Effect Of Antifungals On Physiological Activities Of Some Plant Pathogenic Fungi

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Abstract

Each of the antifungal compounds extracted from each of the antagonistic microorganisms was found to has an inhibitory effect on Rhizoctonia solani and Fusarium oxysporum. Four antifungal compounds were found to be the most inhibitory than the other compounds. The first one from Eupenicillium senticosum, the second from Streptomyces aurantiacus, the third from Curtobacterium pusillum and the last one from Saccharomyces unispora. The first three compounds were active against R. solani, while the last one was active against F. oxysporum. All the four compounds were found to reduce or inhibit the growth of R. solani and F. oxysporum. They also reduce the carbohydrate concentrations, as well as reducing the nitrogen content (total nitrogen, soluble nitrogen and protein nitrogen). Regarding their effect on the enzyme activity, they increase the activity of fumarase, malic dehydrogenase and succinic dehydrogenase, while they reduce the activity of catalase enzyme of both R. solani and F. oxysporum as compared to control.

INTRODUCTION

Nitrogen fractions such as amino-N, peptido-N, ammonia-N, total soluble-N, protein-N as well as total-N of six fungal species exposed to three fungicides were measured. The fungicide Afugan (pyrazofos) induced a significant increase in the total-N of Trichoderma harizanum and Fusarium solani, where as a significant decrease was observed with Aspergillus niger and Penicillium chrysogenum [18].

Biological control has potential for the management of soil-inhabiting fungi. A variety of soil microorganisms have demonstrated activity for control of various soil-borne plant pathogens. Biological control fungi, such as Trichoderma and Gliocladium spp., have been used to control a variety of fungal pathogens, including Rhizoctonia, Pythium, Sclerotinia and Fusarium spp. [14].

Five antifungal agents with different mechanisms of action were compared for their ability to affect mitochondrial dehydrogenase activity and adherence capacity of Candida albicans to polystyrene and extracellular matrix proteins. Only amphotericin B inhibited mitochondrial dehydrogenase activity when the culture medium was supplemented with galactose [10].

Chlorinated macrolides, haterumalide Na, B and NE, and a new haterumalide X were produced by the soil bacterium Serratia plymuthica. Haterumalides NA, B and NE caused complete suppression of apothecial formation in Sclerotia sclerotiorum at a concentration of 0.5 μg ml^{-1}. Ascospore germination of this fungus was inhibited in the concentration range 0.8- 3.0 μg ml^{-1}. Haterumalides Na, B and NE prevented spore germination of several other filamentous fungi as Oomycetes at concentrations ranging from 0.4 to 4.0 μg ml^{-1}, but did not show any effect against the yeast Candida albicans [11].

Biological control is important to reduce the environmental contamination with pesticides. The present investigation aims at understanding the effect of the antifungal compounds, produced by some antagonistic microorganisms, on growth of plant pathogenic fungi via their effect on some physiological activities of these plant pathogens.

MATERIALS AND METHODS

PATHOGENS

Rhizoctonia solani, the cause of damping-off of cotton.

Fusarium oxysporum, the cause of wilt disease of kidney bean.

ANTAGONISTIC MICROORGANISMS

Eupenicillium senticosum, Hansenula arabitolgenes, Streptomyces aurantiacus and Curtobacterium pusillum

which antagonize R. solani and Sacharomyces unispora and Candida steatolytica which antagonize F. oxysporum.

All these antagonistic microorganisms were isolated, identified and screened for their antagonistic activities in previous studies: the first two [4], the second two [8] and the last two [3].

EXTRACTION AND DETECTION OF ANTIFUNGAL COMPOUNDS

The tested antagonistic microorganisms were grown on broth media under the optimum culture conditions for antifungal production. The extraction was carried out using a mixture of chloroform and ethyl acetate 1:1 (v/v). The Rf values of the active components in the culture filtrate of each antagonist were identified by descending paper strip chromatography using the solvent system mixture of 1-butanol, pyridine and water (6:4:3 v/v/v)[19]

IDENTIFICATION OF ANTIFUNGAL COMPOUNDS

The antifungal compounds were identified in previous studies [3,4,8] using mass spectroscopy as in Table (1).

Figure 1

Table 1: Identification of the active components produced by the antagonists.

