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Cultural and Aggressiveness Variability of *Cercospora coffeicola*

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Abstract

Although brown eye spot of coffee, caused by *Cercospora coffeicola*, is important for coffee production in Brazil, there is a general lack of knowledge regarding the disease. In this study, we evaluated the variability of both the cultural and aggressiveness traits of 60 isolates from coffee plants grown under conventional and organic systems in three regions of Minas Gerais State, Brazil. Variability among the isolates was detected with regard to all of the traits and was unrelated to an effect of either the region or cropping system. Mycelial growth, cercosporin production and sporulation were assessed in the laboratory. Of the 60 isolates, 27 did not sporulate at 25°C; the mycelial growth of all of the isolates and cercosporin production by 18 of the isolates linearly increased as the temperature rose from 18 to 26°C. We inoculated six selected isolates on plants of two coffee cultivars ('Catuaí Vermelho IAC44' and 'Catucaí Vermelho 785-15') and evaluated the incubation period (IP), latent period (LP) and disease severity. All three of these traits were affected by temperature postinoculation and KCl amendment. The significant correlations were as follows: IP and LP in both cultivars; severity and leaf fall in both cultivars; and cercosporin production *in vitro* and severity values in 'Catucaí Vermelho 785-15'. In conclusion, we found that (i) *C. coffeicola* is highly variable for both cultural and aggressiveness traits; (ii) laboratory and glasshouse experiments were suitable to assess the pathogen variability; (iii) research protocols should account for the effect of environmental factors, such as temperature and KCl, on the traits evaluated; and (iv) these protocols should include the assessment of the IP instead of the LP, as both are correlated, and the IP is easier to evaluate.

Introduction

Coffee (*Coffea arabica* L.) is an important commodity in Brazil, and studies aimed at minimizing crop losses due to disease are common. Brown eye spot (BES), caused by *Cercospora coffeicola* Berk & Cooke, is an important factor associated with coffee yield losses. Depending on the season and region in Brazil, BES can be the most important disease, leading to losses of up to 30% (Zambolim et al. 1997). The disease occurs from nursery-to-field plantings, and severe epidemics are recorded when management practices are not well conducted. The symptoms of BES are necrotic spots, consisting of a light-coloured centre surrounded by a purplish brown ring with yellowish edges, on the leaves and dark spots, with a dry aspect on the berries (Souza et al. 2011). Losses from BES most commonly result from leaf fall and a reduction in the quality of the diseased berries.

Since the first reports in Brazil in 1901 (Noack 1901), BES was considered for decades to be of secondary importance; however, this scenario has recently changed. Severe epidemics of BES have been reported, and the control of the disease is now a constant concern for producers (Juliatti et al. 2000). Several factors are considered as potential causes of the shift in the importance of BES: the expansion of the Brazilian coffee-growing area into the 'Cerrado' region, where the soil and climate conditions differ from those of traditional areas; the cultivation of new coffee cultivars; changes in cultural practices, mainly sprinkler irrigation, planting density and fertilization; climate change; and shifts in the pathogen population (Fazuoli et al. 2002).

With regard to populations of the pathogen, some key questions include whether there is variability in *C. coffeicola*, which traits are involved and how the experimental conditions affect the variability. To assess the variability, a thorough study was conducted in Minas Gerais, the largest coffee-producing state in Brazil, and the distribution pattern of vegetative compatibility groups of *C. coffeicola* isolates, collected in conventional and organic coffee fields from three geographic regions of Minas Gerais State, was used to infer the variability in the pathogen populations (Martins et al. 2008). The authors found high variability within fields

and pathogen populations that were not structured according to the growing system or geographic regions. The study was informative as an overview of the variability of the population; however, from an epidemiological perspective, the analysis of another set of variables would be more revealing to evaluate the increase in the intensity of BES epidemics. In addition, Martins (2007) reported on the variability of *C. coffeicola* isolates with respect to cercosporin production, a toxin related to the aggressiveness of *Cercospora* spp. (Almeida et al. 2005).

