Acta Biologica Hungarica 61(3), pp. 282–298 (2010)
DOI: 10.1556/ABiol.61.2010.3.5

SCAVENGING OF REACTIVE OXYGEN SPECIES IN APOPLASTIC AND SYMPLASTIC AREAS OF ROLLED LEAVES IN *CTENANTHE SETOSA* UNDER DROUGHT STRESS

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(Received: August 17, 2009; accepted: October 28, 2009)

The correspondence among apoplastic and symplastic antioxidant status, stomatal conductance and water potential was investigated during leaf rolling in *Ctenanthe setosa* (Rosc.) Eichler (Marantaceae) under drought stress. Apoplastic and symplastic extractions of leaf and petiole were performed at different visual leaf rolling scores from 1 to 4 (1 is unrolled, 4 is tightly rolled and the others are intermediate form). In the leaf symplast, the highest changes were found in catalase (CAT) and guaiacol peroxidase (GPX) activities when compared to score 1 during leaf rolling. No significant change was observed in superoxide dismutase (SOD) and ascorbate peroxidase (APX) activities in the symplast of leaf during the rolling. The same phenomenon was also present in the symplast of petiole except APX activity. In the leaf apoplast, the highest increase occurred in APX and GPX activities, whilst a slight increase in CAT and SOD activities. In the apoplast of petiole, the highest increment was found only in GPX activity, while there were small increases in SOD, APX and CAT activities. Hydrogen peroxide content increased up to score 3 in the apoplast and symplast of leaf and petiole but then slightly decreased. Also, superoxide production increased in the leaf and petiole apoplast but its quantity in the apoplast was much more than that of the symplast. On the other hand, NAD(P)H oxidase activity increased in the leaf but no change was observed in the petiole. In conclusion, as a result of water deficit during leaf rolling antioxidant enzymes are induced to scavenging of ROS produced in symplast and apoplast.

Keywords: Antioxidant enzymes – apoplast – *Ctenanthe setosa* – drought – leaf rolling – symplast

INTRODUCTION

Drought stress is one of the major factors limiting the growth of land plants. Drought is probably the most important stress factor determining plant growth and productivity since it brings about a reduction in growth rate, leaf expansion and stomata movements [19]. Plants have a series of non-enzymatic and enzymatic antioxidant systems to cope with drought stress and to avoid photooxidative damage, either by stress avoidance or stress tolerance. When plants are subjected to environmental stresses,

Abbreviations: APX – ascorbate peroxidase; AWF – apoplastic washing fluid; CAT – catalase; G6PDH – glucose-6-phosphate dehydrogenase; GPX – guaiacol peroxidase; RWC – relative water content; g_s – stomatal conductance; SOD – superoxide dismutase; Ψ_{leaf} – leaf water potential

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some reactive oxygen species (ROS) such as superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($^{\bullet}OH$) and singlet oxygen ($^1O_2$) have been produced. These ROSs may initiate destructive oxidative processes such as lipid peroxidation, chlorophyll bleaching, protein oxidation, and damage to nucleic acids [36]. However, enzymatic antioxidant systems such as superoxide dismutase (SOD, EC 1.15.1.1), glutathione reductase (GR, EC 1.6.4.2), catalase (CAT, EC 1.11.1.1), peroxidase (POD, EC 1.11.1.7) and ascorbate peroxidase (APX, EC 1.11.1.11) provide protection against the toxic effects of reactive oxygen species. Modulation in the activities of these enzymes may be an important factor in tolerance of various plants to environmental stresses [34].

Although H_2O_2 is scarcely toxic by itself unless at very high concentrations, it can damage membranes, particularly following its reduction to $^{\bullet}OH$ radical by transition metals (Fenton reaction). H_2O_2 is produced during different metabolic processes such as during photorespiration in chloroplasts or during the formation of lignin in cell walls and moves rapidly across membranes of the cells and organelles [17]. Its cellular levels are strictly linked to the generation of $O_2^{\bullet -}$. Nevertheless, other oxidases such as glycolate oxidases, glucose oxidases, aminoacidoxidases, and sulfite oxidases release H_2O_2 following the oxidation of their respective substrates [3]. In recent years, other enzymatic sources of $O_2^{\bullet -}/H_2O_2$ such as cell wall-bound peroxidases, oxalate, amine and NADPH oxidases have been identified [17]. NAD(P)H oxidase is a plasma membrane-bound enzyme and whose function is to produce superoxide radical that is active in the extracellular spaces [7]. It has been postulated that this activity may have important roles in mediating several important plant responses [4].

The apoplast has been defined as the extraprotoplastic matrix of plant cells including the cell wall. The apoplast is involved in many physiological processes of the cell, for instance nutrition, growth and defence. Also, the apoplast matrix contains many enzymatic and non enzymatic components [14]. The effects of biotic and abiotic stress on the antioxidant systems of the apoplastic space have been studied [14, 28, 45]. Little is known about synthesis of ROS in apoplast and, in this case, the role played by apoplastic antioxidant systems during drought. In addition there is not available data about apoplastic antioxidant changes during leaf rolling resulting from drought stress.

