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Chilling stress leads to increased cell membrane rigidity in roots of coffee (*Coffea arabica* L.) seedlings

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Abstract

Tropical and sub-tropical higher plant species show marked growth inhibition when exposed to chilling temperatures. In root tip segments of coffee seedlings which were subjected for 6 days to temperatures of 10, 15, 20 and 25°C, in darkness, we have detected an increased amount of malondialdehyde formed in the 10°C treatment, accompanied by higher electrolyte leakage. The electron paramagnetic resonance (EPR) technique and the fatty acid spin probes 5-, 12- and 16-doxylstearic acid were used to assess cellular membrane fluidity. At the depth of the 5th and 16th carbon atom of the alkyl chains the nitroxide radical detected more rigid membranes in seedlings subjected to 10°C compared with 15 and 25°C. At the C-12 position of the chains the probe showed very restricted motion and was insensitive to chilling induced membrane alterations. EPR parameters for intact tissues and microsome preparations from root tips showed that the fluidity was essentially the same when evaluated at C-5 and C-16 positions of the chains, and was considerably more fluid for microsomal membranes in the region of the C-12 position of the bilayers. The rotational motion of the nitroxide at C-16 position of the chains experienced a phase transition at about 15°C. The calculated energy barriers for reorientational motion of the probe 16-doxylstearic acid were higher at temperatures of 5–15°C than in the interval of 15–25°C, suggesting that below the phase transition the membrane lipids assume a more ordered and compacted array. Membrane rigidity induced by chilling was interpreted as due to lipid peroxidation that could have been facilitated by higher density of peroxidizable chains below the membrane phase transition.

Keywords: Chilling stress; Membrane fluidity; Lipid peroxidation; EPR; (*Coffea arabica* L.)

1. Introduction

Chilling sensitivity has been looked upon as an important issue in plant ecophysiological and biophysical studies [1], particularly in tropical and sub-tropical species, which show characteristic damage symptoms when subjected to low-above zero temperatures [2,3]. Literature reports have shown that low temperature exposure invariably induces biochemical and physiological changes in sensitive plants, that may be correlated with different degrees of resistance depending on the capacity for acclimation of the particular species [1,4].

Studies in coffee plants revealed that temperatures below 16°C provoke inhibition of vegetative growth

Abbreviations: EPR, electron paramagnetic resonance; 5-DSA, 5-doxylstearic acid; 12-DSA, 12-doxylstearic acid; 16-DSA, 16-doxylstearic acid.

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and decreased net photosynthesis [5], in addition to erratic bean maturation leading to poor yields [6–8]. In plant root tissues chilling temperatures may lead to impaired radicle elongation and cause cortex breakdown [9], as well as alterations in metabolic heat rates, measured by microcalorimetry [10]. In addition, chilling stress commonly induces increased electrolyte leakage, associated with disruption of membrane integrity [11,12].

Numerous experimental evidences have indicated that exposure to low positive temperatures often causes structural changes in membrane lipids [13–15], which have been associated with the damaging symptoms expressed in plant tissues subjected to oxidative stresses [16].

Lipid peroxidation is a free radical-mediated degradative process that involves homolysis of polyunsaturated fatty acids resulting in the formation of a lipid radical [17–20]. Literature reports have shown that free radical-induced peroxidation of membrane lipids might lead to membrane dysfunction through modifications in fluidity [16,21–24]. In this context it has been demonstrated that several biological membrane functions can be influenced by membrane fluidity, such as the selective barrier which is essential for ion transport, selective permeability, enzyme activity and receptor availability. Despite the voluminous literature dealing with lipid peroxidative phenomena in plant tissues, the nature of the chemical and physical changes associated to membrane rigidity and increased ion permeability [12,25–27] deserve further description.

