Pertanika J. Trop. Agric. Sci. 43 (3): 391 - 413 (2020)

PERTANIKA JOURNALS

TROPICAL AGRICULTURAL SCIENCE

Journal homepage: http://www.pertanika.upm.edu.my/

Preliminary Study on the Effect of Nitrogen and Potassium Fertilization, and Evapotranspiration Replacement Interaction on Primary and Secondary Metabolites of *Gynura procumbens* Leaves

Mohamad Fhaizal Mohamad Bukhori[1,2]*, Muhd Kamal Izzat[3], Mohd Zuwairi Saiman[3], Nazia Abdul Majid[3], Hawa ZE Jaafar[1], Ali Ghasemzadeh[1] and Uma Rani Sinniah[1]

[1]Department of Crop Science, Faculty of Agriculture, Universiti Putra Malaysia, Serdang 43400, Selangor, Malaysia
[2]Centre for Pre-University Studies, Universiti Malaysia Sarawak, 94300 Samarahan, Sarawak, Malaysia
[3]Institute of Biological Sciences, Faculty of Science, Universiti Malaya, 50603 Kuala Lumpur, Malaysia

ABSTRACT

Despite progressive reports on pharmacological properties in *Gynura procumbens*, many are overlooking the importance of agronomic requirements for high yields and phytochemical content that vary due to environmental variations. The study was carried out to examine the effects of nitrogen (N), potassium (K), and evapotranspiration replacement **(**ER) on growth and phytochemical content. Treatments affected parameters significantly ($p \leq 0.05$) with a stronger effect on physiological and biochemical attributes ($p \leq 0.0001$). Highest and lowest yield of biomass and phytochemical content were observed under N0K30(70) and N90K0(25), respectively. Treatments interaction was highly significant ($p \leq 0.0001$) in Cond, TPrC, and TFC, ($p \leq 0.05$) in CF and PWP, and not significant ($p > 0.05$) in Photo, TCC, and TPC. The 75% ER had significant ($p \leq 0.05$) output of biomass and phytochemical content. As ER decreased from 100 to 25%, the Photo and CF were reduced. Phytochemical content displayed a significant negative relationship with PWP. Caffeic acid, kaempferol, and ferulic acid demonstrated as lead compounds. Following this, there were correlations between phenolic biosynthesis-regulated gene and gene expressions such as PAL, CHS, and F3H primers.

Keywords: Biosynthesis, flavanone3-hydroxylase, *Gynura*, metabolite, phenylalanine ammonia-lyase, phytochemical

ARTICLE INFO

Article history:
Received: 15 November 2019
Accepted: 04 March 2020
Published: 28 August 2020

E-mail addresses:
mbmfhaizal@unimas.my (Mohamad Fhaizal Mohamad Bukhori)
kamal.izzatmnd@gmail.com (Muhd Kamal Izzat)
zuwairi@um.edu.my (Mohd Zuwairi Saiman)
nazia@um.edu.my (Nazia Abdul Majid)
hawazej@upm.edu.my (Hawa Ze Jaafar)
upmali@yahoo.com (Ali Ghasemzadeh)
umarani@upm.edu.my (Uma Rani Sinniah)
* Corresponding author

ISSN: 1511-3701
e-ISSN: 2231-8542

INTRODUCTION

Enzymes in photosynthesis and metabolite processes require nitrogen (N), potassium (K), and water as cofactors. Several studies have reported associations between single or interaction of N-, K-, and water-dependent enzymes such as pyruvate kinase, starch synthase, nitrate reductase, and Rubisco for specific metabolic regulation (Pant et al., 2015). Due to the increasing establishment of gene expression and protein modification role in metabolic regulation, studies of N, K, and water availability-dependent reactions have also come into the mechanisms. To understand how N, K, and water influence the metabolite, the studies must assess metabolic regulation at different biological levels including photosynthesis, phytochemical, and gene expression to identify target rates of N, K, and water. Concurrently, the functional genes are determined and their roles verified (Mongkhonsin et al., 2016).

Among the parameters, water content is most affecting metabolites composition because water is characterized as a source of energy to synthesize organic compounds in photosynthesis. Water supply has an important role in growth and essential to determine the yield and quality of plants. Deficit or surplus water content is an environmental stress and is a significant factor influencing growth and productivity (Selmar & Kleinwächter, 2013). Identifying growth stages and compounds profile will allow water supply regimes to optimize crop yield and efficiently use of water resources. Rates of water intervals affecting plant performance. Water treatment, water use efficiency, and developmental stages have influenced overall plant performance including growth and metabolic activities. Generally, there are positive and negative effects exhibited by water treatment, depending on target treatment. There will be a target on growth and biomass, or biosynthesis of metabolites is a concern. The amount of water supply and selected rates of N and K will affect plant behavior regarding the biosynthesis of the metabolite (Kleinwächter & Selmar, 2014).

Besides, cellular and molecular regulation influences the biosynthesis and, therefore, affects the metabolites composition. Moreover, metabolites content and composition, and consistency in growth and development are especially susceptible to environmental factors such as water availability and fertilization due to plant heterogeneity. Therefore, it is significant to determine optimum evapotranspiration rate to optimize the productivity of the water unit, and at the same time to determine whether fertilizer supply may enhance or diminish the tolerance of plants to drought and possibly *vice versa*. Therefore, the general objective of the study was to examine the effects of different rates of N, K, and evapotranspiration replacement (ER) interaction on the growth and phytochemical content of *Gynura procumbens* (*G. procumbens*). And, specific objectives were to examine the correlation between total phenolic content biosynthesis and gene expressions such as phenylalanine ammonia-lyase and chalcone synthase to different rates effect of N, K and ER interaction,

and to identify optimum harvesting time for biomass yield and total content of metabolite.

MATERIALS AND METHODS

Treatments

The study was a three-factorial experiment. The first factor was two selected rates of N and K, *viz*. 0 (0.00 g total per plant) and 90 (1.08 g) kg N/ha (N0 and N90), applied in the form of urea and 0 (0.00 g) and 30 (0.36 g) kg K/ha (K0 and K30) applied in the form of muriate of potash. The second factor was four rates of ER, *viz.* 100 (percent, % of water replacement of total water lost volume per plant) as a control, 75 (75%), 50 (50%), and 25 (25%) per plant (100% ER, 75% ER, 50% ER, and 25% ER), applied manually at alternate days of irrigation frequency. The N and K were split into three fertilization phases (three months), and each phase (each month) was about 33.3% of total N and K fertilizer applied in the first week of the month. Meanwhile, the third factor was three harvestings (H) times, *viz*. 4, 8, and 12 weeks after treatment (WAT). These gave combined treatments of 216. Each combined treatment gave 72 plants per block, giving a total of 216 plants.

Total Plant Dry Weight

Three plants per treatment were harvested randomly and separately at 4, 8, and 12 WAT. The plants were separated into leaves, stems, and roots manually. Fresh plant materials were weighed and put in labeled paper bags. Then, dried at 40°C in a forced draft oven until constant weight attained to obtain dry weight (TPDW) (Oyedeji et al., 2014). The biomass of fresh and dry weight was measured using an electronic weighing machine. The unit of weight used was g. Dried samples were ground to a fine powder (0.25 mm) using a grinder and kept until analysis.

Leaf Gas Exchange Rate

The measurement was obtained from LI-COR® Environmental with a closed infrared gas analyzer. The measurements were carried out using fully expanded young leaves numbered three and four from plant apex to record photosynthetic carbon assimilation rate (Photo) and stomatal conductance to water (Cond). The unit of photosynthetic rate and stomatal conductance used was mol H_2O $m^{-2}s^{-1}$ and µmol CO_2 $m^{-2}s^{-1}$, respectively. The data were stored in the console and analyzed by the Photosyn Assistant software (Ibrahim et al., 2014).

Plant Water Potential Rate

The measurement was obtained from the pressure chamber instrument. The measurements were carried out using fully expanded young leaves numbered three and four from plant apex to record water potential rate (PWP). The leaf was cut from the stem and placed in a chamber with the cut petiole surface (0.5 cm) protruding through the rubber chamber lid. The pressure was applied to the leaf in the chamber and reading was taken when the first water appeared at the cut surface of the petiole. The unit of water potential used was Mpa (Jamaludin et al., 2015).

Chlorophyll Fluorescence Rate

The measurements were carried out using fully expanded young leaves numbered three and four from plant apex to record chlorophyll fluorescence rate (CF). Leaves were darkened for 15 min by attaching light-exclusion clips to the central region of the leaf surface. The rate was measured using a portable chlorophyll fluorescence meter. Measurements were recorded up for 5 s. The unit of quantum efficiency rate used was Fv/Fm (Ibrahim et al., 2017).

