International Journal of Botany Studies
ISSN: 2455-541X
Impact Factor: RJIF 5.12
www.botanyjournals.com
Volume 2; Issue 6; November 2017; Page No. 170-175

Screening of biocontrol agent against tomato wilt disease

A Pauline Fatima Mary, R Sagaya Giri
Department of Botany, Kunthavai Naacchiyaar Govt. Arts College (W), Autonomous, Thanjavur, Tamil Nadu, India

Abstract
Tomato is one of the most widely grown vegetable crops across the globe. It is an important source of minerals, vitamins, essential amino acids, sugars and dietary fibers. During the cultivation, tomato crop is susceptible to various kinds of disease and disorders. Among which wilt disease is one of the most severe disease on tomato crop resulting up to 90% yield loss. *Fusarium oxysporum* f. sp. *lycoperscisi* (Sacc.) is a causative organism of *Fusarium* wilt. This pathogen was isolated from infected tomato leaves and also identified by using standard manuals. In This present study, Eight Rhizosphere soil samples of different tomato crop fields from four districts of Tamilnadu were investigated to study the diversity of fungi. Antagonistic activity of some soil fungi against *F. oxysporum* were studied in *in vitro* dual culture technique. A total of 17 species belonging to 7 genera of fungi were isolated and characterized from eight different soil samples such as *Aspergillus, Penicillium and Mucor, Curvularia, Rhizopus* and *Trichoderma.* In the antagonistic activity, the species of *Trichoderma viride* and *Aspergillus flavus* showed maximum inhibition against the pathogen *F. oxysporum* which cause wilt disease.

Keywords: tomato, fungi diversity, antagonistic activity, *Fusarium oxysporum*

Introduction

Diversity of soil fungi

Soils are highly complex systems, with many components playing diverse functions mainly due to the activity of soil organisms. Soil microflora plays a pivotal role in evaluation of soil conditions and in stimulating plant growth (Kiran Singh *et al.*, 1999) [9]. Microorganisms are beneficial in increasing the soil fertility and plant growth as they are involved in several biochemical transformation and mineralization activities in soils. Type of cultivation and crop management practice found to have greater influence on the activity of soil microflora (Gill *et al.*, 1980) [6]. The rhizosphere is the narrow region of soil that is directly influenced by root secretions and associated soil microorganisms. The rhizosphere micro-organisms predominantly help in metabolizing the root exudates. Microorganisms in the Rhizosphere complete both chemical and physical modifications to the soil profile in and around the rhizosphere that affect plants. They can be beneficial to the plant (by pathogen suppression) or detrimental (by competition for nutrients) (Sylvia *et al*., 2005) [20].

Fungi are an important component of the soil microbiota typically constituting more of the soil biomass than bacteria, depending on soil depth and nutrient conditions (Ainsworth and Bishy, 1995) [1]. The role of fungi in the soil is an extremely complex one and it is fundamental to the soil ecosystem. They perform ecological services that strongly impact the quality of human life and have enormous potential for providing economic benefits. Microfungi play a focal role in nutrient cycling by regulating soil biological activity (Arunachalam *et al.,* 1997) [2]. The quantities of organic and inorganic materials present in the soil have a direct effect on the fungal population of the soil. The members and kinds of microorganisms present in soil depend on many environmental factors such as the amount and type of nutrients, moisture, aeration, pH and temperature etc. The aim of the present investigation is to isolate mycoflora from different tomato fields of four districts in Tamilnadu.

Antagonistic activity against Tomato wilt's Pathogen

Tomato (*Lycopersicon esculentum* Mill.) is one of the most widely grown vegetable crops across the globe. It is an important cash crop for small and medium scale commercial farmers. Tomatoes contribute to a healthy, well balanced diet. They are rich in minerals, vitamins, essential amino acids, sugars and dietary fibres. Hence, tomato forms one of the widely used vegetable but during the cultivation, tomato crop is susceptible to various kinds of disease and disorders, among which *Fusarium* wilt is reported to cause one of the most severe disease on tomato resulting in complete damage of crop resulting in yield loss. *Fusarium oxysporum* f. sp. *lycopersici* (Sacc.) W.C. Snyder and H.N. Hansen is the causative organisms of *Fusarium* wilt disease (Snyder and Hansen, 1940) [18]. *Fusarium* spp., causes up to 100 % yield loss in the tomato worldwide (Santos *et al.*, 2002) [15].

