# Evaluating strategies to improve glucosinolate concentration and root yield of field-grown horseradish in a Mediterranean environment: preliminary results

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## Abstract

Horseradish is grown for its enlarged taproot that is widely used as a dish condiment and as a source of horseradish peroxidase. Nowadays, the species is gaining great interest due to the richness in bioactive compounds that besides providing a high nutritional value are tested for innovative applications in different fields. Nevertheless, the effect of crop management on root yield and glucosinolates (GLS) biosynthesis is poorly documented. Aim of this study was to evaluate the root yield and GLS concentration of two field-grown horseradish accessions (Cor and Mon) grown with nitrogen (N) alone and both N and sulphur (S) (-N-S, +N-S and +N+S treatments) and harvested at different times [late autumn (LA), 2011 and 2012, early spring (ES), 2012]. Yield increased throughout the harvests up to 48% on average of the fertilised treatments and 25% in the unfertilised control. Conversely, root GLS concentration significantly declined in the unfertilised control throughout the harvests [from 7.6 in LA_2011 to 1.43 μmol/g dry matter (DM) in LA_2012] while it highly increased in plants grown with N alone and with both N and S by 46 and 98%, respectively, from LA_2011 to ES_2012 (up to 11.9 and 21.1 μmol/g DM, respectively); then it drastically decreased by 80% on average, in the next harvest. Among individual GLS, the concentration of sinigrin and nasturtin similarly varied as effect of the analysed factors, showing the highest values in Cor accession. The data show that although the level of GLS is highly dependent on genotype, fertilisation and harvesting date may play a primary role in determining the yield and GLS concentration in horseradish root.

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## Introduction

Horseradish (*Armoracia rusticana* Gaertner, Meyer & Scherbius) is known since antiquity as a folk medicinal herb, natural preservative and dish condiment. The plant is cultivated for the thick, fleshy and white roots that have a bitter taste due to the richness in glucosinolates, sulphur- and nitrogen-containing secondary metabolites mainly found in the *Brassicaceae* family. 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 collected from the previous year's crop. The plant is popular in Europe and America; USA is now the largest producer of horseradish in the world (1600 ha). In Europe, the main production takes place in Hungary (1200 ha), but Austria, Germany and Poland are also producers. Until 19$^{th}$ century, in the Northern Europe countries there was a large production of horseradish but today only very few producers are left in those (Wedelsbäck Bladh, 2014) as in many other European countries including Italy (Sarli *et al*., 2012), where the species is still cultivated in small areas, or even in home gardens. However, horseradish is getting a growing interest due to the abundance of bioactive compounds, which in addition to providing a high nutritional value, are tested for innovative applications in various sectors (Wedelsbäck Bladh, 2014). Recently, the Food and Drug Administration approved horseradish as seasoning, spice, and flavouring and affirmed it as *generally recognised as safe* (FAO, 2008). Besides to the horseradish peroxidase, an enzyme commonly used as component of clinical diagnostic kits, in the medical research and neuroanatomy, and in targeted cancer therapy (Veitch, 2004), the plant is very rich in ascorbic acid (Davey *et al*., 2000) and glucosinolates (Agneta *et al*., 2013; Nguyen *et al*., 2013). Unlike most other species which contain only one or at the most 2-5 glucosinolates (GLS) (Clarke, 2010), in horseradish Agneta *et al*. (2013, 2014a) recently identified 17 GLS (11 not previously characterised in plant), some of which being present as isomers or occurring in trace amounts. GLS (particularly sinigrin and its breakdown product, the allyl-isothiocyanate) are known to have beneficial effects on human health and have great potential for medical use (*e.g*., nasal and sinus dysfunction, urinary antiseptic drug, cancer protection) and food industry (*e.g*., natural preservatives against bacteria and fish oomycete pathogens, cheaper substitute of wasabi) (Wedelsbäck Bladh, 2014). GLS are the principal source of anticarcinogenic activity in *Brassica* vegetables and this provides strong reason for the manipulation of GLS levels in vegetables for human consumption (Mithen *et al*., 2000). Despite the crosscutting interest towards the bioactive compounds in horseradish, and the need to preserve local biodiversity, the agricultural research has given relatively little attention to this species that is still considered neglected and/or underutilised although is known to have important issues on the national and local levels. Indeed in Basilicata region horseradish has been recently included in the list of

