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Isolation, Identification of *Alternaria alternata* causing Leaf Blight in Chrysanthemum and its Management

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ABSTRACT: *Alternaria alternata* causing Alternaria blight is one of the most widely spread and destructive disease of chrysanthemum (*Chrysanthemum indicum* L.). The pathogen induces leaf blight and leaf spot which accounts for about up to 80% yield losses, in field also in market condition. Hence experiments are conducted to determine the efficacy of fungicides and bioagents in vitro using poison food technique for fungicides and dual culture method for bioagents. Among the non-systemic fungicides, the highest mean growth inhibition was observed with mancozeb (89.73%) followed by copper oxychloride on radial growth and per cent growth inhibition (15.66 mm and 82.60%) and propineb (15.42 mm and 82.87%), respectively. Among systemic fungicides, complete inhibition of growth was noticed in all the tested concentration of propiconazole and carbendazim + mancozeb at 1500 ppm. In case of bioagents, highest inhibition of growth and width of zone of inhibition were observed in *B. subtilis* (83.88% and 3.0 mm) and *T. asperellium* (82.11% and 3.0 mm).

Keywords : *Alternaria alternata*, Chrysanthemum, Fungicides, Mancozeb, *B. subtilis*, per cent inhibition.

INTRODUCTION

Chrysanthemum morifolium (Ramat) is a multi-use flower crop belongs to *Asteraceae* family and gaining more popularity as a cut flower, loose flower and pot plant. It is native to the northern hemisphere chiefly Europe and Asia. It is an important commercial flower next to rose in the international florist's trade and grown throughout the World (Teixeira Da Silva *et al*., 2013).

It is difficult to get good quality exportable blooms, higher yields and long lasting post-harvest life of the cultivars under open conditions. The most important factors responsible for the threatened production of chrysanthemum flower yield is by many diseases.

Alternaria alternata (Fries.) Keissler causing Alternaria blight is one of the most widely spread and destructive disease of chrysanthemum (*Chrysanthemum indicum L.*). The pathogen induces leaf blight and leaf spot which accounts for about up to 80 % yield losses, in field also in market condition. The disease causes symptoms on leaves, stalks, and flower petals. For chrysanthemums used for ornamental purposes, the occurrence of leaf diseases reduces the overall aesthetic feeling of chrysanthemums and damages their ornamental value as well as reduces the yield of chrysanthemums by affecting photosynthesis and plant health. Therefore, a study about chrysanthemum foliar fungal diseases is of great significance. In order to identify the pathogen that caused the foliar diseases and control of the disease, the present investigation was carried out.

MATERIALS AND METHODS

The experiment was done in laboratory of Plant Pathology, Dr. YSR horticultural University, College of Horticulture, Venkataramannagudem during November 2023 to April 2024. Samples of leaf spots in open chrysanthemum fields are collected and bought to laboratory for isolation and identification.

Leaf bits of five mm^2 with healthy and infected leaf portion were cut, surface sterilized using 1% sodium hypo chloride for a minute and rinsed in three changes of sterile distilled water to remove the disinfectant. Leaf bits were blot dried before transferring aseptically on to PDA plates and then incubated at 27±1°C in incubator.

Three-day old mycelial bits developed from incubated bits were aseptically transferred to glass slide and observations were made to confirm their identity based on morphological characters (conidia). The obtained

pathogen cultures were sub-cultured on PDA after confirmation.

A. Identification of pathogen

Cultural and morphological characters of *Alternaria alternata*. Cultural characters like colour of the colony by Munsell colour charts, 1994, growth of the colony (mm/day) and morphological characters like shape of the spores, size of the spores, septation of the spores were recorded. The shape and size (length and width) of conidia produced by the each pathogen were recorded from 10 randomly selected conidia. Micrometric technique was followed to measure length as well as breadth.

Molecular identification of pathogen. Molecular characterization of isolated pathogens was determined using universal primers of Internal Transcribed Spacer (ITS)region *viz.*, ITS1 and ITS4. Standard protocols were adopted for the extraction of DNA by CTAB (Cetyl trimethyl ammonium bromide) method described by Murray and Thompson (1980).