Use of pesticides and other commercially available fungicides have shown a ray of hope on improving the crop yield but at the same time, the large use of these pesticides and fungicides are bound with various limitations such as loss of soil fertility, contamination of both ground and surface water, biomagnifications, health hazards etc., which are reported to have deleterious effects on health of all living organisms of the biosphere. Therefore, alternative strategies are being widely employed. One such practice is use of bio-control agents. Research on bio-control agents have expanded in recent past as ecofriendly management of targeted crops. In the present study to evaluate the antagonistic activity of seven soil fungi against *Fusarium oxysporum* f. sp. *lycopersici,* the causal organism of wilt disease in tomato.

Materials and Methods
Diversity of soil fungi
Collection of soil samples
Rhizosphere soil samples were collected from different tomato field of various Districts in Tamil Nadu (S1, S2, S3, S4, S5, S6, S7 and S8) Table-1. In each field 1kg of soil sample was collected from the surface area reaching about 10 – 15 cm depth and near the rhizosphere region of plants. Soils were collected in sterile polythene bags and sealed on the spot. Samples were stored in laboratory at 4ºC until further analysis.

Isolation of Mycoflora
Dilution plate technique described by Warcup (1951) [22] was used for the isolation of fungi from various rhizosphere soil samples. 10 grams of soil samples were suspended in 90 ml of distilled water. The flasks were shaken thoroughly in order to get uniform distribution of the soil particles. The soil suspensions were diluted in 10 fold increment from 10^{-2} to 10^{-4}. The Volume of 10 ml of soil sample suspension from each serial dilution was pipetted onto different melted, cooled culture media Potato Dextrose Agar (PDA) supplemented with 1% Streptomycin. The pH of the culture media was maintained at 5.5 being optimal for the growth and sporulation in a majority of fungi. Each culture media was prepared in a liter of distilled water and autoclaved at 120ºC at 15 psi for 20 min. 1% Streptomycin was used as an antibiotic for the restrain of bacterial growth. Each colony was sub cultured and maintained on potato dextrose agar slants. The inoculated plates were incubated at room temperature 28±2°C in an inverted position for 5-7 days. Three replicates were maintained for each sample**.**

Data Analysis
Population density is expressed in terms of Colony Forming Unit (CFU) per gram of soil with dilution factors. The percentage contribution of each isolate was calculated by using the following formula (Gaddeyya *et al.,* (2012) [5].
Total No. of CFC* of an

$$\% \text{ contribution} = \frac{\text{Individual species}}{\text{Total No. of CFU* of all Species}} \times 100$$

Antagonistic activity against Tomato wilt's pathogen
Isolation of the *Fusarium* wilt's pathogen from the infected leaf samples.
The infected leaves of *Solanum lycopersicum* leaves were collected (Figure 1) and infected parts were cut into small pieces with the help of sterilized knife. They were washed with distilled water. Then they were surface sterilized with 0.1% mercuric chloride solution by keeping the pieces of leaves in that solution for about 30 to 40 seconds. After surface sterilization the pieces took out carefully and washed atleast 6-7 changes of sterilized distilled water. Then after the bits were blotted to dry. Finally it transferred on to the solidified agar surface of plates. The respective plates were incubated at 28±2^{0}C for 5-7 days.

Identification of *Fusarium* wilt's pathogen
After five days of inoculation period, the organism that grows for the infected specimen over PDA medium. The pure culture of the pathogen were maintained in Potato Dextrose Agar medium by streaking method and stored for further use. The organism was observed under microscope by using Lacto Phenol Cotton Blue Staining technique. The pathogenic fungi were identified by using standard manuals such as Manual of soil fungi (Gillman, 1957) [7].

In vitro antagonistic activity of soil fungus against *Fusarium oxysporum*
The antagonistic activity of various soil fungi against the test pathogen were studied with dual culture plate technique (Morton and Strouble 1955) [11] under *in vitro* condition. The test pathogen *Fusarium oxysporum* f. sp. *lycopersici* and the soil fungi viz., *Aspergillus terreus, A. nige, A. flavus, A. fumigatus, Penicillium notatum, P. chrysogenum and Trichoderma viride* were grown separately on PDA medium. The agar block cut from actively growing margin of individual species of soil fungi and test organism were inoculated opposite to each other approximately 3 cm apart on potato dextrose agar medium in petriplates. Three replicates were maintained for each set. Controls were set in single and dual inoculated cultures at the fungus. The position of the colony margin on the back of the disc was recorded daily. The assessment was made for both organisms. The percentage of inhibition of growth was calculated as follows.

$$\text{Percentage of inhibition of growth} = \frac{r - r'}{r} \text{x}100$$

Where,
r = growth of the fungus from the centre of the colony towards the centre of the plate in the absence of antagonistic fungus.
r ' = growth of the fungus from the centre of the colony towards the antagonistic fungus.
The colony interaction between test pathogen and soil fungi was proposed methods (Skidmore and Dickinson 1976).

