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EVALUATION OF SOME FUNGICIDES AND BIOCONTROL AGENTS FOR CONTROLLING OF ALTERNARIA ROT ON CITRUS FRUITS

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ABSTRACT

In this study, chemical and bio-control agents were evaluated for their efficiency to control of citrus fruit-rot disease caused *Alternaria citri* (K2) both, *in vitro* and *in vivo* trials. The most effective fungicides were score, montro and Iprodione compared with other fungicides depend on Ec50 and Ec90. While, the fungicide Pyraclostrobine had moderate effect on linear growth of *A. citri*. Meanwhile, the Coprax, Coprareekh and Azoxystrobine were the least effective fungicide at Ec90. *Trichoderma harzianum* and *Bacillus subtilis* tested showed antagonistic action effect to a highly pathogenic isolate of *Alternaria citri*, with different degrees of inhibition. *T. harzianum* was the most effective compared with *B. subtilis*, *in vitro* trials. Also, all fungicides were evaluated on incidence of citrus fruit rot disease *in vivo* trials. The disease incidence was reduced, but with different degrees. In addition, using *T. harzianum*, *B. subtilis, B. megaterium* (Bio-ARC) and *Trichoderma album* (Bio-Zeid) decreased incidence of citrus fruit rot disease. On the other side, *T. harzianum* showed the highest suppressive effect against citrus fruit rot pathogen. Generally, all tested fungicides and bio-agents were effective against the causal fungus *in vitro* and *in vivo* trials, but with different degrees.

INTRODUCTION

Citrus trees (*Citrus* sp.) within the genus *citrus* belongs to family Rutaceae sub family Aurantoideae. It is cultivated world wide. Citrus is liable to infection by several fungal, bacterial and viral diseases, in addition to physiological disorders **(El-Zayat, et al 1983, Commonwealth of Australia 2002 and Manner et al 2006).** Fungal diseases, especially Alternaria causing many diseases on citrus trees *i.e.* fruit rot of citrus fruit, leaf spot brown spot, stem end rot and mancha foliar disease **(Brown, 1994; Brown & Eckert, 2000 and Timmer et al 2003).**

Foliar fungicide applications are usually necessary to produce fruit with good external quality in areas where Alternaria brown spot is common. Studied the effect of Copper fungicides are the only material registered for control of this disease, but they are not highly effective. Since captafol has a long residual and is redistributed, few applications were needed for good disease control. However, this product is no longer registered in most areas due to health concerns **(Timmer, 1998 and Vicent et al 2007, 2009).** Iprodione is also very effective for disease control **(Whiteside, 1979; Solel et al 1996; Bhatia et al 2003 and Timmer, et al 2003).** Other fungicides that are effective and registered in Israel such as dithiocarbamates, triazoles, and famoxadon. Also, the strobilurin fungicides have been evaluated and proven effective for control of brown spot. In addition, Azoxystrobin and pyraclostrobin are generally more effective than trifloxystrobin **(Timmer et al 2003; Reis et al 2006 and van-Zyl et al 2013).** The growth of *Alternaria alternata*, *Penicillium digitatum* and *Alternaria citri* fungi were greatly suppressed by Difenoconazole at 150 ppm **(Monir and Salaheldin, 2016).**

Also, studied the effect of *T. harzianum* was the most antagonistic to the tested *Alternaria* isolate causing citrus brown spot. Also, *T. viride* and *T. koningii* exhibited an antagonistic activity more

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than that recorded for *T. album* and *G. roseum. In vitro* trials all *Trichoderma* species namely, *Trichoderma* viz., *Trichoderma viride*, *Trichoderma aureoviride, Trichoderma reesei, Trichoderma koningii* and *Trichoderma harzianum* were significantly reduced the biomass of *Alternaria citri* the causal agent of the citrus black rot disease on a broad range of citrus cultivars. In addition, using *Bacillus subtilis* against species of *Alternaria* was the most antagonistic **(Singh & Deverall 2006; Sharma, et al 2009 and Murtaza et al 2012).** Biological control of plant pathogens by microorganisms has been considered a more natural and environmentally acceptable, safety, active, alternative to the existing chemical treatment methods and economically the bio-agents cost less than fungicides **(Siameto et al 2010).**

This work was planned to test some fungicides and bio-control agents to control fruit rot disease of citrus.

