Bull Environ Contam Toxicol (2007) 79:499–503
DOI 10.1007/s00128-007-9223-6

Comparative Toxicity of the Phytotoxins (8*R*,16*R*)-(–)-Pyrenophorin and (5*S*,8*R*,13*S*,16*R*)-(–)-Pyrenophorol on Aquatic Organisms

M. Chrysayi-Tokousbalides · K. Machera · K. Kyriakopoulou · K. A. Aliferis · K. K. Schrader · I. Tsoutsanis · P. Anastasiadou

Received: 18 June 2006 / Accepted: 22 May 2007 / Published online: 25 September 2007

Abstract The acute toxicities of the fungal phytotoxins (8*R*,16*R*)-(–)-pyrenophorin and (5*S*,8*R*,13*S*,16*R*)-(–)-pyrenophorol on *Vibrio fischeri*, *Oscillatoria perornata*, *Pseudokirchneriella subcapitata*, *Lemna minor* and *Artemia fransiscana* were evaluated. (8*R*,16*R*)-(–)-pyrenophorin was more toxic than (5*S*,8*R*,13*S*,16*R*)-(–)-pyrenophorol to *V. fischeri*, *O. perornata*, *L. minor* and *A. fransiscana*. The highest acute toxicity of (8*R*,16*R*)-(–)-pyrenophorin was exhibited on *V. fischeri* (5 min median effective concentration of 3.57 M 10^{-5}) whereas the corresponding value for (5*S*,8*R*,13*S*,16*R*)-(–)-pyrenophorol was 801 M 10^{-5}. *P. subcapitata* exhibited a lack of sensitivity (median inhibitory concentration of >10 M 10^{-5}) to both phytotoxins.

Keywords Phytotoxin selectivity · Aquatic organisms

Although there is extended scientific information on chemistry and phytotoxicity of fungal phytotoxins on plants (Strobel et al. 1991; Capasso et al. 1996; Duke et al. 2002; Andolfi et al. 2005; Evidente et al. 2005), little is known about the effects of fungal phytotoxins on other organisms (Ishibashi 1962; Bunkers et al. 1990; Capasso et al. 1996; Andolfi et al. 2005; Evidente et al. 2005). Since fungal phytotoxins can be exploited for the development of new herbicides, more information about the effects on non-target organisms needs to be known. Although broad risk assessment is not necessary before the screening of such compounds in technical scale, the effects on organisms belonging to different taxa would expand our knowledge on structure–activity relationships and contribute to the elucidation of subcellular targets of natural phytotoxic compounds.

Pyrenophorin and pyrenophorol are fungal phytotoxins which belong to macrodiolides, the former being the oxidation product of the latter. The isomers (8*R*,16*R*)-(-)-pyrenophorin and (5*S*,8*R*,13*S*,16*R*)-(–)-pyrenophorol were isolated from cultures of a phytopathogenic strain of *Drechslera avenae* and found to differ in their selective phytotoxicity at the plant level (Kastanias and Chrysayi-Tokousbalides 2000, 2005). The aim of this study was to examine the effects of the above phytotoxins on organisms representing different trophic levels of aquatic ecosystems and four major taxonomic groups (bacteria, algae, higher plants and crustaceans). Therefore, the marine bacterium *Vibrio fischeri*, the cyanobacterium *Oscillatoria perornata* (Skuja), the green alga *Pseudokirchneriella subcapitata* (formerly *Selenastrum capricornutum*), the aquatic plant *Lemna minor* and the crustacean *Artemia fransiscana* (formerly *Artemia salina*) were selected as test organisms. The data presented in this paper provide an ecotoxicological evaluation for the impact of these fungal phytotoxins on non-target organisms.

