African Journal of Agricultural Research Vol. 5(13), pp. 1733-1738, 4 July, 2010
Available online at http://www.academicjournals.org/AJAR
ISSN 1991-637X ©2010 Academic Journals

Full Length Research Paper

Evaluation of lyophilized and non lyophilized toxins from *Trichoderma* species for the control of *Ceratocystis paradoxa*

E. I. Eziashi[1]*, N. U. Uma[2], A. A. Adekunle[2], C. E. Airede[1] and E. E. Odigie[1]

[1]Nigerian Institute for Oil Palm Research (NIFOR), Plant Pathology Division, P. M. B. 1030, Benin City, Edo State, Nigeria.
[2]Department of Botany and Microbiology, University of Lagos, Akoka, Yaba, Lagos State, Nigeria.

Accepted 13 May, 2010

Lyophilized and non-lyophilized toxins produced from *Trichoderma* species were investigated against *Ceratocystis paradoxa* mycelial growth at different concentrations *in-vitro*. Lyophilized toxin inhibition on *C. paradoxa* by *T. viride* (28.14 and 28.14%) and *T. polysporum* (28.88 and 28.88%) at 70 and 100% concentrations were significant (p = 0.005) compared with *T. hamatum* (20.37 and 21.48 %) and *T. aureoviride* (20.00 and 20.37%) six days after incubation. Non-lyophilized phytotoxin inhibition on *C. paradoxa* mycelia by *T. viride* (21.85 and 23.335%) and *T. polysporum* (22.22 and 22.59%) at 70 and 100% were also significant (p = 0.005) when compared with *T. hamatum* (16.29 and 18.14%) and *T. aureoviride* (17.03 and 17.77%) 6 days after incubation. The mycelial growth of *C. paradoxa* was stimulated by *T. hamatum* and *T. aureoviride* at 20 and 50% concentrations with lyophilized and non-lyophilized phytotoxins. The lyophilized phytotoxin exhibited better control of *C. paradoxa* compared with non-lyophilized and the control treatments. Using gas chromatography-mass spectrometry, column fraction isolated from *T. viride* R_f value 0.51 was deduced to be 1, 2-benzendicaboxylic acid. It is responsible for the inhibitory action on *C. paradoca* mycelia. This compound produced by *T. viride* depended on type of fungus strain and standard used.

Key words: GC-MS, inhibition, mycelia, 1, 2-benzendicarboxylic acid.

INTRODUCTION

Ceratocystis paradoxa Dade C. Moreau is the causal agent of black rot of sprouted seeds disease of the oil palm. It causes a sudden loss of a high proportion. This eventually results in acute shortage and the upsetting of a long term planting programme (Omamor, 1985). The potential of fungal antagonists for biological control of plant pathogens has been recognized for many years (Chet, 1987), and according to Chet et al. (1998). Series of co-ordinated inoculation of *Trichoderma* spp. leading to successful parasitism by increased active ingredients to control a range of economically important aerial and soil-borne fungal plant pathogens has been reported (Harman, 2000). The antagonistic activity of *Trichoderma* strains is attributed to one or more complex mechanisms, including nutrient competition, antibiosis, the activity of cell wall-lytic enzymes induction of systemic resistance, and increased plant nutrient availability (Naseby et al., 2000; Lorito et al., 1996; Jeffries, 1995). Typical symptoms of most plant diseases revealed the involvement of phytotoxic metabolites, which therefore suggest a role for toxic metabolites secreted by the pathogen in the disease development. Metabolites of many fungi may have adverse or stimulatory effects on plants (Scheffer, 1983).

At present benomyl fungicide is recommended for the control of *C. paradoxa* causing rot of sprouted seeds of the oil palm (Omamor, 1985). However, fungicide treatment is not desirable for disease control due to some adverse effects on the environment and ecosystem (Chet and Inbar, 1994). In addition, fungicide treatment is expensive in comparison with the relative low commodity price of sprouted seeds. The objective of the study was to determine the effect of toxins produced by *Trichoderma*

*Corresponding author. E-mail:eziashius@yahoo.com.

species for the control of *C. paradoxa.*

MATERIALS AND METHODS

Fungal isolate

The *Ceratocystis paradoxa* was isolated from diseased oil palm sprouted seeds collected from NIFOR seed store. The pure culture was maintained in Petri dish of potato dextrose agar medium for 4 days before used.

