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Research article

Effects of different nitrogen forms on growth, phenolics, flavonoids and antioxidant activity in amaranth species

Rozy Munene[1]*, Evans Changamu[2], Nicholas Korir[1] and Gweyi-Onyango Joseph[1]

[1]Department of Agricultural Science and Technology, Kenyatta University, Nairobi, Kenya
[2]Department of Chemistry, Kenyatta University, Nairobi, Kenya

*Corresponding Author: rozymunene@gmail.com
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Abstract: Higher plants, accumulate large number of polyphenolic compounds which are believed to act as defence compounds against different environmental constraints. Nitrogen (N) is a critical element for plant growth, absorbed as (NH_4^+) and (NO_3^-), which affects plant growth and to some extent contributes to secondary metabolites accumulation. Greenhouse experiment was carried out to determine the effect of N forms on growth and phytochemical accumulation in *Amaranthus* species. Two amaranth varieties; AB6 and AB7 constituted the main plot while three N forms; ammonium, nitrate, ammonium nitrate and control (no N form) represented the subplot. Destructive sampling was done and plant height was recorded. Folin-Ciocalteu's and aluminium trichloride methods were used to determine total phenolic content (TPC) and total flavonoid content (TFC) respectively. DPPH (diphenylpicrylhydrazyl) radical scavenging activity assay was used to obtain total antioxidant activity. Nitrogen forms significantly ($p \leq 0.05$) affected plant height between two amaranth varieties. Under nitrate treatment, AB7 exhibited greater height (40.2 cm) than AB6 (35.2 cm). Furthermore, N effect was more evident in AB6 variety, where by compared to the control, NO_3^- as exclusive N source enhanced shoot length by 64% in AB6 and 51% in AB7 which was twice that of the NH_4^+ -N treated plants. Sole NH_4^+ and no N form enhanced accumulation of both TFC and TPC, unlike nitrate and ammonium-nitrate mixture. Compared to NH_4^+ treatment, NO_3^- reduced TFC by 17.4% in AB6- variety and 14.7% in AB7 variety and TPC accumulation by 23% AB6 and 20% AB7 varieties respectively. Correspondingly, NH_4^+ - N form resulted to superior antioxidant DPPH scavenging activity indicated by high scavenging activity and lower IC_{50} value (concentration which scavenged 50% of the DPPH radicals). Plant height displayed a significant negative correlation with TFC and TPC accumulation of r= 0.75 and r= 0.81 respectively. The results indicated that ammonium-induced stress enhanced total flavonoids and phenolics accumulation; a salient phytochemical plasticity observed during plant growth and survival trade-off in a vegetable amaranth.

Keywords: Nitrogen forms - Ammonium- Nitrate - Phenolics - Flavonoids - Amaranth.

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INTRODUCTION

African indigenous vegetables, including leafy amaranth occupy a very important place in the human diet in African communities and they are believed to have some form of medicinal and therapeutic properties (Mavengahama 2013). They are not only excellent host of numerous vitamins and mineral elements (Kwenin *et al*. 2011, Habwe *et al*. 2009) but also reported to contain immense bioactive phytochemical compounds strongly associated with the health and remedial benefits (Amabye 2015). Higher plants portray a considerable metabolic plasticity by increased production and accumulation of myriad phytochemicals such as flavonoid and phenolic compounds, enabling them survive the various biotic and abiotic stresses (Nakabayashi & Saito 2015, Altiok 2010). As the polyphenolic compounds effects positively on human health because of their antioxidative and

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protective properties, it achieved more interest in the last decade. Several studies have shown that phytochemicals have antibacterial (Amabye 2015), anticancer (Mates *et al.* 2011); anti-aging attributes (Xiang *et al.* 2011). Observational epidemiology studies have indicated that significant dietary intake of flavonoids and phenolics are associated with a lower incidence of various cancers (Collins 2005).

