Ammonium-nitrogen Supply Induces Growth, Physiological and Secondary Metabolites Changes in *Centella asiatica* L. Urban

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Abstract

Background: Centella [*Centella asiatica* (L.) Urban], as a herb, is well known for its nutritional and pharmaceutical value. Nitrogen fertilizer plays a significant role in yield and quality of the herb. The present study was aimed to evaluate the effect of mineral nitrogen on the centella plant yield, total chlorophyll, total flavonoid content, antioxidant activities and C/N ratio.

Methods: The experiment was conducted under greenhouse conditions. Mineral nitrogen fertilizer ammonium nitrate (33% N) was applied with 30, 60, 90 and 120 kg N ha^{-1}, with no N (0 kg ha^{-1}) as the control. The dosage was split into three times application at 7, 14 and 21 days after transplanting. All plants were watered 10-days intervals with the nutrient solution.

Result: The results showed that the increasing doses of nitrogen improved growth rate and overall biomass of centella. Among nitrogen treatments, the 60 kg N ha^{-1} were found to yield maximum growth and the maximum values of leaf number, leaf area, rosette diameter and petiole length. The application of nitrogen (30-60 kg N ha^{-1}) increased the total chlorophyll and reduced total flavonoid content, antioxidant activity and the C:N ratio. The application of 60 kg N ha^{-1} of nitrogen should be considered as the optimal amount for reconciling limited yield loss and maintaining the quality of centella.

Key words: *Centella asiatica* L., Chlorophyll pigments, Nitrogen, Secondary metabolites.

Introduction

Centella asiatica (L.) Urban is an indigenous medicinal plant in Vietnam that is used in traditional medicine and cosmetics. As Centella is a leafy vegetable, it is consumed as a juice blend in many Asian countries. Currently, the genus Centella includes more than 50 species distributed in many tropical regions of the world such as Asia and some African countries (Prasad *et al*. 2019). Among medicinal properties, centella is recognized to have some outstanding properties such as anti-inflammatory, anti-oxidative and used to treat a number of diseases such as Alzheimer's diseases and varicose veins. The medicinal value of centella is determined by plant secondary metabolites such as terpenes, phenols, vitamins and flavonoids (Gohil *et al*. 2010).

Plant secondary metabolites are compounds that play an important role in plant adaptation to the environment, resistance to pests and diseases and are also an important material for the pharmaceutical industry (Azaizeh 2012). The synthesis of these compounds in plants is influenced by many environmental factors such as light, temperature, salinity and fertilizer (Kumar *et al*. 2020; Hoang *et al*. 2020). Among these factors, mineral elements affect the growth and development of plants and plant materials through their influence on primary metabolism and the production of secondary metabolites (Montoya-Garcia *et al*. 2018). Nitrogen is a major mineral element of primary metabolism with the role of stimulating plant growth and resulting in increased biomass (Xin *et al.* 2014). It is involved in the synthesis of organic compounds important for plant life such as amino acids, nucleic acids, lipids and enzymes (Pathak *et al*. 2008). The production of secondary metabolites is influenced by nitrogen supply and this effect is variable depending on the compound and plant species. Previous research has shown that the application of nitrogen reduces levels of phenolic compounds in *Chrysanthemum morifolium* Ramat. (Liu *et al.* 2010) and *Moringa oleifera* Lam. (Guillen-Roman *et al*. 2018).

Despite substantial numbers of studies of plant secondary metabolites in *Centella*, there are very few studies which discuss changes in the bioactive compounds after the application of nitrogen fertilizer. We hypothesized that applying nitrogen would increase plant growth, reduce the total flavonoid content and the antioxidant activity in *Centella*. Therefore, the present study was conducted to assess the effect of nitrogen supply on the growth and yield of *Centella*, the accumulation of flavonoid. Additionally, the study would assess changes in the total antioxidant activity of secondary metabolites and illuminate the metabolic mechanism of N control in photosynthetic C allocation.

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MATERIALS AND METHODS

Seeds of *Centella asiatica* L. were collected from *la nho* cultivar at the Quang Tho Commune (Thua Thien Hue province, Vietnam) in late September, 2019. The collected seeds were germinated in trays (40×20 cm) containing a mixture of garden soil and coconut fibers. At the 3rd leaf stage, 20 days after sowing, the seedlings were transplanted to plastic containers (150 × 30 × 15 cm) filled with 15 kg sandy soil. Ten seedlings at the 4th leaf stage were then planted 12 cm apart to a depth of 2 cm.

