

HENARA VALÉRIA MIRANDA CASTRO

**USING A COPPER-POLYPHENOLS COMPOUND AS AN ALTERNATIVE FOR
COFFEE LEAF RUST CONTROL**

Dissertation submitted to the Plant Pathology Graduate Program of the Universidade Federal de Viçosa, in a partial fulfillment of the requirements of the Graduate requirements for the degree of *Magister Scientiae*.

Adviser: Fabrício Ávila Rodrigues

VIÇOSA – MINAS GERAIS

2024


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ABSTRACT

CASTRO, Henara Valéria Miranda, M.Sc., Universidade Federal de Viçosa, August, 2024.
Using a copper-polyphenols compound as an alternative for coffee leaf rust control.
Adviser: Fabrício Ávila Rodrigues.

Epidemics of coffee leaf rust (CLR), caused by the biotrophic fungus *Hemileia vastatrix*, greatly contributes to lower the production of fruit berries. This study investigated the potential of using Mantus[®] [nitrogen (1%) and copper (20%) complexed with a plant-derived pool of polyphenols (10%)] to hamper the infection process of *H. vastatrix* in coffee leaves. This product will be referred to copper-polyphenols compound (CPC) hereafter. A 2 × 2 factorial experiment was arranged in a completely randomized design with four replications (one plant) per sampling time. The factors studied were plants sprayed with water (control), CPC, and fungicide (dicopper chloride trihydroxide) that were non-inoculated or inoculated with *H. vastatrix*. In comparison to the control treatment, urediniospores germination was significantly reduced by 59 and 99% for the CPC (rate of 1 mL/L) and fungicide treatments respectively. Urediniospores did not germinate after being exposed to the rates of 2.5, 5, 10, and 15 mL of CPC/L. The CLR severity (92-99% from 22 to 35 days after inoculation) and the area under coffee leaf rust progress curve (98 to 99%) were significantly lower for plants from CPC and fungicide treatments compared to plants from the control treatment. The incubation period (days) significantly increased by 46 and 53% for CPC and fungicide treatments, respectively, compared to the control treatment. The latent period (days) and the intensity of fungal sporulation were significantly higher by 40% and lower by 55%, respectively, for CPC treatment compared to the control treatment. For the CPC treatment, significant decreases of 45 and 28% were obtained for the area of uredosporic sorus and the number of uredosporic sori per cm² of leaf and the area (µm²) of uredosporic sorus, respectively, compared to the control treatment. The production of urediniospores per cm² of leaf was significantly lower by 96 and 99% for CPC and fungicide treatments, respectively, compared to the control treatment. At the physiological level, the CPC-sprayed and infected plants displayed a better photosynthetic performance (greater values for the maximum photochemical efficiency of photosystem II parameter) linked to the preservation of photosynthetic pigments (chlorophyll *a+b* and carotenoids) compared to water-sprayed and infected ones. In conclusion, the CPC showed great potential to be used for CLR control considering its effect to affect some components of coffee resistance and variables related to the fungal infection process. It is noteworthy in this context that besides the effect of Cu contained in the CPC against the germination of urediniospores from *H. vastatrix*, defense reactions could be boosted in coffee leaves to hamper the colonization of leaf tissues by fungal hyphae. Considering a field coffee-growing condition, the use of CPC could contribute to lowering the CLR epidemic rate and be considered in an integrated disease management program.

Keywords: *Coffea arabica*; Biotrophic pathogen; Histopathology; Cell defense reactions; Photosynthesis

RESUMO

CASTRO, Henara Valéria Miranda, M.Sc., Universidade Federal de Viçosa, agosto de 2024. **Uso de um composto de cobre-polifenóis como alternativa para o controle da ferrugem do café.** Orientador: Fabrício Ávila Rodrigues.

Epidemias da ferrugem do cafeeiro, causada pelo fungo biotrófico *Hemileia vastatrix*, tem reduzido a produção de café. Este estudo investigou o potencial de utilização do Mantus[®] [nitrogênio (1%) e cobre (20%) complexado com uma mistura de polifenóis de origem vegetal (10%)] em afetar o processo infeccioso de *H. vastatrix* nas folhas de cafeeiro. O produto Mantus[®] será denominado de composto contendo cobre e polifenóis (CCP) daqui em diante. O experimento foi instalado em delineamento inteiramente casualizado num esquema fatorial 2 × 2 com quatro repetições (uma planta) por época de avaliação. Os fatores estudados foram plantas pulverizadas com água (controle), CCP e fungicida (oxicloreto de cobre) e plantas não inoculadas ou inoculadas com *H. vastatrix*. Em comparação com o tratamento controle, a germinação dos urediniósporos foi significativamente reduzida em 59 e 99% para os tratamentos CCP (dose de 1 mL/L) e fungicida. Os urediniósporos não germinaram quando expostos às doses de 2,5, 5, 10 e 15 mL de CCP/L. A severidade da ferrugem (92-99% dos 22 aos 35 dias após inoculação) e a área abaixo da curva do progresso da ferrugem (98 a 99%) foram significativamente menores para as plantas dos tratamentos CCP e fungicida em comparação com as plantas do tratamento controle. O período de incubação (em dias) aumentou significativamente em 46 e 53% para os tratamentos CCP e fungicida, respectivamente, em comparação com o tratamento controle. O período latente (em dias) e a intensidade da esporulação do fungo foram significativamente maior em 40% e menor em 55%, respectivamente, para o tratamento CCP em comparação com o tratamento controle. Para o tratamento CCP, houve reduções significativas de 45 e 28% para o número de soros uredospóricos por cm² de folha e a área (μm²) de soros uredospóricos, respectivamente, em comparação com o tratamento controle. A produção de urediniósporos por cm² de folha foi significativamente menor em 96 e 99% para os tratamentos CCP e fungicida, respectivamente, em comparação com o tratamento controle. A nível fisiológico, as plantas pulverizadas com o CCP e infectadas por *H. vastatrix* apresentaram um melhor desempenho fotossintético (maiores valores para o parâmetro máxima eficiência fotoquímica do fotossistema II) e preservação dos pigmentos fotossintéticos (clorofila *a+b* e carotenoides) em comparação com as plantas pulverizadas com água e infectadas pelo fungo. Conclui-se que o CCP apresentou potencial para ser utilizado no controle da ferrugem considerando o seu efeito nos componentes de resistência do cafeeiro à ferrugem e as diferentes variáveis relacionadas com o processo infeccioso de *H. vastatrix*. Além do efeito do CCP na germinação dos urediniósporos de *H. vastatrix* devido à presença do Cu, é possível que respostas de defesa possam ter sido potencializadas nas folhas de café para afetar a colonização dos tecidos foliares pelas hifas do fungo. Em uma condição de cultivo do cafeeiro no campo, o uso do CCP poderia contribuir para reduzir a taxa de progresso da ferrugem e ser considerado em um programa de manejo integrado da doença.

Palavras-chave: *Coffea arabica*; Patógeno biotrófico; Histopatologia; Reações celulares de defesa da planta; Fotossíntese

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1. INTRODUCTION

The production of both Arabic (*Coffea arabica*) and Conilon (*Coffea canephora*) coffee is affected by the occurrence of severe epidemics of coffee leaf rust (CLR), caused by the biotrophic fungus *Hemileia vastatrix* Berkeley and Broome., worldwide (Zambolim et al. 2016; Silva et al. 2022). Reduction in the yield of fruit berries is the result of intense plant defoliation and profound changes in photosynthesis as a result of greater intensity of CLR on coffee trees (Zambolim et al. 2016; Silva et al. 2022). The infection process of *H. vastatrix* takes place with the deposition of urediniospores over the abaxial surface of coffee leaves (Silva et al. 2022). Over the stoma, an appressorium is formed in the germinated urediniospore from which a penetration hypha emerges to form a vesicle in the substomatal cavity from which occurs the differentiation of haustorial mother cell (Silva et al. 2022). The haustorial mother cell gives rise to haustorium in the cytosol of stomatal subsidiary cells with the concomitant intercellular growth of hyphae to form haustoria inside of cells in the spongy and palisade parenchyma as well as upper epidermis (Silva et al. 2022). Haustoria formed inside the cytosols results in visible chlorotic spots (flecks) on the abaxial surface of leaves, followed by the development of protosori in the substomatal cavities (Silva et al. 2022). Depending on the environmental conditions and the basal level of resistance of the coffee cultivar, urediniosporic sori protrude through the stomata in a bouquet shape from 22 to 35 days after urediniospore germination (Silva et al. 2022). Wind and rain splashes contribute to disperse the urediniospores formed in the sori to originate either auto- or allo-infections or being deposited over the abaxial leaf surface that will originate the pustules of orange color (Silva et al. 2022). For CLR control, it is recommended to reduce the density of coffee trees, provide plants with adequate levels of fertilizers, using coffee-resistant cultivars against the multiple races of *H. vastatrix*, and spray copper-based fungicides (contact) combined or not with systemic ones as well as nanoparticles of silver and copper (Zambolim et al. 2016; Leal et al. 2024).

