Available online at www.sciencedirect.com
ScienceDirect
ELSEVIER
Food and Chemical Toxicology 44 (2006) 1922–1931
Food and Chemical Toxicology
www.elsevier.com/locate/foodchemtox

Toxicity assessment of metabolites of fungal biocontrol agents using two different (*Artemia salina* and *Daphnia magna*) invertebrate bioassays ☆

M. Favilla [a], L. Macchia [b], A. Gallo [a], C. Altomare [a,*]

[a] *Institute of Sciences of Food Production, CNR, Via G. Amendola 122, 70125 Bari, Italy*
[b] *Department of Internal Medicine, Immunology and Infectious Diseases, University of Bari, Italy*

Received 10 October 2005; accepted 13 June 2006

Abstract

Fungal biocontrol agents (BCAs) have been marketed for control of crop pests, weeds, and diseases. However, BCAs may produce toxic metabolites, whose presence in the formulated products, in the crops and in the environment should be considered along with the associated risk. Two invertebrate models, *viz*. *Artemia salina* and *Daphnia magna* were used to assess the acute toxicity of seven BCA metabolites, characterized by different chemical nature and mode of action, namely alamethicin (ALA), paracelsin (PCS), antiamoebin (AAM), gliotoxin (GTX), destruxin A (DA), oosporein (OOS), and elsinochrome A (EA). The two invertebrates were very sensitive to all the metabolites examined, except OOS. The LC_{50}s after 24 and 36 h exposures showed the following toxicity ranks: *A. salina*, DA > ALA > EA > GTX > AAM > PCS (LC_{50}s ranging from 9.78 to 40.84 μg/ml at 24 h and from 2.92 to 18.56 μg/ml at 36 h); *D. magna*, DA > GTX = EA > ALA > PCS > AAM (LC_{50}s ranging from 0.20 to 24.41 μg/ml at 24 h and from 0.16 to 11.98 μg/ml at 36 h). LC_{50} of OOS to *D. magna* increased dramatically in 36 h exposure, compared to 24 h exposures (5.84 and 68.40 μg/ml, respectively). *A. salina* and *D. magna* proved to be suitable models for rapid and inexpensive screening of toxicity of BCAs at an early stage of product development.
© 2006 Elsevier Ltd. All rights reserved.

Keywords: Fungi; Mycotoxins; Invertebrate models; Ecotoxicity; Risk assessment

1. Introduction

There is increasing interest in the exploitation of fungi for the biological control of crop pests, weeds and diseases (Butt et al., 2001). A number of biological pesticides (biopesticides) containing fungi as the active ingredients are already marketed in Europe, USA, and developing countries (Copping, 1998), while many other biocontrol fungal strains or formulations are expected to be placed on the world market in the next few years. In 1999, the biopesticide market was estimated to grow at a rate of 10% per year *vs*. 1–2% for chemical pesticides (Hall and Menn, 1999).

Many fungi are known to produce bioactive secondary metabolites, including toxins that may be harmful to humans and animals (Cole and Cox, 1981; Cole and Schweikert, 2003). In particular, some fungal biocontrol agents (BCAs) have been reported to secrete compounds with several different biological activities (Vey et al., 2001). These metabolites serve different functions, depending on the ecological niche of the fungus. For instance, metabolites may be antibiotics to protect the BCA against

Abbreviations: AAM, antiamoebin; ALA, alamethicin; BCA, fungal biocontrol agent; CC_{50}, median cytotoxic concentration; DA, destruxin A; EA, elsinochrome A; GTX, gliotoxin; ISO, International Organization for Standardization; OECD, Organization for Economic Co-operation and Development; OOS, oosporein; LC_{50}, median lethal concentration; PCS, paracelsin.

☆ The first and the last author contributed equally to this work.

* Corresponding author. Tel.: +39 080 5929318; fax: +39 080 5929371. *E-mail address:* claudio.altomare@ispa.cnr.it (C. Altomare).

0278-6915/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.fct.2006.06.024

competing microorganisms, or phytotoxins that may be part of the mechanism of action of plant pathogenic fungi used as mycoherbicides for weed control. However, some metabolites may also be toxic to animals, including mammals. Hence, based on hazard characterization of BCA metabolites, considerations must be made as for the possible presence of toxins in formulated products, in crops and in the environment.

The crustacean *Artemia salina* (brine shrimp) and the cladoceran *Daphnia magna* (water flea) are two invertebrate models that have been widely used for studies of ecotoxicology, as well as of general toxicology of chemicals (Lilius et al., 1995; Barahona and Sánchez-Fortún, 1999; Cleuvers, 2003) and natural compounds (Solis et al., 1993; Caldwell et al., 2003), including mycotoxins (Schmidt, 1989; Hartl and Humpf, 2000). In the present study, these two organisms were used to assess the acute toxicity of bioactive metabolites produced by BCAs in short-term assays. For this purpose, seven metabolites (Table 1), displaying different structural nature and mode of action and produced by mycoparasitic, entomopathogenic or phytopathogenic fungi (used for biological control of fungal plant diseases, pests or weeds respectively) were utilized.

Three of these metabolites, *viz*. alamethicin (ALA), paracelsin (PCS) and antiamoebin (AAM), belong to the family of peptaibols. The peptaibols are short-chain linear peptides, typically made of 15–20 residues, which are

