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Chilling-induced changes in membrane fluidity and antioxidant enzyme activities in *Coffea arabica* L. roots

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Abstract

Exposure of coffee to low temperatures caused growth inhibition, changes in metabolic rates, and membrane alterations. Root tissue exposed to 10 °C evolved significantly lower rates of metabolic heat compared with controls grown at 25 °C, and the values were closely associated with the observed root growth inhibition. Electron paramagnetic resonance spectra of intact tissues showed that the spin probe 5-doxylstearic acid was capable to intercalate within the cellular membrane lipids. Indeed, at the depth of the 5th carbon atoms of the alkyl chains, the nitroxide radical detected more rigid membranes in seedlings exposed to 10 °C compared with 25 °C treated samples. Ascorbate peroxidase and catalase activities did not show appreciable changes under chilling conditions, while guaiacol peroxidase activity increased 55 % compared to the control. On the other hand, glutathione reductase activity decreased, in parallel to a significant decline in the capacity to reduce triphenyl-tetrazolium. Our results showed a marked correlation between lipid peroxidation and root tissue damage, which seemed to be associated with increased membrane rigidity.

Additional key words: coffee, lipid peroxidation, EPR, stress.

Introduction

Several tropical and sub-tropical higher plant species show characteristic damage symptoms when exposed to chilling temperatures (*e.g.*, Raison and Lyons 1986). In addition to shoots, roots are also affected by chilling stress, which causes growth inhibition, root cortex breakdown (Harrington and Kihara 1960) and various

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Abbreviations: EPR - electron paramagnetic resonance; MDA - malondialdehyde; TTC - triphenyl-tetrazolim chloride.
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metabolic alterations (Criddle *et al.* 1991). These responses have been associated with decreased activity of cell membranes, eventually disruption of membrane integrity (Prasad *et al.* 1994b). The effects of chilling on cell membranes have been looked at as an oxidative stress that results in the production of highly reactive substances, such as hydrogen peroxide and oxygen free radicals (Elstner and Oswald 1994, Prasad *et al.* 1994a,b). These compounds oxidize SH groups (Bindoli 1988), and often cause peroxidation of membrane lipids (Gutteridge and Halliwell 1990), denaturation of proteins (Davies 1987), and DNA mutations (Bowler *et al.* 1992, Qiu and Liang 1995). The free-radical-induced lipid peroxidation can alter membrane functioning through changes in fluidity (Merzlyak 1994). Utilizing spin-label EPR spectroscopy, Patterson *et al.* (1978) were able to detect significant differences in lipid-phase separation temperatures in *Passiflora* genotypes, at 1 °C (the chilling tolerant) or 9 °C (the most sensitive one). In general, protection from oxidative damage requires that plants sustain complex detoxifying defense mechanisms against highly reactive compounds such as hydrogen peroxide. These processes include antioxidant enzymes of the ascorbate/glutathione cycle (Smirnoff 1993, Zhang and Kirkam 1996), which function in the removal of hydrogen peroxide from cell compartments (Cakmak *et al.* 1993).

The coffee plant (*Coffea arabica* L.) has been recognized as chilling sensitive species (Levitt 1980). Field and laboratory trials with coffee revealed that temperatures below 16 °C invariably cause impaired vegetative growth (Haarer 1963) and decreased photosynthesis (Bauer *et al.* 1985, 1990). In contrast to majority of chilling-sensitive plants, responses of coffee genotypes to low-above zero temperatures may differ (Bauer *et al.* 1985). Working with nine cultivars from Central Africa, Bauer *et al.* (1990) observed variable rates of recovery of photosynthetic activity at low temperature and, in some cases, very low, or virtually no response to hardening treatments.

The objective of the present work was the investigation of processes which presumably determine susceptibility of coffee roots to chilling temperatures, particularly those associated with alterations in cell membrane integrity. For this, time-course measurements of lipid peroxidation were carried out, in parallel with spin-label EPR evaluation of changes in membrane fluidity under chilling conditions. In addition, under comparable experimental conditions, we measured the activities of antioxidant enzymes, namely ascorbate peroxidase, catalase, guaiacol peroxidase, and glutathione reductase. Tests for tissue viability under low-temperature, utilizing microcalorimetry, and triphenyl-tetrazolium chloride (TTC) reduction, were also included.

