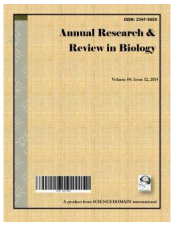

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Effect of Nitrogen Rates on Growth and Quality of Water Spinach (*Ipomea aquatica)*

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Authors' contributions

This work was carried out in collaboration between all authors. Authors MHI and NYAR designed the study, performed the statistical analysis, wrote the protocol and first draft of the manuscript. Author NAMZ managed the analyses of the study. Author MHI managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The study was conducted to investigate the impact of nitrogen fertilization on growth, leaf gas exchange and bio-metabolite accumulation in *Ipomea aquatica*.
Treatment and Experimental Design: *Ipomea aquatica* plants were exposed to four different rates of nitrogen (0, 30, 60 and 90 N kg/ha) using Urea (46% N) as a nitrogen source. The experiment was laid out in Complete Randomize Design (CRD).
Place and Duration of Study: Department of Biology, Faculty of Science, Universiti Putra Malaysia between September to November 2016.
Methodology: Four nitrogen rates were applied (0, 30, 60 and 90 N Kg/ha) using Urea as a nitrogen source. The growth data collections were conducted once a week after the application of

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the treatments for the plant growth parameters. The total chlorophyll content in the leaves was measured using a Soil Plant Analytical Device (SPAD-502) chlorophyll meter. The leaf gas exchange was determined using a LI-6400XT portable photosynthesis system. Total phenolics and flavonoid were determined using Folin-Ciocalteu reagent.

Results: It was found that the highest measurements of growth parameters namely plant height, leaf numbers, branches numbers, total biomass and chlorophyll content were observed at 90 kg N/ha and the lowest at 0 kg N/ha. As for the leaf gas exchange, the positive effect of nitrogen fertilization on kangkung was shown by the increased in photosynthesis rate (A) and stomatal conductance (gs) where the highest measurement recorded at 90 kg N/ha, and the lowest at 0 kg N/ha. However, the water use efficiency (WUE) decreased as the nitrogen rates increased. At lower rates of nitrogen fertilization (30 kg N/ha) produced the highest production of secondary metabolites, where the total phenolics and flavonoids production were enhanced compared to other nitrogen treatments.

Conclusion: In conclusion, as the nitrogen rates increased, the growth and leaf gas exchange properties was enhanced however the production of secondary metabolites was decreased *in I. aquatica*.

Keywords: Nitrogen; Ipomea aquatic; growth; leaf gas exchange; biometabolites production.

1. INTRODUCTION

In Malaysia, agricultural sector contributed about 8.5% to Gross Domestic Products (GDP). About 39% of the contributions originated from the production of food crops, fruits, and vegetables. It is estimated that about 44, 000 hectares of the total area in Malaysia were used for vegetable cultivation [1]. According to Department of Agriculture Malaysia in 2011, *Ipomea Aquatica* is one out of ten types of vegetables that occupied the largest area for vegetable production. This plant is among the most consumed vegetable in Asia. This is because of its low price compared to other types of vegetable. Kangkung air or it's scientific name, *Ipomea aquatica*, is a widely known leafy vegetable, especially in the Asian country. The plant is also commonly known by different local names, such as water spinach, swamp cabbage, or water convolvulus. From its scientific classification, kangkung has been classified under the family Convolvulaceae [2]. According to [3], Convolvulacea family consists of primarily 1650 of tropical species. Moreover, the genus of kangkung which is *Ipomea* has about 500 to 600 different species and it has been the most number of containing species in Convolvulaceae family [4]. This species of the family can nicely be grown at almost anywhere at the higher or lower altitudes. *Ipomea aquatica* is one of the species that is cultivated on the higher land. Besides easy to be grown, Kangkung cultivation is favored due to its quick maturity period and it does not take long time to mature and harvest. It can easily adapt towards it's growing environment and usually unsusceptible to disease. Almost all parts of kangkung plant are edible [3].