M. Chrysayi-Tokousbalides (✉) · K. A. Aliferis · I. Tsoutsanis
Pesticide Science Laboratory, Agricultural University of Athens, 75 Iera Odos, 118 55 Athens, Greece
e-mail: mchrys@aua.gr

K. Machera · K. Kyriakopoulou · P. Anastasiadou
Laboratory of Pesticides Toxicology, Benaki Phytopathological Institute, 8 Stefanou Delta Street, Kifissia, 145 61 Athens, Greece

K. K. Schrader
United States Department of Agriculture, Agricultural Research Service, Natural Products Utilization Research Unit, National Center for Natural Products Research, P.O. Box 8048, University, MS 38677, USA

Materials and Methods

The lyophilized strain NRRL-B-11177 of the bioluminescent bacterium *Vibrio fischeri* (*Photobacterium phosphoreum*) was obtained from Azur Environmental, Carlsbad, CA, USA, and stored at –20°C until used. The 2-methyisoborneol-producing cyanobacterium (blue-green alga) *Oscillatoria perornata* was isolated from water samples obtained from channel catfish (*Ictalurus punctatus*) ponds in west MS, USA. A culture of the green alga *Pseudokirchneriella subcapitata* was obtained from Dr. J. C. Greene, United States Environmental Protection Agency, Corvallis, OR, USA. Cultures of these phytoplankton species were maintained in steady-state growth using continuous culture systems and media as outlined in van der Ploeg et al. (1995) and modified by Schrader et al. (1997). *Lemna minor* culture was kindly provided by LemnaTec GmbH, Germany, and was maintained on modified Steinberg medium (International Organization for Standardization 2003). *Artemia fransiscana* cysts were obtained from Salt Lake Brine Shrimp Inc., UT, USA.

(5*S*,8*R*,13*S*,16*R*)-(–)-pyrenophorol (MW 312) and (8*R*,16*R*)-(–)-pyrenophorin (MW 308) were isolated from cultures of a pathotype of *Drechslera avenae* (Kastanias and Chrysayi-Tokousbalides 2000, 2005). Paraquat (1,1′-dimethyl-4,4′-bipyridinium) (33.5%), mesotrione [2-(4-mesyl-2-nitrobenzoyl)cyclohexane-1,3-dione] (99.7%) and norflurazon [4-chloro-5-methylamino-2-(α,α,α-trifluoro-*m*-tolyl)pyridazin-3(2*H*)-one] (98.0%) were kindly provided by Syngenta Hellas. Diuron [3-(3,4-dichlorophenyl)-1,1-dimethylurea] (99.6%) and oxadiazon [5-*tetr*-butyl-3-(2,4-dichloro-5-isopropoxyphenyl)-1,3,4-oxadiazol-2(*3H*)-one] (99.8%) were kindly provided by Bayer CropScience Germany. Glyphosate [*N*-(phosphonomethyl)glycine] as isopropylamine salt (99.5%) was courtesy of Agan Chemical Manufacturers Ltd, Israel. Reagents and other compounds used were purchased from Merck (2,4-dinitrophenol), Sigma (cycloheximide, kinetin, chloramphenicol), Serva (L-streptomycin) and Instant Ocean® Sea Salt was obtained from Aquarium Systems Inc., OH, USA. Acetone and ethanol were of analytical grade, purchased from Lab Scan Analytical Reagents (Dublin, Ireland).

The Microtox® Analyzer Model 500 (Strategic Diagnostics Inc., DE, USA) was used to conduct the acute toxicity assay for the phytotoxic compounds under study. The Microtox® assay is based on the inhibition of the natural luminescence emitted by the bacterium *V. fischeri* when exposed to test preparations. In the present study, the "90% basic test for pure compound" protocol was followed. The lyophilized culture of *V. fischeri* was rehydrated with a reconstitution solution and incubated at 5.5 ± 1°C for 20 min. Following stabilization, the bacterial suspension was exposed to test compounds and the acute toxicity was expressed as inhibition of bioluminescence with endpoints of 5-min and 15-min median effective concentrations (EC_{50}). Working solutions of pyrenophorin, pyrenophorol, paraquat and glyphosate were made in diluent. Oxadiazon, diuron and mesotrione were first dissolved in a small volume of acetone and then the diluent was added for obtaining the working solutions. Norflurazon was dissolved in ethanol and then the ethanolic solution was taken up in diluent to make final working solutions. The solvent did not exceed 1% in the treated and control samples. The pH of test preparations was adjusted within the range of 6–8. Each treatment was replicated at least three times.

