Chemical Papers
https://doi.org/10.1007/s11696-021-01965-5

SHORT COMMUNICATION

Cercosporin production by *Cercospora coffeicola* isolates: spectrophotometry and HPLC quantification and image analysis

Deila Magna dos Santos Botelho[1] · Mário Lúcio Vilela de Resende[1] · Alexandre Rezende Teixeira[1] · Wilder Douglas Santiago[2] · Edson Ampélio Pozza[1] · Silvino Intra Moreira[1] · Juliana Barros Ramos[1] · Sinara Oliveira de Aquino[2] · Antônio Gilberto Ferreira[3] · Tharyn Reichel[1] · Leonor Guerra-Guimarães[4,5]

Received: 27 June 2021 / Accepted: 2 November 2021

Abstract
Cercosporin has excellent properties of photosensitization that have been widely used in organophotocatalyst and photodynamic therapy as well as an antimicrobial agent. Therefore, there is a need to quantify it accurately with accessible methods. A comparative analysis of cercosporin quantification obtained by spectrophotometry (SPEC) and high-performance liquid chromatography (HPLC) was performed for nineteen *Cercospora coffeicola* isolates from different coffee-producing municipalities in Brazil. Image analysis of cercosporin crystals was performed in isolates with either high or low production of the toxin. Our results show that SPEC and HPLC are equally valid for the cercosporin evaluation of *C. coffeicola* cultures grown in vitro. The isolates with high cercosporin production had a higher crystal number and size when compared to the one with low cercosporin production.

Keywords Microscopy · Toxin · High-performance liquid chromatography · Methods

Introduction

Perylenequinones are a class of photoactivated polyketide mycotoxins produced by fungal plant pathogens that notably produce reactive oxygen species with visible light (Newman and Townsend 2016). The cercosporin produced by *Cercospora* and some *Pseudocercosporella* and *Colletotrichum* species is the best-studied perylenequinone (Jonge et al. 2018; Gunasinghe et al. 2016). Cercosporin is a highly coveted red–purple polyketide produced by fungal hyphae for its excellent properties of photosensitization, being, therefore, widely investigated for its photophysics, photochemistry and photobiology aspects (Guedes and Eriksson 2007; Daub and Chung 2009; Souza et al. 2019; Zhou et al. 2021). Furthermore, it has shown potential in medicine regarding the cytotoxic effect on human tumor cells (Mastrangelopoulou et al. 2019). *Cercospora coffeicola*, the causal agent of brown eye spot in coffee trees—a disease that can cause a reduction yield of up to 30%, is the fungus that produces cercosporin (Pozza et al. 2010; Vale et al. 2019).

A simple and accessible method for the extraction and quantification of cercosporin by spectrophotometry (SPEC) was proposed by Jenns et al. (1989) and is used for cercosporin quantification in several Cercospora species, such as *C. coffeicola* (Vale et al. 2019; Souza et al. 2019). Cercosporin quantification has also been performed by high-performance liquid chromatography (HPLC) for *Cercospora beticola*, *Cercospora* cf. *flagellaris*, *Colletotrichum fioriniae, Pseudocercosporella capsellae* (Gunasinghe et al. 2016; Jonge et al. 2018; Rezende et al. 2020). However, there are no reports of studies comparing the cercosporin concentration in SPEC x HPLC. Therefore, the goal of this study was to perform a comparative quantification of cercosporin

✉ Deila Magna dos Santos Botelho
deilamagna@hotmail.com

1 Departamento de Fitopatologia, Universidade Federal de Lavras, Caixa Postal 3037, Lavras, MG, Brazil

2 Departamento de Química, Universidade Federal de Lavras, Caixa Postal 3037, Lavras, MG, Brazil

3 Departamento de Química, Universidade Federal de São Carlos, São Carlos, SP, Brazil

4 Centro de Investigação das Ferrugens do Cafeeiro, Instituto Superior de Agronomia, Universidade de Lisboa, Lisboa, Portugal

5 Linking Landscape, Environment, Agriculture and Food, Instituto Superior de Agronomia, Universidade de Lisboa, Lisboa, Portugal

Published online: 30 December 2021

produced by different *C. coffeicola* isolates using two different methodologies, SPEC and HPLC. Additionally, cercosporin crystals obtained from representative isolates (with low and high cercosporin production) were displayed by laser confocal microscopy.