Results and Discussion
Diversity of soil fungi
The Present investigation was conducted to find out the fungal diversity in eight different tomato fields. In this study, 181 fungal colonies of 17 fungal species belonging to 7 genera were isolated and identified (Table 2). The maximum fungal species belonging to phyla Ascomycota (160 colonies) followed by Zygomycota (21 colonies) were observed. Seventeen fungal isolates were identified as, *Aspergillus flavus, A. fumigatus, A. niger, A. sydowii, A. terreus, A. versicolor, A. sulphureus, A. candidus, Penicillium chrysogenum, P. citrinum, P. frequestans, P. notatum, Fusarium oxysporum, Mucor sp., Rhizopus stolanifer, Curvularia lunata and Trichoderma viride* based on cultural characters and sporulation structure (Figure 1). The colonies of *Aspergillus and Penicillium* belonging to Ascomycetes were predominant in all soil samples of tomato filed. The

similar and significant report has already been made in all soil samples of crop fields such as sunflower, sesame, capsicum, rice, green gram, sugarcane, ground nut and black gram by Shiny Niharika *et al*., (2013) [16].

Gaddeyya *et al.,* (2012) [5] determined the fungal population and their diversity in agricultural fields of Salur. During the investigation period 173 fungal colonies of 15 fungal species were observed. The maximum fungal species belongs to Deuteromycotina (169 colonies) and Zygomycotina (4 colonies) were observed. Among the isolates the genera *Aspergillus* and *Penicillium* were dominant. Rohilla and Salar (2012) [14] twenty three soil samples were characterized for the incidence of fungal strains from pesticides contaminated agricultural soils. A total of 59 fungal isolates were obtained from the analyses of 23 soil samples taken from pesticide contaminated soils through soil dilution agar plating, Warcups soil plate and Waksman Direct inoculation method.

Antagonistic activity against Tomato wilt's pathogen
Isolation and Identification of *Fusarium* wilt's pathogen

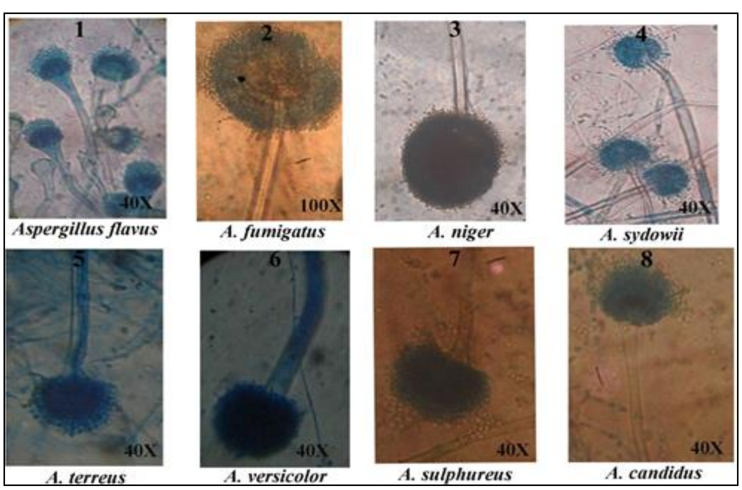
In the present study, the isolated colonies are white in color (Figure-3). The fungus produces three types of spores, microconidia, macroconidia and chlamydospores. Microconidia are borne on simple phialides arising laterally and are abundant, oval ellipsoid, straight to curved, 5-12x2.2-3.5 µm and non septate. Their Macroconidia are fusiform, slightly curved, pointed at the tip, mostly three septate, basal cells pedicellate, 23-54 x 3-4.5 µm (figure-3). Chlamydospores, both smooth and rough walled are abundant and form terminally or on an intercalary basis. They are generally solitary, but occasionally form in pairs or chains. This characteristic pointed to the isolates being of the species *Fusarium oxysporum f. sp. lycopercisi* (Figure 3). Narendra Kumar and Swati Sharma., 2015 [12], Maja Ignjatov *et al.*, 2012 [10], Stevan *et al.*, 2005 [19], reported similar findings. Randall Rowe (1980) [13] isolated *Fusarium oxysporum* from crown and root rot – affected tomato plants in 20 locations in North America and Japan. Vibha and Nidhi (2014) [21], isolated the *Fusarium oxysporum* f. sp. *lycopersici* from wilted tomato plant