According to Susila et al. [5], nitrogen is the primary nutrient that involved in producing a high yield of vegetables. Nitrogen is one of the macro-nutrients that is very crucial especially for a plant to have a proper growth and development [6] such as that required in constructing the matter of the plant cell and tissue [7]. The amount of nitrogen in the soil could be insufficient for the plant to grow. Therefore, the source of nitrogen for plant especially in agriculture field is often found in the form of a fertilizer. Both organic and inorganic nitrogen fertilizer is widely used in agriculture especially in cultivating green crops to keep the source of nutrients for the plant being for supplied [6]. Practically, an appropriate and suitable amount of nitrogen to be given to plant will affect its crop yield. Nitrogen is also very important especially to promote the growth of the plant leaf [8]. Nitrogen is a crucial element not only to promote the growth and plant development, also increase yield and quality in vegetable crops. Increasing level of nitrogen resulting in a number of leaves, leaf length and plant body [8]. Nitrogen also enhancing the size of fruits and vegetables where at an optimum application of N will result in a better size. The metabolic process which stimulated by N by enhance the vegetative and also the reproductive growth in the plant. Besides, high plant biomass can be obtained when there is high N accumulated in a shoot, along with the increasing of root growth in a plant if there is sufficient amount of N supply [9]. However, the lack of N in a plant would cause the reduced in plant development and eventually will lower the crop yield. Plants can take up nitrogen (N) either as inorganic ions (NH^{4+} or NO^{3-}), or as organic N. In leafy vegetables, high uptake of NO3- can cause serious health problems to the consumers [7]. Nitrate has been attributed to have negative effects to human health. Toxicity of nitrate to human can be manifested by headaches, syncope, vertigo and discoloration that manifest in fingers or lips [6].

Nitrogen has been proven to have a strong relationship with photosynthesis process in the plant. Increasing N level leads to higher N content in leaves. N also enhances the leaf chlorophyll and CO2 assimilation which increase in the Rubisco activity [10]. Therefore, increase in the rate of photosynthesis is the most vital biochemical process in plants [11]. According to [12,13], rate of photosynthesis (A) depends on the growth and development of the plant's leaf. The leaf development includes the increase in leaf area, leaf thickness, the surface volume of mesophyll cells, and leaf chloroplast. The photosynthesis rate will be increased as the leaf development also increased [14]. Nitrogen is an element that has a significance role in photosynthesis which involves in the opening of the stomata. The stomatal vent will decrease following the nitrogen deficit which then will decrease the transpiration rate [15].

Secondary metabolites such as phenolic compounds in plants are usually associated with survival of the plant and health benefits for those who consume the plant. Low nitrogen level in the plant has been reported to have more secondary metabolites compare to plant that has high N level [16]. Application of more N level resulting in a decrease of phenolic concentrations based on carbon/nutrients balance (CNB) hypothesis [17]. Flavanoids, also a secondary metabolite, are widely distributed with different functions in plants. The biological functions of flavonoids include defense against UV-B radiation, pathogen infection, nodulation and pollen fertility [18]. A study was done by [8] on leaf mustard where the total phenolics concentration was observed to be decreased as the level of N increased. It is well known that nitrogen application can directly affect the morphological growth and yield of this plant, however, little work has been carried out to look on the impact of nitrogen of the leaf gas exchange properties and previous work have not comprehensively considered the production of secondary metabolites of *I. aquatica* under nitrogen fertilization. The main aim of the research was to investigate the effect of nitrogen fertilization on the growth, leaf gas exchange and production of secondary metabolites of *I. aquatica* and to determine the best nitrogen rates for growth and development of *I. aquatica*. This research will provide the important information for vegetable growers that involved in the cultivation of vegetables in Malaysia.

2. MATERIALS AND METHODS

2.1 Plant Material and Maintenance

The experiment was conducted at the Department of Biology, Universiti Putra Malaysia, Serdang (UPM), Selangor The seeds were pre-germinated in the nursery for two weeks after which they were transplanted into the polybags filled with a mixture of topsoil, organic matter and sand in the ratio of 3:2:1 respectively. All the plants were irrigated using overhead mist irrigation given four times a day or when necessary. Each irrigation session lasted for 7 min. The nitrogen sources used was single fertilizer Urea (46% N). The polybags were arranged in Completely Randomized Design (CRD) with five replications. There were four nitrogen rates were applied (0, 30, 60 and 90 Kg N/ha). The fertilization with nitrogen levels were split into three applications, given at 5, 15 and 25 days after treatments and each phase was about 33.3% of total nitrogen fertilizer. The growth data collections were conducted once a week for four weeks after the application of the treatments. While the destructive analysis and leaf gas exchange of the experiment was conducted at the end of the experiment.

