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Screening of bacterial endophytes isolated from root nodules of lentil for their antagonistic & plant growth promoting activities through some biochemical tests

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Abstract
Lentils are believed to have originated and been consumed since pre-historic times. They are one of the first crops to have been cultivated. Lentil seeds dating back 8,000 years have been found at archeological sites in the Middle East. In this study bacteria from root nodules of lentil (*Lens culinaris* L.) grown in different agroclimatic zones were isolated and their beneficial properties for plants were characterized. Five isolates were obtained from nodules and screened for some biochemical properties such as citrate utilization, amylase production, methyl red test, HCN production for antagonistic activity & phytohormone production such as IAA. Out of five isolates all the isolates showed ability to produce HCN, citrate & phytohormone IAA. Four out of five isolates exhibited positive test for starch hydrolysis & methyl red test.

Keywords: Screening, bacterial, endophytes, isolated, antagonistic, biochemical

Introduction
Soil, a dynamic, living matrix is an important resource for agricultural products. Soil is also a storehouse of microbial activity, which is confined to aggregates with accumulated organic matter, the rhizosphere. The rhizosphere both contacts plant roots and supports high populations of active microorganisms (Nautiyal and DasGupta, 2007) [26]. In the rhizospheric plant soil, diversity and community structure of microorganisms are plant species dependent and differ among varieties or cultivars. This may be affected by both specific plant root exudates and soil type (Kremer *et al*., 1990) [15]. Food legumes account for an important share of food and feed, which are rich in proteins and provide a significant amount of fixed nitrogen useful for cereals, reducing production costs and limiting groundwater pollution by nitrates in fertilizers (Labdi, M. 1991 & Ryan *et al*., 2007) [18, 25]. Lentil (*Lens culinaris* Medik.) is one of the important rabi pulse crop of India, which is cultivated in the different states. It suffers from several microbial diseases including wilt diseases caused by *Fusarium oxysporum.* Exploitation of antagonistic endophytic bacteria for plant growth promotion and biocontrol of phytopathogens has earlier been attempted (Kumar *et al.,* 2011, 2012; Nejad and Johnson, 2000) [16-17]. Rhizospheric organisms can play a role in governing plant growth and development (Napoli *et al*., 2008) [21]. Rhizobacteria that exert beneficial effects on plant development are termed "Plant Growth-Promoting Rhizobacteria" (Kloepper and Schroth, 1978) [14]. PGPR was found to be mainly involved in enhancing plant nutrition, stress tolerance or health (Vacheron *et al*., 2013) [32]. This is mainly due to their effect associated with enhanced availability of nutrients (Lugtenberg and Kamilova, 2009; Drogue *et al*., 2012) [10], phytohormones-mediated stimulation of root system (Somers *et al*., 2004) [19, 29] and induced systemic resistance (Zamioudis and Pieterse, 2012) [23]. Plant growth-promoting rhizobacteria (PGPR) are soil bacteria, which were colonized plant roots and improved their growth by several mechanisms such as phytohormones, siderophore production and solubilization of phosphate, inhibition of pathogenic microorganisms and detoxification of the environment (Gupta *et al*., 2021 & Beauchamp, CJ 1993) [12, 5]. Due to the diversity of Plant growth-promoting rhizobacteria (PGPR), capacity for colonization, mechanisms of action, formulation, their application for the management of agricultural systems should facilitate their development as reliable components.

Materials and Method

Sample collection

Root nodules were taken from freshly uprooted lentil plants from different agroclimatic zones of India such as chhattisgarh & uttarpradesh. Samples were brought to the laboratory and root system was washed with running tap water to remove the adhering soil particles & to get intact nodules for isolation of nodule inhabiting bacteria.

Surface sterilization

After washing with tap water nodules were subjected for surface sterilization in order to remove unwanted and pathogenic epiphytes. Healthy pink nodules were selected for the isolation of nodule associated bacteria (NAB). Nodules were safely separated from the root & surface sterilized with 0.1% $HgCl_2$ for 1 minute and subsequently treated with 70% ethyl alcohol for 30 seconds. Nodules were successively washed 3-4 times with sterile distilled water in order to remove chemical residues from the surface of nodules. All these steps were performed under aseptic condition inside laminar air flow.

