

**STUDIES ON *Fusarium* WILT IN SCENTED  
GERANIUM [*Pelargonium graveolens* (L.) Herit]**

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**BANGALORE**

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**STUDIES ON *Fusarium* WILT IN SCENTED  
GERANIUM [*Pelargonium graveolens* (L.) Herit]**

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*Thesis submitted to the*  
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*in partial fulfilment of the requirements*  
*for the award of the Degree of*

**MASTER OF SCIENCE (Agriculture)**  
**in**  
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**NOVEMBER, 2020**



*Affectionately dedicated to*

*my beloved parents,*

*Sri. Tulajappa*

*Smt. Yallamma*

*Brother, Sister, Friends and*

*Chairperson*




**DEPARTMENT OF PLANT PATHOLOGY  
UNIVERSITY OF AGRICULTURAL SCIENCES  
BANGALORE**

**CERTIFICATE**

This is to certify that the thesis entitled “STUDIES ON *Fusarium* WILT IN SCENTED GERANIUM [*Pelargonium graveolens* (L.) Herit]” submitted by Mr. ARUNKUMAR, ID No. PALB 8315 in partial fulfilment of the requirement for the degree of MASTER OF SCIENCE (Agriculture) in PLANT PATHOLOGY to the University of Agricultural Sciences, Bangalore, is a record of *bona-fide* research work done by him during the period of his study in this university, under my guidance and supervision and no part of the thesis has been submitted for the award of any other degree, diploma, associateship, fellowship or any other similar titles.

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November, 2020

(Arunkumar)

BENGALURU

**STUDIES ON *Fusarium* WILT IN SCENTED GERANIUM  
[*Pelargonium graveolens* (L.) Herit]**

**ARUNKUMAR**

**THESIS ABSTRACT**

Scented geranium is an important, perennial aromatic herb affected by several diseases of which wilt caused by *Fusarium oxysporum* is becoming a major constraint in production. Wilt symptoms such as yellowing, drooping of plants and vascular discoloration were observed in the infected stems. Maximum wilt incidence recorded in Tumakuru district (36.73 %). The wilt causing pathogen was isolated from infected plant samples collected from different geranium growing areas of southern Karnataka and pathogenicity was proved under greenhouse conditions. The re-isolated pathogen was identified as *F. oxysporum* based on its morphological characters by comparing with standard descriptions of *Fusarium* species and also confirmed the pathogen through molecular characterization of isolates by using universal fungal ITS-1 and ITS-4 primers. Sabouraud's dextrose agar (SDA) and corn meal agar (CMA) were found to be ideal for radial mycelial growth and sporulation. The maximum growth and sporulation of the pathogen was found at temperature 25 °C and pH of 6.5. Among the botanicals and bio agents evaluated under *in vitro* condition against *F. oxysporum*, agave (59.31 %), *Trichoderma viride* Tv- 8 (71.12 %), *Bacillus pumilis* (54.66 %) and *B. velezensis* Bv-P<sub>42</sub> (53.61 %) were effective in inhibition of mycelial growth of the pathogen. Among the fungicides evaluated, systemic fungicides carbendazim and propiconazole showed 100.00 per cent, combi products hexaconazole + captan 84.51 per cent and contact fungicide copper oxychloride 56.48 per cent were effective as evidenced by maximum mycelial growth inhibition compared to other fungicides under *in vitro* condition.

November, 2020  
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Major Advisor

ಪನ್ನೀರೆಲೆಯಲ್ಲಿ [ಪೆಲಗೋನಿಯಂ ಗ್ರೇವೋಲೆನ್ಸ್ (ಎಲ್.) ಹೆರಿಟ್] ಫ್ಯುಸೇರಿಯಂನಿಂದ ಉಂಟಾಗುವ  
ಸೊರಗು ರೋಗದ ಅಧ್ಯಯನ

ಅರುಣ್ ಕುಮಾರ್

ಪ್ರಬಂಧ ಸಾರಾಂಶ

ಪನ್ನೀರೆಲೆಯು ಒಂದು ಪ್ರಮುಖ, ದೀರ್ಘಕಾಲಿಕ ಸುಗಂಧ ಬೆಳೆಗಳಲ್ಲಿ ಒಂದಾಗಿದ್ದು, ವಿವಿಧ ರೋಗಗಳಿಗೆ ತುತ್ತಾಗುತ್ತಿರುವುದು ಕಂಡುಬಂದಿರುತ್ತದೆ, ಅವುಗಳಲ್ಲಿ ಫ್ಯುಸೇರಿಯಂ ಆಕ್ಸಿಸ್ಪೊರಮ್ ನಿಂದ ಉಂಟಾಗುವ ಸೊರಗು ರೋಗವು ಅದರ ಉತ್ಪಾದನೆಗೆ ಮಾರಕವಾಗಿದೆ. ಈ ನಿಟ್ಟಿನಲ್ಲಿ ತುಮಕೂರಿನ ಪನ್ನೀರೆಲೆಯ ತಾಕುಗಳಿಗೆ ಭೇಟಿನೀಡಲಾಗಿದ್ದು, ಹಳದಿ ಜೊತೆಗೆ ಸೊರಗುವಿಕೆ ಹಾಗೂ ನಾಳಿಯ ಬಣ್ಣಗಳ ಬದಲಾಗುವಿಕೆಯಂತಹ ರೋಗಲಕ್ಷಣಗಳೊಂದಿಗೆ ಶೇ. ೩೬.೭೩ ರೋಗದತೀವ್ರತೆ ಕಂಡುಬಂದಿದೆ. ರೋಗಾಣುವಿನ ಪ್ರಬೇಧಗಳನ್ನು ದಕ್ಷಿಣ ಕರ್ನಾಟಕದ ವಿವಿಧ ಪ್ರದೇಶಗಳಿಂದ ತಂದು ಹಸಿರು ಮನೆಯಲ್ಲಿ ರೋಗಕಾರಕತೆಯನ್ನು ಸಾಬಿತುಪಡಿಸಲಾಯಿತು ಮತ್ತು ಅದೇ ರೋಗಾಣುವನ್ನು ರೂಪವಿಜ್ಞಾನ, ಫ್ಯುಸೇರಿಯಂ ಪ್ರಭೇದಗಳ ಪ್ರಮಾಣಿತ ವಿವರಣೆಯೊಂದಿಗೆ ಹೊಲಿಸುವ ಮತ್ತು ಅಣುಜೀವಾಣು ಶಾಸ್ತ್ರದ ಪ್ರಯೋಗಗಳಿಗೆ (ಯುನಿವರ್ಸಲ್ ಪ್ರೈಮರ್ಸ್ ಐಟಿಎಸ್-೧ & ಐಟಿಎಸ್-೪) ಒಳಪಡಿಸುವ ಮುಖಾಂತರ ರೋಗಾಣುವನ್ನು ಫ್ಯುಸೇರಿಯಂ ಆಕ್ಸಿಸ್ಪೊರಮ್ ಎಂದು ಗುರುತಿಸಲಾಗಿದೆ. ಸರ್ಬೊಡ್ ಡೆಕ್ಟ್ರೋಸ್ ಅಗಾರ್ (ಎಸ್‌ಡಿಎ) ಮತ್ತು ಕಾರ್ನ್‌ಮಿಲ್ ಅಗಾರ್ (ಸಿಎಎ), ೨೫ ಡಿ. ಸೆಂ. ತಾಪಮಾನದ ಜೊತೆಗೆ ೬.೫ ರಸಸಾರದಲ್ಲಿ ರೋಗಾಣುವು ಅತಿಹೆಚ್ಚು ಕವಚಜಾಲದ ಬೆಳವಣಿಗೆಯೊಂದಿಗೆ ಹೆಚ್ಚು ಬೀಜಕಗಳ ಉತ್ಪಾದನೆಗೆ ಸೂಕ್ತವಾಗಿದೆ ಎಂದು ಪ್ರಯೋಗಾಲಯದಲ್ಲಿ ಕಂಡುಬಂದಿದೆ. ಪ್ರಯೋಗಾಲಯದಲ್ಲಿ ಪರಿಕ್ಷಿಸಲಾದ ವಿವಿಧ ಸಸ್ಯದ ಸಾರಗಳಲ್ಲಿ ಮತ್ತು ಜೈವಿಕ ಜೀವಾಣುಗಳಲ್ಲಿ ಭೂತಾಳೆ (ಶೇ. ೫೯.೩೧), ಜೈವಿಕ ಜೀವಾಣುಗಳಾದ ಟ್ರೈಕೋಡರ್ಮ್ ವಿರೀಡೆ ಟಿವಿ-೮ (ಶೇ. ೭೧.೧೨); ಬ್ಯಾಸಿಲಸ್ ಪುಮಿಲಿಸ್ (ಶೇ. ೫೪.೬೬) ಮತ್ತು ಬ್ಯಾಸಿಲಸ್ ವೆಲೆಜ್‌ನಿಸ್ ಬಿವಿ-ಪಿ೪೨ (ಶೇ. ೫೩.೬೧) ಗಳು ರೋಗಾಣುವಿನ ಬೆಳವಣಿಗೆಯನ್ನು ಪ್ರತಿಬಂಧಿಸುತ್ತವೆ ಎಂದು ತಿಳಿದುಬಂದಿದೆ. ಅಂತರ್ವ್ಯಾಪಿ ಶಿಲೀಂಧ್ರ ನಾಶಕವಾದ ಕಾರ್ಬೆಂಡಜಿಮ್ ಮತ್ತು ಪ್ರೊಪಿಕ್‌ನೋ ನಜೋಲ್ (ಶೇ. ೧೦೦); ಸಂಯುಕ್ತ ಶಿಲೀಂಧ್ರನಾಶಕಗಳಾದ ಹೆಕ್ಸಾಕೋನಜೋಲ್+ಕ್ಯಾಪ್ಟಾನ್ (ಶೇ. ೮೪.೫೧); ಸ್ವರ್ಶ ಶಿಲೀಂಧ್ರ ನಾಶಕವಾದ ಕಾರ್ಪ್ ಆಕ್ಸಿಕ್ಲೋರೈಡ್ (ಶೇ.೫೬.೪೮); ಗಳು ಸೊರಗು ರೋಗದ ರೋಗಾಣುವನ್ನು ಪ್ರಯೋಗಾಲಯದಲ್ಲಿ ಪ್ರತಿಬಂಧಿಸುತ್ತದೆ ಎಂದು ಕಂಡುಬಂದಿದೆ.

ನವಂಬರ್, ೨೦೨೦

ಸಸ್ಯರೋಗಶಾಸ್ತ್ರ ವಿಭಾಗ

ಕೃಷಿ ವಿಶ್ವವಿದ್ಯಾನಿಲಯ, ಬೆಂಗಳೂರು

ಗಾ.ಕೃ.ವಿ.ಕೇ, ಬೆಂಗಳೂರು-೬೫

ಕೆ. ಆರ್. ಶ್ರೀನಿವಾಸ

(ಮುಖ್ಯ ಸಲಹೆಗಾರರು)

# Morphological and molecular characterization of *Fusarium oxysporum* infecting scented geranium [*Pelargonium graveolens* (L.) Herit]



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## INTRODUCTION

Scented geranium (*Pelargonium graveolens* (L.) Herit) is an important, high value, perennial aromatic herb. This crop is prone to diseases like leaf blight, fungal wilt, root rot, bacterial wilt and root knot nematode. Wilt caused by *Fusarium oxysporum* is the major constraint in this crop resulting economic loss.

The disease is soilborne and the chlamydospores of the pathogen survive in the soil for several years. There is need to study the morphological and molecular characterization of the pathogen in order to develop effective management strategies.



Plate 1. Symptoms of wilt disease in scented geranium

## OBJECTIVES:

1. Collection and isolation of pathogen causing wilt in geranium
2. Morphological and molecular characterization of *Fusarium oxysporum* infecting geranium

## MATERIAL AND METHODS

### 1. Collection and isolation of pathogen causing wilt in geranium

- ✓ Samples from geranium plants showing symptoms typical to fusarium wilt were collected from Tumakuru, Bengaluru Rural, Hassan districts.
- ✓ Which was isolated by tissue isolation technique and pure culture was established by single spore isolation (Booth, 1971)
- ✓ Pathogenicity was proved by inoculating mass multiplied giant culture of *F. oxysporum* isolates on healthy rooted geranium cuttings under controlled conditions.
- ✓ The purified fungus was identified based on its mycelial and conidial characteristics by comparing with standard descriptions of *Fusarium* species given by (Booth, 1975), Barnett and Barry (1992), Leslie and Summerell (2006).

Table 1. Isolation and designation of *F. oxysporum* isolates

Sl. No.	Isolate designation	Place of collection	State
1	Isolate GFO 1	Tiptur, Tumakuru	Karnataka
2	Isolate GFO 2	Hosakote, Bengaluru Rural	Karnataka
3	Isolate GFO 3	Arsikere, Hassan	Karnataka

### 2. Morphological and molecular characterization of *F. oxysporum* infecting geranium

- ✓ Morphological characters were recorded from ten days old culture of the isolates grown in Petri plates.
- ✓ Spore characters like production of microconidia, macroconidia, type of conidiophore and their size, shape, no. of septations and apical cell morphology were recorded.
- ✓ Fungal DNA was isolated by CTAB protocol given by Murray and Thompson (1980).

- ✓ Polymerase Chain Reaction (PCR) amplification was done using ITS-1/4 (White *et al.*, 1990)
- ✓ PCR product was sequenced and sequences were edited aligned by ClustalW and phylogenetic tree was constructed with maximum likelihood approach in MEGA X software.
- ✓ Aligned sequences were submitted to NCBI-GenBank.

## RESULTS

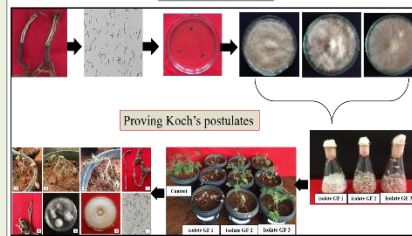


Plate 2. Pathogenicity test under controlled conditions

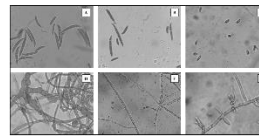


Plate 3. Morphological characters of *F. oxysporum*

(A) Macroconidia (B) Macroconidia and microconidia (C) Oval shaped microconidia (D) Chlamydospores (E) Hyaline, branched septate mycelia (F) Short and plump monophialides [100x]

## Morphological characterization

Table 2. Conidial characters of *F. oxysporum* isolates infecting geranium

Isolates	Size (µm)		Shape	No. of septa	Apical cell (µm)
	Range (L-W)	Average(L-W)			
Macroconidia					
GFO 1	12.5-30.93 × 1.92-8.85	21.12 × 2.46	This walled fusiform, sickle shaped	2-5 (3)	1.73 slightly curved
GFO 2	14-16.17 × 3.4-3.65	15.54 × 3.54	Thin walled straight elongated	1-2 (2)	2.53 blunt end
GFO 3	14-15.92 × 1.8-2.82	14.96 × 2.31	Thin walled straight	1-2 (3)	2.06 blunt end
Microconidia					
GFO 1	8-9.78 × 1.9-2.55	9.44 × 2.30	Oval to elliptical	0	1.54
GFO 2	4.6-6.7 × 1.2-2.1	5.38 × 1.66	Oval to elliptical	1	1.24
GFO 3	5.2-7.57 × 1.5-2.19	6.43 × 1.87	Oval	0	1.59
Chlamydospores					
GFO 1	7-10.48 × 7.94	8.26 × 7.55	Thick walled oval shape	Arrangement	
GFO 2	5.8-7.77 × 4.6-6.68	6.48 × 5.91		Either in chains or in clusters	
GFO 3	6.6-11.5 × 6.1-10.84	8.48 × 7.94		In chains	
				Formed in chains or singly	

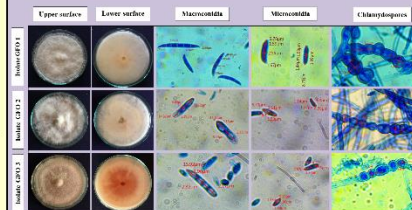


Plate 4: Cultural and spore characters of all isolates on PDA (100x)

- ✓ Pathogen was identified as *F. oxysporum* based on morphology by comparing with descriptions of Booth (1975), Barnett and Barry (1992), Leslie and Summerell (2006).
- ✓ *F. oxysporum* produces macroconidia, microconidia thick walled chlamydospores (Plate 3, 4 and Table 2)

## Molecular characterization

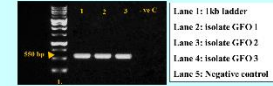


Plate 5: ITS rDNA region amplification of *F. oxysporum* isolates

- ✓ PCR primers of ITS1/4 yielded specific PCR products of approximately 550 bp length (Plate 3).
- ✓ BLAST and phylogenetic results of ITS sequences showed the isolates were 100 per cent similar with *F. oxysporum* isolate Glnrarsnl [JMN055701]
- ✓ ITS sequences of isolates confirmed that causal organism of wilt disease in scented geranium as *Fusarium oxysporum*

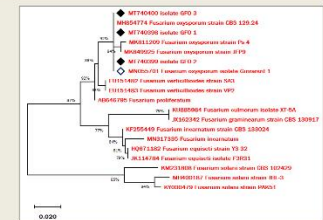


Fig. 1: Phylogenetic tree of ITS

## DISCUSSION

- ✓ Prasad *et al.* (2008) tested the pathogenicity of geranium plants by artificial inoculation using giant culture of pathogen isolates.
- ✓ *F. oxysporum* produces macroconidia that are short to medium length, straight to slightly curved, relatively slender and thin walled, apical cell morphology tapered and curved, sometimes with a slight hook, basal cell foot shaped to pointed, usually 3-septate. Microconidia are oval, elliptical or kidney shaped and usually no septation on short monophialides. Chlamydospores arranged either terminal or intercalary mainly smooth or rough walled Leslie and Summerell (2006).
- ✓ Kuppasamy *et al.*, 2018; Meena and Roy (2019) characterized *Fusarium* species causing wilt disease using molecular tools such as ITS.

## SUMMARY

- Koch's postulates were established for *F. oxysporum* isolates under controlled conditions.
- Based on morphological and conidial characters, causal organism of wilt disease in geranium was identified as *F. oxysporum*.
- Phylogenetic analysis of ITS sequences confirmed the causal organism as *F. oxysporum*

## ADVISORY COMMITTEE

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## I INTRODUCTION

In nature diversity of crops that exists, our mother nature had created many such extraordinary plant species. Among them, aromatic crops are those plants which possess essential oils. Essential oils are secreted in oil glands which are available at least in one or other plant parts. These oils are complex mixture of odoriferous steam volatile compounds that are stored by plants in sub-cuticular spaces, granular hairs, cell organelles, excretory depressions, channels and heart wood. These oils play significant role in our everyday life beginning from birth to death. They are utilized in one or other manner in beautifiers, flavour, perfume and pharmaceutical industries.

Scented Geranium (*Pelargonium graveolens* (L.) Herit) belongs to family geraniaceae which is a perennial, high value and drought tolerant aromatic herb. It was originated in Cape province of South Africa. Earlier, French planter Earnest Sens introduced this herb to Shevaroy hills of South India in 1915. Further, it advanced to Nilgiris in 1954 which grown and established well under the sub-tropical conditions of Southern India. Today, it is a magnificent and top most financial harvest in numerous places in India (Arumugam and Kumar, 1979). In India, this crop is majorly cultivating in Tamil Nadu, Maharashtra, Andhra Pradesh, Uttar Pradesh. Narayana *et al.* (1979) reported that in Karnataka state, this herb was first introduced at lower altitude in the Bengaluru plains and being developed in and around Bengaluru districts as a commercial crop since recent decades.

Geranium is a sub-frutescent bushy plant of about 60 to 90 cm height with ramified stems and leaves are simple, alternate, broadly cordate with small flowers. Algerian/Tunisian and Bourbon/Reunion are two types of geranium which are presently under cultivation in India. Algerian type is growing in extensive areas of Nilgiris having characteristics of slenderness and gives dark pink flowers but this type is not ideal for cultivation in wet conditions. Likewise, Bourbon type is stiff and produce light pink flowers growing in areas of Annamalai and Nilgiris. This type was more preferred to cultivate under plains and is ideal for wet conditions (Rao, 2000).

Geranium essential oil is extensively used in the soap, perfumery and cosmetic industries. The oil possesses fine rosy odour with fruity mint undertone and a sturdy long lasting, sweet rosy dry out. Chief constituents are geraniol 41.6 per cent and citronellol 8.5 per cent (Rao *et al.*, 1986). It is very versatile oil for perfumery due to its exquisite odour and its ability to blend with all floral and oriental bouquets. It is also used for extraction of rhodinol which is used in most high-grade perfumes (Douglas, 1969).

Geranium oil is also used in aromatherapy to cure post-menopausal problems, skin diseases, mental fatigue and anxiety (Ranade, 1998; Singh *et al.*, 1999 and Singh *et al.*, 2000). It has antiseptic (Aggarwal *et al.*, 2000), antifungal, pesticidal and pharmacological properties (Dube *et al.*, 1983; Lis Balchin *et al.*, 1998 Aggarwal *et al.*, 2000).

The total world production of geranium oil is about 800 tonnes every year (Bhaskar *et al.*, 2000). Whereas, India produces about only 5 tonnes per annum (Singh *et al.*, 2001).

World's production of essential oil is estimated at about 1- 1.1 lakh tonnes where India stands at third position with a share of 16 to 17 per cent. In terms of value, India is in second position with worth of 21 to 22 per cent shares due to the mint revolution in north India (Varshney, 2000).

Geranium is prone to many diseases like root rot, leaf blight, fungal wilt, bacterial wilt and root-knot nematodes. Among these *Fusarium* wilt diseases is destructive, affecting wide range of crop plants. Disease results in symptoms like yellowing of leaves, vascular discolouration, retarded growth even plant death, due to the soil borne nature of the pathogen can survive in soil for many days to years and perennial nature of its hosts *Fusarium* managements is a challenge (Joshi, 2018).

Major problem faced in the cultivation of geranium is its high susceptibility to vascular wilt caused by *Fusarium oxysporum* var. *rodolens*, a soil borne fungus which heavily effects the crop in terms of yield under favourable conditions (Sarwar, 1973).

Wilt, a dreaded disease of scented geranium, is threatening the cultivation of the crop particularly in plains. A survey of scented geranium growing areas of India shows that

the disease incidence is wide spread with plant mortality due to the disease ranging from 15-20 per cent in Kodaikanal (high altitude location) to 35-40 per cent in Lucknow (North Indian plains) and 60-70 per cent in Bengaluru (South Indian plains). Different fungi such as *Verticillium albo atrum*, *Fusarium* spp. (Rao *et al.*, 1999).

Scented geranium is a major growing aromatic crop in Karnataka to undertake the investigations into the role of the pathogen responsible in causing wilt disease. Crop subjected to wilt disease will affect the economic part of geranium such as leaf due to attach of wilt pathogen in severe form will affect, destroy the whole crop keeping this in understanding the present study was planned to identify the pathogen causing wilt and also attempts had made to achieve the disease management *in vitro* by the use of botanicals, biocontrol agents and new molecules of fungicides. With this background of information, following objectives were framed.

**Objectives:**

1. Collection and isolation of the pathogen causing wilt in geranium.
2. Cultural and morphological characters of pathogen causing wilt in geranium.
3. Molecular characterization of *Fusarium* sp. infecting geranium.
4. *In vitro* evaluation of botanicals, bio agents and fungicides against *Fusarium* sp. infecting geranium.

## II REVIEW OF LITERATURE

Scented geranium [*Pelargonium graveolens* (L.) Herit] is an important aromatic herb. It is grown in cooler and subtropical climate in Karnataka, this crop is prone to many diseases like leaf blight, root rot, fungal wilt, bacterial wilt and Root knot nematode. Among these diseases, *Fusarium* wilt is most economically important soil-borne disease, limiting geranium cultivation. The disease was observed in Bengaluru Rural, Tumakuru and Hassan districts of Karnataka in severe form during 2019-20. The diseased samples were collected from different fields of geranium growing areas. Isolation of pathogen from diseased plant parts consistently yielded *Fusarium* sp. which was later identified as *Fusarium oxysporum* (Schlecht.) Emend Snyder & Hansen.

The literature pertaining *Fusarium* wilt in scented geranium is scanty, the work done on various aspects of the pathogen and disease on other related crops were reviewed and presented briefly under different headings in this chapter.

### 2.1 History and disease occurrence

Kalra *et al.* (1992) observed wilt disease on scented geranium caused by *F. solani*. However, Rao *et al.* (1999) reported *Fusarium oxysporum* as causative agent of wilt disease on scented geranium (*P. graveolens*) from Uttarakhand. Anonymous (2010) reported occurrence of *F. solani* on Satavari (*Asparagus racemosus*) which started as small brown discoloration on the surface of the roots.

Geranium is attacked by root rot and wilt complex caused by *F. oxysporum* and *Rhizoctonia solani* Kuhn which leads to significant losses every season. The disease has plagued geranium cultivation in Tamil Nadu, Karnataka and Uttar Pradesh. Survey of rose-scented geranium growing area in these states revealed that the root rot and wilt complex disease incidence ranged from 18 to 46 per cent (Prasad *et al.*, 2008).

Rao *et al.* (1999) recorded wilt disease of rose-scented geranium caused by *F. oxysporum* var. *redolens* (Wollenweb.) resulted in plant mortality of 2.6-72.2 per cent

(average 19.3 %) in one-year old plantation and 0-18.0 per cent (average 4.2 %) in three months old plantation.

Gogoi *et al.* (2017) reported root rot disease of patchouli is caused by *Fusarium solani* (Mart.) Sacc. a severe and wide spread disease in India and major constraint to patchouli cultivation in Assam.

## 2.2 Symptomatology

Rao *et al.* (1999) observed symptoms of the wilt disease in scented geranium were exhibited by the leaves of one or more branches of the infected plants through loss of turgidity and drooping irrespective of the soil moisture condition. They failed to recover upon irrigation. Other branches were affected similarly and the entire plant wilted in a matter of few days, the wilted leaves turned yellow, dried, turned black and were shed. Stems and roots turned black, showing browning or blackening of the xylem when split open.

Prasad *et al.* (2008) described symptoms like wilted leaves turn yellow, dry, turn black and are shed. In later stage, the entire plant is wilted within few days. Stems and roots turn black showing browning or blacking of the xylem vessels.

Michielse and Rep (2009) elucidated the symptoms caused by *F. oxysporum* were, initially vascular wilt include vein clearing and leaf epinasty, followed by stunting, yellowing of the lower leaves, progressive wilting, defoliation and, finally, death of the entire plant. On fungal colonization, the vascular tissue turns brown, which is clearly visible in cross-sections of the stem.

The disease symptoms started with yellowing of the older leaves which advanced to the middle & terminal leaves. Later, drying of green parts and wilting though there was sufficient moisture in the soil. The diseased plant showed brown to black discolouration of the stem, root along with disintegration of secondary root surface. The diseased patchouli plants wilted completely at a premature stage and could be pulled off easily from the soil Kalita *et al.* (2012).

Joshi (2018) observed symptoms of wilt caused by *F. oxysporum* shows symptoms like wilting during the day, stunted growth and leaf chlorosis while the internal symptoms of the affected vascular tissues become black or brownish in colour. The pathogen is destructive and has been reported to cause huge field crop losses.

### **2.3 Pathogen**

*Fusarium* sp. is the most destructive soil borne fungus, The genus *Fusarium* is a large group of hyaline filamentous fungi firstly described by Link (1809) and it was accepted in an amended form by Fries (1849) for species with fusiform, septate spores described and clearly illustrated by Martius and Von (1842) According to the recent literature, it comprises approximately 200–300 species belonging to 20–22 species complexes (O'Donnell *et al.*, 2013) *Fusarium* are common soil saprophytes; however, they are also known as phytopathogens and transferred to *Fusarium oxysporum* by Schlechtendal.

Michielse and Rep (2009) reported that *F. oxysporum* is genetically diverse, and pathogenic strains have been assigned to *formae speciales* based on host specificity, with over 120 *formae speciales* currently described.

Gordon (2017) authored that *F. oxysporum* is a soilborne fungus that has been in most agricultural fields around the world. It comprises more than 100 host-specific strains (*formae speciales*), many of which have worldwide distributions. All spores known to be produced by *F. oxysporum*, *i.e.*, macroconidia, microconidia and chlamydospores and having several hosts ranging from herbaceous plant to trees.

Joshi (2018) reviewed that the fungal pathogen affects the plants by producing macro and micro conidia that infect the roots of the plants feeding and infecting the vascular tissues leading to wilting and gradual death of the plants.

### **2.4 Isolation and establishment of pure culture**

Booth (1971) described a procedure of carrying out the isolation from plant material for *Fusarium* spp. and for carrying out the single spore isolation.

Garibaldi *et al.* (2004) isolated *Fusarium* spp. consistently and readily from symptomatic vascular tissue on *Fusarium* selective medium from the wilt affected plants. Colonies were identified as *F. oxysporum* after sub-culturing on potato dextrose agar on the basis of morphological observations. Similarly, Garibaldi and Minuto (2007) isolated *F. oxysporum* repeatedly from gerbera plants showing wilting symptoms grown in a soilless cultivation system.

Rao *et al.* (1999) conducted experiments for isolation and identification of the pathogen causing wilt disease, samples of the diseased tissues were surface sterilized with mercuric chloride (0.01 %) solution for one minute. They were then placed aseptically onto potato dextrose agar (PDA) (250 g peeled potato, 20 g dextrose, 20 g agar and 1,000 mL distilled water) medium containing 100 µg/ mL streptomycin sulphate and incubated at 25±2 °C for 4-5 days. The pathogen was isolated on PDA from developing colony using single spore isolation technique.

## **2.5 Pathogenicity**

Healthy rooted 30 day old plants of gerbera were inoculated by dipping roots into a conidial suspension ( $5 \times 10^7$  conidia per mL) of test isolates of *F. oxysporum* transplanted into pots containing Rockwool based substrate. Wilt symptoms and vascular discoloration in the roots, crown and veins developed within 30 days on each inoculated plant, while non-inoculated plants remained healthy. *Fusarium oxysporum* was consistently re-isolated from infected plants (Garibaldi *et al.*, 2004).