Both the primary and secondary metabolism of higher plants is influenced by mineral nutrition (Caretto *et al.* 2015). Species growing in nutrient-poor habitats often have traits that lead to high nutrient retention and high levels of secondary metabolites (Lillo *et al.* 2008). Deficiency in mineral elements such phosphorous and potassium have been reported to up-regulate the amounts of polyphenols either as existing pools or by inducing their *de novo* synthesis (Kováčik *et al.* 2007, Glynn *et al.* 2008). Nakabayashi *et al.* (2014) observed that increased amount of flavonoids as a consequence of phosphorous and water limitation. Drought often causes oxidative stress (Akula & Ravishankar 2011) and was reported to show increase in the amounts of flavonoids and phenolic acids in *Solanum species* and *Ligustrum vulgare* (Okello 2015, Tattin *et al.* 2004). Phosphorous stress was reported to elevate total phenolic and antioxidant activity in black nightshade (Ogembo 2015).

Nitrogen is a one of the most critical elements affecting plant growth and development (Zhou *et al.* 2011). Unlike other elements nitrogen is metabolized by plants in two ionic forms NH_4^+ andNO_3^- (Olfati *et al.* 2012) which not only affects plant growth but also nutritional quality of higher plants (Sun *et al*. 2014, Sabir *et al.* 2013). Nitrogen nutrition is of great importance as it influences both the primary and secondary metabolic pathways thus secondary plant metabolites accumulation (Chen *et al.* 2011). Deficiency of crucial elements for instance nitrogen has been found to enhance accumulation of phenolics compounds in the plant tissues (Ibrahim *et al.* 2011). Previous studies (Argyropoulou *et al.* 2015, Salahas *et al.* 2011) have demonstrated that different rates of N application can influence phytochemical buildup in plant tissues; however very little has been done on effects of different N forms. Therefore the aim of the present study was to evaluate the effects of N forms on growth and total flavonoids and phenolic concentration in leafy amaranth. The information is relevant in N forms management for optimum derivation of these vital therapeutic components.

MATERIALS AND METHODS

Experimental Design and Treatments

The experiments were laid out in a split plot arrangement on a Randomized Complete Block Design (RBCD) replicated three times. The main plot comprised of two vegetable amaranth varieties *i.e.* Abukusa 6 (AB6) and Abukusa 7 (AB7) while three N-forms (NO_3^-, NH_4^+ and NH_4NO_3) and control (no N form used) constituted the sub-plots. Sole NH_4^+ and NH_4NO_3 were stabilized with padin® as the nitrification inhibitor composed of a mixture of dicyandiamide and 3, 4 methylpyrazole phosphate.

Agronomic practices

Amaranth seeds were obtained from Jomo Kenyatta University of Agriculture and Technology (JKUAT). The seeds were directly sown 2 kg containers and about two grams of Triple superphosphate (TSP) was used as basal fertilizer. Watering of the amaranth plants was done daily.

Harvesting and Preparation of plant samples

Harvesting of the plant samples was done by uprooting the whole plant. The shoots and roots were separated and shoot height recorded in centimeter (cm). Plant shoots (stems and leaves) were oven dried at 60–65 ºC for 72 hours until the weight was constant and dry weight recorded. The dried plant samples were groundusing a grinder-MIKA® to fine powder (0.2 mm) which was kept in zip lock polythene bags, appropriately labeled and stored in the dark awaiting phytochemical analysis.

Extraction of plant material

About 20 g of the powdered plant material was weighed, placed in a flask, 100 ml methanol AR was added and allowed to stand for 48–72 hours. It was then filtered through Whatman filter paper No. 1 and distilled using rotary evaporatorat 65°C until methanol-free paste was obtained (Mibei *et al*. 2012). The resulting extracts were properly labeled and preserved at 5°C in airtight plastic bottles for further use.

Total flavonoids contents analysis

The $AlCl_3$ method (Mervat *et al.* 2009) was used for the determination of total flavonoid content (TFC) of the sample extracts. Portions of 1.5 ml of 1:10 $g.ml^{-1}$ extracts were added to equal volumes of a solution of 2% AlCl3 $6H_2O$. The mixture was vigorously shaken and allowed to stand between 10–15 min. and absorbance read

with a spectrophotometer (Spectro SC labmed inc.) at 425 nm. Flavonoid contents were expressed as mg catchin equivalent (mgCE.g^{-1}) dry weight.

