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Assessment of botanical extracts and biocontrol agents for the management of leaf spot disease of *Withania somnifera* instigated by *Alternaria alternata*

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Abstract
Withania somnifera, commonly known as Ashwagandha, is a prominent medicinal plant recognized for its extensive therapeutic applications in traditional medicine systems such as Ayurveda, Siddha, and Unani. Historically utilized as an aphrodisiac, liver tonic, anti-inflammatory agent, and astringent, it has gained popularity for treating conditions like bronchitis, asthma, ulcers, emaciation, insomnia, and senile dementia. Recent increases in the demand for Ashwagandha-based products highlight its significance in natural healthcare. However, the plant is susceptible to various pathogens, particularly leaf spot disease caused by *Alternaria alternata*, which has been reported in Madhya Pradesh's Grid region. To address this issue sustainably, both biological control agents and botanical extracts have been evaluated. *In vitro* studies revealed that five botanical extracts (Neem leaf extract, Moringa leaf extract, Lantana leaf extract, Garlic bulb extract and Akanda leaf extract) at 5%, 10% and 15% concentrations significantly inhibit *A. alternata*, with Moringa extract showing the highest inhibition rate at 15% (68.40%), followed by Neem extract 15% (61.47%), Garlic bulb extract at15% (49.35%) and lowest inhibition rate at Lantana extract at 5% (2.60%). Additionally, four biocontrol agents demonstrated varying effectiveness, with *Trichoderma viride* exhibiting the greatest inhibition (72.59%), followed by *Trichoderma* spp. (62.10%) and *T. harzianum* (56.17%), while *Pseudomonas fluorescens* showed the least efficacy (39.71%). These findings support the potential of using environmentally friendly strategies to manage leaf spot disease in Ashwagandha cultivation.

Keywords: *Withania somnifera*, *Alternaria alternata*, leaf spot disease, eco-friendly management

1. Introduction
Withania somnifera (L.) Dunal of family Solanaceae, which commonly known as Ashwagantha, Indian ginseng and winter cheery is a green herbaceous perennial medicinal plant. It is grown widely in tropical and subtropical climate of Africa, Middle East and Mediterranean region of the world. It is found throughout the drier parts in subtropical regions and upper Gangetic Plains. In India, ashwagandha is successfully grown in Madhya Pradesh, Gujarat, Uttar Pradesh, Haryana, Rajasthan and Punjab (Bhatia *et al.*, 1987) [3]. It is a well-known herbal plant for its therapeutic properties and immensely used in the Ayurveda, Siddha and Unani systems of medicines (Chopra *et al.*, 1958; Murthy *et al.*, 2008) [6, 12]. The roots of the plants contain several bio active principal compounds which include withaferin-A, withanolides, sterols and phenols etc., (Bhattacharya *et al.*, 2002) [4]. The awareness about the value of natural products for health care has led to increased demand of ashwagandha based products. The herb is cultivated in around 10,780 ha with the production of 8429 tones in India. The annual demand of this herb increased from 7028 tones (2001-2002) to 9127 tones (2004-2005) (Shrivastava and Sahu, 2013) [23]. However, ashwagandha is invaded by many plant pathogens and pests (Gupta *et al.*,1993; Nagraj and Reddy, 1985) [11, 13]. In India, the crop was reported to be infect by *Colletotrichum gloeosporioides* causing leaf spot (Sarkar and Dasgupta, 2017) [20], *Alternaria chlamydospora* causing leaf blight (Vanitha *et al.*, 2006) [24], *Fusarium solani* causing root-rot and wilt (Gupta *et al.*, 2004) [10]. Among them, Alternaria leaf spot caused by *Alternaria alternata* is one of the major diseases found under cultivated conditions (Shivanna *et al.*, 2014) [22]. Alternaria leaf spot causes heavy yield loss of around 50-60% (Pati *et al.*, 2008) [16].

While the medicinal properties of Ashwagandha have been extensively studied, there has been little research on its diseases and environmentally friendly management practices, which can result in considerable yield losses. Although chemical fungicides have been applied for disease control, there is a stronger preference for utilizing biocontrol agents, botanical extracts, and essential oils for managing diseases in this medicinal plant. With this context in mind, our primary objective for the current investigation is to identify effective *in vitro* eco-friendly strategies for managing *Alternaria alternata* disease in Ashwagandha.