Table 1: Soil samples of four different Districts

S. No	Districts	Places
S1	Thanjavur	Arputhapuram
S2		Nagathi
S3	Thiruvarur	Edamalaiyur
S4		Vaduvur
S5	Ariyalur	Allinagaram
S6		Thanrayappan Mettutheru
S7	Pudukkottai	Vennavalkudi
S8		Annai Nagar

Table 2: Occurrence of soil mycoflora in different tomato field from various Districts of Tamilnadu.

Districts	Study Area	Average No. of Individual Colonies																	
		Aspergillus Species								Penicillium Species				F. oxysporum	Mucor sp.	Rhizopus stolanifer	Curvularia lunata	Trichoderma viride	Total no. of colonies
		A. flavus	A. fumigatus	A. niger	A. sydowii	A. terreus	A. versicolor	A. sulphureus	A. candidus	P. chrysogenum	P. citrinum	P. frequestans	P. notatum						
Thanjavur	S1	1	3	5	2	1	2	1	2	2	1	1	1	1	1	2	1	1	28
	S2	2	-	2	1	2	1	2	1	4	2	1	1		1	2	1	-	23
Thiruvarur	S3	1	2	3	1	3	1	-	1	3	1	-	2		4	2	2	1	27
	S4	2	4	3	2	2	2	2	2	2	2	1	1	1	1	-	1	2	30
Ariyalur	S5	2	1	2	1	2	2	2	2	2	-	1	2	1	1	3	-	-	24
	S6	3	2	2	2	1	1	-	-	1	1	-	-		1	-	-	-	14
Pudukkottai	S7	2	1	2	2	3	-	1	1	2	-	1	1		2	-	1	-	19
	S8	-	2	1	1	-	2	1	1	-	1	2	3	1	1	-	-	-	16
Total		13	15	20	12	14	11	9	10	16	8	7	11	4	16	9	6	4	181
% contribution		7.18	8.28	11.04	6.62	7.73	6.07	4.9	5.52	8.83	4.41	3.86	6.07	2.2	6.62	4.9	3.31	2.2	



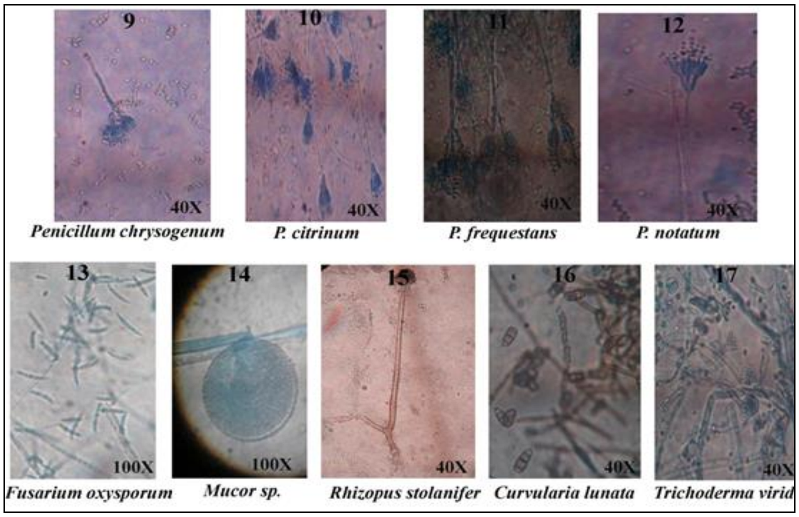


Fig 1: Microscopic view of soil fungi isolated from tomato fields

Fig 2: Infected leaves of Tomato

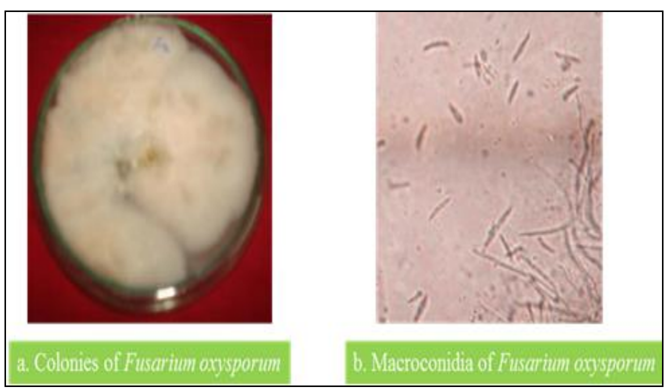


Fig 3: Macroscopic and Microscopic nature of *Fusarium oxysporum*

Table 3: Percentage of Growth inhibition of seven soil fungi against *Fusarium oxysporum f. sp. lycopercisi*

