Antioxidant capacity and chemical composition of *Carum copticum* under PEG treatment

Roya Razavizadeh*[1] and Mozhdeh Karami[2]

1. Department of Biology, Payame Noor University, Tehran, Iran
2. Department of Biology, Payame Noor University, Isfahan, Iran

Abstract

Drought stress is one of the main non-biological factors limiting the growth and yield of plants in dry and semi-dry regions of the world. Plants are the source of much chemicals derived from secondary metabolism. *Carum copticum* is a plant from the Apiaceae family with the seeds containing 2-4% the essential oil which are rich in monoterpenes such as thymol and are widely used as an antibacterial agent. This experiment was conducted in order to evaluate the effects of drought stress on physiological parameters and essential oil properties of seedlings and callus of *C. copticum*. For this purpose, seeds of *C. copticum* were cultured in Murashige and Skoog medium containing 0, 2, 4, 6, and 8% PEG. Also, calluses cultured were in MS medium containing 0.25 mg.L^{-1} 2, 4-Dichlorophenoxyacetic acid, and 1 mg.L^{-1} benzyl amino purine and different levels of PEG. After PEG treatment for 4 weeks, results showed that drought stress decreased chlorophylls and carotenoids contents while it increased anthocyanin, phenolic compounds, protein contents, and CAT and APX activities. Unlike CAT and APX, water deficit induced a significant reduction in superoxide dismutase activity. The content of reducing sugars and proline increased progressively when drought stress increased. Furthermore, PEG changed the essential-oil composition in shoots and calluses. Drought stress increased thymol and p-cymene concentration, though it decreased γ-terpinene. In general, these results showed the high tolerance of *C. copticum* to drought stress and also revealed positive effects of drought on the antioxidant activities and essential oil composition.

***Keywords**: Carum copticum*; polyethylene glycol; callus; tissue culture; essential oil

Abbreviations: 2,4-D: 2,4-Dichlorophenoxyacetic acid; APX: Ascorbate peroxidase; CAT: Catalase; PEG: Polyethylene glycol; SOD: Superoxide dismutase; ROS: Reactive oxygen species

Razavizadeh, R. and M. Karami. 2018. 'Antioxidant capacity and chemical composition of *carum copticum* under PEG treatment'. Iranian Journal of Plant Physiology 8(2), 2321-2331.

Introduction

Drought stress is one of the main abiotic factors which limit the plant growth and crop productivity in most parts of the world. The response of the plants to drought stress depends on the type, severity, and duration of stress and plant species. Plants reduce the harmful effects of water stress by decreasing the amount of metabolism and osmotic regulation (Jaleel et al.,

*Corresponding author
E-mail address: razavi.roya@gmail.com
Received: June, 2017
Accepted: November, 2017

2007). *Carum copticum* is a *Eudicots Angiosperm* plant belonging to the *Apiaceae* family which is commonly known as Ajwain and grows in India, Pakistan, Iran and Egypt. The fruits of Ajwain accumulate up to 4% essential oils such as thymol and delta cymene and are commonly used for its antimicrobial effects (Mirzavand et al., 1992). The first reaction of plants to drought stress is growth reduction because plant growth depends on cell division and cell growth both of them being affected by drought stress. Water deficit reduces rate of photosynthesis. Stomatal and non-stomatal factors limit the photosynthesis under drought stress. Non-stomatal factors reduce or inhibit the synthesis of photosynthetic pigments such as chlorophylls and carotenoids (Flexas et al., 2004). Phenolic compounds such as flavonoids and anthocyanin have antioxidant roles under environmental stress conditions. Under drought stress, plants synthesize compatible ingredients which are soluble substances with low molecular weight such as sugars and proline and do not interact with biochemical reactions of cells. The accumulation of these substances reduces water potential of plant tissues; therefore, water absorption is continued by the plant.

Biological and non-biological stresses lead to the formation of reactive oxygen species (ROS) that cause membrane lipid peroxidation and protein and nucleic acids degradation. Plants have different enzymatic and non-enzymatic mechanisms to reduce the harmful effects of ROS. Antioxidant enzymes such as catalase, superoxide dismutase and peroxidase are involved to scavenge free oxygen radicals (Cruz, 2008). Secondary metabolites are often referred to compounds that have no significant role in life process but play a role in adapting the plant to the surrounding environment stresses and their production is usually less than one percent of the dry weight of the plant. Terpenes are the dominant components of most essential oils. Plant essential oils are aromatic, refractory, concentrated, and volatile compounds that exist in cells and the cortex, secretion glands, and in the surface and internal parts of various organs such as leaves, flowers, fruits, and buds (Tajkarim, 2010).

Plant tissue culture causes to produce and proliferate complete plant from different plant tissues by cultivation of protoplast, cell, tissue, and plant organs in sterile conditions. Plant tissue culture has provided an important source for production of secondary metabolites such as antibiotics and anticancer alkaloids (Razden, 2003).

Polyethylene glycol (PEG) is a flexible and non-toxic polymer that causes negative osmotic pressure and is known as the best treatment for osmotic stress in comparison with other osmolytes such as mannitol, sugar, and salt. It is not absorbed by the plant and its concentration remains constant throughout the stress period. PEG is widely used to create drought stress in tissue culture because it creates a similar conditions to those of natural environments (Kramer and Boyer, 1995). The present study was conducted with the aim of evaluating the effects of drought stress induced by PEG treatment on physiological and biochemical parameters and essential oil production of seedlings and calluses of *C. copticum.* The study also aimed to identify the adaptive mechanisms of *C. copticum* to drought stress.

