Sains Malaysiana 52(1)(2023): 57-69
http://doi.org/10.17576/jsm-2023-5201-05

Drought Stress Induced the Flavonoid Content in Moringa (*Moringa oleifera* Lam.) Leaves

(Stres Kemarau Mengaruh Kandungan Flavonoid pada Daun Moringa (*Moringa oleifera* Lam.))

RIDWAN[1,2], HAMIM[1,*], SUHARSONO[1] & NURIL HIDAYATI[2]

[1]*Department of Biology, Faculty of Mathematics and Natural Sciences, IPB University, Bogor, Indonesia*
[2]*National Research and Innovation Agency, Indonesia*

Received: 26 April 2022/Accepted: 27 October 2022

ABSTRACT

Flavonoid is one of the most widely available bioactive compounds presence in the Moringa (*Moringa oleifera* Lam.) leaves. Osmotic stress is known to induce flavonoid production, however, the water level varies among plant species. This study aimed to examined the effects of water stress levels on the flavonoid content of Moringa leaves. The plants were treated withholding watering at three intervals (I): i.e.,: 1 (as control), 3 and 7 days based on the evaporated water during the treatments. The drought treatments were given within 4 different periods (D) before the plants were harvested, i.e.,: 8, 16, 24 and 32 days, and measured for the growth and biomass, proline (Pro) and chlorophyll (Chl) content, leaf water potential (WP), leaf relative water content (RWC), quercetin (Q) and kaempferol (K) content, and water use efficiency of flavonoid (WUE_f). The results showed that the drought treatments induced different water statuses in the plants by decreases the leaf relative water content (RWC) and leaf water potential (WP) and increases of proline content significantly (up to 3 fold). Growth and biomass production decreased with the increase of water stress, whereas flavonoid content increased when the drought was mild and decreased again under severe drought. The highest content of flavonoids (1314.53 mg/kg leave biomass for Q and 2984.15 mg/kg leave biomass for K) and WUE_f were shown when the plants were treated with 3 days drought for 16 days periods before the plants were harvested (I2D2) with no significant reduction in leaf biomass. This result suggests that the treatment of I2D2 is the best for bioactive production in Moringa.

Keywords: Bioactive compounds; drought; kaempferol; quercetin

ABSTRAK

Salah satu sebatian bioaktif yang paling banyak digunakan yang diperoleh daripada daun Moringa (*Moringa oleifera* Lam.) ialah flavonoid. Kajian ini bertujuan untuk mendorong kandungan flavonoid daun Moringa melalui rawatan tekanan air yang tidak mengurangkan jumlah pengeluaran bioaktif. Rawatan kemarau digunakan dengan menahan penyiraman dengan selang 1 (sebagai kawalan), 3 dan 7 hari. Rawatan kemarau diberikan dalam 4 tempoh (kitaran) yang berbeza sebelum tanaman dituai, iaitu: 8, 16, 24 dan 32 hari. Isi padu pengairan ditentukan berdasarkan perbezaan di antara berat pasu di bawah kapasiti ladang dan di bawah rawatan kemarau sejurus sebelum penyiraman seterusnya. Pemboleh ubah yang diperhatikan ialah pertumbuhan dan biojisim, prolin (Pro), klorofil (Chl), potensi air daun (WP), kandungan air relatif daun (RWC), kandungan kuersetin (Q) dan kaempferol (K) dan kecekapan penggunaan air flavonoid. (WUE_f). Hasil kajian menunjukkan bahawa rawatan kemarau mampu menyebabkan status air yang berbeza antara rawatan yang ditunjukkan oleh penurunan kandungan air relatif daun (RWC) dan potensi air daun (WP) akibat kemarau yang lebih lama, manakala ia menyebabkan peningkatan kandungan prolin dengan ketara. Pertumbuhan dan pengeluaran biojisim menurun dengan peningkatan tekanan air, manakala kandungan flavonoid meningkat apabila kemarau sederhana dan berkurangan semula di bawah kemarau yang teruk. Kandungan flavonoid (Q dan K) dan WUE_f tertinggi ditunjukkan apabila tumbuhan dirawat dengan kemarau 3 hari selama tempoh 16 hari sebelum tanaman dituai (I2D2) tanpa pengurangan ketara dalam biojisim daun. Keputusan ini menunjukkan bahawa rawatan I2D2 adalah yang terbaik untuk pengeluaran bioaktif di Moringa.

Kata kunci: Kaempferol; kuersetin; sebatian bioaktif; tekanan air

Introduction

Flavonoids are one of the polyphenol compounds that have recently been widely studied and used in the health sector. They have potential functions as antiviral/bacterial, anti-diabetic, anti-cancer, anti-inflammatory (Hasym et al. 2021; Wang, Li & Bi 2018), and for the treatment of degenerative diseases, but mainly have a function as antioxidants (Mondal & Rahman 2020). Currently, there are at least 4000 variations of known flavonoid compounds with different levels of antioxidant activity. Quercetin and kaempferol have considerably high antioxidant activity based on the values of HBC (the number of conjugated hydroxyl groups of the B and C rings) (Mondal & Rahman 2020). As phytochemical compounds, flavonoids are not synthesized in the human or animal body. Flavonoid biosynthesis occurs in almost parts of plants, especially in photosynthetic cells (Kumar & Pandey 2013). Moringa (*Moringa oleifera* Lam.) is among the plants that have been known to contain flavonoid compounds with high antioxidant activity (Edwinanto et al. 2018; Lin, Zhang & Chen 2018; Pakade, Cukrowska & Chimuka 2013).

Moringa contains various types of flavonoid compounds such as quercetin, kaempferol, isorhamnetin, apigenin, and myricetin (Edwinanto et al. 2018; Lin, Zhang & Chen 2018). The flavonoid content of Moringa leaves has been reported to be higher than that of other plants, such as spinach, broccoli, and other vegetables (Pakade, Cukrowska & Chimuka 2013). Gonzalez-Romero et al. (2020) also stated that the flavonoid content (catechin equivalent) of Moringa leaves was higher (327.2 ± 13.8 mg/100 g FW) than that of 19 vegetables commonly consumed in a prepackaged salad (3.8 - 191 mg/100 g FW) in Spain. However, the flavonoid content of this plant is highly dependent on environmental conditions, such as temperature, light intensity, and water availability. Wasonowati et al. (2019) reported that Moringa grown in the dry season possessed a higher flavonoid content than in the rainy season. The concentration of flavonoids in Moringa leaf (quercetin and kaempferol) has also been reported to increase when treated with withholding water for 30 days (Brunetti et al. 2018). Those suggests that the flavonoid content of Moringa leaves can be improved by decreasing water availability through water stress treatment.

Increasing plant bioactive compounds through the manipulation of water availability has been carried out in many plants. Azhar et al. (2011) have discovered that soil moisture of 80% and 60% field capacity increased the content of the phenolic compound of *Trachyspermum ammi* by about 82% and 104% than that of 100% field capacity. Sarker and Oba (2018) have also shown that flavonoid and other bioactive compounds of *Amaranthus* increased along with a decrease in water availability. Ahmed et al. (2021) have also reported water stress treatment that can induce higher bioactive compounds in *Populus* leaves. However, many of such studies were focused only on increasing the concentration of bioactive compounds without considering the decrease in biomass production due to the drought treatments. To the best of our knowledge, there was no study that investigated the proper drought treatment that can increase flavonoid content of Moringa without any significant reduction on leaf biomass production. Therefore, the objective of this study was to induce the higher flavonoid content of Moringa leaves through water stress treatments by considering the decrease in biomass production which did not reduce total bioactive production. The results of this study are expected to become an important reference in the cultivation of Moringa to produce leaf biomass with high-quality bioactive compounds, which can be used as a material for functional foods and drugs to treat degenerative diseases.

