



**JULIANA BARROS RAMOS**

**CERCOSPORIOSIS RESISTANCE AND CERCOSPORIN  
PRODUCTION IN COFFEE GENOTYPES**

**LAVRAS – MG  
2022**

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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós- Graduação em Agronomia/Fitopatologia, área de concentração Fitopatologia, para obtenção do título de Doutora.

Dr. Mario Lucio Vilela de Resende  
Orientador

**LAVRAS – MG**

**2022**

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**JULIANA BARROS RAMOS**

**CERCOSPORIOSIS RESISTANCE AND CERCOSPORIN  
PRODUCTION IN COFFEE GENOTYPES**

**RESISTÊNCIA À CERCOSPORIOSE E PRODUÇÃO  
DE CERCOSPORINA EM GENÓTIPOS DE CAFEIEIRO**

Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós- Graduação em Agronomia/Fitopatologia, área de concentração Fitopatologia, para obtenção do título de Doutora.

APROVADA em 29 de Setembro de 2022.

Dr. Cristian David Plaza  
Dra. Deila Magna dos Santos Botelho  
Dra. Juliana Costa de Rezende  
Dr. Paulo Estevão de Souza

UDLA  
UFLA  
EPAMIG  
UFLA

Dr. Mario Lucio Vilela de Resende  
Orientador

**LAVRAS - MG**

**2022**

*Aos meus pais, Maria da Graças e Joaquim, que muito me apoiaram e me  
incentivaram a realizá-lo.*

*Dedico*

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*"Sem a curiosidade que me move, que me inquieta, que me insere na busca,  
não aprendo nem ensino."*

**Paulo Freire**

## RESUMO GERAL

A cercosporiose, doença causada pelo fungo necrotrófico *Cercospora coffeicola* leva a perdas significativas na produção do cafeeiro. Esta doença, causa danos nas mudas e em plantas adultas ocasionando desfolha, além de prejudicar a qualidade da bebida, pela ocorrência de lesões nos frutos. O fungo *C. coffeicola* produz a cercosporina, uma toxina de coloração vermelha que é altamente tóxica aos tecidos vegetais. O controle da cercosporiose é realizado por meio de práticas culturais e aplicação de fungicidas. Porém, a busca por cultivares resistentes à doença caracteriza-se como método de manejo duradouro e eficaz. Desta forma, este estudo teve como objetivos avaliar a resistência de 60 genótipos de cafeeiro a cercosporiose, associando dados de severidade da doença e herdabilidade; determinar os teores de lignina solúvel total e compostos fenólicos solúveis totais em genótipos contrastantes quanto à suscetibilidade à doença, além de quantificar os níveis de cercosporina em folhas de genótipos de *Coffea arabica* e *C. canephora* com sintomas de cercosporiose. Para isso, mudas de 60 genótipos de *C. arabica* provenientes do banco de germoplasma de Minas Gerais foram inoculadas com isolados de *C. coffeicola* obtidos de diferentes regiões cafeeiras do Brasil. A severidade da doença foi quantificada utilizando escala diagramática totalizando cinco avaliações. Para o experimento de quantificação dos teores de compostos fenólicos solúveis totais e lignina solúvel, foram utilizadas mudas dos genótipos MG 1207 Sumatra (menor suscetibilidade à cercosporiose) e MG 0291 e Catuaí Vermelho IAC 144 (maior suscetibilidade à cercosporiose). As coletas foliares foram realizadas às 24, 120, 240, 480 e 720 horas após a inoculação com *C. coffeicola*. Para quantificação de cercosporina, foram utilizados os genótipos de *C. arabica* (Mundo Novo 376/4, Catuaí Vermelho IAC 99, IPR 102, Topázio MG 1190) e *C. canephora* (Conillon clone 213) com sintomas foliares da cercosporiose. A cercosporina foi quantificada utilizando cromatografia líquida de alta eficiência (HPLC). Os resultados mostraram que a população estudada apresentou grande variabilidade genotípica (herdabilidade) quanto fenotípica (severidade da cercosporiose). Os valores de severidade variaram entre 2,37% (genótipo MG 1207 Sumatra) e 32,02% (Catuaí Vermelho IAC 144) e a herdabilidade individual variou de 29 a 88%, entre os 60 genótipos estudados. O genótipo MG1207 Sumatra apresentou resistência parcial a doença e alta herdabilidade, mostrando potencial fonte de resistência para programas de melhoramento de café. Os teores dos compostos fenólicos apresentaram variação nos três genótipos avaliados (MG 1207 Sumatra, MG 0291 e Catuaí Vermelho IAC 144) ao longo do tempo de coleta realizado. Contudo, não houve relação entre os teores de compostos fenólicos, lignina e resistência à cercosporiose, nos genótipos de cafeeiro. A cercosporina foi detectada nas folhas das cultivares Mundo Novo 376/4, Catuaí Vermelho IAC 99, IPR 102, Topázio MG 1190 e Conillon clone 213, indicando a presença da toxina nas lesões foliares de cercosporiose. Além disso, constatou-se a eficiência do método HPLC para a quantificação da cercosporina em folhas de café.

**Palavras-chave:** *Cercospora coffeicola*. Resistência genética. Fitotoxina.

## GENERAL ABSTRACT

Brown eye spot (BES), a disease caused by the necrotrophic fungus *Cercospora coffeicola*, leads to significant losses in coffee production. This disease causes damage to seedlings and adult plants, causing defoliation, in addition to impairing the quality of the beverage, due to the occurrence of lesions in the fruits. The fungus *C. coffeicola* produces cercosporin, a red toxin that is highly toxic to plant tissues. The control of BES is carried out through cultural practices and application of fungicides. However, the search for disease-resistant cultivars is characterized as a long-lasting and effective management method. Thus, this study aimed to evaluate the resistance of 60 coffee genotypes to BES, associating data on disease severity and heritability; to determine the levels of total soluble lignin and total soluble phenolic compounds in contrasting genotypes regarding disease susceptibility, in addition to quantifying cercosporin levels in leaves of *Coffea arabica* and *C. canephora* with symptoms of BES. For this, seedlings of 60 *C. arabica* genotypes from the Minas Gerais germplasm bank were inoculated with *C. coffeicola* isolates obtained from different coffee growing regions in Brazil. Disease severity was quantified using a diagrammatic scale, totaling five assessments. For the experiment to quantify the levels of total soluble phenolic compounds and soluble lignin, seedlings of the genotypes MG 1207 Sumatra (lower susceptibility to BES) and MG 0291 and Catuaí Vermelho IAC 144 (higher susceptibility to BES) were used. Leaf collections were performed at 24, 120, 240, 480 and 720 hours after inoculation with *C. coffeicola*. For the quantification of cercosporin, the genotypes of *C. arabica* (Mundo Novo 376/4, Catuaí Vermelho IAC 99, IPR 102, Topázio MG 1190) and *C. canephora* (Conillon clone 213) with leaf spot symptoms of BES. Cercosporin was quantified using high performance liquid chromatography (HPLC). The results showed that the population studied showed great genotypic (heritability) and phenotypic (severity of BES) variability. Severity values ranged from 2.37% (MG 1207 Sumatra genotype) to 32.02% (Catuaí Vermelho IAC 144) and individual heritability ranged from 29 to 88% among the 60 genotypes studied. The MG1207 Sumatra genotype showed partial disease resistance and high heritability, showing a potential source of resistance for coffee breeding programs. The levels of phenolic compounds showed variation in the three genotypes evaluated (MG 1207 Sumatra, MG 0291, and Catuaí Vermelho IAC 144) throughout the collection period. However, there was no relationship between the levels of phenolic compounds, lignin and resistance to BES, in the coffee genotypes. Cercosporin was detected in the leaves of cultivars Mundo Novo 376/4, Catuaí Vermelho IAC 99, IPR 102, Topázio MG 1190 and Conillon clone 213, indicating the presence of the toxin in leaf spot lesions. In addition, the efficiency of the HPLC method was verified for the quantification of cercosporin in coffee leaves.

**Keywords:** *Cercospora coffeicola*. Genetic resistance. Phytotoxin.

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## PRIMEIRA PARTE

### 1. INTRODUÇÃO GERAL

O cultivo do café no Brasil é de extrema importância para a economia do país, sendo fonte de renda para centenas de municípios, além de gerar milhares de postos de trabalho na agropecuária nacional (MAPA, 2017). Segundo dados da Conab (COMPANHIA NACIONAL DE ABASTECIMENTO, 2022), o país é o maior produtor e exportador do produto. Além de assumir tal posição no *ranking* mundial de produção, o Brasil também possui uma legislação altamente exigente em relação as questões sociais e ambientais (EMBRAPA, 2018). Por isso, tem-se investido em pesquisas aliando o melhoramento genético e a biotecnologia visando à redução no uso de agroquímicos, propiciando uma agricultura cada vez mais sustentável.

A cafeicultura brasileira é ameaçada por vários fatores abióticos e bióticos e, qualquer fator capaz de reduzir a produção, está afetando conseqüentemente a cadeia produtiva do café. Entre os fatores bióticos cita-se a cercosporiose, doença cujo agente etiológico é o fungo *Cercospora coffeicola* Berk. & Cooke. Essa doença leva a perdas significativas na produção devido à queda prematura das folhas, principalmente em mudas nos viveiros e em plantas adultas no campo (POZZA; CARVALHO; CHALFOUN, 2010). Dependendo dos fatores ambientais (localização geográfica e microclima da região) a cercosporiose ocasiona perdas de mais de 30% na produção (SOUZA et al., 2015).

Até recentemente, a doença era de importância secundária e geralmente associada com plantas em estresse nutricional ou hídrico. No entanto, nas últimas duas décadas, produtores e pesquisadores notaram um acréscimo na intensidade da cercosporiose em lavouras de todo o país (PATRÍCIO; BRAGHINI; FAZUOLI, 2010). Esse aumento foi atribuído a vários fatores, tais como a expansão de áreas de cultivo de café na região de Cerrados, o plantio de novas cultivares de café, as mudanças nas práticas culturais e as alterações nas condições climáticas (MARTINS; MAFFIA; MIZUBUTI, 2008).

O progresso da doença é favorecido por vários fatores relacionados ao patógeno e hospedeiro, e principalmente ao ambiente (SANTOS et al., 2008). Em contraste aos fungos patogênicos biotróficos, os quais necessitam de células vivas do hospedeiro, fungos necrotróficos utilizam um arsenal de enzimas para a degradação da parede celular das plantas, bem como toxinas para degradar a célula hospedeira e torná-la fonte de energia.

