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# CHANGES IN THE COMPOSITION OF PHOSPHOLIPID FATTY ACIDS AND STEROLS OF MAIZE ROOT IN RESPONSE TO MONOTERPENES

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**Abstract**—Terpenes are thought to be important in plant–plant interactions because of their phytotoxic action on seed germination and growth. Herein, the effects of five volatile monoterpenes on root sterols and phospholipid fatty acid (PLFA) composition have been studied during maize seedling germination. The investigated monoterpenes (camphor, 1–8 cineole, geraniol, menthol, and thymol) were applied at their respective $IC_{80}$ (concentration causing 80% inhibition). They quantitatively affected free sterols and PLFA composition, thus producing an increase in the percentage of unsaturated PLFAs, stigmasterol of the free sterol fraction, and saturated steryl ester fatty acids. Alcoholic and nonalcoholic monoterpenes appeared to have different modes of action. The former affected unsaturated fatty acid and stigmasterol to a greater extent, and accordingly they could interfere in seedling growth by changes in the membrane lipids.

**Key Words**—Allelopathy, fatty acid, monoterpenes, sterol, *Zea mays*, geraniol, 1,8-cineole, menthol, camphor, thymol, phytotoxicity, membrane.

## INTRODUCTION

Monoterpenes are toxic towards vascular plants (Vaughn and Spencer, 1993, 1996; Scrivanti et al., 2003). An extensive study by Vaughn and Spencer (1993) showed that 18 volatile monoterpenes are inhibitory to the germination and growth of nine different plant species. They were generally phytotoxic to corn, wheat, and alfalfa. Notwithstanding, the mode and mechanism by which these

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compounds affect the growth of other plant species have been poorly examined at chemical levels (Abrahim et al., 2000; Romagni et al., 2000).

In recent years, the lipid composition of various plant tissues has been shown to influence a number of factors crucial to growth and development (Grandmougin-Ferjani et al., 1997; Borst et al., 2000; Hellgren and Sandelius, 2001; Harker et al., 2003). Phospholipids are more than just structural components of membranes; they can be cofactors for membrane enzymes, signal precursors, or signaling molecules themselves (Laxalt and Munnik, 2002). Plant sterols, like other sterols, are primary components of cellular membranes where they regulate fluidity and permeability. This structural role is often described as "bulk" function. Additionally, sterols can participate in the control of membrane-associated metabolic processes; their involvement in signal transduction events has been reported in mammalian cells (Hartmann, 1998). It is known that free and esterified sterols participate in the formation of biomembranes, and during plant development the sterol content is not static (Huang and Grunwald, 1988; Grandmougin et al., 1989; Izzo and Navari-Izzo, 1993; Moreau et al., 1998).

Since monoterpenes are lipophilic compounds, the question arises whether they act on plant growth by affecting lipid composition. This investigation was conducted to evaluate how monoterpenes affect the most abundant free and esterified sterols, i.e., sitosterol, stigmasterol, and campesterol (Dyas and Goad, 1993; Hartmann, 1998) and phospholipid fatty acid (PLFA) composition of roots during maize seedling growth.

## METHODS AND MATERIALS

*Chemicals.* The monoterpenes used were 1,8-cineole, thymol, menthol, geraniol, and camphor. Previous studies indicated these were the most active compounds (Asplund, 1968; Vaughn and Spencer, 1993, 1996; Koitabashi et al., 1997) They were obtained from ICN Pharmaceuticals Co. (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. 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 d at 27 ± 1°C in the dark. At the end of this period, the length of the roots was 6 ± 1 cm, and the seedlings were harvested and transferred to bioassay.

*Bioassay.* Three-d-old seedlings (0 hr 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 beaker. No direct physical contact occurred between the compounds and the seedlings. These amounts 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 inhibitory concentration 80 ($IC_{80}$), defined as the concentration that inhibited 80% of root growth at 96 hr of treatment compared with the control (Zunino and Zygadlo, 2004). Flasks were placed in the dark at 27 ± 1°C and atmospheric pressure for 24, 48, and 96 hr. Then, the plants were harvested, the roots dissected, and the different experimental parameters were measured. The central beakers were left empty in the controls.

*Analysis of Volatile Compounds.* Volatiles from headspace of glass desiccators were trapped by using a 10-ml gastight syringe and were analyzed by gas chromatography (GC) and GC/mass spectrometry (MS). A gas chromatograph (Shimadzu R1A) equipped with a flame ionization detector was used. 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 μm film thickness), and ramped column temperature conditions (60°C for 3 min, increased 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 by using a mass selective detector (Perkin-Elmer Q700 gas chromatograph–mass spectrometer) and co-injection with standards. GC-MS was performed under the same conditions. The ionization potential of MS was 70 eV. The quantity of monoterpenes in the headspace was determined by the external standard method (Vaughn and Spencer, 1993). Standard curves were generated by analysis of known concentrations of each compound dissolved in *n*-$C_6H_{14}$.

