The Pharma Innovation Journal 2023; 12(2): 25-29

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277-7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2023; 12(2): 25-29

www.thepharmajournal.com
Received: 04-12-2022
Accepted: 11-01-2023

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Antifungal efficacy of fungicides, botanicals and bioagents against *Alternaria* leaf blight of chrysanthemum

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DOI: https://doi.org/10.22271/tpi.2023.v12.i2a.18600

Abstract
The present investigation was conducted to test the *in vitro* efficacy of fungicides, botanicals and bio-agents against *Alternaria alternata* causing leaf blight disease of chrysanthemum. Seven fungicides evaluated under *in-vitro* condition showed that Hexaconazole, Difenconazole and Azoxystrobin + Difenconazole at all concentrations *viz.*,0.1, 0.2 and 0.3% completely inhibited fungal mycelial growth followed by Tebuconazole + Trifloxystrobin at 0.3% concentration. Among the botanicals Garlic clove extract @ 10% was found most effective with 83.67% fungal mycelial inhibition against *Alternaria alternata*. Bioagent tested by dual culture technique revealed that *Trichoderma harzianum* and *Trichoderma asperellum* was the best antagonistic against *Alternaria alternata* by inhibiting mycelial growth of *A. alternata* 79.25% and 77.15% respectively.

Keywords: Chrysanthemum, *Alternaria alternata*, fungicides, botanicals, bio-agent

Introduction
Chrysanthemum (*Dendranthema grandiflora*) belongs to the family Asteraceae. Chrysanthemum word derived from Greek word (Chryos = golden and anthus = flower). It is beautiful and oldest flowering plant commercially grown in different parts of the world. Chrysanthemum is also known as Queen of East, Autumn Queen and Guldaudi. It occupies the key position among commercial flower crops which has high demand in both domestic and international markets. It is grown for its aesthetic and commercial value. It is important both as cut flower and as potted plant. Chrysanthemum governs a key position in the floriculture industry and it's the world's second most important floricultural crop after rose (Kalia, 2015) [6].

The major chrysanthemum growing states in India are Karnataka, Tamil Nadu, Maharashtra, Rajasthan, Madhya Pradesh and Bihar. Maharashtra is one of the leading state of the country in flower production. Total area under floriculture in Maharashtra was found to be 11.45 thousand ha with the production of 59.93 thousand Mt of loose flowers and 0.03 thousand Mt of cut flowers. In Maharashtra chrysanthemum is grown on an area of 0.39 thousand ha with the production of 1.65 thousand tonne loose flowers and 0.05 thousand tonnes cut flowers (Anonymous 2018) [1]. In Maharashtra, the leading districts in floriculture production are Ahmednagar, Nashik, Thane, Pune, Satara, Sangali, and Nagpur. However, Ahmednagar district is specialized as growing district of the Maharashtra (Tupe *et al.*, 2017) [13].

Material and Methods
Collection and isolation of pathogen
The leaves of chrysanthemum exhibiting typical symptoms of chrysanthemum blight disease were collected in paper bags and brought to the laboratory for further investigation. Standard tissue isolation procedure was used to isolate the pathogen. The infected plant parts were cut into smaller bits along with healthy portion with the help of sterile blade. The infected small bits were surface sterilized with 0.1% sodium hypochloride solution for 2 min. followed by washing 3-4 times in double distilled water to remove the traces of sodium hypochloride. Then sample bits were dried on sterile blotter paper. Then they were transferred aspetically into sterile petridish containing potato dextrose agar medium.
Inoculated petri plates were incubated at of 27±1 °C for 7 days. The plates were examined regularly. The fungus colonies growing around the each bit were examined and sub cultured. Based on colony character, morphological characters (types of conidia) and published literature the fungi were identified as *Alternaria alternata.*

The pure culture was transferred on PDA slants and maintained for further studies.

