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Herbicidal Activity of Monoterpenes Is Associated with Disruption of Microtubule Functionality and Membrane Integrity

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Aromatic plants and their volatile compounds affect seed germination and plant growth, and therefore hold potential for agriculture uses as plant growth regulators and bioherbicides. In the present study 17 major monoterpenes were selected, and their mechanisms of plant toxicity were elucidated using transgenic *Arabidopsis thaliana* at various growth stages. Microtubulin and the plant cell membrane were identified as the focal targets through which phytotoxicity and herbicidal activity acted. Variability in monoterpene mechanisms was observed. Limonene and (+)-citronellal had strong antimicrotubule efficacy, whereas citral, geraniol, (–)-menthone, (+)-carvone, and (–)-citronellal demonstrated moderate antimicrotubule efficacy. Pulegone, (–)-carvone, carvacrol, nerol, geranic acid, (+)/(–)-citronellol, and citronellic acid lacked antimicrotubule capacity. An enantioselective disruption of microtubule assembly was recorded for (+)/(–)-citronellal and (+)/(–)-carvone. The (+) enatiomers were more potent than their (–) counterparts. Citral, limonene, carvacrol, and pulegone were also tested for phytotoxicity and herbicidal activity. Pulegone had no detectable effect on microtubules or membranes. Citral disrupted microtubules but did not cause membrane damage. Carvacrol lacked a detectable effect on microtubules but incited membrane leakage, and limonene disrupted microtubules and membrane leakage. Therefore, only limonene was herbicidal at the tested concentrations. *In planta* quantification of residues revealed that citral was biotransformed into nerol and geraniol, and limonene was converted into carvacrol, which could explain its dual capacity with respect to microtubules and membrane functionality. The results obtained are an important added value to commercial efforts in selecting appropriate aromatic plants to be sources of bioherbicidal compounds for sustainable weed management with a limited potential for herbicide resistance evolution in weed populations.

Nomenclature: *Arabidopsis thaliana*, citral, limonene, pulegone, carvacrol.

Key words: Allelochemicals, aromatic plants, bioherbicides, microtubule, membrane leakage, mode of action, monoterpene.

Weeds are among the major causes of crop yield loss together with pests and diseases (Dayan and Duke 2014; Pimentel et al. 2005). In commercial production, weeds compete with the crop, reducing its growth rate and productivity. Hence, weed control is an essential practice to limit the negative effects on crop production (Berchielli-Robertson et al. 1990). Various approaches to alternative weed control are available, such as sanitation of plant material and seeds, use of mulch, solarization, hand weeding, heat, use of acids or soaps, etc. (Chappell et al. 2012). However, regardless of the alternatives, commercial production still relies heavily on herbicides with synthetic chemistries. These encompass 91.9% of the new active ingredients registered, whereas 8.1% are synthetic active ingredients derived from natural sources (Cantrell et al. 2012).

The intensive use of synthetic herbicides poses two major challenges in relation to public health, environmental damage (Narwal 1999), and the development of weeds resistant to the currently used chemistries (Dayan et al. 2012). Grana et al. (2012) pointed out environmental concerns, such as interruption of ecological equilibrium, negative influence on human health, and increased incidence of weeds developing resistance. Research has indicated increased risk of cancer and Parkinson's disease following exposure to herbicides (Gorrel et al. 1998; Kettles et al. 1997; Kogevinas et al. 1997); and Dayan et al. (2012) indicated that in the last 20 yr herbicides with mechanisms of action for new target

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sites have not been commercialized, which consistently increases the risk for resistance developing in weed populations. Therefore, there is a considerable need for new chemistries with innovative mechanisms of action.

The public concern with the possible undesirable effects of synthetic herbicides on human health and the environment calls for the use of ecofriendly chemistries. Currently, only 7% of the commercialized chemistries approved by the U.S. Environmental Protection Agency (EPA) are natural bioherbicides (Cantrell et al. 2012; Dayan and Duke 2014), indicating the limited use of natural compounds as herbicides (Seiber et al. 2014). Aromatic plants have been a commercial source of bioactive compounds for many years (Christaki et al. 2012; Gerwick and Sparks 2014; Kala 2015). Compounds produced by aromatic plants, in particular essential oils and their monoterpene constituents, possess insecticidal (Isman 2000), antimicrobial (Abad 2014; Bossele and Juliani 2002), and herbicidal properties (Dudai 1999; Grana 2012, 2013a). In this sense, essential oils and their monoterpene constituents are innovative and ecofriendly chemistries that can potentially be used as bioherbicides.

Essential oils affect plant cells, causing membrane breakage and leakage of macromolecules and induction of oxidative stress by lipophilic compounds (Abrahim et al. 2003; Cox et al. 1988; Di Pasqua et al. 2007; Einhellig 1986; Lambert et al. 2001; Maffei et al. 2001; Sikkema et al. 1995; Singh et al. 2009a; Zunino and Zygadlo 2004). Monoterpenes are one of the largest and most important groups of secondary metabolites found in essential oils, and some of them are phytotoxic, making them potential bioherbicides (Abrahim et al. 2000; Dudai et al. 2000a). Monoterpenes are inhibitors of seed germination and plant growth (Abrahim et al. 2000; Dudai et al. 2000a; Einhellig and Leather 1988; Fischer 1986; Gouda et al. 2016; Grana et al. 2012; Reynolds 1987; Weidenhamer et al. 1994). Anatomical and physiological changes have been recorded in plant seedlings as a result of exposure to monoterpenes in both the vapor and aqueous phases (Dudai et al. 2000b; Einhellig and Leather 1988; Fischer 1986; Grana et al. 2013b; Koitabashi et al. 1997).

In 2010, Chaimovitsh et al. demonstrated inhibition of plant growth by citral. The mechanism underlying this inhibition involved microtubule disruption without a detectable effect on the actin cytoskeleton. Furthermore, in vivo and in vitro tests demonstrated a harmful effect of citral on cell microtubules that was dose and time dependent yet reversible. Nevertheless, under the experimental conditions used, damage to the plasma membrane was not recorded.