Material and methods

Sample preparation

Nineteen monoconidial isolates of *C. coffeicola* were obtained from the fungal specimen collection of the Laboratory of Parasitism Physiology, Federal University of Lavras, Minas Gerais State, Brazil. The isolates were cultured on 9-cm-diameter Petri dishes containing 9 mL of PDA (potato dextrose agar) culture media maintained in a BOD (Biochemical Oxygen Demand) incubator adjusted for a 12-h photoperiod at 25 °C for 12 days (Vale et al. 2019).

Spectrophotometric analysis

Four mycelial plugs (6 mm diameter) collected from each Petri dish colony were immersed in 8 mL of 5 N KOH and maintained in the dark for 4 h (Jenns et al. 1989). Afterward, the SPEC analysis was performed using a spectrophotometer reader PowerWave XS (Biotek®) at an absorbance of 480 ηm (Jenns et al. 1989). The cercosporin concentration was calculated using a molar absorption coefficient (ε) of 23,300 mM^{-1} cm^{-1}.

HPLC analysis

Concerning the HPLC analysis, the equipment used was an LC-20AT pump, DGU-20A5 degasser, SIL-20A HT automatic injector, CTO-20ª, detector UV–Vis SPD-20ª and CBM-20A interface model. The column was Shim-pack CLC-ODS (15 cm × 6.0 mm D.I., 5 µm) connected to precolumn Eclipse XDB-C18 (4.6 × 12.5 mm, 5 µm). The chromatographic method used was gradient mode analysis with a mobile phase composed of 5% (*v*/*v*) acetic acid in water (eluent A), acetonitrile (eluent B) at 1.5 mL min^{-1}. A linear gradient is adapted from Gunasinghe et al. (2016) ranging from 50 to 70% of eluent B for the first eight minutes followed by a change to 100% of eluent B within a period of one minute and maintained for six more minutes. Afterward, a new change to 50% of eluent B with the linear gradient within 10 min was performed for column rebalance. The analytical

