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The effects of nitrogen application on the growth, photosynthesis, and antioxidant activity of *Amaranthus viridis*

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Abstract

Amaranthus viridis is a functional food due to its antioxidant activity. The aim of this study was to investigate the responses of photosynthesis, growth, and antioxidant properties in *A. viridis* to nitrogen (N) applications. *A. viridis* plants were cultivated under low N (LN), medium N (MN), and high N (HN), and harvested at the reproductive phase. The dry mass and plant height of *A. viridis* plants increased with elevated N, and the dry mass of HN was saturated. Net photosynthetic rate, stomatal conductance, and water-use efficiency in the leaves at HN were strengthened. Meanwhile, under HN, chlorophylls (Chl), their precursors, and degradation intermediates in the leaves were highly accumulated, and the minor route of Chl degradation pathway was induced dramatically. However, 2,2-diphenyl-1-picrylhydrazyl radical-scavenging, ferrous iron-chelating, and reducing power in the extracts were reduced under HN. Conclusively, an appropriate N application balanced the yield and antioxidant properties of *A. viridis*.

Keywords: antioxidant activity; chlorophyll biosynthesis; chlorophyll degradation; dry mass; nitrogen fertilization; photosynthesis.

Introduction

Amaranthus viridis, an annual plant in the family Amaranthaceae, is considered a kind of field weed and also an amaranth crop (Reddy *et al.* 2007, Sharma *et al.* 2012). Its native range is tropical America but is widely distributed in tropical climates around the world. Several studies reported that *A. viridis* is an alternative vegetable containing plenty of dietary fiber, amino acids, microelements, carotenoids, vitamin C, and B vitamins (Guil *et al.* 1997, Sena *et al.* 1998, Sharma *et al.* 2012, Datta *et al.* 2019, Silva *et al.* 2021). Furthermore, several

Highlights

- High N promoted photosynthetic capacity, and Chl biosynthesis and degradation in *Amaranthus viridis* leaves
- High N did not increase dry mass significantly and reduced antioxidant activity of *A. viridis* extract
- Medium N input balanced yield and bioactivity in *A. viridis* plants

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Abbreviations: Anth – anthocyanin; Car – carotenoids; Chl – chlorophyll; Chlide – chlorophyllide; DM – dry mass; DPPH – 2,2-diphenyl-1-picrylhydrazyl; *E* – transpiration; g_s – stomatal conductance; MGPP – magnesium protoporphyrin IX; Pchlide – protochlorophyllide; Phe – pheophytin; Pho – pheophorbide; P_N – net photosynthetic rate; PPIX – protoporphyrin IX; TF – total flavonoids; WUE – water-use efficiency.

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phenolic acids and flavonoids (Flv) have been isolated from *A. viridis* (Kumar *et al*. 2009a, Datta *et al*. 2019), and antioxidant, antinociceptive, and antipyretic bioactivity among other processes have been previously confirmed in *A. viridis* (Kumar *et al*. 2009b, Bang *et al*. 2021). *A. viridis* is therefore also used as a medicinal plant. A recent study indicated leaves and inflorescence of *A. viridis* possessed higher phenols and Flv, and antioxidant activities (Silva *et al*. 2021). The composition of phenolic compounds and antioxidant activity of *A. viridis* seeds were also surveyed recently (Popoola 2022), but seeds displayed lower content of phenols and Flv than that of leaves and inflorescence (Silva *et al*. 2021). Previous pharmaceutical studies have revealed additional benefits of *A. viridis*, including antidiabetic, anticholesterolemic, antihyperlipidemic, and antihyperglycemic activity (Girija and Lakshman 2011, Girija *et al*. 2011, Kumar *et al*. 2012, Pandhare *et al*. 2012). Hepatoprotection (Kumar *et al*. 2011) and cardioprotection (Saravanan *et al*. 2013) functions of *A. viridis* were also previously demonstrated. Hence, *A. viridis* would be produced as a nutraceutical and functional food for the promotion of human health.

Nitrogen (N) fertilizer is one of the vital macroelements for crop growth, and nitrogen management is one of the essential practices in agricultural production. The plant height and biomass of amaranth crops exhibit positive correlations with nitrogen contents, and N application significantly promotes yield (Ayodele 2002). A linear increase in amaranth crop yield with nitrogen fertilizer content is also observed under moderate sowing density (Ferreira *et al*. 2014). Even though N application promotes amaranth crop yields effectively, N use is significantly inhibited under nitrogen over-fertilization (Schulte auf'm Erley *et al*. 2005). Moreover, a previous study indicated that the plant height of the amaranth crop is positively correlated with the level of nitrogen fertilization, but its yield is unresponsive to fertilization level (Gélinas and Seguin 2008). Recent research suggests that moderate N fertilization promotes agronomic traits and yield in the amaranth crop (Maseko *et al*. 2019). In addition, a high N supply with temporal variability might increase plant growth dramatically (Wang *et al*. 2022). On the other hand, the accumulation of secondary metabolites, such as phenols, Flv, and their related derivatives in crops also responds to N application (Zhang *et al*. 2017). Previous studies have found that phenylpropanoid and Flv biosynthesis in plants was also induced under limited N application, resulting in higher Flv and anthocyanins (Anth) accumulation (Ibrahim and Jaafar 2011, Zhao *et al*. 2015). Sun *et al*. (2020) revealed that N application reduced carbon/nitrogen (C/N) ratio and carbohydrate, and limited the Flv and phenolic synthetic pathway in plants. However, different species and organs of plants showed diverse responses to N application (Deng *et al*. 2019, Zhao *et al*. 2021). The accumulation of phenols and Flv is correlated to antioxidant activity, including 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging, ferrous iron-chelating, and ferric-reducing power in plants (Olarewaju *et al*. 2018, Silva *et al*. 2021, Zhao *et al*. 2021). As speculated by Chen *et al*. (2021), an appropriate N application balanced yield and accumulation of secondary metabolites for crop production.

