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Evaluation of Biocontrol Agents against *Phytophthora drechsleri* F.sp. *cajani* (Leaf Blight) in Pigeonpea

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A B S T R A C T

Pigeon pea is an important pulse crop of the world which is an important source of protein and other nutrients. Phytophthora leaf blight is a major disease of pigeon pea which is caused by *Phytophthora drechsleri* f.sp. *cajani. In vitro* evaluation of different isolates of *Trichoderma* and a strain of *Pseudomonas fluorescens* against *Phytophthora drechsleri* f.sp. *cajani* was carried out to test the efficiency of these antagonists as biocontrol agents against the test pathogen. Ten isolates of *Trichoderma* spp. and *Pseudomonas fluorescens* were tested against the blight pathogen under dual culture experiments. Volatile and non-volatile metabolites released from the antagonists were also tested against the pathogen. Percent inhibition in the mycelia growth of the pathogen was determined. All *Trichoderma* isolates showed significant inhibitory effect on the pathogen in all the experiments. Under dual culture method maximum and minimum percentage of inhibition was showed by AN-33 (81.7%) and AN-37 (63.4%) respectively. Under volatile experiments AN-48 (62.6%) and AN-6 (46.9%) showed maximum and minimum percent inhibition respectively. Under non-volatile experiments, the highest inhibition of pathogen was observed at 15% concentration of the culture filtrates of the bio agents. At this concentration AN-33 exhibited the maximum (72%) and AN-6 showed the minimum (43.5%) inhibition to the pathogen growth. *Pseudomonas fluorescens* showed significant inhibition in the mycelial growth of the pathogen under dual culture test with percentage inhibition of 43.02%. The study suggests that the *Trichoderma* spp. has a potential to be used as biocontrol agents against Phytophthora blight of pigeon pea. *Trichoderma* isolate AN-33 was identified as the most effective isolate out of the ten isolates studied.

Introduction

Blight (*Phytophthora drechsleri* f. sp. *cajani*) is among the most destructive and widespread disease of Pigeonpea (*Cajanus cajan* L.) Millsp 1994). The Pathogen damage the pith (the water transport system of the plant) resulting water soaked spots, stem become brown to dark brown, stunting and finally plant die. Phytophthora blight (PB) is caused by *Phytophthora drechsleri* Tucker f. sp. *cajani* which was first isolated from wilted pigeonpea plants with stem canker symptoms at the research farm of the Indian Agricultural Research Institute, New Delhi, India and identified by (Williams *et al.,* 1966; Pal *et al.,*

1970). The disease can be managed in many ways. For effective control, growers use a combination of crop rotation, cultivar resistance and fungicides application. Fungicides such as metalaxyl reported the poor efficacy of applied as seed dressing in protecting older pigeonpea plants against PB. Furthermore, the use of fungicides, besides being expensive and involving risks to the environment associated with the application of chemicals, is not totally effective and may lead to the appearance of new, resistant strains of pathogens (Bruin and Edgington, 1980).

It is therefore necessary to develop alternative ways of control. One such alternative is biological control, in which microorganisms are selected for their ability to antagonise pathogens. *Trichoderma* controls Ascomycetous, Deutermycetous and Basidiomycetous fungi, which are mainly soil-borne but also air borne pathogen (Chet and Hadar, 1997). *T. viride* and *Gliocladium virens* have been effective in the control of *Phytophthora* spp. causing cotton root disease (Heller and Hedtrich, 1994). *G. virens* and *Trichoderma* sp. have been used to control *Fusarium oxysporum* and *Fusarium solani* (Zhang *et al.,* 1996) and *Rhizoctonia solani* (Askew and Laing, 1994).

However, little attention has been paid to their ability to control *Phytophthora* spp. *Trichoderma* has several biocide mechanisms; some of them are myco-parasitism (drilling the plant pathogen hyphae wall by production of enzymes like glucanases and chitinase), strangulation of phytopathogen mycelium, competition for space and nutrients and production of secondary metabolites like glyotoxins, viridine, trichodermine, furanone and 6-pentyl-pyrone. Literature reports mycoparasitic fungi displaying lethal mechanisms against phytopathogenic fungi that could be of importance for plant disease biological control (Bélanger *et al.,* 1995; Benhamou and Chet, 1996). Excellent results of integrated control have been attained with strain of *Trichoderma* and Metalaxyl against *Pythium ultimum* infecting cotton (Chet and Hadar,1997). Weindling (1932) reported that the potential of *Trichoderma* species as biocontrol agent of plant diseases was first recognized in early 1930s. The present experiment was undertaken to evaluate the efficacy of the mycelial growth inhibition of *P. drechsleri* f.sp. *cajani* by *Trichoderma* isolates, *Pseudomonas fluorescens* and investigating the mechanisms involved in inhibition under microscopic observation that could be used in the future for *P. drechsleri* f.sp. *cajani* control.

