



Physiological Responses of *Calystegia soldanella* under Drought Stress

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Abstract

This study was conducted to determine the extent of drought resistance based on physiological responses of *Calystegia soldanella* under water deficit. In order to investigate the changes of plant growth, stomatal density, photosynthesis, chlorophyll fluorescence, the contents of chlorophyll and carotenoid, osmolality, total ion contents, the contents of carbohydrate and proline, *C. soldanella* was grown under well watered and drought stressed conditions for 12 days. In this study, water-deficit resulted in remarkable growth inhibition of *C. soldanella*. The effect of water-deficit on plant growth was associated with low osmotic potential of soil. On day 12 after drought treatment, dry weight, relative water contents, number and area of leaves and stem length were lower than those of control. The stomatal conductance and net photosynthetic rate were significantly reduced in water stressed plant to regulate inner water contents and CO₂ exchange through the stomatal pore. Chlorophyll fluorescence and chlorophyll contents were not different in comparison with the control, indicating that the efficiency of photosystem II was not affected by drought stress. This results could be explained that water-deficit in *C. soldanella* limits the photosynthetic rate and reduces the plant's ability to convert energy to biomass. A significant increase in total ion contents and osmolality was observed on day 7 and day 12. Accumulation of proline in leaves is associated with the osmotic adjustment in *C. soldanella* to soil water-deficit. Consequently, this increase in osmolality in water stressed plant can be a result in the increase of ion contents and proline.

Key words: Coastal sand dune plant, chlorophyll fluorescence, photosynthesis, proline, water stress

INTRODUCTION

Plant species on coastal sand dune areas are affected by many environmental stresses that negatively impact plant metabolism and survival. Drought, salt spray, flood, high temperature, low capillary water-holding capacity of the sandy soil, low nutrient and water availability are the important ecological factors (Hesp 1991, Maun 1998, Lawlor and Cornic 2002). In coastal sand dune regions, water-deficit stress is one of the major stresses. The fre-

quent moisture deficiency caused by high evaporative demand limits plant growth, development and viability. General adverse effects of water deficit on plants are the decline in height and total fresh and dry biomass production (Baher et al. 2002, Farooq et al. 2009). Plant growth is largely affected by drought stress, and therefore adaptation on stress conditions is important for plant survival. The reduction of plant height is related to the decrease

<http://dx.doi.org/10.5141/ecoenv.2013.255>



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Received 08 November 2013, Accepted 26 November 2013

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in the cell enlargement and cell expansion due to the low turgor pressure (Bhatt and Srinivasa Rao 2005, Jaleel et al. 2007, Karthikeyan et al. 2007).

Many plants respond to water stress at the physiological and biochemical levels. Water stress is characterized by traits like decreased water and turgor potentials, relative water content (RWC), osmotic adjustment, wilting, high leaf temperature, closure of stomata, and decrease in cell enlargement and growth (Kumar and Singh 1998, Paseban-Islam et al. 2000, Shao et al. 2008). Stress as a result of water deficit changes a range of physiological processes such as photosynthesis, respiration, transpiration, ion uptake, carbohydrate contents, stomatal conductance and electron transport (Acevedo et al. 1971, Angelopoulos et al. 1996, Flexas et al. 1998, Lu and Zhang 1998, Clifton-Brown et al. 2002, Munns 2002, Silva et al. 2007). Under drought stress, to avoid and tolerate stress conditions plants accumulate metabolites as osmolytes (Bartels and Sunkar 2005). Particularly, proline has been suggested to play an important role as an organic osmolyte. Many studies have shown that proline contents in leaves of many plants are increased by many stresses including drought stress (De Ronde et al. 2000, Abdel-Nasser and Abdel-Aal 2002, Parida et al. 2002).

Understanding of the physiological mechanisms of many stressors is important in predicting how disturbances will impact future plant distribution and community pattern. Previous studies have suggested that adaptive features of coastal dune plants include tolerance to high temperatures, and efficient water use under high water-vapor saturation deficit (Monson et al. 1983, Mooney et al. 1983).

Calystegia soldanella is an endemic plant on coastal sand dunes and is located in the foredunes where environmental stresses are stronger. This plant is a perennial rhizomatous herb with stem up to 50-100 cm, and is a representative plant showing ubiquitous distribution on coastal sand dune in Korea. Many wild plants have a tolerance to water stress, but its extent varies from species to species. It is well known that how plants cope with many environmental stresses, but the effect of the drought stress on *C. soldanella* plant has not been studied well. The object of this study is to determine the extent of drought resistance based on physiological responses under water deficit.

