture	20 µl

Table 6- PCR cycling

Stages	Temperature	Time
Initial denaturation	95 ºC	5 min
Denaturation	95 ºC	60 sec
Annealing	56 ºC	60 sec
Extension	72 ºC	90 sec
Final extension	72 ºC	5 min

Table 7- Number of cycles at every stage

Stage	Number of Cycles
Denaturation	1
Start Loop	25
Final extension	1

Isolation of Plasmid DNA: Colonies confirmed positive for carrying insert were used for plasmid DNA isolation. Plasmid DNA isolation kit (QiaGen Science, USA) was used and method followed was described by Chen et al. [35].

Purification of plasmid DNA: Isolated Plasmid DNA was further purified by the PEG method (polyethylene glycol) explained by Li et al. [36].

Sequencing and Data Analysis: Isolated Plasmid DNA was used for cycle sequencing and subsequent sequencing [37]. ABI Prism Applied Biosystems 310 DNA Sequencer was used for sequencing. Sequencing was carried in both directions using forward and reverse primers. DNA sequencing data were subjected to bioinformatics tools for homology search, deduced amino acid sequence, alignment, multiple alignments, and construction of the phylogenetic tree. The data were aligned with related sequences in the databases. The EXPASYsoftware and NCBI Blast (nucleotide and amino acid) at the National Centre of biological information, Expasy, JustBio, and ClustalW Softwares were used. MEGA 6 software was used to construct a phylohenetic tree.

IV. RESULTS AND DISCUSSION

PCR amplification of bacterial nodC:

By using the PCR technique nodC gene was amplified, which was of 575bp in length, and that was isolated from the pea rhizobia. A universal pair of primers (nodCF and

nodC1) was used for this purpose. Reagents of PCR amplification were optimised according to requirements. Gradient PCR was used for the optimisation of temperature at the annealing stage, which was 52°C. The samples were sequenced at the CABB department of UAF, Pakistan. Best results were observed when dNTPs, MgCl2, Primer, and annealing temperature were 2.5 mM, 1Mm, and 54°C, respectively. Amplification of nodC is visible in Figure 1.

Blue-white screening for positive clones:

Amplified Fragments were cloned into the vector after purification of amplified PCR from the gel, and the heat shock method was used for the transformation of the competent cell. LB agar plates were prepared with the different concentrations of ampicillin, X-GAL, and IPTG, and these reagents were in50 µg/ml, 40 mg/ml, and 40 µl, respectively Figure 2. Mini-prep Kit was used by following the procedure instructed by the manufacturer to isolate the Plasmid. For insert check, EcoR1 and BamHI enzymes were used to restrict the clone. The First band was about 2736 bp which was a vector and the lower band is 585 bp which represents the insert Figure 2.

Sequencing of cloned nodC gene:

Using specific primers discussed in materials and methods, the NodC gene fragment was amplified. PCR product was e ectrophoresed, gel eluted, and finally cloned into a cloning vector. Plasmid DNA was isolated and the fragment was sequenced. The deduced nucleotide sequence is presented in Fig 3. Sequencing was conducted from both sides using forward and reverse primers. The deduced sequence was translated into amino acids and presented in Figure 4.

Sequence Homology using BLAST:

In the database, the sequence was compared with the previous sequences that were available. BLAST was used and the *Rhizobium* clone in the NCBI database was compared with the corresponding sequences. The Pea *Rhizobium* clone has been found to have elevated homology more than 99% with related *Rhizobium* clones (*Rlv. trifoli* 0.01283, *Rhizobium sp*. BIHB, *R. phaseoli* and *Rlv. SEMIA Rhizobium sp*. and pea *Rhizobium*) presented in (Figure 5) and (Figure 6). In (Figure 5), the *Rhizobium* clone was compared with the *Rhizobium leguminosarum* isolates PS25-2, and there was complete homology (100%). In Figure 6, homology between the *Rhizobium* clone and *Rhizobium leguminosarum bv. viciae* strain LMR620 was 99%. Multiple sequence alignments were conducted, also are presented in Figure 7.

Phylogenetic analysis using MEGAX:

The phylogenetic tree was constructed using MEGAX is shown in (Figure 8). The phylogenetic data revealed that the sequence was closely related to *Rhizobium*-0.00069, pea *Rhizobium*, Mesorhizobium0.12447, *Rhizobium sp.* BL160.02645, *Rhizobium Burkholderia*, and *Rhizobium sp*. BL210.12273 were distantly related.

Discussion

Atmospheric nitrogen is fixed by the bacteria rhizobium found in the nodules of roots in this crop to enhance soil productivity [38]. Without any expenditure large quantity of nitrogen is accumulated in the soil through the nitrogen-fixing activity of bacteria to increase the growth and production of pea. The number of nodules, cultivar, and several bacterial strains are the main important factor on which phenomena of nitrogen fixation depends. Immunisation of roots of crops with rhizobia is considered a great source of improvement in growth rate and production of pea [39, 40].

Solubilisation of phosphate, nitrogen fixation, production of indole acetic acid, and biofertilisers are the main important methods for improving the crop but the process of biofertilisers has proved most significant as reported by Vessey [41] and Saharan and Nehra [42]. Pea plants obtain nitrogen by 1) Supplemented by ammonia and nitrate manure or fertiliser given to the soil, 2) decomposition of organic matter, 3) nitrogen transformation by natural approaches, and 4) biological nitrogen fixation [43].

Rhizobacteria are essential biological weapons to fix atmospheric nitrogen by increasing the root growth and formation of nodules in the roots [44]. Several research groups/administrations are involved in developing biofertilisers and are trying to enhance the use of biofertilisers [45]. At the molecular level, identification of bacterial species is made by the nodC gene sequencing, which is used in research and examination [46, 47]. Manual methods for identification and distinction between species provide unsuccessful results. These types of results created incorrect data [48]. NodC gene has been found in bacteria, and the study of this gene about its feature and functions has been done using a genetic marker [49]. By collecting information about sequences, genetic distance can be measured between strictly connected species of bacteria, and this way of measurement is reflected as most accurate [50, 51].

