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In vitro evaluation of different fungicides against pomegranate anthracnose caused by *Colletotrichum gloeosporioides*

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Abstract
The cultivation of pomegranate is going to be increase due to high remuneration from its fruits as it is widely used as a nutritive food. Leaf and fruit spot caused by *Colletotrichum gloeosporioides* is contributing factors for the low productivity as well as economic losses among the farmers. Pomegranate leaves and fruits with anthracnose symptoms were collected from the farmer's field. On isolation, they yielded species of *Colletotrichum gloeosporioides* with typical cultural and morphological characters on potato dextrose agar media. Koch's postulates were successfully proved in laboratory by standard method. In laboratory screening of different fungicides against *Colletotrichum gloeosporioides*, tebuconazole 25.9% EC and hexaconazole 5% EC were found to be effective in inhibiting the cent per cent radial growth among systemic group of fungicides, while in non-systemic group of fungicides, captan 50% WP was quite effective and in case of ready mix fungicides azoxystrobin 11% + tebuconazole 18.30% SC, epoxiconazole 50g/l + pyraclostrobin 133g/l, tebuconazole 50% + trifloxystrobin 25% WG, zinab 68% + hexaconazole 4% WP were significantly inhibited the growth of test fungus under *in vitro*.

Keywords: Pomegranate, anthracnose, Fungicide, *Colletotrichum gloeosporioides*

1. Introduction
Pomegranate (*Punica granatum* L.) is an ancient and commercially important fruit crop of both tropical and subtropical countries; belongs to the family: *Lythraceae*. The fruit is very much liked for its cool and refreshing juice. The arils of the well matured fruits are consumed as such and also in processed form like juice or concentrate, syrup and jelly.

Globally India is ranked first with 0.234 million ha area and 2.845 million MT production during 2017-18 (Anon., 2018a) [3]. It is cultivated in different parts of the country such as Maharashtra, Karnataka, Andhra Pradesh, Gujarat, Rajasthan, Tamil Nadu, etc. Maharashtra considered as pomegranate basket of India and leading producer of pomegranate which cover two third of the total cultivated area of the country (Dev and Narendrappa, 2016) [5]. In Gujarat, pomegranate cultivation occupies an area of 30.512 thousand ha with an annual production and productivity of 461.752 thousand MT and 15.13 MT/ha, respectively. It is cultivated intensively in northern parts of Gujarat covering different districts such as Kutch, Banaskantha, Morbi and Mehsana (Anon., 2018a) [3]. Now a day's its cultivation is expanded to Saurashtra region of Gujarat. Although the pomegranate cultivation in Gujarat is gaining popularity, but the production has not increased significantly because it suffers from variety of fungal, bacterial and viral diseases. Pomegranate fruits are more susceptible to pathogens attack due to its high moisture content and rich in nutrients. Among the various fungal diseases, anthracnose caused by *Colletotrichum gloeosporioides* (Penz.) Penz. and Sacc. is the serious disease of pomegranate. In India, anthracnose disease was first reported by McRae (1924) [10]. *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. is an asexual facultative parasite. The fungus comprises *C. gloeosporioides* as anamorph imperfect or asexual state while, *Glomerella cingulata* as sexual (perfect) teleomorph state (Cannon *et al*., 2012) [4]. The fungus prefers warm humid environment for spreading the anthracnose disease uniformly and effectively (Farr *et al.*, 2006) [6]. The symptoms on leaves observed as pinhead size of black to brown water soaked spots with circular margin. In advanced stage, these spots enlarged, coalesced and resulted in bigger patches. In severe case, leaves dried up and drooped down.

On fruits, scattered brown spherical depressed spots occurred on the pericarp. In advanced stage, these spots coalesced to form necrotic patches over the surface of the fruit (Krishnappa, 2010) [8].

2. Materials and Methods

2.1 Isolation and purification of pathogen

Infected fruits and leaves of pomegranate were collected from the farmer's field. These specimens were brought to laboratory, Department Plant Pathology, J.A.U. Junagadh in butter paper bags and used for isolation of the pathogen.

Pomegranate fruits and leaves infected with anthracnose which showed typical symptoms were first microscopically examined to confirm the presence of the fungus. The isolation of the pathogen was made by following standard tissue isolation technique as described below.

