Horseradish (Armoracia rusticana G. Gaertn., B.Mey. & Scherb.) cultivated in Trentino-Alto Adige (Northern Italy) characterized by biometric traits and glucosinolate content.

Journal:	*Plant Genetic Resources*
Manuscript ID	PGR-RA-21-219.R2
Manuscript Type:	Research Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Fusani, Pietro; Consiglio per la ricerca in agricoltura e l'analisi dell'economia agraria, Research Centre for Forestry and Wood Aiello, Nicola; Consiglio per la ricerca in agricoltura e l'analisi dell'economia agraria, Research Centre for Forestry and Wood Giannì, Sergio; Consiglio per la ricerca in agricoltura e l'analisi dell'economia agraria, Research Centre for Forestry and Wood Camin, Federica; Edmund Mach Foundation Department Food Quality and Nutrition; University of Trento, Centre Agriculture Food Environment C3A; International Atomic Energy Agency Pagnotta, Eleonora; Consiglio per la ricerca in agricoltura e l'analisi dell'economia agraria, Research Centre for Cereal and Industrial Crops Bagatta, Manuela; Consiglio per la ricerca in agricoltura e l'analisi dell'economia agraria, Research Centre for Cereal and Industrial Crops
Keywords:	Horseradish, morphological traits, agronomic features, germplasm, glucosinolates
Abstract:	Horseradish is a crop grown for its edible underground parts. The development of new cultivars is hindered by the species' predominant vegetative reproduction, making it essential to evaluate locally cultivated accessions to identify new types suitable for cultivation. To this end, eleven horseradish accessions from family vegetable gardens in Trentino-Alto Adige, Italy, were examined using 26 qualitative and six quantitative morphological descriptors and characterised by the five major glucosinolates (GSLs) present in the rhizome compared to a reference cultivar. A wide range of variability was observed for the considered qualitative morphological traits. The rhizome's top and basal diameters were 9.9 and 6.2 cm, respectively, with an average fresh weight of 521 g. Total GSL content ranged between 79.5 and 133.5 μmol·g-1 dry weight (DW), with sinigrin (SIN) being the primary component at an average content of 110.0 μmol·g-1 DW. Differences among the investigated accessions were noted for quantitative traits describing their productive features and for GSL content. A positive correlation was discovered between the biometric traits of the plant's underground parts and the SIN and total GSL content, suggesting a link between the quality and yield of the edible product. According to the multivariate analysis, accessions were grouped into three main clusters: the largest of the reference cultivar and the majority of accessions with similar productive and qualitative traits; another featuring two with good qualitative and productive characteristics. The investigated accessions proved to be a valuable germplasm source for cultivating the species.

SCHOLARONE™
Manuscripts

Horseradish (*Armoracia rusticana* G. Gaertn., B.Mey. & Scherb.) cultivated in Trentino-Alto Adige (Northern Italy) characterized by biometric traits and glucosinolate content.

Pietro Fusani[a*], Nicola Aiello[a], Sergio Gianni[a], Federica Camin[b], Eleonora Pagnotta[c], Manuela Bagatta[c]

[a] Council for Agricultural Research and Economics, Research Centre for Forestry and Wood, piazza Nicolini 6, 38123 Trento, Italy

[b] Edmund Mach Foundation, Food quality and nutrition Department (DQAN), via Mach 1, 38010 San Michele all'Adige, Italy; Centre Agriculture Food Environment C3A, University of Trento, Via Mach, 1, 38010, San Michele all'Adige (TN), Italy; current address: International Atomic Energy Agency, Vienna International Centre, PO Box 100, A-1400 Vienna, Austria

[c] Council for Agricultural Research and Economics, Research Centre for Cereal and Industrial Crops, Via di Corticella 133, 40128 Bologna, Italy

* Corresponding author. E-mail: *pietro.fusani@crea.gov.it*

For Peer Review

ABSTRACT

Horseradish is a crop grown for its edible underground parts. The development of new cultivars is hindered by the species' predominant vegetative reproduction, making it essential to evaluate locally cultivated accessions to identify new types suitable for cultivation. To this end, eleven horseradish accessions from family vegetable gardens in Trentino-Alto Adige, Italy, were examined using 26 qualitative and six quantitative morphological descriptors and characterised by the five major glucosinolates (GSLs) present in the rhizome compared to a reference cultivar. A wide range of variability was observed for the considered qualitative morphological traits. The rhizome's top and basal diameters were 9.9 and 6.2 cm, respectively, with an average fresh weight of 521 g. Total GSL content ranged between 79.5 and 133.5 µmol·g^{-1} dry weight (DW), with sinigrin (SIN) being the primary component at an average content of 110.0 µmol·g^{-1} DW. Differences among the investigated accessions were noted for quantitative traits describing their productive features and for GSL content. A positive correlation was discovered between the biometric traits of the plant's underground parts and the SIN and total GSL content, suggesting a link between the quality and yield of the edible product. According to the multivariate analysis, accessions were grouped into three main clusters: the largest of the reference cultivar and the majority of accessions with similar productive and qualitative traits; another featuring two with good qualitative and productive characteristics. The investigated accessions proved to be a valuable germplasm source for cultivating the species.

Keywords: Horseradish; morphological traits; agronomic features; germplasm; glucosinolates.

For Peer Review

INTRODUCTION

Horseradish (*Armoracia rusticana* G.Gaertn., B.Mey. & Scherb.) is a herbaceous perennial plant that belongs to the Brassicaceae family. It is native to Southern Russia and Eastern Ukraine and has been naturalised throughout most of Europe (Ball, 1964). Although wild populations of the species are no longer reported, it is still cultivated today as a crop and is appreciated for the edible features of its underground parts (Sampliner and Miller, 2009). The main cultivation areas of horseradish are in Northern and Eastern European countries and the United States (Shehata *et al.*, 2009; Filipović *et al.*, 2015).

Horseradish roots and rhizomes have long been used as a spice in traditional dishes (Courter and Rhodes, 1969; Fahey *et al.*, 2001; Agneta *et al.*, 2013) and as a condiment due to the presence of several compounds in the plant tissues. Glucosinolates (GSLs), which are secondary metabolites of Brassicaceae and precursors of isothiocyanates (ITCs), are responsible for the characteristic pungent and spicy taste obtained from the underground parts of the horseradish plant (Agneta *et al.*, 2013).

Bell *et al.* (2021) characterised the pungent aroma of some Brassicaceae and identified 75 volatile compounds in the headspace of *Armoracia* roots, including 38 previously unreported compounds and GSL hydrolysis products such as allyl ITC and phenetyl ITC, which had the highest relative abundances. Brassicaceae vegetables are a rich source of phytochemicals with high nutritive and nutraceutical value. Plant extracts obtained from Brassicaceae have numerous bioactive properties, including antimicrobial, antioxidant, anti-inflammatory, cardioprotective, immunomodulating, and antimutagenic functions (Favela-González *et al.*, 2020; Peña *et al.*, 2022). Over 130 GSLs have been characterised in plants to date (Blažević *et al.*, 2020), and after crushing or chewing, these secondary metabolites are hydrolysed and release ITCs, which are the main reaction products formed at neutral pH in the presence of the myrosinase enzyme. The presence of GSLs, which have different profiles and contents in all Brassicaceae species (Agerbirk and Olsen, 2012; Blažević *et al.*, 2020), has been shown to have a chemopreventive effect by facilitating the inhibition of mutagenic factors and cancer growth (Velasco *et al.*, 2010; Zhang *et al.*, 2020; Peña *et al.*, 2022).

To date, 17 GSLs have been identified in the roots and sprouts of *A. rusticana* (Agneta *et al.* 2012, 2014b). More recently, Dekić *et al.* (2017) identified, for the first time, the presence of 5-phenylpentyl ITC in the aerial parts of the species. This compound is a probable natural hydrolysis product of a 5-phenylpentyl GSL or glucoarmoracin, as proposed by the authors. It may have interesting antimicrobial and spasmolytic effects. The therapeutic effects of *Armoracia* are mainly attributed to its GSL content, and the plant's medicinal uses, which have been known since

For Peer Review

antiquity (Wedelsbäck Bladh and Olsson, 2011; Walters, 2021), are now limited to traditional uses still surviving in some countries (Sampliner and Miller, 2009; Papp *et al.*, 2018).

Several studies have investigated the health effects of horseradish and reported its ability to prevent cancer proliferation, decrease plasma cholesterol, and exhibit antimicrobial and antioxidant activity (Agneta *et al.*, 2013; Popović *et al.*, 2020; Walters, 2021). Most reports published on the characterisation of horseradish from different origins have mainly focused on its GSL content. Li and Kushad (2004) evaluated 27 accessions of horseradish varieties from North America and Eastern Europe and reported a wide range of variability in the GSL amount in different plant tissues. Wedelsbäck Bladh *et al.* (2013) reported the GSL content of 168 accessions from Nordic European countries, while Ciska *et al.* (2017) evaluated the GSL quantity of four European landraces.

While the bio-agronomic characterisation of accessions is essential for the horseradish germplasm description and classification (Agneta *et al.*, 2013), only a few authors have reported the agronomic and morphological traits of the investigated accessions. Katalyin (2012) described the morphological and agronomic features of 78 cultivated varieties, mainly of Hungarian origin. Wedelsbäck Bladh *et al.* (2014) evaluated the morphological traits, level of genetic diversity, and GSL content of 176 accessions.

