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Manipulating the Difference between the Day and Night Temperatures Can Enhance the Quality of *Astragalus membranaceus* and *Codonopsis lanceolata* Plug Seedlings

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Abstract: *Astragalus membranaceus* Bunge and *Codonopsis lanceolata* Benth. et Hook. f. are two famous medicinal species in Korea, China, and Japan, mainly used for treating diseases including cancer, obesity, and inflammation. Manipulation of the difference between the day and night temperatures (DIF) is an efficient horticultural practice to regulate the growth and development of vegetables in a glasshouse. However, little research has focused on how the DIF influences the plug seedling quality of medicinal plants. In this study, uniform plug seedlings were cultivated in three environmentally controlled chambers under an average daily temperature of 20 °C with negative (−10 °C), zero, or positive (+10 °C) DIFs, and the same relative humidity (75%), photoperiod (12 h), and light intensity (150 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ photosynthetic photon flux density with white LEDs). The results showed that the DIF had a noticeable effect on the growth, development, and morphology of *A. membranaceus* and *C. lanceolata* plug seedlings. The positive DIF (+10 °C) significantly increased the biomass (shoot, root, and leaf), stem diameter, and Dickson's quality index, indicating an enhanced plug seedling quality. Moreover, the contents of primary and secondary metabolites, including soluble sugar, starch, total phenols and flavonoids, were higher with higher DIFs, where the maximum values were found at 0 °C or +10 °C DIF. Furthermore, the increases in the chlorophyll content and stomatal conductance were obtained in a positive DIF, indicating that a positive DIF was favorable to photosynthesis. An analysis of the gene expression showed that a positive DIF (+10 °C) up-regulated the expression of photosynthetic genes, including *GBSS*, *RBCL*, and *FDX*. In conclusion, the results of this study recommend a positive DIF (+10 °C) for enhancing the quality of *A. membranaceus* and *C. lanceolata* plug seedlings.

Keywords: DIF; gene; medicinal plant; metabolite; photosynthesis; stomatal conductance

1. Introduction

Astragalus membranaceus Bunge and *Codonopsis lanceolata* Benth. et Hook. f. are two medicinal species that are widely distributed in East Asian countries, particularly Korea, China, and Japan. These two species have a long history of being used as medicine to treat many diseases including cancer, obesity, and inflammation in Asian countries [1,2]. In addition to having its medicinal values, *C. lanceolata* has been consumed as a high-class vegetable in Asian countries, especially in ancient Korea [3,4]. In recent years, more and more researchers have shown an increased interest in these two

species, since more and more phytochemicals have been identified and applied in medicine for treating diseases [5,6]. However, most of those works focused on how to separate, extract, and characterize the medicinal compounds in *A. membranaceus* and *C. lanceolata*. Not much information is available on the cultivation of those two species in a glasshouse by manipulating the temperature regime.

Temperature is a dominant and controllable component in protected plant production, which strongly influences the crop growth, yield, and quality [7–9]. Manipulation of the difference between the day and night temperature (DIF) is an efficient horticultural practice to control plant morphology [10–12]. DIF is defined as the day temperature minus the night temperature, which means that if the day temperature is higher than the night temperature, it results in a positive DIF, and the opposite for a negative DIF [13]. Previous studies have reported that negative DIFs could be used for inhibiting stem elongation and increasing yield [11,14,15]. However, the opposite results have also been reported. For instance, Lund, et al. [16] found that a highly positive DIF does not seem to increase the elongation growth of *Hibiscus rosa-sinensis* L. Similarly, Lyngved, et al. [17] reported that the optimal values for biomass growth of *Cyclamen persicum* Mill. were under a positive DIF (+10 °C). It was reported that a negative DIF inhibited the growth and development of young cucumber plants [18]. Moreover, the mechanism of how the DIF affects the growth and morphology of plants is not well known [19]. Studies on the effects of the DIF on medicinal plants are limited especially in *A. membranaceus* and *C. lanceolata* plug seedlings.

Photosynthesis is the most important photochemical reaction that produces food for all organisms, using sunlight, water, and CO₂. Many genes and proteins are involved in this process. In the photosynthetic chain, *ferredoxin* (*FDX*) codes one kind of iron–sulfur proteins, named ferredoxin [20]. In the non-cyclic photophosphorylation, protein is the last electron acceptor and transports them to NADP⁺ [21]. *Ribulose biphosphate carboxylase large chain* (*RBCL*) is the key gene for the CO₂ fixture in the CO₂ assimilation [22]. Ribulose biphosphate carboxylase (*RuBisCO*) catalyzes the reaction of ribulose biphosphate and CO₂ at the initial step of photosynthesis, which determines the photosynthetic rate [23]. *Granule-bound starch synthase* (*GBSS*) is an obligato gene for the synthesis of amylose in starch, which plays a role in building the final structure of amylopectin [24–26]. Previous studies have reported the responses of those gene expressions to the temperature [27–29]. However, few studies have focused on how the DIF affects the expression of those genes in medical plant plug seedlings.

