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RESEARCH

Agronomic, Chemical, and Antioxidant Characterization of Grain Amaranths Grown in a Mediterranean Environment

Fabio Gresta, Alessandra Guerrini,* Gianni Sacchetti, Massimo Tacchini, Orazio Sortino, Giuseppe Ceravolo, Andrea Onofri

ABSTRACT

The use of amaranth (*Amaranthus* spp.) in the food and dietary supplements industry is increasing. The aim of this work is to promote the Mediterranean area as a production hot spot, by using multivariate statistical analyses on data obtained in two-year trials and relating to morphological, productive, and qualitative traits and antioxidant activity of 20 accessions of five amaranth species (*A. caudatus* L., *A. cruentus* L., *A. hybridus* L., *A. hypochondriacus* × *hybridus* L., *A. hypochondriacus* L.). *Amaranthus cruentus* accessions (Mexicane and New Mexico) were stable and highly productive (>2.7 t seeds ha^{-1}), while Kinnaury Dhankar accession showed the highest protein percentage (17%). *Amaranthus hypochondriacus* (Orange Giant and Burgundy) possessed the highest oil percentage (always above 5.7%). A high amount in linoleic, *cis*-oleic, and palmitic acids, and an interesting saturated/unsaturated acids ratio ranging from 0.26 (*A. hybridus*) to 0.32 (*A. hypochondriacus*) were detected. The unsaponifiable fraction revealed the abundance of squalene in all species, while total tocopherol concentrations were lower than expected.

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Abbreviations: ABTS^{+}, 2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid; AMMI, additive main effect multiplicative interactions; DAD, diode array detector; DPPH, 1,1-diphenyl-2-picrylhydrazyl; FAME, fatty acids methyl esters; FID, flame ionization detector; GC, gas chromatography; HPLC, high performance liquid chromatography; MS, mass spectrometry; NIST, National Institute of Standards and Technology; PCA, principal component analysis; UV-VIS, ultraviolet-visible.

The globalization of agriculture has brought undoubted benefits to some, but it has also caused some negative side effects, such as monoculture production and reduced genetic diversity. As a consequence, global food security has become increasingly vulnerable. It has been stated that 75% of the world's food is generated from only 12 plants (FAO, 2015). Among these, just three—rice (*Oryza sativa* L.), maize (*Zea mays* L.), and wheat (*Triticum aestivum* L.)—contribute nearly 60% of the calories and proteins obtained by humans from plants.

The reduction in the number of crops being grown has stimulated international organizations and the whole research community to investigate neglected or underexploited crops. In some cases, it has been found that these crops possess favorable nutritional and nutraceutical benefits (Bruni et al., 2001; Barba de la Rosa et al., 2009), and grain amaranth (*Amaranthus* spp.) is one of

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these valuable alternative crops. Amaranth species, which originated from South America, are annual, broad-leaf, herbaceous plants with an erect stem ending in a large, often colored, inflorescence. Along with such plants as maize and sorghum [*Sorghum bicolor* (L.) Moench], they show a C4-photosynthetic pathway, which is highly efficient in high-light-intensity and high-temperature conditions. For these reasons, amaranth plants are drought resistant and thrive in temperatures of 30 to 35°C (Rastogi and Shukla, 2013). They are recognized as invasive and cosmopolitan weeds because of their high capability of germinating in a wide range of environmental and climatic conditions (Cristaudo et al., 2014).

Some species, such as *A. hypochondriacus*, *A. cruentus*, and *A. caudatus*, have been commonly considered grain crops and have been grown since ancient times as sources of food, assuming great importance in the Pre-Columbian era (Caselato-Sousa and Amaya-Farfan, 2012). Grain amaranth might be interesting also nowadays because of its high protein percentages (14 to 17%) and dietary mineral (Fe, Cu, Zn, etc.) contents (Tömösközi et al., 2009) when compared with cereals. Moreover, amaranth exhibits a fatty acid profile similar to that of maize oil and has a valuable antioxidant potential (Caselato-Sousa and Amaya-Farfan, 2012). Likewise, it contributes to the maintenance of normal blood cholesterol level, to cardiovascular health, and to normal formation of red blood cells and hemoglobin (EU Register on Nutrition and Health Claims, 2017), which gives it great potential as a healthy and functional food (Bruni et al., 2001; Marcone et al., 2004; Martirosyan et al., 2007).

Amaranth may nowadays be included among gluten-free pseudo-cereal species and may represent an important source of food for people with celiac disease (Gambuś et al., 2009). All these features make amaranth a crop with high commercial interest: in fact it can act as a wheat substitute for breakfast cereals, bread, biscuits, and other flour-based products. It can also be combined with other grain flours, such as oats and wheat, to make more flavorful food.

Notwithstanding its healthy potential, amaranth is still a niche crop, cultivated on a small scale in Central and South America, India, Pakistan, Nepal, and China. It is unlisted in international agricultural statistics. Although this plant has recently been appreciated both for its healthy and dietary potential, for its wide environmental adaptability, and for its ability to improve crop production in the Mediterranean area (Jacobsen et al., 2012), researchers have paid little attention to the identification of the best species and accessions for high quantity and quality of seed yield in this environment.

With this in mind, the present research aims to assess the quantitative and qualitative performances of 20 different amaranth accessions belonging to five different species (*A. caudatus*, *A. cruentus*, *A. hybridus*, *A. hypochondriacus*, and *A. hypochondriacus x hybridus*), grown in a Mediterranean environment of Southern Italy. Such an evaluation was performed for 2 yr (2005 and 2006) and includes morpho-productive traits and phytochemical investigation of the lipid fraction, focusing on fatty acids, phytosterol derivatives, and tocopherol concentration. Furthermore, antioxidant activity and protein percentage were also considered, to further promote this pseudo-cereal as one of the most important sources of functional compounds for health products and dietary supplements (Bruni et al., 2001; Marcone et al., 2004; Martirosyan et al., 2007).

MATERIALS AND METHODS

Field Experiments and Plant Material

The trials were performed at the experimental field of Cava d'Ispica (230 m a.s.l.) in the southeastern part of Sicily (Italy) in 2005 and 2006. This location is characterized by a Meso-Mediterranean dry climate, with mean annual rainfall of 470 mm (30-yr average), mostly in autumn and winter (73%) and in spring (17%). The mean air temperature is 18.6°C in autumn, 9.6°C in winter, 14.7°C in spring, and 25.5°C in summer. The average minimum and maximum annual temperatures are 12.3 and 21.8°C, respectively. The soil is medium textured, with 37.1% of clay, 26.2% of loam, and 36.7% of sand, well endowed with the main mineral nutrients. Before the experiments, the fields had been cultivated with wheat (*Triticum aestivum* L.), fertilized with 40 Kg N ha^{-1}, 80 Kg P_2O_5 ha^{-1}, and 60 Kg K_2O ha^{-1} and shallow-plowed.

