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ISOLATION AND CHARACTERIZATION OF THERMOTOLERANT MUNGBEAN (*VIGNA RADIATA* L.) RHIZOBIAL ISOLATE AS BIOFERTILIZER

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Abstract

Rhizobia act as primary nitrogen fixing microorganisms in the root of leguminous plants. Efficient rhizobia are helpful in increasing productivity of legumes. In the present investigation a total of twenty rhizobial isolates were retrieved from root nodules of mungbean plants growing in field during summer season. On evaluation of plant growth promoting traits of all rhizobial isolates it was found that rhizobial isolate HSR1 had maximum indole acetic acid (IAA) production (58.83 µg/ml), ammonia excretion (4.78 µg/ml) and phosphate solubilization potential. Other plant growth promoting traits like aminocyclopropane-1-carboxylic acid (ACC) deaminase activity, siderophore production and hydrogen cyanide (HCN) production were also exhibited by isolate HSR1. This isolate was able to survive 45ºC and exhibited plant growth promoting traits at high temperature. Genomic DNA extracted from isolate HSR1 was subjected to amplification of 16S rRNA gene for identification. The similarity searching of the sequence obtained after 16S rRNA gene sequencing showed 99% similarity with *Sinorhizobium* sp. T25, which was confirmed by morphological and biochemical tests. Presence of good plant growth promoting traits makes HSR1 a promising strain to be developed as biofertilizer to improve soil fertility and plant growth.

Introduction

Mungbean (*Vigna radiata* L.), commonly known as green gram, is one of the important pulse crops of India. Mungbean contributes 14% of total pulse area and 7% total pulse production in India. Mungbean is grown during spring, summer and kharif season (Dudeja *et al.* 2012). It has special importance in the intensive crop production system of India for its short growing period, high protein content and nutritive value. Overgrowing population demands high supply of mungbean, which indirectly requires sustainable agriculture practices for higher production and lower expenditure.

Nitrogen and phosphorous are important nutrients for growth of legumes like mungbean. Chemical fertilizers are helpful in fulfillment of essential nutrients for plants but high cost and environmental problems associated with chemical fertilizers restrict their uses. Active cultures of microorganisms also known as biofertilizer being cost effective and environment friendly in nature make a significant approach for meeting nutrients requirement (Elsoni and Osman 2011) Chemical fertilizers provide only nutrients to growing plants but biofertilizers provide resistance to plants against invading pathogens through the volatile compounds secreted by microbes and other mechanisms (Ahuja and Kissen 2012). Unlike chemical fertilizers, biofertilizers overdose do not cause harm to the environment.

Abiotic and biotic stresses are the limiting factors for the leguminous plants in which productivity depends on symbiotic nitrogen fixation. Rhizobia besides fixing nitrogen possess different plant growth promoting traits like phytohormone production, siderophore production, 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity, hydrogen cyanide (HCN) and

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ammonia excretion, phosphate, zinc and potassium solubilization activity which enhance productivity and improve plant health. Rhizobia can provide significant amount of nitrogen to the leguminous plants resulting in increased plant height, number of leaves, percentage of seed filling and seed dry weight. Rhizobia also synthesize IAA, thus increasing plant growth by suppressing expression of ethylene level (Glick 2007, 2012). It has been reported that rhizobial inoculation enhances stomatal conductance thereby increasing photosynthetic rate and grain yield. However, successful deployment of these organisms in adverse environments such as high temperature depends on their ability to withstand and proliferate under stressed ecosystems and exhibit plant growth promoting traits. The present study was aimed to isolate rhizobial strains from root nodules of summer mungbean, evaluate growth and plant growth promoting traits at high temperature and characterize them using molecular and biochemical methods.

