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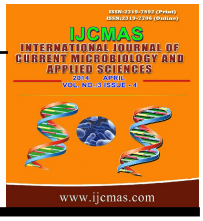

Original Research Article

Screening of *Mesorhizobium* spp. for control of *Fusarium* wilt in chickpea *in vitro* conditions

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A B S T R A C T

Mesorhizobium sp. promote the growth of plants either directly through N_2 fixation, supply of nutrients, synthesis of phytohormones and solubilization of minerals or indirectly as a biocontrol agent by inhibiting the growth of pathogens particularly *Fusarium* wilt of chickpea. Sixteen native isolates *Mesorhizobium* spp. along with reference *Mesorhizobium* sp. (LGR 33) were tested as biocontrol agent *in vitro* against the causative agent of *Fusarium* wilt of chickpea. *Mesorhizobium* spp. were tested for antagonistic effect through production of volatile antifungal compounds, cell wall degrading enzymes, hydrogen cyanide and siderophore. All *Mesorhizobium* spp. inhibited the growth of *Fusarium oxysporum* f. sp. *ciceris*, with growth inhibition varied from 34.2 (LGR 1) – 59.3% (LGR 16) whereas variation in production of volatile antifungal compounds was from 20 (LGR 9) - 46.3% (LGR 16). Out of 17 *Mesorhizobium* spp. 88% each were able to produce siderophores and protease and 64% each were able to produce HCN and cellulose. Three native isolates of *Mesorhizobium* spp. LGR 14, LGR 15 and LGR 16 were able to produce maximum HCN, siderophore, cell wall degradation enzymes, antagonistic effect and volatile antifungal compounds. More extensive study are required to identify the native isolates of *Mesorhizobium* spp. with dual functionality traits as biofertilizer as well as biocontrol agent for sustainable production of chickpea.

Introduction

Chickpea (*Cicer arientum* L.) is high protein legume grown in India. It is grown an about 9.21 million ha with production of 8.88 million ton with productivity of 995Kg/ha (Singh and Sewak 2013). Majority of chickpea affected by seed and soil borne diseases caused by *Ascochyta rabiei, Fusarium oxysporum, Botrytis cinerea* and *Rhizoctonia solani* (Arfaoui *et al* 2006). *Fusarium* wilt caused by *F. oxysporium* f. sp. *ciceris* is a major constrain in chickpea production. Annual chickpea yield losses implicating *Fusarium* wilt vary from 10-15%, but the disease span completely destroy the crop under unfavorable conditions (Cherif *et al*

2007). *Fusarium oxysporum* can survive in soil for several years by chlamydospores. Various chemical and biocontrol agents have been employed for control of *Fusarium* but rhizobia offer great advantage fixing atmospheric nitrogen in legume nodules and also could have antagonistic effects on soil borne pathogens (Arfaoui *et al* 2006; Khalequzzaman and Hossian 2008).

Rhizobium is most extensively explored microbe with N_2 fixing capacity on root of more than 20,000 species of family Fabaceae (Sharma and Gill 2010). Introduction of specific strains for chickpea nodulation is of particular importance since *Mesorhizobium* symbiosis is highly specific.

Mesorhizobium promotes plant growth either directly through N_2 fixation, or indirectly as a antagonist against particularly *Fusarium* wilt of chickpea. The biocontrol effect of rhizobia is also attributed due to the production of secondary metabolites *viz.* cell wall degrading enzyme production (protease and cellulose), Fe chelating siderophore and HCN or to the accumulation of phytoalexins leading to induction of systematic resistance. (Arora *et al* 2001, Arfaoui *et al* 2006 and Deshwal *et al* 2003)

Several bacterial species such *Bacillus*, *Pseudomonas* and *Rhizobium* are being currently examined as alternative to fungicides due to their perceived level of safety and minimal environmental hazards. In present *Mesorhozobium* spp. were being investigated for their antagonistic activity against *Fusarium oxysporium* sp. *ciceris* in chickpea under *invitro* condition.

Materials and Methods

Procurement of cultures

Sixteen native isolates of Mesorhizobia with reference strain LGR 33 were procured from Pulses Microbiology Laboratory, Department of Plant Breeding and Genetics, Punjab Agricultural University, Ludhiana and were maintained on Yeast Extract Mannitol Agar (YEMA) slants at 4ºC for further study.

Isolation of *Fusarium oxysporum*

Wilt infected plants of a highly susceptible variety of chickpea JG 62 were collected and isolation of fungus was carried out. Wilt infected tissue was cut into small pieces and surface sterilized with 0.01% of $HgCl_2$ for 30 sec. and transferred on water agar plates at 28±2°C. After 7 days, white colony of fungus was transferred to Potato Dextrose Agar (PDA) slants. Pure culture was maintained and stored at 4ºC for further use.

Biocontrol activities

Cell wall degrading enzyme production

Chitinase, cellulase and protease enzyme activities (casein degradation) were determined for clear halo zone on chitin, carboxy methyl cellulose (CMC) and skimmed milk agar plates, respectively as described by (Chaiharn *et al* 2008).

