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Drought Hardening Contributes to the Maintenance of Proportions of Non-Embolized Xylem and Cambium Status during Consecutive Dry Treatment in Container-Grown Seedling of Japanese Cedar (*Cryptomeria japonica*)

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Abstract: Climate models in Japan predict that the annual mean air temperature and number of consecutive dry days will increase in the future, leading to high seedling mortality rates. Maintaining high survival rates of *Cryptomeria japonica* seedlings, a commercially important tree species, is therefore, important in terms of appropriate forest management under climate change. Although drought hardening, in which seedlings are acclimated to dry conditions in the nursery prior to planting, contributes to increased survival under drought conditions, little is known about the effective irrigation frequency of drought hardening in *C. japonica* seedlings. In this study, we therefore, examine the effectiveness of different drought-hardening treatments in *C. japonica*. We first clarify the effects on physiological and morphological traits by comparing three drought-hardening treatments [control (*C*): Irrigation once daily; mild (*M*): irrigation once every three days; and severe (*S*): irrigation once every five days] for one month. Next, to confirm the effects during consecutive dry treatment, we stopped irrigation for 13 days and once again compared the physiological traits between the three drought-hardening treatments. Drought hardening reduced whole-plant transpiration (E_{whole}), resulting in conserved water use, and this tendency was particularly evident under the *S* treatment. Moreover, during consecutive dry treatment, the E_{whole}, proportions of non-embolized xylem, and cambium status of basal stem regions were maintained for the longest duration under the *S* treatment, followed by the *M* treatment. Our findings suggest that the efficiency of drought hardening increased with drought severity. Furthermore, one month of drought hardening contributed to both water conservation and the maintenance of cell differentiation under consecutive dry treatment, likely increasing the tolerance and survival of *C. japonica* seedlings under prolonged drought.

Keywords: *Cryptomeria japonica*; drought hardening; drought tolerance; forestry; seedling; embolism; cryo-SEM

1. Introduction

Cryptomeria japonica D. Don (Japanese cedar) is a commercially important tree species in Japan, covering 17.7% of the total forested area [1]. According to afforestation data from 2006 to 2014 for compensation of tree die-off due to natural disasters, the area of afforestation resulting from drought is estimated at 1919 ha (14.9% of the total area of afforestation) [2]. In Japan, the ratio of drought-induced tree death is particularly high in 1- to 5-year-old seedlings, at approximately 38% (drought-induced annual morality rates are 0.27%) [3]. Because 85% of the afforestation costs for C. japonica is spent during 5 years after planting [4], the high rate of drought-induced mortality results in huge economic losses. Furthermore, climate models in Japan predict that the annual mean air temperature will increase from 1 °C to 2.5 °C [5] and consecutive dry days have been on the increase since 1901 [6], further increasing the risk of seedling mortality. Maintaining high rates of survival in *C. japonica* seedlings is therefore important in terms of appropriate forest management under this changing climate.

Drought hardening is the process by which seedlings acclimate to low water conditions in the nursery by the artificial withholding of irrigation to increase the drought tolerance of seedlings prior to planting [7,8]. Woody plants can avoid drought-induced desiccation of their tissues through physiological and morphological responses, such as stomatal closure [9], an increased root-to-leaf area ratio [10–12], and leaf shedding [13,14]. These physiological and morphological traits enable plants to maintain high water content during drought by maximizing water uptake and water storage, and minimizing water loss [15,16]. These responses depend on the level of water deficit of drought hardening, as well as the species [8,17–19].

Effective methods for drought hardening before planting *C. japonica* seedlings have not been established. Nagakura et al. [20] found that whole-plant transpiration in *C. japonica* seedlings decreased after 19.5 weeks of dry treatment compared with wet treatment, even after irrigation, suggesting a link to the maintenance of a high water content. In contrast, Nagakura et al. [21] showed that whole-plant transpiration did not differ from that of control seedlings after 12 weeks of dry treatment. These findings suggest that the physiological response is related to the duration of drought hardening in *C. japonica* seedlings. The root-to-shoot ratio was found to increase in *C. japonica* seedlings under drought conditions [22]. In other tree species, van Den Driessche [7,23] showed that two and a half months of drought-hardening treatment contributes to survival and physiological and morphological acclimation under drought conditions in seedlings of Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco), lodgepole pine (*Pinus contorta* Dougi.), and white spruce (*Picea glauca* (Moench.) Voss). These findings suggest that drought hardening affects not only physiological but also morphological traits.

In the present study, we examined the effectiveness of different levels of drought hardening before planting in *C. japonica* seedlings. First, to clarify the effect on physiological and morphological traits, we carried out comparisons between three drought-hardening treatments for one month ("drought hardening"). Next, to verify the effectiveness of drought hardening during consecutive dry treatment, irrigation was stopped for 13 days and comparisons of physiological traits were once again carried out between the three drought-hardening treatments ("consecutive dry treatment").

2. Materials and Methods

2.1. Plant Materials

Three-year-old container-grown *C. japonica* seedlings (Ringyo-Shubyo Association, Ibaraki, Japan) were used. The seedlings were grown in the field for 1 year and transplanted to 150-cc multi-cavity containers (OY-150; Zenbyoren, Tokyo, Japan) filled with coir peat. They were then periodically fertilized using solid and liquid fertilizer and were irrigated daily in the field for 1 year. After the development of leaves and cessation of xylem development (start of September 2018), the seedlings were transplanted into 200 cc single-cavity pots filled with coir peat and placed in a phytotron chamber under an artificial illumination system (Koitotron KG; Koito Industries, Yokohama, Japan) at the Forestry and Forest Products Research Institute (Tsukuba, Japan; 36°01′N, 140°08′E) for 1 month. The seedlings were maintained at day and night temperatures of 28 °C and 25 °C, respectively, with a linear increase from 25 °C to 28 °C for 1 h followed by 28 °C for 10 h, a linear decrease to 25 °C for 1 h, and then 25 °C for 12 h. Humidity was maintained at 60% in the day and 70% at night, respectively, throughout the experiment, with a linear decrease from 70% to 60% for 1 h followed by 60% for 10 h, a linear increase to 70% for 1 h, and 70% for 12 h. Day and night PPFD was maintained at 1200 and 0 μmo m^{-2} s^{-1}, with a linear increase from 0 to 1200 μmo m^{-2} s^{-1} for 1 h followed by 1200 μmo m^{-2} s^{-1} for 10 h, a linear decrease to 0 μmo m^{-2} s^{-1} for 1 h, and 0 μmo m^{-2} s^{-1} for 12 h. Before drought hardening, the seedlings were fully irrigated and fertilized with liquid fertilizer in the form of 0.5 L 1000× Hyponex solution (22 mg N L^{-1}, 36 mg $PO_4{}^{3-}$ L^{-1}, and 18 mg K L^{-1}; 6:10:5, N:P:K; Hyponex Japan Corp., Ltd., Osaka, Japan) for 1 month to minimize the effects of transplanting and transfer on seedling growth. The position of the seedlings was randomly changed approximately once a week to minimize the effect of micro-environmental factors within the phytotron chamber.

