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Barba de la Rosa, Ana P.; de León-Rodríguez, Antonio; Laursen, Bente; Fomsgaard, Inge S.

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Influence of the growing conditions on the flavonoids and phenolic acids accumulation in amaranth (*Amaranthus hypochondriacus* L.) leaves
Influencia de las condiciones de crecimiento en la acumulación de flavonoids y ácidos fenólicos en hojas de amaranto (*Amaranthus hypochondriacus* L.)

Ana P. Barba de la Rosa[1], Antonio de León-Rodríguez[1], Bente Laursen[2], and Inge S. Fomsgaard[2,‡]

[1] Instituto Potosino de Investigación Científica y Tecnológica (IPICYT). Camino a la Presa San José 2055, Col. Lomas 4 sección. 78216, San Luis Potosí, San Luis Potosí, México.
[2] Aarhus University, Faculty of Science and Technology, Department of Agroecology. AU Flakkebjerg Forsøgsvej 1 4200 Slagelse, Denmark.
‡ Corresponding author (inge.Fomsgaard@agro.au.dk)

SUMMARY

Phytochemicals or phenolic compounds are important natural bioactive molecules that plants accumulate in response to environmental conditions and may exert beneficial effects on health by protecting humans against many diseases. The aim of this work was to analyze the influence of biotic and abiotic stress on the accumulation of flavonoids and phenolic acids on leaves of two cultivars of *Amaranthus hypochondriacus*, which are differentiated by the colour of their leaves (red or green). Phenolic compounds were extracted using the accelerated solvent extraction (ASE) method and their identification was carried out by LC-MS analysis. Rutin was the main flavonoid in amaranth leaves; the highest concentrations were found in green leaves when plants were subjected to stress (9715 μg g^{-1}). Phenolic acids were minor compounds; ferulic acid was only present in red leaves (0.5 μg g^{-1}) and p-coumaric acid only in green leaves (0.7 μg g^{-1}). Our results indicate that leaves from *A. hypochondriacus*, the main species that produce edible seeds, are a good source of phytochemical compounds and their accumulation could be driven by the growing conditions.

Index words: *abiotic stress, accelerated solvent extraction, biotic stress, electrospray mass spectrometry, high performance liquid chromatography, phenolic compounds.*

RESUMEN

Los fitoquímicos o compuestos fenólicos son importantes moléculas bioactivas naturales que las plantas acumulan en respuesta a las condiciones ambientales y que pueden ejercen efectos benéficos para la salud protegiendo a los humanos de muchas enfermedades. El objetivo de este trabajo fue analizar la influencia del estrés biótico y abiótico en la acumulación de flavonoides y ácidos fenólicos en las hojas de dos cultivares de *Amaranthus hypochondriacus* diferenciadas por el color de sus hojas (rojas y verdes). Los compuestos fenólicos fueron extraídos empleando la extracción de solvente acelerado (ESA) y analizados mediante LC-MS. La rutina fue el principal flavonoide en hojas de amaranto; las más altas concentraciones se encontraron en la especie de hoja verde cuando las plantas fueron sometidas a estrés (9715 μg g^{-1}). Los ácidos fenólicos fueron los compuestos minoritarios; el ácido ferúlico sólo se encontró en hojas rojas (0.5 μg g^{-1}) y el ácido p-cumárico solo en hojas verdes (0.7 μg g^{-1}). Los resultados indican que las hojas de *A. hypochondriacus*, la principal especie productora de semillas comestibles, son una fuente rica de compuestos fitoquímicos y su acumulación podría ser dirigida por las condiciones de cultivo.

Palabras clave: *estrés abiótico, extracción acelerada con solvente, estrés biótico, electrospray espectrometría de masas, cromatografía líquida de alta resolución, compuestos fenólicos.*

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INTRODUCTION

Phytochemicals are a heterogeneous group of bioactive compounds; they belong to a class of plant secondary metabolites with a polyphenolic structure (Panche *et al*., 2016). The most studied are flavonoids, alkaloids, glycosides, steroids, tannins, and terpenoids (Cheynier *et al*., 2013). Phytochemicals have several functions in plants; they act as cell wall support material (Neutelings, 2011), as colourful attractants for birds and insects helping seed dispersal and pollination (Stevenson *et al*., 2016). They also are important in plant defence against different environmental stress conditions such as wounding, infection, and excessive light or UV irradiation (Berger *et al*., 2007; Bergquist *et al*., 2007; Altemimi *et al*., 2017).