*Lipid Extraction and Analysis.* Lipids were extracted from the roots (20 ± 5 g fresh weight) with $CHCl_3$:methanol (87:13) in a Soxhlet apparatus for 12 hr. Extracts were dried on dry $Na_2SO_4$, taken to dryness under vacuum, and chromatographed on preparative silica gel thin-layer plates (silica gel 60 G F254) using hexane–ether (4:1 by volume) as the developing system. Sterols were visualized under UV light. Three fractions were eluted corresponding to steryl esters (SE), free sterol (FS), and phospholipids (PL). Cholesteryl myristate, sitosterol, and phosphatidylcholine were used as markers, respectively. The FS fraction was scraped off and eluted in dichloromethane, filtered to remove the residual silica, dried in a rotatory evaporator, and then purified by a bidirectional thin-layer chromatography (TLC; hexane–ether 4:1 and benzene–ether 4:1 as developing system) (Rohmer et al., 1972). SE and PL fractions were saponified with 1 N methanolic KOH to obtain the respective fatty acids. Fatty methyl esters from SE and PL were prepared by transesterification through treatment with 1 N $H_2SO_4$ in dry methanol for 30 min (Grosso et al., 1994) and analyzed by GC. Quantitative determinations of total PLFA were made with

heptadecanoic (C17:0) methyl esters as an internal reference. Analytical GC was performed on a Shimadzu GC-R1A gas chromatograph (FID) fitted with Supelcowax-10 capillary column (30 m × 0.25 mm i.d.). Column temperature was programmed from 180 to 240°C (4°C $min^{-1}$). Injector and detector temperature was 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.

SE fraction sterols were recovered from unsaponifiable matter and together with FS were subjected to GC analysis. Qualitative and quantitative analysis were carried out using a Shimadzu GC-R1A gas chromatograph (FID) fitted with DB5 capillary column (30 m × 0.25 mm i.d.). Column temperature was programmed from 240°C to 290°C (4°C $min^{-1}$). Injector and detector temperature was 300°C, and $N_2$ was used as the carrier gas at a flow rate of 1 ml/min. The injection volume was 2 μl.

Sterol identities were confirmed by comparison with authentic compounds under identical conditions using a GC mass-selective detector.

*Statistical Analyses.* Experimental values are means and SDs of three independent experiments. A two-way analysis of variance (ANOVA) was used to evaluate treatment time (T) and monoterpenes (M) effects together with their interaction (T × M). Significance (at $P \leq 0.05$) was assessed using DGC multiple range test (Di Rienzo et al., 2002).

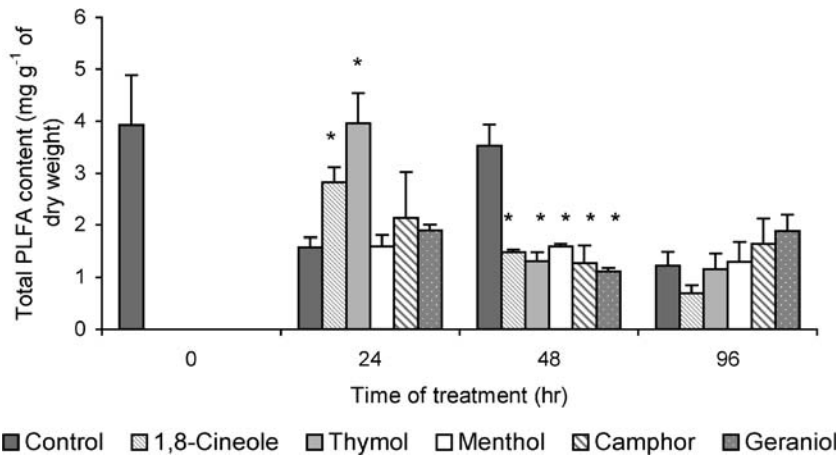


FIG. 1. Effect of monoterpenes on total PLFA content in maize roots. Values are means and SD. *Denotes significant differences between treatment and control for each time of treatment, according to DGC multiple range test at $P \leq 0.05$ ($n = 3$).

## RESULTS

*Effect of Monoterpenes on Phospholipid Fatty Acid Content.* Each treatment and the control reduced the total PLFA content of roots during the period that goes from 0 to 96 hr (Figure 1). Control roots and roots treated with menthol, camphor, and geraniol showed a decrease of total PLFA at 24 hr, but soon after a recovery occurred in control roots at 48 hr (but not with treatments). Otherwise, roots treated with 1,8-cineole and thymol showed a decrease of the total PLFA from 48 hr. Nonetheless, the total PLFA of each treatment and control at 96 hr was lower than that observed at 0 hr (Figure 1).