Photosynthesis is a process to convert light energy into plant biomass, and both chlorophyll (Chl) and carotenoids (Car) are pigments that participate in photosynthesis (Bode *et al*. 2009). Chl content and photosynthetic traits, such as stomatal conductance (g_s), net photosynthetic rate (P_N), and water-use efficiency (WUE), are modulated by N application (Dordas and Sioulas 2008). Meanwhile, a previous study revealed that genes encoding for light-harvesting complex and Rubisco were upregulated by N fertilization (Midorikawa *et al*. 2014, Zaid and Mohammad 2018). Conversely, N limitation or deficiency resulted in the downregulation of genes coding for proteins related to PSI, PSII, ATP synthase, Rubisco, and Chl biosynthesis with a decline in organic acids related to the tricarboxylic acid (TCA) cycle and in C_3 and C_4 carbon metabolism (Amiour *et al*. 2012, Zhao *et al*. 2015, Curci *et al*. 2017). *Amaranthus* species belong to C_4 dicots, which perform at a better photosynthetic efficiency than that of C_3 plants, and C_4 plants presented higher P_N and Chl content with N supply (Tsutsumi *et al*. 2017, Togawa-Urakoshi and Ueno 2022). The study of Hunt *et al*. (1985) revealed that Chl accumulation and P_N in amaranth plants were elevated under high nitrogen contents. The recent study also demonstrated that elevated N benefited the accumulation of biomass, Chl, and photosynthesis and resulted in a higher Chl *a*/*b* ratio in *A. cruentus* along with increases in N applications (Cechin and Valquilha 2019). Deng *et al*. (2019) reported that appropriate N application would influence the internal C/N ratio and balance the photosynthesis and biosynthesis of Flv in *Cyclocarya paliurus*, whereas the responses of photosynthesis and accumulation of Flv in *A. viridis* to N application were rarely studied.

Chl accumulation in plants depends on Chl biosynthesis and/or degradation pathways (Eckhardt *et al*. 2004). Chl precursors, including protoporphyrin IX (PPIX), magnesium protoporphyrin IX (MGPP), and protochlorophyllide (Pchlide), and mole percentages of these precursors, respond to various environmental factors and genetic backgrounds (Huang *et al*. 2014). The expression of genes encoding for Chl biosynthesis-related proteins, such as genomes uncoupled 4 (GUN4) protein, glutamyl-tRNA reductase (HEMA), and protochlorophyllide oxidoreductase (POR), are modulated by nitrogen *via* the regulation of transcription factors *GATA*, *NITRATEINDUCIBLE*, *CARBON-METABOLISM-INVOLVED* (*GNC*), and *CYTOKININ-RESPONSIVE GATA 1/GNC-LIKE* (*CGA1*) (Hudson *et al*. 2011). Chen *et al*. (2021) also indicated that N supply could upregulate the expression of genes encoding for HEMA and POR and increase Chl accumulation. Another previous study illustrated that one Chl biosynthesis-related gene, *magnesium-chelatase subunit H* (*BCHH*), was downregulated under a nitrogen deficiency (Zhao *et al*. 2015). On the other hand, pheophytin (Phe) and chlorophyllide (Chlide) are the products of the first step in the Chl degradation pathway and are catalyzed by Mg-dechelatase and chlorophyllase, respectively;

pheophorbide (Pho) is generated from the breakdown of Phe and Chlide, and then red-colored catabolite is generated from Pho catalyzed by pheophorbide oxygenase (Oda-Yamamizo *et al*. 2016, Hu *et al*. 2021). Our previous studies indicated that different plant species preferred Chl → Phe → Pho or Chl → Chlide → Pho as the major route for Chl degradation, whereas some biotic/abiotic factors affected the degradation pathway in plants (Yang *et al*. 2003, Huang *et al*. 2014, Chen *et al*. 2016). A previous study indicated *chlorophyllase 1* (*CHL1*) was upregulated to accelerate Chl degradation in plants under N deficiency (Zhao *et al*. 2015). However, the responses of the Chl biosynthetic/degradation pathway to N fertilization in *A. viridis* are seldom discussed.

It has been confirmed that the leaf and inflorescence of *A. viridis* would be potential functional foods due to their antioxidant activity (Silva *et al*. 2021). Based on studies by Deng *et al*. (2019) and Sun *et al*. (2020), we hypothesize that N application might induce photosynthesis and Chl synthesis and increase biomass in *A. viridis*, but the accumulation of secondary metabolites, such as Flv and Anth, and antioxidant activity in extracts of *A. viridis* might be reduced with more N application. Optimizing biomass accumulation and antioxidant activity in *A. viridis* with moderate nitrogen application is one approach to functional food production. Hence, in this study, *A. viridis* plants were cultivated under three concentrations of nitrogen and harvested at the reproductive phase. The objective of this study was to investigate the growth, photosynthesis, and antioxidant activity of plant extract in *A. viridis.*

Materials and methods

Plant material and experimental design: A rooftop farming experiment was carried out at the rooftop of the National Research Institute of Chinese Medicine (NRICM) building, Taipei, Taiwan (25°07'11"N, 121°30'53"E). The precipitation and atmospheric temperature during the cropping season are presented in Fig. 1. The *A. viridis* strain AV1601 provided by Taitung District Agriculture Research and Extension Station was used in this study. Nursery pots (9 cm diameter × 8 cm deep) containing a peat–vermiculite–perlite mix (1:1:1 by volume) were prepared. Seeds were sown in nursery pots on 31 May 2018 and raised with regular irrigation and appropriate thinning (1–3 plants per pot) on the rooftop of the NRICM building until the sixth leaf had expanded.