Fungal culture filtrate

A small mycelial block (4 mm) was removed from each of the 7 day-old pure cultures of *T.* species and *C. paradoxa.* Each was transferred to1 L conical flask (Brian and Hemming, 1945) containing medium. Inoculated conical flasks were incubated aerobically and statically 22 ± 2°C for 60 days. Filtrates were harvested by filtering through a muslin cloth twice and then by Whatman filter paper no1 twice. The culture filtrate was again obtained by centrifugation at 5000 x g for 15 min. The first filtrate (250 ml) was concentrated to 10 fold (10 ml) through lyophilization. Second set of filtrate (250 ml) was non-lyophlized.

Experiments

Mycelial growth of *C. paradoxa* in the presence of lyophilized and non-lyophilized toxins from *T. viride, T. polysporum, T. hamatum* and *T. aureoviride* were determined at different concentrations (20, 50, 70 and 100%) and were tested on solidified PDA. Each 0.2 ml of *T.* species toxin was pipetted into 8 mm diameter and a disc of *C. paradoxa* mycelium (4 mm) was inoculated on it. The control plates had medium without toxin, inoculated with *C. paradoxa* agar disc. The *C. paradoxa* mycelia growth was measured daily and the inhibition percentage was also obtained using the formula 1% = [(C2-C1)/ C2)] x 100 (Edington et al., 1971), where C1 means growth of *C. paradoxa* and C2 means growth in control.

Three grams of sticky samples each from *Trichoderma* spp., *C. paradoxa* and the controls (medium with chloramphenicol and medium without chloramphenicol) were sequentially extracted. Extracts were dissolved in 100% methanol at 1 gm/ml. The compounds were spotted on TLC silica gel, sprayed and detected with anilsaldehyde sulphuric acid 0.2 gm (Harborne, 1998) and later kept in an oven for 5 min. at 100°C. Retention factor (R_F) values were calculated as distance by sample/distance moved by the solvent (Harborne, 1998). Only very strong column chromatography fraction from *T. viride* for the control of *C. paradoxa* mycelial growth was extracted from TLC plate. The sample was sent for identification at Lagos State Ministry of Health, drug quality control laboratories. The sample was dissolved in 2 ml of n-hexane. Fraction was analyzed by gas chromatography-mass spectrometry (GC-MS). (Hewlett Packard 5890 series II gas chromatography coupled to Hewlett Packard 5989 A MS engine). The experiment was repeated twice. Data were given as mean ± SEM of measurements from 5 replicates. They were submitted to student's "t" with 1% significance level.

RESULTS

Ceratocystis paradoxa (Dade) C Moreau (Herbarium IMI No 314373), *T. viride*, *T. polysporum*, *T. hamatum* and *T. aureoviride* have been reported to affect oil palm sprouted seed (Eziashi et al., 2006). The study showed that *Trichoderma* species produced toxins in the medium. Production of *Trichoderma* toxins reached to it peak after 60 days of incubation. The initial dull of the broth changed to cream and yellow pigmentations. Lyophilized and non lyophilized toxins extracted on the 2nd, 4th and 6th day after incubation at 70 and 100% concentrations did not show significant differences. Significant differences were noticed with 20 and 50% concentrations. Lyophilized toxin by *T. viride* inhibited *C. paradoxa* (28.14 and 28.14%) and *T. polysporum* (28.88 and 28.88%) at 70 and 100% concentrations which were significant (p = 0.005) when compared with *T. hamatum* (20.37 and 21.48%) and *T. aureoviride* (20.00 and 20.37%) 6 days after incubation. Inhibition with non-lyophilized toxin on *C. paradoxa* by *T. viride* (21.85 and 23.335%) and *T. polysporum* (22.22 and 22.59%) at 70 and 100% were significant (p = 0.005) when also compared with *T. hamatum* (16.29 and 18.14%) and *T. aureoviride* (17.03% and 17.77%) 6 days after incubation, as shown in Table 1.

