Response of *Cannabis sativa*L. to foliar application of 2chloroethyltrimethylammonium chloride

Hakimeh Mansouri[1, 2]* and Monir Rohani[3]

1. Department of Biology, Shahid Bahonar University of Kerman, Kerman, Iran
2. Department of Ecology, Institute of Science and High Technology and Environmental Sciences, Graduate University of Advanced Technology, Kerman, Iran
3. Kerman Agriculture Jahad Organization, Kerman, Iran

Abstract

The growth of medicinal plants and biosynthesis of secondary metabolites is influenced by plant growth regulators. In this study we investigated influence offour levels (0, 500, 1000, and 1500 mg l^{-1}) of2-chloroethyltrimethylammonium chloride (Cycocel), a plant growth retardant, on growth parameters and some biochemical parameters of cannabis plants. Cycocel only at 500 mg l^{-1} decreased shoot length of male plants. The fresh weight of leaves in female plants decreased with 1500 mg l^{-1}Cycocel. The other Cycocel treatments increased the fresh weight of leaves in female and male plants. Root and stem fresh weight of male and female plants showed an increase in most treatments (except for 1500 mg l^{-1}Cycocel). Cycocel at 1000 mg l^{-1} had enhancing effect on the fresh weight of male and female flowers. Also, the plants treated with Cycocel had a higher content of soluble carbohydrates and protein. Malondialdehyde content was decreased in male and female plants by 500 mg l^{-1}Cycocel treatment. Tetrahydrocannabinol (THC) content increased in male plant leaves under 1000 and 1500 mg l^{-1}Cycocel treatment, but in female plants only 500 mg l^{-1}Cycocel caused an increase in THC content in leaves. While Cycocel decreased cannabidiol (CBD) content in male leaves, in female leaves it increased CBD concentration. In fact, only 500 mg l^{-1}Cycocelled to more CBD content in female flowers while the other treatments declined CBD content in female flowers. The findings showed that the response to Cycocel depended on the sex in cannabis plants. Ineffectiveness of Cycocel in reducing shoot length suggests that cannabis is insensitive to Cycocel as an inhibitor of gibberellin biosynthesis and its application at specific concentrations can be used to improve growth.

Keywords: Plant growth retardant; soluble carbohydrate; protein; Malondialdehyde; Cannabinoids

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Introduction

Cannabis (*Cannabis sativa* L.) is a dioecious and annual plant with a variety of applications e.g. as furnishing fiber, oil, medicine, and as narcotics. It is well known that the cannabis plant shows a number of chemical varieties, two typical ones being fiber-type containing low levels of psychoactive material which is an economically important crop for fiber and seeds and drug-type containing high levels of psychoactive material. Cannabinoids are a group of terpenophenolic compounds found in the cannabis. The highest cannabinoid

*Corresponding author
E-mail address: h_mansuori@yahoo.com
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concentrations are found in the resin secreted by the plants' flowering buds. Δ^9-Tetrahydrocannabinol (THC) is the psychoactive component of the hemp plant; other major nonpsychoactive constituents include cannabidiol (CBD) and cannabinol (CBN) (Pellegriniet al.,2005). Cannabis is used in modern medicine for the treatment of emesis in chemotherapy as anti-emetics. Cannabinoids appear to have therapeutic value as antispasmodics, analgesics, anti-emetics, and appetite stimulants and may also have potential in the treatment of epilepsy, glaucoma, and asthma (Guzman, 2003; Howlettet al., 2004). The growth of medicinal plants and biosynthesis of secondary metabolites is influenced by both environmental and plant factors. Changes in primary metabolic processes due to plant growth regulators may play an important role in the regulation of secondary metabolism.

Plant growth retardants are synthetic compounds used to retard the shoot length of plants. This is achieved by reducing cell elongation and by lowering the rate of cell division (Rademacher, 2000). Most plant growth retardants inhibit the formation of active gibberellins and can be used to reduce shoot elongation (Singh, 2004). Cycocel (chlormequat chloride) is a synthetic growth retardant that is extensively used for dwarfing of plants. Studies have indicated that Cycocel is effective in stimulating the production of secondary metabolites (Farooqiet al., 2005; El-Keltawi and Croteau, 1986; Shukla et al., 1992).

