Biological and chemical control of grapevine die-back disease and their effect on defense related enzymes

By

El-Habbaa, G.M.[1]; Abdou, M.M Mahdy[1]; F. G. Mohamed[1] and El-Shaery, S.A. [2]
1.Plant Pathol. Dept., Fac. Agric., Moshtohor, Benha Univ., Egypt
2. Plant Pathol. Res. Instit., Agric. Res. Center (ARC), Giza, Egypt.
Corresponding Author E-mail: ghabaa@yahoo.com

Abstract—Three bio-formulations *i.e.,* Rhizo-N, Plant gaurd and Bio-Zeid with six concentrations were tested *in vitro* against *Lasiodiplodia theobromae*-B isolate the causal of grapevine die-back disease. Rhizo-N followed by Bio-Zeid was respectively more effective than Plant guard in reducing the growth of *L. theobromae*-B isolate. Spraying the three tested bio-formulations on grapevine shoots at the same time of infestation with *L. theobromae-B* isolate was effective in reducing the die-back infection at 7-28 days post infestation. Results indicated also that Rhizo-N was more effective than others when applied at 7 days pre infestation. Also, all tested fungicides were effective in reducing the growth of *L. theobromae*, *in vitro*. The best effective ones were Topsin-M70 and Kema-Z at low and high concentrations while, Bellis and Captan were effective at high concentrations only. On the other hand, all tested fungicides *i.e.* Kema-Z.50%, Topsin M-70 and Kocide-2000 were effective in controlling grapevine die-back infection when sprayed after or before artificial inoculation with *L. theobromae* under greenhouse conditions. Also, all bio-formulations and fungicides treatments increased activities of peroxidase, polyphenoloxidase and Chitinase compared with control treatment.

Key words—Fungicides, biocides, *Lasiodiplodia theobromae*, grapevine, peroxidase, polyphenol oxidase and Chitinase**.**

1 Introduction

Grapevine die-back is one of the most important diseases all over the world which attack twigs of grapevine and cause great losses in the field. *Lasiodiplodia theobromae* (syn. *Botrydiplodia theobromae*, *Diplodia natalensis*) is the pycnidial state of *Botryosphaeria rhodina*, a pathogen that causes cankers and die-back in many woody and herbaceous species. Many investigators studied die-back disease of grape in Egypt (El-Goorani and El-Meleigi, 1972; Farag, 1998; Saber, 1998; Kamhawy, 2001; Abo Rehab *et al.*, 2013 ; Haggag, *et al.*, 2013 and El-Banna *et al.*,2015).

Concerning control of grapevine die-back disease, Farag (1998)reported the growth inhibition of *L. theobromae* completely on PDA medium which contained Topsin M-70 (5 mg/l), Topas (30 mg/l) and Rubigan (50 mg/l). He also added that the best chemical control for die-back disease of grapevine cv. Bez–Anz was achieved when the twigs were sprayed with Rubigan, Topas or Topsin M-70, 24 hours before inoculation with *L. theobromae*. The die-back symptoms were greatly decreased as a result of spray with Topsin M 70, Rubigan, Topas after inoculation with *L. theobromae.* Kamhawy *et al.* (2005) reported that the linear growth of *L. theobromae* was completely inhibited at 10 mg/L of thiophanate-methyl (Hista 70% WP) and carbendazim (Actazime 50% WP) followed by pyrifenox (Dorado 200 EC) at 40 mg/l and propioconazol as (Cornazol 10 EC) at 50 mg/l. Carbendazim and thiophanate-methyl gave the best control followed by propioconazol and pyrifenox. Mahmood *et al.* (2007) found that Precure Combi and Derosal completely inhibited the mycelial growth of *L. theobromae* by food poisoning technique even at 10 mg/l concentration while, Topsin-M produced the same result at 25 mg/L. Topsin-M significantly controlled the disease in Greenhouse experiment and under field conditions when applied after scratching the diseased portion of the infected plants. Javaid *et al.* (2008)evaluated *in vitro* four fungicides viz. Acrobat MZ 75/667WP, Dithane M-45 80% WP, Mancozeb 80% WP and Metalaxyl plus Mancozeb 72% WP against *L. theobromae* Pat., the causal of die-back disease of mango in Pakistan and they found them highly effective even at a very low concentration. Hassan (2015) tested *in vitro* nine commercial fungicides against *L. theobromae* Q1 and *F. moniliforme*. The fungicide Occidor was superior to Antracol, and the other fungicides, Nimrod, Penzaole, Dithane and Topsin M-70, came next in their inhibitory action, Copprotech 50% was not effective, Vectra and Crown were of moderate effect. Safdar *et al*. (2015) showed that any increase in the concentration of the fungicide was positively correlated with the growth inhibition when studied the effect of synthetic fungicides on the radial growth of the *L. theobromaein vitro*. As for biological control, Chakraborty *et al.* (2006) tested different antagonists (*Trichoderma harzianum, T. viride. T. lignorum*and *T. reesei*), for their activity against *L.theobromae*, the causal agent of die-back of bottle brush (*Callistemon citrinus*). Among antagonists, *T. harzianum* showed the highest inhibitory activity against *L. theobromae*, followed by *T. viride* and *T. lignorum*. Sangeetha *et al.* (2009) tested 12 isolates of *Trichoderma* spp. for their effectiveness against *L. theobromae*. Eight isolates of *Trichoderma* spp. inhibited mycelial growth of *L. theobromae*, with reductions ranging from 45.1 to 85.0%. Of these, *T. harzianum*-3 was the most strongly one in inhibiting *L. theobromae* (85.0%) followed