The objectives of this study were to demonstrate how the DIF influences the plug seedling quality of *A. membranaceus* and *C. lanceolata* and to identify an ideal temperature regime for production of high quality plug seedlings. Uniform plug seedlings were cultured under positive (10 °C), zero, and negative (−10 °C) DIFs for four weeks. The growth and morphological responses of plug seedlings to the different DIFs were investigated. The contents of the primary and secondary metabolites were also determined, such as soluble sugar, starch, total phenols and flavonoids. Additionally, the chlorophyll content and stomatal conductance were evaluated. The expression of photosynthesis-related genes including *FDX*, *RBCL*, and *GBSS* were assayed. The data in this study could provide a theoretical and practical basis for enhancing the quality of plug seedlings by manipulating the DIF, and useful information for growers and managers to regulate other medicinal plants.

2. Materials and Methods

2.1. Plant Materials and Treatments

Seeds of *A. membranaceus* and *C. lanceolata* were sown in 200-cell plug trays filled with the Bio Medium (FarmHannong Co. Ltd., Seoul, South Korea). The components of Bio Medium are 20% coir, 60% peat moss, and 20% perlite. After germination, uniform plug seedlings were selected and cultivated in three different environmentally controlled chambers. The average daily temperature in the chambers was 20 °C, with negative (−10 °C), zero, and positive (+10 °C) DIFs: the day/night temperatures were 15/25 °C, 20/20 °C, and 25/15 °C, respectively. Each treatment was set up with the same relative humidity (75%), photoperiod (12 h), and light intensity (150 μmol·m^{−2}·s^{−1} photosynthetic

photon flux density with white LEDs). Before the experiment, the light intensity was adjusted and calibrated using a photo-radiometer (HD2102.1, Delta OHM, Padova, Italy). The experimental design was completely random design with three replications. Each replication included 20 plugs seedlings (total 60 seedlings per treatment). After four weeks of cultivation, some plug seedlings were harvested for measurements of the growth parameters and others were frozen immediately in liquid nitrogen for further analysis. The Dickson's quality index was calculated according to a previous formula [30], which is

$$\text{Dickson's quality index} = \text{Total DW} / ((\text{shoot length} / \text{stem diameter}) + (\text{shoot DW} / \text{root DW})) \quad (1)$$

where "DW" represents the dry weight.

2.2. Contents of Soluble Sugar and Starch

The contents of soluble sugar and starch were determined by the anthrone colorimetric method [31]. In brief, leaf samples (0.2 g) were ground and then extracted in distilled water (14 mL) for 30 min at 100 °C. After a 15-min centrifugation at 3000 rpm, the supernatant was transferred into a new tube for assay of soluble sugar. For measurement of starch, the residue was re-extracted in distilled water mixed with perchloric acid (2 mL, 52%).

The supernatant (0.5 mL) was added to distilled water (1.9 mL) combined with anthrone (0.5 mL, 2%), and concentrated sulfuric acid (5 mL, 98%), followed by 15 min of incubation at 100 °C. The absorbance at 630 nm and 485 nm was recorded using a UV-spectrophotometer (Libra S22, Biochrom Ltd., Cambridge, UK). The calibration curves of soluble sugar and starch were made with standard solutions.

2.3. Contents of Total Phenols and Flavonoids

Total phenols and flavonoids in leaves were extracted with 80% methanol. The contents of total phenols and flavonoids were measured according to previous methods described by Manivannan et al. [32].

2.4. Hydrogen Peroxide Content

To determine the hydrogen peroxide content, leaves (100 mg) were ground and mixed with a TCA extracting solution (0.1%, 1.0 mL). The mixture was centrifuged for 15 min at 12,000 rpm, and then the supernatant was collected. The supernatant (0.3 mL), sodium phosphate buffer (50 mM, 0.5 mL), and potassium iodide (1 M, 0.5 mL) were mixed and then incubated for 30 min in the dark. The absorbance of the mixture was measured and recorded at 395 nm using a UV spectrophotometer (Libra S22, Biochrom Ltd., Cambridge, UK) [31].

2.5. Assessments of the Chlorophyll Content and Stomatal Conductance

The chlorophyll content was measured by using the Plus Chlorophyll Meter (SPAD 502, Konica Minolta Sensing Inc., Osaka, Japan). The stomatal conductance was assessed at 10 am using the Decagon Leaf Porometer SC-1 (Decagon Device Inc., Pullman, WA, USA).