As BES was not a major concern in the past, studies on the biology of the pathogen and on the disease epidemiology and management are scarce. To pursue such studies, basic information on the pathogen/disease needs to be generated. For instance, it is difficult to obtain *in vitro* sporulation of *C. coffeicola* (Del Peloso et al. 1989), and a reproducible methodology was only recently developed (Souza et al. 2005). Moreover, variable results are common when the pathogen is inoculated under controlled conditions.

It is well known that the interactions of the disease triangle – pathogen, environment and host factors – determine the occurrence and intensity of any disease. In fact, temperature is a critical factor in the occurrence and progress of diseases. In fungal diseases, the temperature affects sporulation, lesion expansion and intensity; however, there are no reports to date on the effects of temperature on *C. coffeicola* isolates and BES intensity. BES is also associated with stress conditions in coffee, especially nutritional stress, and some authors correlate potassium application with disease intensity (Pozza et al. 2000, 2004). Although there are no cultivars that are resistant to BES, it is reasonable to expect that coffee genotypes vary with regard to disease intensity (Pozza et al. 2004).

Because an understanding of the variability of *C. coffeicola* is essential to the success of any research project, especially those related to epidemiology and BES management strategies including resistance and chemical control, our goal was to assess the variability of the pathogen. To this end, we compared the isolates collected by Martins et al. (2008) at three geographic regions and under two cropping systems, conventional and organic, in Minas Gerais State through laboratory and glasshouse experiments. A total of 60 isolates were compared with respect to the effects of temperature on cercosporin production, mycelial growth and sporulation. We also compared the aggressiveness of six selected isolates on 'Catuaí Vermelho IAC44' and 'Catucaí Vermelho 785-15' plants grown in substrates with and without potassium chloride and maintained at different temperature levels after inoculation.

Materials and methods

General procedures

The experiments were conducted at the Departamento de Fitopatologia, Universidade Federal de Viçosa (UFV), MG, Brazil. For the laboratory experiments, we used 60 monosporic isolates of *C. coffeicola* from three geographic regions of Minas Gerais State (Zona da Mata, Triângulo Mineiro and Sul de Minas) and from two cropping systems (conventional and organic). These isolates were obtained from a collection by Martins (2007) who sampled BES-diseased leaves of *C. arabica* cultivars, isolated the fungus from the leaf spots, prepared monosporic cultures and confirmed their fungal identify as *C. coffeicola* through microscopy and published taxonomic keys (Chupp 1953; Crous and Braun 2003). For the tests conducted in the glasshouse and growth chambers, six isolates were selected according to sporulation, mycelial growth and cercosporin production characteristics. The isolates were stored on potato dextrose agar (PDA, in test tubes) at 5°C and cultivated on solid V8 (V8S) medium (200 ml V8® juice, 800 ml distilled water and 20 g agar) in 9-cm Petri dishes or in liquid V8 (V8L) medium in 25-ml Erlenmeyer flasks.

The isolates were compared in experiments conducted in the laboratory (sporulation, cercosporin production and mycelial growth) and glasshouse (aggressiveness). Each experiment that is described below was performed twice in a completely randomized design and, unless otherwise specified, included three replicates. An experimental unit consisted of a 9-cm-diameter Petri dish for the laboratory experiments and one coffee plant with two pairs of mature leaves for the glasshouse experiment.

