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VA Chavan
Department of Plant Pathology and Agricultural Microbiology, Post Graduate Institute, MPKV, Rahuri, Maharashtra, India

PB Khaire
Department of Plant Pathology and Agricultural Microbiology, Post Graduate Institute, MPKV, Rahuri, Maharashtra, India

CG Sawant
Central Agricultural University, Imphal, Manipur, India

AG Shinde
Department of Plant Pathology and Agricultural Microbiology, Post Graduate Institute, MPKV, Rahuri, Maharashtra, India

RA Yumlembam
Central Agricultural University, Imphal, Manipur, India

Corresponding Author:
CG Sawant
Central Agricultural University, Imphal, India

In vitro assessment of efficacy of different fungicides against *Macrophomina phaseolina,* a green-gram blight pathogen of Western Maharashtra

VA Chavan, PB Khaire, CG Sawant, AG Shinde and RA Yumlembam

Abstract
The *in vitro* assessment of efficacy of nine systemic, non-systemic and combi-fungicides revealed that, all the systemic fungicides (propiconazole, tricyclazole and carbendazim) significantly inhibited the colonial growth of *Macrophomina phaseolina*, a blight pathogen of green-gram crop. Propiconazole and tricyclazole gave 100% mycelial growth inhibition of test fungus at all the three concentrations (0.05, 0.1 and 0.15%), while carbendazim showed complete inhibition at 0.15% conc. Among the non-systemic and combination fungicides, carbendazim + mancozeb completely restricted the mycelial growth of test fungus at all concentrations (0.15, 0.2 and 0.25%). Mancozeb and thiram were also found effective. The *in vitro* assessment of fungicide is an important aspect in the determination and selection of fungicide for field management of the concern pathogen.

Keywords: *Macrophomina phaseolina*, mung-bean blight, fungicides, disease management

1. Introduction
Mung-bean (*Vigna radiata* L. Wilczek), is one of the most important pulse crops cultivated in countries like Burma, Shri Lanka, South and North America, China, Africa and India. India alone accounts for about 2/3rd of total global production of mungbean. More than 80 per cent of mung-bean production comes from10 states *viz.*, Rajasthan, Madhya Pradesh, Maharashtra, Bihar, Karnataka, Tamil Nadu, Gujarat, Andhra Pradesh, Odisha and Telangana (Anonymous, 2018) [1]. Maharashtra rank third in production of mungbean after Rajasthan and Madhya Pradesh. Mung-bean is primarily grown in kharif season but recently many early varieties are developed which can be grown in spring and summer season.
Numerous maladies that are brought on by nematodes, bacteria, viruses, fungus and other abiotic stressors cause mungbean Khaire *et al*., (2018) [6]. The growth and development of the crop is hampered by a number of diseases, including powdery mildew disease (PMD) (*Erysiphe polygoni* DC), *Cercospora* leaf spot (*C. canescens*, *C. cruenta*) and root disease complex (*Pythium* spp., *Rhizoctonia solani*, *Macrophomina phaseolina* (Khaire *et al*, 2020) [8], *Fusarium* spp.) Among these, leaf blight disease (LBD) causing pathogen (*Macrophomina phaseolina*) is reported to be serious pathogen on mung-bean causing heavy economic losses in Maharashtra states (Zote *et al*., 1983; Khaire *et al*, 2020) [18, 8]. The severity of the disease increases with increase in temperature. Microsclerotia and mycelia of *M. phaseolina* overwinter on the soil or plant debris and serve as primary inoculum. The fungus can survive up to 2-3 years as a mycelium, in asymptomatic seeds, or as microsclerotia in the soil or plant debris. Under dry condition, microsclerotia can survive up-to 15 years in the soil. The fungus cause disease symptom in hot and dry weather. *Macrophomina phaseolina* competes well with other soil pathogens when soil nutrient level is low and soil temperature is high. Plant debris are the niche for the formation of sclerotia that overwinter in dry soil. Seeds produced in the same field by healthy plant can become contaminated with microsclerotia as a latent infection and spread the pathogen to a new field. To manage this important pathogen of mung-bean crop, the selection of proper fungicide is important, and therefore *in vitro* assessment of efficacy of different systemic, non-systemic and combination fungicides were carried out against the pathogen *Macrophomina phaseolina* isolate of western Maharashtra.

