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IN VITRO EVALUATION OF FUNGICIDES, ESSENTIAL OILS AND BIOAGENTS AGAINST *ALTERNARIA* LEAF SPOT OF ASHWAGANDHA (*WITHANIA SOMNIFERA* (L.) DUNAL) CAUSED BY *ALTERNARIA CRASSA*

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ABSTRACT

Ashwagandha (*Withania somnifera* (L.) Dunal) is a prominent medicinal plant of Ayurvedic origin, currently threatened by foliar diseases such as leaf spot caused by *Alternaria crassa*. *In vitro* studies were conducted to assess the efficacy of selected fungicides, essential oils, and bioagents. Among fungicides, Carbendazim 12% + Mancozeb 63% WP at 0.15% concentration recorded the highest inhibition (87.35%) of the pathogen's radial growth. Clove and Eucalyptus oils exhibited maximum antifungal activity among the essential oils, with 77.83% and 76.66% inhibition, respectively. Among bioagents, *Trichoderma viride* recorded the highest inhibition (75.11%) in dual culture assay. The study highlights the potential of integrating chemical and non-chemical treatments for managing *Alternaria* leaf spot in Ashwagandha.
Keywords: Ashwagandha, *Alternaria crassa*, fungicides, essential oils, *Trichoderma*, *In vitro* evaluation.

Introduction

Ashwagandha (*Withania somnifera* (L.) Dunal) is a valued medicinal shrub in Indian systems of medicine. Cultivated widely in arid and semi-arid regions, it contributes significantly to India's herbal exports. However, its production is hampered by foliar diseases, particularly leaf spot caused by *Alternaria crassa*, which can lead to 50–60% yield losses and reduce withanolide content by up to 76%. The disease thrives under high humidity and moderate temperatures, particularly in poorly drained fields post-monsoon (Pati *et al.*, 2008).

Management of *Alternaria* leaf spot in Ashwagandha has predominantly relied on synthetic fungicides. Alongside chemical treatments, biological control agents such as *Trichoderma* spp. and *Pseudomonas fluorescens*, as well as plant-derived essential oils including Neem, Garlic, Clove, and Eucalyptus, have been explored due to their antifungal properties. Given the growing interest in sustainable agriculture and integrated disease management, it is essential to evaluate the comparative efficacy of these chemical, biological, and botanical options against *Alternaria crassa* in Ashwagandha.

Therefore, the present study was conducted to evaluate the *in vitro* efficacy of fungicides, essential oils, and bioagents against *Alternaria crassa*, with the objective of identifying potent and sustainable disease management options.

Materials and Methods

Isolation and Identification of Pathogen

Diseased Ashwagandha leaves exhibiting typical leaf spot symptoms were collected from All India Coordinated Research Project on Medicinal, Aromatic Plants and Betelvine research farm (19.3570199N, 74.6551379E), MPKV, Rahuri. Tissues from lesion margins were surface sterilized in 1% sodium hypochlorite for 30 seconds, rinsed in sterile distilled water, and plated on Water Agar medium. Emerging fungal growth was sub-cultured on PDA and incubated at 30±1°C. Identification was performed based on colony morphology, conidial characters, and confirmed through 18S rRNA, ITS and TEF1-α gene sequencing along with phylogenetic analyses, confirming the identity as *Alternaria crassa*. The gene sequences of *A. crassa* have been submitted to NCBI. The 18S rRNA gene (Accession No. PX405986), ITS region (Accession No. PX406136), and TEF1-α gene (Accession No. PX436131) are all deposited under BioSample SAMN51795668, associated with BioProject PRJNA1332369. The pure culture of *A. crassa* has been deposited at the ICAR–NAIMCC-NBAIM Culture Collection under accession number NAIMCC-F-04667. Further the metadata of the isolate has been registered with Mycobank database.