Materials and Methods

Plant materials and experimental design

Plant materials used in this study were Moringa accession from Deli Serdang Regency, North Sumatera, Indonesia, which has superior characteristics including high leaf biomass production, total flavonoid content, and antioxidant activity (Ridwan et al. 2021). Moringa seeds were washed and then soaked in clean water for 60 min, followed by soaking in a solution of bactericide Agrept 20 Wp and fungicide Masalgin 50 Wp (2 g/L) for 30 min. The seeds were then germinated on seedling trays. Three weeks after sowing (± 7 cm height), the seedlings were then planted to medium consisting of soil, manure, and roasted husks with a ratio of 2:1:1 (v/v/v) in a 45 × 45 cm polybag (14 kg). At the age of 2 months after planting (MAP), pruning was performed at a height of 30 cm to multiply branches and increase the potential of biomass production. Only three branches were allowed to grow and be observed.

The experiment applied two different water treatments: i.e., withholding watering intervals and drought periods. The withholding watering intervals applied were: 1 day as control (I_1), 3 days (I_2), and 7 days (I_3), while the drought periods given were: 8 days

(D_1), 16 days (D_2), 24 days (D_3), and 32 days (D_4) before harvesting. All experimental units were placed in a greenhouse and arranged using a randomized completely block design (RCBD) with 3 replications. The watering volume was determined based on the difference between the weight of the pot under field capacity and that under the drought treatments just before the next watering before the next irrigation (watering).

DETERMINATION OF SOIL MOISTURE CONTENT

Soil moisture content was measured by the gravimetric method. The soil samples were taken at a depth of 5 cm at 3 points around the stem, then mixed and weighed immediately to obtain the soil wet weight (WW). The soil samples were then dried in an oven at 110 °C for 3 days and then weighed again to obtain the soil dry weight (DW). The soil moisture content was expressed in percent dry weight which was calculated according to the following equation (Shukla et al. 2014):

$$m_d = \frac{(weight\ of\ wet\ soil) - (weight\ of\ dry\ soil)}{(weight\ of\ dry\ soil)} \times 100$$

where m_d is the moisture content in dry weight basis.

PLANT GROWTH AND BIOMASS OBSERVATION

Plant growth variables observed were plant height (PH), stem diameter (StD), canopy diameter (CD), leaves number (LN), leaves senescence (Snc) and abscission (Abc), leaflet number (LtN), leaflet area (LtA). Plant height (PH) was observed from the base of the stem to the highest shoot tip. Stem diameter (StD) was observed at a height of 5 cm from the base. Canopy diameter (CD) was observed at the widest point on two perpendicular sides to each other. Leaves Number (LN) was calculated based on the number of leaves that are still attached to the plant. Senescence (Snc) was calculated based on the number of compound leaves that had yellowed 50%, while Abc was calculated based on the number of compound leaves that had separated from the plant. Leaflet number (LtN) and LtA were measured on the 4th compound leaves from the top. Leaflet area (LtA) was measured on 5 leaflets at the base (2 samples), middle (2 samples), and tip (1 samples) of the leaves. The 4th compound leaves were picked and photographed and then observed using Image-J 1.47v software. Biomass observations were performed on leaflet dry weight (LtDW). All compound leaves were picked manually and then washed in clean water, then the leaflets were picked manually as well, and finally dried using oven at 60 °C and weighed. All variables were observed once at the end of the treatment (4 MAP).

DETERMINATION OF LEAF WATER POTENTIAL (WP) AND RELATIVE WATER CONTENT (RWC)

Observation of WP was carried out using the WP4 PotentiaMeter equipment. As many as one to two leaflets (middle leaflets of the 4th compound leaves from the apex of Moringa) were collected in the morning (09.00 - 10.00), sealed in a zip lock and then stored in an ice box. The leaflet samples were cut into small pieces and arranged in the WP4 PotentiaMeter tube (no overlap each other). The WP value was read in the range of Ts-Tb -0.48 to -0.58 and expressed as MPa.

The samples were then weighed to obtain fresh weight (FW), and followed by immersion in distilled water for 24 hours and re-weighed to obtain the turgid weight (TW). The samples were dried in an oven at 60 °C for 3 days and then reweighed to obtain dry weight (DW). The value of RWC was calculated based on the following formula (Zhang et al. 2017):

$$RWC = \frac{FW - DW}{TW - DW} \times 100$$

where RWC is the relative water content (%); FW is the fresh weight (g); DW is the dry weight (g); and TW is the turgid weight (g).

DETERMINATION OF LEAF CHLOROPHYLL CONTENT (Chl)

The 3rd, 4th and 5th compound leaves from the apex were taken and washed in running water. The leaflets were picked and wrapped in aluminium foil, and then frozen in liquid nitrogen. The samples were then taken to the laboratory and stored in a freezer at -30 °C until further use to determine the chlorophyll content, proline content, and flavonoid extraction. Leaf chlorophyll content was observed following the method of Shah Houborg and McCabe (2017) and Sims and Gamon (2002) with slight modifications. Briefly, leaf samples with an area of 1 cm^2 were crushed manually in 2 mL of 80% acetone solution using a mortar and then centrifuged at 10,000 rpm for 3 min. The supernatant was taken and added with the extracting solution to a final volume of 6 mL. The absorbance of the solution was then observed at 663 and 645 nm. The leaf chlorophyll content was determined using the following equation:

Chl_t (μg.cm^{-2}) = [(20.2 × A_{645}) + (8.02 × A_{663})] × mL of $Acetone_{80\%}$/Leaf area (cm^2)

Chl_a (μg.cm^{-2}) = [(12.7 × A_{663}) + (2.6 × A_{645})] × mL of $Acetone_{80\%}$/Leaf area (cm^2)

Chl_b (μg.cm^{-2}) = [(22.9 × A_{645}) + (4.68 × A_{663})] × mL of $Acetone_{80\%}$/Leaf area (cm^2)

DETERMINATION OF PROLINE CONTENT (Pro)

The proline content was observed following the method developed by Bates, Waldren and Teare (1973). As much as 0.25 g of leaf sample and 10 mL of 3% sulfosalicylic acid were ground using mortar. The solution was then centrifuged at 3500 rpm for 10 min. A total of 2 mL of supernatant was mixed with 2 mL of 6 M orthophosphoric acid, 2 mL of ninhydrin solution and 2 mL of glacial acetic acid, and then heated in a 100 °C water bath for 1 hour and suddenly cooled in ice. The solution was then added with 4 mL toluene and shaken vigorously to form chromophore. The absorbance of the chromophore was observed at 520 nm. Standard solutions were made with several graded concentrations, i.e.,: 20, 40, 60, 80, and 100 ppm.