As part of the human diet, flavonoids are claimed to exert protective effects against diseases such as cardiovascular diseases and cancer, among other illnesses. They have several modes of action (antioxidative, antiproliferative, anti-inflammatory or antibacterial) (Harborne and Williams, 2000; Cheynier *et al*., 2013). Phenolic acids are considered to be free radical scavengers, and their antioxidant properties depend on their chemical structure (Jordão *et al*., 2010). The use of plants as a source of phytochemicals has increased in the popularity of plant-based medicines leading to a new segment in horticultural crop production and agriculture to produce these compounds (Dillard and German, 2000; Parr and Bolwell, 2000; Pinhatti *et al*., 2010).

Because bioactive compound profile in plants may change depending on the cultivation site, season, age of plants or species, several strategies have been undertaken to find practical approaches to increase the yields of these molecules (Howard *et al*., 2002, Cho *et al*., 2008; Sarker and Oka, 2018). The applications of cell culture together with elicitation to induce the synthesis of phenolic compounds as well as the selection of plant varieties that accumulate high amount of these bioactives, are some examples of alternatives for plant phytochemical production (Mulabagal and Tsay, 2004; Luna-Palencia *et al*., 2005; Camarena-Rangel *et al*., 2017). Understanding of how environmental factors affect the production of phytochemicals will be at great importance towards optimising field growth conditions for their maximal production (Briskin, 2000; Fraisse *et al*., 2007; Jordão *et al*., 2010).

Edible amaranth leaves are a nutritious vegetable; the flavour of cooked vegetable amaranth is claimed to be equal or better than spinach. Amaranth leaves are high in calcium, fibre, niacin, and vitamin C (Mnkeni *et al*., 2007). Green leaves from amaranth (*Amaranthus cruentus* L.) are considered as medicinal plant mainly due to the content of several flavonoids such as dihydroquercetin (taxifolin), quercetin, rutin, and apigenin. In amaranth leaves are also found phenolic acids such as ferulic, caffeic, p-coumaric, and chlorogenic acids (Kalinova and Dadakova, 2009; Kolesnikov and Gins, 2001). *Amaranthus spinosus* L. is grown throughout India and is used as a medicinal plant for treating malaria and hepatic disorders; it also has anti-inflammatory and diuretic activity. Constituents such as b-sitosterol, stigmasterol, rutin (Olajide *et al*., 2004; Suryavanshi *et al*., 2007), and cinnamoylphenethylamines such as feruloyltyramine and feruloyldopamine have been reported in different amaranth species (Pedersen *et al*., 2010).

Amaranthus hypochondriacus L. is the main specie used to produces edible seeds, which are high quality protein (rich in essential amino acids) and nutraceutical compounds as peptides with several activities such as antihypertensive and cancer preventive (Silva-Sánchez *et al*., 2008; Barba de la Rosa *et al*., 2010). Seeds also contain polyphenols such as rutin and nicotiflorin (Barba de la Rosa *et al*., 2009). Some cultivars of *A. hypochondriacus* have leaves with a brilliant red colour, those leaves are rich in betacyanins, compounds that have high potential as natural food colorants and antioxidants (Cai *et al*., 2006), but there are some cultivars that presents green leaves and no information on the flavonoids and phenolic acid composition of those cultivars have been reported so far, neither the influence on their accumulation due to environmental stresses. Therefore, the objective of this work was to analyse qualitatively and quantitatively the flavonoids and phenolic acids presents in leaves of *A. hypochondriacus* L. cultivars (red and green leaves) and to investigate the effect of the growing conditions on the phenolic compounds accumulation.

MATERIALS AND METHODS

Amaranth Samples

A. hypochondriacus L. cv. Criolla with green leaves and cv. Nutrisol with red leaves were germinated

in small pots contained Sunshine Mix 3 (SunGro Horticultural Ltd., Seba Beach, Canada). Three-week-old seedlings were transferred to grown under four different environmental conditions: 1 = control plants: in fields outdoors under straight sunlight and soils prepared for crop production; 2 = biotic stress: leaves from control plants showing more than 30% damage caused by chewing herbivorous; 3 = biotic stress: plants grown in greenhouse under shade nettings, black with 70% light reduction and UV protection; 4 = biotic stress: plants subjected to soil limitation (plants were grown in small pots of 10 × 10 cm). All Plants were watered normally and leaves were collected in the morning at the full flowering stage (Berger *et al*., 2007). Collected leaves were immediately immersed under liquid nitrogen, stored at -80 °C, and freeze-drying (Labconco Corp., MO, USA).

