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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 ±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.