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Yield and qualitative characterisation of seeds of *Amaranthus hypochondriacus* L. and *Amaranthus cruentus* L. grown in central Italy

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Abstract

Amaranth can be considered a very interesting crop for the Mediterranean region, thanks to its inherent tolerance to disadvantageous growing conditions, along with the high nutritional and nutraceutical value of its seeds. The study aims to evaluate the seed yield, and the oil content and quality of two amaranth genotypes (species *Amaranthus hypochondriacus* L. and *Amaranthus cruentus* L., respectively) grown in central Italy, testing two types of soil (loamy and sandy soil). The two species showed a good performance in the tested environment, with satisfactory seed yield and relative short growth cycle. Significant differences between the two genotypes were observed in terms of seed yield. The crude oil content ranged from 7.5% to 6.0%, with linoleic, palmitic and oleic acids as the major fatty acids of the oil in both genotypes. The unsaponifiable fraction was rich in sterols (campesterol, stigmasterol and β-sitosterol), and interesting levels of squalene were found. This study demonstrated the unique nutraceutical properties of the seeds of two genotypes of *A. hypochondriacus* and *A. cruentus,* grown in central Italy environment, as a source of polyunsaturated fatty acid and squalene. These characteristics make amaranth a valuable alternative crop for specialty oil production in the Mediterranean region.

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Introduction

High evapotranspiration rates, increased variability in rainfall, and intensive summer drought, along with the predominance of cereal-based rainfed cropping systems, can be considered the main constraints to crop production in the Mediterranean region. Under such conditions, the adoption of sustainable agricultural practices and the introduction of new/underutilised crops, able to diversify cropping systems and to mitigate climate changes, can represent very promising strategies in addressing cropping system sustainability, crop improvement and food security.

In such context, amaranth (*Amaranthus* spp.) could be a suitable candidate for the successfully establishment in the Mediterranean region, since its inherent tolerance to adverse climatic conditions (drought, high temperatures and light intensities) (RetaAlemayehu *et al*., 2015). Thanks to its high genetic diversity and its phenotypic plasticity (Emire *et al*., 2012; Rastogi *et al*., 2013), amaranth is well adapted to a wide range of environmental conditions and can be considered a valuable dryland crop for farmers in semi-arid regions (Myers, 1996; Jacobsen *et al*., 2003; Chivenge *et al*., 2015; Pulvento *et al*., 2015).

The *Amaranthus* genus, belonging to the *Amaranthaceae* family, includes more than 60 species (Pisarikova *et al*., 2006), with a worldwide distribution, although most species are found in the warm temperate and tropical regions of the world (Sauer, 1993). Amaranth was cultivated by early civilisations over 2000 years ago, and continues to be used essentially worldwide, even to the present day (Liu and Stützel, 2004). Amaranth can be considered a multipurpose crop and several *Amaranthus* species are cultivated as ornamentals, pseudo-cereals with high nutritive value (*e.g.*, amaranth grain), leaf-vegetables, potherbs and for fodder (Sauer, 1967; Mallory *et al*., 2008). The *Amaranthus* species are grouped into three subgenera; the most economically important is the subgenus *Amaranthus* proper, which includes the three species domesticated for grain production: *Amaranthus hypochondriacus* L., *Amaranthus cruentus* L., and *Amaranthus caudatus* L. (Truccoand Tranel, 2011).

The high nutritional value of both seeds and leaves, and the recent interest for this crop - that may provide high-quality protein, unsaturated oil, and various other valuable constituents - support the use of species from this genus as oilseed crops for specialty oils production, all over the world (Kauffman, 1992; Venskutonis and Kraujalis, 2013). In fact, the seed oil is characterised by high levels of unsaturated fatty acids (UFA) (Hlinková *et al*., 2013), whose important health benefits in lowering the cholesterol and triacylglycerol concentrations, are very well documented (Kris-Etherton *et al*., 1999). It has been demonstrated, in fact, that the consumption of polyunsaturated fatty acids (PUFA) decreases the ratio of total cholesterol to high-density lipoprotein cholesterol, improves insulin resistance (Mensink *et al*., 2003) and reduces systemic inflammation (Summers *et al*., 2002; Ferrucci *et al*., 2006).

Nevertheless, amaranth grain contains significant levels of minerals, vitamins, as well as bioactive components, such as phytosterols, squalene, fagopyritols, saponins, flavonoids, and phenolic acids, with antioxidant properties (Pasko *et al*., 2009; Reta Alemayehu *et al*., 2015). Among these, squalene (2,6,10,15,19,23-hexamethyl-2,6,10,14,18,22-tetracosahexaene) represents a very important component of amaranth-seed oil. Squalene is a biosynthetic precursor to all steroids and an important ingredient in skin cosmetics, due to its photoprotective role and as a lubricant for computer disks due to its thermo-stability (Budin *et al*., 1996; Sun *et al*., 1995). It is hypothesised that the decreased risk for various cancers associated with high olive oil consumption, could be due to the presence of squalene (Smith *et al*., 2000). It has also been suggested that squalene has a chemo preventive effect on colon cancer (Rao and Newmark, 1998). Other beneficial effects on health can be attributed to its hypocholesterolaemic action, in combination with the administration of tocotrienols (Khor and Chieng, 1997). Additionally, the use of squalene alone has been demonstrated to be effective in decreasing serum cholesterol levels and in inhibition of chemically-induced colon, lung and skin tumorigenesis (Miettinen and Vanhanen, 1994; Smith *et al*., 2000; Shin *et al*., 2004).

This study aimed to evaluate the oil qualitative responses of amaranth genotypes (species *A. hypochondriacus* and *A. cruentus*, respectively), testing two types of soil (loamy and sandy soil), in a typical Mediterranean environment of central Italy. Crop development and related biochemical aspects, total biomass and seed yield, oil and protein content, as well as oil quality, in terms of fatty acid composition, squalene and sterols contents, of the two grain amaranth genotypes have been evaluated, assessing as promising novel food resources.