S. No.	Growth response of the antagonist and test fungus	Antagonistic fungi tested						
		A. terreus	A. niger	A. flavus	A. fumigatus	P. notatum	P. chrysogenum	T. viride
1	Colony growth of the pathogen towards antagonist (mm).	20	26	19	20	24	22	17
2	Colony growth of the pathogen away from the antagonist (mm).	19	21	21	21	22	24	23
3	% growth inhibition of the pathogen in the zone of the interaction (mm).	50.8	45.4	62.5	56.4	52.0	58.35	68.88
4	% Colony growth of the antagonist in control ie. Growth towards the centre of plate in the absence of the pathogen (mm).	33	40	40	34	32	33	43
5	Control growth of the antagonist away from the pathogen (mm).	26	19	32	23	25	25	38
6	% growth of inhibition in the zone of interaction.	21.2	52.5	20	32.3	21.8	24.2	11.6

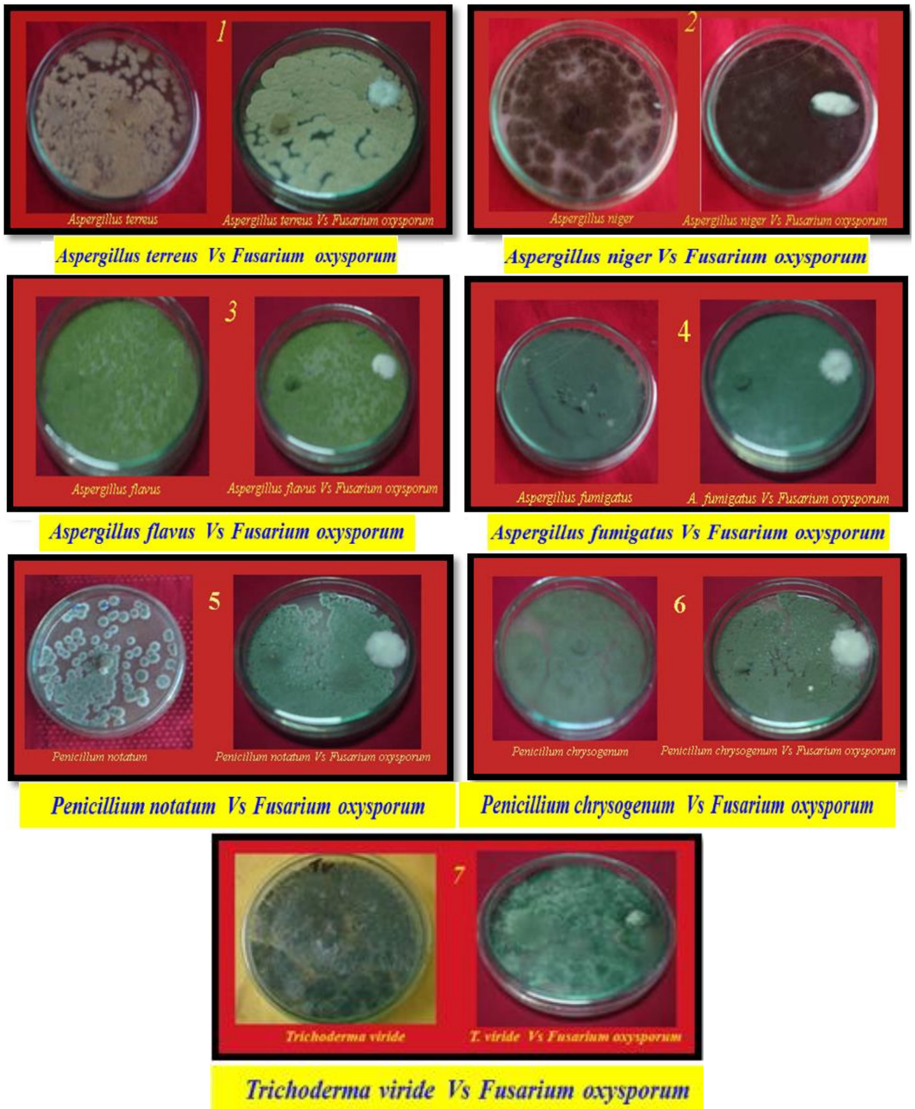


Fig 4

In vitro* antagonistic activity of soil fungus against *Fusarium oxysporum

In the present study, antagonistic activity of *Aspergillus terreus*, *A. niger, A. flavus*, *A. fumigatus*, *Penicillium notatum, P. chrysogenum and Trichoderma viride* against *Fusarium oxysporum* f. sp. *Lycopersici* were studied by *in vitro* dual culture experiment. The species of *Trichoderma* and *A. flavus* showed the ability to inhibit the pathogen. But these species showed variability in the percentage of inhibition (Figure -4). The percentage inhibition of growth of pathogen against *Trichoderma viride*, *Aspergillus flavus and A. fumigatus* were 68.8, 62.5, 58.6 percentage with respectively. Growth inhibition decreased as follows; *T. viride* (68.88%) > *A. flavus* (62.5%) > *P. chrysogenum* (58.35%) > *A. fumigatus* (56.4 %) > *P. notatum* (52.0%) > *A. terreus* (50.8%) > *A. niger* (45.4%) (Table 3).

Gomathi and Ambikapathy 2011 [8], studied the antagonistic activity of the soil fungi viz, *Aspergillus flavus*, *A. fumigatus, A.niger, A.sydowi, A. sulphureus, Penicillium* sp., *T. harzianum*, *T. koeningii and T. viride* against the test pathogen *Pythium debaryanum.* All the species of *Trichoderma* showed the ability to inhibit the pathogen. Ashwani Tapwal *et al.*, (2011) [3] evaluated *Trichoderma viride* against five selected phytopathogens by the dual culture technique. *T. viride* had a marked significantly inhibitory effect on the growth of selected phytopathogens compared to their respective control. Growth inhibition decreased as follows; *F. oxysporum* (67.96%) > *R. solani* (48.67%) > *Curvularia lunata* (44.32%) > *A. solani* (34.76%) > *A. zinniae* (32.44%).

