

Induced responses of *Coffea arabica* to attack of *Coccus viridis* stimulate locomotion of the herbivore

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Abstract

The green scale, *Coccus viridis* (Green) (Hemiptera: Coccidae), is an insect pest of coffee and several other perennial cultivated plant species. We investigated changes in alkaloid and phenolic contents in coffee plants as a response to herbivory by this insect. Greenhouse-grown, 11-month-old coffee plants were artificially infested with the coccid and compared with control, uninfested plants. Leaf samples were taken at 15, 30, 45, and 60 days after infestation, and high-performance liquid chromatography was used to identify and quantify alkaloid and phenolic compounds induced by the coccids at each sampling date. Of the compounds investigated, caffeine was the main coffee alkaloid detected in fully developed leaves, and its concentration in infested plants was twice as high as in the control plants. The main coffee phenolics were caffeic and chlorogenic acid, and a significant increase in their concentrations occurred only in plants infested by *C. viridis*. A positive and significant relationship was found between alkaloid and phenolic concentrations and the infestation level by adults and nymphs of *C. viridis*. Caffeine and chlorogenic acid applied on coffee leaves stimulated the locomotory activity of the green scale, thus reducing their feeding compared to untreated leaves. This is the first study to show increased levels of coffee alkaloids and phenolics in response to herbivory by scale insects. The elevation of caffeine and chlorogenic acid levels in coffee leaves because of *C. viridis* infestation seems to affect this generalist insect by stimulating the locomotion of crawlers.

Introduction

Insects and plants have coexisted for over 350 million years and have developed a series of relationships, among which the most common interaction involves the attack by phytophagous insects and plant defenses against such herbivores (Karban & Agrawal, 2002; Schoonhoven et al., 2005). Whereas some plant species accumulate secondary metabolites as defensive traits, others do not commit resources to the accumulation of defensive compounds but rather allocate energy and matter to evade herbivores or tolerate their attack (Karban & Baldwin, 1997; Gatehouse, 2002).

In terms of their expression, plant defenses against phytophagous insects are divided into constitutive and induced traits, although the compounds or proteins involved are often the same (Price et al., 1980; Karban & Baldwin, 1997; Gatehouse, 2002; Dicke & Hilker, 2003; Schoonhoven et al., 2005).

Coffee phenolics, namely chlorogenic and caffeic acid, and alkaloids, including caffeine and other methylxanthines, are toxic secondary compounds (Akazawa & Wada, 1961; Nathanson, 1984; Frischknecht et al., 1986; Appel, 1993). These compounds may play an important role in the coffee plant's susceptibility to insect pests (Nardini et al., 2002). *Coccus viridis* (Green) (Hemiptera: Coccidae) is a generalist insect pest with a host list spanning 57 plant families including several perennial plant species. It can cause serious damage to *Coffea arabica* L. and *Coffea*

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canephora Pierre ex A. Froehner (Rubiaceae), especially to young plants and those under dense cultivation (Hollingsworth, 2000). Adults and nymphs feed on plant sap and excrete honeydew, which leads to the development of fungi on leaf surfaces and thereby reducing photosynthesis. They are also believed to introduce toxins causing weakening of the plant, leaf fall, and yield reduction (Lepelley, 1968).

Adult *C. viridis* are 3–4 mm long, whereas nymphs (crawlers) are <1 mm long at emergence, and unlike most other soft scale species, they can move along the host plant. The egg-to-egg duration varies from 50 to 70 days (Fredrick, 1943). Eggs hatch into crawlers that wander around the plant or disperse to other hosts. Once a suitable leaf or green shoot is found, the nymphs begin to feed and usually remain in this feeding site until maturity, the phase in which the female coccid becomes immobile in its host plant (Roba, 1936; Fredrick, 1943; Zucchi et al., 1993).

