Elucidating the interactions between the rust *Hemileia vastatrix* and a *Calonectria* mycoparasite and the coffee plant

**Highlights**

*Calonectria* hemileiae was proven to be an effective mycoparasite of coffee leaf rust

*Calonectria* hemileiae reduced significantly the germination and growth of rust spores

Rust severity on coffee reduced by 70-90% by *Calonectria* application

Photosynthetic capacity of coffee unharmed by *Calonectria* but reduced by fungicides
Article

Elucidating the interactions between the rust *Hemileia vastatrix* and a *Calonectria* mycoparasite and the coffee plant

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**SUMMARY**

*Calonectria hemileiae*, a fungus associated with pustules of the coffee leaf rust (CLR, *Hemileia vastatrix*) in Brazil, was tested *in vitro* and *in planta* to assess its biocontrol potential. The fungus inhibited the germination of rust spores by over 80%. CLR severity was reduced by 93% when *Calonectria* was applied to coffee leaf discs inoculated with *H. vastatrix*, whilst a reduction of 70-90% was obtained for *in planta* experiments. Mycoparasitism was demonstrated through the fulfillment of Koch’s postulates. Elucidation of the biochemical interaction between *Calonectria* and *Hemileia* on coffee plants indicated that the mycoparasite was able to increase plant resistance to rust infection. Coffee plants sprayed with *Calonectria* alone showed greater levels of chitinase, β-1,3-glucanase, ascorbate peroxidase and peroxidase. Although effective in controlling the rust, fungicide applications damaged coffee photosynthesis, whereas no harm was caused by *Calonectria*. We conclude that *C. hemileiae* shows promise as a biocontrol agent of CLR.

**INTRODUCTION**

Brazil is the largest coffee producer and exporter in the world (MAPA, 2018). Coffee is one of the top global commodities, generating around 90 billion US dollars a year (Batista et al., 2012; ICO, 2016). Only two species of the genus *Coffea* are of economic importance: *Coffea arabica* (Arabica coffee) and *Coffea canephora* (Robusta coffee or Conilon), representing 66 and 34%, respectively, of commercially planted coffee (Somarriba et al., 2004). Despite its economic and social relevance, coffee cultivation has always been threatened by negative abiotic and biotic factors, especially drought and fungal diseases, both of which can drastically decrease yields (Rodrigues-Junior, 1990; Menezes-Silva et al., 2017).

The most devastating disease affecting the crop is coffee leaf rust (CLR), caused by *Hemileia vastatrix* (, *Mikroneregleriaceae, Pucciniales*; McTaggart et al., 2016). At high incidence, CLR can cause defoliation of up to 50% and yield losses between 30 and 50% (Bhat et al., 2000; Capucho et al., 2013a; Zambolim, 2016), whilst economic losses have been estimated at between t 1-2 billion US dollars annually (Talhinhas et al., 2017).

CLR control is based on the use of resistant varieties, the application of contact and systemic fungicides (Zambolim, 2016), as well as disease escape by establishing highland plantations (McCook, 2004). However, there are limitations to each of these approaches. High *H. vastatrix* variability and the emergence of new races of the rust, as well as the occurrence of a complex of races, challenges the establishment of durable resistance in this crop (Varzea and Marques, 2006; Cabral et al., 2009). The use of fungicides, although effective for the control of CLR (Capucho et al., 2013b), is costly and may be impractical for the challenging terrain of upland plantations. It is also rejected as an option—particularly in the case of systemic fungicides—for the high-value organic coffee market. Additionally, the continuous and repetitive use of systemic fungicides can promote the selection of resistant populations of the rust. Although there is no evidence of fungicide-resistant strains of *H. vastatrix*, there are records of such events for other rust fungi, such as *Phakopsora pachyrhizi*—soybean rust (Godoy, 2012), and as chemical control of CLR presently relies on products belonging to two chemical groups only (azoles and strobilurins), there is the added danger of loss of efficiency for these products. Regular application of broad-spectrum fungicides also brings the
threat of environmental impact and may, for instance, harm populations of beneficial organisms, including bacterial and fungal antagonists of *H. vastatrix* (Capucho et al., 2013b; Honorato et al., 2015a).

A series of alternative control strategies for CLR are reported in the literature, such as the combination of cultivation under shade and adequate nitrogen fertilization, as well as the use of resistant varieties (Toniuitti et al., 2017). Although it has been suggested that there is an untapped potential in biological control for CLR management, published results of studies aimed at rust control are still relatively few and concentrated on the use of antagonistic bacteria, such as *Bacillus thuringiensis*, *B. subtilis*, and *Pseudomonas putida* to be deployed as biopesticides (Shiomi et al., 2006; Mejía, 2015). Examples of promising results with potential bacterial products for control of CLR have been reported (Bettiol et al., 1994; Cristancho, 1995; Costa et al., 2007; Daivasikamani and Rajanaika, 2009; Haddad et al., 2009, 2013, 2014). As compared with bacteria, fungi antagonistic to *H. vastatrix* have been poorly investigated.

The only publication of a systematic survey for mycoparasites of *H. vastatrix* was that of Carrión and Rico-Gray (2002) undertaken in the Mexican state of Veracruz. James et al. (2016) used single-molecule DNA sequencing for evaluating the diversity of fungal communities associated with CLR lesions collected from coffee leaves in Mexico and Puerto Rico. These authors found 69 taxonomic units (putative species), 15 of which were interpreted to be mycoparasitic fungal species belonging to the Cordycipitaceae (Ascomycota) and the Tremellales (Basidiomycota).

No surveys for fungal antagonists of *H. vastatrix* have been conducted in Brazil or Africa until now, and even the ubiquitous “white colony-forming fungi” found on rust pustules have generally been assigned to *Lecanicillium* (now *Akanthomyces*) lecanii without careful examination; missing a significant diversity of these monilaceous fungi, as well as of other fungal groups (Barreto et al., 2015). A project was initiated in 2014, sponsored by World Coffee Research (WCR, 2020), aimed at surveying the natural enemies (fungi) of *H. vastatrix* associated with the genus *Coffeea* in its African cent of origin, as well as in Brazil. Surveys were undertaken in Africa in collaboration with local scientists and yielded 1516 isolates; representing a broad diversity of fungi, arbitrarily treated as belonging to two ecological groups: (i) endophytic mutualists growing inside coffee plant tissues (potentially serving as fungal bodyguards); and (ii) rust pustule colonizers (purported mycoparasites). Their potential for use in biological control is currently under evaluation for selected isolates. Although coffee and the CLR fungus are non-native in Brazil, the brief survey conducted in the Brazilian coffee-growing areas produced an unexpectedly large diversity of mycoparasites. One such CLR pustule-colonizing fungus among those which were encountered belonged to *Calonectria*—a genus not known to include mycoparasitic species—which was found to be new to science and described as *Calonectria hemileiae* S.S. Salcedo, A.A. Colmán, H.C. Evans, and R.W. Barreto, in Crous et al. (2018). During a preliminary screening for potential biocontrol candidates, *C. hemileiae* was shortlisted for more detailed evaluation. Given the promising preliminary results obtained with of *C. hemileiae* as an anti-CLR treatment, the present study aimed at further testing the hypotheses that: (a) *C. hemileiae* is a mycoparasite of *H. vastatrix*; (b) *C. hemileiae* can reduce the severity of CLR; (c) *C. hemileiae* does not interfere with the photosynthetic capacity of the plants; (d) *C. hemileiae* can induce disease resistance. These were tested through a series of experiments described herein. All experiments involved the type strain of *C. hemileiae* (COAD 2544), identified here by our original collecting code AC121.

