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Effects of drought stress on growth and flavonoid accumulation of fish mint (*Houttuynia cordata* Thumb.)

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Abstract

Fish mint (*Houttuynia cordata* Thumb.) is a popular medicinal plant grown primarily because of its pharmacological values. Drought stress has on the relationship between growth and physio-biochemical changes, especially flavonoid content. The impacts of various drought stress conditions on the fish mint development were investigated, including 85% of field capacity (FC), 75% FC, 65% FC and 55% FC in 14, 21 and 28 days. Agronomic, physiological and biochemical parameters during the growth of fish mint plants under drought stress conditions were assessed. According to the results of variance analysis, drought stress results in a considerable drop in the measured parameters (shoot height, leaf number, leaf area and fresh weight). Similarly, all of the above-mentioned parameters were also decreased with increasing the number of drought days. Furthermore, drought period and level caused a drop in respiration, photosynthetic rate, chlorophyll and starch content. The concentration of carotenoids and flavonoids, on the other hand, increased dramatically as drought stress periods and levels increased. In comparison to the control, the drought treatment (65% FC) in 7 days maintained the growth rate and increased flavonoid accumulation from 2.42 mg to 3.04 mg. These findings might give a scientific foundation for growing fish mint plants under drought stress to boost flavonoid content.

Keywords

Accumulation, drought, fish mint, flavonoid, *Houttuynia cordata*, stress

Introduction

Fish mint (*Houttuynia cordata* Thumb.) is a fragrant perennial herb that has been utilized in medicine, functional foods and cosmetics (1). Fish mint is mostly grown in damp environments such as ravines, streamsides, woodlands, wet meadows, roadsides and ditch banks (2). Fish mint has been recognized as one of the most promising wild plant resources for medicinal purposes (3). Fish mint has chemical components (flavonoids, alkaloids and essential oils) and has anti-cancer, anti-oxidative and anti-bacterial properties (4, 5). Natural substances and their uses are gaining popularity and secondary metabolites and bioactive phytochemicals from fish mint have been targeted in drug development (6). Therefore, the demand for fish mint has increased in recent years. On the other hand, natural resources are unable to supply such massive demands, so farmers have been attempting to raise productivity by artificial cultivation.

As a result of global warming and related climatic issues, crop plants are increasingly vulnerable to abiotic stress such as drought, salt and other factors. Drought stress is one of the most severe restrictions on agricultural yield among the abiotic environmental conditions. Drought's damaging effects on agricultural output are determined by the severity and duration of the adverse conditions. Drought may cause the increase of flavonoids, which help plants cope with environmental stresses (7). Drought stress causes flavonoid concentrations to rise, as shown in various species. The augmentation of flavonoid metabolism under drought circumstances was also corroborated by transcriptome data on *Arabidopsis thaliana*. Drought regulates key genes involved in flavonoid production, such as chalcone isomerase, flavonoid 3'-hydroxylase and flavanone 3-hydroxylase.

This research aimed to investigate drought stress affected growth and flavonoid accumulation in fish mint. This research provides a scientific basis for the cultivation and management of fish mint and contributes to our understanding of the acclimation of fish mint to drought stress, using it as a natural antioxidant and increasing its flavonoid content for use in the medical industry.