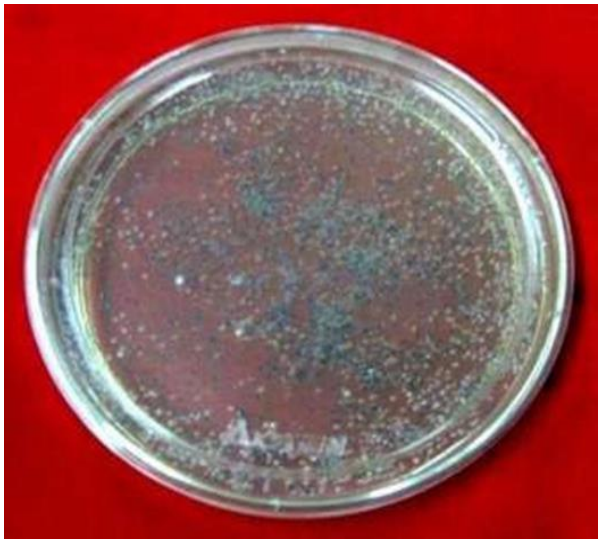

Figure 1. Blue white screening for positive clones

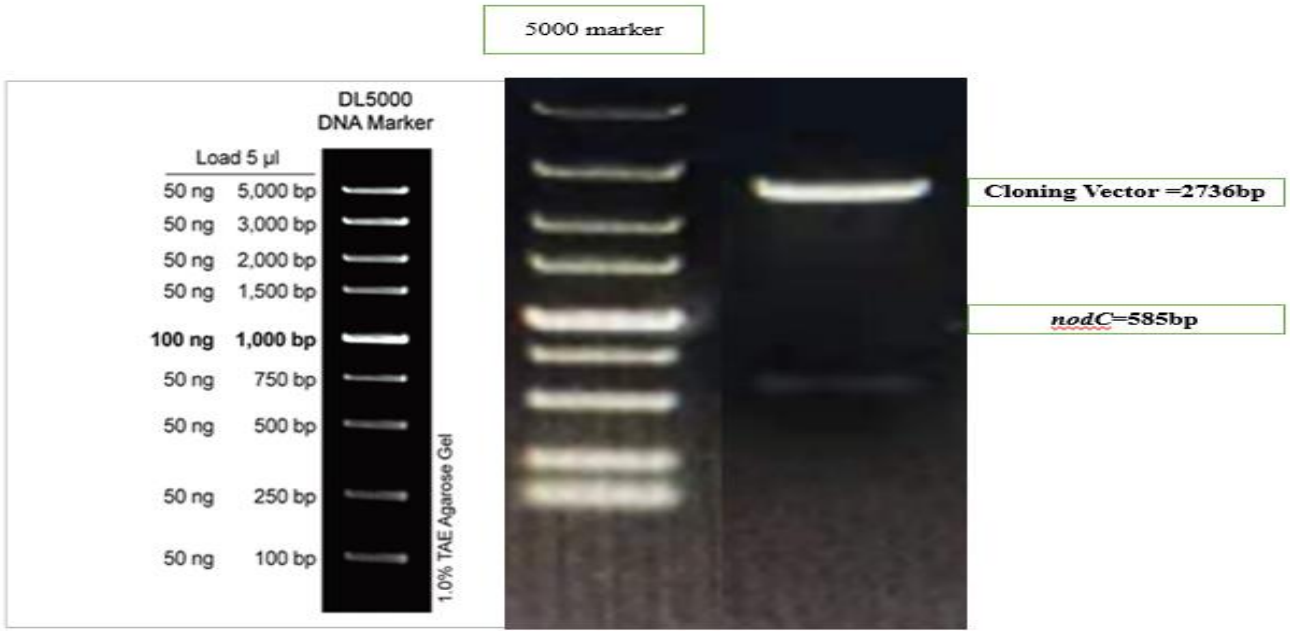

Figure 2. Restriction analysis of rhizobium clone using EcoRI and BamHI enzymes. L lane= ladder (5kb), 1-lane= two bands (upper for vector, lower for insert)

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1-GCCTGCAACGAGGAGCGCGCGGCACAAGCTCGCTTCGGTGCAGTTATGTGTTGCTGCGGC-60
61-CCATGTGCGATGTACCGTCGGTCTGCTATGCTTTCGCTGCTCGATCAGTACGAGACGCAG-120
121-CTTTATCGCGGCAAGCCGAGTGACTTCGGCGAAGATCGCCATTTGACGATTCTCATGCTG-180
181-AGCGCAGGCTTTCGAACTGAGTATGTTCCGAGTGCCATCGCGGCGACAGTCGTTCCAGAC-240
241-ACGATGGGTGTTTATCTACGCCAACAACTACGGTGGGCACGCAGCACCTTTCGGGATACT-300
301-TTGCTTGCGCTTCCTGTACTGCCTGGTCTCGATCGGTATCTCACGCTGGACGCAATAGGG-360
361-CAAAATGTCGGCCTGCTACTTCTTGCGCTGTCGGTATTGACAGGAATTGGCCAGTTTGCG-420
421-CTGACCGCCACACTGCCCTGGTGGACGATCCTGGTCATTGGATCCATGACTCTTGTACGC-480
481-TGTAGCGTGGCTGCCTATCGCGCCCGCGAACTTAGGTTTTTGGGTTTTGCTCTCCACACG-540
541-CTCGTGAACATCTTTCTCTTAATTCCCTTGAAGGC-575
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Figure 3. : Deduced nucleotide sequence of nodC gene from pea rhizobium

