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Variability in aggressiveness, cultural characteristics, cercosporin production and fatty acid profile of *Cercospora piaropi*, a biocontrol agent of water hyacinth

D. J. Tessmann[a]*†, R. Charudattan[a] and J. F. Preston[b]

[a]*Plant Pathology Department, PO Box 110680, University of Florida, Gainesville, FL 32611-0680; and* [b]*Department of Microbiology and Cell Science, PO Box 110700, University of Florida, Gainesville, FL 32611-0700, USA*

The extent of variation in aggressiveness, growth and pigmentation in culture, phytotoxin production and fatty acid profile were determined in a population of 55 isolates of *Cercospora piaropi*, a fungus used as a biocontrol agent of the aquatic weed water hyacinth (*Eichhornia crassipes*). Besides differences in the colour of mycelium and diffusible pigments in culture, isolates of *C. piaropi* grown under standard conditions differed significantly in their ability to produce the phytotoxin cercosporin, as well as in aggressiveness and growth rate. A positive correlation existed between the ability of the isolates to produce cercosporin and their aggressiveness, and a negative correlation between growth rate and cercosporin production or growth rate and aggressiveness. Based on thin-layer chromatographic separation of extracts and comparison with beticolin-1, used as a standard, there was no evidence of production of beticolins. In discriminant analysis, fatty acid methyl ester (FAME) profiles had low resolution for differentiating populations among isolates of the fungus, and the level of resolution was influenced by the age of the colonies. Diffusible pigments in culture and cercosporin production are useful adjuncts to aggressiveness screening for choosing the most effective isolate of *C. piaropi* for biological control.

Keywords: aquatic weed, bioherbicide, *Cercospora rodmanii*, *Eichhornia crassipes*, pathogenic fungi, pathogenic variability, phytotoxin

Introduction

Water hyacinth (*Eichhornia crassipes*), an aquatic plant indigenous to lowland tropical South America, was spread worldwide by man. Its free-floating habit and capacity for rapid vegetative propagation has enabled it to become one of the most prolific aquatic weeds in many tropical and subtropical regions of the world (Pieterse, 1990). Biological control based on plant pathogens and insects has been part of the strategy used for controlling this weed (Charudattan, 1986, 2001; Julien, 2001). The fungal species *Cercospora piaropi* (Tharp, 1917) [syn. *C. rodmanii* (Conway, 1976a)], the only valid *Cercospora* species described on water hyacinth after the emendation by Tessmann *et al*. (2001), was shown to decrease water hyacinth biomass, and in some instances to cause substantial decline of water hyacinth populations (Freeman & Charudattan, 1984; Charudattan *et al*., 1985; Martyn, 1985; Morris, 1990). Pathogenic variability, although presumed to exist, has not been identified in *C. piaropi*. Isolates of *C. piaropi* show a high level of variation in their cultural features, such as mycelial colour, colour and intensity of diffusible pigments, and growth rate. However, it is unknown to what extent these factors are related to pathogenicity or aggressiveness of isolates. Such information would be important for choosing the most effective biocontrol strain and optimizing large-scale inoculum production, both of which are important steps in the bioherbicide strategy to control this weed (Charudattan *et al*., 1985; Charudattan, 1996).

In *Cercospora* spp., two coloured secondary metabolites have been identified as phytotoxins: a red-purple compound, called cercosporin, identified in several species in this genus (Assante *et al*., 1977; Lynch & Geoghegan, 1977; Jenns *et al*., 1989; Upchurch *et al*., 1991), and a yellow compound, identified only in *Cercospora beticola*, which actually corresponds to a group of 20 toxins, named beticolins and formerly known as *C. beticola* toxin (CBT) (Milat & Blein, 1995; Goudet *et al*., 2000).

*E-mail: djtessmann@uem.br

†Present address: State University of Maringá, Agronomy Department, Av. Colombo, 5790, CEP 87020-900, Maringá, PR, Brazil.

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Table 1 Designations and geographic origins of the isolates of *Cercospora piaropi* analysed in this study

Designation[a]	Geographic origin/Collector	Date
WH9R, WH9BR	Rodman Reservoir, FL, USA[b]	1973, 1974
WHK	Kissimee, FL, USA[c]	1981
2619	Suwannee River, FL, USA[c]	1994
SGS25, SGS32, SGS35, SGS49, J2, J6	Marion Co., FL, USA[d]	1996
I75-99, I75-101, I75-102, I75-104	Sarasota Co., FL, USA[d]	1996
I75-108	Lee Co., FL, USA[d]	1996
LJ37	Leon Co., FL, USA[d]	1996
TX2, TX15, TX18, TX20	Lake Conroe, TX, USA[d]	1996
RR4, RR22, RR24, RR27, RR29, RR31	Rodman Reservoir, FL, USA[d]	1996
WH83	Gainesville, FL, USA[b]	1983
BA55, BA57, BA59	Gainesville, FL, USA[d]	1996
BC31	Vero Beach, FL, USA[d]	1997
2702, 2703, 2704	Minas Gerais, Brazil[c]	1994
65-2, 67-1, 69-1, 70-12	Alagoas, Brazil[c]	1995
49-1, 49-2, 61-5, 62-1, 62-2, 62-4	Pernambuco, Brazil[c]	1995
16-1, 18-2	Corumba, Mato G. do Sul, Brazil[c]	1995
28-1	Rio Verde, Mato G. do Sul, Brazil[c]	1995
10	Sao Paulo City, SP, Brazil[c]	1996
34	Rio Grande do Sul, Brazil[c]	1994
2867, 2943	Mexico[c]	1982
WHV	Venezuela[c]	1982
114, 279	South Africa[e]	Unknown
400	Zambia[e]	Unknown

[a]Designation corresponds to notation of isolates used in this study.
Collected by [b]K. Conway, [c]R. Charudattan and [d]D. J. Tessmann, University of Florida; and [e]M. Morris, Plant Protection Research Institute, Stellenbosch, South Africa.