***In vitro* evaluation of fungicides by poison food method**

Antifungal efficacy of seven fungicides *viz*., carbendazim + mancozeb, haxaconazole, tebuconazole + trifloxystrobin, pyraclostrobin, difenconazole, copper oxychloride and azoxystrobin + difenconazole carried out against mycelial growth of *A. alternata*. Experiment was conducted by poisoned food technique (Nene and Thapliyal., 1982) [8]. Three different concentrations were evaluated at 0.1, 0.2 and 0.3% of each fungicides. Required quantity of each fungicide was added separately to sterilized molten medium, mixed thoroughly and poured in sterilized glass Petriplates and allowed to solidify. Three replications were maintained for each treatment. Control was maintained without adding any fungicides to the medium. Each plate was inoculated with 5 mm discs with the help of sterilized cork borer from the edge of the growing fungal culture and incubated at 27±1ºC for 7 days. Colony diameter was recorded in mm and percent mycelial growth inhibition was calculated as per Vincent's (1947) [14] formula.

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

Where,
PI = Percent Inhibition
C = Growth of fungi in control (mm)
T = Growth of fungi in treatment (mm)

***In vitro* evaluation of botanicals by poison food method**

Botanical extracts of Garlic, Neem, Onion, Tulsi and Turmeric were evaluated against *A. alternata*. Aqueous botanicals were prepared by grinding with mortar and pestle. 100 g washed plant part of each plant species in 100 ml distilled water are grinded and filtered through double layered muslin cloth. Each of the filtrate obtained was further filtered separately through Whatman No. 1 filter paper. The final clear extracts obtained formed the standard leaf extracts of 100% concentration and these botanicals were evaluated at 10% concentration against *A. alternata* by applying Poisoned food technique. After an expiry of seven days incubation period the mycelial inhibition was calculated as per formula mentioned in the *In vitro* evaluation of fungicides by poisoned food method.

***In vitro* evaluation of bio-agents by dual culture method**

The lawn culture of test fungi and bio-agents i.e. *Trichoderma asperellum* and *Trichoderma harzianum* were prepared. Twenty ml of sterilized PDA medium was poured aseptically into sterile petriplate and allowed for solidification. The plates were inoculated with the culture of test fungi and bioagents after solidification of media and then plates were incubated at room temperature for seven days.

Bacterial bio-agents, *Bacillus subtilis* and *Psudomonas fluorescence* were prepared by inoculating a loopful culture in sterilized conical flask containing hundred ml of nutrient broth. Broth culture was incubated at room temperature for three days. Ten days old culture of test pathogen was taken and cut into 5 mm disc by using sterile cork borer and placed near the periphery, on one side of PDA plate. Similarly antagonistic fungus disc was placed on other side. For bacterial antagonists test fungus was placed on the center of the plate and the bacterial antagonists' i.e. *Bacillus* and *Pseudomonas* were streaked on both the sides of the test fungus. A plate with pathogen alone without antagonist served as control. All these plates were incubated at room temperature for seven days. After an expiry of seven days incubation period the mycelial inhibition was calculated as per formula mentioned in the *In vitro* evaluation of fungicides by poisoned food method.

Results and Discussion

In vitro* evaluation of fungicides against *Alternaria alternata

The efficacy of seven fungicides was tested *in vitro* at three concentrations *viz*., 0.1, 0.2 and 0.3% against *Alternaria alternata* on PDA medium by poison food technique. Data presented in Table 1 revealed that the fungicides influenced the radial mycelial growth of *A. alternata* greatly and it was found to be decreased steadily with increase in concentrations of the test fungicides. Hexaconazole 05% EC, Difenconazole 25% EC and Azoxystrobin (18.2%) + Difenconazole (11.4%) WW SC at all three test concentrations i.e 0.1%, 0.2% and 0.3% showed complete inhibition of mycelial growth (100%) of the test pathogen followed by Tebuconazole (50%) + Trifloxystrobin (25%) WG (78.78, 85.40 and 100%), Copper Oxychloride 50% WP (72.47, 78.12 and 82.00) and Carbendaizm 12% + Mancozeb 63% WP (66.40, 73.97 and 78.68). Pyraclostrobin 20% WG proved least effective amongst all the tested fungicides recording 34.43, 28.77 and 23.78 mm growth of *Alternaria alternata* thereby inhibiting the growth by 60.87, 67.30 and 72.97% respectively at 0.1, 0.2 and 0.3% concentration.

