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Different agronomic and fertilization systems affect polyphenolic profile, antioxidant capacity and mineral composition of lettuce

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ABSTRACT

The present paper aims to investigate phenolic profiles, antioxidant capacity and mineral composition of lettuce (*Lactuca sativa* L., var. 'Maravilla de Verano') grown under conventional (CON) and an organic (ORG) systems with four different fertilization treatments. The polyphenolic profiles of leaf extracts were determined by ultra-high-performance liquid chromatography (UHPLC), the levels of mineral elements by means of inductively coupled plasma mass spectrometry, whereas total phenolic content and antioxidant capacity were determined spectrophotometrically. Yield, soil and meteorological parameters were measured. In all the fertilization treatments, total phenolic acids and flavonols in CON were significantly higher compared to ORG. A trend parallel to that of single phenols was observed for total phenolic content and total antioxidant capacity. Plant mineral distribution revealed significant changes between CON and ORG systems in some plant macronutrients (N, Mg, S, Na, Fe) and micronutrients (Se, Mn, Mo). The differences among fertilization treatments for all the parameters considered were also discussed. From the overall analysis of the results, the higher content of phenolics observed in CON system could be associated to the presence of more stressful conditions, in terms of plant and/or soil mineral deficits. On the other hand, the adoption of an organic management determined higher yields and a better plant mineral balance.

1. Introduction

Green lettuce (*Lactuca sativa* L.) is primarily consumed as whole heads or fresh-cut product (Romani et al., 2002). As lettuce worldwide consumption has steadily increased in the last decades (Heimler et al., 2012), a series of studies have been recently conducted for studying its nutraceutical and health-promoting compounds (Ribas-Agustí et al., 2011; Heimler et al., 2012; Abu-Reidah et al., 2013; Durazzo et al., 2014; Pepe et al., 2015).

It was demonstrated that lettuce is particularly rich of some classes of polyphenols, plant secondary metabolites with free radical-scavenging properties that have evolved for facing abiotic and biotic stresses experienced by the plants in the surrounding environment (Romani et al., 2002; Oh et al., 2009a). Polyphenols are also important in human diet, preventing cancer and cardiovascular diseases (Manach et al., 2004; Hooper and Cassidy, 2006). The health-promoting properties of lettuce polyphenols were recently tested by Pepe et al. (2015) and Adesso et al. (2016), who demonstrated that lettuce leaf extracts are able to reduce both the inflammatory and oxidative stress in murine monocyte macrophage cells, by decreasing reactive oxygen species, nitric oxide release, inducible nitric oxide synthase and cycloxygenase-2 expression, and promoting the nuclear translocation of the nuclear

Abbreviations: CON, conventional soil management; DPPH•, 2,2-diphenyl-1-picrylhydrazyl radical; ORG, organic soil management; DW, dry weight; FRAP, ferric reducing ability of plasma; FW, fresh weight; GAE, gallic acid equivalents; PAR, photosynthetically active radiation; TE, Trolox equivalents; UHPLC, ultra-high-performance liquid chromatography.

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Table 1
Compost and rock dust applied every year (2011–2013) for the different fertilization treatments.

	Fertilization treatment	Compost (t ha^{-1} yr^{-1})	Rock dust (t ha^{-1} yr^{-1})	Calcium nitrate (kg N ha^{-1} yr^{-1})
A	No fertilization	0	0	0
B	Manure	12	0	0
C	Rock dust	0	28.8	0
D	Manure + rock dust	12	28.8	0
K	Mineral fertilization (control)	0	0	150

factors Nrf2 and NF-κB. Considering that lettuce is a cheap and popular food widely consumed fresh worldwide and that the lack of cooking preserve the degradation of the more thermolabile chemical species and avoid the loss of water-soluble compounds, this vegetable is of key importance for the dietary supply of natural antioxidants (Pepe et al., 2015).

In lettuce, two classes of polyphenols are mainly present: phenolic acids and flavonols (Manach et al., 2004; Baslam et al., 2013; López et al., 2014). Phenolic acids are rarely found in lettuce in the free form, being mostly present as bound forms, mainly glycosylated derivatives or esters (e.g. chlorogenic acid, from the combination of caffeic and quinic acids) (DuPont et al., 2000; Mulabagal et al., 2010; Ribas-Agustí et al., 2011; Mai and Glomb, 2013). Flavonols are very frequent in lettuce, and their main representatives are the glycosylated derivatives of quercetin and kaempferol (Romani et al., 2002; Hooper and Cassidy, 2006).

Greenhouse and soilless lettuce culture systems are today increasingly widespread than open-air-grown lettuce (Romani et al., 2002; Heimler et al., 2012; Durazzo et al., 2014). While this warrants a higher yield and better climatic, phytopathogical, water and nutritional control, it not always ensures a good phytochemical profile in the final product (Tomas-Barberan et al., 1997; Llorach et al., 2008). Indeed, the adverse agronomic and environmental factors experienced by plants grown in open field, such as high or low temperature, ultraviolet light, insect attack, pathogen infection and eventual nutrient deficiency can increase the amounts of phenolics and/or change their profiles, even with a decrease of crop productivity (Tomas-Barberan et al., 1997; Llorach et al., 2004, 2008; Oh et al., 2009b). For instance, flavonols are yellow pigments generally considered to act as UV protectants and free radical scavengers, so their biosynthesis is stimulated in the outer and aerial tissues (stem and leaf epidermis) under excess light (Sofo et al., 2012). In addition, the levels of phenolic acids in lettuce are sensitive to environmental conditions (Liu et al., 2007; Mai and Glomb, 2013). For all these reasons, it is important to find a compromise between convenient agronomic practices and food nutritional properties. On the other hand, to obtain top yields of high quality and preserve environmental sustainability, it is indispensable to adopt sustainable agricultural techniques. Among open field agronomic techniques, organic farming offers more benefits than a conventional approach in terms of sustainability, enhanced physico-chemical and microbiological soil fertility, and absence of synthetic fertilizers and chemical pesticides (Heimler et al., 2012; Durazzo et al., 2014)

Other than phenolics, lettuce chemistry at the elemental level, that includes the content of all mineral nutrients and trace elements, is of key importance (Kelly and Bateman, 2010). Indeed, it is known that some inorganic cations, naturally present at non-toxic concentrations in lettuce, such as Fe, Cu and Zn, participate in the radical-scavenging reactions for their role of cofactors in some antioxidant enzymes, Fe/S proteins and cytochromes. In the case of K, Na, Ca, Mg, S and P, they are essential to the human organism together with some micronutrients (e.g., Mn, Se and Co) that act as cofactors in human vitamins and enzymes.