Materials and Methods

Plant materials, growth condition and induction of stress

Fish mint (*Houttuynia cordata* Thumb.) seeds were collected from the University of Agriculture and Forestry, Ho Chi Minh City, Vietnam. Seeds were soaked in deionized water for 24 hr in the dark condition before sowing them in pots containing garden soil. Experimental soil had the following physical and chemical properties: organic matters of 24.91 g kg^{-1}, total N of 0.165%, available phosphorous of 0.062% and available potassium of 0.93%. The content of Zn, B, Cu and Mo in soil was 733 mg kg^{-1}, 98 mg kg^{-1}, 26 mg kg^{-1} and 0.9 mg kg^{-1} respectively. All pots were irrigated to 85% field capacity from the tap water till the start of drought stress treatments. After seven days, drought stress of varying soil moisture was induced. The plants were subjected to four drought conditions for 28 days: well-watered condition (85% of field capacity; C), mild drought stress (75% of field capacity; MIDS), moderate drought stress (65% of field capacity; MDS), and severe drought stress (55% of field capacity; SDS). To determine the soil field capacity (FC): the 1 kg dry soil (drying at 105 °C till to constant) was watered until saturation. Then, saturated soil was allowed to drain for two days to measure the weight. The weight difference between soil after draining and dry soil (1 kg) was the water to irrigate for 1 kg of dry soil to reach 100% FC. Therefore, the rule of 3 was applied to calculate the desired FC. The effects of the drought period on plant growth were studied by changing the number of well-watered days and the number of moderate drought stress days. There were 5 treatments, namely, 28 C/0 MDS (85% FC in 28 days), 21 C/7 MDS (85% FC in 21 days and 65% FC in 7 days), 14 C/14 MDS (85% FC in 14 days and 14% FC in 7 days) and 28 MDS (65% FC in 28 days). During the drought duration, the water content in pots was kept by a moisture sensor system and drip irrigation system. The experiment was performed at the Department of Plant Physiology, University of Science, Ho Chi Minh City, Vietnam.

Plant growth parameters

After 4 weeks of treatments, plant growth parameters such as shoot height (cm), root length (cm), leaf number, leaf area (cm^2) and fresh weight (FW) were measured. Plants were dried to a constant weight at 80 °C for measuring dry weights (DW). The relative water content of the leaves was calculated according to the formula of Barrs and Weatherley (8).

Determination of chlorophyll and carotenoid

For chlorophyll and carotenoid quantification, 0.5 g leaf sample was extracted with 5 ml of ethanol (95%) and centrifuged at 10000 g for 5 min to take the supernatant. The optical density of the extract was measured at wavelengths 470, 648 and 664 nm. The chlorophyll and carotenoid content were calculated according to the formula of Lichtenthaler (9).

Chlorophyll a = $13.36\ A_{664} - 5.19\ A_{648}$

Chlorophyll b = $27.34\ A_{648} - 8.12\ A_{664}$

Carotenoid = $(1000\ A_{470} - 2.13\ C_{\text{chlorophyll a}} - 97.64\ C_{\text{chlorophyll b}})/209$

Determination of respiration and photosynthesis intensity

The respiration and photosynthesis rate were determined by the CO_2 meter with a non-dispersive infrared sensor (EA80, Extech, USA) connected to an airtight chamber. The leaf chamber temperature was controlled at 28 ± 2 °C, and the light source was controlled at 0 lx (respiration) or 10000 lx (photosynthesis). The respiration rate was calculated based on the amount of CO_2 released instead of the amount of CO_2 absorbed in the photosynthesis rate (10).

Determination of soluble sugar and starch

For soluble sugar and starch quantification, 500 mg leaf sample was hydrolyzed with 5 ml of HCl (2,5 N) for 3 hr and then centrifuged (5000 g for 5 min) to take the supernatant (supernatant 1). The residue was hydrolyzed with 6.5 ml of $HClO_4$ (52%) for 24 hr, centrifuged (5000 g for 10 min) and obtained the supernatant (supernatant 2). Supernatant 1 and supernatant 2 (separately) were assayed with the anthrone reagent (heated for 8 min) to determine total soluble sugar and starch content. Absorbance was read at 630 nm with a spectrophotometer and converted using a standard curve performed with glucose (11).

Determination of proline

For proline quantification, 500 mg of fresh sample was ground with 10 ml of sulfosalicylic acid (3%) and centrifuged at 10000 g for 10 min. Then, 1 ml of ninhydrin reagent was added to 1 ml of the supernatant, which was then incubated at 100 °C for 1 hr. After that, 4 ml of toluene was added. Absorbance was read at 520 nm with a spectrophotometer and converted using a standard curve performed with L-proline (12).