2. Material and Methods

The current investigation was carried out to evaluate the efficacy of biocontrol agents and botanical extracts against *Alternaria alternata* causing leaf spot disease of Ashwagandha under *in vitro* condition. Diseased leaves showing various typical symptoms were collected from the crop cafeteria at ITM University, and then isolation of causal organism from the diseased leaves, purification and the pathogenicity of the causal organism were conducted in Plant Pathology Laboratory, School of Agriculture, ITM University, Gwalior, Madhya Pradesh, during 2023-24.

2.1 Collection and preparation of plant extract

Fresh and healthy leaves and bulbs were collected and washed properly to removed unwanted parts and dust, and then dried under shade. Botanical extracts were prepared by grinding 70 g of dried leaves and bulbs, soaking them overnight in distilled water four times the quantity of botanical extracts to achieve a 25% concentration as stock solution (Begum and Bhuiyan, 2006; Draz *et al*., 2019; Shabana *et al*., 2017) [2, 7, 21]. The extracts were filtered through double layered sterilized muslin cloth and sterilized Whatman's filter paper No. 1.

Table 1: Botanicals, parts and concentration of botanical extracts used in food poison technique.

Common name	Scientific name	Parts used	Concentration	References
Neem	*Azadirachta indica*	Leaves	5%, 10% and 15%	Kathal *et al.*, 2016 [8]
Moringa	*Moringa oleifera*	Leaves	5%, 10% and 15%	Nayak *et al*. (2023) [14]; Regmi *et al.* (2014) [19]
Lantana	*Lantana camara*	Leaves	5%, 10% and 15%	Nayak *et al*. (2023) [14]
Garlic	*Allium sativum*	Bulbs	5%, 10% and 15%	Yadav *et al.* (2020) [26]; Nayak *et al*. (2023) [14]
Akanda	*Calotropis gigantea*	Leaves	5%, 10% and 15%	Regmi *et al.* (2014) [19]

2.2 *In vitro* efficacy of botanicals: Food poison technique

To assess the antifungal activity of different botanicals against pathogen under *in-vitro* condition, respective plant leaf dextrose agar medium was utilized. Five botanicals *viz*., Neem (*Azadirachta indica*), Moringa (*Moringa oleifera*), Lantana (*Lantana camara*), Garlic (*Allium sativum*) and Akanda/Madar (*Calotropis gigantea*) were evaluated using the poisoned food technique (Nene and Thapliyal, 1982) [15] on PDA medium, incubated at 25 ℃. Control plates without any extract addition were also prepared. Various concentrations (5%, 10% and 15%) of botanical extracts were aseptically added to PDA medium to made up to 100 ml, which was then sterilized. To prevent bacterial contamination, Streptomycin sulphate was added when pouring the media into Petri plates. 5mm discs of the pathogens were taken from 5-7 days old culture of test fungus and placed in the centre of the plates. Each treatment, including the control, was replicated three times and incubated at 25 ℃ in BOD. Percent inhibition of mycelial growth was calculated by the following formula given by Vincent (1947) [25],

Inhibition % = [C–T/C] × 100

Where, C = Diameter of fungus colony (mm) in control plate
T = Diameter of fungus colony (mm) in treated plate

2.3 *In vitro* efficacy of biocontrol agents: Dual culture technique

Four biocontrol agents including *Trichoderma* spp., *T. viride*, *T. harzianum*, and *Pseudomonas fluorescens* were sourced from PG Plant Pathology laboratory, ITM University. They were tested for antagonism against *Alternaria alternata* using the dual culture method on PDA in Petri dishes (Fokkema, 1978) [9]. Each antagonistic-pathogen combination was assessed on 20ml of PDA in 9cm Petri plates with three replicate plates per treatment. For the dual culture technique, 0.5cm mycelial discs were taken with cork-borer from 5-7 days old culture on PDA medium of both pathogens and biocontrol agents and placed opposite each other on the periphery of the Petri plates. The dual culture plates were incubated at 25 ℃. For estimation of bio-control activity, the radial growth of the pathogen in treated and control plates was measured after different days of incubation at 25 ℃. Percent inhibition of mycelial growth was calculated by the following formula given by Vincent, 1947 [25].

