RESEARCH ARTICLE

ISOLATION AND CHARACTERIZATION OF ANTAGONISTIC RHIZOSPHERIC BACTERIA FROM LENTIL

Niyaj Ahamad*, Adesh Kumar, Km. Komal, and D.K. Dwivedi

Department of Plant Molecular Biology and Genetic Engineering
Acharya Narendra Deva University of Agriculture and Technology,
Kumarganj, Ayodhya 224229, U.P., India

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Abstract: Sixteen lentil rhizobacteria were isolated at nutrient agar medium. Out of which, twelve isolates showed antagonistic activity against *Fusarium oxysporum* f.sp. *lentis*. All the antagonistic isolates produced diffusible and volatile antifungal metabolites. The isolates 10 and 14 showed a maximum antagonism 72% and 68.6% respectively. All the antagonistic isolates showed PGPR activity such as phosphate solubilization, IAA production and ammonia production. Isolates produced IAA ranging from 1.25 to 3.61 ug/ml. The highest amount of IAA was produced by isolate 14.

Keywords: Antagonistic activity, PGPR, *Fusarium oxysporum* f. sp. *lentis*, PGP traits

INTRODUCTION

Lentil (*Lens culinaris* Medik.) is the second most important grain legume widely cultivated in semi-arid regions of the world (Malik 2005). Cultivation of lentil attributes the soil by enriching the soil nutrients by the help of nitrogen fixation, carbon and organic matter accumulation which promotes proceeding crop production and productivity. The crop production reduces due to suffering from various plant diseases caused by fungi, viruses, nematodes, insect pests and parasitic weeds ultimately resulted in huge economic losses. Lentil *Fusarium* wilt caused by *Fusarium oxysporum* f.sp. *lentis* plays a major role in limiting the yield potential and causes severe damage of leaves, pods, stems and roots (Pouralibaba *et al*. 2015; Singh, 2015). Lentil legume has ability to fix atmospheric nitrogen into a biologically useful form by the symbiotic association of rhizobacteria. Several environmental conditions limits the symbiotic relations resulting reduced nitrogen fixing system, application of plant protective chemicals, fertilizers are harmful for plant and bacteria both (Andres, 2012). The rhizosphere is the narrow zone of soil influenced by the root system of plant and populated with variety of microorganisms and the bacteria colonizing this zone is known as rhizobacteria (Beneduzi*et al*. 2012).The plant growth promoting rhizobacteria have the capability to stimulate the plant growth by the root colonization and exert beneficial effects on the plant (Sulsow*et al*. 1979) by increasing plant-microbe symbiosis, competition for nutrients, root colonization space and limit the activities of plant pathogens (Glick 1995; Lugtenberg 2002). PGPRs have both the mechanisms of direct and indirect. Direct mechanism of PGPR consists of phosphate solubilization, nitrogen fixation, Ammonia production, Siderophore production and procurement of plant hormones like auxins, cytokinins and gibberellins and limiting the ethylene by ACC deaminase activity. In order to indirect mechanism of PGPRs consists of pathogen suppression by means of production of enzymes and compounds like chitinase, protease, cellulose, antibiotics, HCN, ammonia and volatile organic compound *etc*.Plant growth promoting rhizobacteria can biologically fix nitrogen, solubilize phosphate and maintains the proper level of phytohormones such as Idole acetic acid that help in the improvement of lentil (Rani *et al*. 2012).Rhizospheric bacteria can promote root growth and yield and play an alternative role to increase abiotic stress tolerance in legumes (Rashid *et al*., 2012, Sarma and Saikia 2014). Plant growth promoting rhizobacteria can increase growth by variety of mechanisms, such as nitrogen fixation, phosphate solubilization, and phytohormone production (Rashid *et al*., 2012). Some bacteria synthesizes indole acetic acid (IAA) hormone which regulates the growth and development of the plant, act as signal molecule that control the expression of several genes (Duca*et al*., 2014). Plant growth promoting bacteria colonize the roots of plant rhizosphere that enhances the plant growth. Rhizospheric microbial population is different from its surroundings due to root exudates that function as a source of nutrients for microbial growth (Burdman*et al*., 2000). PGPR enhance the plant growth by the control of phytopahtogenic agents, primarily by the production of metabolites that contribute to the antibiosis and antifungal properties that acts as defence systems. The mechanism would involve the production of hydrolytic enzymes, such as chitinase and glucanase producing bacteria could inhibit fungal growth (Kumar *et al*., 2010).

Thus, the identification and characterization of PGPR populations indigenous to leguminous rhizosphere are therefore, to characterize these isolates on the basis of morphology and determination of PGPR properties along with antifungal activity.