2 Materials and Methods
2.1 Symptomology of the LBD
2.1.1 Visual examination: Gradual development of LBD symptoms on mungbean plants were

recorded by visual observation in field and on inoculated plants in glasshouse. Mungbean plants with typical symptoms were collected and brought to the laboratory for examination and isolation.

2.1.2 Microscopic examination
Diseased leaf samples showing LBD symptoms were brought to the laboratory and washed in tap water to remove extraneous material. Free hand sections of the diseased specimens were cut with sharp blade. Also diseased specimens were scrapped and diluted in distilled water. Leaf tissue sections and suspension of scrapping were put in water drop on clean glass slides, covered with cover slip and mounted initially under low power objective lens of the compound microscope (Make: Labomed Vision, 2000) to record the observations for presence of the test pathogen, its mycelium and spores if any.

2. 2 Isolation of LBD pathogen from mung-bean crop grown in western Maharashtra LBD infected samples were washed with running tap water and kept on blotter paper for soaking. In laminar air flow, the PDA medium (previously prepared and sterilized) was poured into sterilized Petri plates and allowed to solidify. The sample was cut with sterilized blade into small pieces keeping half healthy and half diseased portion intact. These pieces were surface sterilized with 0.1% aqueous solution of mercuric chloride ($HgCl_2$) for two minutes and then washed by giving three changes with sterile distilled water to remove traces of mercuric chloride and blot dried. These surface sterilized diseased pieces were aseptically inoculated on the solidified and cooled PDA medium in Petri plates. Inoculated Petri plates then kept for incubation in BOD incubator at 27±2 °C. After 6-8 days growth of fungus was observed. Using single hyphal-tip technique, the fungus was sub-cultured aseptically on the PDA slant in test tubes. Number of slants were prepared and stored in refrigerator for further studies.

2.3 Identification
Identification of isolated fungus was carried out by visual examination and by studying cultural and morphological characters using microscope. The pathogen was identified on the basis of character of the mycelium growth and colour, size and shape of sclerotia.

2.4 Morphological and Cultural Characterization
2.4.1 Morphological characterization
The morphological characteristics of *M. phaseolina viz*., size and shape of sclerotia and mycelium were studied with the help of compound microscope. The slide of the fungus was prepared by staining an actively growing fungus with cotton blue. The size of sclerotia was measured using ocular micrometre (calibrated using stage micrometre) under the compound microscope.

2.4.2 Cultural characterization
The cultural characters of *M. phaseolina* were studied on eight different culture media *viz*., Yeast extract agar, Yeast mannitol agar, Corn meal agar, V8 juice agar, Czapek's dox agar, Oat meal agar, Malt extract agar and Potato dextrose agar. Except PDA, all the media used were readymade. All media were prepared in 100 ml conical flasks and sterilized in autoclave at 15 Lbs / $inch^2$ pressure for 20 min. After sterilization all the media were brought into Laminar air flow. Each media was poured into three Petri plates (20 ml each) and allowed to solidify. After solidification, 5 mm disc of *M. phaseolina* was transferred in all the Petri plates using cork-borer. Three replications of each media were taken. Plates of each media were placed in a separate BOD bag and incubated at 27 ± 2 °C.
The observations on radial mycelia growth/colony diameter (mm), colony colour, colony morphology and sporulation were recorded at 24 hrs interval and continued till one week after inoculation. Sclerotial production was determined on the basis of microscopic observation. - = No sclerotial formation, + = Poor sclerotial formation, + + = Fair sclerotial formation, + + + = Good sclerotial formation, + + + + = Excellent sclerotial formation