Sporulation

We used the mycelial mass drying technique to induce *C. coffeicola* sporulation (Souza et al. 2005): three mycelial discs (each 0.5 cm in diameter) from colony borders were transferred to 10 ml V8L in 25-ml Erlenmeyer flasks and shaken at 120 rpm and 25°C. After 4 days, the content of each Erlenmeyer was poured into agar–water (15 g agar/1 L water) in three open Petri dishes placed 40 cm below a set of Sylvania® 40 W white fluorescent lamps and Sylvania® 40-W Gro-lux lamps, with a 165.3 μmol/s/m^2 light intensity and a 12-h photoperiod, at 25°C. When the liquid medium evaporated, approximately 4 days later, 10 ml of distilled water was poured into each dish, which was then superficially scraped with a glass stick. The resulting suspension was filtered through a layer of cheesecloth, and the concentration of the spores in the filtrate was estimated by averaging the number of spores in two counting fields of a haemocytometer. The experiment included a single factor (isolate).

Cercosporin production

For each isolate, mycelial discs (0.5 cm in diameter) from the borders of colonies on V8S were placed in the centre of a Petri dish with PDA and incubated in growth chambers at 18, 22 or 26°C with fluorescent lamps, as described above. The light intensity in the chambers was 276.3 μmol/s/m^2 under a 12-h photoperiod. After 14 days, the cercosporin production, marked by a red pigmentation on PDA, as reported by Choquer et al. (2005), was evaluated using a

four-grade scale: 1 – no pigment production; 2 – pigment production only under the colony; 3 – pigment covering up to half of the dish; and 4 – pigment covering the entire dish (Brunelli 2004). The experiment was designed in a factorial scheme (3 temperature levels × 60 isolates).

Mycelial growth

Mycelial discs (0.5 cm in diameter) from the borders of colonies on V8S were placed in the centre of a Petri dish with PDA and incubated in growth chambers at 18, 22 or 26°C with fluorescent lamps, as described above. Every 3 days (for 18 days), the colony diameter was measured in two directions, and the average diameter was estimated. The area under the mycelial growth curve (AUMGC) was calculated using the equation for the area under the disease progress curve (Shaner and Finney 1977). The experiment was in a factorial scheme (3 temperature levels × 60 isolates).

Aggressiveness

Six isolates of the 60 compared in the laboratory were selected, including two from each geographic region with different sporulation, cercosporin production and mycelial growth characteristics.

At 6 months of age, plants of 'Catuaí Vermelho IAC44' ('Catuaí') and 'Catucaí Vermelho 785-15' ('Catucaí') from two different nurseries were grown in plastic bags (10 × 20 cm) containing a mixture of soil, sand and cow manure in a proportion of 3 : 1 : 1 (v : v : v). Half of the bags of each cultivar were amended twice with 0.35 g of KCl/plant diluted in 10 ml of distilled water at 12-day intervals (E.A. Pozza, personal communication). The levels of potassium in the soil and coffee leaves were analysed both before the KCl amendment and at the end of the experiment (Table 1).

All of the plants were inoculated at 4 months after sowing, 1 month after the last fungicide treatment and 12 days after the second KCl application. The inoculum suspension was adjusted to 4.5×10^4 conidia/ml and sprayed on both sides of four leaves per plant using a De Vilbiss atomizer. Distilled water was sprayed on the control plants. After inoculation, all of the plants were transferred to growth chambers at 18, 22 or 26°C under fluorescent light and with a 12-h photoperiod. For the first 12 h, the plants were covered with transparent plastic bags sprayed with water; the bags were removed at 48 h after the inoculation, and the plants were transferred to a glasshouse with natural lighting at 25 ± 3°C until the end of the experiment.

Table 1
Levels of potassium in the soil and leaves of coffee plants under the treatments without (–) or with (+) the amendment of potassium chloride (KCl). Data are from two experimental repeats

Coffee cultivar	Run	KCl	Soil (mg/dm^3)	Leaf (dag/kg)
Catuaí	1	–	38	1.054
		+	372	1.294
	2	–	26	0.976
		+	245	1.154
Catucaí	1	–	14	1.294
		+	294	1.414
	2	–	10	1.233
		+	215	1.399