Tested antagonists	Active components
t) *E. senticosum*	1) 2-Nonadecanone,2,4-D.N.P.H. 2) 1,2-benzenedicarboxylic acid, bis (2-ethyhexyl) ester. 3) 2,6-Di-tert-butyl-4-bromomethyl phenol. 4) 1-Octanol,2-butyl.
d) *H. arabitolgenes*	1) Toosendanin. 2) 2-Nonadecanone,2,4-D.N.P.H. 3) 2,6-Di-tert-butyl-4-bromomethyl phenol. 4) 2,6-ditert-4-hydroxymethyl phenol. 5) 2-isopropyl-5-(methy-D_3)-1-cyclohexanon-4,4-D_2.
f) *S. aurantiacus*	1) 2,3-dihydro-3-methoxywithacnistin acetate. 2) 2-bromo-3,3',5,5'-tetra(1-butyl)-biphenyl. 3) 5-hydroxy-2,2-dimethyl-5,6-bis(2-oxo-1-propyl)-1-cyclohexanone. 4) Butanedioic acid, methyl-,dimethyl ester.
y) *C. pusillum*	1) Toosendanin. 2) 1,1,3,3,5,5,7,7,9,9,11,11-dodecamethyl-hexasiloxane. 3) 1,2-benzenedicarboxylic acid, bis(2-ethylhexyl) ester. 4) 2,6-ditert-4-hydroxymethyl phenol. 5) Ethyl 2- hydroxybutyrate.
y) *S. Unispora*	1) Tetratetracontane. 2) Oleic acid, eicosyl ester. 3) 9-Octadecanoic acid(z) tetradecyl ester. 4) 2-Iodoethyl Myristate.
n) *C. steatolytica*	1) Tetratetracontane. 2) Hentetetracontanoic acid. 3) Hexadecanoic acid,hexadecyl ester. 4) Nonaedioic acid,bis(2-methypropyl)ester.

INHIBITORY EFFECT OF ANTIFUNGAL COMPOUNDS

The chloroform extracts were air dried, redissolved in acetone and were assayed for activity against R. solani and F. oxysporum as follows: the extracts were diluted with 1:1 with sterile water, 80 µl aliquots were placed in 7 mm wells cut into PDA plates seeded with hyphal fragments and spore suspension of R. solani and F. oxysporum, respectively. The plates were incubated at 28ºC for 24 hrs in case of R. solani and for 4 days in case of F. oxysporum. After incubation, the diameters of inhibition zones were measured [9].

EFFECT OF ANTIFUNGAL COMPOUNDS ON GROWTH OF PATHOGENIC FUNGI

The most inhibitory antifungal compounds extracted from the culture filtrate of each antagonist were assayed for their effect on growth as follows: one ml of diluted extract of each antifungal compound was added to potato dextrose broth inoculated with spore suspension of each of the two pathogenic fungi and incubated at 28ºC for 48 h in case of R. solani and for 4 days in case of F. oxysporum. After incubation, the cultures were filtered, oven dried and weighed.

ESTIMATION OF CARBOHYDRATE FRACTIONS

Sugars were extracted by overnight submersion of dry tissue in 10 ml of 80% (v/v) ethanol at 25°C with periodic shaking.

ESTIMATION OF GLUCOSE

Glucose contents were estimated using the O-toluidine procedure of Feteris [5] as modified by Riazi et al. [21] as follows: One ml aliquot of the alcoholic extract was heated with 5 ml O-toluidine reagent (60 ml O-toluidine and 2 gm thiourea made to 1000 ml with glacial acetic acid) and incubated for 15 minutes at 97°C. Absorbance was measured at 630 nm wave length using spectronic 21 D spectrophotometer. Glucose contents were calculated by the use of a calibration curve which was obtained using standard pure glucose solution.

ESTIMATION OF NITROGEN CONTENT

Total nitrogen was estimated according to the method of Markham [16] and protein nitrogen was estimated according to that of Thimann and Loos [24]. Soluble nitrogen was then calculated by subtracting protein nitrogen from total nitrogen.

ESTIMATION OF ENZYME ACTIVITY

The enzymes were extracted according to the method of Byrde et al. [1]. Catalase was assayed following the method of Kar and Mishra [13], fumarase activity was estimated by the method of Laki [15], malic dehyrogenase activity was measured by the method of Gale and Stephenson [7], and finally succinic dehydrogenase activity was assayed following the method of McShan and Lardy [17].

