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Developmental and biochemical analyses of *in vitro* drought stress response in ornamental European Bluestar (*Amsonia orientalis* Decne.)

Arda Acemi[1]*, Yonca Avcı Duman[2], Yonca Yuzugullu Karakus[1], Fazıl Özen[1]

[1] Department of Biology, Faculty of Arts and Sciences
Kocaeli University, 41380 İzmit, Kocaeli, Turkey
[2] Department of Chemistry, Faculty of Arts and Sciences
Kocaeli University, 41380 İzmit, Kocaeli, Turkey

ABSTRACT

This study aimed to investigate the effects of drought stress on *Amsonia orientalis*, an endangered ornamental plant with a limited natural distribution in Europe. Effects of polyethylene glycol (PEG)-mediated drought stress (-0.15, -0.49, -1.03 and -1.76 MPa osmotic potentials) were tested on *in vitro* cultures. In general, root lengths and numbers, total protein, chlorophyll a and carotenoid contents were negatively influenced at elevated levels of the stress factor. The successive decrease in the tested osmotic potentials resulted in gradually higher H_2O_2, malondialdehyde (MDA) and proline contents. Activities of the antioxidant enzymes, peroxidase (POD) and catalase (CAT), were found to be enhanced in response to the decreasing osmotic potential tested, whereas increased superoxide dismutase (SOD) activity was observed at the -0.15 MPa osmotic potential. Strong activation of POD enzymes under drought stress suggests that POD enzymes might have a major role in regulating the H_2O_2 content, while CAT has only a supplementary role in *A. orientalis*. These results indicated that although *A. orientalis* is susceptible to long-term drought, the species may survive during mild drought stress because the development of the plant was not totally inhibited but only limited. Nevertheless, the species should be introduced to well-irrigated lands, after evaluation of the soil's water status, in order to ensure the continuation of its generations.

Keywords: antioxidant enzymes, Apocynaceae, polyethylene glycol, *Amsonia* (*Rhazya*) *orientalis*, tissue culture

Abbreviations:

CAT – catalase, MDA – malondialdehyde, PEG – polyethylene glycol, POD – peroxidase, ROS – reactive oxygen species, SOD – superoxide dismutase, TBA – thiobarbituric acid, TCA – trichloroacetic acid

INTRODUCTION

Among the abiotic stress factors, drought which dramatically affects growth and metabolic processes in plants is considered as a rising risk to plant species due to climate change. Owing to the recent global climate change, drought has become one of the major environmental stress factors that can limit plant growth and alter the physiological characteristics of plant species (Wu et al., 2012). Almost all unfavorable environmental conditions induce oxidative stress in plants as an early and rapid

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response (Kubiś and Zając, 2008). In particular, prolonged drought stress may result in oxidative damage due to an over-production of reactive oxygen species (ROS). ROS can oxidize cellular components, damage RNA and DNA, inhibit several enzymes, and cause membrane lipid peroxidation with basically four forms: singlet oxygen ($^1O_2$), superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH•) (Cruz de Carvalho, 2008). Plants have a dual defence system to protect cellular components from the cytotoxic effects of ROS. This defence system consists of enzymatic and non-enzymatic pathways. In the enzymatic pathway, catalase (CAT), peroxidase (guaiacol and ascorbate peroxidase: POD), superoxide dismutase (SOD) and enzymes of the ascorbate-glutathione cycle take part, while carotenoids, glycine betaine, some phenolic compounds, polyamines, proline, and sugar are involved in the non-enzymatic defence pathway. In addition to this, the ROS formation-dependent level of malondialdehyde (MDA) as an endogenous genotoxic product of enzymatic and oxygen-radical-induced lipid peroxidation is an indicator of cellular membrane damage. MDA levels have been commonly reported in plants and are frequently used as indicators of environmental stresses (Dong et al., 2013).

Amsonia orientalis Decne. (syn. *Rhazya orientalis* (Decne.) A. DC.) from the Apocynaceae family is a medicinally important ornamental plant with anti-cancer, anti-tumour and antimicrobial activities (Acemi et al., 2017a). The species is a perennial and rhizomatous plant which prefers a well-drained soil and also needs full sun or partial shade. It can be found along margins of lakes and streams. The species is also known as "European Bluestar" because of its navy-blue flowers. Unlike its limited natural distribution area covering the northwest of Turkey and the northeast of Greece, the species is cultivated in many gardens, mostly in Europe and the United States, for ornamental purposes (Acemi et al., 2016). Although the species has been listed by the European Council (Bern Convention, 1979) as one of the plant species that must be conserved on a European scale, it was declared that the natural distribution of the species in Turkey had decreased to cover only 10 km² in the Balıkesir province (Gürkanlı et al., 2014). Therefore, several conservation studies were conducted to increase the numbers of individuals of this species in nature (Acemi et al., 2012; 2013). Besides human-sourced factors such as industrialization and urbanization, environmental stress factors that affect