Materials and Methods

Mature and sterilized seeds of *C. copticum* were cultured in the MS medium (Murashige and Skoog, 1962) supplemented with different concentrations (0, 2, 4, 6, and 8%) of PEG and kept in the growth chamber (16 h light/8 h dark), at a temperature of 25 °C and a relative humidity of 95%. Four weeks after the treatment, the effects of drought stress were studied on the photosynthetic pigments, secondary metabolites (carotenoid, anthocyanin, and flavonoid), proline, soluble carbohydrates, antioxidant enzymes activities, and essential oil composition of the seedlings.

Callus initiation under drought stress

Four-week-old growing seedlings were used as the source of explants. Stem explants about 0.5 cm were cultured on MS media containing combination of 0.25 $mg.L^{-1}$ 2,4-D (2,4-dichlorophenoxyacetic acid) and 1 $mg.L^{-1}$ BAP (benzyl amino purine). After a few days callus induction was observed. After 4 weeks, callus

explants were sub cultured in the same medium and were kept at darkness and 25 ± 2 °C for 4 weeks. Callus subculture was repeated monthly. After 3 subcultures, calluses were transferred into the medium supplemented with 1 mg. L^{-1} BAP and 0.25 mg. L^{-1} 2,4-D and different concentrations of PEG 0, 2, 4, 6, and 8% respectively. After 4 weeks' essential oil production was analyzed in calluses of *C. copticum* seedlings.

Measuring the amount of chlorophyll and carotenoids

Chlorophyll and carotenoids were determined according to Lichtenthaler's method (1987). A portion (0.2 g) of the frozen leaves of seedlings were homogenized in 15 mL of 80% acetone. This solution contained chlorophyll *a*, *b*, and carotenoids. The absorbance of each sample with three replications was measured at 646.8, 663.2, and 470 nm using a spectrophotometer (U-6305 model; Jenway, Staffordshire, UK) and pigments' concentrations were expressed as mg/g FW.

Measuring amount of flavonoids

The contents of flavonoids were measured by adapting Kotzé and Eloff (2002) method. Disks of leaves were rubbed in a mortar containing ethanol (Ethyl alcohol and glacial acetic acid 1:99 respectively). Extracts were put in 80 °C hot bath after centrifuging for 10 minutes and the absorption rate was measured at 270, 300, 330 nm.

Measuring amount of anthocyanin

Total anthocyanin determined according to modified Wagner's method (1979) using acidified ethanol (Methanol: HCl 99:1 v/v). A portion (0.05 g) of frozen shoot homogenized in 5 mL of acidified ethanol and then kept at 25°C for 24 h in the dark. The extract centrifuged at 4000 Xg for 10 min at room temperature. The absorbance of each supernatant was measured at 550 nm using the spectrophotometer. The extinction coefficient 33000 (mM^{-1} cm^{-1}) was used to calculate the amount of total anthocyanin which was expressed as µM/g FW.

Measuring amount of proline

Proline content was estimated using ninhydrin reaction (Bates et al., 1973). A portion (0.5 g) of shoot was homogenized with 10 mL of 3% (w/v) sulphosalicylic acid and passed through Whatman filter paper (no. 2; whatman, Maidstone, UK). Ninhydrin reagent (2 mL) (Sigma, St. Louis, Missouri, USA) and glacial acetic acid (2 mL) were added to 2 mL of the filtered extract. The mixture was then incubated at 100 °C for 1 h and the reaction was terminated by placing it on ice. The reaction mixture was extracted with 4 mL toluene and the absorption of chromophore was measured at 520 nm, against toluene as blank, using the spectrophotometer. Proline content was calculated using L-proline (Sigma) as a standard curve.

Measuring amount of soluble carbohydrates

Reducing carbohydrate contents was measured by adapting Somogyi-Nelson's method (1952). A portion (0.05 g) of the shoot was extracted with 10 mL of distilled water. The mixture was then boiled in a boiling water bath before it was cooled and filtered. The extract (2 mL) was mixed with 2 mL of alkaline copper tartrate and the reaction mixture was heated for 20 min. Alkaline copper tartrate was prepared by dissolving 4 g of anhydrous sodium carbonate, 0.75 g of tartaric acid, and 0.45 g of hydrated cupric sulphate in 80 mL of distilled water and finally it was made up to 100 ml. Two ml of phosphomolibdate solution was added and the intensity of the blue color was measured at 600 nm using the spectrophotometer. The reducing sugar content was expressed as mg/g FW.

Gas chromatography-mass spectrometry

The gas chromatograph-mass spectrometry was used for the identification of the components of the essential oils of the seedling and callus of *C. copticum.* A Hewlett-Packard 5890 gas chromatograph (Hewlett Packard, Waldbronn, Germany), equipped with a flame ionization detector (HP-5970 mass-selective detector), and 50 m × 0.20 mm HP-5 (cross-linked Phenyl–Methyl Silicon) column with 0.25 µm film thickness were used for this study. The FID was

maintained at 250 °C. In addition, the ionization energy was 70 eV and the temperature program was 100 -250 °C with changes of 4 °C/min. Helium was used as carrier gas and the flow through the column was 1 mL/min and the split ratio was set to 100:1. Identification based on the sample retention time and mass was recorded (Li et al., 2009).

Protein and enzyme extraction and assay

A portion (0.1 g) of the frozen shoot was homogenized in 2 mL of 25 mM sodium phosphate buffer (pH=7). The homogenate was centrifuged at 15,000 X g for 20 min at 4 °C. The supernatant was collected for the measurement of protein and antioxidant enzyme activities.

Measuring of protein concentration

Total protein of shoots was measured according to Bradford (1976) method. Absorption intensity of extractions was determined in wavelength 595 nm and the results were reported according to mg/g fresh weight. Samples containing 0.1 g of the frozen leaves were homogenized in 2 ml of the sodium phosphate buffer solution (25 mM and pH=7). The homogenate was centrifuged at 15,000 X g for 20 min at 4 °C. The supernatant was collected to measure enzyme antioxidant activities.