MATERIALS AND METHODS

Plant Material and Growth Conditions

The seeds of *C. soldanella* were collected from the Golaebul sand dune on the eastern coast in Korea (36°14'90"N, 129°22'50"E) in June 2010. *C. soldanella* was soaked in 98% sulfuric acid for two and a half hours and then washing out repeatedly. Finally, these seeds were soaked in distilled water for 30 min. Selected seeds were germinated separately in a pot (11 cm × 9 cm) filled with sand soil. The soil medium used was originally taken from the Golaebul sand dune. This experiments were conducted in the Kyungpook National University, from August to September 2011 in greenhouse where mean temperatures was about 27°C and relative humidity was about 30%. Natural light from the sun with a mean daytime photosynthetic photon flux density (PPFD) was 460 μmol m⁻² s⁻¹. After sowing, every pot received modified Hoagland's solution (0.5 mM NH₄NO₃, 0.5 mM MgSO₄·7H₂O, 0.5 mM KH₂PO₄, 0.5 mM CaCl₂·2H₂O, 0.5 mM K₂SO₄, 19 mM Fe-EDTA and trace elements) every day with 100 mL for 3 weeks. The experiment was composed of two irrigation treatments (well-watered and drought conditions). The experiment was started when the plants had about 6 leaves after three weeks.

Soil Water Contents and Soil Water Potential

Water contents of the soil were determined by the weight difference between fresh and air-dried. Water potential of the soil was measured using Mini Tensiometer (Skye Instruments Ltd., Llandrindod Wells, UK), and data was collected every day.

Plant Growth and Dimensional Parameters of Leaves

At each harvest (day 7 and day 12), the fresh matter of leaves was weighted, and stem length and total leaves number of plant were determined.

The relative water contents

The relative water contents (RWC) was determined as follows:

$$\text{RWC (\%)} = (\text{FW} - \text{DW}) / (\text{TW} - \text{DW}) \times 100,$$

FW = fresh weight, DW = dry weight, and TW = turgid weight. Dry weight of leaves was measured after 3 days dried in the oven at 80°C, and a turgid weight of leaves was measured after infiltrating the samples for 12 h in distilled water at 4°C (Cameron et al. 1999). Relative growth rate (RGR, per day) of leaves was determined as:

$$\text{RGR} = (\ln DW_2 - \ln DW_1) / (d_2 - d_1),$$

where DW_2 and DW_1 are the dry weight of plants for two successive harvest dates (d_1 and d_2).

Stomatal Density (SD), Photosynthesis and Chlorophyll Fluorescence

SD, photosynthesis and chlorophyll fluorescence of leaves were measured on day 7 and day 12 after drought treatment with three replicates. The area of four expanded leaves was calculated using the image-analyzing program (SCIONIMAGE). Stomatal density (SD) was measured on adaxial leaf surface. Stomata were counted in optical Axioskop 2 plus microscope (Carl Zeiss, Oberkochen, Germany) and photographs of epidermal prints (Boccalandro et al. 2007) were obtained at $\times 200$ magnification from middle portions of leaflet laminae. To determine stomatal density, further analyses were used.

Stomatal density (SD) = no. of stomata per leaf area (mm^2)

Photosynthesis was measured by a LCI portable infrared gas analyzer (IRGA) (ADC BioScientific Ltd., Hoddesdon, UK). Chlorophyll fluorescence was measured with a portable Handy PEA (Hansatech Instruments Ltd., King's Lynn, UK). Chlorophyll fluorescence was measured on the adaxial leaf surface, which had been pre-darkened for at least for 30 min. Measurements were conducted on cloudless days and were done between 10:00 and 14:00.

Contents of Chlorophyll a, Chlorophyll b and Carotenoid

The leaf samples for chlorophyll and carotenoid contents were extracted by DMSO (Dimethyl sulfoxide) at 60°C for 12 h. The contents of chlorophyll a, chlorophyll b and carotenoid were estimated from absorbance at 645 nm, 663 nm and 480 nm with UV mini-1240 spectrophotometer (Shimadzu, Kyoto, Japan). Quantitative estimation of the chlorophyll and carotenoid contents was obtained by using the equation of Holden (1965) and Kirk and Allen (1965), respectively.

