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Research Article

Effects of temperatures on growth, physiological, and antioxidant characteristics in *Houttuynia cordata*

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Abstract

Houttuynia cordata Thunb. (HC) is a traditional medicinal plant with a variety of pharmaceutical activities. The objective of this study was to investigate the growth, photosynthetic parameters, and antioxidant properties of HC plants in response to various temperatures. Pots of HC plants were maintained in day/night temperatures of 15/10 °C, 20/15 °C, 25/20 °C (control), 30/25 °C, and 35/30 °C for two months in each of five growth chambers having a 13.5 h photoperiod at 396, 432, 474, 449, and 619 $\mu mol \cdot m^{-2} \cdot s^{-1}$ radiation, respectively. Eight plants for each temperature were randomly placed in a growth chamber. HC plants survived at 30/25 °C and 35/30 °C treatments and had significantly higher plant heights, leaf numbers, and soil-plant analysis development (SPAD) and normalized difference vegetation index (NDVI) values compared to other treatments. However, long-term 35/30 °C treatment caused reductions in leaf length and width, significantly decreasing shoot and leaf fresh weight (FW) and dry weight (DW) compared to 30/25 °C treatment and controls. These results indicate that HC leaf development was affected during the 35/30 °C treatment, and that both SPAD and NDVI can help in advancing our understanding of the photosynthesis process in HC. Moreover, all plants subjected to 15/10 °C suffered more severely in all traits and parameters than other treatments. Therefore, HC plants tended to be heat-tolerant and exhibited adaptive morphologic plasticity to 30/25 °C conditions. Positive and significant correlations were observed among temperatures and total phenolics (TP), total flavonoids (TF), chlorogenic acid (CGA), and hyperoside (HO) content, and all bioactive contents increased as temperature increased, except that both CGA and HO content were remarkably decreased after 30/25 °C treatment. Thus, 30/25 °C treatment would be more beneficial for high marketability resulting from increased leaf number, DW, and all secondary metabolites compared to other treatments, and for use as a health food and for medicinal purposes. In addition, leaf growth, physiological parameters, and secondary metabolite accumulations in HC plants can be optimized for commercial production via temperature control technologies. This approach may also be applicable to leafy vegetables to produce stable industrial supplies having high leaf yields and metabolite content.

Keywords: flavonoids; medicinal plant; phenolic compounds; spectral reflectance; temperature stress

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Introduction

Houttuynia cordata Thunb. (HC) is an edible and aromatic herb belonging to the family Saururaceae, and has been widely used in medicine, functional food products, cosmetics, and other products (Wu *et al.*, 2021). It is a pungent, perennial, and rhizomatous plant usually grown as a leaf and root vegetable. Despite their unpleasant fishy smell, young leaves of HC are a popular vegetable and used as a fresh herbal garnish and in tea or salads in Taiwan. HC is native to Southeast Asia, cultivated mainly in Mediterranean countries, and restricted to and specialized to grow in moist and shady habitats (Wang *et al.*, 2016). There is growing interest in using natural compounds and their applications in food, nutrition, and medical treatments, and the secondary metabolites and bioactive phytochemicals from these medicinal plants have been explored and targeted in drug discovery (Shingnaisui *et al.*, 2018). Previous studies have shown that HC plants possess a number of pharmacologically important activities, such as being hypoglycemic (Fu *et al.*, 2013), anti-leukemic (Lou *et al.*, 2019), anti-carcinogenic (Kim *et al.*, 2008), anti-inflammatory (Li *et al.*, 2011), antioxidant (Kumar *et al.*, 2014), and others (Tian *et al.*, 2012; Yoon and Shim, 2015).

Plants have developed different adaptive mechanisms to cope with temperatures by inducing physiological responses; hence, understanding the physiological mechanisms of resistant HC in response to high temperatures is important. High temperatures reduce the ability of photosynthesis to utilize incident photons, leading to photoinhibition, a concomitant reduction in the quantum yield of photochemistry, and a decrease in chlorophyll fluorescence (ChlF). ChlF measurements, such as the maximal quantum yield of PSII photochemistry (Fv/Fm), are noninvasive techniques widely used in a range of photosynthetic organisms and tissues to study functional changes in the photosynthetic apparatus under different heat stress conditions in controlled environments and in the field (Lin *et al.*, 2020), but no effort has been made to study photosynthetic indices in response to various temperatures in HC. Reflectance spectroscopy is another underexploited, noninvasive technique that can be used in physiological studies, as it is simple, rapid, and nondestructive. Reflectance spectra are altered when stress occurs, and these alterations can be used to calculate different vegetation indices such as the adjusted normalized difference vegetation index (NDVI), which has been linked to photosynthetic light-use efficiency (Ballester *et al.*, 2018). Soil Plant Analysis Development (SPAD) values have been widely examined in a number of plants to determine injury or tolerance to various environmental stresses, as they assess total Chl contents and photosynthetic capacity (Rahbarian *et al.*, 2011). Previously, we found that SPAD, NDVI, and Fv/Fm indices were accurate proxies of responses to various temperatures and soil water content, and thus can be used to maximize efficiencies in growth and the production of antioxidants and other bioactive compounds in *Perilla* plants under temperature and water stressing (Lin *et al.*, 2020). Therefore, in the present study, we attempt to determine whether these indices exhibit distinguishable differences in the growth, physiological, and antioxidant characteristics of HC plants subjected to various temperatures. The effective management of these parameters in response to temperatures provides a better understanding of the photosynthetic characteristics of HC plants grown in a specific temperature.