by *T. koningii* (76.7%). Haggag *et al.* (2013) reported that *Pseudomonas putida,* was effective in reducing grapevine die-back disease caused by a fungus *L. theobromae.* El-Banna *et al.* (2015) found that the bio-control isolates of *Paeniobacillus polymyxa, Brevibacillus brevis, Pseudomonas putida, Pseudomonas fluorescens* and *Streptomyces griseus* enhanced the resistance against die-back disease caused by *Botryoplodia theobromae* on grapevine transplanting inoculated with pathogen and sprayed with bio-agents in the greenhouse experiment. *P. putida* was the highest effective treatment followed by *P. polymyxa* and *Brevibacillus brevis*, respectively.

Regarding the changes in activities of defense related enzymes as a result of treating grapevine plants with some fungicides or biocides, some plant growth promoting bacteria (PGPB) strains as bio-agents could protect plants by activating gene encoding defense enzymes i.e., peroxidase, chitinase, phenylalanine ammonia-lyase, β-1,3-glucanase and others, involved in synthesis of phytoalexin (M'Piga *et al.*, 1997). Cucumber roots treated with *T. harzianum* showed higher activities of chitinase, β-1, 3-glucanases and peroxidase (Yedidia *et al.,* 2000). Gomathinayagam *et al.* (2008) found that treating rice plants with either benomyl, carbendazim, baycor and calixin inhibited the bacterial multiplication in rice leaves and induced defense-related enzymes such as peroxidase, polyphenol oxidase, phenylalanine ammonia lyase and tyrosine ammonia lyase, which are responsible for host resistance thereby reduced the bacterial leaf blight in rice. Saravanakumar *et al.* (2008) revealed the greater accumulation of defense enzymes, peroxidase, polyphenol oxidase and phenylalanine ammonia lyase in apples treated with Mach1 and challenged with *B. cinerea* compared to untreated controls. Chakraborty *et al.* (2010) revealed that *Serratia marcescens* enhanced activities of defense-related enzymes *i.e.,* peroxidase, chitinase, phenylalanine ammonia-lyase and β-1,3-glucanase as well as total and O-dihydroxyphenols. Prakongkha *et al.* (2013) found that chitosan and benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester (BTH) are active-elicitors that induce protection in grapevine against several diseases. Also increased chitinase, ß-1,3-glucanase and peroxidase activities levels in leaves over non-treated plants. El-Banna *et al.* (2015) tested some bacterial bio-agents against *L. theobromae, in vitro* bioassay. All treatments reduced growth of *L. theobromae* compared to control. They cleared that treating with bacterial bio-agents encourage the secretion of enzymes endo, exo-β-1,3-glucanase, chitinase and protease which be involved in the degradation of fungal cell walls. *B. polymyxa* isolates produced extracellular cellulolytic enzymes (exo-glucanase and endo-glucanase that possibly related to the bio-control process.