Secondary plant metabolites induced by insect feeding may have a role in plant resistance if they cause lethal or sublethal effects on the herbivore (Karban & Baldwin, 1997). Experimentally, these effects can be assessed by applying purified compounds on the plant parts normally colonized by the herbivore and measuring its mortality or change in behavior related to colonization of plant tissues. Some studies have been carried out to identify chemical compounds present in coffee plants and their association with plant resistance to insect pests, especially the coffee leafminer and the coffee berry borer (Guerreiro Filho & Mazzafera, 2000, 2003; Magalhães et al., 2008a). However, there is a paucity of information on changes in plant chemistry involving the interaction between coffee plants and the green scale *C. viridis*, which is the aim of the present study.

Materials and methods

Plant cultivation

Coffea arabica plants of the Catuaí Vermelho variety were grown hydroponically in a greenhouse complex of the Federal University of Viçosa, at 25 ± 5 °C, 50–70% r.h., and L12:D12 photoperiod. Coarse sand (sieved to 2 mm diameter) was used as substrate for the plants. Prior to use, the sand was sterilized for 24 h in a 10% HCl aqueous solution, washed 10 times with tap water to reduce the acidity, and rinsed with distilled water to remove clay, organic matter, and nutrients. A portion of the sand was transferred to plastic trays (80 × 5 × 10 cm), and 20 coffee seeds were sown in each tray. After germination, seedlings were watered daily until reaching the two-cotyledon leaf stage when they were transplanted to 3-l pots filled with the substrate. A bucket of similar size was placed underneath each pot to collect the nutritive solution that eventu-

ally drained. Nutrients were provided daily by applying 0.5 l of nutritive solution containing the following concentrations of macronutrients (mM) 3.0 N, 9.0 K, 1.0 P, 4.0 Ca, and 2.1 S and of micronutrients (μM) 46 B, 0.3 Cu, 60 Fe, 2.0 Mg, 36 Mn, 0.5 Mo, and 0.1 Zn. Distilled water was added to the drained volume to a total of 0.5 l. The pH of the solution was adjusted to the range of 5.5–6.5 using NaOH or HCl at 0.1 mol m^{-3} (Vetec Química Fina, Duque de Caxias, Rio de Janeiro, Brazil), and this final solution was reapplied to the sand in each respective pot. Aluminum foil was placed over the pots to avoid development of algae.

Coccus viridis colony

Nymphs and adults were collected in various coffee plantations ($20^{\circ}32.9'S$, $42^{\circ}53.5'W$; $20^{\circ}33.0'S$, $42^{\circ}53.6'W$; and $20^{\circ}33.7'S$, $42^{\circ}53.7'W$) near the university campus. The insects were transferred to young coffee plants of the Catuaí Vermelho variety that were maintained in pots inside wooden cages (100 × 50 × 90 cm) covered with white organza. Plants were replaced monthly, and the insects were reared for 3–5 generations. The cages were each placed inside a plastic tray (120 × 50 × 10 cm) containing a 30% detergent solution and the bottom of the cage supported by four glass containers to avoid infestation by mutualistic ants and predators.

Experimental treatments

The experiment was set up in a completely randomized design with two treatments (infested and uninfested plants) and nine replications. Eight-month-old coffee plants (ca. 15 cm tall) were infested with nymphs and adults by placing 50 coccids (third instars) on the third fully expanded leaf from the top. The leaves were enclosed by muslin bags tied at the base of the petioles. Control (i.e., non-infested) plants were handled in the same way and separated from the infested plants by a glass wall inside the greenhouse. In the interior of each environment (treatment), a small meteorological station was installed to measure temperature, relative humidity, and solar radiation incidence. The experimental conditions of the infested and non-infested plants by *C. viridis* during the experimental period were 24.5 ± 1.05 and 24.4 ± 1.02 °C, 65.3 ± 10.14 and $65.8 \pm 9.14\%$ r.h., and 25.4 ± 3.57 and $24.9 \pm 1.01 \text{ MJ m}^{-2}$ per day, respectively.