Materials and methods

Plant material and experimental design

The study was conducted at the Experimental Centre of the Department of Agriculture, Food and Environment (DAFE) of the University of Pisa, located at San Piero a Grado, in the Pisa coastal plain (43°40'N lat; 10°19'E long; 1 m above sea level and 0% slope), during 2014 growing season. The area has a Mediterranean climate, with rainfall mainly concentrated in the autumn and spring (mean 948 mm year^{-1}). Total rainfall, during the growing season (from June to the end of September) were exceptionally abundant, accounting for 304 mm. The mean maximum and minimum temperatures, in the same period, were 28.0°C and 14.8°C, respectively.

The seeds of one genotype of *A. hypochondriacus* and one genotype of *A. cruentus* were kindly obtained from Davies company, Germany, to be used in this study. Seeds of each species were planted in a greenhouse in May, and then the resulting 4 week old plants were manually transplanted, when they were about 10 cm in height and had 6-8 pairs of leaves, in two different types of soil: loamy (A) and sandy (B) soil. The physical and chemical characteristics of these soils were presented in Table 1. Total nitrogen was evaluated by the macro-Kjeldahl digestion procedure (Bremner and Mulvaney, 1982), while available phosphorus was determined by colorimetric analysis using the Olsen method (Olsen and Sommers, 1982). Soil organic carbon (SOC) was determined using the modified Walkley-Black wet combustion method (Nelson and Sommers, 1982). Soil organic matter was estimated by multiplying the SOC concentration by 1.724 (Nelson and Sommers, 1982). Soil pH was measured on a 1:2.5 soil:water suspension (McLean, 1982).

The two factors of variability (species and soil type) were randomly scattered among twelve large plastic-made tanks (10 tanks per each soil type; 6 plants per tank per each species) according to a complete randomised design, threefold replicated (1 tank per replicate). The tanks of about 1 m^{3}volume (0.95 m length, 1.15 m width, 1.00 m height) were placed in galvanised steel frames, leaning on bricks displaced in two parallel rows at 0.50 m from the ground. Insulating panels surrounded the tanks, in order to decrease heat exchange between the soil and the air. The tanks were originally drilled on the bottom, and then the drainage hole was covered by a 5 cm thick layer of gravel in order to facilitate drainage. On the top of the gravel layer, a fine maze gauze sheet was laid down to filter out solids from drainage water and to avoid clogging.

The experiment started in June 2014 with fertilisation and transplanting (on 10th June) of the two *Amaranthus* genotypes. Before plant transplanting, nitrogen, phosphorus and potassium were added to all treatments at the rate of 50 kg ha^{-1} of N, 100 kg ha^{-1} of P_2O_5 and 40 kg ha^{-1} of K_2O (equal to 19.23 g N per tank as ammonium nitrate, 21.74 g P per tank as triple per phosphate, and 8.00 g K per tank as potassium sulphate). All tanks were watered to facilitate transplanting recovery. During the trial, the plants were maintained under optimal water supply through a drip irrigation system in order to maintain soil moisture to 75-80% of field capacity. Neither pests nor diseases have been observed during the trial.

Table 1. Physical and chemical characteristics of the two types of soil.

	Soil A	Soil B
Sand (2-0.05 mm, %)	52.0	85.9
Silt (0.05-0.02 mm, %)	32.0	6.3
Clay (<0.002 mm, g kg^{-1})		
Type of soil	16.0 Loamy	7.8 Sandy
pH (H_2O 1:2.5 soil: water suspension; McLean method)	8.5	7.7
Organic matter (Walkley–Black method, %)	0.86	0.84
Total nitrogen (Kjeldhal method, g kg^{-1})	0.47	0.42
Available phosphorus (Olsen method, mg kg^{-1})	3.3	6.7
C/N ratio	10.82	12.03
Cation exchange capacity (method BaCl2, pH 8.1, meq 100 g^{-1})	9.56	6.96
Conductibility (µS cm^{-1})	77.6	29.8

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Crop sampling and measurements during the crop cycle and at harvest

Plant development was monitored by measuring plant height and leaf photosynthetic pigments (chlorophyll *a* chlorophyll *b*, total chlorophyll and carotenoids) through three samplings (24th June, July 9th and July 23th). Plant height was measured from ground to the tip of the tallest shoot of each plant. In the last two dates the dynamic of accumulation of some secondary metabolites, such as polyphenols, flavonoids as well as radical scavenging activity (measured by DPPH assay) have been carried out. At seed maturity, from September to October (September, 3rd and October, 3rd, for *A. hypocondriacus* and *A. cruentus*, respectively), the plants were manually collected from each tank, by cutting plants at soil level, and total fresh above ground biomass was determined. Plants were air-dried in a ventilated oven at 40°C for dry weight determinations of the stems, inflorescences and the total above-ground biomass. The inflorescences were threshed by a fixed machine, using sieves suitable for small seeds, forseed yield and seed moisture evaluation.

Total phenolic content

Dry leaves (0.5 g) of different plant samples were pulverised and homogenised in a mortar with 1 mL of 70% (v/v) methanol to facilitate the extraction. After 30 min of incubation on ice, the extracts were centrifuged at 10,000 g for 10 min at room temperature to collect the supernatant (methanol extract) to be used for the determination of secondary metabolites.

Total soluble polyphenolic compounds were assayed in different sample extracts using the Folin-Ciocalteau's phenol protocol with minor modification (Singleton and Rossi, 1965). 0.5 mL of Folin-Ciocalteau's reagent and 0.45 mL of sodium carbonate (7.5% w/v) were added to 1 mL of total volume sample. After the incubation at room temperature for 2 h, the absorbance at 765 nm of the samples was detected in UV-VIS spectrophotometer (Cintra 101, GBC Scientific equipment LTD, Dandenong, Australia) and referred to a standard curve for chlorogenic acid prepared in the range of 0-50 mg mL^{-1}. All determinations were performed in triplicate.

