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ORIGINAL ARTICLE

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Effect of monoterpenes on lipid oxidation in maize

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Abstract The monoterpenes 1,8-cineole, thymol, geraniol, menthol and camphor strongly inhibited the root growth of *Zea mays* L. seedlings. They induced an oxidative stress as measured by the production of malondialdehyde, conjugated dienes and peroxides. This oxidative stress depended on the length of the exposure and on the monoterpene applied. The total fatty acid content was measured and fatty acid composition was analyzed. Unsaturated fatty acids increased in the treated samples. The alcoholic and non-alcoholic monoterpenes appeared to have different modes of action.

Keywords Allelopathy · Fatty acid · Lipid peroxidation products · Monoterpene · *Zea*

Abbreviations MDA: Malondialdehyde · TFA: Total fatty acid content · FA: Fatty acid · IC_{80}: Concentration causing 80% inhibition

Introduction

The terpenes are the largest group of secondary products and include the monoterpenes. The latter are components of essential oils and are responsible for the biochemical interactions among plants known as allelopathy (Fischer 1986; Einhellig and Leather 1988; Weidenhamer et al. 1994). It is known that monoterpenes and essential oils strongly inhibit seed germination and plant growth (Panasiuk et al. 1986; Bradow and Connick 1988; Tarayre et al. 1995; Abrahim et al. 2000). Moreover, it has also been demonstrated, that monoterpene vapors may cause anatomical and physiological changes in plant seedlings (Fischer 1986; Einhellig and Leather 1988; Fischer 1991; Vaughn and Spencer 1993; Koitabashi et al. 1997; Dudai et al. 2000).

In biological systems, oxygen-derived free radicals have repeatedly been demonstrated to play a role in cellular injury through a chain reaction which leads to lipid peroxidation. Several environmental stresses promote the formation of oxygen's free radicals which mediate in phospholipid degradation, leading to a loss of membrane function (Halliwell and Gutteridge 1984, 1989; Deighton et al. 1993; Koga et al. 1994; Pastori and Trippi 1995).

Thus, we propose that, when applied to maize roots, monoterpenes create an environmental stress and, as a result, affect the fatty acid (FA) composition and induce peroxidation products.

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Materials and methods

Chemicals

The monoterpenes used were: 1,8-cineole, thymol, menthol, geraniol and camphor. They were obtained from ICN Pharmaceuticals (Costa Mesa, CA, USA) and were of high purity.

Plant material

Maize seeds (*Zea mays* L.) were rolled in the upper 3 cm of a 20-cm-long paper towel scroll. The individual scrolls were moistened with 25 ml of distilled H_2O and placed upright in 3-l flasks. The scrolled seeds were germinated for 3 days at 27 ± 1°C in the dark. At the end of this period, when root lengths were 6 ± 1 cm, the seedlings were harvested and bioassayed.

Bioassay

The 3-day-old seedlings (0 hour of treatment) were placed in 3-l desiccator flasks on Whatman No. 1 filter paper wetted with 15 ml of distilled water, and a 5-ml glass beaker was placed in the center. A sample of 2 ml of liquid (1,8-cineole and geraniol) or

2 g of solid (thymol, menthol and camphor) monoterpenes (volatile source) was added to the glass beaker. No direct physical contact occurred between the compounds and the seedlings. The above amounts of monoterpenes saturated the headspace, reaching concentrations of 21.7, 2.0, 1.9, 2.5 and 7.4 mg l^{-1} of 1, 8-cineole, thymol, geraniol, menthol and camphor, respectively. These concentrations corresponded to the IC_{80}, defined as the concentration that inhibited 80% of root growth at 96 h of treatment compared with the control. The flasks were placed in the dark at 27 ± 1°C and atmospheric pressure for 24, 48 and 96 h. Then, the plants were harvested, the roots dissected, and the different experimental parameters measured. The central beakers were left empty in the controls.

