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Elsinochrome A production by the bindweed biocontrol fungus *Stagonospora convolvuli* LA39 does not pose a risk to the environment or the consumer of treated crops

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Abstract

Biological control as an alternative to chemical pesticides is of increasing public interest. However, to ensure safe use of biocontrol methods, strategies to assess the possible risks need to be developed. The production of toxic metabolites is an aspect which has so far largely been neglected in the risk assessment and the registration process for biocontrol products. We have evaluated the risks of elsinochrome A (ELA) and leptosphaerodione production by the fungus *Stagonospora convolvuli* LA39, an effective biocontrol agent used against bindweeds. The toxicity of the two metabolites to bacteria, protozoa, fungi and plants was evaluated in *in vitro* assays. The most sensitive bacteria and fungi were already affected at 0.01–0.07 μM ELA, whereas plants were far less sensitive. Leptosphaerodione was less toxic than ELA. Subsequently, it was investigated whether ELA is present in the applied biocontrol product or LA39-treated bindweed and crop plants. In plants ELA was never detected and in the biocontrol product the ELA concentration was far too low to have toxic effects even on the most sensitive organisms. We conclude that the production of ELA by biocontrol strain LA39 does not pose a risk to the environment or to the consumer.

Introduction

Public concern regarding environmental problems associated with the use of chemical pesticides is growing. Therefore, the development of alternatives such as biological control is of increasing interest. Many fungal plant diseases and also weeds can be controlled by the use of fungal and bacterial biocontrol agents (BCAs). However, to ensure the safe use of biocontrol methods, strategies to assess the risks of BCAs need to be developed. So far there has been considerable uncertainty about the kind of risk assessment studies that would be necessary for the registration of a BCA, and there are as yet no well-defined regulations (Strasser, 2003; Skrobek & Butt, 2005). The Council Directive 91/414/EEC of the European Union (http://europa.eu.int/eur-lex/en/consleg/main/1991/en_1991L0414_index.html), for example, raises concern about environmental safety and the potential of BCA metabolites to enter the food chain. The authors of the SANCO document 10754/2005 (revision 5, 5 April 2005) even claim that the production and toxicity of metabolites has to be assessed at strain level if it is known that different strains from the same species produce distinct metabolites with toxic potential. Several researchers have discussed the need to examine product toxicity as well as the fate of toxic metabolites produced after fungal BCA application (Strasser *et al.*, 2000b; Strasser, 2003; Skrobek & Butt, 2005). Very few methods, strategies or regulations for the risk assessment of metabolites exist, which has prompted several studies focused on secondary metabolites produced by BCAs. Other authors have monitored the distribution of secondary metabolites produced by *Beauveria brongniartii*, a fungal BCA to control *Melolontha melolontha* (Strasser *et al.*, 2000b; Seger *et al.*, 2005). Destruxines, secondary metabolites of the entomopathogenic fungus *Metarhizium anisopliae*, and its crude extracts were tested for toxicity by Skrobek & Butt (2005). A further study focused on the evaluation of different biological test systems to assess the toxicity of metabolites from fungal BCAs (Skrobek *et al.*, 2006).

In this study a general strategy is presented to assess the risks of toxic metabolites produced by fungal BCAs (Fig. 1). We have applied this strategy using the ascomycete *Stagonospora convolvuli* strain LA39 as a model biocontrol strain. LA39 effectively controls the two notorious weeds *Convolvulus arvensis* ([L.] R. Br., field bindweed) and *Calystegia*

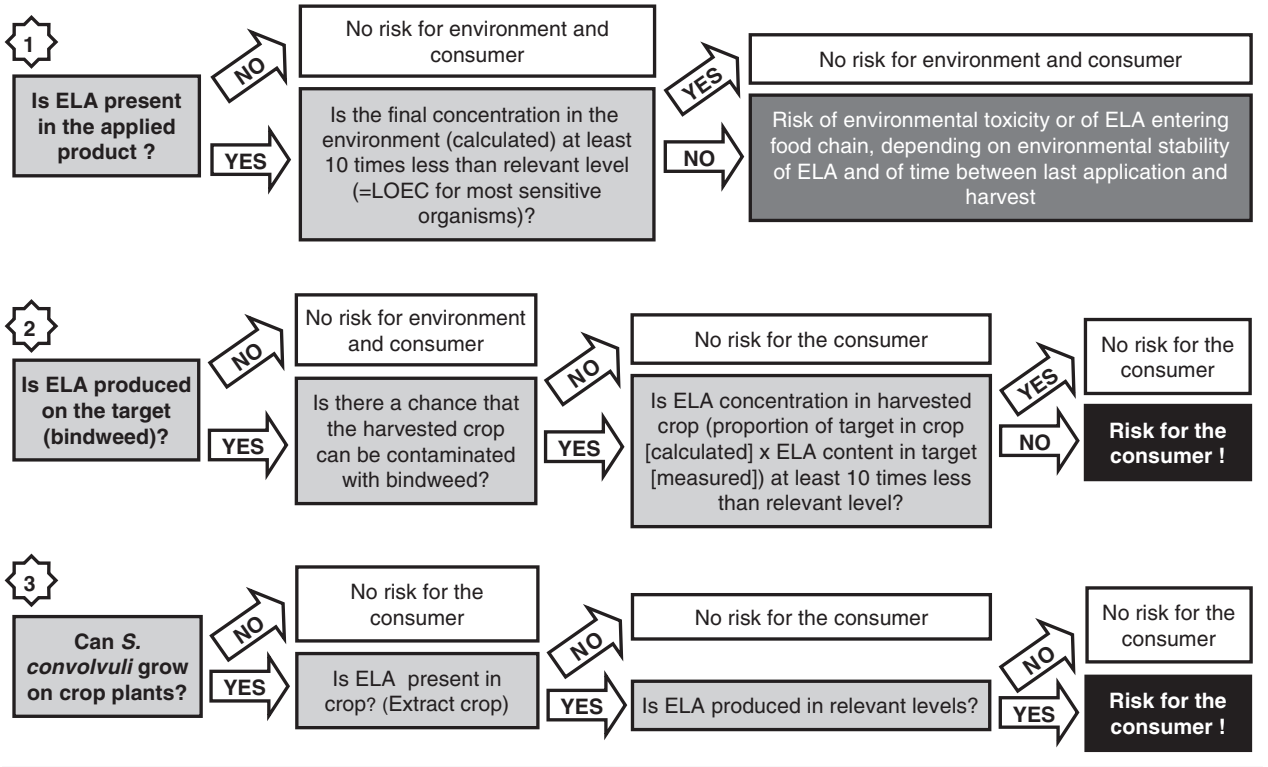

Fig. 1. Decision scheme to evaluate if elsinochrome A produced by biocontrol strain *Stagonospora convolvuli* LA39 poses a risk to the environment or the consumer.

sepium (L., hedge bindweed), which belong to the economically most important weeds worldwide. In recent years, screening for suitable organisms to control bindweeds revealed that *S. convolvuli* is the most promising species (Pfirter *et al.*, 1997, 1999a; Défago *et al.*, 2001). Both bindweeds can effectively be controlled by application of a mycoherbicide containing conidia of *S. convolvuli* LA39 (Guntli *et al.*, 1998; Pfirter & Défago, 1998; Défago *et al.*, 2001). Evaluating different *Stagonospora* isolates for biocontrol capacity showed that all highly aggressive isolates produce the secondary metabolites elsinochrome A (ELA) and most of them also leptosphaerodione (Ahonsi *et al.*, 2005). *Stagonospora convolvuli* LA39 is able to produce both of these (Nicolet, 1999; Nicolet & Tabacchi, 1999). ELA is a perylenequinone, which upon activation by light reacts with molecular oxygen to form reactive oxygen species, notably singlet oxygen and superoxide (Daub & Hangarter, 1983; Ma *et al.*, 2003) and is thus assumed to be of broad spectrum toxicity. Relatively little information is available regarding the mode of action and toxicity of leptosphaerodione. It was found to be phytotoxic to field bindweed and tomato plants (Nicolet, 1999).

