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Variation of glucosinolates concentration and root growth of horseradish as affected by nitrogen and sulphur supply

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ABSTRACT

The aim of the study was to evaluate total and individual glucosinolates concentration, root weight and diameters of horseradish accessions (Cor and Mon) grown by adding nitrogen (N) and sulphur (S) and harvested at the beginning (late autumn) and towards the end (early spring) of the harvest period. The concentration of the 9 glucosinolates (GLS) quantified in roots varied greatly between accessions (6-fold higher in Cor) and with fertilization (N alone led to an increase of 64% of GLS with an additional enhancement of 65% when S was added to N). Root GLS concentration was significantly higher in early spring compared to the previous harvest in late autumn (12.5 vs 8.9 μmol/g DW (dry weight)); a drastic decline of GLS (2.7 μmol/g DW) occurred in root harvested in the following autumn from plants left *ad hoc* in the field. The aliphatic sinigrin was the most abundant (80% of total GLS), followed by the aromatic gluconasturtiin and the indolic glucobrassicin (5.8% and 4.7%, respectively). Root diameter and weight increased in plants treated with fertilizers (19% and 61%, respectively) and throughout the harvests with Cor showing values double with respect to Mon (925 vs 476 g/plant). Relationships between GLS concentration and root weight (R^2 = 0.61) and diameter (R^2 = 0.69) were improved by excluding the roots harvested from plants left *ad hoc* in the field that showed the worst performances.

Keywords: above and below-ground tissues; *Armoracia rusticana*; medicinal herb; natural preservative; secondary metabolites

Horseradish (*Armoracia rusticana* Gaertn., Mey., & Scherb., Brassicaceae) is known since antiquity as a folk medicinal herb, natural preservative and dish condiment. The species is cultivated for the roots that have an intense pungent flavour caused by glucosinolates (GLS). The root system consists of a long, cylindrical or tapering main root with several thin lateral roots. The species is usually propagated by planting sections of side roots separated from the main roots collected from the previous year's crop. The harvest period of the roots usually starts once the foliage has been killed by frost (late October–November), 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). As horseradish is a perennial species, if roots are not harvested and left in the field, multiple sprouts arise on the crown generating the new vegetation and a growing cycle will start. Recently the species, approved as seasoning, spice, and flavouring and affirmed as generally recognized as safe by the Food and Drug Administration (FDA 2008), have been gaining an increasing interest due to the richness in GLS that besides providing a high nutritional value could be employed in numerous fields (Nguyen et al. 2013, Wedelsbäck Bladh 2014). The GLS, S-and N-containing compounds, are widely studied in a number of *Brassica* vegetables for their effects on human health (Björkman et al. 2011) and several other biological activities (Zukalová and Vašák 2002). In horseradish, the predominant GLS is the sinigrin, whose breakdown product, the allyl-isothiocyanate (AITC),

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has a great potential for medical use (e.g., nasal and sinus disfunction, urinary antiseptic drug, cancer protection), pharmaceutic and food industry (e.g., natural preservatives against bacteria and fish oomycete pathogens, cheaper substitute of wasabi) (Wedelsbäck Bladh 2014). Former studies revealed a wide variation of GLS depending on endogenous (e.g., genetic variability, root age, phenological stage, tissues or organs) and exogenous (e.g., climate conditions, agronomic practices) factors (Agneta et al. 2013, Nguyen et al. 2013). Among agronomic practices, the fertilization has been largely studied in numerous Brassicaceae crops to maximize benefits for production and GLS content. Several studies highlighted that S led to increases of GLS in most cases (Falk et al. 2007) while N level, form and application timing exhibited diverse responses. Referring to horseradish, the influence of N and S on GLS content was mostly studied in embryoids, suspension cells and calli, and plantlets grown *in vitro*; in the latter, Alnsour et al. (2012) found that GLS concentrations could be modulated 20-fold by varying the sulphate concentration in the medium. About the yield, Perlaki and Djurovka (2009) showed an increase of the root yield from 8.8 to 22.6 t/ha depending on the amount and kind of fertilizer used. The aim of the study was to evaluate the variation of total and individual GLS concentration and root growth of 2 horseradish accessions (Cor and Mon) grown by adding N and S and harvested at the beginning (late autumn) and towards the end (early spring) of the harvest period in order to advance knowledge to improve the quality of horseradish roots, at least in a Mediterranean environment.

