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Evaluating of Drought Tolerance of Four Afforestation Species

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Abstract

Background and Objective: Drought is a major abiotic stressor that limits plant growth and productivity, and it is very important to select plant species that are resistant to drought. However, the measurement and selection of drought-tolerant species and/or varieties relied on data obtained from field trials, which are difficult to obtain accurate data because there are many unpredictable factors such as soil moisture and physiological conditions. Therefore, a more accurate and scientific method of measuring dry resistance was needed. In this study, four afforestation species were done to accurately assess drought resistance based on physiological and biochemical indicators.

Methodology: Four species of three-year-old seedlings were planted in pots and allowed to acclimate for three months. After drying, the water resistance was measured. First, we examined Days of resistance to drought of stem (DRDS) and days of resistance to drought of leaf (DRDL), Relative water content (RWC) and relative water loss (RWL). Second, the contents of biochemicals such as proline and soluble reducing sugar were investigated. Finally, the degree of death of one cell was measured by Evan's blue staining assay, and recovery after drying was measured by DAB staining.

Result: DRDS and DRDL were different for each species. DRDS and RWC were the highest in *Q. acutissima* and the lowest in *J. regia* and *C. obtusa*. Proline and reducing sugar contents increased after irrigation stopping. Cell death was observed 4 weeks after irrigation stopping, and severe cell death was observed in *J. regia* and *F. rhynchophylla*. *Q. acutissima* and *C. obutusa* recovered quickly after drying.

Conclusion: DRDS and DRDL, RWL and RWC measurement method is good for using drought stress measurement method. As a result, *Q. acutissima* and *C. obtusa* were found to have high drying resistance, while *J. regia* and *F. rhynchophylla* had poor drying resistance. The analysis of the content of proline and reducing sugar can be used to clarify the drying resistance assay. Also, cell level assays can be a very good method for determining resistance to dryness. Visualizing stressed plants is very effective in measuring various inanimate stress tolerances. DAB staining, which detects the most important ROS chemically, is also a useful method for measuring the recovery of drought stressed plants.

Keywords: Afforestation Species; DAB Staining; Drought Tolerance Assessment; Evan`S Blue

Introduction

Among forest species, it was not known which species are suitable for afforestation due to the difficulty of selection. So far, the idea of the right tree on the right site has been the main strategy for efficient afforestation. In order to make sure the strategy, test plantings have been carried out for various species to monitor their growth feature at the afforest site. The prospective afforestation species are determined in consideration of the location conditions (environment, soil and meteorological factors) of the stands [1,2]. However, these test planting is often on the risk of adaptation failure at the test site due to unexpected climate event because these are time-consuming works. In order to increase the efficiency

of afforestation, it is demanded that the idea of the right tree on the right site is implemented not only practical test plantings but also pre-screening for promising species using physiological research data. However, physiological studies of the promising species for afforestation are rarely reported on environmental factors.

Drought stress is one of major inhibition factors for plant growth and productivity, and the forest plants are not the exception. Climate change is considered as cause of drought stress currently even in forests [3]. In Korea peninsula, drought effects on forests has been reported in Gangwon region, Korea [4]. Tree mortality, as an evidence of climate change effects, in natural habitats of *Abies koreana* at Hala Mt., Jeju island, Korea, has been reported [5]. Water stress is a main environmental factor for afforestation in Korea due to annual precipitation cycle. The precipitation in Korea peninsula is concentrated in summer. Thus, plants are often suffered from drought stress in other seasons. Due to this climate feature, a temporary lack of soil moisture often occurs in spring and autumn when transpiration of plant including afforestation species is active [6].

Hinoki cypress (*Chamaecyparis obtusa* S. et Z.) is introduced into Korea from Japan around 1904, mainly in coastal areas in southern part and Jeju island, Korea. In recent years, it has been attracting attention in the field of forest recreation and healing by using unique fragrance and materials besides the use of wood [7].

Ash tree (***Fraxinus rhynchophylla* Hance**) is a deciduous broad-leaved arboreous tree. It is native to all parts of Korea and is distributed widely at an altitude of 100 ~ 1600m. Wood is a species that is hard enough to be used in making furniture, dressing, and traditional crafts, and is a high number of woody species [7].