MATERIALS AND METHODS.

1. Source of pathogenic fungus

Isolate of *A. citri* (code K2) was isolated from infected citrus fruit with typical symptoms of rot disease. Infected fruits were collected from Qaliobiya Governorate**.** The fungus was microscopically identified on the basis cultural and microscopic characteristic also, confirmed by Mycology and Plant Dis. Survey Department, Plant Pathol. Res. Inst. (ARC)**.**

2. Source of bioagents

Isolate of *Trichoderma harzianum* was isolated during the isolation of the causal pathogens of citrus fruit rot disease this isolate was identified by Mycology and Plant Dis. Survey Dept., Plant Pathol. Res. Inst., (ARC) Giza Egypt.

Isolate of *Bacillus subtilis* was obtained from Bacterial Dis., Res., Dept., Plant Pathol. Res. Inst., (ARC) Giza Egypt. Effect of these bioagents against the causal pathogen of citrus fruit rot disease, *i.e.* (*A. citri)*, this pathogenic isolate were isolated from infected citrus fruit and tested *in vitro* and *in vivo* trials.

3. Source of bio-agents formula

Three bioagents namely, *Bacillus subtilis* and *Bacillus* spp., (Omega) which contains $3x10^7$ to $3.1x10^7$ cfu/g and obtained from ARC, *Bacillus megaterium* (Bio ARC) which contains 25 X 10^{6} cfu /g and *Trichoderma album* (Bio Zeid) contains of $10X10^6$ spores/g were provided by Plant Pathol. Res. Inst., (ARC) Giza, Egypt. All these bioagents were evaluated against the tested pathogen and added as suspensions at the rate of 5 g / liter of water.

4. Fungicides

Efficiency of seven fungicides differed in their active ingredients and chemical groups, mentioned in **(Table 1)** to control the causal pathogen of citrus fruit-rot disease *in vitro* and *in vivo* trials.

5. *In vitro*

5.1. Preparation of fungicides concentration

Concentrations they are prepared for each fungicide according to recommended dose two concentrations before and two concentrations after recommended dose were prepared for each fungicide, according to **Sharvell (1962).** The concentrations were added to PDA medium (poisons media) as the described method by Al-Hassan and Najlah, (1982) to determine their ability to inhibit the mycelial growth of the tested pathogen. Reduction percentage of fungal growth was calculated according to the following formula.

$$\boldsymbol{Reduction}\ (\%) = \frac{\boldsymbol{Control-treatment}}{\boldsymbol{Control}}\ \mathbf{x\ 100}$$

Table 1. Tested fungicides against pathogenic isolate of Alternaria rot of citrus fruit disease

Commercial name	Common name	Recommended Dose	Company, type
Coperrarikh 50% WP.	Copper oxychloride	3 g/L	Iccta (contact)
Montoro (30%) EC.	Propaconazol-Difenoconazole	0.4ml/L	Star-chem
Score (250%) EC.	Difenoconazole	0.5 ml/L	Syngenta
Iprodione **(50%) G**	Iprodione	1.5 g/L	PROPLAN
Coprax 77% WP	Copper hydroxide	2.50g/L	
Azoxystrobine **25%SC**	Azoxystrobine	0.5 ml/L	GREEN PLANT
Pyraclostrobine 11.2% EC	**Pyraclostrobine**	2ml/L	BASF

5.2. Preparation inoculum of *T. harzianum* and solid agar bioassay

T. harzianum was grown on PDA medium. To test the antagonistic effect of the two bio-agents under study *in vitro* on the linear growth of the causal pathogen of citrus fruit rot disease, the following method was used. Petri dishes (9 cm in diameter), each contained 20 ml., of PDA medium were inoculated with discs (5 mm in diameter) of the tested pathogen, taken from 7day-old cultures. The discs were placed near of the edge of each Petri-dish. At the same time plates were inoculated with equal discs of *T. harzianum*. Five plates were used as replicates for each treatment. *In-vitro* biological activity of *T. harzianum* on *A. citri* was investigated by double cultures on the potato dextrose agar. They were incubated at 28±1 ^{o}C and the diameter of the colonies was measured for 10 days.