Ctenanthe setosa is a member of family of tropical herbaceous perennials and is cultivated as a greenhouse ornament and house plant. Additionally, *C. setosa* is a plant that evidently shows a leaf rolling response to drought stress [43]. In recent years, some studies have been recorded on resistance to drought stress during leaf rolling in *C. setosa* [22–24, 41–43]. As emphasized in these reports, *C. setosa* could be a model plant to explain the leaf rolling that is one of the mechanisms of resistance to drought stress. Moreover, its leaves show gradual rolling and the duration of this process takes a long time (between 30-40 days) therefore observation and following of leaf rolling are practical. As known, *C. setosa* has a rhizome and a long petiole in upper side of the soil. Moreover, leaf shows the rolling while the petiole has not such a morphologically obvious adaptation response to drought. For this reason, we stud-

ied antioxidant system in two parts of this plant; the lamina (without petiole, we called it leaf or leaves in the text) and the petiole.

Although there are some studies on the relationships between antioxidative enzymes and various environmental stresses such as salt, cold and ozone in apoplastic/symplastic spaces, there is no information about how the balance among antioxidant enzyme activities (SOD, GPX, CAT and APX) and ROS changes under drought stress in apoplastic/symplastic spaces in plants. For this reason, the objective of the present study was to examine the changes in activities of the antioxidant enzymes (SOD, GPX, CAT and APX), and contents of the ROS in apoplastic and symplastic spaces of lamina and petiole at four different leaf rolling scores under drought stress in *C. setosa*.

MATERIALS AND METHODS

Growth of the plants and stress applications

Ctenanthe setosa (Rosc.) Eichler (Marantaceae) plants were vegetatively propagated and grown in plastic pots (14 cm high, 16 cm top and 11 cm bottom diameter) containing peat. The old and wilted leaves were trimmed. Plants were kept well watered and then incubated in a growth chamber with the following parameters: 16 h light and 8 h darkness at 25 °C, relative humidity 70%, photon flux density at the surface of the leaves 300 μmol m^{-2} s^{-1}. The plants at the same age were selected. Old and wilted leaves were trimmed. Some plants were well-watered (control) throughout the experiment while others were subjected to drought stress to achieve different visual leaf rolling scores from 1 to 4 ( : 1,  : 2,  : 3,  : 4) by withholding water through 56 days. Score 1 indicates no rolling (control plants), while score 4 indicates a complete rolling. Visual leaf rolling scores were also used in other studies [31]. Following parameters were measured in apoplastic and symplastic spaces during leaf rolling.

Water status

Relative water content (RWC) of the leaves and petioles was estimated according to the method of Castillo [11] and calculated for each leaf rolling score. Samples (0.5 g) were saturated in 100 ml of distilled water for 24 h at 4 °C in the dark and their turgid weights were recorded. Subsequently, they were dried at 65 °C of oven for 48 h and their dry weights were recorded. RWC was calculated as given below:

RWC (%) = [(FW – DW) / (TW – DW)] × 100, where FW, DW, and TW are fresh weight, dry weight, and turgid weight, respectively.

Leaf water potential (Ψ_{leaf}) was measured with a C-52 thermocouple psychrometer (Wescor, Inc., Logan, UT USA). Six discs about 6 mm in diameter were cut from the youngest fully expanded leaves of plants and sealed in the C-52 psychrometer cham-

ber. Samples were equilibrated for 45 min before the readings were recorded by a Wescor PSYPRO water potential datalogger in the psychrometric mode.

Stomatal conductance

Stomatal conductance (g_s) was monitored on randomly selected six leaves by using a dynamic diffusion porometer (AP4, Delta-T Devices, Burwell, Cambridge, UK) after calibrated with a standard calibration plate following manufacturer's instructions.

H_2O_2 *assay*

Apoplastic washing fluid (AWF) for determination of apoplastic H_2O_2 was extracted from the leaf and petiole by vacuum infiltration according to Takahama and Oniki [39]. Fresh leaf and petiole were cut into 1 cm lengths, washed in deionised water and infiltrated for 20 min with 100 mM KCl solution (pH 5.5). The leaf and petiole were blotted dry with thin paper tissues, placed vertically in barrels in centrifuge vials and centrifuged at 1450 g and 825 g for 15 min at 4 °C, respectively.

H_2O_2 concentration in the AWF was determined by the method of Jiang et al. [21]. The method is highly sensitive and measures low levels of water-soluble hydroperoxide present in the aqueous phase. H_2O_2 was determined by adding 300 µl of H_2O_2 to 200 µl of AWF and 500 µl of assay reagent (500 µM ammonium ferrous sulfate, 50 mM H_2SO_4, 200 µM xylenol orange, and 200 mM sorbitol). Absorbance of the Fe^{+3}-xylenol orange complex (A_{560}) was detected after 45 min. The specificity for H_2O_2 was tested by eliminating H_2O_2 in the reaction mixture with catalase. Standard H_2O_2 curves were obtained for each independent experiment by adding variable amounts of H_2O_2 to 500 µl of assay reagent. Data were normalized and expressed as µmol in the AWF.