2.2 Plant Height, Number of Leaves and Branches

Plant height, was measured from the ground level to the tip of the highest growing point using measuring tape. The leaf and branches number were counted manually per plant and the mean recorded.

2.3 Plant Total Dry Weight Measurement

The plants were first uprooted carefully and were washed with tap water. After that, the shoot and the root parts were separated. All the plants were dried in an oven for 48 hours at temperature of 60°C until constant weight reached. Then, the plant total dry weight was measured by using electronic digital scale.

2.4 Total Chlorophyll Content

SPAD-502 chlorophyll meter was used to measure the total chlorophyll content of the leaves. Three readings were taken at three spot on a leaf of each plant and the average readings were recorded. Time interval between 9.00 a.m and 12.00 p.m was used to measure the chlorophyll content.

2.5 Leaf Gas Exchange Measurement

The leaf gas exchange measurement was obtained after week 4 the treatment was given. The result then was obtained by using the Portable Photosynthesis System machine (LICOR 6400 XT). The IRGA was firstly warm up for at least 30 minutes before the leaf gas exchange was collected with Zero IRGA mode. The optimal condition was set to 400 µmol mol^{-1} carbon dioxide (CO_2), 30°C cuvette temperature, 60% relative humidity with air flow rate set at 500 cm^3 min^{-1}, and 800 $\mu molm^{-2}s^{-1}$ of cuvette condition of photosynthetic photon flux density (PPFD). The time for the measurement were done at the morning of a day. The measurement of photosynthesis rate was taken from the first kangkung leaves starting from the plant apex. The data then were recorded and stored in a console of the system and analyse with Photosyn Assistant Software. The photosynthesis (A), transpiration rate (E), stomata conductance (gs) and water use efficiency (WUE) data was recorded during the measurement.

2.6 Total Phenolics and Flavonoids Quantification

The methods used for extraction and quantification of total phenolics and flavonoids contents followed that described in Ibrahim et al. [19]. A fixed amount of ground tissue samples (0.1 g) was extracted with 80% ethanol (10 mL) on an orbital shaker for 120 min at 50°C. The mixture was subsequently filtered (Whatman™ No.1), and the filtrate was used for the quantification of total phenolics and total flavonoids. Folin–Ciocalteu reagent (diluted 10-fold) was used to determine total phenolics content of the leaf samples. The sample extract at 200 µL was mixed with Folin–Ciocalteau reagent (1.5 mL) and allowed to stand at 22°C for 5 min before adding $NaNO_3$ solution (1.5 mL, 60 g L^{-1}). After two hours at 22°C, absorbance was measured at 725 nm. The results were expressed as mg g^{-1} gallic acid equivalent (mg GAE g^{-1} dry sample). For total flavonoids determination, samples (1 mL) were mixed with $NaNO_3$ (0.3 mL) in a test tube covered with aluminium foil, and left for 5 min. Then 10% $AlCl_3$ (0.3 mL) was added followed by addition of 1 M NaOH (2 mL). The absorbance was measured at 510 nm using a spectrophotometer with rutin as a standard (results expressed as mg/g rutin dry sample).

2.7 Statistical Analysis

Data were analysed using the analysis of variance procedure in SAS version 17. Means separation between treatments was performed using Duncan multiple range test and the standard error of differences between means was calculated with the assumption that data were normally distributed and equally replicated.

3. RESULTS AND DISCUSSION

3.1 Plant Height

Fig. 1 shows the plant height of *I. aquatica* as influenced by differing nitrogen treatments. The plant height of kangkung was mostly affected by different rates of nitrogen treatment in all weeks of measurement ($P \leq 0.05$). In view of the result obtained, as nitrogen levels increased from 0 to 90 kg N/ha the plant height was enhanced in all weeks of measurement. At four weeks after treatment (4 WAT), plant at 0 kg/ha have the average height of 31.02 cm compared to 32.17 cm by 30 kg/ha, 35.61 cm by 60 kg/ha and 37.24 cm in 90 kg/ha. Clearly, as expected, applying higher rates of nitrogen levels would enhance the plant height of *I. aquatica*. The positive effects on plant height caused by the increase of nitrogen rates application may be due to the natural role of nitrogen on vegetative growth performance of plants [6]. The increase in plant height under nitrogen fertilization might be due to well-developed primary growth under high nitrogen fertilization that resulted in taller plant [20]. Besides that, increase in plant height might be associated with the increased of number and length of the internodes by nitrogen [21]. The result obtained agreed with the previous work carried out by [4] and [6] where the increment of nitrogen fertilization rates applied to *I. aquatica* significantly increased the plant height at end of the harvesting period.