In both years, 20 different accessions belonging to five amaranth species (*A. caudatus*, *A. cruentus*, *A. hybridus*, *A. hypochondriacus x hybridus*, and *A. hypochondriacus*; Table 1), obtained from the Kokopelli Seed Foundation, were used in the experiments. Sowing was performed on 27 May 2005 and on 11 May 2006 inside a nursery in expanded polystyrene trays. Plants with four true leaves were transplanted in the field 20 d later, at a density of 10 plants m^{-2}, on rows 0.50-m apart, according to randomized complete block designs, with three replicates and plots of 6 m^2. Just before anthesis, 60 kg N ha^{-1} were applied. Water was supplied with a sprinkler system, aiming at restoring 50% of reference evapotranspiration, as determined by using an A class pan evaporimeter. On the whole, 10 irrigations were performed during amaranth growth, supplying a total amount of 1811 m^3 ha^{-1} in 2005 and 1838 m^3 ha^{-1} in 2006.

Plots were individually hand-harvested at physiological maturity, according to the earliness of each accession; seeds were threshed with a fixed machine, and after harvest were stored at room temperature until chemical analysis.

Air temperatures and rainfall were recorded during crop growth, together with the following crop traits: date of the main phenological stages, plant height, stem diameter, root length, total dry biomass, seed yield, and thousand-seed weight.

Unsaponifiable fraction, phytosterols, tocopherols, and antioxidant activity were determined by the following reported methods, considering for each species a bulk sample characterized by a comparable amount of each accession. For *A. hybridus* and *A. hypochondriacus x A. hybridus* species, the samples were instead analyzed using the single accessions K593 and Guarijio, respectively.

Table 1. Characteristics of plant materials in the two experiments.

Code	Species	Accession
1	*Amaranthus caudatus*	Oscar Blanco
2		Oscar Blanco (Bolivia)
3	*A. cruentus*	Ames 2244
4		Amont
5		Golden Giant
6		KinnauryDhankar
7		Mexican
8		Mexicane
9		Nepalese
10		New Mexico
11	*A. hybridus*	K593
12	*A. hypocondriacus*	Annapurna
13		Annapurna (India)
14		Burgundy
15		MannadeMontagna
16		Opopeo
17		Orange Giant
18		Plaisman
19		Plenitude K436 Rouge
20	*A. hypocondriacus* × *A. hybridus*	Guarijio

Chemical Composition of Amaranth Samples: Protein and Lipidic Percentages

All the seeds obtained from each plot were milled in a blade grinder (0.2-mm mesh; Fritsch) to prepare samples for chemical analysis. Flour milling was performed at a temperature that never exceeded 30°C. After grinding, the flours were stored in the dark at 20°C before the protein and lipidic determination.

The grain protein percentage was computed by Kjeldahl method (N × 6.25).

Fatty Acids

Fatty acids composition was determined by placing each amaranth flour sample (5 g) in *n*-hexane (100 mL) and performing the extraction with the help of sonication. The samples were filtered and centrifuged (3000 rpm, 20 min). The supernatants were dried with Rotavapor and stored at −20°C until gas chromatography (GC) analyses. The fatty acids as methyl esters (FAME) were analyzed by GC after processing the samples through transmethylation using sodium methoxide in the presence of methyl acetate (Bruni et al., 2001). Gas chromatography analyses were performed in triplicate using a Varian GC-3800 gas chromatograph equipped with a Varian MS-4000 mass spectrometer using electron impact and hooked to the National Institute of Standards and Technology (NIST) library.

Fatty acids as methyl esters were identified by comparing their GC retention time and the mass spectrometry (MS) fragmentation pattern with those of 37 pure component FAME mixes (Supelco, Sigma-Aldrich). Operating conditions were as follows: injector temperature, 300°C; carrier (helium) flow rate, 1 mL min^{-1}; and split ratio, 1:50. Oven temperature was increased from 100 to 250°C at a rate of 5°C min^{-1}, followed by 10 min at 250°C. The MS conditions were: ionization voltage, 70 eV; emission current, 10 mAmp; scan rate, 1 scan s^{-1}; mass range, 29 to 600 Da; trap temperature, 150°C; and transfer line temperature, 300°C. One microliter of each sample was injected. Fatty acids methyl esters samples were analyzed in GC–flame ionization detector (FID) for quantitative determination through the normalization method, without using correction factors: the relative peak areas for individual constituents were averaged on three different chromatograms of three independent reactions. The relative percentages were determined using a ThermoQuest GC-Trace gas chromatograph equipped with a FID detector maintained at 300°C; all other GC conditions were the same as the GC–MS method.

Unsaponifiable Fraction and Phytosterols

All the amaranth flour samples considered in the "Field Experiments and Plant Material" section were subjected to solvent extraction in triplicate. Samples of each amaranth flour (5 g) were placed in 100 mL of methanol and subjected to ultrasound treatment in the dark at a constant temperature of 25°C for 30 min. The extracts obtained were then dried in a rotavapor, weighed, and stored in the dark at −20°C until the moment of GC analysis.

Successively, each of the amaranth samples obtained by extraction (40 mg of oil) was weighed into a 10-mL vial with a screw cap and cold-saponified with 5 mL of 1M methanolic KOH, maintained under constant agitation for 24 h at 28°C. Afterward, the solution was extracted twice with 2 mL of hexane and 0.2 mL of ethanol using a separator funnel. The *n*-hexane fraction was then dried under a nitrogen flow, and the unsaponifiable fraction was silanized at room temperature with 2 mL of a silanizing mixture containing pyridine-hexamethyldisilazane-trimethylchlorosilane (5:2:1). After 1 h the liquid was evaporated under a nitrogen flow in a heat bath at 80°C and then extracted with 0.3 mL of hexane. The conical test tube was placed in ultrasound for 2 min, centrifuged, and the supernatant withdrawn for injection into the GC. One microliter of the solution was injected into GC-FID for quantification and in GC–MS for identification of phytosterols. Qualitative data were based on comparison of the retention times and of the mass spectra with NIST library and literature data. Gas chromatography operating conditions were as follows: column Varian FactorFour VF-5ms poly-5% phenyl-95%-dimethyl-siloxane bonded phase (i.d., 0.25 mm; length, 30 m; film thickness, 0.25 μm); injector temperature, 300°C; carrier (helium) flow rate, 1.2 mL min^{-1}; and split ratio, 1:50. Oven temperature was increased from 230 to 320°C at a rate of 5°C min^{-1}, followed by 7 min at 320°C. The MS conditions were: ionization voltage, 70 eV; emission current, 20 mAmp; scan rate, 1 scan s^{-1}; mass range, 29–800 Da; trap temperature, 150°C, transfer line temperature, 320°C.