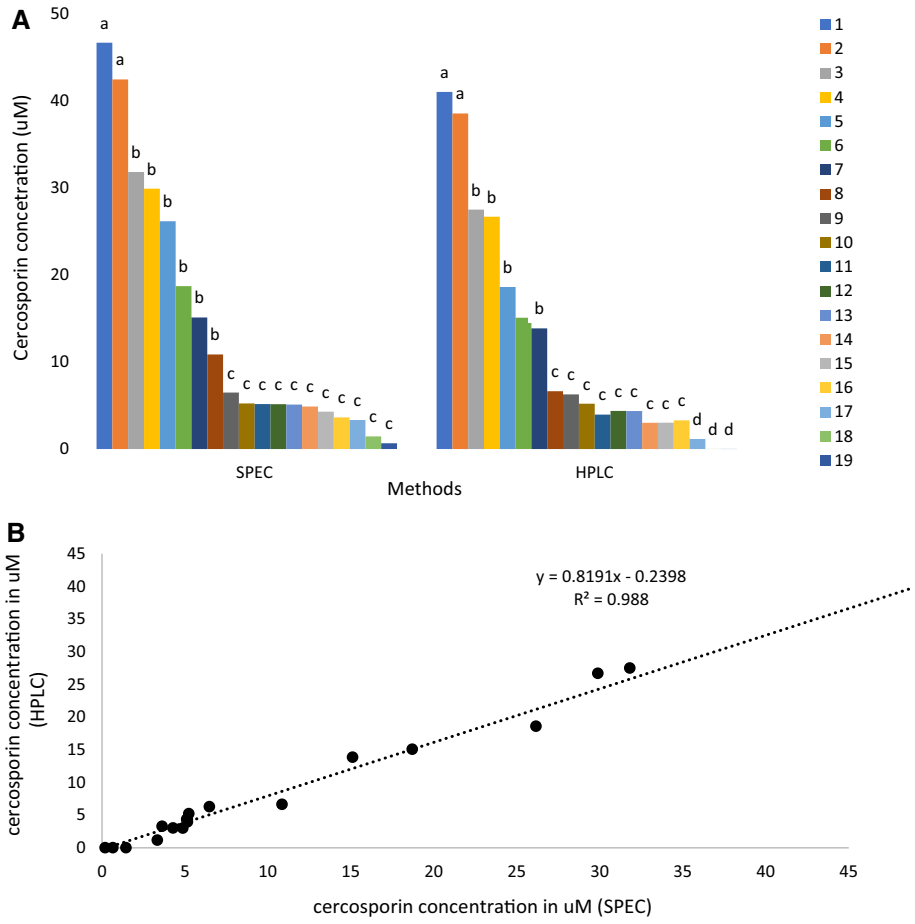

Fig. 1 Comparation cercosporin concentrations in 19 *Cercospora coffeicola* isolates quantified by SPEC and HPLC methods (**a**). Bars followed by the same letters do not differ significantly at Scott Knott's test ($p \leq 0.05$). Correlation cercosporin concentrations of 19 *C. coffeicola* isolates in SPEC method versus HPLC method (**b**)

curve for cercosporin quantification was built using a standard stock solution of this toxin from *Cercospora hayii* (C6696 Sigma-Aldrich) at concentration 4×10^{-4} mol.L^{-1}. Standards and samples were filtered into a 0.45-µm-pored filter syringe (Millipore®) before injection of 20 µL into the chromatograph (in triplicate). The cercosporin quantification in the samples was calculated based on the peak retention time and compared to the standard curve of this toxin.

The linearity was adjusted using the correlation coefficient from the analytical curve (straight line) fitted from cercosporin analytical data in the pre-chosen concentration range.

The limit of detection (LOD) and limit of quantitation (LOQ) were calculated according to the International Conference on Harmonization guidelines: LOD = 3.3 SDb/a and LOQ = 10.0 SDb/a, where SDb represents the standard deviation of the y-intercept and a is the calibration curve slope.

Spectroscopic analysis

The spectroscopic analysis was made with the isolate 19 (higher cercosporin producer). All NMR measurements were done in a Bruker 14.1 T (600 MHz for hydrogen frequency) AVANCE III equipment with 5 mm TCI cryoprobe head, using deuterated chloroform as a solvent and keeping the temperature at 25 °C during all the experiments.

Image analysis of cercosporin crystals

A laser confocal microscope was used for image analysis of cercosporin crystals. Agar plugs containing mycelium of two isolates with contrasting cercosporin production (low and high) were aseptically transferred to freshly prepared malt extract agar MEA (MEA: 20.0 g L^{-1} malt extract, 20.0 g L^{-1} glucose, agar 15.0 g L^{-1} and peptone 1.0 g L^{-1}) and incubated for three weeks in an incubator at 20 °C (Gunasinghe et al. 2016). Fragments with 0.5 cm diameter were positioned above coverslips for observation using an inverted scanning laser confocal Zeiss Observer Z1 LSM780 with the Zen 2012 software. The image acquisition was performed done using EC Plan-Neofluar 100×/1.3 oil M27 objective, 1024 × 1024 resolution, Ch2 detector, beam splitter MBS 543 ηm, pinhole 106 µm, 543 ηm excitation laser at 20%, master gain 600, digital gain 1, digital offset gain − 46 and emission filter range at 600–710 ηm. Bright-field images were acquired using the T-PMT detector, master gain 423, digital gain 1 and digital offset gain − 62. The images were edited using the Fiji-ImageJ and Corel Draw software.