the plant's survival ability by causing several negative outcomes in growth and biochemical characteristics might also be contributing to this decrease in the natural distribution area of the plant. Changes in the parameters such as growth, photosynthetic pigments, protein and proline contents, ROS production, lipid peroxidation level and antioxidant enzyme activities would let us analyze the responses of the defensive system of the plant against drought. Thus, the present study was conducted to shed light on the plant's ability to tolerate this environmental stress factor resulting mainly from climate change, to reveal the main biochemical responses of the plant to drought and to contribute to the reintegration of the plant to adequate habitats.

MATERIAL AND METHODS

Plant material preparation and in vitro stress treatment

Amsonia orientalis is represented by 4 populations (Gaziosmanpaşa, Paşa Alanı, Adnan Menderes and Ömerli) in the Balıkesir Province of Turkey. Only the individuals propagated from the Gaziosmanpaşa population were employed in the experiments because genetic variation had been found among its populations (Gürkanlı et al., 2014). One specimen with both shoots and rhizome was sampled from the species' natural habitat along the Üzümcü Creek near the Gaziosmanpaşa district of the Balıkesir Province in May of 2009, and taken for *ex vitro* conservation in the gardens of Kocaeli University. A voucher specimen from the same habitat was deposited in the herbarium of Uludağ University (BULU, specimen no: 18138). Thenceforth, *in vivo* and *in vitro* propagation efforts using stem cuttings have been carried out to extend the plant's population in Kocaeli University. The nodal explants only from these propagated mature individuals were used to establish *in vitro* primary cultures. The desired number of plant material was obtained after following the shoot multiplication protocol described by Acemi et al. (2013). Excised single node explants from *in vitro* raised shoots were transferred to the MS medium (Murashige and Skoog, 1962) without any treatment (control) and with different additional osmotic potentials (-0.15, -0.49, -1.03 and -1.76 MPa) prepared by using polyethylene glycol (PEG-6000) in defined amounts (Mohammadkhani and Heidari, 2008). The cultures were incubated for 30 days under the same conditions as defined by Acemi et al. (2013). The growth (organ development) and biochemical parameters of the explants were evaluated at the end

of the incubation period. All variants of the medium were supplemented with 30 g dm^{-3} sucrose and 7 g dm^{-3} of plant agar, and the pH of culture media was adjusted to 5.7 prior to autoclaving. Relative water contents of regenerated plants were determined according to Ammar et al. (2014).

A preliminary study had been conducted to determine the osmotic potentials to be applied, and it resulted in total inhibition of root growth when the osmotic potential was below -1.76 MPa. Thus, the lowest level of osmotic potential was determined accordingly.

Determination of protein content

Total soluble protein content was determined following the method of Bradford (1976), and bovine serum albumin was used as the standard. Tissue samples (250 mg) were transferred into a sterile mortar and ground in a cold Tris-HCl buffer (10 mM, pH 6.8), and then the final mixture was centrifuged at 15,000 g for 20 min. The resulting supernatant was used for the determination of soluble protein content. Data were expressed as mg g^{-1} fresh weight (FW).

Determination of free proline content

The method of Bates et al. (1973) was followed to estimate free proline contents of the tissue samples. The collected leaves (0.5 g) were homogenized using 10 ml of 3% sulfosalicylic acid, in a cooled mortar. The extract was filtered through Whatman No. 2 filter paper. The filtrate, acid ninhydrin and glacial acetic acid (1:1:1 v/v) were mixed together and left for incubation at 100°C for 1 h. The resulting mixture was extracted using toluene after the reaction was finalized on ice. The toluene-extracted phase was collected, and its spectrophotometric absorbance was read at 530 nm. The data were expressed as μmol g^{-1} FW.

Determination of photosynthetic pigment quantities

Photosynthetic pigment extraction from the tissue samples was carried out using 80% (v/v) acetone in an ice-cold mortar. The resulting extract was then filtered through Whatman No. 2 filter paper. Chlorophyll a, b and total carotenoid quantities were calculated using the equation described by Lichtenthaler (1987). Data were expressed as mg g^{-1} FW.