Cercosporin is a photosensitizing metabolite with host-non-specific toxicity (Daub & Ehrenshaft, 2000) that acts during pathogenesis (Shim & Dunkle, 2003; Choquer *et al*., 2005): its toxic effects are the result of the production of active oxygen forms. Beticolins are also host-non-specific phytotoxins which have been associated with a broad range of cytotoxic effects (Simon-Plas *et al*., 1996; Goudet *et al*., 2000).

The objective of this study was to determine the extent of variation in aggressiveness and in some physiological characteristics, including the production of the phytotoxins cercosporin and beticolins, in a population of *C. piaropi* isolates collected from several geographic locations. In addition, isolates of *C. piaropi* were characterized based on fatty acid methyl ester profiles (FAME), with the aim of finding useful biochemical markers to differentiate isolates and populations of the fungus. FAME profiles have been used successfully as biochemical markers for characterization and differentiation of species and populations of various fungi (Stevens Johnk & Jones, 1993; Graham *et al*., 1995; Stahl & Klug, 1996; Larkin & Groves, 2003). However, only limited information has been published evaluating the potential of FAME profiles for characterization of species and populations of the genus *Cercospora* (Berger *et al*., 1991). Hence, the knowledge of pathogenic variability can provide valuable information for biological control programmes to select the most effective strains, to infer the stability of these strains, and to define the boundaries of species and populations.

Materials and methods

Fungal isolates and cultural characteristics

The origins, designations, and the names of the collectors of the isolates used in this study are listed in Table 1. All isolates were obtained from water hyacinth leaves showing symptoms and monocultures were obtained from mycelial tips. These isolates have been preserved at the Biological Control of Weeds Laboratory of the Plant Pathology Department, University of Florida. Cultural characteristics were studied on potato dextrose agar (PDA) (Difco Laboratories). Three replicates for each isolate were inoculated with 4-mm-diameter mycelial plugs removed from the margins of 7-day-old colonies growing in 9-cm-diameter Petri dishes containing 20 mL PDA. Inoculated plates were incubated in a growth chamber at a temperature of 24 ± 2°C under cool-white fluorescent light with a 12-h photoperiod, and radial growth was measured 8 days later. The mean diameter was calculated as the average of two perpendicular measurements of colony diameter on each plate. Colony morphology, pigment diffusion, and mycelial pigmentation were also recorded 8 and 14 days after inoculation. The experiment had a completely randomized design

with three replicates for each isolate and was repeated once.

Toxin analysis

Toxin analysis was based on the protocol developed by Milat & Blein (1995). Isolates were cultured in 50-mL test tubes containing 15 mL of sterilized V8 broth medium (200 mL V8 juice, 3 g $CaCO_3$ and 800 mL of tap water). Each tube was inoculated with eight 4-mm-diameter mycelial plugs removed from the margins of 7-day-old colonies growing in 9-cm-diameter dishes containing 20 mL PDA. After incubation for 14 days at 25 ± 2°C under constant cool-white fluorescent light, the mycelium was separated from the broth by filtration using a double-layered cheese cloth, and blended for about 5 s in a Waring blender in ethyl acetate (1 g wet mycelium in 20 mL). The crude extract was separated decanted from the mycelial debris and resolved by thin-layer chromatography (TLC) using pre-coated TLC plates of silica gel without fluorescent indicator (E. Merck). A total of 5 μL crude extract was spotted on the TLC plate for each isolate. Chromatograms were developed using chloroform/methanol/water (80:20:2, v/v) as the elution mixture. The standards used were cercosporin from Sigma Chemical Co. and beticolin-1 obtained from Dr L. Milat (INRA, Dijon Cedex, France). In addition, the spectrum of the crude extract was determined in a spectrophotometer with wavelengths between 280 and 700 nm, and cercosporin was quantified in each sample by reading its absorbance at 473 nm. The amount of cercosporin was calculated using the formula: [(absorbance at 473 nm/31 455) × 534] μg, with 31 455 the molar extinction coefficient at 473 nm and 534 the molecular weight of cercosporin. This formula contained a corrected extinction coefficient with ethyl acetate as solvent, since in the original formula, used by Jenns *et al.* (1989) and Velicheti & Sinclair (1994), 5 N KOH was used as solvent. This correction was needed because the maximum absorption of cercosporin in 5 N KOH was 480 nm, compared with 473 nm in ethyl acetate. Each sample corresponded to an individually grown isolate. The experiment had three replicates in a randomized block design and was performed twice.

