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Screening of Fungicide to Control *Ciboria carunculoides* Under Laboratory Conditions

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Abstract.- *Ciboria carunculoides* is one of the major fungal pathogen that attack mulberry grown worldwide as a crop for silkworm rearing. The use of chemical fungicides is one of the main tactics being used for the management of plant diseases. The effect of 15 fungicides on radial growth of *C. carunculoides* was studied in order to screen out fungicides which are highly effective against this fungus. Carbendazim was proved to the most aggressive showing the lowest EC_{50} value (8.04 mg/l), and glyphosate was the least virulent with a mean EC_{50} value of 8741.78 mg/l. The mixtures of fungicides at different concentration with mulberry juice were tested for *C. carunculoides* growth inhibition, and the results showed that Carbendazim + juice mixture was most aggressive having the lowest EC_{50} value (521.01 mg/l), when compared with Procymidone + juice mixture. The percent conidial germination of *C. carunculoides* was significantly affected by different concentration of Carbendazim and Glyphosate used at different concentrations in soil. Both the fungicides used in this study caused 100% inhibition of fungal germination at the concentration of 40 and 80 mg/l, respectively. Our study has helped to screen some highly effective fungicides for *C. carunculoides* management.

Keywords: Fungicide, *Ciboria carunculoides*, carbendazim, glyphosate.

INTRODUCTION

Mulberry is grown worldwide as a crop for silkworm rearing. Apart from this many parts of mulberry tree have been used as uncooked or processed foods for health care (Hong *et al*., 2007). Mulberry fruits also contain several substances with medical action. Due to increase in fruit demand, growing area of the tree has remarkably increased (Kishi, 1998). According to the index of plant diseases, mulberries are susceptible to many pathogens (Anonymous, 1960). *Ciboria carunculoides* (Siegler and Jenkins) Whetzel, *C. shiraiana* (Henn.) Whetzel and *Sclerotinia shiraiana* (Henn.) Imai have been reported separately as causal agents of the fruit diseases (Kishi, 1998; Kohn and Nagasawa, 1984; Whetzel and Wolf, 1945).

Ciboria carunculoides is one of the major fungal pathogens that attack mulberry fruits by causing disease named as "popcorn disease". Popcorn diseased mulberry fruits have greatly enlarged ovaries with small and succulent calyx lobes in comparison with fleshy lobes of normal fruits (Gray and Gray, 1987). Diseased mulberry fruits have ovaries filled with fungal mycelium rather than having normal plant tissues. The pathogen releases vast number of fungal spores, resembling a waxy substance on outside ovary wall. As a result, normal drupelets formation and maturation are prevented, thereby destroying the mulberries as edible fruit (Siegler and Jenkins, 1922).

The use of chemical fungicides is one of the main tactics being used for the management of plant diseases. Knowledge is scarce about the management of *C. carunculoides* by using chemical fungicides. Chai *et al*. (2005) showed that 25% Amistar SC was more effective on popcorn disease when compared with conventional pesticides carbendazim and thiophanate-methyl under field conditions. Therefore Amistar could be applied in production to control popcorn disease of mulberry. The aim of the present work was to test the impact of different fungicides on growth inhibition, sporulation and other biological parameters of *C. carunculoides* which can provide the basic

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information for future management of *C. carunculoides* under field conditions.

MATERIALS AND METHODS

Fungal strains

Ciboria carunculoides isolate, Cc01, originally isolated from Mulberry, deposited at the collection at Sericulture & Agri-food Research Institute of Guangdong Academy of Agricultural Sciences, identified as Hu *et al*. (2011) by PCR, was used in this study. To produce the inoculum for each assay, *C. carunculoides* was cultured on potato dextrose agar (Potato infusion 200 g/L; Dextrose 20 g/L and Agar 20 g/L) and incubated at 20±2°C for 10 days. Conidia were harvested with distilled water containing 0.03% Tween-80, (Whiga chemicals Shanghai). Conidia were counted in a Fuchs-Rosenthal hemocytometer and a suspension of 1×10^7 conidia/ml was prepared.

Fungicides

List of pesticides used in the study, molecular formula, manufacturer/supplier are given in Table I whereas the concentrations of different fungicides used in this study are given in Table II.