Following collection of AWF, the residual leaves and petioles (1.0 g) were ground in 5 ml of 5% trichloroacetic acid with 0.1 g activated charcoal at 0 °C [2]. The homogenate was filtered, centrifuged at 20.000 *g* for 20 min and the supernatant was adjusted to pH 3.5 with 4N KOH. H_2O_2 content was measured according to the method of Capaldi and Taylor [9] based on the oxidative coupling of 3-methyl-2-benzothiazolinone hydrazone (MBTH) with its formaldehyde azine by H_2O_2 in the presence of horseradish peroxidase which results in the formation of a tetraazapentamethine dye. The sample (200 µl) was added to 100 µl of reagent solution containing 3.4 mM MBTH and 3.32 mM formaldehyde. The reaction was initiated by adding 500 µl of the solution of horseradish peroxidase (0.5 U) in 0.2 M sodium acetate buffer (pH 3.5), and after 2 min it was quenched with 1400 µl of 1N HCl. The absorbance at 630 nm was measured 15 min after quenching. H_2O_2 content was estimated from a standard curve prepared with known concentrations of H_2O_2, and was expressed in µmol g^{-1} FW.

Determination of superoxide radical production

Superoxide released to the medium was determined spectrophotometrically, using the tetrazolium salt XTT {(2.3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide} (Sigma-Aldrich Co., Israel) as described by Frahry and Schopfer [16] with minor modification. For quantitative determination of superoxide radicals in symplast, the leaf and petiole (0.5 g) were cut into small pieces and the leaf and petiole segments were vacuum-infiltrated for 20 min with 5 ml of 10 mM Na-citrate buffer (pH 7.0) containing 500 μM XTT, with/without 3.5 U ml^{-1} superoxide dismutase in dark. Then, samples were totally incubated 2 h in the buffer. The buffer containing $O_2^{\bullet-}$ which was released by the samples was used for symplastic $O_2^{\bullet-}$ determination.

In order to determine the apoplastic superoxide radicals, the leaf and petiole segments were also blotted dry, rolled and placed vertically in a barrel. The barrels were placed in centrifuge vials. Apoplastic extract was collected from the bottom of the vials after leaves and petioles were centrifuged at 1450 g and 825 g for 15 min, respectively. In both symplast and apoplast, the increase in XTT reduction was read at 470 nm in a spectrophotometer. Specific absorbance due to the presence of $O_2^{\bullet-}$ was calculated as the difference in A_{470} between samples with and without SOD. It was transformed into molar concentration using an extinction coefficient of 2.16×10^4 M^{-1} cm^{-1}.

Determination of dilution factors for apoplastic H_2O_2 *and* $O_2^{\bullet-}$

Apoplastic air and water volumes were firstly determined to calculate the dilution factors of apoplastic H_2O_2 and $O_2^{\bullet-}$. For the determination of apoplastic air volume (V_{air}), the leaf and petiole segments were weighted, infiltrated with high-viscosity silicone fluid, for which the plasma membrane is impermeable, under vacuum and reweighed. The leaf surface was blotted dry, and the weight increase (corrected for the density of silicone oil) was used to determine V_{air} [20].

Apoplastic water volume was determined as described previously [20]. The leaf and petiole segments were weighed and infiltrated with 0.05 M indigo carmine (indigo-5,5'-disulphonic acid) which dissolved in the infiltration media. The vacuum infiltration was carried out using a barrel. The leaf and petiole segments were then centrifuged at 1450 g and 825 g for 15 min at 4 °C, respectively, and the extinction of dye before infiltration (E_{inf}) and in the apoplastic washing fluid (E_{AWF}) was measured spectrophotometrically in 150 μl samples at 610 nm. The relative apoplastic water volume (V_{apo}, cm^3 H_2O cm^{-3} tissue) was calculated as:

$$V_{apo} = (E_{inf} \times V_{air} / E_{AWF}) - V_{air}$$

The concentrations of apoplastic H_2O_2 and $O_2^{\bullet-}$ were corrected for dilution with the infiltration media by multiplication with the dilution factor.

$$(F_{dil}): F_{dil} = (V_{apo} + V_{air}) / V_{apo}$$

Extractions of antioxidant enzymes

Apoplastic washing fluid for determination of apoplastic antioxidant enzymes was extracted by vacuum infiltration method by Hernandez et al. [18]. Fresh leaf and petiole segments (2 g) were cut into 1 cm lengths, and rinsed in tap water six times to remove cellular proteins from the cut ends. Then, the leaf and petiole segments were vacuum-infiltrated for 20 min with 50 mM potassium-phosphate buffer (pH 6.5), containing 0.2 M KCl and 0.1 mM $CaCl_2$. The samples were blotted dry, and the apoplastic solutions were collected in centrifuge vials by centrifuging as mentioned above. AWF was kept at –20 °C and used for analysis.