Analysis of volatile compounds

Volatiles from the headspace of glass desiccators were trapped by using a 10-ml gas-tight syringe and were analyzed by GC and GC–MS. A gas chromatograph (Shimadzu R1A) equipped with a flame ionization detector was used to analyze volatile compounds. A split inlet (split ratio 200:1) was used to inject volatiles into a DB-5 capillary GC column (30 m × 0.25 mm i.d., and 0.25°m film thickness), and ramped column temperature conditions (60°C for 3 min, increasing to 240°C at 4°C min^{-1}) were used. Detector temperature was 280°C. The carrier gas was He and it was applied at a constant flow of 0.9 ml min^{-1}. Individual peaks were identified using a mass selective detector (Perkin Elmer Q700 gas chromatograph–mass spectrometer) and co-injection with standards. GC–MS was performed with the same conditions as GC. The ionization potential for MS was 70 eV. The quantity of monoterpenes in the headspace was determined by the external standard method. The standard curves were generated by analysis of known concentrations of each compound dissolved in *n*-C_6H_{14}.

Lipid extraction and analysis

The total lipids were extracted from the roots (5 g FW) with $CHCl_3$:methanol (87:13, v/v) in a Soxhlet apparatus for 6 h. The extracts were dried on dry Na_2SO_4 and then were taken to dryness under vacuum. After the addition of an internal standard (heptadecanoic acid), fatty methyl esters from total lipids were prepared by transesterification by treatment with 1 N KOH in dry methanol for 30 min (Grosso et al. 1994) and then analyzed by GC. Analytical GC was performed on a Shimadzu GC-R1A gas chromatograph (FID) fitted with a Supelcowax-10 capillary column (30 m × 0.25 mm i.d.). Column temperature was programmed from 180°C to 240°C (4°C min^{-1}). Injector and detector temperature were 250°C and N_2 was used as the carrier gas at a flow rate of 20 ml min^{-1}. The injection volume was 2 µl.

Lipid peroxidation products

Among the methods available for the detection of lipid peroxidation products in this work we used malondialdehyde (MDA) content, peroxide values and formation of conjugated dienes.

Thiobarbituric acid assay Schmedes and Holmer (1989) discussed the advantages and limitations of this method. MDA was measured by a colorimetric method (Heath and Packer 1968). Roots (100 mg) were homogenized in 5 ml of ethanol (96% v/v). An equal volume of 0.5% 2-thiobarbituric acid in 10% trichloroacetic acid (TCA) solution was added and the sample incubated at 95°C for 30 min. The reaction was stopped by placing the reaction tubes in an ice bucket. The samples were then centrifuged at 10,000 *g* for 30 min. The supernatant was removed and the absorbance measured at 532 nm (Schmedes and Holmer 1989). The amount of MDA present was calculated from the extinction coefficient $\epsilon = 155$ mM^{-1} cm^{-1} (Kosugi et al. 1989).

Estimation of peroxide values Lipids from 1 g of roots were extracted with $CHCl_3$ by 1 h reflux; after drying under vacuum they were transferred to a test-tube. Then, 25 µl of aqueous ammonium thiocyanate solution and 4.85 ml of $CHCl_3$:methanol (3:5, v/v) were added. After 10 min the absorbance was measured at 505 nm. The peroxide value was calculated as milliequivalents per g dry weight of roots using the extinction coefficient $\epsilon = 55.84$ mM^{-1} cm^{-1} (Chapman and MacKay 1949).

Conjugated dienes Roots (100 mg) were homogenized in 10 ml of ethanol (96% v/v), the brei filtered and the absorbance of the supernatant was read at 234 nm. The content of conjugated dienes was calculated using the extinction coefficient $\epsilon = 2.65 \times 10^4$ M^{-1} cm^{-1} (Boveris et al. 1980).

Statistical analysis

Experimental values are means ± SD or SE of the number of experiments indicated in the legends. A two-way analysis of variance was used. Significance (at $P \leq 0.05$) was assessed using Duncan's multiple range test.