Horseradish is a rhizomatous geophyte (Pignatti, 2017) with an underground part consisting of a rhizome and roots that are often difficult to distinguish. Most authors report roots as the main exploited part of the plant (Nguyen *et al.*, 2013), with a primary or marketable root being distinguished from secondary roots (Walters and Wahle, 2010). The presence of rhizome in horseradish is responsible for the species' characteristic of being propagated by its hypogeous parts, as it rarely produces viable seeds and is commercially propagated by rhizome cuttings (Sampliner and Miller, 2009). However, this characteristic also presents an obstacle to improving the crop by developing new cultivars (Walters *et al.*, 2016). Horseradish cultivars with some degree of variability are grown worldwide (Walters and Wahle, 2010). In Italy, where the species is cultivated or naturalised near vegetable gardens (Pignatti, 2017), Sarli *et al.* (2012) reported the morphological traits of 32 cultivated accessions from Basilicata in Southern Italy. The traditional uses of horseradish in the Basilicata region were reported by Agneta *et al.* (2013), who later evaluated six accessions from the same region for their morphological and productive traits, GSL content, and genetic diversity (Agneta *et al.*, 2014a).

Despite the extensive cultivation or naturalisation of horseradish being reported in the northern regions of Italy (Fiori, 1923; Pignatti, 2017) and particularly in Trentino-Alto Adige (Dalla Torre, 1909; Dalla For, 1969; Aeschimann *et al.*, 2004), to our knowledge, no reports on the

For Peer Review

characterisation of cultivated accessions from this area have been published to date. The interest in this region stems from the presence of typical small-scale mountain farming, known locally as "Maso" (Mori and Hintner, 2013), where farmers grow horseradish in vegetable gardens for self-consumption. The underground parts of the plant are traditionally used to prepare a sauce mainly used as a condiment for meat dishes. Farmers usually propagate horseradish themselves, maintaining and conserving their cultivated accessions. This tradition is mainly preserved by the German-speaking ethnic group living in the region and probably originated from the use of the plant, which has been known since ancient times and is widespread in the areas situated further north of the Alps (Mattioli, 1568). Moreover, recently, the Trentino-Alto Adige region has been the subject of studies on locally cultivated plants (Lucchin *et al.*, 2003; Heistinger and Pistrick, 2007), highlighting the importance of this area for the presence of underutilised plant genetic resources.

This study aims to evaluate horseradish accessions cultivated in vegetable gardens in the Trentino-Alto Adige region by describing their morphological and productive traits and quantifying the main GSLs contributing to defining the quality of the obtained product when compared to a reference cultivar. Characterising the investigated accessions could be important in expanding the existing knowledge on the germplasm consistence of the species and identifying possible new genetic resources suitable for cultivation.

MATERIALS AND METHODS

Plant material

The investigated accessions were collected from local vegetable gardens in the Trentino-Alto Adige region (see Supplementary Figure 1) between 2012 and 2016. Exact details regarding their sites of origin are reported in Table 1 and online in Supplementary Figure 2.

Table 1

The cultivated variety Bagameri 93/1 was obtained from the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK) in Gatersleben, Germany. During the collection of accessions from horseradish growers, ethnobotanical information was acquired through interviews using a set of open-ended questions regarding the characteristics of the interviewee, plant origin, cultivation techniques used, and product uses. The collected accessions were conserved *in vivo*, *ex situ*, at the experimental farm "Nicolini" of the Research Centre for Forestry and Wood of the Council for Agricultural Research and Economics in Trento, Italy (46°02'53'' N, 11°08'51'' E; 370 m a.s.l.), an area characterised by a temperate sub-continental climate and a Hyperskeleti-rendzic leptosol soil

For Peer Review

type (IUSS, 2015). On March 2, 2016, all collected accessions were simultaneously vegetatively propagated and transplanted into a field settled at the same experimental farm, with climatic conditions and soil characteristics representative of the growing sites from which the cultivated accessions originated.

Propagation was carried out using root cuttings that were 20 cm in length and 1 cm in diameter, transplanted at distances of 120 cm between rows and 70 cm within the row. The field was prepared beforehand by creating ground beds of approximately 30 cm in height, where the cuttings were laid at a 45° angle, following the polarity of the roots, with the proximal end (the point of attachment to the mother plant's rhizome) placed on top of the ground. Six plants per accession were transplanted in single rows and cultivated in 2016, following the local cultivation practices for the species, including watering at the time of transplantation and subsequently as needed and manual weed control (Supplementary Figure 3). Fertilisation was carried out one month before transplantation by applying 50 kg·ha^{-1} of N as ammonium nitrate, 50 kg·ha^{-1} of K, 30 kg·ha^{-1} of S as potassium sulfate, and 50 kg·ha^{-1} of P as mineral superphosphate on the entire area. No further manuring was done during cultivation.

Morphological characterisation

Morphological characteristics were recorded following the UPOV guidelines for horseradish TG/191/2 "Guidelines for the Conduct of Tests for Distinctness, Uniformity and Stability of Horseradish (*Armoracia rusticana* Gaertn. Mey. et Scherb.)" (UPOV, 2001), using the complete set of 29 characters. Characters related to aerial parts (UPOV descriptors n° 1–17), except for the height of stems at full flowering, were recorded in the first year of cultivation on all six plants per accession since no missing plants were recorded, on the fourth fully developed leaf and the first incised leaf, following the UPOV guidelines. Characters related to the underground parts were recorded on three plants per accession during the harvest, conducted manually between March 8 and 16, 2017, at the beginning of the vegetative growth phase in the second year after transplant. The harvest was conducted during this period to follow the local practice linked to the traditional use of the product during the Easter festival.

The height of stems at full flowering (UPOV descriptor n°29) was recorded during the second year after transplant for the plants left after harvest. Some of the quantitative morphological traits included in the UPOV descriptor list, related to the dimension of the rhizome (UPOV descriptors n°20, 21, and 22), were measured instead of being described by classes. The total fresh weight of all underground parts of the plant, which is not included in the UPOV guidelines, was also measured as an additional character. The quantitative traits related to the dimension of the leaf,

For Peer Review

including length, width, their ratio, and those of the petiole (respectively, UPOV descriptors n°2, 3, 4, 13, and 14), were measured and transformed into classes by following the method described by Sarli *et al.* (2012), which established the limit values of each class so that the arithmetic mean was the central value, and each class included at least 10% of cases concerning the total.

Sample preparation and GSL analysis

To determine the GSL content, samples of rhizomes from three harvested plants per accession were prepared as follows: immediately after recording the morphological traits of the underground parts, the rhizomes were cleaned with distilled water, dried with a paper towel, and a central portion of their tissue was cut and separated from the rest. The tissue was quickly put in liquid nitrogen, then freeze-dried and stored in sealed paper bags at room temperature until analysis. The residual parts of the rhizome were then dried in a thermostatic ventilated oven at 105°C until steady weight to determine dry matter content. Two hundred milligrams of finely powdered freeze-dried samples were extracted with boiling 70% ethanol. The GSLs were desulfated and analysed by HPLC-UV in compliance with the EU official ISO 9167–1:2019 method, with minor modifications (Pagnotta *et al.*, 2017).

Desulfated-GSLs were detected by monitoring their absorbance at 229 nm and identified based on the retention times and UV spectra of desulfo-GSL standards available in our library (Wathelet *et al.*, 2004; Galletti *et al.*, 2015; Müller *et al.*, 2018). Their content was quantified using isolated epi-Progoitrin [(2S)-2-hydroxy-but-3-enyl-GSL] as an internal standard (250 µl of a solution 0.031 µmol/µl). The epi-Progoitrin standard was isolated from *Crambe abyssinica* Hochs t. ex R. E. Fries (Daubos *et al.*, 1998) at a purity of 96% on a weight basis and stored at -20°C until required. Sulfatase from *Helix pomatia* (Sigma Aldrich, USA) was purified according to ISO 9167-1:2019 and stored at -20°C until required.

Statistical analysis

Quantitative measured traits were subjected to one-way ANOVA, with the percentage data previously transformed into angular values (arcsin/√x), and statistical mean comparisons were calculated following Tukey's (HSD) test. Agglomerative hierarchical clustering (AHC) was performed using the Unweighted Pair Group Medium Average fusion method and the Euclidean distances as criteria. These statistical analyses, principal component analysis (PCA), and correlation analysis were conducted using the XLSTAT software package (Addinsoft, New York, US).

RESULTS

For Peer Review

Ethnobotany and uses

Among the 11 interviewees who grew horseradish, six were male. Only one was under 30 years old, while eight were over 50 years old, and two were over 70 years old. Five of them were professional farmers who owned the typical local mountain farm, called "Maso", while two were professionals in the food business. They all reported cultivating their horseradish accessions since they received the plant from their forefathers. They used the obtained product for self-consumption and grew plants in a limited part of their home garden. Only one interviewee cultivated it on a larger surface (around 100 square meters) to sell locally. The edible parts of the plants consumed in line with tradition were rhizomes and roots, which were used fresh and ground to prepare a sauce by mixing it with various ingredients, including apples (five interviewees), cream (four interviewees), vinegar, bread (four interviewees), salt (two interviewees), sugar, pepper, and cranberry jam (one interviewee).

Two interviewees reported using edible flowers, and one grower mentioned that young leaves were eaten according to tradition. Both flowers and leaves were used for preparing salads. All interviewees stated that spring (March or April) is the optimal period for harvesting horseradish underground parts, consistent with the traditional use of the obtained sauce. Five recalled the custom of combining the sauce with boiled ham, a traditional dish prepared during the Easter period. In some villages of the province of Bolzano, the tradition of bringing the foods, including the horseradish root, consumed during the Easter lunch to the Easter Mass for blessing is still alive (online Supplementary Fig. 4). Three respondents highlighted that roots harvested in autumn are too stringy to prepare a good sauce. One interviewee recalled the medicinal use of horseradish underground parts, applied fresh externally to cure contusions. Another reported the plant protection features of horseradish. It was cultivated under fruit trees to prevent plant pathogens, possibly due to the well-known nematocidal effect of GSLs in the plant's underground parts (Van Dam *et al.*, 2009).

