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Evaluation of Phyto-extracts, Biological Agents and Chemicals against the Development of *Alternaria brassicae in vitro* and *in vivo*

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Authors' contributions

This research work was carried out by the team of authors. Author MKB planned the study, carried out the layout in field and performed the statistical analysis, wrote the methodology and prepared the initial manuscript. Author TG collected the data from the field and laboratory, arranged them systematically, collected the review from different sources and checked the manuscript at final stage. Both authors read and approved the final manuscript.

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ABSTRACT

Alternaria leaf blight caused by *Alternaria brassicae* and *Alternaria brassicola*, is one of the most destructive diseases of mustard (*Brassica campestris, B. juncea* and *B.napus*) in West Bengal, causing considerable damages to the crop. The experiment was conducted under *in vitro* and *in vivo* conditions to see the effect of bio-agents, plant extracts and chemicals (fungicide and SAR compound) against *Alternaria brassicae*. Maximum inhibition in mycelial growth (95.56%) was observed with Mancozeb 75% Wp followed by *Lantana camera* (80%), Salicylic acid (73.33%), *Allium sativum* (54.44%) and *Zingiber officinale* (17.78%) in comparison to control. Foliar spray with fungicide (Mancozeb 75% WP @ 0.2%) was found to be most effective in reducing disease severity (81,23%) and infection rate which increased the yield (77.23%) of mustard over untreated control. Among the plant extracts, *Lantana camera* was found to be excellent in controlling the

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Alternaria blight infection in the field (71.92% reduction in disease severity and 68.18% increase in yield) in comparison to salicylic acid (SAR compound) and bio-agent (*Trichoderma viride*) 48.33% and 36.27% reduction in disease severity respectively.

Keywords: Alternaria brassicae; mustard; salicylic acid; bio-agent; plant extracts; fungicide.

1. INTRODUCTION

Rapeseed-mustard (also called oilseed brassicas) is a group of crops contributing nearly 32% of the total oilseed production in India, and it is the second largest indigenous oilseed crop. Out of 75.55 million tonnes of estimated rapeseed-mustard produced over 30.51 million ha in the world, India produces 7.36 million tonnes from 6.18 million ha with 1190 kg ha^{-1} productivity [1].The production of rapeseed and mustard in India and West Bengal was 7.036 million tonnes and 1.021 million tonnes respectively, in 2010-2011. *Alternaria brassicae* is responsible for major seed yield losses in rapeseed and mustard and this is the most important aspect of its economic impact [2]. It has the ability to survive in seeds for several months at different temperatures and relative humidity [3,4]. There is a wide gap exists between the potential yield and the yield realized at the farmer's field owing to the fact that these crops are exposed to several biotic and abiotic stresses. Among the biotic stresses, Alternaria blight disease caused by *Alternaria brassicae* (Berk.) Sacc. is one of the important diseases of rapeseed-mustard with no proven source of transferable resistance in any of the hosts. The yield loss due to this pathogen is up to 47% in the entire mustard growing area [1].The spots on all *Alternaria brassicae* infected plant parts are always covered with an olive coat, usually composed of concentric zones formed by aggregations of conidiophores with conidia. Each spot is frequently surrounded by a chlorotic halo [5]. The species of *Alternaria brassicae* produce dark brown to olivaceous brown colour, branched, septate mycelium in the host [6]. Kolte [7] however had reported *Alternaria brassicae* to exhibit slow and rudimentary growth in media and to form chlamydospores in less frequency as compared to *A. brassicicola*. According to Ellis [8], *Alternaria* contains 44 species. It has been found that *Alternaria* species are either parasites on living plants or saprophytes on an organic substrate. And the range of hosts of pathogenic *Alternaria* is very broad. In the absence of resistant cultivars, fungicides provide the most reliable means of disease control [9]. Foliar sprays of aqueous bulb extract of *Allium sativum* (garlic) and *Eucalyptus globulus* (Eucalyptus) have been reported to effectively manage the *Alternaria* blight on leaves and pods and could be an eco-friendly substitute for chemical fungicide [1,10]. Foliar application of soil inhabitants isolates of *T. harzianum* and *P. fluorescens* were found effective in the management of *Alternaria* blight [11]. Salicylic acid, Chitosan, etc. one of the important aspects of induced resistance is that it is not underlined by genome alterations (mutations, integration of foreign genetic material), which enhances it's biological safety [12]. With the view of the above an experiment was conducted to developed a suitable package for the control of *Alternaria* blight with the help of bio-agents, plant extracts and chemicals (fungicides and SAR compounds).