RESULTS

Table (2) shows the inhibitory effect of antifungal compounds against the two tested pathogenic fungi. It is shown from the table that three active components showed the most inhibitory effect against R. solani and one active component showed the most inhibitory effect against F. oxysporum.

Figure 2

Table 2: Inhibitory effect of the purified antifungal compounds.

Active components of the selected antagonists	Diameter of inhibition zone (mm)	
	R. solani	*F. oxysporum*
A) E. senticosum:		
1) 2-Nonadecanone,2,4-D.N.P.H.	13.64	
1,2-benzenedicarboxylic acid,bis(2-ethylmethyl)ester.	15.82	
2,6-Di-tert-butyl-4-bromomethyl phenol.	8.56	
1-Octanol,2-butyl.	10.12	
H.arabitolgenes:	6.14	
Toosendanin.	13.64	
2-Nonadecanone,2,4-D.N.P.H.	8.56	
2,6-Di-tert-butyl-4-bromomethyl phenol.	11.16	
2,6-ditert-4-hydroxymethyl phenol.	9.84	
2-isopropyl-5-(methyl-D3)-1-cyclohexanon-4,4-D2.	18.66	
S. aurantiacus:	9.44	
2,3-dihydro-3-methoxywithacnistin acetate.	11.86	
2-bromo-3,3´,5,5´-tetra(1-butyl)-biphenyl.		
5-hydroxy-2,2-dimethyl-5,6-bis(2-oxo-1-propyl)-1-cyclohexanone.	10.14	
Butanedioic acid, methyl-,dimethyl ester.	6.14	
C. pusillum:	12.62	
Toosendanin.	15.82	
1,1,3,3,5,5,7,7,9,9,11,11-dodecamethyl-hexasiloxane.	11.16	
1,2-benzenedicarboxylic acid,bis(2-ethylhecyl)ester.	17.22	19.36
2,6-ditert-4-hydroxymethyl phenol.		10.44
Ethyl 2-hydroxybutyrate.		6.66
S. unispora:		8.12
Tetrtetracontane.		
Oleic acid, eicosyl ester.		19.36
9-Octadecanoic(z) tetradecyl ester.		11.60
2-Iodoethyl Myristate.		13.44
C. steatolytica:		9.84
Tetratetracontane.		
Hentetetracontanoic acid.		
Hexadecanoic acid,hexadecyl ester.		
Nonaedioic acid,bis(2-methylpropyl)ester.		

The most inhibitory four active components were tested for their effect on growth, expressed as dry weight, of both R. solani and F. oxysporum (Table 3). Data in the table show the same previous result and also show that 2,3-dihydro-3-methoxywithacnistin acetate is the most inhibitory one against R. solani.

Figure 3

Table 3: Effect of the purified antifungal compounds on growth of the two pathogenic fungi.

Active components	Dry weight (gm/50 ml broth)			
	R. solani		*F. oxysporum*	
	Control	Treated	Control	Treated
• 2,3-dihydro-3-methoxywithacnistin acetate.	0.48	0.17		
• Ethyl 2-hydroxybutyrate.	0.48	0.19		
• 1,2-benzenedicarboxylic acid,bis(2-ethyhexyl)ester.	0.48	0.22		
• Tetratetracontane.			0.54	0.13

The two tested pathogenic fungi were treated, each with the active components to determine the effect of these active components on carbohydrate contents of pathogenic fungi (Table 4). It was found that the three active components inhibiting the growth of R. solani and the active component inhibiting the growth of F. oxysporum, all led to a decrease in both reducing and non-reducing sugars compared to the untreated ones (control).

Figure 4

Table 4: Effect of the purified antifungal compounds on carbohydrate concentrations of the two Pathogenic fungi.

Active components	Glucose Concentration µg/ml			
	R. solani		*F. oxysporum*	
	Control	Treated	Control	Treated
• 2,3-dihydro-3-methoxywithacnistin acetate.	370	145		
• Ethyl 2-hydroxybutyrate.	370	168		
• 1,2-benzenedicarboxylic acid, bis(2-ethylhexyl)ester.	370	198	480	166
• Tetratetracontane.				