Catalase activity

The CAT activity was assayed based on the rate of H_2O_2 decomposition (with an extinction coefficient of 36 mM^{-1} cm^{-1}) as measured by the decrease in absorbance at 240 nm, following the procedure of Aebi (1974). The reaction mixture contained 2 ml of the sodium phosphate buffer (25 Mm and pH=7), 100 µl of H_2O_2 (37%), and 50 µl of the extraction enzyme. One unit of catalase was defined as the amount of enzyme liberating half the peroxide oxygen from 10 mM/L of H_2O_2 solution in 100 sec at 25 °C.

Ascorbate peroxidase activity

Ascorbate peroxidase activity was determined based on the decrease in the absorbance at 290 nm (extinction coefficient equal to 2.8 mM^{-1} cm^{-1}). Reaction mixture contained 50 mM sodium phosphate buffer (pH=7), 0.5 mM ascorbic acid, 0.2 mM EDTA, 0.1 mM H_2O_2, and 100 µL of the enzyme extract (Nakano and Asada, 1981).

Superoxide dismutase activity

Superoxide dismutase activity was determined by adding 300 µL of the extracts to a mixture containing 50 mM of the sodium phosphate buffer (pH=7.8), 0.1 mM of EDTA, 50 mM of Na_2CO_3, 12 mM of L-methionine, 1 µM of riboflavin, and 75 mM of p-nitro blue tetrazolium chloride (NBT) in dark conditions. The reaction was carried out under illumination (30 W fluorescent lamp) at 25 °C for 10 min. The absorbance was measured at 560 nm. One SOD activity unit (AU) was defined as the amount of enzyme required to inhibit 50% of NBT photo reduction (Beauchamp and Fridovich, 1971) and expressed as unit/g FW.

Statistical Analysis

All experiments were in a completely random design with three replicates and the statistical significance was measured using the one-way analysis of variance test (ANOVA). Duncan's test was used to compare the mean values at $p \leq 0.05$. All statistical analyses were performed with SPSS software version 20.

Results

Drought stress significantly affected the amounts of chlorophylls as well as total carotenoid in the leaves of *C. capticum*. The amounts of chlorophyll and carotenoid in the stressed seedlings decreased during the four weeks of treatments in the stressed seedlings compared to the control (Fig. I. a-c). As shown in Figure (I. a), the amount of chlorophyll a decreased with increasing PEG concentration. However, no

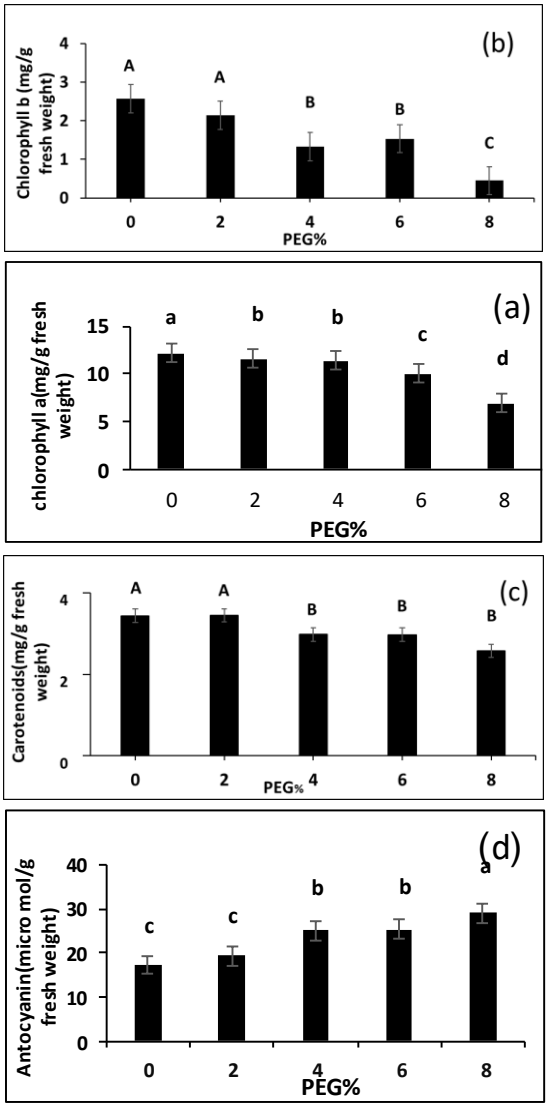

Fig. I. The effect of PEG on chlorophyll a (a), chlorophyll b (b), carotenoids (c), and anthocyanin (d) contents in the leaf of *C. copticum*; values represent the mean of three replicates and the dissimilar letters are significantly different according to Duncan's test (P≤ 0.05).

significant differences were observed in the leaf chlorophyll a contents among the levels of mild drought stresses (2, 4%). Also, chlorophyll b exhibited a significant decrease under 4% and severe drought treatments compared to the control (Fig. I. b). The lowest chlorophyll content was observed in the media containing 8% PEG and the lowest carotenoids were found in 4, 6, and 8% treatments (Fig. I. a-c) Finally, PEG treatments were found to increase the anthocyanin contents invariably under 4% and severe (6, 8%) stress conditions (Fig. I. d).

