Research Article

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Sulfur bentonite-organic-based fertilizers as tool for improving bio-compounds with antioxidant activities in red onion

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Abstract

BACKGROUND: Red onion is popular in cuisines worldwide and is valued for its potential medicinal properties. Red onion is an important source of several phytonutrients such as flavonoids, thiosulfinates and other sulfur compounds, recognized as important elements of the diet. Nowadays, there is the need of producing food enriched in health benefit compounds. In this study, pads of sulfur bentonite (SB) with the addition of orange residue (OR) or olive pomace (OP) were used to improve the quality of red onion. The experiment was conducted for 3 months in the field to evaluate the phytochemicals of differently amended red onion.

RESULTS: Treated plants were better in quality than controls. Antioxidant activity, detected as DPPH, ORAC and ABTS, was highest in plants grown in the presence of SB enriched with agricultural wastes, particularly SB-OR. Polyphenols increased in all treated plants. The volatile fraction was clearly dominated by sulfur compounds that are strictly related to the concentration of the aroma precursors *S*-alkenyl cysteine sulfoxides. The greater amount of thiosulfinates in treated compared with untreated onion evidenced that SB pelletized with agricultural wastes can represent a new formulation of organic fertilizer able to improve the beneficial properties of onion. The results highlighted that the best red onion quality was obtained using SB-OR pads.

CONCLUSION: The use of SB bound with agricultural wastes represents a novel strategy to increase bio-compounds with beneficial effects on human health, to enhance the medical and economic values of sulfur-loving crops, with important consequences on the bio and green economy.

Keywords: agricultural wastes; bio-compounds; fertilizer; glucosinolate; onion; sulfur-bentonite

INTRODUCTION

Sulfur is a macronutrient important for optimal plant growth and for promoting resistance to soil-borne fungal and bacterial diseases.[1] Recently, sulfur has become a requested plant nutrient because of its amount in soil being reduced by: (i) enhanced use of monoammonium and diammonium phosphate as fertilizers that contain less sulfur than superphosphate; (ii) decreased sulfur addition to the soil from atmospheric sources as industrial pollution has been reduced; and (iii) the introduction of higher-yielding crop varieties that remove more sulfur from soils with harvesting. Lastly, sulfur is the nutrient allowed to be used in organic plant production worldwide, therefore recovered sulfur with agricultural wastes can offer improved agronomic benefits with a better end-of-life option for final waste disposal. Elemental sulfur is insoluble in water; for this reason, new products such as sulfur bentonite granules are becoming in demand. They are prepared by using liquid sulfur absorbed by bentonite clay and through solidification and drying; the final product is a granule similar to a lentil.[2] Sulfur bentonite granules are able to increase their volume from three- to fivefold when in contact with soil moisture, breaking into micron-size fragments that slowly solubilize, and sulfur is transformed by soil microorganisms into sulfate, the form taken up by plants. Previous works[3,4] investigated the possibility of using crude orange and olive wastes, produced in great quantities in Mediterranean countries, to make fertilizers. These agricultural wastes, rich in nutrients but poor in sulfur, if used in combination with elemental sulfur, were able to produce fertilizers with a balanced level of nutrients and with organic components useful for maintaining soil biodiversity. In the present investigation, pads of sulfur bentonite with the addition of orange residue and olive pomace were prepared and tested on the nutritive properties of red onion. *Allium cepa* L. (the common onion) is one of the oldest plants cultivated around the world and is consumed as a vegetable and spice. It is also appreciated as a medicinal plant in traditional medicine for its high content of phytochemicals, including polyphenols, flavonoids and sulfur-based compounds. Regular onion bulb intake is reported to have profound radical-scavenging activity, and onion extracts have been recognized to have several beneficial effects on health, such as preventing tumors and cancers,[5]

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cardiovascular diseases,[6] hypertension,[7] hyperglycemia, hyperlipidemia and atherosclerosis.[8–10] The majority of beneficial effects of onion on health are mainly ascribed to the amount and types of bio-compounds present in the bulb,[11] which can vary according to onion variety and cultivation conditions.[12] Among the varieties, it was well demonstrated that *A. cepa* L. var. *tropeana* (red onion) contains more phytochemicals than white onion.[13] Regarding the cultivation conditions, numerous studies demonstrated that onion is a sulfur-loving crop and that sulfur increased bulb yield and improved its quality and flavors.[14–16] Other works demonstrated that onion growth increased by using organic fertilizers with respect to mineral fertilizer. Abolmaaty and Fawaz[17] reported that the application of vermicompost helped in obtaining maximum onion height. Yassen and Khalid[18] showed that organic fertilizer treatments improved not only onion growth but also its essential oil with respect to mineral fertilizer (NPK). Bua *et al.*[19] demonstrated that organic amendment had beneficial effects on onion yield and that there was a different response of onion to different soil organic amendments. Abou-El-Hassan *et al.*[20] showed that compost and vermicompost produced onion with higher bulb quality at harvesting and during storage. On the basis of the above results, and considering that earlier studies of Ullah *et al.*[21] and de Souza *et al.*[22] indicated that sulfur fertilization during cultivation of onion is important for the formation of bioactive compounds, our starting hypothesis was that the use of sulfur-organic-based fertilizers could be able to increase in red onion the production of bioactive organosulfur compounds and antioxidants. Our specific interests were: (i) to produce very low-emission sulfur-organic-based fertilizers by using sulfur residues and organic wastes to reduce the use of chemicals, making the food production chain safe; and (ii) to increase in a sustainable way the quality of red onion, enhancing the synthesis of health-promoting organosulfur compounds.