Determination of lipid peroxidation products

Quantities of lipid peroxidation products were determined in terms of MDA content. The method of Neto et al. (2006) was followed with some modifications. Thiobarbituric acid (TBA) reagent (15% w/v trichloroacetic acid and 0.375% w/v TBA in 0.25 M HCl) and crude extract were mixed (0.4 ml; 1:1) and the mixture was heated up to 95°C for 15 min, then cooled immediately in an ice bath. The resulting mixture was centrifuged at 1,500 g for 15 min. The supernatant was collected and the spectrophotometric absorbance was read at two specific wavelengths (532 nm and 600 nm). The amount of MDA was calculated from the extinction coefficient of 155 mM^{-1} cm^{-1}. The data were expressed as μmol g^{-1} FW.

Determination of H_2O_2 content

The method of Doupis et al. (2011) was followed to determine the H_2O_2 contents of tissue samples. The reaction mixture consisted of 0.1% (w/v) trichloroacetic acid (TCA), 1M KI, 0.5 ml of crude extract and 50 mM sodium phosphate buffer (pH 7.0) in the final volume of 2.5 ml. The reaction solution was kept in darkness for 60 min and then the absorbance was measured at 390 nm using a 0.1% (w/v) TCA solution and pure catalase reagent as a blank, to ensure zero interference. Hydrogen peroxide content was calculated from a standard curve prepared with known H_2O_2 concentrations. The data were expressed as μmol g^{-1} FW.

Preparation of crude extract

The crude extracts were prepared from tissue samples homogenized, using a mortar, in an extraction buffer of 50 mM sodium phosphate (pH 7.0) containing 0.1 mM ethylenediaminetetraacetic acid (EDTA). The resulting homogenates were filtered and then centrifuged at 14,000 g for 15 min at 4°C. The supernatants were collected and subsequently used as crude extracts for analysis of SOD, POD and CAT activities. Extract preparation and enzyme activity assays were done on the same day to eliminate a possible activity loss in the enzymes.

Determination of SOD, POD and CAT activities

SOD activity was determined by following the method of Dhindsa et al. (1981) based on monitoring the inhibition of photochemical reduction of NBT (nitroblue tetrazolium) at 560 nm. One unit of SOD activity was defined as the amount of enzyme required for 50% inhibition.

POD activity was assayed using the pyrogallol oxidation method (Kar and Mishra, 1976). Enzyme activity was determined using the extinction coefficient at 425 nm of 2640 M^{-1} cm^{-1} for pyrogallol,
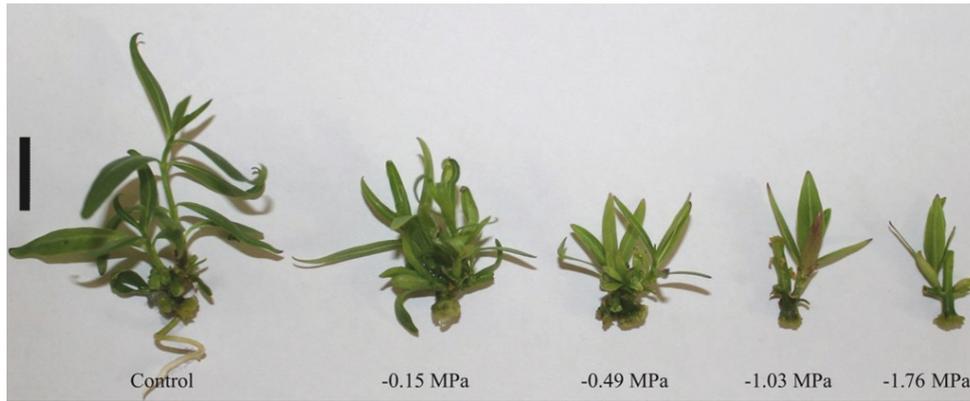
Figure 1. Effects of drought stress on *in vitro* development of *A. orientalis* after 30 d of culture. The numeric values are osmotic potentials of the tested medium. The scale bar represents 1 cm

where one unit of enzyme activity corresponded to the formation of 1 mg of purpurogallin per 5 min.

CAT activity was determined according to the method of Aebi (1974), which is based on the calculation of the decline in absorbance (the rate of H_2O_2 disappearance) at 240 nm ($\varepsilon = 0.039$ cm^2 μmol^{-1}). One unit of CAT activity was defined as the amount of enzyme that catalyzed the decomposition of 1 μmol H_2O_2 per minute.

Data collection and statistical analysis

Each culture vessel contained 5 explants and 30 explants were employed in each replication. The incubation period for the tissue culture experiments lasted 30 days. The growth and biochemical parameters of the explants were evaluated at the end of the incubation period. Shoots and roots were sampled together to obtain the crude extract. All assays were repeated three times, and mean values were compared using Duncan's multiple range test at the $p < 0.05$ significance level. Data were given as mean ± standard deviation (SD). Spectrophotometric assays were done by using a Bio-Rad Smartspect 3000 spectrophotometer. The IBM SPSS Statistics 19 software was used for statistical analysis.