5.3. Efficacy of antagonistic bioagent against *A. citri* (code K2)

A- **In diffusible metabolites,** investigations were maintained with the variants:

1- Placing the fragments of test fungus and pathogen on the separated half of a media. The discs of test fungus and pathogen were placed near the edge of each petri-dish. At the same time plates were inoculated with equal discs of *T. harzianum* on the same distance of appoint side.

B- **In volatile metabolites** investigations, fragments of the tested *Alternaria citri* (Code, K2) and *T. harzianum* was placed in the center of two separate PDA plates sealed with parafilm, so, the *T. harzianum* was in the under plate and, the *Alternaria* isolate was in upper plate. There were two controls (*T. harzianum* and *A. citri)* in an inverted position. After the period of incubation, micro observations were made of the *Alternaria* isolate in the two kinds of double cultures **(Gveroska and Ziberoski, 2012).**

5.4. Preparation inoculum of *B. subtilis*

Isolate of *Bacillus subtilis* was grown on nutrient agar medium (NA). Antagonistic effect of *B. subtilis* on the linear growth of the same pathogen was tested *in vitro*. The tested bacterium was streaked on PDA plate near of the edge of each Petri dish, while the inoculation with the tested pathogen was done as mentioned before in the second half of each dish. All plates were incubated at 28±1 ºC until the growth in control treatment reached the edge of the plates. Reduction percentage of fungal growth was calculated according to the formula as mentioned before.

6. *In vivo*

6.1. Fungicides

The above mentioned fungicides were used sprayed on citrus old-shoots of Navel orange carrying flowers at Qaliubiya governorate. These fungicides were utilized as suspension and sprayed as mentioned before, at their recommended dose per liter of water.

6.2. Biological control

6.2.1. Preparation of antagonists inocula:

These trials were carried out under open field conditions were utilized to evaluate the efficiency of the bioagents *T. harzianum*, Bio-ARC (*B. megaterium*), Bio Zeid (*T. album*) the bioagents Bio-ARC (*B. megaterium* 6% (25x16^{6} bacterial cells /g) or Bio-Zeid (*T. album*, 2.5 % (10x10^{6} spores /g) and (*Bacillus* sp*., B. subtilis*¸ contains 3x10^{7} to 3.1x10^{7} cfu/g) omega for controlling of pathogenic fungus associated with citrus fruit rot disease.

6.2.2. Preparation inoculum of *T. harzianum*

The inoculum of *T. harzianum* (the fungal isolate) was prepared by growing on autoclaved liquid medium (Czapek's liquid medium CLM **(Jakovljević et al 2015).** The inoculation was carried out with 5mm in diameter fungal disks taken from the margin of 7 days old culture. The inoculated flask were incubated at 28±1 ºC for 15 days, *T. harzianum* was sprayed on the tree at the rate of 10^{8} spore/ml^{-1} **(Arrebola et al 2010).**

6.2.3. Preparation inoculum of *B. subtilis*

B. subtilis was prepared by grown on liquid medium (5g peptone, 3g beef extract and 10 glucose per liter) was sprayed on the tree at the rate of 10^{7} cfu/ml^{-1}. Also, a number of trees were inoculated with the bioagents, each alone, and the same number was left free from the bioagent as control treatment.

6.2.4. Preparation inoculum of formula

In the second method, the bioagents were sprayed on the trees during flowering. While, *B. megaterium* (Bio-ARC) *T. album* (Bio-Zeid) and *B. subtilis, Bacillus* spp*.,* (Omega) were sprayed on tree as suspensions at the rate 5 g / liter of water.