The present study evaluates the risks of ELA and leptosphaerodione production by the BCA *S. convolvuli* LA39, which forms the active ingredient of the evaluated biocontrol product. The toxicity of ELA and leptosphaerodione was investigated, and their effects on plants, bacterial proliferation, fungal growth and on the mortality of the protozoan *Tetrahymena pyriformis* were tested. The next step was to investigate whether ELA is present in the applied conidia formulation or is produced on the target (bindweed) or on crop plants. With the data obtained, worst case calculations of possible environmental concentrations were made. The possible risks of ELA contaminating the environment or entering the food chain were assessed.

Material and methods

Bacterial and fungal strains

All bacterial and fungal strains used in this study are detailed in Table 1.

Production of LA39 conidia and bindweed inoculation

Stagonospora convolvuli LA39 was grown on V8 agar plates [100 mL V8-juice (N.V. Campbell Foods Belgium SA), 3 g $CaCO_3$, 12 g agar (Oxoid), 900 mL double-distilled water]. For the induction of asexual spores (conidia) formation plates were incubated under continuous white light

Table 1. Bacterial and fungal strains used for toxicity tests

Organisms	Origin
Bacterial strains	
Pseudomonas aeruginosa PAO1	ATCC 15692
P. fluorescens CHA0	Stutz *et al.* (1986)
P. fluorescens KD	Rezzonico *et al.* (2004)
Escherichia coli K12	ATCC 35695
P. syringae	ATCC 19310
Erwinia carotovora sp.	Switzerland, isolated from carrots
Fungal strains	
Pythium ultimum strain 67-1	Obtained from Allelix Agriculture, Mississauga, Canada
Fusarium graminearum strain GZ3639	Proctor *et al.* (1995)
Trichoderma atroviride strain P1	Kullnig *et al.* (2001)
Stagonospora convolvuli strain LA39	Pfirter & Défago (1998)
Rhizoctonia solani strain 160	Obtained from Ciba Geigy, Basel, Switzerland
Botrytis cinerea strain 25L	Obtained from Maag, Dielsdorf, Switzerland
F. oxysporum f. sp. *radicis lycopersici* strain 22	Obtained from C. Alabouvette, INRA, Dijon, France

(29 μmol photons s^{-1} m^{-2}). Couscous has emerged to be a suitable medium for the mass production of conidia (Pfirter *et al.*, 1999b). For couscous inoculation conidia were harvested by floating the agar plates with autoclaved double-distilled water. The fungus was grown on 300 g of twice autoclaved couscous amended with 250 mL of autoclaved double-distilled water inoculated with LA39 conidia at a concentration of 10^4 mL^{-1}. The culture was grown under continuous white light (29 μmol photons s^{-1} m^{-2}) at 20 °C to induce sporulation. Three-week-old cultures were mixed with 600 mL of double-distilled water using a kitchen mixer. The suspension was filtered through four layers of cheesecloth and the spore suspension was adjusted to the desired spore concentration using a haemocytometer. The applied LA39 product (hereafter referred to as LA39 conidia formulation) consists of 5×10^6 spores mL^{-1} amended with 0.3% (v/v) of the surfactant Genol Plant (Syngenta Agro AG). For greenhouse experiments as well as in the field the mycoherbicide was applied using a 1-L hand sprayer (Birchmeier Super Star 1.25). In the greenhouse plants were sprayed to run-off and subsequently placed in a plastic tent with 100% relative humidity for 24 h. Plants were kept at 70% relative humidity with alternating 16 h of white light (90 μmol photons s^{-1} m^{-2}) at 22 °C and 8 h of darkness at 17 °C. In the field plants were sprayed with LA39 conidia formulation at 40 mL m^{-2} (= 400 L ha^{-1}).

Preparation of compounds, LA39 conidia formulation and LA39 conidia extracts for toxicity tests

High-purity LA39 metabolites (> 97% purity) were kindly provided by R. Tabacchi, University of Neuchâtel, Switzerland: ELA (MW 544 g mol^{-1}) and leptosphaerodione (MW 354 g mol^{-1}) were purified from liquid cultures of *S. convolvuli* LA39. For toxicity tests, ELA and leptosphaerodione were dissolved in acetonitrile/tetrahydrofuran (19 : 1, v/v) or in neat acetonitrile for tests with bacteria and the protozoan *T. pyriformis*.

LA39 conidia formulation: for toxicity tests LA39 conidia were tested at a concentration of 5×10^6 conidia mL^{-1} containing 0.3% Genol Plant as used for field application.

LA39 conidia extracts: LA39 spores were produced and harvested as described above for bindweed inoculation. For preparation of LA39 conidia extracts the spore-containing filtrate obtained from 300 g of couscous was extracted as follows. The filtrate was extracted twice with 600 mL of ethyl acetate. The organic phases were filtered twice through silicon-coated filter papers (Macherey & Nagel), pooled and evaporated to complete dryness. For toxicity tests extracts were dissolved in neat acetonitrile.

In vitro toxicity tests

Several bacterial and fungal strains (Table 1), the protozoan *T. pyriformis*, plant seeds of different plant species and the arthropodan *Artemia salina* and *Daphnia magna* were exposed to different concentrations of ELA and some of them also to leptosphaerodione, LA39 conidia formulation and LA39 conidia extracts. Tests with ELA and conidia extracts were performed in duplicates, one under continuous white light (29 μmol photons s^{-1} m^{-2}) and one in the dark. Toxicity tests with leptosphaerodione were performed in the dark. The solvent content in the medium was 1% except in tests with *Pseudomonas syringae* (0.5%) and plant seedlings (2%). Control treatments without test compounds also contained the same amount of solvent.

Bacteria

Bacterial strains (Table 1) were grown for 5 h in Luria–Bertani broth [LB; 10 g Bacto tryptone (Becton Dickinson), 5 g

Bacto yeast extract (Difco), 0.25 g $MgSO_4 \cdot 7H_2O$, 8 g NaCl, 1000 mL double-distilled water]. Bacterial cultures were then adjusted with 0.9% NaCl solution to an optical density at 600 nm ($OD_{600\,nm}$) of 0.0025 for *Pseudomonas aeruginosa* PAO1 or 0.01 for the other bacterial strains tested. Wells of a 96-well microtitre plate (Greiner) were filled with 190 μL LB medium and amended with different concentrations of ELA and leptosphaerodione. Ten microlitres of the bacterial suspensions were added to each well. Plates were incubated on a rotary shaker at 250 r.p.m. at 27 °C. Bacterial growth was evaluated by measuring the $OD_{600\,nm}$ using a Dynatech ELISA reader model MR5000.

