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염 및 건조스트레스 하에서 포복형 백리향의 생육과 Abscisic Acid 농도변화

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Growth and Abscisic Acid Changes of Creeping Thyme in the Exposure of NaCl and Drought

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ABSTRACT : Experimental purpose was to evaluate growth characteristic and abscisic acid (ABA) responses against salt/drought stresses. In the shoot biomass, creeping thyme was tolerated in mild NaCl stress, ranging 0 to 100 mM, while it was severely reduced in higher salinity. Under constant drought stress, the shoot biomass of creeping thyme showed a worse value compared to that of 100 mM NaCl treatment. Chlorophyll degradation was more severe in immature leaf than mature leaf under salt and drought stresses. In salt stress, immature leaf produced much amounts of ABA compared to mature leaf and also immature leaf showed faster increase of ABA than that of mature leaf. In drought stress, immature leaf responded to stress within 24 hours by the increase of ABA, while mature leaf responded to at 72 hours. Our results recommended that the optimal salinity level of creeping thyme was 50~100 mM NaCl.

Key Words **:** Abscisic Acid, Biomass, Chlorophyll content, Creeping Thyme, Salinity, Water Deficit

INTRODUCTION

Creeping thyme (*Thymus serpyllum* L.), an herbaceous perennial ornamental, is originated from Mediterranean area and cultivated in many countries for the purpose of several agricultural uses, such as essential oil production, additive food, ornamental and ground cover. In the use of an ornamental groundcover, creeping thyme is popularly utilized as a groundcover in home gardens and landscapes because it grows low like a carpet form above the ground and is blooming white flowers during late spring to summer. The plant is a vigorous spreader, emerging new leaves in the early fall after flowering has been completed (Eom *et al*., 2005). Creeping thyme prefers full sun and well-drain soils, but avoids wet area (Armitage, 1997). The yield of creeping thyme is dramatically reduced if weeding is not accomplished in early spring season (Eom *et al*., 2005). In the use of a medicinal herb, essential oils in the plant have been intensively investigated (Loziene *et al*., 1998; Weinkötter *et al*., 2007). Essential oil composition is likely dependent upon cultivating locations (Paaver *et al*., 2008; Kim *et al*., 2008; Kim *et al*., 2008). In the use of a food additive, fresh or dried leaves of creeping thyme that are distinct limy-like fragrance have been used in the source of foods.

Although fundamental information of creeping thyme cultivation is known in terms of light and soil moisture conditions, it is true that physiological and growth responses in creeping thyme have not been investigated minutely under drought and salt exposures. Plant species respond drought and salt stresses with various mechanisms, including increase of abscisic acid (ABA), proline, and glycinebetain, reduced leaf size, and thicker leaf. Plant responses are also differed in the intensity of drought. ABA, a plant hormone, is well known to a defense mechanism against drought stress. Under water deficits, ABA is associated with

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acclimation of plants to stress, by triggering Ca^{2+} activation to close stomata under water deficit conditions (Albinsky *et al*., 1999; Aldesuquy and Ibrahim, 2001; Chen *et al*., 2002; Jia *et al*., 2002; Luan, 2002). Increased ABA content in leaf tissue under stress condition appears to be correlated with leaf area reductions (He and Cramer, 1996).

In this experiment, we intended to explain whether change of ABA can be an indicator as stress symptoms and also to find the limitation of cultivation in creeping thyme under salt or drought condition.

MATERIALS AND METHODS

1. Plant materials

Seeds of creeping thyme were purchased from Jelitto Staudensamen (GmbH, Am Toggraben 3, D-26290 Schwarmstedt, Germany) and propagated in a greenhouse. Creeping thyme was grown in a propagation house for 12 months before NaCl treatment. Periodic fertilization with Hoagland's solution was performed prior to NaCl treatments. Plants were trimming 15 days prior to the initiation of experiment to insure uniformity in plant size.

