International Journal of Pharmacy and Biological Sciences
ISSN: 2321-3272 (Print), ISSN: 2230-7605 (Online)
IJPBS | Volume 9 | Special Issue 1- NCBTIL | 2019 | 51-58
National Conference on Biochemistry –Transcending and Integrating Life Sciences (NCBTIL) -2019
Held @ Kristu Jayanti College, K Narayanapura, Kothanur PO, Bengaluru-560077, January 17th & 18th
| Conference Proceedings | Research Article | Biological Sciences |Open Access |MCI Approved|
|UGC Approved Journal|

Antagonistic Effect of *Aspergillus* and *Penicllium* Against Wilt Disease of Tomato by *Fusarium Oxysporum*

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Received: 10 Dec 2018 / Accepted: 30 Dec 2018 / Published online: 10 Jan 2019
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Abstract

Fusarium oxysporum f. sp. *Lycopersici* is a known pathogen of tomato plant which is an economically important crop. Tomato yield is significantly reduced by *F. oxysporum* f. sp. l*ycopersici* because it can destroy roots of tomatoes at growth stages. Biological control of *Fusarium oxysporum* f. sp. *lycopersici* (FOL) causing wilt disease of tomato was studied in vitro as well as under pot conditions. Dual culture technique showed tha*t Aspergillus* and *Penicillium,* inhibited the radial colony growth of the test pathogen. Maximum seed germination was observed in seeds treated with the biocontrol agents. Under pot conditions, the plant height, fresh and dry weight of plants was found to be increased significantly in all treatments with antagonists' fungi. Maximum control of wilt disease was observed with *Aspergillus* treated plants as compared to FOL inoculated plants.

Key words
Bio control, Wilt disease, Tomato, Antagonists, *Aspergillus, Penicillium*

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INTRODUCTION

Fusarium oxysporum f. sp. *lycopersici* (FOL) is a known pathogen of tomato plant which is an economically important crop (Suárez-Estrella *et al*., 2007). Tomato yield is significantly reduced by *F. oxysporum* f. sp. *lycopersici* because it can destroy roots of tomatoes at growth stages. Numerous strategies have been proposed to control this fungal pathogen (Biondi *et al*., 2004; Ahmed, 2011). Currently, the most effective method in preventing tomato from fusarium wilt is mixing of tomato seeds with chemical fungicides. However, the use of chemical fungicides can be harmful to other living organisms besides reduction of soil microorganisms (Lewis *et al.,* 1996). Therefore, public concern is focused on alternative methods of pest control, which can play a role in integrated pest

management systems to reduce our dependence on chemical pesticides (Sutton, 1996).

Fusarium wilt is a common vascular wilt fungal disease, exhibiting symptoms similar to Venticillium wilt. The pathogen that causes Fusarium wilt is *Fusarium oxysporum*, the species is further divided into forma specialis based on host plant.The fungal pathogen *Fusarium oxysporum* affects a wide variety of hosts of any age. Tomato, tobacco, legumes, cucurbits, sweet potatoes and banana are a few of the most susceptible plants, but it will also infect other herbaceous plants. *Fusarium oxysporum* generally produces symptoms such as wilting, chlorosis, necrosis, premature leaf drop, browning of the vascular system, stunting, and damping-off (Snyder and Hanson, 1940). The most important of these is vascular wilt. Fusarium wilt starts out looking like vein clearing on the younger leaves and drooping of the older lower leaves, followed by stunting of the plant, yellowing of the lower leaves, defoliation, marginal necrosis and death of the plant. On older plants, symptoms are more distinct between the blossoming and fruit maturation stages.

Fusarium oxysporum is split into divisions called formae speciales (singular forma specialis, abbreviated f.sp.). There are over 100 formae speciales divisions, each with one or two different races. Each forma specialis within the species are host-specific (i.e. specific to a certain plant). Fusarium *oxysporum f. sp. lycopersici* causes vascular wilt in tomato. The disease starts out as yellowing and drooping on one side of the plant. Leaf wilting, plant stunting, browning of the vascular system, leaf death, and lack of fruit production also occur.