Total phenolic contents Analysis

Total phenolics content (TPC) was determined by Folin Ciocalteu reagent (Esmaeili *et al*. 2009, Nabavi *et al*. 2008). Dilute solution of amaranthextracts (0.5 ml of 1:10 g.ml^{-1}) or gallic acid (standard phenolic compound) was mixed with Folin Ciocalteu reagent (5 ml, 1:10 diluted with distilled water) and aqueous 5% Na_2CO_3 (4 ml). The mixture was allowed to stand for 15 minutes and absorbance determined at 765 nm with a spectrophotometer (Spectro SC labmed Inc.). The standard curve was prepared by 0, 50, 100, 150, 200, and 250 mg.ml.l^{-1} solutions of gallic acid to determine the total phenolic concentrations. Total phenolic content values are expressed in terms of gallic acid equivalent (mgGAE.g^{-1}) of dry weight.

Anti-oxidant analysis

Diphenylpicrylhydrazyl (DPPH) was used to determine radical-scavenging capacity of samples according to (Mibei *et al.* 2012). Different concentrations 0.05, 0.1, 0.5, 1.0, 2.0 and 5 mg.ml^{-1} of the extracts were prepared, in methanol. One ml of the extract was placed in a test tube, 3ml of methanol added followed by 0.5 ml of 1 mM DPPH in methanol. This was shaken vigorously and left to stand for about five minutes. Concentrations of ascorbic acid were prepared at the same concentrations as the extract and used as standard. Blank solution was prepared with same amount of DPPH and methanol. Absorbance of the solutions was obtained at 517 nm with a spectrophotometer (Spectro SC labmed Inc.). Radical scavenging capacity of the samples determined using formula:

$$\% \text{ inhibition} = \frac{\text{Ab} - \text{Aa}}{\text{Ab}} \times 100$$

Where, Ab = absorption of the blank sample; Aa = absorption of the extract.

Data analysis

Data was subjected to analysis of variance (ANOVA) at 95% confidence level using SAS- computer software (SAS 2015, Version 9.0). Where significant, mean separation was obtained by LSD. Data was presented inform of tables and graphs.

RESULTS

Plant height

Nitrogen forms significantly (P≤ 0.05) affected shoot height in the greenhouse experiment (Fig. 1). Data showed that ammonium depressed plant growth (height) for both varieties compared to nitrate as a sole N source. Variety AB7 had greater height (40.2 cm) than AB6 (35.2 cm) under nitrate treatment. Compared to the control, provision of nitrate as sole source of N enhanced shoot length by 64% in AB6 and 51% in AB7 which was twice that of the ammonium treated amaranth plants.

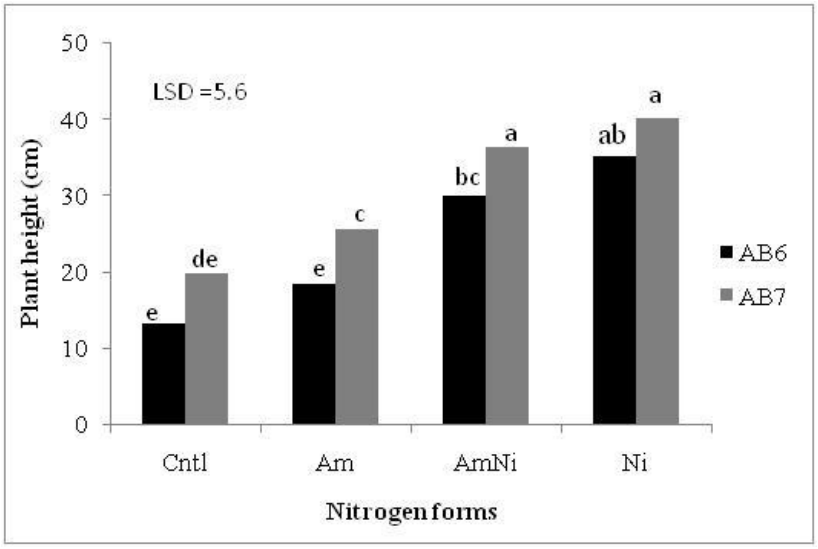

Figure 1. Effects of nitrogen forms on amaranth plant height.