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Gcctgcaacgaggagcgcgcggcacaagctcgcttcggtgcagttatgtgttgctgcggc
 A  C  N  E  E  R  A  A  Q  A  R  F  G  A  V  M  C  C  C  G
ccatgtgcgatgtaccgtcggtctgctatgctttcgctgctcgatcagtacgagacgcag
 P  C  A  M  Y  R  R  S  A  M  L  S  L  L  D  Q  Y  E  T  Q
ctttatcgcggcaagccgagtgacttcggcgaagatcgccatttgacgattctcatgctg
 L  Y  R  G  K  P  S  D  F  G  E  D  R  H  L  T  I  L  M  L
agcgcaggctttcgaactgagtatgttccgagtgccatcgcggcgacagtcgttccagac
 S  A  G  F  R  T  E  Y  V  P  S  A  I  A  A  T  V  V  P  D
acgatgggtgtttatctacgccaacaactacggtgggcacgcagcacctttcgggatact
 T  M  G  V  Y  L  R  Q  Q  L  R  W  A  R  S  T  F  R  D  T
ttgcttgcgcttcctgtactgcctggtctcgatcggtatctcacgctggacgcaatagggg
 L  L  A  L  P  V  L  P  G  L  D  R  Y  L  T  L  D  A  I  G
caaaatgtcggcctgctacttcttgcgctgtcggtattgacaggaattggccagtttgcg
 Q  N  V  G  L  L  L  L  A  L  S  V  L  T  G  I  G  Q  F  A
ctgaccgccacactgccctggtggacgatcctggtcattggatccatgactcttgtacgc
 L  T  A  T  L  P  W  W  T  I  L  V  I  G  S  M  T  L  V  R
tgtagcgtggctgcctatcgcgcccgcgaacttaggttttgggttttgctctccacacg
 C  S  V  A  A  Y  R  A  R  E  L  R  F  L  G  F  A  L  H  T
Ctcgtgaacatctttctcttaattcccttgaaggc
 L  V  N  I  F  L  L  I  P  L  K
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Figure 4. Amino acid sequence of deduced nodC from pea rhizobium. The amino acid sequence is abreviated in the single letter code below the nucleotide sequence. Translator tool 'Justbio software' was used for analysis.

Figure 5. Comparison of the nucleotide sequence of *Rhizobium* Clone with *Rhizobium leguminosarum* isolates PS25-2 using BlastN.

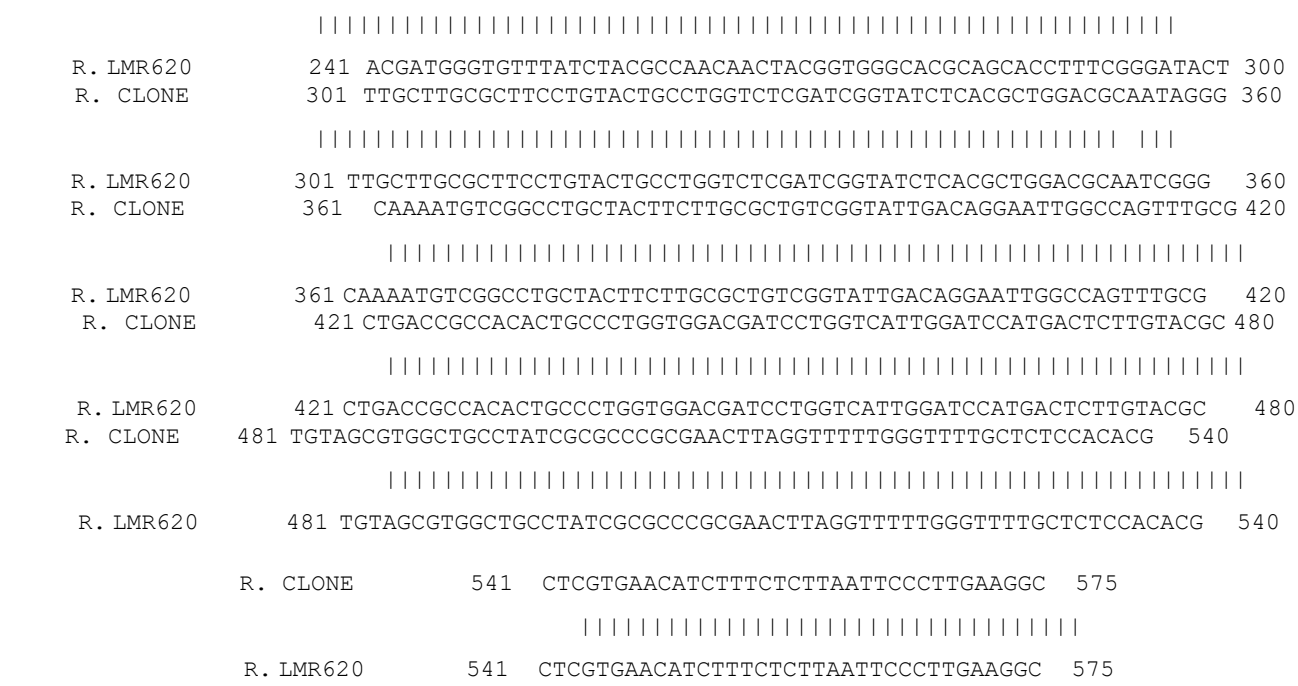

Figure 6. Comparison of the nucleotide sequence of *Rhizobium* Clone with *Rhizobium leguminosarum bv. viciae* strain LMR620 using BlastN

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Rhizobium   clone   GCCTGCAACGAGGAGCGCGCGGCACAAGCTCGCTTCGGTGCAGTTATGTGTTGCTGCGGC31
Pea.Rhizo           GCCTGCAACGAGGAGCGCGCGGCACAAGCTCGCTTCGGTGCAGTTATGTGTTGCTGCGGC31
Mesorhizobium ---------------------------- GCCCAAGCTCGTTTCGGTGCGGTTATGTGCTGCTGTGGC31
BL16 ------------------------------------------- TCGCTTCGGCGCTGTTATGTGTTGCTGCGGT31
Burkholderia ------------------------------------ TCGCTTCGGCGCTGTTATGTGTTGCTGCGGT31
BL21 ------------------------------------------- TCGCTTCGGCGCTGTTATGTGCTGCTGCGGT31
                                                  *** ***** ** ******** ***** **

Rhizobium   Clone   CCATGTGCGATGTACCGTCGGTCTGCTATGCTTTCGCTGCTCGATCAGTACGAGACGCAG91
Pea.Rhizo           CCATGTGCGATGTACCGTCGGTCTGCTATGCTTTCGCTGCTCGATCAGTACGAGACGCAG91
Mesorhizobium       CCATGTGCGATGTATCGCCGGTCGGCCCTGCTTATGGTTCTTGATCAATACGAAACGCAG91
BL16                CCATGTGCCATGTACCGCCGGTCCGCGCTCCTTTTGCTGCTAGATCAGTACGAGACGCAA91
Burkholderia        CCGTGTACCATATACCGACGGTCCGCGCTCCTTCTGCTGCTAGATCAGTACGAGACGCAA91
BL21                CCGTGTACCATATACCGACGGTCCGTGCTTCTTTTGCTGTTAGACCAGTACGAGACACAG91
                     ** *** * ** ** ** ***** *  * ***  * *  * ** ** ***** ** **

