

SUNN HEMP (*CROTALARIA JUNCEA*) NODULATING BACTERIA CAPABLE FOR HIGH ANTAGONISTIC POTENTIAL AND PLANT GROWTH PROMOTION ATTRIBUTES

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ABSTRACT

Rhizobia and plant legumes have been potentially associated by an essential process symbiosis for biological nitrogen fixation (BNF). However, compatibility and responsible factors for symbiosis are poorly understood. The present study revealed the 55 indigenous rhizobacterial isolates from root nodules of *Crotalaria juncea* from three different agricultural regions/locations in North-east Indo Gangetic Plains (NE-IGP) Uttar Pradesh and Uttarakhand. Amongst them, strain NCR5, was screened as a resilient antagonistic isolate against *Macrophomina phaseolina*. Based on 16S rRNA gene sequencing, it was identified as *Rhizobium leguminosarum*. Amplification of *Nod* gene proved it to be a nodulating strain. Phenotypically, NCR5 grew well within the temperature range 10-40 °C and pH 5.5-9.0 as well as survived with 3.0% salt stress. Plant growth promotion characteristics were confirmed it as the best-performing PGP strain. The characteristics included phosphate solubilization, IAA production, siderophore production, HCN productions. This study underwrote the *Rhizobium leguminosarum* NCR5 as a potential applicant for bioformulation to apply in the soil for promoting the PGP traits and. It can also be employed as fungicides against *Macrophomina phaseolina* in adverse soil types and environments.

Keywords: Sun-hemp, rhizobia, antagonism, plant growth promotion, 16S rRNA gene

INTRODUCTION

Sunn hemp (*Crotalaria juncea*) is a tropical to subtropical, multipurpose cover legume crop with great value for farmers due to several characteristics such as fiber crop, high biomass yield, non-host for a large group of pests and pathogens, weed suppressive (Collins *et al.*, 2008). It is a green manure, increases soil organic matter, symbiotic and is an efficient Nitrogen (N) fixer in association with the rhizobacteria (Abdul-Baki *et al.*, 2001). It cultivates widely in India, Asia and in North America and is popular due to short rotations in vegetable and cropping systems (Schomberg *et al.*, 2007) and also used to avoid outbreaks of pests and diseases (Cook *et al.*, 2009).

Balkcom and Reeves (2005) have revealed it to be use it as a potential green manure/cover crop and was also proven by Cherr *et al.* (2006). It is a tropical legume and has the ability to fix the nitrogen by symbiosis with root nodulating rhizobia (Mendonça *et al.*, 2005). Moreover, legumes are well known for the ability to create symbiosis with soil bacteria that belong mainly to the genera *Rhizobium*, *Ensifer*, *Mesorhizobium*, *Bradyrhizobium* and *Azorhizobium*, commonly known as rhizobia. These relationships allow them to be independent of the nitrogen levels in the soil. Under limited nitrogen availability, rhizobia form specific symbioses with legume hosts, elicit root nodules and reduce atmospheric nitrogen inside them. The symbiotic relationship between rhizobia and legumes accounts for around 60% of total biological nitrogen fixation (BNF) inputs in the world agriculture per annum (Herridge *et al.*, 2008). Resultantly, Nitrogen is used to synthesize plant proteins and nucleic acids, including DNA. Phosphorus (P) is the most limiting nutrient after nitrogen (N) in crop yields and is essential factor of soil health and plant growth. The deficiency of Phosphorus directly inhibits the stem and root development, flowering, fruits and seed quality which have resulted in the development of bioinoculants for plant growth and sustainable agriculture.

Currently, diazotrophic bacteria have the potential to uses an alternative to nitrogen fertilizers and therefore used as bioinoculants (Welbaum *et al.*, 2004). Diazotrophic bacteria belonging to Plant Growth Promoting Bacteria (PGPB) can competitively colonize plant root for nodulation, promoting the plant growth, and reduce plant diseases. In the rhizosphere, PGPB may colonize soil that envelops

the roots or adhere to the root surface (Andrews and Harris, 2000). Many members of α -proteobacteria have the capacity to interact with eukaryotic cells and function as endophytic symbionts. Amongst them (*Rhizobacteria*, *Agrobacterium*, *Rickettsia*, *Bartonella* and *Brucella*), few of could fix nitrogen and are therefore agriculturally important (Yuan *et al.*, 2018). Rhizobia have been used in agricultural practices mainly for nitrogen fixation and PGP (Weyens *et al.*, 2009) due to their wide distribution.

