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# Polyphenoloxidase activity in coffee leaves and its role in resistance against the coffee leaf miner and coffee leaf rust

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**Abstract**

In plants, PPO has been related to defense mechanism against pathogens and insects and this role was investigated in coffee trees regarding resistance against a leaf miner and coffee leaf rust disease. PPO activity was evaluated in different genotypes and in relation to methyl-jasmonate (Meja) treatment and mechanical damage. Evaluations were also performed using compatible and incompatible interactions of coffee with the fungus *Hemileia vastatrix* (causal agent of the leaf orange rust disease) and the insect *Leucoptera coffeella* (coffee leaf miner). The constitutive level of PPO activity observed for the 15 genotypes ranged from 3.8 to 88 units of activity/mg protein. However, no direct relationship was found with resistance of coffee to the fungus or insect. Chlorogenic acid (5-caffeoylquinic acid), the best substrate for coffee leaf PPO, was not related to resistance, suggesting that oxidation of other phenolics by PPO might play a role, as indicated by HPLC profiles. Mechanical damage, Meja treatment, *H. vastatrix* fungus inoculation and *L. coffeella* infestation caused different responses in PPO activity. These results suggest that coffee resistance may be related to the oxidative potential of the tissue regarding the phenolic composition rather than simply to a higher PPO activity.


*Keywords:* *Coffea*; Coffee; Rubiaceae; *Hemilea vastatrix*; *Leucoptera coffeela*; Phenolic compounds; Plant resistance; Polyphenoloxidase

## 1. Introduction

Polyphenoloxidase – PPO (EC 1.14.18.1 or EC 1.10.3.2) is an enzyme of broad distribution among plants, that catalyzes the hydroxylation of monophenols to *o*-diphenols and their oxidation to *o*-diquinones (Mayer and Harel, 1979). Quinones are highly reactive molecules that can spontaneously complex various types of molecules into large structures, including proteins, lipids, nucleic acids and carbohydrates.

The physiological function of PPO in plant cells is still unknown. A few reports have suggested a specific physiological function for PPO. Cho et al. (2003) showed that a PPO was responsible for the conversion of (+)-larreatricin into (+)-3′-hydroxylarreatricin in *Larrea tridentata* and that the conversion was specific for the (+)-enatiomer. Most of the reports on PPO indicate a function to defend plants against pathogens and insect attack. Recently, Li and Steffens (2002) have obtained direct evidence of such a role for PPO in plants. These authors observed that transgenic plants of tomato overexpressing PPO had a higher oxidizing capacity and displayed increased resistance to *Pseudomonas syringae* pv. tomato.

The mode of action proposed for PPO is based on its capacity to oxidize phenolic compounds when the tissue is damaged. In this situation, the rupture of plastids, the cellular compartment where PPO is located, leads to the enzyme coming into contact with the phenolic compounds released by rupture of the vacuole, the main storage organelle of these compounds (Mayer and Harel, 1979).

The quinones formed may act in several ways leading to the protection of plants: (1) through their high capacity for reacting with other cellular compounds, quinones can limit

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the development of diseases at the infected sites; accelerating the cellular death of cells close to the infection site; preventing the advance of infection and/or by generating a toxic environment which will inhibit the growth of the pathogen inside the cells (Bi and Felton, 1995); (2) through their ability to alkylate proteins, mainly by becoming covalently linked to amino acids susceptible to alkylation, such as lysine, histidine, cysteine and methionine, thereby reducing the bioavailability of such proteins (Felton et al., 1989, 1992); (3) and also by being able to react with other phenolic compounds; increasing the formation of polymers, covalent linkages and condensation with more proteins, leading to additional barriers that can prevent the pathogens from entering the cell.

The fact that PPO activity can be induced by abiotic and biotic factors, such as damage caused by herbivory, fungal and bacterial infections, mechanical damage, insect regurgitation and by treatments with compounds which signal the octadecanoid pathway, is additional evidence of involvement of PPO in plant defense mechanisms. This response has been observed in expression of other genes related to plant defense (Corbin et al., 1987; Farmer and Ryan, 1992; Warner et al., 1995), and constitutes an evolutionary strategy of plants for defending themselves against predators.

Few studies have related PPO and defense mechanisms in coffee plants. Maxemiuc-Naccache and Dietrich (1985) and Mazzafera et al. (1989) suggested PPO involvement in coffee tree resistance towards the fungus *Hemileia vastatrix*, the causal agent of coffee leaf orange rust, and the root nematode *Meloidogyne incognita*. Ramiro (2003), however, found no evidence of PPO involvement in resistance against the insect *Leucoptera coffeella*, a miner of coffee leaves.

5-Caffeoylquinic acid (5CQA) [chlorogenic acid] (Clifford, 1985) is the most efficient substrate of PPO in coffee (Mazzafera and Robinson, 2000). Its content is fairly high in the coffee plant, where it may account for about 11% and 1% of the dry matter of fruits and leaves, respectively (Clifford, 1985; Sartor and Mazzafera, 2000). In coffee, the constitutive level of PPO is also relatively high when compared to other plant species and PPO activity is induced by treatment with methyl-jasmonate (Meja) and by mechanical damage (Mazzafera and Robinson, 2000). According to Felton et al. (1989), the concomitance of PPO and diphenols is an indication that phenolic oxidation mediated by this enzyme is a potential defense mechanism in plants.

