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Assessment of genetic diversity and symbiotic effectiveness of Pigeon pea (*Cajanus cajan*) Nodulating Bradyrhizobia

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Abstract

The Pigeon pea is an excellent source of organic nitrogen increases soil organic matter, improves soil quality also useful for intercropping and agroforestry. In order to investigate symbiotic effectiveness of Brady rhizobium associated with this plant, a total of eight rhizobial isolates were collected from different locations of eastern U.P. India, were analysed on the basis of colony morphology using congo red medium plates on which strain ETWH23 form largest and GZ2 forms least size colony and then acid/alkaline reaction on BTB plates on which except strain CHAND3 all are acidic in nature. On the growth medium except CHAND3 all grow well in glucose, demonstrating that bradyrhizobia lack uptake and catabolic system for disaccharide. Molecular diversity was checked among all isolates on the basis of RFLP analysis using 16S rRNA and IGS region. The dandrogram obtained from RFLP analysis reveals that two strains ETWH23 and MZP21 were diverse from each other and representative of all rhizobial isolates. On the basis of Blastn analysis of 16S rRNA sequences showed 98% similarity with Bradyrhizobium. Despite all variability, all strains nodulate well and there was a positive correlation between nodulation and nodule dry weight whereas plant dry weight showed positive correlation with acetylene reductase activity as showed by strain ETWH23. The results revealed that expanding bradyrhizobial diversity is a clear indication that Pigeonpea symbiosis is not yet genetically settled and it is also possible to conduct a systematic and scientific investigation on native Pigeonpea nodulating rhizobia.

Introduction

Pigeonpea or Redgram [*Cajanus cajan* (L.) Millspaugh] is an important food legume for the tropical and sub-tropical regions of Indian sub-continent, South-East Asia and East Africa. Pigeonpea is grown on ~5 million hectares (ha), making it the sixth most important legume food crop globally. It accounts for over 16% of the total pulse area and over 20% of the total pulse production in India. Owing to biotic and abiotic stresses, and the fact that pigeonpea is grown in low-input and risk-prone marginal environments, there is a large gap between potential yield (2,500 Kg/ha) and yields obtained in farmer's fields (866.2 kg/ha in Asia and 736.2 kg/ha in Africa) (Mula and Saxena, 2010). However, the crop productivity of pigeonpea has remained stagnant less than 1 ton per hectare for last 40 years as the crop is exposed to several biotic (e.g., Fusarium wilt, sterility mosaic disease, and pod borer) and abiotic (drought, salinity, and water logging) stresses.

The beneficial effect of endosymbionts in agricultural legumes in terms of biological nitrogen fixation has been the main focus in the recent past (Deshwal et al. 2003; Herridge et al. 2008), as it is an important aspect of sustainable food production and long term crop-productivity. Various types of native rhizobia are present in cultivated soils of different geographical and ecological zones of the world, including India. Through inoculation of strains selected from indigenous population, improved symbiotic efficiency can be achieved leading to qualitative and quantitative yield enhancement of pigeonpea. As a consequence of the persistent energy crisis resulting in higher fertilizer cost, biological N fixation (BNF) has become one of the most attractive strategies for the development of sustainable agricultural systems.The role of BNF, especially in legumes, is well established and documented. However, it has been reported that the different varieties or cultivars of grain legumes, namely pigeonpea, faba bean and cowpea, show significantly

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wide ranging differences in their ability to support BNF (Hardarson 1993).

Rhizobium – legume apart from their symbiotic association the complex fixes atmospheric nitrogen and improves the soil fertility. Addition of chemical fertilizers is necessary to correct poor soil fertility by supplying nutrients needed for optimum crop growth (Elsheikh et al. 2005). Chemical fertilizers became the target for criticisms mainly because of the heavy use in the world, where they were suspected of having adverse impact on the environment through nitrate leaching, eutrophication, greenhouse gas emissions and heavy metal uptakes by plants. In contrast, biofertilizers are more beneficial, a substance which contains living microorganisms which, when applied to seed, plant surfaces, or soil, colonizes the rhizosphere or the interior of the plant and promotes growth by increasing the supply or availability of primary nutrients to the host plant (Vessey 2003).