The study was continued in 2012 (Chaimovitsh et al. 2012) identifying, in wheat seedlings, that the mitotic microtubules were more sensitive to citral than the cortical microtubules. Citral inhibited the cell cycle and increased the frequency of wheat root cells and BY2 cells with asymmetric walls. The findings explained the observed phytotoxic effect of citral, at micromolar concentrations, in seed germination inhibition.

In a continuing effort to understand the phytotoxic and herbicidal potential of citral derivatives, the present study examined the hypothesis that the mechanism of citral derivatives and other related monoterpenes is via interference with the cortical microtubules, F-actin functioning, and membrane integrity.

Materials and Methods

Plant Material Used. *Arabidopsis thaliana* ecotype Col-0 was used. Seeds of *Arabidopsis* plants expressing the green fluorescent protein–tubulin α6 (GFP-TUA6) marker were kindly provided by T. Hashimoto (Ueda et al. 1999), and transgenic plants expressing the GFP-mTalin, were generated in the lab with a plasmid kindly provided by N. Chua (Kost et al. 1998).

Volatile Compounds Used. The following 17 compounds were used in this study to examine their effect on plant tissue: citral, (+)/(−)-citronellal, geraniol, nerol, (+)/(−)-citronellol, citral dimethyl acetal, geranic acid, (+)/(−)-citronellic acid, limonene, menthone, (+)/(−)-carvone, pulegone, and carvacrol. All compounds were examined for their effect on microtubulin in *Arabidopsis* seedlings (Table 1). Four compounds—limonene, citral, pulegone, and carvacrol—were also tested for their effect on F-actin and membrane integrity in seedlings and for their effect on membrane leakage and phytotoxicity in mature *Arabidopsis* plants (Table 1). All compounds were purchased from Sigma-Aldrich, Israel.

Analysis of Microtubules, F-actin, and Membrane in Seedlings by Confocal Microscopy. In this test, all 17 compounds were examined for their effect on microtubulin, and citral, limonene, pulegone, and carvacrol were also tested for their effect on F-actin and cell membrane (Table 1).

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Table 1. Effect of 17 monoterpenes and citral derivatives on the activity of microtubulin, membrane, and F-actin in *Arabidopsis* seedlings and on phytotoxicity and plant biomass in mature *Arabidopsis* plants.

Compound	Monoterpene	Citral derivative	Dosage (µl per 20 ml)[a]	Disruptive effect in *Arabidopsis* seedlings[b]			Phytotoxicity and effect on mature *Arabidopsis* plants[b,c]		
				Microtubule	Membrane	F-actin	Membrane leakage	Herbicidal activity	Biomass reduction
Limonene	*		0.75	+	+	–	18.2% (after 30 min.) 29.2% (after 60 min.)	+/–(after 30 min) +(after 60 min)	16% after 60 min
			1.5	+			43.3% (after 30 min.) 49.8% (after 60 min.)	+(after 15 min)	
(+)-Citronellal		*	0.75	+	nt	nt	nt	nt	nt
			1.5	+			nt		
(–)-Citronellal		*	0.75	+/–	nt	nt	nt	nt	nt
			1.5	+			nt		
Citral			0.75	+/–	–	–	–	–	–
			1.5	+			–		
(–)-Menthone	*		0.75	+/–	nt	nt	nt	nt	nt
			1.5	+			nt		
Geraniol		*	0.75	+/–	nt	nt	nt	nt	nt
			1.5	+			nt		
(+)-Carvone	*		0.75	–	nt	nt	nt	nt	nt
			1.5	+			nt		
(–)-Carvone	*		0.75	–	nt	nt	nt	nt	nt
			1.5	–			nt		
Nerol		*	0.75	–	nt	nt	nt	nt	nt
			1.5	–			nt		
(–)-Citronellol		*	0.75	–	nt	nt	nt	nt	nt
			1.5	–			nt		
(+)-Citronellol		*	0.75	–	nt	nt	nt	nt	nt
			1.5	–			nt		

Compound	Monoterpene	Citral derivative	Dosage (µL/20 mL)[a]	Disruptive effect in *Arabidopsis* seedlings[b]			Effect on mature *Arabidopsis* plants[b,c]		
				Microtubulin	Membrane	F-actin	Membrane leakage	Herbicidal activity	Biomass reduction
Citral di methyl acetal		*	0.75	–	nt	nt	nt	nt	nt
			1.5	–					
Geranic acid		*	0.75	–	nt	nt	nt	nt	nt
			1.5	–					
(–)-Citronellic acid		*	0.75	–	nt	nt	nt	nt	nt
			1.5	–					
(+)-Citronellic acid		*	0.75	–	nt	nt	nt	nt	nt
			1.5	–					
Pulegone	*		0.75	–	–	–	–	–	–
			1.5	–			–		
Carvacrol	*		0.75	–	–	–	–	–	–
			1.5	–			30% after 60 min.		

[a] Low (0.75 µl per 20 ml) and high (1.5 µl per 20 ml) dosages.
[b] Effect: +, complete; +/–, partial; –, none; nt, not tested.
[c] Duration (min) of exposure to vapors.

Arabidopsis seeds were sown onto Murashige and Skoog medium, incubated at 4 C in darkness for 4 d, and then transferred to a growth chamber at 24 C under a photoperiod of 16 h light/8 h dark. Then, five 8-d-old seedlings, each weighing approximately 0.05 g (a total of 0.25 g of plant tissue), were transferred into sterile 20 ml scintillation vials and exposed to 0.75 µl (low dose) or 1.5 µl (high dose) of each compound (Table 1) for 30 min at room temperature in darkness.