Walnut tree (*Juglans regia* L.) are deciduous broad-leaved arboreous trees, growing at an average annual temperature of 12°C, and are cultivated from the central part of the temperate to the temperate middle-part. Fruit is used for food, preservation, medicines, etc. Wood is used for furniture and crafts, bark for dyes, medicines [7].

Sawtooth oak (*Quercus acutissima* Carruthers) Oak tree is broad-leaved tree, which accounts for about 8% of the forest area in Korea. It is distributed over a wide area from Jeju Island to South Hamgyeong Province. The forested area is 50 ~ 500m above sea level, and there is a great demand for soil nutrients and a large part of the mountainous area where the effective soil depth is deep. The fruit has been used since ancient times as a kind of horticultural crop, and the wood is used as furniture, highland branches, charcoal, wood vinegar and so on [7].

Drought is the major abiotic stress factors for plants which limits plant growth and productivity [8,9]. In the field of plant breeding, identifying drought-resistant and sensitive genotypes is one of the most important steps to improve drought tolerance [10]. This is because the degree of tolerance of each plant to water defects varies with species although almost plant species have defense mechanisms [11].

Moreover, the development of drought plant species or variety is one of the most important for preparing global warming and water shortages [12]. Although there is no consensus on the availability of water resource-related parameters such as drought tolerance selection criteria [13], the selection criteria related to plant productivity and high heritability under drought stress required to be identified for simple and highly accurate measurement method in large populations.

Plant growth and productivity decreases by drought are caused because changes in plant moisture relationships, decreased CO_2 assimilation, cellular oxidative stress, membrane damage in affected tissues, and in some cases inhibition of enzyme activity. Plants respond to drought stress using the following mechanisms: (1) drought escape, (2) drought avoidance (3) resistance to drought by controlling osmotic pressure and increasing cell wall elasticity; (4) resistance to drought through changes in metabolic changes such as increased antioxidant metabolism. Plants can use the mechanisms mentioned above in response to drought stresses either continuously or simultaneously.

Even though previous studies on measurement and selection of drought tolerant species and/or varieties are often supported by only data obtained from field trials, tests for drought tolerance are required under the controlled condition for accuracy. This is because there are many unpredictable factors in the soil moisture and physiological condition, which makes it difficult to obtain accurate data. It is essential to choose a method that can be evaluated at the cellular level as well as external evaluation. Recent studies have examined the factors that determine the degree of drought tolerance among Korean native plants. Chlorophyll content, relative water content, relative water loss, proline and reducing sugar content were effective to measure and selection of drought tolerant *Asteraceae* [14]. Assessment of tolerance to inanimate stress should be simple and reproducible. In this study, four afforestation species were done to accurately assess drought resistance based on physiological and biochemical indicators.

Materials and Methods

Plant Materials

The species used for experiments were the species recommended by the Korea Forest Service for planting. Timber species were *Chamaecyparis obtusa*, *Quercus acutissima* and *Fraxinus rhynchophylla*. The *Juglone regia* was selected as a representative species of Fruit trees. These plants were collected from Gyeongsangnam-do Forest Environment Research Institute, Korea. Plant identification followed the National Arboretum Standard [15].

For test accuracy, 3-year-old seedlings were used, they were planted in same size pots (top diameter: 16 cm, bottom diameter: 11 cm, height: 17 cm) containing soil composed with peat moss, pearlite and vermiculite (1:1:1, v/v/v). Then the seedlings were acclimated for 3 months in the growth chamber under a photoperiod of 16 hrs illumination with a light intensity of 25 µmol $m^{-2}s^{-1}$and 8hr dark at 25±1 ˚C. The similar size seedlings were collected to avoid experimental errors and used for further experiments.