2.6. Gene Expression Analysis

Leaves of *A. membranaceus* and *C. lanceolata* (mixed sample, three biological replications) were ground into powder in liquid nitrogen and homogenized in a lysis buffer under a RNase-free condition. The total RNA was extracted using a commercial extraction kit (iNtRON Biotechnology, Seoul, Republic of Korea), and cDNA was converted from total RNA using the GoScript Reverse Transcription System (Promega, Madison, WI, USA). The gene expression was determined by employing a Rotor-Gene Q detection system (Qiagen, Hilden, Germany), and the value was calculated by using the $2^{-\Delta\Delta Ct}$

method [33]. All primers were designed using Premier 5.0 (Premier Biosoft Inc., Palo Alto, CA, USA) and the primer sequences are presented in Table 1.

Table 1. The primer sequence for gene expression assay using quantitative real-time polymerase chain reaction.

Gene	Forward Primer	Reverse Primer
18S	5'-CTCAACCATAAA CGATGCCGACC-3'	5'-AGTTTCAGCCTTGGCACCATACTCC-3'
AmGBSS	5'-ATAACATAGCGTATCAGGG-3'	5'-CTCGGTCAGATTCTAACACT-3'
AmRBCL	5'-TGGCTGTTCCTATCGTCA-3'	5'-AAGTAATCTCCCTTTCTCCT-3'
β -Actin	5'-CGAGAAGAGCTACGAGCTACCCGATGG-3'	5'-CTCGGTGCTAGGGCAGTGATCTCTTTGCT-3'
ClFDX	5'-CTTCGGCGTTTCTTCGT-3'	5'-CTGCCAAACCCTTGATAACT-3'
CIRBCL	5'-GCTTACCCATTAGACCTTT-3'	5'-GGGACGACCATACTTGTT-3'

Am, *Astragalus membranaceus*; GBSS, granule-bound starch synthase; RBCL, ribulose biphosphate carboxylase large chain; Cl, *Codonopsis lanceolata*; and FDX, ferredoxin. 18S and β -Actin were used as the housekeeping gene in *A. membranaceus* and *C. lanceolata*, respectively.

2.7. Data Collection and Analysis

The data were collected with three individual biological repeats, and are presented as the mean \pm standard error. A one-way analysis of variance (one-way ANOVA) was performed to estimate the differences among treatments, followed by a Duncan multiple range test ($p < 0.05$) using SPSS (Statistical Package for the Social Sciences, version 21; International Business Machine Corp., Armonk, NY, USA). All figures in this study were prepared using the OriginPro software (version 9.0; OriginLab Corp, Northampton, MA, USA.)

3. Results

3.1. Growth, Development, and Morphology

In this study, the DIF had a significant influence on the growth, development, and morphology of *A. membranaceus* and *C. lanceolata* plug seedlings (Figure 1, Tables 2 and 3). As shown in Figure 1, a morphological distinction of plug seedlings was observed with the increase from $-10\text{ }^{\circ}\text{C}$ DIF to $+10\text{ }^{\circ}\text{C}$ DIF. At the negative DIF ($-10\text{ }^{\circ}\text{C}$), plug seedlings were inferior with a low number of roots, curved shoots and few leaves. In comparison, plug seedlings were stronger and more compact with straight shoots and well-developed roots at the positive DIF ($+10\text{ }^{\circ}\text{C}$). As shown in Table 2, the dry weights of the shoot and root in *A. membranaceus* plug seedlings were 34.8 ± 2.7 and 4.3 ± 0.4 mg at $+10\text{ }^{\circ}\text{C}$ DIF, significantly higher than that at the other two DIFs. Similarly, the maximum stem diameter of 0.90 ± 0.02 mm was observed at $+10\text{ }^{\circ}\text{C}$ DIF. Importantly, the $+10\text{ }^{\circ}\text{C}$ DIF also increased the Dickson's quality index (DQI), which was remarkably greater than that in $-10\text{ }^{\circ}\text{C}$ and $0\text{ }^{\circ}\text{C}$ DIF. Similarly in *C. lanceolata* plug seedlings, the $+10\text{ }^{\circ}\text{C}$ DIF remarkably increased the growth parameters, such as shoot and root dry weights (98.9 ± 10.3 and 20.5 ± 2.2 mg), leaf area ($8.5 \pm 0.5\text{ cm}^{-2}$), stem diameter (2.06 ± 0.07 mm), and DQI ($14.2 \pm 0.8 \times 10^{-4}$), in comparison to those at the $-10\text{ }^{\circ}\text{C}$ DIF (Table 3).