2.2. Drought Hardening and Consecutive Dry Treatment

A total of 36 seedlings [65.6 ± 6.6 (mean ± 1SD) cm in height with a basal stem diameter of 7.6 ± 1.0 mm (mean ± 1SD)] were transferred to the phytotron chamber. All seedlings satisfied the standard height (30 cm height) and basal stem diameter (3.5 mm diameter) for afforestation in Japan. Twelve seedlings were subjected to each of three drought-hardening treatments for 1 month: irrigation once daily [control (*C*) treatment], irrigation once every 3 days [mild drought (*M*) treatment], and irrigation once every 5 days [severe drought (*S*) treatment] ("drought hardening"). To minimize the effects of water distribution heterogeneity in the soil, irrigation was carried out by submerging the pots in water for 45 min. The volumetric soil water content (SWC) was then measured in four pots per treatment using a soil moisture sensor (S-SMC-M005; Onset, Kyoto, Japan). The measurement interval for SWC was 15 min. After drought hardening, irrigation was ceased in all treatments to verify the efficacy of drought hardening on physiological traits during consecutive dry days ("consecutive dry treatment"). Additionally, three seedlings were irrigated on day 8 of the consecutive dry treatment to verify the refilling of embolized tracheids in the basal stem xylem (see below for details).

2.3. Leaf Gas Exchange Traits during Drought Hardening and Consecutive Dry Treatment

Leaf gas exchange traits were measured in the morning on days 1, 16, and 31 of drought hardening after at least 30 min when the seedlings were exposed to full irradiation. The following traits were measured using a portable open gas exchange system (LI-6400; Li-Cor Inc., Lincoln, NE) with a leaf chamber fluorometer (LI-6400-40; Li-Cor Inc.): Net assimilation (A), water vapor stomatal conductance (g_s), and the transpiration rate (E). All measurements were conducted under a photosynthetic photon flux (PPF) of 1200 μmo m^{-2} s^{-1} using red-blue LED lamps, at a leaf temperature of 24.8 °C to 27.1 °C, and a CO_2 concentration of 400 μmol mol^{-1} in the inlet gas stream of the leaf chamber. To measure the response of g_s to leaf-to-air vapor pressure deficit (VPD), VPD inside the leaf chamber was adjusted four points from 0.8 to 3.3 kPa, by gradually increasing the volume of dry air and by adding Drierite (97% $CaSO_4$, 3% $CaCl_2$) to the inlet gas stream. After at least 15 min from the adjustment of VPD, g_s was measured. As g_s decreased exponentially with increasing leaf-to-air VPD, an analysis of stomatal sensitivity was carried out according to Oren et al. [24],

$$g_s = -m \ln (\text{leaf-to-air VPD}) + b \tag{1}$$

where m is the stomatal sensitivity to VPD (mmol m^{-2} s^{-1} ln $(kPa)^{-1}$) and b is the reference stomatal conductance at a leaf-to-air VPD of 1 kPa. The value of m was determined by extrapolation and curve fitting using least squares regression analysis.

Before turning on the lights, all the pots, excluding the above-ground seedling, were covered in a plastic bag to prevent evaporation from the soil surface and the weight of each pot was measured (W_{start}). After turning off the lights (following exposure to light for 12 h), the pots were re-measured (W_{end}) and the plastic bags were removed to minimize the effect on root growth. Whole-plant transpiration(E_{whole}) was then calculated as follows,

$$E_{whole} = (W_{start} - W_{end})/W_{shoot} \tag{2}$$

where W_{shoot} is the shoot dry weight.

2.4. Growth and Biomass Allocation during Drought Hardening

Tree height and the diameter of the basal stem region were measured at both the start and end of drought hardening to determine the growth rate. Three seedlings per drought-hardening treatment were also sampled on days 1, 8, and 13 of consecutive dry treatment. Seedlings were then divided into leaf, stem, coarse-root (>2 mm in diameter), and fine-root (<2 mm in diameter) samples and dried at 70 °C for at least 3 days to determine the dry mass. Shoot and fine-root biomass, the ratio of shoot to whole-plant dry weight [shoot weight ratio (SWR)] and the ratio of fine-root to shoot dry weight (fine-root: shoot ratio) at the end day of drought hardening (start of consecutive dry treatment) were then determined.

2.5. Cryo-SEM Observations of Xylem and Cambium in the Basal Stem Region

To visualize the water distribution in the xylem tracheids and cambium under progressive drought during consecutive dry treatment, cryo-SEM observations of the xylem and cambium in the basal stem region were conducted on days 1, 8, and 13 of the consecutive dry treatment with one frozen basal stem per drought-hardening treatment per sampling day. Additionally, the seedlings that were irrigated on day 8 of consecutive dry treatment were also sampled on day 13 to verify recovery of xylem embolism. All seedlings were placed in a black plastic bag for at least 2 h to prevent transpiration [25] before the top shoot region was removed under running water. The top shoot region was submerged in water for at least 30 min to release negative pressure in the xylem conduits, since non-negligible artifacts have been reported under negative pressure [26,27]. Immediately after sampling, the main stems were submerged in liquid nitrogen for at least 1 min until the stem and water froze. They were stored in a deep freezer at −80 °C until cryo-SEM observation. The transverse face of the frozen sample was cleanly cut using a cryostat microtome (Cryo Star NX70, Thermo Fisher, Shanghai, China) then transferred to the cold stage of the cryo-SEM (JSM-6510 attached to a cryo-SEM unit; JEOL Ltd., Tokyo, Japan) and subjected to etching at −90 °C for 3–5 min. The specimens were then observed using cryo-SEM under an accelerating voltage of 3 kV and a cold stage temperature of approximately −130 °C.

2.6. Statistical Analysis

For statistical analysis of the relationships between the different drought-hardening treatments and the physiological and morphological traits in the *C. japonica* seedlings, generalized linear mixed models (GLMM) were developed and the best fit model was selected based on Akaike Information Criterion (AIC) values. All models were developed with a GLMM, assuming Gaussian error distribution with identity link function. The response variables were the physiological and morphological traits (numeric class), the explanatory variable was the different irrigation frequency during drought hardening (factor class), and the random effect was the holder of 200-cc single-cavity pots used in the phytotron chamber. Physiological traits were the leaf gas exchange parameters (A, g_s, E, and E_{whole}) and morphological traits were the growth traits (growth rates of height and basal stem diameter during 1 month of drought hardening, SWR, and fine-root: shoot ratio). Five models were constructed using dummy explanatory variables as follows: Model 1 [y = 1 (null model)] indicated no difference between irrigation frequencies during drought hardening in any given response variable. Model 2 [y = (*C* treatment + *M* treatment) + *S* treatment − 1] indicated a difference in the response variable under the *S* treatment compared with the *C* and *M* treatments. In this model, (*C* treatment + *M* treatment) and (*S* treatment) were used as the dummy variables "ab" and "c", respectively. Model 3 [y = (*C* treatment + *S* treatment) + (*M* treatment) − 1] indicated a difference in the response variable under the *M* treatment compared with the *C* and *S* treatments, with (*C* treatment + *S* treatment) and (*M* treatment) used as the dummy variables "ac", and "b", respectively. Model 4 [y = (*C* treatment) + (*M* treatment + *S* treatment) − 1] indicated a difference in the response variable under the *C* treatment compared with the *M* and *S* treatments, with (*C* treatment) and (*M* treatment + *S* treatment) used as the dummy variables "a" and "bc", respectively. Model 5 [y = (*C* treatment) + (*M* treatment) + (*S* treatment) − 1] indicated a difference in the response variable between drought hardening treatments. In this model, (*C* treatment), (*M* treatment), and (*S* treatment) were used as the dummy variables "a", "b", and "c", respectively.

