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Lignification response for rolled leaves of *Ctenanthe setosa* under long-term drought stress

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Abstract: Leaf rolling is a dehydration avoidance mechanism for plants under drought stress. To understand how it affects the lignification process in response to long-term drought stress in *Ctenanthe setosa* plants that have the leaf-rolling mechanism, the enzymes in lignification were studied in unrolled leaves as a control and at 2 different leaf rolling indices at days 35 and 47 of the drought period. The results indicated that the activities of phenylalanine ammonia lyase, indole-3-acetic acid oxidase, soluble peroxidase, ionically wall-bound peroxidase, covalently wall-bound peroxidase, and polyphenol oxidase were increased during long-term drought stress. However, nitrate reductase activity was decreased while lignin content was increasing during the period of drought. Lignin content was positively correlated to activities of phenylalanine ammonia lyase, indole-3-acetic acid oxidase, soluble peroxidase, ionically wall-bound peroxidase, and polyphenol oxidase, but it was negatively correlated to activity of nitrate reductase. Furthermore, there was a positive correlation between leaf rolling and lignin content. The results indicate that the leaf-rolling process may be related to the lignification mechanism in alleviating damage from stress in *Ctenanthe setosa* under long-term drought stress.

Key words: *Ctenanthe setosa*, enzyme activity, leaf rolling index, leaf water potential, lignification, long-term drought stress, relative water content

1. Introduction

Drought is one of the major environmental factors limiting crop productivity. Plants evolve different mechanisms to evade drought stress. One of these mechanisms, leaf rolling, is a hydronastic mechanism that reduces light interception, transpiration, and leaf dehydration. *Ctenanthe setosa* 'Grey-maranta', a perennial plant exhibiting leaf rolling to adapt to drought stress conditions, is grown for its attractive foliage. It is a good model plant for leaf-rolling studies because its leaves show gradual rolling, and thus observing its leaf-rolling response is easy (Kadioglu and Terzi, 2007).

Lignins are cell wall phenolic heteropolymers that are covalently bound to cellulose, other polysaccharides, and proteins of the cell wall. The complex structure of lignin provides mechanical support and water transmission, as well as blocking the growth of pathogens and infection. It has been well documented that biotic and abiotic stresses are responsible for the increase in cell wall lignification (Moura et al., 2010). Lignification is a complex process that involves several different phenolic substrates and enzymes (Valentines et al., 2005).

Some of the key enzymes catalyzing the biosynthesis of lignin include peroxidase (POD), polyphenol oxidase (PPO), and phenylalanine ammonia-lyase (PAL). POD within the cell wall, in either the free or bound state, has been shown to be involved in monolignol polymerization and therefore lignifications (Fry, 2004). PPO, involved in the oxidation of polyphenols into quinines, is also associated with lignification of plant cells (Cervilla et al., 2009). PAL is also the entry-point enzyme in the phenylpropanoid biosynthesis pathway and it catalyzes deamination of phenylalanine to *trans*-cinnamate. Phenylalanine and tyrosine, primary metabolites of nitrogen metabolism, are the precursors of the secondary metabolites as phenylpropanes and derivates (Schrader and Hageman, 1967). Nitrogen metabolism in plants is a complex process involving a series of enzymes. Nitrate reductase (NR), the first enzyme in the nitrate assimilation pathway, is a limiting factor for plant growth and development. Nitrates absorbed by the plant are reduced to NH_4^+ with the help of 2 enzymes, NR and nitrite reductase. Several processes generating NH_4^+ in the leaf are nitrate/nitrite reduction, lignin biosynthesis, photorespiration, and protein turnover. In the lignin biosynthetic pathway, a significant amount of NH_4^+ is generated directly in the leaf apoplast (Nakashima et al., 1997). NR is lost with the concurrent onset of lignification and the activation of indole-3-

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acetic acid oxidase (IAA-O) by phenolics (Schrader and Hageman, 1967). IAA-O is thought to influence plant growth by regulating the concentration of endogenous indole-3-acetic acid (IAA). Cross-linking reactions of the cell wall require hydrogen peroxide, which is generated by peroxidases through the oxidation of NADH or IAA by molecular oxygen (Ferrer et al., 1990).

As mentioned above, leaf rolling is a mechanism that protects the plants from hazardous effects of stress by decreasing water loss. It has been recorded that various tolerance mechanisms, such as the antioxidant system and osmolyte accumulation, were induced in rolled leaves of various plant species under stress conditions (Kadioglu and Terzi, 2007). However, the relationship between the lignification mechanism and leaf rolling has not been studied under long-term drought stress in plants. Studies on changes in enzyme activities involved in lignification are limited. The aim of the present study is to investigate the changes in leaf rolling, lignin content, and activities of enzymes involved in lignification mechanism, such as PODs, PPO, PAL, IAA-O, and NR activity, under long-term drought stress in *Ctenanthe setosa* plants.

