EXPERIMENT 3

Objective
To study the effect of monoterpenes namely citronellol, linalool, eugenol and limonene on the content of macromolecules and enzymatic activities of a selected test weed – Cassia occidentalis.

Hypothesis
Since the test monoterpenes were found to affect the germination, growth and physiology of some weeds, it was hypothesized that they may also affect their metabolites and enzyme activities. C. occidentalis, a relatively hardy shrub was chosen for this purpose since a soft herb will not be able to tolerate the stress caused by the monoterpenes.

Parameters studied
Content of water soluble proteins and carbohydrates and specific activities of some related enzymes namely proteases, α-amylases, β-amylases, peroxidases and polyphenol oxidases of C. occidentalis grown in an environment of monoterpenes formed the parameters of study.

Experimental design
Adequate number of healthy and uniform seeds collected from the plants of C. occidentalis growing in Panjab University campus were allowed to germinate in the Petri dishes. The procedure adopted for raising the seedlings in the
Petri dishes was similar to that explained in the section “Materials and Methods”. After four days, seedlings of size 3.5 ± 0.16 cm were grouped into 39 lots including three for control (that were exposed only to water). The rest of the 36 lots were sub divided into four lots - one for each of the four monoterpenes. For each monoterpane, the seeds were exposed to three concentrations and for each concentration, three replicates were maintained at Petri dish level. The selection of these concentrations was based on a pilot experiment to evaluate the phytotoxicity of monoterpenes to select the concentrations in response to which seedlings were neither killed nor remained unaffected. In other words, the concentrations that showed signs of visible injury such as chlorosis or weaker appearance on the seedlings were selected. Based on these observations, the selected concentrations of monoterpenes were 5, 10 and 15 nl / cc. However, in case of citronellol these were 1.5, 2 and 5 nl / cc. In each Petri dish, 10 seeds were subjected to germination. Application of each of the monoterpenes was given on the inner side of the upper lid of the Petri dishes which were then completely sealed with a good quality cellotape and the vapours of monoterpenes were allowed to spread uniformly in these Petri dishes.

**Preparation of material for macromolecules (proteins and carbohydrates)**

From the seedlings raised in each of the Petri dishes, 200 mg of cotyledonary leaves were taken after 24 h from the time of treatment and were crushed in distilled water (details given in “Materials and Methods”). Three replicates were maintained for each treatment. The content of proteins and carbohydrates was measured by the methods of Lowry et al. (1951) and Loewus (1952), respectively.
Preparation of material for measuring enzyme activities

From the seedlings raised in each of the Petri dishes, 200 mg of cotyledonary leaves were taken after 24 h from the time of treatment and were crushed in 0.1 M potassium phosphate buffer, pH 7 (details given in the chapter, “Materials and Methods”). Three replicates were maintained for each treatment. The specific activities of enzymes, proteases, α-amylases, β-amylases was measured by the methods given by Basha and Beevers (1975), Muentz (1977) and Bernfeld (1951), modified by Dure (1960), respectively. While those of peroxidases and polyphenol oxidases were measured by the methods of Mallik and Singh (1980) and Van Lelyveld and Pretorius (1973), respectively.

Statistical analysis

All data were expressed as mean of the respective parameter and the significance of treatment was tested with respect to control applying ANOVA (Analysis of variance) followed by Duncan’s multiple range test using the statistical package of SPSS version 10. Besides, values of correlation coefficient (r) were calculated between the parameter and concentration of each monoterpane.

