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Full Length Article

Effect of Foliar Application of Hydrogen Peroxide on Growth, Yield, Chemical Composition and Antioxidant Compounds of Amaranth Leaf and Seed

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Abstract

Amaranth crops grown in greenhouses have a better yield but a lower nutritional value than open-field crops. To increase the content of secondary metabolites and to improve nutrient composition in plants, elicitors such as hydrogen peroxide have been used. The aim of this study was to evaluate the effect of foliar application of hydrogen peroxide during greenhouse cultivation of *Amaranthus hypochondriacus* L. on growth, chemical composition and antioxidant compounds in leaves at different stages of maturity as well as in seeds. The concentrations tested were 0, 200, 480 and 610 mg/L, with 4 applications throughout the cultivation period. A two-way ANOVA and a LSD test were used to compare the variables of treatment and sampling time (C.I. 95%, $p < 0.05$). The results of proximate analysis, chlorophyll and growth showed no significant difference between the control and treated samples. In antioxidant compound content, increases were observed in leaves but not in seed. H_2O_2 application may be a viable strategy for increasing production of antioxidants without detriment to the amaranth plant's growth. © 2017 Friends Science Publishers

Keywords: Amaranth; Peroxide hydrogen; Proximate analysis; Secondary metabolites; Phenolic compounds; Antioxidant

Introduction

The word *Amaranthus* is derived from the Greek "Anthos" (flower) which means perpetual. Today, amaranth is considered by some to be the crop of the third millennium, perhaps for its nutritional properties and versatility of its cultivation (Rivera *et al*., 2010; Sanz-Penella *et al*., 2013; Muñiz-Márquez *et al*., 2014). There are 400 species in the *Amaranthus* genus distributed throughout the world. The predominantly domestic (Mexican) amaranth exists in 4 seed-producing species, *A. hypochondriacus*, *A. cruentus*, *A. caudatus* and *A. hybridus* (Corke *et al*., 2016). Amaranth (*Amaranthus hypochondriacus*) is a fast-growing annual species, highly adaptable and drought-resistant and all of the plant (roots, stems, leaves and seeds) is fit for and beneficial to human and animal consumption. It also has promising potential in industrial applications, including as a source of bioactive compounds, protein, cosmetics, dyes and biodegradable plastics (Chávez-Servín *et al*., 2017). Part of amaranth's importance lies in its high protein content, which is 13–18% higher than comparable cereals like corn (10.3%), rice (8.5%) and wheat (14%). It also contains starch (58–66%), dietary fiber (9–16%) and lipids (3.1–11.5%) (Akanbi and Togun, 2002; Caselato-Sousa and Amaya-Farfan, 2012). At the same time, in Mexico, encouraging primary sector production is one of the greatest challenges of our time. It is crucial to find ways to increase productivity and monitor important aspects such as the chemical composition of the harvested product.

Using greenhouses for high-potential crops like amaranth is a strategy that has attracted considerable interest in recent years (Chávez-Servín *et al*., 2017). For example, in

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the state of San Luis Potosi, the government developed and launched a pilot program in 2011 to produce amaranth in 20 hectares of greenhouses. Greenhouses can intensify agricultural production if appropriate measures are taken to accelerate the development of crops and increase the amount of biomass per unit of cultivated area compared with open-field conditions (Fuller and Zahnd, 2012).

Amaranth cultivation in greenhouses has the advantage of increasing yield in a controlled environment but emphasis must be placed on improving nutritional quality and obtaining bioactive compounds with potential benefits, for example, by the use of elicitors (Chávez-Servín *et al*., 2017). An elicitor is a factor that stimulates any type of plant defense and causes the induction of phenolic biosynthesis. Elicitors may be biotic or abiotic in origin. In plants, polyphenols act as a defense (against herbivores, microbes, viruses or competing plants) and signal compounds (to attract pollinating or seed dispersing animals) and also protect plants from oxidation. Usually, oxidative damage and increased resistance under environmental stresses can be correlated with the efficacy of the antioxidative defense system and increased stress tolerance (Zhao *et al.,* 2005). Exogenous application of elicitors such as hydrogen peroxide (H_2O_2), jasmonic acid, salicylic acid, methyl jasmonate, chitosan and other related compounds had been used to study the effects these substances have on the production of secondary metabolites in some plants (Chen and Chen, 2000; Kirakosyan *et al*., 2006; Gadzovska *et al*., 2007; Flores *et al*., 2015). It is known that phenolic compounds, tocopherols and carotenoids are a group of secondary metabolites synthesized by plants and increase as a result of the plants' adaptation to stressed conditions (Gorelick and Bernstein, 2014; Natella *et al*., 2016). These compounds have proven to be potential antioxidants that can protect cells from attack by free radicals associated with the oxidation. A study using exogenous applications of salicylic acid in different concentrations (70, 138 and 276 mg/L) in peppermint plants, found increases of 65, 35 and 31% in phenolic content and also increases of 93, 100 and 56%, respectively, in flavonoids (Figueroa *et al*., 2014). In another study (Swieca, 2015) hydrogen peroxide (H_2O_2) was shown to promote an increase in antioxidant capacity and phenolic content in lentil sprouts but was also associated with lower plant growth and development as well as yield. Because elicitors can increase the content of phenolic compounds but can also affect the parameters of growth, development and yield of a plant, it is necessary to analyze the chemical composition of the plant, or the part of interest (leaves and seeds) as well as its yield. There are few studies on the use of hydrogen peroxide in amaranth. In a study (Cao *et al*., 2012), low concentrations (4, 34 and 170 mg/L) of hydrogen peroxide were applied to amaranth seedlings as an elicitor in the species *A. mangostanus*. That study found that hydrogen peroxide had no significant effect on the accumulation of betacyanin. It is therefore necessary to continue testing the effects of H_2O_2 in different plants of interest to humankind. Existing studies on the use of this substance an elicitor are scarce, even though hydrogen peroxide is less expensive than jasmonic acid and methyl jasmonate for use in larger-scale production.

The aim of this study was to evaluate the effect of foliar application of hydrogen peroxide (200, 480 and 610 mg/L) during greenhouse cultivation of *A. hypochondriacus* L on growth (plant height, stem diameter, leaf number and panicle length), chemical composition, yield and antioxidant compounds in leaves at different stages of maturity (60, 80 and 90 days) as well as in seeds.

