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Pathogenic and genetic diversity of soilborne isolates of *Cylindrocladium* from banana cropping systems

Jean-Michel Risède[1] and Philippe Simoneau[2]
[1]*Laboratoire de Pathologie végétale, Station de Neufchâteau, CIRAD-FLHOR, 97130 Capesterre Belle-eau, Guadeloupe (F.W.I) (Fax: +330590868077; E-mail: risede@cirad.fr);*
[2]*UMR de Pathologie Végétale, Faculté des Sciences, Université d'Angers, 2 Bd Lavoisier, F-49045 Angers, France*

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Abstract

Pathogenicity and genetic variation were investigated within a collection of 104 banana-infecting isolates of *Cylindrocladium* (teleomorph *Calonectria*) originating from different countries and representing previously described morphological taxa or species. These root-rot fungi, along with endoparasitic nematodes, have been reported to be causal agents of necrotic lesions that induce root breakage and toppling of banana plants. Little is known about the individual pathogenic effects of the species involved or their genetic diversity. In the present study, among the five morphological taxa found in the banana rhizosphere, only isolates showing an atypical morphology relative to *Cylindrocladium gracile* (named *Cy. gracile*-like isolates) and *Cy. spathiphylli* isolates were pathogenic on banana cultivar Grande Naine. When comparing the latter isolates with others of the same species, but originating from different hosts, an analysis of rDNA spacer polymorphism partitioned isolates of *Cy. spathiphylli* by host into a banana – tea group and a *Heliconia* – *Spathiphyllum* group. Furthermore, isolates from *Heliconia* were not pathogenic on banana. A pathogenicity assessment of representative isolates from the *Cy. gracile*-like and the *Cy. spathiphylli* taxa on six different banana cultivars yielded no evidence of differential interactions between isolates and banana genotypes. Significant differences in susceptibility between banana genotypes were nevertheless detected that could potentially be exploited by breeders. Random amplified polymorphic DNA analysis revealed a genetic similarity ranging from 70% to 100% within *Cy. spathiphylli* isolates from bananas regardless of the geographic origin. Moreover, *Cy. gracile*-like isolates were highly similar but showed only 60% similarity relative to the *Cy. gracile* reference isolates, thus raising questions about their species status.

Introduction

Banana is a herbaceous monocotyledon (genus *Musa*) with edible varieties being diploid (AA, AB) or triploid (AAA, AAB or ABB) (Jenny et al., 1999). Cooking varieties of banana represent a major subsistence food crop for many populations in tropical and subtropical developing countries. Dessert varieties, including cv Grande Naine (AAA, subgroup Cavendish), are also widely used and produce one of the most traded banana on the world market. Banana is grown as a perennial monoculture and is consequently hampered by many recurrent pests and diseases (Jones, 1999; Stover and Simmonds, 1987).Toppling disease is one of the main factors that decrease yields in banana farms (Price, 1995). Brownish to blackish necrotic lesions affect the underground stem and the derived root system consisting of primary fleshy roots which divide into secondary, tertiary or smaller feeding roots (Lassoudière, 1978). These necrotic lesions are caused by a pathogenic complex comprising endoparasitic nematodes along with soilborne root-rot fungi, and lead to a decrease in nutrient uptake, root breakage and plant toppling (Gowen and Queneherve, 1990; Jones, 1999;

Loridat, 1989; Price, 1995). Root-rot fungi within this biotic complex differ in their pathogenic status. Most are considered as secondary lesion invaders that are unable to colonize healthy roots. However, fungi of the genus *Cylindrocladium* have been shown to actively participate in the necrotic process that induces toppling disease and yield loss (Loridat, 1989; Risède, 1994; Semer et al., 1987). This genus contains species which affect plants from many plant families throughout the world (Crous, 2002; Crous and Wingfield, 1994; Peerally, 1991). When produced, teleomorphs belong to the filamentous euascomycete genus *Calonectria* (Nectriaceae, Hypocreales) (Crous and Wingfield, 1994; Rossman, 1979; 1983) and are either homo- or heterothallic.

Cylindrocladium species have been reported as banana root-rotting agents in Central America (Semer et al., 1987), the West Indies (Loridat, 1989; Risède, 1994) and Africa (Castaing et al., 1996; Kobenan, 1991). However, until recently very little research focused on the diversity of species involved. Through morphological analysis, sequence analysis of the amplified internal transcribed rDNA spacer (ITS) region, and restriction fragment length polymorphism of the intergenic rDNA spacer region (IGS RFLP), Risède and Simoneau (2001) determined that two major *Cylindrocladium* taxa were associated with necrotic lesions in bananas. One taxon grouped *Cy. gracile*-like isolates showing atypical *Cy. gracile* morphology (large cylindrical monoseptate conidia and clavate vesicles) but close phenetic clustering with typical *Cy. gracile* reference isolates. Other isolates were shown to be *Cy. spathiphylli*. In addition, three other rare taxa that obviously represented morphological taxa distinct from the two above-mentioned species, were also detected (unpublished results). Based on the same morphological and molecular typing methods, most of these isolates were found to be similar to *Cy. scoparium*. However, one isolate belonged to the *Cy. floridanum* species complex and another was typical of *Cy. gracile*, both originating from Cameroon. The relative incidence of each *Cylindrocladium* taxon in this complex in the banana rhizosphere remains unknown. Moreover, no studies to date have assessed genetic variation within their populations.

The objectives of the present study were twofold. First, the relative pathogenic contribution of each taxon associated with *Cylindrocladium* root-rot lesions of bananas was determined. To facilitate this study, a reproducible pathogenicity assay method for *Cylindrocladium* isolates on banana plants was developed. Secondly, we assessed genetic variation within *Cylindrocladium* taxa that were shown to be pathogenic on bananas. Randomly amplified polymorphic DNA (RAPD) markers and IGS RFLP profiles were used to evaluate intraspecific diversity, and to determine whether there was a relationship between fungal genotypes, geographic origin and pathogenicity.

Materials and methods

Fungal isolates

A collection of 104 isolates from banana cropping systems was established by root-rot or direct soil isolations (Risède, 1994; 1995). Following single-spore transfer, all isolates were stored by cryopreservation at $-80\,^{\circ}$C of pre-colonized banana-leaf fragments in 10% glycerol (v : v). Most isolates belonged to morphological taxa previously described as *Cy. gracile*-like and *Cy. spathiphylli*, while the others showed distinct morphology (Table 1). Six additional field isolates were recovered in Guadeloupe from root lesions of *Heliconia caribaea*, a species whose botanical family (Heliconiaceae) is a sister to that of bananas (Musaceae) within the order Zingiberales. These isolates of *H. caribaea* were similar in morphology to the *Cy. spathiphylli* isolates from banana, so both of them were further examined and compared. A small collection of reference isolates of *Cylindrocladium* species was also used. It included three *Cy. gracile* strains (PC 551197, ATCC 22833, IMI 346843), two *Cy. pteridis* strains (PPRI 4157, IMI 354530) and three *Cy. spathiphylli* strains (ATCC 44730, CBS 53887, IMI 167983). The two former *Cy. spathiphylli* strains were isolated from *Spathiphyllum* sp., and the third one from *Camellia sinensis* (tea).

Banana varieties used in pathogenicity tests

A two-step procedure was adopted for the assessment of pathogenicity. First, whole potted cv Grande Naine banana plants were used to evaluate intraspecific and interspecific pathogenicity variability in a subset of 49 isolates of banana and *H. caribaea* from diverse geographic origins. Second, the reactions of six banana genotypes were also evaluated towards two isolates from banana shown to be pathogenic in step one. These genotypes were selected to represent hybrids with potential commercial interest (IDN 110 (AA), IRFA 914 (AAB)), varieties used as parents in current