MATERIALS AND METHODS

Chemicals

2,2-Diphenyl-1-picrylhydrazyl (DPPH), sodium hydroxide (NaOH), 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS$^{\bullet+}$), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH) were from Sigma Chemical Co. (St Louis, MO, USA). Acetonitrile, methanol and formic acid were of high-performance liquid chromatography (HPLC) grade and from Merck (Darmstadt, Germany). All available standard reference compounds were from Extrasynthèse (Genay, France). All other reagents and chemicals used in this study were of analytical grade and from Sigma-Aldrich GmbH (Steinheim, Germany).

Raw materials: olive pomace and orange waste chemical characterization

pH was measured in a suspension of distilled water (dry material/water 1:2.5 w/v) using a pH meter. Electrical conductivity (EC) was measured in a suspension of distilled water (dry material/water 1:2.5 w/v) using a conductimeter. Organic C was detected and converted to organic matter by multiplying the percentage of C by 1.72.[23] Total N (Kjeldahl method) was assessed as in Bremner and Mulvaney.[24] Phenols were extracted with distilled water following the procedure of Kaminsky and Müller[25,26] Total water-soluble phenols (WSP, including monomeric phenols and polyphenols) were determined with Folin–Ciocalteu reagent using tannic acid as standard. The concentration of WSP was expressed as mg tannic acid equivalent (TAE) kg^{-1} dry weight (DW).[27] Ions were extracted with distilled water (1:10 w/v) and analyzed using a Dionex IC-1100 (Thermo Fisher, Waltham, MA, USA).[28] Chemical characteristics are reported in Tables 1 and 2. Orange residue and olive pomace contained no heavy metals and pathogens.

Fertilizer preparation

The manufacturing process to obtain sulfur pads of 0.75 mm diameter was performed by Steel Belt Systems s.r.l. (Varese, Italy) and consisted of three phases. (1) Elemental S was pelletized with bentonite clay (as support and carrier). (2) A part of elemental S pelletized with bentonite clay was added with two concentrations of orange waste or olive pomace. Elemental S is in percentage the main constituent of the pads. The two different concentrations of agricultural wastes will be indicated as low and high, because the production process is an industrial secret. The mixtures prepared with liquid S, bentonite and/or agricultural wastes were introduced into a special patented rotary pastillator, which deposits the liquid pads on a heat exchanger in continuous steel tape for their solidification. (3) The pads were lastly sent to the conveyors/collectors and to machines for weighing and packaging.

Plant material and experimental conditions

The experiment was conducted in triplicate and was replicated for three consecutive years for 3 months in the field in alkaline

Table 1. pH, electrical conductivity (EC, mS cm^{-1}), water content (WC, g kg^{-1}), organic carbon ($^{g\ Kg\text{-}1\ DW}$), total nitrogen ($^{g\ Kg\text{-}1\ DW}$), carbon/nitrogen (C/N) ratio and water-soluble phenols (WSP, mg TAE kg^{-1} DW) of olive pomace (OP) and orange residue (OR)

Chemical property	OP	OR
pH (H_2O)	5.04 ± 0.1a	5.14 ± 0.2a
EC	12.03 ± 1.1a	10.09 ± 0.9b
WC	867.0 ± 32a	836.0 ± 29a
OC	576.2 ± 19a	456.2 ± 25b
TN	20.4 ± 6a	12.4 ± 3b
C/N	28.24 ± 1.9b	36.8 ± 1.7a
WSP	1.84 ± 0.4a	0.53 ± 0.2b

Data are mean of three replicates ± standard error. Means in the same row followed by different letters are significantly different (Tukey's test at $P < 0.05$).

Table 2. Ion concentrations (g kg^{-1} DW) detected in olive pomace (OP) and orange residue (OR)

Ion	OP	OR
Na^+	1.85 ± 0.5a	0.97 ± 0.2b
NH_4^+	0.26 ± 0.03a	0.33 ± 0.04a
K^+	39.22 ± 2.3b	49.22 ± 2.6a
Mg^{2+}	2.23 ± 0.4b	4.23 ± 0.7a
Ca^{2+}	2.53 ± 0.7b	9.33 ± 1.0a
Cl^-	3.83 ± 0.5a	2.44 ± 0.6b
PO_4^{2-}	2.06 ± 0.4a	1.09 ± 0.3b
SO_4^{2-}	ND	ND

Data are mean of three replicates ± standard error. ND, not detected. Means in the same row followed by different letters are significantly different (Tukey's test at $P < 0.05$).

sandy-loam soil with a pH of 8.5, 110 g kg^{-1} $CaCO_3$, 53.3 g kg^{-1} organic matter and 0.334 g kg^{-1} SO_4^{2-}. The soil was divided into parcels 10 m square. In each parcel, 30 uniform seedlings of red onion/m square were transplanted. Pads of sulfur bentonite (SB) or SB with two percentages (conventionally called high and low) of olive pomace (OP) or orange residue (OR) were used at a concentration of 16 g m^{-2} (corresponding to 476 kg S ha^{-1}), the dose generally used to lower the pH and to replenish S.[29] Non-amended plants were used as control. Elemental sulfur has not be used as internal control, because sulfur is insoluble in water but soluble only in nonpolar organic solvents. OP and OR were not used as such because they are considered by Italian legislation as potentially 'ecotoxic', with immediate or delayed risks for one or more sectors of the environment.

During the experiment, all plants were regularly watered to maintain 70% of field capacity. At harvest time (3 months), onions were collected and stored at −20 °C for chemical and biological analysis. SB and SB with OP or OR produced onions with greater bulb size with respect to the control (data not shown).