During consecutive dry treatment, mean E_{whole} was also compared between measurement days in the three drought-hardening treatments using one-way repeated measures ANOVA followed by Dunnett's test at a significance level of $\alpha = 0.05$, for multiple comparisons between the first day and subsequent measurement days. All statistical analyses were conducted using 'R' (version 2.15.1; Development Core Team), with the "lme4" and "multcomp" packages for model selection and multiple comparisons, respectively.

3. Results

3.1. Volumetric Soil Water Content during Drought Hardening and Consecutive Dry Treatment

During drought hardening, the mean SWC was lower under *S* treatment [0.66 ± 0.22 (mean ± 1 standard deviation (SD)) m^3 m^{-3}] than *M* [0.70 ± 0.20 (mean ± 1 SD) m^3 m^{-3}] and *C* treatment [0.79 ± 0.17 (mean ± 1 SD) m^3 m^{-3}] (Figure 1a). Moreover, the minimum SWC under *S* treatment (0.16 m^3 m^{-3}) was lower than that under *M* (0.31 m^3 m^{-3}) and *C* treatment (0.62 m^3 m^{-3}). During consecutive dry treatment, a similar decrease in SWC with progressive drought was found between all drought-hardening treatments (Figure 1b), and values recovered in all drought-hardening treatments after re-watering.

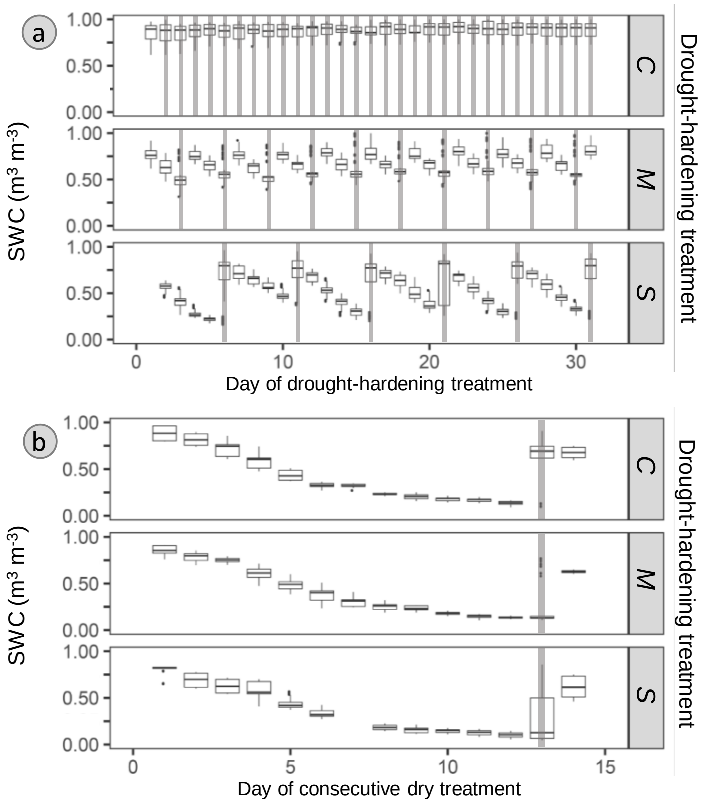

Figure 1. Volumetric water contents in soil (SWC) were compared between drought-hardening treatments [control (*C*), mild drought (*M*), and severe drought (*S*)] during drought hardening (**a**) and consecutive dry treatment (**b**). Boxplots show the median (bar), interquartile range (box), 5th and 95th percentiles (whiskers), and outliers (points) (n of *SWC* was four). Grey bars indicate periods of irrigation.

3.2. Leaf Gas Exchange Traits during Drought Hardening and Consecutive Dry Treatment

The results of model selection in leaf gas exchange traits during drought hardening are shown in Tables 1 and A1. There were no significant differences in *A* and g_s on the first day of drought hardening between drought-hardening treatments. Although, a decrease was observed under the *S* treatment compared with the *C* and *M* treatment on day 16 (the mid-point of drought hardening) and day 31 (the end day of drought hardening) (Figure 2a,b). In contrast, *E* and *m* were lower under the *M* treatment than the *C* and *S* treatments on the first day of drought hardening (Figure 2c,d), while at the end of drought hardening, *E* was highest under the *C* treatment followed by the *M* treatment (Figure 2c). There were no significant differences in *m* between treatments on the final day of drought hardening (Figure 2d). E_{whole} was higher under the *S* treatment than the *C* and *M* treatment on the first day of drought hardening, while at the mid-point to the end of drought hardening, E_{whole} was the lowest under the *S* treatment followed by the *M* treatment (Figure 2e).