Determination of flavonoid

For flavonoid quantification, 500 mg of a dried powdered

Table 1. Effect of drought stress levels on growth of fish mint after 28 days

Treatment	Shoot height (cm)	Leaf number	Leaf area (cm^2)	Fresh weight (g)	RWC (%)
Control (85% FC)	33.20 ± 0.30 [a]	5.83 ± 0.31 [a]	45.50 ± 1.60 [a]	4.42 ± 0.15 [a]	92.43 ± 0.70 [a]
MIDS (75% FC)	28.87 ± 0.25 [b]	5.20 ± 0.30 [b]	37.83 ± 0.35 [b]	4.10 ± 0.15 [b]	84.80 ± 0.56 [b]
MDS (65% FC)	24.83 ± 0.35 [c]	4.47 ± 0.15 [c]	27.77 ± 0.31 [c]	3.73 ± 0.08 [c]	70.00 ± 0.56 [c]
SDS (55% FC)	8.83 ± 0.31 [d]	2.20 ± 0.26 [d]	11.40 ± 0.46 [d]	2.60 ± 0.13 [d]	67.07 ± 0.51 [d]

Values with different letters in a column are significantly different according to Ducan's test (p=0.05)

leaf sample were immersed in 2.5 ml of methanol, shaken for 3 hr (60 °C), centrifuged at 10000 rpm for 10 min and concentrated the supernatant. Then, 50 µl of the extract was mixed with 1 ml of methanol, 0.3 ml of $NaNO_2$ (5%), 0.3 ml of $AlCl_3$ (10%) and 2 ml of NaOH (4%) and the mixture was kept at room temperature for 20 min. Absorbance was measured at 510 nm with a spectrophotometer and converted using a standard curve performed with quercetin (13).

Statistical analysis

A randomized block design (RBD) was used, with 3 replications, on all parameters. All data were analyzed using the SPSS 20.0. Experimental results were represented as mean ± standard deviation (SD). Differences between means were evaluated by Duncan's multiple range tests. Statistical significance was accepted at a level of $p < 0.05$.

Results

Effects of drought stress levels on plant growth

The effect of drought stress levels on the growth of fish mint is presented in Table 1. Growth parameters such as shoot height, leaf number, leaf area and fresh weight were decreased under drought stress when compared with the control (Fig. 1). According to the Duncan's test, drought stress identified 4 groups of fresh weight that decreased according to the increase in drought level. The first was the level FC = 85% averaging 4.42 ± 0.15 g, followed by FC = 75% with a weight of 4.10 ± 0.15 g, then FC = 65% with 3.73 ± 0.08 g and finally FC = 55% with 2.60 ± 0.13 g. Likewise, RWC in fish mint were affected by drought stress levels. The RWC was comparatively lowest in severe drought stress (67.07%), followed by moderate drought stress (70.00%), mild drought stress treated (84.80%) and well-watered condition (92.43%).

Fig. 1. Effect of drought stress levels on growth of fish mint after 28 days. Scale bar = 3 cm.

Fig. 2. Effect of drought stress period on growth of fish mint. Scale bar = 3 cm.

Table 2. Effect of drought stress period on growth of fish mint after 28 days

Drought period	Shoot height (cm)	Leaf number	Leaf area (cm^2)	Fresh weight (g)	RWC (%)
28 C/0 MDS	32.43 ± 0.65 [a]	5.97 ± 0.23 [a]	41.67 ± 0.45 [a]	4.48 ± 0.12 [a]	92.57 ± 0.42 [a]
21 C/7 MDS	28.77 ± 0.31 [b]	5.67 ± 0.15 [a]	40.80 ± 1.18 [a]	4.33 ± 0.08 [a]	91.10 ± 0.92 [b]
14 C/14 MDS	27.83 ± 0.35 [b]	5.17 ± 0.06 [b]	38.57 ± 0.12 [b]	4.08 ± 0.03 [b]	87.47 ± 0.67 [c]
0 C/28 MDS	24.70 ± 0.66 [c]	4.20 ± 0.30 [c]	28.23 ± 0.46 [c]	3.60 ± 0.15 [c]	70.33 ± 0.80 [d]

