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Isolation and characterization of damping off and wilt pathogens of tomato

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Abstract
Tomato is an economically important and commercially worldwide grown crop. Damping off and fungal wilt are the most devastating diseases of tomato which causes considerable damage on yield. The infected samples of tomato were collected from different parts of Tamil Nadu. In the present study, *Pythium* was isolated from infected collar region of seedling and *Fusarium* was isolated from discoloured vascular region of tomato stem. The characterization of *Pythium* and *Fusarium* was performed based on their morphological characters. The hyaline, non- septate mycelium, oospore and lobed sporangia formation were confirmed the isolates as *Pythium aphanidermatum* whereas macro conidia, micro conidia and chlamydospores were confirmed the isolates as *Fusarium oxysporum* f. sp. *lycopersici.* The pathogenicity test for *Pythium* and *Fusarium* isolates were carried out by soil inoculation and stem injection method respectively. The virulent isolates of *Pythium* and *Fusarium* were identified by pathogenicity test. Based on symptom expression highly virulent pathogen was identified among the isolates. The *Pythium* isolate P2 showed disease incidence of 96.67% and *Fusarium* isolate F3 showed 71.43% disease incidence and both the isolates were identified as highly virulent compared to all other isolates.

Keywords: Damping off, *Fusarium* wilt, characterization, pathogenicity
Abbreviations: FOL- *Fusarium oxysporum* f. sp. *Lycopersici*, PDA- Potato Dextrose Agar, DAS- Days after sowing

Introduction
Tomatoes are the most popular and worldwide grown vegetable crop grown in either field or under greenhouse conditions. It has demand throughout the year and it's an economically important crop because of increased commercialization (Hammami *et al.,* 2013) [4]. Tomato ranking second place next to potato in many countries for its importance and tomato production also has a major role in global horticulture (Sharma *et al.,* 2014) [1]. Tomato is a good source of vitamin A, C and lycopene are excellent antioxidant which helps in reducing human diseases (Nahar and Ullah, 2012) [2]. Although tomato is commercially grown across the globe, there is no place where the plant is free from diseases. Fusarium wilt and damping off diseases are the most dangerous diseases which affect the economic importance of the crop in worldwide. Fusarium wilt disease in tomato, caused by *Fusarium oxysporum* f. sp. *lycopersici* (Sacc.) W.C. Snyder and H.N. Hans (Fol), is one of the most devastating diseases of tomato caused major losses in tomato production. The disease is characterized by wilting and browning of the leaves, yellowing, stunted growth and eventual death of the plant. Crop yield is negligible in highly infected tomato plantings. *Fusarium* species are ubiquitous soil-borne pathogens of a wide range of horticultural and food crops (Bodah, 2017). *Fusarium* toxins are the most abundant natural contaminants of diets containing cereals and other grains (Venkataramana *et al.,* 2014, Divakara *et al.,* 2014, Kalagatur *et al.,* 2015, Kumar *et al.,* 2016) [6, 9, 7] and suspected to be implicated in numerous diseases among mammals and other living beings (Nayaka *et al.,* 2010, Venkataramana *et al.,* 2014, Kalagatur *et al.,* 2017, Kalagatur *et al.,* 2018) [11, 6, 10, 8]. Damping off pathogen causes pre and post emergence damping off symptoms on seeds and seedlings respectively. The rotting of seeds and griddling symptom at collar region are the peculiar symptoms (Thakur and Tripathi, 2015) [3].
The control of the soil-borne pathogens is difficult because of their ecological behavior, their extremely broad host range and the high survival rate of resistant forms, such as chlamydospores and oospores under different environ-mental conditions. Many research studies have shown that biological control offers an environment friendly alternative to protect

plants from soil-borne pathogens (Shimon *et al.*, 2004) [5]. Hence characterization of pathogen is highly needed to understand the way of multiplication, survival and symptom expression. The objectives of present study are as follows:

1. Isolation of damping off and Fusarium wilt pathogens from tomato
2. Morphological characterization of damping off and Fusarium wilt pathogens
3. Pathogenicity test for identification of virulent isolates

Materials and Methods

Isolation of damping off, fungal and bacterial wilts pathogens of tomato

The infected tomato plant parts were used for isolation of damping off, Fusarium wilt and bacterial wilt pathogens. The *Pythium* sp. was isolated from the tomato seedlings which has post emergence damping off symptoms at nursery level. The griddled collar region along with healthy portion was cut into small pieces and washed with 0.5% of sodium hypochlorite solution followed by washing with sterile water and then excess moisture was removed using sterilized tissue paper. Then, samples were placed in a Petriplate containing PDA medium and incubated at 25 ± 2 ºC. After 2 days of incubation the hyaline mycelium from the bits was examined under microscope and single hyphae was transferred to another Petridish to get the pure culture of the pathogen.