Gas chromatography–FID was used for quantitative determination through the normalization method, without using correction factors: the relative peak areas for individual constituents were averaged on three different chromatograms of three independent reactions. The relative percentages were determined using a ThermoQuest GC-Trace gas chromatograph equipped with a FID detector maintained at 350°C; all the other GC conditions were the same as the GC–MS method.

Tocopherols Extraction Procedures and Analyses

Tocopherol extractions and analyses were performed following the procedures described in Bruni et al. (2001). In particular, the same amaranth flour amount for each sample (5 g), reported in the "Field Experiments and Plant Material" section, was placed in a flask containing 100 mL of methanol and stirred for 24 h in the dark at a constant temperature of 25°C. The samples were then filtered and centrifuged for 20 min (3000 rpm). The supernatant was recovered, dried with rotavapor, and weighed.

Tocopherol analyses of the extracts were performed employing a modular JASCO high performance liquid chromatography (HPLC) unit characterized by a PU-2089Plus pump, a MD-2010Plus diode array detector (DAD), and a 20-μL sampler loop. Isocratic elution mode (methanol:water; 85:15) was used with a Phenomenex KINETEX PFP column (15 × 0.46 cm, 2.6 μm). Flow rate was 0.8 mL min^{-1} while absorbance was detected at 295 nm. All solvents used were chromatographic grade. Vitamin E stereoisomer peaks (α-, β-, γ-, δ-tocopherols) were identified by comparing their retention time with those of pure standards (Sigma–Aldrich). Integration of the peak areas was performed using a dedicated Borwin software (Borwin–PDA ver. 1.50, JMBS Developments). For each amaranth extract, qualitative and quantitative analyses were performed in triplicate.

Antioxidant Activity

Radical scavenging properties of amaranth methanol extracts were assessed by spectrophotometric evaluation of their ability to bleach the 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid radicals ($ABTS^{\cdot+}$) (Becker et al., 2004; Maietti et al., 2013). This double approach permits the antioxidant effectiveness of a lipidic sample to be more carefully defined, as it is almost impossible to express the antioxidant activity as an absolute value that is universally recognizable, besides being expressed by only one type of assay (Becker et al., 2004).

Each methanol extract was diluted 5-, 10-, 50-, and 100-fold to have concentration ranges comprised between 0.1 and 10 mg mL^{-1}. An aliquot of 100 μL of each solution was added to 2.9 mL of DPPH (1×10^{-4} M in ethanol), shaken vigorously and kept in the dark for 30 min at room temperature. Sample absorbance was measured at 517 nm with an ultraviolet-visible (UV-VIS) spectrophotometer (ThermoSpectronic Helios γ). A stock solution of $ABTS^{\cdot+}$ was instead produced by the reaction of ABTS solution (2 mM) with 70 mM potassium persulfate (both solutions were prepared in bidistilled water) for 12 to 16 h, in the dark at room temperature. The stock solution was diluted in phosphate buffered saline to achieve an absorbance of 0.70 ±0.02 at 734 nm with UV-VIS spectrophotometer (ThermoSpectronic Helios γ). An aliquot of 900 μL of diluted ABTS solution was mixed with 100 μL of amaranth sample. The absorbance at 734 nm was taken exactly 1 min after initial mixing. All the radical scavenging activities were reported as IC_{50} for each sample. A blank was assessed as the solution assay described above without the amaranth extract, instead of which methanol was employed. Butylated hydroxyl anisole and commercial α-tocopherol were used as positive controls and prepared with testing solutions as described above for amaranth samples.

Statistical Analysis

All the data were submitted to mixed effects ANOVA, by considering the fixed effects of years, accessions, and "accession by year" interaction. The "blocks within years" was included as random effect, together with the residual error term. In some cases, chemical determinations were performed on a pooled sample of all accessions for each species. In these cases, the "accession" effect was replaced by the "species" effect on ANOVA.

Owing to the relevant number of variables recorded, we decided to take a graphical-descriptive approach to data analysis based on singular value decomposition and principal component analysis (PCA). The most fundamental traits (seed yield, protein percentage, oil yield, and total tocopherol concentration) were submitted to additive main effects multiplicative interaction (AMMI) analysis, wherein the matrix of residuals from additivity (interaction matrix) is decomposed into the product of scores for accessions-species and scores for years (Onofri and Ciriciofolo, 2007). The results were displayed on AMMI1 biplots, showing both the accessions and the years, by way of their overall means (on the *x* axis) and their "interaction" score (PC1) on the *y* axis. Accessions with a close-to-zero score on the *y* axis were "stable" across years, while those with a high score (in absolute value) were unstable and interacted positively with years characterized by a score of the same sign. For all variables, AMMI1 biplots represented more than 90% of the variability of main effects and interaction and, therefore, provided a good description of the average behavior and across-years stability of accessions.

For all the other investigated traits (morphological traits, fatty acids profile, unsaponifiable fraction composition, tocopherol composition), PCA was performed on the correlation matrix (morphological traits) or on the covariance matrix (fatty acids profile, unsaponifiable fraction composition, tocopherol composition). This decision was taken on the basis of whether the variables submitted to PCA were on different or similar measurement units. For each PCA, the first two principal components were displayed on a distance-based biplot (Onofri and Ciriciofolo, 2007).

Finally, a PCA was attempted on all the main traits, with the aim of exploring the strength of the correlations among all studied variables. Results were displayed on a "correlation" biplot (Onofri and Ciriciofolo, 2007).

All the analyses were performed by using the R statistical software (R Core Team, 2011), with the *lme4* package for ANOVA (Bates et al., 2015), the *vegan* package for PCA (http://CRAN.R-project.org/package=vegan; accessed 21 June 2017), and a user defined script for AMMI analyses (Onofri and Ciriciofolo, 2007).