The objective of the present study was to investigate a possible relationship between PPO activity in coffee leaves and the resistance to *H. vastatrix* and *L. coffeella*. To achieve this, experiments were carried out to evaluate the activity of the enzyme in several coffee species and varieties differing in their resistance to the disease and insects and in response to the treatment with Meja and mechanical damage. Also, evaluations were performed in compatible and incompatible interactions of coffee with the fungus and insect.

## 2. Results

PPO activity was analyzed in leaves of several coffee species differing in their resistance to coffee leaf rust disease caused by *H. vastatrix* and the coffee leaf miner *L. coffeella*. Among the coffee genotypes analyzed, *C. dewevrei, C. salvatrix, C. brevipes, C. stenophylla, C. kapakata, C. racemosa, C. liberica* and *C. eugenioides* are regarded as resistant to the coffee leaf miner (Guerreiro Filho et al., 1991; Guerreiro Filho and Mazzafera, 2000). *Coffea arabica* cv. Mundo Novo and *C. canephora* were susceptible to this pest, and the hybrid lines H1023 and H1036 are susceptible and resistant to the leaf miner, respectively. Regarding leaf rust, *C. arabica* cv. Mundo Novo is susceptible (Eskes, 1982) and the genotype Híbrido do Timor (HT) is considered

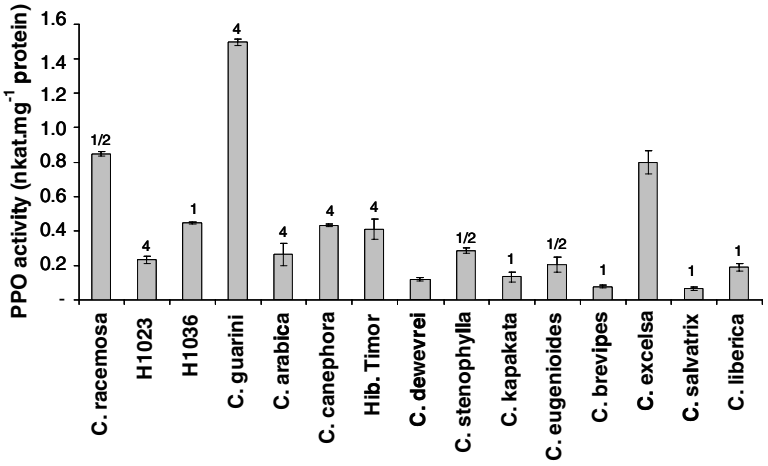


Fig. 1. PPO activity in coffee tree leaves. Mean of three replicates. Bars represent SD. The numbers on the top of the columns indicate the levels of resistance/susceptibility to the coffee leaf miner (Guerreiro Filho and Mazzafera, 2000): 1 = resistant; 2 = moderately resistance; 3 = moderately susceptible; 4 = susceptible.

resistant (Eskes et al., 1990). Mundo Novo is also susceptible to nematodes (Mazzafera et al., 1989).

Fig. 1 shows the constitutive level of the PPO activity observed for the 15 genotypes, ranging from 0.065 in leaves of *C. salvatrix* to 1.496 nkat $mg^{-1}$ of protein in leaves of *C. guarini*. The resistance levels observed by Guerreiro Filho and Mazzafera (2000) against the coffee leaf miner based on the size and type of lesion (scale 1–4, from susceptible to resistant; Guerreiro Filho, 1994) are also indicated in the figures.

PPO was tested for specificity of several substrates and without exception, 5CQA was determined as the best substrate for all tested coffee genotypes. However, good activity was also observed with 4-methylcatechol (4MC), caffeic acid and catechol (Table 1). DOPA, tyrosine, coumaric acid and hydroxyquinone showed some activity with a few species.

A great variation was observed for the phenolic contents in the leaves of the coffee species. The levels of total phenolic compounds varied from 92.9 in *C. racemosa* to

Table 1
Affinity of PPO of coffee tree leaves with different substrates

| Coffee genotype | 5-Caffeoyl quinic acid | 4-Methyl cathecol | Caffeic acid | Cathecol | 3-Hydroxy tyrosine | Tyrosine | Coumaric acid | Hydroxy quinone |
|---|---|---|---|---|---|---|---|---|
| *C. guarini* | 1.496 | 1.210 | 0.235 | 0.753 | 0.054 | 0.017 | 0.010 | – |
| *C. excelsa* | 0.850 | 0.666 | 0.133 | 0.595 | 0.065 | 0.010 | 0.007 | 0.007 |
| *C. racemosa* | 0.848 | 0.505 | 0.102 | 0.376 | – | – | – | – |
| H1036 | 0.447 | 0.422 | 0.095 | 0.172 | 0.039 | – | – | – |
| *C. canephora* cv. Conilon | 0.434 | 0.304 | 0.070 | 0.128 | 0.009 | – | – | – |
| *C. stenophylla* | 0.287 | 0.338 | 0.065 | 0.109 | 0.032 | – | 0.010 | – |
| *C. arabica* cv. Mundo Novo | 0.264 | 0.321 | 0.077 | 0.100 | 0.015 | – | – | – |
| HT | 0.241 | 0.230 | 0.097 | 0.168 | 0.036 | – | – | – |
| H1023 | 0.231 | 0.173 | 0.034 | 0.070 | 0.012 | – | – | 0.012 |
| *C. eugenioides* | 0.206 | 0.138 | 0.032 | 0.075 | 0.010 | – | – | – |
| *C. liberica* | 0.190 | 0.139 | 0.024 | 0.105 | 0.007 | – | – | – |
| *C. kapakata* | 0.133 | 0.383 | 0.105 | 0.265 | 0.029 | 0.014 | – | – |
| *C. dewevrei* | 0.117 | 0.112 | 0.020 | 0.058 | – | – | – | – |
| *C. brevipes* | 0.078 | 0.054 | 0.007 | 0.037 | – | – | – | – |
| *C. salvatrix* | 0.065 | 0.036 | 0.003 | 0.003 | – | – | – | – |

