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α-Pinene Inhibits Growth and Induces Oxidative Stress in Roots

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• *Background and Aims* Determining the mode of action of allelochemicals is one of the challenging aspects in allelopathic studies. Recently, allelochemicals have been proposed to cause oxidative stress in target tissue and induce an antioxidant mechanism. α-Pinene, one of the common monoterpenoids emitted from several aromatic plants including forest trees, is known for its growth-inhibitory activity. However, its mechanism of action remains unexplored. The aim of the present study was to determine the inhibitory effect of α-pinene on root growth and generation of reactive oxygen species, as indicators of oxidative stress and changes in activities of antioxidant enzymes.
• *Methods* Effects of α-pinene on early root growth were studied in five test species, *Cassia occidentalis*, *Amaranthus viridis*, *Triticum aestivum*, *Pisum sativum* and *Cicer arietinum*. Electrolyte leakage, lipid peroxidation, hydrogen peroxide generation, proline accumulation, and activities of the enzymes superoxide dismutase (SOD), ascorbate peroxidase (APX), guaiacol peroxidase (GPX), catalase (CAT) and glutathione reductase (GR) were studied in roots of *C. occidentalis*.
• *Key Results* α-Pinene inhibited the radicle growth of all the test species. Exposure of *C. occidentalis* roots to α-pinene enhanced solute leakage, and increased levels of malondialdehyde, proline and hydrogen peroxide, indicating lipid peroxidation and induction of oxidative stress. Activities of the antioxidant enzymes SOD, CAT, GPX, APX and GR were significantly elevated, thereby indicating the enhanced generation of reactive oxygen species (ROS) upon α-pinene exposure. Increased levels of scavenging enzymes indicates their induction as a secondary defence mechanism in response to α-pinene.
• *Conclusions* It is concluded that α-pinene inhibits early root growth and causes oxidative damage in root tissue through enhanced generation of ROS, as indicated by increased lipid peroxidation, disruption of membrane integrity and elevated antioxidant enzyme levels.

Key words: α-Pinene, radicle growth, lipid peroxidation, hydrogen peroxide, proline content, electrolyte leakage, membrane integrity, antioxidant enzymes, oxidative damage, *Cassia occidentalis*.

INTRODUCTION

Plants synthesize an array of chemical compounds that are involved in a variety of plant–plant, plant–microbe and plant–herbivore interactions. These exhibit a great structural and functional diversity and are produced within plants as a result of secondary metabolism (Hadacek, 2002). Although initially regarded as being functionless, these compounds are now known to play an important role in plant defence mechanisms (Berenbaum, 1995), to provide reproductive fitness (Facchini, 1999) and to impart allelopathic properties (Seigler, 1996); they thus serve as an excellent source of lead compounds for the development of new herbicides (Duke *et al.*, 2000). Among the different classes of natural plant products, monoterpenes—the components of volatile essential oils from a number of plant species—are involved in a variety of ecological interactions and also serve as pollinator attractants and as protectants (Langenheim, 1994; Harrewijn *et al.*, 2001). In addition, they are potent germination inhibitors and suppress early root growth (Muller and Muller, 1964; Abrahim *et al.*, 2000; Romagni *et al.*, 2000; Singh *et al.*, 2002, 2006; Zunino and Zygadlo, 2004; Nishida *et al.*, 2005). However, the exact mode of inhibitory action of monoterpenes remains unknown. Recently, Nishida *et al.* (2005) reported that five volatile monoterpenoids, namely eucalyptol, α- and β- pinene, camphene and camphor (which are present in the foliage, soil and airspace around *Salvia leucophylla*), when used in their purified form (0·1–10 mM) inhibited cell proliferation in roots of *Brassica campestris* by interfering with organelle and nuclear DNA synthesis within the meristem cells. Monoterpenes such as 1,8-cineole, thymol, geraniol and camphor (21, 2·0, 1·9 and 7·4 mg mL^{-1}, respectively, in the headspace) have been reported to inhibit maize root growth and induce oxidative stress by production of malondialdehyde (Zunino and Zygadlo, 2004). Production of reactive oxygen species (ROS), and related oxidative stress in general, has been proposed as one of the major mechanisms of action of the phytotoxins (Weir *et al.*, 2004).

α-Pinene is one of the major components of volatiles released by a wide range of species throughout the world, including those in the tropics (Keller and Lerdau, 1999; Geron *et al.*, 2001, 2002), Mediterranean species (Llusià and Peñuelas, 2000) and northern coniferous forest trees (Geron *et al.*, 2000). Plants emitting high levels include *Eucalyptus* sp., *Pinus* sp. and *Quercus* sp., although thousands of other species also emit monoterpenes, e.g. *Salvia* species. α-Pinene inhibits seed germination

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and primary root growth in maize (Abrahim *et al.*, 2000) and disrupts energy metabolism by acting as an uncoupler of oxidative phosphorylation and inhibiting the electron transport chain (Abrahim *et al.*, 2003). However, not much is known about its exact mode of action, although an increase in malondialdehyde levels in maize roots has been reported (Scrivanti *et al.*, 2003). It is likely that exposure to α-pinene induces oxidative stress in the target tissue and results in the observed inhibitory action. However, details regarding the level and extent of oxidative stress and the induction of anti-oxidative enzyme mechanisms due to α-pinene exposure are lacking. A study was therefore performed to investigate the impact of α-pinene on (1) the germination and early radicle growth of five plant species; (b) the induction of oxidative stress, measured in terms of lipid peroxidation, membrane integrity, hydrogen peroxide generation and proline content; and (c) the levels of induction of antioxidant enzyme mechanisms (in terms of activities of superoxide dismutase, catalase, ascorbate peroxidase, guaiacol peroxidase and glutathione reductase) in the roots.

