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Changes in Anatomical Structure and Levels of Endogenous Phytohormones during Leaf Rolling in *Ctenanthe setosa* under Drought Stress

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Abstract: Leaf rolling occurs as a response to water deficit and its level increases as the drought period is progressing in plants. Changes in anatomical structure and levels of endogenous phytohormones were investigated under drought stress during leaf rolling in *Ctenanthe setosa*. Leaf water potential (Ψ_{leaf}), some anatomical parameters, and the levels of phytohormones were determined at different visual levels of leaf rolling from 1 to 4. Ψ_{leaf}, thickness of mesophyll and lamina, and width/length ratio of hypodermis cells of leaves decreased while leaf rolling level increased. Diameters of xylem vessels and median vein, and length of upper and lower stomatal guard cells also decreased during leaf rolling. The amount of indole-3-acetic acid (IAA) increased up to the 3rd level of leaf rolling but it declined to the value of the 1st level at the 4th level of rolling. The gibberellic acid (GA_3) level changed irregularly during leaf rolling. *Trans*-zeatin and abscisic acid (ABA) amounts also increased during the rolling period. The analysis showed that changes in the levels of these phytohormones may affect the anatomical structure of the leaf during the rolling period under drought stress.

Key Words: Anatomical structure, *Ctenanthe setosa*, drought, leaf rolling, phytohormones

Kuraklık Stresi Koşullarındaki *Ctenanthe setosa*'da Yaprak Kıvrılması Sırasında Anatomik Yapı ve Endojen Fitohormon Seviyelerindeki Değişimler

Özet: Yaprak kıvrılması, bitkilerde su stresine cevap olarak meydana gelir ve seviyesi kuraklık periyodu devam ederken artar. *Ctenanthe setosa*'da yaprak kıvrılması sırasında, kuraklık stresi koşullarında anatomik yapı ve endojen fitohormonların seviyelerindeki değişimler araştırıldı. Yaprak su potansiyeli (Ψ_{yaprak}), bazı anatomik parametreler ve fitohormonların seviyeleri, 1'den 4'e kadar görsel olarak tanımlanan farklı yaprak kıvrılma seviyelerinde belirlendi. Yaprak kıvrılma seviyesi artarken, Ψ_{yaprak}, yaprağın mezofil ve lamina kalınlığı ve hipodermis hücrelerinin genişlik/uzunluk oranı azaldı. Ksilem boruları ve orta damarın çapları ve üst ve alt stomalardaki bekçi hücrelerinin boyları da yaprak kıvrılması sırasında azaldı. İndol-3-asetik asit (IAA) miktarı kıvrılmanın 3. seviyesine kadar arttı fakat kıvrılmanın 4. seviyesinde, 1. seviyedeki değere düştü. Giberellik asit (GA_3) seviyesi yaprak kıvrılması sırasında düzensiz olarak değişti. *Trans*-zeatin ve absisik asit (ABA) miktarları ise kıvrılma periyodu sırasında arttı. Bu fitohormonların seviyelerindeki değişimlerin kuraklık stresi koşullarında meydana gelen kıvrılma periyodu sırasında, yaprağın anatomik yapısını etkilediği sonucuna varıldı.

Anahtar Sözcükler: Anatomik yapı, *Ctenanthe setosa*, kuraklık, yaprak kıvrılması, fitohormonlar

Introduction

Plants are affected by various environmental stresses during their life cycles. Since they are not capable of moving, they can response to environmental stress through morphological and physiological changes. Water deficit causes functional and structural changes in plants.

Although plant species vary in their response to stress, it may be assumed that all plants have different abilities to cope with stress (1). It is known that the tolerance mechanism of plants is a complex phenomenon. Researchers have noted that plants respond to drought by various physiological adaptations. Leaf movements are common adaptive responses to drought stress in plants (2). Among the leaf movements, leaf rolling is an adaptive trait reducing water loss via transpiration, thus controlling plant water metabolism by relieving water stress (3). The rolling also increases drought resistance in cereal crops (4).

