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RESEARCH REPORT

Growth characteristics and bioactive compounds of dropwort subjected to high CO_2 concentrations and water deficit

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Abstract

Here, we determined the effects of mild water deficit (WD) and high CO_2 concentration (HC) on the growth and bioactive compounds of dropwort and proposed culture conditions for producing high-quality plants. Plantlets with two to three offshoots were transplanted to a controlled environment room with artificial light and cultivated for 6 weeks. To investigate the effects of HC, plantlets were grown under relatively low CO_2 concentration (LC; 600 µmol mol^{-1} CO_2) or HC (1000 µmol mol^{-1} CO_2) conditions for 6 weeks (HC_6) or grown under standard conditions for 3 weeks and transferred to HC conditions for the remaining 3 weeks (HC_3). To investigate the effects of WD, control plantlets were subirrigated by keeping the pots in a tray of nutrient solution, whereas WD-treated plants were allowed to absorb water through wicks extending from the pot bottoms to a nutrient solution below. Leaf water potential of WD-treated plantlets was significantly lower than that of controls. Both WD and HC significantly decreased leaf area but did not significantly affect shoot fresh and dry weights. The photosynthetic rates of HC_6- and HC_3-treated plantlets were 53% and 64% greater, respectively, than that of LC; the stomatal conductance and transpiration rate exhibited opposite trends. WD significantly decreased net photosynthetic rate, transpiration rate, and stomatal conductance. The total nonstructural carbohydrate content of HC_6- and HC_3-treated plantlets was 8% and 14% greater, respectively, than that of LC. Total phenolic content and antioxidant capacity of WD plantlets were 17% and 23% greater, respectively, than those of controls. In controls, total phenolic content of HC_6- and HC_3-treated plantlets was increased significantly (by 24% and 34%, respectively) than that of LC plantlets. Phenylalanine ammonia-lyase (PAL) activity of WD-treated plantlets was 14% higher than that of controls; in the controls, PAL activity of HC-treated plantlets increased significantly (by 19%) compared to that of LC plantlets. However, HC did not affect PAL activity under WD. Cyanidin content was increased by both WD and HC treatments. These results indicate that reddish small dropwort (WD treated plantlets) is more useful than greenish dropwort as a functional food and can be easily produced through HC when grown in a closed environment (e.g., a greenhouse or plant factory). Finally, the cultural practice of HC can improve the industrial value of small dropwort grown for food processing.

Keywords Anthocyanin · Drought stress · Elevated CO_2 concentration · *Oenanthe stolonifera* · Primary metabolites · Secondary metabolites

1 Introduction

Dropwort (*Oenanthe stolonifera*) is a perennial herb of the family Umbelliferae, and it is widely distributed in various climatic regions across Korea, China, Japan, Southeast Asia, and Oceania (Rhee et al. 1993). Dropworts are typically classified as either water dropworts, which grow in paddy fields, or small dropworts, which grow in upland fields. Small dropworts bear more leaves and have shorter and harder reddish stems than water dropworts (Hwang et al. 2013), indicating that the morphological characteristics of these species are affected by environmental

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conditions. Moreover, small dropworts show a higher content of carbohydrates, lipids, vitamin C, and various minerals, including calcium, iron, and potassium, than water dropworts (RDA 2011). However, few studies have investigated the environmental factors affecting the growth and nutritional characteristics of dropworts.

Water deficit (WD) is one of the major environmental stresses that reduce crop yield (Boyer 1982). Under WD, plant roots cannot absorb sufficient water, and the stomata are closed to retain water (Kim and van Iersel 2011; Lawlor and Cornic 2002). This closure reduces stomatal conductance, thereby limiting CO_2 diffusion into the leaves and ultimately reducing photosynthesis (Pérez-López et al. 2012). Moreover, WD reduces the electron transport capacity of the photosynthetic electron transport system by damaging the D1 and D2 proteins of photosystem II (Lu and Zang 1999; Reddy et al. 2004). Consequently, the light absorbed by photosystems is not fully utilized for CO_2 assimilation, and this excess excitation energy damages the photosystems (Chaves et al. 2009; Cornic and Briantais 1991; Lawlor and Tezara 2009), primarily through the generation of reactive oxygen species (ROS). However, since plants defend themselves against ROS by synthesizing enzymatic and non-enzymatic antioxidants (Peltzer et al. 2002), moderate WD can enhance the accumulation of plant secondary metabolites, including hypericin in St. John's wort, esculin and quercetin in *Fraxinus ornus*, and ascorbate in mulberry (Fini et al. 2012; Reddy et al. 2004; Zobayed et al. 2007).

CO_2 is a direct source of carbon fixation for photosynthesis. When plants are subjected to CO_2 concentrations at least twice the atmospheric level (~400 µmol mol^{-1}), stomatal conductance is reduced but the photosynthetic rate is enhanced due to a greater CO_2 gradient between the inside and outside of the stomata (Drake et al. 1997; Pérez-López et al. 2012). Such enhanced photosynthetic rates produce nonstructural carbohydrates, and if more carbohydrates are produced than are required for growth, the remainder is used to synthesize carbon-based secondary metabolites (Peñuelas and Estiarte 1998). For instance, Ghasemzadeh et al. (2012) reported that the content of anthocyanins, rutin, and myricetin was significantly increased in ginger in the presence of 800 µmol mol^{-1} ambient CO_2. In addition, high CO_2 levels (700 µmol mol^{-1}) increased the content of pancratistatin and narciclasine in tropical spider lily (Idso et al. 2000). Therefore, when stomatal conductance is reduced under WD, the supply of additional CO_2 may improve the photosynthetic rate and ultimately augment growth and increase the content of secondary metabolites in plants (Pérez-López et al. 2012).

To this end, the objectives of this study were to determine the effects of mild WD and high CO_2 concentration (HC) on the growth and bioactive compounds of dropwort and propose culture conditions for producing high-quality plants.

2 Materials and methods

2.1 Plant materials and growth conditions

Dropwort (*O. stolonifera*) plantlets were vegetatively propagated over a 3-week period in a nursery greenhouse located in Jeungpyeong, Chungcheongbuk-do, Korea. The primary stems of the cultivated plantlets bore three to five leaves and two offshoots, and the plantlets had an average fresh weight of 1.8 ± 0.22 g. These plantlets were transplanted to pots (6.7 × 6.7 × 6 cm^3, L × W × H) containing horticultural growth medium (Myung-Moon; Dongbu hannong, Seoul, Korea). Dropwort plants were cultivated using an irrigation system using a nutrient-stagnant wick culture system (NSW; Oh et al. 2007) in which a wick (length: 12 cm, width: 1.5 cm, thickness: 0.14 cm) was inserted into the bottoms of pots containing the growth medium. To ensure successful rooting, all pots were subirrigated to a depth of 1 cm for 7 days, and the plants were cultivated in an environmental control room with artificial light for 6 weeks under the following conditions: air temperature of 22 °C, relative humidity of 60%, photoperiod of 12 h, and photosynthetic photon flux density (PPFD) (white LEDs; 450 nm, 27% + 555 nm, 65% + 660 nm, 8%) of 270 ± 10 µmol m^{-2} s^{-1}. Electrical conductivity and pH of the nutrient solution (An and Lee 1991) were maintained at 1.5 dS m^{-1} and 6.5, respectively.