MATERIALS AND METHODS

Materials

(–)-α-Pinene of technical grade (purity 98 %) was used in the experiments (Lancaster Synthesis Ltd., Morecambe, UK). Seeds of chickpea (*Cicer arietinum* L. 'GL-470', wheat (*Triticum aestivum* L. 'HD-2329'), and pea (*Pisum sativum* L. 'AP-1') were purchased from Punjab Agricultural University, Ludhiana, India, whilst seeds of coffee weed (*Cassia occidentalis* L.) and green amaranth (*Amaranthus viridis* L.) were collected locally from wild stands. All chemicals used in the enzymatic studies were of technical grade and procured from either Sisco Research Laboratory Pvt. Ltd., India, Sigma Co., St. Louis, USA, Merck Ltd., India or Loba-Chemie Pvt. Ltd., India.

Growth studies

Growth studies were conducted with different concentrations of α-pinene, namely 1, 2·5, 5 and 10 mM (= 0·136–1·36 mg mL^{-1}) under laboratory conditions. Solutions of α-pinene were prepared by dissolving the requisite amount of α-pinene in Tween-80 and making up to final volume with distilled water. The final concentration of Tween-80 in the α-pinene solutions was 100 mg L^{-1} (0·1 %). Tween-80 was used because of its surface activity to dissolve α-pinene in water, and the same amount was added to the distilled water that served as the control. The concentrations of α-pinene used in the study are environmentally relevant and comparable to those reported by previous workers under natural conditions (Wilt *et al.*, 1993; White, 1994; Amaral and Knowles, 1998). Amaral and Knowles (1998) reported that α-pinene concentrations in the range of 5–10 mg g^{-1} soil dry weight are ecologically relevant to those found in natural forest soils, whilst White (1994) and Wilt *et al.* (1993) reported that concentrations of the monoterpenes under natural conditions ranged from 3–5 mg g^{-1} soil dry weight.

Seeds of all test plants were imbibed in distilled water for 6 h except *C. occidentalis*, which were imbibed for 24 h. After imbibition, 15 seeds of each test species (30 for *A. viridis*) were placed in 15-cm diameter Petri dishes lined with two layers of Whatman No. 1 filter paper moistened with 8 mL of α-pinene solution or distilled water (control). The Petri dishes were then sealed with cellotape and Parafilm®. Each treatment was replicated five times. All Petri dishes were placed in a growth chamber maintained at 30/16 °C day/night temperature (± 2 °C) and 16/8 h light/dark, with a photon flux density of approximately 150 μmol photons m^{-2} s^{-1} and relative humidity of 78 ± 2 %. After a week, the number of seeds that germinated was counted and their root length was measured. The entire experiment was repeated. For further studies involving analysis and enzymatic assays, only the roots of *C. occidentalis* were chosen, as this species was affected the most in the growth studies. These were excised and frozen at –80 °C prior to enzyme extraction and other analysis.

Effect on membrane integrity

Loss of membrane integrity (an indicator of cellular damage) was studied in terms of ion (electrolyte) leakage from the roots of *C. occidentalis* by measuring conductivity of the bathing medium containing α-pinene, as per the method of Duke and Kenyon (1993). Root tissue (100 mg) collected from 10-d-old seedlings of *C. occidentalis* (grown under controlled conditions as described above) was dipped in 5 mL of 1 mM MES buffer (2-[N-morpholino]ethanesulfonic acid sodium salt, pH 6·5) containing 2 % sucrose (w/v) and α-pinene (1, 2·5, 5·0 and 10·0 mM) dissolved in Tween-80. A parallel control containing all the materials except α-pinene was also maintained. The conductivity of the bathing medium was measured with a conductivity meter (ECOSCAN CON5; Eutech Instruments Pte. Ltd., Singapore) at regular intervals in the dark (0, 1, 2, 4, 8, 12, 16, 18 and 20 h) followed by exposure to light for a further 10 h (measurements at 22, 24, 26, 28 and 30 h). The root samples were then boiled for 15 min in order to measure the maximum electrolyte leakage. For each treatment there were five replicates, and the experiment was repeated.

Determination of lipid peroxidation

Lipid peroxidation was measured in terms of malondialdehyde content (MDA) as per the method of Heath and Packer (1968). Roots (100 mg) were extracted with trichloroacetic acid (TCA, 0·1 %, w/v) and centrifuged at 10 000 *g* for 10 min. MDA level was used as an index of lipid peroxidation and was expressed as nmol g^{-1} fresh weight (f. wt). One mL of the supernatant was added to 4 mL of 0·5 % thiobarbituric acid (TBA, made in 20 % TCA). The mixture was incubated at 95 °C for 30 min followed by quick cooling over ice, and then centrifuged at 10 000 *g* for 10 min. The absorbance of the supernatant was

determined at 532 nm and corrected for non-specific absorbance at 600 nm. MDA amount was determined using the extinction coefficient of 155 $mM^{-1}cm^{-1}$ and expressed as nmol g^{-1} f. wt.