Pathogenicity test and aggressiveness analysis

A quantitative method of pathogenicity and aggressiveness assessment was developed to compare the isolates under standard conditions. Water hyacinth plants were vegetatively propagated from plants collected from Lake Alice, located on the campus of the University of Florida. The plants were propagated in pots containing tap water supplemented (0·05% w/v) with chelated iron (Keel-Iron, NaFe EDTA 5%; Chase & Company). For pathogenicity tests and screening for aggressiveness, daughter ramets (= offsets or clones) of water hyacinth, with two to three leaves and with approximately equal root mass, were placed in pots containing 125 mL tap water with the supplement described above. Each pot had one plant and the volume of liquid in the pots was maintained at the initial level by daily additions of water. Because of irregular or absent sporulation in culture, the inoculum was prepared from mycelium grown in still liquid cultures. Isolates were cultured in 250-mL flasks containing 50 mL V8 broth medium (as above). Each tube was inoculated with eight 4-mm-diameter mycelial plugs removed from the margins of 7-day-old colonies grown in 9-cm-diameter plates containing 20 mL of PDA. After incubation for 14 days at 25 ± 3°C with 12 h of cool-white fluorescent light per day, the mycelium was separated from the broth by filtration using a double-layered cheese cloth, and blended for about 5 s in a Waring blender at a concentration of 80 mg mycelium mL^{-1} water. The suspension was amended with 0·5% Metamucil (psyllium mucilloid; Procter & Gamble) and 0·05% Silwet L-77 (polyalkyleneoxide-modified heptamethyltrisiloxane, 0·02% v/v, OSI Corp.). A bioassay was developed which consisted of immersing two to three water hyacinth leaves, attached to plants, in a suspension of inoculum. Control leaves were immersed in a solution containing only the amendments. This procedure was used after other inoculation procedures, such as foliar spraying and droplet deposition on leaves, had been evaluated and found to be unsuitable because of runoff and unequal deposition of inoculum. The inoculated and control plants were placed in a dew chamber in darkness, at 25 ± 2°C for 12 h, and then held in a greenhouse for 2 weeks. This experiment was conducted in a well-illuminated quarantine greenhouse, since many of the isolates used in this study were non-native to the USA. Disease severity was assessed 7 and 14 days after inoculation with the aid of a rating scale, where: 1 = no symptoms, 2 = up to 1% of the leaf area exhibiting necrosis and chlorosis, 3 = 2–10% of the leaf area exhibiting necrosis and chlorosis, 4 = 11–25% of the leaf area exhibiting necrosis and chlorosis, 5 = 26–50% of the leaf area exhibiting necrosis and chlorosis, 6 = 51–75% of the leaf area exhibiting necrosis and chlorosis, 7 = more than 75% of the leaf area exhibiting necrosis and chlorosis and 8 = dead lamina. The treatments were arranged in a randomized block design with four replications (pots) per isolate, each pot having one plant with two to three leaves. The experiment was performed three times. Statistical analyses of pathogenic variability, growth rate and cercosporin production were performed with the GLM and CORR procedures of SAS (SAS Institute).

Fatty acid analysis

Fourteen isolates of *C. piaropi*, representing different geographical locations, and three other species of *Cercospora* (outgroups) were included in this study. All fungi were grown in 80 mL modified trypticase soy broth (TSB) (BBL Microbiology Systems, Becton Dickinson and Co.), with 10 g dextrose L^{-1}; in 250-mL flasks in a slow-shake culture (130 r.p.m.) for 4, 5 and 6 days at 24 ± 2°C in the dark before harvest and fatty acid extraction. Four 4-mm-diameter plugs removed from the periphery of 7-day-old cultures in PDA were used as initial inoculum.

Cultures were harvested using a side-arm Erlenmeyer flask fitted with a Buchner funnel filter and attached to a vacuum pump. A nylon-type fabric (polypropylene, mesh opening of 105 μm) was used instead of a filter paper to prevent the sample from becoming contaminated with paper fibres. After harvest, the fungal mycelium was transferred to 15-mL polypropylene tubes and kept in a freezer at –70°C until lyophilization. Samples of fungal tissue (50 g dry weight) were placed in clean screw-cap test tubes (13 × 100 mm; with Teflon cap liners), 2 mL of a saponification reagent (45 g sodium hydroxide in 1 L of 50% methanol) was added and the mixture was homogenized with a vortex mixer for 10 s. The homogenate was then saponified at 100°C in a water bath for 5 min, blended in a vortex mixer for 10 s, kept in a water bath for 25 min at 100°C, and then cooled in a water bath at room temperature. To methylate the liberated fatty acids, 2 mL of 54% 6-N HCl in methanol was added to each tube. Subsamples were then placed in an 80°C water bath for 10 min and immediately cooled to room temperature. To extract fatty acid methyl esters from the aqueous phase, 1·25 mL of 50% hexane-50% methyl tert-butyl ether was added to each tube, and the tubes were rotated end-over-end for 10 min. Next, the aqueous phase (bottom of tube) that contained fungal debris was removed with a Pasteur pipette and 3 mL of 1·2% NaOH in H_2O was added to each tube; the tube was then rotated end-over-end for 5 min. Finally, the organic phase (top of tube) that contained the fatty acid methyl esters was removed from the tubes and placed in a crimp-top gas chromatography vial.

Fatty acid extracts were analysed by gas-liquid chromatography with the Microbial Identification System (MIS) (Microbial ID, Inc.) at the Plant Pathology Department of the University of Florida. The MIS consisted of a chromatographic unit (chromatograph, integrator and autosampler) coupled to a computer system. The gas-liquid chromatograph was equipped with a 25-m × 0·2-mm phenyl-methyl-silicone-fused capillary column (Hewlett-Packard) and a flame ionization detector. Data from chromatographic analysis were sent to the computer system, where fatty acids were identified on the basis of their retention times relative to known standards, and quantified relative to other fatty acids in the sample on the basis of peak width and area data. The system was calibrated with known fatty acid standards when it was started and after every 10th sample. Samples were run through the gas chromatography column for 38 min, long enough for fatty acids up to 28 carbons long to pass through. Fatty acid profiles of individual isolates were based on analysis of the cellular fatty acid content of three independently grown cultures for each of the three harvest days. The value for each fatty acid in a given profile was the mean from all analysed cultures of that isolate. The concentration of each fatty acid was expressed as a percentage of the total fatty acid content. To determine whether the fatty acid compositions of the isolates were statistically different, discriminant analysis, implemented by SAS, was used to test the hypothesis that isolate $x(fa_1, fa_2, fa_3 \ldots fa_n) = y(fa_1, fa_2, fa_3 \ldots fa_n) = \ldots = z(fa_1, fa_2, fa_3 \ldots fa_n)$, where fa is fatty acid (Stahl & Klug, 1996).

