บทความวิจัย (Research Article)

Effects of different nitrogen forms on growth, phenolic content, and antioxidant activity in *Hedychium flavescens* and *H. stenopetalum* (Zingiberaceae)

Janjira Muenrew*

Queen Sirikit Botanic Garden, Mae Rim District, Chiang Mai Province 50180

*Corresponding to: jira_forever@hotmail.com

Naresuan Phayao J. 2020;13(2):18-26.

Received; 9 July 2020; Revised: 27 August 2020; Accepted: 27 August 2020

Abstract

Nitrogen (N) is the one of the most important nutrients needed by plants for their growth and the synthesis of various secondary metabolites. Plants typically obtain inorganic N from two different forms: NH_4^+ and NO_3^-. In this study, the effects of different nitrogen forms on plant growth, phenolic content, and antioxidant activity were investigated in two *Hedychium* species. They were grown in nutrient solutions modified from Smart and Barko with three different N forms: NH_4^+, $NH_4^+NO_3^-$, NO_3^- and control (no N form) for 60 days. Then plants were harvested and their morphology were compared. Total phenolic content (TPC) was evaluated by Folin-Ciocalteu method while the antioxidant activity was evaluated by DPPH and ABTS radical scavenging activity. Different nitrogen forms have significantly ($p \leq 0.05$) affected plant height, root numbers, and total biomass in two *Hedychium* species. *Hedychium stenopetalum* grown in NH_4^+ solution had highest height, root numbers, and total biomass. However, *H. flavescens* had highest height, root length, root number and total biomass in $NH_4^+NO_3^-$ supply. Total phenolic contents in both species were increased plant grown in NH_4^+ supply. The total phenolic content accumulated in the leaves of *H. flavescens* was 117.27 $mg.g^{-1}$ GAE which was higher than in *H. stenopetalum* (82.64 $mg.g^{-1}$GAE). However, the total phenolic content accumulated in the stem of the *H. stenopetalum* was higher than in *H. flavescens*. NH_4^+ supply increased antioxidant activity with DPPH radical scavenging activity and ABTS radical scavenging activity. The results indicated that NH_4^+ induced stress which enhanced phenolics accumulation.

Keywords: Phenolic content, antioxidant activity, nitrogen forms, *Hedychium* species, growth, morphology

Introduction

Hedychium flavescens and *H. stenopetalum* are perennial rhizomatous herb in the family Zingiberaceae. They were commonly called as “ginger lily” or “butterfly lily [1]. These plants were widely used for the cosmetic, and medicinal purpose. Moreover, these plants are rich in antioxidants [2].

Nitrogen (N) is arguably the most important plant nutrient because it influences both the primary and secondary metabolic pathways. Plants grow in nitrogen-poor condition would result in higher levels of secondary metabolites especially phenolic compounds in plant tissues. [3] The plant growing in nitrogen-poor condition is thought to contain more secondary metabolites compounds than plants growing in a nitrogen-rich environment.

Phenolic compounds in plants are mostly synthesized from phenylalanine; it is a common precursor of numerous phenolic compounds which include flavonoids, condensed tannins, lignin, and phenylpropanoid/benzenoid volatile etc. [4]. As the polyphenolic compounds effects positively on human health because of their antioxidative.

Plants typically obtain inorganic N in form of NH_4^+ and NO_3^- which were two lowest energy-consuming forms [5]. However, NH_4^+ nutrition has been partly associated with rhizosphere acidification [6]. The effects of rhizosphere acidification have provided a result of poor plant growth [7]. These, in turn, induced by increased poly-phenolic accumulation. Previous studies [8] have demonstrated that different rates of N application can influence phenolic compounds accumulate in the plant tissue. However, there is little research on the effects of different N forms. Hence, the objective of this study was to examine the effect of different nitrogen forms on growth, phenolic and antioxidant activity in two *Hedychium* species: *Hedychium flavescens* and *H. stenopetalum*).