Materials and methods

Plants: Coffee (*Coffea arabica* L. cv. Catuaí Vermelho) seeds were provided by the Plant Genetics Section, Agronomic Institute of São Paulo, Campinas, SP, Brazil. After removing the fruit endocarp (parchment) the seeds were left to imbibe in distilled water for 48 h, at 25 °C, and sown in plastic pots filled with vermiculite. The

plants were raised in growth cabinets, in the dark, at 25 °C. Water was regularly supplied by twice a week irrigation. The experiments consisted of the exposure of 30-d-old-seedlings (match stage) to continuous temperatures of 5, 10, 15, 20, and 25 °C for 6 d in darkness.

Growth measurements: In order to identify the zone of maximum elongation, the primary roots of 30 d-old-seedlings were marked with indelible Indian ink, at 1 mm intervals starting from the tip. Measurements of the distances between markings allowed calculation of the rate of root expansion in the various temperature treatments. Further, different sets of plants were exposed for 6 d to continuous temperature treatments. Afterwards, the seedlings were transferred to the control temperature (25 °C), and the capacity for resuming growth was measured at each given temperature.

Microcalorimetry: The measurements of metabolic heat rate were carried out in a heat conduction batch-mixing calorimeter (Lovrien *et al.* 1989), similar to the instrument described by Wadsö (1970). The analyses were made at 25 °C and the system was calibrated by neutralizing TRIS with HCl [$\Delta H° = -47.28$ kJ mol^{-1} (H^+ neutralized)] (Grenthe *et al.* 1970). Apical 1-mm root segments were cut, immediately weighed (30 mg f.m.) and put into the sample chamber, containing 1.0 cm^3 of phosphate buffer (pH 7.4). Equilibrium required about 15 min before each run. Manual mixing of the reaction vessel was done at 3 min intervals. The data were reported as heat flow at steady-state level, during 30 min, and the results were expressed as [$\mu W\ mg^{-1}$(f.m.)].

TTC reduction: The capacity to reduce triphenyl-tetrazolium chloride (TTC), was determined according to Burdon *et al.* (1994), with modifications. The collected root segments were washed with distilled water, blotted dry, and 30 mg (f.m.) transferred to 2 cm^3 medium containing 20 mM phosphate buffer, pH 7.0, and 1.0 % TTC. The samples were incubated for 90 min, in darkness, at 25 °C. The TTC was removed and the tissue was homogenized in 3 cm^3 95 % ethanol (v/v), for extraction of formazan produced in the reaction. The homogenate was centrifuged at 500 *g* for 5 min and the absorbance read at 485 nm. TTC reduction was expressed as μmol(formazan) g^{-1}(f.m.) (Steponkus and Lanphear 1967).

Lipid peroxidation was expressed as malondialdehyde (MDA) content, determined by the method of Buege and Aust (1978) of thiobarbituric acid reactive substances. The segments (50 mg) were washed with water, blotted dry, and homogenized in 10 mM HEPES, pH 7.0, at 4 °C. The homogenate was transferred to a medium containing 15 % (m/v) trichloroacetic acid, 0.37 % (m/v) thiobarbituric acid, 0.25 M HCl, and 50 μM butylated hydroxytoluene. The mixture was heated to 80 °C for 15 min, and then quickly cooled in an ice-bath. After centrifuging at 1000 *g* for 10 min, the absorbance of the supernatant was read at 535 nm. The concentration of MDA was calculated using the coefficient of absorbance 1.56×10^{-5} mol^{-1} cm^{-1}, and expressed as μmol(MDA) g^{-1}(f.m.).