Total Chlorophyll Contents

Total chlorophyll content (TChlC) was measured spectrophotometrically using a fresh weight basis (Loh et al., 2002). Only fully expanded young leaves numbered three and four from plant apex were used in the analysis. Leaf disk of 3 mm in diameter was obtained using puncher to acquire 15 consistent sizes. The leaf disks were immersed and incubated in 20 mL of 80% acetone for homogenization in an aluminum foil-covered glass bottle for approximately 24 h at 5°C until all green color had bleached out. Finally, 15 µL solution was transferred into 96 well plates to determine the absorbance of chlorophyll *a* (Chl *a*), *b* (Chl *b*), and carotenoids (Car) using UV–Vis spectrophotometer at wavelengths of 645, 662, and 470 nm optical density (OD), respectively. The Chl *a*, Chl *b*, and Car content was calculated as µg g^{-1} fresh weight as per standard equations recommended:

Chl *a* = [(12.47 × OD at 662) - (3.62 × OD at 645) × 10] / (1000 × wt)

Chl *b* = [(25.06 × OD at 645) - (6.50 × OD at 662) × 10] / (1000 × wt)

Car = [(1000 × OD at 4700 – (1.29 × Chl *a*) – (53.78 × Chl *b*)] / 220

Total Carbohydrates Content

Total carbohydrates content (TCC) was measured spectrophotometrically using the Anthrone and Hofreiter method (Hansen & Moller, 1975). Samples (1g each) were weighed 1 g into a 50 mL conical tube. Then, hydrolyze by keeping it in a boiling water bath for three hours with 5 mL of 2.5 M hydrochloric acid and cool to room temperature. Next, neutralize with solid sodium carbonate until the effervescence ceases. Next, the volume was made up to 50 mL and centrifuged at 5,000 × *g* for 5 min. The supernatant separated and filtered with filter paper. The 1 mL aliquot was taken for analysis. Beforehand, the standards (liquid chromatography-grade glucose) were prepared by taking 0.0 (serves as blank), 0.2, 0.4, 0.6, 0.8, and 1 mL of the working standard. The volume was made up to 1 mL in all tubes including the sample tubes by adding distilled water. Cool the contents of all tubes on ice before adding ice-cold Anthrone reagent. Then, 4 mL of Anthrone reagent was added and heated for 8 min in a boiling water bath. The blanks used were absolute methanol. Finally, cool rapidly, then 15 µL solution was transferred into 96 well plates and read the sample absorbance at

630 nm using a UV–Vis spectrophotometer. From the graph (concentration of standard stock solution versus sample absorbance readings), calculate the quantification of carbohydrate present according to the formulation. The concentration of TCC was calculated according to the equation obtained from the standard glucose graph:

$$y = 0.01x + 0.1813$$

$$R^2 = 0.995$$

The TCC in the sample was expressed as mg glucose equivalent g^{-1} dry sample.

Total Protein Content

Total protein content (TPrC) was measured spectrophotometrically using the Lowry method (John, 1995). A 1g sample was weighed into 50 mL conical tube and extracted with 10 mL of 100% methanol (1:10 w/v) at room temperature for 24 h and centrifuged at 7,000 × *g* for 10 min. The supernatant separated and filtered with filter paper. Then, 0.2 mL of extract was pipette out and the volume was made up to 1 mL with distilled water. The 5 mL of alkaline copper reagent was added to all tubes and allowed it to stand for 10 min. Then, 0.5 mL of Folin's Ciocalteau reagent was added and incubated in the dark for 30 min. The blanks used were absolute methanol. Finally, 15 µL solutions were transferred into 96 well plates to determine the absorbance. The intensity of color developed was read at 660 nm using UV–Vis spectrophotometer. Beforehand, the standards [liquid chromatography-grade bovine serum albumin (BSA)] were prepared by dissolving 20 mg BSA in 10 mL of the same diluents for the samples. Then, dilute to 200, 400, 600, 800, 1,000, and 1,200 µg/mL. From the graph (actual protein content versus absorbance readings), calculate the quantification of protein present in the sample tube. The concentration of TPrC was calculated according to the equation obtained from the standard BSA graph:

$$y = 0.0621x + 0.1554$$

$$R^2 = 0.9904$$

The TPrC in the sample was expressed as mg BSA equivalent g^{-1} dry sample.

Total Lipid Content

Total lipid content (TLiC) was measured using the Folch method (Shams et al., 2015). A 1g well-ground sample was weighed into 50 mL conical tube and extracted with chloroform and methanol (2:1, v/v) (20 mL) (1:20 w/v) for homogenization at room temperature for 24 h and centrifuged at 7,000 × *g* for 10 min. A lipid extract was purified to eliminate contaminants by pouring the extracts into a beaker through filter paper containing activated charcoal to remove coloring matters. A clear supernatant obtained was then further purified with 0.2 mL of aqueous 0.9% (w/v) sodium chloride. Purified lipids were transferred into evaporated and concentrated dryness at 40ºC, and the residue weighed. Quantification of crude lipids was performed based on dry weight determination. The weight of extract gives TLiC which was expressed as mg g^{-1} dry sample.

Total Phenolic Content

Total phenolic content (TPC) was measured spectrophotometrically using the Folin-Ciocalteu colorimetric assay method. A 1g sample was weighed into 50 mL conical tube and extracted with 10 mL of 100% methanol (1:10 w/v) at room temperature for 24 h and centrifuged at 7,000 × *g* for 10 min. The supernatant separated and filtered with filter paper. Then, 1 mL of extract was pipetted out into 15 mL conical tube and 2 mL (10% v/v) of Folin-Ciocalteu reagent was added to the extracted sample and incubated for five min. Later, 1.6 mL (7.5%) of the sodium carbonate solution was added into the sample. The sample mixture was then vortexed and incubated in the dark for one hour at room temperature. The blanks used were absolute methanol. Finally, 15 µL solutions were transferred into 96 well plates to determine the absorbance. The absorbance of the samples was measured at 760 nm using UV–Vis spectrophotometer. Beforehand, a series of standard solutions (liquid chromatography-grade caffeic acid) (0.1 to 2 mg/mL) were prepared. From the graph (actual phenolic content versus absorbance readings), calculate the quantification of the phenolic present in the sample tube (Teoh et al., 2016). The concentration of phenolic compounds was calculated according to the equation obtained from the standard caffeic acid graph:

$$y = 0.0098x + 0.0427$$

$$R^2 = 0.9942$$

The TPC in the sample was expressed as mg caffeic acid equivalents, CAE, in mg/g dry sample.

Total Flavonoid Content

Total flavonoid content (TFC) was measured spectrophotometrically using aluminum chloride complex colorimetric assay method (Mongkhonsin et al., 2016). A 1g sample was weighed 1 g into 50 mL conical tube and extracted with 10 mL of 100% methanol (1:10 w/v) at room temperature for 24 h and centrifuged at 7,000 × *g* for 10 min. The supernatant separated and filtered with filter paper. Then, 1 mL of extract was pipetted out into a 15 mL conical tube, mixed with 5 mL of distilled water and 0.3 mL of 5% sodium nitrite solution. The mixture was mixed well and allowed to stand for 6 min. Then, 0.6 mL of 10% aluminum chloride solution was added. After 5 min, 2 mL of 1M sodium hydroxide was added to the mixture and made up to 10 mL with distilled water. The blanks used were absolute methanol. Finally, 15 µL solutions were transferred into 96 well plates to determine the absorbances. The absorbance of the samples was measured at 510 nm using UV–Vis spectrophotometer. Beforehand, a series of standard solutions (liquid chromatography-grade kaempferol) (0.04 to 1.80 mg/mL) were prepared. From the graph (actual flavonoid content versus absorbance readings), the quantification of flavonoid present in the sample tube was calculated. The concentration of flavonoid compounds was calculated according to the equation obtained from the standard kaempferol graph:

$y = 0.0108x + 0.0435$

$R^2 = 0.9933$

The TFC in the sample was expressed as mg kaempferol equivalents, KE, in mg/g dry sample.

Preparation of Plant Extract

A 1g well-ground sample was weighed into 50 mL conical tube and extracted with 10 mL of 100% methanol (1:10 w/v) at room temperature for 24 h, sonicated at normal mode for 5 min and centrifuged at 7,000 × *g* for 10 min. The supernatant separated and filtered with filter paper. The methanolic-extract was transferred into evaporated and concentrated dryness at 40 ºC using a rotary evaporator. It was weighed, re-dissolved in 1.5 mL liquid chromatography-grade methanol and filtered through a sterile membrane filter, 0.45 µm; 25 mm in 2 mL amber glass HPLC vials and ready for further chromatographic analysis (Li et al., 2016).

Thin Layer Chromatography

The stationary phase used was 20 × 20 cm, 0.25 mm TLC plate pre-coated with silica gel 60 F_{254} on aluminum sheets and the mobile phase used was a mixture of toluene, ethyl acetate and formic acid (5:4:1). Marker or reference compounds used were caffeic acid, cinnamic acid, chlorogenic acid, gallic acid, ferulic acid, and vanillic acid for phenolic compounds. Meanwhile, kaempferol, quercetin, myricetin, and rutin for flavonoid compounds (Ismail et al., 2017). The 10 µL of each tested sample and reference compound was applied as a 6 mm band, 2 mm apart, 10 mm from the lower, upper, left, and right edges of the plate using a microsyringe. In the glass tank, 50 mL developing solvent was poured and allowed to saturate for 5 to 10 min at room temperature. Migration (as in linear ascending development) distance of the developing solvent on the plate is 80 mm from the lower edge of the plate or equivalent to the time allowed for the development and maximal separation of the active compounds present in the samples was 15 to 25 min. The plates were then dried at 100ºC using a forced draft oven for 3 to 5 min. Dried plates were visualized under UV light at 254- and 366- nm. The color and distance of unknown spots were compared with the marker or reference compound. The R_f values were calculated using the formula of migration distance of the sport/migration distance of the solvent.