This study aimed to throw the light on the significant of grapevine die-back disease and how to control it using fungicides or biocides in addition to their effects on defense related enzymes under Egyptian conditions.

2 MATERIALS AND METHODS

Source of grapevine die-back disease pathogen:

Lasiodiplodia theobromae-B isolate which isolated from grapevine twigs showing die-back disease symptoms of collected samples from Beheira (Noubaria) governorate. This isolate was previously tested for its pathogenic potentiality as a highly virulent isolate among the other tested (**un-published data)**. This isolate w used in the control trails.

Disease control:

Biological control

Effect of some bio-formulations on growth of *L. theobromae in vitro:*

This investigation was carried out to study the effect of three bio- formulations which produced as commercial products namely Rhizo-N (*Bacillus subtillus*) Plantgaurd (*Trichoderma harzianum*) and Bio-Zeid 2.5% (*Trichoderma album*) on growth of *L. theobromae*-B isolate using different concentrations (0, 0.5, 1, 1.5 and 2.0 ml. or g/L medium). Each one of the tested concentrations of bio-formulations was added individually to the prepared PDA medium under aseptic conditions directly before solidification by shaking to confirm the entire distribution of the tested bio-formulations then, the medium was poured into the sterilized plates. The plates were inoculated in center with an equal disc (5 mm in diameter) of the test pathogenic fungus (*L. theobromae*-B isolate) then, incubated at 28 °C ±1). Plates free of any one of the tested bio-formulations were used as control. Five Petri plates were used as replicates for each particular treatment. Two perpendicular diameters of the growth fungus were measured when the growth control treatment filled the plates and then, the reduction percentages in growth of the tested fungus were calculated.

Effect of three bio-formulations on grapevine die-back infection under greenhouse conditions.

Efficiency of the three tested bio-formulations i.e., Bio-Zeid 2.5% (as *Trichoderma album,* 10×10^6 spore/g – dose at 250 g/100 L), Plant guard (as *Trichoderma harzianum*, 5×10^6 spore/ml–dose at 250mL/100L) and Rizo-N (as *Bacillus subtilis*, 30×10^6 cfu/g – dose at 400 g/100 L) for controlling die-back disease symptoms incited by *L. theobromae*-B isolate under greenhouse conditions was tested. Inocula of the tested formula were prepared at the recommended dose as described above by the produced company. Grapevine nurslings (cv. Thompson seedless) one year old were inoculated with *L. theobromae*-B isolate using toothpick method according to Yang (1943) as mentioned before and then, sprayed with the tested bio-formulations.

Chemical control:

In this trail, six fungicides were evaluated on growth of the *L. theobromae*-B isolate, the causal of grapevine die-back disease under laboratory conditions. Also, evaluating the best three fungicides among the tested ones in controlling the

grapevine die-back infection on one year old nurslings of cv. Thomposon under greenhouse conditions.

Effect of tested fungicides on growth of L. theobromae-B isolate *in vitro*.

Six fungicides *i.e.*, Kocide-2000 (Cupric hydrocide), Topsin-M70 (Thiophanate-Methyl), Kema-Z (Carbandazim) , Bellis (Boscalid + Pyraclostrobin), Captan (Captan) and Ridomil-Mancozeb (Metalaxyl + Mancozeb) were evaluated to study their effects on growth of the tested fungus. Different concentrations i.e., 0, 1, 5, 10, 100, 200, 400, 600, 800 and 1000 µg/L) were prepared to study their effect on the growth of tested pathogenic fungal isolate. The tested concentrations of fungicides were added individually to the prepared PDA medium under aseptic conditions directly before solidification by shaking to confirm the entire distribution of the tested fungicides then, the medium was poured into the sterilized plates (9cm). The plates were inoculated in center with an equal disc (5 mm in diameter) of the test pathogenic fungus (*L. theobromae*-B isolate) then, incubated at 28 °C±1). Plates free of any one of the tested fungicides were used as control. Five Petri plates were used as replicates for each particular treatment. Two perpendicular diameters of the fungal growth were measured when the growth of control treatment filled the plates and then, the diameter average of linear growth of the tested fungus was recorded.