The experiment was conducted with a completely randomized design with three N concentrations, 120 kg(N) ha^{-1} (low nitrogen, LN), 180 kg(N) ha^{-1} (medium nitrogen, MN), and 240 kg(N) ha^{-1} (high nitrogen, HN), as treatments. All treatments were conducted in triplicate. Combined planter boxes (0.9 cm length × 0.6 m width × 0.2 m deep) contained a mix (88 L) of sandy loam and peat (10:1 by volume) were prepared as plots in this experiment design on 5 July 2018, and the healthy plants were transplanted in planter boxes at 45 × 25 cm plant spacing. Each planter box was regularly irrigated and weeded on the rooftop of the NRICM building.

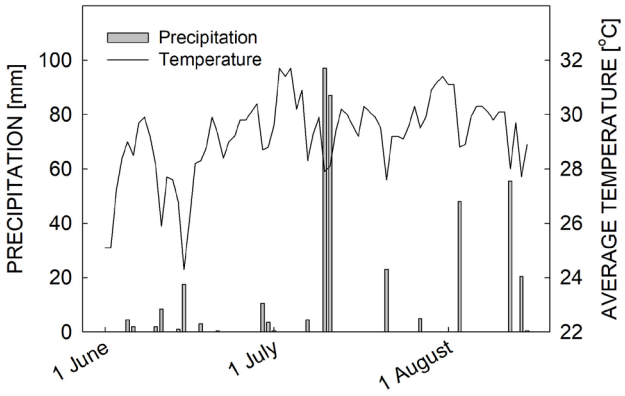

Fig. 1. Daily precipitation and average temperature during the period of cropping season in 2018. *Vertical bars* indicate daily precipitation and *line* indicates the daily average temperature.

Nitrogen (N), phosphorus (P_2O_5), and potassium (K_2O) were applied as ammonium sulfate, calcium superphosphate, and potassium chloride, respectively. The base fertilization containing 105 mg of N, 126 mg of P_2O_5, and 120 mg of K_2O per pot was applied for seedling development before seed sowing. Three N concentrations (120, 180, and 240 kg ha^{-1}) mixed with P_2O_5 (100 kg ha^{-1}) and K_2O (50 kg ha^{-1}) were applied before transplantation. The photosynthetic ability was determined at the reproductive phase on 15 August 2018, followed by whole plants harvested for further analysis.

Photosynthesis: Photosynthetic CO_2 assimilation (P_N), stomatal conductance (g_s), and transpiration (E) of the uppermost fully expanded leaves of cultivated plants were measured at 1,500 μmol(photon) m^{-2} s^{-1} with a CO_2 concentration of 400 μmol mol^{-1} by an open portable photosynthesis system (*LI-6400XT*, *LI-COR*, USA) equipped with a CO_2 injector and LED light source. Water-use efficiency (WUE) was also calculated by the P_N/E ratio. The determined leaves were detached and lyophilized for the determination of photosynthetic pigments.

Plant growth parameters: Plant height was measured from the base to the top of the plant after photosynthesis determination, and shoot and root were harvested to measure fresh mass (FM) and dry mass (DM) with an electronic balance. Harvested plants were lyophilized to determine biomass. Moisture content [%] of shoots was calculated as [1 – (DM/FM)] × 100%. Four plants were sampled from each planter box and their collected data were averaged. Lyophilized plant samples were ground and stored at –20°C for the determination of Anth, total flavonoids (TF), and antioxidant activity assays.

Photosynthetic pigments and Chl-related compounds: The uppermost fully expanded leaves of cultivated plants of each nitrogen concentration were detached, lyophilized, and extracted with 80% acetone. The concentrations of Car, and Chl-related compounds, including PPIX, MGPP,

Pchlide, Chl, Chlide, and Phe, were determined according to Yang *et al*. (1998) with a spectrophotometer (*Hitachi U3010*, Tokyo, Japan). The mole percent of individual porphyrin is defined as [(PPIX, MGPP or Pchlide)/(PPIX + + MGPP + Pchlide)] × 100%. The values of phytylated and/or dephytylated pigments in samples were measured directly at absorbances of 661 and 666 nm [A_{661} and A_{666} g^{-1}(DM)], respectively. A_{661} and A_{666} values could be used to compare the relative concentration of total phytylated (Chl and Phe) and dephytylated (Chlide and Pho) pigments, respectively (Shioi and Sasa 1986).

Anthocyanins and total flavonoids: Anth concentration in sample powder was measured based on the protocol of Mancinelli *et al*. (1975). TF in plants was determined by the method of Djeridane *et al*. (2006). Quercetin was used as a reference standard, and TF concentration was expressed as milligrams of quercetin equivalents per gram of dry mass [mg(QE) g^{-1}(DM)].

Antioxidant activity: A 0.25 g *A. viridis* powder sample was extracted with 5 mL of methanol at room temperature. The sample solution was then collected by a vacuum using filter paper (*Whatman No. 1*) to obtain the crude extract, and aliquots of serial dilutions of 0.05 mL of the methanol extract were used for antioxidant activity assays, including DPPH free radical-scavenging activity, ferrous iron-chelating ability, and the reducing power of sample powder, determined according to the protocol of Nguyen *et al*. (2018). Methanol was used instead of a sample as the control, butylated hydroxytoluene (BHT) was used as the standard for DPPH free radical-scavenging and reducing power assay, and ethylenediaminetetraacetic acid (EDTA) was used as the standard for ferrous iron-chelating assay. The concentrations required for 50% decreases in the absorbance (IC_{50}) of DPPH radicals, ferrous iron, and reducing power were then calculated as the percent inhibition of DPPH, ferrous iron, and reducing power by plotting the percentage of residual DPPH, ferrous iron, and reducing power at a steady state as a function of sample concentration, respectively.