After the collection of AWF, the residual leaf and petiole materials (0.5 g) were homogenized with 1% polyvinylpyrrolidone (PVP) in 5 ml extraction buffer (50 mM potassium-phosphate buffer, 1 mM EDTA pH 7.0). For APX activity, 5 mM ascorbate was added. The symplastic homogenate was centrifuged at 18.000 *g* for 20 min at 4 °C. Residual leaf extracts were decanted into Eppendorf tubes and stored at –20 °C until used for analysis.

Enzyme analysis

All assays were performed using a final volume of 1 ml. Guaiacol peroxidase activity was measured by monitoring the increase in absorbance at 470 nm in 100 mM potassium-phosphate buffer (pH 7.0) containing 0.1 mM EDTA, 5 mM guaiacol, 15 mM H_2O_2 and 50 µl enzyme extract [44]. GPX activity was calculated using and extinction coefficient of 26.6 mM^{-1} cm^{-1} for tetraguaiacol at 470 nm.

Superoxide dismutase activity was determined using the nitroblue tetrazolium (NBT) reduction method of Beauchamp and Fridovich [5]. For activity assay 50 mM potassium-phosphate buffer (pH 7.8) containing 0.1 mM EDTA, 2 µM riboflavin, 13 mM L-methionine and 75 µM NBT was used. Initial absorbance at 560 nm was recorded then samples were exposed to 200 µmol photons m^{-2} s^{-1} for 10 min. Absorbance at 560 nm was then remeasured. The SOD activity was expressed in U; 1 unit corresponding to the amount needed to cause a 50% inhibition in the absorbance of controls performed for each individual assay.

Ascorbate peroxidase activity was determined from the decrease in absorbance at 290 nm, following ascorbate (ASC) consumption [29]. The assay contained 50 mM K_2HPO_4 (pH 7.0), 750 µM ASC, 5 mM H_2O_2. APX activity was calculated by using an extinction coefficient of 2.8 mM^{-1} cm^{-1} for ASC at 290 nm.

Catalase activity was determined from the decrease in absorbance at 240 nm, following the consumption of H_2O_2 [1]. The assay contained 50 mM K_2HPO_4 (pH 7.0), 30 mM H_2O_2 and 20 µl enzyme extract. CAT activity was calculated by using an extinction coefficient of 39.4 mM^{-1} cm^{-1} for H_2O_2 at 240 nm.

NAD(P)H oxidase was extracted with a mortar and pestle from 1.0 g leaf and petiole tissue at 4 °C with 1% PVP and 5 ml of the following extraction solution: 100 mM potassium-phosphate buffer at pH 7.0, 0.1 mM EDTA, and 0.1% Triton. The

extract was centrifuged at 15.000 *g* for 20 min at 4 °C. The supernatant was used for the enzymatic assay. NAD(P)H oxidase was assayed according to Cakmak and Marschner [8]. The reaction mixture contained 0.1 M NADPH. The reaction was initiated by adding the enzyme extract, and the oxidation of NADPH or NADH was recorded by spectrophotometer at 340 nm.

Glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49) activity was used to assess the degree of cytoplasmic contamination of AWF. Activity was measured following the reduction of NADP at 340 nm [27] using 66 mM K_2HPO_4 (pH 7.6), 10 mM $MgCl_2$, 300 µM NADP, 2 mM glucose-6-phosphate (G6P) and 50 µl extract.

Protein content of samples was determined by the Bradford method. Bovine serum albumin was used as a standard [6].

Statistical analysis

All analysis was repeated three times with three replicates. Variance analysis of mean values was performed with Duncan Multiple Comparison test by using SPSS software for Microsoft Windows (Ver. 10.0, SPSS Inc., USA) and significance was determined at the 5% ($P \leq 0.05$) level.

RESULTS

Water status

The leaf rolling began on the 35th day of drought period and relative water content decreased in the leaf and petiole during leaf rolling. Decrease in RWC of petiole was more than that of the leaf. RWC in the leaf and petiole was 95% and 97% at score 1 but 78% and 68% at score 4, respectively (Table 1).

Water potentials of leaves (Ψ_{leaf}) were down from –0.15 MPa at score 1 to –1.18 MPa at score 4 (Table 1).

Table 1

Relative water content (RWC), stomatal conductance and water potential under drought stress condition during leaf rolling. Stomatal conductance was measured in the open areas of rolled and unrolled leaf

Drought period	Visual leaf rolling scores	RWC (%)		Water potential (MPa)	Stomatal conductance (cm s^{-1})
		Leaf	Petiole		
0	1	95.00±0.70 d[a]	97.37±0.54 d	–0.15±0.05 a	0.02±0.002 c
35	2	91.21±2.51 c	93.61±0.90 c	–0.18±0.05 a	0.009±0.001 b
47	3	82.03±0.39 b	79.05±0.07 b	–0.50±0.06 b	0.009±0,001 b
56	4	78.85±0.27 a	68.26±2.17 a	–1.18±0.11 c	0.007±0.001 a

[a]Different letters denote significant differences among means. Mean comparison among drought periods was performed using the ANOVA test at $p \leq 0.05$. The results are the mean ± SD of three replicates.