Contents of Total Ion and Osmolality

The freeze-dried leaves were ground to a homogenous powder and extracted with boiling distilled water for 1 h. Total ion contents (calculated as NaCl equivalents) were determined using a conductivity instrument (Mettler

Check Mate 90; Mettler Toledo, Columbus, OH, USA), and the osmolality of leaf extract solutions was measured by means of cryoscopy (OSMETTE micro-osmometer, model 5004; Precision Systems Inc., Natick, MA, USA).

Contents of Soluble Carbohydrate and Free Proline

Soluble carbohydrate contents of leaves were determined by using the Phenol-Sulfuric acid method. Mix leaf extract of 20 μL and distilled water of 780 μL and mix them with 400 μL of 5% phenol solution and then put concentrated sulfuric acid (98% H_2SO_4) of 2 mL. Leave the reaction solutions undisturbed for 10 min and then shaking for mix. After 30 min, the samples were measured at 490 nm. Soluble carbohydrate was calculated from a standard curve using D-glucose.

Free proline contents were estimated following the method of Bates et al. (1973). Freeze-dried leaves (0.5 g) were extracted in 3% sulphosalicylic acid and the homogenate filtered through filter paper. Filtrate of 2 mL was reacted with 2 mL of acid ninhydrin reagent and glacial acetic acid of 2 mL in a test tube for 1 h at 100°C , and the reaction terminated in an ice bath. The reaction mixture was extracted with 4 mL of toluene and mixed vigorously with a vortex mixture for 15-20 s. The chromophore containing toluene was aspirated from the aqueous phase, warmed to room temperature and the absorbance measured at 510 nm using toluene as blank. Proline concentration was calculated from a standard curve using L-proline.

Statistical Analysis

Statistical analysis of control and drought treatments at each sampling day was conducted using independent samples *t*-test. This test was carried out to determine if significant differences ($P < 0.05$) were found between two groups by using SPSS ver. 18.0 for Windows (SPSS Inc., Chicago, IL, USA). The results are shown as the mean \pm SD of three replicates.

RESULTS and DISCUSSION

Soil Water Contents and Soil Water Potential

Soil water contents were drastically reduced by water stress and the reduction of soil water contents was 89.9% and 96.0% on day 7 and day 12, respectively, in drought

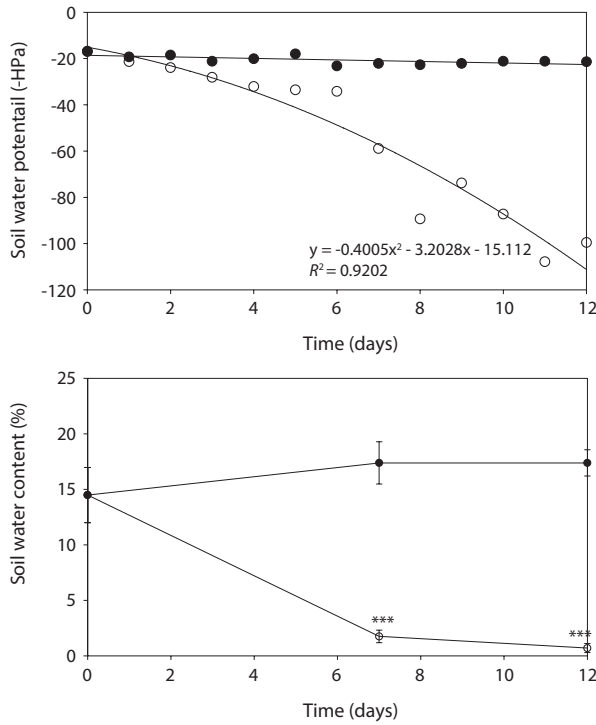


Fig. 1. Change in soil water contents and water potential in pots with well-watered (●) and drought treatments (○). Means values of three replicates with standard deviation (tested with *t*-test; $P = 0.05$). *** $P < 0.001$.

condition as compared to the well-watered (control) condition. During study period, soil water potential decreased continuously by water deficit in drought treatment. Determination of the soil water matric potential (Ψ_m) is significant to characterize and monitor processes such as crop yield production and plant growth (Young and Sisson 2002). During water deficit soil water potential would be decrease and this would negatively affect to water uptake (Loreto et al. 2003). Tensiometer used in this experiment is extensively used instrument for determination of Ψ_m (Or 2001, Young and Sisson 2002). In the present study, Ψ_m was consistently reduced with the decrease of soil water contents during drought period (Fig. 1), which inevitably interfere plant growth and development.