Rhizobium   Clone   CTTTATCGCGGCAAGCCGAGTGACTTCGGCGAAGATCGCCATTTGACGATTCTCATGCTG151
Pea.     Rhizo      CTTTATCGCGGCAAGCCGAGTGACTTCGGCGAAGATCGCCATTTGACGATTCTCATGCTG151
Mesorhizobium       CTGTTTCGTGGGAAGCTGAGCGACTTCGGCGAGGATCGCCATCTCACGATCCTCATGCTG151
BL16                CTTTTTCGCGGGAAGCCGAGCGACTTCGGCGAGGACCGTCATCTGACGATTCTCATGCTG151
Burkholderia        CTTTTTTGCGGGAAACCGAGCGACTTCGGCGAGGACCGCCATCTCACGATTCTCATGCTG151
BL21                TTTTTTCGGGGGAAACCGAGCGACTTCGGCGAGGACCGCCATCTCACGATTCTCATGCTG151
                     * * * * ** ** * *** *********** ** ** *** * ***** *********

Rhizobium   Clone    AGCGCAGGCTTTCGAACTGAGTATGTTCCGAGTGCCATCGCGGCGACAGTCGTTCCAGA210
Pea.Rhizo           AGCGCAGGCTTTCGAACTGAGTATGTTCCGAGTGCCATCGCGGCGACAGTCGTTCCAGA210
Mesorhizobium       AAA-GCAGGGCTTCGAACCGAGTACATTCCGGACGCTATCGCGGCAACGGTCGTGCCGGA210
BL16                ACG-GCAGGTTATCGGACCGTGTACGTTCACGACGCCATTGCGGCGACAGTCGTTCCAGA210
Burkholderia        ACG-GCAGGTTATCGGACCGAGTACGTTCCGGACGCCATTGCGGCGACAGTCGTTCCGGA210
BL21                ACC-GCAGGTTATCGGACCGAGTACGTTCCGGACGCCATTGCGGCGACAGTCGTTCCGGA210
                         *****   *** ** * ***  ***     ** ** ***** ** ***** ** **

Rhizobium   Clone   CACGATGGGTGTTTATCTACGCCAACAACTACGGTGGGCACGCAGCACCTTTCGGGATAC270
Pea.Rhizo           CACGATGGGTGTTTATCTACGCCAACAACTACGGTGGGCACGCAGCACCTTTCGGGATAC270
Mesorhizobium       TCGGCTGCGGCCATATCTACGCCAGCAACTGCGCTGGGCGCGAAGCACCTTCAGAGACAC270
BL16                CAGACTGGGGGCGTATCTGCGCCAACAACTGCGCTGGGCGCGCAGTACGTATCGGGACAC270
Burkholderia        CAGTCTGGGGGCGTATCTGCGCCAACAACTGCGCTGGGCGCGCAGTACCTATCGGGACAC270
BL21                CAGACTGGGGGCGTATCTGCGCCAACAACTGCGCTGGGCGCGCAGTACGTATCGGGACAC270
                          ** *   ***** ***** ***** ** ***** ** ** ** *   * ** **
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Rhizobium   Clone   TTTGCTTGCGCTTCCTGTACTGCCTGGTCTCGATCGGTATCTCACGCTGGACGCAATAGG330
Pea.Rhizo           TTTGCTTGCGCTTCCTGTACTGCCTGGTCTCGATCGGTATCTCACGCTGGACGCAATAGG330
Mesorhizobium       GCTGCTTTCGCTGCGCCTGCTACCCAGCCTCGATCGCTATCTCACCTTGGACGTGGTCGG330
BL16                GTTGCTTTCGCTGCGCCTGCTGCCCCGTCTCGATCGTTACCTTACGCTGGACGTGATCGG330
Burkholderia        GTTGCTTTCGCTGCGCCTGCTGCCCTGTCTCGATCGCTACCTTACGCTGGAGGTGATCGG330
BL21                GTTGCTTTCGCTGCGCCTGCTGCCCTGTCTCGATCGCTACCTTACGCTGGACGTGATCGG330
                       ***** **** *  * ** **  * ******** ** ** **  **** *   * **

Rhizobium   Clone   GCAAAATGTCGGCCTGCTACTTCTTGCGCTGTCGGTATTGACAGGAATTGGCCAGTTTGC390
Pea.Rhizo           GCAAAATGTCGGCCTGCTACTTCTTGCGCTGTCGGTATTGACAGGAATTGGCCAGTTTGC390
Mesorhizobium       GCAAAATGTCGGGCCGCTCCTTCTCGCAGTGTCGGTGGTAACAGGGATCGCGCACCTTGC390
BL16                ACACAATCTGGGCTCGCTATTTCTCGGCTTATCGCTACTAGCGGGTCTCGCGCAGCTCGC390
Burkholderia        ACTGAATCTGGGCTCGCTATTTCTCGCCTTATCGCTTCTAGCGGGGCTCGCGCAGCTTGC390
BL21                ACTGAATCTGGGCTCGCTATTTCTCGCCTTATCGCTTCTAGCGGGGCTCGCGCAGCTCGC390
                      *   *** * **   ***  **** *   * *** *  *  * **  * *  **  * **