Phenolic compounds, particularly flavonoids, are an important group of plant secondary metabolites, and the accumulation of different types of flavonoids helps plants cope with various environmental stresses (Jiang *et al.*, 2016). Generally, plants produce diverse secondary metabolites in response to their environment, and temperatures affect their photosynthesis and have bearing on their chemical composition (Rouphael *et al.*, 2018). Low temperature treatments result in higher total phenolics (TP) content than high temperatures in *Rehmannia glutinosa* under temperature stress, based on the quantification of 16 phenolic compounds (Chung *et al.*, 2006). However, *Polygonum minus* plants under higher temperature treatments produce more flavonoid compounds than lower temperatures (Goh *et al.*, 2016). Several primary and dominant antioxidants and secondary metabolites, such as TP, total flavonoids (TF), chlorogenic acid (CGA), and hyperoside (HO), are chemically identified in HC (Meng *et al.*, 2009; Chou *et al.*, 2009; Nuengchamnong *et al.*, 2009; Tian *et al.*, 2011; Hsu *et al.*, 2016; Nguyen *et al.*, 2020). However, the accumulation of TP, TF, CGA, and HO compounds in HC plants grown under various temperatures has not yet been studied, nor whether these phytochemicals

in HC are affected by drastic changes in climate and environment. Thus, the content of these particular secondary metabolites in the leaves of HC plants were examined in this study. The hypothesis of this study was that the photosynthetic components and production of bioactive compounds in HC leaves might exhibit distinguishable differences under various temperatures. The aims of this study were to investigate the effects of temperature on leaf growth, photosynthetic parameters, and secondary metabolite accumulations in HC leaves, and if the application of photosynthetic indices can efficiently determine temperature-induced changes in TP, TF, CGA, and HO content in HC. This research provides a scientific basis for the cultivation and management of HC, and contributes to our understanding of the acclimation by HC to optimal temperatures, using it as a natural antioxidant, and increasing its bioactive compound content for use in the food industry.

Materials and Methods

Plant materials and cultural practice

Healthy HC plants were obtained from local flower shops in Taipei, Taiwan, on July 19, 2019.HC rhizome cuttings with 3 ~ 4 nodes were collected and placed on wet paper towels in plates containing a thin layer of distilled-deionized (dd) water for 20 days, followed by planting in 3-inch plastic pots containing commercial potting soil with a 3:1(v/v) mixture of peat moss and Perlitefor 20 days. When plants were 2 cm tall with four leaves, they were transplanted into 5-inch plastic pots (one plant per pot) containing the above-mentioned commercial potting soil, and placed in a greenhouse at National Taiwan University (NTU, latitude 25.01°N). The average temperature in the NTU greenhouse was 25 °C, with 12 h day lengths and150 $\mu mol \cdot m^{-2} \cdot s^{-1}$ photosynthetic photon fluxin July and August, 2019. They were evenly spaced to encourage similar growth rates and sizes. Plants were watered twice a week, and a compound fertilizer solution (20N-8.8P-16.6K water-soluble fertilizer at 1 $g \cdot L^{-1}$, Peter's 20-20-20, Marysville, Ohio, USA) was applied weekly throughout the experiment. Plants were grown for 25 days, and those with a uniform size were selected and randomly separated into five groups for temperature experiments in an environmentally controlled growth chamber at NTU. Pots of HC plants were transferred to five growth chambers maintained at 15/10 °C, 20/15 °C, 25/20 °C (control), 30/25 °C, and 35/30 °C for two months (September 2 ~ November 2, 2019) under a 13.5h photoperiod at 396, 432, 474, 449, and 619 $\mu mol \cdot m^{-2} \cdot s^{-1}$ radiation, respectively. Eight replicates (plants) for each temperature group were randomly selected. Both 30/25 and 35/30 °C were considered heat stress temperatures in this study because our previous experiments illustrated that HC plants at 40/35 °C for eight weeks clearly showed heat stress phenotypes with differing levels of severity including plant death (data not shown).

Plant growth measurements

The following horticultural performance indicators were measured at the end of the two-month experimental period:

1. Plant height, measured as the height (cm) above the soil substrate with a Vernier caliper.
2. Number of leaves, recorded as all the fully expanded leaves of a plant.
3. Leaf length, measured as the length (cm) between the base and tip of a fully expanded third leaf with a Vernier caliper.
4. Leaf width, measured as the maximum width (cm) of a fully expanded third leaf with a Vernier caliper.
5. Fresh weight (FW) of shoots and leaves, measured as green shoots and leaves, cut at the soil substrate surface to assess biomass accumulation with an electronic balance.
6. Dry weights (DW) of shoots and leaves, the leaves from each plant being removed from shoots and weighed separately, followed by measuring shoots and leaves after drying in an oven at 70 °C for two days.

