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Biocontrol Mechanisms of Efficient *Azotobacter* Isolates against *Fusarium solani* Causing *Fusarium* wilt of Chilli (*Capsicum annum* L.) under *in vitro* Conditions

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A B S T R A C T

In the present investigation, four efficient *Azotobacter* isolates were screened for various biocontrol traits and evaluated for its biological deterrent activity against *Fusarium solani*. Maximum per cent inhibition of 60.25 was observed in AZT-R_7 isolate followed by AZT-Y_2 (59.17 %), AZT-J_1 (57.77 %) and AZT-G_4 (51.47 %). AZT-R_7 recorded a maximum siderophore, IAA production and highest N_2 fixation of 82.83 per cent, 100.75 µg 50 ml^{-1} and 10.33 mg g^{-1}, respectively. All the four isolates showed positive for the HCN test and the two isolates AZT-R_7 and AZT-G_4 showed positive for the H_2S test and AZT-G_4 for Phosphorus solubilization. Therefore, *Azotobacter* isolate AZT-R_7 can be recommended for commercial production as a potential biocontrol agent and an alternative to *P. fluorescens* against *Fusarium* wilt in chilli grown in Hyderabad Karnataka region.

Introduction

Chilli (*Capsicum annum* L.) is an important vegetable crop and its socio-cultural role is remarkable worldwide. The enormous popularity and demand for chilli is providing a boost to the chilli industry, but its production is increasingly constrained by soil borne diseases. *Fusarium* wilt is the most important soil borne disease caused by *Fusarium sp*. To overcome this, several strategies have been devised, one such a strategy is the use of fungicides. Fungicides being chemical in nature cause havoc in the environment. As an alternative strategy, eco-friendly biological agent such as *Pseudomonas fluorescens, Bacillus sp*., and *Trichoderma sp.*, has been developed to counteract wilt disease.

Use of complex cyst forming *Azotobacter* isolates with high rhizosphere competency will overcome the limitations in commercial production of existing commercial biocontrol agents. The present study aims at the selection of an efficient *Azotobacter* isolate with multiple biocontrol traits and development of

Azotobacter inoculants with commercialization potential, a major challenge hindering the bioinoculant production technology. Such an isolate would be an alternative biocontrol agent to *P. fluorescens* for the management of *Fusarium* wilt of chilli grown in Hyderabad Karnataka region.

Materials and Methods

Isolation of *Azotobacter* isolates and *Fusarium solani*

Azotobacter isolates were isolated by using serial plate dilution technique (Wu *et al.*, 2006). *F. solani* was isolated by tissue segment method (Rangaswamy and Mahadevan, 1999) on Potato Dextrose Agar medium (PDA).

Azotobacter isolates were tested for their inhibitory activity against mycelial growth of *F. solani* by following the dual culture technique (Dennis and Webster, 1971).

Elucidation of biocontrol mechanisms of efficient *Azotobacter* isolates against *F. solani*

The efficient *Azotobacter* isolates were further used in elucidating the mechanisms of biocontrol, such as siderophore production, hydrogen cyanide production, IAA production, *in vitro* N_2 fixation and H_2S production test. N_2 fixation by the isolates was estimated by the method described by Humpries (1956). All the isolates were screened for the production of HCN by adapting the method of Lorck (1948). In order to screen the production of siderophores, the *Azotobacter* isolates were grown in CAS agar medium. The inoculated agar plates were incubated at 37 ^{0} C for 24 h. The observation was made for the change of medium color from blue to reddish yellow to determine the siderophores production. IAA production potential of *Azotobacter* isolates were tested in Ashby′s nitrogen free broth supplemented with 0.005 M concentration tryptophan at 28 ^{0} C. The concentration of IAA in the culture broth after 3 days of incubation was centrifuged at 5,000 rpm for 5 min and determined by spectrophotometric method using Salkowaski's reagent (Mali *et al.*, 2011). Phosphorus solubilization ability of *Azotobacter* isolates were studied on Pikovskaya′s agar plates. The pure culture of *Azotobacter* isolate was spread on agar plates containing Pikovskaya′s medium supplemented with phosphate and these plates were incubated at 25 ^{0} C for 4-5 days. Formation of halo zone was taken as positive for the solubilization of P. Sterilized SIM agar tubes were stab inoculated with the *Azotobacter* cultures and incubated for 48 h at 30 ^{0} C. After incubation, the tubes with cultures observed for the development of black color along the line of the stab.