The *Fusarium oxysporum* f. sp. *lycopersici* was isolated from discoloured vascular region of the stem portion. The infected part with healthy portion was cut into small bits and surface sterilized with 0.1% of mercuric chloride then rinsed three times with sterile water and placed in PDA Petriplate. After 3-5 days of incubation at 28 ± 2 ºC, the yellow to pinkish colour mycelium was transferred to another petridish to get pure culture of wilt pathogen.

Assessing the phenotypic variation of pathogen isolates

The morphological characteristics of different isolates of damping off and Fusarium wilt pathogens were studied by inoculating the cultures in PDA medium. The colony colour, growth rate, size, colour and septation of mycelia and spore were observed after full growth was obtained in plates.

Pathogenicity test

Soil inoculation method

To prove the pathogenicity, the fungal pathogens (*Pythium* sp. and FOL) were mass multiplied in sand maize medium at the ratio of 9:1. A well ground maize and sand was mixed with fifty percent of moisture in order to favour the fungal growth. The mixture was autoclaved at 121 ºC for 20 min under 15 psi pressure for alternate days then fungal culture was inoculated and incubated at room temperature of 28 ± 2 ºC for 10-15 days. In pots, the pathogen inoculum was mixed with the soil and the symptom expression was observed in seedlings and plants for *Pythium* and *Fusarium* disease incidence respectively under glasshouse condition. Disease incidence was recorded by the following formula,

DI (%) = (No. of seeds sown- No. of seeds germinated) + (No. of germinated plants- No. of healthy seedlings) / No. of seeds sown*100

Stem injection method

The spore suspension of FOL was collected and counted under haemocytometer, then diluted to the concentration of 10^6 conidia/ ml for this study. The spores were injected at the stem portion and disease occurrence was monitored at regular interval. Three replications for each isolate were maintained to identify the virulent culture among the isolates. After symptom expression in tomato plant, the pathogen was re-isolated from the infected part to confirm the Koch's postulates. Wilt incidence was calculated by following formula,

Wilt incidence (%) = Number of infected plants/ Total number of plants observed* 100

Statistical analysis

The data were statistically analyzed with the help of SPSS version 160.data were subjected the ANOVA at a significant level (p 0.05) and by using DMRT, the means were compared.

Result and Discussion

Isolation and characterization of damping off and Fusarium wilt pathogens of tomato

Totally five isolates of *Pythium aphanidermatum*, three isolates of *Ralstonia solanacearum* and five isolates of *Fusarium oxysporum* f. sp. *lycopersici* were isolated from the infected tomato seedlings and vascular region of tomato plants. Colony characteristics of *Pythium aphanidermatum* and *Fusarium oxysporum* f. sp. *lycopersici* were studied using PDA medium. All the isolates were stored as a glycerol stock at -20 ºC for long term usage. The isolates diversity of three different pathogens was studied based on their morphological characteristics by observing under light microscope. *Pythium* was identified based on hyaline mycelium, oospore formation and sporangia production. Among the five isolates P3 showed rapid and whitish petal form of fluffy mycelia growth with raised margin appearance. Similarly, isolates P1 and P5 showed rapid growth with aerial mycelium whereas P2 and P4 showed moderate growth rate with dull to whitish mycelium having smooth margin type. The oospore formation was observed higher in P3 isolate followed by P1, P5 and P2 isolates. No oospore formation was observed in P4 isolate on PDA medium (Figure 1; Table 1). Lobed sporangia were also observed in all the isolates that further confirmed the isolates were *Pythium aphanidermatum*. Elshahawy *et al.*, (2018) [18] reported that morphology and cultural character observed in *P. aphanidermatum* isolation of after 5 days of growth on PDA. Similar finding *P. aphanidermatum* culture was 5 days old grown on potato dextrose agar medium isolated from diseases cucumber plants evident the result (Alhussaen, 2019) [17].