Resistant Test Against Drought Stress

For drought stress treatment, watering on the four species of seedlings was stopped. Days of Resistance to Drought of Stem (DRDS) and Days of Resistance to Drought of Leaf (DRDL) under drought condition were counted. Following parameter were recorded for all experiments: endurance days after drought stress affects(T_i), numbers of stem withered plants on day(N_{Si}), number of leaf withered plants on day(N_{Li}) and all number of withered plants (N) were used to calculate Resistant day of stem (DRDS, day) and Resistant day of leaf (DRDL, day) by applying the following formula. The experiment was repeated 5 times.

$$\text{Days of Resistance to Drought of Stem (DRDS, day)} = \sum(T_i N_{Si})/N \cdots \text{Formula 1}$$

$$\text{Days of Resistance to Drought of Leaf (DRDL, day)} = \sum(T_i N_{Li})/N \cdots \text{Formula 2}$$

RWC was calculated by measuring the fresh weight, saturation, and dry weight of the leaves in 7 days' intervals. RWL was measured by weighing the fresh weight of the plant leaves at 7-day intervals, pre-drying at 35 ˚C for 4 hours and then drying in a dryer at 72 ˚C (W4h). RWC and RWL were also surveyed on 10 individuals.

Determination of Biochemical Substances by Applying Dry Stress

Biochemical assays for the application of dry stress were analyzed for proline and reducing sugars. Proline contents were measured by following the method described by Bates, et al. [16]. Fresh weight (0.05g) of leaf tissues were extracted mixed with 5 mL aqueous sulfosalicylic acid(3%w/v). The extracted solution was reacted with an equal volume of glacial acetic acid and ninhydrin reagent and incubated at 100 ˚C for 1hr. The reaction mixture was vigorously mixed with 2mL toluene after in an ice bath. The chromophore was measured by spectrophotometer DR/4000 at 520 nm. The concentration of proline was calculated from a calibration curve plotted with a known concentration of L-proline as a standard with "Y=0.6998x–0.6441(R^2=0.9815)".

The soluble reducing sugar was assessed by the DNS (3,5-dinitrosalicylic acid) method [17]. Fresh weight(0.1g) of leaf tissues were extracted 5ml distilled water at 100 ˚C for 30 min. The extracted solution reacted with 1.0ml DNS (3.5-dinitrosalicylicacid) were mixed and then treated in boiling water for 5min, and absorbance was measured by spectrometer DR/4000 at 546 nm. Various concentration (50, 100, 150 and 200 µg/ml) of glucose was measured for calibration curve with "Y=0.2643x-0.254 (R^2=0.9893)".

Measurement of The Degree of Cell Death by Evan's Blue Staining Assay

Sample leaves (1cm^2) experimented in each condition (ordinary and cold temperature) were incubated with Evan's blue solution (1%, w/v) in for a day. As the positive controls, leaf samples collected from each plant species were boiled water for 1 hour and stained in the same condition described above. Then, sample leaves were extensively washed with distilled water to remove unbounded Evan's blue dye. Photographs of the sample leaves were taken under the microscope (BH-2, Olympus, Japan) with 200 magnifications. To assess dead cells, the stained dye in leaves were dissolved using 2 ml 50% ethanol solution containing 2% SDS. The absorbance values of dissolved dye from the leaves were measured at 500 nm. The inhibition indexes were obtained by below formula (3). As a positive control, plant leaves were heated at a temperature of 70 ˚C or higher for 1 hour and then stained. The negative control stained leaves of plants grown at 25 ˚C without drying treatment.

$$\text{Inhibition index} = \frac{Abs^{H} - Abs^{NC}}{Abs^{PC} - Abs^{NC}} \times 100\% \frac{Abs^{H} - Abs^{NC}}{Abs^{PC} - Abs^{NC}} \times 100\% \cdots \text{Formula 3}$$

Abs^{H}: Absorbance value of the drought-stressed sample, Abs^{NC}: Absorbance value of negative control, Abs^{PC}: Absorbance value of positive control

DAB Staining Analysis for Measuring Recovery of Plants Subjected to Drying

The fresh 3,3′-Diaminobenzidine (DAB) solution was prepared by following the protocol [18]. Sample leaves were collected from each plant species incubated in each normal conditions or drought treatments. DAB solution is taken up into the sample leaves by gently vacuum infiltration for 10 min. Then, sample leaves were placed on a shaker for 4 hrs. at 100 rpm. DAB solution was replaced with bleaching solution (ethanol: acetic acid: glycerol =3: 1: 1), and then sample leave was boiled at 95 ˚C for 30 min to remove chlorophyll. Photographs of the bleached sample leaves were taken under the microscope with 200 magnifications.

