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Molecular Characterization of Rhizobia from Revertants of Non-nodulating Cultivar and Normal Cultivar of Chickpea

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ABSTRACT

The normal nodulating chickpea cultivar (HC5) and revertant of non-nodulating cultivar ICC 4993 NN designated as ICC 4993 (R) were used to study and characterize the rhizobia infecting both cultivars. On the basis of growth characteristic and nodulation, 43 rhizobial isolates from revertant of non-nodulating cultivar and 8 rhizobial isolates from normal nodulating cultivar were selected. Heterogeneity of the rhizobia infecting both of the cultivars was estimated by ERIC (Enterobacterial Repetitive Intergenic Consensus) as well as RFLP (Restriction Fragment Length Polymorphism) analysis of 16S rDNA sequence. Based on the presence of different ERIC profiles, rhizobial isolates from cv. ICC4993 (R) formed eight different clusters and those from cv. HC5 formed three clusters at 80% similarity. A combined dendrogram of all the mesorhizobial isolates from the two cultivars showed two clusters at 70% similarity and eight subclusters at 80% similarity level. Similarly, RFLP patterns showed that rhizobial isolates from cv. ICC4993 (R) formed eleven clusters while those from cv. HC5 formed two clusters at 80% similarity. A combined dendrogram of mesorhizobial isolates from the two cultivars formed twelve clusters at 80% level of similarity. Using both methodologies, heterogeneity (if any) of mesorhizobia nodulating cvs. ICC4993 (R) and HC5 could not be ascertained. Further sequencing of partially amplified 16S rDNA of three rhizobial isolate from cv. ICC4993 (R) and one from cv. HC5 showed more than 98% similarity with *Mesorhizobium muleiense* and *Mesorhizobium mediterraneum*. The phylogenetic analysis of 16S rRNA partial sequence revealed 11 monophyletic clades. The isolates NN78 and HC 1065 were clustered along with *Mesorhizobium mediterraneum* strain PECA20 while NNs13 and NN90 formed a separate cluster.

Keywords: Diversity, ERIC, Nodulation, *Mesorhizobium*, Phylogenetic analysis, RFLP.

INTRODUCTION

Rhizobia are free living Gram-negative bacilli, which are capable of fixing atmospheric nitrogen and form a symbiotic relationship with specific legumes. To enhance biological nitrogen fixation and hence crop productivity, rhizobial inoculants have been considered to be of prime importance (Poustini *et al*. 2007, Keneni *et al*., 2010, Dudeja *et al*., 2011). Till date, more than 120 symbiotic nodulating bacterial species have been identified in 16 genera: *Rhizobium, Ensifer, Mesorhizobium, Phyllobacterium, Bradyrhizobium, Ochrobactrum, Methylobacterium, Azorhizobium, Allorhizobium, Aminobacter, Shinella* and *Devosia* belonging to *α*-proteobacteria, and four genera, namely, *Burkholderia, Microvirga, Cupriavidus* and *Herbaspirillum* belonging to *β*-proteobacteria have been identified (Dudeja *et al.,* 2012; Weir, 2012; ICSP, 2013; Berrada and Fikri-Benbrahim, 2014).

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Chickpea, the most important legume crop of arid zones of India, is one of the oldest known food legumes to the mankind. Chickpea production amounts to the maximum in India, followed by Pakistan and Turkey. It is a self pollinating diploid and the World's third most important food legume and widely grown for its nutritious seeds. Chickpea rhizobia has been grouped under genus *Mesorhizobium*. This genus has 30 species out of which six species including *M. ciceri*, *M. mediterraneum*, *M. temperatum*, *M. tianshanense*, *M.* sp. (*Cicer*) and *Mesorhizobium muleiense* sp. nov. have been reported to form nodules in chickpea (Dudeja *et al*., 2009; Zhang *et al*., 2012; Dudeja and Nidhi, 2014).

