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In vitro efficacy of bio-agents and fungicides against *Alternaria alternata*

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Abstract
Present study revealed that all six bio-agents evaluated and exhibited antifungal activity against test pathogen (*Alternaria alternata*) and significantly inhibited mycelial growth over untreated control. Out of six bio-agents, *Trichoderma viride* showed least mycelial growth of test pathogen (15.70mm) and highest mycelial growth inhibition (77.57%). *T. harzianum* had 31.20 mm of mycelial growth and a growth inhibition of 55.42 %, which was at par to *T. hamatum*, which had 31.80 mm of mycelial growth and a growth inhibition of 54.57 %. The bioagent *Pseudomonas fluorescence* was shown to be the least efficient, with 54.30 mm of mycelial development and a 22.42 % inhibition of the test pathogen. Nine different fungicides at different concentration were evaluated *in vitro* by poisoned food technique. Among these nine fungicides cent per cent growth inhibition of *A. alternata* was recorded in Propiconazole 25% EC @ 0.1 % concentration tested. The next best in order of efficacy was Azoxystrobin 11% + Tebuconazole 18.3% w/w SC @ 0.1% followed by Difinoconazole 25% EC @ 0.05% and Carbendazim 12% + Mancozeb 63% WP @ 0.25 % which reported 88.12%, 84.03% and 80.00% growth inhibition respectively. Tebuconazole + Trifluoxystrobin 25% WG @ 0.06 % showed 77.77% inhibition which was at par with Tebuconazole 25.9% EC @ 0.1 % which was reported 76.03% growth inhibition. The fungicides viz., Propineb 75% WP @ 0.25%, Mancozeb 75% WP @ 0.25% were resulted in 74.08%, and 70.03% inhibition of *A. alternata* respectively. While, Thiophanate methyl 70% WP @ 0.05% was observed to be least effective in per cent growth inhibition of *A. alternata* as compared to other fungicides at their respective concentrations tried *in vitro*.

Keywords: Marigold, Bio-control agents, *Alternaria alternata*, *Trichoderma*, bioagents, Fungicides

Introduction
Marigold is a seasonal flower and can be grown round the year. Marigold flowers gained popularity amongst gardeners and dealers on its easy cultivation and wide adaptability. Marigold as a cut flower and loose flower is extensively used in the social and religious function for internal decoration and garlands. The leaves and flowers are known to possess high phenolic and antioxidant properties can be exploited in pharmaceutical industry Marigold is used as the trap crop in the borders to attract the insects attacking the main crop (Kolambkar *et al.*, 2013) [5]. *Tagetes erecta* and *Tagetes patula* belong to Asteraceae (Compositae) family and it is native to North and South America, but some species now become naturalized around the world. *Tagetes erecta* are the tallest, at three to five feet. Flowers are golden yellow, orange or cream coloured. They are sometimes known as American or African marigolds. *Tagetes patula* is bushy, somewhat smaller plant as compare to *T. erecta* and known as French marigold.

Many diseases affect the crop, including leaf spots and blight (*Altenaria, Cercospora*, and *Septoria sp*.), powdery mildew (*Oidium sp., Levelula taurica*), flower bud rot (*Alternaria dianthi)*, and damping off (*Pythium sp*.). *Alternania alternata* caused leaf spot and flower blight is a serious disease of marigolds, particularly African marigolds (*Tagetes erecta*). The disease is distinguished by circular dark-brown necrotic spots on the leaves, stem, and flowers. The petals and peduncle of the infected flower are discoloured in a distinctive brown, scorched, necrotic pattern. According to estimates, the disease caused a 50-60% reduction in flower yield (Cotty *et al*., 1983) [3]. These diseased flowers are unmarketable, and they cannot even be used to extract Xanthophylls, fatty acids, easters, and oils, which are used in the dye and perfume industries.

Marigold leaf spot and inflorescence blight is a severe disease that occurs in high humidity and rainfall conditions. This disease is also prevalent in marigold-growing areas around Pune. Given the severity of the disease and the economic importance of the crop in the Pune area, the current investigations were undertaken to study the disease's behaviour and to generate

necessary information for suitable management measures to minimise crop losses.

Materials and Methods

Present investigations were carried out in the Department of Plant Pathology and Agricultural microbiology, College of Agriculture, Pune-05.

In vitro* evaluation of bio-agents against *Alternaria alternata

Collection of antagonistic micro-organisms

The potential antagonistic activity of bio-agents *viz. Trichoderma viride, Trichoderma harzianum, Trichoderma hamatum, Trichoderma koningii, Bacillus subtilus and Pseudomonas flurescence* were collected from Biological Nitrogen Fixation Scheme, College of Agriculture, Pune-05.