Leaf sampling and phytochemical extraction

Leaves from the median stratum of each individual plant were collected in batches of 7 g at 15, 30, 45, and 60 days after infestation. The number of adults and nymphs of *C. viridis* present in each experimental unit was also recorded for each date. When taking leaves from infested

plants, the coccids present were gently removed using a fine hair brush and returned to the plant. The leaves were dried at 40 °C for 2 weeks during which they lost on average 70% of their water content. The dried leaves were ground, and 1 g samples was weighed, mixed with 30 ml methanol, and placed in a water bath for 4 h at 60 °C for the phytochemical extraction. The resulting extract was passed through a filter paper (Ø 90 mm; Nalgon, São Paulo, SP, Brazil), concentrated in a rotatory evaporator (MA-120/V; Marconi, Piracicaba, Brazil), and rediluted in methanol to a final volume of 3 ml. The samples were subsequently filtered once again under vacuum in a C₁₈ solid-phase extractor (porous silica 0.45 µm; Waters, São Paulo, Brazil) (Guerreiro Filho & Mazzafera, 2000, 2003; Ramiro et al., 2006).

Chemical standards

To define the peak and retention time for each compound studied, the following standards were used for the chromatographic determinations: chlorogenic acid (5-*O*-caffeoyl-*D*-quinic acid), caffeine (1,3,7-trimethylxanthine), caffeic acid (3,4-dihydroxy-cinnamic acid), theophylline (1,3-dimethylxanthine), theobromine (3,7-dimethylxanthine), and allantoin (2,5-dioxo-4-imidazo-lidinylurea). These chemicals were obtained from Sigma-Aldrich Química Brasil (São Paulo, SP, Brazil).

Identification and quantification of selected alkaloids and phenolics

The extracts obtained as previously described were further diluted to 10 ml with methanol. Aliquots of 0.5 ml were obtained from each sample and diluted in methanol/water (1:1) to a 10 ml volume, and 2 ml of this solution was further diluted to 10 ml with the same solvent mixture (i.e., methanol/water at 1:1). This last solution was filtered using a filtering unit with a polytetrafluoroethylene membrane (0.45 mm mesh and 13 mm in diameter). An aliquot of 20 ml of the filtered solution was injected into the high-performance liquid chromatographer (HPLC) Shimadzu LC-10AD (two pumps) with a SPD-10AV dual detector (Shimadzu, Kyoto, Japan), adjusted to detect alkaloid compounds (i.e., caffeine and related methylxanthines) at $\lambda = 272$ nm in channel 1 and to detect phenolic compounds (i.e., caffeic and chlorogenic acid) at $\lambda = 320$ nm in channel 2. The HPLC was also equipped with a Shimadzu CBM-10A communication system. The compounds of interest were separated with a RP-18 reverse-phase column (Lichrosorb: 250 × 4.6 × 5 mm) using a methanol/water solution with 1.0 mM HCl in elution gradient 0.1–7.0 min (17:83%), 7.1–37.0 min (23:77%), and 37.1–40.0 min (100:0%), at a flow rate of 1.0 ml per min following Magalhães et al. (2008a). The chemical standards were injected individually into the col-

umn and also injected together for determining the retention time. Increasing concentrations of each standard (1, 5, 10, 20, 100, and 200 µg ml⁻¹) were injected in the column for establishing the calibration curve of each standard and eventual quantification in the samples obtained from the coffee leaves by the external standard method. The quantifications were carried out in triplicate for each leaf batch used in the extraction.

Presence of caffeine and chlorogenic acid in *Coccus viridis*

Greenhouse-grown, young plants of *C. arabica* and *Citrus sinensis* (L.) Osbeck (ca. 15 cm tall) were infested with nymphs of *C. viridis* in a completely randomized experiment with six replicates. Citrus plants were used as negative control as they are also host plants for the coccid (Fredrick, 1943). The plants were maintained under the same conditions used previously and infested with 100 *C. viridis* nymphs per plant. After 25 days of feeding, 40 adult coccids were collected from each plant, placed in 5-ml containers with methanol, and grounded with a glass pestle. They were then filtered using filter paper, and alkaloid and phenol contents of these samples were determined as described above.