The applied molar dosages (high and low) of the compounds were as follows: citral: 8.79 M and 4.39 M; (+)/(−)-citronellal: 8.31 M and 4.15 M; geraniol: 8.64 M and 4.32 M; nerol: 8.56 M and 4.28 M; (+)/(−)-citronellol: 8.2 M and 4.1 M, citral dimethyl acetal: 6.73 M and 3.36 M, geranic acid: 8.64 M and 4.32 M, (+)/(−)-citronellic acid: 8.13 M and 4.06 M; limonene: 9.26 M and 4.63 M; menthone: 8.7 M and 4.15 M, (+)/(−)-carvone: 9.58 M and 4.79 M; pulegone: 9.2 M and 4.6 M, and carvacrol: 9.75 M and 4.87 M.

The compounds were separately amended onto Whitman filter paper pieces (1 by 1 cm) that were attached to the inner sides of the vials' caps. Each vial (20 ml) was hermetically sealed, creating a vapor-rich headspace atmosphere. Following exposure, membranes were stained for 5 min with 8 µM of styryl dye FM4-64 (Bolte et al. 2004) to examine membrane integrity. Microtubules and F-actin were determined by live GFP markers. An IX81/FV500 laser-scanning microscope (Olympus) with a Plan Apo 60 ×1.00 WLSM ∞/0.17 objective was used to observe fluorescently labeled plant cells. The filter sets used for observation were 488 nm excitation and BA505-525 for GFP and 488 or 515 nm excitation and BA660 for FM4-64.

Effect on Membrane Leakage and Phytotoxicity in Mature Plants. In this experiment, limonene, citral, pulegone, and carvacrol were tested for their disruption of membrane integrity and phytotoxicity to mature plants (Table 1). *Arabidopsis* plantlets, at the two-leaf stage, were transplanted to 200 ml pots with five plantlets per pot. The soil mixture used was composed of 65% peat TS-1 and 35% vermiculite N-2. The plantlets were grown under short-day conditions (10 h of daylight) in the greenhouse until they reached the 14-leaf stage, when they were transferred to the laboratory to be examined for membrane leakage and phytotoxicity.

In the laboratory, each pot was covered with a 500 ml glass beaker. The tested compounds were each applied to a Whitman's filter paper that was attached to the inner side of the beaker. The plants were exposed to 18.75 µl (low) or 37.5 µl (high) of monoterpene vapor for 0, 15, 30, and 60 min. The dosages were equivalent to 0.75 µl and 1.5 µl previously used in the 20 ml scintillation vials and were (low/high): 4.6/9.2 µmol per 20 ml for limonene, 4.4/9.2 µmol per 20 ml for citral, 4.8/9.7 µmol per 20 ml for carvacrol, and 4.6/9.3 µmol per 20 ml for pulegone. Plants to be examined for phytotoxicity and biomass were retransferred to the greenhouse and were evaluated 2 wk later.

Membrane-leakage quantification was carried out using plants that were exposed to the monoterpene vapors for 30 min following the protocol by Bernstein et al. (2010). Immediately after the 30 min exposure to the vapors, the treated plants with the roots removed were placed in sealed 50 ml tubes containing 20 ml double-distilled water. The tubes were shaken at 200 rpm for 60 min at room temperature, and electrical conductivity (EC) was measured before and after autoclaving. Membrane leakage was expressed as the ratio between the two values, as follows: $\% \text{ EC} = [(EC_f - EC_i)/EC_i] \times 100$, where EC_i = EC measured before autoclaving and EC_f = EC measured after autoclaving.

In Planta Analysis of Monoterpene Concentrations. *Arabidopsis* seedlings exposed to the various monoterpenes in vapor phase were weighed and placed in methyl-*tert*-butyl ether that contained 10 µg ml^{-1} iso-butylbenzene as an internal standard. The samples were shaken at room temperature for 24 h as previously described (Dudai et al. 2009; Lewinsohn et al. 1998). The extract was passed through a small column consisting of a Pasteur pipette containing anhydrous Na_2SO_4 and salicylic acid (Silicagel 60, 230–400 mesh, Merck) to dry it and to remove high-molecular-weight polar substances that could interfere with the gas chromatography (GC) analysis. The samples were analyzed using a GC/mass spectrometer (HP-GCD apparatus) equipped with an HP5 (30 m, 0.25 mm) fused silica capillary column. Helium was used as the carrier in constant-flow mode at 1 ml min^{-1}. The injection temperature was 250 C, and the detector temperature was 280 C. Column conditions were: 70 C for 2 min followed by a 4 C min^{-1} increase to 200 C. The components were identified by co-injection with authentic samples and by comparison with mass spectra from the computerized libraries Wiley7N and HP1600. Following extraction, the plants were dried in an oven at 80 C for 48 h and weighed.

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Statistical Analysis. All trials were carried out in a completely randomized design with five replications per treatment. Data were explored with one-way analysis of variance (ANOVA) followed by mean separation with Tukey's HSD. Values are presented as mean ± standard deviation.

Results and Discussion

Analysis of Microtubules, F-actin, and Membrane by Confocal Microscopy. Eight- day-old *Arabidopsis* seedlings expressing the microtubule marker GFP-TUA6 or the actin marker GFP-mTalin were exposed to 10 citral derivatives and 6 monoterpenes and then stained with the membrane marker FM4-64 (Bolte et al. 2004). Table 1 summarizes the degree of microtubule-disrupting activity in *Arabidopsis* seedlings of the 10 citral derivatives: (+)/(–)-citronellal, geraniol, nerol, (+)/(–)-citronellol, geranic acid, (+)/(–)-citronellic acid, citral dimethyl acetal; and the 6 monoterpenes: limonene, (+)/(–)-carvone, (–)-menthone, pulegone, and carvacrol. Dosages and exposure duration were based on our previous work (Chaimovitsh et al. 2010) and were 0.75 µl (low dose) and 1.5 µl (high dose) in 20 ml scintillation vials for 30 min. The compounds limonene, (+)-citronellal, and (+)-carvone completely disrupted plant microtubules at high and low dosages. Citral, (–)-menthone, geraniol, and (–)-citronellal disrupted microtubules completely and partially at the high and low dosages, respectively. On the other hand, (–)-carvone, nerol, (+)/(–)-citronellol, citral dimethyl acetal, geranic acid, (+)/(–)-citronellic acid, pulegone, and carvacrol did not. Of note was the enantioselective activity recorded for carvone and citronellal, where (+)-carvone and (+)-citronellal disrupted microtubules completely at the low and/or high dosages, whereas (–)-carvone had no effect on microtubules, and (–)-citronellal had a dose dependant activity (Table 1).