Tetrahymena pyriformis

The BACTOX test developed by Schlimme *et al.* (1999) was modified as described previously (Skrobek *et al.*, 2006) to test the toxicity of ELA, leptosphaerodione, LA39 conidia formulation and LA39 conidia extracts to protozoa.

Fungi

Fungal strains (Table 1) were grown for 4–7 days on malt agar [12 g agar, 15 g malt extract (Oxoid), 1000 mL double-distilled water, pH = 7]. For toxicity tests, plugs (diameter: 5 mm) of fungal cultures were placed in small Petri dishes (diameter: 6 cm) containing 3 mL of malt agar supplemented with different amounts of ELA or leptosphaerodione. Experiments were performed in a growth chamber at 20 °C and evaluated by measuring the radial mycelial growth.

Plant seedlings

Four plant species, wheat (*Triticum aestivum* cv. Arina), tomato (*Lycopersicon esculentum* cv. Supermarmande), garden cress (*Lepidium sativum*) and field bindweed (*Co. arvensis*, purchased from MT Valley Seed Service) were tested for their sensibility to ELA. Tomato and wheat seeds were surface sterilized in 1% sodium hypochlorite for 30 min and subsequently rinsed thoroughly with autoclaved water. Wheat seeds were soaked in distilled water for 4 h then incubated in warm water (50 °C) for 10 min before surface sterilization. Field bindweed seeds were first treated with concentrated sulphuric acid for 30 min and were then rinsed thoroughly with distilled water. They were then surface sterilized initially in 5% sodium hypochlorite for 5 min then in 10% hydrogen peroxide for 5 min and were subsequently rinsed with autoclaved water. The seeds were placed on water agar plates (10 g agar, 1000 mL double-distilled water) containing different amounts of ELA and incubated in a growth chamber at 24 °C. The germination rate and the length of the roots were evaluated.

Artemia salina and *Daphnia magna*

Artemia salina and *D. magna* are planktonic branchiopod crustaceans. Test kits were purchased from Vickers Laboratory (Artoxkit MTM, Daphtoxkit FTM Magna). Standard seawater and freshwater were prepared with the ingredients in the kit according to the respective recipes. *Artemia salina* cysts and Ephippia of *D. magna* were hatched at 25 °C for 30 h and at 20–22 °C for 72 h, respectively, under continuous illumination. Wells of the test plates were filled with 1 mL seawater (*A. salina*) or 10 mL of of freshwater (*D. magna*). LA39 conidia formulation or solutions of ELA or LA39 conidia extracts were added. Ten larvae of *A. salina* or five neonates of *D. magna* were added per well and the plates were incubated at 25 °C (*A. salina*) or 20 °C (*D. magna*) in the dark. *Artemia salina* and *D. magna* tests with ELA or LA39 conidia extracts and respective controls were subjected to light activation for 15 min (249 μmol photons s^{-1} m^{-2}) prior to incubation. After 24 h the mortality rate of the crustaceans was evaluated.

Toxicity to plant leaves

Field bindweed (*Co. arvensis*, USA ecotype), hedge bindweed (*Ca. sepium*, Swiss ecotype collected around Zurich) and grape-vines (*Vitis vinifera* cv. Chasselas) were grown in plastic pots containing potting soil (De Baat, the growing power; GVZ Bolter AG) in the greenhouse at 70% relative humidity with alternating 16 h of white light (90 μmol photons s^{-1} m^{-2}) at 22 °C and 8 h of darkness at 17 °C. Plants were watered twice a week and on one application fertilizer (2 mL L^{-1} Wuxal, Aglukon Spezialdünger GmbH) was added.

The leaves of 6-week-old *V. vinifera*, 2.5-week-old *Ca. sepium* or 3-week-old *Co. arvensis* plants were dusted with carborundum 400. Two 20-μL drops of ELA or solutions of LA39 conidia extracts (solvent content 5%) were rubbed onto the leaves using ear buds. One leaf per plant was treated with ELA or LA39 conidia extracts and one with 5% solvent as a control. Plants were incubated in the greenhouse under the same conditions as described above. After 1 week leaves were assessed for chlorosis and necrosis on a scale from 0 to 6 (where 0 = no effect, 1 = up to 5% of leaf area necrotic or chlorotic, 2 = 5–25% of leaf area necrotic or chlorotic, 3 = 25–75% of leaf area necrotic or chlorotic, 4 = 75–95% of leaf area necrotic or chlorotic, 5 = less than 5% green leaf area, 6 = leaf dead).

Detection of ELA in LA39 conidia formulation

LA39 was cultivated on couscous and conidia were harvested and extracted as described above. The dried extracts were dissolved in 1 mL acetonitrile/tetrahydrofuran (4 : 1, v/v), centrifuged (Biofuge pico, Kendro Laboratory Products)

at 16 060 **g** for 2 min to remove insoluble compounds and the supernatants were immediately analysed by high-performance liquid chromatography (HPLC) as described below. The experiment was performed 11 times over a period of 6 months.

To determine the detection limit, samples of 100 g couscous (amount used to produce 10^{11} conidia) suspended in 300 mL water were spiked with ELA in concentrations ranging from 0.01 to 10 $\mu g\,g^{-1}$ couscous. The samples were filtered, extracted and extracts prepared for HPLC analysis as described above. Three replicates per concentration were made.

Detection of ELA in infected bindweed (target)

Greenhouse trial

Plants were grown as described above. Three-week-old field or hedge bindweed plants (24 plants per treatment) were sprayed with LA39 conidia formulation containing the desired spore concentration as used for field application (5×10^6 conidia mL^{-1}) or a 10-fold higher concentration. After 1 week, when first symptoms were visible, and after 2 weeks, when plants were already heavily diseased, 150 leaves were sampled and extracted as described below. The experiment consisted of three repetitions (extractions over time) per bindweed species, spore concentration and sampling time.

Field trial

Field plots of 4×4 m with natural field or hedge bindweed infestations were sprayed four times at 2-week-intervals with LA39 conidia formulation of the desired (5×10^6 conidia mL^{-1}) or at 10-fold higher spore concentration. At three different time points between July and September (first sampling 2 weeks after the first application, last sampling 2 weeks after the last application) samples of 150 leaves were collected and extracted as described below. The experiment consisted of three repetitions (leaf samples) per bindweed species and conidia concentration.

Extraction of leaves

Samples of 150 bindweed leaves were grounded with liquid nitrogen in a mortar. The resulting powder was immediately suspended in 200 mL ice-cold water and stirred for 30 s using a magnetic stirrer. The suspension was then extracted twice with 400 mL of ethyl acetate. The organic phases were filtered and evaporated as described above. The dried extracts were dissolved and centrifuged as described for LA39 conidia extracts and analysed by HPLC as described below.

For determination of the detection limit 4-g leaf samples of were spiked with ELA in amounts ranging from 0.01 to 10 $\mu g\,g^{-1}$. The samples were extracted as described above except that the leaf powder was suspended in 100 mL ice-cold water and was extracted twice by adding 150 mL ethyl acetate. Three replicates per concentration were made.

Detection of ELA in strawberries (crop)

Strawberry plantlets (cv. Elsanta) kindly provided by Peter Knup AG, were grown in the greenhouse under the same conditions as described for bindweed cultivation until the fruits were completely ripe. The plants were supplied with fertilizer grains (Osmocote, Hauert HGB-Dünger AG) on the day of planting and did not receive further fertilizer. Strawberry plants were sprayed three times with the desired (5×10^6 conidia mL^{-1}) or 10-fold higher LA39 spore concentration at different development stages of the plants, starting from flowering until after colour change of the fruits. Control treatments were sprayed with water. One, 3 and 7 days after the last mycoherbicide application strawberry samples of 43 g were collected and extracted as described below. The experiment consisted of three trays per treatment with eight strawberry plants per tray. Three samples per treatment (one per tray) and time point were collected.