Chemicals

The standards: kaempferol-3-O-rutinoside, quercetin-3-O-rutinoside and quercetin-3-O-β-glucopyranoside were purchased from Extrasynthese (Genay, France) and 4-hydroxybenzoic acid, vanillic acid, ferulic acid, p-coumaric acid, sinapic acid, salicylic, 3,4-dihydroxybenzoic acid, gallic acid, syringic acid and caffeic acid from Sigma-Aldrich and Fluka (Denmark).

Flavonoids and Phenolic Acids Extraction

The phenolic compounds extraction was carried out on an automatic Dionex ASE 350 Accelerated Solvent Extractor (Hvidovre, Denmark) as follows: Glowed chemically inert Ottawa sand (5 g) with particle size 20-30 mesh (Fisher Chemicals) were added to the 33 mL extraction cells. Subsequently, 0.1 g of the freeze-dried and homogenised sample was transferred to the extraction cell and another 5 g of sand were added. A filter was placed on the top of the sample, the extraction cell was filled with glowed glass balls, sealed, and placed in the carousel of the ASE extractor. The eluent for flavonoids was 70% MeOH/30% H_2O (v/v) and for the phenolic acids 80% MeOH/1% acetic acid/19% H_2O (v/v/v). The protocol for the ASE extraction was as follows: pre-heat for 5 min, heat for 5 min, static for 3 min, flush 80% vol, purge for 60 s, 4 cycles pressure 10^7 Pa, with temperature 40 °C for the flavonoids and 80 °C for phenolic acids. Extracts were collected in vials and filled to 50 mL with the eluent, then stored at -20 °C until chemical analysis.

Chemical Analysis of Flavonoids and Phenolic Acids

Before the flavonoid analysis, the extracts were filtered on a Sartorius SRP 15 0.45 μm filter (PTFE membrane) and diluted with water in a 1:1 ratio. For the phenolic acids extracts the same filter was used, but they were diluted with a solution of 7% acetonitrile/ H_2O with 20 mM acetic acid in a 1:1 ratio. A liquid chromatography-triple quadrupole mass spectrometer (LC/MS/MS, Agilent 1100/Applied Biosystems MDS Sciex API 2000) with turbo electrospray ionization in a positive multiple reactions monitoring mode was used for chemical analysis of the flavonoids. Each compound was initially optimized in the instrument using flow injection analysis of the pure compound. The chromatographic separation was performed at a flow ratio of 200 μL min^{-1} at 30 °C with an injection volume of 20 μL in a Phenomenex, Synergi 4μ Polar 80A column (250 × 2 mm). The A-eluent contained 7% acetonitrile and 93% milliQ water (v/v) with 20 mmol L^{-1} glacial acetic acid. The B-eluent was 78% acetonitrile and 22% milliQ water with 20 mmol L^{-1} glacial acetic acid. The gradient contained the following: 79% A and 21% B for 19 min followed by 3 min 40% A and 60% B followed by 12 min 100% B and a final equilibration 10 min 79% A and 21% B. The total run of the analysis was 46 min.

For the analysis of the phenolic acids, a HP-1100 series liquid chromatography-mass spectrometer (LC/MS) with an atmospheric pressure ionization-electrospray ionization chamber in a negative single ion-monitoring mode was used. Each compound was initially optimized in the instrument using flow injection analysis of the pure compound. The chromatographic separation was performed at 35 °C with flow ratio of 200 μL min^{-1}; the injection volume was 50 μL. The column was a Synergi 4μ Fusion-RP 80A. The A-eluent contained 7% ACN with 20 mmol L^{-1} acetic acid and the B-eluent contained 78% ACN with 20 mmol L^{-1} acetic acid. The gradient contained the following: 12 min to rise from 15% B to 20% B followed by 3 min to rise to 25% B and then 3 min of a linear gradient of 25% B and subsequently a 4 min ramp back to 15% B (Barba de la Rosa *et al*., 2009). The pure reference compounds were used to

identify the flavonoids and phenolic acids, based on a comparison of fragmentation patterns and retention times (Tables 1 and 2).