Statistical analyses: All measurements were evaluated for significance using an analysis of variance (*ANOVA*), followed by a least significant difference (LSD) test at the $p<0.05$ level. All statistical analyses were conducted using *SAS 9.3* (*SAS Institute*, Cary, NC, USA).

Results

Plant growth: Plant height, shoot and root DM, and shoot moisture content in plants cultivated under different nitrogen concentrations are presented in Table 1, revealing that the N content significantly regulated plant height and dry mass of shoot and root, but not moisture content. The average height of the plants cultivated under LN was 83.19 cm. Plant height was significantly enhanced at MN (91.77 cm) and HN (94.66 cm), whereas shoot DM of MN (31.39 g per hill) and HN plants (30.18 g per hill) was significantly higher than that of LN (19.15 g per hill). A similar trend was also observed in root DM. Moisture content in shoots ranged from 79.4 to 80.0%, and the differences between N concentrations were insignificant.

Photosynthetic characteristics in leaves: Photosynthetic characteristics measured from upper fully expanded and healthy leaves are presented in Fig. 2, indicating that N concentration influenced P_N significantly. P_N in leaves of LN and MN plants ranged from 39.66 to 39.94 μmol(CO_2) m^{-2} s^{-1}, and P_N increased significantly in HN plants (Fig. 2*A*). Nevertheless, N concentration did not show any significant effect on g_s. The g_s in leaves was gradually increasing from 0.19 to 0.26 mol(H_2O) m^{-2} s^{-1} with increasing N (Fig. 2*B*), and g_s of HN was significantly higher than that of LN. Meanwhile, the N content did not influence E significantly. The average E in leaves also showed an upward trend from 4.37 to 5.11 mmol(H_2O) m^{-2} s^{-1} with increasing N, but differences between all N concentrations were insignificant (Fig. 2*C*). In addition, WUE was regulated by N content insignificantly. WUE in leaves of plants cultivated under elevated N was 9.28, 8.57, and 10.93 μmol(CO_2) mmol(H_2O)$^{-1}$ sequentially (Fig. 2*D*), and WUE of HN plants was significantly higher than that of MN.

Photosynthetic pigments and secondary metabolites: Chl concentration and Chl *a*/*b* were calculated from the concentration of Chl *a*, Chl *b*, and their ratio (Table 2). Both Chl and Car were regulated by N concentration significantly, but N content did not affect Chl *a*/*b* at all. Chl

Table 1. The plant height, shoot and root dry mass (DM), and moisture content in shoot of *Amaranthus viridis* plants cultivated under different nitrogen concentrations, low N (LN), medium N (MN), and high N (HN). Within columns, means ± SD ($n = 3$) followed by the same *lowercase letters* are not significantly different, according to *ANOVA* followed by LSD ($p<0.05$). * $p<0.05$ level; ** $p<0.01$ level; ns – no significant difference.

N content	Plant height [cm]	Shoot DM [g per hill]	Root DM [g per hill]	Moisture content [%]
LN	83.19 ± 2.60^b	19.15 ± 0.36^b	3.47 ± 0.29^b	79.43 ± 0.19^a
MN	91.77 ± 2.17^a	31.39 ± 2.39^a	4.67 ± 0.29^a	79.99 ± 0.41^a
HN	94.66 ± 2.40^a	30.18 ± 0.28^a	5.13 ± 0.65^a	80.00 ± 0.20^a
ANOVA				
N content	**	**	**	ns

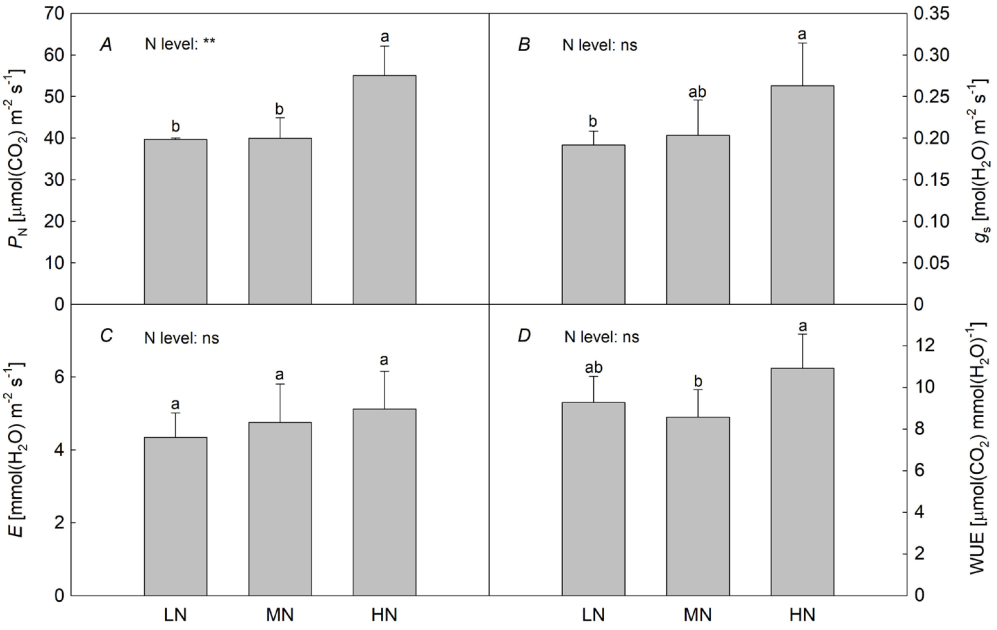

Fig. 2. Net photosynthetic rate (P_N) (*A*), stomatal conductance (g_s) (*B*), transpiration (*E*) (*C*), and water-use efficiency (WUE) (*D*) in leaves of *Amaranthus viridis* cultivated under different nitrogen fertilization levels. Values are the means with standard deviations shown by *vertical bars* (n = 3). *Different lowercase letters* represent statistically significant differences (LSD, p<0.05) according to *ANOVA*. * p<0.05 level; ** p<0.01 level; ns – no significant difference.

in leaves measured for photosynthesis was elevated from 2.33 to 4.82 mg g^{-1}(DM) with an increase of N fertilizer, while Chl *a*/*b* remained at a stable range (4.07–4.22). Car in leaves was significantly augmented under MN [1.43 mg g^{-1}(DM)] and HN [2.04 mg g^{-1}(DM)].