As bases genéticas da resistência quantitativa a fungos necrotróficos são praticamente desconhecidas (MBENGUE et al., 2016). Até o momento, não há relatos de cultivares de

cafeeiro com resistência à *C. coffeicola* (BOTELHO et al., 2017). Alguns fungos fitopatogênicos utilizam estratégias para causar doenças em plantas. Uma estratégia comum envolve a produção de toxinas que matam as células hospedeiras, permitindo a colonização dos tecidos pelo patógeno (SCHEFFER, 1991; BERESTETSKIY, 2008). Muitas espécies do gênero *Cercospora* produzem uma toxina, a cercosporina (OKUBO et al., 1975). A cercosporina é ativada pela luz, gerando espécies reativas de oxigênio (EROs) (DAUB et al., 2013). A produção de EROs tóxicos torna a cercosporina quase universalmente tóxica, com toxicidade comprovada em plantas hospedeiras e não hospedeiras, fungos e bactérias (DAUB et al., 2005). Nas plantas hospedeiras, a cercosporina secretada pelo fungo se acumula nas membranas, levando à peroxidação dos lipídios e morte das células hospedeiras, permitindo a colonização do tecido pelo fungo (DAUB et al., 2013). A via biossintética da cercosporina a produção foi elucidada em *C. nicotianae* (CHOQUER et al., 2005; DEKKERS et al., 2007). Diante do grande número de genótipos de cafeeiro, há a necessidade de intensificar a investigação destes quanto à resistência à cercosporiose, elucidar os mecanismos bioquímicos envolvidos na defesa da planta e quantificar a toxina cercosporina em folhas de cafeeiro. Desta forma, este estudo teve como objetivos avaliar a resistência de 60 genótipos de cafeeiro a cercosporiose, aliando dados de severidade da doença e herdabilidade; determinar os teores de lignina solúvel total e compostos fenólicos solúveis totais em genótipos contrastantes quanto à suscetibilidade à doença e quantificar os níveis de cercosporina em folhas de genótipos de *Coffea arabica* e *C. canephora* utilizando cromatografia líquida de alta eficiência (HPLC).

## **2. REFERENCIAL TEORICO**

### **2.1. A cultura do cafeeiro**

O café é uma das principais fontes de renda para o Brasil, é o maior produtor e exportador desta cultura, responsável por aproximadamente 36% do consumo mundial (INTERNATIONAL COFFEE ORGANIZATION – ICO, 2019). O cafeeiro é um arbusto pertencente à família Rubiaceae, gênero *Coffea*, da qual se conhecem 103 espécies (DAVIS et al., 2006; FERRÃO et al., 2015). Entre as espécies cultivadas, *Coffea arabica* L. e *Coffea canephora* Pierre ex. Froehner são as mais importantes economicamente (ABRAHÃO et al., 2010), responsáveis, por cerca de 75% e 25% da produção total de café do país, respectivamente. A estimativa para a produção nacional na safra de 2020 varia entre 57,15 e 62,02 milhões de sacas beneficiadas. O estado de Minas Gerais é o maior produtor de café

arábica do país e ocupa a maior área plantada, com 1,22 milhão de hectares, correspondente nesta safra a 72,1% da área ocupada dessa espécie no setor nacional (COMPANHIA NACIONAL DE ABASTECIMENTO - CONAB, 2022).

Além da elevada produção, o café brasileiro vem agregando valor ao produto, melhorando em qualidade e nas estratégias de produção, tanto no uso de melhores cultivares como no controle fitossanitário e cuidados pós-colheita (PEREIRA, 2008). Existem numerosas causas capazes de afetar diretamente a produtividade do cafeeiro levando a perdas significativas e, dentre as mais acometidas, estão: condições climáticas adversas, deficiências nutricionais e presença de pragas e doenças (OESTREICH-JANZEN, 2010). A ocorrência de doenças, dependendo da intensidade, pode estar associada a perdas em larga escala, e estas ocorrem desde a fase de viveiro até a produção final. Entre as importantes doenças da cultura, podem-se citar a ferrugem alaranjada (*Hemileia vastatrix* Berk. & Br); cercosporiose (*Cercospora coffeicola* Berk & Cooke); antracnose dos frutos (*Colletotrichum kahawae* Waller & Bridge), considerada praga quarentenária ausente (A1) no Brasil; nematoide das galhas (*Meloidogyne* spp.); mancha de Phoma (*Phoma tarda* (Stewart) Boerema & Bollen); mancha aureolada (*Pseudomonas syringae* pv. *garcae* (Amaral, Teixeira & Pinheiro) Young, Dye & Wilkie), entre outras (ZAMBOLIM; VALE; ZAMBOLIM, 2005; POZZA; CARVALHO; CHALFOUN, 2010; PATRÍCIO et al., 2008; ZAMBOLIM, 2016).

## 2.2. Cercosporiose do cafeeiro

A cercosporiose, conhecida por “mancha-de-olho-pardo” ou “olho-de-pomba”, tem como agente etiológico o fungo *Cercospora coffeicola* Berk. & Cooke, é uma das doenças mais antigas e importante do cafeeiro (CARVALHO; CUNHA; MOURA, 2008; SOUZA et al., 2011). A incidência da cercosporiose tem aumentado, nos últimos anos e a doença encontra-se disseminada em todas as regiões cafeeiras do Brasil e do mundo, ocasionando perdas na produtividade, além de afetar a qualidade do café produzido (MARTINS; MAFFIA; MIZUBUTI, 2008; SOUZA et al., 2015).

Os sintomas da doença ocorrem nas folhas e nos frutos. Nas folhas são caracterizados por manchas circulares de coloração castanho-claro a escuro, com centro branco-acinzentado, quase sempre envolvidas por um halo amarelo, dando à lesão aspecto de olho. Em estágio mais avançado da doença, notam-se pontuações escuras, essas constituem as estruturas de frutificação do fungo. Também, podem ocorrer variações nos sintomas descritos, por ausência

do halo amarelo, sendo a doença denominada em algumas regiões, de cercospora-negra. Já os frutos quando infectados, as lesões se tornam mais presentes próximos a maturação, caracterizadas por lesões deprimidas de coloração marrom ou arroxeada, essas lesões tornam-se escuras e ressecam a polpa do fruto dificultando o processo de despulpamento e provocando queda prematura (SOUZA et al., 2011; LIMA; POZZA; SILVA, 2012; ZAMBOLIM, 2016; BOTELHO et al., 2017).

A cercosporiose ocasiona danos ao cafeeiro desde a fase de mudas em viveiro até plantas adultas no campo. Em condições de viveiro, a incidência do fungo é favorecida por excesso de irrigação ou por deficiência hídrica, desequilíbrio nutricional e insolação. Por conseguinte, as plantas apresentam desfolha intensa, tornando-se raquíticas e impróprias ao plantio (BOTELHO et al., 2005; GARCIA JÚNIOR et al., 2003; POZZA et al., 2001). Já em plantios novos, comumente, ocorre intensa incidência da doença, resultando em desfolha acentuada, prejudicando o crescimento das mudas, principalmente, em lavouras implantadas em terrenos de baixa fertilidade e/ou com adubações desequilibradas. Além da desfolha, a doença promove, também, a queda prematura dos frutos, podendo funcionar ainda como porta de entrada para outros fungos, os quais, irão interferir na qualidade do café (POZZA; CARVALHO; CHALFOUN, 2010; LIMA; POZZA; SANTOS, 2012).

As condições favoráveis para o desenvolvimento do fungo *C. coffeicola* são alta umidade relativa, temperatura em torno de 24 a 30°C (CUSTÓDIO et al., 2011). Além disso, alta luminosidade contribui para o progresso da doença, pois na presença de luz, a cercosporina, toxina produzida por espécies do gênero *cercospora*, é ativada e capaz de reagir com outras moléculas em particular com o oxigênio, o mesmo é convertido em espécies reativas de oxigênio (EROs) altamente tóxicas para a célula do hospedeiro (BERESTETSKIY, 2008; DAUB; HERRERO; CHUNG, 2005).

As medidas de manejo da cercosporiose em caráter preventivo são realizadas na formação de mudas, controlando a irrigação, a luminosidade e adubação equilibrada. O controle químico da doença é realizado com aplicações de fungicidas protetores cúpricos alternando com sistêmicos (PEREIRA et al., 2008; CARVALHO; CUNHA; SILVA, 2012). Porém, altos custos, aumento da resistência do patógeno, como também o impacto ambiental tem levado fitopatologistas a intensificarem os estudos na procura de métodos eficientes no controle da doença (GALDEANO et al., 2010; CARVALHO; CUNHA; SILVA, 2012; COSTA et al., 2014). Apesar dos esforços em busca de resistência completa à cercosporiose em genótipos de cafeeiro, genótipos com resistência completa à doença ainda não foram encontrados

(PATRICIO; BRAGHINI; FAZUOLI, 2010; SOUZA et al., 2011).

Desta forma, verifica-se a necessidade de pesquisas para incremento de tecnologias de controle da doença como o desenvolvimento de cultivares resistentes à cercosporiose do cafeeiro. O uso de genótipos resistentes é uma importante alternativa para o controle da doença.

### **2.3. Resistência à cercosporiose**

A resistência à cercosporiose tem sido pouco estudada, provavelmente porque os esforços das pesquisas vêm se limitando no desenvolvimento de cultivares resistentes à ferrugem, uma das principais doenças da cultura do café, sendo a cercosporiose deixada em segundo plano ou por a mesma ser sempre relacionada com deficiência nutricional. Entretanto, um aumento significativo da doença vem ocorrendo nas regiões cafeeiras (PATRICIO; BRAGHINI; FAZUOLI, 2010; SOUZA et al., 2015).

O desenvolvimento de doenças depende de fatores associados ao patógeno, ao hospedeiro e ao meio ambiente (EASTBURN; MCELTRONE; BILGIN, 2011). O manejo da cercosporiose pode ser realizado com aplicação de fungicidas cúpricos, triazóis e estrobilurinas (PATRICIO; BRAGHINI, 2011) e nutrição equilibrada (GARCIA JUNIOR et al., 2003; POZZA; CARVALHO; CHALFOUN, 2010; BARBOSA JUNIOR, 2019). Porém, o desenvolvimento de uma cultivar resistente à doença é desejável devido à redução e/ ou eliminação da utilização do controle químico como medida de controle. Pozza et al. (2004), observaram mudas de cafeeiro da cultivar Icatu submetidas a inoculação com *C. coffeicola*, foram menos afetadas quando comparadas as mudas das cultivares Catuaí e Mundo Novo. Botelho et al. (2017), verificaram entre os 132 genótipos avaliados com relação a cercosporiose, maior resistência na cultivar Sachimor MG 8840 com relação a todos os demais genótipos avaliados, seguida da cultivar Guatenano. As cultivares comerciais Catiguá MG2 e Paraíso, também apresentaram baixa severidade da doença quando comparadas com os demais genótipos analisados.