Morphological characterisation

Great variability in qualitative traits was observed among accessions, except for the leaf's absent or very weak glaucosity, the mostly smooth surface texture of the rhizome, generally whitish internal colour, and the presence of more than three shoots on the crown of the rhizome in all accessions. Regarding the leaf, the predominant shape was elliptic (66.7%). Most accessions were characterised by medium-sized leaf blades (medium length 58.3%, medium width 75.0%) and petioles (medium length 66.7%, medium width 50%), medium glossiness of the upper side (66.7%), medium twisting of the tip (58.3%), and medium serration (66.7%) of the leaf blade. The undulation of the margin

For Peer Review

was strong in half of the investigated accessions, and the colour of the midrib was whitish in most of them (66.7%), while just two accessions presented anthocyanin colouration at the base of the petiole. The incised leaf's appearance was mostly early (58.3%), and incisions, when present, were weak (25%). As for the rhizome, it was obtriangular (75%), straight (50%), and had weak colouration of the flesh (50%) in the majority of accessions, with a medium density of side roots both at the upper third (66.7%) and at the base (66.7%) (Supplementary Tables S1, S2, and S3). The photographs of the investigated accessions are included in the Supplementary materials (Supplementary Figures 5–16).

Productive traits

The measured biometric traits of the underground parts are given in Table 2.

Table 2

On average, the top diameter of the rhizomes of the accessions was 9.9 cm, and the basal diameter was 6.2 cm. The top diameters of ANT, LAI, and NOV and the basal diameters of BAD and BAG were larger than those of ALD and BED accessions. The average fresh weight of rhizomes was 521.6 g, with BAD having a higher weight (721.6 g) than BED accession (188.1 g). The percentage of the dry matter content of rhizomes (data not shown) was 25.9% on average, with higher values in BAD (28.2%) compared to ALD, ANT, and BED. The mean fresh weight of the underground parts, including rhizomes and roots, was 1485.3 g, with higher weights in BAD, BAG, and LAI than ALD and BED accessions. BAD, LAI, and the reference cultivar BAG showed better productive characteristics, such as larger rhizome diameters and higher fresh weights of rhizomes and whole underground parts, compared to other accessions.

Qualitative traits

Five major GSLs were identified and quantified in the investigated accessions: glucoiberin (GIB), sinigrin (SIN), gluconapin (GNA), glucobrassicin (GBS), and gluconasturtin (GST). Their contents are presented in Table 3, and a representative HPLC-UV profile of GSLs is shown in Supplementary Figure 17.

Table 3

For Peer Review

The total amount of identified GSLs ranged from 133.5 to 79.5 µmol·g^{-1} DW in NOV and BED accessions, respectively, with an average content of 119.9 µmol·g^{-1} DW. SIN was the predominant GSL in all accessions, ranging from 69.5 in BED to 123.9 µmol·g^{-1} in SCE accession, with an average content of 110.0 µmol·g^{-1} DW, followed by GST, GNA, GBS, and GIB. Highly significant differences among investigated accessions resulted in total and single GSL content ($P<0.01$). Eleven out of 12 accessions produced more than 100 µmol·g^{-1} DW of total GSL, and values higher than 120 µmol·g^{-1} DW were found in eight accessions (ANT, BAD, BUR, LAI, NOV, PUS, SCE, and ZIV) which were significantly different ($P<0.01$) from BED, except for PUS. The cultivar BAG showed no difference from the others for the content of total GSL, which was 113.97 µmol·g^{-1} DW. The SIN content of BED was significantly lower ($P<0.01$) compared to half of the investigated accessions (BAD, BUR, LAI, NOV, SCE, and ZIV). Nine accessions produced more than 100 µmol·g^{-1} DW of SIN. In particular, in BAD, BUR, NOV, SCE, and ZIV, more than 120 µmol·g^{-1} DW were extracted. GST content ranged from 3.9 in BAD to 8.2 µmol·g^{-1} DW produced in ALD, accounting for an average of 5.1% of the total GSL. The remaining GSLs were present in amounts lower than 1.5 µmol·g^{-1} DW and with percentages lower than 1.2% of the identified GSLs.

Multivariate analysis

Measured biometric traits of underground parts and rhizome GSL content are the most important traits in defining the attractive quantitative and qualitative characteristics of the obtainable commercial and edible product of the investigated accessions (Agneta *et al.*, 2014a). A principal component analysis (PCA) was carried out using the mean values of these parameters per accession to explore and summarise the existing variability among them for these traits. A correlation matrix was obtained first among the considered traits (online Supplementary Table S4). As expected, a highly significant positive correlation was observed among the different measured biometric characters of the underground parts of the plant. Additionally, SIN and total GSL content were positively correlated with the evaluated biometric traits, except for the lack of correlation between total GSLs and the rhizome basal diameter. Correlation indexes between biometric traits and SIN ranged from 0.624 to 0.762, while those between the same traits and total GSL ranged from 0.709 to 0.745.

Furthermore, GST content was negatively correlated with three rhizome biometric traits: the basal diameter (-0.798), the fresh weight of the rhizome (-0.709), and total underground parts (-0.614). SIN was positively correlated with Total GSLs, with a correlation index of 0.987, which was highly expected since SIN was the main GSL in the rhizomes. A positive correlation was also found between SIN and GNA (0.587) and, consequently, between GNA and Total GSLs (0.609).

For Peer Review

SIN and GNA belong to the Met-derived GSL group, for which studies on the *Arabidopsis* and *Brassica* genus (Liu *et al.*, 2014; Sønderby *et al.*, 2010) revealed that the two GSLs share many steps of the biosynthetic pathway starting from the same precursor amino acid, methionine. Considering that the final step of synthesising these GSLs is catalysed by the same gene (AOP2), even if the metabolic way splits into two different pathways, we could hypothesise that the content of SIN and GNA in *A. rusticana* accessions may be correlated. The results of the PCA are reported in Table 4.

Table 4

The first three principal components (PCs), with an eigenvalue higher than 1, explained 87.13% of the total variability. PC1 was highly correlated with SIN, total GSL content (T.GSL), and all the evaluated biometric traits. PC2 was highly correlated with GBS and, to a lesser extent, with GNA content, while PC3 was positively correlated with GST and negatively with GIB content (see Table 4). In the score plot obtained for the first two PCA components (Fig. 1), most of the investigated accessions positively correlated with PC1 formed a main cluster, almost entirely included in the right quadrants, together with the reference cultivar BAG. Accessions BED and ALD, negatively correlated with PC1, formed a separate cluster.

Figure 1

To better understand the existing similarities among the accessions for the considered traits and to more accurately identify any clusters of accessions with similar traits, AHC was carried out using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) fusion method and the Euclidean distances. The accessions are grouped into three main clusters in the resulting dendrogram (Fig. 2).

Figure 2

The first cluster, comprising accessions ALD and BED, was characterised by lower values of productive traits such as the dimensions and fresh weight of rhizome and whole underground parts, lower amounts of GIB, SIN, GNA, and total GSL, and the highest amount of GST. The second cluster, composed of seven cultivated accessions and the reference cultivar BAG, was characterised by the highest values of productive traits, particularly regarding the fresh weight of rhizome and

For Peer Review

whole underground parts and the highest amounts of SIN and total GSL. The third cluster grouped accessions LAI and PUS and was characterised by the highest amounts of GIB, GNA, and GBS, in addition to sharing with cluster 2 high values of rhizome and underground part fresh weight, as well as high amounts of SIN and total GSL content.

DISCUSSION

A wide range of variability was observed for the considered qualitative morphological traits. The expression level in BAG accession was identical to those reported in the UPOV guidelines for the same reference cultivar, except for the number of rhizome shoots. It was higher than three in this study and lower than three in the UPOV guidelines. In a study focused on the morphological characterisation of *A. rusticana* accessions from the Basilicata region (Sarli *et al.*, 2012), the authors reported the absence of glossiness in the upper side of the leaf, the green colour of the midrib and the absence of anthocyanin colouration at the base of the petiole in all the accessions. However, a certain variability was observed for these characters in our study.

The rhizome biometric data recorded in this study agree with, or in some cases, are higher than, those reported in the literature. However, a comparison was not always easy due to the different criteria used to differentiate the underground parts of the plant. For this purpose, we referred to the UPOV guidelines (UPOV, 2001), distinguishing a central rhizome from side roots. This differs from other authors who distinguished a main root (Rivelli *et al.*, 2017) or a marketable root (Agneta *et al.*, 2014a) from the others.

The rhizome diameters of the accessions investigated in this study comply with the Standards for Grades of Horseradish Roots of the United States Department of Agriculture, for which the minimum diameter of marketable horseradish roots varies from 1.27 to 3.8 cm, depending on the commercial grade (USDA, 2016). The fresh weight of the rhizomes of the accessions fell within and sometimes exceeded the range of 150 and 600 g indicated by Achleitner and Kaufmann (2013) for a commercial standard. In a study investigating the chemical profile of a horseradish accession (Agneta *et al.*, 2012), smaller diameters of the primary root, both at the top (6 cm) and at the base (2 cm), and lower total fresh root weight (1056 g) were reported compared with our results. However, the same author observed a marketable root weight (733 g) comparable to the highest rhizome weight measured in this study. In Agneta *et al.* (2014a), a study on the evaluation of different accessions from the Basilicata region, the marketable root top diameter values varied from 2.6 to 15.3 cm, a wider range compared to our results. However, the basal diameter, with values from 1.8 to 3.4 cm, had a smaller range than our data. The same authors reported fresh weights of marketable roots ranging from 78 to 732 g and total roots from 121 to 1063 g. These

For Peer Review

values were lower than most obtained in the present study in both cases. Rivelli *et al.* (2017), in a similar study focused on two accessions from the same region in Italy, reported the fresh weight of the main root ranging from 189 to 293 g and that of the total roots from 323 to 424 g. In both cases, these values were lower than those reported in this study.