2.5 *In-vitro* evaluation of fungicide against radial mycelial growth of *M. phaseolina*
Nine fungicides were assessed *in-vitro* against *M. phaseolina* by applying poison food technique (Nene and Thapliyal, 1993) [15] using PDA as basal medium. All the fungicides were tested at three different concentrations (0.05% less than of recommended concentration, recommended concentration and 0.05% more than of recommended concentration). Based on active ingredient, requisite quantity of each test fungicide was calculated and mixed thoroughly with autoclaved and cooled (40 °C) PDA medium in conical flasks to obtain desired concentrations of the test fungicides. Fungicide amended PDA medium was then poured aseptically in Petri plates and allowed to solidify at room temperature. After solidification of the medium, all the plates were inoculated aseptically with 5 mm culture disc obtained from a week old actively growing pure culture of *M. phaseolina*. The disc was placed on PDA in the centre of the Petri plate and plates were incubated in inverted position at 27 ± 2 °C. Each of the test fungicide and its concentration was replicated thrice. Petri plates filled with plain PDA (without fungicide) and inoculated with then culture disc of *M. phaseolina* were maintained as control. Colony diameter of the test pathogen was measured after 4, 6 and 8 days of inoculation and sclerotia formation was recorded after 8 days of inoculation. The per cent growth inhibition (PGI) of the pathogen was worked out by using formula given by Vincent (1927) [28].

$$\text{Percent inhibition} = \frac{C - T}{T} \times 100$$

Over control
Where,
C = Mycelial growth in control plate and T = Mycelial growth in treatment plate

Experimental details: Design: CRD**,** Replications: Three**,** Treatments: Ten

3. Result and Discussion
3.1 Symptomology
The typical symptoms of LBD (*M. phaseolina*) were observed on the mungbean plants in field and glasshouse (Fig 1 A, B, C). *M. phaseolina* infected the mungbean plant at all growth stages but maximum disease severity was observed in 4-6 weeks old plants. The disease was characterised by appearance of small, circular to irregular brown to reddish

brown spots started from the margin of the leaf. Later these spots enlarged and coalesced to form big patches all over the leaf. Prolonged warm and humid weather caused drying and defoliation of affected plants. The disease also caused shrivelling of the pods and grains. Tandel *et al*. (2015) [20] reported the symptoms of mungbean blight as small circular to irregular, dark brown to reddish brown or black lesions appears on or near the margin of leaves, which enlarge and coalesced, spot gradually spreading into a large, irregular necrotic spots with brown periphery and grey colour centre. Magar (2009) [21] and Khaire *et al*., 2023 [2] also studied the symptoms of leaf blight of green gram caused by *Macrophomina phaseolina* (Tassi.) Goid and reported that the blight started from the margin of the leaf, initially with small irregular, dark brown spots and proceeded inward. Under moist and warm weather with intermittent rains, these spots coalesced to form bigger patches and the whole leaf was blighted.

3.2 Isolation and Purification of the Pathogen

The fungus was isolated from the diseased leaf samples collected from research field. The surface sterilized diseased bits were inoculated on potato dextrose agar medium in Petri plates and incubated at 27 ± 2 °C. After 5-7 days whitish growth of fungus was appeared on PDA which later became brown to dark (Fig 1 D). Magar (2009) [21] and Khaire *et al*., 2023 [2] isolated *Macrophomina phaseolina* from the blight infected leaves of green gram. Purification of the isolated pathogen was done by single hyphae isolation technique. The purified culture was maintained on potato dextrose agar slants for further use during entire course of investigation (Fig 1 E).