RESULTS

Growth parameters – organ development

The general appearance of *in vitro* regenerated plants is shown in Figure 1. At the end of the incubation period, starting from the highest osmotic potential (-0.15 MPa), the decreasing osmotic potentials gradually reduced the mean shoot lengths. The lowest osmotic potential (-1.76 MPa) in the culture medium caused the largest decrease in shoot length, which was statistically different from that of the control group (Fig. 2A). The root lengths were also negatively influenced by the tested stress factor. All

the tested osmotic potentials dramatically reduced root length (Fig. 2A), while their effects were not statistically different from one another.

The mean shoot numbers were not affected by PEG-induced drought stress. Although osmotic potentials below -0.15 MPa had a slight positive effect when compared to the control group, they were not statistically different (Fig. 2B). In contrast to shoot number, the mean root numbers decreased in response to the decreasing osmotic potentials. A sharp decrease similar to that in root length in response to the tested osmotic potentials was also
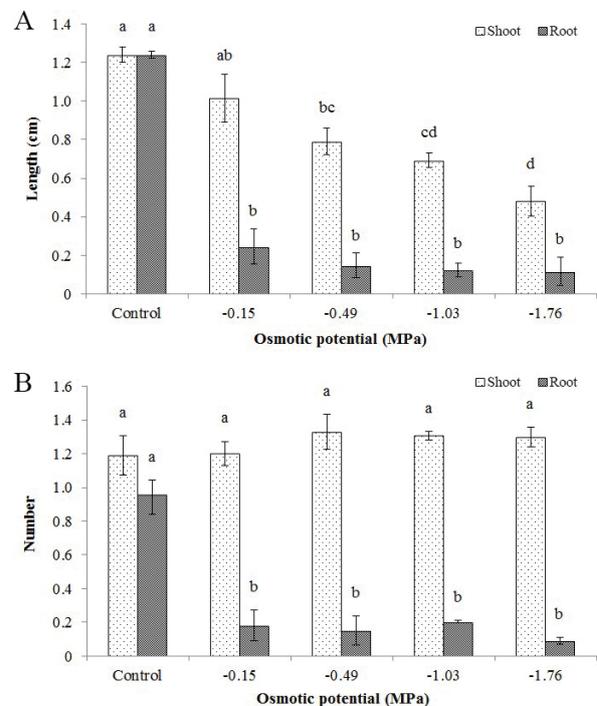

Figure 2. Effects of *in vitro* drought stress on the growth parameters of *A. orientalis*. (A) Mean shoot and root lengths, (B) Mean shoot and root numbers. The values are represented as means ± SD, different letters denote significant differences ($p < 0.05$)

observed in the number of roots. Except for the control group, all the treatments gave statistically the same results for root number (Fig. 2B).

The mean relative water contents of the plants were found to be: 90.01 ± 0.90, 91.67 ± 0.25, 93.10 ± 0.26, 92.03 ± 0.12 and $89.40 \pm 0.26\%$ for the control and osmotic potentials of -0.15, -0.49, -1.03 and -1.76 MPa, respectively.