Inhibition % = [C–T/C] × 100

Where, C = Diameter of fungus colony (mm) in control plate
T = Diameter of fungus colony (mm) in treated plate

2.4 Statistical analysis

The data were analyzed using statistical programme MS-Excel and SPSS. Duncan's Multiple Range Test (DMRT) were used to determined least signific and mean of the replications of the treatments.

3. Result and discussion

3.1 *In vitro* efficacy of botanicals: Food poison technique:

Five plant extracts, namely Neem (*Azadirachta indica)*, Moringa (*Moringa oleifera*), Lantana (*Lantana camara*), Garlic (*Allium sativum*) and Akanda (*Calotropis gigantea*), were tested at concentrations of 5%, 10% and 15% using poison food technique method on PDA media incubated at 27±1 ℃. The inhibitory activity of these plant extracts is attributed to their antimicrobial components; however, the specific chemical constituents and their mechanism controlling *Alternaria alternata* need further clarification.

According to the *in vitro* efficacy experiment with botanicals, Moringa extract at 15% concentration exhibited

the highest inhibition % against *A. alternata* (68.40%), followed by Neem extract 15% (61.47%). The lowest inhibition % was observed with Lantana extract at 5% (2.60%). Moringa consistently showed higher inhibition compared to other plant extracts across all concentrations tested, whereas Lantana demonstrated the least inhibition as represented in Table 2 and Fig 1, 2 and 3. Yadav *et al.* (2020) [26] reported that Garlic cloves extract at 10% (65.45%) exhibited highest inhibition of mycelial growth of *A. alternata* followed by neem extract at 10% (55.12%). Rajeswari and Balasupramani (2020) [17] also reported the same result that garlic clove extract at 5% exhibited high inhibition activity against the same.

Table 2: Radial growth of *Alternaria alternata* and inhibition % in poison food technique.

Treatment	Radial growth of pathogen (cm)					Inhibition % (final day) *
	2DAI	4DAI	6DAI	8DAI	10DAI	
Neem 5%	1.40±0.10^{def}	3.17±0.15^{e}	4.63±0.06^{f}	5.43±0.15^{h}	6.30±0.10^{i}	18.18(25.24) ^{cd}
Neem 10%	1.67±0.06^{g}	2.90±0.20^{de}	4.67±0.06^{f}	5.33±0.15^{gh}	6.50±0.10^{j}	15.94(23.53) ^{c}
Neem 15%	1.23±0.06^{bcd}	1.83±0.12^{a}	2.43±0.06^{a}	2.73±0.06^{b}	2.97±0.06^{b}	61.47(51.63) ^{l}
Moringa 5%	1.27±0.06^{cd}	3.50±0.10^{f}	4.20±0.26^{e}	4.70±0.26^{f}	4.90±0.10^{e}	36.35(37.08) ^{i}
Moringa 10%	1.17±0.06^{bc}	2.87±0.06^{de}	3.50±0.10^{c}	4.13±0.15^{d}	4.27±0.15^{d}	44.82(42.02) ^{j}
Moringa 15%	1.13±0.06^{bc}	1.90±0.10^{a}	2.30±0.10^{a}	2.37±0.15^{a}	2.43±0.06^{a}	68.40(55.80) ^{m}
Lantana 5%	1.93±0.15^{h}	3.83±0.15^{g}	5.60±0.10^{i}	5.83±0.06^{jk}	7.50±0.10^{k}	2.60(9.28) ^{a}
Lantana 10%	1.87±0.06^{h}	3.77±0.06^{fg}	5.37±0.06^{h}	5.50±0.10^{hi}	7.43±0.12^{k}	3.88(11.36) ^{b}
Lantana 15%	1.50±0.26^{efg}	2.57±0.49^{bc}	4.13±0.06^{e}	4.40±0.10^{e}	6.00±0.10^{h}	22.06(28.01) ^{e}
Garlic 5%	1.57±0.06^{fg}	3.00±0.10^{de}	4.03±0.12^{de}	4.87±0.06^{f}	5.13±0.21^{f}	33.33(35.26) ^{h}
Garlic 10%	1.03±0.21^{b}	2.33±0.06^{b}	3.40±0.10^{c}	4.30±0.10^{de}	4.33±0.06^{d}	43.97(41.53) ^{j}
Garlic 15%	0.80±0.00^{a}	1.63±0.12^{a}	3.00±0.10^{b}	3.90±0.10^{c}	3.90±0.10^{c}	49.35(44.63) ^{k}
Akanda 5%	1.60±0.06^{g}	3.83±0.15^{g}	5.10±0.10^{g}	5.93±0.15^{k}	6.23±0.15^{i}	19.04(25.87) ^{d}
Akanda 10%	1.33±0.06^{cde}	3.10±0.10^{e}	4.67±0.15^{f}	5.67±0.06^{ij}	5.73±0.06^{g}	25.86(30.56) ^{f}
Akanda 15%	1.13±0.06^{bc}	2.73±0.15^{cd}	3.90±0.10^{d}	5.23±0.06^{g}	5.23±0.06^{f}	32.03(34.47) ^{gh}
Carbendazim (check)	1.23±0.06^{bcd}	3.50±0.10^{f}	4.23±0.15^{e}	5.40±0.10^{gh}	6.37±0.06^{ij}	29.26(32.75) ^{g}
Control	2.40±0.20^{i}	4.30±0.10^{h}	5.87±0.06^{j}	6.90±0.10^{l}	7.73±0.06^{l}	-
C.D. @5%	0.181	0.262	0.192	0.176	0.176	1.813
SE(m)	0.063	0.091	0.067	0.061	0.061	0.626
SE(d)	0.089	0.128	0.094	0.086	0.086	0.886
C.V.	7.599	5.226	2.762	2.194	2.194	3.282

