Fungitoxicity of Derivatives of *o*-Phenylphenol

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ABSTRACT

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Several isolates of *Diplodia natalensis* and *Penicillium digitatum* were inhibited strongly by *o*-phenylphenol and its esters, 2-biphenylyl acetate and 2-biphenylyl propionate. *o*-Phenylanisole was moderatley inhibitory to *D. natalensis* but was less active against *P. digitatum*. More than 80% of the 2-biphenylyl propionate added to cultures of *D. natalensis* and *P. digitatum* was hydrolyzed to *o*-phenylphenol within 2 hr. Biphenylyl acetate was degraded by *D. natalensis* at a similar rate but was significantly more resistant to attack by *P. digitatum*. Fungitoxicity of the esters of *o*-phenylphenol to both fungi may result from enzymatic hydrolysis of the esters and subsequent accumulation of inhibitory levels of *o*-phenylphenol in the cultures. Neither fungus degraded *o*-phenylphenol during the 24-hr incubation. *D. natalensis* demethylated 10% of the *o*-phenylanisole added to the culture in 24 hr, but *P. digitatum* converted only 1% of the anisole to *o*-phenylphenol during the same period. The toxicity of *o*-phenylanisole to *D. natalensis* was greater then could be accounted for by the accumulation of *o*-phenyphenol in the hyphae. Hyphae of *D. natalensis* bound significantly more *o*-phenylanisole than *P. digitatum*, and this factor may be responsible for the selective fungitoxicity of this compound.

Additional key words: phenol ester, phenol ether.

o-Phenylphenol is used extensively to control postharvest diseases of fresh fruits and vegetables. Despite its broad antimicrobial spectrum, it is too phytotoxic for many potential practical applications (9,11). Selective toxicity to pathogenic microorganisms has been achieved by: (i) maintenance of the treatment solution at pH 12 to convert *o*-phenylphenol to its relatively nontoxic anionic form; (ii) complexing the phenol with hexamethylenetetramine; (iii) incorporation in a "wax" emulsion in which the phenol is partitioned into the nonaqueous phase. Tomkins (23) reported that phytotoxicity could be reduced greatly without loss of antimicrobial effectiveness by esterifying *o*-phenylphenol with lower aliphatic acids such as acetic, propionic, and isobutyric acids. Other investigations have confirmed the effectiveness of esters of *o*-phenylphenol for control of several postharvest diseases—*Botrytis* on grapes (20), *Monilinia* on peaches (19), and *Penicillium* and *Diplodia* on oranges (13).

Derivatives of *o*-phenylphenol, incorporated into potato dextrose agar (PDA), have been tested in our laboratory against the citrus fruit pathogens, *Penicillium digitatum* Sacc. and *Diplodia natalensis* Pole Evans. On a molar basis, 2-biphenylyl acetate (acetyl ester of *o*-phenylphenol) and 2-biphenylyl propionate (propionyl ester of *o*-phenylphenol) were as active as the parent phenol against both species of fungi. *o*-Phenylanisole (methyl ether of *o*-phenylphenol) was somewhat less active than the free phenol against *D. natalensis* but was essentially inactive against *P. digitatum* growing on PDA. The selectivity of *o*-phenylanisole against *D. natalensis* was confirmed with four additional isolates of both fungi. This investigation was undertaken to elucidate the mechanism of fungitoxic action of these derivatives of *o*-phenylphenol. Portions of this work have been reported (10).