Effect of tested fungicides in controlling the grapevine die-back infection under greenhouse conditions:

Three fungicides *i.e.* Kema-Z.50%; Topsin M-70 and Kocide-2000 which showed the highest inhibitory effect on the growth of *L. theobromae*-B isolate were used to study their effects in controlling the grapevine die-back infection on cv. Thompson seedless under greenhouse conditions (*in vivo*). Twenty nurslings one year old were used as four replicates for each treatment of the three tested fungicides. These nurslings were inoculated with the tested pathogenic fungus using toothpick method according to Yang (1943) as mentioned before. Fungicidal treatments were done at the same time of inoculation with the pathogenic fungus, 2 and 7 days before inoculation in addition to 2 and 7 days after inoculation. The average lengths of die-backed areas on infected shoots were measured after 7, 14, 21 and 30 days post inoculation. The obtained data were tabulated and statistically analyzed.

Determination of activities defense related enzymes:

To evaluate the impact of Topsin M-70, Kema-Z and Kocide-2000 as fungicides and also commercial biocides like Bio-Zeid, Rhizo-N and Plant guard on the defense enzymes activities in leaves of grape cv. Thompson Seedless) inoculated or non-inoculated with *L. theobromae*. In this respect, leave samples were collected post 7 days of spraying with tested fungicides and biocides then ground with 0.2 M Tris-HCl buffer (pH 7.8) containing 14 mM β-mercaptoethanol at the rate 1/3 w/v. The extracts were centrifuged at 10,000 rpm for 20 min at 4°C. The supernatant was used to determine enzyme activities (Tuzun *et al.* 1989).

Chitinase activity assay:

The substrate colloidal chitin was prepared from chitin powder according to the method described by Ried and Ogrydziak (1981). Twenty five grams of chitin was milled, suspended in 250 mL of 85% phosphoric acid (H_3PO_4) and stored at 4°C for 24 h, then blended in 2L of distilled water using a warning blender and the suspension was centrifuged. This washing procedure was repeated twice. The colloidal chitin suspension in the final wash was adjusted to pH 7.0 with (1N) NaOH, separated by centrifugation and the pelted colloidal chitin was stored at 4°C. The determination was carried out according to the method of Monreal and Reese, (1969), one mL of 1% colloidal chitin in 0.05 M citrate phosphate buffer (pH 6.6) in a test tube, then one mL of enzyme extract was added and mixed by shaking. Tubes were kept in a water bath at 37°C for 60 minutes, then cooled and centrifuged before assaying. Reducing sugar was determined by adding 1 mL of supernatant with 1 ml of dinitrosalicylic acid and 3 mL distilled water in test tubes and tubes were boiled in water bath for 5 minutes, and then cooled. Optical density was determined at 540 nm. Chitinase activity was expressed as mM N-acetyl glucose amine equivalent released gram fresh weight tissue / 60 minutes.

Peroxidase activity assay:

Peroxidase (PO) assay (based on oxidation of pyrogallol to purpurogallin in the presence of H_2O_2) was determined according to the method described by Allam and Hollis (1972). The reaction mixture contained 0.5 ml of 0.1 M sodium phosphate buffer solution at pH 7.0; 0.3 ml enzyme extract; 0.3 ml 0.05M pyrogallol and 0.1 ml 1.0% H_2O_2. The mixture was completed with distilled water up to 3 ml. Enzyme extract was replaced by distilled water in control blank cuvette. The absorbance of 1 ml was recorded and peroxidase activity was expressed as the change in absorbance (O.D) / minute/gram fresh weight.

Polyphenoloxidase activity:

The activity of polyphenoloxidase was measured as mentioned by Matta and Dimond (1963). The reaction mixture consists of 0.3 mL sample extract, 1.0 mL sodium phosphate buffer (pH 7), 1.0 mL 10- 3 M catechol and completed with distilled water to 6.0 mL. Enzyme extract was replaced by distilled water in control blank cuvette. The polyphenoloxidase activity was assayed as mentioned above and expressed as the change in absorbency per minute per 1.0 g fresh weight.

