

Original Research Article

Efficacy of Botanicals as Biocontrol Agents Against Leaf Spot and Blossom Blight Pathogen of Marigold: A First Report from Longleng District of Nagaland

ABSTRACT

Aims: To study about etiology and organic disease management strategies of *Fusarium oxysporum* induced blossom blight in marigold (*Tagetes* spp.), cultivated in and around Longleng district of Nagaland. The experiment aimed to formulate an effective organic disease management module to combat the disease and assess the efficacy of plant extracts and biocontrol agents in suppressing fungal growth

Study design: A completely randomized design (CRD) was employed for laboratory-based trials, and an exploratory survey was conducted during the winter season of 2024 to monitor disease incidence in marigold-growing areas around Longleng district of Nagaland.

Place and Duration of Study: Field survey has been carried out in Longleng district of Nagaland. All the laboratory works has been carried out in laboratory of Department of Plant Pathology, Assam Agricultural University, Jorhat, Assam. Duration of research is 6 months.

Methodology: The tested phytoextracts at 10% and 20% concentrations included leaves of neem (*Azadirachta indica*), tulsi (*Ocimum sanctum*), garlic (*Allium sativum*), and other local plant species. Biocontrol agents such as *Trichoderma viride*, *Trichoderma harzianum*, and *Pseudomonas fluorescens* were collected from local fields and research institutions for evaluation. Disease incidence and severity were measured based on Percent Disease Intensity (PDI).

Results: The study demonstrated that garlic extract, at both tested concentrations, achieved complete inhibition (100%) of fungal growth. Additionally, several other plant extracts exhibited notable antifungal properties. Among the biocontrol agents, *Pseudomonas fluorescens* showed a strong antagonistic effect against *Fusarium oxysporum* (82.65%). These findings underscore the potential for incorporating phytoextracts and biocontrol agents into effective organic disease management strategies for marigold cultivation

Conclusion: From the study, it is recommended to optimize the application of garlic extract, exploring its effectiveness in field conditions, and evaluating its potential in combination with other biocontrol agents or plant extracts.

Keywords: [Marigold, *Fusarium oxysporum*, Phytoextracts, Bioagents, Efficacy]

1. INTRODUCTION

India has a long tradition of flower cultivation. Encouragement for the commercially potent floriculture has yielded in the blossoming of this field into a viable agri-business option. With globalization and free market economy, floriculture has attained an industrial status and gained tremendous momentum around the world. With changing life styles and increased urban affluence, floriculture has assumed a definite commercial status both in domestic and international market (Kaur et al.,2022).

Marigold was one of the earliest cultivated flowers. Today Marigold is one of the important commercial flowers grown worldwide. In India, it accounts for more than half of the nation's loose flower production. Major growing states are Karnataka, Maharashtra, Gujarat, Haryana, Andhra Pradesh, Uttar Pradesh, Odisha, West Bengal, Tamil Nadu, Jammu and Kashmir, Chhattisgarh, Puduchery, Andaman and Nicobar islands and different parts of North eastern states like Meghalaya, Nagaland etc (Gupta et al.,2022). Marigold has gained popularity because of its adaptability to various soil and climatic condition, longer blooming period and beautiful flower having long shelf-life. It is the important flower used for floral decorations, religious offerings and for making garlands and flower baskets (Mehta et al.,2022).

This flower is native to South-Western Asia as well as Western Europe and the Mediterranean. The marigold is also known as Mary bud, pot marigold, Ruddes, golds, holi gold, gold bloom, Herb of the Sun and the garden marigold. (Suryavansi & Parvez,2014)

The marigold is also known as the *Tagetes* and belong to Asteraceae (Compositae) family and is a genus that includes about 56 species with a mixture annuals and perennials, but the annual is definitely more common than the perennial flower. It is a diploid plant having chromosome number $2n=24$. These flowers have blooms that include colours like gold, orange, white and yellow. The recorded history of marigolds begins with the Aztecs in Mexico, where the flowering plant was used in religious ceremonies and as an herbal medicine. Marigold plant was named for the Virgin Mary -"Mary's Gold in 17th century.