Materials and Methods

Collection, identification and purification of test pathogen and antagonists

Isolation of *Trichoderma* isolate

The isolates of antagonistic (*Trichoderma* isolates, *Pseudomonas fluorescens*) were collected from the Microbial biocontrol lab of National Centre for Integrated Pest Management (NCIPM), New Delhi.

Isolation of *Phytophthora*

P. dreschlera Tucker f.sp. *cajani* (Pal *et al.,* 1970) were identified by distinctive characters mentioned based on the shape and size of, oogonium and oospore formation, temperature requirement and pathogenicity tests. The use of forma specialis was considered appropriate according to the International Rules of Botanical Nomenclature (Stafleu and Bonner, 1972). Then sub culturing, purification and multiplication of the above fungi following hyphal tip technique (Tuite, 1969) were carried out on combined PDA and OMA (1:1) media.

Antagonist activity of *Trichoderma* isolates against the *P.dreschlera* f.sp. *cajani*

In this study, the reduction in growth and inhibition zone in the dual culture technique was used as the criteria to evaluate the *in vitro* antagonistic property of ten isolates of *Trichoderma* isolate (AN12, AN16, AN37, AN38,AN18, AN6, AN44, AN7, AN48, AN48, AN33) (Francisco *et al.*, 2011). All antagonist pathogen combinations were examined on PDA agar in 10 cm Petri dishes. For dual cultures, mycelial discs (5 mm dia), taken from actively growing, 5-day-old cultures of *Phytophthora* and *Trichoderma* isolates, were placed at an extreme of a perti dish. The mix culture was incubated at 25±1°C temperature. Control i.e., without placing the disc of the antagonist only pathogen was kept for comparison. Radial mycelial growth was measured when pathogen's mycelial growth covered whole 9cm petri plates. Observations were taken after two, four and seven days of the inoculation date. The following formula was used for calculation the percentage reduction in growth (Moayedi and Ghalamfarsa, 2011), which is,: (R1-R2)/R1 × 100, where R1 and R2 were the mycelial radial growth of the pathogen in the control and in the presence of the antagonist, respectively.

Antagonist activity of *Pseudomonas fluorescens* against the *P.dreschlera* f.sp. *cajani*

To test their antagonist activity of *Pseudomonas fluorescens* by the dual culture method. *P. fluorescens* was streaked at the centre in the petri plate. A 5mm mycelia disc from the old culture of pathogen was placed on the opposite side in the petri plate. Then these plates were incubated at 28± 2°C for 4 days. The growth of pathogen in the test and controlled were observed until the control covered the 80 % of the petri plate.

Effect of *Trichoderma* volatile substances on *P. dreschlera* f.sp. *cajani*

To determine the effects of volatile metabolites, petri dish with PDA was inoculated in the centre with a 5 mm diameter disc taken from pathogen and antagonist (mycelia disc taken from three days old culture) respectively. *Phytophthora* petri dish were inverted, placed above the *Trichoderma* dish and sealed with parafilm (Ting *et al.*, 2010). These Petri dishes were incubated at 25°C. Growth of the pathogen was recorded by measuring the radius after 3 days. Three replicates were prepared for each strain and plates inoculated only with the pathogen were used as control. Colony diameter of the pathogen was measured at 2, 4 and 6 days after inoculation and the inhibition of mycelial growth determined

Effect of *Trichoderma* non-volatile antibiotics on *P. dreschlera* f.sp. *cajani*

The procedure was as in (Perveen and Bokhari, 2012) with modifications. Two mycelial discs (5 mm diam), taken from actively growing, 5-day-old cultures of each *Trichoderma* isolate were placed into 100 ml PDB broth and incubated at 25°C for 7 days. The cultures were filtered through sterile Whatman filter no 1 paper and then through 0.2 µm millipore filters in order to obtain a sterile liquid from each *Trichoderma* isolate.

