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ORIGINAL ARTICLE

Influence of nitrogen and sulfur fertilization on glucosinolate content and composition of horseradish plants harvested at different developmental stages

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Abstract Horseradish (*Armoracia rusticana*) is a rich source of glucosinolates (GLS), a class of secondary metabolites, nitrogen and sulfur compounds found in Brassicaceae family. Variations of content and composition of nine GLS in horseradish plants grown with N alone and N plus S were evaluated in the above- and below-ground portions at different developmental stages. Total GLS concentration was significantly higher in the above-ground tissues compared to the roots (97.8 vs 11.6 μmol g^{-1} dw); it responded positively to N and S supply in roots (11.5 in N alone and 15.8 μmol g^{-1} dw in N plus S treatments with respect to 7.4 μmol g^{-1} dw of the untreated control) without significant variations in the above-ground tissues. In both portions, total GLS concentration showed the greatest values at the beginning of plant regrowth and then decreased throughout the plant development till the end of the growing period. Among classes, the aliphatic GLS were the most abundant accounting for over 73 and 97 % of the total GLS in roots and above-ground tissues, respectively. Whereas, aromatic and indole GLS were present at roughly equivalent levels in both portions. GLS classes varied differently depending on developmental stage and fertilization, showing the highest percentage increase at the beginning of plant regrowth: aliphatic GLS increased by 150 % with N alone and 400 % with N and S supply, while aromatics and indoles increased both up to 35 % with N alone and 280 and 180 % with N and S, respectively. The results suggest that fertilization led to modulate GLS content and composition in plants in relation to a specific employment.

Keywords Brassicaceae · *Armoracia rusticana* · Fertilization · Glucosinolate · Sinigrin

Abbreviations

4ME	4-Methoxyglucobrassicin
ANOVA	Analysis of variance
BAR	Glucobarbarin and/or epiglucobarbarin
dw	Dry weight
GBN	Glucobrassicanapin
GBS	Glucobrassicin
GCX	Glucocochlearin and/or glucoconringianin
GIB	Glucoiberin
GLS	Glucosinolates
GNA	Gluconapin
HPLC–UV	High performance liquid chromatography and ultraviolet detection
IRMPD	Infrared multiphoton dissociation

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LC-ESI-FTICR	MS liquid chromatography coupled with electrospray ionization and Fourier transform ion cyclotron resonance mass spectrometry
NAS	Gluconasturtiin
SIN	Sinigrin

Introduction

Horseradish (*Armoracia rusticana* P. Gaertner, B. Meyer & Scherbius), member of the Brassicaceae family, is a perennial plant whose edible organs such as leaves and roots have been consumed as food and condiment for a long time and have also been used as a folk medicinal herb (Pieroni and Quave 2005; Sarli et al. 2012; Wedelsbäck-Bladh and Olsson 2011). Native of the temperate regions of eastern Europe and western parts of Russia, horseradish has become naturalized in many parts of the world and can be found cultivated and growing wild. Roots for commercial purposes are usually harvested once the foliage has been killed by frost in late autumn, continues through the winter until the beginning of spring when the soil is not frozen and is dry enough to dig roots (Walters and Wahle 2010). During that period, multiple sprouts can be already formed on the crown of the roots. Then, if roots are not harvested, a new growing cycle will start. As other Brassicaceae, horseradish contains glucosinolates (GLS), a class of nitrogen- and sulfur-containing secondary metabolites, that upon hydrolysis by the enzyme myrosinase liberate a range of bioactive compounds, including isothiocyanates, thiocyanates, and nitriles. GLS and their breakdown products are widely studied in a number of *Brassica* vegetables for their effects on human health (Björkman et al. 2011) and several other biological activities (Mithen 2001). Based on the aminoacid precursor, GLS are classified as aliphatic, aromatic and indole (Ishida et al. 2014) and according to differences in their structure around 132 natural GLS have been documented and estimated by Agerbirk and Olsen (2012). Unlike most of the species which contain only one or at the most 2–5 GLS (Kliebenstein et al. 2001), in horseradish Agneta et al. (2012, 2014a) identified 17 GLS in plant tissues, of which 11 have not previously been characterized in this species, some are present as isomers or in trace amounts. Regardless of the number, GLS pattern differs between species and ecotypes, as well as between and within individual plants (Björkman et al. 2011; Kliebenstein et al. 2005; Rangkadilok et al. 2002) depending on developmental stage (Brown et al. 2003; Cartea et al. 2008) and tissue or organ (Blazevic and Mastelic 2009; Malik et al. 2010; van Dam et al. 2009), several environmental factors and crop management practices (Björkman et al. 2011; Redovniković et al. 2008). A wide range of GLS concentrations varying from 1.7 to 296 μmol g^{-1} dw is reported by several authors depending on genotype (Agneta et al. 2014b; Li and Kushad 2004; WedelsbäckBladh et al. 2013). Concerning the effect of agronomic practices, most of the literature has focused on fertilization, particularly with nitrogen and sulfur, since these nutrients are essential constituents of GLS parent molecules. Several studies have shown that S fertilization leads to an increase in GLS content in most cases (as reviewed by Falk et al. 2007), while N tends to reduce, increase or have no effect on their concentration and composition in several Brassica species (Schonhof et al. 2007; Omirou et al. 2009). Referring to horseradish, Alnsour et al. (2012) found that in plantlets grown in vitro GLS concentrations could be modulated 20-fold by varying the sulfate concentration in the medium (from 0.2 to 21.5 mmol l^{-1}). To our knowledge, no other information is available about the influence of fertilization on GLS in horseradish plants, at least grown in open field conditions. The aim of the study was to evaluate the changes in the content and composition of glucosinolates in the above- and below-ground tissues of horseradish grown with and without nitrogen and sulfur supply and whether fertilization differentially affects the dynamics of each individual GLS throughout plant development.

Materials and methods

Experimental procedure

Field experiment was carried out in 2011 and 2012 at Policoro (Southern Italy, 40°17′30″ N–16°65′16″ E) on alluvial, loamy soil (sand 40 %, silt 37 %, clay 23 %) with 1.25 kg dm^{-3} bulk density, 7.7 pH, total N 1.67 g kg^{-1}, available P_2O_5 26.7 mg kg^{-1}, exchangeable K_2O 227 mg kg^{-1}, total S lesser than 500 mg kg^{-1}, organic matter 36.4 g kg^{-1}, total limestone 15 g kg^{-1}, active limestone 5 g kg^{-1}, 0.95 dSm^{-1} ECe.