There are different varieties of the marigold plant and they are divided into four basic species. These include African/American marigold (*T. erecta*), French marigold (American origin)- *T. patula*, Triploids, and single marigold (*T. tenuifolia*). The two most popular types of marigolds are African or Mexican (*Tagetes erecta*) and French (*Tagetes patula*) marigolds. (Mir et al.,2019).

India's 'flower power' continues to bloom with the country emerging as the second largest grower of flowers around the world, surpassed only by China. About 2,33,000 hectares across the country was used for floriculture, producing 17,29,000 metric tons (MT) of loose flowers and 76,732 lakh cut flowers. Andhra Pradesh leads in loose flowers production with 2,24,410 MT cultivated over 34,850 hectares. followed by Karnataka at 2,07,500 MT cultivated in 29,700 hectares and Tamil Nadu with 3,12,970 MT grown in 28,700 hectares. Total area under flower production in different countries of the world is 3.05.105 ha. Half of the world's cut flower production is from Europe and marigold ranked seventh on the list of the most popular garden plants in world but it ranked 2nd in

India. (Kaur et al.,2022). Total area under marigold production in India 55,890ha and production as loose flower is 511.31MT and cut flower is 4.25 lakh but in Odisha, total area under marigold is 2625ha and production is 2,40,031 MT. So floriculture is one of the fast emerging and rapidly growing sector in Odisha. (De et al.,2019)

Though marigold plant is potential plant in pest management, it is affected by several disease like *Fusarium wilt (Fusarium oxysporum)*, *Verticillium wilt (Verticillium dahliae)*, leaf spot (*Alterariaaria tagetiea*), Blight (*Alternaria tenuissima*), leaf spot and bud rot of marigold (*Alternaria alternata*), leaf and flower blight (*Alternaria zinnia*), bacterial leaf spot (*Pseudomonas syringae pv. tageli*), southern bacterial wilt or brown rot (*Pseudomonas solanaeearum*), *Botrytis blight (Botrytis cinerea)*, *Septoria leaf spot (Septaria ageticola)*, wilt and stem Rot (*Phytophthora cryptogea*), collar Rot (*Phytophthora sp.*, *Pythium sp.*), leaf spot and blight (*Alternaria, Cercospora and Septaria sp.*), powdery mildew (*Oidium spp.*) Flower Bud Rot (*Alternaria dianthi*), Damping Off (*Pythium spp.*). Damping off (*Rhizoctonia solani*), Flower bud rot (*Alternaria dianthi*). collar and root rot (*Pellicularia jilamentosa, Pythium ultimum, (Sclerotinia sclerotiorum)*), leaf blight of fig-marigold (*Rhizoctonia solani*) and a new species of *Alternaria* on seeds of French marigold (*Alternaria patula sp. nov.*). viral diseases like Aster Yellows, Mosaic (cucumber mosaic cucumovirus), little leaf and witches' broom disease (pytoplasma). Insects like aphids (*Aphis gossypii*), bud caterpillars (*Helicoverpu armigera* and *Phycita sp.*), Japanese beetles, leafhopper, plant bug and mite. (Gurjar et al.,2019).

Marigold (*Tagetes spp.*), which is a popular bedding plant, can be used as a cover crop. Marigold produces a substance called alpha-terthienyl, which can aid in the reduction of root-knot nematodes and other disease promoting organisms, such as fungi, bacteria, insects, and some viruses. (Hooks et al.,2010).

Out of several disease and insect pest attack, wilt disease (stem rot, black stem, aster wilt) caused by *Fusarium oxysporum*, is the most serious disease of Marigold in home-yard plantings in Longleng and over most of the world.

In view of severity of marigold disease, it was selected for further research to know the etiology and to formulate organic management strategies. For this efficacy of different plant extracts as well as biological agents have been tested against the causal organism *in vitro*).