PCR amplification of gDNA using ITS primers. The DNA extracted from isolated pathogens was used as template in polymerase chain reaction and amplified in eppendorf thermal cycler using ITS region of universal primers *i.e.* forward and reverse primers, *viz.*, ITS 1 (5' TCCGTAGGTGAACCTGCGG 3') and ITS 4 (5' TCCTCCGCTTATTGATATGC 3'), respectively, synthesized based on conserved 18S and 28S coding regions of the nuclear rDNA were used (White *et al.*, 1990).

Initially, master mix was prepared by adding all the components except template DNA and Taq polymerase. Master mix was transferred to each PCR tube, then template DNA (50 ng/µl) and Taq polymerase were added and spun at 3000 rpm for 30 sec. Amplification was performed in 0.2 ml thin-walled PCR tubes using a thermocycler (Biorad T100 thermocycler, USA). A Gradient PCR between 55 to 65°C was kept to know the annealing temperature at which primers were going to bind to the template DNA and amplify the DNA.

Nucleotide sequencing and phylogenetic analysis. The sequencing of the PCR product was carried out in DNA sequencing facility available with IRA biotech, Hyderabad, India on out sourcing basis. Multiple sequence alignment was produced using the type or ex-type strains sequences retrieved from GenBank and aligned with MAFFT v.7 (Katoh *et al.*, 2017) (http://mafft.cbrc.jp/alignment/server/index.html).

Sequence alignment was deposited to GenBank. Sequence pair distances among related and different fungal pathogens were scored with the Clustral W algorithm in Bioedit (Hall, 1999) and phylogenetic tree analysis was performed with the MEGA-11 software (Kumar *et al.*, 2016).

B. In vitro screening of fungicides and bioagents against A. alternata

***In vitro* evaluation of fungicides.** The details of fungicides evaluated against isolated pathogens were presented in Table 2 and 3. Non systemic fungicides were tested at four different concentrations, *viz.*, 1000 ppm, 2000 ppm, 2500 ppm and 3000 ppm. Systemic fungicides were tested at three different concentrations, *viz.*, 500 ppm, 1000 ppm and 1500 ppm on active ingredient basis using poisoned food technique (Nene and Thapliyal 1993).

Observation on radial growth was recorded by averaging the two diameters of colony at right angles to one another and per cent growth inhibition was calculated by using the formula given by Vincent (1947).

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

Where, I = per cent inhibition, C = growth of the fungus in control (mm) and T = growth of the fungus in fungicide (mm).

***In vitro* evaluation of bioagents.** *In vitro* screening of bio-agents was carried out in completely randomised design (CRD) with six treatments and four replications on PDA medium using dual culture method (Dhingra and Sinclair 1985).

Five mm mycelial disc was made with sterile cork borer from edges of seven days old culture of pathogen as well as from fungal bioagents (*T. harzianum, T. asperellum* and *T. reesei*) and were placed in opposite direction over the solidified PDA in such a way that the distance between each other was approximately 70 mm. While in case of bacterial bioagents, the test pathogen placed at the centre and pure cultures bacterial bioagents was streaked with sterile inoculation loop on both sides of the test pathogen. Control was maintained by inoculating test pathogen at the centre of Petri plate. The inoculated Petri plates were incubated in an incubator at 28 ± 1°C. Antagonistic activity was recorded in terms of per cent inhibition was calculated using the formula of Vincent (1947).

RESULTS AND DISCUSSION

Collection of fungal leaf spots from chrysanthemum plants. Disease samples are collected form open chrysanthemum fields in Dr. YSR Horticultural University, COH, Venkataramannagudem during November 2023. The samples are isolated on potato dextrose agar media. The fungal cultures are identified based on their cultural and morphological characters.

On leaves pathogen produces small brown spots fuses together surrounded by yellow halo, covering most of the surfaces, giving the appearance of blight on the leaves. On flowers, reddish-brown spots appeared on the petals, it turned into large-sized dead spots, which lead to the death of flower petals, grey growth appeared on flower petals, which lead to flower rot. Similar type of symptoms was reported by Adolf and Ali (2023) on chrysanthemum as leaf spot and leaf blight caused by *A. alternata.*

A. Identification of fungal pathogen

Cultural and morphological characters of *Alternaria alternata*. On PDA the isolate produces aerial colourless thick hyphae when young but turned brown to greyish black colour they grew from front view and black colour from back. Mycelium is branched, septate and zonation was absent. Growth of mycelium was observed on first day after incubation and it took seven

days for full growth of mycelium. The fungus is a moderate growing. The growth rate was analysed and ranged from 12.00 to 14.16 mm/day.