In vitro Evaluation Techniques

Fungicides and Essential Oils

The poisoned food technique (Nene and Thapliyal, 1993) was used for evaluating fungicides (Tebuconazole, Propineb, Chlorothalonil, Carbendazim + Mancozeb) and essential oils (Garlic, Eucalyptus, Clove, Neem), incorporated into PDA at specified concentrations.

Bioagents

Dual culture method (Dhingra and Sinclair, 1995) was used for antagonism testing using *Trichoderma viride*, *T. harzianum*, *T. koningii*, and *Pseudomonas fluorescens*.

Colony diameter was measured after 7 days of incubation. Per cent inhibition was calculated using formula by Vincent (1947). All treatments were replicated four times and data were statistically analyzed using CRD.

Per cent Inhibition (%) (I) = ((C–T) /C) × 100

Where:

I = Per cent inhibition of mycelial growth

C = Radial growth (colony diameter) in control (mm)

T = Radial growth (colony diameter) in treatment (mm)

Statistical Analysis

The data generated from *in vitro* experiments were subjected to statistical analysis (Panse and Sukhatme, 1967; Gomez and Gomez, 1984). Data from *in vitro* studies laid out in Completely Randomized Design (CRD) were analyzed using one-way ANOVA. The analyses were performed using the OPSTAT web-based statistical tool (Sheoran *et al.*, 1998). Means were separated using Duncan's Multiple Range Test (DMRT) at significance level $p \leq 0.01$ (Duncan, 1955), using Agricole package in R software (Mendiburu, 2023)

Results and Discussion

In vitro Efficacy of Fungicides

Based on the results (Table 1), the treatment with Carbendazim 12% + Mancozeb 63% WP at 0.15% (T_4) exhibited the lowest mean colony diameter (11.06 mm) and was statistically superior in inhibiting *Alternaria crassa*. Tebuconazole (T_1) and Propineb (T_3) were statistically at par with each other but inferior to T_4, while Chlorothalonil (T_2), although effective, stood apart as less inhibitory than T_4 but better than the untreated control. The untreated control (T_5) recorded the highest colony diameter and showed no inhibition.

Among the tested fungicides, the combination of Carbendazim 12% + Mancozeb 63% WP (T_4) at 0.15% concentration exhibited the highest antifungal activity, resulting in a mean colony diameter of 11.06 mm and an 87.35% inhibition of fungal growth compared to the untreated control. This superior efficacy is consistent with findings by Mathivanan and Prabavathy (2007), who reported complete (100%) inhibition of *Alternaria helianthi* mycelial growth using the same fungicidal combination in sunflower.

Tebuconazole 25.9% EC (T_1) also demonstrated strong inhibitory effects, with a colony diameter of 19.35 mm and 78.13% inhibition rate. This aligns with the study by Meena and Manivel (2020), where Tebuconazole was found to be highly effective against *Alternaria alternata*, achieving up to 89% mycelial growth inhibition.

The untreated control (T_5) recorded the maximum colony diameter of 88.38 mm, indicating unchecked growth of *A. crassa* in the absence of any chemical treatment.

These findings suggest that the combination of Carbendazim and Mancozeb, as well as Tebuconazole, are highly promising fungicidal options for managing leaf spot caused by *A. crassa* under laboratory conditions. The integration of both systemic and contact modes of action in these fungicides likely contributes to their superior efficacy.

Table 1 : *In vitro* efficacy of fungicides against *Alternaria crassa*

Tr. No.	Fungicides	Concentration (%)	Mean colony diameter of *Alternaria crassa* (mm)*	Per cent inhibition of *Alternaria crassa* over Control (%)
T_1	Tebuconazole 25.9% EC	0.10	19.35[c]	78.13
T_2	Chlorothalonil 75% WP	0.20	23.25[b]	73.58
T_3	Propineb 70% WP	0.20	21.70[bc]	75.34
T_4	Carbendazim 12% + Mancozeb 63% WP	0.15	11.06[d]	87.35
T_5	Control	-	88.38[a]	0.00
		S.E. ±	0.89	
		CD at 1%	3.71	

*= The highest superscript represents the least effective treatment.