**RESULTS**

**Effect of Calonectria hemileiae on the germination of urediniospores of Hemileia vastatrix**

Germination of urediniospores ranged from 60 to 75% for the control treatment (Figure 1A). When mixed with conidial suspensions of *C. hemileiae* (Figure 1B), germination dropped to between 0 and 20% (Figure 1C). Therefore, inhibition of germination of urediniospores of 80-100% resulted from exposure to *C. hemileiae* (Figure 1C). The few urediniospores that germinated when exposed to *C. hemileiae*, were rapidly overgrown by the mycoparasite germ tubes and prevented to further develop. In addition, the germ tubes emerging from urediniospores were abnormal—short and inflated (Figure 1C).

**Interactions of Calonectria hemileiae and Hemileia vastatrix on coffee in vitro and in planta**

Based on the in vitro tests (two experiments), the rust disease index (RDI) was significantly reduced by 96, 93 and 96%, respectively, for Ch/72Hv, Ch/Hv, and fungicide treatments when compared to plants that received the rust only (Figure 1J). The presence of pustules and necrotic leaf tissue in the leaf discs treated with the rust alone was evident at 40 days after inoculation (dai) (Figure 1E). Conversely, leaf discs from the
Figure 1. In vitro and in planta evidence of Calonectria hemileiae antagonistic/biocontrol effect on Hemileia vastatrix.

Urediospores of H. vastatrix suspended in sterile distilled water (SDW) and germinating after 6 hr incubation in vitro (A, bar 50 µm). Conidia of C. hemileiae suspended in sterile distilled water (SDW) and forming germ tubes and branching — note germinating conidia of C. hemileiae (black arrows) (B, bar 50 µm). C. hemileiae (Ch) in combined suspension in SDW with urediospores of H. vastatrix — note germinating conidia of C. hemileiae (black arrows) and short and inflated germ tubes emerging from urediospores (white arrows) (C, bar 50 µm). Coffee leaf discs (D to I) and plants (K to P) from experiments that involved C. hemileiae applications at 72 hr before (G and N) or simultaneously (H and O) with inoculation of H. vastatrix (E and L). Effect of fungicides (trifloxystrobin + tebuconazole) (I and P) on H. vastatrix. Leaf discs and coffee plants sprayed with sterile water (D and K) and C. hemileiae (F and M) without inoculation with H. vastatrix used as controls. Rust disease index (RDI) (J) and coffee leaf rust severity (CLRS) (Q) for coffee plants submitted to different treatments. Asterisks (*) indicates significant difference (p ≤ 0.05) by the Dunnet-test. Bars represent the standard deviations. n = 3 and 5, respectively, for experiments involving leaf discs and coffee plants. Scanning electron microscopy micrographs showing a healthy pustule of H. vastatrix producing a group of urediospores (arrowed) on the leaf surface (R, bar 20 µm) and a pustule of H. vastatrix overgrown by C. hemileiae, showing collapsing urediospores (arrowed) (S and T, bar 20 µm) 20 days after spraying with a C. hemileiae conidial suspension.

Ch/72Hv, Ch/Hv, and fungicide treatments showed none or only a few rust pustules (Figures 1G, 1H, and 1I). No visible harm was observed on the leaf discs treated with C. hemileiae alone (Figure 1F). When compared with healthy untreated controls [discs sprayed with sterile distilled water (SDW) only], no abnormalities were observed on leaves treated with C. hemileiae conidial suspension only (Figures 1D and 1F).

Preliminary in vitro results obtained for biocontrol of H. vastatrix with C. hemileiae were highly significant and suggested a high potential for CLR management. To further evaluate C. hemileiae in planta, experiments under controlled conditions were conducted (as described in the supplemental file). Significant reductions in CLR severity were also obtained with reductions of 91 and 73%, respectively, for Ch/72Hv and Ch/Hv treatments (Figure 1Q). Control of CLR was complete for fungicide treatment (Figure 1P). Plants inoculated with the rust and treated with C. hemileiae (Ch/72Hv and Ch/Hv treatments) showed reduced CLR severity in comparison to inoculated plants that remained untreated with Ch (Figures 1N, 1O, and 1I). Inoculated plants that were sprayed with the fungicide did not develop any CLR symptoms or exhibited only minor damage (Figure 1P). Likewise, no damage appeared on the control plants and plants sprayed with C. hemileiae (Figures 1K and 1M).

The in planta experiments further confirmed low levels of CLR severity in the disease progress evaluation for plants treated with the fungicide. Fungicide application reduced disease severity by 90% as compared with the untreated control (Figures 2A, 2B, and 2G). Nevertheless, significant reductions of coffee leaf rust severity (CLRS) were also achieved for Ch/72Hv. When compared with the rust treatment, Ch/72Hv caused reductions of CLRS of 100, 87, 75, and 76% at 28, 36, 42, and 50 dai, respectively (Figures 2A, 2C, and 2E). The Ch/Hv treatment was not as effective as Ch/72Hv for CLR control. In this case, the Ch/Hv treatment led to reductions of CLRS at 28, 36, 42, and 50 dai of 43, 44, 32, and 36%, respectively, as compared with the rust treatment (Figures 2A, 2C, and 2F). Again, no damage to coffee plants was observed as a result of the C. hemileiae applications (Figure 2D).

Evidence of mycoparasitism

Coffee plants sprayed with rust urediospores (Figure 1R), which were also treated with Calonectria hemileiae, exhibited CLR symptoms 30 dai. Typical C. hemileiae colonies were observed growing over the abnormal rust pustules, similarly to those seen in the type material under SEM. Colonization of rust pustules by C. hemileiae led to general disorganization of the uredinia with the collapse of sporogenous fascicles, and disruption of sporogenesis (Figures 1S and 1T). The presence of typical structures of C. hemileiae on diseased CLR pustules was confirmed following microscopic examination. This corresponds to the third step of Koch’s postulates. Finally, isolations made from mycoparasitized colonies resulted in pure, sporulating colonies, which were identified as C. hemileiae—thereby, fulfilling the fourth step of Koch’s postulates and demonstrating that C. hemileiae is a mycoparasite of CLR.

