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Comparative study of osmotic stress effects on the defense mechanisms and secondary metabolites in *Carum copticum* seedling and callus

Roya Razavizadeh[1*], Fatemeh Adabavazeh[1], Fatemeh Rostami[1] and Abbas Teimouri[2]

[1]Department of Biology, Payame Noor University, PO BOX 19395-3697 Tehran, Iran.
[2]Department of Chemistry, Payame Noor University, PO BOX 19395-3697 Tehran, Iran.
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Abstract:

Carum copticum* L. is a medicinal plant of the Apiaceae family with medicinal properties. In this study, the effects of drought stress on the photosynthetic pigments and essential oils as well as the enzymatic and non-enzymatic mechanisms of the seedlings and callus of *C. copticum* were investigated within the framework of a controlled experiment under *in vitro* conditions. For this purpose, the seedlings of *C. copticum* were cultured in the Murashige and Skoog medium containing three levels (0, 3, and 6%) of either sorbitol or mannitol. Furthermore, its calli were independently cultured in the Murashige and Skoog (MS) medium containing 0.25 mg.L^{-1} 2, 4-dichlorophenoxyacetic acid, 1 mg.L^{-1} benzyl amino purine, and different levels (0, 3, and 6%) of either sorbitol or mannitol. Both sorbitol and mannitol were found to reduce the photosynthetic pigments and carotenoid contents but increased the anthocyanin and flavonoid contents significantly. However, these effects were significantly more severe in plants submitted to mannitol, as compared to the sorbitol-treated ones. With the exception of the mannitol-treated leaf samples whose phenolic contents decreased with increasing drought concentration in the culture media, the phenolic contents in the other samples increased relative to those in the control samples. The ascorbate peroxidase activity was found to decrease in all the treated samples while the catalase activity greatly increased, particularly in the mannitol-stressed plants. Compared to the control, drought led to a significant increase in superoxide dismutase activity in all the samples treated with 6% sorbitol and in the calli samples treated with 3% mannitol, whereas the seedlings grown under mannitol treatment showed almost no difference with the control plants. In addition, drought stress changed the essential oil compounds of both the seedlings and the calli of *C. copticum*. Analysis of the essential oil constituents by Gas chromatography–Mass Spectrometry (GC–MS) showed thymol, gamatherpinen, and parasimen to be the main essential oil components which increased in quantity under high stress conditions. These bioactive compounds have many industrial and medicinal applications.

Keywords: Antioxidant enzymes, Callus, *Carum copticum*, Drought stress, Thymol.

Introduction:

Crop production in the modern world is facing increasing challenges as it is strongly affected by both biotic and abiotic stresses. Among the abiotic ones, drought is a major agronomic threat to crop growth and yield, especially in arid and semi-arid areas, as it reduces productivity by inhibiting plant growth and photosynthesis (Taiz and Zeiger, 1998). Reduced photosynthesis is one of the negative effects of water deficit, which is caused by stomata closure, limitations on CO_2 diffusion within the leaf tissues, and mesophyll cell dehydration under drought conditions (Flexas *et al*., 2004).

Under such conditions, plants generate reactive oxygen species (ROS) as one of their earliest responses to stress. ROS act as toxic compounds in the cell causing lipid peroxidation with such dire consequences as membrane injuries, protein degradation, enzyme inactivation, and photosynthesis suppression (Srivastava and Dubey, 2011). Despite their destructive activity, ROS are well-described second messengers in a variety of cellular processes including tolerance to environmental stresses (Yan and Tsuichihara, 2007). They can induce the expression of detoxification and stress protection genes such as heat shock proteins (HSPs), glutathione- S-transferases (GSTs), peroxidases, superoxide, and pathogenesis-related proteins, thus protecting plants from stress damages (Heidarvand and Maali-Amiri, 2013).

Plants detoxify the free radicals and peroxides with such varied protective proteins as dehydrins, antioxidants, and secondary metabolites to prevent damages caused by ROS to other proteins and cell membranes (Pardo, 2010). Secondary metabolites such as anthocyanin, phenolic compounds, and flavonoids have important ecological functions within the defense and protection mechanisms of plants. They are likely to be osmoregulators that, together with compatible solutes such as sugars and proline, not only maintain water homeostasis but also scavenge ROS produced upon abiotic stresses in various plant species (Hughes *et al*., 2013).

*****Corresponding Author, Email: razavi.roya@gmail.com

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The enzymatic systems that detoxify free radicals are divided into two categories: one that reacts with ROS and keeps them at low levels (including peroxidase, superoxide dismutase, and catalase), and one that regenerates the oxidized antioxidants (including ascorbate peroxidase and glutathione reductase) (Smirnoff, 1993).

Carum copticum L. is a member of the Apiaceae family, with a variety of essential oils such as thymol, paracymene, and gamma-terpinene (Yassa *et al*., 2003). Essential oil is one of the most active and effective components of *C. copticum* commonly used for its carminative, antiseptic, amoebiasis expectorant, antimicrobial, antiparasitic, antiplatelet-aggregatory, and antilithiasis effects (Zarshenas *et al*., 2013). This plant is one of the most important aromatic plants cultivated in various regions such as Iran, Egypt, Pakistan, India and Europe (Shojaaddini *et al*., 2008).