Regarding the dry matter content, Agneta *et al.* (2012) reported a percentage of 37%, ranging from 24.9 to 36.4% in a subsequent study (Agneta *et al.*, 2014a). In both cases, the dry matter content was higher than our results. The higher weights and larger dimensions of the rhizomes found in this study, compared to those reported in previous papers, could be due to many factors, such as different environmental conditions and cultivation practices.

Regarding cultivation practices, the supply of fertilisers adopted, nitrogen in particular, may have influenced the development of the plants. However, in a study focused on the effect of fertilisation on horseradish cultivation, Rivelli *et al.* (2016) reported that plants cultivated with an N supply of 100 kg·ha^{-1}, double the dose applied in this study, had a rhizome fresh weight of 824 g and a total root weight per plant of 1042 g. These values were slightly higher and lower compared to our results. The dose of fertilisers used in our study was very low compared to those reported in the literature for *A. rusticana* (Walters and Wahle, 2010; Filipović *et al.*, 2015; Ciska *et al.*, 2017).

The importance of environmental and climatic conditions for the cultivation of horseradish has been reported by various authors (Walters and Wahle, 2010; Filipović *et al.*, 2015). Agneta *et al.* (2012, 2014a) and Rivelli *et al.* (2016, 2017) conducted their experimental trials at cultivation sites situated four degrees of latitude south of the site reported in this study and characterised by a different climate. Rivelli *et al.* (2016) reported a long-term annual average rainfall of 546 mm and mean monthly temperatures during the experimental period ranging from 8 to 26°C, whereas the site of cultivation in this study recorded a mean annual average rainfall of 1127.8 mm over the long term period (1979–2017), and minimum and maximum monthly temperatures of 0.9°C (January 2017) and 23.3°C (July 2016), respectively, throughout the experimental season. The different climatic conditions of the growing sites may have influenced the plants' development, including the underground parts and the dry matter content. The latter parameter could possibly cause the higher fresh weight of the underground parts obtained in this study.

The results reported in this study regarding the productive traits of the accessions refer to soil and climatic conditions similar to the vegetable gardens where the plants are locally cultivated. The genetic background of the cultivated accessions was an important factor in this study and probably played a primary role, as it generally does, in affecting the phenotypic variation of plants and the development of commercially important traits described in this study as the dimensions of the rhizome.

For Peer Review

Regarding the qualitative traits of underground parts, the detected GSLs are consistent with *A. rusticana* GSLs found in previous studies (Li and Kushad, 2004; Agneta *et al.*, 2012, 2014b; Wedelsbäck Bladh *et al.*, 2013; Ciska *et al.*, 2017). Among the investigated horseradish accessions, SIN was the predominant GSL. GST, which is typically the major GSL in Brassicaceae roots and is interesting for its toxic effect on soil-borne organisms due to its hydrolysis products (Van Dam *et al.*, 2009), was confirmed to be the second most abundant GSL in the roots of *A. rusticana*. Several studies have shown that the total GSL content can vary significantly in *A. rusticana*. For example, among 27 horseradish accessions, mainly from Eastern Europe and North America, the total GSLs ranged from 2 to 296 μmol·g^{-1} DW, with an average content of 81.0 μmol·g^{-1} DW, and eight accessions had a total content greater than 150 μmol·g^{-1} DW (Li and Kushad, 2004). In Ciska *et al.* (2017), total GSL contents from 70 to 120 μmol·g^{-1} DW were found in different landraces from Central and Northern Europe. However, Wedelsbäck Bladh *et al.* (2013) reported a total GSL content lower than 50–60 μmol·g^{-1} DW in a screening of 168 Nordic accessions. In Agneta *et al.* (2014a), six local accessions collected in the Basilicata region produced significantly different total GSL contents, ranging from 1.73 to 37.7 μmol·g^{-1} DW, a narrower range with lower values compared to many of those found in previous studies and our investigation.

According to the reports above, SIN accounts for over 80% of the root total GSL content, and its variability depends on SIN content (Li and Kushad, 2004; Wedelsbäck Bladh *et al.*, 2013; Agneta *et al.*, 2014a). In our samples, SIN accounted for an average of 91.5% of the identified GSLs, with a less variable range compared to that (53–87%) reported in Agneta *et al.* (2014a) and similar to the percentage values shown in Wedelsbäck Bladh *et al.* (2013). The SIN content of our accessions was higher than half of the SIN values reported by Li and Kushad (2004) and higher than most values reported by Ciska *et al.* (2017), Wedelsbäck Bladh *et al.* (2013), and Agneta *et al.* (2014a, 2014b).

The range of GST concentrations reported here was similar to the values found in Ciska *et al.* (2017) and Wedelsbäck Bladh *et al.* (2013) and higher than the values indicated in Agneta *et al.* (2014a, 2014b). The range of GNA (1.3–1.5 μmol·g^{-1} DW), GBS, and GIB content (0.8–1.7 μmol·g^{-1} DW and 0.4–1.1 μmol·g^{-1} DW, respectively) found in the investigated accessions was similar to the values reported in previous studies. In many species of the Brassicales order, to which Brassicaceae belongs, GSLs are usually present with different contents and profiles depending on the analysed tissue. Typically, four to five GSLs are predominant, while many others with different structures are present in traces in the same species (Verkerk *et al.*, 2009). Among the seventeen GSLs identified in *A. rusticana*, the five major GSLs found in our samples were included. Our data

For Peer Review

confirmed that A. rusticana has a very rich GSL profile compared to other genera of Brassicaceae (Fahey *et al.*, 2001; Verkerk *et al.*, 2009).

Unlike other species, the roots of horseradish do not have a more diversified GSL profile compared to other plant tissues, where SIN, GIB, and GNA, belonging to the aliphatic GSL group, predominate (about 95%), with SIN accounting for around 90% of the total GSL content. GBS and GST, which belong to the indolic and phenethyl GSL structural groups, are present in much lower concentrations, about 2.6% and 2.4% of the total GSLs, according to Agneta *et al.* (2014b) and the results obtained in this study. Additionally, horseradish has a wide range of total GSL content, reaching some of the highest concentrations measured in root vegetables (Li and Kushad, 2004; Verkerk *et al.*, 2009).

It is well-known that photoperiod, temperature, soil type, and biotic and abiotic factors can affect GSL accumulation (Bijorkman *et al.*, 2011; Burow, 2016). In many species of the Brassica genus, it has been shown that GSL biosynthesis is subject to fluctuations in temperature, with an increase in GSL content induced at temperatures less than or around 10°C and at stress temperatures above 30°C (Engelen-Eigles *et al.*, 2006; Schonhof *et al.*, 2007; Verkerk *et al.*, 2009). Rao *et al.* (2021) reported that in *Brassica rapa* seedlings grown under controlled conditions, the content of aliphatic GSLs could increase during short-term high-temperature stress, while indole GSLs increase during long-term stress or stress recovery. In the roots of four horseradish landraces from Central-North Europe (Ciska *et al.*, 2017), SIN content fluctuated according to the harvest time of the year, reaching the highest concentration in October, with SIN values ranging between 70 and 116 $\mu mol \cdot g^{-1}$ DW, higher than the SIN contents in horseradish from Basilicata (Agneta *et al.*, 2014a, 2014b) and some accessions analysed by Li and Kushad (2004).

Although we found significant differences between the lowest and highest total GSL and SIN content in our samples, which varied almost twofold, the high SIN concentrations detected in most horseradish accessions are consistent with the results reported by Ciska *et al.* (2017). This could be explained by the climate characterising the cultivation site, which is likely similar to that of continental European areas, and by the genetic background of the investigated accessions, considering that they all originated from the Trentino-Alto Adige region in northern Italy. This combined effect of environment and genotype might partially explain the difference in root GSL content between the horseradish accessions evaluated in this study and those analysed in Agneta *et al.* (2014a), which originated from Basilicata, a region in southern Italy characterised by different pedoclimatic traits. Regarding the effect of sulfur fertilisation on sulfur-containing compounds, since GSLs can account for up to 30% of the total sulfur content of plant tissues, sulfur supply in cultivated plants of Brassicaceae species can lead to an increase in GSL content (Falk *et al.*, 2007).

For Peer Review

Thus, besides the genotype effect, GSL root accumulation in the underground parts of *A. rusticana* can also be modulated by fertilisation, particularly affecting SIN content and its degradation product, allyl isothiocyanate (allyl-ITC), which mainly gives a bitter, pungent taste to the roots and contributes to the valued flavour of traditional dishes (Agneta *et al.*, 2013; Rivelli *et al.*, 2016).

In Rivelli *et al.* (2016), a major root yield and an increasing GSL concentration concerning the control were detected in *A. rusticana* plants fertilised with nitrogen and sulfur and harvested during vegetative regrowth in early spring. Consistent with Rivelli *et al.* (2016) and, above all, with the local cultivation practices of the sites where the investigated accessions originated, the underground parts of plants in this study were harvested during early spring vegetative regrowth. Furthermore, the soil had been fertilised before cultivation according to local practices. This fact may raise the question of whether the biosynthesis and accumulation of GSLs were favoured in the first stages of the plant's vegetative cycle. However, it seems unlikely that such fertilisation could have influenced the GSL content of the accessions investigated. It must be taken into account that sulfur in the soil is easily washed away, a low sulfur dose (30 kg·ha^{-1} of S as potassium sulfate) was used in this study, and a long time had passed since fertilisation to harvest, which was over one year. These three factors probably reduced the sulfur effect on the GSL content of the investigated accessions.

