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In vitro study of the efficacy of botanicals, biological agents and fungicides against *Alternaria tagetica* causing leaftspots and flower blight of Marigold

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Abstract
Marigold is one of the most important commercially exploited flowers of the world. Among the diseases reported on marigold, leaf spot and flower blight of marigold caused by *Alternaria tagetica* is one of the most important diseases. The fungus was isolated from an infected marigold plant. The pathogenicity of the isolated fungus was proved by inoculating into the healthy seedling of marigold. Based on typical symptoms shown on the inoculated leaves, microscopic observations and cultural characteristics of the fungus, it was identified as *Alternaria tagetica.* An investigation was carried out *in vitro* to test the efficacy of botanicals, bioagents and fungicides against the isolated fungus. Among the botanical extracts tested, Garlic cloves (20%) extract recorded the highest percent mycelial growth inhibition with 78.25% followed by 10% (73.11%). The results of dual culture technique revealed that *Trichoderma harzianum* gives the highest with 46.82% mycelial growth inhibition. Among the tested fungicides, Mancozeb @ 0.2% showed maximum mycelial growth inhibition with 84.90% against *Alternaria tagetica.* The compatibility test of *T. harzianum* with fungicides revealed that *T. harzianum* was compatible with Mancozeb 75 WP and Copper oxychloride 50 WP but incompatible with Carbendazim 50 WP and Saaf 75WP at all concentrations.

Keywords: *Alternaria tagetica*, botanicals, bioagents, fungicides, *Trichoderma harzianum*

Introduction
Marigold is one of the most important commercially exploited flowers of the world. Marigold belongs to the genus Tagetes and comes under the *Asteraceae* family. It was first discovered by the Portuguese in the 16th century in Central America and is native to North and South America. The area under marigold cultivation in India is 70.74 thousand hectares with a production of loose flower is 712.31 thousand tones and cut flower is 6.59 thousand tones. The states in which it is mainly cultivated are Karnataka, Uttar Pradesh, Tamil Nadu, Andhra Pradesh, Delhi, Himachal Pradesh and Sikkim. In Karnataka, it is cultivated in an area of 8.08 thousand hectare with 73.81 thousand tones production of flower (Anon., 2021).
Marigold plant is affected by various fungal, bacterial and viral diseases despite all the nematicidal, fungicidal, insecticidal properties. Among the fungal diseases of marigold, leaf spot and flower blight of marigold caused by *Alternaria tagetica* become the major constraint in the exploitation of high yielding varieties. Under congenial conditions, the infection can cause premature defoliation and finally death of the marigold plant. The disease resulted in a flower yield loss up to 50-60% (Ratan and Shukla, 2002) [16].
Considering the significance of the crop and the disease, the present study was conducted with isolation, identification, and pathogenicity test, *in vitro* evaluation of botanicals, bioagents, and fungicides against *Alternaria tagetica* and compatibility of *Trichoderma harzianum* with fungicides.

Materials and Methods
Local variety of marigold (*Tagetes sp.*) leaves showing typical symptoms of leaf spots and flower blight were collected from Medziphema town, Nagaland and the pathogen, *Alternaria tagetica* was isolated from the collected samples. The wild strains of *Trichoderma harzianum*, *T. asperellum* and *T. virens* were collected from the department of Plant Pathology, School of Agricultural Sciences, Nagaland University, Medziphema, and Nagaland.

Isolation, Identification of the putative pathogen and Pathogenicity test
The diseased portions of the leaves of marigold were cut into small bits containing 50% healthy tissue and 50% infected tissue. The PDA containing the leaf bits was incubated at 25 ± 2˚C. The pathogen obtained was identified according to its morphological characters, colony, color. The size of the pathogen was measured using the software piximetre 5.2 under compound microscope (Debro microscope DX-600 series).
One month old marigold seedlings were transplanted in pots and was sprayed with the conidial suspension of the concentration 2 x 10^6 /ml on the foliage using hand automizer and was swabbed with moist cotton on to leaves. The fungus was reisolated from the infected leaves after the symptoms appeared, and the obtained culture was compared with the original isolation.

In vitro evaluations of botanicals against *A. tagetica*
The Poisoned food technique (Nene & Thapliyal, 1993) [13] was adopted for evaluating the antifungal potential of the

selected plant extracts (Turmeric, Garlic, Parthenium, Neem, Periwinkle, Bougainvillea, Korpad and Ashoka). The prepared stock solutions mixed in sterilized molten PDA was poured into the plates under aseptic conditions. A 5 days old test culture consisting of 5mm mycelial disc was inoculated aseptically at the center of the plate and then incubated at 25±2˚C. Observations were taken and the data was noted till the untreated control plates had fully grown. The experiment was also conducted in Complete Randomized Design (CRD) and the ten treatments were replicated thrice each.

***In vitro* efficacy of bioagents**

The three bio control agents *viz., Trichoderma harzianum, Trichoderma asperellum* and *Trichoderma virens* were evaluated against *Alternaria tagetica* by dual culture technique (Dennis and Webster, 1971) [5]. 5mm mycelial disc of the fungal antagonists and the test fungus were placed opposite to each other on the same plate and was incubated at 25±2˚C. PDA plate containing only the test pathogen was kept as control. The experiment was conducted in Complete Randomized Design (CRD) and the three treatments were replicated four times each. The percent inhibition was calculated by the formula given by Dennis and Webster (1971) [5]

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

PI= Percent inhibition

C= Average growth of *Alternaria tagetica* in control plates (mm)

T= Average growth of *Alternaria tagetica* in dual culture technique.