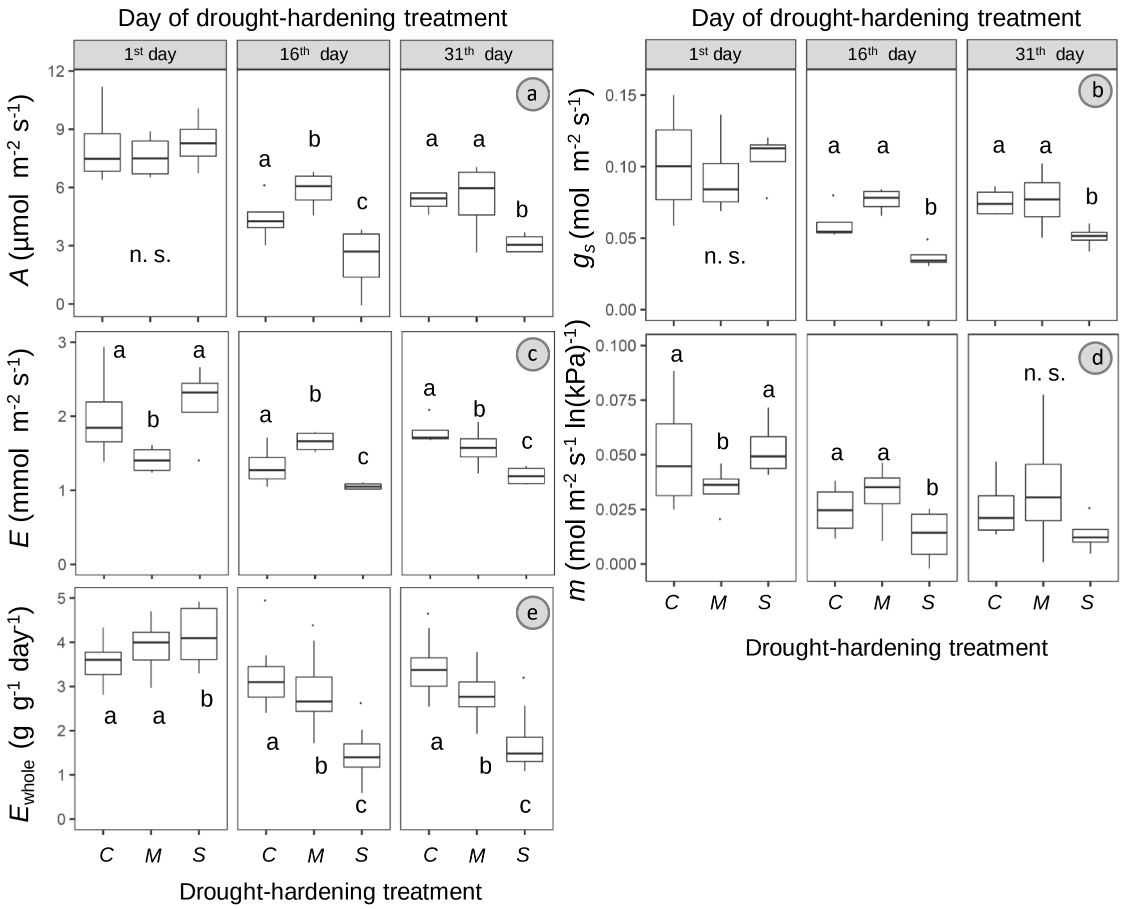

Figure 2. Net assimilation rate (A; **a**), water vapor stomatal conductance (g_s; **b**), transpiration rate (E; **c**), stomatal sensitivity to VPD (m; **d**), and shoot dry mass-based whole-plant transpiration (E_{whole}; **e**) were compared between drought-hardening treatments [control (*C*), mild drought (*M*), and severe drought (*S*)] during drought hardening. Measurements of leaf gas exchange traits were conducted in the morning on days 1, 16, and 31 of drought hardening after full irrigation. Boxplots show the median (bar), interquartile range (box), 5th and 95th percentiles (whiskers), and outliers (points) (n of A, g_s, m, and E were 4, n of E_{whole} was 12). For statistical analysis in the relationships between different drought-hardening treatments and the physiological traits in *C. japonica* seedlings, we developed generalized liner mixed models (GLMM) and then we selected the best fit model between these models based on AIC (Akaike Information Criterion) values. Different letters indicate statistically significant differences (see methods for derails of statistical analysis). “n.s.” means no statistically significant differences.

Table 1. Estimated coefficients, standard error, and AIC values of the selected models in gas exchange traits in shoot. We developed generalized liner mixed models (GLMM) and then we selected the best fit model between these models based on AIC (Akaike Information Criterion) values. In these models, the response variables are leaf gas exchange (A, g_s, E, m, and E_{whole}) (numeric class), the explanatory variable is the different irrigation frequency during drought hardening (factor class) and random effect is the holder of 200-cc single-cavity pots used in the phytotron chamber. In the column of selected model and response variables, *C*, *M*, and *S* means *C* treatment, *M* treatment, and *S* treatment during drought hardening.

Gas Exchange Traits	Unit	Measurement Day	Selected Model	Response Variables	Estimated Coefficients	Standard Error	AIC
A	μmo m^{-2} s^{-1}	1	Null	Intercept	8.03	0.41	48.69
		16	(*C*) (*M*) (*S*)	(*C*)	4.41	0.61	48.64
				(*M*)	5.87	0.06	
				(*S*)	2.29	0.06	
		31	(*C* + *M*) (*S*)	(*C* + *M*)	5.36	0.38	43.60
				(*S*)	3.11	0.53	
g_s	mol m^{-2} s^{-1}	1	Null	Intercept	0.10	0.01	−46.41
		16	(*C* + *M*) (*S*)	(*C* + *M*)	0.07	0.01	−71.10
				(*S*)	0.04	0.01	
		31	(*C* + *M*) (*S*)	(*C* + *M*)	0.08	0.00	−62.74
				(*S*)	0.05	0.01	
E	mmol m^{-2} s^{-1}	1	(*C* + *S*) (*M*)	(*C* + *S*)	2.09	0.16	22.56
				(*M*)	1.41	0.22	
		16	(*C*) (*M*) (*S*)	(*C*)	1.33	0.08	0.23
				(*M*)	1.66	0.08	
				(*S*)	1.05	0.08	
		31	(*C*) (*M*) (*S*)	(*C*)	1.80	0.09	3.44
				(*M*)	1.58	0.09	
				(*S*)	1.20	0.09	
m	mmol m^{-2} s^{-1} ln(kPa)$^{-1}$	1	(*C* + *S*) (*M*)	(*C* + *S*)	0.05	0.01	−56.26
				(*M*)	0.03	0.01	
		16	(*C* + *M*) (*S*)	(*C* + *M*)	0.03	0.00	−64.06
				(*S*)	0.01	0.01	
		31	Null	Intercept	0.02	0.01	−53.66
E_{whole}	g g^{-1}	1	(*C* + *M*) (*S*)	(*C* + *M*)	4.01	0.19	103.64
				(*S*)	5.02	0.26	
		16	(*C*) (*M*) (*S*)	(*C*)	3.36	0.26	88.18
				(*M*)	2.88	0.27	
				(*S*)	1.43	0.27	
		31	(*C*) (*M*) (*S*)	(*C*)	3.39	0.17	70.08
				(*M*)	2.83	0.17	
				(*S*)	1.70	0.17	

During consecutive dry treatment, the effect of the day of treatment on E_{whole} was significant for all drought-hardening treatments (Table A2). E_{whole} remained high for 4 days under the *S* treatment, while E_{whole} remained high for 3 days under the *M* treatment, and under the *C* treatment, E_{whole} remained high for only 2 days (Figure 3). On day 13 of consecutive dry treatment, E_{whole} decreased to a low value in all drought-hardening treatments.

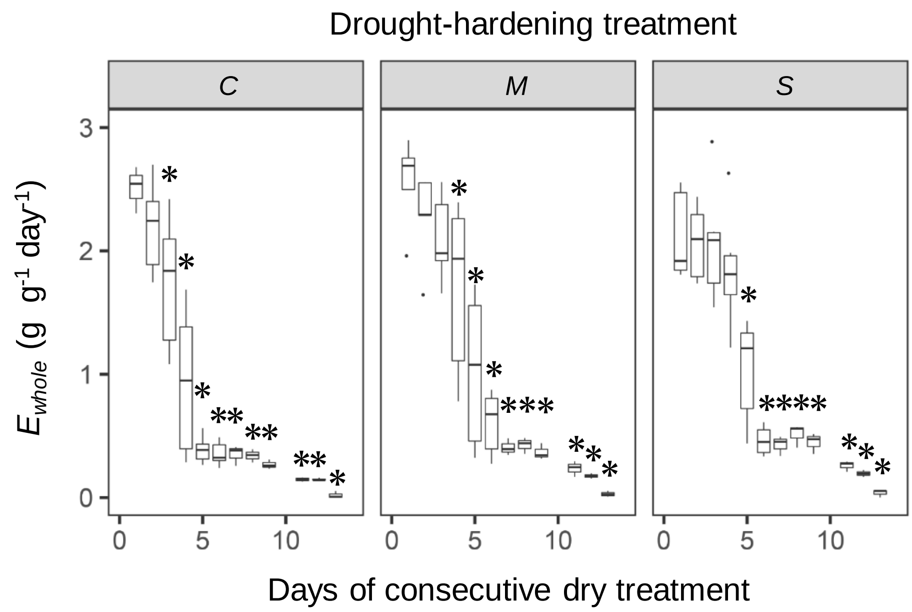

Figure 3. Shoot dry mass-based whole-plant transpiration (E_{whole}) was compared between drought-hardening treatment [control (*C*), mild drought (*M*), and severe drought (*S*)] during consecutive dry treatment. Boxplots show the median (bar), interquartile range (box), 5th and 95th percentiles (whiskers), and outliers (points) (n of E_{whole} was nine from one to eight day and three from eight to thirteen day during consecutive dry treatment). The means of E_{whole} were compared for each measurement days in all drought-hardening treatments using one-way repeated measures ANOVA followed by Dunnett's test at a significance level of $\alpha = 0.05$, for multiple comparisons between the first day and subsequent measurement days. Asterisk indicate statistically significant differences from 1st day.

