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Comparative phytotoxicity of four monoterpenes against *Cassia occidentalis*

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Summary

The effect of four monoterpenes – citronellol, citronellal, cineole and linalool – on the germination, growth and physiology of *Cassia occidentalis* was investigated. All four monoterpenes reduced germination of *C. occidentalis* seeds but to varying extents. Citronellal and linalool completely inhibited germination beyond the concentrations of 55 and 110 μM, respectively, whereas in response to treatment of citronellol no germination was observed beyond 330 μM. Further, the growth of seedlings, measured in terms of seedling length and biomass, was also adversely affected. A reduction in chlorophyll content of the cotyledonary leaves of *C. occidentalis* was also noticed, indicating an adverse effect on photosynthesis. Likewise, respiratory ability of growing seeds was also impaired in response to all four monoterpenes, clearly indicating that monoterpenes affect energy metabolism. On the basis of overall phytotoxicity, potency of monoterpenes was in the order of citronellal > linalool > citronellol > cineole. The results from this study suggest that both citronellal and linalool possess strong phytotoxic potential and can thus serve as lead molecules for the synthesis of bioherbicides.

Key words: Cineole, citronellol, citronellal, linalool, chlorophyll content, cell respiration, structure-activity relationship, bioherbicides

Introduction

One of the recent trends in weed management is to reduce heavy reliance on synthetic herbicides and to move towards low-input sustainable agriculture (LISA) as a part of integrated weed management (Kupatt *et al*., 1993). This is due to increasing awareness of the adverse toxicological effects of synthetic herbicides on environment quality, public health, wildlife and overall ecology. Additionally, resistance of weeds to synthetic herbicides has further renewed interest in LISA. As a result these chemicals have come under tight scrutiny and in many developed countries like the USA their environmental and toxicological impacts are being reassessed (Dayan *et al.,* 1999). Natural products, particularly allelochemicals, are receiving greater attention (Duke & Abbas, 1995; Duke *et al.*, 1998), although they occupy a small niche in the global agricultural market. Advancement in technologies regarding identification, purification, and evaluation of biological activity of natural plant products has simplified protocols for using them (Duke *et al.*, 2000). Even biotechnological tools such as the use of genomics and proteomics are being used to improve the herbicidal properties of natural products/ allelochemicals (Birkett *et al*., 2001). However, before undertaking any further research in this direction, it is essential to determine the physiological and biochemical effects of natural plant products on target weed species (Dayan *et al*., 2000).

Plants are a virtually inexhaustible source of biologically active compounds with great structural diversity. Among several classes of natural plant products volatile monoterpenes have received much attention as strong inhibitors of plant growth, particularly in allelopathic studies (Muller *et al.*, 1964; del Moral & Muller, 1970; Al-Saadawi *et al.*, 1985; Fischer, 1991; Singh *et al*., 1991; Kohli *et al.*, 1998; Kong *et al*., 1999; Vokou, 1999). Monoterpenes are the simplest representatives of the terpenes – the largest group of secondary metabolites (Elakovich, 1988; Abrahim *et al*., 2000). They are constituents of a number of aromatic plants such as *Artemisia* spp. (Ahmad & Misra, 1994), *Cunila spicata* (Manns, 1995), *Cymbopogon citratus, Micromeria fructicosa,* and *Origanum syriacum* (Dudai *et al*., 1999), *Eucalyptus* sp. (Kohli, 1990; Orihara & Furuya, 1994), *Lavandula angustifolia, Rosmarinus officinalis, Salvia fruticosa* and *Origanum vulgare* (Karamanoli *et al.*, 2000), and *Salvia* sp. (Muller, 1965). Besides growth inhibitors, they also play an important role as plant protectants and pollinator attractants (Swain, 1977; Fischer *et al.*, 1994; Paiva, 2000). Of late, their phytotoxic nature towards weed species is being explored so as to use them for weed management purposes (Romagni *et al.*, 2000*a*; Singh *et al*., 2002). Desirable attributes are short-lived persistence in soil due to

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high vapour pressure, almost no leaching to ground water, and low mammalian toxicity (Isman, 2000). Moreover, they often have modes of action different from synthetic herbicides and may serve as prototypes for the synthesis of lead molecules for future herbicides (Dayan *et al*., 1999). However, there is a considerable variation in phytotoxicity of various monoterpenes towards test species, and even closely related compounds, such as 1,4- and 1,8-cineole, have different types of activity (Romagni *et al.*, 2000*a*). Thus, studies on the relative phytotoxicity of monoterpenes should be undertaken to choose the best lead compounds. Keeping this in mind, we selected four monoterpenes *viz.* cineole, linalool, citronellol, and citronellal to find out their comparative phytotoxicity towards *Cassia occidentalis* L. – a weedy species. The objective of the study was to find out their I_{50} concentrations and to select the monoterpenes with greater phytotoxic activity for further studies on herbicidal properties.

