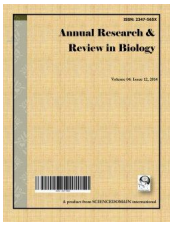

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Impact of Carbon Dioxide and Increasing Nitrogen Levels on Growth, Photosynthetic Capacity, Carbohydrates and Secondary Metabolites in Kacip Fatimah (*Labisia pumila*)

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Authors' contributions

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

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ABSTRACT

Aims: This study was designed to investigate how CO_2 and nitrogen availability alters growth, leaf gas exchange, primary and secondary metabolites production (total phenolics and flavonoid) in three varieties of L. *pumila*. namely var *alata*, var *pumila* and var *lanceolata*. under CO_2 enrichment (1200 µmol mol^{-1}) combined with four levels of nitrogen fertilization (0, 90, 180 and 270 kg N ha^{-1}) for 15 weeks.

Study Design: Three-month old *L. pumila* seedlings of var. *alata*, var. *pumila* and var. *lanceolata* were left for a month to acclimatize in a nursery until ready for the treatments. Carbon dioxide enrichment treatment started when the seedlings reached 4 months old where plants were exposed to 1200 μmol^{-1} mol^{-1} CO_2 and fertilized with four levels of nitrogen concentrations viz. 0, 90, 180 and 270 kg N Ha^{-1}. The fertilization with nitrogen levels were split into three applications. A control at ambient CO_2 (400 μmol^{-1} mol^{-1}) with standard N fertilization (180 kg N ha^{-1}) was included to compare plant responses to high CO_2 combined with different levels of N.

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Place and Duration of Study: Faculty of Agriculture Glasshouse Complex, Universiti Putra Malaysia from February to May 2011.
Methodology: The seedlings were raised in specially constructed growth houses receiving 12-h photoperiod and average photosynthetic photon flux density of 300 µmol m^{-2} s^{-1}. Carbon dioxide at 99.8% purity was supplied from a high–pressure CO_2 cylinder and injected through a pressure regulator into fully sealed 2 m x 3 m growth houses at 2-h daily and applied continuous from 800 to 1000. Total plant biomass was taken by calculating the dry weight of root, stem and leaf per seedling. Total leaf area per plant was measured using a leaf area meter (LI-3100, Lincoln Inc, USA). The measurement of photosynthesis was obtained from a closed system of infra-red gas analyzer LICOR 6400 Portable Photosynthesis System. The response of net CO_2 exchange (A) to changing intercellular CO_2 concentration (C_i) was conducted at 27°C, 50% relative humidity and at light saturating conditions of 800 µmol $m^{-2}s^{-1}$. Anthrone method was used to determine the content of total available carbohydrate. Follin–Ciocalteau reagent was used to determine total phenolics and flavonoid using spectrophotometer.
Results: In all parameters measured there were no significant was observed in the three varieties. As the levels of nitrogen increased from 0 to 270 kg N ha^{-1}, plant growth, photosynthesis and total chlorophyll content was enhanced. However, carbohydrate, total phenolics and flavonoid was reduced with the increase in nitrogen fertilization. It was found that the high application of nitrogen can increased the photosynthetic capacity of *L. pumila* by having high V_{cmax} (RuBisCO CO_2 fixation capacity), A sat (CO_2 saturation point) and low dark respiration rate in the analysis of A/Ci curves. The production of secondary metabolites was related to increased total non-structural carbohydrate (TNC) under low nitrogen condition. Photosynthesis, displayed a significant negative relationship with secondary metabolites production. Total chlorophyll content exhibited very significant negative relationships with total soluble sugar, starch and total non-structural carbohydrate.
Conclusion: This study demonstrated that enrichment with high levels of CO_2 is able to enhance the production of plant secondary metabolites. However, increased nitrogen fertilization can reduce the production of these plant secondary metabolites under CO_2 enrichment. When there were accumulation of TNC in plant leaves and reduction in photosynthesis, the production of plant secondary metabolites might be up-regulated. The increase in the production of plant secondary metabolites was indicated by reduction in chlorophyll contents.

Keywords: Carbon dioxide enrichment; nitrogen fertilization; medicinal plant; growth; primary and secondary metabolites production.

1. INTRODUCTION

Kacip Fatimah (*Labisia pumila* Benth) is a small woody plant. It is usually found at 80 -100 m above sea level in Malaysia tropical rainforest. The subherbaceous plants belongs to the family Mysinaceae and the plant has been categorized into three varieties based on the characteristics of the petioles [1]. The three varieties are *L. pumila* var *pumila*, *L. pumila* var *alata* and *L. pumila* var *lanceolata*. The plant is well accepted by the local Malay traditional practitioners for hasten delivery, regain strength after childbirth, treatment of gonorrhea, dysentery and excessive gas. This herb is in great demand among more than 30 herbal products in Malaysian herbal market. Due to its large uses in the herbal and commercial products [2], and with recent discovery of estrogenic activity demand for *L. pumila* is expected to soar [3]. However, supply of *L. pumila* could not meet demand for raw material as little has been done to domesticate the plant. This situation has forced the propagators to seek plant materials directly from the rainforest, and consequently, the heavy demand for this herb, might endanger existence of the species.

Growth rate of *L. pumila* in natural habitat is very slow, and the propagation from seeds usually takes about 20 to 24 months before the plants can be harvested [4]. The imposition of CO_2 enrichment onto slow growing seedlings may be able to reduce the nursery time as exhibited by reduced nursery time in palm seedlings exposed to elevated CO_2 by at least 42% [5], and in tropical vegetable crop by 45-60% [3] The CO_2-enriched plant is able to enhance its photosynthetic and water use efficiency, and produce extra assimilates that are partitioned to plant organs for stimulation of growth [6]. At elevated CO_2, the rate of photosynthesis

immediately increases, both because CO_2 is a competitive inhibitor of the oxygenation of RuBisCO and therefore photorespiration is depressed, and because the current CO_2 in adequate to saturate Rubisco. Elevated CO_2 therefore leads to increase in substrate binding and increased velocity of carboxylation [7]. As up to 30% of leaf nitrogen is allocated to rubisco, a significant fraction of leaf nitrogen can therefore be reallocated to other photosynthetic or non-photosynthetic processes at elevated CO_2 [8].