 Rhizobium Clone    GCTGACCGCCACACTGCCCTGGTGGACGATCCTGGTCATTGGATCCATGACTCTTGTACG450

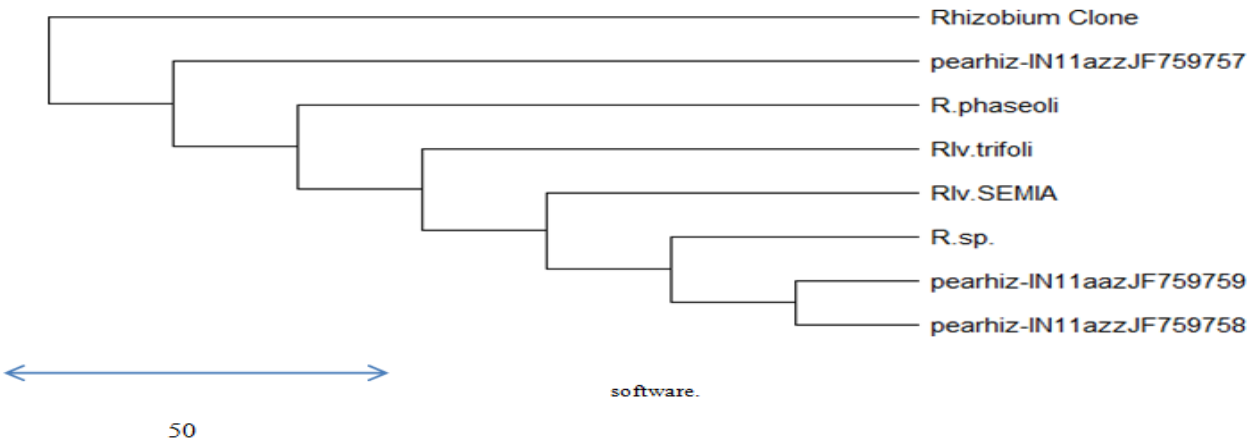
Pea.Rhizo           GCTGACCGCCACACTGCCCTGGTGGACGATCCTGGTCATTGGATCCATGACTCTTGTACG450
Mesorhizobium       GCTGACTGGCGAAGCGCCTTGGTGGACAGGCATGACGATCGCGGCCATGACGCTGATCCG450
BL16                ATTGACAGCGACAGTGCCGTGGTGGACTGCCCTGATAATCGCATCATCGACCATGATCCG450
Burkholderia        ATTGACAGCGACAGTGCCGTGGTGGACTGCCCTGATAATCGCATCATTGACCATGATCCG450
BL21                ATTGACAGCGACAGTGCCGTGGTGGACTGGCCTGACAATCGCATCATTGACCATGATCCG450
                       **** *          *  *** ********   * **   ** *   *   ***  *  * **

Rhizobium   Clone   CTGTAGCGTGGCTGCCTATCGCGCCCGCGAACTTAGGTTTTTGGGTTTTGCTCTCCACAC510
Pea.Rhizo           CTGTAGCGTGGCTGCCTATCGCGCCCGCGAACTTAGGTTTTTGGGTTTTGCTCTCCACAC510
Mesorhizobium       CTGCAGCATGGCAGCGTTTCGTGCTCGCGAATTGCGATTTCTGGGATTTTGTGTCCATAC510
BL16                CTGTAGCGTGGCATCGGTTCGCGCTCGCCAGGTTCGATTTCTTGGCTTTTCTCTTCACAC510
Burkholderia        CTGTAGCGTGGCATCGGTTCGCGCTCGCCAGATTCGATTTCTTGGCTTTTCTCTTCACAC510
BL21                CTGTAGCGTGGCATCGGTTCGCGCTCGCCAGATTCGATTTCTTGGCTTTTCTCTTCACAC510
                       *** *** ****      *   *** ** *** *  *  * *** * ** ***  * * ** **

Rhizobium Clone     GCTCGTGAACATCTTTCTCTTAATTCCCTTGAAGGC ---------------------- 575
Pea.Rhizo           GCTCGTGAACATCTTTCTCTTAATTCCCTTGAAGGC ---------------------- 575
Mesorhizobium       ACCCATCAATCTGTTCCTGTTACTTCCGTTGAAAGCGTATGCGCTTTGCACC       575
BL16                ACCCATTAACCTCTTTCTCTTGCTCCCCCTGAAAGTCTATGCGCTGTGTACATTGAGCAA575
Burkholderia        ACCCATTAACCTCTTTCTCTTGCTCCCGCTGAAAGCCCATGCGCTGGGTACATTGAGCAA575
BL21                ACCCATTAACCTCTTTCTCATGCTCCCCCTGAAAGCCTATGCGCTGTGTACATTGAGCAA575
                       * * * **                  * ** **  *  * **  **** *