The conidiophores were arose singly, straight or flexuous, septate, variable in length, between 21.4-70.8 µm, and brown. The length of spores was ranged from 42.17- 55.78µm. Width of spore was ranged from 9.40-14.48µm. The shape of spore is club shape with medium to long beak length ranged from 12.52-14.29µm. The spores have both horizontal and transverse septa. Average number of longitudinal and transverse septa was ranged 1 to 4 and 4 to 7, respectively.

These characters were in confirmation with the findings of Shamala and Janardan (2015) in chrysanthemum who reported that, initially, the mycelium was hyaline later turned to grey-brownish with multi-celled septate conidia and Priyanka *et al.* (2018) in marigold reported that the fungus produced initially white colony, cottony with profuse aerial mycelium which gradually turned grey colour.

Molecular characterization of *A. alternata.* DNA is isolated by following CTAB protocol and amplified using internal transcribed spacers *viz*., ITS 1 and ITS 2 primers. the sequence of *A. alternata* obtained by Sanger dideoxy sequencing technology. The sequence was submitted to NCBI and obtained unique accession number PQ276939.

Phylogenetic analysis. Dendrogram was constructed using mega XI (plate) software by selecting all the sequences of isolates sequenced and other sequences are also selected from blast sequencing which showed maximum similarity with the isolate sequence, obtained with 1000 bootstrap.

The results of phylogenetic analysis the *A. alternata* isolate AaC formed separate clade. But it showed 46% similarity with OM618618 *A. alternata*isolate AAITS-11 and 38% with MW487251 *A. alternata* isolate of linseed sub clades. In the current study, results were in concur with *the* findings of Ray *et al*. (2022) who reported gene sequences of *A. alternata* (IPL.CIT.A.F1.001) showed satisfactory homology with 16 ex type strains of *A. alternata* sequences from NCBI GenBank data base.

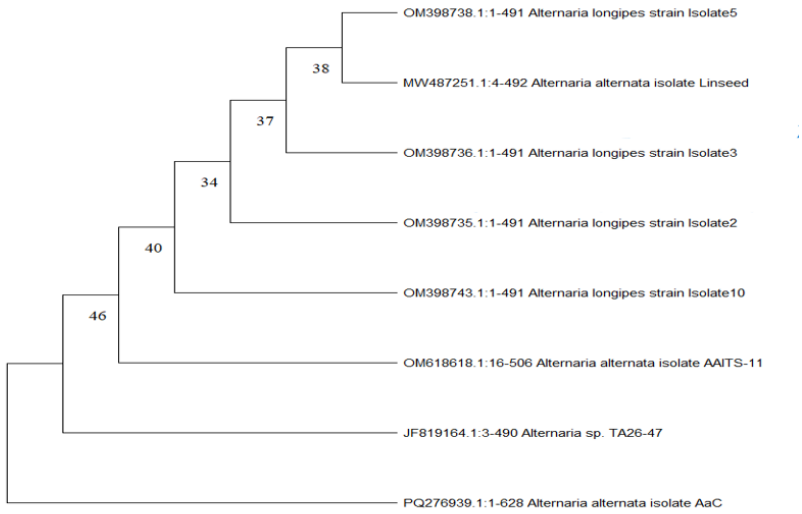


Fig. 1. Dendrogram representing phylogenetic affiliation of *Alternaria alternata* isolate AaC.

B. In vitro evaluation of fungicides and bioagents against Alternaria alternata

Evaluation of non-systemic fungicides against *A. alternata.* Cent per cent growth inhibition was noticed in mancozeb at 2000, 2500 and 3000 ppm, propineb at 2500 and 3000 ppm and copper oxychloride and chlorothalonil at 3000 ppm. The next best treatment in inhibiting the mycelial growth were copper hydroxide at 3000 ppm (86.77%) and copper oxychloride at 2500 ppm (84.22%) but both were statistically different with each other. The lowest growth inhibition was recorded with chlorothalonil at 1000 ppm (23.18%). This indicates that, mancozeb 2000 ppm was good enough in field conditions to avoid the use of higher dose, which in turn results increase in cost of cultivation (Table 1, Plate 1).