Values with different letters in a column are significantly different according to Ducan's test (p=0.05)

Effects of drought stress period on plant growth

Overall, fish mint subjected to the control (28 C/ 0 MDS) and 21 C/7 MDS treatment exhibited the highest leaf number, leaf area and fresh weight, but these parameters decreased gradually as the drought stress period increased (Fig. 2, Table 2). The ANOVA showed that the shoot height varied significantly between drought stress periods. Duncan's test classified effects of drought period on shoot height following the descending order: 28 C/0 MDS > 21 C/7 MDS > 14 C/14 MDS > 0 C/28 MDS. Furthermore, the Duncan's test indicated that the highest RWC was allocated to the 28 C/0 MDS treatment (92.57%), followed by 21 C/7 MDS treatment (91.10%), 14 C/14 MDS treatment (87.47%) and 0 C/28 MDS with 70.33%.

Changes in photosynthetic pigments

Photosynthetic pigments such as chlorophyll a and b decreased in the drought plants (Fig. 3A). Chlorophyll a and b were the highest content (7.87 mg and 6.63 mg) noted in control. Moreover, drought stress increased the level of carotenoid content compared to control. Similarly, the

chlorophyll content decreased, but the carotenoid content increased sharply in the case of increasing the drought period (Fig. 4A).

Changes in gas exchange parameters

Compared with the well-watered condition (85% FC), drought stress conditions (75% FC, 65% FC and 55% FC) dramatically decreased respiration and photosynthesis (Fig. 3B). Similarly, 21 C/7 MDS, 14 C/14 MDS, and 0 C/28 MDS treatments decreased the respiration of fish mint leaves by 6.19%, 15.93% and 25.66% respectively, relative to the control. Fish mint plants grown on control condition (28 C/0 MDS) showed photosynthesis values about 1.57 mg CO_2. $cm^{-2}.h^{-1}$. The photosynthesis rate of 21 C/ 7 MDS treatment was not significantly different compared with the control. However, in 14 C/14 MDS and 0 C/28 MDS treat-