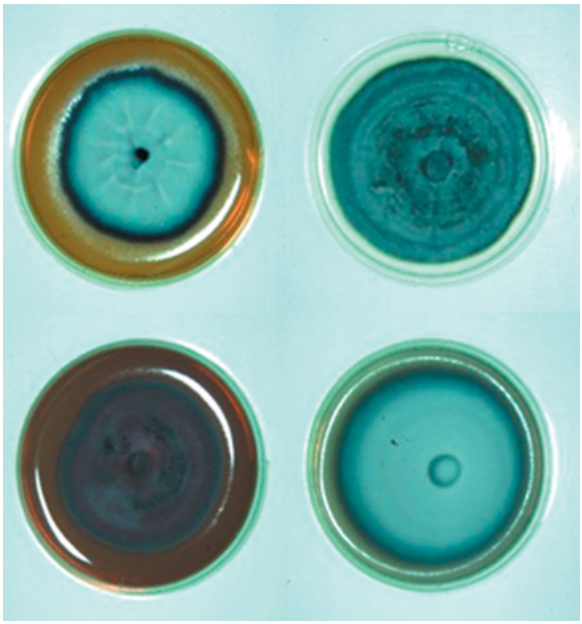

Figure 1 Differences in colony characteristics of *Cercospora piaropi* from water hyacinth after growth for 7 days on PDA. Isolates WHK (upper left), I75–99 (upper right), BA57 (lower left) and WH83 (lower right).

Results

Cultural characteristics

The isolates compared in this study formed colonies on PDA with colours that ranged from pale to dark grey, with pale pink to pink mycelium. The colonies produced reddish-purple or yellow diffusible pigments, with different intensities. In addition, some isolates had dark grey mycelium and these did not produce diffusible pigments (Fig. 1; Table 2).

Reddish-purple pigment was produced by isolates with all the mycelial colours described above, while only isolates with pale pink mycelium produced yellow pigment. The growth rate among the isolates showed significant variation ($P = 0{\cdot}0001$), and ranged from 2·1 to 3·9 mm per day. The data from two independent experiments were combined since the differences between them were not statistically significant ($P = 0{\cdot}5012$) (Table 2).

Toxin analysis and aggressiveness

The isolates showed significant differences ($P \leq 0{\cdot}0001$) in cercosporin production, according to quantification through spectral absorption at 473 nm. The differences in cercosporin production among the isolates varied by up to 22-fold. Data from both experiments were combined for analysis, since the differences between them were not significant ($P = 0{\cdot}5137$).

Table 2 Colour of diffusible pigments produced in axenic culture, mycelial growth rates, and production of cercosporin of *Cercospora piaropi* isolates ranked by disease rating

Isolate	Diffusible pigment colour[a]	Mycelial growth rate (mm day^{-1})[b]	Cercosporin (μg g^{-1})[c]	Cercosporin TLC[d]	Disease rating[e]
BA57	Reddish-purple	2·21 ± 0·10	1·14 ± 0·14	+	6·92
BA59	Reddish-purple	2·52 ± 0·24	0·39 ± 0·04	+	6·71
2867	Reddish-purple	2·57 ± 0·17	0·32 ± 0·03	+	6·67
2619	Reddish-purple	2·17 ± 0·07	0·75 ± 0·10	+	6·54
WH9R	Reddish-purple	2·81 ± 0·17	0·70 ± 0·07	+	6·37
62-2	Reddish-purple	2·13 ± 0·11	0·85 ± 0·04	+	6·37
2703	Reddish-purple	2·71 ± 0·19	0·61 ± 0·11	+	6·25
2702	Reddish-purple	2·46 ± 0·10	0·70 ± 0·10	+	6·04
WHV	Reddish-purple	3·07 ± 0·34	0·47 ± 0·05	+	6·04
I75-101	Reddish-purple	2·77 ± 0·25	0·83 ± 0·14	+	6·04
RR31	Reddish-purple	2·25 ± 0·26	0·59 ± 0·15	+	5·92
SGS49	Reddish-purple	2·34 ± 0·14	0·42 ± 0·06	+	5·75
2943	Reddish-purple	2·67 ± 0·30	0·29 ± 0·04	+	5·96
RR29	Reddish-purple	2·19 ± 0·11	0·67 ± 0·11	+	5·83
67-1	Reddish-purple	2·63 ± 0·20	0·45 ± 0·08	+	5·45
62-4	Reddish-purple	2·32 ± 0·26	0·76 ± 0·09	+	5·33
RR27	Reddish-purple	2·09 ± 0·08	0·56 ± 0·10	+	5·37
BA55	Reddish-purple	2·21 ± 0·10	0·42 ± 0·03	+	5·71
I75-108	Reddish-purple	2·90 ± 0·40	0·37 ± 0·05	+	5·17
I75-102	Reddish-purple	2·52 ± 0·36	0·44 ± 0·05	+	5·17
RR24	Reddish-purple	2·30 ± 0·21	0·25 ± 0·04	NT	5·17
I75-104	Reddish-purple	2·26 ± 0·10	0·42 ± 0·07	+	5·00
RR22	Reddish-purple	2·17 ± 0·16	0·39 ± 0·08	+	4·70
WH83	Reddish-purple	2·88 ± 0·28	0·29 ± 0·03	+	4·41
BC31	Reddish-purple	2·27 ± 0·11	0·31 ± 0·07	+	4·37
10	Reddish-purple	2·84 ± 0·26	0·32 ± 0·07	+	4·21
SGS25	Yellow	2·29 ± 0·46	0·65 ± 0·14	+	4·16
61-5	Reddish-purple	2·40 ± 0·26	0·57 ± 0·07	+	3·87
WHK	Yellow	2·61 ± 0·31	0·31 ± 0·05	+	3·85
RR4	Reddish-purple	2·61 ± 0·31	0·19 ± 0·02	+	3·79
WH9BR	Reddish-purple	2·86 ± 0·17	0·31 ± 0·07	+	3·60
28-1	Reddish-purple	2·69 ± 0·17	0·30 ± 0·04	+	3,60
49-2	Reddish-purple	2·82 ± 0·55	0·20 ± 0·02	+	3·42
J2	Yellow	2·48 ± 0·16	0·23 ± 0·05	NT	3·34
2704	Reddish-purple	3·13 ± 0·93	0·40 ± 0·05	+	3·29
69-1	Yellow	2·74 ± 0·46	0·31 ± 0·05	+	3·08
62-1	Reddish-purple	2·67 ± 0·64	0·20 ± 0·04	+	2·79
400	Reddish-purple	3·04 ± 0·14	0·15 ± 0·03	+	2·58
70-12	Reddish-purple	3·11 ± 0·70	0·21 ± 0·06	+	2·58
TX18	Reddish-purple	2·24 ± 0·16	0·28 ± 0·03	+	2·46
SGS35	Reddish-purple	2·69 ± 0·44	0·23 ± 0·04	NT	2·46
TX20	Reddish-purple	2·27 ± 0·43	0·32 ± 0·09	+	2·35
J6	Yellow	3·00 ± 0·67	+	+	2,10
LJ37	Reddish-purple	3·34 ± 0·11	0·16 ± 0·02	+	1·89
65-2	Reddish-purple	3·59 ± 0·95	0·19 ± 0·04	+	1·67
279	Reddish-purple	3·34 ± 0·42	0·15 ± 0·02	+	1·41
49-1	Reddish-purple	2·48 ± 0·17	0·19 ± 0·04	+	1·41
TX2	Reddish-purple	3·30 ± 0·49	0·15 ± 0·01	+	1·39
TX15	Np	2·55 ± 0·43	0·07 ± 0·01	–	1·00
SGS32	Np	2·82 ± 0·23	0·08 ± 0·02	–	1·00
114	Reddish-purple	3·38 ± 0·17	0·16 ± 0·02	+	1·00
34	Np	3·07 ± 0·61	0·14 ± 0·02	+	1·00
16-1	Np	3·82 ± 0·13	0·08 ± 0·04	–	1·00
18-2	Np	3·49 ± 0·98	0·07 ± 0·04	–	1·00
I75-99	Np	3·60 ± 0·67	0·05 ± 0·02	–	1·00
Control				–	1·00
LSD[f]		–	–	–	0·50
$P > F$[g]		0·0001	0·0001	–	0·0001
Coefficient of variation (%)		6·5	18·7	–	9·3