In the last years, great importance has been devoted to the consumption of lettuce since it is a source of natural antioxidants (Llorach et al., 2008; Kelly and Bateman, 2010; Heimler et al., 2012; Abu-Reidah et al., 2013; Lee and Scagel, 2013). Based on this accepted knowledge, the present paper aims at investigating phenolic profiles, total phenol content, total antioxidant capacity and mineral composition of lettuce grown under two different farming systems (conventional and organic) and under different fertilization treatments in order to establish if these management practices can modify its nutritional quality.

2. Materials and methods

2.1. Experimental site and plant material

The experiment was conducted in 2011, 2012 and 2013 in two open-field farms at Järna, in central Sweden, not distant each other and have similar climatic conditions. The first farm ('Gerstaberg'; CON) was managed conventionally for over 50 years, cultivated with a rotation potato/rye in the last 12 years (fertilization using 35 kg N-NO_3 ha^{-1} yr^{-1} and 20 kg N-NH_4 ha^{-1} yr^{-1}, crop residue shredding, and ploughing twice a year at a soil depth of 30 cm). The second one was an organic farm ('Nibble garden'; ORG) amended with different types of animal manure and without any synthetic fertilizers or chemical pesticides since its inception in 1966, and cultivated with a rotation of with potato/clover/barley in the last 12 years (minimum tillage, crop residues shredded and incorporated into the soil after a light harrowing at 10-cm soil depth).

The lettuce (*L. sativa* L.) variety used in this study was 'Maravilla de Verano'. Starting from 2011, both farms (CON and ORG) were divided in four blocks: one unfertilized control (treatment A) and three fertilization treatments (treatments B, C and D) with different types of soil amendments (Table 1). An additional mineral fertilized treatment (K) was included in CON field whereas it was not possible to include it in the ORG system due to the strict restrictions in the use of synthetic fertilizers.

Cattle manure composted for six months was used for treatment B. Rock dust (treatment C) was obtained by finely grinding (approximately 0.5 mm of diameter) local rocks using a jaw crusher (Model Nordberg C100; Metso Minerals Ltd., Tampere, Finland). Compost mean composition was the following: water 248 g kg FW^{-1}, pH 7.98, total N 18 g kg DW^{-1}, organic C 338 g kg DW^{-1}, organic matter 583 g kg DW^{-1}, humus 104 g kg DW^{-1}, P_2O_5 6.8 g kg DW^{-1}, K_2O 14 g kg DW^{-1}, Zn 0.112 g kg DW^{-1}, Fe 5.53 g kg DW^{-1}, 0.065Cu g kg DW^{-1}, Mn 0.114 g kg DW^{-1}. Rock dust mean composition (% w/w) was the following: SiO_2 65.21, K_2O 11.41, CaO 9.06, Al_2O_3 5.42, MgO 1.96, Na_2O 1.65, P_2O_5 1.28, FeO 1.21, Fe_2O_3 1.02, MnO 0.07, other minerals 1.71. Compost stone meal (treatment D) was prepared by mixing the stone powder with cattle manure (<0.2 mm) from diabase, after which the mixture was composted for 6 months. Fertilizers amounts of the treatment K (Table 1) were calculated on the basis of the values of soil texture and N content (Table 2). At the establishment of the treatments, ten composite soil sample were taken by randomly collecting three soil cores per each farming system and fertilization treatment. On these samples, soil texture was determined, soil N was measured by the analytic kit Reflectoquant 10© (Merck, NJ, USA) combined to the Kjeldahl

Table 2
Soil texture and nitrogen content at 0–30 cm depth in the conventional (CON) and organic (ORG) experimental sites at the beginning of the experiment ($n = 10$).

	CON	ORG
Soil organic matter (% w/w)	2.6	6.7
Clay (% w/w)	45.2	40.0
Silt (% w/w)	40.8	40.0
Sand (% w/w)	11.4	13.3
NO_3-N (kg ha^{-1})	47.4	81.0
NH_4-N (kg ha^{-1})	31.7	12.1

method, and soil organic matter was determined by dry combustion method using a LECO-SC230 apparatus (LECO Instruments, UK). Soil wilting point, field capacity and saturation were estimated from the values of soil texture and soil organic matter using the software SPAWHydrology (version 6.02.74; USDA Agricultural Research Service, Washington, Pullman, WA, USA).

Lettuce was planted in late June of the three experimental years in both the systems. The distance between the plants was 0.25 × 0.25 m. Plants were covered with a nylon cloth to reduce evaporation and protect them against pests. The harvest was carried out in late July in both the systems. A number of 16 lettuce heads per each fertilization treatment of both CON and ORG systems were randomly harvested in the central part of each fertilization block for avoiding boundary effects among the fertilization treatments and placed in white plastic bags to minimize water loss. The bags were immediately transported in the laboratory, where they were weighed. The heads were divided lengthwise in half. One half of each head was put in a cloth bag, weighed and dried at 53 °C for 48 h. The dry matter was weighed, ground and stored in plastic tins for element determination. The remaining halves were used for determining phenol extraction and antioxidant capacity. The number of plants randomly chosen for yield measurements expressed as g DW head^{-1} in each fertilization block was 200. Meteorological variables were monitored by weather stations of the national weather measuring system of Swedish Meteorological and Hydrological Institute, placed at a 50 m from the experimental fields.

2.2. Phenolic extraction and determination

For each plant, central leaves were detached, slightly washed with distilled water to remove eventual residues, and used for the following measurements. Five grams of fresh leaf taken from the central part were collected with a scalpel and placed in an extracting solution of 49 ml methanol + 1 ml HCl 37%, according to Sofo et al. (2012). The solution was covered with parafilm to avoid evaporation, shaken at 100 rpm at 20 °C in the dark for 45 min. The extracts were filtered through 0.20 μm Minisart SFCA sterile filters (Sartorius Stedim Biotech GmbG, Goettingen, Germany), and immediately stored at −20 °C. Leaf extracts were analyzed by high-performance liquid chromatography for the determination of the phenolic compounds. The polyphenols contents of leaf extracts were determined by ultra-high-performance liquid chromatography (UHPLC). All leaf extracts were filtered through 0.45-μm Whatman filters.