Small pieces of tissues from infected leaves and fruits (3 mm) along with some healthy tissues were cut using sterilized scalpel and the cut tissues were surface sterilized with 1 per cent sodium hypochlorite solution for about 30 to 60 seconds. They were then washed four times with sterilized distilled water to remove the excess of sodium hypochlorite. Finally, they were transferred under aseptic condition on to Petriplates containing solidified potato dextrose agar (PDA) and incubated at 28 ± 2^0 C. The resulting fungal cultures were purified by hyphal tip method. Purified cultures of test pathogen maintained on PDA slants by storing it under refrigeration at 4^0 C. To maintain the culture for further studies, periodical transfers were made once in a month. The fungus was isolated, purified and sub cultured in aseptic condition under a laminar flow.

2.2 *In vitro* evaluation of different fungicides against test pathogen

In vitro efficacy of different fungicides against leaf and fruit spot pathogen was evaluated by poisoned food technique (Shravelle, 1961) [16].

2.2.1 Poisoned food technique

Required quantity of fungicide was added in 100 ml of lukewarm PDA media and mixed thoroughly. This solution was poured into Petri plates about 30 ml in each. After solidification of media 5 mm discs of four days old culture of test pathogen were inoculated at the centre of Petri plates and then incubated at 28 ± 2°C. Three repetitions were maintained for each fungicide. Medium without fungicide was kept as control. Per cent inhibition of mycelial growth of test pathogens was calculated by the following formula as suggested by Vincent (1927) [178].

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

Where

I = Per cent inhibition of test pathogen

C = Radial growth (mm) in control

T = Radial growth (mm) in treatments.

Toxicity index for each fungicides were calculated by total of growth inhibition per cent of all concentrations for respective fungicide.

2.2.2 Evaluation of different fungicides

Six systemic fungicides *viz.*, carbendazim 50% WP, tebuconazole 25.9% EC, iprobenfos 48% EC, picoxystrobin 22.52% SC, difenoconazole 250 EC and hexaconazole 5% EC with three concentrations (100, 250 and 500 ppm), six non-systemic fungicides *viz.*, propineb 70% WP, mancozeb 75% WP, copper oxychloride 50% WP, chlorothalonil 75% WP, copper hydroxide 53.8% WP and captan 50% WP with three concentrations (500, 1000 and 2000 ppm) and six ready mix fungicides *viz.*, azoxystrobin 11% + tebuconazole 18.30% SC, carbendazim 25% WP + mancozeb 50% WP, epoxiconazole 50 g/l + pyraclostrobin 133 g/l, cymoxanil 8% WP + mancozeb 64% WP, tebuconazole 50% + trifloxystrobin 25% WG and zineb 68% WP + hexaconazole 4%WP with four concentration (100, 250, 500 and 1000 ppm) were evaluated against test pathogen under laboratory condition by following poisoned food technique.

Experiment was laid out with six treatments and each treatment repeated three times. Completely Randomized block Design with Factorial Concept was used for analyzing the data.

3. Results and Discussion

The fungus was isolated from infected pomegranate fruit and leaf and pure culture was obtained by hyphal tip method, such culture was used for pathogenecity test of Koch's postulates and to evaluate the fungicides also. After successfully proven of pathogenicity the test pathogen used to evaluate the fungicides.

In vitro efficacy of different fungicides against leaf and fruit spot pathogens was evaluated by poisoned food technique (Shravelle, 1961) [16].

3.1 *In vitro* evaluation of systemic fungicides against *Colletotrichum gloeosporioides*

Efficacy of six commonly used systemic fungicides *viz*., carbendazim, tebuconazole, iprobenfos, picoxystrobin, difenoconazole and hexaconazole were evaluated against *Colletotrichum gloeosporioides* at different concentrations *viz*., 100, 250 and 500 ppm using poisoned food technique as described in materials and methods. The data revealed that all the fungicides at all concentrations reduced mycelial growth (Table-2) of *Colletotrichum gloeosporioides* as compared to control (Fig. - 1).