Statistical Analysis

The experiments were conducted for three times with repetitive results. Data were subjected to statistical analysis by using the SPSS software. One-Way Analysis of Variance (ANOVA) was conducted, and means were compared using Duncan's Multiple-Range Test (DMRT) at 0.05 level of probability. Values were represented as the mean ± Standard Deviation (SD).

Results

The External Response of the Plant to Drought Stress

DRDS and DRDL of four species under drought stress were varied greatly from species to species (Table 1). The longest DRDS was shown in *Q. Acutissima* (53 days), and the second was observed in *C. obtusa* (47 days). However, DRDS of *F. rhynchophylla* was only 20 days, which is the shortest duration. Similar results were observed in DRDL. The each DRDL of *Q. acutissima* and *C. obtusa* were 40 days. The DRDL of *F. rhynchophylla* and *J. regia* were 18 days and 14 days, respectively. These results implied that *Q. acutissima* and *C. obtuse* are more tolerant against drouth stress than *F. rhynchophylla* and *J. regia*.

Species	Days of Resistance to Drought of Stem (DRDS, day)	Days of Resistance to Drought of Leaf (DRDL, day)
C. obtusa	47.6±3.2^{b}	39.9±3.8^{a}
F. rhynchophylla	20.3±0.5^{d}	18.2±2.3^{b}
J. regia	37.1±2.5^{c}	14.0±0.9^{b}
Q. acutissima	53.2±1.8^{a}	40.6±2.3^{a}

Table 1: Days of resistance to drying of stem and leaf of each species by drying.

The withering rates of the seedlings by stopping irrigation varied from each other species although they were increased by the duration of drought stress (Figure 1). The seedlings of *J. regia* shown withered phenotypic response in only seven days from stopping irrigation, which was the earliest. The seedlings of *F. rhynchophylla* were begun to wither by 12 days, while the withered phenotypic responses of *C. obtusa*, and *Q. acutissima* were shown in 21 days. However, 70 days after stopping the irrigation, no seedlings of every species were survived.

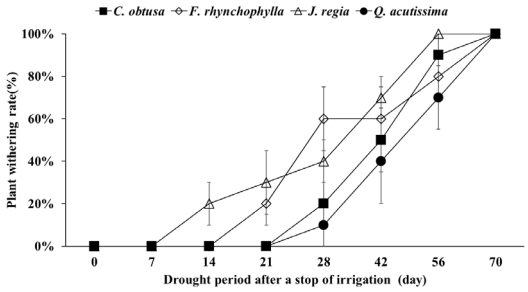

Figure 1: The rate of mortality of four afforestation species according to the period of irrigation stops. The rate of mortality counts the percentage of individuals whose stem has died from among 10 individuals.

RWC and RWL of Four Plant Species Under Drought Stress

It was shown that the RWC of leaves decreased in all plants depending on the days of drying (Figure 2). In the case of *Q. acutissima*, the decrease of RWC was much lower than that of other species. On the other hand, the RWC of *J. regia* and *C. obtusa* trees decreased significantly. Especially, *J. regia* decreased from 88.78% to 27.04% at 4th week.

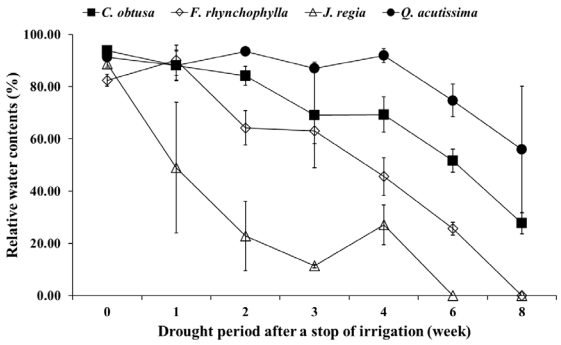

Figure 2: The RWC of four afforestation species by irrigation stop.

As a result of RWC measurements, the RWL of each plant leaves tended to increase by the duration of drought stress (Figure 3). Water loss was abruptly observed from 1 week after the irrigation was stopped, and then the loss of water was shown gradually. *F. rhynchophylla* showed the highest water loss until 6 weeks from irrigation stops, and *J. regia* showed more water loss after that. The RWL of *Q acutissima* was high until 6 weeks of irrigation stops, but it was the lowest after 8 weeks.