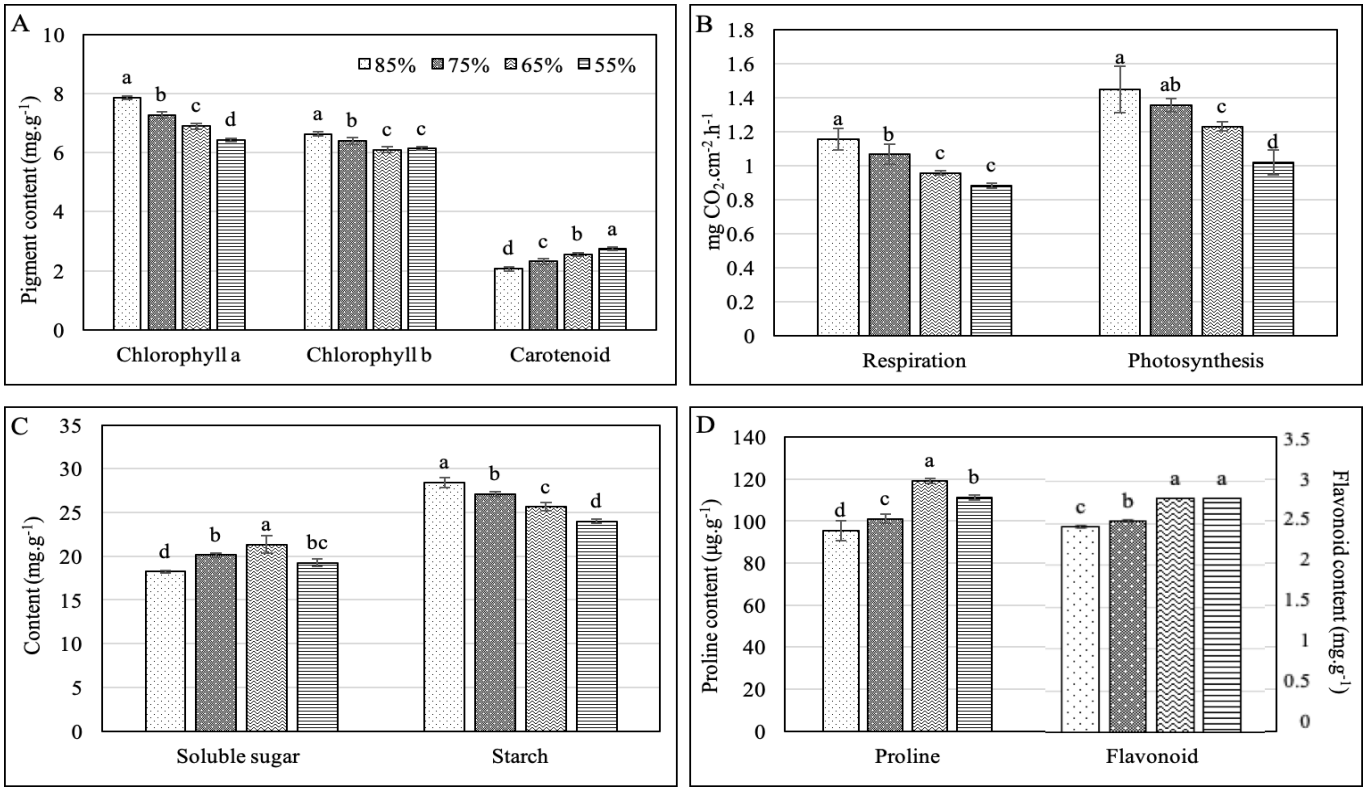

Fig. 3. Effect of drought stress levels (%FC) on physiological and biochemical changes of fish mint after 14 days. (**A**) chlorophyll and carotenoid, (**B**) respiration and photosynthesis, (**C**) sugar and starch, (**D**) proline and flavonoid. Error bars represent standard deviations. The value in line marked with different lower-case letters denote significant differences between samples at $p < 0.05$ (Duncan's multiple range test).

