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ORIGINAL ARTICLE

β-Pinene inhibited germination and early growth involves membrane peroxidation

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Abstract β-Pinene, an oxygenated monoterpene, is abundantly found in the environment and widely occurring in plants as a constituent of essential oils. We investigated the phytotoxicity of β-pinene against two grassy (*Phalaris minor*, *Echinochloa crus-galli*) and one broad-leaved (*Cassia occidentalis*) weeds in terms of germination and root and shoot growth. β-Pinene (0.02–0.80 mg/ml) inhibited the germination, root length, and shoot length of test weeds in a dose–response manner. The inhibitory effect of β-pinene was greater in grassy weeds and on root growth than on shoot growth. β-Pinene (0.04–0.80 mg/ml) reduced the root length in *P. minor*, *E. crus-galli*, and *C. occidentalis* over that in the control by 58–60, 44–92, and 26–85 %, respectively. In contrast, shoot length was reduced over the control by 45–97 % in *P. minor*, 48–78 % in *E. crus-galli*, and 11–75 % in *C. occidentalis* at similar concentrations. Further, we examined the impact of β-pinene on membrane integrity in *P. minor* as one of the possible mechanisms of action. Membrane integrity was evaluated in terms of lipid peroxidation, conjugated diene content, electrolyte leakage, and the activity of lipoxygenases (LOX). β-Pinene (≥0.04 mg/ml) enhanced electrolyte leakage by 23–80 %, malondialdehyde content by 15–67 %, hydrogen peroxide content by 9–39 %, and lipoxygenases activity by 38–383 % over that in the control. It indicated membrane peroxidation and loss of membrane integrity that could be the primary target of β-pinene. Even the enhanced (9–62 %) activity of protecting enzymes, peroxidases (POX), was not able to protect the membranes from β-pinene (0.04-0.20 mg/ml)-induced toxicity. In conclusion, our results show that β-pinene inhibits root growth of the tested weed species through disruption of membrane integrity as indicated by enhanced peroxidation, electrolyte leakage, and LOX activity despite the upregulation of POX activity.

Keywords β-Pinene · Lipid peroxidation · Electrolyte leakage · Membrane disruption · Lipoxygenases · Peroxidases

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Introduction

Higher plants synthesize a great variety of terpenoids that play a multitude of ecological functions (Peñuelas et al. 1995). Among these, monoterpenes—the colorless, lipophilic volatile compounds and the constituents of floral scents, essential oils, and defensive resins—are chemically simple and the most abundant class of terpenoids. They play an important role in plant defense, various ecological interactions (like allelopathy and pollinator attraction), atmospheric chemistry, and plant–plant communications (Dudareva et al. 2006). Their increased synthesis within plants has even been suggested to provide greater thermotolerance (Peñuelas and Staudt 2010). Monoterpenes are also commercially important as these are used as flavoring agents, perfumes, and insecticides since antiquity. Of late, studies have evaluated the use of monoterpenes or essential oils as environmentally safer pesticides and herbicides (Singh et al. 2003; Batish et al. 2008; Dayan et al. 2009; Kaur et al. 2010). However, only 1,4-cineole (Duke et al. 2002) and eugenol-rich clove oil (Bainard et al. 2006) have been used for this purpose. Monoterpene-based commercial herbicide formulations have been recommended for organic

agriculture (Dayan et al. 2009). Therefore, it is worthwhile to explore more monoterpenoids for their phytotoxic effects on weeds with the purpose of using them as active ingredient in herbicide formulations.

Plants emit a vast array of volatile compounds, especially the isoprene and monoterpenes, which alone may account for ~55 % of the emitted volatiles (Guenther et al. 1995). Several studies have shown that monoterpenes inhibit germination and growth of other plants especially weeds and thus may possess good herbicidal potential (Singh et al. 2002a, b, 2006; Kordali et al. 2007; Dayan et al. 2000, 2009). However, among the tested monoterpenes, very little has been done on the phytotoxic effect of β-pinene towards weeds, even though it is a widely detected volatile compound in the environment (Geron et al. 2002). In fact, β-pinene along with α-pinene and sabinene forms the bulk of the monoterpenes emitted in the environment (Bertin et al. 1997). It also occurs naturally in several plants as a constituent of the essential oil (Geron et al. 2000, 2002). It has even been reported from the forest soil under the trees of its potential emitters (Paavolainen et al. 1998). Recently, β-pinene has been reported to inhibit early growth in rice and alter protein and carbohydrate content and related enzymes in the growing seedlings (Chowhan et al. 2011). However, the studies exploring its phytotoxicity against weeds and the possible mechanism/mode of action are largely lacking. Previously, monoterpenes being lipophilic in nature have been shown to alter membrane permeability, induce lipid peroxidation, and cause oxidative burst (Zunino and Zygadlo 2004; Singh et al. 2006). However, nothing has been done to explore such a mechanism of β-pinene. The present investigation was undertaken to assess the phytotoxicity of β-pinene and its possible mechanism of action in terms of effect on membrane integrity. The phytotoxicity was assessed against two graminaceous (*Phalaris minor* and *Echinochloa crus-galli*) and one broad-leaved (*Cassia occidentalis*) weeds in terms of germination, root and shoot length, dry weight, and chlorophyll content. Membrane integrity was evaluated in terms of lipid peroxidation (both quantitative and qualitative), conjugated diene content, electrolyte leakage, and the activity of lipoxygenases and peroxidases.