Numerous physiological and biochemical changes occur in response to drought stress in various plant species. The levels of phytohormones inducing the plant growth change under various stress conditions. Abscisic acid (ABA) induces a group of proteins (e.g. dehydrins, late embryogenesis abundant proteins) that protect cells from water deficit (5). Endogenous indole-3-acetic acid (IAA) and cytokinin levels were reported to decrease or increase in a few plants under drought stress (6,7). It was also noted that the level of gibberellic acid (GA_3) in melon cultivars decreased under drought stress (8).

Anatomical variations in plants under water stress have also been recorded in various studies (9,10). However, there are very few reports on anatomical changes during leaf rolling in plants. Redmann (11) reported changes in levels of leaf rolling, leaf dimensions, and stomatal densities on adaxial and abaxial leaf surfaces in grass species in dry and wet habitats. This is the first report on changes in the levels of endogenous phytohormones and anatomical structure during leaf rolling in plants. *C. setosa* is a convenient model plant in leaf rolling studies because its leaves show gradual rolling and this process takes a long time (30 to 40 days); therefore the observation of leaf rolling is easy (12). The objective of this study was to determine anatomical variations and changes in the levels of endogenous phytohormones during leaf rolling under drought stress.

Materials and Methods

Ctenanthe setosa Rosc. Eichler was vegetatively propagated and grown in plastic pots containing peat and sand (5:1) in a growth chamber with the following parameters: 16 h light and 8 h darkness at 25 °C, relative humidity 70%, and photon flux density at the surface of the leaves 300 µmol m^{-2} s^{-1}. One-year-old plants were used in the experiment. Some of the plants were well watered (tap water) with 2-day intervals during the experiments and so the unrolled (control) 1st level of leaf rolling was obtained. The other plants were subjected to drought stress to achieve different leaf rolling levels from 2 to 4, by withholding water for 35, 47, and 56 days, respectively. The 1st level indicates no rolling, the 4th level indicates complete rolling, and the others indicate intermediate forms. Similarly, visual leaf rolling levels were also used in other studies (13).

Measurement of water potential

Leaf water potential was measured with a C-52 thermocouple psychrometer. Disks about 6 mm in diameter were cut from the leaves of plants and sealed in the C-52 psychrometer chamber. Samples were equilibrated for 45 min in order to condense water on the thermocouple junction before the readings were recorded by a Wescor PSYPRO water potential datalogger in the psychrometric mode.

Anatomical examination

Leaves of similar size were used for anatomical measurements. Cross sections were cut by hand from the same levels of leaves (1 cm below the leaf tip) in all materials. Sections were mounted on an isotonic sugar solution containing 87% glycerol for the leaf cells at each rolling level. Microscopic sections were prepared at each rolling level from 3 different plants.

Light microscopic observations were performed on cross and surface sections of the leaves. Microphotographs were taken with a Nikon microscope. The thicknesses of the lamina, mesophyll, upper and lower epidermis, width/length ratio of hypodermis cells, diameters of xylem vessels and median veins, and lengths of upper and lower stomatal guard cells were measured under a light microscope. All micrometric measurements (n = 10) were performed 3 times.

Extraction, purification, and determination of endogenous phytohormones

The analysis of indole-3-acetic acid and *trans*-zeatin was performed according to Kuraishi et al. (14) and Battal et al. (15). One gram of each frozen leaf sample was powdered in liquid nitrogen. Then cold methanol was added and it was stored at 4 °C for 24 h in the dark. The samples were homogenized in an Ultra Tissue Lysis and

filtered through filter paper (Whatman No. 1). The filtrates were collected. The residue was reprocessed in the same way as mentioned above and combined with the former one in order to minimize the loss of phytohormones. The filtrates were filtered through polytetrafluoroethylene (PTFE) filters (0.45 µm). Methanol was removed under reduced pressure at 35 °C. The extracts were re-dissolved in K_2PO_4 buffer (pH 8.5) and centrifuged at 10,000 × g for 1 h at 4 °C. Then, the supernatants were put in flask (25 ml), each containing 1 g of polyvinylpolypyrolidone (PVPP), well mixed and filtered through Whatman filter paper (No. 1). The filtrates were introduced into Sep-Pak C_{18} cartridges. The hormones were adsorbed by the cartridges and the remnants were removed. The hormones were eluted from the cartridges with 80% methanol and collected in vials. The hormone extracts were injected into a high performance liquid chromatography (HPLC) system to detect *trans*-zeatin and IAA.

