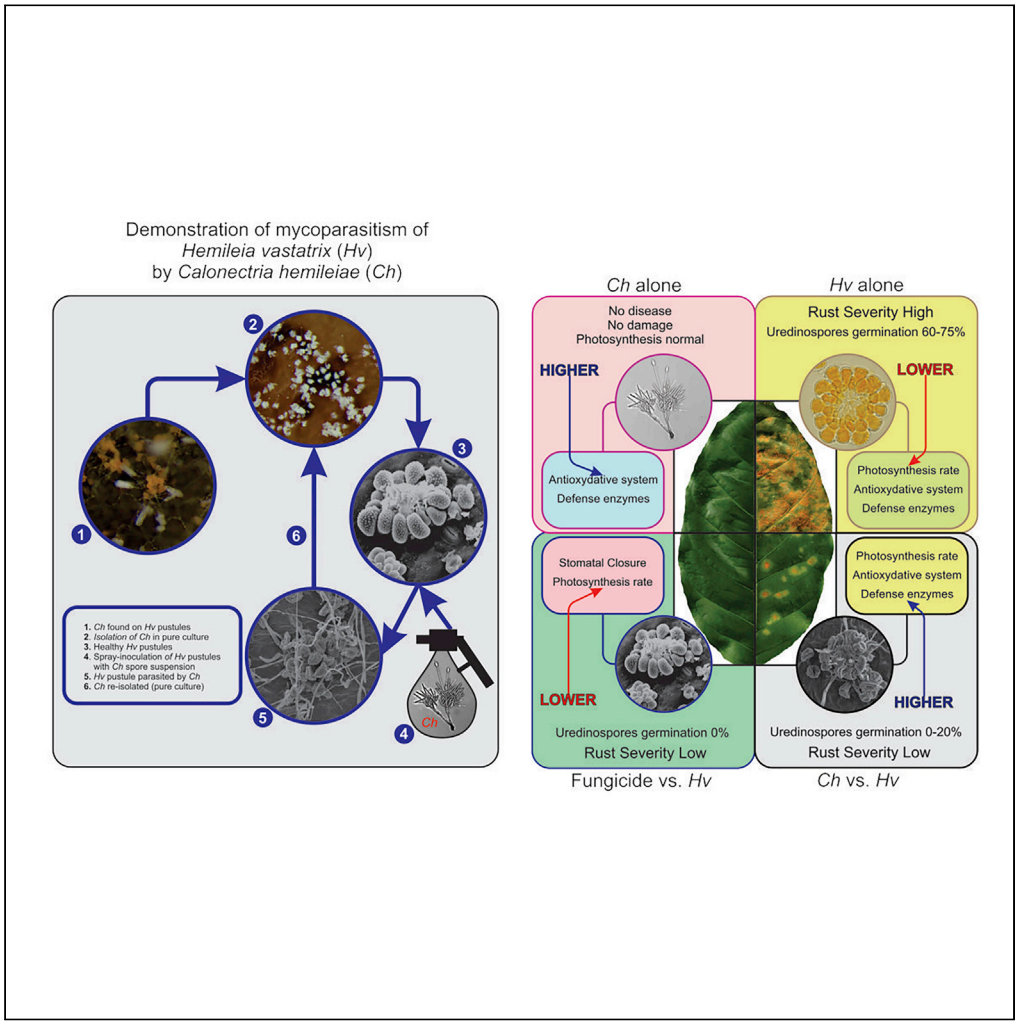
iScience

CellPress
OPEN ACCESS

Article

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



Sara Salcedo-Sarmiento, Carlos E. Aucique-Pérez, Patrícia R. Silveira, ..., Fabrício Á. Rodrigues, Harry C. Evans, Robert W. Barreto

rbarreto@ufv.br

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

Salcedo-Sarmiento et al., iScience *24*, 102352
April 23, 2021
https://doi.org/10.1016/j.isci.2021.102352

iScience

CellPress
OPEN ACCESS

Article

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

Sara Salcedo-Sarmiento,[1] Carlos E. Aucique-Pérez,[1] Patrícia R. Silveira,[1] Adans A. Colmán,[1] André L. Silva,[1] Paloma S. Corrêa Mansur,[1] Fabrício Á. Rodrigues,[1] Harry C. Evans,[1,2] and Robert W. Barreto[1,3,*]

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* (, *Mikronegeriaceae*, *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, 2006). 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 may 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

[1]Departamento de Fitopatologia, Universidade Federal de Viçosa, Viçosa-MG, Brazil

[2]CAB International, UK Centre, Egham, Surrey, UK

[3]Lead contact

*Correspondence: rbarreto@ufv.br

https://doi.org/10.1016/j.isci.2021.102352

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 (Toniutti 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 moniliaceous 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 *Coffea* 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*/72*Hv*, *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