The electron paramagnetic resonance (EPR) of fatty acids spin labels is a useful method that offers the possibility of monitoring the local dynamic properties of the lipid molecules in the membrane bilayer [28]. For this purpose we have recently developed a method for incorporation of fatty acid spin probes into lipid membrane of intact stratum corneum [29,30], which allowed measurements of membrane fluidity directly in the biological material. The survey of pertinent literature has shown that measurements of membrane fluidity in response to exposure of plant tissues to environmental stresses commonly utilize microsomal membrane preparations [31]. However, since microsome isolation is a fairly high tissue-material consuming under particular experimental conditions, we decided to evaluate the viability of employing intact plant tissues as an alternative means for assessing fluidity changes preserving membrane physical properties.

The present research attempted to investigate the way chilling episodes induce root tissue damage in coffee seedlings basically utilizing electron paramagnetic resonance (EPR) technique. The fatty acid spin probes 5-, 12- and 16-doxylstearic acid (5-, 12- and 16-DSA, respectively) were used for measurements of depth-dependent fluidity, as well as its thermotropic behavior, in both intact tissues and microsomal membranes.

2. Materials and methods

2.1. Plant material and growth condition

Coffee seeds (*Coffea arabica* L.) cv. (Catuaí Vermelho) 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 of 1-liter volume 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. Treatments started when the seedlings were 30 days old (‘match stage’).

2.2. Test for chilling tolerance

Chilling tolerance was evaluated by the electrolyte leakage test, described by Jennings and Saltveit (1994) [32]. Apical 1 mm root segments were washed in deionized water, weighed (300 mg) and immediately transferred to glass flasks containing 15 ml mannitol 0.3 M, prepared with water Milli-Q. The segments were incubated in a rotary shaker for 50 min, at 25°C, for conductivity measurements with a Radiometer type CDM conductivity meter. Electrolyte leakage was expressed as μmho g^{-1} (FW).

2.3. Determination of lipid peroxidation

The levels of lipid peroxidation in root-tip segments were expressed as malondialdehyde (MDA)

content, determined by the method of TBARS (thiobarbituric acid reactive substances) of Buege and Aust (1978) [33]. The segments where washed with water, blotted dry, immediately weighed (50 mg) and homogenized in Hepes 10 mM, pH 7.0, at 4°C. The homogenate was transferred to a medium containing trichloroacetic acid, thiobarbituric acid, HCl (15% w/v, 0.37% w/v, 0.25 M) and butylated hydroxytoluene 50 μM. The mixture was heated to 80°C for 15 min, and then quickly cooled in an ice-bath. After centrifuging at $10\,000 \times g$ for 10 min, the absorbance of the supernatant was read at 535 nm. The value of non-specific absorption at 600 nm was subtracted from the reading at 535 nm. The concentration of MDA was calculated using the extinction coefficient of 1.56×10^{-5} M^{-1} cm^{-1}, and expressed as μmol MDA g^{-1} (FW).

2.4. Isolation of microsomal membranes

The preparation of microsomal membranes was carried out at 4°C according to the technique of Basu et al. (1994) [31] with modifications. Approximately 1 g of tissue (root tips) was ground with a mortar and pestle in an ice-cold medium (2:1 ratio) consisting of 50 mM Hepes, 2 μM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT) and 1 mM EDTA (pH 6.5). The homogenate was centrifuged at $20\,000 \times g$ for 20 min, the resulting supernatant centrifuged again at $105\,000 \times g$ for 60 min in a Beckman L8-M ultracentrifuge. The pellet (microsomal fraction) was resuspended in phosphate buffer 0.2 M, pH 7.4, containing 5 mM EDTA. Protein concentration (20 mg/ml) was determined according to Bradford (1976) [34].