It is well known that abiotic or biotic inducers of resistance are capable of providing plants with more rapid and strong defense strategies in response to the infection caused by pathogens of different lifestyles (Siah et al. 2018). This approach fits well with the actual need for finding more environmentally friendly strategies for CLR control, considering the constant changes in the population of *H. vastatrix* in terms of variability and adaptability that will force the supplantation of major S_H genes in the coffee genotypes (Zambolim et al. 2016; Silva et al. 2022). There is a large body of information in the literature showing the potential of different

products [*e.g.*, acibenzolar-S-methyl, plant extracts, yeast extract, exopolysaccharides from bacterial cells of *Xanthomonas campestris*, metabolites produced by some biological control agents (*Pseudomonas putida*, *Bacillus thuringiensis*, and *Bacillus subtilis*), phosphites, and products containing silicon and some nutrients for foliar spray] to stimulate the defense of coffee leaves to cope with the infection by *H. vastatrix* and, consequently, reduce the progress of CLR (Zambolim 2016; Possa et al. 2020; Silva et al. 2022). These scientific efforts are worthwhile to avoid the abusive use of fungicides that may favor the emergence of populations from *H. vastatrix* resistant to them and the negative impact imposed on the environment (Zambolim et al. 2016).

The current study aimed to investigate the potential of using a product containing copper mixed with polyphenols to reduce the CLR severity. This hypothesis was tested by quantifying some components of coffee resistance against infection by *H. vastatrix* (incubation period, latent period, intensity of fungal sporulation, area of uredosporic sori, number of uredosporic sori per cm² of leaf, and production of urediniospores per cm² of leaf) and determining the impairments in photosynthesis (quantification of the parameter maximum photochemical efficiency of photosystem II and the pool of photosynthetic pigments) in coffee plants non-challenged or challenged with *H. vastatrix* that were previously exposed to this product.

2. MATERIAL AND METHODS

2.1 *In vitro* assay

Different volumes of a stock solution (30 mL/L) of Mantus[®] [nitrogen (1%) and copper (20%) complexed with plant-derived pool of polyphenols (10%); FertiGlobal, Larderello, Italy], prepared using deionized water, were separately mixed with 1 mL of a suspension of urediniospores (2×10^5 urediniospores/mL) from *H. vastatrix* to obtain the final concentrations of 1, 2.5, 5, 10, and 15 mL/L. A total of 20 µL of urediniospores suspension containing the different concentrations of Mantus[®] was transferred to a glass slide and covered with a coverslip. A suspension of urediniospores without Mantus[®] or mixed with fungicide solution [1.7 mL/L; Difere[®], dicopper chloride trihydroxide (588 g/L); Oxiquímica Agrociência Ltda, Brazil] corresponded to the control treatments. The glass slides were kept inside of a growth chamber (temperature of 22°C and complete darkness) for 12 h. A total of six glass slides were used per each treatment. After this period, each glass slide received 40 µL of lacto-fuchsin

solution [1:1 mixture of acid fuchsin (1 mg/mL) with lactic acid (85%)] to both stain and stop urediniospores germination. One hundred urediniospores were randomly viewed in each glass slide under a light microscope (Carl Zeiss AxioImager A1) using the bright field at the magnification of 100×. The images for the details of urediniospores germination were acquired digitally (model AxioCam HR and Axion Vision software v. 4.8.1.) at the magnification of 400×. Urediniospore with a germ tube larger than its diameter was considered germinated. The percentage of urediniospores germination was calculated using the six replications (glass slides) per each treatment.

2.2 Plant growth

Coffee seeds from cultivar “Catuaí Vermelho - IAC 44” (the most common coffee cultivar grown in Brazil and highly susceptible to CLR) (Setotaw et al. 2013) were sown in plastic trays (15 × 40 × 10 cm; Ecovaso, Jaguariúna São Paulo, Brazil) containing sterilized fine sand (10 mm). The 55-day-old seedlings (cotyledonary growth stage) were individually transplanted to a plastic pot containing 2 kg of substrate (3:1:1 mixture of soil, manure cow, and sand) mixed with simple superphosphate and potassium chloride (5 and 1 kg/m³). The plant in each pot received (weekly) 25 mL of a nutrient solution composed, in mg/L, of 192 KCl, 104.52 K₂SO₄, 150.37 MgSO₄, 61 CH₄N₂O, 100 NH₄NO₃, 0.27 (NH₄)₆MO₇O₂₄, 1.61 H₃BO₃, 6.67 ZnSO₄, 1.74 CuSO₄, 4.10 MnCl₂, 4.08 FeSO₄, and 5.58 ethylenediaminetetraacetic acid. Plants were kept inside of a greenhouse [temperature of 22 ± 3°C, relative humidity of 80 ± 5%, and natural photosynthetically active radiation (PAR) of 965 ± 150 μmol photons m⁻² s⁻¹ measured at midday)], under shadow, until the formation of four pairs of expanded leaves.

2.3 Spray of coffee plants with Mantus[®] and fungicide and inoculation with *H. vastatrix*

Plants were sprayed with a solution of Mantus[®] (5 mL/L; 10 mL per plant) at 72 h before inoculation with *H. vastatrix*. This product will be referred to as copper-polyphenols compound (CPC). The solution of the CPC was prepared using deionized water. Plants sprayed with deionized water and fungicide [1.7 mL/L; Difer[®], dicopper chloride trihydroxide (588 g/L); Oxiquímica Agrociência Ltda, Brazil] corresponded to the control treatments. The urediniospores of *H. vastatrix* were obtained from the leaves of coffee plants (cultivar “Caturra”) that were previously inoculated with the monopustular isolate UFV-DFP Hv22 (race

II) of *H. vastatrix*. The urediniospores collected were transferred to gelatin capsules and kept inside a desiccator [with three glass vials containing 100 mL of sulfuric acid solution (32% v/v) each] for at least 30 days. The desiccator was kept inside of a refrigerator at 4°C. The abaxial surface of all leaves per each plant was carefully sprayed with a suspension of urediniospores (1×10^5 urediniospores/mL) from *H. vastatrix* using a VL Airbrush atomizer (Paasche Airbrush Co., IL, USA). Non-inoculated and inoculated plants were kept in a moist growth chamber (temperature of $22 \pm 2^\circ\text{C}$, relative humidity of $90 \pm 5\%$, and complete darkness) for 72 h. Thereafter, plants were transferred to a growth chamber (temperature of 22°C , relative humidity of $80 \pm 5\%$, and 12 h photoperiod with fluorescent light yielding a photon flux density of approximately $900 \mu\text{mol m}^{-2} \text{s}^{-1}$ at plant canopy). Non-inoculated plants were kept in a separate growth chamber with these same environmental conditions.

2.4 Processing the infected leaf fragments for light and fluorescence microscopies

2.4.1 Measurement of leaf anatomical traits and colonization of leaf tissues by *H. vastatrix*

A total of six fragments ($\approx 0.5 \text{ cm}^2$) were collected in the central region (without the main vein) from one leaf of the first pair of expanded leaves, from apex to base, of one plant per replication of each treatment at 35 dai. The leaf fragments (*e.g.*, fixation as well as infiltration and embedding in methacrylate resin) were prepared for light microscopy observations following the procedures described by Araujo et al. (2016). Six blocks of resin (two leaf fragments per block) were obtained for each treatment. A total of 40 transversal series sections (2-4 μm thick) were obtained from each block using a Leica RM 2245 rotary microtome (Leica Microsystems®).

For anatomical leaf measurements, a total of 20 transversal series sections were collected randomly. One section was placed in each glass slide and stained with toluidine blue (0.5%) solution for 5 min (Marques and Soares 2022). Each transversal section was photographed in a Carl Zeiss Axio Imager A1 microscope (Carl Zeiss, Germany) in the bright-field mode at the magnification of 200 \times . The vertical measurements (μm) of adaxial epidermis (AdE), abaxial epidermis (AbE), palisade mesophyll (PM), spongy mesophyll (SM), and leaf thickness (from the adaxial to the abaxial surface) in the image of each transversal section were carried out using the AXION VISION v.4.8.1 software. Three different positions (left, middle, and right regions) were selected in each image of the transversal section during the measurements.