[a]Observed in 10-day-old cultures grown on PDA plates at 25 ± 1°C and 12-h photoperiod. Np = nonpigmented.
[b]On PDA plates at 25 ± 1°C and 12-h photoperiod. Values are the means ± SD of three replicates of each isolate. Data combined from two experiments.
[c]Production of cercosporin in cultures quantified by spectrophotometry at 473 nm. The procedures for culture, extraction and quantification are explained under Materials and methods.
[d]Production of *Cercospora* toxins in cultures, detected in thin-layer chromatograms (TLC); +, presence of cercosporin; –, cercosporin not detected; NT, not tested. The toxin beticolin-1 was not detected in any sample. The procedures for culturing, extraction and detection by TLC are explained under Materials and methods.
[e]Means of disease ratings on water hyacinth leaves 14 days after inoculation based on a 1–8 scale, where 1 = no symptoms and 8 = dead lamina. Data were combined from three experiments conducted in a quarantine greenhouse.
[f]LSD = Fisher's least significant difference test ($P = 0·05$).
[g]Probability values associated with *F*-tests.

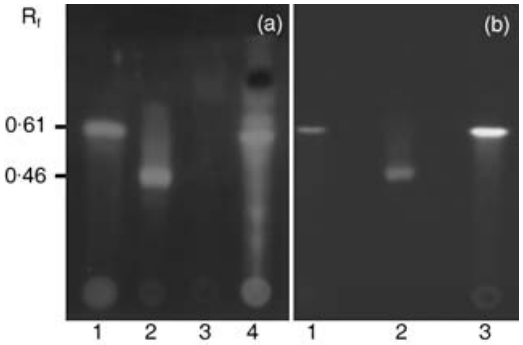

Figure 2 Crude extracts of isolates of *Cercospora piaropi* resolved on thin layer chromatograms and visualized under long-ultraviolet light. (a) Lanes 1–4, standard cercosporin (50 ng), standard beticolin-1 (150 ng), nonpigmented isolate I75–99 and typical yellow-pigment-producer isolate WHK; (b) lanes 1–3, standard cercosporin (25 ng), standard beticolin-1 (75 ng), and typical purple-pigment-producer isolate BA57.

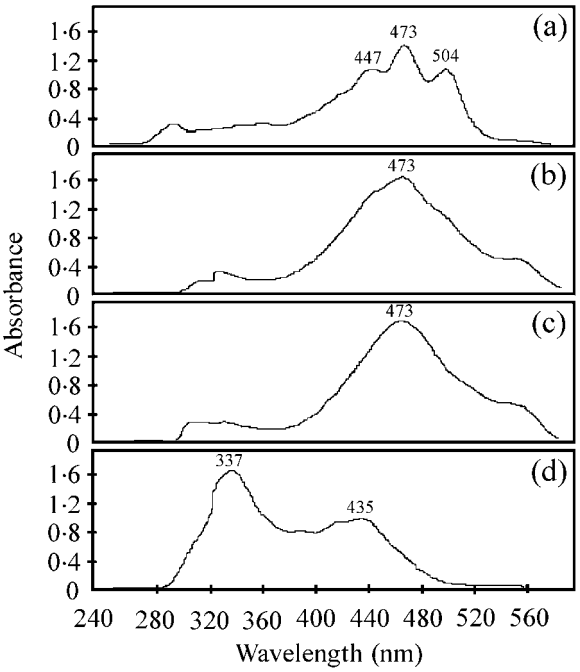

Figure 3 Spectra of crude extracts from the *Cercospora piaropi* yellow-pigment-producer, isolate WHK (a), and the reddish-purple-pigment-producer, isolate BA57 (b), compared to the standards cercosporin (c) and beticolin-1 (d) in ethyl acetate.

