

The Role of Shade and Nitrogen on Physiological Traits and Secondary Metabolites of *Piper betle* L

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Abstract

Piper betle, better known as purple betel has received a universal attention due to its increasing medicinal value. There is enormous potential to discover various new medicinal compounds in this species and an instantaneous need for the techniques to facilitate the production of high quality. In that connection, shade and nitrogen could play a significant role in the development of a phytochemical production. Therefore, the current study utilized the different levels of shade and rates of nitrogen. After harvesting of three months *Piper betle* seedlings, different physio-chemical traits were observed including photosynthetic rate, stomata conductor, transpiration rates, chlorophyll content (a, b, ab) and secondary metabolites (total phenolics contents (TPC) concentration, total flavonoids contents (TFC) concentration and antioxidant activate (DPPH and FRAP). The chlorophyll content was more under 30% and 50% shades with 100 kg/ha of N, whereas full sunlight with 0 kg/ha of N was unsuitable for the *Piper betle* crop as the high light intensity caused scorching on leaves and stunted in growth, accumulation secondary metabolites and Antioxidant activates. The results of the present study revealed the establishment of shade techniques which influenced the production parameters of the piper species. These findings are an overview of the recent advances and could be further helpful in *in vivo* studies of *Piper betel* in regard to their physiological parameters.

Keywords: *Piper betle*; Shade; Nitrogen; Secondary metabolites

Introduction

Plants are potential sources of natural bioactive compounds such as primary and secondary metabolites and antioxidants. Medicinal plants quality is determined by their superior genetic characteristics and great biomass with high and consistent secondary metabolite content [1]. The concentration of these secondary metabolites and chemical profile of plants grown in the field can be influenced by environmental conditions such as temperature, light quality and light intensity [2].

Piper betle L. is grown in hotter and damper parts of the country [3,4]. In betel vine cultivation, light and nutrients play an imperative role in improving the productivity and quality of leaves. It is one of the main limiting factors for phenolic compound biosynthesis in plants [5]. The rate of photosynthesis as a function of light intensity has been studied by numerous workers [6,7] and in most of these studies showed that the rate of photosynthesis increases rapidly with increasing light intensity. Although, photosynthetic rate, stomatal conductance, and transpiration rate also significantly decrease with increasing the shade level [8,9] all these plant components respond differently to light intensity in different plant species. However, light-demanding species are more flexible in both morphology and biomass allocation in response to changes in the light intensity than shade-tolerant species [10].

Nitrogen is also one of the main nutrients required for the plant growth. Its application to plants also supports to utilize the environmental resources like water and light that in turn activate the plant metabolites (amino acids) and their derivatives (enzymes and co-factors), respectively. Plant nutrients are the major important factors in determining the secondary metabolism and antioxidant activity. By increasing fertilizer rate, results could be increased in the concentration of lutein and carotene of parsley and phenolic compounds and carotenoids in lavender. However, in *chrysanthemum morifolium*, heavy nitrogen fertilization was reported to reduce the flavonoids and antioxidant activity of flowers [11].

Furthermore, the levels and composition of phenolics acid and flavonoids in plants also varies according to the genotype, climate factors and agronomical practices [12]. Cultivation factors like soil type, compost, mulching, and fertilization also can affect the plant secondary metabolites and antioxidant activity of the plant. The fertilizer effect on vegetative growth is well documented. However, the influence of fertilizer rates and shade levels on phytochemical of *P. betle* is still lacking. Therefore, the current study was designed to conduct an experiment to observe the effects of shades and N rates on physiological components and secondary metabolites of *P. betle*.

Material and Methods

The experiment was carried out in the field 2 black net houses at the Faculty of Agriculture, 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. Stem cuttings of *P. betle* were propagated for four weeks in small pots (1 kg) and then transferred to white polyethylene bags (45 × 50 cm) filled with a topsoil, coco peat and river sand (ratio 3:2:1). *P. betle* is a semi-shade plant that requires some amount of shade for maximum production. In order to determine the shade level for maximum production, three levels of shade net including 0%, 30%, and 50% were used in order to reduce light intensity in the field. All light measurements were made between 8 am and 7 pm daily by meter light (phonex, ILTI400, Japan). The average light intensity passing through

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in each shading treatment was measured about full sunlight = 790 $\mu\text{mol}/\text{m}^2/\text{s}$, 30% shade = 550 $\mu\text{mol}/\text{m}^2/\text{s}$ and 50% shade 390 $\mu\text{mol}/\text{m}^2/\text{s}$ photosynthetically active radiation (PAR), respectively. The application started after one week from transplanting with a variable rate of N (0, 50, 100, 150 kg/ha), P (100 kg/ha) and K (100 kg/ha). Plants were watered twice daily by spraying irrigation system 7:00 am and 7:00 pm. The plants were harvested after 90 days after transplanting.