3.2 Leaves Numbers

The variation of leaf numbers with different nitrogen fertilization is in *I. aquatica* is depicted in Fig. 2. Generally, leaf number of *I. aquatica* was found to be influenced by the different rates of nitrogen treatments (0, 30, 60 and 90 kg/ha; P≤0.05). Based on Fig. 2, it shows that there were significant effects of nitrogen fertilization rates on the number of leaves in every week of measurements. Overall at 90 N kg/ha as the highest treatments of nitrogen applied, lead to the drastic production in the number of leaves from 1 to 4 WAT. An increase in number of leaf age in plants indicates better plant growth and development. Eventually, the plant production also will increase. Similar trends were observed in [6] and [20] where they found that as the rate of nitrogen increases the *I. aquatica* leaf numbers were also enhances. The increase in leaf number in *I. aquatica* might be due to increase in internodes number with the high application of nitrogen [21]. The high application of nitrogen usually would reduce the apical dominance and stimulated the development of lateral buds that eventually increase the production of plant leaf and simultaneously enhanced the leaf numbers [22].

3.3 Plant Total Dry Weight

Nitrogen application significantly influenced on the total plant dry weight of *I. aquatica* plant as shown in Fig. 3. The graph pattern shows increased in production in total biomass with the higher application of nitrogen fertilization rates.

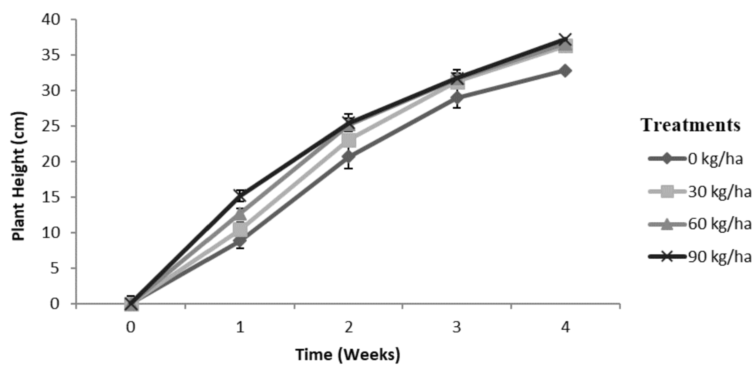

Fig. 1. The impact of different nitrogen rates on the height of *Ipomea aquatica.*
N =10. Bars represent standard error of differences between means (SEM)

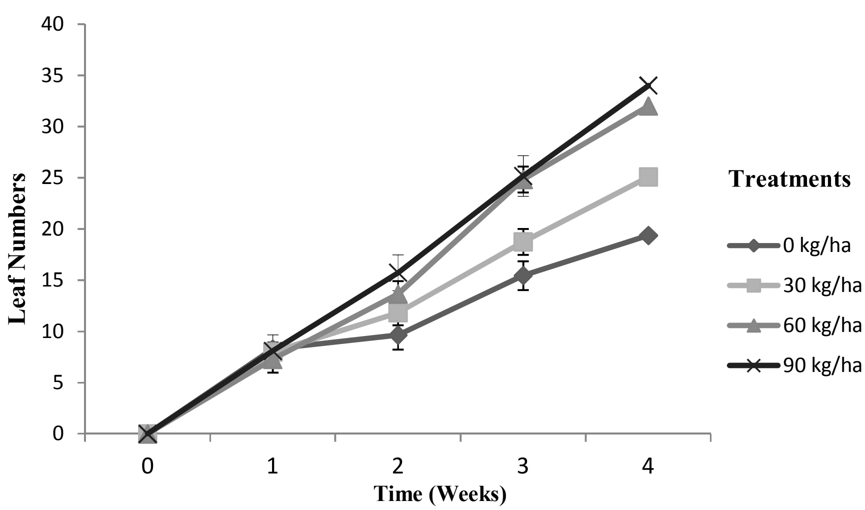

Fig. 2. The impact of different nitrogen rates on the leaves numbers of *Ipomea aquatica*
N =10. Bars represent standard error of differences between means (SEM)