Each plant was assessed daily from the 10th to the 40th day after inoculation. The evaluations comprised the incubation period (IP), the number of days between inoculation and the appearance of the first symptom on a leaf, and the latent period (LP), the number of days between the inoculation and the visualization of the first sporulating lesion on a leaf. For each plant, the IP and LP were calculated by averaging the values of the four inoculated leaves. On the 40th day, the disease severity was evaluated based on a diagrammatic scale (Oliveira et al. 2001) with five grades: 1 – 0%; 2 – >0 to 3%; 3 – >3 to 6%; 4 – >6 to 12%; and 5 – >12 to 25% diseased area. The intermediate severity value between the lower and the upper limits of each grade was used in the statistical analysis. The leaf fall was also evaluated on the 40th day by counting the number of leaves that fell in relation to the initial leaf number.

For each coffee cultivar, we designed the experiment in a factorial scheme (2 KCl levels × 3 temperature levels × 6 isolates). The experiment was repeated twice, with two and three replicates, respectively.

Data analysis

There was a homogeneity of the variances between the two repeats of all of the experiments (Levene's test, $P = 0.05$), and the data from both repetitions were pooled. We also checked for error normality (Shapiro–Wilk test, $P = 0.05$). To fulfil the analysis of variance (ANOVA) assumptions, the sporulation values were transformed to $\log_{10}$ (sporulation).

After analysing the laboratory experiments using ANOVA, the Scott–Knott ($\alpha = 0.01$) test for multiple comparisons was used to cluster the isolates. The data from the glasshouse experiment were not normally distributed and were analysed through a nonparametric approach (Bakeerathan and Samita 2003). Multiple comparisons of the rank means was through the least significant differences (LSD) test ($\alpha = 0.01$). The Spearman's correlation coefficient was used to assess the correlation between the IP, LP, cercosporin production, severity and leaf fall associated with the six isolates compared *in vivo*. The statistical analyses were performed using SAS® v 9.1, with the exception that the Scott–Knott test that was performed using SISVAR® v 5.3 (UFLA, MG, Brazil).

Results

Sporulation

The 60 isolates significantly differed with regard to sporulation ($P < 0.0001$) and were clustered into four groups: A – with 17 isolates (28.3%), sporulation between 2.5×10^4 to 1.3×10^5 conidia/ml; B – 15 isolates (25.0%), with sporulation between 5.4 to 2.3×10^4 conidia/ml; C – 1 isolate (1.7%), with sporulation of

4.7×10^3 conidia/ml; and D – 27 isolates (45.0%) that did not sporulate. In general, the isolates were not clustered according to the region or cropping system.

Cercosporin production

There was a significant effect of the temperature, isolate and the interaction of temperature with isolate on the cercosporin production (all $P < 0.0001$). For 28 isolates (46.7%), the temperature significantly affected the cercosporin production (all $P < 0.0071$); for 18 isolates (30.0%), the cercosporin production linearly increased as the temperature increased.

At each temperature level, five clusters of isolates were found with regard to the cercosporin production (Table 2). More isolates produced intermediate levels (average of >1 and <3.2) at 22°C than at 18 and 26°C. At 18°C, 11.7% of the isolates were high producers (average of ≥ 2.7), whereas 40 and 35% isolates were high producers at 22 and 26°C, respectively. Of the remaining isolates, 20% of the isolates did not produce cercosporin at 18°C (average = 1.0), whereas 10 and 13.3% of the isolates did not produce cercosporin at 22 and 26°C, respectively (Table 2). The isolates were not clustered according to the region or crop system.

Mycelial growth

There was a significant effect of the temperature, isolate, and the interaction of temperature with isolate on the mycelial growth (all $P < 0.0001$). For all of the isolates, as the temperature increased, the AUMGC increased linearly (all $P < 0.0031$).

The isolates were grouped in 3, 5 and 6 clusters at 18, 22 and 26°C, respectively (Table 2). Growth was inhibited at 18°C, as the AUMGC was at most 8.4 cm per day for 90% of the isolates. At 22 and 26°C, the AUMGC was <8.6 cm per day for 6.7 and 5% of the isolates, respectively. Approximately 40% of the isolates exhibited values for the AUMGC ≥ 12.9 cm per day at 26°C (Table 2). The isolates were not clustered according to the region or crop system.