Material and methods

Materials

β-Pinene of technical grade (purity>98 %) purchased from Alfa-Aesar, Lancashire, England, was used in the present study. Seeds of *C. occidentalis* were collected locally from the plants growing in the wastelands on the periphery of Chandigarh, whereas *P. minor* and *E. crus-galli* seeds were collected from agricultural fields on the outskirts of Chandigarh, India. These were surface-sterilized with sodium hypochlorite (0.1 %, *w*/*v*) for 2 min and washed under running tap water (for 5 min) followed by distilled water. Seeds were dried between folds of filter paper and stored in a refrigerator for further use. Prior to use, these were scarified with concentrated sulfuric acid for 2 min (in case of *C. occidentalis*) or 30 s (in case of *P. minor* and *E. crus-galli*) to make the seed coat permeable. The scarification was done with the acid to thin the seed coat, remove the external dormancy, and make the seed germinate under laboratory conditions. The scarified seeds were washed under running tap water for 2 min and then with distilled water twice before use in growth studies.

Growth studies

Growth studies were conducted with β-pinene (0.02, 0.04, 0.08, 0.20, 0.40, and 0.80 mg/ml) in a dose–response manner under the laboratory conditions as per method given in Singh et al. (2009). The selected concentrations of β-pinene are ecologically realistic and comparable to those under natural conditions as reported earlier (White 1994; Amaral and Knowles 1998). β-Pinene solutions were prepared by dissolving the requisite amount in Tween-80 (~0.1 %) and making the final volume with distilled water. Twelve pre-imbibed seeds (in distilled water for 24 h) of test weeds were placed equidistantly in Petri dishes (ϕ=15 cm) lined with a thin layer of cotton and Whatman # 1 filter circle and moistened with 12 ml of β-pinene solution or distilled water (control). Petri dishes were sealed immediately with adhesive tape and Parafilm®. For each treatment concentration and seed species, five replicates were maintained in a randomized block design. All Petri dishes were kept in growth chambers set at 25±2 °C (for *C. occidentalis* and *E. crus-galli*) or 15±2 °C (for *P. minor*), 16/8 h light/dark photoperiod of 240 μmol m^{-2} s^{-1} and relative humidity of 75±2 %. Three such parallel sets of Petri dishes (of treatments and control) were maintained to measure the growth parameters and conduct biochemical studies at different time intervals, i.e., third, fifth, and seventh day. After the third, fifth, and seventh days of treatment, one complete set of control and treated Petri dishes (with five replicates) was used for measuring the root and shoot length of emerging seedlings. However, the total number of germinated seeds, dry weight, and chlorophyll content in each treatment were noted on the seventh day only. Since the inhibitory effect was the greatest on root length of *P. minor*, these roots were harvested for further biochemical studies. For relative electrolyte leakage measurement, fresh tissue was used, whereas for other biochemical analyses, roots were stored at −80 °C until further use.

Effect on membrane integrity

We studied the time-course changes in membrane integrity upon β-pinene exposure in *P. minor* roots as per the method of Duke and Kenyon (1993). It was studied in terms of electrolyte leakage from the roots of test weeds by measuring conductivity of the bathing medium. Electrolyte leakage is an indicator of membrane damage and occurs due to membrane peroxidation (Bajji et al. 2002). Briefly, roots (100 mg) from 7-day-old *P. minor* seedlings (grown under controlled conditions) were dipped in 1 mM MES buffer (2-[N-morpholino] ethanesulfonic acid sodium salt, pH 6.5; 5 ml) containing 2 % sucrose (*w*/*v*) and β-pinene (0.04 and 0.20 mg/ml) dissolved in Tween-80. A parallel control containing all the materials except β-pinene was also maintained. The conductivity of the bathing medium was measured at regular intervals in the dark (0, 1, 2, 4, 8, 12, 16, 18, and 20 h) and then in the light (at 22, 24, 26, 28, and 30 h after treatment) with a conductivity meter (ECOSCAN CON5; Eutech Instruments Pte. Ltd., Singapore). Root tissue was also boiled for 15 min to measure the maximum electrolyte leakage. Five independent tissue replicates were maintained for each treatment.

Lipid peroxidation

Oxidative damage to lipids was measured in terms of malondialdehyde (MDA) and conjugated dienes (CD) content. MDA, a major thiobarbituric acid reactive species, was determined as per Heath and Packer (1968). Briefly, root tissue (100 mg) was homogenized in tricarboxylic acid (TCA; 0.1 %, *w*/*v*, 5 ml) and centrifuged at 10,000×*g* for 15 min. To 1 ml of the supernatant, 4 ml of 0.5 % TBA in 20 % TCA was added. The mixture was heated at 95 °C for 30 min, cooled over ice, and centrifuged at 10,000×*g* for 10 min. The absorbance of the supernatant was recorded at 532 nm and corrected for nonspecific absorbance at 600 nm. MDA content was calculated using an extinction coefficient (ε) of 155 mM^{-1} cm^{-1} and expressed as nanomole per gram fresh weight.

CD content was determined by homogenizing the root tissue in ethyl alcohol (96 %, *v*/*v*), and the absorbance was measured at 234 nm (Singh et al. 2007). CD content was calculated using ε=26.5 mM^{-1} cm^{-1} and expressed as micromole per gram fresh weight.

