



Chlorogenic acid levels in leaves of coffee plants supplied with silicon and infected by *Hemileia vastatrix*

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ABSTRACT

Rust, caused by *Hemileia vastatrix*, is the main disease that decreases coffee production in Brazil. New and enhanced methods to reduce rust intensity that can be integrated with modern genetic and chemical approaches need to be investigated. Considering that many plant species supplied with silicon (Si) show increased resistance to several pathogens, this study examined the possible effect of this element in increasing chlorogenic acid (CA) concentrations in coffee leaves and, consequently, increasing the level of resistance to rust. Plants (cv. "Catuaí Vermelho IAC 44") were inoculated with *H. vastatrix* after growing for 35 days in a hydroponic culture amended with 0 (-Si) or 2 (+Si) mM Si. Concentration of Si in leaf tissues was of 0.36 and 0.42 dag/kg for -Si and +Si treatments, respectively, but without a statistically significant difference. The area under rust progress curve was 154.5 and 119.4 for -Si and +Si treatments, respectively, but without significant statistical difference. For non-inoculated plants, the concentrations of total CA and caffeoyl-quinic acid (CQA) compounds (diCQA) were similar between -Si and +Si treatments. Even though there was an increase of 236.4 and 257.1%, respectively, for total CA and diCQA for +Si when compared to -Si treatment at 30 days after inoculation with *H. vastatrix*, reduction on rust severity was not obtained once the fungus had already colonized the leaf tissues. Therefore, regardless of the increase in the concentrations of chlorogenic acid on leaves, coffee resistance to *H. vastatrix* infection was not potentialized by Si.

Key words: *Coffea arabica*, biotrophic, disease resistance, metabolomics, phenolics, secondary metabolites.

RESUMO

Níveis de ácido clorogênico em folhas de plantas de cafeeiro supridas com silício e infectadas por *Hemileia vastatrix*

A ferrugem, causada por *Hemileia vastatrix*, é a principal doença e a que mais reduz a produção do cafeeiro no Brasil. Novos métodos de controle que podem reduzir a intensidade da ferrugem e serem integrados com a resistência genética e o controle químico precisam ser investigados para aperfeiçoamento do seu manejo. Considerando que várias espécies de plantas supridas com silício (Si) apresentam aumento da resistência a diversos patógenos, este estudo examinou o possível efeito do Si em aumentar a concentração de ácido clorogênico (AC) em folhas de cafeeiro e, conseqüentemente, o nível de resistência à ferrugem. Plantas de cafeeiro (cv. "Catuaí Vermelho IAC 44") crescidas por 35 dias em solução nutritiva contendo 0 (-Si) e 2 mM Si (+Si) foram inoculadas com *H. vastatrix*. A concentração foliar de Si foi de 0,36 e 0,42 dag/kg e a área abaixo da curva do progresso da ferrugem foi de 154,5 e 119,4, respectivamente, para os tratamentos -Si e +Si, mas não houve diferença significativa entre os dois tratamentos. Para plantas não inoculadas com *H. vastatrix*, a concentração foliar de AC total e ácido quínico-cafeoil (AQC) foi similar entre os tratamentos -Si e +Si. Embora tenha ocorrido aumento de 236,4 e 257,1%, respectivamente, para AC total e AQC para o tratamento +Si quando comparado ao tratamento -Si aos 30 dias após inoculação com *H. vastatrix*, a severidade da ferrugem não decresceu, pois o fungo já havia colonizado os tecidos foliares. Assim, independente do aumento na concentração foliar de AC, a resistência do cafeeiro à infecção por *H. vastatrix* não foi potencializada pelo Si.

Palavras-chave: *Coffea arabica*, biotrófico, fenólicos, metabólitos secundários, metabóloma, resistência a doenças.

Coffee leaf rust, caused by *Hemileia vastatrix* Berkeley and Broome, is one of the most serious agronomic problems on coffee (*Coffea arabica*) production in Brazil (Zambolim et al., 2002). This biotrophic fungus enters through the stomata on the adaxial surface of leaves, efficiently obtains nutrients through haustoria formed inside the cells, and finally produces uredia that contain numerous

orange uredospores (Silva et al., 2006). Coffee leaf rust causes premature leaf fall and yield losses ranging from 10 to 40% (Zambolim et al., 2002). Resistant cultivars are the most economical and viable management practice to control coffee leaf rust, and can be obtained quite easily due to the great genetic variability found in the germplasm of the genus *Coffea* (Van der Vossen, 2005). However, their continuous use can cause the loss of vertical resistance due to the rapid genetic changes in the pathogen population (Várzea and Marques, 2005). Protecting and systemic fungicides are also used to reduce coffee leaf rust intensity (Zambolim et al., 2002),

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but several are expensive and hazardous to the environment and human health.