Table 1
Metabolites of fungal biocontrol agents (BCAs) used in this study

Metabolite	Producing BCA(s)	Chemical nature	Toxicity data	References
Alamethicin(s)	*Trichoderma viride*[a]	Polypeptide	Antibacterial	Meyer and Reusser (1967)
			Human erythrocytes, $CC_{50} = 16\ \mu M$	Brückner et al. (1984)
			Human HL-60 cell line, $CC_{50} = 10\ \mu M$	Macchia et al. (2003)
			Human U-937 cell line, $CC_{50} = 3\ \mu M$	Macchia et al. (2003)
			Lepidopteran SF-9 cell line, $CC_{50} = 2\ \mu M$	Macchia et al. (2003)
			Mice, oral administration, $LD_{50} = 80$ mg/kg	Taylor (1986)
Paracelsin(s)	*Trichoderma* spp.[a]	Polypeptide	Antibacterial	Brückner and Graf (1983)
			PC12 cells, $CC_{50} = 21.8\ \mu M$	Abu Raya et al. (1993)
			Human erythrocytes, $CC_{50} = 37\ \mu M$	Brückner et al. (1984)
			Mice, intraperitoneally, $LD_{50} = 5$ mg/kg	Brückner et al. (1984)
Antiamoebin(s)	*Gliocladium catenulatum*[a]	Polypeptide	Antiprotozoal-anthelmintic	Thirumalachar (1968)
			Human erythrocytes, $CC_{50} = 125\ \mu M$	Brückner et al. (1984)
Gliotoxin	*Gliocladium virens*[a]	Epidithiodiketo-piperazine	Antibacterial, antifungal	Johnson et al. (1943)
			Mice, intraperitoneally, $LD_{50} = 25$ mg/kg	Johnson et al. (1943)
			Lepidopteran SF-9 cell line, $CC_{50} = 4\ \mu M$	Fornelli et al. (2004)
			Human lung carcinoma A549 cell line, $IC_{50} = 0.3\ \mu M$	Kreja and Seidel (2002)
			Human HL-60 cell line, $CC_{50} = 100\ \mu M$	Macchia et al. (2003)
			Human U-937 cell line, $CC_{50} = 200\ \mu M$	Macchia et al. (2003)
			Artemia salina, LC_{50} at 16 h = 3.5 μg/ml	Harwig and Scott (1971)
Destruxin A	*Metarhizium anisopliae*[b]	Cyclodepsipeptide	Mice, intraperitoneally, $LD_{50} = 1–1.35$ mg/kg	Kodaira (1961)
			Silkworm larvae, LC_{50} at 24 h = 0.015–0.030 mg/g	Kodaira (1961)
Oosporein	*Beauveria brongniartii*[b]	Hydroxybenzoquinone	Antibacterial	Wainwright et al. (1986)
			Mice and hamsters, intraperitoneally, $LD_{50} = 0.5$ mg/kg	Wainwright et al. (1986)
			Day-old cockerels, oral administration, $LD_{50} = 6.12$ mg/kg	Cole et al. (1974)
Elsinochrome A	*Stagonospora* spp.[c]	Perylenequinone	Monkey embryo R366.4 cell line, CC_{50} at 5 min = 100 μM	Ma et al. (2003)
			Human Hce-8693 cell line, CC_{50} at 5 min = 100 μM	Ma et al. (2003)

[a] BCA(s) of fungal plant diseases (Meyer and Reusser, 1967; Solfrizzo et al., 1994; Jaworski and Brückner, 2000; Aluko and Hering, 1970).
[b] BCA of insects (Kodaira, 1961; Strasser et al., 2000).
[c] BCAs of weeds (Nicolet and Tabacchi, 1999).

produced by a number of fungal genera, including *Trichoderma*, *Gliocladium*, *Acremonium*, *Paecilomyces*, and *Emericellopsis* (Degenkolb et al., 2003). These peptides are characterized by a high proportion of non-proteinogenic amino acids (mostly α, aminoisobutyric acid and isovaline), a C-terminal amino alcohol and an acylated N-terminal group. A most characteristic feature of peptaibols is their pronounced microheterogeneity, which results from a non-ribosomal biosynthesis via the so-called "thio-template mechanism". The single and multiple exchange of amino acids leads to a complex pattern of closely related sequence analogues. The biological activity of peptaibols is due to their ability to form voltage gated ion channel in lipid membranes. The pores so formed are able to conduct ionic species and this conductance leads to the loss of osmotic balance. In addition, by modifying membrane structures, they uncouple the oxidative phosphorylation and the associated effects ultimately result in cell death (Boheim et al., 1978).

Gliotoxin (GTX) is a member of the epipolythiodioxopiperazine family, a class of biologically active fungal metabolites characterized by the presence of a bridged disulphide ring that is essential for activity. GTX has been shown to have immunomodulating effects and induce apoptotic cell death. Beside BCAs belonging to the genus *Gliocladium*, GTX is produced by a number of moulds, including *Aspergillus fumigatus*, a well-known agent of pulmonary diseases in immunosuppressed patients. GTX may be produced *in vivo* during the course of the infection and contribute to the pathogenesis of the disease. GTX appears to act via covalent interaction with proteins through mixed disulphide formation. In addition, GTX was shown to inhibit a number of thiol-requiring enzymes (Waring and Beaver, 1996).

Destruxins are cyclic hexadepsipeptides, composed of an α-hydroxy acid and five amino acid residues. To date, 28 different but structurally-related destruxins have been identified from three different fungal sources, the great majority of which were isolated from cultures of *Metarhizium anisopliae*, a pathogen of diverse insect species. In particular, this species produces the two most active forms, namely destruxin A (DA) and E. Destruxins are related to enniatins and to beauvericin, two mycotoxins with insecticidal, phytotoxic and antimicrobial activities and, like them, their biological activity is linked to ionophoric properties (Pedras et al., 2002; Hinaje et al., 2002), that is the capability to form complexes with cations and induce selective membrane permeability for the complexed ions.

The red-colored dibenzoquinone oosporein (OOS) is produced by a large number of soil fungi and the entomogenous fungi belonging to the genus *Beauveria* (Strasser et al., 2000). OOS is thought to react with proteins and amino acids through redox reactions by altering the SH groups, resulting in enzyme malfunction (Wilson, 1971). In addition, OOS was shown to inhibit the erythrocyte membrane ATPase activity, especially that of Ca^{2+}-dependent ATPases, in a dose-dependent manner (Jeffs and Khachatourians, 1997).

Perylenequinones are photosensitive pigments widespread in nature, which have been isolated from fungi, as well as other organisms (Nicolet and Tabacchi, 1999). The lipid-soluble 4,9-dihydroxy-3,10-perylenequinone derivatives, including the fungal metabolite elsinochrome A (EA), are efficient producers of singlet oxygen ($^1O_2$) in visible light. Due to their excellent photosensitive properties, they are expected to be developed as new phototherapeutic medicines (Chen et al., 1999).

The aims of the present study were: (i) to evaluate the sensitivity of *A. salina* and *D. magna* to bioactive metabolites produced by fungal BCAs in the perspective of their possible use for preliminary toxicological screenings of fungal BCAs and biopesticides; (ii) to generate toxicological data useful for risk assessment of BCA metabolites possibly produced on the crop and released in the environment. The dose–response curve and the median lethal concentration (LC_{50}) of each compound to *A. salina* and *D. magna* were determined.

2. Materials and methods

2.1. Sources of metabolites

ALA (mixture of alamethicin homologs, purity 99.1%), AAM (purity 95%), and GTX (purity 98%), were purchased from Sigma-Aldrich chemicals (St. Louis, MO, USA). PCS (mixture of paracelsin homologs, purity 88.4%) was purchased from Fluka-Chemie (Buchs, CH). Samples of DA (purity > 98%), EA (purity > 97%) and OOS (purity 98%) were supplied by Dr. A. Skrobek, University of Wales, Swansea, Dr. M. Maurhofer, Swiss Federal Institute of Technology, Zürich, and Drs. H. Strasser and C. Seger, Leopold-Franzens-Universität, Innsbruck, respectively.