RESULTS

Weather Pattern

In 2005 the average temperature during the trial (May through October) was 21.9°C, with a minimum temperature of 10.7°C in May and a maximum of 30.8°C in July; rainfall was 105 mm, mainly distributed in August and October. In 2006 the weather was a little warmer and rainier than in 2005: the average temperature was 22.8°C, with a minimum temperature of 10.8°C in May and a

maximum temperature of 35.9°C in July. Rainfall during the trial period was 185 mm, almost exclusively in September and October.

Phenological Traits

For phenological traits, very little "within accessions" variability in both trials was observed, and therefore, no ANOVA could be reliably performed. The biological cycle (from seed to seed) lasted, on average of all the accessions, 151 (SE = 1.29) days in 2005 and 167 (SE = 1.25) in 2006, and it was, on average, the shortest for all accessions of *A. cruentus* and *A. hybridus* (156 d on average, with SE of 8.0) and the longest for the *A. hypochondriacus x hybridus* (169 d, with SE of 7.5). Such variability mainly related to the vegetative phase of the biological cycle (from sowing to flowering), which lasted for 44 d in 2005 and 60 d in 2006 for all accessions, except Annapurna (India), Guarijo, Oscar Blanco (Bolivia), and Plenitude K436 Rouge, which showed a length of 53 d in 2005 and 69 d in 2006. On the other hand, very little variability was noted for the length of the ripening period: indeed, the period from flowering to milk maturity ranged from 45 to 49 d (on average), while the period from milk maturity to waxy maturity was always 15 d, and the period from waxy to physiological maturity ranged from 40 to 48 d (this latter length was observed only in 2006, for all the accessions of *A. hypochondriacus*).

Morphological Traits

For all morphological and yield-related traits (height, stem diameter, stem length, root length, plant dry matter, and thousand-seed weight), the "accession by year" effect was significant ($P < 0.05$). Therefore, the mean values for each accession in the 2 yr were submitted to PCA and displayed on a biplot (Fig. 1A), while the observed means with pooled standard errors are available in Supplemental Tables S1 and S5, respectively.

We clearly see that all results obtained in 2005 lie on the left side of the biplot (Fig. 1A) and are associated to high values (above average) of root length and low values (below average) of height, stem length and diameter, and dry matter percentage at harvest. In this first year, *A. caudatus* (both accessions), *A. hybridus* (K593), most accessions of *A. hypocondriacus* (all but Annapurna India, Orange Giant, and Plenitude K436 Rouge), and only a few accessions of *A. cruentus* (Ames 2244, Amont, and Mexican) showed a high value of seed weight. The second year (2006) was characterized by a higher aerial size of plants, and most of the above-mentioned accessions confirmed a higher seed weight with respect to the others.

Seed Yield and Protein Percentage

The "accession by year" interaction of seed yield was significant and represented 16% of the overall data variability, while the accession was the most important effect, representing 50% of total data variability. The observed means with standard errors are reported on Supplemental Table S2. The AMMI1 biplot may help understand the yield behavior of accessions in the two experimental years. It should be remembered that yield levels are shown along the *x* axis, while PC1 scores for accessions and years are shown along the *y* axis, and roughly speaking, they can be taken as a measure of stability (Fig. 2A). Mexicane and New Mexico (*A. cruentus*) were on average the highest yielding, with high and stable yields in both years (always above 2.8 t ha^{-1}). Amont and Golden Giant (*A. cruentus*), together with Burgundy and Opopeo (*A. hypocondriacus*) were also very stable, but with lower yield levels (around 2.2 t ha^{-1}). Plenitude K436 (*A. hypocondriacus*) and Mexican (*A. cruentus*) gave yields above 2.7 t ha^{-1} only in 2006, while K593 (*A. hybridus*) gave 2.65 t ha^{-1} in 2005 but was very low yielding in 2006. Almost all the other accessions were below average (vertical dotted line in Fig. 2A).

The average protein percentage and its standard error for all accessions are reported on Supplemental Tables S2 and S6, respectively, and shows a marked variability due to the accessions, while the variability across years was smaller than that observed for yield.

As shown in the AMMI1 biplot (Fig. 2B), Kinnaury Dhankar was on average the richest accession in terms of protein percentage, with a good stability across years (16.9 and 17.0, respectively, in 2005 and 2006). Other rich and stable accessions were Golden Giant (16.0 and 16.4), Mexicane (16.1 and 16.6), Oscar Blanco (Bolivia) (16.3 and 16.4), and Annapurna (India) (15.8 and 16.1).

Lipid Fraction

Amaranth seeds were also characterized in terms of their lipid fraction (fatty acids and unsaponifiable fraction), following the method described in Bruni et al. (2001). All the observed means and their standard errors are reported on Supplemental Tables S2 and S6, respectively. Total oil yield was, on average, 4.84%, and the percentage of unsaturated fatty acids was 77%, with a saturated/unsaturated ratio of 0.3. The "accession by year" interaction was always significant, as observed already for all the other traits.

The extraction yield was higher in 2005 than in 2006, with means of 5.24 and 4.48%, respectively (Fig. 2C). Considering the accessions, Orange Giant was, on average, the highest yielding (5.98%), with good stability across years (6.27 and 5.68%, respectively). This accession was followed by Burgundy, Opopeo, Mexican, Plenitude K436 Rouge, and Oscar Blanco, which showed average oil yields above 5.5%. All these accessions were rather stable across years, except Mexican, which showed the highest oil yield in 2005 (6.8%) and dropped remarkably in 2006 (4.6%) (Fig. 2C).