Table 1: Systemic fungicides

	Systemic fungicides	Concentration (ppm)
1.	Carbendazim 50% WP	A = 100 B = 250 C = 500
2.	Tebuconazole 25.9% EC	
3.	Iprobenfos 48% EC	
4.	Picoxystrobin 22.52%SC	
5.	Difenoconazole 250 EC	
6.	Hexaconazole 5% EC	
7.	Control	

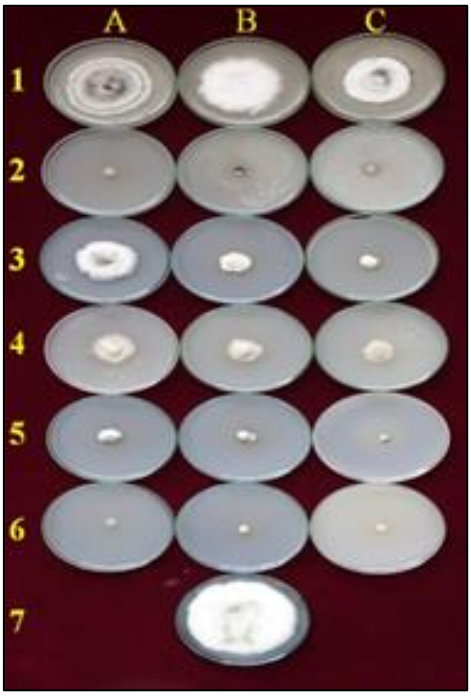

Fig 1: Growth inhibition of *Colletotrichum gloeosporioides* on PDA supplemented with systemic fungicides.

It is evident from the data presented in Table-2 and Fig-1, that cent per cent mean per cent inhibition of *Colletotrichum gloeosporioides* mycelial growth were recorded in tebuconazole and hexaconazole which was followed by difenoconazole (90.61%), picoxystrobin (69.92%) which was at par with iprobenfos (69.73%). Carbendazim was found to be least effective against *Colletotrichum gloeosporioides*. Maximum toxicity index was found in tebuconazole and hexaconazole (299.94) which was followed by difenoconazole (271.84).

Table 2: *In vitro* evaluation of systemic fungicides against *C. gloeosporioides*

Systemic fungicides	Growth inhibition (%)			Mean inhibition (%)	Toxicity index #
	100 ppm	250 ppm	500 ppm		
Carbendazim 50% WP	32.41 (28.74)*	34.20 (31.61)	40.03 (41.38)	35.55 (33.91)	101.72
Tebuconazole 25.9% EC	89.19 (99.98)	89.19 (99.98)	89.19 (99.98)	89.19 (99.98)	299.94
Iprobenfos 48% EC	45.00 (50.00)	60.19 (75.29)	66.36 (83.91)	57.18 (69.73)	209.20
Picoxystrobin 22.52% SC	53.69 (64.94)	56.15 (68.97)	60.57 (75.86)	56.81 (69.92)	209.77
Difenoconazole 250 EC	66.81 (84.48)	69.18 (87.36)	89.19 (99.98)	75.06 (90.61)	271.84
Hexaconazole 5% EC	89.19 (99.98)	89.19 (99.98)	89.19 (99.98)	89.19 (99.98)	299.94
Mean	62.72 (71.35)	66.35 (77.20)	72.42 (83.51)	67.16 (77.35)	-
	Fungicide (F)		**Concentration (C)**		**F x C**
S.Em. ±	0.25		0.17		0.43
C.D at 5%	0.71		0.50		1.22
CV%	1.10				

Maximum toxicity index = 300

* Data outside the parentheses are arcsine transformed whereas inside are re-transformed values

There was positive correlation between concentration and inhibition of growth of pathogen. It is also observed that with increasing concentration of all fungicides, inhibition of growth of pathogen also increased, except tebuconazole (100 ppm) hexaconazole (100 ppm) which give almost cent per cent inhibition at lower concentration. The carbendazim (500 ppm), iprobenfos (500 ppm), picoxystrobin (500 ppm) and difenoconazole (500 ppm) showed 41.38, 83.91, 75.86 and 99.98 per cent inhibition of mycelial growth, respectively which were higher than their lower concentration of 100 and 250 ppm (Fig-1).

Looking to concentration of individual fungicides, all the fungicides differed significantly in inhibition of test pathogen, except tebuconazole and hexaconazole which were found at par at all concentration tested, *i.e.* 100, 250 and 500 ppm.

The effectiveness of tebuconazole, hexaconazole and difenoconazole against *Colletotrichum gloeosporioides* has been reported by Dev and Narendrappa (2016) [5], Ahmadi (2011) [2] and Sharma (2012) [15]. On contrary, Abhishek and Verma (2007) [1] and Vinod *et al.* (2009) [18] recorded carbendazim as a potent inhibitor of *Colletotrichum gloeosporioides.*

In present investigation fungicides of trizole group were found more effective against *Colletotrichum gloeosporioides* as compare to benzimidazole and strobilurin group may be due to their mode of action, inhibition of eargosterol synthesis in pathogen.