Extraction and determination of total anthocyanins

The assessment of total anthocyanin content was carried out by the pH differential method according to the Official Methods of Analysis of AOAC International as previously described by Lee *et al.*[30] Absorbance was measured at 510 and 700 nm in buffers at pH 1.0 and 4.5. Values were expressed as g cyanidin 3-glucoside equivalent kg^{-1} fresh weight (FW) using a molar extinction coefficient of 26 900.

Ethanolic extracts

Small pieces of frozen onion samples (500 mg) were weighed and extracted (three times) at room temperature under continuous stirring for 1.5 h with ethanol (15 mL). The samples were then immediately centrifuged at 2365 × *g* for 15 min and the supernatants were filtered, evaporated to dryness and re-suspended in a final volume of 3 mL with ethanol.

Determination of total phenolic compounds and total flavonoids

The Folin–Ciocalteu assay[31] with some modification was used for evaluating total phenol content. Ethanol extracts (0.04 mL) were added to 1.6 mL of water and 0.1 mL of Folin–Ciocalteu reagent and incubated at 25 °C for 10 min. Then 0.6 mL of 200 g L^{-1} Na_2CO_3 solution was added to each sample and left at 40 °C for 20 min in a water bath, with intermittent shaking. The absorbance of the samples was recorded at 760 nm. A calibration curve was constructed with gallic acid and results were expressed as g gallic acid equivalent kg^{-1} DW.

Total flavonoid content in extracts was measured according to the spectrophotometric method of Djeridane *et al.*[32] Briefly, 1 mL of diluted extract was mixed with 1 mL of 20 g L^{-1} $AlCl_3$ methanolic solution. After incubation at room temperature for 15 min, the absorbance was measured at 430 nm. Flavonoid content was calculated from a calibration curve of rutin and expressed as g rutin equivalent kg^{-1} DW.

Determination of antioxidant activities

The antioxidant activity against DPPH radical was determined according to Papalia *et al.*[33] The DPPH$^{\bullet}$ concentration in the cuvette was chosen to give absorbance values of ∼1.0. The reaction mixtures were composed of 10 µL of each extract, 700 µL of DPPH$^{\bullet}$ and ethanol up to a final volume of 1 mL. A blank without ethanol extract was prepared for each sample. The change in absorbance of the violet solution was recorded at 517 nm after 30 min of incubation at 37 °C. The inhibition *I* (%) of radical-scavenging activity was calculated as $I\ (\%) = [(A_0 - A_S)/A_0] \times 100$, where A_0 is the absorbance of the control and A_S is the absorbance of the sample after 30 min of incubation. Results were expressed as Trolox equivalent (TE).

The ABTS assay was performed according to Re *et al.*[34] with some changes. Solutions of 7 mmol L^{-1} ABTS$^{\bullet+}$ (final concentration) and 2.45 mmol L^{-1} ammonium persulfate (final concentration) in phosphate-buffered saline (PBS) were mixed and kept in the dark at room temperature for 12–16 h. Before use, the absorbance of the ABTS$^{\bullet+}$ solution was fixed at 0.70 ± 0.02 at 734 nm. Aliquots of ethanol extract (25, 50 and 100 µL) were added to 0.5 mL of ABTS$^{\bullet+}$ solution and brought to a final volume of 600 µL with PBS. After 6 min of incubation in the dark at room temperature, the absorbance of the samples was recorded at 734 nm using a UV–visible spectrophotometer. The inhibition *I* (%) of radical-scavenging activity was calculated as $I\ (\%) = [(A_0 - A_S)/A_0] \times 100$, where A_0 is the absorbance of the control and A_S is the absorbance of the sample after 4 min of incubation. Results were expressed as µmol L^{-1} TE using a Trolox (1–50 µmol L^{-1}) calibration curve.

The oxygen radical absorbance capacity (ORAC) assay was performed according to Dévalos *et al.*[35] with some modification. A 20 µL aliquot of extract was added to 120 µL of fresh fluorescein solution (117 nmol L^{-1}). After a pre-incubation time of 15 min at 37 °C, 60 µL of freshly prepared AAPH solution (40 mmol L^{-1}) was added. Fluorescence was recorded every 30 s for 90 min (λ_{ex} 485 nm, λ_{em} 520 nm). A blank using 20 µL of methanol instead of the sample was also analyzed. ORAC values were expressed as µmol TE mg^{-1} FW using a Trolox (10–100 µmol L^{-1}) calibration curve.

HPLC and gas chromatography/mass spectrometry (GC/MS) analysis of volatile organic compounds

For the extraction procedure, frozen onion samples (1 g) cut into small pieces were incubated overnight in absolute methanol at 4 °C. Then the methanol was separated from the pieces of onion and collected in a balloon. The small pieces were further homogenized with absolute methanol (10 mL) in a mortar and left for 30 min under magnetic stirring at room temperature (25 °C). Then the samples were centrifuged and each supernatant was mixed with the other methanol. The precipitates were re-suspended in methanol (10 mL) and the above operations were repeated twice. The methanolic phases were combined, reduced to a volume of 10 mL in a rotary evaporator and stored at −18 °C until use. Methanolic extracts (1 mL) were diluted with dimethylformamide (1 mL) and filtered through an Iso-Disk P-34, 3 mm diameter poly(tetrafluoroethylene) (PTFE) membrane, 0.45 µm pore size, supplied by Supelco. Diode array detection (DAD)-HPLC (Shimadzu, Kyoto, Japan) separation of onion flavonoids was performed according to the method described by Papalia *et al.*[33] The column was a Discovery C18 (250 mm × 4.6 mm i.d., 5 mm) supplied by Supelco and equipped with a 20 mm × 4 mm guard column. The Discovery C18 guard column was placed in a column oven set at 30 °C. The injection loop was 20 µL and the flow rate was 1 mL min^{-1}. The mobile phase consisted of a linear gradient of acetonitrile