EPR spectroscopy: The spin label derivative of stearic acid, 5-doxyl stearic acid (5-DSA) was purchased from *Aldrich Chemical Company* (Milwaukee, USA). This spin label had a nitroxide radical ring attached at C-5 position of acyl chain. A small aliquot of stock spin label dissolved in ethanol (5 mg cm^{-3}) was placed in a glass tube. The solvent was evaporated under nitrogen flux, and samples containing 20 mg (f.m.) of 1 mm root tip segments (approximately 300 segments) suspended in 0.2 cm^3 of phosphate buffer 0.2 M, pH 7.4, 5 mM EDTA, were added to the spin label thin film and incubated for 5 min, with gentle manual agitation. The root tip segments were introduced in a capillary tube utilizing a small paint brush. The final nitroxide concentration in the root segment was estimated between 1 to 3×10^{-4} M.

EPR measurements were performed on a *Varian E-9 spectrometer* (*Varian Assoc.*, Palo Alto, USA) equipped with the rectangular cavity, the temperature being controlled with a nitrogen stream system (*Air Products and Chemicals*, Allentown, USA). EPR spectra were obtained at X-band (9.15 GHertz) with microwave power of 20 mW, modulation frequency 100 kHz and amplitude 2.5 Gauss. The sweep time was 2 min and magnetic field scan 100 Gauss (Alonso *et al.* 1997).

In general, the fluidity of the membrane can be estimated from the order parameter S determined according to Gaffney (1976). The calculation uses an expression which includes the apparent parallel and perpendicular hyperfine splitting parameters of the spectrum (Fig. 3) and an empirical correction factor for the difference between of the true and apparent polarity.

Enzyme assays: Measurement of enzyme activity was carried out according to Burdon *et al.* (1994), with modifications. Approximately 200 mg (f.m.) of root segments were sampled and immediately homogenized, at 4 °C, in 5 cm^3 extraction medium containing 50 mM phosphate buffer, pH 7.8, 0.2 mM EDTA, 0.1 % (m/v) bovine serum albumin (BSA), 0.1 % (m/v) ascorbate, 0.05 % (m/v) β-mercapto-ethanol, 0.2 % (v/v) Triton X-100, and 0.5 % (m/m) polyvinylpyrrolidone. The extract was centrifuged at 10 000 *g* for 10 min. The supernatant was desalted in *Sephadex* column pre-equilibrated with 10 mM phosphate buffer, pH 7.0, containing 0.2 mM EDTA, and utilized for enzyme assays. Protein content in the extract was determined according to Bradford (1976), using BSA as standard.

Ascorbate peroxidase activity (EC 1.11.1.11) was determined by the method of Nakano and Asada (1981), by monitoring the rate of ascorbate oxidation at 290 nm. The reaction medium contained 50 mM phosphate buffer, pH 7.0, 0.5 mM ascorbate, 0.1 mM EDTA, 0.1 mM hydrogen peroxide, and 0.2 cm^3 enzyme extract. The reaction started by addition of ascorbate. The decrease in absorbance was followed from 10 to 180 s. Guaiacol peroxidase activity (EC 1.11.1.7) was measured according to Nakano and Asada (1981), using 18 mM pyrogallol as substrate. The H_2O_2-dependent pyrogallol oxidation was determined following the increase in absorbance at 430 nm for 60 s, at 25 °C. Catalase (EC 1.11.1.6) activity was determined by the method of Cakmak *et al.* (1993). The reaction medium consisted of 25 mM phosphate buffer, pH 7.0, 19 mM H_2O_2 and 0.1 cm^3 of enzyme extract. Decomposition of hydrogen peroxide was determined following the decline in absorbance at 240 nm, for 180 s, at 25 °C. Activity of glutathione reductase (EC 1.6.4.2) was assayed by the

method of Cakmak *et al.* (1993), following the oxidation of NADPH, at 25 °C, through the decline in absorbance at 340 nm. The reaction mixture consisted of 25 mM phosphate buffer, pH 7.8, 0.5 mM oxidized glutathione, 0.12 mM NADPH, and 0.1 cm^3 of enzyme extract.

Results

The analysis of coffee primary root growth showed that the zone of elongation was limited to the first apical millimetre, with an expansion rate of 2.45 ± 0.26 mm d^{-1}. The chilling treatments at 5 and 10 °C, imposed during six consecutive days, completely inhibited root growth, but treatments at 15 and 20 °C only partially affected root expansion, compared to the control at 25 °C (Fig. 1). When chilled plants returned to the control temperature, the 15 and 20 °C treated seedlings were capable of resuming growth, while no growth was detected in the 10 °C-treated plants.