***In vitro* efficacy of fungicides**

Poisoned food technique (Nene & Thapliyal, 1993) [13] was used for evaluating the activity of fungicides against *Alternaria tagetica.* The fungicides were tested at 0.1, 0.2, 0.25% concentrations. The poisoned PDA containing individual fungicides were inoculated with 5mm mycelial disc of 5 days old *Alternaria tagetica*. The Petri plates were then incubated at 25±2˚C and growths were measured till the maximum growth was observed in the control plates. The experiments were conducted in Completely Randomized Design (CRD) and the twelve fungicide treatments were replicated three times each. The percent inhibition was calculated by the formula given by Vincent (1947) [21].

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

PI= Percent inhibition

C= Average growth of *Alternaria tagetica* in control plates (mm)

T= Average growth of *Alternaria tagetica* in poisoned food plates

Compatibility of *Trichoderma harzianum with fungicides*

Evaluations of compatibility of *Trichoderma harzianum* with selected fungicides was carried out using Poisoned food technique (Nene &Thapliyal, 1993) [13] at 0.1%, 0.2% concentrations. PDA poisoned with fungicides was then poured into the sterilized Petri plates which was inoculated with 5mm mycelial disc of *Trichoderma harzianum*. The Petri plates were then incubated at 25 ± 2˚C. The experiment was carried out in Complete Randomized Design (CRD) and each treatment was replicated thrice times. The percent inhibition of growth of *Trichoderma harzianum* was calculated by the formula given by Vincent (1947) [21].

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

PI= Percent inhibition

C= Average growth of *Alternaria tagetica* in control plates (mm)

T= Average growth of *Alternaria tagetica* in poisoned food plates

Statistical analysis

Statistical analysis of laboratory experiment will be done by Completely Randomized Design (CRD) described by Goon *et al.*, 1931[8]. The experimental results were statistically interpreted through calculation of 'Analysis of Variance' (ANOVA)and by using Fisher Schedecor 'F' test the statistical significance will be tested at 0.05 level probability.

Results and Discussions

Isolation, Identification, Pathogenicity Test

The infected marigold plant showing characteristic symptoms of leaf spot and flower blight were collected and the pathogen was isolated in the laboratory as mentioned.

The fungus when cultured *in vitro* showed olivaceous brown to black color after incubation for 7 days (168 hours) and a whitish fluffy mycelial growth were observed on the colony as shown in Plate 1. The fungus took 14 days to cover the full area of Petriplate having 90 mm diameter at a temperature of 25 ± 2º C. The hyphae were brown, smooth, branched at right angle and septate. The conidiophores were simple straight, light brown in color and septate. The conidia were muriform, obclavate to obipyriform, light to dark brown in color with 3 transverse septa and 2 longitudinal septa. The length of the conidium measures upto 245.96µm, 89.61µm breadth and the length of the beak was 78.72µm. The observed colony formed on PDA medium was found to be similar with the observations made by Woodward *et al.*(2007) [23] that the colonies formed by fungus that caused *Alternaria* leaf spot of Cotton were olive brown to black on PDA. The findings were also similar with the report given by Yu Zhan *et al*. (2010) [24] that the conidia are obclavate, ellipsoidal, obpyriform in form and formed singly or in short chains, having a conical or cylindrical beak with transverse and longitudinal septa. Our observations were also in accordance with the observations made by several workers (Maya *et al.*, 2013 and Zhao *et al*., 2016) [11, 25].

Pathogenicity test performed showed a typical symptom of *Alternaria* leaf spot after 15 days of inoculation (Plate 2). Light brown to dark brown spots appeared on the inoculated part of the leaves and stem. These lesions enlarged and coalesced and formed a blighted appearance. The whole plant showed a burnt appearance and died within 2 to 3 weeks after inoculation. The symptoms of leaf spots observed and on re-isolation, the morphological characters

of the pathogen were similar with that of the test pathogen *Alternaria tagetica* isolated from the natural field of marigold plants and thus fulfilled Koch's postulates. Sharma *et al*. (2012) [17] proved pathogenicity of *A. alternata* in pigeon pea by inoculating seedlings with conidial (5 x 10^5 conidial/ml) suspension and incubated at 25±2˚C. After ten days, symptoms on leaves were developed, which were identical to the observed symptoms in the field. Aktar and Shamsi (2014) [1] conducted pathogenicity test by spraying water suspension of the test fungus (*A. alternata*) at 10^4 ml concentration. The pathogen showed the symptoms of disease on all the inoculated plants of *Tagetes spp.*