Observations and Results

Content of water-soluble macromolecules

1. Proteins

The content of water-soluble proteins in control was recorded to be 38.87 ± 0.20 μg / mg and was seen to increase with different concentrations of citronellol (Fig. 3.1a). With the treatment of 1.5 nl / cc, an increase of about 28% to that of control was noticed. There was a further increase of 30.5 and 44.12%, respectively with the treatments of 2 and 5 nl / cc concentrations of citronellol. A similar trend was seen with various treatment concentrations of
linalool where content of water soluble proteins was evaluated to be 42.58 ± 0.37, 53.80 ± 0.19 and 55.71 ± 0.35 µg / mg when exposed to the treatments of 5, 10 and 15 nl / cc compared to 38.87 ± 0.20 µg / mg in control (Fig. 3.1b). The correlation between the content of proteins and different concentrations of linalool was statistically significant and the value of r was measured to be more than 0.963.

With the treatment of eugenol, there was an increase in the content of proteins by 12.43% of that of control in samples exposed to 5 nl / cc concentration (Fig. 3.1c). It increased further with increasing concentrations of eugenol. An increase of 16.65 and 40.31%, respectively was observed with 10 and 15 nl / cc of eugenol treatments. The curve drawn between the content of proteins and concentrations of eugenol indicated a dose-response relationship, with significantly high value of correlation coefficient. In case of limonene, there was almost no change in the content of proteins especially in seedlings exposed to 5 nl / cc, while increase of about 13 and 40% was noticed in samples treated with 10 and 15 nl / cc (Fig. 3.1d). The increase in the content of proteins with different concentrations of limonene was statistically significant over control. Here also, the value of correlation coefficient was significantly high and close to one.

At the concentration of 5 nl / cc, the change in the content of proteins was compared in response to each of the four monoterpenes and maximum increase (of about 45%) in the protein content was noticed in response to citronellol (Fig. 3.4). An increase of about 10 and 12% over control in response to linalool and eugenol, respectively was noticed at this concentration. However, no significant change was observed in protein content in response to 5 nl / cc of limonene (Fig. 3.4). Thus, citronellol is the most effective monoterpene while limonene is the least in this case.
Fig. 3.1: Effect of different concentrations of (a) citronellol (b) linalool (c) eugenol (d) limonene on the water soluble content of proteins and carbohydrates of *C. occidentalis*

There was an increase in the amount of water-soluble carbohydrates with increasing concentrations of monoterpenes. With the treatment of 1.5 nl / cc concentration of citronellol, carbohydrate content was measured to be around

2. **Carbohydrates**

There was an increase in the amount of water-soluble carbohydrates with increasing concentrations of monoterpenes. With the treatment of 1.5 nl / cc concentration of citronellol, carbohydrate content was measured to be around
64 μg / mg compared to 53.93 ± 0.32 μg / mg in case of water treated control (Fig. 3.1a). With the treatments of 2 and 5 nl / cc, it further increased to about 74 and 78 μg / mg, depicting an increase of about 38 and 44%, respectively. The increase in carbohydrate content with various concentrations of citronellol compared to control was statistically significant. A similar trend of increase in the content of carbohydrates was seen with the treatment of linalool (Fig. 3.1b). It was measured to be about 61, 76 and 77 μg / mg when treated with 5, 10 and 15 nl / cc, respectively compared to about 54 μg / mg in control. An increase of 43% over control in carbohydrate content was seen at the highest concentration of linalool. The curves drawn between carbohydrate content and linalool concentrations indicated a dose-response relationship, with high value of correlation coefficient.

When treated with eugenol, the content of carbohydrates was measured to be about 74, 78 and 79 μg / mg at the concentrations of 5, 10 and 15 nl / cc (Fig. 3.1c). Thus, an increase of about 37, 45 and 47%, respectively was measured at 5, 10 and 15 nl / cc concentrations. The data on content of carbohydrates in response to the concentrations of eugenol was put to linear regression analysis and a high value of correlation coefficient (r) was observed i.e. r= 0.876. In case of treatment with limonene, carbohydrate content was measured to be about 68, 72 and 84 μg / mg when treated with 5, 10 and 15 nl / cc compared to 53.93 ± 0.32 μg / mg in case of water treated control, thus exhibiting an increase of 27, 34 and 56% over control, respectively (Fig. 3.1d). The value of r was calculated to be 0.979 indicating a strong correlation.