Regardless of any unverifiable hypotheses, the fact remains that the accessions investigated in this study were all cultivated under the same experimental conditions, and the differences found in their GSL content are thus effective. Plant genetic background plays a primary role in affecting SIN content (Magrath *et al.*, 1993). Studies examining possible effects of the interaction of genotype and environment on GSL content have confirmed that the genotype has a greater influence than climate factors, mainly on aliphatic GSL synthesis (Farnham *et al.*, 2004; Verkerk *et al.*, 2009). In the present study, the variability in quantified GSLs among investigated accessions cultivated under the same experimental and environmental conditions could be attributed to their different genetic profiles.

Finally, some of the results of multivariate analysis, particularly the significant positive correlation between SIN, GSL content, and some of the evaluated biometric traits of underground parts, are partly in contrast with the results of Agneta *et al.* (2014a), who reported that higher GSL concentrations were not always associated with the best root yield traits in six accessions from southern Italy. In our study, the positive correlation between SIN and total GSL content and the main biometric traits of underground parts suggests that the quality and yield of *A. rusticana*'s edible product can be correlated.

For Peer Review

CONCLUSIONS

The accessions examined in this study demonstrated high variability in morphological qualitative and quantitative traits, encompassing both aerial and underground parts. Most of them exhibited biometric traits indicative of productive features, such as rhizome diameters and weights, with values comparable to the reference cultivar and, in some instances, surpassing those found in the existing literature. High variability was also observed in the case of GSL content, where the mean relative percentages aligned with those reported in the literature, while the absolute amounts of GSL often exceeded those documented in prior studies.

GSL content correlated with the rhizome's biometric traits, highlighting their significance in evaluating intraspecific diversity in horseradish. Taking these characteristics into account, the agronomic features of most locally cultivated plants proved to be comparable to those of the reference cultivar. Two types cultivated in the study area, specifically those originating from Laion and Pusteria, distinguished themselves from the others due to their intriguing qualitative and quantitative traits.

By exhibiting notable productive and qualitative features, the accessions cultivated in the Trentino-Alto Adige region serve as a valuable source of germplasm for the cultivation of this species.

ACKNOWLEDGEMENTS

The Ministry of Agriculture, Food and Forestry Policies supported this work under the "Implementation of the FAO International Treaty on Plant Genetic Resources for Food and Agriculture" project. This project aims to facilitate research and experimentation in collecting, characterising, and evaluating plant genetic resources. The authors thank the horseradish growers in Trentino-Alto Adige for generously providing propagation materials from their cultivated accessions. We would also like to thank Heinrich Abraham of the Laimburg Research Centre and the Südtiroler Bäuerinnenorganisation in Bolzano, Italy, for offering valuable information about the study area and granting permission to publish the image of the Easter basket (available in Supplementary Fig. 4 online). Furthermore, we appreciate the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK) in Gatersleben, Germany, for supplying the Bagameri 93/1 reference cultivar. Lastly, the authors express their gratitude to Katharine Ann Gethin for her meticulous revision of the text in English.

For Peer Review

References

Achleitner A and Kaufmann F (2013) Band 5 Arznei und gewurzpflanzen L-Z. In: Gartenbau FH and Hoppe B (Eds), *Handbuch des Arznei- und Gewürzpflanzenbaus*. Bernburg, Germany: Saluplanta V., p148. ISBN: ISBN-13: 978-3935971348.

Aeschimann D, Lauber K, Moser DM and Theurillat JP (2004) *Flora Alpina.* Bologna, Italy: Zanichelli.

Agerbirk N and Olsen CE (2012) Glucosinolate structures in evolution. *Phytochemistry* 77: 16–45. https://doi.org/10.1016/j.phytochem.2012.02.005

Agneta R, Rivelli A, Ventrella, E, Lelario F, Sarli G and Bufo SA (2012) Investigation of glucosinolate profile and qualitative aspects in sprouts and roots of horseradish *(Armoracia rusticana)* using LC-ESI−Hybrid Linear Ion Trap with Fourier Transform Ion Cyclotron Resonance Mass Spectrometry and Infrared Multiphoton Dissociation. *Journal of Agricultural and Food Chemistry* 60: 7474−7482. https://doi.org/10.1021/jf301294h.

Agneta R, Möllers C and Rivelli AR (2013) Horseradish *(Armoracia rusticana),* a neglected medical and condiment species with a relevant glucosinolate profile: a review. *Genetic Resources and Crop Evolution* 60: 1923–1943. https://doi.org/10.1007/s10722-013-0010-4.

Agneta R, Möller C, De Maria S and Rivelli AR (2014a) Evaluation of root yield traits and glucosinolate concentration of different *Armoracia rusticana* accessions in Basilicata region (southern Italy). *Scientia Horticulturae* 170: 249–255. https://doi.org/10.1016/j.scienta.2014.03.025.

Agneta R, Lelario F, De Maria S, Möllers C, Sabino Aurelio Bufo SA and Rivelli AR (2014b) Glucosinolate profile and distribution among plant tissues and phenological stages of field-grown horseradish. *Phytochemistry* 106: 178–187. https://dx.doi.org/10.1016/j.phytochem.2014.06.019.

Ball PW, 1964. Armoracia Gilib. In: Tutin TG, Heywood VH, Burges NA, Moore DM, Valentine DH, Walters SM and Webb DA (Eds.) *Flora Europaea.* 1st ed, Vol I, Cambridge, UK: Cambridge University Press, p. 284.

Bell L, Kitsopanou E, Oloyede OO and Lignou S (2021) Important odorants of four Brassicaceae species, and discrepancies between Glucosinolate profiles and observed hydrolysis products. *Foods* 10 (5): 1055. https://doi.org/10.3390/foods10051055.

Björkman M, Klingen I, Birch A, Bones AM, Bruce TJA, Johansen TJ, Meadow R, Mølmann J, Seljåsen R, Smart LE and Stewart D (2011) Phytochemicals of Brassicaceae in plant protection and

For Peer Review

human health – Influences of climate, environment and agronomic practice. *Phytochemistry* 72: 538–556. https://doi.org/10.1016/j.phytochem.2011.01.014.

Blažević I, Montaut S, Burčul F, Olsen CE, Burow M, Rollin P and Agerbirk N (2020) Glucosinolate structural diversity, identification, chemical synthesis and metabolism in plants. *Phytochemistry* 169: 112100. https://doi.org/10.1016/j.phytochem.2019.112100.

Burow M (2016) Chapter Two - Complex Environments Interact With Plant Development to Shape Glucosinolate Profiles. In: Kopriva S (Ed.) *Advances in Botanical Research*. Academic Press, 80: 15-30. https://doi.org/10.1016/bs.abr.2016.06.001.

Ciska E, Horbowicz M, Rogowska M, Kosson R, Drabińska N and Honke J (2017) Evaluation of seasonal variations in the glucosinolate content in leaves and roots of our european horseradish (*Armoracia rusticana*) landraces. *Polish Journal of Food and Nutrition Sciences* 67(4): 301–308.

Courter JW and Rhodes AM, 1969. Historical notes on horseradish. *Economic Botany* 23(2): 156–164.

Dalla Fior G (1969) *La nostra flora*. Trento, Italy: GB Monauni.

Dalla Torre KW and Sarnthein LG (1909) *Flora der gefürsteten grafschaft Tyrol.* Innsbruck, Austria: Wagnerschen Universitat.

Daubos P, Grumel V, Iori R, Leoni O, Palmieri S and Rollin P (1998) *Crambe abyssinica* meal as starting material for the production of enantiomerically pure fine chemicals. *Industrial Crops and Products* 7(2–3): 87-193. https://doi.org/10.1016/S0926-6690(97)00047-2.

Dekić MS, Radulović NS, Stojanović NM, Randjelović PJ, Stojanović-Radić ZZ, Najman S and Stojanović S (2017) Spasmolytic, antimicrobial and cytotoxic activities of 5-phenylpentyl isothiocyanate, a new glucosinolate autolysis product from horseradish (*Armoracia rusticana* P. Gaertn., B. Mey. & Scherb., Brassicaceae). *Food Chemistry* 232: 329-339. https://doi.org/10.1016/j.foodchem.2017.03.150.

Engelen-Eigles G, Holden G, Cohen JD and Gardner G (2006) The effect of temperature, photoperiod, and light quality on gluconasturtiin concentration in watercress (*Nasturtium officinale* R. Br.). *Journal of Agricultural and Food Chemistry* 54: 328–334. https://doi.org/10.1021/jf051857o.

Fahey JW, Zalcmann AT and Talalay P (2001) The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. *Phytochemistry* 56: 5–51. https://doi.org/10.1016/s0031-9422(00)00316-2.

For Peer Review

Falk KL, Tokuhisa JG and Gershenzon J (2007) The effect of sulphur nutrition on plant glucosinolate content: physiology and molecular mechanisms. *Plant Biology* 9: 573-81. https://doi.org/10.1055/s-2007-965431.

Farnham MW, Wilson PE, Stephenson KK and Fahey JW (2004) Genetic and environmental effects on glucosinolate content and chemoprotective potency of broccoli. *Plant Breeding* 123: 60–65. https://doi.org/10.1046/j.0179-9541.2003.00912.x.

Favela-González KM, Hernández-Almanza AY and De la Fuente-Salcido NM (2020) The value of bioactive compounds of cruciferous vegetables (*Brassica*) as antimicrobials and antioxidants: A review. *Journal of Food Biochemistry* 44: e13414. https://doi.org/10.1111/jfbc.13414.