In the present study the effect of Cycocel was investigated on growth and cannabinoids level in cannabis plants with regards to the differences between male and female plants. The study is a part of a broader study on the effects of plant growth regulators on biosynthesis of cannabinoids.

Materials and Methods

Plant material

The seeds of *C. sativa* L. were sown in pots (20 cm, i.d soil-leaf mold-perlite = 2:1:1) and cultivated in a greenhouse. During the period of experimentation (June to September) the average maximum and minimum temperature were 35° C and was 25°C, respectively and photoperiod was 14 h (in the short days we used artificial light). The plants were fertilized regularly with a Hoagland nutrient solution every week till the productive stage. We applied four levels of Cycocel concentration (0, 500, 1000, and 1500 mg l^{-1}). There were 10 replications for each treatment. The plants were sprayed to drench with 100 ml Cycocel solutions when they had seven pairs of leaves and this was repeated after 10 days. Male and female flowering shoots were harvested after appearance and blooming of flowers on shoots.Leaves were collected, immediately frozen in liquid nitrogen, and stored at -20°C until biochemical analyses.

Growth parameters

Observations were made and recorded on plant height, fresh weight of shoot, root and flowers, and the number of internodes from three male and female plants.

Biochemical parameters determination

Chlorophyll and carotenoids were extracted from leaves with 95% ethanol and quantified by measuring the absorbance at 664, 648 and 470 nm as described by Lichtenthaler et al. (1997).

Reducing sugars were measured according to Somogyi (1952).The extraction and analysis of soluble carbohydrate were carried out as described previously (Roe, 1955).The content of protein was determined by Bradford (1976) method, using bovine serum albumin as a standard.For the measurement of lipid peroxidation in leaves, the thiobarbituric acid (TBA) test, which determines malondialdehyde (MDA) level was applied (Heath and Packer, 1968). Lipid peroxidation was measured in terms of thiobarbutaric acid reactive substance (TBARS) concentration. Leave samples of 0.5 g were homogenized in 10 ml of 0.1% trichloroacetic acid (TCA). Thehomogenate was centrifuged at 15,000 × g for 5 min. Four milliliters of 0.5% thiobarbutaric acid (TBA) in 20% TCA was added to 2 ml of supernatant aliquot. The mixture was heated at 95°C for 30 minand then quickly cooled

in ice bath followed by centrifugation at 10,000 × g for 10 min. Absorbance of the supernatant was recorded at 532 nm for MDA. The values of non-specific absorption at 600 nm were subtracted. The TBARS concentration was calculated using its absorption coefficient of 155 mM^{-1} cm^{-1}.

Female and male samples were collected as flowering tops and leaves separately. In female plants, flowering top samples included bracts and small leaves (small and palmate compound leaves surrounding the flowers) and flowers and pollen grains in males. Mature leaves with almost 7 cm length from male and femaleplants were used for cannabinoid measurement. All samples were dried at room temperature in darkness. Sample material (50 mg) was placed in a test tube with 1 ml chloroform. Sonication was applied for 15 min. After filtration, the solvent was evaporated to dryness and the residue was dissolved in 0.5 ml methanol. Chromatographic separations of cannabinoids were performed on a liquid chromatograph with a quaternary pump and a variable wavelength UV detector as described by Rustichelli et al. (1998). Chromatographic separations were performed at room temperature with a mobile phase consisting of