Considerando a cercosporiose uma doença do cafeeiro cuja importância tem aumentado, especialmente nas regiões de expansão da cafeicultura, como o Cerrado, e na cafeicultura irrigada, a identificação de fontes de resistência a essa doença se torna cada vez mais relevante, podendo ser obtidos materiais com resistências múltiplas.

## 2.4. Mecanismos de Defesa

Para se defenderem contra ataques de patógenos, as plantas apresentam um conjunto de mecanismos de defesa. Esses podem ser estruturais e bioquímicos constitutivos, bem como um sistema de defesa induzível como resposta ao processo de infecção (JONES; DANGL, 2006). A sinalização de defesa atua cooperativamente com inúmeros processos celulares e, juntos, a soma dessas interações confere a capacidade de reconhecer uma vasta gama de ameaças bióticas como patógenos e pragas (SANABRIA et al. , 2008).

As defesas expressas, constitutivamente, são caracterizadas por estruturas como ceras, cutícula, parede celular espessa, adaptações nos estômatos e fibras vasculares, bem como substâncias bioquímicas pré-formadas, como fenóis, alcaloides, glicosídeos fenólicos, glicosídeos cianogênicos, inibidores proteicos e enzimas hidrolíticas (HEMATY; CHERK; SOMERVILLE, 2009; HANKS; NELSON, 2016). Por outro lado, dentre os mecanismos induzidos, podem ser citados a produção de fitoalexinas, proteínas relacionadas à patogênese (PR-proteínas), espécies reativas de oxigênio (EROs), formação de papila, lignificação, camada de cortiça, formação de tiloses e deposição de goma, entre outros (TORRES; JONES; DANGL, 2006; HANKS; NELSON, 2016). Entender como o fitopatógeno altera a fisiologia e bioquímica da célula vegetal pode auxiliar no controle de doenças.

Durante o processo de reações de defesa ocorre também a formação de lignina e compostos fenólicos, provenientes do metabolismo dos fenilpropanóides. A biossíntese da lignina envolve uma série de enzimas, dentre elas a fenilalanina amônia liase (PAL), responsável por catalisar a conversão da fenilalanina em ácido *trans* cinâmico, resultando em fitoalexinas, compostos fenólicos e lignina, e, os mesmos, podem conferir maior resistência à parede celular das plantas durante o ataque de patógenos (NAKAZAWA et al., 2001; TAIZ; ZEIGER, 2004). A lignina é um dos metabólitos secundários mais importantes produzidos pela via metabólica da fenilalanina nas células vegetais é o segundo biopolímero mais abundante que responde por 30% do conteúdo de carbono orgânico na biosfera (RALPH et al., 2004). A lignina e seu metabolismo relacionado desempenham papéis importantes no crescimento e desenvolvimento das plantas. Como polímero fenólico complexo, a lignina aumenta a rigidez da parede celular da planta, propriedades hidrofóbicas e promove o transporte de minerais através dos feixes vasculares da planta (SCHUETZ et al., 2014; LIU; LUO; ZHENG, 2018). A lignificação pode impedir o desenvolvimento do fungo nos tecidos vegetais de várias maneiras,

como o estabelecimento de barreira mecânica ao avanço e o desenvolvimento do patógeno; a modificação da parede celular, tornando-a mais resistente ao ataque de enzimas hidrolíticas e o aumento da resistência das paredes à difusão de toxinas produzidas por patógenos, impede o invasor de utilizar os nutrientes do hospedeiro (CAVALCANTI, 2006; SHADLE et al., 2007). O metabolismo da lignina também pode estar envolvido ativamente em resposta a vários estresses ambientais (MOURA et al., 2010).

## 2.5. A toxina Cercosporina

Muitas espécies do gênero *Cercospora* produzem a cercosporina, uma toxina não seletiva que atua de forma prejudicial a inúmeros organismos. Pertence ao grupo químico perilenoquinona, contém moléculas fotossensibilizadoras capazes de absorver luz e gerar espécies reativas de oxigênio, causando danos à membrana celular (DAUB; CHUNG, 2009).

A produção de tal metabólito *in vitro* ocorre por meio de hifas em meio de cultura, resultando em acúmulo da toxina nas proximidades das hifas e na superfície do meio. A toxina cercosporina apresenta coloração avermelhada, todavia em condições alcalinas como na presença de KOH, a mesma apresenta coloração esverdeada variando de verde claro a verde intenso. Ao realizar testes de absorvância da toxina Jenns et al. (1989) observaram que a mesma apresenta maior absorção a um comprimento de onda de 480 nm.

Daub e Chung (2009) mencionam que um importante fator tanto para a ativação da toxina quanto para sua biossíntese é a presença de luz. Não há relatos sobre os genes envolvidos na produção de cercosporina em *C. coffeicola*. No entanto, foi demonstrado que o sombreamento reduz a penetração de fungos e menos lesões se desenvolvem nas folhas sombreadas (SILVA et al., 2015). Em estudos anteriores (SOUZA et al., 2012), encontraram alta variabilidade na produção de cercosporina por isolados de *C. coffeicola* coletados em diferentes locais do estado de Minas Gerais além da correlação positiva entre a produção de cercosporina *in vitro* e virulência (SOUZA et al., 2012). Posteriormente, Souza et al. (2019) verificaram que a cercosporina pode ser um importante fator de virulência de *C. coffeicola* para a infecção de cafeeiros, e que os esforços para identificar genes envolvidos na resistência à cercosporina podem permitir novas estratégias para o desenvolvimento de café com resistência à cercosporiose (SOUZA et al., 2019).

A cercosporina *in vitro* é produzida pelo patógeno apenas em meios de cultura que favoreçam o crescimento vegetativo, e é inibido em meios nutritivos que induzam o processo de esporulação, como o meio de cultura composto pelo suco V8. Diversos parâmetros

fisiológicos, incluindo temperatura, condições nutritivas, pH, luminosidade e fonte de carbono, afetam a produção do metabólito. A síntese de metabólitos também varia entre espécies e entre populações de isolados de uma única espécie (DAUB; CHUNG, 2007).

Gunasinghe et al. (2016), detectaram a cercosporina a partir de lesões de mancha branca nas folhas de Brassicaceae e, correlação positiva entre o nível de produção de cercosporina e a virulência dos isolados consolidam a informação de que a toxina é um fator de virulência de *Pseudocercospora capsellae*.

Alguns estudos relatam a eficácia da quantificação da cercosporina *in vitro* realizada por cromatografia líquida de alta eficiência (HPLC) em *Cercospora coffeicola*, *C. beticola*, *C. cf. fagellaris*, *Colletotrichum foriniae*, *Pseudocercospora capsellae* (Gunasinghe et al. 2016; Jonge et al. 2018; Rezende et al. 2020, Botelho et al., 2021). No entanto, mesmo a cercosporina sendo considerada um fator de virulência, não existem relatos da quantificação *in vivo* (a partir de folhas de cafeeiro com sintomas da cercosporiose), indicando a necessidade de estudos com este enfoque.

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## **ARTIGO 1 - Screening coffee genotypes for brown eye spot resistance in Brazil**

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### **Authors**

Juliana Barros Ramos<sup>1</sup>; Mario Lucio Vilela de Resende<sup>1</sup>; Deila Magna dos Santos Botelho<sup>1</sup>; Renata Cristina Martins Pereira<sup>1</sup>; Tharyn Reichel<sup>1</sup>; André Augusto Ferreira Balieiro<sup>1</sup>; Gustavo Pucci Botega<sup>2</sup>; Juliana Costa de Rezende Abrahão<sup>3</sup>

### **Institutional Affiliation**

<sup>1</sup>Universidade Federal de Lavras – Dept. de Fitopatologia – C. P. 3037 - 37200-900 – Lavras, MG – Brasil

<sup>2</sup>Universidade Federal de Lavras – Dept. de Genética e Melhoramento de Plantas – C. P. 3037 – 37200-900 – Lavras, MG – Brasil

<sup>3</sup>Empresa de Pesquisa Agropecuária de Minas Gerais – Campus Universitário – C. P. 176 – 37200-900 – Lavras, MG – Brasil

**\*Corresponding author:** E-mail: [julianacosta@epamig.br](mailto:julianacosta@epamig.br)

### **Abstract**

Several researchers have attempted to develop coffee plants that are resistant to brown eye spot (BES); however, no coffee cultivars are resistant to the disease. In the present study, a blend of strains from *Cercospora coffeicola* was inoculated into 19 Brazilian commercial cultivars and 41 accessions from the Germplasm Collection of Minas Gerais to evaluate the genetic resistance

ability within the population and select superior genotypes for the breeding program. After predicting the genotypic values of the evaluated material, the evaluations number necessary for selecting genotypes with accuracy and efficiency was determined based on the data of severity to BES. The action of defense mechanisms plant was also investigated by assessing the levels of total soluble phenolic compounds and soluble lignin in contrasting genotypes for disease susceptibility. Based on the results, the genotype MG 1207 Sumatra, had an intrinsic genetic capacity to maintain low levels of severity to BES. The genotype MG 1207 Sumatra can substantially contribute to the development of new cultivars, which may lead to the reduced use of pesticides. According to the accuracy and efficiency results obtained, four evaluations BES severity are sufficient to achieve accuracy, providing expressive genetic gains. Finally, the levels of lignin and phenolic compounds were not found to be associated with the resistance of coffee genotypes to BES.

**Keywords:** *Coffea arabica*; defense-related compounds; *Cercospora coffeicola*; germplasm collection.

## 1 Introduction

Coffee is a crop of great economic importance in many countries including Brazil, the largest producer and exporter of coffee in the world [1]. The country is internationally recognized for its ability to produce coffee in large volumes and at competitive prices, but, recently, it also stands out as a prestigious origin of specialty coffees. Brazil is also the world's second largest consumer of the beverage, surpassed only by the United States of America (16%) [2,3]. The occurrence of diseases is most important limitations in the coffee production, especially brown eye spot (BES), which is considered a disease economic importance for coffee growers in Brazil and other countries coffee producers as Colombia, Porto Rico, Costa Rica, El-Salvador and Honduras [4]. The disease caused by fungus *Cercospora coffeicola* Berk. & Cooke has host *Coffea* species as *Coffea arabica*, *Coffea canephora*, *Coffea eugenioides*, *Coffea liberica* and *Coffea racemosa* (Echandi, 1959). In the absence of control management, losses may reach up to 30% are registered in Brazil [5]. The BES is characterized by leaf symptoms round necrotic lesions, with a brown outer ring and a gray-white center area [6,7]. Further, BES causes defoliation, stimulates maturation, besides intensifying pulp adherence to the endocarp, which makes it

difficult to carry out de-pulping affecting the quality of the produced coffee [6]. Increases in the productive potential in coffee areas can lead to nutritional imbalance, resulting in a higher susceptibility to disease [7]. Current disease management has been commonly accomplished via the application of chemical products coupled with an accurate water supply and plant nutrition [8]. In Brazil, increases in the incidence of BES is related with the expansion of coffee fields to other regions with different environmental conditions.