Interactions of Calonectria hemileiae, fungicide mixture and Hemileia vastatrix: effects on enzymatic activity in coffee leaves

At 72 h after spraying the coffee plants with C. hemileiae, the chitinase (CHI), β-1,3-glucanase (GLU), peroxidase (POX), and superoxide dismutase (SOD) activities were significantly different among the treatments. Plants treated with C. hemileiae (Ch and Ch/72Hv treatments) showed a CHI activity 5-times higher than that of the controls. GLU activity had a greater increase in plants treated with C. hemileiae as compared with the controls. APX activity was not significant for all treatments (Table 1). There were significant differences between treatments at 72 h after inoculation (hai) for CHI, GLU, and SOD activities. Meanwhile, at 50 hai, significant differences were detected in the CHI, GLU, APX, POX, and SOD activities (Table 1). At 72 hai,
CHI and SOD activities were greater for the Ch/72Hv treatment as compared with other treatments. Significant reductions in the GLU activity were found for treatments involving the application of C. hemileiae, as well as for plants treated with fungicide, in comparison with the rust treatment and the control. SOD activity on plants inoculated with the rust, showed a significant reduction when compared with Ch/72Hv and Ch/Hv treatments (Table 1). At 50 dai, plants sprayed with C. hemileiae (Ch treatment) showed higher CHI, APX, and POX activities in comparison to the other treatments. Conversely, the SOD activity was higher in the Ch/72Hv and fungicide treatments as compared with other treatments, whilst plants inoculated with the rust (Hv treatment) presented a high GLU activity as compared with other treatments.

Interactions of Calonectria hemileiae, fungicide mixture, and Hemileia vastatrix: effects on photosynthetic parameters

Fluorescence images of maximal photosystem II quantum yield ($F_{v}/F_{m}$) parameter clearly show the alterations caused by the rust on the photosynthetic performance of leaves as CLR developed (Figure 3). This was mirrored by the low $F_{v}/F_{m}$ values that were found for inoculated plants (Figure 4A). Additionally, the quantitative analysis of the values of $F_{v}/F_{m}$ indicated that after an asymptomatic period, rust infection harmed the photosynthetic process of the leaves (Figure 4A). In the case of the Ch/72Hv, Ch/Hv and fungicide treatments, the values of $F_{v}/F_{m}$ ($\geq 0.8$) found were not as significantly different from those of healthy non-inoculated plants (Figures 3 and 4A).

From 36 dai, onwards, significant differences in the photochemical yield of photosystem II ($\Phi_{PSII}$) and the electron transport rate (ETR) parameters were detected among the treatments (Figures 4B and 4D). For rust-inoculated plants, significant reductions at 36, 42, and 50 dai occurred for $\Phi_{PSII}$ and ETR as compared with other treatments. In contrast, for these evaluation times, the Ch/72Hv and Ch/Hv treatments yielded $\Phi_{PSII}$ values that stayed at levels that were close to those of coffee plants sprayed only with SDW. At 50 dai, $\Phi_{PSII}$ and ETR showed significant reductions for Ch/72Hv and Ch/Hv treatments (Figures 4B and 4D). With regards to $q_{N}$ parameter, these showed a significant increase for plants from the Ch/Hv and Hv treatments at 36 and 50 dai, respectively, as compared to the control treatment (Figure 4C).

The net CO$_2$ assimilation rate ($A$), stomatal conductance to water vapor ($g_s$), and transpiration rate ($E$) values were significantly reduced from 28 dai for plants inoculated with the rust (Figures 5A, 5B, and 5D). Losses of
up to 70% in the CO₂ fixation at 50 dai were observed, whereas the internal CO₂ concentration (Cᵢ) showed significant increases after 42 dai (Figure 4C). For the Ch/72Hv, Ch/Hv, and for the fungicide treatments, the A, gₛ, and E values were significantly higher as compared with those found for coffee plants inoculated with the rust (Figures 5A–5C). However, at 50 dai, the fungicide treatment produced significant reductions in A, gₛ, and E coupled with significant increases in Cᵢ, as compared with Ch/72Hv and Ch/Hv treatments (Figure 5C). Despite the CLR reduction in the plants treated with fungicide, the A and E values were significantly reduced in fungicide-treated plants as compared to water-sprayed controls. Reductions were of 18 and 34% at 20 dai, 16 and 31% at 28 dai, 42 and 49% at 50 dai. These reductions were in connection with the low gₛ values, which were as follows: 40% at 20 dai, 35% at 28 dai, 52% at 36 dai, 31% at and 53% at 50 dai (Figures 5A, 5B, and 5D). In contrast, for all photosynthetic process of the plants (Figures 4 and 5).

### DISCUSSION

Fungal disease management is one of the most challenging and essential goals in modern agriculture. During the 20th century, the use of fungicides became the dominant method for control of diseases caused by fungi in most crops of economic importance, such as coffee. Nevertheless, genetically homogeneous monocultures and the diminished resistance diversity of crops, became major drivers of change in the genetic profile of pathogen populations and crop resistance to pathogens and fungicide efficacy have been compromised on many fronts (Garrett et al., 2006; Zhan et al., 2014). Different approaches are now needed, and the use of antagonistic fungi and bacteria as plant disease control tools is on the rise (Glare et al., 2012; Qualhato et al., 2013; Nygren et al., 2018), especially against CLR (Shiomi et al., 2006; Haddad et al., 2009, 2013, 2014; Mejia, 2015). Historically, the Central American countries and Colombia escaped CLR through a combination of highland cultivation and chemical control, but global warming has been implicated in recent rust outbreaks at higher altitudes and, since the early 2010s, rust epidemics have devastated coffee plantations in these regions, with crop losses of between 30 and 90% (Avelino et al., 2015). CLR remains by far the worst coffee disease and the cause of great and increasing concern in coffee-producing countries in Central and South America. In countries such as Brazil, planting resistant genotypes and fungicide applications have traditionally been efficient (Zambolim, 2016). However, the burgeoning market demand for pesticide residue-free or organic coffee and the repeated breakdown of resistance, are paving the way for a fresh look at biocontrol for the management of CLR (WCR, 2020).

Here, the recently discovered species Calonectria hemileiae was subjected to in vitro and in planta tests to evaluate its antagonism against H. vastatrix and thus to assess its potential as a biocontrol agent. It was demonstrated that C. hemileiae inhibited the germination of rust urediniospores in vitro, whilst the in planta study revealed that the effect of C. hemileiae was comparable to that of chemical fungicide application.
Calonectria hemileiae was found to induce the production of enzymes known to play a role in the coffee defense system and to reduce the oxidative damage caused by rust infection without causing harmful effects to the plant’s photosynthetic performance. This was in contrast to the marked damage caused by a fungicide mixture. Adverse effects of fungicides on carbon metabolism have been reported previously. In the specific case of the azole fungicides, studies have demonstrated that these compounds can cause photosynthesis inhibition (cyazofamid), stomatal closure (triadimefon), CI increase (cyazofamid and triadimefon), as well as reductions in the oxygen evolution and ETR (epoxiconazole) (Petit et al., 2012).