The photosynthetic rate measurement was estimated in a closed system of infra-red gas analyzer Li-Cor 6400 Portable Photosynthesis system (Li-Cor 6400, Li-Cor, Lincoln, Nebraska, USA) Figure 1. Before warming and calibrating the portable photosynthesis system. In the first step, the initial zeroing process for the built-in flow meter and in the second step, zeroing process for the infra-red gas analyzer was observed. The procedure was followed similarly as previously reported by Jaafar et al. [11]. The measurements were used optimal cuvette conditions such as 1000 μmol photosynthetically active radiation (PAR)m, 400 $\mu\text{mol}/\text{mol}$ carbon dioxide, 30°C leaf temperature, 60% relative humidity with air flow rate of 500 cm^3/min . The measurements of gas exchange were carried out between 9:00 to 11:00 a.m. Measurements were taken before harvest (90 days) from fully expanded leaf (second frond). The leaf surfaces were cleaned and dried before being enclosed in the leaf cuvette. Data for net photosynthesis rate, transpiration rate and stomatal conductance were simultaneously recorded. The operation was automatic, and the data were stored in the Li-Cor 6400 computer within the console and analyzed by "Photosyn Assistant" software.

The actual leaf chlorophyll content was determined by using Coombs method as previously described by Beadle et al. [13]. Leaves were gnawed using cock borer to get four sample area of 1 cm^2 per gnawing. Samples were put into a vial, and 20 ml of 80% (v/v) acetone was poured into a vial and covered with aluminum foil. These samples were kept in the dark place for about three to seven days until extraction of all chlorophyll from leaves. Chlorophyll content was then determined by using Spectrophotometer (Model UV 3101 PC) at wavelengths of 664 nm and 647 nm. Coombs method (Coombs et al.) consisted of following description with formula. The unit of measurement is mg/cm^2 .

$$\text{Chlorophyll a (mg cm}^{-2}\text{)} = 13.19 A_{664} - 2.57 A_{647}$$

$$\text{Chlorophyll b (mg cm}^{-2}\text{)} = 22.10 A_{647} - 5.26 A_{664}$$

$$\text{Total chlorophyll content (mg cm}^{-2}\text{)} = 3.5 \times (\text{chlorophyll a} + \text{chlorophyll b}) / 4$$

$$A_{664} = \text{Value of absorption at wavelength of 664}$$

$$A_{647} = \text{Value of absorption at wavelength of 647}$$

$$3.5 = \text{total of chlorophyll extract in vial}$$

$$4 = \text{area of leaves for chlorophyll extraction}$$

Secondary Metabolites

Extraction and preparation of sample

Secondary Metabolites were estimated from fresh leaves washed with distilled water and dried under shade for seven days and powdered. Later, the extraction was carried out by do slightly modification as suggested by Dhote et al. [14]. Then, the powdered leaves were a bit extracted in 80% methanol (1 g: 20 ml) with the help of cold maceration at room temperature for about 24 h and were frequently shaken. The process of extraction was repeated three times. The solution was filtered using Whatman's filter paper No.1, and the solvent was allowed to evaporate completely to obtain the extract. The extract was stored to use in sterile glass vials at the temperature of 4°C.

To determine the total phenolic content of the leaf extract of *P. betle*, Folin-Ciocalteu Reagent (FCR) was estimated as suggested by Mukherjee et al. [15]. The reaction mixture contained 200 μl of diluted extract, 800 μl of freshly prepared diluted Folin-Ciocalteu reagent and 2 ml of 7.5% sodium carbonate. The final mixture was diluted to 7 ml with deionized water. The mixtures were kept in the dark at ambient conditions for 2 hours to complete the reaction. The absorbance was recorded at 765 nm. Gallic acid was used as a standard, and the results were expressed as mg Gallic acid equivalents (GAE)/g of dry weight.

Aluminum chloride (AlCl_3) was used to determine the total flavonoid content of the extract. A quantity of 0.1 ml methanolic extracts was added to 0.3 ml distilled water followed by 0.03 ml, NaNO_2 (5%) and after 5 min 0.03 ml AlCl_3 (10%) was also added. After 5 minutes, the reaction mixture was treated with 0.2 ml of 1 mM NaOH. Finally, the reaction mixture was diluted to 1 ml with water, and the absorbance was measured at 510 nm. Quercetin was used as a positive control as suggested by Rintu et al. [16]. The antioxidant activities were carried out through two assay techniques.

1,1-Diphenyl-2-picrylhydrazyl (DPPH) assay: This procedure was followed according to the procedure previously reported by Braca et al. [17] in order to execute the antioxidant activity of the crude methanolic. *P. betle* extracts were evaluated against 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radicals. Various concentration of the methanolic extract was added to the methanolic solution (0.004%) of DPPH on a 96 well ELISA plate. In last, after 30 minutes of incubation, absorbance was determined at 517 nm, and the inhibition percent activity was calculated utilizing the following formula:

$$\text{AA\%} = (\text{Absorbance sample} - \text{Absorbance empty sample}) \times 100 / \text{Absorbance controls}$$

However, the blank samples contained 1 ml methanol + 2.5 ml from various concentrations of *P. betle* extracts. Furthermore, control sample contained 1 ml of 0.3 mM DPPH + 2.5 ml from methanol. In optic density of the samples, the control and empty samples were measured in comparison with methanol where tocopherols (α) were positive controls.