Table 1: Colony characteristics of different isolates of *Pythium aphanidermatum* on PDA medium

S. No	Isolates	Radial growth (cm)*		Cultural characteristics on PDA medium	Oospore and sporangia formation in plates
		24 hrs	36 hrs		
1	P1	6.70^{b}	9.00	Rapid and whitish growth with aerial flat mycelium	+
2	P2	7.10^{a}	9.00	Rapid with whitish petal fluffy growth and raised margin	+
3	P3	6.20^{cd}	9.00	Moderate with dull white sparse growth and smooth margin	+

4	P4	5.90[d]	9.00	Moderate with whitish fluffy growth and smooth margin	+
5	P5	6.50[bc]	9.00	Rapid with whitish aerial flat mycelium	+
CD value (0.05%)		0.35			

*Mean of three replications.
The treatment means are compared using Duncan Multiple Range Test (DMRT).

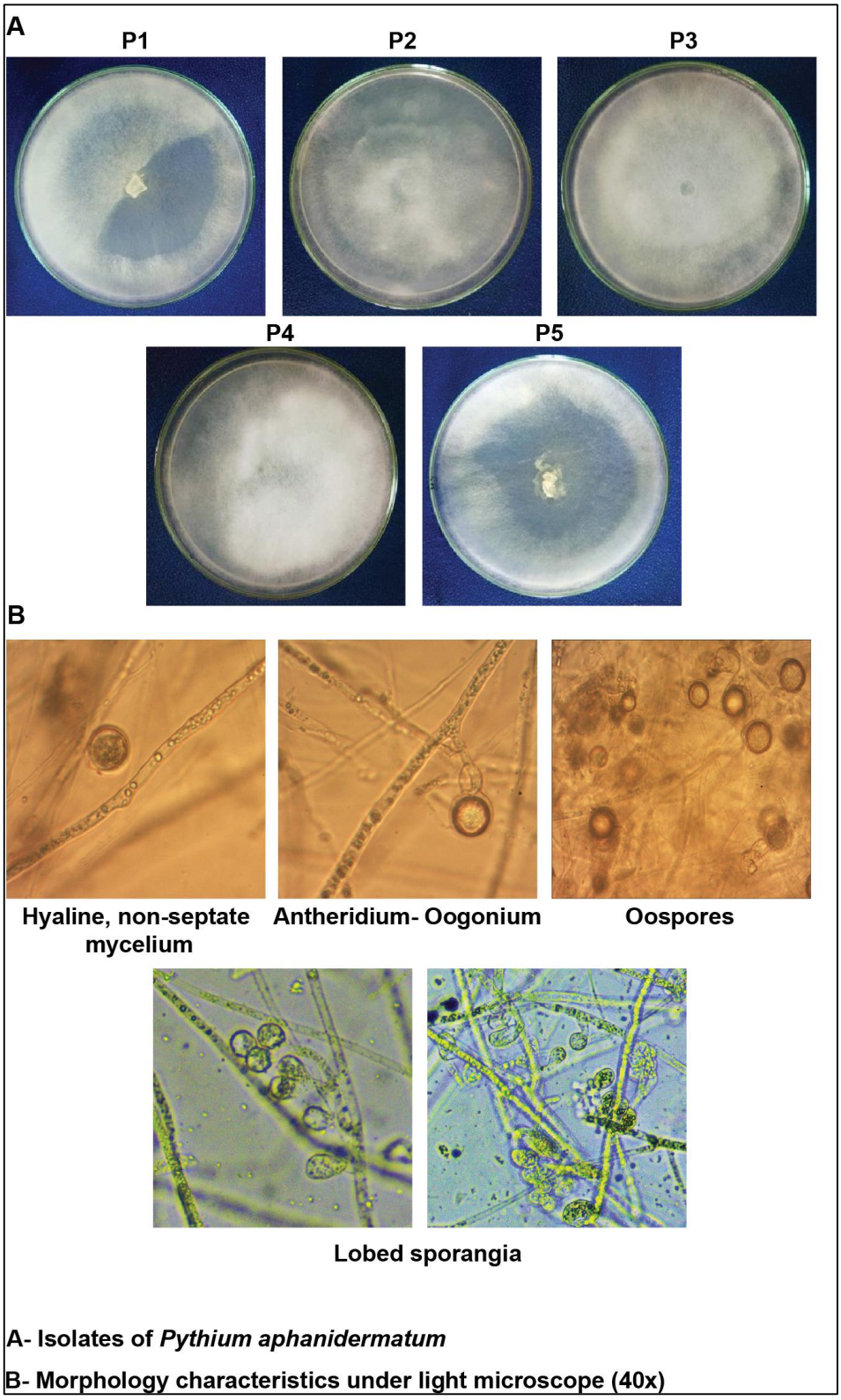


Fig 1: Isolation and morphological characterization of damping off pathogen

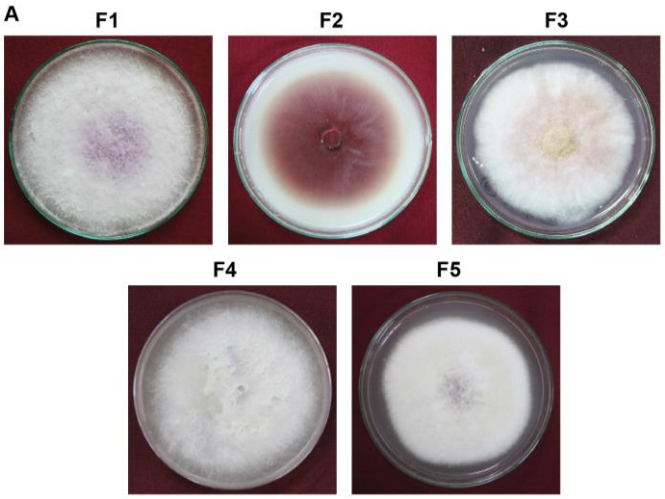
The isolates of *Fusarium oxysporum* f.sp. *lycopersici* was identified based on mycelia colour and conidia production. Upon observation under light microscope they showed septate mycelium with micro conidia, macro conidia and chlamydospores. The colony colour varied from white, white with pink, white with orange and white with yellow tinch. The mycelia was flat to raised and they produced two types of conidia *viz*, micro and macro conidia. Micro conidia were

hyaline, small, oval shaped and single or bicelled. Nirmaladevi and Srinivas (2012) observed that the colour and pigmentation of the isolates on PDA medium varied between white, creamish white to cream, light pink to pink and light purple to violet. The size of the macro conidia ranged from 35.88 µm (F5) to 38.27µm (F2) in length and 8.32 µm (F5) to 10.02 µm (F2) in breath. Macro conidia were fusiform shaped with 3-4 septation. The size of the micro conidia ranged from 12.55 µm (F1) to 14.35µm (F2) in length and 3.74 µm (F1) to 4.98 µm (F2) in breath. Micro conidia were elliptical shaped with 0-1 septation (Figure 2; Table 2). From this above mentioned morphological characters the isolates were identified as FOL.