FLAVONOID EXTRACTIONS

The leaf samples were freeze dried using Eyela FDU-1200 at a temperature of -45 °C and a vacuum gauge of 25 Pa. The extraction was performed according to Vongsak et al. (2013) with minor modifications. Briefly, 5 g of freeze dried leaf samples were ground using a blender Philips HR2115. A total of 3 g of Moringa leaf powder was macerated in 60 mL of 70% ethanol (1:20 w/v) with occasional shaking for 3 days at room temperature. The extract was then filtered using Whatman filter paper size 42, and the dregs were remacerated with the same process. The results of the first and second extractions were mixed and evaporated to obtain viscous extracts. The viscous extract was freeze-dried again to obtain a dried extract.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) ANALYSIS

Analysis of flavonoid content was conducted using HPLC Shimadzu LC-20AD with UV-Vis SPD-20A detector. The mobile phase used was TCA 0.3% in water and acetonitrile (50:50, v/v), which was run at a flow rate of 0.9 mL/min for 13 min (Abdelkawy, Balyshev & Elbarbry 2016). The absorbance was measured at a 254 nm. Standard solutions were made with several graded concentrations, namely 50, 25, 10, 5, 2.5, 1, and 0.5 ppm for quercetin, and 50, 25, 10, 5, 2.5, 1, 0.5, 0.2, 0.1, and 0.05 ppm for kaempferol. A total of 20 μL of standard solution and dried extracts diluted with 70% ethanol were injected into the column for HPLC analysis. The peak area and concentration of standard solutions were regressed to create a standard curve formula. The peak area of the sample was used to determine the quercetin and kaempferol concentration of the sample based on the standard curve formula.

WATER USE EFFICIENCY OF FLAVONOID PRODUCTION (WUE_f) DETERMINATION

Water use efficiency of flavonoid production was determined using a formula (Lima et al. 2018) based on the quercetin and kaempferol content of the plant:

$$WUE_f = \frac{Yield\ (Quercetin\ and\ Kaempferol)}{Total\ Water\ Supply}$$

where WUE_f is the water use efficiency of flavonoid production.

DATA ANALYSIS

The data of relationship of withholding watering intervals and drought periods with soil moisture content were analysed using MS Excel 2010. Analysis of variance (ANOVA) was performed using SPSS for Windows version 16 (SPSS Inc., Chicago, IL, USA) to identify the response of *M. oleifera* to water stress. Differences among means were detected with DMRT (Duncan Multiple Range Test) at α=0.05. Principal component analysis (PCA), biplot analysis, and fold change analysis were conducted using Metaboanalyst 5.0 (https://www.metaboanalyst.ca/). The correlation and path analysis were performed using R-studio program.

RESULTS AND DISCUSSION

SOIL MOISTURE CONTENT

The difference in soil moisture content was more influenced by withholding watering intervals rather than by drought periods (Figure 1(A) and 1(B)). The highest soil moisture was shown by the control at 43.97%, then decreased by about 36.28% (28.02%) and 56.69% (19.04%) at I_2 and I_3, respectively (Figure 1(A)).

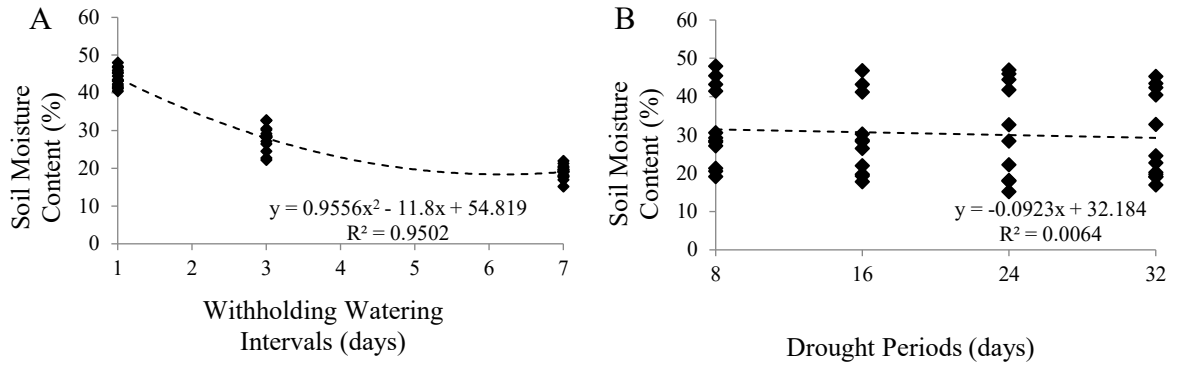

FIGURE 1. Correlation of soil moisture content with watering intervals (A) and treatment periods (B)

This indicated that the treatment given had successfully induced gradually water stress as expected, to affect plant growth and induce plant metabolism. Whereas the drought periods did not cause the significant differences on soil moisture content (Figure 1(B)).

PLANT GROWTH AND BIOMASS

As occur in most of plants, the growth of Moringa is also hampered by water stress. It can be seen by a decrease in plant height (PH), stem diameter (StD), canopy diameter (CD), and leaflet area (LtA), while I caused an increase in leaves senescence (Snc) and abscission (Abc) as well. Some growth variables severely affected by water stress, including CD, LtA, Snc, and Abc, which were affected even in mild stress (I_2), while PH and StD were affected only at severe drought stress (I_3) (Table 1). Several growth variables at I_2 and I_2 were found to be lower than those of the control, but StD, LtA, Snc, and Abc at D_1 (I_2D_1) and I_3D_1) were not significantly different compared to the control. Specifically, Snc and Abc of I_2D_2 showed no significantly different with the control. Leaflet dry weight of Moringa (LtDW) also decreased with increasing withholding watering intervals, however, treatment combination of I_2D_1, I_2D_2, I_3D_1 have no significantly different compared to the control (Table 1). Several growth variables were found to have correlation with leaf biomass production, either positive or negative. Among all variables, Snc and Abs have a highest negative correlation to LtDW (Figure 2(A)). The result of path analysis also showed that Snc had a highest negative direct effect on LtDW (-0.83) (2B), which means that leaf biomass production of Moringa was much affected by Snc. It is understandable because the leaves that were harvested in this study merely the fresh and green leaves.

The decrease in plant growth variables, such as PH, StD, CD, and LtA under water stress conditions can occur due to several possibility, which include: 1) decrease in cell turgidity due to a reduction in cell fluid, therefore cells, tissues, and organs do not develop optimally (Zhang et al. 2017); 2) increase in reactive oxygen species (ROS) level that can cause damage to cellular molecules such as proteins, amino acids, and lipids (Juan et al. 2021); 3) decrease in photosynthetic rate due to stomatal closure and decreased carbon fixation (Malinowska, Donnison & Robson 2020); and 4) the results of carbon assimilation are allocated greater in the roots than in the shoot to maintain water absorption capacity (Kapoor et al. 2020). The increase in Snc and Abc in drought stress conditions is to reduce water loss through transpiration. Leaf senescence and abscission under drought stress conditions is closely related to a decrease in cytokinin concentrations, and an increase in abscisic acid (ABA), ethylene, jasmonic acid, and salicylic acid (Patharkar & Walker 2019; Sade et al. 2018). All those processes directly and/or indirectly affect the LtDW of the plant as well. The decrease in plant growth and biomass under water stress has been reported in Moringa by Boumenjel, Papadopoulos and Ammari (2021), Brunetti et al. (2018), and Hasan et al. (2020), also in other species such as wheat (Thapa et al. 2017), *Maclura pomifera* (Khaleghi et al. 2019), and Miscanthus (Malinowska, Donnison & Robson 2020).