2.2. *A. salina* acute toxicity test

Stock solutions of metabolites were prepared by dissolving pure compounds in methanol (ALA, AAM, PCS, and OOS), ethanol (GTX), methanol:acetonitrile (1:1) (DA), or acetonitrile:tetrahydrofurane (9:1) (EA), to a final concentration of 3.0 mM for ALA, PCS, and AAM, 12.0 mM for GTX, 10.0 mM for DA, and 20.0 mM for EA and OOS. Then, serial twofold dilutions of each compound were made in the same solvent used for the mother solution.

Brine shrimp eggs were obtained from a local pet shop and hatched in Petri dishes containing artificial sea water (3.3% (wt/vol) marine salts in distilled water) and incubated in the dark at 26 ± 1 °C for 36–48 h. Subsequently, the newly hatched brine shrimps (nauplii) were separated from the shells and the remaining cysts, transferred to fresh sea water with a Pasteur pipette and immediately used for bioassays. Assays were performed in 24-well culture plates (Corning, NY, USA). Each well contained 30–40 larvae in 500 μl of marine water. Five microliters of each dilution of the toxins was transferred to the wells to obtain a final solvent concentration of 1% (vol/vol) and compound concentrations in the range of 0.7–70 μg/ml, except EA that was tested at concentrations ranging from 0.4 to 100 μg/ml. The EA solutions were activated by exposure to white light (1600 lux) for 1 h prior to testing. Untreated control and 1% solvent control were assayed along with compound tests. Tests were performed in quadruplicate. In the absence of specific information about the stability in the light of the BCA metabolites under study, the bioassays were carried out in the dark to prevent any possible photodegradation, while the assays

with EA were carried out under continuous white light in order to allow photoactivation. The percentage of larval mortality was determined after exposure to the metabolites for 24 and 36 h at 26 ± 1 °C in the dark. The mortality endpoint for this bioassay was defined as the absence of forward motion of shrimps.

2.3. *D. magna* acute toxicity test

The *D. magna* bioassays were carried out using dormant eggs (ephippia) and the salts for preparation of standard freshwater (ISO formula) contained in the "Daphtoxkit F™" (Creasel BVBA, Deinze, Belgium). Hatching of ephippia and preparation of standard freshwater were performed according to the manufacturer instructions, while the bioassay procedure was modified in order to minimize the amount of compounds needed. The content of vials with *D. magna* ephippia was poured into a microsieve and rinsed with tap water to eliminate all traces of the storage medium. The ephippia were transferred to hatching Petri dishes (10 cm diameter) with 50 ml pre-aerated standard freshwater. The hatching Petri dishes were covered and incubated for 72 h, at 20–22 °C under continuous illumination of 6000 lux. Subsequently, the daphnids were transferred to

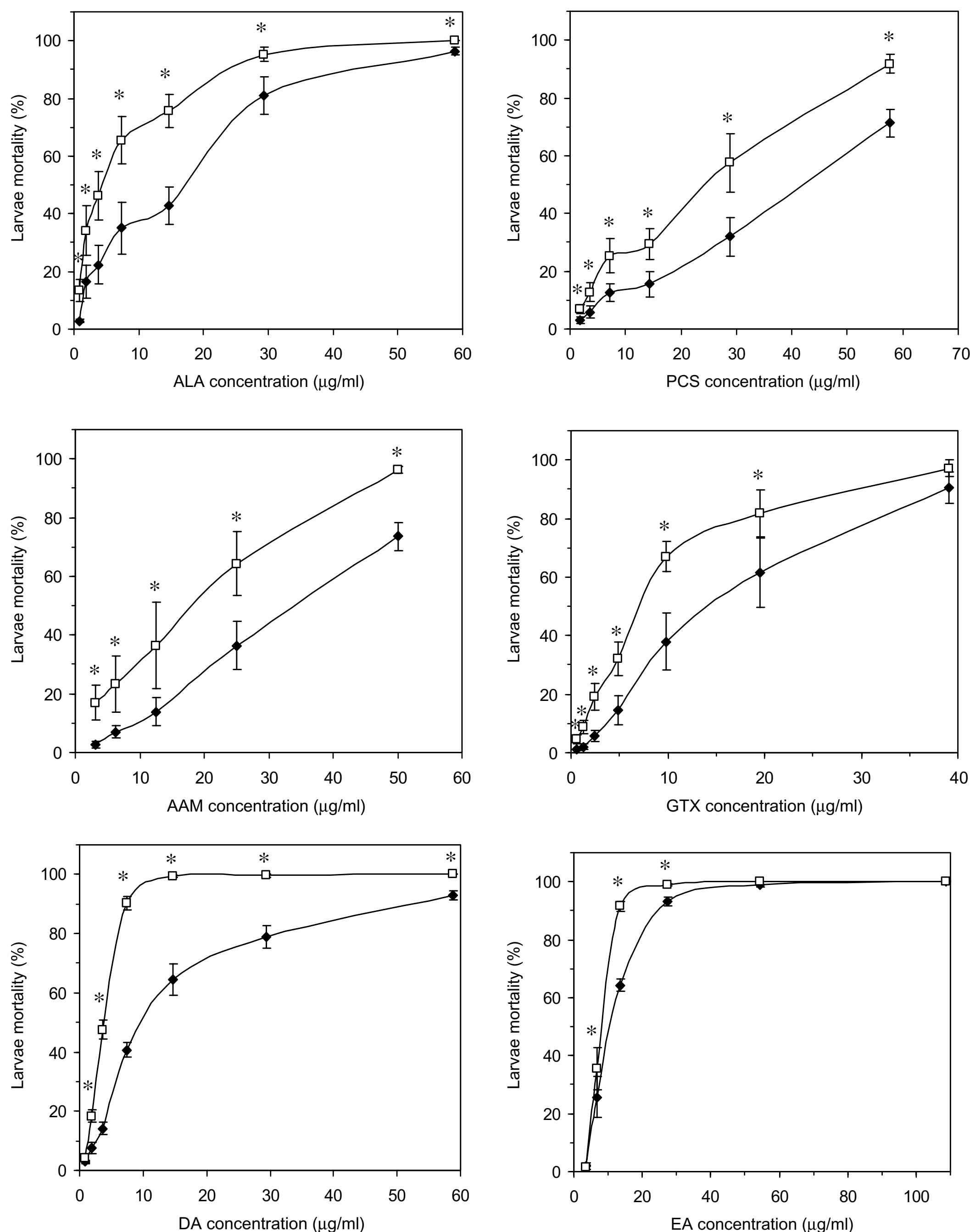

Fig. 1. Dose–mortality curves of brine shrimp (*A. salina*) larvae exposed to ALA, PCS, AAM, GTX, DA and EA for 24 (◆) and 36 h (□). The data are percent mortalities corrected for the natural mortality, as described in materials and methods. Values are the means ± SE of 6 (ALA, PCS, AAM, and GTX) or 3 (DA and EA) independent experiments, each with four replicates. All the data shown are statistically different from controls; asterisks indicate significant differences between 36 and 24 h mortality values (paired Student's *t*-test, $P < 0.05$).

fresh standard water with a Pasteur pipette and immediately used for bioassays.