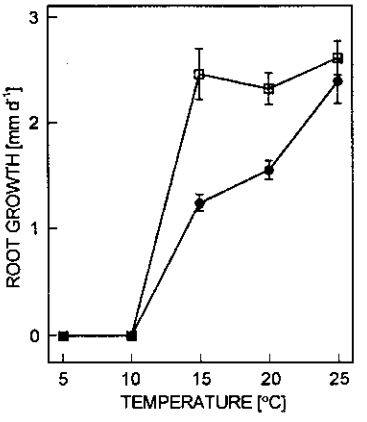

Fig. 1. Root growth rate of 30-d-old coffee seedlings exposed to chilling treatments for 6 d (*closed circles*) and after transfer to 25 °C for another 6 d (*open squares*). Means of 10 replicates; *vertical bars* indicate SD.

Table 1. Metabolic heat rate and TTC reduction in root-tip segments of 30-d-old coffee seedlings exposed to 10 °C for 6 d. Controls were kept at 25 °C. Means ± SD of four experiments. Means followed by the same letter do not statistically differ (Tukey's test, $P < 0.05$).

	25 °C	10 °C
Metabolic heat rate [μW mg^{-1}(f.m.)]	2.410 ± 0.230 a	0.660 ± 0.030 b
TTC reduction [μmol(formazan) mg^{-1}(f.m.)]	0.295 ± 0.037 a	0.116 ± 0.046 b

Metabolic activities in coffee root-tip segments, evaluated by microcalorimetry and formazan production, showed high sensitivity to chilling (Table 1). Under our

experimental conditions, the values of metabolic heat rate, as well as TTC reduction decreased by 73 % and 61 %, respectively, at 10 °C treatment, in comparison with the control at 25 °C.

In previous work (Alonso *et al.* 1997) we have found that roots of coffee seedlings undergo loss in membrane fluidity when exposed to chilling stress (10 °C) for 6 d in darkness. Here, we used the EPR spectroscopy of a spin-labeled stearic acid to follow membrane changes as a function of time of exposure. The EPR spectra at 25 °C of 5-DSA structured in membranes of intact root-tip segments for the control and in 6 d treated samples are typical for natural membranes in which the lipids can interact with the membrane proteins (Fig. 2). The relationships between the Order Parameter S and the time of incubation of plants at 10 °C indicated significant decrease in membrane fluidity from the 5th to the 7th day of incubation (Fig. 3).

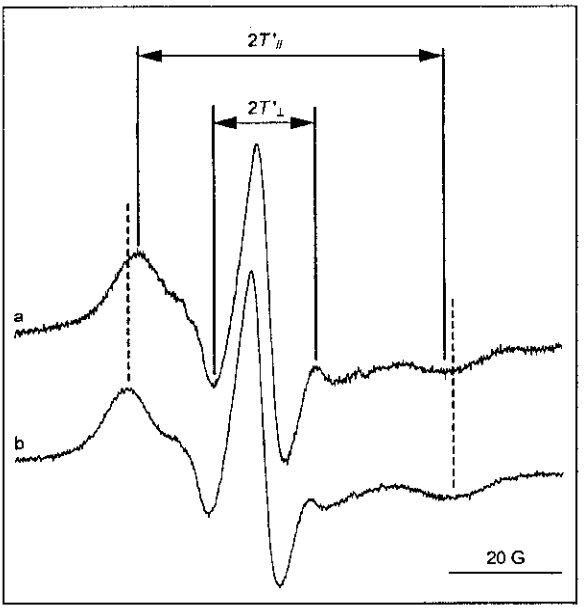

Fig. 2. EPR spectra of spin label 5-DSA in cellular membranes of root tip segments of coffee seedlings at 25 °C (*a*) and at 10 °C (*b*). The measured parameters are indicated.