TABLE 1. Growth and biomass characters of Moringa treated by withholding watering intervals and drought periods

Watering Intervals (days)	Drought Periods (days)	PH (cm)	StD (mm)	CD (cm)	LN	LtN	LtA (cm^2)	Snc	Abc	LtDW (g)
1	8	129.72±4.338 a	1.14±0.078 a	69.50±3.786 a	43.33±2.404	358.33±12.719	3.51±0.636 a	2.00±0.289 d	3.50±1.041 c	18.60±2.055 a
	16	128.89±4.060 a	1.08±0.060 a	68.67±2.351 a	34.00±6.245	311.67±50.363	3.83±0.219 a	3.33±0.601 d	3.83±0.167 c	17.10±1.136 a
	24	133.72±6.620 a	1.12±0.067 a	71.33±4.324 a	34.00±7.234	341.67±48.667	2.67±0.217 a	2.83±0.441 d	2.83±0.167 c	15.80±2.055 ab
	32	145.28±14.730 a	1.19±0.097 a	67.17±5.134 a	34.67±4.807	354.33±25.957	3.23±0.642 a	3.17±0.167 d	3.17±0.577 c	18.17±2.741 a
3	8	129.33±1.711 a	1.08±0.066 a	59.67±2.315 b	40.00±1.528	323.67±23.132	3.06±0.879 a	3.17±0.441 d	3.83±0.441 c	17.20±1.553 a
	16	127.89±1.176 a	1.18±0.103 a	57.33±2.404 b	36.67±3.844	317.00±51.069	1.50±0.132 b	4.50±0.764 cd	4.50±0.866 c	16.90±2.081 a
	24	126.67±8.643 a	1.01±0.020 ab	65.67±2.489 b	44.67±2.848	419.67±45.407	1.42±0.286 b	7.85±0.333 b	8.50±0.764 b	13.10±0.300 b
	32	132.89±11.573 a	1.11±0.093 a	59.17±3.005 b	35.33±5.364	305.67±22.696	1.33±0.282 b	7.50±0.764 b	7.67±0.333 b	12.40±0.404 b
7	8	121.11±3.234 b	1.06±0.052 ab	61.83±1.922 b	38.00±4.041	421.67±32.359	2.44±0.367 a	5.00±1.000 cd	2.67±0.441 c	16.20±0.987 a
	16	121.50±2.557 b	0.99±0.057 b	64.17±6.692 b	26.33±4.910	237.00±49.759	1.57±0.159 b	8.67±0.441 b	9.83±1.453 ab	12.20±1.353 b
	24	117.39 ±7.330 b	0.98±0.037 b	54.50±1.528 b	35.00±6.429	240.33±42.369	1.30±0.112 b	9.17±0.167 b	11.00±0.289 a	10.97±2.378 bc
	32	104.44 ±8.231 b	0.94±0.048 b	52.00±1.756 b	36.00±6.028	285.00±62.000	1.14±0.275 b	12.50±3.041a	10.83±0.882 a	9.00±0.231 c

The values are the means of 3 replications and standard error. Different letters in the same column indicate statistically significant differences between ($p < 0.05$). PH (plant height), StD (stem diameter), CD (canopy diameter), LN (compound leaves number), LtN (leaflet number), LtA (leaflet area), Snc (compound leaves senescence number), Abc (compound leaves abscission number), LtDW (leaflet dry weight)

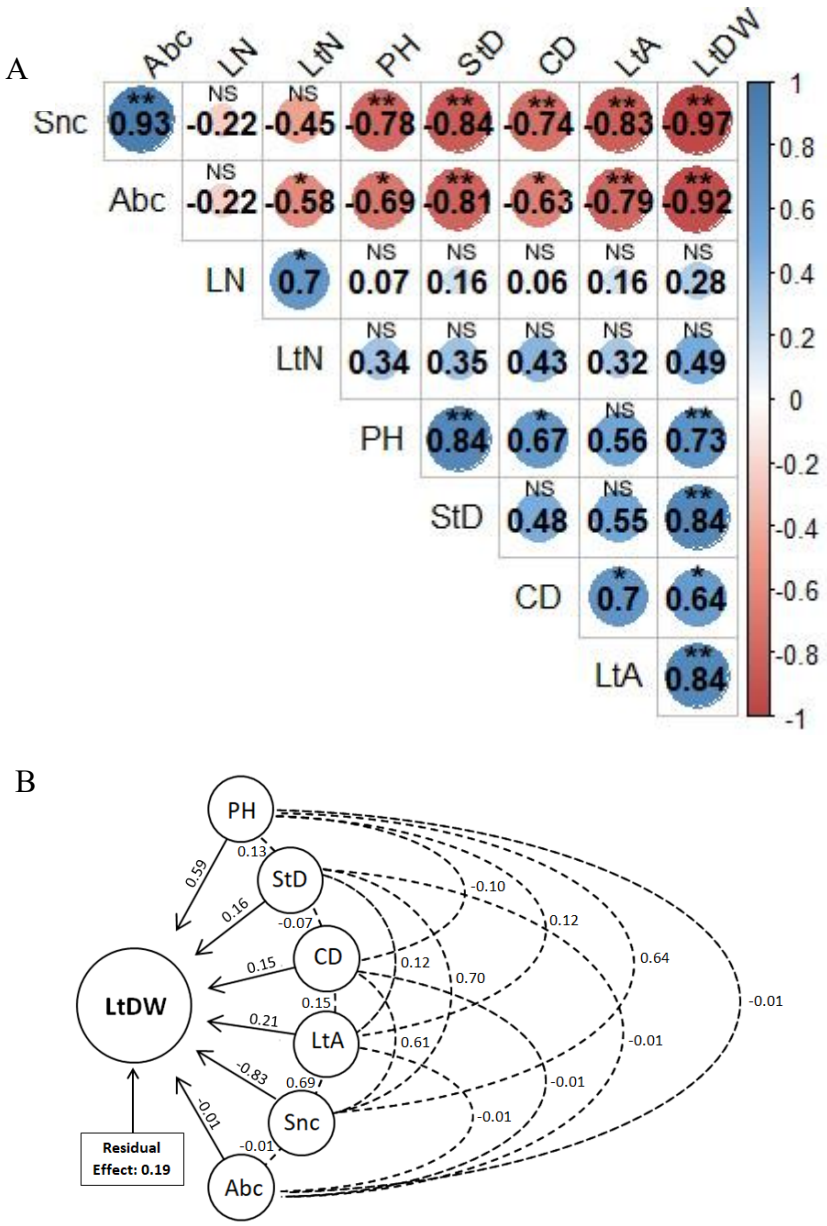

FIGURE 2. Pearson correlation (A) and path analysis (B) of several agronomic characters of *Moringa oleifera* treated by withholding watering intervals and drought periods. LtDW (leaflet dry weight), PH (plant height), StD (stem diameter), CD (canopy diameter), LN (compound leaves number), LtN (leaflet number), LtA (leaflet area), Snc (senescence), Abc (abscission). In path analysis, solid and dotted lines describe the direct effect and indirect effect to LtDW. * Significantly different at $p \leq 0.05$, ** Significant at $p \leq 0.01$, ^{NS}Not significant at $p \leq 0.05$

PHYSIOLOGICAL RESPONSES

The withholding watering treatments decreased water potential (WP), relative water content (RWC), and chlorophyll content (Chl), while they increased proline content (Pro) (Figure 3). Statistical analysis showed that WP and RWC decreased significantly due to withholding watering intervals, and were not affected by drought periods. The I_2 and I_3 caused WP to decrease significantly by about 31.66% and 86.20%, and RWC

by about 12.90% and 27.56% compared to the control (Figure 3(A) and 3(B)). Chl a, Chl b, Chl total, and Pro were affected by the interaction of both withholding watering intervals and drought periods. Chl a, Chl b, Chl total of treatment combination I_2D_1, I_2D_2, I_3D_1 were not significantly different compared to the control, whereas other treatments decreased significantly (Figure 3(C), 3(D), 3(E)). Proline content (Pro) increased along with increasing withholding watering intervals and drought periods. The increase in Pro began at the 3 days withholding watering interval and increased more after the 7 days. Pro also increased due to the increasing drought periods, where the highest Pro was shown by 32 days, followed by 24 days and 16 days, then 8 days (Figure 3(F)).

