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Relationship between aggressiveness of *Stagonospora* sp. isolates on field and hedge bindweeds, and *in vitro* production of fungal metabolites cercosporin, elsinochrome A and leptosphaerodione

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Abstract

Stagonospora convolvuli LA39, an effective biocontrol agent of *Convolvulus arvensis* (field bindweed) and *Calystegia sepium* (hedge bindweed) produces phytotoxic metabolites leptosphaerodione and elsinochrome A. *Stagonospora* isolate 214Caa produces the toxin cercosporin. If toxic metabolite production is not linked to the pathogenic ability of the fungus on bindweeds, selection of aggressive strains with limited or no production of the metabolites would reduce any perceived risk of using strains of the fungus as a mycoherbicide. Therefore, 30 isolates of *Stagonospora* sp. including LA39 and 214Caa were characterised for aggressiveness on both bindweeds, and production of the three metabolites. Nine isolates were more aggressive than LA39 on both bindweeds. Classification of isolates based on metabolite type agreed largely with previous similar characterisation based on polymerase chain reaction-restriction fragment length polymorphism of internal transcribed spacer of ribosomal DNA. Cercosporin producers produced neither leptosphaerodione nor elsinochrome A and together with isolates that produce none of the three metabolites, were less pathogenic on bindweeds. Conversely, there was a positive correlation between elsinochrome A and leptosphaerodione production, and each was positively correlated with aggressiveness of isolates on both bindweeds. Generally, any isolate where elsinochrome A was not detected was not aggressive on any of the two bindweeds. This probably implies that selecting elsinochrome A-negative, but aggressive *Stagonospora* strain(s) may be difficult. However, aggressive isolates may not produce elsinochrome A *in planta* at levels that could constitute any risk in the environment. In a preliminary attempt to determine the levels of elsinochrome A and leptosphaerodione produced in diseased bindweeds, none of the toxins was detected in *Stagonospora* infected bindweed leaves. Detailed investigation focusing on the detection and quantification of *in planta* production of elsinochrome A by *Stagonospora* isolates, and determination of the fate of elsinochrome A in the environment, and its relationship with leptosphaerodione may be essential. Similarly, development of molecular tools to monitor the mycoherbicide following field application is vital.

Introduction

Field bindweed (*Convolvulus arvensis*) and hedge bindweed (*Calystegia sepium*) are important weeds in agriculture and amenity areas in many parts of the world, particularly in Europe and western Asia, north Africa and north America (Davison, 1976; Holm et al., 1977; Schroeder et al., 1993; Boldt et al., 1998; Ammon and Müller-Schärer, 1999; Défago et al., 2001). They are deep-rooted perennial weeds with abundant food reserves in their rhizomes (hedge bindweed) and roots (field bindweed); hence, they are able to escape or survive many chemical and mechanical control

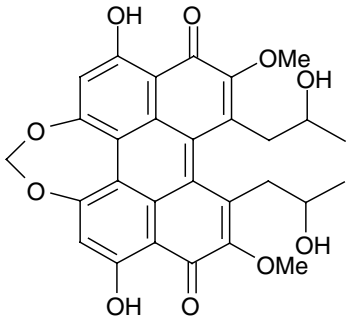
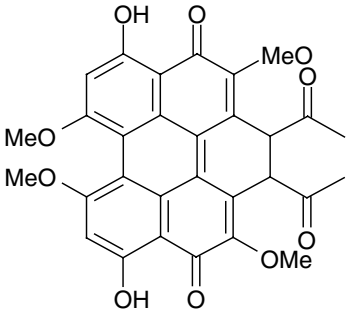
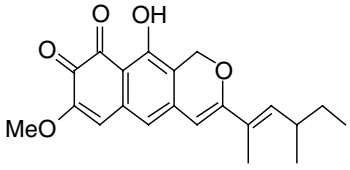
methods (Weaver and Riley, 1982; Westra et al., 1992; Klimeš and Klimešová, 1994). Furthermore, since, in addition to seeds, both bindweeds propagate vegetatively and spread by fragmented underground parts, biocontrol would be a promising alternative both on farmland and in non-cropped areas, such as gardens and parks (Défago et al., 2001).

Pfirter and Défago (1998) tested fungal isolates of the genus, *Stagonospora* for their potential to control field bindweed. One isolate, *Stagonospora convolvuli* LA39 has been selected as an effective biocontrol agent of both bindweeds (Défago et al., 2001). *Stagonospora convolvuli* LA39 has been shown to be an effective biocontrol agent against field and hedge bindweeds in both agricultural systems and in a non-cropped situation (Pfirter et al., 1997; Guntli et al., 1998; Défago et al., 2001).

Biologically active substances with pronounced effects on symptom expression and tissue colonization have been isolated from most phytopathogenic fungi (Daub and Ehrenshaft, 1993, 2000). Like many necrotrophs, *S. convolvuli* LA39 produces phytotoxic compounds in liquid media, notably, elsinochrome A and leptosphaerodione (Table 1) (Nicolet, 1999; Nicolet and Tabacchi, 1999). These authors also identified the toxin cercosporin in another isolate of *Stagonospora*, 214Caa (Table 1).

Cercosporin and elsinochrome A are closely related photosensitizing perylenequinone toxins produced typically by species of *Cercospora* and *Elsinöe*, respectively (Chen et al., 1966; Daub, 1982;

Table 1. Chemical properties and sources of *Stagonospora* toxins[a]

Fungal toxin	Chemical structure	Chemical formula	Molecular weight	Source fungus
Cercosporin		$C_{29}H_{26}O_{10}$	534	*Stagonospora isolate 214Caa*
Elsinochrome A		$C_{30}H_{24}O_{10}$	544	*Stagonospora convolvuli LA39*
Leptosphaerodione		$C_{21}H_{22}O_5$	354	*Stagonospora convolvuli LA39*

[a]Nicolet (1999).