2. Salt stress

Salt treatments were applied in daily solutions to creeping thyme at five NaCl concentrations; 0, 50, 100, 200, and 400 mM. In order to keep salinity concentrations consistently throughout the experiment, the soil mixture was flushed thoroughly with tap water every day and leached for one hour before addition of salt daily. Even though precise measures were taken to keep the NaCl concentrations consistent, it is very difficult to maintain salinity levels by utilizing adequate water for an extended period of time (Chao *et al*., 1999; Mäkelä *et al*., 2003; Wimmer *et al*., 2003). Smaller pots (14 ㎝) were treated with 100 mL of NaCl solution. The amount of retained solution was determined by the rate of leaching following the addition of daily salt solutions. Pot weight was recorded each morning (at 8:00 a.m.) to calculate water loss. Each pot was then irrigated with tap water (200 mL for small pots, 800 ㎖ for large pots) after weights were recorded (9:00 a.m.). After two hours following the irrigation and leaching (11:00 am), the NaCl solutions were added. Pot weights were again recorded after two hours following NaCl treatment (1:00 p.m.) to determine full soil saturation after completed leaching. Daily water loss (or plant water use) data were calculated based on pot weight differences from full saturation in comparison to water loss by 8:00 a.m. the following day. At the termination date of the experiment, 21 days following initiation, shoot and root biomasses were collected and placed in a drying oven at 40℃ for one week, and dry weights were recorded.

3. Drought stress

Pot-grown thymes in a greenhouse were not irrigated from the initial day of drought stress experiment to the twelfth day. The water losses of pot weights were daily measured and during leaf samples were taken for measuring ABA. For a comparative water transpiration measurement, non-planted pots which were filled the same types of soil mixtures were coincidentally applied in the experiment.

4. Sample preparation of ABA analysis

ABA analyses were performed on both immature and mature leaves from creeping thyme, using standard laboratory procedures for ABA determinations. Immature leaves were collected from the stem at position four from the apical meristem, while mature leaves were collected by choosing the largest leaf from the bottom of the stem. Tissue for ABA analysis was taken by punching a 0.7 ㎝ leaf disc from each groundcover used in the salinity test. Leaves were collected each morning prior to leaching of NaCl from the soil and were immediately placed into a 250 μL chilled solution of 80% methanol. Samples were refrigerated at 4℃ for 48 hours before ABA extraction; afterwards, they were dried at room temperature for an additional 48 hours. Extracts were reconstituted in 100 μL of a 30% methanol solution (69% distilled water, 30% methanol, 1% acetic acid) and 20 μL of 0.04% bromecresol green was added. All treatments were replicated five times. The ABA extracts were fractionated by C18 reverse-phase chromatography and analyzed by enzyme-linked immunosorbent assay (Setter *et al*., 2001).

5. C18 column to separate ABA fraction

The C18 column consisted of 96 wells and was initially cleaned by adding 400 μL of 95% EtOH, and by 600 μL of 30% MeOH per well in order. Well plates were loaded with extract, rinsed and ABA was separated by partitioning using C18 column chromatography. For each leaf tissue

sample, 120 μL extract was loaded on the column and collected on the load plates. To rinse residual sample off the column, 80ìl of 30% MeOH was added to the column and collected on the load plate. The load plate was removed and saved for measuring the absorbance of bromecresol green, to successfully confirm chromatographic separation. The collection plate for the wash layer was assembled and the column was washed with 200 μL of 30% MeOH to remove sugars and other hydrophilic compounds from samples. The collection plate for the ABA fraction was assembled, and 200 μL of 65% MeOH was passed through the column to elute ABA. The collection plate for the final EtOH fraction was assembled and collected after adding 200 μL of 95% EtOH to the column. ABA fractions were dried at room temperature and redissolved in 100 μL distilled water for ELISA.