2.2 WD and HC treatments

To investigate the effects of WD, control plantlets were subirrigated by keeping the pots in a tray of water, whereas WD-treated plants were allowed to absorb water through a wick (length, 4 cm) extending from the bottom of the pots to the surface of the nutrient solution, as described previously (Lee and Oh 2017). Using the NSW system, the soil water contents of control plants and WD-treated plants were maintained 32% and 22%, respectively, during the cultivation period. To investigate the effects of HC, three treatments were delivered, namely, relatively low CO_2 concentration (LC), HC_6, and HC_3. The CO_2 concentrations of the LC and HC_6 treatments were set at 600 and 1000 µmol mol^{-1}, respectively, for 6 weeks. Dropwort plantlets subjected to HC_3 treatment were grown under LC conditions for an initial 3 weeks and then transferred to HC conditions for the last 3 weeks. The CO_2 concentration of each treatment was measured every 10 min using a KCD-100 CO_2 sensor (Korea Digital, Seoul, Korea), and data were recorded using a data logger (CR1000; Campbell Scientific, Logan, UT, USA). Furthermore, the CO_2 level was controlled using an on/off relay instrument (AM16/32B;

Campbell Scientific). Dropwort plantlets were grown in two separate cultivation rooms to manipulate two different CO_2 levels, and WD was induced in both rooms (Fig. 1).

2.3 Growth characteristics

Shoot fresh and dry weights and leaf area were measured at 6 weeks after transplanting. At each time point, shoot fresh and dry weights were measured using an electronic scale (Si-234; Denver Instrument, Bohemia, NY, USA), and dry weight was measured after freeze-drying the shoots for 72 h (PVTFD10A; IlShinBioBase, Dongducheon, Korea) at − 50 °C and under vacuum of 7 kPa. Leaf area was measured using LIA 32 ver.0.377 (Kazukiyo Yamamoto, Nagoya University, Japan) after detaching the leaves from the stems.

2.4 Leaf water potential

To confirm the effect of WD induced by the wicks, leaf water potential was measured using a water potential meter (WP4; Decagon Devices, Pullman, WA, USA) at 6 weeks after transplanting. For measurement, four-leaf disks (diameter, 14.5 mm) were collected from the upper leaf of the third or fourth node of each plant using a cork borer and then were immediately placed in a small plastic chamber (diameter, 4 cm). During the measurement, the air temperature was equilibrated between inside and outside the chamber at 25 °C for 10 min.

2.5 Net photosynthetic rate, transpiration rate, and stomatal conductance

The net photosynthetic rate, transpiration rate, and stomatal conductance of dropwort leaves were measured every week after transplanting. These parameters were measured at the upper leaf of the first petiole at the fourth or fifth node using a portable photosynthesis system (LI-6400-40; Li-Cor, Lincoln, NE, USA). Measurements were conducted 3 h after the start of the light period and lasted for 3 h. All measurement conditions were similar to the growth conditions: air flow of 300 µmol s^{-1}; CO_2 concentration of 600 or 1000 µmol mol^{-1}; leaf temperature of 22 °C, and PPFD of 270 µmol m^{-2} s^{-1}.

2.6 Total nonstructural carbohydrates (TNCs)

To analyze the content of starch and total soluble sugars, freeze-dried samples were prepared from the shoots of 6-week-old plants, uniformly ground using a Tube-Mill control (IKA, Wilmington, NC, USA), and stored at 4 °C. Soluble sugars were analyzed according to the method described by González-Rossia et al. (2008), with minor modifications. Briefly, powdered samples (100 mg) were mixed with 10 mL of 80% ethanol in 15-mL conical tubes and sonicated using an ultrasonic bath (SK5210HP; Hangzhou Nade Scientific Instrument, Zhejiang, China) at 25 °C for 1 h. Then, the extracted samples were centrifuged at 13,000 × *g* for 10 min at 4 °C. The resulting pellet was stored at − 80 °C for starch analysis. The resulting supernatants were evaporated at 50 °C using a rotary evaporator (N-1000; EYELA, Tokyo, Japan) and redissolved in 2 mL of deionized water. Then, the redissolved samples were sequentially passed through Sep-Pak C18 cartridges (WAT020805; Waters, Milford, MA, USA) and 0.22-µm UHP (PTFE) syringe filters (SPU0213-1; Woongki, Seoul, Korea). The filtered extracts were then concentrated in a 2-mL microtube for 24 h using a concentrator (Modul 31; Hanil Science Medical, Daejeon, Korea) connected to a freeze dryer (FD8508; Ilshinbiobase, Dongducheon, Korea). Finally, the concentrated sugars from each sample were redissolved in 0.6 mL of deionized water, and the resulting sugar solutions were analyzed using a high-performance liquid chromatography system (UltiMate 3000;

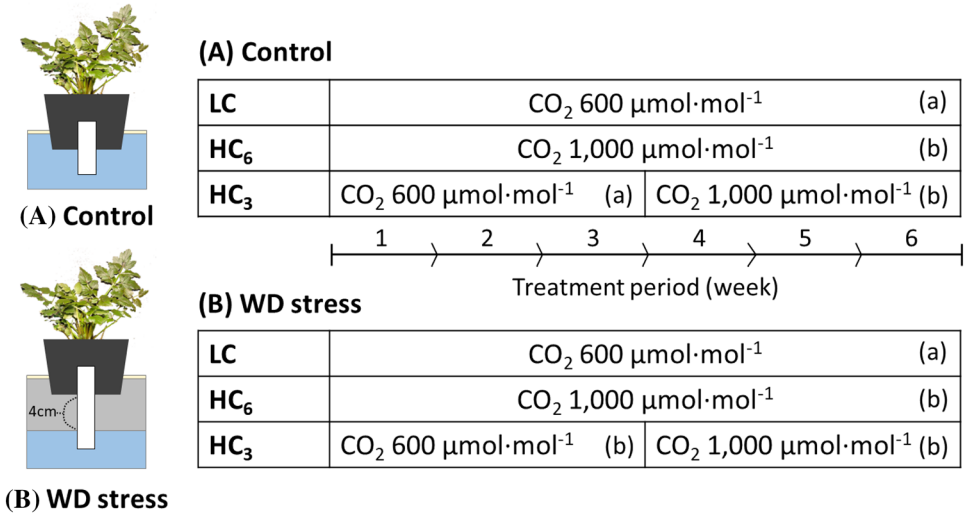

Fig. 1 Dropwort plantlets were grown in two separate cultivation rooms under low CO_2 concentration (LC; 600 µmol mol^{-1}) and high CO_2 concentration (HC; 1000 µmol mol^{-1}) conditions for 6 weeks (HC_6). The HC_3 dropwort plantlets were transferred from cultivation room a to b after 3 weeks. Control (**a**; subirrigation) and water-deficit (**b**; WD) stress plantlets were treated in individual cultivation rooms. Wick length, from the pot bottom to the surface of the nutrient solution, was maintained at 4 cm for the WD treatment

Dionex, Idstein, Germany) equipped with a refractive index detector (Shodex RI-101; Showa Denko, Kanagawa, Japan). Briefly, the extracts were injected into a Sugar-Pak column (6.5 × 300 mm; Waters) at 70 °C; deionized water was used as a solvent, at a flow rate of 0.5 mL min^{-1}. Calibration curves were obtained using sucrose (Sigma-Aldrich, St. Louis, MO, USA), glucose (Junsei Chemical, Tokyo, Japan), and fructose (Sigma-Aldrich) standards, and the soluble sugar content of each sample was expressed as milligrams per gram dry weight of dropwort.