PGP activity of soil microbes has been reported extensively by many researchers (Yang *et al.*, 2018; Singh *et al.*, 2019) but abundance of potent strains is still limited. Reports of current research have revealed that the excessive and indiscriminate use of chemical fertilizers has resulted in increased concentration of nitrate, phosphate and sulphate ions in soil (Adesemoye *et al.*, 2009), which can percolate to ground-water or accumulate in water bodies due to run off, thus creating serious health problem for living beings. The PGP rhizobacteria perform the vital roles in plant growth by nitrogen fixation (Letters, R. B. 2011), phosphate solubilization (Santoyo *et al.*, 2016) and production of phytohormones (Tyc *et al.*, 2017) or diverse lytic enzymes with deleterious properties against pathogenic organisms (Philippot *et al.*, 2013).

Hence, the present investigation was taken up to isolate the root nodule endophytic bacteria from *C. juncea* L. and aimed to screen the isolates having potentiality to inhibit the soil-borne fungal phytopathogen *M. phaseolina* and endowed with the plant growth promoting traits.

MATERIAL AND METHODS

Root nodule Sampling, bacterial isolation, authentication and preservation

Healthy Root nodules from *C. juncea* L. were collected from three different field sites of Gurukul Kangri Vihwavidhyalaya Campus, Haridwar (Uttarakhand); Kankhal (District Haridwar, Uttarakhand); Seohara (District Bijnor, Uttar Pradesh). Briefly, healthy *C. Juncea* plants were uprooted and were placed into a sterilized plastic bag and immediately brought to the laboratory. Plant roots were immersed in running water to remove the adherent soil. Further, healthy nodules surface sterilization was carried out by placing it in 95% ethanol for 5-10 sec.,

then 3% (v/v) sodium hypochlorite (2-4 min.) and finally rinsed in SDW with 4-5 times to remove traces of sterilizing agents. Surface sterilized nodules were plated on Yeast mannitol agar with Congo red dye (YMAcr) at 28±2°C to procure the nodule bacteria.

All the purified isolates were tested for their ability to induce root nodules on *C. juncea* L. with standard procedures (Singh et al., 2016; Zhang et al., 2016). Purified isolates were preserved in to slant (4°C) for routine use and in glycerol stock (-20°C) for long term storage.

Physicochemical Properties of sampling sites

Soils from each sampling sites were collected randomly from 5 locations at a depth of 10 and 5 cm and were composite it after being dried. Crop cultivars and cultivation history were acquired from field owner. Locations were noted down by Android GPS system. Soil type, sand percentage, total N, Total P, Total K, pH and EC of soil were analyzed by standard methods (Sinsiri et al., 2002).

Selection of superlative Antagonist

Soil borne fungal pathogens *Macrophomina phaseolina*, *Fusarium udum* and *Rhizoctonia solani* were obtained from fungal collection of the Department of Botany and Microbiology, Gurukul Kangri Vishwavidyalaya, Haridwar and tested against the procured nodules endophyte. Briefly, a round mycelia mat (5 mm) of soil-borne fungi, was placed on one side of a potato dextrose agar (PDA, HiMedia) and 5µl of log phase cultured broth was spread on the other side. Further, it was placed in to the incubator at 28 °C±2 for 5 to 10 days. Moreover, Cell-free culture supernatant (CFCS) of tested strains (100µl) was also dropped nearby (1.0 cm) fungal disc into wells (0.5 mm) of new plates and incubated as described above. Antagonism property of tested strains and their CFCS was confirmed by zone of inhibition formed along with the cultivation of mycelia. All the experiments were performed in triplicate.

Molecular Identification

Isolation of Genomic DNA (gDNA) were performed through gDNA Purification Kit (HiMedia) and visualized on 0.8% horizontal agarose gel electrophoresis. Nearly full-length 16S rRNA gene was amplified using universal primer pair, fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rp2 (5'-ACGGCTACCTTGTTACGACTT-3') (Weisburg et al., 1991). Amplified gene product was further examined in 1.2% agarose gel and was purified by gel-elution method (HiMedia gel elution kit). Purified PCR products was further sequenced with the same primers. Similarity search and annotation was carried out by NCBI-BLAST_N program and validated sequence was deposited in NCBI Gene Bank. Bootstrap UPGMA phylogeny of tested strain was drawn by MEGA software Version 5.0 (Tamura et al., 2011) under maximum composite likelihood model with 1000 bootstrap replication with 64238seed value.