The activity is expressed in nkat $mg^{-1}$ protein. Mean of three replicates. Dash indicates undetected activity.

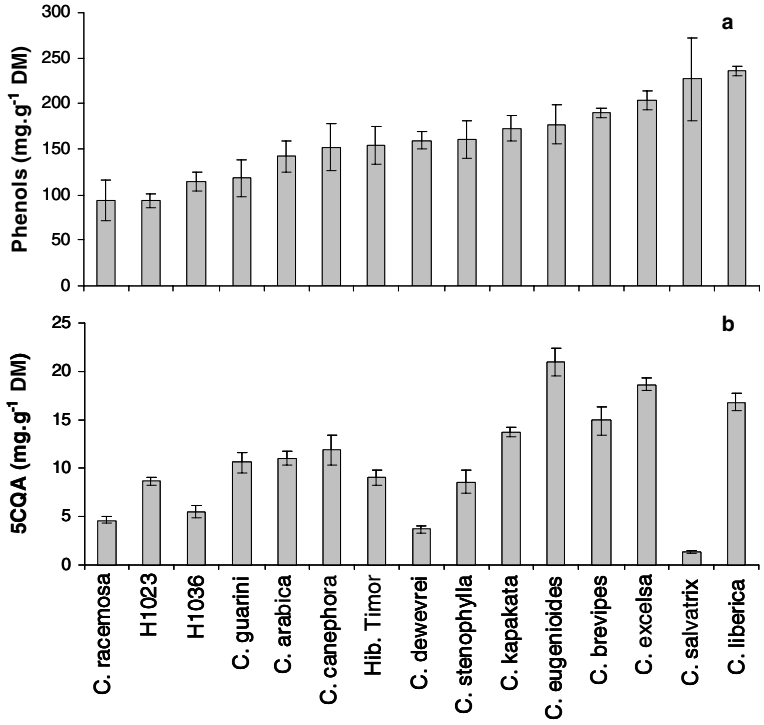


Fig. 2. Level of total phenolic compounds (a) and 5CQA (b) in coffee tree leaves. Mean of three replicates. Bars represent SD.

235.5 mg $g^{-1}$ in *C. liberica* (Fig. 2(a)) and the levels of 5CQA varied from 1.39 mg $g^{-1}$ in *C. salvatrix* to 21.01 mg $g^{-1}$ in *C. eugenioides* (Fig. 2(b)).

Mechanical damage and exposure to Meja in *C. arabica* cv. Mundo Novo, HT, *C. racemosa* and *C. salvatrix* led to varied responses in the PPO activity. These species were chosen for induction treatments firstly because *C. arabica* is susceptible to both rust and leaf miner, HT is resistant to rust and *C. salvatix* and *C. racemosa* are resistant to the leaf miner. Second, because the chromatographic profiles obtained with the methanolic extracts from *C. arabica* cv. Mundo Novo and HT were similar, but they presented marked differences with the profiles obtained for *C. racemosa* and *C. salvatrix* (Fig. 3).

Leaves of *C. arabica* cv. Mundo Novo did not show significant variation with Meja exposure or mechanical damage (Fig. 4(a)). On the other hand, the PPO activity in leaves of *C. racemosa* and HT was significantly induced in at least one of the treatments (Fig. 4(b) and (c)). The variation in relation to the control reached about 30% for *C. racemosa* leaves submitted to mechanical damage and about 45% for HT leaves treated with Meja. In *C. salvatrix*, the variation of activity reached about 85% for damaged leaves and about 225% in leaves treated with Meja (Fig. 4(c)). Still in the same experiment, the contents of total phenolic compounds were different from the controls only in leaves of *C. racemosa* treated with Meja, being higher in the control (Fig. 5).