2.5. EPR spectroscopy

Spin-labeled derivatives of stearic acids, 5-, 12- and 16-doxylstearic acid (5-DSA, 12-DSA and 16-DSA, respectively) were purchased from Aldrich. These spin labels have each one a nitroxide radical ring attached at C-5, C-12 and C-16 positions of acyl chain, respectively. A small aliquot of stock spin label dissolved in ethanol (5 mg ml^{-1}) was placed in a glass tube. The solvent was evaporated under nitrogen flux, and samples containing 20 mg (FW) of 1 mm root tip segments (approximately 300 segments) suspended in 200 μl 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 (25 μl), for EPR experiments, utilizing a small paint brush. Similarly, 20 μl of microsomal suspension (20 mg protein/ml) were spin labeled and introduced in the capillary tube. The final nitroxide concentrations in both samples were estimated between 1 to 3×10^{-4} M.

EPR measurements were performed on a Varian E-9 spectrometer equipped with the rectangular cavity, the temperature being controlled with a nitrogen stream system (Air Products and Chemicals). EPR spectra were obtained at X-band (9.150 GHz) with microwave power of 20 mW, modulation frequency 100 kHz and amplitude 2.5 G. The sweep time was 2 min and magnetic field scan 100 G.

In general, the fluidity of the membrane can be estimated from the order parameter S determined according to Gaffney (1976) [35]. The calculation uses an expression which includes the parameters $T'_{\parallel}$ and $T'_{\perp}$, the apparent parallel and perpendicular hyperfine splitting parameters of the spectrum (Fig. 2), and an empirical correction factor for the difference between the true and apparent polarity. Since the resolution of $2T'_{\perp}$ is very poor at low temperatures, we used the dependence of $2T'_{\parallel}$ to monitor temperature changes for 5-DSA and 12-DSA at all measured temperature intervals, and the order parameter S at temperature intervals where simultaneous resolution of both parameters permitted its calculation.

In the more rapid motion regime, as is the case of 16-SASL, the calculation of S is less reliable since the resolution of the outer features of $2T'_{\parallel}$ becomes very poor and insufficient for precise determination from the experimental spectra (Fig. 2). In this case, the rotational correlation time, τ_c, can be calculated for data analysis. This empirical parameter describes the rate of motion of the probe as it rotates about its long axis. The spectral parameters W_0, the peak-to-peak linewidth of the central line of spectrum ($M_I = 0$) and h_0 and h_{-1}, the intensities of the central and high field lines, respectively, were measured graphically (Fig. 2), and the τ_c was calculated using the formulae of Simon (1979) [36].

The activation energy E_a was also calculated for 16-DSA using the Arrhenius equation:

$$\log \tau_c = E_a/2.3\,RT \quad (1)$$

where R is the gas constant and T the absolute temperature. In practice, the numerical value of E_a was determined from the slope of a plot of log τ_c versus $1/T$ (Arrhenius plot).

3. Results

3.1. Evaluation of chilling tolerance

The rate of ion leakage shown in Fig. 1 significantly increased in root samples of 5 and 10°C incubated plants, compared with the 15, 20 and 25°C treatments. In order to investigate whether the plants affected by chilling (5 and 10°C) were able to recover after being transferred back to ambient temperature, the seedlings were exposed to 25°C for an additional period of 6 days, and a new set of measurements was carried out. It was observed that after returning to normal temperature for growth, the seedlings exposed to 5 and 10°C continued to leak electrolytes suggesting the occurrence of irreversible changes in membrane integrity, a phenomenon frequently observed in chilling-sensitive tissues [37]. The data of Fig. 1 show that root segments subjected to 5 and 10°C lost approx. 55% more electrolytes than the control plants, and leaked 51% more ions when they returned to the control temperature.

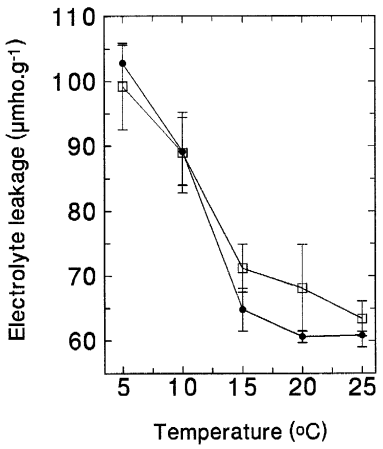

Fig. 1. Electrolyte leakage in root-tip segments of 30 days old coffee seedlings exposed for 6 days to various temperatures (closed circles), in darkness, and after the plants were returned for additional 6 days to 25°C (open squares). Vertical bars indicate S.D. values of three independent experiments.