Experimental design and statistical analysis

The experiment was conducted in a randomized complete block design with 19 treatments (isolates) evaluated in two methods (SPEC and HPLC) and three replicates. The experimental plot consists of three Petri dishes. The means of the treatments were grouped by the Scott–Knott test ($p \leq 0.05$).

Results and discussion

Our results showed that cercosporin produced by the 19 *C. coffeicola* isolates varied substantially among isolates, ranging from 0.2 to 48.65 µM to SPEC and 1.15 to 37.26 µM to HPLC (Fig. 1a). The amount variability of cercosporin produced by *C. coffeicola, C. piaropi, Cercospora cf. alchemillicola* and *Pseudocercosporella capsellae* isolates has already been reported by other authors using the SPEC method (Gunasinghe et al. 2016; Silva 2018; Vale et al. 2019). Cercosporin biosynthesis is affected by external factors such as light, temperature, pH, nutrient supply and incubation time (Jenns et al. 1989; Vale et al. 2019; Zhou et al. 2021). Since the factors mentioned above have been standardized in the present study, possibly the isolates have intrinsic factors that influence the production of the toxin in hyphae, resulting in different cercosporin concentrations. The selection of isolates with the ability to produce cercosporin associated with enhancers (e. *Bacillus velezensis*, *Lysinibacillus* sp.) will allow more potential industrial applications with cercosporin (Zhou et al. 2021).

The cercosporin quantification obtained by SPEC and HPLC showed a significant linear association between the methods, indicating a direct relationship between them (Fig. 1b). The HPLC method was validated since it presented selectivity, linearity, limits of detection (8.8 E-7 mol/L) and limits of quantification (2.9 E-6 mol/L), retention time mean of 4.8 min and robustness, which generated reproducible and reliable results in the quantification of cercosporin (Fig. 2). The spectrophotometry effectiveness for cercosporin quantification must be emphasized for its speed, easy execution and low cost of reagents and equipment compared to the HPLC method (Kumar et al. 2010).

Regarding the SPEC analysis, the most common problems found in applying this methodology are specificity and selectivity since other substances can absorb UV radiation in the same wavelength (Oliveira et al. 2010). Our results indicate that both methods can be used for cercosporin quantification and the chosen one depends on the study objective and available equipment in the laboratory.

The results of spectroscopic analysis of cercosporin from *C. coffeicola*: ^{1}H NMR spectrum (600 MHz, $CDCl_3$) δ 0.65 (d 6.11), 2.91 (dd 6.11,13.25), 3.39 (sext 6.37), 3.59 (dd 7.13, 13.25), 4.21 s, 5.75 s, 7.08 s and 14.83 s. ^{13}C {^{1}H}