At end of the treatments, It was observed that the highest total biomass of kangkung was obtained in 90 kg N/ha, followed by 60 kg N/ha and 30 N kg N/ha that recorded at 3.7 g and 3.26 g respectively. The lowest total biomass was recorded in control treatment 0 kg N/ha that just recorded 3.13 g. The increase of total plant biomass with increasing nitrogen levels can be explained by the increase in plant sink strength with increasing nitrogen levels. As nitrogen uptake increased, more of accumulation of dry biomass will be expected due to increase in plant sink strength that can accommodate initiation of new plant sink There were no significant different occurred in between 0 and 30 N kg/ha treatment ($p \geq 0.05$). The result of the present study was in agreement with the research conducted by [23] where, they found that the dry weight of shoot increased with the increase of nitrogen supplied in *I. aquatica*. This justifies that high availability of nitrogen was important in increasing the dry biomass of *I. aquatica* that was observed in the present study [24,25].

3.4 Number of Branches

Fig. 4 shows the branches number of kangkung plant as affected by nitrogen treatments in all four weeks of treatment. As the higher rate of nitrogen treatments, the branching of plants was enhanced. At the first 2 weeks after the treatments were applied, the number of branches at 60 N kg/ha was higher than plants that were applied with 90 N kg/ha. But then, at week 3 and 4, the opposite results were obtained where the highest number of branches occurred at 90 N kg/ha. The study was in agreement with findings by Nashrin et al. [6] on *I. aquatica*, where the highest branching was obtained under highest nitrogen fertilization. Also, Osman and Abo Hassan [26], observed increased branching of Mangrove as nitrogen rate was increased to 100 kg N/ha. The increased in branching of the plant under high nitrogen fertilization might be due to increase in apical branches with higher nitrogen fertilization. This was due to enhanced vegetative growth under high nitrogen fertilization that enhanced the branching abilities of the plant [27].

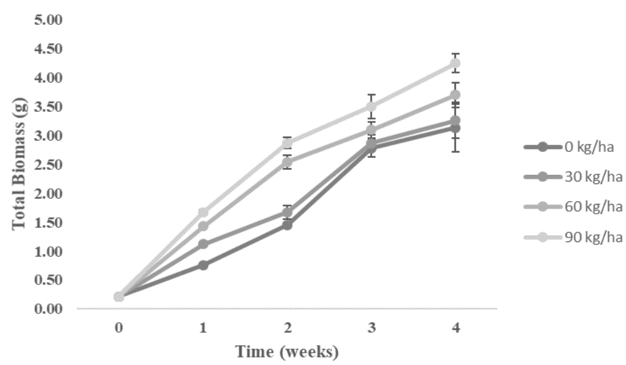

Fig. 3. The impact of different nitrogen rates on total biomass of *Ipomea aquatica*.
N =10. Bars represent standard error of differences between means (SEM)

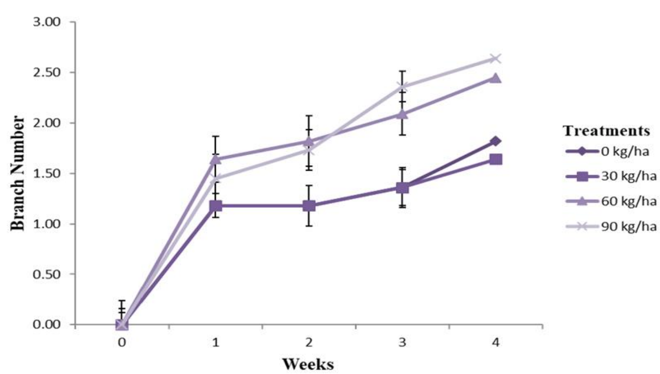

Fig. 4. The impact of different nitrogen rates on the branch number of *Ipomea aquatica*.
N =10. Bars represent standard error of differences between means (SEM)