Filipović V, Popović V and Aćimović M (2015) Organic production of horseradish *(Armoracia rusticana* Gaertn.,Mey., Scherb.) in serbian metropolitan regions. *Procedia Economics and Finance* 22: 105-113. https://doi.org/10.1016/S2212-5671(15)00232-4.

Fiori A (1923) *Nuova Flora analitica d'Italia*. Firenze, Italy: Ricci.

Galletti S, Bagatta M, Branca F, Argento S, De Nicola GR, Cianchetta S, Iori R and Ninfali P (2015) *Isatis canescens* is a rich source of glucobrassicin and other health-promoting compounds. *Journal of the Science of Food and Agriculture,* 95(1): 158-164. https://doi.org/10.1002/jsfa.6697.

Heistinger A and Pistrick K (2007) 'Altreier Kaffee': *Lupinus pilosus* L. cultivated as coffee substitute in Northern Italy (Alto Adige/Su¨ dtirol). *Genetic Resources and Crop Evolution* 54: 1623-1630. http://doi.org/10.1007/s10722-007-9265-y.

IUSS Working Group WRB (2015) *World Reference Base for Soil Resources 2014, update 2015. International soil classification system for naming soils and creating legends for soil maps.* World Soil Resources Reports No. 106. FAO, Rome, Italy.

Katalin IO (2012) Különböző tormafajták és vonalak fajtakörönkénti jellemzése. PhD thesis, Corvinus University of Budapest, Budapest, Hungary. http://phd.lib.uni-corvinus.hu/632/ (accessed 3 November 2023).

Li X and Kushad MM (2004) Correlation of glucosinolate content to myrosinase activity in horseradish *(Armoracia rusticana)*. *Journal of Agricultural and Food Chemistry* 52: 6950-6955. https://doi.org/10.1021/jf0401827.

Liu S, Liu Y, Yang X, Tong C, Edwards D, Parkin IAP, Zhao M, Ma J, Yu J, Huang S, Wang X, Wang J, Lu K, Fang Z, Bancroft I, Yang TJ, Hu Q, Wang X, Yue Z, Li H, Yang L, Wu J, Zhou Q, Wang W, King GJ, Pires JC, Lu C, Wu Z, Sampath P, Wang Z, Guo H, Pan S, Yang L, Min J, Zhang

For Peer Review

633 D, Jin D, Li W, Belcram H, Tu J, Guan M, Qi C, Du D, Li J, Jiang L, Batley J, Sharpe AG, Park BS,
634 Ruperao P, Cheng F, Waminal NE, Huang Y, Dong C, Wang L, Li J, Hu Z , Zhuang M, Huang Y,
635 Huang J, Shi J, Mei D, Liu J, Lee TH, Wang J, Jin H, Li Z, Li X, Zhang J, Xiao L, Zhou Y, Liu Z,
636 Liu X, Qin R, Tang X, Liu W, Wang Y, Zhang Y, Lee J, Kim HH, Denoeud F, Xu X, Liang X, Hua
637 W, Wang X, Wang J, Chalhoub B and Paterson AH (2014) The *Brassica oleracea* genome reveals
638 the asymmetrical evolution of polyploid genomes. *Nature Communications*, 5: 3930.
639 https://doi.org/10.1038/ncomms4930.

640 Lucchin M, Barcaccia G and Parrini P (2003) Characterization of a flint maize (*Zea mays* L. convar.
641 *mays*) Italian landrace: I. Morpho-phenological and agronomic traits. *Genetic Resources and Crop*
642 *Evolution* 50: 315-327. https://doi.org/10.1023/A:1023578207258.

643 Magrath R, Herron C, Giamoustaris A and Mithen R (1993) The inheritance of aliphatic
644 glucosinolates in *Brassica napus*. *Plant Breeding* 111: 55-72. https://doi.org/10.1111/j.1439-
645 0523.1993.tb00607.x.

646 Mattioli PA (1568) I Discorsi nelli sei libri di Pedacio Dioscoride Anazarbeo della materia
647 medicinale. Venezia, Valgrisi (Ed.), Vol II: pp 406-409.

648 Mori E and Hintner W (2013) Il maso chiuso. La sua storia e la normativa vigente. Bolzano, Italy:
649 Fondazione Università Popolare delle Alpi Dolomitiche.

650 Müller C, Schulz M, Pagnotta E, Ugolini L, Yang T, Matthe, A, Lazzeri L and Agerbirk N (2018)
651 The role of the glucosinolate-myrosinase system in mediating greater resistance of *Barbarea verna*
652 than *B. vulgaris* to *Mamestra brassicae* Larvae. *Journal of Chemical Ecology* 44 (12): 1190-1205.
653 https://doi.org/10.1007/s10886-018-1016-3.

654 Nguyen NM, Gonda S and Vasas G (2013) A review on the phytochemical composition and potential
655 medicinal uses of horseradish *(Armoracia rusticana)* root. *Food Reviews International* 29 (3): 261-
656 275. https://doi.org/10.1080/87559129.2013.790047.

657 Pagnotta E, Agerbirk N, Olsen CE, Ugolini L, Cinti, S and Lazzeri L (2017) Hydroxyl and methoxyl
658 derivatives of benzylglucosinolate in *Lepidium densiflorum* with hydrolysis to isothiocyanates and
659 non-isothiocyanate products: substitution governs product type and mass spectral fragmentation.
660 *Journal of Agricultural and Food Chemistry* 65: 3167-3178.
661 https://doi.org/10.1021/acs.jafc.7b00529.

662 Papp N, Gonda S, Kiss-Szikszai A, Plaszkó T, Lőrincz P and Vasas G (2018) Ethnobotanical and
663 ethnopharmacological data of *Armoracia rusticana* P. Gaertner, B. Meyer et Scherb. in Hungary and

For Peer Review

Romania: a case study. *Genetic Resources and Crop Evolution* 65: 1893–1905. https://doi.org/10.1007/s10722-018-0663-0.

Peña M, Guzmán A, Martínez R, Mesas C, Prados J, Porres JM and Melguizo C (2022) Preventive effects of Brassicaceae family for colon cancer prevention: A focus on in vitro studies. *Biomedicine & Pharmacotherapy*, 151: 113145. https://doi.org/10.1016/j.biopha.2022.113145.

Pignatti S (2017) *Flora d'Italia* Milano, Italy: Edagricole – Il sole 24 ore, 2nd ed., Vol 2: 923-924.

Popović M, Maravić A, Čikeš Čulić V, Đulović A, Burčul F and Blažević I (2020) Biological effects of glucosinolate degradation products from horseradish: a horse that wins the race. *Biomolecules* 10(2): 343. https://doi.org/10.3390/biom10020343.

Rao SQ, Chen XQ, Wang KH, Zhu ZJ, Yang J and Zhu B (2021) Effect of short-term high temperature on the accumulation of glucosinolates in *Brassica rapa*. *Plant Physiology and Biochemistry* 161: 222-233. https://doi.org/10.1016/j.plaphy.2021.02.013.

Rivelli AR, Lelario F, Agneta R, Möllers C and De Maria S (2016) Variation of glucosinolates concentration and root growth of horseradish as affected by nitrogen and sulphur supply. *Plant Soil and Environment* 62 (7): 307-313. https://doi.org/10.17221/750/2015-PSE.

Rivelli AR, Caruso MC, De Maria S and Galgano F (2017) Vitamin C content in leaves and roots of horseradish (*Armoracia rusticana)*: Seasonal variation in fresh tissues and retention as affected by storage conditions. *Emirates Journal of Food and Agriculture* 29 (10): 799-806. https://doi.org/10.9755/ejfa.2017.v29.i10.1294.

Sampliner D and Miller A (2009) Ethnobotany of horseradish (*Armoracia rusticana,* Brassicaceae) and its wild relatives (Armoracia spp.): reproductive biology and local uses in their native ranges. *Economic Botany* 63(3): 303–313. https://doi.org/10.1007/s12231-009-9088-1.

Sarli G, Lisi A, Agneta R, Grieco S, Ierardi G, Montemurro F, Negro D andMontesano V (2012) Collecting horseradish *(Armoracia rusticana,* Brassicaceae): local uses and morphological characterization in Basilicata (Southern Italy). *Genetic Resources and Crop Evolution* 59: 889–899. https://doi.org/10.1007/s10722-011-9730-5.

Schonhof I, Kläring HP, Krumbein A, Clauen W and Schreiner M (2007) Effect of temperature increase under low radiation conditions on phytochemicals and ascorbic acid in greenhouse grown broccoli. *Agriculture, Ecosystems and Environment* 119: 103-111. https://doi.org/10.1016/j.agee.2006.06.018.

For Peer Review

694 Shehata A, Mulwa RMS, Babadoost M, Uchanski M, Norton MA, Skirvin R and Walters SA (2009)
695 Horseradish: botany, horticulture, breeding. In: Janick J (Ed.) *Horticultural reviews.* New York, US:
696 Wiley, pp 221–261. https://doi.org/10.1002/9780470593776.ch4.

697 Sønderby IE, Geu-Flores F and Halkier BA (2010) Biosynthesis of glucosinolates-gene discovery
698 and beyond. *Trends in Plant Science* 15: 283–290.

699 UPOV (2001) International Union for the Protection of New Varieties of Plants. Guidelines for the
700 conduct of tests for distinctness, uniformity and stability. Horseradish (*Armoracia rusticana* Gaertn.,
701 Mey. et Scherb.). TG/191/2. https://www.upov.int/edocs/tgdocs/en/tg191.pdf (accessed 3 November
702 2022).

703 United States Department of Agriculture (USDA) (2016) U.S. standards for grades for horseradish
704 roots. https://www.ams.usda.gov/grades-standards/horseradish-root-grades-and-standards (accessed
705 3 November 2022).