Ramaprasadshresti (2005) carried out pathogenicity test for *F. chlamydosporum* by inoculating with spore suspension and homogenized mycelial bits of *F. chlamydosporum* on 30 days old coleus plants. The infected plants were characterized by gradual yellowing and drying of leaves followed by loss of vigour and premature death. The infected plants pulled off from soil showed discolouration of roots and complete destruction of tap and lateral root system. The bark of such plants was easily peeled off and the affected plant was killed finally due to severe infected tubers showed rotting which emitted bad odour.

Garibaldi and Minuto (2007) proved pathogenicity of *F. oxysporum* by dipping healthy roots of gerbera in conidial suspension ( $5 \times 10^7$  conidia per mL), wilt symptoms and vascular discoloration in the roots, crown and veins developed within 30 days on each inoculated plant.

Sreedevi (2007) concluded that artificial inoculation of giant culture of *F. solani* to the sterile soil and planting patchouli seedlings produced characteristic wilt symptoms viz., yellowing of lower leaves after 25 days of inoculation which extended upwards and whole leaves gradually turned brown coloured after 34 days of inoculation. The plant became stunted with plant height 18 cm in inoculated seedlings where as it was 95.6 cm in control at 60 DAS. The death of the plants observed on 60th day and when the infected plant was uprooted dark brown to red discoloration of vascular tissue was observed.

Prasad *et al.* (2008) tested the pathogenicity of the fungi separately on 45 days old geranium plant by artificial inoculation through soil amendment of pathogen isolates by mixing three week old cultures of pathogen grown on autoclaved ragi seeds in steam sterilized soil @ 10g/ pot and observed wilt complex symptoms. Symptoms were manifested as yellowing, stunting, defoliation, and drooping of leaves and branches, roots of affected plants showed severe rotting and lesions developed over the stem at or below the soil line. Finally, the whole plant wilted in a few days.

Bhaskar (2016) tested the pathogenicity of isolated fungus *Fusarium oxysporum* f. sp. *dianthi* (Priel. And Del.) Synd. and Hans. by soil inoculation method. Typical symptoms of the *Fusarium* wilt disease observed after 25-30 days were yellowing of leaves, crook neck shoot, abnormal growth, stunting of young shoots and discoloration of vascular region.

Gogoi *et al.* (2017) experimented pathogenicity test, 30 day old patchouli seedlings were planted in 25 cm diameter plastic pots containing sterilized soil under net-house condition. The mass cultures of *F. solani* were applied to the pots @ 40 g and kept for four days before transplanting of seedlings. Observations were made regularly for the appearance and development of symptoms. After symptom development, re-isolation was

done from the artificially infected plants. The fungus was re-isolated from the artificially inoculated plants and resulting cultures were compared with the original ones.

Meena and Roy (2020) carried out the pathogenicity of isolated *Fusarium* sp. isolate (isbfu-1) was proved by using the fungal inoculum, prepared from actively grown mycelium of the 7 days old culture in sterilized distilled water. A suspension of  $4 \times 10^5$  conidia/mL was adjusted and inoculated in the root zone of 15 days old seedling of *Plantago ovata*.

## **2.6 Cultural studies of the pathogen**

### **2.6.1 Effect of different media**

Reddy and Chaudhary (1985) recorded cultural variations of six isolates of *Fusarium udum* causing wilt of pigeon pea. Radial growth and colony character on different media viz., Potato dextrose agar (PDA), Potato sucrose agar (PSA), Czapek's Dox (CDA) and Oat meal agar (OMA) was recorded. The six isolates differed in their colony characters and were grouped into three groups based upon colony characters on potato sucrose agar (PSA) medium, Group-I showed fluffy and forming concentric rings (isolate-1 and 2), group-II showed cottony, forming sectors (isolate-3) and group-III showed fluffy and sticky growth (isolates-4, 5 and 6).

Khilare and Ahmed (2012) conducted laboratory studies to see the effect of different culture media on mycelial growth of *Fusarium oxysporum* f. sp. *ciceri*. The fungus grew the best on Czapek's Dox Agar and PDA media among six culture media tested.

Kishore (2007) isolated the fungus from wilted carnation plants and was identified as *F. oxysporum* f. sp. *dianthi* (Prill & Declar) Synder & Hans. Potato dextrose agar, Richard's agar, Czapek's Dox agar and oat meal agar supported maximum growth of *F. oxysporum* and maximum dry mycelia weight of *Fusarium oxysporum* was recorded on oat meal broth, Richard's broth and potato dextrose broth.

Chaudhary *et al.* (2018) evaluated the effect of seven media on the growth of *F. udum* which showed that maximum colony diameter (82.0 mm) was recorded on PDA. The next best medium was Richard's agar medium which yielded 79.33 mm colony diameter followed by Czapek's agar medium (56.83 mm). Least colony diameter (45.0 mm) of the test fungus was observed in Coon's agar medium.

Yadav *et al.* (2017) carried out *in vitro* studies to examine the growth of the *Fusarium oxysporum* f. sp. *ciceri* on different solid medium. The best fungal growth was recorded on Potato agar medium followed by Richard's agar medium. Further, the morphological character studies of the fungus carried out on PDA showed that the colony were white, radiating with wine-red pigmentation in the later stage which was clearly visible from the bottom side of the Petri-plate. The mycelium of the fungus was branched, creeping, hyaline, cylindrical, septate and measured 3.2 to 4.6  $\mu$ m in width and microconidia of the fungus were hyaline single celled oval to cylindrical. Macroconidia were ellipsoid oval and cylindrical and possess generally 3- 5 septa and 3.5-4.5 $\times$ 25-65  $\mu$ m, chlamydospores were formed in the old culture at high temperature measured 7.0-8.0 $\times$ 3.5  $\mu$ m.

## 2.7 Morphological characters of the pathogen

Booth (1975) described that most important species among genus *Fusarium* is *Fusarium oxysporum*. In these pathogen produces short, stubby phialides borne laterally on the hyphae or at the apices of short lateral branches. They are much more beautiful and elaborate in *F. solani*, with well-developed conidiophores that branch extensively, and with long delicate phialides often bearing a well-marked apical collarete.

Barnett and Barry (1992) elucidated the morphological characters of *Fusarium* like mycelium extensive and cotton-like in culture, often with some tinge of pink, purple, or yellow in the mycelium on medium. Conidiophores variable, slender, and simple, or stout, short, branched irregularly or bearing a whorl of phialides, single or grouped, conidia (phialospores) hyaline, variable, principally of two kinds, often held in small moist heads. Macroconidia many celled, slightly curved at the pointed ends, typically canoe-shaped.

Microconidia are one-celled, ovoid or oblong, thick-walled chlamydo-spores common in some species.

Kharbanda and Stevens (1988) elucidated the morphology of *F. oxysporum* it produces microconidia in false heads born on short monophialides (conidiophores), macroconidia are delicate looking, slightly curved and apical cell is attenuated.

Leslie and Summerell (2006) described colony morphology on PDA vary widely. Mycelia may be floccose, sparse or abundant and range in colour from white to pale violet. Abundant pale orange or pale violet. *F. oxysporum* usually produces a pale to dark violet or dark magenta pigment in the agar but some isolates produce no pigment at all. Some isolates of *F. oxysporum* mutate readily to the pionnotal form or to a flat “wet” mycelial colony with a yellow to orange appearance when cultured on PDA. Also described spore characters of *F. oxysporum* it produces macroconidia which are short to medium length, straight to slightly curved, relatively slender and thin walled, apical cell morphology tapered and curved, sometimes with a slight hook, basal cell foot shaped to pointed, usually three septate. Microconidia are oval, elliptical or kidney shaped and usually no septations on short monophialides. Chlamydo-spores formed either terminal/intercalary on surface of hyphae which is mainly smooth or rough walled.

Rao *et al.* (1999) showed that the wilt causing pathogen comprised mainly of microconidia formed from phialides on short branches. The microconidia were unicellular, oval to cylindrical shaped, rarely curved and measured 7-14×3.0-4.1 µm. Macroconidia were hyaline, falcate, 3-6 septate and measured 20-25×4.5-5.5 µm. On the basis of cultural characters and formation of macroconidia, microconidia, the pathogen causing wilt disease was identified as *F. oxysporum* var. *redolens* (Wollenweb.).

Zheng *et al.* (2012) described microconidia of the isolates were abundant, generally unicellular, oval to reniform, 5.5-7.5×2.3-5.0 µm, and formed in false heads on short monophialides 5.0-12.8×2.1-3.3 µm. Macroconidia were also abundant, sickle shaped with attenuated apical cells, foot shaped basal cells, thin walled measuring 22.5–50.5×3.0–5.0 µm with 3 to 5 septa (mostly three septate). Chlamydo-spores were abundant, globose,

single celled, terminal or intercalary, produced singly or in pairs and 7.0–10.0 µm in diameter. Dimensions and morphology of microconidia, macroconidia and chlamydospores as well as descriptions of conidiogenesis were similar to those reported by Booth (1971) and Nelson *et al.* (1983).

## **2.8 Physiological studies of the pathogen**

### **2.8.1 Effect of temperature**

Cruz *et al.* (2019) carried out the *in vitro* effect of different temperatures on growth of soybean seedling disease caused by *F. oxysporum*, they tested different temperatures (15, 20, 25 & 30) and the temperature 25 °C shows the maximum mycelium growth.

Yadav *et al.* (2014) tested the effect of different levels of temperature (15, 20, 25, 30 and 35 °C) on growth and sporulation of the pathogen causing basal rot of onion *F. oxysporum* showed maximum growth of mycelia observed at 25 °C (86.50 mm).

Chaudhary *et al.* (2018) examined the effect of different temperatures on the growth of pigeon pea wilt pathogen *F. udum*. The pathogen growth was maximum at 30 °C after seven days of inoculation, which was reduced drastically below 10 °C and above 35 °C.

Pal *et al.* (2019) carried out an *in vitro* experiment to find out the suitable temperature, growth and sporulation of *Fusarium oxysporum* f. sp. *lini*. Study showed that after nine days of incubation, the maximum growth of the fungus was 88.33 mm at 24 °C with highest growth rate of 9.81 mm per day and highest sporulation of  $7.9 \times 10^6$  per mL.

### **2.8.2 Effect of hydrogen ion (pH) concentration**

Srivastava *et al.* (2011) recorded that most suitable pH for the growth of *Fusarium oxysporum* was 6.0 and growth decreased with increase in pH.

*In vitro* studies were conducted to check the effect of pH on the growth and sporulation of *Fusarium oxysporum*. After two weeks incubation of culture in Potato dextrose broth. It was observed that pH level 6.0 is the optimum pH for the growth as well

as sporulation of the fungus. Further increases in the pH level showed retarding effect on the growth and sporulation (Tyagi and Paudel, 2014).

Yadav *et al.* (2014) noted the effect of different levels of pH (5.5, 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5) on growth and sporulation of the pathogen *in vitro*. Maximum dry weight of mycelia and sporulation the pathogen causing basal rot of onion *F. oxysporum* observed at 6.5 (280 mg).

Chaudhary *et al.* (2018) studied the effect of different levels of pH on the growth of pigeon pea wilt pathogen *F. udum* and found that the most suitable pH level for growth of fungus was 6.0 and 6.5 with excellent sporulation.

Pal *et al.* (2019) tested pH for the growth and sporulation of *F. oxysporum* f. sp. *lini*. Study showed that in case of pH, maximum growth of the fungus was 86.33 mm at pH 5.5 with highest growth rate of 9.59 mm per day and highest sporulation of  $8.2 \times 10^6$  per mL after nine days of incubation at  $25 \pm 2$  °C.

Khilare and Ahmed (2012) concluded that the most suitable pH level for growth of fungus was 6.0 and 6.5. The effect of pH was observed highest at 6.0 with sporulation 24.70 conidia/ $\mu$ L. However, it was found that the range from 4.5 to 8.0 was suitable for the growth of *F. oxysporum* f. sp. *ciceri*. The foremost acidic and alkaline pH was not suitable for the growth of pathogen.

Kumar *et al.* (2019) noted that the most suitable pH level for growth of fungus *F. verticillioides* causing crown rot of guava was 6.0 and 6.5 with excellent sporulation.

## **2.9 Molecular characterization**

Zheng *et al.* (2012) first reported *F. oxysporum* infecting coleus (*Coleus forskohli*) in China. They carried out genomic DNA extraction, PCR amplification the internal transcribed spacer (ITS) region of ribosomal DNA and the  $\beta$ -tubulin. Later, gene sequences were confirmed *F. oxysporum* as the causal agent.

Singha *et al.* (2016) isolated *Fusarium* sp. from Assam. Molecular identification of *Fusarium* isolates were done by amplifying the internal transcribed spacer (ITS) region of the conserved ribosomal DNA using primers ITS-1 and ITS-4. All the ITS sequences were compared for gaps and similarity. It was concluded that molecular profiling using ITS is an indispensable method for identification studies.

Meena and Roy (2020) characterized *Fusarium* sp. causing wilt disease on isabgol (*Plantago ovata* Forsk.) using molecular tools such as ITS, translation elongation factor (EF-1 $\alpha$ ) and RNA polymerase II (RPB2) and phylogenetic tree was constructed, which confirmed the association of *Fusarium oxysporum* species complex with the wilt disease of *P. ovata*.

Kuppusamy *et al.* (2018) confirmed *F. oxysporum* isolated from *Allium cepa* by using molecular tools such as genomic DNA isolation, PCR amplification of ITS region was performed by using universal ITS primers.

## **2.10 Management studies**

### **2.10.1 *In vitro* evaluation of botanicals**

Among the ten botanicals tested *in vitro* against *F. oxysporum* f. sp. *gladioli*, neem seed kernel extract at 10 per cent (54.49 %) was found superior followed by custard apple leaf extract at 10 per cent (52.50 %) (Kulkarni Sumithra, 2006).

Among the 11 plants extracts evaluated against *F. oxysporum* f. sp. *dianthi* clove oil found most effective as evidenced by cent percent inhibition of mycelial growth followed by garlic extract. Chilli and neem were less effective and *Clerodendrum inerme*, *Pongamia pinnata* and *Tridax procumbens* were completely failed to inhibit the mycelial growth (Kishore, 2007).

Joseph *et al.* (2008) assessed *in vitro* efficacy of different plant extracts *viz.*, *Azardiachta indica*, *Artemessia annua*, *Eucalyptus globulus*, *Ocimum sanctum* and *Rheum emodi* were tested to control brinjal wilt pathogen. Different concentrations 5, 10, 15 and 20 per cent of plant extracts was used in the study. All the plant extracts showed significant

reduction in the growth of pathogen. Among the different extracts 20 per cent of *A. indica* was found most effective followed by *R. emodi*, *Eucalyptus globulus*, *A. annua* and *O. sanctum*.

Chavan and Hegde (2009) tested *in vitro* efficacy of plant extracts against *F. solani* infecting patchouli, results showed that neem seed kernel extract proved to be the most effective botanical and recorded the maximum inhibition of growth (68.43 %) which was significantly superior to all other plant extracts. Garlic (56.93 %) and eucalyptus (50.68 %) were next best followed by parthenium (47.81 %). Tulsi (39.30 %) and clerodendron (39.15 %) were at par to each other extracts in order of supenoxy in comparison to control were pongamia (37.92 %), bougainvillea (35.57 %) and durantha (31.28 %) which differ significantly to each other. Least inhibition was observed with glyricidia (19.97 %).

Kadam *et al.* (2014) carried out *in vitro* experiments using various plant extracts against the *F. oxysporum* f. sp. *gladioli* and showed that complete inhibition of mycelial growth with 10 % jatropa extract, followed by soapnut (37.00 %), neem seed kernel extract (35.00 %), cassia (26.44 %), neem leaf extract (22.00 %), bulb extract of garlic (17.22 %). Clove, castor, nilgiri, cinnamon and sarpagandha were least effective against the fungus.

Sunderrao *et al.* (2017) evaluated antagonistic activity of different botanicals against *F. oxysporum* f. sp. *dianthi* by poisoned food technique *in vitro*. Least growth of pathogen was recorded in neem leaf extract which show excellent inhibitory effect (78.19 %). Next best in order of merit was eucalyptus extract (75.87 %) followed by ashoka extract (72.48 %) and calotropis extract (65.22 %) and least by others. Among the neem oil cake and datura extract maximum growth inhibition of (62.09 %) and (60.27 %) over control, respectively.

Hegde *et al.* (2017) studied the effect of plant extracts on the fungal growth of which garlic extract (89.44 %) was found effective in inhibiting mycelial growth which was superior over all other plant extracts and it was followed by neem leaf extracts (68.32 %) and pongamia extract (62.58 %). Least inhibition was observed in agave (33.07 %) followed by marigold (41.84 %).

Ghante *et al.* (2019) evaluated the antagonistic activity of botanicals viz., *A. cepa*, *L. camara*, *O. sanctum*, *Gliricidia sepium*, *A. indica*, *Allium sativum*, *Bougainveillia spectabilis*, *Moringa oleifera*, *Eucalyptus globulus*, *Pongamia pinnata*, *Vinca rosea* and *Asparagus racemosus in vitro* for their antifungal activities against wilt of pigeon pea, *F. udum* using poison food technique. Out of which significantly highest inhibition of the fungus was seen in *A. indica* (77.23 %) followed by *A. sativum* (76.11 %), *O. sanctum* (64.08 %), *A. cepa* (59.26 %), *E. globulus* (60.19 %), *L. camara* (57.41 %), *P. pinnata* (56.30 %) and *B. spectabilis* (28.89 %).

### **2.10.2 In vitro evaluation of bio agents**

Chavan and Hegde (2009) evaluated six bio agents against *F. solani* *Trichoderma harzianum* (Dharwad isolate) inhibited maximum (78.11 %) mycelial growth followed by *T. koningii* (75.63 %) which was significantly superior to all other bio agents tested. Next best was *T. viride* (74.92 %) being at par to *T. koningii*. However, *Bacillus subtilis* (57.02 %) and *Pseudomonas fluorescens* (65.10 %) were least effective in inhibiting mycelial growth of the pathogen.

Naik *et al.* (2010) evaluated the ability of bio-control agents in suppressing the growth of *F. oxysporum* f. sp. *vanilla* causing stem rot in vanilla under *in vitro* by employing dual culture technique. *T. harzianum*, *P. fluorescens* and *B. subtilis* inhibited the growth of pathogen. In nature microbial interactions involve competition, hyper parasitism or antibiosis and these phenomena play an important role in striking ecological balance and keeping several plant pathogens in check.

*T. viride*, *T. harzianum* and *P. fluorescens* were evaluated for their antagonistic activity against *F. oxysporum in vitro*. The conidia of *F. oxysporum* were found to be inhibited by all the three antagonistic microorganisms. The highest per cent inhibition of conidial germination was brought out by *T. viride* followed by *T. harzianum* and *P. fluorescens* and inhibition of radial mycelia growth were 86.6, 84.0, 60.0 per cent respectively (Rajeswari and Kannabiran, 2011).

Mahalakshmi and Yesu Raja (2013) tested the antagonistic potential of four isolates of *T. viride*, eight isolates of *T. harzianum* and five isolates of *T. reesei* against *F. oxysporum* f. sp. *dianthi*. *T. harzianum* (Th2) showed the maximum inhibition of mycelial growth of *F. oxysporum* f. sp. *dianthi* (45.33 %) over control followed by *T. harzianum* (Th6) and *T. viride* (Tv3) with 42.11 and 38.66 per cent inhibition respectively.

Kumari *et al.* (2014) studied *in vitro* evaluation of bio agents against mycelial growth of the causal pathogen *F. oxysporum* f. sp. *cubense* by dual culture technique. Among the biocontrol agents *T. viride* completely inhibited test pathogen mycelial growth.

Ajay and Naik (2013) evaluated the efficacy of some biocontrol agents against the isolated wilt pathogen of castor *in vitro*. Among the fungal bio agents examined against *Fusarium oxysporum* f. sp. *ricini*, by dual culture method *T. viride* recorded the maximum inhibition of 92.35 per cent and minimum inhibition of mycelial growth was recorded in *T. harzianum* (84.37 %).

Hegde *et al.* (2017) experimented among the different bio agents, maximum 64.44 per cent reduction in colony growth was observed in *T. harzianum* (UAHS isolate) which is on par with *T. harzianum* (UASD isolate) (62.91 %). Least reduction of colony growth was observed in *P. fluorescens* (UAHS) (47.74 %) against *F. oxysporum* f. sp. *dianthi* causing wilt of carnation.

### **2.10.3 *In vitro* evaluation of fungicides**

Among the systemic fungicides, carbendazim was highly effective in inhibiting the growth of *F. oxysporum* f. sp. *gladioli* in all the concentrations (0.025, 0.05 and 0.1 %). Among the non-systemic fungicides, mancozeb at 0.3 per cent was found significantly superior over other fungicides in inhibiting *F. oxysporum* f. sp. *gladioli* (Kulkarni Sumithra, 2006).

Nel *et al.* (2007) concluded that the fungicides, prochloraz and propiconazole significantly inhibited mycelial growth at concentrations of 1 and 5 µg/mL, respectively. The fungicide benomyl and the DMI fungicides prochloraz, propiconazole and

cyproconazole/ propiconazole significantly reduced the incidence of *Fusarium oxysporum* f. sp. *cubense* with the root dip and soil drench treatments 1 week after planting up to 80.6 per cent.

Prasad *et al.* (2011) found that systemic fungicides, carbendazim + mancozeb and carbendazim alone completely inhibited the mycelial growth even at 100 and 200 ppm, whereas among non-systemic fungicides, thiram was found to be the most effective and gave 52.77, 62.77 and 85.00 per cent inhibition at 500, 1000 and 1500 ppm concentrations, respectively. Thiram was found to be most effective fungicides in inhibiting the both fungi (68.84 and 58.69 %) followed by the indofil M 45 (47.59 and 51.38 %).

Kumari *et al.* (2014) conducted *in vitro* studies on four fungicides mancozeb, saaf, carbendazim and cuprozin in three different concentrations (0.01 %, 0.02 % and 0.03 %) among which carbendazim at its all concentrations was found to be the most effective against the pathogen followed by saaf.

Bhapkar (2016) tested the efficacy of four fungicides *in vitro* against *F. oxysporum* f. sp. *dianthi*. Among fungicides Carbendazim @ 0.1 percent recorded highest mycelial growth inhibition (84.85 %), followed by captan @ 0.2 per cent which accounted 66.28 per cent mycelial growth inhibition.

Hegde *et al.* (2017) evaluated a total of nine fungicides *in vitro* against *Fusarium oxysporum* f. sp. *dianthi* causing wilt of carnation. Out of which systemic fungicides such as Carbendazim, Propiconazole, Difenoconazole were found effective at all concentrations tested and contact fungicide such as Chlorothalonil and Mancozeb were found effective at higher concentrations.

Bhimani *et al.* (2018) evaluated different fungicides against fenugreek wilt caused by *F. oxysporum*. Among the different contact fungicides tested copper oxychloride 50 % WP and copper hydroxide 77 % WP, were found to be effective against mycelial growth of the pathogen.

Desai *et al.* (2017) evaluated different fungicides against *F. oxysporum* among them combination products of fungicides, pyraclostrobin + metiram, captan + hexaconazole were most effective and gave 100 cent per cent inhibition of test fungi.

### III MATERIAL AND METHODS

Studies on *Fusarium* wilt in scented geranium [*Pelargonium graveolens* (L.) Herit] were carried out during 2019-2020 in the glasshouse and laboratory at the Project Coordinating Unit (Small millets), Zonal Agricultural Research Station, Gandhi Krishi Vignana Kendra, Bengaluru. Samples from geranium plants showing symptoms typical to *Fusarium* wilt were collected from different geranium growing areas of Tumakuru, Bengaluru Rural and Hassan districts and further study was carried out. The details of the material and methodologies adopted during the study were described below.

#### 3.1 General laboratory procedure

##### 3.1.1 Cleaning and sterilization of glass wares

The glass wares were cleaned by following usual laboratory method. The glass wares were kept in solution containing 60 g potassium dichromate ( $K_2Cr_2O_7$ ) for about 1 hour and 60 mL of concentrated sulphuric acid ( $H_2SO_4$ ) in one litre of water and cleaned thoroughly with detergent solution followed by washing under running tap water and rinsed twice in distilled water. They were air dried and sterilized in an autoclave at 1.1 kg/cm<sup>2</sup> pressure for 15 minutes at 121 °C and these sterilized glasses were kept in hot air oven at 55 °C for one hour.

##### 3.1.2 Media composition and their preparation

In the present investigation potato dextrose agar (PDA) was used for isolation and further culturing of the pathogen and also for culturing fungal bio agents. Nutrient agar (NA) was used for culturing bacterial bioagents.

###### 3.1.2.1 Media composition was given below

###### A) Potato Dextrose Agar (PDA)

Peeled potatoes	:	200 g
Dextrose	:	20 g
Agar agar	:	20 g
Distilled water	:	1000 mL
pH	:	6.0

## B) Nutrient Agar (NA)

Peptone	:	5.0 g
Beef extract	:	3.0 g
Agar agar	:	15.0 g
NaCl	:	5.0 g
Distilled water	:	1000 mL
pH	:	6.0

### 3.1.2.2 Preparation of media:

#### 1. Potato Dextrose Agar (PDA) medium

Peeled potato pieces of 200 g were boiled in distilled water for half an hour till the pieces get softened and the extract was filtered using a double layered muslin cloth. To this extract 20 g of Dextrose was added and dissolved. In another one-litre beaker 20 g of Agar agar was melted in 500 mL of distilled water. Then both the solutions were mixed and the volume was made to 1000 mL with distilled water. The pH of the medium was adjusted to 6.0 by using 1 N NaOH or 1 N HCl and sterilized in an autoclave.

#### 2. Nutrient agar (NA) medium.

All the components mentioned in the composition were weighed accordingly and mixed in one litre of distilled water and boiled till the contents get dissolved completely and sterilized.

### 3.2 Collection and isolation of the pathogen causing wilt in geranium

#### 3.2.1 Collection, isolation and Purification of the fungus

The plants showing typical wilt symptoms were collected from geranium growing fields of Tumakuru, Bengaluru rural and Hassan districts. The stem and root portion of the infected plants was cut open to examine the presence of mycelia growth in the vascular tissue. Thin sections of the discoloured vascular tissue were kept on a clean glass slide having a drop of lactophenol and then a drop of cotton blue stain was added. The specimen was then covered with a cover slip and observed under microscope and examined for the

confirmation of the fungus. After confirmation of the fungus as *Fusarium* sp. infected tissue was selected and pathogen was isolated by following standard tissue isolation method. Isolates designation, place of collection is listed in below table.

Sl. No.	Isolate designation	Place of sample collection	State
1	Isolate GFO 1	Tiptur, Tumakuru	Karnataka
2	Isolate GFO 2	Hosakote, Bengaluru Rural	Karnataka
3	Isolate GFO 3	Arsikere, Hassan	Karnataka

Infected root and stem portion from collar region were cut into small bits along with healthy tissue and washed with sterilized water. These bits were surface sterilized with sodium hypochlorite (0.1 %) for two minute and subsequently the pieces were washed thrice with sterile distilled water for one minute each time and then the bits were transferred aseptically to Petri plates containing potato dextrose agar. The Petri plates were incubated at  $27\pm 2$  °C temperature in BOD incubator for a week. After the growth of fungus starts, a loop full of mycelia was taken on a glass slide and observed under microscope for the presence of conidia.

The pure culture of the fungus was obtained by a standard procedure of carrying out the isolation from plant material for *Fusarium* sp. and for carrying out the single spore isolation as described by Booth (1971).

This pure culture was maintained on PDA slants in refrigerator at  $5\pm 2$  °C temperature and again sub-cultured once in a month during the course of investigation under aseptic condition to maintain the viability of the pathogen.

### 3.2.2 Identification of the pathogen

The identification of the isolated pathogen was done based on the cultural and morphological characters such as micro and macro conidial characters and the presence of prominent hook at the base of the macroconidia and other spore characters with the help of monograph entitled “ The *Fusarium*” authored by Booth in 1975, Illustrated genera of

Imperfect Fungi (Barnett and Barry, 1992) and CMI descriptions and Also by The *Fusarium* Laboratory Manual (Leslie and Summerell, 2006).

### **3.2.3 Pathogenicity**

Pot inoculation or sick pot method was followed for proving pathogenicity of the fungus in order to fulfil Koch's postulate of the isolated fungus *Fusarium* sp. by establishing its capability to produce typical symptoms on scented geranium cuttings. The pathogen isolates were mass multiplied on sorghum grains as substrate. The conical flasks with semi-cooked sorghum (100 g in 250 mL conical flask) presented in (Plate 4) were sterilized by autoclaving at 15 psi for 20 min at 121 °C. The flasks were inoculated by actively growing fungal mycelial culture and incubated at 28±2 °C for 15 days. The pots were filled with sterile soil mixture, soil: sand: farm yard manure in 3:1:1 ratio. Further, sick soil will be prepared by inoculating 4 g per Kg of giant culture of *Fusarium* sp. isolates which were grown on sorghum to the sterile soil. A control treatment will be maintained without adding the inoculum and plants were watered regularly. Observations will be made regularly for the appearance and development of symptoms. After symptom are development, re-isolation was done from the artificially infected plants. The symptoms observed in inoculated plant and culture obtained was compared with the original symptoms and original culture for confirmation.

## **3.3 Cultural and morphological characters of pathogen causing wilt in geranium**

### **3.3.1 Effect of different media on the growth of *Fusarium* sp.**

The study was conducted to describe the cultural characters *viz.*, colour, type of margin, surface, topography, pigmentation, texture and sporulation of the pathogen and morphological characters such as hyphal, conidiophore and conidial characters on different solid media. Five mm culture discs of the pathogen will be inoculated separately on different media *viz.*, potato dextrose agar, corn meal agar, malt extract agar, Sabouraud's dextrose agar, Richard's synthetic agar, Czapek's Dox agar, rose Bengal agar, oat meal agar, carrot agar, vegetable juice (V<sub>8</sub>) agar and water agar and incubated at 27±1 °C for 7 days. The cultural characteristics and the colony diameter (mm) on each medium will be recorded.

