PHENOLIC COMPOUNDS AND ANTIOXIDANT ACTIVITY IN LEAVES OF THREE AMARANTH SPECIES AS EFFECT OF CULTIVATION LOCATION AND FERTILIZATION

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SUMMARY

The objective was to evaluate the effect of crop location, fertilization, species and genotypes on the content of flavonoids and total polyphenols and on antioxidant activity in the leaves and sprouts of amaranth. Sowing and cultivation were carried out in Huaquechula, Puebla, and Tepetitla, Tlaxcala, Mexico under a factorial arrangement of 16 genotypes of Amaranthus hypochondriacus *L. (2)*, A. hybridus *L. (8) and* A. cruentus *L. (6) and two fertilization conditions (with and without 18N-07P-09K) under a randomized block design with two repetitions. At 40 days after planting, a sample of leaves and sprouts was collected, dried at room temperature and ground; later, the flavonoids, total polyphenols and antioxidant activity (methods DPPH and FRAP) were quantified by UV-visible spectrophotometry and reference standards. The results showed that crop location, use or nonuse of fertilizer, species and genotype had a significant effect on flavonoids, total polyphenols and antioxidant activity. Huaquechula had days with low precipitation and high temperatures; such environmental conditions influenced the content of flavonoids and total polyphenols and antioxidant activity.* A. hybridus *showed high flavonoid and total polyphenol values that were significantly different from the values recorded for* A. hypochondriacus *and* A. cruentus.

Introduction

The genus *Amaranthus* includes 70 species globally, eleven of which are widely distributed in Mexico and Central America: *Amaranthus cruentus*, *A. hybridus*, *A. hypochondriacus*, *A. powellii*, *A. tricolor*, *A. blitum*, *A. viridis*, *A. retroflexus*, *A. palmeri*, *A. dubius* and *A. spinosus* (Espitia-Rangel *et al*., 2010; Das, 2012; Mapes and Basurto, 2016; Ruiz-Hernández *et al*., 2018). In addition, more than 250 native species with edible leaves, flowers and sprouts are used in the same region; in Mexico and in Central America, they are called '*quelites*', and the ones most frequently used since pre-Columbian times are *huauzontle*, *quelite*, *romerito* (seepweed), *verdolaga* (purslane) and amaranth. Amaranth is widely used in the diet of indigenous communities as part of their customs, reflecting the traditional use of regional biodiversity but is an undervalued and underutilized resource in terms of its nutritional contribution and its functional compounds (Castro-Lara *et al*., 2011; Mapes and Basurto, 2016; Santiago-Sáenz *et al*., 2019). For this reason, it is common for cultivated and wild species to be frequently reported in ethnobotanical studies in Mesoamerica (Basurto-Peña *et al*., 1998; Castro-Lara *et al*., 2014; Balcazar-Quiñones *et al*., 2020). However, it remains necessary to document the specific contributions of the nutritional and bioactive compounds of amaranth with regard to improving the health of families.

Obesity, overweight and associated diseases are worldwide problems, often linked to dietary transitions or the abandonment of traditional diets. According to the national survey of health and nutrition conducted in mid-2016 in Mexico, 33.2, 36.3 and 72.5% of children, adolescents and adults, respectively, were considered obese or overweight due to inactivity (82.8, 39.5 and 14.4%, respectively, reported activity <35 min), low consumption of vegetables (22.6, 26.9 and 42.3%, respectively) and an overall increase in meat consumption (86.7%) (Secretaría Salud, 2016). This problem is not exclusive to Mexico but, rather, is a global problem (Polyzos and Mantzoros, 2019). In the context of this food-related public health problem, amaranth leafy greens can play an essential role in health as they provide proteins, amino acids, minerals, vitamins E and C and fiber, and are an important source of bioactive compounds, such as phenolic compounds, flavonoids and pigments (e.g., chlorophylls or betalains) that have high antioxidant activity (Santiago-Sáenz *et al*., 2019). The traditional diet based on local native plants is being revisited through updated information on nutritional and nutraceutical compounds (Gálvez-Mariscal and Peña-Montes, 2015).

The flavonoid content of amaranth leafy greens, such as rutin and quercetin, varies

KEYWORDS / Bioactive Compounds / Environmental Effect / Nutritional-Nutraceutical Potential/ Spectrophotometry /

Received: 01/12/2022. Modified: 12/13/2022. Accepted: 12/14/2022.

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COMPUESTOS FENÓLICOS Y ACTIVIDAD ANTIOXIDANTE EN HOJAS DE TRES ESPECIES DE AMARANTO COMO EFECTO DE LOCALIDAD DE CULTIVO Y FERTILIZACIÓN

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RESUMEN

El objetivo fue evaluar el efecto de localidad de cultivo, fertilización, especies y genotipos en el contenido de flavonoides, polifenoles y actividad antioxidante en hojas y brotes de amaranto. La siembra y cultivo se condujo en Huaquechula, Puebla y en Tepetitla, Tlaxcala, México bajo un arreglo factorial de 16 genotipos de Amaranthus hypochondriacus *L. (2),* A. hybridus *L. (8) y* A. cruentus *L. (6) y dos condiciones de fertilización (con y sin 18N-07P-09K) bajo un diseño de bloques al azar con dos repeticiones. A 40 días de la siembra se recolectó una muestra de hojas y brotes, se secaron a temperatura ambiente y molieron, posteriormente se cuantificó el contenido de flavonoides, polifenoles totales y actividad antioxidante (métodos FRAP y DPPH), mediante espectrofotometría UV-visible y estándares de referencia. Los resultados muestran que, localidad de cultivo, uso o no de fertilizante, especies y genotipos tienen efecto significativo en el contenido de flavonoides, polifenoles totales y actividad antioxidante. Huaquechula presentó días de baja precipitación y altas temperaturas, condición ambiental que influyó en mayor concentración de flavonoides, polifenoles totales y actividad antioxidante.* A. hybridus *presentó valores altos de flavonoides y polifenoles totales significativamente diferentes de los valores registrados para* A. hypochondriacus *y* A. cruentus.