A total of 20 transversal series sections were collected randomly and one section was placed in each glass slide. The methodology of chitin-specific dye wheat germ agglutinin-alexa fluor conjugate (WGA-AF) 488 (Molecular Probes, Karlsruhe, Germany) and calcofluor white stain (catalog number 4193-55-9; Sigma-Aldrich, São Paulo, Brazil) (Marques and Soares 2022) was used to observe the colonization of leaf tissues by hyphae of *H. vastatrix*. Fluorescence images were recorded on a Carl Zeiss Axio Imager A1 microscope using the Zeiss filter sets 1 (ultraviolet; excitation, beam splitter, and emission of 365, 395, and 397 nm, respectively) and 9 (Alexa; 450 to 490 nm excitation, 510 nm beam splitter, and 515 nm emission) separately. The digital images were acquired with an AxioCam HRc Rev. 2 in the Carl Zeiss Axio Imager A1 microscope and combined using the function Add Channels in the AXION VISION v.4.8.1 software.

2.4.2 Quantification of uredosporic sori and determination of their area

A total of five fragments (1 cm²) were collected in the central region (without the main vein) from one leaf of the first pair of expanded leaves, from apex to base, of one plant per replication of each treatment at 35 dai. The leaf fragments were placed in microcentrifuge tubes containing 15 mL of trypan blue (0.4 g/mL; catalog number 72-57-1, Sigma-Aldrich, São Paulo, Brazil) for 1 h at 60°C. The leaf fragments were cleared for 24 h in saturated choral hydrate solution (300 g/mL) (Sigma-Aldrich, São Paulo, Brazil). Cleared leaf fragments were mounted abaxial side up on glass slides containing three drops of modified Hoyer's mounting medium (Rodrigues et al. 2005). Images of the leaf fragments containing the uredosporic sori were acquired digitally (Axio Cam HR; Carl Zeiss) using a Carl Zeiss Axio Imager A1 microscope equipped with differential interference contrast. A total of six images were randomly obtained for each leaf fragment at the magnification of 100×. The sori present in each image were counted and their size (μm²) were determined using the function Measure (area) of the AXION VISION v. 4.8.1 software.

2.5 Evaluation of CLR severity, incubation period, latent period, and intensity of fungal sporulation as well as the quantification of urediniospores

The CLR severity was evaluated at 15, 22, 29, and 35 dai on the first and second pairs of expanded leaves, from apex to base, of one plant per replication of each treatment using the standard area diagram proposed by Belan et al. (2020). Values for CLR severity were used to

calculate the area under coffee leaf rust progress curve (AUCLRPC) according to the formula proposed by Shaner and Finney (1977). At 35 dai, the intensity of fungal sporulation (IFS) in the abaxial surface of the four leaves per each plant was evaluated according to a scale of notes as follows: 1 - pustules with light fungal sporulation (light yellow-colored uredinia), 2 - pustules with weak fungal sporulation (intense yellow-colored uredinia), and 3 - pustules with exuberant fungal sporulation (intense orange-colored uredinia) (Carré-Missio et al. 2012). The appearance of whitish areas (flecks) in the abaxial surface of the first and second pairs of expanded leaves per each plant was monitored every 24 h and started at 7 dai using a hand-held microscope (30×) to determine the incubation period (IP). For the determination of the latent period (LP), a total of ten flecks were randomly selected and marked on each leaf and examined every 24 h with a hand-held microscope (30×) to determine the beginning of fungal formation. In order to quantify the number of urediniospores formed on the abaxial leaf surface, a total of five fragments (1 cm²) were collected in the central region (without the main vein) of the leaf in the first pair of expanded leaves, from apex to base, of one plant per repetition of each treatment at 35 dai. The leaf fragments were placed in microcentrifuge tubes containing 15 mL of fungicide solution [1.7 mL/L; Difere[®], dicopper chloride trihydroxide (588 g/L); Oxiquímica Agrociência Ltda, Brazil] prepared with phosphate buffer (0.2 M, pH 7). The microcentrifuge tubes were vortexed (250 rpm for 30 sec) and a total of 1 mL of the suspension of urediniospores were placed in the hemacytometer. The number of urediniospores were counted and mathematically related to the number of urediniospores per milliliter as well to the area (1 cm²) of each leaf fragment.

2.6 Determination of Cu foliar concentration

A total of three pairs of expanded leaves, from apex to base, of one plant of each treatment (four replications, 4 plants, and 24 leaves) were collected at 35 dai, washed sequentially in deionized water for 1 min, in a solution of neutral detergent (0.1%) for 1 min, and in a solution of HCl (0.3%) for 30 sec. The washed leaves were dried in an oven with forced ventilation for 48 h. The foliar concentration of Cu was determined by the method of nitric-perchloric digestion and inductively coupled plasma-optical emission spectrometry (ICP-OES).

2.7 Imaging of the maximum photochemical efficiency of the photosystem II (F_v/F_m) parameter, quantification of F_v/F_m , and determination of the concentration of photosynthetic pigments

The Imaging-PAM fluorometer and the Imaging Win software MAXI version (Heinz Walz GmbH, Effeltrich, Germany) were used to obtain the images of the parameter F_v/F_m . The first and second pairs of expanded leaves, from apex to base, of each plant per replication of each treatment were collected at 15, 22, 29, and 35 dai. The chlorophyll *a* fluorescence emission transients were captured by a CCD (charge-coupled device) camera with a resolution of 640×480 pixels in a visible sample area of 24×32 mm on each leaf. The images were obtained with a resolution of 640×480 pixels. The leaves were then exposed to a weak and modulated measuring beam ($0.5 \mu\text{mol m}^{-2} \text{s}^{-1}$, $100 \mu\text{s}$, 1 Hz) to determine the initial fluorescence (F_0) when all the PSII reaction centers were open. Next, a saturating white light pulse of $2.400 \mu\text{mol m}^{-2} \text{s}^{-1}$ (10 Hz) was applied for 0.8 s to ensure maximum fluorescence emission (F_m) when all the PSII reaction centers are expected to be closed. The leaves were initially adapted to darkness for 30 min , after which they were carefully and individually fixed in support at a distance of 18.5 cm from the CCD camera. From these initial measurements, the maximum PSII photochemical efficiency of the dark-adapted leaves was estimated through the variable-to-maximum Chl *a* fluorescence ratio as follows: $F_v/F_m = [(F_m - F_0)/F_m]$. The parameter was calculated using the Imaging Win software (Kramer et al. 2004). After obtaining the images of F_v/F_m , five leaf discs (8 mm in diameter) were removed from the first and second pairs of expanded leaves of each plant per replication of each treatment. The leaf discs were placed in glass vials containing 5 ml of dimethyl sulfoxide for 24 h at 25°C and the absorbances of the extracts were read at 480 , 649 , and 665 nm (Santos et al. 2008).

2.8 Experimental design and statistical analysis of data

For the *in vitro* assay, the experiment was arranged in a completely randomized design with three treatments (control and five concentrations of Mantus[®]) and ten replications. Each replication corresponded to one glass slide. The experiment was repeated. A 3×2 factorial experiment was arranged in a completely randomized design with four replications per each evaluation time. The factors studied were plants sprayed with water (control), CPC, and fungicide [named products (P)] and plants that were non-inoculated or inoculated with *H. vastatrix* [named plant inoculation (PI)]. Plants in this experiment were used to assess CLR severity and the F_v/F_m parameter as well as to obtain leaf samples for microscopical analysis and to quantify the concentration of pigments. The experiment was repeated once. Data from variables and parameters obtained were subjected to analysis of variance (ANOVA). Data were checked for normality and homogeneity of variance before ANOVA. The ANOVA was shown per each evaluation time. Comparisons among control, CPC, and

fungicide treatments were made using the Tukey test and between non-inoculated and inoculated plants using the F test ($P \leq 0.05$). The procedures described by Moore and Dixon (2015) were followed to combine the data from the variables and parameters evaluated from the repeated experiments. The Minitab Statistical software was used for the statistical analysis mentioned above (Minitab Inc. 2024).

3. RESULTS

3.1 Analysis of variance

The factor P was significant for UG ($P < 0.032$), CLR severity at 15, 22, 29, and 35 dai ($P < 0.001$) as well as for AUCLRPC, IP, LP, IFS, AUS, NUS, NU, AdE, AbE, PM, SM, and LT ($P < 0.001$). The factors P, PI, and the $P \times PI$ interaction were not significant for F_v/F_m , Chl $a+b$, and Car at 15 dai. For F_v/F_m and Chl $a+b$, the factors P and PI and their interaction were significant ($P < 0.001$) at 22, 29, and 35 dai. For Car, the factor PI, and the $P \times PI$ interaction were significant at 22 dai ($P < 0.001$) while the factors P and PI were significant at 29 and 35 dai ($P < 0.001$).