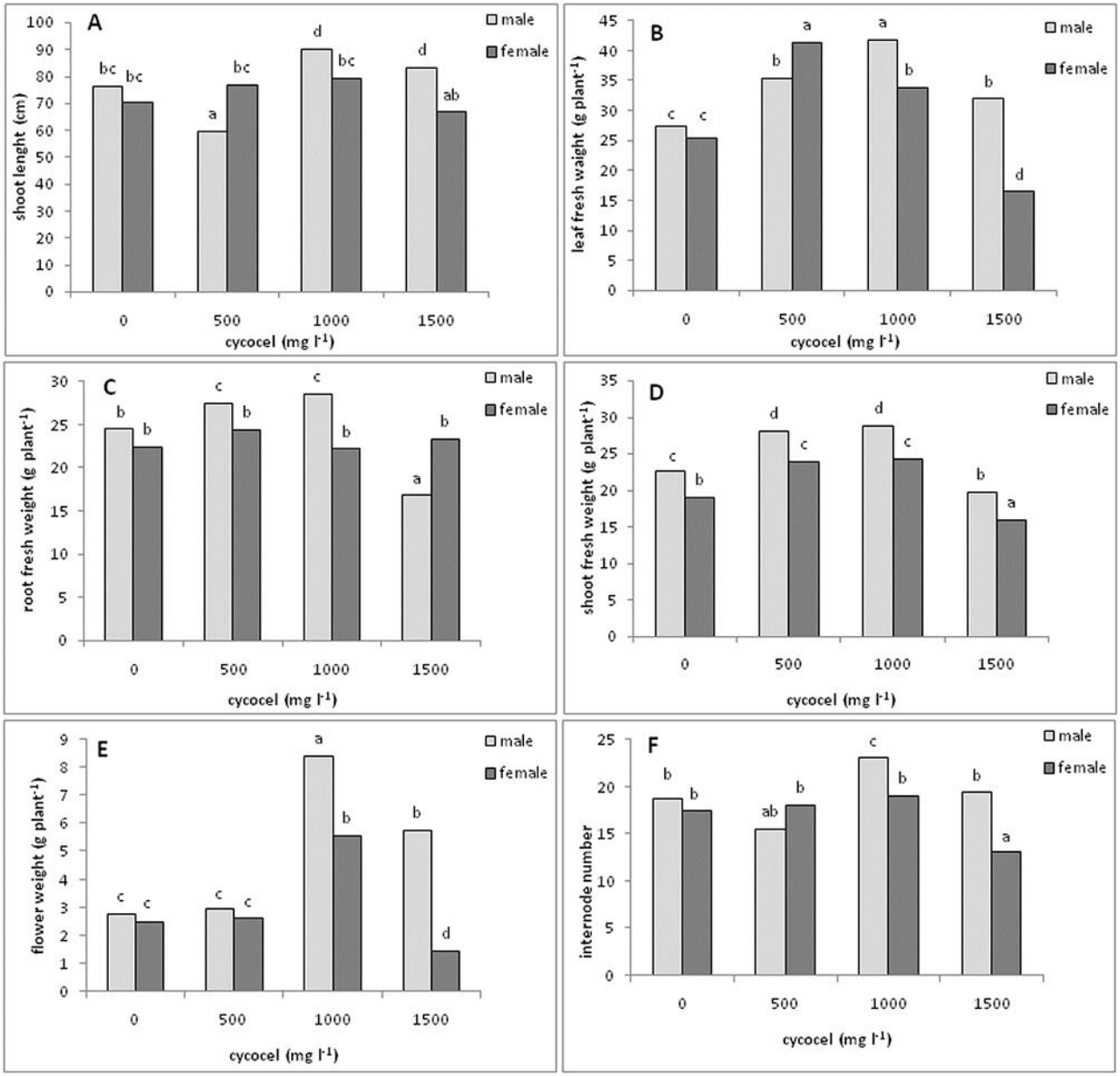

Fig. I. The effects of Cycocel on growth parameters in cannabis plants;values are means of three replications. Means followed by different letters are significantly different (P < 0.05) according to Duncan test.

methanol/water in the ratio 80:20 (v/v); the flow-rate was 1.0 mL min^{-1}. Separations were carried out on a LiChroCART- LiChrospher 100-RP-18 column (125 x 4mm; 5 µm). Cannabinoid peaks were identified by cannabinoids standards (THC and CBD).