Plant Growth and Dimensional Parameters of Leaves

DW, RGR and RWC in leaves of drought treatment were lower than control after day 7, but were not shown significant difference between control and treatment (Fig. 2). A significant decrease in DW, RGR and RWC was observed on day 12 after treatment, and decreased by 64.5%, 89.1% and 25.3% as compared with that in the control,

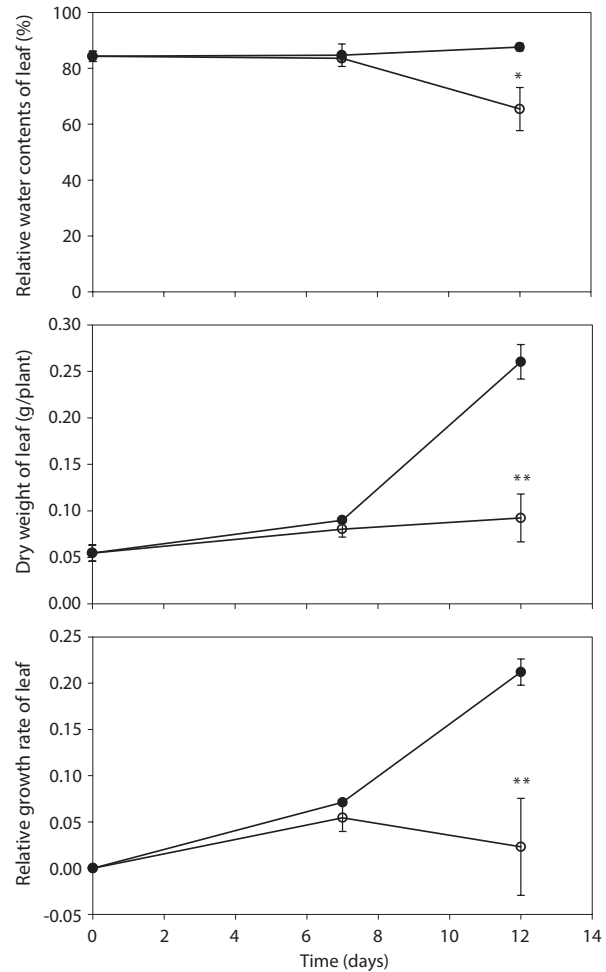


Fig. 2. Change in relative water contents, dry weight and relative growth rate of leaves per plant with well-watered (●) and drought treatments (○). Means values of three replicates with standard deviation (tested with *t*-test; $P = 0.05$). * $P < 0.05$, ** $P < 0.01$.

respectively. After day 12, stem length and leaf numbers per plant in drought treatment were shorter and fewer than control, and decreased by 34.9% and 29.5% as compared to control, respectively (Fig. 3). In drought-stressed plants, RGR and RWC were not shown a significant difference compared with that of control until day 7, but it decreased after day 12. Plant growth inhibition was observed during water deficit. In general, water deficit stress mostly reduced plant growth and development (Thakur and Kaur 2001). According to many studies, stem length was decreased under drought stress (Patel and Golakia 1988, Pita and Pardes 2001), and the reduction in plant dry weight and height is associated with the decrease of stem length (Martiniello and Ciola 1995, Iannucci and Martiniello 1998).

The differences in the leaf area of fully expanded new

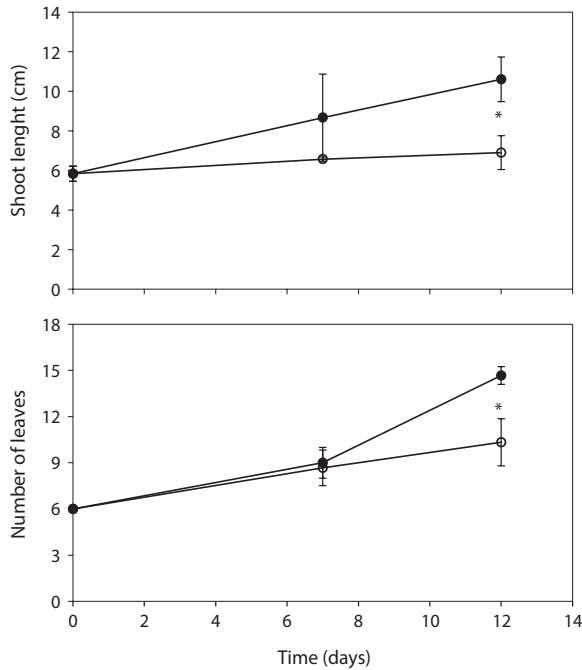


Fig. 3. Change in stem length and number of leaves per plant with well-watered (●) and drought treatments (○). Means values of three replicates with standard deviation (tested with *t*-test; $P = 0.05$). * $P < 0.05$.