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the traditional national food products (Ministerial decree n. 168 of 17 June 2015; Italian Regulation, 2015). While the origin and distribution of the species, and its utilisation as food and medical herb are well documented (Agneta *et al*., 2013; Wedelsbäck Bladh, 2014), little is known about the effect of genotype, environmental conditions and crop management on GLS concentration and yield. Among agronomic practices, the effect of fertilisation that has been mostly studied in other *Brassicaceae* crops is poorly documented in horseradish grown in open field and GLS have been mostly studied in plantlets cultivated *in vitro*, embryoids, suspension cells and calli. Recently, Alnsour *et al*. (2012) showed that in *A. rusticana* grown *in vitro*, GLS concentrations could be modulated 20-fold by varying the sulphate concentration in the medium. About the yield, except for Perlaki and Djurovka's paper (2009), which showed an increase of root yield depending on amount and kind of fertiliser used, there are no previous researches in open field regarding the improvement of the quality and yield of horseradish, at least in Mediterranean environment. Aim of the study was to evaluate the root yield and GLS concentration of two field-grown horseradish accessions grown with nitrogen (N) alone and both N and sulphur (S) and harvested at different times.

## Materials and methods

A field experiment was carried out in 2011 and 2012 at Policoro (Italy, 40° 17' 30" N; 16° 65' 16" E) on alluvial, silty-clay soil (sand 39.8%, silt 37.4%, clay 22.8%) with 1.25 kg dm$^3$ bulk density, 7.7 pH in water, 1.67‰ total N (method Kjeldhal), 26.7 ppm available $P_2O_5$ (method Olsen), 227 ppm exchangeable $K_2O$ (ammonium's method acetate), less than 500 ppm total S, 140 ppm sulphate, 5.5 ppm nitrate, 3.64% organic matter (method Walkley-Black), 6.20% carbonates (method Drouineau), 1.15‰ salinity. During the experimental period, maximum and minimum air temperatures were similar 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; scarce precipitations were recorded during the period June-September in both years and no rain in August 2011. Two accessions of *A. rusticana,* obtained from local nurseries of Corleto Perticara (PZ, 40° 23' 00" N; 16° 03' 00" E, 749 a.s.l.) and Montemurro (PZ, 40° 18' 00" N; 15° 59' 00" E, 723 a.s.l.), named as Cor and Mon, respectively, were grown without adding N and S as control (-N-S), with N only (+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 of ammonium nitrate and ammonium sulphate to provide 45 kg S/ha. The experiment was arranged in a split-plot design with fertilisation treatments as the main plot (each of 8×6 m), accession as subplot and harvesting date as sub-subplot, replicated three times.

Root cuttings (20 cm in length and 1 cm in diameter) were transplanted in single rows (100 cm between rows and 50 cm within the row) on April 6, 2011. The fertiliser application was split into three doses at 23, 37 and 70 days after transplanting giving 30, 35 and 35% of the full dose, respectively. Once the foliage was senescent or killed by frost, three plants per treatment were manually dug out at three different harvesting dates: i) late autumn, December 2011 (LA_2011; corresponding to the end of the first growing cycle when the harvest of roots for commercial purposes usually starts); ii) early spring, March 2012 (ES_2012; corresponding to the beginning of vegetative re-growth when the harvest of roots usually ends); iii) late autumn 2012 (LA_2012; corresponding to the end of the second growing cycle of plants, left in field *ad hoc*).

At each harvest plants were cleaned with tap water, divided into sprouts, taproot including the crown and side roots, and weighted. Afterward, sprouts and roots were processed for GLS analysis following the method recently detailed by Lelario *et al*. (2015). Nine GLS were quantified: 3-(methylsulfinyl)-propyl-GLS (or glucoiberin, GIB), 2-propenyl-GLS (or sinigrin, SIN), 3-butenyl-GLS (or gluconapin, GNA), 1- or 2-methylpropyl-GLS (or glucochlearin, and/or glucocoringianin GCX), 2(S)-hydroxy-2-phenylethyl-GLS or 2(R)-hydroxy-2-phenylethyl-GLS (or glucobarbarin and/or epiglucobarbarin, BAR), 4-pentenyl-GLS (or glucobrassicanapin, GBN), indol-3-ylmethyl-GLS (or glucobrassicin, GBS), 2-phenylethyl-GLS (or gluconaturtiin, NAS), 4-methoxyindol-3-ylmethyl-GLS (or 4-methoxyglucobrassicin, 4ME).

