Effects of Different Nitrogen Forms on Growth, Phenolic Content, and Antioxidant Activity in *Hedychium speciosum* and *H. coronarium*

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Abstract

Nitrogen (N) is the most important nutrient element for plant growth and the synthesis of many secondary metabolites. Plants typically obtain inorganic N in form of NH_4^+ and NO_3^-. Two *Hedychium* species were grown in nutrient solutions modified from Smart and Barko (1985). The experiment consists 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) for 60 days. Destructive sampling was done and morphology was recorded. 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, root number and total biomass between two *Hedychium* species. *H. speciosum* had height new shoot, leaf number, root number and total biomass in NH_4^+ supply. *H. coronarium* had height, leaf number, root number and total biomass in $NH_4^+NO_3^-$ supply. Total phenolic contents both species were increased by NH_4^+ supply. *H. speciosum* were accumulate total phenolic content in the leaves 21.55 mg.g^{-1} GAE was lower than in *H. coronarium* 43.49 mg.g^{-1}GAE, but in stem of the *H. speciosum* were accumulate total phenolic content 25.08 mg.g^{-1}GAE was lower than in *H. coronarium* 20.66 mg.g^{-1}GAE. NH_4^+ supply increased antioxidant activity with DPPH radical scavenging activity and ABTS radical scavenging activity.

Keywords: Phenolic content, Antioxidant activity, Nitrogen forms, *Hedychium* species, Growth, Morphology

Introduction

The genus *Hedychium*, commonly called "ginger lily" in the family Zingiberaceae. They were widely used for aromatic, cosmetic, and medicinal purpose (Hartatia, Sugandaa, & Fidriannya, 2014). Nitrogen is important macronutrient for plant growth. It is a component of amino acids, protein and nucleotide in plant structure. The deficiency of N can cause stunted growth, chlorosis and reduction of yield and quality (Minu, Masroor, & Khan, 2016). Plants grow in nitrogen-poor condition would result in higher levels of secondary metabolites especially phenolic compounds in plant tissues (Ibrahim, Jaafar, Rahmat, & Rahman, 2011). 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. (Saxena, Saxena, Nema, Singh, & Gupta, 2013; Kova, Klejdus, Backor, & Rep, 2007). The natural phenolic compounds have received considerable attention due to being potentially protective factors against cancer and heart diseases (Cartea, Francisco, Soengas, & Velasco, 2011).

Plants typically obtain inorganic N in form of NH_4^+ and NO_3^- (Britto & Kronzucker, 2013). These mineral are absorbed from water or soil through plant root cells as well as others minerals. Generally, most plants usually take up NH_4^+ more than NO_3^- because energy used for the NH_4^+ uptake is lower than for NO_3^- uptake (Tischner, 2000). NH_4^+ induced stress enhanced total flavonoids, phenolics and natural

antioxidants (Munene, Changamu, Korir, Onyango, & Joseph, 2017). NH_4^+ nutrition has been partly associated with rhizosphere acidification (Horchani, Aloui, Brouquisse, & Aschi-Smiti, 2010; Britto & Kronzucker 2002), Which associated with poor plant growth (Gweyi-Onyango, Neumann, & Roemheld, 2009). Previous studies (Munene et al., 2017; Salahas, Papasavvas, Giannakopoulos, Tselios, & Savvas, 2011; Ibrahim et al., 2011) have demonstrated that different rates of N application can influence phenolic compounds accumulate in the plant tissue. *Hedychium speciosum* and *H. coronarium* are a rich source of antioxidants (Suksathan, Puangpradab, Saratan, & Boonvun 2018). *Hedychium* is an aromatic rhizomatous plant which possesses important medicinal properties (Pachurekar & Dixit, 2017). The flowers are used in the treatment of fever, arthritis and eye disease (Jain, Singh, & Singh, 2003). Hence, the objective of this study was to examine the effect of different nitrogen forms on growth, phenolic and antioxidant activity in two *Hedychium speciosum* and *H. coronarium.*

Materials and methods

Preparation of plants material and growth conditions

Hedychium speciosum and H. coronarium were collected from Queen Sirikit Botanic garden is located in Mae Rim district of Chiang Mai, Thailand. Northern Thailand. All rhizomes were placed in shallow water until there were rootlets and new shoot generating from rhizome. All the new plants were grown on nutrient solutions modified from Smart and Barko (1985) for 30 day. Forty new plants were selected and recorded for fresh weight and height before experiment setup. 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). 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 70 days.