Field cultivation of medicinal plants and crops in Iran is in most cases uneconomical due to the dry climate characterized by low precipitation and severe droughts in some areas. Often in the field, plants have to endure different abiotic stresses such as heat, drought, and salinity. This situation warrants the use of new biotechnological methods such as *in vitro* cultured plant tissues and cells to produce drought-resistant plants and, thereby, to enhance crop productivity. Tissue culture techniques and applications in agriculture have become an integral tool in crop improvement, commercial production of natural compounds, and development of new transgenic plants. In addition, it is a novel approach that can be exploited to study the stress tolerance mechanisms under *in vitro* conditions and to provide excellent environments for the investigation of biochemical and metabolic pathways (Karuppusamy, 2009). Simulation of drought stress under *in vitro* conditions constitutes a convenient means of studying the effects of drought on plant biochemical, physiological, and metabolic responses. Mannitol and sorbitol are the two osmotic regulators used in plant tissue culture for the identification of drought-tolerant plants (Hassanein and Dorion**,** 2006). They act as growth retardants by causing osmotic stress to the material under conservation.

Given the fact that identification of plant responses to environmental stresses is the key challenge to modern biological research, the present work was designed and implemented to investigate the effects of drought stress induced by sorbitol and/or mannitol as osmotic agents on the photosynthetic pigments and essential oils as well as the non-enzimatic and enzymatic defense mechanisms in *C. copticum* seedling and callus under *in vitro* culture.

Materials and methods:

Mature and sterilized seeds of *C. copticum* were cultured in the MS medium (Murashige and Skoog, 1962) and kept in the growth chamber (16 h light/8 h dark) at a temperature of 25 °C and a relative humidity of 95%. Stem explants with nodes were excised after 4 weeks from the seedlings to be directly cultured separately on the MS medium supplemented with different concentrations (0, 3, and 6%) of sorbitol or mannitol. Four weeks after the treatment, the effects of drought stress were studied on the photosynthetic pigments, secondary metabolites (carotenoid, anthocyanin, flavonoid, and thymol), and enzymatic systems (Catalase (CAT), Ascorbate peroxidase (APX), and Superoxide dismutase (SOD)) of the leaves.

Callus initiation under drought stress: Four-week-old growing seedlings were used as the source of explants. Stem explants 0.5 cm long were cultured on the MS medium containing a combination of 0/25 $mg.L^{-1}$ 2, 4-D (2, 4-dichlorophenoxyacetic acid) and 1 $mg.L^{-1}$ BAP (benzyl amino purine). Callus induction was observed after a few days. The explants were subcultured after 4 weeks on the same medium and maintained in the dark for 4 weeks at 25 ± 2 ºC. Callus subculture was repeated on a monthly basis not only to increase callus production but also to avoid depletion of essential nutrients and drying out of the gel. Another point of concern was to avoid the accumulation of the metabolites secreted by the callus to toxic levels in the medium. After 3 subcultures, the calli were transferred into the relevant MS medium supplemented with different concentrations (0, 3, and 6%) of either sorbitol or mannitol as required. After four weeks, the essential oils and enzymatic systems (CAT, APX, and SOD) were analyzed in the calli of *C. copticum* according to procedures used for the seedlings. Each trait of interest was measured using the pertinent producer.

Chlorophyll and carotenoid: Chlorophyll and carotenoids were determined according to the Lichtenthaler (1987) method. Briefly, 0.2 g of the frozen leaf from the seedlings was homogenized in 15 ml of acetone 80%. This solution contained chlorophyll a and b as well as carotenoids. The absorbance of each sample with three replications was measured at 646.8, 663.20, and 470 nm using a UV-visible spectrophotometer (U-6305 model, Jenway, UK). The measured amounts of chlorophylls and carotenoids were expressed as mg. g^{-1} fresh weight.

A**nthocyanin:** Total anthocyanin was determined according to the modified Wagner (1979) method using acidified ethanol (Methanol: HCl 99: 1 v/v). For this purpose, samples each containing 0.05 g of frozen leaf and callus were homogenized in 5 ml of acidified ethanol and kept at 25°C for 24 h in the dark. Each extract was centrifuged at 4000 g for 10 min at room temperature. The absorbance of each supernatant was measured at 550 nm using a UV-visible spectrophotometer (U-6305 model, Jenway, UK). The extinction coefficient 33,000 (mM^{-1} cm^{-1}) was used to calculate the amount of total anthocyanin expressed as $\mu mol.g^{-1}$ fresh weight.

Total Phenolic Content: Total phenolic content was determined according to the Sonald and Laima (1999) method. Briefly, samples containing 0.1 g of

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frozen leaf and callus were homogenized in 5 ml of ethanol 95% and kept at 25°C for 24-72 h in the dark. A diluted sample extract (1 mL) was mixed with ethanol 95% (1 ml), 3 ml of distilled water, Folin 50% (0.5 ml), and aqueous sodium carbonate 5% (1 ml). After 1h, the absorbance of each sample was measured at 725 nm using a UV-visible spectrophotometer (U-6305 model, Jenway, UK). Gallic acid was used to construct the standard curve. Results were expressed as mg gallic acid.g^{-1} fresh weight.

Flavonoid: Flavonoid concentration was measured using the spectrophotometric method adapted from the one described in Krizek *et al*. (1998). In this procedure, leaf disks were prepared and ground in mortars filled with acid ethanol (Ethylic alcohol and Acetic acid glacial in 99:1 vol/vol.). The extract was treated for 10 minutes in a warm bath under 80°C upon centrifugation. Absorption spectrum was recorded at the wavelengths of 270, 300, 330 nm using a UV-visible spectrophotometer (U-6305 model, Jenway, UK).

Essential oils: The components present in the essential oils of *C. copticum* were determined by gas chromatography–mass spectrometry (GC–MS) using a Hewlett-Packard 5890 gas chromatograph (GC) equipped with a flame ionization detector (HP-5970 mass-selective detector-USA) and a 50 m × 0.20 mm HP-5 (cross-linked Phenyl–Methyl Silicon) column with a film thickness of 0.25 µm. The flame ionization detector (FID) was maintained at 250 ºC. In addition, the ionization energy was set to 70 eV. The temperature of the program ranged over 100–250 °C changing at a rate of 4 °C/ min. Helium was used as the carrier gas while the flow through the column and the split ratio were set to 1 ml/min and 100:1, respectively. Identification was based on the sample retention time and the recorded mass (Davies, 1990).