The above interaction study suggested that, mancozeb at 2000, 2500 and 3000 ppm, was equally effective to propineb at 2500 and 3000 ppm and copper oxychloride and chlorothalonil at 3000 ppm. It indicates that alternate use of different fungicides for effective management of pathogen and to prevent the development of resistance.

These results were in accordance with the findings of Thaware *et al*. (2010) who reported that complete inhibition with mancozeb and comparatively less inhibition with chlorothalonil.

Evaluation of systemic fungicides against *A. alternata.* Complete inhibition of growth was noticed in all the tested concentration of propiconazole and carbendazim+ mancozeb at 1500 ppm. The next best treatment in inhibiting the mycelial growth were difenconazole and tebuconazole at 1500 ppm (97.17% and 97.10%) but both were statistically at par with each other. The lowest growth inhibition was recorded with azoxystrobin at 500 ppm (38.74%). This indicates that,

propiconazole 500 ppm was good enough in field conditions to avoid the use of higher dose, which in turn results in the increase in cost of cultivation (Table 2, Plate 2).

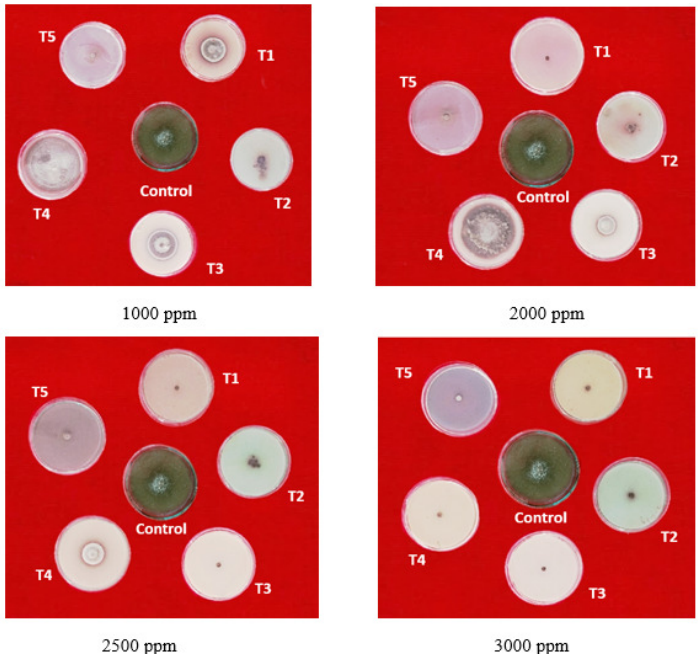
The above interaction study suggested that, propiconazole at 500, 1000 and 1500 ppm, was equally effective to carbendazim+ mancozeb at 1500 ppm. It indicates that alternate use of different fungicides for effective management of pathogen and to prevent the development of resistance.

Table 1: *In vitro* efficacy of non-systemic fungicides against *A. alternata*.

Sr. No.	Treatments	Mycelial growth (mm)*				Mean mycelial growth (mm)	Per cent inhibition*				Mean per cent inhibition
		1000 ppm	2000 ppm	2500 ppm	3000 ppm		1000 ppm	2000 ppm	2500 ppm	3000 ppm	
1.	Mancozeb 75 WP	36.96	0.00	0.00	0.00	**9.24**	58.93 (50.12)**	100.00 (89.97)	100.00 (89.97)	100.00 (89.97)	**89.73 (71.28)**
2.	Copper Oxy Chloride 50 WP	28.70	19.75	14.20	0.00	**15.66**	68.11 (55.60)	78.06 (62.050	84.22 (66.57)	100.00 (89.970	**82.60 (65.32)**
3.	Propineb 70 WP	40.95	20.73	0.00	0.00	**15.42**	54.50 (47.56)	76.96 (61.29)	100.00 (89.970	100.00 (89.970	**82.87 (65.53)**
4.	Chlorothalonil 70 WP	69.13	58.38	24.78	0.00	**38.07**	23.18 (28.77)	35.13 (36.34)	72.46 (58.32)	100.00 (89.97)	**57.69 (49.40)**
5.	Copper hydroxide	28.10	27.13	24.73	11.90	**22.96**	68.77 (56.00)	69.85 (56.67)	72.52 (58.36)	86.77 (68.64)	**74.48 (59.63)**
6.	Control	90.00	90.00	90.00	90.00	**90.00**	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	**0.00 (0.00)**
Mean of concentrations		**48.97**	**36.00**	**25.62**	**16.98**		**45.58 (42.45)**	**60.00 (50.75)**	**71.53 (57.73)**	**81.13 (64.23)**	