Plants of horseradish (*A. rusticana*, Corleto accession) were grown without N and S supply as a control (−N−S), with N alone (+N−S) by applying 100 kg N/ha as ammonium nitrate, and with both N and S (+N+S) by applying 100 kg N/ha as a mixture (1:1) of ammonium nitrate and ammonium sulfate to provide 45 kg S/ha.

The experiment was arranged in a split-plot design with fertilization treatments as the main plot (each of 8 × 6 m) and harvesting time as the sub-plot, replicated three times.

Root cuttings (approximately 20 cm in length and 1 cm in diameter) were transplanted in single rows (100 cm between rows and 50 cm on the row) on April 6, 2011. The

fertilizers were applied at three different times at 23, 37 and 70 days after transplanting giving 30, 35 and 35 % of the full dose, respectively. Irrigation, plant protection and weed control were carried out according to local practices and weather conditions. In both years, mean temperatures ranged, on average, from 8 (Jan–Feb) to 26 (Aug) and 10 °C (Dec); total rainfall was 528 and 446 mm in 2011 and 2012, respectively. Approximately, 25 % of the total rainfall fell in March 2011 and in February 2012; lesser precipitations were recorded during the period June–September in both years, with no rain recorded in August 2011.

Plants were harvested at different developmental stages: (D1) end of the 1st year of growing period, when the foliage of the plant is entirely senescent and the harvest of roots for commercial purposes usually starts (December 2011); (D2) beginning of plant regrowth, when root harvest for commercial purposes usually ends (March 2012); (D3) flowering (May 2012); (D4) silique formation (July 2012); and (D5) end of the 2nd year of growing period of the plants left in the field ad hoc (December 2012). At each harvest, plants were separated into roots and sprouts (at D1, D2 and D5) or young and mature leaves (at D3 and D4), quickly cleaned with distilled water, dried with paper towels, weighed and then frozen at −80 °C to inhibit myrosinase activity until GLS analysis. In the meantime, subsamples of each tissue were dried in ventilated oven at 75 °C until steady weight to determine the dry matter weight (dw).

Analysis of glucosinolate

Plant tissues were processed for qualitative and quantitative analyses following the protocol described by Agneta et al. (2014a) and later detailed by Lelario et al. (2015). In brief, all samples were lyophilized and homogenized into fine powder using a laboratory mill. Afterwards, polypropylene tubes containing 200 mg dry material of each samples, were placed in a water bath, heated to 75 °C for 1 min. For GLS extraction, 2 ml of 70 % methanol (75 °C) and 200 µl of internal standard solution (6 mM glucotropaeolin) were added to each sample and vortexed shortly, incubated for 10 min in a water bath at 75 °C, mixed twice on a vortex mixer, centrifuged (Heraeus Varifuge F) for 5 min at 2400*g* and separated in a supernatant that was decanted into a polypropylene tube and in a remaining pellet that was extracted once again with 2 ml of 10 % methanol as described before. Afterwards, the supernatants were pooled and vortexed, then 500 µl of the extract were transferred onto a small ion-exchange column (Pasteur pipette) containing 20 mg of Sephadex DEAE-A 25 in the formiate form. The column was washed twice with 1 ml of deionized water, then 100 µl sulfatase type H-1 (Sigma S-9626) diluted 1:2.5 were added to achieve the desulfation and incubated overnight at 39 °C. Then, desulfated GLS were eluted with 3 × 500 µl water, collected in 70/12 PP tubes, vortexed shortly and transferred into 1 ml sample vials. Thereafter samples were processed for qualitative analyses by using the liquid chromatography (LC) coupled with electrospray ionization (ESI) and fourier transform ion cyclotron resonance (FT-ICR) mass spectrometry (MS) (Thermo Fisher Scientific, Bremen, Germany) and tandem mass spectrometry by infrared multiphoton dissociation (IRMPD) while quantitative analysis were performed on high performance liquid chromatography and ultraviolet detection (HPLC-UV) analyzer equipped with a photodiode array detector (PDA). The individual GLS were quantified by integration of their peak areas by comparing the peak area of the samples with the peak area of the internal standard. Nine GLS were quantified: glucoiberin GIB, sinigrin SIN, gluconapin GNA, glucocochlearin and/or glucoconringianin GCX, glucobarbarin and/or epiglucobarbarin BAR, glucobrassicanapin GBN, glucobrassicin GBS, gluconaturtiin NAS, 4-methoxyglucobrassicin 4ME. The identification of the GLS quantified was performed by comparison with retention time of individual standards. The total GLS concentration is referred to the sum of the nine GLS quantified in plant tissues.

Chemicals

For qualitative analysis by LC-FTICR MS system: glucoiberin was purchased from C2 Bioengineering (Karlslunden, Denmark); sinigrin monohydrate from horseradish (99 %) was obtained by Sigma-Aldrich (Steinheim, Germany); gluconapin, 4-hydroxyglucobrassicin, glucobrassicanapin, glucobrassicin and gluconasturtiin were separated and identified from rapeseed certified reference material (ERM367R); methanol and acetonitrile (ACN), both LC–MS grade, were obtained from Carlo Erba (Milan, Italy); ultrapure water was produced using a Milli-Q RG system from Millipore (Bedford, MA, USA).

For quantitative analysis by HPLC–UV: sinigrin (SIN), glucotropaeolin (GTL), glucobarbarin and/or epiglucobarbarin (BAR) were isolated and purified as desulfated molecules as described by Thies (1988), from *Brassica nigra*, *Lepidium sativum*, and *Barbarea vulgaris*, respectively; 4-methoxyglucobrassicin (4ME) was synthesized as described in Viaud et al. (1992) and then purified as desulfated molecules; glucoiberin (GIB) was purchased from Carl Roth (Germany) whereas gluconapin (GNA), glucobrassicanapin (GBN), glucobrassicin (GBS) and gluconasturtiin (NAS) were obtained from Phytolab (Germany), each subsequently purified as desulfated molecules.

Statistical analysis

Statistical analysis was performed using M-STAT software (version 2.00). Pearson correlations were used to analyze correlations of GLS concentrations in above-ground tissues and roots. Differences in GLS concentrations throughout the growing season of horseradish grown with and without S and N supply were analyzed by the analysis of variance (ANOVA). Data were compared using the least significant difference (LSD) at the 0.05 significance level.