Harvesting and sample extracts

After 60 days, all plants were harvested and recorded for the number of new shoots, total height, leaf number, root number, root length and rhizome length. Then, the plants were separated into four parts; roots, stems, leaves and rhizomes; and stored in a freezer (temperature - 50 °C) before plant samples were oven dried at 45-50 °C for 2-3 days until the weight was constant and humidity was no more than 5 %. After that their dry weight was measured to obtain final dry weight. 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.

The phenolic contents Analysis

The total phenolic content (TPC) was determined by Folin-Ciocalteu reagent method (Esmaeili, Tavassoli, & Ebrahimzadeh, 2009; Nabavi, Ebrahimzadeh, Nabavi, Hamidinia, & Bekhradnia, 2008). 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 allowed to stand for a further 30 min in the dark and 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 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

The measurement of the DPPH radical scavenging activity was performed according to methodology described in Brand-Williams, Cuvelier, and Berset (1995). The samples were reacted with the stable DPPH radical in a methanol solution. The reaction mixture consisted of adding 0.067 ml of sample and 0.133 ml of DPPH radical solution. After that, the mixture was shaken and incubated at room temperature for 30 minutes. The changes in color (from deep violet to light yellow) were read from the absorbance at 515 nm by using a 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 an assay, modified from Schlesier, Harwat, Bö hm, and Bitsch (2002). 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 sample means were compared by Tukey's test. A difference was considered statistically significant if $p \leq 0.05$.

Results

Effects of different N forms on morphology and biomass productivity

In overall, new shoot, height, leaf number, root length, root number and total biomass of *H. speciosum* were significantly affected by different N forms. Unlike, rhizome length was not significantly affected by different N forms.

The height of *H. speciosum* tended to decrease with grown under nitrogen in the form of NH_4^+ NO_3^-. The leaf numbers tended to increases with grown under nitrogen in the form NH_4^+ was not significantly different from that grown under $NO_3^-$and NH_4^+ NO_3^-.

The new shoot tended to decrease with grown under nitrogen in the form NH_4^+ NO_3^-. While, the root number to increase with grown under nitrogen in the form of NH_4^+. The root length to increase with grown under nitrogen in the form of NO_3^-.

In overall, the total leaf, stem, root, old rhizome and new rhizome dry weight of the plants were significantly affected by different N forms. The total biomass of *H. speciosum* to increase with grown under nitrogen in the form of NH_4^+ as shown in Table 1 and Figure 1.

Table 1 Means, Standard error of morphology and total biomass of *H. speciosum* grown under different inorganic nitrogen forms

	Nitrogen sources				*F-ratio*
	NH_4^+	NO_3^-	NH_4^+ NO_3^-	Control	
Morphology					
New shoot	3.67± 0.33^b	2.00± 0.58^{ab}	1.00± 0.00^a	1.33± 0.33^a	10.13*
Height (cm)	24.33± 2.33^b	22.67± 1.76^b	13.00± 3.00^a	16.00± 0.58^{ab}	6.48*
Leaf number	6.67± 0.33^b	5.33± 0.88^{ab}	5.00± 2.52^{ab}	4.00± 0.00^a	6.76*
Root length (cm)	7.33± 0.33^a	16.00± 2.65^b	11.00± 0.58^{ab}	10.00± 0.58^{ab}	3.97*
Root number	49.33± 2.19^c	22.67± 1.76^b	24.67± 1.45^b	11.33± 0.33^a	101.33***
Rhizome length (cm)	4.00± 0.00	4.33± 0.33	4.67± 1.45	1.33± 0.67	3.49
Total biomass (g)					
Total leaf dry weight	2.57± 0.24^b	0.85± 0.13^a	0.34± 0.08^a	0.41± 0.05^a	53.11***
Total stem dry weight	3.61± 0.19^c	1.77± 0.06^b	0.27± 0.02^a	0.37± 0.08^a	208.58***
Total root dry weight	0.81± 0.08^b	0.43± 0.13^a	0.17± 0.04^a	0.12± 0.00^a	15.42*
Total old rhizome dry weight	0.85± 0.06^c	0.50± 0.07^b	0.15± 0.06^a	0.31± 0.01^{ab}	42.95***
Total new rhizome dry weight	1.83± 0.13^c	0.67± 0.04^b	0.23± 0.07^a	0.22± 0.01^a	99.13***