*Corresponding Author

MATERIALS AND METHODS

Isolation and purification of *Fusarium oxysporum* f. sp. *lentis*

For the isolation of phytopathogen of wilt disease, diseased along with healthy tissues were cut with the help of a sterilized blade. These pieces were washed thoroughly with tap water and placed into 0.1% sodium hypochloride solution for 30 seconds followed by washing thoroughly. Excess water was removed by placing on the folds of sterilized blotting paper. Dried pieces were aseptically transferred into sterilized petri-plates containing potato dextrose agar medium with the help of a sterilized forceps. Petri plates were incubated at 28 ℃in a B.O.D. incubator for 5-6days. The growth of isolated wilt fungus was transferred to the slants.

Isolation and purification of bacteria from lentil rhizosphere

In this study, soil sample were collected from the rhizosphere of different lentil fields. The serial dilution technique was used for the isolation of bacteria. Nutrient agar medium was used to isolate valuable bacteria from the lentil rhizosphere. The colonies with different morphotypes were picked up and streaked on fresh plates. These isolates were repeatedly streaked on respective media until pure cultures were obtained. The slant cultures were prepared on respective media and stored at 4 ℃. Sub culturing was done at the interval of 8 weeks.

Antifungal test of rhizobacteria against *Fusarium oxysporum*

Bacterial isolates were streaked at one side of Petri dish containing PDA. Loopful mycelia from seven days old PDA culture of *Fusarium oxysporum* f. sp. *lentis* were placed at the opposite side of petri dishes perpendicular to the bacterial streak respectively and incubated at 28 ℃for 5-7 days. Observations on width of inhibition zone and mycelial growth of test pathogen were recorded and per cent inhibition of pathogen growth was calculated.

Screening of isolates for PGPR properties

Phosphate solubilization

A loop full of fresh bacterial cultures was streaked on the center of Pikovaskaya agar plate containing insoluble tricalcium phosphate (TCP) and incubated for seven days at 28 ℃ described by King (1932). The presence of halo zone around the bacterial colonies indicated positive phosphate solubilisation ability. The degree of solubilisation by each selected isolates was determined by measuring the zone of solubilisation around the colonies.

IAA Production

Indole acetic acid (IAA) production was qualitatively estimated by Salkowski method Brick *et al*., (1991). Bacterial cultures were grown in the flask showing dense milky white growth tested for purity. Fifty ml of Luria Bertani (LB) broth containing 0.1% DL tryptophan were inoculated with 500 µl of 24 h old bacterial cultures and incubated in refrigerated incubator shaker at 30 ℃ at 180 rpm for 48 h in dark. Fully grown bacterial cultures were centrifuged at 10,000 rpm for 10 min at 4 ℃. Estimation of IAA production in the supernatants was done using colorimetric assay. One ml of 10 mM orthophosphoric acid and 2 ml of the Salkowski reagent (1 ml of 0.5 $FeCl_3$ in 50 ml of 35% $HClO_4$) at 28 for 30 minute development of pink colour in test tubes at the end of the incubation indicated IAA production. Quantification of IAA was measured by the pink colour absorbance at 530 nm after 30 min in UV spectrophotometer.

Ammonia production

Bacterial isolates were tested for ammonia production in peptone water (Peptone 10g, Nacl 5 g in litre, pH-7.0). Freshly grown cultures were inoculated in 10 ml peptone water in each tube and incubated for 48-72 h at 28 ℃. Sebsequently, Nessler's reagent (0.5 ml) was added to the bacterial suspension. Development of brown to yellow colour was a positive test for ammonia production.

RESULTS AND DISCUSSION

The plant rhizosphere constitutes a versatile and dynamic biological habitat for note worthy bacteria. In the present study, it was aimed to find out such bacteria having PGPR activities along with antagonistic property against the pathogen causing wilt disease of lentil *Fusarium oxysporum*f.sp. *lentis.* The hyphal growth inhibition ability of bacterial isolates obtained from the lentil rhizosphere, against *Fusarium oxysporum* was assayed in a dual culture experiment on PDA. Out of sixteen isolates, twelve isolates showed inhibitory effect on the pathogenic fungus and recorded with medium to bigger clearing zone on the test medium. Rest of the isolates showed very low to negative inhibitory effect against the pathogenic fungus (Table 1). Two efficient bacterial isolates namely isolate 10 and 14 have most positive antagonistic effects against *Fusarium oxysporum* f.sp. *lentis* revealed by 72% and 68.5% respectively.