RESULTS AND DISCUSSION

Flavonoids Identification

It is known that colourless leaves have higher flavonoid contents as complementary protective action against ultraviolet radiation (Ng *et al*., 2000; Cai *et al*., 2006). However, it was not found a significant difference in flavonoids accumulation among green and red amaranth leaves. The three flavonoid glycosides tested (Table 1) were detected in *A. hypochondriacus* leaves. Rutin, which is considered as one of the most potent antioxidants (Enogieru *et al*., 2018), was the main flavonoid detected in amaranth leaves (Table 2). There were no differences in rutin concentrations between two amaranths cultivars, in control plants growing under normal conditions values were 7264 μg g^{-1} and 7151 μg g^{-1} for cultivars Criolla (green) and Nutrisol (red), respectively. However, plants subjected to biotic stress, rutin concentrations increased significantly in both amaranth species. In green leaves values were 9715 μg g^{-1} and in red leaves values were 7807 μg g^{-1} (Table 3). Limitation of nutrients was the abiotic stress with more effect on rutin accumulation where in green leaves values were similar to those obtained in biotic stress, in green leaves (9325 μg g^{-1}) and in red leaves (7672 μg g^{-1}). It has been reported that nutrient stress induces the accumulation of flavonoids in plants as soybean and tomato (Harborne and Williams, 2000; Klunklin and Savage, 2017). Rutin concentration in amaranth leaves detected in this work were higher than other plants considered as medicinal such as *Cnidoscolus chayamansa* (2 g rutin g^{-1} dried extract) and *Alpinia zerumbet* (83.2 μg g^{-1}) (Loarca-Piña *et al*., 2010; Victório *et al*., 2010), and several species of *Thymus* where rutin values reported of 21.3 ng g^{-1} (Boros *et al*., 2010). But several controversies about the rutin concentration amongst others amaranth species it was reported. Steffensen *et al*. (2011) reported values of 600 to 3000 μg g^{-1} for *A. mantegazzianus* and *A. hybridus*, respectively. Kalinova and Dadakova (2009) reported values from 2385 μg g^{-1} for *A. tricolor* and 13 950 μg g^{-1} for *A. hypochondriacus,* whilst Kolesnikov and Gins (2001) reported values of 24.5 mg g^{-1} for *A. cruentus*. For *A. spinosus* values of 150 mg g^{-1} were reported (Suryavanshi *et al*., 2007). The great variability of rutin concentration is due to the different amaranth species analysed, the type of extraction, and the place of growing, but in general amaranth leaves could be considered as an excellent source of rutin. Isoquercitrin was also found to be affected by environmental stresses, in green leaves values, values were 407 μg g^{-1} in control plants, and the higher amounts were detected in plants subjected to nutrient stress (930 μg g^{-1}) (Table 3). In similar way, in red leaves isoquercitrin was more accumulated in leaves from plants subjected to abiotic stresses, nutrients and light (611 μg g^{-1} and 686 μg g^{-1}, respectively) (Table 3). Isoquercitrin values from 1.3 to 6.5 g g^{-1} were found in several species of *Thymus* (Boros *et al*., 2010). Kalinova and Dadakova (2009) reported that higher amounts of rutin and quercetin and their derivate were found in old amaranth leaves, so age of plants is also important in phytochemical accumulation.

The higher concentration of nicotiflorin was detected in plants with green leaves subjected to nutrient stress (1397 μg g^{-1}). Bergquist *et al.* (2007)

Table 1. Flavonoids detected in amaranth leaves.

Short name	Systematic name	Cas number	Molar mass	Detection in LC/MS/MS
Rutin	Quercetin-3-O-rutinoside	153-18-4	610.51	611.0/302.9 Rt: 10.1 min
Isoquercitrin	Quercetin-3-O-β-glucopyranoside	482-35-9	464.38	465.0/302.8 Rt: 13.1 min
Nicotiflorin	Kaempferol-3-O-rutinoside	17650-84-9	594.53	595.1/286.9 Rt: 15.1 min

Table 2. Phenolic acids analysed in amaranth leaves.