Citral, limonene, pulegone, and carvacrol were examined further for their effect on microtubules, F-actin, and membrane uniformity. Similar to citral (Chaimovitsh et al. 2010), the three monoterpenes lacked anti – F-actin activity (Table 1; Figure 1). Limonene caused breakage of the plasma membrane, whereas citral, pulegone, and carvacrol, did not (Table 1; Figure 1).

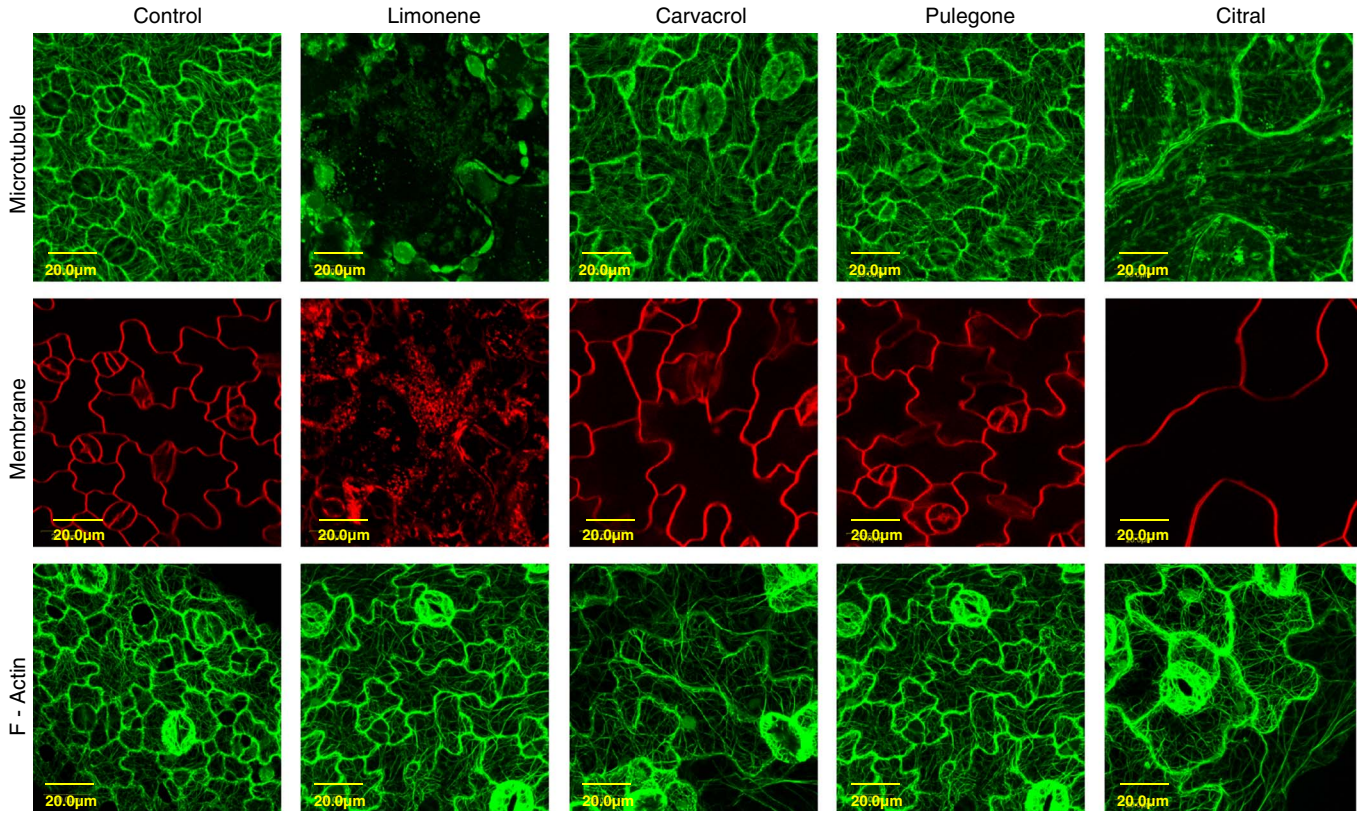

Figure 1. The effect of limonene, pulegone, and carvacrol in relation to citral on microtubules, F-actin, and the plasma membrane in *Arabidopsis* leaf epidermal cells. GFP-TUA6 or GFP-mTalin lines were exposed to the vapors in 20 ml scintillation bottles for 30 min and then stained with FM4-64. Shown are confocal images of the abaxial leaf epidermis.

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Effect on Membrane Leakage and Phytotoxicity to Mature Plants. To compare the influence of the four monoterpenes' vapors on mature plant growth, we examined two dosages of each compound on *Arabidopsis* plants. Following treatment, the plants were transferred to the greenhouse, and growth was monitored for 2 wk. Only limonene had herbicidal activity (Table 1; Figure 2), initiating with phytotoxic reaction expressed as necrotic and dried leaves and eventual plant death. At the low limonene vapor concentration, a time-response effect was observed. The plants were not damaged by 15 min exposure and were only partially damaged after 30 min exposure. However, after 60 min exposure to limonene vapor the plants died (Table 1; Figure 2A). Exposure to the high limonene vapor concentration (9.2 µmol per 20 ml) caused severe wilting after only 15 min (Table 1; Figure 2B), indicating a strong herbicidal potential. Exposure of *Arabidopsis* plants to vapors of citral, carvacrol, and pulegone did not lead to significant wilting (Table 1; Figure 2C), suggesting lack of herbicidal potential at these concentrations.