Table 1
Estimation of lipid peroxidation (malondialdehyde content) in root-tip segments of 30 days old coffee seedlings that were exposed to chilling temperatures for 6 days, in the dark

Temperature (°C)	MDA (μmol g^{-1} (FW))
10	9.8 ± 0.6 A *
15	7.0 ± 0.5 B
25	6.8 ± 1.0 B

Values are means and S.D. of four separate experiments.
* Statistical significance: the means followed by the same capital letter do not differ significantly, with $P < 0.05$ (Tukey's test).

3.2. Chilling induces lipid peroxidation

The results depicted in Table 1 show that root-tips of coffee seedlings contain relatively smaller amounts of thiobarbituric reactive substances, compared with the concentration of malondialdehyde normally found in other higher plant tissues [38]. Nevertheless, our results showed a significant increase of malondialdehyde formed in roots exposed to 10°C for 6 days, and the values were 30% higher for the chilling treatment compared with the control at 25°C. The rise in malondialdehyde content measured in 10°C-treated plants indicated an increased rate of oxidation of membrane lipids which presumably lead to membrane injury.

3.3. EPR spectroscopy

The EPR spectra at 25°C for 5-, 12- and 16-DSA, incorporated in cell membranes of apical root segments of coffee seedlings acclimated at 25°C for 6 days, and in microsomes obtained from this same tissue, are shown in Fig. 2. Due to the large hydrophobic force of these fatty acid spin probes, and proper conditions of preparation, they were able to penetrate the cell wall and intercalate with the membrane lipids. The EPR spectra of coffee root membranes were similar to typical spectra observed in plasma membranes. The spin probes having the paramagnetic fragment attached at three different depths

of the acyl chains showed EPR spectra that indicate large differences in probe mobility and ordering of the molecules in the lipid bilayer, particularly as the nitroxide moves from C-12 to C-16 positions of the carbon atoms in the membrane. The three resonance lines originated by probe tumbling in aqueous solution appeared minimized in the spectra, even under conditions of fully hydrated samples. This behavior occurs due to the proper packing of the root tissues in the capillary tube, or to the high concentration of lipids present in microsomal membrane suspensions (estimated as 30 mg/ml), that cause exclusion of sample's excess water.

The spectra presented in Fig. 2 also show that the spin labels were adequately incorporated in the lipid bilayers of cell membranes and were properly distributed throughout the tissues. The appropriate distribution of spin label was assured by the capacity of samples to get them structured in their lipid bilayers, without allowing magnetic interactions (dipole–dipole and exchange interactions). These magnetic interactions, that cause broadening of resonance lines and reduce spectra resolution, are largely avoided as