Measurements of ChlF, spectral reflectance, and total Chl content

The potted plants were moved into the shade under a cottage before sunrise at 05:30~06:00, and then the ChlF parameters of dark-adapted leaves were measured with a portable fluorometer (MINI-PAM, Walz, Effeltrich, Germany) at ambient temperature after adaptation to the dark for 20 min. The middle portions of fully expanded third leaves of each plant were used for the measurements. Values of the minimal ChlF (Fo) and maximal ChlF (Fm) of dark-adapted samples were respectively determined using the modulated irradiation of a weak LED beam (measuring light) and a saturating pulse. We then calculated the maximum photochemical quantum yield (Fv/Fm), where Fv, the yield of variable fluorescence, was calculated as (Fm - Fo). When measuring Fv/Fm, samples were first acclimated to dark conditions to ensure that all reaction centers were in an open state and there was minimal non-photochemical dissipation of excitation energy (Porcar-Castell *et al.*, 2008). Measurements were recorded using WinControl-3 software (Heinz Walz, Effeltrich, Germany).

Spectral reflectance was measured from mature, healthy, fully expanded third to fourth leaves of each plant at wavelengths of 200~900 nm using an integrating sphere fitted to a scanning spectrophotometer (PolyPen RP 400, Photon Systems Instruments, Prague, Czech Republic). The adjusted normalized difference vegetation index (NDVI) was calculated as (R750 - R705) / (R750 + R705 - 2 × R445) (Weng *et al.*, 2010).

Healthy, fully expanded mature leaves from the middle to upper portion of each plant were used to determine total chlorophyll content using a soil-plant analysis development (SPAD) analyzer (SPAD- 502 Chlorophyll meter reader, Konica Minolta, Tokyo, Japan).

Analysis of TP, TF, CGA, and HO content

The middle portions of fully expanded third to fourth leaves were collected, and first rinsed with running water and then with dd water. Washed leaves were next placed in the chambers of a tray shelf Freeze Dryer (FD-20B3P, Taipei, Taiwan) for three days, and the dried leaves were powered with a grinder.

Ten mg of a freeze-dried powdered leaf sample were immersed in 1 mL of 80% methanol, shaken for 1 h, centrifuged at 9,000 rpm for 5 min, and the supernatant obtained as crude *H. cordata* leaf extract (HLE) used for analysis of TP, CGA, and HO content and concentrations. TP content was determined as previously described (Pourmorad *et al.*, 2006). An aliquot of HLE and standard gallic acid were added to 100 μL of Folin-Ciocalteau reagent (Sigma Aldrich) for 5 min holding, then mixed with 400 μL of 7% Na_2CO_3 for 15 min, and then the supernatant was mixed into each 200 μl sample and allowed to equilibrate for 3 min. Absorbance at 760 nm was measured at room temperature using a 96-plate microplate reader (InterMed, South Portland, ME, USA). A standard curve for gallic acid (20 ~100mg L^{-1}) was used to calculate TP levels. The TP content was expressed as mg of gallic acid equivalent (GAE) per g of DW. The standard curve equation was y = 0.0249x + 0.0595, r = 0.9589. The assay was run in triplicate for each sample.

Similar to TP content analysis, 25 mg of a freeze-dry powdered leaf sample were immersed in 750 μL of 70% ethanol, shaken for 1 h, centrifuged at 9,000 rpm for 5 min, and the supernatant as HLE used for the analysis of TF content and concentration.TF in extracts was determined by the method of Saravanan and Parimelazhagan (2013). Briefly, 1 mL of HLE was mixed with 1 mL of 70% ethanol, 0.3 mL of $NaNO_2$ (5%) and $Al(NO_3)_3$ (10%), and 2 mL of 4% NaOH, and the mixture stirred and kept at room temperature for 20 min. Absorbance was measured at 510 nm on a spectrophotometer. Rutin was used as a reference standard, and TF content was expressed as milligrams of rutin equivalent (RE) per gram of DW (mg RE·g^{-1} DW). The linear range of the calibration curve was 20 ~ 100 mg L^{-1} (r = 0.998).

To separate and identify the individual phenolic compounds CGA and HO in the HLE, reverse-phase C18 high-performance liquid chromatography (HPLC) was used as described previously (Lu *et al.*, 2006; Meng et al., 2009). A Hypersil ODS C18 column (250 x 4.6 mm, 5μm) was connected to an LC-1379 HPLC system (Perkin-Elmer, Waltham, MA, USA). Five μL of the 80% methanol-extracted HLE was used for the HPLC analysis after filtration through a 0.22-μm syringe filter (Millex-GV, Millipore), which was injected and eluted with formic acid and acetonitrile. The flow rate was 1 mL min^{-1} at 30 °C and the injection volume was 5 μL. A 2% formic acid aqueous solution (v/v, solvent A) and acetonitrile (solvent B) were used to establish the gradient

elution as follows: 0-8 min at 12% B; 10-15 min linear gradient from 12% to 15% B; 28-30 min linear gradient from 15% to 100% B; and 33-36 min at 100% B for column equilibration. The eluent's absorbance at 345 nm was scanned with an LC-1315A diode array detector (Hewlett Packard, San Jose, CA, USA). Peaks were identified by comparing the retention time and ultraviolet absorption spectrum of the eluting peaks with authentic standards for CGA and HO (Sigma Aldrich), and were used to identify the phenolic compounds of *H. cordata*. A series of standard solutions dissolved in 80% methanol ranging 25 ~ 200 mg mL^{-1} was tested to determine the calibration curve. Regression equations for CGA and HO were calculated in the form of y = ax + b, where y and x were the peak area and amount of standard injected, respectively, and all calibration curves had coefficients of linear correlation $r^2 > 0.990$.