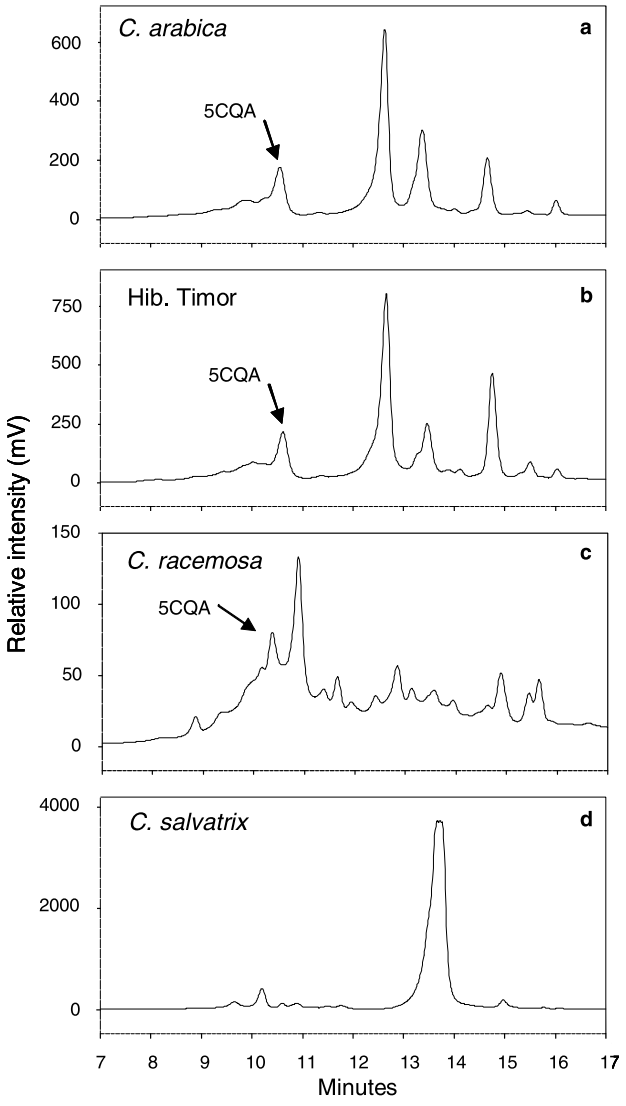


Fig. 3. Chromatographic profile (HPLC) for phenolic compounds extracted from leaves of *C. arabica* (a), Híbrito do Timor (b), *C. racemosa* (c) and *C. salvatrix* (d).

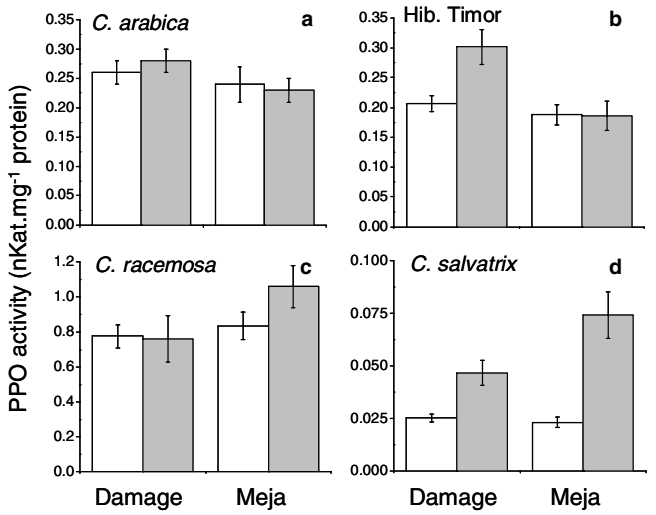


Fig. 4. PPO activity in coffee leaves of *C. arabica* (a), Híbrito do Timor (b), *C. racemosa* (c) and *C. salvatrix* (d) subjected to mechanical damage and treatment with Meja. Controls = open columns. Mean of four replicates. Bars represent SD.

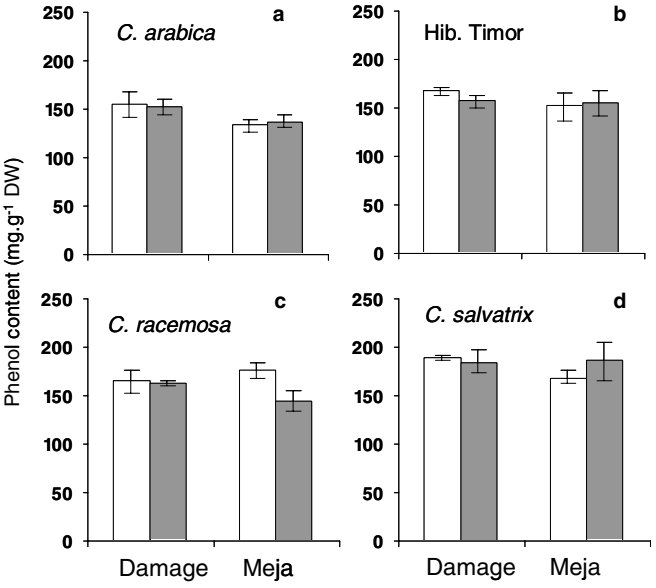


Fig. 5. Levels of total phenolic compounds in coffee leaves *C. arabica* (a), Híbrito do Timor (b), *C. racemosa* (c) and *C. salvatrix* (d) submitted to mechanical damage and treatment with Meja. Controls = open columns. Mean of four replicates. Bars represent SD.