3.3. Growth and Biomass Allocation during Drought Hardening

The results of model selection in terms of growth and biomass allocation during drought hardening are shown in Tables 2 and A3. During 1 month of drought hardening, the height growth rate, shoot dry weight, and SWR remained unchanged between drought-hardening treatments (Figure 4a,c,e), whereas diameter growth of the basal stem region was lower under the *S* treatment than the *C* and *M* treatments (Figure 4b). In addition, fine-root dry weight and the fine-root:shoot ratio were higher under the *S* treatment than the *C* and *M* treatments (Figure 4d,f). These findings suggest that growth and biomass allocation changed from diameter growth of the basal stem region to growth of the fine-roots during severe drought hardening.

3.4. Cryo-SEM Observations of Xylem and Cambium in Basal Stem Region during Consecutive Dry Treatment

No embolism of xylem tracheids was observed under any of the drought hardening treatments on day 1 of consecutive dry treatment (Figure 5a). However, on day 8, embolism was shown under the *C* treatment, even in current-year xylem, while proportions of embolized xylem was small under the *M* and *S* treatments (Figure 5b). On day 13, most tracheids had lost water under the *C* and *M* treatments, while approximately half of tracheids in current-year xylem were filled with water under the S treatment (Figure 5c). In addition, cambium cells remained unchanged under the *S* treatment on day 13 of consecutive dry treatments, whereas cambium cell deformation was observed under the *C* and *M* treatments (Figure 6).

Under the *S* treatment, comparisons of proportions of non-embolized xylem in the basal stem region before and after irrigation treatment on day 8 of consecutive dry treatment revealed no clear recovery of the embolism area even after re-watering (Figure 7). This trend was also observed under the *C* and *M* treatments (data not shown).

Table 2. Estimated coefficients, standard error, and AIC values of the selected models in growth, shoot and fine-root dry weight, the ratio of shoot dry weight to whole-plant dry weight (SWR), and the ratio of fine-root dry weight to shoot dry weight (fine-root:shoot ratio). We developed generalized liner mixed models (GLMM) and then selected the best fit between these models based on AIC (Akaike Information Criterion) values. In these models, the response variables are growth traits (the rate of height and basal diameter, shoot and fine-root dry weight, SWR, and fine-root:shoot ratio) (numeric class), the explanatory variable is the different irrigation frequency during drought hardening (factor class) and random effect is the holder of 200-cc single-cavity pots used in the phytotron chamber. In the column of selected model and response variables, *C*, *M*, and *S* means *C* treatment, *M* treatment, and *S* treatment during drought-hardening.

Morphological Traits	Unit	Selected Model	Response Variables	Estimated Coefficients	Standard Error	AIC
Height growth	mm month^{-1}	Null	Intercept	0.38	0.18	82.71
Diameter growth	mm month^{-1}	(*C* + *M*) (*S*)	(*C* + *M*) (*S*)	2.16 1.40	0.28 0.36	91.69
Shoot dry weight	g	Null	Intercept	10.50	0.81	47.54
Fine−root dry weight	g	(*C* + *M*) (*S*)	(*C* + *M*) (*S*)	2.55 3.65	0.28 0.40	25.91
SWR	g g^{-1}	Null	Intercept	0.42	0.01	−31.19
Fine-root: shoot	g g^{-1}	(*C* + *M*) (*S*)	(*C* + *M*) (*S*)	0.24 0.36	0.02 0.03	−21.03

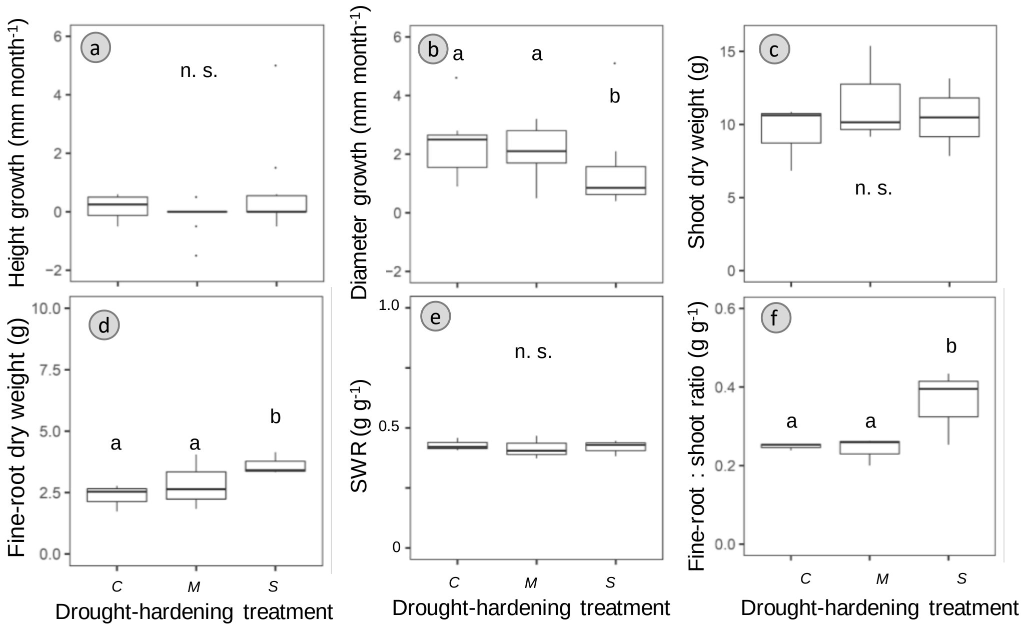

Figure 4. Height (**a**) and diameter growth (**b**) in basal stem region, shoot (**c**) and fine-root biomass (**d**), the ratio of shoot dry weight to whole-plant dry weight (SWR; **e**), and the ratio of fine-root dry weight to shoot dry weight (fine-root:shoot ratio; **f**) were compared between drought-hardening treatment [control (*C*), mild drought (*M*), and severe drought (*S*)] during drought hardening. Boxplots show the median (bar), interquartile range (box), 5th and 95th percentiles (whiskers), and outliers (points) (n of height and diameter growth were 12. n of shoot and fine-root biomass, SWR, and fine-root:shoot ratio were 3). For statistical analysis in the relationships between different drought-hardening treatments and the morphological traits in *C. japonica* seedlings, we developed generalized liner mixed models (GLMM) and then we selected the best fit model between these models based on AIC (Akaike Information Criterion) values. Different letters indicate statistically significant differences (see methods for derails of statistical analysis). "n.s." means no statistically significant differences.