Extraction of strawberries

Strawberry fruit samples of 43 g were mixed with 100 mL ice-cold double-distilled water using a kitchen blender. The resulting suspension was extracted twice with 250 mL ethyl acetate. The organic phases were filtered and evaporated as described above. The dried extracts were dissolved and centrifuged as described for LA39 conidia extracts and analysed by HPLC as described below.

For determination of the detection limit strawberry samples of 43 g were spiked with ELA in amounts ranging from 0.01 to 10 $\mu g\,g^{-1}$. The samples were extracted as described above and the extracts analysed as described below. Three replicates per concentration were made.

HPLC analysis

Extracts of strawberries, bindweeds and formulated LA39 conidia were all analysed using the same method. Twenty-five microlitres of extracts, dissolved in 1 mL acetonitrile/tetrahydrofuran (4 : 1, v/v), were analysed in a Hewlett Packard 1090 liquid chromatograph equipped with a UV-diode-array detector, using a precolumn (4×8 mm) followed by the main column (4×250 mm) both packed with Nucleosil 120-5-C18 (Macherey and Nagel). The temperature of the column was 28 °C. The samples were eluted with a linear gradient of acetonitrile from 80 to 100%

(0–20 min) in 0.043% o-phosphoric acid at a flow rate of 1 mL min^{-1}. ELA was detected by absorption at 230 nm. The retention time of ELA was 5.0 min.

The detection limits for ELA were 0.9 μg g^{-1} on field bindweed leaves, 1.8 μg g^{-1} on hedge bindweed leaves, 0.7 μg g^{-1} on strawberries and 0.3 μg per 10^9 spores, which corresponds to 1.5 ng mL^{-1} in the applied conidia formulation.

Statistical analysis

For statistical analysis the statistic program Systat, version 10.0 (Systat Inc.), was used. Results of independent experiments were first analysed by two-way ANOVA. If this analysis did not reveal significant trial by treatment interactions data of individual experiments were pooled for further statistical analysis. Otherwise the individual experiments were analysed separately. Each experiment was analysed first by one-way ANOVA followed by Fisher's protected least significant difference (LSD) test ($P < 0.05$). Data derived from *T. pyriformis* and plant leaf tests were arcsine transformed [ASIN(SQRT)] prior to statistical analysis.

EC_{50} values (the effective concentration to inhibit bacterial, fungal growth or plant root elongation by 50%), LC_{50} values (the effective concentration to kill 50% of the *T. pyriformis*) and their 95 % confidence intervals were calculated by PriProbit 1.63 (1996–2000 Masayuki Sakuma). EC_{100} and LC_{100} values were defined as the lowest concentration to inhibit growth by 100% or to kill 100% of the test organisms. Lowest observed effect concentrations (LOECs) were defined as the lowest tested concentrations for which a statistically significant difference to the control was observed (Fisher's protected LSD test, $P < 0.05$).

Results

In vitro toxicity tests

When experiments were performed in the dark none of the tested organisms was affected by ELA up to the maximal concentration tested (MCT).

Bacteria

ELA affected the growth of all tested bacteria (Table 2). However, the sensitivity of the different bacterial strains varied widely. The most sensitive bacterium was *P. syringae*. For this bacterium ELA was toxic from 0.05 μM on. The average EC_{50} was 0.08 μM (44 ng mL^{-1}). For *Pseudomonas fluorescens* CHA0, *P. fluorescens* KD, *Erwinia carotovora* and *Pseudomonas aeruginosa* first growth inhibitory effects were observed at concentrations ranging from 1 to 25 μM and average EC_{50} values of 1.5–25 μM were calculated. *Escherichia coli* was the least sensitive and the average EC_{50} for this bacterium was 49 μM. Five out of six bacterial species tested were far more sensitive to ELA than to leptosphaerodione (Table 2). The only exception was *E. coli*, which was 2.5–5 times more sensitive to leptosphaerodione than to ELA. For all other bacteria the EC_{100} obtained for ELA was equal or even lower than the LOEC obtained for leptosphaerodione. For *P. fluorescens* CHA0, *P. fluorescens* KD, *P. syringae* and *P. aeruginosa* no toxic effects were observed up to the MCT of 25 μM leptosphaerodione.

Tetrahymena pyriformis

Tetrahymena pyriformis cells were slightly more sensitive to ELA than to leptosphaerodione (Table 3). Cells were significantly affected by ELA at 0.3 μM and by leptosphaerodione at 0.3–1 μM with LC_{50} values of 0.65 μM for ELA and 1.2–1.6 μM for leptosphaerodione. All cells were killed at 3 μM ELA and 3–10 μM leptosphaerodione.

Fungi

Apart from *S. convolvuli* LA39 all fungi tested were extremely sensitive to ELA with LOECs ranging from 0.01 to 0.3 μM (Table 4). Fungi were 30–300 times less sensitive to leptosphaerodione than to ELA. Initial toxic effects were observed at 3–10 μM leptosphaerodione.

The most sensitive fungus was *Trichoderma atroviride*, which was already affected at 0.01 μM (5 ng mL^{-1}) ELA and 3 μM leptosphaerodione. The EC_{50} of ELA was as low as 0.1 μM. Strain LA39 did not show any growth inhibition caused by ELA and leptosphaerodione up to the MCT of 30 μM.

Plant seedlings

ELA had no effect on seed germination (data not shown) but affected the root elongation of all four plant species tested (Table 5). The most sensitive plant species was garden cress (*Le. sativum*), in which root elongation was already significantly inhibited at 0.3 μM ELA. The EC_{50} values for garden cress ranged from 2.2 to 5.2 μM. On field bindweed (*Co. arvensis*) and wheat (*Triticum aestivum*) seedlings first toxic effects could be observed at 3 μM. However, field bindweed was more sensitive than wheat at higher ELA concentrations. The EC_{50} values for field bindweed were 6.0–9.3 μM, whereas the root elongation of wheat seedlings was only inhibited by 31% at the MCT of 30 μM. Initial effects on tomato (*Ly. esculentum*) seedlings were observed at 3–30 μM ELA and at the MCT of 30 μM root elongation was inhibited by 19–39% (Table 5).