The analysis of GA_3 equivalents was performed according to Fujioka et al. (16), Cakmak et al. (17), and Wang et al. (18) with some modifications. As mentioned above, after methanol extraction, the aqueous residue was adjusted to pH 2.5 with 2 M HCl. This solution was then partitioned with equal volumes of ethyl acetate and the combined organic phases were partitioned with 5% (m/v) sodium bicarbonate (3 × 1/5 volume) and separated GA_3 equivalents were injected into the HPLC system.

The analysis of abscisic acid was also performed according to Unyayar et al. (19) with some modifications. After methanol extraction, the residue was dissolved with 1.5 ml of 0.5 M potassium dihydrogen phosphate (KH_2PO_4) buffer (pH 8.3). The combined organic phases were partitioned 3 times against hexane and 3 times against ethyl acetate at pH 3. The ethyl acetate of the combined organic fractions was removed under reduced pressure at 35 °C. The residue was dissolved in 100% methanol and loaded on a Bondesil DEA column. After the column was washed with 100% methanol, absorbed hormone was eluted with methanol containing 0.5% acetic acid and collected in a vial. The hormone extracts were injected into the HPLC system to detect ABA levels.

The isocratic system was used for HPLC analysis. The HPLC system used for analysis of phytohormones was equipped with a Waters Hichrom 6000 A pump, an ultraviolet detector (Unicam Analytical Systems, Cambridge, UK), and a Bondapak C_{18} column (Waters Hichrom) using acetonitrile (12.00%, pH 4.98) as a mobile phase. The flow rate, pressure, and wavelength were 2 ml min^{-1}, 2000 psi, and 265 nm, respectively. Under these conditions, the retention times of GA_3, *trans*-zeatin, IAA, and ABA were determined as 2.85, 3.88, 7.17, and 20.50 min for the standards, respectively.

Statistical analysis

Measurements of anatomical sections and phytohormonal analysis were performed 30 and 6 times, respectively. Variance analysis of the means was performed with Duncan's multiple comparison test using SPSS for Microsoft Windows (Ver. 10.0, SPSS Inc., USA) and significance was determined at $P < 0.05$.

Results

Leaf water potential

In *C. setosa*, leaf water potential decreased while the level of visual leaf rolling increased under drought stress. It was determined that Ψ_{leaf} was -0.15, -0.18, -0.50, and -1.18 MPa at the 1st, 2nd, 3rd, and 4th levels of leaf rolling, respectively (Table 1).

Anatomical Structure

The structural changes in stressed leaves of *C. setosa* were studied. Thickness of mesophyll and lamina, and width/length ratio of hypodermis cells decreased while

Table 1. Changes in leaf water potential (Ψ_{leaf}) and endogenous phytohormone levels (µg g^{-1} fw) during leaf rolling in *Ctenanthe setosa*.

Period of drought (day)	Level of leaf rolling	Ψ_{leaf} (MPa)	IAA	*trans*-zeatin	GA_3	ABA
0 (control)	1 (unrolled)	-0.15 ± 0.05^{a} *	1.6 ± 0.4^{a}	1.1 ± 0.3^{a}	9.67 ± 1.5^{b}	0.9 ± 0.2^{a}
35	2	-0.18 ± 0.05^{a}	4.6 ± 1.2^{b}	2.6 ± 1.3^{ab}	11.99 ± 1.9^{b}	0.5 ± 0.2^{a}
47	3	-0.50 ± 0.06^{b}	7.4 ± 0.8^{c}	3.4 ± 0.9^{b}	5.08 ± 1.1^{a}	1.5 ± 0.6^{a}
56	4	-1.18 ± 0.11^{c}	1.6 ± 0.5^{a}	5.5 ± 1.1^{c}	9.1 ± 1.7^{b}	3.5 ± 1.2^{b}

* Means ± standard deviation of 6 replicates. Different letters in each column represent significant differences at $P < 0.05$.

thickness of epidermis (both upper and lower) did not significantly change as a result of water stress during leaf rolling (Table 2, Figure 1 A-D). Vessel diameter, diameter of median vein, and length of upper and lower stomatal guard cells also decreased during leaf rolling (Table 2, Figure 2 A-D, Figure 3 A-D). However, vessel diameter was greater at the 3rd level of leaf rolling compared to the 2nd level. In addition, lengths of upper and lower stomatal guard cells were greater at the 4th level of leaf rolling compared to the 3rd level (Table 2, Figure 3-A-D).