MATERIALS AND METHODS

Inhibitors. *o*-Phenylphenol, 2-biphenylyl acetate, and 2-biphenylyl propionate were supplied by the Dow Chemical Co., Midland, MI 48640. The first two compounds were recrystallized from isooctane and melted at 57 C and 63.5–64.5 C, respectively. 2-Biphenylyl propionate was a clear liquid that distilled at 197–198 C at 0.25 mm Hg. The free phenol in the esters was measured by extracting isooctane solutions of the esters with 1 N NaOH and measuring the absorbance of the alkaline solution of sodium *o*-phenylphenate at 307 nm. The samples of 2-biphenylyl acetate and 2-biphenylyl propionate contained, respectively, a maximum of 4.8% and 1.4% *o*-phenylphenol. Attempts to reduce the amount of *o*-phenylphenol in the samples by distillation or extraction were unsuccessful and were abandoned after a preliminary experiment revealed that this level of contamination was insignificant compared with the phenol that would accumulate in the cultures through the hydrolysis of the esters by the fungi.

o-Phenylanisole was obtained from Eastman Organic Chemicals, Rochester, NY 14650. The oil was dissolved in 100 ml of diethyl ether and washed three times with 0.1 N NaOH and with water. The ether solution was dried with anhydrous $MgSO_4$ and filtered and the ether evaporated. An equal volume of anhydrous ethanol was added to the *o*-phenylanisole oil, and the solution was refrigerated. The crystals that formed had a melting point of 30 C. The crystalline sample contained unacceptable traces of free *o*-phenylphenol and was, therefore, refluxed with 1 N NaOH for 8 hr, washed repeatedly with water, and finally dried with $MgSO_4$. The final sample used in these investigations contained less than 0.001% *o*-phenylphenol, an amount below the detectable limits in our experiments.

o-Phenylphenol and its derivatives were dissolved in 95% ethanol containing 1 g of Tergitol XD/ L. Tergitol XD is a nonionic surfactant (polyalkalene glycol ether) supplied by Union Carbide Corp., New York 10017. Solutions of this nonaromatic surfactant are optically transparent in the region of the UV spectrum used for the analysis of the phenol derrivatives. All of the phenolic inhibitors were added to the fungus cultures in 0.5 ml of Tergitol solution. The final concentration of Tergitol and ethanol in the cultures did not significantly influence the growth of either fungus.

Culture conditions. Spores of *P. digitatum* (isolate M6R) were washed from a slant culture into a 2-L Erlenmeyer flask containing 500 ml of orange juice medium (2). The culture was incubated on an orbital shaker (200 oscillations per minute) at 26 C for 3 days. The hyphae were filtered onto a glass fiber filter (Whatman GF/A), rinsed with water, and weighed. The mycelial mat was dispersed in a volume of 0.05 M KH_2PO_4, calculated to provide a hyphal suspension of 10 mg of dry wt per milliliter, by means of a Waring Blendor operating at low speed for 60 sec.

Hyphae of *D. natalensis* (isolate S2C) were produced similarly except for minor variations necessitated by the slower growth and

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slimy character of the hyphae of this fungus. Hyphal fragments from a slant culture of *D. natalensis* were introduced into a 250-ml screw-cap Erlenmeyer flask containing 50 ml of potato dextrose broth (250 g of potatoes, 20 g of glucose, in 1 L of water). The stationary culture was incubated at 25 C for 3 days, shaken vigorously to disperse the hyphae, and then poured into a 2-L flask containing 500 ml of the orange juice medium. After 3 days' shaking incubation, the culture of *D. natalensis* was transferred to 200-ml bottles and centrifuged at 2,000 rpm for 10 min. The supernatant was decanted and the hyphae were washed with an equal volume of 0.05 M KH_2PO_4. The mycelial pellet was transferred to a Büchner funnel fitted with a circular piece of brass strainer cloth (150 μm) and a cover with a neoprene-sheet dam. The excess liquid was forced from the hyphae and the mycelial mat was dispersed in phosphate buffer in the manner described for *P. digitatum*.

Experiments on fungal growth inhibition and metabolism were conducted by transferring 20 ml of the hyphal suspension (200 mg, dry weight) with an agar pipette to a 500-ml Erlenmeyer flask containing 180 ml 1.1X synthetic medium (2). After a 1-hr recovery, an appropriate weight of phenolic compund in 0.5 ml of Tergitol-ethanol was added to the culture, which was then incubated at 26 C on an orbital shaker (200 oscillations per minute) for 24 hr. Growth of the hyphae was determined periodically by filtering portions of the culture through Whatman 541 paper or by filtering the entire culture as described above. Samples of the cultures were iced and stored at 1 C for analysis a few hours later.