Effects of different fungicides

Radial growth

Potato dextrose agar (PDA) was sterilized at 121°C at 15 psi for 25 minutes. Freshly prepared fungal suspension (100 µl of 1×10^6 conidia ml^{-1}) was inoculated in the centre of plates using a micro applicator and was spread to cover the whole plate. The plates were incubated at 20 ± 2 °C, 80 ± 5% R.H., and L14:D10 h for 2-3 days. Mycelial discs together with medium (Ø1 cm) were removed and cultured on PDA having the different concentrations of fungicides (Tables II, III), respectively, and incubated at 20 ± 2°C. The same medium without fungicides served as a control. There were 10 Petri dishes for each concentration of different fungicides. Colony diameters were measured after 4-5 days. All the treatments were replicated three times. The average diameter of every colony was calculated as (long diameter + short diameter) / 2 (Ali *et al*., 2009). Inhibitory percentage on mycelia growth was calculated as:

$$\% \text{ Inhibition of mycelial growth} = [(Mc-Mt)/Mc]*100$$

where Mc=Dimeter of mycelium in control and Mt=Diameter of mycelium in treatment.

Table I.- Fungicides used in the study.

Fungicide (crude)	Molecular formula	Manufacturer/ supplier
Tebuconazole	$C_{16}H_{22}ClN_3O$	Bayer
Nicotinamide	$C_6H_6N_2O$	BASF
Metiram	$C_{12}H_{12}N_6S_{16}Zn$	BASF
Thiophanate-Methyl	$C_{12}H_{14}N_4O_4S_2$	BEST
Carbendazim	$C_2H_5NO_2$	Jiangshu Nanfong Co.
Iprodione	$C_{13}H_{13}Cl_2N_3O_3$	Bayer
Dimetachlone	$C_{10}H_7Cl_2NO_2$	Zhejian Heyipest.Co.
Iprovalicarb	$C_{18}H_{28}N_2O_3$	BEST
Zineb	$C_4H_6N_2S_4Zn$	Shenzhen Noposion
Mancozeb	$[C_4H_6MnN_2S_4]xZny$	Dow AgroSciences
Prochloraz	$C_{15}H_{16}Cl_3N_3O_2$	Jiangshu Huifong Co.
Procymidone	$C_{13}H_{11}Cl_2NO_2$	Sumitomo Chemical
Triadimefon	$C_{14}H_{16}ClN_3O_2$	Jiangshu Qizhounushe Chemical
Difenoconazole	$C_{19}H_{17}C_{l2}N_3O_3$	Shandong United Pesticide Industry Co.
Glyphosate	$C_3H_8NO_5P$	Monsanto Company

Table II.- Fungicides and concentrations used in this study.

Fungicides	Concentrations (mg/l)				
Carbendazim	5	10	20	40	80
Iprodione	300	600	1200	2400	4800
Prochloraz	15.63	31.25	62.5	125	250
Dimetachlone	25	50	100	200	400
Procymidone	35	70	140	280	560
Glyphosate	3380	6750	13500	27000	54000

The curves of (log concentration – probit line (LC-p)) were calculated and tested by chi-square test, median lethal concentrations (LC_{50}) and their confidence intervals were calculated by probit

analysis using SPSS (Statistical Package for Social Science) 8.0 for windows (SPSS, 1997).

Germination of C. carunculoides

Six different fungicides were selected based on previous section (2.3). Liquid culture medium was prepared with KNO_3 10 g, KH_2PO_4 5 g, $MgSO_4$ 2.5 g, $FeCl_3$ 0.02 g, Sucrose 50 g dissolved in deionized water 1000 ml and then the medium was sterilized at 121°C at 15 psi for 25 minutes. Different fungicides were added to the basal medium at three different concentrations (according to the EC_{95} values of experiment 2.4, Table V) while the basal medium without any fungicide served as control. *Ciboria carunculoides* (1×10^4 conidia/ml) was added to each flask. The flasks were incubated at 20±2°C. Percent germination was calculated after 36-48 h of shaking at 200 rpm by placing one ml of suspension on cavity slides. Three separate fields were observed for germination at 400 X magnifications for each treatment and 100 conidia were observed randomly in each field. Conidia with germ tubes equal to or greater than the width were considered to have germinated. The entire experiment was replicated three times on different dates.

Effect of different fungicides mixed with mulberry juice on radial growth of C. carunculoides

The effects of different mixtures at six different concentrations on radial growth were studied by using the method of 2.3. The freshly prepared PDA medium having different concentrations of fungicides (Table VI) was added to 9 cm petridish followed by covering a layer of mulberry leaf juice (300 µl, having 60 µg/ml Ampicillin). Mycelial discs together with medium (Ø1 cm) were removed to each flask. The flasks were incubated at 20±2°C. The same medium without the mixture of fungicide and juice served as a control. The fresh mulberry leaf with flexible branch was washed with deionized water three times, and then was put into a small mill for fresh mulberry juice.