The rapid bioassay of Schrader et al. (1997) was used to evaluate the toxicity of pyrenophorol and pyrenophorin towards *O. perornata* and *P. subcapitata*. Stock solutions of the phytotoxins were made at 2,000, 200, 20, and 2 μM in 99.9% (v/v) methanol. Absorbance measurements (650 nm) of microplate wells were obtained at 24-h intervals for 4 days using a Packard model SpectraCount microplate photometer (Packard Instrument Company, Meriden, CT, USA). Mean values of the absorbance measurements for each concentration (n = 3) and controls (n = 3) were graphed, and graphs were used to determine the 50% inhibition concentration (IC_{50}), lowest-observed-effect concentration (LOEC), and lowest-complete-inhibition concentration (LCIC) for each test compound.

L. minor cultures were grown on modified Steinberg medium in plastic beakers of 25 mL capacity and 4 cm in height. Ten colonies with 3–4 fronds each were placed on the medium (10 mL) in each vial. A plastic lid with five punctures (1–2 mm) was placed on each vial. Paraquat and 3,5-dichlorophenol were used as reference chemicals. Working solutions of test compounds were made in Steinberg medium, with the exception of 3,5-dichlorophenol the solutions of which were made in ethanol. Final concentration of ethanol did not exceed 1%. The cultures were kept in a growth chamber at 24 ± 1°C, relative humidity of 85% ± 5% and 16 h photoperiod. The light source used was cool white fluorescent lamps (Sylvania) providing illumination of approximately 90 μmol m^{-2} s^{-1}. Colonies were examined for visual signs of phytotoxicity 5 days after treatment. Each experiment was replicated three times. The EC_{50} values were calculated using linear regression analysis.

A. fransiscana cysts were placed in artificial seawater (Instant Ocean) with a salinity of 35‰. After a 24-h incubation at 25°C under aeration in the light, first instar nauplii were transferred to fresh seawater. The nauplii were further incubated for 24 h and then transferred to fresh water in 12-well plates for the bioassays (5 nauplii/0.3 mL/well). Sensitivity of *A. fransiscana* to the test compounds was examined according to the Organisation for Economic Co-operation and Development (OECD) guideline (1997)

and previous studies (Machera et al. 1996) by counting dead nauplii following a 48-h incubation at 25°C in the dark. The observed mortality in the untreated was less than 10%. Each treatment was replicated four times. The median lethal concentrations (LC_{50}) were calculated using linear regression analysis.

All data were analyzed using ANOVA JMP IN 4.0 except those of Table 2 for which ANOVA-Microsoft Excel was used.

Results and Discussion

The results from the Microtox® assay are presented as acute 5 and 10 min EC_{50} values in Table 1. Differences were observed between treatments in regards to EC_{50} values but also to exposure time required for complete inhibition of *V. fischeri*. As the data in Table 1 show, pyrenophorin had the lowest EC_{50} values (3.57 and 0.97 M 10^{-5}) exhibiting higher activity than those of the herbicides included in the tests. Pyrenophorin strongly inhibited the bacterial bioluminescence, and this effect was almost complete within 5 min of exposure with a toxicity threshold of 36.35 M 10^{-5}. On the contrary, pyrenophorol was much less toxic than pyrenophorin with EC_{50} values of 801 and 400.64 M 10^{-5} at 5 and 15 min, respectively. The low level of toxicity of pyrenophorol towards *V. fischeri* combined with the observation that such effect had not been completed within 5 min (toxicity threshold >576.72 M 10^{-5}) indicates that differences in cell delivery and/or toxicity mechanism may exist between the above phytotoxins. The inhibition of bioluminescence of *V. fischeri* by the tested herbicides did not reach a maximum in 5 min, with the exception of mesotrione in which the effect appeared to be completed within 5 min.