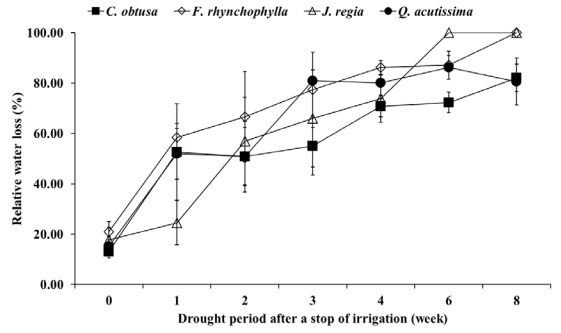

Figure 3: The RWL content of four afforestation species by irrigation stop.

Determination of Biochemical Substances by Applying Dry Stress

The content of proline in leaves increased after stop of irrigation but showed a large difference according to species (Figure 4). Proline content gradually increased after irrigation stops. The proline content in *C. obtusa* increased rapidly after 3 weeks. The

proline content in *Q. acutissima* was not changed until 6 weeks after the irrigation stops. The proline content of *F. rhynchophylla* remained unchanged after the water supply interruption. The proline content of J. regia began to increase rapidly from one week to four weeks after stopping irrigation, and then sharply decreased thereafter.

Reducing sugar contents were not change significantly, and only slight increment was shown by the duration of drought stress (Figure 5). The reducing sugar contents in *J. regia* and *F. rhynchophylla* tended to increase slightly, one in *C. obtusa* was changed significantly. However, the Reducing sugar content in *Q acutissima* was decreased until 2 weeks from stopping water supply, but it was begun to increase rapidly.

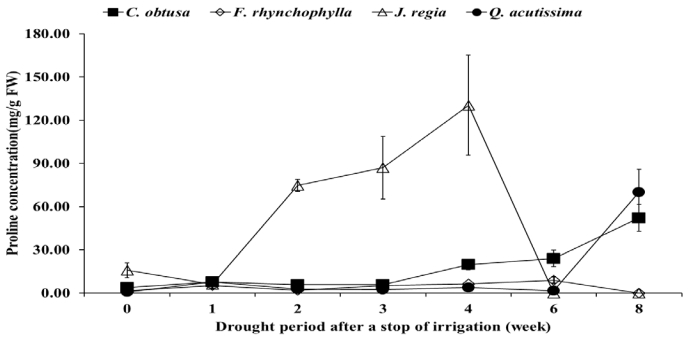

Figure 4: Proline content of four afforestation species with irrigation stopped.

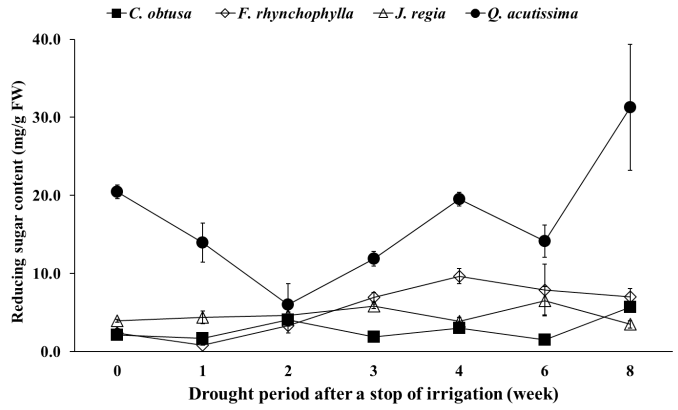

Figure 5: Reducing sugar contents of four afforestation species by irrigation stop.

Observation of Cell Death by Drought Stress

Damage caused by drought stress in the leaves of the four species seedlings was assessed by Evan's blue, a blue-colored reagent deposited on dead cells (Figure 6). By the duration of drought stress, the degree of apoptosis increased. *Q. acutissima* and *C. obtusa* were not significantly affected by drought stress until the 4th week, but dead cells were observed after 6 weeks. On the other hand, in the case of the *J. regia* and *F. rhynchophylla*, the dead cells were found from the 1st week, and the leaves were all lost after the 4th week. Assay results showed a difference of about 13% compared to the negative control group, and the damage to drying stress was high.

Evan's blue inhibition index varied from species to species (Figure 7). The inhibition index increased with increasing drying days. The species with the highest inhibition index was *J. regia* and *Q. acutissima* was the lowest.