Hydrogen peroxide (H_2O_2) content

H_2O_2 content was determined using the method given by Velikova *et al.* (2000). Root tissue (100 mg) was extracted with 5 mL of 0·1 % TCA and centrifuged at 12000 *g* for 15 min. Then 0·5 mL of supernatant was mixed with 0·5 mL of 10 mM phosphate buffer (pH 7·0) and 1 mL of 1 M potassium iodide and the absorbance was determined at 390 nm. The amount of H_2O_2 read using the extinction coefficient 0·28 μM^{-1} cm^{-1} and expressed as nmol g^{-1} f. wt.

Determination of free proline content

Proline content was measured following the method described by Bates *et al.* (1973). Dried and powdered root tissue (100 mg) was digested in 3 mL of 3 % sulfosalicylic acid for 30 min at 100 °C followed by centrifugation at 2000 *g* for 5 min at 25 °C. To 0·2 mL of the extract was added 0·4 mL of distilled water and 2 mL of the reagent mixture (consisting of 30 mL glacial acetic acid, 20 mL distilled water and 0·5 g Ninhydrin). The samples were boiled for 1 h, cooled and extracted with 6 mL of toluene. The absorbance of the toluene phase was determined at 520 nm and proline content was calculated from a standard curve and expressed as mg g^{-1} f. wt.

Preparation of enzyme extract

Enzyme extract was prepared by homogenizing 200 mg of frozen root tissue (from each treatment or control) in 10 mL of sodium phosphate buffer (0·1 M, pH 7·0). The homogenate was filtered through a triple layer of cheesecloth and centrifuged at 15 000 *g* at 4 °C. The supernatant was collected, stored at 4 °C and used as the enzyme extract for analysis of superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6), ascorbate peroxidase (APX; EC 1.11.1.11), guaiacol peroxidase (GPX; EC 1.11.1.7) and glutathione reductase (GR; EC 1.6.4.2). Total protein content was determined using a 0·5-mL aliquot of the enzyme extract as described by Lowry *et al.* (1951) using bovine serum albumin as standard.

Activities of antioxidant enzymes

Superoxide dismutase (SOD) was assayed following the method of Beauchamp and Fridovich (1971) by measuring its ability to inhibit photochemical reduction of nitro blue tetrazolium chloride (NBT). The reaction mixture (4 mL) contained 63 μM NBT, 13 mM methionine, 0·1 mM EDTA (ethylene diamine tetraacetic acid), 13 μM riboflavin, 0·05 M sodium carbonate and 0·5 mL enzyme extract (0·5 mL distilled water in the case of the control). Test-tubes were kept under two 15 W fluorescent lamps for 20 min and then transferred to the dark for 20 min. The absorbance was determined at 560 nm and activity was expressed as enzyme units mg^{-1} protein. One unit of the enzyme activity was defined as the enzyme required for 50 % inhibition of the reduction of NBT in comparison with the tubes lacking the enzyme.

Catalase (CAT) activity was measured as per the method of Cakmak and Marschner (1992). The reaction mixture (2 mL) consisted of 25 mM phosphate buffer (pH 7·0), 10 mM H_2O_2 and 0·2 mL of enzyme extract. The activity was determined by measuring the rate of disappearance of H_2O_2 for 1 min at 240 nm, and calculated using an extinction coefficient of 39·4 mM^{-1} cm^{-1} and expressed as enzyme units g^{-1} f. wt. One enzyme unit was defined as the amount of enzyme required to oxidize 1 μM of H_2O_2 min^{-1}.

Glutathione reductase (GR) activity was determined spectrophotometrically by monitoring GSSG (glutathione oxidized)-dependent oxidation of nicotinamide adenine dinucleotide phosphate (NADPH) at 340 nm as per the method of Foyer and Halliwell (1976). The reaction mixture (2 mL) contained 25 mM phosphate buffer (pH 7·0), 0·1 mM EDTA, 0·5 mM GSSG, 0·12 mM NADPH and 0·2 mL enzyme extract. Absorbance at 340 nm was read immediately after addition of the enzyme extract at time zero and after 5 min. The enzyme activity was measured in terms of NADPH left unoxidized using an extinction coefficient 6·224 mM^{-1} cm^{-1}, and expressed as enzyme units g^{-1} f. wt. One enzyme unit was defined as the amount of enzyme required to oxidize 1μM of NADPH min^{-1}.

Ascorbate reductase (APX) was assayed as per the method of Nakano and Asada (1981). The reaction mixture (2 mL) consisted of 25 mM phosphate buffer (pH 7·0), 0·1 mM EDTA, 0·25 mM ascorbate, 1·0 mM H_2O_2 and 0·2 mL enzyme extract. The enzyme activity was determined using an extinction coefficient of 2·8 mM^{-1} cm^{-1} by measuring the decrease in absorbance at 290 nm for 1 min. It was expressed as enzyme units g^{-1} f. wt. One enzyme unit was defined as the amount of enzyme required to oxidize 1 μM of ascorbate min^{-1}.