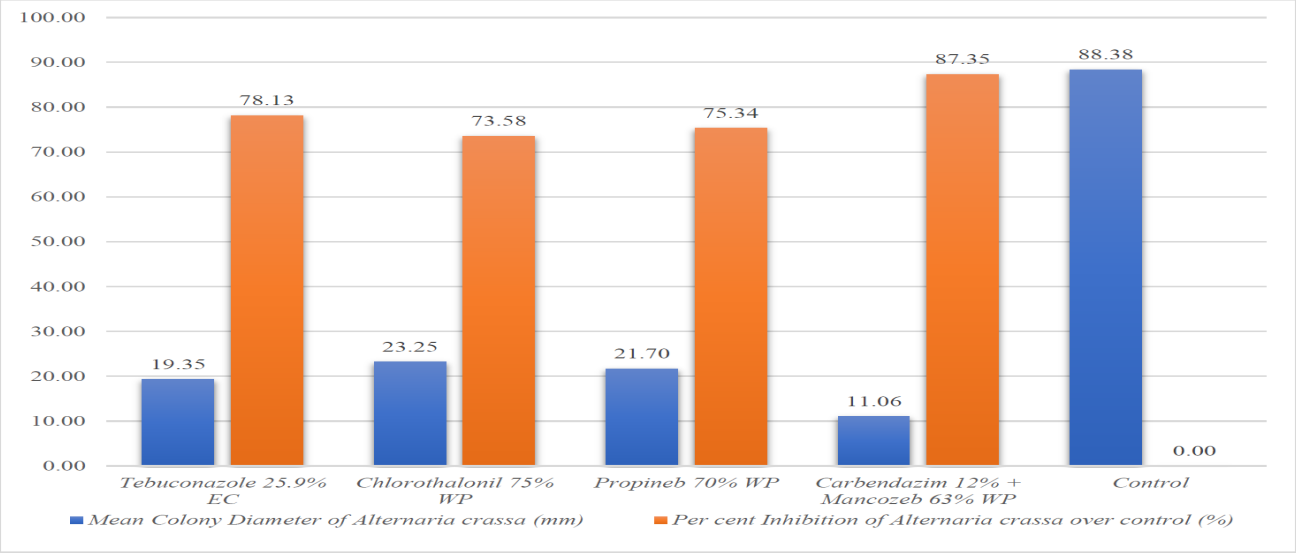

Fig. 1 : *In vitro* efficacy of fungicides against *Alternaria crassa*

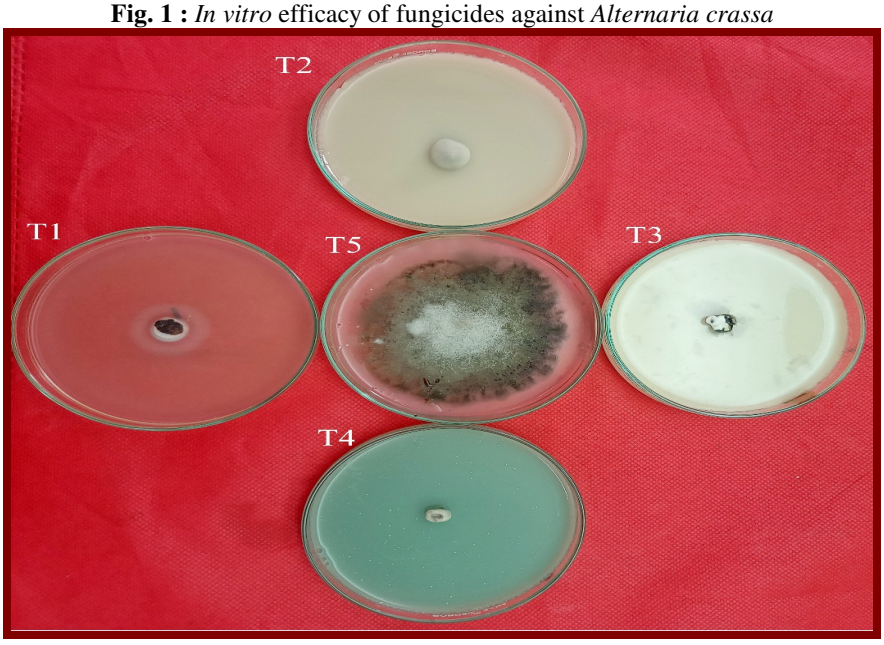

Plate 1 : *In vitro* efficacy of fungicides against *Alternaria crassa*

In vitro Efficacy of Essential Oils

Among the essential oils tested, Clove oil (T_3) and Eucalyptus oil (T_2) recorded the lowest mean colony diameters and were statistically at par, indicating superior efficacy against *Alternaria crassa*. Garlic oil (T_1) showed moderate inhibition and was significantly better than the control but inferior to T_2 and T_3. Neem

oil (T_4) was the least effective among treatments, though still significantly better than the untreated control (T_5), which exhibited the highest colony diameter.

In the present study, Clove oil (T_3) and Eucalyptus oil (T_2), each applied at a 5% concentration, exhibited the highest antifungal activity against *Alternaria crassa*. They recorded mean colony diameters of 19.00 mm and 20.00 mm, corresponding to 77.83% and 76.66% inhibition of mycelial growth, respectively. These findings align with previous research demonstrating the potent antifungal properties of Clove and Eucalyptus oils against various fungal pathogens (Diánez and Gea, 2018) (Khalse and Simon, 2017) (Liñán-Atero and Hadidi, 2024) (Čmiková and Kačániová, 2023).

The antifungal efficacy of Clove oil is primarily attributed to eugenol, its major active component, which disrupts fungal cell membranes and inhibits ergosterol biosynthesis, leading to compromised cell integrity and function (Hammer and Riley, 1999). Similarly, Eucalyptus oil contains compounds like 1,8-cineole (eucalyptol), which have been shown to inhibit fungal growth by affecting membrane permeability and inducing oxidative stress (Hammer and Riley, 1999).