Aggressiveness

The six isolates selected for the experiment were as follows (see Table 3 for explanations of the names): MO53 (clusters 'B' for sporulation, 'E' at 18, 22 and 26°C for cercosporin and 'A' at 18, 22 and 26°C for growth); MC56 (clusters 'B' for sporulation, 'B' at 22 and 26°C and 'C' at 18°C for cercosporin production and 'C' at 22 and 26°C and 'B' at 18°C for growth); TO02 (clusters 'A' for sporulation, 'E' at 18 and 22°C and 'D' at 26°C for cercosporin production and 'A' at 18 and 22°C and 'C' at 26°C for growth); TC07 (clusters 'A' for sporulation, 'D' at 18 and 26°C and 'B' at 22°C for cercosporin production, and 'B' at 18 and 26°C and 'C' at 22°C for growth); SO40 (clusters 'A' for sporulation, 'C' at 18, 22 and 26°C for cercosporin production and 'C' at 22 and 26°C and 'B' at 18°C for growth); and SC31 (clusters 'B' for sporulation, 'C' at 22 and 26°C and 'D' at 18°C for cercosporin production and 'D' at 22 and 26°C and 'C' at 18°C for growth).

For both cultivars, the KCl amendment and isolate affected all of the variables (all $P \leq 0.0014$), and the temperature affected the IP, LP and SEV (all $P < 0.0001$) but not the leaf fall ($P > 0.6$ for both cultivars). Significant interaction effects were found for the following: KCl × isolate on severity for 'Catuaí' ($P < 0.0001$); KCl × temperature on severity for 'Catucaí' ($P = 0.0086$); and isolate x temperature on severity for both 'Catuaí' ($P = 0.0052$) and 'Catucaí' ($P = 0.0029$).

For 'Catuaí', the IP lasted 23.5 days without KCl and 21.8 days with KCl and 22.1 days at 18°C, 21.9 days at 22°C and 23.8 days at 26°C. The LP lasted 33.7 days without KCl and 31.0 days with KCl and 31.7 days at 18°C, 31.8 days at 22°C and 33.23 days at 26°C. The leaf fall was 0.4 without KCl and 1.3 with KCl. For 'Catucaí', the IP lasted 22.4 days without KCl and 21.2 days with KCl amendment and 21.7 days at 18°C, 20.6 days at 22°C and 23.1 days at 26°C. The LP lasted 32.6 days

Table 2
Clusters of *Cercospora coffeicola* isolates grown on potato dextrose agar at 18, 22 or 26°C and evaluated with regard to the cercosporin production and the area under mycelial growth curve (AUMGC). A total of 60 isolates were evaluated, and the Scott–Knott test ($\alpha = 0.01$) was used to cluster the isolates and define the ranges at each temperature

		Temperature					
		18°C		22°C		26°C	
Evaluation	Cluster	Range	Isolates (%)	Range	Isolates (%)	Range	Isolates (%)
Cercosporin production (average note)	A	3.5	1 (1.7)	3.2–3.5	3 (5.0)	3.6–3.8	4 (6.7)
	B	2.8–3.0	6 (10.0)	2.7–3.0	21 (35.0)	2.8–3.2	17 (28.3)
	C	2.5–2.6	7 (11.7)	2.3–2.5	14 (23.3)	2.5–2.7	9 (15.0)
	D	2.0–2.3	34 (56.6)	2.0–2.2	16 (26.7)	2.0–2.3	22 (36.7)
	E	1.0	12 (20.0)	1.0	6 (10.0)	1.0	8 (13.3)
AUMGC (cm·day)	A	8.8–9.8	6 (10.0)	12.4–13.9	10 (16.7)	14.1–15.5	10 (16.7)
	B	7.2–8.4	30 (50.0)	11.2–12.2	15 (25.0)	12.9–13.8	13 (21.7)
	C	5.3–6.9	24 (40.0)	9.6–11.0	26 (43.3)	11.4–12.4	14 (23.3)
	D	–*	–	8.7–9.2	5 (8.3)	10.5–11.3	17 (28.3)
	E	–	–	7.8–8.2	4 (6.7)	9.9–10.2	3 (5.0)
	F	–	–	–*	–	8.1–8.6	3 (5.0)