Total flavonoid content

The total flavonoid content was determined using the colorimetric method of Kim *et al*. (2003). Methanol extract of different samples was added to the solution of 5% (w/v) sodium nitrite ($NaNO_2$) and incubated for 5 minutes with the 10% (w/v) of aluminium chloride ($AlCl_3$) solution; after 5 min were added 0.5 mL of 1 M sodium hydroxide (NaOH). The absorbance of the samples was detected after 15 min at 510 nm with the UV-VIS spectrophotometer, and referred to a calibration curve done with quercetin (1 mg mL^{-1}) as standard. Each analysis was repeated three times.

2,2-diphenyl-1-picrylhydrazyl radical-scavenging assay

The antiradical activity of different samples was determined by using the stable 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) (Brand-Williams *et al*., 1995). One mL of sample at different concentrations (0.25, 0.50, 1 mg mL^{-1}) was added to 0.50 mL of a DPPH methanol solution 0.25 mM (w/v) and incubated at room temperature in the dark for 30 min. The activity was measured as a decrease in absorbance at 517 nm using the UV-VIS spectrophotometer. The percent inhibition of the DPPH radical by the samples was calculated according to the formula: % inhibition = (A_{blank} – A_{sample} / A_{blank}) × 100, where A_{blank} is the absorbance of the DPPH and A_{sample} is the absorbance of the samples. The extract concentration (μg mL^{-1}) providing 50% of antioxidant activities (IC_{50}) was calculated by plotting in a graph inhibition percentage against extract concentration. All determinations were performed in triplicate.

Leaf photosynthetic pigment determination

Total chlorophyll and carotenoid contents were determined using the method described by Lichtenthaler (Lichtenthaler, 1987). Six fresh leaf discs 10 mm diameter per treatments were extracted in 5 mL of pure methanol and kept at 4°C in the dark for 24 h. The absorbance of the extracts at 665, 652, and 470 nm was measured using the UV-Vis spectrophotometer and the content of total chlorophyll and carotenoids were expressed as μg cm^{-2} fresh leaves. The presented data are the mean of five independent replications.

Seed chemical composition

Samples were analysed for dry matter (DM) and lipid content according to official AOAC methods (AOAC, 2000). Ether extract system was used for oil extraction from powdered samples, by an ANKOM model XT10 extractor. In a typical extraction process, 1 g-powdered samples were immersed in boiling petroleum ether for 60 min to dissolve most of the soluble material.

Fatty acids composition

Total lipids (TL) were extracted by means of a chloroform/methanol solution (2:1, v/v), according to Rodriguez-Estrada *et al*. (1997). An acid trans methylation was used to prepare fatty acids for the analysis. Fatty acid methyl esters (FAME) were prepared pouring 30 mg of oil and 4.5 mL of 10% HCl methanolic solution into a 20 mL vial and mixed with a vibration mixer for 60 s. After 8 h, 5 mL of n-hexane were poured into the vial and the mixture was shaken for 1 min. The layers were allowed to separate and the hexane fraction was injected to gas-chromatographic (GC) analysis. For the analysis was used a GC2010 Shimadzu gas chromatograph (Shimadzu, Columbia, MD, USA) equipped with a flame-ionisation detector and a high polar fused-silica capillary column (Chrompack CP-Sil88 Varian, 152 Middelburg, the Netherlands; 100 m, 0.25 mm i.d.; film thickness 0.20 μm). Hydrogen was used as the carrier gas at a flow of 1 mL min^{-1}. Split/splitless injector was used with a split ratio of 1:40. An aliquot of the sample was injected under the following GC conditions: the oven temperature started at 40°C and held at that level for 1 min; it was then increased to 163°C at a rate of 2°C/min, and held at that level for 10 min, before being once again increased to 180°C at 1.5°C/min and held for 7 min, and then to 187°C at a rate of 2°C/min; finally the temperature was increased to 220 °C with a rate of 3°C/min and held for 25 min. The injector temperature was set at 270°C and the detector temperature was set at 300°C. Individual FA methyl esters were identified by comparison with a standard mixture of 52 Component FAME Mix (Nu-Chek Prep Inc., Elysian, MN, USA).

Squalene and phytosterols composition

Unsaponifiable matter was obtained according to Sander *et al*. (1989). Briefly, 300 mg of TL were cold saponified adding 4.5 mL of ethanolic KOH (4.8% w/v) solution and incubated at room temperature. The unsaponifiable matter was isolated by two washes with 4.5 mL of water and 9 mL of hexane. The polar phase (upper phase) was transferred in a fresh tube and dried by nitrogen gas. Finally, the samples were re-suspended with 1 mL of methanol. Before the saponification, 100 μL of a solution of dihydrocholes-

terol in chloroform (2 mg/mL) as internal standards for phytosterol was added to TL. Phytosterols and squalene were identified by GC-FID (GC 2000 plus, Shimadzu, Columbia, MD, USA) equipped with a VF 1-ms apolar capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness; Varian). For cholesterol determination, a 2 μL sample in hexane was injected into the column with the carrier gas (hydrogen) at a flow rate of 1 mL min^{-1}. The split ratio was 1:10. The run was carried out in constant pressure mode. The oven temperature was held at 250°C for 1 min, increased to 260°C over 20 min at a rate of 0.5°C min^{-1}, then increased to 325°C, over 13min at a rate of 5°C min^{-1} and kept at 325°C for 15 min. The injector and detector temperatures were both set at 325 °C. Chromatograms were recorded with LabSolution software (Shimadzu). Cholesterol content were calculated by comparing the areas of samples and internal standards and expressed as g $100g^{-1}$ of total lipids.