The growth stimulation of the plants under elevated CO_2 could be enhanced more by the application of nitrogen to the seedling under elevated CO_2 enrichment. It is reported that nitrogen availability is a critical factor limiting plant growth and increasing the response to elevated CO_2 conditions. It has been reported that at elevated CO_2 concentration at low N supply could limit photosynthesis subsequently, diminished plant nitrogen availability for long time [9]. A reduction in leaf nitrogen under elevated CO_2 is a result of dilution by carbohydrate accumulation and increased in plants requirement for nitrogen [10]. Moreover, dilution of nitrogen may cause immobilization of carbon in sources tissues, leading to carbohydrate accumulation. This implies a physiological a physiological adjustment conducive with a higher photosynthetic nitrogen use efficiency, i,e an increase in the rate of carbon assimilation per unit nitrogen in the foliage. An accumulation of carbon leading to a decrease in specific leaf area would also lower leaf nitrogen content per unit dry mass [11]. It has been proposed that plant adjust physiologically to low nitrogen by reducing growth rate and accumulating a high concentration of carbon based secondary metabolites due to increase in carbon relative to nitrogen [12]. Previous studies have shown decreases in nitrogen concentration of plant grown under elevated CO_2 at various nitrogen availabilities [13]. The changes in nitrogen concentration and C/N ratio in plant tissues will likely affect plant –herbivore interactions and litter decomposition rates [14].

The up-regulation of growth under elevated CO_2 are dependent on the ability of the plant to develop new sinks or expand existing ones. Thomas and Strain [15] have proposed that when plants exposed to elevated CO_2 have constraints increasing carbon sink strength, plant tend to decrease their photosynthesis to balance carbon source activity and sink capacity. The reduction in photosynthesis rates would not be occur if the plant has the ability to develop new sinks or to expand the storage capacity or growth rate of existing sinks [16]. Ainsworth et al. [17] suggested, that the down-regulation is the consequence of an insufficient sink plant capacity that been caused by limited nitrogen supply to the plants. The increase in plant productivity in response to elevated CO_2 is largely dictated by photosynthesis, respiration, carbohydrate production and their different allocation between plant organs and the subsequent incorporation into biomass. For this reason, many studies have investigated the effects of elevated CO_2 on primary metabolites but relatively few studies have investigated the response of plant secondary metabolites concentrations to increasing CO_2 and its interaction with nitrogen availability It is well established that environmental factors can influence the production of secondary metabolites in plant (Waterman and Mole, 1989). For example, in nutrient deficient conditions levels of non-nitrogenoues metabolites derived from the shikimic acid pathway such as phenolics acids, lignin, hydrolysable tannins and proanthocyanidins usually increases in woody plants [18]. The increase in carbon based secondary metabolites frequently occurs when environmental conditions also promote an accumulation of carbohydrates in plants [19]. Elevated CO_2 often increases carbohydrates concentrations in plants and possibly stimulates secondary metabolism, although experimental results have not always indicated a relationship as predictable as that seen with nutrient deficiency [20].

Bryant et al. [21] suggest that the carbon/nutrient balance of individual plants strongly affects their allocation of resources to primary and secondary metabolites. They predict that fertilization with growth limiting nutrients e.g., nitrogen, will increase the nutrient concentrations in the leaves, thereby stimulating leaf growth more than photosynthesis. Subsequently, concentrations of carbohydrates and carbon based secondary metabolites (total phenolics and flavonoid) in leaves would decline. However, some researchers did not found the relationship might true some species [22]. The question that the research wants to answer is whether growth stimulation with increases nitrogen application under elevated CO_2 will also enhanced the production of plant secondary metabolites. The aims of this study were to investigate how CO_2 and nitrogen availability alters growth, leaf gas exchange, primary metabolites (total available

carbohydrate) and secondary metabolites production (total phenolics and flavonoid) in three varieties of *L. pumila*. namely var *alata*, var *pumila* and var *lanceolata*.

2. MATERIALS AND METHODS

2.1 Experimental Location, Plant Materials and Treatments

The experiment was carried out under growth house at Ladang 2, Faculty of Agriculture Glasshouse Complex, Universiti Putra Malaysia (longitude 101° 44' N and latitude 2° 58'S, 68 m above sea level) with a mean atmospheric pressure of 1.013 kPa. Three-month old *L. pumila* seedlings of var. *alata*, var. *pumila* and var. *lanceolata* were left for a month to acclimatize in a nursery until ready for the treatments. Carbon dioxide enrichment treatment started when the seedlings reached 4 months old where plants were exposed to 1200 μmol^{-1} mol^{-1} CO_2 and fertilized with four levels of nitrogen concentrations viz. 0, 90, 180 and 270 kg N Ha^{-1}. The fertilization with nitrogen levels were split into three applications. A control at ambient CO_2 (400 μmol^{-1} mol^{-1}) with standard N fertilization (180 kg N ha^{-1}) was included to compare plant responses to high CO_2 combined with different levels of N. This factorial experiment was arranged in a split plot using a randomized complete block design with nitrogen levels being the main plot, and varieties as the sub-plot replicated three times. Each treatment consisted of ten seedlings.

2.2 Growth House Microclimate and CO_2 Enrichment Treatment

The seedlings were raised in specially constructed growth houses receiving 12-h photoperiod and average photosynthetic photon flux density of 300 µmol m^{-2} s^{-1}. Day and night temperatures were recorded at 30±1.0°C and 20±1.5°C, respectively, and relative humidity at about 70% to 80%. Vapor pressure deficit ranged from 1.01 to 2.52 kPa. Carbon dioxide at 99.8% purity was supplied from a high–pressure CO_2 cylinder and injected through a pressure regulator into fully sealed 2 m x 3 m growth houses at 2-h daily and applied continuous from 0800 to 1000 a.m. The CO_2 concentration at different treatments was measured using Air Sense ™ CO_2 sensors designated to each chamber during CO_2 exposition period. Plants were watered three to four times a day at 5 min per session to ensure normal growth of plant using drip irrigation with emitter capacity of 2 L hr^{-1}. The experiment lasted for 15 weeks from the onset of treatment.

2.3 Growth Analysis

Total plant biomass was taken by calculating the dry weight of root, stem and leaf per seedling. Destructive plant analysis was carried out every three weeks for 15 weeks. The plant part parts were placed in paper bags and oven dried at 80°C until constant weight was reached using electronic weighing scale (Metler-Toledo Model B303-S, Swizerland). Total leaf area per plant was measured using a leaf area meter (LI-3100, Lincoln Inc, USA). The leaves were arranged within the field of view, and overlapping of adjacent leaves was avoided.

2.4 Chlorophyll Content

Total chlorophyll content was measured by method from [23] using fresh weight basis. Prior to each destructive harvest each seedling was analyzed for the leaf chlorophyll relative reading (SPAD meter 502, Minolta Inc, USA). The leaves of *L. pumila* with different greenness (yellow, light green and dark green) were selected for analysis and total leaf chlorophyll content was analyzed. For each type of leaf greenness, the relative SPAD value was recorded (5 points/leaf) and the same leaves sampled for chlorophyll content determination. Leaf disk 3 mm in diameter was obtained from leaf sample using a hole puncher. For each seedling, the measurement was conducted on the youngest fully expanded leaves on each plant, generally the second or third leaf from the tip of the stem was used. The leaf disks were immediately immersed in 20 ml of acetone in aluminum foil-covered glass bottle for approximately 24 hours at 0°C until all the green colour had bleached out. Finally, 3.5 ml of the solution was transferred to measure at absorbance of 664 and 647 nm using a spectrometer (UV-3101P, Labomed Inc, USA). After that the least squares regression was used to develop predictive relation between SPAD meter readings and pigment concentrations (mg / g fresh weight) obtained from the chlorophyll destructive analysis.