This plant height was same for both accessions when no N was supplied but once ammonium was supplied, AB7's performance was statistically superior as compared to control plants. Nitrate treatment led to 2 folds increase in height when compared to control for AB7 variety while the increase was almost 4 folds for AB6. The accession AB7 grows better than AB6 but what is significant is that responds better to N in nitrate and ammonium-nitrate N source.

Total flavonoids and phenolics concentration

Nitrogen forms had a significant ($P \leq 0.05$) effect on the total shoot flavonoids and phenolics content. The results revealed a stimulatory effect of sole ammonium on shoot TFC and TPC accumulation in amaranth plants. Compared to nitrate, ammonium increased TFC by 17.4% in AB6 and 14.7% in AB7. Ammonium increased TPC by 23% in AB6 and 20% in AB7 as opposed to when nitrate was added as N source. Amaranth plants not treated with N form (control) had comparatively higher TFC to ammonium form while accumulation of TPC in the plants treated with ammonium form was statistically at par with the control for AB6 variety (Table 1).

Table 1. Effects of nitrogen forms on total phenolic content (TPC) and total flavonoid content (TFC) accumulation.

Treatments		**Total flavonoids Contents (mg.g^{-1} GAE)**	**Total phenolics contents (mg.g^{-1} GAE)**
AB6	**Cntl**	28.2^{b}	72.6^{b}
	Am	25.2^{d}	75.5^{b}
	AmNi	23.1^{e}	61.4^{d}
	Ni	20.8^{f}	58.1^{e}
AB7	**Cntl**	30.6^{a}	74.7^{b}
	Am	26.7^{c}	79.9^{a}
	AmNi	23.0^{e}	64.2^{d}
	Ni	22.8^{e}	63.9^{d}
P value		0.001	0.001
LSD		1.1	3.0
N x V		*	NS

Interesting, AB7 was still superior to AB6 variety (Fig. 1 and Table 1). Though the ammonium treatment elicited greater accumulation of TFC and TPC, AB7 was superior. Naturally it would be expected that production of these phytochemicals incur some energy costs and hence the variety accumulating less should grow slowly (shorter).

Antioxidant DPPH Radical-Scavenging Activity

The IC_{50} (concentration which scavenged 50% of the DPPH radicals) values of the amaranth extracts ranged from 0.06 mg.ml^{-1} to 1.80 mg.ml^{-1}. Ammonium as sole N source had superior antioxidant DPPH scavenging activity indicated by lower IC_{50} value for AB7 (0.06 mg.ml^{-1} and AB6 (0.3 mg.ml^{-1}) compared to nitrate treatment for AB7 (0.7 mg.ml^{-1}) and AB6 (1.8 mg.ml^{-1}) (Table 2); an indication of ammonium treated plants to possess great antioxidative capacity as compared to nitrateand ammonium nitrate mixture which require a bit bigger volumes to scavenge 50% of DPPH radicals.

Table 2. IC_{50} and maximum percentage inhibition values for the 2 amaranth extracts.

Treatments		**IC_{50} (mg.ml^{-1})**	**Max Inhibition (%)**	**Concentration (mg.ml^{-1})**
AB6	Cntl	0.40	82.0	2
	Am	0.30	72.8	2
	AmNi	1.00	60.9	5
	Ni	1.80	65.0	5
AB7	Cntl	0.09	75.0	2
	Am	0.06	86.1	1
	AmNi	0.60	70.1	2
	Ni	0.70	64.3	5
Ascorbic acid		0.07	89.5	5

In addition ammonium form extract revealed a remarkable maximum inhibition percentage 72.8% for AB6 and 86.1% for AB7 at a lower concentration of 2 mg.ml^{-1} unlike nitrate (65% for AB6 and 64.3% for AB7) and ammonium nitrate mixture (60.9% for AB6 and 70.1%) which with maximum inhibition of 5 mg.ml^{-1} which was 2.5 folds higher than that of ammonium. Control had equally superior antioxidant capacity as ammonium

(Table 2). Maximum inhibition of ascorbic acid (89.5%) was higher than that of amaranth extract, however at a higher concentration (5 $mg.ml^{-1}$) than that of ammonium treated amaranths and the control and indication of notable stimulatory antioxidative effect of ammonium form.