Statistical analysis:

The experiment was arranged in a randomized complete blocks design and the obtained data were subjected to analysis of variance and significant differences among means according to Snedecor and Cochran (1980). In addition significant differences among means were distinguished according to the Duncan multiple test range Duncan (1955).

3 Results and Discussion

Disease control:

Biological Control:

In vitro:

In this trail, three bio-formulations *i.e.*, Rhizo-N, Plant guard and Bio-Zeid with six concentrations were tested *in vitro* against *L. theobromae*-B, the causal of grapevine die-back disease. Data in Table (1) indicate that increasing the concentration of the three tested bio-formulations from 0.5-2 mL or g/L increased gradually their effects in inhibiting the growth of *L. theobromae*-B isolate. It is clear that using the high concentration 2g or 2mL of the tested bio-formulations was the best where it completely inhibited the growth of the fungus. Also, Rhizo-N followed by Bio-Zeid was respectively more effective than Plant-guard in reducing the growth of *L. theobromae*-B isolate.

Under greenhouse conditions

In this trail, the three tested bio-formulations i.e., Rhizo–N, Bio-Zeid and Plant guard were tested for their effects against grapevine die-back infection caused by *L. theobromae-B* isolate. Data in the Table (2) indicate that the three tested bio-formulations were effective when sprayed on the grapevine shoots at the same time of infestation with *L. theobromae-B* isolate, the causal pathogen and at 7 days post infestation where the average of die-backed infected areas were zero at all incubation periods which ranged between7-28 days. On the other hand, spraying grapevine shoots with the tested bio-formulations, 7 days pre infestation with the causal pathogen of die-back was low effective at all incubation periods comparing with control treatment. Results indicate also that Rhizo-N was more effective than others when applied at 7 days pre infestation. These results are in agreement with the findings of Abo-Rehab *et al*. (2013) who found that the bio-agents i.e., Bio-Zeid (*Trichoderma album*), Bio-ARC (*Bacillus megaterium*) and Rhizo-in (*Bacillus subtilis*) were efficient in suppressing fungal pathogens cause graft failure on grapevine. The bio-fungicides reduced disease incidence when applied at the same time of pathogen inoculation. Considering the efficiency against *P. viticola, L. theobromae* and *F. solani*, Bio-Zeid (*Trichoderma album*) was the most effective in decreasing the percentage of disease incidence by 40%. The disease incidence was reduced by 27 and 25% with Rhizo-in (*Bacillus subtilis*) and Bio-ARC (*Bacillus megaterium*), respectively. Also, Haggag *et al*. (2013) reported that PGPR with wide scope for commercialization includes *Pseudomonas putida*, was effective in reducing die-back caused by a fungus *L. theobromae* and arm death, caused by a fungus, *Phomopsis viticola* of grapevine *in vitro* and *in vivo*. *Pseudomonas putida* showed optimum siderophore pseudobactin production at 72 h, and growth peak at 120 h. glycerol as a source of carbon and yeast as a source of nitrogen. *Pseudomonas putida* was very effective as bio-control agent to reduce the die-back and arm death disease on grapevine.

Table (1): Effect of some bio-formulations on growth of *L. theobromae*-B isolate in vitro.

Tested Bio-fungicides	Linear growth of *L. theobromae*-B (mm) Concentration ml or g / Liter						
	0	0.5	0.75	1	1.5	2	Mean
Rhizo - N	90.00a	30.00e	25.00f	15.00g	0.00i	0.00i	26.66
Plant-guard	90.00a	50.00b	45.00c	25.50f	5.00h	0.00i	35.91
Bio-Zeid 25%	90.00a	43.30c	35.00d	16.00g	0.00i	0.00i	30.70
Mean	90.00	41.10	35.00	18.83	1.66	0.00	

Means within a column having the same letters are not significantly different according to Duncan's Multiple Range Test at 5% level.

Table (2): Effect of some bio-formulations on grapevine die-back infection (cv. Thomson Seedless) under greenhouse conditions.