Garlic oil (T_1) also showed moderate inhibitory effects, registering a mean colony diameter of 35.00 mm and 59.15% inhibition. The antifungal properties of Garlic oil are attributed to Allicin, a bioactive sulfur compound known to interfere with fungal enzymatic activities and membrane integrity (Devi and Basu, 2013).

Neem oil (T_4), although widely recognized for its pesticidal and antifungal properties (Devi and Basu, 2013), was relatively less effective in the current study, recording only 39.19% inhibition with mycelial growth of 52.10 mm. This outcome aligns with findings from (Sagoua and Loiseau, 2008), who observed significant inhibition of banana pathogens using Neem oil at various concentrations, suggesting that higher concentrations may enhance antifungal efficacy. The control treatment (T_5), recorded maximum radial growth (85.69 mm), signifying unhindered fungal development in the absence of any treatment.

These results underscore the potential of Clove and Eucalyptus oils as eco-friendly biocontrol alternatives to synthetic fungicides in the integrated management of leaf spot disease caused by *Alternaria crassa*.

Table 2 : *In vitro* efficacy of essential oils against *Alternaria crassa*

Tr. No.	Essential Oils	Concentration (%)	Mean colony diameter of *Alternaria crassa* (mm)*	Per cent inhibition of *Alternaria crassa* over Control (%)
T_1	Garlic	5	35.00^{c}	59.15
T_2	Eucalyptus	5	20.00^{d}	76.66
T_3	Clove	5	19.00^{d}	77.83
T_4	Neem	5	52.10^{b}	39.19
T_5	Control	-	85.69^{a}	0.00
		S.E. ±	0.50	
		CD at 1%	2.09	

*= The highest superscript represents the least effective treatment.