Table 2. Effect of *Stagonospora convolvuli* LA39 metabolites on bacterial growth

			Elsinochrome A				Leptosphaerodione		
Bacterial strain*	Incubation time (h)	Exp.	LOEC (μM)†	EC50 (μM)‡	EC100 (μM)§	Inhibition at MCT (100 μM) (%)¶	LOEC (μM)	EC50 (μM)	Inhibition at MCT (25 μM) (%)
P. fluorescens CHA0	24	1	10	13.8 (9.7; 20.7)**	25	100	No toxic effects observed		
		2	5	6.6 (nr)	10	100			
		3	5	6.3 (4.1; 9.6)	25	100			
P. fluorescens KD	24	1	2.5	6.9 (4.5; 10.5)	25	100	No toxic effects observed		
		2	2.5	5.0 (2.9; 8.3)	10	100			
		3	2.5	1.5 (1.5; 1.5)	2.5	100			
E. coli K12	24	1	50	50.3 (27.2; 115.2)	> MCT	89.6 ± 9.8			
		2	25	40.9 (35.2; 47.8)	> MCT	76.5 ± 6.5	10	> MCT	16.9 ± 8.7
		3	25	54.7 (23.8; 870.0)	> MCT	61.5 ± 17.1			
P. aeruginosa PAO1	18	1	25	30.7 (nr)	50	100			
		2	25	24.7 (15.8; 37.1)	50	100	No toxic effects observed		
			10	19.8 (14.3; 27.0)	50	100			
E. carotovora sp.	42	1	2.5	3.1 (nr)	5	100	5	> MCT	18.8 ± 3.9
		2	1	6.8 e^{-1} (6.8 e^{-1}; 6.8 e^{-1})	1	100	5	> MCT	7.2 ± 5.7
		3	2.5	7.0 e^{-1} (nr)	2.5	100	5	> MCT	12.4 ± 2.3
P. syringae 19310	18	1	0.05	5.8×10^{-2} (5.8×10^{-2}; 5.8×10^{-2})	0.1	100			
		2	0.1	7.9×10^{-2} (7.9×10^{-2}; 7.9×10^{-2})	0.25	100	No toxic effects observed		
		3	0.05	9.5×10^{-2} (8.5×10^{-2}; 1.1×10^{-1})	0.25	100			

*Bacterial growth was assessed by measuring the OD at 600 nm after cultivation in LB liquid medium amended with different amounts of ELA and leptosphaerodione at 27 °C for 16–40 h.

†LOEC, lowest concentration at which significant growth inhibition effects were observed (Fisher's protected LSD test, $P < 0.05$, Systat).

‡EC_{50}, concentration to inhibit bacterial proliferation by 50%; calculated using PriProbit 1.63 ($P < 0.05$). 95% confidence intervals are given in brackets.

§EC_{100} (observed), concentration at which 100% growth inhibition had occurred.

¶MCT, maximal concentration tested, 100 μM for ELA and 25 μM for leptosphaerodione.

**If individual experiments could not be pooled (two-way ANOVA, Systat, version 10.0, Systat Inc.) the three experiments were analysed separately (Fisher's protected LSD test, $P < 0.05$, Systat, version 10.0) and means of six (ELA) or four (leptosphaerodione) replicates are presented. Otherwise experiments were pooled and means of 18 (ELA) or 12 (leptosphaerodione) replicates are presented.

nr, confidence intervals not reliable.

Table 3. Effect of Stagonospora convolvuli LA39 metabolites on Tetrahymena pyriformis

Metabolite*	Exp.	LOEC (μM)†	LC_{50} (μM)‡	LC_{100} (μM)§
Elsinochrome A	1–3	0.3¶	6.5×10^{-1} (5.8×10^{-1}; 7.3×10^{-1})	3
Leptosphaerodione	1	0.3	1.3 (0.4; 4.7)	3
	2	1	1.6 (7.8; 3.3)	10
	3	1	1.2 (nr)	3

*Test substances were added to 4×10^4 *T. pyriformis* cells mL^{-1} suspended in 2 mL water. After 4 h of incubation the percentage of dead cells was evaluated.

†LOEC, concentration at which first dead cells were observed (Fisher's protected LSD test, $P < 0.05$, Systat).

‡LC_{50}, concentration at which 50% of the test organisms were killed; calculated using PriProbit 1.63 ($P < 0.05$). 95% confidence intervals are given in brackets.

§LC_{100} (observed), concentration at which 100% cell death had occurred.

¶Experiments with leptosphaerodione could not be pooled (two-way ANOVA, Systat, version 10.0, Systat Inc.). Therefore, the three experiments were analysed separately (Fisher's protected LSD test, $P < 0.05$, Systat, version 10.0). Means of three replicates are presented. Experiments with ELA could be pooled and were analysed by Fisher's protected LSD test (Systat, version 10.0). Means of nine replicates are presented.

nr, confidence interval not reliable.

Table 4. Effect of elsinochrome A on the mycelial growth of different fungi

		Elsinochrome A					Leptosphaerodione			
Fungus*	Incubation time (days)	Exp.	LOEC (μM)†	EC_{50} (μM)‡	EC_{100} (μM)§	Inhibition at MCT (30 μM) (%)¶	Exp.	LOEC (μM)	EC_{50} (μM)	Inhibition at MCT (30 μM) (%)
B. cinerea 25L	4	1–3	0.03**	3.1 (1.5; 8.2)	> MCT	68.5 ± 7.2			Not determined	
P. ultimum 67-1	1	1	0.1	17.5 (10.8; 32.5)	> MCT	47.4 ± 7.0				
		2	0.1	3.4 (1.8; 7.1]	30	100.0 ± 0.0	1–3	10	> MCT	12.2 ± 4.4
		3	0.3	22.6 (9.0; 113.3)	> MCT	51.0 ± 8.2				
R. solani 160	3	1	0.1	15.4 (6.4; 63.8)	> MCT	47.2 ± 1.9				
		2	0.1	3.2 (2.3; 4.4)	> MCT	76.3 ± 1.5			Not determined	
		3	0.1	5.0 [3.4; 7.9)	> MCT	62.2 ± 3.6				
F. oxysporum	7	1	0.1	1.6 (1.3; 2.1)	> MCT	82.7 ± 1.8				
f. sp. *radicis*		2	0.1	1.9 (1.5; 2.5)	> MCT	84.0 ± 1.4			Not determined	
lycopersici 22		3	0.1	2.8 (2.2; 3.7)	> MCT	78.9 ± 1.4				
F. graminearum	7	1	0.1	30.3 (8.6; 375.6)	> MCT	40.2 ± 2.0				
GZ 3639		2	0.03	9.9 (5.8; 19.6)	> MCT	57.5 ± 1.5	1–3	3	> MCT	8.0 ± 0.5
		3	0.03	24.6 (13.6; 54.3)	> MCT	50.9 ± 1.2				
Trichoderma	2	1	0.01	9.8×10^{-2}	10	100.0 ± 0.0				
atroviride P1				(7.8×10^{-2}; 1.2×10^{-1})						
		2	0.01	8.7×10^{-2}	> MCT	99.3 ± 1.5	1–3	3	> MCT	7.5 ± 1.1
				(6.8×10^{-2}; 1.1×10^{-1})						
		3	0.01	1.0×10^{-1}	> MCT	97.2 ± 2.3				
				(7.9×10^{-2}; 1.3×10^{-1})						
S. convolvuli LA39	7	1–3		No toxic effects observed				1–3	No toxic effects observed	

*Fungi were grown on malt agar plates containing different amounts of purified ELA or leptosphaerodione at 20 °C. After 1–7 days the diameter of the fungal mycelium was measured.

†LOEC, lowest concentration at which significant growth inhibition occurred (Fisher's protected LSD test, $P < 0.05$, Systat).

‡EC_{50}, concentration to inhibit growth by 50%; calculated using PriProbit 1.63 ($P < 0.05$). 95% confidence intervals are given in brackets.

§EC_{100} (observed), concentration at which growth is inhibited by 100%.

¶MCT, maximal concentration tested = 30 μM.