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

($NH_4^+NO_3^-$) (NH_4^+)

Figure 1 The morphological characteristics of *H. speciosum* grown under three nitrogen forms and control (NH_4^+, $NH_4^+NO_3^-$ and NO_3^-)

(NO_3^-) Control

Figure 1 (Cont.)

The height, leaf number and root number and total biomass of *H. coronarium* were significantly affected by different N forms. Unlike, new shoot, root length and rhizome length were not significantly affected by different N forms. The height, leaf number and root number of *H. coronarium* to increase with grown under nitrogen in the form of NH_4^+ NO_3^-. The leaf numbers tended to increase with grown under nitrogen in the form $NH_4^+NO_3^-$.

The total leaf, stem, root, old rhizome and new rhizome dry weight of the plants were significantly affected by different N forms. The total biomass including root number, the total leaf, stem, root dry weight, total old and new rhizome dry weight of *H. speciosum* to increase with grown under nitrogen in the form of NH_4^+ NO_3^- as shown in Table 2 and Figure 2.

Table 2 Means, Standard error of morphology and total biomass of *Hedychium coronarium* grown under different inorganic nitrogen forms

	Nitrogen sources				*F-ratio*
	NH_4^+	NO_3^-	NH_4^+ NO_3^-	Control	
Morphology					
New shoot	6.00± 1.33	2.67± 0.33	7.00±0.58	2.33± 0.33	0.27
Height (cm)	26.00± 3.00^b	22.70± 1.20^a	35.00±2.00^c	23.67± 1.45^a	27.21***
Leaf number	6.00± 1.00^b	6.7± 0.67^b	10.67±1.20^c	5± 0.67^{ab}	23.10***
Root length (cm)	6.00± 1.00^a	12.3± 0.58^b	12.67±1.45^b	7.00± 1.73^a	4.03
Root number	20.70± 1.73^b	13.00± 1.73^a	26.70±1.00^c	13.30± 2.08^a	36.00***
Rhizome length (cm)	3.30± 0.91	2.60± 0.10	3.7± 2.15	2.60± 0.58	0.45
Total biomass (g)					
Total leaf dry weight	2.41± 0.08^b	0.51± 0.03^a	2.56± 0.03^b	0.44± 0.11^a	269.39***
Total stem dry weight	2.43± 0.12^b	0.55± 0.05^a	2.67± 0.13^b	0.70± 0.09^a	125.04***
Total root dry weight	0.26± 0.02^a	0.17± 0.01^a	0.42± 0.03^b	0.23± 0.01^a	21.46***
Total old rhizome dry weight	1.56± 0.14^b	0.61± 0.05^a	3.68± 0.16^c	0.72± 0.03^a	162.40***
Total new rhizome dry weight	1.39± 0.04^b	0.37± 0.08^a	2.34± 0.01^c	0.18± 0.01^a	527.83***