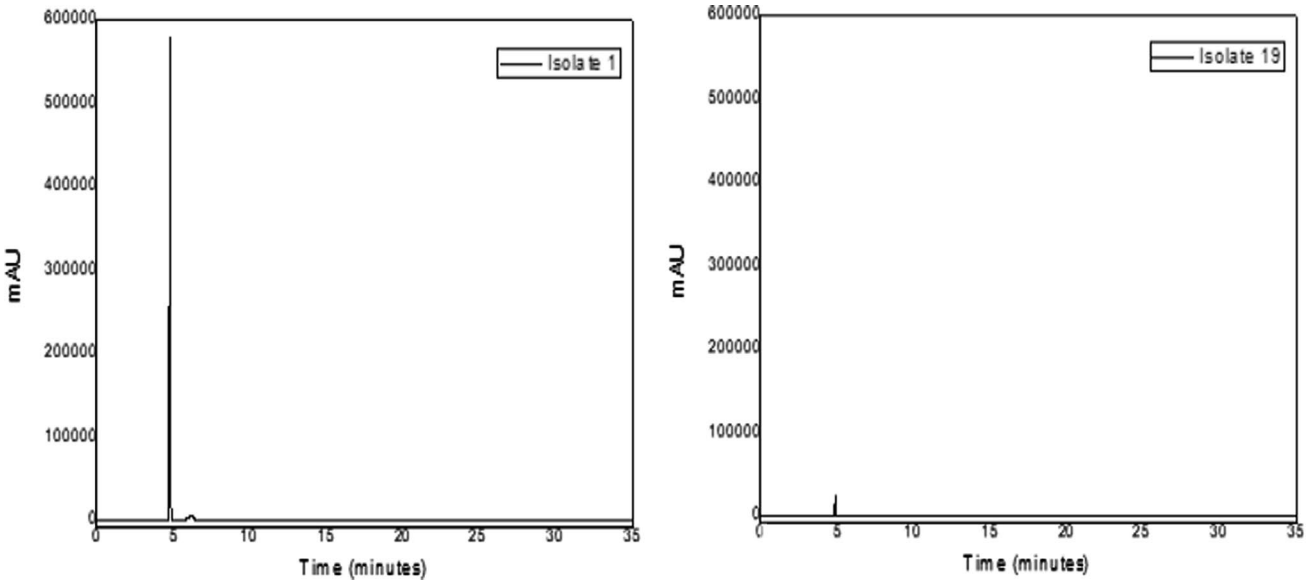

Fig. 2 Chromatograms obtained from HPLC analysis for isolate 1 (high producer cercosporin) and isolate 19 (low producer cercosporin)