Measurement of MDA content during 6 consecutive days showed that exposure of seedlings to 10 °C did not affect the rate of lipid peroxidation during the first five days of treatment, but experienced a sharp rise in MDA production between 5th and 6th day, then becoming stable in an apparent steady-state (Fig. 4).

The activities of hydrogen peroxide scavenging enzymes, ascorbate peroxidase, guaiacol peroxidase and catalase, responded differently to chilling stress (Table 2). No changes in ascorbate peroxidase and catalase were detected in plants exposed for 6 d at 10 °C, compared to the control. In contrast, guaiacol peroxidase presented a 55 % increased in activity, at 10 °C. On contrary, glutathione reductase activity

decreased, concomitantly with a noticeable decrease in the capacity for TTC reduction (Table 1).

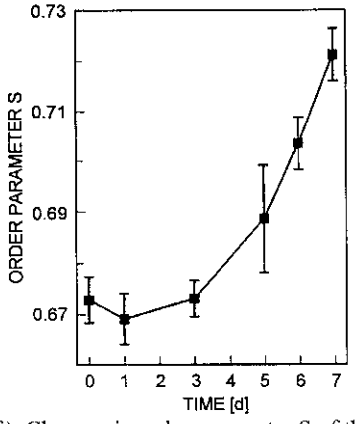

Fig. 3 (*left*). Changes in order parameter S of the spin label 5-DSA in cellular membranes of root tip segments of coffee seedlings as a function of time of exposure to 10 °C. Means and SD of 3 experiments.

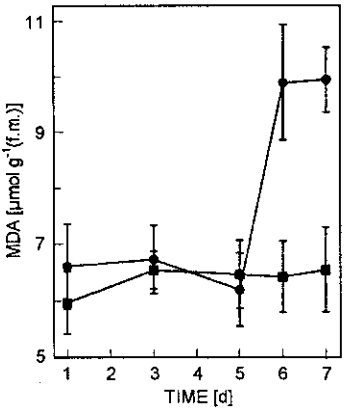

Fig. 4 (*right*). Changes in malondialdehyde (MDA) content of root tip segments of coffee seedlings as a function of time of exposure to 10 °C (*circles*) and 25 °C (*squares*). Means and SD of 4 experiments.

Table 2. Activity of ascorbate peroxidase, guaiacol peroxidase, catalase, and glutathione reductase and protein content in root-tip segments of coffee seedlings exposed to 10 and 25 °C, for 6 d in darkness. Means ± SD of 4 experiments. Means followed by the same letter do not statistically differ (Tukey's test, $P < 0.05$).

	25 °C	10 °C
Ascorbate peroxidase [nmol(ascorbate) mg^{-1}(protein) s^{-1}]	5.25 ± 0.93 a	4.53 ± 0.18 a
Guaiacol peroxidase [nmol(purpurogalin) mg^{-1}(protein) s^{-1}]	8.70 ± 0.10 a	13.50 ± 1.17 b
Catalase [nmol(H_2O_2) mg^{-1}(protein) s^{-1}]	0.152 ± 0.023a	0.164 ± 0.055a
Glutathione reductase [nmol(NAPH) mg^{-1}(protein) s^{-1}]	0.244 ± 0.011a	0.222 ± 0.012b
Protein content [mg g^{-1}(f.m.)]	58.0 ± 7.0 a	54.0 ± 3.0 a

Discussion

Coffee root tip tissues are very sensitive to chilling temperatures below 15 °C. Under the experimental conditions used in this work, root expansion was markedly reduced and did not recover when 10 °C treated seedlings returned to 25 °C. In parallel, a marked inhibition of aerobic metabolism occurred (Table 1), in addition to a significant decrease in the fluidity of cell membranes and an increase in the amount of MDA formed (Fig. 3 and 4).

