Citronellol Disrupts Membrane Integrity by Inducing Free Radical Generation

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Citronellol, an oxygenated monoterpene, is found naturally in the essential oils of several aromatic plants and has been reported to exhibit growth inhibitory and pesticidal activities. However, its mechanism of action is largely unexplored. We investigated the effect of citronellol, which is lipophilic in nature on membrane integrity in terms of lipid peroxidation, conjugated dienes content, membrane permeability, cell death, and activity of the enzyme lipoxygenase in roots of hydroponically grown wheat. Citronellol (50–250 μM) caused a significant inhibition of root and shoot growth. Furthermore, exposure to citronellol enhanced the solute leakage, increased the malondialdehyde content and lipoxygenase activity, and decreased the conjugated diene content. This indicates that citronellol induces generation of reactive oxygen species (ROS) resulting in lipid peroxidation and membrane damage. This was confirmed by *in situ* histochemical studies indicating cell death and disruption of membrane integrity. We conclude from this study that citronellol inhibits the root growth by ROS-mediated membrane disruption.

Key words: Lipid Peroxidation, Electrolyte Leakage, Cell Death

Introduction

Plants produce a myriad of secondary compounds that perform a wide range of important ecological and biological functions. Among these, monoterpenes are the simplest constituents of the essential oils released by a number of aromatic plant species (Singh *et al*., 2009). These play an important role in plant defense mechanisms and serve as attractants for pollinators and seed dispersers (Dudareva *et al*., 2006), provide thermotolerance (Sharkey *et al*., 2001), and act as signalling molecules (Yuan *et al*., 2009). Additionally, they provide donor plants with allelopathic properties and suppress seed germination and growth of other plants (Batish *et al*., 2008; Singh *et al*., 2006a). Essential oils from eucalypt (*Eucalyptus* spp.), clove (*Eugenia caryophyllus*), citronella and lemon grass (*Cymbopogon* spp.), peppermint (*Mentha piperata*), and red stem wormwood (*Artemisia scoparia*) have been found to possess herbicidal activity (Singh *et al*., 2005; Batish *et al*., 2007, 2008; Dayan *et al*., 2009; Kaur *et al*., 2010). This property of essential oils bears a great significance in view of the environmental and health implications and herbicidal resistance of synthetic compounds.

Essential oils possess novel mode(s) of action, different from those of synthetic herbicides, and are environmentally benign in nature, thus do not persist in the environment (Dayan *et al*., 2009). Individual monoterpene constituents of essential oils, such as citronellal, eugenol, cineole, menthol, linalool, β-myrcene, and limonene, have been found to suppress seed germination and growth of weeds (Singh *et al*., 2002a, b, 2009; Dayan *et al*., 2009).

Citronellol, an oxygenated monoterpene, is found in the essential oils of several aromatic plants, including species of *Eucalyptus*, *Pelargonium*, *Geranium*, *Cymbopogon*, and *Rosa*. It is a colourless or slightly pale oil with a rose smell and is widely used in perfumeries and fragrance industry. Furthermore, it is also used as a mosquito repellent (Ritter, 2006). The commercial miticide BIOMITE™ that repels mites harmful to agricultural crops and ornamental plants has citronellol as an active ingredient (USEPA, un-

dated). Citronellol has been considered a GRAS (generally regarded as safe) compound that easily metabolizes into harmless substances and has no effect on non-target species (USEPA, undated). Earlier studies have reported that citronellol inhibits seed germination and radicle emergence of some weedy species such as *Ageratum conyzoides* and *Cassia occidentalis* (Singh *et al*., 2002a, b).

For applicability of citronellol as a bioherbicide, it is desirable to understand its mode of action. A few recent studies have shown that monoterpenes, being lipophilic in nature, alter membrane permeability and fluxes, and cause oxidative burst in the treated tissues (Maffei *et al*., 2001; Singh *et al*., 2009). However, no study has been conducted to evaluate such an effect of citronellol. We therefore investigated the effect of citronellol on membrane integrity in terms of lipid peroxidation, conjugated dienes content, membrane permeability, cell death, and activity of the enzyme lipoxygenase in roots of hydroponically grown wheat.