Statistical analysis

The data were analyzed statistically and analysis of variance (ANOVA) for randomizedblock design was performed. The data were recorded in three replications. The treatment means in each trait were compared statistically using Duncan multiple range tests at 5% level of significance.

Results

Effect of Cycocel on Growth parameters

The average shoot length of male plants sprayed with 500 mg l^{-1}Cycocel was lower than that in control male plants (Fig. I. A). The male plants which received foliar application of Cycocel at 1000 and 1500 mg l^{-1} concentrations showed an increase in shoot length (Fig. I. A). Spraying female plants with Cycocel at different concentrations did not show any significant change in shoot length.Male plants treated with all concentrations of Cycocel showed an increase in fresh weight of leaves in comparison to control (male) plants (Fig. I. B). The maximum increase was observed in 1000 mg l^{-1} Cycocel treatment. The fresh weight of leaves in female plants increased with 500 and 1000 mg l^{-1} Cycocel and decreased with 1500 mg l^{-1}Cycocel treatment (Fig. I. B). Root and stem fresh weight in male plants treated with 500 and 1000 mg l^{-1}Cycocel increased and in those treated with 1500 mg l^{-1}Cycocel decreased in comparison with control male plants (Figs. I C and D). Root fresh weight of female plants showed significant decrease only in female plants sprayed with 1500 mg l^{-1}Cycocel (Fig. I. C). Treatments with 500 and 1000 mg l^{-1}Cycocel increased stem fresh weight of female plants while this was decreased in the treatment with 1500 mg l^{-1}Cycocel (Fig. I. D). Cycocel was more effective in increasing fresh weightof male flowers especially at 1000 mg l^{-1}Cycocel (Fig. I. E). The female plants also showed increase in fresh weight of flowers when treatment with 1000 mg l^{-1}Cycocel, but treatment with 1500 mg l^{-1}Cycocel decreased flowers fresh weight in female plants.The numbers of internodessignificantlyincreased in male plants sprayed with 1000 mg l^{-1} Cycocel and decreased in female plants treated with 1500 mg l^{-1} (Fig. I. F).

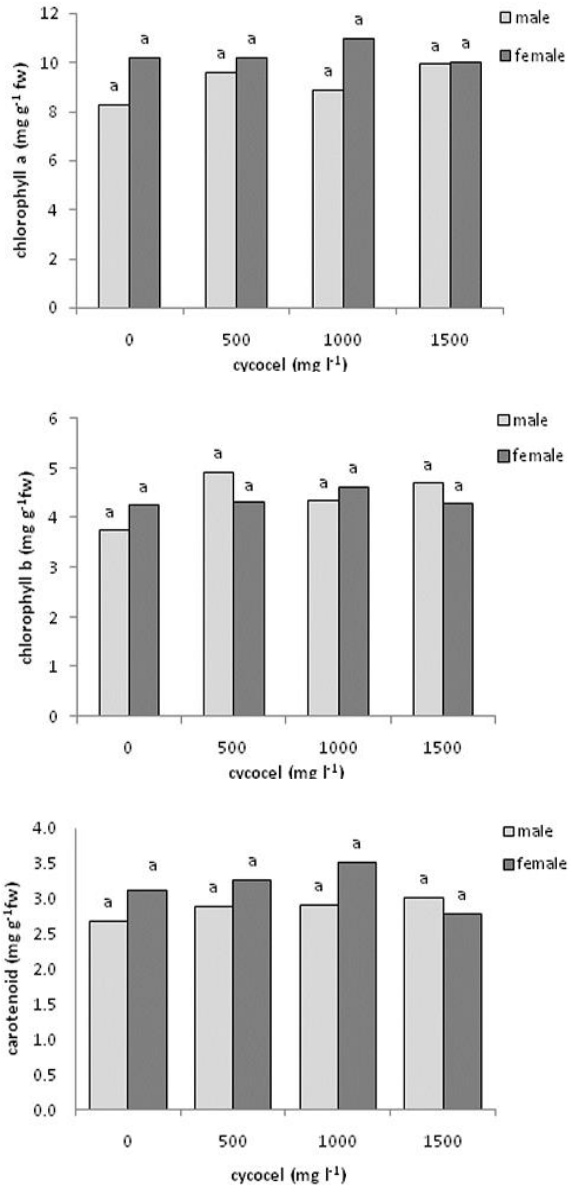

Fig. II. The effects of Cycocel on chlorophylla, b and carotenoids; values are means of three replications difference.Means followed by different letters in a column are significantly different (P < 0.05) according to Duncan test.