In addition some researchers recorded carbendazim as a potent inhibitor of pathogen, but maybe it is possible that now pathogen developed resistance towards carbendazim which is known to inhibit cell division of pathogen.

3.2 *In vitro* evaluation of non-systemic fungicides against *Colletotrichum gloeosporioides*

Efficacy of six commonly used non-systemic fungicides *viz*., propineb, mancozeb, copper oxychloride, chlorothalonil,

copper hydroxide and captan were evaluated against *Colletotrichum gloeosporioides* at different concentrations *viz*., 500, 1000 and 2000 ppm using poisoned food technique as described in materials and methods. The data revealed that all the fungicides at all concentrations reduced mycelial growth (Table 4) of *Colletotrichum gloeosporioides* as compared to control (Fig.-2).

Table 3: Non-systemic fungicides

	Non-systemic fungicides	Concentration (ppm)
1.	Propineb 70%WP	A = 500
2.	Mancozeb 75% WP	
3.	Copper oxychloride 50% WP	B = 1000
4.	Chlorothalonil 75% WP	
5.	Copper hydroxide 53.8% WP	C = 2000
6.	Captan 50% WP	
7.	Control	

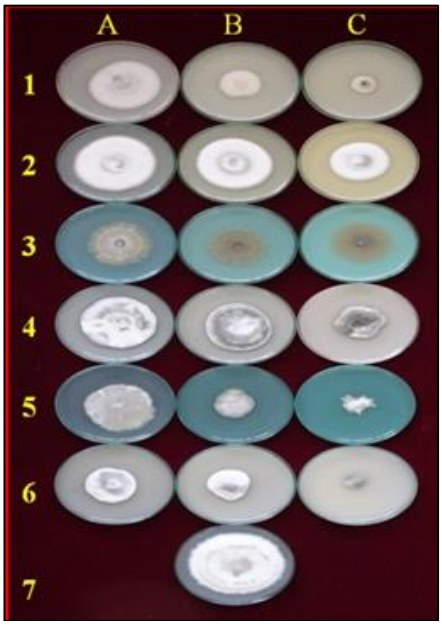

Fig 2: Growth inhibition of *Colletotrichum gloeosporioides* on PDA supplemented with non-systemic fungicides.

It is inferred from the data presented in Table-4 and Fig.-2 that all the fungicides are significantly effective in inhibition of test pathogen.

The perusal of data presented in Table-4 and Fig.-2 revealed that mean per cent growth inhibition of *Colletotrichum gloeosporioides* mycelial growth was maximum in captan (58.49%) followed by copper hydroxide (51.20%) which was at par with propineb (50.98%). The copper oxychloride (37.47%), chlorothalonil (33.77%) and mancozeb (25.49%) were found moderately effective. On contrary, Kumari *et al.* (2017) [9] and Ahmadi (2011) [2] recorded mancozeb as a most effective fungicide against *C. gloeosporioides.* Captan was found superior to suppress the mycelial growth of test pathogen may be due to their mode of action on respiration of the organism while mancozeb found least effective is may be due to possibly the organism developed resistance by alter the site of action (lipid metabolism). Moreira *et al.* (2017) [11] classified 21.4% of the isolates as resistant or highly resistant and 35.7% of isolates moderately resistant based on EC_{50} among 39 isolates of Glomerela Leaf Spot (*Colletotrichum* Spp.) in apple to the mancozeb.

Table 4: *In vitro* evaluation of non-systemic fungicides against *C. gloeosporioides*

Non systemic fungicides	Growth inhibition (%)			Mean inhibition (%)	Toxicity index #
	500 ppm	1000 ppm	2000 ppm		
Propineb 70%WP	29.89 (24.84)*	48.57 (56.21)	57.99 (71.90)	45.48 (50.98)	152.95
Mancozeb 75% WP	23.30 (15.69)	28.12(22.22)	38.39 (38.56)	29.93 (25.49)	76.46
Copper Oxychloride 50% WP	32.43 (28.76)	39.54 (40.52)	41.05 (43.14)	37.67 (37.47)	112.42
Chlorothalonil 75% WP	29.44 (24.18)	30.74 (26.14)	45.56 (50.98)	35.25 (33.77)	101.30
Copper hydroxide 53.8% WP	32.84 (29.41)	48.19 (55.56)	55.94 (68.63)	45.66 (51.20)	153.60
Captan 50% WP	42.94 (46.41)	46.11 (51.94)	61.43 (77.12)	50.16 (58.49)	175.47
Mean	31.81 (28.21)	40.21 (42.10)	50.06 (58.39)	40.69 (42.90)	
	Fungicide (F)		**Concentration (C)**		**F x C**
S.Em. ±	0.30		0.21		0.51
C.D at 5%	0.85		0.60		1.48
CV%	2.19				

Maximum toxicity index = 300

*Data outside the parentheses are arcsine transformed, whereas inside are re-transformed values

There was positive correlation between concentration and inhibition of growth of pathogen. It is also observed that with increasing concentration of all fungicides, inhibition of growth of pathogen also increased.