2. Materials and methods

2.1. 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 in top diameter, 11 cm in bottom diameter) containing peat and sand (5:1). Plants were kept adequately watered and then incubated in a growth chamber with a 16/8 h light/dark period at 25 °C with 70% relative humidity and 300 μmol m^{-2} s^{-1} photosynthetic photon flux density at the surface of the leaves. The plants were subjected to a 47-day drought period by withholding water in order to achieve visual leaf rolling. Leaf samples were taken on days 35 and 47 of the drought period together with samples from the watered controls.

2.2. Leaf-rolling index and leaf water potential

Leaf-rolling index (LRI) was estimated by using the method of Shi et al. (2007). The measurement is:

$$LRI\ (\%) = (Lw - Ln) / Lw \times 100,$$

where Lw = the greatest width of the leaf blade when the leaf blade is expanded and Ln = natural distance of the leaf blade margin. Leaf water potential (Ψ_{leaf}) was measured with a thermocouple psychrometer (PSYPRO, Wescor, Inc., Logan, UT, USA).

2.3. Lignification-related enzyme assays

PODs (EC 1.11.1.7) were extracted following the method of Pandolfini et al. (1992) and determined in 3 fractions: the soluble (SPO), ionically (IPO) bound, and covalently (CPO) bound fractions.

PAL (EC 4.3.1.5) was extracted following Havir et al. (1971) with some modifications.

PPO analysis (EC 1.14.18.1) was determined using the method of Espin et al. (1995).

IAA-O analysis (EC 1.11.1.8) was determined using the method of Beffa et al. (1990).

2.4. Measurement of nitrate reductase enzyme activity

NR activity (EC 1.6.6.1) was determined using the method of Yandow and Klein (1986).

2.5. Determination of lignin

Lignin content was measured by the modified acetyl bromide procedure (Iiyama and Wallis, 1990).

Protein content was determined according to the method of Bradford (1976) with bovine serum albumin as the standard.

2.6. Statistical analysis

All analysis was repeated 3 times with 3 replications. The data recorded in figures are means of the values with standard deviations (SDs). The analysis of variance was performed with the Duncan multiple comparison test using SPSS 15.0 for Windows (SPSS Inc., USA), and significance was determined at the 5% ($P < 0.05$) level. Correlation analysis was used to determine the significance of relationships among the measured variables.

3. Results

3.1. LRI and leaf water potential

The leaf rolling began at day 35 of the drought period, and the LRI gradually increased in *C. setosa* as the drought period continued. To understand the relationship between plant water status and lignification, we measured the water potential of leaves during the drought period. Water potential of leaves (Ψ_{leaf}) also decreased during drought stress. For example, Ψ_{leaf} was down from −0.17 MPa in control leaves to −0.50 MPa in stressed leaves at day 47 of the drought period (Table).

3.2. Changes in activities of lignification-related enzymes

SPO activity increased during the drought period and was significant ($P \leq 0.05$) for all LRIs. As compared with their controls, activity had increased by 14% and 26% at days 35 and 47 of drought, respectively. IPO activity increased gradually over the drought period. IPO activity was significantly increased: 40% and 138% at days 35 and 47, respectively. A similar trend was found in CPO activity during the drought period; the activity was increased by 25% and 82% at days 35 and 47, respectively (Figure 1). The data show significant correlations between lignin content and IPO ($r = 0.867^{**}$, $P < 0.01$) and SPO ($r = 0.817^{**}$, $P < 0.01$) activities.

Changes in PAL activity are presented in Figure 1. The trend was similar to those of wall-bound peroxidase activities. PAL activity rapidly increased (especially at day 47) during the drought period. The increase of PAL activity was approximately 1.5- and 16-fold respectively compared

Table. Leaf rolling index (LRI), relative water content (RWC), and leaf water potential (MPa) under drought stress conditions.

Applications	LRI	RWC (%)	MPa
Control 1	0	95.00 ± 0.70 c	–0.15 ± 0.05 b
Day 35 of drought	26 ± 2.99 a	91.21 ± 2.51 b	–0.18 ± 0.05 b
Control 2	0	96.00 ± 0.24 c	–0.17 ± 0.04 b
Day 47 of drought	54 ± 3.16 b	82.03 ± 0.39 a	–0.50 ± 0.06 a

Different letters denote significant differences among means. Mean comparison among drought periods was performed using the ANOVA test at $P \leq 0.05$. Data are means ± SD.

to the controls. In addition, PAL activity was significantly correlated with lignin content ($r = 0.883^{**}$, $P < 0.01$).