Effect of cabendazim and glyphosate against C. carunculoides *in soil*

Three different concentrations of two fungicides (carbendazium and glyphosate) were selected based on experiment 2.4. The soil collected from the vicinity of mulberry tree mixed with *C. carunculoides* (10 ml of 1×10^3 conidia ml^{-1}) was put into a plastic box with (30×15×8cm). Different concentrations of fungicides (Table VII) were sprayed with 500ml sprayer to soil surface while the box without any fungicide served as control. The plastic boxes were incubated at 20±2°C for 10 days. Inhibitory percentage on germination was calculated as:

$$\% \text{ inhibition} = [(G_c-G_t)/Gc]*100$$

where G_c = germination in control and G_t = germination in treatment.

The entire experiment was replicated three times on different dates.

Statistical analysis

All experiments were carried out in triplicate and the results were expressed as average of triplicate determinations. Radial growth, inhibition growth and inhibition germination data were analyzed by Analysis of variance (ANOVA) and treatment means were compared by using Tukey's HSD test for mean comparisons at 5% level of significance. All statistical analysis was performed using SAS 8.01(2000).

RESULTS

Effect of different fungicides

Radial growth of C. carunculoides

The effect of 15 fungicides on radial growth of *C. carunculoides* was studied to screen out fungicides which are highly effective against this fungus. The average radial growth of *C. carunculoides* was significantly affected by different fungicides. Maximum radial growth (1.3±0.2 cm) was observed for Thiophanate-Methyl whereas no radial growth was observed for Tebuconazole, Nicotinamide, Prochloraz, Carbendazim, Dimetachlone, Difenoconazole and Glyphosate (Table III).

The fungicides of Carbendazim, Iprodione, Dimetachlone, Prochloraz, Procymidone and Glyphosate were used to study further on the basis of their economics and active mechanisms on controlling pathogen in crop field. The

half-maximal effective concentration (EC_{50}) of different fungicides against *C. carunculoides* is shown in Table IV.

Carbendazim was proved to the most aggressive showing the lowest EC_{50} value (8.04 mg/l), and Glyphosate was the least virulent with a mean EC_{50} value of 8741.78 mg/l. Statistically similar EC_{50} values were obtained for Dimetachlone, Prochloraz and Procymidone (Table IV).

Table III.- Effect of different fungicides on radial growth of *C. carunculoides* (4d).

Fungicide	Concentration (g/L)	Radial growth (cm) (Mean± S.E)
Tebuconazole	8.6	0.0 ± 0.0 d
Nicotinamide	10	0.0 ± 0.0 d
Metiram	14	0.9 ± 0.0 ab
Thiophanate-Methyl	14	1.3 ± 0.2 a
Carbendazim	10	0.0 ± 0.0 d
Iprodione	10	0.3 ± 0.1 c
Dimetachlone	8	0.0 ± 0.0 d
Iprovalicarb	14	0.9 ± 0.2 ab
Zineb	13	1.1 ± 0.2 a
Mancozeb	16	0.7 ± 0.0 b
Prochloraz	5	0.0 ± 0.0 d
Procymidone	2	0.8 ± 0.1 b
Triadimefon	4	0.7 ± 0.1 b
Difenoconazole	2	0.0 ± 0.0 d
Glyphosate	80	0.0 ± 0.0 d
		F =96.25, df=14, P<0.0001

Means ± SE in the same column followed by different letters are significantly different (Tukey's HSD Test, P<0.05)

Germination of C. carunculoides

The percent conidial germination of *C. carunculoides* was significantly affected by different fungicides used at different concentrations (based on EC_{95} value of above experiment) as shown in table V. All the fungicides used in this study caused 100% inhibition of fungal germination at the highest concentrations whereas the germination inhibition differed at lower concentrations of different fungicides. The highest germination inhibition (96.2%) at the lowest concentration was caused by carbendazim when used at 60 mg/l. The lowest germination inhibition (92.1%) at the lowest concentration was observed for Procymidone when used at 650 mg/l (Table V).

Effect of different fungicides mixed with mulberry juice

Radial growth of C. carunculoides

The effect of mixtures of fungicides at different concentration with mulberry juice on growth inhibition of *C. carunculoides* is shown in Table VI. The average radial growth of *C. carunculoides* was significantly affected by different treatments. The highest growth inhibition (97.96%) among treatments was observed for mixture of carbendazim 4000 mg/l and mulberry juice whereas the lowest growth inhibition was observed for the mixture having Procymidone 625 mg/l+ mulberry Juice (Table VI).