Table 1. Origins of *Cylindrocladium* field isolates used in this study

Isolates	Origin	
	Country	Host/ substrate
Cy. gracile (small cylindrical 1-septate conidia, clavate vesicle)		
Cam14[s]	Cameroon	Banana*
Cy. gracile-like (large cylindrical 1-septate conidia, clavate vesicle)		
Mar1, Mar2, Mar3, Mar4, Mar5, Mar8, Mar9, Mar10, Mar11, Mar14, Mar15, Mar16, Mar17, Mar18, Mar19, Mar20, Mar21, Mar22, Mar23, Mar24, Mar25, Mar26, Mar27, Mar28, Mar29, Mar30, Mar31, Mar32, Mar33, Mar34, Mar35, Mar36, Mar37, Mar38, Mar39	Martinique	Banana
Gua8, Gua9, Gua10, Gua11, Gua12[s], Gua24[s], Gua25, Gua26, Gua27, Gua28, Gua29	Guadeloupe	Banana*
Slu1, Slu2, Slu3, Slu4, Slu5, Slu6, Slu7	Saint Lucia	Banana
Cy. spathiphylli (large cylindrical 1- to 3-septate conidia, globose vesicle)		
Mar7	Martinique	Banana
Cam1, Cam2[s], Cam3, Cam4, Cam5, Cam6[s], Cam7, Cam8[s], Cam15, Cam16, Cam17, Cam18, Cam19	Cameroon	Banana*
Cor1, Cor2, Cor3, Cor4, Cor5, Cor6, Cor7	Costa Rica	Banana
Dom1, Dom2, Dom4, Dom5, Dom6, Dom7	Dominica	Banana
Gua1, Gua2, Gua3, Gua4, Gua5, Gua14, Gua15, Gua16, Gua17, Gua18, Gua19, Gua20, Gua21, Gua22, Gua23	Guadeloupe	Banana
Hel2A, Hel2B, Hel2C, Hel7A1, Hel7A2, Hel8A1	Guadeloupe	*Heliconia*
Cy. scoparium-like (small cylindrical 1-septate conidia, pyriform vesicle)		
Mar12, Mar13[s]	Martinique	Banana*
Cam9[s], Cam10[s], Cam11, Cam12	Cameroon	Banana*
Gua13[s]	Guadeloupe	Banana*
Cy. floridanum-like (small cylindrical 1-septate conidia, sphaero-pedonculate vesicle)		
Cam13[s]	Cameroon	Banana*

*Strains with a name followed by the letter 's' were isolated from soil of banana plots. Strains from banana were isolated from AAA cultivars, except Cam2 from AAB cv. Strains with an underlined name were used in pathogenicity tests.

breeding programs (Pisang Madu (AA), Kunnan (AB), PKW (BB)) and a worldwide grown cultivar (Grande Naine (AAA)).

Micropropagated banana plants were weaned for 4 weeks on peat moss in small pots (4 cm × 4 cm × 4 cm) under greenhouse benches (23–28 °C; 80–90% RH; daily drip fertilization). They were grown for 4 additional weeks in 0.6-l pots on the same substrate under greenhouse conditions.

Inoculum preparation and inoculation procedure

For inoculum production, intense conidiogenesis was obtained by growing cultures on banana leaf agar (BLA) (Risède and Simoneau, 2001) under continuous fluorescent cool white and near-ultraviolet lights, at 25 °C for 7 days. Cultures were flooded with sterile water amended with Triton X 100 surfactant ($250\,\mu l\,l^{-1}$), and shaken gently. The resulting conidial suspension was filtered through a 32 μm sieve and concentrated by centrifugation. The conidial concentration was adjusted to 2×10^3 conidia ml^{-1}. Just before inoculation, peat was gently removed from the root system of 8-week-old banana plants under running tap water. The root system of each plant was dipped for 20 s in 250 ml of the conidial suspensions. Banana plants were left to drain for 1 min, before transplantation into 2-l pots filled with a balanced mixture of sand and perlite. Eighteen plants were inoculated for each of the 49 isolates tested (Table 1). Controls were performed using the same procedure except that the conidial suspension was replaced by sterile water amended with Triton X 100 ($250\,\mu l\,l^{-1}$). The Gua5 isolate was previously found to be pathogenic on bananas (Declerck et al., 2002) and was therefore used as positive control. Following inoculation, plants were maintained in a growth chamber for the remainder of the experiment (temperature: 26 °C (day) and 22 °C (night); 80–90% relative humidity; daily photoperiod: 12 h) according to a completely randomized design. This experiment was repeated once.

Disease assessment

Root lesion severity was assessed at 4, 8 and 12 days after inoculation on six randomly selected plants per

date and per isolate. After gentle removal of potting mixture from the root system under running tap water, each primary root and its lateral daughters (the whole unit further considered as a 'bundle') was detached from the underground stem. Each root bundle was visually rated using the following 0–5 scale: 0, no lesions; 1, 1–20% of root area is necrotic; 2, 21–40%; 3, 41–60%; 4, 61–80% and 5, more than 80%. A root necrotic potential (RNP) value was determined for each isolate on each plant and at each sampling date. RNP represents a global measure of isolate pathogenicity and is quantified according the following formula: RNP = (0.105 × number of bundles rated '1') + (0.305 × number of bundles rated '2' + ····· + (0.905 × number of bundles rated '5')/total number of bundles, where 0.105, 0.305, 0.505, 0.705 and 0.905 are the central values of the five root necrotic area classes. At each date, a mean RNP value was calculated for each isolate from the six replicate plants. Mean RNP changes were monitored 4, 8 and 12 days after inoculation.

RNP data were submitted to variance analysis using SAS GLM procedures (SAS Institute, Cary, NC). The residuals of the data met the assumption of normality with the $(RNP)^{1/2}$ transformation. Tukey's Studentized Range Test was used for comparisons and groupings among isolates.

Sexual compatibility tests

Sexual compatibility among *Cy. spathiphylli* isolates was tested by mating, in all possible combinations, isolates Hel2A and Hel2B from *Heliconia*, isolates Dom4 and Cor4 from bananas and the two reference isolates of *Cy. spathiphylli* ATCC 44730 and IMI 167983. Pairwise cultures of these isolates were grown on BLA and incubated at 25 °C for 4–5 weeks under continuous fluorescent cool white and near-ultraviolet lights. Five replicates were performed for each mating combination. Cultures were examined weekly under a binocular stereo microscope for perithecial development. Perithecia were considered fertile when ascospores were able to germinate and grow on 1% malt-extract agar.

Genetic characterization

For each isolate, Petri dishes of malt-extract agar (2%) overlaid with a sterile cellophane membrane sheet were inoculated with a mycelial suspension. Forty-eight to sixty hours later, mycelia were scraped off the membrane from a 2 cm^2 area and stored at −80 °C. Genomic DNA was extracted using a microwave miniprep method (Goodwin and Lee, 1993). The final DNA pellet was re-suspended in 100 µl of TE buffer (10 mM Tris–HCl, 0.1 mM EDTA, pH 8) and stored at −20 °C.

rDNA spacer polymorphism was investigated to assess genetic diversity among *Cy. spathiphylli* isolates from bananas and *Heliconia*. The whole transcribed ITS region of three randomly chosen isolates from *H. caribaea* was amplified with the universal primers ITS1 and ITS4 (White et al., 1990). PCR products were sequenced with an automatic sequencer (373XL Applied BioSystems) by Genome Express (Grenoble, France). Sequencing was performed on both DNA strands with the same ITS primers used in the initial amplification step. Nucleotide sequences were deposited in GenBank with the following accession numbers: AY147189 (isolate Hel7A1), AY147190 (isolate Hel8A1) and AY147191 (isolate Hel2B). Using the multiple-alignment program CLUSTAL W (Thompson et al., 1994), nucleotide sequences of isolates from *H. caribaea* were aligned and compared with the published sequences of *Cy. spathiphylli* reference isolates. IGS RFLP was also investigated after PCR amplification with primers CNL12 and NS21 (Duchesne and Anderson, 1990) as described previously (Risède and Simoneau, 2001).

For the RAPD analyses, 24 decamer primers among Operon Kits E, BH, H and S (Operon Technologies Inc., Alameda, CA., USA) were chosen randomly and tested on genomic DNA from 16 geographically diverse isolates from banana and *H. caribaea*, from the major morphological taxa. After testing to optimize the reaction conditions, six primers that showed informative and reproducible banding patterns were selected: OPE-02: 5′-GGTGCGGGAA-3′; OPE-03: 5′-CCAGATGCAC-3′; OPE-04: 5′-GTGACATGCC-3′; OPBH-03: 5′-GG AGCAGCAA-3′; OPBH-04: 5′-ACCTGCCAAC-3′ and OPBH-06: 5′-TCGTGGCACA-3′. The first five were used to evaluate genetic diversity among *Cy. gracile* isolates and similar taxa from the field. The last five primers were used to detect polymorphism among field and reference *Cy. spathiphylli* isolates. Each PCR amplification was performed with a M. J. Research PTC 100 thermal cycler in 50 µl total volume containing 1 unit of *Taq* DNA Polymerase (Eurobio, France) in an appropriate PCR reaction buffer, 1.5 mM $MgCl_2$, 200 µM of each nucleotide, 2 µM of primer and 25 ng of genomic DNA. Thermal cycling parameters were set at 92 °C

for 30 s (denaturation), 37 °C for 30 s (annealing) and 72 °C for 1 min (extension). After 40 cycles, PCR products were resolved by electrophoresis through 1.5% agarose gels in TBE buffer (90 mM Tris–Borate, 1 mM EDTA pH 8.0). A 100-bp ladder (MBI Fermentas Ltd) was used as DNA size marker. Each isolate was characterized by the combined fingerprint patterns obtained with the five primers. Without taking its intensity into account, the presence (1) or absence (0) of a band was scored as two alleles at a single genetic locus. Dissimilarity binary matrices were constructed to estimate the genetic distance between pairs of isolates according to the coefficient of Sokal and Michener (1958). The data were used for cluster analyses using the unweighted pair-group method with arithmetic averages (UPGMA) and the Phylogeny Inference Package (PHYLIP ver 3.55c, Felsenstein, 1995). Dendrograms were drawn using the NJPLOT software program (Perrière and Gouy, 1996). Their statistical support was assessed by performing 1000 bootstrap replicates for each analysis.