Rupela (1994) at ICRISAT (International Crops Research Institute for the Semi-Arid-Tropics), Hyderabad, developed a non-nodulating line of chickpea (ICC4993 NN) to estimate the quantum of nitrogen fixed by the chickpea plant and benefit of nodulation trait. However, over the years, very low reversion frequency of this non-nodulating line to nodulating line was observed, particularly at Hisar location in Haryana. Pure lines of these chickpea revertants cv. ICC4993 (R) were developed at CCS Haryana Agricultural University, Hisar, and these are also of great interest to the researchers. Few reports are available regarding the rhizobia nodulating chickpea cultivars in Indian soils, particularly in Haryana, a northern state. Earlier, one of the efficient chickpea strains, i.e. Ca181, was identified as *M. ciceri* from chickpea (Arora, 2010; Dogra *et al*., 2013), however, mesorhizobial isolates from the same field after 25 years were identified as *Mesorhizobium mediterraneum* (Dudeja and Singh, 2008). None of the isolates belonged to *M. ciceri*. This was accompanied by a shift in the soil pH from 7.4 to 8.4 (Nandwani and Dudeja, 2013), indicating a shift in mesorhizobial species infectivity. Now, under the same field conditions, non-nodulating chickpea line was reverted back to nodulation. Thus, it was interesting to investigate whether some new rhizobial specie was responsible for this reversion or the host itself reverted back. Therefore, the present investigation was planned to assess the heterogeneity of the rhizobia infecting the reverted non-nodulating cultivar [cv. ICC4993 (R)] in comparison to a normal nodulating chickpea cultivar (cv. HC 5).

MATERIALS AND METHODS

Isolation of Mesorhizobia from Normal and Reverted Non-nodulating Lines

Reverted non-nodulating line of cv. ICC4993NN showing nodulation cv. ICC4993 (R) and normal nodulating cv. HC5 were grown under field conditions at Chaudhary Charan Singh Haryana Agricultural University, Hisar Research Farm. The plants were uprooted at 60 days of growth. The healthy nodules were removed, surface sterilized and rhizobia were isolated on YMA plates and purified further (Vincent, 1970). Single rhizobial colonies were picked up from the plates, maintained on YMA slants, and stored at 4°C in a refrigerator for further studies.

Genotypic Heterogeneity of Mesorhizobia

Based on growth characteristics, 66 rhizobial isolates with 56 from cv. ICC4993 (R) and 10 from normal nodulating cv. HC5 were selected for authentication, using plant infectivity test by coffee cups (Giri and Dudeja 2013) and their efficiency was also determined (Suneja-Madan and Dahiya, 2013). The authenticated and efficient mesorhizobial isolates were grown in flasks containing 25 mL of sterilized modified TY broth. The log phase cells were harvested and total genomic DNA was isolated by standard phenol-chloroform extraction method (Ausubel *et al*., 2001). After quantification, DNA was stored at -20°C. Genotypic heterogeneity of 51 authenticated mesorhizobial isolates was assessed by

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amplification of genomic DNA using ERIC primers as well as RFLP of the amplified 16S rDNA. Genomic DNA of 43 mesorhizobial isolates from cv. ICC4993 (R) and 8 mesorhizobial isolates from cv. HC5 were amplified by ERIC-1R (5' ATGTAAGCTCCTGGGGATTCAC3') and ERIC-2R (5' AAGTAAGTGACTGGGGTGA GCG 3') primers. PCR amplification reactions were performed in 23.5 µL volume per reaction (Dudeja and Singh, 2008). Reaction mixture included 18.25 µL of millipore water ; 2.5 µL PCR buffer (10x) ; 0.375 µL of $MgCl_2$ (25 nm) ; 0.25 µL of dNTP mixture (10mM) ; 0.5 µL of each primers ERIC-1R (100 ng μL^{-1}) and ERIC-2R (100 ng μL^{-1}) ; 0.25 µL of Taq DNA polymerase(3U μL^{-1}) and 2.5 µL of template DNA (50 ng μL^{-1}approx.). The conditions for DNA amplification included initial denaturation at 94 °C for 3 minutes; denaturation at 94 °C for 45 seconds; annealing at 48/45°C for 40/45 seconds; extension at 72°C for 1 minutes; these steps were repeated 40 times; final extension at 72°C for 10 minutes; holding at 4°C. The profiles of DNA fragments generated after amplification were used for determining the heterogeneity of mesorhizobia.