**If individual experiments could not be pooled (two-way ANOVA, Systat, version 10.0, Systat Inc.) the three experiments were analysed separately (Fisher's protected LSD test, $P < 0.05$, Systat) and means of four replicates are presented. Otherwise experiments were pooled, statistically analysed (Fisher's protected LSD test, $P < 0.05$, Systat, version 10.0) and means of 12 replicates are presented.

Toxicity to plant leaves

ELA rubbed onto leaves caused necrosis on all three plant species tested (Table 6). However, both bindweed species were more sensitive than grape-vine. On bindweed leaves ELA caused initial visible necrosis at a concentration of 30 μM, and on grape-vine at a concentration of 30–100 μM. At the MCT of 300 μM 50–73% of the bindweed leaf area was necrotic, compared with only 9–27% of the grape-vine leaf area. LA39 conidia extracts did not cause any necrosis on all three plant species up to the MCT of 300 μg mL^{-1} (data not shown).

Effect of LA39 conidia formulation and its extracts

The toxicity of the LA39 conidia formulation as well as of conidia extracts was tested on *T. pyriformis*, *A. salina*, *D. magna* and *P. syringae* (only extracts). Neither LA39 conidia extracts up to the MCTs of 300 μg mL^{-1} (*P. syringae* and *T. pyriformis*) or 100 μg mL^{-1} (*A. salina*, *D. magna*) nor LA39 conidia formulation showed any toxic effects (data not shown).

Detection of ELA in LA39 conidia formulation, bindweed leaves infected with LA39 and strawberries treated with LA39

In order to test whether ELA could be present in the LA39 conidia formulation, conidia were produced on couscous, harvested as for field application and extracts of the conidia were prepared. ELA could only be detected in two of 11 extracts. The amounts detected were 4.9 and 2.0 μg per 10^9 conidia. The final ELA concentration in the applied LA39 conidia formulation prepared with these conidia would be 25 ng mL^{-1} (0.05 μM) and 10 ng mL^{-1} (0.02 μM).

ELA was never detected in LA39-infected bindweed leaves, neither when leaves were sampled in the field nor when they were collected in the greenhouse. The detection

Table 5. Effect of elsinochrome A on root elongation of different plant seedlings

Plant species*	Exp.	LOEC† (μM)	EC_{50} (μM)‡	Inhibition of root elongation at MCT (%)§
Co. arvensis	1	3¶	6.4 (2.8; 21.3)	70.1 ± 6.7¶
	2	3	6.0 (3.0; 14.8)	70.6 ± 9.7
	3	3	9.3 (7.7; 11.4)	76.5 ± 2.3
L. esculentum	1	30	> MCT	19.4 ± 8.6
cv. Supermarmande	2	3	> MCT	38.8 ± 4.6
	3	10	> MCT	26.3 ± 3.2
L. sativum	1	0.3	3.2 (1.5; 7.2)	79.7 ± 2.1
	2	0.3	2.2 (1.1; 4.6)	77.9 ± 1.1
	3	0.3	5.2 (4.1; 7.0)	72.0 ± 1.6
Triticum aestivum cv. Arina	1–3	3	> MCT	31.2 ± 5.6

*Seeds were surface sterilized and germinated on water agar containing different amounts of ELA at 24 °C for 2–3 days.

†LOEC, minimal concentration at which statistical significant inhibition of root elongation could be observed (Fisher's protected LSD test, $P < 0.05$, Systat).

‡EC_{50}, concentration to inhibit root elongation by 50%; calculated using PriProbit 1.63 ($P < 0.05$). 95% confidence intervals are given in brackets.

§MCT, maximal concentration tested = 30 μM.

¶Mostly the data of the three experiments could not be pooled (two-way ANOVA, Systat, version 10.0, Systat Inc.). Therefore, individual experiments were analysed separately using Fisher's protected LSD test ($P < 0.05$, Systat). Means of three replicates (plates) with 20 (*Co. arvensis*) or 24 (*Ly. esculentum, Le. sativum*) seeds per plate are presented. In the case of *Triticum aestivum* experiments were pooled and the mean of nine replicates with 20 seeds per replicate is presented.

Table 6. Effect of elsinochrome A on the development of leaf necrosis on field bindweed, hedge bindweed and grape vine

		Necrotic or chlorotic leaf area (%)*				
Plant species	Exp.	0 μM	10 μM	30 μM	100 μM	300 μM
Co. arvensis	1	0.5 a†	1.3 a	8.8 b	38.3 c	63.8 d
	2	1.1 a	1.3 a	42.1 b	44.2 b	52.5 b
	3	1.0 a	3.8 ab	6.3 b	42.1 c	70.0 d
Ca. sepium	1	0.7 a	1.3 a	24.6 b	38.3 c	50.0 d
	2	0.8 a	3.8 a	38.3 b	38.3 b	50.0 b
	3	1.1 a	1.7 a	44.2 b	61.7 c	73.3 d
V. vinifera	1	0.1 a	0.4 a	0.8 a	2.1b	8.8 c
cv. Chasselat	2	0.3 a	1.7 ab	10.0 bc	12.5 c	26.7 d
	3	0.3 a	0.8 a	2.9 ab	4.2 b	20.8 c

*Leaves of 3-week-old *Co. arvensis*, 2.5-week-old *Ca. sepium* and 6-week-old *V. vinifera* were treated with elsinochrome A solutions of different concentration. After 7 days leaves were assessed for necrosis and chlorosis.

†The data of the three experiments could not be pooled (two-way ANOVA, Systat, version 10.0, Systat Inc.). Therefore, individual experiments were analysed separately. Means of six plants per concentration are presented. Values for the same plant species within the same row followed by the same letter are not significantly different at $P < 0.05$ according to Fisher's protected LSD test (Systat, version 10.0).

limit was 0.9 μg g^{-1} for field bindweed, and 1.8 μg g^{-1} for hedge bindweed. Even when bindweed plants were treated with the 10-fold higher concentration of the desired LA39 conidia concentration ELA could not be detected in the leaves at any development stage of the disease.

In order to investigate whether an agricultural product treated with LA39 could be contaminated with ELA strawberry plants were treated with LA39 conidia formulation at different stages of fruit development. The harvested fruits were extracted and assessed for ELA contamination. The detection limit of ELA in strawberries was 0.9 μg g^{-1}. In none of the strawberry samples could ELA be detected, even when they were treated with the 10-fold higher spore concentration. Moreover, LA39 did not grow on or infect strawberry stems, leaves or fruits.

Discussion

Stagonospora convolvuli LA39 produces metabolites with toxic properties

The effect of the two major *S. convolvuli* LA39 metabolites ELA and leptosphaerodione was investigated on a wide array of organisms, including bacteria, fungi, plants, protozoa and arthropods. ELA activated by light is known to produce reactive oxygen species (Daub & Hangarter, 1983; Ma *et al.*, 2003). For this reason the compound could be expected to be of universal toxicity. Indeed, our results showed that ELA is a highly toxic compound. The most sensitive organisms (*Trichoderma atroviride*, *Botrytis cinerea*, *Fusarium graminearum*, *P. syringae*) were already affected by concentrations of 0.01–0.07 μM ELA. All tested organisms were sensitive to ELA; however, considerable differences between the different groups of test organisms were observed. Generally fungi were the most sensitive organisms. Bacteria and protozoa were less sensitive and plants the least sensitive. In other studies it has been shown that ELA also affects insect cells, human leukaemic cells and human colorectal carcinoma cells. Initial toxic effects on these cells were observed at 0.1–1 μM ELA (Ma *et al.*, 2003; Skrobek *et al.*, 2006).