Considering the fatty acids profile in more detail (Supplemental Tables S3 and S7), linoleic acid (C18:2) was,

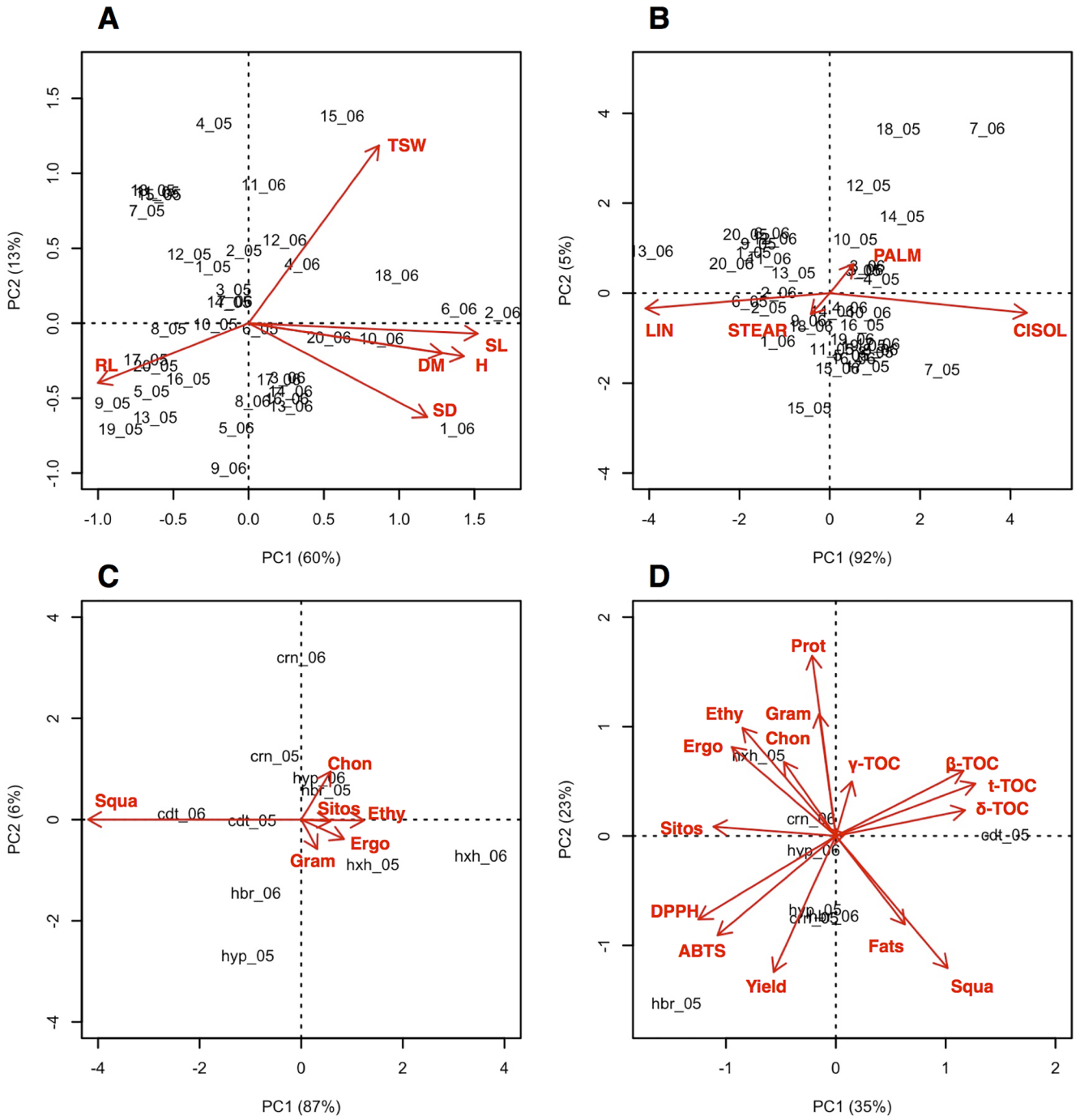

Fig. 1. Biplot from principal component analysis (PCA). (A) PCA Biplot on morphological traits, as observed in 20 accessions of *Amaranthus* spp. in 2005 and 2006 (SL: stem length; H: height; DM: plant dry matter; SD: stem diameter; RL: root length; TSW: thousand-seed weight). The first two digits of the codes represent the accessions (Table 1), while the last two digits represent the years (2005 and 2006). (B) PCA Biplot on fatty acids profile, as observed for 20 accessions of *Amaranthus* spp. in 2005 and 2006 (LIN: linoleic acid, C18:2; CISOL: *cis* oleic acid, C18:1; PALM: palmitic acid, C16:0; STEAR: stearic acid, C18:0). The first two digits of the codes represent the accessions (Table 1), while the last two digits represent the years (2005 and 2006). (C) Biplot from PCA on the unsaponifiable fraction, as observed for five species of amaranth in two experimental years (Squa: squalene; Chon: chondrillasterol; Ethy: 24-Ethylidencholest-7-en-3-ol; Sitos: sitosterol D7; Ergo: ergost-7-en-3-ol; Gram: Gramisterol). The first three characters of the codes represent the species (cdt: *A. caudatus*; hyp: *A. hypochondriacus*; hxh: *A. hypochondriacus x hybridus*; crn: *A. cruentus*; hbr: *A. hybridus*), while the last two digits represent the years (2005 and 2006). (D) Summary PCA biplot on all the main traits, as observed for five species of amaranth, in two experimental years. The first three characters of the codes represent the species (cdt: *A. caudatus*; hyp: *A. hypochondriacus*; hxh: *A. hypochondriacus x hybridus*; crn: *A. cruentus*; hbr: *A. hybridus*), while the last two digits represent the years (2005 and 2006). Squa: Squalene; Fats: total oil percentage; Sitos: sitosterol D7; Ergo: ergost-7-en-3-ol; Ethy: 24-Ethylidencholest-7-en-3-ol; Chon: chondrillasterol; Gram: Gramisterol; Prot: protein percentage; γ-TOC: γ-tocopherol; β-TOC: β-tocopherol; t-TOC: total tocopherol concentration; δ-TOC: δ-tocopherol.