Stomatal conductance

In *C. setosa*, stomatal conductance also gradually decreased while visual leaf rolling scores were increasing under drought stress condition. Exposure to drought stress resulted in decreasing of stomatal conductance in 55%, 57% and 66% at scores 2, 3 and 4 in leaf, respectively (Table 1).

ROS production in AWF

The air and apoplastic water volume are important parameters for the calculation of apoplastic H_2O_2 and $O_2^{\bullet-}$ concentration from AWF. Due to the fact that the infiltration of the apoplastic air space leads to a dilution of the apoplastic fluid, the solute concentrations in the AWF have to be corrected by dilution factor. Dilution factors used for calculation of H_2O_2 were 1.8 and 2.2 in the leaf and petiole, respectively. As for $O_2^{\bullet-}$, they were 1.2 and 1.5 in the leaf and petiole, respectively.

Apoplastic H_2O_2 content significantly increased up to score 3 in the leaf and petiole compared to score 1 but slightly declined at score 4 compared to score 3. Ratio of the increase at score 3 compared to score 1 was 2.1- and 2-fold in the leaf and petiole, respectively (Table 2).

Apoplastic $O_2^{\bullet-}$ production in the leaf and petiole increased up to score 3 compared to score 1 but a little declined at score 4. At score 3, the increase in the leaf and petiole was found as 5.0- and 4.6-fold in comparison with score 1, respectively (Table 2).

ROS production in residual leaf and petiole

Symplastic H_2O_2 content significantly increased up to score 3 in the leaf and petiole but it declined at score 4 as similar to apoplastic H_2O_2 content. Ratio of the increase at score 3 compared to score 1 was 1.4- and 2.1-fold in the leaf and petiole compared to score 1, respectively (Table 2). Also, symplastic H_2O_2 contents of leaf and petiole were higher than those of their apoplasts.

Symplastic $O_2^{\bullet-}$ production significantly increased in both leaf and petiole during leaf rolling. The increase of $O_2^{\bullet-}$ production at score 4 was 2.1- and 1.3-fold as compared to score 1 in the leaf and petiole, respectively. Also, symplastic $O_2^{\bullet-}$ quantities in the leaf and petiole were lower than those of their apoplasts (Table 2).

Apoplastic antioxidant enzymes

G6PDH activity as a cytoplasmic marker enzyme was measured to determine the cytoplasmic contamination of each apoplastic sample. Our results showed that AWF was free from symplastic contamination. G6PDH activity in AWF was below the

Table 2
The changes in H_2O_2 content and $O_2^{\bullet -}$ production during leaf rolling

Visual leaf rolling scores	H_2O_2 (µmol g^{-1} fw)				$O_2^{\bullet -}$ (nmol min^{-1}g fw)			
	Leaf		Petiole		Leaf		Petiole	
	Symplast	Apoplast	Symplast	Apoplast	Symplast	Apoplast	Symplast	Apoplast
1	3.8 ± 0.6 a[a]	1.5 ± 0.4 a	6.7 ± 0.1 a	1.3 ± 0.4 a	31.8 ± 1.6 a	101.6 ± 30.9 a	27.3 ± 4.4 a	24.0 ± 22.3 a
2	4.8 ± 0.4 ab	1.6 ± 0.2 a	9.7 ± 0.1 b	1.4 ± 0.3 a	37.3 ± 0.9 b	246.0 ± 14.8 b	32.9 ± 1.2 b	71.6 ± 15.5 b
3	5.5 ± 0.7 b	3.1 ± 0.3 b	14.3 ± 0.6 c	2.6 ± 0.3 b	41.1 ± 1.2 b	504.2 ± 13.7 c	31.4 ± 0.4 ab	110.2 ± 15.6 c
4	4.7 ± 0.3 ab	1.7 ± 0.4 a	9.4 ± 0.3 b	1.7 ± 0.3 b	67.9 ± 5.1 c	284.2 ± 26.2 b	35.5 ± 0.8 b	61.4 ± 10.2 b

[a]Different letters denote significant differences among means. Mean comparison among drought periods was performed using the ANOVA test at $p \leq 0.05$. The results are the mean ± SD of three replicates.

limits of detection (1%) in *C. setosa*. It was found 0.075, 0.070, 0.061, 0.086% in the leaf, 0.19, 0.15, 0.32, 0.47% in the petiole at scores 1, 2, 3 and 4, respectively. Exact values for the contamination of each sample were obtained, and this allowed an accurate determination of cytoplasmic contamination in each apoplastic sample.

The results showed that GPX, SOD, CAT and APX were present in the apoplastic spaces of the leaf and petiole. GPX activity significantly increased in the leaf and petiole during leaf rolling. The activity of GPX in the leaf was higher than that of the petiole. The activity in apoplast also increased 23%, 228% and 295% in the leaf and 32%, 96% and 206% in the petiole at scores 2, 3 and 4, respectively (Fig. 1A).

SOD activity in the leaf and petiole increased at all rolling scores compared to score 1 but slightly declined at score 4 compared to score 3 in the leaf and petiole (Fig. 1B). SOD activity in the apoplast of leaf was higher than that of the petiole. For example, at score 1, SOD activity was found about 0.55 U/mg protein in the apoplast of leaf while it was about 0.21 U/mg protein in the apoplast of petiole.