*There were three and five clusters at 18 and 22°C, respectively.

Table 3
Brown eye spot severity, incubation period, latent period and the number of fallen leaves of the plants of two coffee cultivars grown in substrates either amended or not amended with KCl and inoculated with six isolates of *Cercospora coffeicola* and then maintained for 48 h after inoculation at 18, 22 or 26°C. In this factorial experiment, the interactions of isolate with either KCl amendment or temperature were significant for severity, whereas only the effect of the isolate was significant for the incubation period, latent period and the number of leaves dropped

		Severity (%)							
		KCL		Temperature (ºC)			Incubation period (days)	Latent Period (days)	Fallen leaves (number)
Cultivar	Isolate*	Without	With	18	22	26			
Catuaí	MC56	5.05a†	10.65ab	9.13a	8.73a	5.69a	21.63c	30.87bc	1.54ab
	SC31	3.30ab	12.87a	7.52ab	9.02a	7.73a	21.93c	30.77bc	1.80a
	SO40	3.20ab	12.99ab	8.63a	8.72a	6.95a	20.67c	30.30c	0.63bc
	MO53	4.40ab	7.63b	3.80ab	9.48a	4.77a	23.13b	32.80b	1.00cd
	TC07	2.83b	7.18b	6.55ab	5.38a	3.08ab	23.33b	33.23b	0.37bcd
	TO02	1.50c	2.94c	5.38b	1.58b	1.80b	25.75a	37.20a	0.33d
Catucaí	SC31	6.57a	10.29a	9.24a	9.59a	6.47a	19.70c	28.80c	1.41ab
	SO40	5.05ab	8.77a	8.47ab	6.79ab	5.47a	21.40bc	30.37bc	1.74ab
	MC56	4.24b	9.81a	8.45ab	6.89ab	5.74a	21.40bc	30.53bc	1.89a
	MO53	2.18c	4.33b	2.29c	3.62bc	3.85ab	22.57b	31.93b	0.80b
	TC07	3.99bc	8.93a	5.62b	8.80ab	4.98a	21.67b	31.57b	1.06bc
	TO02	0.60d	2.03c	1.50d	1.43c	2.33b	25.15a	36.95a	0.00c

*For each isolate, the letters represent the region of origin (S = Sul de Minas, T = Triângulo Mineiro or M = Zona da Mata) and cropping system (C = Conventional or O = Organic), and the number identifies the sequence of sampling.
†For each column and cultivar, the means followed by the same letter are not different according to the Fisher's LSD test ($\alpha = 0.01$). The means shown are the actual values, whereas the statistical comparisons were made using the ranked values based on a nonparametric approach (see 'Data analysis').

without KCl and 30.3 days with KCl and 31.5 days at 18°C, 29.8 days at 22°C and 32.8 days at 26°C. The leaf fall was 0.37 without KCl and 1.12 with KCl.

For both cultivars, the severity values differed among the isolates, depending on the combination of KCL and temperature. For all combinations of treatment and cultivar, isolate SC31 induced a higher severity, higher leaf fall and smaller values for the IP and LP. Similar results were found for isolates MC56 (except for 'Catucaí' without KCl, with an intermediate value of severity) and SO40 (except for 'Catuaí', with intermediate value of leaf fall). For isolates MO53 and TC07, high severity values were recorded in certain treatment–cultivar combinations, with intermediate values for the IP and LP in all combinations and a smaller amount of leaf fall in almost all of the combinations. For isolate TO02, a lower severity and lower leaf fall as well as higher values for the IP and LP were observed for all of the treatment–cultivar combinations (Table 3).