Heterogeneity of selected chickpea mesorhizobial isolates (28 from cv. ICC4993 (R) and 8 from cv. HC5) was also assessed by RFLP of amplified 16S rDNA sequences with 1,000 bp ladder as molecular weight marker. The amplification of 16S rDNA sequences was carried out by polymerase chain reaction using primer fD1 (5' AGAGTTTGATCCTGGCTCAG 3') and rD1 (5'AAGGAGGTGATCCAGC CGCA 3'). The same PCR conditions were used as in case of ERIC, however, the annealing temperature of 50/55°C was used for some strains. The amplified product of 16S rDNA was subjected to three restriction endonucleases: *MspI*, *Csp6I* and *RsaI*. *MspI* restriction endonuclease was used alone whereas *Csp6I* and *RsaI* were used together. Digestion mixture for *MspI* consisted of 6 µL milli pore water; 2 µL Y^+/Tango buffer (2x) ; 1 µL *MspI* (3U μL^{-1}); 9 µL 16S rDNA amplified product. Digestion mixture for *Csp6I* and *RsaI* consisted of 5 µL milli pore water ; 2 µL Y^+/Tango buffer (2x) ; 1 µL *Csp6I*(3U μL^{-1}) ;1 µL *RsaI* (3U μL^{-1}) ; 9 µL 16S rDNA amplified product.

The amplified products were resolved on 1.5% agarose gel in TBE buffer with the constant voltage of 40 volts for 1.5-2.0 hours. The molecular marker 100 bp ladder was also loaded on the gel. Gels were stained with ethidium bromide and visualized under gel documentation system and images were analyzed in order to prepare binary matrix. The profiles of isolates were made on the basis of band size by comparing it with the standard marker. The 0-1 matrix was prepared upon the presence or absence of a particular band. These similarity matrices were analyzed by UPGMA (unweighted pair grouping with mathematical average) cluster analysis using NTSYS programme (Version2.1: Exeter software, Setauket, NY) and dendrograms were constructed depending upon the genetic similarity between different mesorhizobia (Rohlf, 1998).

Sequencing of Selected Chickpea Mesorhizobia from Different Clusters

To identify chickpea rhizobial isolates HC 1065, NN s13, NN 90, and NN 78 representing different clusters (VI, VII, XI and II) were amplified using 16S rDNA primers. The products of amplification were purified and sequenced from Merck Millipore DNA Sequencing Services, Bangalore, India. For each isolate, there were two replicas of PCR products. One was sequenced with the forward primer and the other with the reverse primer. Sequence data were analyzed by comparison to 16SrRNA genes in the GenBank database. The nearest relatives of each organism were obtained by BLAST searches (Altschul *et al*., 1990). BLAST algorithm was used to produce a tree from given distances (or dissimilarities) between pair of sequences and alignments using Neighbor Method (Desper and Gascuel, 2004; Saitou and Nei, 1987; Tamura *et al*., 2013).

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RESULTS

The non-nodulating cv. ICC4993NN, its revertant line cv. ICC4993 (R) and normally nodulating cv. HC5 of chickpea are shown in Figure 1. Out of 56 mesorhizobial isolates from cv. ICC4993 (R), 43 were authenticated, on the basis of nodulation and symbiotic effectiveness in terms of shoot biomass as detailed earlier (Suneja-Madan and Dahiya, 2013). Similarly, 8 out of 10 mesorhizobial isolates from cv. HC5 were authenticated and all are given in Figure 1.