Materials and Methods

Experimental Details and Treatments

Reagents biological materials: DPPH (2,2-diphenyl-1-picrylhydrazyl) 98% pure, 99% Sodium acetate, Sodium Carbonate 99%, Ascorbic acid 99%, Gallic acid 98%, Ferric chloride97%, Vanillin 99%, Catechin, Triphenyltriazine (TPTZ) - 2,4,6-tris (2-pyridyl) -1,3,5-triazine ≥ 98% and Folin-Ciocalteu reagent 98% pure from Sigma-Aldrich Corp. (St. Louis, MO, USA), Hydrochloric acid, Ethanol and Methanol from JT Baker (Center Valley, USA) were used. The "Revancha" seed variety of the *A. hypochondriacus* species, certified by the National Institute of Forestry, Agriculture and Livestock (INIFAP) was used.

Location and type of greenhouse: The study was conducted at the Amazcala Engendering Faculty, in the municipality of El Marques, in the state of Queretaro, Mexico. Amazcala, located at 20° 42' 20" north and 100° 15' 37" west, at 1,921 m above sea level, in a semi-arid region. A single 100 m^2 Gothic-style greenhouse was used, equipped with an electric ventilator (50 inches and 0.5 hp). The greenhouse is oriented north-south. The cladding material was a single layer of long-lasting polyethylene plastic.

Study design and treatment with H_2O_2: This was a randomized experimental study. Six randomized blocks (6 repetitions) with 6 plants per treatment (36 plants) and a total of 4 hydrogen peroxide concentrations (0, 200, 480 and 610 mg/L) were established. That is, each treatment was applied to 36 plants, for a total of 144 plants distributed in a 100 m^2 greenhouse. Cultivation began on August 30 by transplanting 15 cm high seedlings and the harvest ended with amaranth seed being collected on November 28, 2015. The seedlings were placed in 20 L black polyethylene bags. The bags were filled with a substrate of tezontle (50%), sand (25%) and clay soil (25%), a mixture that provides porosity, electrical conductivity, water and nutrient retention. The universal solution (Steiner, 1984) was used at 40% for the nutrient base solution, applied three weeks from germination of the plants until the beginning of inflorescence. Hydrogen peroxide concentrations were prepared in the plant physiology laboratory at the Amazcala campus. Four concentrations of hydrogen peroxide were prepared, of 0, 200, 480 and 610 mg/L. The elicitor was

applied 40 days after germination and 4 times during the cultivation period, every 7 days. They were applied by foliar spray to drip point between 09:00 and 10:00 h. A spray atomizer was used for the application of the hydrogen peroxide solution. The atomizer was placed at a distance of approximately 30 cm from the leaves and sprayed to drip point. Approximately 10 mL per 10 leaves were used. Each of the pots was irrigated manually. At the start, 200 mL of water were applied every third day during the first month. Subsequently, that amount was gradually increased according to environmental conditions, reaching 2000 mL every third day starting in the second month.

Sample selection: During the study, leaf samples were collected at 60, 80 and 90 days after planting. In each of the samplings, 10 true leaves were collected in a randomized manner from the middle part of each of the plants. The seed was harvested 90 days after planting. Samples were collected in paper bags and taken immediately to the laboratory, where they were dried in an oven (Shel lab Fx 1375, USA) at 40°C for 24 h. The samples were ground in a mill (Thomas Wiley Model 4 Scientific, USA) with a 0.5 mm-diameter sieve. The powdered samples were then stored in an ultra-freezer (REVCO last II, USA) to -80°C until analysis.

Extraction of phenolic compounds: The powdered samples were subjected to extraction using a methanol:water solution, 80:20 (v/v) at a solid-liquid ratio of 1:10 (w/v). To assist in the extraction, a digital orbital stirrer (model OM10E, brand OVAN) was used at 100 rpm and at room temperature overnight without light. The resulting solution was filtered through 0.20 μm filter paper prior to analysis.

Analytical Determinations

Proximate analysis: The content of moisture, ash, crude fat extract, protein and crude fiber content was measured in amaranth seeds and fresh leaves following AOAC official methods of analysis (2000) (934.01, 923.03, 920.39, 960.52, and 920.86, respectively).

Chlorophyll absorbance: Chlorophyll absorbance was measured 5 times during the cultivation cycle (at 30, 35, 55, 60 and 70 days after planting). The determination was made directly on the leaves of the amaranth plants. A SPAD 502 Chlorophyll meter (Konica Minolta, USA) was used. The absorbance was measured in dimensional values from 0 to 199 in Soil Plant Analysis Development (SPAD) units. The determinations were made according to the manufacturer's technical guide (SüB *et al*., 2015).

Plant height: Plant height was measured 7 times during the cultivation period (30, 35, 55, 60, 70, 80 and 90 days after planting). This variable was measured in centimeters (cm) using a 5 m Mark Truper measuring tape, FH-3m (Truper, Taiwan), with an accuracy of 0.01 mm, placed vertically on the substrate surface. The measurement was taken at the apical meristem.

Stem diameter: The stem diameter was measured 7 times during the cultivation period (30, 35, 55, 60, 70, 80 and 90 days after sowing), using a stainless hardened 190 mm vernier caliper (Metromex 222-A, Mexico). The unit of measurement was millimeters (mm). The stem diameter was measured on the main stem of the plant, 1 cm above the substrate.

Number of leaves: The total number of leaves on each amaranth plant (36 determinations per group) was counted five times during the cultivation period (30, 35, 55, 60 and 70 days after planting).

Panicle length: Panicle length was measured using a tape measure (Truper, Mexico) at the end of the cultivation period. The results were expressed in centimeters.

Determination of DPPH radical scavenging activity: A DPPH assay was performed as reported by Brand-Williams *et al* (1995), with some modifications, using a microplate reader. Aliquots of 280 μL of 100 μM DPPH/methanol solution per well were placed in the same row of a 96-well plate and then 20 μL of extracts, diluted to different concentrations, was added to each well to complete 300 μL. Aliquots of 300 μL of methanol were placed in the first row as a blank. The plates were incubated for 30 min in the dark and measurements were taken at 490 nm.