2. MATERIAL AND METHODS

2.1. Site of survey for disease occurrence and location of laboratory research work

The survey for disease occurrence was conducted in 2024 winter in several marigold fields across different villages within Longleng district, where farmers grow marigold for traditional purposes. The marigold plants in these areas were Observed for symptoms of of leaf spot and blossom blight, both of which are known to be caused by *Fusarium oxysporum*. The sites selected for the survey were primarily located in areas with temperate conditions conducive to the development of fungal diseases. Farmers in these areas reported significant crop loss due to these diseases, which prompted the need for this study. The survey aimed to assess the prevalence and severity of the diseases and evaluate potential biocontrol and plant extract-based solutions, which align with the region's traditional agricultural practices of avoiding chemical pesticides.

All the Laboratory research works have been carried out in the laboratory of Department of Plant Pathology, Assam Agricultural University, Jorhat, Assam.

2.2. Isolation, characterization, identification and pathogenicity test of the causal fungus:

The affected Marigold samples such as leaves and flower buds showing disease symptoms were collected from the different locations in and around Longleng during the period of survey. Standard isolation techniques were followed to isolate the in sighted fungus. On thorough examination of morphological and cultural characters to identify the causal organism had been done which were then subjected to pathogenicity test following the guidelines of Koch's postulates.

2.3. Preparation of Crude extracts

The fresh plant parts were collected from field and washed with tap water followed by distilled water. The plant parts were dried for few minutes. 100 g of each plant part was weighed and ground using grinder with addition of equal volume (w/v) of ethanol. These extracts were then filtered through double layered muslin cloth and kept in 100 ml conical flasks. The content was mixed thoroughly and centrifuged at 5500 rpm for 10 minutes and the supernatant was filtered through Whatman filter paper no.1 and after filtration the contents were used for further study.

2.4. Poisoned food technique

The bio-efficacy of plant extracts (Table-1) were evaluated by poisoned food technique (Nene and Thaplial, 1973) in two different concentrations i.e. 10% and 20%. Required amount of crude extracts were mixed with 90 and 80 ml of sterilized molten potato dextrose agar medium so as to get 10% and 20% concentration respectively in laminar air flow chamber. Control was maintained without any plant extracts. 20 ml of media was poured into Petri dishes and allowed to solidify. 8 mm culture disc was put on the middle of the solidified petri dishes. All the Plates were incubated at room temperature. Mycelia growth was measurement when fungal growth was completely covered control plate. The growth of mycelium on other Plates was compared with the control plate. The efficacy of plant extracts was expressed as percentage inhibition of mycelia growth over control. The per cent inhibition over control was calculated according to formula of Vincent as follows.

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

Where, I = Percent inhibition of radial growth

C= Radial growth on control plate

T= Radial growth on treatment plate

Table-1 : List of plant extracts used in management of the causal fungus

Sl. No.	Common name	Scientific name	Plant parts used	Concentrations used
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1.	Tulsi	<i>Ocimum sanctum</i>	Leaf	10% and 20%
2.	Sadabahar	<i>Vinca rosea</i>	Leaf	10% and 20%
3.	Adhatoda	<i>Adhatoda vasica</i>	Leaf	10% and 20%
4.	Morning glory	<i>Ipomea spp.</i>	Leaf	10% and 20%
5.	Begonia	<i>Vitex negundo</i>	Leaf	10% and 20%
6.	Custard apple	<i>Annona reticulate</i>	Leaf	10% and 20%
7.	Big-sage	<i>Lantana camara</i>	Leaf	10% and 20%
8.	Neem	<i>Azadirachta indica</i>	Leaf	10% and 20%
9.	Milk weed	<i>Calotropis gigantean</i>	Leaf	10% and 20%
10.	Karanj	<i>Pongamia pinnata</i>	Leaf	10% and 20%
11.	Bael	<i>Aegle marmelos</i>	Leaf	10% and 20%
12.	Onion	<i>Allium cepa</i>	Bulb	10% and 20%
13.	Garlic	<i>Allium sativum</i>	Clove	10% and 20%

All the experiments were conducted in Completely randomized design (CRD) and statistical analysis was done by using online statistic software, OPSTAT. (14.139.232.166/opstat/default.asp)

2.5 In vitro evaluation of biocontrol agents

The efficiency of biocontrol agents against the causal organism of disease were tested by dual culture method. Biocontrol agents like *Trichoderma viride*, *Trichoderma harzianum*, *Trichoderma hamatum*, *Pseudomonas spp.* and *Bacillus sp* were collected from different places as mentioned below (Table-2) and were tested against the pathogen. The fungal antagonists were grown in potato dextrose agar to get fresh and active culture for the experiment.