The starch content of the pellets obtained after extracting the soluble sugars was analyzed using the dinitrosalicylic acid method (Miller 1959; Araújo et al. 2004) with slight modifications. Briefly, the pellets were dissolved in 2 mL of distilled water and autoclaved for 30 min to gelatinize the starch particles. Then, 1 mL of 0.2 M Na-acetate (pH 5.5) buffer, 1 mL of 30 U amyloglucosidase (Sigma-Aldrich), and 1 mL of 10 U β-amylase (Sigma-Aldrich) were added for hydrolysis. After centrifugation at 13,000 × *g* for 10 min, the resulting supernatants (50 µL) were mixed with 0.5 mL of dinitrosalicylic acid reagent and heated at 100 °C in distilled water for 5 min. After complete cooling, 0.9 mL of distilled water was added to 0.1 mL of each reaction solution, and absorbance was measured at 525 nm using a spectrophotometer (UV-1800; Shimadzu, Kyoto, Japan). The starch content of each sample was expressed as milligrams of glucose (Sigma-Aldrich) per gram dry weight of dropwort.

2.7 Mineral content

To analyze the mineral content, dropwort shoots were digested using a wet digestion method described by Havlin and Soltanpour (1980) with minor modifications. Briefly, freeze-dried samples (1 g) were digested with 15 mL of 70% nitric acid (HNO_3) at 125 °C for 1.5 h. Then, H_2O_2 (7.5 mL) was added, and the solutions were heated at 125 °C for 1 h. The same volume of H_2O_2 was added, and the solutions were heated at 200 °C for 2 h. After cooling for 24 h, the solutions were mixed with 45 mL of 2% HNO_3 and resuspended for 3 h. The solutions were adjusted to approximately 90 g using triple distilled water and filtered through 125 mM quantitative filter paper (Quantitative ashless, Hyundai Micro, Seoul, Korea). The content of P, K, Ca, Mg, S, Cu, Fe, Zn, Mn, and B was measured using an ICP-OES spectrophotometer (Optima 7300 DV, Perkin Elmer, Waltham, MA, USA).

2.8 Total phenolic content and antioxidant capacity

The total phenolic content was measured using the Folin–Ciocalteu method (Ainsworth and Gillespie 2007) with slight modifications. Briefly, freeze-dried samples (40 mg) were extracted in 5-mL tubes using 4 mL of 80% acetone with 15 min of ultrasonication. The resulting extracts were incubated at 4 °C for 12 h in the dark and then centrifuged at 1000 × *g* for 2 min (1730R; Gyrozen, Daejeon, Korea). The resulting supernatants were analyzed as described by Son and Oh (2013). The total phenolic content was expressed as milligrams of gallic acid (Acros Organics, Geel, Belgium) per gram dry weight of dropwort.

The antioxidant capacity was measured according to the method described by Miller and Rice-Evans (1996), with minor modifications, using 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (Sigma-Aldrich). The samples were extracted using the same method to analyze total phenolic content and incubated at − 20 °C for 12 h in the dark. The extracts were then centrifuged at 1000 × *g* and 4 °C for 2 min, and the resulting supernatants were analyzed according to the method described by Son and Oh (2013). The antioxidant capacity was expressed as Trolox (mM) equivalent antioxidant capacity (TEAC) per gram dry weight of dropwort.

2.9 PAL activity

PAL activity was measured according to the method described by Boo et al. (2011). Upper leaf tissues (0.5 g) from the first petiole of the fourth or fifth node were sampled at 4 weeks after transplanting, frozen in liquid nitrogen, and stored at − 70 °C until analysis. Frozen samples were ground in a mortar with liquid nitrogen and then mixed with 10 mL of 25 mM borate buffer (pH 8.8) and 2 mL of 3 mM β-mercaptoethanol (Sigma-Aldrich). The extracted samples were transferred to 2-mL microtubes and centrifuged at 15,000 × *g* for 20 min. The resulting supernatants (0.5 mL) were reacted with 2.5 mL of 25 mM borate buffer (pH 8.8) and 2 mL of 10 mM L-phenylalanine (Sigma-Aldrich) at 40 °C. After 2 h, the reaction was stopped by adding 100 µL of 5 N HCl. The absorbance of the reacted solutions was measured at 290 nm using a spectrophotometer and compared to a standard curve of trans-cinnamic acid (Sigma-Aldrich). PAL activity was expressed as the trans-cinnamic acid (mM) equivalent per hour per gram fresh weight of dropwort.

2.10 Anthocyanin content

Qualitative and quantitative analyses of individual anthocyanins were performed according to the methods described by Jeon et al. (2017). Standard curves were established using delphinidin 3-*O*-D-glucoside (D3G; Sigma-Aldrich), cyanidin 3-*O*-glucoside (C3G; Sigma-Aldrich), and pelargonidin 3-*O*-glucoside (P3G; Sigma-Aldrich). The content of individual anthocyanins was expressed as milligrams per gram shoot dry weight.

2.11 Statistical analysis

The experiment was conducted using a split plot design. Four replicates were used per treatment, with a single sample collected per replicate ($n=4$). All data were analyzed using SAS 9.2 (SAS Institute, Cary, NC, USA). The effects of the two main factors, namely, WD and HC, on each parameter were evaluated using two-way analysis of variance, and the significance of differences in the means among treatments was assessed using Tukey's honestly significant difference test.

3 Results

3.1 Growth characteristics

The shoot fresh and dry weights were not significantly affected by either WD or HC (Table 1). In the absence of WD, while the leaf area of the HC_6-treated plantlets was significantly reduced (by 41.2%) compared with that of LC, that of the HC_3-treated plantlets was not significantly different from that of LC. WD and HC significantly affected the leaf water potential, though the two-way interaction between WD and HC was not significant.