The composition of different solid media used during the study was given below:

**1. Czapek's Dox Agar:**

Sucrose	: 30 g
Sodium nitrate	: 20 g
Dipotassium hydrogen Phosphate.	: 1 g
Magnesium sulphate.	: 0.5 g
Ferrous sulphate	: 0.01 g
Potassium chloride	: 0.5 g
Agar	: 15 g
Sterile distilled water	: 1000 mL

All the chemical ingredients excluding agar were dissolved in 500 mL water and agar was melted separately in distilled water 500 mL. Both the solutions were mixed thoroughly and mixed solution was sterilized at 121 °C and 1.1 kg/cm<sup>2</sup> pressure for 15 min and preserved for further use.

**2. Carrot Agar:**

Fresh carrots	: 200.0 g
Agar	: 20.0 g
Dextrose	: 20.0 g
Distilled water (Volume to make up)	: 1000.0 mL

Carrot were peeled off and cut into small pieces and were boiled in 400 mL of distilled water and extract was filtered through the muslin cloth. Then 20 g of dextrose was dissolved in the extract. 20 g of Agar agar was dissolved in another 500 mL distilled water and mixed with the extract and the volume was made to 1000 mL and sterilized at 121 °C and 1.1 kg/cm<sup>2</sup> pressure for 15 min.

### 3. Malt Extract Agar:

Malt extract powder	: 25 g
Agar agar	: 20 g
Sterile distilled water	: 1000 mL

Malt extract powder was dissolved in 500 mL of distilled water and 20 g of agar was melted separately in 500 mL of distilled water. Both the solutions were mixed thoroughly and mixed solution was sterilized at 121 °C and 1.1 kg/cm<sup>2</sup> pressure for 15 min and preserved for further use.

### 4. Oat Meal Agar:

Oat meal powder	: 30 g
Agar agar	: 20 g
Sterile distilled water	: 1000 mL

Oat meal powder was dissolved in 500 mL of distilled water. Agar (20 g) was melted separately in 500 mL distilled water. Both the solutions were mixed thoroughly and mixed solution was sterilized at 121 °C and 1.1 kg/cm<sup>2</sup> pressure for 15 min and preserved for further use.

### 5. Potato Dextrose Agar:

Potato dextrose broth	: 24 g
Agar agar	: 20 g
Sterile distilled water	: 1000 mL

Ready to use 24 g of potato dextrose broth was dissolved in 500 mL of distilled water. Agar was melted separately in 500 mL of distilled water. Both the solutions were mixed thoroughly and mixed solution sterilized at 1.1 kg/cm<sup>2</sup> pressure for 15 min and preserved for further use.

## 6. Richard's Synthetic Agar:

Sucrose	: 50 g
Potassium dihydrogen Phosphate	: 5 g
Potassium nitrate	: 10 g
Magnesium sulphate	: 2.5 g
Ferrous chloride	: 0.02 g
Agar agar	: 20 g
Sterile distilled water	: 1000 mL

All the ingredients except potassium dihydrogen phosphate were dissolved in 450 mL distilled water. Agar melted in 500 mL of distilled water was mixed with the above solution and sterilized at 121 °C and 1.1 kg/cm<sup>2</sup> pressure for 15 min and preserved for further use. Potassium dihydrogen phosphate dissolved separately in 50 mL water and mixed together with the sterilized medium at the time of pouring to plates.

## 7. Rose Bengal Agar:

Peptone	: 5 g
Dextrose	: 10 g
Magnesium sulphate	: 0.5 g
Potassium phosphate	: 1 g
Rose Bengal	: 0.05 g
Chloramphenicol	: 0.1 g
Agar agar	: 15 g
Sterile distilled water	: 1000 mL

All the ingredients excluding agar were dissolved in 500 mL of distilled water and agar was melted separately in 500 mL of distilled water. Both the solutions were mixed thoroughly and sterilized at 121 °C and 1.1 kg/cm<sup>2</sup> pressure for 15 min and preserved for further use.

### 8. Saboraud's Dextrose Agar:

Peptone	: 10 g
Dextrose	: 40 g
Agar agar	: 15 g
Sterile distilled water	: 1000 mL

All the ingredients were dissolved one by one in 500 mL distilled water and agar was dissolved separately in 500 mL distilled water and mixed with the above solution and sterilized at 121 °C and 1.1 kg/cm<sup>2</sup> pressure for 15 min and preserved for further use.

### 9. Water Agar:

Agar agar	: 20 g
Sterile distilled water	: 1000 mL

Twenty grams of agar was melted in 500 mL of distilled water. The volume was made up to 1000 mL with distilled water and sterilized at 121 °C and 1.1 kg/cm<sup>2</sup> pressure for 15 min and preserved for further use.

### 10. Vegetable (V8) juice Agar:

V8 juice (100 mL)	: 8.3 g
L-Asparagine	: 10 g
Yeast extract	: 2 g
Calcium carbonate	: 2 g
Glucose	: 2 g
Agar	: 2 g
Sterile distilled water	: 1000 mL
Final pH (at 25 °C)	: 5.7±0.2

Suspend 44.3 grams of agar mixture in 1000 mL of distilled water. Heat just to boiling. Sterilize by autoclaving at 15 lbs. pressure, 121 °C for 15 minutes and preserved for further use.

## 11. Corn Meal Agar:

Corn meal, infusion form	: 50 g
Agar agar	: 15 g
Sterile distilled water	: 1000 mL
Final pH (at 25 °C)	: 6.0 ± 0.2

Suspend 17 grams in 1000 mL purified/ distilled water. Heat to boiling to dissolve the medium completely. If desired add 1 per cent polysorbate 80. Sterilize by autoclaving at 15 lbs. pressure (121 °C) for 15 minutes.

Twenty mL of each solid media was poured into 90 mm diameter Petri plates. Such medium was replicated thrice. Five mm culture disc of pathogen was taken from the periphery of 10 days old colony grown on PDA and placed at the Centre of the plate. Such plates were incubated at 27±1 °C for 15 days. The sporulation was graded as follows

Sl. No	Grade	Conidia per microscopic field under 40x	Indication
1	Excellent	>100	++++
2	Good	50-100	+++
3	Moderate	20-50	++
4	Poor	1-20	+
5	No sporulation	-	-

### 3.3.2 Morphological characters of *Fusarium* sp.

#### 3.3.2.1 Morphological characters

All morphological related study of the fusarium isolates was performed from 10 days old single spore isolated cultures on PDA. For morphological studies initial identification of isolates involved examination of hyphae under a compound microscope (at magnifications 10 X, 40 X, 100 X) with hyphae mounted in water or stained with lactophenol-cotton blue. Later, observations were made on spore characters like production of microconidia, macroconidia type of conidiophore and their size, shape, no. of septations

and apical cell morphology were recorded by considering at least average of 10 conidia and measurements obtained on various characters of spores was tabulated and they were compared with the standard descriptions.

### **3.3.2.2 Spore germination studies**

Spore suspension of *Fusarium* sp. was prepared separately by using double distilled water. Germination of spore, time taken for germination and length of germ tube was measured at different intervals of time *i.e.*, from 1 hour to 5 hours and type of germination was also recorded, after germination of spore by following hanging drop technique where edges of coverslips were sealed with Vaseline using toothpick to prevent evaporation of water.

### **3.3.3 Physiological studies**

#### **3.3.3.1 Effect of temperature on the growth of *Fusarium* sp.**

This study was conducted to observe the influence of different temperatures on growth and sporulation of pathogen. The fungal growth was tested at 20, 25, 30, 35 and 40 °C. For each treatment, three replications were maintained. Twenty-five mL of potato dextrose broth (PDB) was added into each of 100 mL conical flask and sterilized at 1.1 kgcm<sup>-2</sup> pressure for 15 minutes at 121 °C. The flasks were allowed to cool after sterilization. Later, the flasks were inoculated with 5 mm disc of fungus which was collected from 15 days old culture and incubated at respective temperatures. The mycelial mat was harvested by filtering through Whatman No.1 filter paper of 9 cm diameter and dried. The dry mycelial weight was recorded. Similarly, 15 to 20 mL of PDA was poured into petri plate three replications were maintained in case of solid media study they were incubated at different temperatures by setting the temperature in the incubators. After that radial mycelial growth of test fungus were recorded at 10 days after inoculation and these results were analysed statistically.

### **3.3.3.2 Effect of hydrogen ion concentration (pH) on the growth of *Fusarium* sp.**

This study was conducted to observe the influence of pH on growth and sporulation of pathogen. Potato dextrose broth and Potato dextrose agar medium was adjusted by using digital pH meter to various pH levels *viz.*, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0 and 7.5 and with 1N Hydrochloric acid (HCl) and 1N Sodium hydroxide (NaOH). Each treatment was replicated thrice. The pH adjusted media was autoclaved at 121 °C and 15 lbs. pressure for 15 minutes. 15 to 20 mL of PDA was poured into petri plate in case of solid media study. The media was seeded with 5 mm disc of 15 days old mycelial culture and mycelial growth was observed periodically. Similarly, in case of liquid media 20-25 mL of broth was prepared in 100 mL conical flask, the media was seeded with 5 mm disc of 15 days old mycelial culture and mycelial growth was observed periodically at the end of incubation period, growth was harvested and filtered by using previously weighed Whatman No.1 filter paper and it was dried at 60 °C for two days in hot air oven and dry weights were recorded repeatedly until the readings become constant. The difference in weight of mycelia was averaged and analysed statistically. Radial mycelial growth of test fungus at various pH were recorded at 10 days after inoculation and these results were analysed statistically.

### **3.4 Molecular characterization of *Fusarium* sp. infecting geranium.**

Fungal DNA of *Fusarium* isolates were extracted from the established pure culture by using standard CTAB protocol and genomic DNA of the *Fusarium* sp. were amplified in PCR using fungal universal primers (ITS1/4) and sequencing was done. Based on the sequence, phylogenetic analysis was carried out.

### 3.4.1 Extraction of fungal DNA by CTAB method.

#### i) Stock solutions

**Table 1. List of reagents and their composition used to make stock solution for DNA extraction**

Sl. No.	Reagents	Composition
1	1 M Tris-HCl (pH-8)	Dissolve 121.1 g of Tris hydroxyl methane aminomethane in 800 mL distilled H <sub>2</sub> O. Adjust pH to 8.0 with NaOH. Make up to 1 liter. Dispense into aliquots. Sterilize by autoclaving.
2	0.5 M EDTA (pH 8.0)	Add 186.1 g of sodium ethylene diamine tetra-acetate. 2 H <sub>2</sub> O to 500 mL of H <sub>2</sub> O. Stir vigorously on a magnetic stirrer. Adjust the pH to 8.0 with NaOH. Make up to 1 liter. Dispense into aliquots and sterilize by autoclaving.
3	5M NaCl	Dissolve 292.2 g of NaCl in d H <sub>2</sub> O and make up to 1 liter. Dispense into aliquots sterilize by autoclaving.
4	3M Sodium acetate (pH 4.8-5.2)	Dissolve 408.1 g of sodium acetate in 800 mL of distilled water. Adjust the pH to 4.8 to 5.2 with glacial acetic acid. Make up to 1 liter. Dispense into aliquots. Sterilize by autoclaving.
5	2 % CTAB	Dissolve 2 g of CTAB in 100 mL of double distilled water.
6	CTAB Buffer (100 mL)	Following chemicals were mixed to prepare 100 mL of CTAB buffer 5M NaCl – 28 mL 1M Tris HCl (pH.8) – 10 mL 0.5M EDTA (pH.8)– 4 mL 2 % CTAB – 2 g β-mercaptoethanol – 200 μL Final volume was maintained to 100 mL by adding 55.8 mL of double distilled water.
7	70 % Ethanol	70 mL of 99.9 % ethanol was mixed with 30 mL of double distilled water and kept in refrigerator for cooling.
8	TE buffer	1 M Tris HCl (pH.8) – 10 mL. 0.5 M EDTA (pH.8)– 2 mL Final volume makeup to 1 Lt with ddH <sub>2</sub> O.
9	Phenol: Chloroform: Isoamyl alcohol (25:24:1)	25 mL of equilibrated phenol and 24 mL of Chloroform was mixed with 1 mL of Iso amyl alcohol to make 100 mL solution of 25:24:1 (P:C: I).
10	Chloroform: Isoamyl alcohol (24:1)	96 mL of Chloroform was mixed with 4 mL of Isoamyl alcohol to make 100 mL solution of 24:1 (C: I).

## ii) Extraction methodology of fungal genomic DNA

Genomic DNA was extracted following the protocols developed by Murray and Thompson (1980) with slight modifications.

- For DNA extraction, the cultures were grown in potato dextrose broth (PDB; pH 5.5) for 5 to 7 days at  $28 \pm 1$  °C in a shaker incubator at 100 rpm.
- Filtered mycelium (200 to 500 mg) was ground to fine powder in liquid nitrogen.
- Transferred the grinded mycelium in 2 mL Eppendorf tube with 1 mL of CTAB buffer and kept at 65 °C for one hour with occasional stirring in between every 15 minutes.
- The samples will then centrifuge at 10,000 rpm for 10 min at room temperature.
- Supernatant will be collected in another 2 mL Eppendorf tube.
- Equal volume of Phenol: Chloroform: Isoamyl alcohol (25:24:1) was added to tube, followed by centrifugation at 10,000 rpm for 10 min.
- Supernatant will be again collected in another 2 mL Eppendorf tube and equal volume of Chloroform: Isoamyl alcohol (24:1) mixed, followed by centrifugation at 10,000 rpm for 10 min.
- Pipette out the upper aqueous phase in a fresh 1.5 mL Eppendorf tube and mixed with 0.1 volume of sodium acetate (3M) and 0.6 volume of ice-cold isopropanol for DNA precipitation.
- Samples were incubated at -20 °C/ -80 °C for 30 minutes.
- After thawing of samples at room temperature centrifuged again at 13,000 rpm for 15 minutes to pellet the DNA.
- Supernatant was decanted and DNA pellet washed with 70 % ethanol, dried at room temperature was finally dissolved in 50-100  $\mu$ L TE (10mM Tris-HCl, pH 8.0; 1mM EDTA) buffer for further use.
- If the pellets are not dissolved properly in TE buffer, then it is advised to keep the tube in water bath for 5 to 10 min at 65 °C and then store it in -20 °C.
- Check the quality and quantity of DNA with Nano drop spectrophotometer as well as running it on 0.8 % agarose gel electrophoresis.

### 3.4.2 Qualitative and quantitative analysis of DNA

Quality and quantity of DNA was checked by two methods.

#### 1. Agarose Gel Electrophoresis (AGE):

Agarose gel about 0.8 per cent was prepared by dissolving 0.8 g Agarose in 100 mL TAE buffer. After cooling, 5.0  $\mu$ L EtBr (Ethidium Bromide) was added. Gel was casted in gel casting tray. Wells were formed by inserting comb in the gel casting tray. DNA samples together with tracking dye (6X Methanol blue) were loaded in the well. Gel was run in the electrophoretic unit for 45 min at 70W. After 45 min, gel was taken out carefully from gel casting tray and placed into InGenius3 gel Doc-Syngene. Gel picture was taken using InGenius3 software and saved in jpg format. Another method, Nano drop spectrophotometer for confirmation of DNA in the sample was used.

#### 2. Absorbance at 260/280 nm:

Thermoscientific DS-11 FX+ spectrophotometer/ fluorometer was used to check the DNA quality and quantity in sample. In this method, 1  $\mu$ L DNA sample was used to measure absorbance at 260/280 nm.

##### a) Qualitative analysis

A sample of dsDNA was diluted 50X. Absorbance of diluted sample was recorded using Thermoscientific DS-11 FX+ spectrophotometer at OD<sub>260</sub>. According to Barbas *et al.*, 2007, pure DNA has an OD<sub>260</sub>/OD<sub>280</sub> ratio of ~1.8: pure RNA has an OD<sub>260</sub>/OD<sub>280</sub> ratio of ~2.0 and low ratio denote protein or phenol contamination. O.D. of all the isolates were recorded as per above information.

##### b) Quantitative analysis

To determine the concentration of DNA in the original sample, following calculation was performed: dsDNA concentration (mg/mL) = 50  $\mu$ g/mL  $\times$  OD<sub>260</sub>  $\times$  dilution factor (Barbas *et al.*, 2007) For a 1-cm pathlength, the optical density at 260 nm (OD<sub>260</sub>) equals 1.0 for the following solutions: a 50  $\mu$ g/mL solution of dsDNA.

### 3.4.3 Polymerase chain reaction (PCR) for identification of ITS in the isolates

Following work was conducted in Project Coordinating unit, AICRP on small millets, molecular lab ZARS., UAS, GKVK, Bengaluru.

**Table 2. Thermal conditions, components and Nucleotide sequence of the primer used for PCR amplification of ITS region**

<b>I. Thermal conditions for amplification (ITS)</b>		
<b>Reactions</b>	<b>Temperature</b>	<b>Time</b>
Initial denaturation	95 °C	3 min
Denaturation	94 °C	30 sec
Annealing	52 °C	30 sec
Extension	72 °C	1 min
Final extension	72 °C	10 min
No. of cycles	35	
<b>II. Components of PCR reaction mixture</b>		
<b>Particular</b>	<b>Quantity (µL)</b>	
TAKARA master mixture	10.0	
Forward primer	1.0	
Reverse primer	1.0	
PCR water	6.0	
Template	2.0	
<b>III. Nucleotide sequence of the primer used for PCR amplification of ITS region</b>		
<b>Region</b>	<b>Primer sequence</b>	<b>Reference</b>
ITS	ITS-1: 5'-TCCGTAGGTGAACCTGCGG-3' ITS-4: 5'-TCCTCCGCTTATTGATATGC-3'	White <i>et al.</i> , 1990

### **Identification of amplified genes using AGE**

- Agarose gel 1.0 % was prepared by dissolving 1.0 g agarose in 100 mL TAE buffer.
- After cooling, 75 µL EtBr (Ethidium Bromide) was added.
- Gel was casted in gel casting tray. Wells were formed by inserting comb in the gel casting tray.
- DNA samples together with tracking dye (6X Methanol blue) were loaded in the well.
- Gel was run in the electrophoretic unit for 45 min at 100W. After 45 min, gel was taken out carefully from gel casting tray and placed into InGenius3 gel Doc-Syngene.
- Gel picture was taken using Gene sys software and saved in jpg format.

### **Sequence alignment and phylogenetic analysis**

PCR product was sent to sequencing for pathogen confirmation later, the sequences were submitted to NCBI GenBank and by comparing with other related species sequences phylogenetic analysis was done.

### **3.5 *In vitro* evaluation of botanicals, bio agents and fungicides against *Fusarium* sp. infecting geranium.**

#### **3.5.1 *In vitro* evaluation of botanicals**

*In vitro* evaluation of twelve plant extracts at three different concentrations *viz.*, 10, 15 and 20 per cent were studied for the effect on growth and sporulation of *Fusarium* sp. by the poison food technique (Nene and Thapliyal, 1979). The different botanicals with plant parts used for extraction are listed in table 3.

The PDA media has been prepared and sterilized in an autoclave. The medium was cooled to 40 °C. The stock solution of 100 per cent botanicals was made based on weight per volume and stock solution of each plant extract were filtered through a muslin cloth. An appropriate amount of botanical stock solution was added to the medium, to obtain a required concentration of 10, 15 and 20 per cent and the conical flasks were gently shaken to completely disperse the botanical solution. About 15-20 mL of poisoned media was

poured into 90 mm Petri dishes. The active growth culture was taken by using an aseptic cork borer and transferred to the centre of each Petri dish containing the poisoned medium. A control was maintained in which the fungal pathogen was grown under similar conditions on agar medium without any plant extract. The inoculated plates were incubated at 27±1 °C for ten days and radial growth of the wilt pathogen was recorded in three directions and the average diameter was calculated. The percentage of inhibition of growth over control was calculated with the formula given by Vincent (1947).

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

Where., I = Per cent inhibition. of mycelium

C= Growth of mycelium in control

T = Growth of mycelium in treatment

**Table 3. List of the botanicals used for evaluation against *Fusarium sp.***

Sl. No.	Common name	Scientific name	Family	Plant part used
1	Calotropis	<i>Calotropis gigantea</i> (L.) Dryand.	Apocynaceae	Leaves
2	Eucalyptus	<i>Eucalyptus obliqua</i> L'Her.	Myrtaceae	Leaves
3	Ginger	<i>Zingiber officinale</i> R.	Zingiberaceae	Rhizome
4	Parthenium	<i>Parthenium hysterophorus</i> L.	Asteraceae	Leaves
5	Turmeric	<i>Curcuma longa</i> L.	Zingiberaceae	Rhizome
6	Karanja	<i>Millettia pinnata</i> (L.) Panigrahi.	Fabaceae	Leaves
7	Agave	<i>Agave americana</i> L.	Asparagaceae	Leaves
8	Aloe vera	<i>Aloe vera</i> (L.) Brum. f.	Asphodelaceae (Liliaceae)	Succulent leaves
9	Garlic	<i>Allium sativum</i> L.	Amaryllidaceae	Bulb
10	Gliricidia	<i>Gliricidia sepium</i> (J.) Stedu.	Fabaceae	Leaves
11	Datura	<i>Datura metel</i> L.	Solanaceae	Leaves
12	Neem	<i>Azadirachta indica</i> A. Juss.	Meliaceae	Leaves

### 3.5.2 *In vitro* evaluation of bio agents

Bio agents were evaluated under laboratory conditions against *Fusarium* sp. through dual culture technique. Bio agents used and their sources were listed in table 4.

**Table 4. List of the bio agents used for *in vitro* evaluation against *Fusarium* sp.**

Sl. No.	Bio agent	Isolate/ Strain	Source
<b>Fungal Bio agents</b>			
1	<i>Trichoderma viride</i>	-	Mycology lab, UAS, GKVK, Bengaluru
2	<i>T. viride</i> -1	Tv1	Mycology lab, UAS, GKVK, Bengaluru
3	<i>T. viride</i> -3	Tv3	Mycology lab, UAS, GKVK, Bengaluru
4	<i>T. viride</i> -8	Tv8	Plant pathology lab, IIHR, Bengaluru
5	<i>T. harzianum</i> -10	Th10	Plant pathology lab, IIHR, Bengaluru
6	<i>T. harzianum</i> -14	Th14	Mycology lab, UAS, GKVK, Bengaluru
7	<i>T. harzianum</i> -41	Th41	Mycology lab, UAS, GKVK, Bengaluru
8	<i>T. harzianum</i> -55	Th55	Mycology lab, UAS, GKVK, Bengaluru
9	<i>T. harzianum</i> -56	Th56	Mycology lab, UAS, GKVK, Bengaluru
10	<i>T. harzianum</i> - B <sub>2</sub>	ThB <sub>2</sub>	Mycology lab, UAS, GKVK, Bengaluru
11	<i>T. harzianum</i> - 4B	Th4B	Plant pathology lab, IIHR, Bengaluru
12	<i>T. harzianum</i> - GJ 16B	ThGJ 16B	Plant pathology lab, IIHR, Bengaluru
<b>Bacterial Bio agents</b>			
13	<i>Pseudomonas fluorescens</i>	-	Bacteriology lab, UAS, GKVK, Bengaluru
14	<i>Bacillus megaterium</i>	-	Bacteriology lab, UAS, GKVK, Bengaluru
15	<i>B. pumilis</i>	-	Bacteriology lab, UAS, GKVK, Bengaluru
16	<i>B. velezensis</i> -A <sub>6</sub>	A <sub>6</sub>	Bacteriology lab, UAS, GKVK, Bengaluru
17	<i>B. velezensis</i> -P <sub>42</sub>	P <sub>42</sub>	Bacteriology lab, UAS, GKVK, Bengaluru
18	<i>B. subtilis</i>	-	Bacteriology lab, UAS, GKVK, Bengaluru
19	<i>Azotobacter chroococcum</i>	-	Dept. of Agril. Microbiology, UAS, GKVK, Bengaluru
20	<i>Azospirillum brasilense</i>	-	Dept. of Agril. Microbiology, UAS, GKVK, Bengaluru

## Dual culture technique

In the dual culture technique, sterilized PDA media was cooled and about 20 mL of media was poured into sterile Petri plates. Fungal antagonists were evaluated by inoculating the blight pathogen at one peripheral end of Petri plate and the antagonist on the opposite peripheral end of the same plate by leaving 3 to 4 cm gap. Similarly, for bacterial antagonists, fungal disc was placed at one peripheral end of Petri plate and bacterial antagonists were streaked at other end of same plate. Each treatment was replicated three times. After 10 days of incubation period the radial growth of pathogen was measured. Per cent inhibition over control was worked out according to the equation of Vincent (1947) which mentioned earlier.

### 3.5.3 *In vitro* evaluation of fungicides

A total of ten systemic, three combination product and four contact (non-systemic) fungicides were tested against *Fusarium* sp. infecting geranium under this study. The systemic fungicides were tried at 50, 100 and 150 ppm concentrations while combi-product fungicides were tried at 150, 250 and 500 ppm. Similarly, contact fungicides were tried at 500, 750 and 1000 ppm concentrations. The various fungicides evaluated were listed in table 5.

Poisoned food technique (Nene and Thapliyal, 1979) was followed to test the efficacy of the above-mentioned fungicides. The fungus, *Fusarium* sp. was grown on PDA medium for 10 days prior to setting the experiment. Fungicides suspension was prepared in PDA medium by adding required quantity of fungicide to obtain desired concentration on basis of active ingredient present in the chemical. Twenty mL of poisoned medium was poured in each sterilized Petri plates. Control was maintained without addition of fungicide to PDA medium. Five mm mycelial disc taken from the periphery of 10 days old colony was placed in the centre and incubated at  $28 \pm 1$  °C for 10 days. Each treatment was replicated thrice. The diameter was measured in three directions and average was recorded. Per cent inhibition of radial growth was calculated by using formula given by Vincent (1947) which mentioned earlier.

**Table 5. List of fungicides used for *in vitro* evaluation against *Fusarium sp.***

Systemic fungicides				
Sl. No.	Common name	Trade name	Chemical name	Fungicide group
1	Cyzoamid	Ranman top (34.5 % SC)	4-Chloro-2-cyano-N,N-dimethyl-5-(p-tolyl)-1H-imidazole-1-sulfonamide	Imidazole
2	Difenoconazole	Score (25% EC)	1-((2-(2-Chloro-4-(4-chlorophenoxy)phenyl)-4-methyl-1,3-dioxolan-2-yl) methyl) -1H-1,2,4-triazole	Triazole
3	Penconazole	Topaz (10 % EC)	1-[2-(2,4-dichlorophenyl) pentyl]-1H-1,2,4-triazole	Triazole
4	Kresoxim methyl	Ergon 44.3% SC	Methyl (αE)-α-(methoxyimino)-2-(2-methylphenoxy)methyl phenyl) acetic acid	Strobilurins
5	Isoprothiolane	Fujita (40 % EC)	Diisopropyl-2-(1,3-dithiolan-2-yliden) malonat	Dithiolane
6	Thifluzamide	Amistar (23 % SC)	Methyl(2E)-2-{2-[6-(2-cyanophenoxy) pyrimidin-4-yloxy] phenyl}-3-methoxyacrylate	Strobilurins
7	Carbendazim	Bavistin (50 % WP)	Methyl 1Hbenzimidazol2ylcarbamate	Benzimidazoles
8	Propiconazole	Tilt (25 % EC)	(±)-1-[2-(2,4-dichlorophenyl)-4-propyl-1,3-dioxolan-2-ylmethyl]-1H-1,2,4-triazole	Triazoles
9	Pyraclostrobin	Headline (20 % WG)	Methyl (2-(((1-(4-chlorophenyl)-1H-pyrazol-3-yl) oxy) methyl) phenyl) (methoxy) carbamate	Strobilurins
10	Dimethomorph	Lurit (50 % WP)	3-(4-chlorophenyl)-3-(3,4-dimethoxyphenyl)-1-morpholin-4-ylprop-2-en-1-one	Morpholines
Combi-product fungicides				
Sl. No.	Common name	Trade name	Chemical name	Fungicide group
1	Tebuconazole 50 % + Trifloxystrobin 25 % WG	Nativo 75 % WG	Manganese ethylene bis dithiocarbonate plus zinc + N-(methoxyacetyl)-N-(2,6-xylyl)-DL-alaninate	
2	Azoxystrobin 20 % + Difenoconazole 12.5 % SC	Amistar top 35.5 % SC	Methyl(2E)-2-(2-(6-(2-cyanophenoxy) pyrimidin-4-yloxy) phenyl)-3-methoxyacrylate + Cis, trans-3-chloro-4(4-methyl-2(1H-1, 2, 4- traizole-1-yl, methyl)-1, 3-dioxonlan-2yl) Phenyl 4chlorophenyl ether	
3	Hexaconazole 5 % + Captan 70 % WP	Taqat 75 % WP	N-trichloromethyl mercapta 4-cyclohexene-1, 2-dis carboximide N- trichloromethyl thiotetra hydro othalamide +RS) -2-(2,4-dichloro phenyl) -1- (14-1,2,4-triazole-1yl) hexane-2-ol	
Contact (Non systemic) fungicides				
Sl. No.	Common name	Trade name	Chemical name	Fungicide group
1	Captan	Captan (50 % WP)	(3aR,7aS)-2-[(Trichloromethyl)sulfanyl]-3a,4,7,7a-tetrahydro-1H-isindole-1,3(2H)-dione	Phthalimide
2	Chlorothalonil	Kavach (75 % WP)	2,4,5,6-Tetrachlorobenzene-1,3-dicarbonitrile	Organic compound
3	Mancozeb	Indofil M-45 (75 % WP)	Manganeseethylene bisdithiocarbamate Polymeric complex with zinc salts	Dithiocarbamate
4	Copperoxy chloride	Blitox (50 % WP)	Dicopperchloridetrihydroxide	Organic compound

### **3.6 Statistical analysis**

The data generated by different experiments were analysed using the WASP (Web Based Agricultural Statistics Software Package) software developed by ICAR- Central Coastal Agricultural Research Institute, Goa and the inferences were made with a probability of one percent for laboratory experiments.