The involvement of ELA in plant infection processes is not known. Our results showed that ELA rubbed onto bindweed leaves causes necrosis. This suggests that ELA might play a role in the infection process and disease development by generating reactive oxygen species which destroy the membranes of host plants. Such a mechanism is suggested for cercosporin, a perylenquinone similar to ELA (Daub & Ehrenshaft, 2000), which is produced by *Cercospora* (Daub, 1982) and *Stagonospora* species (Ahonsi *et al.*, 2005). Regarding the broad spectrum toxicity of ELA, it is essential to investigate thoroughly whether this compound poses a risk to the environment or the consumer after treatment of crops with an ELA-producing BCA.

The second tested metabolite, leptosphaerodione, is clearly less toxic than ELA. For four of the six tested bacterial species no toxic effects could be observed. Moreover, for all tested fungi the LOEC of leptosphaerodione was at least 100 times higher than that of ELA. Taking into account first that ELA is of higher toxicity than leptosphaerodione and second that all *Stagonospora* isolates that are highly aggressive to bindweed produce ELA but not necessarily leptosphaerodione in liquid culture (Ahonsi *et al*., 2005), we mainly focused on ELA in further risk assessment studies.

It is possible that LA39 produces toxic metabolites not specifically detected and tested here. Therefore, crude conidia extracts were included in toxicity tests. In the case of LA39, crude extracts did not have any toxic properties so that the presence of any toxic metabolite in relevant levels in the LA39 conidia formulation can be excluded. In general we propose that for the risk assessment of a biocontrol product it would certainly be useful to test not only the product itself but also product extracts, which contain major metabolites. Testing the toxicity of crude extracts may also take into account interactions between different metabolites (Skrobek & Butt, 2005).

ELA content in the biocontrol product is far too low to pose a risk to the environment or the consumer of treated crops

There are two ways for ELA to enter the environment or the food chain: first by means of the LA39 conidia formulation if it contains ELA, and second via the possible production of ELA by the BCA after its release into the environment.

HPLC analysis of LA39 conidia extracts revealed that only in two of 11 samples was ELA detected. The maximal content was 25 ng mL^{-1} and can thus be assumed to be the worst case concentration in the applied product. Worst case scenarios for environmental contamination, for example for the contamination of the top soil, can now be roughly calculated. We assume that the LA39 conidia formulation applied at 400 L ha^{-1} three times per season is washed out by rain into the first 10 cm of the top soil. This results in an ELA concentration of 0.03 ng mL^{-1} soil, a concentration which is 167 times lower than the LOEC for the most sensitive organism (*Trichoderma atroviride*, 5 ng mL^{-1}). Therefore a risk for any soil organisms which could get in contact with ELA after application of LA39 can be excluded.

If the biocontrol product contains a toxic metabolite like ELA it also has to be evaluated whether the treated crop contains ELA residues in relevant amounts at harvest. Bindweeds can cause severe problems in the production of berries. Therefore we have calculated a worst case scenario for ELA residues in treated strawberries. If strawberry cultures were treated immediately before harvest with the normal dosage (400 L ha^{-1}) of LA39 conidia formulation containing the highest ever detected ELA concentration of 25 ng mL^{-1}, fruits would be covered by *c.* 1.6 μL conidia formulation g^{-1} fruits; thus the maximal content in the fruits would be about 0.04 ng g^{-1}. This concentration is 125 times lower than the LOEC for the most sensitive organism (*Trichoderma atroviride*, 5 ng mL^{-1}), and thus far too low to be of any risk for the consumer. When estimating the risk of residues in agricultural products it must also be taken into account that normally the last mycoherbicide treatment is applied several weeks before harvest and that ELA is rapidly degraded by sunlight, as was demonstrated by Ahonsi *et al*. (2006). They observed that ELA applied to an inert surface was degraded rapidly with a half-life of 14–99 h depending on the actual weather conditions. Our results (D. Boss, unpublished data) showed that the degradation of ELA when applied to plants is even 10–20 times faster than the degradation on inert surfaces. We therefore assume that *in planta* ELA is quickly degraded under natural conditions and will not accumulate.

ELA production after LA39 application does not pose any risk

As discussed above, the ELA content of an LA39-based mycoherbicide does not pose any risk either to the environment or to the consumer of treated crops. However, the possibility that ELA could be produced by the BCA in the environment/crop plants after application of the biocontrol product must still be taken into consideration. We can definitely exclude the possibility that ELA is produced on a crop directly. Pfirter & Défago (1998) showed that strain LA39 is highly specific and only infects plants belonging to the family *Convolvulaceae*. Furthermore, results obtained in this study revealed that strain LA39 is not able to grow even on ripe fruits of treated strawberry plants. We therefore conclude that the only possible location in the environment where LA39 can develop and possibly produce toxic metabolites such as ELA is on the target plant, namely the bindweed itself.

The methods used in this study allowed us to detect and quantify ELA in bindweed leaves down to concentrations of 0.9 μg g^{-1} (field bindweed) and 1.8 μg g^{-1} (hedge bindweed). However, ELA was not detected in any of the LA39-infected bindweed leaf samples, either when they were collected in the field or when they were collected in greenhouse experiments. If ELA was still produced *in planta* the concentrations would thus be below the detection limits. We thus assume a maximal possible ELA contamination of about 2 μg g^{-1} bindweed. For the calculation of worst case scenarios additional information is needed: the maximal possible bindweed content/contamination of a given crop. In the case of berries a bindweed contamination of the crop is unlikely owing to the harvesting techniques, but in the case of cereals

this could be possible. Assuming a maximal ELA content of $2\,\mu g\,g^{-1}$ in the bindweed and a maximal maize contamination with bindweed of $100\,\mu g\,g^{-1}$ maize, the resulting worst case ELA content in the silage would be $0.2\,ng\,g^{-1}$. The daily maize silage uptake of cattle is at most 5% of bodyweight. The maximal daily ELA dose for a cow would thus be as low as $0.01\,ng\,g^{-1}$ bodyweight. We therefore conclude that the risks of ELA being present in animal or human food are negligible.

Are all these studies necessary for the registration of a biocontrol product?

The results of this study allow us to estimate the risks of one specific toxic metabolite produced by one specific fungal BCA following the decision scheme shown in Fig. 1. Are such extensive studies on toxic metabolites really necessary for each commercially used biocontrol organism? Most microbial BCAs are probably able to produce metabolites with toxic properties, taking into consideration that a BCA has to survive and to compete against other microorganisms in the environment (Cook *et al.*, 1996), even if they live in small niches, such as on bindweeds. Furthermore for several fungal BCAs toxic metabolites have been described (Gillespie & Claydon, 1989; Hajek & St Leger, 1994; Vey *et al.*, 2001) and some of them are known to be important for the infection process and disease development. However, it is certainly neither realistic nor possible to conduct studies to the extent presented here for every toxic metabolite of every biocontrol strain. If regulations for registration demanded such risk studies for individual metabolites, this would exceed the time and costs for companies developing biocontrol products, and thus would certainly be a major obstacle for biocontrol in general (Strasser *et al.*, 2000a). The study presented here is a model by which the whole risk scenario has been calculated for a specific case. If several model studies such as this were available, estimating the risks for other fungal BCAs would become easier. A reasonable approach to solve the problem of extensive risk assessment of individual BCAs is proposed in the *EFSA Journal* Volume 226 (2005, pp. 1–12) where the authors suggest the establishment of a qualified presumption of safety (QPS) status that could be applied to selected groups of microorganisms. If it can be verified that a new BCA belongs to a taxonomic unit already granted QPS status the only requirement in relation to the safety assessment would be provision of product-specific safety data.