In contrast, Anth and TF concentration in shoots did not show an increasing trend like Chl and Car, but N concentration influenced Anth significantly (Table 2). The Anth concentration of MN [2.12 μg g^{-1}(DM)] was significantly lower than that under HN [3.32 μg g^{-1}(DM)]. Furthermore, N concentration affected TF insignificantly. TF concentration ranged from 13.67–19.21 mg(QE) g^{-1}(DM), and the differences between N concentrations were insignificant.

Porphyrins and mol percentages in leaves: Chl precursors, including PPIX, MGPP, and Pchlide, in leaves measured for photosynthesis were determined in this study, and then porphyrins and mol percentages were calculated from these Chl precursors (Fig. 3). N content stimulated porphyrins significantly. Porphyrins in leaves ranged from 0.38–0.40 mmol g^{-1}(DM) in LN, while porphyrins of HN were significantly increased to 0.79 mmol g^{-1}(DM) (Fig. 3*A*). Under LN and MN, the mole percentages of PPIX, MGPP, and Pchlide were 76.5–80.7%, 19.3–23.1%, and 0.0–0.4%, respectively. The mole percentage of PPIX significantly declined to 54.4%, and the mole percentages of MGPP and Pchlide were raised to 30.6 and 15.0%, respectively (Fig. 3*B*). These Chl precursors were all promoted significantly in HN (Fig. 3*A*).

Chl degradation intermediates in leaves: The dynamics of Phe, Chlide, and their ratio are presented in Fig. 4, showing that Phe, Chlide, and their ratio were regulated by the N concentration significantly. Phe concentration was higher than that of Chlide under each N concentration (Fig. 4*A*,*B*); therefore, leaves of *A. viridis* took Chl → Phe → Pho as the major route in the Chl degradative pathway, and Chl → Chlide → Pho as the minor route. Phe content in leaves gradually accumulated from 0.90 to 1.78 mg g^{-1}(DM) with elevated N (Fig. 4*A*). Chlide ranged from 0.17 to 0.20 mmol g^{-1}(DM) under LN and MN, and abruptly raised to 0.83 mmol g^{-1}(DM) under HN (Fig. 4*B*). Moreover, Phe/Chlide remained at 5.85–5.97 under LN and MN, but declined to 2.17 under HN (Fig. 4*C*). The results of phytylated and dephytylated pigments and their ratios also showed a similar trend to the Phe/Chlide ratio (Fig. 5). N concentration influenced phytylated and dephytylated pigments and their ratios significantly. Phytylated pigment in leaves gradually increased from 108 to 210 A_{661} g^{-1}(DM) with an increase of N (Fig. 5*A*). Dephytylated pigments ranged from 7 to 9 A_{666} g^{-1}(DM) under LN and MN, and suddenly enhanced to 30 A_{666} g^{-1}(DM) under HN (Fig. 5*B*). Phe/Chlide stayed at 16.14–16.41 under LN and MN but dropped to 2.17 under HN (Fig. 5*C*).

Antioxidant activities in methanol extracts: The IC_{50} of DPPH free radical-scavenging activity, ferrous iron-chelating ability, and ferric-reducing power assay in methanol extracts of *A. viridis* plants cultivated under three N concentrations are listed in Table 3, and the value

Table 2. Chlorophylls (Chl), Chl *a*/*b* ratio, carotenoids (Car), anthocyanins (Anth), and total flavonoids (TF) concentration of *Amaranthus viridis* cultivated under different nitrogen fertilization levels. Within columns, means ± SD (n = 3) followed by the same *lowercase letters* are not significantly different, according to *ANOVA* followed by LSD (p<0.05). * p<0.05 level; ** p<0.01 level; ns – no significant difference. QE – quercetin equivalent.

N content	Chl [mg g^{-1}(DM)]	Chl *a*/*b*	Car [mg g^{-1}(DM)]	Anth [µg g^{-1}(DM)]	TF [mg(QE) g^{-1}(DM)]
LN	2.33 ± 0.38^{b}	4.22 ± 0.08^{a}	1.09 ± 0.18^{c}	2.54 ± 0.27^{ab}	19.21 ± 5.84^{a}
MN	3.08 ± 0.33^{b}	4.07 ± 0.19^{a}	1.43 ± 0.11^{b}	2.12 ± 0.55^{b}	13.67 ± 1.55^{a}
HN	4.82 ± 0.52^{a}	4.10 ± 0.14^{a}	2.04 ± 0.18^{a}	3.31 ± 0.71^{a}	18.33 ± 4.72^{a}
ANOVA					
N content	**	ns	**	*	ns