The fungicides propineb, mancozeb, copper oxychloride, chlorothalonil, copper hydroxide and captan showed higher 71.90, 38.56, 43.14, 50.98, 68.63 and 77.12 per cent inhibition of mycelial growth at 2000 ppm, respectively as compared to their lower concentration of 1000 and 500 ppm (Fig.-2). Maximum toxicity index was found in captan (175.47) which was followed by copper hydroxide (153.60). The effectiveness of captan against *Colletotrichum gloeosporioides* has been recorded by Krishnappa (2010) [8], Sharma (2012) [15] and Kumari *et al.* (2017) [9].

Looking to concentration of individual fungicides, all the fungicides differed significantly in inhibition of test pathogen.

3.3 *In vitro* evaluation of ready mix fungicides against *Colletotrichum gloeosporioides*

The results of the laboratory evaluation of ready mix fungicides on the radial growth of *Colletotrichum gloeosporioides* are presented in the Table 4 and Fig.-3. The results of the experiment showed that all selected ready mix fungicides at all four *viz.*, 100, 250, 500 and 1000 ppm concentrations inhibited the radial growth of tested pathogen.

Table 5: Ready mix fungicides

	Ready mix fungicides	Concentration (ppm)
1.	Azoxystrobin 11% + Tebuconazole 18.30% SC	A = 100 B = 250 C = 500 D = 1000
2.	Carbendazim 12% + Mancozeb 63% WP	
3.	Epoxiconazole 50 g/l + Pyraclostrobin 133 g/l	
4.	Cymoxanil 8% + Mancozeb 64% WP	
5.	Tebuconazole 50% + Trifloxystrobin 25% WG	
6.	Zineb 68% + Hexaconazole 4%WP	
7.	Control	

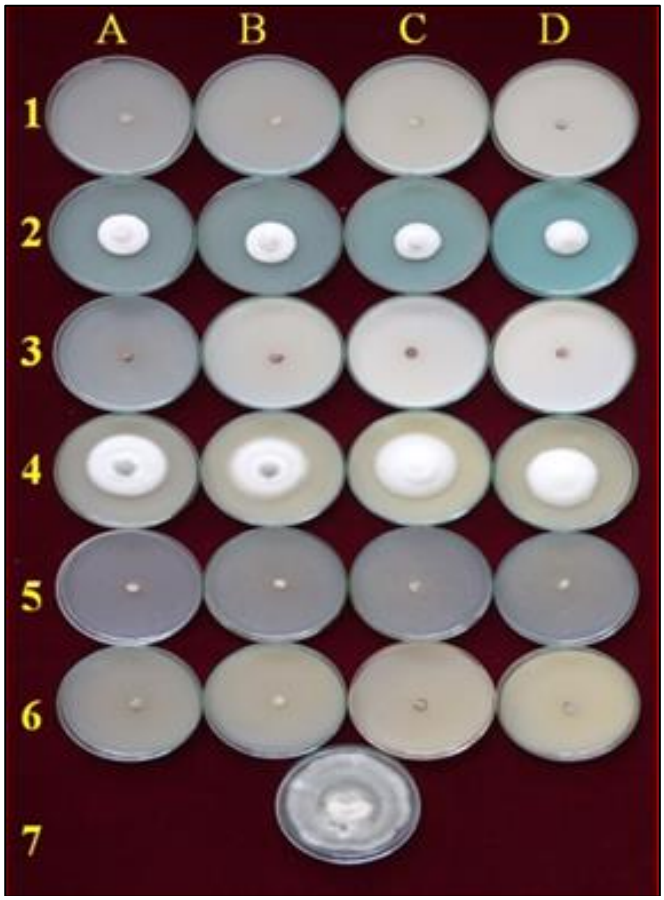

Fig 3: Growth inhibition of *Colletotrichum gloeosporioides* on PDA supplemented with non-systemic fungicides.