Results

Chemical and trivial names, molecular formulae and retention time of 17 individual GLS detected in the tissues of horseradish, grouped as aliphatic, aromatic and indole GLS are reported in Table 1. For each glucosinolate identified using LC-ESI-FTICR MS, details regarding the accurate m/z ratio, their monoisotopic exact values as $[M-H]^-$ ion (m/z) and the mass error (ppm), the main infrared multiphoton dissociation mass spectra (IRMPD) product ions (accurate m/z) and mass error (ppm) have been previously reported in Agneta et al. (2012, 2014a). The number of GLS varied among plant organs with 17 GLS identified both in roots and sprouts, 15 in the young leaves and only 12 in the mature ones (Table 1). In particular, the two aliphatic GLS 4-mercaptobuthyl and 7-(methylthio)heptyl were missing in young leaves, while mature leaves were also lacking the aliphatic GLS 1-methylpropyl, the indole 4-hydroxyindol-3-ylmethyl and its unidentified isomer.

Nine GLS (each labeled with an abbreviation in Table 1) have been quantified in variable concentrations in the above- and below-ground tissues. The sum of concentrations of those GLS, henceforth referred to as total, significantly varied throughout the developmental stages as the effect of nitrogen and sulfur supply in both portions (Fig. 1). Considering the above-ground tissues (Fig. 1a), the total GLS concentration in the sprouts was unaltered passing from D1 to D2 stage, except in +N+S treatment in which it was reduced by about 20 %. During flowering at D3 stage, it was reduced by about 50 % in leaves (118 vs 60 μmol g^{-1} dw, on average) and as observed at D2 it was higher in +N−S than in +N+S treatment. Later at the D4 stage, GLS in leaves settled around 80 μmol g^{-1} in almost all treatments (on average). At the last stage D5, the concentration of GLS in the young sprouts already formed was about 100 μmol g^{-1} dw, with values significantly higher than 20 % in the +N+S treatment. Meanwhile, in the roots (Fig. 1b) total GLS concentration was approximately 15.2 μmol g^{-1} dw (on average) at D1 stage, with values tending to be higher in +N+S treatment. At D2 stage, the concentration significantly increased by 150 and 380 % in the +N−S and +N+S treatments, respectively; then, it gradually decreased during plant development to 6.2 μmol g^{-1} dw (on average) at the last stage. Among classes, the aliphatic GLS, which were always predominant both in the above-ground tissues (accounting for over 98 % of the total GLS) and roots (accounting for over 73 % of the total GLS) followed the trend of the total concentration. Aromatic and indole GLS which were present at roughly equivalent levels in both portions (each representing an average of about 1 % in above-ground tissues and 8 % in roots), were significantly reduced throughout the plant development and significantly increased only in roots as the effect of fertilization (data not shown). Figure 2 showed the percentage variation of aliphatic, aromatic and indole GLS in the fertilized treatments with respect to the untreated control. In the above-ground tissues (Fig. 2a), fertilization supply led to an increase less than 20 % in all GLS classes; in roots (Fig. 2b) pronounced differences have been observed as the effect of fertilization: aliphatic GLS increased up to 150 % in the treatment with N and up to 400 % in the plants treated with both N and S (D2 stage) while aromatic and indole GLS increased both by about 35 % in the +N−S treatment, and 280 and 180 %, respectively, in the +N+S treatment.

Each of the nine GLS quantified in the tissues contributed differently to the variation of the total concentration. Among them, the major glucosinolate was the aliphatic sinigrin (SIN); the minor GLS (GLS present in smaller amounts) were the aliphatic glucocochlearin and/or glucoconringianin (GCX), the aromatic gluconasturtiin (NAS) and the indole glucobrassicin (GBS); the GLS in trace amounts (whose concentrations were less than 0.60 μmol g^{-1} dw) were the aliphatics glucoiberin (GIB), gluconapin (GNA) and glucobrassicanapin (GBN), the indole 4-methoxyglucobrassicin (4ME) and the aromatic glucobarbarin (BAR). In the above-ground tissues (Table 2), the fertilization × developmental stage interaction significantly affected SIN, GBN and GNA among the aliphatic GLS and GBS between the indoles. SIN which represented about 95 % of the total GLS, as expected varied similarly to the total concentration; GBN which represented only 0.2 % of the total GLS, significantly increased at D1 stage in the +N+S treatment (130 % increase) and at D2 stage in the +N−S treatment (45 % increase with respect to the untreated control), without significant differences among treatments in the following three developmental stages; GNA remained quite similar at D1, D2 and D5 stages, decreasing by about 50 % during flowering and silique forming stages (D3 and D4). Between the indole GLS (Table 2), GBS was quantitatively more representative; it increased in −N−S and +N−S treatments passing from D1 to D2 stage and drastically

Table 1 Chemical class, chemical and trivial name, molecular formulae and retention time of the glucosinolates detected in the tissues of horseradish plants

Class	Chemical name	Trivial name	Abbreviation[a]	Molecular formulae	Retention time	Plant tissue
Aliphatic	3-(Methylsulfinyl)propyl	Glucoiberin	GIB	$C_{11}H_{21}NO_{10}S_3$	4.3	Root, sprout, young and mature leaves
	2-Propenyl	Sinigrin	SIN	$C_{10}H_{17}NO_9S_2$	4.4	Root, sprout, young and mature leaves
	2-Methylsulfonyl-*oxo*-ethyl	–	–	$C_{10}H_{17}NO_{12}S_3$	4.6	Root, sprout, young and mature leaves
	3-Butenyl-GLS	Gluconapin	GNA	$C_{11}H_{19}NO_9S_2$	5.5	Root, sprout, young and mature leaves
	1-Methylpropyl and/or	Glucococchlearin	GCX	$C_{11}H_{21}NO_9S_2$	6.2	Root, sprout and young leaves
	2-Methylpropyl	Glucoconringianin		$C_{11}H_{21}NO_9S_2$	6.4	Root, sprout, young and mature leaves
	4-Mercaptobuthyl	Glucosativin	–	$C_{11}H_{21}NO_9S_3$	6.5	Root and sprout
	7-(Methylsulfinyl)heptyl	Glucoibarin	–	$C_{15}H_{29}NO_{10}S_3$	7.3	Root, sprout, young and mature leaves
	4-Pentenyl	Glucobrassicanapin	GBN	$C_{12}H_{21}NO_9S_2$	7.8	Root, sprout, young and mature leaves
	7-(Methylthio)heptyl	–	–	$C_{15}H_{29}NO_9S_3$	16.7	Root and sprout
Aromatic	2(*S*)-Hydroxy-2-phenylethyl-GLS and/or 2(*R*)-hydroxy-2-phenylethyl-GLS	Glucobarbarin and/or epiglucobarbarin	BAR	$C_{15}H_{21}NO_7S$	7.8	Root, sprout, young and mature leaves
	Benzyl-GLS	Glucotropaeolin	–	$C_{14}H_{19}NO_9S_2$	8.5	Root, sprout, young and mature leaves
	2-Phenylethyl-GLS	Gluconasturtiin	NAS	$C_{15}H_{21}NO_9S_2$	12.1	Root, sprout, young and mature leaves
Indole	4-Hydroxyindol-3-ylmethyl-GLS	4-Hydroxyglucobrassicin	–	$C_{16}H_{20}N_2O_{10}S_2$	7.4	Root, sprout and young leaves
	Unidentified isomer of 4-hydroxyindol-3-ylmethyl-GLS	–	–	$C_{16}H_{20}N_2O_{10}S_2$	7.5	Root, sprout and young leaves
	Indol-3-ylmethyl-GLS	Glucobrassicin	GBS	$C_{16}H_{20}N_2O_9S_2$	10.3	Root, sprout, young and mature leaves
	4-Methoxyndol-3-ylmethyl-GLS	4-Methoxyglucobrassicin	4ME	$C_{17}H_{22}N_2O_{10}S_2$	12.7	Root, sprout, young and mature leaves