Material and Methods

Materials

Citronellol (94%; M_r 156.27) was procured from Lancaster (Morecambe, UK). All other chemicals were of analytical grade and procured from Sisco Research Laboratory Pvt. Ltd. (Mumbai, India), Sigma-Aldrich Co. (St. Louis, MO, USA), Merck Ltd. (Mumbai, India), or Loba-Chemie Pvt. Ltd. (Mumbai, India). Healthy and certified seeds of wheat (*Triticum aestivum* var. PBW 502) were purchased locally from the seed store.

Experimental set-up

Wheat seeds were disinfected with 0.1% (w/v) sodium hypochlorite for 15 min followed by washing under running water and then with distilled water. Pre-imbibed (in water for 6 h) wheat seeds were allowed to germinate in plastic trays (23 cm × 14 cm × 7 cm) lined with two layers of Whatman # 1 filter paper moistened with distilled water. These trays were placed in a growth chamber maintained at day/night temperature of (20/12 ± 2) °C, a relative humidity of (74 ± 2)%, and a 14-h photoperiod of ~240 μmol photons $m^{-2}\,s^{-1}$ photon flux density. After 48 h, when the entire seedlings were ~3 cm long, they were transferred to a nylon mesh kept over 500-ml glass beakers containing distilled water for acclimatization. After another 24 h, distilled water was replaced by different concentrations (50, 100, or 250 μM) of citronellol (dissolved in Tween-80) for 24, 48, and 72 h. In the control, instead of citronellol, distilled water (containing the equivalent amount of Tween-80) was used. For each treatment including control, 12 such beakers were maintained. 24, 48, and 72 h after treatment, the seedlings were removed and their root and shoot lengths measured. Furthermore, the root tissue was cut with a fresh blade and used for further biochemical assays.

Lipid peroxidation

Lipid peroxidation was determined in terms of malondialdehyde (MDA) content as described by Singh *et al*. (2007). Briefly, roots were homogenized in trichloroacetic acid (TCA; 0.1%, v/v) and the mixture centrifuged at 15,000 × *g* for 30 min. To 1 ml supernatant, 4 ml of thiobarbituric acid (TBA; 0.5%, w/v, in 20% TCA) were added and the mixture incubated at 95 °C for 30 min. Thereafter, the reaction was stopped by quick cooling in an ice bath and the mixture centrifuged at 10,000 × *g* for 10 min. The absorbance of the supernatant was read at 532 nm and corrected for non-specific absorbance at 600 nm. The MDA content was determined using an extinction coefficient of 155 $mM^{-1}\,cm^{-1}$ and expressed as nmol g^{-1} fresh weight (FW).

Conjugated dienes content

Fresh roots (50 mg) were homogenized in 96% ethanol (5 ml), and the absorbance of the mixture was read at 234 nm (Singh *et al*., 2007). The conjugated dienes content was determined using an extinction coefficient of 26.5 $mM^{-1}\,cm^{-1}$ and expressed as μmol g^{-1} FW.

Determination of membrane integrity

Membrane integrity, an indicator of membrane damage, was estimated in terms of electrolyte leakage from roots by measuring the conductivity of the bathing medium (Singh *et al*., 2007). For this, 100 mg roots were immersed in distilled water for 30 min, and the initial conductivity (C_1) was measured with a conductivity meter (ECOSCAN CON5; Eutech Instruments Pvt. Ltd., Singapore). Then, the samples were boiled for 15 min and the

conductivity (C_2) was measured again. *REL* (relative electrolyte leakage) was determined as follows: % $REL = (C_1 / C_2) \cdot 100$.

Lipoxygenase (LOX) activity

The activity of LOX was determined as the rate of oxidation of linoleic acid (Axelford *et al.*, 1981). For this, roots (200 mg) were homogenized in 10 ml of chilled phosphate buffer (0.1 M, pH 7), and the homogenate was centrifuged at 15,000 × *g* at 4 °C for 30 min. The supernatant (enzyme extract) was collected and stored at 4 °C. The reaction mixture contained 0.575 mM linoleic acid in 0.1 M phosphate buffer (pH 7.4), 0.2% (v/v) Tween-80, and enzyme extract. The LOX activity was determined at 234 nm using an extinction coefficient of 25 mM^{-1} cm^{-1} and expressed as enzyme unit (EU) mg^{-1} protein.