2. MATERIALS AND METHODS

2.1 Preparation of Pure Culture

Isolates of *Alternaria brassicae* were obtained from infected leaf samples of rapeseed and mustard collected from the field. Blighted leaf pieces (2 mm) were surface sterilized with 0.1% Mercuric chloride ($HgCl_2$) for one minute, rinsed in sterile water for 1 minutes for three times and then finally placed on Potato Dextrose Agar (PDA) plates. Fungal growth was observed after 5-7 days of incubation at 25ºC. Thereafter, growing mycelia from the margin of apparently distinct colonies of the leaf spot pieces on the medium were aseptically transferred into another Petri plate containing PDA medium, where it was grown for 7 days at 23±2°C in the BOD incubator. On the basis of their conidiophores and conidial morphology as described by Simmons [13], the pathogen was identified as *Alternaria brassicae* (Berk.) Sacc. These isolates were purified and preserved as PDA slants at 4ºC [14].

2.2 Evaluation of Phyto-extract, Biological Agent, and Chemicals against *Alternaria* Leaf Blight of Mustard

2.2.1 *In vivo* evaluation

Phyto-extract were prepared from, bulb of garlic (*Allium sativum* L), rhizome of ginger (*Zingiber*

officinale Rose) and leaves of *Lantana camera* by washing with tap water followed by distilled water at the rate of one gm of tissue in one ml of water (1:1w/v) and filtered through double layers of cheesecloth. This formed the standard solution (100%). The Phyto-extracts were sprayed at the rate of 10% prepared from standard solution. Fungal bio-agent *Trichoderma viride* at a rate of 20 g inoculum per liter of water and salicylic acid at a ratio of 2 ppm were sprayed before the appearance of the disease [15,16]. While phyto-extracts and Dithane M-45 at a ratio of 0.2% were sprayed after the appearance of the first symptoms in the field. Unsprayed plots were kept as control. The experiment was conducted in the field under a natural condition in a randomized block design with four replications. The second spray was made after 15 days of the first spray.

2.2.2 Preparation of fungicidal spray solution

The spray solution of the desired concentration was prepared by adopting the following formula given by Edward et al [17].

$$V = \frac{C \times A}{\%a.i.}$$

Where,

V = Volume / Weight of commercial fungicide ml or g
C = Concentration required
A = Volume of Solution to be prepared
% a.i. = percentage of active ingredient in commercial product

2.2.3 Percent decrease in PDI

Percent decrease in PDI was calculated by using this formula given by Vincent [18].

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

Where,

C = PDI observed in control treatment
T = PDI observed in different treatments

2.2.4 *In vitro* evaluation

The fungitoxicity of the chemicals and phyto-extracts were tested by poisoned food technique [19]. For *in vitro* evaluation of plant extract, 100 gm of fresh leaf materials of *Lantana camera,* bulb of garlic (*Allium sativum* L.) and rhizome of ginger(*Zingiber officinale* Rose) were harvested, washed thoroughly with running tap water, rinsed with distilled water, air dried and macerated separately with 100 ml of distilled water in a Waring blender. The extract was filtered through double-layered muslin cloth and centrifuged at 4000 rpm for 30 minutes. The supernatant was collected and filtered through Whatman No.1 filter paper. Five ml of each plant extract was incorporated in 50 ml of potato dextrose agar medium (PDA) and autoclaved for 20 minutes at 1.41 kg/cm^2 pressure. After sterilization, the molten media were poured into sterilized glass petriplates. After solidification, all the plates were inoculated individually with a 3mm diameter culture disc of *Alternaria brassicae*. Mancozeb (Dithane M-45) @ 0.02% and salicylic acid @ 2 ppm were dissolved in 50 ml of sterilized molten PDA prior to inoculation of *Alternariabrassicae*. PDA plates without chemicals and plans extract but inoculated with *Alternariabrassicae* served as control. Four replications were maintained for all the treatments and plates were incubated in BOD incubator at a temperature of 22-25°c. The colony diameter of the fungus was measured on 3rd, 4th and 5th day of incubation and compared with the colony growth of the fungus in control.

