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## Rhizobium pusense strain isolated from chickpea important for the control of Fusarium wilt of chickpea varieties

**Abstract:** Present investigation was carried out to find out an efficient Rhizobium strain for two chickpea varieties like Fe efficient (GG1) and Fe inefficient (ICCC-4), most effective against control of wilt disease of chickpea varieties. **Major research findings:** Among three test isolates, R-16 was found best for the control of Fusarium wilt of chickpea followed by R-12, R-19 and standard culture IC-75. They showing 50% possible reduction of wilt disease of chickpea in pots. **Brief experimental methods:** Molecular characterization indicated that Rhizobium isolates have shown nifH gene presence. 16SrDNA sequencing identified isolate R-16 as Rhizobium pusense (NCBI Accn no: JX266311.1).Data of the pot studies indicated that prevention of Fusarium wilt by Rhizobium pusense in Fe inefficient variety is low as compared to Fe efficient variety of chickpea, which is able to produce HCN, siderophore, organic acid.

### Keywords: Rhizobium pusense, HCN, siderophore organic acid, nif H, HPLC

Fusarium wilt is a biotic stress of chickpea which is major reason for the yield losses in chickpea.. The contributory agent of this Fusarium wilt disease has been classified as Fusarium oxysporum f. sp. ciceris (Foc). This fungus can also survive in infected crop in soil and other chickpea species can also be affected under non-natural inoculation conditions (Barve, 2003). Disease plants of chickpea found a light green colour of the leaves, rapid slumped of the petioles, rachis and leaves. (Nene et al.1978).

**Objective of the research**: Wilted plants of chickpea can be prevent by the use of Rhizobium inoculation. Among the Rhizobium group, Rhizobium leguminosarum, Sinorhizobium meliloti, and Bradyrhizobium japonicum, Rhizobium pusenses, Rhizobium aggregatum, can prevent wilt disease of chickpea plant. Rhizobium has multiple actions that allow them to reduce pathogens. These mechanisms include, fight for iron by production of siderophores, organic acid production, hydrogen cyanide production and fungal cellwall degrading enzyme produce. (Carrillo and Del Rosario, 1992; Arora et al., 2001).

### **MATERIALS AND METHODS:**

### 2.1 Source of cultures

Chickpea isolates (R-12, R-16, R-19 and one standard IC-75) were isolated from chickpea root nodules. **2.2Molecular characterization 2.2.1Amplification of nifH gene** 

Amplification of nifH gene was conceded out using next two primers which was constructed by MWG Biotech Pvt. Ltd. at Banglore, India.

Fragments of nifH genes were amplified by two PCR reactions. PCR reaction mixture was prepared from the stock solutions of each individual component. All the reagents were mix carefully by a short spin using microfuge.

Every one of the PCR reaction were carried out in 200  $\mu$ l slim walled PCR tubes. PCR tubes contain reaction combination were tap gently. The amplification was carried out with Master cycler personal (Eppendorf, Germany).

PCR products were analyzed by gel electrophoresis. If PCR products showed multiple bands than re-PCR of amplified product was performed a 25  $\mu$ l reaction mixture using 0.5  $\mu$ l of the primary PCR product as the DNA template. PCR amplification condition were used except that time, denaturation 30 sec. at 94°C; annealing 30 sec. at 55 °C; extension 30 sec. at 72°C. The PCR products were than analyzed by agarose gel electrophoresis.

#### **2.3PGPR traits**

### 2.3.1Organic acid production 2.3.1.1Qualitative and Quantitative analysis of Organic acids production in liquid media by HPLC

Organic acid qualitative and quantitative analysis was performed by High Performance Liquid Chromatography (HPLC) technique as described by Dinkci et al. (2007).

#### 2.4 Siderophore production

Production of siderophore by isolates was assessed through plate assay. Chrome Azurol S blue agar medium (CAS) was used to detect siderophore production by the isolates as per method described by Alexander and Zuberer (1991). CAS medium (1000ml) was arranged by dissolve 60.5 mg Chrome Azurol S (CAS) (HiMedia) in 50 ml of distilled water and mix by 10 ml iron (III) solution (1 mM FeCl<sub>3.6</sub>H<sub>2</sub>O in 10 mM HCl). With constant stirring, the clarification was slowly added to 72.9 mg hexadecyl trimethyl ammonium bromide (HDTMA) dissolve in 40 ml water. The resultant dark blue liquid was autoclaved.

### 2.5 Antagonism of Rhizobium against wilt disease causing by fungus Fusarium.

In vitro antagonisms tests are to be performed on NA-PDA in 9 cm Petri plates by apply following a dual culture technique Rhizobium isolates will be put in the middle of the plate. At the each end of the plate place 5mm disc of 7 day old culture of Fusarium for the antagonist effect. The distance beteen the two microorganisms will be kept 2.5 cm. plates are to be incubated at 25° C for one week. Growth inhibition of wilt disease was calculated by the formula of whipps ;(R1-R2)/R1\*100, where, R1 is the farthest away radial space (measured in mm) grown foc after 7 days of incubation and R2 is the space of the fungal growth from the point of inoculation to the colony margin in the direction of the antagonist. (Arfaoui 2005)

#### **RESULTS & DISCUSSIONS:**

### 3.1 1 6SrDNA gene sequencing and identification of native most promising Rhizobium isolate ch16

16S rRNA partial gene sequence of ~ 1500 bp was carried out by gene sequencing of Rhizobium isolates.