Ferric reducing antioxidant: Power (FRAP) Assay: Ferric reducing the antioxidant potential of the extract was analyzed using the method proposed by Firuzi et al. [18]. FRAP solution was prepared by adding 200 ml of 300 mM acetate buffer (which was adjusted to pH of 3.6 by the adding of acetic acid) to 20 ml of 20 mM ferric



Figure 1: Measurement photosynthesis rate, stomatal conductance, and transpiration rate by Li-Cor 6400 Portable Photosynthesis system.

chloride hexahydrate (which was dissolved in distilled water) and 20 ml of 10 mM 2,4, 6-tri-(2-pyridyl)-triazine (TPTZ) (dissolved in 40 mM HCl). Ferrous sulphate standard graph was prepared by taking a different concentration of ferrous sulphate (0.1-1 mM). 120 µl distilled water/standard/sample was added to 4 ml of the FRAP solution and absorbance was taken at 593 nm after 4 minutes. Ferrous sulphate equivalent concentration in mM was calculated from the standard graph and expressed as mmol ferrous sulphate equivalent/g on a dry weight basis (mmol Fe+2/g DW).

The experiments were nested and carried out in a Randomized Complete Block Design (RCBD) with three replications and analyzed using analysis of variance (ANOVA). The results were presented with Mean ± S.D, and Tukey test at 0.05 probabilities was utilized to separate the means with significant differences. Meanwhile, the correlation was used to investigate the relationship between all the physiological parameters. All the analysis was done utilizing Statistical Analysis Software version 9.3 (SAS Institute Inc. 2009).

Results

Net photosynthetic rate (PN) was significantly higher ($P < 0.05$) in those plants which were grown under 30% shade. PN of plant increased with a decrease light intensity (Figure 2). In general, the photosynthesis rate declined in the following order 30% > 50% > 0% and so as well nitrogen rate 100 > 150 > 50 > 0 kg/ha, respectively. The plants under 30% shade with 100 kg N/ha showed the highest P_N 10.13 $\mu\text{mol m}^{-2}\text{s}^{-1}$ followed by 8.69 $\mu\text{mol m}^{-2}\text{s}^{-1}$ at 150 kg N/ha under the same shading. However, the similar result (8.68 $\mu\text{mol m}^{-2}\text{s}^{-1}$) with no significant different ($P > 0.05$) was also observed with 100 kg N/ha under 50% shade. The plants under full sunlight had the low P_N as compared to those plants which were kept under 30% and 50% shades. It was due to the exposure of the full sun which severely damaged the plants from leaf scorching and had stunted growth as compared to other treatments.

The stomata conductance (G_s) increased with increasing shading. G_s with the application of N at 100 kg N/ha showed significantly highest ($P < 0.05$) with a mean value of 0.13 $\mu\text{mol m}^{-2}\text{s}^{-1}$ under 30% shade, however, 150 and 100 kg N/ha under 30% and 50% shade showed similar results with no significant difference. The results were lowest under 0% shade in control (Figure 3).

The transpiration rate (E) was significantly highest ($P < 0.05$) at 30% shade with the application of nitrogen at the rate of 100 kg N/ha. Meanwhile, there was no significant difference in transpiration rate in plants those were kept under 30% and 50% shades with 50 and 150 N kg/ha. The results were lowest under 0% shade in control (Figure 4).

In the present study, chlorophyll concentration was significantly influenced ($P < 0.05$) by the different shade levels and nitrogen rates. Increasing light intensities with decreasing shade levels and nitrogen rates enhanced the amount of chlorophyll (a, b) and total chlorophyll (a+b). In total chlorophyll content increased with increasing shade and nitrogen rate (Figure 5). Meanwhile, chlorophyll was affected with the highest value at 100 kg/ha under 30% shade, whereas no significant difference between 100 and 150 N kg/ha under 50% shade and the lowest value was found in the control treatment.

The effect of different light intensities and nitrogen rates on the chlorophyll (b) of *P. betle* (fresh leaves) also showed a statistically significantly different. The highest chlorophyll (b) content (1.83 mg/cm²) with 100 kg of N/ha was recorded at the lowest light intensity (50% shade). These results were not different statistically for chlorophyll content b (1.76 mg/cm²) with the application of N at a rate of 50 and

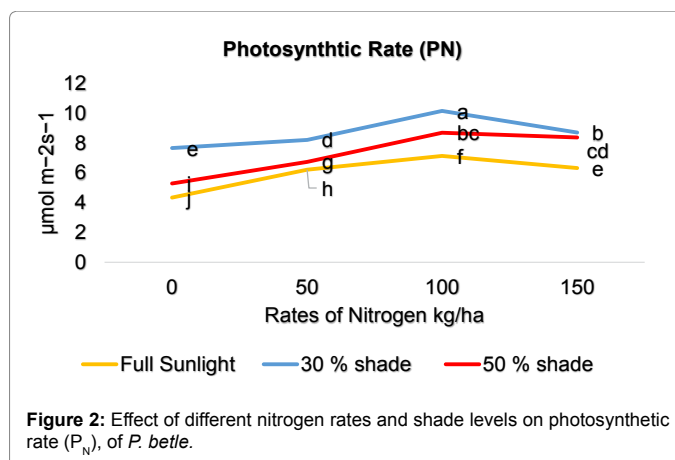


Figure 2: Effect of different nitrogen rates and shade levels on photosynthetic rate (P_N), of *P. betle*.