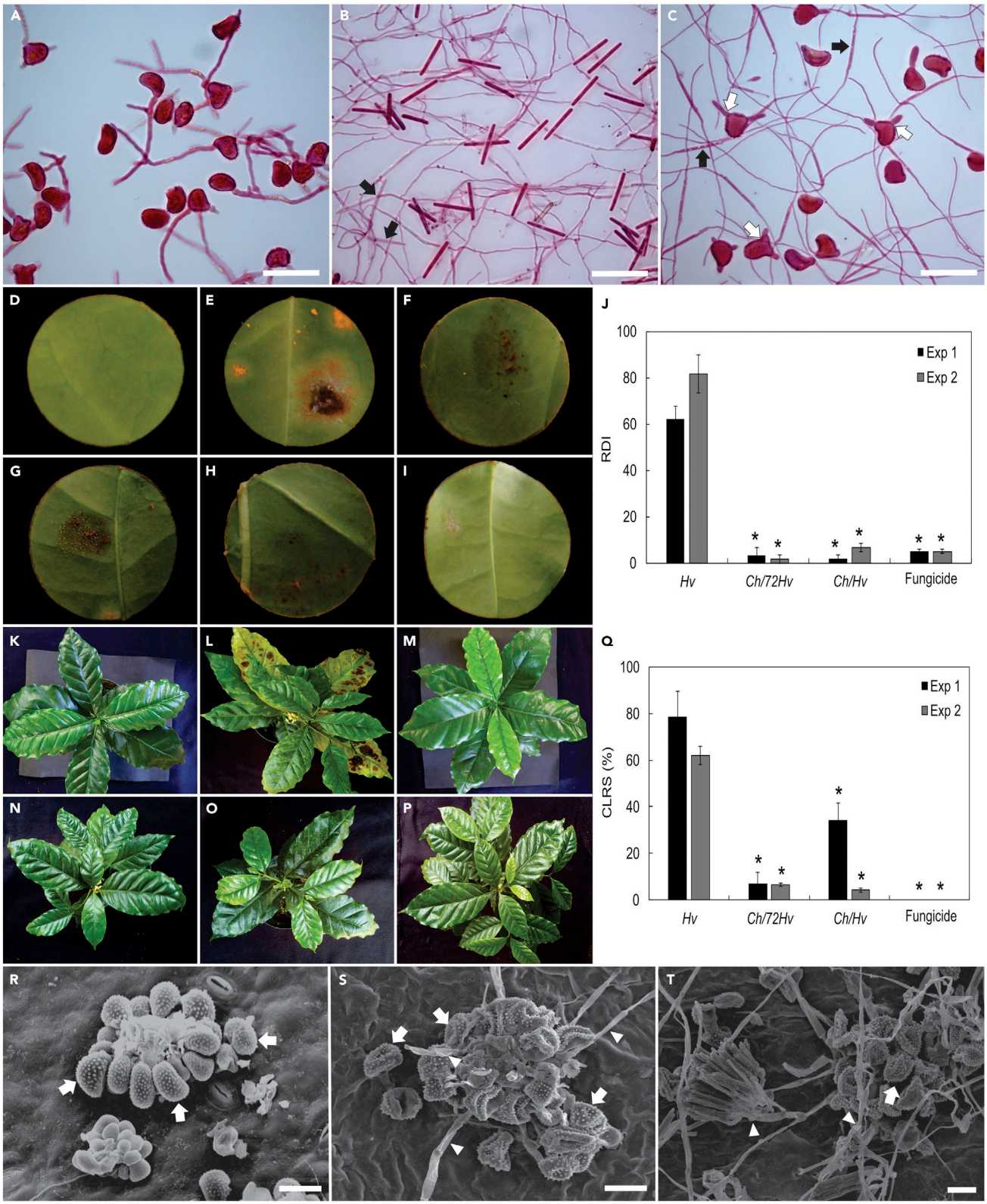

A
B
C
D
E
F
G
H
I
J
100
80
60
40
20
0
RDI
Exp 1
Exp 2
*
Hv
Ch/72Hv
Ch/Hv
Fungicide
K
L
M
N
O
P
Q
100
80
60
40
20
0
CLRS (%)
Exp 1
Exp 2
*
Hv
Ch/72Hv
Ch/Hv
Fungicide
R
S
T

Figure 1. *In vitro* and *in planta* evidence of *Calonectria hemileiae* antagonistic/biocontrol effect on *Hemileia vastatrix*.
Urediniospores 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 urediniospores of *H. vastatrix*—note germinating conidia of *C. hemileiae* (black arrows) and short and inflated germ tubes emerging from urediniospores (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 + tebuconazol) (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 ($p \leq 0.05$) 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 urediniospores (arrowed) on the leaf surface (R, bar 20 μm) and a pustule of *H. vastatrix* overgown by *C. hemileiae*, showing collapsing urediniospores (arrowed) (S and T, bar 20 μm) 20 days after spraying with a *C. hemileiae* conidial suspension.

Ch/72*Hv*, *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*/72*Hv* 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*/72*Hv* and *Ch*/*Hv* treatments) showed reduced CLR severity in comparison to inoculated plants that remained untreated with *Ch* (Figures 1N, 1O, and 1L). 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*/72*Hv*. When compared with the rust treatment, *Ch*/72*Hv* 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*/72*Hv* 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 urediniospores (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*/72*Hv* 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 dai, significant differences were detected in the CHI, GLU, APX, POX, and SOD activities (Table 1). At 72 hai,

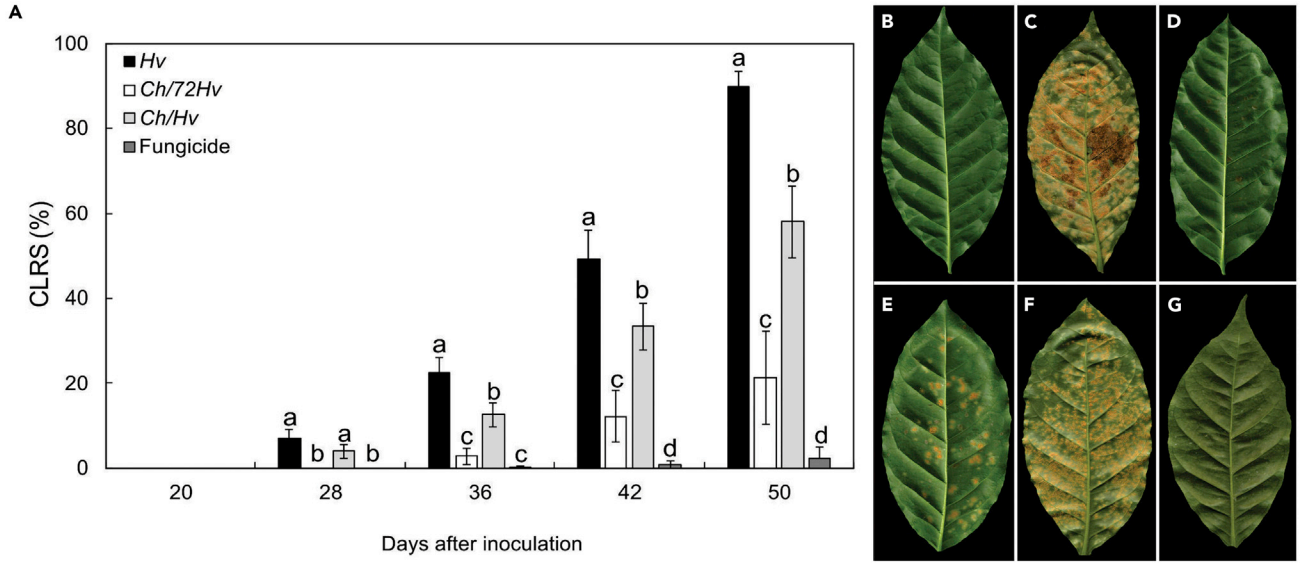


Figure 2. *Calonectria hemileiae* reduced coffee leaf rust severity (CLRS) on coffee plants inoculated with *Hemileia vastatrix*.

CLRS of coffee plants inoculated with *H. vastatrix* at 20, 28, 36, 42, and 50 days after inoculation. Treatments were, respectively: inoculation with *H. vastatrix* and spraying of plants with antagonist (*C. hemileiae*) 72 h before (*Ch*/72*Hv*) or simultaneously (*Ch*/*Hv*) with the inoculation with *H. vastatrix* (*Hv*) and treated with fungicide (A). Abaxial surface of leaves represnting: control (B), *Hv* (C), *Ch* only (D), *Ch*/72*Hv* (E), *Ch*/*Hv* (F), and fungicide treatment (trifloxystrobin + tebuconazol) (G). Pictures taken 50 days after inoculation with *H. vastatrix* took place. Treatment means followed by the same letter, for each sampling time, are not significantly different ($p \leq 0.05$) as determined by Tukey's test. Bars represent the standard deviation. $n = 5$.