COMPOSTOS FENÓLICOS E ATIVIDADE ANTIOXIDANTE EM FOLHAS DE TRÊS ESPÉCIES DE AMARANTO COMO EFEITO DO LOCAL DE CULTIVO E DA FERTILIZAÇÃO

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RESUMO

O objetivo foi avaliar o efeito do local de cultivo, fertilização, espécies e genótipos sobre o teor de flavonoides, polifenóis e atividade antioxidante em folhas e brotos de amaranto. A plantação e cultivo foram realizados em Huaquechula, Puebla e Tepetitla, Tlaxcala, México sob um arranjo fatorial de 16 genótipos de Amaranthus hypochondriacus *L. (2),* A. hybridus *L. (8) e* A. cruentus *L. (6) e duas condições de fertilização (com e sem 18N-07P-09K) seguindo um desenho de blocos casualizados com duas repetições. Aos 40 dias após a plantação foi coletada uma amostra de folhas e brotos, secas em temperatura ambiente e moídas, posteriormente foram quantificados os teores de flavonoides, polifenóis totais e atividade antioxidante (métodos FRAP e DPPH), mediante espectrofotometria UV-visível e padrões de referência. Os resultados mostram que, local de cultivo, uso ou não de fertilizantes, espécies e genótipos têm efeito significativo sobre o teor de flavonoides, polifenóis totais e atividade antioxidante. Huaquechula apresentou dias de baixa precipitação e altas temperaturas, condição ambiental que influenciou na maior concentração de flavonóides, polifenóis totais e atividade antioxidante.* A. hybridus *apresentou valores altos de flavonóides e polifenóis totais significativamente diferentes dos valores registrados para* A. hypochondriacus *e* A. cruentus.

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depending on the part of the plant that is consumed; for example, flavonoid content is greater in leaves than in flowers or stems and varies according to the crop cycle from year to year and according to the variety and species of amaranth (Kalinova and Dadakova, 2009). Nana *et al*. (2012) claim that the polyphenol and flavonoid content and antioxidant activity are higher in *A. hybridus* than in *A. cruentus*. A similar finding was reported by Niveyro *et al*. (2013), who concluded that the concentration of the flavonoid nicotiflorin is higher in *A. mantegazzianus*; *A. hypochondriacus* has increased concentrations of rutin and iso-quercetin; and *A. cruentus* has the lowest levels of these three compounds. Differences in bioactive compounds, minerals and antioxidant activity among species have been widely reported in different studies (Khanam and Oba, 2013; Li *et al*., 2015; Jiménez-Aguilar and Grusak, 2017; Schröter *et al*., 2018). However, the effect of mineral fertilization-crop location interactions on the content of bioactive-functional compound and on antioxidant activity is less known. It is also noteworthy that bioactive compounds have different preventive effects on metabolic disorders and diseases due to their anticancer and antitumor (Joshua *et al*., 2010; Baskar *et al*., 2012), antidiabetic and hypolipidemic (Girija *et al*., 2011), anti-inflammatory (Bihani *et al*., 2013), antibacterial, anticholesterolemic and cardiovascular protective effects (Jimoh *et al*. 2019).

In rural communities in southeastern Mexico and Central America, the consumption of fresh amaranth (*Amaranthus* spp.) collected from the wild or from plants sown for the production of grain is common. Previous works concluded that there are substantial differences among species in terms of bioactive compounds and that these compounds also change according to the developmental phase of the plant. In this context, the objective was to evaluate the changes in flavonoids, total polyphenols and antioxidant activity in leaves and sprouts of *Amaranthus hypochondriacus*, *A. hybridus* and *A. cruentus* influenced by crop location and fertilizer use.

Material and Methods

Species and genotypes evaluated

Flavonoids, total polyphenols and antioxidant activity were evaluated in 16 genotypes of three species of amaranth:

AV25, AV28, AV7, AV3, CP30, Areli, Laura and Nutrisol of the species *Amaranthus hypochondriacus*; AV17, AV8, AV29 and AV19 of *A. hybridus*, which came from Zapotitlán de Méndez, Puebla; and CP38, CP39, CP15 and Benito belonging to *A. cruentus* from Tochimilco, Puebla, and Huazulco, Morelos, Mexico. More details on the origin and grain characteristics of the evaluated genotypes were reported in Ortiz-Torres *et al.* (2018).

Experimental design and management

In Huaquechula, Puebla (18°45'58.3"N, 98°33'25.14"W, 1577m altitude, climate semi-hot subhumid, soil type loam-clay-sandy, vegetation subdeciduous to temperate (17.93%), forest (7.27%), another (4.76%) and pasture (3.49%), planting September 8), and Tepetitla, Tlaxcala (19°16'31.31"N, 98°23' 20.07"W, 2228m altitude, climate temperate subhumid, soil type loam-clay-sandy, vegetation agriculture and pasture (0.91%), planting 24 August), Mexico, during the 2018 spring-summer cycle, two experiments were established using a 16 x 2 factorial treatment arrangement in a randomized complete block design with two repetitions. The genotype factor consisted of 16 genotypes, and the fertilization factor consisted of two levels, with and without 18N-07P-09K at sowing using urea [CH_4N_2O], diammonium phosphate (18-46-0) [$(NH_4)_2HPO_4$] and potassium chloride [KCl] as sources. The experimental plot consisted of two rows that were 5m long and 0.8m wide. The temperature and precipitation at each experimental site were recorded (Figure 1). At the time of sowing, soil samples were taken at a depth of 30cm and a general fertility analysis was performed (Table I).

The experiments were conducted under rain-fed conditions and without irrigation. Weed control was performed mechanically and manually, and cypermethrin was applied at the edges to control grasshoppers (*Sphenarium purpurascens* Charp.). The plants were harvested 40 days after sowing and leaves and sprouts were separated and dried in the shade for 15 days. Subsequently, the samples were placed in a forced air-drying oven (Thermo Scientific) for 48 h at a temperature of 48°C and constant humidity. Once dried, they were placed in vials, kept in a dry place and isolated from light until analysis.