Physiological characters have been widely used as indicators of a plant experiencing water stress (Ali et al. 2019; Brunetti et al. 2018; Hasan et al. 2020; Jabeen et al. 2019; Thapa et al. 2018). Several physiological variables including WP, RWC, and Pro

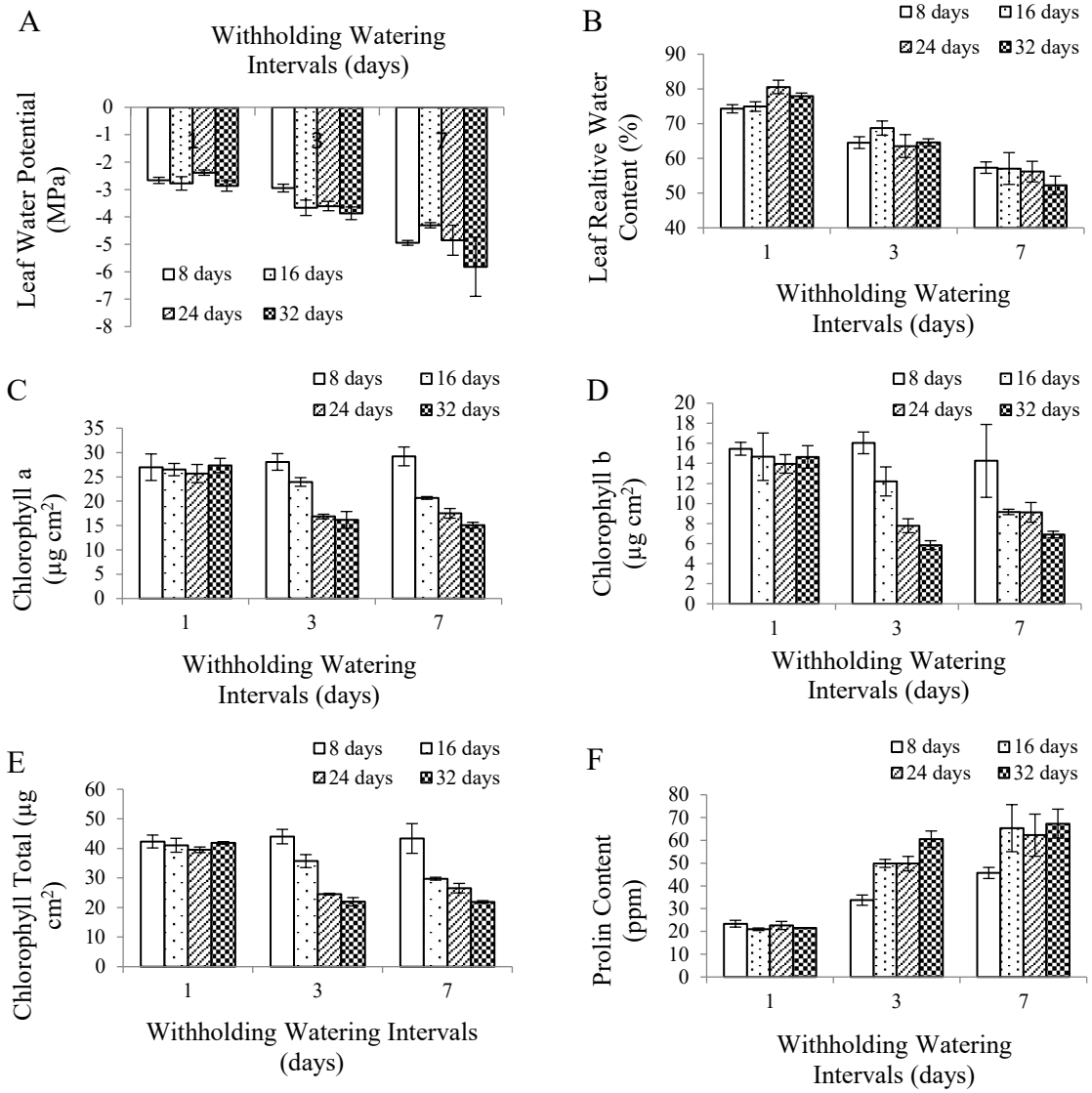

FIGURE 3. Physiological characteristics of *Moringa oleifera* treated by 1, 3, 7 days of withholding watering intervals and 8, 16, 24, 32 days of drought periods. Leaf water potential (A), leaf relative water content (B), chlorophyll a (C), chlorophyll b (D), chlorophyll total (E), and proline (F)

values in *Maclura pomifera* changed under water stress conditions compared to normal conditions. WP decreased progressively in the treatment of water stress of 75% and 30% FC by about 23% and 67%, while RWC decreased by about 26.58% and 42.01% at 50% and 30% FC, whereas Pro increased by about 20% and 40% at 50% and 30% FC (Khaleghi et al. 2019). Chl was also been reported to decrease gradually under gradual water stress conditions (5, 10, and 15 days without irrigation) in *Populus* (Ahmed et al. 2021). The decrease in WP and RWC under water stress conditions was mainly caused by higher transpiration compared to water absorption by roots (Ratzmann, Zakhrova & Tietjen 2019; Sallam et al. 2019). Transpiration still occurs as a compensation for carbon fixation in photosynthesis; on the other hand, the soil water availability is low due to the water molecules being strongly adsorbed by soil particles. Chl decreased under water stress conditions, possibly due to degradation of the photosynthesis apparatus by overproduction of ROS (Karuppanapandian et al. 2011). The increase in Pro is one of the adaptation mechanisms by plants through osmotic adjustment. It leads to the osmotic potential in plant tissues to be remaining lower than the soil solution, therefore water remains able to be absorbed by the roots. Additionally, the synthesis of proline may also part of plant mechanism to inhibit ROS formation due to the excess of energy from the photosynthesis light reaction process under water stress conditions (maintaining NADPH/NADP+ balance) which increase the concentration of proline in plant tissues (Furlan et al. 2020; Hauer 2010).

FLAVONOID CONTENT AND WATER USE EFFICIENCY OF FLAVONOID PRODUCTION (WUE_f)

The Q and K in Moringa leaves were influenced by the interaction of both the withholding watering intervals and the drought periods. Quercetin (Q) and K increased in the plants treated with I_2, but they decreased significantly on the I_3. At the treatment combination I_2D_1, and I_3D_1 did not increase, but K increased by about 77% and 24% compared to the control, respectively. At the treatment combination I_2D_2, Q and K experienced the highest increase by about 173% and 126%, respectively, but at the treatment combination I_3D_2, they decreased drastically and even lower than those of the control plants (Figure 4(A) and 4(B)). At the treatment combination I_2D_3, Q and K increased by about 150% and 55%, but decreased significantly at the treatment combination I_3D_3. At the treatment combination I_2D_4, Q was not significantly different from the control, but at the treatment combination I_3D_4, it was 32% lower than the control. The K decreased by about 43% and 99% at the treatment combinations I_2D_4 and I_3D_4, respectively. From those data we can conclude that the optimum induction to increase the maximum content of Q and K in Moringa using water stress was the treatment combination I_2D_2.