6. ELISA for ABA analysis

A round-bottom 96-well microtiter plate (Coster High Binding #3366, Dynatech Immulon-2, Corning High-binding #25802 plates) was coated by dispensing 200 μL conjugate into wells. The conjugate solution per a 96 well plate was made by adding 10ìl of ABA-BSA conjugate stock solution (Bach 1 in glycerol stock) to 21 g bicarbonate/carbonate buffer. The plate was sealed by a flexible dimpled plate mat and incubated at 5℃ overnight for 24 hours, after which the coating solution from the plate was discarded. The plate was rinsed four times with 1X TBST (Tris-buffered saline plus Tween 20) using a manifolder dispenser.

ABA standards for prediction of ABA concentrations were placed into the middle and terminal rows of the plate. For comparison, a dilution series of (+) ABA standards was prepared daily using common stock solution at concentrations covered a linear range over which samples were evaluated for ABA.

Primary antibody (100 μL monoclonal antibody, mAb) was then added to each well. Wells were then sealed, and incubated at 5℃ overnight for 24 hours. The primary antibody was prepared as follows: for 96 wells, 100 μL of 100X primary antibody solution was added to 10.6 mL of 1X MBSA (methylated bovine serum albumin). Primary monoclonal antibody stock solution (100X) contained 2 ㎎ PHYTODETEK mAb, 10 ㎖ TBS, and 10 mL glycerol and was stored at −20℃ in small aliquots. Each well thus contains a final dilution of 1 ㎍ protein/μL. After 24 hours, the solution was discarded and the plate was rinsed four times by TBST using a manifolder dispenser.

Secondary antibody tracer (180 μL 2°Ab) was then added into each well, sealed, and incubated at 5℃ overnight for 24 hours. The 2°Ab tracer was made using following steps: for 96 wells, 20 μL of 2°Ab (anti-mouse IgG-alkaline phosphatase conjugate, Sigma product A-3562) was mixed with 19 g of tyrodes bovine serum albumin (TBSA) and 180 μL was placed in each well. After 24 hours, the solution from the plate was discarded and the plate was rinsed four times with TBST using a manifolder dispenser.

The substrate *para*-nitrophenylphosphate (PNPP, 180 μL) was added into each well and incubated approximately 60-90 minutes at room temperature. The substrate was prepared by adding 20 mL of frozen PNPP reagent to diethanolamine (DEA) buffer.

After 30 minute incubation, absorbance of each sample and the ABA standards were read at 405 ㎚. The absorbance data were evaluated in comparison to the standard curve, using calculated regressions to predict concentrations.

7. Chlorophyll measurement

Leaf samples were collected in 15 days after experimental initiation. Chlorophyll analysis was performed on young, immature leaves located in position one to four from the growing tip, and also from fully expanded leaves. Fresh leaf tissue (30 ㎎) was extracted in 3 mL of N,N-dimethylformamide (DMF) for 48 hours at 4℃. Extracts were placed in culture tubes (13 × 100 ㎜, Durex™ Brosilicate Glass. VWR Scientific Inc.) and absorbance was recorded at both 647 ㎚ and 664 ㎚ using a Spectronic® 20 Genesys™ spectrophotometer (Spectronic instruments, Rochester, NY, USA). Chlorophyll contents were presented as proportion on the basis of absorbance value in non-stress controls.

8. Statistic analysis

Data including plant biomass, water use, and chlorophyll content are presented as treatment means with standard errors. ABA analysis using ELISA was repeated over time with collected samples. Means of all data were subjected to standard ANOVA procedures. Significant differences among data were determined at the 5% level based on Fisher's least significant difference (LSD) test.

Table 1. Biomass of *T. serpyllum* under salt and drought stresses.