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

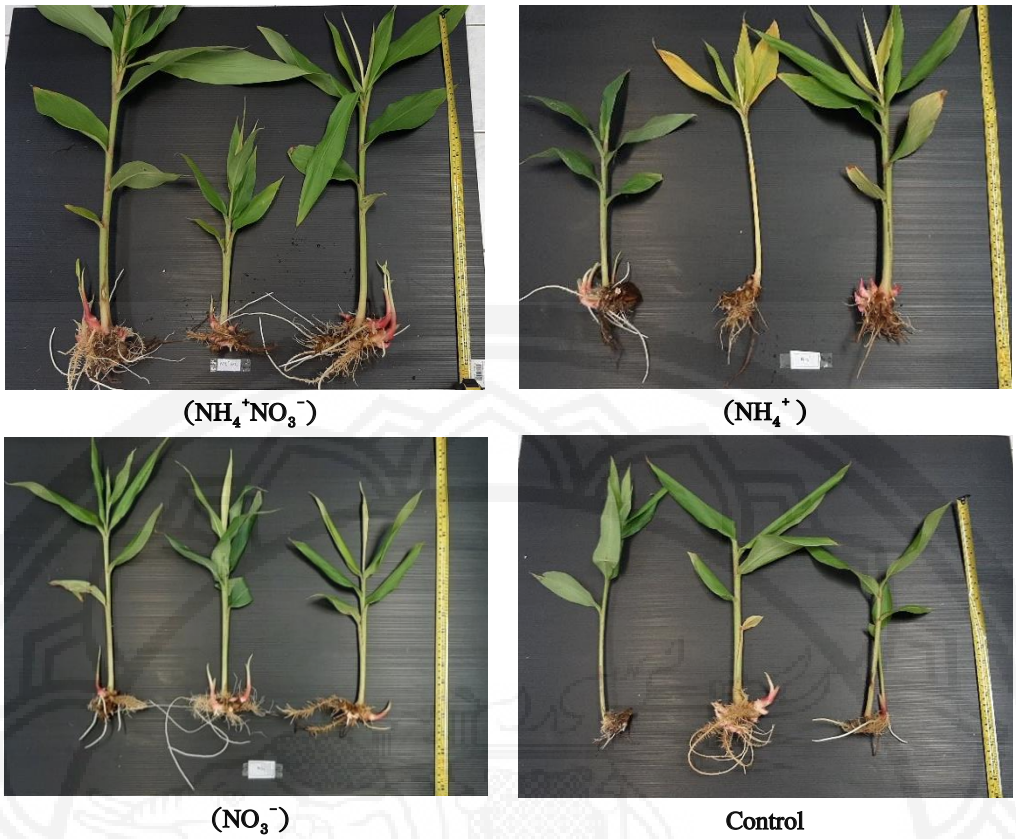

Figure 2 The morphological characteristics of *H. coronarium* grown under three nitrogen forms and control (NH_4^+, $NH_4^+NO_3^-$ and NO_3^-)

Total phenolic contents

The phenolic contents of *H. speciosum* and *H. coronarium* 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. speciosum*, the level of TPC in NH_4^+ was 53% and 63% 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 the highest level of TPC comparing to other solution, NH_4^+ had higher level of TPC by 6 % and 16 % comparing to NO_3^- and NH_4^+ NO_3^-, respectively. In root, the accumulation of TPC in root extract treated with NH_4^+ NO_3^- form had comparatively lower. Both old and new rhizome treated with $NH_4^+NO_3^-$ and NO_3^- form had comparatively lower TPC. In *H. coronarium*, the level of TPC in NH_4^+ was 15% higher than the level in NO_3^- solution. In stem, similar trend occurred. Plants in NH_4^+ solution accumulated the highest level of TPC comparing to other solution, NH_4^+ had higher level of TPC by 3 % and 5% comparing to NO_3^- and NH_4^+ NO_3^-, respectively. In root, the accumulation of TPC in root extract treated with NO_3^- form had comparatively lower, while old rhizome treated with NH_4^+ form had higher level of TPC as shown in Figure 3.