The effect on biomass (Table 1; Figure 3) correlated with time and dose responses described in Figure 2. The biomass decreased from 0.29 g plant^{-1} for the untreated control to less than 0.05 g plant^{-1} after 60 min of exposure to either the low or the high concentration of limonene (Figure 3A). Exposure to carvacrol, pulegone, or citral did not cause a significant reduction in plant biomass compared with the untreated plants at any of the time settings (Figure 3C, D).

To determine the influence of limonene, citral, carvacrol, and pulegone on the cell membrane leakage of the mature plants, we used the experimental setup described in Figure 3. Exposure of the plants to limonene at 4.6 µmol per 20 ml (0.75 µl per 20 ml) led to 18.2 and 29.2% leakage after 30 and 60 min exposure, respectively. Exposure to limonene at 9.2 µmol per 20 ml (1.5 µl per 20 ml) led to 43.3 and 49.8% leakage after 30 and 60 min, respectively. Membrane leakage in control untreated plants was only 11.5% (Figure 4A). Exposure of the plants to citral, carvacrol, and pulegone vapors did not incite a significant membrane leakage (Figure 4A, B), except for exposure to the high dose of carvacrol (1.5 µl per 20 ml) for 60 min, which caused 30% leakage (Figure 4B). Interestingly, the plants tolerated this apparently harmful effect, demonstrating a normal appearance after the treatment (Figure 2C).

In Planta Analysis of Monoterpene Concentrations. Monoterpene residues were quantified in *Arabidopsis* plants using the experimental setup described in Figure 2. Treated plants were extracted

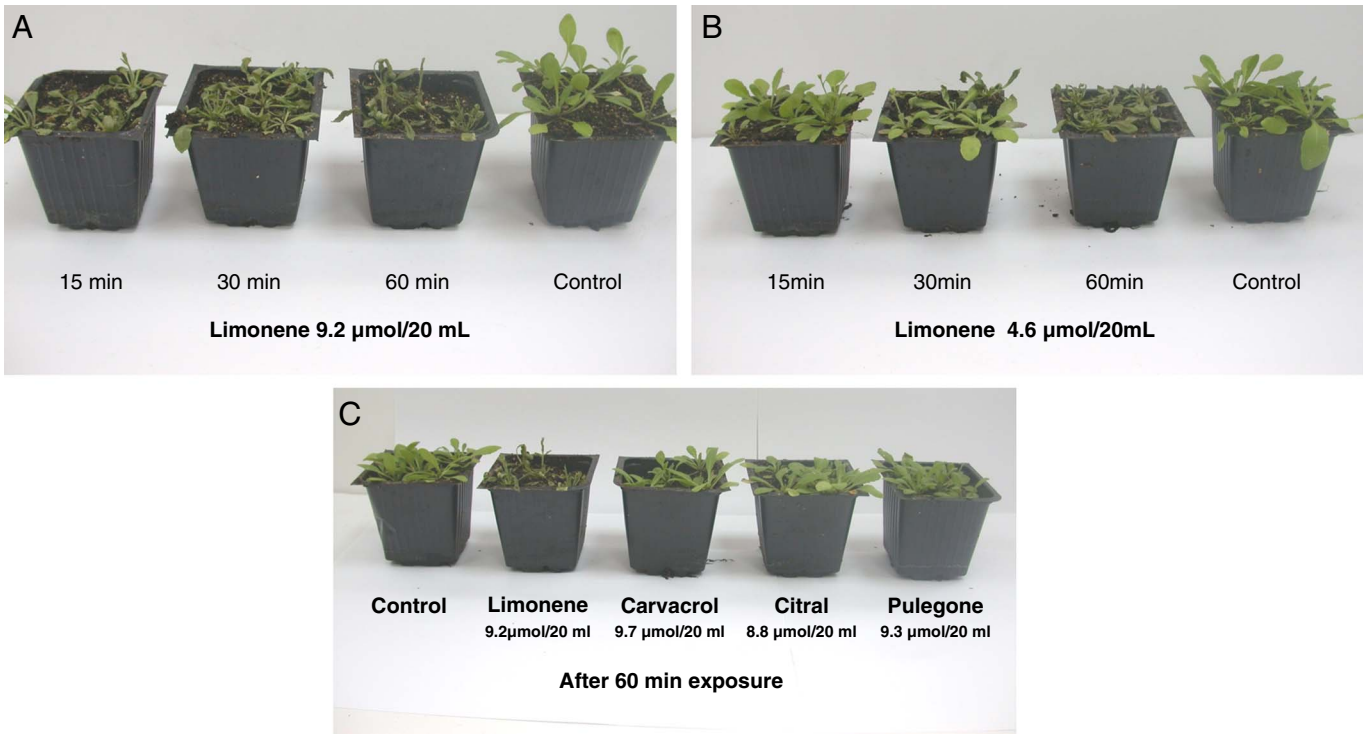

Figure 2. Phytotoxicity of limonene, pulegone, citral, and carvacrol to *Arabidopsis* plants. Note: the plants were exposed to monoterpene vapors for 0, 15, 30, and 60 min, and the herbicidal potential was examined 14 d postexposure.