Statistical analysis was performed by M-STAT software (version 2.00; Michigan State University, East Lansing, MI, USA). All variables were tested with multifactor analysis of variance followed by least significance difference test to separate the means.

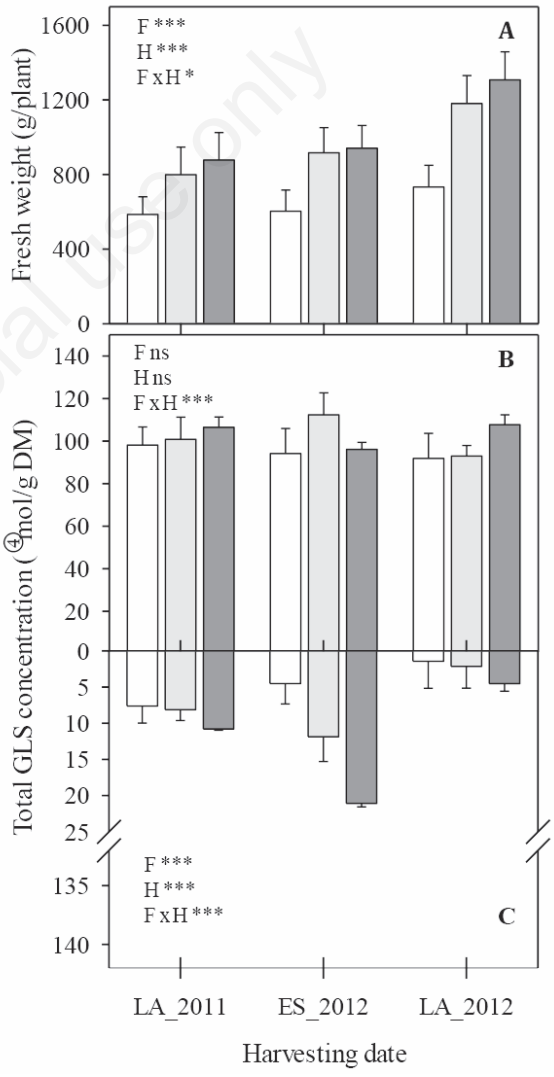


**Figure 1. Effect of fertilisation (-N-S, +N-S, +N+S, respectively white, grey and black bars) and harvesting date (LA_2011, ES_2012 and LA_2012) on root fresh weight (A), and total glucosinolate concentration in sprouts (B) and roots (C) of horseradish. Values are means (n=6) ± standard error. In each graph, ns, *, ***, mean not significant and significant at P≤0.05 and 0.001, respectively. F, fertilisation; H, harvesting date; GLS, glucosinolates; DM, dry matter; LA, late autumn; ES, early spring.**