The half-maximal effective concentration (EC_{50}) of different fungicides and juice mixtures against *C. carunculoides* is shown in Table VII. Carbendazim + juice mixture was proved more aggressive showing the lowest EC_{50} value (521.01 mg/l), when compared with Procymidone + juice mixture with a mean EC_{50} value of 1322.55 mg/l (Table VII).

Effect of Cabendazim and Glyphosate against C. carunculoides *in soil*

The percent conidial germination of *C. carunculoides* was significantly affected by different concentration of Carbendazim and Glyphosate used at different concentrations in soil as shown in Table VIII. Both the fungicides used in this study caused 100% inhibition of fungal germination at highest concentrations whereas the germination inhibition differed at lower concentrations of different fungicides. The lowest germination inhibition (82.1%) at lowest concentration was caused by carbendazim when used at 10 mg/l. The lowest germination inhibition (80.7%) at lowest concentration was observed for glyphosate when used at 40 mg/l (Table VIII).

DISCUSSION

Progressive increase of production and application of chemicals fungicides for agriculture as well as for plant protection has converted the

problem of environmental pollution into national and international issue. (Plimmer, 1990; Mathys, 1994). Therefore, more efforts are being directed towards using selective chemicals as well assessment and usage of their minimum concentration required for disease management (Wania and Mackay, 1996; Larson *et al*., 1997). Initially, screening bioassays were carried out to determine most suitable fungicides for the management of mulberry popcorn disease on the basis of their effectiveness as well as economics. All the chemicals used in this study effectively reduced the radial growth of *C. carunculoides.* The concentrations of different fungicides were selected on the base of reduction in radial growth. In order to observe inhibition of spore germination and mycelial growth in subsequent experiments, EC_{50} and EC_{95} concentrations of fungicides were calculated. EC_{50} and EC_{95} values of carbendazim (8.04 and 57.09 mg/l) calculated in this

Table IV.- Regression analysis of probit Inhibition growth and log-concentration data of bioassay with fungicides against *C. carunculoides* (4d).

Fungicide	Regression equation (R^2)	EC_{50} (mg/l) (Mean± S.E)	EC_{95} (mg/l) (Mean± S.E)
Carbendazim	Y=3.2813+1.8987X (0.9862)	8.04 ± 0.8	59.09 ± 1.4
Iprodione	Y=0.1824+1.5571X (0.9984)	1241.54 ± 11.3	14137.06 ± 20.6
Dimetachlone	Y=1.5386+2.0265X(0.9950)	51.06 ± 1.2	330.93 ± 9.3
Prochloraz	Y=2.2099+1.8054X(0.9908)	35.10 ± 2.1	286.05 ± 8.7
Procymidone	Y=1.7241+1.7464X(0.9941)	75.12 ± 6.2	657.08 ± 9.2
Glyphosate	Y=-4.2311+2.3420X (0.9819)	8741.78 ± 23.5	44051.32 ± 27.9

Table V.- Germination percentages of *C. carunculoides* conidia suspended in nutritional liquid containing varying concentration of different fungicides (36 - 48h, 20±2°C).

Fungicide	Concentration (mg/l)	Inhibition germination % (Mean± S.E)
Carbendazim	60	96.2 ± 1.2 b
	100	100.0 ± 0.0 a
	120	100.0 ± 0.0 a
		F =51.76, df=2, P<0.0001
Iprodione	14500	92.7 ± 4.7 c
	16000	99.5 ± 3.1 b
	20000	100.00 ± 0.0 a
		F=72.34, df=2, P<0.0001
Prochloraz	280	95.9 ± 2.9 c
	300	98.5 ± 3.4 b
	320	100.0 ± 0.0 a
		F=65.28, df=2, P<0.001
Dimetachlone	350	94.1 ± 5.2 b
	500	100.0 ± 0.0 a
	600	100.0 ± 0.0 a
		F=80.41, df=2, P<0.001
Procymidone	650	92.1 ± 5.2 b
	800	100.0 ± 0.0 a
	900	100.0 ± 0.0 a
		F=92.13, df=2, P<0.0001
Glyphosate	45000	95.3 ± 4.7
	55000	99.4 ± 1.8
	65000	100.0 ± 0.0
		F=63.70, df=2, P<0.001

Means ± SE in the same column followed by different letters are significantly different (Tukey's HSD Test, P<0.05)

Table VI.- Effect of different mixtures at six different concentrations on radial growth of *C. carunculoides* (4d).