CHI and SOD activities were greater for the *Ch*/72*Hv* 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*/72*Hv* 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*/72*Hv* 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*/72*Hv*, *Ch*/*Hv* and fungicide treatments, the values of F_v/F_m (≥ 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 (Φ_{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 Φ_{PSII} and ETR as compared with other treatments. In contrast, for these evaluation times, the *Ch*/72*Hv* and *Ch*/*Hv* treatments yielded Φ_{PSII} values that stayed at levels that were close to those of coffee plants sprayed only with SDW. At 50 dai, Φ_{PSII} and ETR showed significant reductions for *Ch*/72*Hv* 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

Table 1. Defense and antioxidative response in coffee leaves sprayed with *Calonectria hemileiae* and inoculated with *Hemileia vastatrix*

	72 hbi					72 hai					50 dai				
Treatments	CHI	GLU	APX	POX	SOD	CHI	GLU	APX	POX	SOD	CHI	GLU	APX	POX	SOD
Control	2.8b	3.1b	15.1a	0.10b	1.6a	5.2ab	5.7a	19.6a	0.2a	1.8ab	5.4ab	1.6b	26.8b	0.3ab	0.6b
Hv	–	–	–	–	–	5.0ab	4.7a	21.9a	0.2a	0.7b	7.3ab	5.0a	28.2b	0.2b	0.6b
Ch	12.7a	8.6a	22.2a	0.07b	1.5a	2.2c	1.4b	22.2a	0.2a	1.5ab	9.7a	2.3b	49.4a	0.4a	0.6b
Ch/72Hv	10.7a	5.4ab	29.1a	0.21a	0.8b	6.7a	2.6ab	24.4a	0.2a	2.1a	2.0b	0.9b	2.6c	0.2ab	1.4a
Ch/*Hv*	–	–	–	–	–	4.5abc	2.6ab	27.9a	0.3a	2.0a	5.1ab	2.3b	29.9b	0.2b	0.5b
Fungicide	–	–	–	–	–	2.5bc	2.7ab	24.1a	0.2a	1.6ab	4.1ab	0.4b	17.4bc	0.2ab	1.6a
F Values	26.8**	4.56*	2.43^{ns}	33.6**	15.4**	6.95*	4.52*	1.47^{ns}	1.53^{ns}	3.76*	3.66*	10.9**	17.1**	3.69*	8.90**

Activities of chitinase (CHI, μmol min^{-1} mg^{-1} protein), β-1,3-glucanase (GLU, μmol min^{-1} mg^{-1} protein), ascorbate peroxidase (APX, μmol min^{-1} mg^{-1} protein), peroxidase (POX, μmol min^{-1} mg^{-1} protein), and superoxide dismutase (SOD, unit min^{-1} mg^{-1} protein) in the leaves of coffee plants that were treated with *C. hemileiae* at 72 h before as well as at 72 h and 50 days after inoculation with *H. vastatrix* and coffee plants that were treated with fungicides (trifloxystrobin + tebuconazol). Plants sprayed with sterile water and *C. hemileiae* without inoculation with *H. vastatrix* served as the control treatments. Treatment means followed by the same letter, within each experiment, are not significantly different ($p \leq 0.05$) as determined by Tukey's test. *, ** = significant differences at 0.05 and 0.01, respectively. ^{ns} = not significant. $n = 5$.

up to 70% in the CO_2 fixation at 50 dai were observed, whereas the internal CO_2 concentration (C_i) showed significant increases after 42 dai (Figure 4C). For the *Ch*/72*Hv*, *Ch*/*Hv*, and for the fungicide treatments, the *A*, g_s, 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*, g_s, and *E* coupled with significant increases in C_i, as compared with *Ch*/72*Hv* and *Ch*/*Hv* treatments (Figure 5C). Despite the CLRS 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, 36 and 43% at 36 dai, 20 and 17% at 42 dai, and 48 and 49% at 50 dai. These reductions were in connection with the low g_s 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 the fluorescence and leaf gas exchange parameters evaluated, *C. hemileiae* did not interfere with the 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; Mejía, 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.

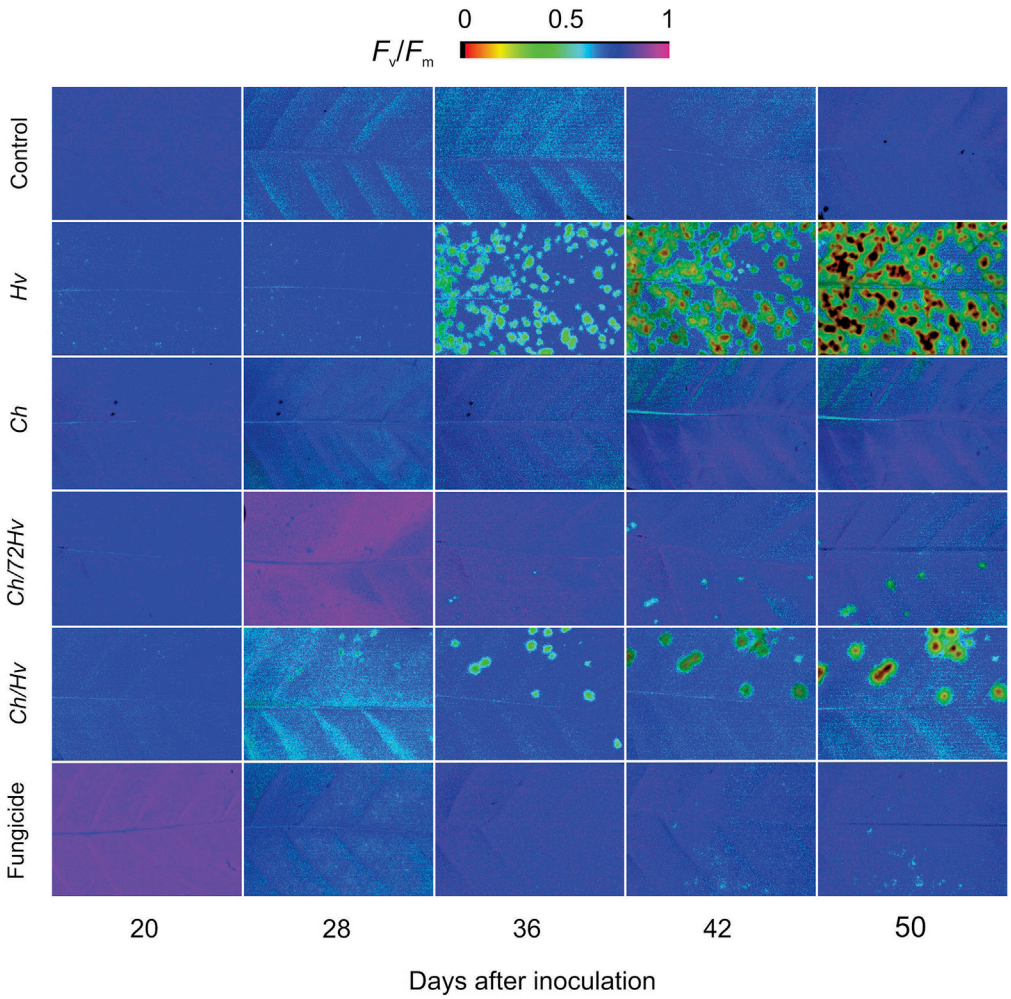


Figure 3. Photosythetic capacity of coffee plants sprayed with *Calonectria hemileiae* or fungicide mixture was not affected when plants were inoculated with *Hemileia vastatrix*.