3.2 *In vitro* assays

The germination of urediniospores was significantly reduced by 59 and 99% for the CPC (rate of 1 mL/L) and fungicide treatments, respectively, compared to the control treatment. The urediniospores exposed to the rates of 2.5, 5, 10, and 15 mL of CPC/L did not germinate.

3.3 Fungal sporulation on coffee leaves, CLR severity, AUCLRPC, IP, LP, IFS, area of US, number of US, and production of urediniospores

At 35 dai, the sporulation of *H. vastatrix* was intense and the presence of suprastomatal, bouquet-shaped, orange-colored uredinia was abundant on the abaxial surface of leaves from water-sprayed plants (Fig. 1A and D) compared to the leaves from CPC-sprayed plants (Fig. 1B and E). Fungal sporulation was not noticeable on the abaxial surface of fungicide-sprayed leaves (Fig. 1C and F). The severity of CLR was significantly lower by 93% for fungicide-sprayed plants compared to water-sprayed plants at 15 dai (Fig. 2A and C). Significant

decreases of 92 and 95% at 22 dai, of 99 and 99.7% at 29 dai as well as 99 and 99.7% at 35 dai on CLR severity were obtained for CPC and fungicide treatments, respectively, compared to the control treatment (Fig. 2A-C). The AUCLRPC was significantly lower by 99 and 99.9% for CPC and fungicide treatments, respectively, compared to the control treatment (Fig. 2A-C). The IP significantly increased by 46 and 53% for CPC and fungicide treatments, respectively, compared to the control treatment (Fig. 2D). For fungicide treatment, the IP significantly increased by 14% compared to the CPC treatment (Fig. 2D). The LP and IFS were significantly higher by 40% and lower by 55%, respectively, for CPC treatment compared to the control treatment (Fig. 2E and F). For the CPC treatment, significant decreases of 28 and 45% were obtained for the area of US and the number of US per cm² of leaf, respectively, compared to the control treatment (Fig. 2G and H). The production of urediniospores per cm² of leaf was significantly lower by 96 and 99% for CPC and fungicide treatments, respectively, compared to the control treatment (Fig. 2I). For fungicide treatment, the production of urediniospores per cm² of leaf was significantly lower by 80% compared to the CPC treatment (Fig. 2I).

3.4 Microscopical evaluations

At 35 dai, the abaxial epidermis and the spongy mesophyll of leaf fragments obtained from water-sprayed plants were massively colonized by hyphae of *H. vastatrix* and the suprastomatal uredosporic sorus was very well developed compared to the CPC-sprayed leaf fragments (Fig. 3A-B). Hyphae of *H. vastatrix* were not noticed on the leaf fragments obtained from fungicide-sprayed plants (Fig. 3C).

The cells in the abaxial epidermis as well as in both spongy and palisade mesophylls in the leaf fragments obtained from water-sprayed plants were smaller and the leaf tissue seemed to be more compacted compared to the leaf fragments from CPC- and fungicide-sprayed plants (Fig. 4A-C). The vertical thickness of adaxial epidermal cells was significantly lower by 12% for water-sprayed plants compared to the fungicide-sprayed ones (Table 1). For the CPC treatment, the vertical thickness of abaxial epidermal cells, palisade mesophyll, and sponge mesophyll, as well as the leaf thickness, were significantly higher by 12, 19, 22, and 16%, respectively, compared to the control treatment (Table 1). The vertical thickness of abaxial epidermal cells, palisade mesophyll, and sponge mesophyll, as well as the leaf thickness, were significantly higher by 29, 16, 17, and 15%, respectively, for the fungicide treatment compared to the control treatment (Table 1).

3.5 Foliar concentration of Cu

For non-inoculated plants, the foliar Cu concentration was significantly higher by 87 and 98% for CPC and fungicide treatments compared to the control treatment (Fig. 5A). The foliar Cu concentration was significantly higher by 86% for fungicide-sprayed and non-inoculated plants compared to the CPC-sprayed and non-inoculated plants (Fig. 5A). For inoculated plants, the foliar Cu concentration significantly increased by 97 and 96% for CPC and fungicide treatments, respectively, compared to the control treatment (Fig. 5B). The foliar Cu concentration was significantly higher by 23 and 79% for inoculated plants from the control and CPC treatments, respectively, compared to their counterparts (Fig. 5A-B). The foliar Cu concentration was significantly higher by 32% for fungicide-sprayed and non-inoculated plants compared to fungicide-sprayed and inoculated ones (Fig. 5A-B).

3.6 Physiological evaluations

3.6.1 Imaging and quantification of F_v/F_m

There was no visual difference on the images for F_v/F_m among control, CPC, and fungicide treatments for non-inoculated plants (Fig. 6). For inoculated plants, visual changes on the images for F_v/F_m progressed from 15 to 35 dai, the loss of the photosynthetic capacity more evident for water-sprayed leaves compared to CPC- and fungicide-sprayed leaves (Fig. 6). For non-inoculated plants, there was no significant difference among the control, CPC, and fungicide treatments for F_v/F_m regardless of the evaluation time (Fig. 7A). For inoculated plants, F_v/F_m significantly increased by 8, 21, 23, and 41% at 15, 22, 29, and 35 dai, respectively, for CPC-sprayed plants compared to water-sprayed ones (Fig. 7B). Significant increases of 6, 21, 23, and 43% at 15, 22, 29, and 35 dai, respectively, were obtained for fungicide-sprayed plants compared to water-sprayed ones (Fig. 7B). The F_v/F_m values were significantly higher for non-inoculated and water-sprayed plants compared to inoculated and water-sprayed ones from 15 to 35 dai (Fig. 7A-B).

3.6.2 Photosynthetic pigments

There was no significant difference among the control, CPC, and fungicide treatments for both Chl *a+b* and carotenoids concentrations regardless of plant inoculation and evaluation time (Fig. 8A-C). For the concentrations of Chl *a+b* and carotenoids at 15 dai and the concentration of carotenoids at 29 dai, there was no significant difference among the control, CPC, and

fungicide treatments (Fig. 8B and D). The concentration of Chl *a+b* was significantly higher for CPC (56, 51, and 39% at 22, 29, and 35 dai, respectively) and fungicide (55, 45, and 39% at 22, 29, and 35 dai, respectively) treatments compared to the control treatment (Fig. 8B). There were significant increases for carotenoids concentration for CPC (38 and 27% at 22 and 35 dai, respectively) and fungicide (29 and 23% at 22 and 35 dai, respectively) treatments compared to the control treatment (Fig. 8D).

4. DISCUSSION

Although the spray of fungicides provides acceptable levels of CLR control, new alternatives more aligned with the context of sustainable coffee production need to become available to growers, especially those dedicated to the production of organic coffee. It is noteworthy in this context that the risks imposed on the environment due to an abusive use of fungicides will decrease along with a reduction in the production costs and less chance that populations of *H. vastatrix* become resistant to them. To bring scientific support to this concept, the present study provides microscopical and physiological shreds of evidence for using a product containing Cu mixed with polyphenols, named CPC, to hamper the infection process of *H. vastatrix* in coffee leaf tissues and, therefore, slowing the CLR progress. Interestingly, the germination of urediniospores from *H. vastatrix* was greatly reduced by the CPC *in vitro* with a level of efficiency comparable to the obtained for the contact fungicide. It is plausible to postulate that the Cu contained in the formulation of CPC may have affected the germination of urediniospores. The foliar spray of fungicides containing Cu (*e.g.*, copper sulfate, copper hydroxide, copper oxychloride, and cuprous oxide) or certain products containing soluble Cu is currently used for CLR control (Zambolim et al. 2016). Based on the known fungicidal or fungistatic properties of Cu, the different homeostatic processes taking place inside the fungal hyphae and the functionality of the cell membrane and the synthesis of proteins are dramatically affected along with DNA disruption and an increase in the production of reactive oxygen species (Cioffi et al. 2004; Krumova et al. 2009; Rai et al. 2018). The sensitivity of the germ tube to Cu during spore germination can be increased due to the weakening of the cell wall as the fungal hyphal growth takes place by the enzymatic action of glucanases and disulfide reductase (Bartnicki-Garcia 1968). Moreover, the multisite effect of Cu provides its great potential to form complexes with sulfhydryl, hydroxyl, amino, or carboxyl groups, to interfere with numerous enzymatic reactions, denaturing proteins, and blocking electron transport in

respiratory activities (Pozza et al. 2024). The concentration of Cu^{2+} in coffee leaves is usually less than 70 mg/kg, but due to the intensive spray of fungicides-containing Cu (from 1 to 2 kg of metallic Cu per hectare per spray) this concentration can be up to 150 mg/kg and be highly toxic against the urediniospores of *H. vastatrix* due to its redox properties (Leal et al. 2024). The inhibition of urediniospores germination from *H. vastatrix* by CuO-nanoparticles was greater than 90% (Leal et al. 2024). Interestingly, Rodrigues et al. (2023) reported a significant reduction in germination of *Phakopsora pachyrhizi* urediniospores using the same CPC compared to the control treatment (Water). Other pathogens such as *Alternaria alternata*, *Botrytis cinerea*, *Monilia fructicola*, *Verticillium dahliae*, *Colletotrichum gloeosporioides*, *Fusarium oxysporum* f. sp. *radices-lycopersici*, and *Fusarium solani* had their growth greatly affected by the fungistatic effect of Cu (Malandrakis et al. 2019).