One control method is to improve soil conditions because *F.oxysporum* spreads faster through soils that have high moisture and bad drainage. Other control methods include planting resistant varieties, removing infected plant tissue to prevent overwintering of the disease, using soil and systemic fungicides to eradicate the disease from the soil, flood fallowing, and using clean seeds each year. Applying fungicides depends on the field environment. It is difficult to find a biological control method because research in a greenhouse can have different effects than testing in the field. The best control method found for *F. oxysporum* is planting resistant varieties, although not all have been bred for every forma specialis. Antagonism is hostility that results in active resistance, opposition, or contentiousness. Antagonism refers to the action of any organism that suppresses or interfere the normal growth and activity of a plant pathogen, such as the main parts of bacteria or fungi. An antagonist is an organism which has inhibitory relationship with other organism. Many studies revealed that the antagonistic activity has often been associated with production of secondary metabolites (Haggag and Mohamed, 2007; Larkin and Fravel, 1998). Thus proving that there are variety biological control agents to control Fusarium wilt pathogen such as *Trichoderma harzianum*, *Pythium oligandrum*, *Achromobacter xylosoxydans,Penicillium oxalicum* and non-pathogenic *F. oxysporum*.

MATERIALS AND METHODS

Isolation of *Fusarium oxysporum* and pathogenicity testing

Tomato seeds were treated with 0.1% Sodium Hypochlorite solution for surface sterilization. The seeds were plated onto wet blotter disc (ISTA, 2003). The plates were incubated for 7 days at 22°C and varying dark and light conditions. After incubation, fungi developed on each seed were examined and identified. The identification was based on the characteristic growth of the fungus on seeds and the morphological characters of conidia. Pure cultures were maintained on Potato Dextrose Agar (PDA) slants at 4°C. Under greenhouse conditions the pathogenicity test of the isolated strain was conducted (Asha *et al*., 2011).

Isolation and maintenance of fungal native antagonists from tomato rhizosphere soil

Rhizosphere soil from different locations were collected and identified for*Aspergillus* and *Penicillium* antagonists by serial dilution technique.The isolate was maintained in the pure form in PDA slants and stored at 4°C (Elad and Chet, 1983).

Antagonism studies

Antagonistic studies of*Aspergillus* and *Penicillium* isolates were screened against the wilt pathogen *Fusarium oxysporum*by employing the dual culture technique. The interaction was studied on a petri plate containing sterile PDA. The isolates were inoculated, and the plates were incubated for 2 days at 15°C. A 5mm diameter agar disc of *F.oxysporum*from a week old culture was placed on to the centre of the plate.

After a week of incubation at 28°C, the zone of inhibition was measured for each isolate and the inhibition was calculated according to the formula I=100-(100 R2/R1) Where 'I' is the degree inhibition of vegetative growth of the fungi, R1 is the radius of the control colony in mm, and R2 is the distance travelled by the *F.oxysporum* colony (Ahmed, 1999). The experiment was conducted in triplicate and was repeated twice.

Dual culture test

The antagonistic fungi *Aspergillus* and *Penicillium* were tested to inhibit the growth of *F. oxysporum f. sp. lycopersici* by using dual culture test (Soytong,1992). The experiment was designed in completely randomized design (CRD) with four trials. Pathogenic *F. oxysporum* was used and the tested dual culture plates were incubated at room temperature (28°C). The data were collected as colony diameter and conidial number of pathogenic fungus. The colony diameter and conidia of pathogen were measured and calculated using the following formula: % Inhibition = [(colony diameter or conidial number of pathogens in control – colony diameter or conidial number of pathogens in dual culture plate) colony diameter or conidial number of pathogens in control)] x 100. The experiment was repeated two times.

Extracts of Antagonistic Fungi's Activity

Using Hexane, ethyl acetate and methanol as solvents, crude extracts from *Aspergillus* and *Penicillium* were obtained. After extraction the experiment was carried out in rotary evaporator to remove the solvents from the extracts. The crude extracts were then tested for inhibitory activity against the growth of plant pathogens on PDA at concentrations of 0, 10, 50, 100, 500 and 1000 µg/ml. The experiment was Completely Randomized Design with four replications. Data were collected as colony diameter (cm) and spore production. The statistical analysis of variance (ANOVA) was determined using the Duncan's multiple range test (DMRT) at P=0.01 and effective dose of ED_{50} (Kanokmedhakul *et al*., 2006).

Cellulase activity assay

The antagonistic fungi were grown on yeast extract peptone agar medium (yeast extract 0.1 g, peptone 0.5 g, agar 16 g, congo red 0.2%, distilled water1000 ml) supplemented with 0.5% Na-carboxymethyl cellulose (CMC). The clear zone surrounding the colonies (cm) which indicated the cellulolytic activity was observed at 5 days. The experiment was done by using Completely Randomized Design with four replications (Ghose, 1987).