2.2.5 Assessment of disease severity

Assessment of disease severity was done by scorecard method following a 5 point scale (0-5) for scoring leaf spot disease. From each plot, 25 plants were randomly selected and tagged. Disease scoring was done at 45, 60 and 75 days after sowing.The rating scales used for the assessment of disease severity [20] are mentioned below,

0 => No symptoms
1 => 1-10% leaf area covered
2 => 11-25% leaf area covered
3 => 26-50% leaf area covered
4 => 51-75% leaf area covered
5 => >75% leaf area covered

Percent of disease index was calculated by using the techniques of Mc.Kinney [21] and the formula is,

[PDI = (Sum of all numerical ratings/ No. of leaflets observed X maximum rating used) X 100]

2.2.6 Apparent infection rate

The interval between the date of sowing and the appearance of first symptoms in different varieties and the interval between first incidence and final incidence of the disease were also recorded. The apparent infection rate of spread of the disease was calculated according to the following formula given by Vander Plank [22].

$$R = \frac{2.3}{t_2 - t_1} \left\{ \text{Log}\left(\frac{X_2}{1-X_2}\right) - \text{Log}\left(\frac{X_1}{1-X_1}\right) \right\}$$

Where,

r = Apparent infection rate at exponential growth stage
t_1 = First day of observation
t_2 = Last date of observation
X1 = Production of the disease on the first day of observation
X_2 = Production of the disease on last day of observation

3. RESULTS AND DISCUSSION

3.1 Effect Phytoextract, Biological agent, and Chemicals on the Development of *Alternaria* Leaf Blight of Mustard in Field

The effects of different treatments on the development of *Alternaria* leaf blight and the data obtained on various parameters are presented in Table 1.

The data showed the interaction between treatments and *Alternaria* blight severities, infection rates and yield of mustard. Foliar sprays with fungicide (Mancozeb 75% WP @ 0.2%) proved to be the most effective in reducing disease severity, infection rate and increased the yield over untreated control. Among the phyto-extracts, minimum *Alternaria* blight (15.23%) and maximum yield (1657.60 kg/ha) was recorded from *Lantana camera* followed by *Allium sativum* (24.25% PDI and yield 1422.40 kg/ha) and *Zingiber officinale* (42.25% PDI and yield 1142.4 kg/ha) which were significantly superior than untreated control (54.25% PDI and yield 985.6 kg/ha). Fungal antagonist i.e. *Trichoderma viride* was also found to be effective in controlling *Alternaria* blight of mustard (34.57 % PDI) and contributed significantly toward increasing the yield (1210.10 kg/ha). The resistance of plant against *Alternaria* blight of mustard was reported with the application of SAR compound (Salicylic acid). A reduction of 48.33 % in disease severity, and 38.63% increase in yield have been reported with salicylic acid (Figs. 1 & 2).