3.2. Contents of Soluble Sugar and Starch

As shown in Figure 2A,B, increases in the soluble sugar and starch contents were found in *A. membranaceus* and *C. lanceolata* seedlings with higher DIFs. The maximum soluble sugar and starch contents were found at $+10\text{ }^{\circ}\text{C}$ DIF, where they were 10.8 and $24.6\text{ mg}\cdot\text{g}^{-1}$ FW in *A. membranaceus*, and 10.9 and $25.6\text{ mg}\cdot\text{g}^{-1}$ FW in *C. lanceolata*, respectively—significantly higher than those in $-10\text{ }^{\circ}\text{C}$ DIF.

Table 2. The effects of the day and night temperatures (DIF) on the growth parameters of *A. membranaceus* plug seedlings.

DIF ^z (°C)	Length (cm)		Dry Weight (mg)			Leaf Area (cm ⁻²)	No. of Leaves	Stem Diameter (mm)	Root DW: Shoot DW Ratio	Shoot Dry Weight Per Shoot Length (g·m ⁻¹)	Root Dry Weight Per Root Length (g·m ⁻¹)	Dickson's Quality Index (×10 ⁻⁴)
	Shoot	Root	Shoot	Root	Leaf							
-10	9.2 ± 0.3 b ^y	3.3 ± 0.2	18.9 ± 1.4 c	2.0 ± 0.1 c	4.2 ± 0.3 c	3.5 ± 0.3 c	3.0 ± 0.0 c	0.62 ± 0.03 c	0.10 ± 0.01	0.21 ± 0.01 b	0.06 ± 0.00 b	1.3 ± 0.1 c
0	8.8 ± 0.3 b	4.3 ± 0.2	28.0 ± 0.5 b	2.8 ± 0.3 b	6.4 ± 0.4 b	4.6 ± 0.3 b	3.5 ± 0.2 b	0.78 ± 0.03 b	0.10 ± 0.01	0.32 ± 0.01 a	0.07 ± 0.01 b	2.5 ± 0.2 b
10	10.2 ± 0.2 a	4.4 ± 0.5	34.8 ± 2.7 a	4.3 ± 0.4 a	7.5 ± 0.4 a	6.2 ± 0.3 a	3.8 ± 0.2 a	0.90 ± 0.02 a	0.13 ± 0.02	0.34 ± 0.02 a	0.10 ± 0.02 a	3.2 ± 0.2 a
F-test	**	NS	***	***	***	***	**	***	NS	***	*	***

^z DIF, difference between day and night temperatures. ^y Different letters indicate significant separation within columns by Duncan's multiple range test at a 0.05 level. NS, *, **, and ***, represent no significant difference, significant difference at $p = 0.05$, 0.01, or 0.001, respectively.

Table 3. The effects of the DIF on the growth parameters of *C. lanceolata* plug seedlings.

DIF ^z (°C)	Length (cm)		Dry Weight (mg)			Leaf Area (cm ⁻²)	Specific Leaf Weight (g·m ⁻²)	Stem Diameter (mm)	Root DW: Shoot DW Ratio	Shoot Dry Weight Per Shoot Length (g·m ⁻¹)	Root Dry Weight Per Root Length (g·m ⁻¹)	Dickson's Quality Index (×10 ⁻⁴)
	Shoot	Root	Shoot	Root	Leaf							
-10	16.5 ± 0.4	3.9 ± 0.4	56.6 ± 3.5 b ^y	11.1 ± 1.8 b	8.3 ± 0.8 b	6.0 ± 0.5 b	13.8 ± 0.5 b	1.51 ± 0.07 c	0.19 ± 0.03	0.34 ± 0.02 b	0.28 ± 0.04 b	5.9 ± 0.4 c
0	17.0 ± 0.5	3.9 ± 0.3	77.7 ± 8.1 ab	17.3 ± 2.7 ab	10.5 ± 1.2 b	7.4 ± 0.8 b	14.1 ± 0.7 b	1.82 ± 0.09 b	0.22 ± 0.02	0.45 ± 0.04 a	0.44 ± 0.05 a	9.8 ± 1.6 b
10	17.9 ± 0.5	4.9 ± 0.4	98.9 ± 10.3 a	20.5 ± 2.2 a	14.4 ± 1.3 a	8.5 ± 0.5 a	16.8 ± 0.8 a	2.06 ± 0.07 a	0.21 ± 0.02	0.55 ± 0.05 a	0.47 ± 0.03 a	14.2 ± 0.8 a
F-test	NS	NS	**	*	**	*	*	***	NS	**	*	***

^z DIF, difference between day and night temperatures. ^y Different letters indicate significant separation within columns by Duncan's multiple range test at a 0.05 level. NS, *, **, and ***, represent no significant difference, significant difference at $p = 0.05$, 0.01, or 0.001, respectively.