MATERIAL AND METHODS

Field experiment was carried out in 2011–2012 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 in water, 1.67‰ total N (Kjeldhal method), 26.7 ppm assimilable P (Olsen method), 227 ppm exchangeable K (ammonium's method acetate), total S lesser than 500 ppm, 2.1% organic carbon (Walkley Black method), 6.20% carbonates (Drouineau method), 0.95 dS/m electrical conductivity (ECe). The climatic conditions along the experimental period were characterized by annual rainfall of 528 mm and 446 mm in 2011 and 2012, respectively, against 546 mm as long-term average (1959–2009). The most rainy period goes from January to March (255 and 190 mm vs 167 mm long-term mean value) while from June to September the most dry conditions occur (40 and 46 mm vs 59 mm long-term mean value). In both years, mean temperatures ranged, on average, from 8 (Jan–Feb) to 26 (Aug) and 10°C (Dec), which do not differ from the long-term average (Figure 1).

Root cuttings (20 cm in length and 1 cm in diameter) of 2 accessions of *A. rusticana*, obtained from local nurseries, named as Cor and Mon were transplanted in single rows (100 cm between rows and 50 cm on the row) on April 6, 2011. Plants were grown without N and S supply as a control (-N-S), with N only (+N-S) by applying 100 kg N/ha as NH_4NO_3, and both N and S (+N+S) by applying 100 kg N/ha as a mixture (1:1) of NH_4NO_3 and $(NH_4)_2SO_4$ to provide 45 kg S/ha. Plants were harvested in three different times: (H1) late autumn (December 4,

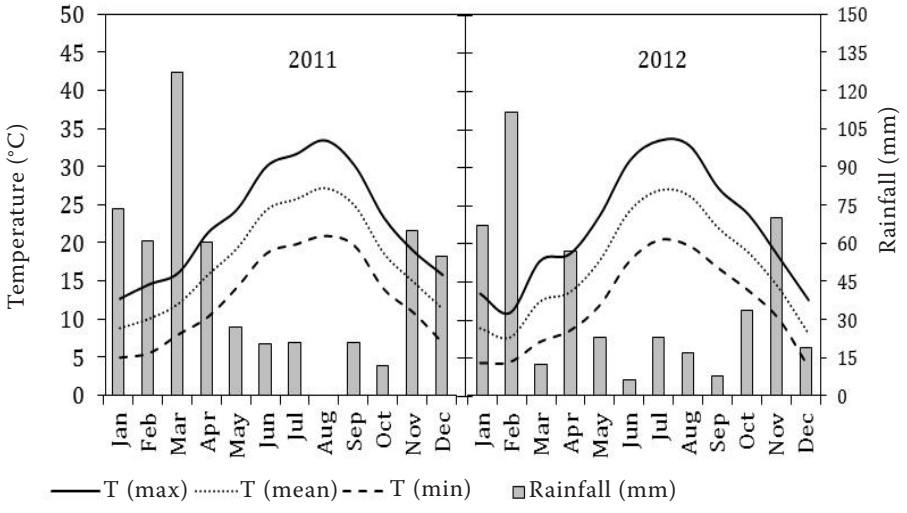

Figure 1. Air temperature and rainfall recorded during the experimental period

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2011) end of the growing cycle when the harvest period of roots for commercial purposes usually starts; (H2) early spring (March 10, 2012) when the harvest usually ends; (H3) late autumn (December 15, 2012) end of the second growing cycle of plants, left *ad hoc* in the field. A split-split-plot design was arranged with fertilization as the main plot (each 8 × 6 m), accession as the subplot and harvesting time as the sub-subplot. At each harvest, 3 plants per treatment were manually dug out, cleaned and divided in main root (including the crown with young sprouts) and side roots. Then, selected morphological descriptors (equatorial and polar diameter at the top and at the base of the main root, number of side roots) were recorded following the UPOV guidelines (2001) and fresh and dry weight (FW and DW) were measured. Samples of the middle portion of the main root and sprouts were processed for GLS quantification by the method described by Agneta et al. (2014a) and later detailed by Lelario et al. (2015). Nine GLS were quantified: glucoiberin (GIB); sinigrin (SIN); gluconapin (GNA); glucochlearin (GCX); glucobarbarin and/or epiglucobarbarin (BAR); glucobrassicanapin (GBN); glucobrassicin (GBS); gluconaturtiin (NAS); 4-methoxyglucobrassicin (4ME).