Material and method

Experimental Design and Treatments

Living plants of *Hedychium flavescens* and *H. stenopetalum* were collected from Queen Sirikit Botanic Garden, Chiang Mai, Thailand. Whole plants were placed in shallow water until new rootlets and shoot grown out from rhizome. These new plants were grown on nutrient solutions modified from [9] for 30 day. Forty new plants were selected and recorded for fresh weight and height. All similar plants (N=3) were placed in black bucket containing 10 liter of nutrient solution. The experiment consisted of three N forms; NH_4^+, $NH_4^+NO_3^-$, NO_3^- and control (no N form) with the same concentration of nitrogen (500 µM). pH of solutions was adjusted to 6.5 ± 0.2 by using hydrochloric acid (HCl) and sodium hydroxide (NaOH). The nutrient solutions were changed every 5 days. The period of experiment was 60 days. During experiment period, the light regime was approximately 12 h light/12 h dark and the temperature range was 25-31 ºC: 18-21 ºC (day: night)

Figure 1 The new plants for experiment.

Harvesting and sample extracts

After 60 days, all plants were harvested and recorded for the number of new shoots, height, leaf number, root number, root length and rhizome length. Then, plants were separated into four parts; roots, stems, leaves and rhizomes; and stored in a freezer (temperature -50 °C). To acquire plant dry weight, plant samples were dried in the oven at 45–50 °C for 2–3 days, until the weight was stable and humidity was less than 5%. Then their weigh was measured. The dried samples of plant parts were cut into small pieces and weighed for 200 mg each. The samples were kept in zip lock polythene bags for father investigation.

The phenolic contents Analysis

The total phenolic content (TPC) was determined by Folin-Ciocalteu reagent method [10]. The reaction mixture consisted of adding 0.02 ml of sample and 0.1 ml of Folin-Cioclateu's phenol reagent in 96-well plate which was incubated at room temperature for a minute, followed by the addition of 0.08 ml of 20% (w/v) sodium carbonate (Na_2CO_3). The mixture was kept in the dark room for 30 min then the absorbance was measured at 765 nm. The total phenolic content was calculated from the calibration curve for gallic acid. All data are expressed as mg/g of gallic acid equivalents (GAE) in milligrams per gram (mg GAE/g) of dry extract.

Antioxidant analysis

The free radical scavenging from the extracts of different parts of the *Hedychium* spp. (leaves, stem and rhizome) were determined using DPPH and ABTS radical scavenging activity.

DPPH radical scavenging activity

DPPH radical scavenging activity was investigated according to methodology described in [11]. In brief, the samples were mixed with the stable DPPH radical in a methanol solution. This mixture consisted of 0.067 ml of sample and 0.133 ml of DPPH radical solution. Then, the mixture was shaken and incubated at room temperature for 30 minutes. The changes in color (from deep violet to light yellow) were measured from the absorbance at wavelength 515 nm by UV spectrophotometer. The level of remaining DPPH in the reaction medium was calculated using the following equation:

% radical scavenging = $[1- (A_{sample}/A_{control})] \times 100$ where A_{sample} = the absorbance of the sample solution + DPPH solution and $A_{control}$ is the absorbance of the control reaction. The concentration of solution and % radical scavenging obtained was used to plot graph to calculate the IC_{50}.

ABTS radical scavenging activity

The measurement of the ABTS radical scavenging activity was determined with a modified assay [12]. The preparation of ABTS stock solutions was prepared by allowing the ABTS solution to react with the potassium persulfate ($K_2S_2O_8$) solution (final concentration: 2.45 mM) for 16-18 h in the dark at room temperature. The preparation of working ABTS solution by allowing the ABTS stock solutions to diluted in ethanol absolute. After that, the absorbance was measured at 734 nm is a value between 0.7 - 0.9. The reaction mixture consisted of adding 0.0019 ml of sample, 0.0075 ml of Abs. ethanol and working ABTS 0.1906 ml. The mixture was shaken and incubated at room temperature for 5 minutes and absorbance was measured at 734 nm and then the calculated % inhibition and the TEAC (Trolox equivalent antioxidant capacity).