Statistical analysis

Data for the measurements of phenotypic traits, spectral reflectance, and antioxidant ability were analyzed by analysis of variance (ANOVA) according to a completely randomized experimental design. Means were separated for each parameter using Fisher's least significant difference (LSD) test at $p < 0.05$ by CoStat 6.4 (CoHort Software, Monterey, CA). To investigate the relationship between temperatures and TP, TF, CGA, and HO content, the coefficients of r^2 were examined by regression analyses. Model datasets were developed based on 24 leaves per temperature treatment group, and temperatures were recorded at 15/10 °C, 20/15 °C, 25/20 °C, 30/25 °C, and 35/30 °C for two months (September 2 ~ November 2, 2019) in each of five growth chambers. Two models were tested, with linear and nonlinear regression models being selected as the best interpretations of the relationships between TP and TF content and CGA and HO content, respectively. All models were evaluated for goodness of fit by graphical analysis of residuals in computing r^2.

Results

Temperature effects on the growth traits of HC plants

Table 1 illustrates how plant height, leaf number, leaf length, leaf width, and both DW and FW of shoots and leaves differed in five different temperature treatments after eight weeks of cultivation. Plant heights with daytime 35 °C treatment (11.03 cm) and 30 °C treatment (14.04 cm) were significantly taller than with other temperature treatments (range, 3.83 ~ 9.33 cm), indicating that high temperatures increased plant height (Figure 1). In addition, leaf numbers per plant subjected to both 35 and 30 °C treatments (11.1 and 11.4, respectively) were also significantly more than in other temperature treatments (ranging 8.2 ~ 10.4), suggesting that the increase in leaf numbers was caused by changes in plant height in response to increasing temperatures. However, when plants were subjected to 25 and 20 °C treatments, plants had significantly longer leaf lengths (respectively 9.2 and 9.4 cm) and leaf widths (7.6 cm) than other temperature treatments. Shoot FW under 30 °C treatment was significantly higher (14.51 g) than with other temperature treatments (ranging 9.09 ~ 13.26 g). Similar trends and rates of FW in leaves were also observed, and maximal and significant increases in leaf FW occurred in the 30 °C treatment with 11.43 g/plant compared to other treatments (ranging 7.47 ~ 10.59 g/plant). Significantly higher shoot and leaf DW were detected in both the 30 °C treatment (2.22 and 1.78 g/plant, respectively) and 25 °C treatment (2.06 and 1.70 g/plant, respectively) compared to other temperature treatments.

Table 1. Effects of temperature on plant height, leaf number, leaf length, leaf width, shoot fresh and dry weights, and leaf fresh and dry weights of *H. cordata* plants in a growth chamber over a 60-day period after temperature treatment

Day/night temperature (°C)	Plant height (cm)	Number of leaves (per/plant)	Leaf length (cm)	Leaf width (cm)	Shoot		Leaf	
					Fresh weight (g/plant)	Dry weight (g/plant)	Fresh weight (g/plant)	Dry weight (g/plant)
35/30	11.03 b	11.1 a	7.0 d	6.2 c	11.23 c	1.79 b	8.99 c	1.44 b
30/25	14.04 a	11.4 a	8.2 c	6.8 b	14.51 a	2.22 a	11.43 a	1.78 a
25/20	9.33 c	10.4 b	9.4 a	7.6 a	13.26 b	2.06 a	10.59 b	1.70 a
20/15	6.29 d	9.4 c	9.2 a	7.6 a	12.04 c	1.70 b	9.73 c	1.43 b
15/10	3.83 e	8.2 c	8.6 b	7.0 b	9.09 d	1.15 c	7.47 d	0.99 c

Means with the same lowercase letter within a column do not significantly differ by the least significant difference (LSD) test at $p < 0.05$. n =8

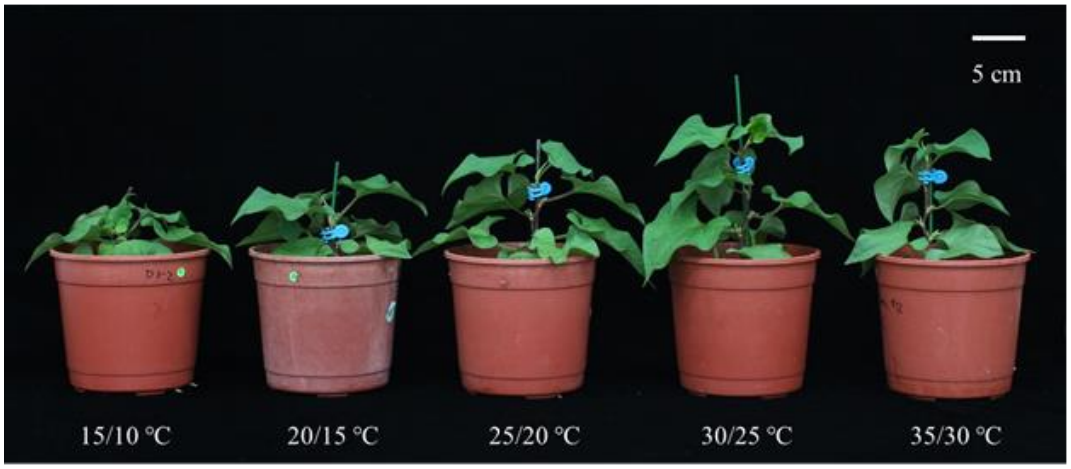

Figure 1. Effects of different day/night temperatures on the morphological appearance of *Houttuynia cordata* in a growth chamber over a 60-day period after temperature treatments. White bar indicates 5 cm