Mother solutions and serial twofold dilutions of ALA, AAM, PCS, OOS, GLT, DA, and EA were prepared as described for the *A. salina* bioassay, with the exception of OOS, whose concentration in the mother solution was 40.0 mM. In some cases, after preliminary experiments it was necessary to prepare further intermediate dilutions in order to refine the dose–response curve. Assays were carried out in 24-well plates. In each well, 195 μl of standard water plus 5 μl of test compound solution were pipetted. By using a micropipette, daphnids were aspired with 60 μl of standard water and transferred in the wells. In the end, each well contained five daphnids in 500 μl of standard solution with 1% (vol/vol) of test compound solution. Freshwater controls and 1% solvent blanks were included in every test. Tests were performed in quadruplicate. The plates were covered and incubated at 20 °C, in the dark, except EA, whose bioassays were incubated under continuous white light (1600 lux). After 24 h and 36 h incubation, the number of dead and immobilized neonates *vs* that of the actively swimming daphnids was recorded and the percent mortality was calculated.

2.4. Data analysis

Mortality data from 3 to 6 independent experiments were used to construct the dose/response curves and determine the LC_{50} of each compound to *A. salina* and *D. magna*.

In dose/response curves, the concentrations tested were plotted against the averaged percent mortalities corrected for the natural mortality ± standard error (SE). After arcsine transformation of the mortality values, the unpaired Student's *t*-test was used to determine the significance of differences between controls and treatments, while the paired *t*-test was used for comparison between 36 and 24 h mortality values, with $P < 0.05$.

The LC_{50} with 95% confidence limits of each of the BCAs metabolites was calculated by using probit analysis (Finney, 1971), performed with the computer program POLO-PC (LeOra Software, 1987). Percentage mortalities were corrected for the mortality observed in the solvent controls according to Bliss (1935). The number of individuals used in a particular test were multiplied by the proportion alive in the control, to give the net number of organisms actually exposed. The number of surviving individuals was subtracted from the net number of exposed individuals and the result (multiplied by 100) was divided by the net number of exposed individuals to give the percentage killed.

3. Results

3.1. *A. salina* acute toxicity test

The results of the bioassays performed in the *A. salina* model with pure bioactive metabolites produced by fungal BCAs are shown in Fig. 1 and Table 2.

A. salina proved to be sensitive to all the toxins examined, with the exception of OOS (which was tested in a range of concentrations from 0.78 to 200 μM, equal to 0.2–61.2 μg/ml), regardless of the chemical nature of the compound. Dose–response experiments indicated that brine shrimp sensitivity to BCA metabolites was dose- and time-dependent. In most cases, the data of 36 h exposure assays showed a lower variability compared to data from the 24 h exposure experiments, resulting in a narrower 95% confidence interval. The most toxic compounds were ALA, GTX, and DA, which showed an LC_{50} at 36 h around 5 μg/ml, followed by EA ($LC_{50} > 5 < 10$ μg/ml) and AAM and PCS ($LC_{50} = 13.8$ and 18.6 μg/ml, respectively). Among the peptaibols, PCS and AAM were 4–5 times less toxic than ALA. In the case of EA, it was not possible to calculate the LC_{50} at 36 h by using probit analysis, since the heterogeneity factor exceeded 100, indicating that the dose–mortality curve did not fit the probit analysis model (Finney, 1971). In fact, in brine shrimps exposed to EA for 36 h, a steep increase in mortality (from 1.6% to 91.6%) was observed between the concentrations of 3.4 and 13.6 μg/ml. This made the data deviate from a sigmoid distribution. Nevertheless, it was possible to infer the LC_{50} at 36 h of EA from the dose–mortality curve, yielding an estimate of 8.3 μg/ml. The highest concentration of OOS tested affected 1.32% of larvae at 24 h and 32.58% at 36 h. Consequently, the LC_{50} values of this toxin were not determined.

Table 2
LC_{50} of fungal BCA toxins to brine shrimps (*A. salina*)

Compound	MW	Exposure (h)	LC_{50} (95% confidence) (μM)	Slope	LC_{50} (μg/ml)
Alamethicin	1960	24	5.32 (4.19–6.87)	1.697	10.43
		36	1.96 (1.53–2.46)	1.678	3.84
Paracelsin	1921	24	21.26 (16.53–29.58)	1.603	40.84
		36	9.66 (7.63–12.73)	1.675	18.56
Antiamoebin	1671	24	19.79 (15.49–27.31)	1.954	33.07
		36	8.25 (6.14–11.67)	1.806	13.79
Gliotoxin	326	24	39.49 (27.96–61.90)	1.990	12.87
		36	20.44 (17.28–24.44)	1.874	6.66
Destruxin A	578	24	16.92 (13.40–21.67)	1.597	9.78
		36	5.05 (3.87–6.61)	2.459	2.92
Oosporein	306	24	ND[a]	–	ND[a]
		36	ND[a]	–	ND[a]
Elsinochrome A	544	24	20.18 (18.81–21.64)	3.705	10.98
		36	15.26[b]		8.30[b]

[a] Not determined. The maximal concentration tested (200 μM = 61.2 μg/ml) was not toxic at 24 h and gave 32.58 ± 1.6% mortality at 36 h.
[b] Inferred from dose–mortality curve (see text).