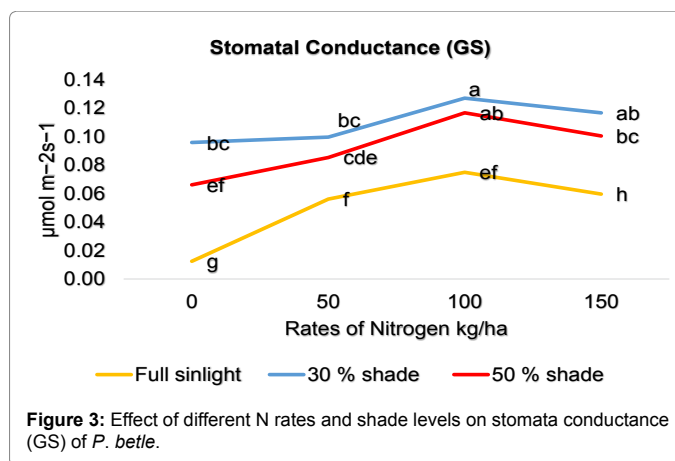


Figure 3: Effect of different N rates and shade levels on stomata conductance (GS) of *P. betle*.

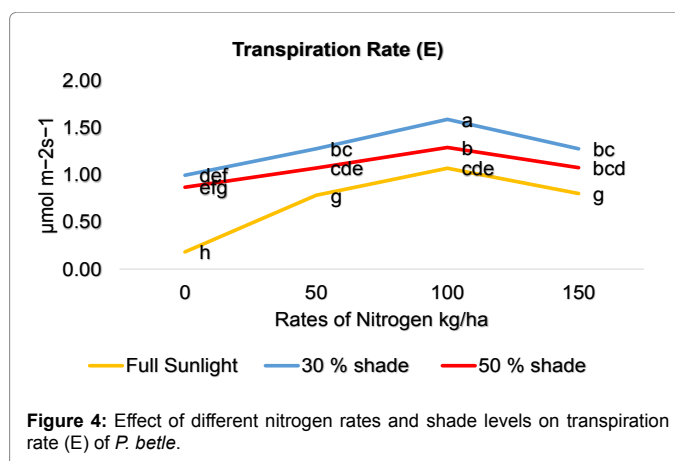


Figure 4: Effect of different nitrogen rates and shade levels on transpiration rate (E) of *P. betle*.

150 kg of N/ha under the same level of shade but were different at 30% shade. Beside the chlorophyll content b (1.55 and 1.49 mg/cm²) with the application of N rates under 30% shade showed statically no difference between 50 and 100 kg/ha (Figure 6).

The overall total chlorophyll concentration significantly increased with increasing light intensity under 0% to 50% shade and with increasing nitrogen rates from 0 < 50 < 100 < 150 kg/ha. A high concentration of chlorophyll (a + b) 3.81 and 3.76 mg/cm² was obtained under 30 and 50% shade respectively with 100 kg N/ha (Figure 7).

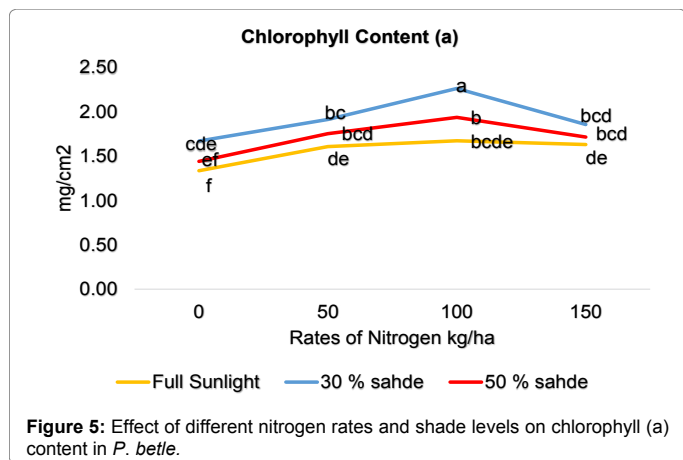


Figure 5: Effect of different nitrogen rates and shade levels on chlorophyll (a) content in *P. betle*.

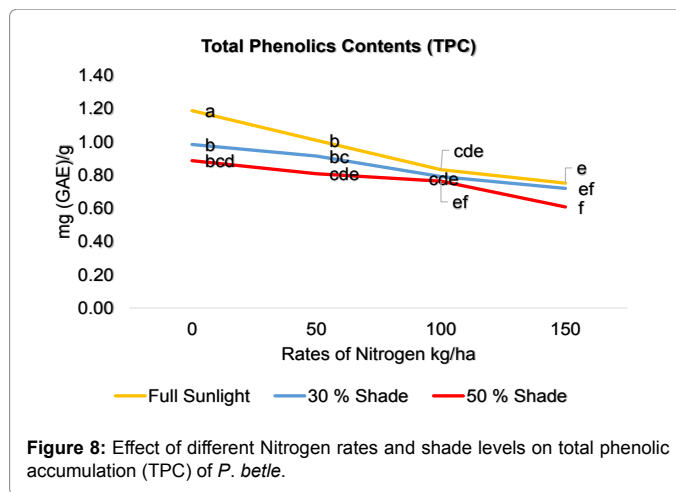


Figure 8: Effect of different Nitrogen rates and shade levels on total phenolic accumulation (TPC) of *P. betle*.