Guaiacol peroxidase (GPX) activity was measured using the method of Egley *et al.* (1983). The reaction mixture (2 mL) consisted of 25 mM phosphate buffer (pH 7·0), 0·05 % guaiacol, 1·0 mM H_2O_2, 0·1 mM EDTA and 0·2 mL of the enzyme extract. Increase in absorbance was measured at 470 nm due to oxidation of guaiacol. The enzyme activity was calculated using an extinction coefficient of 26·6 mM^{-1} cm^{-1} and expressed as enzyme units g^{-1} f. wt. One enzyme unit was the amount of enzyme that catalyses oxidation of 1μM guaiacol min^{-1}.

Statistical analysis

For each treatment five replicates were maintained in a completely randomized manner and repeated. Five tissue sample replicates were used for enzymatic assay and all other analyses. Data is presented as mean ± s.e. and was analysed by one-way analysis of variation followed by separation of treatment means from the control at $P < 0·01$ and 0·05 by *post hoc* application of Dunnett's test.

TABLE 1. *Effect of α-pinene on the germination percentage of test species measured after 7 d*

Concentration (mM)	Plant species				
	C. occidentalis	*A. viridis*	*P. sativum*	*C. arietinum*	*T. aestivum*
0	100 ± 0	95·5 ± 3·85	100	100	100
1	100 ± 0^{ns}	93·3 ± 0^{ns}	100^{ns}	100^{ns}	100^{ns}
2·5	77·8 ± 4·87**	91·1 ± 3·85^{ns}	100^{ns}	100^{ns}	100^{ns}
5	55·6 ± 12·73**	86·7 ± 0**	100^{ns}	100^{ns}	0**
10	50·0 ± 8·33**	84·5 ± 3·85**	100^{ns}	100^{ns}	0**

Values are means ± s.e. (n =5).
*, ** indicate significant difference from controls at $P < 0·05$ and $P < 0·01$, respectively, after applying Dunnett's test; ns: non-significant.

TABLE 2. *Effect of α-pinene on radicle growth (cm) of test species measured after 7 d*

Concentration (mM)	Plant species				
	C. occidentalis	*A. viridis*	*P. sativum*	*C. arietinum*	*T. aestivum*
0	3·77 ± 0·28	4·68 ± 0·35	5·83 ± 0·09	10·46 ± 0·05	12·42 ± 0·22
1	3·55 ± 0·02^{ns}	4·03 ± 0·19*	5·23 ± 0·35^{ns}	9·29 ± 0·26**	10·31 ± 0·75**
2·5	2·74 ± 0·04**	3·12 ± 0·02**	3·78 ± 0·17**	8·20 ± 0·23**	5·52 ± 0·39**
5	0·61 ± 0·05**	2·37 ± 0·44**	1·98 ± 0·14**	4·37 ± 0·29**	0**
10	0·41 ± 0·12**	1·26 ± 0·10**	1·29 ± 0·07**	3·40 ± 0·35**	0**

Values are means ± s.e. (n = 5).
*, ** indicate significant difference from controls at $P < 0·05$ and $P < 0·01$, respectively, after applying Dunnett's test; ns: non-significant.

RESULTS AND DISCUSSION

The results indicate that α-pinene inhibited seed germination of *C. occidentalis*, *A. viridis* and *T. aestivum*, and the effect was species- as well as concentration-dependent. However, there was no effect on seed germination of *P. sativum* and *C. arietinum* (Table 1). At higher concentrations of α-pinene (5 or 10 mM), the germination of *T. aestivum* was completely suppressed (Table 1), whilst the germination of *A. viridis* and *C. occidentalis* was significantly reduced ($P < 0·01$). Regardless of the effect on seed germination, α-pinene inhibited radicle elongation in all the plant species tested (Table 2). The inhibitory effect on radicle length was statistically significant ($P < 0·01$), except in *C. occidentalis* and *P. sativum* where the reduction was insignificant at 1·0 mM α-pinene concentration. At 5·0 mM concentration, radicle growth was reduced in the range of 49–66 % in *A. viridis*, *C. arietinum* and *P. sativum*, whereas in *C. occidentalis* nearly 89 % reduction was observed (Table 2). Reduction in germination and radicle elongation in maize caused by α-pinene has been reported previously by Abrahim *et al.* (2000) and Scrivanti *et al.* (2003). Several studies have shown that volatile monoterpenes are potent inhibitors of seed germination and root elongation. For example, Vaughn and Spencer (1993) reported that several monoterpenes, particularly oxygenated ones, when used as a headspace volatile (in the concentration range 20–350 μM) decreased germination of four weed species (*Abutilon theophrasti*, *Lolium multiflorum*, *Amaranthus retroflexus* and *Digitaria sanguinalis*). Romagni *et al.* (2000) observed that cineoles (both 1,4- and 1,8-) at concentrations ranging from 10–1000 μg g^{-1} in sand inhibited germination and radicle elongation of barnyard grass (*Echnichloa crus-galli*) and sicklepod (*Cassia obtusifolia*). Singh *et al.* (2006) reported that citronellal at concentrations ranging from 5–100 μg g^{-1} in sand severely affected the germination and early growth of six weeds, namely billy goat weed (*Ageratum conyzoides*), common lambsquarters (*Chenopodium album*), ragweed parthenium (*Parthenium hysterophorus*), prickly malvastrum (*Malvastrum coromandelianum*), coffee weed (*Cassia occidentalis*) and littleseed canarygrass (*Phalaris minor*). The concentrations of monoterpenes used by these workers are similar to the range observed under natural environmental conditions (see below). Although the exact mechanism for the observed inhibitory activity of terpenes remains largely unknown, some studies have shown that volatile terpenes, namely 1,8-cineole (at 25 μg g^{-1} in sand; Romagni *et al.*, 2000) and citronellal (at 2500 μM, ≈ 25 μg g^{-1} in sand; Singh *et al.*, 2006) inhibit mitotic activity in growing root tips of onion. α-Pinene (at 1000 μM concentration) inhibits mitotic activity in root tip cells of onion (H. P. Singh *et al.*, unpubl. data). Nishida *et al.* (2005) have reported that volatile monoterpenes such as α- and β-pinene, eucalyptol and camphor in their purified form inhibited root growth of *Brassica campestris* by inhibiting cell proliferation in root apical meristems, and decreased the mitotic index at concentrations ranging from 200–1250 μM.