Effects of caffeine, chlorogenic acid, and caffeic acid on *Coccus viridis*

Because the caffeine, chlorogenic acid, and caffeic acid levels were considerably elevated in coccid-infested coffee plants, their potential effects on the green scale were tested in a toxicity test and in a behavioral assay. To perform the two bioassays, leaves of the middle third of the coffee canopy (fourth pair from the top) were immersed for 5 s in distilled water with surfactant (0.02% calcium dodecylbenzene sulfonate) and the purified compounds. Control leaves were treated with distilled water + surfactant. Final concentrations (% wt/vol) of chlorogenic acid, caffeine, and caffeic acid in the solution were 3.1, 1.2, and 2.3, respectively (based on the maximum concentrations detected in our leaf determinations, i.e., 806, 175, and 663 mg kg⁻¹, respectively). The leaves were subsequently dried for 2 h at room temperature and placed in Petri dishes (9 cm in diameter).

A bioassay was carried out in a completely randomized experimental design with six replicates. Each replicate consisted of a 9-cm Petri dish with the bottom covered by leaves treated as described above. Twenty first instars of *C. viridis* were transferred to each dish and placed in an incubator at 25 ± 0.5 °C, 75 ± 5% r.h., and 12-h photophase. After a 24-h exposure, coccid mortality was assessed: insects were considered dead if they did not move when prodded with a fine hair brush.

Behavioral tests were carried out between 07:00 and 18:00 hours in a room with artificial, incandescent light

and average temperature of 25 ± 3 °C. One first-instar green scale was released on the leaf in the center of each Petri dish (9 cm diameter \times 2 cm high), and after 30 s of contact with the treated leaf, the time that each nymph remained immobile (and feeding) or walking was recorded during 15 min. Ten replicates, each consisting of a single insect, were used for the experiments. The coffee leaf was replaced for each trial or replicate.

Statistical analysis

Results of the phytochemical quantification were subjected to multivariate repeated measures analysis of variance (ANOVA) (Proc GLM; SAS, 2002) to test the null hypothesis of lack of differences between treatments over time for all of the compounds studied. Subsequently, the concentration of each compound was analyzed across dates using univariate repeated measures ANOVA (Proc MIXED; SAS, 2002). The ANOVA model included the main effects of time, treatment, and their interaction. The antedependence covariance structure was used given that it had an Akaike information criterion smaller than that of other models and a significant log likelihood ratio test ($P < 0.05$) relative to the compound symmetry covariance structure (Littell et al., 2006). If the interaction treatment*time was significant, linear regression analyses were used to analyze the trends of the phytochemical concentrations in each treatment through time. Moreover, the relationships between the numbers of adults and nymphs of coccids and the concentrations of the phytochemicals in coffee leaves were analyzed using linear regression analysis (Proc REG; SAS, 2002).

The data of the toxicity and behavioral assays were analyzed by one-way univariate ANOVA, and the means were separated using Tukey's test ($\alpha = 0.05$). For all analyses, the assumptions of normality and homogeneity of variance were checked (Proc UNIVARIATE, Proc GPLOT; SAS, 2002), and no transformation was necessary.

Results

The HPLC allowed identification and quantification of allantoin, theobromine, caffeine, teophylline, chlorogenic acid, and caffeic acid at retention times of 13.54, 10.85, 12.01, 14.02, 15.05, and 16.71 min, respectively. Among these compounds, theobromine, teophylline, and allantoin were detected in low concentrations in infested (3.36 ± 1.21 , 0.41 ± 0.15 , and 1.91 ± 0.60 $\mu\text{g kg}^{-1}$, respectively) and non-infested plants (3.31 ± 1.79 , 0.70 ± 0.37 , and 0.63 ± 0.39 $\mu\text{g kg}^{-1}$, respectively) and were not considered in the subsequent analyses.