Preparation of samples for analysis

From each experimental plot, 0.2g of powdered sample was taken; 20ml of 80% ethanol was added for the determination of total polyphenols and flavonoids; and 20ml of 80% methanol was used for the analysis of antioxidant activity using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) and Ferric ion Reducing Antioxidant Power (FRAP) methods. Samples were homogenized in an Ultra Turrax disperser (IKA T75 Digital) for 90s and centrifuged at 11,000rpm (5810R Eppendorf refrigerated centrifuge) for 15min at a temperature of 4°C. The supernatants were separated for analysis. All extractions were performed in triplicate.

TABLE I
DESCRIPTION OF SOIL TEXTURE AND FERTILITY IN CROP LOCATIONS AT PUEBLA AND TLAXCALA, MEXICO

Location descriptors	Huaquechula, Puebla	Tepetitla, Tlaxcala
Climatic condition	Semi-hot subhumid	Temperate subhumid
Soil type	Loam-clay-sandy	Loam-clay-sandy
pH	6.90	6.86
Organic matter (%)	0.99	2.97
P-Bray (ppm)	17.8	340.0
K (ppm)	257.0	1213.0
Ca (ppm)	2078.0	1337.0
Mg (ppm)	491.0	432.0
Na (ppm)	27.5	35.0
Fe (ppm)	15.0	17.90
Zn (ppm)	0.69	6.02
Mn (ppm)	19.4	2.79
Cu (ppm)	1.37	0.80
B (ppm)	0.17	0.45
S (ppm)	1.38	20.70
$N\text{-}NO_3$ (ppm)	30.2	494.0

Evaluation of flavonoids, total polyphenols and antioxidant activity

The flavonoid content was determined using the method described by Lin and Tang (2007). To 500µl of the extract, 1.5ml of 95% ethanol, 100µl of aluminum chloride, 100µl of potassium acetate and 2.8ml of deionized water were added; the solution was vortexed and left to stand for 40 min at room temperature. Absorbance was then recorded at a wavelength of 415nm using a UV-visible spectrophotometer (Shimadzu UV-1800, Kyoto, Japan). For the quantification of flavonoids, a calibration curve with a quercetin standard was used as a reference at a range of 0.01 to 0.07mg·ml^{-1} (r^2= 0.999). The results were expressed in mg of quercetin (Q) equivalents (E) per g of dry weight (dw).

The determination of total polyphenols was performed according to the method proposed by Singleton and Rossi (1965). To 400µl of extract, 1 ml of deionized water and 200 µl of Folin-Ciocalteu reagent were added. The mixture was stirred and incubated for 5 min. Subsequently, 2ml of Na_2CO_3 at 7% and 1.4ml of deionized water were added, and the solution was vortexed and left to stand for 1 h at room temperature. Next, absorbance readings were taken at a wavelength of 750nm

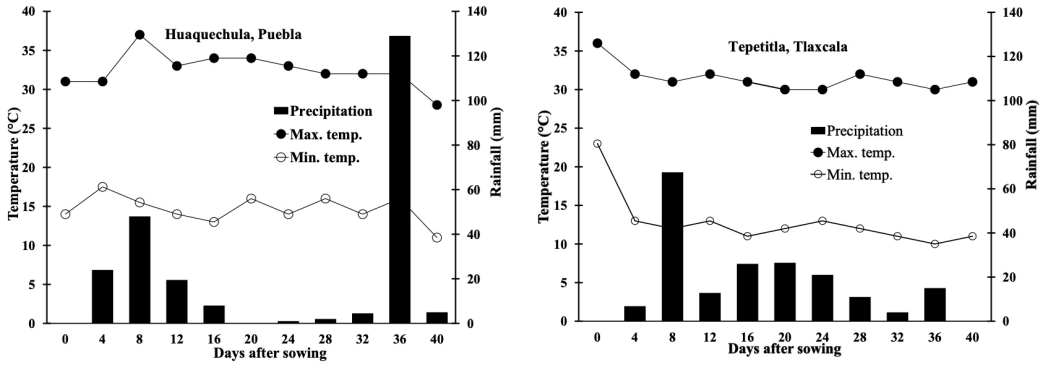

Figure 1. Maximum and minimum temperatures (°C) and rainfall (mm) at the crop locations from Huaquechula, Puebla, and Tepetitla, Tlaxcala, Mexico.

with a UV-visible spectrophotometer; quantification was based on a calibration curve using a gallic acid standard at a concentration range of 0.021 to 0.165 mg·ml^{-1} (r^2= 0.999). The results were reported in mg of gallic acid (GA) equivalents (E) per g of dw.

Antioxidant activity was determined by DPPH using the method formulated by Brand-Williams *et al*. (1995). To 100 µl of the extract, 2.9ml of DPPH reagent was added. The solution was stirred and incubated for 30min at room temperature. Subsequently, readings were taken at a wavelength of 517nm with a UV-visible spectrophotometer. Antioxidant activity was recorded based on a calibration curve of Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) from 0.133 to 1.33 µmol ·ml^{-1} and expressed in µmol TE ·g^{-1} dw.

Antioxidant activity was analyzed using FRAP according to the method described by Benzie and Strain (1996). To 100µl of the methanolic extract, 3ml of FRAP reagent was added. The solution was incubated in a water bath at 37°C for 30min and absorbance was recorded at a wavelength of 593nm. The quantification of antioxidant activity was based on a Trolox calibration curve of 50 to 1000 µmol·L^{-1} and the results were expressed as µmol TE ·g^{-1} dw.

Statistical analysis

Based on the experimental field design, the analysis of combined variances was performed using a linear model of randomized complete blocks with repetitions nested in crop locations and genotypes nested in species. Complementarily, multiple comparisons of means were performed using Tukey's test ($p \leq 0.05$). Analyses were performed using the SAS statistical package (SAS Institute, 2002, Inc. Cary, North Carolina, USA). The location-genotype and fertilization-genotype interactions are presented graphically.