Total RNA Extraction

Samples were ground into fine powder in liquid nitrogen. About 100 mg of ground samples were transferred into 1.5 mL Eppendorf tube®. Total RNA was extracted from each sample using the Universal Plant Total RNA Extraction Kit (Spin-column) (BioTeke®, China) according to the manufacturer's protocol. The 1 mL Lysis Buffer PL™ was added into the tube and incubated for 15 min at 65ºC. The mixture was centrifuged at 12,000 × *g* for 10 min at 4ºC and the supernatant was pipette into RNase-free Filtration Column. The column was centrifuged at 12,000 × *g* for 10 min

and the flow-through was transferred into 1.5 mL Eppendorf tube®. One volume of 70 % ethanol was added, and mixtures were thoroughly mixed. Mixtures were then pipetted into Spin-column AC and centrifuged at 10,000 × *g* at 4ºC for 45 s. Flow-through was discarded and both Spin-column AC and collection tubes were reused. The 500 µL of Buffer RE was pipette to the center of Spin-column AC and the column was centrifuged at 10,000 × *g* for 45 s. Flow-through was discarded. Seven hundred (700) µL of Buffer RW was added and the column was centrifuged at 12,000 × *g* for 60 s followed by flow through the disposal. Five hundred (500) µL of Buffer RW was again added and the column was centrifuged at 12,000 × *g* for 60 s. Flow-through was discarded. Spin-column AC was replaced into the collection tube and spin for 2 min to remove excess ethanol. Spin-column AC was placed on 1.5 mL Eppendorf tube®. The 50 µL of pre-heated (at 75ºC) RNase-free water was applied into the center of Spin-column and left at room temperature for 2 min. The tube was centrifuged at 12,000 × *g* for 1 min. The RNA extracts were stored at -80ºC for further analysis.

RNA Quantification

The RNA extracts were quantified and characterized for purity using Nanophotometer Pearl UV-Vis spectrophotometer (IMPLEN™, Germany).

Synthesis of Complementary DNA (cDNA)

Synthesis of cDNA was carried out using TransScript® II One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen Biotech™, China). The method was conducted according to the manufacturer protocol, consisting of 200 ng of each RNA samples, 1 µL anchored oligo $(dT)_{20}$ primer (0.5 µg/µL), 10 µL 2X TS II reaction mix, 1 µL TransScript® II RT/RI enzyme mix, 1 µL gDNA remover, and nuclease-free water. The total volume of the reaction is 20 µL. The mixtures were incubated for 15 min at 50ºC and the reaction was terminated by heating the samples at 85 ºC for 5 s. The 5 µL of stock cDNA was aliquot into 45 µL of dH_2O to be used for RT-PCR runs.

Real-time PCR Analysis

The analysis was carried out using the KAPA SYBR® FAST qPCR Master Mix (2X) Kit (Kapa Biosystems™, USA). The primers sequence of *Gynura bicolor* (*Gb*) phenylalanine ammonia-lyase (PAL) (*Gb*PAL), chalcone synthase (CHS) (*Gb*CHS), flavanone3-hydroxylase (F3H) (*Gb*F3H) and one globular protein gene as a control gene (Tubulin) were used according to Fukuoka et al. (2014) (Table 1). The primer sequence was checked and verified using Primer3 software before synthesis. The qPCR samples were prepared to reach a final volume of 10 µL. None template control (NTC) was also prepared but without a cDNA template. The qPCR analysis was

Table 1
The primer used for qPCR

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')	Size of product (bp)
PAL	CTTACTTGACCGGCGAAAAGG	TTTGCACATAGCCGTGAACAC	2181
CHS	CCTTGACACAAGCCTTTACTCCTT	AGGGTGCGCGATCCAA	1197
F3H	ACCTTGTTGCTTCAGGACCAA	ATCCAACTCTTGCCACCATCA	1068
Tubulin	TGGAGGAGACCTGGCTAAGGT	CGGGAGAAGACTTCAGCAACA	275

Note. PAL = phenylalanine ammonia-lyase; CHS = chalcone synthase; F3H = flavanone-3-hydroxylase; Tubulin = globular protein; bp = base pair

conducted using the QuantStudio 12K Flex Real-Time PCR System. The qPCR method was set up for 40 cycles.

Statistical Analysis

The collected data were subjected to analysis of variance (ANOVA) and correlation using SAS® 9.4 software. The analyses were done in triplicate and expressed as mean (n=3) ± standard error (SE) from the dependent treatments (Jaafar et al., 2012). All the variables from measurements were analyzed using the General Linear Model with N, K, and ER supply management. Any differences between treatment means were analyzed by two-way analysis and compared using Duncan's multiple range test (DMRT) at *p*-value ≤0.05 levels. The regression model that best fitted the data, evaluated by an F-test, was chosen.

RESULTS

Total Plant Dry Weight, Leaf Gas Exchange Rate, Plant Water Potential Rate, Chlorophyll Fluorescence Rate, and Total Chlorophyll Contents

The effect of N, K, and ER rates as well as harvest time on TPDW, Photo, Cond, PWP, and CF were recorded in Tables 2, 3, and 5. Meanwhile, the effect on TChlC was listed in Tables 2, 4, 5, and 6.

Total Carbohydrates Content

The TCC was statistically significant in all N and K interaction treatments, increasing with increasing harvesting time and significantly different with decreasing rate of ER including the control plants ($p \leq 0.05$). The content was highest at Week 12 compared to Week 4 (Figure 1, Tables 2 and 4). The TCC are significantly correlated with Photo, TPrC, TPC, and TFC at r=-0.360; $p \leq 0.01$, r=0.745; $p \leq 0.0001$, r=0.515; $p \leq 0.0001$ and r=0.262; $p \leq 0.05$, respectively. However, the TCC is not significantly correlated with TLiC at r=0.218; $p > 0.05$ by a linear function (Tables 5 and 6). The contents of soluble sugar vary in plants and are subjected to different moisture conditions and nutritional status (Cheng et al., 2014). And, drought stress can also increase organic compounds required for cell osmotic adjustment, such as soluble sugars. The report is following the result when TCC was high at Week 12 compared to Week 4 with no significant difference ($p > 0.05$) in decreasing the rate of

Table 2
Probability of greater F (P>F) for the ANOVA on the effect of nitrogen, potassium and evapotranspiration rates, and harvest times on primary and secondary metabolite variables

Source	NK	ER	H	NK x ER	NK x H	ER x H	NK x ER x H	CoV
df	1	3	2	3	2	6	6	
TPDW	ns	ns	****	*	ns	ns	ns	28.399
Photo	ns	ns	****	*	**	ns	ns	41.464
Cond	**	****	****	***	****	****	****	15.191
PWP	****	****	****	ns	ns	ns	*	17.713
CF	****	****	***	ns	**	**	*	8.765
TChlC	****	****	****	ns	**	****	****	2.737
TCC	*	**	****	ns	ns	*	ns	9.729
TPrC	****	****	****	****	****	****	****	3.814
TLiC	ns	**	*	**	ns	ns	ns	48.295
TPC	ns	ns	****	*	****	****	ns	14.454
TFC	***	****	****	****	****	****	****	7.082

Note. All analyses are mean ± standard error of mean (SEM), N = 72 using DMRT. * significant at $p \leq 0.05$; ** significant at $p \leq 0.01$; *** significant at $p \leq 0.001$; **** significant at $p \leq 0.0001$; ^{ns} not significant at $p > 0.05$; N = nitrogen rates; K = potassium rates; ER = evapotranspiration replacement rate; H = harvest time; CoV = coefficient of variation; df = degree of freedom; TPDW = total plant dry weight; Photo = photosynthetic rate; Cond = stomatal conductance rate; PWP = plant water potential rate; CF = chlorophyll fluorescence rate; TChlC = total chlorophyll content; TCC = total carbohydrate content; TPrC = total protein content; TLiC = total lipid content; TPC = total phenolic content; TFC = total flavonoid content

Table 3
Effect of treatments and harvest times on growth and physiology variables