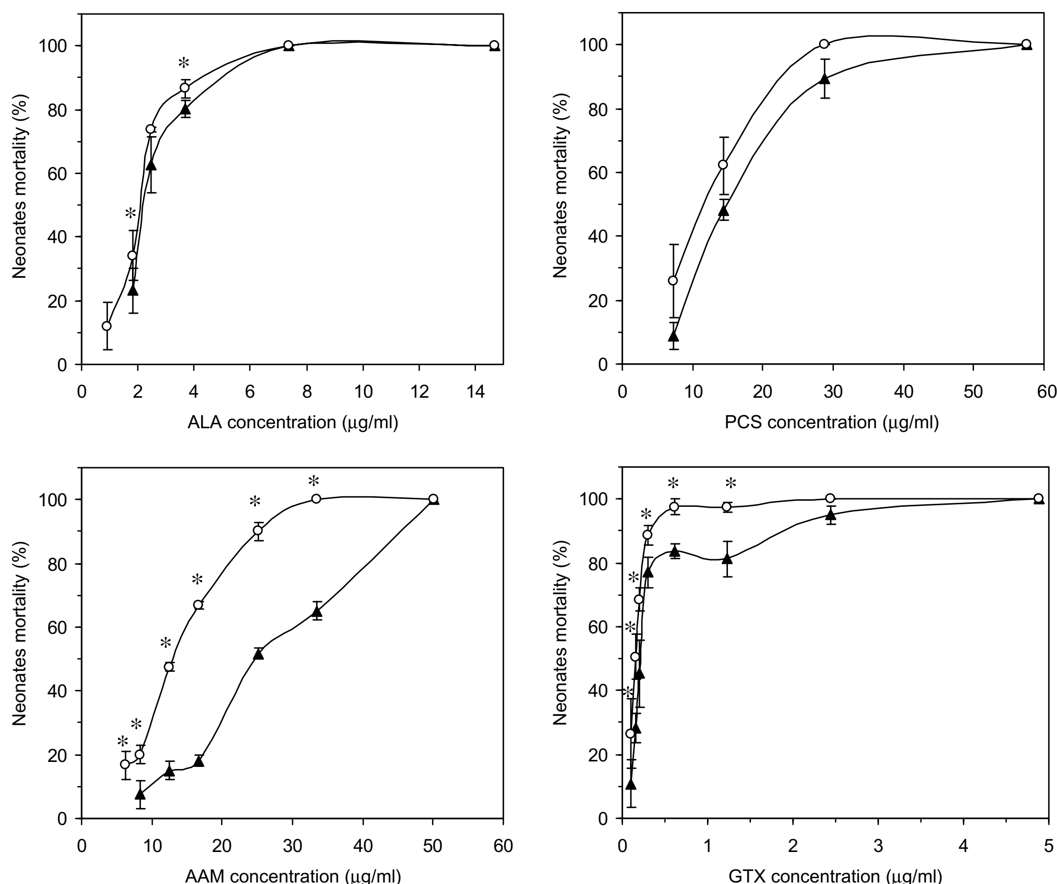

Fig. 2a. Dose–mortality curves of water flea (*D. magna*) neonates exposed to ALA, PCS, AAM, and GTX for 24 (▲) and 36 h (○). Values are the means ± SE of 5 (GTX), 4 (ALA) or 3 (PCS, and AAM) independent experiments, each with four replicates. All the data shown are statistically different from controls; asterisks indicate significant differences between 36 and 24 h mortality values (paired Student's *t*-test, $P < 0.05$).

3.2. *D. magna* acute toxicity test

The results of the toxicity tests with *D. magna* are shown in Figs. 2a and 2b, and Table 3. The standard protocol of *D. magna* acute toxicity test, as prescribed by various international organizations (*e.g.* OECD guideline 202 or ISO EN UNI 6341) for water pollutants, is carried out in 10 ml of water containing five daphnids. In our study, the bioassay protocol was modified in order to spare pure compounds that were available only in limited amounts. The bioassays were performed in a 0.5 ml volume containing different concentrations of metabolite and 1% of solvent. Preliminary experiments, carried out with the reference toxicant potassium dichromate ($K_2Cr_2O_7$) using the dilution series 3.2, 1.8, 1.0, 0.56, 0.32 mg/l, showed that the reduced test volume did not affect the bioassay over a 36 h observation. In addition, the natural mortality that was determined in either freshwater or solvent controls was usually 0%, and never exceed 5%.

D. magna was very sensitive to all the toxins examined, including OOS. Differences in mortalities caused by AAM and GTX at 24 and 36 h were less conspicuous than those found in *A. salina*, while no statistically significant differences were found between 24 and 36 h exposures to PCS. However, a dramatic increase in mortality at 36 h compared to 24 h was observed with OOS. Moreover, the toxic effects of OOS at 36 h occurred in a rather restricted concentration range, since neonate mortality was minimal (0%) at 1.9 µg/ml but already 77.5% at 7.7 µg/ml, as indicated by the steep profile of the dose–response curve (Fig. 2b). DA, GTX and EA proved to be the most toxic compounds ($LC_{50} = 0.20$, 0.28 and 0.29 µg/ml at 24 h and 0.16, 0.16 and 0.22 µg/ml at 36 h, respectively). ALA was confirmed as the most toxic among the peptaibol toxins, while PCS and AAM were 6 to 10 times less toxic.

4. Discussion

Recent years have seen the development of a number of toxicity tests in which the response has been measured in invertebrates. These tests have the virtue of being inexpensive, reproducible, easy to carry out, and environmentally relevant (Calow, 1993). Invertebrates are already used in tests that are required by some regulatory authorities for the environmental risk assessment of pesticides, chemicals and pollutants (Commission of the European Communities, 1991, 1992; United States Environment Protection Agency, 2002). In this study, the acute toxicity of seven different bioactive metabolites produced by BCAs and