Statistical analysis was performed by using M-STAT software (version 2.00, East Lansing, USA). Data expressed in percentage were normalized by using arc sin transformation and the variables were tested with ANOVA followed by *LSD* (least significant difference) test to separate the means. Significance levels in the Tables were expressed as $^{*}P < 0.05$; $^{**}P < 0.01$ and $^{***}P < 0.001$.

RESULTS AND DISCUSSION

Root weight and biometric characteristics of horseradish significantly differed between ac-

Table 1. Root weight and diameters of horseradish as affected by accession, fertilization and harvesting time

Source of variance	Main root					Side roots		Total root
	FW (g/plant)	DW (%)	equatorial Ø at the top	polar Ø at the top	basal Ø	number (*n*/plant)	DW (%)	FW (g/plant)
			(cm)					
Accession (A)								
Cor	925^{a}	33.0^{a}	13.6^{a}	10.4^{a}	7.2^{a}	14.6^{a}	36.2^{a}	1213^{a}
Mon	476^{b}	30.3^{b}	9.2^{b}	6.7^{b}	3.7^{b}	6.0^{b}	31.1^{b}	564^{b}
	***	***	***	***	***	***	***	***
Fertilization (F)								
-N-S	497^{b}	30.5	10.1^{b}	7.5^{b}	4.9^{b}	7.9^{b}	32.0	641^{b}
+N-S	778^{a}	32.3	11.6^{ab}	8.6^{ab}	5.8^{a}	11.2^{a}	34.7	983^{a}
+N+S	824^{a}	32.2	12.5^{a}	9.5^{a}	5.6^{a}	11.7^{a}	34.4	1042^{a}
	***	ns	*	*	*	**	ns	***
Harvesting time (H)								
H1	531^{c}	35.0^{a}	9.7^{b}	6.9^{b}	5.7	12.2^{a}	37.7^{a}	754^{b}
H2	699^{b}	31.4^{ab}	10.6^{b}	8.1^{b}	5.2	10.4^{ab}	31.5^{b}	820^{b}
H3	871^{a}	28.7^{b}	13.9^{a}	10.6^{a}	5.7	8.2^{b}	31.9^{b}	1091^{a}
	***	***	***	*	ns	**	***	***
***F*-probability**								
A × F	***	ns	ns	ns	ns	ns	ns	***
A × H	***	ns	ns	**	***	ns	ns	***
F × H	**	ns	ns	**	ns	ns	ns	*
A × F × H	ns	ns	ns	ns	ns	**	ns	*

Within a column, the factor values followed by different letters are significantly different according to $LSD_{0.05}$. FW – fresh weight, DW – dry weight; H1 – late autumn (December 4, 2011); H2 – early spring (March 10, 2012); H3 – late autumn (December 15, 2012). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; ns – not significant

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Table 2. Individual and total glucosinolate (GLS) concentrations in roots of horseradish as affected by accession, fertilization and harvesting time

Source of variance	Glucosinolates (μmol/g of DW)									
	aliphatic					aromatic		indolic		Total GLS
	GIB	SIN	GNA	GCX	GBN	BAR	NAS	GBS	4ME	
Accession (A)										
Cor	0.22	11.2^{a}	0.033	0.33^{a}	0.044	0.09	0.76^{a}	0.59^{a}	0.12^{a}	13.4^{a}
Mon	0.19	1.7^{b}	0.028	0.22^{b}	0.040	0.07	0.18^{b}	0.17^{b}	0.07^{b}	2.7^{b}
	ns	***	ns	**	ns	ns	***	***	**	***
Fertilization (F)										
-N-S	0.23	3.3^{b}	0.023^{b}	0.24	0.030^{b}	0.09	0.30^{b}	0.24^{b}	0.10	4.5^{b}
+N-S	0.18	6.0^{ab}	0.032^{ab}	0.24	0.035^{b}	0.06	0.41^{b}	0.36^{b}	0.08	7.4^{b}
+N+S	0.21	10.1^{a}	0.038^{a}	0.35	0.060^{a}	0.08	0.71^{a}	0.55^{a}	0.09	12.2^{a}
	ns	***	*	ns	*	ns	**	***	ns	**
Harvesting time (H)										
H1	0.27^{a}	6.8^{b}	0.036^{a}	0.40^{a}	0.075^{a}	0.12^{a}	0.54^{a}	0.45^{a}	0.13^{a}	8.9^{b}
H2	0.22^{ab}	10.4^{a}	0.044^{a}	0.35^{a}	0.026^{b}	0.07^{b}	0.68^{a}	0.59^{a}	0.09^{ab}	12.5^{a}
H3	0.13^{b}	2.1^{c}	0.013^{b}	0.08^{b}	0.025^{b}	0.04^{b}	0.19^{b}	0.10^{b}	0.06^{b}	2.7^{c}
	***	***	***	***	***	***	***	***	***	***
***F*-probability**										
A × F	ns	***	***	ns	*	ns	***	**	ns	***
A × H	ns	***	***	***	ns	*	***	***	ns	**
F × H	ns	***	***	ns	***	*	***	**	ns	***
A × F × H	ns	***	**	ns	ns	ns	**	ns	ns	***