In vitro* evaluation of botanicals against *A. tagetica

Since the botanical extracts are abundant, cheaply available and hold promises in disease management, therefore the efficacy of eight plant extracts were evaluated against the pathogen *A. tagetica* at 10 % and 20% concentrations *in vitro* by using poison food technique. Most of the plant extracts were observed to have higher inhibitory action with higher concentration against the mycelia growth of the pathogen as presented in Table 1 and Plate 3. Among the eight plant extracts tested, Garlic clove (20%) was found the highest percent inhibition of 78.25% followed by 10% (73.11%), Turmeric (10%), Parthenium (20%) with percent inhibition of 61.94 % and 60.20% respectively. The least inhibition of mycelia growth was recorded in Ashoka (10 %) 20.00% followed by Ashoka (20%) 30.00%. The fungistatic action of the botanicals might be due to the presence of several antifungal compounds like phenols, alkaloids, tannins. The antifungal compound in Garlic(allicin), Turmeric(curcumenol, curdione, isocurcumenol, curcumin, curcumol, curzerene, germacrone, β-elemene), Parthenium (terpenoids, saponins, flavanoids, tannins and alkaloids), Periwinkle (2,3 dihydroxybenzoicacid), bougainvillea (alkaloids, flavanoids, phlobatannins and terpenoids), Korpad (anthraquinones,dihydroxyanthraquinones, saponins), Neem(terbinafine), Ashoka(alkaloids,flavanoids, tannins, saponins, glycoside) plays role in inhibiting the growth of the mycelia of the pathogen. The present investigations were in agreement with the observations and reports given by several workers like Singh and Majumdar (2001),Gangopadhay *et al.* (2010), Ranaware *et al*.(2010) [15, 19].The findings were also in coordination with the work of Apet *et al.(*2014) [4].

In vitro* evaluations of bioagents against *A. tagetica

The data pertaining to the effect of bioagents on the fungal growth ranged from 25.12% to 29.75% inhibition at (7 days) after inoculation and 60.77% to 63.88% inhibition at (14 days) after inoculation (Table.2, Plate 4). Among the three fungal biocontrol agents tested *T. harzianum* showed the maximum percent inhibition of 46.82% followed by *T. virens* (45.40%) and the least percent inhibition was found in *T. asperellum* with 42.95 % inhibition. The inhibitory effect of the bioagents may be due to the mechanism of antibiosis, mycoparasitism, lysis, competition for food and space. Since the bioagents overgrew the tested fungus, it may be interpreted that *Trichoderma spp.* produce enzymes like chitinases, lipases, β-(1-3)-glucanases, protease that degrade the cell wall of the pathogens which may attribute to another mode the mycoparasitic effect. The results were in agreement with the work of Lewis and Lumsden (2001), who reported that the genus *Trichoderma sp.* are capable of inhibiting the growth of pathogenic fungi by the mechanism of mycoparasite, antibiosis, competition for food and space. The observations obtained also correlate with the findings of Ambuse *et al*. (2012), Jaiswal *et al*. (2018) and Jackson and Kumar (2019) [2, 9, 10] as *T. harzianum* was the most effective in inhibiting the pathogen growth

In vitro* evaluation of fungicides against *A. tagetica

The evident from the data (Table 3, Plate 5) indicates that among the evaluated fungicides, Mancozeb at all concentrations gives the maximum inhibition percentage against *Alternaria tagetica.* Mancozeb 0.2% concentration gives the highest inhibition of 84.90% which was followed by 0.25% and 0.1% with percent inhibition of 83.68% and 82.68 % respectively. Carbendazim 0.1% was found to be the least inhibitory effect of 52.10%. The present investigations were also indicated that higher the concentrations, higher the inhibition percentage irrespective of fungicides. The fungicide Mancozeb was significantly superior in inhibiting mycelial growth as reported by Godika *et al*. (2001) [7] who observed that mancozeb and copper oxychloride were effective in controlling the disease. Wagh *et al.* (2017) and Prasad *et al.* (2018) [14, 22] also concluded that Carbendazim + Mancozeb combination gives the maximum inhibition percent of *Alternaria sp*.

***In vitro* evaluation of compatibility of *T. harzianum* with fungicides**

The potential fungal biocontrol agent *T. harzianum* was selected for fungicidal compatibility studies since it has shown the maximum inhibition of *A. tagetica* growth in dual culture studies when compared to *T. asperellum* and *T. virens*. Compatibility of *T. harzianum* was evaluated against the four most effective fungicides *viz*., Mancozeb, Copper oxychloride, Carbendazim and Saaf at the concentrations of 0.1%, 0.2% by poison food technique. The percent inhibition over the control plates were calculated after 96 hours and 168 hours as shown in the Table.4, Plate 6. The data thus obtained showed that the two contact fungicides *viz*., Mancozeb and Copper oxychloride were 100% compatible with *T. harzianum* at all concentrations (0.1%, 0.2%) except in 0.2% concentration of Mancozeb which exhibited 5.55% inhibition. Both 0.1% and 0.2% concentrations of both Carbendazim and Saaf showed 100% inhibition. Thoudam and Dutta (2014) [20] found that *T. harzianum* is incompatible with carbendazim at 0.1% and compatible with mancozeb at copper oxychloride at 0.1 and 0.2%. This is due to the fact that *T. harzianum* has the ability to degrade fungicides and used their by-products carbon and nitrogen for its growth and development (Mohamed & Radwan, 2017). Shashikumar *et al*. (2019) [9, 12] also reported that mancozeb 75%WP was found compatible with *T. harzianum* but incompatible with carbendazim 50%WP among the tested fungicides.