The most notable effect of the CPC was the changes occurring for all components related to both host resistance and the infection process of *H. vastatrix* evaluated. The palisade and spongy mesophylls in the abaxial leaf surface of CPC-sprayed plants were less colonized by hyphae of *H. vastatrix* resulting, therefore, in less sporulation. On top of that, the IP and LP (time needed to noticed the flecks and urediniospores, respectively, in the abaxial leaf surface) linked to variables that indirectly reflected the lower capacity of *H. vastatrix* to reproduce (uredosporic sori formed per cm^2 of leaf, number of urediniospores produced per cm^2 of leaf, and area of each uredosporic sorus) were affected in the CPC-sprayed leaves. Longer IP and LP along with changes in the values of variables intrinsically related to the reproduction of the pathogen (*e.g.*, reduced infectious period and less frequency of infection) greatly contribute to slowing the epidemic rate of a certain disease decreasing, therefore, the number of secondary cycles of inoculum produced by the pathogen allowing to control the disease more efficiently (Zadoks 1971; Parlevliet 1979). In the leaf tissues of CPC-sprayed leaves, the histopathological observations indicated that the changes in both format and volume of cells were less drastic due to less internal compression occurred in the abaxial epidermis as well as in both spongy and palisade mesophylls as a result of less fungal colonization. In this scenario, the internal pressure exerted by fungal stroma in the substomatic cavity towards the sporulation seemed less strong in the leaf tissues of CPC-sprayed leaves. Reduced colonization of leaf tissues by fungal hyphae and, consequently, less sporulation (*e.g.*, less abundant suprastomatal, bouquet-shaped, orange-colored uredinia) is a plausible explanation for the less alterations in the vertical thickness of abaxial epidermal cells, palisade mesophyll, and sponge mesophyll, as well as the leaf thickness noticed for CPC-sprayed plants in contrast to water-sprayed ones at 35 days after plant inoculation with *H. vastatrix*.

The foliar supply of nutrients helps maintain an adequate nutritional status on the plant canopy in situations of deficiency resulting in better physiological performance and better growth and resilience against different types of stress (Niu et al. 2021; Ishfaq et al. 2022). In fact, the increased availability of nutrients, especially the micronutrients, in the plant cytosol will help the different biochemical pathways to function more appropriately for the production of antimicrobial compounds (Rengel et al. 2022; Tubana and Cruz 2023). For instance, Cu is a co-factor of several enzymes involved in the photosynthesis, respiration, antioxidative (*e.g.*, superoxide dismutase and ascorbate oxidase), carbohydrate, and nitrogen metabolisms as well in the synthesis of aromatic metabolites and lignin production (*e.g.*, polyphenol oxidases) (Elmer et al. 2023; Tubana and Cruz, 2023). In the present study, the higher foliar concentration of Cu for CPC-sprayed plants may have helped them to face the infection by *H. vastatrix* more efficiently considering its indirect involvement in host defense reactions. Interestingly, the foliar Cu concentration was lower for CPC-sprayed and non-inoculated plants compared to CPC-sprayed and inoculated plants. It is tempting to speculate that impairment on its redistribution among leaf tissues of the same leaf and between leaves was an effect of higher CLR severity that impacted coffee plants growth during the time course evaluated.

Alterations in the photosynthetic apparatus will impact the translocation of photoassimilates across the different organs is predispose plants to become more susceptible to infection by pathogens of different lifestyles considering the less efficiency of the defense reactions (Debona et al. 2014; Rios et al. 2014; Silveira et al. 2015; Dias et al. 2020; Sterling and Melgarejo 2021). The images of the F_v/F_m parameter, as well as the values obtained from its quantification, reflect the physiological status of plant tissues facing different types of either abiotic or biotic stress before any symptom becomes noticeable by naked eye (Aucique-Pérez and Ramos 2024). In the coffee leaves, the efficiency of the photosystem II was profoundly lowered due to the infection by *H. vastatrix* based on the lower F_v/F_m values (Honorato Júnior et al. 2015; Salcedo-Sarmiento et al. 2021; Aucique-Pérez and Ramos 2024). For other pathosystems such as soybean-*P. pachyrhizi*- and -*Corynespora cassiicola*, wheat-*Pyricularia oryzae*, and rice-*Bipolaris oryzae*, the F_v/F_m values were greatly lower indicating extensive damage to the photosynthetic apparatus (Rios et al. 2017; Fortunato et al. 2018; Aucique-Pérez et al. 2020; Hawerroth et al. 2023; Aucique-Pérez and Ramos 2024). In the present study, higher F_v/F_m values were obtained for CPC-sprayed and infected leaves from 15 to 35 dai compared to water-sprayed and infected ones. Interestingly, the values of F_v/F_m values were quite similar between infected leaves of both CPC and fungicide treatments due to reduced damage to the PSII reaction centers and, consequently, less photoinhibition of photosynthesis. The prompted

functioning of the photosynthetic apparatus of leaves infected by pathogens of different lifestyles was greatly affected due to the lower pool of photosynthetic pigments (Aucique-Pérez et al. 2014; Silveira et al. 2015; Dias et al. 2018; Rios et al. 2018; Einhardt et al. 2020; Silva et al. 2023). Light absorption and energy flux to the centers of reaction where the photochemical phase of photosynthesis occurs are intrinsically linked to the concentrations of chlorophylls *a* and *b* as well as carotenoids (Taiz and Zeiger 2010). In the present study, the pool of Chl *a+b* (at 22, 29, and 35 dai) and carotenoids (at 22 and 35 dai) were kept higher for CPC-sprayed and infected leaves compared to water-sprayed and infected leaves. Altogether, these physiological findings point out that the photosynthetic apparatus of CPC-sprayed and infected leaves were exceptionally preserved with less degradation of Chl *a+b* and carotenoids as a consequence of reduced CLR severity and other variables linked to the infection process (*e.g.*, leaf tissue colonization and reproduction) of *H. vastatrix*.

5. CONCLUSION

The CPC showed great potential in reducing the progress of CLR based on the quantification of several variables related to host resistance and fungal infection process (*e.g.*, IP, LP, IFS, area of uredosporic sorus, number of uredosporic sori per cm² of leaf, and number of urediniospores produced per cm² of leaf) and a more preserved photosynthetic apparatus. The fungistatic effect of Cu in the CPC against the germination of urediniospores from *H. vastatrix* must be taken into account to explain the reduction in CLR severity. In a field condition scenario, the CPC could be an interesting approach to lower the initial inoculum of *H. vastatrix* that will contribute to a severe CLR epidemic considering that the level of CLR control using this product was quite remarkable in comparison to the Cu-based fungicide. The number of fungicide sprays is expected to be reduced or even interspersed with the CPC within the context of integrated CLR management in different coffee-producing regions.

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7. LIST OF TABLE AND FIGURES

Table 1. Vertical measurements (μm) of the adaxial epidermis (AdE), abaxial epidermis (AbE), palisade mesophyll (PM), spongy mesophyll (SM), and leaf thickness (from the adaxial to the abaxial surface) in transverse sections of coffee leaves obtained from plants sprayed with water (control), copper-polyphenol compound (CPC), and fungicide at 35 days after inoculation with *Hemileia vastatrix*.

Treatments	AdE	AbE	PM	SM	LT
Control	19.92 b	15.63 b	31.57 b	88.86 b	159.45 b
CPC	21.51 ab	17.76 a	39.05 a	114.35 a	188.67 a
Fungicide	22.52 a	22.00 a	37.44 a	106.98 a	188.95 a

For each vertical measurement, means within a column followed by the same letter are not significantly different ($P = 0.05$) as determined by Tukey's test.