Considering the difficulties in coffee leaf rust control, investigations aimed at developing alternate control methods that can be integrated with genetic and chemical approaches are justified. Silicon (Si) may provide a viable alternative as its direct or indirect beneficial effects to plants under biotic and/or abiotic stresses have been reported in a wide variety of crops such as barley, corn, cucumber, grape, rice, rye, strawberry, and wheat (Datnoff et al., 2007). In coffee, Pereira et al. (2009) reported no significant difference between coffee plants sprayed or not sprayed with potassium silicate, a soluble source of Si, for leaf rust severity. Indeed, potassium silicate spray did not increase activity of the enzymes chitinases, β -1,3-glucanases, peroxidases, polyphenol oxidases, phenylalanine ammonia-lyases, and lipoxygenases. In another study, Carré-Missio et al. (2009) grew coffee plants in a nutrient solution containing 0 (-Si) or 2 mmol Si/L (+Si) and inoculated them with *H. vastatrix*. According to these authors, Si concentration in leaf tissues of plants supplied with Si did not increase and there was no significant difference between -Si and +Si treatments for incubation period, latent period, number of pustules per leaf, pustule size, pustule expansion, and area under rust progress curve. The activity of chitinases, β -1,3-glucanases and peroxidases increased after inoculation with *H. vastatrix*, but their activities were not higher on leaf tissues of Si-supplied plants. Coffee plants can uptake Si through their roots (Silva et al., 2010), but translocate it inefficiently to shoots (Carré-Missio et al., 2009), depriving them of the multiple positive benefits brought by this element.

Insoluble and soluble Si in plant tissues is associated with an increase in host resistance to several root and foliar diseases (Datnoff et al., 2007). Rice plants supplied with Si and inoculated with *Pyricularia grisea* showed an increase in the production of phenolic-like compounds and two momilactones phytoalexins (Datnoff et al., 2007). *Coffea* species are rich in caffeine, chlorogenic acid, caffeic acid, and hydroxyhydroquinone, which help to reduce the level of scavenger reactive oxygen species (Butt & Sultan, 2011). Chlorogenic acid is an ester formed between caffeic acid and quinic acid with three isomerisations of the quinic acid in positions 3 (3-CQA), 4 (4-CQA), and 5 (5-CQA) (Clifford et al., 2003). Chlorogenic acid is widespread in almost all higher plants, but *Coffea* species have the highest known content namely: about 1 and 11% dry matter of leaves and fruits, respectively (Clifford, 1985; Clifford et al., 2003). Chlorogenic acid plays an important role in the response of several plant species to fungal infections (Nicholson and Hammerschmidt, 1992). No previous study has investigated the role of Si on coffee leaf rust at the phenolic concentration level. This paper includes the first exploration of the effect of this element on the concentration of chlorogenic acid on coffee leaves and its possible involvement in increasing coffee resistance to infection by *H. vastatrix*.

The nutrient solution was prepared based on Clark (1975) with modifications. The nutrient solution included, with concentrations in mmol/L, 5.7 N-NO₃⁻, 1 N-NH₄⁺, 0.1 P-H₂PO₄⁻, 2.4 K, 1.2 Ca²⁺, 0.6 Mg²⁺, 0.7 S-SO₄²⁻, and, in μ mol/L, 35 Fe, 0.8 Cu, 1.5 Zn, 5 Mn, 17 B, 0.1 Mo, and 25 EDTA disodium. Silicon was supplied as monosilicic acid, which was prepared by passing potassium silicate through a cation exchange resin (Amberlite IR-120B, H⁺ form; Sigma-Aldrich, São Paulo, Brazil) (Dallagnol et al., 2009). The Si concentrations used were 0 or 2 mM. The pH of the nutrient solution was not altered by the addition of silicic acid. The aerated nutrient solution was changed every seven days and the pH kept around 5.5 by using solutions of HCl or NaOH (1 M). Coffee seeds from cultivar “Catuaí Vermelho IAC 44”, susceptible to *H. vastatrix*, were surface sterilized in 10% (v v⁻¹) NaOCl for 1.5 min, rinsed in sterilized water for 3 min, and sown at the rate of 35 seeds per plastic tray containing washed and sterilized sand. Four seedlings at cotyledonary leaf stage (Hess et al., 1997) were transplanted to each of the plastic pot containing 4 liters of nutrient solution.