In maize roots, the FAs 16:0, 16:1, 18:0, 18:1, 18:2, and 18:3 were the main contributing ones (Grandmougin et al., 1989; Bohn et al., 2001). The percentage of FA 18:2, 18:1, and 16:0 was higher than the other FAs, 16:1, 18:0, and 18:3 (Table 1). Saturated FAs did not show any significant changes

TABLE 1. EFFECT OF 1,8-CINEOLE, THYMOL, MENTHOL, GERANIOL, AND CAMPHOR ON PLFA COMPOSITION OF MAIZE (*Zea mays*) ROOTS

| | | PLFA (% of total) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Monoterpenes | Time (hr) | 16:0** | 16:1* | 18:0* | 18:1* | 18:2* | 18:3** | Unsaturated/ saturated* |
| Control | 0 | $27.63^{1}$ | $1.43^{a}$ | $1.46^{a}$ | $9.17^{a}$ | $56.41^{b}$ | $3.90^{1}$ | $2.5^{b}$ |
| | 24 | $29.92^{b1}$ | $1.08^{a}$ | $1.77^{a}$ | $9.47^{a}$ | $53.82^{b}$ | $4.12^{a1}$ | $2.2^{b}$ |
| | 48 | $28.39^{a1}$ | $1.81^{a}$ | $1.83^{a}$ | $7.77^{a}$ | $55.88^{b}$ | $4.32^{a1}$ | $2.3^{b}$ |
| | 96 | $27.68^{a1}$ | $2.60^{a}$ | $7.57^{b}$ | $18.40^{b}$ | $39.27^{a}$ | $4.49^{a1}$ | $1.8^{a}$ |
| 1,8 Cineole | 24 | $24.80^{a1}$ | $5.30^{b}$ | $2.03^{a}$ | $20.72^{b}$ | $44.23^{a}$ | $2.92^{a1}$ | $2.8^{b}$ |
| | 48 | $28.80^{a1}$ | $4.90^{b}$ | $3.55^{a}$ | $13.03^{a}$ | $45.85^{a}$ | $3.87^{a1}$ | $2.1^{b}$ |
| | 96 | $31.25^{a1}$ | $2.11^{a}$ | $3.41^{a}$ | $10.91^{a}$ | $47.19^{a}$ | $5.14^{a1}$ | $1.6^{a}$ |
| Thymol | 24 | $25.12^{a1}$ | $1.07^{a}$ | $2.60^{a}$ | $15.80^{b}$ | $51.7^{b}$ | $3.70^{a1}$ | $2.6^{b}$ |
| | 48 | $28.19^{a1}$ | $1.93^{a}$ | $2.73^{a}$ | $8.80^{a}$ | $54.17^{b}$ | $4.18^{a1}$ | $2.2^{b}$ |
| | 96 | $30.26^{a1}$ | $1.04^{a}$ | $2.76^{a}$ | $6.73^{a}$ | $54.06^{b}$ | $5.16^{a1}$ | $2.0^{b}$ |
| Menthol | 24 | $30.14^{b1}$ | $1.75^{a}$ | $2.04^{a}$ | $7.48^{a}$ | $55.01^{b}$ | $3.58^{a1}$ | $2.1^{b}$ |
| | 48 | $27.63^{a1}$ | $1.86^{a}$ | $1.87^{a}$ | $8.30^{a}$ | $56.22^{b}$ | $4.12^{a1}$ | $2.4^{b}$ |
| | 96 | $29.70^{a1}$ | $1.79^{a}$ | $2.24^{a}$ | $7.02^{a}$ | $55.04^{b}$ | $4.21^{a1}$ | $2.1^{b}$ |
| Geraniol | 24 | $25.06^{a1}$ | $1.23^{a}$ | $1.66^{a}$ | $8.30^{a}$ | $59.90^{b}$ | $3.86^{a1}$ | $2.7^{b}$ |
| | 48 | $28.69^{a1}$ | $1.24^{a}$ | $2.18^{a}$ | $7.53^{a}$ | $56.60^{b}$ | $3.77^{a1}$ | $2.2^{b}$ |
| | 96 | $29.19^{a1}$ | $2.62^{a}$ | $2.61^{a}$ | $7.72^{a}$ | $54.03^{b}$ | $3.84^{a1}$ | $2.2^{b}$ |
| Camphor | 24 | $25.66^{a1}$ | $2.28^{a}$ | $3.77^{a}$ | $11.95^{a}$ | $52.19^{b}$ | $4.15^{a1}$ | $2.4^{b}$ |
| | 48 | $28.42^{a1}$ | $3.74^{b}$ | $2.50^{a}$ | $11.18^{a}$ | $50.70^{b}$ | $3.47^{a1}$ | $2.2^{b}$ |
| | 96 | $25.64^{a1}$ | $4.40^{b}$ | $3.28^{a}$ | $14.51^{b}$ | $46.99^{a}$ | $5.18^{a1}$ | $2.5^{b}$ |

Results are the means of three independent experiments.

*ANOVA with interaction. Values having different letters are significantly different from each other according to DGC multiple range test at $P \leq 0.05$.