Water stress has been proven to increase the concentration of secondary metabolites. This is part of the plant's mechanisms to deal with stress conditions and protect cells and its organelles from free radical, which is usually overproduced under stress conditions. Under water stress conditions, some of the carbon fixed from the atmosphere are used to form secondary metabolites using excess energy from light reaction of photosynthesis (Al-Gabbiesh, Kleinwächter & Selmar 2015; Zhang et al. 2017). In this study, the flavonoid (Q and K) content of Moringa leaves increased under mild water stress, but then decreased under severe water stress. Increased plant flavonoid concentration under water stress has been widely reported, such as Zhang et al. (2017) on *Stelaria dichotoma*, Sarker and Oba (2018) on *Amaranthus* leaves, and Ahmed et al. (2021) on *Populus*. However, based on the results of this study, the increase of flavonoid concentration was not always linear, but increased to a certain level of water stress. Yuan et al. (2012) reported that the baicalin (flavone) content of *Scutellaria baicalensis* leaves significantly decreased under water stress conditions for 70 days after increasing significantly on 50 days of treatment. Hodaei et al. (2018) also reported that concentrations of quercetin, apigenin, and luteolin in *Chrysanthemum morifolium* L. cultivar Azita increased significantly under 5 days of water stress, but decreased significantly under 7 days of water stress. In peanuts, Kubra et al. (2021) also reported that the flavanol content of 6 susceptible and tolerant to drought stress genotypes decreased significantly after 14 days of water stress treatment. The decrease of flavonoid concentrations (Q and K) under severe water stress conditions in this study was probably due to the decrease of enzymes involved in the synthesis of flavonoid compounds such as CHS (chalcone synthase). CHS enzyme is known as an enzyme that plays an important role in flavonoid biosynthesis, especially at the stage of converting Coumaryl-CoA to naringenin chalcone before becoming naringenin, which is known as an intermediate compound (Saito et al. 2013). CHS has been reported to decrease after increasing in mild stress in *Scutellaria baicalensis* (Yuan et al. 2012).

The non-linear trend of bioactive compounds in response to drought treatments gives an opportunity to find the best treatment for bioactive induction in Moringa. Among the water stress treatments applied, treatment combination I_2D_2 was the most effective and efficient treatment to induce the synthesis of Q and K in Moringa leaves as presented from the highest value of WUE_f (Figure 4(C) & 4(D)) and the value of fold change, which reached 2.5 and 2-fold for Q and K, respectively (Figure 5). For every liter of water given, as much as 0.75 mg Q and 1.65 mg K were produced. The efficiency of water uses in this treatment increased by about 178% for Q and 123% for K compared to the control. This is supported by the results of PCA and the biplot analysis, which showed that treatment combination I_2D_2 was clustered according to Q and K characters and adjacent to the control group which was clustered according to several plant growth characters including LtDW (Figure 6). This indicated that the treatment combination I_2D_2 not only had the highest concentrations of Q and K but also produced high leaf biomass. It means that the treatment can increase the concentration of flavonoids, especially Q and K, without significantly reducing biomass. This result also in line with the induction of other plants such as *Achillea* sp. and *Stelaria dicotoma*. Gharibi et al. (2016) recommended the moderate drought stress treatment (50% of field capacity) as the optimum condition to obtain appreciable total phenolic and flavonoid compounds from *Achillea* species. Zhang et al. (2017) also found that the moderate water stress (60-70% or 80-90% of field capacity) was suitable for biomass formation and bioactive accumulation in *Stelaria dicotoma*.

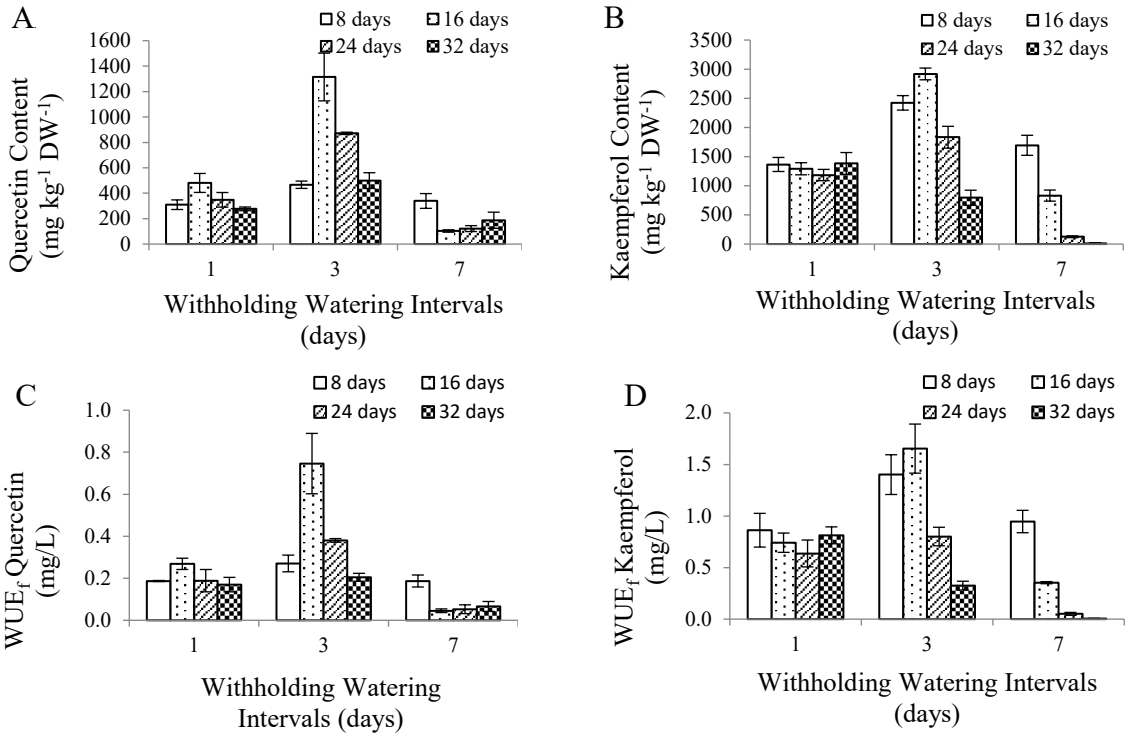

FIGURE 4. Flavonoid content and water use efficiency of flavonoid (WUE_f) based on quercetin and kaempferol of *Moringa oleifera* treated by 1, 3, 7 days of withholding watering intervals and 8, 16, 24, 32 days of drought periods. Quercetin content (A), Kaempferol content (B), WUE_f Quercetin (C), and WUE_f Kaempferol (D)

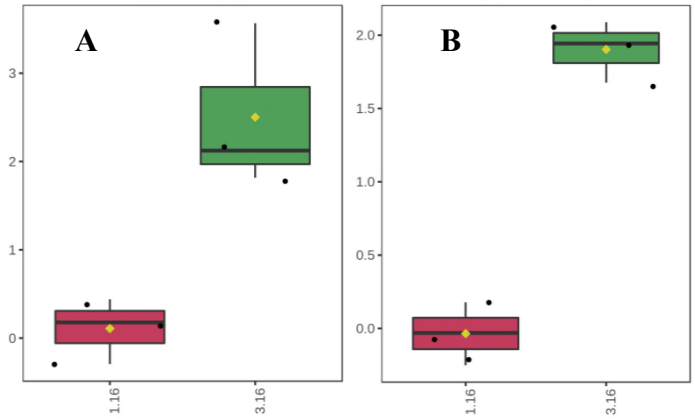

FIGURE 5. Fold change of flavonoid content of *Moringa oleifera* treated by 3 days of withholding watering intervals and 16 days of drought periods (I_2D_2) compared to the control (I_1D_2). Quercetin (A) and Kaempferol (B)