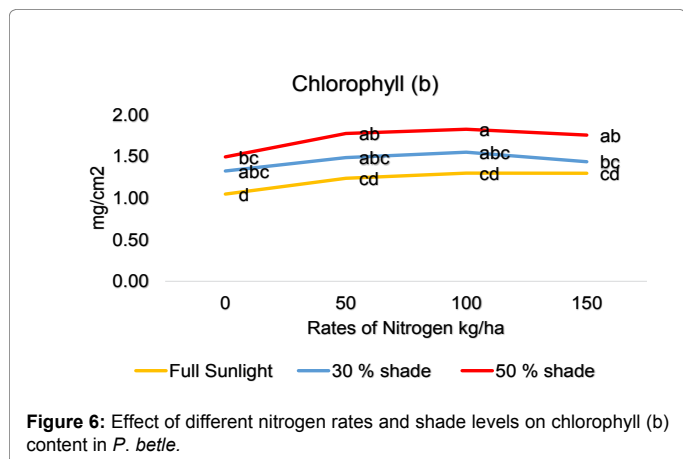


Figure 6: Effect of different nitrogen rates and shade levels on chlorophyll (b) content in *P. betle*.

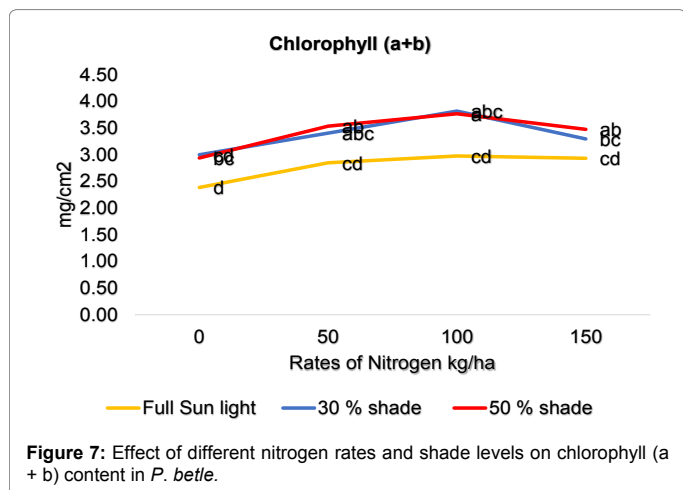


Figure 7: Effect of different nitrogen rates and shade levels on chlorophyll (a + b) content in *P. betle*.

The total phenolic accumulation (TPC) at different shade levels and nitrogen rates were observed significantly different ($P < 0.05$) (Figure 8). The highest TPC of 1.19 mg (GAE)/g was recorded under full sunlight. The lowest TPC accumulation of 0.61 ± 0.03 mg gallic acid equivalent (GAE)/g dry weight (DW) was recorded for plants grown under 50% shade with 150 kg N/ha. Meanwhile, no variation in results was recorded in 100 kg/ha N under all levels of shade. These findings clearly indicated that the light intensity and the fertilizers application particularly nitrogen appeared a negative effect on the concentration of TPC in *P. betle* leaves.

Total flavonoids contents (TFC) concentration of *P. betle* was affected by the different light intensities (Figure 9). The different light intensity and rates of nitrogen had a significant effect compared with other treatment, and the effect of TF content was similar as it was observed in TP content. The results displayed that the *P. betle* had the highest TF (128.13 mg Quercetin/g) under 0% shade at 0 kg N/ha. Meanwhile, the lowest result (56.80 mg Quercetin/g dry weight) was noted under 50% shade with the application of 150 kg/ha N. Flavonoids are the most readily-produced phenolics in the epidermal cells of plants exposed to high light intensity. They are antioxidants, and their production is considered as a response toward protecting the plant against oxidative damage. The present studies have shown an increase in flavonoid content of various plant species grown under high light conditions compared to those in the shade or low light.

1,1- Diphenyl-2-picryl-hydrazyl (DPPH) is one of the important plant components that reported as free radicals and is mostly used for examining the preliminary radical scavenging activity of a coupon or plant extracts. The results showed that 1,1- Diphenyl-2-picryl-hydrazyl (DPPH) under different shade levels and nitrogen rates had a negative effect, the result observed a highest (85.15%) under control treatment (full sunlight / 0 nitrogen rate). Meanwhile, the lowest results (22.00%) was found under 50% shade / 150 kg/ha of N. Therefore, increasing antioxidant activity (1,1- Diphenyl-2-picrylhydrazyl DPPH) in *P. betle* may be related to increasing and total phenolic content and total flavonoid content accumulation.

The results regarding Ferric Reducing Antioxidant Power (FRAP) through assay (Figures 10 and 11) showed that the ferric reducing antioxidant potential of *P. betle* increased with increasing the light intensity and nitrogen rates. A significant difference was observed under full sunlight treatment. According to the TF and TP results under this light intensity, synthesis of TF and TP increased. Therefore, increasing antioxidant activity in *P. betle* may be related to increasing TF and TP accumulation.