 Rhizobium Clone    ----   575
 Pea.Rhizo          ----   575
 Mesorhizobium      ----   575
 BL16               CAGC   575
 Burkholderia       CAGC   575
 BL21               CAGC   575
```

Figure 7. Multiple alignments of constructed nucleotide sequence of *Rhizobium* Clone with associated sequences in the data base using ClustalW



Rlv. trifoli 0.01283, *Rhizobium* sp. BIHB, *R. phaseoli* and *Rlv. SEMIA Rhizobium* sp.

Figure 8. Phylogenetic tree of *nodC* of pea strains with other rhizobial strains, phylogenetic analysis done with MEGAX.

A symbiotic relationship is present between the bacteria *Rhizobium*, which is present in the nodules of root and crop [52]. The symbiotic relationship proved very healthy and fruitful for crop production and soil fertility [53]. One of the reasons for the adverse effects of co-inoculating PGPR with *Rhizobium* may be the production of antibiotics and areas of competitive attachment to roots surface. [54, 55, 56, 57].

The use of bioinformatics tools for cloning and sequencing of DNA for bacterial identification and analysis has been described as more faithful and dependable [58]. Molecular techniques used for amplification and sequencing of this gene and using different kinds of software for this analysis have proved that the nodC gene sequence is well preserved between the same species and the same genus [59]. Previous research on rhizobia has shown that rhizobia sequestered from various parts of the world is genetically more diverse [60]. DNA was isolated from *Rhizobium* found in nodules of the root of pea and after sanitisation of bacteria; a PCR technique was used for the amplification of nodC gene by using different types of primers [59]. High homology is expressed by this DNA sequencing between associated sequences in data [61]. Vessey and Chemining'wa [62] have already proved these types of same conclusions. Amplification by PCR provided a sequence of nucleic acid, which proved very helpful for constructing the phylogenetic tree [63]. Phylogenetic tree data tells us the close relationship of isolated sequences to *Rhizobium leguminosarum*. The same results of nodC gene have been seen by using these techniques [64].

V. CONCLUSION AND FUTURE SCOPE

Root nodule of pea crop was used to obtain the rhizobium and then sterilization performed. Crushing of nodules and then culturing was done and again re-cultured it. After this DNA extraction was performed. Technique of polymerase chain reaction used to amplify selected nodC gene by using specific primers. Gel electrophorese was performed on the product of PCR and desired fragment was isolated from gel. Then cloning and sequencing of fragments was accomplished then this sequence was used to search the homology and phylogenetic tree formation. Reason of choosing pea as a crop sample was its high production rate and rich source of important minerals, vitamins and protein. A high value of homology resemblance was found between the amplified sequence and the other sequence present in the database. The clones AY665788, KR154711, KR154710, KR154716, KR154714, KR154626, and KP760600 found in the database showed more than 98% homology.

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REFERENCES

[1] B. Vanlauwe, M. Hungria, F. Kanampiu, K.E. Giller, "The role of legumes in the sustainable intensification of African smallholder agriculture: Lessons learnt and challenges for the future", *Agriculture, Ecosystems & Environment*, Vol.**284,** pp.**106583**, **2019**.

[2] J.I. Sprent, J. Ardley, E.K. James, "Biogeography of nodulated legumes and their nitrogen‐fixing symbionts", *New Phytologist*, Vol.**215,** Issue.**1,** pp.**40-56**, **2017**.

[3] M.H. Sarwar, M.F. Sarwar, M. Sarwar, N.A. Qadri, S. Moghal, "The importance of cereals (Poaceae: Gramineae) nutrition in human health: A review", *Journal of cereals and oilseeds*, Vol.**4,** Issue.**3,** pp.**32-35**, **2013**.

[4] K.-H. Jung, G. An, P.C. Ronald, "Towards a better bowl of rice: assigning function to tens of thousands of rice genes", *Nature Reviews Genetics*, Vol.**9,** Issue.**2,** pp.**91-101**, **2008**.

[5] J. Burstin, P. Marget, M. Huart, A. Moessner, B. Mangin, C. Duchene, B. Desprez, N. Munier-Jolain, G. Duc, "Developmental genes have pleiotropic effects on plant morphology and source capacity, eventually impacting on seed protein content and productivity in pea", *Plant physiology*, Vol.**144,** Issue.**2,** pp.**768-781**, **2007**.

[6] R. Bora, C. Sarma, "Effect of gibberellic acid and cycocel on growth, yield and protein content of pea", *Asian Journal of Plant Sciences*, **2006**.

[7] C. Masson-Boivin, E. Giraud, X. Perret, J. Batut, "Establishing nitrogen-fixing symbiosis with legumes: how many rhizobium recipes?", *Trends in microbiology*, Vol.**17,** Issue.**10,** pp.**458-466**, **2009**.

[8] E.D. Bobo, P. Jinga, N. Muzvidzwa, G. Zingoni, P. Munosiyei, E. Zingoni Assessment of Nodulation Potential of *Bradyrhizobium japonicum* and *Bradyrhizobium elkanii* on *Glycine max* L. in Acidic Clay and Loam Soils, *International Journal of Scientific Research in Biological Sciences*, Vol.**7,** Issue.**3,** pp.**47-51**, **2020**.

[9] G. Rajendran, S. Mistry, A.J. Desai, G. Archana, "Functional expression of Escherichia colifhuA gene in Rhizobium spp. of Cajanus cajan provides growth advantage in presence of Fe 3+: ferrichrome as iron source", *Archives of microbiology*, Vol.**187,** Issue.**4,** pp.**257**, **2007**.

[10] G. Rajendran, F. Sing, A.J. Desai, G. Archana, "Enhanced growth and nodulation of pigeon pea by co-inoculation of Bacillus strains with Rhizobium spp", *Bioresource technology*, Vol.**99,** Issue.**11,** pp.**4544-4550**, **2008**.

[11] Y. Zhou, Y. Zhang, J. Wei, Y. Zhang, J. Li, W. Wang, H. Duan, J. Chen, "Cloning and analysis of expression patterns and transcriptional regulation of RghBNG in response to plant growth regulators and abiotic stresses in Rehmannia glutinosa", *Springerplus*, Vol.**4,** Issue.**1,** pp.**60**, **2015**.

[12] E.M. Lodwig, A.H. Hosie, A. Bourdes, K. Findlay, D. Allaway, R. Karunakaran, J. Downie, P.S. Poole, "Amino-acid cycling drives nitrogen fixation in the legume–Rhizobium symbiosis", *Nature*, Vol.**422,** Issue.**6933,** pp.**722-726**, **2003**.