Materials and Methods

Chemicals and biological material

Uniform, healthy seeds of coffeeweed (*Cassia occidentalis* L.) were collected locally from wild stands on the campus of Panjab University, Chandigarh India. Technical grade citronellol, citronellal, linalool, and 1,4-cineole were obtained from Sigma Co., St. Louis, USA, Fluka Chemie GmBH, Switzerland, and Lancaster, UK.

Dose-response study

Effects of different concentrations of all the four monoterpenes were studied on the germination of *C. occidentalis* in a laboratory bioassay. Seeds were divided into 40 groups (10 for each monoterpene) of 20 seeds each and placed in distilled water for 16 h to allow imbibition prior to germination trials. Twenty seeds each were then equidistantly placed in 15 cm diameter Petri dish (approximate area 500 cm^2) lined with two layers of moistened Whatman no. 1 filter paper. The filter paper was treated with different monoterpenes so as to have concentrations of 2.75, 5.5, 11, 22, 55, 110, 220, 330, 440 and 550 µM. After the addition of monoterpenes, Petri dishes were sealed with a Parafilm M® (American National Can™, Neenah, WI.). Treatment in a similar manner with distilled water instead of monoterpenes served as control. For each monoterpene concentration there were five replications. Petri dishes were placed in a growth chamber at 25 ± 2°C temperature, a 16/8 h light/dark photoperiod, photon flux density of approximately 150 µmol m^{-2} s^{-1} and relative humidity of around 75%. After 8 days, the number of seeds that had germinated was counted. Based on the growth experiments, I_{50} concentrations (the concentration at which 50% inhibition occurs) were determined. The whole experiment was performed on two occasions.

Growth experiments

In another set of experiments, the effect of eight concentrations (13.75, 27.5, 55, 110, 220, 330, 440 and 550 µM) of the four test monoterpenes was studied on the early growth of *C. occidentalis*. Fifteen seeds of *C. occidentalis* were allowed to germinate and grow in a 15 cm diameter Petri dish lined with a Whatman no. 1 filter paper treated with different concentrations of respective monoterpene as described in dose-response studies. For each treatment there were five replicates. Treatment with distilled water in a similar manner served as control. The entire set-up was kept in a growth chamber at 25 ± 2°C temperature, 73 ± 2% relative humidity, 16/8 h light/dark photoperiod and photon flux density of approximately 150 µmol m^{-2} s^{-1}. After 10 days, seedling length (from tip of root to tip of shoot), and seedling dry weight were measured. The experiment was repeated.

Estimation of chlorophyll content

Chlorophyll was extracted from 25 mg of cotyledonary leaves of *C. occidentalis* (treated with different concentrations of monoterpenes) in 4 ml of dimethyl sulphoxide following the method of Hiscox & Israelstam (1979). Chlorophyll concentration was determined spectrophotometrically using the equation of Arnon (1949) and expressed in terms of dry weight of the tissue as suggested by Rani & Kohli (1991).

Determination of cell respiration

Respiratory values were determined from the fresh plant tissue indirectly using 2,3,5-triphenyl tetrazolium chloride according to the method of Steponkus & Lanphear (1967). This is an indirect measurement of the cell respiration whereby formation of red formazan traps the oxygen molecules released through the respiratory chain. The values of treated samples were expressed as percent respiration with respect to control.

Statistical analysis

All the experiments were performed in a randomised block design with at least five replications and the entire experiment was repeated. The dose-response experiment for germination studies has been presented on a log scale (conc.) to make the data more linear. Likewise, values of seedling length and weight, chlorophyll content and cell respiration have been analysed on log concentration scale *vs* response and subjected to linear regression. All the statistical analysis was performed using SPSS/PC software version 10.0.