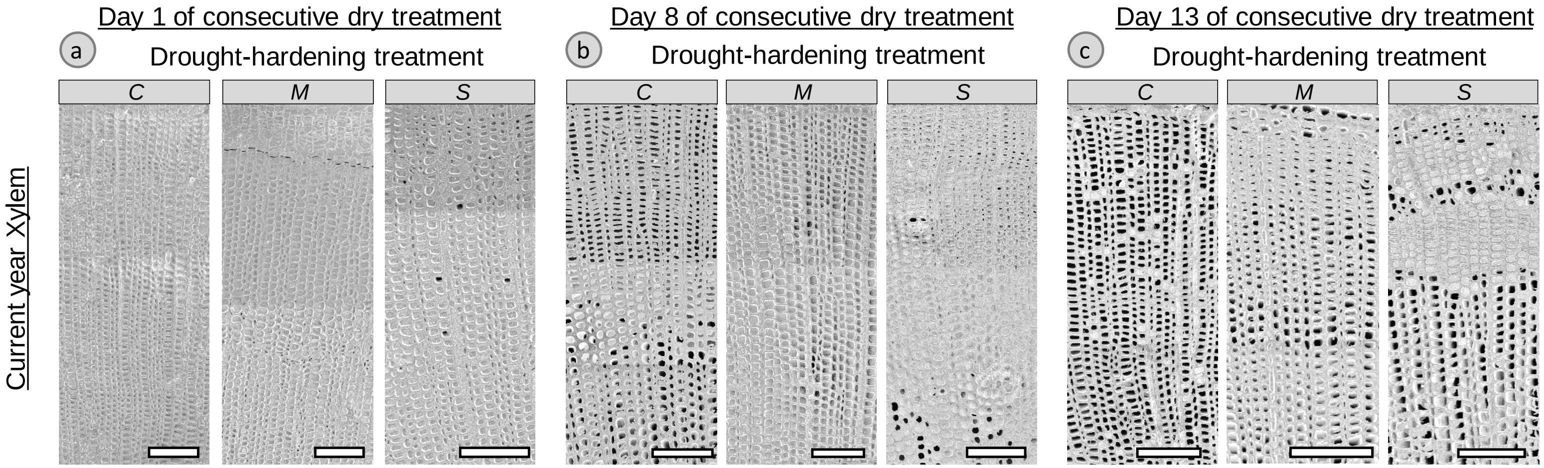

Figure 5. Cross-sections of xylem in basal stem region of seedlings of *Cryptomeria japonica* observed with cryo-SEM at the day 1 (**a**), 8 (**b**), 13 (**c**) of consecutive dry treatment. Seedlings were exposed to three different drought-hardening treatments [control (*C*), mild drought (*M*), and severe drought (*S*)]. Notice differences in proportions of embolized tracheids between individual treatments, which are characterized by dark lumina. Scale bars indicate 100 μm.

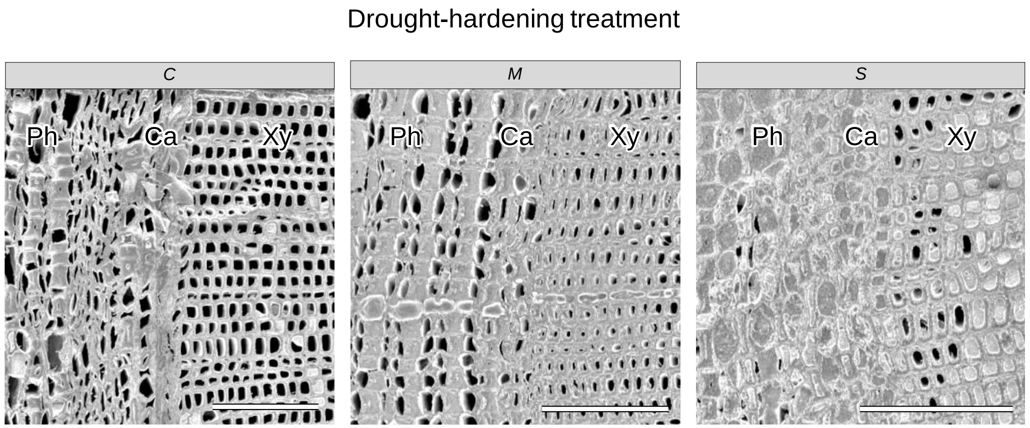

Figure 6. Cross-sections of cambium in basal stem region of seedlings of *Cryptomeria japonica* observed with cryo-SEM at day 13 of consecutive dry treatment. Seedlings were exposed to three different drought-hardening treatments [control (*C*), mild drought (*M*), and severe drought (*S*)]. Abbreviations: Ca = cambium; Ph = secondary phloem; Xy = xylem. Scale bars indicate 100 μm.