Determination of FRAP activity: A FRAP (Ferric Reducing Ability of Plasma) assay was performed at 37°C and pH 3.6. Ferric (Fe^{3+}) to ferrous (Fe^{2+}) ion reduction causes formation of an intense blue-colored ferrous-tripyridyl-s-triazine complex (TPTZ) with absorbance maximum at 593 nm. Absorbance was measured after 30 min and was proportional to the combined ferric reducing/antioxidant power of the antioxidants in the extracts. The final results were expressed in grams of ascorbic acid (AA) equivalents per 100 g of dry matter (Benzie and Strain, 1996).

Determination of total phenolic compounds: The total phenolic content of the amaranth samples was measured using the Folin–Ciocalteu colorimetric method (Singleton and Rossi, 1965) and the results were expressed in milligrams of gallic acid (GA) equivalents per 100 g of dry matter.

Condensed tannins: The condensed tannin content was measured by a vanillin hydrochloric acid test (Deshpande and Cheryan, 1985) and the results were expressed in mg of catechin equivalents per 100 g of dry matter.

Anthocyanins: Anthocyanins were measured by the method developed by Abdel and Hucl (1999). The results were expressed in mg equivalents of cyanidin 3-glucoside per 100 g of dry matter.

Statistical Analysis

Experimental data for scale variables were submitted to analysis of variance (ANOVA) followed by a *post hoc* Tukey test for significant differences between treatment concentrations (0, 200, 480 and 610 mg/L of H_2O_2) and between sampling times (60, 80 and 90 days). A two-way ANOVA and a LSD test were used to compare among the treatment concentration and sampling time variables.

Pearson correlations between phenolic compounds and antioxidant capacity were performed. A confidence interval of 95% and a level of significance ($p < 0.05$) was used. SPSS V23 for Windows was used.

Results

Proximate Analysis

Proximate analysis (Table 1) was used to measure the percentage of moisture, ash, crude fiber, crude protein and fat. Average percentages of 76.7 ± 1.4%, 3.7 ± 0.2%, 2.6 ± 0.2%, 3.9 ± 0.2% and 0.3 ± 0.1%, respectively, were observed in amaranth leaves at 60 days. In seed, the averages were 11.2 ± 1.2%, 2.9 ± 0.2%, 5.9 ± 0.2%, 17.1 ± 0.1% and 5.4 ± 0.3%, respectively. No significant difference ($p > 0.05$) was found between the control and hydrogen peroxide treatment groups. Furthermore, no significant differences ($p > 0.05$) were observed between the different sampling times (60, 80 and 90 days). The proximate composition was not found to be adversely affected by the use of hydrogen peroxide in the doses and applications used in this study. According to the growth parameters evaluated in this study, the development of the amaranth plants was not negatively affected by the application of hydrogen peroxide. In analyzing plant height, stem diameter, leaf number and panicle length throughout the period of greenhouse cultivation, no statistical difference ($p > 0.05$) was found between the control and hydrogen peroxide treatment groups in this study.

Amaranth Growth

Phenology is the visible external changes during the plant's development. These changes are the result of environmental conditions: if the environmental conditions are favorable for the plant, the plant will grow and develop properly; otherwise, it may grow and mature more slowly. During the study, the well-developed (morphologically) true leaves were quantified. These leaves are the site of marked photosynthetic activity. This is evident in the color of the amaranth leave: green toward the front and red toward the back. Besides quantifying the number of leaves, chlorophyll content, plant height, stem diameter and panicle length were also determined. Samples were taken according to the growth and development of the plant. Table 2 presents different growth parameters from 30 days (post-emergency phase, two true leaf phase and four true leaf phase). At 30 days, more than 6 true leaves were observed. At 35 days the branching phase began with more than 9 true leaves. By 40 days there were more than 30 leaves and at 60 days more than 100 leaves. At 60 days the panicle was observed (between 50 and 60 days: the beginning of the panicle and the panicle, respectively). The elicitor was applied between the branching phase and the panicle phase, beginning at 40 days after planting, every 7 days with a total of 4 applications. Between 60 and 90 days, maturation took place. The amaranth plants had an initial average height of 10 cm and 30 days after planting, they reached an average height of 118 cm at harvest. No significant differences ($p > 0.05$) were found between the control and treatment groups (Table 2). The stem diameter was measured at 0.17 cm at the start of determinations and reached 1.19 cm by the end of the cultivation cycle. No significant differences ($p > 0.05$) were found between the control and treatment groups (Table 2). The total number of leaves on each plant was counted 5 times during the cultivation period. No significant differences ($p > 0.05$) were found between the control and treatment groups (Table 2). At 70 days, a decrease was observed in the number of leaves of all plants because of leaf sampling for laboratory analysis performed at 60 days. Panicle length was measured when the amaranth seed was harvested. No significant differences ($p > 0.05$) between the control and treatment groups were observed. Length values were observed in the range of 99 to 102 cm (Table 2).

Chlorophyll

No significant differences were found between the control and hydrogen peroxide treatment groups. In the first chlorophyll determination (30 days after planting) SPAD values of 32.5 ± 3, 31.5 ± 4, 31.6 ± 4 and 32.2 ± 4 were observed with treatments of 0, 200, 480 and 610 mg/L (H_2O_2), respectively. In the last chlorophyll measurement (70 days after planting) SPAD values between 49.6 ± 5 to 51.8 ± 4 were obtained. Eventually chlorophyll levels increased in the leaves, attributable to the normal physiological growth and development of amaranth plants. A linear increase was observed in chlorophyll values in all plants throughout the amaranth cultivation period, although no statistical difference was found between the control and the hydrogen peroxide treatment groups.

Yield

The amount of seeds produced in each experimental group (0, 200, 480 and 610 mg/L of hydrogen peroxide) was determined. The amount of seeds per plant was 20.1, 19.8, 20.0 and 19.9 g respectively, with no differences between groups. Assuming a density of 10 plants per m^2, i.e., 100,000 plants per hectare, there would be yields of: 2.01, 1.98, 2.00 and 1.99 ton/ha. These values are superior to the majority of studies reported in the open field (Peiretti and Gesumaria, 1998; García-Pereyra *et al*., 2009; Ramírez *et al*., 2010) and similar to greenhouse (Chávez-Servín *et al*., 2017). It should be mentioned that the extrapolations are not entirely valid, because in the present study, to have a greater control of variability, amaranth was planted in bags with the same amount of substrate, whereas in the above-mentioned open-field studies, they were cultivated directly in the soil.