Hydrogen peroxide (H_2O_2) content

It was determined as per the details given in Singh et al. (2007). Briefly, root tissue (100 mg) was homogenized in TCA (0.1 %, *w*/*v*, 5 ml) in an ice bath, and the homogenate was centrifuged at 12,000×*g* for 15 min. To 0.5 ml of root extract, 0.5 ml of 10 mM PO_4^{3-} buffer (pH=7.0) and 1 ml of 1 M potassium iodide were added. The absorbance of the mixture was measured at 390 nm and H_2O_2 content was calculated using ε=0.28 μM^{-1} cm^{-1} and expressed as nanomole per gram fresh weight.

In situ detection of membrane peroxidation

Lipid peroxidation was histochemicallly detected in vivo using Schiff's reagent (Kaur et al. 2012). Root plasma membrane integrity was detected by incubating the roots in Evans blue solution (0.025 %, *w*/*v*, in 100 μM $CaCl_2$) for 30 min (Kaur et al. 2012). The stained roots were washed four times with distilled water and viewed under a Trinocular Stereo Zoom Microscope (Model RSM-9; Radical Instruments, Ambala Cantt, India) fitted with a digital imaging system (Nikon Coolpix 4500, Japan).

Enzyme extraction and assay

Root tissue (200 mg) was homogenized in pre-chilled 100 mM PO_4^{3-} buffer (pH=7.0) under ice-cold conditions. The homogenate was filtered through three layers of cheesecloth and centrifuged at 15,000×*g* for 30 min at 4 °C rotor temperature. The supernatant was stored at 4 °C until used for assaying the activities of lipoxygenases (LOX) and peroxidases (POX). An aliquot (0.5 ml) of the supernatant was used for protein estimation using bovine serum albumin as standard.

Activity of POX was estimated at 430 nm using H_2O_2 (0.2 M) as substrate and expressed as katals per second per milligram protein (Chowhan et al. 2011). Briefly, the reaction mixture contained 100 mM PO_4^{3-} buffer (pH=6.5), *o*-dianisidine (4 mM in methanol), and 0.5 ml of enzyme extract.

LOX activity was estimated at 234 nm in terms of the rate of oxidation of linoleic acid (Axelrod et al. 1981). The reaction mixture contained 0.575 mM linoleic acid in 100 mM PO_4^{3-} buffer (pH=7.4), Tween 80 (0.2 %, *v*/*v*), and 0.2 ml enzyme extract. LOX activity was determined using ε=25 mM^{-1} cm^{-1} for linoleic acid and expressed as enzyme unit (EU) per milligram protein.

Statistical analyses

The experiments were conducted in a randomized design with five replicates per treatment including control. For each biochemical and enzymatic assay, there were five replicated (independent) tissue samples. The experiments and analyses were repeated. The presented data are means of two experiments. The data for germination were analyzed by nonlinear fit model: log inhibitor vs. response–variable slope by plotting the concentration (on the *x*-axis, log scale) and percent germination (on the *y*-axis) using the software GraphPad

Prism® (ver. 5). The data on growth studies (root and shoot length, dry weight, and chlorophyll content) and biochemical analyses were analyzed by linear regression models.

Results and discussion

β-Pinene (≥0.04 mg/ml) significantly inhibited the germination in seeds of test weeds in a dose-dependent manner (Fig. 1). Among the three test weeds, the inhibition in seed germination was the greatest in *P. minor* and the minimum in *C. occidentalis.* In *P. minor*, none of the seed could germinate at 0.40 mg/ml β-pinene. At 0.20 mg/ml β-pinene, seed germination was reduced by 33.6, 42.8, and 27.3 % in *P. minor*, *E. crus-galli*, and *C. occidentalis*, respectively (Fig. 1). The observed variations in the response of different weeds towards β-pinene could be attributed to seed size as suggested by Williams and Hoagland (1982). In our study, the small-seeded *P. minor* was the most susceptible species and the large-seeded *C. occidentalis* was least affected. This is supported by earlier findings that 2-benzoxazoline (hydroxamic acid) inhibited small-seeded species without affecting large-seeded crops (Burgos and Talbert 2000).

Further, β-pinene significantly reduced the seedling growth measured in terms of root and shoot length of the test weeds in a dose- and time-dependent manner (Fig. 2). In general, the inhibitory effect of β-pinene was greater on root and shoot length of the grassy weed *P. minor* than on the other tested weeds, which were less affected. In general, root growth after the third, fifth, and seventh day of β-pinene exposure declined by ~24–88, 29–90, and 41–92 % in *E. crus-galli*; ~40–91, 42–95, and 45–96 % in *P. minor*; and by 3–82, 7–84, and 7–85 % in *C. occidentalis*, respectively, over the respective controls (Fig. 2). Upon exposure to 0.08 mg/ml β-pinene, the root length was reduced over the control by 56–62 % in *E. crus-galli*, 69–72 % in *P. minor* and 25−39 % in *C. occidentalis* upon 3–7 days of β-pinene exposure. At 0.20 mg/ml β-pinene, the root length in *P. minor* was reduced by 91–96 % over that in the control upon 3–7 days of β-pinene exposure. However, in *E. crus-galli* and *C. occidentalis* plants, the root length was less affected at 0.20 mg/ml β-pinene and was reduced by 72–75 and 40–54 %, respectively, during 3–7 days of β-pinene exposure (Fig. 2).