The effect of the active components on the nitrogen content (total nitrogen, soluble nitrogen and protein nitrogen) of the two tested pathogenic fungi is shown in Table (5).It is revealed from the table that the active components reduce the nitrogen content of the treated pathogenic fungi compared to control.

Figure 5

Table 5: Effect of the purified antifungal compounds on the nitrogen content of the two Pathogenic fungi (mg/gm fresh weight).

Active components	*R. solani*					
	Control			Treated		
	T N	S N	P N	T N	S N	P N
• 2,3-dihydromethoxywithacnistin acetate.	31.69	17.04	14.65	14.82	6.16	8.66
• Ethyl 2-hydroxybutyrate.				15.75	6.33	9.42
•1,2-benzenedicarboxylic acid,bis(2-ethylhexyl)ester.	31.69	17.04	14.65	17.31	6.49	10.88
• Tetratetracontane.	31.69	17.04	14.65			

Active components	**F. oxysporum**					
	Control			Treated		
	T N	S N	P N	T N	S N	P N
• 2,3-dihydromethoxywithacnistin acetate.						
• Ethyl 2-hydroxybutyrate.						
•1,2-benzenedicarboxylic acid,bis(2-ethylhexyl)ester.					4.99	6.45
• Tetratetracontane.	21.37	10.52	10.85	11.44		

Where
T N: total nitrogen
S N: soluble nitrogen
P N: protein nitrogen

Finally, the effect of the active components on the enzyme activity of the treated pathogenic fungi is shown in Table (6). It is shown from the table that the active components led to an increase in the activity of fumarase, malic dehydrogenase and succinic dehydrogenase, while the activity of catalase was reduced as compared to control.

Figure 6

Table 6: Effect of the purified antifungals on enzyme activity of pathogenic fungi.

Active components	Enzyme activity as µmole of substrate/ gm fresh weight/ hour R. solani							
	Fumarase		Malic dehydrogenase		Succinic dehydrogenase		Catalase	
	C	T	C	T	C	T	C	T
1	2760	3540	1.8	2.4	1.4	2.4		210
2	2760	3230	1.8	2.1	1.4	1.9	420	260
3	2760	3120	1.8	2.2	1.4	1.8		285
4							420	
							420	

Active componets	Enzyme activity as µmole of substrate/ gm fresh weight/ hour F. oxysporum							
	Fumarase		Malic dehydrogenase		Succinic dehydrogenase		Catalase	
	C	T	C	T	C	T	C	T
1								
2								
3								
4	2460	3540	1.2	2.4		2.1		
					1.2		660	270

Where
C: Control (untreated)
T: treated
1: 2,3-dihydro-3-methoxywithacnistin acetate.
2: Ethyl 2-hydroxybutyrate.
3: 1,2-benzenedicarboxylic acid,bis(2-ethylhexyl) ester.
4: Tetratetracontane.

DISCUSSION

Treatment of the tested pathogenic fungi with the antifungal compounds led to a reduction in their growth. This may be due to the effect of these antifungal compounds on spore germination leading to its inhibition [2]. The above results may also be due to the effect of these antifungals on cell wall altering its permeability [22, 24,25], or the antifungals suppressed the early stages of mycelial growth [9].

The treatment of pathogenic fungi with the antifungal compounds resulted in several distinguishable alterations in their physiology as the observed decline in carbohydrate contents. These changes are attributed to the altered metabolism of the treated fungi such as abnormal translocation or inhibition of carbohydrate synthesis [20]. The above result may also be due to the inhibitory effect of antifungals on the activity of enzymes involved in gluconeogensis [22].

The inhibitory effect of antifungals on the nitrogen content of the tested pathogenic fungi may be due to their inhibitory effect on nitrate reductase system [2,13].

The increase in the activity of fumarase, malic dehydrogenase and succinic dehydrogenase is usually correlated with the increase in the rate of carbohydrate and other substrate catabolism and consequently a decrease in the rate of growth [13]. On the other hand, the activity of catalase enzyme was reduced by the effect of antifungals. Catalase is implicated in the beak down of hydrogen peroxide. Therefore, hydrogen peroxide was accumulated and toxicity increased leading to a decrease in growth [6]

CONCLUSION

It was concluded from the present investigation that the antifungal compounds exert their effect in a fungicidal way.

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