## IV RESULTS AND DISCUSSION

This chapter includes the experimental results in detail that have been carried out in the present work “Studies on *Fusarium* wilt in scented geranium [*Pelargonium graveolens* (L.) Herit]” conducted during 2019-20. All possible attempts had made to find the causal organism with reference to isolation, identification of the pathogen, proving pathogenicity, cultural, morphological and molecular characterization of the pathogen in detail, further work carried out with emphasis on management aspects like testing the efficacy of botanicals, various chemicals as well as the antagonistic activity of many bio agents against the pathogen under *in vitro* condition. Results were analysed statistically wherever needed, critically examined and discussed with the available review of literature on all the aspects of present study are presented here under.

### 4.1 Symptomatology

The infected plants of geranium showing symptoms of wilt caused by *Fusarium* such as yellowing with interveinal necrosis in a moderate to severe form later stages the entire leaf become necrotic and hangs down. Lower leaves drop down leaving only few upper leaves, giving the plant sick appearance. The infected plants completely dried up gives a wilted appearance, upon progressive wilting finally death of entire plants (Plate 1.). Later stages of infection resulted in blackening of the stem as well as roots, and brown to black vascular discoloration seen when the stem is split open (Plate 2.). Similar descriptions of the symptoms were given by Rao *et al.* (1999), Prasad *et al.* (2008) and Michielse and Rep (2009).

### 4.2 Collection and isolation of the pathogen causing wilt in geranium

#### 4.2.1 Isolation of the pathogen

Scented geranium plants showing typical wilt symptoms were collected from geranium growing areas of Tumakuru, Bengaluru Rural and Hassan districts of Karnataka. Wilt incidence and locations of major geranium growing areas of Southern Karnataka are presented (Table 6, Fig. 1.) and the pathogen was isolated by following tissue isolation technique as described under materials and methods (Section 3.2.1). Collected samples

hand sections were made from infected stems with sterile blade and placed on PDA media which produce mycelia after 2 days that have observed under light microscope for presence of conidia to identify the causal organism. Sickle shaped macroconidia, oval to kidney shaped microconidia observed in the compound microscope and confirmed as *F. oxysporum*. The isolates obtained were further purified by single spore isolation and were stored at 10 °C in refrigerator and maintained by periodical transferring to PDA slants and plates containing fresh media for further use. Pure culture of *F. oxysporum* of all the isolates were depicted in Plate 3a.

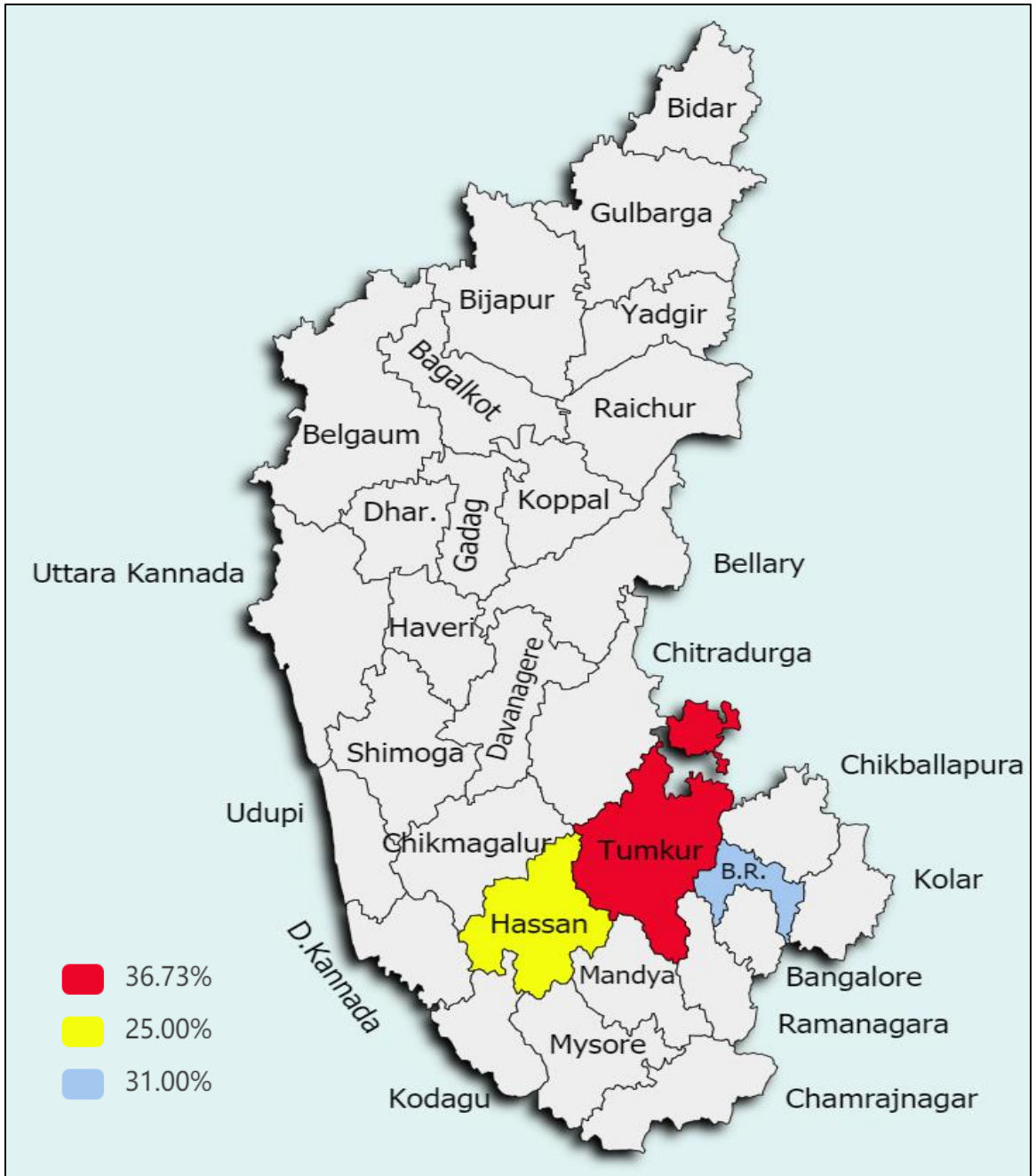
**Table 6. Wilt incidence in major geranium growing areas of Southern Karnataka**

Sl. No.	District	Taluk	Village/ town	Wilt incidence (%)
1	Tumakuru	Tiptur	Hullukatte	42.20
			Koppa	38.00
			Echanur	30.00
<b>Mean</b>				<b>36.73</b>
2	Bengaluru Rural	Hosakote	Sulibele	32.00
		Devanahalli	Devanahalli	30.00
<b>Mean</b>				<b>31.00</b>
3	Hassan	Arsikere	Arsikere	<b>25.00</b>

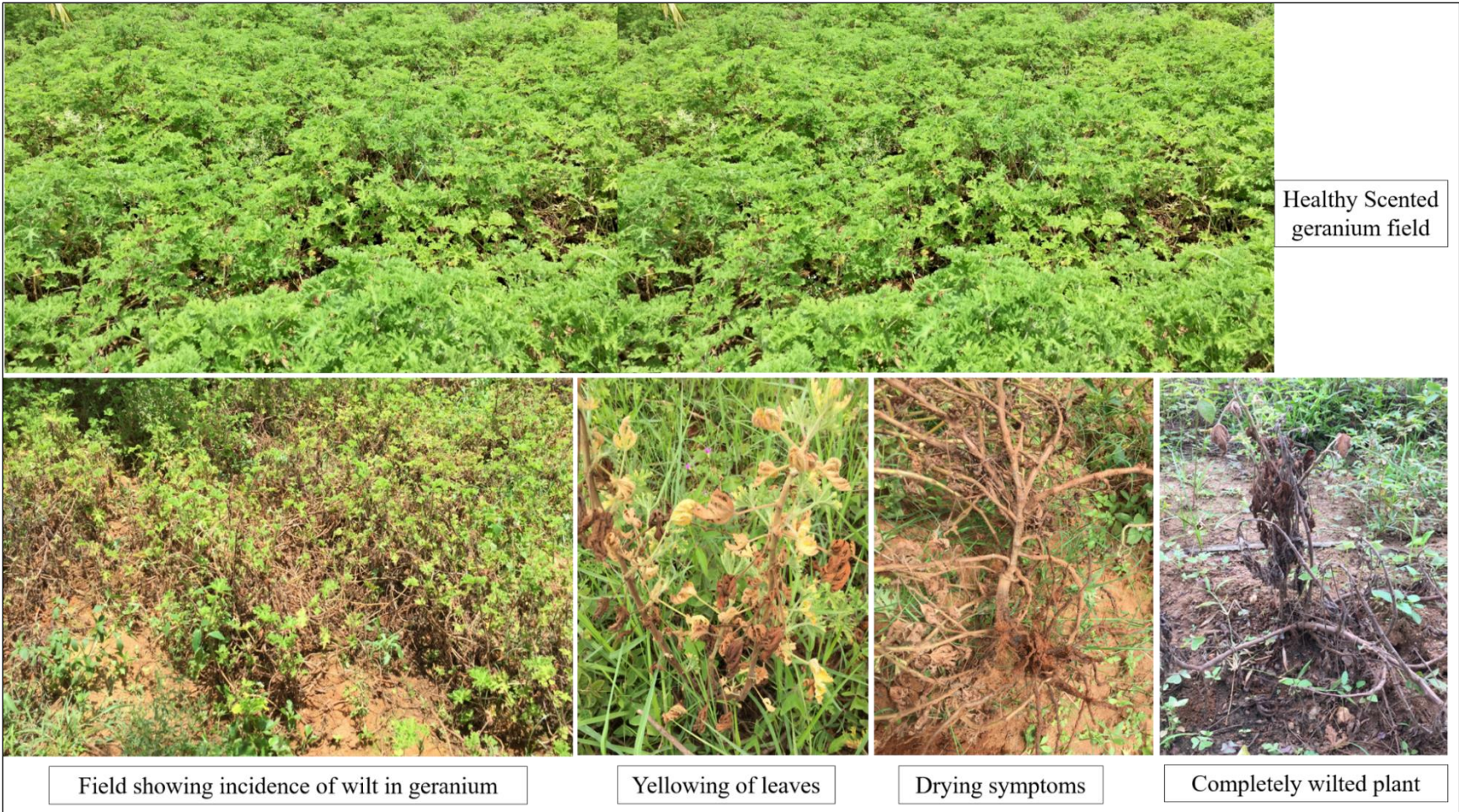
#### 4.2.2 Identification of the pathogen

The cultural and morphological characters *viz.*, mycelial growth pattern, macroconidia, microconidia and chlamydospores of the pathogen were studied by making slides of the respective isolates.

The fungus on PDA produced white cottony mycelial growth with orange, pinkish to purple pigmentation, the mycelia turned to dark purple when old in some isolates such as isolate GFO 2 & 3 microscopic observation of the young culture showed the presence of both micro and macroconidia. Chlamydospore formation was seen in the two-week old culture. The fungus produced hyaline, thin, septate and branched hyphae which bear simple and short slender conidiophores. Microconidia were hyaline, ovoid to ellipsoid in shape



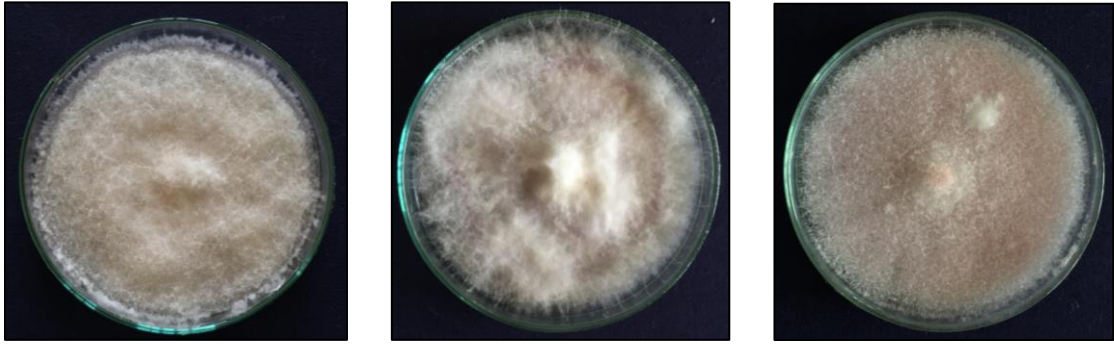
**Fig. 1: Location of major geranium growing areas of Southern Karnataka**



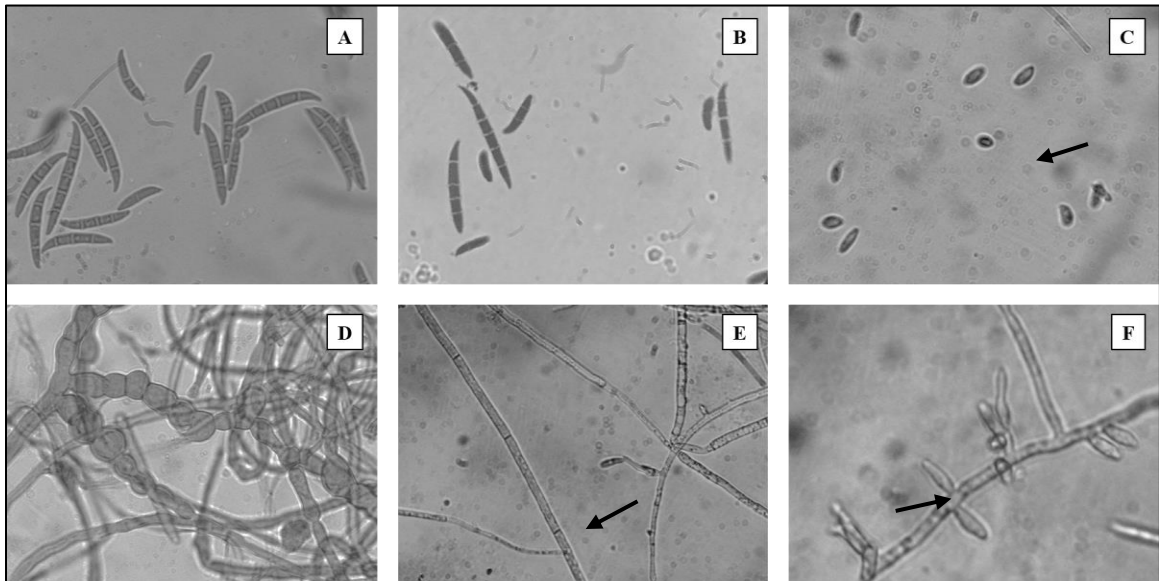
**Plate 1. Symptoms of *Fusarium* wilt disease in geranium field**



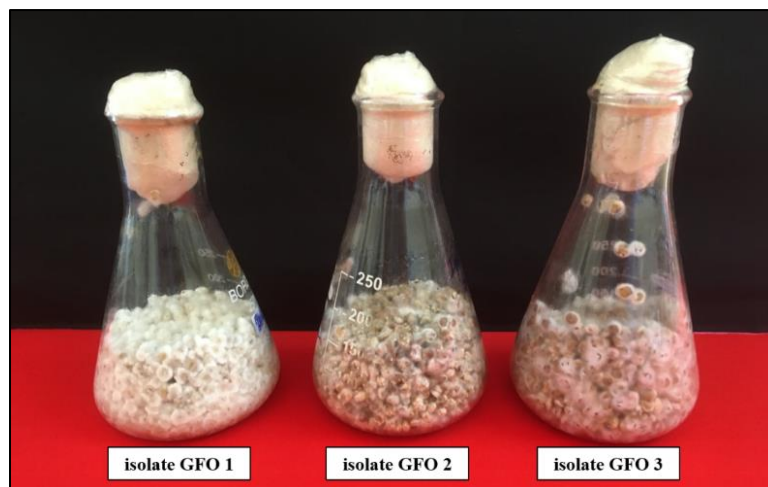
**Plate 2. Longitudinal sectioning showing typical wilt symptoms**



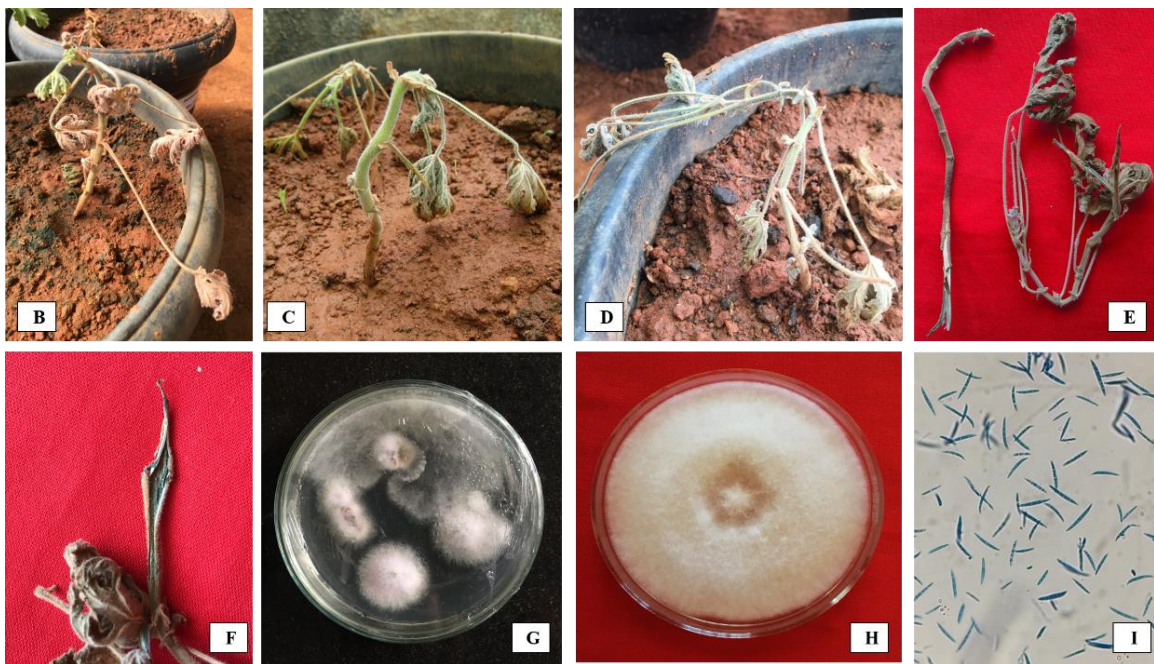
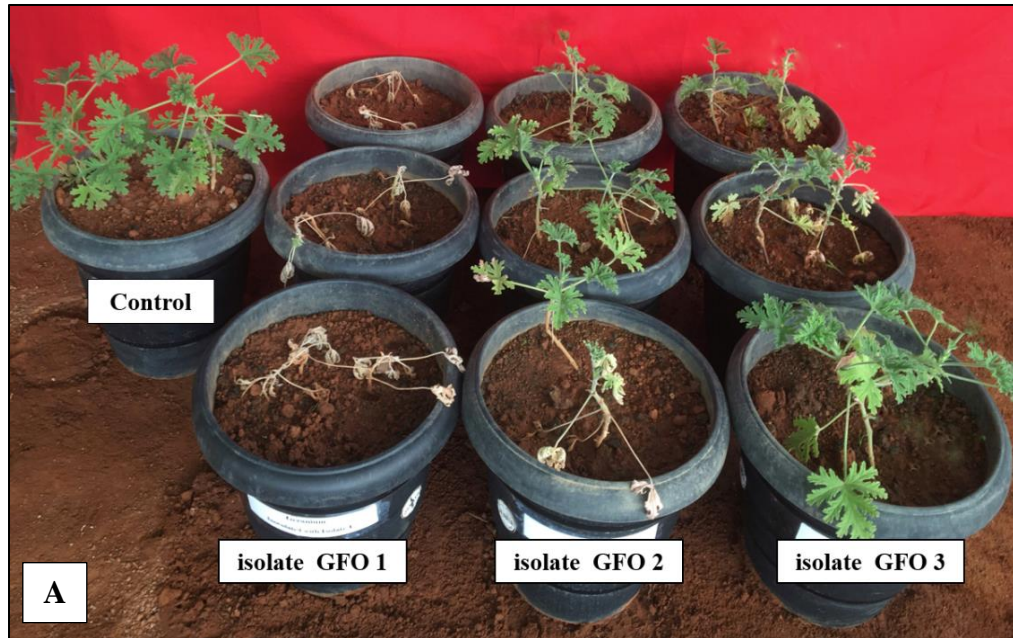
**Plate 3a. Pure culture of all three isolates of *F. oxysporum* on PDA**



**Plate 3b. Microscopic images showing spore and mycelial characters of *Fusarium oxysporum*** (A) Macroconidia; (B) Macroconidia and microconidia; (C) Oval shaped microconidia; (D) Chlamydospores; (E) Septate mycelia; (F) Short and plump short monophialides [100x]



**Plate 4. Mass multiplication of fungal inoculum on sorghum grains**



**Plate 5. Pathogenicity test of *F. oxysporum* isolates infecting geranium**  
**(A)** Pathogenicity by sick pot method; Wilt symptoms of isolate GFO 1, 2, 3 **(B,C,D)** respectively; **(E)** Complete wilted plant; **(F)** Vascular discolouration of stem from infected sample; **(G)** Re-isolated culture; **(H)** 10 days old culture; **(I)** Re-isolated

with no septation and are produced in abundance. Morphology of spore characters presented in Plate 3b. Kharbanda and Stevens (1988) elucidated the morphology of *F. oxysporum* it produces microconidia in false heads born on short monophialides (conidiophores). Macroconidia were few in number, sickle shaped with 2-3 septa. Both terminal and intercalary chlamydospores were formed either singly or in chains.

Based on these cultural and morphological characteristics the isolated fungus was identified as *Fusarium oxysporum*. The above description of the fungus with respect to mycelium, conidia and conidiophores are in agreement with the original description given by Booth (1975), Barnett and Barry (1992) and Leslie and Summerell (2006). After confirming the pathogen, the isolates of *F. oxysporum* were designated as *F. oxysporum* isolate GFO 1, *F. oxysporum* isolate GFO 2 and *F. oxysporum* GFO 3.

#### **4.2.3 Proving pathogenicity**

Pathogenicity test was carried out to prove pathogenicity of the causal organism, by following pot inoculation method or sick pot method as detailed in the materials and methods (Section 3.2.3). The inoculated plants showed wilting symptoms such as drooping, yellowing of older leaves two weeks after inoculation. Among three isolates inoculated the *F. oxysporum* isolate GFO 1 was found virulent over the other, isolates as evidenced by wilting and complete collapse of the plants within 20 days after inoculation where other isolate GFO 2 and GFO 3 showed wilt symptoms after 25 days of inoculation, while no wilting or death of the seedling was recorded in un inoculated pots (Plate 5). The re-isolated pathogen from the diseased seedlings had similar cultural and morphological characters of the originally isolated culture. The findings were in conformity with Prasad *et al.* (2008), Bhapkar (2016) and Gogoi *et al.* (2017). So, for the further study isolate GFO 1 was used since it is very virulent over other isolates tested.

### **4.3 Cultural and morphological characters of pathogen causing wilt in geranium.**

#### **4.3.1 Influence of different culture media on growth and sporulation of *F. oxysporum***

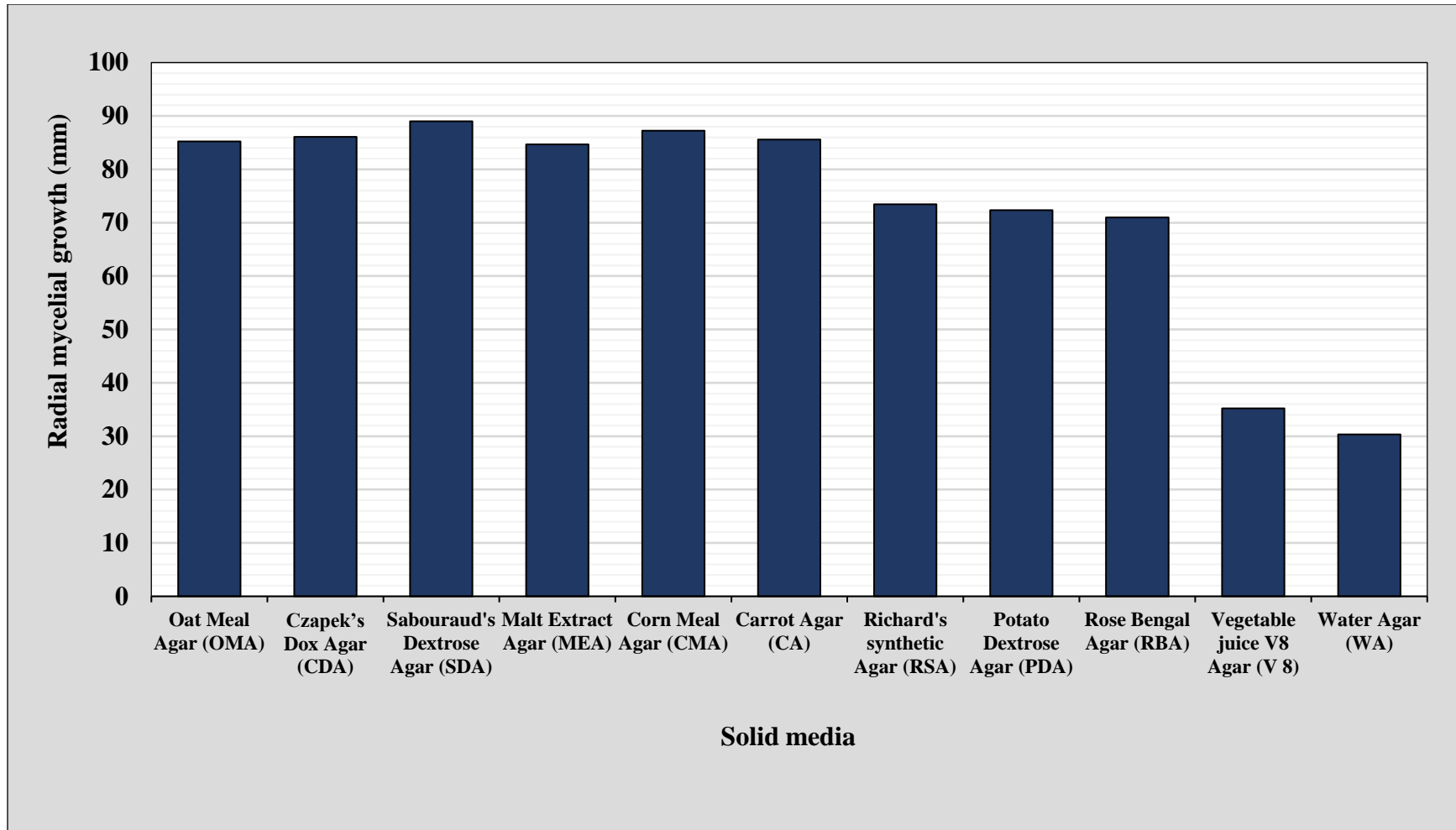
Eleven different solid media were used in this study as described in material methods (Section 3.3.1). Results are presented in table 7, fig. 2 and plate 6. *F. oxysporum*

showed moderate variation in growth and sporulation on different solid media which revealed that maximum radial mycelial growth was observed in Sabouraud's dextrose agar (89.00 mm) which is statistically on par with corn meal agar (87.22 mm) at seven days after inoculation. The other solid media showed varied growth ranged from 35.22 to 86.10 mm. Lowest growth was recorded in water agar (30.33 mm) which formed as a thin mat that spread over media. Likewise, maximum sporulation of *F. oxysporum* was observed in Sabouraud's dextrose agar (> 100 conidia per microscopic field). Whereas lowest sporulation was noticed on malt extract agar (1-20 conidia per microscopic field). No sporulation was recorded in vegetable juice V8 agar, carrot agar and water agar. All media except water agar showed pale white to cottony white colony colour.

**Table 7. Effect of different solid media on growth and sporulation of *F. oxysporum***

Sl. No.	Media	Radial mycelial growth (mm) *	Sporulation**
1	Oat Meal Agar (OMA)	85.22	+++
2	Czapek's Dox Agar (CDA)	86.10	+++
3	Sabouraud's Dextrose Agar (SDA)	89.00	++++
4	Malt Extract Agar (MEA)	84.66	+
5	Corn Meal Agar (CMA)	87.22	+++
6	Carrot Agar (CA)	85.55	-
7	Richard's Synthetic Agar (RSA)	73.44	+++
8	Potato Dextrose Agar (PDA)	72.33	++
9	Rose Bengal Agar (RBA)	70.99	+++
10	Vegetable Juice V8 Agar (V 8)	35.22	-
11	Water Agar (WA)	30.33	-
	<b>S. Em ±</b>	<b>0.76</b>	
	<b>CD (p 0.01)</b>	<b>2.01</b>	

**Note:** \*Means of three replications; \*\*(-) No sporulation- 0 conidia per microscopic field; (+) Poor- 1-20 conidia per microscopic field; (++) Moderate- 20-50 conidia per microscopic field; (+++) Good- 50-100 conidia per microscopic field; (++++) Excellent - > 100 conidia per microscopic field.



**Fig. 2: Effect of different solid media on mycelial growth of *F. oxysporum***

*F. oxysporum* exhibited varying cultural characteristics like different colony colours *i.e.*, white, pale white, and colony texture ranges smooth to cottony. All media were exhibited dull luster of mycelial growth with regular or irregular margin and among them PDA, OMA and SDA shows umbonate type of topography (Table 8).

Among the eleven media evaluated for radial growth and spore production of *F. oxysporum* infecting geranium, Sabouraud's dextrose agar and corn meal agar are found suitable for maximum growth and SDA alone was found ideal for better sporulation.

Results obtained are not in accordance with these findings. However, Khilare and Ahmed (2012) reported that *F. oxysporum* f. sp. *ciceri* grew best on Czapek's Dox agar and PDA media. *F. oxysporum* f. sp. *dianthi* showed PDA, Richard's agar, Czapek's Dox agar and oat meal agar supported maximum growth (Kishore, 2007).