GLS are grouped in: aliphatic (GIB – glucoiberin; SIN – sinigrin; GNA – gluconapin; GCX – glucococchlearin; GBN – glucobrassicanapin), aromatic (BAR – glucobarbarin and/or epiglucobarbarin; NAS – gluconasturtiin) and indolic (GBS, glucobrassicin 4ME, 4-methoxyglucobrassicin) classes. Within a column, the factor values followed by different letters are significantly different according to $LSD_{0.05}$. DW – dry weight; H1 – late autumn (December 4, 2011); H2 – early spring (March 10, 2012); H3 – late autumn (December 15, 2012). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ns – not significant

cessions (Table 1) with Cor always showing values greater than Mon. The main root, which includes the crown with the young sprouts and represented approximately 72% of the total root weight, was double in Cor in comparison to Mon (925 vs 476 g/plant, respectively) with diameters larger than 40% (on average). The accessions also differed in response to fertilization and harvesting time (as highlighted by the interactions in Table 1), with values always significantly higher in Cor (data not shown). A wide variability among genotypes based on morphological root and leaf descriptors, root yield as well as on GLS is largely described (Agneta et al. 2014b, Wedelsbäck Bladh 2014). Regardless of the accessions, the N and S supply significantly improved the FW of the main root that increased by about 60% in the fertilized treatments, which did not differ from each other. In addition, the weight of the main root resulted 28% higher at the end of the harvesting period in early spring (H2), compared to the previous harvest in late autumn (H1) with a further increase of 33% harvesting the roots in the following autumn (H3) from plants left in the field *ad hoc* (Table 1). As above-mentioned describing the plant, if the roots are not harvested and are left in the field, a new growing cycle starts and roots continue to grow varying in diameter and weight. Indeed, similar to the weight of the main root, equatorial and polar diameters significantly differed based on fertilization and harvesting time adopted (Table 1); while roots DW of both main and side roots were unaffected by fertilization and slightly decreased over time. Regardless of the treatments, the root weight recorded fell within the range reported in the literature (Perlaki and Djurovka 2009, Agneta et al. 2014b).

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Considering the glucosinolates, 9 different GLS have been detected in variable concentrations in sprouts and roots of horseradish. The sum of those GLS, henceforth referred to as total, showed significant differences as effect of the analysed factors for both roots (Table 2 and Figure 2) and young sprouts (Figure 2). In roots (Figure 2), total GLS gradually decreased in -N-S passing from the harvest H1 to H3 in both accessions whereas, in plants treated with fertilizers it highly increased from H1 to H2 (by about 37% and 96% in Cor and 100% and 84% in Mon, in +N-S and +N+S treatments, respectively) and drastically decreased at the last harvest H3, particularly in Cor accession. In sprouts (Figure 2), the interaction among factors was highly significant ($P \leq 0.001$) showing that in Cor GLS concentration remained almost similar in H1 and H2 harvests and slightly decreased in the last (H3), contrarily to Mon in which it tended to slightly increase over time. As overall mean values across all treatments, the sprouts separated from the main root contained a surprising amount of total GLS up to 12-fold higher than roots (100 vs 8.0 μmol/g DW) (Figure 2), with SIN accounting for 94% of the total GLS (data not shown). In literature, investigations of GLS in horseradish have largely focused on the root tissues while the capability of the above-ground material to synthesize GLS has hitherto received little attention. In roots a wide range of GLS concentration (from 1.7 up to 296 μmol/g DW) depending on genotype is reported by several authors (Li and Kushad 2004, Agneta et al. 2013, Wedelsbäck Bladh 2014). Besides genotype, sulfur fertilization led to enhanced glucosinolate content in a number of *Brassica* species, and increases of over 10-fold were reported (Falk et al. 2007). On wasabi, whose flavor as for horseradish comes from the liberation of the allyl-isothiocyanate (AITC) by the hydrolysis of precursor SIN, Sultana et al. (2002) found that fertilization with ammonium sulphate produced the highest-quality rhizomes with an increase of 72% in AITC yield, while N alone reduced it by up to 15%. Regardless of fertilization and accession, GLS differed depending on harvesting time; GLS were much higher in roots harvested in early spring probably due to the subsequent effect of the winter frost and the following air temperature increases that stimulate the growth of photosynthesizing young sprouts. The lower values observed in autumn, particularly in H3, could be caused by the winter frost effect and the dilution of GLS in tissues. Also Velasco et al. (2007) found that plants sampled during the winter had lower levels of GLS concentration.