Enzyme extraction and assay: Samples containing 0.1 g of 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 × 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 of 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 mmol/L of H_2O_2 solution in 100 sec at 25 °C.

Ascorbate peroxidase activity: This activity was determined based on the decrease of 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), 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: This activity was determined by adding 300 µL of the extracts to a mixture containing 50 mM of the soduim 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 µM of p-nitro blue tetrazolium chloride (NBT) in dark conditions according to the procedure described in Giannotolitis and Ries (1997). The reaction was carried out under illumination (a 30 W fluorescent lamp) at 25 °C for 10 min. Absorbance was measured at 560 nm. One SOD activity unit (AU) was defined as the amount of enzyme required to inhibit 50% of NBT photoreduction (Beauchamp and Fridovich, 1971) and expressed as unit.g^{-1} fresh weight.min^{-1}.

Statistical analysis: All the experiments were performed in a completely randomized design with three replicates. A one-way ANOVA was used for the treatment assay and the Duncan tests were used to compare the mean values.

Results:

Drought significantly affected the amounts of chlorophylls a and b as well as total carotenoid in the leaves of *C. caticum*. The amounts of chlorophyll and carotenoid in the stressed seedlings decreased during the four weeks of treatment in the stressed seedlings compared to the control ones in which they either showed no change or increased (Fig. 1a-d). As shown in Figure 1a, the amount of chlorophyll a decreased with increasing mannitol or sorbitol concentration. However, no significant differences were observed in the leaf chlorophyll a contents among the different levels of drought stress. Neither was any difference observed between mannitol and sorbitol treatments with respect to this parameter. While chlorophyll b exhibited a significant decrease in the mannitol-treated seedlings under severe drought treatments, no difference was observed in the chlorophyll content with different concentrations of sorbitol (Fig. 1b). Even though both treatments led to reduced total chlorophyll, this effect was significantly more severe in the seedlings subjected to mannitol treatment. The highest total chlorophyll content was observed in the control seedlings (Fig. 1c). Finally, both sorbitol and mannitol treatments were found to reduce the carotenoid content invariably under the mild (3%) and severe (6%) stress conditions (Fig. 1d).Under the control treatment, leaf anthocyanin content remained low but exhibited an increasing trend during the whole treatment period. A significant difference was found between the mannitol and sorbitol treatments so that the highest anthocyanin was observed in the mannitol-treated plants (Fig. 2a). A similar trend in anthocyanin content was observed in the callus

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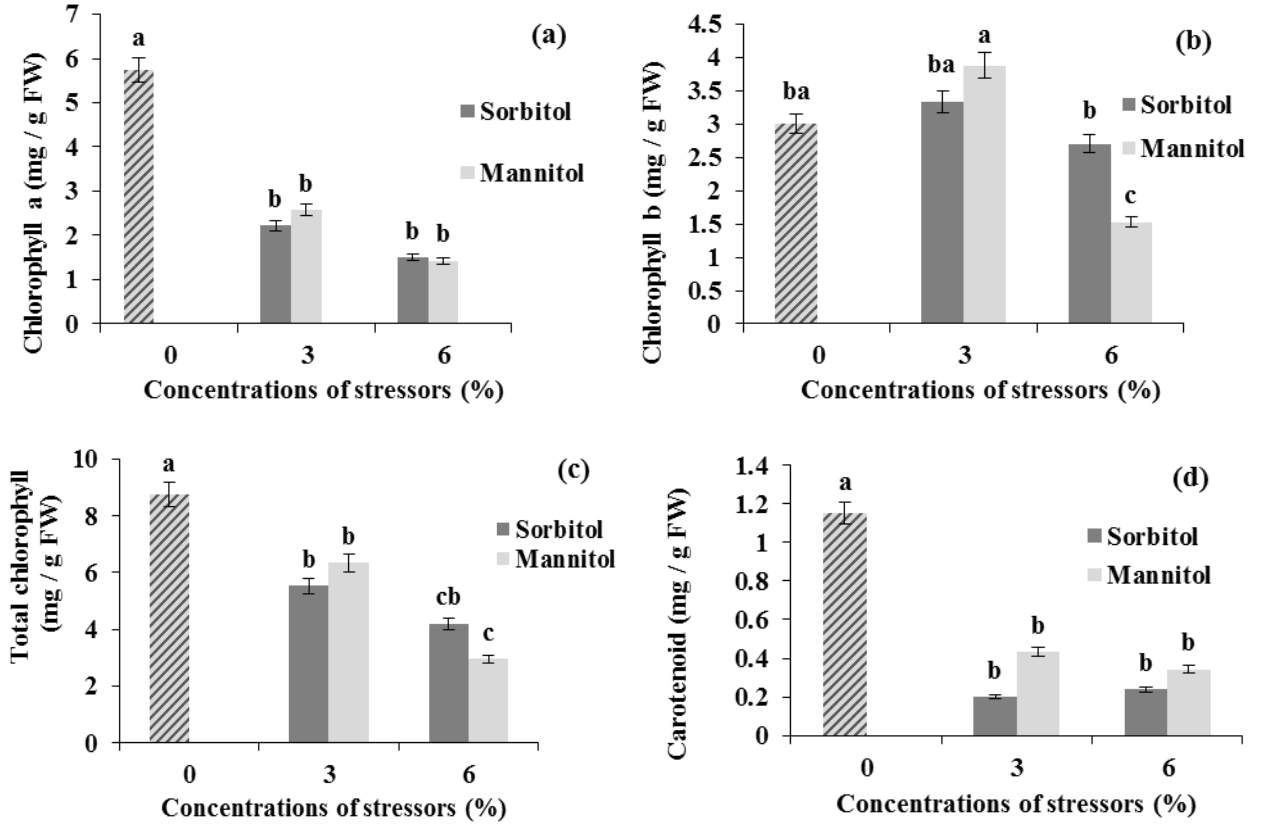


Figure 1. Effects of sorbitol and mannitol on chlorophyll a (a), chlorophyll b (b), total chlorophyll (c), and carotenoids (d) of *C. copticum*. Values represent means of three replicates and dissimilar letters are significantly different based on the Duncan's test (P ≤ 0.05).