Based on the presence of yellow metabolites in some cultures of *C. piaropi* isolates, it was hypothesized that the yellow colour could be related to the toxins beticolins, formerly called the CBT. However, the analyses based on thin-layer chromatograms, resolved under long-UV light and having a standard beticolin-1 for comparison, did not show the presence of beticolins in the samples, but only cercosporin with an R_f value of 0·61 (e.g. isolate WHK in Fig. 2a). According to Milat & Blein (1995), the six beticolin toxins would be expected to have R_f values below that of cercosporin, as shown by the standard for beticolin-1, which had an R_f value of 0·46. Figure 2b shows a thin-layer chromatogram of a crude extract from an isolate that produced a typical reddish-purple pigment and had a band corresponding to cercosporin. The toxin cercosporin was present in the crude extract of most isolates analysed, but the toxin beticolin-1 was not detected in any. Cercosporin was not detected by the TLC method in nonpigmented isolates; however, spectrophotometric analysis revealed absorbance at 473, indicating that this toxin was present in low levels (Table 2).

The ultraviolet spectra of crude extracts for typical yellow and reddish-purple isolates, compared with the spectra of the standards for cercosporin and beticolin-1 in ethyl acetate are shown in Fig. 3. The absorption spectrum of the crude extract of the isolate producing yellow pigment had a maximum peak at 473 nm (Fig. 3a). However, it also showed two extra peaks at 447 and 504 nm compared to the spectrum of the reddish-purple isolate (Fig. 3b), which was very close to the spectrum of pure cercosporin (Fig. 3c). Beticolin-1 was expected to be detected through the UV spectrum, having maximum absorption at 337 nm (Fig. 3d); however, its presence was not evident in the samples analysed with a spectrophotometer. The isolates that were the strongest cercosporin producers, such as BA57, BA59, 2867 and others, were those with more intense reddish-purple diffusible pigments in culture.

The presence of two peaks in WHK at 447 and 504 suggested the presence of two unidentified metabolites in this isolate. This isolate, along with four others from the USA and Brazil, was unique in the production of a yellow pigment in culture. All produced cercosporin in culture, but were not among the most aggressive isolates (Table 2).

The aggressiveness of 55 isolates of *C. piaropi* from several geographical locations was evaluated in three independent experiments and the combined data are presented in Table 2. Significant differences ($P < 0·05$) were observed in the ability of the isolates to cause disease (i.e. difference in aggressiveness) among the isolates. The most aggressive isolates caused necrosis of almost 75% of the leaf area by 14 days after inoculation. Most isolates showed intermediate levels of aggressiveness and some isolates were nonpathogenic to water hyacinth. There was no relation between degree of aggressiveness and geographic origin of the isolates. Highly aggressive as well as nonpathogenic isolates were found among collections from inside and outside South America, the geographic centre of origin of water hyacinth. The ranking of the isolates for level of aggressiveness was conserved among the experiments, with some variation caused by uncontrolled factors. The disease scores of the third experiment were lower than those of the first and second experiments. This variation may have been the result of ambient temperature, since the third experiment was run at a cooler

Table 3 Correlation coefficients[a] among some physiological traits of 55 isolates of *Cercospora piaropi* from several geographical locations

	Aggressiveness experiment 1	Aggressiveness experiment 2	Aggressiveness experiment 3	Cercosporin production experiment 1	Cercosporin production experiment 2	Mycelial growth experiment 1
Aggressiveness experiment 2	0·93	...	...	...	...	...
Aggressiveness experiment 3	0·92	0·91	...	...	...	...
Cercosporin production experiment 1	0·78	0·72	0·89	...	...	...
Cercosporin production experiment 2	0·80	0·73	0·85	0·87	...	...
Mycelial growth experiment 1	−0·60	−0·61	−0·66	−0·62	−0·54	...
Mycelial growth experiment 2	−0·55	−0·55	−0·61	−0·57	−0·50	0·81

[a]$P \leq 0{\cdot}0003$.

Table 4 Fatty acid composition and canonical discriminant analysis of *Cercospora* spp. isolates based on fatty acid profiles of 4-day-old mycelia