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## Results and discussion

Root fresh weight and total glucosinolate concentration of horseradish is shown in Figure 1. Since the two accessions tested (Cor and Mon) had similar response to fertilisation and harvesting date, data are shown as average values across accessions. Fresh matter (FM) per plant ranged from 585 (in -N-S treatment in LA_2011) to 1307 g/plant (in +N+S in LA_2012). In each harvest both fertiliser supplies led to an increase of FM, whereby FM tended to be higher when both, S and N were added (+N+S). The fertilisation induced root FM gain in comparison to the untreated control ranged from 43% in LA_2011 to 54% in ES_2012 and 70% in LA_2012 (as average of +N-S and +N+S treatments). Such gains corresponded to the increases in root diameter measured at the top of the taproot over time (data not shown). Usually the harvest of this species covers a long period of time starting once the foliage has been killed by frost in late autumn and is continued through the winter and the early months of the spring when the soil is not frozen and is dry enough to dig roots (Bratsch, 2009; Walters and Whale, 2010). Since horseradish is a perennial crop, if the root is not harvested and maintained in the field, above the ground multiple sprouts on the root crown generate the new vegetation. Thus, a new growing cycle starts and roots continue to grow in diameter and weight. In our case, delaying harvesting date up to early spring (2012), we found FM increases of 3 and 10% in the unfertilised and fertilised treatments, respectively; additional increases of 22 and 48% respectively, were found when the roots were harvested in the following late autumn 2012. The greater FM increase observed in LA_2012 in fertilised in comparison to not fertilised plants was due to the larger root diameter and higher number of sprouts (data not shown) recorded at the end of the first year of growing, that was associated with a greater number of leaves (and leaf area index) during the second growing cycle (data not shown). Horseradish is a crop that needs a lot of nutrients and its powerful root system drains large amounts of soil moisture and nutrient reserves (Perlaki and Djurovka, 2009). For *A. rusticana* general fertilisation recommendations are about 100-200 kg N/ha, 100-150 of P, 100-150 of K and 15-50 of S; nevertheless, until today not many detailed studies about types and quantities of fertilisers, and effects of fertilisation on root yield and glucosinolates composition are available. Perlaki and Djurovka (2009) comparing mineral and organic fertilisers in a three-years experiment in open field found a wide range of yields from 8.8 to 22.6 t/ha depending on amount and kind of fertiliser used. In our case, root FM did not significantly differ between plants fertilised only with N and plants fertilised with both, N and S. However, fertilisation strongly influenced GLS concentration (Figure 1). Indeed, by adding both, S and N, GLS in roots increased by about 33, 77 and 110% in plants harvested in LA_2011, ES_2012 and LA_2012, respectively, compared to adding N alone. In addition, root GLS increased from LA_2011 to ES_2012 by about 46 and 98% in +N-S and +N+S treatments and drastically declined in the next harvest in late autumn 2012 by about 80%, while in unfertilised control it gradually decreased throughout the harvests [from 7.6 in LA_2011 to 1.43 μmol/g dry matter (DM) in LA_2012]. These results suggest that it could be negative to leave the roots in the field for a further growing cycle because in the face of rising yield, the concentration of GLS may decrease in roots that may also have a too large diameter which are generally not appreciated from consumers. At each harvest roots had already formed new young sprouts, which had the highest concentration of GLS, up to 12-fold higher than that of roots (100 *vs* 8.0 μmol/g DM, as overall mean values across all treatments) (Figure 1), with SIN accounting for 94% of the total GLS (data not shown). In sprouts, GLS concentration remained almost constant over time and similarly to the roots they benefited from the fertilisation. It has been reported that sulphur fertilisation allowed an increase in GLS concentration in most cases, even by over 10 times, suggesting that there are substantial opportunities to manipulate the GLS concentration in plants to enhance their organoleptic and health properties, or their value as biofumigants (Falk *et al*., 2007). On wasabi, whose flavour, as for horseradish comes from the liberation of the allyl-isothiocyanate (AITC) by the hydrolysis of precursor SIN, Sultana *et al*. (2002) found that fertilisation with ammonium sulphate produced the highest-quality rhizomes with an increase by 72% in AITC yield, while nitrogen fertiliser alone reduced the AITC yield by up to 15%, showing the importance of sulphur in improving the AITC concentration. Besides the effect of fertilisation and harvesting date, it is well known that genetic variability may play a primary role in determining GLS concentration. However, variations in the composition of GLS among accessions in reference to the number, amount of individual GLS and their percentage of the total are still poorly documented. The

**Table 1. Effect of accession (Cor and Mon), fertilisation and harvesting date on sinigrin, nasturtin, glucobrassicin, glucocochlearin, and trace glucosinolates (as sum of glucoiberin, gluconapin, glucobrassicanapin, epibarbarin and 4-methoxyglucobrassicin) concentration in horseradish roots.**