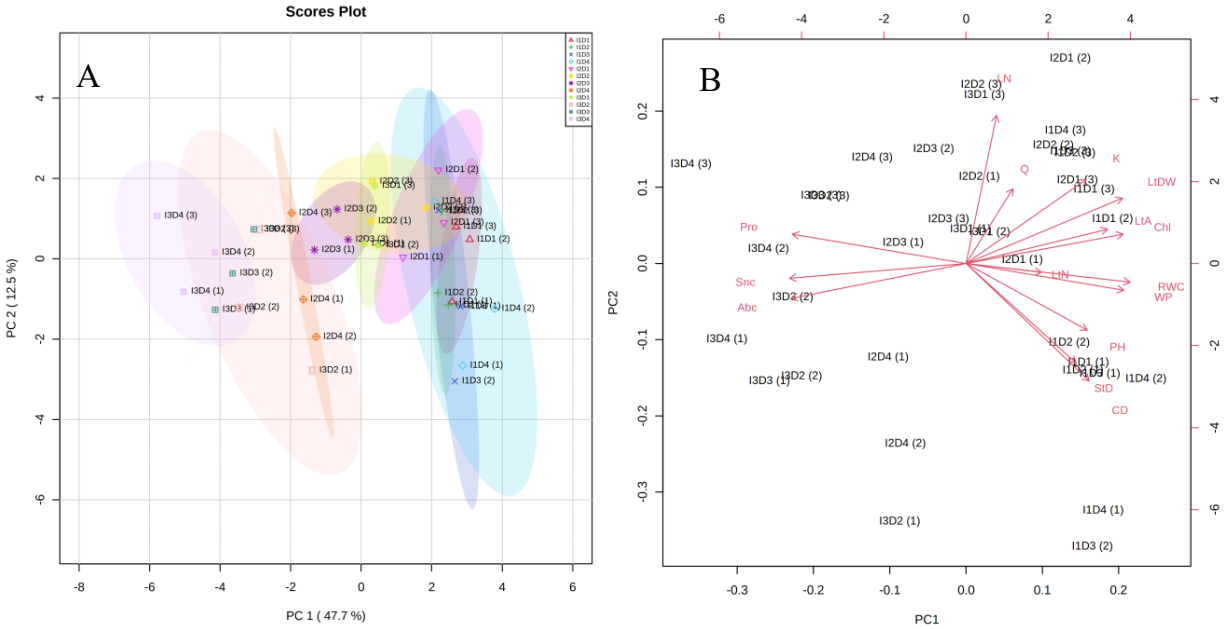

FIGURE 6. PCA of *Moringa oleifera* is based on the characteristics of agronomy, physiology, and the content of flavonoids (quercetin and kaempferol). 2D-Scores plot (A) and biplot (B). PH (plant height), StD (stem diameter), CD (canopy diameter), LN (leaves number), LtN (leaflet number), LtA (leaflet area), Snc (senescence), Abc (abscission), LtDW (leaflet dry weight), WP (water potential), RWC (relatif water content), Chl (chlorophyll), Pro (proline), Q (quercetin), and K (kaempferol)

Conclusion

The treatments given in this study have succeeded in inducing water stress in Moringa plant which affected physiological characters, growth, biomass production, and flavonoid content. The content of leaf quercetin and kaempferol in Moringa can be effectively increased by mild drought stress treatment. The treatment combination of I_2D_2 (3 days withholding watering intervals with 16 days of drought periods) could be recommended as an effective and efficient irrigation method in Moringa cultivation to produce high-quality leaf biomass, which can be used as a material for functional foods and drugs to treat degenerative diseases. However, further research still needed to apply this result in the field when the plants are grown on the land without intensive irrigation system.

ACKNOWLEDGEMENTS

This research was funded by The Ministry of Research and Technology/National Research and Innovation Agency through Doctoral Dissertation Research Program, with the Grant number of 1/E1/KP.PTNBH/2021, and Indonesian Institutes of Sciences 2021. Special appreciate to Indra gunawan for his technical assistance.

REFERENCES

Abdelkawy, K.S., Balyshev, M.E. & Elbarbry, F. 2016. A new validated HPLC method for the determination of quercetin: Application to study pharmacokinetics in rats. *Biomedical Chromatography* 31(3). doi: 10.1002/bmc.3819

Ahmed, U., Rao, M.J., Qi, C., Xie, Q., Noushashi, H.A., Yaseen, M., Shi, X. & Zheng, B. 2021. Expression profiling of flavonoid biosynthesis genes and secondary metabolites accumulation in populus under drought stress. *Molecules* 26: 5546. https://doi.org/10.3390/molecules26185546

Al-Gabbiesh, A., Kleinwächter, M. & Selmar, D. 2015. Influencing the contents of secondary metabolites in spice and medicinal plants by deliberately applying drought stress during their cultivation. *Jordan Journal of Biological Sciences* 8(1): 1-10.

Ali, E., Iqbal, A., Hussain, S., Shah, J.M., Said, F., Imtiaz, M., Jalal, F. & Khan, M.A. 2019. Selection criteria to assess drought stress tolerance in wheat genotypes using physiological and biochemical parameters. *Biosciences Biotechnology Research Asia* 16(4): 751-762.

Azhar, N., Hussain, B., Ashraf, M.Y. & Abbasi, K.Y. 2011. Water stress-mediated changes in growth, physiology and secondary metabolites of desi ajwain (*Trachyspermum ammi* L.). *Pakistan Journal of Botany* 43: 15-19.

Bates, L.S., Waldren, R.P. & Teare, L.D. 1973. Rapid determination of free proline for water-stress studies. *Plant Soil* 39: 205-207.

Boumenjel, A., Papadopoulos, A. & Ammari, Y. 2021. Growth response of *Moringa oleifera* (Lam) to water stress and to arid bioclimatic conditions. *Agroforestry Systems* 95: 823-833.

Brunetti, C., Loreto, F., Ferrini, F., Gori, A., Guidi, L., Remorini, D., Centritto, M., Fini, A. & Tattini, M. 2018. Metabolic plasticity in the hygrophyte *Moringa oleifera* exposed to water stress. *Tree Physiology* 38(11): 1640-1654.

Edwinanto, L., Septiadi, E., Nurfazriah, L.R., Anastasya, K.S. & Pranata, P. 2018. Phytochemical features of *Moringa oleifera* leaves as anticancer. *Journal of Medicine and Health* 2(1): 680-688.

Furlan, A.L., Bianucci, E., Giordano, W., Castro, S. & Becker, D.F. 2020. Proline metabolic dynamics and implications in drought tolerance of peanut plants. *Plant Physiology and Biochemistry* 151: 566-578.

Gharibi, S., Tabatabaei, B.E.S., Saeidi, G. & Goli, S.A.H. 2016. Effect of drought stress on total phenolic, lipid peroxidation, and antioxidant activity of achillea species. *Applied Biochemistry and Biotechnology* 178: 796-809.

González-Romero, J., Arranz-Arranz, S., Verardo, V., García-Villanova, B. & Guerra-Hernández, E.J. 2020. Bioactive compounds and antioxidant capacity of moringa leaves grown in Spain versus 28 leaves commonly consumed in pre-packaged salads. *Processes* 8(10): 1297.

Hasan, M.M., Alharby, H.F., Uddin, N., Ali, M.A., Anwar, Y., Fang, X.W., Hakeem, K.R., Alzahrani, Y. & Hajar, A.S. 2020. Magnetized water confers drought stress tolerance in moringa biotype via modulation of growth, gas exchange, lipid peroxidation and antioxidant activity. *Polish Journal of Environmental Studies* 29(2): 1625-1636.

Hasym, A.M.O.M., Nor, N.M., Adnan, L.H.M., Ahmad, N.Z.B., Septama, A.W., Najihah, N.N., Lwin, O.M. & Simbak, N. 2021. Effects of apigenin, luteolin, and quercetin on the natural killer (NK-92) cells proliferation: A potential role as immunomodulator. *Sains Malaysiana* 50(3): 821-828.

Hauer, B. 2010. Role of proline in plant response to drought and salinity. *Handbook of Plant and Crop Stress*. 3rd edition, Chapter 9. Boca Raton: CRC Press.