Results

Pathogenicity characteristics of Cylindrocladium *field isolates*

In most cases, inoculation of banana cv Grande Naine with isolates of *Cylindrocladium* yielded root necrotic lesion symptoms that differed in intensity. When present, symptoms were similar to those obtained in previous experiments with the *Cy. spathiphylli* isolate Gua5 (positive control), and resembling those described by Loridat (1989). Symptom types did not vary among isolates. Necrotic lesions generally started as individual 1–5 mm long flecks that gradually enlarged in length, width and depth to become well-defined oblong lesions that coalesced to rot large portions of roots. They affected the fine lateral roots, as well as all other root types including secondary and primary roots (Figure 1a). For some isolates, necrotic lesions were already prominent as early as 4 days after inoculation. Symptoms were not found

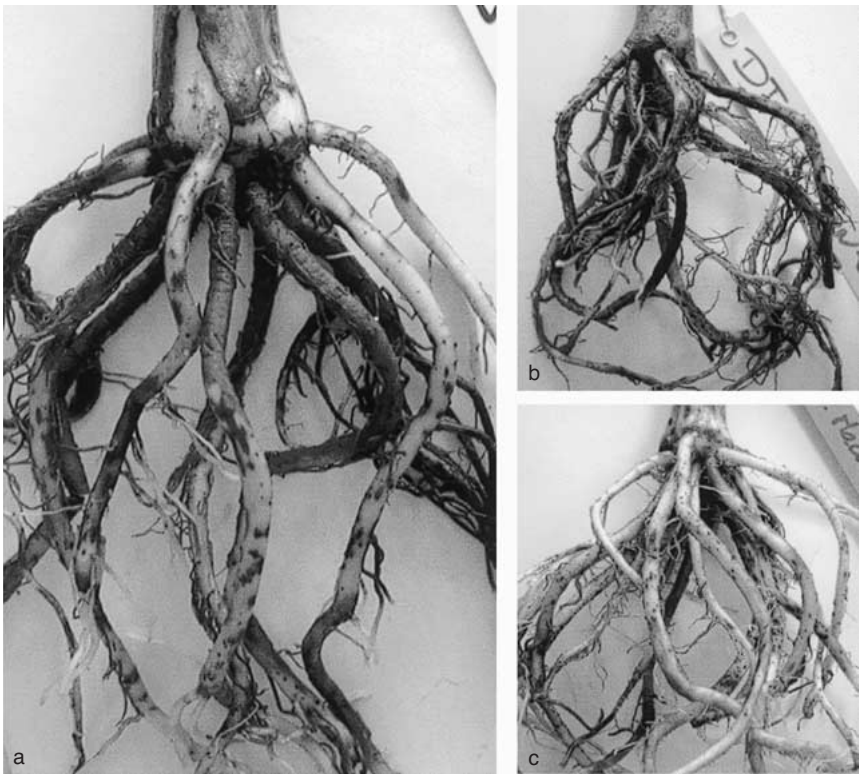

Figure 1. Necrotic lesions on banana roots induced by a *Cy. spathiphylli* isolate from the banana rhizosphere (Gua5); (a) cv Grande Naine (AAA) 8 days after inoculation, (b) cv Kunnan (AB) 12 days after inoculation and (c) cv Pisang Madu (AA) 12 days after inoculation.

on noninoculated control plants. Analysis of variance (ANOVA) on root square-transformed RNP to normalize variance highlighted significant differences in disease severity among isolates (Table 2). Mean RNP comparisons with the Tukey's test revealed a continuous variation with many overlapping groups. Nevertheless, two main severity categories of isolates could be observed (Figure 2a).

Table 2. ANOVA on pathogenicity on banana cv Grande Naine measured by RNP among 49 *Cylindrocladium* field isolates from banana (43) and from *Heliconia* (6). Data were root square-transformed prior to the analysis to normalize variance

Date	Source of variation	DF[a]	Mean square	Pr > F[b]
4 days after inoculation	Isolate	48	0.10594716	<0.0001
	Error	242	0.00129684	
8 days after inoculation	Isolate	48	0.20376438	<0.0001
	Error	239	0.00132279	
12 days after inoculation	Isolate	48	0.27838807	<0.0001
	Error	239	0.00150136	

[a]Degrees of freedom.
[b]Probability values associated with the F-tests.

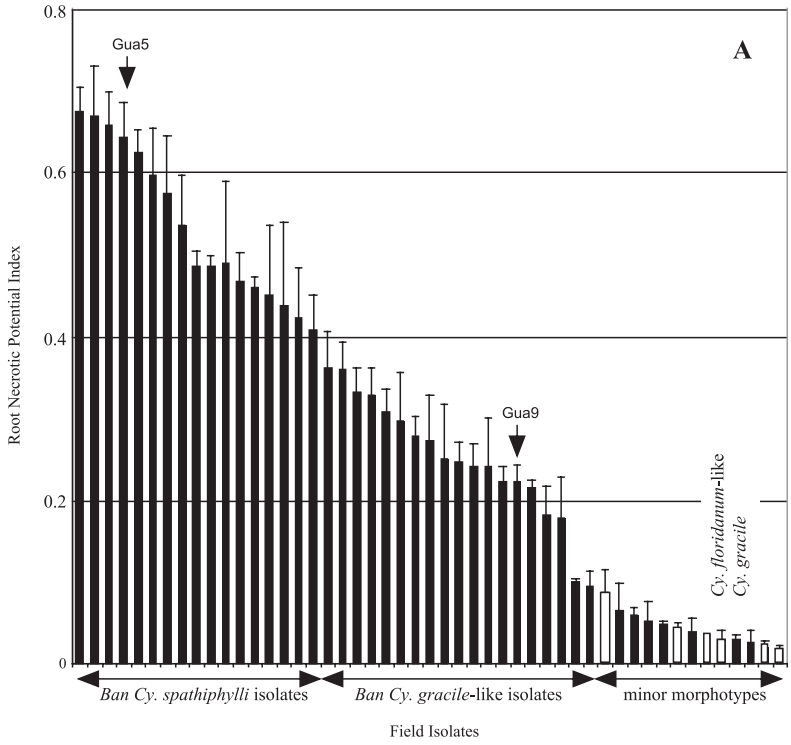

Figure 2. Pathogenicity on banana cv Grande Naine (AAA) of field isolates. Pathogenicity of each isolate was evaluated by a RNP index (0–1) based on an assessment of rot lesions severity as defined in the Materials and methods. RNP are represented by mean values. Error bars indicate standard deviation. (a) RNP values after 12 days are given for 49 field isolates derived from banana (black bars) or *Heliconia* (white bars) plants. Minor morphological taxa correspond to *Cy. scoparium*-like isolates and the two African *Cy. floridanum*-like and *Cy. gracile* isolates. The two latter isolates are identified above corresponding bars. (b) RNP values after 4 (black bars), 8 (white bars) and 12 (stripped bars) days are given for 14 selected isolates representing the five morphological taxa found in the banana and *Heliconia* rhizospheres and various geographic origins.

The first category grouped isolates that were clearly pathogenic, and encompassed all *Cy. spathiphylli* isolates from banana and *Cy. gracile*-like isolates. These isolates exhibited a RNP that steadily increased with time, with greater than 80% isolation frequency from inoculated banana plants (data not shown). *Cy. spathiphylli* isolates from banana were highly pathogenic whereas *Cy. gracile*-like isolates generally showed medium pathogenicity (Figure 2b).

The second category grouped the single isolate showing a typical *Cy. gracile* morphology, the single *Cy. floridanum*-like isolate, along with *Cy. scoparium*-like isolates and isolates from *H. caribaea*. All of these isolates were re-isolated from inoculated banana plants with frequencies of less than 30%. They were weakly pathogenic to almost nonpathogenic on banana cv Grande Naine, and had a mean RNP score of less than 0.1, which remained very low even 12 days after inoculation (Figure 2).

