*Differences among five amaranth varieties (*Amaranthus *spp.) regarding secondary metabolites and foliar herbivory by chewing insects in the field*

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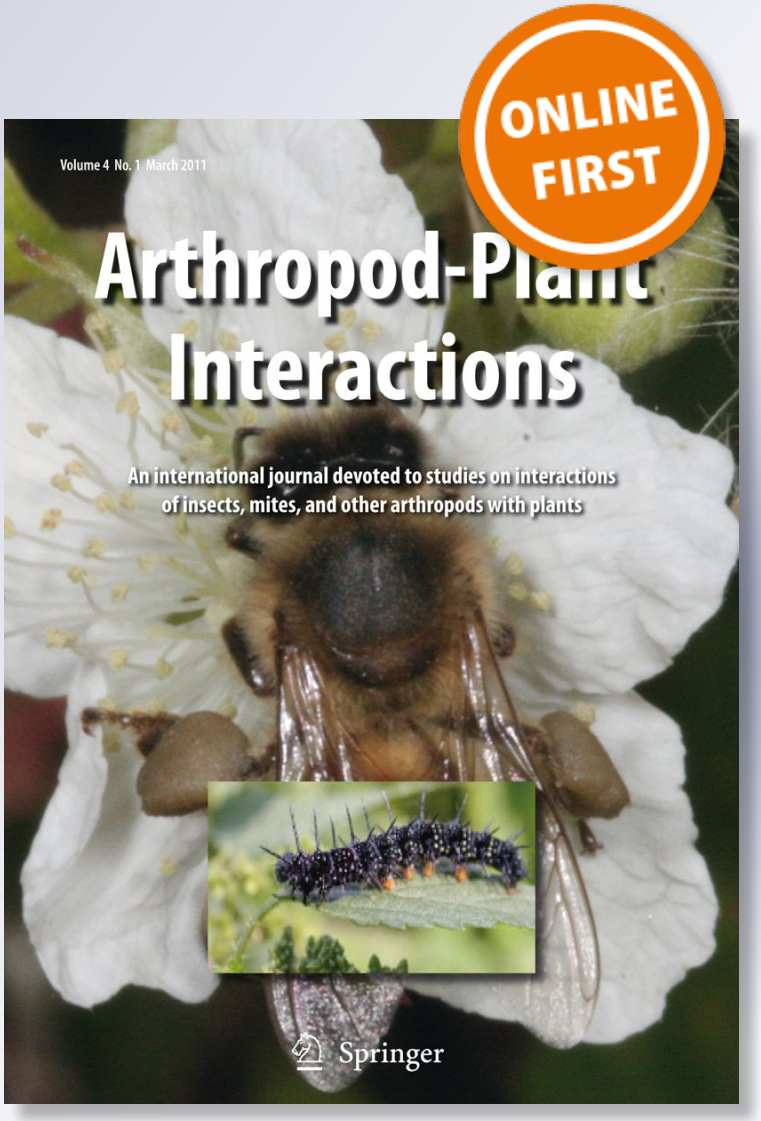

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ORIGINAL PAPER

Differences among five amaranth varieties (*Amaranthus* spp.) regarding secondary metabolites and foliar herbivory by chewing insects in the field

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Abstract In this study, we determined the abundance of secondary metabolites present in leaves of five varieties of *Amaranthus*, described the community of chewing insects observed in the foliage and also quantified damage by folivore insects in the field. Three flavonoid glucosides (rutin, nicotiflorin and isoquercitin), nine phenolic compounds (coumaric, vanillic, caffeic, syringic, ferulic, sinapic, protocatechuic, salicylic and 4-hydroxybenzoic acid) and three betalains (amaranthine, iso-amaranthine and betanin) were found to be present in amaranth leaves. Flavonoids appeared in of all varieties analyzed, with rutin being the most important. Betalains occurred only in some varieties and at different proportions, and nine phenolic acids were observed in all the varieties, with the exception of sinapic acid. Significant differences in the chemical composition of the varieties were noted. A total of 17 species of chewing phytophagous insects were observed through visual counting in *Amaranthus* plants, with the order Coleoptera being the most important and having the highest diversity of species. The degree of herbivory differed significantly among the varieties. Multivariate regression analysis indicated that the eight analyzed compounds detected in the plants had significant linear relationships with herbivory in the field. However, to draw any conclusions relating the amount of any compound to the degree of herbivory damage is premature at this stage of the research.

Keywords Flavonoids · Phenolic acids · Betalains · Herbivory · Amaranth · Stem borer · Amaranthine · Insects

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S. L. Niveyro (✉)
Facultad de Agronomía, Universidad Nacional de La Pampa, Ruta 35 Km 334, Santa Rosa 3300, La Pampa, Argentina
e-mail: niveyro@agro.unlpam.edu.ar

A. G. Mortensen · I. S. Fomsgaard
Faculty of Agricultural Sciences, Department of Integrated Pest Management Research Centre, Flakkebjerg, Aarhus University Forsøgsvej, 1, 4200 Slagelse, Denmark

A. Salvo
Centro de Investigaciones Entomológicas de Córdoba, IMBIV/CONICET, Facultad de Ciencias Exactas Físicas y Naturales, Universidad Nacional de Córdoba, Vélez Sarsfield 1611, Córdoba, Argentina

Introduction

Amaranths, *Amaranthus* spp., are a group of non-grass plants belonging to the Amaranthaceae family. Plants of this genus are valuable, not only for their properties as both vegetable and grain crops (Teutonic and Knorr 1985; Yánez et al. 1994), but also because they represent an alternative source of vegetable protein for dry-land agriculture. Grain *Amaranthus* have been recognized for their ability to thrive under abiotic stress (Brenner et al. 2000; Johnson and Henderson 2002), and most of the amaranth plants are able to grow in poor soils with moderate salinity levels (Liu and Stützel 2002; Omami and Hammes 2006). Also, plant presents C_4 photosynthesis, which confers on them high photosynthetic efficiency under conditions such as bright light, water deficiency and high temperatures (Grubben 1976).