Antioxidant Capacity

DPPH: Three leaf samplings were carried out during the

Table 1: Proximate analysis results in amaranth leaf and seed

Sample (time)	Treatments H_2O_2 (mg/L)	Moisture (%)	Crude ash[a] (%)	Crude fiber[a] (%)	Crude protein[b] (%)	Ether extract[a] (%)
Leaf	0	76.66 ± 1.4	3.73 ± 0.2	2.60 ± 0.2	3.94 ± 0.2	0.30 ± 0.1
(60 days)	200	77.24 ± 1.5	3.74 ± 0.1	2.64 ± 0.2	3.91 ± 0.2	0.40 ± 0.2
	480	77.30 ± 1.0	3.70 ± 0.1	2.62 ± 0.2	3.95 ± 0.2	0.35 ± 0.2
	610	76.60 ± 1.0	3.84 ± 0.1	2.65 ± 0.2	3.91 ± 0.2	0.37 ± 0.1
Leaf	0	75.70 ± 0.5	3.71 ± 0.1	2.49 ± 0.1	3.97 ± 0.1	0.35 ± 0.1
(80 days)	200	76.96 ± 1.5	3.67 ± 0.3	2.59 ± 0.1	3.94 ± 0.2	0.42 ± 0.2
	480	76.59 ± 1.0	3.72 ± 0.2	2.64 ± 0.2	3.85 ± 0.1	0.40 ± 0.1
	610	76.75 ± 1.5	3.77 ± 0.1	2.68 ± 0.1	3.87 ± 0.1	0.35 ± 0.1
Leaf	0	76.60 ± 1.5	3.68 ± 0.1	2.59 ± 0.3	3.92 ± 0.2	0.36 ± 0.1
(90 days)	200	76.64 ± 1.0	3.70 ± 0.2	2.51 ± 0.2	3.92 ± 0.2	0.37 ± 0.2
	480	76.75 ± 1.5	3.72 ± 0.1	2.55 ± 0.2	3.84 ± 0.1	0.48 ± 0.2
	610	76.54 ± 1.0	3.74 ± 0.2	2.60 ± 0.1	3.96 ± 0.2	0.32 ± 0.2
Seed	0	11.20 ± 1.2	2.96 ± 0.2	5.89 ± 0.2	17.13 ± 0.1	5.37 ± 0.3
	200	10.20 ± 1.0	2.81 ± 0.2	5.90 ± 0.2	17.15 ± 0.1	5.52 ± 0.3
	480	9.30 ± 1.0	3.00 ± 0.1	5.94 ± 0.2	17.12 ± 0.1	5.40 ± 0.2
	610	9.70 ± 1.5	2.94 ± 0.1	6.06 ± 0.1	17.17 ± 0.1	5.43 ± 0.1

The results are shown as the average of 3 measurements ± one standard deviation. No statistical difference was found ($p > 0.05$) in any of the studied variables; [a](% dry matter); [b](% dry matter N x 6.25)

Table 2: Results of the growth parameters of amaranth

Treatments H_2O_2 mg/L	30 days	35 days	55 days	60 days	70 days	80 days	90 days
			Plant height* (cm)				
0	10.64 ± 1.2	19.02 ± 3.2	80.66 ± 15.1	100.66 ± 15.1	112.25 ± 11.9	117.0 ± 12.9	117.66 ± 12.2
200	10.16 ± 1.4	18.68 ± 2.1	85.02 ± 12.7	101.47 ± 13.2	110.80 ± 12.7	119.42 ± 14.7	119.05 ± 14.9
480	10.06 ± 1.5	18.28 ± 2.5	84.97 ± 10.7	99.26 ± 12.2	110.53 ± 13	116.30 ± 12.3	116.17 ± 12.9
610	10.02 ± 1.6	18.54 ± 3.3	84.01 ± 12.9	98.99 ± 11.6	111.93 ± 12.7	115.72 ± 11.3	115.13 ± 13.4
			Stem diameter* (cm)				
0	0.17 ± 0.5	0.37 ± 0.07	1.07 ± 0.1	1.08 ± 0.1	1.07 ± 0.1	1.08 ± 0.1	1.15 ± 0.2
200	0.22 ± 0.3	0.39 ± 0.1	1.09 ± 0.2	1.09 ± 0.1	1.08 ± 0.1	1.12 ± 0.1	1.19 ± 0.2
480	0.17 ± 0.5	0.37 ± 0.1	1.09 ± 0.2	1.15 ± 0.2	1.09 ± 0.1	1.12 ± 0.2	1.15 ± 0.1
610	0.17 ± 0.1	0.36 ± 0.8	1.07 ± 0.1	1.12 ± 0.1	1.14 ± 0.1	1.19 ± 0.1	1.19 ± 0.1
			Number of leaves				
0	6.19 ± 0.9	8.80 ± 1.9	80.60 ± 21.0	118.08 ± 21.3	104.53 ± 30.4		
200	6.36 ± 1.0	9.08 ± 1.9	84.28 ± 19.2	126.14 ± 25.1	102.75 ± 22.1		
480	6.25 ± 0.8	9.05 ± 2.2	87.97 ± 18.5	115.75 ± 22.5	95.42 ± 24.7		
610	6.28 ± 0.9	8.88 ± 1.5	84.08 ± 16.6	124.60 ± 29.9	110.80 ± 26.8		
			Panicle lenght* (cm)				
0							101.60 ± 15.8
200							100.23 ± 11.8
480							101.94 ± 12.3
610							98.72 ± 11.2