Hodaei, M., Rahimmalek, M., Arzani, A. & Talebi, M. 2018. The effect of water stress on phytochemical accumulation, bioactive compounds and expression of key genes involved in flavonoid biosynthesis in *Chrysanthemum morifolium* L. *Industrial Crops & Products* 120: 295-304.

Jabeen, M., Akram, N.A., Ashraf, M. & Aziz, A. 2019. Assessment of biochemical changes in spinach (*Spinacea oleracea* L.) subjected to varying water regimes. *Sains Malaysiana* 48(3): 533-541.

Juan, C.A., de la Lastra, J.M.P., Plou, F.J. & Pérez-Lebeña, E. 2021. The chemistry of reactive oxygen species (ROS) revisited: Outlining their role in biological macromolecules (DNA, lipids and proteins) and induced pathologies. *International Journal of Molecular Sciences* 22: 4642.

Kapoor, D., Bhardwaj, S., Landi, M., Sharma, A., Ramakrishnan, M. & Sharma, A. 2020. The impact of drought in plant metabolism: How to exploit tolerance mechanisms to increase crop production. *Applied Sciences* 10: 1-19.

Karuppanapandian, T., Moon, J.C., Kim, C., Manoharan, K. & Kim, W. 2011. Reactive oxygen species in plants: Their generation, signal transduction, and scavenging mechanisms. *Australian Journal of Crop Science* 5(6): 709-752.

Khaleghi, A., Naderi, R., Brunetti, C., Maserti, B.E., Salami, S.A. & Babalar, M. 2019. Morphological, physiochemical and antioxidant responses of *Maclura pomifera* to drought stress. *Scientific Reports* 9: 1920.

Kubra, G., Khan, M., Munir, F., Gul, A., Shah, T., Hussain, A., Caparrós-Ruiz, D. & Amir, R. 2021. Expression characterization of flavonoid biosynthetic pathway genes and transcription factors in peanut under water deficit conditions. *Frontiers in Plant Science* 12: 1-18.

Kumar, S. & Pandey, A.K. 2013. Chemistry and biological activities of flavonoids: An overview. *The Scientific World Journal* 2013: 162750.

Lima, V.M., de Lima Junior, J.A., Santos, H.C.A., da Silva, A.L.P., de Oleiveira, P.D., de Brito Neto, J.F. 2018. Growth of *Capsicum chinense* Jacq. subjected to different irrigation depths in the northeastern region of Pará, Brazil. *Jaboticabal* 46(1): 30-37.

Lin, M., Zhang, J. & Chen, X. 2018. Bioactive flavonoids in *Moringa oleifera* and their health-promoting properties. *Journal of Functional Food* 47: 469-479.

Malinowska, M., Donnison, I. & Robson, P. 2020. Morphological and physiological traits that explain yield response to drought stress in Miscanthus. *Agronomy* 10: 1194.

Mondal, S. & Rahman, S.T. 2020. Flavonoids: A vital resource in healthcare and medicine. *Pharmacy & Pharmacology International Journal* 8(2): 91-104.

Pakade, V., Cukrowska, E. & Chimuka, L. 2013. Metal and flavonol contents of *Moringa oleifera* grown in South Africa. *South African Journal of Science* 109(3-4): 7.

Patharkar, O.R. & Walker, J.C. 2019. Connections between abscission, dehiscence, pathogen defense, drought tolerance, and senescence. *Plant Science* 284: 25-29.

Ratzmann, G., Zakhrova, L., Tietjen, B. 2019. Optimal leaf water status regulation of plants in drylands. *Scientific Reports* 9: 3768.

Ridwan, Hamim, Suharsono, Nuril Hidayati & Indra Gunawan. 2021. Drumstick (*Moringa oleifera*) variation in biomass and total flavonoid content in Indonesia. *Biodiversitas* 22(1): 491-498.

Sade, N., Rubio-Wilhelmi, M.d.M., Umnajkitikorn, K. & Blumwald, E. 2018. Stress-induced senescence and plant tolerance to abiotic stress. *Journal of Experimental Botany* 69(4): 845-853.

Saito, K., Yonekura-Sakakibara, K., Nakabayashi, R., Higashi, Y., Yamazaki, M., Tohge, T. & Fernie, A.R. 2013. The flavonoid biosynthetic pathway in Arabidopsis: Structural and genetic diversity. *Plant Physiology and Biochemistry* 72: 21-34.

Sallam, A., Alqudah, A.M., Dawood, M.F.A., Baenziger, P.S. & Börner, A. 2019. Drought stress tolerance in Wheat and Barley: Advances in physiology, breeding and genetics research. *International Journal of Molecular Sciences* 20(13): 3137.

Sarker, U. & Oba, S. 2018. Drought stress enhances nutritional and bioactive compounds, phenolic acids and antioxidant capacity of *Amaranthus* leafy vegetable. *BMC Plant Biology* 18(1): 258.

Shah, S.H., Houborg, R. & McCabe, M.F. 2017. Response of chlorophyll, carotenoid and SPAD-502 measurement to salinity and nutrient stress in Wheat (*Triticum aestivum* L.). *Agronomy* 7: 61.

Shukla, A., Panchal, H., Mishra, M., Patel, P.R., Srivastava, H.S., Patel, P. & Shukla, A.K. 2014. Soil moisture estimation using gravimetric technique and FDR probe technique: a comparative analysis. *American International Journal of Research in Formal, Applied & Natural Sciences* 8(1): 89-92.

Sims, D.A. & Gamon, J.A. 2002. Relationships between leaf pigment content and spectral reflectance across a wide range of species, leaf structures and developmental stages. *Remote Sensing of Environment* 81: 337-354.

Thapa, S., Reddy, S.K., Fuentealba, M.P., Xue, Q., Rudd, J.C., Jessup, K.E., Devkota R.N. & Liu, S. 2018. Physiological responses to water stress and yield of winter wheat cultivars differing in drought tolerance. *Journal of Agronomy and Crop Science* 204(4): 347-358.

Vongsak, B., Sithisarn, P., Mangmool, S., Tongpraditchote, S., Wongkrajang, Y. & Gritsanapan, W. 2013. Maximizing total phenolics, total flavonoids content and antioxidant activity of *Moringa oleifera* leaf extract by the appropriate extraction method. *Industrial Crops and Products* 44: 566-571.

Wang, T.Y., Li, Q. & Bi, K.S. 2018. Bioactive flavonoids in medicinal plants: Structure, activity and biological fate. *Asian Journal of Pharmaceutical Sciences* 13(1): 12-23.

Wasonowati, C., Sulistyaningsih, E., Indradewa, D. & Kurniasih, B. 2019. Morphophysiology and the yield of two types of moringa (*Moringa oleifera* Lamk) cultivated in two different regions in Madura. *IOP Conf. Series: Earth and Environmental Science* 250: 012004.

Yuan, Y., Liu, Y., Wu, C., Chen, S., Wang, Z., Yang, Z., Qin, S. & Huang, L. 2012. Water deficit affected flavonoid accumulation by regulating hormone metabolism in *Scutellaria baicalensis* Georgi roots. *PLoS ONE* 7(10): e42946.

Zhang, W., Cao, Z., Xie, Z., Lang, D., Zhou, L., Chu, Y., Zhao, Q., Zhang, X. & Zhao, Y. 2017. Effect of water stress on roots biomass and secondary metabolites in the medicinal plant *Stellaria dichotoma* L. *var. lanceolata* Bge. *Scientia Horticulturae* 224: 280-285.

*Corresponding author; email: hamim@apps.ipb.ac.id