Chemical analyses of *Amaranthus* plants indicate that they have noticeable quantities of secondary metabolites. Although these substances are not directly essential for basic photosynthetic or respiratory metabolism, they are thought to be required for plant survival in the environment in different ways, as signal and defense compounds and

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also by protecting plants from ultraviolet radiation and oxidants (Lattanzio et al. 2006).

Different flavonoids and phenolic compounds have been reported in leaves of *Amaranthus* (Cai et al. 2005; Suryavanshi et al. 2007; Steffensen et al. 2011), with most of these being considered beneficial for human health due to their nutritional and antioxidant properties (Ferguson et al. 2001; Srinivasan et al. 2006; Robaszkiewicz et al. 2007). The occurrence of secondary metabolites in human food plants is not only interesting because of their nutritious properties, but also because of their interactive role between plants, herbivorous organisms and plant pathogens (Harborne and Grayer 1994; Simmonds 2003; Carlsen and Fomsgaard 2008). Chemicals in the group of the phenolic acids have shown negative effects on insects, acting as deterrent or even being toxic to non-adapted insects (Simmonds 2003). It has been proposed that these compounds induce oxidative stress in herbivores (Summers and Felton 1994), but the effectiveness of some phenolic is also enhanced by a reduction in the digestibility and nutritional value of the plant (Bi et al. 1997; Lattanzio et al. 2006). In some cases, phenolic compounds might alter the insect fitness (Oberdorster et al. 2001; Bi et al. 1997), which will also depend on the ability of insects to detoxify and metabolise ingested plant metabolites.

Other compounds, however, stimulate the feeding rate (Green et al. 2003) and may even increase the insect fitness. Flavonoids have been previously mentioned as phagostimulants for polyphagous insects (Lattanzio et al. 2006), and in some cases, these insects can sequester these compounds into their body cuticle for protection against predators, or into their wings for visual communication (Burghardt et al. 2000; Simmonds 2003; Lattanzio et al. 2006). As mentioned above, the role of secondary metabolites in the insect–plant interactions is rather complex, as they might function as deterrents or phagostimulants in insect feeding. This may depend on their concentrations in plant tissues (Blaney and Simmonds 1983) or/and even on the occurrence of other chemical compounds in the plant (Green et al. 2003; Ruuhola et al. 2001). In *Amaranthus,* however, little is known about the defense mechanisms, whether constitutive or inducible, that plants might employ to reduce insect-derived damage (Sánchez-Hernández et al. 2004; Delano-Frier et al. 2004, 2011).

Studies focusing on the role of secondary metabolites in amaranth-insect interactions are still scarce. However, the larvicide effects on *Anopheles stephensi* Liston, a vector insect of malaria, were recently demonstrated for the raw extract of *Amaranthus oleracea* (Sharma et al. 2009). The aim of this study was to assess the herbivory by chewing insects in the foliage of five crop varieties of *Amaranthus* and, to evaluate the chemical composition of leaves damaged by folivores, in terms of quality and quantity of secondary metabolites.

Materials and methods

Field experiments

The study was performed in the experimental field of the Faculty of Agronomy, UNLPam Santa Rosa, La Pampa (36°37′00″S, 64°16′60″W) from the end of November 2007 to May 2008. The trial was carried out with a Latin Square design of 5 × 5 plots ($n = 25$), with each plot (43.75 m^2) being sown with eight rows of 12 m at a spacing of 0.50 m. Plots were separated from each other the next by a distance of 1 m. The sowing density was 3.5 kg of seeds per hectare, and plants did not receive any insecticide application with all weeding being done manually.

Five varieties were sown: *Amaranthus cruentus* cv. Don León, *Amaranthus hypochondriacus* San Antonio, *A. hypochondriacus* FK 280-FH1, *A. hypochondriacus* cv. Artasa 9122 and *Amaranthus mantegazzianus* cv. Don Juan. In order to simplify the description of the results and discussion, varieties are named Cruentus, Hyp SA; Hyp 280; Hyp Artasa and Mantegazzianus, respectively. The genotypes were chosen on the basis of their good agronomic performances (Covas 1987; Niveyro, unpublished data). These had different levels of pigments in the foliage, stems and panicles, as shown in Table 1.

The climatic conditions during the period of study were mean air temperature between 16 and 23 °C, precipitation between 44.6 and 110.6 mm and an average solar radiation of 19.4 MJ/m2/day.