A dendrogram of mesorhizobial isolates on the basis of ERIC profiles, from both cultivars, showed two clusters at 70% similarity (Figure 2). Cluster I included the maximum number of isolates whereas cluster II was having only four isolates. All the mesorhizobial isolates from cv. HC5 were present in the cluster I. At 80% similarity level, mesorhizobial isolates from both cultivars (ICC4993 (R) and HC5) formed eight sub clusters. Out of eight, seven isolate from cv. HC5 were present in sub cluster I. The isolate HC1014 was 100% similar to isolates NN506, NN250, NN218, and NN212. The isolates HC1049, HC1059, HC1063, HC1070, HC1080 from cv. HC5 were 100% similar and showed 100% similarity to isolates NN14, NN15, NN37, NN54, NN501, NN222, NN219, NN502, NN520, NNs16, NN512, NN511, NN510, and NN507 from cv. ICC4993 (R).The isolate HC 1065 was present in sub-cluster VI along with isolate NN 53, but formed a separate sub-cluster above 80% similarity level.

Heterogeneity of 28 mesorhizobial isolates from cv. ICC4993 (R) and 8 from cv. HC5 was also assessed by RFLP of amplified 16S rDNA sequences with the three restriction endonucleases: *MspI, Csp6I* and *RsaI*. In total, 20 bands of varying molecular weights were observed in all the isolates. The different rhizobial isolates showed 2-6 reproducible bands with each restriction endonuclease. Dendrogram of similarity coefficients between different chickpea mesorhizobial isolates of cvs. ICC4993 (R) and HC5 showed that all the mesorhizobial isolates from cv. ICC4993 (R) formed 11

(a) (b) (c)

Figure 1. Non-nodulating chickpea cv. ICC4993NN (a), Non-nodulating revertant of chickpea cv. ICC4993NN(R) designated as NN (isolates of 2, 3, 6, 9, 11, 12, 13, 14, 15, 17, 18, 19, 24, 37, 41, 42, 44,49, 51, 53, 54, 74, 78, 90, 94, 95, 96, 501, 502, 504, 506, 507, 510, 511, 512, 513, 518,519, 520, 523,201, 212, 218, 219, 220, 221, 222, 224, 229, 230, 250, 282, s13, s14, s16, s17 were isolated) (b), and normally nodulating cv. HC5 designated as HC (isolates of 1010, 1014, 1029, 1049, 1059, 1062, 1063, 1065, 1070, 1080 were isolated) (c).

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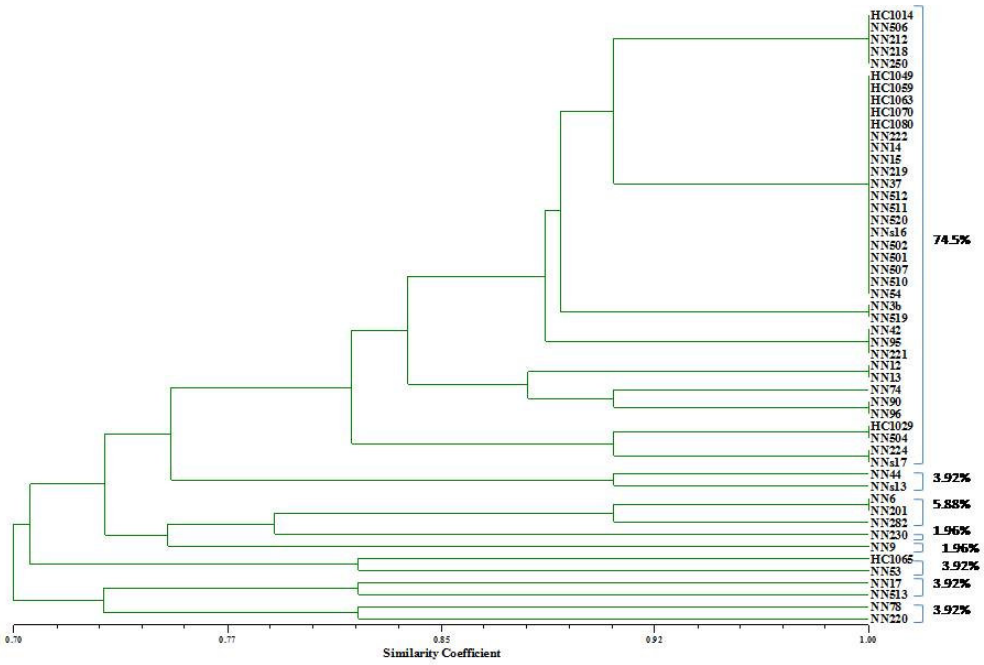