Statistical Analysis

All statistics were carried out by SPSS statistics, version 17.0. The data were analyzed by one-way analysis of variance (ANOVA) and Tukey's test as post hoc. A difference was considered statistically significant if $p \leq 0.05$.

Results

Effects of different N forms on growth and morphology

Overall, morphological characteristics of *H. flavescens* including height, leaf number, root length and number were significantly affected by different N forms. The height of *H. flavescens* was lowest when grown in NO_3^- solution. While, the root number and root length of plants grown in NH_4^+ and NO_3^- were higher than plant grown in other solutions. Leaf numbers of *H. flavescens* grown under NO_3^- and NH_4^+ NO_3^- were higher than other forms. However, the height and root number of *H. stenopetalum* were decrease when grown under nitrogen in the form of NO_3^- and $NH_4^+NO_3^-$. The leaf number showed a decrease with grown under nitrogen in the form of $NH_4^+NO_3^-$. However, new shoot and rhizome length of plants were not significantly affected by different N forms.

In overall, the total leaf, stem, root, old rhizome and new rhizome dry weight of the plants were significantly affected by the inorganic nitrogen forms. The total biomass of *H. flavescens* was highest when grown under nitrogen in the form of $NH_4^+NO_3^-$. While, the total biomass of *H. stenopetalum* was highest when grown under nitrogen in the form of NH_4^+. **Table 1**

Table 1 Means morphological parameters and total biomass of *H. flavescens* and *H.stenopetalum* under different inorganic nitrogen forms. Different letters above columns indicate significant differences between treatments.

	Nitrogen sources				*F-ratio*
	Control	NH_4^+	NH_4^+ NO_3^-	NO_3^-	
H. flavescens					
Morphology					
New shoot	2.00±0.00	4.67±1.20	6.00±2.00	2.67±1.33	1.87
Height (cm)	30.67±0.33^a	37.67±0.33^b	35.33±0.33^b	30.33±1.45^a	21.15***
Leaf number	3.00±0.00^a	3.33±0.33^a	6.67±0.33^b	7.00±0.00^b	26.20***
Root length (cm)	18.10±0.31^a	17.67±0.88^a	23.67±0.67^b	20.00±1.00^a	49.83***
Root number	15.33±0.67^a	27.67±1.45^b	83.67±2.73^d	36.00±1.00^c	325.04***
Rhizome length (cm)	6.33±1.20	6.67±0.67	7.33±1.20	6.33±0.67	0.24
	Total biomass (g)				
Total leaf dry weight	1.67±0.09^a	3.56±0.20^b	11.63±0.11^c	1.41±0.04^a	1487.43***
Total stem dry weight	2.75±0.00^{ab}	3.46±0.22^b	12.36±0.93^c	1.18±0.03^a	111.53***
Total root dry weight	1.57±0.23^a	1.59±0.19^a	7.50±0.14^b	1.26±0.09^a	316.23***
Total old rhizome dry weight	1.43±0.02^b	2.51±0.14^c	5.75±0.08^d	0.87±0.03^a	703.79***
Total new rhizome dry weight	1.38±0.04^a	3.56±0.23^a	17.11±0.99^b	3.29±0.08^a	203.46***
H. stenopetalum					
Morphology					
New shoot	2.67±0.33	3.33±1.33	2.67±0.33	3.00±0.58	0.18
Height	24.67±1.45^a	45±3.00^c	21.67±1.20^a	35±2.00^b	27.21***
Leaf number	7.67±0.67^{ab}	8.8±1.00^b	4.67±0.67^a	7.67±1.20^{ab}	23.10***
Root length	12.00±10.15^a	14.00±1.00^{ab}	18.67±1.45^b	11.00±0.58^a	4.03*
Root number	19.00±2.08^a	42.00±1.73^c	25.00±1.73^a	34.00±1.00^b	36.00***
Rhizome length	5.00±0.58	3.53±0.91	3.20±0.10	4.27±2.15	0.45