Among the various treatments tested for their efficacy against the *Alternaria* blight of mustard, the fungicide 75% WP @ 0.2%) was found to be most effective and reduced the severity of the disease up to 81.34%. Similar results with mancozeb (0.2%) against *Alternaria* blight were also reported by [23,24]. *Lantana camera* was the most effective plant extract and reduced the rate of infection considerably, followed by *Allium sativum* and *Zingiber officinale*. Extract of *Zingiber officinale* was less effective in comparison to other extract, Fungal antagonist (*Trichoderma viride*) and SAR compound (Salicylic acid were significantly superior to control in reducing the disease severity, rate of infection and grain yield.The difference in the effectiveness of extract may be due to variation in composition of anti-fungal compounds in different plants. The findings of [25,26] reporting efficiency of leaf extract of *Lantana camera* and *Alliums ativum* on disease severity and yield of mustard corroborates with the present results. The significant role of SAR compound (Salicylic acid) in controlling disease severity of *Alternaria* blight of mustard was probably due to the accumulation of PR protein, lignification, and production of callose –containing papillae in plants. Similar findings with salicylic acid @ μg/ml. against *Alternaria brassicae* were also reported by Atwal et al. [27].

3.2 *In vitro* Evaluation of Phyto-extracts and Chemicals against *Alternaria brassicae*

Phyto-extracts (*Lantana camera, Allium sativum* and *Zingiber officinale*), biological agent (*Trichoderma viride*), fungicide (Mancozeb 75% WP) and SAR compound (Salicylic acid) were tested *in vitro* for their efficacy against the pathogen (*Alternaria brassicae*). The inhibitory effect of individual treatment in terms of radial growth of mycelium was recorded at regular intervals and the data obtained are presented in Table 2.

Table 1. Effect of phyto-extract, biological agent and chemicals on the development of *Alternaria* leaf blight of mustard in the field

Sl. no	Treatment	Concen-tration	Percentage disease index	Apparent infection rate	Reduction of PDI over control (%)	Yield Kg./Ha	Increase in yield over control (%)
1.	Mancozeb75%WP	0.02%	10.12	0.1186	81.34	1747.20	77.27
2.	Salicylic acid	2ppm	28.03	0.1782	48.33	1366.40	38.63
3.	*T.viride*	4%	34.57	0.1649	36.270	1210.10	22.77
4.	*Lantana camera*	10%	15.23	0.1284	71.92	1657.60	68.18
5.	*Zingiber officinale*	10%	42.25	0.2416	22.11	1142.40	15.90
6.	*Allium sativum*	10%	24.25	0.1718	55.29	1422.40	44.31
7.	Control	--	54.25	0.2308	0.00	985.60	0.00
SE(treatment mean)=			**0.583**	**0.010**		**55.634**	
CD at (5%) =			**1.734**	**0.010**		**2.750**	

Table 2. *In vitro* evaluation of phyto-extracts and chemicals against *Alternaria brassicae*

Sl. no	Treatment	Concentration	Radial growth of mycelium 10 days after inoculation (mm)	Percentage inhibition in mycelial growth
1	Mancozeb75%WP	0.02%	4	95.56
2	Salicylic acid	2 ppm	24	73.33
3	*Lantana camera*	10%	18	80
4	*Zingiber officinale*	10%	74	17.78
5	*Alliums ativum*	10%	41	54.44
6	*Trichoderma viride*	4%	37	58.88
7	Control	--	90	--
SE(treatment	mean)=		0.822	
CD at (5%)=			2.44	