**ANOVA without interaction. For each monoterpene, values in columns followed by different numbers and for each time value in rows followed by different letters are significantly different according to DGC multiple range test at $P \leq 0.05$.

with treatments with the exception of FA 18:0 that increased from 48 to 96 hr in control roots. At 24 hr, roots treated with 1,8-cineole, thymol, geraniol, and camphor showed lower percentage of FA 16:0 than controls. For each monoterpene, the percentage of FA 18:0 was lower than controls at 96 hr. Among unsaturated FAs, there was a decrease from 0 to 96 hr in the percentage of 18:2 for controls, 1,8-cineole, and camphor. Compared with the untreated control, the composition of 18:2 at 96 hr was higher in each treatment, except in the 1,8-cineole and camphor treatments where their composition was similar to that of the controls (Table 1). A significant increase in the FA 18:1 was observed at 96 hr in the control and camphor treatments. This was not observed with the other treatments, which showed lower percentages of 18:1 than those at 96 hr. At 24 hr, 1,8-cineole and thymol treatments showed higher values of 18:1 than controls. FA 18:3 showed changes neither in time nor with treatments. Whereas FA 16:1 increased only with camphor during treatments (showing

TABLE 2. EFFECT OF 1,8-CINEOLE, THYMOL, MENTHOL, GERANIOL AND CAMPHOR ON SEFA COMPOSITION OF MAIZE (*Zea mays*) ROOTS

| | | SEFA (% of total) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Monoterpenes | Time (hr) | 16:0** | 16:1* | 18:0* | 18:1** | 18:2* | 18:3** | Unsaturated/ saturated** |
| Control | 0 | $19.96^{1}$ | $3.01^{a}$ | $5.48^{a}$ | $21.83^{1}$ | $49.71^{b}$ | $Tr^{1}$ | $3.4^{2}$ |
| | 24 | $12.44^{a1}$ | $1.35^{a}$ | $2.25^{a}$ | $29.27^{b1}$ | $54.68^{b}$ | $Tr^{a1}$ | $5.8^{b2}$ |
| | 48 | $14.88^{a1}$ | $0.98^{a}$ | $6.43^{a}$ | $27.91^{a1}$ | $49.80^{b}$ | $Tr^{a1}$ | $4.0^{b2}$ |
| | 96 | $16.40^{a1}$ | $4.31^{a}$ | $8.87^{a}$ | $23.09^{a1}$ | $47.32^{b}$ | $Tr^{a1}$ | $3.1^{b2}$ |
| 1,8 Cineole | 24 | $17.54^{a1}$ | $2.29^{a}$ | $4.98^{a}$ | $33.41^{b1}$ | $41.79^{b}$ | $Tr^{a1}$ | $3.5^{a2}$ |
| | 48 | $28.89^{b1}$ | $1.79^{a}$ | $9.02^{a}$ | $31.60^{a1}$ | $28.70^{a}$ | $Tr^{a1}$ | $1.6^{a2}$ |
| | 96 | $17.62^{a1}$ | $2.93^{a}$ | $9.97^{a}$ | $28.61^{a1}$ | $40.88^{b}$ | $Tr^{a1}$ | $2.7^{b2}$ |
| Thymol | 24 | $19.42^{a1}$ | $4.56^{a}$ | $8.61^{a}$ | $22.32^{a1}$ | $45.09^{b}$ | $Tr^{a1}$ | $3.2^{a2}$ |
| | 48 | $15.15^{a1}$ | $4.1^{a}$ | $5.68^{a}$ | $22.89^{a1}$ | $52.18^{b}$ | $Tr^{a1}$ | $4.0^{b2}$ |
| | 96 | $18.23^{a1}$ | $5.76^{a}$ | $6.16^{a}$ | $21.77^{a1}$ | $48.08^{b}$ | $Tr^{a1}$ | $3.1^{b2}$ |
| Menthol | 24 | $22.36^{a1}$ | $9.06^{b}$ | $11.56^{a}$ | $20.14^{a1}$ | $36.87^{b}$ | $Tr^{a1}$ | $2.1^{a2}$ |
| | 48 | $23.78^{b1}$ | $6.69^{a}$ | $9.12^{a}$ | $19.05^{a1}$ | $43.59^{b}$ | $Tr^{a1}$ | $2.2^{a2}$ |
| | 96 | $24.49^{b1}$ | $9.75^{b}$ | $11.39^{a}$ | $27.87^{a1}$ | $24.84^{a}$ | $6.68^{b2}$ | $1.7^{a2}$ |
| Geraniol | 24 | $17.97^{a1}$ | $6.58^{a}$ | $8.03^{a}$ | $30.39^{b1}$ | $36.44^{b}$ | $1.77^{b1}$ | $2.9^{a2}$ |
| | 48 | $29.49^{b1}$ | $9.26^{b}$ | $19.00^{b}$ | $16.35^{a1}$ | $25.07^{a}$ | $1.66^{b1}$ | $1.1^{a1}$ |
| | 96 | $27.62^{b1}$ | $8.90^{b}$ | $15.31^{b}$ | $22.72^{a1}$ | $24.45^{a}$ | $4.01^{b2}$ | $1.3^{a1}$ |
| Camphor | 24 | $27.44^{a1}$ | $6.90^{a}$ | $14.34^{b}$ | $20.57^{a1}$ | $30.74^{a}$ | $Tr^{a1}$ | $1.6^{a1}$ |
| | 48 | $25.38^{b1}$ | $5.38^{a}$ | $20.41^{b}$ | $21.96^{a1}$ | $25.69^{a}$ | $2.38^{b2}$ | $1.0^{a1}$ |
| | 96 | $32.97^{b1}$ | $5.02^{aa}$ | $20.83^{b}$ | $22.20^{a1}$ | $18.98^{a}$ | $Tr^{a1}$ | $1.1^{a1}$ |