Phytohormones

It was determined that the amount of *trans*-zeatin increased during leaf rolling. The amount of IAA increased up to the 3rd level of leaf rolling but at the 4th level it declined to the value of the 1st level. Amount of GA_3 increased at the 2nd level of leaf rolling but the increase was not significant. However, it was significantly decreased at the 3rd level of leaf rolling compared to the 1st level. At the 4th level of leaf rolling, GA_3 again increased and reached the value of the 1st level. In addition, amount of abscisic

Table 2. Changes in some anatomical parameters during leaf rolling in *Ctenanthe setosa*.

Anatomical parameters	Level of leaf rolling			
	1 (unrolled)	2	3	4
Thickness of lamina (μ)	202.9 ± 3.9[b] *	159.3 ± 1.6[a]	157.6 ± 1.5[a]	152.1 ± 1.9[a]
Thickness of mesophyll (μ)	86.3 ± 1.3[b]	70.9 ± 0.7[a]	69.6 ± 1.1[a]	65.8 ± 1.4[a]
Thickness of upper epidermis (μ)	7.8 ± 0.41[a]	8.0 ± 0.3[a]	8.3 ± 0.4[a]	8.4 ± 0.4[a]
Thickness of lower epidermis	12.3 ± 0.3[a]	13.2 ± 0.4[a]	12.3 ± 0.4[a]	12.7 ± 0.4[a]
Width/length ratio of hypodermis cells	0.6[b]	0.5[a]	0.5[a]	0.4[a]
Vessel diameter (μ)	45.4 ± 0.6[c]	39.7 ± 1.1[a]	42.8 ± 1.0[b]	38.8 ± 1.1[a]
Diameter of median vein (μ)	1244.0 ± 35.4[b]	1214.7 ± 6.0[b]	1249.3 ± 8.2[b]	1160.0 ± 4.1[a]
Length of upper stomatal guard cells (μ)	28.4 ± 0.2[b]	27.8 ± 0.2[b]	26.8 ± 0.4[a]	27.8 ± 0.3[b]
Length of lower stomatal guard cells(μ)	26.4 ± 0.2[b]	25.9 ± 0.2[ab]	25.7 ± 0.2[a]	26.3 ± 0.2[b]

* Means ± standard error of 30 replicates. Different letters in each line represent significant differences at $P < 0.05$.