The activity of CAT in the leaf and petiole significantly increased at all leaf rolling scores compared to score 1. The activity in apoplast increased 25%, 56% and 41% and 10%, 11% and 13% in petiole at scores 2, 3 and 4, respectively (Fig. 1C).

The highest activity of APX in apoplast was found in the leaf. APX activity increased in the leaf and petiole at all rolling scores compared to score 1. Moreover, the highest APX activity in apoplast was observed at score 4 (Fig. 1D).

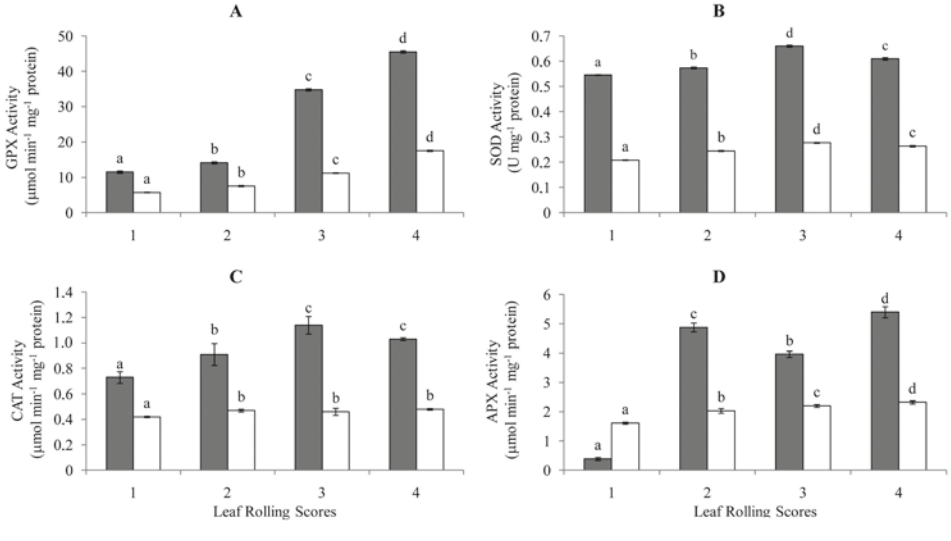

Fig. 1. Enzyme activities (A–D) measured in apoplastic washing fluid isolated from the leaf and petiole of *C. setosa*. (A) GPX, guaiacol peroxidase; (B) SOD, superoxide dismutase; (C) CAT, catalase; (D) APX, ascorbate peroxidase. The vertical bars represent standard deviation of the means of three replicates. Leaf or leaves were used for lamina (without petiole) (■) leaf apoplast; (□) petiole apoplast

Antioxidant enzymes of residual leaf and petiole

GPX activity significantly increased in the leaf and petiole during leaf rolling. The highest activity was found in the symplast of leaf. The activity increased 7%, 13% and 31% in the symplast of leaf and 7%, 15% and 26% in the symplast of petiole at scores 2, 3 and 4, respectively (Fig. 2A).

The activity of SOD in the leaf and petiole did not significantly change during leaf rolling (Fig. 2B).

CAT activity in the leaf and petiole increased during leaf rolling. These increases were statistically significant ($P \leq 0.05$) at all rolling scores in the leaf but only at score 3 and score 4 in the petiole. The activity increased 206%, 252% and 247% in the leaf and 2%, 16% and 56% in the petiole at scores 2, 3 and 4, respectively (Fig. 2C).

APX activity in the leaf did not change at all leaf rolling scores. The activity increased in the petiole at score 2 and score 4 compared to score 1 but no change was found at score 3 during drought period (Fig. 2D).

