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Molecular Characterization and Identification of Biocontrol Isolates of *Azotobacter* sp. Antagonistic to *Fusarium solani* in Chilli Crop

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ABSTRACT

Chilli is one of the most important commercial crop in India and it needs proper care for getting higher yields since it is susceptible to many of the soil diseases. Majority of the farmers will go for chemical sprays which are not at all advisable; conversely we have lot of biocontrol agents which will effectively suppress the activity of the pathogens under different agro-climatic conditions. In search of new and native biocontrol agents, we have investigated 75 Azotobacter isolates from the rhizospheric soils obtained from the chilli grown areas in Raichur, Kalaburgi, Bellary and Koppal region. All the isolates were evaluated for their biological deterrent activity against Fusarium solani, and we found four Azotobacter isolates were effectively inhibited the mycelial growth of F. solani under in vitro conditions. Maximum per cent inhibition of 60.25 was observed with AZT-R_7 isolate followed by AZT-Y_2 (59.17 %), AZT-J_1 (57.77 %) and AZT-G_4 (51.47 %). Further these isolates were characterized by using ten RAPD primers, in Jacob coefficient analysis Azotobacter isolates formed two main clusters viz, AZT-R_7 and AZT-Y_2; AZT-G_4 and AZT-J_1. Both the clusters were related by 27 per cent only. However, within the clusters, the AZT-R_7 and AZT-Y_2 were related by 44 per cent whereas, AZT-G_4 and AZT-J_1 by 45 per cent. These potential isolates can be recommended for the commercial production as a biocontrol agent.

Key words: *Azotobacter, Biocontrol, Chilli, F. solani, RAPD etc.*

INTRODUCTION

Microorganisms in soil play vital roles in soil fertility and primary production through organic matter decomposition and nutrient cycling. The genus *Azotobacter* comprises large, Gram-negative bacteria, mainly found in neutral to alkaline soils, where they constitute a major fraction of the free-living N_2-fixing population that contributes to nitrogen cycling in the biosphere[15].

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Azotobacter is able to colonize the rhizosphere and root surfaces of many agronomically important plants and establish non-endosymbiotic root association that leads to plant growth promotion[3], fix nitrogen[12], and secrete stimulating hormones like Gibberellins, Auxins and Cytokinins in different crops[7]. Chilli (*Capsicum annum* L.) is an important vegetable crop and its socio-cultural role is remarkable worldwide but its production is increasingly constrained by soil borne diseases. *Fusarium* wilt is the major soil borne disease of chilli caused by *Fusarium sp*. Over the decades many biocontrol agents were used to control *Fusarium,* such as *Pseudomonas fluorescens, Bacillus subtilis*, and *Trichoderma sp.,* has been developed to counteract wilt disease. The major limitation experienced by the manufacturers in commercialization of these bacterial biocontrol agents being incapable of producing resting spores as in case of *Pseudomonas sp.*, inferior in exerting direct effect on high pathogenic inoculums in soil as in case of *Bacillus sp.*, and failure in terms of efficacy due to poor viability of conidia, short time for activity and prevalence of high disease pressure in soil as in case of *Trichoderma sp.*, complicating commercial production of these biocontrol agents.

In order to overcome the drawbacks of these biocontrol agents, *Azotobacter* can be best recommended due to its high rhizosphere competency, rapid multiplication rate and complex cyst forming capacity under stress conditions. Inoculation of these bacteria competitively colonizes the roots of the plant and can act as biofertilizers and/or antagonists (biopesticides) or simultaneously both. In view of this, the present study was undertaken to investigate the biocontrol activity of strains *Azotobacter* isolated from different crop soils and used as potential biocontrol agent.

MATERIAL AND METHODS

Isolation of *Azotobacter* isolates and *Fusarium solani*

Azotobacter isolates were isolated from rhizosphere soil of chilli grown in Raichur, Kalaburgi, Bellary and Koppal region by using serial plate dilution technique. One gram of soil sample was suspended in 9 ml sterilized distilled water, and serially diluted up to 10^{-5}. From 10^{-4} and 10^{-5} dilutions, 0.1 ml of soil suspension was spread on Waksman No.77 N-free agar plates[17]. Then these plates were incubated in inverse position at 28±2 ^{0}C for 4-6 days in BOD incubator. Simultaneously, *F. solani* was isolated from the root and shoot samples of chilli plants showing characteristic wilt symptoms collected from Raichur by tissue segment method[13] on Potato Dextrose Agar medium (PDA). Reference strain of *Azotobacter* was obtained from the Department of Agricultural Microbiology, College of Agriculture, UAS, Raichur.