The present findings are in agreement with Phapale *et al*., (2010) [10], Thejakumar and Devappa (2016) [12], and Ghule *et al*., (2021) [4] who reported that hexaconazole and difenconazole was most effective fungicides against *Alternaria alternata*. Ginoya and Ghohel (2015) [5] also found that hexaconazole, difenconazole and azoxystrobin + difenconazole was the most effective fungicide in controlling *A. alternata* by 100% in seven day after inoculation. Arunkumar and Kamanna (2009) [2] observed fungicidal activity of nine fungicides against *A. alternata* and within that hexaconazole (5 EC) at 0.1, 0.2 and 0.3% concentrations recorded cent per cent (100%) inhibition of the mycelial growth of *Alternaria alternata*.

The results presented in the (Table 2.) indicated that all the tested botanicals showed significant differences compared with control. Among the plant extracts, Garlic clove extract @ 10% recorded maximum inhibition 83.67% of mycelial growth of test fungus and was significantly superior to rest of the treatments. This was followed by turmeric rhizome extract and onion bulb extract recorded 71.50% and 60.44% mycelial growth inhibition of *A. alternata* respectively. Rest of the plant extract *viz*. tulsi leaf extract and neem leaf extract recorded 49.16% and 46.08% mycelial growth inhibition which was least effective against test pathogen.

These results are almost similar to those of Pareek *et al*., (2012) [9] observed the mycelial inhibition of *Alternaria alternata* due to Garlic clove extract by 82.0% in cucumber. Wagh *et al*., (2017) [15] founded 88.33% growth reduction of *Alternaria carthami* with garlic clove extract. Similarly Zade *et al*., (2018) [17] reported highest inhibition of *A. alternata* by clove extract of Garlic (87.50%). Sharma *et al*., (2021) [11] who noticed that clove extracts from Garlic was the most effective against *A. alternata*. The fungicidal activity of plant extracts in the present study might be due to antifungal metabolites present in different plant species.

Table 1: Effect of fungicides on radial mycelial growth of *Alternaria alternate*

Tr. No	Fungicides	Radial growth (mm)*at concentration			% Inhibition at concentration		
		0.1%	0.2%	0.3%	0.1%	0.2%	0.3%
T1	Carbendaizm 12% + Mancozeb 63% WP	29.56	22.90	18.76	66.40	73.97	78.68
T2	Hexaconazole 05% EC	0	0	0	100	100	100
T3	Tebuconazole 50% + Trifloxystrobin 25% WG	18.67	12.84	0	78.78	85.40	100
T4	Pyraclostrobin 20% WG	34.43	28.77	23.78	60.87	67.30	72.97
T5	Difenconazole 25% EC	0	0	0	100	100	100
T6	Copper Oxychloride 50% WP	24.22	19.25	15.84	72.47	78.12	82.00
T7	Azoxystrobin 18.2%WW + Difenoconazole 11.4% WW SC	0	0	0	100	100	100
T8	Control	88.00	88.00	88.00	0	0	0
	F test	Sig.	Sig.	Sig.			
	S.E(m)±	0.30	0.28	0.25			
	CD (P= 0.01)	1.22	1.17	1.05			

In vitro* evaluation of botanicals against *Alternaria alternata

Five plant extracts were studied for their antifungal activity against *Alternaria alternata.* (Table 2)

Table 2: Efficacy of botanicals against *Alternaria alternata* by poisoned food technique

Sr. No.	Botanicals	Plant part used	Conc. used	Mean radial mycelial growth (mm)	Per cent mycelial inhibition (%)
1	*Allium sativum*	Clove	10%	14.69	83.67
2	*Azadirachta indica*	Leaves	10%	48.52	46.08
3	*Allium cepa*	Bulb	10%	35.60	60.44
4	*Ocimum sanctum*	Leaves	10%	45.75	49.16
5	*Curcuma longa*	Rhizomes	10%	25.65	71.50
6	Control		-	90.00	00.00
F test	-		-	Sig.	-
S.E(m)±	-		-	0.45	-
CD (P= 0.01)	-		-	1.84	-