Figure 2. UPGMA dendrogram showing similarity between ERIC profiles of mesorhizobia isolated from cvs. ICC4993 (R) and HC5.

clusters at 80% level of similarity. Many of the isolates i.e. 28.57 % belonged to cluster VII. The mesorhizobial isolates NN 51 and NN 53 were 100% similar. Isolates from cv. HC5 formed two clusters at 80% similarity. Cluster I had maximum number of mesorhizobial isolates i.e. 62.5%, while cluster II had 37.5% of isolates. Mesorhizobial isolates HC1080 and HC 1029 exhibited 100% similarity.

The combined dendrogram of mesorhizobial isolates from both of the cultivars, resulting from RFLP of amplified 16S rDNA sequence, showed twelve clusters at 80% level of similarity (Figure 3). Mesorhizobial isolates from cv. ICC4993 (R) were distributed in almost all the clusters. Mesorhizobial isolates from both cvs. ICC4993 (R) and HC5 were intermixed in all clusters. Isolates NN6 and HC1059 were present in the same cluster V, but diverged from each other at 85% similarity level, thus forming separate sub clusters. HC 1063 and HC1065 were present in cluster VI along with NN507 at 80% similarity level but diverged from NN507 at 87% similarity level and formed separate subcluster. All the other mesorhizobial isolates from cv. HC5 were present in cluster XI along with NN90 from cv. ICC4993 (R) at 80% similarity level. Isolates HC1014 and HC 1049 diverged from other isolates at 85% similarity level, from each other at 94.7% and formed separate sub clusters. Isolates HC1029, HC1080, HC1070 diverged from NN90 at 86% similarity level and formed a separate sub cluster. The isolates HC1029, HC1080, HC1070 were present in the same sub cluster up to 94.7% similarity level. However, the isolate HC1070 diverged from the other two isolates at 94.7% similarity level and formed a separate sub-cluster, but isolates HC1029 and HC1080 showed 100% similarity.

The heterogeneity of mesorhizobial isolates from cvs. ICC4993 (R) and HC5

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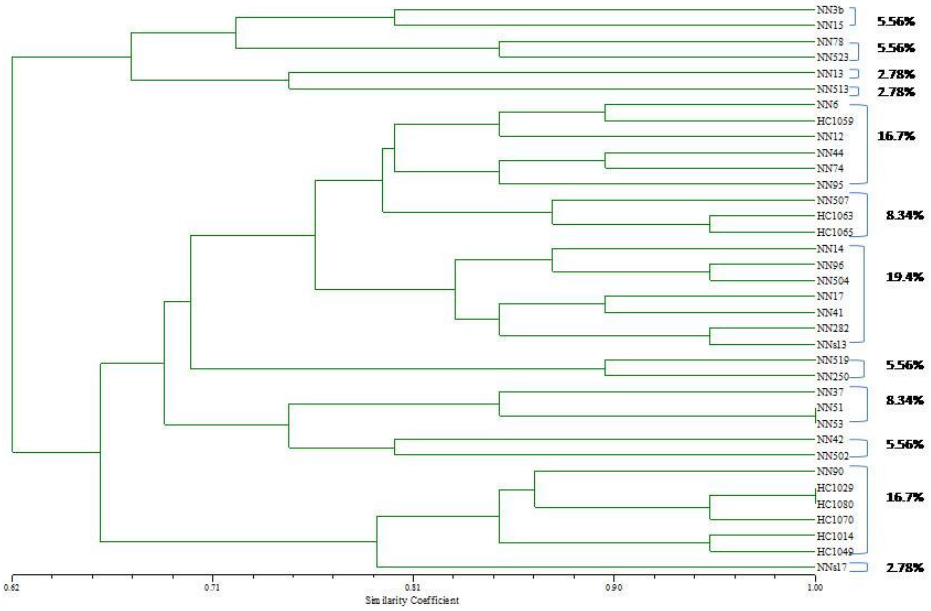