The experiment was conducted in a greenhouse in the Huong So commune in Thua Thien Hue Province, Vietnam (16°29′28.6″N 107°34′05.1″E) between October, 2019 and February 2020. The experimental design was a complete randomized design (CRD) with three containers which were prepared in three replications for each treatment. All plants were watered 10-days intervals with the nutrient solution (300 mg/L KH_2PO_4, 120 mg/L $CaSO_4$, 120 mg/L $MgSO_4$, 9.4 mg/L EDTA-Fe ,1 mg/L KCl). Ammonium nitrate (33% NH_4-N) was applied in the five rates: 0 (control) without supplemental N, 12, 24, 36 and 48 mg N kg^{-1} which corresponded to 0, 30, 60, 90 and 120 kg N ha^{-1}. Application of nitrogen fertilizer was done 3 times at 7, 12 and 17 days after transplanting. The plants were watered daily (ECi: 0.03 dS m^{-1}). The experiment was terminated at 35 days after transplanting and the plants were assayed for plant growth and phytochemical analysis.

Plant traits including the number of leaves, petiole length, leaf area and specific leaf area were recorded. The total leaf area was measured using the millimeter graph paper method (Pandley and Sign 2011). Specific leaf area was calculated as the ratio of leaf area to dry mass. After harvesting, the stem, leaves and root dry weight was determined after oven drying at 70°C for 60 h.

Colorimetric aluminum chloride method was used for flavonoid determination (Zhishen *et al*., 1999). The antioxidant activity was determined by DPPH radical scavenging assay following the procedure of Shimada *et al*. (1992).

Roots, stalks and leaves were separated and then dried (70°C) and ground. Then 0.5 mg of samples was warped up with tin can (2 × 5 mm) and the total carbon and nitrogen concentration were determined by combusting in an element analyzer (EA3000, Euro Vector, Italy).

For determination of the total chlorophyll content, 100 mg of leaves were ground in 8 mL 80% acetone (v/v) using a pre-chilled mortar and pestle. The extractant was filtered and the final volume was made up to 10 mL by adding diluted acetone. The absorbance of the extract was read at 663 and 645 nm on a spectrophotometer and the total chlorophyll content was calculated using the equation of Lichtenthaler (1987).

Analysis of variance (ANOVA) tests were performed using the Statistical Package for the Social Sciences (SPSS) software version 12. In addition, the F-test was applied to test significance and means were compared using the least significant difference (LSD) test at a 5% probability level.

RESULTS AND DISCUSSION

Plant growth and biomass

The growth and the yield of *C. asiatica* were significantly improved when nitrogen fertilizer was used (Table 1). An increase in the leaf number was observed in 30 and 60 kg N ha^{-1} treatment, ranging from 15.9% to 38.4%, whereas no significant differences were observed between the other N treatments. Maximum leaf area, rosette diameter and petiole length were observed in the 60 kg N ha^{-1} treatment sample, whereas the minimum values were observed for the control treatment. The highest specific leaf area was observed in the control and was lower for all the other treatments. Centella produced the highest dry matter and fresh yields for 60 kg N ha^{-1}, although they did not differ from the 90 and 120 kg N ha^{-1} treatments. The lowest herbal yields were obtained in the control.

The present study demonstrated that N application can increase growth parameters to a certain extent (60 kg N ha^{-1}) but has a negative effect at higher levels. The optimum nitrogen application can be differed depends on plants. Radušienë *et al*. (2019) reported that *Hypericum pruinatum* had maximum yield at 90 kg N ha^{-1} treatment whereas Dhaka *et al.* 2020 observed that it happened to *Cajanus cajan* at 40 kg N ha^{-1}. The maximum growth of *Centella asiatica* at 60 kg N ha^{-1} could be attributed to increased cytokinin production, which subsequently affected cell wall elasticity, number of meristematic cells and cell growth (Lawlor 2002). On other hand, reduced growth at higher N above than optimum might be related to toxicity. The NH^{4+} nutrition reduces uptake of many inorganic cations relative to anions even uptake of NH^{4+} itself increases to toxic levels that disturb intracellular pH which is associated with its toxicity (Kosegarten *et al*. 1997).

Table 1: Growth and yield responses of *Centella asiatica* L. at different nitrogen levels.