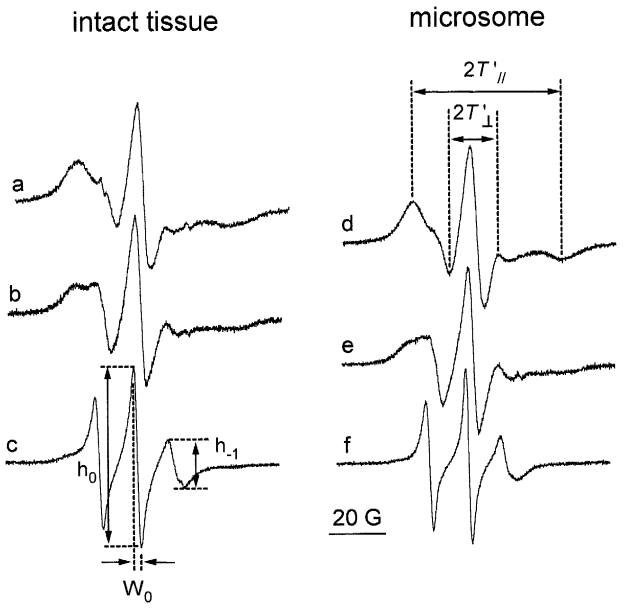

Fig. 2. EPR spectra at 25°C of the spin labels 5-, 12- and 16-DSA in excised root segments (a,b,c) and in microsomal membranes of coffee seedlings (d, e, f), both suspended in phosphate buffer (0.2 M, pH 7.4, 5 mM EDTA). The measured parameters are indicated.

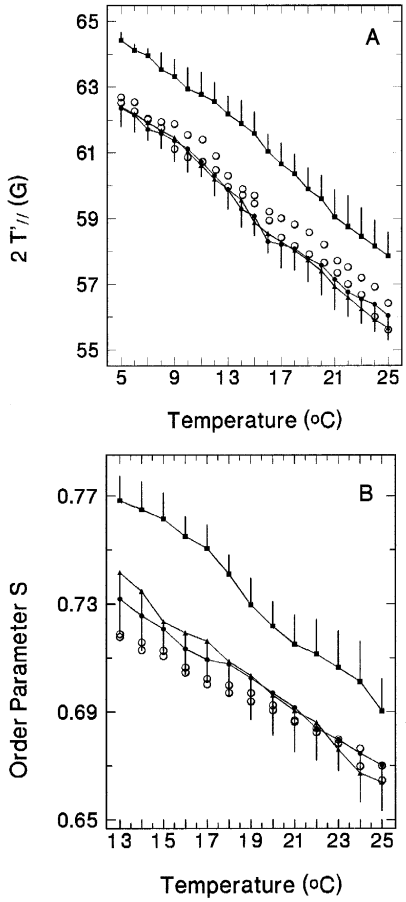

Fig. 3. EPR parameters of the spin label 5-DSA in cellular membranes of excised root-tip segments of coffee seedlings and in microsomal fraction as a function of temperature. The symbols indicate three acclimation temperatures for intact tissue: 10°C (square), 15°C (triangle) and 25°C (circle). The microsomal membranes (open circle) were obtained from plants acclimated at 25°C. The means and S.D. were obtained from three independent experiments.

the preparation contains 150 or more membrane lipids for each spin label [39].

Figs. 3 and 4 show the dependence of the parameters $2T'_{\parallel}$ and S as a function of temperature for 5-DSA and 12-DSA structured in cell membranes of root segments acclimated at 10, 15 and 25°C, and in root microsomal membranes of plants exposed to 25°C. In the case of probe 5-DSA, the values obtained for both parameters in microsomes and intact tissues at 25°C, were similar, since we estimated an experimental error of 0.5 G for $2T'_{\parallel}$ and of 0.02 for the order parameter. The plants exposed to 10°C