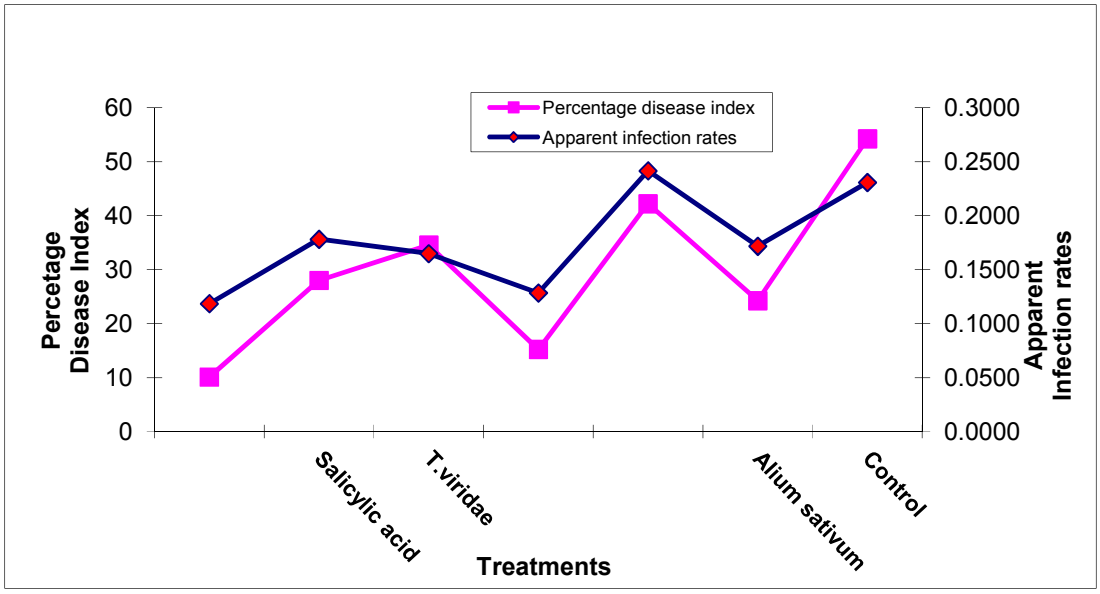

Fig. 1. Effect of Phyto-extract, biological agent and chemicals on diseas severity and apparent infection rate

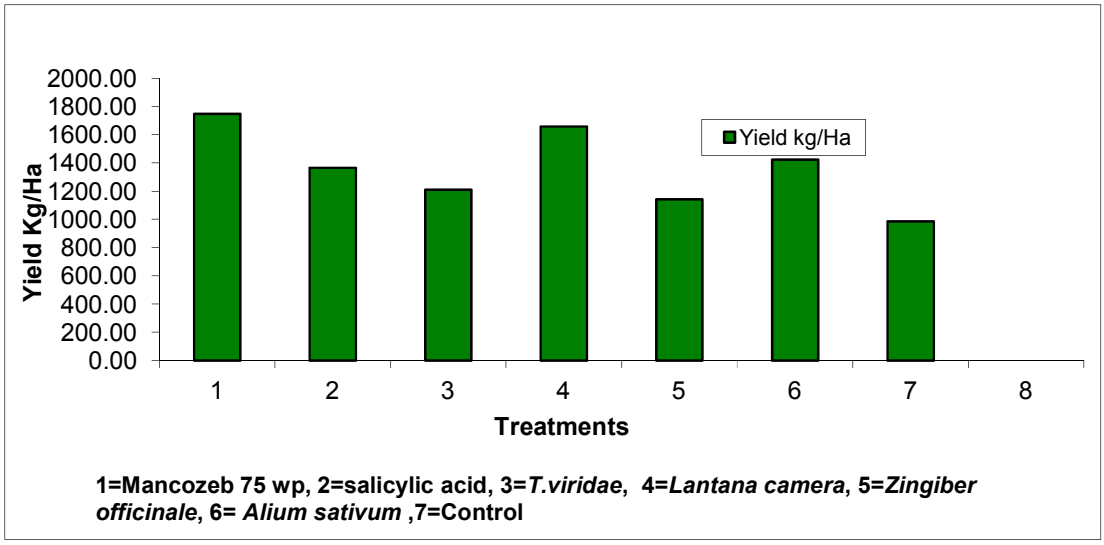

Fig. 2. Effect of phyto-extract, biological agent and chemicals on yield of mustard