Uredospores of *H. vastatrix* were obtained from naturally rusted leaves from coffee plants (cv. “Catuaí 44 vermelho IAC”) kept in the greenhouse. A suspension of uredospores (viability 35%) of *H. vastatrix* (1 mg/mL) was applied as a fine mist to the abaxial leaves of each plant until runoff using a VL Airbrush atomizer (Paasche Airbrush Co., Chicago, IL). Plants were inoculated with *H. vastatrix* after 35 days in hydroponic culture with and without Si. Immediately after inoculation, plants were transferred to a mist chamber (25 \pm 0.5°C; relative humidity of 95 \pm 5%) and kept in the dark for 48 h. After this period, plants were transferred to a growth chamber at 22°C with a 12 h photoperiod under a photon irradiance of approximately 225 μ mol m⁻² s⁻¹ provided by cool-white fluorescent lamps. Leaf rust was scored on two leaves per plant at 5, 10, 15, 20, and 30 days after inoculation (dai) by using a scale based on the percentage of diseased leaf area (Carré-Missio et al., 2009). Area under rust progress curve (AURPC) for each leaf on each plant was computed using the trapezoidal integration of rust progress curve over time, with the formula proposed by Shaner & Finney (1977).

For sample preparation, extraction, and LC-ESI-MS analysis for chlorogenic acid concentration on coffee leaves, two leaves per plant for five replications were collected at 5, 10, 15, 20, and 30 dai. Leaves collected from non-inoculated plants served as control treatment. Leaf samples from inoculated and non-inoculated plants were placed in plastic bags, kept in liquid nitrogen during sampling, and stored at -80°C. Leaf samples were freeze-dried (Labconco Corporation, Kansas City, MO) for approximately 72 h, blended to a fine powder, and transferred to plastic tubes which were stored at -80°C until further analysis. For single step extraction analysis, a total of 0.5 g of each sample was weighed and placed in a scintillation vial containing 4 mL of methanol. The vials were capped and wrapped with sealing tape, sonicated for 15 min at room temperature, and

allowed to stand at room temperature overnight. An aliquot was removed from each vial and filtered through a 0.45 μM nylon 66 filter for HPLC analysis.

The HPLC analysis was conducted on a Shimadzu LC-20 HPLC system (LC-20AT quaternary pump, DGU-20A5 degasser, SIL-20A HT auto-sampler, and a SPD M20A photodiode array detector, running under Shimadzu LCSolutions version 1.22 chromatography software, Columbia, MD, USA). The column used was an Inertsil ODS-3 reverse phase C-18 column (5 μm , 250 \times 4.6 mm from Varian). For phenolic compound analysis, the initial conditions were 20% methanol and 80% water with 0.05 M phosphoric acid at a flow rate of 1 ml per min. The effluent was monitored at 325 nm on the VWD. After injection (25 μL), the column was held under the initial conditions for 2 min, then developed to 100% methanol in a linear gradient over 55 min. A standard curve of chlorogenic acid (CA) (Sigma-Aldrich, São Paulo, Brazil), in nanomoles, was generated. The molar extinction coefficient for CA was used to quantitate caffeoyl-quinic acid compounds (dicQA). The degree of substitution on the quinic acid was confirmed by purification and LC-MS.

Samples were also run on an Applied Biosystems/MDS Sciex QStar Elite Q-TOF mass spectrometer with a Turboionspray electrospray source and an Agilent 100 series HPLC system (G1379A degasser, G1357A binary capillary pump, G1389A auto-sampler, G1315B photodiode array detector, and a G1316A column oven) all running under Applied Biosystems Analyst 2.0 (build 1446) LC-MS software. The MS was calibrated at least daily with a standard calibration mixture recommended by Applied Biosystems, and the signal detection was optimized as needed. Data were acquired in the MOF MS mode negative. The MS parameters were as follows: accumulation time - 1 sec, mass range 200 to 1000 daltons, source gas 1 - 50 units, source gas 2 - 35 units, curtain gas - 25 units, ion spray voltage 4500, source heater - 400 degrees, declustering potential -80, focusing potential - 265, declustering potential 2 - 15, ion release delay - 6, ion release width - 5. The column was a 3 mm \times 150 mm inertsil reverse phase C-18, ODS 3, 3 μm column (Metachem, Torrance, CA). The initial solvent system was 20% methanol and 80% water with 0.25% formic acid at a flow rate of 0.25 mL per min. After injection (15 μL), the column was developed with a linear gradient to 100% methanol over 50 min. The column effluent was monitored at 280 nm in the PDA detector. The software package was set to collect mass data between 150-1000 AMUs. Generally the most significant sample ions generated under these conditions were $[\text{M}^-]$ and $[\text{M}+\text{HCOO}]^-$.