To date, as the physiology of plant responses underlying genetic resistance is quite complex, few studies have been carried out on BES resistance in coffee species [9, 10, 11]. In this context, Dell'Acqua et al. [9] and Patricio et al. [10] used the phenotypic data from BES severity evaluation under greenhouse conditions to carry out selection based on a test of means. These researchers considered the studied genotypes as fixed effects, without estimating the genetic variance and heritability of the phenotypic variation. This strategy may not be effective in crop breeding programs that aim to develop materials resistant to BES as it does not assess the potential effects of selection on the studied characters.

Plants have a set of defense mechanisms to defend themselves against pathogen attacks, which can either be constitutively structural or biochemical [12]. Among the induced mechanisms, the production of phenolic compounds and lignin is commonly observed during defense reactions, which are produced via the metabolism of phenylpropanoids [13], thereby providing higher resistance to the plant cell wall during pathogen attack. Understanding how infection process for *C. coffeicola* affects the biochemical aspects of plant cells can shed light on a further mechanism to control BES.

This study aimed to select superior *Coffea arabica* genotypes for resistance to BES and quantify the total soluble phenolic compounds and soluble lignin in contrasting genotypes for disease susceptibility. The possibility of selecting coffee plants with higher resistance to this disease is extremely important; however, the success of this strategy depends on the existence of genetic variation within studied characteristics, and a high heritability rate.

## **2 Materials and Methods**

### **2.1 BES severity**

The experiment was carried out under greenhouse conditions at the Department of Phytopathology of the Universidade Federal de Lavras - UFLA, Brazil. Coffee seedlings of 19 commercial cultivars and 41 accessions from the Germplasm collection of the Agricultural

Research Corporation of the State of Minas Gerais (EPAMIG) in Patrocínio, MG, were evaluated to determine their resistance to BES (Table S1). The genotypes were selected according to their characteristics of yield, drink quality, and/or resistance to other diseases of economic importance.

Seeds were sown in 5 L plastic trays containing autoclaved sand. The germination chamber was adjusted to 30 °C and 80% relative humidity. The seedlings were transplanted into punched-black polyethylene pots (0.11 x 0.20 m) at the phase of cotyledons. The substrate consisted of 300 L of cattle manure and 700 L of soil mix extracted from the 0.4 to 0.8 m layer of Dystrophic Red Latosol and fertilized with 5 kg of simple superphosphate and 500 g of potassium chloride.

The seedlings were inoculated at the stage of three pairs of true leaves. The inoculum consisted of a blend of different isolates of *C. coffeicola* obtained from coffee leaves with symptoms of BES collected in the municipalities of Marechal Floriano (ES), Cachoeirinha, Ervália, Lavras, and Patrocínio (MG). Different isolates were employed to include a higher variability of the fungus [6, 11].

The sporulation of the isolates was performed as described by Souza et al. [14], with adaptations. Eight mycelial discs (6 mm in diameter) were removed from the colony borders (on day 15 of growth) of different isolates of *C. coffeicola*. Discs were macerated in 400 µL of sterile distilled water. The macerated mycelium from each isolate was placed in Erlenmeyer flasks containing 20 mL of liquid V8 culture medium (100 mL of V8 in 900mL of distilled water) under shaking at 100 rpm for 12 d at room temperature. The liquid containing the mycelium was transferred to plates containing a water-agar medium. The plates were kept in a BOD incubator (Bio-Oxygen Demand), with a photoperiod of 12 h at 25 °C. After culture medium dehydration (approximately 5 days of incubation), 10 mL of sterile water was added to each plate and the conidia were removed with a Drigalski spatula. The suspension was filtered with sterile gauze to remove residues and the conidia were subsequently quantified in a Neubauer chamber. The suspension used for inoculation was adjusted to  $5 \times 10^4$  conidia·mL<sup>-1</sup> and sprayed on the abaxial side of the leaves of all seedlings using a manual sprayer. Thereafter, the seedlings were placed in a humid chamber for 72 h.

Temperature and relative humidity data were collected over the experimental period with a Datalogger HT-500, Instrutherm®. Weekly assessments of disease severity were performed on the first two pairs of true leaves throughout the five weeks, starting from the onset of symptoms (approximately 15 d after inoculation). The severity of BES in different coffee

genotypes was quantified using a diagrammatic scale with six classes of the proportion in the infected area by BES [15]: class 1: 0.1-3.0%; class 2: 3.1-6.0%; class 3: 6.1-12.0%; class 4: 12.1-18.0%, class 5: 18.01-30.0%, and class 6: 30.1-50.0%. The experiment was repeated twice. The distribution of the phenotypic segregation was evaluated in each genotype within the six classes described. Based on this assessment, the resistance level was determined: class 1- resistant (R); class 2- partially resistant (PR); class 3- moderately susceptible (MS); class 4- susceptible (S); classes 5 and 6- highly susceptible (AS).

The severity dates were used to calculate the area under disease progress (AUDPC), as previously proposed by Shaner and Finney [16].

$$AUDPC = \sum_{i=1}^{n-1} \left( \frac{si+si+1}{2} \right) (ti + 1 - ti) \quad (1)$$

where AUDPC is area under disease progress curve;  $S_i$  is disease severity in the time of evaluation; and  $t_i$  is the time of evaluation  $i$ .

## 2.2 Total soluble phenolic compounds and soluble lignin

Both total soluble phenolic compounds and soluble lignin levels were quantified in the leaves of MG 1207 accession (presenting low disease severity in the present study) and MG 0291 and Catuaí Vermelho IAC 144 genotypes (presenting high disease severity). The Catuaí Vermelho IAC 144 genotype was used as a control of susceptibility, according to Patricio et al. [10] and Botelho et al. [11].

The samples consisted of 2<sup>nd</sup> and 3<sup>rd</sup> pairs of fully expanded leaves, collected at 24, 120, 240, 480, and 720 h after inoculation (hai) of *C. coffeicola*. Samples of non-inoculated plants with the pathogen were also collected at 24 and 720 h to confirm that the inoculation influences the levels of total soluble phenolic compounds and soluble lignin. After collection, the samples were immediately stored in liquid nitrogen and kept in an ultra-freezer until sample processing.

The macerated samples were lyophilized and approximately 30 mg of the material was homogenized in 80% methanol. The solution was centrifuged at room temperature for 5 min at 14000 rpm. The supernatant and the precipitate were used to quantify total soluble phenolic compounds and soluble lignin, respectively.

The levels of total soluble phenolic compounds were determined as described by Spanos and Wrolstad [17], with modifications. The supernatant was homogenized with 0.25 N Folin-

Ciocalteau reagent, 1 M Na<sub>2</sub>CO<sub>3</sub>, and distilled water. The reaction was standardized in 200 µL and quantified using a spectrophotometer at 725 nm. Based on the standard curve of chlorogenic acid, the levels of total soluble phenolic compounds were calculated.

Lignin was quantified as proposed by Doster and Bostock [18]. The precipitate was homogenized in 80% methanol and centrifuged as described for phenolic compounds. The contents were evaporated in an oven at 45 °C overnight and mixed with thioglycolic acid and 2 M HCl (ratio 1:10) in a water bath at 100 °C for 4 h. After centrifugation and solubilization in 0.5 M NaOH, the supernatant was homogenized with HCl P.A., and kept at 4 °C for 4 h before centrifugation. The precipitate was homogenized in 0.5 M NaOH. A 200-µL aliquot of this solution was used for the reaction, and the absorbance was assessed in a Power Wave XS microplate spectrophotometer (Biotek®) at 280 nm. Based on a standard curve of lignin, the soluble lignin content was subsequently estimated. The quantification of total soluble phenolic compounds and soluble lignin contents was performed in triplicate.

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### 2.3 Statistical analysis

The experiment BES severity were conducted in a randomized complete block design, with 60 treatments (genotypes), eight replicates, and five evaluations. The experimental plot comprised two coffee plants. A mixed model analysis was accomplished using the Selegen REML/BLUP software [19]. For the randomized completely randomized design, the following equation was used:  $y = Xm + Zg + Tp + e$ , where  $y$  is the data vector;  $m$  is scalar referring to the general average of fixed effect;  $g$  is the vector of random genetic effects;  $p$  is the vector of the random-effects of blocks within replications and experiment; and  $e$  is the vector of random errors.  $X$ ,  $Z$ , and  $T$  are incidence matrices of referred effects. The variance components were subjected to the likelihood ratio test at 5% probability.

The experiment of quantification of total soluble phenolic compounds and soluble lignin in coffee genotypes, were conducted in a randomized complete block design with three replicates and two plants per plot. Analysis of variance was conducted in a 3 x 5 factorial scheme, with three evaluated genotypes and five collection times (24, 120, 240, 480, and 720 h) after inoculation (hai) of *C. coffeicola*. The data were subjected to analysis of variance and the means were compared by Tukey's test at 5% probability.

## 3 Results

### 3.1 Genotype selection

The analysis based on mixed models provided a simultaneous estimation of both individual heritability and plot repeatability (Fig 1, Table S1). The heritability and genetic variation coefficients were significant, thereby indicating the possibility of selection. The selection among families was high (98%) and should be recommended. The coefficient of permanent effect determination showed a low magnitude ( $<0.00$ ), revealing that variations in the environment among measurements do not affect such responses. The overall average of the

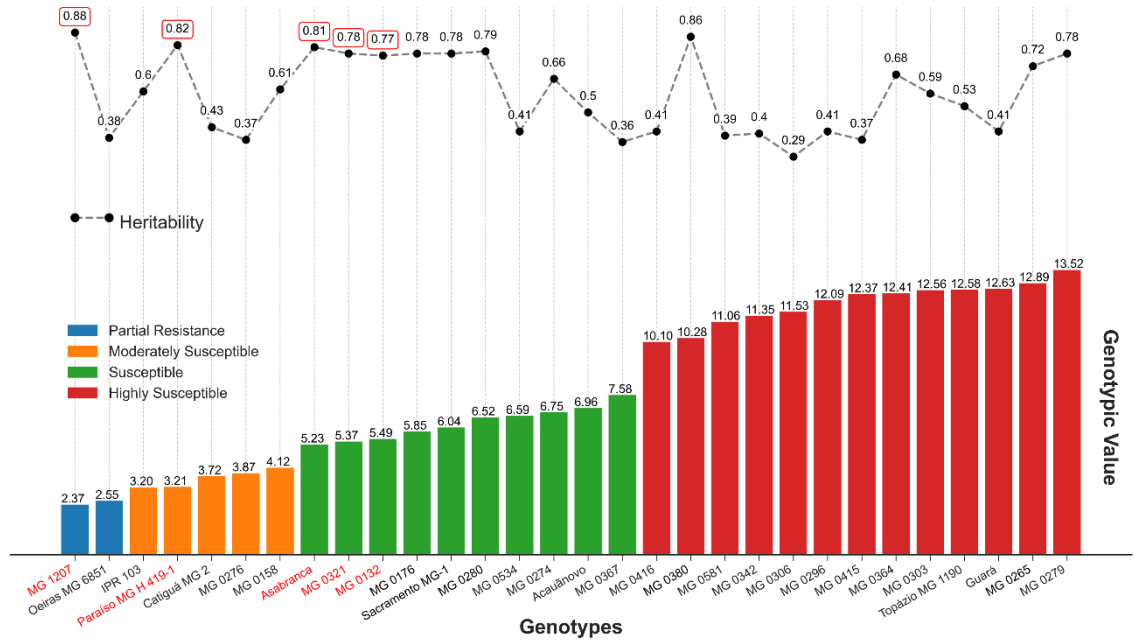
experiment was over 14.50% of severity (Table S1).

