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In vitro antagonism of soil and chickpea nodule isolates against *Fusarium oxysporum* f. sp. *ciceris* in chickpea

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***Fusarium oxysporum* f. sp *ciceris* (Foc) causes *Fusarium* wilt disease of chickpea (*Cicer arietinum* L.) resulting in severe losses in the irrigated belt of Punjab. The most effective and practical method is the use of fungicides or resistant cultivars to control *Fusarium* wilt in chickpea. Rhizobia application could be used as an alternative way to control *Fusarium* wilt of chickpea. *Rhizobium* spp. are effective biological control agents as they promote plant growth directly by effecting symbiotic N_2 fixation, nodulation or nodule occupancy in chickpea. Twenty soil and chickpea nodule isolates along with reference *Mesorhizobium* spp. (LGR 33) were tested as biocontrol agent *in vitro* against the causative agent of *Fusarium* wilt of chickpea via production of cell wall degrading enzymes and volatiles. Out of 20 soil and chickpea nodule isolates including LGR 33, 43 and 33% were able to produce siderophores and HCN respectively, whereas 62 and 57% produced cellulase and protease enzymes respectively. Soil and chickpea nodule isolates revealed maximum inhibition of 45% followed by 35 and 30% for control of *F. oxysporum* strains 1, 3 and 2, respectively. Three native isolates of soil and chickpea nodule (LGR 46, LGR 50 and LGR 52) including LGR 33 were able to produce volatiles, cell wall degradation enzymes and antagonistic effect. Further extensive research is required to understand the mechanism of potential isolates of soil bacteria and chickpea in controlling *Fusarium* wilt disease of chickpea. Selection of rhizobia with twin functional traits (N_2 fixation and biocontrol agent) can be exploited as future biofertilizer in chickpea.**

Key words: Biocontrol, Chickpea, *Fusarium oxysporum.*

INTRODUCTION

Legumes are important source of dietary proteins with a unique property of maintaining and restoring soil fertility through biological nitrogen fixation (BNF) as well as conserving and improving physical properties of soil. Of the different legumes grown around the world, chickpea (*Cicer arietinum* L.) is one of the most widely grown legumes. It is a major legume crop contributing 38% of national pulse production in India. India stands first in terms of area of 68% and production of 70%. Globally, chickpea is cultivated on about 13.20 Mha and produces 11.62 million tonnes with an average productivity of 880 kg/ ha (Singh and Sewak, 2013). *Fusarium* wilt caused by *Fusarium oxysporum* f. sp. *ciceris* is a major constraint to chickpea production throughout the world which can

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cause upto 100% yield losses annually (Pande et al., 2010). *F. oxysporum* can survive in soil several years by means of chlamydospores. *Fusarium* wilt is soil or seed borne and causes reduction in seedling emergence as well as wilt of affected plants. The most practical method of control worldwide is application of fungicides or resistant cultivars. Regular use of fungicides can pose a risk to the agro-ecosystem. Application of biological control agents for plant diseases can be used as an alternative to synthetic pesticides due to environmental impacts. Recently, rhizobia are employed as biocontrol agents in legumes due to their great advantage of symbiotic N_2fixation in legumes as well as to several mechanisms including competition for iron by production of siderophores, competition for nutrients, production of antibiotics and cell wall degrading enzymes or HCN production (Arfaoui et al., 2006). Among the *Rhizobium* genera, *Rhizobium leguminosarum, Sinorhizobium meliloti and Bradyrhizobium japonicum* have been used successfully against fungal pathogens belonging to genera *Macrophomina, Rhizoctonia and Fusarium* (Ozkoc and Deliveli, 2001 and Arfaoui et al., 2006)

The aim of the present work was to characterize and select soil and chickpea nodule isolates with antagonistic activity against wilt caused by *F. oxysporum* f. sp. *ciceris*.

MATERIALS AND METHODS

Origin of *Mesorhizobium*

Twenty soil and chickpea nodule isolate samples were obtained from chickpea growing areas of Punjab. Reference strain LGR 33 was obtained from Pulses Microbiology Laboratory, Department of Plant Breeding and Genetics, Punjab Agricultural University, Ludhiana. All isolates were identified by using morphological and biochemical characteristics and stored at 4°C on slants and maintained through sub-culturing. The isolates were characterized by Gram staining, Methyl Red, Voges Proskauer, Citrate Utilization, Nitrate reduction, 3-Ketolactose test, oxidase test and catalase test as per Bergey's Manual of Systemic Bacteriology.