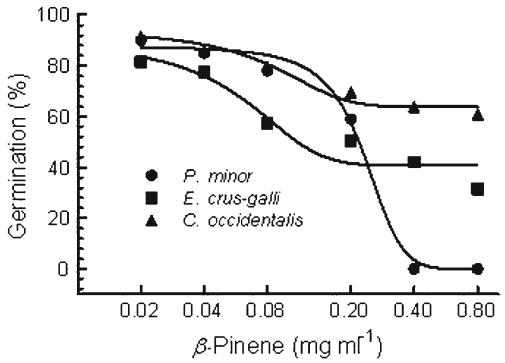

Fig. 1 Dose–response curves showing the effect of β-pinene on percent germination in test weeds measured after 3 days. Dose–response curves were derived by plotting the concentration (on the *x*-axis) and percent response (on the *y*-axis) and analyzed by nonlinear fit model: log inhibitor vs. response–variable slope using GraphPad Prism version 5

The reduction in shoot growth after the third, fifth, and seventh days of β-pinene exposure was in the range of ~18–69, 18–76, and 18–78 % in *E. crus-galli* and 2–61, 23–74, and 18–75 % in *C. occidentalis*, respectively, over the respective controls (Fig. 2). At 0.20 mg/ml, β-pinene shoot growth was drastically reduced in *P. minor* by 82–97 % upon 3–7 days of β-pinene exposure. However, the reduction in shoot growth was lesser in *E. crus-galli* and *C. occidentalis* plants, and it decreased by 55–61 and 30–55 %, respectively, at 0.20 mg/ml β-pinene, upon 3–7 days of β-pinene exposure (Fig. 2).

β-Pinene exposure reduced the dry weight in all the test weeds and the reduction was the maximum in *P. minor* (Fig. 3a). Dry weight declined by ~8–84 % in *P. minor,* ~6–83 % in *E. crus-galli*, and ~9–39 % in *C. occidentalis* in response to 0.02–0.80 mg/ml β-pinene (Fig. 3a). β-Pinene (0.02–0.80 mg/ml) negatively affected the chlorophyll content in all the three test weeds. Chlorophyll content declined by ~15–90, 3–80, and 7–81 % in *P. minor*, *E. crus-galli*, and *C. occidentalis*, respectively, upon exposure to 0.02–0.80 mg/ml β-pinene (Fig. 3b).

The inhibitory effect of β-pinene was greater on root growth than on the shoot/hypocotyl growth. A recent study has attributed the relatively greater effect of 1,8-cineole on the roots than on the shoots to either its more concentration around the roots and/or higher permeability of roots to the chemical since the lesser part of the root is covered by cuticle than the shoots/hypocotyls, which are more protected by cuticle (Yoshimura et al. 2011). The observed inhibitory effect of β-pinene is supported by earlier studies reporting growth inhibitory effect of oxygenated monoterpenes like 1,4- and 1,8-cineole, citronellal, limonene, thymol, geraniol, linalool, β-myrcene, and α-pinene (Romagni et al. 2000; Zunino and Zygadlo 2004; Nishida et al. 2005; Singh et al. 2006, 2009; Kordali et al. 2007; Singh et al. 2009). Based on the literature, it could be attributed to the possible interference of monoterpenes with the root mitotic activity and related changes such as DNA synthesis and cell proliferation in the root apical meristem (Romagni et al. 2000; Nishida et al. 2005; Singh et al. 2006).

Earlier studies have reported that essential oils per se and their constituent terpenes inhibit plant growth through disruption of membrane integrity (Tworkoski 2002; Zunino and Zygadlo 2004; Singh et al. 2006, 2009). Experiments were therefore undertaken to explore the possible role of β-