At the common concentration of 5 nl / cc, the content of carbohydrates in samples treated with citronellol was recorded to increase by about 44% over control (Fig. 3.4). At the same concentration of linalool, an increase of 13% over control was observed. An increase of 37 and 27%, respectively in response to eugenol and limonene at the concentration of 5 nl / cc over control was recorded. Thus, citronellol was the most effective in this case.
followed by eugenol and limonene while linalool was found to be least effective (Fig. 3.4).

Specific activities of enzymes

1. Proteases

A decrease in the specific activity of proteases was observed with treatment of monoterpenes. The specific activity of proteases in case of water treated control was measured to be $0.56 \pm 0.006$ mg / h / mg protein.

With the treatment of 1.5 nl / cc concentration of citronellol, the specific activity of proteases was measured to be 95% of that of control (Fig. 3.2a). It was further reduced by 29% and 43% with the treatments of 2 and 5 nl / cc, respectively. A strong reciprocal correlation between protease activity and different citronellol concentrations was also observed. With the treatment of 5 nl / cc concentration of linalool, protease activity was measured to be $0.47 \pm 0.003$ mg / h / mg compared to $0.56 \pm 0.006$ mg / h / mg in water treated control (Fig. 3.2b). With the treatment of higher concentrations of linalool, a further decrease in protease activity was observed (Fig. 3.2b). Here also, a strong correlation between concentrations of linalool and observed parameter could be seen.

A similar trend followed in case of treatment with eugenol where reductions of 9, 30 and 39% were noticed from that of control with the treatment of 5, 10 and 15 nl / cc, respectively (Fig. 3.2c). The decrease in protease activity with increasing concentrations of eugenol was statistically significant. With the treatment of 5 nl / cc of limonene, the activity of proteases was seen to reduce by 39% compared with control. At still higher concentrations, a further decrease in the enzyme activity was noticed i.e. reduction of about 46 and 48% at 10 and 15 nl / cc, respectively compared to control (Fig. 3.2d). The curves drawn between activity of proteases and different concentrations of
limonene indicated a dose-response relationship with significant value of correlation coefficient (r) (Fig. 3.2d).

Fig. 3.2: Effect of different concentrations of (a) citronellol (b) linalool (c) eugenol (d) limonene on the specific activities of proteases, α-amylases and β-amylases of *C. occidentalis*. Values in control: Proteases: 0.56 ± 0.01 mg / h / mg protein, α-amylases: 21.46 ± 0.33 mg / h / mg protein, β-amylases: 99.32 ± 1.62 μg / min / mg protein

![Graphs showing effects of different concentrations of citronellol, linalool, eugenol, and limonene on specific enzyme activities of proteases, α-amylases, and β-amylases](image)

Similar symbols along each curve in each figure represent insignificant difference among each other at P < 0.05 applying DMRT. r represents value of correlation coefficient.
The specific activity of proteases in samples treated with 5 nl / cc of each of the four monoterpenes was evaluated (Fig. 3.5b). Here also, citronellol was responsible for decrease of about 43% (maximum reduction), followed by limonene (about 40% reduction), linalool (16% reduction) and lastly, eugenol (least reduction of only 9%) (Fig. 3.5b).

2. \(\alpha\)-amylases

The specific activity of \(\alpha\)-amylases in control was measured to be 21.46 ± 0.33 \(\mu\)g / min / mg protein. Compared to this, a decrease in the activity was noticed with the treatment of different concentrations of citronellol and at 5 nl / cc, the specific activity of \(\alpha\)-amylases was 55% compared to control (Fig. 3.2a). In case of linalool treated samples, decrease of 20%, 37% and 50% in the activity of \(\alpha\)-amylases were noticed upon treatments with 5, 10 and 15 nl / cc concentrations, respectively (Fig. 3.2b) thus showing a consistent decrease over control.