Time of treatment	Bio-agents	Die-backed area (mm) at days				
		7	15	21	28	Mean
7days pre-infestation	Bio-Zeid2.5%	25j	31i	37gh	42ef	33.6
	Rhizo-N	30i	35h	40fg	45e	37.5
	Plant guard	40fg	42ef	50d	55c	40.8
	Control	50d	55c	70b	100a	68.8
At the time	Bio-Zeid2.5%	0k	0k	0k	0k	0.0
	Rhizo-N	0k	0k	0k	0k	0.0
	Plant guard	0k	0k	0k	0k	0.0
	Control	50d	55c	70b	100a	68.8
7dys post infestation	Bio-Zeid2.5%	0k	0k	0k	0k	0.0
	Rhizo-N	0k	0k	0k	0k	0.0
	Plant guard	0k	0k	0k	0k	0.0
	Control	50d	55c	70b	100a	68.8

Means within a column having the same letters are not significantly different according to Duncan's Multiple Range Test at 5% level.

Chemical control:

In vitro

In this trail, six fungicides *i.e.* Bellis, Kema-Z., Topsin M-70, Ridomil-Mancozeb, Captan and Kocide-2000 were tested in vitro at the concentrations 1, 5, 10, 100, 200, 400, 600, 800 and 1000 µg/ml against *L. theobromae*the causal of grapevine die-back disease. Data in Table (3) indicate that all tested fungicides were effective in reducing the growth of *L. theobromae*, the causal of grapevine die-back disease when tested *in vitro*. The best effective ones wereTopsin-M70 and Kema-Z where both of them were able to inhibit completely the growth of the tested fungus at all tested concentrations which ranged between 1-1000 µg/ml. On the other hand, Kocide-2000, Bellis and Captan were effective respectively at the higher concentrations only. Also, Ridomil-Mancozeb fungicide was the least effective one among all tested fungicides.

Table (3): Effect of some fungicides on growth of *L. theobromae in vitro*.

Tested Fungicides	Linear growth (mm) at different concentrations (µg/ml)									
	1	5	10	100	200	400	600	800	1000	Control
Topsin - M70	0.0b	0.0b	0.0b	0.0b	0.0b	0.0b	0.0b	0.0b	0.0b	90.0a
Bellis	86.4b	42.7c	30.0d	20.3e	15.7f	9.4g	7.9g	0.0h	0.0h	90.0a
Captan	90.0a	86.5b	85.0b	73.5c	71.0c	51.0d	29.9e	13.9e	12.4f	90.0a
Kema-Z	0.0b	0.0b	0.0b	0.0b	0.0b	0.0b	0.0b	0.0b	0.0b	90.0a
Kocide -2000	90.0a	90.0a	90.0a	50.0b	25.0c	12.0d	0.0e	0.0e	0.0e	90.0a
Ridomil-Mancozeb	90.0a	90.0a	90.0a	90.0a	90.0a	90.0a	65.0a	60.0c	50.0d	90.0a

Means within a column having the same letters are not significantly different according to Duncan's Multiple Range Test at 5% level.

Under greenhouse conditions:

In this trail, three fungicides *i.e.* Kema-Z.50%; Topsin M-70 and Kocide-2000 having the most effect among the other tested fungicides *in vitro* against *L. theobromae* were applied under greenhouse conditions to test their effects in controlling grapevine die-back infection. In this respect, data in Table (4) show that, all tested fungicides were effective in controlling grapevine die-back infection when sprayed after or before artificial inoculation with *L. theobromae* under greenhouse conditions. All the three tested fungicides suppressed completely the die-back infection when sprayed2-days pre and post inoculation as well as, when sprayed at the same time of inoculation and 7 days post inoculation with causal pathogen. On the other hand, spraying the grapevine shoots 7 days before inoculation with *L. theobromae,* the causal pathogen of die-back was the least effective treatment in controlling the disease under greenhouse conditions. It was noticeable that die-back disease incidence % was increased gradually by increasing the incubation period from 7- 30 days in case of spraying the grapevine shoots 7 days before inoculation with the causal pathogen. The obtained results of *in vitro* or *in vivo* trails could be discussed in light the findings of Abo Rehab *et al.* (2013)who found that Bellis, Saprol, Syllet and Conazol were the most effective fungicides among 6 tested in inhibiting the spore viability and mycelial growth of *P. viticola* when tested *in vitro* and reduced the percentage of infection by 75, 70, 62 and 55%, respectively when tested under greenhouse conditions. Also, Adeniyi and Olufolaji (2014) assayed six fungicides i.e., Mancozeb 80% WP, Carbendazim 50% WP, Copper-1-oxide 60% + metalaxyl M 6%, Copper hydroxide (57%), Copper-1-oxide 60% + metalaxyl 12% WP and Cuprous oxide (86%) *in vitro* against *L. theobromae* isolate at 0.1, 0.25, 0.5, 1.0, 1.5, 2.0 and 2.5 µg/ml, Carbendazim 50% WP with the minimum inhibitory concentration (MIC) at 0.25 µg/mL gave the highest (16.67mm) zone of inhibition; mancozeb 80% WP at 1.5µg/mL gave 12.33mm zone of inhibition however, other fungicides have zero zone of inhibition. Mancozeb 80% WP at 2.5µg/ml and 2.0µg/ml and carbendazim 50% WP at 0.1µg/mL caused the highest reduction of mycelia growth of *L. theobromae*. The highest percent inhibition of 94.12% was recorded in mancozeb 80% WP at 2.5µg/ml and 2.0µg/ml at 2 days after inoculation (DAI) and the least of 2.35% recorded in Copper-1-oxide 60% + metalaxyl 12% WP at 0.1µg/ml. The inhibition percent of the tested fungicides increased with the increase in concentration with the exception of carbendazim 50% WP in both 2 and 4 DAI. Also, Rehman *et al.* (2014) evaluated five systemic fungicides against *L. theobromae*, they found that thiophanate-methyl was significantly superior to all the other fungicides with inhibition zones of 0.15, 0.30, 0.43 and 1.16 cm at 100, 75, 50 and 25 µg mL^{-1} concentrations, respectively. The results of difenoconazole were at par with carbendazim with the inhibition zone of mycelial growth of *L. theobromae* at different concentrations. In the case of diethofencarb, there was a significant decrease in the colony diameter of *L. theobromae* as compared to control. Pyrachlostrobin was the least effective among the systemic fungicides. While, copper oxychloride showed inhibition to some extent at 100 and 75 µg ml^{-1} with the inhibition of mycelial growth of *L. theobromae* to 0.29 and 3.29 cm, but statistically no significant difference was found. Mancozeb was the least effective among all the fungicides tested on *L. theobromae*.

Changes in activities of defense related enzymes in treated grape plants with some fungicides and biocides.

Data in Table (5) show that all fungicides treatments increased activities of peroxidase, polyphenol oxidase and Chitinase compared with control treatment. As for polyphenol oxidase, the highest activities were recorded with Kocide-2000 followed by Kema-Z and Topsin-M70 treatments respectively compared with treated control. On the other hand, the highest activities of peroxidase were recorded with Topsin-M70 followed by Kocid-2000 respectively compared with control treatment and inoculated plants only. However, Kema-Z fungicide treatment was the least effective one on peroxidase activity compared with the other fungicide treatments and inoculated plants only. Also, Topsin-M70 treatment was the highest effective one in increasing the activity of chitinase, while the rest of tested fungicides decreased the activities of Chitinase. The highest decrease in Chitinase activity was recorded with Kocid-2000 followed by Kema-Z respectively compared with treated control.

As for the tested biocides, data in Table (6) show that generally all treatments increased polyphenol oxidase, peroxidase and chitinase activities compared with control treatments. As for polyphenol oxidase, the highest activity was recorded with treatments of Rhizo-n followed by Bio-Zied respectively compared with control treatments. While plant guard decreased the activities of polyphenol oxidase compared with other biocide treatments and inoculated plants only. Also, the highest activity of peroxidase was recorded with Rhizo-N followed by Bio-Zeid and plant guard treatments respectively compared with control treatments.

Table (4): Effect of different fungicides of the length of infected parts in table grape twigs (mm) inoculated with the causal pathogen.