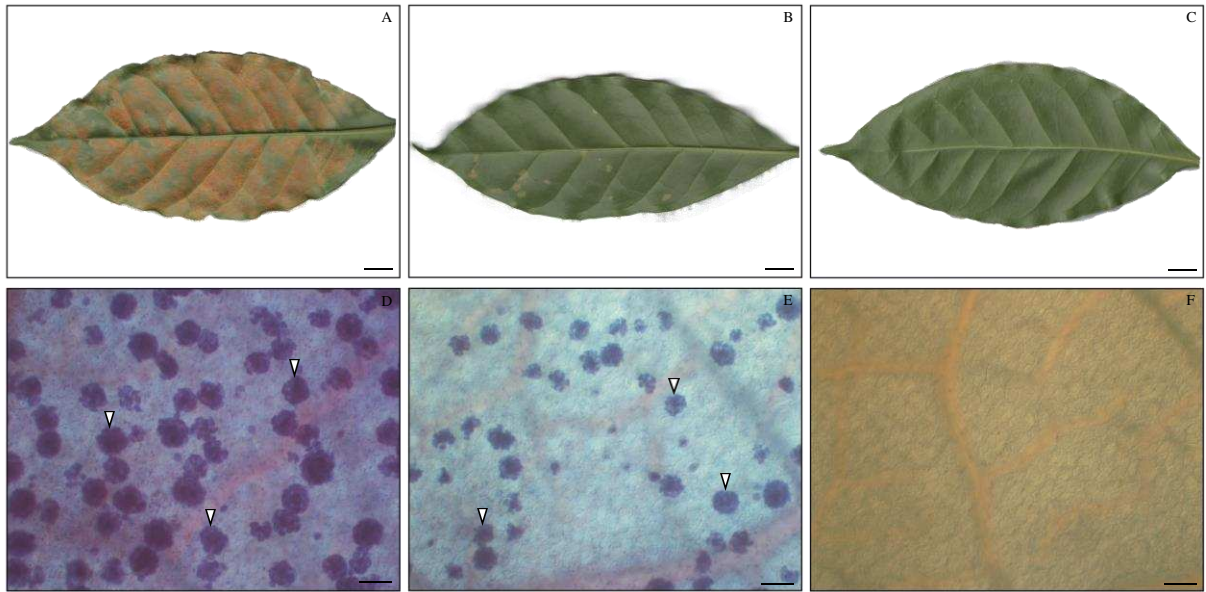


Figure 1. Sporulation of *Hemileia vastatrix* (A, B, and C) and uredosporic sori (arrowheads) formed on stomata (D, E, and F) in the abaxial surface of coffee leaves from plants sprayed with water (control) (A and D), copper-polyphenol compound (B and E), and fungicide (C and F) at 35 days after inoculation with *H. vastatrix*. Scale bars = 1 cm (A-C) and 100 μm (D-F).

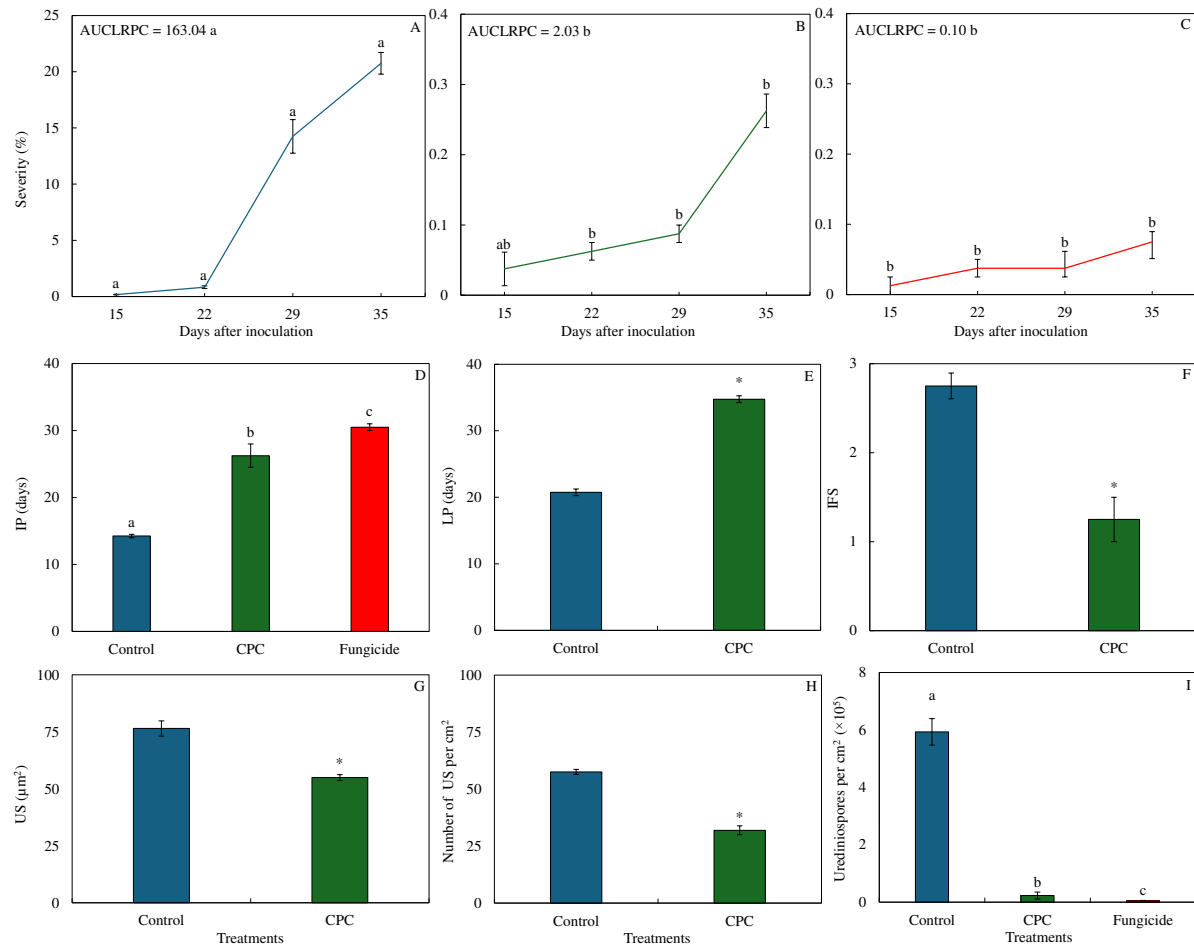


Figure 2. Severeity of coffee leaf rust and area under coffee leaf rust progress curve (AUCLRPC) control (A), CPC (B), and fungicide(C), incubation period (IP) (D), latent period (LP) (E), intensity of fungal sporulation (IFS) (F), area of uredosporic sorus (US) (G), number of uredosporic sori (US) (H), and number of urediniospores (I) for coffee plants sprayed with water (control), copper-polyphenol compound (CPC), and fungicide. Means for control, CPC, and fungicide treatments followed by different letters [in graphs A, B, and C (at each evaluation time), D, and I] are significantly different ($P \leq 0.05$) according to the Tukey test. Means for control and CPC treatments (in graphs E, F, G, and H) followed by an asterisk (*) are significantly different ($P \leq 0.05$) according to the F test. Bars represent the standard error of the means.

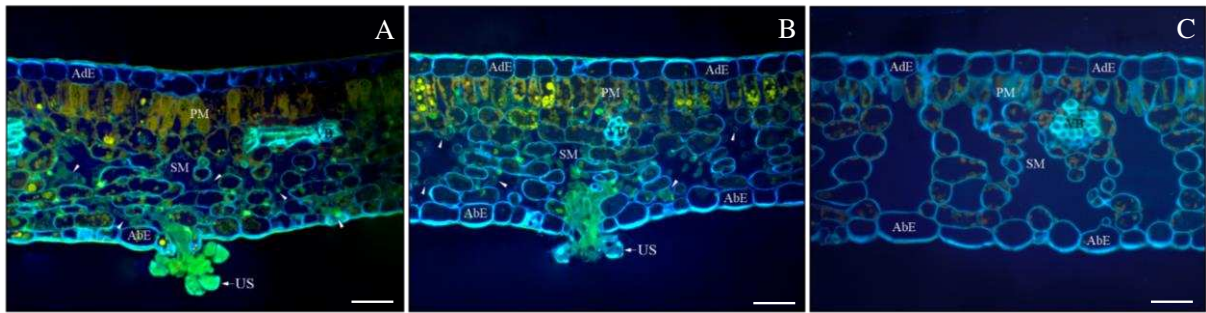


Figure 3. Fluorescence micrographs of transverse sections of leaves obtained from coffee plants sprayed with control (A), CPC (B), and fungicide (C) at 35 days after inoculation with *Hemileia vastatrix*. The palisade and sponge mesophylls in the leaf section from water-sprayed plants were massively colonized by fungal hyphae that were stained with the WGA-AF 488 (green fluorescence; arrowheads) in contrast to the leaf sections of CPC- and fungicide-sprayed plants. Adaxial leaf epidermis (AdE), abaxial leaf epidermis (AbE), palisade mesophyll (PM), sponge mesophyll (SP), vascular bundle (VB), and uredosporic sorus (US). Scale bars: 50 μ m.