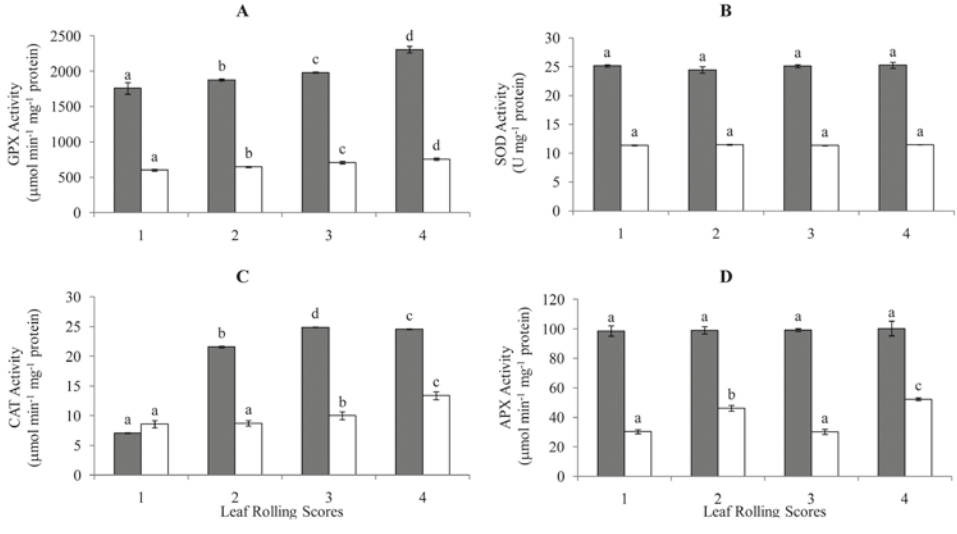

Fig. 2. Enzyme activities (A–D) measured in residual leaf extracts from the leaf and petiole of *C. setosa*. (A) GPX, guaiacol peroxidase; (B) SOD, superoxide dismutase; (C) CAT, catalase; (D) APX, ascorbate peroxidase. The vertical bars represent standard deviation of the means of three replicates. Leaf or leaves were used for lamina (without petiole) (■) leaf symplast; (□) petiole symplast

NAD(P)H oxidase activity

NAD(P)H oxidase activity significantly increased at scores 3 and 4 compared to score 1 in the leaf. These increases were statistically significant ($P \leq 0.05$) at scores 3 and 4. The increase of activity was 1.2-fold at score 4 compared to score 1. However, in the petiole, the activity did not change at all leaf rolling scores (Fig. 3).

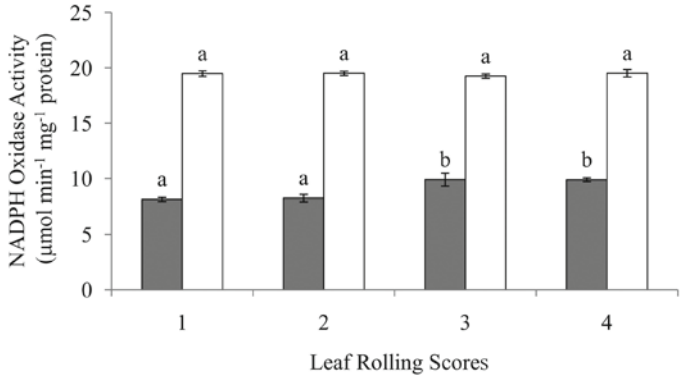

Fig. 3. NAD(P)H oxidase activity in the leaf and petiole. The vertical bars represent standard deviation of the means of three replicates. Leaf or leaves were used for lamina (without petiole) (■) leaf; (□) petiole

DISCUSSION

In the present study, RWC and water potential (Ψ_{leaf}) of the leaf declined in response to drought stress (Table 1). As known, RWC and Ψ_{leaf} are good indicators for drought stress [38]. As indicated before, *C. setosa* has mechanism of leaf rolling decreasing transpiration and water loss during drought stress. Moreover, this plant resists drought a long time (about 60 days) since decrease in RWC and Ψ_{leaf} are not high during the drought period as compared to less drought resistant plant species. There were good negative correlations between leaf rolling and RWC ($r = -0.99$, $P < 0.01$) and water potential ($r = -0.90$, $P < 0.05$) in the leaf. The minor changes in water potential and RWC in the leaf during drought period may strongly be related to an adaptation response of the plant to the extent of drought stress.

In the present study, g_s was approximately 2-fold lower in the rolled leaves than that of the unrolled leaves (Table 1). It is clear that stomata progressively close during leaf rolling. However, no notable change was observed between scores 2 and 4 since the conductance is steady at these scores. We can suppose that leaf rolling may contribute to creating a cooler area inside of the leaf in which some of the stomata can still take CO_2 from air to maintain photosynthesis. In addition, variations in stomatal conductance cause changes in leaf water potential by changing transpiration rate [37].

G6PDH was used as a cytoplasmic marker enzyme to determine the degree of contamination of the apoplastic extracts by components originating from within the cells. G6PDH activity in AWF was below the limits of detection (1%) in *C. setosa*. Similarly, the activity of G6PDH in apoplast of barley leaves was below 1% [32].

In the present study, H_2O_2 content increased up to score 3 but later, decreased in the apoplast and symplast of leaf and petiole (Table 2). We also found that content of $O_2^{\bullet-}$ enhanced 2.1- and 1.3-fold in the symplast of leaf and petiole during leaf rolling,

respectively. So, we can say that leaf rolling does not exactly prevent ROS production although it decreases water loss. Moreover, in the apoplast, the increase in $O_2^{\bullet-}$ was higher than that of the symplast. Increased superoxide in apoplast may convert to H_2O_2 with dismutation and cause lignification of the cell wall [30]. Superoxide is produced in electron transport chain which includes a number of enzymes on the reducing (acceptor) side of photosystem I: Fe-S centers, reduced thioredoxin, and ferredoxin. These electron transport components are auto-oxidizable and superoxide radical can be formed under conditions limiting the availability of NADP [12, 15]. Indeed, in the present study, NAD(P)H oxidase activity increased in the leaf, whereas did not change in the petiole during the rolling (Fig. 3). Thus, in the petiole, superoxide may be mostly produced by the other ways and the other enzymes such as xanthine oxidase.