2.5 Leaf Gas Exchange Measurement

The measurement was obtained from a closed system of infra-red gas analyzer LICOR 6400 Portable Photosynthesis System (IRGA: LICOR

Inc. Nebraska, USA). Prior to use, the instrument was warmed for 30 minutes and calibrated with ZERO IRGA mode. Two steps are required in the calibration process: first, the initial zeroing process for the built-in flow meter; and second, zeroing process for the infra-red gas analyzer. The measurements used optimal conditions of 400 µmol/mol CO_2 30°C cuvette temperature, 60% relative humidity with air flow rate set at 500 cm^3 /min, and modified cuvette condition of 800 µmol m^{-2} s^{-1} photosynthetically photon flux density (PPFD). The measurements of gas exchange were carried out between 0900 to 1100 a.m. using fully expanded young leaves numbered three and four from plant apex to record net photosynthesis rate (A). The operation was automatic and the data were stored in the LI-6400 console and analyzed by "Photosyn Assistant "software (Version 3, Lincoln Inc, USA). Several precautions were taken to avoid errors during measurements. Leaf surfaces were cleaned and dried using tissue paper before enclosed in the leaf cuvette. The response of net CO_2 exchange (A) to changing intercellular CO_2 concentration (C_i) was conducted at 27°C, 50% relative humidity and at light saturating conditions of 800 µmol $m^{-2}s^{-1}$. The objective was to infer changes in the underlying photosynthetic capacity of the leaves. Net CO_2 exchange was measured 10-15 minutes after CO_2 supply and when stable chamber conditions were reached. Measurement of A was made starting at 400 µmol mol^{-1} CO_2 surrounding the leaf for control leaves and CO_2 was decreased stepwise to 50 µmol mol^{-1} CO_2. The CO_2 was set again to 400 µmol mol^{-1} and increased stepwise to 1000 µmol mol^{-1} [24]. From the A-Ci curve plotted, Rubisco CO_2 fixation capacity, V_{cmax} and maximum electron transfer rate, J_{max} were calculated using the equations of Farquhar et al, [25].

2.6 Total Available Carbohydrate

Anthrone method was used to determine the content of total available carbohydrate in the samples as explained by [26]. One gram of dried ground sample was weighed into the 250 ml conical flask and added with 10 ml of distilled water and 13 ml of 52% perchloric acid. The mixture was then shaken using orbital shaker for 20 minutes. Later, the mixture was transferred into a 100 ml volumetric flask and graduated to 100 ml with distilled water. After that, it was filtered into a 250 ml volumetric flask and graduated to 100 ml distilled water in another 100 ml volumetric flask. One ml of sample was mixed with 5 ml of anthrone reagent in a test tube. The tube was then placed in water bath at 100°C for 12 minutes to obtain a dark green solution. The tube was immediately cooled under running tap water and the absorbance was read at 630 nm by using spectrophotometer.

2.7 Total Phenolics and Total Flavonoid Quantification

The method of extraction and quantification for total phenolics and flavonoids contents followed after Jaafar et al. [27]. An amount of 0.1 g of grounded tissue samples was extracted with 10 ml of 80% ethanol on an orbital shaker for 120 minutes 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 the total phenolics content of the leaf samples. Two hundred µl of the sample extract was mixed with 1.5 ml of Follin–Ciocalteau reagent and allowed to stand at 22°C for 5 minutes before adding it with 1.5 ml of $NaNO_3$ (60 g L^{-1}) solution. 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 dry sample). For total flavonoids determination, 1 ml sample was mixed with 0.3 ml $NaNO_3$ in a test tube covered with aluminium foil, and left for 5 minutes. Then 0.3 ml of 10% $AlCl_3$ was added followed by addition of 2 ml of 1 M NaOH was added and the absorbance was measured at 510 nm using rutin as a standard (mg rutin/ g dry sample).

2.8 Statistical Analysis

Data were analyzed using analysis of variance by SAS version 17. Mean separation test between treatments was performed using Duncan multiple range test and 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 Total Plant Biomass and Leaf Area

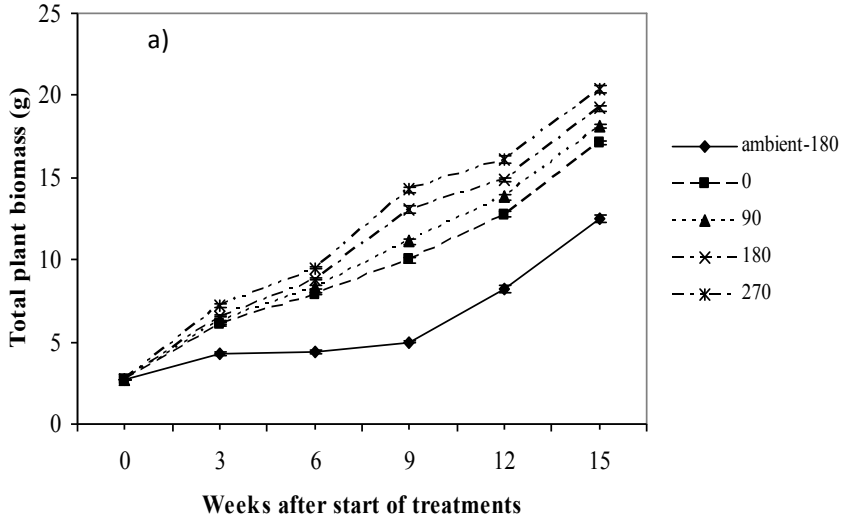
Total plant biomass was influenced by the nitrogen levels applied ($P<0.05$). At recommended nitrogen (N) rate and ambient CO_2 level, total plant biomass remained significantly lower in all of the weeks measured. Under elevated CO_2 270 kg N ha^{-1} gave the highest total plant biomass and 0 kg N ha^{-1} was the lowest. At 15 Weeks after treatment (WAT)