Total biomass (g)					
Leaves	10.68±0.43^{b}	35.30±0.28^{d}	6.42±0.19^{a}	19.19±1.51^{c}	252.47***
Stems	17.95±0.60^{b}	46.14±1.25^{d}	9.11±0.64^{a}	25.53±2.03^{c}	155.66***
Roots	3.04±0.24^{a}	11.93±0.15^{c}	2.26±0.13^{a}	6.82±1.25^{b}	47.07***
Old rhizome	20.02±0.45^{b}	35.23±1.64^{c}	23.75±0.19^{b}	12.63±6.81^{a}	74.96***
New rhizome	9.51±1.23^{a}	32.46±0.74^{d}	16.99±0.48^{b}	21.57±0.41^{c}	149.98***

*p<0.05, **p<0.01, ***p<0.001

Total phenolic contents

The total phenolic contents (TPC) of *H. flavescens* and *H. stenopetalum* were significantly affected by the different inorganic nitrogen forms. The level of TPC accumulation in leaves and stems were highest in NH_4^+ solution for both species. In *H. stenopetalum,* the level of TPC in NH_4^+ was 61% and 75% higher than the level in NO_3^- and NH_4^+ NO_3^- solution, respectively. While the TPC level in *H. flavescens,* was 60% and 44% higher than the level in NO_3^- and NH_4^+ NO_3^- solution, respectively.

In stem, similar trend occurred. Plants in NH_4^+ solution accumulated more TPC than other solutions. The level of TPC accumulated in *H. stenopetalum* grown in $NH4^+$ was 59% and 63% higher than plants which grown in NO_3^- and NH_4^+ NO_3^- solution, respectively. The increments were lower in *H. flavescens*, the level of TPC of plant grown in $NH4^+$ was 6 % and 15 % higher than plant grown in NO_3^- and NH_4^+ NO_3^- solution, respectively.

In contrast, the accumulation of TPC in root extract of *H. flavescens* treated with NH_4^+ form was comparatively low. However, the accumulation of TPC in root extract of *H. stenopetalum* was lowest in the plants treated by $NH_4^+NO_3^-$. Both old and new rhizome of *Hedychium flavescens* and old rhizome of *H. stenopetalum* treated with NO_3^- form also had low TPC. For new rhizome, the accumulation of TPC was lowest in plants treated with $NH_4^+NO_3^-$ form **(Figure 1).**

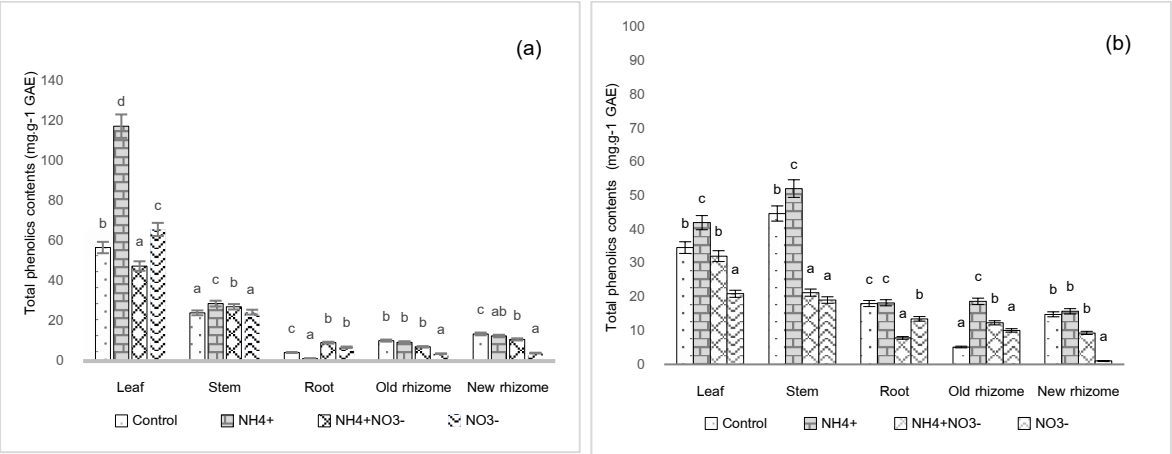

Figure 1 Effects of different nitrogen forms on total phenolic contents (TPC) in *H. flavescens* (a) and *H. stenopetalum* (b)(Different letters above columns indicate the significant differences at p<0.05).