Materials and Methods

Mungbean root nodules were collected from CCS Haryana Agricultural University, Hisar fields (latitude- 29°10'N and longitude- 75°46'E) during summer season. Healthy pink nodules were selected and immersed in freshly prepared 0.1% $HgCl_2$ solution for two min followed by washing with 70% ethanol and finally rinsed with sterilized water for 3 to 5 times. The surface sterilized nodules were crushed in sterilized Petri plates and the suspension was streaked on plates of Congo red containing yeast extract mannitol agar (CR-YEMA) medium. Plates were incubated at 30°C for 3 to 6 days and single colonies were picked and surface-streaked several times until purification. Isolated colonies of rhizobial isolates were streaked on YEMA slants and kept in refrigerator for further experiments.

Temperature tolerance of different rhizobial isolates was tested by spotting 100 µl active cultures on yeast extract mannitol agar medium plates, incubated at 30, 35, 40 and 45°C for 3 days and growth was recorded. The growth of thermotolerant rhizobial isolate in yeast extract mannitol broth incubated at different temperatures was also observed in terms of optical density at 600 nm after 72 hr of incubation.

Nodulation test for thermotolerant rhizobial isolate in summer season was performed under sterilized conditions in Leonard jar assembly (Vincent 1970). Dry river sand was sterilized with hydrochloric acid and acid was removed from the sand by washing with tap water. The sand was dried in an oven at 80°C for 3 to 4 days and autoclaved twice for 30 min at 121°C at an interval of 24 hrs. Healthy seeds of mungbean variety MH 421 were surface sterilized by immersing in 1% $HgCl_2$ and then washed thoroughly with five changes of sterilized water. Surface sterilized seeds were treated with rhizobial culture and four seeds were sown in Leonard jars. Uninoculated seeds were kept as control and allowed to germinate. After germination, plants were watered with sterile distilled water daily and Sloger's solution was added once in a week. Three plants for each replicate were maintained and at the time of 50% flowering, plants were uprooted from the assembly very carefully without any root damage, washed with water and observed for nodulation.

The thermotolerant mungbean rhizobial isolate was tested for plant growth promoting traits *viz.* IAA production, ammonia excretion, phosphate solubilization, ACC deaminase activity, siderophore production and HCN production at different temperatures *i.e.,* 30, 35, 40 and 45°C. The ability to produce IAA by thermotolerant rhizobial isolate at different temperatures was tested by growing in tryptophan supplemented broth at different temperatures. The appearance of pink color on testing with salkowaski reagent indicated IAA production (Gordon and Weber 1951).

Thermotolerant rhizobial isolate was observed for ammonia excretion in peptone broth as per the method described by Chaney and Marbach (1962) and observed for the development of red color after incubation for 72 hrs at different temperatures.

Phosphate solubilization potential of thermotolerant rhizobial isolate was checked by spotting 3 µl of log phase grown culture on Pikovskaya's agar plates (Pikovskaya 1948). The plates were incubated at different temperatures for 3-4 days. P-solubilization activity was observed as formation of solubilization zone and calculation of solubilization index (P-SI) by following the formula of Premono *et al*. (1996).

ACC deaminase activity of thermotolerant rhizobial isolate was tested for utilization of ACC by spotting 3 days old culture on minimal medium agar plates supplemented with 2 mM ACC and growth was observed at different temperatures (Penrose and Glick 2003). Thermotolerant rhizobial isolate was checked for siderophore production on chrome azurol S (CAS) assay plates and observed for orange halo zone formation after 72 hr of incubation at different temperatures (Schwyn and Neilands 1987). HCN production potential of rhizobial isolate at different temperatures was evaluated by inoculation in peptone broth containing filter paper strips hanging loosely to the test tube. Development of dark red brown color after incubation indicated HCN production (Alstrom and Burns 1989). Biochemical characterization of rhizobial isolate was carried out as per standard methods described in Bergey's Manual of Determinative Bacteriology (Holt *et al*. 1994).