Figure 3. UPGMA dendrogram showing similarity between RFLP of PCR amplified 16S rDNA profiles of mesorhizobia isolated from cvs. ICC4993 (R) and HC5 by three restriction endonucleases, *Csp*6I, *Rsa*I and *Msp*I.

were further assessed by partial sequencing of 16S rDNA. The amplified products of 16S rDNA of four isolates, namely, HC1065 isolated from cv. HC5 and isolates NNs13, NN90, and NN78 isolated from revertant of non nodulating cv. ICC 4993 NN were sequenced. To confirm the phylogenetic relationship of the isolates, a neighbour-joining phylogenetic tree was constructed on the basis of 16S rRNA partial gene sequences of NN78, HC1065, NN s13 and NN90 by Jukes-Cantor nucleic acid substitution model and 1,000 bootstrap replicates using MEGA 6. (Figure 4). The consensus phylogenetic tree thus obtained reflected a total of 11 monophyletic clades. The first two mesorhizobial isolates formed a unique lineage sharing 99% 16S rRNA partial gene sequence similarity with *Mesorhizobium muleiense* strain CCBAU 83963 and hence were classified as *Mesorhizobium muleiense*. Isolate NNs 13 and NN 90 formed a completely different cluster and thus were classified as *Mesorhizobium* sp. nov. The estimated value of the shape parameters for the discrete gamma distribution was 15.1249 [40,41] . The estimated transition/translation bias (R) was 0.92. The substitution matrix was calculated by the probability of substitution (r) from one base to another base. The nucleotide frequency was also calculated using this matrix. This whole analysis involved 28 nucleotide sequences and all positions containing gaps and missing data were eliminated. There was a total of 116 positions in the final datasets.

DISCUSSION

Isolation efficiency of rhizobia from chickpea nodules was low as reported earlier (Chaudhary *et al*., 2001). Surprisingly, out

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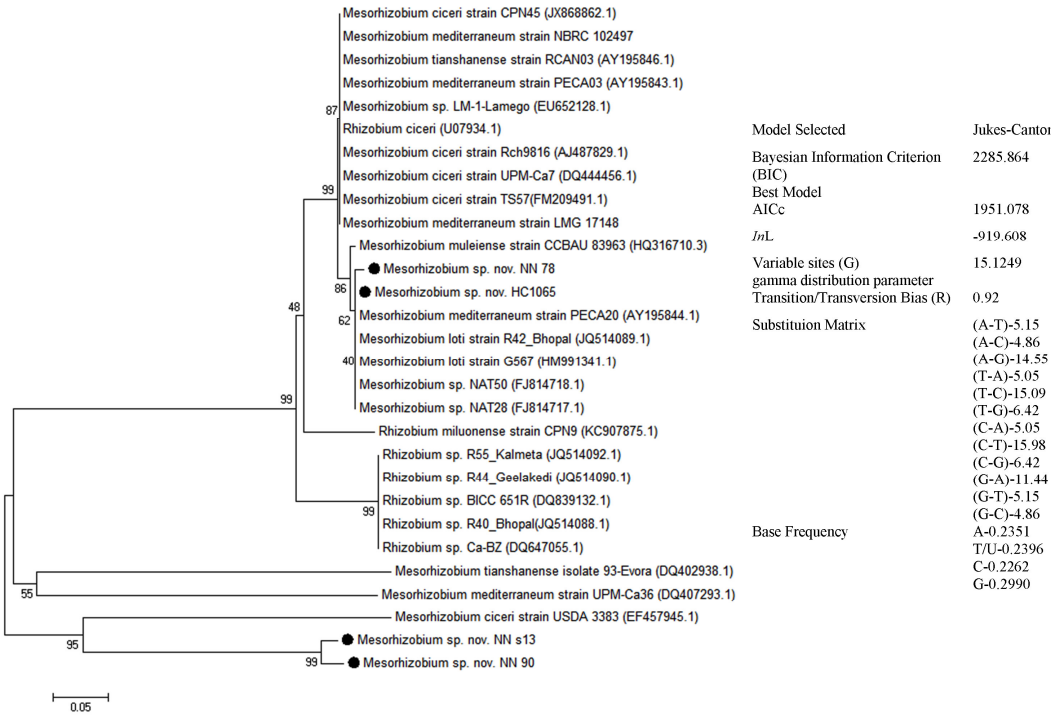