Weise et al., 1987; Daub and Ehrenshaft, 2000). Photosensitizing perylenequinones are chemical substances that absorb light and become energetically activated, reacting with molecular oxygen to form both radical and non-radical species of activated oxygen, which have near-universal toxicity (Daub and Ehrenshaft, 1993, 2000). Cercosporin which is the most studied has been shown to generate both singlet oxygen ($^1O_2$) and superoxide (O_2^-) *in vitro* when illuminated, but the former is primarily responsible for its high toxicity (Daub and Hangarter, 1983). Cercosporin is known to have near-universal toxicity to living cells, with toxic effects well documented against mice, many bacteria and fungi, plants, and human cells at micromolar concentrations (Balis and Payne, 1971; Yamazaki et al., 1975; Daub, 1982; Tamaoki and Nakano, 1990). Similarly, elsinochromes such as B1, B2, C1 and C2 have been shown to have photodynamic inhibitory effects and are known to inhibit proteinkinase C (Arnone et al., 1993). The photodynamic inhibitory activity of elsinochrome A on cells was recently reported for the first time (Ma et al., 2003). These authors found elsinochrome A concentrations as low as 1 and 10 μM to induce up to 41% and 53% apoptosis on the human cell line Hce-8693 after 20 min irradiation.

Leptosphaerodione is less known compared to cercosporin and elsinochrome A. It was first isolated from the marine ascomycetes, *Leptosphaeria oraemaris* and described in 1991 (Guerriero et al., 1991). Little is known about its biological activity and role in pathogenicity, but it was found to be phytotoxic to field bindweed (*Convolvulus arvensis*) and tomato plants (*Lycopersicon esculentum*) at micromolar concentrations in a greenhouse study by Nicolet (1999).

The production of these metabolites by isolates of *Stagonospora* (Nicolet, 1999; Nicolet and Tabacchi, 1999) could arouse attention in two major directions. Effectiveness of the biocontrol agent may be enhanced by increasing the production of the phytotoxins. Conversely, production of such compounds by the biocontrol agent could generate some biosafety concerns that may delay its registration as a mycoherbicide. Therefore, the objectives of this study were to: (1) determine the intra- (and inter-) specific variation among 30 isolates of *Stagonospora* with regard to their aggressiveness on both bindweeds, and production of cercosporin, elsinochrome A and leptosphaerodione; and (2) determine the relationship between aggressiveness and toxin production. The purpose was to determine the possibility of selecting aggressive biocontrol *Stagonospora* isolates with limited or no production of these metabolites, as a way of reducing any potential risk in the environment.

Materials and methods

Fungal isolates

The 30 isolates of *Stagonospora* used for this study are described in Table 2. The isolates were collected from field bindweed (*Convolvulus arvensis*) and hedge bindweed (*Calystegia sepium*) in different places in Europe (Table 2).

Characterisation of Stagonospora *isolates for aggressiveness on field and hedge bindweeds*

Seed coats of field bindweed (USA ecotype) and hedge bindweed (Switzerland ecotype) were digested with concentrated sulphuric acid for 30 min. The seeds were then washed twice with double distilled water (ddH_2O) and germinated in the dark at 24 °C on water agar plates for 2 days. The seedlings were transplanted to 9 cm diam. plastic pots containing 150–200 g of 3:1 mixture of soil (De Baat, the growing power; GVZ Bolter AG, 8048 Zurich) and 1.5–2.2 mm quartz sand. The plants were grown for 3 weeks in a glasshouse with 70% relative humidity (RH) and alternating 16 h of light (90 μmol s^{-1} m^{-2}) at 22 °C and 8 h of darkness at 17 °C.

Depending on the best medium for spore production determined prior to experimental set up, isolates were grown on potato glucose agar (PDA) or 10% V8-juice agar (V8 agar) in 9 cm diam. Petri dishes for 3 weeks at 20 °C under continues white light (17 μmol s^{-1} m^{-2}). V8 agar plates were poured from an autoclaved solution containing 100 ml V8-juice (N.V. Campbell Foods Belgium SA, Belgium), 3 g $CaCO_3$, 12 g agar (Oxoid) and 900 ml ddH_2O. Spores were harvested by flooding the culture with ddH_2O. The surface of the agar was gently scraped with a glass slide, and the spore suspension was collected through sterile glass wool. Spores were counted under the microscope using a haemocytometer, and the suspension was adjusted to a