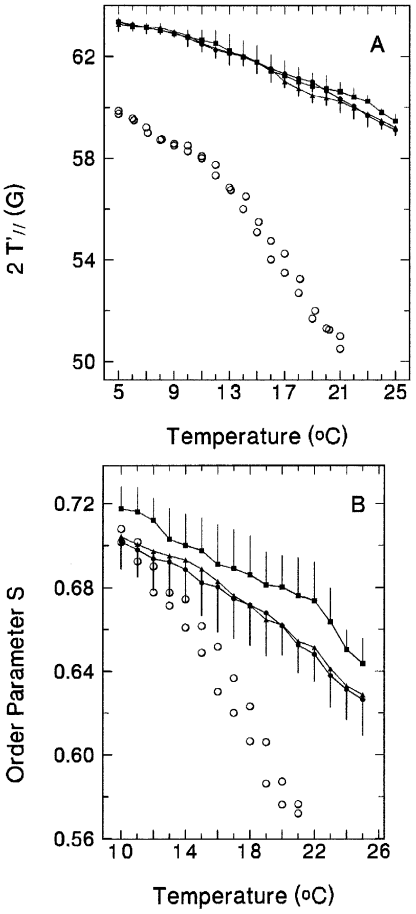

Fig. 4. EPR parameters of the spin label 12-DSA in cellular membranes of root-tip segments of coffee seedlings and in microsomal membranes as a function of temperature. The symbols indicate three acclimation temperatures for intact tissue: 10°C (square), 15°C (triangle) and 25°C (circle). The microsome membranes (open circle) were obtained of plants acclimated at 25°C. The means and S.D. were obtained from three independent experiments.

presented more rigid membranes compared with those acclimated at 15 and 25°C, as observed by the higher values of $2T'_{\parallel}$ and S. For the 12-DSA, the calculated parameters showed a marked difference in membrane fluidity in both microsomes and intact tissues (Fig. 4A). This difference tended to disappear at temperatures below 17°C, when evaluated by the order parameter (Fig. 4B). The probe 12-DSA showed very low sensitivity to alterations in membrane fluidity induced by the chilling treatments. The parameter $2T'_{\parallel}$ did not show significant changes among the three temperature treatments, and the values of the order parameter were somewhat higher for plants acclimated at 10°C (Fig. 4B), the differences being non-significant ($P < 0.05$) for most of the measured temperatures.

The curves of $2T'_{\parallel}$ versus temperature for the probe 12-DSA (Fig. 4A) showed different slopes at temperatures below and above 11°C, suggesting a membrane phase transition at that temperature. The change in slope was more pronounced in microsomal samples, which showed a steep increase in fluidity starting at approximately 12°C, followed by a continuous rise until the complete loss of $2T'_{\parallel}$ resolution at 22°C (Fig. 4A and 4B).

It is worth noticing that the values of $2T'_{\parallel}$ for the probe 12-DSA in intact tissue are higher than the values for the probe 5-DSA, in plants acclimated at 15 and 25°C, the difference becoming larger as temperatures rise above 10°C. This was considered an unexpected result for the fluidity generally increases as the nitroxide moves away from the polar region and approaches the hydrophobic core of the membrane. We believe that some protein present in intact root membranes, and apparently absent in microsomes, might have been the cause for this hindered motion of probe 12-DSA in root membranes, and its increased motional freedom in microsomal membranes above 10°C.

The values of rotational correlation time, τ_c, for the nitroxide 16-DSA, are presented in Fig. 5 as a function of the reciprocal absolute temperature, in intact root membranes and root microsomal membranes. This probe, located deeper inside the membrane, also detected more rigid membranes in plants acclimated at 10°C, as seen by the higher values of τ_c for these samples. It was also observed that the rigidity effect upon the membrane was greater at the lowest temperatures. Conversely, we did not find any significant difference in τ_c measured in plants exposed to 15 and 25°C. In the case of microsomal samples, the values of rotational correlation time were similar to those obtained for root membranes, at treatment temperatures of 15 and 25°C, in the temperature range of 5–20°C, and values a little smaller in root membrane in the range of 20–25°C.

The curves presented in Fig. 5 show discrete differences in slope coefficients in the temperature intervals of 5 to about 15°C, and from approximately 15 to 25°C. The changes in slopes, that were more