In vitro screening of multiple functional traits of soil and chickpea nodule isolates

HCN Production

Cyanide production was detected according to Bakker and Schippers (1987). Petri plates containing YEMA supplemented with 4.4 g of glycine per litre was inoculated with the different soil and chickpea nodule isolates and inverted with filter paper impregnated with 0.5% picric acid and 2% sodium carbonate in the lid of each Petri plate. Petri plates were incubated at 28±2°C for 3-7 days along with control plate without inoculum. A change in colour from yellow to orange brown on the filter paper indicated cyanide production.

Siderophore production

Siderophore production was done using Chrome azurol S (CAS) agar (Schwyn and Neilands, 1987). CAS dye (60.5 mg) was dissolved in 50 ml deionised water, and then mixed with 10 ml of a FeIII solution (1 mmol/L $FeCl_3.6H_2O$ in 10mmol/L HCl). This mixture was mixed with 72.9 mg of Hexa-decyl-Trimethyl-Ammonium Bromide (HDTMA) dissolved in 40 ml water. The resulting mixture was autoclaved, cooled to 50- 60°C and mixed with 900 ml succinate medium. The medium was poured into Petri plates and different soil and chickpea nodule isolates were inoculated. Appearance of halo zones around the colony due to chelation of iron indicated the production of siderophore.

Cell wall degrading enzyme production

Soil and chickpea nodule isolates was screened for cellulase and protease activities on carboxy methyl cellulose (CMC) agar and skimmed milk agar plates, respectively. The agar plates were prepared and spot inoculated with the test soil and chickpea nodule isolates and incubated at 28±2°C for 3-7 days. Production of halo-zone around the colony was considered as positive for cell wall degrading enzyme production (Chaiharn et al., 2008).

Antagonistic effect against Fusarium Wilt

In vitro antagonism was performed on Potato Dextrose Agar (PDA) in Petri plates by using dual culture technique (Sadfi et al., 2001) with slight modification. Soil and chickpea nodule isolates were streaked across the two edges of plate and a disc of fungus cut from the edge of a 7 day old culture of *Fusarium* sp. was placed in the centre of plate. Plates were incubated at 25°C for 7 days. Percent growth inhibition after 7 days was calculated by using the formula:

$$\% \text{ Inhibition} = (R - r)/R \times 100$$

Where, R is the radius of the fungal colony opposite to the antagonist and r, is the radius of the fungal colony towards the antagonist.

RESULTS

On the basis of morphological characters, all the isolates produced circular shape, entire margin, milky-to-watery translucent features on Congo Red Yeast Extract Mannitol Agar medium. All the colonies were odourless and designated as LGR 41, LGR 42, LGR 43, LGR 44, LGR 45, LGR 46, LGR 47, LGR 48, LGR 49, LGR 50, LGR 51, LGR 52, LGR 53, LGR 54, LGR 55, LGR 56, LGR 57, LGR 58, LGR 59, LGR 60 and LGR 33 (Reference). All soil and chickpea nodule isolates were tentatively assigned to genera *Rhizobium* on the basis of morphological characters. All soil and chickpea nodule isolates were found to be rod shaped and gram negative on the basis of Gram's reaction. Biochemical characterization was done on the basis of biochemical tests *viz* oxidase, catalase, citrate utilization, methyl red, Voges Proskauer, nitrate reduction and 3-Ketolactose production. All the isolates were positive for oxidase, catalase, citrate utilization, nitrate reduction, while found negative for methyl red, Voges Proskauer and 3-Ketolactose production tests (Table 1).

Cyanide production was assessed on YEMA medium

Table 1. Biochemical characterization of soil and chickpea nodule isolates.

Characteristics	% of isolates showing reactions
Oxidase	100
Catalase	100
Citrate utilization	100
Methyl red (MR)	0
Voges Proskauer (VP)	0
Nitrate reduction (NR)	100
3-Ketolactose production	0

Plate 1. HCN Production by isolate LGR 46.

supplemented with glycine and inverted with filter paper impregnated with picric acid and sodium carbonate placed in the lid of each Petri plate. Change in colour after incubation from yellow to light brown or reddish brown indicated HCN production (Plate 1). Out of 20 soil and chickpea nodule isolates along with reference *Mesorhizobium* spp. LGR 33 tested, 33% isolates were found positive for HCN production (Table 2). Siderophore production was tested by streaking soil and chickpea nodule isolates on CAS agar medium. Appearance of clear halo zones around the colony due to chelation of iron bound to CAS dye indicated the production of siderophore zone after 48 h of incubation (Plate 2). Out of 20 isolates of soil and chickpea nodules including LGR 33 (reference), 43% were able to produce siderophores (Table 2). Out of 20 isolates of soil and chickpea nodules 57% (12) were able to produce protease on skim milk agar

Table 2. Screening of soil and chickpea nodule isolates for multi-functional traits.