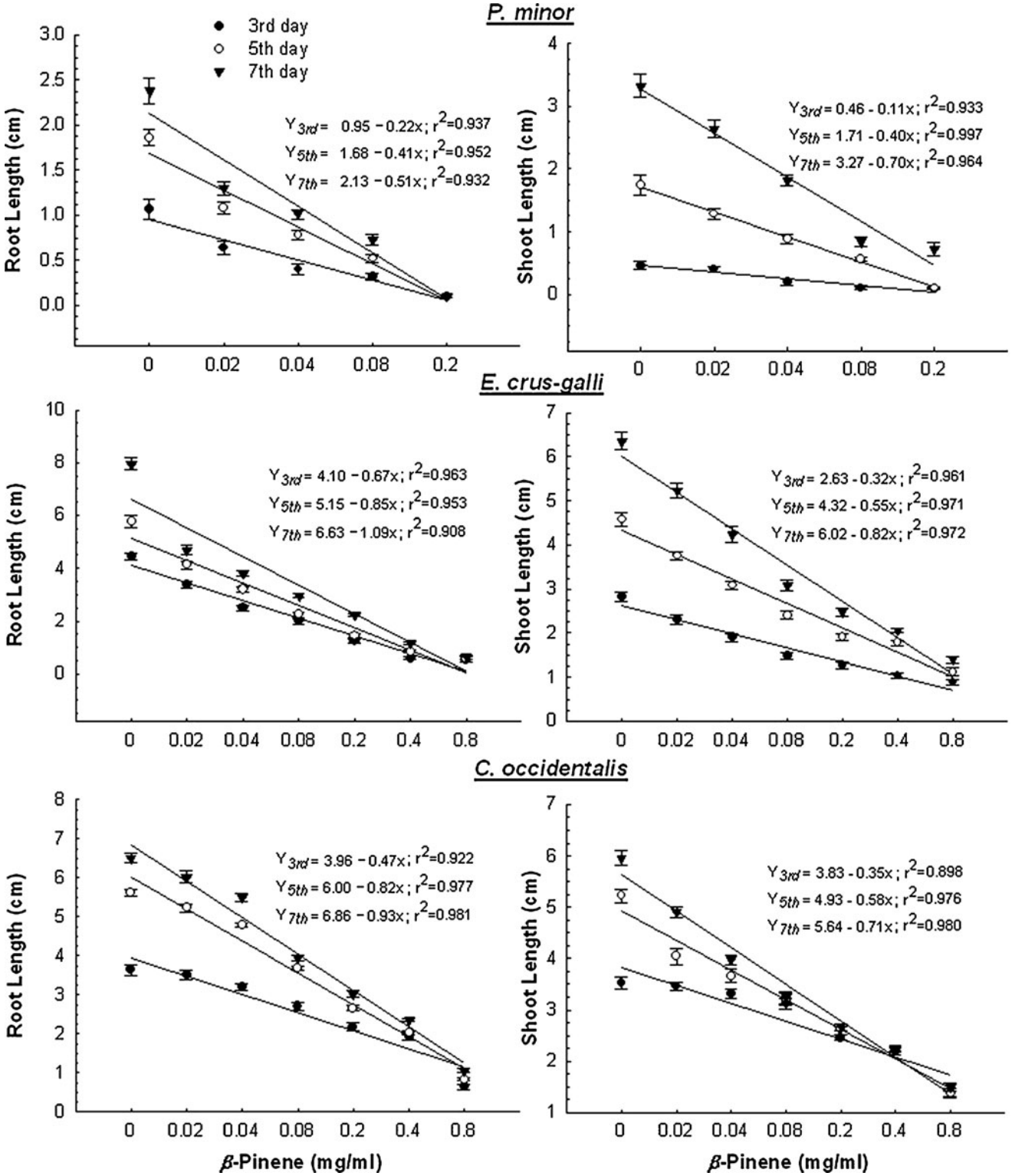

Fig. 2 Effect of β-pinene on the root (*left panel*) and shoot length (*right panel*) of *Phalaris minor*, *Echinochloa crus-galli*, and *Cassia occidentalis* measured on the third, fifth, and seventh day after exposure. Data were analyzed by linear regression model and r^2 represents the correlation coefficient. *Vertical bars* along each data point represent the standard error

pinene in altering membrane permeability that could possibly be its primary target. β-Pinene significantly enhanced electrolyte leakage in the root tissue of *P. minor*, as indicated by enhanced conductivity of the bathing medium (Fig. 4).

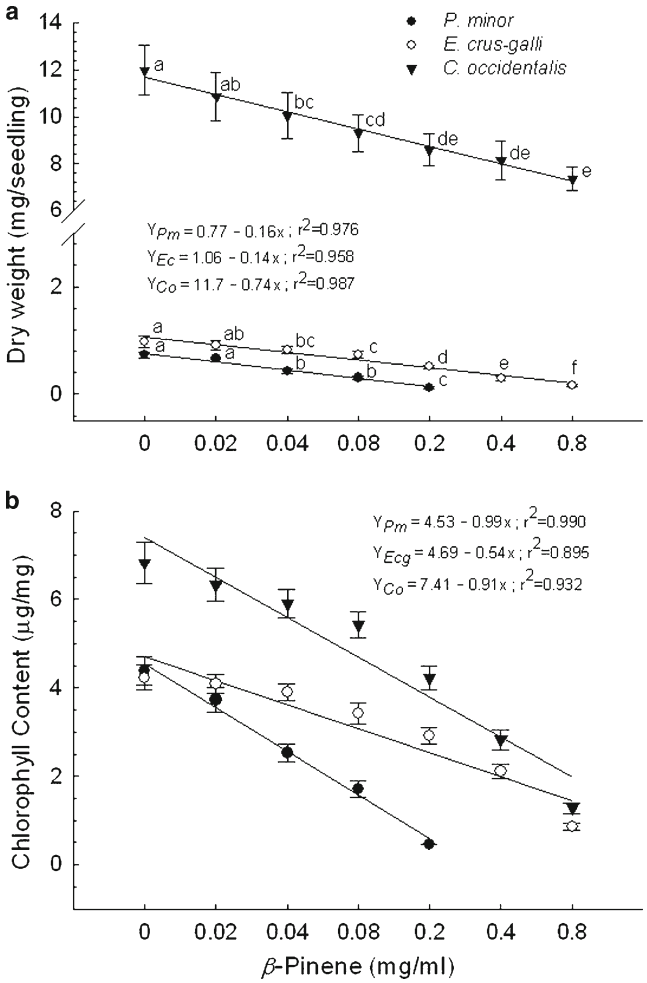

Fig. 3 Effect of β-pinene on **a** dry weight and **b** total chlorophyll content of *Phalaris minor*, *Echinochloa crus-galli*, and *Cassia occidentalis* measured on the seventh day after exposure. Data were analyzed by linear regression model and r^2 represents the correlation coefficient. *Vertical bars* along each data point represent the standard error. *Different letters* along each curve in dry weight figure represent significant difference at $p<0.05$