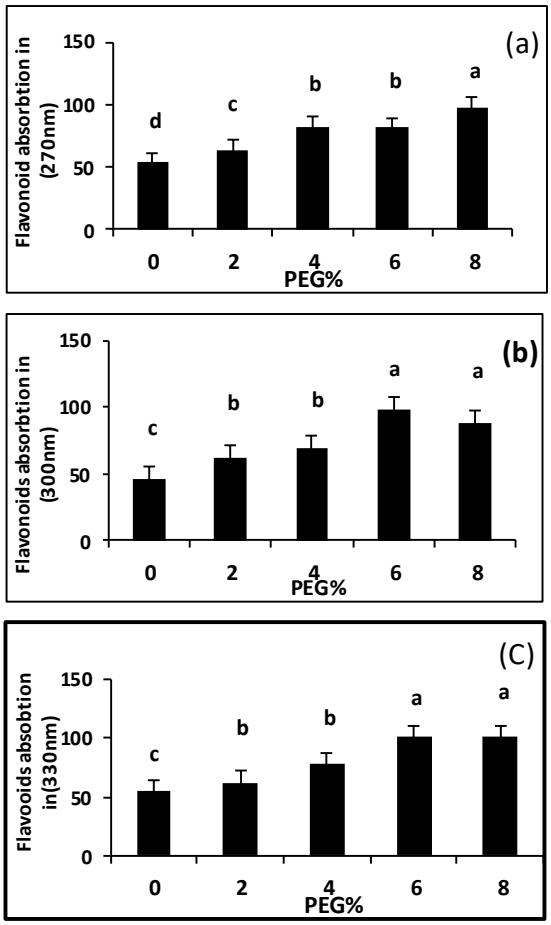

Fig. II. The effect of PEG on flavonoids in 270 nm (a), 300nm (b), and 330nm (c) in leaves of *C. copticum*; values represent the mean of three replicates and the dissimilar letters are significantly different according to Duncan's test (P ≤ 005).

Effects of polyethylene glycol on flavonoid contents

Flavonoid contents were investigated only in leaf samples. Based on the experimental data obtained, leaf flavonoid contents increased significantly at 270, 300, and 330 nm under osmotic stress conditions compared to the control. The highest leaf flavonoid content was observed at 8% PEG at 270 nm and the highest leaf flavonoid content was recorded at 6% and 8% PEG at wavelength of 300 and 330 nm (Fig. II).

Effects of polyethylene glycol on reducing sugar and proline and protein content

Reducing sugar and proline contents as compatible osmolytes were measured in the shoot

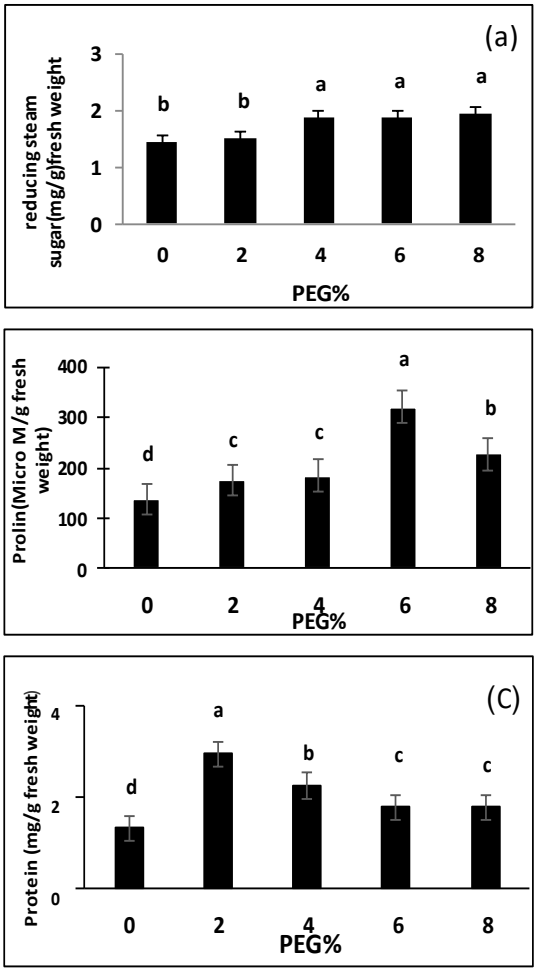

Fig. III. The effect of PEG on reducing sugar (a), proline (b), and protein (c) in leaves of *C. copticum*; values represent the means of three replicates and the dissimilar letters are significantly different according to Duncan's test (P ≤ 0.05).

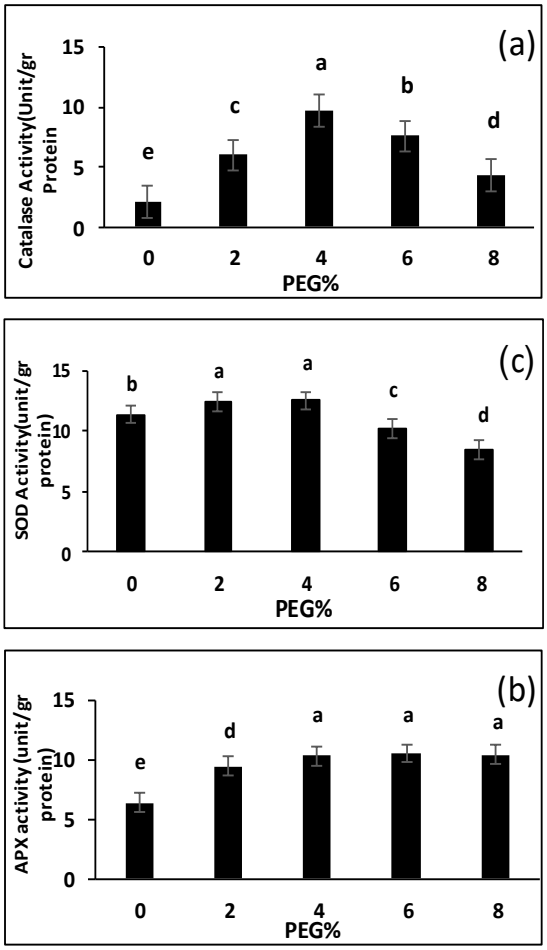

Fig. IV. The effect of PEG on CAT (a), APX (b), and SOD (c) in leaves of *C. copticum*; values represent the means of three replicates and the dissimilar letters are significantly different according to Duncan's test (P ≤ 005).