Isolation & storage of bacteria

The nodules were kept in 1.5 ml microfuge tubes containing 0.5 ml N-saline. Further nodules were crushed with the help of sterile forceps and a liquid suspension thus obtained was streaked on YEMA (Yeast extract mannitol agar) plates with the help of a sterile inoculation loop. All the plates were incubated at 28 ± 2°C. Single colonies were picked after 48 hours and stored in a YEMA slant for further investigation. Cultures were maintained in the P.G.P.R. Laboratory of Prof. Ramesh Kumar Singh, Department of Genetics and Plant Breeding, Institute of Agricultural Sciences, Banaras Hindu University Varanasi- India.

Biochemical Characterization of bacterial isolates

Biochemical characterization of bacterial isolates was done on the basis of citrate utilization, starch hydrolysis, HCN production, Methyl red test & IAA Production as per the standard methods.

Citrate utilization test

The bacterial isolates were spot inoculated on Simmons agar medium containing per liter of distilled water, magnesium sulphate (0.200g), ammonium dihydrogen phosphate (1g), dipotassium phosphate (1g), sodium citrate (2g), sodium chloride (5g) bromothymol blue (0.08g). Test tubes were incubated at 28°C for 24-48 hours. At the end of incubation period, the change in colour from green to blue indicates citrate utilizing bacteria whereas citrate negative bacteria does not show any growth and the medium remains green (Simmons, 1926) [27].

Starch hydrolysis

The bacterial isolates were spot inoculated on starch agar medium containing per liter of distilled water, beef extract 3 g, peptone 5 g, soluble starch 20 g and agar 15 g. Plates were incubated at 28°C for 72 hours. At the end of incubation period, the plates were flooded with iodine solution, kept for a minute and then poured off. After few minutes blue colour faded rapidly and a hollow zone around the bacterial colonies appeared. The colour less zone surrounding the colonies indicated the production of amylase (Collins *et al*., 1995) [6].

Methyl red (MR) test

The MR test is used to determine conversion of glucose to acidic products like lactate, acetate and formate. Glucose peptone broth (peptone 5.0g; potassium dehydrogenate phosphate 5.0g; glucose 10.0g; distilled water 1 lit, pH 7.4) was inoculated with the isolates and incubated at 28°C for 72 hours to confirm. In methyl red test, development of colour was checked after adding 5 drops of methyl red indicator (methyl red 5.0g; dissolved in 30ml of ethanol (95%) and diluted to 50 ml with distilled water), the development of a stable red colour shows positive test. (Das Gupta *et al*., 2015) [7].

IAA production

Bacterial isolates were grown in yeast extract mannitol broth supplemented with 50 µg/ml tryptophan and incubated at 28±2 ^{0}C for 3 days under shaking condition. The broth was then centrifuged at 10,000 rpm for 20 min at 4 ^{0}C to collect the supernatant. The supernatant (2 ml) was mixed with two drops of orthophosphoric acid and 4 ml of the Salkowski's reagent (50 ml, 35% of perchloric acid, 1 ml 0.5 M $FeCl_3$ solution). Development of dark brown to pinkish red colour indicates IAA production (Gordon and Paleg, 1957) [11]. Absorbance was taken at 530 nm.

Standard curve of IAA

Standard curve was made by using IAA solution in 0-100 µg/ml concentration. After making volume to 1ml using distilled water followed by adding Salkowski's reagent (2 ml), total volume was made to 4 ml and incubated for 25 minutes at room temperature. Standard curve was plotted with the different readings obtained by taking absorbance at 530 nm.

The production of IAA was calculated by equation (y = mx + c) in µg /ml.

Where

Y = O.D. of *Rhizobium* and PGPR culture.

m = O.D. of blank solution

x = amount of IAA produced by *Rhizobium* and PGPR strains

c = Zero (constant)

HCN production

HCN production by bacterial isolates was determined by colour change of filter paper following Alstrom and Burns (1989) [1]. One hundred microliter of bacterial suspension was inoculated on nutrient agar medium containing 4.4 gL^{-1} glycine. Filter paper soaked in a reagent solution (2.5 g picric acid and 0.125 g sodium carbonate, 1000ml distilled water) was placed in the upper lid of petridishes. To prevent volatilization, the plates were sealed with parafilm and incubated at 28°C for four days. The plates without bacterium served as control. A change in colour of the filter paper from yellow to light brown, brown or reddish brown was recorded as weak (+), moderate (++) or strong (+++) reactions, respectively.