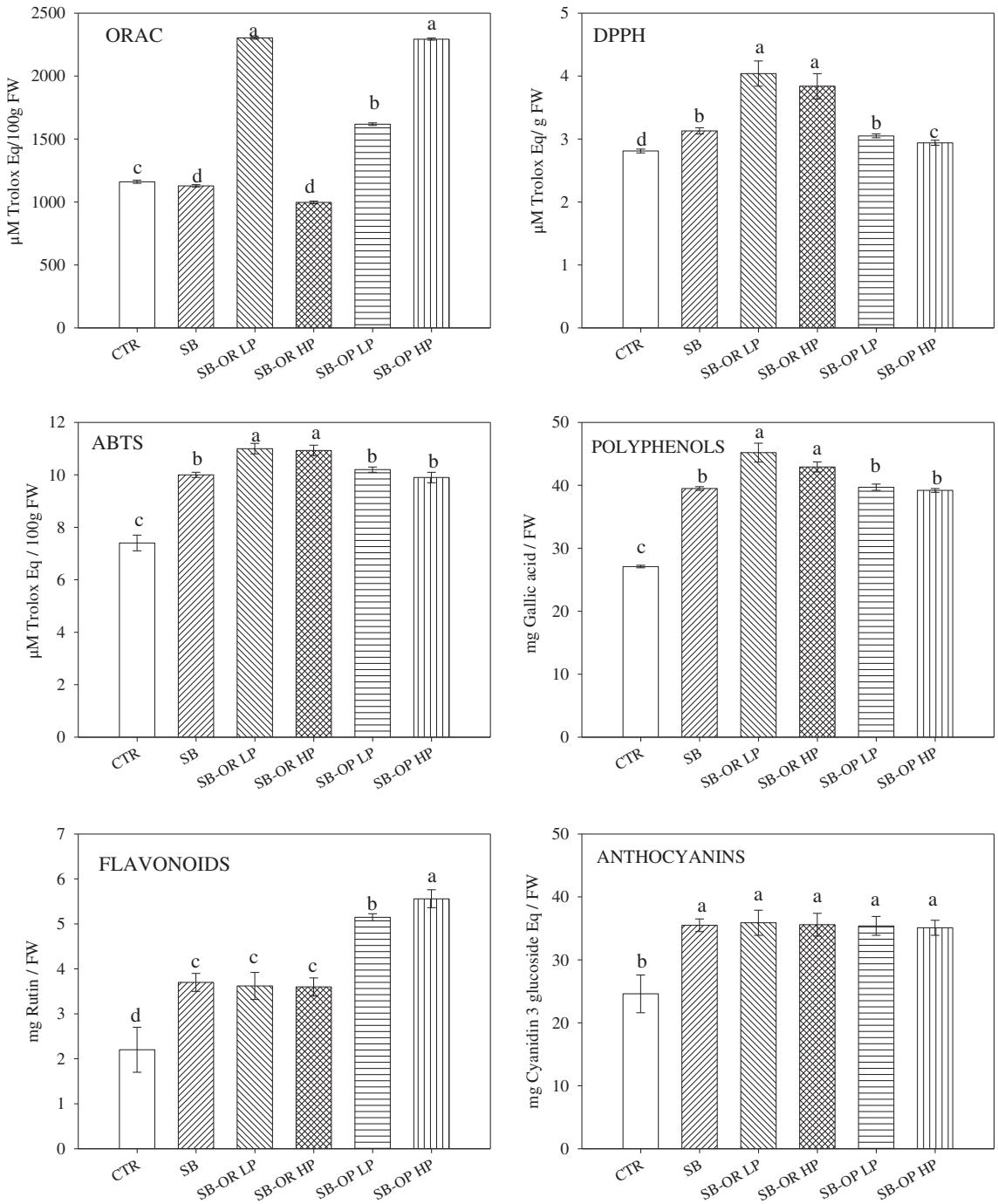

Figure 1. Antioxidant activities (ORAC, DPPH and ABTS) and polyphenol, flavonoid and anthocyanin amounts in red onion bulbs grown for 3 months in soils differently treated: control (CTR), sulfur bentonite (SB), sulfur bentonite-low percentage orange residue (SB-OR LP), sulfur bentonite-high percentage orange residue (SB-OR HP), sulfur bentonite-low percentage olive pomace (SB-OP LP), sulfur bentonite-high percentage olive pomace (SB-OP HP). Data are mean of three replicates ± standard error. Means followed by different letters are significantly different (Tukey's test at $P < 0.05$).

in water as follows: 5–20% (0–15 min); 20–30% (15–20 min); 30–50% (20–30 min); 50–100% (30–35 min); 100% (35–40 min); 100–5% (40–50 min); 5% (50–55 min). UV spectra were recorded between 200 and 450 nm and simultaneous DAD was performed at 350 nm.