of control and PEG-treated seedlings. Drought induced a significant increase in the reducing sugar content. However, since a significant decrease in the tissue water content occurred in plants subjected to osmotic stress, the content of reducing sugars of shoots increased progressively with an increase in drought (Fig. III. a). Moreover, PEG induced an increase in the shoot proline content. Proline accumulation progressed during the osmotic treatments in relation to the severity of the drought stress. The highest increase in proline was observed at 6%. Unexpectedly, in plants under severe stress (8%), proline contents significantly decreased but this amount was still higher than the control (Fig. III. b). Protein contents in PEG-treated shoots increased significantly in comparison with control (Fig. III. c). Although, the results showed a reduction in protein content at 6% and 8%, still this was higher than the control and the highest amount was observed in 2% treatment.

Effects of polyethylene glycol on antioxidant enzymes activity

Drought stress induced a significant increase in the CAT activity of PEG-treated plants at 2, 4, 6 and 8% (Fig. IV. a). The lowest and the highest CAT activities were observed at the stressor concentrations of 0 and 4%, respectively (Fig. IV. a). Results showed that the osmotic stress led to a significant increase in the APX activity of *C. capticum* (Fig. IV. b). As shown in Figure IV. B), minimum APX activity in the PEG-treated seedlings was observed at control, which then

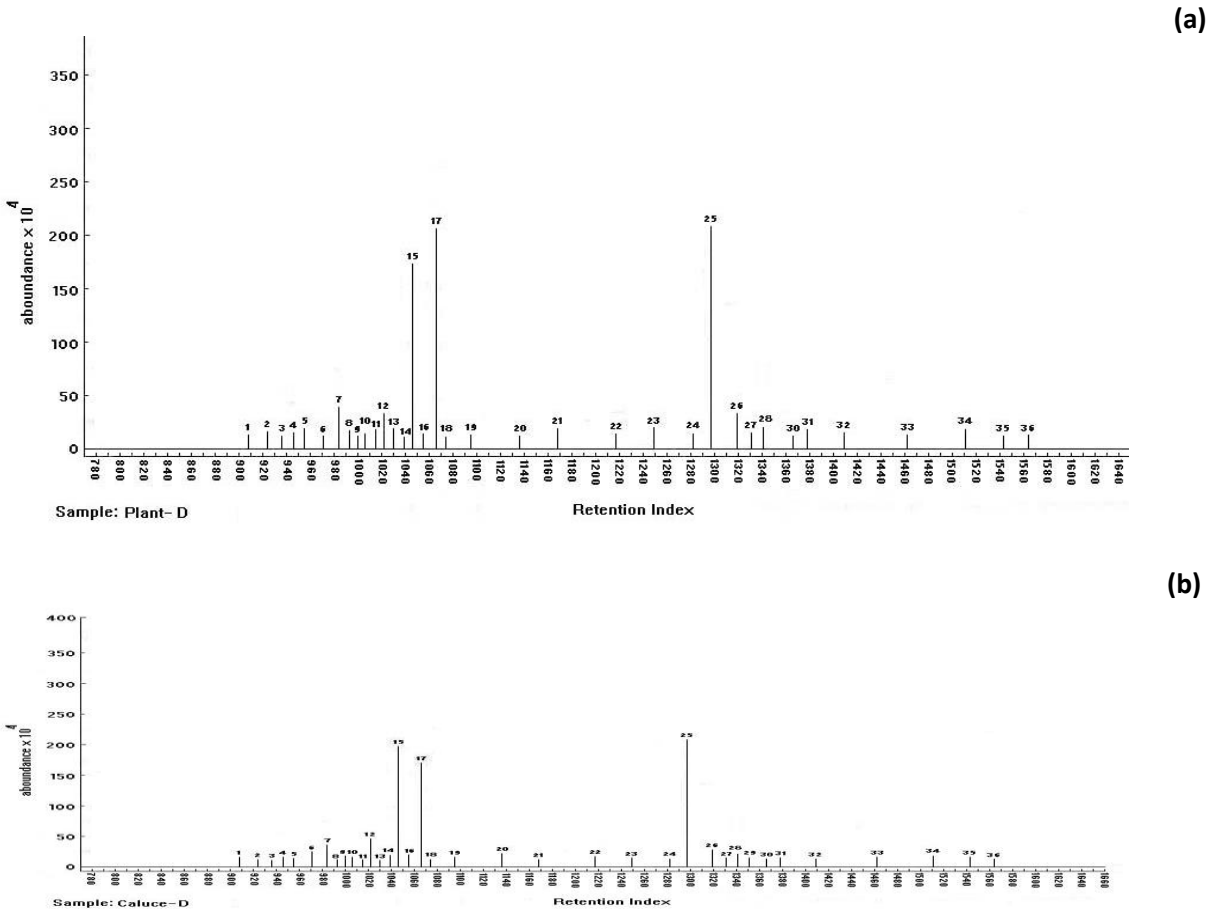

Fig. V. The effect of polyethylene glycol on essential oil constituents of *C. copticum* in plant (a) and callus (b) by GC MS method

increased at the other treatments (2, 4,6,8%). Drought stress increased the SOD activity in leaves at the stressor concentrations of 2 and 4% and decreased it at 6 and 8% compared to the control (Fig. IV. c).