### **3.2 Detection of nif H gene**

Isolates positive for nif H gene was detect by PCR amplification of region coding for Fe – protein (component II) of nitrogenase enzyme complex using degenerate primers **(fig:1)**. This primer pair selectively amplified Fe – protein coding region (ranging from 250 – 500 bp) depending upon the organism and its nif gene sequence (Gonzalez et al., 2011). All the checked isolates and one standard IC-75 gave product size of ~ 347 representing these isolates have capability to fix atmospheric nitrogen

Dubey et al. (2010) isolated 8 strains of Rhizobium from Pigeon pea (Cajanus cajan),out of which five isolates (KCC1 to KCC5) were investigated for genetic diversity using ARDRA and were placed into different clusters.

Specific primers nifH1 and nifH2 were used for amplification of nifH and compare with low range DNA ruler, 386 bp long amplified products.

### 3.3 Screening of isolates for organic acid production

To unveil the mechanism of phosphate solubilization, two selected isolates viz. Isolate R-16 and R-12 showing highest solubilization zones were subjected to organic acid production profile on solid media as well as in liquid media trough HPLC analysis.

### 3.3.1 Qualitative and quantitative examination of organic acids amount in liquid media by HPLC

HPLC chemical analysis of culture filtrate was conceded out to identify and quantify the organic acids produced during solubilisation of TCP by selected four Rhizobium cultures at 3, 5 and 7 DAI. During TCP solubilisation, it was observed that the major organic acids produced by bacteria are oxalic, gluconic, tartaric, malonic, acetic, pyruvic, propionic, lactic and gluconic acids.(Fig. 2,3)

At 3 DAI Isolate R-16 produced tartaric acid as major product (1521.0  $\mu$ g/ml) and Malonic acid (798.3  $\mu$ g/ml).

At 5 DAI isolate R-16 produced oxalic (1210.0µg/ml), malic acid(227.4), acetic acid(223.24) followed by citric acid (83.72µg/ml).

At 7 DAI the concentration of oxalic acid  $(970.7\mu g/ml)$  citric acid  $(79.5\mu g/ml)$  and acetic acid  $(82.17\mu g/ml)$  production was reduced. **(FIG: 5)** 

Similar trend was observed for isolate R-12 with maximum production of gluconic acid ( $3518.7 \mu g/ml$ ) while it was not observed on 5 and 7 DAI. In this culture instead of oxalic acid production, acetic acid (29.66). Malic acid (192.20) was found in at 3 DAI which is decreased after 5 DAI 135.06(**FIG:4**) This organism was also able to produce citric acid (132.5) and Succinic acid (23.92) at 7 DAI. Total organic acid production was recorded ( $3740.56\mu g/ml$ ) after 3 DAI and ( $5055.66\mu g/ml$ ) after 5DAI, whereas it was reducing after 7 day.

Similar trend was observed for standard check IC-75 although the total organic acid production was low in comparison to tested isolates. In case of IC-75 additional production of gluconic acid was also recorded at 3 and 5 DAI (9500.0  $\mu$ g/ml) and 886.3 $\mu$ g/ml) which was not measured at 7 DAI.(**FIG:6**).

## 3.4 Siderophore production on solid CAS agar medium

Among all the isolates 16 ( $2.5\pm0.4$ ), 12 ( $1.8\pm03$ ) and IC-75 ( $1.9\pm04$ ) produced larger halo on CAS agar medium compared to 19 isolate **(Table 2, Fig. 7, 8)** 

## 3.5 In vitro siderophore production activity of isolates

## 3.5.1 Siderophore production in liquid CAS broth

As indicative in the (**Table 4**), the entire isolates found positive for siderophore production in CAS liquid medium at 3 and 5 DAI. Isolate R-12 (22.34, 32.25  $\mu$ g/ml), R-16 (17.81, 29.70  $\mu$ g/ml) and R-19 (16.6,24.10  $\mu$ g/ml) produced higher amount of siderophore as compared to other isolates on 3<sup>rd</sup> and 5<sup>th</sup> day respectively, while standard cultures IC-75 (15.9, 29.50  $\mu$ g/ml) showed siderophore production.

### **3.6 HCN production**

From the three cultures and one standard culture were tested for their ability to produce HCN. In the qualitative assay, all the isolates have transformed the colour of sodium picrate impregnated filter paper strips, which undoubtedly indicated that they are cyanogenic Rhizobium. However, the intensity of brown or reddish brown has diverse between the isolates. All these 3 culture and 1 standard culture were grown in nutrient broth in test tubes for the quantification of HCN. This study had revealed that isolates R-16 (72.6), R-12 (54.8) produced higher amounts of HCN than the other standard culture IC-75 (50.6), and other culture **(Table 6 fig: 10)**.