For molecular characterization, genomic DNA of rhizobial isolate was extracted using standard method and purified by RNase treatment (Leonard *et al*. 1986). Genomic DNA was subjected to PCR amplification of 16S rRNA gene using the forward primer 27F and reverse primer 1492R. The PCR reaction for amplification of 16S rRNA gene consisted of 1µl DNA, 4 µl d NTP (2.5 mM each), 10 µl of 10xTaq DNA polymerase assay buffer, 1µltaq DNA polymerase (3U/ µl), 400 ng forward and reverse primers. PCR conditions for the amplification 16S rRNA gene were: 5 min initial denaturation at 96°C, followed by 35 cycles of denaturation at 94° C for 30 sec, annealing at (50°C) for 30 sec and extension at 60°C for 1.30 min followed by extension for 7 min at 72°C. PCR product was separated by electrophoresis on 1.5% agarose gel stained with ethidium bromide and photographed under UV illumination using gel doc (Fig. 1). The partial sequence of 16S rRNA gene of the thermotolerant rhizobial isolate was compared with the sequences already submitted by National Centre for Biotechnology Information (NCBI) database using the BLASTN program. Sequence analysis was performed by construction of Phylogenetic tree using mega software 7 through neighbor joining method (Saitou and Nei 1987).

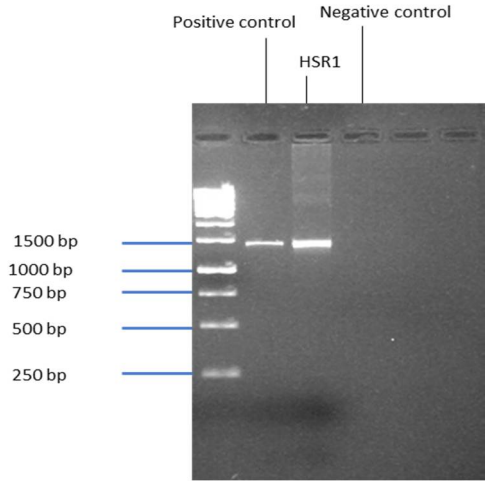

Fig. 1. PCR amplification of 16S rDNA fragment from bacterial sample loaded on 1.5 %Agarose gel.

Results and Discussion

A total of 20 rhizobial isolates (HSR1, HSR3, HSR5, HSR 7, HSR9, HSR11, HSR13, HSR 15, HSR17, HSR 19, HSR 21, HSR 23, HSR 25, HSR 27, HSR 29, HSR 31, HSR 33, HSR 35, and HSR 37and HSR 39) were retrieved from root nodules of summer mungbean growing in CCSHAU farm (Monika and Wati 2016).

The temperature range best suited for growth of most rhizobial was 25-28°C. At 30°C all the rhizobial isolates were growing well but with increase in temperature to 35 and 40°C and further to 45°C their growth retarded. The isolate HSR1 showed good growth at 40°C and moderate growth at 45°C (Fig.2). It was also able to nodulate mungbean variety MH 421 in summer season under leonard jar conditions as shown in (Fig. 3). Results of present study were in corroboration with other researchers' findings. It has been reported that higher temperature limits for different rhizobia differ within species and strains. Bansal *et al.* (2014) reported that 84% mungbean rhizobial isolates grew at 40^0C but only 26% could grow at 45^0C. Manasa *et al*. (2017) screened 15 rhizobial isolates for their ability to grow at high temperature and found two strains able to grew up to 45^0C.

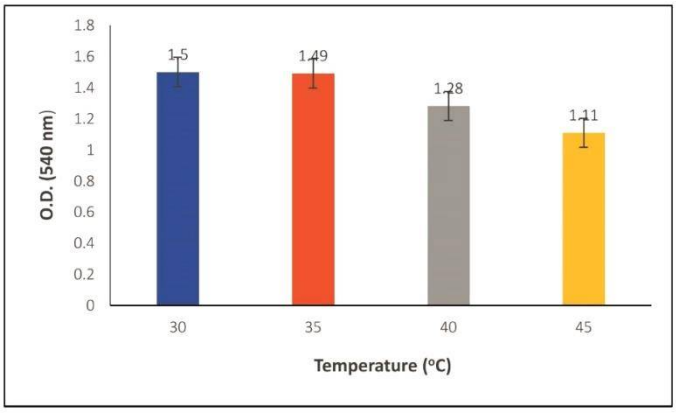

Fig. 2. Growth of rhizobial isolate HSR1 at different temperatures.