Similarly, IAA-O activity increased significantly during the drought period. The activity increased by 16% and 142% at days 35 and 47, respectively, compared with the controls (Figure 1). There was a positive significant correlation between IAA-O and lignin content ($r = 0.917^{**}$, $P < 0.01$).

There was a significant increase in PPO activity during the drought period. The activity was increased 27% and 32% at days 35 and 47 compared to the controls, respectively (Figure 1). In addition, PPO activity was positively related to lignin content during the stress period ($r = 0.750^{*}$, $P < 0.05$).

3.3. Activity of nitrate reductase

NR activity significantly decreased during the drought period. The activity decreased by 81% and 85% at days 35 and 47, respectively, compared to the controls (Figure 2). There was a significant negative correlation between NR and lignin content ($r = –0.667^{*}$, $P < 0.05$).

3.4. Lignin content

Lignin content increased gradually throughout the drought period. The lignin content was enhanced by 7% and 39% at days 35 and 47, respectively, as compared to the controls (Figure 3). There was a positive correlation between leaf rolling and lignin content ($r = 0.763^{*}$, $P < 0.05$).

4. Discussion

Plants can protect themselves against environmental stresses such as drought, UV irradiation, wounding, and pathogen attacks. Lignin, one of the cell wall components, plays an important role as a defense mechanism (Moura et al., 2010). Thus, lignification-related enzymes deserve much attention during periods of water deficit in plants.

Leaf water potential is a useful means for assessing the physiological water status of plants. In the present study, a significant reduction of leaf water potential was observed in leaves exposed to drought stress. For example, water potential of leaves was reduced from –0.17 MPa at day 0 (control) to –0.50 MPa at day 47 of the drought period. As indicated before**,** *Ctenanthe setosa* has a mechanism for decreasing transpiration and water loss during drought stress. Moreover, this plant resists drought for a long time because decreases in relative water content (RWC) and Ψ_{leaf} are not high during the drought period compared to those of less drought-resistant plant species. This indicates that minor changes in water potential and RWC in leaves during a drought period may be strongly related to an adaptation response of the plant to the extent of drought stress (Saruhan et al., 2012).

To estimate a relationship between lignification and leaf rolling, we examined certain lignification-related enzymes. PAL, IAA-O, soluble and wall-bound PODs, and PPO are important enzymes for catalyzing lignin biosynthesis. PAL is considered to be responsible for the conversion of phenylalanine to *trans*-cinnamic acid, a key intermediary in the biosynthesis pathways of phenolic and lignin (Rivero et al., 2001). In our study, PAL was activated by drought stress. It was shown that PAL is generally stimulated in plant tissues exposed to several environmental stresses. Some authors indicated that PAL enhancement in stress conditions is due to H_2O_2 generation, which occurs as a primary reaction in response to stress (Jouili and El Ferjani, 2003). Increased PAL activity can be related to the implication of this enzyme in the plant lignification response to drought stress. Similarly, IAA-O activity markedly increased during the drought period in *Ctenanthe setosa*. In accordance with the present results, Pujari and Chandra (2002) reported that the induction of IAA-oxidase and POD activities may be a defense mechanism to protect the plant from abiotic stress; it is known that IAA-O activity is related to POD-generating capacity. PODs catalyze the covalent cross-linking of wall polymers, promoting the tightening of the wall as well as oxidizing IAA (Fry, 1986). It can thus be said that increasing IAA-O activity during drought stress could lead to an increase in the lignification process.

PODs catalyze the polymerization of monolignol to complete the lignification process (Lee et al., 2007). PODs catalyzing lignification leads to decreased cell wall plasticity. Therefore, this process represents part of a