#### **4.3.2 Morphological characters of *F. oxysporum* isolates**

As described in the material and methods (Section 3.3.2.1) In this study we used ten days old single spore isolated cultures on PDA of isolates of *F. oxysporum* for morphological studies. Micromorphological characters *viz.*, macroconidia, microconidia and chlamydospores were observed under a compound microscope at magnifications (10 X, 40 X, 100 X) presented in plate 8. Later the conidial characters were observed under higher magnifications in which we can clearly observe the conidial size, shape, septations, apical cell morphology also the pigment production in ten days old single spore isolated culture of all three isolates showing different pigmentations such as *F. oxysporum* isolate GFO 1 shows white to pale orange colour pigmentations. Whereas, *F. oxysporum* isolate GFO 2 and *F. oxysporum* isolate GFO 3 shows light pinkish to purple and red tinge pigmentation (Plate 7.).

Under this study, *F. oxysporum* showing thin walled fusiform sickle shaped macroconidia size ranging from 12.50-30.93  $\mu\text{m}$  length and 1.90-2.85  $\mu\text{m}$  width and these are having 2- 5 septate on an average of 3 septations and 1.7  $\mu\text{m}$  average apical width with slightly curved.

**Table 8. Cultural characteristics of *F. oxysporum* on different solid media**

Sl. No.	Media	Days to cover full plate	Colony colour	Colony texture	Surface and topography	Margin
1	Oat Meal Agar (OMA)	7	Cottony white	Smooth cottony growth	Smooth and umbonate	Regular and filamentous
2	Czapek's Dox Agar (CDA)	7	Pale White	Cottony growth	Rough surface and raised	Regular and uniform
3	Sabouraud's Dextrose Agar (SDA)	7	White	Smooth fluffy growth	Smooth and umbonate	Uniform margin
4	Malt Extract Agar (MEA)	8	White	Rough and cottony	Rough surface and convex	Even
5	Corn Meal Agar (CMA)	8	Pale white	Cottony growth	Rough surface and raised	Regular and uniform
6	Carrot Agar (CA)	8	White	Rough and cottony	Rough surface and convex	Even
7	Richard's Synthetic Agar (RSA)	10	Cottony white	Rough and cottony	Rough surface and raised	Uneven /undulate
8	Potato Dextrose Agar (PDA)	8	White	Cottony	Rough surface and umbonate	Filamentous margin
9	Rose Bengal Agar (RBA)	10	Cottony white	Rough and cottony	Rough surface and raised	Uniform margin
10	Vegetable Juice V8 Agar (V 8)	10	White	Thin	Sparsely	Uneven
11	Water Agar (WA)	-	No proper growth, very thin insignificant mycelia was developed			

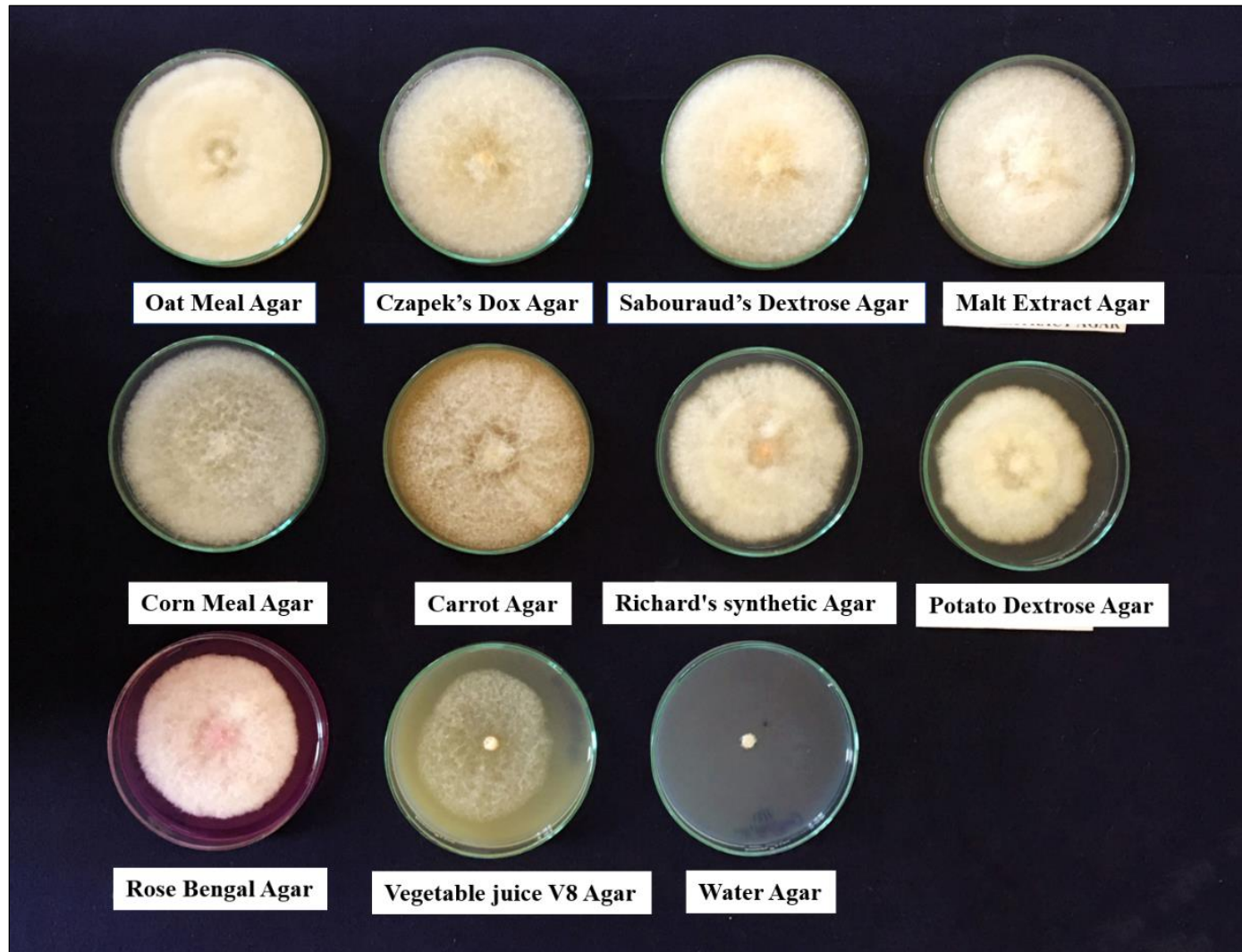


Plate 6. Effect of different culture media on growth of *F. oxysporum*

Microconidia were abundant oval to elliptical in shape, sized from 8.00-9.78  $\mu\text{m}$  length 1.99-2.55  $\mu\text{m}$  in size with no septations, average width of apical cell is 1.64  $\mu\text{m}$ . It produces chlamydospores in the old cultures. They are thick walled oval shaped ranging from 7.00-10.48 $\times$ 7.94  $\mu\text{m}$  in size and arranged singly, chains or in clusters (Plate 9c.) Morphological characteristic data of all three isolates is presented in table 9.

Results obtained from this study were in accordance with Booth (1975) who described that most important species among genus *Fusarium* is *F. oxysporum* with short, stubby phialides borne laterally on the hyphae or at the apices of short lateral branches. Barnett and Barry (1992) described *F. oxysporum* produces macroconidia several-celled, slightly curved or bent at the pointed ends, typically canoe-shaped. Microconidia are one celled, ovoid or oblong. Leslie and Summerell (2006) studied *F. oxysporum* that producing macroconidia are short to medium length, straight to slightly curved, relatively slender and thin walled, apical cell either tapered or curved and sometimes with a slight hook, the basal cell was foot shaped to pointed, usually three septate. Microconidia are oval, elliptical or kidney shaped and usually non septate arising on short monophialides. The pathogen was isolated and morphologically identified as *F. oxysporum* based on the conidial, chlamydosporial and cultural characters. The size of microconidia ranged between 5.97 to 8.60  $\mu\text{m}$  in to 2.02 to 4.07  $\mu\text{m}$  in width, most of the isolates did not produce macro conidia (Peeran *et al.*, 2019). Chlamydospores may be either terminal or intercalary in aerial, submerged or surface hyphae mainly smooth or rough walled and produced singly or in pairs and 7.0–10.0  $\mu\text{m}$  in diameter (Zheng *et al.*, 2012).

#### **4.3.3 Spore germination studies of *F. oxysporum***

Spore germination was carried out as mentioned in material and methods (Section 3.3.2.2). These studies were carried out on the virulent isolate *F. oxysporum* isolate GFO 1 which showed maximum germination. The results obtained from these studies after observing spores under compound microscope at different time intervals from 1 h to 5 h after germination of macroconidia. Macroconidia at 0 h initiation of germ tube formation after 1 h it produces germ tube with the length of 7.04  $\mu\text{m}$ . At 2 h and 5 h it produces length of 21.68  $\mu\text{m}$  and 48.20  $\mu\text{m}$  respectively and also observed different types of macroconidia germination (Plate 9a and 9b.).

**Table 9. Morphological characters of *F. oxysporum* isolates infecting geranium**

Isolates	Size ( $\mu\text{m}$ ) *		Shape	No. of septa	Apical cell ( $\mu\text{m}$ )
	Range (L×W)	Average (L×W)			
<b>Macroconidia</b>					
GFO 1	12.50-30.93 × 1.9-2.85	21.12×2.46	Thin walled fusiform, sickle shaped	2-5 (3) **	1.73 slightly curved
GFO 2	14.00-16.17× 3.4-3.65	15.54×3.54	Thin walled straight elongated	1-2 (2) **	2.53 Blunt end
GFO 3	14.00-15.92× 1.8-2.82	14.96×2.31	Thin walled straight	1-3 (3) **	2.06 Blunt end
<b>Microconidia</b>					
GFO 1	8.00-9.78×1.99-2.55	9.44×2.30	Oval to elliptical	No	1.64
GFO 2	4.6-6.7×1.2-2.1	5.38×1.66	Oval to elliptical	1	1.24
GFO 3	5.2-7.57× 1.5-2.19	6.43×1.87	Oval	0	1.69
<b>Chlamyospores</b>				<b>Arrangement</b>	
GFO 1	7.00-10.48× 7.94	8.26×7.55	Thick walled oval shape	Either in chains or in clusters	
GFO 2	5.80-7.77× 5.4-6.68	6.48×5.91	Thick walled oval spores	In chains	
GFO 3	6.60-11.5× 6.10-10.84	8.48×7.94	Thick walled oval shape	Formed in chains or singly	

**Note:** \*All parameters are considered after observing minimum of ten conidia; \*\*Average number of septations.

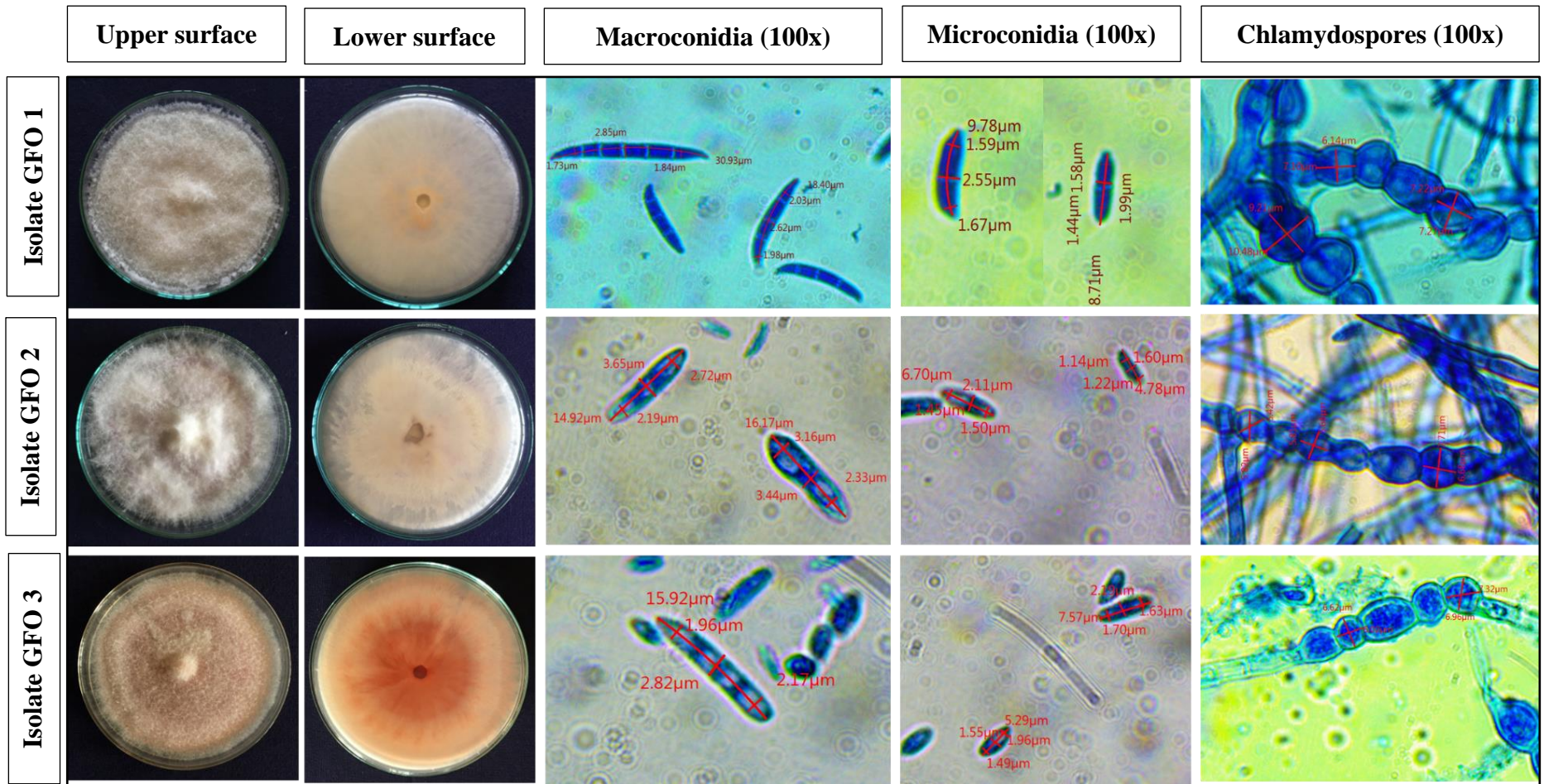
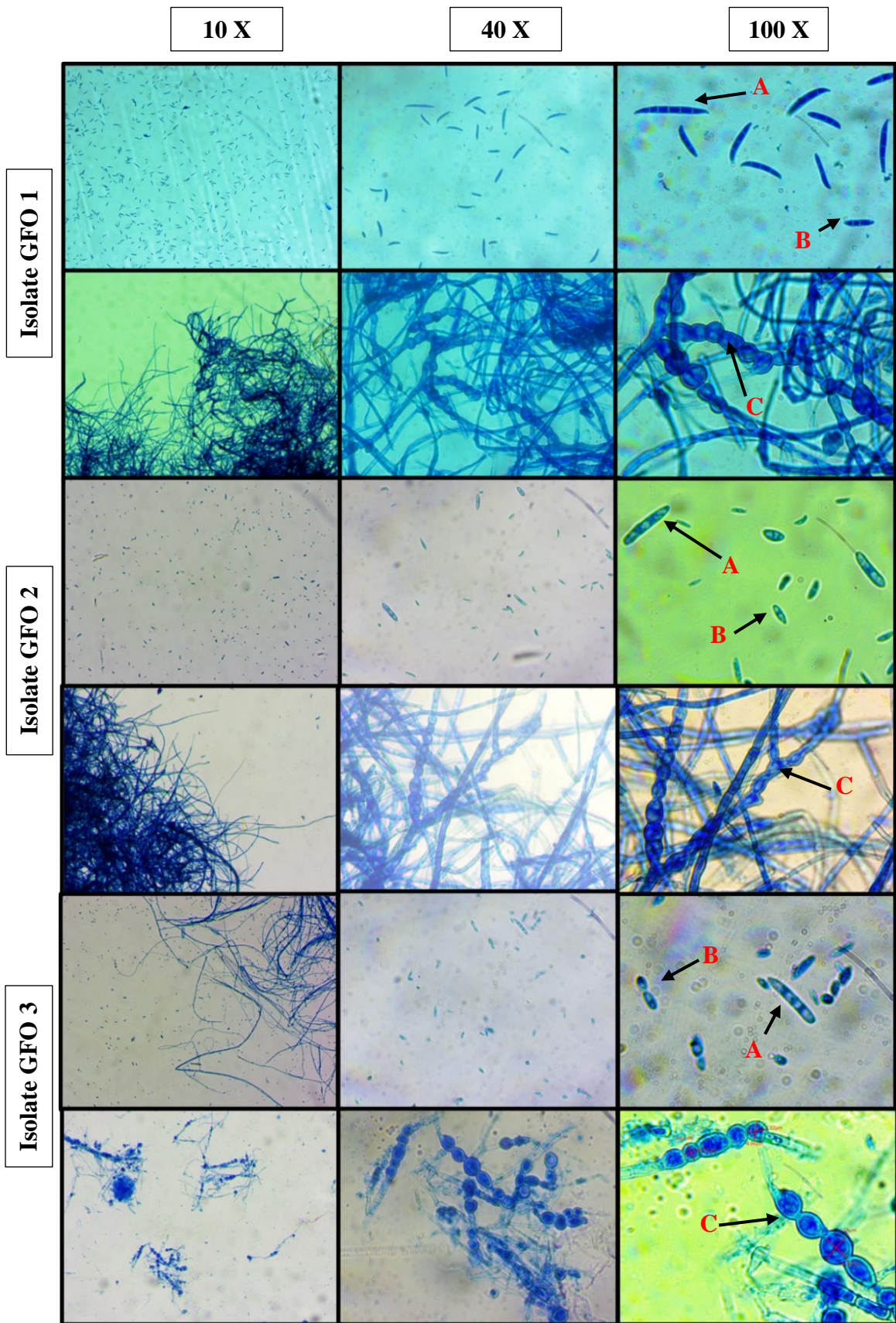
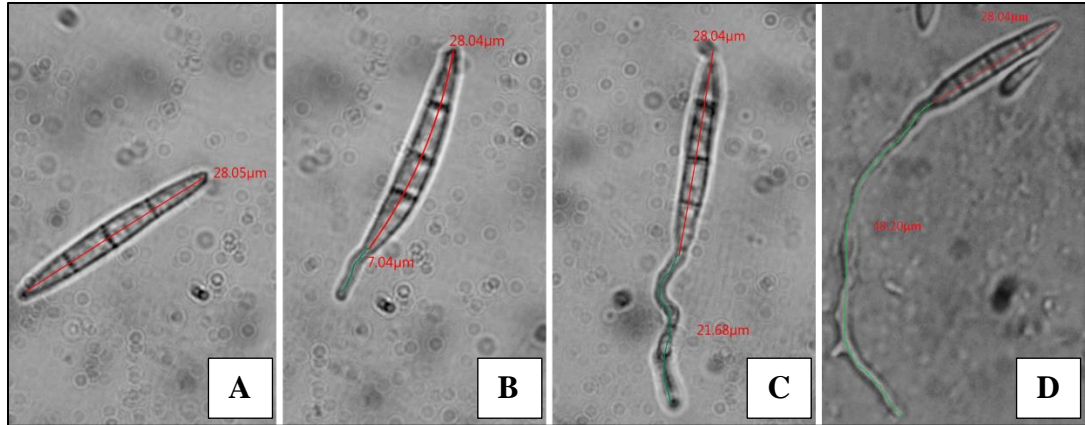


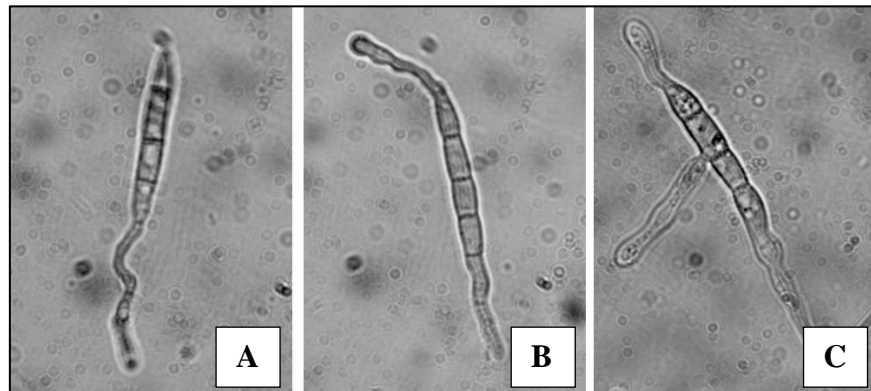
Plate 7. Morphological characters of *F. oxysporum* isolates infecting scented geranium



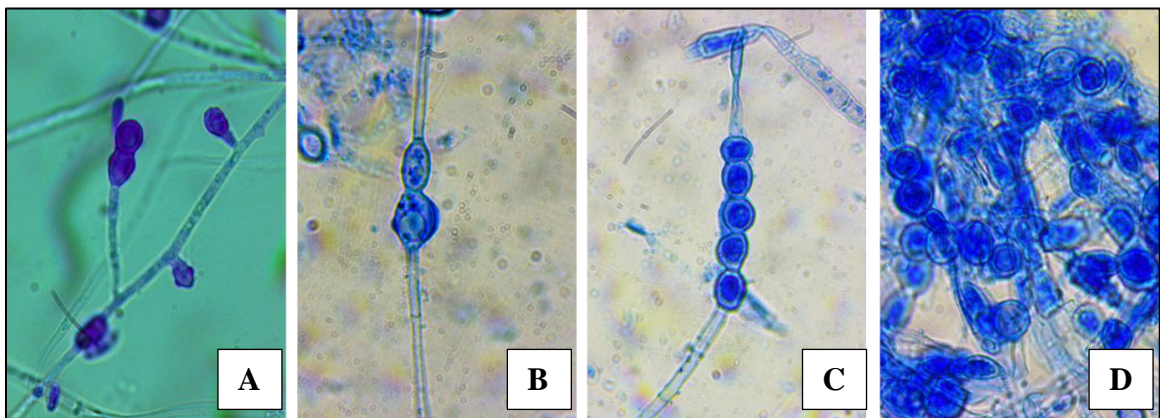
**Plate 8. Microscopic images showing different types of spores produced by *F. oxysporum* isolates (A- Macroconidia; B- Microconidia; C- Chlamydoconidia) at different magnifications under compound microscope (10 X, 40 X, 100 X)**



**Plate 9a. Germination of Macroconidia in distilled water at different time intervals (A- 0 h; B- 1 h; C- 2 h; D- 5 h)**



**Plate 9b. Different types of spore germination (A-Polar; B-Bipolar; C-Bipolar and intercalary cell)**



**Plate 9c. Different types of chlamydospores arrangements (A- Singly/ terminal; B- Intercalary; C- In chains; D- Clusters)**

## 4.4 Physiological studies

### 4.4.1 Influence of different temperatures on mycelial growth and sporulation of *F. oxysporum* in solid and liquid media.

Solid (PDA) and liquid media (PDB) were used to study the effect of temperature on growth of *F. oxysporum* as described in material and methods (Section 3.3.3.1)

The maximum radial mycelial growth was recorded at 25 °C (88.77 mm) which is on par with 30 °C (87.77 mm) other temperatures varied from 43.33 to 74.22 °C. Whereas, least growth was recorded at 40 °C (10 mm) in solid media and in case of liquid media maximum biomass production was significantly higher at 25 °C (0.67 g 100 mL<sup>-1</sup>) other temperatures the biomass was ranged from 0.31 to 0.44 g 100 mL<sup>-1</sup> and no growth and sporulation was observed at 40 °C. Maximum sporulation was recorded at 25 °C both in solid and liquid media (Table 10, Fig. 3, 4 and Plate 10a, 10b)

Results showed the ideal temperature to culture *F. oxysporum* causing geranium wilt was 25-30 °C in solid media and 25 °C in liquid media. Ideal temperature for sporulation in solid and liquid media was observed at 25 °C. Similarly, Pal *et al.* (2019) observed maximum 88.33 mm growth and highest sporulation of *F. oxysporum* f. sp. *lini* at 24 °C and Cruz *et al.* (2019) observed that at 25 °C *F. oxysporum* showed the maximum mycelium growth. Yadav *et al.* (2014) observed maximum growth of mycelia (86.50 mm) of *F. oxysporum* causing basal rot of onion at 25 °C.

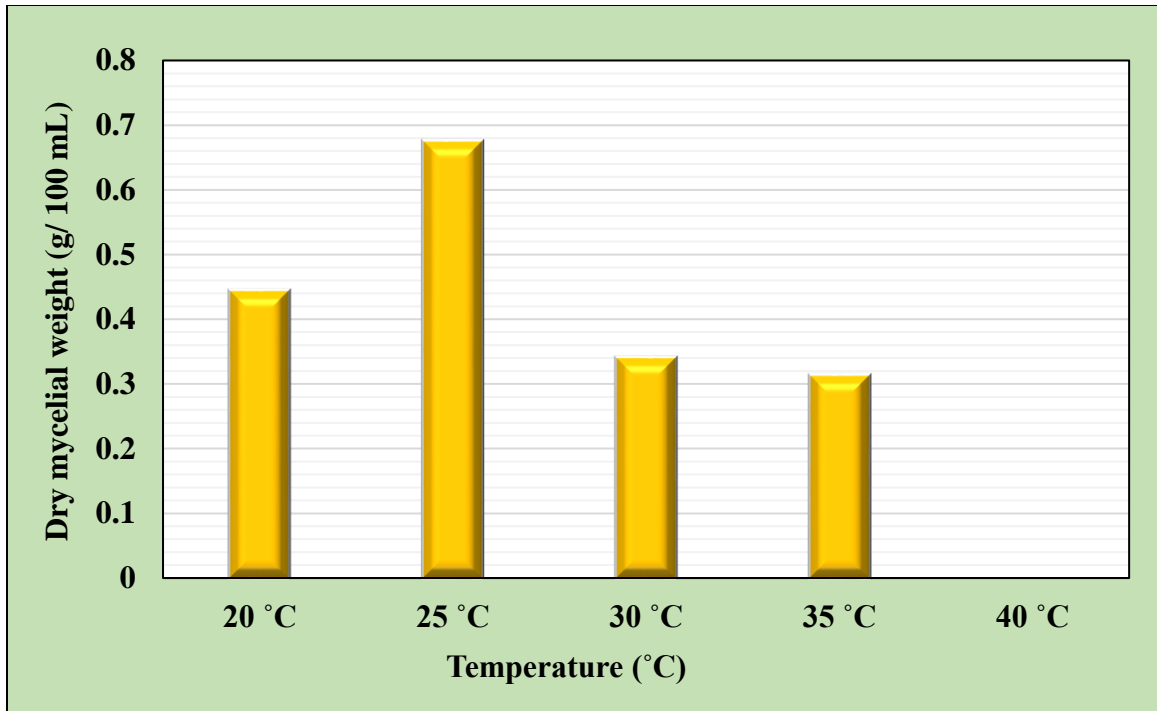
### 4.4.2 Influence of pH on mycelial growth and sporulation of *F. oxysporum* in solid and liquid media.