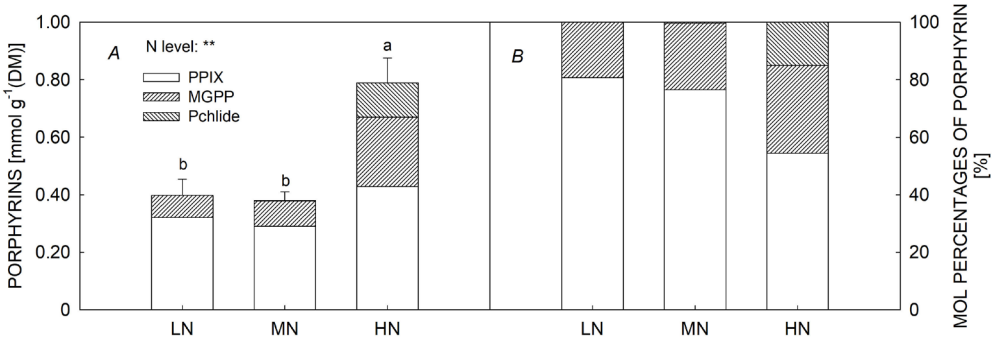

Fig. 3. Porphyrins (*A*) and mol percentages of protoporphyrin IX (PPIX), magnesium protoporphyrin IX (MGPP), and protochlorophyllide (Pchlide) (*B*) in leaves of *Amaranthus viridis* cultivated with different nitrogen concentrations. Values are the means with standard deviations shown by *vertical bars* (n = 3). *Different lowercase letters* represent statistically significant differences (LSD, p<0.05) according to *ANOVA*. * p<0.05 level; ** p<0.01 level; ns – no significant difference.

of the standard for each assay is also presented there. All treatments significantly influenced all antioxidant activities (Table 3). The IC_{50} of DPPH free radical-scavenging activity in *A. viridis* extracts gradually rose with elevated N concentration, and the IC_{50} of DPPH scavenging in HN was significantly higher than that in LN. The IC_{50} of ferrous iron-chelating ability in HN was significantly higher than that in LN and MN. The IC_{50} of the reducing power also showed an upward trend with the elevated N concentration.

Discussion

As a functional food and/or medicinal plant, *A. viridis*' nutritional components, comprising amino acids, fatty acids, Car, vitamins, and mineral content, were analyzed previously (Guil *et al*. 1997, Sena *et al*. 1998, Datta *et al*. 2019, Silva *et al*. 2021), and the composition and total content of phenols and Flav in *A. viridis* were also profiled by Datta *et al*. (2019) and Silva *et al*. (2021). Antioxidant activity, comprising DPPH-scavenging activity, ferrous iron-chelating ability, and the reducing power of *A. viridis* extracts, were also investigated recently (Datta *et al*. 2019, Bang *et al*. 2021, Silva *et al*. 2021). Leaves and inflorescence contained more phenols and Flav and better antioxidant activities among all organs of *A. viridis* (Silva *et al*. 2021). Olarewaju *et al*. (2018) suggested that not only TF content in *A. viridis* but also antioxidant activity of plant extracts were responsive to N application. Thus, for the purpose of functional food cultivation, the response of antioxidant properties and growth of *A. viridis* to N application should be considered simultaneously.

N application enhanced morphological traits and yields of amaranth crops (*A. hybridus* and *A. hypochondriacus*) effectively, whereas the optimum rate of N fertilization had to be amended for the demand of each amaranth crop (Ayodele 2002). As speculated by Maseko *et al*. (2019), the optimum rate of N fertilizer promotes the development and yield of the amaranth crop and reduces N loss, while Gélinas and Seguin (2008) implied that N application just modulated plant height rather than yield in *A. caudatus*. The growth parameters of *A. viridis* cultivated at all N concentrations showed that N application increased plant height and shoot and root DM significantly (Table 1), but the promotion of growth parameters in MN and HN was insignificant. A similar result of the study by Schulte auf'm Erley *et al*. (2005) exhibited high N application did not promote yield significantly and resulted in lower N-utilization efficiency. Ferreira *et al*. (2014) demonstrated that planting density and N application rate both modulated

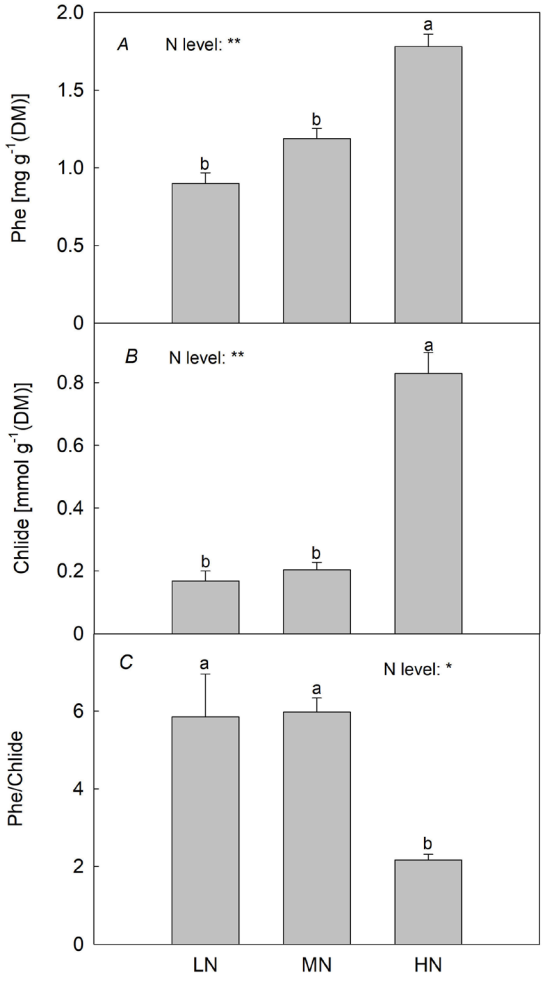

Fig. 4. Pheophytin (Phe) (*A*), chlorophyllide (Chlide) (*B*) pigments and their ratio (*C*) of leaves of *Amaranthus viridis* cultivated under different nitrogen concentrations. Values are the means with standard deviations shown by *vertical bars* (n = 3). *Different lowercase letters* represent statistically significant differences (LSD, $p<0.05$) according to *ANOVA*. * $p<0.05$ level; ** $p<0.01$ level; ns – no significant difference.