The represented data are mean ± standard deviation, and mean in the column followed by the same letter are not significantly different according to DMRT (p<0.05). DAI- days after inoculation.
* Figures in parenthesis are arcsine/angular value

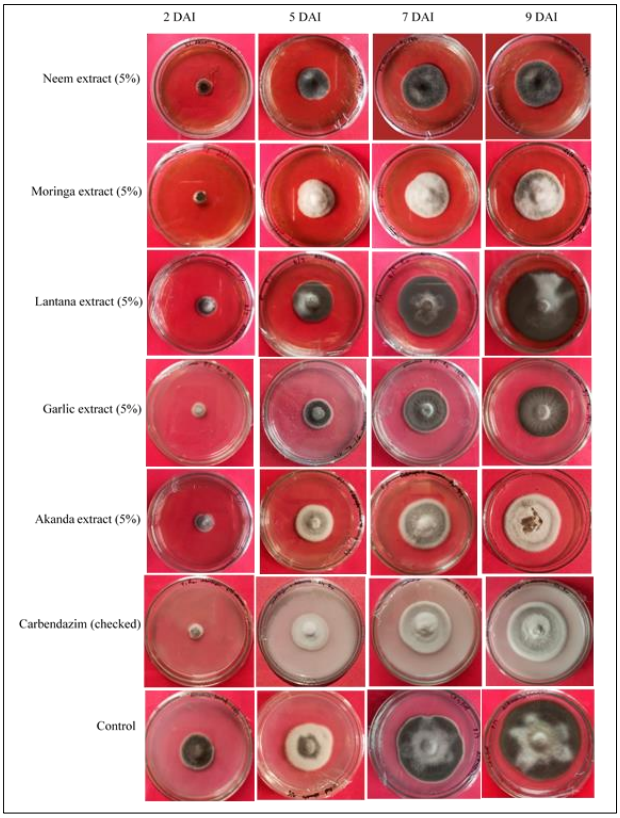

Fig 1: Poison Food technique for evaluation of efficacy of Plant extracts at 5% concentration against *Alternaria alternata in vitro*. DAI denotes days after inoculation