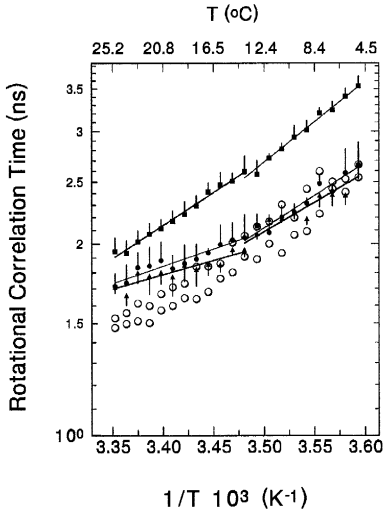

Fig. 5. Arrhenius plot of the rotational correlation time, τ_c, of 16-DSA structured in cellular membranes of coffee root-tip segments and in microsome preparations as a function of temperature. The symbols indicate three acclimation temperatures for intact tissue: 10°C (square), 15°C (triangle) and 25°C (circle). The microsome membranes (open circle) were obtained from plants acclimated at 25°C. Regression analyses were made considering the temperature intervals 25–15 and 15–5°C. The means and S.D. were obtained from three independent experiments.

evident in plants treated at 15 and 25°C, suggest a membrane phase transition occurring at temperatures around 15°C. The calculated activation energies for reorientation of the probe 16-DSA in the membrane (Eq. (1)) for three independent experiments, are presented in Table 2. In the three acclimation temperatures the probe detected essentially the same energy barrier (4.5–5.5 kcal/mol) for its reorientational motion in the temperature range of 5–15°C. Between 15–25°C, the activation energy did not change in plants of the 10°C-treatment, but was significantly reduced (2.0–2.5 kcal/mol) in plants acclimated at 15 and 25°C. Therefore, while the 10°C-treated plants maintained the same apparent membrane phase in the whole range of temperatures, plants subjected to 15 and 25°C undergo an apparent phase transition at temperatures close to 15°C (Fig. 5). The observed transition represented a change from a more ordered membrane to a more fluid one, where the probe has higher motional freedom.

Table 2
Activation energy for rotational diffusion of 16-DSA in membranes of coffee root segments

Temperature treatment (°C)	ΔE (kcal/mol)	
	25–15°C	15–5°C
10 [a]	4.8 ± 0.7 [A,b]	5.4 ± 0.5 [A]
15	2.2 ± 0.6 [B]	4.5 ± 0.7 [A]
25	2.5 ± 0.5 [B]	4.9 ± 0.8 [A]

The means and S.D. of three independent experiments were calculated from data presented in Fig. 5.
[a] Plants acclimated at indicated temperature for 6 days.
[b] Statistical significance: the means indicated with different capital letters are statistically different with $P < 0.01$ (Student's *t*-test).

4. Discussion

The membrane fluidity in root segments subjected to chilling treatments for 6 days was not significantly altered at 15°C, when evaluated by the nitroxide moiety of the three positional isomers spin probes in the temperature range of 5–25°C. However, under similar conditions the treatment at 10°C revealed a more rigid membrane when analyzed by the segmental motion at the C-5 position of the alkyl chains (Fig. 3), or by the rotational motion at 16-DSA position (Fig. 5). On the other hand, the 12-DSA tied in the membrane did not distinguish any chilling-induced membrane alteration.

Comparing the EPR parameters for microsomal membranes and intact root tissues we verified that, (a) the fluidity was essentially the same when evaluated by the probe 5-DSA and the parameters $2T'_{\parallel}$ and S (Fig. 3), (b) the parameter $2T'_{\parallel}$ of the 12-DSA revealed a lipid bilayer considerably more fluid in isolated microsomes throughout the whole range of temperatures (Fig. 4A), the order parameter S indicating the same fluidity for both samples in the temperature range from 5 to 16°C, and a pronounced increase in microsomal fluidity compared with intact tissue in the range of 17–25°C (Fig. 4B), and (c) the values of rotational correlation time of the 16-DSA indicated fluidity differences solely in the temperature interval of 20–25°C, where the microsomal samples were slightly more fluid, compared with intact root membranes (Fig. 5).

Our results showed that when the acclimation temperature dropped below 15°C, electrolyte leakage in the chilled root tissues increased (Fig. 1) in parallel

with the rate of malondialdehyde produced (Table 1). The significant reduction in membrane fluidity was presumably associated with lipid peroxidation of root cell membranes, caused by the chilling treatment. Indeed, a decrease in membrane fluidity or molecular order following peroxidation, has been reported for model and biological membranes using fluorescence probes [40,41] and spin probes [42–44].