3.4 Identification of Pathogen

The identification of fungus was carried out by studying the cultural and morphological characters such as mycelium, sclerotia and growth patterns. The pathogen produced mycelium and sclerotia (Fig 1 F, G). Pycnidiospore formation was not found in the culture. The mycelium was septate and initially white and later became dark (Fig 1 G). Sclerotia were black with various shapes and size. The studies on morphological and cultural characters of test pathogen showed its similarity with *M. phaseolina* as described by Tandel *et al.* (2015) [20] and Khaire *et al*., 2023 [2]. Pathogenicity test was carried out by Koch's postulates and fungus isolated was confirmed as *Macrophomina phaseolina.*

3.5 Morphological and Cultural Characters of Pathogen

3.5.1 Morphology of *M. phaseolina*

Mycelium

The colony of pathogen (*Macrophomina phaseolina*) grew rapidly on PDA and achieved a full growth within 7 days at 27±2 °C temperature. Initially the pathogen produced white mycelium with linear and profuse growth (Fig 1 D). Later the colour of mycelium gradually turned brown to black due to the formation of sclerotia. The mycelium was hyaline, branched and septate with diameter ranging from 1.85 – 6.90 µm (Fig 1 F, G). The morphological characters reported shows its similarity with the morphological characters of *M. phaseolina* described by Agrawal (1989) [22] and Tandel (2004) [17]. Suryawanshi *et al.*, (2008) [23] and Khaire *et al*., 2023 [2] reported that the mycelium of *M. phaseolina* was superficial, hyaline to brown, septate and tree like in form and the sclerotia produced by *Macrophomina phaseolina* were black to brown in colour, round to oblong of irregular as shape.

Sclerotia

Abundant sclerotial formation was observed in the 10 days old culture. Size, shape and colour of sclerotia was recorded under microscope. The sclerotia were dark brown to black in colour and hard. Sclerotia varied in shape from irregular to oval or spherical, more or less round measuring from 66.40 to 188.75 µm in diameter (Fig 1 F). Pycnidia were not produced in the culture. Tandel (2004) [17] and Khaire *et al*., 2023 [2] reported the diameter of sclerotia of *M. phaseolina* as 68.20 to 196.15 and 67.92 to 195.18 µm respectively.

Fig 1: Symptomatology of LBD and isolation and identification of *M. Phaseolina* (A) Blightening of leaves (B) Shrivelling of pods (C) Completely dried plant (D) Pure culture in Petri plate, (E) In test tubes (F) Sclerotia bodies (G) Mycelium

3.6 Effect of Culture Media on Mycelial growth of *M. phaseolina*

Cultural characteristics of *M. phaseolina* were studied *in-vitro* on eight different synthetic and semisynthetic media. The observations recorded on mycelial growth, colony colour, colony diameter and sporulation etc. are presented in Table 1 and Fig 2.

3.6.1 Colony characters

The mean radial mycelial growth of the fungus on different solid media ranged between 34.60 mm (Corn meal agar) to 90 mm (PDA) (Fig 2). The result shows that, of the eight-culture media tested, Potato dextrose agar was most suitable and encouraged maximum radial mycelial growth (90.00 mm). Oat meal agar (84.30 mm), Yeast mannitol agar (82.26 mm) and V8 juice agar (78.13 mm) also recorded significant growth. This was followed by Malt extract agar (51.03 mm), Yeast extract agar (37.40 mm) and Czapek's dox agar (36.33 mm). Whereas Corn meal agar showed least mycelial growth (34.60 mm).

3.6.2 Colony colour and sporulation

The pathogen exhibited variety of colours on different media up to ten days. Fungus produced brown to black colour on Potato dextrose agar, Oat meal agar, Corn meal agar media, while on Yeast mannitol agar and Yeast extract agar media, it showed grey to black colour. Pathogen exhibited whitish to dirty white colour on Czapek's dox agar and Malt extract agar. On the other hand, it showed pale green to grey colony on V8 juice agar media (Fig 2).