Recovery of Plants Against Drought Stress

The recover ability of each species from drought stress was observed by DAB staining (Figure 8). Plants recovered after 2 weeks of irrigation. However, the degree of recovery varied depending on species. H_2O_2 was detected in *F. rhynchophylla* and *J. regia* up to 10 days under recovery condition. However, after 4 days no more H_2O_2 was detected in the *Q. acutissima* and *C. obtusa*. Accordingly, it seems that *Q. acutissima* and *C. obtusa* is relatively fast recovered from drought stress.

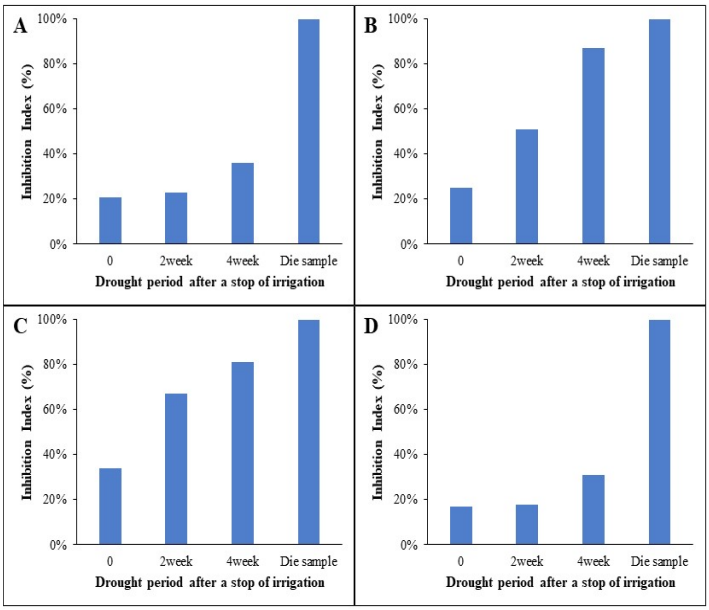

Figure 6: Evan's blue inhibition index in dry stress leaf tissues A: *C. obtusa*, B: *F. rhynchophylla*, C: *J. regia*, and D: *Q. acutissima*.