Exposure to α-pinene caused significant excessive ion leakage from *C. occidentalis* roots, as measured by increased conductivity of the bathing medium (MES buffer). This indicates that α-pinene disrupts membrane

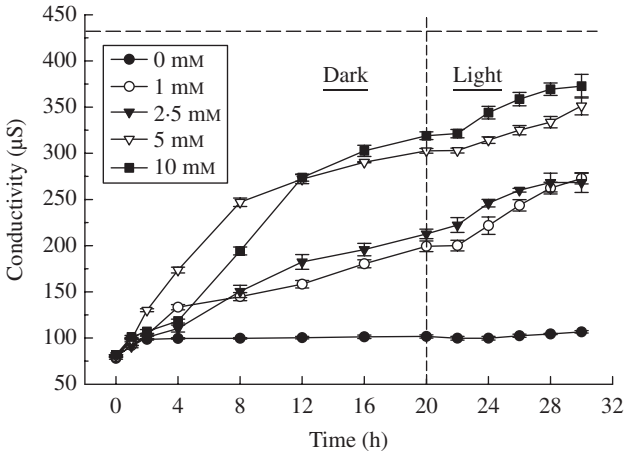

FIG. 1. Effect of α-pinene on electrolyte leakage (measured as conductivity) of *C. occidentalis* roots determined one week after treatment. The dashed horizontal line indicates maximum ion leakage (432·05 μS) achieved after boiling samples; the vertical line at 20 h indicates the point of transition from dark to light conditions. Bars indicate s.e. ($n = 5$).

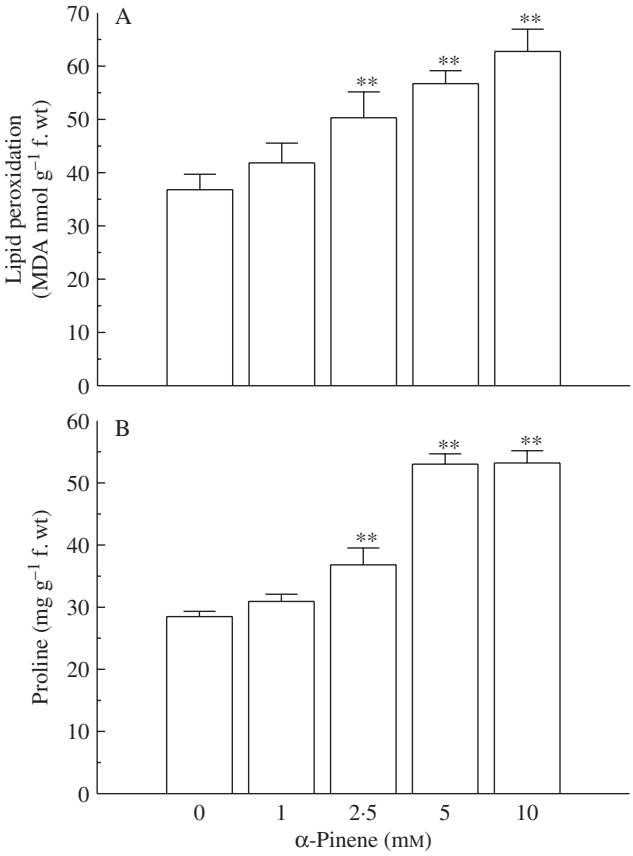

FIG. 2. Effect of α-pinene on (A) lipid peroxidation and (B) proline accumulation in *C. occidentalis* roots ($n = 5$) determined a week after treatment. Bars indicate s.e.; ** indicates significance from control at $P < 0·01$ applying Dunnett's test.

permeability resulting in solute leakage, and the effect was concentration dependent (Fig. 1). Solute leakage increased steadily with time up to 20 h in the dark and then for another 10 h in the light. After 30 h, the conductivity of the bathing medium containing 5·0 or 10 mM α-pinene was 80–86 % of the maximum (approx. 432·05 μS) as obtained upon boiling the tissue (Fig. 1). The ion leakage was irrespective of light or dark conditions and such a response indicates occurrence of enhanced respiration and oxidative stress (Dayan *et al.*, 2000). There was, however, not much change in electrolyte leakage in either the 1·0 or 2·5 mM α-pinene treatment (Fig. 1). Increased conductivity levels indicate that α-pinene caused stress resulting in disruption of membrane integrity. Membrane disruption by monoterpenoids, as one of the mechanisms for their fungicidal and bactericidal activity resulting in cell death, has previously been suggested (Harrewijn *et al.*, 2001). A decrease in membrane permeability could be due to peroxidation of polyunsaturated fatty acids in the biomembranes resulting in the formation of several byproducts, including malondialdehyde (MDA; Kappus, 1985; Maness *et al.*, 1999). In order to explore this, the amount of MDA as an indicator of lipid peroxidation was measured (see below). Enhanced lipid peroxidation and electrolyte leakage resulting in loss of membrane integrity are among the key factors that determine cellular injury.