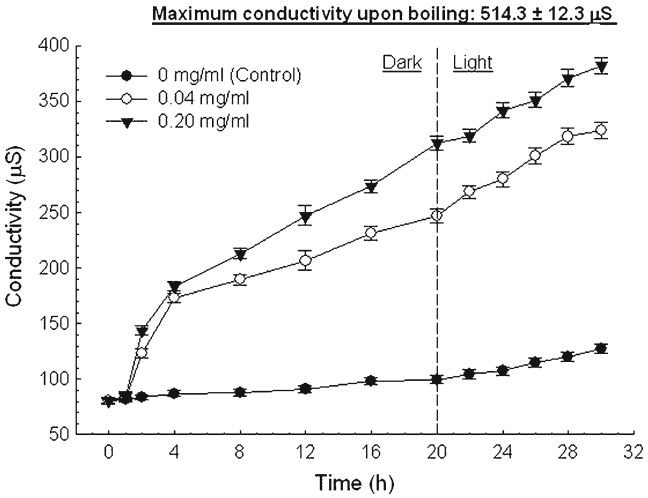

Fig. 4 Time-dependent relationship between β-pinene exposure and electrolyte leakage measured as conductivity in the roots of 1-week-old of *P. minor*. *Vertical dotted line* at the 20-h stage indicates the point of transition from dark to light conditions

The effect was concentration- and exposure period- dependent. Solute leakage increased with time and was irrespective of the dark and light. After 30 h, the conductivity of the bathing medium containing 0.20 mg/ml β-pinene was nearly 75 % of the maximum obtained upon boiling the tissue.

β-Pinene caused loss of membrane permeability, and cell death was confirmed by in situ staining with Evans blue (plasma integrity indicator), wherein β-pinene-exposed roots stained dark blue than the unexposed control roots (Fig. 5a).

Increased ion leakage suggested disruption of plasma membrane integrity resulting in greater release of electrolytes (Duke and Kenyon 1993; Singh et al. 2009). The decreased membrane permeability correlates positively to peroxidation of polyunsaturated fatty acids in membranes, thereby resulting in formation of malondialdehyde (Maness et al. 1999). Previously, β-pinene has been reported to alter biochemical properties of the membrane and impair electron transport (Klingler et al. 1991). Uncoupling of electron transport results in reactive oxygen species (ROS) generation (Badger 1985) and lipid peroxidation (Elstner 1982). Monterpenes, (−)-menthone and (+)-pulegone, inhibit mitochondrial respiration through their interference with cell walls and plasma membrane (Mucciarelli et al. 2001). Excessive leakage of ions could be attributed to peroxidation of polyunsaturated fatty acids or lipids in the biomembranes resulting in the formation of several byproducts, including

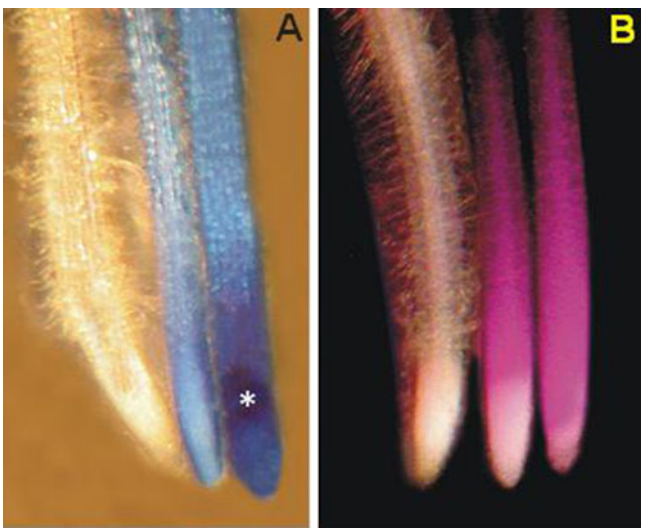

Fig. 5 Photographs showing histochemical localization of **a** the loss of membrane integrity and **b** lipid peroxidation in the roots of *Phalaris minor* upon β-pinene exposure. In each photograph, from *left* to *right*: 0 (control), 0.04, and 0.20 mg/ml β-pinene treatments. *Asterisk* indicates enhanced stain indicating greater accumulation of Evans blue dye

conjugated dienes (CD) and malondialdehyde (MDA). We, therefore, measured the amounts of MDA, CD, and H_2O_2 content in response to β-pinene.

Exposure to β-pinene significantly ($p \leq 0.05$) enhanced MDA content in the roots of *P. minor* in a dose- and time-dependent manner. In general, MDA content increased by 9–43, 10–55, and 13-67 % after the third, fifth, and seventh day of exposure to 0.02-0.20 mg/ml β-pinene. Exposure to 0.04 mg/ml β-pinene enhanced MDA content by 15–25 % over that in the control. It further increased with concentration and was 43–67 % greater over that in the control at 0.20 mg/ml β-pinene (Fig. 6a). β-Pinene-induced accumulation of MDA was also evident from greater staining of treated roots than the untreated control roots. Roots from β-pinene treatments stained dark pink with Schiff's regent in a concentration-dependent manner (Fig. 5b).

These observations suggest that β-pinene cause peroxidation of lipids in the membranes resulting in enhanced MDA content. Lipid peroxidation can alter membrane lipid bilayers and permeability resulting in cellular dysfunction (Nigam and Schewe 2000). Our results are in agreement with earlier studies reporting membrane peroxidation and damage induced by essential oils and monoterpenes in relation to root growth inhibition (Zunino and Zygadlo 2004; Singh et al. 2006, 2009; Mutlu et al. 2011).