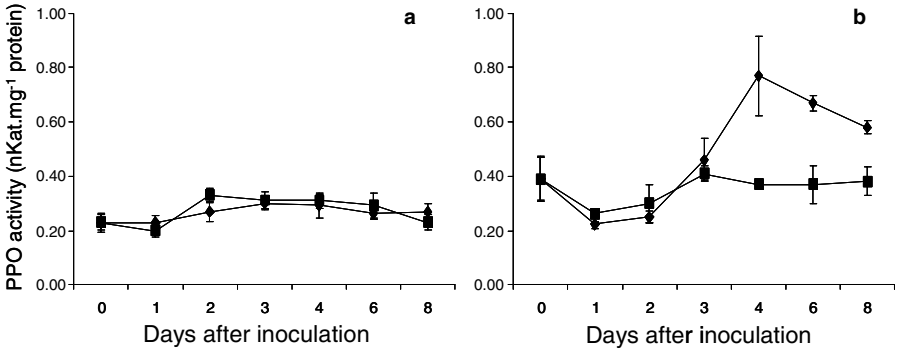


Fig. 6. PPO activity in coffee leaves of *C. arabica* (a) and Híbrito do Timor (b) inoculated (◆) and non-inoculated (■) with spores of *H. vastatrix*. Mean of four replicates. Bars represent SD.

When leaves of *C. arabica* cv. Mundo Novo and HT were inoculated with spores of *H. vastatrix* (Fig. 6), PPO activity was significantly induced only in the later genotype (Fig. 6(b)). This was observed from the fourth day on after inoculation.

The infestation of leaves of *C. arabica* cv. Mundo Novo and the hybrids H1023 and H1036 with the leaf miner did not cause a consistent increase of the PPO activity (Fig. 7).

It is interesting to observe that in both inoculation with rust and infestation with the leaf miner there was an unexpected trend to decrease PPO activity in *C. arabica* cv. Mundo Novo and HT. This was also observed with the control leaves.

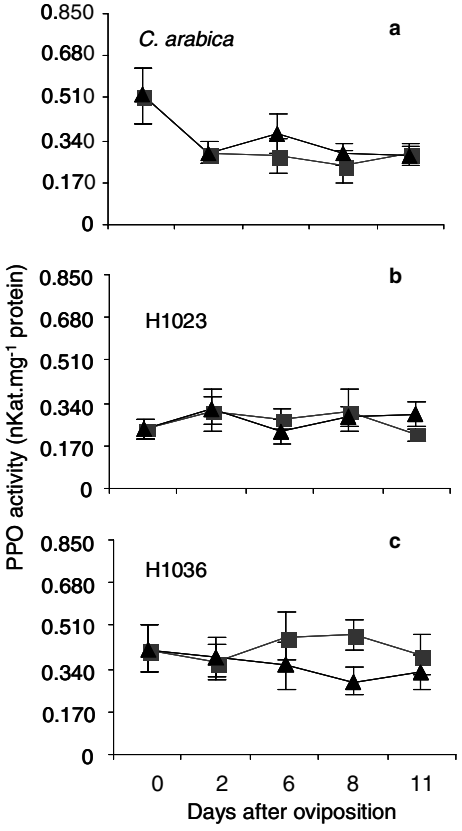


Fig. 7. PPO activity in coffee leaves of *C. arabica* (a) and the hybrids H1023 and H1036 infested (▲) and uninfested (■) with *L. coffeella*. Mean of five replicates. Bars represent SD.

## 3. Discussion

In several studies, higher levels of PPO activity have been associated with the resistance of plants to insects and pathogens (Thipyapong et al., 1995; Constabel et al., 2000; Haruta et al., 2001; Li and Steffens, 2002; Shimizu, 2004). This has been discussed both in terms of the constitutive levels of activity as well as in relation to the induction of enzyme activity.

In the coffee genotypes studied here, a great variation in the constitutive levels of the PPO activity was observed. From the lowest to the highest value of activity observed, there was a 10-fold variation (Fig. 1). Considering the characteristics of these genotypes in relation to the resistance to the coffee leaf miner and coffee leaf orange rust no relationship could be established with the constitutive PPO activity. That is, the genotypes with some degree of resistance reported for the coffee rust or leaf miner (*C. dewevrei*, *C. salvatrix*, *C. brevipes*, *C. stenophylla*, *C. eugenioides*, *C. liberica*; *C. kapakata* and *C. racemosa*, HT and hybrid H1036) showed PPO activity among the lowest as well as among the highest values. This is also true for the susceptible genotypes (*C. arabica* cv. Mundo Novo, *C. guarini*, *C. canephora* cv Conilon and hybrid H1023).

For most of the species studied here we used the same species used by Guerreiro Filho and Mazzafera (2000) in their study on the role of caffeine in the resistance of coffee to the coffee leaf miner. Selecting only the common plants, an analysis of the correlation between PPO activity and the damage caused by the leaf miner (data drawn from Guerreiro Filho and Mazzafera, 2000), resulted in $r$(Pearson) = 0.44, $p = 0.1749$. These results suggest that PPO is not involved in the resistance against the coffee leaf miner at least when the constitutive activity is considered.

The mode of action proposed for PPO, however, is based on the capacity of that enzyme to oxidize *o*-dihydroxyphenols to *o*-quinones when the tissue is attacked. The quinones, in their turn, may act in several ways leading

to the protection of the plants. Thus, the efficient action of PPO depends on other factors such as the levels of its most active substrates and the quantity and quality of proteins present in the plant, which ultimately will lead to a higher effect of the formed quinones (Felton et al., 1992).