Effects of PEG on essential oil of callus and seedlings

The composition of the essential oil of calluses and seedlings of *C. copticum* under 0 and 8% PEG treatments included 36 components, the main ones included γ-terpinene, thymol, and p-cymene. The effect of water deficit stress on the essential oil components of *C. copticum* shoots and calluses was variable (Table 1). Among these main aroma constituents of *C. copticum*, the content of γ-terpinene decreased in the treated calluses and seedlings, as compared to control while thymol and p-cymene increased at 8% PEG in seedlings and calluses. As seen in Table 1, Methyl Eugenol was not detected in seedlings under drought stress (8% PEG). Moreover, PEG caused a significant increase in some constituents of seedlings exposed to PEG and reduced β-Caryophyllene and sabinene in the treated calluses. By contrast, some constituents such as Hexa decanoic acid and α-Fenchen increased in the PEG-treated calluses while they decreased in the treated seedlings.

Discussion

The aim of this study was to investigate the effects of drought stress induced by PEG on growth, secondary metabolites, accumulation of some solutes, and enzymatic mechanisms in the *C. copticum*. Chlorophyll contents decreased significantly by increasing severity of drought stress. The lowest chlorophyll *a* and *b* accumulations were observed at 8%. Severe drought stress in two *Populus cathayana* populations showed chlorophyll contents usually decreases under drought stress due to their slow

Table 1
Constituents of *C. copticum* essential oil using mass spectrometry (GC-MS)

	Pic. No	RI	Callus Control	Callus PEG		Seedlings Control	Seedlings PEG	
1,3-octadien	1	909	a0.8	1.2	⇑	1.0	0.9	⇓
Pyridine	2	924	a1.1	0.9	⇓	0.7	1.1	⇑
α-Thujune	3	936	1.2	0.8	⇓	1.1	0.8	⇓
α-Pinene	4	947	1.3	1.1	⇓	1.8	1.0	⇓
Comphene	5	955	0.9	1.0	⇑	0.8	1.2	⇑
Sabinene	6	971	1.0	1.4	⇑	1.7	0.8	⇓
β-Pinene	7	984	4.5	2.1	⇓	5.6	3.7	⇓
a3-Ocatnone	8	993	1.1	0.8	⇓	1.3	1.2	⇓
Myrcene	9	999	0.8	1.1	⇑	0.9	0.8	⇓
3-Carene	10	1007	1.3	1.0	⇓	1.2	1.0	⇓
1,8-Cineol	11	1015	1.1	0.7	⇓	0.9	1.2	⇑
α-Phellandrene	12	1023	2.5	3.8	⇑	2.3	2.4	⇑
α-Fenchen	13	1030	1.0	0.8	⇓	1.1	1.3	⇑
α-Terpinene	14	1039	0.7	1.1	⇑	1.0	0.8	⇓
p-cymene	15	1046	15.3	19.8	⇑	13.4	17.6	⇑
Limonene	16	1055	1.6	1.4	⇓	1.2	1.0	⇓
γ-Terpinene	17	1066	23.3	17.8	⇓	25.2	21.0	⇓
Sabinene hydrate	18	1074	0.9	0.8	⇓	1.0	0.8	⇓
Terpinolene	19	1095	1.2	1.0	⇓	1.6	1.1	⇓
Linalool	20	1136	0.9	1.4	⇑	1.1	0.9	⇓
Menta-3-8-diene	21	1168	0.8	0.9	⇑	1.0	1.3	⇑
α-Compheonelal	22	1217	1.0	1.1	⇑	1.4	1.0	⇓
Verbenone	23	1249	1.4	1.0	⇓	0.9	1.4	⇑
Borneol	24	1282	1.1	0.8	⇓	1.2	1.0	⇓
Thymol	25	1297	18.3	23.9	⇑	15.5	21.2	⇑
Carvacrol	26	1319	3.1	2.1	⇓	3.6	3.2	⇓
Piperitenon oxide	27	13٢2	1.5	1.0	⇓	0.8	1.1	⇑
Candinol	28	1341	0.9	1.4	⇑	1.7	1.4	⇓
Methyl Eugenol	29	1352	1.3	1.1	⇓	1.3	--	
β-Caryophyllene	30	1366	0.8	0.9	⇑	1.2	0.8	⇓
Hexadecanoic acid	31	1378	1.2	1.0	⇓	0.9	1.3	⇑
α-Hummulene	32	1409	1.1	0.8	⇓	1.1	1.0	⇓
γ-Cadinene	33	1463	0.9	0.8	⇓	1.0	0.9	⇓
Germacrene D	34	1511	0.8	1.2	⇑	0.8	1.2	⇑
Caryophyllen epoxide	35	1543	1.0	1.0		0.9	0.8	⇓
α-Bisabolol	36	1564	1.2	0.7	⇓	1.1	1.0	⇓

synthesis or fast breakdown. The other factor which reduces the content of chlorophyll of the plants under drought stress is reactive oxygen species production that causes lipid peroxidation and chlorophyll degradation. In this study, proline was increased by increasing PEG concentrations to protect the enzymes and cytoplasm proteins. These results agreed with the previous studies that reported proline was enhanced in sunflower under drought stress. Proline, which is a compatible solute, regulates osmotic pressure, protects protein molecules and cell membrane, and integrates and scavenges free radicals. Also, the amount of soluble sugar increased with increasing PEG concentration. Soluble sugars, which are compatible solutes, not only protect cells by osmotic adjustment but also cause stability of the cell membranes and protect proteins through the formation of hydrogen bonds between their carboxyl groups and polar chains of proteins (Ingram and Bartels, 1996). Soluble sugar content increased under water deficient in *Melissa officinalis* (Nadiu and Naraly, 2001), *Brassica* (Hisao, 1973), and *Pigeon pea* (Ingram and Bartels, 1996). There are some reports that show complex carbohydrates may break down into simple carbohydrates under drought stress. Also under drought stress conditions, increase in the ratio of sucrose to insoluble sugars, decomposition of insoluble sugars and reduction in the transfer of sucrose from leaves result in an increase in carbohydrates content in plants which plays an important role in adjusting osmotic pressure of leaves in response to drought stress in many plants (Pereira et al., 1993). Active oxygen radicals change amino acid positions in protein

strands and facilitate the effect of protein degrading enzymes on them. Drought stress affects nitrogen metabolism in plants and other studies have shown that under drought stress the process of hydrolysis of proteins begins, which leads to an increase in amino acids in the plant (Kramer, 1983).