Table-2 : List of bio agents with their place of collection

SI. NO.	Bio control agents	Place of collection
1	<i>Trichoderma viride</i>	Rhizospheric soil of Marigold, Hukphang Village, Longleng, Nagaland

2	<i>Trichoderma harzianum</i>	Rhizospheric soil of Marigold , Pongo village, Longleng, Nagaland
2	<i>Trichoderma hamatum</i>	From Rhizosphere of Marigold, Longleng, Nagaland.
4	<i>Pseudomonas fluorescens</i>	Department of Plant Pathology, College of Agriculture, OUAT, Bhubaneswar, Odisha
5	<i>Bacillus subtilis</i>	Central Tropical Mushroom Research and Training centre, AAU, Jorhat, Assam

2.6. Dual culture method

About 20 ml of potato dextrose media was poured into petri dishes and allowed to cool down. The fungal mycelial disc (5mm) was transferred to one end of the Plate and fungal antagonist culture disc placed opposite to it leaving 5-6 mm distance from the periphery of the plates. In case of bacterial antagonist, spore suspension of bacteria was mixed in the molten media at 38°C and thoroughly mixed and plated immediately. Eight mm fungal disc was put in the centre after solidification and cooling. The radial growth of the fungus was measured. Fungal disc was also put in the petri plate without bacterial suspension as control. Each treatment was replicated three times. The inoculated Plates were incubated at room temperature. After five days, observations were taken. The efficacy of bio-control agents were expressed as percentage inhibition of mycelia growth over control. The Per cent inhibition over control was calculated according to Vincent's formula as follows

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

Where, I = Percent inhibition of radial growth

C= Radial growth on control plate

T=Radial growth on treatment

3. RESULTS AND DISCUSSION

3.1. SURVEY OF DISEASE INCIDENCE

The survey indicated the presence of five foliar as well as soil borne fungal diseases namely, Leaf spot (*Alternaria tagetica*), Powdery mildew (*Oidium spp.*), Botrytis Blight (*Botrytis cineraria*). Blossom Blight (*Fusarium oxysporum*) and wilt (*F.oxysporum*). However, the most commonly encountered disease was diagnosed as Blossom blight of marigold caused by *Fusarium oxysporum* affecting leaf, flowers and flower buds (Fig-1). The same organism was found to be associated with the wilt disease in few instances. Low to moderate incidence of powdery mildew and botrytis blight were recorded (Table-3) at all the surveyed locations.

Table 3 : Survey on disease incidence of marigold

SI.NO.	Name of the disease	Causal organism	Percent Disease Intensity (PDI)
1	Leaf spot and blossom blight	<i>Fusarium oxysporum</i>	52.3%
2	Wilt	<i>Fusarium oxysporum</i>	17.6%
3	Botrytis Blight	<i>Botrytis cinerea</i>	43.4%
4	Powdery Mildew	<i>Oidium spp.</i>	12.3%
5	Leaf spot	<i>Alternaria tagetica</i>	37.9%



Fig-1. Blossom blight of marigold caused by *Fusarium oxysporum*

3.2 Isolation, characterisation, identification and pathogenicity of the causal fungus:

In marigold, *Fusarium* blossom blight of the floral organs is rather uncommon and has not been reported from the marigold growing areas of the country particularly from Nagaland. The affected samples were collected from the growing areas in and around Longleng during the period of survey. Standard isolation techniques were followed to isolate the fungus involved. On thorough examination of morphological and cultural characters, the causal fungus was identified as *Fusarium oxysporum*. The fungus produced light pink to white cottony growth on potato dextrose agar plates and macro as well as microconidia. The taxonomic position of the fungus has been given below. (Booth C, 1971)