Statistical analysis

Data were analysed with the following mixed linear models:

$$y_i = \mu + S_i + \varepsilon_i \quad (1)$$

where y_{ijz}= dependent variables (dry matter, lipid content, fatty acids composition, phytosterols composition and squalene content), S_i= fixed effect of the ith soil treatment (soil A and soil B); ε_{ijz}= random residual.

$$y_i = \mu + A_i + \varepsilon_i \quad (2)$$

where y_{ijz}= dependent variables (dry matter, lipid content, fatty acids composition, phytosterols composition and squalene content), A_i= fixed effect of the ith *Amaranthus* species treatment (*A. hypochondriacus, A. cruentus*); ε_{ijz}= random residual.

Results and discussion

Crop growth and biochemical traits

According to the growing season and the experimental conditions here tested, the two amaranth genotypes showed good agronomic performance, with a relative short cycle in spring-summer period and satisfactory seed yields. The plants of both genotypes showed a rapid growth during the vegetative phase until to the start of the reproductive phase. The plant height registered a rapid and linear increment from June to July, with significant differences between the genotypes in the second (Figure 1, H2, July 9th) and in the third sampling date (Figure 1, H3, July 23th). A higher plant height was registered for *A. hypochondriacus* genotype in comparison with *A. cruentus* one. The genotype belonging to *A. hypocondriacus* displayed a shorter growth cycle than the other one, reaching a faster seed maturity in 2014 growing season (85 *vs* 115 days from transplanting, data not shown). In *A. cruentus* genotype, the panicles were developed on the apices of the single main stem, with low degree of scaling with maturation and a reduction of seed natural dispersal. This characteristic is very important since amaranth is prone to grain shattering and losses due to wind. On the contrary, the *A. hypocondriacus* genotype showed several panicles branched from the axils of the leaves, with a higher scalarity during seed maturation.

Rivelli *et al*. (2008), in a pot trial carried out in south Italy, found that *A. hypochondriacus* accessions were characterised by the shortest vegetative growth period and a long period from inflorescence emission to seed ripening. Wu *et al.* (2000) observed a great variability among amaranth species/genotypes in growth cycle, endorsed to the day-length sensitivity and similarity of climate between the site of origin of the genotypes and the target area for cultivation.

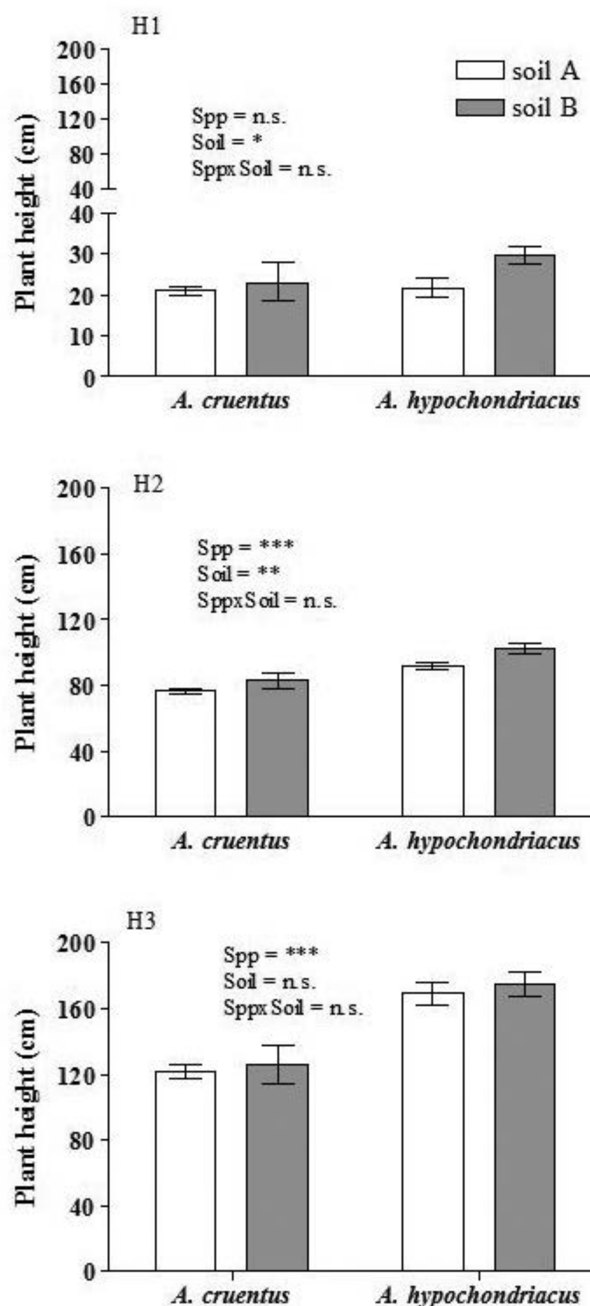

Figure 1. Plant height of the two amaranth genotypes (species *A. hypochondriacus* and *A. cruentus,* respectively) grown in two kinds of soil (A = loamy; B = sandy), measured during crop development: H1, June 24th; H2, July 9th; H3, July 23th. ***P < 0.001 level; **P<0.01 level; *P<0.05; ns, not significant.

The kind of soil significantly affected the plant height in H1 and H2, with the highest values recorded for the plants grown in the sandy soil (Figure 1). These preliminary results seem to indicate that the sandy soil can be more favorable to the amaranth development in the early growth period, confirming the important role of soil texture in affecting crop growth and development.

The relative chlorophyll and carotenoid content of the two *Amaranthus* genotypes grown in two different kinds of soil were measured through three samplings, during the vegetative phase until flowering (Table 2). The two genotypes exhibited not significant differences of photosynthetic pigments, with the exception of chlorophyll *a*. The choice of soil determined a significant difference in the production of carotenoids and chlorophyll *b* in both species. On the other hand, harvest time and the interaction between harvest time and species significantly affected all the investigated parameters, showing that the time of harvesting is a key factor in determining the photosynthetic pigment concentration. In particular, highest concentrations were recorded during the second harvest (July 9th). In the last harvest (end of July), when the plants were fully flowered, the pigment concentration decreased. It has been shown that the chlorophyll content increases during leaf expansion and then gradually declines, and more drastically during leaf senescence (Simova–Stoilova *et al*., 2001; Imai *et al*., 2005). By examining the overall three parameters (harvest time, species and soil), only chlorophyll *b* resulted in not significant values, while the chlorophyll *a*, total chlorophyll and carotenoid amounts were significantly affected by harvest time × Spp. × Soil interaction, with the highest values in *A. cruentus*, when the leaves were collected atH1 and H2. In the latest harvest time, the concentration of photosynthetic pigments were lower in the genotype belonging to *A. cruentus*.