Table 2: Colony characteristics of different isolates of *Fusarium oxysporum* f. sp. *lycopersici* on PDA medium

S. No	Isolates	Pigmentation	Colony characteristics	Spore characters	Covered entire Petriplate (Days)*	Spore size
1	F1	Whitish pink colour	Raised fluffy growth with pink mycelium	Macro conidia- Fusiform shape, blunt end, some cylindrical shape, 3 septate Micro conidia- Elliptical shape, 0-1 septate	7^{b}	Macro conidia- 37.18x9.23 µm Micro conidia- 12.55x3.74 µm Chlamydospore- 9.93x9.34 µm
2	F2	Pink colour	Flatted mycelium growth with tiny white droplets	Macro conidia- Fusiform shape, tapering end, 3-4 septate Micro conidia- Elliptical shape, 0-1 septate	5^{d}	Macro conidia- 38.27x10.02 µm Micro conidia- 14.35x4.98 µm Chlamydospore- 10.04x10.25 µm
3	F3	Dull white to orange colour	Raised fluffy growth with light orange mycelium	Macro conidia- Fusiform shape, tapering end, 3-4 septate Micro conidia- Elliptical shape, slightly curved, 0-1 septate	8^{a}	Macro conidia- 36.51x8.92 µm Micro conidia- 12.13x4.10 µm Chlamydospore- 9.20x9.11 µm
4	F4	White with dull yellow colour	Raised fluffy growth with small yellowish droplets on mycelium	Macro conidia- Fusiform shape, tapering end, 3 septate Micro conidia- Elliptical shape, 0-1 septate	6^{c}	Macro conidia- 38.05x10.47 µm Micro conidia- 14.11x4.65 µm Chlamydospore- 10.10x10.03 µm
5	F5	Dull white with yellow colour	Suppressed fluffy mycelium with yellow patches	Macro conidia- Fusiform shape, blunt end, 3 septate Micro conidia- Elliptical shape, slightly curved, 0-1 septate	8^{a}	Macro conidia- 35.88x8.32 µm Micro conidia- 13.67x4.84 µm Chlamydospore- 9.34x9.21 µm
				CD value (0.05%)	0.31	