[a] The abbreviation is referred to the GLS quantified in the tissues

decreased over 90 % at D3 and D4 stages. The remaining aliphatic GLS, GIB and GCX, the indole 4ME and both the aromatics (BAR and NAS) were significantly affected only by plant developmental stages ($P < 0.001$) showing values significantly lower at D3 and D4 stages, except GIB that decreased at the D5 stage. As observed in the above-ground tissues, SIN, GNA, GBN, and GBS were significantly influenced by fertilization × developmental stage interaction also in the roots (Table 3); in addition, the aromatic NAS was affected by the interactions of the two factors.

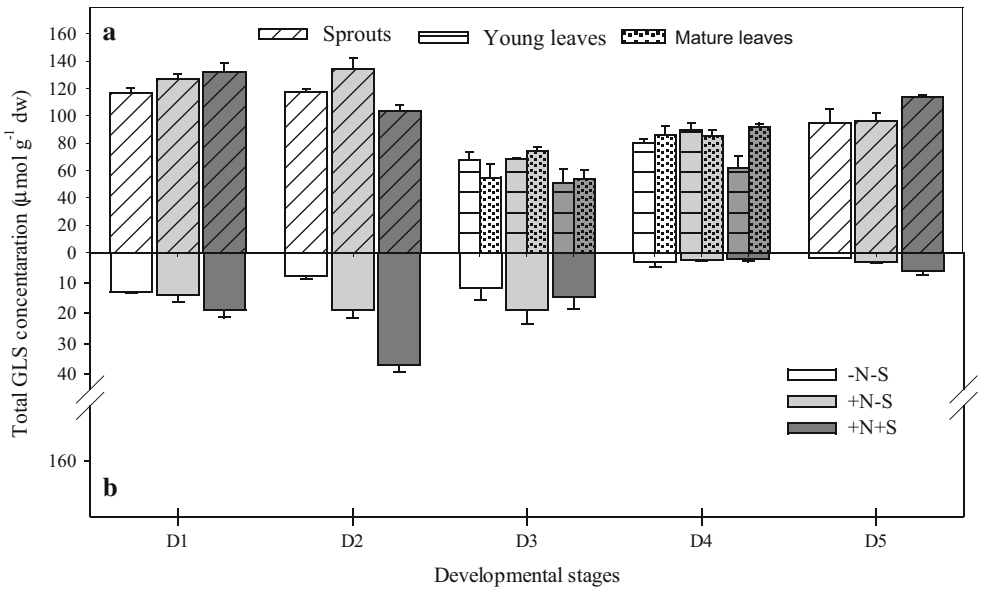

Fig. 1 Total glucosinolates concentration in the above-ground tissues (*a*) and roots (*b*) of horseradish grown with and without fertilization (*−N−S*, *+N−S*, and *+N+S* untreated control, *N* alone, both *N* and *S*, respectively) and collected at different developmental stages (*D1*, *D2*, *D3*, *D4*, *D5*: end of 1st year of growing period, beginning of plant regrowth, flowering, silique formation, end of 2nd year of growing period, respectively). Data were analyzed independently by factorial ANOVA followed by LSD test (0.05). Values of LSD tests are 16.5 for sprouts, 15.5 and 18.3 for young and mature leaves, respectively, and 5.5 for roots