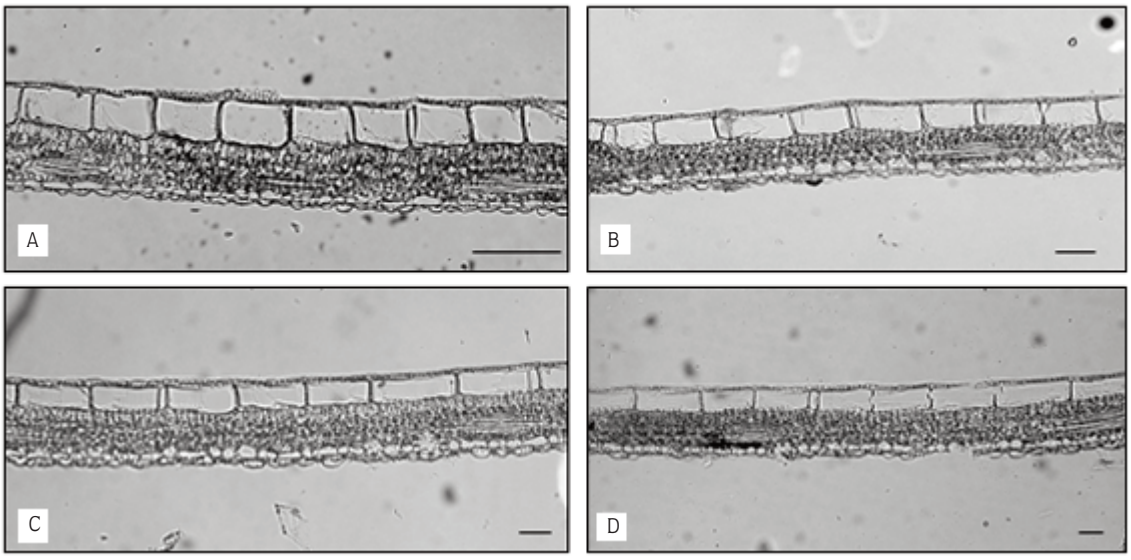

Figure 1. Micrographs of leaf cross-sections illustrating differences on the thickness of mesophyll and lamina, and width/length ratio of hypodermis cells under the light microscope (A: 1st level, B: 2nd level, C: 3rd level, D: 4th level of leaf rolling). Scale: 50 μ.

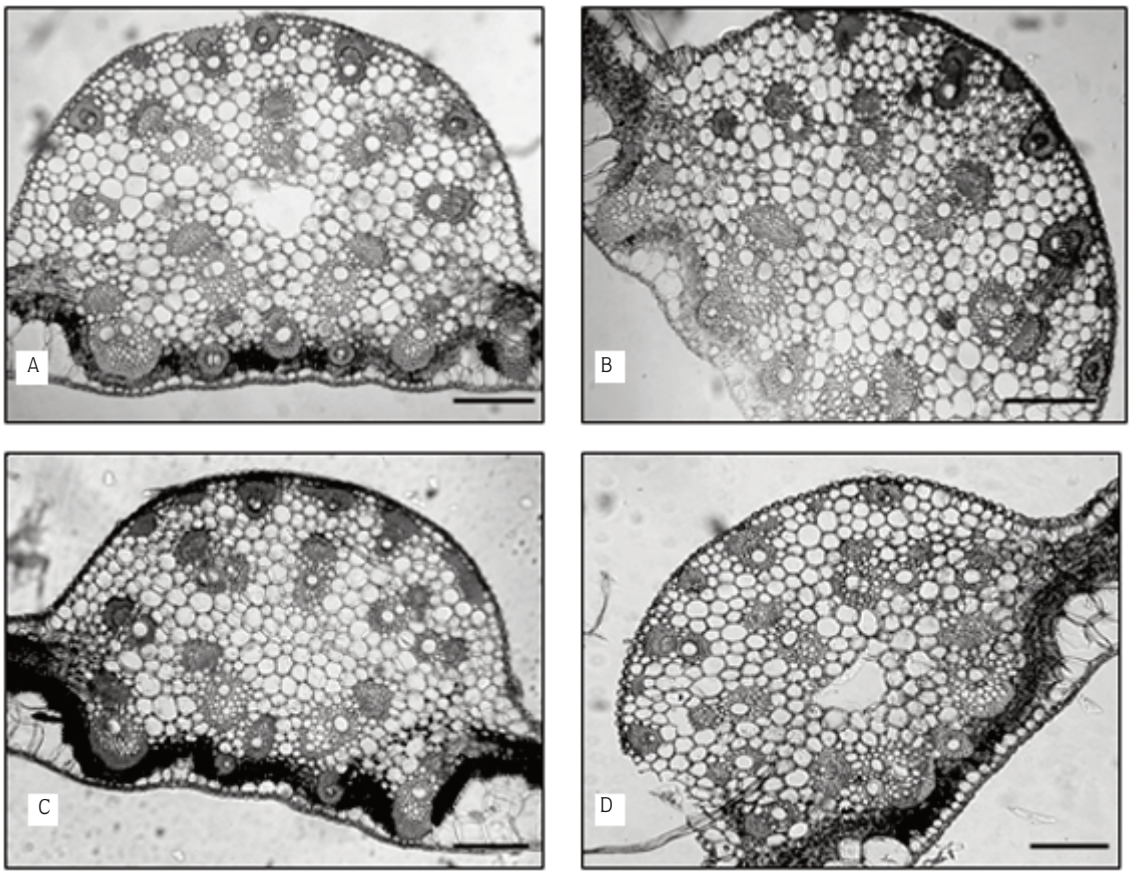

Figure 2. Micrographs of leaf cross-sections illustrating differences on the diameter of median vein under the light microscope (A: 1st level, B: 2nd level, C: 3rd level, D: 4th level of leaf rolling). Scale: 200 µ.

acid did not significantly change up to the 3rd level of leaf rolling but it increased at the 4th level (Table 1).