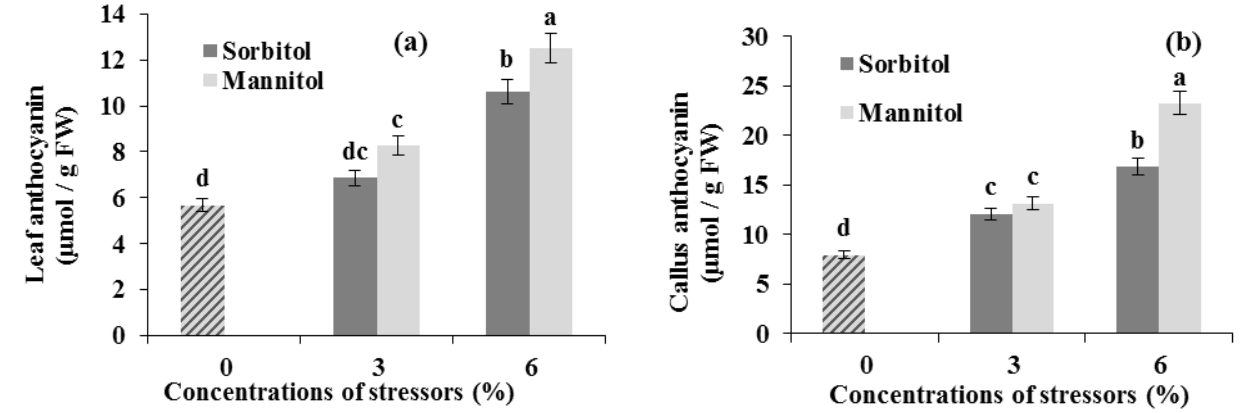


Figure 2. Effects of sorbitol and mannitol on anthocyanin content in leaf (a) and callus (b) of *C. copticum*. Values represent means of three replicates and dissimilar letters are significantly different based on the Duncan's test (P ≤ 0.05).

samples (Fig. 2b).

Fig (3) shows the variations in the total phenolic contents of *C. copticum* callus and seedling samples exposed to different concentrations of mannitol or sorbitol Compared to the control plants, leaves in the sorbitol treatment recorded a significant increase in their total phenolic content, with the highest observed with a sorbitol concentration of 3%. In contrast, increasing mannitol concentration in the culture media led to reduced phenolic content in the leaf (Fig 3a). Compared to the control, the calli also showed a significant increase in their total phenolic content with increasing drought concentrations in the culture media (Fig 3b).

Flavonoid content was investigated only in leaf samples. Based on the experimental data obtained, leaf flavonoid content increased significantly at 270, 300, 330 nm under osmotic stress conditions compared to the control conditions. At all the three wavelengths tested, the highest leaf flavonoid content was observed at a stressor concentration of 6% and at a wavelength of 270 nm in the sorbitol-treated plants and at 300 nm in the mannitol-treated ones; at 330 nm, however, no significant difference was observed between the two treatments (Fig. 4).

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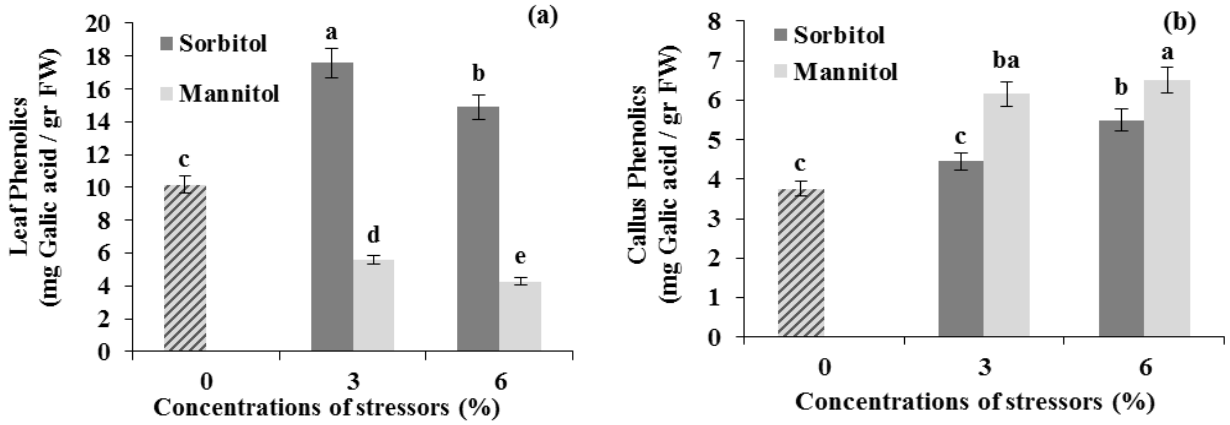


Figure 3. Effects of sorbitol and mannitol concentrations on the phenolic content of leaf (a) and callus (b) of *C. copticum*. Values represent means of three replicates and dissimilar letters are significantly different based on the Duncan's test (P ≤ 0.05).