Table 1: Effect of different culture media on radial mycelial growth, cultural characteristics and sporulation of *M. phaseolina*\

Sr. No.	Media	Colony Diameter * (mm)	Cultural Characteristics	Sclerotial production
1	YEA	37.40	Colony colour initially grey, later turned to dark. Mycelial growth dense.	+++
2	YMA	82.26	Grey to black colony. Mycelial growth dense and flat.	+++
3	CMA	34.60	Brown to black colony colour. Thin and poor mycelial growth.	+
4	V8 JA	78.13	Initially pale green colony later turned grey, uniform mycelium growth.	+++
5	CDA	36.33	Colony colour dirty white, spares mycelium with irregular margin.	++
6	OMA	84.30	Light black colony, mycelial growth flat and fine.	++++
7	MEA	51.03	Whitish colony with black margins, thick, cottony and raised mycelium growth.	++
8	PDA	90	Colony initially white then slowly turned brown to black. Thick, aerial and good mycelial growth.	++++

Sclerotial production: (Excellent - ++++), (Good - +++), (Fair - ++), (Poor - +), (No sporulation -)
*-Mean of three replications.

All the eight-culture media tested exhibited a wide range of sporulation of test fungus. Potato dextrose agar and Oat meal agar recorded excellent (+ + + +) sporulation. Good sporulation (+++) occurred on V8 JA, YMA and YEA. CDA and MEA exhibited fair (++) sporulation, while CMA showed poor (+) sporulation. This result on effect of culture media on growth and sporulation of *M. phaseolina* coincide with those reported by Tandel (2012) [24] and Priya Santosh (2006) [25].

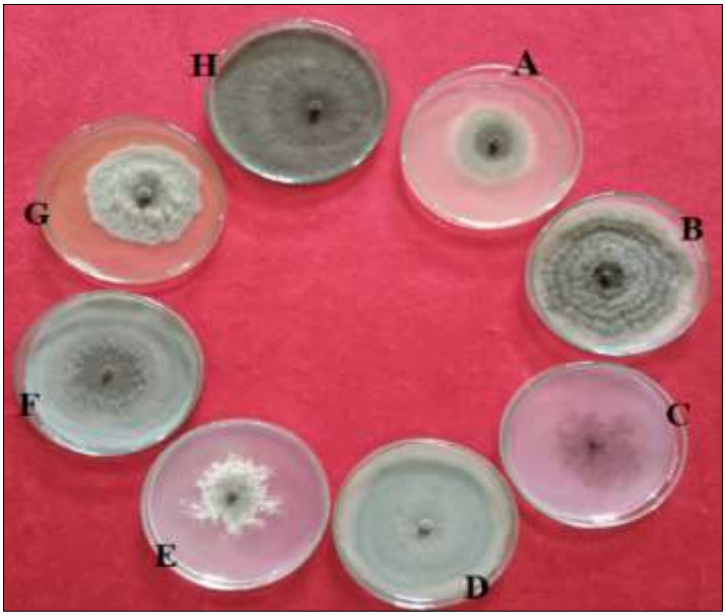

Fig 2: Cultural characteristics of the *M. phaseolina* on different media A- YEA, B-YMA, C-CMA, D-V8 JA, E-CDA, F-OMA, G- MEA and H-PDA

3.7 *In vitro* evaluation of fungicides against *M. phaseolina*

Nine fungicides were evaluated *in vitro* for their efficacy in inhibiting mycelial growth of *M. phaseolina.* The fungicides (systemic, non-systemic and comb.) were tested at three different conc. (0.05% less than of recommended conc., recommended conc. and 0.05% more than of recommended conc.). The results on the per cent mycelial growth inhibition of the fungus are presented in Table 2, 3, 4 and Fig. 3 (A, B & C).