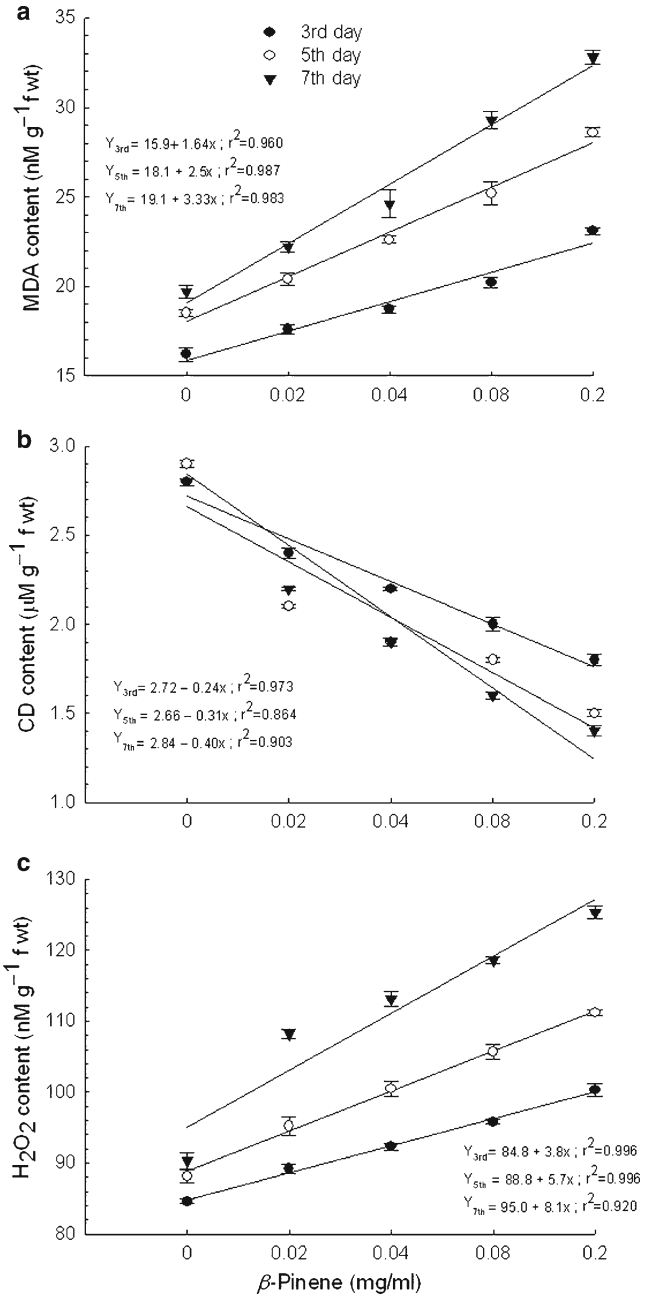

Fig. 6 Effect of β-pinene on lipid peroxidation in terms of **a** malondialdehyde (MDA) and **b** conjugated diene (CD) content, and **c** hydrogen peroxide (H_2O_2) accumulation in the roots of *Phalaris minor* measured on the third, fifth, and seventh day upon exposure. Data were analyzed by linear regression models and r^2 represents the correlation coefficient. *Vertical bars* along each data point represent the standard error

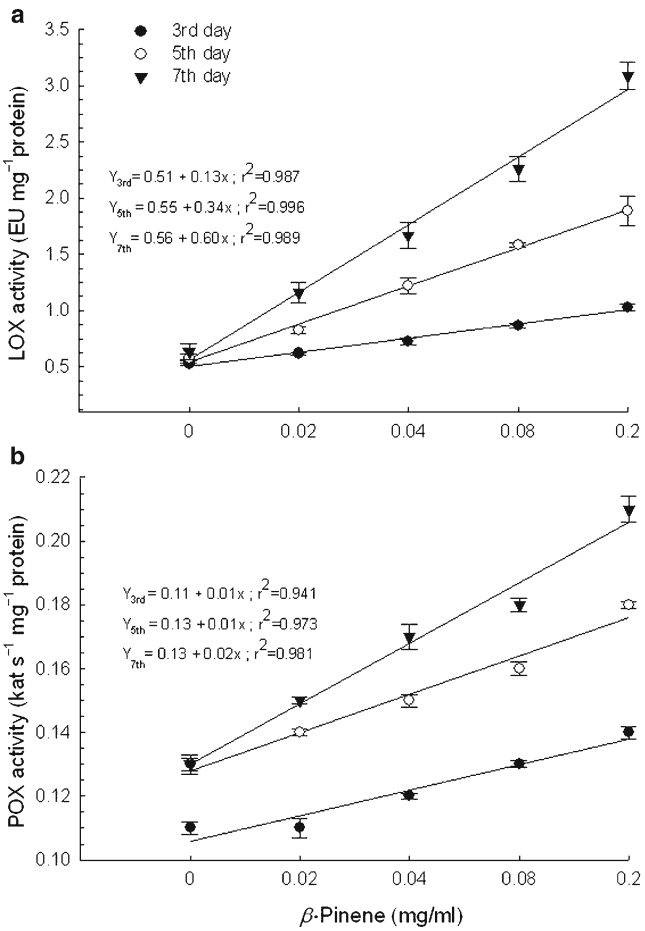

Fig. 7 Effect of β-pinene on the activity of **a** lipoxygenases (LOX) and **b** peroxidases (POX) in the roots of *Phalaris minor* measured on the third, fifth, and seventh day upon exposure. Data were analyzed by linear regression models and r^2 represents the correlation coefficient. *Vertical bars* along each data point represent the standard error