Conversely, other fungicides (paclobutrazol and triadimefon) have been shown to stimulate photosynthesis (Kasele et al., 1995; Gopi et al., 2005; Kishorekumar et al., 2006). It appears that the physiological response of plants to the azole fungicides depends on the compound type, the dosage, and the plant species involved. For the strobilurin fungicides, a highly negative influence on photosynthesis has been reported (Nason et al., 2007). These authors tested five different strobilurin fungicides on barley, soybean, and wheat and found that these caused drastic reductions in the gs (stomatal closure) leading to loss of CO2 influx to carboxylation sites in the chloroplasts (<A), as well as a low transpiration rate for the test plants.
et al. (2016) found that rice plants grown under unstressed conditions and sprayed with azoxystrobin had reductions of 19, 36, and 28%, respectively, in A, gs, and E values; confirming the negative impact of this particular strobilurin type on the photosynthetic capacity of rice plants. Here, our results also indicate that adverse effects on the photosynthesis of coffee plants can result from the use of a mixture of tebuconazole and trifloxystrobin.

The plant defense mechanisms against pathogen infection involve pathogenesis-related proteins (PR-proteins), including CHI and GLU, which are responsible for the catalysis of the degradation process of polysaccharides in the pathogens’ cell wall (Robert et al., 2008). These enzymes are abundant in many plant species after infection by pathogens of different lifestyles (Ebrahim et al., 2011). For plants sprayed only with C. hemileiae, CHI and GLU activities were greater than for the controls at 72 hr before rust inoculation. The results suggest that C. hemileiae elicited activation of the genes producing these enzymes, especially CHI, and triggered the plant’s defense mechanisms against rust infection. There are examples in the literature of non-pathogenic fungi promoting defense reactions in plants that can lead to their protection against fungal pathogens. For instance, it was found that spraying Curvularia inaequalis conidial suspension on sorghum leaves reduced significantly the severity of anthracnose caused by Colletotrichum sublineolum (Resende et al., 2015). These authors attributed this response to significant increases in the CHI and GLU activities as compared with sorghum plants not treated with C. inaequalis before C. sublineolum inoculation.

In addition, several studies have confirmed that CHI and GLU activities could be elicited in plants due to the presence of microbes (Koike et al., 2001). Proteomic analysis studies of susceptible vs. resistant coffee cultivars indicated increased accumulation of PR-proteins, such as chitinases, osmotin, and a cysteine-rich repeat secretory protein, as a feature present in the resistant cultivar only. This may be related to the induction of the basal defense responses, possibly regulated by salicylic acid (Guerra-Guimarães et al., 2015).

The reactive oxygen species (ROS) production in plants, either during the colonization by pathogenic agents or by symbiotic organisms, is usually very similar in the first stage of the interaction as both can induce oxidative burst (Fester and Hause, 2005; Torres, 2010). In the plant-pathogen interaction, ROS production is activated in the plant for limiting pathogen infection, either directly or indirectly (Møller et al., 2007). However, progressive ROS accumulation may impact on the structure and functions of the plant cells and, in order to avoid such problems, the cell has enzymatic and non-enzymatic mechanisms aimed at helping the metabolism of ROS and hence to ensure cellular homeostasis (Kumar et al., 2009). In our study, SOD activity increased in coffee leaves sprayed with C. hemileiae and inoculated with the rust, especially at 72 hai and 50 dai. This suggests that SOD can actively participate in the \( \mathrm{O}_2^* \) metabolism in response to rust infection. SOD may reduce cellular intoxication by this molecule. A previous study of the maize-Piriformospora indica interaction and the biocontrol agent Fusarium verticilloides posited that induction of SOD by F. verticilloides application may lead to the recognition of \( P. \) indica by the plant tissues and consequent activation of plant defense responses (Kumar et al., 2009). Proteomic analysis of the coffee-H. vastatrix interaction demonstrated that the accumulation of copper-zinc superoxide dismutase occurs in tissues of coffee plants that are susceptible to the rust at 48 hai, suggesting that these “PR-like” proteins may co-regulate basal defenses in coffee (Guerra-Guimarães et al., 2015).

APX and POX are involved in \( \mathrm{H}_2\mathrm{O}_2 \) metabolism (Torres, 2010); APX functions at the level of chloroplasts, peroxisomes, and mitochondria using ascorbate as a specific electron donor to reduce \( \mathrm{H}_2\mathrm{O}_2 \) to water (Quan et al., 2008); whilst POX is an enzyme with an essential role in plant defense against pathogens due to its participation in lignin biosynthesis (Rauyaree et al., 2001). In our study, increases in APX and

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**Figure 4.** Photochemical and non-photochemical energy dissipation pathways were preserved on coffee plants inoculated with Hemileia vastatrix but treated with Calonectria hemileiae or fungicide mixture. Maximal photosystem II quantum yield (\( F_{\text{v}}/F_{\text{m}} \)) (A), photochemical yield of photosystem II (\( \Phi_{\text{PSII}} \)) (B) quenching non-photochemical (\( q_{\text{p}} \)) (C) and electron transport rate (ETR) (D) on leaves of coffee plants at 20, 28, 36, 42, and 50 days after inoculation with H. vastatrix. The treatments used were: plants sprayed with C. hemileiae before at 72 h (Ch/72Hv) and simultaneously with H. vastatrix (Ch/Hv). The Ch and Hv treatments were considered as controls for antagonist and pathogen, respectively. Trifloxystrobin + tebuconazole was used as the fungicide treatment. Coffee plants sprayed with sterile distilled water (Control). Treatments means with the same letters are not significantly different (p ≤ 0.05) as determined by Tukey’s test. Bars represent the standard deviations of the means. n = 5.
Studies have demonstrated that CLR causes reduction in the photosynthetic capacity of coffee plants and premature defoliation of trees with direct damage to yield (Avelino et al., 2015; Honorato et al., 2015a, 2015b, 2015c). In the present study, a progressive increase in CLR led to detrimental losses in the photosynthetic capacity of the plants. However, our results revealed that coffee plants sprayed with Clonostachys hemileiae and inoculated with H. vastatrix showed no damage in the photochemical phase (analyzed as $F_v/F_m$) of photosynthesis as compared with plants not treated with C. hemileiae. Additionally, $\Phi_{PSII}$ and ETR values suggested that there was a preservation of the functional integrity of the photosynthetic machinery in the plants sprayed with C. hemileiae and infected by the rust. In this context, the use of C. hemileiae did not alter the values of $A$, $g_a$, $C_i$, and $E$ compared to control plants. This is in sharp contrast to the treatment where the fungicide mixture (chosen as representing commercial products commonly used in coffee plantations for CLR control) was applied. The fungicide treatment was the most effective at reducing CLR.