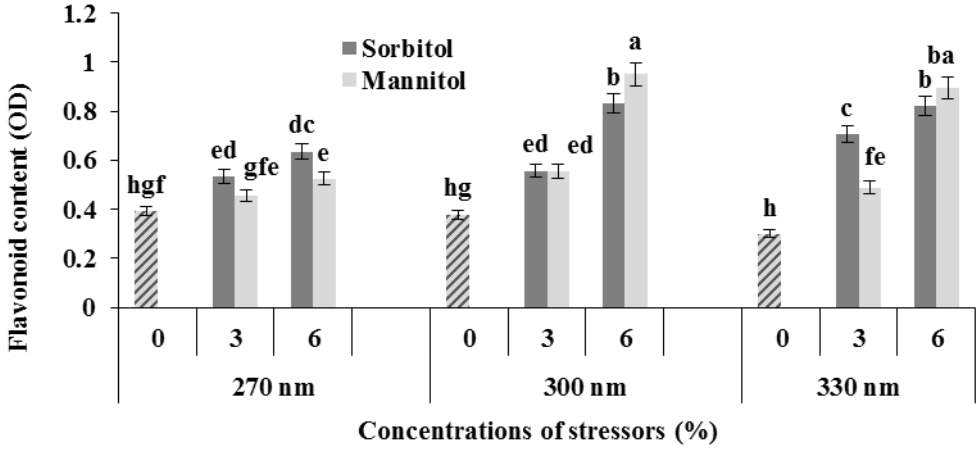


Figure 4: Effects of sorbitol and mannitol treatment on leaf flavonoid content in *C. copticum*. Values represent means of three replicates and dissimilar letters are significantly different based on the Duncan's test (P ≤ 0.05).

The CAT activity of mannitol-treated plants was significantly different from that of the sorbbitol-treated ones. Drought induced a significant increase in the CAT activity of sorbitol-treated plants at 3%, whereas it increased in plants treated with 6% mannitol (Fig. 5a). Both mannitol and sorbitol gave rise to significant increases in the catalase activity of *C. capticum* calli. This effect was, however, significantly more severe in plants exposed to mannitol. The lowest and the highest activities were observed at the stressor concentrations of 0 and 6%, respectively (Fig. 5b).

The results of means comparisons revealed that osmotic stress led to a significant decrease in the APX activity of *C. capticum*. As shown in Figure 6a, maximum APX activity in the sorbitol-treated seedlings was observed at a mild stress (3%), which then decreased at a more severe stress (6%). In the mannitol-treated seedlings, APX activity exhibited a significant decrease under both mild and severe stress conditions (Fig. 6a). Based on the experimental data obtained, APX activity in the calli treated with sorbitol showed a significant decrease relative to that observed in the control. Compared to control calli, those treated with 3% mannitol exhibited a significantly reduced APX activity, but no variation was observed at a stressor concentration of 6% (Fig. 6b).

Compared to the control treatment, the drought stress induced by sorbitol increased the total SOD activity in leaves at a stressor concentration of 6%. This is while the mannitol treatment led to an almost identical activity as that observed in the control plants (Fig. 7a). Figure 7b shows the changes in the SOD activity of callus samples exposed to different stressor concentrations. The highest SOD activity was observed in the callus samples exposed to 3% mannitol while no significant differences were observed between the mild and severe stresses in the sorbitol-treated calli.

The results of comparison of means indicate that the osmotic stress had a significant effect on the essential oils of both the calli and seedlings of *C. copticum* Thymol and γ-terpinene were positively affected while p-cymene was negatively affected by increasing water deficit. It is clear from Figs. 8a and b that the highest amounts of thymol and γ-terpinene were produced at the highest sorbitol concentration (6%) while the amount of p-cymene decreased at this concentration. Moreover, significantly more severe effects were observed in the

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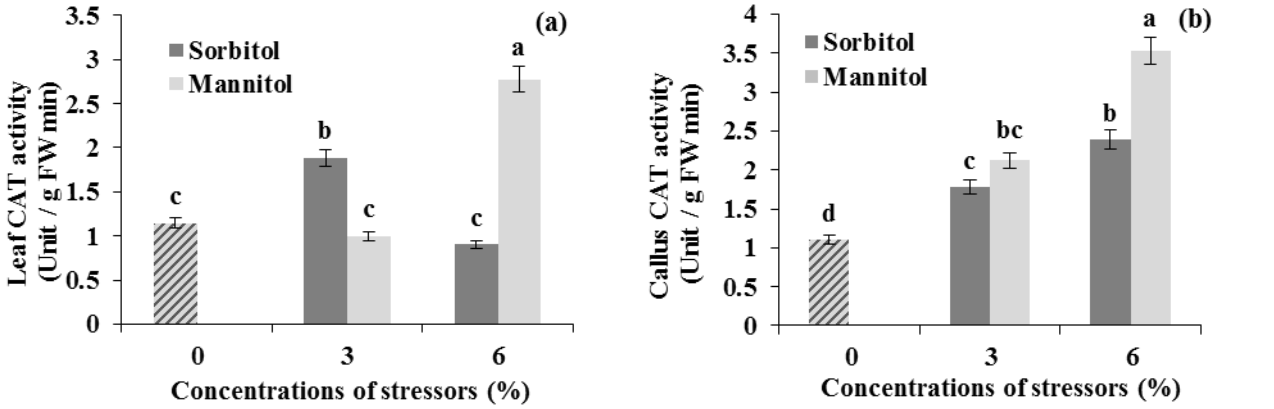


Figure 5. Effects of sorbitol and mannitol on CAT activity in the leaf (a) and callus (b) of *C. copticum*. Values represent means of three replicates and dissimilar letters are significantly different based on the Duncan's test (P ≤ 0.05).