Maintenance of culture

The antagonistic fungal microorganisms were grown on PDA slants stored at 6 °C in refrigerator and sub-culturing was done consequently at an interval of thirty days in order to retain virulence of the fungal bio- agents for their further study.

***In vitro* evaluation of bio-agents**

The effectiveness of antagonists against the pathogen was assessed by means of dual culture technique (Dennis and Webster, 1971) [4] on PDA medium.

Dual culture technique

About 20 milliliter of Potato dextrose agar medium was added into sterile Petri plate and allowed to solidify. The 10 days old fungal culture was taken and cut into five millimeter circular disc by means of sterile cork borer and kept nearby the periphery, on one side of PDA plate. Likewise antagonistic circular disc was kept on other side. A plate with pathogen only without antagonist served as control. The incubation was done at 27±1 ºC for time period of 7 days. Each treatment was replicated four times. After the period of incubation, when the growth in the control plate reached maximum (90 mm diameter), the radial growth of the pathogen was measured and per cent inhibition over control was found out as per equation suggested by Vincent (1947) [9].

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

Where,

I = Per cent inhibition of fungal growth.

C = Growth/colony diameter of the pathogen in control plate (mm).

T = Growth /colony diameter of the pathogen in dual culture plate (mm).

In vitro* evaluation of fungicides against *Alternaria alternata

The fungicides utilized in the present assessment along with particulars of trade name, ingredient of the chemical in formulation and source of supply were presented in Table 1. The efficacy of all following fungicides was assayed by using poisoned food technique on PDA as basal medium.

Poisoned food technique

The required quantity of specific fungicide was added separately into molten and cooled potato dextrose agar in order to get the desired concentration of fungicides. After that, 20 milliliter of the poisoned medium was added into sterile petri plates. Mycelial circular discs of 5 millimeter size from vigorously growing culture of the fungus were cut out by means of sterile cork borer and one circular disc was kept at the centre of each agar plate. Without adding any fungicides to the medium, Control plate was maintained. Each treatment was replicated thrice. The incubation was done at 27±1 ºC temperature for time period of 8 days and radial fungal colony growth was determined. The effectiveness of a fungicide was assessed by calculating per cent inhibition of mycelial growth over control by utilizing the formula suggested by Vincent (1947) [9].

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

Where,

I = Per cent inhibition of fungal growth

C = Growth/colony diameter of the pathogen in control plate (mm)

T = Growth/colony diameter of the pathogen in treatment plate (mm)

Table 1: Particulars of fungicides used in the investigation/study.

Sr. No.	Common Name	Trade Name	Active Ingredient	Conc(%)	Manufacturer
1.	Propiconazole	Tilt	25% EC	0.1	Syngenta Ltd, Mumbai
2.	Tebuconazole + Trifloxystrobin	Nativo	50 + 25% WG	0.06	Bayer India L.td, Mumbai
3.	Tebuconazole	Orius	25.9 % EC	0.1	Adama India Pvt Ltd
4.	Difenoconazole	Score	25 % EC	0.05	Syngenta Indian Ltd, Mumbai
5.	Mancozeb	Dithane M-45	75 % WP	0.25	Indofil Chemical Co. Ltd, Mumbai
6.	Thiophanate methyl	Topsin-M	70 % WP	0.05	Rocco Ltd, Mumbai
7.	Propineb	Antracol	70 % WP	0.25	Bayer India Ltd, Mumbai
8.	Carbendazim + Mancozeb	Saaf	12 + 63 % WP	0.25	UPL Ltd
9.	Azoxystrobin+ Tebuconazole	Custodia	11%+18.3% SC	0.1	Adama India Pvt Ltd

Results and discussion

In vitro* evaluation of bio-agents against *Alternaria alternata

A total six bio-agents which includes four fungal antagonistic *viz. T. viride, T. harzianum, T. koningii* and *T. hamatum and* two bacterial *Bacillus subtilis and Pseudomonas fluorescens* bioagents/antagonists were evaluated *in-vitro* for their bio-efficacy against *Alternaria alternata* by applying dual culture technique and using PDA as basal medium. The result obtained on mycelial growth and per cent growth inhibition of test pathogen with bio-agent are presented in Table 2, Fig 1 & 2 and PLATE 1.