Determination of *o*-phenylphenol and derivatives in cultures. Samples of the culture or mycelium were acidified, and the phenolic compounds were steam-distilled into a Clevenger trap. Approximately 700 ml of distillate was collected from each sample. The distillate from samples that contained *o*-phenylphenol and *o*-phenylanisole was neutralized and then made alkaline by addition of another 5 ml N NaOH. The alkaline distillate then was extracted with 50 ml of isooctane. The aqueous phase was transferred to another flask and acidified. *o*-Phenylphenol in the acidified sample was determined colorimetrically with the 4-amino-antipyrene reagent (18) or extracted into isooctane for measurement of the absorbance at 282 nm. The procedure for handling distillates containing the esters of *o*-phenylphenol was modified slightly to avoid hydrolyzing the esters under alkaline conditions. Distillates containing the esters first were extracted with isooctane to quantitatively transfer the esters and a part of the *o*-phenylphenol to the isooctane phase. Then, 5 ml N NaOH was added to the aqueous phase and the two phases shaken again to quantitatively move *o*-phenylphenate back into the aqueous phase. *o*-Phenylphenol was determined as described above, the isooctane phase was washed with water, and the esters were determined by absorbance of the isooctane phase at 238 nm. Recovery of all phenolic compounds from samples fortified before steam distillation exceeded 90%.

In experiments on the uptake of *o*-phenylanisole by hyphae of *P. digitatum* and *D. natalensis,* cultures were centrifuged after the appropriate exposure periods, and the mycelial pellet was transferred to a Büchner funnel for removal of most of the extracellular fluid by vacuum filtration. The mycelial mat was peeled from the filter paper and discarded, because preliminary experiments showed that the hyphae contained natural substances that absorbed at 282 nm, the absorption maximum of *o*-phenylanisole. The centrifuge bottle was rinsed with 95% ethanol, which then was passed through the filter paper in the Büchner funnel and collected in the filter flask. The alcohol rinses were combined with the culture supernatant and the mycelial filtrate in a separatory funnel. The pH was adjusted to 12 by addition of 5 N NaOH, and the *o*-phenylanisole was extracted from the aqueous phase with isooctane. The concentration of *o*-phenylanisole in the isooctane phase was measured by absorbance at 282 nm.

RESULTS

Fungitoxicity of *o*-phenylphenol and derivatives. Table 1 shows similar ED_{50} values for *o*-phenylphenol, 2-biphenylyl acetate, and 2-biphenylyl propionate against 24-hr growth of both *D. natalensis* and *P. digitatum* in liquid culture, although the ED_{50} for *o*-phenylanisole against *P. digitatum* is about twice that for *D. natalensis.*

About 80–85% of the 2-biphenylyl propionate added to the cultures of both fungi was hydrolyzed to *o*-phenylphenol within 2-hr incubation at 26 C (Fig. 1). 2-Biphenylyl acetate was hydrolyzed at almost the same rate by *D. natalensis* but at a significantly lower rate by *P. digitatum*. Control cultures with heat-killed hyphae of *D. natalensis* or *P. digitatum* did not contain significant amounts of *o*-phenylphenol after 24-hr incubation with either of the esters. These results indicate that the fungitoxicity of 2-biphenylyl acetate and 2-biphenylyl propionate to *D. natalensis* and *P. digitatum* can be explained solely by the accumulation of inhibitory concentrations of *o*-phenylphenol in the cultures as a result of the enzymatic action of the fungi on the phenol esters.