Maximal photosystem II quantum yield (F_v/F_m) determined 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/72*Hv*) and simultaneously with *H. vastatrix* (*Ch*/*Hv*). The *Ch* and *Hv* treatments were considered as controls for antagonist and pathogen, respectively. Trifloxystrobin + tebuconazol was used as the fungicide treatment. Plants sprayed with sterile distilled water served as the control treatment. The color bar represents the scale from near zero (dark orange) to near one (dark violet).

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), C_i 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 g_s (stomatal closure) leading to loss of CO_2 influx to carboxylation sites in the chloroplasts (<*A*), as well as a low transpiration rate for the test plants. Debona

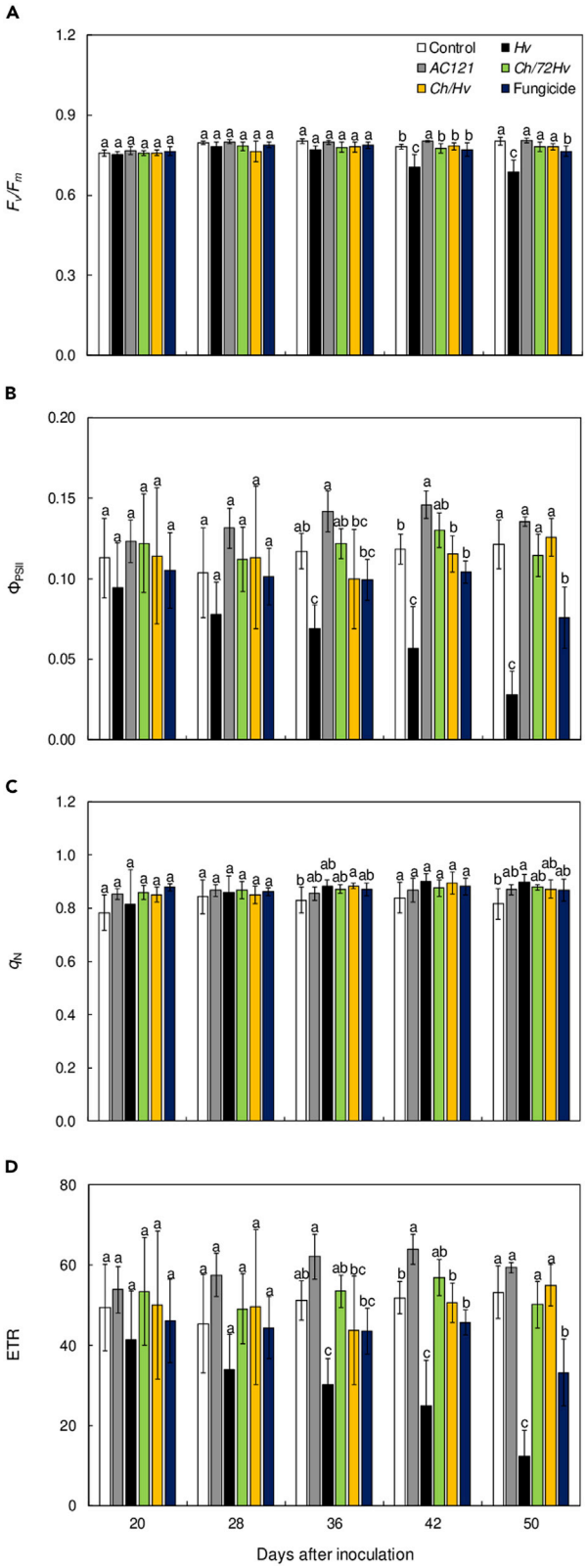

A
B
C
D
Control
Hv
AC121
Ch/72Hv
Ch/Hv
Fungicide
F_v/F_m
Φ_{PSII}
q_N
ETR
Days after inoculation
20
28
36
42
50

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_v/F_m) (A), photochemical yield of photosystem II (Φ_{PSII}) (B) quenching non-photochemical (q_N) (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*/72*Hv*) and simultaneously with *H. vastatrix* (*Ch/Hv*). The *Ch* and *Hv* treatments were considered as controls for antagonist and pathogen, respectively. Trifloxystrobin + tebuconazol was used as the fungicide treatment. Coffee plants sprayed with sterile distilled water (Control). Treatments means with the same letters are not significantly different ($p \leq 0.05$) as determined by Tukey's test. Bars represent the standard deviations of the means. $n = 5$.

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*, g_s, 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 (Roberti 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 $O_2^{\bullet-}$ metabolization 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 verticillioides* posited that induction of SOD by *F. verticillioides* 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. vastratrix* 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 H_2O_2 metabolism (Torres, 2010); APX functions at the level of chloroplasts, peroxisomes, and mitochondria using ascorbate as a specific electron donor to reduce H_2O_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