Total soluble protein content

Assay results for the total soluble protein content are shown in Figure 3A. The highest protein content was found in the untreated plants. The osmotic potentials below -0.15 MPa led to a gradual decrease in the total soluble protein content. Although there was also a slight decrease found in plants grown in the medium with the -0.15 MPa osmotic potential, the result was found to be statistically the same as that for the control group. The decline in the protein content was more evident in the plants grown in the media with the -1.03 and -1.76 MPa osmotic potentials.
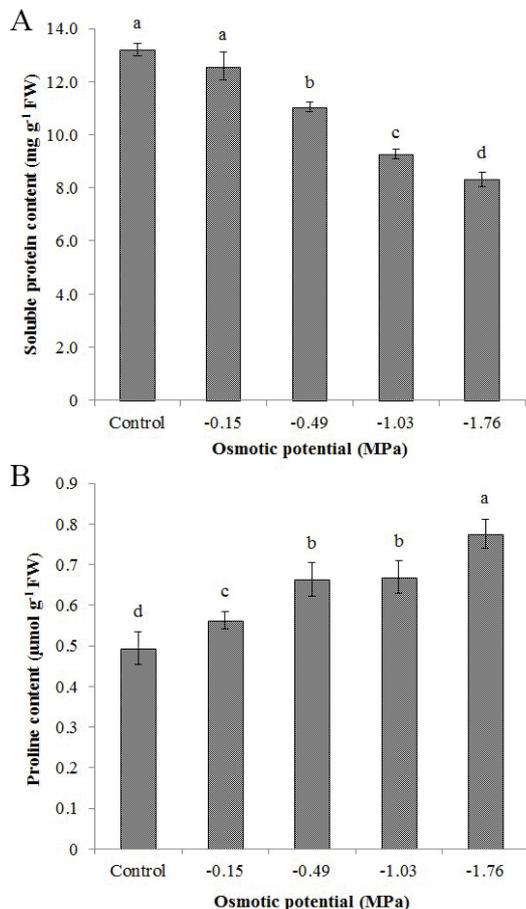
Figure 3. Changes in soluble protein (A) and proline (B) contents of *A. orientalis* due to *in vitro* drought stress. The values are represented as means ± SD, different letters denote significant differences ($p < 0.05$)
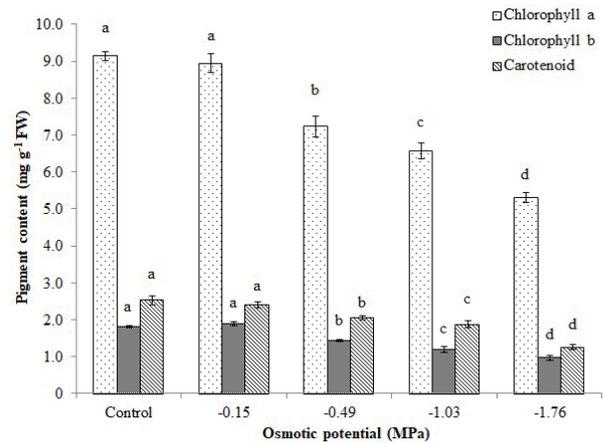
Figure 4. Changes in photosynthetic pigment contents of *A. orientalis* due to *in vitro* drought stress. The values are represented as means ± SD, different letters denote significant differences ($p < 0.05$)

Free proline content

Free proline assay results are shown in Figure 3B. All the tested osmotic potentials caused a significant increase in proline content compared to the control group, whereas the results for the -1.03 and -0.49 MPa osmotic potential treatments were statistically the same. The highest proline value was found in the plants grown in the medium with the osmotic potential of -1.76 MPa, while the control group had the minimum value for proline accumulation.

Photosynthetic pigment contents

Assay results for the photosynthetic pigment content are shown in Figure 4. The highest pigment contents were observed in the control and -0.15 MPa group. Below -0.15 MPa, the decreasing osmotic potentials caused statistically different reductions in the quantities of all the pigments, when compared to the control group, whereas the effects of the -0.15 MPa osmotic potential and the control group remained statistically the same. Additionally, the chlorophyll a, b and carotenoid contents showed a similar downward trend starting from the -0.49 MPa osmotic potential.

Lipid peroxidation

Lipid peroxidation assay results are shown in Figure 5A. The highest MDA accumulation was found in the plants treated with the -1.76 MPa osmotic potential. Generally, the MDA levels showed an upward trend for the tested osmotic potentials, compared to the control group. However, the -0.49 MPa osmotic potential caused a slightly lower MDA level than that for the -0.15 MPa osmotic potential. Nevertheless, the accumulation levels caused by the
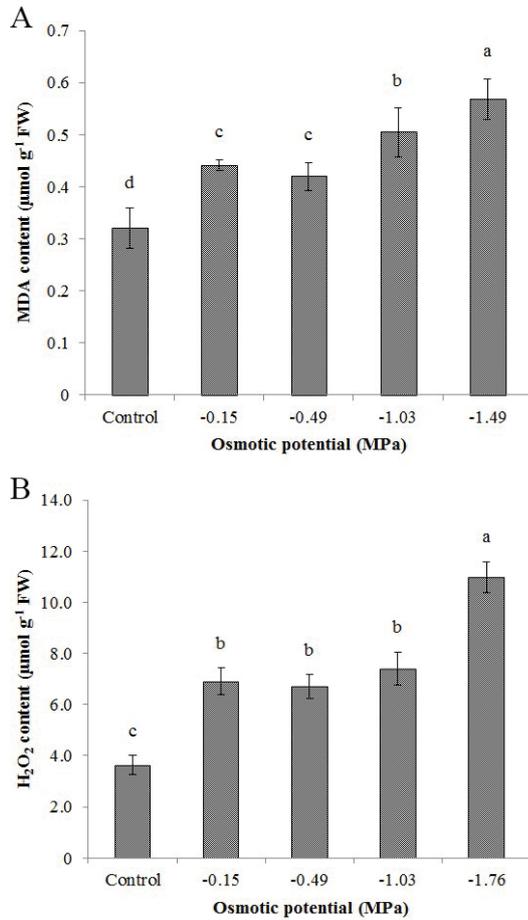
Figure 5. Changes in MDA (A) and H_2O_2 (B) contents of *A. orientalis* due to *in vitro* drought stress. The values are represented as means ± SD, different letters denote significant differences ($p < 0.05$)

-0.15 MPa and -0.49 MPa osmotic potentials were not statistically different.