In situ histochemical analyses

In situ detection of lipid peroxidation was done by staining freshly harvested root tips in Schiff's reagent for 60 min until a pink colour appeared (Pompella *et al.*, 1981). Extra stain was removed by rinsing in potassium metabisulfite solution (0.5%, w/v, in 0.05 M HCl). The root tips were observed under a trinocular stereo zoom microscope (Model RSM-9; Radical Instruments, Ambala Cantt, India) and photographed with a digital imaging system Nikon (Tokyo, Japan) Coolpix 4500 fitted to the microscope.

The loss of membrane integrity was histochemically analysed by incubating roots in Evans blue solution (0.025%, w/v, in 100 μM $CaCl_2$, pH 5.6) for 30 min (Yamamoto *et al.*, 2001). Roots were then washed four times with distilled water, analysed under the microscope, and photographed (as explained above).

Statistical analyses

For each treatment including control, there were four independent beakers (as replicates) arranged in a randomized block design. For each enzymatic analysis, there were five independent tissue samples to serve as replicates. The data are presented as mean ± SE (standard error) and analysed by one-way ANOVA followed by the comparison of mean values using post-hoc Tukey's test at $P \leq 0.05$.

Results and Discussion

Citronellol inhibited the root growth of wheat in a concentration- and time-dependent manner (Table I). After 24, 48, and 72 h of exposure, the root length decreased in the range of 16–46, 3–52, and 10–63%, respectively (Table I). Likewise, shoot elongation also declined in response to citronellol application; however, the reduction in shoot length was smaller than in root length. Citronellol caused inhibition of the shoot length in the range of ~8–25, 12–16, and 4–22% after 24, 48, and 72 h of treatment (Table II). These observations agree with earlier studies reporting that monoterpenes are potent inhibitors of seed germination and seedling growth (Singh *et al.*, 2006a, b; Kordali *et al.*, 2009). However, the exact mechanism involved in inducing root growth inhibition is largely unknown. Nevertheless, the previous studies indicated that monoterpene-mediated inhibition of the mitotic activity in root tips is one of the reasons for growth suppressing effects of monoterpenes (Dayan *et al.*, 2000; Singh *et al.*, 2006b).

The exposure to citronellol enhanced the levels of MDA, a major TBA-reactive species and an indicator of lipid peroxidation, in wheat roots

Table I. Effect of citronellol on root length (± SE) of hydroponically grown *T. aestivum* measured 24, 48, and 72 h after treatment.

Concentration [μM]	Root length [cm]		
	24 h	48 h	72 h
0	6.71 ± 0.05 a,A	7.90 ± 0.06 a,B	10.71 ± 0.03 a,C
50	5.62 ± 0.06 b,A	7.71 ± 0.05 a,B	9.62 ± 0.05 b,C
100	4.64 ± 0.07 c,A	5.33 ± 0.05 b,B	6.05 ± 0.07 c,C
250	3.63 ± 0.05 d,A	3.86 ± 0.08 c,B	3.91 ± 0.08 d,B

Different letters (lower case in a column and upper case in a row) represent significant difference at $P < 0.05$ applying Tukey's test.

Table II. Effect of citronellol on shoot length (± SE) of hydroponically grown *T. aestivum* measured 24, 48, and 72 h after treatment.

Concentration [μM]	Shoot length [cm]		
	24 h	48 h	72 h
0	5.73 ± 0.05 a,A	8.37 ± 0.05 a,B	10.00 ± 0.05 a,C
50	5.36 ± 0.06 b,A	7.36 ± 0.05 b,B	9.62 ± 0.04 b,C
100	4.91 ± 0.07 c,A	6.73 ± 0.05 c,B	8.92 ± 0.05 c,C
250	4.30 ± 0.06 d,A	7.02 ± 0.03 b,B	7.81 ± 0.12 d,C

Different letters (lower case in a column and upper case in a row) represent significant difference at $P < 0.05$ applying Tukey's test.

Table III. Effect of citronellol on lipid peroxidation in terms of MDA content (± SE) in roots of hydroponically grown *T. aestivum* measured 24, 48, and 72 h after treatment.