Morpho-phenotypes and PGP attributes

Abiotic environmental tolerance (pH and NaCl) was evaluated by inoculating the 5µl log phase bacterial culture to the 100ml nutrient broth in conical flasks. The intrinsic tolerance of the isolate was measured after adding the NaCl 0-5% (w/v) separately while pH tolerance was measured at range 4.0 to 10.0 by adding the 0.1M HCl or NaOH. Without NaCl and HCl or NaOH was worked as control. Bacterial Growth was measured spectrophotometrically and plotted against control to evaluated the tolerance capability. Further, above described inoculating

conical flask and media plate was incubated at 28±2°C for 72h to 120h. Temperature tolerance was measured after examined the growth of spotted culture on NA media at the temperature 10, 15, 20, 25, 30, 35, 40, 42 and 45°C. Plate spot method (on Pikovaskya agar) was followed for the phosphate utilization test and was evaluated on the basis of halo zone formation nearby inoculation colony (Pikovskaya, 1948). IAA production was assessed as described by Brick et al. (1991). Briefly, pure culture of tested isolates was grown in nutrient broth (with and without L-Tryptophan) for 7 days at 120 rpm and 30°C. After that, grown cell broth was harvested for supernatant by centrifugation at 4500 rpm and then Salkowski reagent (50 ml, 35% of perchloric acid, 1ml 0.5M FeCl₃ solution) was added individually. Further, O.D. (optical density) was measured by spectrophotometer and culture supernatants IAA concentration was speculated through extrapolate against standard curve of pure IAA (10-100 µg/ml). Ammonia, HCN and Siderophore production test was carried out according to Cappuccino and Sherman (1992), Lorck (1948) and Alexander and Zuberer (1991), respectively.

Seedling test and Pot Assay

Seedling test of *C. juncea* L. was performed according to Singh et al. (2019) to evaluate the potential of screened strain with and without fungal pathogen *M. phaseolina*. Uninoculated seeds were treated as control. Briefly, healthy seed of *C. juncea* L. (surface sterilized) were treated with log phase culture (100µl) and placed on sterilized 0.5% water agar poured petri-plate and covered with UV-sterilized blotting paper sheet. Plates were incubated in laboratory at room temperature. After 10 days, root length and numbers, shoot length and plant weight were measured. Each set of experiment was carried out in five replicates. Nodulation efficiency of screened strain was evaluated by pot assay that were carried out according to Singh et al. (2015) with partial modification. Briefly, germinated Sun-hemp seedlings were taken and inoculated with 2 ml broth (log phase) of tested isolate and were transferred to clay pot containing sterilized sand. Un-inoculated pot was treated as negative control. All the experimental setups were kept in net house of Gurukul Kangri Vishwavidyalaya and were watering regularly by sterilized nitrogen free Sloger's solvation. After, 30 days, plants were uprooted carefully, washed gently with cleaned water and measured the root length, root fresh and dry weight, shoot length, shoot fresh and dry weight and nodule number. All the experiments performed in 5 replicates of each. All the data were subjected to analysis of variance using one-way ANOVA and means were compared by Duncan's multiple-range test (Duncan, 1955).

RESULTS AND DISCUSSION

Plant adaptation, growth promotion and protection against the phytopathogens in various types of environment are fundamentally correlated to soil microorganism symbiosis (Compant et al., 2010; Yang et al., 2019; Singh et al., 2019). With the aim to isolate the *C. juncea* root nodulating bacteria, three different cropping sites were sampled for healthy plants of Sun-hemp. Selected sites had never been inoculated with any bio-inoculant. Physicochemical characterization of sampled sites is described in Table 1 and S-1. A total of 55 bacteria has procured from healthy root nodule. All the isolates were formed mucilaginous rhizoid and creamy whitish semitransparent colonies on YMAcr plate that proved it as rhizobia. Most of the isolates were fast growing to moderately fast-growing ones with a Generation time (GT) between 3.5 and 4-6 h respectively.

Table 1 Physio-chemical Properties of Rhizospheric soil Associated with *C. juncea* L.