#### 3.7 In vitro growth inhibition category of Rhizobium isolates

Among the three Rhizobium isolates and one standard(IC-75) were tested in dual culture, all the isolates and standard inhibited the growth of Fusarium in vitro condition more than 30% and belonged to growth inhibition categories 2 and 3. Isolates R-12, R16, and R-19 were the most effective in vitro and caused more than 50% growth inhibition.R-12 and R-16,R-19 and IC-75 produced a halo on dicalcium phosphate agar plates **(Table 6, Fig.9)**.All three isolates, R- 12,R-16,R-19 and IC 75 were positive for cyanide production and phosphate solubilisation.

#### 3.8 Greenhouse experiments.

In pot trial these isolates were shows reduction of the wilt diseases of the two chickpea(GG1,ICCC-4) varieties. **(Table: 7)**, after sowing of chickpea seed, 12 weeks, there was more disease (78% of plants wilted) in the inefficient cultivar ICCC-4 than in the efficient cultivar GG1 (67.5%). Treatment of chickpea seeds with Rhizobium isolates before sowing with isolates R-12, R-16, R-19 and IC-75 considerably prevent the wilt disease of two chickpea varieties ,one is iron efficient and other is iron inefficient. This percentage ranged from 25 to 82.5% in GG1 and from 39 to 83.4% in ICCC 4. The better wilt control was obtained with isolate R-16, followed by R-12, IC-75 and R-19.

### CONCLUSION

Chickpea is a very important legume crop, but the reduction of the yield of the plant is due to the biotic stress and abiotic stresses. This loss is reduced by the use of Rhizobium inoculants as it increases the yield by adding nitrogen to the plant as well as HCN and organic acid are important for the control of wilt disease of chickpea plant.

Isolate	Length of 16S rRNA gene sequenced	GeneBank accession no.	Most closely related organisms*			
			Species	Accession description	% Gene identity	% Query coverage
1	761	KF888659	Rhizobium pusense strain R- 16	JX266311.1	99	100

#### Table: 1 Identification of Rhizobium isolates

#### Table: 6 In vitro growth inhibition category of Rhizobium isolates.

Rhizobium isolates	Growth Inhibition zone (mm)	GI category	Phosphate solubilization	Cyanide Production(mg)
R-12	19.75	2	4	33.8

R-16	21.42	3	12	49.3
R-19	13.17	2	7	29.6
IC-75	18.25	2	6	40.2
Pseudomonas	19.55	3	12	35.4
S.Em. ±	0.40		0.42	0.36
CD (P=0.05)	1.16		1.23	1.29
CV%	6.08		4.41	7.25

(+) = Percent growth inhibition compared to uninoculated control was determined after 7 days of incubation using Whipps' (1987) formula. Values assigned on a scale from 0 to 3 = were: 0 = no growth inhibition; 1 = 1 to 25%; 2 = 26 to 50%; 3 = 51 to 75%.

Table: 7 Effect of Rhizobium isolates on wilt incidence in chickpea cultivars GG1 (efficient) and ICCC-4(inefficient) inoculated with Fusarium oxysporum f.sp. ciceri under greenhouse conditions at 12 weeks after sowing

Treatment	Disease incidence (% of wilted plants) (In green house)		
	GG1(efficient)	ICCC 4(inefficient)	
Control	0.0	0.0	
FOC	82.5	83.4	
R-12	48.5	55.6	
R- 16	25	39	
R- 19	67.5	78	
IC-75	57.5	60.2	
Pseudomonas	45	49	

Table: 2 Siderophore productions in CAS agar medium

Isolates	Siderophore production on CAS agar medium	Diameter of halo (in cm)
R-12	++	1.8±0.2
R-16	+++	2.5±0.4
R-19	++	1.9±0.3
IC-75	+++	1.9±04

Table: 4 Siderophore productions in CAS liquid medium

Isolates	Siderophore production in CAS liquid medium			
15014105	3 DAI	5 DAI		
R-12	22.34	32.25		
R-16	17.81	29.70		
R-19	16.6	24.10		
IC-75	15.9	29.50		

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S.Em. ±	0.20	0.32
CD (P=0.05)	1.36	1.25
CV%	7.28	6.50



Fig: 4 Chromatograms of organic acid profile of isolate R-12 at 3, 5 and 7 DAI



### Fig: 5 Chromatograms of organic acid profile of isolate R- 16 at 3, 5 and 7 DAI



## Fig: 6 Chromatograms of organic acid profile of isolate IC-75 at 3, 5 and 7 DAI





Fig:9 Inhibition zone of *Fusarium oxysporum fv. ciceri* by *Rhizobium* (A)isolate R-16(B)IC-75 1000 bp 300bp 100bp 100b

Fig: 1 nif H gene amplification profile of Rhizobium isolates(R-12,R-16,R- 19) and IC-75 (standard culture)

#### FIG: 10 HCN productions by Rhizobium isolates



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