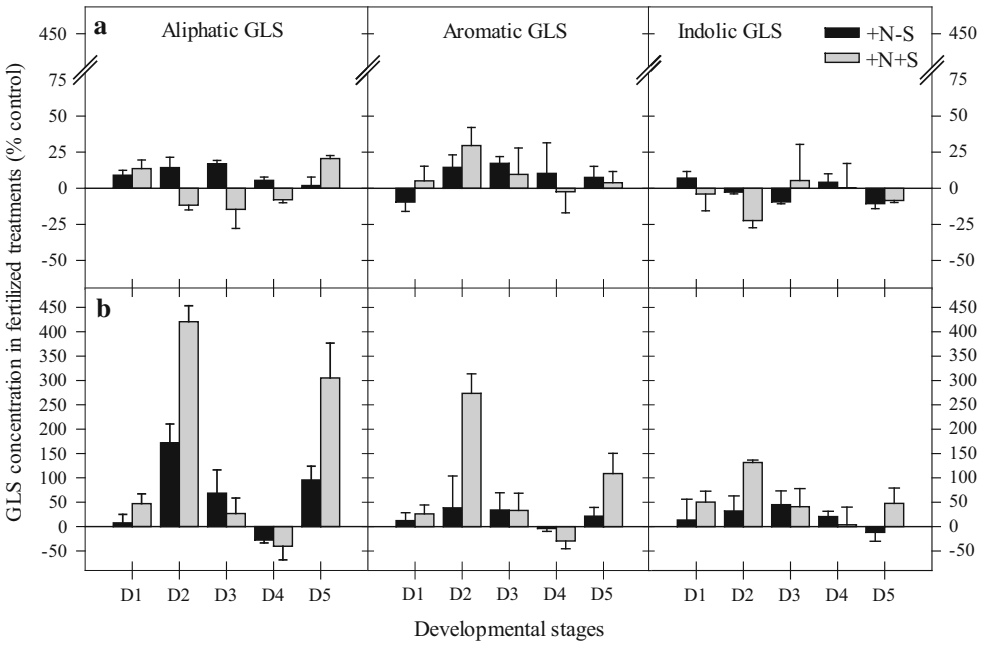

Fig. 2 Percentage variation of aliphatic, aromatic and indole glucosinolates in the fertilized treatments (*+N−S* and *+N+S*) relative to the untreated control in the above-ground tissues (*a*) and roots (*b*) of horseradish at different developmental stages (*D1*, *D2*, *D3*, *D4*, *D5* end of 1st year of growing period, beginning of plant regrowth, flowering, silique formation, end of 2nd year of growing period, respectively). SE were calculated from the variation in fertilization-treated plants relative to the mean of the untreated control. Data were analyzed independently by factorial ANOVA followed by LSD test. Values of LSD tests are 20.7 for aliphatic GLS in above-ground portion and 157.2 and 141.9 for aliphatic and aromatic GLS, respectively, in roots

Both NAS and SIN, that accounted for more than 5 and 80 % of the total GLS, respectively, showed a pattern of variation similar to that above described for the total concentration. The aliphatics GNA and GBN always showed the lower concentrations; furthermore, GNA increased passing from D1 to D2 in +N+S treatment (0.04

Table 2 Concentration (μmol g^{-1} dw) of aliphatic, aromatic and indole GLS in the above-ground tissues of horseradish as affected by developmental stages and fertilization

Developmental stage[a]	Fertilization[b]	Aliphatic GLS					Aromatic GLS		Indole GLS	
		GIB[c]	SIN	GNA	GCX	GBN	BAR	NAS	GBS	4ME
D1	−N−S	0.36^{bc} (0.3)	111.9^{ac} (96.2)	0.33^{ab} (0.3)	1.36^{bc} (1.2)	0.18^{cd} (0.2)	0.10^{ac} (0.1)	0.84^{bc} (0.7)	1.05^{d} (0.9)	0.28^{a} (0.2)
	+N−S	0.37^{bc} (0.3)	121.9^{ab} (96.2)	0.31^{b} (0.2)	1.62^{ab} (1.3)	0.18^{cd} (0.1)	0.10^{ac} (0.1)	0.75^{c} (0.6)	1.20^{cd} (1.0)	0.23^{bc} (0.2)
	+N+S	0.45^{ab} (0.3)	126.7^{a} (96.1)	0.36^{ab} (0.3)	1.72^{ab} (1.3)	0.43^{bc} (0.3)	0.09^{cd} (0.1)	0.90^{bc} (0.7)	1.06^{d} (0.8)	0.23^{bc} (0.2)
D2	−N−S	0.38^{bc} (0.3)	111.9^{ac} (95.3)	0.38^{a} (0.3)	1.33^{bc} (1.1)	0.61^{ab} (0.5)	0.11^{a} (0.1)	0.79^{bc} (0.7)	1.73^{a} (1.5)	0.24^{ab} (0.2)
	+N−S	0.42^{bc} (0.3)	127.6^{a} (95.3)	0.32^{ab} (0.2)	1.78^{ab} (1.3)	0.88^{a} (0.2)	0.11^{a} (0.1)	0.92^{bc} (0.7)	1.70^{a} (1.3)	0.22^{bc} (0.2)
	+N+S	0.36^{bc} (0.3)	99.0^{cd} (95.3)	0.32^{ab} (0.3)	1.33^{bc} (1.3)	0.18^{cd} (0.2)	0.08^{f} (0.1)	1.09^{b} (1.1)	1.38^{b} (1.3)	0.15^{d} (0.1)
D3	−N−S	0.36^{bc} (0.6)	59.9^{gh} (97.8)	0.11^{d} (0.2)	0.49^{d} (0.8)	0.08^{d} (0.2)	0.02^{h} (0.0)	0.13^{d} (0.2)	0.10^{e} (0.2)	0.07^{e} (0.1)
	+N−S	0.57^{a} (0.8)	69.8^{fg} (97.6)	0.15^{cd} (0.2)	0.63^{d} (0.9)	0.08^{d} (0.1)	0.01^{h} (0.0)	0.17^{d} (0.2)	0.09^{e} (0.1)	0.04^{e} (0.1)
	+N+S	0.56^{a} (1.1)	50.9^{h} (97.1)	0.11^{d} (0.2)	0.44^{d} (0.8)	0.05^{d} (0.1)	0.02^{h} (0.0)	0.15^{d} (0.3)	0.10^{e} (0.2)	0.05^{e} (0.1)
D4	−N−S	0.37^{bc} (0.5)	81.3^{ef} (97.8)	0.19^{c} (0.2)	0.55^{d} (0.7)	0.15^{cd} (0.2)	0.02^{h} (0.0)	0.24^{d} (0.3)	0.22^{e} (0.3)	0.07^{e} (0.1)
	+N−S	0.37^{bc} (0.4)	85.4^{df} (97.5)	0.22^{c} (0.3)	0.78^{cd} (0.9)	0.23^{cd} (0.3)	0.02^{h} (0.0)	0.26^{d} (0.3)	0.23^{e} (0.3)	0.07^{e} (0.1)
	+N+S	0.31^{cd} (0.4)	74.8^{eg} (97.7)	0.18^{cd} (0.2)	0.61^{d} (0.8)	0.11^{d} (0.1)	0.01^{h} (0.0)	0.24^{d} (0.3)	0.23^{e} (0.3)	0.06^{e} (0.1)
D5	−N−S	0.19^{d} (0.2)	88.9^{de} (93.8)	0.38^{ab} (0.4)	1.87^{ab} (2.0)	0.22^{cd} (0.2)	0.08^{f} (0.1)	1.49^{a} (1.6)	1.49^{b} (1.6)	0.19^{cd} (0.2)
	+N−S	0.21^{d} (0.2)	90.5^{de} (93.9)	0.31^{ab} (0.3)	2.02^{a} (2.1)	0.19^{cd} (0.4)	0.09^{e} (0.1)	1.60^{a} (1.7)	1.34^{bc} (1.4)	0.15^{d} (0.2)
	+N+S	0.19^{d} (0.2)	108.1^{bc} (95.2)	0.35^{ab} (0.3)	1.53^{ab} (1.3)	0.17^{cd} (0.2)	0.09^{e} (0.1)	1.35^{a} (1.4)	1.34^{bc} (1.2)	0.19^{bd} (0.2)
F probability										
Fertilization (*F*)		n.s	n.s	n.s	n.s.	*	n.s.	n.s.	n.s.	n.s.
Developmental stage (*D*)		***	***	***	***	***	***	***	***	***
$F \times D$		n.s	***	*	n.s.	***	n.s.	n.s.	*	n.s.