Concentration [μM]	MDA content [nmol g^{-1} FW]		
	24 h	48 h	72 h
0	0.15 ± 0.01 a,A	0.15 ± 0.02 a,A	0.16 ± 0.01 a,A
50	0.17 ± 0.01 a,A	0.21 ± 0.01 b,B	0.24 ± 0.02 b,C
100	0.24 ± 0.02 b,A	0.29 ± 0.02 c,B	0.31 ± 0.01 c,C
250	0.28 ± 0.02 c,A	0.34 ± 0.02 d,B	0.37 ± 0.02 d,C

Different letters (lower case in a column and upper case in a row) represent significant difference at $P < 0.05$ applying Tukey's test.

Table IV. Effect of citronellol on conjugated dienes content (± SE) in roots of hydroponically grown *T. aestivum* measured 24, 48, and 72 h after treatment.

Concentration [μM]	Conjugated dienes content [μmol g^{-1} FW]		
	24 h	48 h	72 h
0	3.01 ± 0.02 a,A	3.01 ± 0.02 a,A	3.04 ± 0.01 a,A
50	2.93 ± 0.01 ab,A	2.90 ± 0.02 ab,A	2.86 ± 0.02 b,B
100	2.94 ± 0.01 b,A	2.69 ± 0.03 b,B	2.52 ± 0.01 c,C
250	2.54 ± 0.02 c,A	2.46 ± 0.04 c,B	2.35 ± 0.02 d,C

Different letters (lower case in a column and upper case in a row) represent significant difference at $P < 0.05$ applying Tukey's test.

(Table III). After 24 h, the MDA content had increased by 13% in response to 50 μM citronellol. It further rose to 60 and 87% at 100 and 250 μM citronellol, respectively. After 48 and 72 h of 250 μM citronellol treatment, the MDA content was 127 and 131% of the control, respectively (Table III). Unlike MDA, the conjugated dienes (hereafter CDs) content decreased in response to citronellol treatment in a time- and concentration-dependent manner. After 24 h of exposure to 250 μM citronellol, the CDs content was reduced by 16% over the control, and it further declined by 18 and 23% over the control at 48 and 72 h of exposure (Table IV).

In contrast, a significant increase was observed in the relative electrolyte leakage upon exposure to citronellol. After 24 h of exposure, the relative electrolyte leakage was 17, 40, and 46% greater over the control in response to 50, 100, and 250 μM citronellol, respectively. It further increased in the range of 26–54 and 28–76% (compared to the control) after 48 and 72 h of exposure (Table V). Parallel to increased relative electrolyte leakage, there was a significant increase in the LOX activity compared to the control. Upon exposure to 250 μM citronellol, the activity of LOX was approximately 3-, 4-, and 5-times that of the control after 24, 48, and 72 h of exposure, respectively (Table VI).

Lipid peroxidation in roots was also evident from the *in situ* histochemical studies. Upon staining with Schiff's reagent (a marker of lipid per-

Table V. Effect of citronellol on relative electrolyte leakage (± SE) in roots of hydroponically grown *T. aestivum* measured 24, 48, and 72 h after treatment.

Concentration [μM]	Relative electrolyte leakage (%)		
	24 h	48 h	72 h
0	20.07 ± 0.20 a,A	21.05 ± 0.24 a,A	22.04 ± 0.34 a,A
50	23.54 ± 0.31 ab,A	26.45 ± 0.26 ab,A	28.17 ± 0.38 b,B
100	28.15 ± 0.11 b,A	30.23 ± 0.31 b,B	34.78 ± 0.21 c,C
250	29.28 ± 0.30 c,A	32.34 ± 0.22 c,B	38.85 ± 0.42 d,C

Different letters (lower case in a column and upper case in a row) represent significant difference at $P < 0.05$ applying Tukey's test.

Table VI. Effect of citronellol on LOX activity (± SE) in roots of hydroponically grown *T. aestivum* measured 24, 48, and 72 h after treatment.