Short name	Systematic name	Cas number	Molar mass	Detection in LC/MS
Vanillic acid	4-Hydroxy-3-methoxybenzoic acid	121-34-6	168.15	Mass: 167 Rt: 9.9 min
Ferulic acid†	Trans-4-Hydroxy-3-methoxycinnamic acid	1135-24-6	194.19	Mass: 193 Rt: 17.1 min
p-Coumaric acid†	Trans-4-Hydroxycinnamic acid	501-98-4	164.16	Mass:163 Rt: 15.1 min
4-HBA†	4-Hydroxybenzoic acid	99-96-7	138.12	Mass: 137 Rt: 9.0 min
Syringic acid	4-Hydroxy-3,5-dimethoxybenzoic acid	530-57-4	198.17	Mass: 197 Rt: 10.4 min
Protocatechuic acid	3-4-Dihydroxybenzoic acid	99-50-3	154.12	Mass: 153 Rt: 6.7 min
Caffeic acid†	3,4-Dihydroxy-cinnamic acid	331-39-5	180.16	Mass: 179 Rt: 10.8
Salicylic acid†	2-Hydroxybenzoic acid	69-72-7	138.12	Mass: 137 Rt: 22.3
Gallic acid	3,4,5-Trihydroxybenzoic acid	149-91-7	170.12	Mass: 169 Rt: 5.2
Sinapic acid	3,5-Dimethoxy-4-hydroxycinnamic acid	530-59-6	224.21	Mass: 223 Rt: 21.9

† Phenolic acids detected in amaranth leaves. 4-HBA = hydroxybenzoic acid.

indicated that shade nettings had no effect on flavonoid concentration in baby spinach, which is in agreement with the behaviour showed in amaranth leaves. In potato tubers nicotiflorin values ranged from 15 to 29 μg g^{-1} (Kröner *et al*., 2012). Nicotiflorin have been isolated at 96.5% purity from partially purified extracts from flowers of *Edgeworthia chrysantha* L. (Tong *et al*., 2009) and from aerial parts of *Peucedanum aucheri* Boiss (Dehaghani *et al*., 2017). The function of nicotiflorin in plants is not fully understood, it has been reported that its presence is correlated with resistance to potato tubers pathogens, while rutin does (Kröner *et al*., 2012).

It has been demonstrated that quercetin (the aglucone of rutin), isoquercitrin, and nicotiflorin have important biological functions such as: antitumor, antiinflammatory, antiallergenic, antiviral, and neuroprotective (Schroeter *et al*., 2001; Li *et al*., 2006; Russo, 2007; Srinivasan *et al*., 2007). Nicotiflorin also is known to have a protective effect against memory dysfunction and oxidative stress in multi-infarct dementia model rats (Huang *et al*., 2007).

Phenolic Acids

Ferulic acid was detected (0.5 μg g^{-1}) only in *A. hypochondriacus* cultivar Nutrisol (red leaves) subjected to nutrient stress, whereas coumaric acid was only detected (0.7 μg g^{-1}) in cultivar Criolla (green leaves) when plants were subjected to abiotic stress (Table 4). The higher concentration of caffeic acid of 29.9 μg $g^{-1}$and 33.3 μg g^{-1} for green and red leaves, respectively, were detected in plants collected in fields (Table 4). Only nutrients deficit causes a significantly reduction, until not detectable values, in both cultivars (Table 4). Values of caffeic acid have been reported in

Table 3. Concentration of flavonoids detected in *Amaranth hypochondriacus* L. leaves grown under different conditions.

Sample	Flavonoids		
	Rutin	Isoquercitrin	Nicotiflorin
	- - - - - - - - - - - - - - - μg g^{-1} - - - - - - - - - - - - - - -		
A. hypochondriacus cv Criolla (green leaves)			
Control	7264 c,d†	407 d	639 d
Biotic stress (insect damage)	9715 a	561 c	816 c
Abiotic stress (light limitation)	4343 e	526 c	785 c
Abiotic stress (nutrient limitation)	9236 a	930 a	1397 a
A. hypochondriacus cv Nutrisol (red leaves)			
Control	7151 d	371 d	622 d
Biotic stress (insect damage)	7807 b	401 d	523 e
Abiotic stress (light limitation)	3441 f	686 b	582 d,e
Abiotic stress (nutrient limitation)	7672 b,c	611 b,c	1014 b

† Means in the same column with different letter are significantly different at $P < 0.05$.

peels of fruit ranged from 130 to 24.25 μg g^{-1} (Zhang *et al*., 2010). In roots of red beetroot, the amount of caffeic acid was 0.012 mg g^{-1}, and this value increased to 0.396 mg g^{-1} when produced in extracts from hairy root cultures (Georgiev *et al*., 2010).