706 Van Dam NM, Tytga TOG and Kirkegaard JA (2009) Root and shoot glucosinolates: a comparison
707 of their diversity, function and interactions in natural and managed ecosystems. *Phytochemistry*
708 *Reviews* 8: 171-186. https://doi.org/10.1007/s11101-008-9101-9.

709 Velasco P, Francisco M and Cartea ME (2010) Glucosinolates in Brassica and cancer. In: Watson
710 RR, Preedy VR, (eds.) *Bioactive Foods and Extracts. Cancer Treatment and Prevention.* Boca Raton,
711 FL, USA: CRC Press (1st ed.), pp. 3-29.

712 Verkerk R, Schreiner M, Krumbein A, Ciska E, Holst B, Rowland I, De Schrijver R, Hansen M,
713 Gerhäuser C, Mithen R and Dekker M (2009) Glucosinolates in Brassica vegetables: The influence
714 of the food supply chain on intake, bioavailability and human health. *Molecular Nutrition and Food*
715 *Research* 53: S219-S219. https://doi.org/10.1002/mnfr.200800065.

716 Walters SA and Wahle EA (2010) Horseradish production in Illinois. *HortTechnology* 20(2): 267-
717 276. https://doi.org/10.21273/HORTTECH.20.2.267.

718 Walters SA, Bernhardt P, Joseph M and Miller AJ (2016) Pollination and sterility in horseradish.
719 *Plant Breeding* 135: 735-742. https://doi.org/10.1111/pbr.12413.

720 Walters SA (2021) Horseradish: A Neglected and Underutilized Plant Species for Improving Human
721 Health. *Horticulturae* 7: 167. https://doi.org/10.3390/horticulturae7070167.

722 Wathelet JP, Iori R, Leoni O, Rollin P, Quinsac A and Palmieri S (2004) Guidelines for glucosinolates
723 analysis in green tissues used for biofumigation. *Agroindustria* 3: 257–266.

For Peer Review

724 Wedelsbäck Bladh K and Olsson KM (2011) Introduction and use of horseradish *(Armoracia*
725 *rusticana)* as food and medicine from antiquity to the present: emphasis on the nordic countries.
726 *Journal of Herbs, Spices & Medicinal Plants* 17 (3): 197-213.
727 https://doi.org/10.1080/10496475.2011.595055.

728 Wedelsbäck Bladh K, Olsson KM and Yndgaard F (2013) Evaluation of glucosinolates in nordic
729 horseradish (Armoracia rusticana). *Botanica* 19(1): 48-56. https://doi.org/10.2478/botlit-2013-0009.

730 Wedelsbäck Bladh K, Liljeroth E, Poulsen G, Yndgaard F and Brantestam AK (2014) Genetic
731 diversity in Nordic horseradish, *Armoracia rusticana*, as revealed by AFLP markers. *Genetic*
732 *Resources and Crop Evolution* 61: 383-394. https://doi.org/10.1007/s10722-013-0042-9.

733 Zhang Z, Garzotto M, Davis EWI, Mori M, Stoller WA, Farris PE, Wong CP, Beaver LM, Thomas
734 GV, Williams DE, Dashwood RH, Hendrix DA, Ho E and Shannon J (2020) Sulforaphane
735 bioavailability and chemopreventive activity in men presenting for biopsy of the prostate gland: a
736 randomized controlled trial. *Nutrition and Cancer* 72: 74-87,
737 https://doi.org/10.1080/01635581.2019.1619783.

For Peer Review

738 **Tables, Figure legends**

739 **Table 1.** Identification code and geographical origin (municipality, province, altitude and
740 geographic coordinates) of *A. rusticana* accessions investigated in this study.

741 **Table 2.** Morphological quantitative traits of underground parts of *A. rusticana* accessions
742 (abbreviated codes are indicated in Table 1).

743 **Table 3.** Glucosinolate content in rhizome of *A. rusticana* accessions (GIB = glucoiberin; SIN =
744 sinigrin; GNA= gluconapin; GBS = glucobrassicin; GST = gluconasturtin; reported values
745 expressed as $\mu mol \cdot g^{-1}$ dry weight).

746 **Table 4.** The principal component analysis results of the *A. rusticana* accessions evaluated with the
747 10 morphological quantitative traits (first three principal components); abbreviated codes of
748 considered traits are indicated in Tables 2 and 3.

749 **Figure 1.** Score plot for the 12 evaluated *A. rusticana* accessions (abbreviated codes are indicated
750 in Table 1).

751 **Figure 2.** UPGMA dendrogram based on the relative scores for the three extracted PCs
752 (abbreviated codes are indicated in Table 1).

753 **Supplementary Figure S1.** One of the vegetable gardens from which the investigated accessions
754 were sourced.

755 **Supplementary Figure S2.** Map of Trentino-Alto Adige region, Italy, showing the localities where
756 the cultivated *A. rusticana* accessions examined in this study originated.

757 **Supplementary Figure S3.** Field trial for the evaluation of *A. rusticana* accessions.

758 **Supplementary Figure S4.** The Easter basket contains the foods consumed during the lunch to be
759 brought to the Easter Mass for blessing, horseradish included.

760 **Supplementary Figure S5.** Cultivated plant of Aldino accession.

761 **Supplementary Figure S6.** Cultivated plant of Anterivo accession.

762 **Supplementary Figure S7.** Cultivated plant of Avigna accession.

763 **Supplementary Figure S8.** Cultivated plant of Badia accession.

764 **Supplementary Figure S9.** Cultivated plant of Bagameri cultivar.

765 **Supplementary Figure S10.** Cultivated plant of Bedollo accession.

766 **Supplementary Figure S11.** Cultivated plant of Burgusio accession.

767 **Supplementary Figure S12.** Cultivated plant of Laion accession.

768 **Supplementary Figure S13.** Cultivated plant of Nova Levante accession.

769 **Supplementary Figure S14.** Cultivated plant of Pusteria accession.

770 **Supplementary Figure S15.** Cultivated plant of Scena accession.

771 **Supplementary Figure S16.** Cultivated plant of Zivignago accession.

For Peer Review

Supplementary Figure S17. Representative chromatogram of glucosinolate profile of *A. rusticana* rhizome (Avigna accession) with the indication of the identified desulfoglucosinolates 1–5, retention time (r.t), UV spectrum of each desulfoglucosinolate, and chromatogram and UV spectrum of Internal standard (I.S.), epiprogoitrin.

For Peer Review

Table 1. Identification code and geographical origin (municipality, province, altitude and geographic coordinates) of *A. rusticana* accessions investigated in this study.

ID code	Denomination	Municipality	Province	Altitude (m a.s.l.)	Latitude (N)	Longitude (E)
ALD	Aldino	Aldino	Bolzano	1194	46°22'11" N	11°21'11" E
ANT	Anterivo	Anterivo	Bolzano	1139	46°16'42" N	11°22'04" E
AVI	Avigna	San Genesio Atesino	Bolzano	1022	46°34'14" N	11°21'40" E
BAD	Badia	San Martino in Badia	Bolzano	1138	46°40'57" N	11°53'44" E
BAG	Bagameri 93/1	-	-	-	-	-
BED	Bedollo	Bedollo	Trento	1049	46°09'40" N	11°17'58" E
BUR	Burgusio	Malles	Bolzano	1154	46°42'23" N	10°31'30" E
LAI	Laion	Laion	Bolzano	1100	46°36'34" N	11°33'58" E
NOV	Nova Levante	Nova Levante	Bolzano	1046	46°25'46" N	11°30'44" E
PUS	Pusteria	Vandoies	Bolzano	1156	46°50'39" N	11°42'57" E
SCE	Scena	Scena	Bolzano	448	46°40'36" N	11°11'19" E
ZIV	Zivignago	Pergine Valsugana	Trento	520	46°03'55" N	11°15'04" E

For Peer Review

Table 2. Morphological quantitative traits of underground parts of *A. rusticana* accessions (abbreviated codes are indicated in Table 1).

Accessions	Traits							
	R.TD (cm)		R.BD (cm)		R.FW (g)		T.FW (g)	
ALD	5.9 ± 1.2	B	3.4 ± 0.7	B	218.6 ± 65.8	AB	626.2 ± 240.6	BC
ANT	12.1 ± 1.4	A	5.8 ± 1.5	AB	586.0 ± 123.5	AB	1749.6 ± 301.0	AB
AVI	9.5 ± 2.2	AB	7.4 ± 3.1	AB	519.4 ± 244.6	AB	1178.3 ± 548.0	AC
BAD	10.3 ± 1.6	AB	8.5 ± 0.6	A	721.6 ± 142.1	A	2038.2 ± 311.8	A
BAG	11.0 ± 0.4	AB	8.6 ± 1.3	A	631.3 ± 252.5	AB	1990.7 ± 82.2	A
BED	6.5 ± 1.1	B	2.8 ± 0.6	B	188.1 ± 58.3	B	582.2 ± 243.4	C
BUR	9.3 ± 0.2	AB	6.6 ± 1.4	AB	536.1 ± 86.1	AB	1304.8 ± 283.6	AC
LAI	12.1 ± 0.9	A	6.1 ± 1.1	AB	690.3 ± 126.2	AB	1914.6 ±223.8	A
NOV	12.1 ± 1.7	A	5.9 ± 1.1	AB	562.4 ± 191.7	AB	1718.2 ± 361.0	AC
PUS	7.8 ± 1.8	AB	5.7 ± 0.7	AB	342.3 ± 94.3	AB	1304.6 ± 543.7	AC
SCE	10.9 ± 1.2	AB	7.1 ± 0.4	AB	631.8 ± 112.1	AB	1718.0 ±161.5	AC
ZIV	10.8 ± 2.6	AB	7.0 ± 1.2	AB	630.8 ± 99.0	AB	1698.0 ± 318.5	AC
Mean	*9.9 ± 2.4*		*6.2 ± 2.0*		*521.6 ± 210.7*		*1485.3 ± 551.3*	

Mean values (N=3) ± SD followed by different letters are significantly different at P<0.01 according to Tukey (HSD) Test. *R.TD* Rhizome top diameter; *R.BD* Rhizome basal diameter; *R.FW* Rhizome fresh weight; *T.FW* Total rhizome and root f.w.