Leaves of the two genotypes were also evaluated for their content of polyphenols, flavonoids and antioxidant activity, following the dynamic of accumulation of these secondary metabolites in the last two sampling dates (Table 3). The *A. cruentus* exhibited higher amount of polyphenols and flavonoids as well as antioxidant activity than the *A. hypochondriacus* in both kinds of soil. Significant correlations between the bioactive compounds and antioxidant activity have been observed (r^2=0.881 and P=0.0006, for phenols *vs* antioxidant activity; r^2=0.724 and P=0.0074, for flavonoids *vs* antioxidant activity), suggesting the contribution of individual compounds to the antioxidant capacity. Araceli López-Mejía *et al*. (2014), analysing the antioxidant capacity of *A. hypochondriacus* leaves and seeds, found that the antioxidant capacities of the studied extracts are not only due to phenolic compounds. The same authors also observed that the amaranth leaves exhibited more antioxidant capacity than that recorded from seeds.

Statistical differences have been observed for each variability factor (harvest time, species and soil), but considering the three factors together, only the flavonoids were statistically affected. In

Table 2. Chlorophyll *a*, chlorophyll *b*, total chlorophyll and carotenoids in the leaves of two *Amaranthus* genotypes (species *A. hypochondriacus* and *A. cruentus,* respectively) grown in two kinds of soil (A = loamy; B = sandy) and collected in three harvesting time (H1= June 24th, H2 = July 9th; H3 = July 23th).

Harvesting time	Spp.	Soil	Chlorophyll *a* (μg cm^{-2})	Chlorophyll *b* (μg cm^{-2})	Total Chlorophyll (μg cm^{-2})	Carotenoids (μg cm^{-2})
H1	*A. hypochondriacus*	A	11.02±1.89	4.80±2.11	15.81±2.42	1169.84±138.62
		B	11.94±3.96	7.29±0.92	17.55±3.99	1106.46±1988.69
	A. cruentus	A	14.87±0.31	7.39±2.07	21.64±0.63	1431.01±37.95
		B	13.13±1.32	8.10±2.42	21.00±1.78	1269.20±133.67
H2	*A. hypochondriacus*	A	15.07±1.17	6.24±2.65	21.31±1.62	1410.70±61.87
		B	11.20±1.81	6.66±2.24	17.50±1.92	1218.78±94.08
	A. cruentus	A	15.24±1.76	6.12±0.97	21.31±2.52	1422.23±111.41
		B	14.39±2.26	8.69±1.30	22.35±3.12	1412.79±166.70
H3	*A. hypochondriacus*	A	8.50±1.28	5.31±2.21	20.35±3.01	867.84±103.71
		B	6.06±1.68	5.88±1.86	16.60±3.25	680.98±104.52
	A. cruentus	A	4.14±0.97	0.66±1.47	5.31±2.05	279.56±75.09
		B	8.06±1.70	0.06±0.14	13.2 5±4.10	505.19±144.32
Mean H1			12.74±1.87	6.90±1.88	19.00±2.21	1244.13±127.23
Mean H2			13.98±1.75	6.93±1.79	20.62±2.29	1366.12±108.52
Mean H3			6.69±1.41	2.98±1.42	13.88±3.10	583.39±106.52
Mean *A. hypochondriacus*			10.63±1.97	6.03±2.00	18.19±2.70	1075.77±116.92
Mean *A. cruentus*			11.64±1.39	5.17±1.40	17.48±2.37	1053.33±111.52
Mean Soil A			11.47±1.23	5.09±1.91	17.62±2.04	1096.86±88.11
Mean Soil B			10.80±2.12	6.11±1.48	18.04±3.03	1032.23±140.33
Prob>F	Harvest time		<0.0001*	<0.0001*	<0.0001*	<0.0001*
Prob>F	Spp		0.0425*	0.0770 n.s.	0.3139 n.s.	0.4802 n.s.
Prob>F	Soil		0.1680 n.s.	0.0355*	0.5526 n.s.	0.0459*
Prob>F	Harvest time × Spp		0.0079*	<0.0001*	<0.0001*	<0.0001*
Prob>F	Harvest time × Soil		0.0390*	0.3104 n.s.	0.1382 n.s.	0.1786 n.s.
Prob>F	Spp × Soil		0.0252*	0.7828 n.s.	0.0015*	0.0116*
Prob>F	Harvest time × Spp × Soil		0.0016*	0.2031 n.s.	0.0007*	0.0071*

*P<0.05; ns, not significant.

addition, the obtained results showed as the harvest time was an important factor in defining the flavonoid content, and the antioxidant activity in amaranth, with the highest values recorded in the leaves collected at H3, during the flowering stage. The influence of soil was less significant between the two genotypes, since only the flavonoid content was affected. Variation of phenolic concentration during plant growth, states the influence of phenological stages on production of these metabolites, so far scarcely examined in the leaves of such species. Khanam and Oba (2013) measured the level of total polyphenols and total flavonoids in the leaves of *A. tricolor* and *A. hypochondriacus* at edible stage (4 week-old seedlings). These authors demonstrated a correlation between these bioactive compounds and the species examined. Moreover, the effect of light intensity seems to be also important for the amount of the bioactive compounds in *A. tricolor* (Ali *et al*., 2010).