Antioxidant activities

The antioxidant activities were evaluated by two method, ABTS and DPPH radical scavenging activity. The ABTS radical scavenging activity of *H. flavescens* and *H. stenopetalum* were significantly affected by the different inorganic nitrogen forms. The extracts of most different parts from both species which grown in NH_4^+ solution had the highest level of ABTS scavenging activity, comparing to other solutions (Fig. 2). However, the roots of *H. flavescens* which grown under NH_4^+ NO_3^- solution had the highest ABTS scavenging activity level (**Figure 2**)

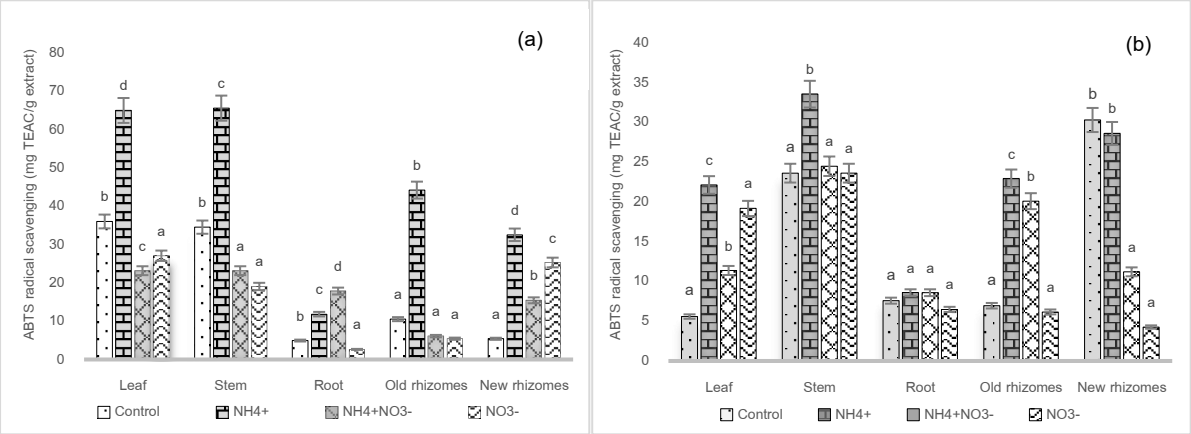

Figure 2. Effects of nitrogen forms on ABTS radical scavenging (mg TEAC/g extract) of *H. flavescens* (a) and *H. stenopetalum* (b) were grown under different inorganic nitrogen forms. Different letters above columns indicate significant differences between treatments.

DPPH radical scavenging activity of *H. flavescens* and *H. stenopetalum* were significantly affected by the inorganic nitrogen forms. The IC_{50} values of leaf extracts from the *H. flavescens* and *H. stenopetalum* were ranged from 0.76 - 1.12 $mg.ml^{-1}$ and 1.21-2.48 $mg.ml^{-1}$, respectively. NH_4^+ as sole N source had superior antioxidant DPPH scavenging activity indicated by lower IC_{50} value for leaves of *H. flavescens* and *H. stenopetalum* 0.76 $mg.ml^{-1}$ and 1.21 $mg.ml^{-1}$ respectively. Similarly, the old rhizome and new rhizome had the lower IC_{50} value 8.55 and 11.27 $mg.ml^{-1}$ for *H. flavescens* and IC_{50} value 8.33 and 8.51 for *H. stenopetalum* as shown in **Figure 3.**