Effects of different temperatures on photosynthetic parameters in HC plants

Table 2 shows the different responses towards ChlF and spectral reflectance values for different temperature treatments. SPAD values with 35 °C treatment was significantly higher (55) than other temperature treatments (ranging 48.2 ~ 51.5). NDVI showed significantly higher values with 35 °C treatment (0.658) and 30 °C treatment (0.665) compared to other temperature treatments (ranging 0.626 ~ 0.644). It is noteworthy that NDVI values increased in all plants as the temperature increased, suggesting that long-term high temperature treatments increased these values in HC plants. Nevertheless, no significant differences in Fv/Fm values were observed in any temperature treatments, suggesting that Fv/Fm is not a sufficient parameter to determine the adaptability of these tested plants to temperatures.

Influences of temperature on secondary metabolites in HC

Table 3 presents the concentrations and content of TP, TF, CGA, and HO in extracts of HC leaves grown under various temperatures, and shows that different temperatures displayed variations in secondary metabolic levels. TP concentrations in these plants under 35 °C and 30 °C treatments at 30.4 and 25.5 mg GAE (g DW)$^{-1}$, respectively, were significantly higher than in other temperature treatments, ranging 16.5 ~ 19.9 mg GAE (g DW)$^{-1}$. When TP content was calculated on the plant level, plants also had significantly higher TP content in response to 35 °C and 30 °C treatments (respectively 45.4 and 4.1 mg GAE per plant) compared to other treatments (ranging 16.3 ~33.6 mg GAE per plant). TP concentration and content increased as temperature increased. TF levels in HC plants showed similar trends and rates to TP levels under various

temperatures. For example, compared to 25 °C , 20 °C, and 15 °C treatments, plants subjected to 35 °C and 30 °C treatments contained significantly higher TF concentrations (respectively 68.4 and 53.8 mg RE $(g\ DW)^{-1}$) and TF content (respectively 98.7 and 96.2 mg RE per plant). In particular, HC plants under 35 °C treatment displayed a significant three-fold increase compared to the 15 °C treatment (31.4 mg RE per plant). Furthermore, significantly higher CGA concentration and content were observed in the 30 °C treatment (57. 0 mg $(g\ DW)^{-1}$ and 111.8 mg per plant, respectively) compared to other treatments, and they were also remarkably higher (a three-fold increase) than in the 15 °C treatment (37.0 mg per plant). The patterns and trends in the HO concentration and content of HC appeared to be similar to those of TP and TF concentration and content under various temperature treatments. All tested plants had significantly higher HO concentration and content in the 35 °C treatment (respectively 41.1 mg $(g\ DW)^{-1}$ and 61.0 mg per plant) and 30 °C treatment (respectively 36.4 mg $(g\ DW)^{-1}$ and 64.8 mg per plant) compared to other treatments.

Table 2. Effects of temperature on soil-plant analysis development (SPAD), normalized difference vegetation index (NDVI), and maximum photochemical quantum yield (Fv/Fm) values of *H. cordata* plants in a growth chamber over a 60-day period after temperature treatments

Day/night Temperature (°C)	SPAD	NDVI	Fv/Fm
35/30	55.0 a	0.665 a	0.81 a
30/25	51.5 b	0.658 a	0.82 a
25/20	48.6 c	0.644 b	0.82 a
20/15	48.2 c	0.638 b	0.82 a
15/10	49.6 bc	0.626 c	0.82 a

Means with the same lowercase letter within a column do not significantly differ by the least significant difference (LSD) test at $p < 0.05$. n= 8

Table 3. Effects of temperature on total phenolics concentration and content, total flavonoids concentration and content, total chlorogenic acid concentration and content, and total hyperoxide concentration and content of *H. cordata* plants in a growth chamber over a 60-day period after temperature treatments

Day/night temp. (°C)	Total phenolics		Total flavonoids		Chlorogenic acid		Hyperoside	
	Conc. (mg GAE/g dw)	Content/ plant (mg GAE)	Conc. (mg RE/g dw)	Content/ plant (mg RE)	Concentra-tion (mg/g dw)	Content/ plant (mg)	Concentra-tion (mg/g dw)	Content/ plant (mg)
35/30	30.4 a	45.4 a	68.4 a	98.7 a	50.8 b	78.5 b	41.1 a	61.0 a
30/25	25.5 b	44.1 a	53.8 b	96.2 a	57.0 a	111.8 a	36.4 b	64.8 a
25/20	19.9 c	33.6 b	39.9 c	67.6 b	47.3 c	80.6 b	30.9 c	53.7 b
20/15	16.9 d	24.3 c	33.4 d	47.8 c	35.6 d	44.8 c	24.8 d	35.1 c
15/10	16.5 d	16.3 d	31.9 d	31.4 d	37.5d	37.0 c	28.7 c	30.1 c

Means with the same lowercase letter within a column do not significantly differ by the least significant difference (LSD) test at $p < 0.05$. n=8

Relationships between temperatures and TP, TF, CGA, and HO content

The relationships among temperatures and TP and TF content were linear (Figures 2A and B), but between CGA and HO content were nonlinear (Figures 1C and D). Regression analysis showed that TP, TF, CGA, and HO content were highly and significantly correlated with temperature, with $r^2 = 0.87$, 0.90, 0.86, and 0.87 ($p < 0.001$), respectively. The content of TP and TF increased as temperature increased, suggesting that temperatures measured by TP and TF content can be used for predicting TP and TF content in HC leaves. While CGA and HO content increased with increasing temperatures from 15 to 30 °C, their content beyond this temperature started to decline, indicating that optimal CGA and HO content were obtained in the 30 °C treatment.