| Harvesting date | Fertilisation | Glucosinolates (μmol/g DM) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | SIN | | NAS | | GBS | | GCX | | Trace GLS | |
| | | Cor | Mon | Cor | Mon | Cor | Mon | Cor | Mon | Cor | Mon |
| LA_2011 | -N-S | 10.5$^{c}$ | 0.96$^{ef}$ | 0.80$^{bd}$ | 0.12$^{g}$ | 0.56 | 0.13 | 0.41 | 0.36 | 0.63 | 0.84 |
| | +N-S | 11.3$^{c}$ | 1.28$^{ef}$ | 0.92$^{bc}$ | 0.18$^{fg}$ | 0.71 | 0.17 | 0.38 | 0.30 | 0.63 | 0.42 |
| | +N+S | 15.6$^{b}$ | 1.36$^{df}$ | 1.06$^{b}$ | 0.19$^{fg}$ | 0.97 | 0.18 | 0.46 | 0.51 | 0.67 | 0.59 |
| ES_2012 | -N-S | 5.7$^{d}$ | 0.74$^{f}$ | 0.49$^{df}$ | 0.12$^{g}$ | 0.51 | 0.11 | 0.40 | 0.12 | 0.53 | 0.31 |
| | +N-S | 16.6$^{b}$ | 3.47$^{df}$ | 0.72$^{ce}$ | 0.35$^{fg}$ | 0.75 | 0.33 | 0.42 | 0.26 | 0.54 | 0.29 |
| | +N+S | 32.2$^{a}$ | 3.88$^{df}$ | 2.06$^{a}$ | 0.32$^{fg}$ | 1.49 | 0.35 | 0.64 | 0.26 | 0.61 | 0.39 |
| LA_2012 | -N-S | 1.1$^{ef}$ | 0.61$^{f}$ | 0.16$^{fg}$ | 0.12$^{g}$ | 0.07 | 0.09 | 0.11 | 0.04 | 0.30 | 0.26 |
| | +N-S | 2.5$^{df}$ | 0.85$^{ef}$ | 0.21$^{fg}$ | 0.08$^{g}$ | 0.09 | 0.07 | 0.05 | 0.05 | 0.26 | 0.19 |
| | +N+S | 5.3$^{de}$ | 2.15$^{df}$ | 0.40$^{eg}$ | 0.20$^{fg}$ | 0.18 | 0.11 | 0.09 | 0.11 | 0.31 | 0.28 |
| ANOVA sources | | | | | | | | | | | |
| A | | *** | | *** | | *** | | ** | | ns | |
| F | | *** | | ** | | *** | | ns | | * | |
| H | | *** | | *** | | *** | | *** | | *** | |
| A×F×H | | *** | | ** | | ns | | ns | | ns | |

DM, dry matter; SIN, sinigrin; NAS, nasturtin; GBS, glucobrassicin; GCX, glucocochlearin; GLS, glucosinolates; LA, late autumn; N, nitrogen; S, sulphur; ES, early spring; ANOVA, analysis of variance; A, accession; F, fertilisation; H, harvesting date. ns, not significant; *,**,***Values are significant at P≤0.05, 0.01 and 0.001, respectively. $^{a-g}$Values followed by different letters are significantly different for P≤0.05 according to least significant difference test.

concentration of the individual glucosinolates SIN, GCX, NAS and GBS and trace GLS (as sum of GIB, GNA, GBN, BAR and 4ME) as effect of the interaction among factors (accession × fertilisation × harvesting date) is shown in Table 1. Sinigrin is the major GLS found in horseradish representing about 83 and 63% of the total GLS in Cor and Mon, respectively. Its concentration was always higher in Cor than in Mon and varied following the trend of the total. Similar response were observed for NAS, while GBS, GCX and trace GLS were unaffected by the three-way interaction. However, GBS increased in the treatment +N+S only in Cor accession and, like GCX and trace GLS, declined over time.

Such results suggest a wide diversity within the same species in terms of metabolic responses to the environmental factors and cultivation practices. In our previous study (Agneta *et al*., 2014b) on six horseradish accession grown in a Mediterranean environment, we found total GLS concentrations ranging from 1.73 to 35.67 μmol/g DM, with SIN representing from 53% to 87% of the total GLS, NAS accounting from 5% to 15% and GBS from 4.7% to 8.6% of the total GLS. Also Wedelsbäck Bladh (2014), on a total of 168 Nordic accessions of horseradish found that SIN levels varied between 10 and 45 μmol/g DM, NAS between 1.3 and 7.4 μmol/g DM and GBS between 0.1 and 2.6 μmol/g DM, with accessions showing high levels of both SIN and NAS. In our case, SIN and NAS, the most abundant GLS in horseradish, reached values up to 32.2 and 2.1 μmol/g DM, respectively, in Cor (at ES_2012 in +N+S treatment) compared to Mon in which values were quite lower (3.9 and 0.3 μmol/g DM, respectively, in the same treatment). Since GLS give the bitter taste to horseradish, the large variation in GLS concentration could be manipulated to satisfy chefs and consumers, who look for specific flavour (mild to strong) when root is used as a condiment in food.

## Conclusions

In comparison to fertilisation with only N, adding both, sulphur and nitrogen, greatly improved the productivity and quality of the horseradish root. Although the level of GLS is highly dependent on genetic factors, fertilisation and harvesting date may play a primary role in determining the composition and amount of GLS. The best quantity and quality of roots was obtained by delaying the harvest from autumn to early spring, that curiously is the traditional period of higher consumption of the fresh root in the Mediterranean environment (as the root is usually consumed to prepare traditional dishes during the carnival and Easter period).

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