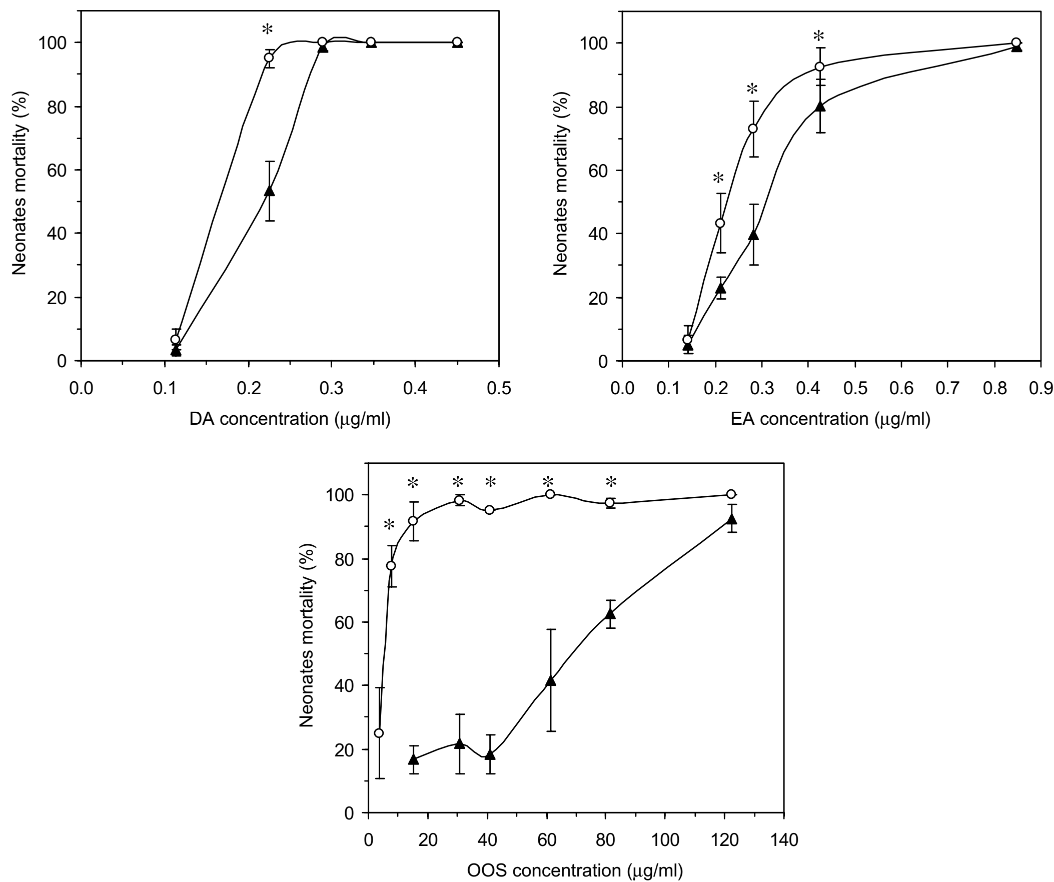

Fig. 2b. Dose–mortality curves of water flea (*D. magna*) neonates exposed to DA, EA, and OOS for 24 (▲) and 36 h (○). Values are the means ± SE of 4 (EA) or 3 (DA, and OOS) independent experiments, each with four replicates. All the data shown are statistically different from controls; asterisks indicate significant differences between 36 and 24 h mortality values (paired Student's *t*-test, $P < 0.05$).

Table 3
LC_{50} of fungal BCA toxins to water flea (*D. magna*)

Compound	MW	Exposure (h)	LC_{50} (95% confidence) (μM)	Slope	LC_{50} (μg/ml)
Alamethicin	1960	24	1.19 (1.08–1.30)	4.854	2.33
		36	0.99 (0.84–1.15)	3.753	1.94
Paracelsin	1921	24	7.70 (6.83–8.77)	4.516	14.79
		36	5.60 (4.72–6.77)	3.933	10.76
Antiamoebin	1671	24	14.61 (11.96–19.33)	3.246	24.41
		36	7.17 (6.43–8.02)	3.613	11.98
Gliotoxin	326	24	0.85 (0.67–1.06)	2.104	0.28
		36	0.50 (0.39–0.62)	3.535	0.16
Destruxin A	578	24	0.35 (0.31–0.38)	9.103	0.20
		36	0.27 (0.25–0.29)	9.472	0.16
Oosporein	306	24	223.54 (166.30–357.93)	1.985	68.40
		36	19.10 (11.51–27.40)	2.745	5.84
Elsinochrome A	544	24	0.53 (0.42–0.69)	3.978	0.29
		36	0.40 (0.33–0.49)	4.620	0.22

characterized by diverse chemical nature and modes of action were investigated by means of the *A. salina* and *D. magna* short-term bioassays. LC_{50}s of BCA metabolites were determined for 24 and 36 h exposures. Determination of LC_{50} at 36 h was preferred over LC_{50} at 48 h since *Artemia* mortality assessment at 48 h is affected by higher variability in both treatments and controls, possibly as a consequence of starvation of nauplii.

Both the organisms utilized were sensitive to BCA metabolites. Generally, sensitivity of *D. magna* was higher

and, in some cases (GTX, OOS), much higher than *A. salina*. Moreover, in *D. magna* bioassays a lower variability in mortality response and less marked differences between 24 h and 36 h responses were observed, compared to *A. salina*. This is probably due to the use of more standardized materials for the *D. magna* assay. Since test animals hatching from ephippia are of similar age, genotype and physiological conditions, test variability is greatly reduced. On the other hand, *A. salina* cysts are easily available commercially and inexpensive and hence this assay may be useful in situations where rapidity and low cost make it practical to test large number of samples for preliminary toxicity screening.

In both test organisms, a delayed toxic effect of OOS was observed, resulting in a low LC_{50} at 24 h, followed by a dramatic increase in mortality of the 36 h observations. Therefore, for the evaluation of the ecotoxicological risks of this toxin, long exposure times are perhaps advisable.

There has been some success in relating the toxicity data for certain invertebrates to toxicity in vertebrates in the case of a number of compounds. For instance, Lagarto Parra et al. (2001) found a good correlation between the *in vivo* tests of various plant extracts in mice and *in vitro* tests with *A. salina* ($r = 0.85$, $P < 0.05$). They concluded that *A. salina* bioassay was an useful tool for predicting oral acute toxicity of plant extracts. A good correlation has been shown between toxicity to *Daphnia* and toxicity to fish for a group of 42 industrial chemicals (Walker et al., 1991). Also, a strong correlation between the acute toxicity of 54 different compounds to *D. magna* and rats was found by Guilhermino et al. (2000). In several instances, good correlations were found also with cytotoxicity. McLaughlin et al. (1993) were able to determine a positive correlation between brine shrimp lethality by plant extracts and cytotoxicity towards 9KB cells. Solis et al. (1993) found the *A. salina* test predictive of KB cell cytotoxicity, except for compounds requiring metabolic activation, since brine shrimps lack the necessary cytochrome P-450 enzyme. Logrieco et al. (1996) reported that the median cytotoxic concentration (CC_{50}) of the fungal toxin fusaproliferin to the human B-lymphocyte cell line IARC/LCL 171 and the insect cell line SF-9 were approximately the same as the LC_{50} found in *A. salina* assay. A comparison of our results with cytotoxicity data of some BCAs metabolites retrieved from the literature (see Table 1) corroborates the above findings. In particular, even if different cell lines may exhibit different sensitivity to one particular metabolite, CC_{50} of ALA PCS, and GTX in the SF-9 cell line model, are in the same order of magnitude as the LC_{50} to *A. salina* or *D. magna*. Interestingly, the two human cell lines HL-60 and U-937, appear much less sensitive to GTX than invertebrates (Macchia et al., 2003). LC_{50}s at 24 h of the tested metabolites to *A. salina* were in the range of 10–40 μg/ml. Compared with some fungal toxins found as contaminants of foods and feeds, such as aflatoxins, fumonisins and trichothecenes, these metabolites appear to have low toxicity (Harwig and Scott, 1971; Schmidt, 1989; Hartl and Humpf, 2000). However, their toxicity is in the same order of magnitude of the fungal nephrotoxin ochratoxin A and the recently discovered fusaproliferin (Harwig and Scott, 1971; Logrieco et al., 1996).