Fig. 3. Nodulation by rhizobial isolate HSR1 under sterile conditions.

In present study, the rhizobial isolate HSR1 was able to produce IAA in tryptophan supplemented broth incubated at different temperatures that however decreased with increase in temperature above 40^{0}C (Fig. 4). IAA production by rhizobia isolated from legumes has also been reported by Ghosh *et al.* (2015). Decrease in indole acid production with increasing temperature corroborates the results of Modi and Khanna (2018) where they reported increase in IAA production by rhizobacterial isolates from 30 to 40°C but sharp decrease at 50°C.

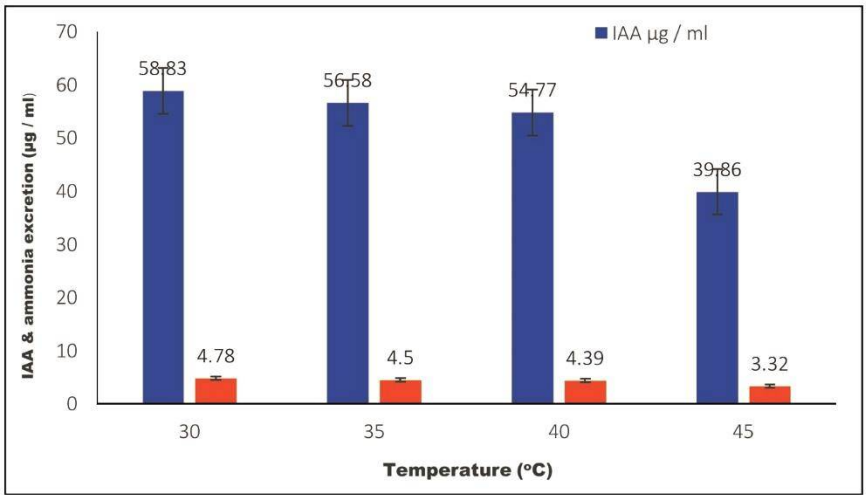

Fig. 4. Effect of different range of temperatures on IAA production and ammonia excretion by rhizobial isolate HSR1.

In present investigation, the rhizobial isolate HSR1 grown in peptone broth at different temperatures was found to excrete ammonia as indicated by developing a red color with Nessler's reagent. The maximum ammonia excretion by the isolate HSR1 was observed at 30^{0}C *i.e.*, 4.78 µg/ml that decreased with increase in temperature above 40°C (Fig. 4). Similarly, in another study by Nagoma *et al.* (2012) out of 23 rhizobial isolates obtained from chick pea plants 11 isolates were able to produce ammonia while 12 isolates were unable to do so. Rhizobial isolate HSR1 showed a clear zone on Pikovskaya agar indicating phosphate solubilizing activity and PSI value 3.3 at 30^{0}C that however decreased with increase in temperature (Table 1). Similarly, Manasa *et al*. (2017) reported phosphate solubilizing ability in 45% rhizobial isolates having PSI ranged between 1.0-2.5 while Routray and Khanna (2018) reported PSI of rhizobactrial isolates ranged between 1.8-3.1 at 35°C.

The rhizobial isolate HSR1 was tested for siderophore production ability on CAS assay medium plates. It showed good growth on iron deficient medium, at 30, 35 and 40°C which was moderate at 45°C (Table 1). Similarly, Modi and Khanna (2018) reported significant siderophore production by rhizobacterial isolates up to 40^{0}C. Rhizobial isolate HSR1 was able to synthesize HCN at all temperatures (Table 1) with change in color of filter paper from yellow to reddish brown which agrees with the findings of Manasa *et al* (2017). HSR1 grew on ACC supplemented medium showing its ability to produce ACC-deaminase. The growth of HSR1 was high at 30 and 35°C and moderate at 40 and 45°C (Table 1). Othman and Tamimi (2016) reported that 15% of rhizobial isolates isolated from *Vicia faba* were able to utilize ACC which was supplemented in minimal medium plates.