Herbivory measures

For each plot, on six sampling dates throughout the development of the crop, herbivory by chewing phytophagous

Table 1 Plant tissue pigmentation in five varieties of *Amaranthus*

Plant part	Cruentus	Hyp SA	Variety Hyp 280	Hyp Artasa	Mantegazzianus
Leaf	Green	Green	Reddish	Reddish green	Green
Stem	Green	Reddish green	Reddish	Reddish green	Green
Panicle	Yellowish green	Red	Red	Red	Yellow

insects was measured by estimating the leaf damage percentage. To carry this out, on each sampling date, 30 leaves were taken from each plot at random, and the foliar damage was estimated visually in the field by assigning a percentage of leaf area lost by herbivory on a 0–9 scale with the following categories—0: 0 %, 1: 1–5 %, 2: 6–10 %, 3: 11–20 %, 4: 21–30 %, 5: 31–40 %, 6: 41–50 %, 7: 51–65 %, 8: 66–75 % and 9: 76–95 % (Fig. 1). In order to avoid repetition through the estimation of foliar damage in the same leaves on different dates, leaves were always chosen for each sampling date at a lower level on the plant than in the previous sampling.

Sampling of insects

Insect sampling was conducted weekly throughout crop development in all varieties of the trials. For each plot and sampling date, 10 plants in three inner rows were randomly selected and revised carefully. Observations were conducted during the day from 10:00 to 15:00 hours, when most of the insects that feed on *Amaranth* were active and conspicuous. All chewing insects observed for 15 min (discounting handling time) were counted, collected and conserved in entomological boxes (adults) or conserved in 70 % alcohol (soft insects and larvae) for subsequent identification in the laboratory.

Chemical analysis

In order to analyze the content of secondary metabolites, twenty leaves from each plot were randomly cut at the end of April (end of the flowering stage) and stored at −80 °C until being freeze-dried. Leaves were collected near the harvest, after 4 months of exposition to insects, in order to evaluate the compounds potentially induced by herbivory. Plants at this state showed no signs of senescence.

Reagents and chemicals

The following chemicals were purchased from commercial sources: HPLC-grade methanol (Rathburn Microlab, Denmark), glacial acetic acid (J.T. Baker, VWR, Denmark), acetonitrile (Rathburn Microlab, Denmark), nicotiflorin (Extrasynthese, France), isoquercitrin (Extrasynthese, France), rutin (Extrasynthese, France), protocatechuic acid (Sigma, Denmark), *p*-hydroxybenzoic acid (Sigma, Denmark), gallic acid (Fluka Sigma, Denmark), vanillic acid (Fluka Sigma, Denmark), caffeic acid (Sigma, Denmark), syringic acid (Sigma, Denmark), *p*-coumaric acid (Fluka Sigma, Denmark), sinapic acid (Fluka Sigma, Denmark), ferulic acid (Fluka Sigma, Denmark), salicylic acid (Sigma, Denmark) and ammonium acetate (Merck, VWR, Denmark).

Extraction of flavonoids and phenolic acids

The freeze-dried samples were crushed and homogenized with a Waring blender before extraction on an accelerated solvent extraction (ASE) 200 system (Dionex). For each sample, two replicate extractions were made. Five grams of glowed chemically inert Ottawa sand (particle size 20–30 mesh, Fisher Chemicals, Denmark) was added to the 33-mL extraction cells. Subsequently, 0.1 g of the freeze-dried and homogenized sample was transferred to the extraction cell, five more grams of glowed chemically inert Ottawa sand was added, a filter was placed on top of the sample, and the extraction cell was filled with glowed glass balls. The eluent was 70 % MeOH (Rathburn. Mikrolab, Denmark) with 30 % water. The protocol for the ASE extraction was the following: preheat 5 min, heat for 5 min, static for 3 min, flush 80 %, purge for 60 s, four cycles, pressure 1,500 Pa and temperature 80 °C. Extracts were collected in vials and stored at −20 °C for chemical analysis.

Chemical analysis of flavonoids and phenolic acids

The extracts were diluted with water at a 1:1 ratio. An Applied Biosystems 3200 QTrap liquid chromatography triple quadruple mass spectrometer (LC/MS/MS) (ABSciex) with turbo electrospray ionization in a negative multiple reaction monitoring mode (MRM) was used for the chemical analysis. The chromatographic separation was performed at a flow rate of 0.2 mL/min at 30 °C with an injection volume of 20 μL. The column used was a Phenomenex Synergi Polar–RP801 (2.00 mm × 250 mm, 4 μm). The A-eluent contained 7 % acetonitril (Rathburn. Mikrolab, Denmark) and 93 % filtered milliQ water (v/v) with 20 mM of glacial acetic acid (J.T.Baker, VWR, Denmark). The B-eluent was acetonitril 78 and 22 % filtered milliQ water with 20 mM of glacial acetic acid. The gradient contained the following: 16 % B for 1 min followed by linear gradient to 18 % B for 4 min; a linear gradient to 30 % B for 17 min; a linear gradient to 100 % B for 8 min; isocratic elution for the next 5 min; a 4 min ramp back to 16 % B and re-equilibration for 11 min. The total run time of the analysis was 48 min. The mass-to-charge ratio (m/Z) used for identification of the flavonoids and phenolic acids was as follows; (Q1/Q3): nicotiflorin (593.5/284), isoquercitrin (463.3/300.4), rutin (609.5/300.7), protocatechiuc acid (153.1/109), *p*-hydroxybenzoic acid (137.2/92.8), gallic acid (169.1/169.1), vanillic acid (167.2/152), caffeic acid (179.0/135), syringic acid (197.2/197.2), *p*-coumaric acid (163.2119.2), sinapic acid (223.1/164), ferulic acid (193.0/149) and salicylic acid (137.2/92.8).