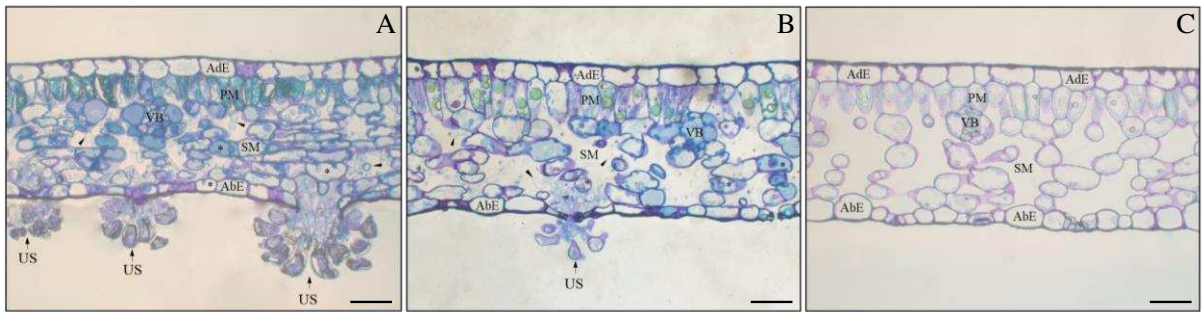


Figure 4. Light micrographs of transverse sections of leaves obtained from coffee plants sprayed with control (A), CPC (B), and fungicide (C) at 35 days after inoculation with *Hemileia vastatrix* that were used for the anatomical measurements of the adaxial leaf epidermis (AdE), abaxial leaf epidermis (AbE), palisade mesophyll (PM), and sponge mesophyll (SM). US = uredosporic sorus. Scale bars: 50 μ m.

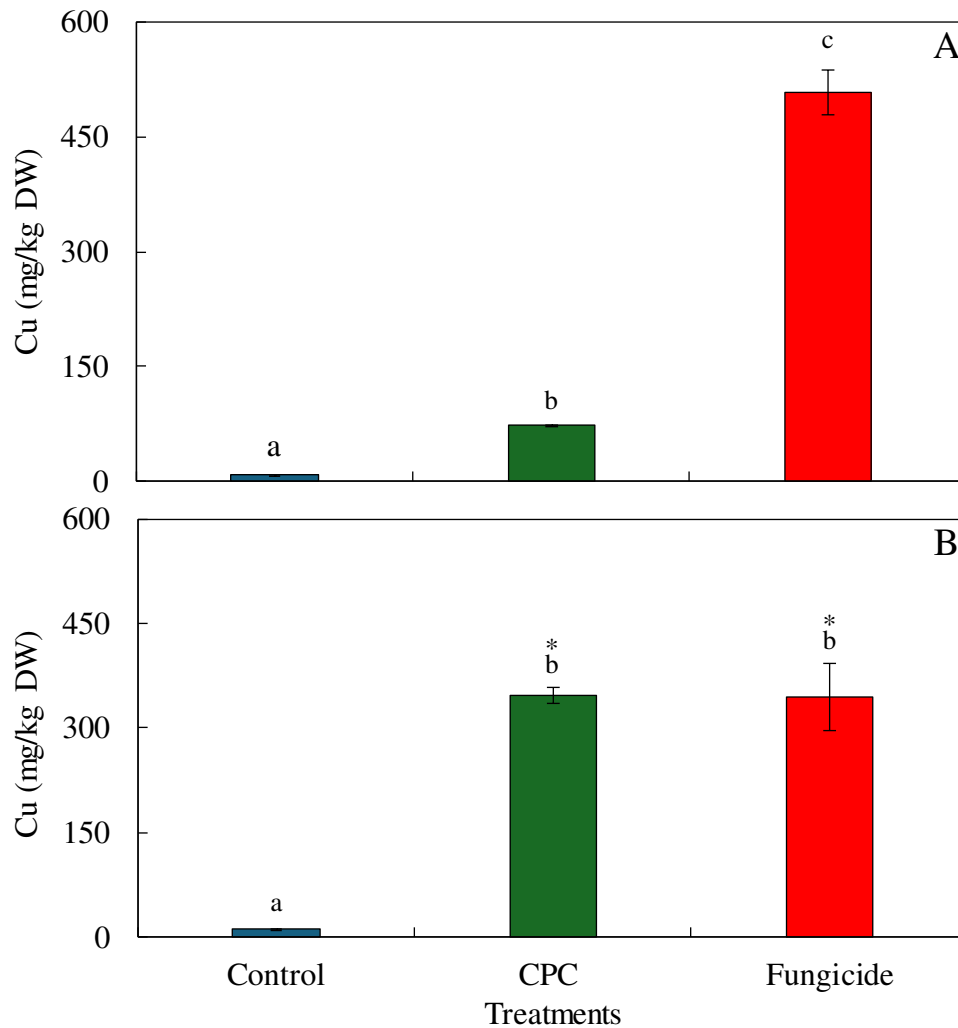


Figure 5. Foliar concentration of copper (Cu) for coffee plants sprayed with water (control), copper-polyphenol compound (CPC), and fungicide that were non-inoculated (NI) (A) or inoculated (I) (B) with *Hemileia vastatrix*. Means from control, CPC, and fungicide treatments followed by different letters (in graphs A and B) and for NI and I plants, for each treatment, followed by an asterisk (*), are significantly different ($P \leq 0.05$) according to Tukey and *F* tests, respectively. Bars represent the standard error of the means. DW = dry weight.