Results and Discussion

The nutritional and nutraceutical potential or properties of amaranth consumed as a *quelite* were defined using robust information regarding the content of bioactive compounds. In this case, the combined analysis of variance showed significant differences ($p \leq 0.01$) in flavonoid and total polyphenol contents and antioxidant activity according to crop location, use of fertilizer, species and genotype within species and all interactions of principal effects; the exception was the interaction effect of location-fertilization on antioxidant activity evaluated using the FRAP method (Table II). The differences between the principal factors and interactions indicate that the crop location, use or nonuse of fertilizer, species and genotype and the interactions of these factors influence the flavonoids, total polyphenols and antioxidant activity in the leaves and sprouts of amaranth, which are the parts consumed as *quelites*.

The concentrations of flavonoids, total polyphenols and antioxidants differed according to crop location, and significantly higher values were determined in plants from Huaquechula, Puebla, than in those from Tepetitla, Tlaxcala, Mexico. This indicates that the growing environment determines the concentration of bioactive compounds. In the present study, the quantity and distribution of rainfall differed between crop locations: a period of water stress was recorded in Huaquechula from 16 to 32 days after sowing but this was not the case in Tepetitla. A similar pattern was observed for minimum and maximum temperatures (Figure 1) and nitrogen content in crop soil (Table I); thus, this stress condition, among other factors, led to higher bioactive compound content and antioxidant activity in the plants grown in Huaquechula than in those grown in Tepetitla and confirms the environmental effect on the biosynthesis of secondary metabolites (Table III). A similar pattern was reported by Sarker and Oba (2018a) when evaluating the effect of three water deficit regimens (low to severe) in *A. tricolor*; the researchers concluded that the polyphenol and flavonoid content and antioxidant activity evaluated by DPPH increased as water stress became more severe. Even the severity of the stress generated different concentrations of specific phenolic compounds, such as benzoic acids, cinnamic acids and quercetin (Sarker and Oba, 2018a; Sarker and Oba, 2018b).

When growing amaranth for grain, it is common to apply fertilizers to improve and increase yield; however, when it is used for *quelite* or *quintonil*, fertilizers are traditionally not applied. In the present study, the application of 18-07-09 (N-P-K) fertilizer generated a significant increase in flavonoid, total polyphenol and antioxidant activity compared with no fertilizer use (Table III). Conversely, Onyango *et al*. (2012) did not find significant differences in the phenol content of *A. hypochondriacus* leaves when nitrogen was applied. In contrast, regarding antioxidant activity, our results coincided with the findings of Skwaryło-Bednarz and Krzepiłko (2019) in leaves of *A. cruentus*.

Regarding the location-fertilization interaction, we observed that in Huaquechula, Puebla, the application of fertilizer generated higher flavonoids, total polyphenols and antioxidant activity (evaluated

TABLE II
SIGNIFICANCE OF SQUARE MEANS FROM THE ANALYSIS OF VARIANCE OF FLAVONOIDS, TOTAL POLYPHENOLS AND ANTIOXIDANT ACTIVITY IN AMARANTH LEAFY VEGETABLES

Sources of variation	Flavonoids (mg QE. g^{-1})	Total polyphenols (mg GAE.g^{-1})	Antioxidant activity (µmol TE.g^{-1})	
			DPPH	FRAP
Locations (L)	491.62**	6985.8**	108268.1**	187927.6**
Fertilization (F)	50.72**	23.0**	110.6**	1058.8**
Species (S)	8.09**	13.9**	1507.4**	2709.3**
Genotype (G)/Specie (S)	8.29**	16.5**	488.3**	790.5**
L x F	1.72**	65.9**	547.6**	3.8^{ns}
L x S	17.30**	34.8**	990.5**	3028.1**
F x S	17.65**	28.9**	150.1**	281.3**
L x G/S	3.64**	26.4**	575.3**	829.4**
F x G/S	4.84**	29.3**	535.1**	894.6**
L x F x S	4.13**	72.8**	85.4**	87.7**
L x F x G/S	7.56**	20.9**	672.0**	979.4**
Repetitions/L	0.02^{ns}	0.41**	5.39^{ns}	18.2**
Replicate in Lab./Repetitions	0.01**	0.02^{ns}	9.60^{ns}	0.52^{ns}
Error	0.02	0.03	3.40	1.64
Coefficient of variation (%)	3.1	2.5	6.4	4.4

^{ns}Not significant ($p > 0.05$); **Significant at $p \leq 0.01$.

TABLE III
EFFECT OF CROP LOCATION, FERTILIZATION AND LOCATION-FERTILIZATION INTERACTION ON FLAVONOIDS, TOTAL POLYPHENOLS AND ANTIOXIDANT ACTIVITY IN AMARANTH LEAVES AND SPROUTS

Main factors and interactions	Flavonoids (mg QE. g^{-1})	Total polyphenols (mg GAE.g^{-1})	Antioxidant activity (μmol TE.g^{-1})	
			DPPH	FRAP
Locations†				
Huaquechula, Pue.	5.11 a	10.76 a	42.8 a	47.1 a
Tepetitla, Tlax.	3.29 b	3.64 b	14.7 b	10.7 b
Fertilization†				
With fertilizer	4.54 a	7.46 a	29.4 a	30.1 a
Without fertilizer	3.86 b	6.94 b	28.2 b	27.7 b
Location-fertilization interactions†				
Huaquechula, Pue.				
With fertilizer	5.44 a	11.48 a	42.7 a	48.5 a
Without fertilizer	4.78 b	10.03 b	42.9 a	45.8 b
Tepetitla, Tlax.				
With fertilizer	3.64 c	3.44 d	16.0 b	11.8 c
Without fertilizer	2.94 d	3.84 c	13.5 c	9.7 d

†Among locations, fertilization and location-fertilization interactions, the means with the same letter are not significantly different (Tukey's test, $p \leq 0.05$).