Extraction of betalains

One hundred milligrams of freeze-dried sample was put in vials. Subsequently, 2.5 mL of 70 % MeOH/H_2O (v/v) and 47.5 mL of 10 mM ammonium acetate (Merck, VMR, Denmark) (pH 6.6) were added. This mixture was shaken for 30 min, after which the extracts were collected and stored in flasks at −4 °C until chemical analysis. The extracts were filtered on a Sartorious SRP 15 0.45 μM filter (PTFE membrane) and put in injection vials. The analyses were carried out using a LC/MS Hewlett Packard 1,100 series with a DAD detector. Two replicates were analyzed for each sample.

Detection of betalains

The chromatographic separation was performed at a flow rate of 0.2 μL/min at 25 °C with an injection volume of 50 μL. The column was Phenomenex Synergi Fusion–RP 80A (250 mm × 2.00 mm, 4 μm). The A-eluent contained 1 % MeOH in 99 % ammonium acetate 10 Mm, and the B-eluent consisted of 90 % MeOH and 10 % ammonium acetate 10 mM. The gradient contained the following: 3 % B for 1 min; a linear gradient to 10 % B for 5 min; a linear gradient 15 % B for 9 min isocratic elution for 3 min; a 1 min ramp back to B; equilibration for 6 min.

The absorbance of four betalains was measured in a DAD detector at 538 nm. The identities of amaranthine and isoamaranthine were confirmed by single ion monitoring mass spectrometry (SIM-MS) with an m/z value of 727 $[M + H]^+$. The identities of betanin and isobetanin were confirmed with an m/z value of 551 MS $[M + H]^+$. The quantity of the betalains was determined on the basis of their molar absorptivities as described by Cai et al. (2001, 2005).

Statistical analysis

An analysis of variance (ANOVA) and the LSD test a posteriori were used to determine the significant differences in secondary metabolite content, density and percentage of insect herbivory among amaranth varieties. When necessary, data were logarithmically or square root-transformed in order to meet the assumptions of the parametric tests.

Discriminant analysis (DA) and multivariate analysis of variance (MANOVA) were used to analyze similarities in chemical compound content among varieties.

The relationship between herbivory (response variable) and chemical compounds (explicatory variables) was evaluated using multiple regression analysis and the selection of the linear regression model determined by a backward elimination method. In both analyses, regression and DA data were standardized.

Results

Chemical analysis

Our results showed that three flavonoid glucosides (rutin, nicotiflorin and isoquercitin), nine phenolic compounds (coumaric, vanillic, caffeic, syringic, ferulic, sinapic, protocatechuic, salicylic and 4-hydroxybenzoic acid) and three betalains (amaranthine, iso-amaranthine and betanin) were present in amaranth leaves (Table 2). Flavonoids occurred in leaves of all the varieties analyzed, with rutin being the most common. Betalains were present only in the varieties Hyp 280, Hyp Artasa and Mantegazzianus, at different concentrations (Table 2). The nine phenolic acids were observed for all the varieties, with the exception of sinapic acid. There were significant differences among the chemical composition of the varieties (Table 2).

The results of the discriminant analysis (DA) indicated that Hyp 280 differed from all the other varieties studied, whereas Cruentus–Hyp SA and Mantegazzianus–Hyp Artasa were similar to each other ($F_{(60,123)}$ of Wilks = 5.52; $p < 0001$) (Fig. 2). Ninety-four percent of the samples were correctly assigned to the varieties, and the first two axes of the ordination absorbed 86 % of the data variance.

Herbivory measures

Herbivory rates (percentage of leaf area damaged) caused by chewing phytophagous insects varied between 18 and 29 % (Fig. 3) with the degree of herbivory differing significantly among the varieties (ANOVA $N = 25$, $df = 4$, 20 $F = 3.86$, $p = 0.030$). The highest values were observed in Hyp SA and Hyp Artasa, whereas the lowest were in Mantegazzianus (Fig. 3).

The multivariate regression analysis indicated a significant relationship between secondary metabolites and herbivory rates found in the field ($R^2 = 0.80$, $N = 42$, $p < 0.0001$). Out of fifteen compounds analyzed, eight were significant predictors in the constructed model (Table 3).

Sampling of insects

Seventeen chewing herbivory species were observed in *Amaranthus* plants (Table 4). Of these, the Order Coleoptera was the most important, with the highest diversity of species. Most of the genera observed had been previously reported by other authors (Weber et al. 1990; Wilson 1990). However, some insects that had been mentioned as

Table 2 Mean concentration (μg/g dry weight) ± standard errors of secondary metabolites detected in leaves of *Amaranthus* varieties