The required volume of culture filtrate was added with known volume of melted PDA to obtain final concentration of 5%, 10% and 15% (v/v) culture filtrate. The amended media was poured into petri dish. After the agar had solidified, mycelial discs of *Phytophthora dreschlera* f.sp. *cajani* (6 mm diameter), obtained from actively growing colonies were placed in the centre of the agar plates. The Petri dishes were incubated at 28 ± 1°C for 4 days until the control treatment covered the

petri dish .The PDA medium without addition of culture filtrate of antagonist was served as control. After this time, the percentage of *P. dreschlera* f.sp. *cajani* inhibition was determined. The radial mycelial growth of test pathogen was measured and per cent inhibition of mycelial growth of pathogen was calculated as mentioned earlier.

Statistical analysis

All experiments were performed in triplicate, and all statistical analyses were performed using SAS version 8.0 software. Differences in mean values were considered significant when $P < 0.05$.

Results and Discussion

Antagonist activity of *trichoderma* isolates against the *P.dreschlera* f.sp. *cajani*

Differential action of the biocontrol agents was noticed on mycelial growth of the *P. dreschlera* f.sp. *cajani* (Fig. 1a & Fig. 2a). A reduction in the growth of *P. dreschlera* f.sp. *cajani* was evidenced when it was paired with antagonists. Among the ten isolates of *Trichoderma* maximum percentage of inhibition (81.7%) was recorded with AN-33 isolate followed by AN-12(75.6%), AN-38(74.7%), AN-44(68.6%), AN-18(68.6%), AN-48(66.0%), AN-16-(64.3%) and AN-37(63.4%), whereas the isolate AN-6 (60.8%) was recorded with least effective in parasitization of mycelial growth of pathogen.

In the present study was also concluded that *P. fluorescens* maximum inhibition of mycelia growth of pathogen was 43.02%. The result showed that antagonist effect of the antibiotic, chitinase of *Pseudomonas fluorescens* which helps to degrade the fungal cell wall and inhibit the fungus growth under in vitro condition.

Effect of volatile metabolites of *trichoderma* spp. on mycelia growth of *P. drechsleri* f.sp *cajani*

The inhibition of *P. drechsleri* f.sp *cajani* mycelia growth induced by the volatile compounds produced by the *Trichoderma* isolate ranged between 62.6% to 46.9% (Fig.1b & Fig. 2b). The major effect was observed with the *Trichoderma* isolate AN-48 and the least with *Trichoderma* isolate AN-6. It was observed that the ten *Trichoderma* isolate were able to produce volatile compounds with inhibitory properties against *P. drechsleri* f.sp *cajani.*

Effect of *tichoderma* isolate non-volatile substances against *P. drechsleri* f.sp. *cajani*

The results of effect of non-volatile compounds revealed that among the different isolates of *Trichoderma* evaluated against *Phytophthora* (Fig.1c & Fig.2c). In general, maximum inhibition (69.40%) of the mycelial growth of *Phytophthora* was observed with the culture filtrate of *Trichoderma* isolate, AN-33 at 15% concentration. AN-38 (69.40), isolate of *Trichoderma* isolate caused maximum growth inhibition followed by isolate of AN-12 (65.9%), AN-44 (62.3%*)* AN-48 (59.2%), AN-18(58.0%), AN-16 (56.9%), AN-7 (53.7%), AN-37 (49.0%) and least percentage inhibition was observed with the isolate AN-6 by 43.5%. with statistically at par result.

Phytophthora is one of the yield limiting factors of pigeonpea across the country. The advantage of the use of biocontrol includes environmental safety, cost effective. In this studied it found the *Trichoderma* isolates and *Pseudomonas fluorescens* showed the higher percentage of inhibition against *P. dreschlera* f.sp. *cajani.*