Scientific Classification

Kingdom - Fungi
Division - Ascomycota
Class - Sordariomycetes
Order - Hypocreales
Family - Nectriaceae
Genus - *Fusarium*
Species - *F.oxysporum*

3.3. Symptomatology

The disease symptom was observed mostly in the flower and flower buds of the host plant. The petals were discoloured light brown to black initially and flowers droop. Usually the disease was seen to affect all the petals of the flower, but partial infection was also common at few locations. As the disease advanced pinkish-white mycelial mat was often observed on the affected floral parts. In severe condition the flowers drooped down wards and the plants exhibited a sickly appearance. The plants having affected flowers didn't exhibit the wilt syndrome as observed in the survey.

3.4. Pathogenicity test

The Pathogenicity of the test fungus was proved on potted marigold plants following the postulations demonstrated by Robert Koch (1887) and the procedure described under "materials and methods". Test fungus was inoculated by pinpricking leaf epidermis and by swabbing the spore suspension on the injured leaves with a cotton swab. The inoculated plants were covered with polythene bags in order to maintain Favourable microclimate for disease appearance. The inoculated plants started to exhibit the typical disease symptoms on the floral parts at 7 days of inoculation and the flowers completely blighted at 10 days of inoculation. However, the controlled plants maintain for the purpose of comparison did not show any.



Fig.2 Pathogenicity test on healthy plants following Koch's Postulates.

3.5. Morphological studies

The relevant morphological character were studied from 7 days old petriplate culture after isolation of the pathogen from the affected plant part. The available literature was referred for taxonomic identification of the pathogen as and when required. The detailed cultural characteristics of the species was studied. The fungus produced hyaline, sickle shaped and septate macro conidia and oval to oblong micro conidia with or without septation. The dimension of the macro conidia (Fig-3) was in the range of 12.25-

Fig.4: Micro conidia of *Fusarium oxysporum*

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3.6. Efficacy of different phytoextracts in inhibiting the radial growth of *Fusarium oxysporum*

Among the tested phytoextracts at two different concentrations, it was observed (Table-4) that the extracts at twenty per cent were significantly superior over ten per cent concentration. The garlic extract was found to be superior in comparison to the other tested extracts showing cent per cent inhibition of diametric growth of both the concentrations. Among the extracts at ten per cent concentration Garlic-T₁₃ (100%) was found to be most effective followed by Sadabahar-T₂ (98.49%) and Onion-T₁₂ (95.56%). Complete inhibition of radial growth was also retarded by extracts of Sadabahar-T₂ (100%), Basanga-T₃ (100%), Begonia-T₅ (100%), Custard apple-T₆ (100%), Karanj-T₁₀ (100%) as well as Onion (100%) at twenty per cent concentration. Bael plant extract was found to be least effective (68.85%) against radial growth of *Fusarium oxysporum*.

Our p study is in concurrence with the findings of Kamdi et al., (2012) and Hossain et al. (2013). The inhibitory effect of garlic bulb crude extract on fungal growth is primarily attributed to allicin, which serves as the main antibacterial, antifungal, and antiviral compound. (Miron et al., 2000).

Table-4: Effect of Phytoextracts on mean diametric growth and percent inhibition over control

Treatment	Plant Extracts	Plant part used	Mean diametric Growth(mm)		Per cent inhibition over control	
			Concentration (%)		Concentration (%)	
			10	20	10	20
T ₁	Tulsi (<i>Ocimum sanctum</i>)	Leaf	25 (5.05)	5.93 (2.52)	67.67	92.27