Nevertheless, the photosynthetic performance of fungicide-treated coffee plants was negatively affected. Studies with pecan-nut plants showed that the application of fungicides containing strobilurins significantly reduced the leaf gas exchange parameters, especially $A$ (Wood and Bock, 2017). Nevertheless, Honorato et al. (2015a, 2015b) concluded that the use of the fungicides triazole and strobilurin did not cause negative physiological alterations in coffee plants. This remains an important issue that needs to be urgently addressed since this fungicide combination has become important for the management of fungal crop diseases. Their use may have a “hidden cost” on plant metabolism, which may not be limited to coffee and pecan-nuts. The maintenance of the photosynthetic process on coffee plants sprayed with C. hemileiae may be attributed to lower CLR severity. Such an effect from antagonistic fungi has been demonstrated previously. In rice, for example, several isolates of Trichoderma asperellum were able to reduce the size of leaf scald lesions and the area under the disease progress curve, minimizing the harmful effects of this disease on $A$, $g_a$, $C_i$, and $E$, as well as on Chl a fluorescence parameters and in the antioxidative metabolism (Bueno et al., 2017). Resende et al. (2015) have also shown that, besides ensuring a reduction in the anthracnose levels in sorghum plants, the application of C. inaequalis did not impair the photosynthetic capacity of the infected plants.

**Conclusions**

Applying C. hemileiae in advance of the rust promoted host defense responses in the leaves of coffee plants as revealed by high CHI, APX, and POX activities, resulting in reduced CLR severity. CLR control, similar to those of chemical control, was achieved but without any evidence of damage to the photosynthetic capacity of coffee plants. Interestingly, the best CLR control occurred when C. hemileiae was sprayed before rust inoculation (72 hr), for both in vitro and in planta experiments, indicating that C. hemileiae was able to protect the coffee plants against CLR. It may be acting on two fronts, both as a mycoparasite of the rust and by inducing host resistance – as the evidence presented here indicates—and which has been demonstrated for other fungal taxa used in disease biocontrol, such as Trichoderma and Clonostachys (Qualhato et al., 2013; Nygren et al., 2018).
The induction of protection of plants as promoted by biological control agents or their metabolites, is a non-specific form of disease resistance that involves the synthesis and accumulation of several antimicrobial compounds—such as CHI, GLU, callose deposition, and the production of phenolics and phytoalexins—added to a high antioxidant activity triggered through a combination of different modes of host defense against pathogens (Cohen et al., 1993; Kohl et al., 2019). Carrión and Rico-Gray (2002) claimed that the six mycoparasitic fungi found colonizing pustules of *H. vastatrix* in Mexico were capable of destroying the reproductive structures of the rust and conjectured that these might have an impact on rust populations by decreasing the inoculum potential. Unfortunately, their study did not progress toward a practical biocontrol solution, such as the development of a biofungicide (Loguercio et al., 2009). At present, there are no such products designed explicitly for CLR control. Further studies are underway to determine if *Calonectria hemileiae* can fit the bill and become a biocontrol tool for use against *Hemileia vastatrix*.

Limitations of the study

This study identifies *C. hemileiae* as a promising biocontrol agent of CLR based on laboratory and greenhouse screening. There are other hurdles to overcome before its actual potential can be fully realized. Most notably is the need for a pest risk assessment, especially since the fungus belongs to a genus containing important plant pathogens. Our results, thus far, have shown that *C. hemileiae* is non-pathogenic to coffee but wider host-range screening is needed to establish whether or not it poses a risk. If *C. hemileiae* is confirmed to be ecologically restricted to a mycoparasitic lifestyle, then the next phase will be to test its efficacy in the field.

Resource availability

**Lead contact**
Further information and requests for resources and materials should be directed to and will be fulfilled by the lead contact, Prof. Dr. Robert W, Barreto (rbarreto@ufv.br)

**Materials availability**
This study did not generate new unique reagents.

**Data and code availability**
Source data for the figures published in this the paper are available per request.

**METHODS**

All methods can be found in the accompanying transparent methods supplemental file.

**SUPPLEMENTAL INFORMATION**
Supplemental information can be found online at https://doi.org/10.1016/j.isci.2021.102352.

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**AUTHOR CONTRIBUTIONS**
S.S.-S., C.E.A.-P., F.A.R., and R.W.B designed the experiments. S.S.-S., A.A.C., A.L.S., and P.S.C.M. isolated, multiplied, and inoculated the plants with *C. hemileiae* and *H. vastatrix*. S.S.-S., C.E.A.-P., A.A.D., A.L.S., and P.S.C.M. evaluated the in vitro and in plant experiments. S.S.-S., C.E.A.-P., A.L.S., and P.S.C.M. performed the leaf gas exchange and chlorophyll a fluorescence measurements. S.S.-S., P.R.S., and P.S.C.M. performed the biochemical analysis. S.S.-S and A.A.D. collected and prepared the leaf samples for examination in the scanning electron microscope. S.S.-S., C.E.A.-P., F.A.R., H.C.E., and R.W.B.
analyzed the data of the experiments, drafted the manuscript, and prepared its final version for publication. All authors interpreted the results and approved the final version of the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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Supplemental information

Elucidating the interactions between the rust *Hemileia vastatrix* and a *Calonectria* mycoparasite and the coffee plant

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Supplemental Information

A fungus-eat-fungus world – *Calonectria hemileiae* vs. *Hemileia vastatrix*: physiological responses of coffee plants, mycoparasitism and biocontrol

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Transparent Methods

Materials and Methods

**Inoculum of *Calonectria hemileiae***

An isolate of *C. hemileiae*, stored in the culture collection at the “Universidade Federal de Viçosa (UFV) – Coleção Octávio Almeida Drummond” (Acc. No COAD 2544) was grown on potato-dextrose-agar (PDA) plates for 7 days and incubated at 22 ±2°C under a 12 h daily light regime (light provided by two daylight fluorescent lamps and one near-UV lamp placed 35 cm above the plates). Subsequently, the surface of colonies was gently scraped with a soft brush and left in the same conditions for an additional 2 days to induce sporulation. After this period, sporulation was abundant. A concentrated conidial suspension was prepared by flooding each plate with 10 mL of a 0.05% Tween 20 solution and scraping the surface of colonies with a rubber spatula. The concentration of the conidial suspension was adjusted to $1 \times 10^5$ conidia mL⁻¹ with the help of a hemocytometer to be used in all experiments.
Inoculum of *Hemileia vastatrix*