by FRAP); this finding was the opposite of the interaction response determined for Tepetitla, Tlaxcala and for plants grown without fertilizer. In particular, in Tepetitla, the application of fertilizer yielded a lower total polyphenol content than that without fertilizer (Table III). Munene *et al*. (2017) determined lower amounts of phenolic compounds and flavonoids in amaranth leaves when they added nitrogen fertilizer (NO_3^-). Urea or another nitrogen source, produces greater plant biomass (Motasim *et al*., 2022; Ordoñez-Monroy *et al*., 2022), under a normal condition without stress, and not increase substantially the concentration of phenolic compounds, PAL enzymatic activity and the PAL gene, and suppresses MYB transcription factor (regulators of phenylpropanoid metabolism) (Sun *et al*., 2020; Liu *et al*., 2015), but any N-deficiency during the period of greatest demand causes nutritional stress, reduction of soluble proteins, increase availability of amino acid phenyl alanine, and stimulate biosynthesis of secondary plant metabolites (Kováčik and Bačkor 2007; Ibrahim *et al*., 2011). Moreover, in *Labisia pumila* plants, the application of urea caused a reduction in the concentration of phenolic compounds and total flavonoids, but, in contrast, the absence or low concentrations of nitrogen increased L-phenylalanine ammonium lyase (PAL, EC 4.3.1.5) enzyme activity and consequently the production of phenolic compounds and flavonoids (Ibrahim *et al*., 2011). Taken together, these findings confirm that the phenolic compounds and antioxidant activity in amaranth leaves are strongly influenced by crop location and growing environment, especially stress conditions, and they present a pattern similar to the environmental effect that has been widely documented for grain composition (Venskutonis and Kraujalis, 2013). However, according to López-García *et al*. (2018), the flavonoid and polyphenol content of *A. hybridus* change from one growing season to the next.

Modi (2007) noted that temperature has a significant effect on the nutritional quality of amaranth leaves, high content of total protein and amino acid concentration in the leaves is associated with cool environmental conditions, and mineral nutrients, Ca and Fe increase in the leaves in response to increasing growth temperature.

Among *A. hybridus*, *A. hypochondriacus* and *A. cruentus*, there were significant differences in flavonoids, total polyphenols and antioxidant activity in leaves and sprouts. *A. hybridus* showed high values that were significantly different from those recorded for *A. hypochondriacus* and *A. cruentus*. However, the highest antioxidant activity, based on FRAP and DPPH methods, was observed for *A. cruentus* (which has reddish leaves and reddish inflorescences), followed by *A. hypochondriacus* (which has ash-colored leaves with reddish tones and reddish inflorescences) and *A. hybridus* (which has light green leaves, sometimes with reddish margins and ribs), which had the lowest values (Table IV). Jiménez-Aguilar and Grusak (2017) also determined significant differences between *A. cruentus* and *A. hybridus* in terms of flavonoids, polyphenols and antioxidant activity, although the present study recorded slightly higher values. Together, these findings indicate that species respond differently to environmental conditions based on their genetic constitution or genealogical origin.

Within each species, it was observed that the various genotypes differed significantly. For example, in terms of flavonoid content, the genotypes of *A. hybridus* varied from 3.2 to 5.2 mg QE $\cdot g^{-1}$ dw and were significantly different from those of *A. cruentus* (3.3 to 4.6 mg QE $\cdot g^{-1}$) and *A. hypochondriacus* (4.2 to 4.6 mg QE $\cdot g^{-1}$). A similar pattern was seen in total polyphenol content, which varied from 7.0 to 7.6, 6.4 to 8.2 and from 6.2 to 7.7mg GAE $\cdot g^{-1}$ among genotypes of *A. hypochondriacus*, *A. hybridus* and *A. cruentus*, respectively (Table IV). Among four varieties of *A. bicolor*, Sarker and Oba (2018a) determined high variation in polyphenols, flavonoids and antioxidant activity, although they found significantly higher values than those reported in the current study; a similar pattern was also determined by Sarker *et al.* (2020) among 12 genotypes of *A. bicolor*. The genotypes with the highest flavonoid and total polyphenol content were AV-17 and AV-19 for *A. hybridus* and CP-38 for *A. cruentus*. Conversely, the lowest flavonoid content was found for AV-25 (*A. hybridus*) and CP-15 (*A. cruentus*), and AV-28 and AV-8 (*A. hybridus*) had the lowest total polyphenol content. The genotypes with the highest antioxidant activity were CP-38 and CP-39 of the *A. cruentus* species and AV-19 and AV-3 of *A. hybridus*.

In terms of fertilization-genotype interaction, the genotype Nutrisol (*A. hypochondriacus*) and genotypes AV-17, AV-29 and AV-3 (*A. hybridus*) showed similar concentrations of flavonoids with or without fertilizer. This pattern was also found for total polyphenol content for the genotypes AV-19 and AV-29 of *A. hybridus* and CP-39 of *A. cruentus*. Regarding both compounds, *A. hybridus* was more stable than *A. hypochondriacus* and *A. cruentus*. In contrast, in terms of antioxidant activity, *A. cruentus* was more stable than *A. hybridus* or *A. hypochondriacus*, and the most stable genotypes were Benito, CP-15,

TABLE IV
FLAVONOIDS, TOTAL POLYPHENOLS AND ANTIOXIDANT ACTIVITY IN *A. hybridus*, *A. hypochondriacus* AND *A. cruentus* LEAVES AND SPROUTS