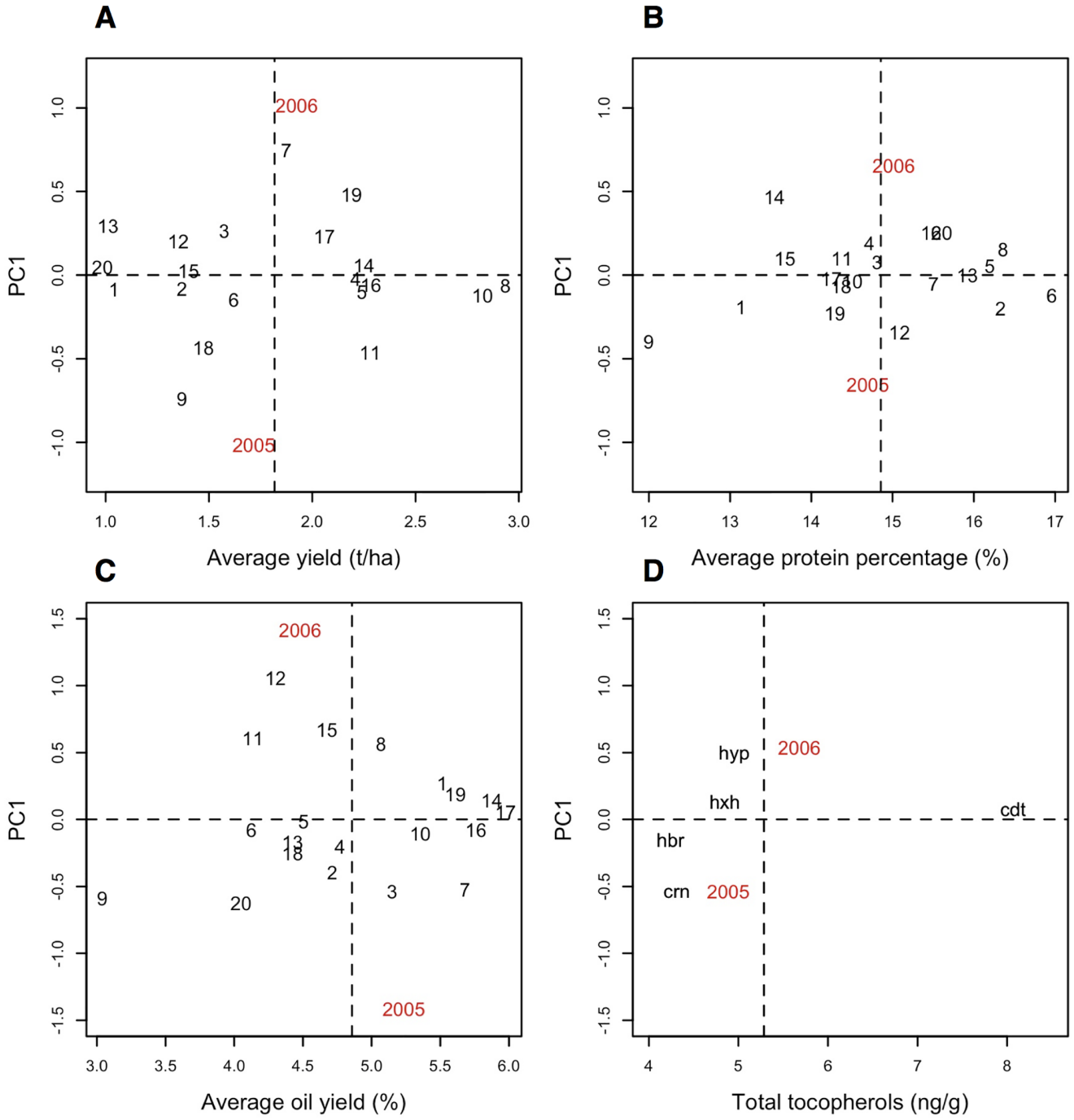

Fig. 2. Additive main effect multiplicative interactions (AMMI)1 biplot. (A) AMMI1 biplot for grain yield of 20 accessions of *Amaranthus* spp., as observed in 2005 and 2006. Codes for accessions are reported in Table 1. The vertical dotted line represents the grand mean (1.82 t ha^{-1}). (B) AMMI1 biplot for protein percentage in seeds (%) of 20 accessions of *Amaranthus* spp., as observed in 2005 and 2006. Codes for accessions are reported in Table 1. The vertical dotted line represents the grand mean (14.85%). (C) AMMI1 biplot for total oil percentage in seeds (%) of 20 accessions of *Amaranthus* spp., as observed in 2005 and 2006. Codes for accessions are reported in Table 1. The vertical dotted line represents the grand mean (4.84%). (D) AMMI1 biplot for total tocopherol concentration of five species of amaranth, as observed in 2005 and 2006. Codes for species are: cdt, *A. caudatus*; hyp, *A. hypochondriacus*; hxh, *A. hypochondriacus x hybridus*; crn, *A. cruentus*; hbr, *A. hybridus*). The vertical dotted line represents the grand mean (5.28 ng g^{-1}).

on average, the most abundant (40.6%), followed by *cis*-oleic acid (C18:1; 35.6%) and palmitic acid (C16:0; 18.8%). The other detected fatty acids were stearic acid (C18:0; 3.6%) and arachidic acid (C20:0; 0.6%), while other acids were detected only sporadically (always lower than 1%). In particular, heptadecanoic acid (C17:0) was detected in Oscar Blanco (Bolivia) in 2005 and Ames 224, Annapurna (India), Plaisman, and K593 in 2006. Palmitoleic acid (C16:1) was detected only in Nepalese and Plaisman in 2006. *Cis*-eicoesanoic acid was identified in traces only in Guarijo (2005) and Plenitude K436 Rouge (2006).

A PCA biplot was drawn for the most abundant acids (linoleic, *cis* oleic, palmitic, and stearic; Fig. 1B). It shows that, with respect to the overall average, Annapurna, Burgundy, and Plaismanin 2005 were particularly high in palmitic acid (>19%), together with Mexican in 2006 (25.6%). Considering the *cis*-oleic acid, Mexican reached high amounts in both years (52.4 and 55.4%). Annapurna (India) was high in linoleic acid in 2006 (64.0%), while Manna de Montagna was rich in stearic acid (5.1%) and exhibited a low percentage of palmitic acid (16.8%) in 2005 (Fig. 1B).

The unsaponifiable fraction revealed a remarkable abundance of squalene (overall average: 53.0%), followed by the other dominant sterol chondrillasterol (overall average 16.4%) (Supplemental Tables S4 and S8). The other minor sterols, in order of abundance, were ergost-7-en-3-ol (9.8%), sitosterol (8.28%), 24-ethylidencholest-7-en-3-ol (7.1%), gramisterol (2.6%), citrostadienol (1.5%), 24-methylene cycloartenol (0.9%,), and campesterol (0.5%). In general, the variability across years was small for these traits, while the variability among species and the interaction "year by species" was always rather high and significant ($p < 0.05$).

It may be useful to look at the PCA biplot, to obtain an overview of the behavior of species in the 2 yr. *Amaranthus caudatus*, in both the years, showed the highest values of squalene (Fig. 1C), reaching up to 57% in 2005. On the other side, chondrillasterol was particularly abundant (above average) in *A. cruentus* in both years (17.0 and 20.3%, respectively), while *A. hypochondriacus x hybridus* showed a slightly different quantitative profile with respect to the other amaranth species, and it was high in gramisterol in both years (4.26 and 4.46%) and in ergosterol (13.8%) and 24-ethylidencholest-7-en-3-ol (11.0%) only in 2006 (Fig. 1C).