T ₂	Sadabahar (<i>Vincarosea</i>)	Leaf	1.17 (1.23)	0 (0.71)	98.49	100
T ₃	Basanga (<i>Adhatodavasic a</i>)	Leaf	10.67 (3.34)	0 (0.71)	86.21	100
T ₄	Morning glory(<i>Ipoma spp.</i>)	Leaf	16.57 (4.13)	3.4 (1.94)	78.58	95.57
T ₅	Begonia (<i>Vitex negundo</i>)	Leaf	5.43 (2.43)	0 (0.71)	92.97	100
T ₆	Custard apple (<i>Annona reticulate</i>)	Leaf	18.6 (4.37)	0 (0.71)	75.95	100
T ₇	Big-sage (<i>Lantana camara</i>)	Leaf	23.3 (4.88)	2.27 (1.65)	69.87	97.05
T ₈	Neem (<i>Azadiracht aindica</i>)	Leaf	16.4 (4.11)	8.77 (3.04)	78.79	88.58
T ₉	Milk weed (<i>Calotropis gigantea</i>)	Leaf	18.4 (4.35)	9.6 (3.17)	76.21	87.49

T ₁₀	Karanj (<i>Pongamia pinnata</i>)	Leaf	13.57 (3.75)	0 (0.71)	82.46	100
T ₁₁	Bael (<i>Aegle marmelos</i>)	Leaf	37.33 (6.15)	10.77 (3.35)	51.72	85.97
T ₁₂	Onion (<i>Allium Cepa</i>)	Bulb	3.43 (1.98)	0 (0.71)	95.56	100
T ₁₃	Garlic (<i>Allium sativum</i>)	Clove	0 (0.71)	0 (0.71)	100	100
T ₁₄	Control		77.33 (8.82)	76.77 (8.79)		
SE(m) ±			0.096	0.116		
CD at 0.05			0.281	0.339		

values in the parentheses indicate $\sqrt{}$ transformed values

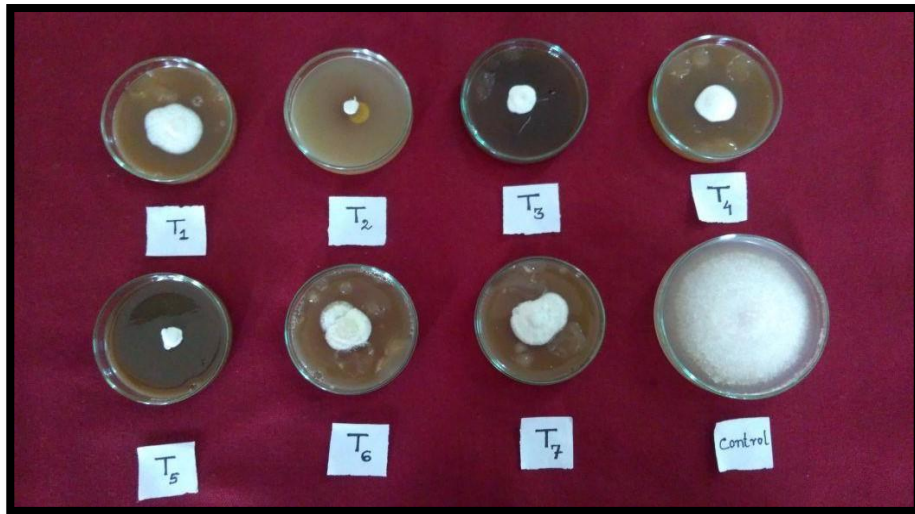


Fig.5: T1: Tulsi, T2: Sadabahar, T3: Adhatoda, T4: Morning glory, T5: Begonia, T6: Custard apple, T7: Big-sage, Control