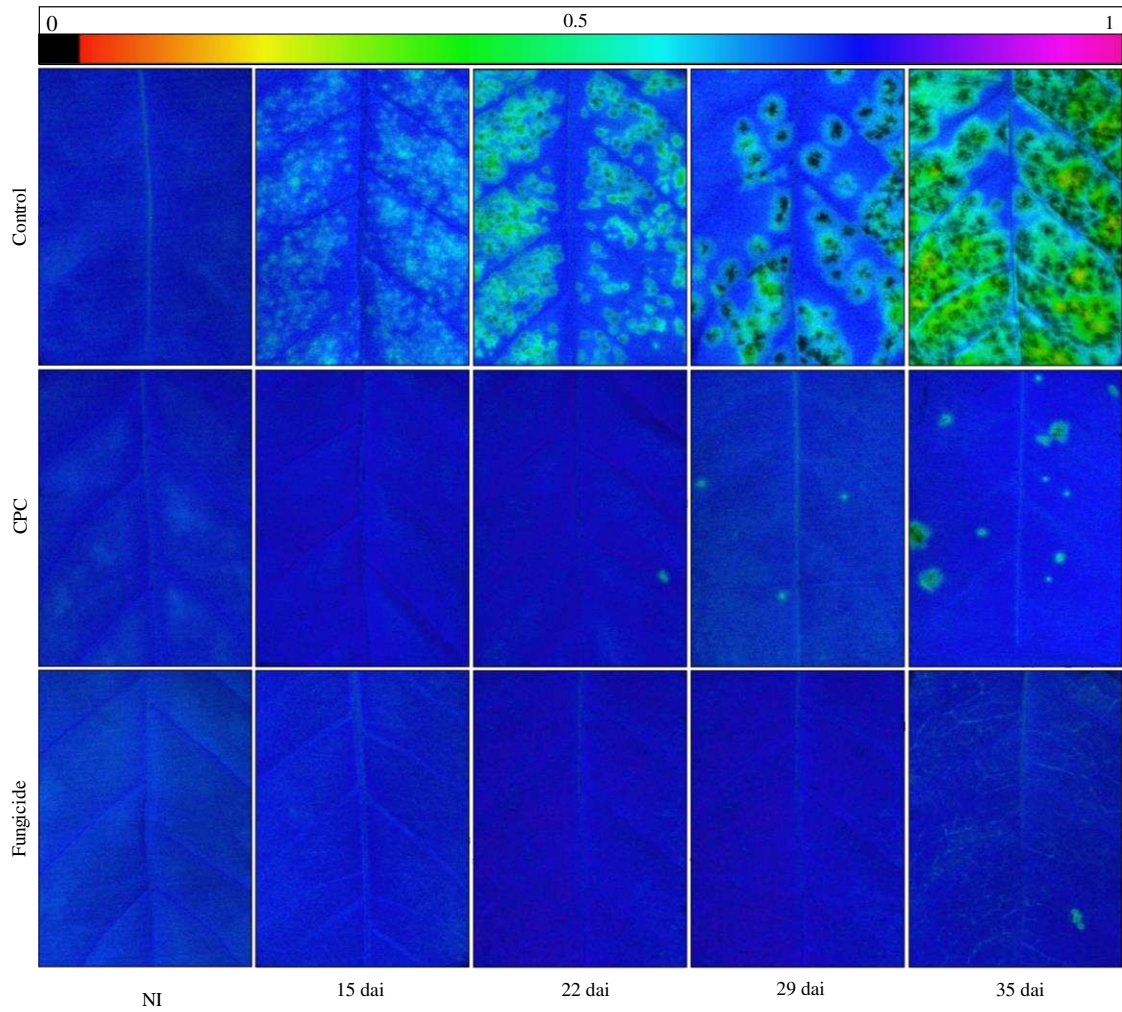


Figure 6. Images for maximum photochemical efficiency of photosystem II (F_v/F_m) parameter determined on the leaves of coffee plants sprayed with water (control), copper-polyphenol compound (CPC), and fungicide and non-inoculated or at different days after inoculation (dai) with *Hemileia vastatrix*.

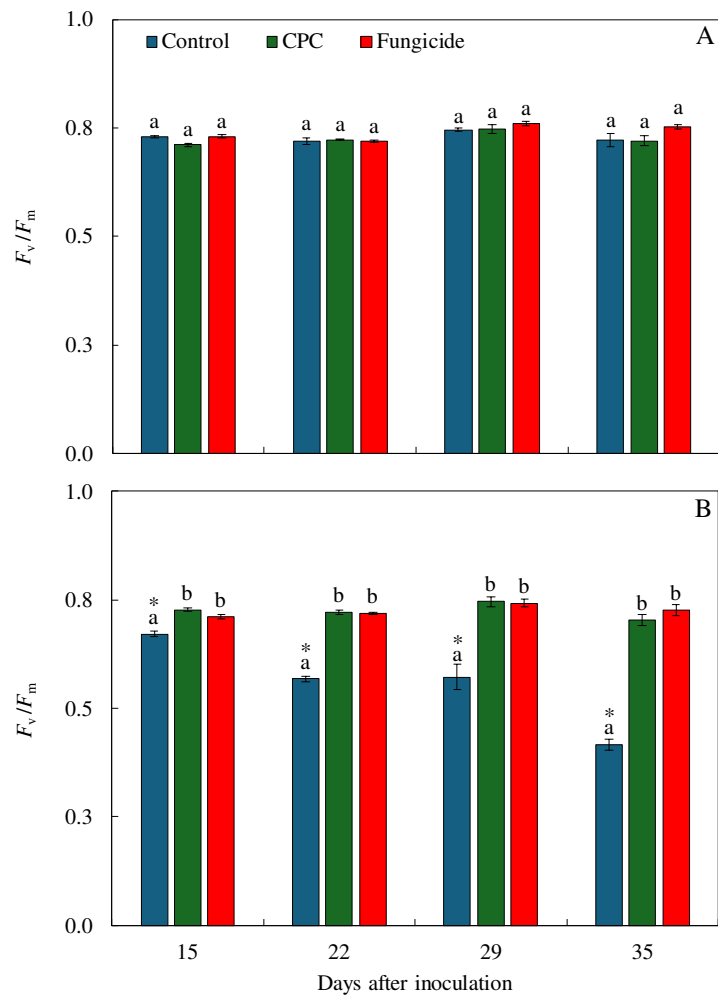


Figure 7. Maximum photochemical efficiency of photosystem II (F_v/F_m) parameter determined on the leaves of coffee plants sprayed with water (control), copper-polyphenol compound (CPC), and fungicide and non-inoculated (NI) (A) or inoculated (I) (B) with *Hemileia vastatrix*. For each evaluation time, means from control, CPC, and fungicide treatments followed by different letters and for NI and I plants, for each treatment, followed by an asterisk (*), are significantly different ($P \leq 0.05$) according to Tukey and F tests, respectively. Bars represent the standard error of the means.

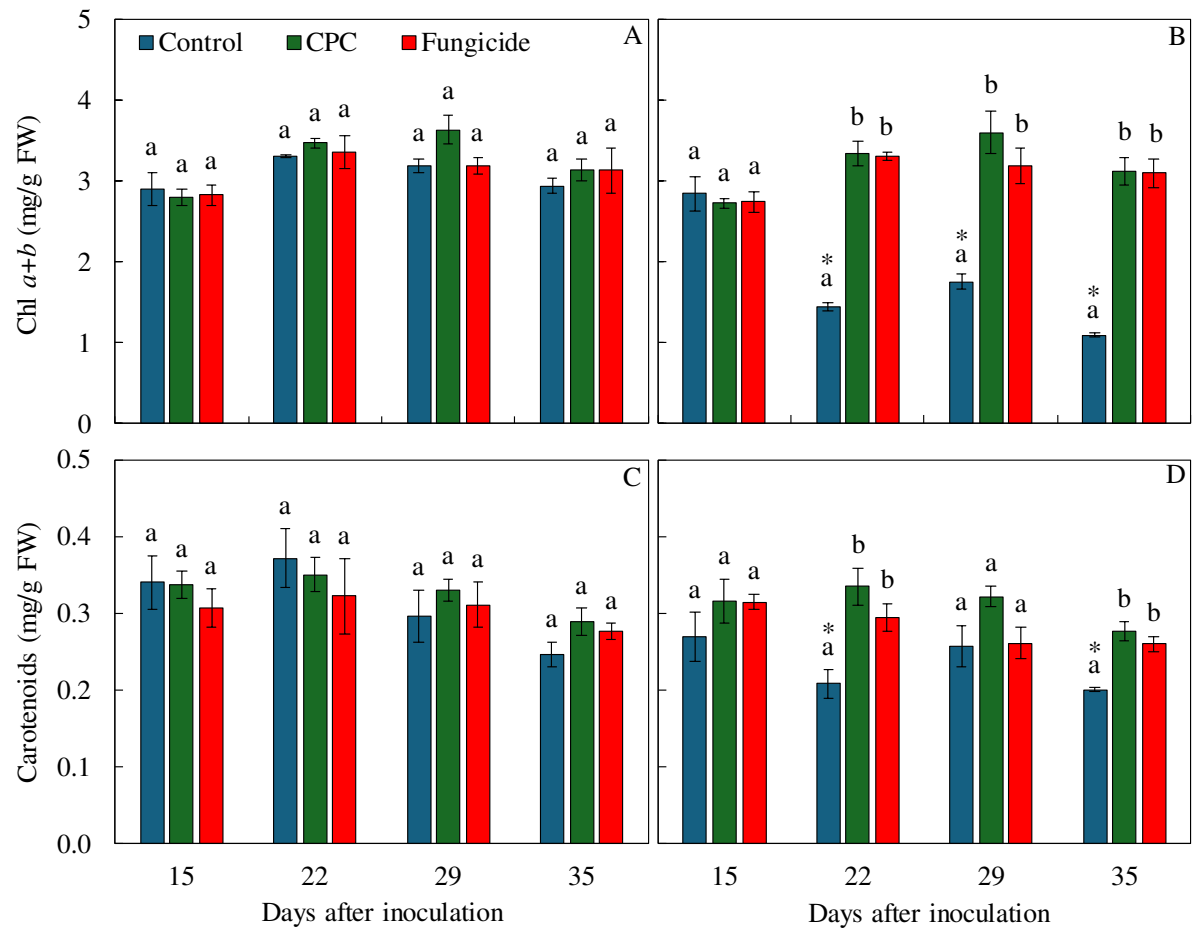


Figure 8. Concentrations of total chlorophyll (Chl *a+b*) (A and B) and carotenoids (C and D) determined on the leaves of coffee plants sprayed with water (control), copper-polyphenol compound (CPC), and fungicide and non-inoculated (NI) (A and C) or inoculated (I) with *Hemileia vastatrix* (B and D). For each evaluation time, means from control, CPC, and fungicide treatments followed by different letters and for NI and I plants, for each treatment, followed by an asterisk (*), are significantly different ($P \leq 0.05$) according to Tukey and *F* tests, respectively. Bars represent the standard error of the means. FW = fresh weight.