Results

Effect of monoterpenes on maize root growth

All monoterpenes inhibited root growth (Fig. 1). At 24 h the bioassays with 1,8-cineole, thymol and geraniol revealed strong inhibition. Interestingly, during the time period from 24 to 96 h the seedlings treated with 1,8-cineole and thymol showed a significant ($P \leq 0.05$) increase in the growth of their roots, although they were always shorter than those of the control samples. In contrast, roots treated with the other monoterpenes (menthol, geraniol and camphor) did not show significant development from 24 to 96 h (Fig. 1).

Effect on FA content and percentage composition

All the treatments reduced the total fatty acid content (TFA) of roots over the period from 0 to 96 h (Fig. 2). It is know that light stimulates FA synthesis, probably by providing both ATP and carbon skeletons (Willms et al. 1999), so this diminution of TFA may possibly have been due to the growth of seedlings in darkness. Under these conditions the plants would not have been able to photosynthesize and would have exhausted their reserves during growth. It is known too that in plants growing in prolonged darkness the β-oxidation cycle necessary for carbohydrate synthesis from FAs is reactivated (Poirier et al. 1999). Hooks et al. (1995) analyzed acyl-CoA oxidase activities in extracts of different tissues (roots among them) of dark-grown maize seedlings and in isolated peroxisomes. They showed higher acyl-CoA oxidase activities in roots and peroxisomes isolated from maize root tips incubated for 24 h without an exogenous carbohydrate supply, when compared to peroxisomes isolated from freshly excised root tips. In our study, the roots treated with 1,8-cineole showed an increase in

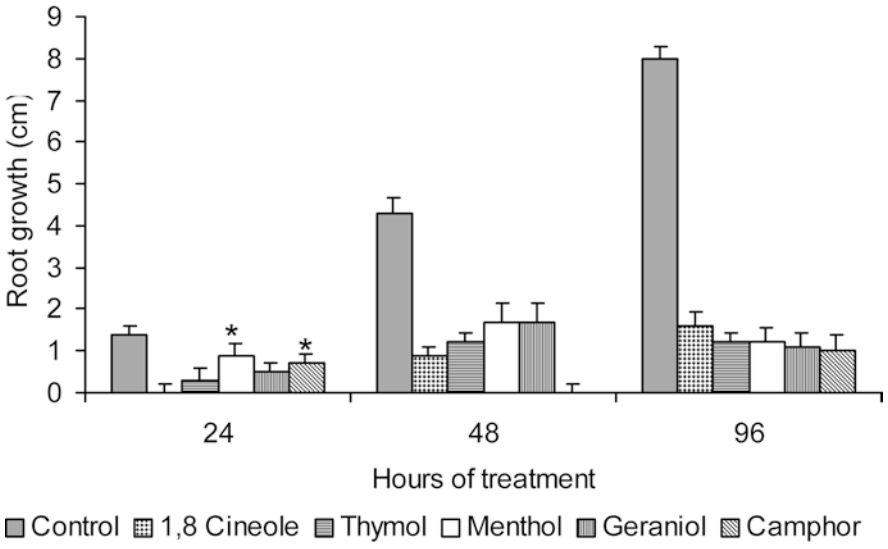

Fig. 1 Effect of monoterpenes on maize (*Zea mays*) root growth. Values are means and SE. * Denotes no significant difference between treatment and control for each time of treatment, according to Duncan's multiple range test at $P \leq 0.05$ ($n = 20$)