The vitamin E concentration was checked through HPLC-DAD with reference to α-, β-, γ-, and δ-tocopherol (Bruni et al., 2001). Considering total tocopherols concentration, the "species by year" interaction was significant, and the AMMI1 biplot (Fig. 2D) shows that seeds of *A. caudatus* were, on average, the richest (8.07), with good stability across the 2 yr (7.63 and 8.51 μg g^{-1}). Lower values were detected for the other species—*A. hypochondriacus*, *A. hypochondriacus x hybridus*, *A. cruentus*, *A. hybridus*—which ranged from 3.93 μg g^{-1} (*A. hybridus*, 2005) to 5.61 μg g^{-1} (*A. hypochondriacus*, 2006).

The qualitative profile (Table 2) was characterized by the presence of the four tocopherols in all the amaranth species, except for *A. hybridus*, which lacked γ-tocopherol. The average concentrations for α-, β-, γ-, and δ-tocopherol were 0.29, 3.75, 0.23, 1.02 ng g^{-1}, respectively. Apart from β-tocopherol, the other isomers were in minor abundance, reflecting always the same quantitative pattern in both the years for *A. caudatus*, *A. hybridus* (β- > δ- > α- > γ-tocopherol), and *A. hypochondriacus x hybridus* (β- > δ- > γ- > α-tocopherol) (Table 3). On the contrary, *A. cruentus* and *A. hypochondriacus* showed different quantitative profiles in 2005 and 2006. In particular, the ranking for *A. cruentus* was β- > δ- > α- > γ-tocopherol in 2005 and β- > δ- > γ- > α-tocopherol in 2006 (Table 3). Similarly, the ranking for *A. hypochondriacus* was β- > γ- > α- > δ-tocopherol in 2005 and β- > δ- > γ- > α-tocopherol in 2006.

With the objective of finding possible correlations between vitamin E concentration and bioactivity and trying to appraise the Sicilian amaranth seeds under a functional point of view, antioxidant capacity of methanol extracts was assessed through spectrophotometric DPPH

Table 2. Tocopherol concentration (μg g^{-1}) in seeds for five species of *Amaranthus*, during two experiments in Mediterranean conditions.

Species	Year	Tocopherols (μg g^{-1})				
		Total	α-	β-	γ-	δ-
Amaranthus caudatus	2005	7.64	0.08	4.75	0.01	2.8
	2006	8.51	0.4	4.94	0.39	2.77
A. cruentus	2005	4.23	0.37	3.32	0.01	0.53
	2006	4.43	0.01	3.92	0.36	0.14
A. hybridus	2005	3.93	0.15	3.08	n.d.†	0.7
	2006	4.56	0.26	3.85	n.d.	0.45
A. hypochondriacus × *hybridus*	2005	4.38	0.22	2.95	0.36	0.85
	2006	5.32	0.22	4.05	0.32	0.74
A. hypochondriacus	2005	4.29	0.49	3.07	0.51	0.23
	2006	5.61	0.59	3.6	0.48	0.94
Pooled standard error of a mean (SEM)		0.007	0.006	0.01	0.005	0.007

† n.d., not detected.

Table 3. Antioxidant activity (milligrams per milliliter) of amaranth methanol extracts expressed as IC_{50} with 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic (ABTS) acid assays.

Species	Year	DPPH test	ABTS test
Amaranthus caudatus	2005	2.26	1.17
	2006	1.32	0.77
A. cruentus	2005	2.85	1.80
	2006	2.74	1.44
A. hybridus	2005	4.02	2.94
	2006	2.87	1.61
A. hypochondriacus × *hybridus*	2005	2.95	2.13
	2006	2.66	1.32
A. hypochondriacus	2005	3.20	2.05
	2006	2.95	1.40
SEM		0.051	0.044
A. caudatus	Avg.	1.79	0.97
A. cruentus	Avg.	2.80	1.62
A. hybridus	Avg.	3.45	2.27
A. hypochondriacus × *hybridus*	Avg.	2.90	1.73
A. hypochondriacus	Avg.	3.07	1.72
SEM		0.036	0.031
Trolox		0.0049	0.0024
α-tocopherol		0.0078	0.0053

and ABTS tests and expressed as IC_{50} (milligrams per milliliter; Table 3). With both the tests, *A. caudatus* extracts were on average the most effective, while *A. hybridus* showed the least active performances. All species gave better results in 2006, and it should be pointed out that such an increase of antioxidant capacity was really important, in some instances. Compared with 2005 values for the most bioactive *A. caudatus*, the increase in 2006 was 41.5% with DPPH and 34.2% with ABTS. For the other samples and with reference to DPPH and ABTS, respectively, *A. cruentus* evidenced an increase of 3.9 and 20.0%, *A. hypochondriacus* of 28.6 and 45.2%, *A. hypochondriacus x hybridus* of 9.8 and 38.0%, and *A. hypochondriacus* of 7.8 and 31.7%.

With an omnicomprehensive approach, some relationships among variables studied can be outlined (see Fig. 1D and Table S9). An interesting correlation can be observed between grain yield and antioxidant activity (DPPH and ABTS), while grain yield seems negatively related with tocopherol fractions (α-, β-, γ-, and δ-tocopherol). Antioxidant activity was also positively correlated to sitosterol. Other interesting correlations involve oil and protein percentage: total oil percentage strictly related to squalene as well as protein percentage to gramisterol.

DISCUSSION

As is well known, amaranth is a C4 species able to tolerate the high temperatures and light intensity levels that often occur in summer Mediterranean climates. Because of their wide adaptation capability and the large quantity of seed produced, several species of this genus may be found all over the world as weeds, though they might have a potential interest as crops. Several valuable features may in fact be the cornerstones for their introduction into cropping systems with an extremely wide range of environments.

Many studies have demonstrated the wide degree of within-species or even within-population polymorphism and variability (Jacobsen and Mujica, 2003; Sogbohossou and Achigan-Dako, 2014). Indeed, compared with the legume crops conventionally grown in Mediterranean environments, amaranth species showed a greater variability of grain yield, which may be advantageous for breeding processes, although at the same time it may be a disadvantage, resulting in unpredictable yield levels. Another limit is represented by the lack of registered herbicides and the consequent need for the adoption of mechanical weed control methods, while chemical weed control represents a hinge management practice in field legume crops (Avola et al., 2008).