*Results are expressed as the average of 36 determinations ± standard deviation. No statistical difference was found ($p > 0.05$) in any of the studied variables. The different treatments are compared in each of the columns

cultivation period (60, 80 and 90 days). In the second (80 days) and third sampling (90 days), significant differences ($p < 0.05$) were observed between the control group and the hydrogen peroxide treatment group (Table 3). At 80 days of cultivation, increases of 19% and 30% in antioxidant capacity were observed with treatments of 200 and 610 mg/L (H_2O_2), compared to the control group. At 90 days of cultivation a 10.6% increase in antioxidant capacity was observed with the treatment of 200 mg/L H_2O_2. In analyzing the seeds, significant differences ($p < 0.05$) were observed between the control and treatment groups but these differences were the opposite of what was expected. Hydrogen Peroxide is applied as elicitor to stimulate the production of phenolic compounds and antioxidant capacity, as an adaptation response to stress conditions. However, not only did the antioxidant capacity measured by DPPH in the seeds fail to increase upon foliar application of the elicitor in the amaranth plant, it actually decreased and this decrease was more pronounced (66%) in the 610 mg/L concentration. Interestingly, treatment with higher concentrations of H_2O_2 (610 mg/L) resulted in the lowest observed concentration of antioxidant capacity in the amaranth seed evaluated by DPPH.

FRAP: Antioxidant capacity in amaranth leaf and seed was also measured using the FRAP method. In the first and second leaf samplings, increased antioxidant capacity was found. At the time of the last cut, increases of 16% and 18% in antioxidant capacity were observed with treatments of 200 and 480 mg/L (H_2O_2), respectively, compared to the control group. In amaranth seed a 60% increase in antioxidant capacity was observed with the 610 mg/L treatment (H_2O_2) (Table 3). In this study the observed antioxidant capacity was different in the leaves, where the hydrogen peroxide was applied directly, compared to the

Table 3: Results of the antioxidant capacity by DPPH and FRAP methods in amaranth leaf and seed

	Treatment H_2O_2				
Sample (day)	0 mg/L	200 mg/L	480 mg/L	610 mg/L	Total*
	DPPH, expressed as mg Equivalent of Ascorbic Acid/ 100g dry matter				
Leaf (60 days)	$297.58 \pm 21.9^{a,1}$	$296.20 \pm 3.8^{a,1}$	$288.49 \pm 1.8^{a,1}$	$246.21 \pm 10.8^{b,1}$	284.82 ± 24.4^{1}
Leaf (80 days)	$211.14 \pm 4.5^{a,2}$	$252.31 \pm 9.8^{b,2}$	$230.39 \pm 14.7^{a,2}$	$276.29 \pm 13.4^{b,2}$	242.57 ± 30.1^{2}
Leaf (90 days)	$432.09 \pm 11.7^{a,3}$	$478.03 \pm 2.8^{b,3}$	$425.26 \pm 10.8^{a,3}$	$456.48 \pm 8.5^{a,3}$	453.63 ± 23.1^{3}
Total*	313.60 ± 95.9^{a}	340.19 ± 99.2^{b}	314.72 ± 85.9^{a}	326.33 ± 97.5^{c}	
Seed**	283.42 ± 6.8^{a}	189.46 ± 3.4^{c}	228.35 ± 3.7^{b}	94.96 ± 6.1^{d}	
	FRAP, expressed as g Equivalent of Ascorbic Acid/ 100g dry matter				
Leaf (60 days)	$3.46 \pm 0.0^{a,1}$	$3.35 \pm 0.1^{a,1}$	$3.42 \pm 0.0^{a,1}$	$3.43 \pm 0.0^{a,1}$	3.39 ± 0.1^{1}
Leaf (80 days)	$3.48 \pm 0.0^{a,1}$	$3.51 \pm 0.0^{a,1}$	$3.47 \pm 0.0^{a,1}$	$3.52 \pm 0.0^{a,1}$	3.49 ± 0.1^{2}
Leaf (90 days)	$3.25 \pm 0.1^{a,1}$	$3.77 \pm 0.4^{b,2}$	$3.86 \pm 0.1^{b,2}$	$3.15 \pm 0.0^{a,2}$	3.55 ± 0.3^{3}
Total*	3.40 ± 0.1^{a}	3.53 ± 0.2^{b}	3.59 ± 0.2^{b}	3.37 ± 0.2^{a}	
Seed **	0.53 ± 0.0^{a}	0.71 ± 0.1^{a}	0.67 ± 0.0^{a}	0.85 ± 0.0^{b}	

The results are shown as the average of four determinations ± one standard deviation. Different letters indicate a significant difference ($p < 0.05$) comparing different H_2O_2 treatments (0 vs. 200 vs. 480 vs. 610 mg/L) in the same row. Different numbers indicate a significant difference ($p < 0.05$) comparing the time (60 vs. 80 vs. 90 days) in each of the treatments in the same column.* The interaction between the sampling time and the treatment of hydrogen peroxide is significant ($p < 0.05$). ** In the seed, comparisons were made only between the different treatments of hydrogen peroxide

Table 4: Results of the content of total phenolic compounds by Folin Ciocalteau method in amaranth leaf and seed expressed as mg Equivalent of gallic acid/ 100g dry matter

	Treatment H_2O_2				
Sample (day)	0 mg/L	200 mg/L	480 mg/L	610 mg/L	Total*
Leaf (60 days)	$654.93 \pm 3.2^{a,2}$	$610.55 \pm 1.0^{b,1}$	$611.17 \pm 1.4^{b,1}$	$527.18 \pm 14.0^{c,1}$	600.96 ± 48.2^{1}
Leaf (80 days)	$514.55 \pm 9.7^{a,2}$	$558.40 \pm 7.7^{b,2}$	$564.07 \pm 11.0^{b,2}$	$516.35 \pm 16.4^{a,1}$	538.35 ± 25.9^{2}
Leaf (90 days)	$523.05 \pm 15.5^{a,2}$	$467.19 \pm 8.9^{b,3}$	$498.77 \pm 12.3^{a,3}$	$499.55 \pm 9.1^{a,2}$	497.14 ± 23.1^{3}
Total*	564.18 ± 67.8^{a}	545.39 ± 62.2^{b}	558.00 ± 48.9^{a}	514.36 ± 17.1^{c}	
Seed**	125.91 ± 3.8^{a}	130.86 ± 11.0^{a}	140.57 ± 7.5^{a}	147.85 ± 5.4^{a}	