Discussion

In this study, changes in the levels of endogenous phytohormones and anatomical structure were investigated during leaf rolling under drought. Water potential of leaves declined in response to drought stress (Table 1). As is known, Ψ_{leaf} is used to determine the severity of stress. Depending on plant species and growth conditions, mild, moderate, and severe stress are from 0 to -1 MPa, from -1 to -2 MPa, and greater than -2 MPa of Ψ_{leaf}, respectively (20). Therefore, we can say that the plant is under moderate stress through the leaf rolling mechanism at the highest level of rolling.

The width/length ratio of hypodermis cells was found to decrease significantly during leaf rolling. It is known that the change in hypodermis volume controls leaf rolling in *C. setosa* (12). However, leaf rolling occurs as a consequence of shrinking of bulliform cells in the other species that have rolling in the leaves (21). The thickness of mesophyll and lamina was found to decrease significantly in the rolled leaves (Table 2). The decrease in mesophyll thickness of the rolled leaves may arise from a reduction in intercellular space. The decrease in lamina thickness can also be related to shrunken volumes of hypodermis and mesophyll in the rolled leaves. Similarly, Todd et al. (22) observed that leaf thickness had decreased as a result of water stress.

Epidermis is the first tissue exposed to drought in leaves. Therefore, it is usual to observe a thickening epidermis under severe drought stress (23). However, the present study showed that the variation in epidermal thickness with increasing leaf rolling under drought stress was not significant ($P < 0.05$). This result is probably related to the leaf rolling mechanism decreasing the effects of drought stress. In accordance with our result, Sam et al.