Results and Discussion

From the results it is clear that different concentrations of all four monoterpenes (citronellol, citronellal, cineole and linalool) reduce both germination and growth of *Cassia occidentalis*. The I_{50} (concentration of monoterpenes required for 50% inhibition of seed germination) values of citronellol, citronellal, cineole and linalool were calculated to be 259.8, 50.10, 341.84 and 56.54 μM, respectively (Fig. 1). The determination of I_{50} for each monoterpene is important for determination of the concentration needed to do more definitive studies (Dayan *et al.*, 2000). In the case of citronellal, no seed could germinate beyond 55 μM, whereas for linalool such a response was observed beyond 110 μM. Not only the germination, but seedling growth, in terms of seedling length and biomass, was also adversely affected (Figs 2 and 3). At 330 μM, citronellol reduced seedling length by over 95%, whereas in response to treatment with linalool and citronellal it was inhibited by nearly 75% and 65%, respectively, at 55 μM (Fig. 2). On the other hand, in the treatment with cineole, no complete inhibition of germination was observed, even at the highest concentration of 550 μM, although seedling length at this concentration was too small (< 2 mm) to be measured (Fig. 2). However, at 440 μM of cineole, the seedling length was reduced by more than 95%. Likewise, the dry weight of *C. occidentalis* was drastically reduced in response to all the treatment concentrations of the four test monoterpenes (Fig. 3). Here, nearly 44% reduction was observed in response to 110 μM of linalool, whereas an almost similar reduction was observed at the 440 μM of cineole.

Although, the exact mechanism of growth inhibition could not be determined from the present experiments, it could be associated with the inhibition of mitosis. Vaughn & Spencer (1993), Baum *et al.* (1998) and Romagni *et al.* (2000*a*) have reported that cineoles are inhibitors of mitosis. Romagni *et al.* (2000*a*) observed that most of the cells of onion treated with 1,8-cineole remained in interphase with only a few cells in any other phase of division. Thus, disruption of mitotic activity may be responsible for observed inhibition/reduction of germination and growth of *C. occidentalis* in the present study. Romagni *et al.* (2000*b*) have also reported that 1,4-cineole is a potent inhibitor of the enzyme asparagine synthetase, which plays an important role in nitrogen mobilisation.

Treatment with the monoterpenes also caused a reduction in chlorophyll content of *C. occidentalis* (Fig. 4). At 55 μM of linalool, the chlorophyll content was reduced by over 80%, whereas at similar concentrations of citronellal, citronellol, and cineole, it was reduced by nearly 50%, 76%, and 39% (Fig.

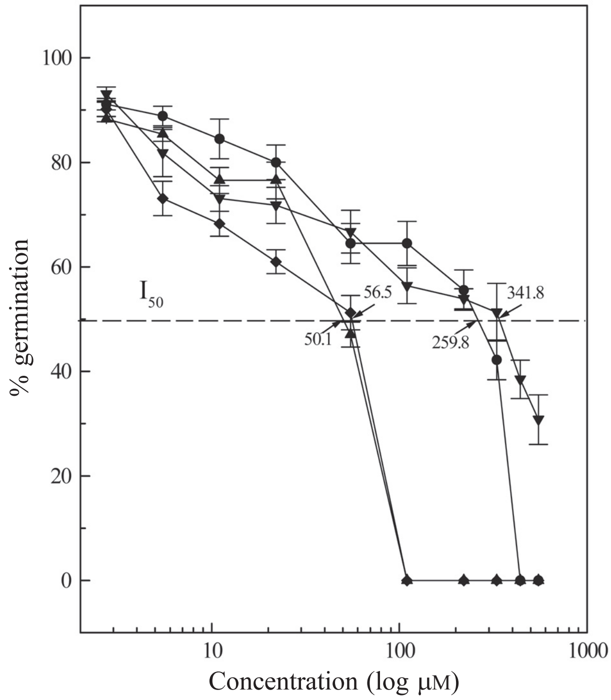

Fig. 1. Dose-response curve between different concentrations of monoterpenes and percent germination of *C. occidentalis*. Bars represent ± SD. Horizontal line indicates I_{50} concentration. Values along arrows indicate I_{50}. r = correlation coefficient. ●, Citronellol; ▲, Citronellal; ▼, Cineole; ◆, Linalool.