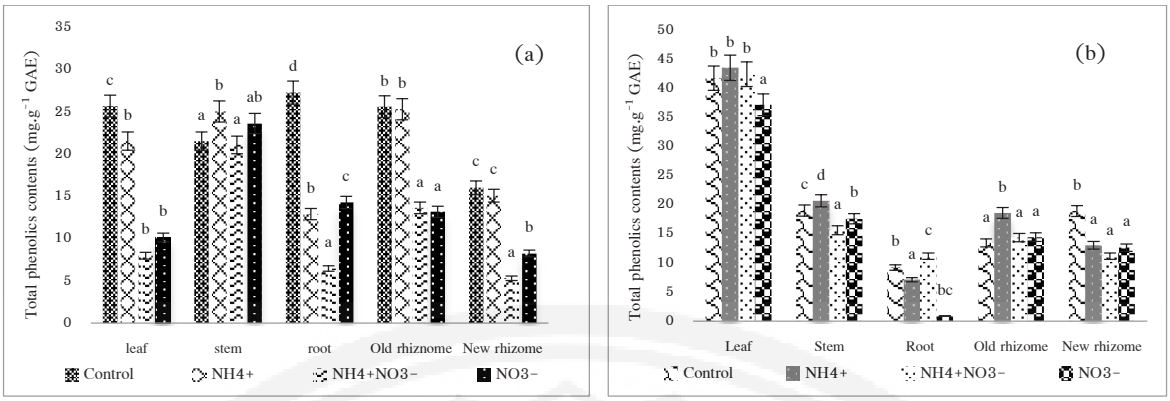

Figure 3 Effects of nitrogen forms on total phenolic contents of *H. speciosum* (a) and *H. coronarium* (b) were grown under different inorganic nitrogen forms

Antioxidant activities

The antioxidant activities were evaluated by two method, ABTS and DPPH radical scavenging activity. The ABTS radical scavenging activity of *H. speciosum* and *H. coronarium* 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. However, the roots of *H. speciosum* which grown under NH_4^+ solution had the lowest ABTS scavenging activity level. Also, the roots and old rhizome of *H. coronarium* which grown under NH_4^+ solution had the lowest ABTS scavenging activity level as shown in Figure 4.

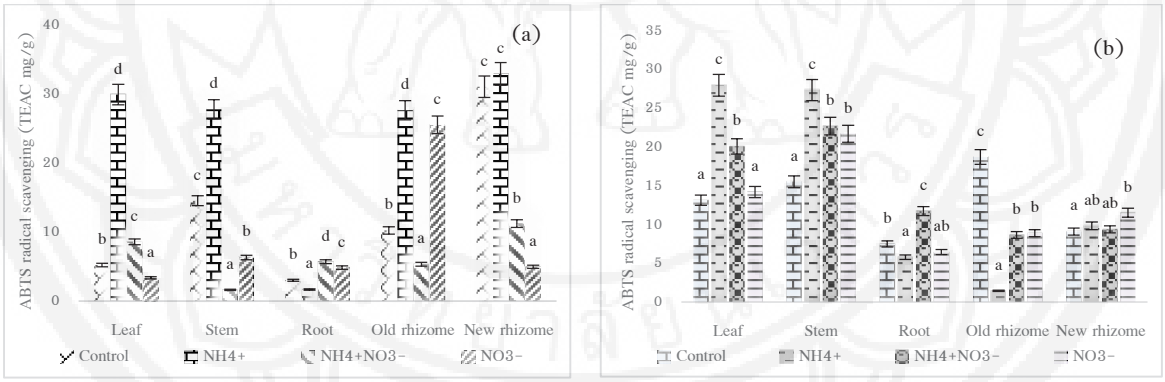

Figure 4 Effects of nitrogen forms on ABTS radical scavenging (mg TEAC/g extract) of *H. speciosum* (a) and *H. coronarium* (b) were grown under different inorganic nitrogen forms

DPPH radical scavenging activity of *H. speciosum* (a) and *H. coronarium* were significantly affected by the inorganic nitrogen forms.