total biomass for 270, 180, 90 and 0 kg N ha^{-1} was 20.42 g, 19.21 g, 18.15 g and 17.13 g respectively compared to only 12.51 g for ambient-180 kg N ha^{-1}. At 15 weeks after treatments (WAT), the total biomass for respective 270, 180, 90 and 0 kg N ha^{-1} was 62%, 54%, 44.7% and 36.7% higher than the ambient-180 kg N ha^{-1} (Fig. 1a). Total leaf area was enhanced by the N levels applied ($p \leq 0.05$). As the weeks progressed, total leaf area has shown to increase until end of 15 WAT. The total leaf area started to show significant difference start from 9 WAT. It was found that the highest total leaf area was obtained in 270 kg N ha^{-1} followed by 180, 90, 0 and ambient-180 kg N ha^{-1} (Fig. 1b). At 15 WAT, the total leaf area of ambient-180 kg N ha^{-1} (254.59 cm^2) was 27%, 51%, 67% and 81% less than 356.71 cm^2, 384.04 cm^2, 425.55 cm^2 and 458.13 of 0, 90, 180 and 270 kg N ha^{-1} respectively. Plant growth was enhanced under increased N fertilization under CO_2 enrichment. This was showed by increased total plant biomass and leaf area in weeks 3, 6, 9, 12, and 15. Under elevated treatment condition the increase in N from 0 – 270 kg N ha^{-1} have enhanced the total plant biomass and total leaf area drastically. During the 4 months periods, total plant biomass and leaf area of *L. pumila* under ambient and standard N fertilization (ambient-180 kg N ha^{-1}) was the lowest in all weeks of measurement. These results are expected for plant that are treated with high CO_2 although the N content was low (0 and 90 kg N ha^{-1}) than standard fertilization N fertilization at ambient condition (ambient-180 kg N ha^{-1}), the total biomass and leaf area of plant under high CO_2 was higher. This suggest that plant total biomass and leaf area was enhanced under elevated CO_2 compared to the ambient condition (ambient-180 kg N ha^{-1}) regardless the nitrogen levels. The enhancement of total biomass and leaf area under elevated CO_2 might due increase plant sink strength with enhancement of N fertilizer. According to Bowler and Press [28] an increase in nitrogen, would enhance plant sink strength and the stimulation of the sink strength are high under CO_2 enrichment when N are not limited. Strong significance positive was found between total biomass and leaf area (R^2 = 0.871; $P \leq 0.05$; Table 1),

Table 1. Pearson correlation between all measured parameters in the experiment

Parameters	1	2	3	4	5	6	7
1. T. Biomass	1.00						
2. Leaf area	0.871*	1.000					
3. T. chlorophyll	0.592*	0.685*	1.000				
4. Photosynthesis	0.258	0.388	0.771*	1.000			
5. Carbohydrate	0.189	0.073	-0.549*	-0.865*	1.000		
6. T. Phenolic	0.34	0.131	0.775*	-0.790*	0.861*	1.000	
7. T. Flavonoid	-0.28	-0.34	-0.731*	-0.622*	0.755*	0.863**	1.000

** and ** significant at P ≤0.05 and P≤0.01 respectively*



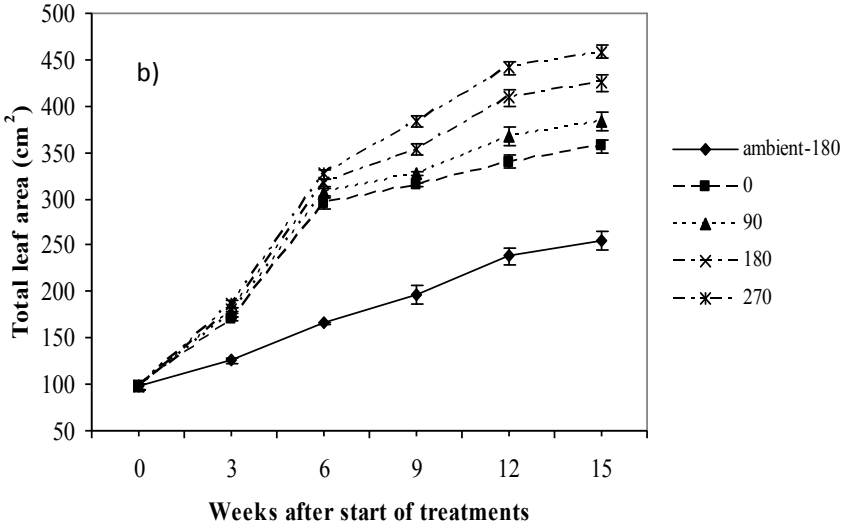


Fig. 1. The effects of nitrogen levels (kg N ha^{-1}) on total plant biomass (a) and leaf area (b) of *L.pumila* under CO_2 enrichment (1200 µmol mol^{-1}). Ambient -180 (*L. pumila* under standard nitrogen fertilization and ambient CO_2 concentration; control). N = 15

this suggest that the increase in total biomass of *L. pumila* seedlings under high nitrogen levels and CO_2 was due to increase in interception of light due to increase in leaf area that stimulated the biomass production [5].

3.2 Total Chlorophyll Content

The nitrogen level applied have significantly influenced the total chlorophyll content of *L. pumila* ($P<0.05$). Start from 3 -6 WAT, the total chlorophyll content of ambient-180 kg N ha^{-1} was higher than all elevated nitrogen treatment. However, from 9 -15 WAT 270 kg N ha^{-1} maintained the highest total chlorophyll content. The lowest total chlorophyll content was on 0 kg N/ha followed by 90 and 180 kg N ha^{-1}. At 15 WAT, total chlorophyll content of ambient-180 kg N ha^{-1} was 2.75%, 16.19% and 27% higher than 180, 90 and 0 kg N ha^{-1} respectively (Fig. 2). However total chlorophyll content was 10% lower in ambient-180 kg N ha^{-1} compared to 270 kg N ha^{-1}. Total chlorophyll content was influenced by the nitrogen levels applied to *L. pumila*. The increase in N fertilization have augment the total chlorophyll content in the plant under CO_2 enrichment and ambient treatments (ambient-180 kg N ha^{-1}). The enhancement of total chlorophyll content might due to increased nitrogen fertilization that enhanced the production the light harvesting protein [29]. The increase in total chlorophyll content of plant enriched with high CO_2 and N fertilization was observed by [30] when they observed 27% increased in total chlorophyll content production of soybean was under CO_2 and nitrogen fertilization rather than enrichment with CO_2 alone. The enhancement of total chlorophyll content might up regulate net photosynthesis (R^2 = 0.771;$P\leq0.05$; Table 2) due to enhanced uptake of nitrogen under elevated CO_2. However, negative relationship observed between total chlorophyll content with carbohydrates (R^2 = -0.549;$P\leq0.05$ Table 2;), total phenolics (R^2 = -0.775 ;$P\leq0.05$; Table 2) and total flavonoid (R^2 = -0.731; $P\leq0.05$; Table 2) might suggest that production of total chlorophyll content might be down-regulated under accumulation of carbohydrates that might be channeled to the production of plant secondary metabolites [31,32].