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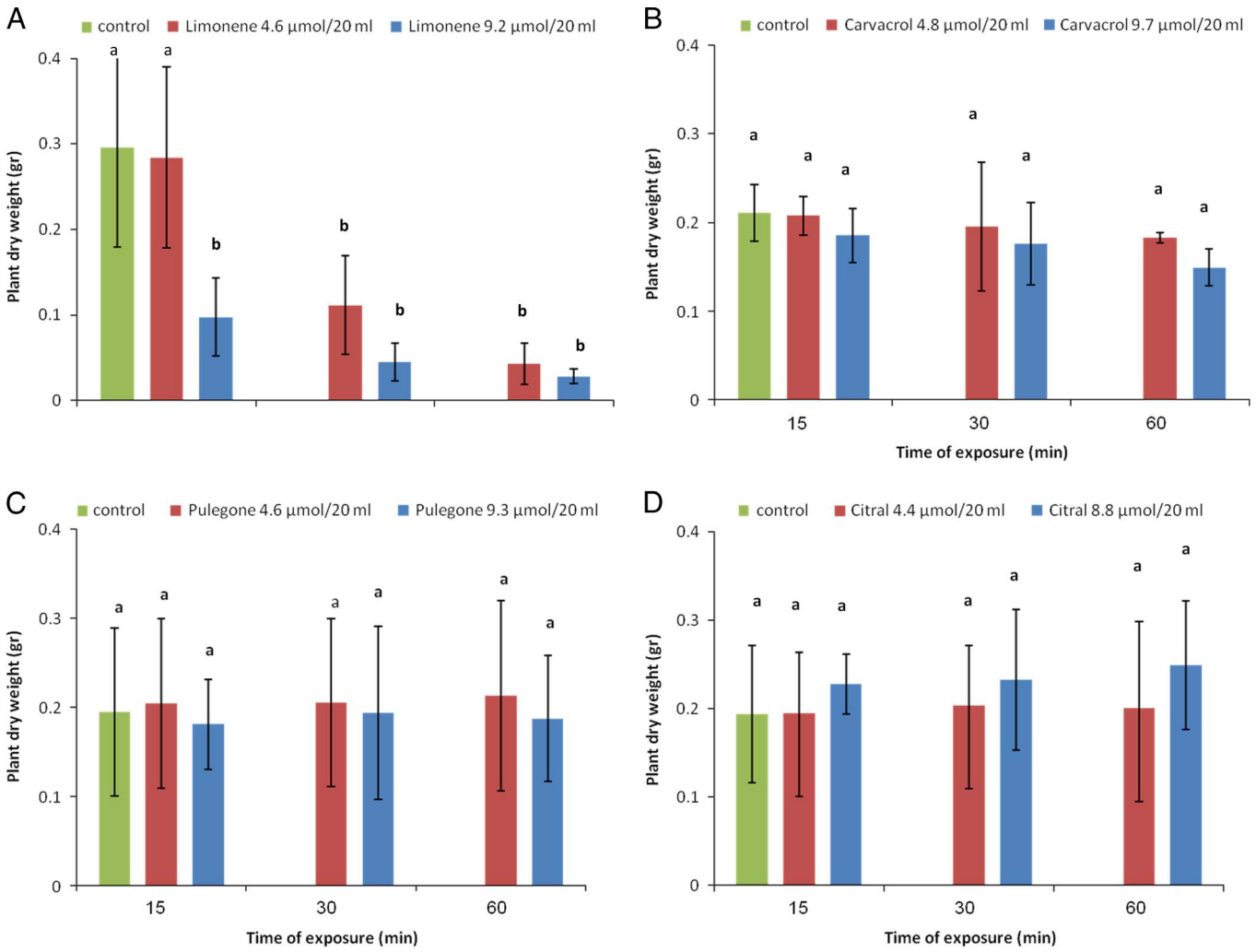

Figure 3. Effect of time and dose on fresh weight (g plant^{-1}) of *Arabidopsis* plants exposed to limonene (A), carvacrol (B), pulegone (C), and citral (D) at vapor phase for 15, 30, and 60 min. Lowercase letters represent treatments that were significantly different than the control as determined by ANOVA followed by Tukey's HSD post hoc test.

and analyzed by GC–mass spectrometry to measure the amounts of limonene, carvacrol, pulegone, and citral absorbed by the plants. As reflected in Table 2, a positive correlation was recorded between the residue level in the tissue and the combined compound concentration and exposure duration interaction. In other words, the higher the concentration and the longer the exposure duration were, the higher the residue level detected in the tissue. Of note was the outcome for citral and limonene. Plants that absorbed citral biochemically reduced it to nerol and geraniol; plants that absorbed limonene converted part of it to carvacrol. The levels of carvacrol and pulegone remained stable within the plants (Table 2).

Previous studies conducted by our research team (Chaimovitsh et al. 2010, 2012) shed light on the mode of action of citral. We found that microtubules were the immediate target of citral in plant and animal cells. Microtubules were disrupted in the presence of micromolar concentrations of citral, whereas F-actin and cell membrane remained intact (Chaimovitsh et al. 2010). In the present study we provide evidence that only a subset of monoterpenes disrupt microtubules. Furthermore, we confirm our previous observation (Chaimovitsh et al. 2010) that citral causes detectable damage to the plasma membrane under the same experimental conditions in which microtubule disruption was observed. Interestingly, plants exposed to citral exhibited cell swelling, inhibition of polar cell growth, zigzagged cell walls, and irregular phragmoplasts, all directly related to microtubule deformation (Grana et al. 2013a).