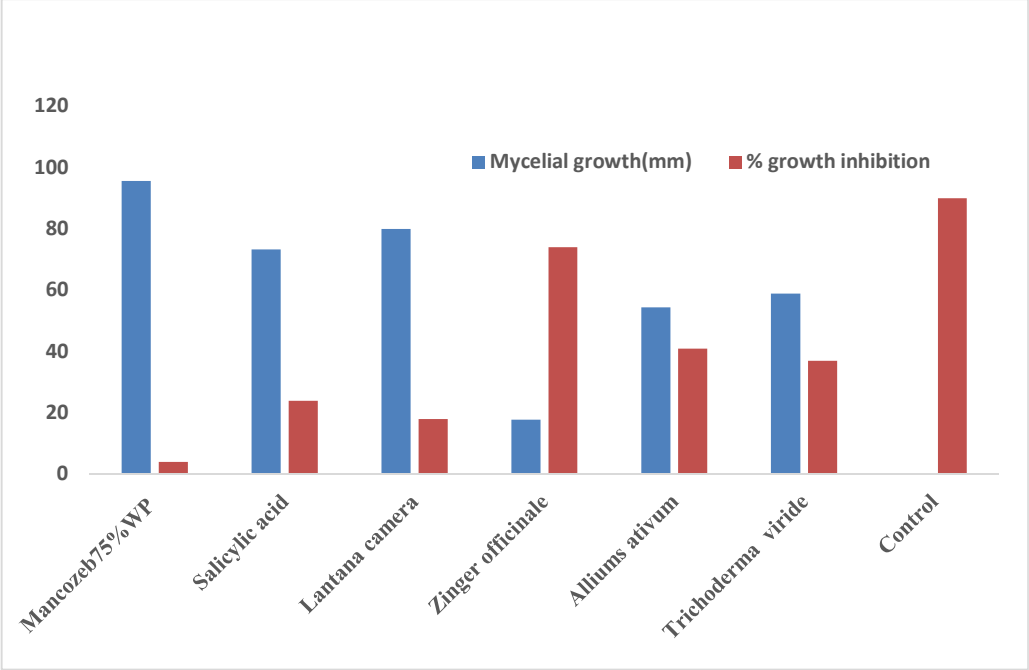

Fig. 3. *In vitro* evaluation of phyto-extract,fungicide, bioagent and salicylic acid against *Alternaria brassicae*

The average radial growth of *Alternaria brassicae* in PDA amended with various extracts, boi-agent and chemicals was greatly influenced and was significantly superior than control (Fig. 3, Plates 1 and 2). Minimum radial growth (4 mm) and maximum mycelium growth inhibition (95.56%) was observed with Mancozeb 75% WP. Among different plant extracts, *Lantana camera* exhibited less radial growth (18 mm) compared to *Allium sativum* (41 mm) and *Zingiber officinale* (74 mm) which was found 54.44% and 80% growth inhibition respectively. Extract of *Zingiber officinale* showed minimum mycelium (17.78%) growth. Salicylic acid, which was exhibited moderate effect *in vivo*, performed well in inhibiting the mycelium growth of fungi

(*Alternaria brassicae) in vitro* and showed 24 mm radial growth and 73.33% mycelium growth inhibition. The only bioagent *Trichoderma viride* exhibited 37 mm growth and 58.88% growth inhibition. Significant differences were exist among the phyto-extracs, bioagent and fungicide (Mancozeb 5% WP). However, the effect of *Lantana camera* and Salicylic acid did not differ significantly to each other. All the treatments were found statistically superior than control. The antifungal activity of Mancozeb 75% WP against *Alternaria brassicae* was further confirm the findings of Chattopadhyay et al. [24]. Leaf extract of *Lantana camera* was found to be equally effective in inhibiting the growth of *Alternaria brassicae* was probably due to the toxic compound contained in extract. Presence of excessive sugar content in PDA was probably determined the effect of salicylic acid against the growth of *Alternaria brassicae* as it decrease the starch content in host. The results of bio- agent further confirm the findings of [27,28].