H_2O_2 content

H_2O_2 content assay results are shown in Figure 5B. H_2O_2 accumulation was induced under drought stress; however, the results for the plants grown in the media with the -0.15, -0.49 and -1.03 MPa osmotic potentials were found to be statistically the same. Moderate osmotic potentials produced close effects on H_2O_2 accumulation. Although the lowest osmotic potential (-1.76 MPa) resulted in a more substantial increase, even the relatively moderate osmotic potentials (-0.15, -0.49 and -1.03 MPa) led to a more than twofold increase in H_2O_2 accumulation.

Effects of drought stress on antioxidant enzyme activities

The effects of prolonged drought stress on antioxidant enzyme activities of *Amsonia orientalis* are summarized in Figure 6. All the tested osmotic potentials increased SOD activity to different levels. The highest osmotic potential triggered SOD activity intensely. Therefore, this increase was found to be statistically significant compared to the results of the other treatments (Fig. 6A). Although the -0.49 and -1.03 MPa osmotic potential treatments resulted in a slight increase compared to the control, their results remained statistically the same.

Higher POD activities were found as results of all the treatments (Fig. 6B). The elevated POD activity was observed to be stable and statistically the same regardless of the tested osmotic potentials. The increased activity observed due to the tested osmotic potentials was more than three times as high as that in the control. The osmotic potential-dependent stimulations of POD activity seemed to be more pronounced than those of CAT and SOD activities.
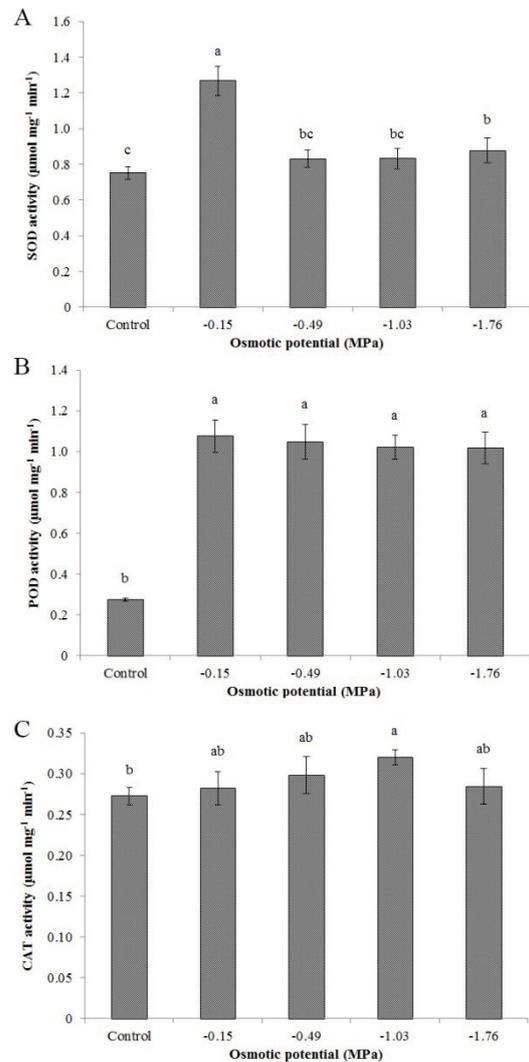

Figure 6. Changes in activities of antioxidant enzymes (A – SOD, B – POD, C – CAT) of *A. orientalis* due to *in vitro* drought stress. The values are represented as means ± SD, different letters denote significant differences ($p < 0.05$)

The results of CAT activity assays were found to be close to one another for all the treatments. The lowest CAT activity was in the control group. The osmotic potentials of -0.15, -0.49 and -1.76 MPa slightly triggered the enzyme activity but caused statistically similar responses. However, the -1.03 MPa osmotic potential in the culture medium led to a significant increase in CAT activity (Fig. 6C).

Among the investigated antioxidant enzymes, SOD and POD activities were stimulated more after the treatment with the highest level of osmotic potential, while CAT activity was found to be induced significantly only after the -1.03 MPa osmotic potential treatment.