Factors	C.D@0.1 %	SE (m)	SE (d)	C.D@0.1 %	SE (m)	SE (d)
T	0.37	0.10	0.45	0.11	0.03	0.24
C	0.23	0.08	0.4	0.11	0.03	0.24
T x C	0.71	0.19	0.62	0.26	0.07	0.37

*Average of three replications ** Figures in parentheses are angular transformed values



T1-Mancozeb 75% WP; T2-Copper Oxy Chloride 50% WP; T3-Propineb 70% WP; T4-Chlorothalonil 70 % WP; T5-Copper hydroxide 53.8% DF

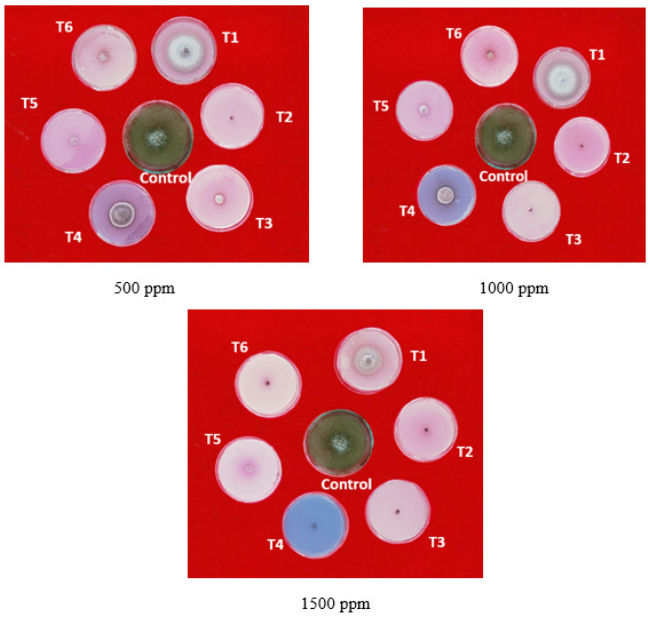
Plate 1. *In vitro* evaluation of non-systemic fungicides at different concentrations against *A. alternata*.

These results were in line with the findings of Arun Kumar and Kamanna (2009); Thaware *et al*. (2010); Apet *et al*. (2014); Thejakumar and Devappa (2016); Shindhe *et al.* (2018); Vijayalakshmi and Rao (2018); Glory *et al.* (2022) who reported that different group of triazole fungicides like propiconazole, tebuconazole, difenconazale and hexaconazole were effective in inhibiting the mycelial growth of test pathogen.

Table 2: *In vitro* evaluation of systemic fungicides against *A. alternata.*

Sr. No.	Treatments	Mycelial growth (mm)*			Mean mycelial growth (mm)	Per cent inhibition*			Mean per cent inhibition
		500 ppm	1000 ppm	1500 ppm		500 ppm	1000 ppm	1500 ppm	
1.	Azoxystrobin 25% SC	55.13	53.83	38.17	**49.04**	38.74 (38.48)**	40.18 (39.32)	57.58 (49.34)	**45.50 (42.40)**
2.	Propiconazole 25 EC	0.00	0.00	0.00	**0.00**	100.00 (89.97)	100.00 (89.97)	100.00 (89.97)	**100.00 (89.97)**
3.	Tebuconazole 25.9 EC	8.40	3.01	2.61	**4.67**	90.66 (72.18)	96.65 (79.42)	97.10 (80.16)	**94.80 (76.79)**
4.	Carbendazim 25% + Mancozeb 50% WS	36.77	25.99	0.00	**20.92**	59.14 (50.25)	71.12 (57.47)	100.00 (89.97)	**76.75 (61.15)**
5.	Zineb 68% + Hexaconazole 4% WP	11.13	10.34	5.14	**8.87**	87.63 (69.38)	88.51 (70.16)	94.28 (76.13)	**90.14 (71.67)**
6.	Difenconazole 25 EC	14.66	9.07	2.54	**8.76**	83.71 (66.17)	89.92 (71.46)	97.17 (80.28)	**90.27 (71.80)**
7.	Control	90.00	90.00	90.00	**90.00**	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	**0.00 (0.00)**
Mean of concentrations		**30.87**	**27.46**	**19.78**		**65.70 (54.13)**	**69.48 (56.44)**	**78.02 (62.02)**	
Factors		C.D@0.1%	**SE(m)±**	**SE (d)**		**C.D@0.1%**	**SE(m)±**	**SE (d)**	
T		0.60	0.16	0.57		0.41	0.11	0.47	
C		0.37	0.10	0.45		0.26	0.07	0.37	
T x C		1.05	0.28	0.75		0.71	0.19	0.62	