3.3 Net Photosynthesis (A)

Net photosynthesis was influenced by nitrogen levels ($p\leq0.05$). It was shown from the figure that as the levels of N increases the net photosynthesis showed increasing trends. From 3 – 15 WAT, as the levels of nitrogen increased from 0 to 270 kg N ha^{-1} net photosynthesis showed to be statistically significant from each other start from 9 WAT. For 180 and 270 kg N ha^{-1} as the nitrogen levels increased net photosynthesis increased until end of 15 WAT. However, for 0 and 90 kg N ha^{-1} it started to decrease from 9 WAT. The net photosynthesis

for ambient-180 kg N ha^{-1} remained lowered throughout all the weeks of measurement (Fig. 3). It was found that net photosynthesis (A) was influenced by the nitrogen levels applied to the *L. pumila* seedlings. It was observed that as the levels of nitrogen increased the rate of A increase throughout the weeks of measurement. In all weeks of measurement, A was the highest for 270 kg N ha^{-1} followed by 180 kg N ha^{-1}, 90 kg N ha^{-1}, 0 kg N ha^{-1} and ambient-180 kg N ha^{-1} (Fig. 7). Furthermore, correlation table have shown that A and leaf nitrogen are significantly positive related (R^2 = 0.764; P≤0.05; Table 2). The figure also showed that A can be enhanced under high elevated CO_2 without any N fertilization (0 kg N ha^{-1}). This implied that elevated CO_2 can enhanced A of not well fertilized plant [33]. However, for not fertilized plant down-regulation of A start to be expressed at 9 WAT. The decrease might due to reallocation of N away from the photosynthetic apparatus [34]. The enhanced in A in high N fertilized might due to increased sink strength of the plant fertilized with high N that can distribute the carbohydrate to other plant parts. For the plant that cannot distribute carbohydrate the carbohydrate there will accumulation of carbohydrate that can repressed the A [35]. In the present study, the highest accumulation of carbohydrates was found on low N fertilized plant (0 and 90 kg N ha^{-1}) that might support the hypothesis that increased carbohydrate in leaves might down-regulate A due to accumulation of carbohydrate in leaves. Furthermore, correlation table in the experiment showed that A and total available carbohydrate are significant negatively related (R^2 = -0.865; P≤0.05).

3.4 Net Assimilation Rate to Intercellular CO_2 (A/C_i) Curves

Fig. 4 showed the influence of N levels on A/Ci properties of *L. pumila*. As intercellular CO_2 increased, seedlings treated with high N levels showed increasing net assimilation rate than ambient -180 kg N ha^{-1} treatments. However, from the graph 0 kg N ha^{-1} have the lowest A/C_i peak. A sat (CO_2 saturation point) was enhanced by 217%, 163%, 129 and 27% in the 270 kg N ha^{-1}, 180 kg N ha^{-1}, 90 kg N ha^{-1} and 0 kg N ha^{-1} respectively compared to the ambient-180 kg N ha^{-1}. Meanwhile nitrogen levels resulted significant higher RuBP carboxylation efficiency (V_{cmax}). Seedling exposed to high N under elevated CO_2 have 243%, 195%, 146% and 23% in 270, 180, 90 and 0 kg N ha^{-1} efficiency on V_{cmax} compared to the ambient-180 kg N ha^{-1}. In contrast, respiration rate was when nitrogen levels reduced in elevated treatments. It was found that the highest respiration occurred at ambient-180 kg N ha^{-1} (7.36 µmol $m^{-2}s^{-1}$), followed by 0 kg N ha^{-1} (6.88 µmol $m^{-2}s^{-1}$),

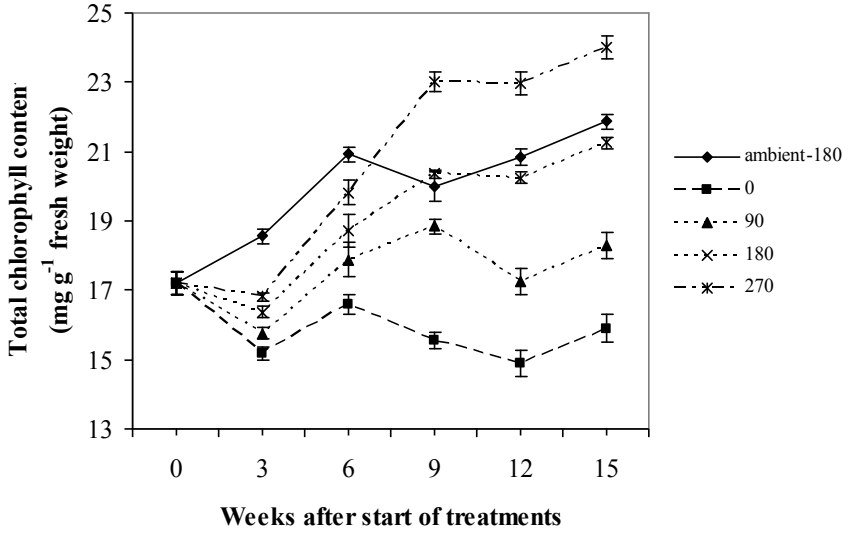


Fig. 2. The effects of nitrogen levels (kg N ha^{-1}) on total chlorophyll content of *L.pumila* under CO_2 enrichment (1200 µmol mol^{-1}). Ambient -180 (*L. pumila* under standard nitrogen fertilization and ambient CO_2 concentration; control). N = 15

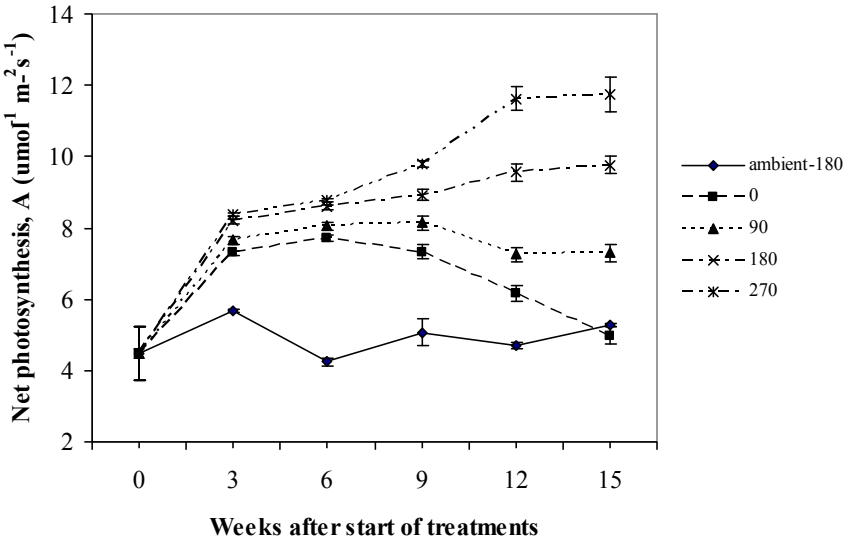


Fig. 3. The effects of nitrogen levels (kg N ha^{-1}) on net photosynthesis of *L.pumila* under CO_2 enrichment (1200 µmol mol^{-1}). Ambient -180 (*L. pumila* under standard nitrogen fertilization and ambient CO_2 concentration; control). N = 15