Results and Discussion

Isolation of *Azotobacter* isolates *F. solani*

After 4-6 days of incubation at 30 ^{0} C, the isolates formed small water droplets like white glistening colonies on Waksman No. 77 agar plates, later turned to brown to black colored colonies (Gibbs and Shapton, 1968).

The chilli plants showing typical wilted symptoms were used for the isolation of *F. solani*. The culture was developed on PDA medium in the Petri plates and observed the presence of fungal spores under the microscope. It was identified as *F. solani* based on the mycelial and conidial (microconidia, macroconidia and chlamydospores) characteristics through standard mycelial keys; the results are in agreement with the findings of Barnett and Hunter (1972) (Plate 1 and 2).

Table.1 Per cent inhibition of mycelial growth of *F. solani* by efficient isolates of *Azotobacter*

Sl. No.	*Azotobacter* isolate	Per cent inhibition
1	Control	0.00
2	AZT-Y_2	59.17 (50.28)[a]
3	AZT-R_7	60.25 (50.91)[a]
4	AZT-J_1	57.77 (49.47)[b]
5	AZT-G_4	51.47 (45.54)[d]
6	Ref. *Azotobacter*	53.43 (46.97)[c]
SEm ±		0.14
CD (1 %)		0.58

Note: Figures in the parentheses are arc sine transformed values

Table.2 Elucidation of biocontrol mechanisms of efficient *Azotobacter* isolates against *F. solani*

Sl. No.	*Azotobacter* isolate	*Siderophore production (%)	*In vitro* N_2 fixation (mg N_2 fixed g^{-1} of mannitol)	IAA (µg 50 ml^{-1})
1	Control	1.99 (8.11)[c]	0.00	0.00
2	AZT-Y_2	81.51 (64.54)[a]	9.13[b]	93.37[b]
3	AZT-R_7	82.83 (65.54)[a]	10.33[a]	100.75[a]
4	AZT-J_1	79.98 (63.44)[ab]	9.02[b]	81.00[cd]
5	AZT-G_4	77.44 (61.65)[b]	8.27[c]	60.87[e]
6	Ref. *Azotobacter*	79.73 (63.24)[ab]	8.56[bc]	82.00[c]
SEm ±		0.52	0.06	0.40
CD (1 %)		1.62	0.27	1.63

* Figures in parenthesis are arc sine transformed values

Plate.1 Growth of *Azotobacter* isolate on Waksman No. 77 plate

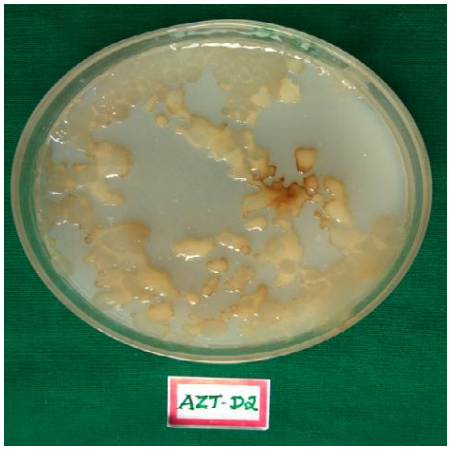


Plate.2 Growth of *Fusarium solani* on PDA plate

Plate.3 Inhibition of mycelial growth of *F. solani* by the efficient *Azotobacter* isolates after five days of incubation

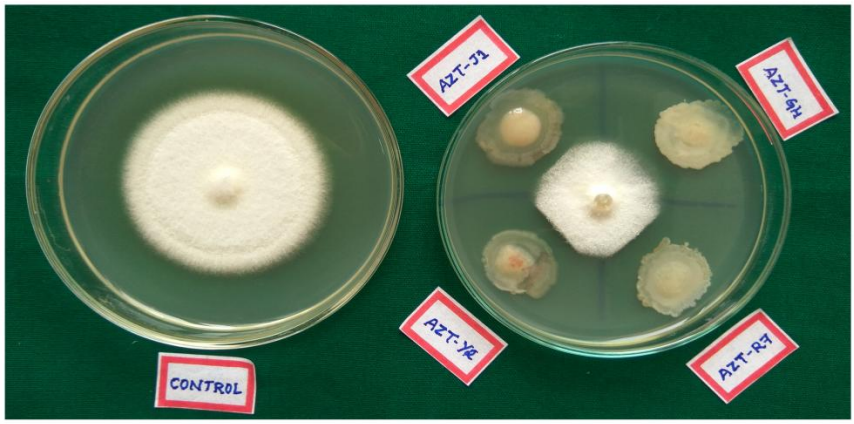


Plate.4 Change in the color of CAS agar medium from blue to reddish yellow indicating the production of siderophore by AZT-R_7