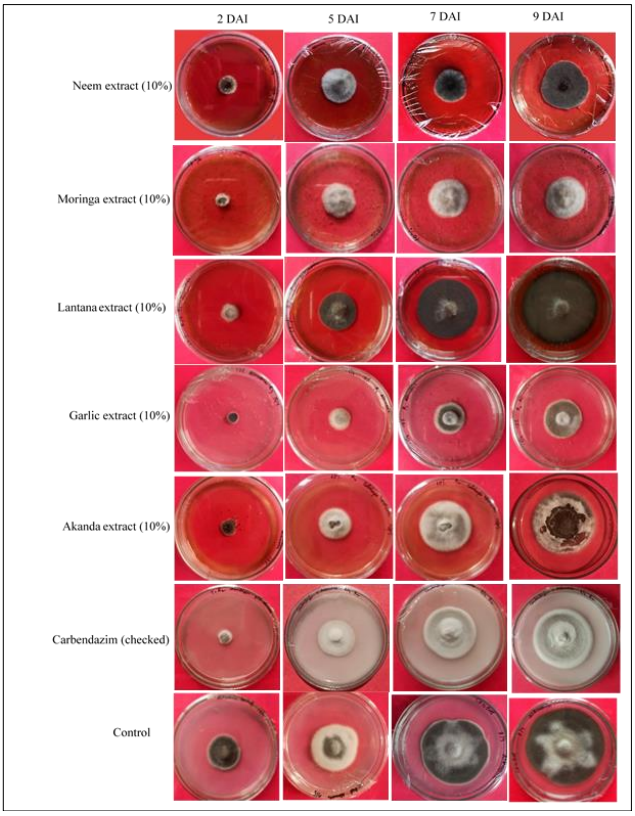

Fig 2: Poison Food technique for evaluation of efficacy of Plant extracts at 10% concentration against *Alternaria alternata in vitro* DAI denotes days after inoculation