[13] G. Centenaro, C. Hudek, A. Zanella, A. Crivellaro, "Root-soil physical and biotic interactions with a focus on tree root systems: A review", *Applied Soil Ecology*, Vol.**123,** pp.**318-327**, **2018**.

[14] R. Rivas, M. Laranjo, P. Mateos, S. Oliveira, E. Martínez‐Molina, E. Velázquez, "Strains of Mesorhizobium amorphae and Mesorhizobium tianshanense, carrying symbiotic genes of common chickpea endosymbiotic species, constitute a novel biovar (ciceri) capable of nodulating Cicer arietinum", *Letters in applied microbiology*, Vol.**44,** Issue.**4,** pp.**412-418**, **2007**.

[15] C. Silva, P. Vinuesa, L.E. Eguiarte, E. Martínez-Romero, V. Souza, "Rhizobium etli and Rhizobium gallicum nodulate common bean (Phaseolus vulgaris) in a traditionally managed milpa plot in Mexico: population genetics and biogeographic implications", *Applied and environmental microbiology*, Vol.**69,** Issue.**2,** pp.**884-893**, **2003**.

[16] P. Garcia-Fraile, D. Mulas-Garcia, A. Peix, R. Rivas, F. Gonzalez-Andres, E. Velazquez, "*Phaseolus vulgaris* is nodulated in northern Spain by *Rhizobium leguminosarum* strains harboring two nodC alleles present in American Rhizobium etli strains: biogeographical and evolutionary

implications", *Canadian journal of microbiology*, Vol.**56,** Issue.**8,** pp.**657-666**, **2010**.
[17] G. Bena, A. Lyet, T. Huguet, I. Olivieri, "Medicago–Sinorhizobium symbiotic specificity evolution and the geographic expansion of Medicago", *Journal of Evolutionary Biology*, Vol.**18,** Issue.**6,** pp.**1547-1558**, **2005**.
[18] F. Debelle, C. Rosenberg, J. Denarié, "The Rhizobium, Bradyrhizobium, and Azorhizobium NodC proteins are homologous to yeast chitin synthases", *MOLECULAR PLANT MICROBE INTERACTIONS*, Vol.**5,** pp.**443-443**, **1992**.
[19] F. Mus, M.B. Crook, K. Garcia, A.G. Costas, B.A. Geddes, E.D. Kouri, P. Paramasivan, M.-H. Ryu, G.E. Oldroyd, P.S. Poole, "Symbiotic nitrogen fixation and the challenges to its extension to nonlegumes", *Applied and environmental microbiology*, Vol.**82,** Issue.**13,** pp.**3698-3710**, **2016**.
[20] J. Angelini, S. Castro, A. Fabra, "Alterations in root colonization and nodC gene induction in the peanut–rhizobia interaction under acidic conditions", *Plant Physiology and Biochemistry*, Vol.**41,** Issue.**3,** pp.**289-294**, **2003**.
[21] B. Gourion, F. Berrabah, P. Ratet, G. Stacey, "Rhizobium–legume symbioses: the crucial role of plant immunity", *Trends in plant science*, Vol.**20,** Issue.**3,** pp.**186-194**, **2015**.
[22] R.F. Fisher, J.K. Tu, S.R. Long, "Conserved Nodulation Genes in *Rhizobium meliloti* and *Rhizobium trifolii*", *Applied and Environmental Microbiology*, Vol.**49,** Issue.**6,** pp.**1432-1435**, **1985**.
[23] N. Peters, D. Verma, "Phenolic compounds as regulators of gene expression in plant-microbe interactions", *Mol Plant-Microbe Interact*, Vol.**3,** pp.**4-8**, **1990**.
[24] X. Perret, C. Staehelin, W.J. Broughton, "Molecular basis of symbiotic promiscuity", *Microbiology and Molecular Biology Reviews*, Vol.**64,** Issue.**1,** pp.**180-201**, **2000**.
[25] R. Poupot, E. Martinez-Romero, J.C. Prome, "Nodulation factors from Rhizobium tropici are sulfated or nonsulfated chitopentasaccharides containing an N-methyl-N-acylglucosaminyl terminus", *Biochemistry*, Vol.**32,** Issue.**39,** pp.**10430-10435**, **1993**.
[26] R. Poupot, E. Martinez-Romero, N. Gautier, J.-C. Promé, "Wild type Rhizobium etli, a bean symbiont, produces acetyl-fucosylated, N-methylated, and carbamoylated nodulation factors", *Journal of Biological Chemistry*, Vol.**270,** Issue.**11,** pp.**6050-6055**, **1995**.
[27] P. Rahi, R. Kapoor, J. Young, A. Gulati, "A genetic discontinuity in root‐nodulating bacteria of cultivated pea in the Indian trans‐Himalayas", *Molecular Ecology*, Vol.**21,** Issue.**1,** pp.**145-159**, **2012**.
[28] J.M. Vincent, "A manual for the practical study of the root-nodule bacteria", *A manual for the practical study of the root-nodule bacteria*, **1970**.
[29] N.D. Marsudi, A.R. Glenn, M.J. Dilworth, "Identification and characterization of fast-and slow-growing root nodule bacteria from South-Western Australian soils able to nodulate Acacia saligna", *Soil Biology and Biochemistry*, Vol.**31,** Issue.**9,** pp.**1229-1238**, **1999**.
[30] H. Sambrook, "Molecular cloning: a laboratory manual. Cold Spring Harbor, NY", **1989**.
[31] G. Laguerre, S.M. Nour, V. Macheret, J. Sanjuan, P. Drouin, N. Amarger, "Classification of rhizobia based on nodC and nifH gene analysis reveals a close phylogenetic relationship among Phaseolus vulgaris symbiontsThe GenBank accession numbers for the sequences reported in this paper are AF217261 through AF217272 for nodC and AF218126, AF275670 and AF275671 for nifH", *Microbiology*, Vol.**147,** Issue.**4,** pp.**981-993**, **2001**.
[32] F. Silva, J. Simões-Araújo, J. Silva Júnior, G. Xavier, N. Rumjanek, "Genetic diversity of Rhizobia isolates from Amazon soils using cowpea (Vigna unguiculata) as trap plant", *Brazilian Journal of Microbiology*, Vol.**43,** Issue.**2,** pp.**682-691**, **2012**.
[33] Z. Topcu, "An optimized recipe for cloning of the polymerase chain reaction-amplified DNA inserts into plasmid vectors", *Acta Biochimica Polonica*, Vol.**47,** Issue.**3,** pp.**841-846**, **2000**.
[34] L. Lakatos, G. Szittya, D. Silhavy, J. Burgyán, "Molecular mechanism of RNA silencing suppression mediated by p19 protein of tombusviruses", *The EMBO journal*, Vol.**23,** Issue.**4,** pp.**876-884**, **2004**.
[35] X. Chen, X. Ding, W.-Y. Song, "Isolation of Plasmid DNA rescued from single colonies ofAgrobacterium tumefaciens by means of rolling circle amplification", *Plant Molecular Biology Reporter*, Vol.**21,** Issue.**4,** pp.**411-415**, **2003**.
[36] J.-F. Li, E. Park, A.G. von Arnim, A. Nebenführ, "The FAST technique: a simplified Agrobacterium-based transformation method for transient gene expression analysis in seedlings of Arabidopsis and other plant species", *Plant Methods*, Vol.**5,** Issue.**1,** pp.**6**, **2009**.
[37] M.A. Graham, K.A.T. Silverstein, S.B. Cannon, K.A. VandenBosch, "Computational Identification and Characterization of Novel Genes from Legumes", *Plant Physiology*, Vol.**135,** Issue.**3,** pp.**1179-1197**, **2004**.