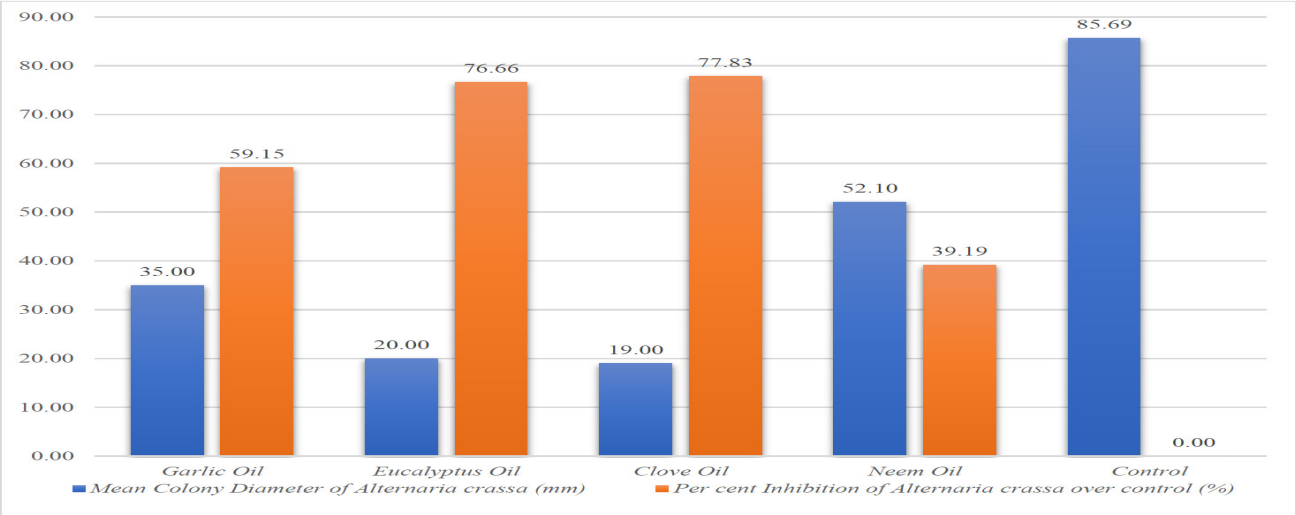

Fig. 2 : *In vitro* efficacy of essential oils against *Alternaria crassa*

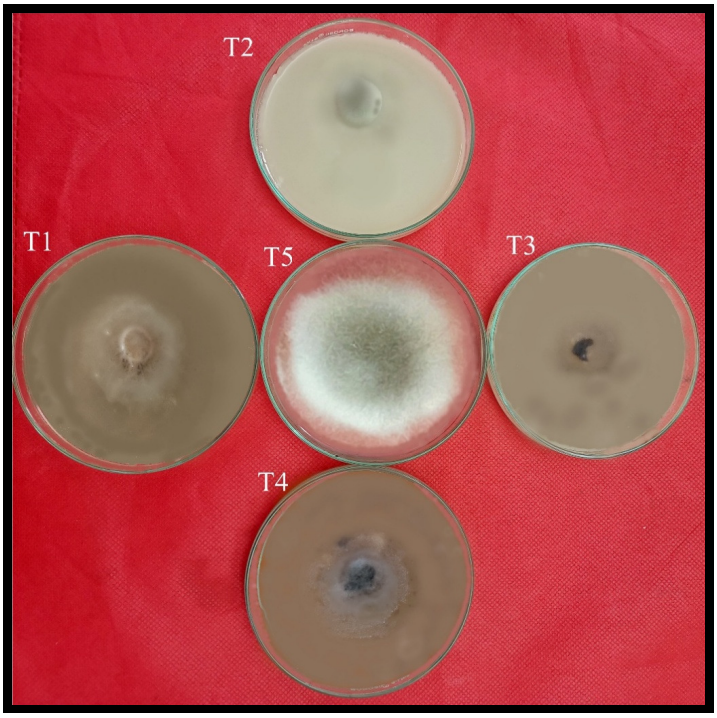

Plate 2 : *In vitro* efficacy of essential oils against *Alternaria crassa*

In vitro Efficacy of Bioagents

Trichoderma viride (T_1) demonstrated the highest antagonistic effect against *Alternaria crassa*, with the lowest mean colony diameter and was statistically superior to all other bioagents. *Trichoderma koningii* (T_3) and *Trichoderma harzianum* (T_2) were statistically distinct yet less effective than T_1, while *Pseudomonas fluorescens* (T_4) showed the least inhibition among the treatments but remained significantly better than the untreated control (T_5), which recorded the maximum colony diameter.