We further studied two isolates of the 'pathogenic category' by inoculating them on six different banana genotypes. Isolates Gua9 and Gua5, representing the *Cy. gracile*-like taxon and *Cy. spathiphylli*, were chosen for their different levels of pathogenicity on cv Grande Naine. All banana genotypes expressed symptoms in response to both isolates 4 days after inoculation, but with different levels of lesion severity (Figure 3). Root damage increased 8 or 12 days after inoculation to various extents in the different cultivars. Indeed at these two dates, ANOVA performed to assess lesion severity (expressed as RNP) revealed significant isolate and genotype effects (Table 3). No isolate × genotype interaction could be detected 8 days after inoculation.

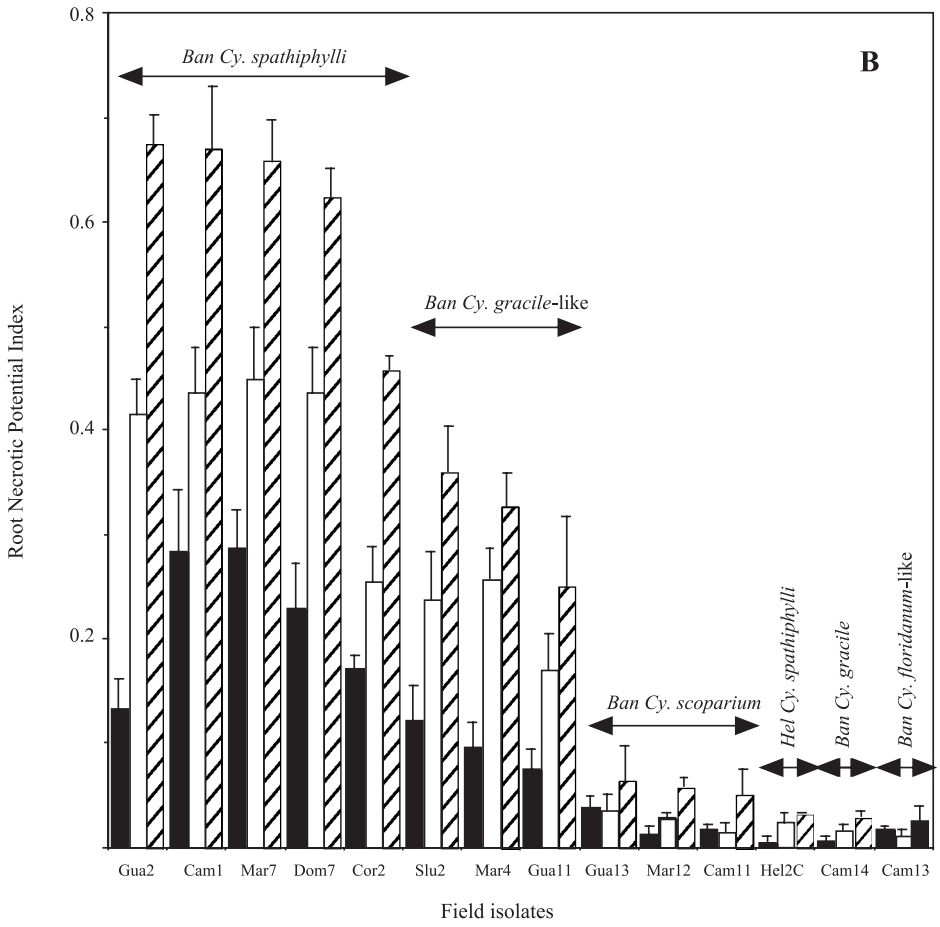

Figure 2. (*Continued*)

Isolates were ranked by banana genotypes in the same manner, with Gua5 always being more pathogenic than Gua9. Genotypes were also ranked by the two isolates in an identical way. Tukey's Studentized Range Test applied on mean RNP scores indicated that IDN 110 (AA) was significantly ($P = 0.05$) the most susceptible banana genotype 8 days after inoculation (Table 4). It was followed by IRFA 914 (AAB) and Kunnan (AB) which also revealed high susceptibility (Figure 1b). Grande Naine differed from this second group and exhibited a medium susceptibility. In contrast, both PKW (BB) and P. Madu (AA) were significantly less susceptible than any of the four other banana genotypes. At 8 days, with a RNP of less than 0.2, P. Madu showed the lowest susceptibility towards isolates Gua5 and Gua9 (Figure 1c). Twelve days after inoculation, although a significant isolate × genotype interaction was detected, its contribution to the total variation was much lower than that of isolates or even of genotypes (Table 3). At this date, the mean square for the isolate

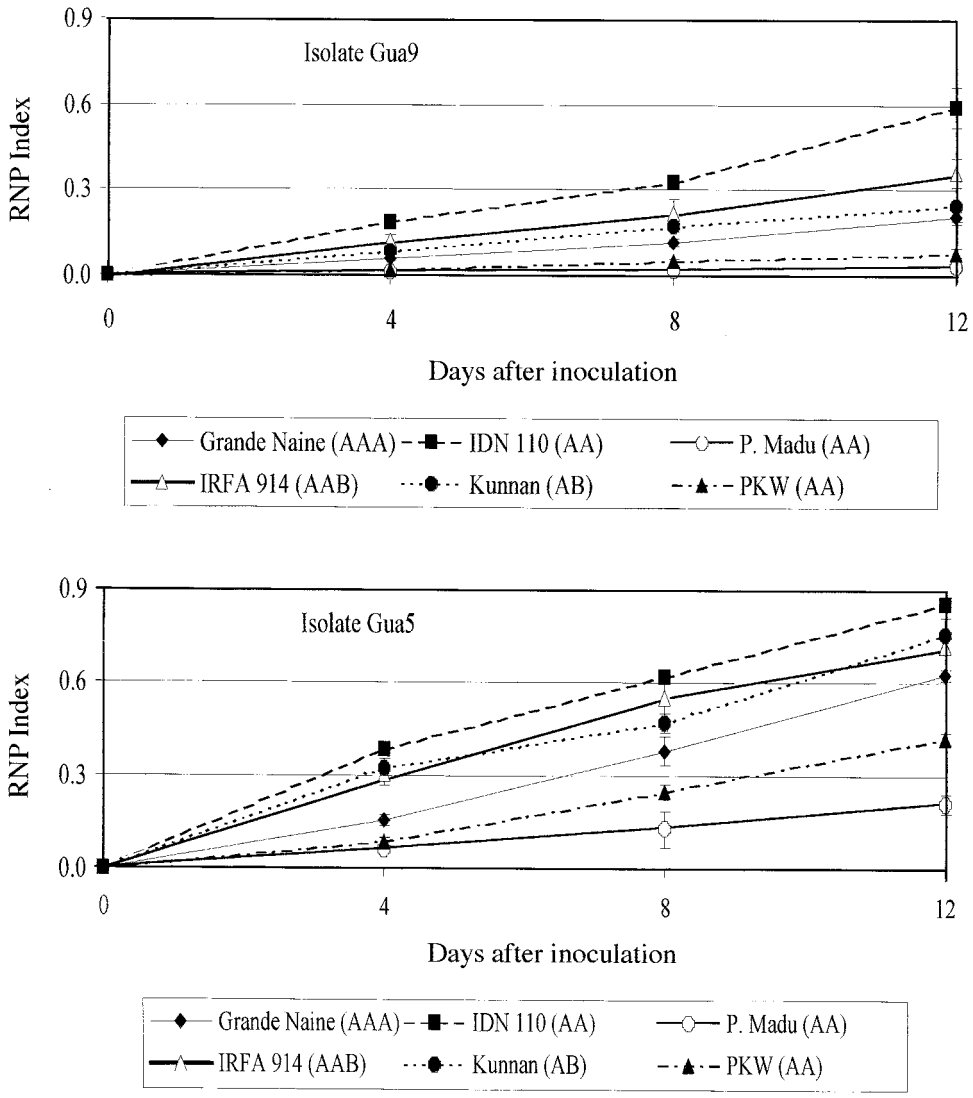

Figure 3. Pathogenicity of a *Cy. gracile*-like isolate (Gua9) and a *Cy. spathiphylli* isolate (Gua5) on six banana genotypes. These two strains were isolated from banana fields. Pathogenicity was measured by RNP, as defined in the Materials and methods. Error bars indicate standard deviation.

Table 3. ANOVA describing effects of isolate, banana genotype and interaction on RNP, 8 or 12 days after inoculation of two *Cylindrocladium* isolates (Gua9 or Gua5) on six banana genotypes. Data were root square-transformed prior to the analysis to normalize variance

Date	Source of variation	DF[a]	Mean square	Pr > F[b]
8 days after inoculation	Isolate	1	1.04560239	<0.0001
	Genotype	5	0.27535182	<0.0001
	Isolate × genotype	5	0.00278337	0.1128*
	Error	53	0.00147792	
12 days after inoculation	Isolate	1	1.31800308	<0.0001
	Genotype	5	0.39531587	<0.0001
	Isolate × genotype	5	0.01828277	<0.0001
	Error	51	0.00109387	

[a]Degrees of freedom.
[b]Probability values associated with the F-tests.
*Nonsignificant.