It seems unrealistic to look for absolute risk-free BCAs but the risks connected with their use should be far lower than the risks posed by chemical pesticides used for the same purpose. In this study the risks of using the fungus *S. convolvuli* LA39 as a BCA against bindweeds proved to be negligible.

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References

Ahonsi MO, Maurhofer M, Boss D & Défago G (2005) Relationship between aggressiveness of *Stagonospora* sp. isolates on field and hedge bindweeds, and *in vitro* production of fungal metabolites cercosporin, elsinochrome A and leptosphaerodione. *Eur J Plant Pathol* **111**: 203–215.

Ahonsi MO, Boss D, Maurhofer M & Défago G (2006) Potential environmental fate of elsinochrome A, a perylenequinone toxin produced in culture by bindweed biocontrol fungus *Stagonospora convolvuli* LA39. *The Environmentalist* **26**: 183–193.

Cook J, Bruckart WL, Coulson JR *et al.* (1996) Safety of microorganisms intended for pest and plant disease control: a framework for scientific evaluation. *Biol Control* **7**: 333–351.

Daub ME (1982) Cercosporin, a photosensitizing toxin from *Cercospora* species. *Phytopathology* **72**: 370–374.

Daub ME & Hangarter RP (1983) Light-induced production of singlet oxygen and superoxide by the fungal toxin, cercosporin. *Plant Physiol* **73**: 855–857.

Daub ME & Ehrenshaft M (2000) The photoactivated *Cercospora* toxin cercosporin: contributions to plant disease and fundamental biology. *Annu Rev Phytopathol* **38**: 461–490.

Défago G, Ammon HU, Cagan L *et al.* (2001) Towards the biocontrol of bindweeds with a mycoherbicide. *Biocontrol* **46**: 157–173.

Gillespie AT & Claydon N (1989) The use of entomogenous fungi for pest-control and the role of toxins in pathogenesis. *Pestic Sci* **27**: 203–215.

Guntli D, Pfirter HA, Moënne-Loccoz Y & Défago G (1998) *Stagonospora convolvuli* LA39 for biocontrol of field bindweed infesting cotoneaster in a cemetery. *Hortscience* **33**: 860–861.

Hajek AE & St Leger RJ (1994) Interactions between fungal pathogens and insect hosts. *Annu Rev Entomol* **39**: 293–322.

Kullnig CM, Krupica T, Woo SL, Mach RL, Rey M, Benitez T, Lorito M & Kubicek CP (2001) Confusion abounds over identities of *Trichoderma* biocontrol isolates. *Mycol Res* **105**: 770–772.

Ma L, Tai H, Li C, Zhang Y, Wang ZH & Ji WZ (2003) Photodynamic inhibitory effects of three perylenequinones on human colorectal carcinoma cell line and primate embryonic stem cell line. *World J Gastroenterol* **9**: 485–490.

Nicolet B (1999) Isolement et identification de métabolites secondaire de deux souches de champignon *Stagonospora* sp., agent pathogène du liseron des haies (*Calystegia sepium*) et du liseron des champs (*Convolvulus arvensis*). PhD thesis, Faculté de Sciences, University of Neuchâtel, Neuchâtel.

Nicolet B & Tabacchi R (1999) Secondary metabolites produced by *Stagonospora* sp., a potential biocontrol agent against bindweeds. *Modern Fungicides and Antifungal Compounds*, *Vol. 2* (Lyr H, Russell P & Sisler H, eds), pp. 469–476. Intercept Ltd, Andover.

Pfirter HA & Défago G (1998) The potential of *Stagonospora* sp. as a mycoherbicide for field bindweed. *Biocontrol Sci Technol* **8**: 93–101.

Pfirter HA, Ammon H-U, Guntli D, Greaves MP & Défago G (1997) Towards the management of field bindweed (*Convolvulus arvensis*) and hedge bindweed (*Calystegia sepium*) with fungal pathogens and cover crops. *Integr Pest Manage Rev* **2**: 61–69.

Pfirter HA, Marquis F & Défago G (1999a) Genetic and pathogenic characterization of different *Stagonospora* sp. isolated from bindweed. *Biocontrol Sci Technol* **9**: 555–566.

Pfirter HA, Guntli D, Ruess M & Défago G (1999b) Preservation, mass production and storage of *Stagonospora convolvuli*, a bioherbicide candidate for field bindweed (*Convolvulus arvensis*). *Biocontrol* **44**: 437–447.

Proctor RH, Hohn TM & McCormick SP (1995) Reduced virulence of *Gibberella-zeae* caused by disruption of a trichothecene toxin biosynthetic gene. *Mol Plant Microbe Interact* **8**: 593–601.

Rezzonico F, Défago G & Moënne-Loccoz Y (2004) Comparison of ATPase-encoding type III secretion system hrcN genes in biocontrol fluorescent pseudomonads and in phytopathogenic proteobacteria. *Appl Environ Microbiol* **70**: 5119–5131.

Schlimme W, Marchiani M, Hanselmann K & Jenni B (1999) BACTOX, a rapid bioassay that uses protozoa to assess the toxicity of bacteria. *Appl Environ Microbiol* **65**: 2754–2757.

Seger C, Erlebach D, Stuppner H, Griesser UJ & Strasser H (2005) Physicochemical properties of oosporein, the major secreted metabolite of the entomopathogenic fungus *Beauveria brongniartii*. *Helvetica Chimica Acta* **88**: 802–810.

Skrobek A & Butt TM (2005) Toxicity testing of destruxins and crude extracts from the insect-pathogenic fungus *Metarhizium anisopliae*. *FEMS Microbiol Lett* **251**: 23–28.

Skrobek A, Boss D, Défago G, Butt TM & Maurhofer M (2006) Evaluation of different biological test systems to assess the toxicity of metabolites from fungal biocontrol agents. *Toxicol Lett* **161**: 43–52.

Strasser H (2003) *Future Needs in Risk Assessment of Fungal Biological Control Agents, in Insect Pathogens and Insect Parasitic Nematodes*, *Vol. 26* (Papierok B, ed), pp. 5–8. IOBC/wprs Bulletin.

Strasser H, Vey A & Butt TM (2000a) Are there any risks in using entomopathogenic fungi for pest control, with particular reference to the bioactive metabolites of *Metarhizium*, *Tolypocladium* and *Beauveria* species? *Biocontrol Sci Technol* **10**: 717–735.

Strasser H, Abendstein D, Stuppner H & Butt TM (2000b) Monitoring the distribution of secondary metabolites produced by the entomogenous fungus *Beauveria brongniartii* with particular reference to oosporein. *Mycol Res* **104**: 1227–1233.

Stutz EW, Défago G & Kern H (1986) Naturally-occurring fluorescent pseudomonads involved in suppression of black root-rot of tobacco. *Phytopathology* **76**: 181–185.

Vey A, Hoagland R & Butt TM (2001) Toxic metabolites of fungal biocontrol agents. *Fungi as Biocontrol Agents: Progress, Problems and Potential* (Butt TM, Jackson C & Magan N, eds), pp. 311–346. CABI Publishing, Wallingford, UK.