Anthocyanins were extracted from frozen onion tissues (0.5 g) homogenized in a mortar with 10 mL of methanol containing 1 mL L^{-1} HCl at room temperature for 2 h. The extracts were filtered through an Iso-Disk P-34, 3 mm diameter PTFE membrane of 0.45 µm pore size (Supelco) and utilized for HPLC analysis. HPLC separation was carried out using a Spherisorb S5 ODS2, Merck KGaA, Darmstadt, Germany (250 mm × 4.6 mm i.d., 5 µm) as described by Fossen *et al.*[36] with some modification. The column was equilibrated in 80% solvent A (100 mL L^{-1} formic acid) and 20% solvent B (methanol/water/formic acid 50:40:10 v/v/v). At 4 min after injection, a linear gradient of 22 min up to 80% solvent B allowed the elution (monitored at 520 nm) of all anthocyanin compounds. Quantification of

Table 3. Two-way ANOVA results for effects of SB pads combined with different concentrations (Conc.) of two raw materials (RM) on antioxidant activities (ORAC, DPPH and ABTS) and on content of polyphenols, flavonoids and anthocyanins in red onion bulbs after 3 months of treatment

Item	ORAC	DPPH	ABTS	Polyphenols	Flavonoids	Anthocyanins
R^2	1.000	0.997	0.974	0.965	0.958	0.023
F *ratios*						
Conc.	8425***	172***	31***	23***	30***	NS
RM	1548***	1144***	108***	75***	143***	NS
Conc. × RM	12256***	288***	27***	21***	37***	NS

Significance: ***$P < 0.001$; NS, not significant.

Table 4. Phenolic acids and flavonols (mg per 100 g FW) found in red onion bulbs grown for 3 months in soils differently treated: control (CTR), sulfur bentonite (SB), sulfur bentonite-low percentage orange residue (SB-OR LP), sulfur bentonite-high percentage orange residue (SB-OR HP), sulfur bentonite-low percentage olive pomace (SB-OP LP), sulfur bentonite-high percentage olive pomace (SB-OP HP)

	Phenolic acids				Flavonols	
ID	Gallic	Caffeic	*p*-Coumaric	Chlorogenic	Kaempferol	Quercetin
CTR	ND	0.19 ± 0.01c	0.011 ± 0.01b	2.22 ± 0.09c	20.3 ± 0.5d	17.1 ± 0.2c
SB	0.11 ± 0.01b	0.21 ± 0.01b	0.033 ± 0.01a	2.61 ± 0.10b	25.2 ± 1.2b	19.5 ± 0.2a
SB-OR LP	0.17 ± 0.01a	0.26 ± 0.02a	ND	3.98 ± 0.30a	31.9 ± 2.1a	19.5 ± 0.4a
SB-OR HP	0.16 ± 0.01a	0.21 ± 0.01b	ND	2.20 ± 0.03c	26.1 ± 1.5b	19.5 ± 0.3a
SB-OP LP	0.17 ± 0.01a	0.20 ± 0.01b	ND	2.16 ± 0.05c	22.3 ± 0.5c	18.4 ± 0.2b
SB-OP HP	0.17 ± 0.01a	0.21 ± 0.01b	ND	2.03 ± 0.01d	22.1 ± 0.8c	18.3 ± 0.3b

Data are mean of three replicates ± standard error. ND, not detected. Means in the same column with the same letter are not significantly different (Tukey's test, $P \leq 0.05$).

single compounds was achieved by a calibration curve obtained using pure cyanidin or delphinidin (Extrasynthèse) as standard.

To detect *S*-methyl-L-cysteine sulfoxide (SMCSO), small pieces of frozen red onion (250 mg) were homogenized with 5 mL of distilled water and filtered through filter paper. SMCSO was quantified by HPLC after derivatization with *o*-phthalaldehyde according to the method of Ziegler and Sticher.[37] A 20 µL aliquot of the onion extract was mixed with 180 µL of 2.8 mg mL^{-1} *o*-phthalaldehyde solution in 50 mmol L^{-1} sodium borate (pH 9.5) containing 100 mL L^{-1} methanol. A 20 µL aliquot of the mixture was injected into the HPLC system. The HPLC column was a Spherisorb ODS2 (250 mm × 4.6 mm i.d., 5 µm). Isocratic elution was done with a mixture of 50 mmol L^{-1} sodium phosphate (pH 7.15) and methanol (32:68 v/v). The flow rate was 1 mL min^{-1} and the column was maintained at room temperature. The absorbance at 339 nm was monitored.[38]

Red onion volatile organic compound (VOC) analysis was performed using a Thermo Fisher gas chromatograph (TRACE 1310) equipped with a single-quadrupole mass spectrometer (ISQ LT). The capillary column was a TG-5MS (30 m × 0.25 mm, 0.25 µm). The carrier gas was helium at a flow rate of 1 mL min^{-1}. Injector and source temperatures were set at 250 and 260 °C respectively. A 1 g sample of fresh plant material was incubated for 1 min at 40 °C, and 1 µL of the head space was injected in split mode with a split ratio of 50. The following temperature program was

Table 5. Glycosylated flavonols (mg per 100 g FW) of red onion bulbs grown for 3 months in soils differently treated: control (CTR), sulfur bentonite (SB), sulfur bentonite-low percentage orange residue (SB-OR LP), sulfur bentonite-high percentage orange residue (SB-OR HP), sulfur bentonite-low percentage olive pomace (SB-OP LP), sulfur bentonite-high percentage olive pomace (SB-OP HP)