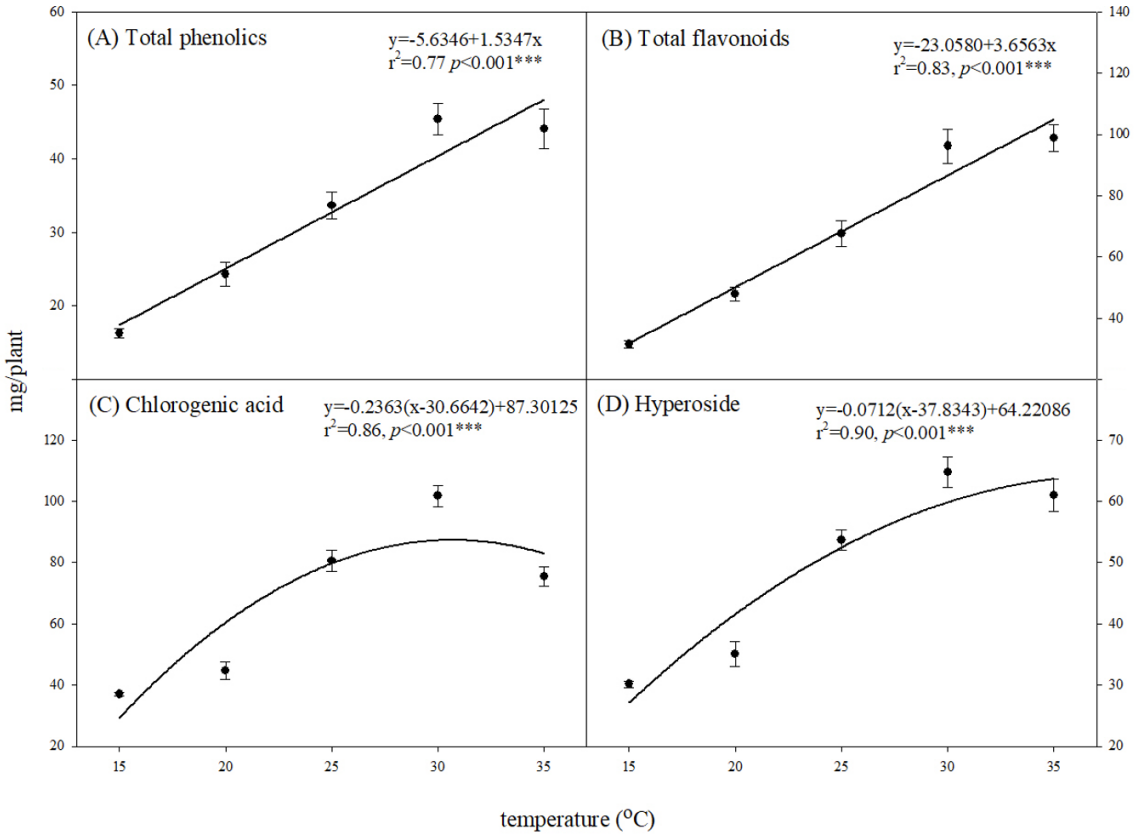

Figure 2. The relationship between temperature and total phenolics (A), total flavonoids (B), chlorogenic acid (C) and hyperoside (D) content of *H. cordata* plants

Measurements were recorded at 15 °C , 20 °C, 25 °C, 30 °C, and 35 °C for two months (September 2 ~ November 2, 2019) in each of five growth chambers. Each data point represents the mean ± standard deviation of three leaves from eight replicates for each temperature treatment. Each chemical content was calculated using temperature data from the model's validation datasets, and the determination coefficient (r^2) provides a measure of regression model fit with the model *p*-values of model significance at $p < 0.001$ denoted with ***

Discussion

Many types of morphologic stresses occur when plants encounter a high temperature stress, and the tested plants' visual appearances did not show obvious changes after eight weeks of 35 and 30 °C treatments (Figure 1). Visual observations indicated that these temperatures produced HC plants with acceptable leaves. Most of the upper and middle portions of these leaves appeared to have a healthy green color and revealed no visible signs of high-temperature damage (e.g., shoot burn or leaf necrosis) when grown under both 35 and 30 °C conditions compared to other temperatures for two months. Thus, those plants tended to be unaffected and exhibited adaptive morphologic plasticity. Nevertheless, the leaves of 15 °C treated plants turned reddish and withered. Moreover, the growth of 15 °C treated plants displayed retardation, and plant height increased only 0.87 cm in the two-month period. In addition, the growth of the HC plants tended to be more sensitive to 35 and 30 °C treatments than to 25 °C controls, showing significantly decreased leaf length and width in the upper and middle portions of their leaves. These differences can possibly be attributed to the evolutionary adaptations of various organs or tissues for growth under different temperatures.