The research work "Identification, characterization and symbiotic evaluation of pigeonpea (*Cajanus cajan*) nodulating bradyrhizobia" was carried out in Department of Genetics and Plant Breeding, Institute of Agricultural Sciences, Banaras Hindu University. Rhizobium strains that were used in this research work, were obtained from the Department. The procured rhizobial strains were authenticated on Pigeonpea plant grown under controlled condition. Infective rhizobial strains were isolated from the nodules formed on pigeonpea plant after inoculation. Nodules were surface sterilized with acidified 0.1% mercuric chloride solution for 1-2 minutes followed by 70% alcohol for 1 minute and then rinsed 4-5 times with sterilized distilled water. Each nodule was crushed on the glass slide with the help of sterilized forcep. The rhizobial strains were grown and maintained in yeast-mannitol (YM) medium (Vincent, 1970). Agar-agar at a concentration of 1.5% was used to solidify the medium wherever necessary. Rhizobial clones were isolated by streaking one loop full suspension on YMA plates which were incubated in a growth chamber at 28 ± 1^0C. Mother cultures were maintained on YMA slants containing 0.3% $CaCO_3$.

2.02 Reaction to Congo red and Bromothymol blue

Stock solution of congo red (CR) and bromothymol blue (BTB) were prepared by dissolving 250mg of the dye in 100ml of water and ethanol, respectively. Ten ml of stock solution was added to one litre of YMA in order to get required concentration (25µg/ml). A loopful of exponentially growing culture of each strain was streaked on three different agar plates (YMA, YMA+CR and YMA+BTB). All plates were incubated for 5-7 days in growth chamber at 28^0Ċ . Clear individual 10 colonies were taken for recording colony morphology data of each strain. In addition, change in colour around individual colonies on YMA+BTB plates was considered for acid (yellow) or alkali (blue) production by rhizobial strain.

Methods

2.01*Rhizobium*Strain:- The research work "Identification, characterization and symbiotic evaluation of pigeonpea (*Cajanus cajan*) nodulating bradyrhizobia" was carried out in Department of Genetics and Plant Breeding, Institute of Agricultural Sciences, Banaras Hindu University. Rhizobium strains that were used in this research work, were obtained from the Department. The procured rhizobial strains were authenticated on Pigeonpea plant grown under controlled condition. Infective rhizobial strains were isolated from the nodules formed on pigeonpea plant after inoculation. Nodules were surface sterilized with acidified 0.1% mercuric chloride solution for 1-2 minutes followed by 70% alcohol for 1 minute and then rinsed 4-5 times with sterilized distilled water. Each nodule was crushed on the glass slide with the help of sterilized forcep. The rhizobial strains were grown and maintained in yeast-mannitol (YM) medium (Vincent, 1970). Agar-agar at a concentration of 1.5% was used to solidify the medium wherever necessary. Rhizobial clones were isolated by streaking one loop full suspension on YMA plates which were incubated in a growth chamber at 28 ± 1^0C. Mother cultures were maintained on YMA slants containing 0.3% $CaCO_3$.

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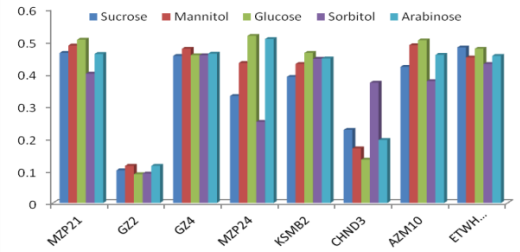
2.03Growth under different carbon sources

The growth of rhizobial strains was assessed in synthetic medium (SM) of O'gara and Shanmugam (1976) in presence of different carbon sources (mannitol, glucose, arabinose, maltose and sucrose) at a concentration of 1g/l (fig .1). Experiments were performed in 30 ml culture tubes (JSGW, India) containing 10 ml medium at 28± 2°C for 7 days. The data of optical density at 600nm were compared with digital spectrophotometer (Electronic India 305).