Species and genotypes	Flavonoids (mg QE. g^{-1})	Total polyphenols (mg GAE.g^{-1})	Antioxidant activity (µmol TE.g^{-1})	
			DPPH	FRAP
Amaranthus hybridus				
AV-17	5.2 a†	8.1 a	26.9 f	28.4 h
AV-19	4.2 d	8.2 a	31.4 b	33.7 b
AV-29	4.6 b	7.5 c	28.4 e	26.9 i
AV-8	3.9 f	6.5 h	21.7 h	20.4 k
Average	***4.4 A†***	***7.3 A***	***26.1 C***	***26.4 C***
Amaranthus hypochondriacus				
Areli	4.2 d	7.6 b	30.0 c	30.0 g
AV-25	3.2 h	5.8 j	20.8 h	19.4 l
AV-28	3.8 g	6.4 h	25.3 g	24.6 j
AV-3	4.4 c	7.4 d	32.5 b	33.6 b
AV-7	4.0 f	7.2 e	30.1 c	31.5 e
CP-30	3.9 f	7.5 b	24.6 g	21.2 k
Laura	4.4 c	6.2 i	29.4 d	28.7 h
Nutrisol	4.6 b	7.0 f	28.9 d	27.7 h
Average	***4.1 B***	***7.0 C***	***28.3 B***	***27.6 B***
Amaranthus cruentus				
Benito	4.1 e	6.7 g	30.4 c	31.1 f
CP-15	3.3 h	6.8 f	30.1 c	32.2 d
CP-38	4.6 b	7.7 b	32.2 b	32.6 c
CP-39	4.1 e	7.7 b	37.9 a	40.8 a
Average	***4.0 C***	***7.2 B***	***32.6 A***	***34.2 A***

†In columns, among genotypes and among species, the means with the same letter (normal or capital) are not significantly different (Tukey's test, $p \leq 0.05$).

CP-38 and CP-39 from *A. cruentus* and AV-3 from *A. hybridus* (Figure 2). The described patterns show that the genotypes respond differently to the application of fertilizer, as reflected in the bioactive compounds and antioxidant activity observed in leaves and some patterns indicate that the application of fertilizer does not substantially improve the final content. That is, the nutritional composition of AV-17, CP-39 and AV-29 for consumption remained high and constant, with or without the application of fertilizer. However, in another group of genotypes (e.g., AV-25), concentrations remained low despite the addition of fertilizer.

In the interaction of location-collection, variety or species, it was observed that the Areli genotype (*A. hypochondriacus*) and AV-17 and AV-29 (*A. hybridus*) had stable total polyphenol and flavonoid contents regardless of crop location. The genotypes AV-25 and AV-28 (*A. hybridus*) were also stable but had low total polyphenol and flavonoid contents. Alternatively, AV-8 (*A. hybridus*) performed better in Tepetitla, Tlaxcala, in terms of flavonoid content, and CP-39 (*A. cruentus*) showed the highest values of total polyphenols in Huaquechula, Puebla. Genotypes AV-19 (*A. hybridus*), Benito, Laura, CP-38 and CP-39 (*A. cruentus*) presented stable antioxidant activity across crop locations (Figure 3). These results show the considerable heterogeneity of responses among genotypes in response to environmental or crop location effects and that genotype-environment interactions are significant. In terms of total polyphenol and flavonoid content, *A. hybridus* genotypes performed better, but in terms of antioxidant activity, *A. cruentus* genotypes stood out, with *A. hypochondriacus* having intermediate responses between the two.

In the triple interaction crop location-fertilization-genotypes or species, the patterns of bioactive compounds and antioxidant activity in amaranth leaves were different. Generally, the fertilized genotypes showed higher flavonoid and total polyphenol contents and antioxidant activity in Huaquechula, Puebla (location 1), than in Tepetitla, Tlaxcala (location 2), with or without fertilizer. Specifically, in Huaquechula, AV-17 (*A. hybridus*), CP-38 (*A. cruentus*) and Areli (*A. hypochondriacus*) had higher flavonoid content without the application of fertilizer than with fertilizer. An opposite pattern was observed for total polyphenols in genotypes AV-19 (*A. hybridus*), AV-7, AV-3 and CP-30 (*A. hypochondriacus*) grown with fertilizer in Huaquechula. The lowest values of flavonoids, total polyphenols and antioxidant activity were found in the unfertilized plants from Tepetitla. The genotypes with the highest values were AV-19 for *A. hybridus*, CP-38 and CP-39 for *A. cruentus*, and AV-3, Laura and Areli for *A. hypochondriacus* (Table V). Consequently, fertilization does not guarantee the induction of major concentrations of bioactive compounds, particularly when the environment has restrictive conditions, such as limited rainfall.

The flavonoid and total polyphenol content and antioxidant activity in amaranth leaves are strongly influenced by crop location, use of fertilizers, species and genotypes, with double and triple interactions of these factors (Table II). The significant interaction effects showed that total polyphenols and flavonoids, products of secondary metabolism, changed or increased under certain stressful conditions. In the present study, water stress (days without rain or with low rainfall in the case of Huaquechula, Puebla) yielded higher concentrations of total polyphenol compounds and antioxidant activity, and higher concentrations were recorded with the application of fertilizers (N-P-K:18-07-09), mainly in genotypes of the species *A. hybridus* and *A. cruentus*, throughout two annual cultivation cycles. These results are consistent with the findings of Sarker and Oba (2018b) in *A. tricolor* under three conditions of water stress. Venskutonis and Kraujalis (2013) reported that fertilization and environmental factors influence total polyphenol concentration in amaranth leaves. However, it seems important to remark that species and/or genotype play a key role (Jiménez-Aguilar and Grusak, 2017; Sarker and Oba, 2018a; Sarker and Oba, 2019a; Sarker and Oba, 2019b).