Definitively, in amaranth leaves, the levels of bioactive compounds (phenols and flavonoids), as well as the amounts of leaf photosynthetic pigments, seemed to be related with the phenological stage. It is also important to consider that the presence of polyphenols and flavonoids in the amaranth leaves could be a precondition for considering this species as potential antioxidant source according to the consumer tendencies towards new or innovative natural ingredients.

Biomass and seed yield

Significant differences between the two genotypes were also observed for the seed yield, whereas no effect of soil type on grain yield was recorded (Table 4). These observations can suggest that,

Table 3. Total polyphenols, total flavonoids and antioxidant activity (expressed by IC_{50} value) in the leaves of two *Amaranthus* genotypes (species *A. hypochondriacus* and *A. cruentus*, respectively) grown in two kind of soils (A = loamy; B = sandy) and collected in two harvesting time (H2= July 9th; H3 = July 23th).

Harvesting time	Spp.	Soil	Total polyphenols (mg g^{-1} DW)	Total flavonoids (mg g^{-1} DW)	IC_{50} (μg DW mL–1)
H2	*A. hypochondriacus*	A	24.88±2.86	23.28±1.26	0.423±0.004
		B	25.06±0.24	24.04±0.55	0.437±0.009
	A. cruentus	A	32.87±5.11	31.54±0.13	0.381±0.024
		B	28.41±4.11	25.89±1.40	0.406±0.040
H3	*A. hypochondriacus*	A	32.56±8.29	26.79±1.63	0.287±0.020
		B	29.71±3.23	27.43±1.40	0.420±0.036
	A. cruentus	A	45.19±7.79	43.43±1.71	0.117±0.074
		B	33.25±5.71	28.36±1.70	0.273±0.051
Mean H2			27.81±3.75	26.19±3.73	0.410±0.024
Mean H3			35.18±6.85	31.50±7.98	0.270±0.124
Mean *A. hypochondriacus*			28.05±3.74	25.38±2.03	0.392±0.070
Mean *A. cruentus*			34.93±7.19	32.31±7.77	0.294±0.132
Mean Soil B			29.11±3.38	26.43±1.89	0.384±0.075
Mean Soil A			33.88±8.40	31.26±8.79	0.302±0.136
Prob>F	Harvest time		0.0035*	<0.0001*	<0.0001*
Prob>F	Spp		0.0057*	<0.0001*	<0.0001*
Prob>F	Soil		0.0420*	<0.0001*	<0.0001*
Prob>F	Harvest time × Spp		0.5830 n.s.	0.0036*	0.0014*
Prob>F	Harvest time × Soil		0.2410 n.s.	0.0005*	0.0012*
Prob>F	Spp × Soil		0.1312 n.s.	<0.0001*	0.5940 n.s.
Prob>F	Harvest time × Spp × Soil		0.6127 n.s.	0.0006*	0.8592 n.s.

*P<0.05; ns, not significant.

Table 4. Stem and inflorescences plus seeds dry yields, and seed yield, expressed as g m^{-2}, of the two Amaranthus genotypes (species *A. hypochondriacus* and *A. cruentus*, respectively) grown in two kinds of soil (A = loamy; B = sandy) and collected at seed maturity (September 3rd and October 3rd, for *A. hypocondriacus* and *A. cruentus*, respectively).

Spp.	Stem dry yield (g m^{-2})			Inflorescences + seeds dry yield (g m^{-2})			Seed yield (g m^{-2})		
	Soil A	Soil B	Mean Spp.	Soil A	Soil B	Mean Spp.	Soil A	Soil B	Mean Spp.
A. hypochondriacus	219.07±41.82	272.86±30.00	245.97±38.04	350.08±71.44	381.14±20.83	365.61±21.96	163.36±27.94	184.48±17.42	173.92±14.93
A. cruentus.	250.28±11.36	240.66±9.07	245.47±6.81	302.55±10.30	344.49±27.57	323.52±29.66	144.36±4.67	148.59±6.01	146.47±2.99
Mean soil	234.68±22.07	256.76±22.77		326.31±33.61	362.82±25.91		153.86±13.44	166.53±25.38	
Prob>F Spp.		0.9751 n.s.			0.1059 n.s.			0.0227*	
Prob>F Soil		0.1905 n.s.			0.1528 n.s.			0.2301 n.s.	
Prob>F Spp. × Soil		0.0741 n.s.			0.8197 n.s.			0.412 n.s.	

*P<0.05; ns, not significant.

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in amaranth, seed yield is quantitatively inherited and influenced by genotype/species. At the same time, the high genetic variability and the phenotypic plasticity, well documented for this versatile crop (Kulakow and Hauptli, 1994; Brenner *et al*., 2000; Khanam and Oba, 2014), allowed the plants to grow well under the two types of soil, readily adapting to different growth conditions, with good performance in terms of seed yield.

Our results pointed out that the genotype belonging to *A. hypochondriacus* species was characterised, during the growing season analysed, by the highest seed yield, with values of 174 g m^{-2} (Table 4). This genotype showed also a higher harvest index than *A. cruentus* genotype (0.28 and 0.26, as mean value over the soil type). Casini and La Rocca (2014), in a two-year field experiment carried out in central Italy (Arezzo, Tuscany) compared different genotypes of *A. hypochondriacus* and *A. cruents*. They found that *A. cruentus* exhibited the best growth in the tested environment in comparison to *A. hypochondriacus*. This latter species, in fact, showed negligible yields in the tested environment, especially due to drought and high average temperatures during the summer period, differently to what occurred in our experimental conditions, where the growing period was characterised by abundant rainfall, mainly concentrated in July (194 mm). On the other hand, it is known that the grain yield strongly varies depending, not only on genotype, but also on site, pedo-climatic conditions, growing season and agronomic practices (Gimplinger *et al*., 2008; Rivelli *et al*., 2008). However, the genotypes here investigated, exhibited good potential levels of grain yield in both soil types, reaching values consistent with those reported in literature (Myers, 1996; Kaul *et al*., 2000; Pospiŝil *et al*., 2006; Gimplinger *et al*., 2008; Rivelli *et al*., 2008; Casini and La Rocca, 2014), even if further investigation are needed in order to confirm the observed results.