leaves between control and drought treatment was not shown on day 7. However, the leaf area on 12 days after drought treatment decreased by 52.2% compared with the control (Fig. 4). In the present study, water deficit stress reduced plant growth such as stem length, number of leaves and leaf area. The reduction in plant growth by water deficit is associated with the reduction of plant biomass and RGR. Leaf area plasticity is an important means by which a drought-stressed crop maintains control of water use and the leaf growth was used as a physiological trait to estimate acclimation to water deficit (Rucker et al. 1995, Blum 1996, Shubhra et al. 2003). The maintenance of higher RWC can be used as an indicator of drought resistance mechanisms in plants under soil drying conditions (Chylinski et al. 2007). Also, decrease in RWC reflects a loss of turgor causing of limited water availability (Ndayiragije and Lutts 2006).

Stomata Density, Photosynthesis and Chlorophyll Fluorescence

On day 7 and day 12, Stomata density was not significantly different compared with that of control (Fig. 5). Stomata aperture and stomatal density mainly control the stomatal conductance of CO_2 which vary with changes of

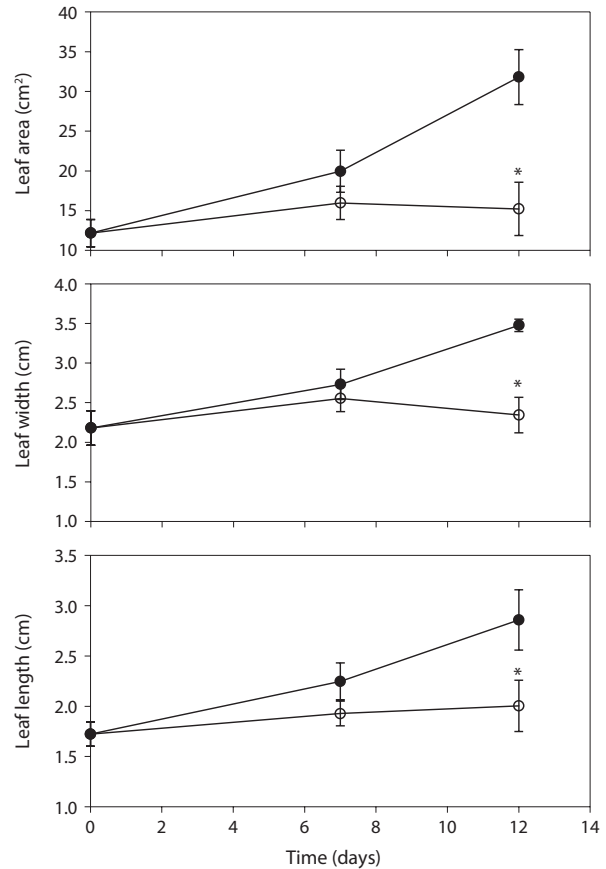


Fig. 4. Change in dimensional parameters of four expanded leaves with well-watered (●) and drought treatments (○). Means values of three replicates with standard deviation (tested with *t*-test; $P = 0.05$). * $P < 0.05$.

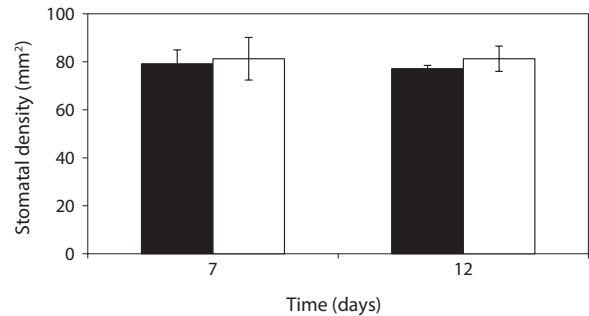


Fig. 5. The difference in stomatal density between two conditions on day 7 and day 12 with well-watered (■) and drought treatments (□). Means values of three replicates with standard deviation (tested with *t*-test; $P = 0.05$).

leaf area in response to water availability and other environmental factors (Casson and Gray 2008, Boccalandro et al. 2009). Under stress conditions many plants increased stomatal density with the decrease of leaf area that enhanced gas exchange per leaf area and biomass yield