Effect of Cycocel on biochemical parameters

According to the results, no significant difference was observed in chlorophyll a, b, and carotenoid contents between male and female plants treated with Cycocel and control plant (Fig. II).

The amount of reducing sugars in male and female plants sprayed with Cycocel at different concentrations did not show any significant change (Fig. III. A). Moreover, all Cycocel concentrations increased soluble carbohydrates in male and female plants relative to control plants (Fig. III. B). On the other hand, there was no significant difference between applied concentrations of Cycocel.

Protein content of male plants showed considerable increases in 500 and 1500 mg l^{-1} Cycocel treatments (Fig. III. C). Male plants sprayed with 1500 mg l^{-1} Cycocel did not show any difference with control male plants in case of protein content. The amount of protein in female plants enhanced with all Cycocel concentrations. Male and female plants treated with 1000 mg l^{-1}Cycocel had the maximum content of protein.

There was an increase in malondialdehyde content in male and female plants treated with 500 mg $l^{-1}$and 1500 mg l^{-1}Cycocel (Fig. III. D). The highest level of malondialdehyde was observed in female plants treated with 500 mg l^{-1}Cycocel. Malondialdehyde content was decreased in male and female plants by 500 mg l^{-1}Cycocel treatment.

The amounts of THC and CBD were analyzed as the most important cannabinoids in leaves and flowers of cannabis plants. Comparison of male and female leaves in control plants showed that the amount of THC in female plant leaves was more than that ofmale plants. THC content was enhanced in male plant leaves by 1000 and 1500 mg l^{-1} Cycocel treatment, but in female plants only 500 mg l^{-1}Cycocel caused an increase in THC content in leaves hitting the maximum levelamong all treatments (Fig. IV. A). Female flowers also had more THC in comparison with male flowers. Again maximum THC was observed in female plant flowers treated with 500 mg l^{-1} Cycocel while the other treatments