2.05DNA isolation, PCR amplification and Restriction Digestion of Amplified 16S rRNA and IGS (16S-23S rRNA) regions

Total genomic DNA of each strain was extracted from bacterial cells grown in YEM broth until late exponential phase (10^9cells/ml). Extraction of DNA was performed by a standard protocol (Ausubel et al. 1994). Purified DNA was dissolved in 10 mM Tris-HCL buffer containing 1 mM EDTA (pH 8.0). PCR reaction was carried out in final volume of 25µl reaction mixtures containing 12 µl PCR master mix (Fermentas, USA), 1µl of each primer (10pM) of Operon USA, 1µl (50 ng) genomic DNA and 10 µl sterilized water. The forward primer (5'GGAGAGTTAGATCTTGGCTC3') and reverse primer (5'CACCGCTACACCAGGAATTC3') of Operon, USA were used to amplify nearly 650 bp of 16S rRNA genes and IGS (16S-23S) region was amplified using primers FGPS 1490-72 (5'TGCGGCTGGATCCCCTCCTT3') and FDPS132-38 (5'CCGGGTTTCCCCATTCGG3') (Integrated DNA technology, USA) with standard protocol of Laguerre et.al. (1996). Amplification was done by using a standard temperature profile, in a Thermo cycler, GeneAmp PCR system 2720 (Applied Biosystem, USA), including initial denaturation at 94°C for 1 min, annealing at 57°C for 1 min , and extension at 72°C for 2 min , and a final extension at 7 min. The Amplified products were visualized on 1.5% of agarose gel.The endonucleases AluI, *Hind*III, HinfI, TaqI and MspI of Fermentas USA were used separately to digest the amplified 16S and 16S-23S rRNA region.The restriction fragments were analysed by electrophoresis in 2.5% agarose gel supplied with ethidium bromide (0.5µg/ml).The rRNA gene restriction patterns were visualized and photographed under UV light. Strains with identical restriction patterns were defined as belonging to the same ITS type.

2.06Sequencing and Phylogenetic Analysis of 16S rRNA

The amplified product of 16S rRNA fragement was purified using GeneJET PCR purification kit (Fermentas LifeSciences, India) and sequenced according to the manufacturer's instructions by ABI 3010 (Applied Biosystems automated sequence analyzer). All nucleotide sequences were blast using the National Center for Biotechnology Information (NCBI) server at http://www.ncbi.nlm.nih.gov/blast/Blast.cgi. The sequences were deposited in GenBank to get the Accession number. The phylogenetic tree derived from the 16S rRNA sequences of the strains was constructed with standard bradyrhizobial 16S rRNA sequences obtained from GenBank. Sequence alignment was performed with the ClustalX2 software. Aligned sequences were analyzed using MEGA software, version 4.1 (Tamura et al. 2007). Phylogenetic analysis of 16S rRNA sequences was performed by using theUPGMA method, involving 1000 replication
Bootstrapped of each sequence(Fig 2)

2.07RFLP Cluster analysis of 16S-23S rRNA region

Only distinct, well resolved, and unambiguous bands were scored, discarding faint bands. The digested fragments were scored as (1) at the presence and (0) at the absence of homologous bands. Thereafter, the similarity of strains tested was evaluated by simple matching Jaccard's coefficient. With the help of NTSYSpc 2.1 software (Rohlf 1992), a dendrogram was constructed from the distance matrix by the mean of unweighted pair group method with arithmetic mean algorithm (UPGMA)(Fig 3).

2.08Plant Culture and Symbiotic Effectiveness test

Plants were grown on nutrient–agar slant in glass tube. Nitrogen free Fahraeus N-free plant growth medium (Fåhraeus, 1957) was used for plant culture.

Healthy Pigeon pea seeds were surface sterilized with acidified mercuric chloride (0.2% w/v) for 3-5 minutes and 70% ethyl alcohol for 1-2 minutes and then thoroughly rinsed with sterile distilled water for 4-5 times. Surface sterilized seeds were germinated inside petri dish containing water agar (1% w/v). Following germination (1.0 cm long radical), the seed coat were removed and seedlings were transferred to nutrient agar slants inside glass tubes (200×38mm). Each seedling was fixed on agar slant in such a way

that the root system could grow downward along the agar surface. Bacterial suspension (1 ml containing about 10^8 cells) was inoculated after 24h of seedling growth. Plants were grown in a culture room at 28^0C under fluorescent light (intensity of 3 K lux) with 14/10h light/dark cycle. Uninoculated plants served as control.