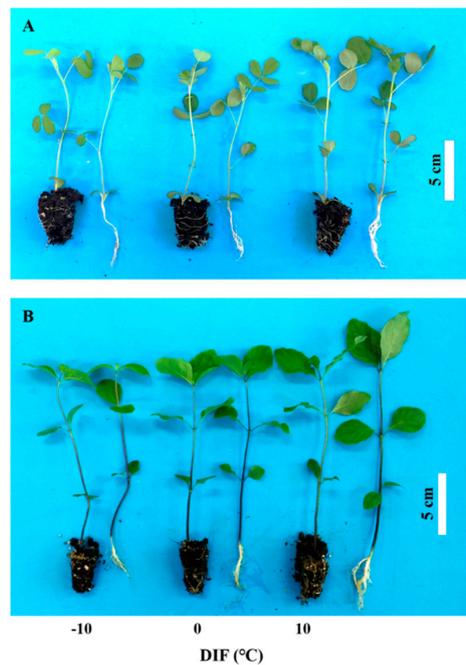


Figure 1. Morphological characteristics of *A. membranaceus* (A) and *C. lanceolata* (B) plug seedlings affected by the difference between the day and night temperatures (DIF).

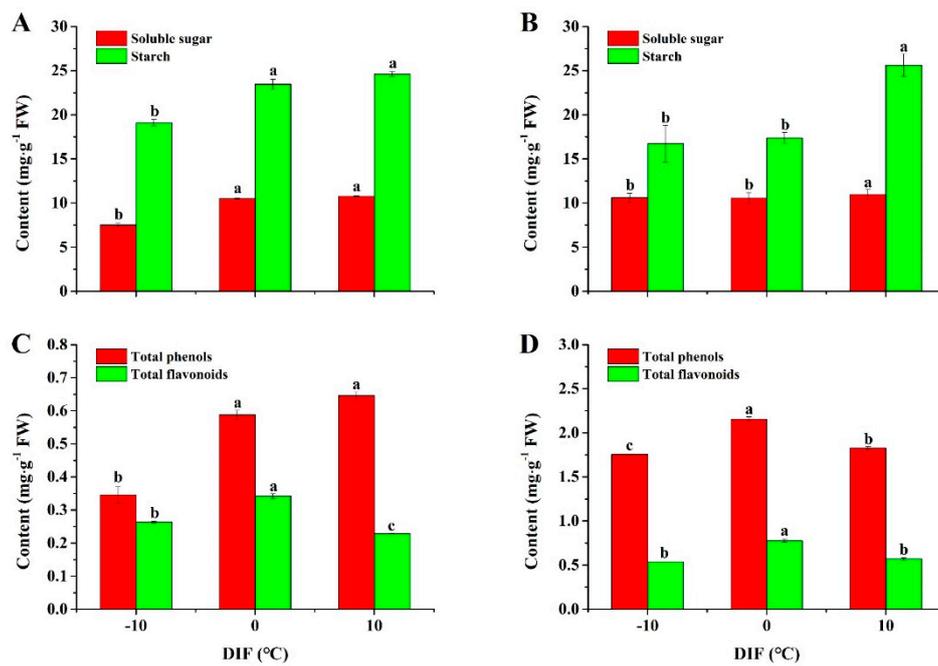


Figure 2. Contents of soluble sugar, starch, total phenols, and flavonoids in *A. membranaceus* (A,C) and *C. lanceolata* (B,D) plug seedlings affected by the DIF. Different letters (a, b, and c) indicate significant differences among treatments by Duncan's multiple range test at a 0.05.

3.3. Contents of Total Phenols and Flavonoids

In *A. membranaceus* plug seedlings, the content of total phenols gradually increased with the increase in the DIF, whereas the content of total flavonoids went up first and then declined, as shown in Figure 2C. The highest contents of total phenols and flavonoids were $0.65 \pm 0.1 \text{ mg}\cdot\text{g}^{-1} \text{ FW}$ (10 °C DIF) and $0.34 \pm 0.1 \text{ mg}\cdot\text{g}^{-1} \text{ FW}$ (0 °C DIF), respectively. In *C. lanceolata*, the DIF had the same effects on

the contents of total phenols and flavonoids. The maximum values of total phenols and flavonoids were 2.15 ± 0.03 and $0.78 \pm 0.02 \text{ mg}\cdot\text{g}^{-1}$ FW in 0°C DIF, followed by those in $+10^\circ\text{C}$ DIF (Figure 2D).

3.4. Hydrogen Peroxide Content

As shown in Figure 3, the H_2O_2 content in both species decreased significantly with the increase in DIF. The lowest H_2O_2 content was $0.119 \pm 0.001 \text{ mg}\cdot\text{g}^{-1}$ for *A. membranaceus* and $0.251 \pm 0.004 \text{ mg}\cdot\text{g}^{-1}$ FW for *C. lanceolata*, both under a positive DIF.