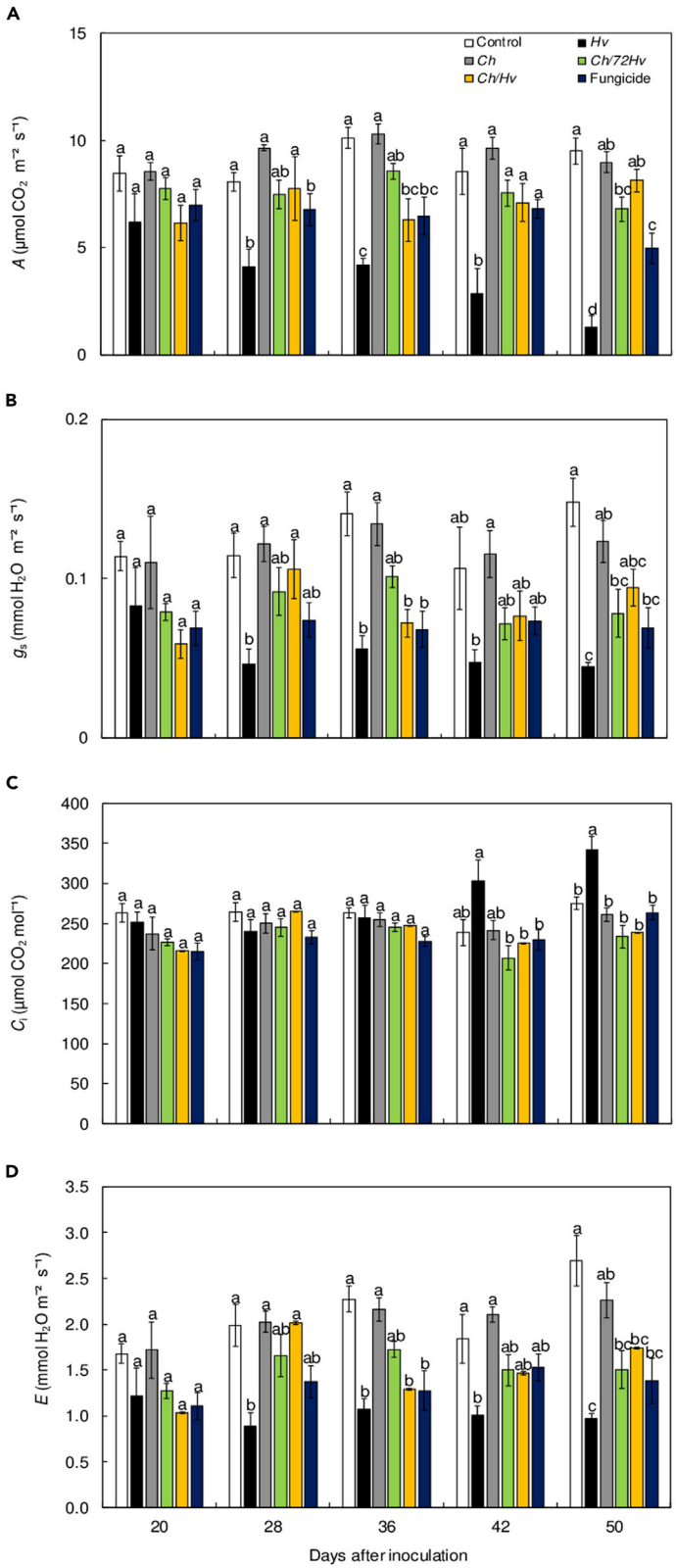

A
B
C
D
Control
Hv
Ch
Ch/72Hv
Ch/Hv
Fungicide
A (μmol CO2 m-2 s-1)
gs (mmol H2O m-2 s-1)
Ci (μmol CO2 mol-1)
E (mmol H2O m-2 s-1)
20
28
36
42
50
Days after inoculation

Figure 5. *Calonectria hemileiae* application did not harm the photosynthetic capacity of the coffee plants whereas spraying with fungicide mixture reduced the photosynthetic capacity.

Net CO_2 assimilation rate (*A*) (A), stomatal conductance to water vapor (g_s) (B), internal CO_2 concentration (C_i) (C), and transpiration rate (*E*) (D) determined on the 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 + tebuconazol was used as the fungicide treatment. Plants sprayed with sterile distilled water served as the control treatment. Treatments means with the same letters are not significantly different ($p \leq 0.05$) as determined by Tukey's test. Bars represent the standard deviations of the means. $n = 5$.

POX activities occurred for coffee plants sprayed with *C. hemileiae* alone at 50 dai, suggesting that the fungus can activate the antioxidant system, keeping it in a "state of alert" to the threat of rust colonization. It is important to note that no sign of damage or disease symptoms appeared as a result of *C. hemileiae* applications to the coffee plants. In the treatment tested with *C. hemileiae* combined with the rust, the APX and POX activities were at lower levels as compared with *C. hemileiae* application alone. The presence of the rust may alter the context of APX and POX activities creating ambiguous scenarios. Biochemical studies have shown that several oxidase enzymes are involved in resistance responses to CLR, constituting one of the components at play and limiting infection by *H. vastatrix* (Guerra-Guimarães et al., 2009, Guerra-Guimarães et al., 2013).

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). In the present study, a progressive increase in CLRS led to detrimental losses in the photosynthetic capacity of the plants. However, our results revealed that coffee plants sprayed with *C. 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, Φ_{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_s, 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_s, 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; Köhl 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.

ACKNOWLEDGMENTS

This work represents part of a research project submitted to the Departamento de Fitopatologia, Universidade Federal de Viçosa (UFV, MG, Brazil) by S.S.-S. as part of the requirement for a DSc in Phytopathology. This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior—Brasil (CAPES)—Finance Code 001. S.S.-S. was supported by the "Programa de Estudante-Convênio de Pós-Graduação" (PEC-PG) from CAPES. We thank Prof. Luiz Antônio Dias of the Departamento de Agronomia (UFV) for allowing us the use of scientific equipment. This study was supported by grants from World Coffee Research (WCR), the Coordenacão de Aperfeiçoamento do Pessoal de Nível Superior (CAPES), and the Conselho Nacional do Desenvolvimento Científico e Tecnológico (CNPq).

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. hemileia* 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.

Received: January 18, 2021
Revised: February 26, 2021
Accepted: March 19, 2021
Published: April 23, 2021

REFERENCES

Avelino, J., Cristancho, M., Georgiou, S., Imbach, P., Aguilar, L., Bornemann, G., Läderach, P., Anzueto, F., Hruska, A.J., and Morales, C. (2015). The coffee rust crises in Colombia and Central America (2008–2013): impacts, plausible causes and proposed solutions. Food Sec. *7*, 303–321.

Barreto, R.W., Colmán, A.A., and Evans, H.C. (2015). Fungal natural enemies of *Hemileia vastatrix* and their potential for use in classical biological control of coffee rust. In Congress International Botany 2015 - Edmonton, Alberta - July 25–29. http://2015.botanyconference.org/engine/search/index.php?func=detail&aid=1235.

Batista, K.D., Araujo, W.L., Antunes, W.C., Cavatte, P.C., Moraes, G.A.B.K., Martins, S.C.V., and DaMatta, F.M. (2012). Photosynthetic limitations in coffee plants are chiefly governed by diffusive factors. Trees *26*, 459–468.

Bhat, S.S., Naidu, R., Daivasikamani, S., and Nirmala, K. (2000). Integrated disease management in coffee. In IPM System in Agriculture – Cash Crops, R.K. Upadhyay, K.G. Mukerji, and O.P. Dubey, eds. (Aditya Books Private Limited), pp. 233–250.

Bettiol, W., Saito, M.L., and Brandão, M.S.B. (1994). Controle da ferrugem do cafeeiro com produtos à base de *Bacillus subtilis*. Summa Phytopathol. *20*, 119–122.

Bueno, A.C.S.O., Castro, G.L.S., Rêgo, M.C.F., Batista, T.F.V., Filippi, M.C.C., and da Silva, G.B. (2017). *Trichoderma* reduces scald and protects the photosynthetic apparatus in rice plants. Biocontrol Sci. Technol. *27*, 449–460.

Cabral, P.G.C., Maciel-Zambolim, E., Zambolim, L., Lelis, T.P., Capucho, A.S., and Caixeta, E.T. (2009). Identification of a new race of *Hemileia vastatrix* in Brazil. Australas. Plant Dis. Notes *4*, 129–130.