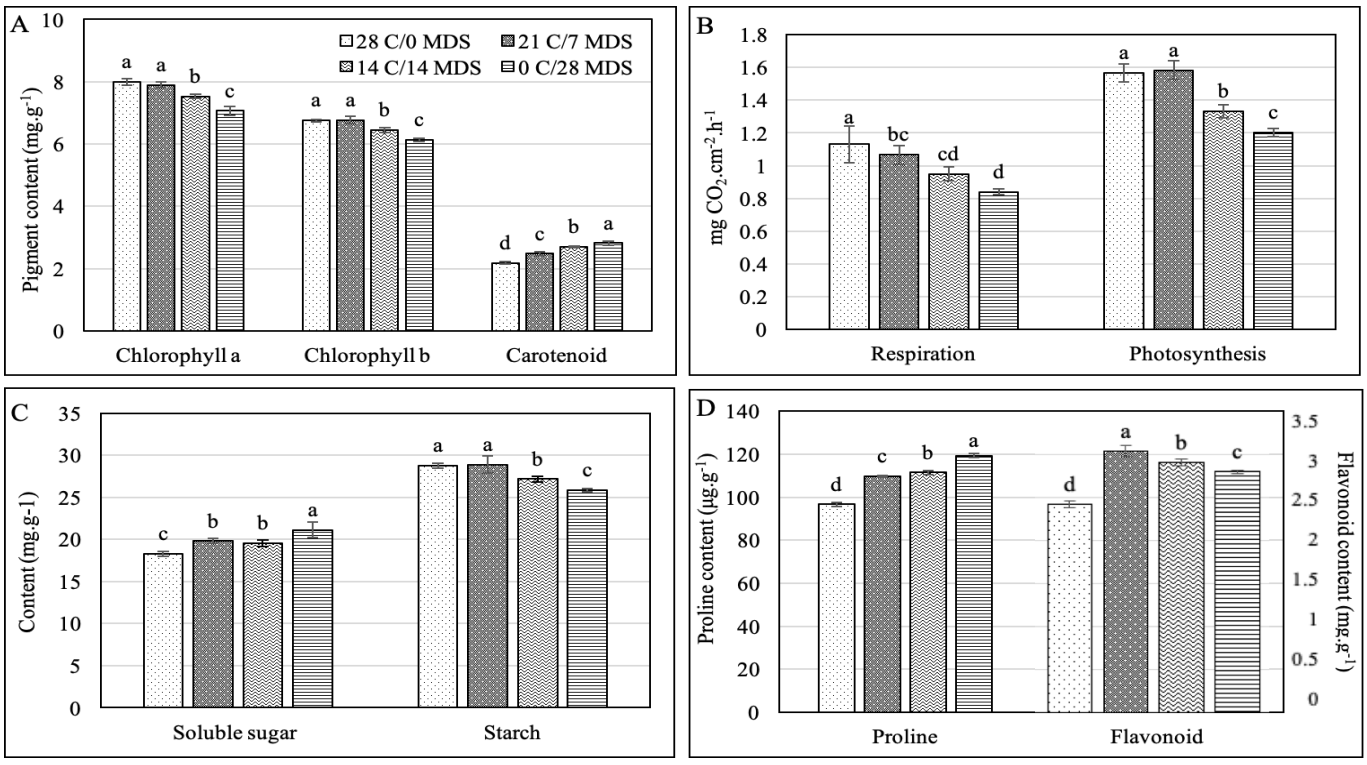

Fig. 4. Effect of drought stress period on physiological and biochemical changes of fish mint after 14 days. (**A**) chlorophyll and carotenoid, (**B**) respiration and photosynthesis, (**C**) sugar and starch, (**D**) proline and flavonoid. Error bars represent standard deviations. The value in line marked with different lower-case letters denote significant differences between samples at $p < 0.05$ (Duncan's multiple range test).

ments, the rate of photosynthesis was reduced by 14.74% and 23.07% respectively, compared to the control (Fig. 4B).

Changes in soluble sugar, starch and proline content

Results showed that the effects of drought stress levels on sugar, starch and proline content were significant (Fig. 3C-D). Plants grown under moderate drought stress (65% FC) had the highest levels of sugar and proline. Sugar and proline levels increased considerably beginning with mild drought stress (75% FC), but these content dropped during severe drought stress (55% FC) (Fig. 1). In contrast, starch content in leaves continually decreased with increasing levels of drought stress. According to Ducan's test, the highest starch content (28.43 mg) was obtained with FC = 85%, followed by the value 27.10 mg for the level FC = 75%, then FC = 65% with 25.63 mg and finally FC = 55% with 24.00 mg. The accumulation of sugars and proline was proportional to the number of drought days (Fig. 4C-D). Among treatments, 0 C/28 MDS and 28 C/0 MDS had the highest and the lowest sugar and proline content respectively.

Changes in flavonoid content

The content of flavonoids in fish mint significantly increased with increasing drought stress levels. The total flavonoid increased from 2.46 mg for the 85% FC to the highest content of 2.80 mg for 65% FC and 55% FC (Fig. 3D). Similarly, drought stress periods had a significant effect on the flavonoid concentration. Ducan's test indicated that the improvement in flavonoid content was particularly crucial for the 21 C/7 MDS treatment (3.04 mg), which was significantly higher than for the 28 C/0 MDS treatment (2.42 mg) and 14 C/14 MDS treatment (2.90 mg) (Fig. 4D).