Table 4. Tukey grouping of six banana genotypes based on expression of RNP of two *Cylindrocladium* isolates (Gua9 or Gua5), 8 days after inoculation. Data were root square-transformed prior to the analysis to normalize variance

Mean*	*N*	Genotype
0.68103[a]	10	IDN 110 (AA)
0.59257[b]	11	IRFA 914 (AAB)
0.54909[b]	12	Kunnan (AB)
0.49122[c]	11	Grande Naine (AAA)
0.35804[d]	10	P.K.W. (BB)
0.23629[e]	11	P. Madu (AA)

*Means followed by the same letter are not significantly different ($P = 0.05$) according to Tukey Studentized Range Test.

effect was higher than for the banana genotype effect, therefore illustrating primacy of variation between the two considered isolates.

Sexual compatibility among Cy. spathiphylli *isolates*

Cultures that were tested individually (self crosses) yielded no perithecia. Conversely, typical *Calonectria* perithecia were obtained from crosses between the banana *Cy. spathiphylli* isolates Dom4 or Cor4 and the reference strain IMI 167983, therefore confirming previous results (Risède and Simoneau, 2001). In turn, the *Cy. spathiphylli* isolates from banana were able to induce fertile perithecia when mated with the two isolates from *H. caribaea* Hel2A and Hel2B. Fertile perithecia were also obtained when the two *Cy. spathiphylli* reference strains (ATCC 44730 and IMI 167983) were mated.

rDNA spacer polymorphism between Cy. spathiphylli *isolates*

Following PCR amplification, ITS sequences were determined for Hel7A1, Hel8A1 and Hel2B. Using CLUSTALW they were aligned and compared with the previously published sequences of the *Cy. spathiphylli* field isolates Cam1, Cor4 and Dom4 (GenBank accession numbers: AF268481–AF268483) from banana (Risède and Simoneau, 2001), but also with the ITS sequences of three *Cy. spathiphylli* isolates from *Spathiphyllum* (AF124345-AF124347, Schoch and Crous, 1999), and those of two *Cy. spathiphylli* reference isolates, the ex-type strain ATCC 44730 from *Spathiphyllum* (AF124344) and the strain IMI 167983 from tea (AF261738). These 11 sequences differed by only one nucleotide substitution in position 112 (numbering as in Jeng et al., 1997) and therefore fell into two groups. One group encompassed *Cy. spathiphylli* isolates from *Spathiphyllum* or *Heliconia* (nucleotide at position 112 is a C), while the other clustered all the banana *Cy. spathiphylli* isolates and the single *Cy. spathiphylli* reference isolate from *C. sinensis* (nucleotide at position 112 is an A).

When studying polymorphism in the amplified IGS region, all *Cy. spathiphylli* isolates showed identical *Pst* I, *Rsa* I and *Mva* I profiles. Only the endonuclease *Ava* II revealed polymorphism within this species, with two patterns (Figure 4). The first

pattern was shared by *Cy. spathiphylli* isolates from *Spathiphyllum* and *Heliconia*, while the second was shared by *Cy. spathiphylli* isolates from banana and *C. sinensis*.

Genetic variation within Cy. gracile*-like and* Cy. spathiphylli *isolates using RAPD markers*

Two RAPD analyses were conducted. The first one concerned 52 *Cy. gracile*-like isolates from banana, the single isolate with typical *Cy. gracile* morphology, three *Cy. gracile* reference strains and two *Cy. pteridis* reference isolates. This analysis was conducted with the five primers OPE 02–04, OPBH03 and OPBH04. These primers generated three to seven reproducible PCR products, each ranging in size from 200 to 1200 bp. When combining the banding patterns from these five primers, 58 fragments were scored as polymorphic markers. Nevertheless, the detected polymorphism was mainly due to a distinct genetic divergence between *Cy. gracile*-like isolates on one hand, and the single *Cy. gracile* isolate along with the *Cy. gracile* reference strains on the other. *Cy. gracile*-like isolates indeed exhibited almost uniform banding patterns (Figure 5) irrespective of the different tested primers, thus revealing a very low intragroup polymorphism. Cluster analysis based on UPGMA further confirmed that these two groups were distinct entities (I and II) that both remotely clustered with *Cy. pteridis*, a morphologically similar species (Figure 6). Within group I, isolates showing *Cy. gracile*-like morphology were found to share about 95% similarity. In the second group (II), the isolate showing typical *Cy. gracile* morphology was found to closely cluster with reference strains of this species, thus supporting a possible conspecificity. Isolates from the two groups shared only 60% similarity. The second RAPD analysis focused on the extent of genetic variation among *Cy. spathiphylli* isolates, by examining 40 *Cy. spathiphylli* from banana and five from *Heliconia*, two *Cy. spathiphylli* reference strains from *Spathiphyllum* (ATCC 44730 and CBS 538.87) and another one from *C. sinensis* (IMI 167983). The selected primers, OPE03, OPE04, OPBH 03, 04 and 06 each generated 4–7 reproducible PCR products ranging from fewer than 200 to more than 1000 bp. Divergence

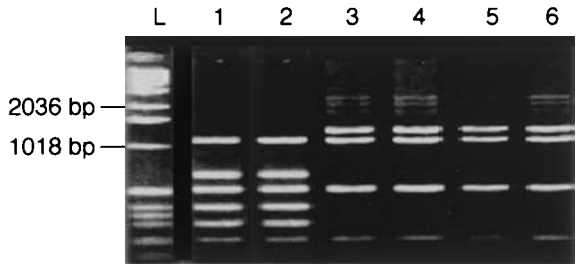

Figure 4. *Ava* II RFLP patterns of the intergenic spacer of *Cy. spathiphylli* and *Cy. spathiphylli*-like isolates from different hosts. *L* = 100 bp DNA ladder. Lane 1: *Cy. spathiphylli* ATCC 44730 from *Spathiphyllum*, lane 2: Hel2B *Cy. spathiphylli*-like isolate from *Heliconia*, lane 3: *Cy. spathiphylli* IMI 167983 from tea, lanes 4–6: *Cy. spathiphylli* isolates from bananas. Isolate CBS 538.87 from *Spathiphyllum* is as in lane 1. Other isolates from *Heliconia* are as in lane 2, while all *Cy. spathiphylli* isolates from bananas are as in lanes 4–6.

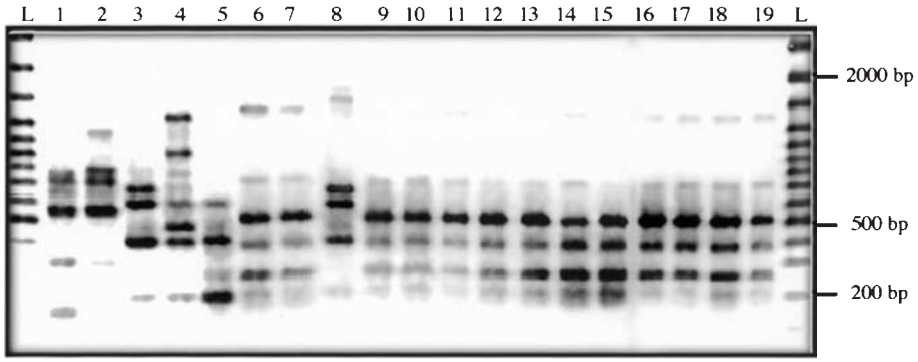

Figure 5. RAPD profiles of *Cylindrocladium* isolates with clavate vesicles generated by the OPE02 primer. Lane L: 100 bp DNA ladder, lane 1: *Cy. pteridis* PPRI 4157, lane 2: *Cy. pteridis* IMI 354530, lane 3: *Cy. gracile* PC551197, lane 4: *Cy. gracile* ATCC 22833, lane 5: *Cy. gracile* IMI 346843, lanes 6, 7 and 9–19: *Cy. gracile*-like isolates from banana (Mar14, Mar15, Mar1, Mar3, Mar20, Mar24, Gua9, Gua12, Gua25, Gua28, Slu1, Slu4, Slu5, respectively) and lane 8: *Cy. gracile* isolate from banana (Cam14). A negative view of the original stained gel is shown.