Table 1. Plant growth promoting traits of rhizobial isolate HSR1 at different temperatures.

Character	Temperature (ºC)			
	30	35	40	45
Phosphate solubilization (P- SI)	3.3	3.0	2.8	2.3
Siderophore production	+ + +	+ + +	+++	++
HCN production	+ + +	+ + +	+++	++
ACC-utilization	+ + +	+ + +	+++	++

++ and +++ represent moderate and high growth.

On the basis of promising PGP traits at high temperature isolate HSR1 was used for biochemical and molecular characterization. The rhizobial isolate HSR1 formed white gummy colonies on Congo red containing YEMA medium and stained Gram negative (Table 2). Similar colony characters were reported for rhizobial isolates from *Cajanus cajan* by Dubey *et al.* (2010). The isolate HSR1 showed negative results for growth on Glucose peptone agar medium and Ketolactose production. Some other researchers also reported the similar conditions for *Rhizobium* species (Deka and Azad 2006).

Table 2. Morphological and Biochemical characterization of rhizobial isolate HSR1.

Test	Result
Gram Staining	Gram –ve
Shape	Rod
Colony color	Watery translucent
Indole production	-
Voges Proskauer	-
Citrate utilization	-
H_2S production	-
Urease production	-
Gelatinase	-
Glucose	+
Mannitol	+
Arabinose	+
Glucose peptone agar medium	-
Ketolactose test	-

In present study, the sequenced PCR product of genomic DNA of isolate HSR1showed 99% similarity with *Sinorhizobium* sp. T 25 after matching with available gene sequence in NCBI gene database. The phylogenetic analysis performed by constructing a phylogenetic tree revealed that the isolate HSR1 fell within *Sinorhizobium* sp. (Fig. 5). The partial 16S rDNA sequence of strain HSR1 has been deposited in the GeneBank database under accession number KY575143. Identification of *Sinorhizobium* sp. from legumes using 16s rRNA sequencing has been supported by many researchers. Kumar and Ram (2016) isolated five *Sinorhizobium* strains from *Vigna*

tribola root nodules, on 16S rDNA analysis one strain among five found belonging to *Sinorhizobium* sp. MRR 101. Similarly, Favero *et al*. (2021) also isolated 101 rhizobial isolates from mungbean and three of which belonged to *Sinorhizobium* sp.

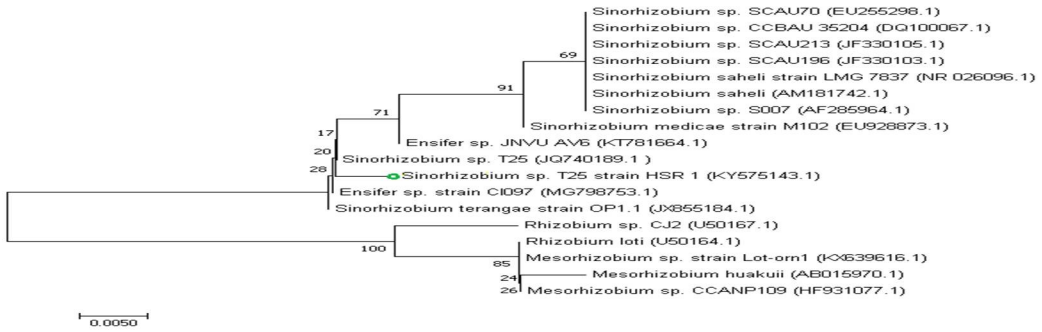

Fig. 5. Phylogenetic tree of rhizobial isolate HSR1 based on 16S rRNA gene sequences

The possession of plant growth promoting activities favors the use of microorganisms as biofertilizers for legume crops. The thermotolerant rhizobial isolate HSRI showed all the potential plant growth promoting traits that will be of great advantage for its use as biofertilizer in sustainable agriculture resulting in plant growth promotion, disease suppression and further increase in crop yield.

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