Table 2. Isolates of *Stagonospora* used in the study

Isolate	Geographic origin	Weed host	RFLP-type [a]	Reference [b, c]
172Caa	Staffelfelden, FR	*Calystegia sepium*	C	[b]
174Coa	Guebwiller, FR	*Convolvulus arvensis*	Not described	[c]
174Cob	Guebwiller, FR	*Convolvulus arvensis*	B	[b]
175Caa	Nambsheim, FR	*Calystegia sepium*	C	[b]
175Cab	Nambsheim, FR	*Calystegia sepium*	Not described	[c]
177Cab	Säckingen, DE	*Calystegia sepium*	A	[b]
178Caa	Säckingen, DE	*Calystegia sepium*	C	[b]
182Caa	Eschikon, CH	*Calystegia sepium*	C	[b]
213Coa	Brno, CZ	*Convolvulus arvensis*	B	[b]
214Caa	Brno, CZ	*Calystegia sepium*	C	[b]
92Coa	Zürich, CH	*Convolvulus arvensis*	B	[b]
A7	Yeorile, UK	*Calystegia sepium*	A	[b]
B4	Yeorile, UK	*Calystegia sepium*	A	[b]
B7	Yeorile, UK	*Calystegia sepium*	Not described	[c]
B8	Yeorile, UK	*Calystegia sepium*	A	[b]
C5	Yeorile, UK	*Calystegia sepium*	B	[b]
D6	Yeorile, UK	*Calystegia sepium*	A	[b]
LA10A	Long Ashton, UK	*Convolvulus arvensis*	B	[b]
LA10B	Long Ashton, UK	*Convolvulus arvensis*	B	[b]
LA24	Long Ashton, UK	*Convolvulus arvensis*	A	[b]
LA30B	Long Ashton, UK	*Convolvulus arvensis*	B	[b]
LA31	Long Ashton, UK	*Convolvulus arvensis*	A	[b]
LA311	Long Ashton, UK	*Convolvulus arvensis*	Not described	[c]
LA35	Long Ashton, UK	*Convolvulus arvensis*	A	[b]
LA36	Long Ashton, UK	*Convolvulus arvensis*	Not described	[c]
LA37	Long Ashton, UK	*Convolvulus arvensis*	Not described	[c]
LA39	Long Ashton, UK	*Convolvulus arvensis*	A	[b]
LA51	Long Ashton, UK	*Convolvulus arvensis*	A	[b]
LA53	Long Ashton, UK	*Convolvulus arvensis*	A	[b]
LA57	Long Ashton, UK	*Convolvulus arvensis*	A	[b]

[a] Fragment pattern type based on Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) of the ITS region of rDNA (Pfirter et al., 1999).
[b]Pfirter et al. (1999).
[c]This study.

concentration of 5×10^7 conidia ml^{-1}. This was added to 10% vegetable oil emulsion (Pfirter and Défago, 1998) to a final inoculum concentration of 5×10^6 conidia ml^{-1} and mixed thoroughly using a hand-held electronic kitchen mixer (MIO STAR Swissline, 50 Hz 200 W, Migros, Switzerland).

Leaves of 3 weeks old field or hedge bindweed were sprayed to run-off at low pressure (0.3 bars) with fungal inoculum. Immediately after inoculation, plants were placed in the glasshouse under 70% RH with alternating 16 h of light (90 μmol s^{-1} m^{-2}) at 22 °C and 8 h of darkness at 17 °C. Control treatments were sprayed with 10% vegetable oil emulsion. Each treatment was replicated eight times (eight pots) in a randomized complete block design (RCBD). The experiment was repeated at two later dates, giving a total of three experimental runs.

At 1, 2 and 3 weeks after inoculation of plants, disease severity was assessed on each inoculated leaf, using a scale of 0–6, where 0 = 0% infection (leaf healthy), 1 = 1–5% infection (first tiny lesions), 2 = 6–25% infection (clearly visible lesions and or chlorosis), 3 = 26–75% infection (>25%, but up to 75% of leaf either necrotic or chlorotic), 4 = 76–95% infection (<25% green area left on leaf), 5 = 96–100% infection (no green area left on leaf, completely chlorotic or necrotic, but still turgid), 6 = 100% infection (leaf dead). To estimate

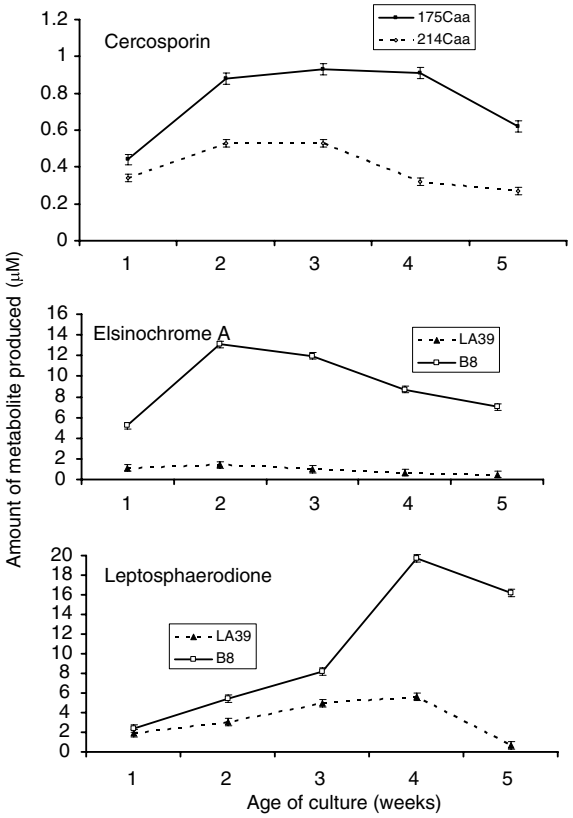


Figure 1. Time course for the production of cercosporin and elsinochrome A in V8 broth and leptosphaerodione in potato glucose broth by two isolates of *Stagonospora* Isolates LA39 and B8 were used for elsinochrome A and leptosphaerodione production, while 175Caa and 214Caa were used for cercosporin production. Cultures were extracted with ethyl-acetate, extracts dissolved in acetonitrile and analysed by high performance liquid chromatography using a reverse phase nucleosil – C18 column. There were three replications and the experiment was repeated once.

percentage infection on a plant, average severity score of the total number of inoculated leaves on the plant was calculated and the median % infection corresponding to the severity score was taken.

Characterisation of Stagonospora *isolates for metabolite production*

Stagonospora isolates were characterised for the type and amount of metabolite (cercosporin, elsinochrome A, or leptosphaerodione) produced. Several preliminary tests were conducted to adapt and optimise culture conditions, the extraction method and chromatographic analyses of metabolites described by Nicolet (1999) prior to characterisation of the 30 isolates. The time course of production of each of the three metabolites was developed (Figure 1) using two isolates identified for producing the respective metabolite.