In general, various types of environmental stresses (including abiotic, xenobiotic and herbicidal) mediate their impact through oxidative stress caused by generation of reactive oxygen species, ROS (Smirnoff, 1995; 1998; Blokhina *et al.*, 2003). ROS, such as singlet oxygen ($^1O_2$), superoxide radicles (O_2^-), hydroxyl radicles ($OH^·$) and hydrogen peroxide (H_2O_2), are highly reactive and toxic molecules that can cause oxidative damage to membranes, DNA, proteins, photosynthetic pigments and lipids (Apel and Hirt, 2004). Recently, ROS generation and related oxidative stress has been proposed as one of the modes of action of plant growth inhibition by allelochemicals (Weir *et al.*, 2004). However, very little is known about the action of allelochemicals/phytotoxins in inducing ROS-mediated oxidative damage. Bais *et al.* (2003) reported that (–)-catechin, a putative phytotoxin, inhibits plant growth due to a severe oxidative burst in root tips, resulting in cell death. To explore whether α-pinene induces a similar response, various non-enzymatic indicators (such as membrane leakage, lipid peroxidation, proline content and hydrogen peroxide) and enzymatic mechanisms linked with oxidative stress were assessed in roots of *C. occidentalis*.

Levels of MDA (the main thiobarbituric acid-reactive species, or TBARS) increased in roots of *C. occidentalis* upon exposure to α-pinene. An increase in the levels of TBARS is an indicator of lipid peroxidation and membrane damage. The increase was significant ($P < 0·01$) at 2·5 mM or higher concentrations (Fig. 2A). Heath and Packer (1968) observed that environmental stress caused enhanced MDA levels in the target tissue due to ROS generation and resulted in lipid peroxidation. The results in the current study match previous work that has shown that monoterpenes, including α-pinene, enhance lipid peroxidation (Scrivanti *et al.*, 2003; Zunino and Zygadlo, 2004). Increased lipid peroxidation therefore indicates that

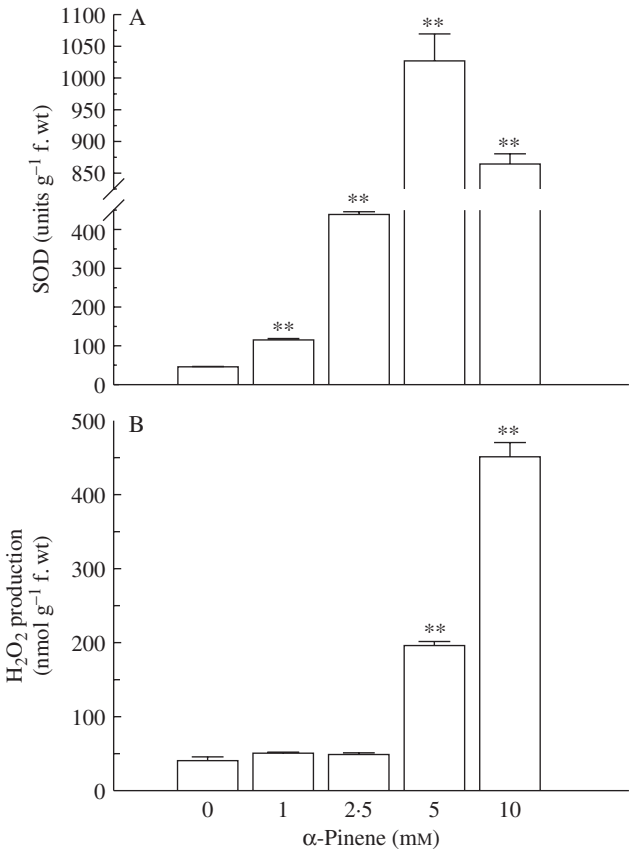

FIG. 3. Effect of α-pinene on (A) activity of superoxide dismutase (SOD) and (B) hydrogen peroxide production in roots of *C. occidentalis* determined one week after treatment. Bars indicate s.e. (n = 5); ** indicates significant difference from the control at $P < 0{\cdot}01$, after applying Dunnett's test.

α-pinene exposure results in oxidative stress due to generation of ROS species and causes a loss of cell integrity. Under a variety of abiotic stresses, plants accumulate higher levels of proline (Upadhyaya *et al.*, 1989) and this indicates induction of oxidative damage. Treatment with α-pinene significantly ($P < 0{\cdot}01$, except at 1 mM) increased the amount of endogenous proline in root tissue of *C. occidentalis* (Fig. 2B). Proline content increased by nearly 1·3-fold at 2·5 mM α-pinene concentration compared with the control, whereas at 5·0 mM concentration the increase was nearly 1·9-fold (Fig. 2B). Proline acts as an electron acceptor and prevents damage to membranes (Ain-Lhout *et al.*, 2001). It also provides protection against ROS-induced disruption of photosystems (Hare *et al.*, 1998).