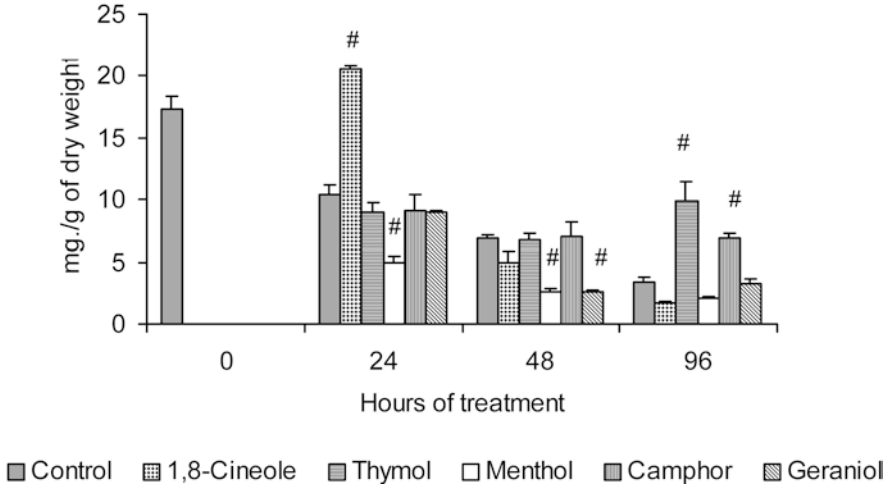

Fig. 2 Effect of monoterpenes on total fatty acid content in maize roots. Values are means and SE. # Denotes significant difference between treatment and control for each time of treatment, according to Duncan's multiple range test at $P \leq 0.05$ ($n = 3$)

TFA from 0 to 24 h of treatment. This behavior is not very clear; nevertheless, at 96 h the TFA was lower than that observed at 24 h. With other treatments the TFA decreased starting at 0 h. At 96 h the roots treated with thymol and camphor showed values of TFA higher than both the control and other treatments, but lower than at 0 h (Fig. 2).

In maize roots, the percentage of FAs 18:2, 18:1 and 16:0 was higher than that of the other FAs studied, 16:1, 18:0 and 18:3 (Table 1). The effects of monoterpenes on the percent composition of FAs were characteristic for the particular monoterpene. Thus, for saturated FAs, 1,8-cineole and thymol did not increase the percentage of 16:0 during the time of treatment. On the other hand, as a result of camphor, menthol and geraniol treatments the amount of 16:0 increased as a function of time from 16.1% (0 h) to 21.4%, 35.4% and 32.3%, respectively, while the control value increased from 16.1% to 44.1% (96 h). The percentage of FA 18:0 increased with time in the control and in the treated samples, although the increase was smaller in the latter than the former (Table 1).

Among unsaturated FAs, there was a diminution with time in the contents of 18:2 for the control and all treatments except 1,8-cineole and camphor. However, contents for all treatments at 96 h were higher than that of the control (Table 1).

Another important unsaturated FA affected was 18:1. In the control, the percentage of FA 18:1 decreased with time of treatment. In seedlings treated with the monoterpenes 1,8-cineole and thymol, the content of 18:1 in roots increased from 24 to 96 h, while the percentage of 18:1 in menthol-, geraniol- and camphor-treated samples did not change. However, for all monoterpene treatments at 96 h, the contents of 18:1 were above that of the control (Table 1).

Although the changes observed in the percent composition of FAs induced by the treatments with the different terpenes were dissimilar, the ratio of unsaturated to saturated FAs decreased by 50% more over time in the control and with exposure to menthol and geraniol than with exposure to the other monoterpenes. Moreover, the ratio of unsaturated to saturated FAs was

Table 1 Effect of five monoterpenes on the FA composition of maize (*Zea mays*) roots. Results are the means of three independent experiments. Values having different letters are significantly different from each other according to Duncan's multiple range test at $P \leq 0.05$ ($n = 3$). *tr* Trace (< 0.5%)