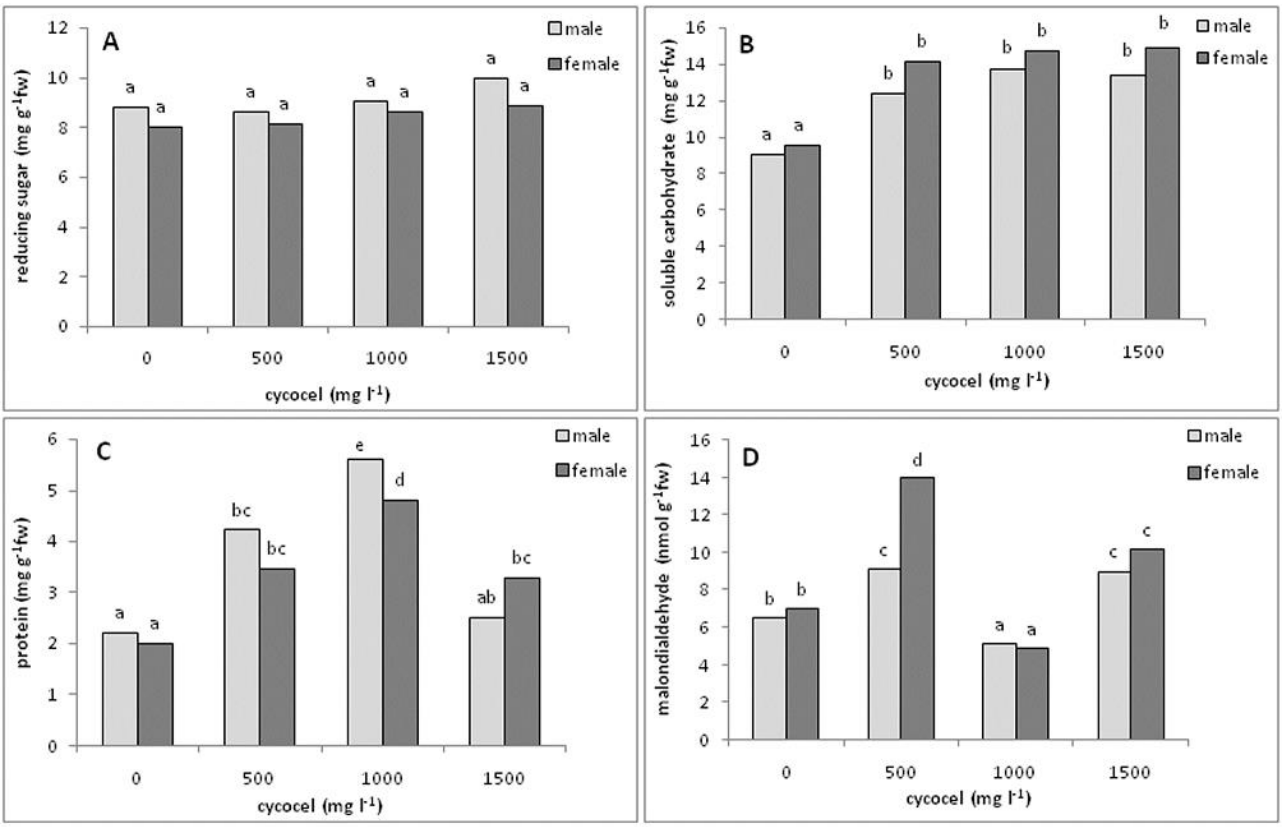

Fig. III. The effects of Cycocel on reducing sugar (A), soluble carbohydrate (B), protein (C) and malondealdehyde (D) content of leaves in cannabis plants; values are means of three replications difference.Means followed by different letters in a column are significantly different (P < 0.05) according to Duncan test.

decreased THC content in female and male flowers (Fig. IV. B).

The amount of CBD in male leaves was about 12-folds in comparison with control plants. Cycocel treatment decreased CBD content in male leaves especially in 1000 mg $l^{-1}$and 1500 mg l^{-1}concentrations (Fig. IV. C). However, in female leaves CBD concentration increased in plants sprayed with Cycocel. The amount of CBD in male flowers was more than female flowers but Cycocel treatment decreased CBD accumulation in male flowers (Fig. IV. D). In female flowers of plants treated with 500 mg l^{-1}Cycocel, there was more CBD content in comparison withthe female control plants but the other treatments declined CBD content in female flowers (Fig. IV. D).

Discussion

The results showed that shoot length of male plants increased with increasing Cycocel concentration. Cycocel had no measurable effect on the growth of the treated female plants. Therefore, this suggests that the response of cannabis plants to Cycocelissex-linked. Most of the studies showed that Cycocel reduced the plant height (Pateli et al., 2004; Farooqi et al., 2005; Bhat etal., 2011; Lodetaet al., 2010; Ziauka and Kuusiene, 2010). The physiological basis of plant height retardation by Cycocel involves its inhibitory effect on gibberellin biosynthesis (Berova and Zlatev, 2000). Yet, Cycocel and other plant growth retardants may influence other metabolic pathways and respond differently in different plant species (Rademacher, 2000). Therefore, it is important to investigate the responses of particular plant species to these compounds. Application of inhibitors can sometimes promote a metabolic pathway for example mevinolin (especially inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase) induced the accumulation of this enzyme in Tobacco Bright Yellow-2 Cells (Hemmerlin et al., 2003). Ziauka and Kuusiene (2010) reported that Cycocel promoted shoot growth *Populustremula* explants in Petri dish.