All 30 isolates were screened in both potato glucose broth (PDB) and V8 broth (content as in V8 agar, but without agar) for cercosporin, elsinochrome A and leptosphaerodione production. There were three replications over time, using inoculum from a fresh V8 agar plate of a 2-week-old culture (temporarily stored at 3 °C) each time. Erlenmeyer flasks (500 ml) containing 300 ml of PDB or V8 broth were inoculated and incubated on a rotary shaker running at 130 rpm at 20 °C in continuous light (17 µmol s^{-1} m^{-2}). Since it was found that comparing production of the three metabolites at the same culture age would be best at 3 weeks (Figure 1), the cultures were grown for 3 weeks before metabolite extraction. Extraction of the fungal culture was done by filtering culture suspension through four layers of cheesecloth. Each filtrate was adjusted to pH 5.0 with 2 M HCl and extracted twice with the same volume of ethyl-acetate (Erne AG, Dällikon, Switzerland). The organic phase was filtered through silicone-coated filter paper (Folded filters, Macherey-Nagel GmbH & Co. KG, D-d2313 Düren, Germany). The organic filtrate was evaporated to complete dryness *in vacuo*. The dried extract was dissolved in 1 ml acetonitrile (HPLC gradient grade; ≥99.9% (GC), Fluka Chemie AG, Buchs, Switzerland) and immediately analysed by high performance liquid chromatography (HPLC). Fungal extracts (in acetonitrile) were analysed in a Hewlett Packard 1090 Liquid Chromatograph equipped with a diode-array detector. A reverse phase column, CC 250 × 4 mm Nucleosil 120-5 C18 (Macherey-Nagel, Germany) was used, and the temperature of the column was 28 °C. Injection volume was 10 µl, and the extracts were eluted with 80% acetonitrile, 20% ddH_2O (acidified with 0.045% H_3PO_4) for 15 min at a flow rate of 1ml min^{-1}. Cercosporin and elsinochrome A were detected at 230 nm, while leptosphaerodione was detected at 254 nm. Metabolites were identified by comparing retention time and UV absorption spectra with those of the reference substances. Cercosporin, elsinochrome A and leptosphaerodione were detected at 2.9–3.1, 4.9–5.1, and 6.7–6.9 min, respectively (Figure 2).