Ultrapure water was obtained by a Milli-Q system (Millipore, Milan, Italy). All the chemicals used were purchased by Sigma-Aldrich (Milan, Italy). The UHPLC analysis was performed on a Shimadzu Nexera UHPLC system, consisting of a CBM-20A controller, two LC-30AD dual-plunger parallel-flow pumps, a DGU-20 A5 degasser, an SPD-M20A photo diode array detector (equipped with a semi-micro flow cell of 2.5 μl), a CTO-20A column oven, a SIL-30AC autosampler. The UHPLC system was coupled online to an LCMS-IT-TOF mass spectrometer through an ESI source (Shimadzu, Kyoto, Japan). LC–MS data elaboration was performed by the LCMSsolution® software (Version 3.50.346, Shimadzu). Mobile phases were: A = 0.1% (v/v) HCOOH in water; B = 0.1% (v/v) HCOOH in acetonitrile. Analyses were performed in gradient elution as follows: 0–2.90 min, 15–20% B; 2.90–3.10 min, 20% B; 3.10–3.80 min, 20–27% B; 3.80–5.30, 27–35% B, 5.30–6.60 35–55% B, 6.60–10 55–100% B. Column oven temperature was set to 48 °C. Injection volume was 2 μl of methanolic extract. The following PDA parameters were applied: sampling rate, 40 Hz; detector time constant, 0.160 s; cell temperature, 40 °C. Data acquisition was set in the range 190–400 nm and chromatograms were monitored at 330 and 350 nm at the maximum absorbance of the compounds of interest. UHPLC system was coupled on-line to a hybrid IT-TOF instrument, flow rate from LC was splitted prior of the ESI source by means of a stainless steel Tee union (1/16 in., 0.15 mm bore; Valco Instruments Company Inc., Houston, TX, USA). Resolution, sensitivity, and mass number calibration of the ion trap and the TOF analyzer were tuned using a standard sample solution of sodium trifluoroacetate. MS detection was operated in negative ionization mode with the following parameters: detector voltage, 1.53 kV; Interface voltage, −3.5 kV, CDL (curve desolvation line) temperature, 200 °C; block heater temperature, 200 °C; nebulizing gas flow (N_2), 1.5 l min^{-1}, drying gas pressure, 100 kPa. Full scan MS data were acquired in the range of 200–900 m/z ion accumulation time, 40 ms; IT, (repeat = 2). MS/MS experiments were conducted in data dependent acquisition, precursor ions were acquired in the range 150–800 m/z; peak width, 3 Da; ion accumulation time, 60 ms; CID energy, 60%, collision gas 50%, repeat = 1; execution trigger (BPC) intensity, at 95% stop level.

Quantification of individual phenolic compounds was performed by photodiode array detection (DAD) at the maximum absorbance of the compounds of interest. Stock solution (1 mg mL^{-1}) were prepared in methanol, the calibration curves were obtained in a concentration range of 1–100 μg mL^{-1} with six concentration levels (1, 5, 10, 25, 50, 100 μg mL^{-1}) and triplicate injection of each level were run. Phenolic acids derivatives were quantified by comparison with an external standard of caffeic acid where flavonols were quantified as quercetin 3-*O*-glucoside. The quantification of the compounds for which there were not a standard available was made using the calibration curve of the compound that was included in their structure. Peak areas of each standard were plotted against corresponding concentrations. The amount of the compounds in the sample was expressed as milligram per gram of extract, linear regression was used to generate calibration curve, R^2 values were ≥0.999, retention times and areas repeatability was also evaluated showing RSD% values below 0.80 and 8.68 respectively, further confirming the precision of the method. Identification of polyphenols was carried out using both DAD spectra and MS/MS data, comparing the fragmentation pattern with data, when present, in literature. Molecular formula was calculated by the Formula Predictor software (Shimadzu), setting a low tolerance so that most of the identified compounds were in position 1 in the list of the possible candidates. Two different column setup were employed in this work: a Kinetex C18 150 × 2.1 mm, 2.6 μm (Phenomenex, Castle Maggiore, Italy) for UHPLC–MS/MS qualitative analyses, and a Kinetex C18 150 × 4.6 mm, 2.6 μm for UHPLC-PDA quantitative analysis.

Total phenolic content was determined by the Folin-Ciocalteu colorimetric method using gallic acid as a standard. Each reaction mixture contained 100 μl sample solution, 6 ml distilled-deionized water, 500 μl Folin-Ciocalteu reagent and 0.15 ml of Na_2CO_3. For the blank, acetone-water (1:1, v/v) replaced gallic acid. After 2 h of reaction at 20 °C, the absorbance was measured at 765 nm using a Jasco V-530 UV–vis spectrophotometer (Jasco Corp., Tokyo, Japan). Total phenolic content was expressed as mg gallic acid equivalents (GAE) per gram of fresh weight of leaves.

2.3. Total antioxidant capacity

The ability of leaf extracts to scavenge the DPPH• (2,2-diphenyl-1-picrylhydrazyl) radical was measured according to Brand-Williams et al. (1995). Aliquots (20 μl) of leaf extract were added to 3 ml of DPPH• solution (6×10^{-5} mol L^{-1}) and the absorbance was spectrophotometrically determined at 515 nm every 5 min until the steady state.

The antioxidant potential of leaf extracts was also determined using a FRAP assay (Ferric Reducing Ability of Plasma). A solution of 10 mmol L^{-1} 2,4,6-tripyridyl-s-triazine (TPTZ) in 40 mmol L^{-1} HCl and 12 mmol L^{-1} $FeCl_2$ was diluted in 300 mmol L^{-1} sodium acetate buffer (pH 3.6) at a ratio of 1:1:10. Aliquots (20 μl) of leaf extract were added to 3 ml of the FRAP solution and the absorbance was spectrophotometrically determined at 593 nm every 5 min until the steady state was reached. For each antioxidant assay, a Trolox aliquot was used to develop a standard curve and all data were expressed as Trolox equivalents (μmol TE g^{-1} FW).

2.4. Element determination

An aliquot (50 g) of the same leaves used for phenol determination was digested in a HNO_3:H_2O_2 solution (5:1, v/v) using a high performance microwave digestion unit (MLS-1200 Mega, Milestone Inc., CT, USA). Two milliliters of HNO_3 (0.1 M) were added and then the solution was made up to a 10 ml with ultra-pure distilled water. The levels of elements were determined by means of quadrupole inductively coupled plasma mass spectrometry, ICP-QMS (Elan DRC II, PerkinElmer SCIEX, CT, USA). Operational parameters were the following: sample uptake rate, 1 ml min^{-1}; sample introduction system, Meinhard nebulizer with cyclonic spray chamber; gas flow rates L min^{-1}: plasma, 15; auxiliary, 1.0; nebulizer, 0.85; dwell time, 50 ms; interface, Pt cones; extraction lens voltage, optimized for maximum detector response (^{56}Fe). High purity He (99.9999%) and H_2 (99.9995%) were used, in order to minimize the potential problems caused by unidentified reactive contaminant species in the cell. The instrument was equipped with an octopole ion guide enclosed in a collision/reaction cell. Moreover, the instrument was operated in an air-conditioned laboratory (20–22 °C) equipped with a filter to remove dust particles. Non-metallic devices were always used to collect and transport the samples. Considering that the instrument used is a simultaneous ICP-QMS, having an array of photo multiplier tubes positioned to look at a fixed set of elements (wavelengths), the reference wavelengths for each metal and metalloid were automatically chosen by the instrument software in order to avoid interferences with the other elements analyzed. Before use, all glassware and plastic containers were cleaned by washing with 10% ultra-pure grade HNO_3 for at least 24 h, and then rinsed copiously with ultra-pure water before use. The calibration solutions were prepared from multi-elemental standard stock solutions of 1000 mg L^{-1}, and the calibration curves were obtained by using at least 6 calibration solutions. Reagent blanks containing ultra-pure water were additionally analysed in order to control the purity of the reagents and the laboratory equipment.