The mycelial growth of *C. paradoxa* was inhibited by *T. hamatum* (10.74 and 12.96%) and *T. aureoviride* (11.48 and 13.33%) at 20 and 50% concentrations with non-lyophilized toxin. The lyophilized toxin exhibited better control of *C. paradoxa* mycelial growth compared with non-lyophilized toxin. The *T.* species were significant when compared with the control treatments (0.00 and 0.00) as shown in Tables 2 and 1. Many UV-absorbing compounds were detected in different species of *Trichoderma* and *Ceratocystis*. Colors such as yellow, brown and grey were obtained immediately as the anisaldehyde-sulphuric acid was sprayed on ethyl acetate-methanol extracts of *Trichoderma* species and *Ceratocystis paradoxa* metabolites. *T. viride* pure column fraction had strong inhibition on the mycelial growth of *C. paradoxa*. The R_f value inhibition (spotted as brown color) of *T. viride* was at 0.51 with (+++) very strong activity. The R_f value inhibition (spotted as grey color) of *T. polysporum* on the pathogen was at 0.29 with (+++) very strong activity (Table 3).

On the basis of analysis by gas chromatography-mass spectrometry (GC-MS), the tentative name of the phytotoxic compound in which column fraction was isolated from *T. viride* R_f value 0.51, was deduced to be 1, 2-benzendicarboxylic acid. Other names are Bis (2-propylheptyl) phthalate; Phthalic acid; bis (2-propylheptyl) ester. The retention (RT) minutes and compound were identified by matching GC-MS data. The peak absorption RT minutes of the compound (R_f value 0.51) was 68.49 (Figure 1).

DISCUSSION

Trichoderma species and *C. paradoxa* produced toxins in the medium. Cream and yellow pigments produced by *Trichoderma* species, suggests that the pigments might

Table 1. Response of non-lyophilized toxin produced by *Trichoderma* species on *C. paradoxa* mycelial growth.

Days	Treatment	Phytotoxin (250 ml) of *T.* spp. and mean radial growth of *C. paradoxa* mycelia (cm) in *T.* spp. phytotoxins				Mean (%) radial growth inhibition on *C. paradoxa* mycelia by *T.* species phytotoxins			
		20%	50%	70%	100%	20%	50%	70%	100%
	T. viride	3.4	2.9	2.6	1.8	16.16±1.12e	18.68±1.09d	20.20±1.07c	24.24±1.13a
	T. polysporum	3.5	2.9	2.3	1.5	15.65±1.12e	18.68±1.09d	21.71±1.09b	25.75±1.19a
2.	*T. hamatum*	4.4	3.6	2.7	2.8	11.11±1.02h	15.15±1.12e	19.69±1.07c	19.19±1.07d
	T. aureoviride	4.2	3.8	2.6	2.9	12.12±1.04g	14.14±1.12f	20.20±1.07c	18.68±1.07d
	Control	6.6	6.6	6.6	6.6	0.00.00i	0.00±00i	0.00±00i	0.00±00i
	T. viride	4.3	3.6	3.1	2.7	17.40±1.15e	20.00±1.07c	21.85±1.09b	23.33±1.13a
	T. polysporum	4.1	3.9	3.0	2.9	18.14±1.09d	18.88±1.09d	22.22±1.13b	22.59±1.13b
4.	*T. hamatum*	5.9	5.3	4.6	4.1	11.48±1.02h	13.70±1.12f	16.29±1.12f	18.44±1.09d
	T. aureoviride	5.7	5.4	4.3	3.9	12.22±1.04g	13.33±1.12f	17.40±1.15e	18.88±1.09d
	Control	9.0	9.0	9.0	9.0	0.00±0.00i	0.00±0.00i	0.00±0.00i	0.00±0.00i
	T. viride	4.5	3.6	3.1	2.7	16.66±1.12e	20.00±1.07c	21.85±1.09b	23.33±1.13a
	T. polysporum	4.4	4.1	3.0	2.9	17.03±1.06e	18.14±1.09d	22.22±1.13b	22.59±1.13b
6.	*T. hamatum*	6.1	5.5	4.6	4.1	10.74±1.01h	12.96±1.04f	16.29±1.12e	18.14±1.09d
	T. aureoviride	5.9	5.4	4.4	4.2	11.48±1.02h	13.33±1.12f	17.03±1.06e	17.77±1.04d
	Control	9.0	9.0	9.0	9.0	0.00±00i	0.00±00i	0.00±00i	0.00±00i

Values are mean ± SEM of five replicate results. Concentration with the highest growth inhibition was compared to others using the student's t-test.

Table 2. Response of lyophilized toxin produced by *Trichoderma* species on *C. paradoxa* mycelial growth.