Values in each column not sharing the same letter are significantly different (LSD test, $P < 0.05$). Level of significance: * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; *n.s.* not significant. Values in parenthesis are expressed as percentage of the total GLS concentration

[a] Developmental stage: D1, D2, D3, D4, D5: end of 1st year of growing period, beginning of plant regrowth, flowering, silique formation, end of 2nd year of growing period, respectively

[b] Fertilization: −N−S, +N−S, and +N+S: untreated control, N alone, both N and S, respectively

[c] GIB, glucoiberin; SIN, sinigrin; GNA, gluconapin; GCX, glucocochlearin and/or glucoconringianin; GBN, glucobrassicanapin; BAR, glucobarbarin and/or epiglucobarbarin; NAS, gluconasturtiin; GBS, glucobrassicin; 4ME, 4-methoxyglucobrassicin

Table 3 Concentration (μmol g^{-1} dw) of aliphatic, aromatic and indole GLS in the root tissues of horseradish as affected by developmental stages and fertilization

Developmental stage[a]	Fertilization[b]	Aliphatic GLS					Aromatic GLS		Indole GLS	
		GIB[c]	SIN	GNA	GCX	GBN	BAR	NAS	GBS	4ME
D1	−N−S	0.26^{ad} (2.0)	10.5^{bc} (81.3)	0.03^{be} (0.2)	0.41^{ab} (3.2)	0.06^{bc} (0.5)	0.12^{a} (0.9)	0.80^{be} (6.2)	0.56^{bd} (4.4)	0.17 (1.3)
	+N−S	0.31^{ac} (2.2)	11.3^{bc} (81.1)	0.03^{be} (0.2)	0.38^{ac} (2.8)	0.07^{b} (0.5)	0.11^{ab} (0.8)	0.92^{bd} (6.7)	0.71^{bc} (5.1)	0.12 (0.8)
	+N+S	0.28^{ad} (1.5)	15.6^{b} (83.2)	0.04^{bd} (0.2)	0.46^{ab} (2.5)	0.11^{a} (0.6)	0.10^{ac} (0.5)	1.06^{bc} (5.6)	0.27^{b} (5.2)	0.13 (0.7)
D2	−N−S	0.21^{ad} (2.7)	5.7^{ce} (74.9)	0.03^{be} (0.4)	0.40^{ab} (5.2)	0.03^{bd} (0.4)	0.09^{ac} (1.2)	0.49^{be} (6.4)	0.51^{be} (6.7)	0.17 (2.2)
	+N−S	0.25^{ad} (1.3)	16.6^{b} (87.2)	0.05^{b} (0.3)	0.42^{ab} (2.2)	0.02^{d} (0.1)	0.09^{ac} (0.5)	0.71^{be} (4.6)	0.76^{bc} (4.0)	0.14 (0.7)
	+N+S	0.32^{ab} (0.9)	32.2^{a} (87.0)	0.09^{a} (0.2)	0.64^{a} (1.7)	0.03^{cd} (0.1)	0.10^{ac} (0.3)	2.06^{a} (5.5)	1.49^{a} (4.0)	0.08 (0.2)
D3	−N−S	0.27^{ad} (2.4)	9.4^{bd} (81.0)	0.04^{bc} (0.3)	0.29^{be} (2.3)	0.02^{d} (0.2)	0.12^{a} (1.1)	0.85^{be} (7.3)	0.48^{ce} (4.2)	0.12 (1.0)
	+N−S	0.34^{a} (1.8)	16.1^{b} (84.7)	0.04^{bd} (0.2)	0.33^{bd} (2.1)	0.02^{d} (0.1)	0.12^{a} (0.6)	1.19^{b} (6.5)	0.78^{bc} (4.1)	0.08 (0.4)
	+N+S	0.35^{a} (2.4)	11.9^{bc} (80.2)	0.03^{be} (0.2)	0.38^{bc} (2.4)	0.03^{bd} (0.2)	0.12^{a} (0.8)	1.17^{b} (7.8)	0.71^{bc} (4.8)	0.13 (0.9)
D4	−N−S	0.15^{bd} (4.7)	2.3^{de} (71.6)	0.02^{de} (0.5)	0.12^{ce} (4.5)	0.03^{cd} (0.9)	0.07^{ac} (2.2)	0.29^{de} (11.5)	0.14^{de} (4.2)	0.10 (3.1)
	+N−S	0.19^{ad} (7.5)	1.6^{de} (61.7)	0.02^{de} (0.8)	0.09^{de} (3.7)	0.04^{bd} (1.4)	0.08^{ac} (3.1)	0.27^{de} (10.7)	0.14^{de} (5.6)	0.15 (5.7)
	+N+S	0.20^{ad} (9.4)	1.3^{e} (60.9)	0.01^{e} (0.5)	0.08^{de} (4.4)	0.02^{d} (1.0)	0.07^{ac} (3.4)	0.19^{de} (10.1)	0.14^{de} (6.5)	0.11 (5.4)
D5	−N−S	0.11^{d} (6.5)	1.1^{e} (63.8)	0.01^{e} (0.5)	0.11^{de} (6.2)	0.02^{d} (1.1)	0.06^{bc} (3.3)	0.16^{e} (8.8)	0.07^{e} (4.0)	0.10 (5.8)
	+N−S	0.14^{cd} (4.4)	2.5^{de} (79.8)	0.01^{e} (0.3)	0.05^{e} (1.7)	0.01^{d} (0.4)	0.05^{c} (1.5)	0.21^{de} (6.8)	0.09^{de} (3.0)	0.06 (1.9)
	+N+S	0.12^{d} (2.0)	5.3^{ce} (84.4)	0.01^{de} (0.2)	0.09^{de} (1.5)	0.04^{bd} (0.7)	0.05^{c} (0.8)	0.40^{ce} (6.3)	0.18^{de} (2.8)	0.08 (1.3)
F probability										
Fertilization (*F*)		*	**	*	n.s.	**	n.s	*	**	n.s
Developmental stage (*D*)		***	***	***	***	***	***	***	***	n.s.
$F \times D$		n.s.	***	***	n.s.	*	n.s.	*	**	n.s