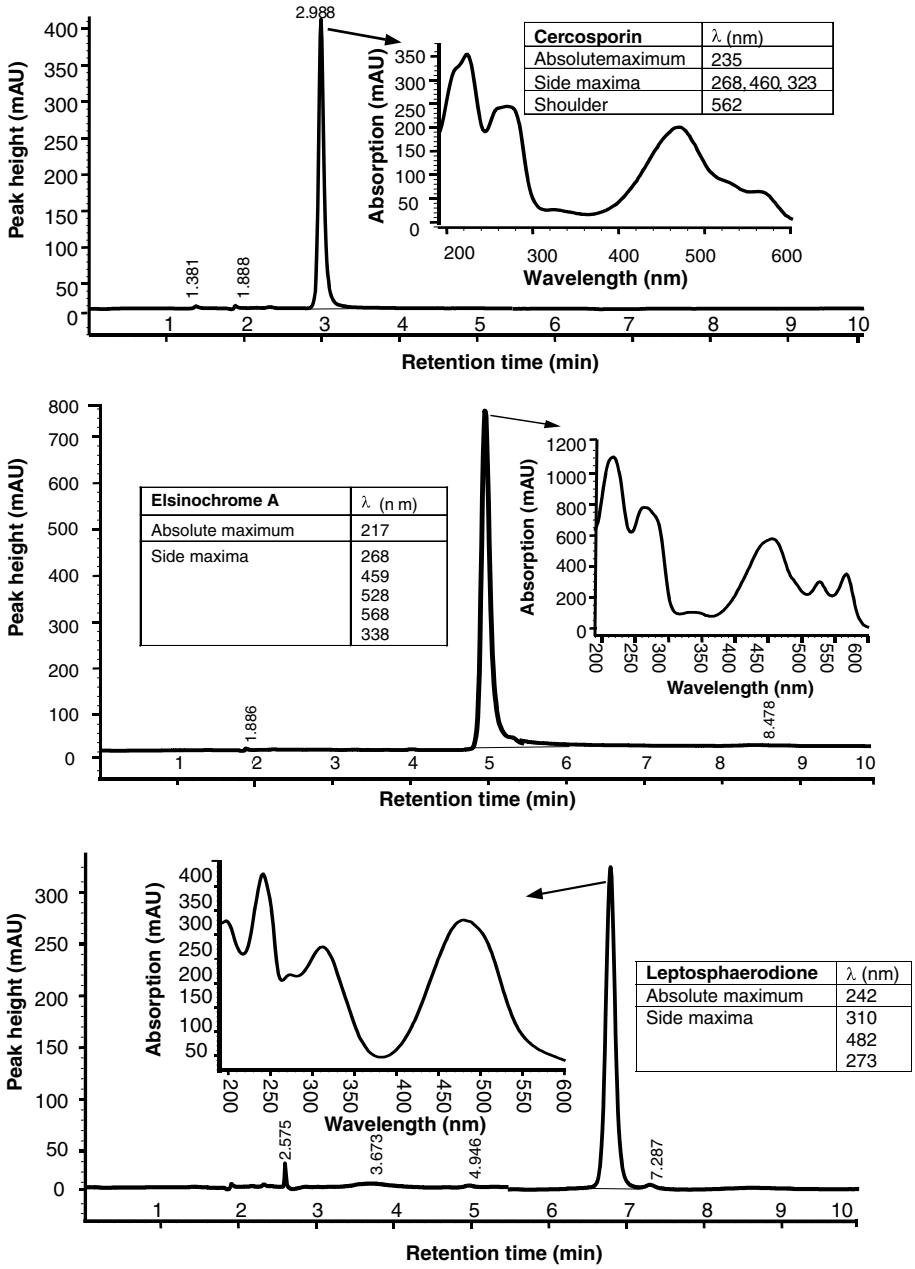


Figure 2. Peak and UV absorption spectrum of cercosporin, elsinochrome A and leptosphaerodione in high performance liquid chromatography chromatogram. A reverse phase nucleosil – C18 column was used. Two nmol of pure cercosporin, 14.7 nmol of pure elsinochrome A and 16.9 nmol of pure leptosphaerodione were analysed. Cercosporin and elsinochrome A were detected at 230 nm, and leptosphaerodione was detected at 254 nm.

Quantification of metabolite was based on the standard peak area – concentration relationship derived from injection of known amounts of the reference substances. Pure cercosporin (≥99.9% purity grade) used as a reference was obtained from Fluka AG, Buchs, Switzerland. Pure elsinochrome A and leptosphaerodione were not commercially available for purchase. Hence, the two substances were purified for reference from *S. convolvuli* LA39 extracts in collaboration with

Prof. R. Tabacchi of the Institute of Chemistry, University of Neuchâtel, Switzerland. The purity grades of elsinochrome A and leptosphaerodione as verified by mass spectrometry (MS) and nuclear magnetic resonance (NMR) were ≥97%.

Statistical analyses

All data were analysed using SAS (SAS® System for windows, Release 8.2, SAS® Inst., Cary, NC, USA). The highest variation in isolate aggressiveness on both bindweeds was observed 2 weeks after inoculation. Hence, the data obtained at this assessment time were used in statistical analyses to differentiate isolates. Percentage infection (median %) data were arcsine (ASIN (SQRT)) transformed (Snedecor and Cochran, 1989) and subjected to analysis of variance using linear mixed model procedure 'Proc MIXED' (Littell et al., 1996). Since variances from repeated experiments were not significantly different from each other, both experimental run and replication were treated as random effects in the model statement, while isolate was taken as the fixed effect variable. Means were estimated as least square means (lsmeans) and separated using the orthogonal contrasts option. For mean comparison, the standard error of difference between means (generated by the orthogonal contrasts) is presented since the large number of isolates to compare made it impossible to present contrast estimates and their significances.

Statistical analysis of the metabolite production data was also performed in SAS using the linear mixed model procedure 'Proc MIXED' to estimate lsmeans and standard error of difference between means as described for analysis of aggressiveness data. To determine the relationship between isolate aggressiveness on both bindweeds and production of either cercosporin, elsinochrome A, or leptosphaerodione, correlation analyses were performed between the two sets of data using the 'merge' command and correlation procedure 'Proc CORR' in SAS. Correlation between each toxin and infection on weed separately and combined, as well as toxin–toxin production correlations were analysed. To show correlation results graphically, scatter gram was invoked in SAS by the 'Gplot' procedure.

Results and discussion

Characterisation of Stagonospora *isolates for aggressiveness on field and hedge bindweeds*

The ability to infect the two bindweeds as quantified visually by the extent of leaf necrotic lesions and chlorosis varied ($P < 0.0001$) among the 30 isolates of *Stagonospora* evaluated (Table 3). More isolates were aggressive (≥40% infection) on field bindweed than hedge bindweed, as some isolates were aggressive on one bindweed but not aggressive or less aggressive on the other (Table 3). On both bindweeds, the 10 most aggressive isolates were 177Cab, LA51, LA57, LA53, B8, B4, LA24, LA31, LA35 and LA39 (Table 3). Ten isolates were significantly more aggressive on field bindweed than on hedge bindweed, while eight isolates were more aggressive on hedge bindweed than on field bindweed (Table 3). Infections of six of the isolates on field bindweed were not significantly different ($P > 0.05$) from the 0% infection in the control treatments, and except for isolate LA36, the same held for aggressiveness of each of these isolates on hedge bindweed (Table 3).

This study confirms the efficacy of *S. convolvuli* LA39 on field and hedge bindweeds (Défago et al., 2001) and also indicates that there are a few other isolates of *Stagonospora* in our collection that are more aggressive than LA39 on both bindweeds. Results do not indicate any definite relationship between the geographic origin of isolates or bindweed from which they were isolated and pathogenic abilities of isolates on either of the bindweeds (Tables 2 and 3). However, except isolate 177Cab which originated from Säckingen Germany, all the 10 most aggressive isolates across bindweeds, originated from the UK. While eight of the 10 isolates that were more aggressive on field bindweed were isolated from this bindweed, only three of the eight isolates that were more aggressive on hedge bindweed were isolated from hedge bindweed (Tables 2 and 3).