The studied population presented a high genotypic variability for the severity of BES. The predicted additive values were between 2.37% (accession MG 1207) and 32.02% (Catuaí Vermelho IAC 144); while values for the individual heritability were between 29 and 88% among 60 genotypes (Table S1).

Based on the analysis of frequency-severity, the genotypes were mainly clustered into 4, 5, and 6 classes (Table 1), and were thus considered susceptible or highly susceptible. The accessions, MG 1207 Sumatra and Oeiras MG 6851, displayed injured leaf area by approximately 3.1 to 6.0%, and were classified as partially resistant (class 2). The accessions MG 0276 and MG 0158, and the cultivars, Catiguá MG2, Paraíso MG H 419-1, and IPR 103, were considered moderately susceptible (class 3). None of the genotypes studied were clustered in the first class (0 to 3% of injured area).

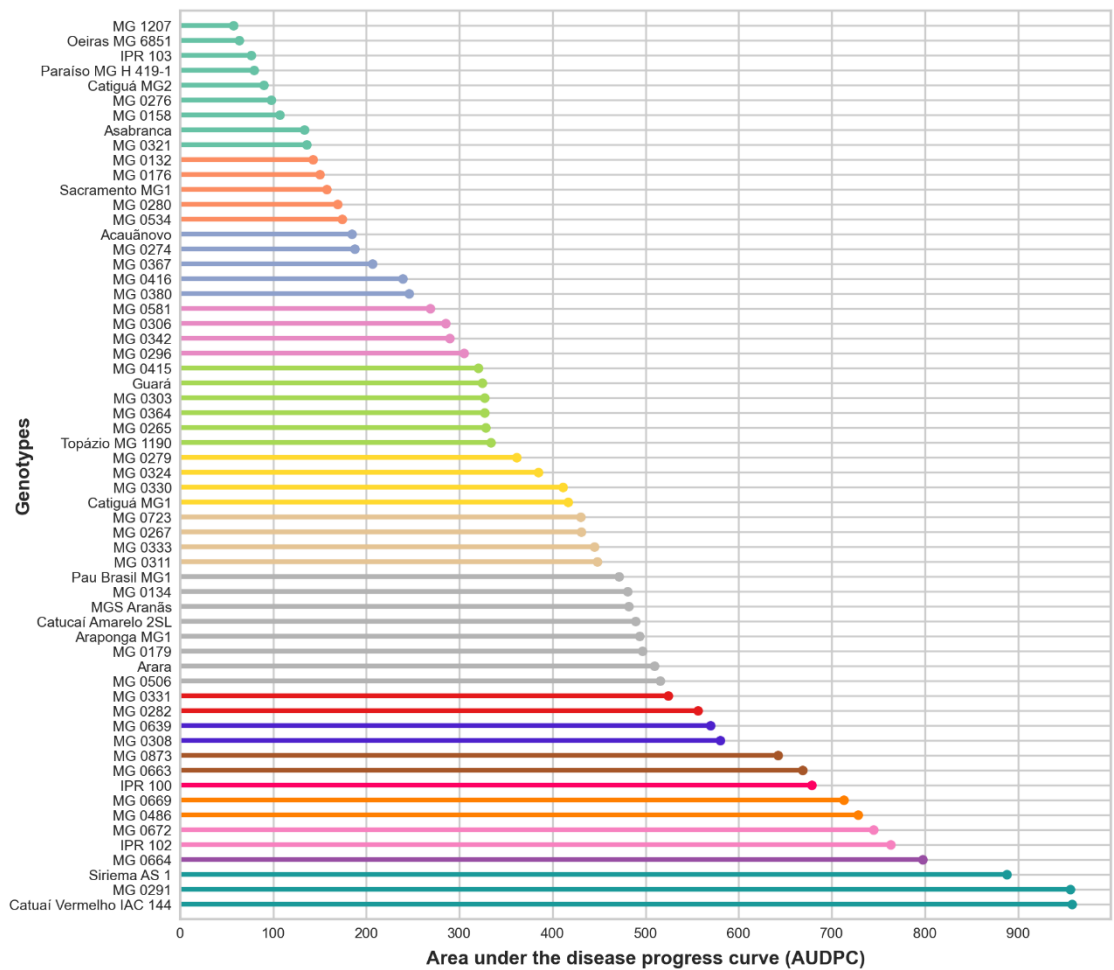
Among the studied population, approximately 38% presented heritability (H) of high magnitude (higher than 70%), namely MG 1207, MG 0321, MG 0132, MG 0176, MG 0280, MG 0380, MG 0265, MG 0279, MG 0324, MG 0267, MG 0723, MG 0333, MG 0134, MG 0179, MG 0282, MG 0663, and MG 0291 accessions, besides the cultivars Paraíso MG H 419-1, Asabranca, Araponga MG1, Sacramento MG1 and Catucaí Amarelo 2SL and Siriema AS 1. Within this population, there was a high variation in the severity of BES, with a phenotypic value ranging from 4.6 to 50.

We selected five genotypes with the highest predicted additive values (between 2.37 and 5.49) and the highest magnitudes of individual heritability (H) (between 77 and 88%), such as the MG 1207, MG 0321, and MG 0132 accessions, and the commercial cultivars, Paraíso MG H 419-1 and Asabranca, which presented low magnitude for their genotypic value to reduce severity, suggesting further probabilities of genetic progress through these evaluations. The selection of five such genotypes resulted in a remarkable selection gain of 70.16% to reduce the severity of BES.



**Fig 1. Genotypes classification based on phenotypic mean, genotypic values (u + g), genotype heritability, calculated on BES severity in coffee seedlings. The genotypes in red color represents heritability presented heritability (H) of high magnitude (higher than 70%).**

Based on the phenotypic data, the outputs from AUDPC severity for BES were significantly different among the genotypes. Otherwise, by considering the heritability parameter, the selection via AUDPC highlighted MG 1207, Oeiras MG 6851, IPR 103, Paraíso MG H 419-1, and Catiguá MG 2 genotypes, which showed the lowest disease progress during the evaluated timeframe (Fig 2). Genotype MG 0291, Catuaí Vermelho IAC 144, and Siriema AS 1 were the most susceptible among them, with AUDPC values of 958.04, 956.24, and 888.08, respectively. By comparing the genotypes that demonstrated the highest and lowest AUDPC, a difference in disease severity of 901.71% was obtained.



**Fig 2. Area under disease progress curve (AUDPC).** \*Bars with the same color do not differ from each other by the Scott-Knott test ( $p \leq 0.05$ ).

Accuracy can be improved using more rigorous analysis methods, such as by increasing the number of severity measurements per plant. In this context, Table 1 presents the accuracies that would be achieved using a higher number of measurements. Considering the estimated individual repeatability, the adoption of four measurements leads to accuracies around 99%.

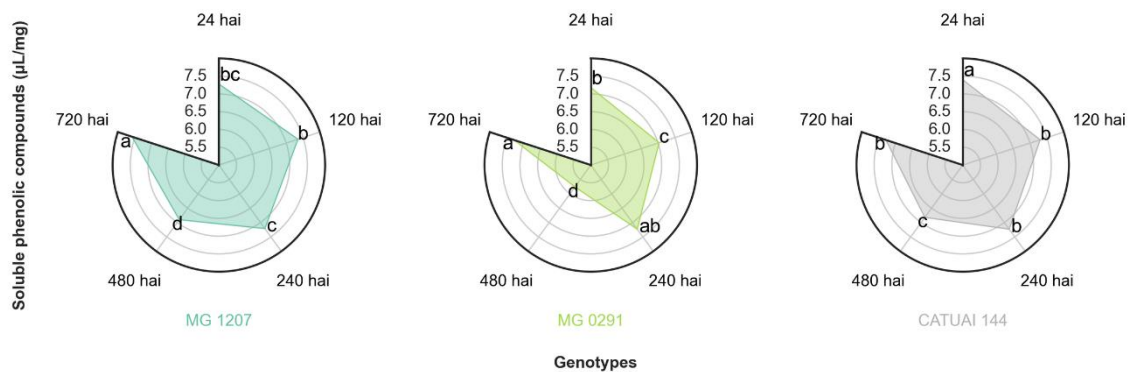
**Table 1. Average heritability, accuracy, and efficiency based on different number of measurements for the severity of brown eye spot.**

Measurements	1	2	3	4	5	6	7	8	9	10
Heritability	0.90	0.95	0.97	0.97	0.98	0.98	0.98	0.99	0.99	0.99
Accuracy	0.95	0.97	0.98	0.99	0.99	0.99	0.99	0.99	0.99	0.99
Efficiency	1.00	1.14	1.20	1.23	1.25	1.27	1.28	1.29	1.30	1.30

### 3.2 Total soluble phenolic compounds and soluble lignin content in coffee genotypes

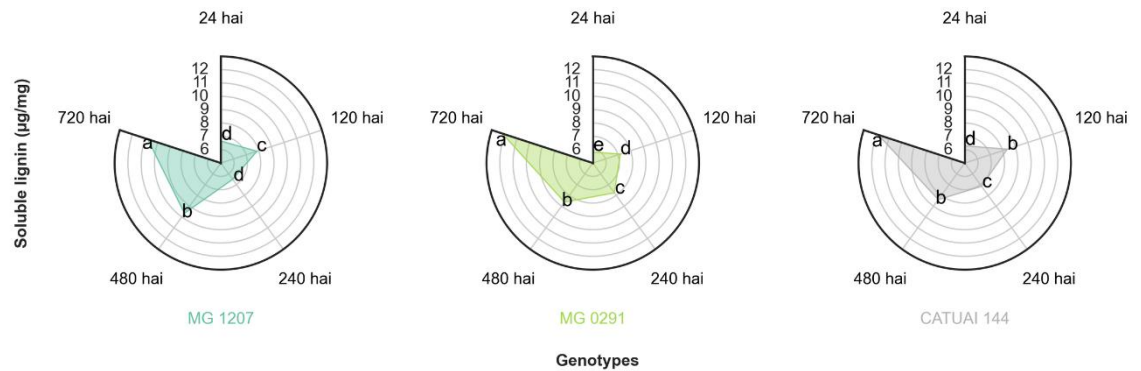
Based on the results of BES severity, the genotype from the Sumatra group (code MG 1207-low severity), the accession MG 0291 (high severity) as well as the susceptibility control (Catuaí Vermelho IAC 144 cultivar) were selected for quantification of total soluble phenolic compounds and soluble lignin. A significant interaction was found between the time of collection for both the contents of phenolic compounds (Fig 3) and soluble lignin (Fig 4).