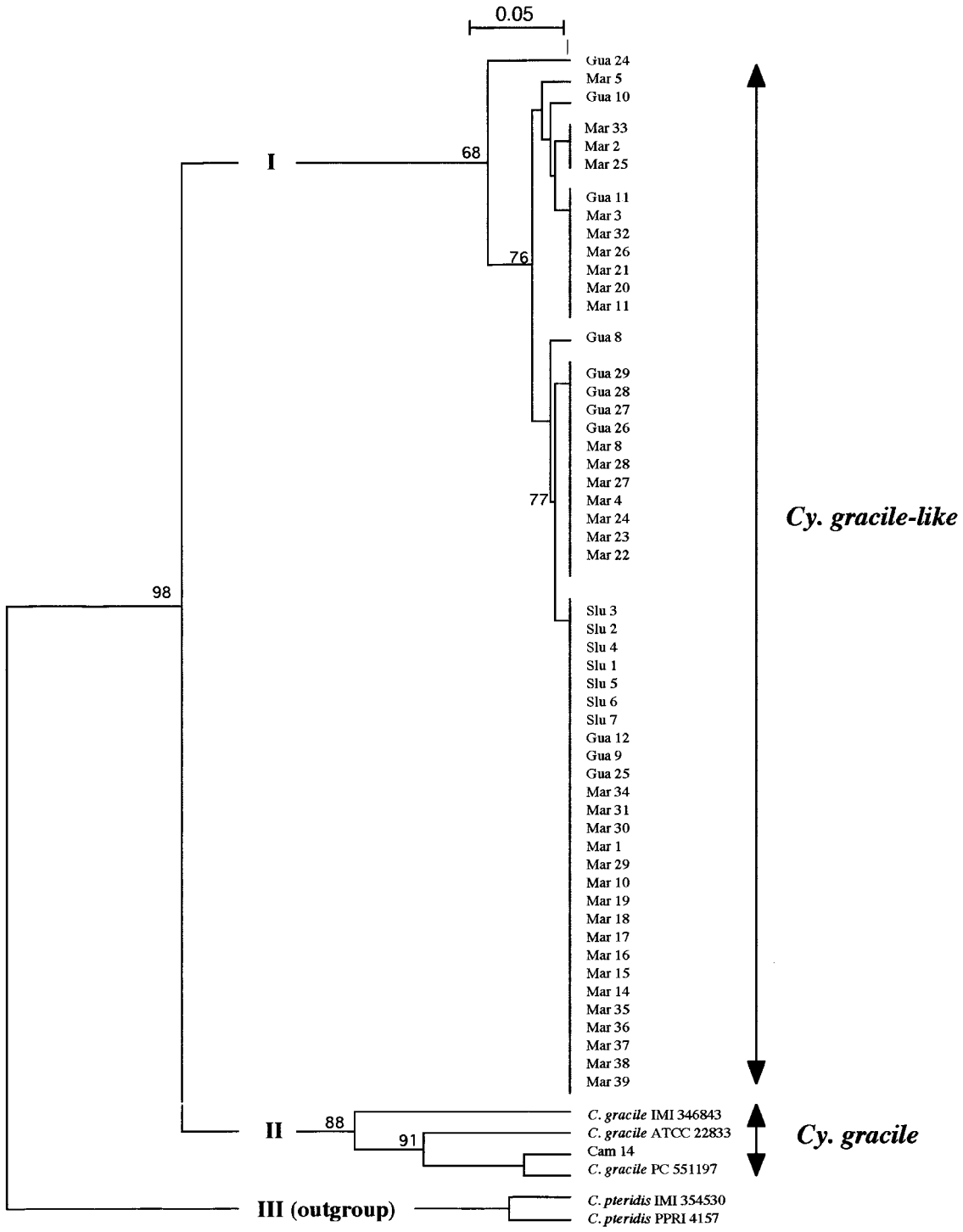

Figure 6. Dendrogram based on UPGMA analysis of: 52 *Cy. gracile*-like field isolates from bananas, one *Cy. gracile* field isolate from bananas, three *Cy. gracile* reference isolates and two *Cy. pteridis* reference isolates used as outgroups. Genetic distances were calculated using Sokal and Michener (1958) coefficient from banding patterns showing 58 polymorphic band positions generated by five RAPD primers. Bootstrap values greater than 50% from 1000 bootstrap replications are indicated.

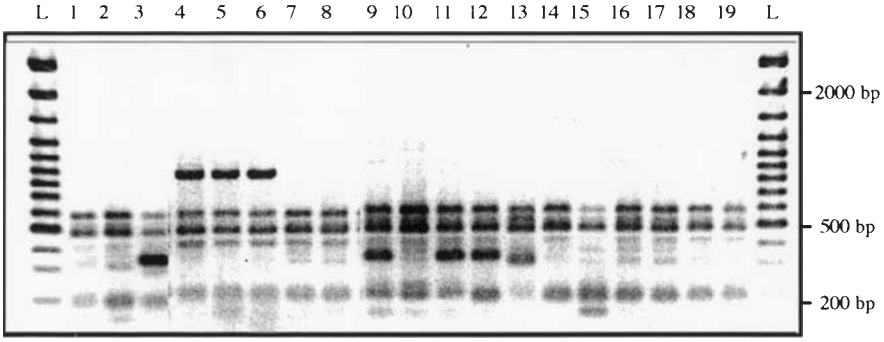

Figure 7. RAPD profiles of *Cy. spathiphylli* isolates generated by the OPE03 primer. Lane L: 100 bp DNA ladder, lane 1: ATCC 44730 (*Spathiphyllum*), lane 2: IMI 167893 (tea), lane 3: CBS 538.87 (*Spathiphyllum*), lane 4: Hel8A1 (*Heliconia*), lane 5: Hel2b (*Heliconia*), lane 6: Hel2c (*Heliconia*), lane 7: Gua4 (banana), lane 8: Gua3 (banana), lane 9: Cor1 (banana), lane 10: Cor2 (banana), lane 11: Cor5 (banana), lane 12: Cor3 (banana), lane 13: Cor4 (banana), lane 14: Cor6 (banana), lane 15: Cor7 (banana), lane 16: Cam2 (banana), lane 17: Cam1 (banana), lane 18: Cam8 (banana), lane 19: Cam19 (banana). A negative view of the original stained gel is shown.

among RAPD profiles was low and mainly originated from isolates: from *H. caribaea* and some isolates from Costa Rica (Figure 7). When subjected to UPGMA analysis, the total data set generated a dendrogram with isolates from banana and *H. caribaea* falling into one large (I) and two smaller clusters (II and III) and the single isolate Cor4 (Figure 8). The separation of isolates into three clusters was supported by high bootstrap values. Cluster I grouped about 90% of the studied isolates. It encompassed all the banana isolates from Cameroon and the West Indies, some from Costa Rica, and the single Maurician reference isolate IMI 167983 isolated from tea. Genetic similarity was higher than 90% within this cluster, although it included many subclusters whose statistical supports were sometimes low. Cluster II showed two subgroups. The first was composed of three isolates from *H. caribaea*, and the second of the two reference strains isolated from *Spathiphyllum*. These subgroups shared about 86% similarity. Cluster III pooled the two other isolates from *H. caribaea* and two banana isolates from Costa Rica. Although differing, the isolates from banana and *H. caribaea* shared about 92% similarity in this cluster. Finally, the banana isolate Cor4 from Costa Rica revealed only 70% similarity with all other *Cy. spathiphylli* isolates used in this study.

Discussion

Banana-infecting *Cylindrocladium* isolates were first reported 15 years ago, in Costa Rica and in the West Indies, as parasitic soilborne fungi able to consistently rot the banana root system and cause a toppling disease, together with endoparasitic nematodes (Loridat, 1989; Semer et al., 1987). Their implication as a species complex in banana cropping systems was highlighted by Risède and Simoneau (2001). Analysis of the *Cylindrocladium* species complex on bananas resulted in the identification of five morphologically different taxa. However, no studies to date have aimed at evaluating the pathogenic status of the species involved and their genetic variation.

In the present study, isolates from the two small-spored taxa with pyriform to sphaeropedunculate vesicles, i.e. *Cy. scoparium*- and *Cy. floridanum*-like isolates, were only slightly pathogenic on banana cv Grande Naine. In the light of this observation and their low re-isolation frequency after artificial inoculation, in the field, fungi of these taxa are probably secondary invaders of necrotic lesions caused by other root pathogens such as parasitic nematodes.