In contrast, CD content declined by 14–55 % ($p \leq 0.05$) during 3–7 days of exposure to 0.02-0.20 mg/ml β-pinene over that in the control (Fig. 6b). It declined over the control by 14–36, 28–48, and 29–55 % after the third, fifth, and seventh day of exposure to 0.02-0.20 mg/ml β-pinene. In our study, we observed greater MDA content but reduced CDs in response to β-pinene exposure. These findings are supported by an earlier study of Zunino and Zygadlo (2004) who reported a decreased CD content in maize roots in response to menthol and geraniol, two oxygenated monoterpenes. CDs are the initial products of lipid peroxidation and react further with ROS resulting in the formation of MDA, which is the final product (Halliwell and Gutteridge 1989).

β-Pinene (0.02-0.20 mg/ml) exposure enhanced ($p \leq 0.05$) H_2O_2 content by 5–39 % (Fig. 6c) during 3–7 days of exposure. H_2O_2 content increased over the control by 9–25, 13–31, and 19–39 % during 3–7 days of exposure to 0.04, 0.08, and 0.20 mg/ml of β-pinene, respectively. H_2O_2 acts as a signaling molecule, aids in cellular defense, and provides tolerance against stress at low concentration, whereas at high concentrations, it induces cellular damage (Stone and Yang 2006). H_2O_2 has been reported to hinder the activity of~SH group containing enzymes, affect the biochemical processes, and thereby reduce plant growth (Stone and Yang 2006). Earlier, a similar increase in H_2O_2 content was observed in *C. occidentalis* in response to α-pinene exposure (Singh et al. 2006).

Disruption of membrane integrity was also confirmed by the enhanced activity of LOX (Fig. 7a). LOX activity enhanced by 17–383 % during 3–7 days of exposure to 0.02-0.20 mg/ml β-pinene over that in the control. It enhanced by 17–94, 43–226, and 81-383 % on the third, fifth, and seventh day of exposure to 0.02-0.20 mg/ml β-pinene. At 0.04 mg/ml β-pinene, LOX activity enhanced over that in the control by 38–161 % (Fig. 6a). LOX is a ubiquitous enzyme in eukaryotic organisms and catalyzes the oxidation of membrane lipids (Siedow 1991). Enhanced LOX activity has been related to peroxidation of lipids resulting in membrane damage and loss of membrane integrity (Maalekuu et al. 2006). Enhanced LOX activity in *C. occidentalis* roots upon β-pinene exposure correlates positively with the lipid peroxidation and electrolyte leakage confirming membrane disintegration. Our results are supported by the results of Song et al. (2001) on the positive correlationship between LOX activity and lipid peroxidation in cotton seedlings.

Enhanced lipid peroxidation and membrane disintegration suggest that β-pinene causes oxidative stress in the roots of *P. minor*. To control ROS generation and regulate lipid peroxidation, cells possess both enzymatic and nonenzymatic mechanisms (Foyer et al. 1994). In this regard, we estimated the activity of POX in response to β-pinene exposure. POX are widely distributed in plant tissues and are one of the major groups of antioxidant enzymes that protect the cells from destructive influence of toxic oxygen radicals (Santos et al. 2004). Exposure to β-pinene (0.02-0.20 mg/ml) significantly ($p \leq 0.05$) increased the activity of POX by 9–62 % over that in the control during 3–7 days of exposure. POX activity increased by 9–31, 18–39, and 27–62 % in response to 3–7 days of exposure to 0.04, 0.08, and 0.20 mg/ml β-pinene, respectively, over that in the control (Fig. 7b). Our findings are supported by those of Amora et al. (2000) who reported increased total peroxidase activity under anoxia treatment. Additionally, the activity of POX correlates negatively with the stiffening of cell walls and thus reduced root elongation (Andrews et al. 2002). However, we did not observe the alterations in the antioxidant status (enzymatic and nonenzymatic) in response to β-pinene. The studies in this regard are under way.

In conclusion, our results show that β-pinene inhibits root growth of the weed species through disruption of membrane integrity as indicated by enhanced peroxidation and electrolyte leakage and greater LOX activity, despite the upregulation of POX activity. However, further studies are required to elucidate the exact cascade of events resulting in the generation of reactive oxygen species vis-à-vis the induced oxidative damage and the alteration in the antioxidant machinery in response to β-pinene. The present study is of immense ecological significance since VOCs are involved in mediating plant–plant interactions (Kegge and Pierik 2010) and intra- and interspecific communications (Owen and Peñuelas 2005). In natural communities, β-pinene upon release/emission from the plant accumulate in the soil (moves downward being heavier than air) where it may affect germination and growth of other plants depending upon the sensitivity of recipient species and concentration in the soil. This would, however, depend upon the type of plant community and climatic conditions of the area. Previous studies have reported that monoterpenes, including β-pinene, play a significant role in determining vegetational patterning and community structure (Muller 1965; Nishida et al. 2005) and regulate soil nitrogen cycling (White 1991).

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