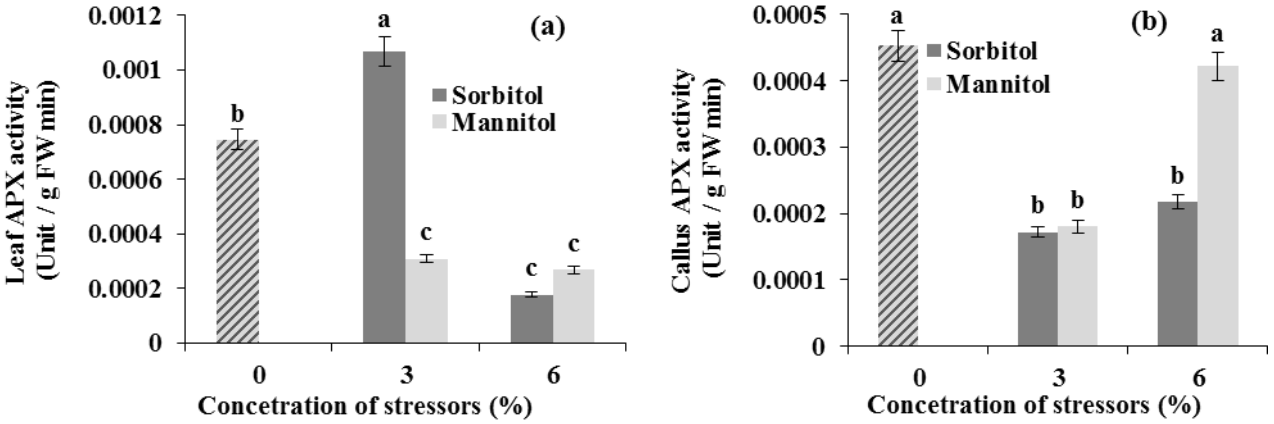


Figure 6. Effects of sorbitol and mannitol concentrations on APX activity in the leaf (a) and callus (b) of *C. copticum*. Values represent means of three replicates and dissimilar letters are significantly different based on the Duncan's test (P ≤ 0.05).

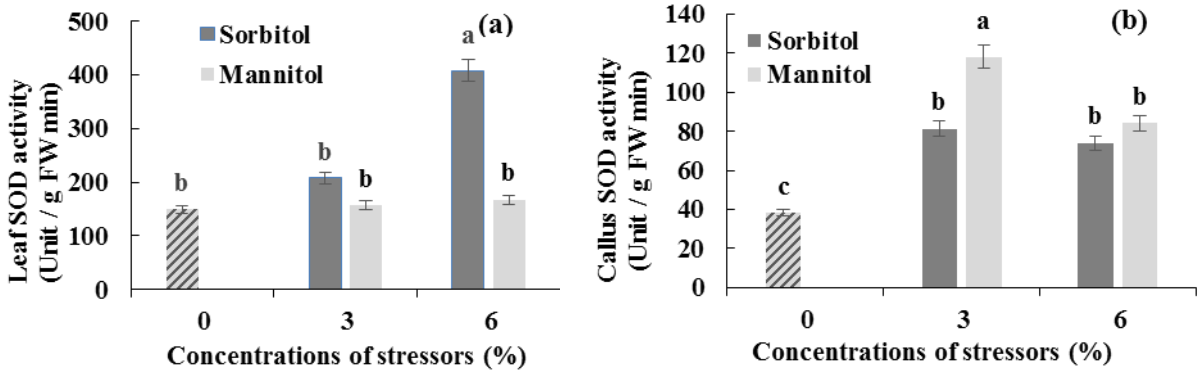


Figure 7. Effect of sorbitol and mannitol concentrations on SOD activity in the leaf (a) and callus (b) of *C. copticum*. Values represent means of three replicates and dissimilar letters are significantly different based on the Duncan's test (P ≤ 0.05).

calli exposed to sorbitol.

Discussion:

Water availability is the main environmental factor affecting plant growth and function. Photosynthetic responses to water stress have been extensively studied over the past few decades. The present study investigated the effects of low water availability induced by sorbitol and mannitol on the photosynthetic pigments and defense mechanisms in the seedlings and calli of *C. copticum.* Results indicated that osmotic stress led to a significant reduction in the pigment content of the samples. Moreover, chlorophyll contents were found to decrease significantly with increasing severity of drought stress. Sorbitol and mannitol treatments in this experiment were shown to have identical effects on the chlorophyll content of the species. In many water-stress situations, photosynthetic reductions can be explained by stomatal closure, limitations to CO_2 diffusion within leaf tissues, and mesophyll cell dehydration, all damaging the photosynthetic machinery (Flexas *et al.*, 2004, 2007)..