	Percentage of total fatty acid content (mean ± SD)						
Isolate/species	14:0	Sum feature 4[a]	16:0	Sum feature 6[b]	18:1 cis 9	18:0	Mean of CAN1[c]
C. oenotherae	0·35 ± 0·04	0·97 ± 0·09	25·59 ± 0·26	40·89 ± 0·71	25·69 ± 0·51	6·63 ± 0·67	23·6582 a
WHK	0·00 ± 0·00	0·74 ± 0·05	24·92 ± 2·03	39·54 ± 2·38	27·41 ± 0·23	7·21 ± 0·88	18·2067 b
C. *beticola*	0·24 ± 0·03	0·87 ± 0·11	25·43 ± 0·49	35·24 ± 0·36	30·26 ± 0·57	7·71 ± 0·69	15·4921 c
Cercospora sp.[d]	0·00 ± 0·00	0·56 ± 0·02	25·41 ± 1·73	27·74 ± 2·40	37·61 ± 1·38	8·60 ± 0·74	−0·2662 d
TX18	0·00 ± 0·00	0·83 ± 0·08	19·33 ± 2·16	47·65 ± 3·08	28·42 ± 0·89	3·78 ± 0·32	−1·0336 de
WHV	0·00 ± 0·00	1·15 ± 0·11	15·63 ± 0·27	50·61 ± 1·22	29·31 ± 1·05	3·31 ± 0·01	−1·0593 def
TX20	0·00 ± 0·00	1·03 ± 0·12	18·79 ± 1·33	51·92 ± 1·43	24·63 ± 0·99	3·62 ± 0·67	−2·3032 efg
2867	0·00 ± 0·00	1·06 ± 0·22	18·39 ± 2·44	48·07 ± 1·24	27·35 ± 1·40	4·96 ± 0·43	−2·5297 efg
WH9BR	0·00 ± 0·00	0·97 ± 0·13	26·00 ± 5·54	39·58 ± 8·59	26·72 ± 1·98	6·60 ± 1·16	−2·7637 fg
2619	0·00 ± 0·00	1·00 ± 0·20	22·77 ± 1·38	45·39 ± 2·43	25·19 ± 0·52	5·40 ± 0·41	−3·1041 g
28-1	0·00 ± 0·00	0·79 ± 0·05	16·46 ± 0·99	53·46 ± 1·48	24·85 ± 0·51	4·03 ± 0·19	−3·2342 g
62-2	0·00 ± 0·00	1·35 ± 0·28	23·08 ± 5·90	42·56 ± 7·05	26·60 ± 0·51	6·02 ± 0·55	−3·4212 gh
114	0·00 ± 0·00	0·83 ± 0·09	15·00 ± 0·49	53·51 ± 1·29	25·75 ± 1·34	4·38 ± 0·64	−3·5484 gh
WH83	0·00 ± 0·00	0·79 ± 0·01	19·36 ± 3·11	49·00 ± 6·10	24·66 ± 1·83	6·06 ± 0·96	−3·6664 gh
400	0·00 ± 0·00	0·96 ± 0·08	16·97 ± 0·46	54·63 ± 0·62	23·28 ± 0·34	3·58 ± 0·39	−3·7964 gh
2943	0·00 ± 0·00	0·86 ± 0·14	16·29 ± 2·28	53·59 ± 3·43	24·18 ± 1·38	4·60 ± 0·63	−3·9350 gh
10	0·00 ± 0·00	0·94 ± 0·19	20·89 ± 2·86	45·42 ± 4·17	25·64 ± 1·56	6·80 ± 0·84	−4·0222 gh
C. gossypina	0·00 ± 0·00	0·61 ± 0·08	19·24 ± 1·38	*51·78 ± 2·62*	21·33 ± 1·17	6·91 ± 0·25	−5·0734 h

[a]Sum feature 4 is an unresolved mixture of 16:1 cis 7c15 iso 20H.

[b]Sum feature 6 is an unresolved mixture of 18:2 cis 6, 9c/18:0 anteiso.

[c]Canonical variable #1, which was created from the fatty acid profiles. CAN1 was the variable that best separated the isolates/species ($R^2 = 0{\cdot}99$; $P \leq 0{\cdot}0001$). Means with the same letter are not significantly different according to Fisher's least significant difference (LSD) test ($P = 0{\cdot}05$).

[d]From *Jasminum* sp.

temperature (May) than the first and second experiments (June/July).

All highly aggressive isolates were also strong producers of diffusible purple pigments, while the producers of yellow pigment were weak to moderate in aggressiveness. The non-pigment-producers were nonpathogenic. The aggressiveness of the isolates was positively correlated with their ability to produce cercosporin, presenting correlation coefficients ranging from 72 to 89% ($P < 0{\cdot}0001$; Table 3). Additionally, aggressiveness was negatively correlated with mycelial growth rate, with correlation coefficients ranging from –55 to –66% ($P < 0{\cdot}0001$), and cercosporin production and mycelial growth were negatively correlated (coefficients of –50 to –62%; $P < 0{\cdot}0003$).

Fatty acid analysis

The *C. piaropi* isolates analysed contained the fatty acids palmitic acid (16:0), oleic acid (18:1 cis 9), stearic acid (18:0) and unresolved mixtures named sum feature 4 (16:1 cis 7c15iso2OH) and sum feature 6 (18:2 cis 6, 9c/18:0 anteiso). In addition, two of the outgroup species also contained the fatty acid myristic acid (14:0). The mean fatty acid composition with standard deviation for each isolate is presented in Table 4. The isolates of *C. piaropi* did not differ in the number and kind of fatty acids present, but differed in the relative concentration of each type. The relative concentration of an individual fatty acid ranged from less than 1% of the total fatty acid content to over 50%. FAME profiles from 4-day-old mycelium

differentiated more of the isolates of *C. piaropi* than the profiles from 5- and 6-day-old mycelia (data from 5- and 6-day-old mycelia not presented). The differentiation of isolates of *C. piaropi* did not relate to the geographical origin of the isolates. In addition, some of the species of *Cercospora* used as the outgroup did not differ from the isolates of *C. piaropi*.

The analysis of correlation between fatty acids content and cercosporin production or aggressiveness among the 14 *C. piaropi* isolates included in the FAME analysis showed only a fair positive correlation between the content of sum feature 4 and cercosporin production ($R^2 = 0{\cdot}57–0{\cdot}72$; $P \leq 0{\cdot}0321$) and a poor positive correlation between the content of sum feature 4 and aggressiveness ($R^2 = 0{\cdot}52$; $P = 0{\cdot}0539$). The FAME contents of the other isolates were not correlated with cercosporin production or aggressiveness.