Phenolic compounds content showed variation among the three genotypes over time (Fig 3), with the lowest levels at 480 hai, presenting mean values between 5.76 and 6.90  $\mu\text{L}/\text{mg}$ . The highest levels of phenolic compounds were observed at 24 hai in Catuaí Vermelho IAC 144 (7.40  $\mu\text{L}/\text{mg}$ ), and 720 hai in MG 1207 (7.58  $\mu\text{L}/\text{mg}$ ) and MG 0291 (7.28  $\mu\text{L}/\text{mg}$ ). The highest lignin contents were found in genotypes at 720 hai, which ranged from 10.79 to 12.17  $\mu\text{g}/\text{mg}$ . Otherwise, the lowest levels of this compound were observed in MG 1207 (6.51  $\mu\text{g}/\text{mg}$ ) at 240 hai, and MG 0291 (5.82  $\mu\text{g}/\text{mg}$ ) and Catuaí Vermelho IAC 144 (6.29  $\mu\text{g}/\text{mg}$ ) at 24 hai.



**Fig 3. Contents of total soluble phenolic compounds ( $\mu\text{L}/\text{mg}$ ) quantified in the leaves of the genotypes MG 1207, MG 0291, and Catuaí Vermelho IAC 144 collected at 24, 120, 240, 480, and 720 h after inoculation of *C. coffeicola*. \*Averages followed by the same lowercase letter in the same genotype do not differ by the Tukey's test ( $p \leq 0.05$ ).**

By analyzing the soluble lignin contents at different collection times, the MG 0291 accession was found to display an increased content of this compound over the timeframe (Fig 4), unlike the MG 1207 and Catuaí Vermelho IAC 144. The lignin content in such genotypes at 24 and 240 hai was lower than that observed at 120 and 480 hai, respectively. Between 480 and 720 hai, the same profile was verified in two genotypes: the last collection time was associated with a higher lignin content than previous collection times.



**Fig 4: Soluble lignin content ( $\mu\text{g}/\text{mg}$ ) in the leaves of MG 1207, MG 0291, and Catuai Vermelho IAC 144 collected at 24, 120, 240, 480, and 720 h after inoculation of *C. coffeicola*. \*Averages followed by the same lowercase letter in the same genotype do not differ by the Tukey's test ( $p \leq 0.05$ ).**

## 4 Discussion

Due to the environmental condition that benefits BES development in most coffee growing regions, besides the lack of resistant cultivars, this disease remains a remarkable challenge faced by Brazilian coffee growers, even after 100 years of introduction of the disease in the country [20], or 40 years after the first epidemic reports [21]. In this context, several efforts have been made by researchers worldwide to develop plants that are resistant to different *Cercospora* species; however, studies on *C. coffeicola* are still incipient. The increases in the productive potential in coffee areas can lead to nutritional imbalance, resulting in a higher susceptibility to disease, which makes it difficult for breeders to select such plants in the field [7]. Thus, this study aimed to highlight strategies for the early selection of genotypes that allow genetic gains in a short timeframe, to further provide materials for the genetic breeding program to achieve resistance under field conditions.

The genotype MG 1207 Sumatra, and the cultivar, Oeiras MG 6851, were considered partially resistant, while accessions MG 0276 and MG 0158 and cultivars Catiguá MG 2, Paraíso MG H 419-1, and IPR 103 were considered moderately susceptible. All other genotypes were susceptible because they presented an injured leaf area greater than 12.0%. In the present study, no genotype was classified as resistant. The existence of partially resistant phenotypes in our study indicated that severity to BES is a quantitatively inherited character and presents an action conditioned by many genes with small individual effects; consequently, it is markedly influenced by environmental factors. In such cases, it is essential to use statistical methods

based on estimates of genetic parameters, as heritability estimates enable both the development of more efficient selection strategies and prediction of the selection gain [22].

By using different approaches to analyze the outputs from severity data, only two genotypes (MG 1207 and Paraiso MG H 419-1) were found to match between both selection methods based on five genotypes selected by AUDPC (MG 1207, MG 0321, MG 0132, Paraiso MG H 419-1 and Asabranca) and five genotypes selected by the highest genotypic values and estimated heritability (MG 1207, Oeiras MG 6851, IPR 103, Paraiso MG H 419-1, and Catiguá MG 2). By estimating the heritability of each genotype, environmental effects are expected to be attenuated. Thus, the genotypes, MG 0321 and MG 0132, and Asabranca, selected by the AUDPC, presented a heritability lower than 70%, which indicates that the expression of the severity of BES in such materials has a low genetic influence, causing major responses to environmental conditions [23]. Such genotypes are not expected to maintain their phenotypic behavior of low severity to BES over time, which makes experimental replication difficult, and may be a false positive source of resistance. Only 20 of the 60 genotypes evaluated showed high magnitude heritability (Table S1). By considering the fixed model (Fig 2), only nine of these genotypes would have a satisfactory heritable fraction of genotypic variance - approximately 70% or more - (MG 1207, Paraiso MG H419-1, Asabranca, MG 0321, MG 0132, MG 0176, Sacramento MG1, MG 0280, MG 0380), if 20 genotypes of low severity to BES are selected. These results indicate that selection without observing the heritability of each genotype individually may not be ideal, and may lead to an inefficient method for breeding programs that seek to achieve resistance to BES.

Genetic gain is inversely proportional to the selection intensity, which was 8.3% (five genotypes) in the present study, thereby allowing higher efficiency in the subsequent steps of selection [24]. The accessions MG 1207, MG 0321, and MG 0132, and the commercial cultivars, Paraiso MG H 419-1 and Asabranca, had a lower magnitude of genotypic value to reduce the severity of BES, suggesting suitable possibilities of genetic progress in the sequence of evaluations (Table 1). Based on the severity of BES, the new predicted mean based on the selection of these five genotypes was approximately 4.33, with a remarkable selection gain of 70% to reduce the severity (Fig 1). The accessions MG 1207 and MG 0132 refer to the Sumatra group, and are parental lines of the cultivar, Mundo Novo; while accession MG 0321 is the Híbrido de Timor UFV 432-09. The cultivar, Paraiso MG H 419-1, represents the interbreeding between Catuaí Amarelo IAC 30 and the Híbrido de Timor UFV 445-46 [25]. Finally, the cultivar, Asa Branca, results from interbreeding between

Sarchimor 1668 and the World New 379-19 [26]. Therefore, these last three genotypes are resistant to the orange rust fungus of the coffee tree, *H. vastatrix*.

Accuracy is a measure closely related to the precision of selection and holds the property of establishing the evaluation reliability and predict genotype genetic value based on its magnitude [23]. In the present study, the assumption of four assessments resulted in values above or close to 99% (Table 1) by considering the estimated individual repeatability, which indicates a very high precision. Such finding suggests that there are low absolute deviations between the true genotypic values and those estimated or predicted. Such outputs make it easier to achieve expressive genetic gains. The efficiency of using four measurements compared to one measurement is approximately 1.23 or 23% for this character. These results align with the number of measurements used in Arabica coffee trees to accomplish agronomic data [27]. By doubling this number (i.e., eight measurements), efficiency only increases by 6%.

In this study, no significant differences were observed in total soluble phenolic compounds and soluble lignin levels among the inoculated and non-inoculated genotypes. Similar results are reported elsewhere on this pathosystem [28, 29]. As phenolic compounds and lignin are produced as a physical and chemical defense mechanism against pathogen attacks [30, 31, 32], the inoculated genotypes are expected to present higher levels of these metabolites than non-inoculated genotypes.

As the levels of metabolites were not found to be associated with coffee tree resistance to BES, this level may be associated with phenylpropanoid pathway behavior, from which phenolic compounds are synthesized via lignin monomers [33, 34]. By facing stress, the expression of genes involved in this pathway may be altered to produce soluble phenolic compounds, which also present functions of defense and antioxidant properties, and may not be directly involved in lignin biosynthesis [35].

The crosstalk among the biosynthesis of phenolic compounds/lignin, growth, reproduction, and plant defense are other factors that can justify the fact that the inoculated and non-inoculated genotypes of coffee plants did not differ in metabolite levels. Plant defense and growth are negatively correlated, such as the activation of defense processes that negatively affect plant growth and reproduction [32]. Thus, in this study, plants may have triggered other physiological processes rather than the accumulation of phenolic compounds and lignin (plant defense). As lignification is a process tightly controlled by various regulatory levels, it will only occur at the appropriate time and location of lignin deposition [34,35].

Leaf tissue necrosis is caused by the oxidation and polymerization of o-diphenols [36,

37]. In the present study, decreases in the content of phenolic compounds observed between 240 and 480 hai may be related to the use of such compounds for the synthesis of other products, such as tannins and lignans, as the time of 480 hai is close to the period of symptom manifestation of BES. However, increases in the content of phenolic compounds, verified between 480 and 720 hai, are characterized as a generic response to insects, fungi, or bacteria attacks [38]. Decreases in phenol content followed by successive increases were demonstrated elsewhere with the resistance of olive cultivars to *Verticillium dahliae* [39].

Lignin plays an important role in plant growth and development, as it promotes increased cell wall rigidity, and its metabolism is involved in the response to different biotic and abiotic stresses [40]. Accordingly, environmental stresses change the content and composition of this compound in plants, which explains the difference observed in lignin content at the different times of collection in this study.

Based on the outputs presented in this study, the genotype MG 1207 Sumatra, was classified as partially resistant, which is in close agreement with the results of Botelho et al [11]. This genotype can substantially contribute to the development of a new cultivar to reduce the use of pesticides. Herein, the assumption of four assessments for the severity of BES was found to be sufficient for the parameters of accuracy and efficiency, leading to expressive genetic gains. Finally, the levels of lignin and phenolic compounds were not found to be associated with the resistance of coffee genotypes to BES.