Values in each column not sharing the same letter are significantly different (LSD test, $P < 0.05$). Level of significance: * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; *n.s.* not significant. Values in parenthesis are expressed as percentage of the total GLS concentration

[a] Developmental stage: D1, D2, D3, D4, D5: end of 1st year of growing period, beginning of plant regrowth, flowering, silique formation, end of 2nd year of growing period, respectively

[b] Fertilization: −N−S, +N−S, and +N+S: untreated control, N alone, both N and S, respectively

[c] GIB, glucoiberin; SIN, sinigrin; GNA, gluconapin; GCX, glucocochlearin and/or glucoconringianin; GBN, glucobrassicanapin; BAR, glucobarbarin and/or epiglucobarbarin; NAS, gluconasturtiin; GBS, glucobrassicin; 4ME, 4-methoxyglucobrassicin

Table 4 Correlation matrices between glucosinolates both in the above-ground tissues and roots

GLS	Pearson's correlation index[a]											
	GIB	SIN	GNA	GCX	GBN	BAR	NAS	GBS	4ME	ALI	ARO	INDO
Above-ground tissues												
GIB[b]	–											
SIN	−0.13	–										
GNA	**−0.47**	**−0.47**	–									
GCX	**−0.31**	**0.68**	**0.68**	–								
GBN	0.02	**0.62**	**0.49**	**0.42**	–							
BAR	−0.24	**0.85**	**0.81**	**0.83**	**0.53**	–						
NAS	**−0.58**	**0.54**	**0.78**	**0.74**	0.26	**0.71**	–					
GBS	**−0.38**	**0.77**	**0.88**	**0.81**	**0.58**	**0.91**	**0.81**	–				
4ME	−0.20	**0.82**	**0.77**	**0.73**	**0.47**	**0.93**	**0.62**	**0.82**	–			
ALI	−0.14	**1.00**	**0.81**	**0.69**	**0.62**	**0.85**	**0.55**	**0.78**	**0.82**	–		
ARO	−0.57	**0.58**	**0.80**	**0.76**	0.28	**0.75**	**1.00**	**0.83**	**0.65**	**0.58**	–	
INDO	−0.37	**0.79**	**0.88**	**0.82**	**0.58**	**0.94**	**0.80**	**1.00**	**0.86**	**0.80**	**0.82**	–
Total	−0.16	**1.00**	**0.82**	**0.70**	**0.62**	**0.86**	**0.57**	**0.80**	**0.83**	**1.00**	**0.61**	**0.82**
Roots												
GIB	–											
SIN	**0.68**	**–**										
GNA	**0.59**	**0.90**	**–**									
GCX	**0.62**	**0.84**	**0.76**	–								
GBN	0.29	0.23	0.12	**0.38**	–							
BAR	**0.76**	**0.56**	**0.50**	**0.71**	**0.33**	–						
NAS	**0.77**	**0.88**	**0.73**	**0.74**	0.26	**0.60**	–					
GBS	**0.78**	**0.95**	**0.85**	**0.88**	**0.38**	**0.68**	**0.87**	–				
4ME	0.27	0.02	0.05	0.25	0.27	**0.37**	−0.10	0.20	–			
ALI	**0.69**	**1.00**	**0.90**	**0.85**	0.24	**0.57**	**0.88**	**0.95**	0.02	–		
ARO	**0.79**	**0.88**	**0.74**	**0.75**	0.27	**0.63**	**1.00**	**0.88**	−0.08	0.88	–	
INDO	**0.79**	**0.91**	**0.83**	**0.88**	0.40	**0.70**	**0.83**	**0.99**	0.33	0.92	0.84	–
Total	**0.71**	**1.00**	**0.89**	**0.85**	0.25	**0.58**	**0.89**	**0.96**	0.03	1.00	0.90	0.93

[a] Numbers in bold denote a significant coefficient correlation ($P < 0.001$ for all correlations except for GIB vs GCX ($P < 0.05$), GIB vs GBS and GCX vs GBN ($P < 0.01$) in the above-ground tissues)

[b] GIB, glucoiberin; SIN, sinigrin; GNA, gluconapin; GCX, glucococochlearin and/or glucoconringianin; GBN, glucobrassicanapin; BAR, glucobarbarin and/or epiglucobarbarin; NAS, gluconasturtiin; GBS, glucobrassicin; 4ME, 4 methoxyglucobrassicin; ALI, aliphatic GLS; ARO, aromatic GLS; INDO, indole GLS; Total, sum of all GLS

vs 0.09 μmol g dw^{-1}) then decreased in the remaining developmental stages without differences between fertilized and unfertilized plants. Whereas, the concentration of GBN was higher at D1 stage in fertilized plants, then it decreased as observed for GNA. The indole GBS exhibited the lowest values overall in the unfertilized treatment, with a drastic reduction of the concentration at D4 and D5 stages. Concerning the remaining GLS, GIB increased as the effect of fertilization in each developmental stage, and similar to GCX and BAR, was strongly reduced at D4 and D5 stages.

In addition to the changes of each individual GLS as the effect of fertilization supply throughout the plant development, we investigated whether there was any relationship among GLS. Therefore, a correlation matrix among individual GLS has been performed both for the above-ground tissues and the roots. In the above-ground tissues (Table 4) all the GLS were correlated with each other, except GIB that was correlated only to GNA, GCX, NAS and GBS. SIN was negatively correlated with GNA and highly positively correlated to both the indole GLS (GBS and 4ME) and the aromatic BAR, with which shared more than 70 % of the variability. Among classes, the indole GLS were highly correlated with aliphatic (0.80) and aromatic (0.82) GLS. In roots (Table 4), all GLS were positively correlated with each other, except 4ME (weakly correlated only to BAR) and GBN (slightly correlated to GCX, GBS and BAR). SIN shared with GNA, GCX, GBS,

and NAS more than 70 % of the variability. The GLS classes were highly correlated to each other also in the roots.