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

Azotobacter isolates were tested for their inhibitory activity against mycelial growth of *F. solani* by following the dual culture technique[5]. Mycelial disc of 5 mm diameter of seven days old culture of *F. solani* was placed in the middle of the Petri plate containing 15 ml PDA medium. Twenty four hour old culture of each *Azotobacter* isolates was placed on either side of the fungal disc (3 cm away from the disc). The plates were incubated at room temperature (28±2 ^{0}C) for 8-10 days. The plates with only *F. solani* disc without bacterial inoculant served as control. Each treatment was replicated three times. After incubation, *i.e.,* when control plates reached 90 mm diameter, the radial growth of pathogen was measured. Per cent inhibition over control was calculated by using the formula of Vincent[16] as follows;

$$I = \frac{(C - T)}{C} \times 100$$

I = Per cent inhibition of mycelium
C= Growth of mycelium in control
T= Growth of mycelium in treatment

Molecular characterization of efficient *Azotobacter* isolates

The polymerase chain reaction (PCR) is the *in-vitro* amplification of particular DNA sequences using random or specific primers and a thermostable DNA polymerase[9]. The diversity analysis as well as finger printing of individuals can be performed with the help of PCR based RAPD markers.

Azotobacter isolates were grown in Waksman No.77 nitrogen free broth medium at 28 ± 2 ^{0}C overnight and genomic DNA was extracted according to the method of Sambrook *et al.*[14].

DNA extraction procedure

1.5 µl of culture was spun for 7 min in a micro centrifuge tube and the supernatant was discarded. To the pellet 567 µl of TE buffer, 3 µl of 20 mg ml^{-1} proteinase-K and 30 µl of 10 per cent (w/v) SDS were added and incubated for 1 h at 37 ^{0}C. To this 100 µl of 5 M NaCl and 80 µl of 3 per cent (w/v) CTAB solution was added and incubated for 10 min at 65 ^{0}C. The solution was centrifuged at 10,000 rpm for 10 min at 4 ^{0}C and the supernatant was transferred to a fresh tube. An equal volume of chloroform: isoamyl alcohol was added to the supernatant and centrifuged at 10,000 rpm for 10 min at 4 ^{0}C. The aqueous phase was transferred to a fresh tube and spun with an equal volume of phenol: chloroform: isomyl alcohol at 10,000 rpm for 10 min at 4 ^{0}C until a clear supernatant was formed. To the extract, equal volume of ice-cold isopropanol was added, mixed gently and incubated at -20 ^{0}C overnight and centrifuged at 10,000 rpm for 10 min at 4 ^{0}C. The DNA pellet obtained was washed with 70 per cent ethanol and the tubes were inverted on blotter paper to dry the pellet. The DNA was dissolved in 100 µl T_{10} E_1 buffer and stored at -20 ^{0}C for further study.

PCR Analysis

The RAPD primers of *Azotobacter* sequence and their amplicon size are shown in table 2. PCR was carried in 25 µl final volume reaction mixture with Taq DNA polymerase (Sigma) 50 µM of each forward and reverse primer, 200 mM of each dNTP and 2 µl DNA. The master mix of 25 µl was added to PCR tubes and was given a short spin to mix the contents. The tubes were placed in the thermal cycler for amplification. The PCR reaction was carried out using master cycler gradient (Eppendorf thermal cycler gradient 5331) with the following thermal cycling conditions 94 °C for 5 minutes followed by 8 cycles of 94 °C for 45 sec, 36 °C for 45sec and 72 °C for 1 minute, with a final extension step of 10 min at 72 °C. The PCR product was analyzed on a 3 % agarose gel. The gel was run for one hour in 1X TAE buffer at 75V. The gels were visualized by ethidium bromide staining under UV light (254–366 nm) and pictures were captured using UV- Gel Documentation System. 1kb DNA ladder was used as DNA molecular weight marker.

RESULTS AND DISCUSSION

Isolation of *Azotobacter* isolates and *F. solani*

A total of 75 *Azotobacter* isolates were isolated from rhizosphere soil of chilli grown in Hyderabad Karnataka region as per the standard procedure given by Wu *et al.*[17]. After 4-6 days of incubation at 28±2 ^{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[8]. The chili 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[2].