Mycelial Growth Inhibition of *Alternaria alternata*

All six bio-agents evaluated and exhibited antifungal activity against test pathogen (*Alternaria alternata*) and significantly

inhibited mycelial growth over untreated control. Out of six bio-agents, *T. viride* was shown to be the most effective of the six bioagents examined, with the least linear mycelial growth (15.70 mm) and the highest mycelial growth inhibition (77.57 %) of the test pathogen compared to the untreated control (70.00 mm and 0.00 percent, respectively). *T. harzianum* had 31.20 mm of mycelial growth and a growth inhibition of 55.42 %, which was at par to *T. hamatum*, which had 31.80 mm of mycelial growth and a growth inhibition of 54.57 %. The bioagent *Pseudomonas fluorescence* was shown to be the least efficient, with 54.30 mm of mycelial development and a 22.42 % inhibition of the test pathogen. (Table 2, Fig 1 & 2 and PLATE 1).

The findings of this study are consistent with those of several other researchers, such as Kumar (2008) [6] and Balai *et al.* (2011) [2], while Pareek *et al.* (2012) [7] showed *T. harzianum* to be the most effective antagonist against *A. alternata*, followed by *T. viride* and *A. niger*.

Table 2: *In vitro* efficacy of bio-agents against mycelial growth inhibition of *A. alternata*

Treatment No.	Bioagents	Av. colony diameter* of test pathogen (mm)	Per cent growth inhibition
T1	*Trichoderma viride*	15.70	77.57 (61.73)
T2	*Trichoderma harzianum*	31.20	55.42 (48.11)
T3	*Trichoderma koningii*	35.00	50.00 (45.00)
T4	*Trichoderma hamatum*	31.80	54.57 (47.62)
T5	*Bacillus subtilis*	48.10	31.28 (34.01)
T6	*Pseudomonas fluorescens*	54.30	22.42 (28.26)
T7	Control	70.00	-
	S. E (m) ±	0.47	-
	C. D at 1%	1.97	-

* Mean of four replications.

Note: 1) Numbers in parenthesis indicate arcsine transformed values.

Plate 1: Growth inhibition of *Alternaria alternata*by different bio-agents

Bioagents

1. *Trichoderma viride*
2. *Trichoderma harzianum*
3. *Trichoderma koningii*
4. *Trichoderma hamatum*
5. *Bacillus subtilis*
6. *Pseudomonas fluorescence*
7. control

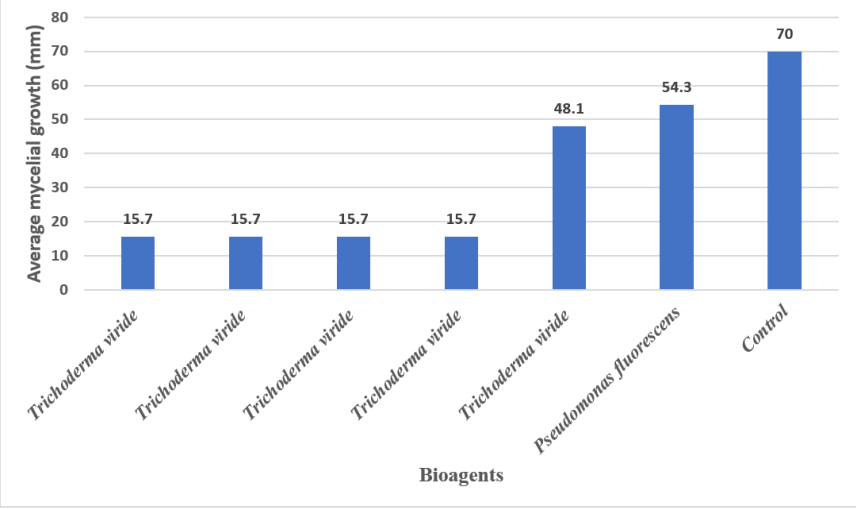

Fig 1: *In vitro* effect of different bio-agents on mycelial growth of *Alternaria alternata*