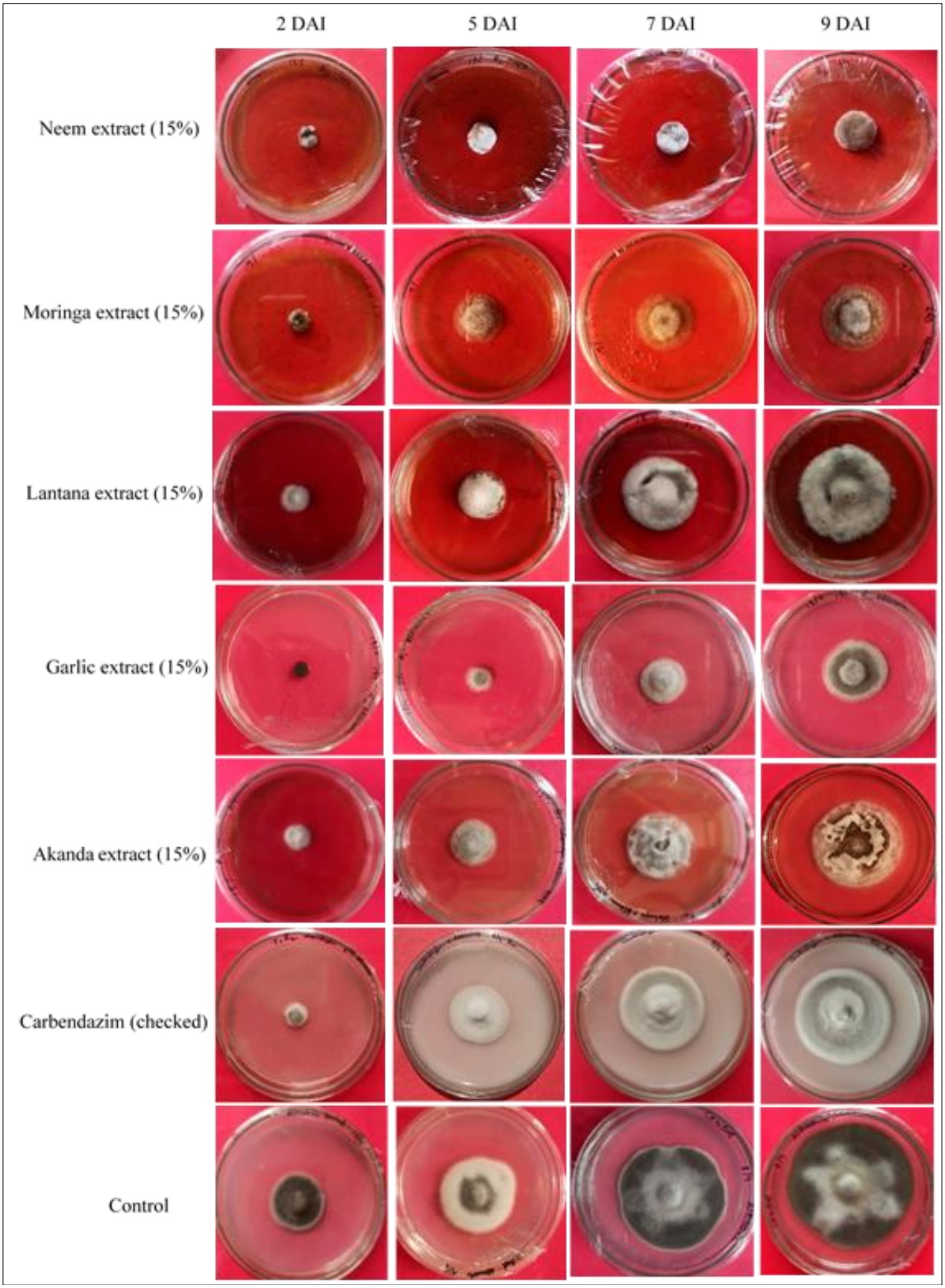

Fig 3: Poison Food technique for evaluation of efficacy of Plant extracts at 15% concentration against *Alternaria alternata in vitro* DAI denotes days after inoculation

3.2 *In vitro* efficacy of biocontrol agents: Dual culture technique

Four biocontrol agents, namely *Trichoderma* spp., *T. viride*, *T. harzianum* and *Psuedomonas fluorescence*, were evaluated *in vitro* for their antagonistic activity against *Alternaria alternata* of Ashwagandha using the dual culture test method.

The results of *in vitro* efficacy of biocontrol agents against *A. alternata* indicated that *Trichoderma viride* exhibited the highest inhibition % of radial growth of the pathogen at 9

days after inoculation (DAI) (72.59%), followed by *Trichoderma* spp. (62.10%) and *Trichoderma harzianum* (56.17%), while *Pseudomonas flourescens* showed the lowest inhibition (39.71%) as shown in Table 3 and Fig 4. In a related investigation, Akbari and Parakhi (2007) [1] documented that *T. viride*-I and *T. hamatum*-IV&V strains exhibited significant antagonism towards *Alternaria alternata*, the pathogen responsible for sesame blight. Similarly, Rani *et al.*, (2018) [18] also reported that *Trichoderma viride* provided 80.36% inhibition of growth of *Alternaria alternata*.

Table 3: Radial growth of *Alternaria alternata* and inhibition % in Dual culture technique

Treatment details	Radial growth of pathogen (cm)				Inhibition % (final day) *
	3 DAI	5 DAI	7 DAI	9DAI	
Trichoderma spp.	1.93±0.14^{d}	2.00±0.14^{b}	2.07±0.17^{b}	2.07±0.17^{b}	62.10(52.00) ^{c}
Trichoderma viride	1.47±0.10^{a}	1.50±0.14^{a}	1.50±0.18^{a}	1.50±0.18^{a}	72.59(58.43) ^{d}
Trichoderma harzianum	1.57±0.10^{b}	2.37±0.10^{c}	2.40±0.10^{c}	2.40±0.08^{c}	56.17(48.54) ^{b}
Pseudomonas flourescens	1.73±0.10^{c}	2.17±0.06^{b}	2.50±0.08^{c}	3.30±0.16^{d}	39.71(39.06) ^{a}
Control	2.57±0.06^{e}	3.30±0.10^{d}	4.20±0.10^{d}	5.47±0.06^{e}	-
C.D. @5%	0.152	0.169	0.198	0.190	2.58
SE(m)	0.050	0.056	0.065	0.063	0.83
SE(d)	0.071	0.079	0.0.92	0.089	1.17
C.V.	5.528	4.958	5.154	4.486	3.46

The represented data are mean ± standard deviation, and mean in the column followed by the same letter are not significantly different according to DMRT (P<0.05). DAI- Days after inoculation.
***** Figures in the parenthesis are arcsine value.