*Average of three replications ** Figures in parentheses are angular transformed values



T1-Azoxystrobin 23% SC; T2-Propiconazole 25 EC; T3-Tebuconazole 25.9 EC; T4-Carbendazim 25% + Mancozeb 50% WS; T5-Zineb 68% + Hexaconazole 4% WP; T6-Difenconazole 25% EC

Plate 2. *In vitro* evaluation of systemic fungicides at different cconcentrations against *A. alternata.*

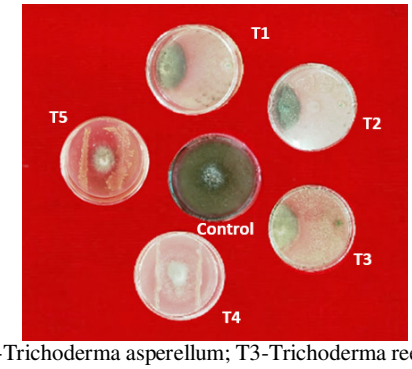
Evaluation of bioagents against *A. alternata.* The results revealed that the highest inhibition of growth and width of zone of inhibition were observed in *B. subtilis* (83.88% and 3.0 mm) and *T. Asperellium* (82.11% and 3.0 mm) and both were significantly different with each other. Per cent growth inhibition in case of *T. asperellium* (82.11%) and *T. reesei,* (82.00%) but both were at par with each other and least growth inhibition was observed in *P. fluorescens* (75.11 %) and zone of inhibition was not noticed (Table 3, Plate 3).

The results were conformity with findings of Ghosh *et al*. (2002); Vijayalakshmi and Rao (2018) who reported that *B. subtilis, T. viride* and *T. harzianum* were effective and inhibited the mycelial growth.

Table 3: *In vitro* evaluation of bioagents against *A. alternata.*

Treatments	Zone of inhibition (mm)	Mean mycelial growth (mm)*	Mean per cent inhibition	Mechanism of biocontrol
T. harzianum	2.00	16.90	81.22 (64.29)**	Hyperparasitism
T. asperellum	3.00	16.20	82.00 (64.87)	Hyperparasitism
T. reesei	1.00	16.10	82.11 (64.95)	Hyperparasitism
P. fluorescens	0.00	22.40	75.11 (60.05)	Antibiosis
B. subtilis	3.00	14.50	83.88 (66.30)	Antibiosis
Control		90.00	0.00 (0.00)	
C.D (0.01)		0.051	0.39	
SE (m)±		0.016	0.13	
SE (d)		0.023	0.51	
C.V(%)		0.173	0.68	

*Average of three replications ** Figures in parentheses are angular transformed values



T1-Trichoderma harzianum; T2-Trichoderma asperellum; T3-Trichoderma reesei; T4-Pseudomonas fluorescens T5-Bacillus subtilis

Plate 3. *In vitro* evaluation of bioagents against *A. alternata.*

CONCLUSIONS

Alternaria alternata causing Alternaria blight on chrysanthemum is common and destructive disease causing 80% yield loss. The pathogen was isolated in PDA media and proved its pathogenicity. Later, the pathogen was identified based on cultural, morphological and molecular characteristics. Testing of different fungicides and bioagents was done *in vitro* to test their efficacy to inhibit growth of pathogen. In this experiment, mancozeb at 2000, 2500 and 3000 ppm, was equally effective to propineb at 2500 and 3000 ppm and copper oxychloride and chlorothalonil at 3000 ppm, all the tested concentration of propiconazole and carbendazim+ mancozeb at 1500 ppm are found to be effective in maximum inhibition of growth of the pathogen.

FUTURE SCOPE

• Surveys need to be conducted in chrysanthemum field for other pathogens.
• To assess the losess caused by floral fungal pathogens.
• To screen the varieties or to find out resistant sources.

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Conflicts of Interest. None.

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