Conclusions

Based on the results of the present study, flavonoids, total

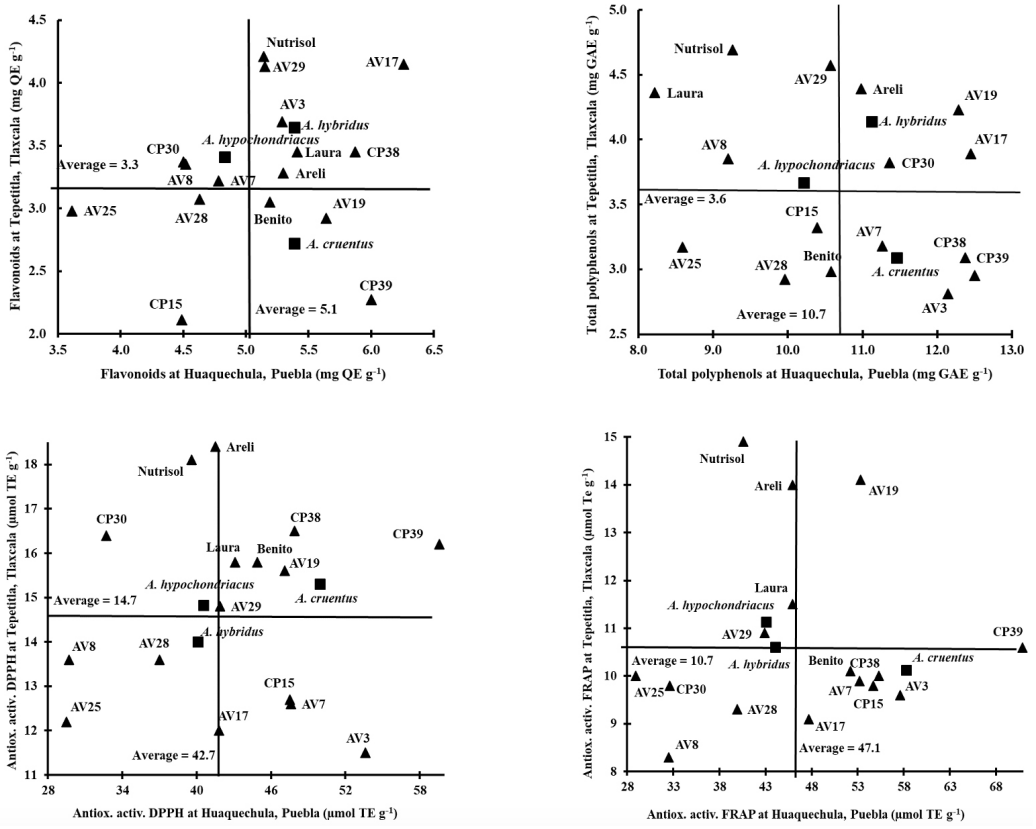

Figure 2. Dispersion of amaranth genotypes as a function of the interaction location-genotype and species with respect to the flavonoids, total polyphenols and antioxidant activity of leaves and sprouts.

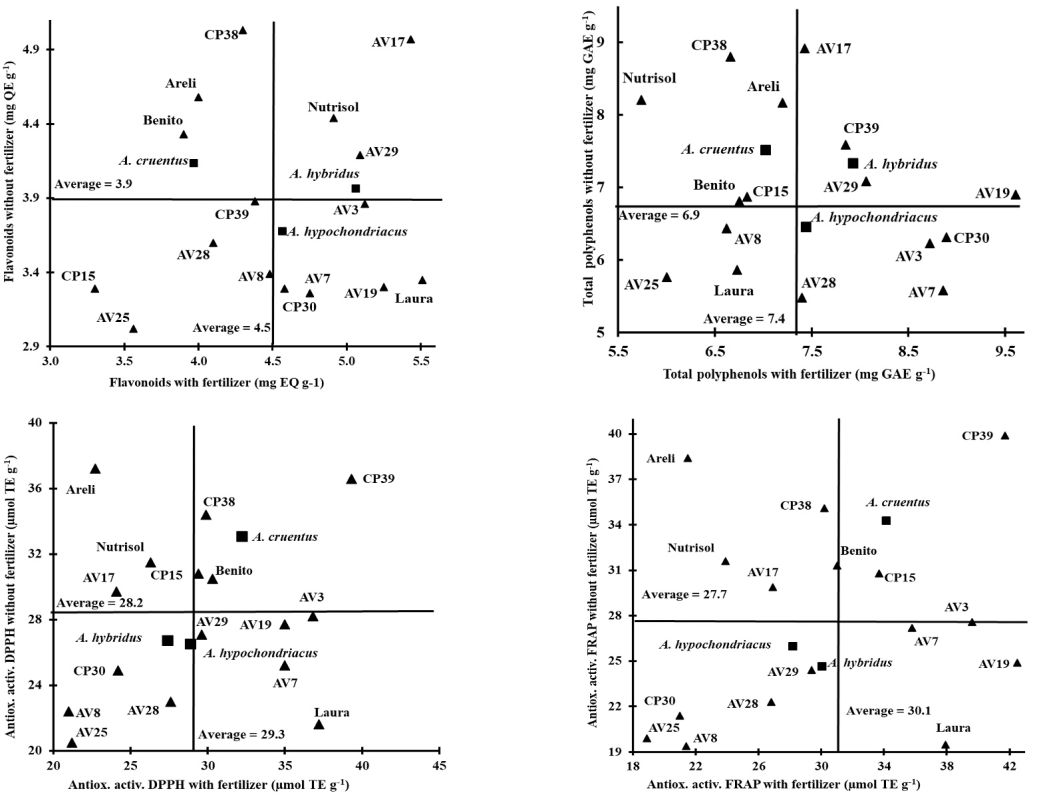

Figure 3. Dispersion of amaranth genotypes as a function of the interaction of fertilization-genotype and species in relation to bioactive compounds and antioxidant activity in leaves and sprouts.

polyphenols and antioxidant activity were significantly influenced by crop location, fertilizer use, species and genotype. In addition, bioactive compounds and antioxidant activity were modified or altered by location-fertilization, location-species or genotype, fertilization-species or genotype interactions and by interaction of location-fertilization-genotype. In Huaquechula, Puebla, there were days without rain or with little precipitation and high temperatures, environmental conditions that influenced, among other factors, the presentation of higher concentrations of phenols and flavonoids, which, in turn, influenced major antioxidant activity. The consumption of amaranth as a *quelite* (leaves and sprouts) is an important source of bioactive compounds, and these compounds differ from species to species. *A. hybridus* showed high values that differed significantly from those recorded for *A. hypochondriacus* and *A. cruentus*. However, the highest antioxidant activity evaluated by the FRAP and DPPH methods was observed for *A. cruentus*, followed by *A. hypochondriacus* and *A. hybridus*. The genotypes with the highest flavonoid and phenol content were AV-17 and AV-19 of the species *A. hybridus* and CP-38 of the species *A. cruentus*; those with the highest antioxidant activity were CP-38 and CP-39 from *A. cruentus* and AV-19 and AV-3 from *A. hybridus*.