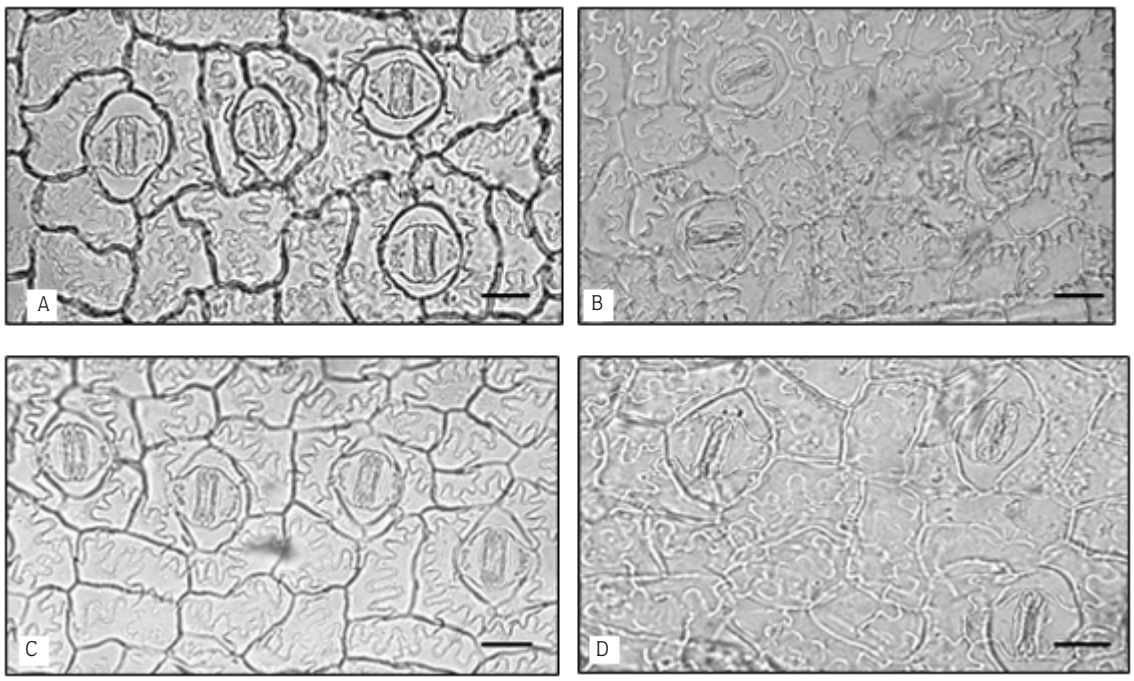

Figure 3. Micrographs of leaf surface-sections illustrating differences in the stomatal guard cells under the light microscope (A: 1st level, B: 2nd level, C: 3rd level, D: 4th level of leaf rolling). Scale: 25 µ.

(24) showed that water deficit had little effect on the length and width of epidermal cells.

Lengths of upper and lower stomata at the 3rd level of rolling were significantly less than those of the unrolled leaves in the present study (Table 2, Figure 3 A-D). Similarly, Croker et al. (25) reported that stomata of plants responded rapidly to environmental changes including soil drying. In addition, Cutler et al. (26) reported that stomata under water stress were smaller than those of well-watered plants. The findings obtained in this measurement can be associated with a trend of increased ABA levels during the rolling. Indeed, Bradford et al. (27) showed that ABA-sprayed tomato leaves had small stomatal dimensions. However, lengths of upper and lower stomatal guard cells increased again at the 4th level of leaf rolling compared to the 3rd level (Table 2, Figure 3 A-D). This change may be related to an increase in GA_3 level at the 4th level of rolling or an antagonistic interaction between the GA_3 level and the high level of ABA at this rolling level.

In our experiment, vessel diameter in the rolled leaves was smaller than that of the unrolled leaves. During leaf rolling, the decrease in vessel diameter may be due to water loss and may be regarded as an adaptation to stress. Similarly, Ristic and Cass (28) reported that vascular tissue area decreased as a result of low soil moisture. However, our study showed that vessel diameter was greater at the 3rd level of leaf rolling compared to the 2nd level. This increase may be related to increases in amounts of IAA and cytokinin or their synergistic effects. In addition, auxin alongside cytokinin is known to induce the differentiation of tracheary elements (29,30). At the 4th level of rolling, the decrease in vessel diameter may also be attributed to decreases in IAA and increases in ABA levels. Some reports support this idea. Little and Savidge (31) reported that ABA antagonized the promontory effect of IAA on tracheid radial expansion under water stress. Similarly, Doley and Leyton (32) stated that water potential and the interaction between this and IAA concentration affected vessel formation, while GA_3 and its interaction with IAA had little or no effect on the vessels.

Although drought generally causes a reduction in cytokinin, IAA, and GA_3 levels, there are very few reports about increases in phytohormones during water stress (7). Our results showed that amounts of IAA (except at the 4th level of leaf rolling), cytokinin, and ABA increased, while the level of GA_3 changed irregularly during leaf rolling. Similarly, Lopez-Carbonell et al. (6) reported that *Rosmarinus officinalis*, a Mediterranean shrub,

accumulated significantly higher levels of IAA and zeatin riboside in response to stress. High amounts of ABA are related to the characters that allow the plant to survive under severe stress (33). In addition, ABA accelerates adaptation of cultured tobacco cells to stress (34). In the present study, the increase in ABA level may be related to the adaptation mechanism during leaf rolling in *C. setosa*. Furthermore, water stress had no effect on GA_3 levels in sunflower shoots or roots (35). In contrast, Bensen et al. (36) provided evidence that low water potential might cause a reduction in endogenous GA_3 concentrations in hypocotyls of soybean. Although some similar results are present in the literature, there are serious differences between our results and the previous studies during drought. The differences may arise from relief in the effects of drought through the leaf rolling mechanism in *C. setosa*. This hypothesis is also supported by the anatomical changes mentioned above.

Consequently, *Ctenanthe setosa* may have tolerance to drought stress via some anatomical and phytohormonal changes alongside leaf rolling. Endogenous phytohormones may have distinct or interactive effects in anatomical responses of plants to drought stress during leaf rolling.

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