Figure 4. Neighbour-Joining tree based on 16S rRNA gene sequences showing the phylogenetic relationships between strains NN78, NN s13, NN90 and HC1065 under the JC model. Bootstrap support values (percentages of 1000 replicates) of the dataset are shown at the corresponding nodes.

of 66 rhizobial isolates from nodules, only 51 rhizobia were able to nodulate chickpea; indicating that apart from other non-rhizobial genera in nodules of chickpea and other legumes, endophytic rhizobia were also present in chickpea nodules (Dudeja *et al*., 2012; Saini *et al*., 2014; Kumar *et al*., 2013). Large number of epiphytic bacteria on chickpea nodules were also observed (Suneja-Madan *et al*., 2014). The genetic heterogeneity of rhizobia infecting revertant of non-nodulating cv. ICC 4993 NN and normal nodulating cv. HC 5 was determined by using PCR amplifications of ERIC sequences. The use of ERIC-PCR technique was preferred as it was quite simple and has been successfully used elsewhere in differentiating the rhizobial strains (Tajima *et al*., 2000; Saldana *et al*., 2003; Dudeja and Singh, 2008). Clustering of different rhizobial isolates into different groups depending upon the homogeneity of one strain with other using ERIC amplification showed immense molecular diversity among the rhizobia infecting non nodulating chickpea plants and normal nodulating cultivar. Though diversity of rhizobia isolated from revertant of non-nodulating chickpea cultivar has not been reported in the literature, rhizobia isolated from different varieties, locality, or species wide heterogeneity has been reported in native rhizobial populations by other workers using random ERIC and REP sequences (de Bruijn, 1992; Chen *et al*., 2000; Tajima *et al*., 2000; Jarabo-Lorenzo *et al*., 2003; Saldana *et al*., 2003; Dudeja and Singh, 2008; Nandwani and Dudeja, 2009; Yadav *et al*., 2013). Similarly, RFLP analysis using three restriction enzymes showed that chickpea mesorhizobia from cv. ICC4993 (R) formed eleven clusters and from cv. HC5 formed two clusters at 80% similarity. Such considerable genetic diversity among chickpea rhizobia has also been reported from different parts of the world by using

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different methodologies. It was shown that not only soil type but also plant genotype, individual plants within a cultivar, nodulation variants of the same cultivar, and soil management regimes affected the diversity of the rhizobial population (Paffetti *et al*., 1996; Chaudhary *et al*., 2001, 2002; Carelli *et al.,* 2000; Dudeja and Singh, 2008; Nandwani and Dudeja, 2009; Dogra *et al*., 2013). Mesorhizobial clusters formed by both methodologies were different, though few isolates were in the same cluster, but main outcome was the same as isolates from both cultivars were intermixed and didn't form exclusive clusters.

The combined dendrogram of mesorhizobial isolates from both of the cultivars based on both methodologies showed that all the isolates formed twelve clusters and mesorhizobial isolates from both of the cultivars were grouped into common clusters. This indicated that the already existing mesorhizobial strains in the Hisar soils nodulated the non-nodulating pure lines of chickpea and no new species of mesorhizobia was identified to be associated with these revertant lines. Notably, about 30 years back, mesorhizobia infecting chickpea being grown in Hisar of Haryana state were isolated and, particularly, strain Ca181 was identified as *M. ciceri* (Arora, 2010) and the pH of the soil was 7.4 at that time. But, at present, the same fields soils have pH> 8.4 and isolation of mesorhizobia from these fields showed the presence of *M. mediterraneum*. Unexpectedly, the revertant was nodulated by the same species of mesorhizobial indicating that either the present methodology is unable to pin point the difference (if any) or the plants have been reverted back to nodulation.