Monoterpene	Hours	Fatty acids (% of total)						Unsaturated/ saturated
		Saturated		Unsaturated				
		16:0	18:0	16:1	18:1	18:2	18:3	
Control	0	16.1^{a}	1.2^{bc}	1.7^{c}	24.5^{efgh}	55.0^{fghi}	1.4^{abc}	4.8
	24	24.8^{f}	5.7^{h}	3.4^{f}	28.2^{i}	36.8^{bc}	1.2^{ab}	2.3
	48	21.6^{de}	2.7^{def}	3.7^{f}	23.6^{defg}	46.6^{e}	1.8^{bcd}	3.1
	96	44.1^{i}	16.1^{j}	2.5^{e}	13.3^{a}	22.9^{a}	1.2^{ab}	0.7
1,8-Cineole	24	14.5^{a}	0.9^{ab}	2.9^{e}	22.3^{cde}	58.0^{i}	1.4^{abc}	5.5
	48	21.1^{cd}	3.8^{g}	4.7^{g}	28.8^{i}	39.5^{cd}	2.1^{cd}	3.0
	96	14.9^{a}	1.9^{bcde}	1.0^{b}	28.5^{i}	52.6^{fg}	1.1^{ab}	4.9
Thymol	24	18.8^{bc}	2.4^{cdef}	1.6^{c}	27.7^{hi}	47.8^{e}	1.7^{abc}	3.7
	48	16.5^{ab}	2.3^{cdef}	2.2^{d}	26.1^{fghi}	51.6^{f}	1.3^{abc}	4.3
	96	16.3^{a}	3.2^{fg}	1.0^{b}	43.1^{j}	35.2^{b}	1.2^{ab}	4.1
Menthol	24	21.7^{de}	1.9^{bcde}	tr^{a}	20.6^{bcd}	54.7^{fghi}	1.2^{ab}	3.2
	48	23.7^{ef}	2.8^{ef}	2.8^{e}	22.1^{bcde}	46.4^{e}	2.4^{d}	2.8
	96	35.4^{h}	7.5^{i}	tr^{a}	19.3^{bc}	36.6^{bc}	1.2^{ab}	1.3
Geraniol	24	18.8^{bc}	1.3^{bc}	tr^{a}	21.6^{bcde}	57.1^{hi}	1.2^{ab}	4.0
	48	20.4^{cd}	2.2^{cdef}	2.6^{e}	21.3^{bcde}	52.5^{fg}	1.0^{a}	3.4
	96	32.3^{g}	5.3^{h}	tr^{a}	18.7^{b}	40.6^{d}	3.0^{e}	1.7
Camphor	24	19.4^{cd}	1.6^{bcd}	tr^{a}	22.5^{cdef}	55.5^{ghi}	1.1^{ab}	3.8
	48	18.8^{bc}	tr^{a}	tr^{a}	26.2^{ghi}	54.0^{fgh}	1.1^{ab}	4.3
	96	21.4^{de}	2.3^{cdef}	2.9^{e}	19.3^{bc}	52.1^{fg}	2.0^{cd}	3.2

higher for all monoterpene treatments than for the control, particularly at 24 and 96 h (Table 1).

Effect on lipid oxidation

Malondialdehyde (MDA) content

In control roots, a lipo-oxidation process due to the senescence of the plant growing in darkness was evidenced by the rise in the MDA content from 0 to 96 h (Table 2; Dhindsa et al. 1981; Jiménez et al. 1998; Pastori and del Rio 1994). From 0 to 24 h of monoterpene treatment, the MDA contents were higher than in the control, suggesting increased lipid peroxidation. However, the levels of MDA at 48 and 96 h were lower than in the control (Table 2).

Content of conjugated dienes

The diene content of control roots rose from 0 to 48 h and then decreased by 96 h (Table 3). However, in 1,8 cineole-treated seedlings the root diene content was lower than in the control over the whole treatment period. On the other hand, diene contents in the presence of the monoterpenes geraniol and menthol were lower than in the control at 24 and 48 h, but they increased by 96 h to higher values than the control. The diene content for samples treated with camphor was higher than for the control at 24 h and then decreased.

Peroxide values

From 0 to 48 h of treatment, peroxides increased in the control roots, as well as in those of seedlings treated with menthol and camphor. However, in the treatments with geraniol, thymol and 1,8-cineole, peroxide values at 0 h were as high as those found at 48 h. For all the monoterpene treatments, peroxide values at 96 h were as high as at 0 h, with the exception of the treatment with thymol and the control, for which values at 96 h were lower than at 0 h (Table 4).

At 24 h all the treatments showed values higher than the control but only the values obtained with menthol and camphor were significantly different. At 48 h, only the thymol treatment showed peroxide values significantly ($P \leq 0.05$) lower than that of the control. At 96 h the treatments with menthol and geraniol showed values higher than for the control (Table 4), suggesting lipid peroxidation.