After the termination of the experiments, leaves were collected from plants of each replication per treatment, washed in deionized water, dried for 72 h at 65°C, and ground to pass through a 40-mesh screen with a Thomas-Wiley mill (Thomas Scientific, Swedesboro, NJ). Silicon concentration in leaf tissues was determined by colorimetric analysis on

0.1 g of dried and alkali-digested tissue (Dallagnol et al., 2009).

A 2 \times 2 factorial experiment, consisting of nutrient solution with and without Si (0 and 2 mM, hereafter referred as to -Si and +Si treatments, respectively), and non-inoculated and inoculated plants, was arranged in a completely randomized design with five replications. Each experimental unit consisted of one plastic pot with four plants. The experiment was repeated once. Data from AURPC and Si concentration on leaf tissues from the two experiments were pooled for statistical analysis because homogeneity of variances was confirmed by Cochran's test. Moreover, the experiment-treatment interactions were not significant ($P = 0.05$) when compared to the main effects of treatments. AURPC data were analyzed by analysis of variance (ANOVA) and treatment mean comparisons by t -test using SAS version 6.12 (SAS Institute, Inc., Cary, NC). Data from concentration of total CA, dicQA, and total CQA (total CA + dicQA) on leaf tissues were presented as a mean of three readings per sample.

Concentrations of Si in leaf tissues were 0.36 and 0.42 dag/kg for -Si and +Si treatments, respectively, but without significant difference ($P = 0.05$) between them. Leaf Si concentrations for plants supplied with this element were lower than those reported for rice, a Si accumulator plant, with Si concentration up to 8% (Dallagnol et al., 2009). There was no significant difference ($P = 0.05$) between -Si and +Si treatments for rust severity for all evaluations (Figure 1). Values for AURPC were 154.5 and 119.4 for -Si and +Si treatments, respectively, but without significant difference ($P = 0.05$) between them. It is plausible that Si concentration in leaf tissues was not sufficient, based on the low innate physiological capacity of the coffee plants to take up this element from the nutrient solution leading to the absence of a negative impacts on the infectious process of *H. vastatrix* in leaves. In contrast, Silva et al. (2010) found an increase of 152% in Si concentration in root tissues of coffee plants grown in a Si-deficient soil amended with calcium

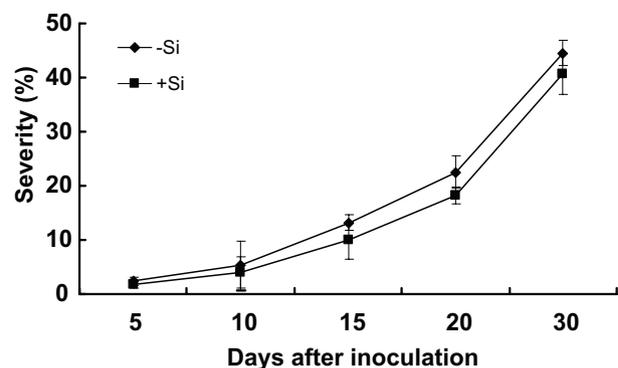


FIGURE 1 - Progress of rust on leaves of coffee plants grown in hydroponic culture containing 0 (-Si) or 2 (+Si) mM silicon. Error bars represent standard deviation of means from two pooled experiments ($n = 10$).

silicate. The authors reported that the galls and egg numbers of *Meloidogyne exigua* decreased in association with high concentration of lignin-thioglycolic acid derivatives and greater activities of peroxidase, polyphenoloxidase, and phenylalanine ammonia lyase.