N application (kg ha^{-1})	Number of leaves	Leaf area (cm^2)	Rosette diameter (cm)	Specific leaf area (cm^2 g^{-1})	Petiole length (cm)	Fresh weight (g/plant)	Dry weight (g/plant)
0	19.5±3.1c*	23.4±2.6c	12.3±1.8c	324.2±21.2a	9.0±2.5c	72.1±18.0d	9.8±1.5d
30	22.6±2.3b	24.8±2.2b	14.5±2.0b	290.1±39.3b	11.3±3.1b	98.6+13.5c	13.4±2.0c
60	24.8±4.5a	30.5±3.5a	16.2±3.3a	230.9±27.9c	12.5±4.5a	120.3±14.3 a	16.3±2.5a
90	23.9±2.5a	29.5±4.1a	15.9±2.4a	216.7±35.5d	12.1±5.6a	120.0±13.4a	16.1±1.8a
120	24.1±2.2a	30.2±3.6a	15.7±2.8a	229.3±28.2d	11.8±5.4a	119.8±12.1a	15.8±4.1a

*Values with a common letter in the same column are not significantly different using LSD at 5% level.

Total chlorophyll content

The total chlorophyll contents in the leaves of *Centella* increased significantly with the 30 and 60 kg N ha^{-1} treatments. However, these contents were significantly similar between other N treatments.The lowest total chlorophyll content was observed in the control plants.

The chlorophyll molecule contains nitrogen, meaning that it is a factor in its biosynthesis. The increase in total chlorophyll content under low to high nitrogen supply (Fig 1) may be associated with an increase in stromal and thykaloid proteins in leaves (Filho *et al*. 2011) to promote the synthesis of chlorophyll pigment.

The C, N content and C/N ratio

The total C content in the leaf, stalk and root were relatively stable under the different N levels, while the total N content increased significantly as the N fertilization increased (Table 2). The highest N content (3.15%) was found in leaf at treatment 120 kg N ha^{-1}. The increase in N content had lead to reduced plant C/N ratio in leaf, stalk and root. The highest C/N ratio was observed in the leaf (19.94), root (37.13) and stalk (73.72) in the control treatment (without supplemental N). Treatment 120 kg N ha^{-1} reduced the the C:N ratio by 49% in the root, 57% in the stalk and 39% in the leaf.

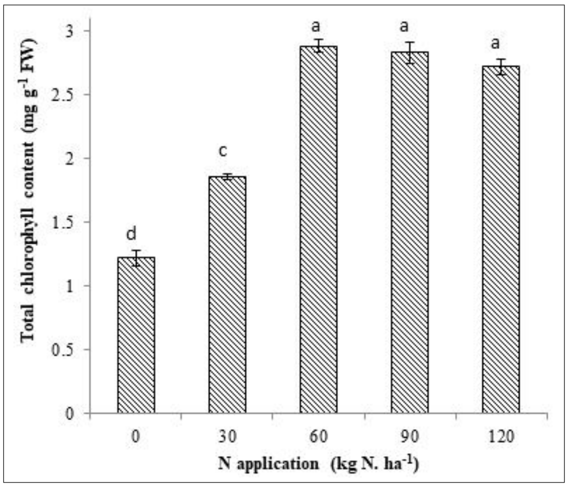

Fig 1: Effects of nitrogen levels on the total chlorophyll content (mg. g^{-1} FW) of *Centella asiatica*. Values with a common letter in the same column are not significantly different using LSD at 5% level.

According to Ibrahim *et al*. (2011), the C/N ratio had a significant positive relationship with total flavonoids and total phenolics compound signifying a good direct association between the C/N ratio and plant secondary metabolites. High N application leads to the reduction of total flavonoids and total phenolics content. Deng *et al*. (2019) also reported that when C/N ratio decreased with nitrogen application, a greater proportion of carbon were allocated to primary metabolisms of plant and less C was used to secondary metabolism.

Total flavonoid content and antioxidant activity

Total flavonoid content was significantly influenced by N levels (Fig 2a). The application of nitrogen reduced the accumulation of flavonoids and antioxidant activity with its lowest level found in 90 and 120 kg N ha^{-1} treatments (Fig 2a, 2b). The highest antioxidant activity was recorded in the control treatment, with up to 95% scavenging activity.

The highest flavonoids concentrations were found in the control treatment. Environmental factors such as soil nutrition, light intensity, temperature etc significantly influenced the synthesis and accumulation of secondary compounds in plants including flavonoids. Previous studies have shown the effect of fertilizers on the flavonoids content of plants (Arena *et al*. 2017; Ibrahim *et al*. 2010).

Nitrogen fertilization is thought to affect the levels of plant secondary metabolites. According to the carbon/nutrient balance hypothesis (Bryant *et al*. 1983), low nitrogen levels in the soil limit plant growth more than photosynthesis. Therefore, the excess carbon that is not used for growth will be allocated to the formation of secondary compounds. The growth differentiation balance hypothesis argues that a trade-off between growth and defense restricts primary metabolism, which induces secondary metabolism in response to stress (Herms and Watson 1992). The total phenolic content increased under nutrient deficiency and at the same time, an increased accumulation in free proline as cellular response to reactive oxygen species (ROS), followed by enhanced antioxidant activity (Lattanzio *et al*. 2009) supports this evident.