2.5. Statistical analysis

For each management system and fertilization treatment, data of polyphenols, total antioxidant capacity and mineral levels were represented as the means of measurements on 16 different plants per treatment per year ($n = 48$), while yield values were represented as the means of measurements on 100 plants per treatment per year ($n = 300$). Data were analyzed using SAS version 9.2 (SAS Institute Inc., Cary, NC, USA). Analysis of variance (ANOVA) was performed using the PROC GLM procedure of the software, that uses the method of least squares to fit general linear models. PROC GLM enables to specify any degree of interaction (crossed effects) and nested effects, and also provides for polynomial, continuous-by-class, and continuous-nesting-class effects. All the values of the three experimental years were considered as replications, as no significant difference were observed between the years. Correlation analysis was performed to determine the relationship between the parameters independent of the agronomic and fertilization treatments using the CORR procedure, computing Pearson correlation coefficients as parametric measure of the linear relationship between the variables. Significant differences among means were determined at $P \leq 0.05$, according to Fisher's LSD test.

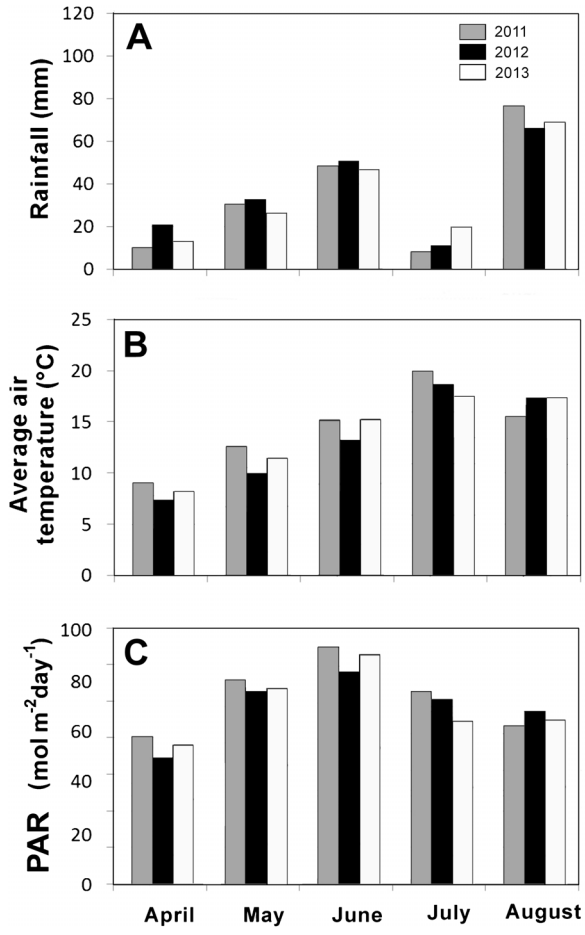

Fig. 1. (A) Rainfall, (B) average air temperature, and (C) mean daily PAR in 2011 (grey bars), 2012 (black bars) and 2013 (white bars).

3. Results

3.1. Climatic variable, soil properties and yield parameters

In the period April-August (mean 2011–2013), there was 204 mm of rainfall, with only 13 mm in July (Fig. 1a). The average temperatures reached a peak of 19 °C (mean 2011–2013) in August (Fig. 1b). The mean daily PAR (photosynthetically active radiation) increased from April to June and successively decreased during the last part of the summer in all the three experimental years (Fig. 1c).

On the basis of the parameters of soil texture and soil organic matter (Table 2), CON soil was a silty clay having 26.8% (v/v) wilting point, 40.9% (v/v) field capacity and 52.3% (v/v) saturation, whereas ORG soil was a silty clay with 24.9% wilting point, 39.9% field capacity and 58.0% saturation. Soil organic matter and total soil N (NO_3-N + NH_4-N) were higher in the ORG system (Table 2).

Table 3
Yield in conventional (CON) and organic (ORG) management systems. The fertilization treatments are indicated in Table 1. Mean values in the period 2011–2013 ($n = 300$) ± SD followed by different letters are significantly different at $P \leq 0.05$, according to Fisher's LSD test.

	Yield (g DW head^{-1})					
	CON			ORG		
	Value	SD	Stat	Value	SD	Stat
A	4.55	2.36	d	12.41	0.69	b
B	10.74	1.25	c	13.46	1.84	ab
C	9.35	1.18	c	13.42	1.99	ab
D	7.57	3.16	cd	15.12	2.70	a
K	8.39	0.99	cd	–	–	–

For all the fertilization treatments, the values of yield were significantly higher in ORG than in CON (Table 3). The unfertilized treatment (A) of ORG had significantly higher yield (12.41 g DW head^{-1}) than the mineral fertilized treatment (K), while A-CON showed very low yield values (Table 3). Considering all the treatments together, excluding K, in all of the three years of experiment, yield was significantly higher in the ORG system (Table 7).

3.2. Levels of phenolic acids and flavonols

The phenolic acids and flavonols revealed in the lettuce samples, sorted in the order of the peaks found in the UHPLC chromatograms, are showed in Table 4. In all the fertilization treatments, the levels of total phenolic acids of CON fertilization treatments were significantly higher compared to ORG. In the CON system, total phenolic acids in the unfertilizated treatment A were significantly higher than in the fertilization treatments including manure and/or rock dust (B, C, and D), wheres this trend was not observed in the ORG system. Compared to the other treatments, the total concentration of phenolic acids under mineral fertilization (treatment K) was 0.845 mg g^{-1} FW, a value significantly lower respect to all the CON fertilization treatments. Feruloylquinic acid, caffeoylferuloyquinic acid, and methylcaffeoylferuloyltartaric acid isomer were found to be the major phenolic acids present, accounting on average for 32, 35 and 19%, respectively, of the total content in phenolic acids.