The freshweight of leaves was increased in the plants sprayed with Cycocel except for

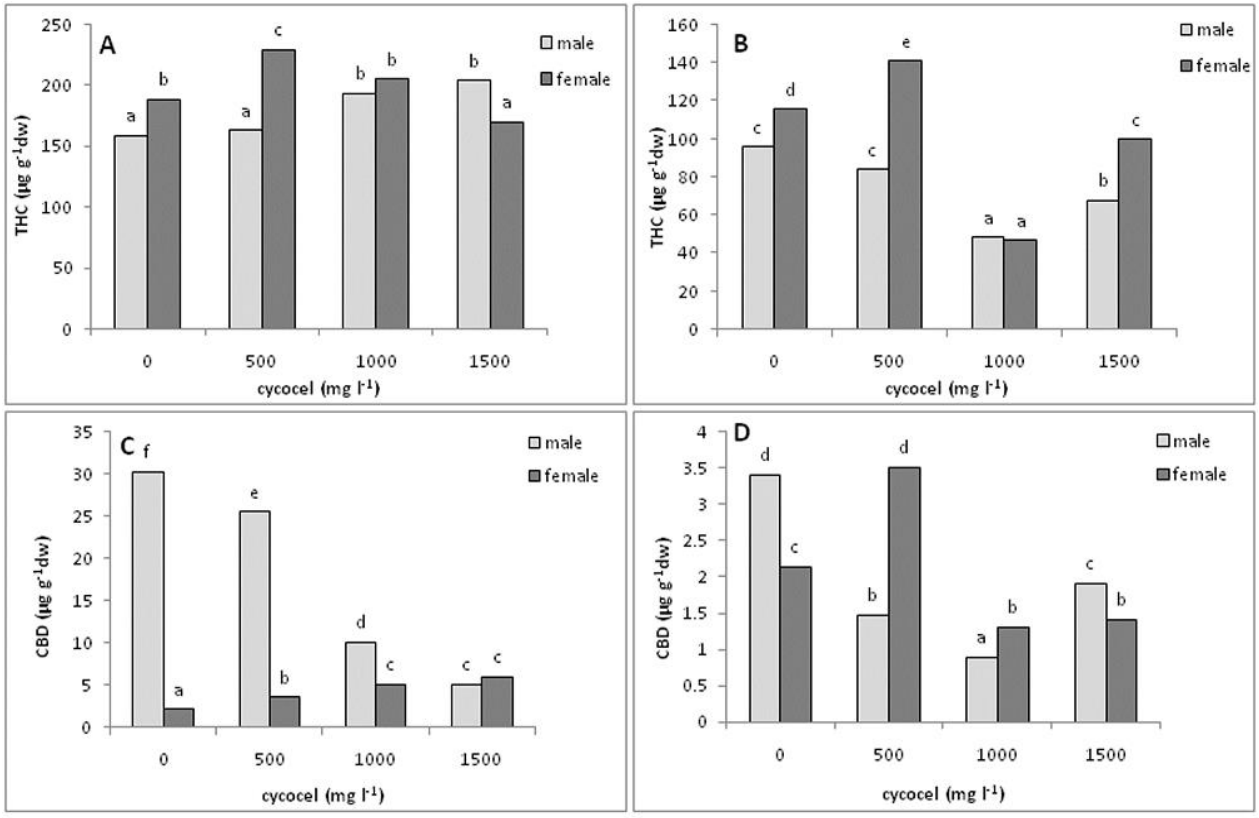

Fig. IV. The effects of Cycocel on THC and CBD content; THC content of leaves (A), flowers (B) CBD content of leaves (C) and flowers (D) in cannabis plants. Values are means of three replications difference.Means followed by different letters in a column are significantly different ($P < 0.05$) according to Duncan test.

female plants sprayed with 1500 mg l^{-1}Cycocel. Like results of the present study, the application of Cycocel significantly increased herbage yield in stressed plants of *Cymbopogonwinterianus* (Farooqi et al., 2005). Bhat et al. (2011) reported opposite results in *Erysimummarshallii*. Also Cycocel promoted root and stem weight in male plants and stem weight in female plants.