In case of treatment with 5 nl / cc of eugenol, the activity of \(\alpha\)-amylases decreased by 15% compared to control. With further increase in concentrations, the activity of enzyme also decreased (Fig. 3.2c). With the treatment of limonene also, a similar trend of decrease in the activity of \(\alpha\)-amylases was noticed. In case of the samples treated with 5 nl / cc of limonene, \(\alpha\)-amylases activity was 56% compared to control (Fig. 3.2d). The activity was further reduced and was measured to be 47 and 45% compared to control with 10 and 15 nl / cc limonene treatments.

The data on \(\alpha\)-amylase activity in response to the four monoterpenes was also put to linear regression analysis in order to obtain the values of correlation coefficient. It is clear from Fig. 3.2 (a, b, c and d) that the correlation coefficient between concentrations of four monoterpenes used and activity of \(\alpha\)-amylases was statistically significant (where values were about -
0.9 in all cases) thereby indicating a strong correlation between concentration and α-amylase activity.

The decrease in the specific activity of α-amylases was also compared in response to each of the four monoterpenes at the concentration of 5 nl / cc. Maximum reduction of 45 and 44% was observed in response to citronellol and limonene, respectively. There was a decrease of 20% with the treatment of linalool. Least reduction of 15% was observed with eugenol treatment (Fig. 3.5a). Thus, citronellol was most effective while eugenol was the least.

3. β-amylases

In case of β-amylases also, a decrease in the specific activity was observed in response to increase in the concentration of monoterpenes treatments. The specific activity in water treated control was measured to be 99.32 ± 1.62 μg / min / mg protein. With the treatment of citronellol, the activity of β-amylases was measured to be 82.49 ± 2.49 μg / min / mg at 1.5 nl / cc, 55.01± 1.12 μg / min / mg at 2 nl / cc and 37.95 ± 0.28 μg / min / mg at 5 nl / cc compared to 99.32 ± 1.62 μg / min / mg in case of water treated control (Fig. 3.2a). When treated with linalool, β-amylase activity decreased compared to control (Fig. 3.2b). At 15 nl / cc treatment, the specific activity of β-amylases was reduced by 69% compared to control.

Likewise, with the treatment of eugenol or limonene, the β-amylase activity decreased compared to control. The decrease was maximum at the highest treatment concentration of either of the monoterpenes. In case of each monoterpenes, the value of correlation coefficient between treatment concentration and the activity of β-amylases was significant and close to −1 (Fig. 3.2 a, b, c and d).

The specific activity of β-amylases was evaluated at the concentration of 5 nl / cc of each of the four monoterpenes (Fig. 3.5a). Here also, citronellol caused maximum decrease of over 60%, followed by limonene (33%) and linalool...
(25%). Least reduction was observed in response to eugenol (a decrease of about 20% over control).

4. Peroxidases

The specific activity of peroxidases, a type of oxido-reductases enzymes, increased with the increasing concentrations of test monoterpenes. In this case, the specific activity in control was measured to be $0.172 \pm 0.03 \mu$Kat/sec/mg protein. With the treatment of 1.5 nl/cc concentration of citronellol, activity of peroxidases increased by 140% (Fig. 3.3a). The activity further increased by 174% with 2 nl/cc and by 178% with 5 nl/cc citronellol concentration. The increase in peroxidase activity with increasing concentration of citronellol was statistically significant with respect to control and the value of correlation coefficient, $r$ was calculated to be 0.767 (Fig. 3.3a). Upon treatment with linalool (Fig. 3.3b), peroxidase activity increased by 165% and 232% with the treatments of 5 and 10 nl/cc, respectively. It was further enhanced by 324% with 15 nl/cc linalool treatment. Here also, the value of $r$ was measured to be strong i.e. 0.981 (Fig. 3.3b).