As for total phenolic acids, statistical differences in total flavonols were revealed among CON and ORG fertilization treatments. In the CON system, the levels of total flavonols were significantly higher in the unfertilized treatment (A) compare to the three fertilized treatments (B, C, and D), showing significant differences respect to the lowest value (0.381 mg g^{-1} FW) found in plants subjected to mineral fetilization (treatment K). Considering on average all the treatments, quercetin 3-*O*-glucoside was the most abundant flavonol, accounting for 38% of total flavonols.

Excluding the treatment D (manure + rock dust), the total phenolic content calculated by the sum of phenolic acids and flavonols resulted to be significantly always higher in CON fertilization treatments than in ORG ones.

3.3. Total phenol content and total antioxidant capacity

As for phenol levels determined by UHPLC, the values of total phenol content measured by the Folin-Ciocalteu colorimetric method was higher in all the CON fertilization treatments compared to ORG, showing the highest levels in the A-CON treatment (2.25 mg GAE g^{-1} FW) (Table 5). Considering all the treatments together, excluding K, in all of the three years of experiment total phenol content was significantly higher in the leaves of plants from the CON system (Table 7). A trend parallel to that of total phenols was observed for total antioxidant capacity determined by DPPH• and FRAP assays, with peaks found in A/C-CON treatments (Table 5). The differences in antioxidant capacity between CON and ORG management systems were all significant (Table 5).

3.4. Element content

Mineral distribution of macroelements revealed that ORG fertilization treatments determined significant increases in N, Mg, S, Na, and Al in all the treatments compared to the respective CON fertilization treatments, whereas no differences in C, K, Ca, P and Fe were observed (Table 6). Regarding microelements, ORG fertilization treatments had significantly higher levels of Cu and Se, and lower concentrations of Mn, Cd and Mo, whereas no changes were found for Zn, Ni, Cr and Co (Table 6). These trends were generally confirmed, excepting for some exceptions in the unfertilized tretments (A). The ORG fertilization treatments that included compost and/or rock dust application (B/C/D) presented a value of N content comparable to that found in the treatment K with mineral fertilization (Table 6). Considering all the treatments together, excluding K, in all of the three years of experiment total N content was significantly higher in plants from the ORG system (Table 7).

4. Discussion

4.1. Phenolic profiles

The extraction method and the determination by UHPLC coupled with LCMS-IT-TOF here used allowed to detect clearly 12 phenolic compounds (eight phenolic acids and four flavonols), a number comparable to that obtained by other authors (Ribas-Agustí et al., 2011; Abu-Reidah et al., 2013). Considering all the treatments and the controls (K), ferulic acid derivatives were the largest group of phenolyc acids, accounting on average for 56% of total phenolic acid content (Table 4). As found by Ribas-Agustí et al. (2011) and Abu-Reidah et al. (2013), the levels of chicoric acid, important for human health due to its strong antinflammatory and antimicrobic actions, and to its anti-cancer and anti-diabetic properties (Manach et al., 2004; Llorach et al., 2008; Mulabagal et al., 2010; Lee and Scagel, 2013), represents a good fraction of phenolic acids (on average 8% for both the compounds). Among flavonols, the levels of quercetin, a strong anti-tumoral agent (DuPont et al., 2000; Llorach et al., 2008), was comparable to those found by other authors in different varieties of conventionally-cultivated lettuce (approximately 100 μg g^{-1} FW) (Ribas-Agustí et al., 2011; Abu-Reidah et al., 2013) (Table 4).

4.2. Effects of crop management system

Generally, environmental stresses, such as heat shock, chilling or high light intensity, cause a higher synthesis and/or accumulation of phenols in lettuce (Romani et al., 2002; Oh et al., 2009a,b). In other cases, as depicted by Ordidge et al. (2010) for lettuce cultivated under different levels of UV radiation determined by the use of plastic films, the reduction of the stressors do not produce decreases in phenolic content, allowing agronomic advantages without phytochemical penalty. The effects of agronomic practices on phenol content of lettuce are not always statistically significant and often milder than those determined by abiotic stressors (Heimler et al., 2012; Durazzo et al., 2014). For instance, in the work of Durazzo et al. (2014), no differences in some phenolic acids (chlorogenic acid and caffeic acid) were found between organic and conventional lettuce cultivated under greenhouse conditions. In our opinion, the missing link of many researches on phenols in cultivated plants is the study of the effects of agronomic practices on plant's nutritional balance, that in turn can affect phenol biosynthesis and accumulation. For all these reasons, we tried to

Table 4
Phenolic acids and flavonol in leaves of green lettuce grown under conventional (CON) and organic (ORG) management systems. The fertilization treatments are indicated in Table 1. Mean values in the period 2011–2013 ($n = 48$) ± SD followed by different letters are significantly different between rows at $P \leq 0.05$, according to Fisher's LSD test. Legend: 1 = chlorogenic acid; 2 = feruloyl tartaric acid; 3 = feruloylquinic acid; 4 = caffeic acid; 5 = chicoric acid; 6 = caffeoylferuloyquinic acid; 7 = methylcaffeoylferuloyltartaric acid; 8 = methylcaffeoylferuloyltartaric acid isomer; 10 = chicoric acid methyl ester; 9 = quercetin 3-*O*-glucoside; 10 = isorhamnetin 3-*O*-glucuronide; 11 = kaempferide 3-*O*-glucuronide; 12 = quercetin.