3.2 Net photosynthetic rate, transpiration rate, and stomatal conductance

Regardless of the water status, the stomatal conductance and transpiration rate of the plantlets were reduced by HC until 3 weeks after transplanting; however, there were no significant differences in either parameter between plantlets grown under the two CO_2 concentrations after 3 weeks (Fig. 2b, c, e, f). In the absence of WD, HC decreased the stomatal conductance but did not change the net photosynthetic rate compared with LC at 3 weeks after transplanting (Fig. 2a, b). In the absence of WD, the net photosynthetic rate of the HC_3-treated plantlets increased by 61.9% within the first week after the change in the CO_2 level (Fig. 2a). Meanwhile, at that same time, in the WD, HC increased the net photosynthetic rate of the HC_3-treated plantlets, irrespective of changes in stomatal conductance (Fig. 2d).

The stomatal conductance and transpiration rate of the WD-treated plantlets did not change during the first 3 weeks after transplanting but were lower than those of controls at 4 weeks and thereafter (Fig. 2b, c, e, f). However, under LC conditions, the net photosynthetic rate of the WD-treated plantlets was almost similar to that of controls at any point during the growth stage, except at 4 weeks after transplanting (Fig. 2a, d). In contrast, under HC_6 conditions, the net photosynthetic rate of the WD-treated plantlets was 14.3% greater than that of controls at 4 weeks after transplanting. Under HC_3 conditions without the WD stress, the net photosynthetic rate increased by 18.8% within a week, though this increasing trend was not observed under WD (Fig. 2a, d).

3.3 Total nonstructural carbohydrates (TNCs)

The nonstructural carbohydrate content of dropwort was affected by both WD and HC treatments (Table 2). In the absence of WD, the starch content of the HC_3-treated

Table 1 Effects of water deficit and CO_2 enrichment on the growth characteristics and leaf water potential of dropwort plants at 6 weeks of transplanting ($n=4$)

Irrigation	CO_2 concentration treatment[z]	Shoot fresh weight ($g \cdot plant^{-1}$)	Shoot dry weight ($g \cdot plant^{-1}$)	Leaf area ($cm^2 \cdot plant^{-1}$)	Leaf water potential (MPa)
Control[y]	LC	57.5 ± 8.1 a[x]	7.0 ± 1.2 a	1077.0 ± 118.1 a	-6.8 ± 0.3 a
	HC_6	47.1 ± 5.5 a	5.0 ± 0.5 a	632.9 ± 90.9 b	-6.8 ± 0.1 a
	HC_3	56.8 ± 6.1 a	6.9 ± 0.5 a	1189.0 ± 55.9 a	-6.6 ± 0.3 a
Water deficit	LC	60.3 ± 11.6 a	7.9 ± 1.7 a	994.0 ± 110.3 a	-7.9 ± 0.1 ab
	HC_6	45.5 ± 6.1 a	5.7 ± 0.7 a	736.2 ± 50.5 a	-9.2 ± 0.5 b
	HC_3	36.8 ± 9.5 a	5.4 ± 1.2 a	777.8 ± 111.1 a	-7.3 ± 0.5 a
Significance[w]					
Irrigation (I)		NS	NS	NS	***
CO_2 enrichment (C)		NS	NS	**	*
I × C		NS	NS	NS	NS

[z]CO_2 concentrations of LC and HC_6 were 600 and 1000 μmol mol^{-1}, respectively, for 6 weeks, and CO_2 concentration of HC_3 was changed from 600 to 1000 μmol mol^{-1} at 3 weeks of transplanting. LC: low CO_2 concentration. HC: high CO_2 concentration

[y]Subirrigation

[x]Different letters within columns indicate significant differences ($p \leq 0.05$, Tukey's test)

[w]NS, not significant; *, **, and ***, significant $p=0.05$, 0.01, and 0.001, respectively