In the present study 10 derivatives of citral were screened. Some are known allelochemicals, such as geraniol (Martino et al. 2010; Zunino and Zygadlo 2004), citronellol (Martino et al. 2010;

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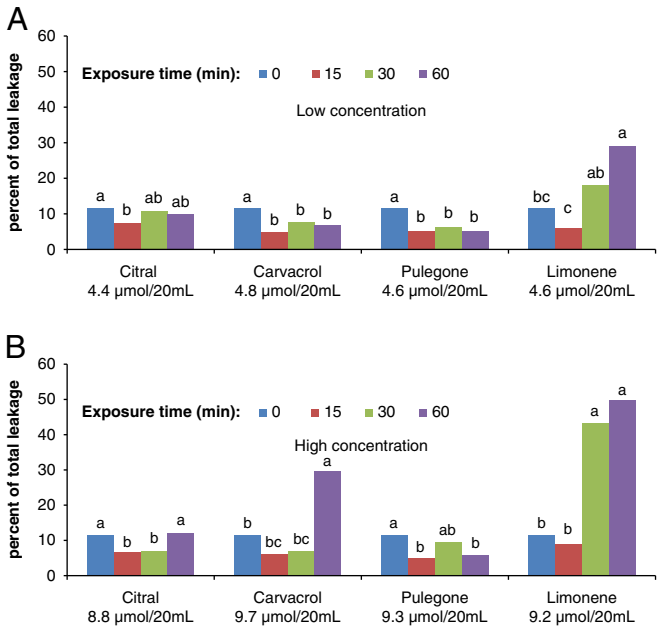

Figure 4. Effect of citral, carvacrol, pulegone, and limonene on membrane leakage in *Arabidopsis* plants exposed to (A) low (0.75 µl per 20 ml) and (B) high (1.5 µl per 20 ml) dosages for 15, 30, and 60 min. Lowercase letters represent treatments that were significantly different than the control as determined by ANOVA followed by Tukey's HSD post hoc test.

Singh et al. 2002), citronellal (Singh et al. 2002), and citral itself (Dudai et al. 1999; Martino et al. 2010; Sousa et al. 2010). The list also included two different enantiomers of three compounds: (+)/(−)-citronellal, (+)/(−)-citronellol, which are abundant in Java citronella (*Cymbopogon winterianus* Jowitt) and cymbopogon [*Cymbopogon nardus* (L.) W. Watson] (Kreis and Mosandl 1994b); and (+)/(−)-citronellic acid, which are abundant in common balm (*Melissa officinalis* L.) (Kreis and Mosandl 1994a). Other derivatives, such as geraniol and geranic acid, are present in sweet scented geranium (*Pelargonium graveolens* L'Hér. ex Aiton); nerol is abundant in cymbopogon (Kreis and Mosandl 1994b); and finally, citral dimethyl acetal is an artificial derivative of citral (Sigma-Aldrich catalog no. 7549-37-3).

Six highly active allelochemical monoterpenes were also screened. These included two enantiomers (+)/(−) of carvone (Martino et al. 2010; Zunino and Zygadlo 2004), limonene (Abrahim et al. 2000; Martino et al. 2010; Singh et al. 2009b), menthone (Maffei et al. 2001; Martino et al. 2010; Mucciarelli et al. 2001), pulegone (Dudai et al. 1999, 2009;

Table 2. Monoterpene residues and their derivatives in *Arabidopsis thaliana* tissue.

Monoterpene	Exposure time (min)	Quantity applied (µmol per 20 ml)	Monoterpene and derivative residues in plant tissue (mol g dry weight^{-1})[1]					
			Citral	Nerol	Geraniol	Limonene	Carvacrol	Pulegone
Citral	15	4.4	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	—	—	—
	30	4.4	6.91 ± 5.0	0.0 ± 0.0	17.83 ± 6.97	—	—	—
	60	4.4	45.38 ± 21.99	5.79 ± 0.56	52.15 ± 17.6	—	—	—
	15	8.8	13.97 ± 4.27	0.0 ± 0.0	9.49 ± 2.66	—	—	—
	30	8.8	21.26 ± 5.67	0.0 ± 0.0	40.34 ± 3.76	—	—	—
	60	8.8	71.39 ± 19.84	8.74 ± 0.52	67.56 ± 23.31	—	—	—
Limonene	15	4.6	—	—	—	60.04 ± 11.4	7.74 ± 2.12	—
	30	4.6	—	—	—	512.84 ± 92.29	14.46 ± 4.15	—
	60	4.6	—	—	—	875.76 ± 75.7	44.51 ± 5.42	—
	15	9.2	—	—	—	264.51 ± 12.3	13.46 ± 3.31	—
	30	9.2	—	—	—	562.39 ± 23.36	25.76 ± 2.76	—
	60	9.2	—	—	—	937.1 ± 62.3	128.02 ± 15.73	—
Carvacrol	15	4.8	—	—	—	—	7.26 ± 3.05	—
	30	4.8	—	—	—	—	82.95 ± 4.15	—
	60	4.8	—	—	—	—	106.99 ± 17.85	—
	15	9.7	—	—	—	—	15.2 ± 5.76	—
	30	9.7	—	—	—	—	95.06 ± 11.08	—
	60	9.7	—	—	—	—	124.42 ± 16.11	—
Pulegone	15	4.6	—	—	—	—	—	39.37 ± 7.34
	30	4.6	—	—	—	—	—	112.63 ± 33.86
	60	4.6	—	—	—	—	—	302.0 ± 32.67
	15	9.3	—	—	—	—	—	42.88 ± 11.14
	30	9.3	—	—	—	—	—	142.17 ± 40.46
	60	9.3	—	—	—	—	—	312.68 ±32.59

Note: values are mean ± standard deviation of five replicated *Arabidopsis thaliana* plants.

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Maffei et al. 2001; Mucciarelli et al. 2001), and carvacrol (Dragoeva et al. 2008; Dudai et al. 1999). It was found that in addition to citral, limonene, (+)-citronellal, menthone, geraniol, and (+)-carvone have the ability to disrupt plant microtubules. Based on our results, the monoterpenes examined in this work can be classified into three main groups: (1) monoterpenes with strong antimicrotubule activity, i.e., limonene and (+)-citronellal; (2) monoterpenes with moderate anti-microtubule activity, i.e., citral, geraniol, (–)-menthone, (–)-citronellal, and (+)-carvone; and (3) monoterpenes lacking antimicrotubule activity, i.e., (–)-carvone, nerol, (+)/(–)-citronellol, geranic acid, (+)/(–)-citronellic acid, citral dimethyl acetal, pulegone, and carvacrol.