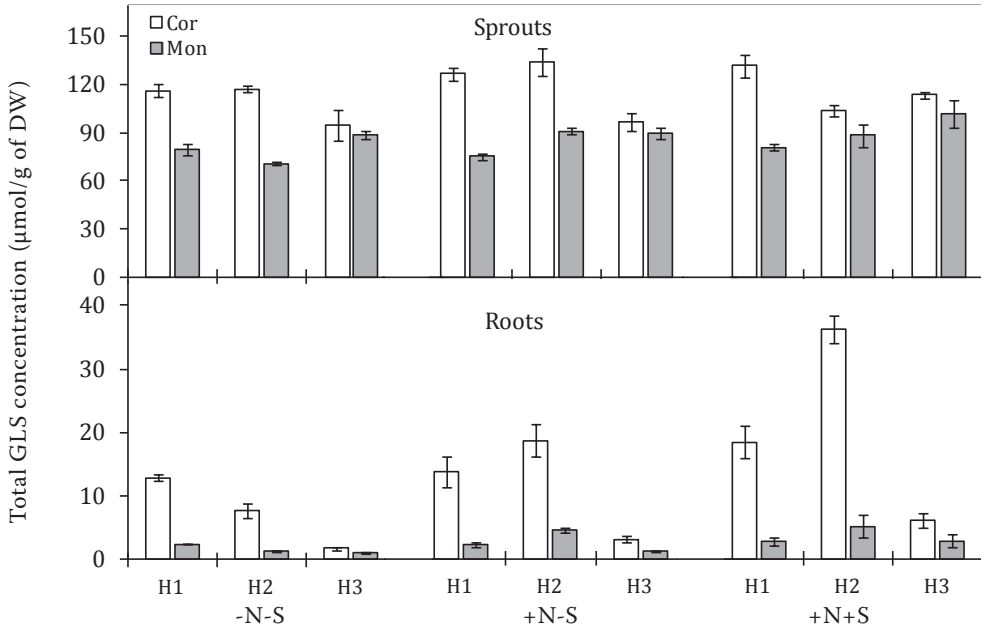

Figure 2. Total glucosinolate (GLS) concentration in sprouts and roots of horseradish as effect of the interaction among accession (Cor and Mon), fertilization (-N-S; +N-S; +N+S) and harvesting time (H1, H2, H3). Values of $LSD_{0.05}$ tests are 4.5 for sprouts and 16.9 for roots. DW – dry weight; H1 – late autumn (December 4, 2011); H2 – early spring (March 10, 2012); H3 – late autumn (December 15, 2012)

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Each of the nine GLS quantified in the tissues (Table 2) differently contributed to the variation of the total GLS in plant. In addition to SIN, NAS and GBS, the main GLS found in horseradish and usually reported in literature, minor GLS, that are rarely quantified, are also reported in Table 2. The aliphatic GLS (GIB, SIN, GNA, GCX, GBN) were always the biggest class, due to the contribution of SIN that was the main GLS, representing on average 80% of the total GLS. The aromatic (BAR, NAS) and indolic (GBS, 4ME) classes, instead, contributed in similar percentage to the total GLS concentration (5.9% and 6.9%, respectively). Changes of those 9 GLS throughout plant development and relationships among them have been already described (De Maria et al. 2016). Comparing the accessions, the aliphatics SIN and GCX, the 2 aromatics BAR and NAS and the indolic GBS resulted significantly higher in Cor than in Mon, whereas no differences were found in the remaining GLS. The +N+S treatment significantly affected SIN, GNA and GBN among the aliphatic GLS, NAS between the aromatics, and GBS between the indolics. In addition, the GLS concentration changed differently throughout the 3 harvests: from the first to the second harvest SIN significantly increased, GBN and BAR significantly decreased, while the remaining GLS resulted unchanged; whereas, all GLS drastically declined at the last harvest (Table 2). Such results suggest that it may be negative to leave the roots in the field for a further growing cycle because despite an increase in root yield and diameters, a drastic decline of GLS concentration can occur in plant.