3.7.1 Mycelial growth inhibition

3.7.1.1 At 0.05% less than of recommended concentration

From the data presented in Table 2 and Fig. 3 (A), it is revealed that, the mean per cent mycelial growth inhibition ranged from 3.18 to 100% (Fig 3. A). Among systemic fungicides, propiconazole and tricyclazole showed complete (100%) inhibition of the test pathogen, while carbendazim also gave good mycelial growth inhibition (94.33%). In case of non-systemic and combination fungicides, carbendazim + mancozeb given 100% mycelial growth inhibition over control. This was followed by mancozeb (82.92%), thiram (65%), captan (59.71%) and propineb (39.44%). However, copper oxychloride showed least (3.18%) mycelial growth inhibition.

3.7.1.2 At recommended concentration

At recommended conc. all the fungicides showed near about similar pattern of mycelial growth inhibition (Table 2) which was observed at 0.05% less than of recommended conc. Mean per cent mycelial growth inhibition ranged from 7.00 to 100% (Fig 3. B). Out of all systemic fungicides, tricyclazole and propiconazole was found most effective which recorded 100% mycelial growth inhibition of test pathogen. Fungicide, carbendazim also found effective with 96.55% mycelial growth inhibition. Among non-systemic and combi. fungicides, carbendazim + mancozeb completely restricted (100%) the growth of pathogen. However, fungicide mancozeb (91.96%) and thiram (83.15%) also significantly inhibited the growth of test pathogen over untreated control. This was followed by captan (63.66%) and propineb (48.44%). However, copper oxychloride recorded less (7.00%) mycelial growth inhibition.

Table 2: *In vitro* efficacy of systemic, non-systemic and combination fungicides against mycelial growth of *M. phaseolina* (0.05% less than of recommended conc.)

Sr. No.	Test Fungicide	Concentration (%)	Colony diameter* (mm)	Per cent inhibition of mycelial growth
1	Thiram	0.15	31.50 (34.12)	65.00 (53.07)
2	Carbendazim	0.05	05.10 (13.03)	94.33 (76.19)
3	Captan	0.2	36.26 (37.01)	59.71 (50.57)
4	Copper oxychloride	0.25	87.13 (68.95)	3.18 (10.20)
5	Mancozeb	0.15	15.36 (23.06)	82.92 (65.56)
6	Tricyclazole	0.05	0.00 (0.00)	100 (90.00)
7	Propiconazole	0.05	0.00 (0.00)	100 (90.00)
8	Propineb	0.05	54.50 (47.56)	39.44 (38.88)
9	Carbendazim + Mancozeb	0.15	0.00 (0.00)	100 (90.00)
10	Control	-	90.00 (71.53)	0.00 (0.00)
	S.E. ±		0.31	0.34
	C.D. at 5%		0.93	1.03

*: Mean of three replications
Figures in parenthesis are arc sine transformed value

Table 3: *In vitro* efficacy of systemic, non-systemic and combination fungicides against mycelial growth of *M. phaseolina* (recommended conc.)

Sr. No.	Test Fungicide	Conc. (%)	Colony diameter * (mm)	% inhibition of Mycelial growth
1	Thiram	0.2	15.16 (22.90)	83.15 (65.73)
2	Carbendazim	0.1	3.10 (9.88)	96.55 (79.52)
3	Captan	0.25	32.70 (34.86)	63.66 (52.91)
4	Copper oxychloride	0.3	83.70 (6.17)	7.00 (15.25)
5	Mancozeb	0.2	7.23 (15.56)	91.96 (73.52)
6	Tricyclazole	0.1	0.00 (0.00)	100 (90.00)
7	Propiconazole	0.1	0.00 (0.00)	100 (90.00)
8	Propineb	0.1	46.40 (42.91)	48.44 (44.08)
9	Carbendazim + Mancozeb	0.2	0.00 (0.00)	100 (90.00)
10	Control		90.00 (71.53)	0.00 (0.00)
	S.E. ±		0.56	0.63
	C.D. at 5%		1.68	1.87