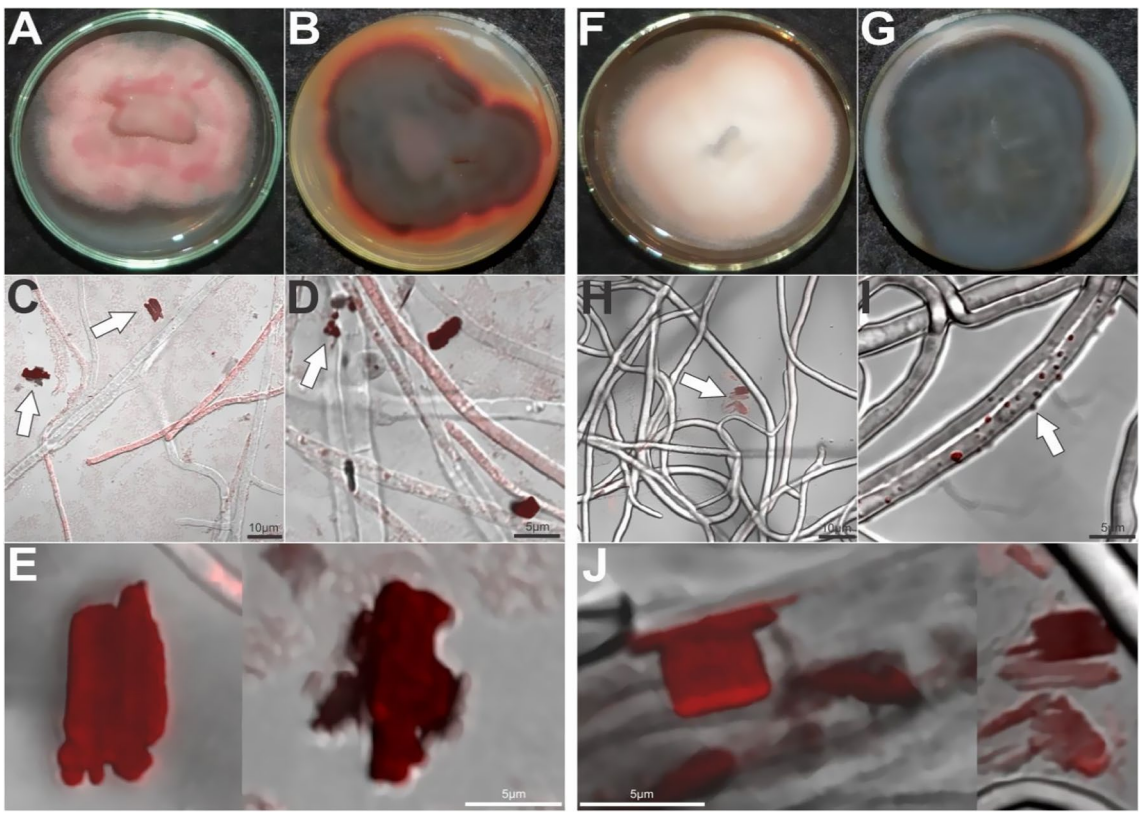

Fig. 3 *Cercospora coffeicola* isolate 1 with high-cercosporin production (**a**–**e**) and isolate 19 with a low-cercosporin production (**f**–**j**) after incubation at 20 °C under cool fluorescent white light for 3 weeks in MEA medium. **a**–**b** and **f**–**g**: Colonies morphology. Laser scanning confocal microscopy: **c**–**d** and **h**–**i**: aerial mycelia and cercosporin crystals (arrows). **e** and **j**: cercosporin crystals magnification

(125 MHz, $CDCl_3$) 23.4, 42.2, 61.2, 68.1, 92.6, 108.2, 109.3, 112.9, 127.9, 130.5, 135.2, 152.8, 163.4, 167.5 and 181.8 (Supplemental Figs. 1 and 2). The NMR data are in agreement with the values reported by Morgan et al. (2010).

Two representative *C. coffeicola* strains with high or low cercosporin production were selected for imaging characterization using laser confocal microscope. For the high producer isolate (1) crystals dispersed in media and adhered to the hyphae surface were observed, with 1.5–5 µm width and 3–10 µm length and with 0.5–1 µm diameter, respectively (Fig. 2a–e). Regarding isolate 19, cercosporin crystals with 1.5–3.5 µm width and 3–5 µm length were dispersed in the culture medium and lower crystals (0.5–1.5 µm diameter) were found adhered to the hyphae surface (Fig. 3d and i, arrow). After release, crystals are deposited in media, and subsequently can aggregate and give rise to larger forms (Fig. 3c and h, arrow). *C. coffeicola* showed similar cercosporin crystals to *Pseudocercosporella capsellae* (Gunasinghe et al. 2016) and the quantification of cercosporin showed relation with presence crystals.

Conclusions

HPLC and SPEC methodologies are equally valid for the cercosporin evaluation from cultures of *C. coffeicola* grown in vitro. The isolate with high cercosporin production had a higher crystal number when compared to the one with low cercosporin production.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11696-021-01965-5.

Acknowledgements Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Consórcio Brasileiro de Pesquisa e Desenvolvimento do Café, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG) and Instituto Nacional de Ciência de Tecnologia do Café (INCT-Café) for supporting the research.

Authors' contributions LGG and MLVR conceived and planned the experiments. WDS and ART carried out HPLC analysis. SIM carried out image analysis of cercosporin crystals. DMSB, JBR and SOA carried out *C. coffeicol*a isolates in vitro and spectrophotometric analysis. AGF was responsible for the NMR measurements and $^{1}H/^{13}C$ attributions. TR carried out samples to spectroscopic analysis. EAP provided material and contributed to interpretation of results. LGG, DMSB and MLVR analyzed the data, finalized the figures and drafted the manuscript. All authors read and approved the final manuscript.

Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

References

Daub ME, Chung KR (2009) Photoactivated perylenequinone toxins in plant pathogenesis. In: Deising HB (ed) The mycota, vol 5. Springer, Berlin, pp 201–219

Guedes RC, Eriksson LA (2007) Photophysics, photochemistry, and reactivity: molecular aspects of perylenequinone reactions. Photochem Photobiol Sci 6:1089–1096

Gunasinghe N, You MP, Cawthray GR, Barbetti MJ (2016) Cercosporin from *Pseudocercosporella capsellae* and its critical role in white leaf spot development. Plant Dis 100:1521–1531. https://doi.org/10.1094/PDIS-10-15-1192-RE

Jenns AE, Daub ME, Upchurch RG (1989) Regulation of cercosporin accumulation in culture by medium and temperature manipulation. Phytopathology 79:213–219

Jonge R, Ebert MK, Huitt-Roehl CR, Pal P, Suttle JC, Spanner RE, Neubauer JD, Jurick WM, Stott KA, Secor GA, Thomma BPHJ, Van de Peer Y, Townsend CA, Bolton MD (2018) Gene cluster conservation provides insight into cercosporin biosynthesis and extends production to the genus *Colletotrichum*. PNAS 115:459–466. https://doi.org/10.1073/pnas.1712798115

Kumar AKH, Sudha V, Swaminathan S, Ramachandran G (2010) Comparison of HPLC & spectrophotometric methods for estimation of antiretroviral drug content in pharmaceutical products. Indian J Med Res 132:390–394

Mastrangelopoulou M, Grigalavicius M, Berg K, Ménard M, Theodossiou TA (2019) Cytotoxic and photocytotoxic effects of cercosporin on human tumor cell lines. Photochem Photobiol 95:387–396. https://doi.org/10.1111/php.12997

Morgan BJ, Mulrooney CA, Kozlowski MC (2010) Perylenequinone natural products: evolution of the total synthesis of cercosporin. J Org Chem 75(1):44–56. https://doi.org/10.1021/jo9013854

Newman AG, Townsend CA (2016) Molecular characterization of the cercosporin biosynthetic pathway in the fungal plant pathogen *Cercospora nicotianae*. J Am Chem Soc 138(12):4219–4228. https://doi.org/10.1021/jacs.6b00633

Oliveira DM, Markman BEO, Uessugui O, Wu EM, Magnelli RF (2010) Ensaio de dissolução de cápsulas de fluconazol: problemas encontrados na determinação por espectrofotometria na região do UV. Rev Ciênc Farm Básica Apl 31(2):211–213

Pozza EA, Carvalho LC, Chalfoun SM (2010) Sintomas de injúrias causadas por doenças em cafeeiro. In: Guimarães RJ, Mendes ANG, Baliza DP (eds) Semiologia do cafeeiro: sintomas de desordens nutricionais, fitossanitárias e fisiológicas. Editora UFLA, Lavras, pp 68–106

Rezende JS, Zivanovic M, Novaes MIC, Chen ZY (2020) The AVR4 effector is involved in cercosporin biosynthesis and likely affects the virulence of *Cercospora* cf. *flagellaris* on soybean. Mol Plant Pathol 21:53–65. https://doi.org/10.1111/mpp.12879

Silva HR, Pozza EA, Souza PE, Ferreira MA, Freitas AS, Moreira SI (2018) Cercospora leaf spot in Toona ciliata: epidemiology and infection process of *Cercospora cf. alchemillicola*. For Pathol. https://doi.org/10.1111/efp.12451

Souza AGC, Herrero S, Daub ME (2019) The toxin cercosporin is a virulence factor for infection of coffee by *Cercospora coffeicola*. BioRxiv. https://doi.org/10.1101/818328

Vale PAS, Resende MLV, Botelho DMS, Pozza EA, Ogoshi C, Monteiro ACA, Costa BHG, Vasconcelos VAM (2019) Temperature, incubation time and virulence of *Cercospora coffeicola* in the production of cercosporin. J Phytopathol 167:371–379. https://doi.org/10.1111/jph.12802

Zhou T, Yu S, Hu Y, Zhang Y, Song Y, Chu J, Liu C, Rao Y (2021) Enhanced cercosporin production by co-culturing Cercospora sp. JNU001 with leaf-spot-disease-related endophytic bacteria. Microb Cell Fact 20(1):1–12. https://doi.org/10.1186/s12934-021-01587-2