Relationshipbetween plant growth and phytochemical accumulation

Enhancement of TFC and TPC accumulation was observed under reduced plant shoot growth (height) exhibited by negative correlation coefficient (r= 0.75 and r= 0.81) respectively (Table 3).

Table 3. Pearson correlations coefficient between measured plant growth parameters and phytochemical accumulation and antioxidant DPPH scavenging activity.

	1	2	3	4	5	6
Shoot fresh weight	1					
Root fresh weight	0.75**	1				
Shoot height	0.91*	0.86*	1			
TFC	-0.75**	-0.66*	-0.75*	1		
TPC	-0.85*	-0.64*	-0.81**	0.68**	1	
Ant. activity	-0.65*	-0.60*	-0.69*	0.63*	0.78**	1

Note: * and ** significant at $P \leq 0.05$ or $P \leq 0.01$ respectively. TFC- Total flavonoids compounds, TPC-Total phenolics compounds, Anti= Antioxidant.

In this study polyphenolics (TFC and TPC) concentration in vegetable amaranth related negatively with plant growth (shoot and root fresh weight) with correlation coefficient r = -0.75 and -0.85 respectively. As expected antioxidative activity of the amaranth extracts had a significant positive relationship with TFC and TPC which implies a higher possibility that DPPH antioxidative capacity is as a result of higher accumulation of flavonoids and phenolics.

DISCUSSION

Plant height

Depression in shoot height agrees with previous work by Borgognone *et al.* (2013) who observed that plant height was sharply decreased when NH_4^+ was the dominant N source. The restriction in shoot height for the plants supplied with sole ammonium compared to sole nitrate and mixed form (ammonium nitrate) may probably be related to decline in cell number and cell size, which is manifested by impaired root- to- shoot translocation of cytokinins as indicated by (Walch-Liu *et al.* 2000) on tobacco. Working with tomatoes Rahayu *et al.* (2005) reported superiority among nitrate treated plants as compared to ammonium treated plants which were even inferior to ammonium-nitrate-N plants. Nitrate N was associated with induction of phytohormonal cascade transduction from root to shoot, leading to expansive leaf growth.

This is in concurrence with findings of Lobit *et al.* (2006), who found that a 49% reduction in the shoot length under dominant NH_4^+ nutrition. These results were also in agreement with the findings of Gweyi-Onyango *et al.* (2009) who observed a higher relative growth rate under nitrate even with lower concentrations as compared to ammonium.

Total flavonoids and phenolics

The current study revealed up-regulated build-up of TFC and TPC that when no N form (control) was supplied to the amaranth plants (Table 1). This is in concurrent with results of other workers (Kováčik & Bačkor 2007, Ibrahim *et al.* 2011) which show that the accumulation of polyphenolic components in plant tissues is often enhanced under conditions of restricted nitrogen nutrition. Salahas *et al.* (2011) observed that N-deficiency stimulated biosynthesis of secondary plant metabolites, such as total phenolics and betacyanins in red beet. Previous findings by Argyropoulou *et al.* (2015) further revealed that total phenolics concentration significantly increased in N-starved plants, indicating that biosynthesis of secondary plant metabolites is stimulated by nitrogen deficiency, and this is in agreement with the report of Scheible *et al.* (2004). Lower levels of phenolic and flavonoid compounds in plants grown under high N supply have been reported for apple trees (Leser & Treutter 2005). Ammonium-N source was superior in poly-phenolic accumulation compared to other sources. Application of plants with sole ammonium source leads to acidification of rhizosphere (Sabir *et al.* 2013), which associated with poor plant growth (Gweyi-Onyango *et al.* 2009). This in turn induces plant defense mechanisms by increased poly-phenolic accumulation (Caldwell *et al.* 2003) as defense mechanism against nutritional stress. This may be supported by carbon nutrient balance (CNB) hypothesis which anticipates