	TPDW	Photo	Cond	CF	PWP
NK					
N0K30	1.087^{a}	3.131^{a}	0.090^{a}	0.525^{b}	0.767^{b}
N90K0	1.047^{a}	3.627^{a}	0.081^{b}	0.599^{a}	0.982^{a}
ER					
100	1.004^{a}	3.362^{a}	0.088^{a}	0.772^{a}	0.725^{c}
75	1.064^{a}	3.342^{a}	0.093^{a}	0.660^{b}	0.810^{bc}
50	1.206^{a}	3.144^{a}	0.071^{b}	0.506^{c}	0.911^{b}
25	0.992^{a}	3.668^{a}	0.088^{a}	0.311^{d}	1.053^{a}
H					
Week 4	0.517^{c}	4.817^{a}	0.049^{c}	0.561^{b}	0.890^{a}
Week 8	0.748^{b}	2.461^{b}	0.057^{b}	0.596^{a}	0.760^{b}
Week 12	1.935^{a}	2.859^{b}	0.149^{a}	0.529^{c}	0.975^{a}
Interaction					
NK	0.578^{ns}	0.140^{ns}	0.005^{**}	$<.0001^{****}$	$<.0001^{****}$
ER	0.144^{ns}	0.733^{ns}	$<.0001^{****}$	$<.0001^{****}$	$<.0001^{****}$
H	$<.0001^{****}$	$<.0001^{****}$	$<.0001^{****}$	0.0001^{***}	$<.0001^{****}$
NKxER	0.013^{*}	0.043^{*}	0.0003^{***}	0.167^{ns}	0.336^{ns}
NKxH	0.410^{ns}	0.004^{**}	$<.0001^{****}$	0.009^{**}	0.287^{ns}
ERxH	0.289^{ns}	0.845^{ns}	$<.0001^{****}$	0.005^{**}	0.158^{ns}
NKxERxH	0.072^{ns}	0.666^{ns}	$<.0001^{****}$	0.013^{*}	0.015^{*}

Note. All analyses are mean ± SEM, N = 72 using DMRT. ^{a,b,c,d} Means with the same letter vertically within each factor are not significantly different ($p > 0.05$)

Table 4
Effect of treatments and harvest times on biochemical assay variables

	TChlC	TCC	TPrC	TLiC	TPC	TFC
NK						
N0K30	5.10036^{b}	182.912^{a}	26.3667^{a}	20.750^{a}	242.330^{a}	61.2039^{a}
N90K0	5.40067^{a}	172.856^{b}	23.9453^{b}	25.972^{a}	228.309^{a}	57.0561^{b}
ER						
100	5.42556^{a}	184.548^{a}	25.2528^{b}	20.222^{b}	238.37^{a}	61.116^{a}
75	5.22928^{b}	167.331^{b}	24.4800^{c}	22.722^{b}	239.06^{a}	61.157^{a}
50	5.07211^{c}	186.898^{a}	26.2978^{a}	18.444^{b}	241.03^{a}	61.033^{a}
25	5.27511^{b}	172.759^{b}	24.5933^{c}	32.056^{a}	222.81^{a}	53.214^{b}
H						
Week 4	7.96933^{a}	157.724^{c}	20.2771^{c}	19.208^{b}	186.220^{c}	51.616^{c}
Week 8	3.79367^{c}	172.062^{b}	24.2454^{b}	22.875^{ab}	215.884^{b}	60.473^{b}
Week 12	3.98854^{b}	203.866^{a}	30.9454^{a}	28.000^{a}	303.853^{a}	65.301^{a}
Interaction						
NK	<.0001****	0.018*	<.0001****	0.056^{ns}	0.087^{ns}	0.0001***
ER	<.0001****	0.003**	<.0001****	0.004**	0.358^{ns}	<.0001****
H	<.0001****	<.0001****	<.0001****	0.033*	<.0001****	<.0001****
NKxER	0.354^{ns}	0.064^{ns}	<.0001****	0.001**	0.044*	<.0001****
NKxH	0.007**	0.067^{ns}	<.0001****	0.105^{ns}	<.0001****	<.0001****
ERxH	<.0001****	0.022*	<.0001****	0.281^{ns}	<.0001****	<.0001****
NKxERxH	<.0001****	0.363^{ns}	<.0001****	0.122^{ns}	0.268^{ns}	<.0001****

Note. All analyses are mean ± SEM, N = 72 using DMRT. ^{a,b,c,d} Means with the same letter vertically within each factor are not significantly different (p>0.05)

Table 5
Correlation of growth and physiology variables

	H	TPDW	Photo	Cond	CF	PWP	TChlC	TCC	TPrC	TLiC	TPC	TFC
H	1.000											
TPDW	0.811 ****	1.000										
Photo	-0.430 ***	-0.282 *	1.000				0.556 ****	-0.360 **	-0.357 **	-0.046 ns	-0.121 ns	-0.365 **
Cond	0.729 ****	0.590 ****	-0.037 ns	1.000								
CF	-0.070 ns	-0.087 ns	-0.0002 ns	-0.048 ns	1.000		0.034 ns	-0.062 ns	-0.095 ns	-0.230 ns	0.074 ns	0.105 ns
PWP	0.139 ns	0.232 *	0.190 ns	0.169 ns	-0.419 ***	1.000						

Note. All analyses are mean ± SEM; N = 72 using DMRT

Table 6
Correlation of biochemical assay variables

	H	TChlC	TCC	TPrC	TLiC	TPC	TFC
H	1.000						
TChlC	-0.831 ****	1.000					
TCC	0.634 ****	-0.465 ****	1.000				
TPrC	0.857 ****	-0.663 ****	0.745 ****	1.000			
TLiC	0.252 *	-0.158 ns	0.218 ns	0.131 ns	1.000		
TPC	0.685 ****	-0.455 ****	0.515 ****	0.761 ****	0.120 ns	1.000	
TFC	0.401 ***	-0.327 **	0.262 *	0.300 *	0.117 ns	0.188 ns	1.000

Note. All analyses are mean ± SEM; N = 72 using DMRT

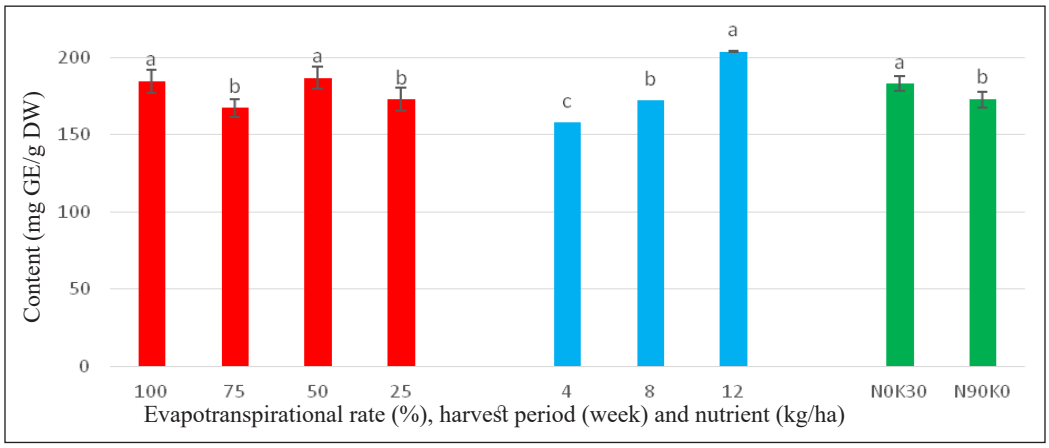

Figure 1. Effect of N, K and ER rates, and H times on total carbohydrate content

ER (Figure 1). However, N deficiencies had increased the content of soluble sugars when TCC was high in N0 than in N90 where ER was not significant (p>0.05) (Figure 1). Meanwhile, K deficiency had inhibited the growth of more than carbon assimilation, which resulted in the accumulation of carbohydrates in the leaves. This response may increase the substrate available for secondary metabolism. However, the results were less significant (p≤0.05) in K30 (182.912 mg GE/g DW) and K0 (172.856 mg GE/g DW) (Figure 1). Carbohydrates are the main organic solutes involved in osmotic adjustment which may lead to a decrease in leaf osmotic potential to maintain turgor.

This is an important adaptive mechanism in plants subjected to deficit irrigation (Wang et al., 2015). The accumulation of soluble TCC is also due to a reduction in soluble sugar transportation under water stress, however, the result reported less significant ($p \leq 0.05$) to TCC and ER.

Total Protein Content

The TPrC was statistically significant in all treatments with increasing harvesting time ($p \leq 0.0001$) (Figure 2, Tables 2 and 4). The 50% ER in N0K30 treatment and 25% ER in N90K0 had shown the highest protein content, meanwhile, 25% ER in N0K30 and N90K0 have shown the lowest (Figure 2). The content was highest under 50% ER in N0K30 at Week 12 compared to 25% ER in N90K0 at Week 4 (Figure 2 and Table 4). The TPrC was significantly correlated with Photo, TCC, TPC, and TFC at r=-0.357; $p \leq 0.01$, r=0.745; $p \leq 0.0001$, r=0.761; $p \leq 0.0001$ and r=0.300; $p \leq 0.05$, respectively. However, it was not significantly correlated with TLiC at r=0.131; p>0.05 by a linear function (Tables 5 and 6). The contents of proline (a proteinogenic amino acid in the biosynthesis of proteins) vary in plants and are subject to different moisture conditions and nutritional status (Mohd Zain & Ismail, 2016). And, drought stress can also increase organic compounds required for osmotic