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authors read and approved the final manuscript.

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**S1 Table: Genotypes and their respective distribution of leaf lesions frequency into six classes, class 1: 0.1-3.0; class 2: 3.1-6.0; class 3: 6.1-12.0; class 4: 12.1-18.0; class 5: 18.1-30.0; class 6: 30.1-50% of the leaf surface affected by brown eye spot, phenotypic mean of the genotypes (M), classification (C) of the accessions based on the phenotypic mean, predicted additive breeding values (u + g), heritability (H) of genotypes, genetic parameters, general mean and estimated gain relative to the severity of brown eye spot in 5 selected genotypes. PR: partially resistant, MS: moderately susceptible, S: susceptible, AS: highly susceptible. \*Cultivar used as a control. CV – Catura Vermelho; HT – Híbrido de Timor  $h^2_m$ : heritability of genotype averages.  $h^2_i$ : broad-sense heritability individually for each plot, or the total genotypic effects.  $c^2_{perm}$ : coefficient of determination of the permanent environmental effects.  $M_0$ : Genotypic mean of 60 genotypes before selection.  $M_S$  Mean of the five selected genotypes. SG: gain selection.**

Access	Genotype	1	2	3	4	5	6	M	C	u + g	H
MG 1207	Sumatra	0	100	0	0	0	0	4.6	PR	2.37	0.88
-	Paraíso MG H 419-1	0	0	100	0	0	0	6.8	MS	3.21	0.82
-	Asabranca	0	0	0	100	0	0	12.1	S	5.23	0.81
MG 0321	HT UFV 432-09	0	0	0	100	0	0	12.8	S	5.37	0.78
MG 0132	Sumatra Amarelo Pl 01	0	0	0	100	0	0	12.1	S	5.49	0.77
MG 0176	Amphillo x H. Natural MR 36-349	0	0	0	100	0	0	13.6	S	5.85	0.78
-	Sacramento MG-1	0	0	0	100	0	0	13.6	S	6.04	0.78
MG 0280	Híbrido Timor UFV 376-14	0	0	0	100	0	0	15.1	S	6.52	0.79
MG 0380	HT UFV 445-70	0	0	0	0	75	25	31.5	AS	10.28	0.86
MG 0265	Durandé Arabica x Canephora	0	0	0	0	75	25	31.5	AS	12.89	0.72
MG 0279	HT UFV 376-31	0	0	0	0	75	25	31.5	AS	13.52	0.78

MG 0324	HT UFV 433-01	0	0	0	0	75	25	31.5	AS	14.46	0.72
MG 0267	HT UFV 377-34	0	0	0	0	75	25	31.5	AS	15.76	0.75
MG 0723	CV x CIFIC H79/1UFV 339-02	0	0	0	0	100	0	30	AS	15.9	0.79
MG 0333	HT UFV 437-10	0	0	0	0	25	75	34.5	AS	16.16	0.74
MG 0134	Sumatra Palma	0	0	0	0	75	25	31.5	AS	17.15	0.73
-	Araponga MG1	0	0	0	0	100	0	30	AS	17.68	0.8
MG 0179	Catuaí Vermelho x Amphillo MR 2161	0	0	0	0	0	100	36.1	AS	17.75	0.74
-	Catuaí Amarelo 2SL	0	0	0	0	0	100	36.1	AS	17.88	0.88
MG 0282	HT UFV 376-12	0	0	0	0	100	0	30	AS	19.42	0.88
MG 0663	CV x CIFIC H276/2UFV 310-08	0	0	0	0	100	0	30	AS	23.93	0.73
-	Siriema AS 1	0	0	0	0	0	100	50	AS	30.06	0.8
MG 0291	HT UFV 379-07	0	0	0	0	0	100	50	AS	31.97	0.81
MG 0364	HT UFV 442-42	0	0	0	0	100	0	30	AS	12.41	0.68
-	IPR 100	0	0	0	0	100	0	30	AS	24.2	0.67
MG 0274	HT UFV 377-05	0	0	0	100	0	0	12.1	S	6.75	0.66
-	Pau Brasil MG1	0	0	0	0	50	50	33	AS	16.75	0.63
-	Catuaí Vermelho IAC 144*	0	0	0	0	0	100	50	AS	32.02	0.62
MG 0158	Maragogipe	0	0	100	0	0	0	8.3	MS	4.12	0.61
MG 0672	CV x S12 Kaffa UFV 331-02	0	0	0	0	100	0	30	AS	26.05	0.61
-	IPR 103	0	0	100	0	0	0	7.6	MS	3.2	0.6

MG 0664	CV x CIFC H276/2UFV 310-14	0	0	0	0	0	100	50	AS	27.54	0.6
MG 0303	HT UFV 427-09	0	0	0	0	75	25	31.5	AS	12.56	0.59
-	Arara	0	0	0	0	100	0	30	AS	18.46	0.57
MG 0330	HT UFV 437-03	0	0	0	0	50	50	33	AS	15.05	0.55
-	Topázio MG 1190	0	0	0	0	100	0	30	AS	12.58	0.53
-	Catiguá MG1	0	0	0	0	0	100	36.1	AS	15.52	0.52
-	IPR 102	0	0	0	0	0	100	50	AS	26.58	0.52
MG 0331	HT UFV 437-06	0	0	0	0	100	0	30	AS	19.88	0.51
-	Acauãno	0	0	0	100	0	0	15.1	S	6.96	0.5
MG 0486	K 7 x HT UFV 358-05	0	0	0	0	100	0	30	AS	25.59	0.45
MG 0506	F 840 x HT UFV 457-34	0	0	0	0	100	0	30	AS	19.3	0.44
-	Catiguá MG 2	0	0	100	0	0	0	9.1	MS	3.72	0.43
-	MGS Aranãs	0	0	0	0	100	0	30	AS	17.34	0.43
MG 0639	CV x KP 423 UFV 308-11	0	0	0	0	100	0	30	AS	21.16	0.42
MG 0308	HT UFV 427-55	0	0	0	0	100	0	30	AS	21.44	0.42
MG 0534	BE 5 W W x UFV 366-08	0	0	0	100	0	0	14.3	S	6.59	0.41
MG 0416	HT UFV 451-42	0	0	0	0	75	25	31.5	AS	10.1	0.41
MG 0296	HT UFV 408-11	0	0	0	0	50	50	33	AS	12.09	0.41
-	Guará	0	0	0	0	50	50	33	AS	12.63	0.41
MG 0342	HT UFV 439-11	0	0	0	0	100	0	30	AS	11.35	0.4
MG 0581	Dilla Alghe x HT UFV 400-06	0	0	0	0	50	50	33	AS	11.06	0.39



## **ARTIGO 2 – Quantification of cercosporin from coffee leaves by *Cercospora coffeicola***

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### **Authors**

Juliana Barros Ramos<sup>1</sup>, Mário Lúcio Vilela de Resende<sup>1</sup>, Maria Eduarda Rodrigues Andrade<sup>1</sup>, Alexandre Rezende Teixeira<sup>1</sup>, Wilder Douglas Santiago<sup>1</sup>, Edson Ampélio Pozza<sup>1</sup>, Tharyn Reichel<sup>1</sup>, Deila Magna dos Santos Botelho<sup>1\*</sup>

### **Institutional Affiliation**

<sup>1</sup>Department of Plant Pathology, Federal University of Lavras (UFLA), Lavras 37200-900, Brazil

**\*Corresponding author:** E-mail: [deilamagna@hotmail.com](mailto:deilamagna@hotmail.com)

### **Abstract**

Cercosporin, a nonspecific toxin produced by *Cercospora* spp., *Pseudocercospora* spp. and *Colletotrichum* spp., is a photosensitizing compound that rapidly kills plant cells in the presence of light. Although this toxin is a virulence factor, its quantification in coffee leaves has not previously been reported. This study aimed to standardize and quantify cercosporin in the leaves of *Coffea arabica* cultivars (Mundo Novo 376/4, Catuaí Vermelho IAC 99, Topázio

MG 1190, and IPR 102) and *Coffea canephora* (Conilon- clone 213) with brown eye spot symptoms using high-performance liquid chromatography (HPLC). Cercosporin was detected in the lesions of all examined cultivars. Thus, HPLC is a valid method for quantifying cercosporin in coffee leaves.

**Keywords:** fitotoxin, high-performance liquid chromatography, brown eye spot, *Coffea arabica*, *Coffea canephora*.

## 1 Introduction

Cercosporin was first extracted from the dry mycelia of *Cercospora kikuchii* in 1957 and identified as perylenequinone (Kuyama and Tamura 1957). This toxin can absorb light and convert to an energetically activated triplet state, which can react with oxygen, resulting in the generation of toxic reactive oxygen species (Daub and Chung 2007). The photosensitized cercosporin can cause membrane lipid peroxidation, causing membrane damage and cell death (Daub 1982; Daub and Briggs 1983). This process contributes to nutrient leakage and promotes the growth and spread of fungal hyphae intracellularly (Daub 1982; Daub and Ehrenshaft 2000).

Cercosporin is produced by *Cercospora* spp., and some members of the genera *Pseudocercospora* and *Colletotrichum* (Jonge et al. 2018; Gunasinghe et al. 2016; Santos Rezende et al., 2020). The genus *Cercospora* includes numerous species that cause diseases in many crops. One important pathogen of the *Cercospora* genus is *Cercospora coffeicola*, the causal agent of brown eye spot (BES) in coffee plants, which can lead to yield losses of up to 30%. It can infect plants at different developmental stages, from seedlings to adults, as well as different plant parts, such as leaves and berries (Pozza et al. 2010; Souza et al. 2011, 2015; Paula et al. 2019).

In *Cercospora* spp., gene disruption mutants are unable to produce cercosporin and display reduced virulence, which underlines the importance of necrosis induction in the infection process (Souza et al. 2019). Although this toxin is considered a virulence factor and has been associated with lesion formation in coffee leaves (Souza et al. 2019), the quantification of cercosporin from *C. coffeicola* has only been evaluated *in vitro* (Silva et al. 2016; Souza et al. 2019; Vale et al., 2019). Therefore, this study aimed to standardize a procedure to extract cercosporin from coffee leaves infected by *C. coffeicola*.

## 2 Material and Methods

Extraction of cercosporin from coffee leaves with BES symptoms was performed according to the method reported by Gunasinghe et al. (2016) with some modifications. Forty leaves each *Coffea arabica* genotypes (Mundo Novo 376/4, Catuaí Vermelho IAC 99, IPR 102, Topázio MG 1190) and *C. canephora* (Clone 213) with BES symptoms were collected (Supplemental Fig. 1). The lesions were cut, separated from the rest of the leaf, and freeze-dried. Samples (2 g each) were taken from each genotype and ground separately in 5 ml chloroform, using a chilled mortar and pestle. The mixture was incubated overnight at 10 °C. Following centrifugation (2,800 rpm for 30 min) the supernatant was collected and cercosporin was detected and quantified by high-performance liquid chromatography (HPLC).