Among the tested bioagents, *Trichoderma viride* (T_1) was the most effective, exhibiting a mean colony diameter of 21.88 mm and achieving 75.11% inhibition of *A. crassa*. This strong antagonistic activity may be attributed to the production of volatile and non-volatile metabolites, competition for nutrients and space, and mycoparasitism, which are well-established mechanisms of *Trichoderma* spp (Khan and Li, 2020). The characteristic overgrowth of *T. viride* on the pathogen was also distinctly visible in the dual culture interaction.

Trichoderma koningii (T_3) and *Trichoderma harzianum* (T_2) followed with 63.70% and 55.74% inhibition, with mycelial growth of 31.90 mm and 38.90 mm respectively. These strains are known to secrete lytic enzymes such as chitinases and β-1,3-glucanases that degrade the cell walls of phytopathogens (Ayyandurai and Revathy, 2024). The moderate efficacy of these two agents in the present study suggests strain-specific differences and highlights the superior biocontrol performance of *T. viride* under the test conditions.

Pseudomonas fluorescens (T_4) demonstrated the least antagonistic activity among the treatments, inhibiting only 47.01% of the pathogen's growth, showing 46.57 mm of colony growth. Though bacterial antagonists like *P. fluorescens* are known for producing antibiotics (e.g., 2,4-diacetylphloroglucinol), siderophores, and lytic enzymes, their effect is often less pronounced in *in vitro* assays compared to fungal bioagents (Raaijmakers and De Souza, 2002). The untreated control (T_5) recorded the maximum colony diameter (87.90 mm), signifying unrestrained fungal growth in the absence of antagonists.

These results reaffirm the efficacy of *Trichoderma viride* as a promising biocontrol agent against *A. crassa* and suggest its potential integration in the eco-friendly management of leaf spot disease in Ashwagandha.

Table 3 : *In vitro* efficacy of bioagents against *Alternaria crassa*

Tr. No.	Bioagents	Mean colony diameter of *Alternaria crassa* (mm)*	Per cent inhibition of *Alternaria crassa* over Control (%)
T_1	*Trichoderma viride*	21.88[e]	75.11
T_2	*Trichoderma harzianum*	38.90[c]	55.74
T_3	*Trichoderma koningii*	31.90[d]	63.70
T_4	*Pseudomonas fluorescens*	46.57[b]	47.01
T_5	Control	87.90[a]	0.00
S.E. ±		0.44	
CD at 1%		1.82	

*= The highest superscript represents the least effective treatment.