The correlation coefficient between photosynthetic rate (P_N) and stomata conductance (GS), transpiration rate (E), chlorophyll (a), chlorophyll (ab) positively was highly significant (Table 1). The relationship between (P_N) and chlorophyll b was positive and significant. Meanwhile, the relationship between photosynthetic rate (P_N) and total phenolics content (TPC), total flavanones content (TFC), DPPH, FRAP was negatively significant. The correlation coefficient between stomata conductance (GS) and transpiration rate (E), chlorophyll (a),

Correlations	P _N	G _s	E	Chl a	Chl b	Chl a+b	TPC	TFC	DPPH	FRAP
P _N	1	-	-	-	-	-	-	-	-	-
G _s	.921**	1	-	-	-	-	-	-	-	-
E	.907**	.957**	1	-	-	-	-	-	-	-
Chl a	.888**	.818**	.869**	1	-	-	-	-	-	-
Chl b	.518**	.623**	.595**	.424*	1	-	-	-	-	-
Chl a+b	.830**	.852**	.865**	.839*	.848**	1	-	-	-	-
TPC	-.615**	-.623**	-.629**	-.486**	-.636**	-.636**	1	-	-	-
TFC	-.624**	-.730**	-.720**	-.541**	-.779**	-.779**	.850**	1	-	-
DPPH	-.684**	-.687**	-.653**	-.561**	-.742**	-.742**	.896**	.833**	1	-
FRAP	-.662**	-.682**	-.701**	-.601**	-.680**	-.680**	.884**	.809**	.901**	1

** Correlation is significant at the 0.01 level (2-tailed).

* Correlation is significant at the 0.05 level (2-tailed).

P_N = Photosynthetic Rate, G_s = Stomata Conductance, E = Transpiration Rate, Chl a = Chlorophyll a, Chl b = Chlorophyll b, Chl a + b = Chlorophyll a + b, TPC = Total Phenolics Content, TFC = Total Flavanones Content, DPPH = 1,1-Diphenyl-2-picryl-hydrazyl, FRAP = Ferric Reducing Antioxidant Power.

Table 1: Correlation coefficient between measured physiological parameters.

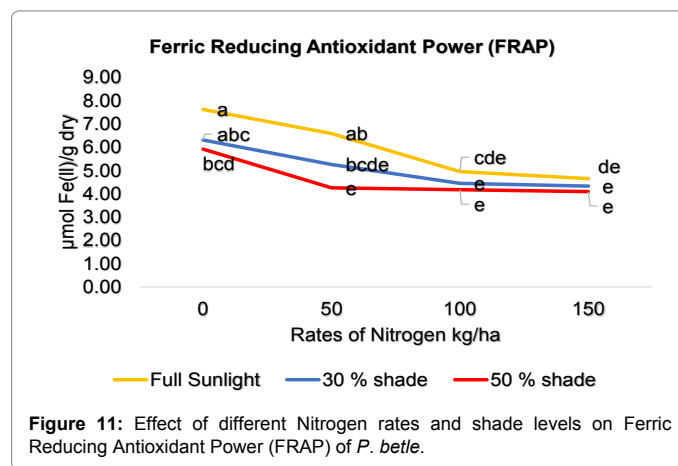
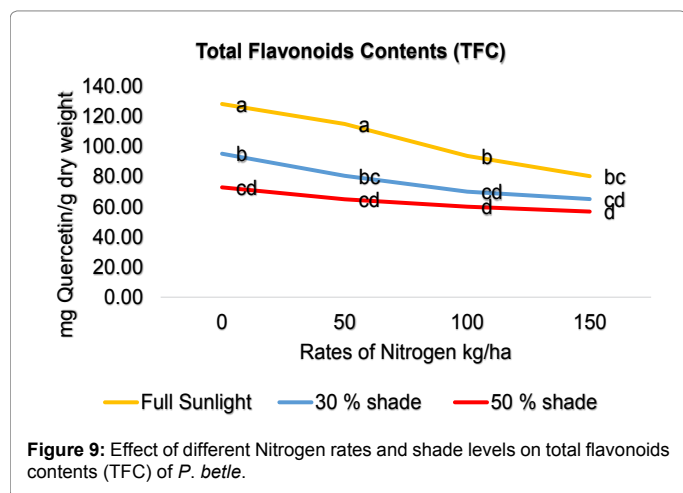


Figure 11: Effect of different Nitrogen rates and shade levels on Ferric Reducing Antioxidant Power (FRAP) of *P. betle*.

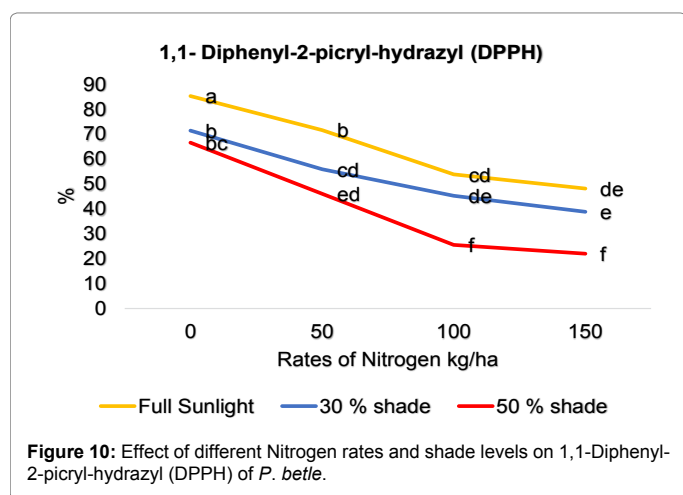


Figure 10: Effect of different Nitrogen rates and shade levels on 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) of *P. betle*.