Seven-month-old healthy coffee plants (cv. “Caturra”) grown in 3 L plastic pots containing pasteurized soil, manure and sand (1.5:1:0.5) were selected and placed in a growth chamber at a relative humidity of 85 ± 5%, 22°C and a 12 h light regime (light provided by white fluorescent lamps) for 15 days before inoculation with *H. vastatrix*. An original stock of the rust (race II) maintained by the team of the BIOCAFE Laboratory (“Laboratório de Biotecnologia do Cafearo – UFV”) was used for inoculating healthy coffee plants (Cabral et al., 2016), and the protocol described in this publication was followed to mass-produce rust urediniospores for later use. Expanded leaves of all 40 plants in the batch were inoculated with *H. vastatrix* by spraying a suspension of urediniospores (1 × 10^5 mL^-1) containing 0.05% Tween 20. A suspension prepared, as described, was used in all experiments. An atomizer (Paasche Airbrush Co., Chicago) was used for spraying coffee leaves of test plants until runoff. After inoculation, plants were kept in a dew chamber in the dark at 22°C for 48 h. Subsequently, the plants were left in the growth chamber bench under the same conditions mentioned above. After 30-45 days, when sporulation was intense, urediniospores were collected by carefully scraping the surface of the leaves with gelatin capsules and then transferred to 1.5 mL plastic microtubes. Half of these microtubes containing the urediniospores were kept in a desiccator at -4°C for short-term storage until the first experiment. The other half was placed in a desiccator and submitted to vacuum and left drying for 2 h and immediately transferred to a deep freezer at -80°C, as a backup, for up to 90 days. After 90 days, a rapid drop in urediniospore viability was found to occur, and new batches of urediniospores were prepared as described above.

**Plant material and growth conditions**
Coffee plants (cv. “Catuai-Vermelho IAC 144”) were grown under the same conditions described above for “Caturra”. Plants were irrigated regularly and fertilized every 15 days, until the end of the experiment, with 25 mL of a nutrient solution (Novais et al., 1991). Then, 15 days before the experiment started, the plants to be used were transferred to a controlled temperature room at 26 ± 2°C, 75 ± 5% relative humidity, and under a 12 h light regime (light provided by white fluorescent lamps yielding a photon flux density of approximately 350 μmolm⁻² s⁻¹ at plant canopy height).

*Calonectria hemileiae* × germination of *H. vastatrix* urediniospores

Two groups of new microscope slides, cleaned with 70% ethanol, (4 each) were placed in plastic boxes (11 × 11 × 3.5 cm) containing a layer of foam saturated with sterile distilled water. One 15 μL drop of a $1 \times 10^5$ mL⁻¹ suspension of urediniospores containing 0.05% Tween 20 was placed centrally on 4 slides used as controls. The other group of 4 slides received a drop of 15 μL of urediniospore suspension centrally and a drop of 15 μL of *C. hemileiae* (COAD 2544) suspension ($1 \times 10^5$ conidia mL⁻¹ and 0.05% of Tween 20), which was placed at the same point and mixed with the tip of the micropipette. The two groups of plastic boxes with slides were kept in the dark for 6 h. After that period, to stop the germination, a 15 μL drop of lactofuscin was added to each droplet. The percentage of germinated urediniospores was determined by examination of 5 fields at 200 × magnification. The urediniospores were considered germinated when their germ tubes had the same length or were longer than the diameter of the urediniospores (Capucho et al., 2009).
Calonectria hemileiae \times \text{rust on coffee leaf discs in vitro}

Leaves (2\textsuperscript{nd} and 3\textsuperscript{rd} pair - from stem’s apex to bottom) were taken from healthy plants of the cultivar “Catuaí-Vermelho IAC 144”, grown in a greenhouse, and 2 cm diameter discs were removed from the lamina along the midrib with a cork punch and immediately used. Three transparent plastic boxes, previously disinfected with 70% ethanol, were lined with a sterile layer of foam saturated with sterile distilled water and covered with a sterile square plastic (PVC) grid. Twelve coffee leaf discs were evenly distributed over the grid with their abaxial surfaces facing up (Eskes, 1989). These leaf discs received a 25 μL drop of urediniospore suspension and/or were also treated with a 25 μL drop of COAD 2544 conidial suspension placed centrally in one of the following treatments: 1) COAD 2544 conidial suspension application 72 h before inoculation with the rust; 2) COAD 2544 conidial suspension deposited immediately after inoculation with the rust; 3) fungicide treatment against \textit{H. vastatrix} on inoculated leaf discs, four days after inoculation (dai) [fungicide – tebuconazole (200 g. i. L\textsuperscript{-1}) and trifloxystrobin (100 g a.i. L\textsuperscript{-1})], and 4) Rust urediniospore suspension only (control). Immediately after preparation of each box, they were wrapped in a PVC plastic film, and the boxes with the urediniospore suspensions were placed in a controlled temperature room (CTR) at 22 ± 2ºC in the dark for 24 h. After that period, boxes were maintained in the CTR but under a 12 h daily light regime, 350 μmol photons m\textsuperscript{-2} s\textsuperscript{-1} (light provided by cool white fluorescent tubes).

After 35 days of incubation under such conditions, each box was taken from the CTR and unwrapped to evaluate the CLR severity using a scale from 1 to 5 based on the percentage of leaf area containing pustules as follow: 1 = 0%; 2 = 1-25%; 3 = 26-50%; 4 = 51-75%; and 5 > 75% of leaf area (Eskes, 1989). Rust disease index (RDI) was calculated according to the following equation:
\[ \text{RDI} (\%) = \left[ \sum (r \times a) / (R \times A) \right] \times 100 \]

Where \( r \) is the rating value, \( a \) is the number of infected leaves with a rating of \( r \), \( R \) is the maxim rating value, and \( A \) is the total number of leaves used.

**Calonectria hemileiae × rust on coffee plants under controlled conditions**

Thirty healthy, six-month-old coffee plants (cultivar “Catuaf-Vermelho IAC 144”), cultivated as previously described, were used in this experiment. The plants were placed in a growth chamber at 85 ± 5% relative humidity, 22°C, and under a 12 h photoperiod under fluorescent white light (yielding a photon flux density of approximately 350 \( \mu \)molm\(^{-2}\)s\(^{-1}\) at plant canopy height) for 15 days, before inoculation with *H. vastatrix*, *C. hemileiae* conidia and rust urediniospore suspensions, prepared as described before, were sprayed separately on the abaxial side of the 2\(^{\text{nd}}\), 3\(^{\text{rd}}\), and 4\(^{\text{th}}\) leaf-pairs (from the stem’s apex to the bottom) following the procedure previously described. This experiment had the following treatments: 1) plants inoculated with the rust only (control); 2) plants treated with *C. hemileiae* 72 h before inoculation with the rust (*Ch/72Hv* treatment); 3) plants treated with *C. hemileiae* and immediately afterward inoculated with the rust (*Ch/Hv* treatment); 4) fungicide treatment against the rust on inoculated plants, four days after inoculation (dai) [fungicide – tebuconazole (200 g. i. L\(^{-1}\)) and trifloxystrobin (100 g a.i. L\(^{-1}\))] applied with a hand sprayer at the recommended commercial dose of 1 L p. c ha\(^{-1}\) using the equivalent of an application volume at 500 L ha\(^{-1}\); 5) antagonist only – healthy plants not exposed or inoculated with the rust and sprayed with *C. hemileiae* alone (*C. hemileiae* treatment); and 6) absolute control – healthy plants sprayed with SDW only. All plants were taken to the CTR after being treated and kept there until the end of the experiment under growth conditions as described above. Plants were irrigated four times a week and examined daily for symptom emergence and sporulation for 50 days.
At 50 dai, the first pair of expanded leaves of each plant for treatment was collected and scanned (HP SCANJET G2410 at 300 dpi resolution) to obtain the images. Images were processed with QUANT software (Vale et al., 2003) to obtain the values of coffee rust severity (CRS). Additionally, the plants in each of the treatments were photographed with a digital camera (Sony Cyber-Shot DSC-TX1) to illustrate the effect of each treatment. For greater detail of the spatial-temporal development of the CRS during the next 20 dai, the first pair of expanded leaves of each plant/treatment was marked and followed for CRS determination. A diagrammatic scale (Capucho et al., 2011) was utilized. The same pair of expanded leaves in each treatment was evaluated for its CRS at 28, 36, 42, and 50 dai.