Capucho, A.S., Zambolim, L., Cabral, P.G.C., Maciel-Zambolim, E., and Caixeta, E.T. (2013a). Climate favorability to leaf rust in Conilon coffee. Australas. Plant Pathol. *24*, 511–514.

Capucho, A.S., Zambolim, L., and Milagres, N. (2013b). Chemical control of coffee leaf rust in *Coffea canephora* cv. Conilon. Australas. Plant Pathol. *42*, 667–673.

Carrión, G., and Rico-Gray, V. (2002). Mycoparasites on the coffee rust in Mexico. Fungal Divers. *11*, 49–60.

Cohen, Y., Gisi, U., and Niderman, T. (1993). Local and systemic protection against *Phytophthora infestans* induced in potato and tomato plants by jasmonic acid and jasmonic methyl ester. Phytopathology *3*, 1054–1062.

Costa, M.J.N., Zambolim, L., and Rodrigues, F.R. (2007). Avaliação de produtos alternativos no controle da ferrugem do cafeeiro. Fitopatol. Bras. *32*, 147–152.

Cristancho, A.M.A. (1995). Efecto protector de la bactéria *Bacillus thuringiensis* em plants de café contra el desarrollo de *Hemileia vastatrix*. Cenicafé *46*, 140–151.

Crous, P.W., Luangsa-Ard, J.J., Wingfield, M.J., Carnegie, A.J., Hernández-Restrepo, M., Lombard, L., Roux, J., Barreto, R.W., Baseia, I.G., Cano-Lira, J.F., et al. (2018). *Calonectria hemileiae*. Fungal Planet description sheets: 823. Persoonia *41*, 324–325.

Daivasikamani, S., and Rajanaika, A. (2009). Biological control of coffee leaf rust pathogen, *Hemileia vastatrix* Berkeley and Broome using *Bacillus subtilis* and *Pseudomonas fluorescens*. J. Biopest. *2*, 94–98.

Debona, D., Nascimento, K.J.T., Gomes, J.G.O., Aucique-Pérez, C.E., and Rodrigues, F.A. (2016). Physiological changes promoted by a strobilurin fungicide in the rice- *Bipolaris oryzae* interaction. Pest Bioch. Physiol. *130*, 8–16.

Ebrahim, S., Usha, K., and Singh, B. (2011). Pathogenesis related (PR) proteins in plant defense mechanism. Sci. Against Microb. Pathol. *2*, 1043–1054.

Fester, T., and Hause, G. (2005). Accumulation of reactive oxygen species in arbuscular mycorrhizal roots. Mycorrhiza *15*, 373–379.

Garrett, K.A., Dendy, S.P., Frank, E.E., Rouse, M.N., and Travers, S.E. (2006). Climate Change effects on plant disease: Genomes to ecosystems. Annu. Rev. Phytopath. *44*, 489–509.

Glare, T., Caradus, J., Gelernter, W., Jackson, T., Keyhani, N., Köhl, J., Marrone, P., Morin, L., and Stewart, A. (2012). Have biopesticides come of age? Trends Biotechnol. *30*, 250–258.

Godoy, C.V. (2012). Risk and management of fungicide resistance in crop in the Asian soybean rust fungus. In Fungicide Resistance in the Crop Protection: Risk and Management, T.S. Thind, ed. (CABI Publishing), pp. 87–95.

Gopi, R., Sridharan, R., Somasundaram, R., Lakshmanan, G.M.A., and Panneerselvam, R. (2005). Growth and photosynthetic characteristics as affected by triazoles in *Amorphophallus campanulatus* Blume. Gen. App. Plant Physiol. *31*, 171–180.

Guerra-Guimarães, L., Cardoso, S., Martins, I., Loureiro, A., Bernardes, A.S., Varzea, V., and Silva, M.C. (2009). Differential induction of superoxide dismutase in *Coffea arabica–Hemileia vastatrix* interactions. In Proceedings of the 22nd International Conference on Coffee Science (ASIC), pp. 1036–1039.

Guerra-Guimarães, L., Tenente, R., Pinheiro, C., Chaves, I., Silva, MdoC., Cardoso, F.M.H., Planchon, S., Barros, D.R., Renaut, J., and Ricardo, C.P. (2015). Proteomic analysis of apoplastic fluid of *Coffea arabica* leaves highlights novel biomarkers for resistance against *Hemileia vastatrix*. Front. Plant Sci. *6*, 478.

Guerra-Guimarães, L., Vieira, A., Chaves, I., Queiroz, V., Pinheiro, C., and Renaut, J. (2013). Integrated cytological and proteomic analysis of *Coffea arabica - Hemileia vastatrix* interactions. In Proceedings of the 24th International Conference on Coffee Science, AISC (ASIC), pp. 1414–1418.

Haddad, F., Maffia, L.A., Mizubuti, E.S.G., and Teixeira, H. (2009). Biological control of coffee leaf rust by antagonistic bacteria under field conditions in Brazil. Biol. Control *49*, 114–119.

Haddad, F., Saraiva, R.M., Mizubuti, E.S.G., Romeiro, R.S., and Maffia, L.A. (2013). Antifungal compounds as a mechanism to control *Hemileia vastatrix* by antagonistic bacteria. Trop. Plant Pathol. *38*, 398–405.

Haddad, F., Saraiva, R.M., Mizubuti, E.S.G., Romeiro, R.S., and Maffia, L.A. (2014). Isolation and selection of *Hemileia vastatrix* antagonists. Eur. J. Plant Pathol. *139*, 763–772.

Honorato, J.J., Zambolim, L., Aucique-Pérez, C.E., Resende, R.S., and Rodrigues, F.A. (2015a). Photosynthetic and antioxidative alterations in coffee leaves caused by epoxiconazole and pyraclostrobin sprays and *Hemileia vastatrix* infection. Pest Biochem. Physiol. *123*, 31–39.

Honorato, J.J., Zambolim, L., Duarte, H.S.S., Aucique-Pérez, C.E., and Rodrigues, F.A. (2015b). Effects of epoxiconozale and pyraclostrobin fungicides in the infection process of *Hemileia vastatrix* on coffee leaves as determined by chlorophyll *a* fluorescence imaging. J. Phytopathol. *163*, 968–977.

International Coffee Organization (ICO) (2016). Statistics 2016. http://www.ico.org/trade_statistics.asp.

James, T.Y., Marino, J.A., Perfecto, I., and Vandermeer, J. (2016). Identification of putative coffee rust mycoparasites via single-molecule DNA sequencing of infected pustules. Appl. Environ. Microbiol. *82*, 631–639.

Kasele, I.N., Shannahan, J.F., and Nielsel, D.C. (1995). Impact of growth retardants on corn leaf morphology and gas exchange traits. Crop Sci. *35*, 190–194.

Kishorekumar, A., Jaleel, C.A., Manivannan, P., Sankar, B., Sridharan, R., Somasundaram, R., and Panneerselvam, R. (2006). Differential effects of hexaconazole and paclobutrazol on the foliage characteristics of Chinese potato (*Solenostemon rotundifolius* Poir., J.K. Morton). Acta Biol. Szegediensis *50*, 127–129.