In the last decades, several studies have been conducted to isolates biocontrol agents with PGP activities. In most cases, isolated bacteria with biocontrol properties belong to the genera *Bacillus* and *Pseudomonas* (Edwards *et al*., 1994). Landa*et al*., 1997 isolated several bacteria and shoed that approximately 32 % of 74 bacterial isolates from the chickpea rhizosphere inhibited in vitro growth of *Fusarium oxysporum* f. sp. *ciceris* in dual culture. Recently, Shankar *et al*., (2019) evaluated the biocontrol property of three rhizobacterial strains against *Fusarium* wilt of chickpea under glass house and field condition and found that *Pseudomonas chlororaphis* reduced the disease by 80.7 % (glasshouse) and 70.18 % in the field condition. Seventeen *Fusarium oxysporum* inhibiting rhizobacteria isolates were.

When all the bacteria were subjected to the Phosphate solubilizing ability test, all the isolates found positive. Six out of sixteen isolates demonstrated maximum comparable level of solubilization, whilefour were medium phosphate solubilizer and remaining six were fewer solubilizer. (Table 2). Mulissa *et al.,* (2016) isolated several Phosphate solubilizing bacterial strains like *Bacillus flexus, Pseudomonas flurescence, Enterobacter* sp., *Enterobacter sakazaki,* and *Enterobacter* sp. and studied their effect on the growth of chickpea in pot culture. There were an increased number of root nodules, shoot dry matter, nitrogen and phosphorus concentration of shoot was demonstrated. Recently, isolation of phosphate solubilizing bacteria from wheat or other crops having a profound effect on phosphorus solubility has also been reported (Majeed*et al*., 2015 and Di Benedetto*et al.,* 2019).

The results related to quantitative IAA production by isolates revealed that all the sixteen isolates have the ability to synthesize IAA (Table 3) obtained in the range of 10-100 µg/ml. The pink colour development indicated IAA production. Evidence suggests that bacterial IAA, in conjunction with plant IAA, can govern specificstages of plant growth (Gamalero and Glick, 2015). Furthermore, this feature is relevant to the stress phase, as IAA alters ACC deaminase production, which can modify ethylene content and hence the stress response. According to Etesami*et al.,* (2015) in addition to enhanceing plant development and playing important role in stress tolerance, IAA can promote biomass production, enhance root elongation, and augment root exudates. These effects because a decline in soil pH, helps in the release of chelators, vary redox potential leading to the solubility of few nutrients and increased availability of iron and phosphorus.

Selected and tested for their ammonia-producing potential. Out of sixteen, six were high ammonia producer, eight were medium and remaining two was very poor ammonia producer (Table2). One of the fundamental properties associated to plant growth promotion in PGPR's ability to produce ammonia, which indirectly influences plant growth Yadav *et al.,* (2010) isolated various bacterial species from the chickpea rhizosphere belonging to different genera and reported that most of the bacteria belonging to genus *Bacillus* and *Pseudomonas* were positive for ammonia production. Ammonia-producing PGPRs have also been isolated from the rhizosphere of other plants for example- recently. Bhattacharyya *et al.,* (2020) isolated ammonia-producing bacteria from the tea rhizosphere.

Table 1. Qualitative screening of rhizobacterial isolates against *Fusarium oxysporum* f. sp. *lentis*

Isolate	Inhibition zone (cm)	Inhibition %
1.	0.96	32.00
2.	0.81	27.00
3.	0.36	12.00
4.	0	0.00
5	0.67	22.00
6.	0	0.00
7.	0	0.00
8.	0	0.00
9.	0.6	20.00
10.	2.16	72.00
11.	1.01	33.00
12.	1.46	48.60
13.	1.41	47.00
14.	2.06	68.60
15.	1.65	35.00
16.	1.71	57.00

Table 2. Qualitative screening of rhizospheric bacteria for phosphate solubilization and Ammonia Production

Sr. No.	Phosphate Solubilization	Ammonia Production
1.	+	++
2.	+	++
3.	+	+++
4.	+	++
5.	+++	++
6.	+++	++
7.	+++	++
8.	++	+++
9.	++	++

10.	+++	+++
11.	+	+++
12.	++	+++
13.	+	+++
14.	+++	+++
15.	+++	+++
16.	++	+++

Table 3. Qualitative screening of rhizobacterial isolates for Indole acetic acid production

Isolate Number	IAA Production (µg/ml)
1.	1.25
2.	1.42
3.	2.71
4.	1.37
5.	1.32
6.	1.48
7.	1.31
8.	2.62
9.	1.58
10.	3.15
11.	2.72
12.	2.95
13.	2.75
14.	3.61
15.	3.41
16.	3.17
Control	0.00
C.D.	0.001

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