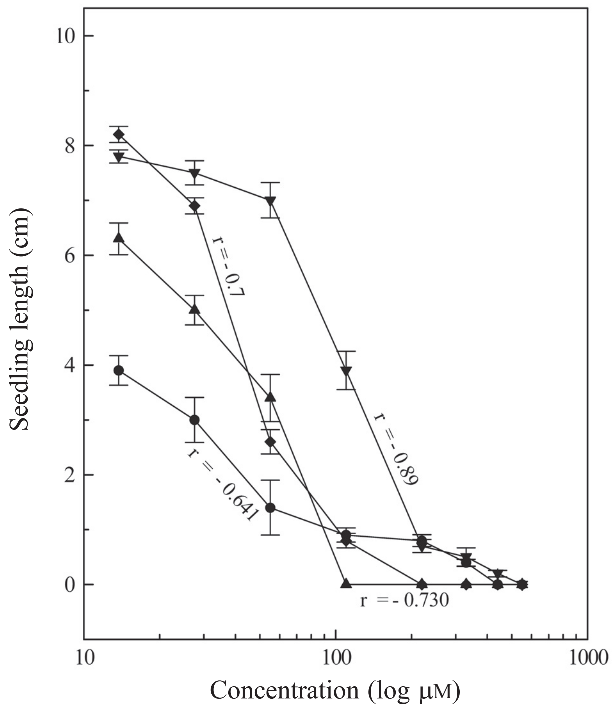

Fig. 2. Effect of monoterpenes on the seedling growth (cm) of *C. occidentalis*. Bars represent ± SD. ●, Citronellol; ▲, Citronellal; ▼, Cineole; ◆, Linalool.

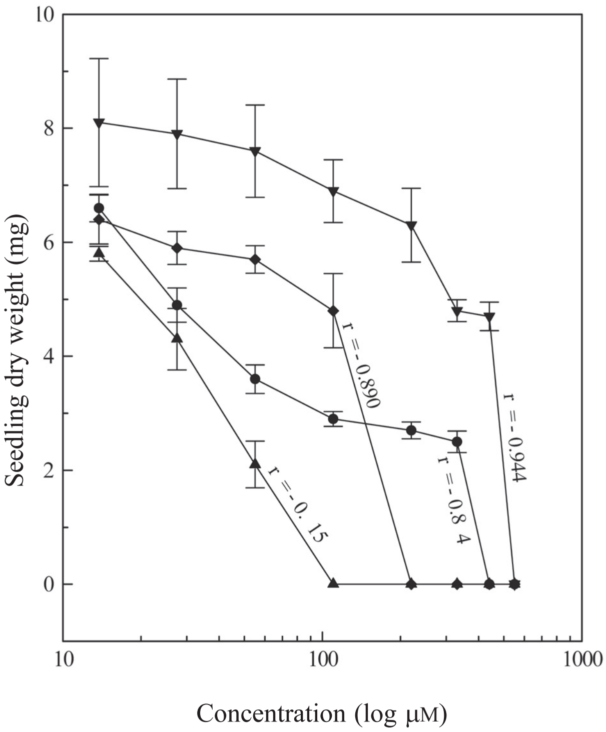

Fig. 3. Effect of monoterpenes on the dry weight (mg) of 1-wk old seedling of *C. occidentalis*. Bars represent ± SD. r = correlation coefficient. ●, Citronellol; ▲, Citronellal; ▼, Cineole; ◆, Linalool.

4). However, there is no evidence of direct inhibition of chlorophyll synthesis by the monoterpenes in the present study. Nevertheless, the loss of chlorophyll affects photosynthesis. Singh *et al*. (2002) and Romagni *et al.* (2000*a*) have also reported that both 1,4-cineole and 1,8-cineole exert physiological stress leading to decreased photosynthetic efficiency in target plants. In the present study, all the four monoterpenes also significantly affected cell respiration as measured by 2,3,5-triphenyl tetrazolium chloride (TTC) and thus caused changes in cellular energy production (Fig. 5). Peñuelas *et al*. (1996) observed that interference of monoterpenes with mitochondrial respiration accounts for their inhibitory effects on germination and growth. Abrahim *et al*. (2000) reported that the monoterpenes α-pinene, limonene, eucalyptol and camphor affected respiration by acting as uncouplers of oxidative phosphorylation due to their high degree of lipophilicity. In the present study too, the inhibitory effect of monoterpenes on the respiratory ability of the *C. occidentalis* seedlings could adversely affect production of cellular energy. This may in turn have serious effects on other metabolic and physiological processes associated with the plant growth such as synthesis of macromolecules and water permeability (Kramer, 1983). However, the intensity of the adverse effect depends upon the