In vitro* evaluation of bioagents against *Alternaria alternata

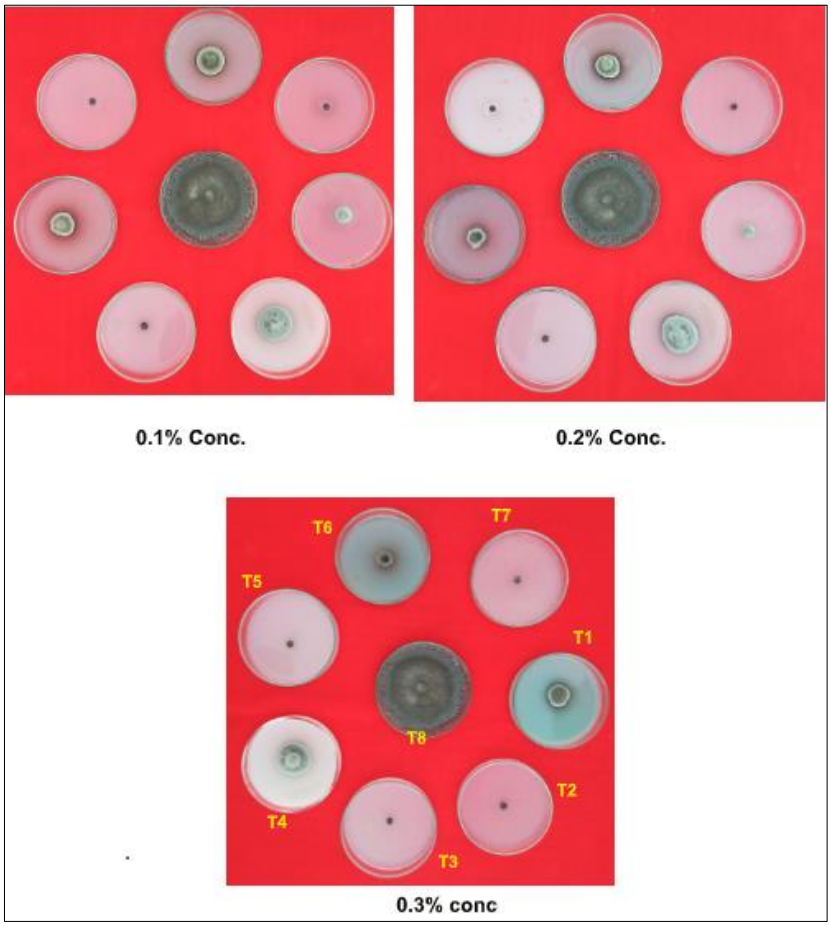
Antagonistic activity of bio-control agents namely *Trichoderma asperellum, Trichoderma harzianum, Pseudomonas fluorescens* and *Bacillus subtilis* were investigated by using the dual culture technique.

Table 3: Efficacy of bio-agents against *Alternaria alternata* by dual culture technique

S. No.	Bio-agents	Mean colony diameter of test pathogen (mm)	Per cent mycelial Inhibition (%)
1	*Trichoderma asperellum*	20.56	77.15
2	*Trichoderma harzianum*	18.67	79.25
3	*Bacillus subtilis*	48.71	45.87
4	*Pseudomonas fluorescens*	43.57	51.58
5	Control	90.00	0.00
	F test	Sig.	-
	S.E(m)±	0.27	-
	CD (P=0.01)	1.14	-

All the bio-agents tested showed the significant effect compared with control in Table 3. Antagonist *Trichoderma harzianum* gave the better effect against *Alternaria alternata* forming maximum per-cent mycelial inhibition 79.25% and decreased the mycelial growth from 90 to 18.67mm. *Trichoderma asperellum* was next best recorded 77.15% inhibition while the least mycelial inhibition was observed in *Pseudomonas fluorescence* 51.58% and *Bacillus subtilis* 45.87%. In present experiment fungal antagonist found effective than bacterial known antagonist. Their findings are conformity with the results of Zade *et al.*, (2018) [17] who reported that *Trichoderma harzianum* and *Trichoderma asperellum* was most effective against *Alternaria alternata* as compared *Pseudomonas fluoroscens* and *Bacillus subtilis*. Waghe *et al*., (2015) [16] recorded that *Trichoderma harzianum* was most effective against *Alternaria* fungus with significant mycelial inhibition (72.22%) followed by *Trichoderma viride* (70.27%) and *Pseudomonas fluorescence* (48.60%). The similar observation was also reported by Naik *et al*., (2020) [7] who recorded that *Trichoderma harzianum* was most effective against *Alternaria spp*. with significant mycelial inhibition 85.13% followed by *Trichoderma viride* 80.67%, and *Pseudomonas fluorescence* 72.87%.