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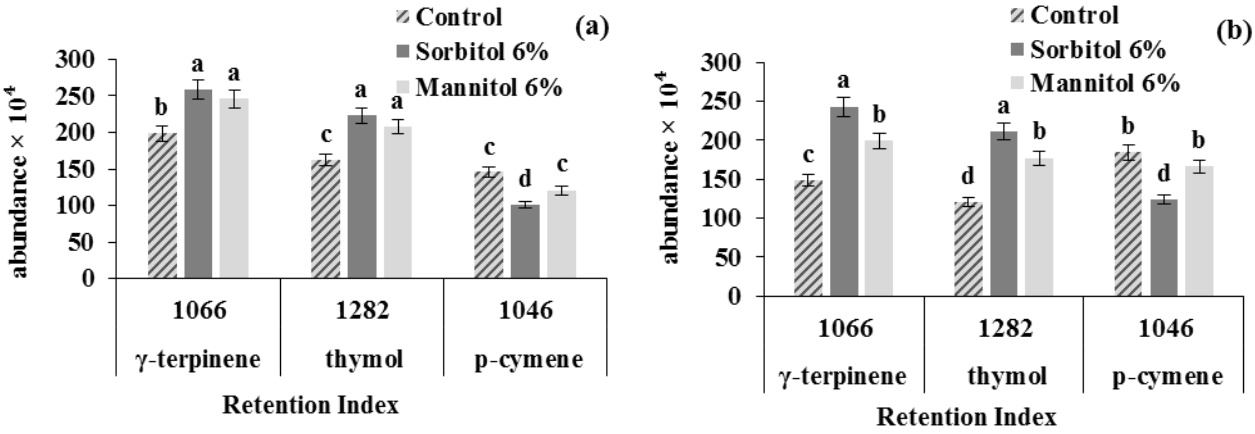


Figure 8. Effects of sorbitol and mannitol concentrations on the main essential oil constituents of *C. copticum* in leaf (a) and callus (b).

One reason claimed for this reduction is the enhanced activity of the chlorophyllase enzyme, especially induced under stress conditions (Ranjan *et al.,* 2001). It seems that the osmotic stress induced by mannitol and sorbitol increases the levels of growth inhibitory substances such as ABA, which initiate a series of signaling events related to the expression of many stress-related genes and activate signal transduction pathways (Zou *et al*., 2010). ABA stimulates chlorophyllase and degrades chlorophyll (Loggini *et al.*, 1999). Also, drought stress has been shown to impair the electron transport system, leading to the formation of activated oxygen. Oxidative molecules initiate damages in the chloroplast and cause a cascade of damaging effects including chlorophyll destruction, lipid peroxidation, and protein loss (Zhang and Kirkham, 1994). Similar results have been reported for *Camellia sinensis* L. (Damayanthi *et al.*, 2010) and *Crocus sativus* L. (Sabet Timori *et al.*, 2010).

Reactive oxygen species produced during stress are thought to play an important role in inhibiting plant growth. Plants need to scavenge ROS for the maintenance of their normal growth. Hence, plants resort to both non-enzymatic and enzymatic defense systems for scavenging ROS (Zabalza *et al*., 2007). Non-enzymatic systems examined in this study included carotenoids, anthocyanins, phenolic compounds, and flavonoids. It is now well documented that carotenoids are involved in the protection of the photosynthetic apparatus against photo-inhibitory damage by singlet oxygen produced by the excited triplet state of chlorophyll. Carotenoids can directly deactivate singlet oxygen and may also quench the excited triplet stat of chlorophyll, thus indirectly reducing the formation of singlet oxygen species to fulfill, thereby, their antioxidant role (Inze and Montagu, 2000). In the present study, the results obtained were found to repudiate claims concerning the protective role of carotenoids. In other words, the carotenoid content of *C. capticum* was observed to decrease under drought stress, thus failing to function as an accessory pigment. Reduction in the levels of photosynthetic pigments, including carotenoids, on exposure to biotic or abiotic stressors have been observed in many species and it seems that reactive oxygen decreases carotenoid concentrations by oxidizing carotenoids and damaging cell membranes (Thao and Yanyun, 2005; Lau *et al*., 2006).

Anthocyanin accumulation in *C. capticum* is enhanced in response to drought stress. It seems that the presence of ROS leads to the significantly enhanced synthesis of anthocyanins, which provides them in adequate amounts to counteract the enhanced rates of H_2O_2 production and to scavenge drastically the oxidative free radicals (Chalker-Scott, 2002). Previous research has demonstrated that abiotic stress enhances anthocyanin synthesis in reaction to drought in rice and *Arabidopsis* (Basu *et al.,* 2010; Sperdouli and Moustakas, 2012). Also, selective application of water deficit has been shown to increase anthocyanin accumulation in grape skins and genes of the corresponding anthocyanin biosynthesis pathway (Castellarin *et al*., 2007).

Phenolic compounds are known to be secondary metabolic substances not only with anti-oxidant and anticancer properties but also with the capability to offer resistance to environmental disasters (Chung *et al.*, 2006). The results of our experiments with *C. capticum* leaves and calli under the sorbitol treatment demonstrated the efficacy of the treatments for extracting phenolic compounds. This is confirmed by results reported elsewhere (Mohdaly *et al*., 2009), suggesting that abiotic constraint may enhance the biosynthesis of phenolic compounds as a response to the oxidative stress. However, in the mannitol-treated plants, phenolic compounds decreased in response to drought although they increased in the calli of *C. capticum* in response to drought treatment with mannitol. It is thought that the decrease in phenolic compounds might result from a decline in the activities of the key enzymes involved in their biosynthesis (Chung *et al.*, 2006). Some of these findings are similar to those of Sin and Sil (2008) who reported a decrease in the phenolic content of red pepper seeds or those of

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Toor and Savage (2005) in tomato. Flavonoids are typical phenolic compounds that act as potent metal chelators and free radical scavengers. Compared to the control leaves, the experimental leaf samples of *C. capticum* exhibited significantly increasing amounts of flavonoids under the osmotic stress conditions. This is in agreement with the findings of Jung (2004) who reported enhanced flavonoid contents in *Arabidopsis thaliana* leaves in response to drought treatment. Reportedly, these substances are capable of scavenging the reactive oxygen species produced due to abiotic stresses (Doshi *et al.*, 2006).