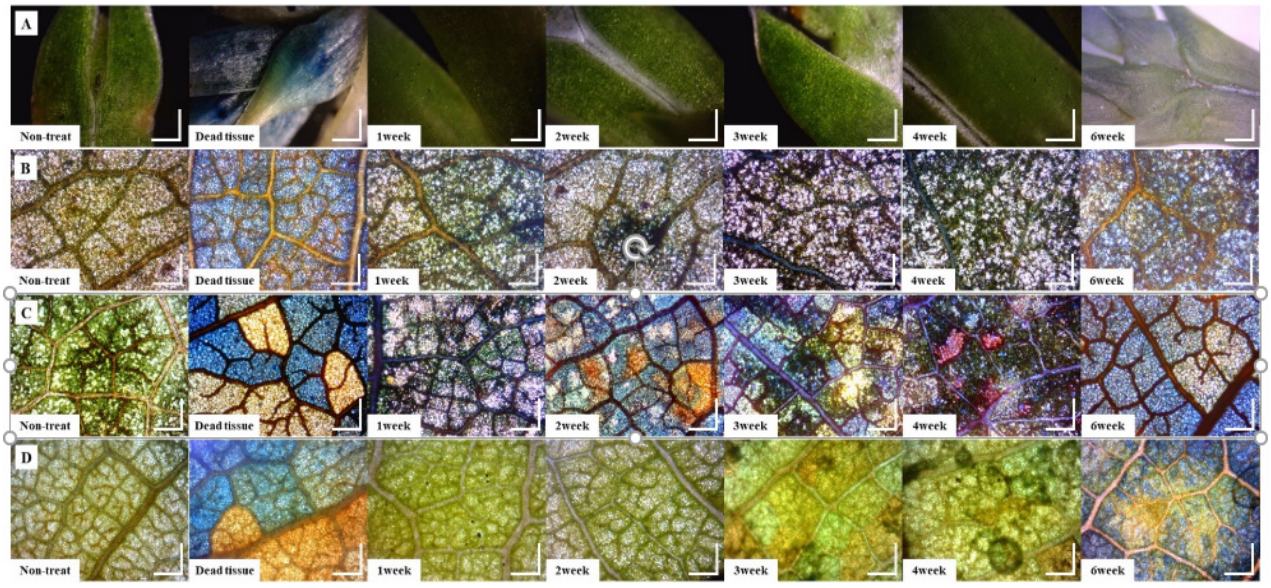

Figure 7: The degree of cell death in the follicles of major plant species treated with dry stress. The sample leaves (1 cm^2) tested under each condition (normal and low temperature) were incubated with Evan's blue solution (1%, w / v) for one day. Leaf samples taken from each plant species are boiled for 1 hour, then stained, and washed with distilled water to remove unlimited Evan blue pigment. Photographs of the sample leaves were taken under a microscope and the dyed dyes of the leaves were lysed using 2 ml of a 50% ethanol solution containing 2% SDS to evaluate dead cells. The absorbance values of the dyes dissolved in the leaves were measured at 500 nm. A: *C. obtusa*, B: *F. rhynchophylla*, C: *J. regia*, and D: *Q. acutissima.* Scale bar: 0.5mm.

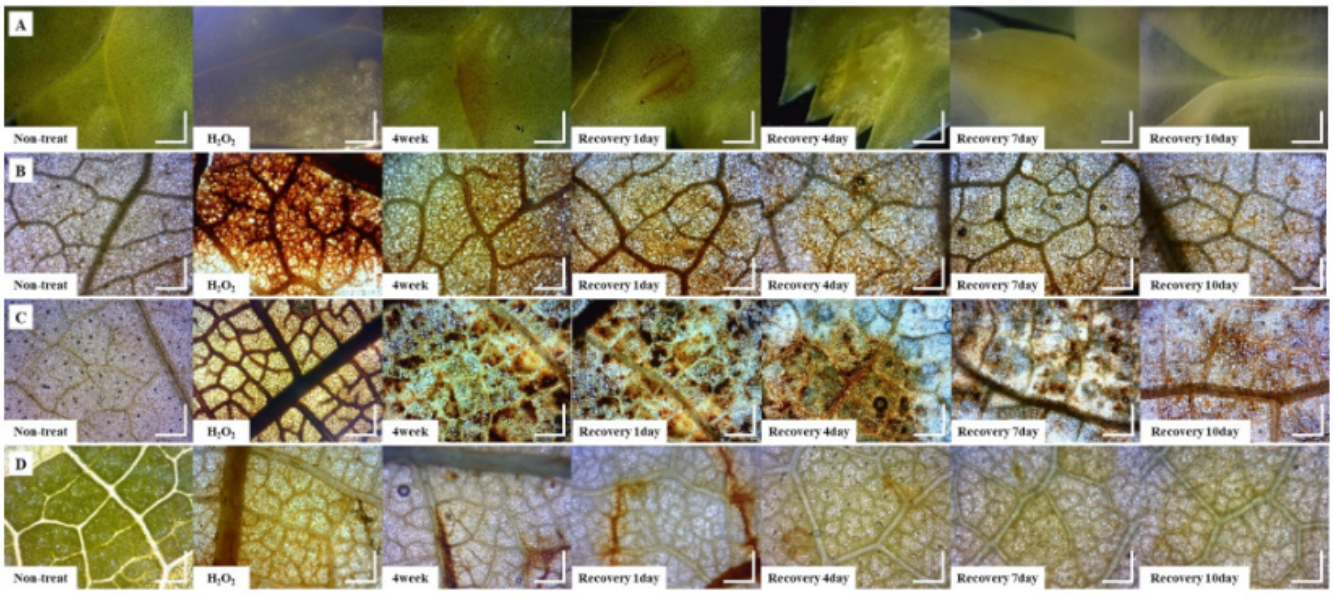

Figure 8: Recovery of different species by re-irrigation after drought stress treatment by DAB staining. The sample leaves were incubated under each normal condition or drought treatment and the DAB solution was gently vacuum infiltrated for 10 minutes to absorb on the sample leaves. After the DAB solution was added, the sample was boiled at 95 ˚C for 30 minutes to remove chlorophyll. Photographs of the bleached sample leaves were taken with a microscope 200 times. A: *C. obtusa*, B: *F. rhynchophylla*, C: *J. regia*, D: *Q. acutissima.* Scale bar: 0.5mm.