Table 1 Median effective concentrations (EC_{50}) in M 10^{-5} obtained by *Vibrio fischeri* bioassay for pyrenophorin, pyrenophorol and six herbicides for two exposure periods

Compound	EC_{50} (M 10^{-5})	
	5 min	15 min
Pyrenophorin	3.57[a]	0.97[a]
Pyrenophorol	801[b]	400.64[b]
Mesotrione	187[c]	117.89[c]
Diuron	>386	>386.1
Norflurazon	>296	>296.34
Oxadiazon	>261	141.95[c]
Paraquat	626[d]	132.19[c]
Glyphosate	>1,064	>1,064.46

Means within the same column followed by the same letter are not significantly different ($p \leq 0.05$)

As the data in Table 2 show, pyrenophorin was found to be more toxic towards *O. perornata* than pyrenophorol based upon IC_{50}, LOEC and LCIC results. Pyrenophorin at a concentration of 10 M 10^{-5} resulted in the disappearance of the filaments of *O. perornata*, but at 1 M 10^{-5} the filaments tended to clump together. Such symptoms appear to be the expression of a "stress" response of *O. perornata* and are usually associated with the loss of integrity of cellular membranes. *O. perornata* was more sensitive than *P. subcapitata* to pyrenophorin. Interestingly, *O. perornata* was found to be less sensitive than *P. subcapitata* to a large number of natural compounds and herbicides, especially to protoporphyrinogen oxidase (Protox) inhibitors (Schrader and Harries 2001). However, it has also been found that a water-soluble anthraquinone derivative, paraquat and other reactive oxygen species (ROS) generating compounds are more toxic to *O. perornata* than to *P. subcapitata* due to the inability of the former organism to scavenge ROS (Schrader et al. 1998a, b, 2005). Such a deficiency in *O. perornata* that is not present in *P. subcapitata* might be responsible for the selectivity of pyrenophorin and the phytotoxicity that has been associated with ROS production (Aliferis and Chrysayi-Tokousbalides 2006). On the contrary, the photosystem II (PSII) inhibitor diuron was found to have low selectivity with the same LCIC values (1 M 10^{-6}) towards *O.* cf. *chalybea* (previous taxonomic designation of *O. perornata*) and *P. subcapitata* (Schrader et al. 1998a).

Treatment of *L. minor* cultures with pyrenophorin resulted in intensively discoloured fronds and a reduction

Table 2 Mean 50% inhibition concentration (IC_{50}), lowest-observed-effect concentration (LOEC), and lowest-complete-inhibition concentration (LCIC) values obtained from repeated bioassays using *Oscillatoria perornata* and *Pseudokirchneriella subcapitata*

	O. perornata			*P. subcapitata*		
Compound	IC_{50} (M 10^{-5})	LOEC (M 10^{-5})	LCIC (M 10^{-5})	IC_{50} (M 10^{-5})	LOEC (M 10^{-5})	LCIC (M 10^{-5})
Pyrenophorin	1.3[a,*]	1[b,*]	10[a,*]	>10[b,*]	>10[b,*]	>10[a,*]
Pyrenophorol	>10[a,**]	10[a,**]	>10[a,*]	>10[a,*]	>10[a,*]	>10[a,*]

Means comparing measured variables between test organisms that are followed by the same letter are not significantly different ($p \leq 0.05$). Means in columns with different number of asterisks are significantly different ($p \leq 0.05$) when comparing pyrenophorin with pyrenophorol

Table 3 Comparative toxicity of pyrenophorin, pyrenophorol, 3,5-dichlorophenol (3,5-DP) and paraquat on *Lemna minor*

Compound	EC_{50} (M 10^{-5})		NOAEL (M 10^{-5})	
	Rhizoids	Fronds	Rhizoids	Fronds
Pyrenophorin	4.53[a]	6.10[a,c]	0.7[a]	0.7[a]
Pyrenophorol	3,059[b]	287[b]	12.8[b]	12.8[b]
3,5-DP	7.44[a]	7.43[a]	0.6[c]	0.6[c]
Paraquat	0.00053[a]	0.107[c]	0.000054[d]	0.000054[d]