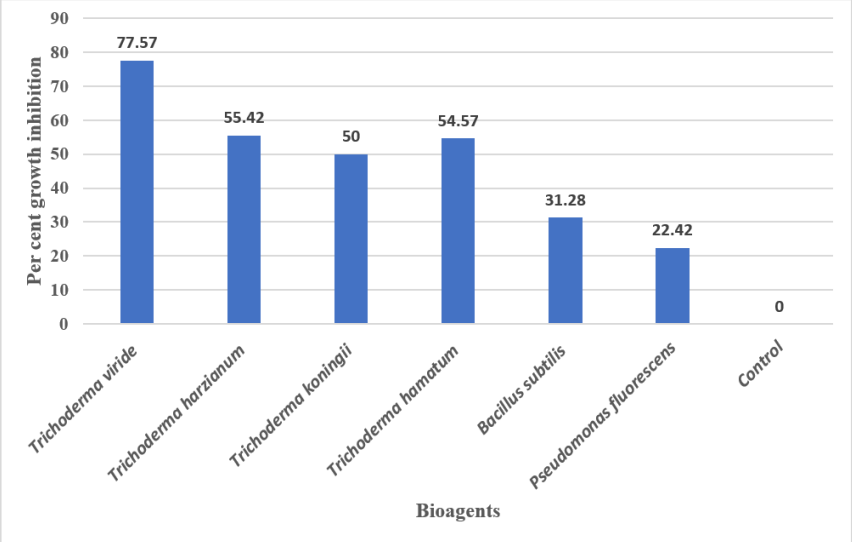

Fig 2: Per cent growth inhibition of *Alternaria alternata* by different bioagents

In vitro* evaluation of fungicides against *Alternaria alternata

Nine fungicides namely, Contact Fungicides- Mancozeb 75%WP @ 0.25%, Propineb 70%WP @ 0.3%, Contact and Systemic Fungicides- Carbendazim 12%+Mancozeb 63%WP @ 0.25% Systemic Fungicides- Tebuconazole 25.9%EC @ 0.1%, Difenoconazole 25%EC @ 0.05%, Propiconazole 25 % EC @ 0.1%, Azoxystrobin 11% + Tebuconazole 18.3%SC @ 0.1%, Tebuconazole 50% + Trifloxystrobin 25%WG @ 0.06% and Thiophanate methyl 70% WP @ 0.05 were evaluated against *Alternaria alternata* by poison food technique and per cent inhibition in mycelial growth was observed.

The result presented in Table-5, Plate-VIII and depicted in Fig- 4 and 5. The efficacy of different fungicides, concentrations and their interaction on per cent inhibition of mycelial growth of *A. alternata* differed significantly and the inhibition of the growth of the fungus ranged from 38.03 to 100.00 per cent.

Nine different fungicides at different concentration were evaluated *in vitro* by poisoned food technique. Among these nine fungicides cent per cent growth inhibition of *A. alternata* was recorded in Propiconazole 25% EC @ 0.1 % concentration tested. The next best in order of efficacy was Azoxystrobin 11% + Tebuconazole 18.3% w/w SC @ 0.1% followed by Difinoconazole 25% EC @ 0.05% and Carbendazim 12% + Mancozeb 63% WP @ 0.25 % which reported 88.12%, 84.03% and 80.00% growth inhibition respectively. Tebuconazole + Trifluoxystrobin 25% WG @ 0.06 % showed 77.77% inhibition which was at par with Tebuconazole 25.9% EC @ 0.1 % which was reported 76.03% growth inhibition. The fungicides *viz*., Propineb 70% WP @ 0.25%, Mancozeb 75% WP @ 0.25% were resulted in 74.08%, and 70.03% inhibition of *A. alternata* respectively. While, Thiophanate methyl 70% WP @ 0.05% was observed to be least effective in per cent growth inhibition of *A. alternata* as compared to other fungicides at their respective concentrations tried *in vitro*.

Propiconazole at 250, 500, and 1000 ppm, as well as Difenconazole at 500 and 1000 ppm, completely inhibited the mycelial development and spore generation of *A. alternata* isolated from marigold, according to Arunkumar (2006) [1]. Shindhe *et al*. (2018) [8] also shown that Propiconazole was the most effective fungicide, with the most reduction in fungus mycelial development (100%) in all concentrations, outperforming all other fungicides. Thiophanate methyl, according to Pareek *et al*. (2012) [7], was the least effective against mycelial development and sporulation. The current findings were consistent with those reported by previous researchers.