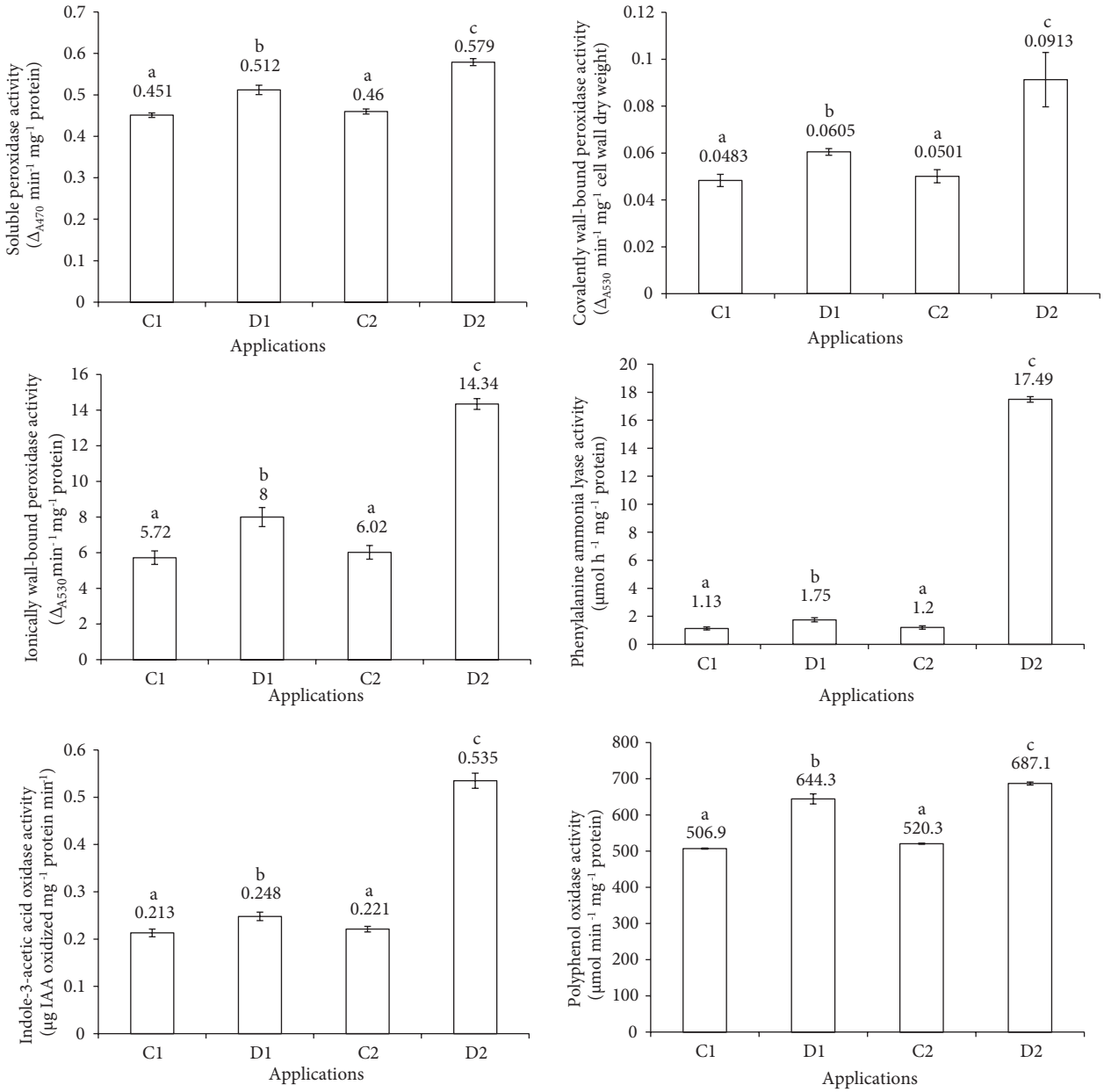

Figure 1. Changes in SPO, IPO, CPO, PAL, IAA-O, and PPO in drought-stressed leaves of *C. setosa*. Different applications represent the following: adequately watered plants up to day 35 of drought period (control, C1); drought-stressed plants on day 35 (drought 1, D1); adequately watered plants up day 47 of drought period (control, C2); drought-stressed plants on day 47 (drought 2, D2). The vertical bars indicate standard deviation and different letters represent significant differences among the applications at $P < 0.05$.

mechanism for adaptation to stress (Schützendübel et al., 2001). In line with this hypothesis, we found that soluble, ionically wall-bound, and covalently wall-bound POD was significantly increased during the drought period as compared to the control. Increasing activities of soluble and cell wall-bound POD were linked with a rise in lignin content during the drought period. Chen et al. (2000) reported that the increase in cell wall POD activity was due to a response to copper stress. As expected, the increased cell wall-bound POD activity in *Ctenanthe setosa* may play a role in the lignification process and contribute to tolerance for drought stress during leaf rolling. A role for PODs in cell wall stiffening through the formation of phenolic cross-links is generally accepted (Fry, 1986). Like NADPH oxidase, cell wall-bound peroxidases could also trigger the oxidative burst in plants (Bolwell et al., 1998).