Table 2 Effect of monoterpenes on MDA content of maize roots from 0 to 96 h of treatment. Values (means ± SD) having different letters are significantly different from each other according to Duncan's multiple range test at $P \leq 0.05$ ($n = 5$)

Terpene	MDA [μmol (g DW)$^{-1}$]			
	0 h	24 h	48 h	96 h
Control	0.84 ± 0.03^{abc}	0.88 ± 0.04^{abc}	1.20 ± 0.07^{fgh}	1.36 ± 0.06^{hi}
1,8-Cineole	–	1.11 ± 0.07^{defg}	1.01 ± 0.04^{cde}	0.78 ± 0.05^{a}
Thymol	–	1.08 ± 0.09^{def}	0.80 ± 0.07^{ab}	1.12 ± 0.09^{defg}
Geraniol	–	1.14 ± 0.11^{efg}	0.81 ± 0.09^{ab}	0.98 ± 0.27^{bcde}
Menthol	–	1.28 ± 0.18^{gh}	1.07 ± 0.09^{def}	0.78 ± 0.20^{a}
Camphor	–	1.49 ± 0.16^{i}	0.87 ± 0.16^{abc}	0.94 ± 0.21^{abcd}

Table 3 Effect of monoterpenes on the content of conjugated dienes in maize roots from 0 to 96 h of treatment. Values (means ± SD) having different letters are significantly different from each other according to Duncan's multiple range test at $P \leq 0.05$ ($n = 8$)

Terpene	Dienes [μmol (g DW)$^{-1}$]			
	0 h	24 h	48 h	96 h
Control	50.5 ± 4.1^{efg}	64.5 ± 8.9^{i}	75.6 ± 8.1^{j}	41.5 ± 6.5^{cd}
1,8-Cineole	–	48.2 ± 3.6^{def}	43.2 ± 5.1^{cd}	28.5 ± 4.8^{a}
Thymol	–	57.2 ± 5.1^{gh}	44.4 ± 4.0^{cdef}	41.6 ± 3.6^{cd}
Geraniol	–	46.2 ± 4.7^{cdef}	33.2 ± 0.8^{b}	59.4 ± 8.1^{hi}
Menthol	–	50.6 ± 1.8^{fg}	40.6 ± 3.3^{c}	57.2 ± 6.3^{gh}
Camphor	–	72.0 ± 6.0^{j}	48.0 ± 2.3^{def}	43.4 ± 5.5^{cde}

Table 4 Effect of monoterpenes on peroxide values of maize roots from 0 to 96 h of treatment. Values (means ± SD) having different letters in the same column and different numbers in the same row are significantly different from each other according to Duncan's multiple range test at $P \leq 0.05$ ($n = 4$)

Terpenes	Peroxide [milliequivalents (g DW)$^{-1}$]			
	0 h	24 h	48 h	96 h
Control	1.88 ± 0.20^{2}	1.69 ± 0.44^{2a}	3.00 ± 0.07^{3bc}	1.13 ± 0.30^{1a}
1,8-Cineole	1.88 ± 0.20^{12}	1.77 ± 0.55^{12ab}	2.30 ± 0.60^{2ab}	1.17 ± 0.34^{1a}
Thymol	1.88 ± 0.20^{2}	2.29 ± 0.22^{2abc}	1.70 ± 0.81^{12a}	1.04 ± 0.27^{1a}
Geraniol	1.88 ± 0.20^{1}	2.82 ± 1.11^{1abc}	2.37 ± 0.32^{1ab}	2.72 ± 0.91^{1b}
Menthol	1.88 ± 0.20^{1}	3.03 ± 0.68^{23c}	3.51 ± 0.86^{3c}	2.28 ± 0.41^{12b}
Camphor	1.88 ± 0.20^{12}	2.88 ± 0.87^{23bc}	3.53 ± 0.97^{3c}	1.23 ± 0.27^{1a}

Discussion

Many stresses that disrupt the cellular homeostasis of cells enhance the production of reactive oxygen intermediates (ROIs). These ROIs act as signals for the activation of stress-response and defense pathways (Mittler 2002).