McLean and Hagaman (1992) [45] pointed out that the rate of peroxidation of arachidonic acid in dimyristoylphosphatidylcholine (DMPC) liposomes, induced by the iron-ascorbate oxidation system, was faster below the phase transition temperature of the host membrane. Those authors also observed a marked decrease in the rate of peroxidation at concentrations of arachidonic acid below ≈ 2 mole%, suggesting that phase separation of the fatty acids, or higher local concentration of peroxidizable chains, promoted the propagation of the oxidation process. In coffee roots, membrane rigidity and increased rates of MDA formed, as well as the rise in ion leakage (Fig. 1), occurred at temperatures below the membrane phase transition temperature, measured at 15°C (Fig. 5). These results indicate that the events occurring at temperatures below the phase transition can be regarded as important determinants of lipid peroxidation. In fact, below the phase transition, lipids assumed a more ordered and compacted array in which the probe 16-DSA experienced a higher energy barrier for its reorientational motion (Fig. 5 and Table 2). In this situation, the distances of the peroxidizable chains are smaller and consequently more favorable for lipoperoxidation [45].

Working with rat liver microsomal membranes, Curtis et al. (1984) [42] showed that the unsaturated/saturated fatty acid ratio decreased after lipid peroxidation NADPH-dependent, in the presence of the chelate ADP-Fe^{3+}. Indeed, the loss of unsaturation as a result of lipid peroxidation might have accounted for the observed decrease in fluidity [43]. Unsaturated acyl chains tend to render the membrane more fluid compared with saturated fatty acids, due to the lesser packing of fatty acid chains with cis configuration of double bond in the membrane [46–48]. However, we believe that either the loss of double bonds, or other membrane compositional changes, are not the main determinants for the decrease in membrane fluidity after peroxidation. It is well known that in both biological and artificial membranes, permeability increases with the increase in fluidity (for a review Ref. [49]), suggesting a correlation between membrane transport and lipid fluidity. However, in peroxidized membranes it is often observed a decrease in fluidity accompanied by increased membrane leakiness [12,25–27]. There are evidences that membrane rigidity following peroxidation is mainly due to the formation of covalent bonds between adjacent lipid radicals [41,42,50], that would lead to decreased motional freedom of the fatty acid chains. These cross-linking interactions in the lipid chains of the membrane would also cause the disruption of the arrangement of the lipid packing, thus, contributing to increased membrane leakiness [21,26] (Fig. 1).

Data depicted in Fig. 5 show that the chilling treatment at 10°C reduced the rotational motion of the probe 16-DSA but maintained its activation energy at temperatures below the membrane phase transition (Table 2). It is conceivable that oxidatively altered lipids and proteins might promote cross-linking interactions in the membrane, that exert certain spatial hindrances on the spin label moiety and decrease the length of its diffusional step. If the energy barrier for the reorientational motion of the probe does not change when the membrane peroxidation occurs, then the decrease of the diffusional step length could explain the reduction of its rotational motion. It is relevant to notice that, while the non-altered membranes subjected to temperatures of 15 and 25°C showed a phase transition at about 15°C, the membranes that undergo lipid peroxidation (10°C-treated) did not present phase changes in the range of temperatures from 5 to 25°C, suggesting that membrane alterations due to peroxidation might have caused some spatial constraints to rearrangement of lipid packing so that the membranes were unable to experiment the thermal transition in the temperature range of 5–25°C.

In this work with coffee root tissues we showed that the EPR technique employed to assess membrane fluidity changes induced by chilling stress is a sensitive method, less biological material consuming (ca. 25-fold), and less invasive compared with microsomal membranes. In addition, we found that the spin label EPR approach can provide precise evaluation of molecular ordering in lipid membranes of plant tis-

sues subjected to environmental factors, particularly low temperature stress.

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