Discussion

Water status is an important indicator for plants' healthiness under drought condition. In this study, the DRDS and DRDL of *Q. acutissima* and *C. obtusa* were quite later than those of *J. regia* and *F. rhynchophylla.* These results indicate that *Q. acutissima* and *C. obtusa* are tolerant more than *J. regia* and *F. rhynchophylla.* The supportive evidences were obtained through measurement of RWL and RWC. RWCs of *Q. acutissima* and *C. obtusa* were higher than those of *J. regia* and *F. rhynchophylla*, and RWLs of *Q. acutissima* and *C. obtuse* were less than those of the others. It is suggested that *Q. acutissima* and *C. obtusa* are better for ability of holing water homeostasis under drought stress than than *J. regia* and *F. rhynchophylla.* This reaction can be attributed because thick leaf surface and relatively smaller pore size which allow higher water use efficiency. It is known that the evaporation of oak trees is hardly carried out under drought stress, and this is because leaf because thick leaf surface and relatively smaller pore size [19].

In this study, it was assumed that measuring RWL and RWC is the efficient method for observing ability of holing water homeostasis under drought stress. Higher RWCs and lower RWLs of plants are commonly found under drought stress. Drought-tolerant genotype of wheat showed higher RWCs and lower RWLs under dry conditions [20]. The measuring RWC and/or RWL is a widely-used and non-destructive way to understand the water status of plants under drought conditions [21,22]. These methods can be used for screening other drought-tolerant afforestation species.

Experiments using biological markers provide a clearer picture of plant stress response. Among them, proline, a type of amino acid, is known as a representative substance that responds to dry stress [23]. Plants make proline to prevent stress-induced protein and membrane destruction [24]. In our study, proline content of *J. regia*, which was weak in withering, was increased by drought stress. However, in the case of *Q. acutissima*, there was no change in the content of proline even with drought stress.

Cell level assays can be a very good method for determining resistance to dryness. There are various methods for immediately monitoring the stress of plants through cell-level analysis. Because visualizing damaged tissue can suggest obvious evidence, Evan`s blue staining and assay method was used in this study. Evan's blue dye was usually used to test cell viability in that it stains dead cells and damaged tissues [25]. This staining method visualizes death cells and tissues under severe stress condition [26]. In this study, the leaf segments of the drought-sensitive plants were stained more than those of the drought-tolerant plants. It was confirmed through performing Evan's blue assay. Thus, this method is highly reproducible and can be used for obtaining accurate evidence of physiological responses of plants under drought stress.

Also, the physiological response changes of plants in the recovery phase are important for the select of stress-tolerant plants. Plants increase reactive oxygen species under adverse conditions [27], and the accumulation of ROS causes cell and tissue damaged (Bailey-Serres and Mittler). Because visualizing changes in ROS can directly show the extent of plant recovery, the DAB staining method was used in this study. DAB is oxidized in the presence of some haem-containing proteins, such as peroxidases, to generate a dark brown precipitate, which can be visualized using an optical microscope. These characteristics can be used to observe changes in ROS during the recovery phase. In this study, the leaf segments of the drought-sensitive plants made more ROS and were stained longer period than those of the drought-tolerant plants. Until now, there have been no examples of the stress recovery of plants by DAB staining, and this method can be used to observe the recovery of plant.

Conclusion

In this study, four afforestation species were subjected to various tests to understand their responses under arid conditions. As a result, *Q. acutissima* and *C. obtusa* were found to have high drying resistance, while *J. regia* and *F. rhynchophylla* had poor drying resistance. Between the tolerant and sensitive species, obvious differences were observed. It was shown that the ability for holding water homeostasis of the tolerant species is higher than those of the sensitive species through DRDS and DRDL, RWL and RWC measurement. The analysis of the content of proline and reducing sugar can be used to clarify the drying resistance assay. Evan's blue staining & assay and DAB staining test also suggested supportive evidences their resistances against drought stress. These methods were rarely used for screening drought-tolerant afforestation species. Because the accuracy of these methods, these can be applied to conduct selection breeding for drought-tolerant genotype of afforestation species in the future studies.

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