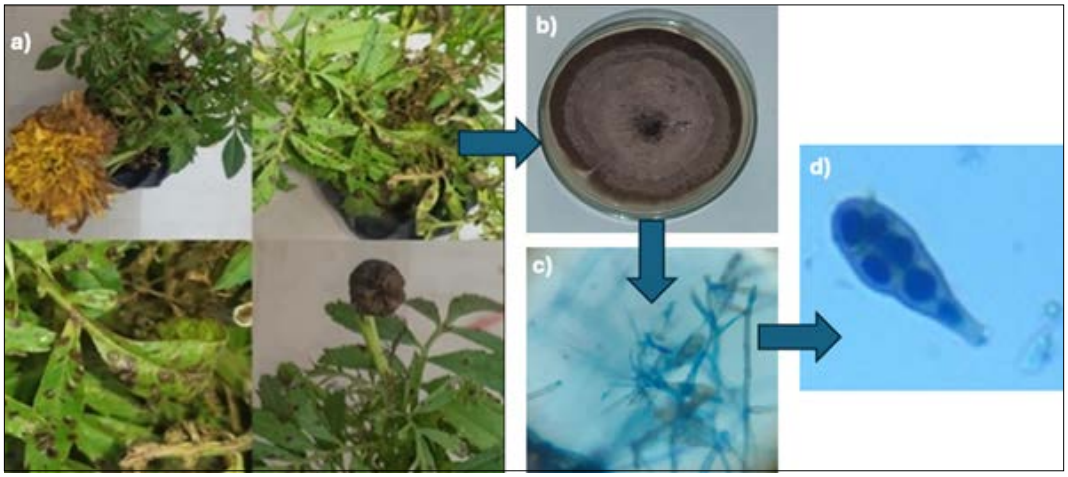

Plate 1: a) Symptoms of *Alternaria* leaf spot and flower blight of Marigold b) Pure culture of *A tagetica* on PDA media c) Mycelia and Conidia of *Alternaria tagetica* d) Conidia of *Alternaria*