chlorophyll (ab) was positively high significant and the relationship between stomata conductance (G_s), and chlorophyll (b) was positively high significant. Meanwhile, the correlation coefficient between stomata conductance (G_s) and total phenolics content (TPC), total flavanones content (TFC), DPPH, FRAP was negatively high significant. The correlation coefficient between transpiration rate (E) and chlorophyll a (Chl a), chlorophyll (Chl ab) was positively high significant, meanwhile the relationship between (E) and the relationship between chlorophyll

b, was positively high significant ($r = 0.595$, $P < 0.01$). In addition, the correlation coefficient between (E) and total phenolics content (TPC), total flavanones content (TFC), DPPH, FRAP was negatively high significant. The correlation coefficient between chlorophyll a (Chl a) and chlorophyll b (Chl b), chlorophyll was positively significant, meanwhile the relationship between (Chl a) and total phenolics content (TPC) was negatively significant, meanwhile the correlation coefficient between (Chl a) and total flavanones content (TFC), DPPH, FRAP was negatively significant. The correlation coefficient between chlorophyll b (Chl b) and chlorophyll ab (Chl ab) was positively high significant and very strongly. Meanwhile the relationship between (Chl b) and total phenolics content (TPC), total flavanones content (TFC), DPPH, FRAP was negatively significant. The correlation coefficient between chlorophyll ab (Chl ab) and total phenolics content (TPC), total flavanones content (TFC), DPPH, FRAP was negatively significant. The correlation coefficient between total phenolics content (TPC) and total flavanones content (TFC), DPPH, FRAP was positively significant. The relationship between total flavanones content (TFC) and DPPH, FRAP was positively significant. The last positively and the very strong relationship was between DPPH and FRAP.

Discussion

Rates of nitrogen and different shade levels had significant effects on chlorophyll content, photosynthetic capacity and secondary metabolite accumulation in *P. betle* plants. 30% shade with 100 kg/ha of N showed a better growth in *P. betle* in term of photosynthetic rate (P_N), stomata conductance (G_s), transpiration rate (E), chlorophyll

content. The increase in P_N under different shade levels has also been previously suggested in regard to its attribution to increase G_s and E [19,20].

Stomatal conductance is important in water and CO_2 exchange between plants and the atmosphere. An increase in the transpiration caused by light intensity increases the amount of water that further increase the photosynthesis in the leaves. Stomatal conductance and transpiration with increasing light intensity has previously been also reported in several plants including *Zingiber officinale* [9,21], *Orthosiphon stimaneus* [22] and *Tacca integrifolia* [23]. An application of 100 kg/ha N had the highest chlorophyll (a) under 30% shade that showed a similar trend as net photosynthesis rate in treated plants. The results further demonstrated that the plants balance light absorption for photosynthesis by regulating the chlorophyll synthesis and in many plants, different light intensity may effect on the physiological responses at the level of leaf and chloroplast [24]. The significant result of chlorophyll (b) showed under 50 % shade at 100 and 150 kg/ha N. The results were also in agreement with the previous findings from several reports on tropical trees and conifers [25,26]. High chlorophyll content (b) in the plant grown with 100 and 150 kg/ha N (1.83, 1.76 mg/cm²) was probably due to the release of nutrients and their beneficial effects on the soil in chemical fertilizer improvement. Compost and manure improved the physical, chemical and biological impact of soil and helped to overcome the loss of nutrients through processes. This was important in the production of healthy herbs which are to be used as natural remedies [27].

Even though the nitrogen is the most important mineral element in the process of chlorophyll biosynthesis, adding nitrogen to the soil can have negative as well as positive effects [28]. The lesser chlorophyll content in plants with 150 kg nitrogen application could be related to lesser photosynthetic electron rate (ETR) with the increase of nitrogen rate. Nitrogen concentration in green vegetation is related to chlorophyll content, and therefore indirectly to one of the basic plant physiological processes i.e. photosynthesis [26,29]. Photosynthetic rate (PN) was positively correlated with stomata conductance (GS), transpiration rate (E), chlorophyll (a). This implicated that the higher plant biomass production maybe due to higher photosynthetic rates when plants were grown 30% shade. Similar results were also reported by various researchers [30,31]. Light is one of the most extensively studied environmental factors in the phenolic metabolism [32]. Flavonoid and phenolic biosynthesis require light or enhanced by light. Flavonoid formation is absolutely light-dependent and its biosynthetic rate was related to light intensity and density [33]. The results indicated that total phenolic accumulation significantly affected by different treatments of shade 30 and 50%. The results showed that in *P. betle*, higher TPC was obtained under 0% shade compared with other shade 30% and 50%. Similar increased in TP with decreased shade was reported in two varieties of *Zingiber officinale* (cv. Halia bentong and Halia Bara; [21]. Total phenolic content in *P. betle* also increased but less when compared to other herbs, such as Calotropis, Hibiscus, Parthenium [34]. The result showed that there was a high relationship between TFC, DPPH, FRAP and total phenolic content TPC ($P \leq 0.01$).