[38] M.K. Litvaitis, "A molecular test of cyanobacterial phylogeny: inferences from constraint analyses", *Hydrobiologia*, Vol.**468,** Issue.**1,** pp.**135-145**, **2002**.
[39] M. Yadegari, H.A. Rahmani, G. Noormohammadi, A. Ayneband, "Evaluation of bean (Phaseolus vulgaris) seeds inoculation with Rhizobium phaseoli and plant growth promoting rhizobacteria on yield and yield components", *Pakistan journal of biological sciences : PJBS*, Vol.**11,** Issue.**15,** pp.**1935-1939**, **2008**.
[40] Minaxi, L. Nain, R.C. Yadav, J. Saxena, "Characterization of multifaceted Bacillus sp. RM-2 for its use as plant growth promoting bioinoculant for crops grown in semi arid deserts", *Applied Soil Ecology*, Vol.**59,** pp.**124-135**, **2012**.
[41] J.K. Vessey, "Plant growth promoting rhizobacteria as biofertilizers", *Plant and Soil*, Vol.**255,** Issue.**2,** pp.**571-586**, **2003**.
[42] B. Saharan, V. Nehra, "Plant growth promoting rhizobacteria: a critical review", *Life Sci Med Res*, Vol.**21,** Issue.**1,** pp.**30**, **2011**.
[43] C.P. Vance, "Symbiotic nitrogen fixation and phosphorus acquisition. Plant nutrition in a world of declining renewable resources", *Plant physiology*, Vol.**127,** Issue.**2,** pp.**390-397**, **2001**.
[44] N. Parmar, K. Dadarwal, "Stimulation of plant growth of chickpea by inoculation of fluorescent pseudomonads", *J Appl Microbiol*, Vol.**86,** pp.**36-44**, **2000**.
[45] M. Naveed, I. Mehboob, M.A. Shaker, M.B. Hussain, M. Farooq, "Biofertilizers in Pakistan: initiatives and limitations", *International Journal of Agriculture and Biology*, Vol.**17,** Issue.**3,** pp.**411-420**, **2015**.
[46] J.E. Clarridge, "Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases", *Clinical microbiology reviews*, Vol.**17,** Issue.**4,** pp.**840-862**, **2004**.
[47] C. Petti, C.R. Polage, P. Schreckenberger, "The role of 16S rRNA gene sequencing in identification of microorganisms misidentified by conventional methods", *Journal of clinical microbiology*, Vol.**43,** Issue.**12,** pp.**6123-6125**, **2005**.
[48] S. Mignard, J.-P. Flandrois, "16S rRNA sequencing in routine bacterial identification: a 30-month experiment", *Journal of microbiological methods*, Vol.**67,** Issue.**3,** pp.**574-581**, **2006**.
[49] D.A. Casamatta, J.R. Johansen, M.L. Vis, S.T. Broadwater, "Molecular and morphological characterization of ten polar and near‐polar strains within the Oscillatoriales (Cyanobacteria) 1", *Journal of phycology*, Vol.**41,** Issue.**2,** pp.**421-438**, **2005**.
[50] P. Rajaniemi, J. Komárek, R. Willame, P. Hrouzek, K. Kaštovská, L. Hoffmann, K. Sivonen, "Taxonomic consequences from the combined molecular and phenotype evaluation of selected Anabaena and Aphanizomenon strains", *Algological Studies*, Vol.**117,** Issue.**1,** pp.**371-391**, **2005**.
[51] M.M. Svenning, T. Eriksson, U. Rasmussen, "Phylogeny of symbiotic cyanobacteria within the genus Nostoc based on 16S rDNA sequence analyses", *Archives of microbiology*, Vol.**183,** Issue.**1,** pp.**19-26**, **2005**.

[52] S. Chakravorty, D. Helb, M. Burday, N. Connell, D. Alland, "A detailed analysis of 16S ribosomal RNA gene segments for the diagnosis of pathogenic bacteria", *Journal of microbiological methods*, Vol.**69,** Issue.**2,** pp.**330-339**, **2007**.
[53] H.H. Zahran, "Rhizobia from wild legumes: diversity, taxonomy, ecology, nitrogen fixation and biotechnology", *Journal of Biotechnology*, Vol.**91,** Issue.**2-3,** pp.**143-153**, **2001**.
[54] V.K. Chebotar, C.A. Asis, S. Akao, "Production of growth-promoting substances and high colonization ability of rhizobacteria enhance the nitrogen fixation of soybean when coinoculated with Bradyrhizobium japonicum", *Biology and Fertility of Soils*, Vol.**34,** Issue.**6,** pp.**427-432**, **2001**.
[55] A. Valverde, Y. Okon, S. Burdman, "cDNA-AFLP reveals differentially expressed genes related to cell aggregation of Azospirillum brasilense", *FEMS microbiology letters*, Vol.**265,** Issue.**2,** pp.**186-194**, **2006**.
[56] B.S. Mirza, M.S. Mirza, A. Bano, K.A. Malik, "Coinoculation of chickpea with Rhizobium isolates from roots and nodules and phytohormone-producing Enterobacter strains", *Australian Journal of Experimental Agriculture*, Vol.**47,** Issue.**8,** pp.**1008-1015**, **2007**.
[57] K. Raverkar, B. Konde, "Effect of Rhizobium andAzospirillum lipoferum inoculation on the nodulation, yield and nitrogen uptake of peanut cultivars", *Plant and soil*, Vol.**106,** Issue.**2,** pp.**249-252**, **1988**.
[58] K. Rudi, M. Zimonja, B. Kvenshagen, J. Rugtveit, T. Midtvedt, M. Eggesbø, "Alignment-independent comparisons of human gastrointestinal tract microbial communities in a multidimensional 16S rRNA gene evolutionary space", *Applied and environmental microbiology*, Vol.**73,** Issue.**8,** pp.**2727-2734**, **2007**.
[59] V. Ventorino, M. Chiurazzi, M. Aponte, O. Pepe, G. Moschetti, "Genetic diversity of a natural population of Rhizobium leguminosarum bv. viciae nodulating plants of Vicia faba in the Vesuvian area", *Current Microbiology*, Vol.**55,** Issue.**6,** pp.**512-517**, **2007**.
[60] L.A. Mutch, S.M. Tamimi, J. Young, "Genotypic characterisation of rhizobia nodulating Vicia faba from the soils of Jordan: a comparison with UK isolates", *Soil Biology and Biochemistry*, Vol.**35,** Issue.**5,** pp.**709-714**, **2003**.
[61] L.A. Mutch, J.P.W. Young, "Diversity and specificity of Rhizobium leguminosarum biovar viciae on wild and cultivated legumes", *Molecular Ecology*, Vol.**13,** Issue.**8,** pp.**2435-2444**, **2004**.
[62] J.K. Vessey, G.N. Chemining'wa, "The genetic diversity of Rhizobium leguminosarum bv. viciae in cultivated soils of the eastern Canadian prairie", *Soil Biology and Biochemistry*, Vol.**38,** Issue.**1,** pp.**153-163**, **2006**.
[63] G.J. Olsen, C.R. Woese, "Ribosomal RNA: a key to phylogeny", *The FASEB journal*, Vol.**7,** Issue.**1,** pp.**113-123**, **1993**.
[64] Z. Zhang, R.C. Willson, G.E. Fox, "Identification of characteristic oligonucleotides in the bacterial 16S ribosomal RNA sequence dataset", *Bioinformatics*, Vol.**18,** Issue.**2,** pp.**244-250**, **2002**

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