The estimation of median effective concentrations (EC_{50}) in M 10^{-5} and no observed adverse effect level (NOAEL) was based on observations on rhizoid number and frond discoloration 5 days after exposure. Means within the same column followed by the same letter are not significantly different ($p \leq 0.05$)

in rhizoid number. The EC_{50} estimated for frond development did not differ from that required for rhizoid inhibition being at the same order of magnitude with 3,5-dichlorophenol (Table 3). On the contrary, the phytotoxin pyrenophorol was not only less toxic than pyrenophorin to *L. minor* but also less active on roots than on fronds with EC_{50} values of 3,059 and 287 M 10^{-5}, respectively. Species of *Lemna* have been characterized by sensitivity to the herbicide paraquat (Fairchild et al. 1997; Michel et al. 2004). Although in our tests *L. minor* showed high sensitivity to paraquat, neither phytotoxin was found as toxic to *L. minor* as paraquat (Table 3).

In regards to *A. fransiscana* sensitivity, cycloheximide, 2,4-dinitrophenol and the phytotoxin pyrenophorin exhibited the highest acute toxicity on *A. fransiscana* larvae with LC_{50} values of 4.7, 6.7 and 14.7 M 10^{-5}, respectively. All mean values are significantly different ($p \leq 0.05$). On the other hand, pyrenophorol caused an increase in nauplii mortality at higher concentration levels (64 M 10^{-5}) but not as high as those required for streptomycin (93 M 10^{-5}) and chloramphenicol (197 M 10^{-5}). It has been reported that other fungal phytotoxins are toxic to *A. salina* at concentrations varying from 10^{-7} to 10^{-3} M (Bunkers et al. 1990; Bunkers and Strobel 1991; Capasso et al. 1996; Evidente 2005). Interestingly, members of eremophilane group produced by the fungus *Drechslera gigantea* induce "green islands" (Bunkers et al. 1990; Bunkers and Strobel 1991), which is also a symptom caused by pyrenophorin on *Avena sterilis*. Also, the degree of oxidation plays a role in bioactivity of the eremophilane phytotoxins against monocots and *A. salina* (Bunkers et al. 1990). The phytotoxicity of pyrenophorin on *A. sterilis* has been associated with plant processes related to oxidative stress and the production of ROS (Aliferis and Chrysayi-Tokousbalides 2006). Such action might be responsible for toxic effect of the phytotoxin on *A. salina* which has been found to be sensitive to menadione bisulfite, a toxic compound associated with superoxide generation (Matthews 1995).

Although the data in hand are not sufficient to explain the differences in the activity of those two phytotoxins, it can be concluded that pyrenophorin seems to be less selective than pyrenophorol in regards to unrelated organisms. The data produced could be useful as indications for the possible effects of the fungal phytotoxins against non-target organisms and as a base for further risk assessment. The observed differences between pyrenophorin and pyrenophorol selectivity towards aquatic organisms suggest additional ecotoxicological studies before using such compounds as chemical templates for the development of new herbicides.

References

Aliferis KA, Chrysayi-Tokousbalides M (2006) On the mode of action of the phytotoxin (8*R*,16*R*)-(–)-pyrenophorin. Pestic Biochem Physiol 86:7–14

Andolfi A, Boari A, Evidente A, Vurro M (2005) Metabolites inhibiting germination of *Orobanche ramosa* seeds produced by *Myrothecium verrucaria* and *Fusarium compactum*. J Agric Food Chem 53:1598–1603

Bunkers GJ, Strobel GA (1991) A proposed mode of action for green island induction by the eremophilane phytotoxins produced by *Drechslera gigantean*. Physiol Mol Plant Pathol 38:313–323

Bunkers G, Kenfield D, Strobel G, Sugawara F (1990) Structure–activity relationships of the eremophilanes produced by *Drechslera gigantea*. Phytochemistry 29:1471–1474

Capasso R, Evidente A, Cutignano A, Vurro M, Zonno MC, Bottalico A (1996) Fusaric and 9,10-dehydrofusaric acid and their methyl esters from *Fusarium nygamai*. Phytochemistry 41:1035–1039