The GLS concentration and the weight and diameters of roots were analysed together to find relationships between parameters. Considering all data, a weak relationship was found between total GLS concentration and root weight (R^2 = 0.14; P = 0.006) and root equatorial diameter (R^2 = 0.21; P = 0.013), as displayed in the insert of Figure 3a and 3b, respectively. Instead, by excluding the data referred to the last harvest H3 (which showed the worst performances), the parameters are more closely related, optimizing the relationships between total GLS concentration and root weight (R^2 = 0.61; P < 0.001) (Figure 3a) and, even more with the equatorial diameter (R^2 = 0.69; P < 0.001) (Figure 3b). According to USDA guidelines, horseradish is commercialized in three grades based on root diameter and length; Ku et al. (2015) highlighted that higher grades display better quality, with higher sinigrin and AITC contents. Our results suggest that glucosinolates content and composition, root weight and diameter are parameters that could be matched for the production of high-quality horseradish root based on the destination of the product e.g., fresh market, pharmaceutical and industrial purposes. In addition, according to several authors, the large variation in GLS could be

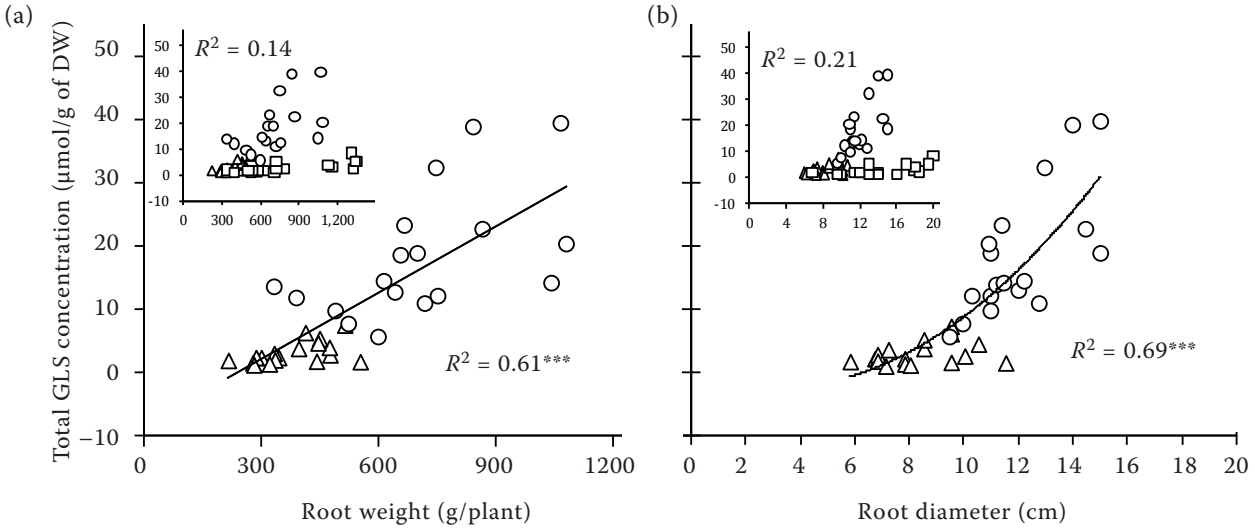

Figure 3. Relationships between total glucosinolate (GLS) concentration and root weight (a) and root equatorial diameter at the top (b) harvested at H1 (triangle) and H2 (circle). Inserts show the same data for the first two harvests plus the data of the last harvest (H3, square). Each data point represents measurements from an individual plant. DW – dry weight; H1 – late autumn (December 4, 2011); H2 – early spring (March 10, 2012); H3 – late autumn (December 15, 2012)

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also manipulated to satisfy chefs and consumers, that look for specific flavor (mild to strong) when the root is used as a food and dish condiment. The genotype and the fertilization particularly with sulphur, should be accurately considered when agricultural practices are specifically targeted to the accumulation of functional metabolites in plants to enhance their organoleptic and health properties, or their value as biofumigants.

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