The changes in metabolic heat evolution and TTC reduction strongly suggested that low temperature treatment might have led to oxidative stress-inducing

dysfunction in cell membranes and consequently, inhibition of respiratory activity (Burdon *et al.* 1994, Prasad *et al.* 1994b). In addition, numerous evidences have shown that changes in membrane functioning, such as decreased fluidity might be the consequence of lipid peroxidation, as detected by spin-label EPR spectroscopy (Bruch and Thayer 1983, Watanabe *et al.* 1990), and fluorescence probes (Chen and Yu 1994). Different authors have detected that presence of hydroperoxides in the inner membrane spaces might induce various changes in membrane organization due to their polarity. This fact can, in turn, affect membrane fluidity and permeability, and cause breakdown of lipid ordering in the membrane, contributing to solute leakage (Dhindsa *et al.* 1981, Rice-Evans and Burdon 1993).

These results support our previous suggestion (Alonso *et al.* 1997), that coffee seedlings are highly sensitive to oxidative stress caused by exposing plants to chilling temperatures (10 to 15 °C). The decrease in membrane fluidity detected at 10 °C (Fig. 3), is presumably due to increased levels of lipid peroxidation. After stress periods of more than 3 d, the antioxidant enzymes, in addition to other radical-scavenging compounds, were not efficient enough to protect membranes against oxidative damage.

In this work, we observed that ascorbate peroxidase and catalase did not change at chilling temperature, in contrast with activity of guaiacol peroxidase, which increased. Recently, Prasad *et al.* (1995) reported mitochondrial guaiacol peroxidase induction in maize seedlings exposed to 14 °C, and suggested that it might perform similar role as catalase does, in the protection of organelle from oxidative injury. Independent of structural and functional differences between ascorbate peroxidase and guaiacol peroxidase, their substrates and products are allowed to interact in order to warrant availability of reduced phenols to the cells. Chen and Asada (1990) showed that guaiacol peroxidase extracted of spinach leaves can catalyze oxidation of *p*-aminophenol by hydrogen peroxide, generating the aminophenoxy radicals, which further oxidize ascorbate, producing monodehydroascorbate and dehydroascorbate radicals. In a recent review, Mehlhorn *et al.* (1996) reported that, under particular conditions, turnover between ascorbate and phenols may occur, suggesting that ascorbate peroxidase and guaiacol peroxidase are not mutually exclusive as proposed earlier. Thus, in some instances, guaiacol peroxidase may show ascorbate peroxidase activity and *vice-versa*. While the guaiacol peroxidases such as horseradish peroxidase prefer phenolic compounds as substrates, they can become effective ascorbate-dependent H_2O_2-scavenging enzymes if ascorbate is available in sufficient concentrations. This property certainly confers a high degree of flexibility for the antioxidant defenses to operate, and allow important metabolic changes associated with lignin biosynthesis and capture of excess hydrogen peroxide to occur.

Several reports indicated increased production of phenolic compounds at low temperature (Graham and Patterson 1982), and the flavonoids might express antiradical properties, directed mostly toward ($OH^{\cdot}$, $O_2^{\cdot}$ and $^1O_2$; Foyer *et al.* 1994), as well as peroxyl and alcoxyl radicals (Saija *et al.* 1995).

The results of this study with chilling stressed coffee roots suggested that a similar mechanism as that described by Chen and Asada (1990), might be operating, leading to the consumption of ascorbate for detoxification of hydrogen peroxide directly, and

for regeneration of phenolic compounds to be utilized as substrate for guaiacol peroxidase. Under certains conditions, if the rate of hydrogen peroxide scavenging by phenols becomes too fast, the availability of reduced phenols may fall to such a low level that complete regeneration of ascorbate might become chemically unfeasable. The presented data showed lowered activity of glutathione reductase at 10 °C, compared with the control at 25 °C, suggesting that chilling temperatures affected the redox system glutathione-ascorbate in such a way as to inhibit the supply of GSH to the cells. This proposal might be conceivably validated by the results of TTC reduction and metabolic heat production (Table 1). These data suggest that availability of GSH decreased due to the lack of reducing power.

The data presented above indicate that exposure of root-tip tissues to chilling temperature impose oxidative stress, characterized by reduction of metabolic activity, and in consequence decreased production of reducing power required for the operation of the antioxidant systems. The high activity of guaiacol peroxidase measured in coffee root tissues further suggest that phenolic compounds may play a role in the detoxification of hydrogen peroxide in chilled stressed cells.

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