ACKNOWLEDGMENTS

The authors are grateful for the financial support provided by CONACYT-Problemas Nacionales (project no. 2015-1-1119) and the Instituto Politécnico Nacional (project nos. 20201270 and 20201269).

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TABLE V
INTERACTION OF CROP LOCATIONS, FERTILIZATION AND GENOTYPES OF THREE AMARANTH SPECIES IN RELATION TO FLAVONOIDS, TOTAL POLYPHENOLS AND ANTIOXIDANT ACTIVITY IN LEAVES AND SPROUTS

Genotypes and species of amaranth	Phenolic compounds							
	Flavonoids (mg QE.g^{-1})				Total polyphenols (mg GAE.g^{-1})			
	Loc-1[1]		Loc-2		Loc-1		Loc-2	
	W/F[2]	O/F	W/F	O/F	W/F	O/F	W/F	O/F
AV-17	5.7	6.8	5.1	3.2	11.6	13.3	3.3	4.5
AV-19	7.1	4.2	3.4	2.4	15.5	9.0	3.7	4.8
AV-29	5.5	4.8	4.6	3.6	11.8	9.3	4.3	4.8
AV-8	5.0	4.0	4.0	2.8	10.4	8.1	2.8	4.8
A. hybridus	***5.8***	***5.0***	***4.3***	***3.0***	***12.3***	***9.9***	***3.5***	***4.7***
Areli	4.4	6.2	3.6	2.9	10.1	11.9	4.3	4.5
AV-25	3.6	3.6	3.5	2.4	8.7	8.4	3.3	3.1
AV-28	5.4	3.8	2.8	3.3	11.8	8.1	3.0	2.8
AV-3	6.3	4.3	3.9	3.5	14.6	9.6	2.8	2.8
AV-7	5.8	3.8	3.7	2.8	14.4	8.2	3.4	3.0
CP-30	5.2	3.8	4.0	2.7	14.1	8.6	3.6	4.0
Laura	7.2	3.6	3.8	3.1	9.6	6.8	3.8	4.9
Nutrisol	5.2	5.1	4.6	3.8	7.9	10.6	3.6	5.8
A. hypochondriacus	***5.4***	***4.3***	***3.7***	***3.1***	***11.4***	***9.0***	***3.5***	***3.9***
Benito	5.0	5.4	2.8	3.2	10.1	11.0	3.4	2.6
CP-15	4.4	4.5	2.2	2.0	10.4	10.3	3.2	3.4
CP-38	4.8	6.9	3.8	3.1	9.8	14.8	3.5	2.7
CP-39	6.2	5.8	2.5	2.0	12.7	12.3	3.0	2.9
A. cruentus	***5.1***	***5.7***	***2.8***	***2.6***	***10.8***	***12.1***	***3.3***	***2.9***
HSD-Tukey[3]		***0.25***				***0.36***		

Species and genotypes of amaranth	Antioxidant activity							
	DPPH (µmol TE g^{-1})				FRAP (µmol TE g^{-1})			
	Loc-1		Loc-2		Loc-1		Loc-2	
	W/F	O/F	W/F	O/F	W/F	O/F	W/F	O/F
AV-17	34.0	49.6	14.2	9.8	43.3	52.3	10.6	7.5
AV-19	53.8	40.5	16.3	14.9	65.6	41.1	19.4	8.8
AV-29	41.7	42.2	17.6	12.0	45.5	40.3	13.3	8.4
AV-8	28.5	31.0	13.6	13.8	34.5	30.5	8.3	8.4
A. hybridus	***39.5***	***40.8***	***15.4***	***12.6***	***47.2***	***41.0***	***12.9***	***8.2***
Areli	24.4	58.6	21.0	15.9	24.2	67.7	18.8	9.2
AV-25	27.8	31.2	14.6	9.8	29.2	28.5	8.7	11.2
AV-28	41.5	32.6	13.8	13.4	43.8	36.0	9.9	8.7
AV-3	62.9	44.4	10.8	12.1	67.8	47.5	11.4	7.8
AV-7	55.0	40.3	15.0	10.2	63.3	43.0	8.3	11.5
CP-30	31.7	33.7	16.8	16.1	30.7	34.6	11.4	8.2
Laura	56.6	29.6	17.9	13.7	63.1	28.7	12.8	10.2
Nutrisol	34.1	45.2	18.6	17.7	31.4	49.7	16.3	13.5
A. hypochondriacus	***41.8***	***39.5***	***16.1***	***13.6***	***44.2***	***42.0***	***12.2***	***10.0***
Benito	41.7	48.2	18.9	12.8	50.9	53.6	11.1	9.1
CP-15	45.9	49.1	12.9	12.6	58.6	50.8	8.8	10.7
CP-38	41.1	54.7	18.8	14.1	50.3	60.2	10.1	10.0
CP-39	62.6	56.7	16.0	16.5	74.1	67.8	9.3	12.0
A. cruentus	***47.8***	***52.2***	***16.7***	***14.0***	***58.5***	***58.1***	***9.8***	***10.5***
HSD-Tukey[3]		***3.60***				***2.49***		

[1]Loc-1 = Huaquechula, Puebla; Loc-2 = Tepetitla, Tlaxcala; [2]W/F: with fertilizer; O/F: without fertilizer; [3]diferences between means with values major than difference honest significant (HSD, honestly significant difference), are different statistically (Tukey's test, $p \leq 0.05$).

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