3.5 Total Chlorophyll Content

Fig. 5 showed the impact of nitrogen fertilization on total chlorophyll content (TCC) of *I. aquatica* in 4 weeks of treatments. There were significant differences were observed for TCC in every week of measurement ($P \leq 0.05$). The chlorophyll content increased after week 1 and reached it's maximum WAT content at week 3 as shown in Fig. 5. In 1 WAT to 4 WAT, As the rate increased from 0 to 90 kg/ha, The TCC was steadily enhanced with the increasing nitrogen rates. In 2 -4 WAT there was no significant difference observed between 60 and 90 kg/ha in TCC. The study was in agreement with findings of According to Bojović and Marković [28] where the higher application of nitrogen increased the TCC in wheat, where establishes a linear relationship between the rates of nitrogen and the chlorophyll content in plants. The plant that has been treated with high N level will result in higher chlorophyll content where this might be due to the immediate absorbance of nitrogen in plant [29]. Since N is important for the structural element of chlorophyll and protein molecules, low N level will affect the formation of chloroplasts and the accumulation of chlorophyll in the plant [22]. Furthermore, as the plant age increased or getting mature, the N level tend to decrease and get mobilized to another part of the plant [29]. It can be concluded that in the present study, the higher rates of nitrogen application have increases the TCC in *I. aquatica.*

3.6 Photosynthesis Rate (A)

The photosynthetic rate of *I. aquatic*a was affected by four different nitrogen treatments. It was clearly observed that from the graph pattern, as the nitrogen rate become higher (0>90 kg/ha), the rate of photosynthesis also enhances (Fig. 6). The highest A was observed in 90 kg/ha nitrogen, followed by 60 and 30 kg/ha, with the means of 3.91, 3.42, and 2.69 umol/m^2/s respectively. The lowest A was observed in 0 kg/ha where it just recorded 2.31 umol/m^2/s. The increase in A under high nitrogen level might be due to increases in leaf area that correspondingly enhanced photosynthetic activity per plant [30]. The result was also in agreement with Boussadia et al. [31] where higher nitrogen content has shown to enhance the photosynthesis rate in olive plants. The nitrogen and photosynthesis activity is linked together because of the Calvin Cycle protein which represents the nitrogen in leaf [32]. At lower N level, the rate of photosynthesis was low. This might be due to the greater resistance and low biochemical of chloroplast [33]. According to Makino et al. [34], the increase in the rate of nitrogen leads to a greater N allocation to Rubisco. Rubisco is the primary CO_2 for enzyme fixation where the amount of this enzyme can drastically affect the photosynthesis rate. Besides, high N is needed in Rubisco protein due to the low rate of catalysis in Rubisco. It can be concluded that, enhanced application of nitrogen would enhance rubisco production that enhanced the net photosynthesis of *Ipomea aquatica* that was observed in the present study.

3.7 Stomatal Conductance (gs)

Based on Fig. 7, it is distinctly observed that different rates of nitrogen had greatly affected the measurement of stomatal conductance. The higher the treatment concentrations (0,30,60,90 kg/ha), the rate of stomatal conductance have shown to increase. The stomatal conductance measurement was 70%, 167% and 260% respectively higher in 30, 60 and 90 kg/ha Nitrogen compared to the control that recorded only 0.33 mmol m^{-2} s^{-1}. The present result was in agreement with the findings of [35,36], where they found that the increase in photosynthesis rate and stomatal conductance are correlated to increase in nitrogen application to the plants. Despite nitrogen, the size of the leaf can be important for certain plant species as it helps for greater conductance through the high number opening of the stomata [37]. This indicates that stomata conductance was enhanced with high levels of nitrogen applied to *I. aquatica.*

3.8 Water Use Efficiency (WUE)

Water use efficiency (WUE) was illustrated in Fig. 8. as it was influenced by the nitrogen treatments ($P \leq 0.05$). Plant with the highest concentration of nitrogen (90 kg/ha) has the lowest measurement recorded in water use efficiency with the mean of 1.46 µmol CO_2/H_2O transpired. While the highest measurement in water use efficiency was recorded in the plant that was applied with 0 Kg/Ha nitrogen with a mean of 1.97 µmol CO_2/H_2O transpired. The current result was contradicting with the findings of stewart [38] in cotton where the highest nitrogen application has shown to enhance the WUE in the plant. The increased of WUE is usually, attributed to the increase of the transpiration rate and showed plant under water stress condition. The current result showed that higher application of nitrogen rates in *I. aquatica* can reduce the plant stress by having lower WUE. [22]. A similar result was obtained by Artur et al. [39] where the increase of N has reduced the WUE in Marandu grass that showed a high application of nitrogen can reduce stress in *I. aquatica*.