Results and discussion

Citrate utilization test

Citrate utilization as a carbon source was examined by replacing mannitol from YEM agar with equal amount of sodium citrate and inorganic ammonium salts ($NH_4H_2PO_4$) as the sole source of nitrogen with the indicator Bromothymol blue (25 mg/l). All of the five isolates were detected positive

for citrate utilization (Table1 & figure1). Datta *et al*., 2015 also found that citrate utilization as a carbon source was positive in *Rhizobium phaseoli* and *Rhizobium trifolii*.

Starch hydrolysis

The starch is hydrolyzed by the amylase enzyme which is produced by different types of micro-organism. Selected five bacterial isolates were tested for starch hydrolysis. All the isolates were tested positive for starch utilization except for 20NII. Where isolate LUP2 showed strong (+++) starch hydrolysis followed by UP4N, LN2 & 19N which showed moderate (++) starch hydrolysis activity (Table1). Datta *et al*., (2015) have reported positive result for starch hydrolysis by *Rhizobium* strains. Positive results were also obtained by Singh *et al*., (2008) from the starch hydrolysis assay.

Methyl red test

The methyl red test is used to test the ability of an organism to produce and maintain acid end products from glucose fermentation. Methyl red is a pH indicator, which detects the acid-producing bacteria. All the five bacterial isolates were examined for their ability to produce acid from glucose. Among five isolates four isolates showed positive result for methyl red test, while one isolate did not produce acid from glucose. Isolates UP4N, LN2, 19N & 20NII were found to be weak (+) acid producer (Table1 & figure 1). Panwar *et al*., (2012) [24] reported positive methyl red test for *Rhizobium* isolates obtained from fenugreek. Nushair *et al*., (2017) [23] also characterized indigenous *Rhizobium* strain isolated from lentil in which negative result was shown by *Rhizobium species* which is in close agreement with present result.

HCN Production

Hydrogen cyanide (HCN) is a volatile, secondary metabolite which inhibits several metallozymes, in particular, copper containing cytochrome C oxidases. Hence, it suppresses the growth and development of many phytopathogenic microorganisms (Martínez-Viveros *et al*. 2010) [20]. In the present study five isolates were tested for their ability to produce HCN. All the isolates were able to produce HCN in *in vitro* condition however there was considerable differences among the isolates for HCN production. Isolate 20NII showed strong (+++) HCN production ability followed by LUP2, UP4N, LN2 & 19N (Table1 & figure 1). Although there are several reports, rhizobia are relatively less efficient in HCN production (Deshwal *et al*. 2003) [9]. Beauchamp *et al*. (1991) [4] and Antoun *et al*. (1998) [2] have found 12.5 and 3% of the total strains screened respectively to be HCN producers. Arfaoui *et al*. (2006) [3] reported six rhizobial strains which were positive for HCN production and showed antagonistic activity towards *F. oxysporum f. sp. ciceri* under *in vitro* and *in vivo* conditions.