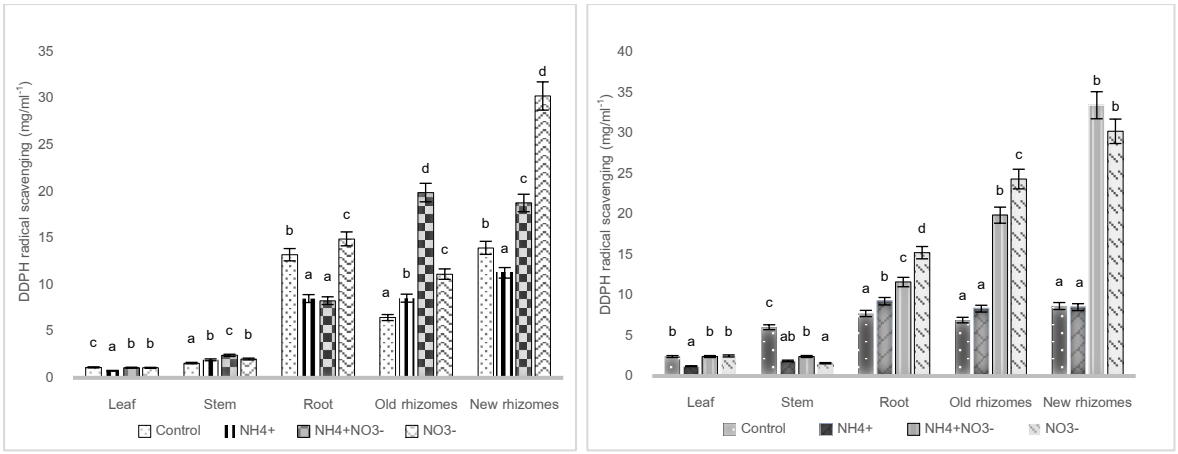

Figure 3. Effects of nitrogen forms on DPPH radical scavenging (mg/ml^{-1}) of *H. flavescens* (a) and *H. stenopetalum*, (b) were grown under different inorganic nitrogen forms. Different letters above columns indicate significant differences between treatments.

Discussion

The addition inorganic nitrogen could increase the height and root number and total biomass because plants could have more nutrient need for their growth. In this study, we found that NH_4^+ NO_3^- solution have increased the root number and root length of *H. flavescens* better than NH_4^+ and NO_3^- solutions. The reason is that plants could absorb nitrogen from either NO_3^- or NH_4^+ forms. Therefore, the plants in NH_4^+ NO_3^- solutions have higher biomass. In this study, the root length of *H. flavescens* were decreased when grown in NH_4^+ solution. Although the maximum concentration of the N in this experiment was 500 μM-N which was the concentration that plants start to be harmed by N toxicity such as reduced growth, leaf chlorosis, and root shortening. This could result in negative effects on nutrient uptake. The high NH_4^+ concentration could restrict root development resulting in shorten root length in many plant species such as *Hordeum vulgare* L. cv Klondike, *Oryza sativa* L., *Ipomoea aquatica* Forssk. [13-15] In this study, we found that NH_4^+ solution have increased height and root numbers of *H. stenopetalum* better than NH_4^+ NO_3^- and NO_3^- solutions. Therefore, the plants in NH_4^+solutions have higher biomass. In overall, other studies have found that many species prefer NH_4^+ rather than NO_3^- due to the lower energy requirement for NH_4^+ assimilation in the roots [16] resulted in more accumulation of NH_4^+ in root which caused the toxicity.

The phenolic contents of *H. flavescens* and *H. stenopetalum* were significantly affected by the different inorganic nitrogen forms. Plants in the sole NH_4^+ solution have highest level of TPC in leaves and stem extracts. The plant uptake with sole NH_4^+ source leads to acidification of rhizosphere which associated with poor plant growth [17]. On the other hand, plant defense mechanism to increased poly-phenolic accumulation. NH_4^+ nutrition stimulates the presence of high levels of polyamines which are also precursors for secondary metabolites. Increased carbon skeletons for NH_4^+ assimilation and hence increases polyphenol oxidase. Similarly, study showed increased phenolics under $NH4^+$ source in green basil. [18]

The level of ABTS and DDPH antioxidant activities from different N form solutions were similar to the TPC accumulation, plants supplied with NH_4^+ exhibited superior scavenging capacity

than other (NO_3^- and NH_4^+/ NO_3^-). It has been reported that phenolic compounds act as antioxidants shows that the leaf extract has antioxidant activity, which is related to the high phenolic compounds. This is in concurrent with [12] which show that the accumulation of phenolic compounds in plant resulting in a high antioxidant activity.