Solid (PDA) and liquid media (PDB) were used to study the effect of pH on growth of *F. oxysporum* as described in material and methods (Section 3.3.3.2). The growth of the pathogen was observed in a range of 4.5-7.5 pH in both solid and liquid media. In case of solid media radial mycelial growth was significantly maximum at pH 6.5 (87.22 mm) while other pH mycelial growth was varied from 29.33 to 83.11 mm and least growth was observed at pH 4.5 (25.11 mm). Whereas, in liquid media significant maximum biomass production was observed at pH 6.5 (0.69 g 100 mL<sup>-1</sup>) whereas, other pH the biomass

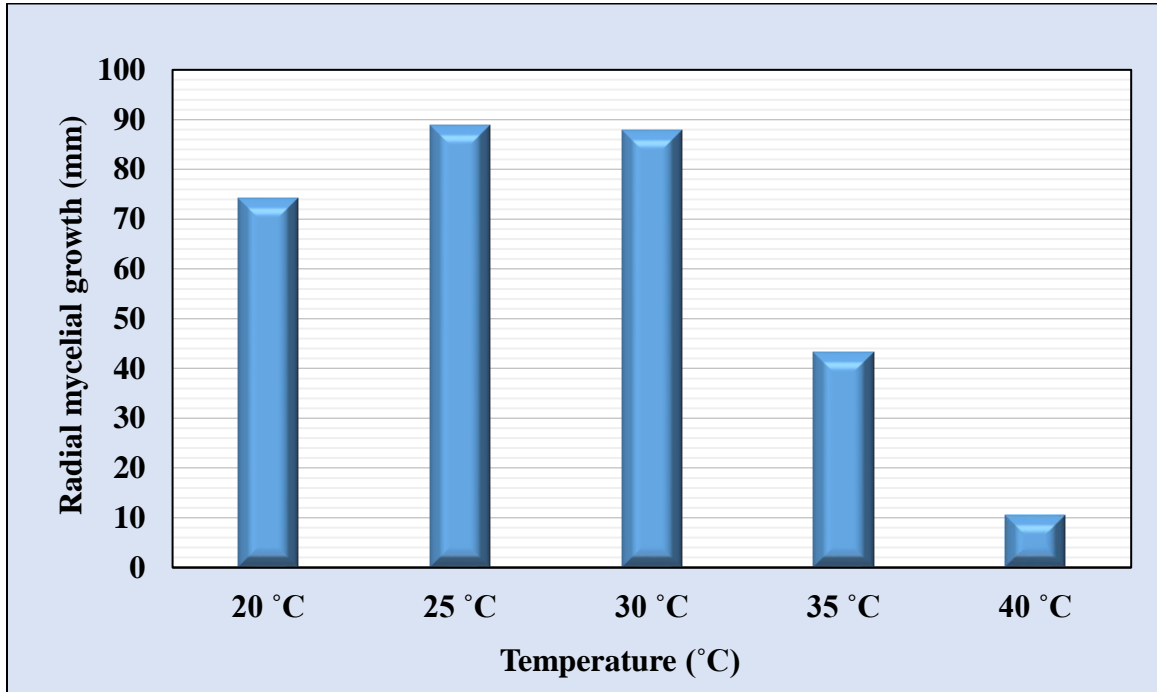
**Table 10. Effect of different temperatures on growth and sporulation of *F. oxysporum* in solid and liquid media**

Sl. No.	Temperature	Solid media (PDA)		Liquid media (PDB)	
		Radial mycelial growth (mm) *	Sporulation **	Dry weight of mycelia (g/100 mL) *	Sporulation **
1	20 °C	74.22	++	0.44	+
2	25 °C	88.77	+++	0.67	+++
3	30 °C	87.77	++	0.34	++
4	35 °C	43.33	+	0.31	+
5	40 °C	10.55	-	0.00	-
	<b>S. Em ±</b>	<b>1.27</b>		<b>0.002</b>	
	<b>CD (p 0.01)</b>	<b>2.92</b>		<b>0.12</b>	

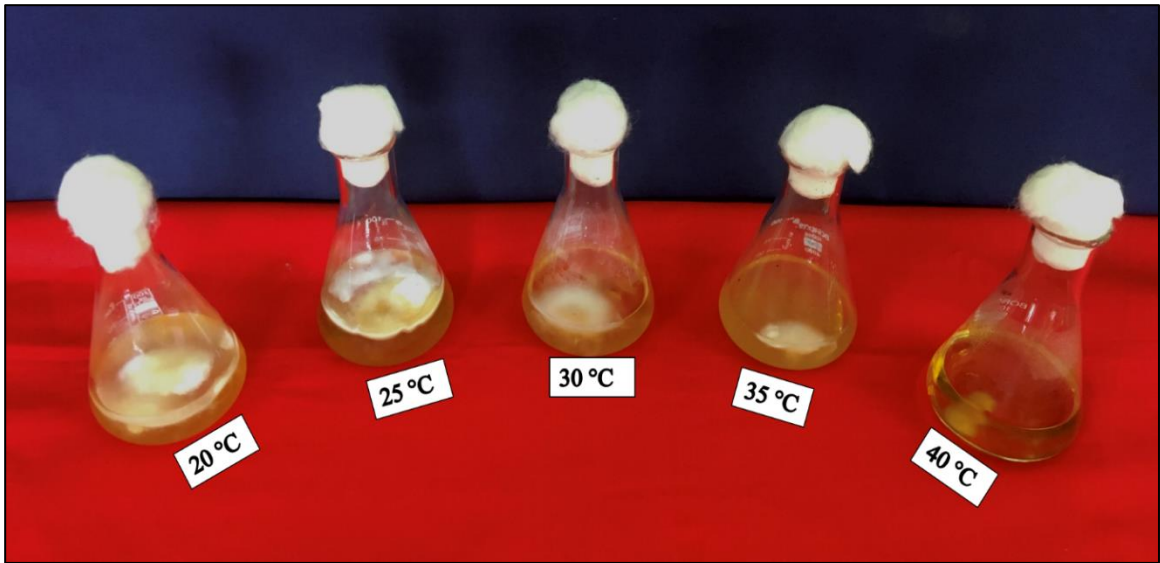
**Note:** \*Means of three replications; \*\*(-) No sporulation- 0 conidia per microscopic field; (+) Poor- 1-20 conidia per microscopic field; (++) Moderate- 20-50 conidia per microscopic field; (+++) Good- 50-100 conidia per microscopic field; (++++) Excellent - > 100 conidia per microscopic field.



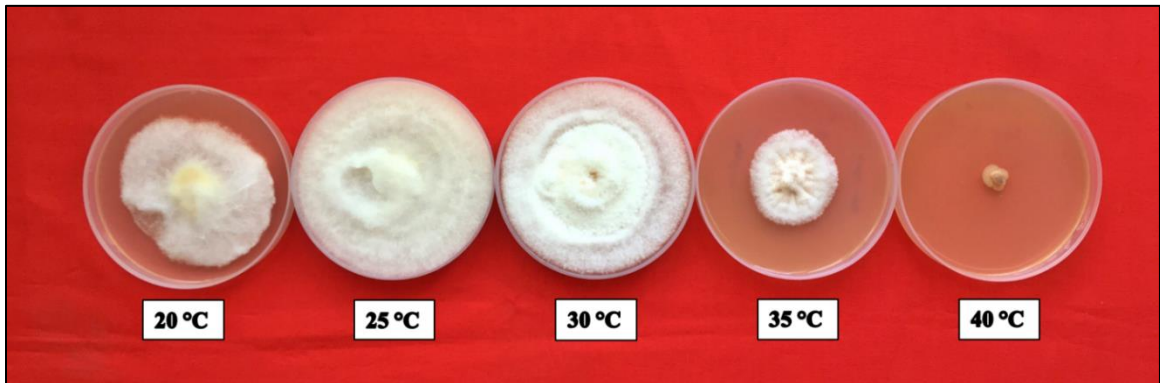
**Fig. 3: Effect of different temperature on biomass production of *F. oxysporum* in liquid media (PDB)**



**Fig. 4: Effect of different temperatures on mycelial growth of *F. oxysporum* in solid media (PDA)**



**Plate 10a. Effect of temperatures on growth of *F. oxysporum* in liquid media (PDB)**



**Plate 10b. Effect of temperatures on growth of *F. oxysporum* in solid media (PDA)**

production is varied from 0.41 to 0.62 g 100 mL<sup>-1</sup> and the least growth was observed at pH 7.5 (0.40 g 100 mL<sup>-1</sup>), sporulation was maximum at pH 6.5 both in liquid and solid media (Table 11, 12, Fig. 5, 6 and Plate 11a, 11b.).

Both results clearly revealed that suitable pH to culture *F. oxysporum* causing geranium wilt was pH 6.5 for mycelial growth and for sporulation 6.5 pH was ideal. The foremost acidic and alkaline pH was not suitable for the growth of pathogen. Similarly, Yadav *et al.* (2014) noted the effect of different levels of pH on maximum dry weight of mycelia and sporulation of the pathogen causing basal rot of onion *F. oxysporum* at pH 6.5 (280 mg).

#### **4.5 Molecular characterization of *Fusarium* sp. infecting geranium.**

*F. oxysporum* isolates were characterized based on morphological and conidial characteristics for further confirmation at molecular level molecular studies were conducted for all isolates by using universal ITS primers as mentioned in material and methods (Section 3.4.1).

##### **4.5.1 PCR amplification and sequencing**

The detailed PCR amplification is mentioned in material and methods (Section 3.4.3) PCR primer pairs of ITS1/ITS4 yielded specific PCR products of approximately 550 bp (ITS). PCR product was gel eluted by using Medauxin® gel extraction kit and then sanger sequenced the genes (Plate 12.).

DNA sequences for ITS1/ ITS4 gene obtained were compared by using NCBI BLAST programme. Based on ITS sequences the results of BLAST showed that all the *F. oxysporum* isolates were showed highest per cent identity with *F. oxysporum* Gnrarsnl 1 causing rhizome rot of ginger which showed 100 per cent homology with the reference GenBank accession number is [MN055701.1] and other strains showing homology with *F. oxysporum* infecting geranium are [MH854774] *F. oxysporum* strain CBS 129.24, [MK811209] *F. oxysporum* strain Ps-4 and [MK849925] *F. oxysporum* strain JFP9.

**Table 11. Effect of hydrogen ion concentration (pH) on growth and sporulation of *F. oxysporum* in liquid media**

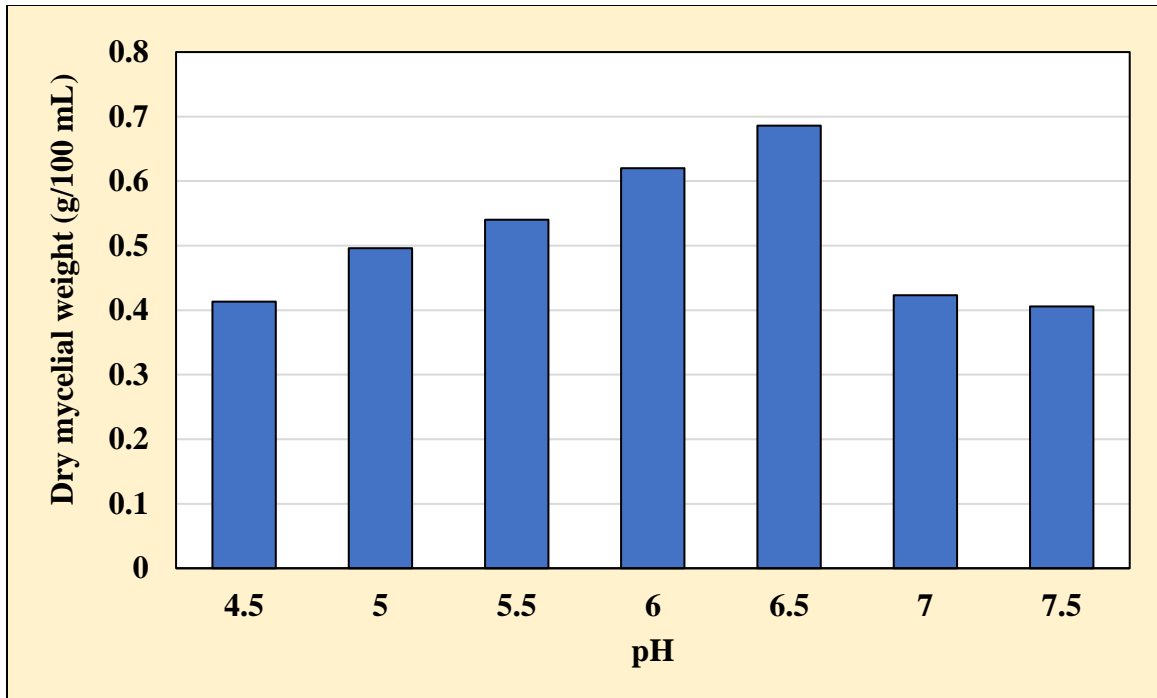
Sl. No.	pH	Dry weight of mycelia (g/100 mL) *	Sporulation**
1	4.5	0.41	+
2	5.0	0.50	++
3	5.5	0.54	++
4	6.0	0.62	+++
5	6.5	0.69	++++
6	7.0	0.42	+
7	7.5	0.40	++
	<b>S. Em ±</b>	<b>0.001</b>	
	<b>CD (p 0.01)</b>	<b>0.06</b>	

**Note:** \*Means of three replications; \*\*(-) No sporulation- 0 conidia per microscopic field; (+) Poor- 1-20 conidia per microscopic field; (++) Moderate- 20-50 conidia per microscopic field; (+++) Good- 50-100 conidia per microscopic field; (++++) Excellent - > 100 conidia per microscopic field.

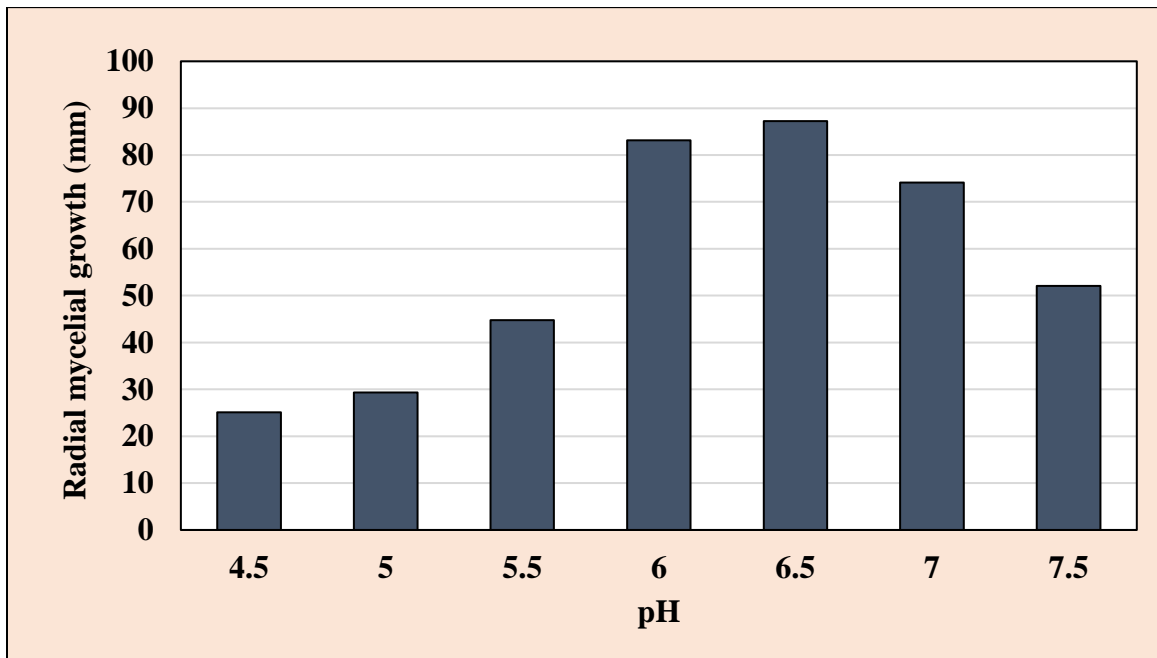
**Table 12. Effect of hydrogen ion concentration (pH) on growth and sporulation of *F. oxysporum* in solid media**

Sl. No.	pH	Radial mycelial growth (mm)*	Sporulation**
1	4.5	25.11	+
2	5.0	29.33	+
3	5.5	44.77	++
4	6.0	83.11	+++
5	6.5	87.22	++++
6	7.0	74.11	+
7	7.5	52.06	+
	<b>S. Em ±</b>	<b>0.90</b>	
	<b>CD (p 0.01)</b>	<b>2.31</b>	

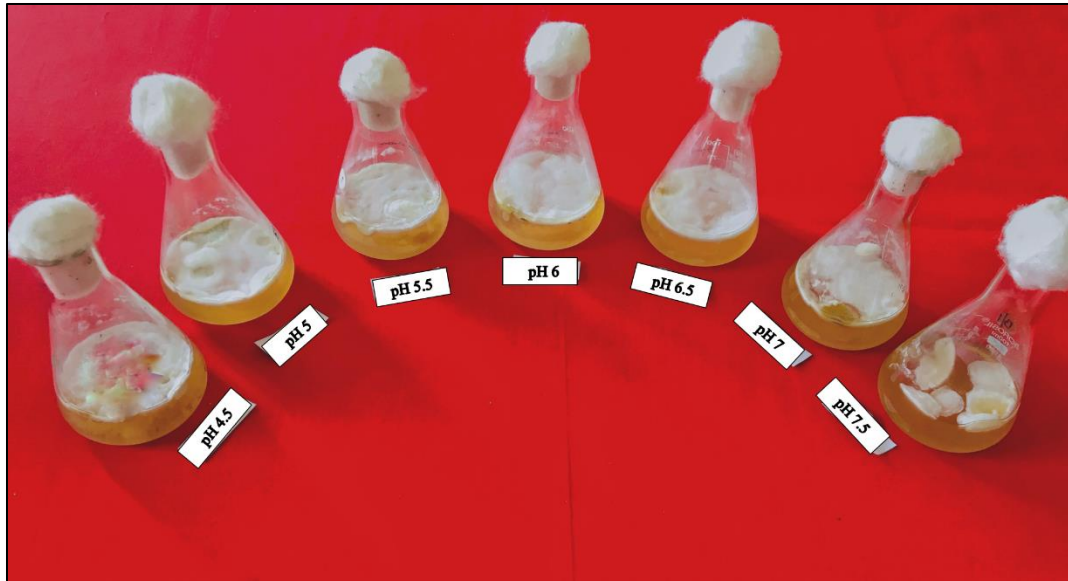
**Note:** \*Means of three replications; \*\*(-) No sporulation- 0 conidia per microscopic field; (+) Poor- 1-20 conidia per microscopic field; (++) Moderate- 20-50 conidia per microscopic field; (+++) Good- 50-100 conidia per microscopic field; (++++) Excellent - > 100 conidia per microscopic field.



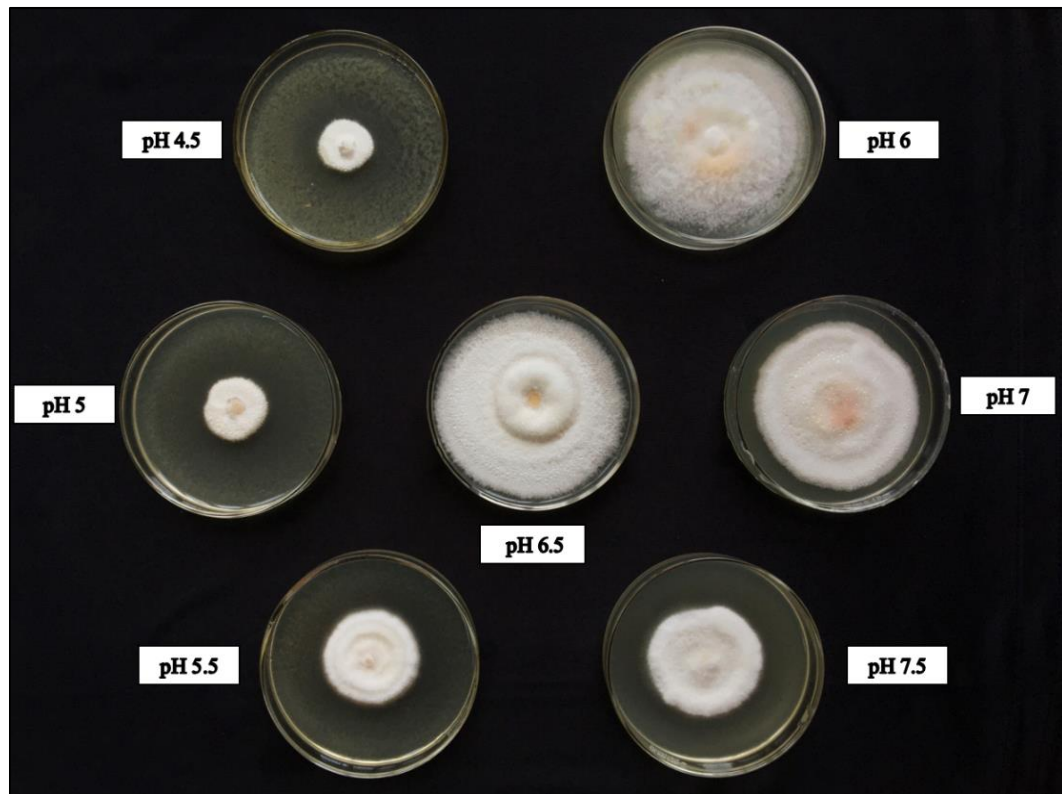
**Fig. 5: Effect of hydrogen ion concentration (pH) on biomass production of *F. oxysporum* in liquid media (PDB)**



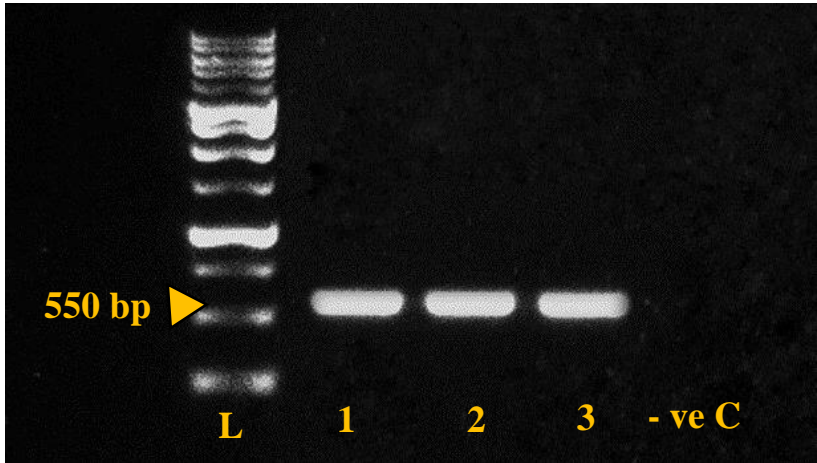
**Fig. 6: Effect of hydrogen ion concentration (pH) on mycelial growth of *F. oxysporum* in solid media (PDA)**



**Plate 11a. Effect of different hydrogen ion concentration (pH) in liquid media (PDB) on growth of *F. oxysporum***



**Plate 11b. Effect of different hydrogen ion concentration (pH) in solid media (PDA) on growth of *F. oxysporum***



Lane 1: 1kb ladder  
 Lane 2: isolate GFO 1  
 Lane 3: isolate GFO 2  
 Lane 4: isolate GFO 3  
 Lane 5: Negative control

Plate 12. Gel banding pattern of ITS rDNA region of *F. oxysporum* isolates

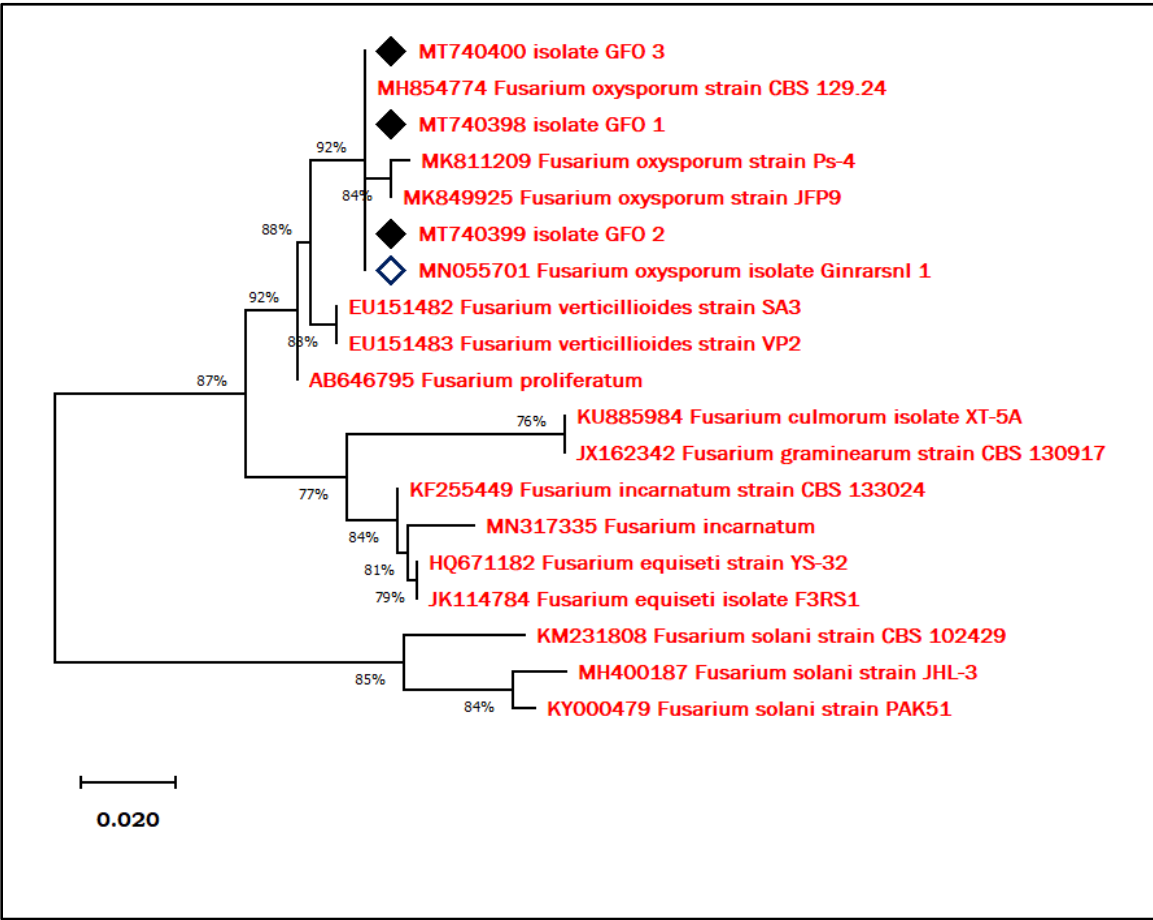


Fig. 7: Molecular evolutionary analysis of Internal Transcribed Spacer (ITS) sequences using maximum likelihood approach with other reference sequences of *Fusarium* species gathered from GenBank

#### **4.5.2 Sequence alignment and phylogenetic analysis**

All sequences were aligned for ITS1/ ITS4 along with other reference sequences of *Fusarium* species and constructed the phylogenetic tree with maximum likelihood approach by using MEGA X software. Phylogenetic analysis showed the sequences of all isolates matched with *F. oxysporum* (Fig. 7).

ITS sequences of isolates confirmed that *F. oxysporum* as causal organism for wilt disease in scented geranium.

Sequences of ITS were submitted to NCBI GenBank and the accession numbers of three isolates were [MT740398] *F. oxysporum* isolate GFO 1, [MT740399] *F. oxysporum* isolate GFO 2 and [MT740400] *F. oxysporum* isolate 3. These sequences showed 100 per cent homology with [MN055701.1] *F. oxysporum* Ginrarsnl 1 reference strain were found by Kumar *et al.* (2019); Khilare and Ahmed (2012); Chaudhary *et al.* (2018) and (Tyagi and Paudel, 2014).

The species distinction results were similar with Singha *et al.* (2016) isolated *Fusarium* sp. from Assam. Amplified the internal transcribed spacer (ITS) region of the conserved ribosomal DNA using primers ITS-1 and ITS-4. All the ITS sequences were compared for gaps and similarity. It was concluded that molecular profiling using ITS was an indispensable method for identification studies. Peeran *et al.* (2019) characterized the pathogen causing root and stem rot of vanilla using molecular tools such as 18s rDNA or ITS region amplification and sequenced. Phylogenetic tree was constructed using maximum likelihood which shows *F. oxysporum* was the causal organism. Results were also in accordance with Zheng *et al.* (2012) and Kuppusamy *et al.* (2018).

#### **4.6 In vitro evaluation of botanicals, bio agents and fungicides against *Fusarium* sp. infecting geranium.**

##### **4.6.1 Evaluation of botanicals under laboratory conditions**

Twelve plant extracts belong to various families were tested by using poisoned food technique to find the efficacy to inhibit the mycelial growth of test fungus as detailed in

material and methods (Section 3.5.1). These plant extracts were screened at three concentrations (10, 15 and 20%) and the obtained data is presented in table 13, fig. 8 and depicted in plate 13. Among the botanicals, highest significant mean per cent inhibition of mycelial growth was recorded in agave (59.31 %) whereas no inhibition was noticed in parthenium (0.00 %). In the other plant extracts, the inhibition was moderate and variable. Amongst the concentrations, 20 per cent showed maximum (18.51 %) inhibition of mycelial growth with the least in 10 per cent (6.58 %).

With respect to interaction of botanicals and concentrations, agave showed 66.47 per cent mycelial growth inhibition which was recorded at 20 per cent. Agave was observed to be effective even at lower concentration than other botanicals at higher concentration parthenium at all the concentrations, datura, garlic, calotropis at 10 and 15 per cent and karanja and turmeric at 10 per cent showed no (0.00 %) inhibition. Inhibition of mycelial growth was possibly due to inhibition of spore production, inhibition of spore germination and deformed conidia. Those botanicals that didn't affect the mycelial growth of *F. oxysporum* probably affected the conidia or sporulation. Agave was observed to be effective even at lower concentration than other botanicals at higher concentration.

Among the different extracts 20 per cent of *A. indica* was found most effective followed by *R. emodi*, *E. globulus*, *A. annua* and *O. sanctum* Joseph *et al.* (2008). Least growth of *F. oxysporum* f. sp. *dianthi* was recorded in neem leaf extract which showed excellent inhibitory effect of (78.19 %) reduction over control.

Neem leaf extract shows best inhibitory action against *F. udum* causing wilt of pigeon pea (Ghante *et al.*, 2019). Among the tested botanicals against *F. oxysporum* f. sp. *cepae* causing onion basal rot NSKE (43.95 %) was found effective in inhibiting mycelial growth which was followed by prickly chaff flower (34.81 %) (Anupama *et al.*, 2019). However, results of present study were not in accordance with these findings.