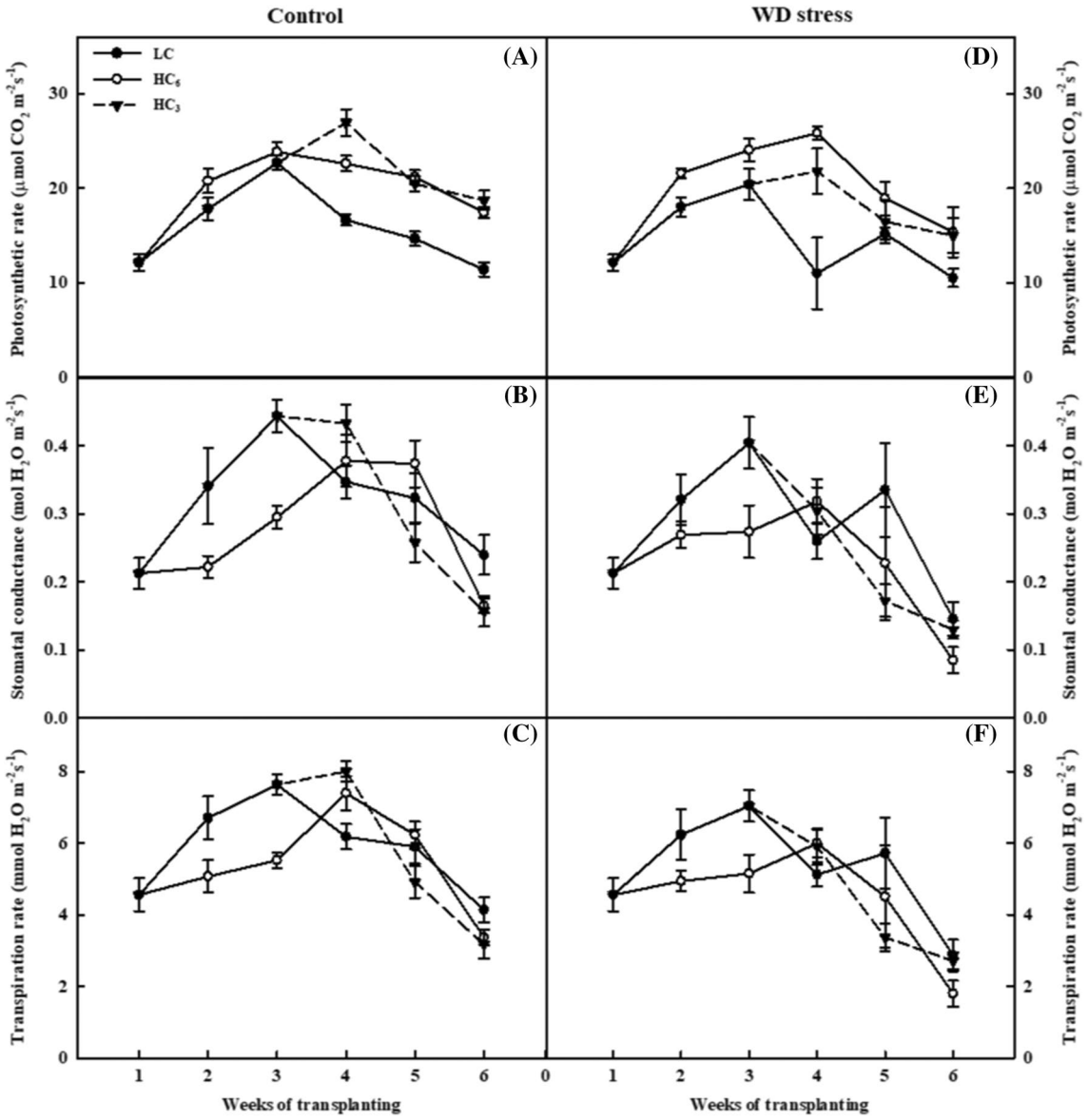

Fig. 2 Photosynthetic rate (**a**, **d**), stomatal conductance (**b**, **e**), and transpiration rate (**c**, **f**) of dropwort plants grown under subirrigation (Control; a, b, c) or water-deficit (WD; d, e, f) and high-CO_2 concentration (1000 μmol mol^{-1}) conditions for 6 weeks ($n=4$). LC: low CO_2 concentration (600 μmol mol^{-1}) for 6 weeks. HC_6: high CO_2 concentration (1000 μmol mol^{-1}) for 6 weeks. HC_3: high CO_2 concentration (1000 μmol mol^{-1}) for the last 3 weeks

plantlets was significantly greater (12%) than that of LC. Meanwhile, in the WD, the starch contents of the HC_6- and HC_3-treated plantlets were significantly increased by 4% and 12%, respectively, compared with that of LC. The contents of sucrose, glucose, fructose, and total soluble sugar were not significantly different among the HC treatments, whereas the TNC content, defined as the sum of the starch and total soluble sugar content, was significantly affected by the HC treatments. Regardless of WD, the TNC contents of the HC_6- and HC_3-treated plantlets were significantly higher than that of LC.

3.4 Mineral content

In the absence of WD, the S contents of the HC_6- and HC_3-treated plantlets were significantly decreased by 24% and 21%, respectively, compared with that of LC (Table 3). Meanwhile, under HC treatments, the contents of Cu, Zn, and B were increased significantly, whereas those of P, K, Ca, and Mg were increased slightly. In particular, the Zn contents of the HC_6- and HC_3-treated plantlets were significantly increased by 49% and 48%, respectively, compared with that of LC. Meanwhile, under WD, there were

Table 2 Effects of water deficit and CO_2 enrichment on the starch, soluble sugar, and total nonstructural carbohydrates content of dropwort plants at 6 weeks of transplanting ($n=4$)

Irrigation	CO_2 concentration treatment[z]	Starch (glucose mg·g^{-1} DW)	Sucrose (mg·g^{-1} DW)	Glucose (mg·g^{-1} DW)	Fructose (mg·g^{-1} DW)	Total soluble sugars (mg·g^{-1} DW)	TNCs[y] (mg·g^{-1} DW)
Control[x]	LC	281.9 ± 2.2 b[w]	11.4 ± 1.8 a	3.2 ± 0.3 a	4.4 ± 0.2 a	18.9 ± 2.2 a	300.8 ± 4.0 c
	HC_6	299.4 ± 4.7 ab	15.7 ± 1.2 a	4.1 ± 0.2 a	7.5 ± 0.7 a	27.2 ± 1.5 a	326.6 ± 3.5 b
	HC_3	317.7 ± 7.1 a	15.7 ± 1.1 a	3.9 ± 0.5 a	7.5 ± 1.3 a	27.0 ± 1.6 a	344.7 ± 6.2 a
Water deficit	LC	282.8 ± 4.3 c	15.4 ± 1.1 a	2.9 ± 0.4 a	5.0 ± 1.1 a	23.3 ± 1.5 a	306.1 ± 4.4 c
	HC_6	293.9 ± 2.5 b	17.9 ± 1.1 a	3.3 ± 0.1 a	6.3 ± 0.9 a	27.5 ± 1.1 a	321.4 ± 2.0 b
	HC_3	315.9 ± 6.4 a	19.4 ± 1.4 a	3.5 ± 0.4 a	6.9 ± 1.3 a	29.8 ± 2.9 a	345.6 ± 3.9 a
Significance[v]							
Irrigation (I)		NS	*	NS	NS	NS	NS
CO_2 enrichment (C)		***	**	NS	**	**	***
I × C		NS	NS	NS	NS	NS	NS

[z]CO_2 concentrations of LC and HC_6 were 600 and 1000 µmol mol^{-1}, respectively, for 6 weeks, and CO_2 concentration of HC_3 was changed from 600 to 1000 µmol mol^{-1} at 3 weeks of transplanting. LC: low CO_2 concentration. HC: high CO_2 concentration

[y]TNCs: total nonstructural carbohydrates, sum of starch, sucrose, glucose, and fructose

[x]Subirrigation

[w]Different letters within columns indicate significant differences ($p \leq 0.05$, Tukey's test)

[v]NS, not significant; *, **, and *** significant at $p=0.05$, 0.01, and 0.001, respectively

no significant changes in the mineral contents following HC treatments.

3.5 Total phenolic content, antioxidant capacity, and PAL activity

The total phenolic content and antioxidant capacity of the plantlets at 6 weeks after transplanting were affected by both WD and HC (Table 4 and Fig. 3). Regardless of the HC treatments, the total phenolic content and antioxidant capacity of the WD-treated plantlets were 18% and 23% higher, respectively, than those of the controls (Fig. 3). In the absence of WD, the total phenolic contents of the HC_6- and HC_3-treated plantlets were significantly increased by 24% and 35%, respectively, compared with that of LC (Fig. 3a). In addition, under WD, the total phenolic contents of the HC_6- and HC_3-treated plantlets were significantly higher than that of LC. Furthermore, in both control and WD-treated plantlets, the antioxidant capacity showed a trend similar to that of the total phenolic content (Fig. 3b).

At 6 weeks after transplanting, the activity of PAL, a key gateway enzyme of the phenylpropanoid pathway, was significantly affected by WD but not by HC (Table 4 and Fig. 4). The mean PAL activity values of the WD-treated plantlets were 12% greater than that of controls. In the absence of WD, the mean PAL activity values of the HC_6- and HC_3-treated plantlets were significantly 19% greater than that of controls.

3.6 Anthocyanin content

D3G, C3G, and P3G were detected in the plantlets, with C3G being the most prevalent anthocyanin (Fig. 5). Regardless of HC, the contents of the three anthocyanins were increased with WD, albeit non-significantly (Table 4 and Fig. 5). In the absence of WD, the C3G contents of the HC_6- and HC_3-treated plantlets were significantly higher (99% and 166%, respectively) than that of controls. The contents of the other two anthocyanins, namely, D3G and P3G, were also increased by HC treatments, irrespective of WD, albeit non-significantly. The anthocyanin content, which was influenced by both HC and WD, was confirmed using photographs of dropwort leaves collected from the same positions (Fig. 6). The leaves of plants grown under WD and/or HC were more reddish than those of controls under LC. The leaves of plants grown under WD and HC_3 showed the greatest amount of red coloration (Fig. 6f).