Except for *C. racemosa* and H1036, the other resistant plants showed higher levels of total phenolic compounds while susceptible plants showed lower levels (Fig. 2(a)). The correlation coefficient – *r*(Pearson) – between the level of total phenolic compounds observed and the resistance to the leaf miner (data drawn from Guerreiro Filho and Mazzafera, 2000) was $-0.443$, $p = 0.1726$. However, when *C. racemosa* was not included in the analysis, $r$ was $-0.746$, $p = 0.0132$, indicating differentiated behavior for that genotype. Since the constitutive levels of PPO activity in coffee are relatively higher than in other species (Mazzafera and Robinson, 2000), the absolute level of PPO activity may be irrelevant with regard to resistance in coffee. However, PPO action becomes important when analyzed in terms of the oxidative capacity of the tissue, based on the levels of total phenolic compounds and resistance.

Despite the greater preference for 5CQA as a substrate, other phenolics were good substrates for PPO (Table 1). On the other hand, there was no significant relationship between the levels of total phenolic compounds and 5CQA ($r$(Pearson) $= 0.38$, $p = 0.1618$). This suggests that other phenols may have greater importance than 5CQA in the oxidation of phenols by PPO in the process of resistance to pest and diseases. The different chromatographic profiles of the methanolic extracts obtained by HPLC suggest that (Fig. 3) even if 5CQA were the best PPO substrate, other phenols present in higher quantity may have a higher relative importance with regard to resistance. The identification of these compounds such as, for example, in the case of *C. salvatrix* (Fig. 3), which produced virtually a single peak in the phenolic extracts could corroborate or strengthen this suggestion.

In general, PPO is codified by more than one gene (Newman et al., 1993; Thygesen et al., 1995) and isoforms of this enzyme are observed in several plants (Constabel et al., 2000; Wang and Constabel, 2003). Substrate specificity can be a distinctive characteristic between the isoforms and, as suggested by Wang and Constabel (2003), this characteristic may be associated with functional differences of the enzyme, including a role in defense mechanisms of plants. These authors studied the expression of two PPO isoforms of the *Populus tremuloides* leaf and observed that beside different specificity for substrate, the isoform with lower specificity was expressed only after induction by mechanical damage.

It was observed that the PPO activity of coffee leaves can be induced in some genotypes but not in others. It was also observed that the induction is dependent on the inducer (Fig. 4). In *C. racemosa*, HT and *C. salvatrix*, genotypes that presented some sort of resistance, at least one treatment led to induction of PPO. In *C. arabica* cv. Mundo Novo, a genotype characteristically susceptible to orange rust and the coffee leaf miner, no response was observed by wounding or exposing detached leaves to Meja.

However, one may argue that the leaf detachment itself might have caused an increase of coffee PPO masking the inducers effect. In a similar way, Mazzafera and Robinson (2000) wounded or exposed coffee leaves attached to the plants to Meja observing a limited increase of PPO. Among four plants exposed to Meja only one showed an increase of PPO activity. On the other hand, wounded leaves showed a discrete but consistent increase of activity as we observed here for most of the treated coffee species, except *C. racemosa*.

Leaf detachment might also affect the natural resistance of the coffee leaves and, therefore, in some way influence the results observed with the leaf miner infestation and rust infection (Figs. 6 and 7). Guerreiro Filho et al. (1991) and Guerreiro Filho (1994) used several coffee species to validate results obtained with detached coffee leaves by comparisons with field and nursery evaluations for leaf miner resistance. These authors carried out these evaluations by infesting the coffee leaves with the pest and measuring the leaf area damaged by the larvae. The data were positively correlated with a scale ranging from 1 (resistant) to 4 (susceptible). In the same studies it was demonstrated that *C. arabica* cv. Mundo Novo and *C. canephora* were susceptible to this pest. The same procedure also showed that the hybrid lines H1023 and H1036 are susceptible and resistant to the leaf miner, respectively (Guerreiro Filho, personal communication).

*Coffea arabica* cv. Mundo Novo is regarded as being susceptible to the leaf rust (Eskes, 1982) while the genotype HT is considered resistant (Eskes et al., 1990). For such evaluation plants were inoculated in the field and in the nursery and the results were positively correlated with leaf disks inoculated in the laboratory and maintained in plastic boxes as we used here.

The inoculation of coffee tree leaves with spores of fungus *H. vastarix* caused an increase in PPO activity in the resistant genotype HT (Fig. 6). An unequivocal role of PPO against a disease was recently shown by overexpressing (Li and Steffens, 2002) or suppressing PPO (Thipyapong et al., 2004) in tomato. Tomato overexpressing a potato PPO or carrying an antisense potato PPO cDNA showed a respective increase of resistance or susceptibility to *P. syringae*. Other studies have shown a possible role of PPO against bacterial diseases (Bashan, 1986; Bashan et al., 1987; Khirbat and Jalali, 1998), however, few were carried out with fungi and a strong correlation between resistance and PPO activity could not be established (Montalbini et al., 1981).