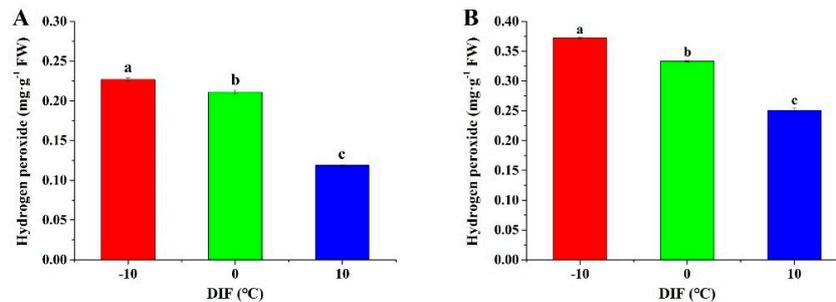


Figure 3. Hydrogen peroxide content in *A. membranaceus* (A) and *C. lanceolata* (B) plug seedlings affected by the DIF. Different letters (a, b, and c) indicate significant differences among treatments by Duncan's multiple range test at a 0.05 level.

3.5. Chlorophyll Content and Stomatal Conductance

The chlorophyll content in *A. membranaceus* and *C. lanceolata* plug seedlings was affected by the DIF. As shown in Figure 4A,B, a positive DIF resulted in much higher chlorophyll content than zero or negative DIF did in both species. The highest value was 33.9 ± 0.4 SPAD for *A. membranaceus* and 33.2 ± 0.6 SPAD for *C. lanceolata*. The data showed that the stomatal conductance increased with the increase in DIF, and the maximum was 594.9 ± 7.8 and $611.2 \pm 71.1 \text{ mmol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ in *A. membranaceus* and *C. lanceolata* plug seedlings, respectively (Figure 4C,D).

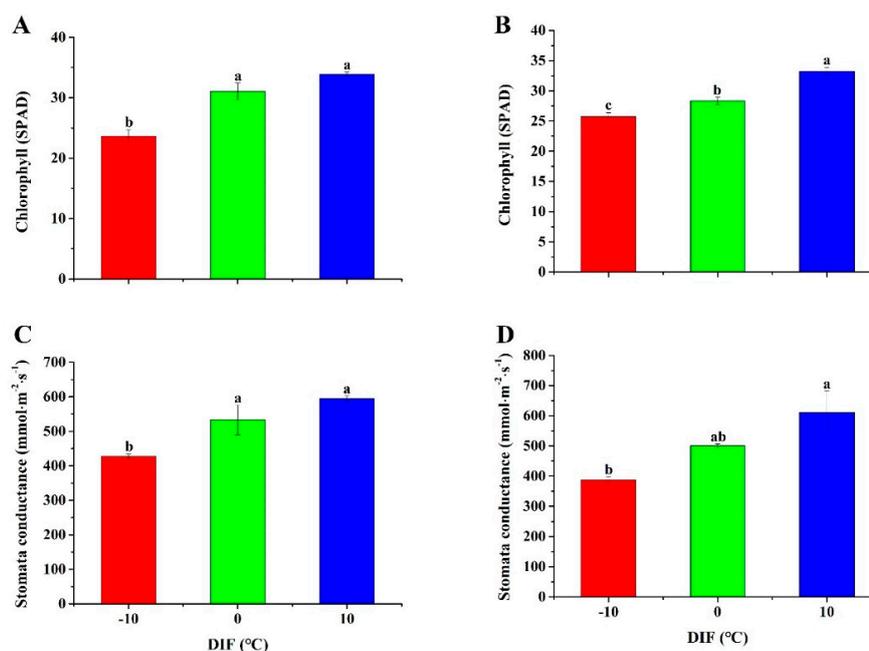


Figure 4. The effects of the DIF on the chlorophyll content and stomatal conductance in *A. membranaceus* (A,C) and *C. lanceolata* (B,D) plug seedlings. Different letters (a, b, and c) indicate significant differences among treatments by Duncan's multiple range test at a 0.05 level.

3.6. Gene Expression Analysis

The DIF had a significant influence on the expression of photosynthetic genes in both *A. membranaceus* and *C. lanceolata* (Figure 5). In *A. membranaceus*, expressions of the *GBSS* and *RBCL* genes were greatly upregulated in the 10 °C DIF than in the two other treatments, which were 5.7- and 1.8-fold higher than that in −10 °C DIF, respectively (Figure 5A). In *C. lanceolata*, the expressions of *RBCL* and *FDX* were significant higher in 10 °C DIF than those in −10 °C and 0 °C DIF, about 2.2 and 1.6 times larger than that in 0 °C DIF (Figure 5B).