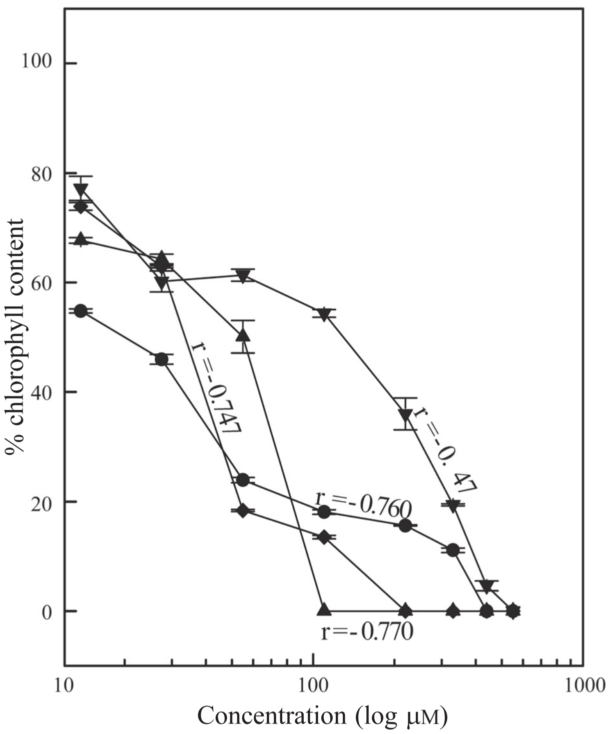

Fig. 4. Effect of different concentrations of monoterpenes on % chlorophyll content of *C. occidentalis*. Bars represent ± SD. r = correlation coefficient. ●, Citronellol; ▲, Citronellal; ▼, Cineole; ◆, Linalool.

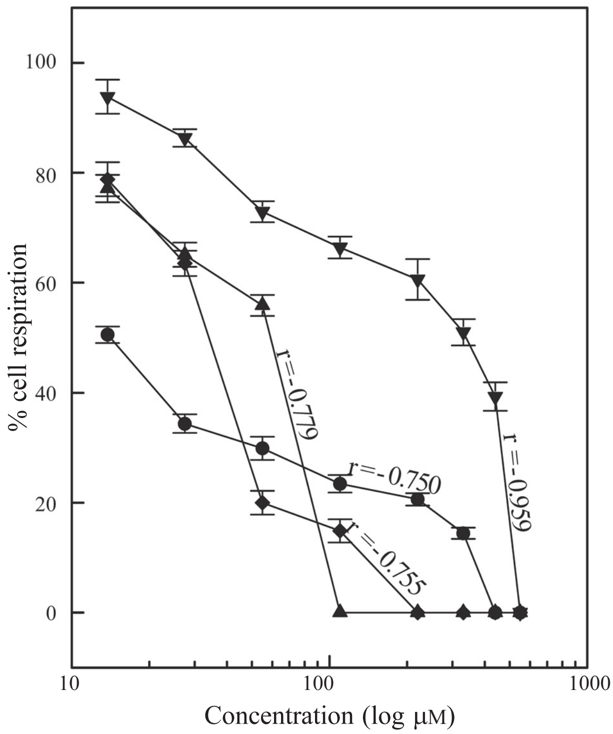

Fig. 5. Percent Respiratory activity of *C. occidentalis* in response to different concentrations of monoterpenes. Bars represent ± SD. r = correlation coefficient. ●, Citronellol; ▲, Citronellal; ▼, Cineole; ◆, Linalool.

relative uptake of the monoterpenes by the growing seedlings

Thus, from the present study, it is clear that volatile monoterpenes exert an overall inhibitory effect on the germination, growth and physiology of *C. occidentalis.* The relative overall potencies of the monoterpenes tested in our study is citronellal > linalool > citronellol > cineole. Cineole surprisingly was least effective, probably due to its limited effects on dicots (Romagni *et al.*, 2000*a*). Citronellal and linalool were observed to possess a high degree of phytotoxicity leading to complete inhibition of germination beyond 55 and 110 μM, respectively, a characteristic of ideal natural herbicides. Both linalool and citronellal possess a double bond in their structure, which offers a potential site for attachment of functional groups for chemical modifications that could improve the physiochemical and biological properties of the molecule as a herbicide. For example, monoterpenes are generally too volatile to be used as herbicides, but modification could reduce volatility to an acceptable level. Cinmethylin, a commercial herbicide based on 1,4-cineole, is a much less volatile compound than 1,4-cineole. Further, as regards the solubility of these monoterpenes and their resulting toxicity, Weidenhamer *et al.* (1993) pointed out that monoterpenes are toxic at concentrations well below their solubility. However, water solubility is not an absolute requirement of commercial herbicides, as many of the best herbicides have very low water solubility.

Thus, based on the present study, it could be concluded that citronellal and linalool, on the basis of their overall phytotoxic impact on *C. occidentalis*, are good candidates for the synthesis of bioherbicides for future sustainable weed management programmes.

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