		Phenolic acids									Flavonols ($\mu g\,g^{-1}$ FW)					
		1 ($\mu g\,g^{-1}$ FW)	2	3	4	5	6	7	8	Totalphenolic acids ($mg\,g^{-1}$ FW)	9 ($\mu g\,g^{-1}$ FW)	10	11	12	Total flavonols(mg) ($mg\,g^{-1}$ FW)	Total phenols(mg)
A	CONValue	28.01	38.41	564.90	26.71	31.74	314.89	42.34	176.08	1.223	257.88	136.48	181.65	113.79	0.690	1.913
	SD	1.32	2.13	3.16	3.94	1.63	23.67	10.22	6.96		16.53	15.35	13.32	5.55		
	Stat	a	a	a	c	a	a	a	a	a	a	a	a	a	a	a
	ORGValue	19.96	33.41	339.82	40.42	27.83	237.85	34.78	141.27	0.875	177.94	71.28	62.59	39.05	0.351	1.226
	SD	2.73	2.21	15.32	2.47	4.95	24.88	5.63	21.00		12.72	5.26	4.86	8.29		
	Stat	b	b	b	a	b	b	a	b	c	b	b	b	c	d	c
B	CONValue	20.39	24.50	432.16	52.73	31.95	230.04	31.37	130.22	0.953	111.26	66.35	183.01	119.79	0.480	1.434
	SD	3.84	3.94	23.48	8.75	2.19	11.01	5.02	8.66		20.05	3.32	14.97	5.93		
	Stat	b	c	ab	a	a	b	a	b	b	c	b	a	a	c	b
	ORGValue	22.51	36.49	331.40	25.57	30.02	252.46	27.14	143.59	0.869	192.05	73.13	78.99	44.05	0.388	1.257
	SD	1.33	4.45	10.73	5.52	2.62	40.39	5.91	27.23		7.74	5.85	2.25	10.25		
	Stat	b	a	b	c	a	b	a	b	c	b	b	a	c	d	c
C	CONValue	22.38	31.46	507.76	35.44	31.29	262.97	33.42	156.30	1.081	148.64	90.28	180.53	89.15	0.509	1.590
	SD	1.63	1.36	20.65	5.01	3.56	13.44	2.53	19.65		8.25	9.43	11.73	5.46		
	Stat	b	b	a	b	a	b	a	ab	b	bc	ab	a	b	bc	b
	ORGValue	20.35	33.65	332.37	53.85	26.29	241.25	34.74	171.81	0.914	196.87	66.10	63.27	39.78	0.366	1.280
	SD	3.83	4.35	15.93	4.30	7.21	35.97	6.93	9.70		12.60	9.30	3.54	18.10		
	Stat	b	b	b	a	b	b	a	a	c	ab	ab	b	c	d	c
D	CONValue	28.43	32.02	519.36	26.80	32.83	255.50	43.15	140.33	1.078	183.21	107.85	156.91	114.59	0.563	1.641
	SD	7.08	1.13	39.09	9.22	8.20	19.98	9.12	9.03		16.3	3.75	12.19	6.06		
	Stat	a	b	a	c	a	b	a	a	b	b	ab	a	a	b	ab
	ORGValue	20.38	34.22	350.59	25.10	27.48	244.62	36.81	169.83	0.909	233.42	82.38	73.32	26.23	0.474	1.553
	SD	3.99	1.38	13.46	4.35	4.09	30.85	8.43	10.96		6.30	12.43	4.80	10.64		
	Stat	b	ab	b	c	b	b	a	a	c	a	b	b	d	c	b
K	CONValue	19.40	40.62	257.31	34.80	22.45	281.63	37.68	151.11	0.845	97.87	46.79	162.28	74.39	0.381	1.226
	SD	3.34	1.45	35.71	4.31	2.14	22.51	4.13	11.73		7.28	9.08	14.62	8.36		
	Stat	b	a	c	b	a	b	a	ab	c	d	c	a	b	d	c

Table 5
Total phenol content (Folin-Ciocalteu assay) and total antioxidant capacity (DPPH• and FRAP assays) in leaves of green lettuce grown under conventional (CON) and organic (ORG) management systems. The fertilization treatments are indicated in Table 1. Mean values in the period 2011–2013 ($n = 48$) ± SD followed by different letters are significantly at $P \leq 0.05$, according to Fisher's LSD test. GAE: gallic acid equivalents. TE: Trolox equivalents.

	Total phenols (mg GAE g^{-1} FW)					
	CON			ORG		
	Value	SD	Stat	Value	SD	Stat
A	2.25	0.03	a	1.25	0.07	bc
B	2.18	0.02	b	0.61	0.10	d
C	1.76	0.16	b	0.85	0.10	d
D	1.30	0.14	bc	0.79	0.07	d
K	1.14	0.07	c		–	–
	DPPH• (μmol TE g^{-1} FW)					
	CON			ORG		
	Value	SD	Stat	Value	SD	Stat
A	40.26	0.29	a	28.35	0.27	c
B	33.20	0.28	b	19.99	0.24	d
C	40.25	0.30	a	24.43	0.41	d
D	28.35	0.28	c	20.00	0.23	d
K	20.01	0.23	d	–	–	–
	FRAP (μmol TE g^{-1} FW)					
	CON			ORG		
	Value	SD	Stat	Value	SD	Stat
A	28.35	0.28	a	18.27	0.25	c
B	25.31	0.41	b	14.00	0.17	cd
C	26.31	0.38	a	14.01	0.16	cd
D	18.50	0.33	c	10.42	0.30	d
K	8.28	0.22	d	–	–	–

better define the relationhips between soil physico-chemical properties (Table 2), fertilization plans (Table 1) and plant mineral status (Table 6).

Focusing on the levels of the phenols detected by UHPLC, different trends were observed: the levels of phenolic acids and total phenols were significantly affected by the choice of the farming system, being higher in CON than in ORG for all the fertilization treatments, whereas flavonols did not significantly change between the two systems (Table 4). Interestingly, the total phenolic trends observed (Table 4) are similar to those found by Romani et al. (2002) in a comparison between greenhouse and oper-air-grown lettuce, with this latter showing a higher total phenolic content, presumably because more subjected to different kinds of abiotic stresses.

The trends of total phenols determined by the Folin-Ciocalteu colorimetric method (Table 5) appeared to be similar to that of total phenolics obtained by the sum of the single phenolic compounds (phenolic acids + flavonols) (Table 4). Considering the normalization due to dry weight conversion, concentration ranges and leaf amounts analyzed, these values are comparable to those found by other authors that applied similar cultural conditions (on average 1–2 mg GAE g^{-1} FW) (Heimler et al., 2007; Liu et al., 2007; Oh et al., 2009b). The results of DPPH• method for the assay of total antioxidant capacity were parallel to that obtained by FRAP method, with the highest values in CON treatments (Table 5). This suggests that the total radical scavenging activity of lettuce samples was correlated to the phenol content, as demonstrated by other authors (Llorach et al., 2004; Heimler et al., 2007; Llorach et al., 2008; Heimler et al., 2012), and here confirmed by the positive relationship between the two parameters ($R^2 = 0.85$) (Fig. 2a). This does not exclude that other important non-phenolic antioxidants of lettuce could take part in the antioxidant capacity. For example, it is known that some minerals, such as Fe, Cu and Zn can strongly contribute to radical scavenging in plant cells (Abu-Reidah et al., 2013). This notwithstanding, no significant correlation was found between [Fe + Zn + Cu] content and antioxidant capacity ($R^2 = 0.33$) (Fig. 2b).