Plate 2: Pathogenicity test of *Alternaria tagetica* on marigold plants

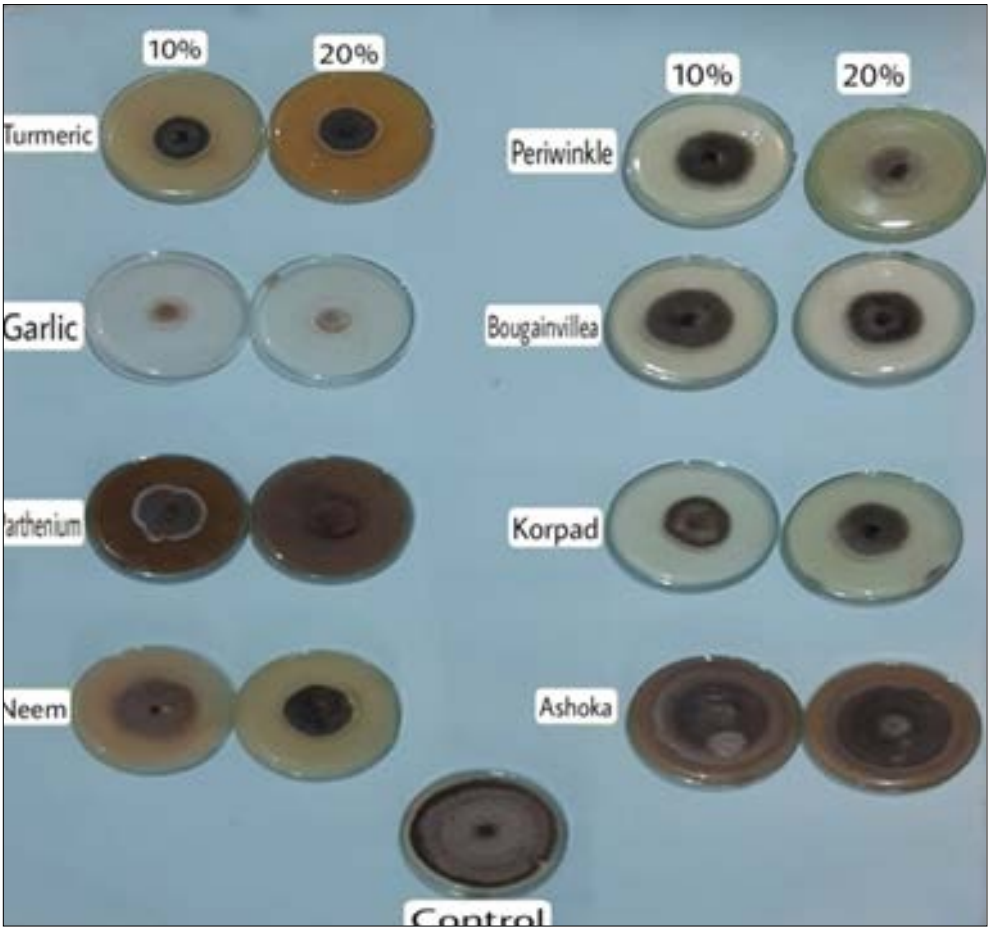

Plate 3: *In vitro* efficacy of botanicals against *A. tagetica*

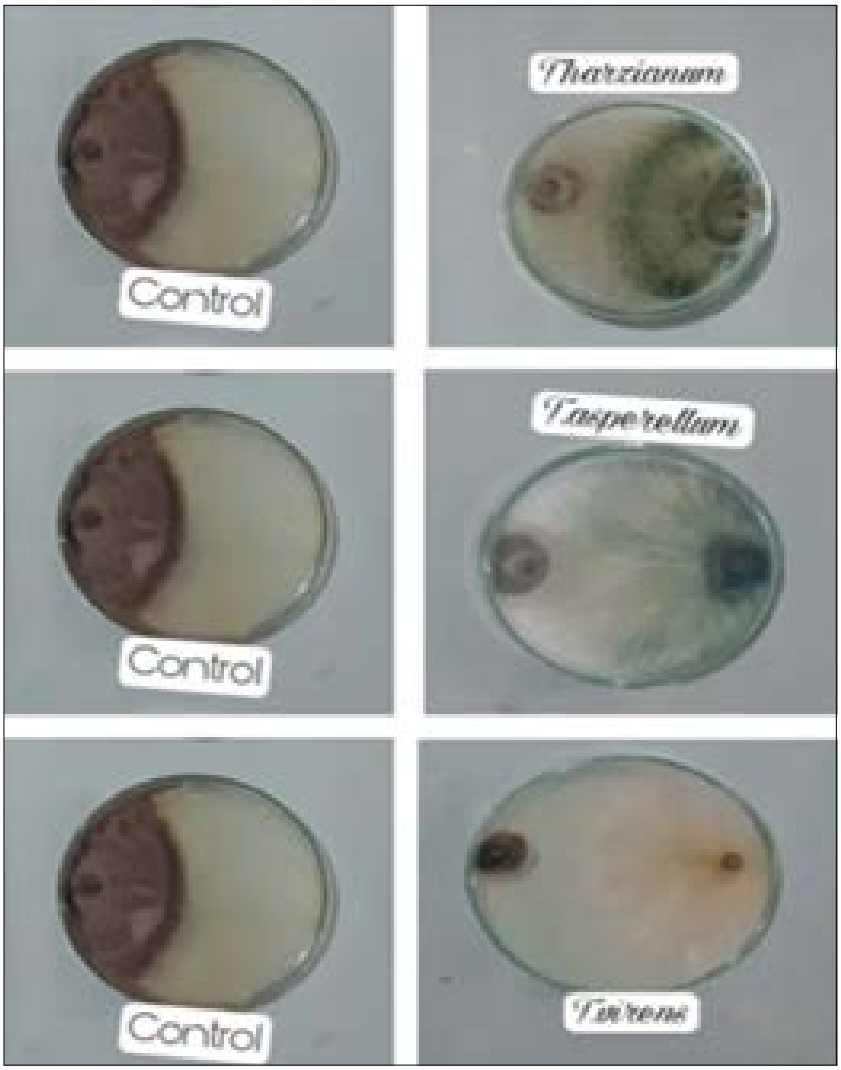

Plate 4: *In vitro* efficacy of bioagents a) *T. harzianum* b) *T. asperellum* c) *T. virens* against *A. tagetica*