Repeated measures multivariate ANOVA indicated a significant main effect of treatment (Wilk's $\lambda = 0.003$;

$F_{3,14} = 168.7$, $P < 0.001$), time (Wilk's $\lambda = 0.003$; $F_{8,9} = 382.38$, $P < 0.001$), and treatment*time interaction in the overall phytochemical concentrations in coffee leaves (Wilk's $\lambda = 0.003$; $F_{8,9} = 383.21$, $P < 0.001$). Subsequent results of the repeated measures ANOVA and regression analyses for each phytochemical confirmed that their concentrations varied differently in infested and non-infested plants over time (Table 1). Infested plants increased the concentrations of caffeic and chlorogenic acid and caffeine over time as indicated by the significant ($P < 0.05$) regression lines (Figure 1). However in the control plants, the concentrations of the compounds did not change or only increased slightly over time (Figure 1).

The population density of adults and nymphs of *C. viridis* varied among infested plants during the course of the experiment. Plants had adult numbers ranging from 1 to 17 and immature stages of the coccid ranging from 1 to 100. Importantly, a positive and significant relationship was obtained between concentrations of the phenolics caffeic acid and chlorogenic acid and the alkaloid caffeine on the one hand, and the numbers of nymphs ($R^2 = 0.79$, 0.96 , and 0.47 , respectively; all $P < 0.01$) and adults ($R^2 = 0.48$, $P = 0.03$; $R^2 = 0.58$, $P < 0.01$; and $R^2 = 0.65$, $P < 0.01$, respectively) of *C. viridis* in coffee plants on the other hand. In the coccids that fed on coffee plants, caffeine (3.7 ± 0.15 $\mu\text{g g}^{-1}$ fresh weight) and chlorogenic acid (1.9 ± 0.03 $\mu\text{g g}^{-1}$ fresh weight) were found, whereas neither of these compounds could be detected in coccids fed on citrus leaves.

Coccid mortality was similar in leaves that were treated or untreated with any of the phytochemicals tested ($F_{3,20} = 1.62$, $P = 0.13$). Caffeine and chlorogenic acid applied on coffee leaves stimulated the locomotory activity

Table 1 Repeated measures ANOVA for concentrations of caffeine, caffeic acid, and chlorogenic acid in leaves of coffee plants infested by *Coccus viridis* or kept uninfested

Source of variation	d.f.	F	P
Caffeine			
Treatment	1,16	115.07	<0.0001
Time ¹	3,48	17.55	<0.0001
Treatment*time	3,48	20.27	<0.0001
Caffeic acid			
Treatment	1,16	109.09	<0.0001
Time ¹	3,48	30.60	<0.0001
Treatment*time	3,48	3.48	0.037
Chlorogenic acid			
Treatment	1,16	4797.69	<0.0001
Time ¹	3,48	513.27	<0.0001
Treatment*time	3,48	503.02	<0.0001

¹15, 30, 45, and 60 days.