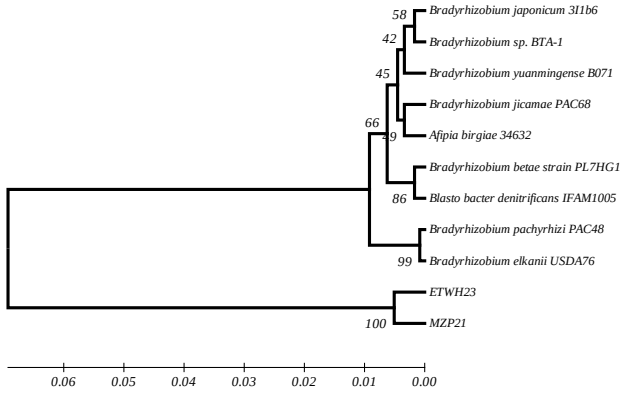


Fig 2: Phylogenetic analysis of 16s rDNA sequences

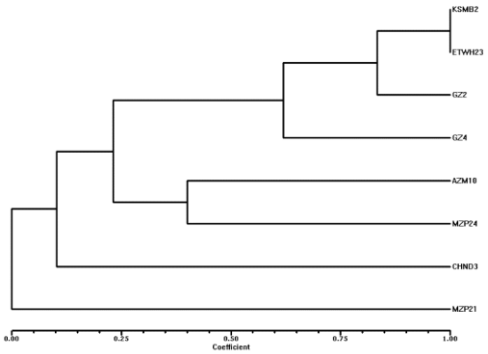


Fig 3: Dendrogram generated by RFLP of IGS region

2.09Evaluation of Symbiotic Effectivity

Following parameters were used for evaluation of symbiotic effectiveness in host × *Rhizobium* combination experiments.

(a) Nodulation: Observations were made on nodule number and nodule dry weight at specified days after sowing/planting.

(b) Nitrogenase Activity: Nitrogenase activity in nodules was determined by acetylene reduction technique followed by Somasegaran and Hoben (1994). Nodulated root was placed in 30 ml assay tube, incubated with 10% acetylene. After one hour of incubation, 0.5 ml gas sample of each flask was analyzed through Gas Chromatography (Nucon, model 5765) equipped with a hydrogen flame detector. The values were expressed as µmol of C_2H_4 produced per plant, calculated as follows:

$$Nitrogenase\,activity = \frac{Ethylene\ Produced}{Time\,(h)\,x\,No.of\ plants}$$

Total FID units through the instrument (GC) = Peak height of C_2H_4 gas × Attenuation

1 fid unit of the gas chromatograph calibrated from standard ethylene gas = 8.2×10^{-3} n mol C_2H_4.

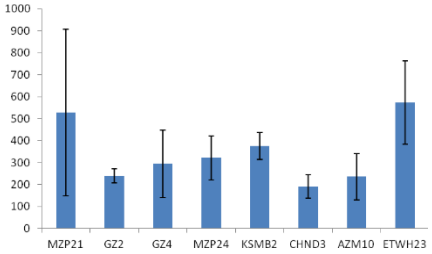
When the volume of incubation vessel was 30 ml and incubation time was 1 hr, then

µmol C_2H_4 produced $plant^{-1}h^{-1}$ = (8.2×10^{-3}) × total FID units × 30 $\times10^{-3}$.

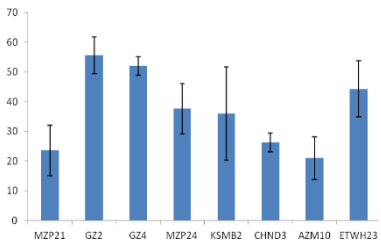
(c) Plant dry weight: Each plant was put separately in a paper bag and dried for 2 days at 60°C. Total plant weight was taken on electric balance.