Organic management ameliorated the mineral status of plants (Tables 6 and 7). Particularly, mineral nitrogen content in plants was likely the main responsible for the higher yields, as shown in Fig. 2c ($R^2 = 0.86$). Conversely, total phenols (values obtained by UHPLC data) were negatively correlated with [Fe + Zn + Cu] content, N content and yield ($R^2 = 0.90$, 0.82 and 0.79, respectively) (Fig. 2d–f, respectively), suggesting that in the plants with a better nutritional status, the synthesis and/or accumulation of phenols was reduced. The correlation between plant mineral content and phenol biosynthesis and accumulation is not surprising. The activity of phenylalanine ammonia lyase (PAL) polyphenol oxidase and some peroxidase isoforms, the key regulatory enzymes for the biosynthesis of plant phenolic compounds, and the transcription of many PAL-encoding genes, is generally increased by adverse environmental conditions, such as low nutrient levels, particularly N and metal microelements (Ruiz et al., 2003; Velicković et al., 2014; Wada et al., 2014). According to Gershenzon (1984), nitrogen stress accompanied by reduced growth and yield (Table 2) could lead, through the deamination of phenylalanine due to PAL catalysis, to an enhanced production of secondary phenolic compounds and to the use of the released NH_3 for other purposes. The same authors pointed out that the rate of protein synthesis slows under condition of N deficiency, and the unused carbohydrate could be diverted to phenolic synthesis. In this study, CON plants showed by reduced yield (Tables 3 and 7), phenolics (Tables 4, 5 and 7) and N (Tables 5 and 7) compared to ORG plants, confirming this hypothesis.

4.3. Effects of mineral fertilization treatment

The significantly low phenols and antioxidant capacity of the treatment K, compared to the other fertilization treatments (A, B,

Table 6
Mineral content in leaves of green lettuce grown under conventional (CON) and organic (ORG) management systems. The fertilization treatments are indicated in Table 1. Mean values in the period 2011–2013 ($n = 48$) ± SD followed by different letters are significantly different between rows at $P \leq 0.05$, according to Fisher's LSD test.

			C (mg g^{-1} DW)	N	K	Ca	P	Fe	Mg	S	Na	Al	Zn (μg g^{-1} DW)	Mn	Cu	Ni	Cr	Cd	Mo	Co	Se	Total (mg g^{-1} DW)
A	CON	Value	395.04	18.41	12.70	15.40	4.58	3.82	3.55	2.55	1.13	0.84	50.40	40.65	7.40	1.81	1.98	1.39	0.65	0.44	0.71	458.13
		SD	9.07	4.35	3.21	2.80	0.22	0.52	0.61	0.30	0.86	0.50	7.23	18.73	1.46	0.60	1.28	0.43	0.30	0.29	0.45	
		Stat	a	d	b	a	a	a	b	b	b	a	b	a	b	a	a	a	b	a	a	b
	ORG	Value	392.86	37.33	9.43	13.14	4.78	3.89	4.41	3.35	3.43	0.91	55.11	20.31	11.24	1.54	1.60	0.80	0.92	0.38	0.79	473.62
		SD	7.85	2.03	2.74	1.51	0.33	0.41	0.74	0.09	1.85	0.16	2.05	0.39	1.57	0.26	0.17	0.11	0.15	0.08	0.42	
		Stat	a	b	b	a	a	a	a	a	a	a	b	b	a	a	a	b	b	a	a	a
B	CON	Value	381.00	25.67	37.00	13.94	4.96	3.58	3.20	2.56	1.43	0.59	44.34	35.76	7.21	1.64	1.56	1.49	1.67	0.34	0.18	474.02
		SD	10.49	4.94	5.45	2.06	0.43	0.69	0.22	0.46	0.11	0.20	5.27	3.94	1.44	0.39	0.64	0.08	0.38	0.07	0.08	
		Stat	a	c	a	a	a	a	v	b	b	b	b	a	b	a	a	a	a	a	b	b
	ORG	Value	382.85	44.35	29.47	12.64	4.72	3.82	3.99	3.13	2.33	0.84	50.18	20.48	10.23	1.47	1.54	0.90	0.89	0.37	0.56	468.22
		SD	3.46	1.81	5.10	0.78	0.54	0.69	0.59	0.14	0.75	0.67	5.19	8.79	1.18	0.64	1.18	0.08	0.08	0.30	0.31	
		Stat	a	a	a	a	a	a	a	a	a	a	b	b	a	a	a	b	b	a	a	a
C	CON	Value	384.72	24.89	32.12	12.81	4.81	3.39	2.84	2.36	1.26	0.43	38.66	32.65	6.81	1.21	0.83	1.34	1.34	0.23	0.20	469.72
		SD	8.20	4.15	3.88	1.06	0.48	0.80	0.22	0.46	0.13	0.25	7.67	2.47	0.81	0.17	0.38	0.14	0.53	0.08	0.06	
		Stat	a	c	a	a	a	a	b	b	b	c	b	a	b	a	a	a	a	a	b	b
	ORG	Value	378.82	43.52	30.97	14.03	4.67	3.53	4.60	3.21	2.86	0.59	51.43	19.13	10.64	1.39	1.24	0.95	0.97	0.32	0.50	486.87
		SD	3.26	3.12	2.11	1.19	0.23	0.86	0.35	0.09	0.76	0.18	3.14	3.09	1.43	0.16	0.14	0.05	0.12	0.04	0.19	
		Stat	a	a	a	a	a	a	a	a	a	b	b	b	a	a	a	b	b	a	a	a
D	CON	Value	390.73	21.41	25.97	16.50	4.61	3.52	3.13	2.10	1.12	0.56	53.42	37.42	7.35	1.84	1.73	1.74	1.40	0.46	0.34	469.76
		SD	5.74	3.23	6.36	0.95	0.32	0.48	0.12	0.32	0.06	0.20	17.58	2.26	2.11	0.68	0.98	0.43	0.45	0.27	0.15	b
		Stat	a	c	a	a	a	a	v	b	b	b	b	a	b	a	a	a	a	a	b	
	ORG	Value	391.99	40.85	17.64	13.36	4.50	3.96	4.42	3.07	3.08	0.99	48.31	23.92	9.47	1.82	2.72	0.81	1.16	0.45	0.67	483.95
		SD	4.15	2.47	4.12	1.63	0.28	0.43	0.68	0.20	0.63	0.47	3.01	6.68	0.57	0.42	1.17	0.07	0.18	0.14	0.31	
		Stat	a	a	ab	a	a	a	a	a	a	a	b	b	a	a	a	b	b	a	a	a
K	CON	Value	407.07	39.01	8.01	13.24	4.85	3.64	4.37	3.68	2.02	0.63	65.65	46.35	11.17	2.61	2.08	1.74	0.99	0.45	0.41	486.66
		SD	2.97	2.74	2.57	1.30	0.27	0.31	0.42	0.44	0.56	0.27	3.86	3.92	0.62	0.46	0.83	0.22	0.68	0.16	0.20	
		Stat	a	a	b	a	a	a	a	a	ab	b	a	a	a	a	a	a	ab	a	ab	a

Table 7
Yield, total phenol content (Folin-Ciocalteu assay), and mineral nitrogen content in leaves of green lettuce grown under conventional (CON) and organic (ORG) management systems (period 2011–2013). For both CON and ORG, the values are means ($n = 400$ for yield and $n = 64$ for total phenols and N) ± SD of the fertilization treatments A, B, C and D (indicated in Table 1). Values followed by different letters are significantly at $P \leq 0.05$, according to Fisher's LSD test. GAE: gallic acid equivalents.