Discussion

The bioherbicidal strategy to control weeds requires large amounts of inoculum to be delivered to the target weed (Templeton *et al*., 1979; Charudattan, 1988). As a result of technical limitations in the production of large quantities of conidia as inoculum, and because *Cercospora* does not readily sporulate *in vitro*, the biological control strategy developed to control water hyacinth with *C. piaropi* has been based on the use of mycelial mass mixed with conidia as inoculum (Conway, 1976b; Conway & Freeman, 1977; Charudattan *et al*., 1985; Charudattan, 1986). Taking into account these limitations, this study was conducted to determine whether there was a difference in aggressiveness among a collection of isolates of *C. piaropi*, using mycelial suspension as inoculum. Even though hyphae from germinating conidia, as well as vegetative mycelium, can infect water hyacinth by penetration through stomata (Freeman & Charudattan, 1984), the differences between the infection efficiency of conidial versus mycelial suspension is still unknown and probably difficult to quantify. It is reasonable to suspect that when mycelial suspension is used as initial inoculum for leaf pathogens, some steps of the infection process may be missed, compared to when conidia are used. However, the use of mycelial suspension is not unprecedented for pathogenicity studies with *Cercospora* spp. For instance, Upchurch *et al*. (1991) used fungal plug and mycelial inoculation techniques to evaluate pathogenicity of mutants of *C. kikuchi* for cercosporin production in soyabean leaves, because of lack of sporulation in these mutants. Shim & Dunkle (2003) also used mycelial mats and small mycelial fragments to evaluate the pathogenicity of a *C2K3*-disrupted mutant of *C. zeae-maydis*.

Based on a comparison of 55 isolates grown under strictly controlled conditions, isolates of *C. piaropi* differed significantly in their ability to produce cercosporin. Indeed, a positive correlation existed between the ability of the isolates to produce cercosporin and aggressiveness. There were also negative correlations between growth rate and cercosporin production and growth rate and aggressiveness (Table 3). Use of strictly controlled conditions is essential for this kind of study in order to avoid the effect of medium, temperature and light on cercosporin accumulation, as pointed out by Jenns *et al*. (1989). This study showed that the isolates that were nonpigmented and produced the lowest levels of cercosporin remained nonpigmented even when they were grown in three different media. In addition, some isolates apparently did not produce cercosporin and were nonpathogenic. Possibly, these nonpathogenic isolates are cercosporin-minus spontaneous mutants such as Upchurch *et al*. (1991) found among isolates of *C. kikuchi*. These authors found that unlike the UV-induced mutants, cercosporin production by spontaneous mutants of *C. kikuchi* was strongly medium-regulated. However, the nonpigmented isolates in the present study did not produce pigment, even when grown on V8 juice agar and oatmeal medium, in addition to PDA.

The study presented herein provides correlative evidence that cercosporin is a aggressiveness factor in the *E. crassipes*-*C. piaropi* pathosystem. It is well known that cercosporin, a photosensitizing secondary metabolite produced by several *Cercospora* species, has an important role in pathogenesis (Daub & Ehrenshaft, 2000). Recent studies, based on gene disruption, showed that it is an important factor in *C. zeae-maydis* (Shim & Dunkle, 2003) and *C. nicotianae* (Choquer *et al*., 2005) during pathogenesis in maize and tobacco, respectively, being required only after the early stages of infection for extensive colonization and lesion expansion (Shim & Dunkle, 2003).

Shim & Dunkle (2003) and Choquer *et al*. (2005) used mycelia and conidia, respectively, as inocula. In this study, mycelial suspension of *C. piaropi* was used as inoculum because some isolates do not produce sufficient conidia for inoculation. It remains to be properly addressed as to what extent the results here might be limited by this type of primary inoculum.

The possible occurrence of the toxins beticolins in isolates of *C. piaropi* can still not be ruled out. Even though in this study standard procedures defined by Milat & Blein (1995) were used, no known beticolin producer was detected among the water hyacinth isolates and hence no positive control was used. However, the protocol used was straightforward and no beticolin band was detected in any of the more than 330 samples by TLC.

The multivariate discriminant analysis based on FAME profiles had low resolution for differentiating populations among isolates of *Cercospora* from water hyacinth. Even the isolate WH9BR, which belongs to the former species *C. rodmanii* (Tessmann *et al*., 2001), did not differ significantly from most *C. piaropi* isolates (Table 4). Indeed, it is possible to differentiate groups in *Cercospora* by FAME profiles, but these groups may not have any relation to the current species definition of *Cercospora*. In fact, this has been already shown by Berger *et al*. (1991), who compared more than 50 *Cercospora* spp. from 40 different hosts, and observed that different *Cercospora* spp. may have similar fatty acid profiles.

The age of the culture affected the level of resolution of *Cercospora* isolates obtained using FAME profiles. The resolution observed in FAME profiles obtained from 4-day-old mycelia was greater than the resolution obtained from 5- and 6-day-old mycelia. Possibly, this was the result of differences in growth rates among the isolates. The effect of culture age on fatty acid composition was observed also by Stevens Johnk & Jones (1993) and Stahl & Klug (1996). According to the latter authors, the cultures should be used at late lag phase to avoid interference resulting from different growth rates.

This study presented limited evidence of correlation between the FAME content of isolates and cercosporin production or aggressiveness. Correlation among these variables would be expected to occur, since genes having predicted functions in fatty acid metabolism and secondary metabolism can be expressed in cercosporin-inducing conditions, as demonstrated by Shim & Dunkle (2003). However, although the medium used in FAME analysis was based on a standard protocol, it was different from the medium used in cercosporin and aggressiveness analysis and this difference in the gene-expression conditions may have masked differences in the results.

The screening procedure for aggressiveness presented here may also have biotechnological applications, such as the production of crude cercosporin for bioherbicidal formulations, as well as mixing different *C. piaropi* isolates that are known to be high cercosporin-producers in bioherbicidal formulations.

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