Finally, regarding stem and inflorescences, no changes in dry yield were observed, neither in function of the species nor in function of the soil type and their reciprocal interaction (Table 4). An interesting aspect was the appreciable production of biomass, which can be regarded as a potentially valuable by-product after seed harvesting, according to a cascading approach of biomass utilisation and/or valorisation.

Oil content and fatty acids composition

The crude oil content of the two *Amaranthus* genotypes ranged from 7.5% to 6.0 %, with an average of 6.7% (Tables 5 and 6). These results are consistent with those found by Budin *et al*. (1996), in a study involving eight *Amaranthus* species, where a total crude fat range from 5.2% to 7.6% has been reported. No differences were observed between plants cultivated on different soils, while *A. hypochondriacus* showed a significantly higher level of fat content than *A. cruentus*. These results underlined that the principal factor affecting oil content in amaranth is the species/genotype, as proposed by Prakash and Pale (1992).

Table 5. Fatty acids composition in relation to soil type (A = loamy; B = sandy).

		Soil A	Soil B	Standard error	P-value
Dry matter	g 100 g^{-1} of seeds	89.06	90.02	0.53	0.325
Lipids	g 100 g^{-1} of seeds	6.88	7.09	0.89	0.879
Satured fatty acids	g 100 g^{-1} of TL	19.54	17.59	0.22	0.025
C14:0	g 100 g^{-1} of TL	0.27	0.19	0.04	0.274
C16:0	g 100 g^{-1} of TL	14.63	13.18	0.24	0.050
C17:0	g 100 g^{-1} of TL	0.08	0.07	0.01	0.699
C18:0 iso	g 100 g^{-1} of TL	0.56	0.48	0.16	0.754
C18:0	g 100 g^{-1} of TL	2.33	2.18	0.28	0.721
C20:0	g 100 g^{-1} of TL	0.62	0.57	0.02	0.327
C22:0	g 100 g^{-1} of TL	0.31	0.27	0.01	0.183
C24:0	g 100 g^{-1} of TL	0.28	0.24	0.02	0.295
Monounsatured fatty acids	g 100 g^{-1} of TL	27.27	25.23	3.88	0.746
C16:1c9	g 100 g^{-1} of TL	0.16	0.13	0.01	0.299
C18:1c9	g 100 g^{-1} of TL	25.73	23.81	4.05	0.769
C18:1c11	g 100 g^{-1} of TL	1.18	1.03	0.21	0.681
C18:1c12	g 100 g^{-1} of TL	0.12	0.15	0.01	0.299
Polyunsatured fatty acids	g 100 g^{-1} of TL	38.43	35.51	7.42	0.806
C18:2c9c12	g 100 g^{-1} of TL	37.39	34.59	7.33	0.813
C18:3c9c12c15	g 100 g^{-1} of TL	0.86	0.76	0.07	0.424
n6/n3		38.52	40.77	4.19	0.741
Phytosterols	mg 100 g^{-1} of TL	1017.48	743.39	89.473	0.163
Campesterol	mg 100 g^{-1} of TL	29.47	17.74	4.75	0.223
Stigmasterol	mg 100 g^{-1} of TL	67.07	30.22	15.81	0.241
β-sitosterol	mg 100 g^{-1} of TL	921.01	695.43	69.02	0.147
Squalene	g 100 g^{-1} of TL	6.33	5.42	0.48	0.321

TL, total lipids.

Regarding fatty acids composition (Tables 5 and 6), our results showed that the major fatty acids in amaranth oil samples were linoleic (C18:2c9c12), palmitic(C16:0), and oleic acid (C18:1c9). These results are in agreement with other works (Singhal and Kulkarni, 1988; Prakash and Pal, 1992; Prakash *et al*., 1995; He and Corke, 2003; Hlinková *et al*., 2013).

The effect of different type of soil did not alter the fatty acid composition of *Amaranthus* oil fraction, while significant differences were recorded between the two genotypes.

Total saturated fatty acid did not show significantly differences between the two genotypes, though the content of stearic acid (C18:0) and C18:0 iso was higher and lower in *A. hypochondriacus* and *A. cruentus*, respectively. Conversely, unsaturated fraction was significantly affected by species origin. *A. hypochondriacus* showed a higher level of monounsatured fatty acids, while *A. cruentus* was characterised by a high level of polyunsatured fatty acids. The higher content of monounsatured fatty acids principally depended by the higher level of oleic acid (C18:1c9) in *A. hypochondriacus* (+39%). On the contrary, the higher level of polyunsatured fatty acids in *A. cruentus* was related to linoleic acid (C18:2c9c12) (+50.74%), while α-linolenic acid (C18:3c9c12c15) was higher in *A. hypochondriacus* oil (+19%).

The ratio of *n*6/*n*3 was similar between the two genotypes, with a level lower than 50:1, as reported by Gebhardt *et al.* (2007), because of the high level of linoleic acid (*n*6) and the low level of α-linolenic acid (*n*3). This ratio is an important determinant in decreasing the risk for coronary heart disease; both in its primary and secondary preventions (Simopoulos, 2008). The ratio is still much higher than that recommended, *i.e.* 4:1, to prevent cardiovascular disease, but the low level of α-linolenic acid, approaching 1%, seems to be sufficient to increase the healthy property of this oil source (Raiciu *et al*., 2016).

So, amaranth seeds represent a very important source of polyunsatured fatty acids and their positive role on human health may be considered, especially for the reduction of LDL-cholesterol and prevention of cardiovascular diseases (Martirosyan *et al*., 2007).