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

Out of 75 *Azotobacter* isolates isolated from 16 different locations, only 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*. Percent inhibition zone was ranging from 51.47 to 60.25 per cent in dual culture technique (Table 1). Results were in accordance with, Chauhan *et al.*,[4], who reported that isolates of *Azotobacter* inhibit the mycelial growth of fungal pathogen in dual cultures. Similarly, Dragana *et al.*,[6] reported its antifungal activity against *Helminthosporium* and *Macrophomina*, which ranged from 10-48 per cent.

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

Molecular characterization of the efficient isolates of *Azotobacter*

A total of ten RAPD primers *viz*, OPAO-08, OPC-10, OPC-07, OPC-15, OPAC-03, OPM-07, OPA-02, OPU-19, OPC-12 were used for the amplification of DNA of four efficient *Azotobacter* isolates. Except OPC-12, all the primers amplified the DNA of all four *Azotobacter* isolates (Fig 2 and 3). A total number of 160 amplicons were produced from the ten RAPD primers ranging from 250 to 6500 bp, where maximum of 29 amplicons were produced from OPC-10 and the minimum of 8 amplicons were produced from OPAO-08. The Jacob coefficient analysis formed four *Azotobacter* isolates in to two main clusters *viz*, AZT-R_7 and AZT-Y_2; AZT-G_4 and AZT-J_1. Both the clusters related by 27 percent only. However, within the clusters, the AZT-R_7 and AZT-Y_2 were related by 44 per cent and AZT-G_4 and AZT-J_1 by 45 per cent (Fig 1).

Similar findings were obtained from a study conducted by Kasa *et al.*[10], who worked on 10 isolates from rhizospheric soils. The isolated samples were inoculated on *Amaranthus spinosus* and *Stevia rebaudiana.* RAPD analysis was performed using 16sr DNA forward and reverse primers for 10 strains. A total of 41 bands were scored, out of which 35 bands were found to be 85.59 percent polymorphic in *Azotobacter*. Abdel-Hamid *et al.*[1] collected 4 soil samples from different locations in Egypt. The molecular test of RAPD analysis was used to compare the similarity pattern of the selected isolates and the reference strain of *Azotobacter chroococcum* using it as identification tool. Five RAPD primers were used against the reference strain *Azotobacter chroococcum* (S) (NNRL-B-14346) and the 4 confirmed strains. Hence, the 5 RAPD primers were able to differentiate all the isolates and were polymorphic as well.

Lenart *et al.*[11] confirmed the genetic diversity of *Azotobacter* spp isolated from industrial and agricultural soils of Cracow-Nowa Huta steelworks (Poland) using RAPD. The results from their findings revealed high level of population diversity. ANOVA revealed that most of the diversity was attributed to within population variation (76-85 %) and only 3.78-6.18 per cent was associated with among group variation.

Table 2: RAPD Primers of *Azotobacter* and their sequences

Sl. No.	Primer	Sequence (5´-3´)	Number of Amplicons	Amplicons size
1	OPC-10	TGTCTGGGTG	29	400-6500
2	OPC-07	GTCCCGACGA	20	300-4000
3	OPM-07	CCGTGACTCA	21	250-2500
4	OPAC-03	CACTGGCCCA	17	250-2500
5	OPC-15	GACGGATCAG	18	300-2000
6	OPA-02	TGCCGAGCTG	9	300-2000
7	OPU-19	GTCAGTGCGG	16	300-2000
8	OPC-03	GGGGGTCTTT	22	400-6500
9	OPC-12	TGTCATCCCC	-	-
10	OPAO-08	ACTGGCTCTC	8	300-2000

(These RAPD primers were obtained from the Molecular department, College of Agriculture, Raichur).