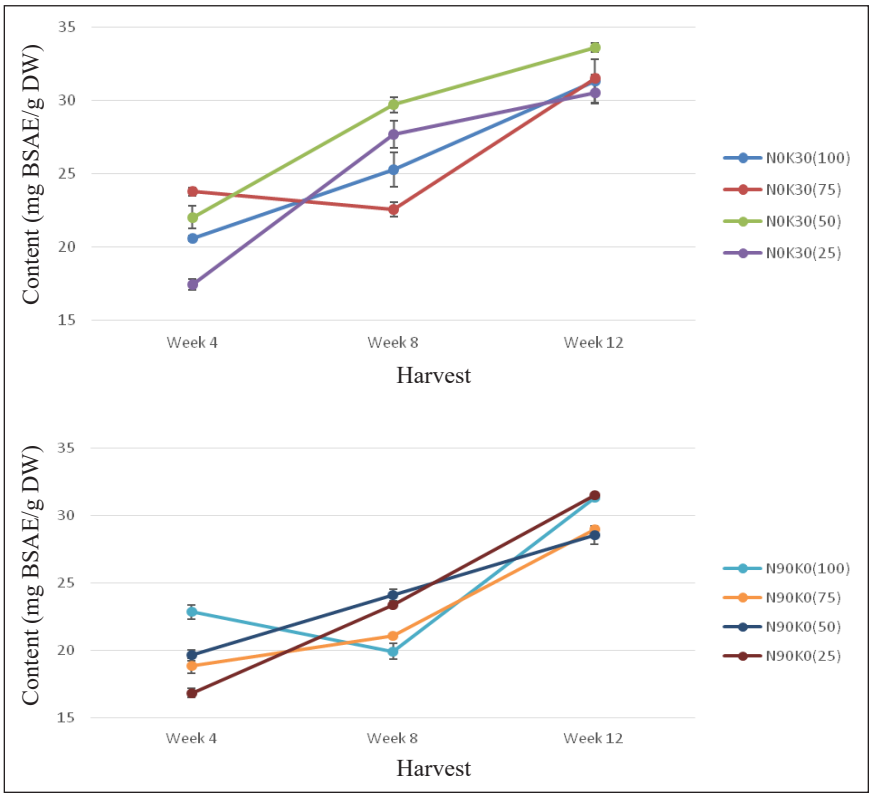

Figure 2. Changes in total protein content as affected by the interactions between rates of N, K and ER, and H times

adjustments, such as proline. The report is following the result when TPrC was high at Week 12 compared to Week 4, especially in N90K0 when 25% ER was reported high (Figure 2). Additionally, K is actively regulated in solute transport, protein synthesis, and enzyme activation point to a close relationship between K and metabolism (Armengaud et al., 2009). Potassium increases the plant's drought resistance through its functions in protein synthesis when TPrC was generally high in N0K30 (17–34 mg BSAE/g DW) compared to N90K0 (16–32 mg BSAE/g DW) (Figure 2).

Total Lipid Content

The TLiC was statistically not significant in all N and K interaction treatments, increasing with increasing harvesting time and significant difference with decreasing rate of ER including the control plants ($p \leq 0.05$). Total lipid content was highest in 25% ER at Week 12 compared to the lowest in 50% ER at Week 4 (Figure 3, and Tables 2 and 4). The TLiC was not significantly correlated with Photo, TCC, TPrC, TPC, and TFC at r=-0.046; p>0.05, r=0.218; p>0.05, r=0.131; p>0.05, r=0.120; p>0.05 and r=0.117; p>0.05, respectively by a linear function (Tables 5 and 6). As water stress rates increased, the oxidative stress in cells and tissues was enhanced, thus implying the occurrence of lipid peroxidation under high water stress (Jaafar et al., 2012) in the respective treatments, 20.222 mg/g DW (100% ER), 22.722 mg/g DW (75% ER), 18.444 mg/g DW (50% ER), and 32.056 mg/g DW (25% ER) (Figure 3). The formation of Malondialdehyde (MDA) was considered as a measurement of lipid peroxidation induced by a high-water stress rate.

Total Phenolic Content

The TPC was statistically consistent in all N and K interaction treatments, increased with increasing harvesting time, and no significant difference with decreasing rate of ER including the control plants ($p \leq 0.05$).

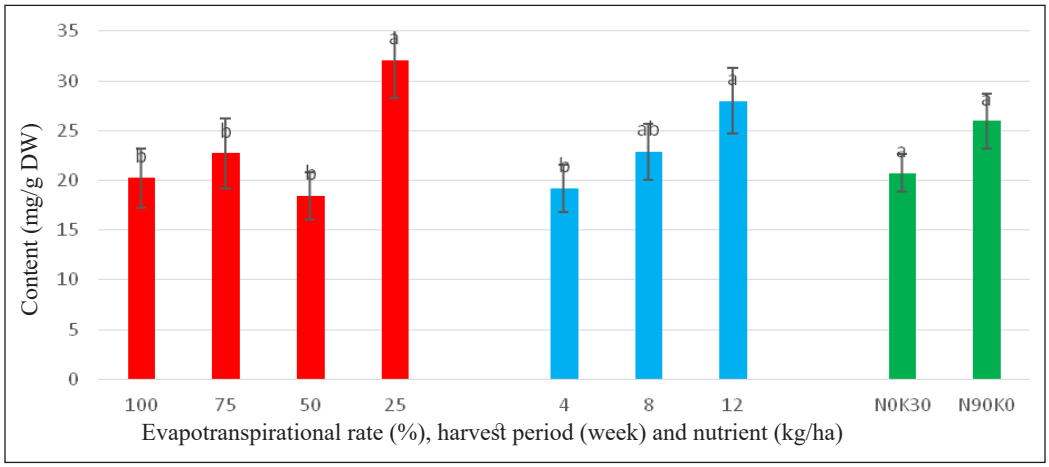

Figure 3. Effect of N, K and ER rates, and H times on total lipid content

Total phenolic content was highest at Week 12 compared to Week 4 (Figure 4, Tables 2 and 4). The TPC are significantly correlated with TCC and TPrC at r=0.515; p≤0.0001 and r=0.761; p≤0.0001, respectively. However, not significantly correlated with Photo, TLiC, and TFC at r=-0.121; p>0.05, r=0.120; p>0.05 and r=0.188; p>0.05, respectively by a linear function (Tables 5 and 6). There were studies which reported the effects of long-term and constant N limitation on carbon allocation for growth and synthesis of phenolics as well as N deficiency increases phenolic content in plants (Caretto et al., 2015). It was following the results when TPC was high in N0 at Week 12 compared to N90 when ER treatment was not significant (p≤0.05) (Figure 4). The increased content of phenolics in plant tissues was either as existing pools or by inducing their *de novo* synthesis (Romagni, 2009). Additionally, the accumulation of TCC had provided a signal of an increase in the production of secondary metabolites. However, less significant content of TCC in this study was translated into non-significant production of TPC corresponded (Figures 1 and 4, Table 6).

Total Flavonoid Content

The TFC was statistically significant in all treatments with increasing harvesting time (p≤0.0001) (Figure 5, Tables 2 and 4). The 75% ER in N0K30 treatment and 50% ER in N90K0 had shown the highest flavonoid content, meanwhile, 25% ER in N0K30 and 75% ER in N90K0 have shown the lowest. The content was highest under 50% ER in N90K0 at Week 12 compared to 75% ER in N90K0 at Week 4 (Figure 5). The TFC was significantly correlated with Photo, TCC and TPrC at r=-0.365; p≤0.01, r=0.262; p≤0.05 and r=0.300; p≤0.05, respectively. However, not significantly correlated with TLiC and TPC at r=0.117; p>0.05 and r=0.188; p>0.05, respectively by a linear function (Tables 5 and 6). The event was probably because, in resource-limited environments, carbon partitioning to constitutive secondary metabolism often increases, which enhances

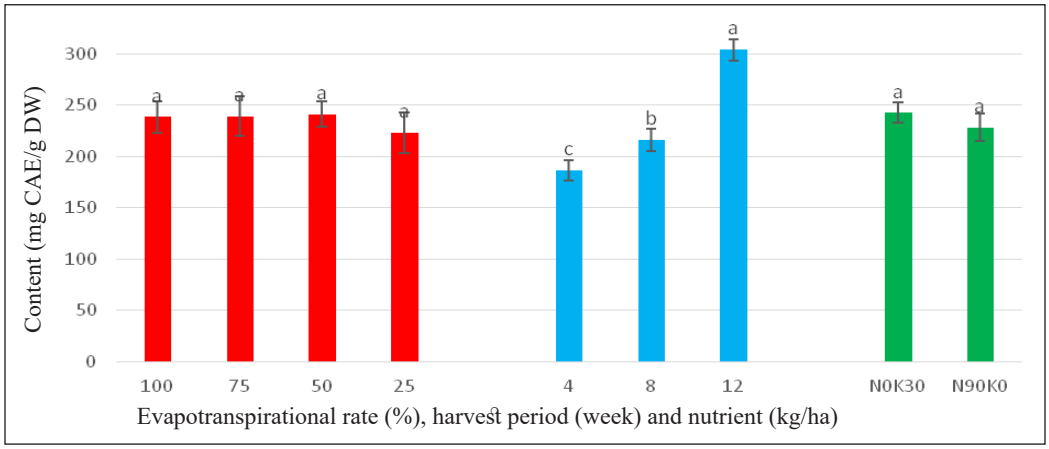

Figure 4. Effect of N, K and ER rates, and H times on total phenolic content

resistance to attack by pathogens and stress tolerance (Caretto et al., 2015). The results indicated the limitations in plant growth were generally accompanied by higher secondary metabolite content. And, N deficiency induces the synthesis of ascorbic acid, flavonoids, and flavonols. The result was following the report when TFC was generally high in N0 (40–80 mg KE/g DW) compared to N90 (20–80 mg KE/g DW). Coinciding with this prediction, the TFC increased with K starvation (N90K0), and an appropriate K rate (N0K30) (Figure 5) could maintain the concentration of flavonoids. In addition, there was an increase in TFC (70 mg KE/g DW and 83 mg KE/g DW) under high water stress (50% ER and 25% ER) (Figure 5), respectively to an accumulation of soluble TCC in plants is also as a result of reduced transportation of soluble sugar under water limitation (Figure 1) (Kleczewski et al., 2010). Possibly, there was a reduction in maximum quantum yield (0.01–0.49 Fv/Fm) (Tables 2 and 3) with increasing content of secondary metabolites as water field capacity being reduced (25% and 50% ER) again demonstrated the possible production of secondary metabolites under increasing water stress (Figure 5).