4 Discussion

4.1 Growth characteristics

In this study, we used an NSW system in which the plants were supplied the nutrient solution through capillary action (Oh et al. 2007) to induce WD. Our previous study, using an NSW system in dropwort plants, revealed that specific soil water levels could be maintained and WD could be induced (Lee and Oh 2017). The soil water

Table 3 Effects of water deficit and CO_2 enrichment on the mineral content of dropwort plants at 6 weeks of transplanting ($n=4$)

Irrigation	CO_2 concentration treatment[z]	Macronutrients (mg·g^{-1} DW)					Micronutrients (μg·g^{-1} DW)				
		P	K	Ca	Mg	S	Cu	Fe	Zn	Mn	B
Control[y]	LC	9.9 ± 0.7 a[x]	67.2 ± 2.5 a	14.1 ± 1.5 a	4.8 ± 0.2 a	11.6 ± 0.6 a	22.8 ± 1.9 b	114.1 ± 24.3 a	80.1 ± 5.6 b	250.4 ± 33.5 a	77.5 ± 7.2 b
	HC_6	11.8 ± 0.4 a	69.5 ± 1.5 a	16.2 ± 1.6 a	5.5 ± 0.2 a	8.8 ± 0.3 b	30.6 ± 1.5 a	74.5 ± 1.7 a	119.7 ± 4.6 a	170.8 ± 15.0 a	113.1 ± 7.3 a
	HC_3	10.8 ± 0.5 a	69.4 ± 1.3 a	14.3 ± 1.0 a	4.9 ± 0.2 a	9.2 ± 0.4 b	26.7 ± 1.6 ab	92.7 ± 6.7 a	118.4 ± 3.9 a	217.9 ± 15.7 a	104.1 ± 11.6 ab
Water deficit	LC	9.0 ± 0.5 a	65.2 ± 3.3 a	11.3 ± 1.4 a	4.2 ± 0.3 a	8.2 ± 0.9 a	25.0 ± 2.2 a	74.3 ± 8.7 a	74.3 ± 4.4 c	154.8 ± 18.1 a	104.8 ± 14.7 a
	HC_6	10.1 ± 0.8 a	65.8 ± 2.6 a	14.5 ± 1.5 a	4.1 ± 0.3 a	7.8 ± 0.6 a	23.4 ± 2.0 a	63.2 ± 8.2 a	167.7 ± 7.9 a	180.4 ± 20.0 a	95.7 ± 14.7 a
	HC_3	9.6 ± 0.5 a	61.4 ± 2.2 a	13.8 ± 1.1 a	4.7 ± 0.6 a	7.4 ± 0.3 a	27.9 ± 2.4 a	82.4 ± 8.2 a	136.8 ± 7.6 b	192.4 ± 25.0 a	115.8 ± 16.2 a
Significance[w]											
Irrigation (I)		*	NS	NS	NS	*	NS	NS	***	NS	NS
CO_2 enrichment (C)		*	*	NS	*	***	NS	*	***	NS	NS
I × C		NS	NS	NS	NS	NS	NS	NS	***	NS	NS

[z]CO_2 concentrations of LC and HC_6 were 600 and 1000 μmol mol^{-1}, respectively, for 6 weeks, and CO_2 concentration of HC_3 was changed from 600 to 1000 μmol mol^{-1} at 3 weeks of transplanting. LC: low CO_2 concentration. HC: high CO_2 concentration

[y]Subirrigation

[x]Different letters within columns indicate significant differences ($p \leq 0.05$, Tukey's test)

[w]NS, not significant; * and *** significant at $p=0.05$ and 0.001, respectively

Table 4 Effects of water availability and CO_2 enrichment on the total phenolic content, antioxidant capacity, and phenylalanine ammonia-lyase (PAL) activity of dropwort plants at 6 weeks of transplanting ($n=4$)

Significance[z]	Total phenolic content	Anti-oxidants capacity	PAL activity	Delphi-nidin 3-*O*-β-D-glucoside	Cyanidin 3-*O*-gluco-side	Pelargonidin 3-*O*-gluco-side
I[y]	***	**	*	NS	NS	NS
C[x]	***	**	NS	*	***	*
I×C	NS	NS	NS	NS	NS	NS

[z]NS, not significant; *, **, and ***, significant at $p=0.05$, 0.01, and 0.001, respectively

[y]Irrigation

[x]CO_2 enrichment

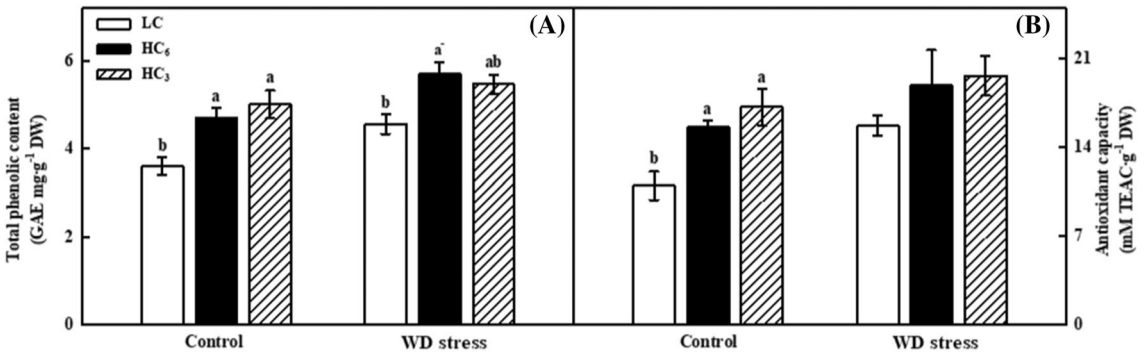

Fig. 3 Total phenolic content (**a**) and antioxidant capacity (**b**) of dropwort plants grown under subirrigation (control) or water-deficit (WD) conditions at 6 weeks after transplanting ($n=4$). LC: low CO_2 concentration (600 μmol mol^{-1}) for 6 weeks. HC_6: high CO_2 concentration (1000 μmol mol^{-1}) for 6 weeks. HC_3: high CO_2 concentration (1000 μmol mol^{-1}) for the last 3 weeks. Different letters within each set of columns indicate significant differences ($p \leq 0.05$, Tukey's test)