Risk assessment procedures are necessary for the introduction and use of BCAs (Blum et al., 2003). In Europe, the Council Directive 91/414/EEC and its successive amendments identify the requirements to be submitted by an applicant for the authorization of production and marketing of pesticides, including those whose active substance is a BCA (Commission of the European Communities, 2004). In particular, the Directive requires the provision of information on short-term toxicity, as well as ecotoxicity, of any relevant metabolites (*i.e.* metabolites of toxicological, ecotoxicological, and/or environmental concern) formed by the candidate BCA. The study presented herein provides toxicological data that are of importance to assess the ecotoxicological relevance of the seven investigated BCA metabolites, but also points out the possible use of invertebrate bioassays for testing the toxigenicity of BCAs and for the preliminary evaluation of the general toxicity of their metabolites. In fact, BCA strains are able to synthesize a plethora of bioactive metabolites with different structures and modes of action (Taylor, 1986), some of which may be still unknown. Moreover these metabolites may have additive or synergistic toxic effects. Therefore, it is conceivable that the toxicological risk associated to a particular BCA would be better foreseen by assaying mixtures of metabolites, like those in crude culture extracts, on test organisms characterized by sensitivity to a large spectrum of different molecules, instead of assessing the toxicity of single metabolites. Based on this approach, *A. salina* or *D. magna* bioassays could be used by the industry for inexpensive and rapid screening of new candidate BCAs at an early stage of development. If appreciable toxicity is found, it might be considered not to proceed with the development of a BCA before any toxicity testing is carried out in appropriate vertebrate models. Based on the findings presented herein, *D. magna* appears to be a more sensitive and reproducible invertebrate model for testing BCA toxins than *A. salina*, and therefore it may be preferable for regulatory purposes.

Acknowledgements

This work was supported by the Quality of Life and Management of Living Resources Programme of the European Commission, Key Action 1 on Food, Nutrition and Health, QLK1-2001-01391 (RAFBCA). We thank Dr. M. Maurhofer, Dr. C. Seger, Dr. A. Skrobek, and Dr. H. Strasser for gifts of BCA metabolites.

References

Abu Raya, S., Trembovler, V., Shohami, E., Lazorovici, P., 1993. Cytolisins increase intracellular calcium and induce eicosanoids release by pheochromocytoma PC12 cell cultures. Nat. Toxins 1, 263–270.

Aluko, M.O., Hering, T.F., 1970. The mechanisms associated with the antagonistic relationship between *Corticium solani* and *Gliocladium virens*. Trans. Br. Mycol. Soc. 55, 173–179.

Barahona, M.V., Sánchez-Fortún, S., 1999. Toxicity of carbamates to the brine shrimp *Artemia salina* and the effect of atropine, BW284c51, iso-OMPA and 2-PAM on carbaryl toxicity. Environ. Pollut. 104, 469–476.

Bliss, C.I., 1935. The calculation of the dosage-mortality curve. Ann. Appl. Biol. 22, 134–167.

Blum, B., Ehlers, R.U., Haukeland-Salinas, S., Hokkanen, H., Jung, K., Kuhlmann, U., Menzler-Hokkanen, I., Ravensberg, W., Strasser, H., Warrior, P., Wilson, M., 2003. Letter to the editors – Biological control agents: safety and regulatory policy. BioControl 48, 474–487.

Boheim, G., Irmscher, G., Jung, G., 1978. Trichotoxin A-40, a new membrane-exciting peptide. Part B. Voltage-dependent pore formation in bilayer lipid membranes and comparison with other alamethicin analogues. Biochim. Biophys. Acta 507, 485–506.

Brückner, H., Graf, H., 1983. Paracelsin, a peptide antibiotic containing α-aminoisobutyric acid, isolated from *Trichoderma reesei* Simmons. Part A. Experientia 39, 528–530.

Brückner, H., Graf, H., Bokel, M., 1984. Paracelsin; characterization by NMR spectroscopy and circular dichroism, and hemolytic properties of a peptaibol antibiotic from the cellulolytically active mold *Trichoderma reesei*. Part B. Experientia 40, 1189–1197.

Butt, T.M., Jackson, C.W., Magan, N., 2001. Fungi as Biocontrol Agents: Progress, Problems and Potential. CABI Publishing, Wallingford, UK.

Caldwell, G.S., Bentley, M.G., Olive, P.J.W., 2003. The use of a brine shrimp (*Artemia salina*) bioassay to assess the toxicity of diatom extracts and short chain aldehydes. Toxicon 42, 301–306.

Calow, P., 1993Handbook of Ecotoxicology, vol. 1. Blackwell, Oxford, UK.

Chen, C.L., Tai, H.L., Zhu, D.M., Uckun, F.M., 1999. Pharmacokinetic features and metabolism of calphostin C, a naturally occurring perylenequinone with antileukemic activity. Pharm. Res. 16, 1003–1009.

Cleuvers, M., 2003. Aquatic ecotoxicity of pharmaceuticals including the assessment of combination effects. Toxicol. Lett. 142, 185–194.

Cole, R.J., Cox, R.H., 1981. Handbook of Toxic Fungal Metabolites. Academic Press Inc., New York, NY, USA.

Cole, R.J., Schweikert, M.A., 2003. Handbook of Secondary Fungal Metabolites. Academic Press Inc., New York, NY, USA.

Cole, R.J., Kirksey, J.W., Cutler, H.G., Davis, E.E., 1974. Toxic effects of oosporein from *Chaetomium trilaterale*. J. Agric. Food Chem. 22, 517–520.

Commission of the European Communities, 1991. Protection of waters against pollution caused by nitrates from agricultural sources. EEC Directive 91/676/EEC.

Commission of the European Communities, 1992. Seventh amendment of Directive 67/548/EEC; Annex V, Part C, Methods for the determination of ecotoxicity, C2: Acute toxicity for *Daphnia*. EEC Directive 92/32/EEC.

Commission of the European Communities, 2004. Council Directive of 15 July 1991, Concerning the placing of plant protection products on the market, Consolidated Text. Office for Official Publications of the European Communities, Luxembourg, LU.

Copping, L.G., 1998. The BioPesticide Manual: A world compendium. British Crop Protection Council, Farnham, UK.

Degenkolb, T., Berg, A., Gams, W., Schlegel, B., Gräfe, U., 2003. The occurrence of peptaibols and structurally related peptaibiotics in fungi and their mass spectrometric identification via diagnostic fragment ions. J. Pept. Sci. 9, 666–678.

Finney, D.J., 1971. Probit Analysis, third ed. Cambridge University Press, Cambridge, UK.

Fornelli, F., Minervini, F., Logrieco, A., 2004. Cytotoxicity of fungal metabolites to lepidopteran (*Spodoptera frugiperda*) cell line (SF-9). J. Invertebr. Pathol. 85, 74–79.

Guilhermino, L., Diamantino, T., Carolina Silva, M., Soares, A.M.V.M., 2000. Acute toxicity test with *Daphnia magna*: an alternative to mammals in the prescreening of chemical toxicity? Ecotoxicol. Environ. Saf. 46, 357–362.

Hall, F.R., Menn, J.J., 1999. Biopesticides: Use and Delivery. Humana Press, Totowa, NJ, USA.

Hartl, M., Humpf, H.-U., 2000. Toxicity assessment of fumonisins using the brine shrimp (*Artemia salina*) bioassay. Food Chem. Toxicol. 38, 1097–1102.

Harwig, J., Scott, P.M., 1971. Brine shrimp (*Artemia salina* L.) larvae as a screening system for fungal toxins. Appl. Microbiol. 21, 1011–1016.