An enantioselective activity was also recorded for isomers. The (+)-carvone and (+)-citronellal isomers were more potent than their (–)-carvone and (–)-citronellal counterparts in disrupting plant microtubules (Table 1), a finding which is in agreement with Altshuler et al. (2013), who showed enantioselective effects of (+)- and (–)-citronellal in animal and plant microtubules. In that work (Altshuler et al. 2013), (+)-citronellal disrupted microtubules, whereas (–)-citronellal at the same concentration did not. Interestingly, positive enantiomers of (+)-α pinene and (+)-β pinene were found to have stronger antimicrobial activity than their (–) counterparts (Rivas da Silva et al. 2012). In addition, (+)-limonene and (+)-carvone had stronger activity to different bacteria and dermatophytic fungi than (–)-limonene and (–)-carvone (Aggarwal et al. 2002).

In the present study we focused on four allelochemicals—citral, limonene, carvacrol, and pulegone—which are known as seed-germination inhibitors (Dudai et al. 1999, 2009). These four monoterpenes had differential modes of action in *Arabidopsis* plants. Limonene was highly active, with strong microtubule- and membrane-disrupting activity. In contrast, carvacrol exhibited only membrane-disrupting activity that was dependent on long exposure at high concentration. Citral exhibited microtubule- but not membrane-disrupting activity; and pulegone lacked an effect on either microtubules or cell membrane.

Limonene inflicted visible damage on the plants and demonstrated herbicidal activity. Its effect was significantly more visible than that of the three other monoterpenes. Its damage was characterized by a range of symptoms, from phytotoxicity in individual leaves to toxicity in all leaves leading to plant death. At the low dose, limonene's herbicidal activity was time dependent, while at the high dosage it was herbicidal at all exposure durations. In contrast, citral, carvacrol, and pulegone were nonherbicidal to the plants even at extended exposures to high dosages.

The effect of monoterpenes on biomass and phytotoxicity was examined using mature, 14-d-old *Arabidopsis* plants. A relationship between phytotoxicity and biomass reduction was observed in the case of limonene. Yet exposure of plants to the high dose of carvacrol did not show any significant biomass differences between exposed and untreated plants, and there were no clear phytotoxicity symptoms. Interestingly, citral, which is a known microtubule-disrupting agent did not incite phytotoxicity or biomass reduction, which was in contrast to its previously described effect on young seedlings (Chaimovitsh et al. 2010). This could be due to the ability of *Arabidopsis* to recover within hours of exposure to citral (Chaimovitsh et al. 2010). Furthermore, pulegone had no phytotoxic effect on mature *Arabidopsis* plants in this work. This outcome prevents clarification of its mode of action, indicating further study is needed.

We described here the influence of limonene, citral, pulegone, and carvacrol on membrane leakage in *Arabidopsis* plants and on the accumulation of these four monoterpenes in the plant tissue. Accumulation of limonene in the plant tissue increased dramatically with increasing dose and exposure time. The amount of limonene that was absorbed by the plants was 10-fold higher than that of citral, 8-fold higher than that of carvacrol, and 3-fold higher than that of pulegone. This might be due to limonene's higher physical volatility. The higher concentrations that accumulated in the plants could explain the stronger antimicrotubule and antimembrane activities, hence its herbicidal potential. The observed conversion of limonene to carvacrol by *Arabidopsis* plants is a novel finding. Carvacrol accumulated following exposure to the high dose of limonene and showed a slight membrane leakage effect. The metabolic pathway that underlies this conversion is currently unclear. Yet the observations in this study suggest that the mode of action of carvacrol as an allelochemical is via membrane leakage at high dosages and extensive exposure duration.

Accumulated data show that growth-inhibiting effects of different monoterpenes depend on their chemical structure. Monoterpenes that contain oxygen show higher inhibitory activity toward seed germination and plant growth than hydrocarbon

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monoterpenes (Elakovich 1988; Reynolds 1987; Vaughn and Spencer 1993). Weidenhamer et al. (1993) showed a connection between inhibitory activity and water solubility of monoterpenes. Ketones were more soluble and more active than alcohols, which were more active and soluble than hydrocarbons. In addition, the bioactivity of monoterpenes seems to be inversely correlated to lipophilicity (Abrahim et al. 2000). Zunino and Zygadlo (2004) concluded that most phytotoxic monoterpenes are from the group of alcohols and phenols. The work presented here examined 17 monoterpenes for microtubule-disrupting activity, and no direct correlation was found between monoterpene structure and microtubule-disrupting activity. The most active monoterpene was limonene, a hydrocarbon that belongs to the less active group described by Weidenhamer et al. (1993). Monoterpenes from the ketone group showed differential activities toward microtubules; (+)-carvone and menthone exhibited microtubule-disrupting activity, and pulegone did not. Monoterpenes from the alcohol group also exhibited differential microtubule-disrupting activity; geraniol exhibited antimicrotubule activity, whereas citronellol and nerol did not. The monoterpenes citral and citronellal from the aldehyde group were found to exhibit microtubule-disrupting activity, but it was weaker than that of the hydrocarbon limonene.

We observed a bioconversion of citral into its derivatives nerol and geraniol in *Arabidopsis* plants. A similar conversion was previously described in wheat seeds and was explained as a mechanism for detoxification of citral by the seeds through reduction to less bioactive derivatives (Dudai et al. 2000b). Interestingly, while geraniol was found to have antimicrotubule activity similar to that of citral, nerol did not.

In this study, no specific effects of pulegone on microtubulin and membrane leakage in the plants were observed. Therefore, the experimental setup used here did not provide evidence for its mode of action. It is probable that higher doses of this compound are required to affect mature *Arabidopsis* plants.

In conclusion, the present study sheds new light on the modes of action of various monoterpenes known as allelochemicals, demonstrating that some of the monoterpenes have isomeric-specific activity and pointing out limonene as possessing high herbicidal potency. These findings could assist in designing new, natural, and ecofriendly herbicides.

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