ID	Quercetin 3,4′-*O*-diglucoside	Isorhamnetin 3,4-*O*-glucoside	Quercetin 3′-*O*-glucoside	Quercetin 4′-*O*-glucoside	Isorhamnetin 4′-*O*-glucoside
CTR	7.93 ± 3.2a	2.00 ± 0.9a	1.84 ± 0.5a	20.46 ± 2.6a	5.20 ± 1.00a
SB	7.39 ± 0.2b	0.40 ± 0.2b	ND	1.11 ± 0.03c	0.24 ± 0.01e
SB-OR LP	ND	ND	ND	1.00 ± 0.02d	0.48 ± 0.02d
SB-OR HP	7.15 ± 1.2bc	0.45 ± 0.3b	0.20 ± 0.05c	1.85 ± 0.20b	0.75 ± 0.03b
SB-OP LP	6.76 ± 0.2c	ND	ND	0.45 ± 0.02e	0.21 ± 0.01f
SB-OP HP	6.80 ± 0.1c	0.43 ± 0.2b	0.22 ± 0.06b	1.86 ± 0.3b	0.69 ± 0.01c

Data are mean of three replicates ± standard error. ND, not detected. Means in the same column with the same letter are not significantly different (Tukey's test, $P \leq 0.05$).

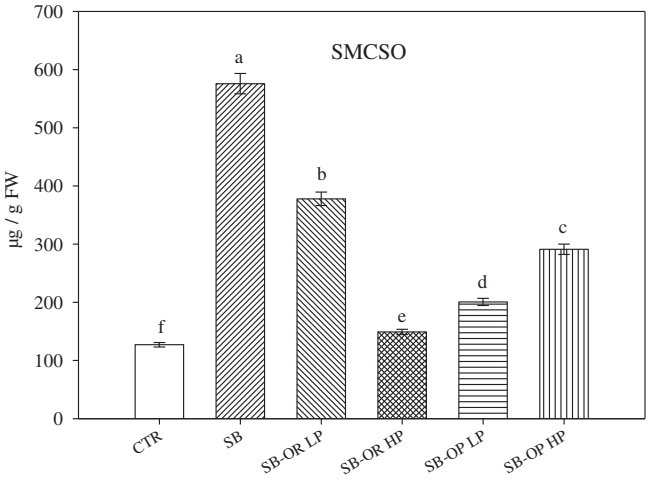

Figure 2. *S*-Methyl-L-cysteine sulfoxide (µg g^{-1} FW) in red onion bulbs grown for 3 months in soils differently treated: control (CTR), sulfur bentonite (SB), sulfur bentonite-low percentage orange residue (SB-OR LP), sulfur bentonite-high percentage orange residue (SB-OR HP), sulfur bentonite-low percentage olive pomace (SB-OP LP), sulfur bentonite-high percentage olive pomace (SB-OP HP). Data are mean of three replicates ± standard error. Means followed by different letters are significantly different (Tukey's test at $P < 0.05$).

used: 45 °C for 7 min, first ramp 10 °C min^{-1} to 80 °C, second ramp 20 °C min^{-1} to 200 °C, then 200 °C for 3 min. Mass spectra were recorded in electronic impact (EI) mode at 70 eV, scanning in the range *m/z* 45–500. Compound identification was carried out by comparing relative retention times and mass spectra of detected compounds with those of standard libraries (NIST 2005, Wiley 7.0, etc.).

Statistical analysis

Statistical analyses were performed using one-way analysis of variance (ANOVA) and mean comparisons were made using Tukey's test ($P < 0.05$). All data were analyzed using SYSTAT 13.0 for Windows (SPSS Inc.). Two-way ANOVA was used to test the effects of the factors (treatments and concentrations) on the antioxidant activity and bio-compounds of red onion.

RESULTS AND DISCUSSION

The analysis of the chemical properties of the raw materials (RM) used to produce the sulfur bentonite (SB)-based pads (Table 1) showed that olive pomace (OP) contained a significantly greater amount of organic carbon (OC), total nitrogen (TN) and water-soluble phenols (WSP) than orange residue (OR); no significant differences in pH and water content were detected between the two RM. The greatest C/N ratio was found in OR (Table 1). Regarding ion content, OP had more sodium, chloride and phosphate than OR, while OR had the greatest amount of calcium, magnesium and potassium (Table 2). The chemical properties of both RM were in any case in line with the ranges commonly reported in the literature for these materials.[39,40] Data showed the greatest amount of carbon and nitrogen in OP but the highest quantity of available macronutrients, important for the mineral nutrition of red onion, in OR. All three treatments, SB, SB-OP and SB-OR, influenced positively but differently the quality of red onion with respect to the control (CTR). The effects of pads containing both low and high percentages of the two agricultural wastes were more positive on red onion quality than those of SB pads and CTR, which could be due to the presence of organic components in the pads. There is evidence that cultivation with organic fertilizers is associated with a greater amount of phytochemicals in onion. Ren *et al.*[41] evidenced a greater level of flavonoids in organically grown Welsh onions than in conventionally cultivated ones, and Hallmann and Rembiałkowska[42] found similar results in organically grown red onion. Significantly high amounts of phenolics, total flavonoids and anthocyanins as well antioxidant activities were found by Ren *et al.*[43] in two different onion varieties grown under organic production. Panuccio *et al.*,[44] Sharma *et al.*[45] and D'Evoli *et al.*[46] confirmed the positive effects of organic fertilization on secondary metabolite production in different crop species, suggesting that plant resource reallocation from primary to secondary metabolism produces phytochemicals useful to induce stress tolerance in crops, but also to preserve human health with intake. However, it is not yet clear if the increase in phytochemicals is a nutrient stress effector or if other factors, including effects of the soil microbiome, are involved. Our results related to the *in vitro* antioxidant capacity determined by DPPH, ABTS and ORAC assays showed that red onion grown with SB pads and with SB with agricultural wastes at both percentages (LP and HP) had higher antioxidant activities with respect to CTR (Fig. 1). Specifically, ORAC was highest in bulbs grown with SB-OR at low percentage and with SB-OP at high percentage, while DPPH and ABTS were always highest with SB pads with OR at both percentages. Polyphenols, flavonoids and anthocyanins, known as potent antioxidants, increased in treated red onion bulbs in comparison with CTR (Fig. 1). The greatest amount of polyphenols was detected in red onion bulbs grown with SB-OR, while SB and SB-OP pads increased the amount of polyphenols to the same extent as CTR, but less than SB-OR pads. Conversely to polyphenols, flavonoids increased mostly in red onion grown with SB-OP pads (Fig. 1). In red onion bulbs, anthocyanins were equally enhanced by all treatments with respect to CTR (Fig. 1). ANOVA was carried out only on samples grown with SB pads added with the two agricultural wastes at different concentrations (Table 3). The results pointed out that the type of waste, regardless of the concentration used, positively influenced total antioxidant activities and polyphenol and flavonoid contents, while the effect due to the interaction of the two factors (concentration × waste) was less significant. Differently, anthocyanin content was not affected by the two treatments (OR and OP) at all concentrations (Table 3).