The two *Cylindrocladium* taxa with one-septate conidia and clavate vesicles exhibited different levels of pathogenicity on bananas. The single small-spored isolate showing a typical *Cy. gracile* morphology (isolate Cam14) was weakly pathogenic on cv Grande Naine. Conversely, *Cy. gracile*-like isolates with larger conidia than Cam14 exhibited a medium but significant RNP that increased with time and a high isolation frequency after inoculation. They may thus represent one of the key pathogenic taxa responsible for necrotic lesions on banana root systems. RAPD analysis of Cam14 and the *Cy. gracile* reference strains clearly revealed a high genetic similarity between these isolates, thus suggesting conspecificity. *Cy. gracile* (syn. *Cy. clavatum*) is a species known to be widely distributed and, due to its high level of pathogenicity

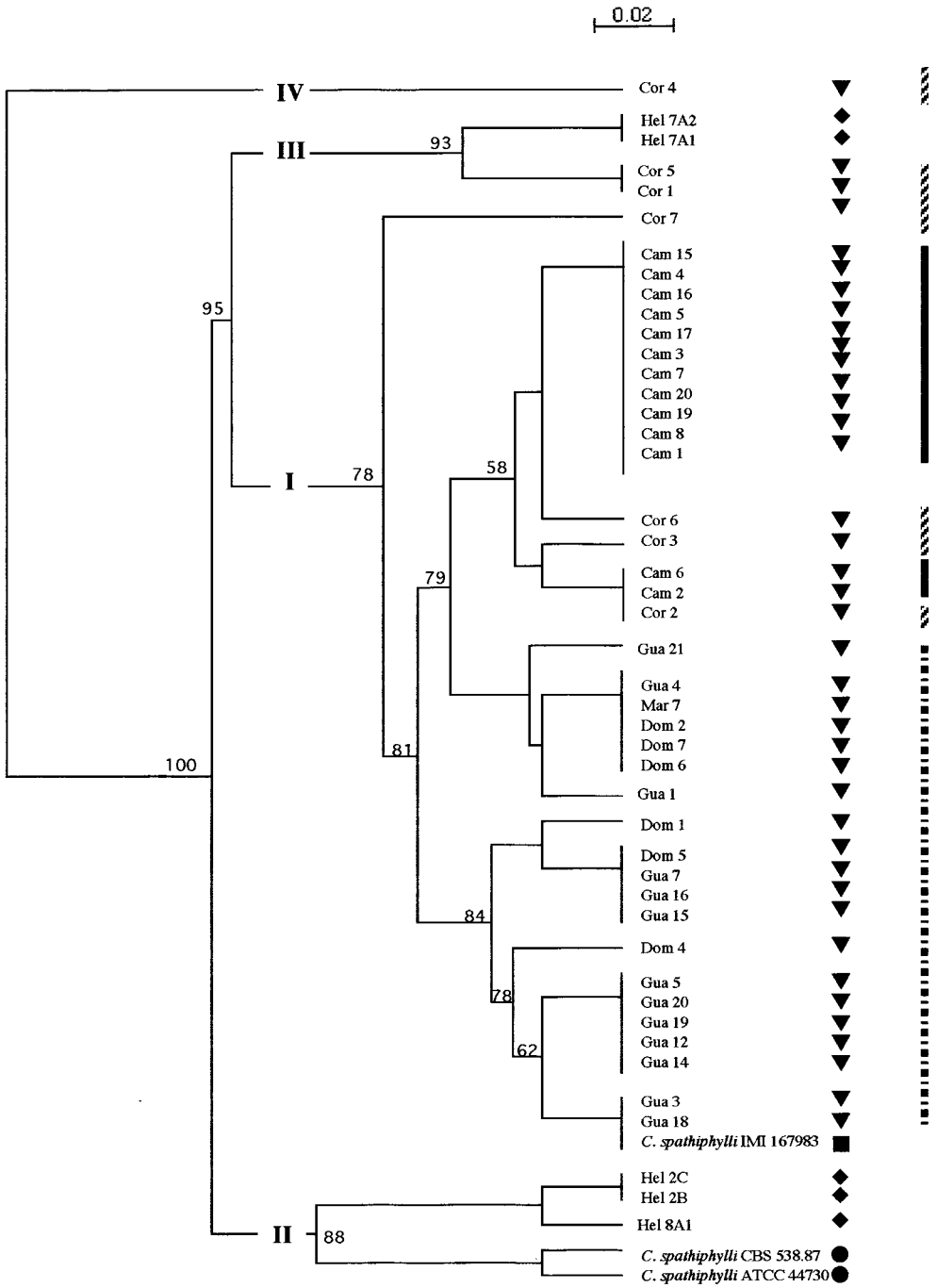

Figure 8. Dendrogram based on UPGMA analysis of 40 *Cy. spathiphylli* field isolates from bananas (▼), two *Cy. spathiphylli* reference isolates from *Spathiphyllum* (●), one *Cy. spathiphylli* reference isolate from tea (■) and five *Cy. spathiphylli* field isolates from *Heliconias* (♦). Genetic distances were calculated using the Sokal and Michener (1958) coefficient from banding patterns showing 25 polymorphic band positions generated by five RAPD primers. The origins of banana field isolates are indicated as follows: solid bar for isolates from Cameroon, hatched bar for isolates from Costa Rica and interrupted bar for isolates from the West Indies. Bootstrap values greater than 50% from 1000 bootstrap replications are indicated.

on some plants, is considered to be an important leaf or root pathogen on various legumes, forest- or fruit-crops (Crous and Wingfield, 1994). However, according to our results, this species may not be considered as an important pathogen for bananas, even if this needs confirmation by the study of a larger set of similar isolates from banana fields. RAPD analysis showed that *Cy. gracile*-like isolates were a genetically homogenous group with only 60% similarity relative to typical *Cy. gracile* strains. Considering that they have atypical conidial morphology in comparison to *Cy. gracile*, along with their pathogenicity on bananas and distinct genetic status, these isolates thus represent an original and unique *Cylindrocladium* taxon. It would be of interest to further analyze their species status through a multilocus approach with additional molecular data sets such as β-tubulin or histone sequence polymorphism (Kang et al., 2001; Schoch et al., 2001). At the moment, this new *Cylindrocladium* taxon has only been identified in the West Indies, on bananas, and its geographical distribution and host range therefore require to be further addressed.

Cylindrocladium spathiphylli is the final species of the *Cylindrocladium* complex that induces root necrotic lesions in banana. This species, although it has only recently been reported on bananas (Risède and Simoneau, 2001), has been known to cause disease symptoms mainly on various ornamentals (Crous, 2002; Crous and Wingfield, 1994; Peerally, 1991). Tropical flower and foliage markets are in particular affected by *Cy. spathiphylli*. Originally found on *Spathiphyllum* in 1978 in Florida, this species was firstly described by Schoulties et al. (1982). It was thereafter reported to be highly pathogenic to *Spathiphyllum*, causing above- as well as under-ground disease symptoms including severe collar, corm and root-rots (Schoch and Crous, 1999; Uchida, 1989) that leads to damping-off of young seedlings or deep quality depreciation of marketable plants (Uchida and Aragaki, 1992). In the late 1980s, *Cy. spathiphylli* was also pointed out as the cause of the '*Heliconia* decline' in Hawaii, where it affected several commercial fields and nurseries, and induced leaf spots, sheath and petiole blights, and severe rhizome and root-rots (Uchida et al., 1989). In the current study, we used isolates of *Cy. spathiphylli* originating either from banana or *Heliconia* and detected significant intraspecific differences in pathogenicity on banana cv Grande Naine. Isolates of *Cy. spathiphylli* from banana, which had a high RNP that rose with time and showed a high isolation frequency after inoculation, were the most pathogenic isolates within the *Cylindrocladium* species complex in the banana rhizosphere. By contrast, we found that *Cy. spathiphylli* isolates from *H. caribaea* were weakly pathogenic on banana. Similar host specificity was reported by Uchida et al. (1989) for *Cy. spathiphylli* isolates from diseased *Heliconia* that were found nonpathogenic on *Spathiphyllum* while being very destructive on commercial *Heliconia* plants. In turn, *Cy. spathiphylli* isolates from *Spathiphyllum* were nonpathogenic to *Heliconia*. Standing on this host specificity along with morphological and physiological traits, these authors tentatively named the causal agent of the '*Heliconia* decline', *Cy. spathiphylli* f. sp. *heliconiae*. This denomination was however never confirmed. In our study, we showed that the pathogenic discrimination between isolates of *Cy. spathiphylli* from banana and *H. caribaea* was correlated with divergent rDNA spacer polymorphism. Single nucleotide polymorphism in the ITS 1 region and *Ava* II restriction polymorphism in the IGS region clearly separated *Cy. spathiphylli* isolates recovered from *Heliconia* and *Spathiphyllum* from those derived from banana or tea, thus highlighting a genetic intraspecific distinction according to the host. When further assessing genetic variation within the *Cy. spathiphylli* species using RAPD markers, there was a less marked partition between isolates according to the original host of isolation. Although no partition according to geographic origin was noted, only the Costa Rican isolates showed noticeable genetic divergence within the banana isolates, some of them even clustering with some of the isolates from *Heliconia*. This cannot yet be explained, but could be considered in the light of the following two points. First, in culture, Costa Rican *Cy. spathiphylli* strains sometimes exhibited an unusual microconidial state. This phenotypic character has been associated with long-term preservation (Crous and Peerally, 1996), but its significance may require further investigation. Secondly, Costa Rica is one of the top world producers of dessert bananas, while it is also a major grower of tropical flowers, including *Heliconia*. Banana and *Heliconia* belong to the same botanical order (Zingiberales). Under laboratory conditions, mating isolates of *Cy. spathiphylli* from banana and *Heliconia* resulted in the production of fertile *Calonectria* perithecia, so it may be hypothesized that mating between isolates of *Cy. spathiphylli* from banana and *Heliconia* may occur in a favorable field environments such as neighboring *Heliconia* and

banana fields. To further test this hypothesis, it would be necessary to find the *Calonectria* teleomorph in the field and to analyze the genetic diversity of a larger number of *Cy. spathiphylli* isolates from Costa Rica sampled from the two sister plants.