*Mean of three replications.
The treatment means are compared using Duncan Multiple Range Test (DMRT).



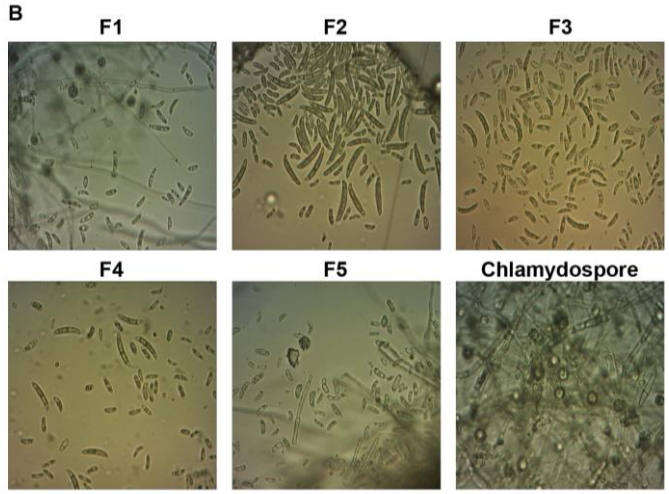


Fig 2: Isolation and morphological characterization of Fusarium wilt pathogen

Proving of pathogenicity test

The various isolates of damping off and Fusarium wilt pathogens were taken to prove pathogenicity test. For that pot culture experiment was conducted under glass house condition. Sand maize medium (95:5) was prepared for *Pythium* and *Fusarium* isolates to get fungal inoculums. Pathogen inoculums were inoculated in soil and observed for symptom expression at 15 DAS for *Pythium* and 60 DAS for FOL. The *Pythium* showed both pre and post emergence damping off symptoms in all the treatments. In pre emergence damping off, seeds were rotted and in post emergence damping off, seedlings showed toppled over symptom due to infection at collar region. The maximum disease incidence of 96.67% was recorded at isolate P2 followed by 94.44% disease incidence in isolate P3. Similarly *Pythium* Species differed in pathogenicity and virulence on seedlings resulting in pre- emergence damping off and some post– emergence damping off. Navi *et al.,* (2019) proven under greenhouse condition there was increased damping off disease incidence, among the 10 isolates maximum incidence of 79.80 per cent was observed *P. ultimum* var. *Ultimum.* In Fusarium wilt, the plant showed vascular discolouration, yellowing of leaves and wilting of entire plant. The FOL enters the epidermis of root, later spreads through the vascular tissue and inhabits the plant xylem vessels, resulting in vessel clogging, and severe water stress as a result wilt like symptoms appear (Singh *et al.,* 2017) [16]. The disease is morphologically identified by wilted plants bearing yellow colored leaves with minimal or absent crop yield. The dormant chlamydospore of FOL in infested soil can survive indefinitely in the absence of host (Khan *et al.,* 2017, Cha *et al.,* 2016) [13, 15]. Colonization of the vessels leads to disease development and the characteristic wilting of the host plant (Di *et al.,* 2016) [14]. The maximum disease incidence of 71.43% was recorded in isolate F3 followed by 62.50% in isolate F4. Among the different isolates tested all the isolates showed disease symptoms of respective pathogens. Based on symptom expression highly virulent pathogen was identified among the isolates. The *Pythium* isolate P2 and *Fusarium* isolate F3 identified as highly virulent isolates.

In this present study we concluded that the isolation and morphological characterization of pathogens considered as a basic study which will help us to know the better understanding on pathogens life cycle and characters for quick identification and pathogenicity test revealed the infective nature and symptom expression by the isolates collected from various parts. The virulent isolates screened in this experiment were used for further study.