Exposure of maize seedlings to monoterpenes resulted in a stress state during the first 24 h. This is observed in the values of MDA, for all the treatments (Table 2), in the values of conjugated dienes for the treatment with camphor, and in the values of peroxides for treatments with camphor and menthol (Tables 3 and 4, respectively). However, at 96 h of treatment the oxidation values as evaluated by the different methods indicate that all the treatments lead to a lower state of oxidation than in the control. Nevertheless, the treatments with geraniol and menthol favour oxidation processes starting at 48 h, as suggested by the values for conjugated dienes and peroxides (Tables 3 and 4, respectively). This could be possibly due to the deactivation of the plant's antioxidative defense system (Jiménez et al. 1998; Mittler 2002; Foyer and Halliwell 1976; Fridovich 1986) and would facilitate the continuation of the oxidation processes in the presence of these two terpenes after a short pause at 48 h. However, this re-activation of the oxidation processes was not observed in the case of MDA production at 96 h with geraniol and menthol, despite the fact that the concentration of FA 18:3 (a precursor of MDA) was higher with the former monoterpene and equal with the latter monoterpene if compared with the control values. Presumably, the MDA content at 96 h declines because it is further oxidized (Beuge and Aust 1978) or it can be readily metabolized by mitochondria, as was reported by Muscari et al. (1990).

The treatment with camphor did not show oxidation values at 96 h higher than those of the control, as evaluated through the determination of conjugated dienes and peroxide; nevertheless, it produced higher values of 18:3 (Table 1) but lower values of MDA than the control at this time. This would indicate that the plant's antioxidant processes have not been affected. This last explanation can be extended to the other treatments, cineole and thymol, for which the 18:3 concentrations were equal to the control but the values of MDA lower. All these explanations are supported by the FA composition (Table 1).

The monoterpenes, being lipophilic compounds, could act physically as perturbing agents, altering the packing, fluidity, and/or physical arrangement of the phospholipids in the membrane (Sikkema et al. 1995; García et al. 1995; Perillo et al. 1999; Turina and Perillo 2003). These effects on membrane organization may trigger changes in the activity of the lipolytic enzymes (Perillo et al. 1994, and references therein). So, we suggest that the increase in the percentage of unsaturated FAs provoked by the monoterpenes (Table 2) could be a response of the plant to their penetration into membrane. It has been suggested that an increase in FA unsaturation would tend to maintain the liquid–crystalline phase of the membrane and prevent lipid peroxidation, and it has also been suggested that oxygen free radicals can promote degradative reactions causing a lost of membrane phospholipids in the absence of changes in FA unsaturation (McKersie et al. 1990). So, the increase in the unsaturated FAs might be associated with an impairment of membrane function due to an increase in membrane fluidity (Karp 1987, p 168), above that required to maintain homeostatic viscosity (Vigh et al. 1979; De Santis et al. 1999).

Pastori and Trippi (1995) also showed a higher ratio of unsaturated to saturated FAs in maize and wheat leaves resistant to water and oxygen stress.

Asplund (1968), Bradow and Connick (1988, 1990), Romagni et al. (2000), and Abrahim et al. (2000) studied the relationship between chemical structure and toxicity of volatile compounds. Asplund (1968) and Bradow and Connick (1990) showed that the most toxic compounds were those having an oxygenated functional group and, among them, the carbonyl group was the most toxic. Our results showed that the most phytotoxic compounds were the alcoholic and phenolic monoterpenes because they showed the lowest IC_{80} (see Materials and methods).

Thus, from our results, we suggest that monoterpenes are able to cause oxidative stress in roots, a property which could explain some physiological and lipid composition changes caused by those compounds in intact tissues (Lorber and Muller 1976; Maffei et al. 2001). Moreover, the alcoholic monoterpenes appear to have a different mode of action compared with the other monoterpenes studied. They produced high values of oxidation at 96 h and the lowest ratio of unsaturated to saturated FAs at this time.

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