Results are the means of three independent experiments. Tr, Trace (<0.5%).

*ANOVA with interaction. Values having different letters are significantly different from each other according to DGC multiple range test at $P \leq 0.05$.

**ANOVA without interaction. For each monoterpene, value in columns followed by different numbers and for each time value in rows followed by different letters are significantly different, according to DGC multiple range test at $P \leq 0.05$.

higher values than controls at 48 and 96 hr), 1,8-Cineole also produced higher values of 16:1 than controls, occurring at 24 and 48 hr (Table 1).

Regarding the unsaturated to saturated FA ratio, a decrease from 0 to 96 hr was shown in control roots and the 1,8-cineole treatment. Nevertheless, the rest of the monoterpenes did not show changes over time but showed higher unsaturated to saturated ratio values than controls at 96 hr (Table 1).

*Effect of Monoterpenes on Composition of Sterol Ester Fatty Acids.* The main FA of SE, like PL, were 16:0, 18:1, and 18:2 (Table 2). The saturated FA 16:0 showed higher values than controls at 48 hr with 1,8-cineole, and at 48 and 96 hr with menthol, geraniol, and camphor. FA 18:0, on the other hand, increased with geraniol at 48 and 96 hr, and with camphor treatment at all time points studied (Table 2). Among unsaturated FAs, 16:1 and 18:3 showed increases preferentially with menthol and geraniol monoterpenes. With camphor, FA 18:3 increased at 48 hr. In contrast, the percentage of the major compounds FA 18:1 and 18:2 showed lower values than controls: 18:1 at 24 hr with monoterpenes thymol, menthol, and camphor, and FA 18:2 with 1,8-

TABLE 3. EFFECT OF 1,8-CINEOLE, THYMOL, MENTHOL, GERANIOL, AND CAMPHOR ON FS COMPOSITION OF MAIZE (*Zea mays*) ROOTS

| | | FS (% of total) | | |
|---|---|---|---|---|
| Monoterpenes | Time (hr) | Campesterol | Stigmasterol | Sitosterol |
| Control | 0 | 19.74[b] | 41.56[a] | 38.74[d] |
| | 24 | 25.57[b] | 36.50[a] | 37.93[d] |
| | 48 | 17.74[b] | 53.90[b] | 28.36[c] |
| | 96 | 25.17[b] | 42.03[a] | 32.70[c] |
| 1,8 Cineole | 24 | 21.27[b] | 45.87[a] | 32.90[c] |
| | 48 | 15.70[a] | 59.14[b] | 25.17[c] |
| | 96 | 30.40[c] | 50.67[a] | 18.97[b] |
| Thymol | 24 | 22.23[b] | 47.25[a] | 30.55[c] |
| | 48 | 13.68[a] | 55.84[b] | 30.48[c] |
| | 96 | 24.35[b] | 48.88[a] | 26.77[c] |
| Menthol | 24 | 20.35[b] | 61.93[b] | 17.72[b] |
| | 48 | 22.73[b] | 57.79[b] | 19.48[b] |
| | 96 | 28.20[c] | 63.60[b] | 8.19[a] |
| Geraniol | 24 | 21.38[b] | 64.91[b] | 13.71[b] |
| | 48 | 17.85[b] | 58.23[b] | 23.92[c] |
| | 96 | 17.59[b] | 67.78[b] | 14.63[b] |
| Camphor | 24 | 26.51[b] | 50.35[a] | 23.14[c] |
| | 48 | 23.13[b] | 60.23[b] | 16.64[b] |
| | 96 | 23.40[b] | 56.86[b] | 19.74[b] |

Results are the means of three independent experiments.
ANOVA with interaction. Values having different letters are significantly different from each other according to DGC multiple range test at $P \leq 0.05$.

cineole (at 48 hr), menthol (at 96 hr), geraniol (at 48 and 96 hr), and camphor (at all times). These changes in FA composition are shown in the unsaturated to saturated ratios, which were lower than controls at every time studied for all monoterpenes—with the exception of 1,8-cineole at 96 hr and thymol at 48 and 96 hr (Table 2).