90 kg N ha^{-1} (5.14 µmol m^{-2}s^{-1}), 180 kg N ha^{-1} (3.75 µmol m^{-2}s^{-1}) and 270 kg N ha^{-1} (0.237 µmol m^{-2}s^{-1}) respectively. The A/Ci curves in *L. pumila* was influenced by the N levels applied to the seedlings. It was shown that the net assimilation rate was higher in plant that fertilized with high N. As the levels of N fertilization increases from 0 – 270 kg N ha^{-1} the net assimilation rate become higher with increasing intercellular CO_2 (Fig. 4). The analysis from the A/Ci curves (Table 2) revealed that plant that fertilized with high N have higher Maximum V_{cmax}, Maximum net assimilation rate (J_{max}) and lowered dark respiration rate than plant with low N fertilization. The same result was also obtained by [36] in *Quercus suber* with increasing N fertilization under CO_2 enrichment. The decreased response of A/Ci for plant under N limited fertilization was due to reduction in RuBisCO protein in leaves due to low N content in leaf [37]. In the present study the reduction in RuBisCO was characterized by low RuBp Carboxylation efficiency (V_{cmax}) and low A_{sat} that showed the reduction in RuBp regeneration under low N condition [38]. In the current study, the same observation has been found in *Chenopodium album*, *Triticum aestivum* and cotton [39-41]. The result from the A/Ci curves implied that the enhancement of $V_{cmax,}$ A sat and reduction on dark respiration was due to increase in production of RuBisCO protein that enhances under high N levels applied to the plants under CO_2 enrichment.

3.5 Total Available Carbohydrate and Profiling

Total carbohydrate content was influenced by the nitrogen levels applied (P≤0.01; Fig. 5). Total carbohydrate was enhanced as weeks progressed. From 5 -12 WAT, *L. pumila* plants with the lowest nitrogen have the highest total available carbohydrate. However, *L. pumila* with the highest N (270 kg N ha^{-1}) accumulated the lowest total available carbohydrate. At 15 WAT total available carbohydrate of 0 kg N ha^{-1} was 42.7 mg g^{-1} dry weight, 90 kg N ha^{-1} 37.45 mg g^{-1} dry weight, 180 kg N ha^{-1} 32.64 mg g^{-1} dry weight, 270 kg N ha^{-1} 23.80 mg g^{-1} dry weight and ambient -180 kg N ha^{-1} was 24.03 mg g^{-1} dry weight. The total available carbohydrate for 0 kg N ha^{-1} was 75% more than ambient-180 kg N ha^{-1} in 15 WAT (Fig. 5a). It was shown that total available carbohydrate was higher in leaves, followed by root and stem parts. The data implied that most accumulation of total available carbohydrate was on aboveground than belowground. From the Fig. 5b also it showed that total available carbohydrate was higher under less N fertilized than high N fertilization on all part of *L. pumila*. From the correlation data (Table 1) strong positive relationship was found between total carbohydrate with total phenolics (R^2 = 0.861; p≤0.05) and total phenolics (R^2 =0.755; p≤0.05) and negative strong relationship with leaf nitrogen (R^2 = -0.811; p≤0.05) and net

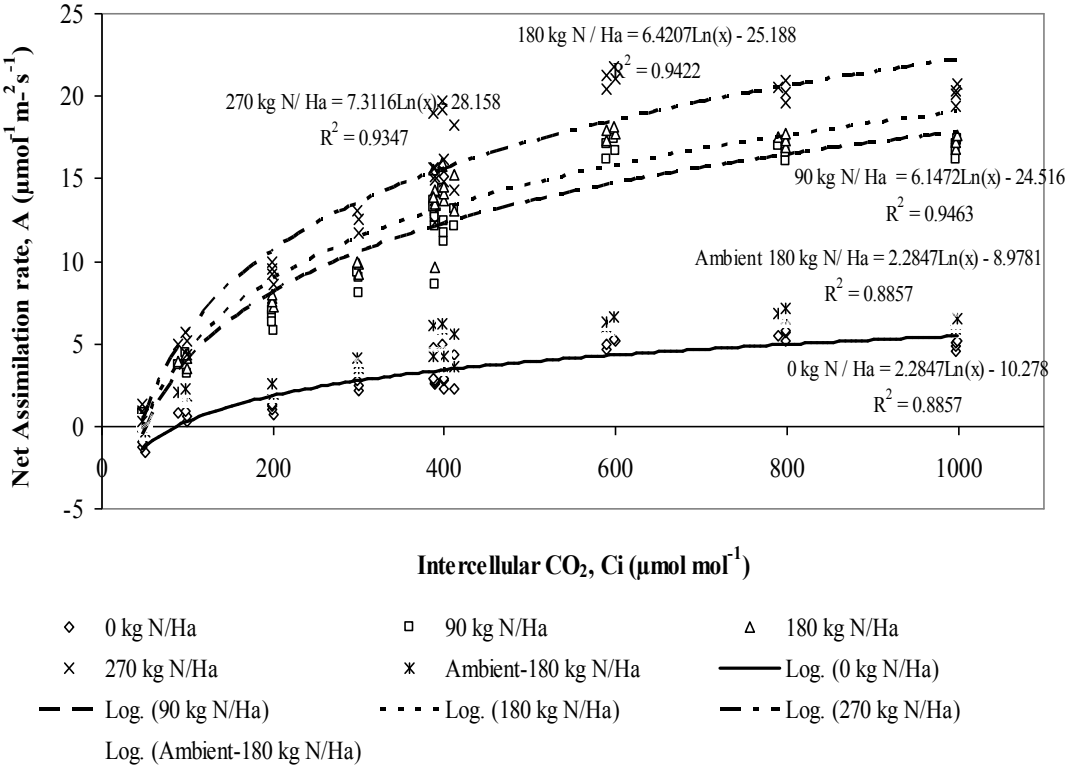


Fig. 4. The effects of nitrogen levels on net assimilation rate to intercellular carbon dioxide levels (A/Ci)

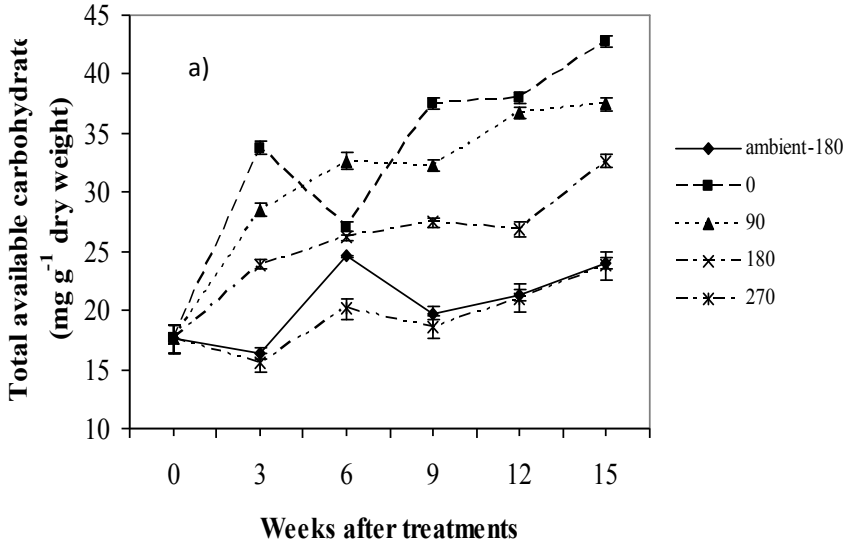
Table 2. A/Ci curves properties of *L. pumila* under different nitrogen fertilization