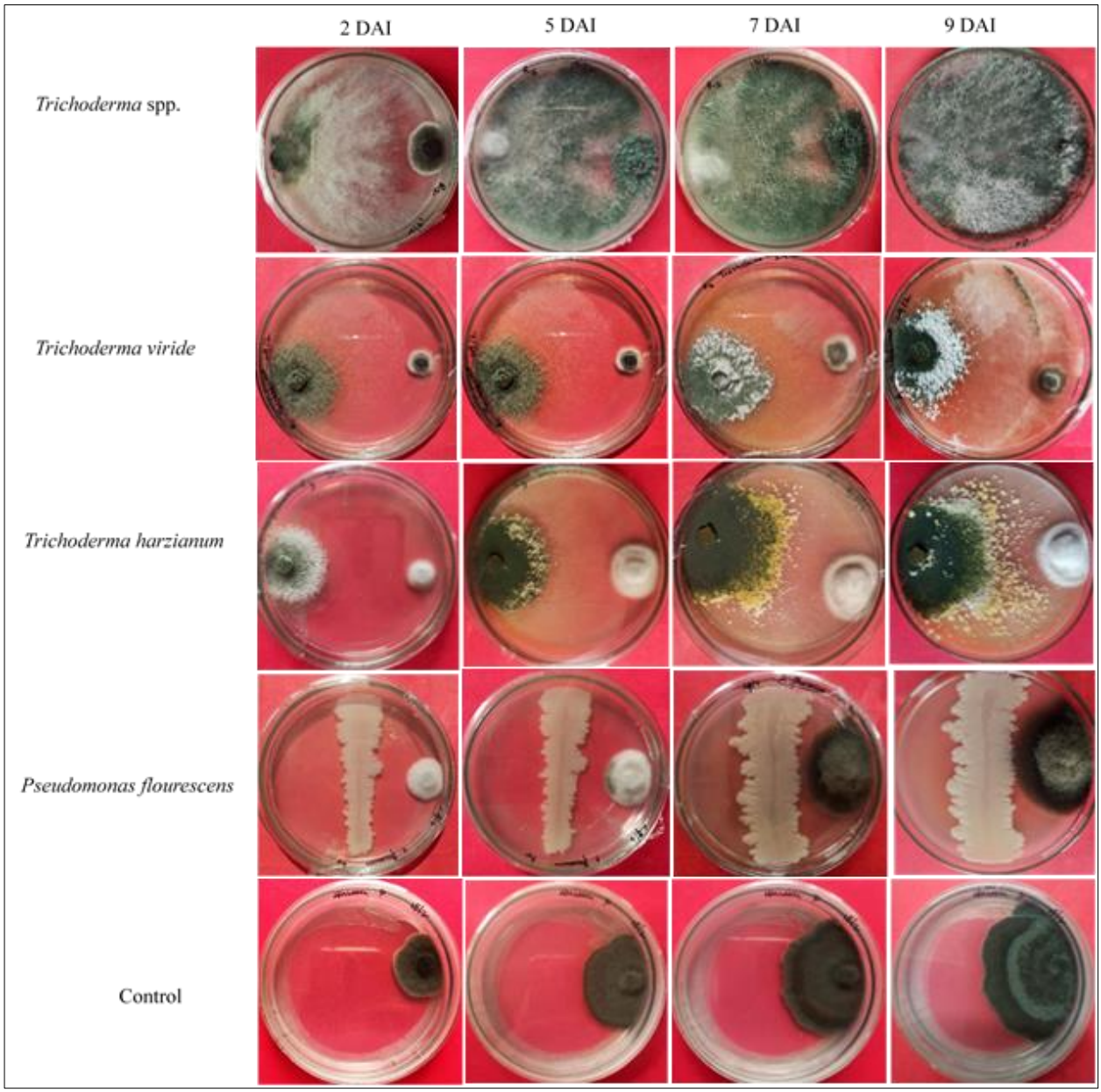

Fig 4: *In vitro* efficacy of biocontrol agents (DAI-days after inoculation)

4. Conclusion

Alternaria alternata has been identified as the causal agent of leaf spot disease affecting Ashwagandha in the Grid region of Madhya Pradesh in the current investigation. In terms of disease management strategies explored in this study, botanical extracts and biocontrol agents showed varying degrees of efficacy against *A. alternata*. Moringa extract at 15% concentration exhibited the highest inhibition of fungal growth (68.40%), followed by Neem extract at 15% (61.47%). Among biocontrol agents tested, *Trichoderma viride* demonstrated the highest inhibition (72.59%), followed by *Trichoderma* spp. (62.10%), whereas *Pseudomonas fluorescens* exhibited the lowest inhibition (39.71%). These findings underscore the significance of integrated disease management approaches using botanicals, and biocontrol agents to mitigate the impact of Alternaria leaf spot disease on Ashwagandha cultivation. Further research is warranted to optimize these strategies under varying environmental conditions and validate their practical application in the field.

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