The results are shown as the average of four determinations ± one standard deviation. Different letters indicate a significant difference ($p < 0.05$) comparing different H_2O_2 treatments (0 vs. 200 vs. 480 vs. 610 mg/L) in the same row. Different numbers indicate a significant difference ($p < 0.05$) comparing the time (60 vs. 80 vs. 90 days) in each of the treatments in the same column.* The interaction between the sampling time and the treatment of hydrogen peroxide is significant ($p < 0.05$). ** In the seed, comparisons were made only between the different treatments of hydrogen peroxide

Table 5: Contents of condensed tannins in amaranth leaf and seed expressed as mg Equivalent of catechin/ 100g dry matter

	Treatment H_2O_2				
Sample (day)	0 mg/L	200 mg/L	480 mg/L	610 mg/L	Total*
Leaf (60 days)	$3366.33 \pm 16.5^{a,1}$	$3349.95 \pm 63.1^{a,1}$	$3032.09 \pm 47.4^{b,1}$	$3055.30 \pm 16.3^{b,1}$	3200.92 ± 166.8^{1}
Leaf (80 days)	$3073.44 \pm 14.6^{a,2}$	$3075.64 \pm 25.4^{a,2}$	$3379.51 \pm 39.2^{b,2}$	$3949.91 \pm 22.1^{c,2}$	3369.62 ± 369.9^{2}
Leaf (90 days)	$4122.73 \pm 29.1^{a,3}$	$3257.45 \pm 28.7^{b,3}$	$3942.43 \pm 29.3^{c,3}$	$4566.46 \pm 22.8^{c,3}$	3972.30 ± 487.1^{3}
Total*	3520.84 ± 462.1^{a}	3227.70 ± 125.1^{b}	3451.35 ± 393.4^{c}	3857.23 ± 648.2^{d}	
Seed**	607.00 ± 11.1^{a}	243.26 ± 5.5^{b}	463.08 ± 15.7^{c}	417.90 ± 6.3^{c}	

The results are shown as the average of four determinations ± one standard deviation. Different letters indicate a significant difference ($p < 0.05$) comparing different H_2O_2 treatments (0 vs. 200 vs. 480 vs. 610 mg/L) in the same row. Different numbers indicate a significant difference ($p < 0.05$) comparing the time (60 vs. 80 vs. 90 days) in each of the treatments in the same column.* The interaction between the sampling time and the treatment of hydrogen peroxide is significant ($p < 0.05$). ** In the seed, comparisons were made only between the different treatments of hydrogen peroxide

seeds, which had no contact with the hydrogen peroxide sprayed. This could indicate that the effect of the elicitor (hydrogen peroxide) differs when acting by direct contact, or as a signaling molecule.

Total Phenolic Compounds

Increases of 8% and 9% in total phenolic compounds were found in the second leaf sampling with treatments of 200 and 480 mg/L (H_2O_2), respectively. For the seed, no significant differences ($p > 0.05$) between the control and treatment groups were observed, although it is important to note that a linear increase in the content of total phenolic compounds was observed as the hydrogen peroxide concentration was increased (Table 4).

Condensed Tannins

In the second leaf sampling, condensed tannins were found to have increased 10% and 28%, with hydrogen peroxide treatments of 480 and 610 mg/L, respectively. Also, in the third leaf sampling, a 10% increase against the control group was observed with the treatment of 610 mg/L (H_2O_2). In the seed, significant differences ($p < 0.05$) were observed between the control group and all treatments. At 90 days, an increase in tannin content was observed in all treatments (Table 5).

Anthocyanins

This group of secondary metabolites provided the highest responses. An increase (though not linear) in anthocyanins

Table 6: Contents of anthocyanins in amaranth leaf and seed expressed as mg Equivalent of cyanidin-3-glucoside/100g dry matter

	Treatment H_2O_2				
Sample (day)	0 mg/L	200 mg/L	480 mg/L	610 mg/L	Total*
Leaf (60 days)	$1001.26 \pm 3.4^{a,1}$	$1032.62 \pm 2.2^{b,1}$	$1040.35 \pm 1.6^{b,1}$	$1016.03 \pm 3.6^{b,1}$	1024.79 ± 15.8^{1}
Leaf (80 days)	$931.80 \pm 5.1^{a,2}$	$926.96 \pm 0.3^{a,2}$	$951.31 \pm 0.2^{b,2}$	$833.32 \pm 0.9^{c,2}$	914.05 ± 47.2^{2}
Leaf (90 days)	$753.24 \pm 7.3^{a,3}$	$790.26 \pm 4.8^{b,3}$	$740.94 \pm 1.2^{c,3}$	$754.54 \pm 1.2^{a,3}$	765.70 ± 19.4^{3}
Total*	895.44 ± 109.2^{a}	916.57 ± 103.6^{b}	910.86 ± 131.1^{c}	867.97 ± 114.4^{d}	
Seed**	153.75 ± 0.8^{a}	116.25 ± 0.3^{b}	186.90 ± 1.2^{c}	165.92 ± 0.1^{d}	

The results are shown as the average of four determinations ± one standard deviation. Different letters indicate a significant difference ($p < 0.05$) comparing different H_2O_2 treatments (0 vs. 200 vs. 480 vs. 610 mg/L) in the same row. Different numbers indicate a significant difference ($p < 0.05$) comparing the time (60 vs. 80 vs. 90 days) in each of the treatments in the same column.* The interaction between the sampling time and the treatment of hydrogen peroxide is significant ($p < 0.05$). ** In the seed, comparisons were made only between the different treatments of hydrogen peroxide

was observed in all three samples of leaf and seed. Anthocyanins increased by 3, 4 and 1% in the first leaf sample with hydrogen peroxide treatments of 200, 480 and 610 mg/L, respectively. In the second sampling, a significant difference was found between the control group and the 480 mg/L (H_2O_2) treatment group, with an increase of 2%. In the third leaf sample, a 5% increase in anthocyanins was observed with the 200 mg/L (H_2O_2) treatment. In amaranth seed, significant differences were observed in the 480 and 610 mg/L (H_2O_2) treatments compared to the control group: increases of 21 and 8%, respectively (Table 6).