Identification of *o*-phenylphenol as a metabolite of *o*-phenylanisole in cultures of *D. natalensis*. Preliminary experiments revealed that cultures of *D. natalensis* incubated with *o*-phenylanisole contained a steam-distillable substance that formed a red color upon reaction with 4-aminoantipyrene, a characteristic of certain phenols including *o*-phenylphenol. Fifteen 200-ml cultures of *D. natalensis* containing 50 μg/ml of *o*-phenylanisole were incubated for 24 hr, and the volatile compounds distilled with steam. The distillates were partitioned between isooctane and 1 N NaOH, and the latter phase was analyzed by the 4-amino-antipyrene procedure. The combined distillates contained 13 mg of *o*-phenylphenol, assuming that this phenol was the sole aminoantipyrene-positive substance in the distillates. The distillates were acidified with H_2SO_4 and extracted with diethyl ether. The ether extract was dried with anhydrous $MgSO_4$ and evaporated to dryness. The resulting oil was crystallized in the cold, yielding 4 mg. The crystals were identical to authentic *o*-

TABLE 1. Inhibition of mycelial growth of *Penicillium digitatum* and *Diplodia natalensis* in liquid culture by *o*-phenylphenol and related compounds[a]

Inhibitor	ED_{50} (M × 10^5)	
	P. digitatum	*D. natalensis*
o-Phenylphenol	13	11
2-Biphenylyl acetate	14	14
2-Biphenylyl propionate	13	13
o-Phenylanisole	54	24

[a]Liquid cultures prepared with about 1 mg dry weight hyphae per milliliter of synthetic medium and incubated at 26 C for 24 hr.

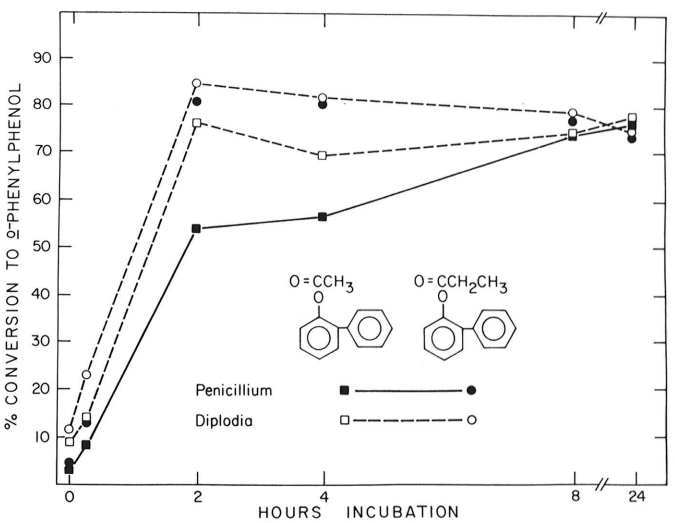

Fig. 1. Conversion of 2-biphenylyl acetate and 2-biphenylyl proprionate to *o*-phenylphenol by *Penicillium digitatum* and *Diplodia natalensis* in liquid culture incubated at 26 C.

phenylphenol by the criteria of the infrared spectrum, TLC mobility in benzene on a silica gel plate, and mixed melting point with authentic *o*-phenylphenol.

Selective toxicity of *o*-phenylanisole to *D. natalensis*. Several possible explanations for the selective toxicity of *o*-phenylanisole to *D. natalensis* were considered: (i) *D. natalensis* might covert *o*-phenylanisole to *o*-phenylphenol at a rate sufficient to cause the accumulation of an inhibitory concentration of the phenol in the hyphae, (ii) *P. digitatum* might detoxify *o*-phenylphenol as it was formed in the culture, (iii) *D. natalensis* might accumulate *o*-phenylanisole to a greater extent than *P. digitatum,* and (iv) *P. digitatum* might not possess an *o*-phenylanisole-sensitive site or reaction sequence.