*-Mean of three replications, Conc-Concentration
Figures in parenthesis are arc sine transformed value

3.7.2 At 0.05% more than of recommended concentration

Result presented in Table 3 clearly show that, the mean per cent mycelial growth inhibition ranged from 14.30 to 100% (Fig.1 C). All systemic fungicides i.e., carbendazim, tricyclazole and propiconazole exhibited complete (100%) mycelial inhibition of test pathogen and out of all non-systemic and combi. fungicides, carbendazim + mancozeb was found most effective which recorded 100% mycelial growth inhibition. The fungicide mancozeb (93.44%), thiram (85.26%), captan (68.71%) and propineb (54.11%) showed significant reduction in mycelial growth of pathogen over control, whereas copper oxychloride (14.30%) found less effective in inhibiting the mycelial growth of pathogen.

Thus, in present investigation carbendazim + mancozeb, propiconazole, tricyclazole and carbendazim found most effective fungicides in inhibiting the radial mycelial growth of *M. phaseolina*. However, other fungicides tested were also found fungistatic to test pathogen and significantly inhibited mycelial growth over untreated control.

Table 4: *In vitro* efficacy of systemic, non-systemic and combination fungicides against mycelial growth *M. phaseolina* (0.05% more than of recommended conc.)

Sr. No.	Test Fungicide	Conc. (%)	Colony diameter * (mm)	% inhibition of Mycelial growth
1	Thiram	0.25	13.26 (21.34)	85.26 (67.40)
2	Carbendazim	0.15	0.00 (0.00)	100 (90.00)
3	Captan	0.3	28.16 (32.03)	68.71 (55.96)
4	Copper oxychloride	0.35	77.13 (61.41)	14.30 (22.19)
5	Mancozeb	0.25	5.90 (14.06)	93.44 (75.16)
6	Tricyclazole	0.15	0.00 (0.00)	100 (90.00)
7	Propiconazole	0.15	0.00 (0.00)	100 (90.00)
8	Propineb	0.15	41.30 (39.97)	54.11 (47.33)
9	Carbendazim + Mancozeb	0.25	0.00 (0.00)	100 (90.00)
10	Control		90.00 (71.53)	0.00 (0.00)
	S.E. ±		0.42	0.46
	C.D. at 5%		1.25	1.39

*Mean of three replications, Figures in parenthesis are arc sine transformed value

Fig 3: *In vitro* efficacy of fungicides against mycelial growth of *M. phaseolina* A) @ 0.05% less than of recommended conc. B) @ recommended conc. C. @ 0.05%more than of recommended Conc

The effectiveness of fungicides against this pathogen prevalent in different geographical region were studied by different researcher. Thombre *et al.* (2018) reported, carbendazim 12% + mancozeb 63% WP and carbendazim 50% WP as most effective fungicides which completely inhibited (100%) the growth of *Macrophomina phaseolina*. The effectiveness of tricyclazole 75% WP against *M. phaseolina* was reported by Tripathy *et al.* (2018) [26]. Khamari and Patra (2018) [27] observed the complete mycelial inhibition (100%) of *M. phaseolina* at 0.05 and 0.10% of carbendazim 50% WP and propiconazole 25% EC. Khaire *et al*, 2018 [6] reported that, highest average mycelial inhibition was recorded with the fungicides carbendazim (100%). The next best fungicide found effective in mycelial inhibition was hexaconazole (99.97%) This was followed by propiconazole (85.98%), carboxin (83.75%), and tebuconazole (80.99%), penconazole (76.79%) and thiophanate methyl (72.59%). The fungicide difenconazole found less effective with 66.16 per cent inhibition, of *Macrophomina phaseolina*. The growth of isolate of *Macrophomina phaseolina* prevalent in Western Maharashtra was effectively checked with carbendazim+mancozeb (SAAF), trycyclazole and propiconazole fungicides which can be included in plant protection schedule of this pathogen.

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