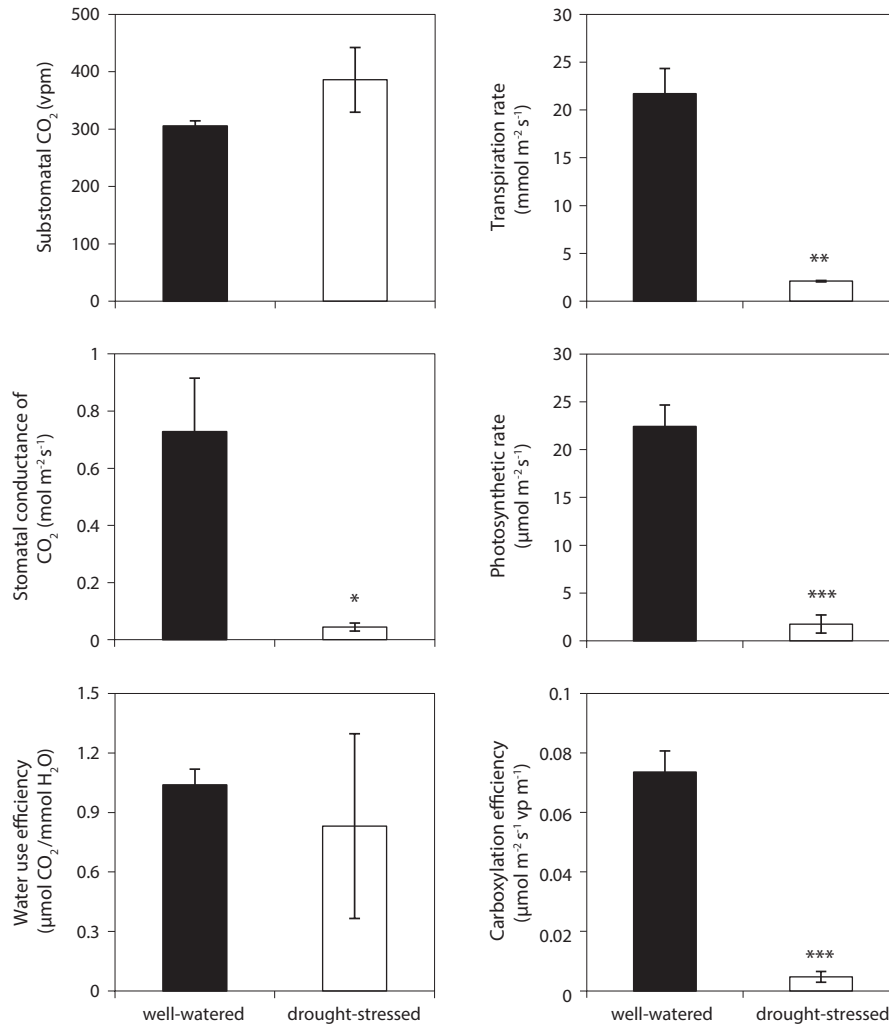


Fig. 6. The difference in photosynthesis between two conditions on day 12 with well-watered (■) and drought treatments (□). Means values of three replicates with standard deviation (tested with *t*-test; *P* = 0.05). **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

production (Yang and Wang 2001, Zhang et al. 2006, Giordano et al. 2011) whereas the number of stomata per leaf decreases (Quarrie and Jones 1977). For the stomatal density per leaf area, *C. soldanella* did not show significant difference between control and treatment. It is suggested that the stomatal density of *C. soldanella* is no largely affected by drought stress.

Photosynthesis was measured at the end of the final stress periods, day 12. Understanding the physiological mechanisms of many stressors is crucial to anticipating how physiological interruption will affect future plant developments. The well-watered plants showed higher net photosynthetic rate, transpiration rate, stomatal conductance of CO₂, and carboxylation efficiency than those of drought-stressed plants (Fig. 6). However, there was no significant difference to the value of substomatal CO₂ and

water use efficiency between treatments on day 12 after drought stress. In the present study, *C. soldanella* showed decrease in photosynthetic rate and stomatal conductance of CO₂ to a similar degree, indicating that photosynthetic rate is largely reduced due to the reduction in stomatal conductance of CO₂ which is highly sensitive to soil-water deficit. Stomatal closure inhibited leaf photosynthetic capacity in drought stressed plants. According to many studies, stomatal conductance of CO₂ declined before leaf water contents are influenced, and photosynthetic rate was mostly dependent on stomatal aperture (Farquhar et al. 1989, Cornic and Briantais 1991). As well as the decline in photosynthetic rate and stomatal conductance of CO₂, the value of the transpiration rate was lower in drought-stressed plants than that of control. Under stressed conditions transpiration rate is largely correlated