In spite of these limitations, amaranth seems a potential alternative crop for Mediterranean environments (Jacobsen et al., 2012), resulting in a seed yield level often higher than 2 t ha^{-1}. Experiments performed in other countries showed a lower yield level. For example, in a 2-yr study performed in Canada, the accessions K593 (*A. hybridus)* and Plaisman (*A. hypocondriacus*) showed a plant height ranging from 132 to 143 cm and a seed production ranging from 0.65 to 0.99 t ha^{-1} for K593 and from 0.75 to 1.20 t ha^{-1} for Plaisman (Gélinas and Seguin, 2008). Comparable yields were obtained in North Dakota by Henderson et al. (1998), while these same varieties in our trials yielded, on average during the 2 yr, 1.47 t ha^{-1} and 2.28 t ha^{-1}, respectively.

In our study, *A. cruentus* was, on average, more productive and more stable and had a higher protein percentage over the 2-yr study compared with the other species. The same observation on yield has been shown by Henderson et al. (2000), comparing *A. cruentus* and *A hypocondriacus x hybridus*. On the contrary, a study performed in Argentina showed that *A. hypocondriacus* produced a higher grain yield than *A. cruentus* and *A. caudatus* (1.2, 0.99, and 0.66 t ha^{-1}, respectively, on average over 3 yr) (de Troiani et al., 2004).

Average protein percentage ranged between 13.1% (*A. hypocondriacus*-Burgundy) and 17.0% (*A. cruentus*-Kinnauridhanka), with large within-species variability. These protein percentage values are in agreement with those obtained by Pospišil et al. (2006) on *A. cruentus* and *A. hypocondriacus*. In contrast, Gimplinger et al. (2007), in a 2-yr study on *A. hypocondriacus* (Mittlerer), obtained a slightly higher protein percentage (18.0%).

As far as the oil percentage is concerned, total extraction yields observed in both years are in line with those reported in the literature (Bruni et al., 2001; Budin, Breene, & Putnam, 1996; He, Cai, Sun, & Corke, 2002), with the exception of some low values recorded in 2006 with *A. hybridus* and *A. hypochondriacus x hybridus*, which showed a remarkable decrease with respect to 2005 (−33.10 and −47.46%). The reason for such a decrease could relate to genetic causes, as cropping conditions were the same for all species and accessions (Henderson et al., 2000; Sogbohossou and Achigan-Dako, 2014).

The fatty acids profile in both years was substantially similar for all species, showing linoleic acid (C18:2) as the most abundant component, followed by *cis*-oleic acid (C18:1) and palmitic acid (C16:0). The chemical quantitative and qualitative composition pattern was similar to what is currently reported in the literature (Budin et al., 1996; Bruni et al., 2001; He et al., 2002).

Other fatty acids were detected, that is, stearic acid (C18:0), arachidic acid (C20:0), and heptadecanoic acid (C17:0), while palmitoleic acid (C16:1) and *cis* eicosanoic acid were detected only in traces. These results regarding less abundant fatty acids partially confirm the data available in the literature, which, however, show the presence of other minor compounds, such as myristic acid (C14:0), linolenic acid (C18:3), behenic acid (C22:0), and lignoceric acid (C24:0) (Budin et al., 1996; Jahaniaval et al., 2000; Bruni et al., 2001;).

.The saturated/unsaturated acids ratio in both experiments ranged from 0.26 (*A. hybridus*) to 0.32 (*A. hypochondriacus*). However, all the species showed saturated/unsaturated acids values similar to those reported for rice (*Oryza sativa* L.) bran and wheat germ oils and just slightly higher than those of buckwheat (*Fagopyrum esculentum*) and soybean [*Glycine max* (L.) Merr.] and lupin (*Lupinus albus*) oils, which are renown for their dietetic properties (Jahaniaval et al., 2000; Bruni et al., 2001; He et al., 2002; Chiofalo et al., 2012).

The unsaponifiable fraction revealed the remarkable abundance of squalene in all the amaranth species in both years of cultivation, particularly in the case of *A. caudatus*, confirming the results reported in the literature (Bruni et al., 2001; He et al., 2002).

It has already been pointed out that the high percentage in squalene for amaranth seeds makes this pseudocereal one of the most important food and/or dietary supplements for preventing cardiovascular diseases (Marcone et al., 2004; Martirosyan et al., 2007).

Total tocopherol concentrations in this study were 5- to 10-fold lower than those reported in the literature (Budin et al., 1996; Bruni et al., 2001), which was a surprising result. Regarding the single isomers, in all our samples β-tocopherol was the most abundant, confirming what was reported by previous studies (Budin et al., 1996; Bruni et al., 2001; Gimplinger et al., 2007), but with important differences, which reflect the quantitative gap mentioned for the total vitamin E amounts. Some yearly variations in terms of isomers ranking for the same species could probably be related to the highest maximum temperature recorded in the second year compared with the previous one (35.9 vs. 30.8°C). However, similar situations regarding the variations of antioxidant compounds (tocopherols and total phenolic concentration) related to different temperature conditions are well known in the literature (Britz and Kremer, 2002; Howard et al., 2003).

With reference to the antioxidant capacity of amaranth extracts, the increased bioactivity of all the samples from 2005 to 2006 represents an important incentive for supporting amaranth cultivations in the Mediterranean area, with particular reference to the species *A. caudatus* as the best performing in both the assays. The positive correlation between sitosterol and antioxidant results pointed out the involvement of this compound in the bioactivity. The apparently lower involvement of the vitamin E isomers, in contrast with the literature, could instead be due to need for checking different in vitro antioxidant methods, overtaking the misinterpretation of results caused, for example, by polar paradox phenomena (Becker et al., 2004).

CONCLUSION

Our study shows that there is an interesting potential for amaranth cultivation in the Mediterranean area. Indeed, in agreement with other results in the literature about productive traits, chemical characterization, and biological assays, our research confirmed that amaranth species are good sources of substances important for health products and dietary supplements. In addition, our experiments showed that amaranth plants grown in Mediterranean regions possess a distinct chemical and antioxidant profile, in contrast to amaranth grown in other regions.

Supplemental Material Available

Supplemental material is available with the online version of this article.

Conflict of Interest Disclosure

The authors declare that there is no conflict of interest.

Acknowledgments

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