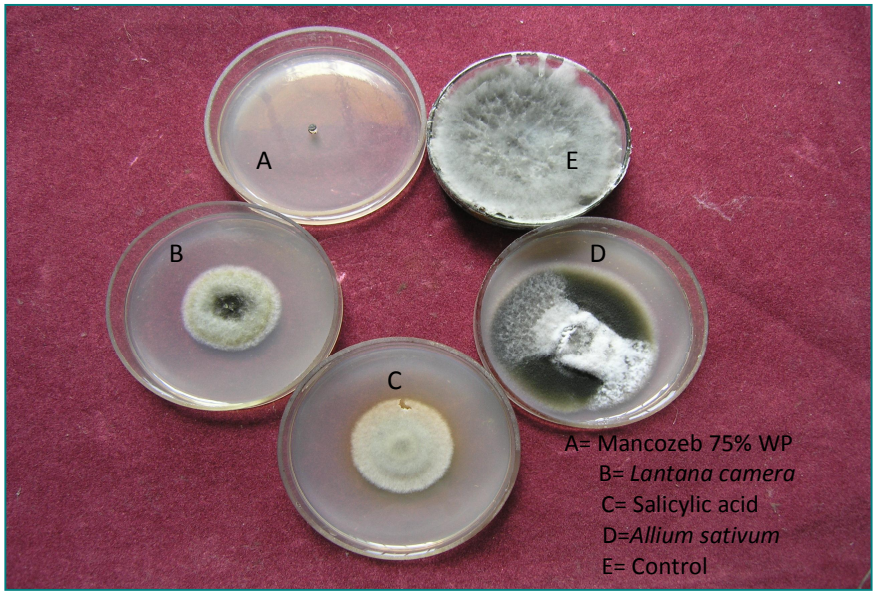

Plate 1. Inhibitory effect of treatments against the mycelial growth of *Alternaria brassica*e (upper view)

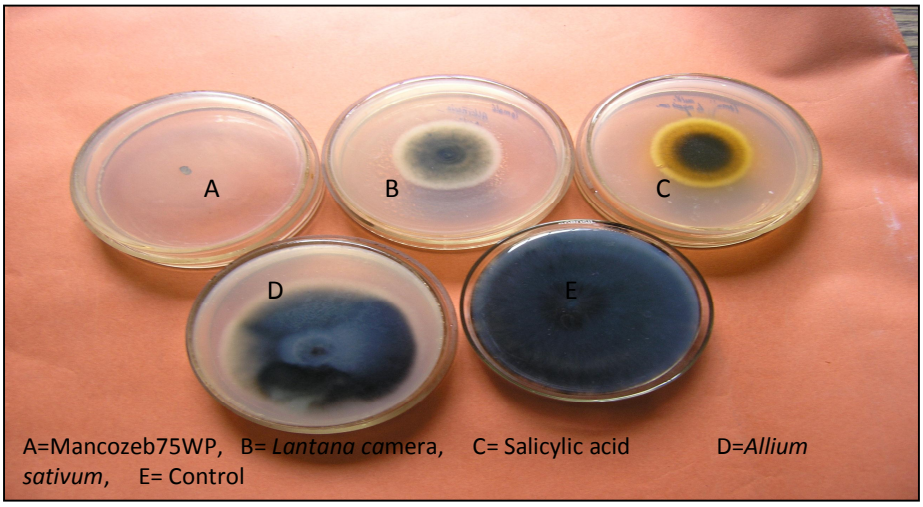

Plate 2. Inhibitory effect of treatments against the mycelial growth of *Alternaria brassica*e (back view)

4. CONCLUSION

One of the major constraints of mustard is that, the crop is infected by large number of diseases of which *Alternaria* blight is very common and destructive in West Bengal causing considerable damages to the crop. The aim of our study is to control the disease with the help of phytoextracts and other eco-friendly products. Fungicide (Mancozeb 75% WP @ 0.02%) proved most effective in reducing disease severity, infection rate, and increasing yield over untreated control. Among the phyto-extracts, minimum *Alternaria* blight infection in leaf (15.23%) and maximum yield (1657.60 kg/ha) was recorded in *Lantana camera*. *Trichoderma viride,* @ 2% and SAR compound, salicylic acid @ 2% were found less effective than Mancozeb and *Lantana camera* in controlling the *Alternaria* blight infection in field. *In vitro* evaluation maximum mycelium growth inhibition (95.56% followed by) was observed with Mancozeb 75% WP. Among the phytoextracts, *Lantana camera* exhibited less radial growth (18 mm) and higher mycelium growth inhibition (80.0%). This information will help the farmers for choosing an alternative method of disease control which reduces environmental pollution.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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