Discussion

Drought can influence many aspects of plants' morphological and anatomical characteristics. A decrease in leaf number and size are some alterations that occur in plants exposed to drought condition (14). Fish mint plants had the same problem. The morphological changes occur when plants encounter drought stress after fourteen days (Fig. 1-2). Visual observations indicated that the increase in drought period and levels resulted in fish mint plants with a low leaf number and area (Table 1-2). Moreover, relative water content (RWC) is a key element in plant-water connections under drought stress, and it was found to be significantly lower compared with the control in this study (Table 1, 2). Low RWC causes stomatal closure by lowering the leaf water potential (15). Plants require proper leaf area development and stomatal openness to achieve optimal photosynthesis (16). Therefore, the photosynthetic rate in water-stressed plants is lowered (Fig. 3B, 4B). The reduction of photosynthetic intensity may be the cause of reducing starch and fresh weight under drought stress (Fig. 3B-4B). Furthermore, the decrease in photosynthesis could be attributed to a drop in chlorophyll concentration, which is a common indication of drought stress. When compared to mild and moderate drought stress (75% FC and 65% FC), the content of chlorophyll *a* and *b* of fish mint plants growing under severe drought stress (55% FC) was sharply reduced (Fig. 3A). In addition, the amount of chlorophyll lost during drought stress is dependent on the drought duration. The treatments (14 C/14 MDS and 0 C/28 MDS) reduced chlorophyll *a* content compared to the control (Fig. 4A). According to one report, carbon dioxide limits caused by the prolonged stomatal closure result in the accumulation of photosynthetic electron transport components (17). These substances can deplete molecular oxygen, resulting in the generation of reactive oxygen species (ROS) such as superoxide, hydroxyl radicals, and H_2O_2, which can cause oxidative damage in chloroplasts (17). Nonetheless, drought treatment resulted in a considerable increase in carotenoid content compared to the control (Fig. 3A, 4A). Carotenoids, unlike chlorophylls, are less responsive to water stress, according to many reports. Carotenoids play a protective role in plants under drought stress, which is involved in the ROS detoxification by the xanthophyll cycle (18, 19). Similar to photosynthesis, the respiration rate of fish mint plants decreased dramatically in drought conditions (Fig. 3B, 4B). Drought tolerance is a costly phenomenon for plants and the amount of energy expended to cope with it is massive. The tricarboxylic acid cycle and ATP production are negatively affected by drought stress, resulting in a lower respiration rate (20).

In this study, biochemical analysis shows that drought stress resulted in significant increases in sugar and proline (Fig. 3D, 4D). A well-known mechanism for plant resistance to drought stress is the accumulation of suitable solutes to offer osmotic adaptation. Compatible solutes have a low molecular weight and may accumulate in large amounts without harming cell components and metabolism. One of the standard amino acids known as osmoprotectants is proline. Drought increase proline content in cells in two ways: by boosting proline production and lowering the activity of enzymes that break it down. Proline accumulation causes cells to have a lower water potential, which aids water uptake from the soil during drought (21, 22). According to one report, the fish mint leaf contains a lot of flavonoids and the total content of flavonoid compounds extracted varies depending on the environmental conditions (23). In this study, moderate and severe drought stress had the highest flavonoid content (2.80 mg), followed by mild drought stress (2.53 mg). Furthermore, the 21 C/7 MDS treatment (3.04 mg) had the highest flavonoid content, followed by the 14 C/14 MDS treatment (2.90 mg) and the 0 C/28 MDS treatment (2.80 mg). Flavonoid variability could be linked to differences in growth conditions. A variety of environmental factors influence plant growth and have a direct impact on biosynthetic pathways, affecting bioactive compound secondary metabolism (24). Different drought condi-

tions during plant growth affect certain biosynthetic pathways, resulting in variations in individual secondary metabolites and it is critical to maintain a balance between biomass yields and metabolic compound concentrations to maximize economic benefits (24). The findings of this study can be used to optimize fish mint plant growth and flavonoid accumulation under drought stress.

Conclusion

In the present study, the level and period of drought stress negatively impacted the growth of fish mint in many parameters such as physiological activities (respiration and photosynthesis) and biochemical metabolites (chlorophyll and starch). However, moderate drought stress (65% field capacity) induced carotenoids, osmoprotectants (sugar and proline) and flavonoid accumulation. Furthermore, the drought stress treatment (65% field capacity) in 7 days showed the maintenance of the growth rate and an increase in flavonoid level compared with the control.

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Authors contributions

NNTL carried out the drought stress studies and biochemical changes. TTT conceived of the study and participated in manuscript editing, its design and coordination. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: **:** Authors do not have any conflict of interests to declare.

Ethical issues: None.

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