Fungicide	Concentration (mg/l)	Inhibition growth (%) (Mean± S.E)
Carbendazim+Juice	250	32.65 ± 3.2 e
	500	43.88± 2.7 d
	1000	66.33 ± 4.1 c
	2000	85.31 ± 5.3 b
	4000	97.96 ± 2.9 a
		F=154.30, df=4, P<0.0001
Procymidone + Juice	625	27.84 ± 2.2 e
	1250	46.39 ± 3.1 d
	2500	67.01 ± 4.5 c
	5000	88.66 ± 1.2 b
	10000	97.94 ± 3.5 a
		F=127.45, df=4, P<0.0001

Means ± SE in the same column followed by different letters are significantly different (Tukey's HSD Test, P<0.05)

study were higher EC_{50} of carbendazim against *Botryosphaeria berengriana* (0.108 mg/l) observed by Li *et al.* (2009). This difference in EC_{50} values can be due to the possible nature of the pathogen against which the chemical is being used about which very little information is available to date.

Table VII.- Regression analysis of probit Inhibition growth and log-concentration data of bioassay with mixtures against *C. carunculoides*.

Fungicide	Regression equation (R^2)	EC_{50} (mg/l)	EC_{95} (mg/l)
Carbendazim + Juice	Y=-0.5898+2.0574X (0.9803)	521.01 ± 9.4	3283.48 ± 27.4
Procymidone + Juice	Y=-1.7990+2.1782X(0.9930)	1322.55 ± 21.5	7526.32 ± 36.7

Table VIII.- Effect of Cabendazim and Glyphosate against *C. carunculoides* in soil (10d).

Fungicide	Concentration (mg/l)	Inhibition germination % (Mean± S.E)
Carbendazim	10	82.1 ± 5.2 b
	20	96.9 ± 4.9 a
	40	100.0 ± 0.0 a
		F=98.14, df=2, P<0.0001
Glyphosate	40	80.7 ± 6.3 b
	60	97.5 ± 3.1 a
	80	100.0 ± 0.0 a
		F=102.36, df=2, P<0.0001

Means ± SE in the same column followed by different letters are significantly different (Tukey's HSD Test, P<0.05)

The disease management strategies are mainly pre-emergence and post emergence. Pre-emergence disease management is carried out at initial stage and contact poisons are used for such disease management whereas after plant growth and initial establishment of disease post emergence strategies are used with systemic fungicides (Galloway, 2008). In this study, the purpose of using six fungicides including contact and systemic fungicides was to find out any possible way of controlling disease (Table V), at the same time, the EC_{95} concentrations of six fungicides were selected to test the maximum control efficacy. It is necessary to use systemic fungicides after disease infected plant internal organs and established in plant. During these studies Carbendazim and Procymidone which are well known systemic fungicides were combined with mulberry juice for possible disease management after the establishment of *C. carunculoides* on mulberry branches and fruits. The average radial growth of *C. carunculoides* was affected by different treatments. Highest growth inhibition (97.96%) was observed for mixture of carbendazim 4000 mg/l and mulberry juice whereas lowest growth inhibition (27.84%) was observed for the mixture having Procymidone 625 mg/l+ mulberry Juice showing that carbendazim is more effective for management of this disease. In order to imitate the effect of the internal environment of plant on fungicide, the project of fungicide and mulberry juice mixture was designed. High concentrations of two systemic fungicides were used to test the efficacy of Carbendazim and Procymidone based on the low efficacy at low concentration of these two fungicides in early experiment (Table VI).

In last part of our studies, efforts were made to imitate the field conditions by applying the *C. carunculoides* to the soil already collected from the vicinity of mulberry tree. After establishment of *C. carunculoides*, Carbendazim and Glyphosate were applied to this culture at different concentrations. Both the fungicides used in this study caused 100% inhibition of fungal germination at the highest concentrations whereas the germination inhibition differed at lower concentrations of different fungicides showing the possibility of using these chemicals for *C. carunculoides* at initial stage or during dormant stage when the pathogen stays in soil for its growth and survival.

Our results have showed that the variation in effectiveness of different chemicals against *C. carunculoides* is dependent upon different unknown factors. Carbendazim was proved to be most effective chemical for *C. carunculoides* management at different stages of disease establishment. We hope that the findings of the

current studies will provide basic information for *C. carunculoides* management in field conditions.

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