with stomatal conductance of CO₂ according to a higher adaptability of stomata (Gratani and Ghia 2002, Niu et al. 2006). Abscisic acid (ABA), a plant stress hormone, induces the closure of leaf stomata, thereby reducing water loss through transpiration, and decreasing the rate of photosynthesis. These responses improve the water-use efficiency of the plant (Waseem et al. 2011). The water use efficiency by stomatal regulation is an imperative feature for plant species to survive in coastal sand dunes.

Maximum quantum use efficiency (F_v/F_m) in dark-adapted leaves corresponds to the ratio $(F_m - F_0)/F_m$, where F_m is the maximal fluorescence yield of a dark-adapted sample, with all PSII reaction centres fully closed, and F_0 is the minimum fluorescence yield of a dark-adapted sample, with all PSII reaction centres fully open. F_v/F_m has been commonly used to identify changes in the photosynthetic apparatus as a result of stress (Baker and Rosenqvist 2004, Resco et al. 2008). Previous studies suggest that chlorophyll fluorescence parameters tend to be strongly correlated with plants mortality to react environmental stresses (Wakrim et al. 2005).

In the present study, F_v/F_m were not significantly different under well-watered and drought conditions (Table 1). According to other previous studies, some plants did not show any remarkable change of F_v/F_m under drought stress whereas plant growth was rapidly decreased (Munns et al. 2010). *C. soldanella* photochemical activity

was resistant to water stress and the electron transport chain was maintained under water deficit conditions, even though photosynthetic rate and stomatal conductance of CO₂ were strongly limited.

Contents of Chlorophyll a, Chlorophyll b and Carotenoid

On day 7, chlorophyll a, total chlorophyll and carotenoid contents showed small increase 15.7%, 15.5% and 16.3%, respectively, in drought treatment as compared to the control. On day 12, chlorophyll b, total chlorophyll and carotenoid contents of the stressed plants were lower than those of controls, but there were no significantly different (Table 2).

Water stress leads to a decline in photosynthetic activity and change of the chloroplast capacity (Martinez et al. 2003, Jaleel et al. 2007, Massacci et al. 2008). Decrease of chlorophylls and carotenoid contents by stressed conditions was reported in several plant species (Loggini et al. 1999, Agastian et al. 2000). In the present study, contents of chlorophyll and carotenoid under water stress were decreased after 12 days, but there was no significant difference compared to control. It is suggested that the capacity of chloroplast does not get much damage from drought conditions.

Contents of Total Ion, Osmolality, Soluble Carbohydrate and Proline

The total ion contents and osmolality of drought-stressed plants had higher than control plants during study period. The value of total ion contents and osmolality gradually increased and it is significantly different than those of control. Total ion contents in the leaf of drought-stressed plant were increased by 19.7% and 36.3%, and osmolality was increased by 41.5% and 32.3% as compared to control on the day 7 and day 12, respectively.

Soluble carbohydrate contents were gradually increased during drought stress. The value of the soluble

Table 1. Change in chlorophyll fluorescence parameters (F_0 , F_m and F_v/F_m) with well-watered and drought treatments

	Day 7		Day 12	
	well-watered	drought	well-watered	drought
F_0	341 ± 10.6	386 ± 55.8	350 ± 36.9	435 ± 87.9
F_m	1614 ± 124	1438 ± 112	1461 ± 124	1483 ± 95
F_v/F_m	0.79 ± 0.02	0.73 ± 0.05	0.76 ± 0.02	0.71 ± 0.05

F_0 , F_m and F_v/F_m mean the minimum fluorescence intensity, the maximal fluorescence intensity and the maximum quantum efficiency of PSII photochemistry, respectively. Means values of three replicates with standard deviation (tested with *t*-test; $P = 0.05$).