Site	N (%)	P (mg/gm)	K (mg/gm)	Ca (mg/gm)	Mg (mg/gm)	Cu (mg/gm)	Fe (mg/gm)	Ni (mg/gm)	Zn (mg/gm)	TOC (%)	Con (µs)	pH
1	0.03	95.7	659.74	3614	2578	21.39	196.32	5.74	26.3	0.2	243	8.18
2	0.16	1998.7	2787.3	3568	3099	25.32	212.73	16.33	105.34	1.87	409	7.47
3	1.8	1381.3	1667.7	5471	3984	20.74	307.1	14.75	77.3	3.76	354	7.30

Abbreviations: Values represent mean of three values; N Nitrogen content; p Phosphate content; K Potassium content; Ca Calcium content; Mg Magnesium content ; Cu Copper content; Fe Ferrous content; Ni Nikil content; Zn Zinc content; TOC Total Organic Carbon; Con Conductivity; pH number of H ions that explain acidity/basicity of a sample; Soil was collected from around rhizosphere of plant site in triplicate and composite it; Soil texture varied from place to place from clay to clay loam; Site 1 Gurukul Kangri Campus (Haridwar); Site 2 Kankhal (Haridwar) Site 3 Seohara (Bijnor)

World-wide crop yield has been significantly affected by soil-borne fungal pathogens but the management strategies of plant diseases basically depends on the chemical fungicides. Using an antagonistic bacterium against phytopathogens can be remarks as one of the most attractive and alternatives tools. Several green house and field studies have revealed the positive antagonistic effects of bacteria such as *Bacillus*, *Pseudomonas*, *Rhizobium* group and *actinobacteria* on soil-borne plant pathogenic fungi (Nelson, 2004; Siddiqui, 2006; Santoyo et al. 2016). *M. phaseolina*, a soil born fungi causes charcoal root rot in about 500 plant species of more than 100 families around the world (Ashok et al., 2009; Gupta et al., 2012). Moreover, it is infected to human beings via environmental exposure. Hence, all the procured isolates were tested for their antagonism potential against *M. phaseolina*. Amongst all the procured root nodule isolates,

NCR5 was screened as superlative strain. The inhibition of fungus *M. phaseolina* by NCR5 was revealed by the high radial growth inhibition percentage. In dual culture test, inhibition started after 48h incubation and increased linearly up to 120h, after this the period it became constant. The average radial growth inhibition percentage against *M. phaseolina* was recorded about 75.14% (r₂= 0.7286) while cell free culture filtrate was displayed the 72.68% (r₂= 0.7492) (Fig. 1). Scanning electron micrographs confirmed the mycelial abnormalities in *M. phaseolina* with appearance of broken mycelium, coagulation and leakage of cytoplasm (Fig.1).

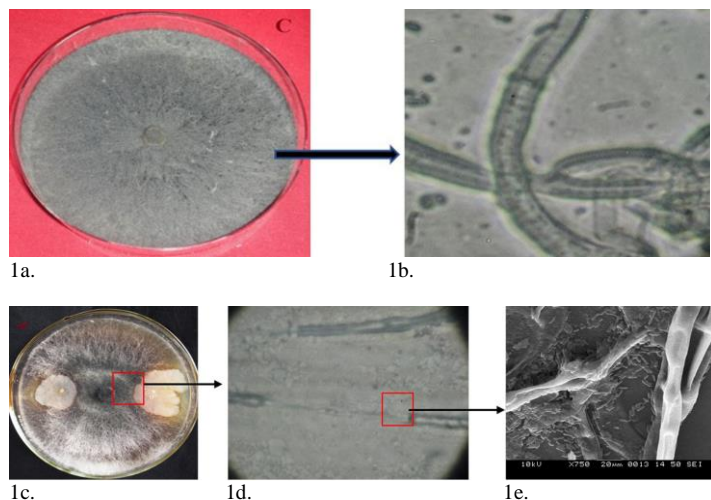


Figure 1 (a-e). Healthy mycelia of *M. phaseolina* (a-b); Plate assay, microscopic and SEM visualization of inhibition of *M. phaseolina* (c-e)