Hemi cellulase (xylanase) activity assay

The antagonistic fungi were cultured on xylan agar medium (xylan 1 g, rice bran 5 g, yeast extract 1 g, agar 16 g, distilled water 1000 ml). After incubation, xylan utilization on the medium was observed for clear zone. Dilute iodine solution was used to stain the agar plates and a yellow-opaque area around colonies indicated the xylan degradation while the reddish-purple colour indicated for the undegraded xylan (Bailey *et al.,* 1992). The experiment was done by using Completely Randomized Design The experiment was further conducted to find the hemi cellulose activity by growing the antagonistic fungi in liquid medium consist of KH_2PO_4 1 g,K_2HPO_4 0.4 g, $MgSO_4.7H_2O$ 0.5 g, $CaCl_2.2H_2O$ 0.013 g, L-asparagine 1.5 g,NH4NO3 0.5 g and rice bran 5 g, 1 ml of trace element solution ($ZnSO_4.7H_2O$0.0264 g, $MnCl_2.4H_2O$ 0.02 g, $CaCl_2.4H_2O$ 0.004 g, $CuSO_4.5H_2O$ 0.4 g, distilled water 1000 ml), at pH 7. The 0.3 cm diameter plug of fungal colony was transferred into the flask of liquid medium, then incubated for 9 days at room temperature (28°C) under static condition. Hemi cellulase activity was analysed at 3, 6- and 9-day intervals by using the method of Nelson (1944) and Somogyi (1952). The rate of release of reducing sugars was determined by using xylan as substrate and D-xylose as standard. Reducing sugars was determined using a spectrophotometer at 540 nm. One unit of enzyme was defined as the amount of enzyme releasing 1 µ mole of glucose equivalent one minute (min^{-1}).

Determination of Seed Germination

Steam sterilized sand was inoculated with *F. oxysporum* before the seeds were sown. Surface sterilized (0.1% sodium hypochlorite) tomato seeds were sown in each pot. Inoculum of *Penicillium*, *Aspergillus*, was prepared in the form of a conidial suspension. Tomato seedlings were raised with one of the antagonist organisms. Seedlings without any inoculation served as the control. Plants were watered regularly and after few days the seed germination was observed. Pots were surface sterilized with 1% sodium hypochlorite and were filled with autoclaved soil. Sand was mixed in the ratio of 5:1. The Mycelial mat of *F. oxysporum* grown on PDA was scrapped (5g/kg) and mixed in sterilized distilled water and mixed with

sterilized sand added to the pots. The pure inoculum of *Apergillus*, *Penicillium*, was mixed with infected soils. Pots were arranged in the lab on rack and watered with sterile water as per the requirement (Perveen *et al.,*2007). Height, fresh weight and dry weight were recorded. 25 ml Hoagland's solution was added to each pot once a week. The percentage of seeds emerging up to 45 days after sowing, were recorded. One more set of treated and control seeds was sown in pots.

RESULTS AND DISCUSSION

Fusarium oxysporum was isolated from infected seeds which showed 78% disease incidence on tomato plants. A pure culture of *F.oxysporum* was maintained on PDA slants at 4°C.Two important biocontrol agents *Aspergillus* and *Penicillium* were screened against *F.oxysporum.* A maximum zone of inhibition 2.8cm was measured for *Penicillium*. Similarly, 2.6cm was measured for *Aspergillus* (Figure- 1& 2) These two fungal strains were found to be effective in reducing the tomato wilt under greenhouse conditions when applied as a seed treatment. This may have been due to their ability to inhibit the growth of *F.oxysporum* in the rhizosphere, which we demonstrated in our *in vitro* antagonism assay. The fresh culture significantly increased seed germination such plant growth promotion activities of *Aspergillus* and *Penicillium.* In plants, induced resistance is the resistance that develops after pre-inoculation of plants with various biological agents or after pre-treatment with various chemical or physical agents (Rabie, 1998). Induction of systemic resistance is suggested as the mechanism of disease suppression (Chandanie *et al*., 2006). The fresh cultures of the biocontrol agent were found to be best in reducing fusarium wilt under greenhouse conditions. The fresh cultures contain fungal mycelium are in the active state and, after application they immediately colonize the emerging root. However, in the case of formulations, the growth of biocontrol agents is negligible; it should be stimulated by root exudates (Aziz *et al.,* 1997) The study on the Mycelial and conidial inhibition of antagonistic fungi (*Aspergillus* and *Penicillium*) on *Fusarium oxysporum* were carried out isolate in dual culture test. After 30 days. The results were effective (Table-1), with *Aspergillus* conidial inhibition with 92.34% and with *Penicillium* 92.52%. Whereas the mycelial inhibition with *Aspergillus* was 78.14% when compared to *Penicillium, it* was 88.09%.