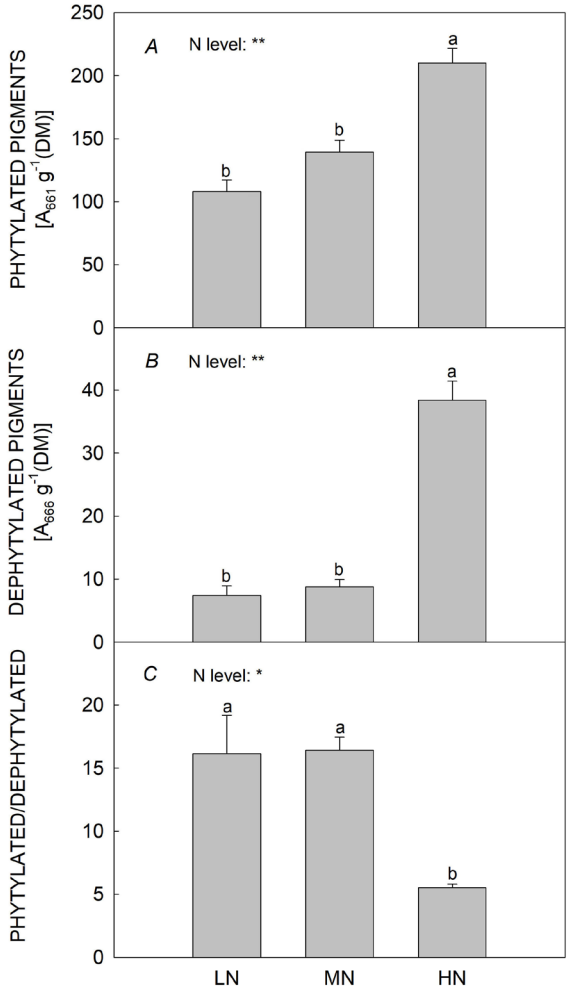

Fig. 5. Phytylated (*A*) and dephytylated (*B*) pigments and their ratio (*C*) of leaves of *Amaranthus viridis* cultivated under different nitrogen concentrations. Values are the means with standard deviations shown by *vertical bars* (n = 3). *Different lowercase letters* represent statistically significant differences (LSD, $p<0.05$) according to *ANOVA*. * $p<0.05$ level; ** $p<0.01$ level; ns – no significant difference.

growth and yield of amaranth crops. Wang *et al*. (2022) indicated high N supply with a pattern of variability, rather than consistency, stimulated a higher plant height and more DM in *A*. *palmeri*. Therefore, HN with temporal variability supply might also promote plant growth of *A*. *viridis*.

The increase in plant DM and growth rate with elevated N content would be ascribable to augmented photosynthesis (Hunt *et al*. 1985). A field study of safflower (*Carthamus tinctorius*) reported that promoted yield and higher Chl content and photosynthetic parameters, including P_N, g_s, and WUE, were concurrent under elevated N application (Dordas and Sioulas 2008). However, the study of *Cyclocarya paliurus* (Deng *et al*. 2019) revealed both P_N and Chl in leaves declined under highest N concentration. In the present study, more accumulation of photosynthetic pigments and better performance in P_N, g_s, and WUE in *A. viridis* leaves were observed under HN (Table 2, Fig. 2). This finding agrees with Cechin and Valquilha (2019) who reported similar results for photosynthetic pigments and characteristics. Tsutsumi *et al*. (2017) investigated 12 *Amaranthus* species (NAD-malic enzyme-type C_4 dicots) and stated that P_N was highly correlated with the Rubisco activity. Furthermore, Togawa-Urakoshi and Ueno (2022) also implied that N supply promoted P_N and Chl content significantly, but did not influence WUE strongly. In accordance with the report of Midorikawa *et al*. (2014), N application would upregulate genes participating in photosynthesis (such as Chl *a*/*b*-binding protein) and carbon fixation (such as Rubisco large subunit and small subunit), and could improve photosynthetic ability. In the study of *Mentha arvensis* (Zaid and Mohammad 2018), the activity of Rubisco was also induced by N supply,

Table 3. The 50% inhibitory concentration (IC_{50}) values of 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical-scavenging activities, ferrous iron-chelating abilities, and ferric-reducing power assays in methanol extracts of *Amaranthus viridis* cultivated under different nitrogen concentrations. Within columns, means ± SD (n = 3) followed by the same *lowercase letters* are not significantly different, according to *ANOVA* followed by LSD (p<0.05). * p<0.05 level; ** p<0.01 level; ns – no significant difference.

N level	DPPH-scavenging activity [mg mL^{-1}]	Fe^{2+}-chelating ability [mg mL^{-1}]	Reducing power rate [mg mL^{-1}]
LN	2.48 ± 0.89^{b}	8.46 ± 0.44^{b}	12.16 ± 3.15^{b}
MN	3.17 ± 0.12^{ab}	8.30 ± 0.13^{b}	17.67 ± 5.85^{ab}
HN	3.40 ± 0.23^{a}	9.70 ± 0.73^{a}	19.20 ± 2.84^{a}
Standard	0.04 ± 0.00	0.17 ± 0.00	0.05 ± 0.00
ANOVA			
N level	**	**	**

and N fertilization altered the expression of transcription factors *GNC* and *CGA1*, followed by modulating both HEMA and POR proteins that were involved in Chl biosynthesis (Hudson *et al*. 2011). The study conducted by Chen *et al*. (2021) also demonstrated that the expression of genes encoding for HEMA and POR was upregulated by N application. In the present work, higher Chl content with a sudden accumulation in porphyrins and dramatically shifted composition of PPIX, MGPP, and Pchlide were observed in *A. viridis* plants cultivated under HN (Table 2, Fig. 3).