In the present study, we determined that the apoplast had an important activity of antioxidant enzymes during leaf rolling. Chiefly, GPX activity was high in both apoplastic and symplastic areas of leaf and petiole (Figs 1A, 2A). In addition, APX and CAT were the second and third enzymes which had high activity in symplast and apoplast during leaf rolling, respectively. We suggest that the activities of these enzymes increased to scavenge toxic H_2O_2 in symplast and apoplast during leaf rolling.

As indicated above, GPX and APX activities in the apoplast of leaf highly enhanced under drought stress (Figs 1A, 1D). These results are consistent with the findings of Diaz-Vivancos et al. [13] reporting H_2O_2 scavenging enzymes (APX and POD) increased in the apoplast of peach and apricot leaves**.** In earlier studies, it was recorded that peroxidases protect cells against damaging effects of H_2O_2 during an oxidative-burst response [7]. Similarly, in the present study, increase in apoplastic GPX and APX enzymes may contribute to tolerance to drought stress during leaf rolling. In our study, in addition to apoplast, one of the highest changes in antioxidant enzymes in symplastic area was also found in GPX activity during the rolling in the leaf. However, in this work, symplastic APX activity in the leaf did not change but increased in the petiole (except score 3). This increase probably results from high increase in H_2O_2 level in the symplast of petiole as compared to the leaves. In the leaves, H_2O_2 was also scavenged by increase in CAT and GPX. According to these results, it can be said that GPX and APX have a main role in scavenging of H_2O_2 in both apoplast and symplast during leaf rolling under drought. Thus, the decrease in H_2O_2 content at score 4 as compare to score 3 can arise from the increases in GPX, APX and CAT activities in the apoplast and symplast of leaf and petiole.

In our study, apoplastic and symplastic CAT activities increased in the leaf and petiole during the rolling (Figs 1C, 2C). Similar results on apoplastic CAT activity were reported in other plants [33, 45]. The results, in the present study, indicated that CAT activity could be involved in the control of regulation of H_2O_2 in extracellular spaces [33].

Superoxide level increased in the apoplast of leaf and petiole in our experiment. Superoxide produced in the symplast may be transported to extracellular space

instead of superoxide dismutation by SOD in the symplast in this plant. As remembered, SOD activity in the symplast of both plant parts during leaf rolling did not change. Indeed, it is known that in plants, the superoxide radical is permeable across membrane in hydroperoxyl form [35].

In our study, SOD was also found to be located in the apoplast of leaves of *C. setosa*, as it has previously been reported in some plants [10, 28]. We also found that apoplastic SOD activity significantly increased at all rolling scores in the leaf and petiole. SOD activity may serve to reduce the risk of hydroxyl radical formation by resulting from an enhanced $O_2^{\bullet-}$ production. The results supported that apoplastic SOD is induced much more than symplastic SOD to catalyze the synthesis of H_2O_2 during the oxidative burst in the rolling process. Recently, Karlsson et al. [26] have reported that a role for apoplastic SOD in regulation of H_2O_2 required for the development of the secondary cell wall. Moreover, the increased apoplastic SOD activity in *C. setosa* may play a role in lignification, since apoplastic SOD has been associated with cell wall lignification [30].

It is well known that various environmental stresses cause an over-production of ROS leading to lipid peroxidation, which ultimately causes membrane damage [40]. In the previous study, it was found that MDA content, a final product of lipid peroxidation increased at score 2 but later, was down to the level of score 1 in leaf of *C. setosa* [41]. As reminded, a sharp decrease in stomatal conductance occurred at score 2. This situation may induce ROS production and subsequently membrane damage because of declining of CO_2 under light conditions. Lower MDA content at the last scores (score 3 and score 4) may be closely related to the increase in the activities of the antioxidant enzymes. Higher activities of the antioxidative enzymes in *C. setosa* effectively protected the plant tissues from membrane damage induced by drought stress. Moreover, increased ratio of H_2O_2 in the petiole was higher than that of the leaf. Similarly, in the previous study, increase in MDA content of petiole was higher than that of the leaf and continued up to score 3. This difference is probably the result of the decrease of RWC in petiole more than that of the leaf.

Although there were many similarities between the leaf and petiole concerning with antioxidant system, we also observed some differences. Although decrease of RWC in the petiole was higher than that of the leaf during the rolling, the activities of antioxidant enzymes in petiole were not higher than those of the leaf. These results showed that antioxidant enzymes in the leaf and petiole were induced in a different manner by ROS. The differences could be associated with different structures of two plant parts or rolling behavior of leaf not petiole. On the other hand, high concentration of H_2O_2 in the petiole can be a possible sign in stress signaling. Various studies suggest that H_2O_2 can function as a mobile signal [25].

In conclusion, all results showed that leaf rolling did not provide enough contribution to drought tolerance, completely prevent ROS production and thus, the antioxidant defense system was stimulated in apoplast and symplastic areas due to decreasing water potential. Moreover, leaf rolling provides a protection for not only leaf but also petiole.

ACKNOWLEDGEMENT

This work is supported by Turkish National Science Foundation (COST FA 0605 Action) Project No: 105T354.

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