Days	Treatment	Phytotoxin concentration (10 ml) of *T.* spp. and mean radial growth of *C. paradoxa* mycelia (cm) in *T.* spp. phytotoxins				Mean (%) radial growth inhibition on *C. paradoxa* mycelia by *T.* spp. phytotoxins			
		20%	50%	70%	100%	20%	50%	70%	100%
	T. viride	0.5	0.3	0.2	0.2	30.80±1.20c	31.81±1.85b	32.32±1.11a	32.32±1.11a
	T. polysporum	0.5	0.4	0.3	0.1	30.80±1.20c	31.31±1.20c	31.81±1.85b	32.82±0.80a
2.	*T. hamatum*	0.7	0.4	0.4	0.2	29.79±1.33d	31.31±1.20c	31.31±1.20c	32.32±1.11a
	T. aureoviride	0.8	0.7	0.4	0.5	29.29±1.33d	29.79±1.33d	31.31±1.20c	30.80±1.20c
	Control	6.6	6.6	6.6	6.6	0.00±0.00f	0.00±0.00f	0.00±0.00f	0.00±0.00f
4.	T. viride	1.7	1.6	1.3	1.4	27.03±1.14c	27.40±1.11b	28.51±1.19a	28.14±1.15a
	T. polysporum	1.8	1.6	1.2	1.2	26.66±1.14c	27.40±1.11b	28.88±1.15a	28.88±1.15a

Table 2. Continued.

	T. hamatum	2.9	2.4	2.1	2.1	22.59±1.20f	24.44±1.13e	25.55±1.19d	25.55±1.19d
	T. aureoviride	3.2	2.9	2.4	2.5	21.48±1.11g	22.59±1.20f	24.44±1.13e	24.07±1.13e
	Control	9.0	9.0	9.0	9.0	0.00±00h	0.00±00h	0.00±00h	0.00±00h
	T. viride	1.7	1.6	1.4	1.4	27.03±1.14d	27.40±1.11c	28.14±1.15b	28.14±1.15b
	T. polysporum	1.8	1.6	1.2	1.2	26.66±1.14d	27.40±1.11c	28.88±1.15a	28.88±1.15a
6.	*T. hamatum*	3.5	3.2	2.9	3.1	20.37±1.07h	21.48±1.11g	22.59±1.20e	21.85±1.09f
	T. aureoviride	3.6	3.5	3.1	3.1	20.00±1.03h	20.37±1.07h	21.85±1.09f	21.85±1.09f
	Control	9.0	9.0	9.0	9.0	0.00±0.00i	0.00±0.00i	0.00±0.00i	0.00±0.00i

Values are mean ± SEM of five replicate results. Concentration with the highest growth inhibition was compared to others using the student's t-test

Table 3. *Trichoderma* spp. R_f values and activities of pure column chromatography fractions on *C. paradoxa* mycelial growth in PDA well.

	Solvents			
Fungal sample	**Ethyl acetate-methanol**		**Chloroform-methanol**	
(0.2 ml/well)	**R_f value**	**Activity**	**R_f value**	**Activity**
T. viride	0.51	+++	0.53	+
	0.56	++		
T. polysporum	0.59	-	0.29	+++
			0.32	+++
T. hamatum	0. 47	+		
	0. 63	-		
T. aureoviride	NT	NT	NT	NT
Control	-	-	-	-

have toxicogenic activities. This agrees with the hypothesis that, isolates, which produced pigments in liquid culture, were more efficient in producing biological active culture filtrate (Duarte and Archer, 2003). The effects of inhibitory actions on *C. paradoxa* mycelia by *T. viride, T. polysporum* and *T. hamatum* appeared to be associated with toxic compounds produced by them. The actual effect and mechanism of inhibition of mycelium by the toxin is not known, However, *Trichoderma* species are known to produce a range of metabolites that may affect the growth of