*Effect of Monoterpenes on Composition of Free Sterols*. The main FS constituent in the FS maize root fraction was stigmasterol (36.5–67.8%), as observed in the control and all monoterpene treatments (Table 3). Treatment with 1,8-cineole and menthol showed an increase in campesterol at 96 hr, whereas at 48 hr it decreased with 1,8-cineole and thymol monoterpenes. Stigmasterol values increased from 0 to 48 hr, decreasing at 96 hr with controls and 1,8-cineole and thymol treatments. At 24 and 96 hr, there were increases in stigmasterol with the menthol and geraniol treatments. With camphor, this FS increased at 96 hr, too (Table 3). In the control and every monoterpene studied, sitosterol decreased through time. At 96 hr, only with thymol were there no significant differences from control, and at 48 hr this FS was unchanged with

TABLE 4. EFFECT OF 1,8-CINEOLE, THYMOL, MENTHOL, GERANIOL, AND CAMPHOR ON SE COMPOSITION OF MAIZE (*Zea mays*) ROOTS

| | | SE (% of total) | | |
|---|---|---|---|---|
| Monoterpenes | Time (hr) | Campesterol | Stigmasterol | Sitosterol |
| Control | 0 | 14.66[a] | 41.87[b] | 43.47[a] |
| | 24 | 17.44[a] | 27.12[a] | 55.44[b] |
| | 48 | 16.36[a] | 14.53[a] | 69.11[c] |
| | 96 | 20.70[b] | 28.17[a] | 51.10[b] |
| 1,8 Cineole | 24 | 19.21[a] | 20.52[a] | 60.14[b] |
| | 48 | 13.37[a] | 15.80[a] | 70.83[c] |
| | 96 | 22.88[b] | 22.32[a] | 54.80[b] |
| Thymol | 24 | 18.22[a] | 21.82[a] | 60.02[b] |
| | 48 | 19.27[a] | 17.83[a] | 62.90[c] |
| | 96 | 25.72[b] | 28.29[a] | 45.99[a] |
| Menthol | 24 | 18.69[a] | 26.36[a] | 54.96[b] |
| | 48 | 14.06[a] | 19.66[a] | 66.28[c] |
| | 96 | 23.43[b] | 23.82[a] | 52.74[b] |
| Geraniol | 24 | 17.30[a] | 18.37[a] | 64.32[c] |
| | 48 | 21.89[b] | 24.15[a] | 53.95[b] |
| | 96 | 13.83[a] | 20.03[a] | 66.14[c] |
| Camphor | 24 | 16.01[a] | 25.51[a] | 58.48[b] |
| | 48 | 18.66[a] | 23.71[a] | 57.63[b] |
| | 96 | 23.91[b] | 41.15[b] | 34.94[a] |

Results are the means of three independent experiments.
ANOVA with interaction. Values having different letters are significantly different from each other according to DGC multiple range test at $P \leq 0.05$.

1,8-cineole, geraniol, and thymol treatments. The rest of the studied monoterpenes showed lower sitosterol values than those of control. This is shown especially with menthol treatment, which produced the small value of 8.19% (Table 3).

*Effect of Monoterpenes on Composition of Sterols of Sterol Esters.* The main sterol in the SE maize root fraction observed in all treatments and controls was sitosterol (43.5–70.8%; Table 4), which is in accordance with the levels expected for this sterol in maize roots (Kemp et al., 1967). Sitosterol increased with time, reaching a maximum level at 48 hr in the control experiment, as well as in the 1,8-cineole, thymol, and menthol treatments. It decreased significantly