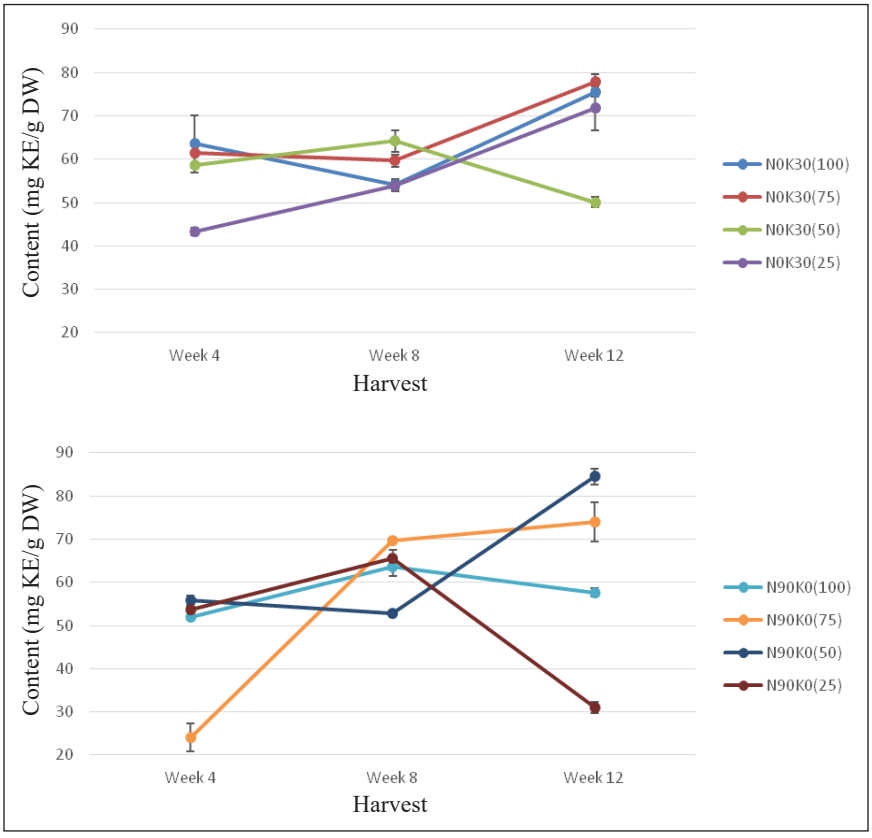

Figure 5. Changes in total flavonoid content as affected by the interactions between rates of N, K and ER, and H time

Thin Layer Chromatography

Qualitative analysis of the leaves methanol extract was performed using a mixture of toluene: ethyl acetate: formic acid (5:4:1 v/v) was used as the mobile phase (Sasidharan et al., 2011) yielded a good resolution of caffeic acid and ferulic acid with bands at R_f=0.64 and 0.69, respectively (Figure 6) for phenolic content. Meanwhile, the analysis also yielded a good resolution

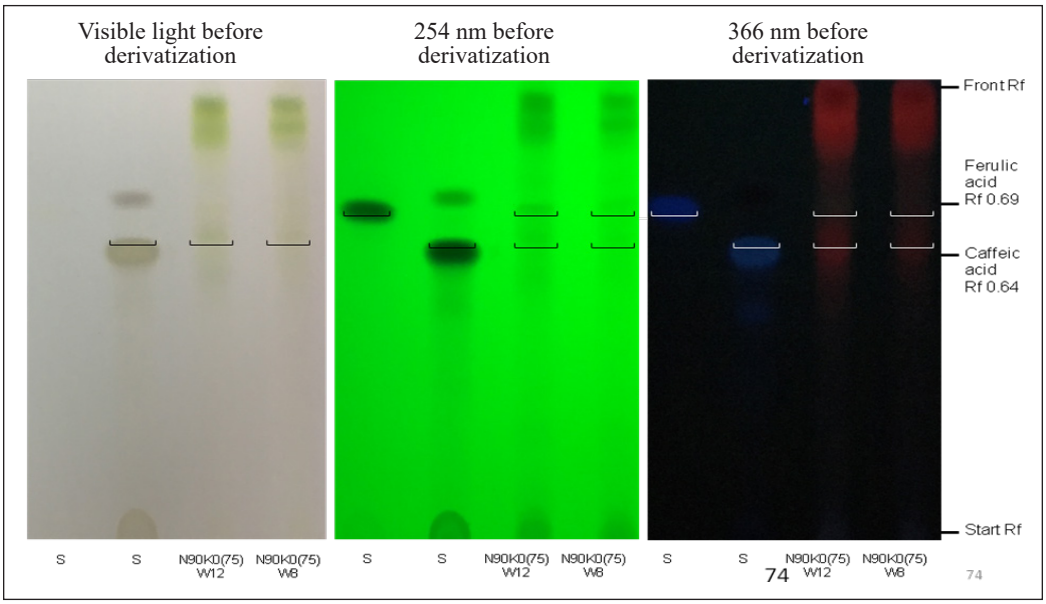

Figure 6. Thin-layer chromatography developed-profiles of caffeic and ferulic acid standard solution

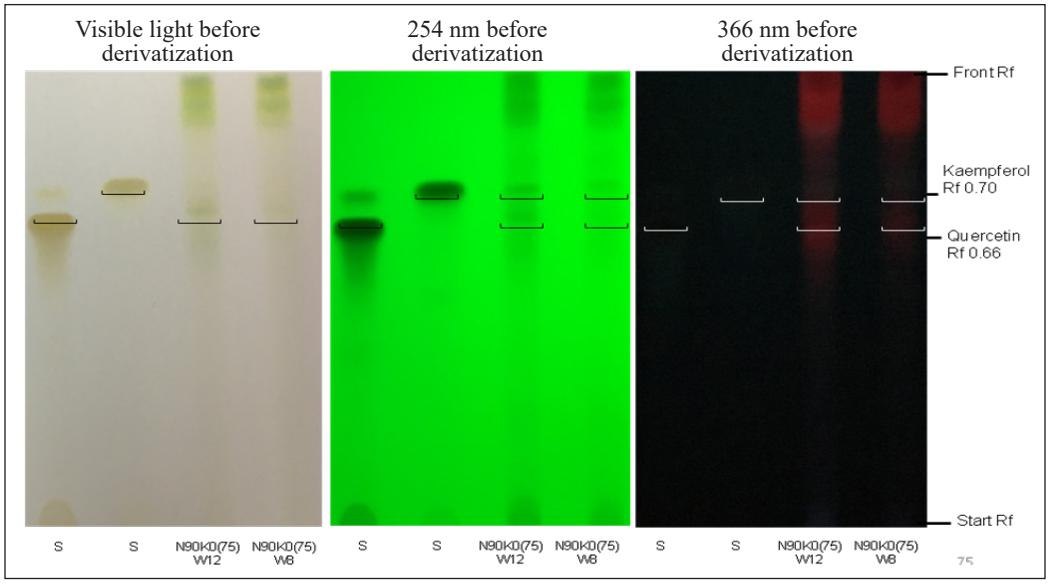

Figure 7. Thin-layer chromatography developed-profiles of kaempferol standard solution

of kaempferol with bands at R_f=0.70 (Figure 7) for flavonoid content. Caffeic acid, ferulic acid, and kaempferol were identified and quantified at visible light, 254 and 366 nm OD. The results revealed the methanol extract at 1 g/10 mL of 100% methanol contained caffeic acid and ferulic acid in accordance with TPC analysis, highest in N90K0(75) treatments at Week 12 compared to N90K0(75) at Week 8 (Figure 4). Meanwhile, kaempferol in accordance with TFC analysis, was highest in N90K0(75) at Week 12 compared to N90K0(75) at Week 8 (Figure 5). This TLC procedure can be used as a fast phytochemical markers screening method for *G. procumbens* leaf samples and herbal formulations.