There were significant correlations between the IP and LP for both 'Catuaí' ($r = 0.88$, $P < 0.0001$) and 'Catucaí' ($r = 0.90$, $P < 0.0001$) between the severity and leaf fall for both 'Catuaí' ($r = 0.82$, $P = 0.001$) and 'Catucaí' ($r = 0.86$, $P = 0.0004$) and between the cercosporin production and severity in 'Catucaí' ($r = 0.59$, $P = 0.0024$).

Discussion

Despite the increasing importance of BES to coffee production (Juliatti et al. 2000), there are few studies on the disease, including research on culturing methodology for *C. coffeicola* and basic aspects of the biology of the pathogen. For instance, only recently were the events of coffee leaf penetration by the fungus clarified (Souza et al. 2011). Another aspect that requires investigation is the variability of *C. coffeicola*, which is crucial for research related to disease epidemiology and management.

We detected the variability among the 60 isolates from the state of Minas Gerais, with regard to all of the variables assessed under both laboratory and glasshouse conditions. As in other *Cercospora* species (Cai and Schneider 2005), the multicellular conidia of *C. coffeicola* allow heterokaryosis to occur, and thus, pathogen variability is expected to be high. Although the 60 coffee isolates originated from three geographic regions and two production systems, their variability was not related to either the region or cropping system. A high variability of *C. coffeicola*, as it relates to vegetative compatibility, was also reported (Martins et al. 2008), but it was suggested that the variability was not due to the effect of the geographic region or cropping system because coffee is continuously planted throughout Minas Gerais and *C. coffeicola* is wind dispersed. Therefore, regardless of the cropping system, it is expected that coffee plantations in the state will share common isolates.

The 60 isolates were clustered into four groups with regard to sporulation. Previously, Souza et al. (2005) found that more than 400 isolates collected in Minas Gerais also differed with regard to sporulation. Regarding the many *Cercospora* species, *C. coffeicola* does not sporulate well *in vitro*, and a protocol to obtain proper amounts of conidia was only recently developed (Souza et al. 2005). Although this protocol was followed in the present study, 27 (45%) of the 60 isolates tested did not sporulate. Interestingly, Martins (2007) collected

and characterized these isolates (data not published) and were able to achieve sporulation using the same methodology. As we expected difficulties in eliciting sporulation, we only assessed sporulation at 25°C; in contrast, mycelial growth and cercosporin production were assessed under variable temperature.

The isolates differed in mycelial growth at the three temperature levels, linearly increasing as the temperature rose, a trend that appears to be common among *Cercospora* spp. (Carisse et al. 1993). At 26°C, the *C. coffeicola* isolates displayed more profuse growth and were more distinguishable (six groups) than at 18°C (three groups). Therefore, we recommend growing the pathogen at 26°C *vs.* lower temperatures.

The isolates were also variable with regard to cercosporin production, a variability that also occurs in other *Cercospora* species (Jenns et al. 1989; Almeida et al. 2005). Temperature affects cercosporin toxin production, which we found to be higher at 22 and 26°C than at 18°C. In addition, the production optimum was 22.5°C for *C. ricinella* grown between 10 and 35°C, and a high cercosporin production by other *Cercospora* species was between 20 and 30°C (Fajola 1978; Jenns et al. 1989). Considering both the present and previous observations, *C. coffeicola* should be grown at 22 to 26°C to achieve more cercosporin production. It would be interesting to conduct studies to compare the growth, sporulation and cercosporin production under *in vivo* conditions to confirm these trends; such studies would also be helpful in clarifying why certain slow-growing isolates (such as SC25 and SC34) produce more cercosporin, whereas fast-growing isolates (such as MO53, SC35 and TO02) do not produce detectable cercosporin (data not shown).