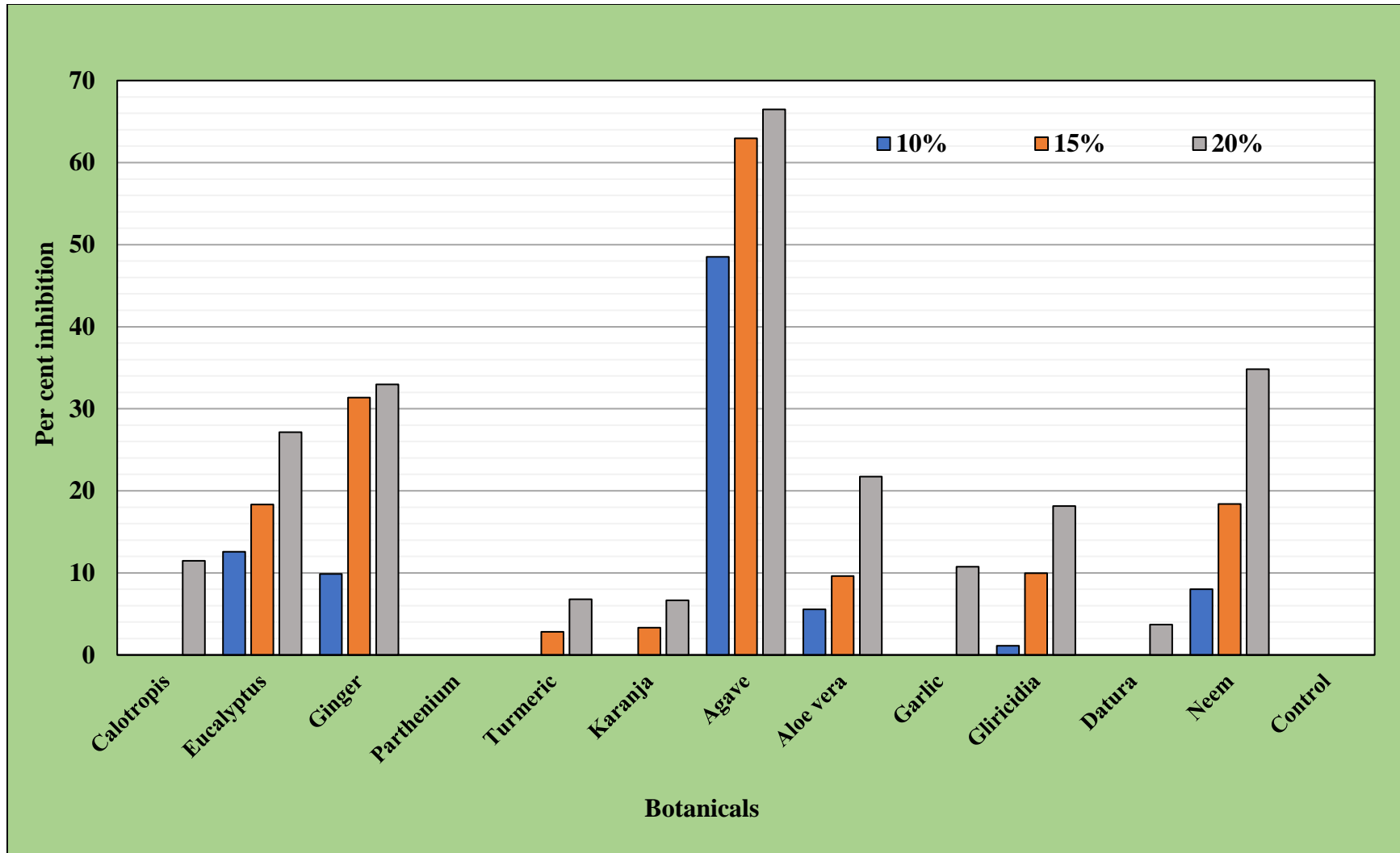
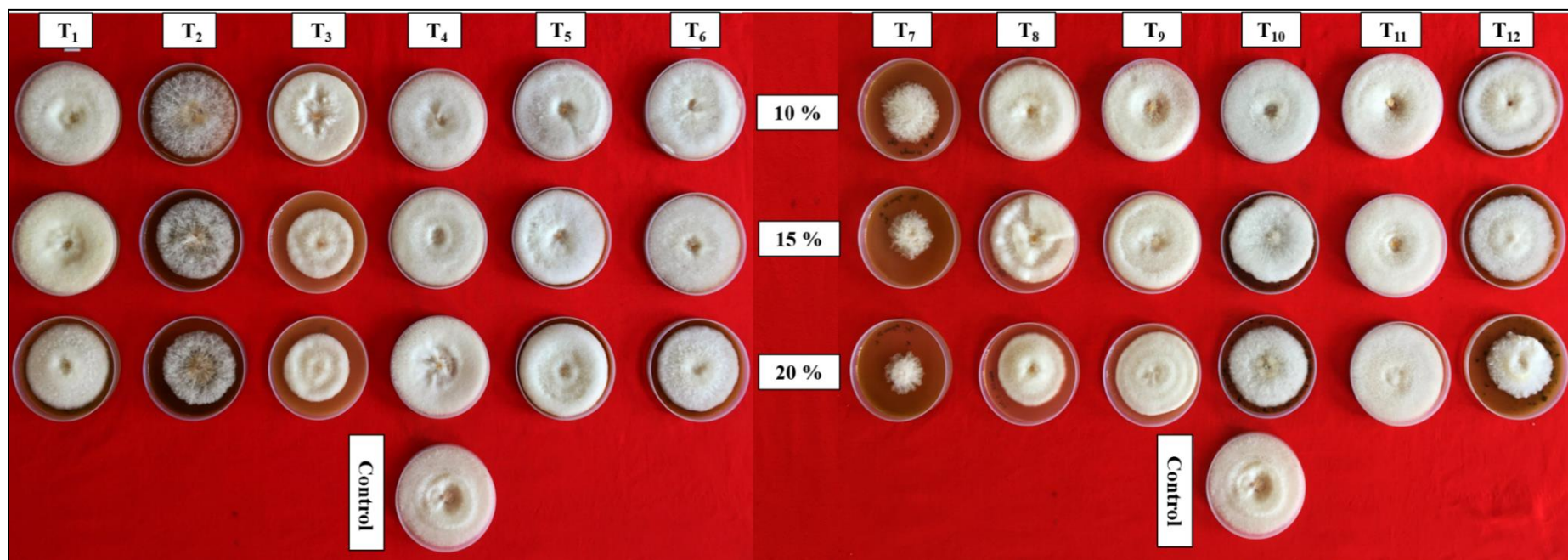


Fig. 8: *In vitro* efficacy of botanicals on mycelial growth inhibition of *F. oxysporum*



**T1- Calotropis**

**T2- Eucalyptus**

**T3- Ginger**

**T4- Parthenium**

**T5- Turmeric**

**T6- Karanja**

**T7- Agave**

**T8- Aloe vera**

**T9- Garlic**

**T10- Gliricidia**

**T11- Datura**

**T12- Neem**

**Plate 13. *In vitro* evaluation of botanicals against *F. oxysporum***

**Table 13. *In vitro* efficacy of botanicals on growth and sporulation of *F. oxysporum* infecting geranium**

Sl. No.	Botanical	Per cent inhibition*			Mean	Sporulation**
		10 % (w/v)	15 % (w/v)	20 % (w/v)		
1	Calotropis	0.00 (0.30)	0.00 (0.30)	11.48 (19.80)	3.82 (6.80)	++
2	Eucalyptus	12.59 (20.77)	18.32 (25.34)	27.16 (31.34)	19.35 (25.82)	++
3	Ginger	9.87 (18.29)	31.36 (34.05)	32.96 (35.03)	24.73 (29.12)	+
4	Parthenium	0.00 (0.30)	0.00 (0.30)	0.00 (0.30)	0.00 (0.30)	++
5	Turmeric	0.00 (0.30)	2.83 (9.66)	6.78 (15.09)	3.20 (8.35)	++
6	Karanja	0.00 (0.30)	3.33 (10.41)	6.66 (14.92)	3.33 (8.54)	-
7	Agave	48.51 (44.14)	62.96 (52.51)	66.47 (54.62)	59.31 (50.42)	-
8	Aloe vera	5.55 (13.58)	9.62 (18.04)	21.72 (27.78)	12.3 (19.80)	+
9	Garlic	0.00 (0.30)	0.00 (0.30)	10.74 (19.12)	3.58 (6.57)	++
10	Gliricidia	1.11 (4.97)	9.95 (18.37)	18.14 (25.20)	9.73 (16.18)	+++
11	Datura	0.00 (0.30)	0.00 (0.30)	3.70 (11.06)	1.23 (3.88)	++
12	Neem	8.01 (16.43)	18.39 (25.39)	34.81 (36.15)	20.40 (25.99)	-
13	Control	0.00 (0.30)	0.00 (0.30)	0.00 (0.30)	0.00 (0.30)	
	<b>Mean</b>	6.58 (9.25)	12.06 (15.02)	18.51 (22.36)		
		<b>Botanicals (B)</b>	<b>Concentration (C)</b>	<b>B×C</b>		
	<b>S. Em ±</b>	<b>0.08</b>	<b>0.49</b>	<b>0.04</b>		
	<b>CD (p 0.01)</b>	<b>1.40</b>	<b>0.67</b>	<b>2.43</b>		

**Note:** Figures in the parenthesis are arcsine transformed values; \*Means of three replications; \*\*(-) No sporulation- 0 conidia per microscopic field; (+) Poor- 1-20 conidia per microscopic field; (++) Moderate- 20-50 conidia per microscopic field; (+++) Good- 50-100 conidia per microscopic field; (++++) Excellent - > 100 conidia per microscopic field.

#### 4.6.2 Assessment of bio agents against *F. oxysporum*

A total of 12 fungal bio agents and 8 bacterial bio agents were evaluated under *in vitro* by dual culture technique against *F. oxysporum* as detailed in material and methods (Section 3.5.2)

##### 4.6.2.1 Fungal bio agents

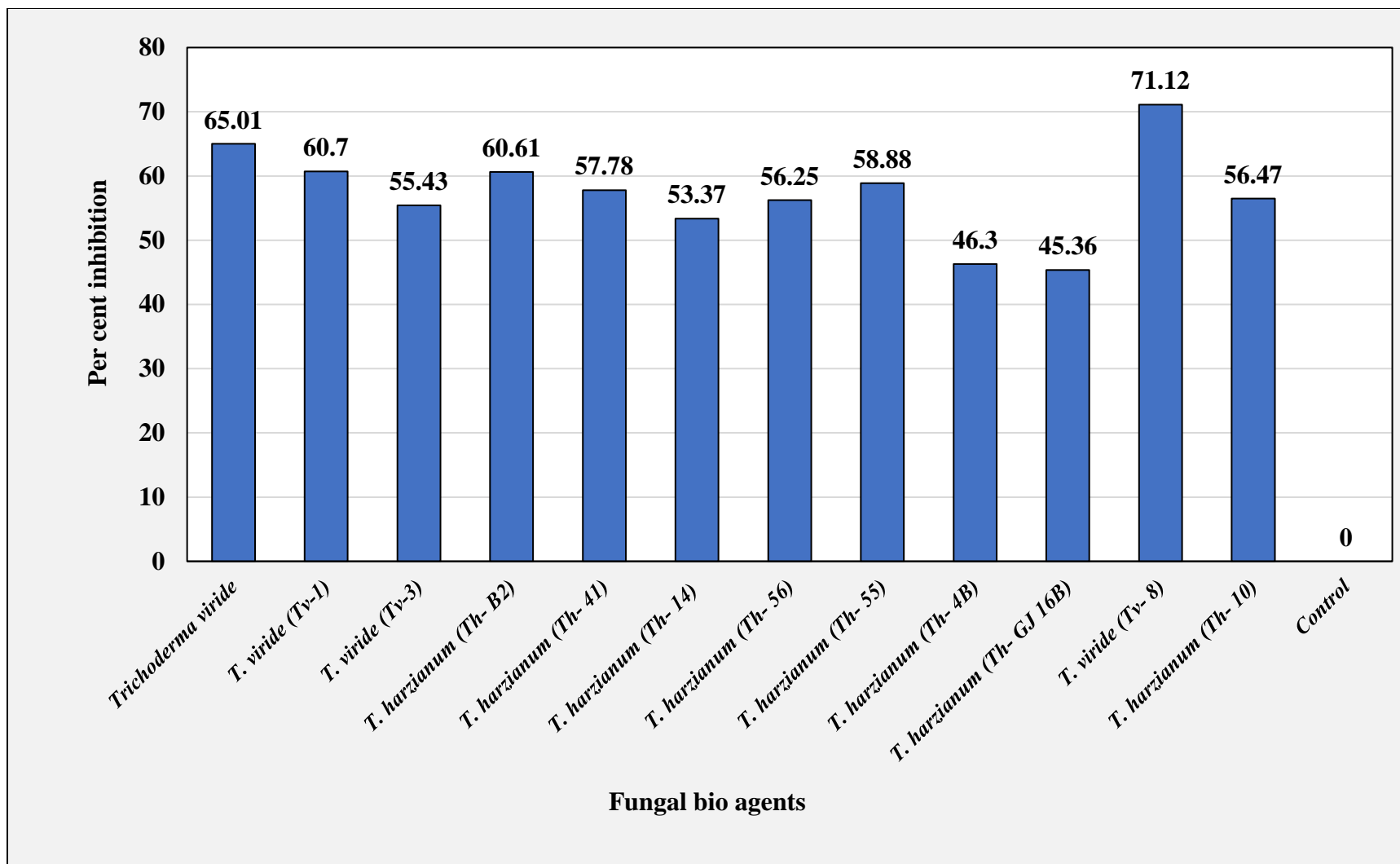
A total of 12 fungal bio agents of *Trichoderma* spp. were evaluated to know their antagonistic effect against *F. oxysporum*. Results presented in table 14, fig. 9 and displayed in plate 14 revealed that the maximum inhibition of mean mycelial growth was observed in *T. viride* (Tv- 8) 71.12 per cent where the other bio agents showed lesser and varied inhibition. Lowest inhibition was observed by *T. harzianum* (Th-GJ 16B) 45.36 per cent. Fungal bio agents inhibited the test fungus growth by means of myco/hyper parasitism (over growth on pathogen) and inhibition zone formation and production of volatile and non-volatile organic compounds or metabolites. The actual control might also be by antibiosis, cell wall degradation, production of enzymes, etc.

##### 4.6.2.2 Bacterial bio agents

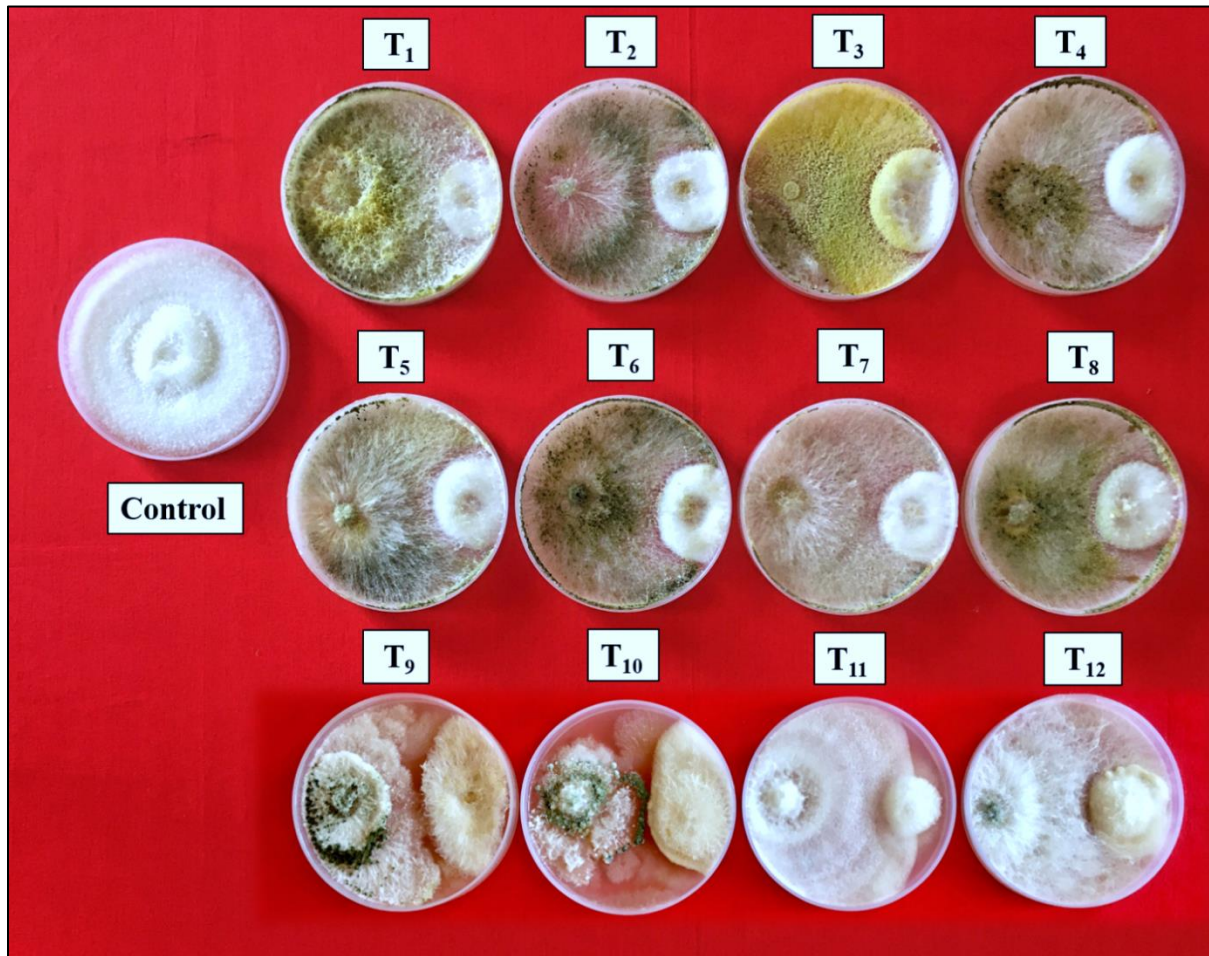
Among all the bacterial bio agents, maximum per cent inhibition of mean mycelial growth was by *B. pumilis* (54.66 %) which is on par with *B. velezensis* (Bv-P<sub>42</sub>) (53.61 %) and the remaining bio agents showed varied inhibition from 16.07-39.97 per cent. Whereas, *Pseudomonas fluorescens* exhibited least inhibition of 11.95 per cent (Table 15, Plate 15 and Fig. 10).

The inhibition of *F. oxysporum* possibly due to production of volatile organic compounds (VOCs) by bacterial bio agents and cell wall degrading volatile enzymes or compounds by fungal bio agents. Here, *F. oxysporum* growth was affected morphologically either by inhibition of sporulation or hyphal bulging at regular or irregular intervals. Fungal bio agents having better efficacy against *F. oxysporum* than bacterial bio agents.

*T. viride*, *T. harzianum* and *P. fluorescens* and *Bacillus* spp. strains were evaluated for their antagonistic activity against *Fusarium oxysporum in vitro*. The conidia of



**Fig. 9: *In vitro* efficacy of fungal bio agents on mycelial growth inhibition of *F. oxysporum***



**T<sub>1</sub>- *Trichoderma viride***

**T<sub>2</sub>- *T. viride* (Tv-1)**

**T<sub>3</sub>- *T. viride* (Tv-3)**

**T<sub>4</sub>- *T. harzianum* (Th- B2)**

**T<sub>5</sub>- *T. harzianum* (Th- 41)**

**T<sub>6</sub>- *T. harzianum* (Th- 14)**

**T<sub>7</sub>- *T. harzianum* (Th- 56)**

**T<sub>8</sub>- *T. harzianum* (Th- 55)**

**T<sub>9</sub>- *T. harzianum* (Th- 4B)**

**T<sub>10</sub>- *T. harzianum* (Th- GJ 16B)**

**T<sub>11</sub>- *T. viride* (Tv- 8)**

**T<sub>12</sub>- *T. harzianum* (Th- 10)**

**Plate 14. *In vitro* evaluation of fungal bio agents against *F. oxysporum***

**Table 14. *In vitro* efficacy of fungal bio agents on growth and sporulation of *F. oxysporum* infecting geranium.**

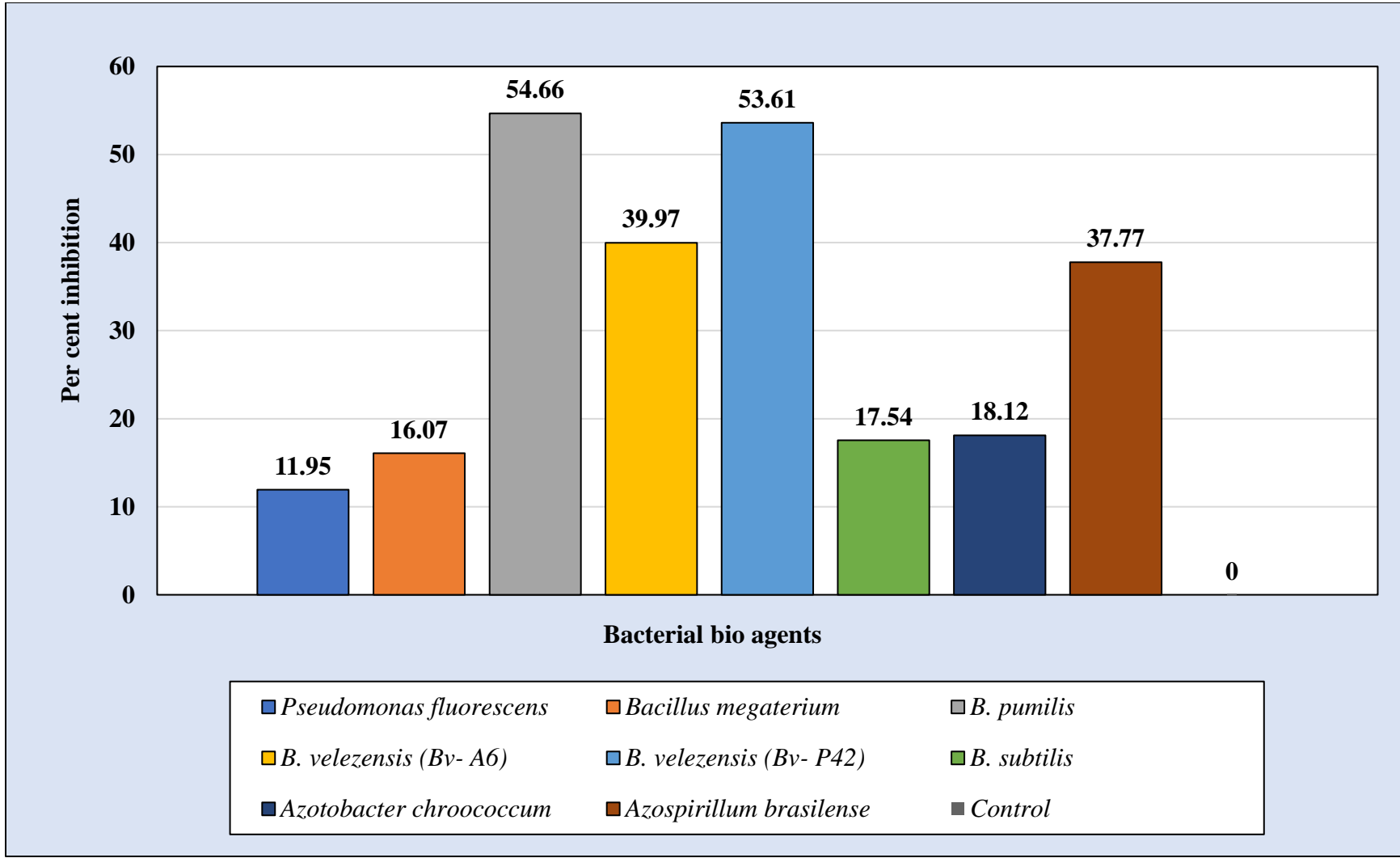
Sl. No.	Bio agent	Per cent inhibition of mycelial growth*	Over Growth***	Sporulation**
1	<i>Trichoderma viride</i> (Tv)	65.01 (53.73)	HP (15 mm)	+
2	<i>T. viride</i> (Tv-1)	60.70 (51.18)	IZ (10 mm)	++
3	<i>T. viride</i> (Tv-3)	55.43 (48.11)	HP (5 mm)	+
4	<i>T. harzianum</i> (Th- B <sub>2</sub> )	60.61 (51.13)	HP (10 mm)	+
5	<i>T. harzianum</i> (Th- 41)	57.78 (49.48)	HP (5 mm)	++
6	<i>T. harzianum</i> (Th- 14)	53.37 (46.93)	IZ (5 mm)	+
7	<i>T. harzianum</i> (Th- 56)	56.25 (48.59)	IZ (8 mm)	+
8	<i>T. harzianum</i> (Th- 55)	58.88 (50.12)	HP (10 mm)	+
9	<i>T. harzianum</i> (Th- 4B)	46.30 (42.87)	IZ (5 mm)	++
10	<i>T. harzianum</i> (Th-GJ 16B)	45.36 (42.34)	IZ (5 mm)	++
11	<i>T. viride</i> (Tv- 8)	71.12 (57.49)	HP (18 mm)	-
12	<i>T. harzianum</i> (Th- 10)	56.47 (48.71)	IZ (5 mm)	+
13	Control	0.00 (0.30)	-	-
	<b>S. Em ±</b>	<b>1.34</b>		
	<b>CD (p 0.01)</b>	<b>2.64</b>		

**Note:** Figures in the parenthesis are arcsine transformed values; \*Means of three replications; \*\*(-) No sporulation- 0 conidia per microscopic field; (+) Poor- 1-20 conidia per microscopic field; (++) Moderate- 20-50 conidia per microscopic field; (+++) Good- 50-100 conidia per microscopic field; (++++) Excellent - > 100 conidia per microscopic field; \*\*\* HP-Hyper parasitism; IZ- Inhibition zone

**Table 15. *In vitro* efficacy of bacterial bio agents on growth and sporulation of *F. oxysporum* infecting geranium.**

Sl. No.	Bio agent	Per cent inhibition of mycelial growth *	Sporulation**
1	<i>Pseudomonas fluorescens</i>	11.95 (20.18)	++
2	<i>Bacillus megaterium</i>	16.07 (23.62)	+
3	<i>B. pumilis</i>	54.66 (47.67)	-
4	<i>B. velezensis</i> (Bv- A <sub>6</sub> )	39.97 (39.21)	-
5	<i>B. velezensis</i> (Bv- P <sub>42</sub> )	53.61 (47.07)	-
6	<i>B. subtilis</i>	17.54 (24.75)	++
7	<i>Azotobacter chroococcum</i>	18.12 (25.19)	++
8	<i>Azospirillum brasilense</i>	37.77 (37.92)	+
9	Control	0.00 (0.30)	-
	<b>S. Em ±</b>	<b>1.32</b>	
	<b>CD (p 0.01)</b>	<b>2.46</b>	

**Note:** Figures in the parenthesis are arcsine transformed values; \*Means of three replications; \*\*(-) No sporulation- 0 conidia per microscopic field; (+) Poor- 1-20 conidia per microscopic field; (++) Moderate- 20-50 conidia per microscopic field; (+++) Good- 50-100 conidia per microscopic field; (++++) Excellent - > 100 conidia per microscopic field.



**Fig. 10: *In vitro* efficacy of bacterial bio agents on mycelial growth inhibition of *F. oxysporum***



- T<sub>1</sub>- *Pseudomonas fluorescens*
- T<sub>2</sub>- *Bacillus megaterium*
- T<sub>3</sub>- *B. pumilis*
- T<sub>4</sub>- *B. velezensis* (Bv- A6)
- T<sub>5</sub>- *B. velezensis* (Bv- P42)
- T<sub>6</sub>- *B. subtilis*
- T<sub>7</sub>- *Azotobacter chroococcum*
- T<sub>8</sub>- *Azospirillum brasilense*

Plate 15. *In vitro* evaluation of bacterial bio agents against *F. oxysporum*

*F. oxysporum* were found to be inhibited by all the three antagonistic microorganisms. The highest per cent inhibition of conidial germination was brought out by *T. viride* followed by *T. harzianum* (Rajeswari and Kannabiran, 2011). *T. viride* completely inhibited the mycelial growth of *F. udum* in dual culture and fungal bio agents were found superior over bacterial bio agent by Ram and Pandey (2011). Ali and Nadarajah (2014) documented that the principle cause for inhibition of the mycelial growth of the pathogen by *Trichoderma* was coiling of pathogen hyphae by *Trichoderma*. Kalaivani *et al.* (2014) reported that the cell wall degrading enzyme endochitinase produced by *Trichoderma* spp. is responsible for breakdown of the cell wall (Chitin) of the pathogen.

The results obtained in case of fungal bio agent are in agreement with the results obtained by (Ajay and Naik, 2013). Similarly, *T. viride* and *T. harzianum* were best against inhibition of *F. oxysporum* has given by Kumari *et al.* (2014), Mahalakshmi and Yesu Raja (2013).

In this study, *B. velezensis* and *B. pumilis* were found superior over other bacterial species in controlling the *F. oxysporum*. This may due to the production of several antibiotic-like substances such as Kanamycin, lipopeptide and Zwittermicin- A (Stabb *et al.*, 1994).

#### **4.6.3 *In vitro* evaluation of fungicides against *F. oxysporum***

Fungicides belongs to different groups (four contact, three combi product and 10 systemic fungicides) were collected and screened against *F. oxysporum* under laboratory conditions using poisoned food technique as detailed in material and methods (Section 3.5.3).