It is evident from the data presented in Table 5 and Fig.-3 all the fungicides were significantly effective in inhibiting growth of pathogen. The cent per cent growth inhibition were recorded in four fungicide combinations such as, azoxystrobin 11% + tebuconazole 18.30% SC, epoxiconazole 50 g/l + pyraclostrobin 133 g/l, tebuconazole 50% + trifloxystrobin 25% WG and zineb 68% WP + hexaconazole 4%WP followed by carbendazim 12% + mancozeb 63% WP (64.65%) and cymoxanil 8% WP + mancozeb 64% WP (45.69%). They were statistically differed to each other. Maximum toxicity index was found in azoxystrobin 11% + tebuconazole 18.30% SC, epoxiconazole 50 g/l + pyraclostrobin 133 g/l, tebuconazole 50% + trifloxystrobin 25% WG and zineb 68% WP + hexaconazole 4%WP (399.92) which was followed by carbendazim 12% + mancozeb 63% WP (258.61). Here again reflect from previous results that trizole group of fungicides alone or in combination found more effective, while carbendazim and mancozeb showed poor results and alone also as reflected in previous study.

Table 6: *In vitro* evaluation of ready mix fungicides against *C. gloeosporioides*

Ready mix Fungicides	Growth inhibition (%)				Mean inhibition (%)	Toxicity index #
	100 ppm	250 ppm	500 ppm	1000 ppm		
Azoxystrobin 11% + Tebuconazole 18.30% SC	89.19 (99.98)*	89.19 (99.98)	89.19 (99.98)	89.19 (99.98)	89.19 (99.98)	399.92
Carbendazim 12% + Mancozeb 63% WP	50.97 (60.34)	52.33 (62.64)	54.39 (66.09)	56.50 (69.54)	53.55 (64.65)	258.61
Epoxiconazole 50 g/l + Pyraclostrobin 133 g/l	89.19 (99.98)	89.19 (99.98)	89.19 (99.98)	89.19 (99.98)	89.19 (99.98)	399.92
Cymoxanil 8% WP + Mancozeb 64% WP	40.37 (41.95)	42.36 (45.40)	43.02 (46.55)	44.34 (48.85)	42.52 (45.69)	182.75
Tebuconazole 50% + Trifloxystrobin 25% WG	89.19 (99.98)	89.19 (99.98)	89.19 (99.98)	89.19 (99.98)	89.19 (99.98)	399.92
Zineb 68% WP + Hexaconazole 4%WP	89.19 (99.98)	89.19 (99.98)	89.19 (99.98)	89.19 (99.98)	89.19 (99.98)	399.92
Mean	74.68 (83.70)	75.24 (84.66)	75.69 (85.43)	76.27 (86.39)	75.47 (85.04)	-
	Fungicide (F)			**Concentration (C)**		**F x C**
S.Em. ±	0.11			0.09		0.23
C.D at 5%	0.32			0.26		0.62
CV%	0.52					

Maximum toxicity index = 400

*Data outside the parentheses are arcsine transformed, whereas inside are re-transformed values

Cent per cent inhibition of *C. gloeosporioides* was recorded by Pavithra and Benagi (2017) [12] and Dev and Narendrappa (2016) [5] with zineb 68% WP + hexaconazole 4%WP and tebuconazole 50% + trifloxystrobin 25% WG. Ranjitha *et al.* (2019) [14] also recorded that the mycelial inhibition of *C. gloeosporioides* by tebuconazole + trifloxystrobin and zineb + hexaconazole was 94.86% and 85.06%, respectivey. On contrary with our results Prashanth *et al.* (2008) [13], Krishnappa (2010) [8] and Jagtap *et al.* (2015b) [7] recorded maximum inhibition with carbendazim 12% + mancozeb 63% WP.

There was positive correlation between concentration and inhibition of growth of pathogen. It is also observed that with increasing concentration of all fungicides, inhibition of growth of pathogen also increased, except azoxystrobin 11% + tebuconazole 18.30% SC, epoxiconazole 50 g/l + pyraclostrobin 133 g/l, tebuconazole 50% + trifloxystrobin 25% WG and zineb 68% WP + hexaconazole 4%WP which give almost cent per cent inhibition at lower concentration. carbendazim 12% + mancozeb 63% WP and cymoxanil 8% + mancozeb 64% WP at 1000 ppm concentration showed 69.54 and 48.85 per cent inhibition of mycelial growth, respectively which were higher as compared to their lower concentration of 100, 250 and 500 ppm (Fig.-3).

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