Treatments	Rubp carboxylation efficiency, V_{cmax} (µmol m^{-2}s^{-1})	Maximum net assimilation rate, Jmax (µmol m^{-2}s^{-1})	Dark respiration rate, R_d (µmol m^{-2}s^{-1})
Amb-180 kgN Ha^{-1}	0.0155±0.0004^{d}	6.60± 0.24^{d}	7.36± 0.22^{a}
0 kg N Ha^{-1}	0.0121±0.0012^{e}	6.78±0.43^{d}	6.88± 0.33^{b}
90 kg N Ha^{-1}	0.0298±0.0012^{c}	15.46±0.52^{c}	5.14±0.21^{c}
180 kg N Ha^{-1}	0.0358±0.0006^{b}	17.87±0.27$_{b}$	3.75± 0.21^{d}
270 kg N Ha^{-1}	0.0416±0.0005^{a}	21.50±0.40^{a}	2.38± 0.19^{e}

All analyses are mean ± standard error of mean (SEM). Means not sharing a common letter were significantly different at P≤ 0.05.
**Control at ambient CO_2 levels (400 µmol^{-1} mol^{-1}) and standard nitrogen fertilization rate (180 kg N ha$^{-1;}$ Amb-180 kg N Ha^{-1})*

photosynthesis (R^2 = -0.865; p≤0.05). It was found that total available carbohydrate was influenced by the nitrogen level applied to the *L. pumila*. The highest accumulation of total available carbohydrate was found on 0 kg N ha^{-1} followed by 90 kg N ha^{-1}, 180 kg N ha^{-1} and 270 kg N ha^{-1}. The accumulation of carbohydrate in *L. pumila* was more pronounced in leaves followed by root and stem in ambient-180 kg N ha^{-1} and elevated CO_2 (Fig. 5). The data suggest that the accumulation of carbohydrate was more on low N fertilized than well N fertilized plants. The accumulation of carbohydrate in nitrogen limited plants might due to these plants cannot generate new sinks for carbohydrates to increases photosynthetic supply [42]. These

findings are in agreement with other researcher on CO_2 and Nitrogen fertilization [43-45]. The increase in carbohydrate might up-regulate the production of *L. pumila* secondary metabolites, where in the present study it was significantly positively correlated (R^2 = 0.861; P≤0.05 and R^2 = 0.755; P≤0.05; Table 1) respectively for total phenolics and flavonoid). The same observation was found by Gebauer et al. [46] on *Pinus taeda* where they found strong positive correlation between carbohydrate and secondary metabolites production. The data implied that when there was accumulation of carbohydrates in low N fertilized plants the up- regulation in secondary metabolites might be occurring [47-51].



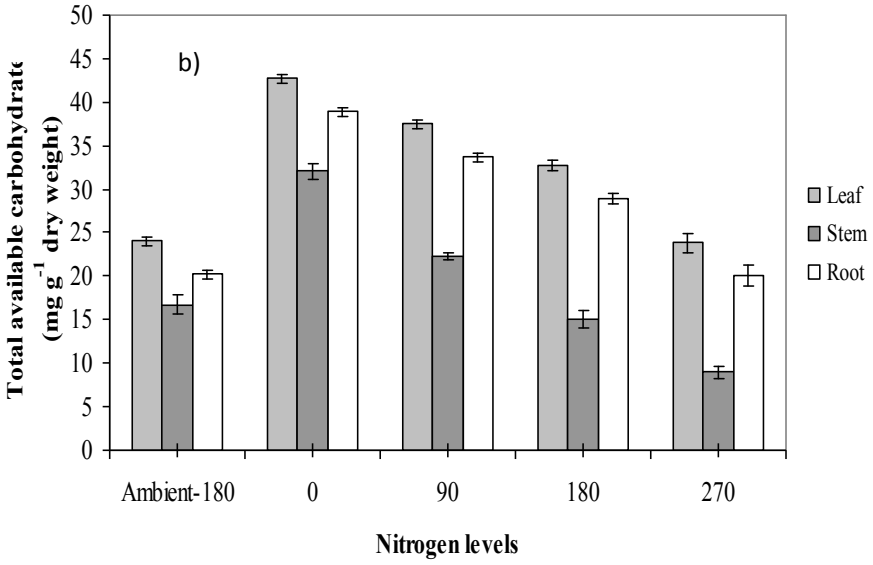


Fig. 5. The effects of nitrogen levels (kg N ha^{-1}) on total available carbohydrates (a) and profiling (b) of *L.pumila* under CO_2 enrichment (1200 µmol mol^{-1}). Ambient -180 (*L. pumila* under standard nitrogen fertilization and ambient CO_2 concentration; control). N = 15

3.6 Total Phenolics and Flavonoids Profiling

Total phenolics and flavonoids was influenced by the nitrogen levels applied to *L. pumila* ($P<0.05$). The plant under the highest nitrogen levels produced the lowest total phenolics throughout the weeks, however under 0 kg N ha^{-1} total phenolics was the highest. As the levels of nitrogen increase (0- 270 kg N ha^{-1}) 5 less total phenolics was produced. This was shown at 15 WAT where 0 kg N ha^{-1} produced the highest total phenolics (1.43 mg g^{-1} GAE; Gallic acid Equaivalent) followed by 90 kg N ha^{-1} (1.359 mg g^{-1} GAE), 180 kg N ha^{-1} (1.25 mg g^{-1} GAE), ambient-180 kg N ha^{-1} (1.16 mg g^{-1} GAE) and 270 kg N ha^{-1} (0.715 mg g^{-1} GAE) (Fig. 6a). In all elevated CO_2 nitrogen treatments, it was observed that total phenolics were higher in leaf followed by root and stem. In ambient -180 kg N ha^{-1} the highest total phenolics were observed in leaf where total phenolics in stem and roots are almost balanced (Fig. 6b).