HPLC analysis was performed using a Shimadzu UFLC, equipped with the following modules: pump (LC-20AT), degasser (DGU-20A5), an automatic injector (SIL-20 A HT), column oven (CTO-20 A), an SPD-20 A UV-Vis detector, and CBM-20 A interface. A Shim-pack CLC-ODS (15 cm × 6.0 mm D.I., 5 µm) column connected to a pre-column (Eclipse XDB-C18; 4.6 × 12.5 mm, 5 µm) was used for the analysis. The analysis was performed in gradient mode, with the mobile phase composed of 5% (v/v) acetic acid in water (eluent A) and

acetonitrile (eluent B) run at a flow-rate of 1.5 mL min<sup>-1</sup>.

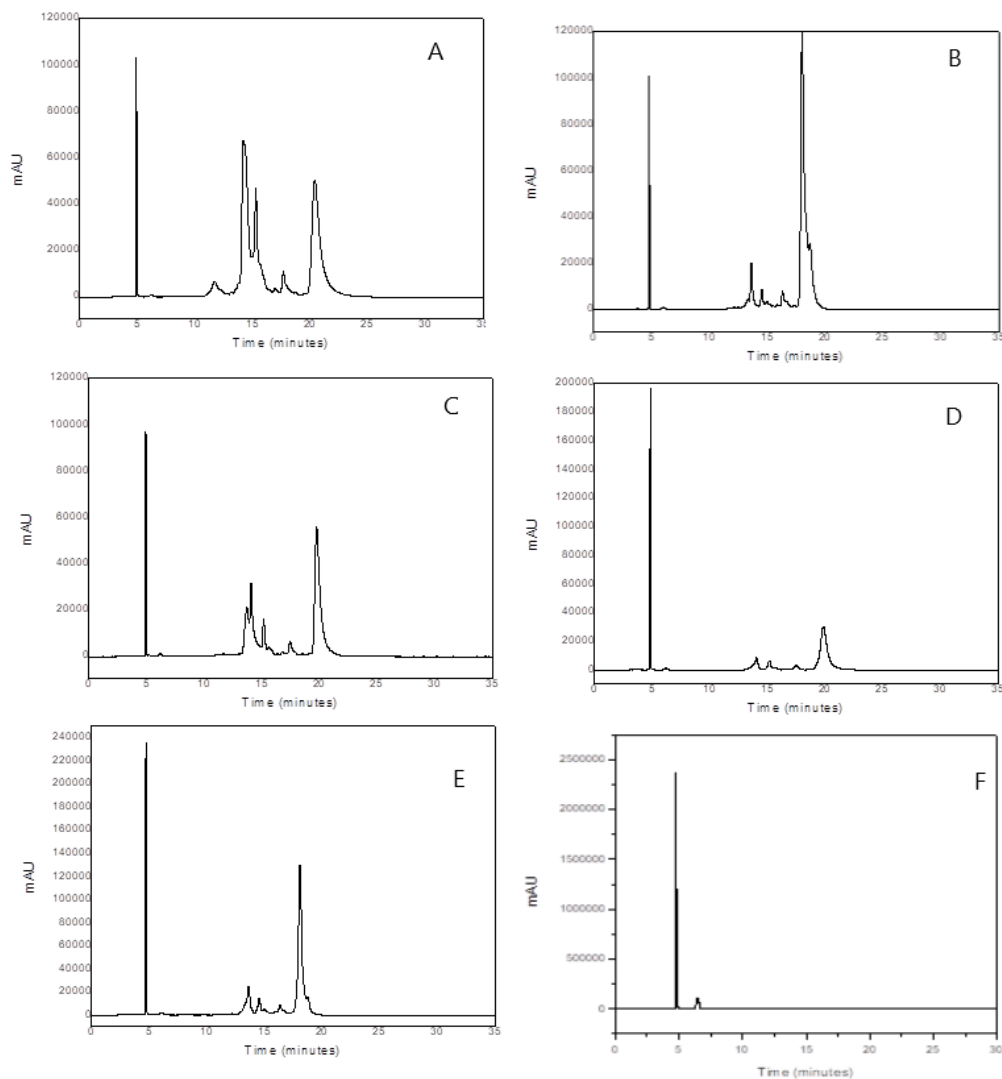
A linear gradient ranging from 50 to 70% of eluent B was used in the first 8 min, followed by a change to 100% of eluent B in 1 min (Gunasinghe et al. 2016); 100% acetonitrile (eluent B) was maintained for 6 min. The mobile phase was changed to 50% of eluent B (linear gradient in 10 min) for column equilibration.

The analytical curve for cercosporin quantification was constructed using a standard stock solution of this toxin from *Cercospora hayii* (C6696 Sigma-Aldrich) at a concentration of  $4 \times 10^{-4}$  mol. L<sup>-1</sup>. We injected 20  $\mu$ L of samples after filtering them through a 0.45- $\mu$ m syringe filter (Millipore®). Cercosporin concentration in the samples was calculated based on the peak retention time, which was to that of the standard curve of this toxin. Linearity was adjusted using the correlation coefficient from the analytical curve (straight line) fitted from the cercosporin analytical data in the pre-determined concentration range. The limit of detection (LOD) and limit of quantification (LOQ) were calculated according to the International Conference on Harmonization guidelines:  $LOD = 3.3 \cdot SD\ b/a$  and  $LOQ = 10.0\ SD\ b/a$ , where SD b represents the standard deviation of the y-intercept and a is the calibration curve slope. The experiment was performed completely randomized design with five treatments (genotypes) and four replicates (samples 2 g each). The means of the treatments were grouped by the Tukey test ( $p \leq 0.05$ ).

Cercosporin was detected in all extracts from different coffee genotypes with BES lesions (Fig. 1 A-E). The retention time resembled that of the standard (4.8 min) (Fig. 1 F). The HPLC method was validated since it presented selectivity, linearity ( $y = 4\ E10\ x + 65,345$ ), R<sup>2</sup> value of 0.99, LOD  $8.8\ E^{-7}$  mol/L, LOQ  $2.9\ E^{-6}$  mol/L, retention time 4.8 min, and robustness, which generated reproducible and reliable results for cercosporin quantification.

### 3 Results and Discussion

The detection of cercosporin in the BES lesions on coffee leaves confirms the findings by Daub et al. (2005) that cercosporin can be isolated from lesions on infected plants, verifying its production during disease development. The necrosis observed on coffee leaves with BES is characteristic of cercosporin activity (Supplemental Fig. 1). According to Daub and Ehrenshaft (2000), the production of the necrosis-inducing toxin cercosporin by *Cercospora* spp. allows the fungus to obtain nutrients required for growth and sporulation from the host.

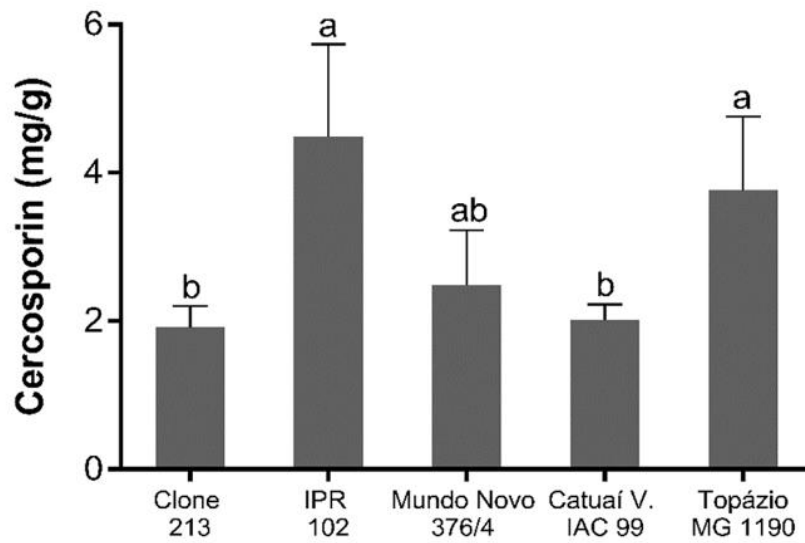


**Fig 1.** Chromatograms obtained from HPLC analysis from coffee leaves of genotypes Catuaí

Vermelho IAC 99 (A), Clone 213 (B), Mundo Novo 376/4 (C), IPR 102 (D) Topázio MG 1190 (E) infected with BES caused by *C. coffeicola* besides the standard cercosporin (F) extracted in chloroform.

Unlike the *in vitro* quantification of cercosporin (from cultures of *C. coffeicola* isolates), where only cercosporin is extracted, resulting in a single peak (Botelho et al., 2021), the *in vivo* analysis by HPLC showed other peaks at different retention times when compared to the retention time of the standard sample (Fig. 1 A-F). Phytopathogenic fungi can secrete a variety of cell-wall-degrading enzymes, which degrade plant cell walls (Moctezuma-Zárate et al. 2013). However, it is possible that infection of coffee by *C. coffeicola* produces other substances (besides cercosporin), which cause necrosis in coffee leaves.

Although all genotypes evaluated in this study were susceptible to BES, IPR 102 and Topázio MG 1190 showed higher cercosporin content than the Mundo Novo 376/4 and Catuaí Vermelho IAC 99 cultivars and Clone 213 (Fig. 2). The interaction of coffee plants with *C. coffeicola* can be specific to each coffee genotype, resulting in higher or smaller secretion of cercosporin during the colonization process in the plant (Batchvarova et al., 1992). According to Souza et al. (2019), mutant *CTB1* of *C. coffeicola*, characterized by the absence of cercosporin production, induced fewer lesions and took longer to manifest symptoms than the wild-type isolate.



**Fig 2.** Quantification of cercosporin (mg/g) from coffee leaves infected by *Cercospora coffeicola*. Bars followed by the same lowercase letter not differ by the Tukey's test ( $p \leq 0.05$ ).

In summary, this study provides information regarding the presence of cercosporin in BES lesions as well as the efficiency of HPLC to quantify it from coffee leaves. These results can help us understand the importance of cercosporin in pathogenesis and may assist in the development of resistance breeding programs.

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### Declarations

### Conflict of Interest

The authors declare that they have no conflict of interest.

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**Supplemental Figure 1.** Coffee leaves with brown eye spot symptoms from Catuaí Vermelho IAC 99 (A), Clone 213 (B), IPR 102 (C), Topázio MG 1190 (D) and Mundo Novo 376/4 (E) genotypes.