Mesorhizobium spp.	Siderophore	HCN	Cellulase	Protease
LGR 41	-	-	+	-
LGR 42	-	-	-	-
LGR 43	+	+	+	+
LGR 44	-	-	-	+
LGR 45	-	-	-	-
LGR 46	+	+	+	+
LGR 47	-	-	+	-
LGR 48	-	+	-	-
LGR 49	+	+	-	+
LGR 50	+	+	+	+
LGR 51	+	-	-	-
LGR 52	+	+	+	+
LGR 53	-	-	+	+
LGR 54	+	-	+	-
LGR 55	-	-	-	+
LGR 56	-	-	+	-
LGR 57	-	-	+	+
LGR 58	-	-	+	-
LGR 59	-	-	-	+
LGR 60	+	-	+	+
LGR 33	+	+	+	+

+ Present, - Absent.

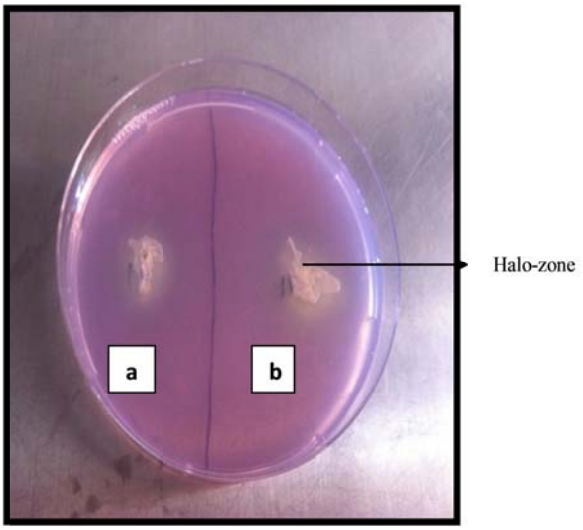

Plate 2. Siderophore production by isolates (a) LGR 46 (b) LGR 50.

and 62% (13) were able to produce cellulase on CMC agar media (Table 2).

The antagonistic effect of 20 soil and chickpea nodule isolates collected from chickpea rhizosphere along with

Plate 3. Anatagonistic effect of soil and chickpea nodule isolates against *Fusarium oxysporum* sp. *ciceris.* (a) Control growth *Fusarium oxysporum* sp. *ciceris.* (b) Inhibition of *Fusarium oxysporum* sp. *ciceris* by isolate LGR 50

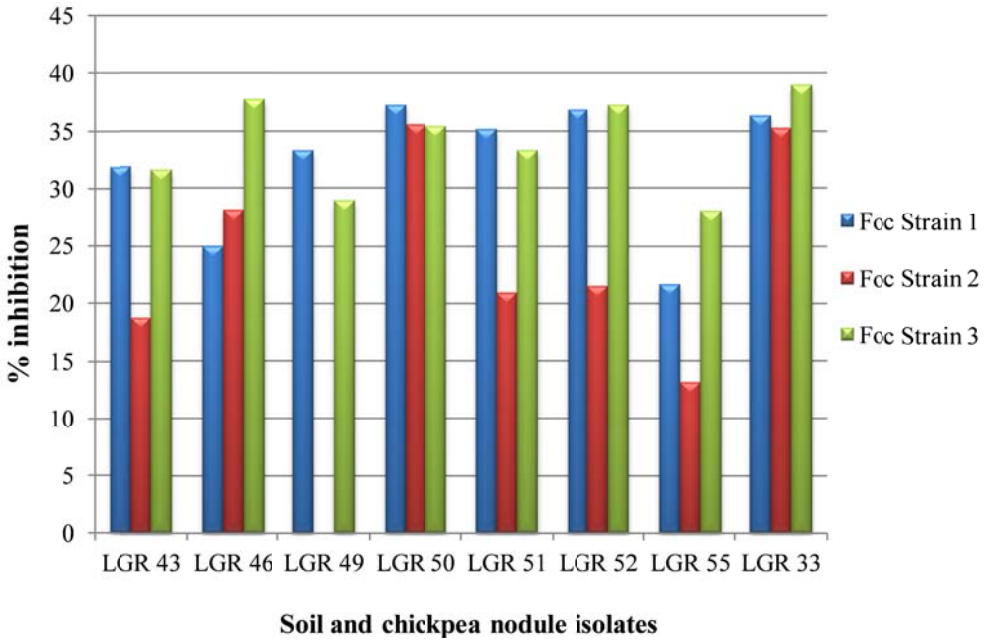

Figure 1. Percentage growth inhibition by soil and chickpea nodule isolates against *Fusarium oxysporum* f. sp. *ciceris.*