Due to limitations of space and materials, we selected six of the 60 isolates assessed in the laboratory to evaluate the differences in aggressiveness. Differences in aggressiveness are common in *Cercospora* spp., as previously observed with *C. coffeicola* in coffee (Lombardi 2002) and *C. zeae-maydis* in corn (Brunelli 2004) and in the present study. There are also reports that coffee genotypes differ with regard to BES intensity (Pozza et al. 2004) and that variability exists among *C. coffeicola* isolates from different regions in Colombia (González et al. 2000). With the exception of observations presented at scientific meetings, this is the first report of differences among Brazilian isolates of *C. coffeicola* with regard to aggressiveness components in two coffee cultivars. As in other systems, host genotype affects pathogen traits; therefore, standardizing the coffee genotype is important for studies of variability of *C. coffeicola*. In both 'Catuaí' and Catucaí', isolates MC56, SC31 and SO40 were the most aggressive, and TO02 was the least aggressive. The aggressiveness of isolates MO53 and TC07 was intermediary and varied between the cultivars, depending on the trait evaluated and the KCl and temperature conditions.

Amendment with KCl tended to increase the disease intensity. The intensity of BES is related to plant nutrition (Pozza et al. 2004), and potassium predisposes plants to the pathogen because it affects the amount and activity of other elements in the plant that are important for plant defence responses (Pozza et al. 2000). We found that KCl application resulted in higher K and lower N and Ca contents in the leaves (data not shown). Considering these reports and the results presented here, plant nutrition, including K amendments, must also be standardized when defining protocols to evaluate both pathogen aggressiveness and host resistance.

Apparently, the temperature postinoculation affected the disease intensity, as the disease severity tended to be higher and the incubation/latent periods lower at 18 and 22°C than at 26°C. The effect of temperature regimes on BES needs to be better understood: in previous experiments, no disease symptoms developed in coffee plants that were maintained in growth chambers at a steady temperature after inoculation (data not shown). Defining the temperature regime postinoculation will also be helpful in defining protocols to evaluate the resistance of coffee genotypes and the aggressiveness of *C. coffeicola* isolates.

It is interesting to view the results for the cercosporin production and aggressiveness of isolates MC56, MO53 and TO02. Among the six isolates tested, MC56 was the highest cercosporin producer and one of the most aggressive, whereas isolates MO53 and TO02 produced no detectable cercosporin and were less aggressive. Furthermore, we found a significant correlation between cercosporin production *in vitro* and severity values in 'Catucaí'. A significant correlation between cercosporin production and the severity of diseases caused by *Cercospora* spp. was previously reported (Almeida et al. 2005; Choquer et al. 2005), and the role of cercosporin in the aggressiveness of *C. nicotianae* has been demonstrated (Choquer et al. 2005). It would be interesting to correlate the cercosporin production and aggressiveness of a larger number of *C. coffeicola* isolates to confirm these results, with the goal of utilizing the cercosporin production as an aggressiveness marker.

The results presented here provide basic and important knowledge about BES in coffee. Through laboratory and glasshouse experiments, we detected *C. coffeicola* variability with regard to physiological and aggressiveness traits among a population from Minas Gerais, and these traits were unrelated to the state region or cropping system. However, the BES intensity was related to the pathogen variability and was affected by potassium amendment, the temperature and the coffee cultivar. Therefore, these factors must be standardized to obtain more reliable results when studying pathogen variability and disease resistance. In experiments regarding both the latent and incubation periods, it is suggested that only the incubation period be evaluated because it is easier to quantify and is correlated with the incubation period. The knowledge gained on the variability of *C. coffeicola* isolates and the traits we measured is an important

step towards defining protocols aimed at understanding the *C. coffeicola*–coffee pathosystem. We expect that these findings could be used to advance the studies on BES in coffee and, most importantly, to provide practical strategies to manage the disease.

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