Table 2 shows that *D. natalensis* demethylated approximately 10% of the *o*-phenylanisole in the culture to *o*-phenylphenol during the 24-hr incubation. Cultures containing hyphae of *P. digitatum* or heat-killed hypae of *D. natalensis* contained less than 0.5 μg *o*-phenylphenol per milliliter after 24-hr incubation. Neither fungus metabolized a significant amount of *o*-phenylphenol in 24 hr. These results suggest that *o*-phenylphenol could contribute to the inhibition of growth of hyphae of *D. natalensis* incubated with *o*-phenylanisole, but that detoxification of *o*-phenylphenol was not a factor in the tolerance of *P. digitatum* to *o*-phenylanisole.

An experiment was conducted to evaluate quantitatively the significance of *o*-phenylphenol, derived by demethylation of *o*-phenylanisole, on the inhibition of growth of *D. natalensis* in cultures with the anisole added. *D. natalensis* hyphae (10 mg/ml) were incubated with concentrations of *o*-phenylphenol and *o*-phenylanisole, which substantially inhibited growth. The total amount of *o*-phenylphenol in the culture and the amount in the culture filtrate were determined after 8 and 24 hr incubation. Hyphae of *D. natalensis* incubated with 20 μg *o*-phenylphenol per milliliter accumulated 1.4 μg *o*-phenylphenol per milligram of dry weight hyphae and were inhibited 46% after 8-hr incubation (Table 3). An identical culture incubated with 50 μg *o*-phenylanisole per milliliter was inhibited 61% but had no detectable *o*-phenylphenol associated with the hyphae. Even after 24-hr incubation, the culture incubated with *o*-phenylanisole was inhibited to essentially the same degree as the one with *o*-phenylphenol, yet the amount of *o*-phenylphenol associated with the hyphae in the former culture was only one-half that in the latter culture. Evidently, the selective toxicity of *o*-phenylanisole to *D. natalensis* cannot be explained solely by the accumulation of metabolically derived *o*-phenylphenol in the hyphae, as in the case of esters of *o*-phenylphenol.

The uptake of *o*-phenylanisole by *P. digitatum* and *D. natalensis* over 24 hr is recorded in Table 4. The amount of the anisole associated with the hyphae of both fungi after 1 and 24 hr incubation was proportional to the amount of *o*-phenylanisole added to the culture. The higher concentration of anisole in the hyphae at the 1-hr sampling probably reflects the greater anisole/hyphae ratio early in the incubation period. Growth inhibition of both fungi was directly related to the amount of anisole associated with the hyphae. The amount of *o*-phenylanisole associated with the hyphae of *D. natalensis* was higher than that for *P. digitatum* at all dosages and both sampling times. This experiment was repeated with similar results.

TABLE 2. Conversion of *o*-phenylanisole to *o*-phenylphenol in cultures of *Diplodia natalensis* and *Penicillium digitatum* and the resistance of *o*-phenylphenol to metabolism by these fungi[a]

Anisole or phenol added to culture	Fungus	*o*-Phenylphenol (μg/ml) found in culture after incubation (hr)		
		0	8	24
None	*P. digitatum*	0.09	···	0.21
	D. natalensis	0.39	···	0.57
o-Phenylanisole 50 μg/ml	*P. digitatum*	0.05	0.26	0.04
	D. natalensis	0.36	1.20	4.70
	D. natalensis (heat-killed)	···	···	0.16
o-Phenylphenol 10 μg/ml	*P. digitatum*	10.00	9.41	9.80
	D. natalensis	10.00	9.40	9.90

[a] Liquid cultures prepared with about 1 mg dry weight hyphae per milliliter of synthetic medium and incubated at 26 C for 24 hr.

TABLE 3. Inhibition of mycelial growth in relation to the conversion of *o*-phenylanisole (OPA) to *o*-phenylphenol (OPP) by *Diplodia natalensis*[a]

Incubation (hr)	Inhibitor added to culture	μg OPP in:		Growth inhibition (%)
		Culture[b]	Hyphae[c]	
0	20 μg OPP/ml	4,000	1.3	···
	50 μg OPA/ml	27	0	···
8	20 μg OPP/ml	4,000	1.4	46
	50 μg OPA/ml	219	0	61
24	20 μg OPP/ml	4,000	1.5	46
	50 μg OPA/ml	1,127	0.84	60

[a] Cultures prepared with about 10 mg dry weight hyphae per milliliter of synthetic medium and incubated at 26 C.
[b] By analysis.
[c] OPP in whole culture minus OPP in culture filtrate.