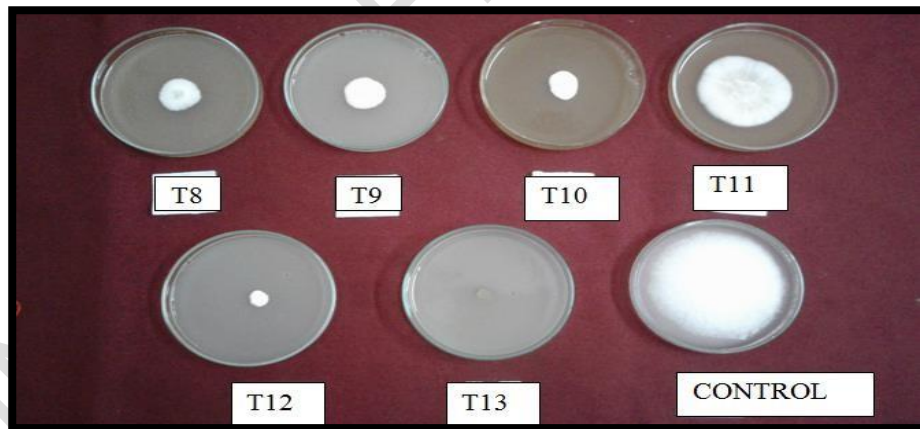


Fig.:6 T8: Neem, T9: Milk weed, T10: Karanj, T11: Bael, T12: Onion, T13: Garlic,Control
(Efficacy of plant extracts against F.oxysporum 10% concentration)

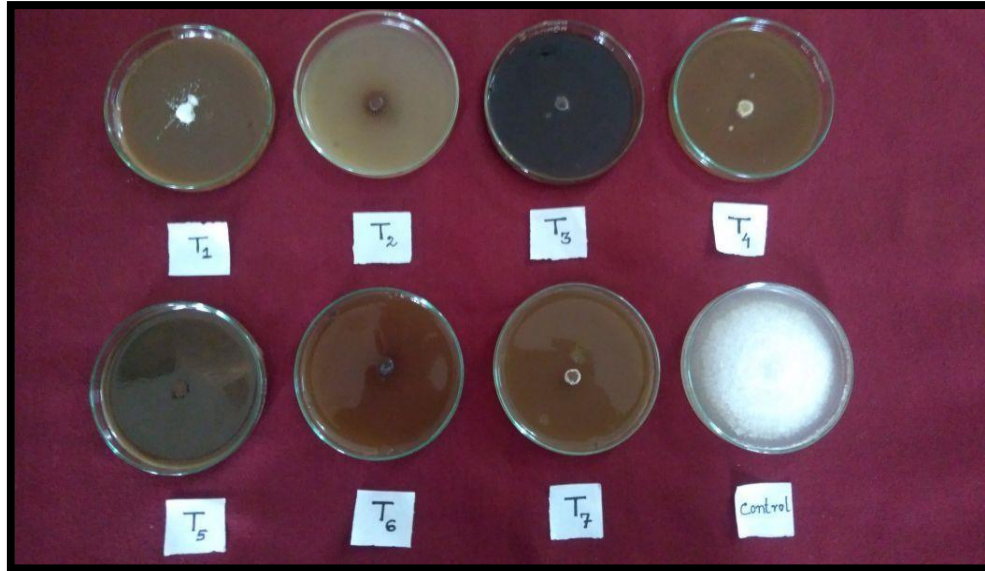


Fig.7- T1: Tulsi, T2: Sadabahar, T3: Adhatoda, T4: Morning glory,
T5: Begonia, T6: Custard apple, T7: Big-sage, Control



Fig.8: T8: Neem, T9: Milk weed, T10: Karanj, T11: Bael, T12: Onion,
T13: Garlic, Control
(Efficacy of plant extracts against *F.oxysporum* 20% concentration)

3.7. Efficacy of fungal and bacterial antagonists against radial growth of *F. oxysporum*

The antagonistic effects of various fungal and bacterial antagonists were evaluated (Table-5) for their impact on the diametric growth of *Fusarium oxysporum*. All fungal bioagents similarly reduced the pathogen's growth, with *Trichoderma harzianum* (T3) showing the highest inhibition, reducing diametric growth by 33.48%, followed by *Trichoderma hamatum* (29%) and *Trichoderma viride* (27.75%) in dual culture. Among bacterial antagonists, *Pseudomonas fluorescens* was the most effective, reducing radial growth by 82.65%, followed by *Bacillus subtilis* (59.65%). Similarly, Kumar et al., (2017) studied the efficacy of plant extracts, bioagents, and fungicides against *Fusarium udum* and reported that among the bioagents, seed treatment with *Trichoderma viride* resulted in the highest control of wilt incidence.