Discussion

As for the proximate analysis in the variables analyzed, i.e. moisture, ash, crude fiber, crude protein and fat content, no statistical difference was found between the control and the hydrogen peroxide treatment groups. One study (Barba de la Rosa *et al*., 2009) involving four varieties of amaranth (Nutrisol, Tulyehualco, DGETA and Gabriela) in the same species reported the results of proximate analysis for seed. This study reported crude fiber values for the four varieties between 1.9 and 2.5%, while our results showed 5.9%, which is a difference of between 2.4 and 3.1 times in our results. Likewise, compared to that same study, our crude protein values are 9.8% higher. Our results are also consistent with those reported in another study (López-Mejía *et al*., 2014) which analyzes the leaves and seeds of the same amaranth species. In our study, we report 1.5% more crude fiber and 2.3% more crude protein in seeds. Regarding grain yield, assuming a density of 100,000 plants per hectare, our yields for the treatments of hydrogen peroxide (0, 200, 480 and 610 mg/L) obtained values of 2.01, 1.98, 2.00 and 1.99 ton/ha. García-Pereyra *et al*. (2009) studied different genotypes of *A. hypochondriacus* with different densities (from 31,250 to 125,000 plants/ha in spring-summer and autumn-winter). They reported that the best grain yield was obtained with a density of 125,000 plants/ha in all genotypes. Also, the best yields for the different *A. hypochondriacus* genotypes were obtained during the spring–summer cycle (1.3–2.2 ton/ha) compared to autumn-winter cycles (0.07–1.3 ton/ha). In other amaranth studies a maximum yield of 1.67 ton/ha has been reported and for the *A. hypochondriacus* (Revancha variety), a yield of 1.44 ton/ha (Ramírez *et al*., 2010) and 1.5 ton/ha (Peiretti and Gesumaria, 1998). Note that comparisons made with other studies are difficult to extrapolate because, in the case of grain yield, crop density has been observed to affect seed yield and in our experiment the plants were grown in individual bags. Neither the use of hydrogen peroxide in our study nor any other treatment was found to affect performance compared to the control group, nor were their observed differences between the treatments. It is known that using elicitors for the production of secondary metabolites, such as the increase in phenolic compounds, can adversely affect different growth parameters and yield. Generally, elicitation to produce secondary metabolites generates a state of stress in the plant that induces it to modify its metabolism, to respond and to adapt to the stress conditions. In this sense, a good elicitor will be one that does not adversely affect the growth parameters or performance of the plant but does induce an increase in bioactive compounds of interest.

Table 7: Pearson correlations among total phenolic compounds, tannins, anthocyanins and antioxidant capacity in amaranth

Parameters	DPPH	FRAP
Total phenolic compounds	0.409**	0.947**
Tannins	0.662**	0.928**
Anthocyanins	0.332*	0.938**

*<0.05; **<0.005

The effect on phenolic compounds and antioxidant capacity in response to the use of elicitors has been poorly studied in seeds and further study is required. Turning our attention to the values of antioxidant capacity in the amaranth leaf at different sampling times, antioxidant capacity in the control group decreased by 30% in the second leaf sampling (day 80) and subsequently a significant increase of 104% was observed in the third leaf sampling (day 90) (Table 3). The increase in antioxidant capacity (DPPH) at 90 days of cultivation may be due to a leaf cutting effect. That is, at 80 days a leaf cut was made, and 7 days later (90 days), the last leaf sample was taken for the analysis. It has been reported that the mechanical damage of cutting of leaves in a plant itself causes a stress in the plants and that these may respond by generating more secondary metabolites in response to the induced damage (Cheynier *et*

al., 2013). In the present study, all plants were cut in the same manner in all groups (control and treatments), so the increase in antioxidant capacity observed in samples at 90 days in all groups may be in part attributed to the previous cutting of the amaranth plant leaves.

It has been reported that hydrogen peroxide plays a role as a signaling molecule in plant growth and development. (Kocsy *et al*., 1997; Foyer *et al*., 2002). Note also that while the antioxidant capacity of the seeds analyzed by DPPH showed a 66% decrease with the highest treatment of hydrogen peroxide (610 mg/L), by the FRAP method the opposite effect was observed: a 62% increase in antioxidant capacity upon treatment with the highest amount of hydrogen peroxide (610 mg/L) showed (Tables 3 and 4). The methods differ according to the oxidizing agent, the substrate used and the technique used, as well as in the possible interactions of the sample with the reaction medium. It is common for antioxidant capacity values to differ from technique to technique. This is why usually more than one technique is used. In the DPPH assay, the purple chromogen radical is reduced by antioxidant/reducing compounds to the corresponding pale yellow hydrazine. The procedure involves measuring the decrease in absorbance of DPPH at its absorbance maxima of 517 nm, which is proportional to the concentration of free radical scavenger added to DPPH reagent solution. The steric accessibility of DPPH radical is a major determinant of the reaction, since small molecules that have better access to the radical site have relatively higher antioxidant capacity. On the other hand, many large antioxidant compounds that react quickly with peroxyl radicals may react slowly or may even be inert in this assay. Likewise, the ferric reducing ability of plasma (FRAP) assay measures the ability of plasma to reduce ferric ions, in the form of ferric 2,4 and 6-tripyridyl-s-triazine (TPTZ). This assay reaction occurs by electron transfer. Furthermore, not all antioxidants are able to reduce Fe, antioxidants that act by H atom transfer are not detected (Pyrzynska and Pekal, 2013). This could explain the different behavior observed in the antioxidant capacity as determined FRAP and DPPH in the present study. The antioxidant potential of the plant material usually appears to correlate with the phenolic content. The analyses presented in this work of the correlation between phenolic content and antioxidant activity measured in the two assays (FRAP and DPPH) are consistent with previous research in which phenolic compounds and antioxidant capacity of different foliar extracts are analyzed (Amensour *et al*., 2010; Das *et al*., 2014; Zlotek *et al*., 2016). The observed correlation between Phenolic compounds (Total phenolic compounds, condensed tannins and anthocyanins) and antioxidant capacity assays (DPPH and FRAP) suggest that phenolic compounds are dominant contributors to the antioxidant activity. The results are presented in Table 7 and show that the highest positive correlation for the extract is present with FRAP analysis and there is a significant correlation with Total phenolic compounds, anthocyanines and tannins. Anthocyanines and tannins are well identified as compounds that contribute to antioxidant capacity (Skrovankova *et al*., 2015).