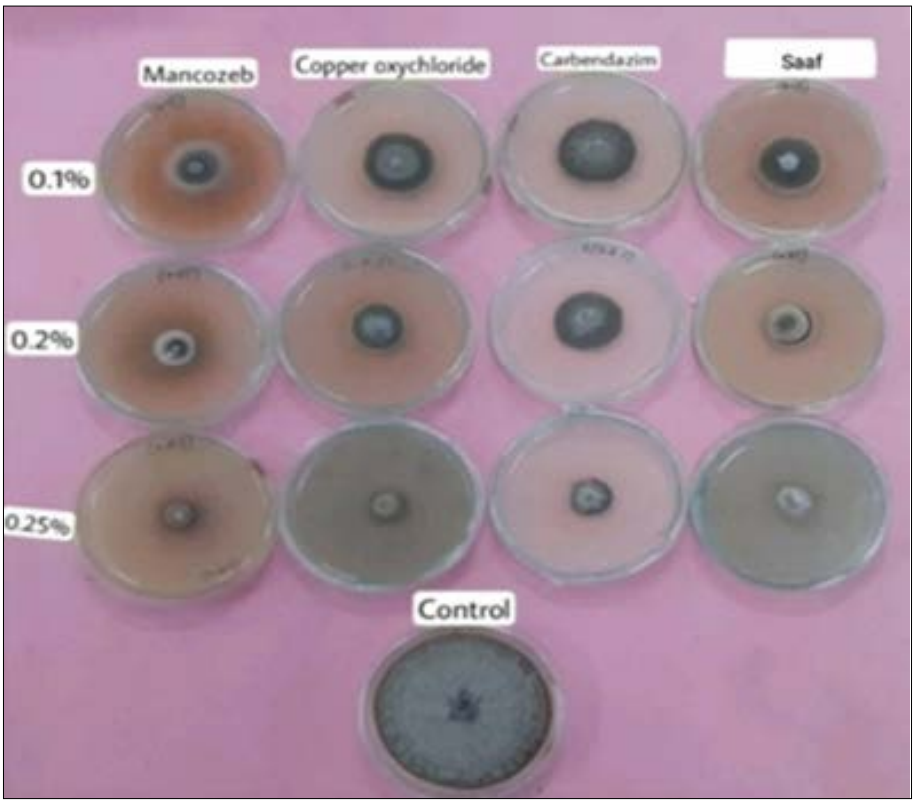

Plate 5: *In vitro* efficacy of fungicides against *A. tagetica*

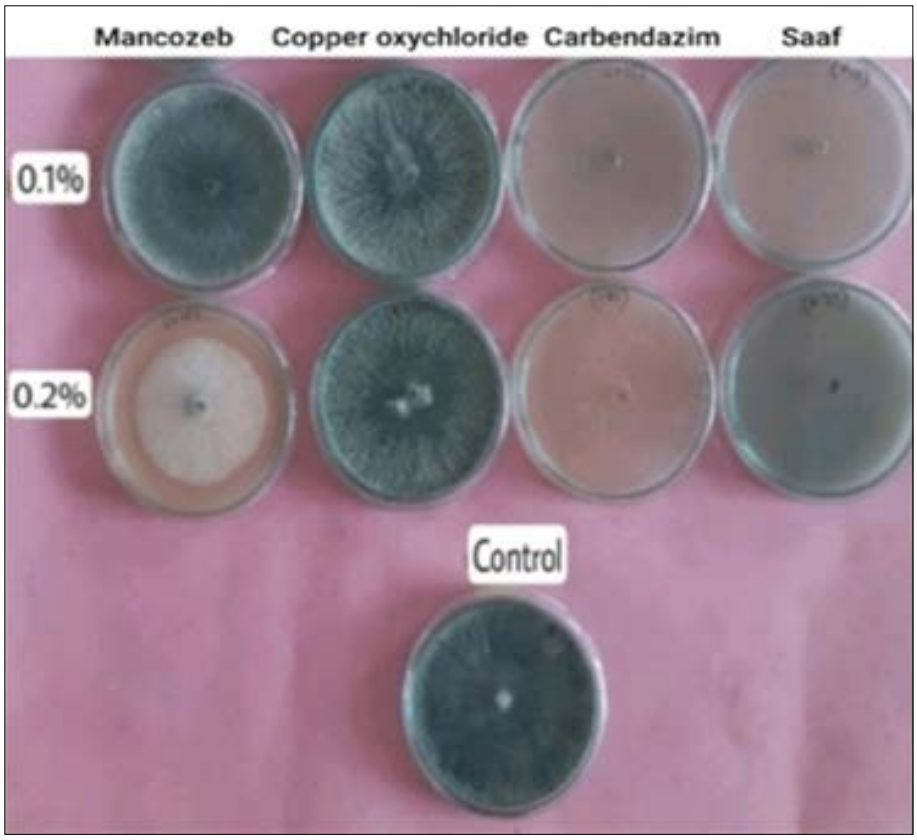

Plate 6: *In vitro* compatibility of T. harzianum with fungicides

Table 1: *In vitro* evaluation of botanicals against *A. tagetica*

Treatments	Conc (%)	Percent Inhibition (%)		
		168 hours	336 hours	Mean
T_0 (Control)		0(0)	0(0)	0(0)
T_1 (Turmeric)	10	62.74(52.38)	61.14 (51.44)	61.94 (51.91)
	20	58.88 (50.11)	58.14(49.68)	58.51(49.90)
T_2 (Garlic)	10	73.99 (59.39)	72.22 (58.19)	73.11 (58.76)
	20	78.73 (62.54)	77.77 (61.87)	78.25 (62.20)
T_3 (Parthenium)	10	54.59 (47.63)	53.33(46.91)	53.96 (47.27)
	20	61.26 (51.50)	60.74 (51.20)	60.99 (51.35)
T_4 (Neem)	10	32.96(35.03)	33.33(35.26)	33.145(35.14)
	20	43.33(41.17)	44.44 (41.81)	43.88 (41.49)
T_5 (Periwinkle)	10	42.96 (40.95)	42.96(40.95)	42.96 (40.95)
	20	54.44 (47.55)	54.44 (47.55)	54.44 (47.55)
T_6 (Bougainvillea)	10	42.59(40.74)	42.59(40.74)	42.59 (40.74)
	20	48.88 (44.36)	48.00 (44.36)	48.44(44.11)
T_7 (Korpad)	10	47.44 (43.30)	47.03(43.30)	47.23 (43.41)
	20	44.44(41.81)	44.44(41.81)	44.44(41.81)
T_8 (Ashoka)	10	20.00 (26.56)	20.00 (26.56)	20.00(26.56)
	20	30.00 (33.21)	30.00 (33.21)	30.00 (33.21)
S. Em (±)		0.43	0.15	
C.D (p =0.05)		1.24	0.44	

*Data in parentheses are arc sine transformed values
**Data are mean of three replications

Table 2: *In vitro* evaluation of bioagents against *A. tagetica*

Treatments	Percent Inhibition (%)		
	96 hours	168 hours	Mean
T_0 (Control)	0(0)	0(0)	0(0)
T_1 (*T. harzianum*)	29.75 (33.05)	63.88(53.06)	46.82(43.17)
T_2 (*T. asperellum*)	25.12 (30.08)	60.77(51.22)	42.95(40.94)
T_3 (*T. virens*)	28.04(31.97)	62.77(52.40)	45.41(42.36)
S Em (±)	0.28	0.15	
C.D (p =0.05)	0.86	0.45	

*Data in parentheses are arc sine transformed values
**Data are mean of three replications