	Yield (g DW head^{-1})					
	CON			ORG		
	Value	SD	Stat	Value	SD	Stat
2011	7.85	2.67	b	12.15	3.12	a
2012	8.10	3.12	b	13.11	4.89	a
2013	8.21	3.98	b	15.53	3.20	a
	Total phenols (mg GAE g^{-1} FW)					
	CON			ORG		
	Value	SD	Stat	Value	SD	Stat
2011	1.99	0.43	a	0.96	0.27	c
2012	1.76	0.14	ab	0.92	0.19	c
2013	1.87	0.62	a	0.76	0.12	cd
	N (mg g^{-1} DW)					
	CON			ORG		
	Value	SD	Stat	Value	SD	Stat
2011	19.29	3.35	c	39.28	3.16	ab
2012	24.30	3.87	c	40.01	2.09	ab
2013	24.20	2.52	c	45.23	3.22	a

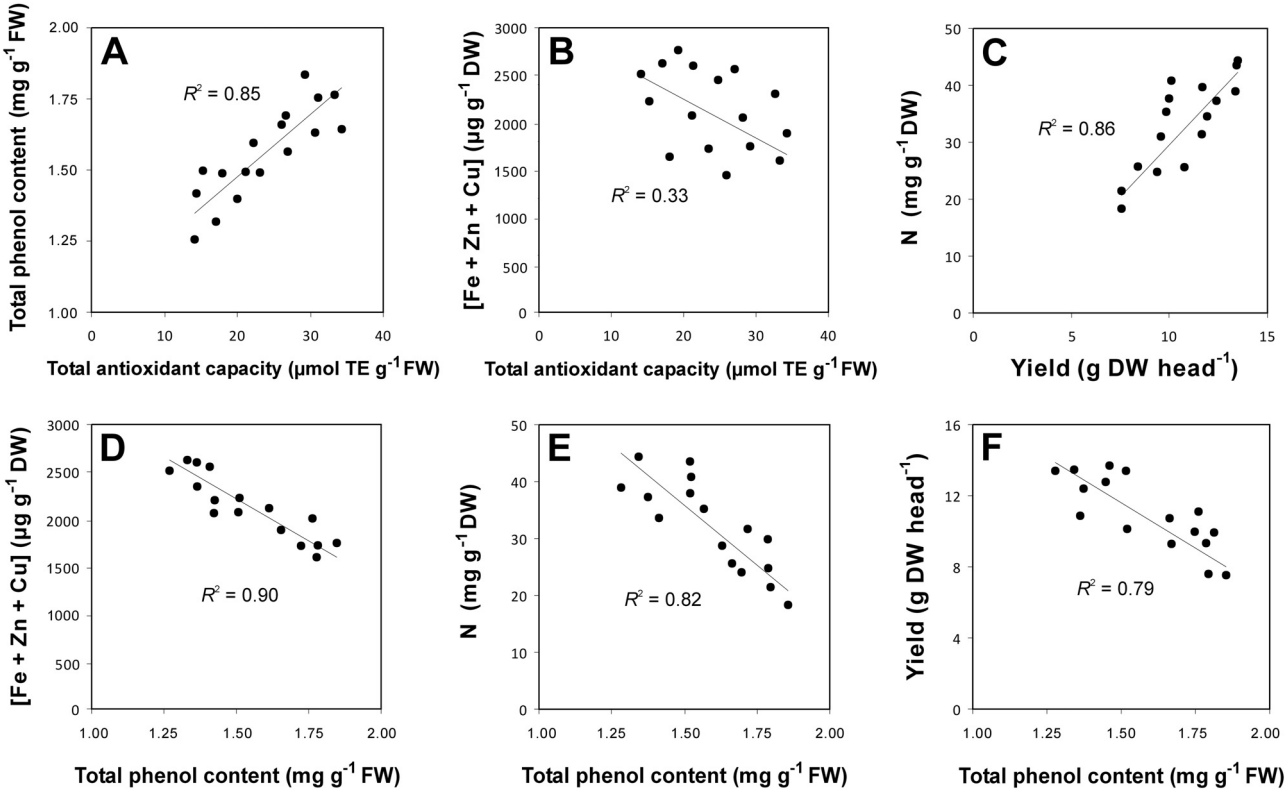

Fig. 2. Regression analysis ($n = 48$; only the means are shown) between (A) total antioxidant capacity and total phenol content. (B) total antioxidant capacity and [Fe + Zn + Cu] content. (C) yield and nitrogen content. (D) total phenol content and [Fe + Zn + Cu] content. (E) total phenol content and nitrogen content. and (F) total phenol content and yield. The values of R^2 are indicated in every subfigure. Total phenol contents were obtained by UHPLC data. TE: Trolox equivalents.

C, and D) of the CON system (Tables 4 and 5) seems to confirm that a strong mineral fertilization (Table 1) was able to increase N and total mineral levels (Table 6) but not that of plant phenols (Tables 4 and 5). In support of this hypothesis, the unfertilized treatment (A) of the CON system maintained a significantly high total phenol content and antioxidant potential compared to the other treatments, including K (Tables 4–6). On the other hand, the most fertilized treatment D-CON (manure + rock dust) had an opposite behavior (Tables 4–6). The situation in the four ORG treatments regarding phenols and antioxidant capacity seems to be more homogeneous, with less marked differences, compared to CON (Tables 4 and 6), likely due to the high levels soil organic matter and N contents at the beginning of the experiment (Table 2).

Almost all the inorganic minerals are naturally present in soil particles and/or soil solution but their levels can be strongly affected by the fertilization treatment adopted in the field (Kelly

and Bateman, 2010; Sofo et al., 2012). This was confirmed by the high plant N content in the treatments with manure and rock dust alone or in combination (B, C, D), compared to the unfertilized one (A) in both the systems (Table 6).

5. Conclusions

From the overall analysis of the results, the higher content of phenolics observed in the conventional treatments could be associated to the presence of more stressful conditions, in terms of plant and/or soil mineral deficits. On the other hand, the adoption of an organic management determined higher yields and a better plant mineral balance. These soil parameters, together with the fluctuations of nutrients in plants, crop yield, and indirect environmental costs should be taken into consideration before scheduling lettuce farming systems.

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