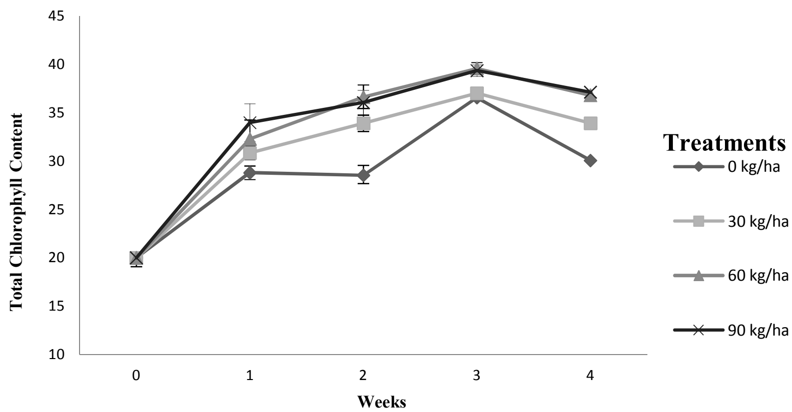

Fig. 5. The impact of different nitrogen rates on the total chlorophyll content of *Ipomea aquatica*

N =10. Bars represent standard error of differences between means (SEM)

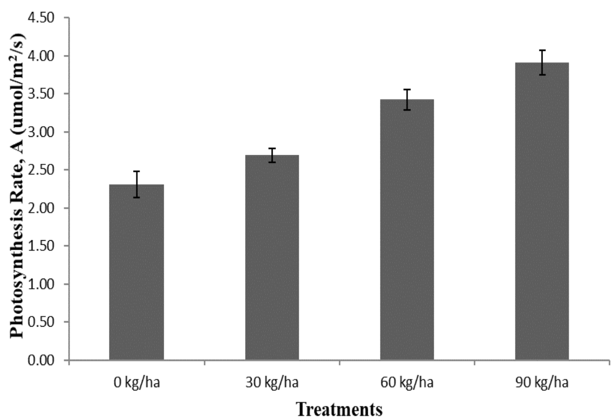

Fig. 6. The impact of different nitrogen rates on the photosynthesis rate of *Ipomea aquatica*

N =10. Bars represent standard error of differences between means (SEM)

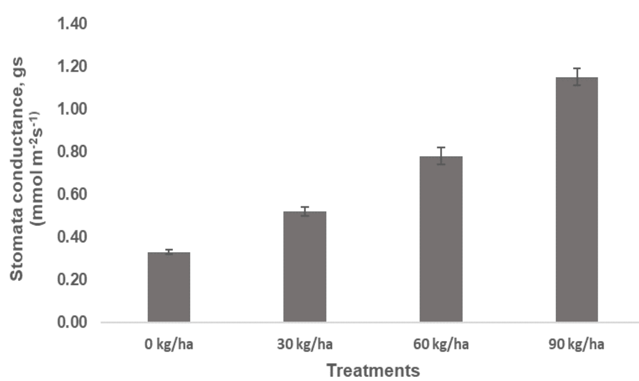

Fig. 7. The impact of different nitrogen rates on the stomatal conductance of *Ipomea aquatica*

N =10. Bars represent standard error of differences between means (SEM)

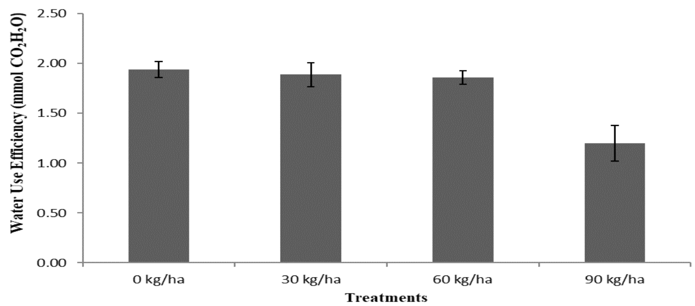

Fig. 8. The impact of different nitrogen rates on the water use efficiency of *Ipomea aquatica*
N =10. Bars represent standard error of differences between means (SEM)