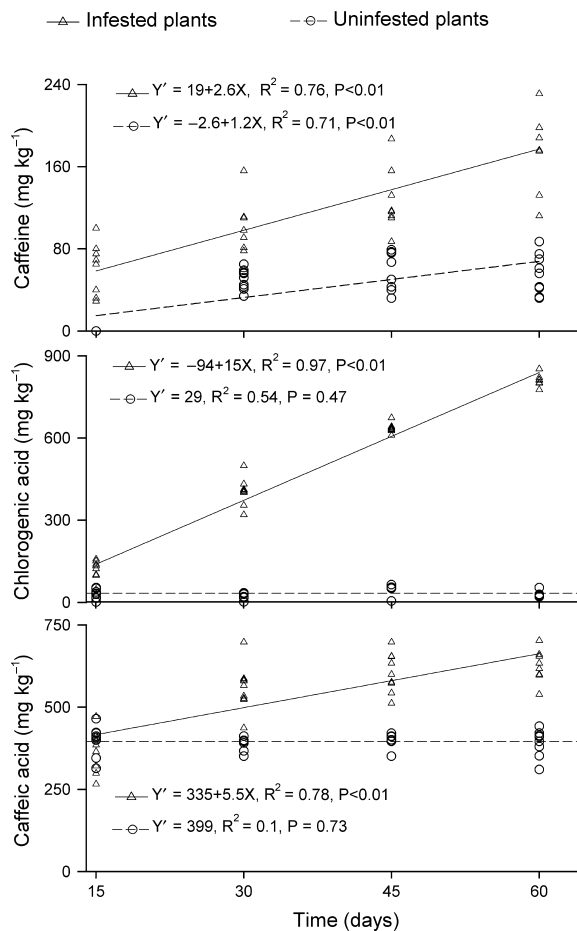


Figure 1 Changes in concentration of the alkaloid caffeine and the phenolics chlorogenic and caffeic acid, in leaves of young *Coffea arabica* plants infested by *Coccus viridis* or of uninfested plants.

of green scales, reducing their feeding on treated leaves relative to controls ($F_{3,36} = 20.71$, $P < 0.001$). No change in the locomotory behavior was observed in the coccids placed on leaves treated with caffeic acid relative to the control (Figure 2).

Discussion

Coffee plants infested with *C. viridis* accumulated higher contents of caffeine, caffeic acid, and chlorogenic acid than uninfested control plants over a period of 45 days after infestation. To our knowledge, this is the first study to document the induction of secondary coffee metabolites by a scale insect. The ability of plants to produce and accumulate secondary metabolites in response to insect feeding was discovered by biochemists and ecologists in 1970, and since then, it has interested entomologists, plant physiolo-

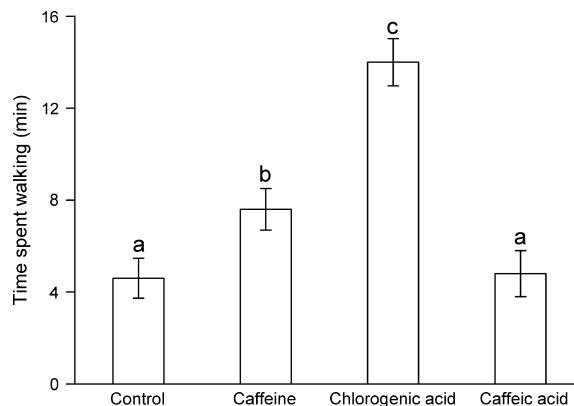


Figure 2 Time (mean \pm SE) that *Coccus viridis* first instars spent walking on coffee leaves impregnated with caffeine, chlorogenic acid, or caffeic acid. Control leaves were impregnated with distilled water + surfactant. Bars followed by the same letter are not significantly different (Tukey's test: $P > 0.05$; $n = 10$ replicates).

gists, and molecular biologists (Karban & Baldwin, 1997; Agrawal et al., 1999). Several plants species have shown induced responses to attacks of phytophagous insects (Kessler & Baldwin, 2002). In coffee, however, little is known on induced responses to insect feeding. Nevertheless, it was shown that the coffee leafminer, *Leucoptera coffeella* (Guérin-Méneville), leads to increased levels of chlorogenic acid and polyphenoloxidase activity in *C. racemosa* (Ramiro et al., 2006), but not in *C. arabica* plants and two hybrids of these species (Melo et al., 2006).

In the present study, we infested coffee plants with nymphs of *C. viridis* and harvested leaves repeatedly over time to determine the secondary compounds. As we did the same with the control plants, which were kept uninfested, the considerably elevated levels observed for the alkaloid caffeine, and the phenolics chlorogenic acid and caffeic acid were clearly induced by coccid infestation. This was especially true for the phenolics, which did not show any change in concentration over time in the uninfested plants (see Figure 1). For caffeine, however, it is likely that the significant increase in concentration over time observed in control plants was induced by the mechanical removal of leaves rather than by the coccids.