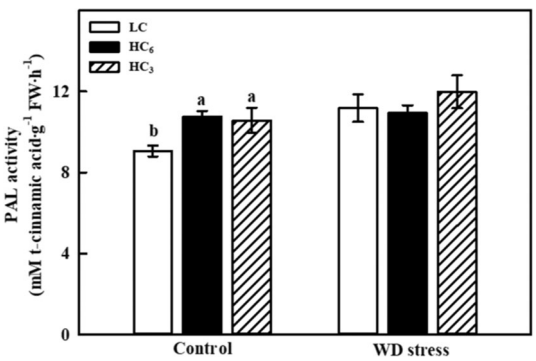

Fig. 4 Phenylalanine ammonia-lyase (PAL) activity of dropwort plants grown under subirrigation (control) or water-deficit (WD) conditions at 6 weeks of transplanting ($n=4$). LC: low CO_2 concentration (600 μmol mol^{-1}) for 6 weeks. HC_6: high CO_2 concentration (1000 μmol mol^{-1}) for 6 weeks. HC_3: high CO_2 concentration (1000 μmol mol^{-1}) for the last 3 weeks. Different letters within each set of columns indicate significant differences ($p \leq 0.001$, Tukey's test)

content can be manipulated by changing the length of the wick between the bottom of the pot and the surface of the nutrient solution; in this study, the wick lengths of the control and WD treatments were 0 and 4 cm, respectively. Consequently, the soil water content of the WD treatments was 10% lower than that of control treatments (i.e., subirrigation; data not shown), and the leaf water potential was reduced, albeit only slightly. This result indicates that mild WD was successfully achieved. Specifically, the leaf water potentials of the WD- and HC-treated plants were lower than that of control plants under LC. In general, plants subjected to HC can maintain a constant water content by closing their stomata (Robredo et al. 2007). In this study, however, the plants exposed to WD and HC had noticeably decreased water contents in leaves owing to the limited amount of water absorbed by the roots. The correlation between plant responses to WD and HC has been reported by Centritto et al. (1999).

Despite the decreased leaf water potential due to WD and HC, the shoot fresh and dry weights were not significantly decreased. Similarly, in our previous study, shoot fresh and dry weights were not significantly decreased under WD (Lee and Oh 2017). However, in the subirrigated plants (without the WD stress), the leaf area was significantly decreased under HC_6 but not under HC_3 treatment, compared to that under LC treatment. Therefore, exposure to HC during early stages might inhibit growth, and the effects of HC likely depend on the growth stage.

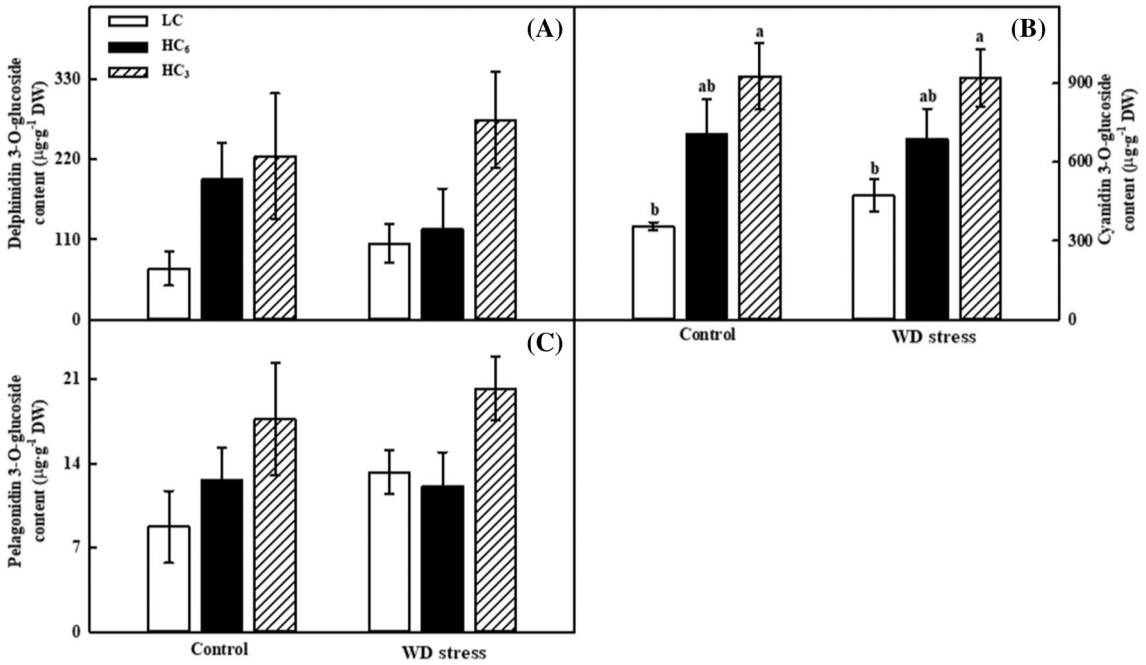

Fig. 5 Delphinidin 3-*O*-β-D-glucoside (**a**), cyanidin 3-*O*-glucoside (**b**), and pelargonidin 3-*O*-glucoside (**c**) content of dropwort plants grown under subirrigation (control) or water-deficit (WD) conditions at 6 weeks of transplanting ($n=4$). LC: low CO_2 concentration (600 μmol mol^{-1}) for 6 weeks. HC_6: high CO_2 concentration (1000 μmol mol^{-1}) for 6 weeks. HC_3: high CO_2 concentration (1000 μmol mol^{-1}) for the last 3 weeks. Different letters within each set of columns indicate significant differences ($p \leq 0.05$, Tukey's test)

Fig. 6 Leaves of dropwort plants subjected to combinations of water-deficit (WD) and high CO_2 concentration (HC) treatments at 6 weeks of transplanting. **a** Control + low CO_2 concentration (LC), **b** Control + HC_6, **c** Control + HC_3, **d** WD + LC, **e** WD + HC_6, and **f** WD + HC_3. LC: 600 μmol mol^{-1} CO_2 for 6 weeks. HC_6: high CO_2 concentration (1000 μmol mol^{-1}) for 6 weeks. HC_3: high CO_2 concentration (1000 μmol mol^{-1}) for the last 3 weeks

4.2 Net photosynthetic rate, transpiration rate, and stomatal conductance

The elevated CO_2 concentration increases the photosynthetic rate, which increases plant growth (Drake et al. 1997; Pérez-López et al. 2012). Regardless of WD, the net photosynthetic rate was increased by HC treatments through the whole period of the treatments, but the stomatal conductance and transpiration rate seemed to be unaffected by HC from 4 weeks of transplanting (Fig. 2). Therefore, stomatal conductance might be sensitive to HC during early growth regardless of WD, and the net photosynthetic rate may increase irrespective of stomatal conductance or transpiration rate. Similar results have been reported by Robredo et al. (2007) and Pérez-López et al. (2012), who investigated the combined effects of WD and HC in barley. Elevated atmospheric CO_2 greatly increases the CO_2 gradient between the inside and outside of the stomata, thereby increasing the CO_2 diffusion rate. This augmented CO_2 supply might increase the rate of Rubisco carboxylation in the chloroplasts and ultimately improve CO_2 assimilation, regardless of impaired stomatal conductance (Pérez-López et al. 2012).

Under LC conditions, neither the stomatal conductance nor the transpiration rate was significantly different between the WD-treated plantlets and controls. However, the net photosynthetic rate of the WD-treated plantlets decreased sharply in the latter half of the growth period. Lee and Oh (2017) reported that the net photosynthetic rate of dropwort was decreased by WD stress. Meanwhile, under HC, the net photosynthetic rate of the WD-treated plantlets was higher than that of controls during the latter half of the growth period. These results are consistent with a previous report of the promotion of carbon cycling by HC in dry soil than in wet soil (Robredo et al. 2007). Therefore, our results imply that HC treatment may alleviate the decreased net photosynthetic rate of dropwort by WD stress.