To avoid the cellular damage due to ROS generation, plants produce a number of antioxidant enzymes that are induced and provide secondary protection against oxidative stress (Mittler, 2002; Apel and Hirt, 2004; Mittler *et al.*, 2004). Increased ROS generation due to α-pinene is also indicated by enhanced activities of scavenging enzymes such as SOD, CAT, APX and GPX. Activity of SOD in *C. occidentalis* roots increased significantly in response to α-pinene compared with controls ($P < 0{\cdot}01$; Fig. 3A). The increase was concentration-dependent and ranged from nearly 2·5-fold over controls at 1·0 mM to a nearly 23-fold increase at 5 mM α-pinene. However, there was a decrease in SOD activity at 10 mM α-pinene compared with the 5 mM concentration (Fig. 3A). SOD is the major scavenger of superoxide (O_2^-) to form H_2O_2 and O_2, and plays an important role in defence activity against the cellular damage caused by environmental stress (Meloni *et al.*, 2003). Increased levels of SOD activity indicate an induction of oxidative stress caused by excessive generation of O_2^-, presumably resulting from α-pinene exposure. An increase in SOD activity in response to phenolic allelochemicals contained in root exudates of cucumber (*Cucumis sativus*) has also been reported (Yu *et al.*, 2003).

The content of H_2O_2 also increased in response to α-pinene treatment compared with the control, and the increase was significant ($P < 0{\cdot}01$) at 5 mM or higher concentration (Fig 3B). At 5·0 mM concentration, there was a nearly 4·5-fold increase in H_2O_2 amount, whereas the increase was nearly 11-fold at 10 mM concentration. Increased levels of H_2O_2 further enhance lipid peroxidation and oxidative stress levels in the target tissues. In the chloroplasts, H_2O_2 interferes with the activities of SH-group-containing enzymes such as fructose-1,6-biphosphatase and inhibits photosynthetic activity (Takeda *et al.*, 1995). Among different ROS produced in response to environmental stresses, H_2O_2 acts as a major signalling molecule and serves as an effective mode of defence (Foyer *et al.*, 1997). H_2O_2 is further reduced to H_2O by CAT in the peroxisomes, by APX in the chloroplasts and cytosol, and by GPX in the cell wall (Blokhina *et al.*, 2003), and APX is the most important peroxidase detoxifying H_2O_2 to water (Noctor and Foyer, 1998).

Activity of CAT increased steadily with α-pinene concentration and it nearly doubled in response to 1 mM α-pinene (Fig. 4). At 5·0 mM α-pinene concentration, the increase was nearly 2·5-fold and it further increased to nearly 3·2-fold at 10 mM. Likewise, APX activity also increased significantly in response to α-pinene exposure: it showed an increase of nearly 25 % (significant at $P < 0{\cdot}05$) over the control at 1 and 2·5 mM α-pinene. In response to 5·0 and 10·0 mM α-pinene, it increased by nearly 2·5 and 3·3-fold (significant at $P < 0{\cdot}01$; Fig. 4). In contrast, there was not much increase in the activity of GPX at the lower concentrations (1 and 2·5 mM) compared with the control. However, at higher concentrations GPX activity showed a significant ($P < 0{\cdot}01$) increase that was nearly double at 5·0 mM α-pinene compared with the control.

A similar trend was observed with the GR assay as with SOD, APX, GPX and CAT. GR, a flavoenzyme in the ascorbate–glutathione cycle, uses NADPH as an electron donor and converts oxidized glutathione (GSSG) to the reduced form, GSH (Noctor and Foyer, 1998) and provides protection against oxidative damage (Aono *et al.*, 1995). Activity of GR increased steadily upon exposure to α-pinene and this increase was statistically significant at $P < 0{\cdot}01$, except at 1 mM concentration. Activity increased by nearly 2·4 times at 2·5 mM α-pinene compared with the control, whereas at 5 mM α-pinene exposure the increase was nearly 13-fold over the control (Fig. 4). Increased