The pattern of accumulation of these three phenolic acids is in agreement with the values observed for *A. hybridus* and *A. mantegazzianus* (Steffensen *et al*., 2011). However, these phenolic acids are in slightly higher concentration than detected for *A. hypochondriacus*, which is the species with the higher rutin concentrations.

Salicylic acid was detected in green leaves (0.9 μg g^{-1}) only in samples subject to biotic stress. In red leaves, was detected in all plants growing under biotic or abiotic stresses (Table 4). This is

Table 4. Concentration of phenolic acids detected in *Amaranto hypochondriacus* L. red and green leaves grown under different conditions.

Sample	Phenolic acids				
	Ferulic	Coumaric	Caffeic	SA	4-HBA
	- - - - - - - - - - - - - - - - - - μg g^{-1} - - - - - - - - - - - - - - - - - -				
A. hypochondriacus cv Criolla (green leaves)					
Control	0	0	29.9 a,b	0	5.1 b
Biotic stress (insect damage)	0	0	30.5 a,b	0.9 a	0
Abiotic stress (light limitation)	0	0.7 a	0	0	6.6 b
Abiotic stress (substrate limitation)	0	0	0	0	0
A. hypochondriacus cv Nutrisol (red leaves)					
Control	0	0	33.3 a,b	0	0
Biotic stress (insect damage)	0	0	34.6 a	0.8 a	9.9 a
Abiotic stress (light limitation)	0	0	25.6 b	0.7 a	0.2 c
Abiotic stress (substrate limitation)	0.5 a	0	0	0.5 b,c	10.2 a

SA = salicylic acid. 4-HBA = 4-hydroxybenzoic acid. Means in the same column with different letter are significantly different at $P < 0.05$. The value 0 indicates that the result was below the limit of detection, determined as three times the standard deviation of the lowest standard.

interesting and correlates with the literature that has been established that salicylic acid is rapidly produced in some plants as a signal molecule to induce defence responses against insects, fungi, bacteria and viruses (Senaratna *et al*., 2000; Bodenhausen and Reymond, 2007). The effect of salicylic acid is still unclear, but its role in the induction of tolerance to several abiotic stresses is still being studied (Khan *et al*., 2015). Salicylic or acetylsalicylic acid increased the drought tolerance of tomato, bean and *Phillyrea angustifolia* plants (Munné-Bosch and Peñuelas, 2003), and is a key signalling molecule that mediates plant defence against a variety of pathogens. Its accumulation is required for the establishment of local and systemic required resistance (SAR) responses (Senaratna *et al*., 2000).

The analogue to salicylic acid, the 4-hydroxybenzoic acid (4-HBA), was found in cultivar Criolla (green leaves) in control plants and plants grown under light stress with values of 5.1 μg g^{-1} and 6.6 μg g^{-1}, respectively (Table 4). In cultivar Nutrisol (red leaves) the highest values (10.2 μg g^{-1}) were found in plants subjected to nutrient deficit (Table 4). 4-HBA is referred to as a biologically inactive compound; the physiological role of its accumulation in plants is not clear (Horváth *et al*., 2007). In lentils the levels of 4-HBA ranged from 15.8 to 44.9 g g^{-1} (Xu and Chang, 2010).

CONCLUSIONS

The three flavonoids tested (rutitn, isoquercetin, nicotiflorin) in *Amaranthus hypochondriacus* leaves were higher in cultivar Criolla, which has green leaves than in cultivar Nutrisol (red leaves). In both cultivars, rutin was the main flavonoid with concentrations higher than those values reported for traditional medicinal plants. The accumulation of this flavonoid is increased when plants were grown under abiotic stress other than UV radiation. Ferulic acid was only detected in red leaves, while coumaric acid was found only in green leaves. Caffeic acid was the main phenolic acid in both red and green leaves and UV radiation seems to be one stress factor that directs its production. Salicylic acid was present in green leaves when they were subjected to biotic or abiotic stresses. In red leaves salicylic acid was found when plant grown under limitation of light and biotic stress. These findings indicate that the production of flavonoids and phenolic acids in amaranth leaves could be directed by growing conditions. The generated data support that *A. hypochondriacus*, which is the main specie used for edible seed production, could also be used as vegetable source because its leaves are rich source of phytochemicals.

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