Another enzyme involved in phenol and lignin metabolism is PPO, which catalyzes the oxidation of

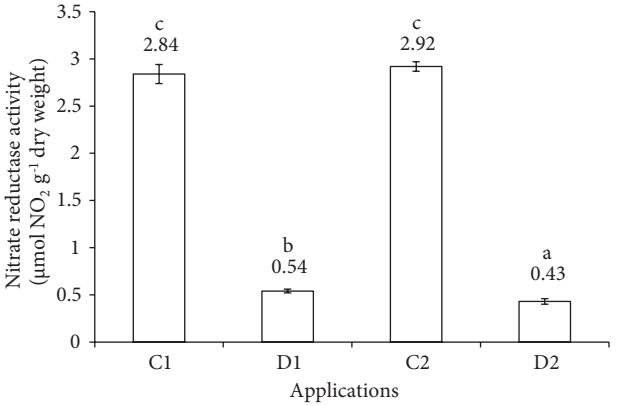

Figure 2. Changes in NR in drought-stressed leaves of *C. setosa*. The vertical bars indicate standard deviation and different letters represent significant differences among the applications at $P < 0.05$.

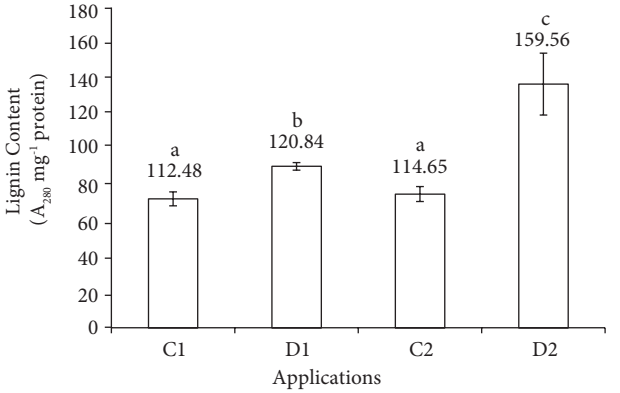

Figure 3. Changes in the lignin content in drought-stressed leaves of *C. setosa*. The vertical bars indicate standard deviation and different letters represent significant differences among the applications at $P < 0.05$.

polyphenols into quinines (Cervilla et al., 2009). In our investigation, there was a similar trend of increased enzymes in PPO activity during the drought period. Cervilla et al. (2009) demonstrated that PPO increased in response to several abiotic stresses such as water deficit and heavy metals. Thus, PPO activity may regulate the redox state of phenolic compounds and become involved in the phenylpropanoid pathway (Nakayama et al., 2000).

One of the enzymes indirectly involving in lignin metabolism is NR, which is the first enzyme in the pathway of nitrate assimilation. It is well documented that NR in the leaves of higher plants is very sensitive to changes in the water status of the plant. In our study, the activity of NR was affected by declining leaf water potential and decreased up to day 47 of the drought period. Similarly, Stewart et al. (1990) reported that there was a negative relationship between NR activity and lignin content. Decreased activity of NR may be due to the breakdown of proteins under water stress conditions (Kalarani and Jeyakumar, 1998). Additionally, in the lignin biosynthetic pathway, a significant amount of NH_4^+ is generated directly in the leaf apoplast (Nakashima et al., 1997), and thus NR activity can be inhibited due to long-term drought stress.

The responses of the enzymes involved in lignin biosynthesis to drought might differ, depending on the length of the period for which the plants are exposed to drought stress. In this study, results indicated that lignin contents and leaf rolling were increased during the drought period. Lignin content has been reported in plants that are exposed to heavy metal, and it could be associated with an increase in the activity of lignifying PODs (Schützendübel et al., 2001). H_2O_2 is produced during different metabolic processes, such as during the formation of lignin in cell walls. The accumulation of H_2O_2 under water deficit stress was suggested as a signaling molecule triggering lignification (Lee et al., 2007). On the other hand, the high level of H_2O_2 in apoplastic space may accelerate strengthening of cell walls (Zarra et al., 1999). In a previous work, we have shown that drought stress caused a significant increase in H_2O_2 in *Ctenanthe* leaves during the leaf-rolling period (Saruhan et al., 2009). In this sense, our results show that there is an interaction between H_2O_2 and lignin content in *Ctenanthe setosa* leaves subjected to drought stress.

Based on the previous results, we suggest that the increase of drought stress could induce lignification in rolled leaves of *Ctenanthe setosa*. The induction of lignification-related enzymes may also be a defense mechanism to protect the plants from long-term drought stress. Finally, there is a positive relationship between the leaf-rolling process and the lignification mechanism. In addition to leaf rolling, it can be concluded that plants have a lignification mechanism to protect themselves from the hazardous effects of long-term drought stress.

Acknowledgments

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