Table-5: Effect of Bio agents on mean diametric growth and percent inhibition over control

Treatments	Fungal bioagents	Mean colony diameter (mm)	Per cent Inhibition
T ₁	<i>Trichoderma viride</i>	27.33	27.75
T ₂	<i>Trichoderma harzianum</i>	25.17	33.48
T ₃	<i>Trichoderma hamatum</i>	26.83	29.07
T ₄	Control	37.83	
Treatments	bacterial bioagents	Mean colony diameter (mm)	Per cent inhibition
T ₁	<i>Pseudomonas fluorescens</i>	13.33	82.65
T ₂	<i>Bacillus subtilis</i>	31	59.65
T ₃	Control	76.83	



Fig.9- T1: *Trichoderma viride*, T2: *Trichoderma harzianum*, T3: *Trichoderma hamatum*, T4:Control

(Efficacy of different fungal antagonists against *F.oxysporum*)

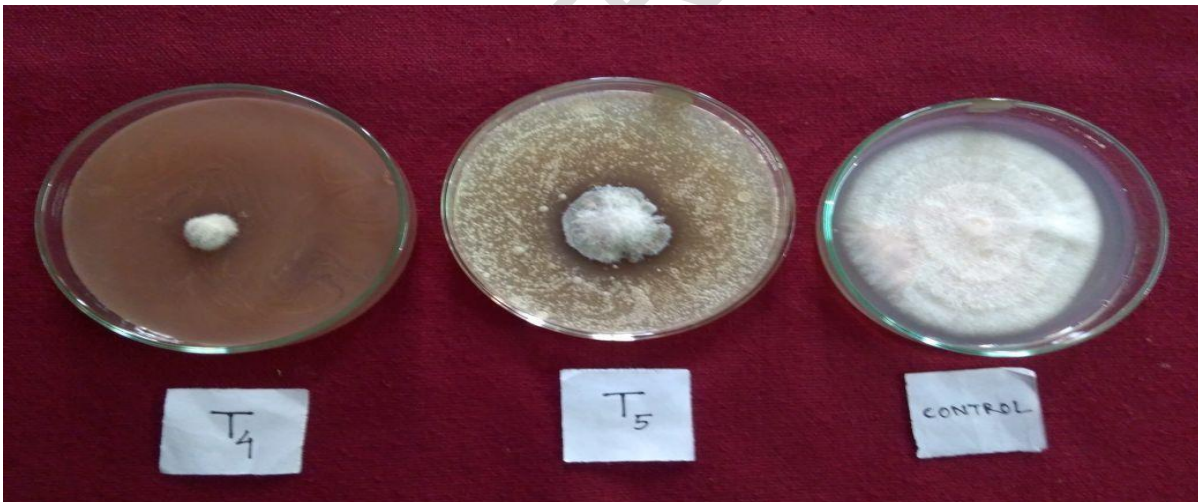


Fig.10- T1: *Pseudomonas fluorescens*, T2: *Bacillus subtilis*,
(Efficacy of different bacterial antagonists against
F.oxysporum).

4. CONCLUSION

This research contributes valuable insights into sustainable alternatives for managing *F. oxysporum* in marigold cultivation. The use of garlic extract as a biocontrol agent not only offers an effective solution to the disease but also aligns with the region's agricultural practices, promoting environmentally friendly pest management. For future research, it is recommended that studies focus on optimizing the application of garlic extract, exploring its effectiveness in field conditions, and evaluating its potential in combination with other biocontrol agents or plant extracts. Additionally, there is a need for more extensive field trials to assess long-term effects and practicality in local farming systems. For farmers in Longleng, the application of garlic extract could serve as a promising, low-cost, and sustainable approach to managing *F. oxysporum*, improving marigold production while reducing reliance on harmful chemicals. Educational efforts to disseminate these findings and train farmers in its proper use would be beneficial for enhancing agricultural practices and promoting environmental sustainability in the region.

COMPETING INTERESTS

The authors declare that there are no competing interests related to the research presented in this paper. There are no financial, personal, or professional relationships with individuals or organizations that could have influenced or biased the results of this study. No funding, patents, or consultancies related to the work were involved, and the authors have not received any honoraria or paid expert testimony. The research was conducted independently, and all necessary ethical guidelines were followed.

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