The biosynthesis pathways of plant secondary metabolites are different depending on their growing environments. Several studies can explain the effect of nitrogen on the trade-off between plant growth and the production of carbon-based secondary metabolites, which

Table 2: The carbon, nitrogen and carbon-to-nitrogen ratio (C/N) in the root, stalks and leaves of *Centella asiatica* L. at different nitrogen rate.

	Root (%)			Stalk (%)			Leaf (%)		
N application (kg ha^{-1})	Carbon	Nitrogen	C/N	Carbon	Nitrogen	C/N	Carbon	Nitrogen	C/N
0	38.2±2.8a	1.0±0.5c	37.1±2.4a	39.8±2.8a	0.5±0.1a	73.7±4.4a	40.1±3.5a	2.0±0.3c	19.94±2.1a
30	37.1±3.4a	1.5±0.1b	25.6±3.2b	39.9±3.2a	0.8±0.3ab	51.2±1.9b	41.2±2.1a	2.7±0.4b	15.27+2.5b
60	37.2 ±5.3a	1.6±0.7ab	22.8±2.5c	40.1±2.5a	1.0±0.2a	41.3±2.6c	40.3±3.2a	2.6±0.1b	15.24±2.7b
90	39.0±3.1a	2.1±0.9a	18.8±1.4d	39.7±3.1a	1.3±0.4a	30.3±3.2d	40.5±1.8a	2.8±0.6b	14.58±3.2b
120	38.6±3.5a	2.0±0.7a	19.0±2.7d	40.7±2.3a	1.2±0.3a	31.5+3.1d	38.2±3.1b	3.2±0.8a	12.12±3.7c

Note: Values with a common letter in the same column are not significantly different using LSD at 5% level.

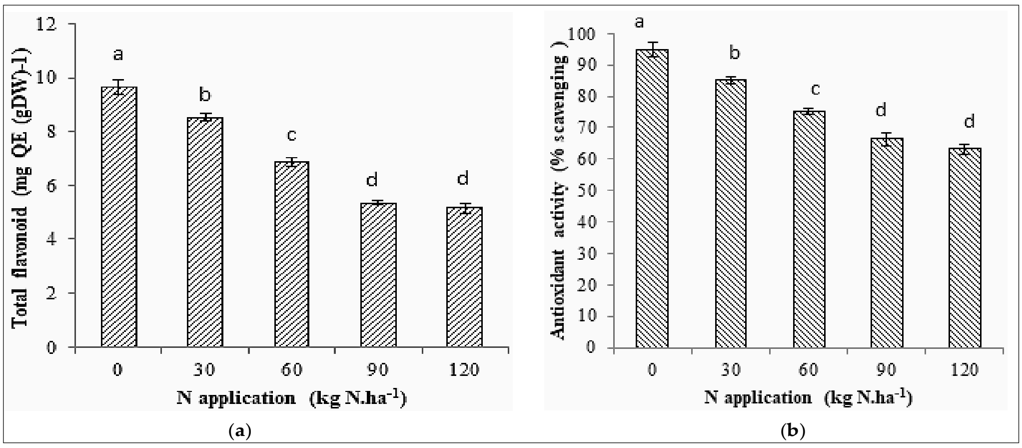

Fig 2: Effects of nitrogen levels on the total flavonoid content (a) and antioxidant activity (b) of *Centella asiatica*.
Values with a common letter in the same column are not significantly different using LSD at 5% level (QE: Quercetin; DW: Dry weight).

include changes in the partitioning of carbon skeletons between primary and secondary metabolism. Therefore, when plant biomass increases in response to the availability of nitrogen, concentrations of secondary metabolites decline due to increased carbon demand for primary metabolites associated with reduced carbon partitioning into secondary metabolites (Matsuki 1996; Mattson *et al*. 2005). This is also evident from increase in secondary metabolites contents in *C. asiatica* of present study at low N.

CONCLUSION

Nitrogen application up to 60 kg/ha increased growth and yield of *Centella asiatica* and is an important factor in secondary metabolism. Additional nitrogen adversely affected the total chlorophyll. The addition nitrogen at high levels reduced abundance of total flavonoid and antioxidant activity to support centella growth requirements. Nonetheless, application of 60 kg N ha^{-1} can be considered as the optimal for reconciling limited yield loss and maintaining sufficient quality of centella.

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Conflict of interest: None.

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