	NaCl concentrations (mM)					Drought
	0	50	100	200	400	
Shoot biomass (g d.w./pot)	6.10 a[z]	6.24 a	6.42 a	4.86 c	4.24 d	5.23 b
Root biomass (g d.w./pot)	2.87 ab	3.07 a	2.74 b	2.76 b	1.86 c	2.76 b
R/S ratio[y]	0.47 bc	0.49 b	0.43 d	0.56 a	0.44 d	0.53 ab

[z]Represents values with the same alphabet in the same row are not significantly differed at $p=0.05$ in Tukey's Range Test.
[y]Represents root to shoot ratio.

RESULTS AND DISCUSSION

1. Effect of salt and drought stresses on the biomass of creeping thyme

Salinity was over than 100 mM of NaCl to decrease shoot growth in creeping thyme. As shown Table 1, low salinity did not reduce the shoot growth, rather slightly increased in the saline level although data were not significantly differed. Shoot growth in high salinity (≥ 200 mM) was dramatically decreased by 20% at 200 mM and 31% at 400 mM, respectively. In root growth, 50 mM NaCl slightly increased when it compared to control. Root growth was not significantly decreased among saline levels from zero to 200 mM. Highest salinity (400 mM) applied in this experiment decreased root growth by 36%. In drought stress, the inhibition of shoot growth was ranged between 100 and 200 mM NaCl treatments and that of root growth was similar with below 200 mM NaCl treatments. Osmotic stress symptom is likely similar in either drought or salt stress (Zhu, 2002). However, salt stress seems to be more damageable to plant growth because of ion toxicity by saline salts including sodium and chloride. Numerous studies proved that Na^+ and Cl^- accumulation in soil inhibit plant growth (Tester and Davenport, 2003; White and Broadley, 2001). Na^+ influx into roots occurs rapidly by a symplastic pathway utilizing pumps, carriers, and channels. The influx is associated with an electrochemical gradient from the soil rhizosphere to the cytosol in plant cells. Thus, uptake of Na^+ is variable in dependent of plant species. Certain salt tolerant species inhibit uptake of toxic ions in root levels so as to Na^+ concentration is generally more variable in shoot tissues than in root tissues (Tester and Davenport, 2003).

One of the typical symptoms of drought and salt stresses on plant growth is that the ratio of root to shoot (R/S ratio) tends to increase (Debouba *et al*., 2006; Jiang *et al*., 2006). However, R/S ratio in salt stress decreases in certain species such as *Dianthus japonicus* and barley (Heo *et al*., 2007; Suhayda *et al*., 1992). In the case of creeping thyme, R/S ratios by stress intensities were variable (Table 1).

2. Chlorophyll content of creeping thyme

Although the absorbent values of the chlorophylls are different, ratio of chlorophyll contents was highly correlated in foliar tissues of creeping thyme under the concentrations of NaCl treatments.

Two types of creeping thyme, *Thymus praecox* and *Thymus serpyllum*, are popularly cultivated in horticultural area such as home gardens and landscape settings. Morphologically the creeping thymes are similar. However, *Thymus praecox* produces abundant hairy trichomes in leaves, but *Thymus serpyllum* contains less of them. Previously, Eom *et al*. (2007) reported that chlorophyll content in the mature leaf of *Thymus praecox* was significantly decreased in 50 mM NaCl treatment, but maintained it in 100 mM NaCl as compared with non-NaCl treatment control. However, in this experiment (Table 2), mature leaves of *Thymus serpyllum* did not decrease chlorophyll content in 50 mM NaCl treatment, while it decreased the content in 100 mM NaCl treatment. Even though detailed studies are required, the salt responses may be differently processed between two species which are different morphological leaf structures. In drought stress, the deterioration of chlorophyll content in immature leaves was similar with that of 50 ~ 100 mM NaCl concentration. Otherwise, the chlorophyll content in mature leaves exhibited similar value with 100 mM NaCl concentration (Table 2).

3. Abscisic acid content of creeping thyme

Periodical ABA contents during salt and drought stresses are shown in Fig. 2. Leaf ABA contents were higher in immature leaf than in mature leaf among all stress treatments. In NaCl treatments, ABA was shown the highest

Table 2. Relative chlorophyll content (%) under salt and drought stresses in *Thymus serpyllum*.