Periods		Die-backed area (mm) at days					
		Spray pre inoculation 7	Spray pre inoculation 2	Spray with Inoculation	Spray post inoculation 2	Spray post inoculation 7	Control
Topsin – M-70	7	6.0d	0.0e	0.0e	0.0e	0.0e	7.0d
	14	7.2d	0.0e	0.0e	0.0e	0.0e	10.2d
	21	9.5d	0.0e	0.0e	0.0e	0.0e	20.7c
	30	34.7b	0.0e	0.0e	0.0e	0.0e	70.0a
	Mean	14.3b	0.0c	0.0c	0.0c	0.0c	26.9a
Kema-Z	7	6.0b	0.0c	0.0c	0.0c	0.0c	7.0d
	14	7.2b	0.0c	0.0c	0.0c	0.0c	10.2d
	21	9.3b	0.0c	0.0c	0.0c	0.0c	20.7c
	30	36.7a	0.0c	0.0c	0.0c	0.0c	70.0a
	Mean	14.8a	0.0b	0.0b	0.0b	0.0b	26.9a
Koside-2000	7	6.5b	0.0c	0.0c	0.0c	0.0c	7.0d
	14	8.5b	0.0c	0.0c	0.0c	0.0c	10.2d
	21	8.8b	0.0c	0.0c	0.0c	0.0c	20.7 c
	30	41.7a	0.0c	0.0c	0.0c	0.0c	70.0a
	Mean	16.4	0.0	0.0	0.0	0.0	26.9

Means within a column having the same letters are not significantly different according to Duncan's Multiple Range Test at 5% level.

On the other hand, all tested biocides decreased the activities of chitinase compared with inoculated plants only as control treatment. The highest decrease was recorded with plant guard followed by Bio-Zeid and Rhizo-N treatments respectively compared with inoculated plants only as control treatment. These results could be interpreting in light the findings of Reuveni *et al.* (1992) who found that peroxidase activity in uninfected muskmelon plants was used to predict the resistance and susceptibility of 527 plants as cultivars or breeding lines and crosses of susceptible and resistant plants. Peroxidase activity increased with time in susceptible and resistant plants. Also, Ahmed (2001) reported that infection of soybean with *Colletotricum dematium* resulted in an increase in peroxidase and polyphenol oxidase activity. The highest increase in the enzyme activity occurred in the resistant and moderately susceptible cultivars. Ali *et al.* (2003) stated oxidative enzymes activity provides a positive correlation between the pathogenicity of the tested fungi and the infection of strawberry leaf spots. Susceptible strawberry cultivars produced such oxidative enzymes which were higher in the leaf tissues of the resistant cultivar. On the other hand, reduction in disease incidence was associated with increased levels of polyphenol oxidase (PPO), peroxidase (PO) and total phenols. PO activity was several times more as compared with PPO-specific activity and increased markedly after infection either with *R. solani* or *R. bataticola*. Also, Biotic inducers increased many PR-proteins such as isozymes of peroxidase, β-1,3-glucanase and chitinase. Induction of resistance using biotic agents triggers defense mechanisms nevertheless the pathogen itself. Biotic inducers increased phytoalexin accumulation after the initial suppression points towards an extra stimulation of host cell metabolism. In addition to phytoalexin accumulation this leads to gum formation and compositional changes in the cell wall. It now appears that changes in host cell wall composition apparently are more prominent or start earlier than an increase in phytoalexin accumulation (Al-Sohaibani *et al.*, 2011).

Table (5): Changes in enzyme activities in treated grape plants with some fungicides.

Treatment	PPO	PO	Chitinase
Topsin-M70	118.80	171.11	124.20
Kema-Z	119.70	122.14	67.50
Kocide-2000	176.42	125.30	69.30
Inoculated treatment	76.62	123.20	125.40
Control	46.62	54.54	61.80

PPO= polyphenol-oxidase, PO= peroxidase

Table (6): Changes in enzyme activities in treated grape plants with the tested biocides.

Treatment	PPO	PO	Chitinase
Bio-Zeid	85.95	139.31	81.60
Rhizo-N	147.60	170.23	88.80
Plant Guard	47.88	134.78	63.30
Inoculated treatment	76.62	123.20	125.40
Control	46.62	54.54	61.80

PPO= polyphenol-oxidase, PO= peroxidase

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