Squalene and sterols content

In amaranth oil, the content of total sterols was 6 g $100g^{-1}$ of total lipids (Tables 5 and 6), while the total of sterol esters was slightly lower. However, the most remarkable characteristic of the amaranth sterols is the structure of their major compounds. Tables 5 and 6 showed the set of 3 quantified sterols (campesterol, stigmasterol and β-sitosterol). Species and soil factor did not affect the phytosterols composition (Tables 5 and 6). Sterols are the major constituents of the unsaponifiable fraction of most edible oils (Kiritsakis and Christie, 2000; León-Camacho *et al*., 2001). Their consumption is positively related to the human blood cholesterol level. According to numerous reports, if phytosterols are consumed regularly at 1-3 g/day dose, phytosterols may reduce blood cholesterol levels by 10 to 15% (EFSA, 2008). Other studies have shown that phytosterols may prevent cancer, atherosclerosis and inflammations (Ryan *et al*., 2005).

Table 6. Fatty acids composition in relation to the two *Amaranthus* genotypes (species *A. hypochondriacus* and *A. cruentus*, respectively).

		A. hypochondriacus	*A. cruentus*	Standard error	P-value
Dry matter	g 100 g^{-1} of seeds	89.97	89.13	0.58	0.413
Lipids	g 100 g^{-1} of seeds	7.87	6.11	0.13	0.011
Satured fatty acids	g 100 g^{-1} of TL	18.51	18.62	0.99	0.941
C14:0	g 100 g^{-1} of TL	0.20	0.26	0.04	0.467
C16:0	g 100 g^{-1} of TL	13.76	14.05	0.75	0.811
C17:0	g 100 g^{-1} of TL	0.08	0.06	0.01	0.095
C18:0 iso	g 100 g^{-1} of TL	0.36	0.67	0.05	0.048
C18:0	g 100 g^{-1} of TL	2.53	1.98	0.08	0.041
C20:0	g 100 g^{-1} of TL	0.58	0.61	0.03	0.476
C22:0	g 100 g^{-1} of TL	0.28	0.31	0.02	0.591
C24:0	g 100 g^{-1} of TL	0.28	0.24	0.02	0.295
Monounsatured fatty acids	g 100 g^{-1} of TL	30.14	22.38	1.04	0.034
C16:1c9	g 100 g^{-1} of TL	0.13	0.16	0.01	0.299
C18:1c9	g 100 g^{-1} of TL	28.82	20.72	0.98	0.028
C18:1c11	g 100 g^{-1} of TL	0.89	1.32	0.08	0.045
C18:1c12	g 100 g^{-1} of TL	0.15	0.12	0.01	0.299
Polyunsatured fatty acids	g 100 g^{-1} of TL	29.59	44.34	1.69	0.025
C18:2c9c12	g 100 g^{-1} of TL	28.71	43.28	1.63	0.024
C18:3c9c12c15	g 100 g^{-1} of TL	0.74	0.88	0.05	0.048
n6/n3		35.51	43.79	1.31	0.045
Phytosterols	mg 100 g^{-1} g of TL	791.56	969.31	137.44	0.456
Campesterol	mg 100 g^{-1} g of TL	19.09	28.11	6.05	0.403
Stigmasterol	mg 100 g^{-1} g of TL	62.97	64.32	18.53	0.354
β-sitosterol	mg 100 g^{-1} g of TL	739.51	876.94	112.98	0.481
Squalene	g 100 g^{-1} of TL	5.72	6.03	0.64	0.726

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The level of squalene in the *Amaranthus* oil (Tables 5 and 6) is comparable with data reported in other works (Czaplicki *et al*., 2012). This level of squalene is higher than usually found in oils from several cereal grains (Gamel *et al*., 2007). Squalene content was not different in the oil fraction of *A. hypochondriacus* and *A. cruentus*. Similarly, no differences were observed after cultivation in the two types of (soil A*vs* soil B). *Amaranthus* squalene level is surely lower than the traditional sources, like shark (*Centrophorus squamosus*) and whale (*Physeter macrocephalus*) liver oil (Jahaniaval *et al*., 2000), but may be considered a good alternative. In fact, the use of squalene in cosmetic applications is limited by the uncertainty of its availability as a result of international concern for the protection of marine animals. In addition, the presence of similar compounds, such as cholesterol, in the oils from marine animal liver can make squalene purification difficult. There is interest in other potential sources of squalene, and plant sources are being widely prospected. Squalene contents in olive, wheat germ, and rice bran oils are in the 0.1-0.7% range (Schnetzler and Breene, 1994), which is not high enough for them to be considered as a viable resource. It was reported that squalene was present in up to 0.46% of the leaf dry weight of Macaronesian *Echium* plants (Guil-Guerrero *et al*., 1998), and that squalene was found in minute amounts in all European *Echium*species. *Amaranthus* oil is potentially an important source of squalene since contains larger amounts of squalene (2.4-8.0%) than other common vegetable oils (Lehmann, 1996; Sun *et al*., 1997).

Conclusions

There is an urgent need, by scientific community, to find solutions to increase cropping system sustainability, crop production and food security, in view of the ongoing climate change. This preliminary study showed that amaranth could be a valuable alternative crop for specialty oil production in the Mediterranean region. The overall results, in fact, demonstrated that the two amaranth genotypes (*A. hypochondriacus* and *A. cruentus species*) showed satisfactory seed yield and relative short growth cycle in the investigated environment of Central Italy. Along these promising agronomic characteristics, the study underlined different and interesting compositional aspects of leaves, seeds and fixed oil of the two amaranth species. In particular, fatty acids profile of the seeds showed very high content of polyunsaturated fatty acids, with consequent potential health benefits in lowering the cholesterol and triacylglycerol concentrations. In addition, our data demonstrated that the amaranth oil can be considered a good source, not only of PUFA, but also of squalene, making amaranth a good alternative of the traditional sources of this bioactive compound. The unique nutraceutical properties of amaranth seeds, due to the high squalene content, open interesting perspective to this crop as sources of food supplements or nutraceuticals for food and pharmaceutical industries, meeting the consumer demands in favor of more nutritious and healthful products.

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