To resist oxidative damages, both antioxidant enzymes and certain metabolites play important roles in the adaptation and ultimate survival of plants under stress conditions (Verma and Dubey, 2003). Accumulation of H_2O_2 has been reported to function as an intercellular signal that stimulates a number of genes and proteins involved in the generation of such stress responses as superoxide dismutase, catalase, ascorbate peroxidase (APX), and peroxidase (POX) (Dynowski *et al.*, 2008). Ample evidence has shown that the amounts and activities of the enzymes involved in scavenging ROS are altered by environmental stresses such as drought, chilling, and salt. Our results indicated that the activity levels of such antioxidant enzymes as SOD, CAT, and APX were variously affected by sorbitol and mannitol treatments.

Another finding of the present research indicated that mannitol had no significant effects on the SOD activity of leaves while it enhanced SOD activity in the other samples exposed to drought stress. Enhanced SOD activity represents the first line of defense in protection against the formation of superoxide radicals from the reduction of O_2. This involves a group of metalloenzymes that convert the superoxide resulting from the oxidative stress to H_2O_2 and O_2 and offers protection in cells against the superoxide-induced oxidative stress (Mittler, 2002). Similar results have been reported on *Juniperus oxycedrus* (Alguacil *et al.*, 2006) and *Pistacia khinjuk* (Mirzaei *et al.*, 2013). Hojati *et al*. (2011) reported that CAT and SOD activities increased under drought stress in *Carthamus tinctorius*. Our findings suggest that an increased H_2O_2 generation in the drought-stressed leaves and calli of *C. capticum* may be induced by SOD.

Osmotic stress was found to lead to a significant increase in the CAT activity of the leaves and calli of *C. capticum.* It has been suggested that H_2O_2 accumulation caused by various environmental stresses results in a combination of CAT and APX activities aimed at protecting plant cells (Malekzadeh *et al*., 2014). CAT can reduce H_2O_2 to water, but it has a very low affinity for H_2O_2 (Graham and Patterson, 1982). A number of investigations have been dedicated to describing enhanced CAT activity under drought conditions (e.g., Jung, 2004). It may thus be claimed that enhanced CAT, SOD, and POX activities during stress, indeed, form the defensive mechanism in plants against free oxygen radicals; the mechanism serves to detoxify and decompose the hydrogen peroxide produced by the cells while it also prevents the breakdown of plant proteins (Ariano *et al*., 2005).

APX catalyzes the elimination of SOD toxic products at the expense of oxidizing ascorbate to monodehydroascorbate (Smirnoff, 1993). Contrary to our expectation, plant exposure to drought resulted in a reduced APX activity. Although APX activity increased in our plants treated with 3% sorbitol, it decreased under severe stress conditions. These findings are consistent with those reported recently (Tejera *et al.*, 2004). Sharma and Dubey (2007) reported an increase in the APX activity of rice under drought stress whereas Simirnoff and Colombe (2000) reported no effects of drought on APX activity. Salwa Jebara *et al*., (2004) even showed the inhibition of APX activity under drought treatment. These discrepancies seem to stem from differences in plant species, plant tissue development stage, stress intensity, and the time elapsed after stress treatment. Enzyme activity may also vary with stressor type and concentration as well as plant part and species (Sharma and Dubey, 2007).

In our study, essential oils were found to be strongly influenced by drought stress. Results showed that thymol, γ-terpinene, and p-cymene account for the main components of the essential oils in *C. copticum.* This is in agreement with those reported by Akbarinia *et al.*, (2005) despite the differences reported in the percentages of the major oil constituents of *C. copticum*.

Thymol has found important cosmetic, culinary, and pharmaceutical applications (Letchamo and Gosselin, 1995). Many investigations have explored the antimicrobial and antioxidant properties of this component (Olasupo *et al.*, 2003). Drought stress was found to have the advantage of increasing the quantities of essential oils in our treated samples relative to those obtained from the control plants. Pitarevic *et al.*, (1985) reported that a long dry season should give a high oil production. Similar results have been reported by Petropoulos *et al.*, (2008) and Aziz *et al.*, (2008). In his evaluation of water stress effects on the essential oils of the two herbal plant species of *Ocimum basilicum* L. (sweet basil) and *Ocimum americanum* L. (American basil), Khalid (2006) reported that both the percentages of essential oils and their main constituents increased in both species under water stress. Finally, Aliabadi Farahani *et al.* (2009) reported drought stress to lead to enhanced essential oil percentages in most medicinal plants because they found more metabolites produced in such conditions to prevent oxidization in cells.

Conclusion:

Overall, *C. capticum* presents a highly efficient antioxidant defense system to minimize the effects of stress, as for example under drought conditions. In our experiment, in *C. capticum* was found to be capable of efficiently adapting to water deficit and accumulating certain metabolites such as phenolic compounds, flavonoid, and anthocyanin. Based on our results, it

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seems that part of the drought tolerance of *C. capticum* stems from its capacity for modulating its phenolic and anthocyanin contents as measures to combat the oxidative stress caused by water deficit. Antioxidants and phenolics are able to act as ROS scavengers or ROS chain breakers, thus vigorously extinguishing oxidative free radicals. Increased CAT and SOD activities appear to play the key roles in the antioxidant defense response of *C. capticum* seedlings and calli when exposed to drought stress. Drought stress was, thus, observed to enhance the thymol content of *C. copticum* under *in vitro* culture. These effects were significantly more severe in plants exposed to mannitol than those treated with sorbitol. This capacity makes the species especially useful for the *in vitro* production of pharmaceuticals and other beneficial substances. Interestingly, due to their higher tolerance of drought conditions compared to the seedlings, the calli of *C. caticum* were found capable of serving as important explants for use in the production of secondary metabolites and as renewable sources of chemicals, especially medicinal compounds.

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