4.3 TNCs

HC affected carbohydrate accumulation in dropwort (Table 2). The TNC content, including starch and soluble sugars (sucrose, glucose, and fructose), was significantly increased by HC treatments under both subirrigated plants and WD-treated plants, with the starch content accounting for 91–93% of the TNCs. Increased starch accumulation due to elevated CO_2 concentrations has also been observed in greater plantain, alpine meadow grass, and oil palm (Baxter et al. 1997; Fonseca et al. 1997; Ibrahim and Jaafar 2012).

Photosynthates are directly used for growth or as raw materials for lipid and protein synthesis (Ghasemzadeh and Jaafar 2011), and the accumulation of primary metabolites under HC is mainly caused by the increased photosynthetic rate due to the increased availability of carbon in the chloroplasts (Ibrahim and Jaafar 2012). Therefore, the increased net photosynthetic rate, but not shoot dry weight, under HC could be attributed to increased TNC production.

4.4 Mineral content

CO_2 enrichment has become an important factor in plant production during the past few years. The inhibitory effects of HC on the mineral content have been reported by Lee and Lee (1994). HC could decrease the foliar mineral content by decreasing stomatal conductance and transpiration rate (Kim et al. 2007). Consistently, our results showed that HC decreased the macronutrient content by decreasing stomatal conductance and transpiration rate (Table 3 and Fig. 2). CO_2 enrichment can increase agricultural yield but may also alter the chemical composition of plants (Loladze 2002). Microelements in plants are vital for human health, though little is known about their response to HC. Högy et al. (2009) reported that elevated CO_2 levels increased the macronutrient content, whereas we observed that the micronutrient content decreased under HC. Nonetheless, HC significantly enhanced the Zn content. Our findings suggest that the positive and negative implications for minerals depend on the experimental conditions employed and cultivars studied. Further studies are warranted to determine the mineral composition of dropwort under HC and WD stress.

4.5 Total phenolic content, antioxidant capacity, and PAL activity

Among primary metabolites, carbohydrates are involved in the growth of plants as well as in the production of secondary metabolites, such as the synthesis of phenolic compounds via the shikimic acid pathway (Ibrahim and Jaafar 2012). Herms and Mattson (1992) suggested that plants with elevated photosynthetic rates due to HC can accumulate carbohydrate levels that exceed their growth requirements, thus making them available for allocation to the carbon-based secondary metabolite biosynthetic pathways, i.e., the growth and differentiation balance model. In this study, the total phenolic content and antioxidant capacity of the HC-treated plantlets were significantly greater than that of the LC-treated plantlets, regardless of WD treatments (Fig. 3). Previous studies reported that HC increased the carbon-based secondary metabolite content and antioxidant capacity (Ghasemzadeh and Jaafar 2011; Ibrahim and Jaafar 2012; Mattson et al. 2005). Therefore, our results suggested that TNC accumulation under HC might enhance the allocation of carbon compounds to carbon-based secondary metabolite production.

Meanwhile, WD increases ROS production, thereby directly or indirectly activating the biosynthesis of secondary metabolites with antioxidant properties (Ksouri et al.

2007; Oh et al. 2010; Sánchez-Rodríguez et al. 2011). In this study, the total phenolic content and antioxidant capacity of the WD-treated plantlets were significantly greater than those of subirrigated controls (Fig. 3). Oh et al. (2010) found that mild WD enhanced the content of phenolics, specifically chicoric acid, in lettuce. Sánchez-Rodríguez et al. (2011) reported that WD significantly increased the levels of flavonols, such as quercetin and kaempferol, in cherry tomatoes. In addition, our results suggest that NSW systems, which can easily control water content of growing medium (Oh et al. 2007), can be efficiently used to increase the total phenolic content and antioxidant capacity of dropwort.

In the shikimic acid pathway, PAL converts cinnamic acid to L-phenylalanine and is the first key enzyme of the phenylpropanoid pathway (Liu et al. 2006). Since various secondary metabolites, including phenolic compounds, are biosynthesized through the phenylpropanoid pathway (Jones 1984), PAL activation is a basic and important metric through which the effects of both WD and HC on secondary metabolite accumulation can be confirmed. In the absence of WD, the PAL activity values of the HC_6- and HC_3-treated plantlets were higher than that of the LC-treated plantlets, suggesting that elevated CO_2 concentrations stimulated secondary metabolite production through PAL activation (Fig. 4). Similar results were reported by Ibrahim and Jaafar (2012) in ginger. However, the PAL activity of the WD-treated plantlets was not affected by HC treatments, probably because PAL had already been activated by WD. Indeed, the PAL activity of the WD-treated plantlets was significantly greater than that of controls. The roles of the PAL gene and enzyme in phenolic compound accumulation under WD were reported previously (Oh et al. 2010; Sánchez-Rodríguez et al. 2011). These results were consistent with our previous report that the total phenolic content, antioxidant capacity, and PAL activity of dropwort plants grown under WD were significantly higher than those of controls (Lee and Oh 2017). This study also found that the PAL activity by HC and WD stress has a positive correlation with total phenolic content and antioxidant capacity in dropwort.

4.6 Anthocyanin content

Anthocyanins are water-soluble pigments that give many fruits and vegetables their red or violet color (Ghasemzadeh et al. 2012). Previous studies have reported that anthocyanin biosynthesis was activated by high salinity (Eryılmaz, 2006) and WD (Balakumar et al. 1993; Hughes et al. 2010). In this study, the effects of HC on anthocyanin accumulation were more pronounced than those of WD (Fig. 5). The content of C3G, which is a major anthocyanin in dropwort, was significantly increased by HC treatments; in particular, HC_3 treatment had more influence on the anthocyanin increase than HC_6 treatment. This result suggests that CO_2 enrichment, especially when treated in the late growth stage, is more effective than water restriction to augment anthocyanin accumulation in dropwort.

5 Conclusion

In this study, we evaluated the effects of mild WD and HC on the accumulation of primary and secondary metabolites in dropwort. HC improved the net photosynthetic rate but did not affect biomass accumulation. Instead, the content of TNCs and secondary metabolites increased by both WD and HC. Excess TNCs by HC might be used to synthesize secondary metabolites through PAL activation. Collectively, our results indicate that reddish small dropwort is more useful than greenish dropwort as a functional food and can be easily produced using CO_2 enrichment when grown in environmental control systems, such as greenhouses and plant factories with artificial light. In addition, the cultural practice of CO_2 enrichment can improve the industrial value of small dropwort cultivated for food processing.

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Author contribution Lee JY carried out the measurements, data analysis and drafted the manuscript. Lee JH and Son KH were involved in the manuscript refinement. Oh MM made a substantial guide about experiment design and critically revised the manuscript.

Data availability The data are available within the article.

Declarations

Conflict of interest The authors declare no conflict of interest.

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