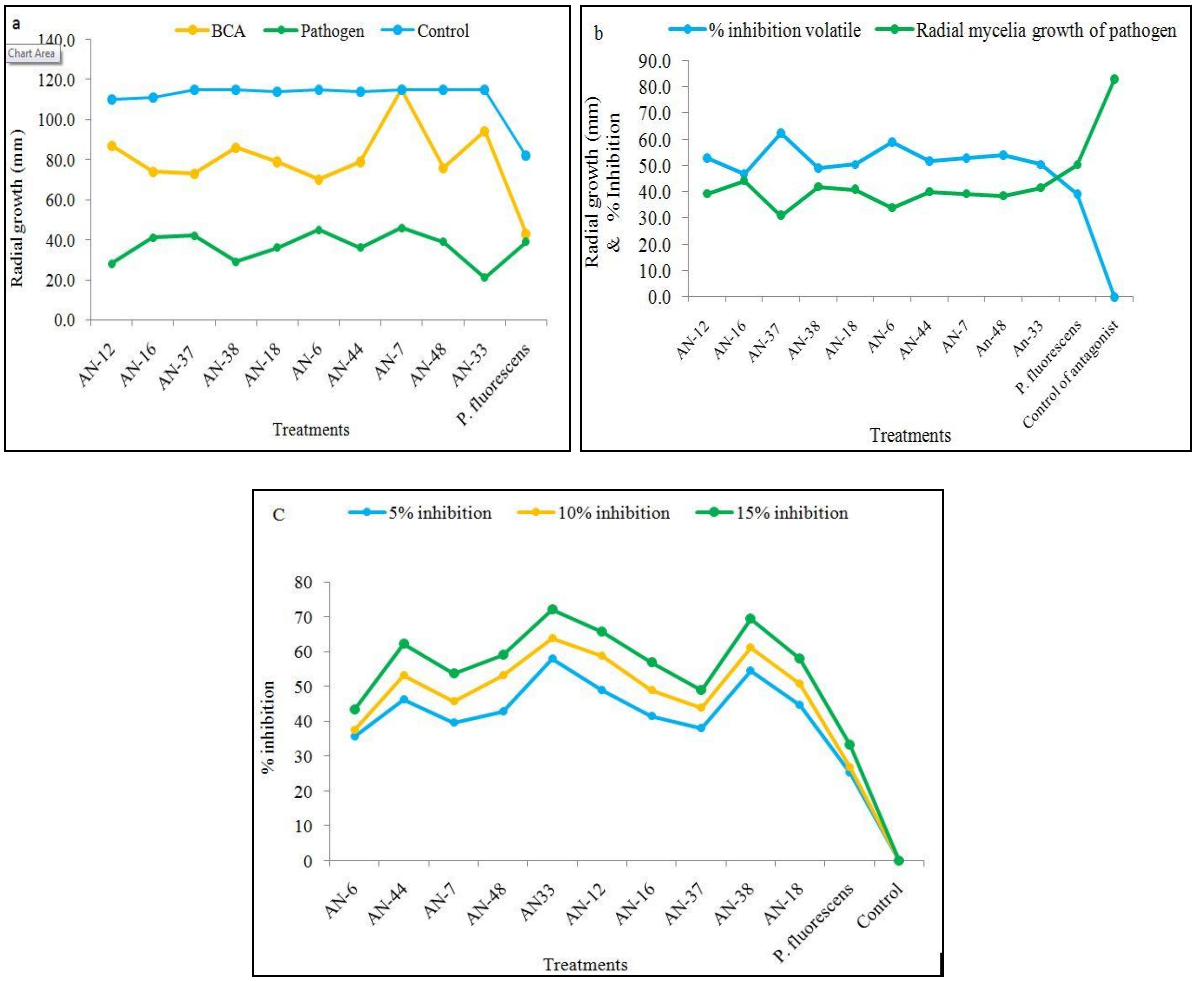

Fig.1 Growth pattern of *P.dreschlera* f.sp. *cajani* and the biological control agents (BCA) in terms of radius of the colony (a) Interaction of biocontrol agents against pathogen – Dual culture method (b) Volatile activity of biocontrol agents against pathogen (c) Non- volatile activity of biocontrol agents against pathogen. All the data is significant *P* value ($P \leq 0.05$)