Table 2. Change in chlorophyll and carotenoid contents with well-watered and drought treatments

	Day 7		Day 12	
	well-watered	drought	well-watered	drought
Chla (mg/g)	6.82 ± 0.30	7.89 ± 0.30*	6.42 ± 0.29	6.50 ± 1.26
Chlb (mg/g)	1.41 ± 0.01	1.61 ± 0.21	1.20 ± 0.27	0.68 ± 0.43
Total Chl (mg/g)	8.23 ± 0.32	9.50 ± 0.19**	7.62 ± 0.55	7.18 ± 0.92
Carotenoid (mg/g)	416 ± 13.62	483 ± 10.31**	408 ± 19.85	375 ± 48.41

* $P < 0.05$, ** $P < 0.01$. Means values of three replicates with standard deviation (tested with *t*-test; $P = 0.05$).

Table 3. Change in total ion, osmolality, soluble carbohydrate and free proline contents

Day	Ion contents ($\mu\text{eq/g}$ plant water)		Osmolality ($\mu\text{osm/g}$ plant water)	
	well-watered	drought	well-watered	drought
0	262 \pm 0.69		275 \pm 1.23	
7	233 \pm 16.34	279 \pm 9.87 [*]	235 \pm 8.65	333 \pm 11.90 ^{***}
12	240 \pm 25.27	327 \pm 3.22 [*]	291 \pm 29.18	386 \pm 23.68 [*]

Day	Carbohydrate ($\mu\text{mole/g}$ plant water)		Proline ($\mu\text{mole/g}$ leaf weight)	
	well-watered	drought	well-watered	drought
0	14.27 \pm 6.71		0.83 \pm 0.37	
7	21.17 \pm 5.91	18.35 \pm 0.47	0.83 \pm 0.30	1.88 \pm 0.47 [*]
12	30.26 \pm 3.22	24.83 \pm 1.12	0.47 \pm 0.44	8.75 \pm 1.53 ^{**}

^{*} $P < 0.05$, ^{**} $P < 0.01$, ^{***} $P < 0.001$. Means values of three replicates with standard deviation (tested with t -test; $P = 0.05$).

carbohydrate contents was similar between control and treatment. Control was litter higher than drought treatment, but it is no significant difference. Proline synthesis was greatly enhanced in drought-stressed plants (Table 3). Proline contents in the drought-stressed plants on the day 7 and day 12 were 2.27 and 18.6 times greater than those of controls. It is indicated that the increase of osmolality in stressed plants was due to the accumulation of osmolytes by water deficit. To prevent water loss, drought-stressed plants try to promote with an increase in osmolality (Chaves et al. 2003). Plant capacity for osmotic adjustment could be important to maintain water absorption in sand dune plant species under water deficit (Nilsen et al. 1984). For these reasons, plants accumulate other compatible solutes such as sugar, glycerol, betaines, proline and inorganic ions under water stress conditions (Bray 1997, Hare et al. 1998, Chaves et al. 2003), and also degree of proline accumulation correlates with the change of water potential and RWC (Knipp and Honermeier 2006).

In the present study, total ion contents and osmolality are higher than those of control, and which are continuously increased with water stress. Also, increase of proline contents in leaves induced by drought stress agreed with previous studies, and accumulation of proline is one of the general adaptive mechanisms in many plants (Hare et al. 1998, Abdel-Nasser and Abdel-Aal 2002). However, soluble carbohydrate did not show any remarkable difference compared to control plants. It may be *C. soldanella* use other ions as compatible solutes rather than soluble carbohydrates. In order to enhance water uptake and prevent water loss, *C. soldanella* accumulate ions and particularly proline to tolerate and survive on sand dune conditions of drought.

In conclusion, physiological responses of *C. soldanella* to water-deficit were related to the drought duration. After day 7 of drought treatment, the inhibition of plant growth and the maximum efficiency of photosystem II (PSII) photochemistry (F_v/F_m) was not observed, but the contents of total ion and proline increased, indicating that the accumulation of ion and osmotic solutes in *C. soldanella* may help to maintain plant growth under the water stress condition. The photosynthetic rate and stomatal conductance in drought stressed *C. soldanella* plant for 12 days were decreased by 92.2% and 93.9%, respectively, with a low leaf RWC of 25.3%, but F_v/F_m was not affected under drought condition, indicating the photosynthesis down regulation may mainly derive from stomatal limitation for this species. The water use efficiency by stomatal regulation is an imperative feature for plant species to survive in coastal sand dunes. The increase of solute, mainly ion and proline indicated that *C. soldanella* plant under severe drought condition is the responses to overcome low osmotic potential of soil.

ACKNOWLEDGMENTS

This work was supported by the Eco-Innovation project and Long Term Ecological Research (LTER) of the Ministry of Environment, Korea.

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