HCN Production

Exponentially grown mesorhizobial isolates were separately streaked on YEMA medium supplemented with glycine (4.4g/litre) and simultaneous supplementation of a filter paper impregnated with picric acid (0.5%) in

sodium carbonate (2%) in the upper lid of Petriplate along with control plate without inoculum. A change in colour from yellow to orange brown on the filter paper indicated cyanide production (Bakker and Schippers 1987).

Siderophore production

Siderophore production was done using Chrome azurol S (CAS) agar (Schwyn and Neilands 1987). CAS dye (60.5 mg) 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 Hexa-decyl-Trimethyl-Ammonium Bromide (HDTMA) dissolved in succinate media. Different *Mesorhizobium* spp. were inoculated on succinate media plates. Appearance of clear halo zones around the colony due to chelation of iron bound to CAS dye indicated the production of siderophore.

Antagonistic effect against *Fusarium* Wilt

In vitro antagonism was performed on Potato Dextrose Agar (PDA) in Petriplates by using dual culture technique (Sadfi *et al* 2001) with slight modification. Isolates of *Mesorhizobium* sp. 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 center of plate. 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.

Production of volatile antifungal compounds by potential *Mesorhizobium* spp.

Production of volatile antifungal compounds by *Mesorhizobium* sp. was analysed by growth inhibition (GI) zone (Fiddman and Rossal 1993). Two hundred microlitre culture of *Mesorhizobium* sp. was spread from YEM broth in a Petridish and second plate containing PDA was inoculated with a plug of fungus in the center of plate, inverted and placed over bacterial culture. Two plates were sealed together with parafilm and incubated at 28±2°C for 24-48 hrs along with control. Petriplate containing agar medium without bacteria placed over the PDA medium inoculated with fungus. Fungal growth was measured as increase in radial growth of fungus for a period of 5 days.

Results and Discussion

Production of fungal cell wall degrading enzymes

Out of 16 isolates of *Mesorhizobium* spp. alongwith reference (LGR33) 88% (15) were able to produce protease on skim milk agar and 64% (11) were able to produce cellulase on CMC medium (Table 1). None of *Mesorhizobium* spp. were able to produce chitinase on chitin agar medium. Our results are in accordance with Yildez *et al* (2012) who reported that out of 216 isolates, 8 were able to produce protease, while no isolate was able to produce chitinase. Robledo *et al* (2008) also reported production of cellulase enzyme by *Rhizbium* sp. and cleavage of glycosidic bonds in plant cell wall polymers considered as a general phenomenon among various species of *Rhizobium* (Antoun and Kloepper 2001). Similarly, Chaiharn *et al* (2008) reported

the production of fungal cell wall degrading enzymes *viz.* cellulase, chitinase and protease by rhizobacteria.

HCN production

Change in colour after incubation on YEMA medium supplemented with glycine from yellow to light brown or reddish brown indicated HCN production (Fig.1). Out of 16 *Mesorhizobium* sp. along with reference *Mesorhizobium* sp. LGR 33 tested, 64% isolates were found positive for HCN production (Table 2). Our results are in agreement with study conducted by Wani and Khan (2013) who reported the production of HCN by *Mesorhizobium* sp. isolates in chickpea and it is known to play an important role in biological control (Kucuk *et al* 2013). HCN affects the respiratory system of pathogenic fungi and results in their growth inhibition (Viveros *et al* 2010 and Verma *et al* 2012). Thus HCN producing strains could be safely used as biocontrol agents of *Fusarium* wilt as these strains do not have adverse affect on plant growth (Goel *et al* 2002).

Siderophore production

Siderophore production was tested by formation of orange halo zone around the inoculated bacterial colonies on CAS agar indicated siderophore production (Fig. 2). All isolates except LGR 4 and LGR 10 were able to form halo zone after 48 hrs incubation (Table 2). Out of 17 isolates of *Mesorhizobium* sp. 88% were able to produce siderophores

Similarly, Raychaudhuri *et al* (2005) also reported the production of siderophores by *Mesorhizobium* sp. isolated from chickpea. Siderophore production has also been reported earlier in various *Rhizobium* spp. (Carson *et al* 2000, Arora *et al* 2001 and Kucuk *et al* 2013) also documented the production of siderophores by *Rhizobium* sp. as a protector against phytopathogenic fungi. Siderophores are known to bind to the available form of iron Fe^{+3} in the chickpea rhizosphere thus making it unavailable to the phytopathogens and consequently protects the plant health (Wani and Khan 2013).