It should nevertheless be noted that genetic similarity is globally high within each of the two main pathogenic *Cylindrocladium* taxa infecting banana. RAPD analysis illustrated genetic similarity as high as 95–100% within the *Cy. gracile*-like isolates, and 70–100% within *Cy. spathiphylli* isolates from banana. This situation may possibly be attributed to a potential predominant asexual propagation of *Cylindrocladium* isolates, favored by the fact that in this genus, microsclerotia are the main survival and dissemination structures in soil and infected host tissue (Thies and Patton, 1970). In bananas, *Calonectria* teleomorphs have indeed never been observed in the field, even on old roots or corms. The fact that bananas have been disseminated for decades by corms and suckers (vegetative reproduction) potentially infected by *Cylindrocladium* fungi could have facilitated the spread of a limited number of genotypes of this fungal genus.

Finally, using two isolates, i.e. one *Cy. gracile*-like and one *Cy. spathiphylli* isolate, we further investigated pathogenicity using six different banana genotypes. Irrespective of the banana genotype tested, the *Cy. spathiphylli* isolate was more pathogenic than the *Cy. gracile*-like isolate. Pathogenicity differences between these two isolates were expressed quantitatively without interaction between isolates and banana genotypes. The banana cvs Kunnan (AB), IRFA 914 (AAB) and above all IDN 110 (AA) showed high lesion severity. The popular cv Grande Naine (AAA), which is widely grown throughout the world, exhibited medium root necrotic damage. Interestingly, the cvs PKW (BB) and P. Madu (AA) were less susceptible. These two diploid cultivars, which are already used as genitors in breeding programs searching for resistance to *Mycosphaerella musicola*, the causal agent of Yellow Sigatoka, could therefore also represent clones of interest for breeding *Cylindrocladium*-resistant bananas.

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References

Castaing V, Beveraggi A, Fouré E and Fogain R (1996) Detection of a *Cylindrocladium* sp. in Cameroon. Studies on pathogenic activity and interactions with *R. similis*. Infomusa 5: 4–7

Crous PW (2002) Taxonomy and Pathology of *Cylindrocladium* (*Calonectria*) and Allied Genera, APS Press, 294 p, St. Paul, Minnesota, USA

Crous PW and Peerally A (1996) *Gliocladiopsis irregularis* sp. nov. and notes on *Cylindrocladium spathiphylli*. Mycotaxon 65: 119–128

Crous PW and Wingfield MJ (1994) A monograph of *Cylindrocladium* including anamorphs of *Calonectria*. Mycotaxon 51: 341–435

Declerck S, Risede JM, Rufyikiri G and Delvaux B (2002) Effects of arbuscular mycorrhizal fungi on severity of root rot of bananas caused by *Cy. spathiphylli*. Plant Pathology 51: 109–115

Duchesne LC and Anderson JB (1990) Location and direction of transcription of the 5S rRNA in *Armillaria*. Mycological Research 94: 266–269

Felsenstein J (1995) PHYLIP (Phylogeny Inference Package) version 3.57c. Seattle: University of Washington, Department of Genetics, USA. Distributed by the author

Goodwin DC and Lee SB (1993) Microwave miniprep of total genomic DNA from fungi, plants, protists and animals for PCR. Biotechniques 15: 438–444

Gowen S and Queneherve P (1990) Nematode parasites of bananas, plantains and abaca. In: Luc et al. (eds) Plant Parasitic Nematodes in Subtropical and Tropical Agriculture (pp 431–460) CAB International, Wallingford, Oxon, UK

Jeng RS, Dumas M, Liu FH, Wang CL and Hubbes M (1997) DNA analysis of *Cylindrocladium floridanum* isolates from selected forest nurseries. Mycological Research 101: 285–291

Jenny C, Carreel F, Tomekpe K, Perrier X, Dubois C, Horry JP and Tezenas du Montcel H (1999) Les bananiers. In: Hamon et al. (eds) Diversité génétique des Plantes Cultivées (pp 113–139) Montpellier, France

Jones DR (1999) Diseases of Banana, Abaca and Enset, CAB International, Wallingford, Oxon, UK

Kang JC, Crous PW and Schoch CL (2001) Species concepts in the *Cylindrocladium floridanum* and *Cy. spathiphylli* complexes (*Hypocreaceae*) based on multi-allelic sequence data, sexual compatibility and morphology. Systematic and Applied Microbiology 24: 206–217

Kobenan K (1991) Parasites et ravageurs des bananiers en Côte d'Ivoire. Fruits 46: 633–641

Lassoudière A (1978) Quelques aspects de la croissance et du développement du bananier Poyo en Côte d'Ivoire. Le système radical. Fruits 33: 314–338

Loridat P (1989) Etude de la microflore fongique et des nématodes associés aux nécroses de l'appareil souterrain du bananier en Martinique. Mise en évidence du pouvoir pathogène du genre *Cylindrocladium*. Fruits 44: 587–597

Peerally A (1991) The classification and phytopathology of *Cylindrocladium* species. Mycotaxon 40: 323–366

Perrière G and Gouy M (1996) WWW-Query: An on-line retrieval system for biological sequence banks. Biochimie 78: 364–369

Price NS (1995) Banana morphology – Part I: Roots and rhizomes. In: Gowen Chapman et al. (eds) Bananas and Plantains (pp 179–189) London, UK

Risède JM (1994) Eléments de caractérisation de *Cylindrocladium* sp. agent de nécroses racinaires du bananier en Martinique. Fruits 49: 167–178

Risède JM (1995) Spatial and temporal distribution in soils of *Cylindrocladium* sp. fungal pathogen of bananas. Phytopathology 85: 1563

Risède JM and Simoneau P (2001) Typing *Cylindrocladium* species by analysis of ribosomal DNA spacers polymorphism: Application to field isolates from the banana rhizosphere. Mycologia 93: 494–504

Rossman AY (1979) *Calonectria* and its type species, *C. daldiniana*, a later synonym of *C. pyrochroa*. Mycotaxon 8: 321–328

Rossman AY (1983) The phragmosporous species of *Nectria* and related genera. Mycological Papers 150: 1–164

Schoch CL and Crous PW (1999) First report of *Cylindrocladium* root and petiole rot to *Spathiphyllum* in South Africa. South African Journal of Botany 65: 208–211

Schoch CL, Crous PW, Wingfield BD and Wingfield MJ (2001) Phylogeny of *Calonectria* based on comparisons of β-tubulin DNA sequences. Mycological Research 105: 1045–1052

Schoulties CL, El-Gholl NE and Alfieri SA (1982) *Cy. spathiphylli* sp. nov. Mycotaxon 16: 265–272

Semer CR, Mitchell DJ, Mitchell ME, Martin FR and Alfenas AC (1987) Isolation, identification and chemical control of *Cylindrocladium musae* sp. nov. associated with toppling disease of banana. Phytopathology 77: 1729

Sokal RRR and Michener CD (1958) A statistical method for evaluation of systematic relationships. University of Kansas Science Bulletin 38: 1409–1438

Stover RH and Simmonds NW (1987) Bananas, 3rd edn. 468 p, Longman Scientific & Technical, Harlow, UK

Thies WG and Patton RF (1970) Biology of *Cylindrocladium scoparium* in Wisconsin forest tree nurseries. Phytopathology 60: 1662–1668

Thompson JD, Higgins DG and Gibson TJ (1994) CLUSTALW: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research 22: 4673–4680

Uchida JY (1989) *Cylindrocladium* rot of *Spathiphyllum*. Hawaii Institute of Tropical Agriculture and Human Resources Brief 78: 1–4

Uchida JY and Aragaki M (1992) Further characterization of *Cy. spathiphylli* from *Spathiphyllum* in Hawaii and Florida. Mycologia 84: 810–814

Uchida JY, Aragaki M and Yahata PS (1989). Heliconia root rot and foliar blight caused by *Cylindrocladium*. Hawaii Institute of Tropical Agriculture and Human Resources Brief 85: 1–2

White TJ, Bruns T, Lee S and Taylor J (1990) Amplification and direct sequencing of fungal ribosomal genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ and White TJ (eds) PCR Protocols: A Guide to Methods and Applications (pp 315–322) Academic Press, San Diego