Statistical analysis

Data were statistically analyzed using complete randomized design according to **Gomez and Gomez, (1984).** The bioassay average *in vitro* trial was compared by least significant differences (LSD) test at level 5 %. Chemical and biological *in vivo* trials the percentage data were transformed into arcsine angles to produce approximately constant variance before carrying out variance (ANOVA). The significance of various treatments was evaluated by Duncan's multiple range tests (p< 0, 05%) **(Duncan, 1955).**

RESULTS

a. Effect of chemical control, *in vitro*.

Data presented in **Table (2)** indicate that all the tested fungicides were affected on the linear growth of the tested fungi. Generally, the magnitude of effect was increased by increasing the concentration of all the tested fungicides. The most significantly effective fungicides were Score, Montro and Iprodione and the reduced the growth of *Alternaria citri* isolate(code K2) which inoculated on (PDA medium) plate was reached around recommended dose for each fungicide was used against *Alternaria citri* isolate (code K2) under study. Also, the results in **Table (2)** show that, Score, Montro and Iprodione fungicides were the most effective fungicides against *Alternaria* isolate (code K2) based on Ec. 90, where the linear growth the fungus 96.89, 78.42 and 329.01 respectively. Whilst, Pyraclostobine fungicide was moderate effective fungicide against *Alternaria* isolate (code K2) at Ec. 90, where the linear growth the fungus 5429.62. While, Coprax, Coprekh, and Azoxystrobine were the lowest effective fungicides compared with other fungicides against *Alternaria* isolate (code K2) at Ec. 90,where the result were found to be 4741.49, 3165.96 and 1000.00, respectively.

b. Effect of bio-agents, *in vitro*

1. Interaction between the bio-agents with *A. citri* isolate (K2) by direct contact

The obtained data in **Table (3)** show that *T. harzianum* was the most effective bio-agents against *A. citri* isolate (code K2) causing citrus fruit rot disease compared with *B. subtilis*. The reduction percentage was 86.66% when *T. harzianum* was used with K2. Whilst, the reduction percentage was 69.44% when *B. subtilis* was used with K2. On the other side, obtained data show that *B. subtilis* was the lowest effective than *T. harzianum* with *A. citri* isolate (code K2). Also, data presented in **Table (3)** revealed that, volatile (metabolism) of *T. harzianum* was more effect on linear growth of *A. citri* the causal pathogen of citrus fruit rot than *B. subtilis* but, less than when *T. harzianum* inoculated in the same plate with *A. citri.* While, the antagonistic action was highly significant and occurred by *T. harzianum* with K2 isolate of *A. citri* the causal pathogen of citrus fruit rot disease and the reduction percentage was 86.44%.

Table 2. Effect of using some fungicide on the mycelial growth of *A. citri* (Code K2) the causal pathogen of citrus fruit rot disease *in vitro* trial.

Fungicides	Ec. 50	Ec. 90	Reg. Equation	R^2
Score	**20.44**	**96.89**	Probit= 1.894 x -2.513	0.865
Montro	**16.04**	**78.42**	Probit= 1.857 x-2.762	0.883
Iprodione	**74.03**	**329.01**	Probit= 1.976 x +1.306	0.819
Coprarrekh	**1511.39**	**5419.62**	Probit= 2.308 x -2.338	0.968
Coprax	**1522.86**	**4741.49**	Probit= 2.595 x-3.259	0.993
Pyraclostrobine	**160.79**	**3165.96**	Probit= 0.989 x+2.818	0.953
Azoxystrobine	**268.27**	**1000.00**	Probit= 2.24 x-0.440	0. 583

Reg. Equation=Regression equation between Log concentration (Log Y) and the probit of the % of growth inhibition (X).
Coeff. Error (R^2) = Correlation coefficient of Y and X

Table 3. Effect of *Trichoderma harzianum* and *B. subtilis* as bio agents on mycelial growth of *A. citri* isolate the casual pathogen of citrus fruit rot disease *in vitro* trial.