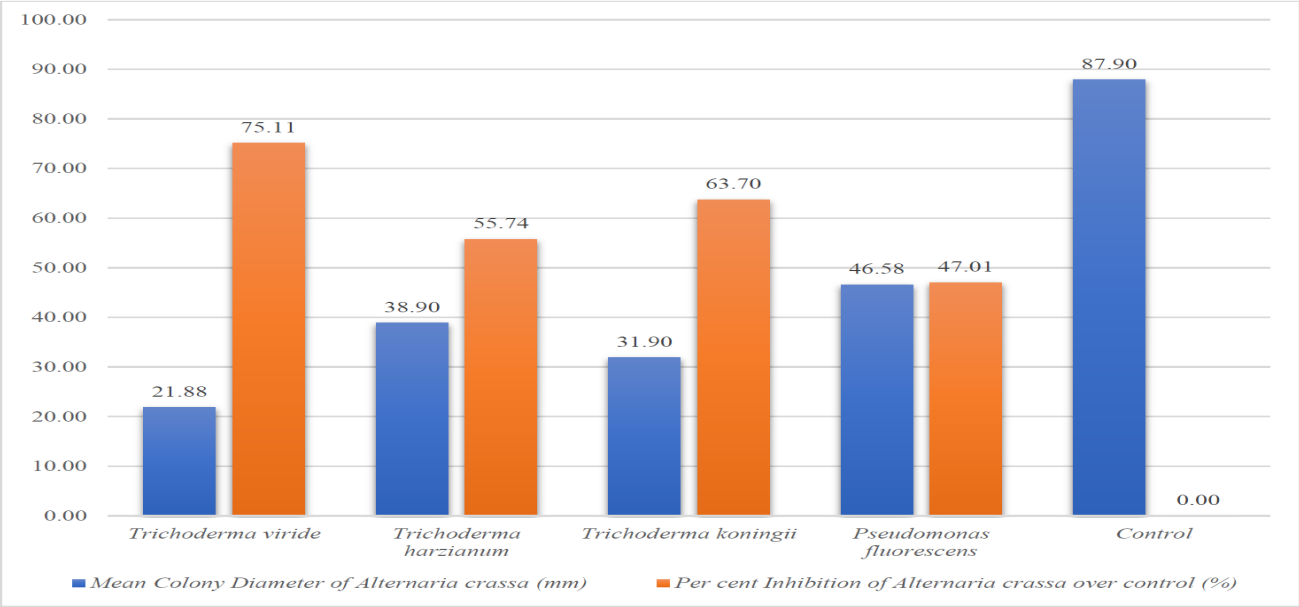

Fig. 3 : *In vitro* efficacy of bioagents against *Alternaria crassa*

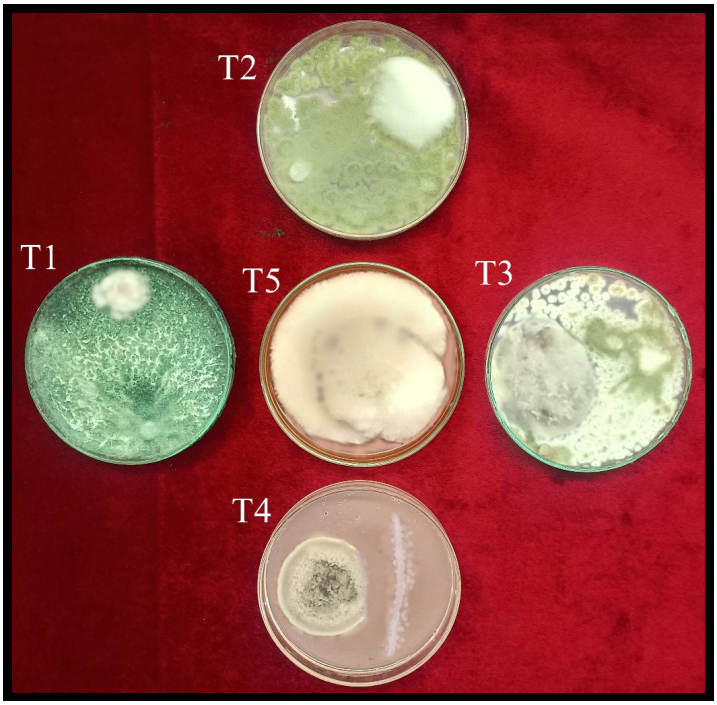

Plate 3 : *In vitro* efficacy of bioagents against *Alternaria crassa*

Conclusion

The study identified potent treatments for the *in vitro* management of leaf spot disease in Ashwagandha. Carbendazim 12% + Mancozeb 63% WP was the most effective fungicide, while Clove and Eucalyptus oils emerged as viable plant-derived alternatives. *Trichoderma viride* exhibited strong antagonism against the pathogen. These findings support the use of integrated strategies combining chemical, botanical, and biological approaches for sustainable disease management in Ashwagandha cultivation.

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