Table 3: *In vitro* evaluation different fungicides on mycelial growth of *A. tagetica*

Treatments	Conc. (%)	Percent Inhibition (%)		
		168 h	336 h	Mean
T_0 (Control)		0(0)	0(0)	0(0)
T_1 (Mancozeb)	0.1	82.22(65.06)	83.14(65.76)	82.68(65.41)
	0.2	85.55(67.66)	84.25(66.62)	84.90(67.13)
	0.25	86.22(68.21)	81.14(64.60)	83.68(66.17)
T_2(Copper oxychloride)	0.1	68.88(56.10)	70.37(57.02)	69.62(56.55)
	0.2	75.70(60.45)	75.92(60.61)	75.81(60.54)
	0.25	77.40(61.62)	79.25(62.91)	78.33(62.22)
T_3 (Carbendazim)	0.1	51.62(45.93)	52.59(46.48)	52.10(46.21)
	0.2	60.37(50.98)	60.74(51.20)	60.55(51.10)
	0.25	78.73(62.54)	77.77(61.87)	78.25(62.20)
T_4 (Saaf)	0.1	72.22(58.19)	74.63(59.75)	73.42(58.97)
	0.2	80.15(63.54)	77.77(61.87)	78.96(62.70)
	0.25	81.85(64.78)	79.80(63.29)	80.82(64.03)
SEm (±)		0.10	1.03	
CD (p =0.05)		0.29	3.02	

*Data in parentheses are arc sine transformed values
**Data are mean of three replications

Table 4: *In vitro* evaluation of compatibility of *T. harzianum* with fungicides

Treatments	Conc (%)	Percent Inhibition (%)			C/NC
		96 h	168 h	Mean	
T_0 (Control)		0(0)	0(0)	0(0)	-
T_1 (Mancozeb)	0.1	0(0)	0(0)	0	C
	0.2	4.81(12.65)	6.28(14.50)	5.55(0.24)	C
T_2(Copper oxychloride)	0.1	0(0)	0(0)	0(0)	C
	0.2	0(0)	0(0)	0(0)	C
T_3 (Carbendazim)	0.1	100(90)	100(90)	100(90)	NC
	0.2	100(90)	100(90)	100(90)	NC
T_4 (Saaf)	0.1	100(90)	100(90)	100(90)	NC
	0.2	100(90)	100(90)	100(90)	NC
SEm (±)		0.17	0.15		
CD (p =0.05)		0.52	0.46		

*Data in parentheses are arc sine transformed values
**Data are mean of three replications