	Variety				
	Cruentus	Hyp SA	Hyp 280	Hyp Artasa	Mantegazzianus
Phenolic acids					
Hydroxybenzoic	8.7 ± 1.6 a	8.8 ± 1.0 a	16.7 ± 2.5 ab	16.4 ± 6.4 ab	23.4 ± 11.3 b
Protocatechuic	13.8 ± 2.5 bc	30.8 ± 3.5 cd	45.0 ± 18.4 d	13.9 ± 6.1 ab	3.2 ± 2.1 a
Coumaric	12.5 ± 2.9 b	9.3 ± 0.9 b	26.1 ± 2.5 c	5.2 ± 5.1 a	0.4 ± 0.3 a
Vanillic	28.0 ± 5.4 a	30.8 ± 6.9 a	36.2 ± 6.2 a	63.2 ± 29.9 a	49.8 ± 30.8 a
Caffeic	7.2 ± 3.0 a	14.6 ± 3.2 a	113.9 ± 22.1 b	18.6 ± 18.0 a	1.9 ± 1.5 a
Syringic	1.0 ± 1.1 ab	23.5 ± 6.3 b	22.2 ± 13.2 b	0.7 a ± 0.6	3.2 ± 3.1 ab
Ferulic	96.2 ± 18.3 b	115.8 ± 10.1 b	112.3 ± 34.6 b	25.9 ± 24.9 a	12.8 ± 12.5 a
Sinapic	0.00	0.00	0.00	0.01 ± 0.01 a	0.6 ± 0.6 a
Salicylic	1.8 ± 0.5 ab	3.4 ± 0.7 bc	4.5 ± 1.4 c	0.7 ± 0.7 a	0.3 ± 0.2 a
Flavonoids					
Rutin	7,317.4 ± 1630.7 a	8,671.3 ± 1,104.7 ab	4,798.5 ± 1,333.9 a	15,531.0 ± 2,658.1 b	9,217.3 ± 4,633.7 ab
Isoquercitrin	27.8 ± 1.8 a	110.8 ± 75.7 ab	279.5 ± 63.3 c	186.6 ± 107.9 bc	36.8 ± 16.1 a
Nicotiflorin	1,281.5 ± b	812.9 ± 51.2 b	141.6 ± 52.4 a	977.1 ± 313.9 b	1,156.9 ± 645.8 b
Betalains					
Amaranthine	0.00	0.00	448.4 ± 222.8 b	318.6 ± 50.6 b	2.1 ± 1.0 a
Isoamaranthine	0.00	0.00	145.1 ± 58.8 b	69.2 ± 16.6 b	0.5 ± 0.5 a
Betanin	0.00	0.00	6.3 ± 2.7 b	4.1 ± 2.0 b	0.2 ± 0.1 a

Mean followed by different letters in the same row are significantly different according to the LSD test ($p < 0.05$)

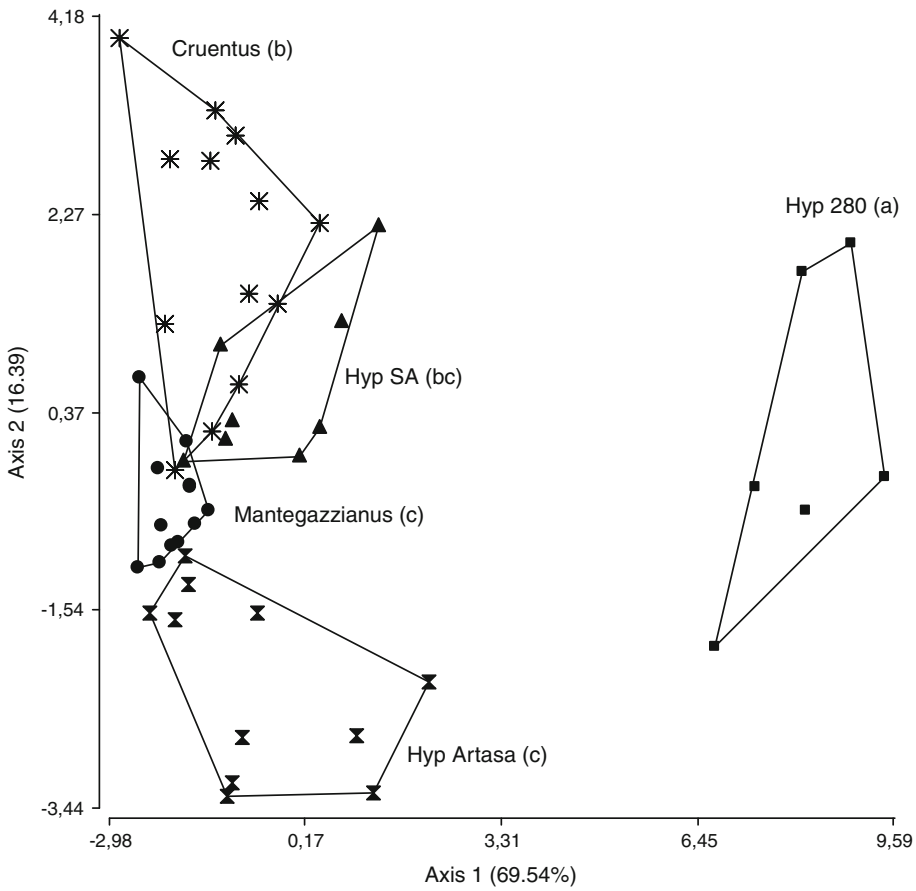

Fig. 1 *Amaranthus* leaves with different leaf areas removed by chewing phytophagous insects. **a** 1–5 % **b** 6–10 % **c** 11–20 % **d** 21–30 % **e** 31–40 % **f** 41–50 % **g** 51–65 % **h** 66–75 % **i** 76–95 %