Köhl, J., Kolnaar, R., and Ravensberg, W.J. (2019). Mode of action of microbial biological control agents against plant diseases: relevance beyond efficacy. Front. Plant Sci. *10*, 845.

Koike, N., Hyakumachi, M., Kageyama, K., Tsuyumu, S., and Doke, N. (2001). Induction of systemic resistance in cucumber against several diseases by plant growth promoting fungi: lignification and superoxide generation. Eur. J. Plant Pathol. *107*, 523–533.

Kumar, M., Yadav, V., Tuteja, N., and Johri, A.K. (2009). Antioxidant enzyme activities in maize plants colonized with *Piriformospora indica*. Microbiology *155*, 780–790.

Loguercio, L.L., de Carvalho, A.C., Neilla, G.R., de Souza, J.T., and Pomella, A.W.V. (2009). Selection of *Trichoderma stromaticum* isolates for efficient biological control of witches' broom disease in cacao. Biol. Control *51*, 130–139.

McCook, S. (2006). Global rust belt: *Hemileia vastatrix* and the ecological integration of world coffee production since 1850. J. Glob. Hist. *1*, 177–195.

McTaggart, A.R., Shivas, R.G., van der Nest, M.A., Roux, J., Wingfield, B.D., and Wingfield, M.J. (2016). Host jumps shaped the diversity of extant rust fungi [Pucciniales]. New Phytol. *209*, 1149–1158.

Mejía, L. (2015). Microbiomas y control biológico como alternativa de manejo de la roya anaranjada del cafeto. In Memorias del seminario científico internacional: Manejo Agroecológico de la Roya del Café (FAO), pp. 47–53.

Menezes-Silva, P.E., Sanglard, L.M.P.V., Ávila, R.T., Morais, L.E., Martins, S.C.V., Nobres, P., Patreze, C.M., Ferreira, M.A., Araújo, W.L., Fernie, A.L., and DaMatta, F.M. (2017). Photosynthetic and metabolic acclimation to repeated drought events play key roles in drought tolerance in coffee. J. Exp. Bot. *68*, 4309–4322.

Ministério da Agricultura, Pecuária e Abastecimento (MAPA). (2018) www.agricultura.gov.br.

Møller, I.M., Jensen, P.E., and Hansson, A. (2007). Oxidative modifications to cellular components in plants. Annu. Rev. Plant Biol. *58*, 459–481.

Nason, M.A., Farrar, J., and Bartlett, D. (2007). Strobilurin fungicides induce changes in photosynthetic gas exchange that do not improve water use efficiency of plants grown under conditions of water stress. Pest Manag. Sci. *63*, 1191–1200.

Nygren, K., Dubey, M., Zapparata, A., Iqbal, M., Tzelepis, G.D., Durling, M.B., Jensen, D.F., and Karlsson, M. (2018). The mycoparasitic fungus *Clonostachys rosea* responds with both common and specific gene expression during interspecific interactions with fungal prey. Evol. Appl. *11*, 931–949.

Petit, A.N., Fontaine, F., Vatsa, P., Clément, C., and Vaillant-Gaveau, N. (2012). Fungicide impacts on photosynthesis in crop plants. Photosyn. Res. *111*, 315–326.

Qualhato, T.F., Lopes, F.A.C., Steindorff, A.S., Brandão, R.S., Jesuino, R.S.A., and Ulhoa, S.J. (2013). Mycoparasitism studies of *Trichoderma* species against three phytopathogenic fungi: evaluation of antagonism and hydrolytic enzyme production. Biotechnol. Lett. *35*, 1461–1468.

Quan, L.J., Zhang, B., Shi, W.W., and Li, H.Y. (2008). Hydrogen peroxide in plants: a versatile molecule of the reactive oxygen species network. J. Integr. Plant Biol. *50*, 2–18.

Rauyaree, P., Choi, W., Fang, E., Blackmon, B., and Dean, R. (2001). Genes expressed during early stages of rice infection with the rice blast fungus *Magnaporthe grisea*. Mol. Plant Pathol. *2*, 347–354.

Resende, R.S., Milagres, C.A., Rezende, D., Aucique-Perez, C.E., and Rodrigues, F.A. (2015). Bioprospecting of saprobe fungi from the semi-arid north-east of Brazil for the control of anthracnose on sorghum. J. Phytopathol. *163*, 787–794.

Roberti, R., Veronesi, A., Cesari, A., Cascone, A., Di Berardino, I., Bertini, L., and Caruso, C. (2008). Induction of PR proteins and resistance by the biocontrol agent *Clonostachys rosea* in wheat plants infected with *Fusarium culmorum*. Plant Sci. *175*, 339–347.

Rodrigues-Junior, C.J. (1990). Coffee rust: history, taxonomy, morphology, distribution and host resistance. Fitopatol. Bras. *15*, 5–9.

Shiomi, H.F., Silva, H.S.A., Melo, I.S., Nunes, F.V., and Bettiol, W. (2006). Bioprospecting endophytic bacteria for biological control of coffee leaf rust. Sci. Agric. *63*, 32–39.

Somarriba, E., Harvey, C.A., Samper, M., Anthony, F., Gonzalez, J.E., Staver, C., Rice, R., Schroth, G., da Fonseca, G.A.B., Gascon, C., et al. (2004). Biodiversity conservation in neotropical coffee (*Coffea arabica*) plantations. In Agroforestry and Biodiversity Conservation in Tropical Landscapes, G. Schroth, G.A.B. da Fonseca, C.A. Harvey, C. Gascon, H.L. Vasconcelos, and A.M.N. Izac, eds. (Island Press), pp. 198–226.

Talhinhas, P., Batista, D., Diniz, I., Vieira, A., Silva, D.N., Loureiro, A., Tavares, S., Pereira, A.P., Azinheira, H.G., Guerra-Guimarães, L., et al. (2017). The coffee leaf rust pathogen *Hemileia vastatrix*: one and a half centuries around the tropics. Mol. Plant Pathol. *18*, 1039–1051.

Toniutti, L., Breitler, J.C., Etienne, H., Campa, C., Doulbeau, S., Urban, L., and Bertrand, B. (2017). Influence of environmental conditions and genetic background of *Arabica* coffee (*C. arabica* L.) on leaf rust (*Hemileia vastatrix*) pathogenesis. Front. Plant Sci. *8*, 2025.

Torres, M.A. (2010). ROS in biotic interactions. Physiol. Plant *138*, 414–429.

Varzea, V.M.P., and Marques, D.V. (2006). Population variability of *Hemileia vastatrix* vs. coffee durable resistance. In Durable Resistance to Coffee Leaf Rust, L. Zambolim, E. Maciel-Zambolim, and V.M.P. Várzea, eds. (UFV, Viçosa), pp. 53–74.