3.9 Total Phenolics

Total plant phenolics contents were influenced by nitrogen fertilization ($P \leq 0,05$; Fig. 9). As levels of nitrogen enhanced, the total phenolics content was seemed to be reduced. Total phenolics was 203%, 41% and 13% higher in 30 kg/ha, 60 kg/ha and 0 kg/ha respectively compared to 90 kg/ha treatments. The previous study had shown that when the level of nitrogen decreased, the phenolic compound increased in Brocolli [40]. Another result obtained by Stewart et al. [41], also prove that the phenolic content increased as the plant faced deficiency in nitrogen level. The result obtained in this study suggested that at lower nitrogen fertilization i.e. 30 kg N/ha the production of total phenolics in Ipomea aquatica was enhanced. According [42], when a plant undergoes N deficiency, the process of distributing carbon-based secondary compounds will increase, thus, decreasing the synthesis of nitrogen-based secondary compounds. Besides, Ibrahim et al. [19] stated that the increase in total phenolics production under low N level also might be due to the increase of total carbohydrate structural production that enhanced the production of carbon- based secondary metabolites.

3.10 Total Flavanoids

The total flavonoids of *Ipomea aquatica* were observed to be affected by the different rates of nitrogen treatments (Fig. 10; $P \leq 0.05$). The production of total flavonoids has the same trends with total phenolics production content where plants which applied with 30 N kg/ha treatments has the highest total flavonoids content (1.05 mg Rutin/g dry weight) compared to 90 kg/ha that only recorded 0.27 mg rutin/ g dry weight. The same observation was obtained by [43] (2012) in Yaupon where the flavonoid content reduces when applied with high N rate. According to [44] the flavonoids content in plant tissues can be increased when having lower nitrogen content in the plant tissues. The increases in synthesis of flavonoid at lower nitrogen level might be due to increases in phenylalanine availability that enhances the phenylalanine lyase (PAL) activity that simultaneously enhanced the production of secondary metabolites [45]. It can be concluded in the present study, that under high nitrogen level the production of total phenolics and flavonoids was reduced in *I. aquatica.*

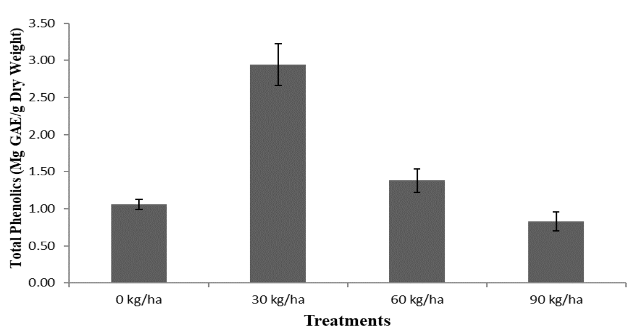

Fig. 9. The impact of different nitrogen rates on total phenolics of *Ipomea aquatica*
N =10. Bars represent standard error of differences between means (SEM)

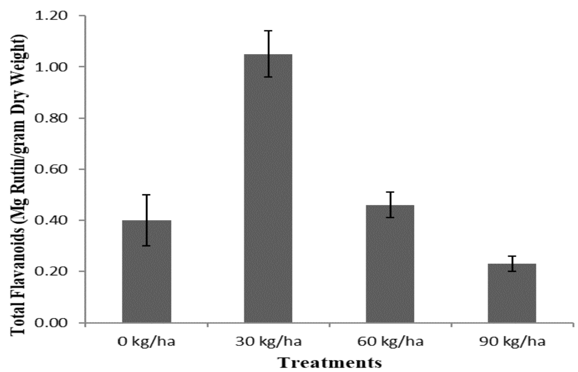

Fig. 10.The impact of different nitrogen rates on total flavonoids of *Ipomea aquatica*

N =10. Bars represent standard error of differences between means (SEM)

4. CONCLUSION

In the present work It was found that as the nitrogen rates increased, the growth and leaf gas exchange properties of *I. aquatica* was enhanced. However, the production of phenolics and flavonoids of kangkung was reduced with high levels of nitrogen application as both total phenolics and flavonoid reached the highest content at 30 kg N /ha. This work gives support that high nitrogen fertilization to *I. aquatica* can reduce the production of secondary metabolites although the growth parameters were enhanced with high nitrogen fertilization.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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