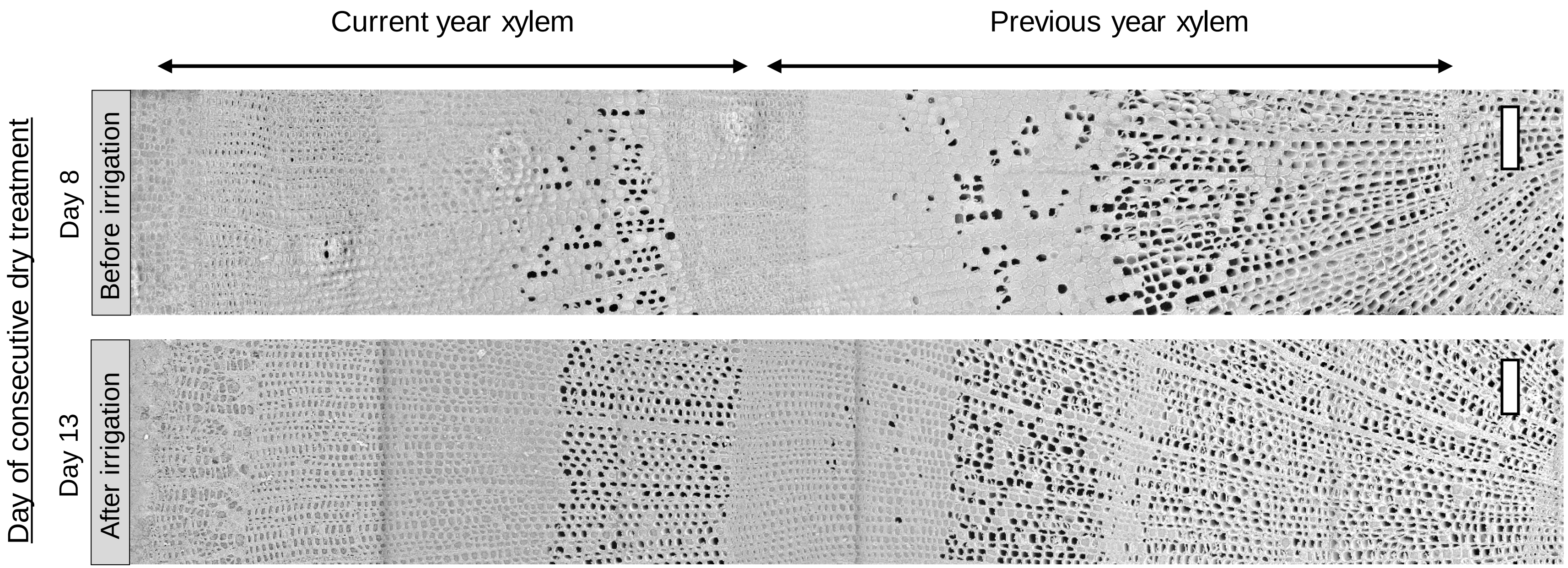

Figure 7. Cryo-SEM images of the transverse surface of the basal stem xylems were observed before (day 8 of consecutive dry treatment; upper panel) and after irrigation (day 13 of consecutive dry treatment; lower panel) during consecutive dry treatment. Prior to the consecutive dry treatment, the seedlings were exposed to the severe drought-hardening treatment (*S*). Notice embolized tracheids are characterized by dark lumina. Scale bars indicate 100 μm.

4. Discussion

One month of drought hardening enhanced water conservation under consecutive dry treatment without inducing leaf shedding in *C. japonica* seedlings. Mild and severe drought hardening (*M* and *S* treatment, respectively) promoted conserved water use through a decrease in whole-plant transpiration (E_{whole}) (physiological response) even after full irrigation during drought -hardening treatment (Figure 2e). This tendency was particularly evident under *S* treatment, whereas E_{whole} for the *C* and *M* treatments on days 1 and 16 of the drought-hardening treatment could be underestimated because shoot biomass may increase, considering the results for diameter growth (Figure 4b). Therefore, our methodological approach needs to be interpreted with attention regarding the normalization of E_{whole}. In addition, *S* treatment promoted a shift in carbon allocation from diameter growth of the basal stem region to fine-root growth (morphological response) (Figure 4b,d,f). These physiological and morphological responses to drought hardening probably contributed to the maintenance of both E_{whole} (Figure 3) and proportions of non-embolized xylem (Figure 5b,c) during consecutive dry treatment. E_{whole} and proportions of non-embolized xylem were maintained longer under *S* treatment than *M* treatment (Figures 3 and 5). Considering that the seedlings were subjected to the drought-hardening treatment after the cessation of xylem development, the maintenance of E_{whole} and proportions of non-embolized xylem during consecutive dry treatment may reflect conserved water use rather than the properties of xylem such as pit ultrastructure. Therefore, our results suggest that the effect of drought hardening on physiological and morphological traits depends on the frequency of irrigation of drought hardening.

The decrease in E_{whole} as a result of drought hardening was inconsistent with the results of a previous study of acclimation to drought in 1.5-month-old *C. japonica* seedlings [21]. Nagakura et al. [21] found that the whole-plant transpiration rate remains unchanged, compared with control seedlings, following acclimation to dry conditions (drought hardening) for 12 weeks. In this previous study, watering was performed when the soil water content reached 0.30 m^3 m^{-3}, similar to the drought hardening *M* treatment in our study (Figure 1). We used three-year-old container-grown seedlings, and E_{whole} under the *M* treatment was already lower than that under the *C* treatment on day 16 of drought hardening, even after full irrigation (Figure 2e). Therefore, the effect of drought hardening on conserved water showed a decrease in E_{whole}, which may depend on the age and/or size of the seedling.

Our findings also suggest that our drought-hardening treatment has the potential to shorten the duration of drought hardening prior to planting for afforestation. Nagakura et al. [20] found that 19.5 weeks of drought hardening, which was 15.5 weeks longer than that in our study, decreased E_{whole} in *C. japonica* seedlings. Additionally, for seedlings of Douglas fir, lodgepole pine, and white spruce, the duration of drought hardening is about 10 weeks [7], which is six weeks longer than in our study. The methodological differences between these previous studies and our study, included differences in the tree species and tree body size, pot size, irrigation interval, and soil type, which are related to the soil water holding capacity. Although, the age and size of *C. japonica* seedlings, soil types, and tree species considered in our study were limited, our drought-hardening treatment is probably applicable to seedlings up to the standard for height and basal stem diameter in *C. japonica* for afforestation in Japan.

Maintenance of xylem transport capacity is important for survival under consecutive drought in seedlings [28]. Embolized tracheids in the xylem are related to hydraulic failure, which leads to rapid or gradual dysfunction of both physiological and metabolic traits, including decreases in photosynthesis and respiration [29–31], which in turn leads to death if xylem embolism is not repaired. The repair of xylem embolism has been explained by both refilling of the xylem, which results in short-term repair (within hours or days) [32], and regrowth of new xylem, which provides long-term repair [33]. We did not detect the refilling of embolized tracheids five days after full irrigation during consecutive dry treatment in *C. japonica* seedlings (Figure 7), which is consistent with a previous study in the *Cupressaceae* family [34]. These findings suggest that species in the *Cupressaceae* family, including *C. japonica*, need to produce new xylem to recover xylem transport capacity after drought-induced hydraulic failure, due to an inability to refill their embolized tracheids. Moreover, Abe et al. [34] found that both the number of tracheids and mean radial diameter of tracheids decreased due to cambium cell distortion under water deficits. This report suggests that maintaining the cambium status is important for survival under drought conditions [33]. Our data showed that drought hardening contributes to the maintenance of the cambium status (Figure 6), and this could be caused by a reduction in E_{whole} (Figure 3). Therefore, drought hardening contributes to the maintenance of cell differentiation related to the production of hydraulically functional xylem, thereby increasing drought tolerance under prolonged drought.

5. Conclusions

Drought hardening promoted water use conservation, which is related to the maintenance of xylem transport capacity and the cambium status during consecutive dry treatment. This is expected to increase the tolerance and survival of *C. japonica* seedlings under prolonged drought. Our results suggest that the effect of drought hardening on water use depends on stomatal regulation under drought conditions, but we cannot exclude impacts of other factors, such as modifications in root morphology (fine roots and root hairs), aquaporin expressions (both in roots and leaves), osmotic adjustment, etc. As our findings are based only on *C. japonica* seedlings, and are focused on stomatal regulation, we will examine the efficacy of drought hardening with a focus on variation in stomatal control and other factors, such as aquaporin expressions, under drought conditions in a wider range of tree species (including isohydric and anisohydric tree species) [35] to validate the generalizability of our findings.

Due to the high genetic divergence between elite trees of *C. japonica* in Japan, differences in the physiological and morphological responses to environmental conditions associated with genetic variation may limit the scope of our findings. Hiraoka et al. [36] reported that annual height increments differ among 12 genotypes of *C. japonica* in a common garden, suggesting that the efficacy of drought hardening is influenced by genotype. Further studies, focusing on the roles of genetic factors, are needed to establish effective methods for drought hardening in different genotypes in *C. japonica*.