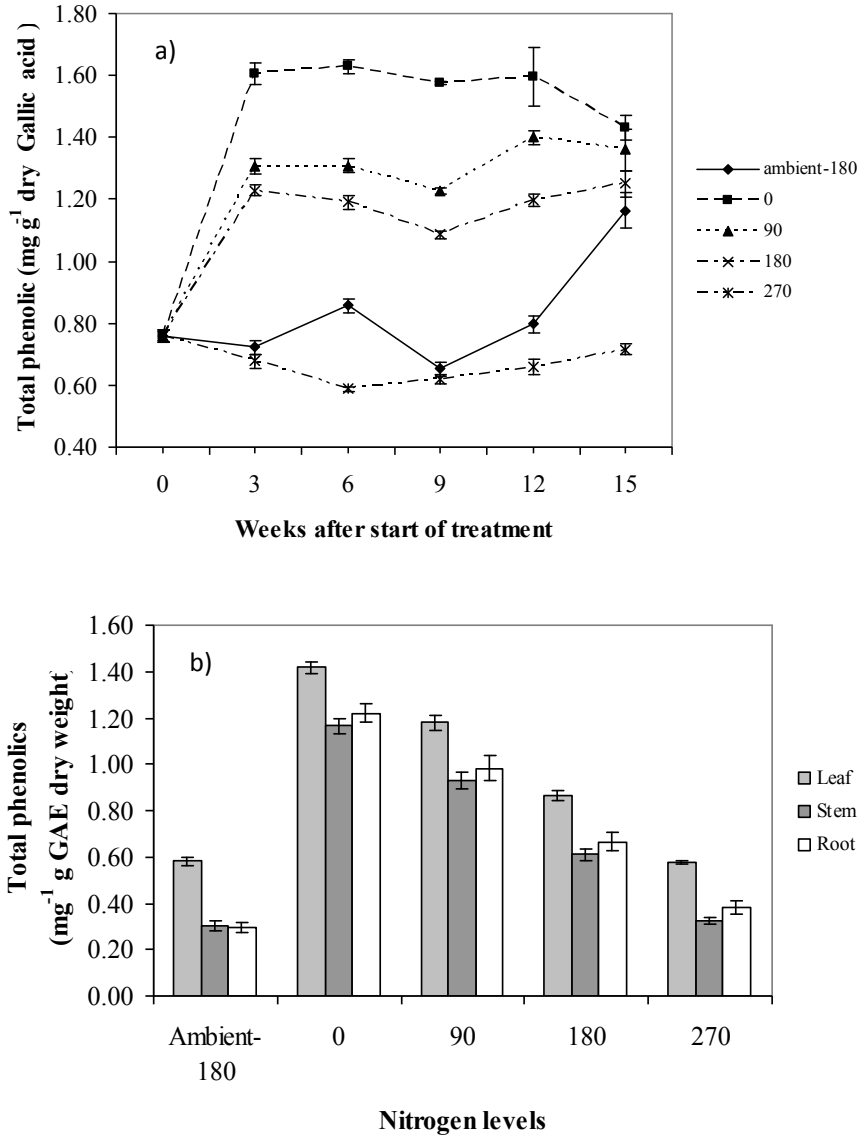


Fig. 6. The effects of nitrogen levels (kg N ha^{-1}) on total phenolics (a) and profiling (b) of *L.pumila* under CO_2 enrichment (1200 µmol mol^{-1}). Ambient -180 (*L. pumila* under standard nitrogen fertilization and ambient CO_2 concentration; control). N = 15

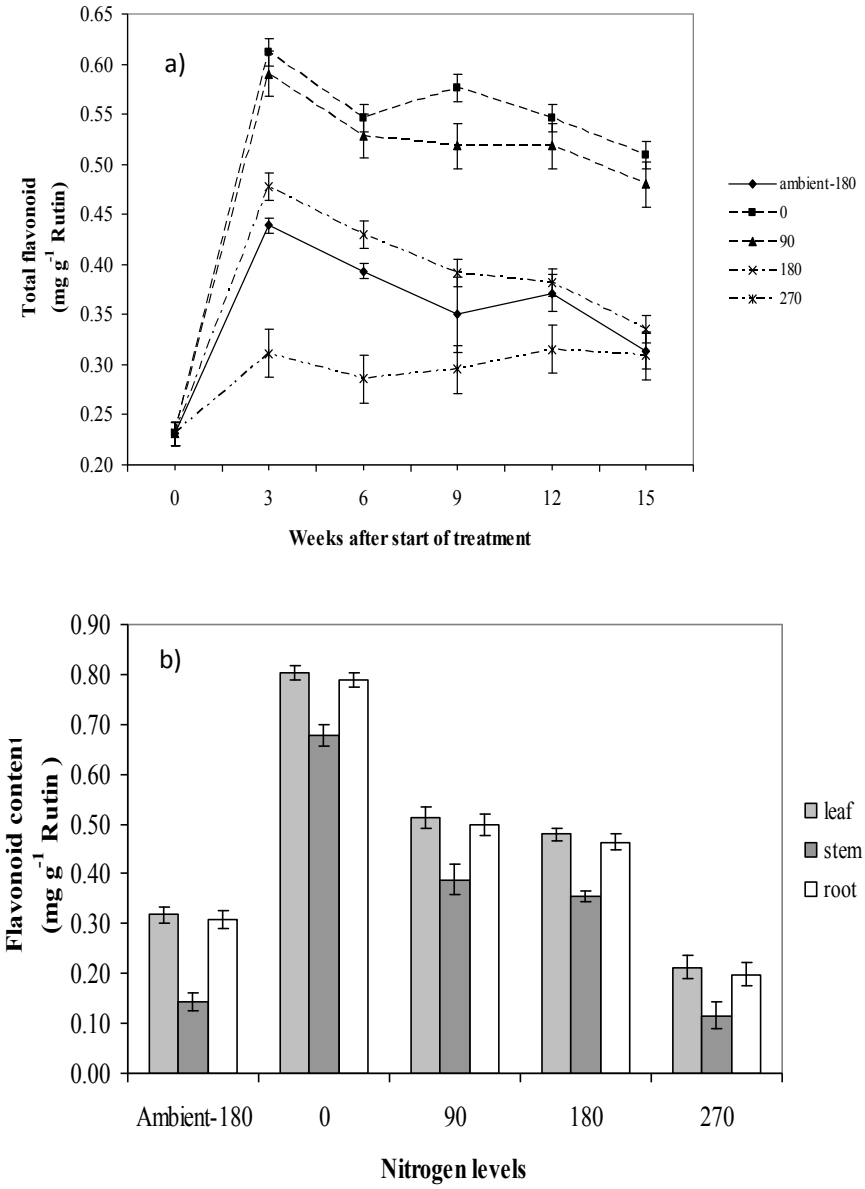


Fig. 7. The effects of nitrogen levels (kg N ha^{-1}) on total flavonoid (a) and profiling (b) of *L.pumila* under CO_2 enrichment (1200 µmol mol^{-1}). Ambient -180 (*L. pumila* under standard nitrogen fertilization and ambient CO_2 concentration; control). N = 15

4. CONCLUSION

This study demonstrated that enrichment with high levels of CO_2 is able to enhance the production of total phenolics and flavonoids in *L. pumila* however, increased in nitrogen fertilization can reduce the production of these plant secondary metabolites under CO_2 enrichment. It is observed, when there was accumulation of TNC in plant leaves and reduction in photosynthesis and chlorophyll content, the production of these metabolites might be up-regulated.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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