For Peer Review

Table 3. Glucosinolate content in rhizome of *A. rusticana* accessions (GIB= glucoiberin; SIN=sinigrin; GNA=gluconapin; GBS= glucobrassicin; GST= gluconasturtin; reported values expressed as μmol·g^{-1} dry weight).

Accession	GIB	SIN	GNA	GBS	GST	Total
ALD	0.77 ± 0.42 AB	94.97 ± 2.78 AB	1.33 ± 0.03 BC	1.29 ± 0.27 AB	8.23 ± 0.81 A	106.53 ± 2.69 AB
ANT	0.70 ± 0.20 AB	110.94 ± 7.54 AB	1.40 ± 0.02 AC	1.21 ± 0.17 AB	7.33 ± 1.76 AB	128.22 ± 5.63 A
AVI	0.97 ± 0.12 AB	97.31 ± 11.02 AB	1.34 ± 0.04 BC	0.96 ± 0.26 AB	4.67 ± 1.65 AB	105.24 ± 10.05 AB
BAD	0.90 ± 0.17 AB	121.14 ± 7.47 A	1.31 ± 0.00 BC	0.98 ± 0.05 AB	3.86 ± 0.52 B	128.22 ± 7.89 A
BAG	0.81 ± 0.17 AB	104.62 ± 25.37 AB	1.41 ± 0.07 AC	1.47 ± 0.22 AB	5.65 ± 0.46 AB	113.97 ± 25.95 AB
BED	0.40 ± 0.20 B	69.50 ± 13.54 B	1.27 ± 0.06 C	1.17 ± 0.23 AB	7.10 ± 2.26 AB	79.52 ± 11.91 B
BUR	0.70 ± 0.10 AB	122.56 ± 13.31 A	1.39 ± 0.01 AC	0.78 ± 0.43 B	5.79 ± 0.98 AB	131.20 ± 11.95 A
LAI	1.10 ± 0.17 A	117.15 ± 4.38 A	1.52 ± 0.03 A	1.65 ± 0.13 A	5.01 ± 0.62 AB	126.44 ± 5.12 A
NOV	0.83 ± 0.06 AB	123.68 ± 9.51 A	1.40 ± 0.05 AC	1.40 ± 0.22 AB	6.16 ± 0.70 AB	133.47 ± 10.32 A
PUS	1.13 ± 0.06 A	112.11 ± 16.66 AB	1.44 ± 0.04 AB	1.39 ± 0.32 AB	6.11 ± 1.00 AB	122.19 ± 15.60 AB
SCE	0.63 ± 0.06 AB	123.89 ± 12.80 A	1.42 ± 0.04 AB	1.18 ± 0.16 AB	5.34 ± 0.28 AB	132.49 ± 13.28 A
ZIV	0.61 ± 0.11 AB	122.54 ± 9.00 A	1.38 ± 0.06 AC	0.80 ± 0.10 B	6.13 ± 0.61 AB	131.46 ± 9.31 A
Mean	*0.80 ± 0.25*	*110.03 ± 18.94*	*1.38 ± 0.07*	*1.19 ± 0.33*	*5.95 ± 1.50*	*119.91 ± 18.63*

Mean values (N=3) ± SD followed by different letters are significantly different at P<0.01 according to Tukey (HSD) Test.

For Peer Review

Table 4. Results of the Principal Component Analysis (PCA) of the *A. rusticana* accessions evaluated with the 10 morphological quantitative traits (first three principal components); abbreviated codes of considered traits are indicated in Tables 2 and 3.

Trait	PC1	PC2	PC3
Factor loadings:			
R.TD	0.872	0.014	0.203
R.BD	0.832	-0.340	-0.312
R.FW	0.946	-0.208	-0.011
T.FW	0.946	-0.014	0.013
GIB	0.477	0.547	-0.508
SIN	0.872	-0.066	0.317
GNA	0.655	0.635	0.185
GBS	0.079	0.909	-0.108
GST	-0.684	0.289	0.607
T.GSL	0.851	-0.015	0.410
Eigenvalue	5.856	1.776	1.080
Variability (%)	58.565	17.764	10.803
Accumulated variability (%)	58.565	76.328	87.131

For Peer Review

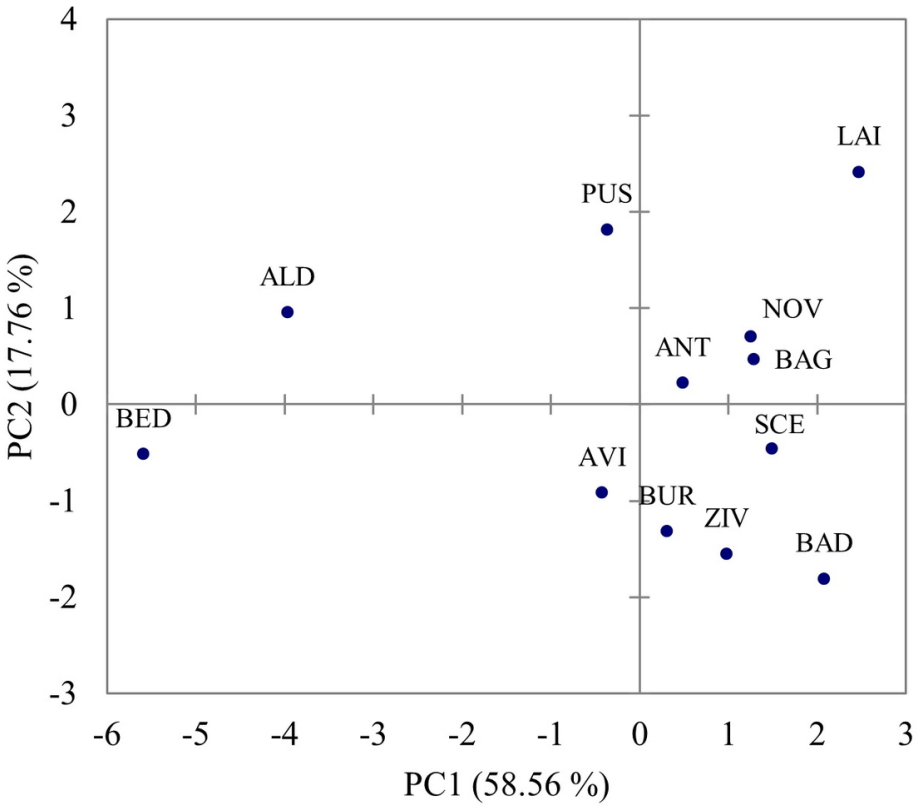


Figure 1. Score plot for the 12 evaluated *A. rusticana* accessions (abbreviated codes are indicated in Table 1).

101x87mm (300 x 300 DPI)

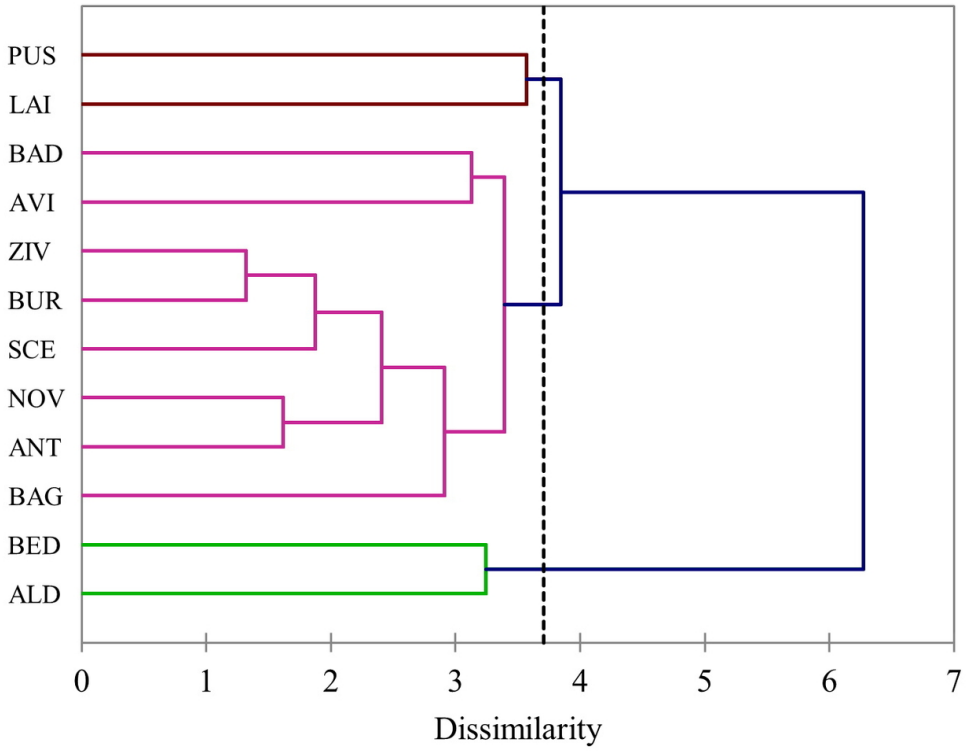


Figure 2. UPGMA dendrogram based on the relative scores for the three extracted PCs (abbreviated codes are indicated in Table 1).

107x87mm (300 x 300 DPI)