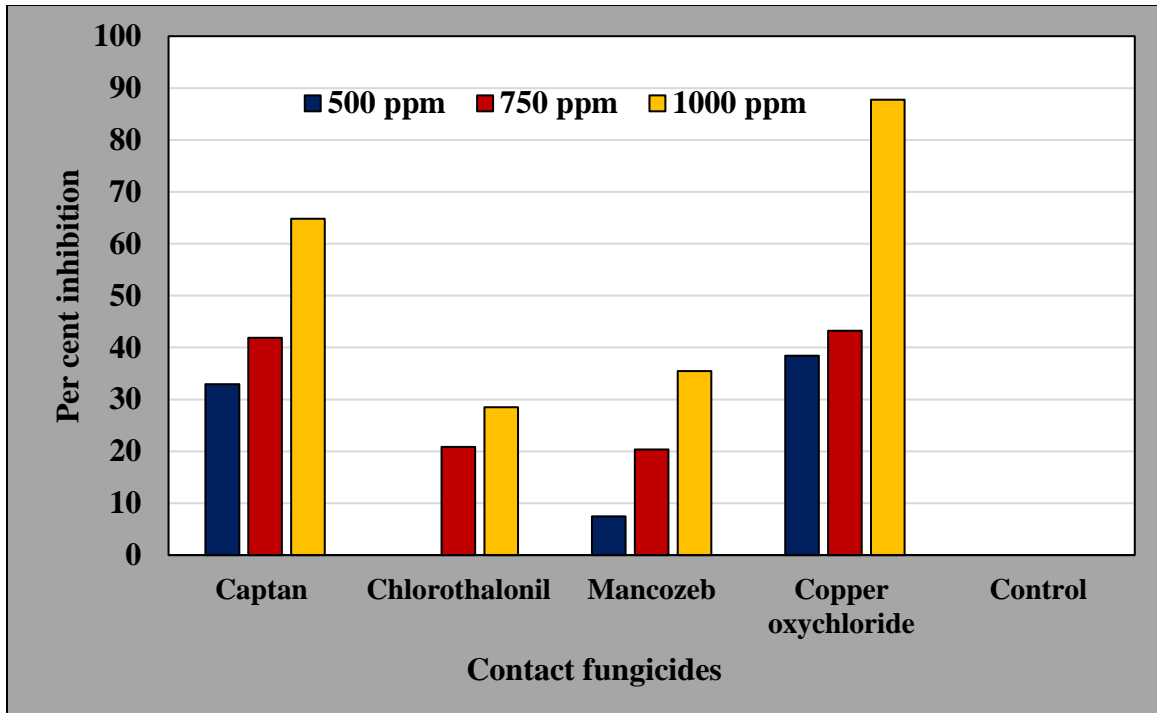
##### **4.6.3.1 Efficacy of contact fungicides**

Among the four contact fungicides, copper oxychloride (56.48 %) recorded the significant maximum inhibition. Other fungicides like mancozeb and captan showed varied per

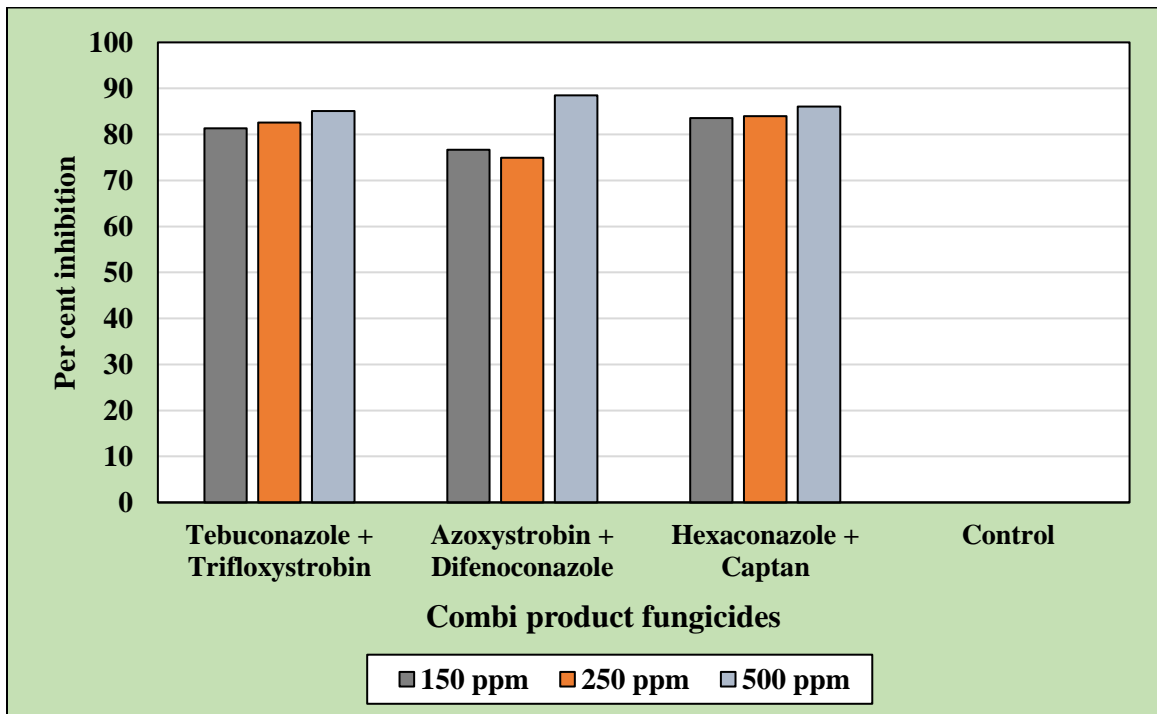
**Table 16. *In vitro* efficacy of contact fungicides on growth and sporulation of *F. oxysporum* infecting geranium**

Sl. No.	Contact fungicide	Per cent inhibition*			Mean	Sporulation**
		500 ppm	750 ppm	1000 ppm		
1	Captan	32.95 (35.03)	41.85 (40.30)	64.81 (53.61)	46.53 (42.98)	++
2	Chlorothalonil	0.00 (0.302)	20.86 (27.17)	28.51 (32.27)	16.45 (19.91)	++
3	Mancozeb	7.45 (15.84)	20.38 (26.83)	35.43 (36.52)	21.08 (26.40)	+++
4	Copper oxy chloride	38.43 (38.31)	43.25 (41.12)	87.77 (69.54)	56.48 (49.66)	-
5	Control	0.00 (0.30)	0.00 (0.30)	0.00 (0.30)	0.00 (0.30)	
	<b>Mean</b>	15.27 (17.95)	25.27 (27.14)	43.30 (38.45)		
		<b>Fungicides (F)</b>	<b>Concentration (C)</b>	<b>F×C</b>		
	<b>S. Em ±</b>	<b>0.24</b>	<b>0.49</b>	<b>0.12</b>		
	<b>CD (p 0.01)</b>	<b>0.86</b>	<b>0.66</b>	<b>1.49</b>		

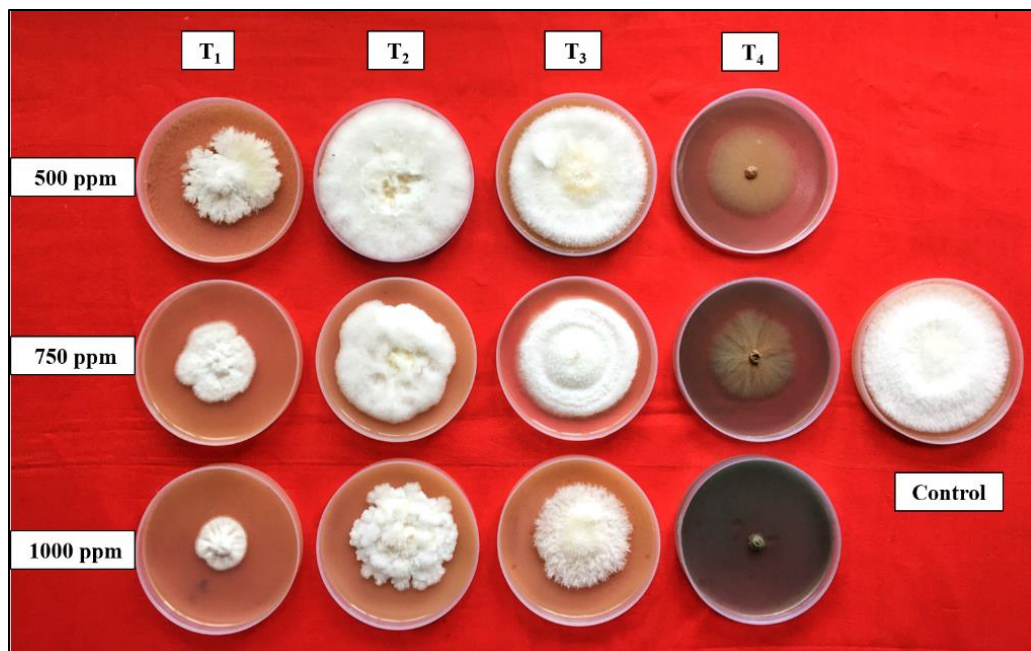
**Note:** Figures in the parenthesis are arcsine transformed values; \*Means of three replications; \*\*(-) No sporulation- 0 conidia per microscopic field; (+) Poor- 1-20 conidia per microscopic field; (++) Moderate- 20-50 conidia per microscopic field; (+++) Good- 50-100 conidia per microscopic field; (+++++) Excellent - > 100 conidia per microscopic field.



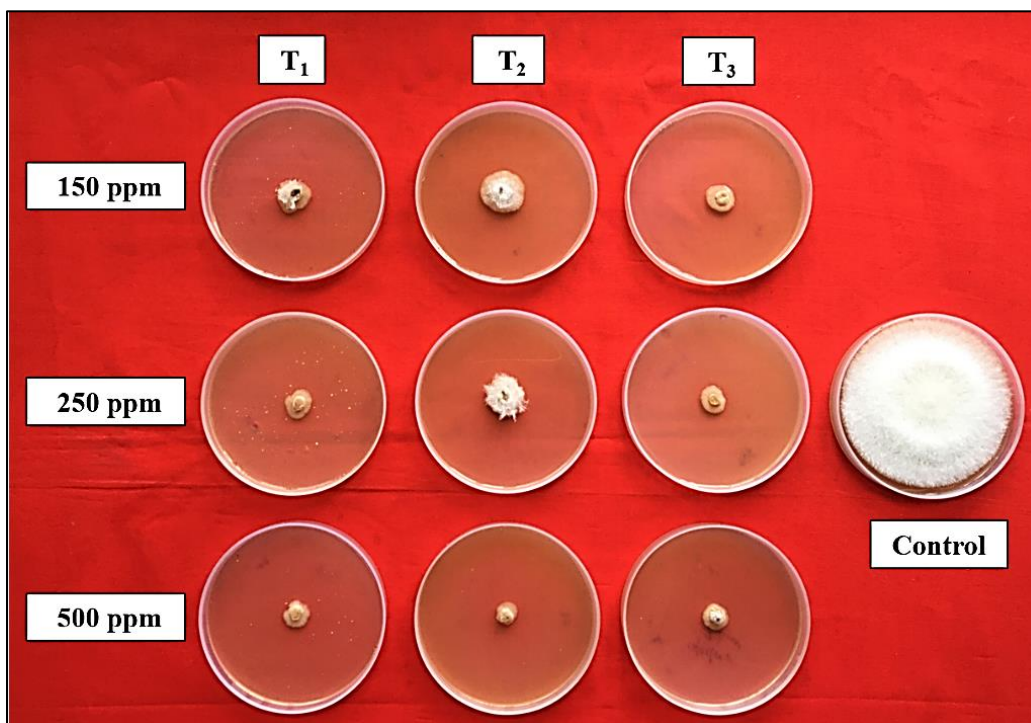
**Fig. 11:** *In vitro* efficacy of contact fungicides on mycelial growth inhibition of *F. oxysporum*



**Fig. 12:** *In vitro* efficacy of combi product fungicides on mycelial growth inhibition of *F. oxysporum*



T<sub>1</sub>- Captan; T<sub>2</sub>- Chlorothalonil; T<sub>3</sub>- Mancozeb; T<sub>4</sub>- Copper oxychloride  
 Plate 16. *In vitro* evaluation of contact fungicides against *F. oxysporum*



T<sub>1</sub>- Tebuconazole + Trifloxystrobin; T<sub>2</sub>- Azoxystrobin + Difenconazole  
 T<sub>3</sub>- Hexaconazole + Captan

Plate 17. *In vitro* evaluation of combi fungicides against *F. oxysporum*

**Table 17. *In vitro* efficacy of combi product fungicides on growth and sporulation of *F. oxysporum* infecting geranium.**

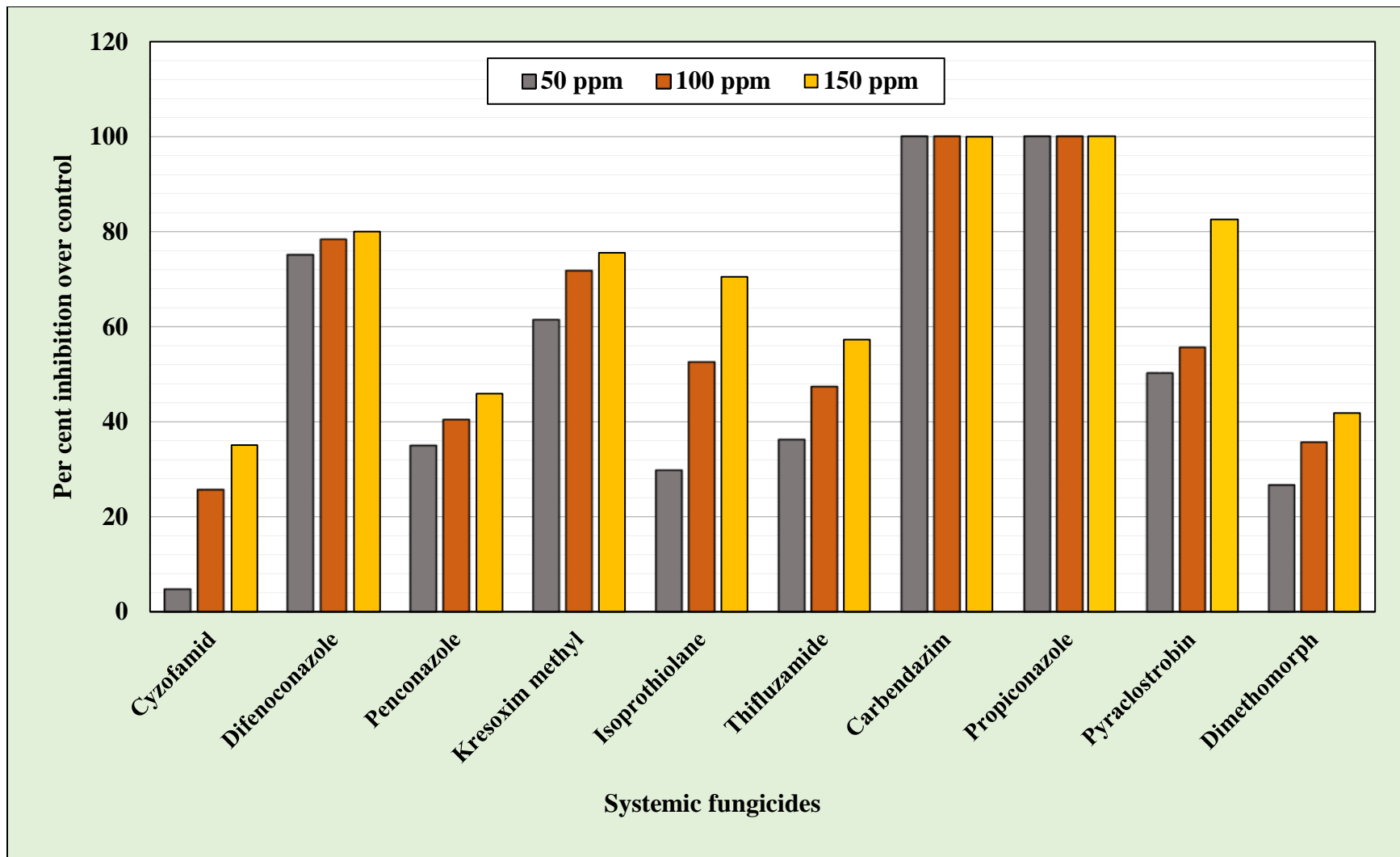
Sl. No.	Combi product fungicide	Per cent inhibition *			Mean	Sporulation**
		150 ppm	250 ppm	500 ppm		
1	Tebuconazole + Trifloxystrobin	81.35 (64.42)	82.59 (65.34)	85.05 (67.26)	83.00 (65.67)	-
2	Azoxystrobin + Difenconazole	76.66 (61.11)	74.93 (59.95)	88.51 (70.19)	80.03 (63.75)	-
3	Hexaconazole + Captan	83.57 (66.09)	83.94 (66.38)	86.02 (68.04)	84.51 (66.84)	+
4	Control	0.00 (0.30)	0.00 (0.30)	0.00 (0.30)	0.00 (0.30)	
	Mean	60.39 (47.98)	60.36 (47.99)	64.89 (51.45)		
		<b>Fungicides (F)</b>	<b>Concentration (C)</b>	<b>F×C</b>		
		<b>S. Em ±</b>	<b>0.33</b>	<b>0.49</b>	<b>0.16</b>	
		<b>CD (p 0.01)</b>	<b>0.80</b>	<b>0.69</b>	<b>1.38</b>	

**Note:** Figures in the parenthesis are arcsine transformed values; \*Means of three replications; \*\*(-) No sporulation- 0 conidia per microscopic field; (+) Poor- 1-20 conidia per microscopic field; (++) Moderate- 20-50 conidia per microscopic field; (+++) Good- 50-100 conidia per microscopic field; (++++) Excellent - > 100 conidia per microscopic field.

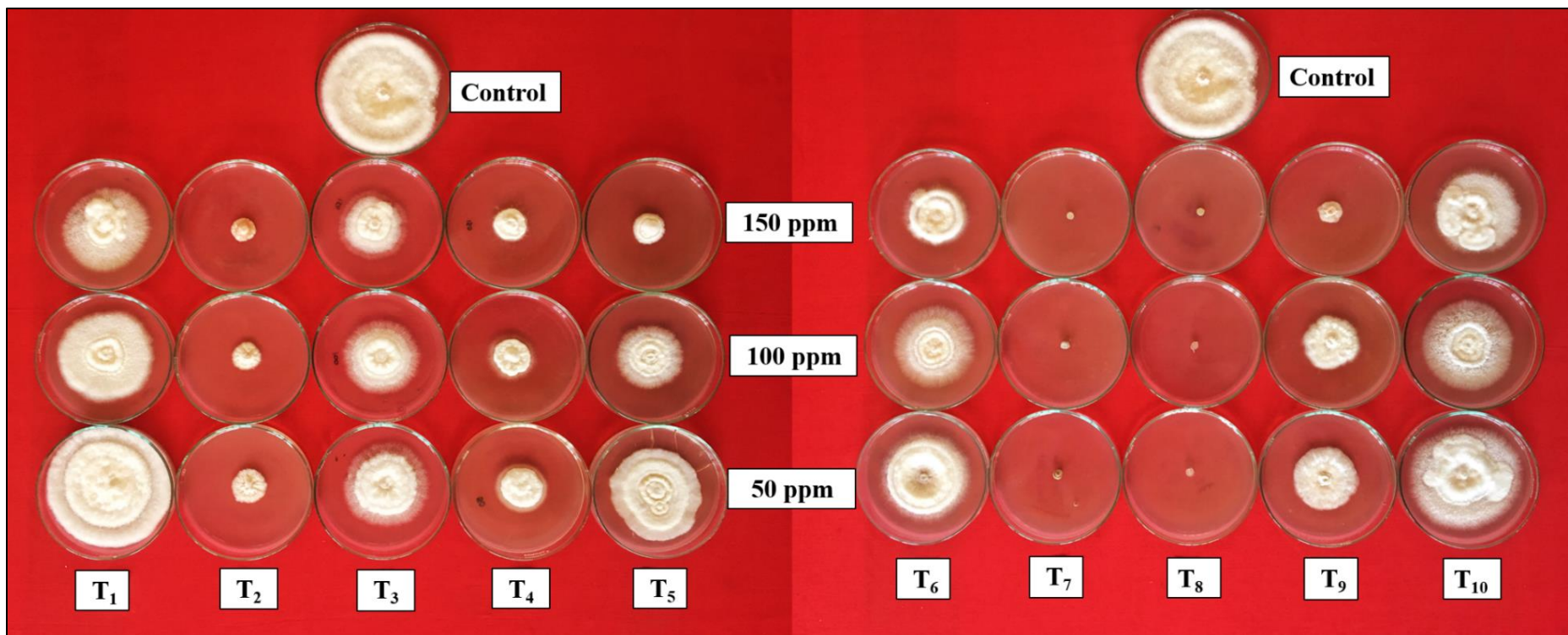
**Table 18. *In vitro* efficacy of systemic fungicides on growth and sporulation of *F. oxysporum* infecting geranium**

Sl. No.	Systemic fungicide	Per cent inhibition over control*			Mean	Sporulation**
		50 ppm	100 ppm	150 ppm		
1	Cyazofamid	4.81 (12.66)	25.93 (30.61)	35.06 (36.30)	21.93 (26.52)	++
2	Difenoconazole	75.18 (60.12)	78.39 (62.30)	80.00 (63.43)	77.85 (61.95)	+
3	Penconazole	35.18 (36.37)	40.61 (39.59)	45.93 (42.66)	40.57 (39.54)	+
4	Kresoxim methyl	61.59 (51.70)	71.85 (57.95)	75.55 (60.36)	69.66 (56.67)	+
5	Isoprothiolane	30.00 (33.21)	52.71 (46.55)	70.49 (57.10)	51.06 (45.62)	++
6	Thifluzamide	36.42 (37.10)	47.53 (43.58)	57.28 (49.18)	47.07 (43.29)	+
7	Carbendazim	100.00 (90)	100.00 (90)	100.00 (90)	100.00 (90)	-
8	Propiconazole	100.00 (90)	100.00 (90)	100.00 (90)	100.00 (90)	-
9	Pyraclostrobin	50.36 (45.21)	55.74 (48.29)	82.47 (65.25)	62.86 (52.92)	-
10	Dimethomorph	26.91 (31.24)	35.92 (36.82)	41.85 (40.30)	34.89 (36.12)	++
	<b>Mean</b>	52.04 (48.70)	60.87 (54.51)	68.86 (59.40)		
		<b>Fungicides (F)</b>	<b>Concentration (C)</b>	<b>F×C</b>		
	<b>S. Em ±</b>	<b>0.10</b>	<b>0.49</b>	<b>0.04</b>		
	<b>CD (p 0.01)</b>	<b>1.13</b>	<b>0.59</b>	<b>1.97</b>		

**Note:** Figures in the parenthesis are arcsine transformed values; \*Means of three replications; \*\*(-) No sporulation- 0 conidia per microscopic field; (+) Poor- 1-20 conidia per microscopic field; (++) Moderate- 20-50 conidia per microscopic field; (+++) Good- 50-100 conidia per microscopic field; (+++++) Excellent - > 100 conidia per microscopic field.



**Fig. 13: *In vitro* efficacy of systemic fungicides on mycelial growth inhibition of *F. oxysporum***



T<sub>1</sub>- Cyzofamid

T<sub>2</sub>- Difenoconazole

T<sub>3</sub>- Penconazole

T<sub>4</sub>- Kresoxim methyl

T<sub>5</sub>- Isoprothiolane

T<sub>6</sub>-Thifluzamide

T<sub>7</sub>- Carbendazim

T<sub>8</sub>- Propiconazole

T<sub>9</sub>- Pyraclostrobin

T<sub>10</sub>- Dimethomorph

Plate 18. *In vitro* evaluation of systemic fungicides against *F. oxysporum*

cent inhibition of 21.08 and 46.53 per cent, respectively. Whereas, least mean mycelial inhibition was noticed in chlorothalonil (16.45 %) (Table 16, Fig. 11 and Plate 16). In the three concentrations tested, 1000 ppm (43.30 %) showed significant highest inhibition and least by 500 ppm (15.27 %).

With respect to fungicide and concentration interaction, Copper oxychloride exhibited maximum inhibition of 87.77 per cent at 1000 ppm while no growth inhibition was noticed in chlorothalonil at 500 ppm. Other interactions exhibited moderate variation in inhibition of radial growth.

#### **4.6.3.2 Efficacy of combi product fungicides**

Among the three combi fungicides, hexaconazole + captan (84.51 %) was recorded the significant maximum inhibition. Other fungicides like tebuconazole + trifloxystrobin and azoxystrobin + difenoconazole shows slight varied inhibition of mycelial growth. Whereas, least mean mycelial inhibition was noticed in azoxystrobin + difenoconazole (80.03 %). In the three concentrations tested, 500 ppm (64.89 %) showed significant highest inhibition. Whereas, 150 and 250 ppm showed on-par results of per cent inhibition.

With respect to fungicide and concentration interaction, azoxystrobin + difenoconazole exhibited maximum inhibition of 88.51 per cent at 500 ppm while least was exhibited by azoxystrobin + difenoconazole (74.93 %) at 250 ppm. Other interactions exhibited slight variation ranging from 76.66-86.02 per cent inhibition of radial growth (Table 17; Fig. 12 and Plate 17).

#### **4.6.3.3 Efficacy of systemic fungicides**

Efficacy of ten systemic fungicides were tested against *F. oxysporum* at three different concentrations (50, 100 and 150 ppm). Obtained data is presented in table 18, fig. 13 and plate 18 revealed that, out of ten fungicides, carbendazim and propiconazole showed cent per cent significant inhibition of mean mycelial growth. Other fungicides showed varied per cent inhibition ranged from 34.89-77.85 per cent. Whereas, least mean mycelial inhibition was noticed in cyzofamid (4.81 %). In the three concentrations tested, 150ppm (68.86 %) showed significant highest inhibition and least by 50 ppm (52.04 %).

With respect to fungicide and concentration interaction, carbendazim and propiconazole exhibited maximum inhibition of 100 per cent at all three concentrations. Whereas, least mycelial growth inhibition was noticed in cyzofamid (4.81 %) at 50 ppm. Other interactions exhibited varied inhibition of radial growth ranged from 26.91-82.47 per cent.

Effect of all (contact, systemic and combi product) groups fungicides on test fungus growth inhibition was achieved by different means viz., inhibition of sporulation, spore germination. Results revealed that among the contact fungicides copper oxychloride, combi products hexaconazole + captan and among systemic fungicides carbendazim and propiconazole were found effective against *F. oxysporum*.

These results were in accordance with Bhapkar (2016) who tested the efficacy of four fungicides *in vitro* against *F. oxysporum* f. sp. *dianthi*. Among fungicides carbendazim @ 0.1 per cent recorded highest mycelial growth inhibition (84.85 %), followed by captan @ 0.2 % (66.28 %). Among the systemic fungicides, carbendazim was highly effective in inhibiting the growth of *F. oxysporum* f. sp. *gladioli* in all the concentrations (0.025, 0.05 and 0.1 %) (Kulkarni Sumithra, 2006). Hegde *et al.* (2017) evaluated nine fungicides *in vitro* against *F. oxysporum* f. sp. *dianthi* causing wilt of carnation. Out of which systemic fungicides such as carbendazim, propiconazole, difenoconazole were found effective. Similar results obtained by Nel *et al.* (2007), Kumari *et al.* (2014). Similarly, Bhimani *et al.* (2018) reported that copper oxy chloride was found effective. Desai *et al.* (2017) evaluated different fungicides against *F. oxysporum* among the combination products of fungicides, pyraclostrobin + metiram, captan + hexaconazole and carbendazim+ mancozeb were most effective and gave 100 cent per cent inhibition.

#### **Future line of work:**

1. Eco-friendly management strategies to control *Fusarium* wilt under field conditions.
2. Effect of wilt disease on oil yield and quality of scented geranium.
3. Detailed investigations on the epidemiology of the wilt disease.
4. Association of other organisms associated with geranium wilt disease

## V SUMMARY

Scented geranium is affected by wilt disease caused by *F. oxysporum* is a potential threat for the commercial cultivation of the crop in the country since the disease can result in reduction in yield loss. The pathogenic variability and virulence exhibited by the fungus resulted in its easy spread and establishment even in the new areas of cultivation. In this context, the present study on *Fusarium* wilt of geranium was carried out on different aspects like isolation, identification, proving pathogenicity, cultural, morphological studies, molecular characterization and *in vitro* evaluation of botanicals, bio agent and fungicides was also carried. The results obtained are summarised here under.

The *F. oxysporum* was isolated from the wilt affected geranium plants collected from different geranium growing areas of Southern Karnataka using standard isolation technique and purification of fungus by single spore isolation method. The maximum wilt incident was recorded in Tumakuru district (36.73 %).

*F. oxysporum* on PDA produced white fluffy mycelia with regular uniform margin, producing pale white, purple pigmentation. Microscopic studies indicated the existence of hyaline short and plump conidiophores bearing ovoid microconidia (0-1 septate) and sickle shaped macroconidia (2-5 septate). Chlamydo spores were formed in a month-old culture.

Pathogenicity was proved using healthy rooted geranium cuttings. Artificial inoculation of pathogen was done by sick pot method. The symptoms like yellowing and drying of older leaves, drooping of the plant at later stages, complete wilting and collapse of plant were observed. The re-isolation on PDA showed colony growth and morphology similar to the originally isolated fungus. Based on the pathogenicity and also morphological characters of the fungus, it was identified as *F. oxysporum*.

Colony characteristics and morphology of *F. oxysporum* on different culture media revealed that the growth was comparatively good in Sabouraud's dextrose agar and CMA but maximum sporulation was observed in SDA. The colony colour, substratum pigmentation, colony texture and morphology showed significant variation on different media tested.

Morphological studies of all *Fusarium* isolates exhibited different size, shapes of conidia and arrangements of chlamydospores. *F. oxysporum* isolates produce sickle shape to elliptical macroconidia, oval to ellipsoid microconidia and thick walled chlamydospores. And morphology of culture ranges from pale white or slight orange colony colour to purple or red colour on PDA. Spore germination studies of *F. oxysporum* revealed the length of germ tube increases as time passes this shows the virulence of the pathogen and these characters confirmed the identity of *F. oxysporum*.

Ideal temperature for growth of *F. oxysporum* is 25 °C as evidenced by 88.77 mm mycelial growth and biomass of 0.67 g 100 mL<sup>-1</sup> in solid and liquid media respectively with maximum sporulation.

The highest biomass production (0.69 g 100 mL<sup>-1</sup>) and mycelial growth (87.22 mm) of *F. oxysporum* was found at pH of 6.5 with maximum sporulation.

Molecular characterization of isolates by using universal fungal ITS primers showed PCR amplification at 550 bp. Isolates *F. oxysporum* isolate GFO 1 [MT740398], *F. oxysporum* isolate GFO 2 [MT740399] and *F. oxysporum* isolate 3 [MT740400]. These sequences show 100 per cent homology with [MN055701.1] *F. oxysporum* Ginrarsnl 1 reference strain. Phylogenetic analysis by using maximum likelihood approach showed the sequences of all isolates matched with *F. oxysporum*. So, ITS sequences confirmed that *F. oxysporum* as causal organism for wilt in scented geranium.

Among 12 botanicals tested against *F. oxysporum* showed that significant maximum mean per cent inhibition of mycelial growth was recorded in agave 59.31 per cent reduction over control.

Among the 12 fungal bio agents tested against antagonist effect, highest mycelial growth inhibition of 71.12 per cent was observed in *T. viride* (Tv- 8). In case of bacterial antagonists *B. pumilis* 54.66 per cent and *B. velezensis* (P42) 53.61 per cent showed a maximum per cent inhibition over the rest. However bacterial antagonists were found inferior to fungal bioagents.

Among four contact fungicides tested *in vitro* copper oxychloride recorded the maximum mean inhibition of 56.48 per cent and among three combi product fungicides tested maximum mean inhibition of 84.51 per cent was observed in hexaconazole + captan. Whereas, among ten systemic fungicides tested cent per cent mean inhibition was recorded in carbendazim and propiconazole. So, among these fungicides systemic fungicides and combination product fungicides were found effective against *F. oxysporum*.

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\*Originals not seen