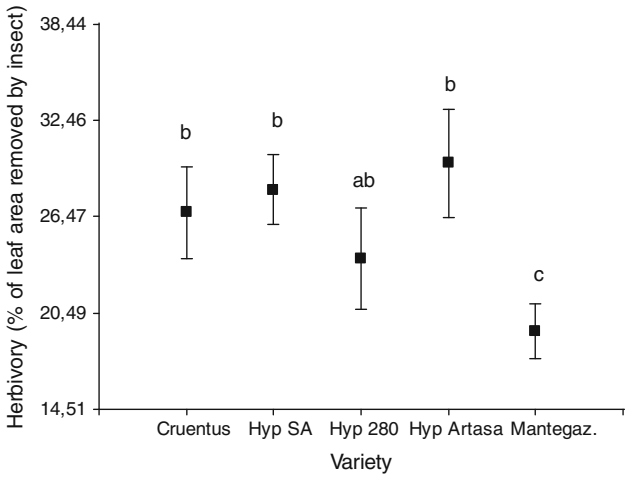

Fig. 2 Discrimination of samples of different *Amaranthus* varieties based on the composition of fifteen secondary metabolites. Ninety-four percent of the samples were classified to the correct variety. *Different letters* following the variety names indicate significant differences according to MANOVA ($p < 0.0001$)

common pests on amaranth plants, such as the tarnished plant bugs *Lygus lineolaris* (Palisot de Beauvois) and *Diabrotica speciosa* (German), were not observed. Different species of weevils were recorded in this study, among which were two stem borers. An extensive list of species belonging to this latter group from around the world has been previously reported (Low et al. 1995, among others). In our study, however, only two species of *Conotrachelus* spp. were present, whose adults cause leaf damage. The total density of insects per plant was analyzed by ANOVA, and significant differences among varieties were found (Fig. 4).

Discussion

In this study, we determined the abundance of secondary metabolites present in leaves of five varieties of *Amaranthus*, described the community of chewing insects observed in the foliage and also quantified insect damage in the field. However, it should be borne in mind that our results regarding the chemical composition of leaves only represent a "snapshot" at a particular stage of development (end of season); thus, no information is provided regarding the way phenolic composition might vary in younger amaranth plants, which are usually more susceptible to herbivory and more responsive to external stimuli.

The rutin and quercetin contents in different plant parts of *Amaranthus* species and varieties have been previously described (Kalinova and Dadakova 2009). In our study, we analyzed not only flavonoids, but also phenolic acids and betalains, which allowed us to show that flavonoids were the main group of compounds found in *Amaranthus* leaves, with rutin being the most abundant among these. In fact, the rutin concentration greatly exceeded that of the rest of the tested compounds, in agreement with observations on other species of *Amaranthus* (Kalinova and Dadakova 2009). The plant age may affect the rutin levels, which tend to be lower in younger plants in comparison with those observed in the mature plants (Steffensen et al. 2011). The relatively high values of rutin detected in mature *Amaranthus* plants by Kalinova and Dadakova (2009) might be attributed to different environmental conditions as the dependence of rutin content on the environmental conditions experienced by plants has been clearly demonstrated (Li et al. 1993; Steffensen et al. 2011).

The nicotiflorin content greatly exceeded that of Isoquercitrin by a large amount in most of the varieties tested (except Hyp 280) which is consistent with levels reported for younger plants (Steffensen et al. 2011). In fact, the high levels of Isoquercitrin in Hyp 280 and rutin observed in Hyp Artasa were the most marked differences in the flavonoids among the five varieties. Isoquercitrin occurrence seemed to be dependent on the amaranth species, with Cruentus and Mantegazzianus being the varieties with lowest quantities of this compound. In agreement with plant coloration (Table 1), the reddish-leaved plants Hyp Artasa and Hyp 280 had signified higher contents of the three betalains analyzed

Table 3 Results of multiple regression analysis of herbivory (leaf damage) and secondary metabolite content in leaves (*df* degree of freedom, *β* standardized coefficient, *SE* standard error)

	Predictor variable	*df*	*β*	SE	*F*	*P*
Herbivory	Nicotiflorin	1.42	−4.60E−03	1.10E−03	17.83	0.0002
	Ferulic acid	1.42	0.05	1.00E−02	39.44	<0.0001
	Rutin	1.42	1.10E−03	1.40E−04	54.43	<0.0001
	Amaranthine	1.42	−0.02	3.70E−03	16.91	0.0002
	Iso-amaranthine	1.42	0.08	0.01	29.74	<0.0001
	Caffeic acid	1.42	−0.06	0.02	16.26	0.0003
	Vanillic acid	1.42	−0.02	0.01	6.56	0.0151
	Syringic acid	1.42	0.08	0.03	7.38	0.0103

Table 4 Species of chewing phytophagous insects observed in *Amaranthus* plants, indicating the stage of the cycle and the part of the plant in which they were observed

Order/family/species	Stage		Plant part		
	Adult	Larvae	Stem	Panicle	Leaf
Coleoptera					
Curculionidae					
Naupactus verecundus Hustache	X				X
Pantomorus viridisquamosum (Boheman)	X				X
Pantomorus auripes Hustache	X				X
Pantomorus ruizi (Brethes)	X				X
Naupactus leucoloma Boheman	X				X
Conotrachelus histrio Boheman	X	X	X		X
Conotrachelus cervinus Hustache	X	X	X		X
Meloidae					
Epicauta adspersa (Klug)	X				X
Melyridae					
Astylus atromaculatus Blanch.	X			X	
Cantharidae					
Chauliognahtus scriptus (Horn)	X				X
Chrysomelidae sp	X				X
Cassidinae					
Chelymorpha varibilis var *crucifera*	X				X
Lepidoptera					
Noctuidae					
Spodoptera frugiperda Smith	X	X			X
Rachiplusia nu (Guenée)	X	X			X
Loxostege sp.	X	X			X
Orthoptera					
Acrididae					
Dichroplus elongatus G. Tos	X				X
Dichroplus maculipennis (Blanch)	X				X