With the treatment of eugenol, there was an increase of 177% compared to control at the concentration of 5 nl/cc (Fig. 3.3c). This further increased with the increasing concentration of the monoterpene and the increase was statistically significant at each treatment concentration compared to control. Here also, the value of $r$ was measured to be strong and positive (Fig. 3.3c). The specific activity of peroxidases increased with the treatment of different concentrations of limonene. However, here, the magnitude of increase was less. Nevertheless, the value of $r$ was measured to be 0.985 and was thus statistically strong.

The increase in the specific activity of peroxidases was compared for each of the four monoterpenes at 5 nl/cc (Fig. 3.5b). In this case, citronellol and eugenol were responsible for an increase of about 178% over control,
followed by linalool (165% increase). Limonene was observed to be least effective causing an increase of 23% over control (Fig. 3.5b).

Fig. 3.3: Effect of different concentrations of (a) citronellol (b) linalool (c) eugenol (d) limonene on the specific activities of peroxidases and polyphenol oxidases of *C. occidentalis*.
5. Polyphenol oxidases (PPO)

Unlike the peroxidases, the specific activity of PPO decreased with the treatment of monoterpenes. With the treatment of 1.5 nl / cc citronellol concentration, a decrease of 22% was measured compared to specific activity of $37.6 \pm 3.0 \mu$Kat / sec / mg protein in case of control (Fig. 3.3a). A further decrease was noticed with increasing concentrations of citronellol. With the treatment of linalool, PPO activity decreased by 35% with the treatment of 5 nl / cc concentration (Fig. 3.3b). It was further decreased by 52% and 72% with higher concentrations of 10 and 15 nl / cc, respectively. It is also clear from Fig. 3.3b that the correlation between PPO activity and linalool concentrations was strong.

Fig. 3.4: Comparative effect of four monoterpenes on the macromolecular content of C. occidentalis at the concentration of 5 nl / cc.

Upon treatment with eugenol, PPO activity was measured to be 68% compared to control with the treatment of 5 nl / cc and it further decreased to 33% and 23.3% compared to control with the treatments of 10 and 15 nl / cc, respectively. (Fig.3.3c). The decrease in PPO activity with increasing
concentrations of eugenol was statistically significant with high value of correlation coefficient i.e. r, presented along the curve (Fig. 3.3c). In case of samples treated with limonene, a decrease of 58% was noticed with the treatment of 5 nl / cc (Fig. 3.3d). With higher concentration treatments i.e. at concentrations of 10 and 15 nl / cc, a further decrease in the activity of PPO was noticed and there was a reduction of 72% and 74.3%, respectively. A high value (-0.879) of correlation coefficient (r) was obtained.

Fig. 3.5: Comparative effect of four monoterpenes on the specific enzyme activities of (a) α-amylases, β-amylase and polyphenol oxidases (PPO) (b) proteases and peroxidases of C. occidentalis

Similar symbols in each bar of the figure represent insignificant difference at P<0.05 applying DMRT

On comparing the specific activity of polyphenol oxidases in response to 5 nl / cc of each of the four monoterpenes, limonene and citronellol were observed to be most effective, causing a decrease of about 58% (Fig. 3.5a). These were followed by linalool (35% decrease) and eugenol (32% decrease) (Fig. 3.5a).
Discussion

A number of studies are reported on allelopathic properties of plants or the biological action of allelochemicals (Rice, 1984; 1995, Kohli et al., 1998a). Though these studies have established the phytotoxicity of allelochemicals towards test plants, yet little attempt has been made to understand their mode of action. This is especially so in case of monoterpenes. Thus, the pathways of action of monoterpenes remain by and large unknown. Nevertheless, some studies have established the mode of action of some monoterpenes.