After spore germination, the hypha of coffee rust spores penetrates the leaf by the stomata aperture. Under optimal temperature conditions a branched mycelium ramifies through the intercellular spaces of the mesophyll cells 96 h post-inoculation and plant cell disruption occurs in susceptible coffee plants (Coutinho et al., 1993). On other hand, in resistant coffee plants fungal growth does not proceeds after haustoria formation (Silva et al., 2002).

PPO is known to be activated by cell damage; however, even after 8 days of rust inoculation, it was not possible to observe an increase in enzyme activity in leaves of susceptible *C. arabica* cv. Mundo Novo. On the other hand, an increase was observed with the resistant HT after four days inoculation (Fig. 7(b)). PPO in tomato is coded by 7 genes and Thipyapong et al. (2004) observed that expression of four of them was induced by infection with *P. syringae*. We have isolated 14 PPO cDNAs from coffee leaves, roots and seeds which could be clustered in four groups according with their similarities (Mazzafera and Robinson, unpublished results). Studies on their expression might provide some information on the role of PPO in the HT resistance to the coffee leaf rust. However, preliminary semi-denaturing PAGE with activity staining for PPO did not show any modification in the banding profile in this coffee hybrid (data not shown).

The induction of PPO activity by abiotic and/or biotic factors has been considered as evidence of PPO action in defense mechanisms of plants (Mayer and Harel, 1979; Felton et al., 1989; Thipyapong et al., 1995; Constabel and Ryan, 1998; Constabel et al., 2000; Haruta et al., 2001). Constabel and Ryan (1998) analyzed the induction of PPO by mechanical damage and by Meja in 18 plant species and they observed a great variation in the responses among the species. For these authors, this is indicative of specialization of mechanisms. For example, cotton plants (*Capsicum annum*) and pea plants (*Medicago sativa*) accumulated significant levels of protease inhibitors, a defense factor, when damaged or treated with Meja. However, the PPO activities in these species were not induced under by Meja or mechanical damage.

The variation observed here for PPO induction by abiotic and biotic factors is probably related to different mechanisms of action involving the enzyme. This may explain the values of PPO activity and total phenolic compounds observed for *C. racemosa* and H1036. Contrary to the other resistant genotypes, they showed high levels of PPO and low levels of total phenolic compounds.

## 4. Conclusion

Based on the results observed here, we concluded that the coffee tree resistance may be related to the oxidative potential of the tissue regarding the phenolic composition rather than simply to a higher PPO activity, and that among the genotypes there may be a specialization of resistance mechanisms involving PPO action.

## 5. Experimental

### 5.1. Plant material

Coffee leaves were collected from coffee plants of the Germoplasm Bank of the Agronomic Institute of Campinas, Campinas, São Paulo State, Brazil. These include the species *C. arabica* cv. Mundo Novo, *C. kapakata*, *C. dewevrei*, *C. salvatrix*, *C. racemosa*, *C. canephora* cv. Conilon, *C. brevipes*, *C. excelsa*, *C. guarini*, *C. liberica*, *C. eugenioides* and *C. stenophylla*. The Hybrid of Timor (HT), a natural hybrid of the species *C. arabica* and *C. canephora*, was also analyzed as well as two hybrids resulting from the cross *C. arabica* × *C. racemosa*, denominated H1023 and H1036.

### 5.2. PPO activity in leaves of different genotypes

Leaves from *C. arabica* cv. Mundo Novo, *C. kapakata*, *C. dewevrei*, *C. salvatrix*, *C. racemosa*, *C. canephora* cv. Conilon, *C. brevipes*, *C. excelsa*, *C. guarini*, *C. liberica*, *C. eugenioides*, *C. stenophylla*, HT, H1023 and H1036 were used in this experiment. For each genotype, leaves from the second and third fully-expanded pairs were collected and protein extracts obtained on the same day of collection. The evaluations were carried out in triplicate, each replicate being composed of three leaves. All leaves were collected from a single plant.

### 5.3. PPO activity in leaves submitted to mechanical damage and treated with Meja

Freshly collected leaves from *C. arabica*, *C. racemosa*, *C. salvatrix* and HT were submitted to mechanical damage and to treatment with Meja. Mechanical damage was inflicted with a pin, producing perforations on both surfaces of the leaves with approximately 15 perforations per $cm^2$. The damaged and undamaged leaves were placed in humidity chambers kept under laboratory conditions. The humidity chambers were made out of plastic trays containing moist foam and covered with a glass plate. Forty-eight hours after being damaged, the leaves were collected and the protein extracts obtained.

In the treatment with Meja, the leaves were placed in humidity chambers as described, but without the glass cover. These chambers were placed inside plastic bags, which were then sealed. Meja (50 μl) was applied to a small piece of cotton wool fixed with adhesive tape to the inner wall of the plastic bag. Meja was applied at the beginning of the experiment and again 24 h later (another 50 μl). Subsequently, 24 h after the second application of Meja, that is, 48 h after the beginning of the treatment, the leaves were removed and the protein extracts obtained. As a control, leaves were submitted to the same conditions, that is, placed in humidity chambers, but with no application of Meja.

In this trial, four replicates were carried out, each being composed of 3 leaves. All leaves were collected from a single plant.