Wood, B.W., and Bock, C.H. (2017). Influence of fungicides on gas exchange of pecan foliage. Plant Pathol. *62*, 265–276.

World Coffee Research (2019). Biocontrol of coffee leaf rust. https://worldcoffeeresearch.org/work/biocontrol-coffee-leaf-rust/.

Zambolim, L. (2016). Current status and management of coffee leaf rust in Brazil. Trop. Plant Pathol. *41*, 1–8.

Zhan, J., Thrall, P.H., and Burdon, J.J. (2014). Achieving sustainable plant disease management through evolutionary principles. Trends Plant Sci. *19*, 570–575.

iScience, Volume *24*

Supplemental information

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

Sara Salcedo-Sarmiento, Carlos E. Aucique-Pérez, Patrícia R. Silveira, Adans A. Colmán, André L. Silva, Paloma S. Corrêa Mansur, Fabrício Á. Rodrigues, Harry C. Evans, and Robert W. Barreto

Supplemental Information

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

Sara Salcedo-Sarmiento[1], Carlos Eduardo Aucique-Pérez[1], Patrícia Ricardino Silveira[1], Adans Agustin Colman[1], André Luiz Silva[1], Paloma Stefany Corrêa Mansur[1], Fabrício Ávila Rodrigues[1], Harry Charles Evans[1, 2], Robert Weingart Barreto[1]*

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 ±2ºC 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 1×10^5 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 Cafeeiro – 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. “Catuaí-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 1×10^5 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 (1×10^5 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 lactofuschin 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* × rust on coffee leaf discs *in vitro

Leaves (2nd and 3rd 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 *H. vastatrix* on inoculated leaf discs, four days after inoculation (dai) [fungicide – tebuconazole (200 g. i. L^{-1}) and trifloxystrobin (100 g a.i. L^{-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^{-2} s^{-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:

$$\text{RDI (\%)} = [\Sigma(r \times a)/(R \times A)] \times 100$$

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 "Catuaí-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 2nd, 3rd, and 4th 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*/72*Hv* 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 ± 2ºC, 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 lactofuschin 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 H_2O_2, 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 Selitrennikoff, 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 H_2O_2 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 CO_2 assimilation rate (A), stomatal conductance to water vapor (g_s), intercellular CO_2 concentration (C_i), 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 targetting 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 CO_2 concentration of 400 µmol CO_2 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 (F_0) 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 (F_m), 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 (Φ_{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), stablishing 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 (*Hv*, *Ch*/72*Hv*, *Ch*/*Hv*, and Fungicide) with three and five replications, respectively, for the *in vitro* and *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 *Calonectria hemileiae* and fungicide mixture × *H. vastatrix;* effects on enzymatic activity in coffee leaves section. The experiment was arranged in a completely randomized design with five replications.

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

The F_{max} test was applied for data obtained for the *in vitro* and *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).

Supplemental References

Asada, K. (1992). Ascorbate peroxidase: a hydrogen peroxide-scavenging enzyme in plants. Physiol. Plant. *85*, 235–241.

Baker, N.R. (2008). Chlorophyll fluorescence: a probe of photosynthesis *in vivo*. Annu. Rev. Plant Biol. *59*, 89–113.

Beauchamp, C., and Fridovich, I. (1971). Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal. Biochem. *44*, 276–287.

Bradford, M.N. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. *72*, 248–254.

Cabral, P.G.C., Maciel-Zambolim, E., Oliveira, S.A.S., Caixeta, E.T., and Zambolim, L. (2016). Genetic diversity and structure of *Hemileia vastatrix* populations on *Coffea* spp. Plant Pathol. *65*, 196–204.

Capucho, A.S., Caixeta, E.T., Maciel-Zambolim, E., and Zambolim, L. (2009). Herança da resistência do Híbrido de Timor UFV 443-03 à ferrugem-do-cafeeiro, Pesqui. Agropecu. Bras. *44*, 276–282.

Capucho, A.S., Zambolim, L., Duarte, H.S.S., and Vaz, G.R.O. (2011). Development and validation of a standard area diagram set to estimate severity of leaf rust in *Coffea arabica* and *C. canephora*. Plant Pathol. *60*,1144–1150.

Chance, B., and Maehley, A.C. (1955). Assay of catalases and peroxidases. Methods Enzymol. *2*, 764–775.

Eskes, A.B. (1989). Resistance. In Coffee rust: epidemiology, resistance, and management, Kushalappa, A. C., and Eskes, A.B., eds (CRC Press, Boca Raton, Florida. EE. UU., 1989), pp 171–291.

Del Longo, O.T., González, C.A., Pastori, G.M, and Trippi, V.S. (1993). Antioxidant defences under hyperoxygenic and hyperosmotic conditions in leaves of two lines of maize with differential sensitivity to drought. Plant Cell Physiol. *34*, 1023–1028.

Giannopolitis, C.N., and Ries, S.K. (1977). Superoxide dismutases I. Occurrence in higher plants. Plant Physiol. *5*, 309–314.

Harman, G.E., Hayes, C.K., Lorito, M., Broadway, R.M., Pietro, A., and Peterbauer, C. (1993). Chitinolytic enzymes of *Trichoderma harzianum*: purification of chitobiosidase and endochitinase, Phytopathology *83*, 313–318.

Honorato, J.J., Zambolim, L., Aucique-Pérez, C.E., Resende, R.S., and Rodrigues, F.A. (2015a). Photosynthetic and antioxidative alterations in coffee leaves caused by epoxiconazole and pyraclostrobin sprays and *Hemileia vastatrix* infection. Pest Biochem. Physiol. *123*, 31–39.

Kar, M., and Mishra, D. (1976). Catalase, peroxidase, and polyphenoloxidase activities during rice leaf senescence. Plant Physiol. *57*, 315–319.

Lever, M. (1972). A new reaction for colorimetric determination of carbohydrates. Anal. Biochem. *47*, 273–279.

Maxwell, K., and Johnson, G.N. (2000). Chlorophyll fluorescence—a practical guide. J. Exp. Bot. *51*, 659–668.

Nakano, Y., and Asada, K. (1981). Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. Plant Cell Physiol. *22*, 867–880.

Novais, R.F., Neves, J.C.L., and Barros, N.F. (1991). Ensaio em ambiente controlado. In Métodos de pesquisa em fertilidade do solo, Oliveira, A.L., Garrido, W.E., Araújo, J.D., and Lourenço, S., eds (EMBRAPA-SEA, Brasília, 1991), pp 153–189.

Roberts, W.K., and Selitrennikoff, C.P. (1988). Plant and bacterial chitinases differ in antifungal activity. J. Gen. Microbiol. *134*, 169–176.

Vale, F.X.R., Fernandes-Filho, E.I., and Liberato, J.R. (2003). QUANT: a software plant disease severity assessment. In Proceedings of the 8th International Congress of Plant Pathology, Close, R., Braithwaite, M., and Havery, I., eds, (Horticulture Australia, Sydney), p. 105.