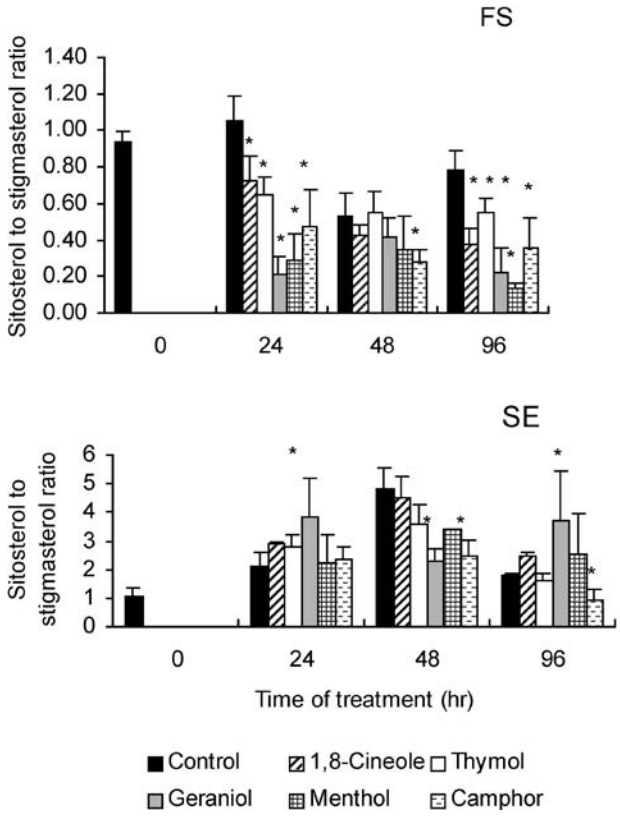


FIG. 2. Effect of monoterpenes on sitosterol to stigmasterol ratio of free sterol (FS) and steryl ester (SE) fractions in maize roots. Values are means and SD. *Denotes significant differences between treatment and control for each time of treatment, according to DGC multiple range test at $P \leq 0.05$ ($n = 3$).

in only the thymol and camphor treatments to levels of 45.99% and 34.94%, respectively, at 96 hr (Table 4). A different result was observed with geraniol treatments. It showed maximum values at 24 and 96 hr (64.32% and 66.14%, respectively) that were higher than controls.

Meanwhile, stigmasterol decreased from 0 to 96 hr in controls, and with all monoterpene treatments showed no significant differences. Only with camphor did this sterol increase at 96 hr—similarly to 0 hr (Table 4).

Geraniol treatment increased campesterol at 48 hr and decreased later (Table 4).

*Effect of Monoterpenes on Sitosterol to Stigmasterol Ratio.* The SE fraction presented a higher sitosterol to stigmasterol ratio (values >1) than that of FS fraction (values <1) (Figure 2), which is in agreement with previously

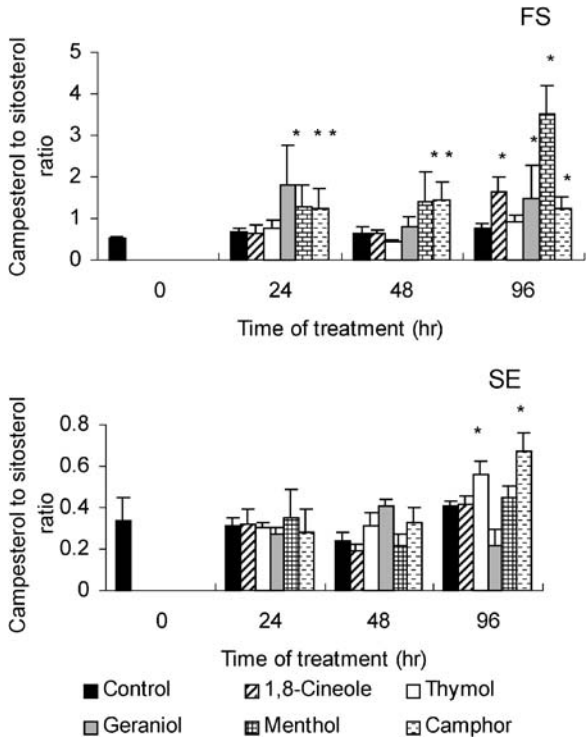


FIG. 3. Effect of monoterpenes on campesterol to sitosterol ratio of free sterol (FS) and steryl ester (SE) fractions in maize roots. Values are means and SD. *Denotes significant differences between treatment and control for each time of treatment, according to DGC multiple range test at $P \leq 0.05$ ($n = 3$).

published data and documents the discriminating character of sterol esterification in plants (Kemp et al., 1967, 1968; Dyas and Goad, 1993). The sitosterol to stigmasterol ratio was more affected by monoterpene treatments in the FS than in the SE fraction. In the FS fraction, this ratio decreased in the studied monoterpenes, mainly at 24 and 96 hr due to an increase in stigmasterol and decrease in sitosterol percentages (Figure 2). In the SE fraction, monoterpenes produced a different result, only geraniol and camphor treatments showing significant differences from controls (Figure 2).

*Effect of Monoterpenes on Campesterol to Sitosterol Ratios.* The FS fraction produced a higher campesterol to sitosterol ratio than that of the SE fraction (values <1) (Figure 3). Only thymol and camphor treatments increased this sterol ratio in the SE fraction at 96 hr, whereas in the FS fraction it was increased mainly by geraniol, menthol, and camphor. These terpenes increased by about 166%, 92%, and 84%, respectively, at 24 hr. Menthol treatment at 96 hr caused a 351% increase in that sterol ratio (Figure 3).

## DISCUSSION

The growth of seedlings in darkness could explain the low content shown in the total PLFA fraction of the untreated control roots (Figure 1) because lipid biosynthesis slows in the dark (Somerville and Browse, 1991). Moreover, plants growing in prolonged darkness reactivate the β-oxidation cycle necessary for carbohydrate synthesis from fatty acids (Poirier et al., 1999). The fluctuation observed in control roots, in which the total PLFA content decreased at 24 hr and then increased to almost the original level at 48 hr (Figure 1), could be explained by the stress that the plant suffers when transferred to the bioassay.