reference *Mesorhizobium* spp. LGR 33, was tested against *F. oxysporum* f. sp. *ciceris* in dual culture under *in vitro* conditions (Plate 3). Inhibition zone was clearly visible on 5th day after incubation. Soil and chickpea nodule isolates LGR 42, LGR 43, LGR 46, LGR 49, LGR 50, LGR 51, LGR 55, LGR 56 and LGR 60 inhibited the growth of *F. oxysporum* f. sp. *ciceris*, with growth inhibition varied from 13 - 37.8%. Out of 20 isolates maximum inhibition was shown with *F. oxysporum* strain 1 (45%) followed by *F. oxysporum* strain 3 (35%) and *F. oxysporum* strain 2 (30%) (Figure 1). Consequently, these strains could be used as biocontrol agents against root rot and wilt caused by fungal pathogens of chickpea.

DISCUSSION

A total of 20 soil and chickpea nodule isolates were tentatively assigned to genera rhizobia on the basis of morphological and microscopic observations. Similarly, Gauri et al. (2012) and Rai et al. (2013) also characterized mesorhizobial isolates on the basis of their

colony shape, colour and texture. Our results are in concordance with those of Gauri et al. (2012) who have also reported that microscopic examination of *Rhizobium* strain were gram negative rod shaped bacteria. On the basis of biochemical observations results are in well agreement with earlier findings of Gauri et al. (2012), Singh et al. (2013) and Wani and Khan (2013) who have obtained positive results for catalase and oxidase activities and was negative for 3-ketolactose test.

Sustainable and efficient disease control strategy demand for eco-friendly technology. In the present study, we have shown that tentative *Rhizobium* sp. can be exploited as biological agent for control of *Fusarium* wilt in chickpea. Chickpea form specific symbiosis with its cognate N_2 fixing bacterium *Mesorhizobium* that convert atmospheric N_2 to ammonia and helps in replenishing nitrogen in soils and simultaneously can be applied as biological seed treatment for control of *Fusarium* wilt in chickpea. *In vitro* reduction of fungal growth by certain *Rhizobium* sp. with the formation of inhibition zone were presumably due to the metabolites released by bacteria into culture medium. Previous studies have implicated *Rhizobium* sp. for release of anti-fungal secondary metabolites for the control of phytopathogens (Wani and Khan, 2013). In the present study isolates from soil and chickpea nodules were able to produce siderophores, HCN, cell wall degrading enzymes,volatiles and P-solubilization (data not shown) and earlier observations also suggested that bacteria possessing these traits can enhance plant growth (Kucuk et al., 2013). *Pseudomonas putida* strain with cyanide production results in reduction of leaf rust and bacterial wilt in wheat and common bean respectively. Our results are in well agreement with the findings of Arfaoui et al. (2006) who observed 8 *Rhizobium* isolates significantly inhibited fungal growth in dual culture technique by production of volatiles. Our results are in accordance with those of Ahemad and Khan (2011) and Kucuk et al. (2013) who have also reported the production of siderophores by *Mesorhizobium* spp. Siderophore producing bacteria are promoting plant growth indirectly by sequestering the iron in the rhizosphere, especially in neutral and alkaline soils, and thereby its availability for the growth of pathogens (Wani and Khan, 2013). Our results reveal that four isolates of soil and chickpea nodule isolates LGR 46, LGR 50, LGR 52 along with LGR33 were able to produce siderophore, HCN, cellulase and protease enzymes. These isolates also showed maximum inhibition of 45%, followed by 35% and 30% for the control of *F. oxysporum* strain1, 3 and 2 respectively. Siddiqui and Singh (2004) reported improved plant growth, better nodulation and lower wilting index in chickpea plants infected with Foc and inoculated with rhizobia. Further field studies must be conducted to analyse the real potential of soil and chickpea nodule isolates for the control of *Fusarium* wilt in chickpea. Further, in future identification of soil and chickpea nodule isolates as *Mesorhizobium* can be considered as an alternative or a supplemental way of reducing the use of chemicals (fertilizers and fungicides) in chickpea.

Conflict of interests

The authors did not declare any conflict of interest.

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