In this study, the highest concentration of Cycocel (1500 mg l^{-1}) showed anegative effect on root and stem fresh weights. Also a decreasein root and stem weight was reported in *Erysimummarshallii* treated with Cycocel (Bhat et al.,2011). The weight of male and female flowers increased in some Cycocel concentrations. This results are consistent with some other reports. High concentrations of Cycocel significantly increased single flower weight in *Chrysanthemum cinerariaefolium* (Haqueet al., 2007). Zhao et al. (2011) reported that spraying Cycocel significantly increased the number of female strobili in *Pinustabuliformis*. Of course there was also inconsistent results in this case and a decrease in the number of flowers per plants treated with Cycocel was also reported by Bhat et al.(2011).

In this investigation foliar application of Cycocel had no measurable effect on the amount of chlorophyll and carotenoid.Leaves of the plants were chlorotic after treating with Cycocel but with the passage of time this chlorosiswas disappeared.Unlike theresults of this investigation, an increase in chlorophyll content concomitant with a reduction in growth as a result of applying Cycocel were reported by Bora and Sarma (2006)in wheat, Krishnamoorthy et al. (1987) in gram,and Prakash and Ramachandran (2000) in Brinjal plants.

The amount of soluble carbohydrates was enhanced by Cycocel treatment in male and female cannabis plants. According to our results, total carbohydrates were significantly increased in the Cycocel treatment of grafted grapevine rootlings (Tedicet al., 2005). Cycocel treatment promoted protein biosynthesis in cannabis plants. These results are confirmed by those obtained by Prakash and Ramachandran (2000) in Brinjal plants, Kimenov et al. (1983) in maize, and Sawan et al. (2001) in cotton.

It has been confirmed that malondialdehyde content is an indicator for membrane damage related to oxidative stress. In this experiment Cycocel with 1000 mg l^{-1}concentration decreased the amount of malondialdehyde in male and female plants. It is suggested that this concentration could be useful for improving plant growth in stress condition such as drought, salinity, and cold stress with concomitant oxidative stress. Decreased malondialdehyde content with other growth retardants such as paclobutrazol was reported in wheat seedlings by Berovaet al. (2002). The effect of Cycocel on lipid peroxidation of membrane could be by gibberellins. In previous work, we reported that gibberellic acid increased phytosterols in the treated plants (Mansouri et al., 2009). Cycocel inhibits gibberellin biosynthesis in plant, and therefore decreases phytosterols levels. As phytosterols play important roles in integrity of membranes, it is reasonable to assume that Cycocel increase membrane damage.

It is found that the levels of secondary metabolites increased in plant under stress. In this condition, plant growth decreased and it needed substrate for biosynthesis of primary metabolites shift to secondary metabolites. We expected that Cycocel with inhibition of gibberellin biosynthesis, cause a decrease in growth and result in an increase in the plants' secondary metabolites including cannabinoids. Application of some concentrations of Cycocel resulted in an increase in THC content in leaves and flowers, but this increase was not considerable. It is interesting that where there was the maximum increase in the weight of flowers we observed the minimum level of THC and this result can confirm our opinion. The amount of CBD increasedonly in female leaves and flowers.Similar to our results, El-Keltawi and Croteau (1986) have indicated that Cycocel could influence monoterpenes biosynthesis due to their direct effect on enzymes involved. Also a stimulation effect of Cycocel on terpenoid accumulation was reported by Haque et al. (2007) in *Chrysanthemum cinerariaefolium* and by Pan et al. (2010) in *Catharanthusroseus*. However, in those studies increase in terpenoid content was accompanied by a decrease in plant

growth and this was not the case with our experiment where reduction in growth was not considerable.

Conclusion

Based on our investigation, response to Cycocel depends on the sex in cannabis plants. Ineffectiveness of Cycocel in reducing shoot length showed that cannabis is not sensitive to Cycocel as an inhibitor of gibberellin biosynthesis and it can be used to improve the plant growth in applied concentrations. Furthermore, the considerable decrease in malondialdehyde content at 1000 mg l^{-1} treatment suggests that utilization of this concentration of Cycocel could be useful for improving growth indexesunder stress conditions.

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