Hinaje, M., Ford, M., Banting, L., Arkle, S., Khambay, B., 2002. An investigation of the ionophoric characteristics of destruxin A. Arch. Biochem. Biophys. 405, 73–77.

Jaworski, A., Brückner, H., 2000. New sequences and new fungal producers of peptaibol antibiotics antiamoebins. J. Pept. Sci. 6, 149–167.

Jeffs, L.B., Khachatourians, G.G., 1997. Toxic properties of *Beauveria* pigments on erythrocyte membranes. Toxicon 35, 1351–1356.

Johnson, J.R., Bruce, W.F., Dutcher, J.D., 1943. Gliotoxin, the antibiotic principle of *Gliocladium fimbriatum*. I. Production, physical and biological properties. J. Am. Chem. Soc. 65, 2005–2009.

Kodaira, Y., 1961. Biochemical studies on the muscardine fungi in the silkworms, *Bombyx mori*. J. Fac. Text. Sci. Technol., Sinshu Univ., Sericult. 5, 1–68.

Kreja, L., Seidel, H.-J., 2002. On the cytotoxicity of some microbial volatile organic compounds as studied in the human lung cell line A549. Chemosphere 49, 105–110.

Lagarto Parra, A., Silva Yhebra, R., Guerra Sardiñas, I., Iglesias Buela, L., 2001. Comparative study of the assay of *Artemia salina* L. and the estimate of the medium lethal dose (LD_{50}) in mice in order to determine oral acute toxicity of plant extracts. Phytomedicine 8, 395–400.

LeOra Software, 1987. POLO-PC a user's guide to probit or logit analysis. LeOra Software, Berkeley, CA, USA.

Lilius, H., Hästbacka, T., Isomaa, B., 1995. A comparison of the toxicity of 30 reference chemicals to *Daphnia magna* and *Daphnia pulex*. Environ. Toxicol. Chem. 14, 2085–2088.

Logrieco, A., Moretti, A., Fornelli, F., Fogliano, V., Ritieni, A., Caraffa, M.F., Randazzo, G., Bottalico, A., Macchia, L., 1996. Fusaproliferin production by *Fusarium subglutinans* and its toxicity to *Artemia salina*, SF-9 insect cells, and IARC/LCL 171 human B lymphocytes. Appl. Environ. Microbiol. 62, 3378–3384.

Ma, L., Tai, H., Li, C., Zhang, Y., Wang, Z.H., Ji, W.Z., 2003. Photodynamic inhibitory effect of three perylenequinones on human colorectal carcinoma cell line and primate embryonic stem cell line. World J. Gastroenterol. 9, 485–490.

Macchia, L., Calo', L., Caiaffa, M.F., Altomare, C., Tursi, A., 2003. Toxicity to mammalian cell lines by gliotoxin and alamethicin, toxic metabolites of fungal biological control agents. Abstracts XIII Congresso Nazionale della Società Italiana di Tossicologia, Urbino, Italy, January 22–25, 2003, p. 76.

McLaughlin, J.L., Chang, C., Smith, D.L., 1993. Simple bench-top bioassays (brine shrimp and potato discs) for the discovery of plant antitumor compounds. In: Kinghorn, A.D., Balandrin, M.F. (Eds.), Human Medicinal Agents from Plants, ACS Symposium, vol. 534. American Chemical Society, Washington, DC, USA, pp. 112–137.

Meyer, C.E., Reusser, F., 1967. A polypeptide antibacterial agent isolated from *Trichoderma viride*. Experientia 23, 85–86.

Nicolet, B., Tabacchi, R., 1999. Secondary metabolites produced by *Stagonospora* sp., a potential biocontrol agent against bindweeds. In: Lyr, H., Russell, P.E., Sisler, H.D. (Eds.), Modern Fungicides and Antifungal Compounds II. Intercept Limited, Andover, UK, pp. 469–476.

Pedras, MS., Irina Zaharia, L., Ward, D.E., 2002. The destruxins: synthesis, biosynthesis, biotransformation, and biological activity. Phytochemistry 59, 579–596.

Schmidt, R., 1989. The application of *Artemia salina* L. bioassay for screening of *Fusaria* toxins. In: Chełkowski, J. (Ed.), *Fusarium:* Mycotoxins Taxonomy and Pathogenicity. Elsevier, Amsterdam, NL, pp. 121–130.

Solfrizzo, M., Altomare, C., Visconti, A., Bottalico, A., Perrone, G., 1994. Detection of peptaibols and their hydrolysis products in cultures of *Trichoderma* species. Nat. Toxins 2, 360–365.

Solis, P.N., Wright, C.W., Anderson, M.M., Gupta, M.P., Phillipson, J.D., 1993. A microwell cytotoxicity assay using *Artemia salina* (brine shrimp). Planta Med. 59, 250–252.

Strasser, H., Vey, A., Butt, T.M., 2000. Are there any risks in using pest control, with particular reference to the bioactive metabolites of *Metarhizium*, *Tolypocladium* and species? Biocontrol Sci. Technol. 10, 717–735.

Taylor, A., 1986. Some aspects of the chemistry and biology of the genus *Hypocrea* and its anamorphs, *Trichoderma* and *Gliocladium*. Proc. N. S. Inst. Sci. 36, 27–58.

Thirumalachar, M.J., 1968. Antiamoebin, a new antiprotozoal-anthelmintic antibiotic. I. Production and biological studies. Hindustan Antibiot. Bull. 10, 287–289.

United States Environment Protection Agency, 2002. Methods for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms, EPA/821/R-02-012.

Vey, A., Hoagland, R.E., Butt, T.M., 2001. Toxic metabolites of fungal biocontrol agents. In: Butt, T.M., Jackson, C.W., Magan, N. (Eds.), Fungi as Biocontrol Agents: Progress, Problems and Potential. CABI Publishing, Wallingford, UK, pp. 311–346.

Wainwright, M., Betts, R.P., Teale, D.M., 1986. Antibiotic activity of oosporein from *Verticillium psalliotae*. Trans. Br. Mycol. Soc. 86, 168–170.

Walker, C.H., Greig-Smith, P.W., Crossland, N.O., Brown, R., 1991. Ecotoxicology. In: Balls, M., Bridges, J., Southee, J. (Eds.), Animals and Alternatives in Toxicology. Macmillan, Basingstoke, UK, pp. 223–252.

Waring, P., Beaver, J., 1996. Gliotoxin and related epipolythiodioxopiperazines. Gen. Pharmac. 27, 1311–1316.

Wilson, B.J., 1971. Miscellaneous *Aspergillus* toxins. In: Ciegler, A., Kadis, S., Aje, S.J. (Eds.), Microbial Toxins: A Comprehensive Treatise, vol. 6. Academic Press, New York, NY, USA, pp. 288–289.