Previous studies have indicated that the differences in the light levels were able to change the production of secondary metabolites [35]. This simultaneously can affect the medicinal compounds in this plant Gu et al. It seemed that the different light intensity had a direct effect on antioxidant activities in plants resulting in increased total phenolics and flavonoids content [36,37]. Increasing the shade from 0% to 50% resulted in an enhancement in phenolics such as

gallic acid and caffeic acid as well as flavonoid compounds such as quercetin, rutin, myricetin, kaempferol and naringin in the leaves of all three varieties of *L. pumila* Benth [11] Flavonoids are the most readily-produced phenolics in the epidermal cells of plants exposed to high light intensity. They are antioxidants and their production is considered as a response toward protecting the plant against oxidative damage. Studies have shown an increase in flavonoid content of various plant species grown under high light conditions compared to those in the shade. In hemlock, the concentration of various phenolics has been found to be lower in plants grown under shade than those found in full sun [38]. The result on the TFC of *P. betle* indicated that TF was considerably affected by the different shade levels and different shade with different nitrogen rates had significant effect on TFC. Similar trend of increasing TFC content *Labisia pumila* [36] increasing shade was observed in leads to an increase TFC. Decreasing shade level increases the primary photosynthate, which in phenolic concentration in the plant [39]. Light intensity provided plants with the energy required for photosynthesis and Carbon assimilation [40]. In addition, light intensity modulates several steps in phenolic metabolism, so that a higher phenolic content may be affiliated with exposure to high light intensities [41]. An elevated carbon to nitrogen (C/N) ratio is associated with an increased photosynthetic rate in plant tissue, which may be due to exposure to high light intensity [42]. Result showed the best levels of shade was under 30% for most morphological parameters, the concentration of various phenolics had been found lower in the plants grown under shade 30% and 50%, and the highest of it was found in full sunlight [38]. However, the different plant species are found to have different levels of sensitivity to light intensities. Bergquist et al. [43] demonstrated that the production of flavonoid concentration and composition in baby spinach under shade netting is more effective and acceptable. Most of the flavonoids are phenolic components and may be responsible for the antioxidant activity of many plants [44]. Similarly, the analyzed data showed a correlation between TFC and TPC and radical scavenging activity (DPPH) of *P. betle*. The 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) plants which grown under full sunlight and containing high accumulation of TF and TP was more than from plants which were grown under 30% and 50 % shade. The plants under shade were found with low levels of TF and TP, that phenolic and flavonoid compounds act as hydrogen donors in that reaction mixture and therefore, the formation of hydroperoxides were decreased [45]. The free radical scavenging of phenolic compounds was mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors and singlet oxygen quenchers [46]. Polyphenols, tannins and flavonoids are very valuable plant constituents possessing scavenging action due to their several phenolic hydroxyl groups [47]. It seemed that the role of TP in *P. betle* was very important for scavenging of free radicals [36], reported the antioxidant activity of *L. pumila* grown under different light intensity were affected which the best antioxidant activity accumulation was under high light intensity. In the present study leave extracts of *P. betle* showed a good potential of antioxidant activities. Measuring of the total capacity of herbs, including FRAP assay, which we have adopted in this study. Further, the ferric reduced the antioxidant potential of *P. betle* that increased with increasing shade. The FRAP values for the methanolic extracts of the *P. betle* was lower under 30% and 50% shade. The ferric reducing ability (FRAP assay) is widely used to evaluate the antioxidant component in dietary polyphenols [48]. The FRAP activity of *P. betle* was significantly enhanced with increasing the light level in 0% shade. According to the TF and TP results, under full sunlight, synthesis of TF increased. Therefore, increasing antioxidant activity in *P. betle* related to increasing TF and TP accumulation. These findings further

supported the idea positive relationship between phenolic compounds and flavonoids with antioxidant varieties of plants. However, antioxidant activity is found with phenolic compounds and especially flavonoids. Oktay et al. [48] reported strong positive relationships between total flavonoids contents and antioxidant activity, which appeared to be the trend in many plant species. Karimi et al. [36] found that higher activity with increasing total phenolics and flavonoids in all plant organs under 0% shade (sunlight) and without nitrogen (0 kg/ha N). Briskin et al. [49] also reported a significant and positive relationship between production of total phenolics and antioxidant activities in plants. It seems that different light intensities had direct effect on antioxidant activities in plant which resulted in increase total phenolics and flavonoids contents.

Conclusion

The significant variation in regard to photosynthetic rate, stomatal conductance, transpiration rate and chlorophyll (a) was observed as these plant increased under 30% shade with 100 kg/ha of nitrogen. Plants preferred some shade or semi-shaded environmental condition. Full sunlight 0 kg/ha of N was unsuitable for the crops as the high light intensity caused scorching on leaves, and stunted in growth, accumulation of total phenolic content (TPC), total flavonoids content (TFC) and antioxidant activity like 1,1-diphenyl-2-picryl-hydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) in the plants. *P. betle* had the highest accumulation of chlorophyll b and ab these increased under 50% shade with 100 and 150 kg/ha of nitrogen.

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