Characterisation of Stagonospora *isolates for metabolite production*

Cercosporin and elsinochrome A were detected in extracts of V8 broth cultures, while leptosphaerodione was detected in extracts of PDB cultures. In rare cases, small amounts of cercosporin could be

Table 3. Aggressiveness of 30 isolates of *Stagonospora* on field and hedge bindweeds, and amounts of metabolites (cercosporin, elsinochrome A and leptosphaerodione) detected in extracts of the fungal culture filtrates by high performance liquid chromatography

Stagonospora isolate	% infection on weed[a, b]		Amount of metabolite (μM)[c]		
	field bindweed	hedge bindweed	Cercosporin[d] (in V8)	elsinochrome A[e] (in V8)	leptosphaerodione[f] (in PDB)
172Caa	3 ^{ns}	11	nd	nd	nd
174Coa	49	19 ***	nd	nd	nd
174Cob	63	35 ***	nd	nd	nd
175Caa	45	28 **	1.07	nd	nd
175Cab	51	17 ***	nd	nd	nd
177Cab	80	74	nd	2.01	nd
178Caa	7 ^{ns}	13	0.13	nd	nd
182Caa	2 ^{ns}	6 ^{ns}	0.36	nd	nd
213Coa	42	13 ***	nd	1.81	nd
214Caa	7 ^{ns}	17	0.75	nd	nd
92Coa	51	10 ***	nd	12.12	nd
A7	13	18	nd	0.99	3.79
B4	63	62	nd	3.41	1.43
B7	37	52 *	nd	7.68	4.87
B8	66	73	nd	8.89	11.20
C5	47	79 ***	nd	0.59	nd
D6	21	41 **	nd	nd	nd
LA10A	66	22 ***	nd	2.00	nd
LA10B	51	25 ***	nd	0.57	nd
LA24	64	58	nd	4.13	5.25
LA30B	59	27 ***	nd	3.84	nd
LA31	73	53 ***	nd	3.15	2.97
LA311	43	67 ***	nd	7.39	4.23
LA35	63	74 **	nd	2.41	1.61
LA36	7 ^{ns}	30 ***	nd	nd	nd
LA37	3 ^{ns}	5 ^{ns}	nd	nd	nd
LA39	54	59 *	nd	0.56	3.66
LA51	78	79	nd	0.76	1.44
LA53	70	81 **	nd	0.56	4.53
LA57	71	75	nd	5.12	4.38
Standard error of difference	5.38	5.35	0.0393	1.2990	1.3844

^{ns} Not significantly different from absolute zero value at $P \leq 0.05$ i.e. no significant infection.
*, **, and *** significantly different from infection on field bindweed at $P \leq 0.05$, $P \leq 0.01$, and $P \leq 0.001$ respectively.
nd = not detected (no toxin detected by HPLC).
[a]Values are means derived from arcsine (ASIN(SQRT)) transformed data of three experiments, each replicated eight times.
[b]Only % infection data at 2 weeks after inoculation were used since the highest variation in aggressiveness of isolates was observed at this assessment time.
[c]Values are means of three replications over time (three experimental runs).
[d]Limit of detection (LOD) = 0.623 nmol (in 10 μl injection volume).
[e]Limit of detection (LOD) = 0.188 nmol (in 10 μl injection volume).
[f]Limit of detection (LOD) = 2.82 nmol (in 10 μl injection volume).

detected in PDB, but there was no detection of elsinochrome A in PDB or leptosphaerodione in V8. There was a significant variation in the type and amount of metabolite detected in extracts of the 30 *Stagonospora* isolates screened (Table 3). Cercosporin was detected only in four isolates, elsinochrome A and leptosphaerodione were detected in extracts of 19 and 12 isolates, respectively, while seven isolates did not produce any of the three metabolites (Table 3). No isolate produced leptosphaerodione in PDB culture without also producing elsinochrome A in V8 broth culture, but

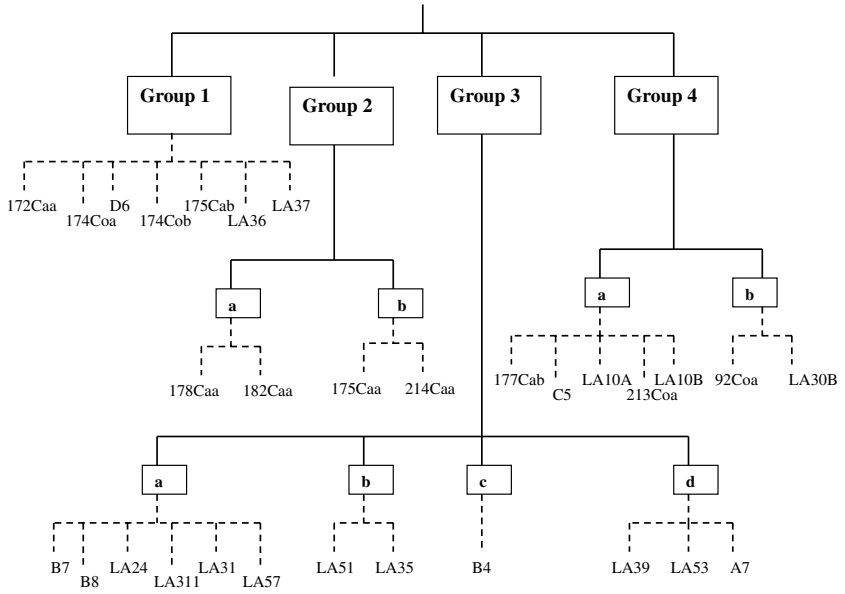


Figure 3. Classification of 30 isolates of *Stagonospora* based on type and amount of metabolite detected by high performance liquid chromatography in extracts of V8 broth and potato glucose broth cultures. Metabolite production data (Table 3) were used to classify isolates into groups. *Group* 1: Do not produce detectable amounts of cercosporin, elsinochrome A, or leptosphaerodione. Group 2: Produce detectable amounts of cercosporin a: Low producers (< 0.5 μM) b: High producers (≥ 0.5 μM). Group 3: Produce detectable amounts of both elsinochrome A and leptosphaerodione a: High producers of elsinochrome A (≥ 2.5 μM) and leptosphaerodione (≥ 2.5 μM) b: Low producers of elsinochrome A (< 2.5 μM) and leptosphaerodione (< 2.5 μM) c: High producers of elsinochrome A, but low producers of leptosphaerodione d: Low producers of elsinochrome A, but high producers of leptosphaerodione. Group 4: Produce detectable amounts of only elsinochrome A a: Low producers b: High producers.