Concentration [μM]	LOX activity [EU mg^{-1} protein]		
	24 h	48 h	72 h
0	1.20 ± 0.02 a,A	1.41 ± 0.07 a,B	1.53 ± 0.08 a,C
50	1.31 ± 0.03 a,A	2.74 ± 0.06 b,B	3.17 ± 0.23 b,C
100	2.84 ± 0.04 b,A	4.63 ± 0.10 c,B	5.88 ± 0.31 c,C
250	3.92 ± 0.05 c,A	5.21 ± 0.20 d,B	6.84 ± 0.52 d,C

Different letters (lower case in a column and upper case in a row) represent significant difference at $P < 0.05$ applying Tukey's test.

oxidation), citronellol-treated wheat roots turned pink, and the pink colour intensified with the increase in citronellol concentration and time of exposure (Fig. 1). These histochemical observations were concomitant with the biochemical observations for lipid peroxidation in the roots in terms of the MDA content. Citronellol-induced cell death in roots was also evident from the *in situ* detection using Evans blue staining. In contrast to control roots, roots from citronellol-exposed wheat seedlings turned dark blue, thereby indicating a loss of plasma membrane integrity. The intensity of the blue colour increased with the increase in the concentration of and exposure time to citronellol (Fig. 2).

Enhanced MDA content and increased electrolyte leakage upon exposure to citronellol indicated the generation of reactive oxygen species

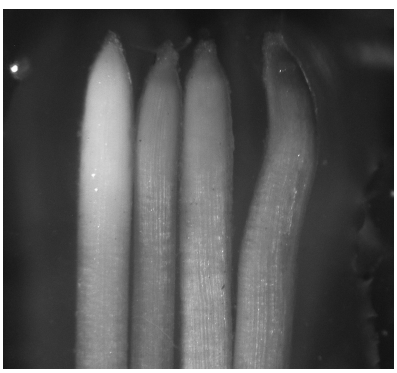

Fig. 1. *In situ* histochemical localization of lipid peroxidation in *T. aestivum* roots exposed to different concentrations of citronellol detected after 72 h. Roots from left to right indicate 0 (control), 50, 100, and 250 μM citronellol.

Fig. 2. *In situ* histochemical localization of loss of membrane integrity (cell death) in *T. aestivum* roots exposed to different concentrations of citronellol detected after 72 h. Roots from left to right indicate 0 (control), 50, 100, and 250 μM citronellol.

(ROS) in response to citronellol application and an alteration in the membrane structure. These observations are parallel to earlier observations that monoterpenes affect membrane structure and function (Zunino and Zygadlo, 2004; Singh *et al*., 2006a). Monoterpenes being lipophilic in nature distort the physical arrangement of phospholipids in the membranes and thus affect membrane functioning (Zunino and Zygadlo, 2004). In the present study, citronellol exposure induced a significant increase in lipid peroxidation and relative electrolyte leakage thus altering the structure and permeability of membranes leading to increased solute leakage. Oxygen-free radicals generated under abiotic stresses induce degradation of phospholipids and subsequently biological membranes leading to cellular injury (Halliwell and Gutteridge, 1999). Histochemical localization of lipid peroxidation and loss of membrane integrity further support impairment of the membrane structure upon citronellol exposure. In fact, the disruption of membranes by monoterpenes has been suggested as one of the mechanisms for their biological activity (Harrewijn *et al*., 2001). A loss in membrane integrity is an indicator of membrane damage due to generation of ROS and thus oxidative stress (Dayan *et al*., 2000; Singh *et al*., 2006a). Of late, generation of free radicals leading to oxidative stress has been suggested as one of the mechanisms of action of plant growth inhibition by volatile allelochemicals (Maffei *et al*., 2001; Zunino and Zygadlo, 2004; Singh *et al*., 2009). The reduced CDs content observed in the present study further indicates free radicals-induced membrane damage.

The damaging effect of citronellol on the membrane integrity and permeability may also affect the activity of LOX – a lipolytic enzyme catalyzing the oxygenation of polyunsaturated fatty acids of membranes – which plays a key role in abiotic stresses (Brash, 1999). In the present study, citronellol stress also resulted in up-regulation of the LOX activity. This clearly indicates that citronellol may stimulate the LOX activity producing hydroperoxide derivatives which destroy cellular membranes.

In conclusion, the present study indicates that citronellol induces the generation of ROS, which cause oxidative stress and damage to biological membranes.

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