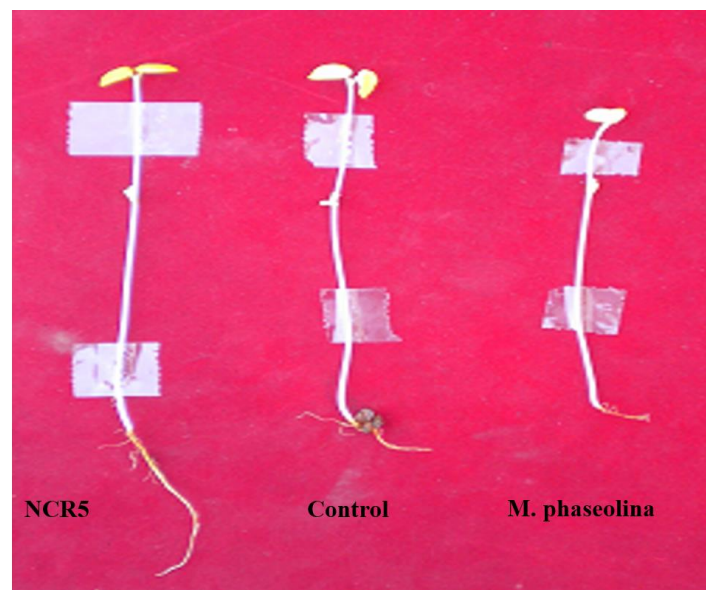


Figure 2 Seedling assay of *C. juncea* inoculated with NCR5, control (Uninoculated) and inoculated with phytopathogen *M. phaseolina*

Endophytic bacterial based benefits to their hosts via PGP activity and reducing the harshness of soil borne diseases are well known (Glick B.R. 2014; Le et al. 2016; Prajakta et al. 2019). The bacterial application for PGP of crops, including good seed emergence, healthy crop growth and high yields, tolerance to biotic and abiotic stress and controls to plant disease has been studied well (Gururani et al. 2013; Masciarelli et al. 2014; Santiago et al., 2017). In this work, the seedling assay of NCR5 displayed the positive PGP effect on Sun hemp growth (Fig. 2). Mean value of five replicates of each set experiments showed that root length, root number and shoot length of NCR5 inoculated seedlings was 3.85±0.09cm, 4.83±0.11 and 8.96±0.12, respectively while control set was measured as 1.93±0.11, 2.84±0.07 and 7.9±0.19, respectively. Seeds inoculated with *M. phaseolina* had drastic alteration in the measured values of root length, root number and shoot length as 1.27±0.10, 1.71±0.12 and 4.92±0.15, respectively.

Nodulation test of studied strain NCR5 displayed the effective nodulation with *C. juncea* in subsequent inoculation tests, as evidenced by the healthy pink color inside the nodules and natural green leaves of the plants. The negative controls had grown poorly with yellow-green leaves and lack of nodules in root that might be inferred the nitrogen fixation capacity of tested strain (Table 2). Interestingly, all the tested parameters were significantly higher than the control and had efficient nodulation as nodule number was 17.362 (Mean of replicates) but growth in root length was found insignificant. For the facilitation of consistent monitoring, molecular identification of the bacteria is the major demand for accurate taxonomy (Subhashini et al. 2017, Singh et al. 2019).

Identification and phylogenetic discrimination were done by amplification and sequencing of 16S rRNA gene. Blast report of curated gene sequence showed the 99.5% identity with *R. leguminosarum*. Identified gene sequence was deposited to NCBI database under the accession number JX124224. The reconstructed UPGMA phylogeny of the homogenous gene sequences showed that NCR5 is clustered with a common sub-group along with identifier strains of *R. leguminosarum* *R. anhuiense* and *R. sophorae* (Fig. 3) and was grouped with *A. rhizogenes* LMG142, *Arthrobacter viscosus* LMG16473T.