Phenolic Gene Expression Profile

The analysis was to determine and identify *G. procumbens* phenolic gene expression and relation with selected phenolic biosynthetic genes. The analysis involved mother plant (control sample), and high and low phenolic content samples at Week 8 and 12 after treatment. The samples were selected based on the TPC analysis (Figure 4). The samples were subjected to real-time PCR analysis to substantiate the metabolites content is matched in quantification measurement with a spectrophotometer as well as to identify the responsible gene in modulating the metabolites synthesis. The control plants have shown the lowest content (Relative quantification, RQ 1) of phenolic content compared to other treatment samples. Phenolic content at Week 8 (RQ 1.482 and 0.974) were under the TPC analysis, however, in Week 12 (RQ 3.926 and 8.37) had contradicted the TPC analysis, where low and high TPC had shown a reversal in phenolic content RQ (Figure 8).

The phenolic metabolism was assessed by monitoring the leaf phenolic content together with the expression of three phenolic biosynthetic genes regulation [phenylalanine ammonia-lyase (PAL), chalcone synthase (CHS), and flavanone3-hydroxylase (F3H)] and one globular protein gene as a control gene (Tubulin). The mechanisms underlying plant adaptation may involve several processes, such as accumulation of signaling proteins and transcription factors or epigenetic modification (de Rosas et al., 2017). Although the transcript levels of phenolic biosynthesis genes were repressed during fertilizer and water re-supply, the decrease in phenolic content in tissues was slower and appeared to be metabolite specific. From a mechanistic viewpoint, the response to fertilizer and water deficiency implies an early reprogramming of gene expression that starts within the first month of limitation, whereas the effects on growth and metabolic content have been proven to be significant between two and three months later.

The increase in the content of secondary metabolites in the present work could be related to an increase in PAL activities under low water field capacity replacement. Suggesting up-regulation of secondary metabolite production with increased PAL activity. This is basically due to PAL is an enzyme, which synthesizes a precursor for total phenolics and flavonoids

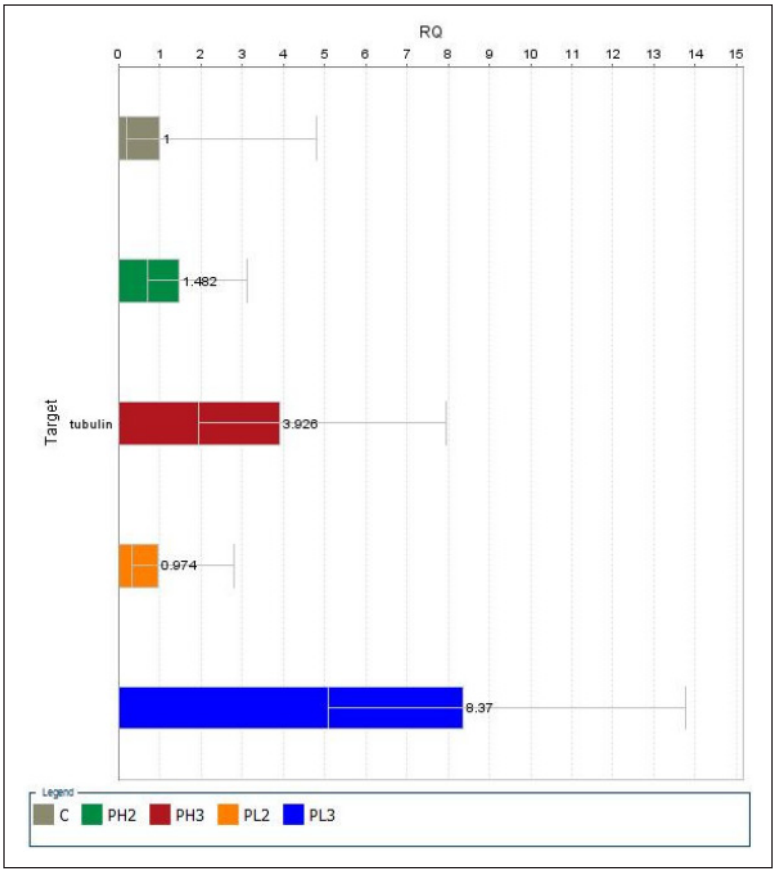

Figure 8. RT-qPCR relative quantification of phenolic compounds gene expression on four samples (PH2 to PL3) and one control sample (C) extracted with the spot method. Serial dilutions of crude extraction were used to calculate the relative quantification using the relative standard curve method. Results from each sample were normalized to the highest value (from sample PL3) and expressed as relative quantification (RQ). Bars represent the standard error of differences between the means at $p \leq 0.05$. C = control plants (mother plants); PH2 = high phenolic (8 WAT); PH3 = high phenolic (12 WAT); PL2 = low phenolic (8 WAT), and PL3 = low phenolic (12 WAT)

biosynthesis (Koyama & Goto-Yamamoto, 2008). High water stress in the present study may have increased the availability of phenylalanine pool as less protein is used for plant maintenance under high water stress, hence, more phenylalanine is available to produce secondary metabolites. These results suggest the up-regulation of secondary metabolites production in *G. procumbens* under high water stress may be due to an increase in PAL activity due in turn to the increased availability of phenylalanine under stress conditions. However, the synthesis of phenolics in plant tissues depends on genetics, the organ, and the developmental stage and is also greatly affected by environmental factors including N, K, and water availability (Turnbull et al., 2018).

DISCUSSION

Water is crucial for productivity and quality. However, the requirements are differed according to plant types and growing media. Deficit or surplus water will induce water stress-related metabolic responses, and due to reduced Cond, the uptake of CO_2 decreases significantly (Tables 2 and 3). Thus, the consumption of reduction equivalents ($NADPH^+H^+$) for CO_2 fixation via the Calvin cycle declines, accordingly, generating large oxidative stress and over-supply of reduction equivalents. Consequently, metabolic processes shifted towards biosynthetic activities which consume reduction equivalents. Accordingly, the synthesis of reduced compounds, such as phenols, is enhanced (Figure 4 and Table 4). As stress-related metabolic changes affect all plant processes extensively, the synthesis of secondary metabolites also is affected (Ren et al., 2014). The present study has shown a discrete effect on growth, physiology, and metabolite content in the following manner, N0K30>N90K0 and ER 75>50>100>25%. The highest and lowest yield of biomass and metabolite content was recorded under N0K30(70) and N90K0(25), respectively. The results also showed the influence of treatments are highly significant ($p \leq 0.0001$) in Cond, TChlC, TPrC, and TFC, ($p \leq 0.05$) in CF and PWP, and not significance ($p > 0.05$) in Photo, TCC, TLiC, and TPC (Tables 2, 3 and 4). Meanwhile, 75% of ER was significantly affected biomass, primary, and secondary metabolites content in all treatments (Tables 2, 3, and 4).

Total biomass and phenolic content negatively interacted with physiology, primary metabolic, and polyphenolic content (flavonoid) (Tables 5 and 6). The study has shown no-significant difference ($p > 0.05$) in Photo has caused TPDW to be significantly affected (Tables 5). Meanwhile, maintaining the productivity of Cond, TChlC, and CF had affected the TCC, TPrC, TLiC, and TFC (Tables 5 and 6). The study suggesting the source (Photo) and sink (metabolites) invariably recorded during the plant growth and development was negatively interacted according to the GDB hypothesis in water stress and selected rates of N and K study (Le Bot et al., 2009). The justification proposes trade-off between primary (growth) and secondary (defense/ stress tolerance) metabolism emerging from developmental constraints in growing cells, and in direct competition for resources between primary and secondary metabolism in mature cells. Therefore, when moderate resource limitation constrains relative growth rate to a greater degree than the net assimilation rate, photosynthate accumulates in tissues and becomes available to support secondary metabolic processes, resulting in higher levels of constitutive secondary metabolites in tissues (Shitan, 2016). These variations may be due to the inherent physiological abilities of the treated plants to absorb and utilize the given fertilizers and water rates with the requirements for growth and development.

In this study, Photo was decreased significantly ($p \leq 0.05$) under water deficit

condition. The decrease is probably due to low CO_2 availability as a result of reduced stomatal and mesophyll conductance. The limited CO_2 assimilation of the leaf tissues may result in an increased allocation of photoassimilates to the secondary metabolites production (Ren et al., 2014). The increase in secondary metabolites production under low Photo was due to upregulated shikimic acid pathway activity under stressed conditions. Suggesting up-regulation of the shikimic acid pathway has involved in secondary metabolites production was under down-regulated Photo. Under high water stress, there is a limit on the translocation of carbon to sinks, with the remaining carbon accumulates as carbohydrates, leading to an increase in carbon pool allocated for secondary metabolism, with little or no competition with growth and development. However, in this study, the result has shown less significance of TCC with ER treatment and in harvest time (Tables 2 and 4). Therefore, the study relatively agrees that water stress usually enhances the production of secondary metabolites (Kleinwächter & Selmar, 2014). This is following plant resource allocation for growth influenced phenolic content in plant tissue, by affecting the plant CNB. When conditions are favorable, plants preferentially allocate carbon for growth. On the other hand, when water or fertilizers are low, carbon will often accumulate, and used for secondary metabolite synthesis (Soubeyrand et al., 2014).

CONCLUSION

The lowest rate of N, moderate of K and 75% ER had produced optimum biomass for required optimum primary and secondary metabolites content. Meanwhile, caffeic acid, kaempferol, and ferulic acid were demonstrated as lead compounds in this study. High phenolic content is probably due to the induction of phenolic-related biosynthesis genes regulation including PAL, CHS, and F3H, and attributed to defense response against ER rates. The N, K, and ER modulation are effective to promote phenolic content because the expressions of phenolic-related biosynthesis genes are high as a result of favorable abiotic influence.