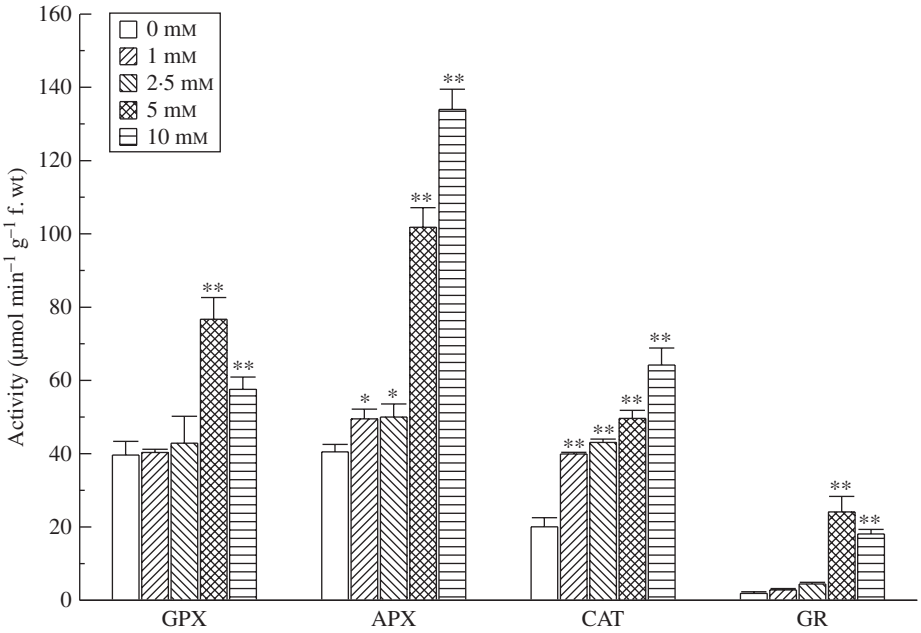

FIG. 4. Effect of α-pinene on activities of GPX, APX, CAT and GR in roots of *C. occidentalis* estimated one week after treatment. Bars indicate s.e. ($n = 5$); *, ** indicate significant difference from the control at $P < 0{\cdot}05$ and $P < 0{\cdot}01$, respectively, after applying Dunnett's test.

activity of GR indicates that α-pinene induces oxidative stress since GR is involved in providing protection from oxidative damage against abiotic stresses (Aono *et al.*, 1995).

A number of reports indicate that oxidative stress induces an increase in the responses of enzymatic systems linked to ROS-scavenging process (Apel and Hirt, 2004; Jones and Smirnoff, 2005). The results obtained in the present study showed that among the antioxidant enzymes assayed, activities of CAT, GPX and APX showed an increase in the range of 2- to 3·2-fold upon α-pinene exposure compared with controls. The activity of GR increased by nearly 13-fold over the control. APX and GR are the main components of the ascorbate–glutathione cycle that provides one of the main defences against oxidative damage in plants (Smirnoff, 1996; Becana *et al.*, 2000). In fact, GR is a key enzyme in providing protection against a variety of environmental and abiotic stresses (Aono *et al.*, 1995; Dalton, 1995; Romero-Puertas *et al.*, 2006). Activity of GR and hence GSH production is generally elevated in plants upon exposure to xenobiotics and various environmental stresses (Foyer *et al.*, 1991). GSH is an essential thiol-containing molecule providing defence against oxidative damage induced by abiotic stresses. It is used by glutathione-*S*-transferases to detoxify xenobiotics (Edwards *et al.*, 2000).

The results obtained in the present study indicate that α-pinene inhibits root growth and induces oxidative stress. However, under natural field conditions the effect depends upon its availability in the soil. Although a lot of information is available regarding foliar release of volatile monoterpenes into the atmosphere from forests and vegetation, information about their release from the roots and underground plant parts is largely unknown. Roots synthesize and accumulate a variety of volatile monoterpenes and sesquiterpenes that enter the rhizosphere soil upon release (Wichtmann and Stahl-Biskup, 1987; Ji *et al.*, 1993; Kovacevic *et al.*, 2002). Monoterpenes enter into the soil medium by several mechanisms, such as by leaching from litter (Angelini *et al.*, 2003) or foliar parts (Weidenhamer *et al.*, 1994), by root exudation (Janson, 1993; Napierala-Filipiak *et al.*, 2002), by rhizo-deposition of volatilized monoterpenes (Muller, 1970), and also by direct release. Among these, leaching from litter is the main source of monoterpenes in the soil (Wood, 1996). Many plant roots also release volatile monoterpenes into the soil; for example, roots of ponderosa pine (*Pinus ponderosa*) release monoterpenes including α-pinene (Latta *et al.*, 2000), 1,8-cineole is released from roots of *Arabidopsis thaliana* (Steeghs *et al.*, 2004) and sesquiterpene (*E*)-β-caryophyllene is released from roots of corn (Rasmann *et al.*, 2005). However, little is known about the amount and persistence of these monoterpenes in the soil.

The concentrations of monoterpenes used in the present study (1–10 mM or 0·136–1·36 mg mL^{-1}) are ecologically relevant in view of the reported levels of monoterpenes under natural conditions. For example, Paavolainen *et al.* (1998) observed that the concentration of monoterpenes was very high in the soil micro-air (1·00–2·06 mg g^{-1} soil) of *Picea abies* forests and α-pinene accounted for nearly 38 % of it. These workers ascribed these high levels to their release from plant roots. The concentration of monoterpenes is much higher in the senescent litter layer (White, 1994), which may contain concentrations in the range of nearly 3–5 mg g^{-1} (Wilt *et al.*, 1993; White, 1994); these are released through leaching and decomposition into the soil system. Forest litter is the main source of release of monoterpenes into the soil in such forests and, as a result,

the superficial layers of forest soil have a very high concentration of these compounds (White, 1994).

In conclusion, the present study shows that α-pinene (at ecologically relevant concentrations) inhibited the germination and radicle growth of the test species examined. Exposure to α-pinene induced oxidative stress through the enhanced generation of ROS, which was accompanied by membrane damage, enhanced lipid peroxidation levels, proline accumulation and by activation of antioxidant enzyme systems. Increased levels of scavenging enzymes indicate their induction as a secondary defence mechanism in response to α-pinene. However, the genetic mechanisms that are involved bringing about such responses to α-pinene remain unknown.

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