Leaf Chl content is considered an appropriate criterion for evaluating the physiological status of plants, and many theoretical models based on leaf reflectance have been developed to predict leaf Chl content and other variables associated with vegetative structure (Hernandez-Clemente *et al.*, 2011). NDVI values is not

only used to monitor plant growth condition, but also illustrates Chl content (Peñuelas *et al.*,1995) and reveals different abilities and specificities in photosynthetic light-use efficiency in plants (Ballester *et al.*, 2018). HC plants survived at high temperatures and had higher plant height, leaf number, SPAD, and NDVI values compared to other treatments, indicating that this species persists under heat stress conditions, whereas cultivation in a cooler temperature (15 °C) was not favorable for these growth traits. High temperature stress acts on primary signaling molecules for regulating plant height and new leaves. Our results suggest that physiological plasticity to different temperatures, which helps to maintain a high level of Chl under conditions of high temperatures, thereby allows HC plants to survive and function during 35 and 30 °C treatments. Within the range of plasticity possible at each temperature level, plants adjust their Chl levels and photosynthetic light-use efficiency in response to temperature, and high temperature treatments may alter photosynthesis in HC species. Therefore, HC was able to acclimatize to high temperatures. It seems to be a heat-tolerant species showing adaptability to heat. Nevertheless, long-term 35 °C treatment caused reductions in HC leaf length and width, and shoot and leaf FW and DW significantly decreased compared to controls, indicating that the leaf development of these plants was affected during 35 °C treatment.

Heat causes many problems for plants that include leaf wilting, increased respiration, decreased photosynthesis reactions, and overproduction of reactive oxygen species (Kreslavski *et al.*, 2009). The need for heat-tolerant plant cultivars is increasing because of rising global temperatures. One of the objectives of this study was to employ nondestructive measurements to determine the total leaf Chl and photosynthetic values and develop accurate, integrated, and quantitative measurements of HC under various temperature conditions which could help create better agricultural methods in relation to current global warming predictions. In trying to understand the responses to high temperature, leaf-monitoring photosynthetic parameters were identified and characterized after heat stress, and the effects of high temperatures on the appearance and physiological characteristics of HC were examined in this study. These different photosynthetic parameters are highly sensitive indicators representing the physiological status of stressed plants and provide a quick means of identifying a plant's physiological condition (Ambrosio *et al.*, 2006). In particular, both SPAD and NDVI were heat stress specific and not expressed solely in response to an increasing excess of photosynthesis, but were suitable for evaluating the morphology subjected to specific temperature treatment. This indicates that they can be used for the rapid monitoring and detection of various temperature-sensitive signals during the vegetative growth stage to screen for individual plants exhibiting tolerance to high temperatures. Thus, they also enable a more efficient use of land when evaluating new material in the field when used for the rapid monitoring and early detection of heat injury in vegetative stages. Applying a combination of tools enables the exploration of and explanation for temperature responses; for instance, combining SPAD (> 50) and NDPI (> 0.650) values after high temperature treatment in a growth chamber comprehensively measures plant and leaf growth and allows for selection against the most susceptible plants by developing indices for the nondestructive Chl estimation of plant leaves and indicating plant photosynthesis. This means that many hundreds of individual plants grown under heat stress can be cost-effectively screened per day, providing more opportunities to discover individuals that manifest plant and leaf growth and development indicators and exhibit greater photosynthesis. Therefore, our results can be applied to improve the heat stress tolerance of HC plants and aid their effective cultivation for farming in areas with hot climates. However, our data still reflect the physiological attributes that contribute to our perception of plant eco-physiology and subsequent growth in outdoor planting sites. To develop a practical technique for manipulating temperature requires experimentation with more cultivars, and further studies are needed to confirm specific goals.

Photosynthesis is sensitive to environmental changes, and under natural conditions photosynthesis is biochemically regulated in response to environmental changes to maintain a balance between the rates of component processes and secondary metabolite levels (Habibi, 2018). Temperature affects the accumulation of secondary metabolites. Increases in all tested metabolic concentrations and contents of HC plants clearly occurred under 35 °C and 30 °C treatments compared to 25 °C, 20 °C, and 15 °C treatments, indicating that the former plants can be used for health foods and medicinal purposes due to their high secondary metabolite

biosynthesis. HC is native to moist and shady locations, so high temperatures may induce this species to produce more secondary metabolites from higher Chl levels when under heat stress. Positive and significant correlations were observed among temperatures and TP, TF, CGA, and HO content, and all metabolites increased in content as temperature increased, except that both CGA and HO content were remarkably decreased after 30 °C treatment. A strong correlation is also observed between temperature and the production of phenolics in strawberry (Wang and Zheng, 2001). Levels and fluctuations in metabolite content are a plant's responses to temperatures and part of an adaptive strategy leading to tolerance to heat stress. The 30 °C treatment was beneficial for HC plants in increasing plant height, leaf number, shoot and leaf FW, and all secondary metabolite levels compared to controls, whereas 20 °C treatment increased leaf length and width but was not beneficial to leaf number, shoot and leaf FW and DW, and the synthesis of secondary metabolites from lower Chl levels. The HC leaf is a popular vegetable that is treated as an herb, a functional food, and a nutraceutical product. Nguyen *et al.* (2020) reported that the HC leaf contains abundant polyphenol compounds, and the influence of its extraction solvent on the total content of phenolic compounds extracted is different. The TP content in ethanol was highest (97.98 ± 1.77 mg GAE/g), followed by diethyl ether (32.18 ± 2.64 mg GAE/g) and aqueous dd water (22.22 ± 2.00 mg GAE/g). Moreover, TF content was highest in ethanol (35.72 ± 1.23 mg QE/g), followed by diethyl ether (31.65 ± 1.07 mg QE/g) and aqueous (4.06 ± 0.54 mg QE/g). In our study, the utilization of HLE offers the possibility for being a natural food additive and food preservative, and it could be developed as a functional food and for nutraceutical use. Notably, wide variations in secondary metabolite levels were observed in HC plants possessing relatively higher TF and CGA levels than TP and HO in each individual temperature treatment. This is possible because the plant has a specific temperature requirement for the maximum production of TF and CGA compounds, and temperature changes can induce cellular responses and lead to alterations in plant metabolism resulting to composition changes in plant metabolites (Chaves *et al.*, 2011). Alternatively, flavonoid biosynthesis is temperature dependent, and high temperatures may stimulate the synthesis of phenols and flavonoids to protect living plants. However, isolating, purifying, and characterizing the functions of its active phytochemicals as protective mechanisms against temperature changes are needed. In addition, it is also necessary to better understand the factors regulating the synthetic balance of the four metabolites in these metabolic biosynthetic pathways.