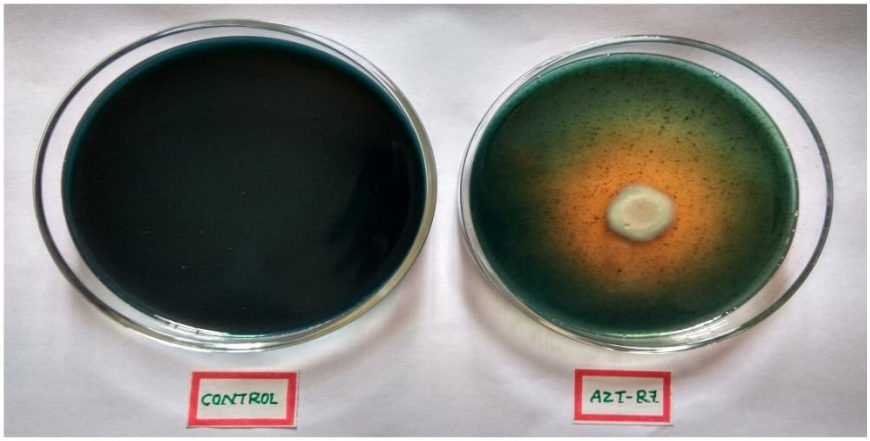


Plate.5 Development of red color in Ashby's N free medium supplemented with tryptophan indicating positive for the IAA production test

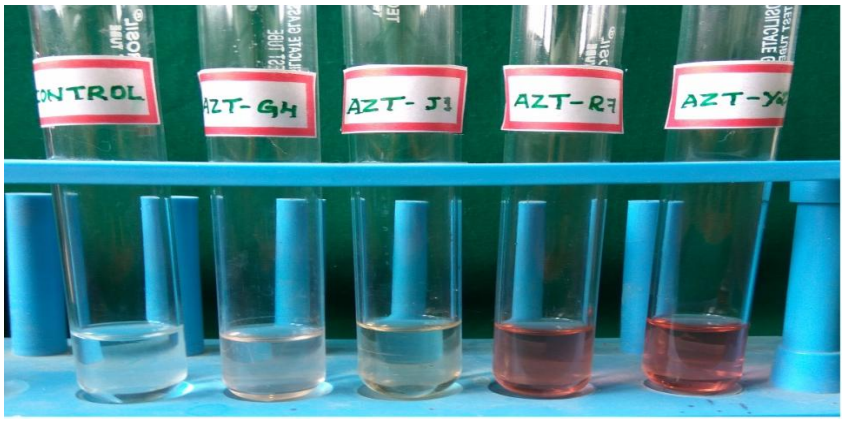
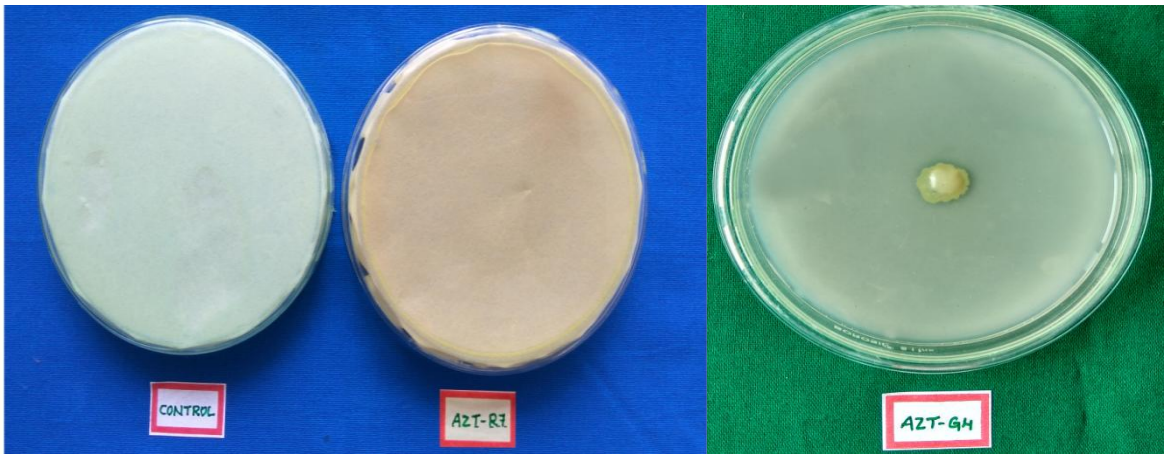