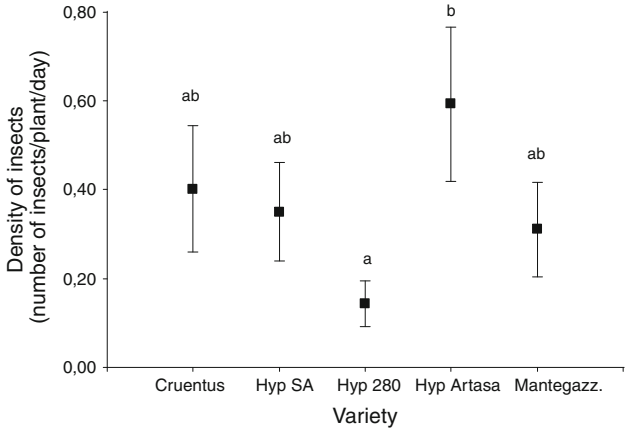

Fig. 3 Mean herbivory levels (and standard errors) recorded in plants of *Amaranthus* varieties. *Different letters* above means indicate significant differences according to the LSD test ($p = 0.030$)

than Mantegazzianus, whereas no betalains were detected in the other two varieties Cruentus and Hyp SA (Table 2). Differences in color of plant tissues may be attributed to pigments such as anthocyanins or betalains. Related to this, betalains are nitrogenous chromoalcaloids, with their presence excluding that of anthocyanins, as observed in Caryophyllales (Clement and Mabry 1996). It is also known that betalains are responsible for the red appearance of members belonging to Amaranthaceae (Cai et al. 2001; Strack et al. 2003). However, the relatively low amounts of red pigments occurring in Mantegazzianus seem not to cover the chlorophyll color, and thus plants kept their green color as it has been previously mentioned in relation to Anthocyanin content (Manetas 2006). Our results are in agreement with studies dealing with betacyanin (red pigment) content in red and yellow plants of *Celosia argentea*, an amaranthaceous plant species with a higher content of amaranthine and its isoform in reddish leaves than in yellow ones (Schliemann et al. 2001).

In addition to the differences occurring in the betalain content, we also found that the phenolic acid content varied widely amply among species and even among the three varieties within the *A. hypochondriacus* species (Table 2). In addition, Hyp 280 was different from all other varieties with respect to its metabolite content, with its samples being completely separated from those samples of the other four varieties in the first axis of the ordination in the discriminant analysis (Fig. 2). Moreover, this variety had a significantly lower content of nicotiflorin (one of the two predominant compounds in all samples analyzed) than the other varieties. In addition, caffeic and coumaric acids were present with significantly higher contents in Hyp 280. According to the same analysis, samples of Hyp Artasa were also completely separated from those of other varieties, as in the case of Hyp 280, with all its samples being correctly assigned to the same group. The absence of betalains was a feature shared by samples of the Cruentus and Hyp SA varieties, while small amounts of sinapic acid were only found in Mantegazzianus and Hyp Artasa. The Mantegazzianus samples were rather homogeneous, but in some cases, these were erroneously assigned to Hyp SA and

Cruentus, as these three varieties were not so clearly differentiated.

The sampling of chewing insects in the field indicated that Hyp Artasa was the variety with the highest density of insects, followed by Cruentus, Hyp SA and Mantegazzianus (Fig. 4). However, the herbivory by insects observed in the plants was significantly lower in Mantegazzianus than in other varieties, with approximately 10 % less foliar area being removed. Hyp 280 showed intermediate values of herbivory and the lowest of insect density, whereas Cruentus, Hyp SA and Hyp Artasa had the highest levels of foliar damage (Fig. 1).

Evaluation of the metabolites occurring in plants that are growing in the field is rather complex because compounds with antioxidant properties are highly influenced by environmental conditions such as temperature and UV radiation, among others (Close and McArthur 2002). The results from our study revealed differences in the contents of secondary metabolites in plants under natural insect folivory. Eight analyzed compounds detected in the plants presented a significant linear relationship with herbivory (Table 3). However, given the methodology employed, we were not able to affirm that the greater quantities of these metabolites in the damaged leaves were a consequence of their phagostimulant effects or just a response to herbivory as an inducible defense against herbivores. Further experimental tests should be carried out in order to elucidate this point.

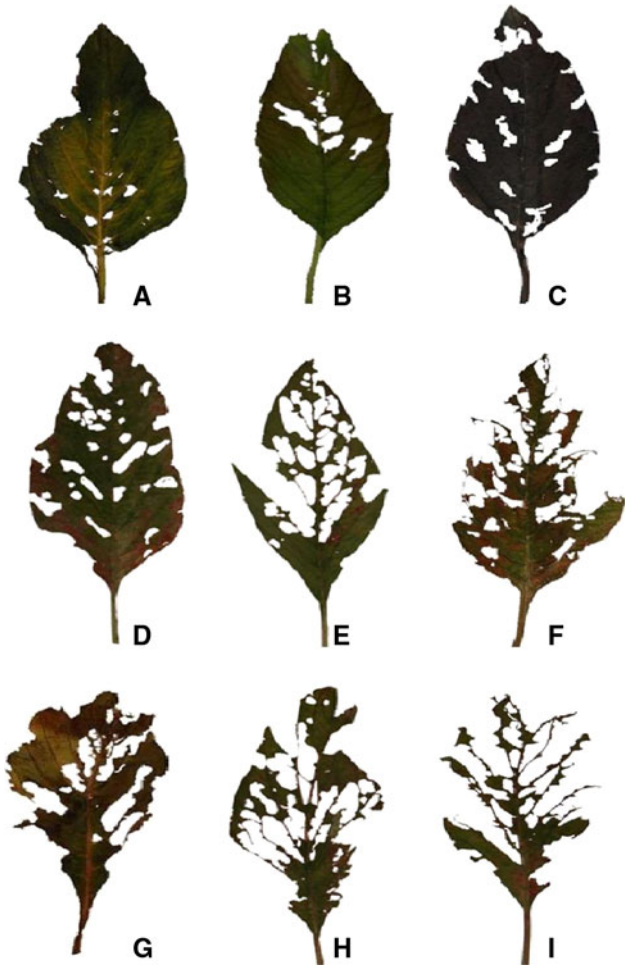

Fig. 4 Mean density of insects (and standard errors) recorded in plants of *Amaranthus* varieties. *Different letters* above means indicate significant differences according to the LSD test ($p = 0.030$)