### 5.4. PPO activity in interactions with the fungus Hemileia vastatrix

In a study of interactions with *H. vastatrix*, leaves from HT (resistant) and *C. arabica* cv. Mundo Novo (susceptible)

were collected from adult plants growing in the field using the second and third fully-expanded pairs, and inoculated with a suspension of spores (1 mg $ml^{-1}$) of the fungus presenting 60% of germination in distilled water (Eskes, 1982). The germination test was performed mixing a small amount of spores with about 100 μl of distilled $H_2O$ placed on a microscopic slide. The slide was then placed in a humidity chamber in the dark for 24 h when the germination of spores was evaluated by counting with the aid of a stereomicroscope. Spores with the emission of well-developed germination tubes were considered germinated.

Inoculation was carried out by spraying the spore suspension in water on the abaxial surface of the leaves. After inoculation, the leaves were placed in humidity chambers, as previously described, and kept in the dark during the first 24 h (Mazzafera and Magalhães, 1989). Thereafter, the chambers were kept under a 12 h photoperiod at room temperature. During the period of the experiment, the leaves were sprayed daily with distilled $H_2O$ and the humidity of the chamber maintained by adding $H_2O$ to the foam. Controls were carried out with non-inoculated leaves and submitted to the same conditions as the inoculated ones. Samples for protein extraction were collected at 0, 2, 3, 4, 6 and 8 days after inoculation.

Four replicates were used, each composed of three leaves. All leaves were collected from a single plant.

### 5.5. PPO activity in interactions with Leucoptera coffeella

Freshly collected leaves from *C. arabica* cv. Mundo Novo and from the hybrids H1036 and H1023 were placed in an insect growth cage overnight for oviposition (Guerreiro Filho et al., 1999). During this period the peduncle of the leaves was kept immersed in Eppendorf tubes filled with $H_2O$. The next day the leaves were removed from the cages and placed in humidity chambers, which were kept under a 12 h photoperiod at room temperature throughout the experimental period. Controls were carried out with leaves not submitted to infestation. Protein extraction of the leaves was carried out on samples collected at times 0, 2, 6, 8 and 11 days after oviposition. Five replicates were used, each composed of one leaf. All leaves were collected from a single plant.

### 5.6. Extraction and determination of PPO activity

The extraction and the assays of the PPO activity were carried out following the method described by Mazzafera and Robinson (2000). The leaves were cut in small pieces and ground in a Polytron (Kinematika GmbH) homogenizer, at speed 5 for 10 s, in extraction buffer (100 mM Na–Pi buffer, pH 7, containing 2% ascorbic acid, 5 mM dithioerythritol and 20% PVPP, using 5 ml per 1 g of leaves). After centrifugation (25,000*g* for 20 min at 4 °C), 2.5 ml of the supernatant were desalted on mini PD-10 Sephadex G25 columns (Pharmacia), using 50 mM Na–Pi buffer, pH 6, as the elution buffer. Protein concentration in the extracts was determined using an Amersham-Pharmacia ready-to-use Bradford reagent (Bradford, 1976). Bovine serum albumin was used as standard.

The PPO activity was determined measuring $O_2$ consumption with an $O_2$ electrode (Hansatech) at 25 °C. The reaction mixture, with a final volume of 1.0 ml, contained 1 mM 5-Cafeoylquinic acid (5CQA), 3.5 mM SDS and 50 mM Na–Pi buffer, pH 6. The reaction was started by the addition of 5CQA and the rate of $O_2$ consumption over the first minute was used to calculate the enzyme activity.

Reactions were also performed in which the caffeic acid, 4-methylcatechol, catechol, L-DOPA (3-hydroxytyrosine), tyrosine, *p*-coumaric acid and hydroquinone were used as substrates. All these substrates were used at a final concentration of 1 mM. The activity was expressed in nkat $mg^{-1}$ protein.

### 5.7. Extraction and analysis of total phenolic compounds and 5CQA

In some assays, the levels of total soluble phenolic compounds and 5CQA were also analyzed in the samples. Freshly (1 g) collected leaves were ground in a Polytron homogenizer with 5 ml of 70% MeOH and the extracts left for 24 h at room temperature (20–25 °C) before centrifugation at 15,000*g* for 10 min. Carrez reagent (Ky et al., 1997) was added to 1 ml of the supernatant and after centrifugation of the mixture, the clear supernatant was used for phenol determination. Total phenols were determined by the method of Swain and Hillis (1959) and 5CQA was used as standard.

5CQA content in the samples was determined by HPLC. The 70% MeOH extract was diluted in distilled $H_2O$ and 50 μl injected in the chromatography system using a Microsorb C18 column (5 μm, 4.6 × 250 mm). The compounds were separated using a linear 40 min gradient (0–80% of solvent B) at a flow rate of 1 ml $min^{-1}$. Solvent A was 0.5% aqueous HOAc and solvent B was MeOH. The compounds eluting from the column were detected by a diode array detector and 5CQA quantified at 326 nm, by comparing known amounts of pure standard used in separate chromatographic runs. In addition, chromatographic profiles of the MeOH extracts were obtained in separate runs using a 15 min gradient of 10–80% of solvent B in solvent A.

Additional samples were dried and a relative value of the dry mass was calculated in relation to the fresh mass. That value was used to calculate the dry mass of each sample analyzed multiplying it by the fresh mass used the each sample used for phenol extraction.

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