Duke SO, Dayan FE, Rimando AM, Schrader KK, Aliotta G, Oliva A, Romagni JG (2002) Chemicals from nature for weed management. Weed Sci 50:138–151

Evidente A, Andolfi A, Vurro M, Fracchiolla M, Zonno MC, Motta A (2005) Drazepinone, a trisubstituted tetrahydronaphthofuroazepinone with herbicidal activity produced by *Drechslera siccans*. Phytochemistry 66:715–721

Fairchild JF, Ruessler PS, Haverland PS, Carlson AR (1997) Comparative sensitivity of *Selenastrum capricornutum* and *Lemna minor* to sixteen herbicides. Arch Environ Contam Toxicol 32:353–357

International Organization for Standardization (2003) Water quality-determination of toxic effect of water constituents and waste water to duckweed (*Lemna minor*)-Duckweed growth inhibition test. ISO/DIS 20079 (draft), Geneva, Switzerland

Ishibashi K (1962) Studies on antibiotics from *Helminthosporium* sp. fungi. Part VIII. Effects of ophiobolin, zizanin, pyrenophorin and siccanin on spore germination and growing mycelium of *Trichophyton mentagrophytes*. J Agric Chem Soc Jpn 36: 645–648

Kastanias MA, Chrysayi-Tokousbalides M (2000) Herbicidal potential of pyrenophorol isolated from a *Drechslera avenae* pathotype. Pest Manag Sci 56:227–232

Kastanias MA, Chrysayi-Tokousbalides M (2005) Bioactivity of the fungal metabolite (8*R*,16*R*)-(–)-pyrenophorin on graminaceous plants. J Agric Food Chem 53:5943–5947

Machera K, Cotou E, Anastasiadou P (1996) Fenbutatin toxicity on *Artemia* nauplii: effects of sublethal concentrations on ATPase activity. Bull Environ Contam Toxicol 56:159–164

Matthews RS (1995) *Artemia salina* as a test organism for measuring superoxide-mediated toxicity. Free Radic Biol Med 5:919–922

Michel A, Johnson RD, Duke SO, Scheffler BE (2004) Dose–response relationships between herbicides with different modes of action and growth of *Lemna paucicostata*: an improved ecotoxicological method. Environ Toxicol Chem 23:1074–1079

Organisation for Economic Co-operation and Development Guidelines for testing of chemicals, Section 2 (1997) Effects on biotic systems, Paris

Schrader KK, Harries MD (2001) Compounds with selective toxicity toward the musty-odor cyanobacterium *Oscillatoria perornata*. Bull Environ Contam Toxicol 66:801–807

Schrader KK, de Regt MQ, Tidwell PR, Tucker CS (1997) Rapid bioassay for selective algicides. Weed Technol 1:767–774

Schrader KK, de Regt MQ, Tidwell PD, Tucker CS, Duke SO (1998a) Compounds with selective toxicity towards the off-flavor metabolite-producing cyanobacterium *Oscillatoria* cf. *chalybea*. Aquaculture 163:85–99

Schrader KK, de Regt MQ, Tidwell PR, Tucker CS, Duke SO (1998b) Selective growth inhibition of the musty-odor producing cyanobacterium *Oscillatoria* cf. *chalybea* by natural compounds. Bull Environ Contam Toxicol 60:651–658

Schrader KK, Dayan FE, Nanayakkara NPD (2005) Generation of reactive oxygen species by a novel anthraquinone derivative in the cyanobacterium *Planktothrix perornata* (Skuja). Pestic Biochem Physiol 81:198–207

Strobel GA, Kenfield D, Bunkers G, Sugawara F, Clardy J (1991) Phytotoxins as potential herbicides. Experientia 47:819–826

van der Ploeg M, Dennis ME, de Regt MQ (1995) Biology of *Oscillatoria* cf. *chalybea*, a 2-methylisoborneol producing blue-green alga of Mississippi catfish ponds. Water Sci Technol 31:173–180