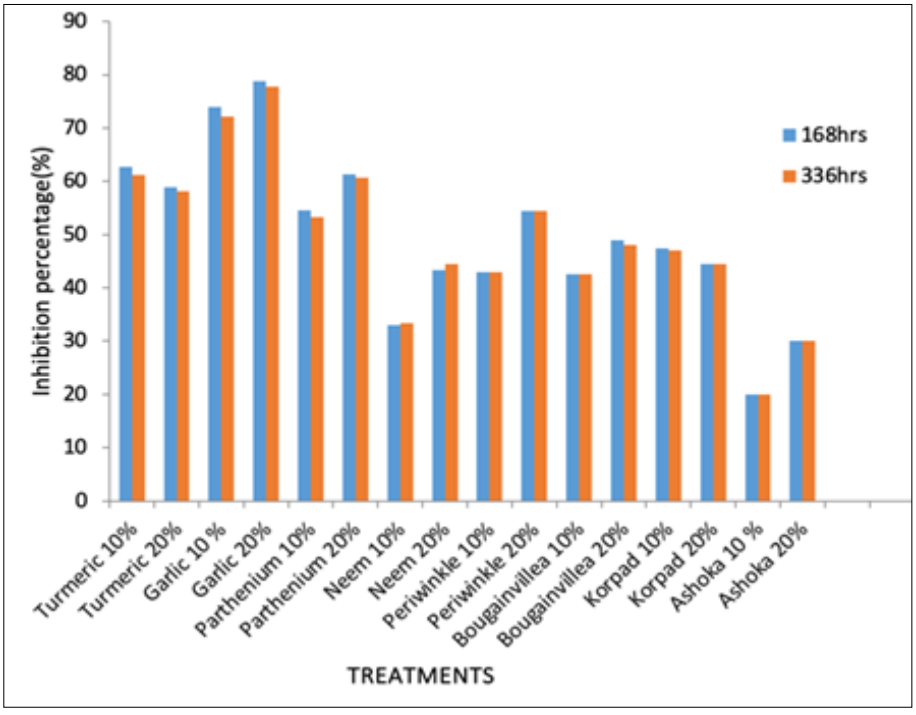

Fig 1: *In vitro* evaluation of botanicals against *A. tagetica*

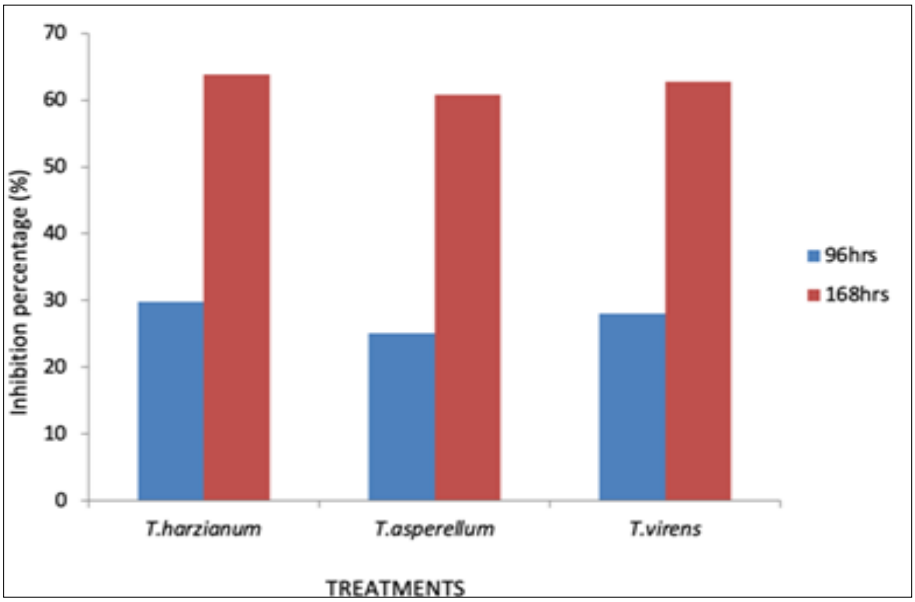

Fig 2: *In vitro* evaluation of bioagents against *A. tagetica*

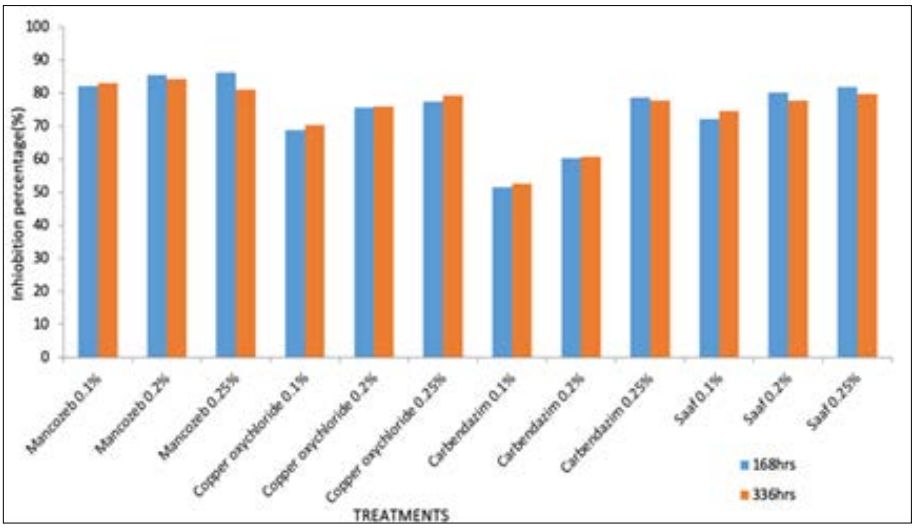

Fig 3: *In vitro* evaluation of chemicals against *A. tagetica*

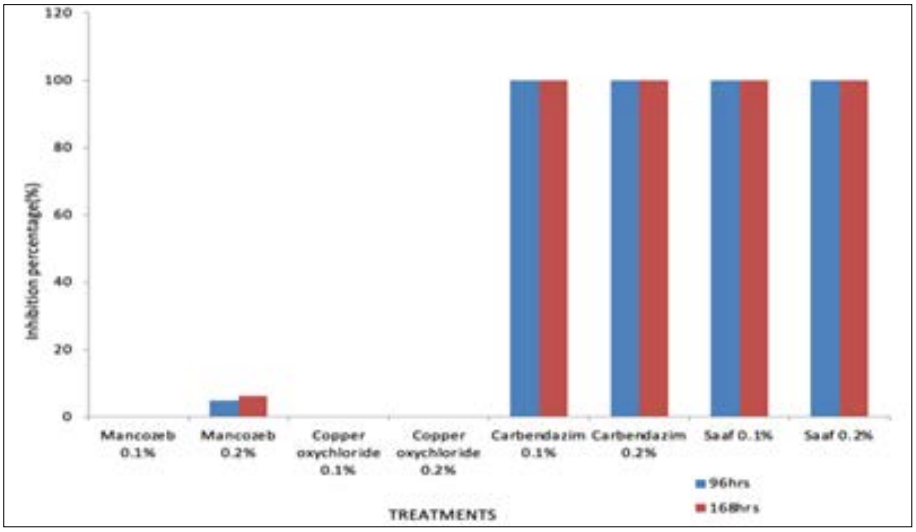

Fig 4: *In vitro* evaluation of compatibility of *T. harzianum* with fungicides

Conclusion

The results from the present investigation can be concluded, by integrating fungicides, botanical extracts and bioagents against the leaf spots and flower blight of marigold caused by *Alternaria tagetica* can be effectively managed and reduced. The present studies also conclude that it is possible

to incorporate certain fungicides with some bio control agents. Combination of fungicides with bioagents is necessary to avoid resistance against chemicals that could develop by certain pathogens due to continuous and repeated applications of chemicals. However, in future, further studies and research may be required in field to give a clear-cut recommendation about the dose and time of application in the integrated management strategy on *Alternaria* leaf spot and flower blight of marigold.

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