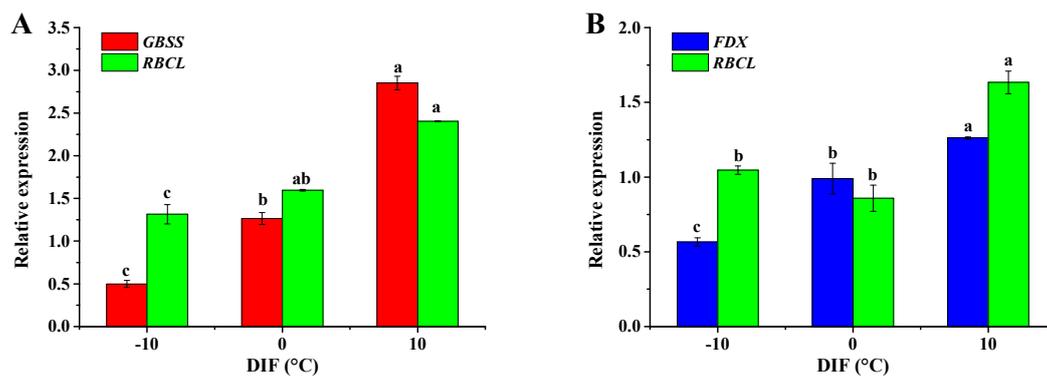


Figure 5. The effects of the DIF on the gene expression in *A. membranaceus* (A) and *C. lanceolata* (B) plug seedlings. *GBSS*, granule-bound starch synthase; *RBCL*, ribulose biphosphate carboxylase large chain; and *FDX*, ferredoxin. Different letters (a, b, and c) indicate significant differences among treatments by Duncan's multiple range test at a 0.05 level.

4. Discussion

This study shows that DIFs significantly affect the growth of *A. membranaceus* and *C. lanceolata* plug seedlings. Morphologically, compact plug seedlings with straight shoots and well-developed roots were observed when grown in a positive DIF (+10 °C). The data also proved that +10 °C DIF upgraded the quality of plug seedlings with significantly increased biomass (shoot and root) and stem diameter. Importantly, the DQI was greater in seedlings grown with the +10 °C DIF than those in the other two treatments, implying the ability of the +10 °C DIF to yield high-quality plug seedlings. The biomass, stem diameter, and DQI are important parameters used to evaluate the quality of seedlings [34–36]. These results were in accordance with previous studies in tomato [19,37] and sweet pepper [38]. However, Bachman and McMahon [39] found that a negative DIF increased the stem diameter in petunia plants which differed from results in this study. The probable reason is the difference in the photoperiod. In their study, an 18-h photoperiod was employed—6 h longer than ours—which meant a lower consumption of carbohydrates during the night. Another reason may be the difference in species, which may exhibit different responses to different temperature regimes.

The DIF had a remarkable effect on the primary and secondary metabolism in both *A. membranaceus* and *C. lanceolata*. The highest contents of soluble sugar and starch observed at the 10 °C DIF, suggests that a positive DIF significantly promotes the accumulation of carbohydrates. As the main photosynthetic products, soluble sugar and starch were synthesized in leaves and then transported to accumulate where they were used for consumption in new tissues and organs. Hence, sufficient soluble sugar and starch produced by photosynthesis are of importance for the growth and development of plug seedlings. The positive DIF (10 °C) increased the contents of soluble sugar and starch, which benefited the growth and development of plug seedlings. Similar results were reported in tomato [19] and cucumber [7]. Moreover, William, et al. [40] reported that negative DIF regimes decreased the total soluble carbohydrates by 39% and 46% in the leaf and stem, respectively, compared to those in positive DIF regimes. The reason is the higher day temperature increases the activities of photosynthetic enzymes, including *RBCL* and *FDX*, while a lower night temperature inhibits the

activities of respiration-related enzymes [41–43]. The data also proved that secondary metabolites were influenced by the DIF. Compared to 0 °C or +10 °C DIF, the –10 °C DIF inhibited the accumulation of total phenols and flavonoids. As photosynthetic products, carbohydrates are important substances for synthesizing secondary metabolites [44,45]. A lower concentration of soluble sugar and starch in –10 °C DIF is a negative factor for biosynthesis and accumulation of total phenols and flavonoids. As non-enzymatic antioxidants in nature, total phenols and flavonoids have the capacity to scavenge reactive oxygen species and maintain the homeostasis in plants [46]. The higher contents of total phenols and flavonoids observed in 0 °C or +10 °C DIF were more helpful to plug seedlings in coping with abiotic stresses [47], compared to the lower concentrations in –10 °C DIF. As important secondary metabolites, the higher contents of total phenols and flavonoids also suggested a higher medicinal value to humans [48].