2.10Statistical Analysis

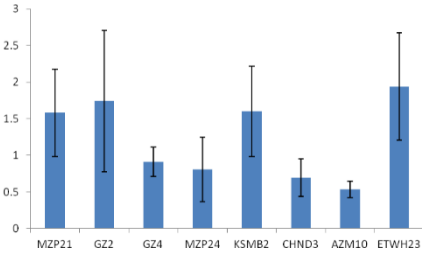
The data were analysed using randomized block design(Fig 4). From analysis of variance, a standard error was utilized to make comparisons among the means at the level of significance of *P* (0.01)



4a) Plant dryweight (mg)



4b) Noduleno.per plant



4c) µ mol ethylene produced perhr

Result and discussion

The pigeonpea rhizobia which were collected from different locations formed distinct colonies on YMA plates. The colonies produced by CHND3 and MZP21 were very small, opaque compact and dome shaped

whereas AZM10 and MZP24 appeared as white gummy colonies but GZ4 and KSMB2 formed slightly round and mucilaginous colonies and ETWH23 grew with highly mucilaginous flattened watery colonies, however, GZ2 formed small white colonies on YM containing agar plates . Colony size varied from 3mm (ETWH23) to 1.75mm (GZ2) in diameter after 8 days of growth on YMA plates (Table 1). Except CHND3 all isolates were acidic in nature which was marked by the change in colour of BTB containing YMA plate from green to yellow whereas CHND3 change the colour from green to blue that shows alkali producing properties of the isolate.

Growth of these rhizobial strains was compared in complex (YM) and synthetic (SM) medium. Result revealed that complex medium was highly supportive in bacterial growth in comparision to synthetic medium. Relative growth of all isolates was compared in presence of different carbon (sucrose, mannitol, glucose, sorbitol and arabinose) sources in synthetic medium and data were recorded after 10 days of growth. All isolates grew well in presence of glucose except CHAND3 which had maximum growth in presence of sorbitol whereas sucrose became least supportive in growth of all isolates. Isolate GZ2 and GZ4 showed similar growth rate in presence of all tested carbon sources .

The amplified PCR product of 16S rRNA and IGS region showed a single band on ethidium bromide treated agarose gel after electrophoresis. The PCR amplified band of 16S rRNA region appeared to be ~ 650 bp. The restriction patterns of 16S rRNA region following digestion with four (*Hind*III, *Alu*I, *Hinf*I, *Msp*I) restriction endonucleases (RE) were compared. The monomorphic bands were observed with all tested restriction endonuclease (RE). Among all four tested RE, only *Alu*I produced highest 3 bands for each isolates. The PCR amplified product of IGS region was ~ 950 bp. The PCR amplified product of IGS region was digested with 3 (*Alu*I, *Msp*I and *Taq*I) RE. Among all three RE, *Alu*I produced polymorphic band whereas *Msp*I, *Taq*I produced monomorphic band . Total 65 digested bands varying from 50 to 500 bp were identified. Of the 65 restriction digested fragements, 49 showed polymorphic bands. The number of restriction pattern was highly observed in *Alu*I (5 pattern) and *Taq*I (5 pattern) whereas the least observed in *Msp*I (1 pattern). The dendrogram generated from RE digested bands or RFLP pattern of IGS region formed four major clusters. Cluster 1 formed with four strains (KSMB2, ETWH23, GZ2 and GZ4) and cluster 2 consisted of two strains i.e. AZM10 and MZP24 whereas cluster 3 and cluster 4 comprised of single strain CHAND3 and MZP21, respectively. In cluster 1 KSMB2 and ETWH23 were very close to each other i.e. they were100% similar. The nucleotide sequence of 16S rRNA region of MZP21 and ETWH23 (both were highly diverse at RFLP pattern of IGS region) were analysed through ABI automated sequence analyzer. Considering the Blastn, both strains had highest 98% similarity to the genus *Bradyrhizobium* sps. Both strains were aligned and used to build the phylogenetic tree along with the reference strains. Phylogenetic analysis positioned the both strains within the *Bradyrhizobium*. The accession number for the strains MZP21 and ETWH23 was JX110119 and JX110120, respectively.