The different effects of SB-OR and SB-OP pads on red onion quality could be related also to their different mineral composition. Numerous studies have evidenced the importance of single macronutrients in stimulating the onion antioxidant system. Kleiber *et al.*[47] showed that increasing the amount of magnesium also increased the amount and type of antioxidants in red onion. This was ascribed to the fact that magnesium is an important regulator of many biochemical processes and can activate primary and secondary metabolic pathways. Other works[48,49] highlighted the importance of calcium and potassium in inducing the production of antioxidant compounds in crops, with consequent increase in antioxidant activities. Phenolic (in particular caffeic, gallic and chlorogenic acid) contents were higher in red onion bulbs treated with all types of SB pads with respect to CTR (Table 4). The greatest content of chlorogenic and caffeic acids was obtained with SB-OR LP pads. Chlorogenic and

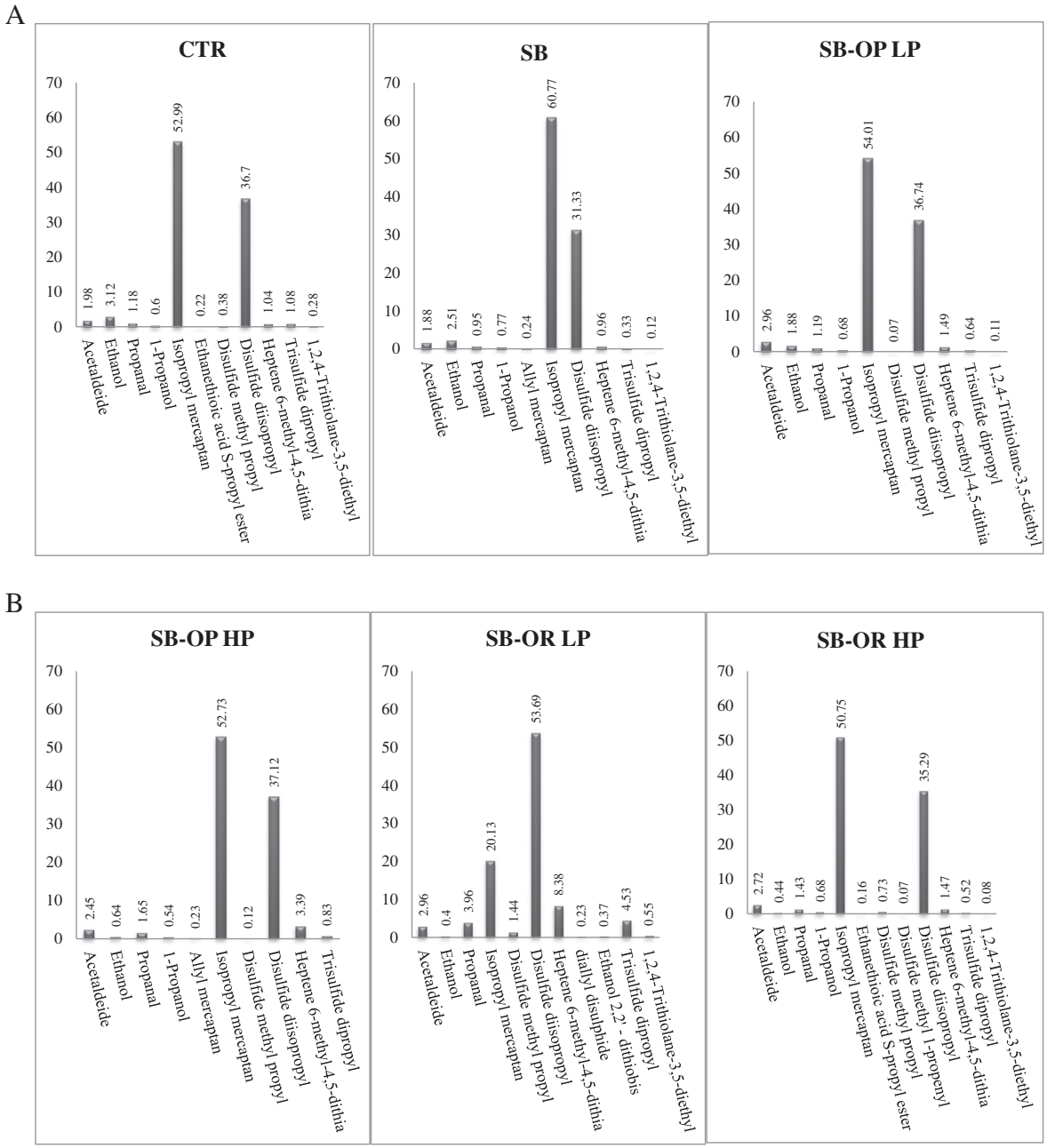

Figure 3. Volatile compounds in red onion bulbs grown for 3 months in soils differently treated: control (CTR), sulfur bentonite (SB), sulfur bentonite-low percentage orange residue (SB-OR LP), sulfur bentonite-high percentage orange residue (SB-OR HP), sulfur bentonite-low percentage olive pomace (SB-OP LP), sulfur bentonite-high percentage olive pomace (SB-OP HP). Data are mean of three replicates and are expressed as RAP% (relative area percentage, peak area relative to total peak area %).

caffeic acids are well known as antioxidants with an important role in prevention of cardiovascular disease. Olthof *et al.*[50] demonstrated that one-third of chlorogenic acid and almost all of caffeic acid were absorbed in the small intestine of humans, highlighting their important role in inhibiting the formation of mutagenic and carcinogenic *N*-nitroso compounds, since they are inhibitors of the *N*-nitrosation reaction *in vitro*, as also shown by Kono *et al.*[51] Among the flavonols, kaempferol and quercetin are two of the most important bio-compounds in onion, which ranks the highest among 28 vegetables and nine fruits.[52] Levels of quercetin in red onion were found to be 14-fold higher than in garlic and 2-fold higher than in white onion. All types of SB-based pads significantly increased the content of both quercetin and kaempferol, enhancing the nutraceutical and therapeutic values of our treated red onions (Table 4). Quercetin is known for its action on anti-proliferation, cell cycle arrest and apoptosis of cancer cells,[53–55] while kaempferol has multiple pharmacological uses as an antioxidant, anti-inflammatory, antimicrobial and antidiabetic and is also recognized as an important antitumor agent for its action on cancer cells from different organs.[56] Considering the glycosylated flavonols found in red onion bulbs, we observed a significant reduction in all conjugated quercetins *versus* an increase in the free form in red onion grown in the presence of SB alone and of SB in combination