Further, the sequences of all the four mesorhizobial isolates from cvs. ICC4993 (R) and HC5 were identified and these belonged to *Mesorhizobium.* Some were closely related to *Mesorhizobium muleiense* and *Mesorhizobium mediterraneum*. However, two predominant mesorhizobial isolates, namely, LN 707b and LN7007 isolated from different nodulation variants of chickpea from the same field, were identified by amplification and sequencing of 16S rRNA gene and showed more than 98% and 99% similarity with *Mesorhizobium mediterraneum*. Complete genome sequences or the use of other methodologies may reveal some differences, but by using ERIC or RFLP and partial sequencing of 16S rDNA we could not differentiate (if any difference exits) the mesorhizobial from revertant lines and normal nodulating cultivars. Possibly, the non-nodulating lines selected from the nodulating population, though considered and reported to be stable, have now reverted back to normal nodulation in Hisar soils.

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تعیین ویژگی های مولکولی ریزوبیوم ها از طریق کولتیوارهای برگشته (Revertant) غیر گره ساز و کولتیوار نرمال نخود

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چکیده

در این پژوهش، کولتیوار گره ساز(nodulating) و نرمال نخود با نماد (HC5)وکولتیوار برگشته (Revertant) غیر گره ساز (ICC 4993 NN)که با نماد(R) ICC 4993 مشخص شده بود برای مطالعه و تعیین ویژگی های ریزوبیوم هایی که هر دو کولتیواررا تلقیح می کنند استفاده شدند. به این منظور، بر اساس ویژگی های رشد و گره سازی ریزوبیوم ها، ۴۳ جدایه از کولتیوار برگشته وغیر گره ساز و ۸ جدایه از کولتیوار نرمال و گره ساز انتخاب شدند. برای برآورد نا متجانسی ریزوبیوم هایی که هر دو کولتیوار را تلقیح می کردند از تجزیه تحلیل ERIC (Enterobacterial Repetitive Intergenic Consensus) وآر.اف.ال.پی از توالی 16S rDNAاستفاده شد. سپس، بر اساس وجود پروفیل های مختلف ERIC ، جدایه های ریزوبیوم مربوط به کولتیوار(R) ICC4993هشت خوشه متفاوت تشکیل دادند و جدایه های کولتیوار HC5درسه خوشه با ۸۰٪ تشابه قرار گرفتند. نیز، نمودار شجره ای (دندروگرام) ترکیبی از همه جدایه های مزو-ریزوبیوم ها از هر دو کولتیوار، دو خوشه با ۷۰٪ تشابه و ۸ زیر خوشه (subcluster) با تشابه ۸۰٪نشان داد. همچنین، طرح های آر.اف.ال.پی چنین نشان می داد که جدایه های ریزوبیومی از کولتیوار(R) ICC4993 تعداد ۱۱ خوشه تشکیل دادند در حالی که جدایه های کولتیوارHC5 دو خوشه با تشابه ۸۰٪ داشتند. نیز، نمودار شجره ای (دندروگرام) ترکیبی از همه جدایه های مزو-ریزوبیوم ها از هر دو کولتیوار ۱۲ خوشه با تشابه ۸۰٪ تشکیل دادند. با وجود کاربرد هر دو روش تجزیه، تعیین نامتجانسی(احتمالی) مربوط به مزو-ریزوبیوم های کولتیوارهای گره ساز (R) ICC4993و HC5 مقدور نشد. ادامه توالی یابی 16S rDNAکه به طور جزیی تکثیر شده بود در مورد سه جدایه ریزوبیوم کولتیوار ICC4993 (R)و یک جدایه از کولتیوار HC5 حاکی از ۹۸٪ تشابه با *Mesorhizobium muleiense*و *Mesorhizobium mediterraneum* بود. تجزیه تبارزایی (phylogenetic) از توالی جزیی 16S rRNA، تعداد ۱۱ واحد طبقه بندی (monphylatic clades) را آشکار ساخت. جدایه های NN78و HC1065همراه با ریسه *Mesorhizobium mediterraneum* PECA20 در یک خوشه قرار گرفتند در حالی که NNs13و NN90خوشه دیگری تشکیل دادند.

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