In this study, protein content increased with drought stress and it also increased in *Lippia citriodora* (Mohammadi et al., 2014) and *Triticum aestivum* L (Saeidi et al., 2011*)* under drought stress*.* Flavonoids are one of the most important phenolic groups that can prevent oxidative stresses because they have the potential to purify active oxygen species. In this study, the amount of flavonoids increased significantly as PEG concentration increased. Flavonoids also increased as a secondary metabolite under water stress conditions in *Brassica napus* (Sangtarash et al., 2009) and *Eucalyptus camaldulensis* (Schwabach et al., 2008). When the plants are exposed to environmental stresses such as drought, the balance between the production and elimination of active oxygen species such as superoxide, hydrogen peroxide, hydroxyl radicals, and single oxygen disturbed. ROS affect lipid peroxidation processes and damage proteins and DNA. In plant cells, ROS scavenging enzymes such as SOD, CAT, and APX regulate the levels of intracellular H_2O_2. In this study, superoxide dismutase was higher at 4 and 2% PEG than control because superoxide molecules increased and so did the expression of superoxide dismutase gene. There was a significant reduction in SOD activity at 8% PEG because superoxide enzyme failed to decompose hydrogen peroxide or the cell was not successful in producing more enzymes. In wheat, the superoxide dismutase activity increased or remained unchanged at the beginning of the stress period but it decreased in more severe stress conditions (Zhang et al., 2010). The prolongation of drought stress reduced the activity of superoxide dismutase and catalase in leaves of three species of *Agrostis stolonifera* (Dacosta and Huang, 2007). Catalase and ascorbate peroxidase are the most important enzymes that collect hydrogen peroxide in the cell and reducing their activities leads to the accumulation of hydrogen peroxide, reduction of the Calvin cycle enzymes such as ribulose monophosphate kinase and phosphatase, and increase in the active oxygen species which damage biomolecules, such as lipids (Mittler et al., 2004).

In this study, catalase and ascorbate peroxidase enzymes increased significantly in all concentrations compared to the control group. Studies on sunflower seedlings showed that CAT activity in drought stress conditions was more than in normal conditions (Manivann et al., 2008). In water stress condition, CAT increased to decompose hydrogen peroxide which was increased by SOD enzyme activity.

Secondary metabolites biosynthesis is regulated through genetic pathways but environmental factors affect their biosynthesis and function. A hypotheses have been developed about the effects of environmental conditions on plant secondary metabolites. Accordingly, the balance between photosynthesis and growth and indicates when a factor limits growth more than photosynthesis. For example, moderate drought, moderate nutrient limitation, and low temperature will increase the carbon pool available for allocation to secondary metabolism.

In this study, essential oil percentage was affected by drought stress applied by PEG treatments on seedling and calluses. The present investigation showed that thymol, γ-terpinene, and p-cymene are the main components of the essential oils in *C. copticum* seedling and calluses. 8% PEG treatment significantly increased the amounts of thymol and γ-terpinene and decreased the concentration of p-cymene. Drought stress was found to increase the levels of essential oils in the treated seedling and calluses comparedto the control. Stresses and stimuli had a significant effect on phenol levels in tissue culture. In this work, a total of 36 metabolites were identified in seedlings and calluses of *C. copticum* and some of these substances were significantly increased under PEG treatment. The elevated compounds belonged to terpenoids indicating an increase in the metabolic activity within mevalonate pathways.

According to the results, the use of PEG under *in vitro* culture can increase the pharmaceutical compounds of *C. copticum*. In conclusion, *C. copticum* is relatively successful to combat drought stress. Drought stress influenced

secondary metabolite production in this plant. Therefore, *C. copticum* is useful for the *in vitro* production of pharmaceuticals and other beneficial substances by plant tissue culture techniques under drought stress.

Conclusion

In the present research, PEG induced some adaptive defense mechanisms in *C. copticum.* The amounts of flavonoids, protein, proline, and sugar increased under PEG treatments. Also, the activities of CAT and APX enzymes increased in all concentrations of PEG in comparison to the control. In addition, this study indicated that adding PEG increased thymol and some essential oil components in seedlings and calluses of *C. copticum*.

References

Aebi, H., 1984. 'Catalase in vitro'. *Method Enzym*e, 105: 121-126.

Bates, L. S., R. P. Waldern and **I. D. Teare,** 1973. 'Rapid determination of free proline for water stress studies'. *Journal of Plant Nutrition and Soil Science*, 39 (1): 205-207.

Beauchamp, C. and **I. Fridovich,** 1971. 'Superoxide dismutase: Improved assay applicable to acrylamide gels'. *Annual Biochemistry*, 44: 276-287.

Bradford, M. M., 1976. 'A rapid sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding'. *Analytical Biochemistry*, 72: 248-254.

Cruz, F. J. R., G. L. S. Castro, D. D. S. Júnior and **H. A. Pinheiro,** 2013. 'Exogenous glycine betaine modulates ascorbate peroxidase and catalase activities and prevent lipid peroxidation in mild water stressed *Carapaguianensis* plants'. *Photosynthetica*, 51: 102–108.