As found in our data and also mentioned in the literature, high values of flavonoids (especially rutin) are always present in various organs of amaranth plants, particularly in leaves (Paśko et al. 2008; Kalinova and Dadakova 2009). Moreover, increased levels of herbivory by insects have been attributed to greater levels of flavonoids in plants (Burghardt et al. 2000), with rutin having been mentioned as a phagostimulant for some lepidopteran species such as *Heliothis virescens*, *Spodoptera littoralis, Spodoptera exigua* and *Spodoptera exempta*, and for some locusts such as *Schistocerca americana, Spodoptera albolineata* and *Melanoplus differentialis* (Bernays et al. 1991; Blaney and Simmonds 1983). In our study, most varieties with higher levels of rutin also presented higher densities of insects and leaf damage, with the exception of Mantegazzianus, which had a high content of rutin and was the least affected by insects.

The function of red pigments such as anthocyanins has been mentioned as being a defense against herbivores, either through direct toxicity, as a warning, or by cryptic coloration. Different hypotheses to explain this have been postulated, but the evidence is rather contradictory. Even though the amaranth plant does not contain anthocyanins, similarities in the spectral and antioxidant properties between anthocyanins and betalains have given rise to speculation as to whether betalains could play similar roles to those of anthocyanins (Manetas 2006). However, research dealing with betalain functions is scarce (Ibdah et al. 2002).

Plant color has been found to affect insect preference in both consumption and oviposition, in favor of green leaf plants (Furuta 1986; Karageorgou and Manetas 2006), despite the fact that insects do not easily distinguish reddish plants due to the greater sensitivity of the eyes of folivorous insects occurring from 300 to 550 nm (Matic 1983; Kelber et al. 2003). Nevertheless, in the case that reddish plants could be seen, insects would tend to avoid them because the red color indicates that plants are defended (Hamilton and Brown 2001; Lev-Yadun 2001). Also, it is probable that red compounds are accompanied by other colorless phenolics, which may also affect the insects (Close et al. 2001; Karageorgou and Manetas 2006).

Our results show that high levels of betalains and phenolic acids were registered in Hyp 280 (Table 2). However,

this did not indicate that plants with betalains were any more protected against herbivores, since varieties with the highest content of betalains (Hyp 280 and Hyp Artasa) showed similar herbivory levels as those without pigments (Hyp SA and Cruentus). There exist, nevertheless, the possibility that the accumulation of betalains in other parts of the plant, such as stems or panicles, might exert defense against other guilds of insects.

Other compounds detected in our study such as phenolic acids have been previously reported to have a protective role against insects. In particular, syringic, ferrulic, caffeic and salicylic acids seem to increase after the plant is attacked by insects (Bi et al. 1997; Clement and Mabry 1996; Heidel and Baldwin 2004). These compounds, as well as all the phenolic acids mentioned above (with exception of salicylic), were good predictors of herbivory in *Amaranthus* leaves (Table 3).

Salicylic acid, an important signaling compound in plant defense (Heidel and Baldwin 2004), was detected at lower concentrations in Mantegazzianus and Hyp Artasa, with the former being the least damaged variety in our study. Although Hyp 280 and Mantegazzianus showed differences with respect to the other varieties in most of the variables measured, the great variability in the amounts of the compounds analyzed (particularly for phenolic acids across varieties) did not allow a clear enough pattern to be revealed in order to connect their differences with herbivory.

It should not be ruled out that other compounds not analyzed in this study may also be influencing differences in herbivory (e.g., oxalates). Oxalates have been reported in amaranth leaves (e.g., *Amaranthus gangeticus* L.) in different forms, as soluble forms such as potassium or magnesium oxalate, and also in the insoluble form such as calcium oxalate (Vityakon and Standal 1989). This latter form of oxalate has been demonstrated to have a defensive role through several modes of action against chewing insects in other types of plants (Franceschi and Nakata 2005; Korth et al. 2006), whereas other guilds of insects are affected by oxalic acid, the precursor of calcium oxalate (Yoshihara et al. 1980). In the latter case, it needs to be considered that this mineral is undesirable in plants for human consumption when present at high concentrations (Vityakon and Standal 1989; Gélinas and Seguin 2007). Thus, the possible role of secondary metabolites in the control of herbivorous insects of *Amaranthus* should be studied as an interesting way to use inherent plant mechanisms as a means of defense in crops, never losing sight of the role that these compounds may have as part of the human diet.

Summing up, we have analyzed differences in the chemistry of the varieties and found differences in the degree of natural insect folivory, but to draw any conclusions about the relation between both is premature at this stage of the study. It is our hope that this study encourages future research in order to elucidate the role of chemical compounds in *Amaranthus* in the defense against insects.

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