Table- 1: Mycelial and conidial inhibition of antagonistic fungi (*Aspergillus* and *Penicillium*) on *Fusarium oxysporum* isolate in dual culture test after 30 days.

Antagonistic fungi used	Mycelial inhibition (%)	Conidial inhibition (%)
Aspergillus	78.14± 0.12	92.34± 0.01
Penicillium	88.09± 0.22	92.52± 0.12

Average of four trials,the values are not significantly different according to Duncan's multiple range test at P = 0.05.

Table-2: Spore production of *Fusarium oxysporum* crude extracts from antagonistic fungi. Number macroconidia of *F. oxysporum* (x 10 ^{6} spore/ml) at each concentration (µg/ml)

Antagonistic fungal

Solvent extracts	**0**	**10**	**50**	**100**	**500**	**1000**	**ED_{50}**
Penicillium Hexane	10.89	10.28	10.34	7.41	4.62	4.13	209
Penicillium Ethyl Acetate	10.95	13.10	10.47	8.00	7.47	3.78	212
Penicillium Methanol	13.26	10.75	8.35	7.22	6.14	5.87	232
Aspergillus Hexane	11.39	5.93	4.82	2.41	1.76	1.72	157
Aspergillus Ethyl Acetate	9.49	8.26	6.83	4.18	3.14	1.69	188
Aspergillus Methanol	10.26	4.67	4.43	1.87	1.48	1.48	192

Statistically not significant, different as determined by Duncan multiple range test at *p* = 0.01. ED_{50}(µg/ml)

The Spore production of *Fusarium oxysporum* crude extracts from antagonistic fungi (Table- 2). Three solvents were used for the study, Hexane, ethyl acetate and Methanol with different concentrations (0, 10, 50, 100, 500 & 1000µg/ml). The effect was analyzed based on the number of Macroconidia

produced (x 10 ^{6} spore/ml) at each concentration. Among them all the three extracts of Aspergillus proved to be effective when compared to *Penicillium* against the pathogen in 1000 µg/ml concentration. The ED_{50} for the pathogen was calculated based on the inhibition and it was clearly evident that *Aspergillus* extract especially hexane extract is more effective than any other (157 µg/ml).

Fungal extracts showed effective inhibition of Fusarium wilt pathogen. These tested antagonistic fungi showed antibiotic mechanism to inhibit growth of Fusarium wilt pathogen which was supported by Soytong (1992) who stated that antagonistic substance from fungi. Could inhibit growth and also break the cells of *F. oxysporum* f.sp. *lycopersici*. Moreover, Park *et al.,* (2005) stated that liquid culture of certain fungus could suppress the development of disease more than 80% and can exhibit anti-fungal activity against *Phytophthora infestans* in tomato at moderate level.

The crude extracts further elucidated that the chemical structures that may play an important role of mechanism of biocontrol in term of antibiosis. Suwan *et al*., (2000) stated that certain group of saprophytic forms produces peptibols that could inhibit plant pathogen and also promote plant growth. The current study suggests that possible control mechanism of those promising antagonists in term of inhibition of growth, enzymes that may release from those antagonists. Enzyme activity assays of antagonistic fungi on agar medium plates were carried out. *Aspergillus* showed remarkably a great enzymatic activity such as cellulase and xylanase. *Penicillium* was found to be showing lesser activity (Table -3). Results indicate that cellulase and hemi cellulase produced by both antagonistic fungi tested. *Aspergillus* and *Penicillium* was seen the highest diameter of clear zone (cm) on cellulase activity (Table 3). From the results, it was observed that both strains had the highest diameter of clear zone (cm) on cellulose activity that was showed the yellow-opaque area around colonies indicated highest xylan degradation. As the report of Kucuk and Kivanc (2002) stated fungal isolates from soil could inhibit *F.oxysporum* and produced chitinase to degrade cell wall of tested plant pathogen. It is suggested that biological control of plant pathogens may play an important role of antibiosis and lysis. *A. niger, P. citrinum, T. harzianum* have almost similar effect on wilt severity and increase in plant growth Whipps and Mc Quilken in 1993 concluded that *A. niger,A. terreus, G. virens, P. citrinum, T. harzianum* and species of *Bacillus* control soil-borne diseases.) observed that *A. flavus, A. niger,* and *T.viride* amended in soil suppressed the growth of *F.oxysporum* f. sp. *ciceri*(Bashar and Rai, 1994) and exhibited strong fungistatic activity against germination of conidia of test pathogen. The fungal mat *Aspergillus* and *Penicillium* significantly reduced disease incidence up to 20-30%. The highest plant height (15.5cm) was resulted from seeds treated with *Aspergillus* (Table-4). Seed treatment with *Penicillium* as well significantly increased plant height, fresh weight and dry weight over control. Vidyasekaran *et al.,* (1997) reported the use of *P.fluorescencens* strains that inhibited mycelial growth of *Fusarium*, successfully through seed treatment.