Table 5: Bio-efficacy of various fungicides against *A. alternata in vitro*

Sr. No.	Fungicides	Conc (%)	Average colony diameter ⁕(mm)	Per cent inhibition over control
1	Propiconazole 25% EC	0.1	0.00	100 (90.00)
2	Tebuconazole 50% + Trifloxystrobin 25% WG	0.06	17.77	77.77 (61.87)
3	Tebuconazole 25.9% EC	0.1	19.17	76.03 (60.69)
4	Difenoconazole 25% EC	0.05	12.50	84.03 (66.45)
5	Mancozeb 75% WP	0.25	23.97	70.03 (56.81)
6	Thiophanate methyl 70% WP	0.05	49.30	38.03 (38.07)
7	Propineb 70% WP	0.3	20.73	74.08 (59.39)
8	Carbendazim 12% + Mancozeb 63% WP	0.25	16.00	80.00 (63.43)
9	Azoxystrobin 11% + Tebuconazole 18.3% w/w SC	0.25	9.50	88.12 (69.84)
10	Control	-	80.00	00
	S.E (m) ±	-	0.34	-
	C. D at 1%	-	1.38	-

⁕ Mean of three replication

Note: Figures in parenthesis indicate arc sin transformed values

Plate II: Bio-efficacy of various fungicides against *A. alternata*

Treatments

1. Propiconazole 25% EC @ 0.1%
2. Tebuconazole50% + Trifloxystrobin 25% WG @ 0.06%
3. Tebuconazole 25.9%EC @ 0.1%
4. Difenoconazole 25% EC @ 0.05%
5. Mancozeb 75% WP @ 0.25 %
6. Thiophanate methyl 70% WP @ 0.05 %
7. Propineb 70 WP @ 0.3 %
8. Carbendazim 12% + Mancozeb 63% WP @ 0.25 %
9. Azoxystrobin 11% + Tebuconazole 18.3% w/w SC @ 0.1%
10. Control

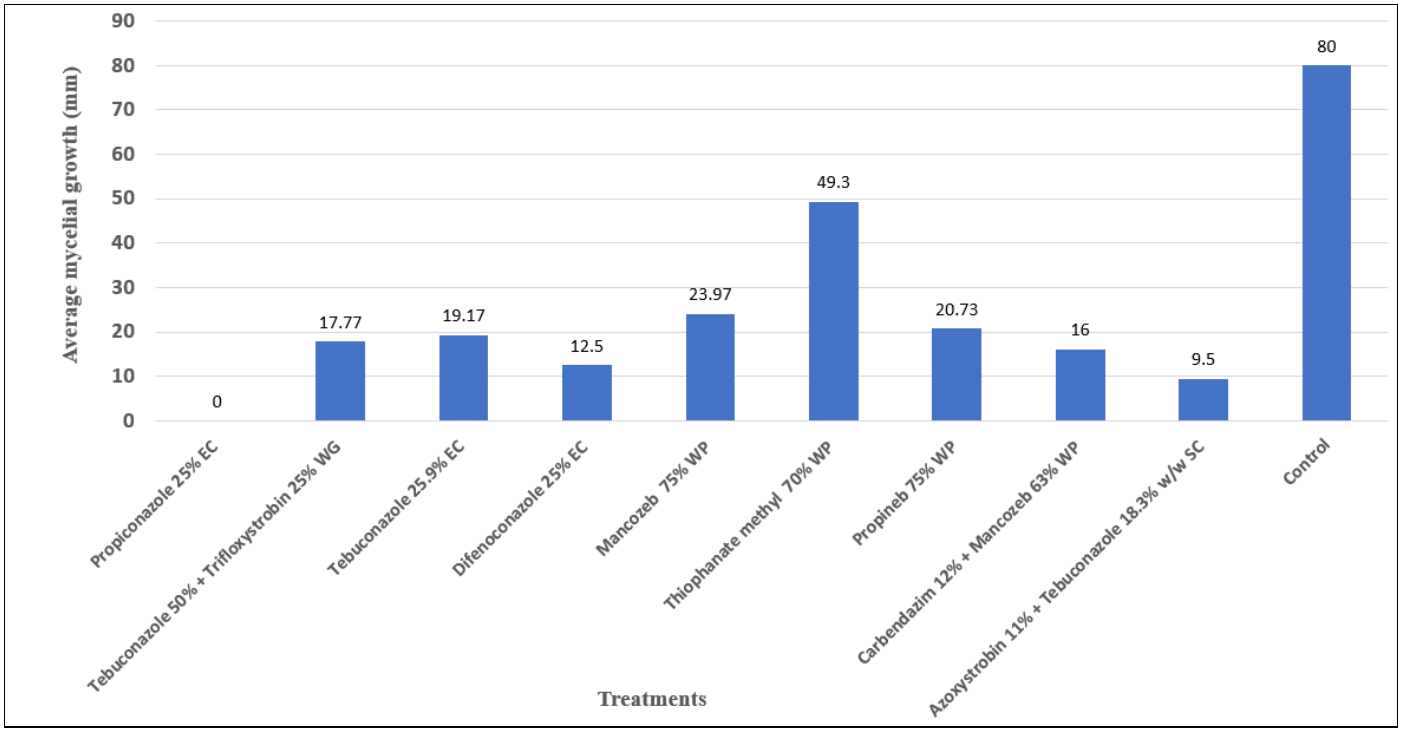

Fig 2: Effect of different fungicides on mycelial growth of *Alternaria alternata in vitro*