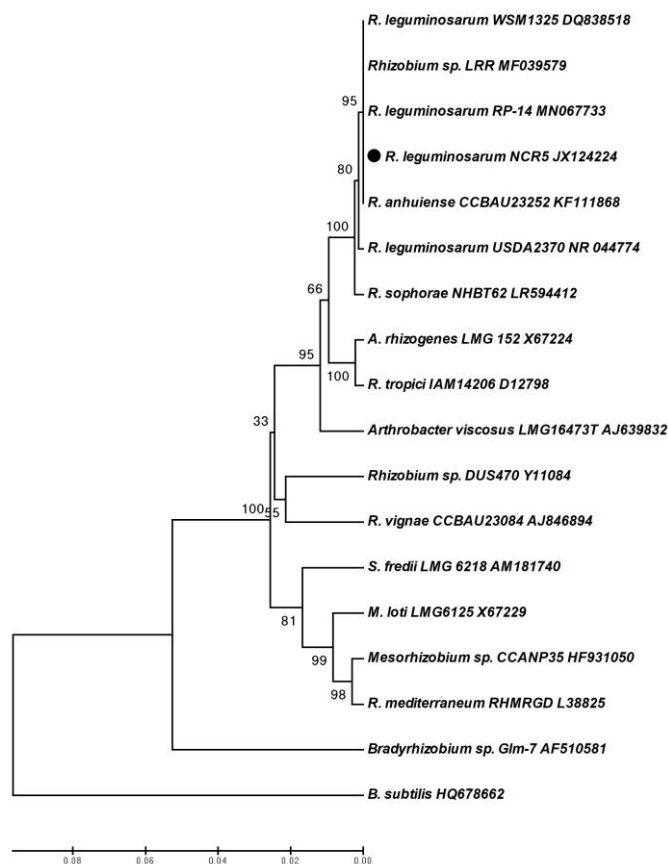


Figure 3 16S rRNA gene phylogeny of *R. leguminosarum* NCR5



4a. Phosphate Solubilization
4b. Siderophore Control
4c. Siderophore production

Figure 4 PGP traits of *R. leguminosarum* NCR5

Changes in pH, temperatures, salinity of soils can restrict the symbiosis and nitrogen fixation of legume and rhizobia and thus the strains that have the capability to tolerate these stresses would survive (Berrada et al., 2012). Isolate NCR5 was able to grow over a salinity ranges from 0 to 3% NaCl concentration (w/v) with optimum at 2.5% NaCl. pH tolerance ability favored with alkaline log scale as colonies were grown well at pH 9.0 (28°C) while no growth was occurred below the pH5.5. Temperature tolerance ability was recorded as slightly moderate to moderate from 10°C to 21°C, favorable from 22°C to 36°C while moderate at 37-40°C.

For legumes nodule biomass can shape the plant growth, yield and nitrogen fixation and is strongly correlated to available P (Hellsten and Huss Danell, 2001). In our study, clear halo zone on NCR5 spotted Pikovskaya agar showed the P-solubilizing ability of the isolate. Similarly, yellow zone production along with NCR5 growth on CAS agar plate revealed it as siderophore producing strain. The siderophores biosynthesis in microorganisms is persuaded by intracellular iron deficiency and are secreted by the cell to scavenge the iron from nature (Wandersman and Delepelaire, 2004). Siderophore synthesis is positively correlated with other stress factors like pH, temperature, carbon source and other metals utilization (Winkelmann G. 2007). PGP traits of strain NCR5

displayed it as a potential producer (Fig. 4). The IAA production was $198.03 \pm 0.01 \mu\text{gml}^{-1}$ and $231.71 \mu\text{g} \pm 0.01 \mu\text{gml}^{-1}$ in absence and presence of tryptophan, respectively. Likewise, Egamberdieva et al. (2016) reported the phytohormone indole-3-acetic acid production by *S. rhizophila* and it was positively correlated with the salinity (up to 4%NaCl) which did not inhibit its auxin production. Moreover, it has been documented well in previous reports that bacterial IAA increased the root surface area and resultantly big surfaces area for

uptake the water and essential nutrients from the soil and have direct effect on plant growth (Berg et al. 2010; Egamberdieva et al., 2016). This statement has been positively displayed and correlated in seedlings assay and pot experiment of strain NCR5. Qualitative estimation of ammonia production and HCN were also detected in the tested strain. Overall results revealed the NCR5 as a best PGPB as well as PGPR strain according to Bashan and Holguin (1998) classification.

Table 2 Pot assay depicting the Vegetative Parameters of *C. juncea* L. (After 30 Days)

Treatment	Shoot			Root			
	Length (cm)	Fresh weight (g)	Dry weight (g)	Length (cm)	Nodule Number (NN)	Fresh weight (g)	Dry weight (g)
T1 (NCR5)	63.0**	13.27**	1.790**	5.8ns	17.362**	0.1149**	0.0536**
Control	56.7	12.28	1.386	5.27	00	0.0743	0.0133

Abbreviation: Values are mean of five replicates; * significant at 0.01 level of analysis of variance (ANOVA); ** significant at 0.01 level of LSD as compared to control ; * significant at 0.05 level of LSD as compared to control; ns = not significant at 0.05 level of LSD as compared to control

CONCLUSION

Besides evaluating their carrier based bioformulations which may induce plant growth and development of *C. juncea* L., many previous studies have shown that PGPR activities of a symbiotic bacteria can be enhanced by co-inoculation with other microorganisms however, nodule endophyte of *Crotalaria juncea* L. have not yet been specifically studied. In this study, we have collected and characterized nodule endophytic bacterial isolates from *C. juncea* L. In our study was to isolate the *C. juncea* nodule bacteria and screened the best antagonistic strain against *M. phaseolina*, a soil borne fungal pathogen. Furthermore, the study identified their phylogenetic place, morpho-phenotypic characterization and PGP potential exploration for agro-economical uses.

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