Bioagent	Reaction	Growth (cm)	*Red. %
T. harzianum	Direct contact	1.20	86.66
	Indirect contact	1.22	86.44
Bacillus subtilis	Direct contact	2.75	69.44
Control		9.00	0.00
LSD. at level 5% for treatment		(*****T)= 0.51553	(*****B) = 0.84802

* Red. = Reduction percentage

* T= *T. harzianum* *B= *Bacillus subtilis*

c. Effect of chemical control, *in vivo* studies

The aforementioned fungicides were tested for their efficiency to control the disease under field conditions. Results in **Table (4)** show that these fungicides differed in their effect on *A. citri* isolate (K2). *Alternaria citri* was highly affected by using selective fungicides namely, Score, Montoro and Iprodione and the percentage of the disease incidence in the field was decreased and the average of the disease incidence was reach about (4, 4 and 8%) respectively, also, the percentage of disease incidence was reach about (5.31, 5.31 and 10.63%), respectively.

Table 4. Effect of some fungicides on disease incidence of citrus fruit rot *in vivo*

Treatment	Dis., Incid. Average	In T	*R. %	H T
Coprareekh	20	23.79 bc	80.00	66.21 ab
Iprodione	8	10.63 c	92.00	79.37 a
Coprax	20	23.79 bc	80.00	66.21 ab
Score	4	5.31 c	96.00	84.69 a
Montoro	4	5.31 c	96.00	84.69 a
Pyraclostrobine	12	13.16 c	88.00	76.84 a
Azoxystrobine	36	36.47 b	64.00	53.53 b
Cont A. with W	76	63.68 a	24.00	26.32 c

* The data were transformed using arcsine formula.

*R= Reduction percentage of the disease incidence

*In T= Transformed data for disease incidence

*H T = Transformed data for fruits healthy unit

While, Pyraclsotrobin was moderate effective when used against the pathogenic *Alternaria* isolate and the reduction percentage of disease incidence was (88%) and the disease incidence average was (12%)**.** Whilst, Coprax, Coprareekh, and Azoxystrobin were the lowest effective fungicides against the highly pathogenic isolate of *Alternaria* (K2) were selective under study.

d. Effect of bio-agents *in vivo*:

Data presented in **Table (5)** reveal that, *T. harzianum* was the most effective bio-agent against tested casual pathogen of citrus fruit rot disease Followed by *B. subtilis*. While, *Bacillus* sp*., B. subtilis* (omega) and *B. megaterium* (Bio-ARC) and *T. album* (Bio-Zeid) were the lowest bioagents, respectively. Application of bioagents led to decrease of the disease incidence to 84% with *T. harzianum* followed by *B. subtilis* whereas the reduction percentage of disease incidence was76%. Whilst, *B. megaterium* (Bio-ARC), *T. album* (Bio-Zeid), and *Bacillus* sp*., B. subtilis* (omega) were the lowest effective bio-agents against *A. citri* isolate causing citrus fruit rot disease, whereas the percentage of disease reduction was reach about (60, 68 and 72%) respectively.

Table 5. Effect of bioagents on the disease incidence of citrus fruit rot, under field conditions

Treatment	Dis., Incid. Average	*In T	*R.%	*H T
Bacillus	24	26.32 bc	76	63.68 ab
Trichoderma	16	18.47 bc	84	71.53 ab
Omega	28	28.63 bc	72	61.38 ab
Bio-zied	32	33.94 bc	68	56.06 ab
Bio-arc	40	39.01 ab	60	51.00 bc
Cont A. With water	68	55.84 a	32	34.17 c

* The data were transformed by using arcsine formula.