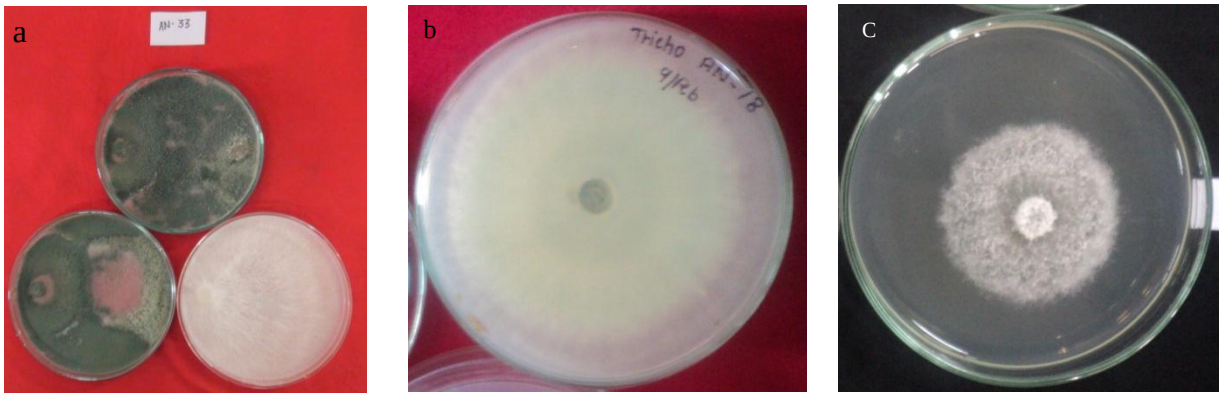

Fig.2 Morphology of antagonism produced by BCA. (a) Antagonist activity due to dual culture technique (b) Effect of volatile metabolites (c) Non-volatile substance produces (15% concentration)

The inhibition percentages of these isolates are differing from each other due to their antagonist activity by differential secretion of antifungal substance. The highest growth is showed by *Trichoderma* isolate AN 33 in dual culture due to direct contact of mycelia hyphae lysed and *P. fluorescens* was also effective as an antagonist against *P.dreschlera* f.sp. *cajani* under dual culture test (Paul and Sarma, 2005) studied. The antagonistic effects of metabolites of *P. fluorescens* strain on the different growth phases of *Phytophthora capsici*, foot rot pathogen of black pepper (Barnett and Binder, 1973) and (Elad *et al.,* 1983) who observed inhibitions is and parasitism by *Trichoderma* spp. of some species of *Phytophthora. dreschlera* f.sp. *cajani.* But in volatile or non-volatile these isolates produces various toxic and antibiotics metabolites (Claydon *et al.,* 1987; Dennis and Webster, 1971a & b; Lorito, 1994) and enzymes (Lorito, 1993) which are involved in the inhibition and lysis of pathogenic fungi.

In this studied showed that *Trichoderma* isolate AN-48 produced highly volatile metabolites that were inhibitory effect on *P. dreschlera* f.sp. *cajani.* Non-volatile metabolites also produced by AN-33 of our *Trichoderma* isolates inhibited the growth of the plant pathogens tested. *In vitro* and *in vivo* studies of the behavior of *Phytophthora* spp. in the presence of antagonistic fungi such as *T. harzianum, Gliocladium* spp. (Smith *et al.,* 1990) or *Pythium nunn* and *Penicillium funiculosum* (Fang and Tsao., 1995) have shown the biocontrol capacity of these fungi on *Phytophthora* spp.

To test the *in vitro* screening showed that bio-antagonists effective against soil borne pathogens is a simplistic and economic approach to control the diseases. Hence, work is needed towards a better understanding and development of technology that allow the biocontrol agent to spread and proliferate in soil. However, (Papavizas, 1985) research indicated the improvement of strains of biological agents that are more capable of becoming established and surviving under adverse field conditions. Thus, it is obvious that biological control offers, durable environmentally safe and cost effective alternative to chemical for the efficient management of plant disease.

The present studies exhibit ten isolates of *Trichoderma* and *Pseudomonas fluorescens* are produce antifungal metabolites against the *P. dreschlera* f.sp. *cajani.* This showed a real alternative to chemical application. This research focused on the antagonistic property of ten isolates of *Trichoderma* and *P. fluorescens* against *P. dreschlera* f.sp. *cajani.* All the isolates of the fungal antagonist caused significant reduction in the mycelium growth of pathogen *in vitro,* in all the three experiment. In the dual culture experiment, *Trichoderma* islolate AN-33 exhibited the maximum percent inhibition (81.7) to the mycelia growth of pathogen. The effectiveness of *P. fluorescnes* on *P. dreschlera* f.sp. *cajani.* was evaluated by conducting dual culture test and reduced the mycelia growth of the pathogen. This has resulted in a good understanding to direct contact of bioagents with the pathogen and effect of metabolites in suppression of the pathogen. These biocontrol are not only beneficial for controlling a disease but also it enhances the plant growth. Biocontrol is very effective management for disease management. The antimicrobial activity of *Trichoderma* and *P. fluorescens* are very effective for controlling the disease.

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