DISCUSSION

Physiological processes in plants, including cell growth, are known to be sensitive to water deficiency. Under long-term drought stress, plant cells decrease osmotic potential to maintain turgor due to impaired water flow through the xylem, which can inhibit cell elongation in higher plants (Farooq et al., 2009). Therefore, the decrease in shoot and root lengths might be explained by the partial interruption of water transport through the xylem. Similar results for shoot and root reduction were also obtained in *Vicia faba* (Ammar et al., 2014) and *Simmondsia chinensis* (Roussos, 2013). Also, significant amounts of PEG can cause several side effects such as reduced phosphorus uptake, translocation and the O_2 content of nutrient solutions (Tingey and Stockwell, 1977). For that reason, the reduced growth observed in *Amsonia orientalis* grown under drought stress might also be associated with phosphorus deficiency since it limits total plant growth, including root growth. However, survival of the plants under osmotic stress might be related to the maintenance of the relative water status of tissues.

Among phytohormones, abscisic acid and cytokinins have opposing roles in drought stress. Under long-term and/or extensive stress, the level of endogenous abscisic acid is increased while that of cytokinin is decreased to close the stomata, which limits water loss, whereas a short-term or mild stress could trigger a temporary elevation of cytokinin level and/or signaling (Ha et al., 2012). After *in vitro* salt treatment in *A. orientalis*, the lowest concentration of NaCl increased shoot numbers, which supports this hypothesis on shoot growth (Acemi et al., 2017b). However, shoot numbers remained statistically the same even after long-term drought in our study. On the other hand, Ivanchenko et al. (2013) had stated that ROS could be produced in response to auxin

because they had found that an increased auxin level triggered H_2O_2 production in the root tips of *Solanum lycopersicum*. However, the increase in H_2O_2 content of *A. orientalis* does not seem to be a result of auxin production because root development is reduced. These results suggest that photohormone modulation during stress differs according to the type of stress factor applied to *A. orientalis*. Nevertheless, this suggestion is not enough to reveal the whole mechanism behind this modulation, thus it should be supported by further experiments.

Although the plants treated with the osmotic potential of -0.15 MPa had statistically the same results as the control, the decreasing osmotic potentials reduced the total protein contents. Likewise, after PEG-induced drought stress, the roots and leaves of two *Zea mays* cultivars exhibited lower protein contents (Mohammadkhani and Heidari, 2008). Also, Behnamnia et al. (2009) stated that a 5-day drought stress caused lower protein contents in *Lycopersicon esculentum* plants. In plants, oxidative stress caused by the accumulation of reactive oxygen species can increase the oxidative modification of cellular molecules such as proteins which are then not repaired and thus removed by proteolytic degradation (Stadtman and Levine, 2000). Therefore, the reduction in the total protein content of *A. orientalis* might be attributed to this protein denaturation via an oxidation process.

Proline is an osmoregulator α-amino acid which plays a role in membrane stabilization during several environmental stresses in plants. In *A. orientalis*, proline accumulation triggered by drought stress was followed by MDA and H_2O_2 accumulations in a dose-dependent manner. Zhu (2002) indicated that free proline can take part in the stabilization of macromolecules, play a role as a sink for excessive reductant, and it can be used as a carbon and nitrogen store which can be used after relief of water deficit. However, to clearly reveal the osmoregulation mechanism in *A. orientalis*, soluble sugars, sugar alcohols and glycine betaine should also be considered. Proline is produced from glutamate in the cytosol or chloroplasts of plants (Krasensky and Jonak, 2012). Therefore, most of the proline production in *A. orientalis* under severe stresses might be cytosol-sourced because the tested stress factor at higher intensities (i.e. at lower osmotic potentials) caused a reduction in the amounts of photosynthetic pigments, which may have been due to chloroplast deformation. This finding supports the major role of glutamate pathway in proline production in plants under osmotic stress.

Several environmental stress factors can have damaging effects on the photosynthetic capacity of plants. In particular, chloroplasts are vulnerable to abiotic factors (Petrov et al., 2015). Similar to our findings, a decline in photosynthetic pigment contents under drought stress was observed in *Tagetes erecta* (Liao et al., 2012) and *Lycium ruthenicum* (Guo et al., 2016). In our experiments, the reduced amounts of photosynthetic pigments due to increased stress might be attributed to oxidative damage as indicated by high values of MDA contents. Ammar et al. (2014), who had reported similar findings in drought-stressed *Vicia faba*, attributed this reduction to the destruction of chloroplasts through the dilation of the thylakoids, breakdown of the envelope and destabilization of the pigment protein complexes. Additionally, without any acclimatization process, *in vitro* propagated plants after several subcultures are prone to water loss when they are taken out of culture vessels. Therefore relatively high protein and pigment contents might be related with this loss in fresh weight.