Photosynthesis is a fundamental and important physiological process, greatly influencing the quality and yield of crops. This process is regulated by a series of genes, and affected by the day and night temperatures. In this study, an up-regulation of expressions of *GBSS*, *RBCL*, and *FDX* was found in the 10 °C DIF, compared to those in the –10 °C and 0 °C DIFs (Figure 5). The *FDX* gene codes the ferredoxin protein and is an important gene in the electron transport chain in photosynthesis. *RBCL* plays a key role in the CO₂ fixture in dark and influences the photosynthetic rate. *GBSS* codes the granule-bound starch synthase and determines the presence of amylose in reserve starches [49]. A high expression of those genes in the 10 °C DIF suggested that a positive DIF had a beneficial effect on photosynthesis and carbohydrate accumulation. This partly explains the higher contents of soluble sugar and starch in plug seedlings grown with the 10 °C DIF. Moreover, the H₂O₂ content was significantly lower in the positive DIF than in the negative and 0 °C DIF. As one kind of reactive oxygen species, H₂O₂ is generated when plants fall into an unfavorable condition with abiotic or biotic stresses. Consequently, a suppressive expression of photosynthetic genes and degradation of proteins occur since H₂O₂ destroys the structure of nucleic acids and proteins and inhibits their function [50]. The data implies that the 10 °C DIF exposed the plug seedlings to a lower level of stresses, contributing to the higher expression of photosynthetic genes, such as *RBCL*, *FDX*, and *GBSS*. This partly explains the high quality of plug seedlings grown with the 10 °C DIF, compared to that with the other two treatments.

In photosynthesis, chlorophyll is a core component of the light-harvesting complex, playing a key role in capturing and transferring photons to the reaction center of the photosystem in the primary reaction. The data showed that the 10 °C DIF increased the chlorophyll content more than the –10 °C and 0 °C DIF did in both species, implying that a positive DIF enhanced photosynthesis by elevating chlorophyll contents. The results coincided with those of previous studies. Xiao, et al. [51] found that chlorophyll a and chlorophyll b contents in *Solanum lycopersicum* L. increased gradually with increasing DIF. Yuan [52] also found that chlorophyll a and b contents were significantly elevated under a positive DIF, and were lower with a negative DIF. Similarly, Bachman and McMahon [39] reported that chlorophyll a content was lower in a negative DIF (–6 °C) compared to the control. Additionally, Agrawal, et al. [53] found that the photosynthetic rate and chlorophyll content were lower at a negative DIF (–10 °C) than at a positive DIF (10 °C).

Stomata is the main channel for the air exchange between plants and the ambient conditions [54]. Carbon dioxide goes into the cell through the stomata and takes part in photosynthesis. Thus, stomatal conductance is an indicator of the stomata opening, which greatly influences the photosynthetic rate by controlling the carbon dioxide concentration in cells. The maximum stomatal conductance was observed at 10 °C DIF, suggesting that a positive DIF was beneficial for photosynthesis in *A. membranaceus* and *C. lanceolata* by promoting the stomata opening and carbon dioxide exchange. This was in accordance with the increased expression of the *RBCL*, *FDX*, and *GBSS* genes, and the chlorophyll content. These results are supported by previous studies. For instance, Xiao et al. [51] also reported that a high or low DIF decreased the stomatal conductance and the maximum was observed in 6 °C DIF at a daily mean temperature of either 18 °C or 25 °C. Yuan [52] showed that the stomatal conductance in *Solanum lycopersicum* significantly increased under a positive DIF, and decreased with a

negative DIF. Moreover, the maximum stomatal conductance observed with the positive DIF (10 °C) was also in agreement with the highest content of carbohydrates, since photosynthesis was enhanced by an elevated stomatal conductance [55–57].

5. Conclusions

As an important horticultural practice, the DIF had a great influence on the growth, yield, and quality of plug seedlings. In this study, the effects of the DIF on the growth, morphology, and physiology of medicinal plant plug seedlings were demonstrated. The data showed that a positive DIF (+10 °C) had enhanced the quality of *A. membranaceus* and *C. lanceolata* plug seedlings by increasing the biomass (shoot, root, and leaf), stem diameter, and Dickson's quality index. Additionally, the contents of soluble sugar, starch, total phenols and flavonoids were significantly enhanced with an increased DIF and their respective maximum values were found in elevated DIFs (0 °C or +10 °C). Furthermore, an increase in the chlorophyll content and stomatal conductance was obtained at +10 °C DIF, indicating that the positive DIF was favorable to photosynthesis. An analysis of the gene expression showed that photosynthesis-related genes, such as *GBSS*, *RBCL*, and *FDX*, were up-regulated by the positive DIF (+10 °C). In conclusion, the results of this study suggest that a positive DIF (10 °C) is a suitable method for enhancing the quality of *A. membranaceus* and *C. lanceolata* plug seedlings.

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