The IC_{50} values of leaf extracts from the *H. speciosum* and *H. coronarium* were ranged from 1.35 – 2.25 $mg.ml^{-1}$ and 0.92–1.30 $mg.ml^{-1}$, respectively. NH_4^+ as sole N source had superior antioxidant DPPH scavenging activity indicated by lower IC_{50} value for leaves of *H. speciosum* and *H. coronarium* 1.35 $mg.ml^{-1}$ and 0.92 $mg.ml^{-1}$ respectively. Similarly, the old rhizome had the lower IC_{50} value 4.25 $mg.ml^{-1}$ for *H. speciosum* and *H. coronarium* as shown in Figure 5.

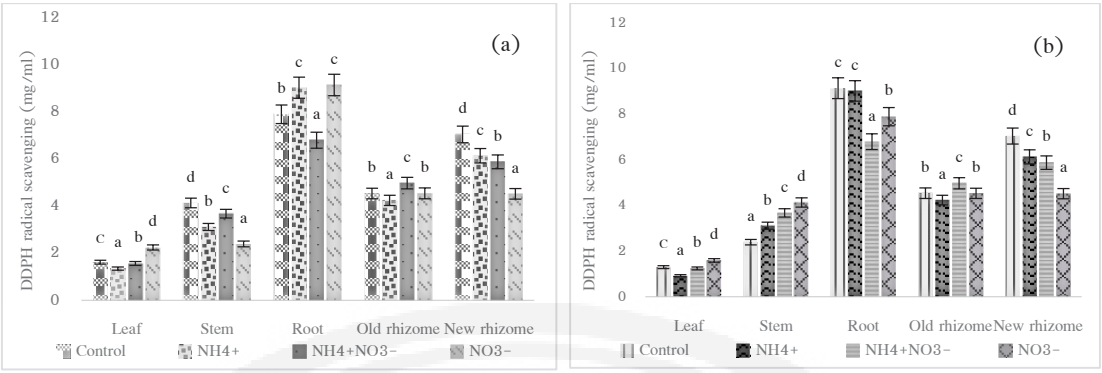

Figure 5 Effects of nitrogen forms on DPPH radical scavenging (mg/ml^{-1}) of *H. speciosum* (a) and *H. coronarium* (b) were grown under different inorganic nitrogen forms

Conclusion and Suggestion

The addition inorganic nitrogen could increase the height, root length and root number because plant uptake nutrient for plant growth (Razaq, Zhang, Shen, & Salahuddin, 2017). In this study, we found that NH_4^+ solution have increased the new shoot, leaf number, root number of *H. speciosum* better than NH_4^+ NO_3^- and NO_3^- solutions. The reason is that plants could absorb nitrogen from NH_4^+ forms. Therefore causing the plant to receive NH_4^+ have a high 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 (Tischner, 2000). In this study, we found that $NH_4^+NO_3^-$ solution have increased the height, leaf number, root number and total biomass of *H. coronarium* 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 causing the plant to receive NH_4^+ NO_3^- has a high biomass. In this study, the root length showed a decrease with grown under nitrogen in the form of NH_4^+. However, the maximum concentration of the N in this experiment was 500 μM-N which did not significantly affect the roots and toxicity symptoms such as reduced growth and leaf chlorosis.

The phenolic contents of *H. speciosum* and *H. coronarium* 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. This result was consistent to the results found in Amaranth species (Munene et al., 2017). The plant uptake with sole NH_4^+ source leads to acidification of rhizosphere which associated with poor plant growth (Sabir et al., 2013). On the other hand, plant defense mechanism to increased poly-phenolic accumulation (Caldwell et al., 2003). ABTS and DDPH antioxidant activities were similar to the TPC accumulation plants supplied with NH_4^+ exhibited superior scavenging capacity unlike other (NO_3^- and NH_4^+ NO_3^-). NH_4^+ as sole N source had superior antioxidant DPPH scavenging activity indicated by lower IC_{50} value for leaves of *H. speciosum* and *H. coronarium* 1.35 $mg.ml^{-1}$ and 0.92 $mg.ml^{-1}$ respectively. 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 results of other workers (Munene et al., 2017) which show that the accumulation of phenolic compounds in plant resulting in a high antioxidant activity.

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