The MDA content indicates ROS-mediated cellular damage concentrated on membrane lipids of stress-exposed plants. In our study, MDA contents were found to be increased under elevated drought stress. Also, the observed increase in ROS levels coincided with the increased MDA levels in *A. orientalis*. Similarly, treatment of *Triticum aestivum* seedlings with elevated levels of PEG-induced drought stress (Kang et al., 2013) had resulted in higher MDA contents. In *A. orientalis*, it seems that the enhanced proline production was not enough to stabilize cell membranes against oxidation because a continuous accumulation of MDA was observed. Also, POD and CAT activities were found to be increased to remove ROS and to limit MDA production in *A. orientalis*.

The reactive oxygen species can peroxidize the cellular membrane lipids and lead to the degradation of enzyme proteins and nucleic acids. H_2O_2, which causes the cellular damage, acts as a signal molecule to the plant's defence system (Gong et al., 2001). Under the tested stress factor, an increase in H_2O_2 and MDA levels indicates this cellular damage in *A. orientalis*. Several cellular protectants can be accumulated to regulate the cellular redox status under drought stress due to excessive H_2O_2 production (Catola et al., 2016). Also, activities of ROS-scavenging enzymes are triggered by H_2O_2 production. However, continuously increasing H_2O_2 content followed by elevated CAT and POD activities in *A. orientalis* means that the enzymatic defence

system of the plant does not seem to be capable of scavenging all of the ROS produced.

As a defence mechanism against environmental stress factors, plants favour the production of antioxidant enzymes. Maintenance of the antioxidant defence system to cope with ROS plays a significant role in keeping the cell membranes stabilized. Antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD) are widely distributed in all higher plants and are involved in the decomposition of different forms of ROS (Foyer and Noctor, 2000). SOD is located in chloroplasts, mitochondria, peroxisomes and the cytosol; the CAT enzyme is present only in peroxisomes, and POD is distributed in vacuoles, cell walls and the cytosol (Wu et al., 2012). Excessive production of O_2^- triggers SOD enzyme activity, which converts superoxide radicals into either O_2 or H_2O_2, while excessive accumulation of H_2O_2 is prevented by enzymes of the ascorbate-glutathione cycle (Ma et al., 2014). Like in our study, elevation of SOD activity under drought stress had also been reported before in *Triticum aestivum* (Hameed et al., 2013). In our study, mild drought stress induced SOD activity, but later reduced it due to decreased osmotic potential. This finding indicates that the SOD enzyme leaves its scavenging role to the other antioxidant enzymes under increased drought stress because elevated SOD activity also contributed to H_2O_2 generation in the plant. In the same conditions, elevated POD activity and nearly unchanged CAT activity catalyzed the transformation of H_2O_2 to H_2O and protected *A. orientalis* against drought stress. POD is involved in processes such as lignification and tolerance to environmental stresses in higher plants. Increasing POD activity was also reported in *Trifolium repens* under drought stress, while SOD activity decreased simultaneously (Li et al., 2013). The increase in drought stress-induced POD activity was more pronounced than in CAT activity. This finding suggests a supplementary role of CAT in regulating H_2O_2 in *A. orientalis* exposed to drought stress. A similar role of CAT was also observed during an *in vitro* salt stress in *A. orientalis* (Acemi et al., 2017b). The main function of CAT is to decompose mainly subcellular H_2O_2 to H_2O and O_2. Also, cooperation between POD and CAT has been declared, since CAT removes the bulk H_2O_2 in the cell, whereas POD scavenges H_2O_2 which has not been decomposed by CAT (Willekens et al., 1997). Increased CAT activity during drought stress was also reported in *Olea europea* (Proietti et al., 2013). However, in *Brassica juncea*, it was not affected

by drought stress (Alam et al., 2013). In this study, the results showed that SOD and POD activities in *A. orientalis* are highly sensitive to drought stress.

CONCLUSIONS

The present study has revealed that although plant growth parameters were negatively affected, *A. orientalis* may partly overcome deleterious effects of long-term mild drought stress because plant growth was not totally inhibited but only reduced. Nevertheless, *A. orientalis* should be planted on well-irrigated lands as part of a proper conservation study to ensure the continuation of its generations. Also, this study is important in that it shows the primary role of POD and a supplementary role of CAT in regulating H_2O_2 accumulation in *A. orientalis* during an environmental stress. Other possible abiotic stress factors affecting *A. orientalis* such as pollutant contamination, radiation and temperature will also be investigated in future studies.

AUTHOR CONTRIBUTIONS

A.A. – designed and conducted the *in vitro* experiments and also performed the statistical analyses; Y.D. and Y.Y.K. – designed and conducted the biochemical assays; A.A., Y.D. and Y.Y.K. – wrote the manuscript; F.Ö. – supplied the plant material and edited the manuscript.

CONFLICT OF INTEREST

Authors declare no conflict of interest.

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