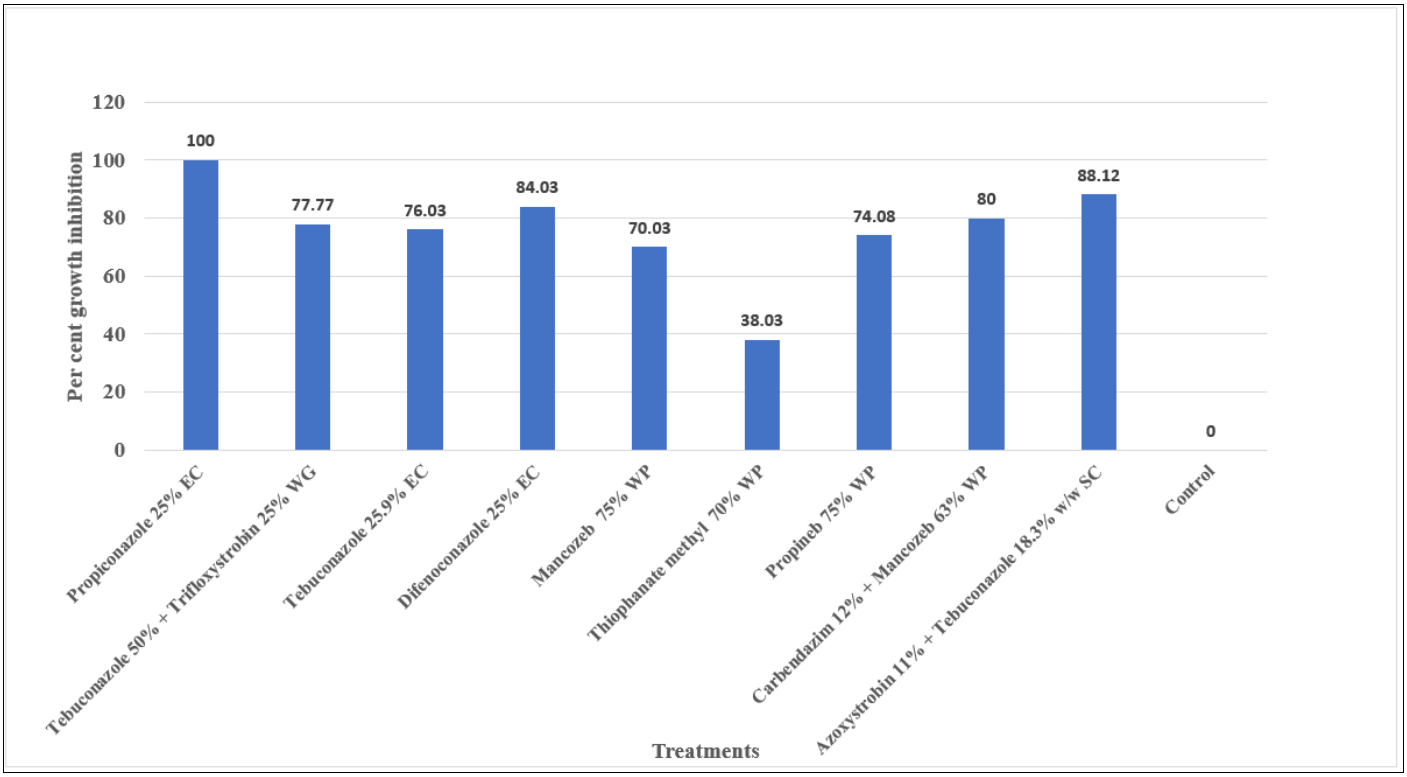

Fig 2: Effect of different fungicides on mycelial growth inhibition *of Alternaria alternata in vitro*

Conclusion

The fungicides *viz*., Propiconazole 25% EC % 0.1%, Azoxystrobin 11% + Tebuconazole 18.3% w/w SC @ 0.1% followed by Difinaconazole 25% EC @ 0.05% and bio-agent *Trichoderma viride*, *Trichoderma harzianum* were found effective in *in-vitro* studies against *Alternaria alternata.*

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