Variability in plant TP and TF could be associated with differences in growth conditions, genetic backgrounds, and methodological differences. Myriad environmental factors influence plant growth and directly impact biosynthetic pathways, thus affecting the secondary metabolism of bioactive compounds (Lu *et al.*, 2018). Various temperature conditions during plant growth affect certain biosynthetic pathways leading to variabilities in individual secondary metabolites, and it is important to keep a balance between biomass yields and metabolic compound concentrations to maximize economic benefits (Lu *et al.*, 2018). It is reported that higher growing temperatures and CO_2 levels increase flavonoid content and the concentrations of phenolic compounds (Wang *et al.*, 2013). In addition, the higher production of TF and TP compounds in sugarcane sprouts at higher temperature treatments have been demonstrated (Wahid, 2007). Therefore, quantifying the optimal stress level of a single environmental factor is crucial for the actual production of HC plants in a controlled environment, and greenhouse cultivation is an effective method for the steady production of HC plants because temperature conditions can be suitably controlled for plant growth and quality. It is possible that concentrations of bioactive compounds in HC plants can be increased through temperature control in greenhouses. Recently, we reported that wide variations occurred in TP, TF, orientin (OR), and isovitexin (IV) content in two *Passiflora* varieties, with '*P. suberosa*' containing higher TP and TF content than the 'Tainung 1' variety in 100% light intensity (LI-100), 50% light intensity (LI-50), and 15% light intensity (LI-15) treatments, but the IV content of '*P. suberosa*' was lower than that of 'Tainung 1' in the LI-15 treatment. Moreover, there were clear increases in TF, OR, and IV content in 'Tainung 1' and '*P. suberosa*' in LI-50 and LI-100 treatments compared to the LI-15 treatment (Ni *et al.*, 2020). In the present study, temperature influences plant growth, Chl content, and different responses by bioactive compounds in HC leaves, information which can be used to optimize the growth and development of plants in controlled-temperature

settings. HC plants subjected to various temperatures can be used as health foods and for medicinal purposes due to their high secondary metabolite content; therefore, they could be used to develop physiological markers to select germplasm through genetic manipulation. In the cases of the highest CGA content (111.78 mg per plant) under 30 °C treatment and TF (98.7 mg per plant) under 35 °C treatment, they share an initial common biosynthetic pathway that is normally present and is suggested for further breeding/cultivation and bioactivity study when it comes to producing natural antioxidants and bioactive compounds beneficial in the food and pharmaceutical industries.TP, TF, CGA, and HO content may be the rate-limiting factors of metabolites for use as genetic tools for developing an effective method of selecting HC individuals to improve the species' adaptability to high temperature, and a better understanding of the relationships of leaf growth traits and physiological parameters with TP, TF, CGA, and HO will stimulate more efficient HC breeding. These may be useful in screening for heat-tolerance plants and achieving commercial HC plant production by utilizing rapid, large-scale, precise management practices, and also provide the theoretical basis for selecting cultivars with specific high temperature tolerances as a pre-harvest factor that positively and significantly increases secondary metabolite content in HC.

Conclusions

Temperature has large effects on the growth, physiological, and antioxidant characteristics of HC. Under 30 °C treatment, all plants exhibited markedly higher plant height, leaf number, shoot and leaf FW and DW, SPAD and NDVI values, and TP, TF, CGA, and HO content compared to 25, 20, and 15 °C treatments. SPAD and NDVI developed in this study for evaluating and screening plant growth and photosynthesis of HC plants under 30 and 35 °C treatments are non-destructive and readily applicable for assessing early growth on a large scale in a controlled environment or indoor farming. High temperature treatment can also be an efficient way to enrich TP, TF, CGA, and HO content, and the cultivation of HC plants for use as a crude medicinal material is proposed. This information will be very useful for HC producers when selecting varieties and applying temperatures strategies to achieve their goals in terms of leaf production, physiological parameters, and the accumulation of desired phenolic compounds.

Authors' Contributions

YSL performed the experiments and collected all data sets. YSC designed the full experiment. CWW and YSC provided laboratory facilities for analysis of antioxidant characteristics in *Houttuynia cordata* Thunb. and interpreted the data. YSL and KHL wrote the manuscript and reviewed the final manuscript for journal submission. All authors read and approved the final manuscript.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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