Reference

1. Rika Hartatia R, Sugandaa AG, Fidriannya I. Botanical, phytochemical and pharmacological properties of *Hedychium* (Zingiberaceae): A review. Procedia Chem. 2014: 3:150-63.
2. Suksathan R, Puangpradab R, Saratan N, Boonvun D. Bioactive compounds and antioxidant properties of four *Hedychium* flowers for flavoured tea. Acta Hortic. 2018:1194:1053-6.
3. Chen L, Liu QQ, Gai JY, Zhu YL, Yang LF, Wang C. Effects of nitrogen forms on the growth and polyamine contents in developing seeds of vegetable soybean. J Plant Nutr. 2011;2:(34):504–21.
4. Margna U. Control at the level of substrate supply - An alternative in the regulation of phenylpropanoid accumulation in plant cells. Phytochem. 1977; 16:419–26.
5. Tischner R. Nitrate uptake and reduction in higher and lower plants. Plant Cell Environ. 2000; 23:1005-1024.
6. Zha Y, Houqing Z, Qirong S, Guntur S. Interplay among NH4+ uptake, rhizosphere pH and plasma membrane H+- ATPase determine the release of BNIs in sorghum roots - possible mechanisms and underlying hypothesis. Plant Soil. 2012;358:131-41.
7. Xiaojuan W, Caixian T. The role of rhizosphere pH in regulating the rhizosphere priming effect and implications for the availability of soil-derived nitrogen to plants.2012. Ann Bot. 2012;121:143-51.
8. Munene R, Changamu E, Korir N, Onyango Joseph G. Effects of different nitrogen forms on growth, phenolics, flavonoids and antioxidant activity in amaranth species. Trop Plant Res. 2017;4(1):81-9.
9. Smart RM, Barko JW. Laboratory culture of submersed freshwater macrophytes on natural sediments. Aquat Bot. 1985;21:251-63.
10. Ronald LP, Xianli W, Karen S. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. J Agric Food Chem. 2005;53:(10):4290-302.

11. Brand-Williams W, Cuvelier ME, Berset C. Use of a free radical method to evaluate antioxidant activity. Food Sci Technol-Leb. 1995;28(1):25-30.
12. Schlesier K, Harwat M, Böhm V, Bitsch R. Assessment of antioxidant activity by using different in vitro methods. Free Radic Res. 2002;36(2):177-87.
13. Britto DT, Kronzucker HJ. NH_4^+ toxicity in higher plants: a critical review. Plant Physiol. 2002;159(6):567-84.
14. Chen G, Guo S, Kronzucker H, Shi W. Nitrogen use efficiency (NUE) in rice links to NH_4^+ toxicity and futile NH_4^+ cycling in roots. Plant Soil. 2013;369(1):351-63.
15. Chairuangsri S, Whangchai N, Jampeetong, A. responses of water spinach (*Ipomoea aquatic* Forssk.) on growth, morphology, uptake rate and nutrients allocation under high ammonium concentration. Chiang Mai J Sci. 2014;41(2):324-33.
16. Tischner R. Nitrate uptake and reduction in higher and lower plants. Plant Cell Environ. 2000;23:1005-24.
17. Ruan JY, Zhang F, Wong MH. Effect of nitrogen form and phosphorus source on the growth, nutrient uptake and rhizosphere soil property of *Camellia sinensis* L. Plant Soil. 2000;223(1):65-73.
18. Prinsi B, Negrin N, Morgutti S, Espen L.Nitrogen Starvation and Nitrate or Ammonium Availability Differently Affect Phenolic Composition in Green and Purple Basil. Agronomy. 2020;10:498.