Table 3: Assessing the virulence of pathogen isolates by pathogenicity test

S. No	Pathogen isolates	Disease incidence (%)*
I	*P. aphanidermatum* isolates	
	P1	85.56 (67.76)^{b}
	P2	96.67 (79.50)^{a}
	P3	97.78 (77.72)^{a}
	P4	78.89 (62.66)^{c}
	P5	94.44 (76.41)^{a}
	Uninoculated control	0.00 (0.28)^{d}
	CD value (0.05%)	3.52
II	FOL isolates	
	F1	42.86 (40.89)^{d}
	F2	57.14 (49.10)^{c}
	F3	71.43 (57.69)^{a}
	F4	62.50 (52.25)^{b}
	F5	28.57 (32.31)^{e}
	Uninoculated control	0.00 (0.28)^{f}
	CD value (0.05%)	1.24

*Mean of four replications.

The treatment means are compared using Duncan Multiple Range Test (DMRT).

Figures in the parentheses are arc sine transformed values

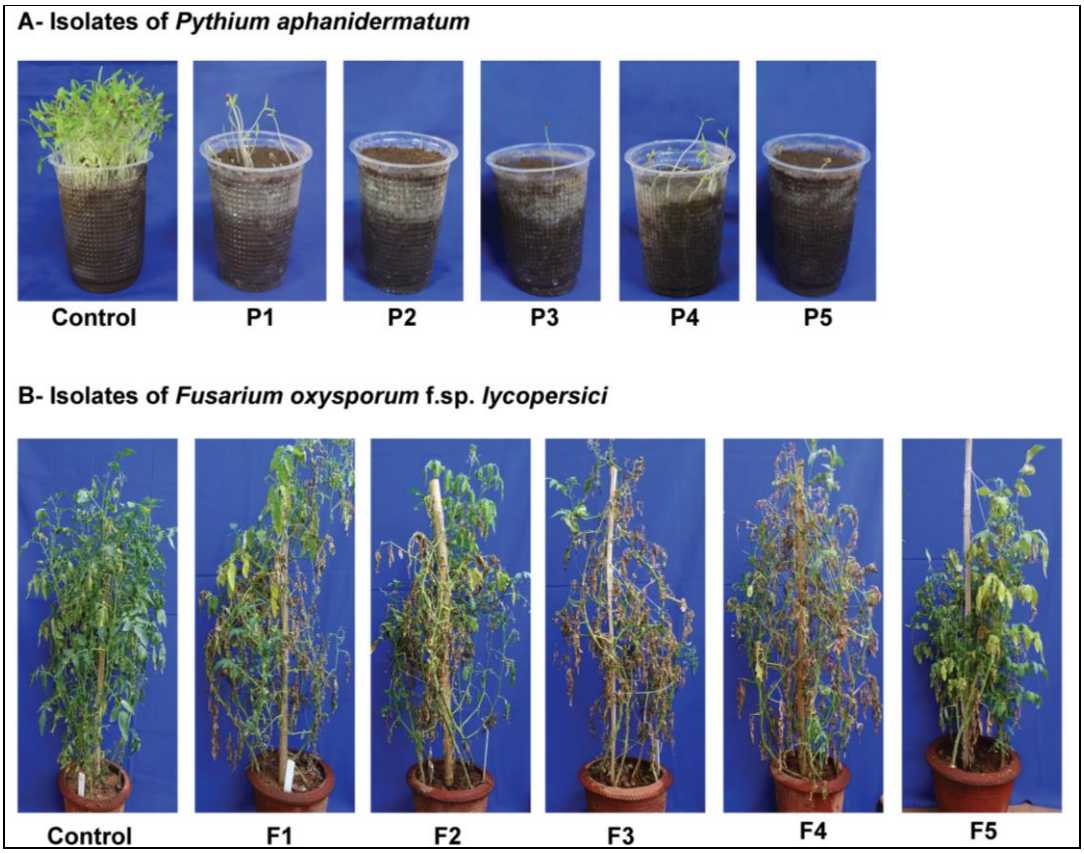


Fig 3: Pathogenicity test

Disclosure of interest

The authors have not supplied their declaration of conflict of interest.

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