Evidence of mycoparasitism

Five plants with pustules on their leaves that remained from the experiment described above (treatment 1) were used. These plants were individually sprayed with a conidial suspension of *C. hemileiae* (suspension obtained by following the steps described in the previous experiment and having the same concentration) until runoff. Immediately afterward, these were left in a CTR under the same conditions described for earlier experiments. The coffee plants were kept under growth conditions at 22 ± 2°C, 70% relative humidity, and 12 h of light regime 350 µmol photons m⁻² s⁻¹ (light provided by cool white fluorescent tubes) for 20 days. After this 20 day-period, two leaves were collected from each plant. Each leaf was scanned under a dissecting microscope (Olympus SZX7), and rust pustules were examined for the presence of mycoparasite colonies. Whenever mycoparasitized pustules were observed, microscopic mounts were prepared by scraping the fungal structures in the colonies for observation. Typical *C. hemileiae* colonies were observed growing over the rust pustules (10 coffee leaves).
Conidia of *C. hemileiae* were collected from colonies formed on pustules, as previously described, and transferred to PDA plates with a sterile fine pointed needle. All colonies obtained on plates had the colony morphology of COAD 2544. Slides were mounted with leaf pieces, containing fungal sporulation taken from such colonies, in lactofuscin and, following observation under a light microscope Olympus BX 51, it was confirmed that COAD 2544 was the only fungus recovered from parasitized CLR pustules.

To document the colonization of the rust by the mycoparasite, selected pieces of herbarium samples of the holotype (VIC 2544) bearing pustules colonized by *C. hemileiae* were obtained. The pieces were further dried by mounting them on stubs with double-sided adhesive tape and leaving them overnight in a desiccator. These specimens were gold-coated using a Balzer’s FDU 010 sputter coater. A Carl-Zeiss Model LEO VP 1430 scanning electron microscope (SEM) was used, operating at 10 Kv with a working distance ranging from 10 to 30 mm to analyse the specimens and generate representative electromicrographs of the colonization event.

*Calonectria hemileiae* and fungicide mixture × *H. vastatrix*; effects on enzymatic activity in coffee leaves

A study of the biochemical responses to *Ch* and fungicide treatment on *H. vastatrix* was conducted, coupled with the experiment described above. Samples consisted of the first pair of expanded leaves of each repetition of each of the treatments. The samples were taken between 12:30 and 13:30 h to standardize the highest metabolic activity of the leaves. All samples were flash-frozen by immersing in liquid nitrogen, packed in aluminum foil bags, and then stored in an ultra-freezer at -80°C and kept under these conditions for later processing. To determine the activities of ascorbate peroxidase (APX, EC 1.11.1.11), chitinase (CHI, EC 3.2.1.14), β-1,3-glucanase (GLU, EC 3.2.1.39),
peroxidase (POX, EC 1.11.1.7), and superoxide dismutase (SOD, EC 1.11.1.6), a total of 0.3 g of leaf tissue (obtained from two leaves collected from the first pair of expanded leaves per replication of each treatment) was ground into a fine powder in a mortar and pestle with liquid nitrogen. The fine powder was homogenized in an ice bath in 2 mL of a solution containing 100 Mm potassium phosphate buffer (pH 6.8), 0.1mM ethylenediaminetetraacetic acid (EDTA), 1mM phenylmethylsulfonyl fluoride (PMSF), 1% polyvinyl-pyrrolidone (PVP) (w v⁻¹) and 4% (w v⁻¹) polyvinylpolypyrrolidone (PVPP). The homogenate was centrifuged at 15.000 g for 25 min at 4°C, and the supernatant was used as crude enzyme extract (Honorato et al., 2015a). The reaction was performed twice for each enzyme. The concentration of total soluble protein in the extracts was measured using bovine serum albumin as the standard protein (Bradford, 1976).

The APX activity was determined (Nakano and Asada, 1981). The reaction consisted of a mixture of 50 mM potassium phosphate buffer (pH 6.8), 1 mM H₂O₂, and 0.8 mM ascorbate in a volume of 265 μL. The reaction was started after the addition of 5 μL of the crude enzyme extract. The APX activity was measured by the rate of ascorbate oxidation at 290 nm for 6 min at 25°C. An extinction coefficient of 2.8 mM⁻¹ cm⁻¹ was used to calculate the APX activity, which was expressed as μmol min⁻¹ mg⁻¹ of protein (Nakano and Asada, 1981).

The CHI activity was determined as in (Roberts and Selitreinikoff, 1988) later modified (Harman et al., 1993), which was used as a substrate p-nitrophenyl-β-D-N-N-diacetilquitobiose (PNP) (Sigma-Aldrich, São Paulo). The reaction medium (250 μL) consisted of a mixture of 50 mM sodium acetate buffer (pH 5.0), 0.1 mM PNP, and the crude enzyme extract (5 μL). Subsequently, the reaction was incubated at 37°C for 2 h, and the reaction was stopped by adding 125 μL of 0.2 M sodium carbonate. The control
samples received 125 µL of 0.2 M sodium carbonate immediately after the addition of the crude enzyme extract to the reaction mixture. The final product released by CHI was measured at 410 nm, and the extinction coefficient of 70 mM⁻¹ cm⁻¹. The CHI activity was expressed as µmol min⁻¹ mg⁻¹ of protein.

The GLU activity was also determined (Lever, 1972). 5 µL of crude enzyme extract was added to a reaction mixture containing 50 mM sodium acetate buffer (pH 5.0) and laminarin (1 mg mL⁻¹). The reaction medium was incubated at 45°C for 1 h. Subsequently, 50 µL of this mixture was added to a reaction mixture of dinitrosalicylic acid (DNS). This reaction mixture was then incubated for 15 min at 100°C and then cooled in an ice bath until it reached 25°C. The absorbance was measured at 540 nm. A similar procedure was used for the control samples except that the first incubation was excluded. The GLU activity was expressed as µmol min⁻¹ mg⁻¹ of protein.