Symbiotic efficiency of these eight bradyrhizobial isolates was evaluated on cv. ICPL7035, long duration crop of pigeon pea under controlled condition. Isolates were screened in glass tubes containing N-free Fahraeus medium. A significant difference of all bradyrhizobial isolates in nodule number, nodule dry weight, acetylene reduction activity and plant dry weight was observed. In terms of nodule number strain GZ2 gave highest number of nodules per plant followed by GZ4, ETWH23, MZP24, KSMB2, CHND3, MZP21 and AZM10. Strain GZ2 was able to form 62% more nodule in compare to AZM10 (least nodulator). However, in terms of nodule dry weight strain KSMB2 exhibited highest nodule dry weight and AZM10 displayed similar trend of nodule number i.e. least nodule dry weight per plant, whereas other isolates except KSMB2 and AZM10, all were almost comparable. In terms of total plant dry weight, strain ETWH23 was more effective in comparision to other strains. Second most effective strain was MZP21, whereas in least effective ones CHND3 was 59% lesser in dry weight in comparision to ETWH23 . Result on acetylene reduction activity (ARA) presented in Fig.4cshowed that strain ETWH23 was able to reduce highest acetylene, followed by GZ2, MZP21, KSMB2, GZ4, MZP24, CHND3 and AZM10.

Pigeon pea was considered for long as an orphan legume crop, but now substantial amount of genomic resources have been generated. It forms effective symbiosis with a wide range of rhizobial strains belonging to the ‘cowpea miscellany’ (People and Herridge 1990). In present study all bradyrhizobial isolates formed distinct colonies on YMA plates. The growth rate mean generation time (MGT) of all rhizobial isolates varied from 7-13 h. Therefore, these apparently fall in the category of slow grower, because as per the report of Fred (1932) and Rabic El Akhal et al. (2009), that rhizobia which have MGT of more than 6h are considered as slow grower.

The observation that both (large and small) type of colonies were found by pigeonpea nodulating bradyrhizobia, is contrast to the result of Bromfield and Kumar Rao (1983) that fast growing pigeonpea rhizobia form large and slow grower form small colonies on YMA plates.

All strains grew well with all carbon sources, but growth was observed maximally with glucose suggesting that all bradyrhizobia appear to lack uptake

system and catabolic enzyme for dissacharide (Glenn and Dilworth 1981).

All eight rhizobial strains showed high genetic diversity after RFLP analysis of IGS region. RFLP analysis of IGS formed 5 types of pattern which provided the strength of discrimination among chromosomally closely related strains (Laguerre et al. 1996). The dandrogram generated after combined data of various restriction enzymes, enables estimation of relatedness between the strains. Therefore, it has been proven that restriction digestion of ribosomal DNA is a powerful tool to characterize rhizobial isolates obtained from root nodules (Santamaria et al. 1997; Shamseldin et al. 2009; Jaiswal et al. 2012). Phylogenetic and 16S rRNA sequence analysis indicated that they belong with *Bradyrhizobium* sp. This result is in agreement with Ramsubhag et al. (2002) that pigeonpea (*Cajanus cajan*) is also nodulated by *Bradyrhizobium* sp.

The efficient exploitation of biological nitrogen fixation to improve agricultural productivity requires that the population of indigenous rhizobia is adequately characterized. Present study detected that genetic diversity occurred under aseptic conditions, showing that the legume-bradyrhizobia symbiosis tends to promiscuity. An interesting point is that, despite all variability observed between strains, all plants nodulated satisfactorily, and there was a positive correlation between nodulation and nodule dry weight (Torres et al. 2012) whereas plant dry weight showed high correlation with acetylene reduction activity which is parallel to the result of Jaiswal and Dhar (2011), demonstrating that, despite variability, the symbiosis per se is very stable.

Conclusion: The expanding diversity of bradyrhizobia is a clear indication that the pigeonpea symbiosis is not yet genetically settled and it indicates that it is possible to conduct a systematic and scientific investigation on native pigeonpea rhizobia from various cultivated fields of Uttar Pradesh, India for obtaining a reliable understanding of the existing bradyrhizobial diversity in this region and conveys the massage that selection of rhizobial strains, appropriated for the newly released cultivar, will certainly provide a great potential for improvement of pigeonpea production in the country

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