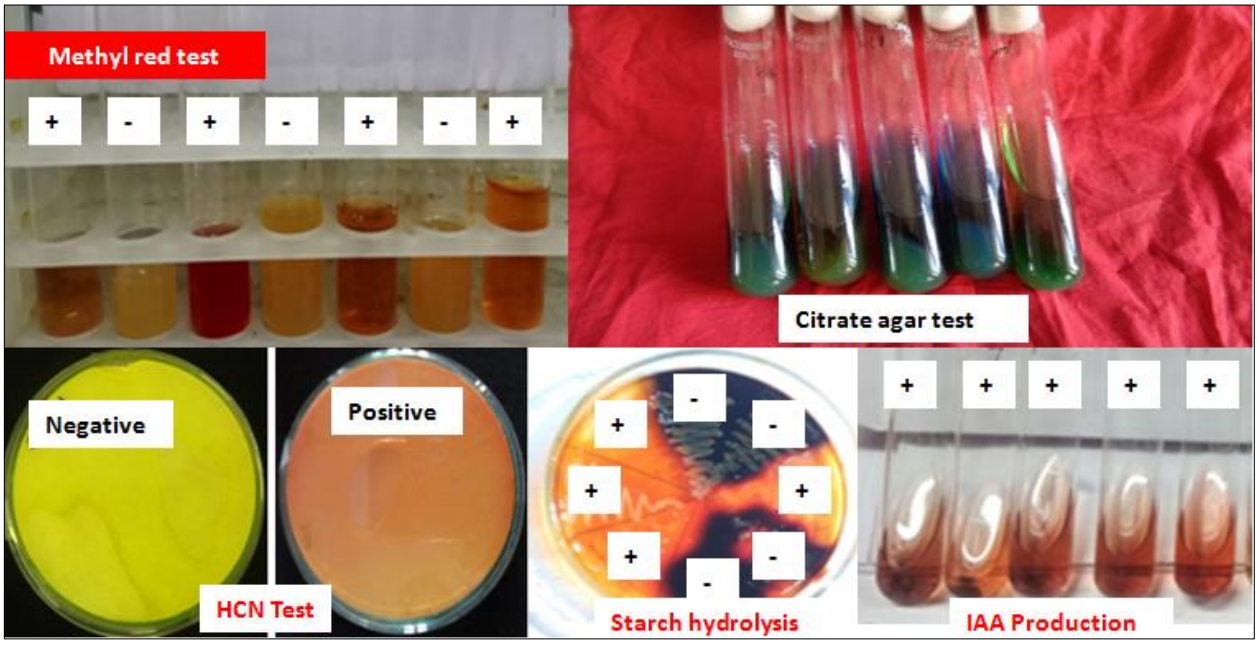

Fig 1: Bacterial isolates showing different biochemical and PGPR activities

Table 1: Bacterial isolates showing different biochemical and PGPR activities

STRAINS	HCN	Citrate utilization	Starch hydrolysis	Methyl red test	IAA production
LUP2	+	+	+++	-	+ (2.45 µg/ml)
UP4N	+	+	++	+	+++ (36.84 µg/ml)
LN2	+	+	++	+	+ (3.09 µg/ml)
19N	+	+	++	+	++ (27.68 µg/ml)
20NII	+++	+	-	+	+++ (30.14 µg/ml)

Production of Indole-3-Acetic-Acid

The physiologically most active auxin in plants is Indole-acetic acid (IAA) which is known to stimulate rapid cell elongation and cell divison and differentiation in plant (Hayat *et al*. 2010) [13]. Inoculation with IAA producing PGPR has been used to stimulate seed germination, accelerate root growth and modify the architecture of the root system, and increase the root biomass (Martinez-viveros *et al*. 2010) [20]. Ability to produce IAA in the presence of tryptophan is considered as IAA producing rhizobacteria. Considerable

variation in the ability to produce IAA by different rhizobacterial isolates was observed. IAA production ranged from 2.45-36.84 µg/ml in presence of tryptophan (Table 1 & figure 1). This result is in accordance to Spaepan and Vanderleden, 2011, who found that higher IAA production was observed in the presence of precursor L-tryptophan and there was significant difference in the potential production of IAA amongst the isolates. Highest IAA production was shown by isolate UP4N *i.e.* 28.3 µg/ml in the presence of L-tryptophan followed by 20NII (30.14 µg/ml), 19N (27.68 µg/ml), LN2 (3.09µg/ml) & LUP2 (2.45µg/ml) (Table 1). A number of *Rhizobium* isolates have been reported to produce high concentration of IAA when grown in medium containing tryptophan in culture (Tsavkelova *et al.*, 2005) [31].

Conclusion

PGPR influences plant growth and nutrition in very specific ways involving bacterial components inducing plant responses. PGPRs can affect both plant growth and nutrition. In the present study, five bacterial isolates from lentil plant from different agroclimatic zones were obtained and their biochemical & plant growth promoting traits were characterized. Based on biochemical characterization isolates LUP2, UP4N, LN2, 19N & 20NII were found to be positive for HCN production & citrate utilization. Isolates LUP2, UP4N, LN2 & 19N showed positive result for starch hydrolysis whereas isolate 20NII was unable to hydrolyze starch. Isolates UP4N, LN2, 19N & 20NII indicated positive result for methyl red test while LUP2 showed negative result. Phytohormone production by bacterial isolates ranged from 2.45 - 36.84 µg/ml tryptophan concentration. Highest IAA production was recorded for the isolate UP4N followed by 20NII, 19N, LN2 & LUP2. The mechanisms by which the microbes act could be key in future applied PGPR development. They can possibly be suggested as microbial inoculants for lentil plant growth promotion as PGPRs. Among bacterial isolates, 20NII showed strong (+++) HCN production ability which could be used as a biological control agent to protect lentil from fungal pathogens.

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