there were seven isolates that produced elsinochrome A in V8 broth without producing leptosphaerodione in PDB (Table 3). Neither elsinochrome A nor leptosphaerodione was detected in isolates where cercosporin was detected (Table 3). Apart from 177Cab which produced only elsinochrome A, all the 10 most aggressive isolates (including *S. convolvuli* LA39) across the two bindweeds, produced both elsinochrome A and leptosphaerodione (Table 3). Another isolate, C5 produced only elsinochrome A, but was among the most aggressive isolates on hedge bindweed, and on field bindweed, its aggressiveness was not significantly different from that of LA39 (Table 3).

The 30 isolates could be separated into four groups based on the type of metabolite produced (in detectable amounts) in the two media tested (Figure 3). Interestingly, this grouping (Figure 3) agrees by over 85% with characterisation by Pfirter et al. (1999) based on PCR-RFLP of ITS region of the nuclear rDNA of the isolates (Table 2). It may therefore be inferred that *in vitro* production of certain metabolites such as cercosporin, elsinochrome A, and leptosphaerodione may serve as a useful supplementary chemo-taxonomic parameter for *Stagonospora* species. Of particular importance is the indication that *in vitro* production of elsinochrome A for instance, may be a useful biomarker for the fast screening of *Stagonospora* isolates to select effective mycoherbicides for field and hedge bindweeds. It may even be possible to increase the speed of screening by using molecular tools such as the RFLP of ITS region to quickly detect and select toxin-producing (potential biocontrol) isolates.

Relationship between in vitro *metabolite production by isolates and aggressiveness of isolates on field and hedge bindweeds*

The amounts of elsinochrome A and leptosphaerodione detected in extracts of culture filtrate of *Stagonospora* isolates by HPLC were positively correlated with the aggressiveness of isolates on both bindweeds (Table 4 and Figure 4). Even a stronger positive correlation between elsinochrome

Table 4. Correlation between amounts of metabolites (cercosporin, elsinochrome A or leptosphaerodione) detected in extracts of culture filtrates of *Stagonospora* isolates by high performance liquid chromatography and percentage infection caused by the isolates on field or hedge bindweed

Relationship[a]	Pearson correlation coefficient (r)	Test of significance ($P > r$ under Ho: Rho = 0)
Cercosporin – infection on field bindweed	−0.27172	< 0.0001
Cercosporin – infection on hedge bindweed	−0.24136	< 0.0001
Cercosporin – infection across weeds (combined)	−0.30162	< 0.0001
Elsinochrome A – infection on field bindweed	0.14897	< 0.0001
Elsinochrome A – infection on hedge bindweed	0.11359	0.0018
Elsinochrome A – infection across weeds (combined)	0.15601	< 0.0001
Leptosphaerodione – infection on field bindweed	0.18977	< 0.0001
Leptosphaerodione – infection on hedge bindweed	0.37495	< 0.0001
Leptosphaerodione – infection across weeds (combined)	0.33923	< 0.0001
Cercosporin – elsinochrome A	−0.20375	< 0.0001
Cercosporin – leptosphaerodione	−0.17559	< 0.0001
Elsinochrome A – leptosphaerodione	0.51621	< 0.0001

[a]Aggressiveness of isolates and metabolite production data were subjected to Pearson correlation analysis in SAS to find the relationship between aggressiveness of isolates and amounts of each of the three metabolites produced, and between the production of each metabolite.

A and leptosphaerodione production was found (Table 4). However, cercosporin production was negatively correlated with aggressiveness of isolates on both bindweeds (Table 4 and Figure 4). Similarly, production of cercosporin correlated negatively with production of elsinochrome A or leptosphaerodione (Table 4). The positive correlation between aggressiveness and leptosphaerodione production may be attributed to the positive correlation found also between the latter metabolite and elsinochrome A production, since no isolate produced leptosphaerodione exclusively of elsinochrome A (Table 3). There seems to be a stronger link between aggressiveness and production of both leptosphaerodione and elsinochrome A than production of elsinochrome A alone. However, isolate 177Cab produces elsinochrome A only (in detectable amounts), yet was highly aggressive on both field and hedge bindweeds. Some isolates, such as C5, LA10A, LA10B, LA30B and 92Coa produced elsinochrome A only but were highly aggressive on only one of the bindweeds (Table 3). Except for isolates 174Coa and 174Cob that were moderately aggressive on field bindweed but not on hedge bindweed, any isolate where elsinochrome A was not detected (elsinochrome A-negative) was not aggressive on either of the two bindweeds (Table 3). Therefore, of the three metabolites, elsinochrome A seems to be the most important in *Stagonospora* pathogenesis.

These results suggest that the production of cercosporin, a putative pathogenicity determinant in the highly successful phytopathogens of the fungal genus *Cercospora* (Fajola, 1978; Upchurch, et al., 1991; Daub and Ehrenshaft, 2000) may not be a determinant of pathogenicity in the *Stagonospora*-bindweed pathosystem. Nevertheless, results generally tend to agree with the presumptions of Daub and Ehrenshaft (2000) that production of photosensitizing perylenequinones and other light-activated toxins may be a common pathogenicity factor in many phytopathogenic fungi. Different necrotrophic fungal genera may use different photoactive perylenequinone compounds as a pathogenesis mechanism (Stack et al., 1986; Hartman et al., 1989; Daub et al., 1992). *Stagonospora* may use the production of elsinochrome A and not cercosporin as the pathogenicity mechanism.