The POX activity was assayed following the colorimetric determination of pyrogallol oxidation (Kar and Mishra, 1976). The reaction mixture contained 50 mM potassium phosphate (pH 6.8), 20 mM pyrogallol, and 20 mM H₂O₂ in a volume of 250 µL. The reaction was started after the addition of 10 µL of the crude enzyme extract, and the POX activity was determined through the absorbance of colored purpurogallin recorded at 420 nm for 6 min at 25°C. The extinction coefficient of 2.47 mM⁻¹ cm⁻¹ was used to calculate POX activity, which was expressed as mmol of purpurogallin produced min⁻¹ mg⁻¹ of protein (Change and Maehley, 1955).

The SOD activity was determined by measuring its ability to photochemically reduce the p-nitrotetrazole blue (NTB) (Del Longo et al., 1993). The reaction was started after the addition of 10 µL of the crude enzyme extract to 250 µL of a mixture containing 100 mM potassium phosphate buffer (pH 7.8), 13 mM methionine, 75 µM NTB, 0.1 mM EDTA, and 2 µM riboflavin. The reaction occurred at 25°C under a 15 W lamp. After 10
min of light exposure, the light was turned off, and the production of formazan blue, which resulted from the photoreduction of NTB, was monitored by the increase in absorbance at 560 nm in a spectrophotometer (Giannopolitis and Reis, 1977). The reaction mixture for the control samples was kept in darkness. The values obtained were subtracted from the values obtained from the samples of the replications of each treatment exposed to light. One unit of SOD was defined as the amount of enzyme necessary to inhibit NBT photoreduction by 50% (Beauchamp and Fridovich, 1971).

**Calonectria hemileiae and fungicide mixture × H. vastatrix; effects on photosynthetic parameters**

The net CO₂ assimilation rate (A), stomatal conductance to water vapor (gₛ), intercellular CO₂ concentration (Cᵢ), and transpiration rate (E) were determined using a portable open-flow infrared gas exchange analyzer (IRGA) systems (LI-6400XT; Li-Cor Inc., Lincoln, NE). All the determinations were performed targeting the first pair of expanded leaves at 20, 28, 36, 42, and 50 dai from 8:00 to 12:00 h (solar time), which is when A is at its maximum. Data was recorded for each leaf while submitted to 5 min of saturate condition of photosynthetic active radiation (PAR = 1000 µmol photons m⁻² s⁻¹) at a CO₂ concentration of 400 µmol CO₂ mol⁻¹ air and at 25°C and a vapor pressure deficit of approximately 1.0 kPa.

The IRGA coupled with chlorophyll (Chl) a fluorescence chamber was utilized on leaves adapted to the dark after a period of 60 min to calculate the initial fluorescence (F₀) through a weak and rapid light pulse (0.03 µmol m⁻² s⁻¹). Immediately, a white light pulse of 8,000 µmol photons m⁻² s⁻¹ was applied for 0.8 s to ensure maximum fluorescence emissions (Fₘₗ), from which the variable-to-maximum Chl fluorescence ratio, \( F_v/F_m = [(F_m - F_0)/F_m] \), was calculated (Maxwell and Johnson, 2000). The steady-state
fluorescence yield ($F_s$) was measured on illuminated leaves, following a saturating white light pulse (8,000 µmol m$^{-2}$ s$^{-1}$, 0.8 s) that was applied to achieve the light-adapted maximum fluorescence ($F_{m'}$) and finally, the far-red illumination was applied (2 µmol m$^{-2}$ s$^{-1}$) in order to measure the light-adapted initial fluorescence ($F_0'$). Other indicators were calculated using these parameters, namely: i) the capture efficiency of the excitation energy by the open PSII reaction centers ($F_{v'}/F_{m'}$), ii) the coefficient for photochemical quenching ($q_P$), iii) the non-photochemical quenching (NPQ), iv) the actual quantum yield of PSII electron transport ($\Phi_{PSII}$), and v) the electron transport rate (ETR) as proposed by Maxwell and Johnson (2000).

The imaging of the fluorescence parameters were determined by using an Imaging-PAM M-Series chlorophyll fluorometer and the software Imaging WIN version 2.32 (Heinz Walz GmbH, Effeltrich, Germany) by following the methodology proposed by Honorato et al., (2015a). The plants of all treatments were dark-adapted for 60 min. Subsequently, leaves were exposed to a light pulse with an intensity of 0.5 µmol m$^{-2}$ s$^{-1}$ (1 Hz), establishing the minimum fluorescence image ($F_0$). Next, a saturating pulse of blue light (470 nm) with an intensity of 2,400 µmol m$^{-2}$ s$^{-1}$ (10 Hz) was delivered for 0.8 s to obtain the maximum fluorescence image ($F_m$). The software was used to perform the calculation and imaging of the fluorescence quantum efficiency ($F_{v'}/F_{m'} = (F_m - F_0)/F_m$) (Baker, 2008).

**Experimental design and statistical analysis**

Six experiments were carried out with different goals. The *in vitro* and *in plant* experiments aimed to determine the biocontrol potential of *C. hemileiae* against *H. vastatrix* aiming to reduce CLR. These experiments were arranged in completely
randomized designs with four treatments (\textit{Hv, Ch/72Hv, Ch/Hv}, and \textit{Fungicide}) with three and five replications, respectively, for the \textit{in vitro} and \textit{in planta} experiments.

For the biochemical analysis associated with the defense and antioxidative systems in healthy coffee plants (control) were compared with plants from the treated as listed in \textit{Calonectria hemileiae} and fungicide mixture \textit{× H. vastatrix}; effects on enzymatic activity in coffee leaves section. The experiment was arranged in a completely randomized design with five replications.

In the \textit{Calonectria hemileiae} and fungicide mixture \textit{× H. vastatrix}; effects on photosynthetic parameters section, the effect of treating coffee plants with \textit{C. hemileiae} on the parameters of leaf gas exchange and Chl \textit{a} fluorescence was compared to the other treatments. This experiment was arranged in a completely randomized design with six treatments (\textit{Hv, Ch, Ch/72Hv, Ch/Hv, fungicide and control}) and five replications.

The $F_{\text{max}}$ test was applied for data obtained for the \textit{in vitro} and \textit{in planta} experiments to determine the degree of variance homogeneity between the repetitions of each of the experiments and separately analyzed by ANOVA and means from the treatments were compared with Dunnet’ tests ($p \leq 0.05$). Data from all variables and parameters obtained from sections 2.8 and 2.9 were analyzed by ANOVA, and means from the treatments were compared with Tukey’ test ($p \leq 0.05$). All data were processed using SAS (version 6.12; SAS Institute, Inc., Cary, NC).

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