Monoterpenes, which are lipophilic compounds, could alter the packing, fluidity, and/or physical arrangement of phospholipids in the membrane (García et al., 1995; Sikkema et al., 1995; Perillo et al., 1999; Turina and Perillo, 2003). Plants treated with monoterpenes showed an increase in the unsaturated to saturated PLFA ratio at 96 hr, with the exception of 1,8-cineole. This increase is mainly due to the high percentage of FA 18:2 and reduction of FA 18:0 (Table 1). Variations in PLFA unsaturation may produce drastic effects on physical and functional membrane properties, and membrane fluidity may increase (Stubbs and Smith, 1984; Karp, 1987; Borst et al., 2000).

Monoterpenes increased the total unsaturated FA of maize root (Zunino and Zygadlo, 2004). In contrast, saturated FAs of the SEFA fraction were increased at 96 hr, mainly in the menthol, geraniol, and camphor treatments (Table 2). This increase might be related to the specificity and increased activity of the acyltransferase enzyme, which esterifies sterols (Dyas and Goad, 1993).

Most of the higher plant sterols are found as FS, which serve as membrane components and reside predominantly in the plasma membranes (Hartmann and Benveniste, 1987; Schaller, 2003), whereas the greatest amount of SE in *Z. mays* roots are located in mitochondrial and microsomal fractions and in the nucleus (Dyas and Goad, 1993). The SE could operate as a supply of FS, as a transport form of sterols in terms of both intracellular movement and movement between tissues, and as a stored form in which sterols present in amounts greater than immediately needed by the plant are sequestered. There is a freely interconvertible pool of FS and SE (Dyas and Goad, 1993; Gondet et al., 1994).

In both the FS and the SE fractions, there was a high negative correlation between sitosterol and stigmasterol in time (see values in Tables 3 and 4). This is indicative of the biosynthetic relationship of these sterols (Kemp et al., 1967; Benveniste, 1986; Izzo and Navari-Izzo, 1993). The major changes observed in the sterol fraction corresponded to the FS fraction, which produced a decrease in the sitosterol to stigmasterol ratio (Figure 2) and an increase in the campesterol to sitosterol ratio (Figure 3). These changes were shown principally with geraniol, menthol, and camphor monoterpenes.

From the "membrane environment" point of view, an appropriate composition of sterols in the cell membranes is crucial for optimal enzymatic activity, ion and metabolite transport or channeling, protein–protein and protein–lipid interactions, signal transduction, and finally to face fluctuating environmental conditions (Schaller, 2003). It is known that membrane fluidity and permeability is regulated by the relative proportion of lipids, as well as by the length and the unsaturation of fatty acids, and that sterols can also participate in the control of membrane-associated metabolic processes (Ros et al., 1990; Schuler et al., 1991; Hartmann, 1998; Hellgren and Sandelius, 2001). Our results indicate that the treatment of maize roots with monoterpenes induces a modification of the sterol proportions, mainly in the FS fraction, and a change in the unsaturation of fatty acids. So it can be suggested that alcoholic monoterpenes (geraniol and menthol) together with camphor would be the most important monoterpenes affecting membrane permeability and fluidity according to the lipid changes shown, i.e., increased percentages of unsaturated PLFA at 96 hr (Table 1) together with an increase on stigmasterol of FS fraction (Table 3).

It has been shown that during senescence the stigmasterol to other sterols ratio increases (McKersie et al., 1978; Lees and Thompson, 1980), and several environmental stresses alter the FS composition as well (Hellgren et al., 2001). Equally, the sitosterol to stigmasterol and/or the stigmasterol to campesterol ratios are modified (Navari-Izzo et al., 1989; Mansour et al., 1994; Hellgren et al., 2001). The central role of campesterol as a precursor of the plant-growth regulators, called brassinosteroids, has been established (Yokota, 1997; Schaller, 2003). The ratio of campesterol to sitosterol is associated with growth and development modifications and with the activity of the enzyme sterol

methyltransferase 2 (SMT2) (Schaeffer et al., 2001). The campesterol to sitosterol ratio in our FS fraction of menthol, geraniol, and camphor treatments increased two times, mainly at 24 and 96 hr (note that this ratio in menthol treatment increased 4.5 times compared to controls) (Figure 3). Thus, the activity of the enzyme SMT2 might be reduced with the above-mentioned treatments. Additionally, the synthesis of 24-ethyl-sterols (sitosterol and stigmasterol) is kinetically favored over 24-methyl-sterols (campesterol) at some stages of corn development (Guo et al., 1995), and, thus, the first sterols are the principal ones. At 96 hr, the campesterol percentage was higher than that of sitosterol with every monoterpene studied, except thymol (Table 3). It may be that the reduced activity of SMT2 is favorable to the seedlings to support some physiological concentration of campesterol.

In summary, from the results obtained, it appears that every monoterpene affects the maize root lipid composition in a different way.

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