The implication of the relationship between toxic metabolite production and aggressiveness of *Stagonospora* on both bindweeds is that it may be difficult to select an atoxigenic isolate aggressive enough as a biocontrol agent of these globally important weeds. What seems possible is the selection of isolates such as LA51, LA35, and 177Cab that are highly aggressive on both bindweeds but produce relatively low amounts of leptosphaerodione and elsinochrome A or elsinochrome A only (in the case of isolate 177Cab). However, the imperfect linear relationship between amounts of

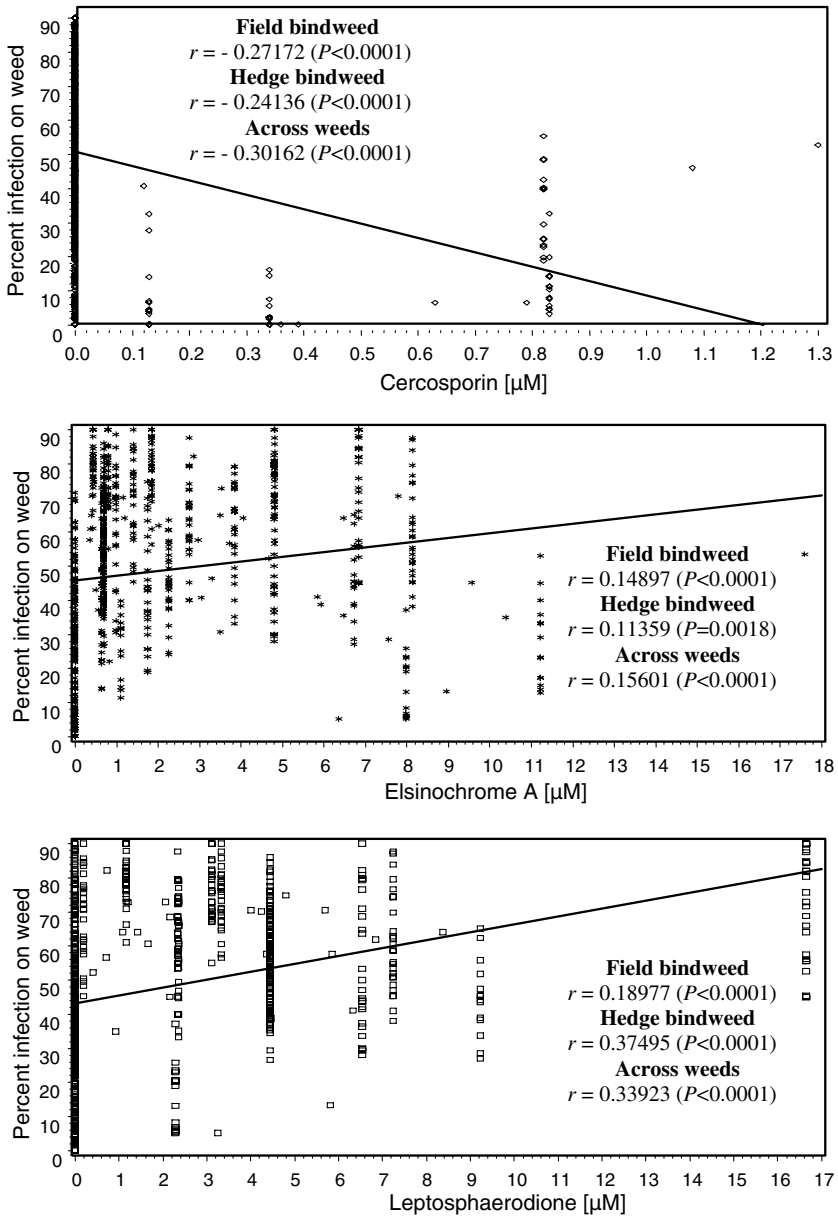


Figure 4. Scatter gram showing correlation between cercosporin, elsinochrome A or leptosphaerodione production *in vitro* by *Stagonospora* isolates and aggressiveness of isolates on field and hedge bindweeds.

leptosphaerodione and elsinochrome A or elsinochrome A only produced in culture by isolates, and aggressiveness of isolates on bindweeds, suggests a more complex situation. Differential regulation of toxin genes in isolates grown in culture media may result in differential amounts of toxin produced. For example, in addition to temperature and light effects, medium components and carbon/nitrogen ratios are reported to play a role in cercosporin biosynthesis, often with the same conditions enhancing production in one isolate while having a negative or no effect on another (Fajola, 1978; Lynch and Geoghegan, 1979; Jenns et al., 1989). Therefore, production of toxins *in planta* may differ greatly from *in vitro* production.

We have extracted bindweed leaves infected with *S. convolvuli* LA39 and isolate B8 to determine the levels of elsinochrome A or leptosphae-

rodione produced *in planta*, but so far have not detected any of the metabolites in the extracts derived from diseased leaves (data not shown). Metabolite production *in planta* may be very minimal and diluted to concentrations below the limit of detection. This implies that aggressive *Stagonospora* isolates may not produce elsinochrome A or leptosphaerodione *in planta* at levels that could constitute any risk in the environment. However, more detailed studies exploiting different techniques to detect and quantify elsinochrome A production *in planta* by *Stagonospora* isolates as mycoherbicides of field and hedge bindweeds may clarify this. In addition, determination of the fate of elsinochrome A in the environment, and its relationship with leptosphaerodione, and the development of molecular tools to monitor the mycoherbicide following field application, are likely to be important.

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