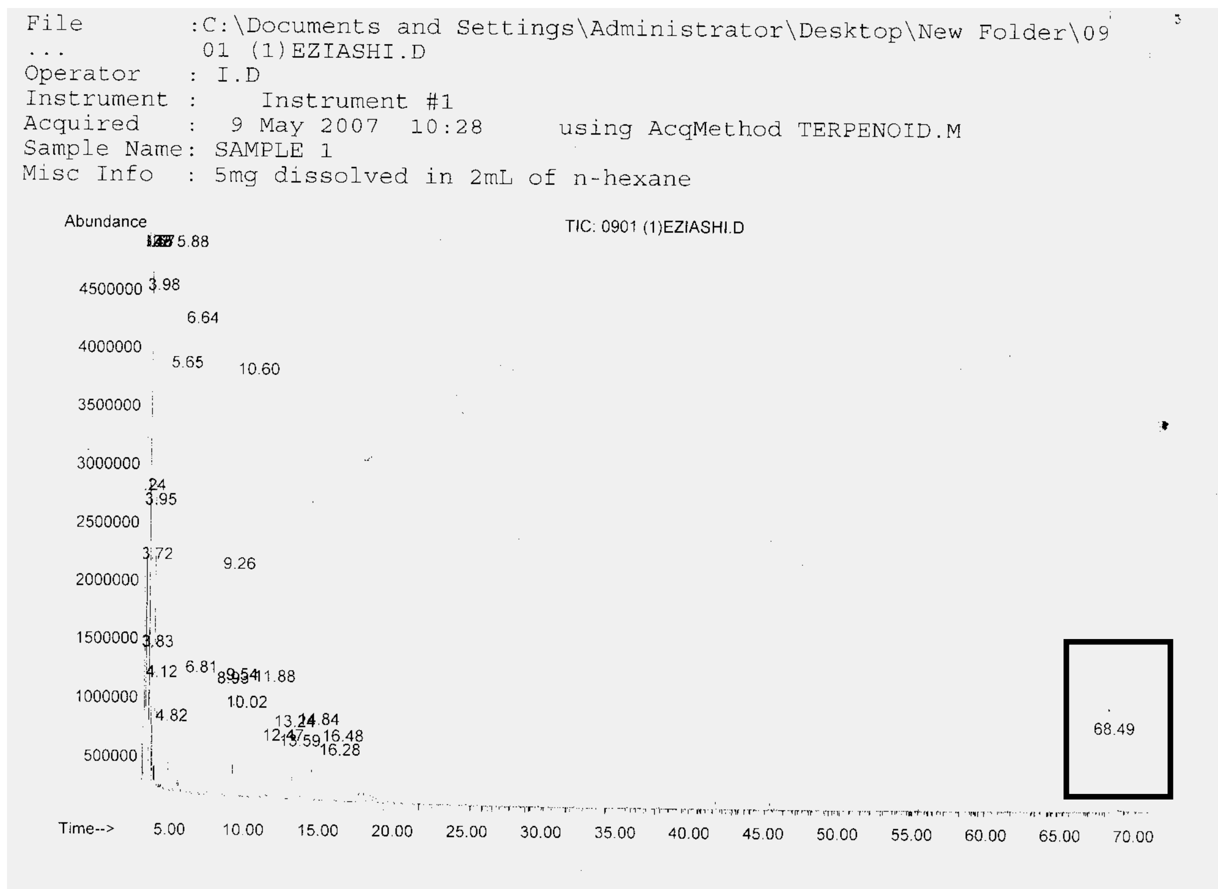

Figure 1. TLC profile of n-hexane developed with chloroform-methanol extract of *Trichoderma viride.* The peak absorption (retention minutes) of 1, 2-benzendicarboxylic (R_f value 0.51) was 68.49.

microorganisms and plants. The production of antibiotics such as viridin, (Grove et al., 1996) trichoder-min, ergokonin, (Kumeda et al., 1994) and viridin fungin A, B and C (Harris et al., 1993) produced by different isolates of *T. viride* have been involved in biological control.

Trichoderma species and *C. paradoxa* extracts spotted on thin layer chromatography (TLC) silica gel showed that species of *Trichoderma* produced brown, grey and yellow colors under ultra-violet light. This indicates that, they contain phenolic compounds (De Rosa, 2003). The GC-MS confirmed 1, 2-benzendicarboxylic as a phytotoxic metabolite from *T. viride*. It is an allelopathic bioagent, which could be used for the control of *C. paradoxa*. This agreed with Chou (1992), who reported 1, 2-benzendicarboxylic as an allelochemical. It was supported by Mattice et al. (1998) as one of the compounds present in rice samples in a study on the effect of allelopathic rice varieties on ducksalad. Rimando et al. (2001) showed that isolated 1, 2-benzendicarboxylic acid from the root of Taichung Native 1 rice was an allelochemical, which inhibited the germination of lettuce seedlings. This compound produced by *T. viride* depends on the type of fungus strain and standard used.

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