Oguchi and Fujibe [6] projected that consecutive dry days increase by around 1.0 day per year over the wide area of Japan. Moreover, the average length of consecutive dry days is around 20 days, with annual fluctuations [6]. These projections indicate that seedlings for afforestation are expected to be exposed to unexperienced severe drought in the future. We showed that the proportions of non-embolized xylem and the phloem status, under the *S* treatment, were maintained for 13 days without irrigation, which is at least five days longer the duration for the *C* and *M* treatments (Figures 5 and 6, respectively). However, we only examined the effectiveness of different levels of drought hardening before planting in *C. japonica* seedlings. Therefore, we intend to examine the effect of drought hardening on drought tolerance using planted seedlings after drought hardening for validation of our findings.

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

Table A1. AIC (Akaike Information Criterion) values of all generalized liner mixed models (GLMM) in gas exchange traits in shoot. In these models, the response variables are leaf gas exchange (A, g_s, E, m, and E_{whole}) (numeric class), the explanatory variable is the different irrigation frequency during drought hardening (factor class) and random effect is the holder of 200-cc single-cavity pots used in the phytotron chamber. In the column of selected model and response variables, *C*, *M*, and *S* means *C* treatment, *M* treatment, and *S* treatment during drought hardening. Gray colored cells indicate the best fit models based on AIC values.

Gas Exchange Traits	Unit	Measurement Day	Model of Grouping	AIC
A	μmo m^{-2} s^{-1}	1	Null	48.69
			(C + M) (S)	50.40
			(C + S) (M)	50.15
			(C) (M + S)	50.65
			(C) (M) (S)	52.11
		16	Null	52.56
			(C + M) (S)	49.26
			(C + S) (M)	51.37
			(C) (M + S)	54.52
			(C) (M) (S)	48.64
		31	Null	48.29
			(C + M) (S)	43.60
			(C + S) (M)	49.29
			(C) (M + S)	49.56
			(C) (M) (S)	45.58
g_s	mol m^{-2} s^{-1}	1	Null	−46.41
			(C + M) (S)	−44.65
			(C + S) (M)	−44.83
			(C) (M + S)	−44.44
			(C) (M) (S)	−42.87
		16	Null	−66.30
			(C + M) (S)	−71.10
			(C + S) (M)	−67.29
			(C) (M + S)	−64.55
			(C) (M) (S)	−70.62
		31	Null	−58.47
			(C + M) (S)	−62.74
			(C + S) (M)	−57.52
			(C) (M + S)	−57.16
			(C) (M) (S)	−60.77
E	mmol m^{-2} s^{-1}	1	Null	24.86
			(C + M) (S)	25.10
			(C + S) (M)	22.56
			(C) (M + S)	26.57
			(C) (M) (S)	24.25
		16	Null	5.54
			(C + M) (S)	3.95
			(C + S) (M)	2.73
			(C) (M + S)	7.53
			(C) (M) (S)	0.23
		31	Null	7.97
			(C + M) (S)	4.03
			(C + S) (M)	9.90
			(C) (M + S)	7.14
			(C) (M) (S)	3.44

Table A1. *Cont.*

Gas Exchange Traits	Unit	Measurement Day	Model of Grouping	AIC
E_{whole}	g g^{-1}	1	Null	107.61
			(*C* + *M*) (*S*)	103.64
			(*C* + *S*) (*M*)	109.42
			(*C*) (*M* + *S*)	107.47
			(*C*) (*M*) (*S*)	104.87
		16	Null	95.32
			(*C* + *M*) (*S*)	88.76
			(*C* + *S*) (*M*)	97.07
			(*C*) (*M* + *S*)	95.36
			(*C*) (*M*) (*S*)	88.18
		31	Null	77.81
			(*C* + *M*) (*S*)	73.01
			(*C* + *S*) (*M*)	79.69
			(*C*) (*M* + *S*)	77.30
			(*C*) (*M*) (*S*)	70.08
m	mmol m^{-2} s^{-1} ln(kPa)$^{-1}$	1	Null	−55.75
			(*C* + *M*) (*S*)	−54.56
			(*C* + *S*) (*M*)	−56.26
			(*C*) (*M* + *S*)	−54.14
			(*C*) (*M*) (*S*)	−54.29
		16	Null	−62.46
			(*C* + *M*) (*S*)	−64.06
			(*C* + *S*) (*M*)	−62.90
			(*C*) (*M* + *S*)	−62.78
			(*C*) (*M*) (*S*)	−62.78
		31	Null	−46.02
			(*C* + *M*) (*S*)	−53.62
			(*C* + *S*) (*M*)	−53.27
			(*C*) (*M* + *S*)	−51.67
			(*C*) (*M*) (*S*)	−52.11

Table A2. The results of one-way repeated measures ANOVA for the effect of day within consecutive day on E_{whole}. *C*, *M*, and *S* means *C* treatment, *M* treatment, and *S* treatment during drought-hardening treatments.

Drought-Hardening Treatment	df	*F* Values	*p* Values
C	11	3.389	<0.001
M	11	6.798	<0.001
S	11	3.942	<0.001

Table A3. AIC (Akaike Information Criterion) values of all generalized liner mixed models (GLMM) in growth, shoot and fine-root dry weight, the ratio of shoot dry weight to whole-plant dry weight (SWR), and the ratio of fine-root dry weight to shoot dry weight (fine-root:shoot ratio). In these models, the response variables are growth traits (the rate of height and basal diameter, shoot and fine-root dry weight, SWR, and fine-root:shoot ratio) (numeric class), the explanatory variable is the different irrigation frequency during drought hardening (factor class) and the random effect is the holder of 200-cc single-cavity pots used in the phytotron chamber. In the column of selected model and response variables, *C*, *M*, and *S* means *C* treatment, *M* treatment, and *S* treatment during drought-hardening treatments. Gray colored cells indicate the best fit models based on AIC values.

Morphological Traits	Unit	Model of Grouping	AIC
Height growth	mm month^{-1}	Null	82.71
		(*C* + *M*) (*S*)	82.73
		(*C* + *S*) (*M*)	83.61
		(*C*) (*M* + *S*)	84.53
		(*C*) (*M*) (*S*)	84.62

Table A3. *Cont.*

Morphological Traits	Unit	Model of Grouping	AIC
Diameter growth	mm month^{-1}	Null	92.39
		$(C + M)$ (S)	90.09
		$(C + S)$ (M)	94.23
		(C) $(M + S)$	92.17
		(C) (M) (S)	91.15
Shoot dry weight	g	Null	47.54
		$(C + M)$ (S)	49.54
		$(C + S)$ (M)	48.63
		(C) $(M + S)$	48.65
		(C) (M) (S)	50.32
Fine-root dry weight	g	Null	27.91
		$(C + M)$ (S)	25.91
		$(C + S)$ (M)	29.83
		(C) $(M + S)$	27.18
		(C) (M) (S)	27.07
SWR	g g^{-1}	Null	−31.19
		$(C + M)$ (S)	−29.21
		$(C + S)$ (M)	−29.34
		(C) $(M + S)$	−29.47
		(C) (M) (S)	−27.49
Fine-root: shoot	g g^{-1}	Null	−15.61
		$(C + M)$ (S)	−21.03
		$(C + S)$ (M)	−15.30
		(C) $(M + S)$	−14.69
		(C) (M) (S)	−19.07

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