that the accumulation of excess carbon in response to nutrient stress leads to the increased production of carbon-based secondary metabolites (CBSM) and their precursors (Yongke *et al*. 2005). Ammonium nutrition stimulates build-up of high levels of polyamines (Chen *et al.* 2011, Gill & Tuteja 2010), which act as precursors of secondary metabolites biosynthesis (Smith 1990). Increased carbon skeletons for ammonium assimilation, might have stimulated shikimic acid pathway activities which in turn could have enhanced the production of plant secondary metabolites (Fan *et al.* 1998) under sole ammonium treatment. Reduction in sink size to some extend reduces translocation of carbohydrates to other plant parts (Reddy *et al.* 1996.) and extra carbohydrates might be channeled towards secondary metabolism (Tognetti &Johnson 1999).

DPPH Anti-oxidant Activity

Similar to the TFC and TPC accumulation plants supplied with NH_4^+-N exhibited superior scavenging capacity unlike other (NO_3^- and NH_4^+/NO_3^- mixture). Maisarah *et al*. (2013) observed that phytochemicals such as flavonoids and phenolics constitute a major group of compounds that act as primary antioxidants. Plants exposed to high NH_4^+ concentration as N source accumulate low molecular osmolytes among them polyamines (Claussen *et al.* 2006) which enhances the plants tolerance to stresses (Tassoni *et al.* 2008, Yamaguchi *et al.* 2007). Moreover polyamines to some extent are involved in detoxification of nitrogen stress acting as nitrogen reservoirs, and free radical scavengers maintaining the integrity of membranes, consequently protecting cells from nitrogen toxicity (Chen *et al.* 2011). Flavonoids have been reported to possess strong antioxidant activity, indicated by their ability to chelate metals, scavenge singlet oxygen radicals and inhibit oxidation of low density lipoprotein *in vitro* studies (Kandaswami & Middleton 1994). Anthocyanins; which are flavonoids (Kumar & Pandey 2013) lower the accumulation of ROS in vivo under oxidative stress (Nakabayashi *et al*. 2014). Previous work has been done to support total phenols as effective antioxidants or free radical scavengers (Ogembo 2015, Okello 2015) in leafy vegetables.

Relationship between plant growth and TFC and TPC concentration

Pearson correlation indicated a strong negative relationship between plant height and TFC and TPC which is in line with the findings of (Ogembo 2015, Sousa *et al*. 2008) who demonstrated higher levels of total phenolics were accompanied by lower plant growth in *Solanum* and *Brassica species*. Phenolics and flavanoids (quarcetin) concentrations increase was observed under inhibited plant growth in *Amaranthus hypochondriacus* (Onyango *et al*. 2012). High level of poly-phenolic compounds may be associated with nutritional-induced stress under the sole ammonium and no –N form due to resource allocation trade-off between plant growth and defense for survival, therefore suppressed plant growth. This is in parallel with results of Donaldson *et al.* (2006) who reported that nutrient limitation decreased growth, leaf mass ratio, and photosynthesis but augmented leaf condensed tannin concentrations. Negative relationship between growth and condensed tannins indicated an additional indirect cost of allocation to secondary metabolites (Donaldson *et al*. 2006).

CONCLUSION

Amaranth plants responded differently to different N forms. Ammonium N form restricted plant growth which correlated negatively with polyphenols (total flavonids and phenolics) accumulation. Sole NH_4^+ induced stress enhanced total flavonoids, phenolics and natural antioxidants with effective antioxidative capacity; a striking metabolic plasticity observed during plant growth and survival trade-off in vegetable amaranth. Variety AB7 was superior to AB6 both in terms of growth and accumulation of TFC and TPC which are important for health and hence this would be recommended to farmers as well as consumers.

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