Da Costa, M. and **B. Huang, 2006.** 'Minimum water requirements for creeping, colonial and velvet bent grasses under fairway conditions'. *Crop Science Society of America*, 46: 81–89.

Flexas, J., J. Bota, F. Loreto, G. Cornic and **T. D. Sharkey,** 2004. 'Diffusive and metabolic limitations to photosynthesis under drought and salinity in C3 plants'. *Plant Biology*, 6: 1–11.

Herms, D. A. and **W. J. Mattson,** 1992. 'The dilemma of plants: to grow or defend'. *The Quarterly Review of Biology*, 67: 283 – 325.

Hsiao, T. C., 1973. 'Plant responses to water stress'. *Annual Review Plant Physiology*, 24: 519-570.

Ingram, J. and **D. Bartels,** 1996. 'The molecular basis of dehydration tolerance in plants'. *Annual Review of Plant Physiology and Plant Molecular Biology*, 47: 377- 403.

Jaleel, C. A., B. Sankar, P. V. Murali, M. Gomathinayagam, G. M. A. Lakshmanan and **R. Panneerselvam,** 2008. 'Water deficit stress effects on reactive oxygen metabolism in *Catharanthus roseus*; impacts on ajmalicine accumulation'. *Colloids and Surface Bio Interfaces*, 62: 105–111.

Kramer, P. J. and **J. S. Boyer.** 1995. *Water Relations of Plant and Soils*.San Diego: Academic Press.

Kotzé, M. and **J. N. Eloff,** 2002. 'Extraction of antibacterial compound from *Combretum microphyllum* (combretaceae)'. *South African Journal of Botany*, 68: 62-67.

Li, C. Z. and **G. X. Wang,** 2004. 'Interactions between reactive oxygen species, ethylene and polyamines in leaves of *Glysyrrhiza inflate* seedlings under root osmotic stress'. *Journal of Plant and Growth Regulation*, 42: 55-60.

Lichtenthaler, H. K., 1987. 'Chlorophylls and carotenoids: pigments of photosynthetic bio membranes'. *Methods Enzymology*, 148: 350-382.

Manivannan, P., C. A. Jaleel, R. Somasundaram and **R. Panneerselvam,** 2008. 'Osmoregulation and antioxidant metabolism in drought-stressed *Helianthus annuus* under triadimefon drenching. *Comptes Rendus Biologis*, 59: 150-157.

Mirzavand, S. 1992. *Evaluation and comparison macroscopic, microscopic and phytochemical properties of Anise, Foeniculum vulgare and Trachyspermum copticum Fruits*. M. Sc. thesis, Isfahan University of Medical Science., Isfahan, Iran.

Mittler, R., S. Vanderauwera, M. Gollery and **F. V. Breusegem,** 2004. 'Reactive oxygen gene network of plants'. *Trends in Plant Science*, 9: 490-498.

Murashige, T. and **F. Skoog**, 1962. 'A revised medium for rapid growth and bioassays with tobacco tissue cultures'. *Physiologia Plantarum*, 15: 473-497.

Nadiu, T. and **A. Naraly,** 2001. 'Screening of drought tolerance in green gram (*Vina radiata* L. *Wilczeek*) genotypes under receding soil moisture'. *Indian Journal of Plant Physiology*, 6 (2): 197-201.

Nakano, Y. and **K. Asada,** 1981. 'Hydrogen peroxide is scavenged by ascorbate peroxidase in spinach chloroplasts'. *Plant Cell Physiology*, 22: 867 – 880.

Pereira, J. S., M. M. Chaves, F. Fonseca, M. C. Araujo and **F. Torres,** 1992. 'Photosynthetic capacity of leaves of *Eucalyptus globulus* (Labill.) growing in the field with different nutrient and water supplies'. *Tree Physiology*, 11: 381–389.

Razdan, M. K., 2003. *Introduction to Plant Tissue Culture*. Science Publishers Inc.

Sangtarash, M. H., M. M. Qaderi, C. C. Chinnappa and **D. M. Reid,** 2009. 'Carotenoid differential sensitivity of canola (*Brassica napus*) seedlings to ultraviolet -b radiation, water stress and abscisic acid'. *Environmental and Experimental Botany*, 66 (2): 212-219.

Schwambach, J., C. M. Ruedell, M. R. de Almeida, R. M. Penchel, de Araújo and **A. G. Fett-Neto,** 2008. 'Adventitious rooting of *Eucalyptus* glubus×maidennii mini-cutting derived from mini-stumps grown in sand bed and intermittent flooding trays comparative study'. *New Forests*, 36 (3): 261-271.

Somogyi-Nelson, M., 1952. 'Notes on sugar determination'. *Journal Biology Chemistry*, 195: 19-29.

Tajkarim, M. M., S. A. Ibrahim and D. O. Cliver, 2010. 'Antimicrobial herb and spice compounds in food'. *Food Control*, 21: 1199-18.

Vahidi, H., M. Kamalinejad and **N. Sedaghati,** 2002. 'Antimicrobial properties of *Croccus sativus* L'. *Iranian Journal of Pharmaceutical Research*, 1: 33-35.

Wagner, G. J., 1979. 'Content and vacuole/extra vacuole distribution of neutral sugars, free amino acids and anthocyanin in protoplast'. *Plant Physiology,* 68: 88-93.

Zhang, K. M., H. J. Yu, K. Shi, Y. H. Zhou, J. Q. Yu and **X. J. Xia**, 2010. 'Photo protective roles of anthocyanins in *Begonia semperflorens*'. *Plant Science*, 179 (3): 202-208.