Antagonistic effect against *Fusarium* Wilt

Antagonistic potential of 16 *Mesorhizobium* spp. collected from chickpea rhizosphere along with reference *Mesorhizobium* sp. LGR 33, were tested against *Fusarium oxysporum* f. sp. *ciceris* in dual culture under *in vitro* conditions. All *Mesorhizobium* sp, inhibited the growth of *Fusarium oxysporum* (Table 3).The diameter of fungal growth ranged from 4-5 cm in dual culture as compared to 7 cm in the control (Fig. 3). Thus the potential antagonists were able to inhibit the growth of *Fusarium oxysporum* f. sp. *ciceris* by 2-3 cm as compared to control. Inhibition zone was clearly visible on 5th day after incubation. The percent growth inhibition was found to be in range of 34.2 to 59.3%. Isolates LGR 2, LGR 3, LGR 7, LGR 8, LGR13, LGR14, LGR15 and LGR 16 were most effective *in vitro* and caused reduction in growth of fungus in the range of 46.5-51%.

These results are in close agreement with the findings of Kucuk *et al* (2013) and Subhani *et al* (2013) who also revealed reduced *Fusarium* wilt of chickpea by *Rhizobium* sp. explained their potential as biocontrol agents. Our findings are also in accordance with Inam-ul Haque *et al* (2003) who observed that rhizobia isolated from chickpea are effective in controlling

Table.1 Screening of *Mesorhizobium* sp. for fungal cell wall degrading enzymes production

Mesorhizobium sp.	Cellulase	Protease
LGR1	+	+
LGR2	+	+
LGR3	+	+
LGR4	-	+
LGR5	-	-
LGR6	+	-
LGR7	+	-
LGR8	+	+
LGR9	+	+
LGR10	+	+
LGR11	+	-
LGR12	+	+
LGR13	+	-
LGR14	+	-
LGR15	+	+
LGR16	+	+
LGR33(Reference)	+	+

+, Positive ; - , Negative

Table.2 Screening of *Mesorhizobium* sp. for functionality traits in chickpea

Mesorhizobium sp.	Siderophore production	HCN production
LGR1	**+**	**+**
LGR2	**+**	**-**
LGR3	**+**	**+**
LGR4	**-**	**+**
LGR5	**+**	**-**
LGR6	**+**	**+**
LGR7	**+**	**-**
LGR8	**+**	**+**
LGR9	**+**	**+**
LGR10	**-**	**-**
LGR11	**+**	**+**
LGR12	**+**	**+**
LGR13	**+**	**-**
LGR14	**+**	**+**
LGR15	**+**	**+**
LGR16	**+**	**+**
LGR 33 (Reference)	**+**	**-**

+, Positive, - , Negative

Table.3 Effect of *Mesorhizobium* sp. on growth of *Fusarium oxysporum* f. sp. *ciceris*

Mesorhizobium sp.	Growth opposite to antagonist (cm)	Growth towards the antagonist (cm)	Growth inhibition (%)
LGR1	4.1	2.7	34.2
LGR2	4.5	2.4	46.7
LGR3	4.2	2.2	47.6
LGR4	4.7	2.8	40.4
LGR5	5.2	3.1	40.4
LGR6	3.7	2.1	43.2
LGR7	4.5	2.1	53.3
LGR8	4.0	2.1	47.5
LGR9	3.8	2.2	42.1
LGR10	4.2	2.4	42.9
LGR11	3.5	2.2	37.1
LGR12	5.0	2.8	44.0
LGR13	5.1	2.5	50.9
LGR14	4.5	2.1	53.3
LGR15	4.5	1.9	57.8
LGR16	5.4	2.2	59.3
LGR 33 (Reference)	3.7	2.4	35.1

Table.4 Production of volatile antifungal compounds by *Mesorhizobium* sp**.**

Mesorhizobium **sp.**	Volatile antifungal compound produced (%)
Uninoculated Control	5.2
LGR1	22.2
LGR2	ND
LGR3	31.9
LGR4	28.4
LGR5	ND
LGR6	23.4
LGR7	ND
LGR8	22.6
LGR9	20.0
LGR10	ND
LGR11	ND
LGR12	ND
LGR13	22.4
LGR14	ND
LGR15	44.2
LGR16	46.3
LGR 33 (Reference)	24.6

ND- Not detected

Fusarium wilt. Inhibition of *Fusarium* wilt is likely due to excretion of essential metabolites produced in excess by the *Mesorhizobium* sp. in a medium where it is in limited supply is also well supported with the findings of Arfaoui *et al* (2005).

Production of volatile antifungal compounds

Out of 16 *Mesorhizobium* spp. tested along with reference *Mesorhizobium* sp. LGR 33, only 10 (58%) were found to produce volatile antifungal compounds (Table 4). A reduction in the radial growth of the test fungus due to production of volatile antifungal compounds by mesorhizobia was observed after 5 days of incubation (Fig. 4). Highest volatile antifungal compounds were produced *in vitro* by *Mesorhizobium* sp. LGR-16 (46.3%) and LGR-15 (44.2%). Sharif *et al* (2003) who reported the rhizobia present in the rhizosphere of plants might be prevented by the contact of pathogenic fungi by covering hyphal tip of fungus and by parasitizing it. Two native isolates *viz*. LGR-15 and LGR-16 were identified as potential bioagent for control of Fusarium wilt in chickpea. Selection of ideal rhizobia with dual functional traits can be exploited as potential biofertilizer in chickpea.

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