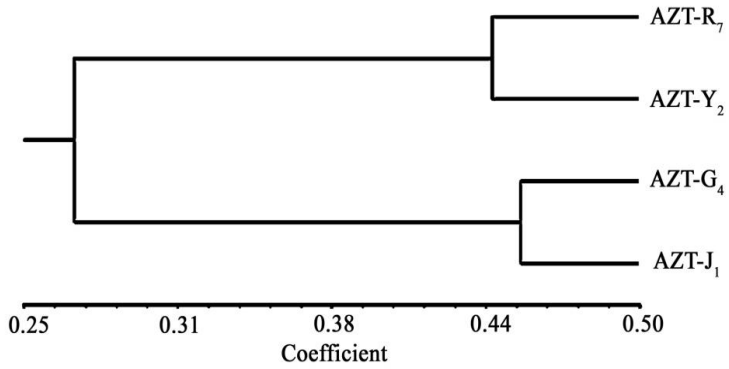

Fig. 1: Dendrogram depicting the polymorphism between AZT-J_1, AZT-G_4 and AZT-Y_2, AZT-R_7

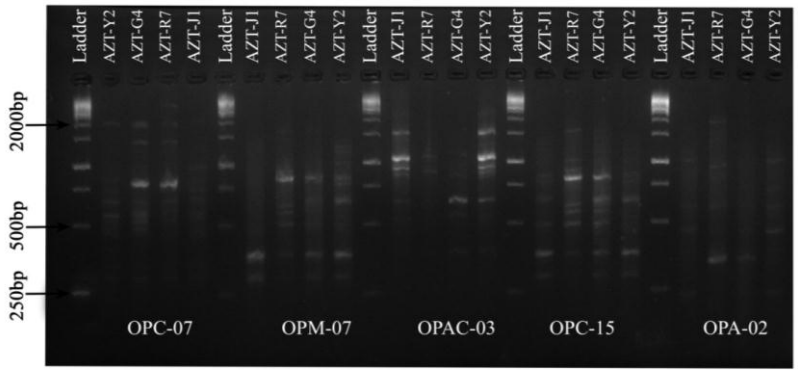

Fig. 2: Amplification of AZT-Y_2, AZT-G_4, AZT-R_7 and AZT-J_1 DNA by using OPC-07, OPM-07, OPAC03, OPC-15 and OPA-02 primers

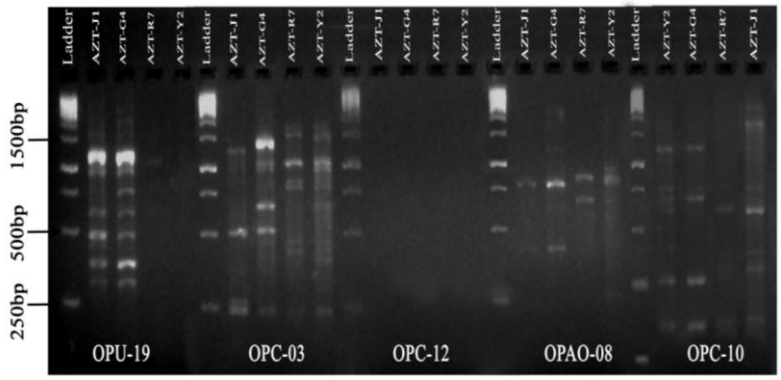

Fig. 3: Amplification of AZT-J_1, AZT-G_4, AZT-R_7 and AZT-Y_2 DNA by using OPU-19, OPC-03, OPC-12, OPAO-08 and OPC-10 primers

CONCLUSION

Biocontrol is considered to be an age old process of controlling pest and diseases, though there is effectiveness which on par with the chemical control. Although, there is a lack of attention due to indebt in deep scientific studies and knowledge. Biological control of soil borne pathogens was a tough task due to their survivability, competition for niche and nutrients in oligotrophic conditions *i.e*, soils with pathogens was a big enigma for existing commercial biocontrol agents. *Azotobacter* is a cyst forming plant growth promoting asymbiotic nitrogen fixer in chilli and other commercial crops in addition to biocontrol activity which was not explored since now. In our present study, we identified four isolates of *Azotobacter* which proved to inhalate the mycelial growth of *Fusarium* under *in vitro* conditions. In addition to this, they are also positive for the production of siderophores, IAA, Gibberellic acid, chitinase, Vit-B, PHB, peroxidase and beta-1, 3 glucanase enzymes and ACC deaminase activity. This study will further help us in achieving eco-friendly approach for controlling soil borne pathogens in various crops with dual functions *i.e*, a biofertilizer with biocontrol properties, which can be exploited in mere futuristic sustainable agriculture. Therefore, more advanced research is required for deeper gene level understanding of these efficient *Azotobacter* isolates for their biocontrol activities.

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