DISCUSSION

Lower alkyl esters of substituted phenols show essentially the same antifungal activity as the parent phenol (3,6–8,17). This relationship usually is attributed to the facile hydrolysis of the esters to the parent phenols by esterases of the test fungi or of the host plant (5–8). In our investigation, 2-biphenylyl acetate and 2-biphenylyl propionate were hydrolyzed rapidly to *o*-phenylphenol by both *D. natalensis* and *P. digitatum.* Thus, the inhibitory activity of the esters of *o*-phenylphenol appears to derive entirely from the fungitoxicity of the free phenol that is released into the culture.

o-Phenylanisole, which is selectively toxic to *D. natalensis,* was cleaved to *o*-phenylphenol to a greater extent by this fungus than by *P. digitatum,* which is relatively insensitive to the anisole. However, the amount of *o*-phenylphenol that accumulated in hyphae of cultures of *D. natalensis* containing *o*-phenylanisole was not sufficient to explain the fungitoxicity of *o*-phenylanisole in a manner analogous to the esters of *o*-phenylphenol. The fungitoxicity of phenyl ethers is usually much less than that of the parent phenols and their alkyl esters (6,7,12,17,21,22). This suggests that the phenol function is essential to antifungal activity

TABLE 4. Uptake of *o*-phenylanisole (OPA) by *Penicillium digitatum* and *Diplodia natalensis*[a]

OPA in culture, (μg/ml)	Fungus	OPA uptake, μg/mg dry hyphae[b]		Growth inhibition (%)
		1 hr	24 hr	
20	*P. digitatum*	9.5	4.6	23
	D. natalensis	13.6	5.7	38
40	*P. digitatum*	24.9	10.8	38
	D. natalensis	36.6	15.2	67
80	*P. digitatum*	56.6	28.0	48
	D. natalensis	77.0	44.3	88

[a] Cultures prepared with about 10 mg dry weight hyphae per milliliter of synthetic medium and incubated at 26 C.
[b] Total OPA in culture medium before addition of hyphae minus amount found in culture filtrate after incubation with hyphae for indicated time period.

and that most fungi cannot demethylate the phenol ethers at a rate sufficient to generate a fungitoxic concentration of the free phenol in the culture. Demethylation of methyl ethers of phenols (anisoles) has been observed in cultures of *Aspergillus* (4), *Alternaria* (16), *Hormodendrum* (14), *Penicillium* (14), and *Rhizoctonia* (15). Hock and Sisler (15) described the demethylation of 1,4-dichloro-2,5-dimethoxybenzene (chloroneb) to 2,5-dichloro-4-methylphenol by *Rhizoctonia,* but they attributed inhibition of growth of this fungus to chloroneb rather than to the phenol metabolite. Similarly, *o*-phenylanisole appears to be the main fungitoxicant involved in the inhibition of *D. natalensis* in cultures incubated with this compound. Methyl ethers of orthosubstituted phenols such as *o*-phenylphenol appear to be more resistant to enzymatic demethylation than meta and parasubstituted phenols (1).

Hyphae of *D. natalensis* accumulated significantly more *o*-phenylanisole than hyphae of *P. digitatum* in the present investigation. This fact, coupled with slow rate of ether cleavage, suggests that the greater toxicity of *o*-phenylanisole to *D. natalensis* than to *P. digitatum* is because of its greater accumulation by hyphae of *D. natalensis.* The possibility of selective accumulation at a specific intercellular site or the existence of an intrinsically sensitive metabolic process in *D. natalensis* cannot be ruled out, however.

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