* R. = Reduction percentage of the disease incidence

* In T= Transformed data for disease incidence

* H T = Transformed data for fruits healthy unit

DISCUSSION

Citrus fruit rot disease incited by *A. citri* is one of the serous diseases which attack citrus cultivars causing severe damage whereas causing many diseases on citrus, *i.e.* black fruit rot, stem end rot, brown spot and leaf spot **(Farooqi et al 1985; Solel, 1991; Brown and Eckert, 2000; Katoh et al 2006 and Kono, et al 2015).** Chemical control results indicated that, *A. citri* was more affected fungus with three fungicides namely, Score, Montoro and Iprodione were the best fungicides based on (Ec. 50 and Ec. 90) and no observed growth of the causal fungus when these selective concentrations were used *in vitro* whereas at Ec. 90 were found to be 96.89, 96.89 and 329.01 respectively, and the same trend was observed *in vivo* trials and the reduction percentage of the disease incidence was 96.00, 96.00 and 92.00 % respectively, while, coprax, coprareekh, and azoxystrobine were the lowest effective fungicides against isolate of *A. citri* (K2) causing citrus fruit rot disease *in vitro* and the same trend was observed *in vivo* trials and the reduction percentage of the disease incidence was 80.00, 80.00 and 64.00% respectively. While, Pyraclsotrobin was moderate effective when used against the pathogenic *Alternaria* isolate and the reduction percentage of disease incidence was (88%) and the disease incidence average was (12%) when was used *in vivo* trial level**.** This result agrees with the authors **(Timmer & Zitko, 1992, 1994, 1997; Solel et al 1997 and Swart et al 1998),** they found that, Iprodione is very effective against Alternaria disease control. Other fungicides that are effective and registered in Israel are the dithiocarbamates, triazoles, and famoxadon. Azoxystrobine and pyraclostrobine are generally more effective than trifloxystrobin. The strobilurin fungicides are single site of action fungicides and also, have been evaluated and proven effective for control of Alternaria brown spot **(Timmer et al 2003).** *In vitro* and *in vivo* trials *Alternaria alternata*, *Penicillium digitatum* and *Alternaria citri*, was greatly suppressed by Difenoconazole at 150 ppm **(Monir and Salaheldin, 2016).**

Biological control is widely used in controlling soil-borne and fruit rot disease in many fruit crops **(Jefferson et al 2000; Arrebola et al 2010 and Ferdousi-Begum et al 2010).** The obtained data showed that, the tested bioagents were effective against the tested causal pathogen of citrus fruit rot disease. *In vitro* studies proved that *Trichoderma harzianum* isolated was found highly effective in suppressing as abioagent against the tested causal pathogenic *Alternaria* isolate (Code, K2) followed by volatile of *Trichoderma harzianum* then Bacillus *subtilis* the reduction percentage of the growth was 86.44, 86.66and 69.44% respectively. *In vivo* studies also, showed that *T. harzianum* was the most effective bioagent against Alternaria isolate (Code, K2) followed by *Bacillus subtilis* then omega whereas the reduction percentage of disease incidence was 76, 84 and 72%. Whilst, *B. megaterium* (Bio-ARC) and *T. album* (Bio-Zeid) were the least effective bioagent against Alternaria isolate (Code, K2) the causal pathogen of citrus fruit rot, reduction percentage of disease incidence was 68 and 60% respectively. These results are in agreement with **Rachniyow and Jaenaksorn (2008)** who found that, *Trichoderma* spp., are common inhabitants of the rhizosphere as biocontrol against soil-borne plant pathogens. Commendable amount of researches have been focused on the mycoparasitic nature of genus *Trichoderma* and its contribution to plant health. Several mechanisms have been considered to be key factors in antagonistic interactions, *i.e.* lysis of host cell walls, antibiosis, competition for nutrients, induced resistance in plants and inactivation of host enzymes. Mixed cultures of the microbial antagonists appear to provide better control of postharvest diseases. At the international level, different microbial antagonists *i.e. Debaryomyces hansenii, Cryptococcus laurentii B. subtilis* and *T. harzianum* were used against and suppress the activity of post-harvest pathogens in fruit and vegetables **(Sharma et al 2009).**

This may be lead to disease escape or to higher tolerance against soil-borne and air-borne pathogens. *B. megaterium*, *B. cereus* and *B. subtilis* have been used for the biocontrol purpose. The activity of biocontrol agents against soil-borne disease is important to achieve successful control activity **(Lee et al 2008 and Hye-Sook et al 2009).**

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