	Immature leaves		Mature leaves	
	Chl. a[z]	*Chl*. b	*Chl*. a	*Chl*. b
Non-stress	100.0 a[y]	100.0 a	100.0 ab	100.0 ab
50 mM NaCl	76.9 b	77.5 b	101.4 a	103.3 a
100 mM NaCl	67.8 bc	65.5 bc	89.9 b	90.7 b
200 mM NaCl	57.5 c	57.6 bc	81.7 bc	82.5 c
Drought	71.4 b	72.5 b	90.0 b	90.6 b

[z]Represents *Chl*.a is chlorophyll a and *Chl*.b is chlorophyll b.
[y]Represents values with the same alphabet in the same column are not significantly differed at $p=0.05$ in Tukey's Range Test.

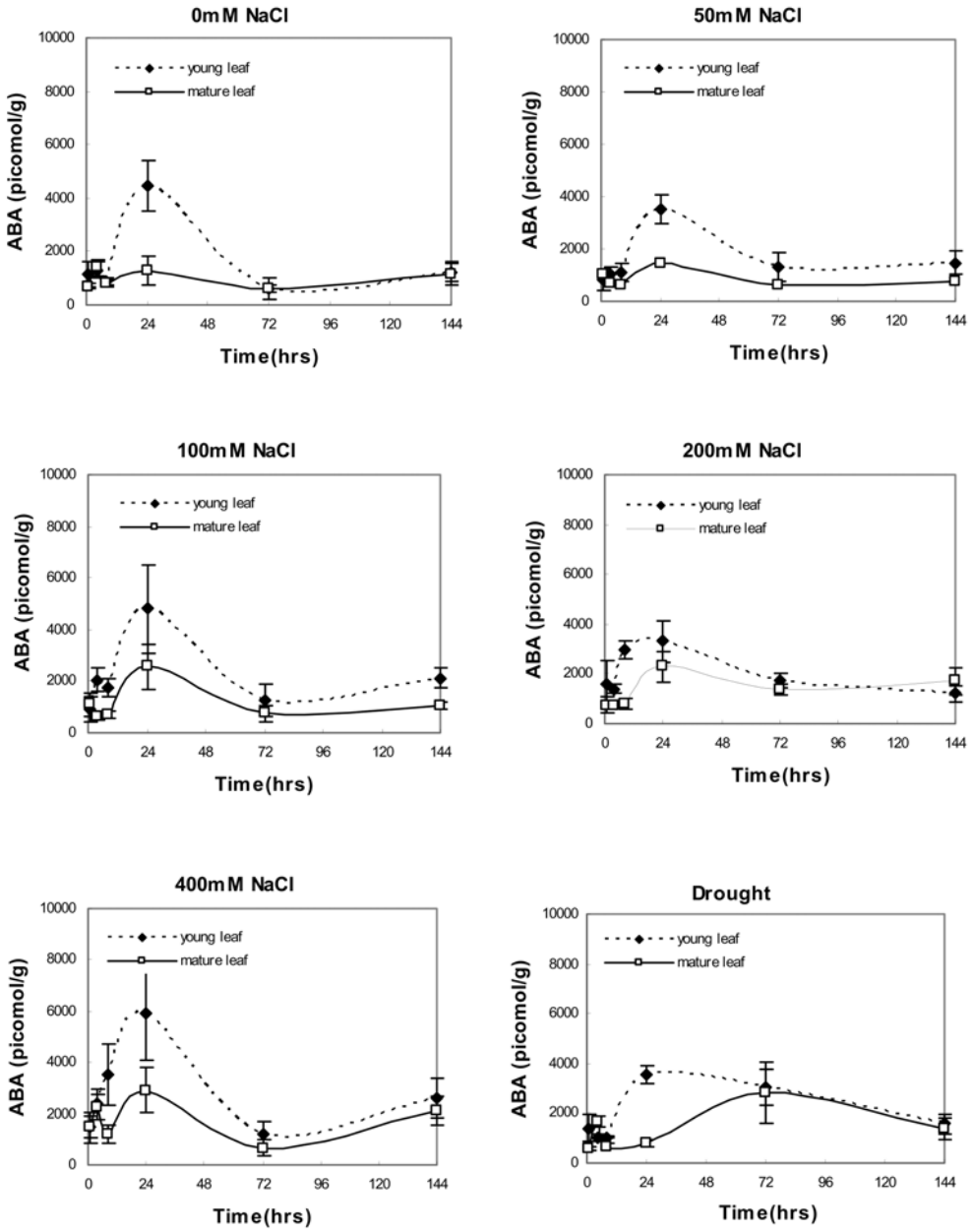

Fig. 1. Periodical ABA responses under salt and drought stresses.

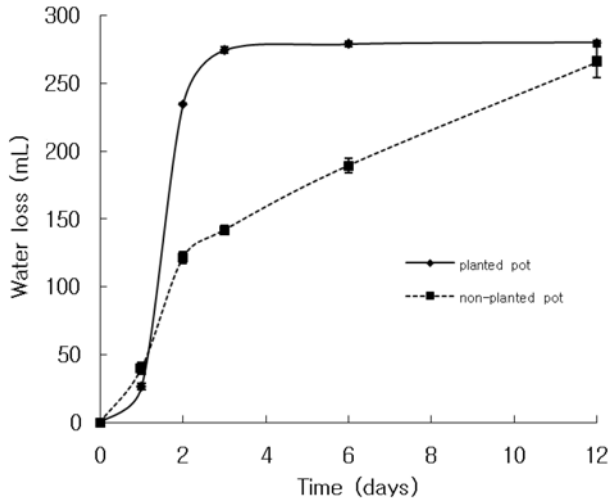

Fig. 2. Water loss patterns between planted and non-planted pots in non-irrigation condition.

content at 24 hours in immature leaf. The increased ABA content in mature leaf was not significantly differed with non-NaCl treatment control. Thus, ABA increase of immature leaf in first step of stress may be caused by water deficit rather than NaCl toxicity. However, increase of ABA was faster (≥ 8 hours) in higher NaCl treatments of above 200 mM than lower NaCl treatments of below 100 mM. ABA contents in immature leaf tissues were maintained relatively high levels in NaCl treatments during a long term evaluation. Mature leaf was less sensitive to NaCl stress compared to immature leaf in terms of ABA content. However, ABA contents in mature leaf tissues were distinctly indicated that increase of ABA was observed in NaCl treatment within 24 hours, but not in drought and non-NaCl treatment control. Increased ABA inhibits plant growth by series of mechanism relating stomatal conductivity. In salt stress, the relations between plant growth and roles of ABA are hardly explained because plant growth was not significantly differed in 0 to 100 mM NaCl concentrations (Table 1), while ABA was sensitively responded in the NaCl concentrations (Fig. 1). Thus, further experiment will be needed.

In drought stress, distinct increase of ABA was observed at 24 hours in immature leaf tissues and 72 hours in mature one (Fig. 1). It is supposed that growing leaves are more sensitively responded to drought by increase of ABA than fully expanded mature leaves. Fig. 2 showed that water in planted pots did not remain in the pot. The ABA increase leaf was maximized in that time when plants undergo severe water stress. The creeping thyme was survived in which water was re-supplied at 3 days. Whereas, the plants did not survive at later irrigation than 3 days (data not presented). However, as shown in Fig. 2, high amount of water loss in planted pots was observed between 1 and 2 days. Therefore, an adequate irrigation time may be determined at the point. Although creeping thyme could survive in 3 day irrigation, that time may be damageable to the growth of the plant.

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