Conversely, N deficiency inhibited the expression of photosynthesis-related genes, reduced metabolites in carbon metabolism, and upregulated the *CHL1* gene, which then led to Chl degradation in plants (Amiour *et al*. 2012, Zhao *et al*. 2015, Curci *et al*. 2017). Studies from Hunt *et al*. (1985) and Deng *et al*. (2019) claimed that Chl content and photosynthesis ability of *A. powellii* and *Cyclocarya paliurus* were reduced as they were raised under N limitation. Another previous study demonstrated that *BCHH*, a Chl biosynthesis gene, was downregulated under nitrogen deficiency (Zhao *et al*. 2015). In this study, the decline in Chl content was not significant under LN, and Chl *a*/*b* was stable at all N concentrations (Table 2). Meanwhile, photosynthetic characteristics (P_N, g_s, and WUE) under LN were not significantly lower than those under MN (Fig. 2). Notably, more accumulations of Chlide and Phe were observed under HN than LN, and the Phe/Chlide ratio was also reduced under HN (Fig. 4). Phytylated and dephytylated pigments showed a similar trend (Fig. 5). These results suggest that LN did not have an N-limited status for *A. viridis* in this study. Furthermore, the dynamics of the ratio of Phe/Chlide and phytylated/dephytylated pigments suggest that an elevated N application would modulate the Chl degradation pathway (Figs. 4*C*, 5*C*). However, Chen *et al*. (2021) revealed that N supply insignificantly regulated the expression of the gene encoding for pheophorbide a oxygenase, resulting in the high accumulation of Chl degradation intermediates (Figs. 4, 5). Moreover, in our work, a boosted photosynthetic ability with stimulated Chl biosynthetic and degradation pathways under HN (Table 2, Figs. 2, 3, 4, 5) did not promote DM accumulation (Table 1), so this situation would be considered nitrogen over-fertilization for *A. viridis* cultivation, and it might lead to a reduced internal C/N ratio and a limited Flv biosynthesis in the plants (Deng *et al*. 2019, Sun *et al*. 2020).

Several studies demonstrated that N deficiency or lower N content induced more accumulation of secondary metabolites in wheat (*Triticum aestivum*), tea (*Camellia sinensis*), *Labisia pumila,* and *Cyclocarya paliurus* (Ibrahim and Jaafar 2011, Zhang *et al*. 2017, Deng *et al*. 2019, Chen *et al*. 2021). N deficiency or lower N content trigger ethylene signaling and upregulating transcription factor *MYB12* to stimulate genes involved in phenylpropanoid and Flv biosynthesis, including L-phenylalanine ammonia-lyase and chalcone synthase (Ibrahim and Jaafar 2011, Zhao *et al*. 2015, Deng *et al*. 2019). However, Zhao *et al.* (2021) demonstrated that N application induced total phenols in *Allium fistulosum*. A meta-analysis conducted by Sun *et al*. (2020) suggested N application promoted biomass, but reduced the internal C/N ratio, and then resulted in a lower biosynthesis of secondary metabolites, such as phenolic acid, Anth, Flv, and tannins.

Phenolic compounds and Flv can protect plants against biotic or abiotic stresses and also be the source of antioxidants in functional foods and nutraceutical products; these metabolites were highly and positively correlated with antioxidant activity as well (Olarewaju *et al*. 2018, Silva *et al*. 2021, Zhao *et al*. 2021, Popoola 2022). Free radicals derived from stress may cause oxidative damage or lipid peroxidation, and one of the antioxidant mechanisms is free radical scavenging by donation of the hydrogen atom from antioxidants, thus DPPH-scavenging activity assays were conducted in this study (Nguyen *et al*. 2018). In addition, the lowest IC_{50} value of DPPH-scavenging activity was observed in LN, indicating that LN plants had the best ability for free radical scavenging. The extracts of LN and MN plants provided better iron-chelating ability to reduce the formation of ferrozine complex from ferrous ions (Fe^{2+}) of IC_{50} value of the ferrous iron-chelating ability. Moreover, the extract of LN plants displayed the best ability electron-donating of IC_{50} of the reducing power. Overall, the antioxidant activity

in plant extract declined with elevated N concentrations (Table 3). However, Anth and TF content in the harvested shoot were irresponsive to N content (Table 2). Silva *et al*. (2021) revealed that leaves and inflorescence of *A. viridis* displayed higher total phenols and TF and better antioxidant activity as well, whereas stem presented lower content of these compounds. Our preliminary results indicated that N supply stimulated the development of lateral bud in *A. viridis* plant (data not shown), and it might result in a divergent proportion of organs in the plant. The accumulation and composition of secondary metabolites in each organ of *A. viridis* are worthy of further investigation. In this study, better antioxidant properties and more DM were observed in harvested plants of *A. viridis* under MN (Tables 1, 3).

Conclusion: The high rate of nitrogen fertilization used in this study significantly enhanced photosynthesis characteristics (P_N, g_s, and WUE) in *A. viridis* leaves. A more Chl content with a high accumulation of porphyrins and shifted composition of porphyrins in *A. viridis* leaves under HN indicated that Chl biosynthesis was boosted by N application. In addition, leaves of *A. viridis* underwent Chl → Phe → Pho as the major route in the Chl degradation pathway, and Chl → Chlide → → Pho as the minor route. N application accelerated Chl degradation in leaves of *A. viridis*, but a minor route was induced dramatically under HN. However, the shoot DM of *A. viridis* under HN was saturated. A high N application might boost the photosynthetic ability and Chl biosynthesis in leaves, whereas a better performance of photosynthetic traits would not necessarily contribute to plant growth and yield. Furthermore, degenerated antioxidant properties of HN extracts indicated high N application inhibited the antioxidant activity of *A. viridis*' extract. Consequently, in cultivating *A. viridis* as functional food, an appropriate N application would balance photosynthetic traits, growth, and antioxidant properties.

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