Plate.6 Production of HCN by AZT-R_7 isolate

Plate.7 P-solubilization by AZT-G_4 isolate



In vitro screening of *Azotobacter* isolates against *Fusarium solani* by dual culture technique

Four isolates *viz.*, AZT-R_7, AZT-G_4, AZT-J_1 and AZT-Y_2 were found highly efficacious in inhibiting the mycelial growth of the *F. solani*. It ranged from 51.47 to 60.25 per cent in dual culture technique. Similarly, Chauhan *et al.*, (2012), reported that isolates of *Azotobacter* inhibit the mycelial growth of fungal pathogen in dual cultures. Dragana *et al.*, 2015 reported its antifungal activity against *Helminthosporium* and *Macrophomina*, which ranged from 10-48 per cent (Table 1 and Plate 3).

Elucidation of biocontrol mechanisms of efficient *Azotobacter* isolates against *F. solani*

All the isolates fixed N_2 in the Waksman no. 77 broth medium. It ranged from 8.27 to 10.33 mg g^{-1} of mannitol source used after 7 days of incubation. The results of this findings correlates with the findings of Akhter *et al.*, (2012), Murumkar *et al.*, (2012), Jnawali *et al.*, (2015) (Table 2). In this study efficient *Azotobacter* isolates were stab inoculated in to the SIM agar tubes and incubated for 48 h at 28 ^{0} C and observed for the development of black colour along the line of stab due to the reaction of ferrous ion

with H_2S to give ferrous sulphide, were considered as positive for the test and those who do not developed black colour were scored negative. The two isolates, AZT-G_4 and AZT-J_1 showed positive results, whereas AZT-R_7 and AZT-Y_2 showed negative results (Abdel-Hamid *et al.*, 2010; Kasa *et al.*, 2016). All the efficient *Azotobacter* isolates *viz.*, AZT-R_7, AZT-G_4, AZT-J_1 and AZT-Y_2 have produced HCN, but the intensity in changing the color varied from isolate to isolate. HCN is a broad spectrum antimicrobial compound involved in the suppression of root diseases (Plate 4–6). The cyanide ion is exhaled as HCN and metabolized to lesser degree into other compounds. It inhibits the electron transport and the energy supply to cell is disrupted leading to death of the cell. HCN synthesis by the *Azotobacter* isolates acts as the inducing agent of systemic resistance in chilli and also it has got potentiality to not get repressed by the fusaric acid produced by the *Fusarium*. On the Chrome Azurol S (CAS) blue media all the four isolates have produced the orange colonies, indicating the activity of iron chelaters, the colonial zone varied with the isolate. Further they are quantified using CAS shuttle assay of Payne (1994), it ranged from 82.83 per cent (AZT-R_7) to 77.44 per cent (AZT-G_4). This character of iron chelation is an important factor for the *Azotobacter* isolates, as it deprives the pathogen from the available iron in the surrounding (Muthuselvan *et al.*, 2013). All the isolates produced IAA. It ranged from 60.87 to 100.75 µg 50 ml^{-1}. The results obtained are in agreement with the findings of several reports (Vikram Patil, 2011; Sivasankari *et al.*, 2016 and Monokoane *et al.*, 2016). The two isolates AZT-R_7 and AZT-G_4 were positive for the H_2S test and only one isolate AZT-G_4 solubilized TCP in the Pikovskaya's medium. Tejera *et al.*, (2005) reported similar kind of biochemical characters of *Azotobacter* isolates which were isolated from sugarcane rhizospheric soils.

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