with agricultural wastes (Table 5). All this may be an advantage, because it was well demonstrated in rats that free quercetin is the most absorbable form in the intestine. It enters the cells more readily than other forms because of its planar molecule, resulting in its high concentration in enterocytes, with an enhancement in the rates of conjugation and transfer of conjugated forms into the intestinal lumen.[57,58] Among the anthocyanins (ANTs), conjugated cyanidin increased only in red onion cultivated with SB and SB-OP HP, while delphinidin 3-glucoside increased in red onion cultivated with all SB-based pads (Table 5). Although ANTs have low absorption and high metabolism, their regular intake has beneficial effects on human health, since they act as antioxidants capable of protecting the vascular system, preventing endothelial dysfunction and atherosclerosis.[59] *Allium cepa* contains also a great amount of organosulfur compounds with beneficial effects on human health, and *S*-alkenyl cysteine sulfoxides are sulfur-containing amino acid compounds with antidiabetic and antihyperlipidemic effects. Various researchers have suggested that *Allium* vegetable consumption may have a strong impact on stomach cancer prevention, and the effects have been ascribed to the content of organosulfur compounds such as SMCSO that are able to inhibit colon and renal carcinogenesis.[60,61] Mechanisms of protection range from induced cancer cell apoptosis[62] and gene transcription inhibition[63] to protection against UV-induced immunosuppression.[64] SMCSO increased in all treated red onions with respect to CTR, confirming that sulfur-based fertilization is important to improve the synthesis of sulfur-based compounds (Fig. 2). The greatest increase in SMCSO was detected in the presence of SB followed by SB-OR LP and SB-OP pads (Fig. 2). The minor SMCSO synthesis in the presence of SB-OR HP and SB-OP pads can be explained by the presence of other nutrients that can compete with sulfur uptake, as already demonstrated for other plants by Eppendorfer.[65] Volatile sulfur compounds found in red onion increased in the case of all treatments, but to different extents (Fig. 3). Pads of SB and SB-OP LP increased the amount of isopropyl mercaptan, while SB-OR LP pads increased significantly the amount of diisopropyl disulfide; both compounds have well-recognized antimicrobial and antioxidant activities.[58,66] Our data showed in brief that the fertilization of red onion with recycled sulfur entrapped in bentonite pads increased its quality, and the positive effects were much more and differently enhanced when agricultural wastes were added.

CONCLUSIONS

The application of sulfur in various forms to crops was up to now recommended for compensating its deficiency in soils, in particular in alkaline soils for correcting the soil basicity. Now, the use of specific fertilizers containing sulfur associated with organic wastes can be considered, from another point of view, as an enhancer of plant performance in terms of production of bioactive compounds. Treatment with sulfur may provide sulfur-containing bonuses, enhancing in many species antioxidant and anticancer actions, in particular in sulfur-loving crops. Thus the use of sulfur-organic fertilization with fertilizers obtained by wastes may provide a novel strategy to diminish the emission of gas in the atmosphere and to use cyclical production chains that recycle and reduce wastes in a sustainable way. Increasing bio-compounds with beneficial effects on human health enhances the medical and economic values of sulfur-loving crops, with important consequences on the bio and green economy.

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