Although we know little about *C. viridis* feeding behavior, its small body size and excretion of honeydew is indicative of a phloem feeder. Plant responses following attack by these insects have been shown to be typical of pathogen attacks (Kaloshian & Walling, 2005), although to date, specific elicitors have not been identified from any phloem-feeding insects (Howe & Jander, 2008). Stylet penetration by the coccids should cause modest or barely perceptible

mechanical damage, but their salivary secretions could be recognized by the plant (Kaloshian & Walling, 2005). Hemipteran saliva contains several compounds (Miles, 1999; Cherqui & Tjallingii, 2000), including detoxifying enzymes and other compounds that can facilitate movement of the stylet among cells and maintenance of continuous feeding and even overcome plant responses (Howe & Jander, 2008; Walling, 2008). Further research is needed to investigate elicitors, components of the plant defense-signaling pathways, and additional metabolic responses that are induced by *C. viridis* attack.

Phenolic compounds including chlorogenic and caffeic acid, which were strongly induced in the present study, are known for their role in plant resistance (Bennett & Wallsgrove, 1994). Chlorogenic and caffeic acid and the alkaloid caffeine are regarded as pesticidal compounds (Akazawa & Wada, 1961; Nathanson, 1984; Frischknecht et al., 1986; Appel, 1993). Recently, Leiss et al. (2009) identified chlorogenic acid as a resistance factor for thrips in chrysanthemum. Caffeine is a purine alkaloid found in several plant species (Ashihara & Suzuki, 2004), and its presence in fully developed coffee leaves is likely to result from translocation from young leaves, where it is synthesized and remains sequestered in vacuoles (Aerts & Baumann, 1994; Fujimori & Ashihara, 1994; Ashihara, 2006). The plant location and biological activity of caffeine from coffee led Frischknecht et al. (1986) to suggest a defensive role causing behavioral and lethal effects (Nathanson, 1984) by multiple mechanisms (Fredholm et al., 1999; Fisone et al., 2004), but more recent evidence indicates that it may also be a signaling molecule (Kim & Sano, 2008), stimulating endogenous defense systems of plants through direct or indirect activation of gene expression.

As expected, caffeine and chlorogenic acid were detected in homogenates of coccids that fed on coffee plants, indicating that these compounds are likely to be present in the phloem. These results are in agreement with previous studies reporting the presence of these compounds in the xylem and phloem of coffee plants (Hamidi & Wanner, 1964; Mazzafera & Gonçalves, 1999; Mondolot et al., 2006).

Although no lethal effect of the induced phenolics and alkaloids was observed on *C. viridis* within a 24-h exposure, first instars (crawlers) were irritated (Lockwood et al., 1984) by these compounds as evidenced by the longer time that they moved around on leaves treated with caffeine and chlorogenic acid relative to the control. Calatayud et al. (1994) verified a deterrent effect of caffeine on *Phenacoccus manihoti* Matile-Ferrero in cassava plants as did Dreyer & Jones (1981) for *Schizaphis graminum* (Ron-dani) and *Myzus persicae* (Sulzer). In coffee, caffeine is not associated with resistance against *L. coffeella* or the curcu-

lionid *Hypothenemus hampei* Wood & Bright (Guerreiro Filho & Mazzafera, 2000, 2003), although this alkaloid and other phenolics may play other roles in the interaction between coffee plants and their herbivorous insects (Magalhães et al., 2008a,b, 2010). Specifically for *C. viridis*, chlorogenic acid and caffeine seem to affect this generalist insect by stimulating the locomotion of crawlers, thus reducing their feeding, as we observed in our tests. Additionally, these behavioral changes may affect the population dynamics of the green scale by increasing the risks of predation and death by other natural mortality factors in coffee agroecosystems (Pereira et al., 2007; Jha et al., 2009).

In summary, this research shows that *C. viridis* induces responses in coffee plants, which ultimately increases the concentrations of caffeine, caffeic acid, and chlorogenic acid. These alkaloid and phenolic compounds do not cause coccid mortality, but rather they seem to affect this generalist insect by stimulating the locomotion of crawlers. This study is the first to show the induction of coffee secondary metabolites by scale insects, and our findings should open up future research related to this induced response, especially regarding the mechanisms involved in the induction, its role in plant defense against *C. viridis*, and its interaction with other organisms associated with the coffee agroecosystem.

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