Table-3: Enzyme activity assays of antagonistic fungi on agar medium plates

Antagonistic Strains used	Cellulase activity	Xylanase activity	Cellulase (diameter of clear zone; cm in 5 days)
Aspergillus	2.28±0.14	2.83± 0.22	4.03± 0.12*
Penicillium	2.11±0.12	2.74 ± 0.22	4.16 ± 0.02*

***Enzyme Activity is expressed in units;**
1 unit = 1 nmol substrate utilized/ mg of protein /min.
Values are mean ± SD of triplicates.
Statistically significant (P<0.05)
***not significantly different as determined by Duncan multiple range test at p = 0.01**

Table- 4: Determination of Seed Germination

Treatment	No. of days of seed germination	No. of seed germinated	Plant Height (cm)	Plant fresh weight (g)	Plant dry weight (g)	Disease incidence (%)
Control	2	5	16± 0.10	0.60± 0.04	0.06± 0.10	0.00
Seed + *Fusarium*	11	1	9.8± 0.15	0.10± 0.02	0.01± 0.15	78 ± 0.01
Seed + *Aspergillus*	4	4	15.5± 0.17	0.23± 0.02	0.05± 0.10	48 ± 0.01
Seed + *Penicillium*	4	3	11.9± 0.15	0.15± 0.05	0.02± 0.12	53 ± 0.01

Values are average of three observations ± SD
Statistically significant (P<0.05) compared to untreated seeds

Figure-1: Antagonistic activity of *Aspergillus* against *Fusarium*

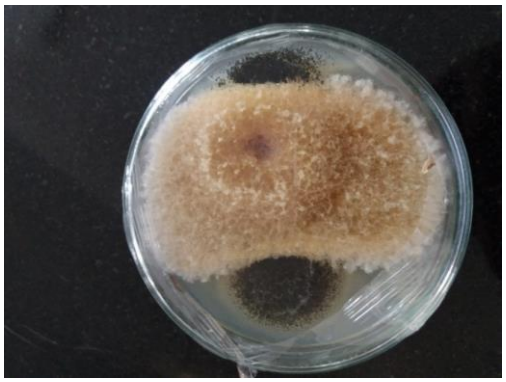
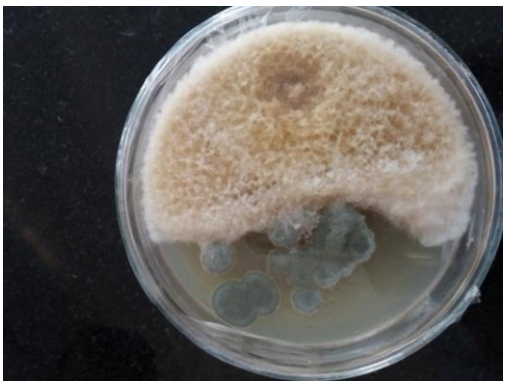


Figure-2: Antagonistic activity of *Penicillium* against *Fusarium*



Bacterial and Fungal control agents can be recommended to the farmers as one of the crop protection strategies for the management of Fusarium wilt of tomato and this practice. Mondal *et al,* (2000) reported that two metabolites isolated from *A. niger*, had increased germination, shoot length, root length and biomass of cauliflower seedlings. It is evident that the plant's height and their fresh and dry weight have increased in all the treated plants. All the treatment showed significant increase in plant height as compared to *F. oxysporum* inoculated control. Maximum plant height, fresh and dry weight was observed in plants treated with biocontrol agents. Dry weight of plants treated was increased as compared to control. Under pot condition, maximum control of wilt disease was observed with both the fungal strains. These tested antagonistic fungi showed antibiotic mechanism to inhibit growth of Fusarium wilt pathogen. Other studies proved that cyanobacteria also control the plant pathogenic fungi, particularly soil borne disease (Kim and Kim, 2008). Recent developments in commercialization of biological control products have accelerated the approach of fungal antagonists (Fravel *et al.,* 2003). Many studies

evaluate the advantage of fungi and their associations with wilt pathogen. Always it had a positive effect on yield of tomato and against wilt. These fungal inoculants likely have biofertilizer effects in terms of disease control, plant growth and enzymatic activity

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