In the present study, the test monoterpenes namely citronellol, linalool, eugenol and limonene affected the macromolecular content and enzymatic activities of growing seedlings of C. occidentalis. An appreciable and significant increase in both proteins and carbohydrates was seen, indicating thereby, the reduced rate of metabolism in response to the treatment of monoterpenes. This increase may be attributed to the possible stress caused by monoterpenes on the growing seedlings. Studies are available in literature which indicate that stress caused by chemicals may enhance the macromolecular content in plants (Verma and Dubey, 2001). There are also reports available which indicate changes in the content of sugars / starch with various kinds of stresses including allelochemicals. Iljin (1927) and Henrici (1945) reported an increase in the sugar content in response to wilting while an increase in the sugar content on exposure to draught was reported by Vassiliev and Vassiliev (1936), Clements (1937a), Julander (1945). Carbohydrate content has been reported to increase with drought stress (Clements, 1937a,b; Grandfield, 1943; Eaton and Ergle, 1948). However, the increase or decrease varies with the plant and the kind / concentration of the stress / allelochemical.

Likewise, the specific activities of enzymes are also likely to be affected. In the present study, the increase in the amount of water soluble proteins was followed by concomitant decrease in the specific activities of related enzymes, the proteases, indicating thereby less hydrolysis of proteins into
amino acids or peptides. Such an observation has also been made by some other workers working on different chemicals. For example, Angelova et al. (2002) made a similar observation in *Zea mays* seedlings when treated with abscisic acid. On the other hand, there are reports which indicate that both protein content and the activity of proteases increase under stress conditions in the germinating seedlings (Zayed and Zeid, 1998). Gauch and Eaton (1942) reported that salt stress decreases the synthesis of new proteins in cotton seedlings while increases the hydrolysis of the storage proteins. Maximum increase in the content of proteins was caused by citronellol treatment while minimum effect was caused by limonene in the present study. Maximum decrease in protease activity was caused by citronellol while eugenol had the least effect.

Monoterpenes also affected carbohydrate metabolism as observed in the present study i.e. the content of carbohydrates increased while the specific activities of enzymes, α- and β- amylases decreased. α- amylases breaks down starch into short dextrins and reducing sugars while β- amylases catalyses the successive removal of maltose units from non-reducing end of α-1,4-D glucan. It is involved in the hydrolysis of short chains produced by the action of α- amylases. Decrease in the activity of these enzymes indicates the slowing down of carbohydrate metabolism in response to monoterpenes. Maximum increase in the content of carbohydrates was caused by citronellol whereas minimum increase was caused by linalool. In case of α- and β- amylases, minimum reduction was caused by eugenol while maximum by citronellol.

Besides proteases, α- and β- amylases, the specific activities of peroxidases and polyphenol oxidases were also assayed as these influence an array of physiological responses involved in the growth and development of plants. The activity of peroxidases (that are involved in functions like lignification, wound-healing and disease-resistance) increased. A few other reports have also made similar observations where peroxidase activity increases in young
seedlings in response to either metal ion stress or salt stress (Simonovicova et al., 2004; Jan et al., 2001). The increase in the specific activity of peroxidases could be visualized in the light of the inhibitory effects of monoterpenes and the test plants try to resist this effect. Maximum increase in peroxidase activity was caused by citronellol, closely followed by eugenol while minimum increase occurred in response to limonene.

Another enzyme, polyphenol oxidases was also studied as it is known to be affected by infection by micro-organisms or mechanical injury. Its activity was found to decrease with the treatment of different concentrations of monoterpenes. Maximum decrease in polyphenol oxidase activity occurred in response to limonene closely followed by citronellol treatment while minimum decrease was caused by eugenol. This shows that monoterpenes affect the overall enzymatic activities in the plant system that in turn, affects their growth and establishment.

Another important observation that was made from the present study was that all monoterpenes either increased or decreased the metabolic activities in the same manner. These did not exhibit any variation with regards to their action, despite structural variations in them.

From the above studies, it is clear that different monoterpenes affected the amounts of proteins and carbohydrates as well as specific activities of enzymes, proteases, α- and β-amylases, peroxidases and polyphenol oxidases. Though these do not provide any specific information regarding their mode of action, yet these observations can serve as important clues in determining the mode of action of monoterpenes in the future studies.