Discussion

Studies carried out on the qualitative and quantitative profile of the glucosinolates in plants have shown that considerable differences occur among and within species and genotypes (Fahey et al. 2001; Kliebenstein et al. 2001), between tissues and organs, and phenological stages of the plants (Agneta et al. 2014a; Brown et al. 2003). The accession of horseradish analyzed is not an exception to this generalization. In our case, the above-ground tissues showed the highest GLS concentrations up to ten-fold higher than roots during the period in which the roots are usually harvested (when buds and young sprouts are already developed on the root crown) and are fivefold higher later when the foliage of the plants is fully developed. In literature, investigations of GLS in horseradish have mainly focused on roots while the above-ground tissues have been poorly analyzed. Li and Kushad (2004) found that GLS concentration may be greater in leaves or in roots depending on the accession. In our study, we found that differences between portions are mainly due to the greater content of aliphatic GLS in young sprouts or leaves in comparison to roots. Indeed, the aromatic and indole classes were present at roughly equivalent levels in all tissues of the plant, showing even higher concentrations in the roots depending on developmental stage and fertilization. The variations pattern between above- and below-ground tissues could be associated to a different regulation of GLS biosynthesis and turnover in different organs, as suggested also by Van Dam et al. (2009). By performing a correlation matrix between above-ground and roots for each glucosinolate, we found no correlations (except for GIB; data not shown) between portions, indicating that the biosynthesis of GLS might be independently regulated in each tissue, as suggested also by Li and Kushad in horseradish plant (2004). Considering separately the variations of GLS in each tissue, almost all GLS were correlated with each other (Table 4) suggesting a co-regulation of their biosynthesis or degradation depending on the plant tissue. The dynamic changes of glucosinolate levels in any particular tissue depend upon the regulation of de novo biosynthesis, degradation and mobilization of GLS (Chen and Andreasson 2001), with the capacity for the de novo synthesis varying according to the type of tissue concerned (Bellostas et al. 2007).

Changes of GLS concentration as the effect of N and S has previously been studied in various Brassica species neglecting several other species including horseradish. Omirou et al. (2009) for broccoli plant tissues reported that GLS concentration increased significantly with increasing N supply rate. In contrast, Schonhof et al. (2007) in broccoli florets found that an increase in N fertilization resulted in a significant reduction of GLS. In our case, the response of horseradish to N was generally positive and overall N supply led to an increase of total GLS concentration by about 55 % in roots and 9 % in the epigeous tissues. By adding S to N, total GLS concentration in roots increased furthermore by 36 %, while in the above-ground, it remained almost constant. The increase in GLS content as the effect of fertilization was particularly higher at the beginning of plant regrowth (D2). Such results may probably be linked to the reduction of primary metabolism occurring during the winter. Indeed, if primary metabolism in relation to plant growth and protein synthesis is reduced, a shift toward secondary metabolism of C-, N-, or S-containing phytochemicals, such as GLS, might occur (Groenbäk et al. 2014; Rosa et al. 1996). Instead, during the period of maximum vegetative growth, from stage D2 onwards, there was a drastic reduction of GLS concentration. In leaves of *Arabidopsis thaliana* Brown et al. (2003) suggested that decreases of GLS content during the growing cycle could be associated with the expansion of cellular tissues that determined a GLS dilution. In addition, GLS may contain up to 10–30 % of the plant's total sulfur in some tissues and may serve as a storage of sulfur as well as acting as an expendable reservoir of this nutrient in times of sulfur deprivation (Falk et al. 2007). As GLS were strongly reduced in roots at the maximum vegetative growth (flowering and silique forming stages) it is possible that plants may catabolize the GLS accumulated in the roots and use the released sulfur to assist the primary metabolism in photosynthesizing tissues. Indeed, the greater reduction was observed in plants treated with both nitrogen and sulfur (more than 50 % decrease in the total concentration passing from D2 to D3) which showed the higher number of leaves and the greater leaf area index (compared to the unfertilized plants or fertilized with N alone; data not shown) that contributed to increasing the primary metabolism probably at the expense of the GLS. On the whole, dynamic changes as the effect of fertilization are attributable mainly to the aliphatic GLS, that among classes showed the highest percentage of variations in the treated plants with respect to the untreated control. In rocket leaves, Omirou et al. (2012) found that aliphatic GLS responded negatively to N fertilization, particularly during the first month of cultivation, whereas indole GLS showed a positive response. It has also been reported that the application of S to S-deficient plants caused the proportion of aliphatic GLS to increase more than the proportion of indoles, probably due to the fact that some aliphatic GLS are derived from the sulfur-containing amino

acid methionine (Falk et al. 2007). In horseradish, we found that, although different GLS classes showed diverse responses to N or N and S fertilization, all increased as the effect of fertilization supply. Among aliphatic GLS, we found that overall SIN is the major GLS in all tissues. Its concentration falls in the wide range reported in the literature varying from 1.0 to 260 μmol g^{-1} dw, depending on genotypes (Agneta et al. 2014b; Li and Kushad 2004; WedelsbäckBladh et al. 2013). Sinigrin and its breakdown products, the allyl-isothiocyanate, has a great potential for medical use (e.g., nasal and sinus disfunction, urinary antiseptic drug, cancer protection) and food industry (e.g., natural preservatives against bacteria and fish oomycete pathogens, cheaper substitute of wasabi) (Wedelsbäck-Bladh and Olsson 2011). We found a large variation of its concentration depending on plant developmental stages and tissues; its concentration increased by providing N and even more providing also S particularly at D1 and D2 stages (period of root harvesting), suggesting that there is a space to increase its production in plants for specific purposes. Concerning the percentage of the minor GLS, we found that in the above-ground tissues SIN is followed by GCX, NAS and GBS, representing on average 1.4, 1.2 and 1 % of the total GLS, respectively. Whereas, a reverse order was observed in roots in which NAS (7 % of the total GLS) was more quantitatively present after SIN, followed by GBS and GCX (4.1 and 3 %, respectively) (Table 3). It has been described that major GLS and their relative proportions are relatively stable for particular species (Kirkegaard and Sarwar 1998). The relative proportion of each GLS in the different tissues could be associated to their biological function that still remains to be clarified.

In conclusion, results of this study indicate that GLS concentrations in horseradish increased as the effect of both N alone and N plus S; they highly varied during plant development showing the highest values at the beginning of plant regrowth. Both above- and below-ground portions contained the highest amount of aliphatic glucosinolates, which represent more than 85 % of the total GLS with sinigrin that is the major GLS present in horseradish. Based on the results, aliphatic and aromatic GLS can be modulated in plants in order to produce horseradish roots with the highest health-promoting glucosinolate content and composition in relation to specific use or application, i.e., fresh market, pharmaceutical and industrial sectors.

Author contribution statement S. De Maria, R. Agneta, A. R. Rivelli: research concept, experiment design and manuscript preparation; F. Lelario: identification of GLS by LC-ESI-FTICR MS and chemical data analysis; C. Möllers: quantification of GLS by HPLC–UV and chemical data analysis. All of the authors revised and approved the final manuscript.

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