In a previous study, low concentrations of hydrogen peroxide were applied to amaranth seedlings as an elicitor in the species *A. mangostanus* (Cao *et al*., 2012). That study found that hydrogen peroxide (at concentrations of 3.4, 35 and 170 mg/L) had no significant effect on the accumulation of betacyanin (a coloring substance present in the aqueous extract of the root) compared to the control group. An earlier study (Sepulveda-Jimenez *et al*., 2005), reported that hydrogen peroxide is a positive stimulant for production of betalains (a secondary metabolite of plants that contributes red and yellow pigment) in the red beet (*Beta vulgaris*). In a study of peppermint plants (*Mentha piperita*), foliar applications of low hydrogen peroxide concentrations (1.7, 3.4 and 0.17 mg/L) were made 2 times throughout the growing season. The results indicated no significant effect on the size of the leaf. The study also reported that with the 17 mg/L hydrogen peroxide concentration, a slight increase was observed (from 10% to 12%) in the content of total phenolic compounds using the Folin Ciocalteu method. An analysis of antioxidant capacity (DPPH and ABTS), found no significant differences ($p > 0.05$) between the control and treatment groups (Figueroa *et al*., 2014). Phenolic compounds are involved in various plant processes, such as growth and reproduction. They are also synthesized as a defense mechanism in response to biotic or abiotic stress (Talhaoui *et al*., 2015). Therefore, production can be improved through treatment with elicitors (Ferrari, 2010). In one study, two doses of hydrogen peroxide (510 to 5100 mg/L) were tested on lentil sprouts (Swieca, 2015). With these two concentrations, total phenolic compounds were higher than in the control group. The 510 mg/L hydrogen peroxide treatment, however, resulted in the greatest increase in total phenolic compounds using the Folin Ciocalteu method, about 65% compared to the control group and 44% with treatment of 5100 mg/L. The 510 mg/L concentration of the elicitor had no effect on the performance of the germinated lentil. Prior to this study, other concentrations of hydrogen peroxide (680 to 6800 mg/L) in lentil sprouts were analyzed and an increase in total antioxidant capacity was observed two days after application (Swieca and Baraniak, 2014). It has been reported that low concentrations of hydrogen peroxide can induce the synthesis of secondary metabolites in plants, such as phenolic compounds (Moskova *et al*., 2009; Kumar *et al*., 2010). Another study reported that the accumulation of capsidiol (a terpenoid compound categorized under the broad term phytoalexin, a class of low-molecular-weight plant secondary metabolites) in peppers (*Capsicum annuum L*.) could be induced by hydrogen peroxide (Arreola-Cortes *et al*., 2007). The accumulation of phenolic compounds under biotic and abiotic stress was reported to be accompanied by increased enzyme phenyl ammonium lyase (PAL). Plant defense and related pathways such as glutathione-S-transferase (GST), antioxidant enzymes, PAL, defense proteins and transcription factors

were also reportedly influenced by hydrogen peroxide (Kovtun *et al*., 2000; Hung *et al*., 2005; Habibi, 2014). Although some reports suggest that exogenous application of hydrogen peroxide can affect the production of secondary metabolites, no study has been conducted to clarify the relationship between the content of phenolic compounds and the elicitor. More research is needed to determine the biological significance of increases in the metabolites analyzed and the antioxidant capacity observed in the present study. Foliar application of hydrogen peroxide in plants may be a viable strategy for increasing production of secondary metabolites. Each plant species responds differently to the application of hydrogen peroxide, however and even-as this study shows-at different sampling times, and for each part of interest (leaf and seed). Elicitors induce the production of secondary metabolites but the concentration of the elicitor, the number of applications throughout of the cultivation cycle and the time of application, among other factors, are all involved in the plant's cellular activities. Elicitation is a tool whose main objective is manipulation of metabolism to increase the content of secondary metabolites in food, which may have various health benefits. Ideally, an elicitor used to spur an increase in secondary metabolite production does not adversely affect the nutritional composition, product performance or growth parameters, as appropriate to the use of the crop (whether for production of leaf, seed, fodder or some combination of these. In the present study, overproduction of phenolics in response to the treatments varied. Different graded concentrations of stressors do not always cause a linear increase in desirable features. Hydrogen peroxide is relatively inexpensive and easy to apply and seems like a good alternative for improving the levels of some secondary metabolites of interest but its use must be analyzed for each particular situation.

Conclusion

Foliar applications of hydrogen peroxide in amaranth were found to be effective in increasing antioxidant compounds in amaranth plants. The growth parameters, chlorophyll content, yield and the proximate analysis showed no significant differences between the control and treatment groups (200, 480 and 610 mg/L H_2O_2). This could be explained by the fact that the concentrations of hydrogen peroxide and/or the frequency of its application are small enough not to adversely affect the growth parameters or yield of the plant, but are sufficient to produce a certain level of stress that promotes the increase in certain secondary metabolites. In the laboratory analysis of amaranth leaf, an increase in antioxidant capacity, total phenolic compounds, condensed tannins and anthocyanins was observed across different sampling times and different treatments compared to the control group. The increase observed in the amaranth seed was smaller than in the leaf, but the level of anthocyanins and antioxidant capacity (by FRAP) were found to increase significantly in the seed. The content of condensed tannins increased without any treatment. Anthocyanins showed the best response to hydrogen peroxide. Elicitation did not affect all analyzed secondary metabolites or antioxidant capacity equally. In amaranth leaves, we found that the response to the 200 mg/L hydrogen peroxide treatment was greater than to the other treatments and the greatest changes were observed at 90 days. The results also show that amaranth leaves responded more strongly than seeds. No dose-dependent response was observed. This follows, because there was no increase in production of secondary metabolites or antioxidant capacity in treatments with higher H_2O_2 concentrations. Hydrogen peroxide concentrations (200, 480 and 610 mg/L) applied 4 times over the amaranth cultivation period promoted an increase in specific antioxidant compounds, without detriment to amaranth plant growth. It is necessary to test higher concentrations of hydrogen peroxide to evaluate if there is a greater increase of the secondary metabolites without diminishing the yield.

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