ACKNOWLEDGEMENTS

The authors would like to thank the MoHE Malaysia, UNIMAS, PBRL UM, and UPM.

REFERENCES

Armengaud, P., Sulpice, R., Miller, A. J., Stitt, M., Amtmann, A., & Gibon, Y. (2009). Multilevel analysis of primary metabolism provides new insights into the role of potassium nutrition for glycolysis and nitrogen assimilation in *Arabidopsis* roots. *Plant Physiology*, *150*(2), 772–785.

Caretto, S., Linsalata, V., Colella, G., Mita, G., & Lattanzio, V. (2015). Carbon fluxes between primary metabolism and phenolic pathway in plant tissues under stress. *International Journal of Molecular Sciences*, *16*(11), 26378–26394.

Cheng, X., Liu, J., Shu, J., & Yu, M. K. (2014). Effects of different light intensity on the growth and nutrients content of *Gynura*. *Advanced Materials*

Research, *955–959*, 3766–3769. doi: 10.4028/www.scientific.net/AMR.955-959.3766

de Rosas, I., Ponce, M. T., Malovini, E., Deis, L., Cavagnaro, B., & Cavagnaro, P. (2017). Loss of anthocyanins and modification of the anthocyanin profiles in grape berries of Malbec and Bonarda grown under high temperature conditions. *Plant Science*, *258*, 137–145.

Fukuoka, N., Suzuki, T., Minamide, K., & Hamada, T. (2014). Effect of shading on anthocyanin and non-flavonoid polyphenol biosynthesis of *Gynura bicolor* leaves in midsummer. *HortScience*, *49*(9), 1148-1153.

Hansen, J., & Moller, I. B. (1975). Analysis of starch and soluble sugars with Anthrone reagent. *Analytical Biochemistry*, *68*, 87–94.

Ibrahim, M. H., Ismail, A., Omar, H., Mohd Nadzir, M. N. H., & Mohd Zain, N. A. (2017). Primary, secondary metabolites, biochemical and antioxidant activity of *Orthosiphon staminues* Benth. (Misai Kucing) under cadmium exposure. *Annual Research and Review in Biology*, *19*(1), 1–14.

Ibrahim, M. H., Jaafar, H. Z. E., Karimi, E., & Ghasemzadeh, A. (2014). *Allocation of secondary metabolites, photosynthetic capacity, and antioxidant activity of kacip fatimah (*Labisia pumila *Benth.) in response to CO_2 and light intensity*. Retrieved October 23, 2019, from https://www.hindawi.com/journals/tswj/2014/360290/

Ismail, N. Z., Arsad, H., Samian, M. R., & Hamdan, M. R. (2017). Determination of phenolic and flavonoid contents, antioxidant activities and GC-MS analysis of *Clinacanthus nutans* (Acanthaceae) in different locations. *AGRIVITA Journal of Agricultural Science*, *39*(3), 335–344.

Jaafar, H. Z. E., Ibrahim, M. H., & Fakri, N. F. M. (2012). Impact of soil field water capacity on secondary metabolites, phenylalanine ammonia-lyase (PAL), maliondialdehyde (MDA) and photosynthetic responses of Malaysian kacip fatimah (*Labisia pumila* Benth.). *Molecules*, *17*(6), 7305–7322.

Jamaludin, D., Abd Aziz, S., Ahmad, D., & Jaafar, H. Z. E. (2015). Impedance analysis of *Labisia pumila* plant water status. *Information Processing in Agriculture*, *2*(3-4), 161–168.

John, E. C. (1995). *Current protocols in protein science*. London, United Kingdom: John Wiley and Sons.

Kleczewski, N. M., Herms, D. A., & Bonello, P. (2010). Effects of soil type, fertilization and drought on carbon allocation to root growth and partitioning between secondary metabolism and ectomycorrhizae of *Betula papyrifera*. *Tree Physiology*, *30*(7), 807–817.

Kleinwächter, M., & Selmar, D. (2014). New insights explain that drought stress enhances the quality of spice and medicinal plants: Potential applications. *Agronomy for Sustainable Development*, *35*(1), 121–131.

Koyama, K., & Goto-Yamamoto, N. (2008). Bunch shading during different developmental stages affects the phenolic biosynthesis in berry skins of "Cabernet Sauvignon" grapes. *Journal of the American Society for Horticultural Science*, *133*(6), 743–753.

Le Bot, J., Bénard, C., Robin, C., Bourgaud, F., & Adamowicz, S. (2009). The "trade-off" between synthesis of primary and secondary compounds in young tomato leaves is altered by nitrate nutrition: Experimental evidence and model consistency. *Journal of Experimental Botany*, *60*(15), 4301–4314.

Li, F., Gao, J., Xue, F., Yu, X., & Shao, T. (2016). Extraction optimization, purification and physicochemical properties of polysaccharides from *Gynura medica*. *Molecules*, *21*(4), 1–13.

Loh, F. C. W., Grabosky, J. C., & Bassuk, N. L. (2002). Using the SPAD 502 meter to assess chlorophyll and nitrogen content of benjamin

fig and cottonwood leaves. *HortTechnology*, *12*(4), 682-686.

Mohd Zain, N. A., & Ismail, M. R. (2016). Effects of potassium rates and types on growth, leaf gas exchange and biochemical changes in rice (*Oryza sativa*) planted under cyclic water stress. *Agricultural Water Management*, *164*, 83–90.

Mongkhonsin, B., Nakbanpote, W., Hokura, A., Nuengchamnong, N., & Maneechai, S. (2016). Phenolic compounds responding to zinc and/or cadmium treatments in *Gynura pseudochina* (L.) DC. extracts and biomass. *Plant Physiology and Biochemistry*, *109*, 549–560.

Oyedeji, S., Animasaun, D. A., Bello, A. A., & Agboola, O. O. (2014). *Effect of NPK and poultry manure on growth, yield, and proximate composition of three Amaranths*. Retrieved October 23, 2019, from https://www.hindawi.com/journals/jb/2014/828750/

Pant, B. D., Pant, P., Erban, A., Huhman, D., Kopka, J., & Scheible, W. R. (2015). Identification of primary and secondary metabolites with phosphorus status-dependent abundance in *Arabidopsis*, and of the transcription factor PHR1 as a major regulator of metabolic changes during phosphorus limitation. *Plant, Cell and Environment*, *38*(1), 172–187.

Ren, J., Guo, S., Xu, C., Yang, C., Ai, W., Tang, Y., & Qin, L. (2014). Effects of different carbon dioxide and LED lighting levels on the anti-oxidative capabilities of *Gynura bicolor* DC. *Advances in Space Research*, *53*(2), 353–361.

Romagni, J. G. (2009). Biosynthesis of chemical signals - *De novo* synthesis and secondary metabolites. In J. D. Hardege (Ed.), *Chemical ecology* (pp. 393 - 415). Oxford, United Kingdom: Encyclopedia of Life Support Systems (EOLSS) Publishers Co. Ltd.

Sasidharan, S., Chen, Y., Saravanan, D., Sundram, K. M., & Latha, L. Y. (2011). Extraction, isolation and characterization of bioactive compounds from plants extracts. *African Journal of Traditional Complementary and Alternative Medicine*, *8*(1), 1–10.

Selmar, D., & Kleinwächter, M. (2013). Stress enhances the synthesis of secondary plant products: The impact of stress-related over-reduction on the accumulation of natural products. *Plant and Cell Physiology*, *54*(6), 817–826.

Shams, K. A., Abdel-Azim, N. S., Tawfik, W. A., Hassanein, H. D., Saleh, M. A., & Hammouda, F. M. (2015). Green extraction techniques: Effect of extraction method on lipid contents of three medicinal plants of Apiaceae. *Journal of Chemical and Pharmaceutical Research*, *7*(4), 1080–1088.

Shitan, N. (2016). Secondary metabolites in plants: Transport and self-tolerance mechanisms. *Bioscience, Biotechnology and Biochemistry*, *80*(7), 1283–1293.

Soubeyrand, E., Basteau, C., Hilbert, G., van Leeuwen, C., Delrot, S., & Gomès, E. (2014). Nitrogen supply affects anthocyanin biosynthetic and regulatory genes in grapevine cv. Cabernet-Sauvignon berries. *Phytochemistry*, *103*, 38–49.

Teoh, W. Y., Wahab, N. A., Richardson, J. S., & Sim, K. S. (2016). Evaluation of antioxidant properties, cytotoxicity and acute oral toxicity of *Gynura procumbens* (Compositae). *Sains Malaysiana*, *45*(2), 229–235.

Turnbull, J. M., Yang, L., Wen, K. S., Ruan, X., Zhao, Y. X., Wei, F., & Wang, Q. (2018). Response of plant secondary metabolites to environmental factors. *Molecules*, *23*(4), 1–26.

Wang, M., Fu, Y., & Liu, H. (2015). Nutritional status and ion uptake response of *Gynura bicolor* DC. between Porous-tube and traditional hydroponic growth systems. *Acta Astronautica*, *113*, 13–21.