Dwivedi and Enespa (2013) [4] studied the antagonistic activity of the *Trichoderma* spp., (*T. koningii* and *T. viride*), *Penicillium spp*., (*P. italicum*, *P. citrinum*) and *Aspergillus spp*., (*Aspergillus flavus*, *A. niger*, *A. luchuensis*) were isolated from the tomato and brinjal crop field against the pathogenic fungus, *Fusarium oxysporum* f. sp. *lycopersici* and *Fusarium solani* were isolated from the wilt affected tomato and brinjal plants and from soil also. The *Aspergillus spp.* showed best ability to inhibit the pathogens compared to *Trichoderma* and *Penicillium* species. Anil Kumar and RajKumar, (2013) studied the antagonistic activity of totally eight *Trichoderma* isolates viz. *T. harzianum* (2 isolates), *T. viride* (2 isolates), *T. hamatum* (2 isolates) and *T. virens* (2 isolates) against *F.*

oxysporum f. sp. *lycopersici* (FLO) by dual culture technique. As a result, *T. harzianum (Thz-1), T. harzianum (Thz-2), T. viride (Tv-1), T. viride (Tv-1)* and *T. hamantum* (Th-1) proved to be best antagonist inhibiting 83.33% and 80.33% over control, respectively, followed by *Trichoderma hamantum* (Th-2), *Trichoderma virens* (Ts-1) and *Trichoderma virens* (Ts-1) which showed 64.48 %, 55.85 % and 60.73 % inhibition respectively.

Conclusion

In this study, 181 fungal colonies of 17 fungal species belonging to 7 genera were isolated and identified from eight different rhizosphere soil samples. *Fusarium oxysporum* f. sp. *lycopersici* is the causative organisms of *Fusarium* wilt disease. The fungus colonies are white in color and produce three types of spores, microconidia, macroconidia and chlamydospores. In antagonistic activity, *Trichoderma viride, Aspergillus flavus* had a marked significantly inhibitory effect on the growth of selected phytopathogens compared to their. The use of these bio-agents are not only safe for the farmers and consumers but also eco-friendly, cose effective, easy to produce and easy to apply the formulation. These studies form the base for eco-friendly management of plant disease which will minimize the usage of pesticide and chemical fungicide.

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