The inhibition of fungal growth due to *Trichoderma spp*. may have been due to secretion of extracellular cell degrading enzymes such as chitinase β-1, 3-glucanase, cellulose and lectin, which may have helped myco-parasites in the colonization of their host. The inhibition of pathogen may also be attributed to the production of secondary metabolites by antagonists such as glioviridin, viridian and gliotoxin (Behairy *et al.*, 2014) [3].

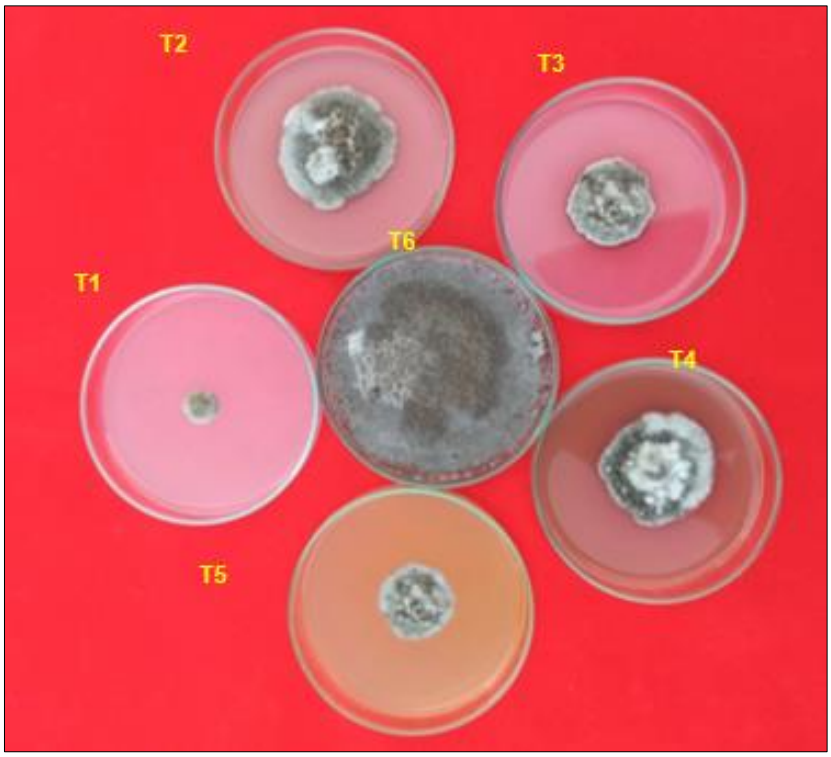


T_1: Carbendaizm 12% + Mancozeb 63% WP T_2: Hexaconazole 05% EC
T_3: Tebuconazole 50% + Trifloxystrobin 25% WG
T_4: Pyraclostrobin 20% WG
T_5: Difenconazole 25% EC
T_6: Copper Oxychloride 50% WP
T_7: Azoxystrobin 18.2%WW + Difenoconazole 11.4% WW SC T_8: Control.

Plate 1: Efficacy of fungicides against *Alternaria alternata* at different concentrations

T_1: *Trichoderma asperellum* T_4: *Pseudomonas fluorescens*
T_2: *Trichoderma harzianum* T_5: Control (Untreated)
T_3: *Bacillus subtilis*

Plate 2: Efficacy of bio-agents against *Alternaria alternata* by dual culture method



T_1: *Allium sativum* T_4: *Ocimum sanctum*
T_2: *Azardirachta indica* T_5: *Curcuma longa*
T_3: *Allium cepa* T_6: Control

Plate 9: Efficacy of botanicals against *Alternaria alternata* by poison food method

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