For the non-inoculated plants, the concentrations of CA total, diCQA, and, consequently, total CQA were quite similar between -Si and +Si treatments (Figure 2A-B). The concentrations of CA total and diCQA peaked at 5 dai for both -Si and +Si treatments with the highest concentrations occurring for the +Si treatment. There was an increase of 236.4 and 257.1%, respectively, for CA total and diCQA for +Si as compared to -Si treatment at 30 days after inoculation with *H. vastatrix* (Figure 2A-B). One mechanism involved in Si-mediated host resistance, especially in the rice-*P. grisea* pathosystem, is the deposit of Si below the cuticle that delays fungus penetration and lesion expansion (Datnoff et al., 2007). However, probing more deeply into how Si affects blast disease in rice, a model plant used in the studies involving Si found that plants supplied with this element responded more promptly to *P. grisea* infection by increasing production of phenolic compounds and momilactones phytoalexins in association with a strong activation of some PR-genes, such as peroxidases and PR-1 (Datnoff et al., 2007). Chlorogenic acid plays an important role in the increased resistance of several plants species to pathogens infections (Bennett & Wallsgrove, 1994).

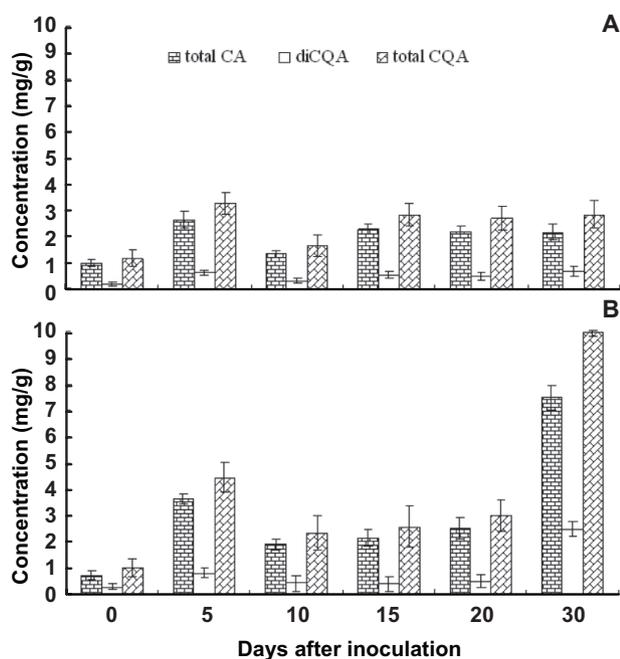


FIGURE 2 - Concentrations of total chlorogenic acid (total CA), caffeoyl-quinic acid compounds (diCQA), and total CQA (total CA + diCQA) in leaves of coffee plants grown in hydroponic culture containing 0 (A) or 2 mM silicon (B) as a function of time after inoculation with *Hemileia vastatrix*. Error bars represent standard deviation of means ($n = 5$).

Bostock et al. (1999) demonstrated that some phenolic acids, such as catechin, procyanidin B3, chlorogenic acid (CGA; 5-O-caffeoylquinic acid), neochlorogenic acid, and caffeic acid in peach fruits, decreased cutinase activity produced by *Monilinia fructicola* and increased their resistance against fungal infection. According to Villarino et al. (2011), chlorogenic acid and its isomer, neochlorogenic acid, were found to be the major phenolic compounds in the flesh and peel of immature peach fruits resistant to brown rot caused by *Monilinia laxa*, possibly by interfering with fungal melanin production. A decrease in the concentration of these two phenolic compounds in maturing fruits increased their susceptibility to infection by *M. laxa*. Even though the concentrations of CA total and diCQA increased at 30 days after inoculation with *H. vastatrix*, especially on leaves of plants supplied with Si, there was no decrease on rust severity once the fungus had already colonized the leaf tissues. Silicon can affect the production of phenolic compounds upon pathogen attack (Carver et al., 1998; Datnoff et al., 2007). However, the results from the current study strengthen the idea that an increase in the concentration of phenolic compounds potentiated by Si must be associated with its continuous uptake by roots and efficient transportation to shoots, as reported by Dallagnoll et al. (2009).

Even though the concentration of chlorogenic acid on coffee leaves of plants supplied with Si was found to be high only at a late stage of *H. vastatrix* infection, a desirable level of host resistance to rust could not be achieved. It is possible that this high concentration of chlorogenic acid on leaves is a host response to stress in close association with fungal infection. Considering that for the present study only one reproductive cycle of *H. vastatrix* was evaluated, it is plausible that a high concentration of chlorogenic acid may help to reduce rust symptoms in epidemics occurring under field conditions.

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