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Hot Spots of Bitter Compounds in the Roots of *Gentiana lutea* L. subsp. *aurantiaca:* Wild and Cultivated Comparative

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Abstract: *Gentiana lutea* L. subsp. *aurantiaca* M. Lainz is a plant endemic to the north-western mountainous areas of the Iberian Peninsula. Its roots are widely used mainly because of the high content of bitter compounds. The occurrence of these valuable bitter compounds in the roots is rather inhomogeneous, resulting in fluctuating root quality. Methanolic extracts obtained from different parts and tissues of wild and cultivated gentian, in and out of its natural environment, were analysed using HPLC chromatography to investigate the variation in the concentration of amarogentin, gentiopicroside, sweroside and swertiamarin. The distribution patterns of these compounds in the different analysed fractions showed that the concentration of bitter compounds varies significantly. Amarogentin is much more highly concentrated in the secondary roots, and all of the analysed compounds were found in a significantly higher content in the root cortex than in the vascular tissues. Roots cultivated in the natural habitat showed much higher concentrations in amarogentin and more biomass, while in those cultivated out of the natural environment, sweroside concentration was higher. These results allow us to understand that, when cultivated, the variability in the concentration of the different bitter compounds is linked with the edaphoclimatic conditions, but more importantly that it is linked with the dominating kind of tissues and the root system structure, especially when analysing the content of amarogentin and sweroside. The selection of plants with an optimal root system structure for breeding may increase the yield in bitter compounds and contribute to developing the commercial cultivation of this protected plant.

Keywords: *Gentiana lutea* L. subsp. *aurantiaca*; roots; bitter compounds; medicinal plant

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1. Introduction

The global use of herbs in medical practices is increasing, as was stated in a report carried out by the World Health Organization [1]. The global botanical drugs market had a value of USD 178.23 million in 2023 and is expected to rise, reaching around USD 3221 million in 2031 [2]. Mostly, plants used for medicinal purposes are wild collected. Therefore, the increasing global demand frequently results in the over-exploitation and degradation of the natural habitats of many species. *G. lutea*, *Arnica montana*, L. *Rheum tanguticum* and *Rhodiola rosea* L. are some examples of this issue [3,4].

Gentiana lutea L. (Gentianaceae), commonly denominated as yellow gentian or bitterwort, is a herbaceous hemicryptophyte perennial plant growing in the alpine and sub-alpine

pastures of central and southern Europe, usually on calcareous soils [5–7]. Due to the bitter substances contained in their succulent roots (mainly secoiridoid glycosides), *G. lutea* subspecies have been collected and used for a variety of purposes. These include the stimulation of appetite in the treatment of dyspepsia and anorexia problems, traditional medicine and liquor production [8–11]. In recent years, an increasing number of investigations have focused on the bioactive potential of the phytochemicals contained in gentians such as anti-mutagenic, lipid-lowering, antitumor, hepatoprotective, anti-inflammatory, osteoprotective, antifibrotic, healing, antifungal, antibacterial and antioxidant [12–18].

The subspecies *G. lutea* L. subsp. *aurantiaca* M. Lainz is an endemic plant distributed in the north-western part of the Iberian Peninsula. Its most prominent characteristic is the colour of the flowers ranging from yellowish to almost red [19], caused by the increased content of pelargonidin glycosides in the petals [20]. Furthermore, other differences such as the habitat, genetics and concentration of bitter compounds in the root allow for differentiating subsp. *aurantiaca* from the other subspecies of *G. lutea* [21–24].

Although protected, gentian roots can be collected legally obtaining a license in the region of Castilla y León. Its roots are paid for by weight rather than by quality factors such as the richness in bitter compounds. This results in the harvesting of roots from older and more developed wild gentian plants, which are the ones with the highest stress resilience and reproductive capacity. Their removal can threaten the survival of the population.

Gentian roots traders do not differentiate between subspecies in the raw material; however, many gentian processing industries demand roots from the north-west of Castilla y León where the *aurantiaca* subspecies grow (own data). Therefore, this subspecies is of particular importance.

Quality control provides homogeneous and objective evaluation criteria, which ensures an adequate payment to collectors or producers, guaranteeing the traceability and thus controlling the product.

Different organs, for example leaves, of *G. lutea* [25–28] and other species of gentian such as *G. cruciate* [29], have been investigated; however, the root system of gentian is of particular interest due to its economic relevance. Nevertheless, to date there is no systematic study of how the bitter compounds are distributed in the main parts and tissues that the root system consists of, neither are there any studies about the bitter compounds content comparing wild and cultivated *Gentiana lutea* from subsp. *aurantiaca*. Furthermore, there is no such study comparing cultivated *aurantiaca* subspecies within and outside of its natural environment.

The main goal of the present research was to investigate the variations in the concentration of amarogentin, gentiopicroside, sweroside and swertiamarin between the different parts and tissues of the root system of wild *G. lutea* subsp. *aurantiaca* collected in the Cantabrian Mountains (Iberian Peninsula) using high performance liquid chromatography (HPLC). Furthermore, the content of bitter compounds was compared to the ones obtained from analysed full root systems (smaller size and weight) of gentian cultivated within and outside of its natural habitat during four and five years. The results obtained show significant differences between analysed parts. This allows the establishment of objective quality evaluation criteria of gentian roots rather than remuneration based on root weight. Also, the results provide a basis for the selection of plants with optimal root systems and facilitates the cultivation of gentian, an innovative crop, in local mountainous areas where the growing conditions allow for high quality roots in terms of the concentration of bitter compounds, thus protecting the natural populations of this plant.

2. Materials and Methods

2.1. Plant Material

2.1.1. Wild Gentian

Root samples of *G. lutea* L. subsp. *aurantiaca* were collected from two wild populations located in the regions of Laciana and Babia (León, Spain), a part of the Cantabrian Mountains where high amounts of gentian roots are traditionally collected. Those populations

were LEB_GERM 19 (42°50′07″ N 006°11′25″ W, 1724 m) and LEB_GERM 20 (42°59′18″ N 006°25′26″ W, 1880 m), selected for being a representative sample of the largest area where *aurantiaca* variety grows in León and because their environmental conditions and genetic diversity are similar. Voucher specimens (LEB-GERM) are deposited at the University of León, in the herbarium Jaime Andrés Rodríguez.

Twenty plants from every studied population were sampled randomly. Sampled plants were at least five meters apart, to avoid sampling the same individual. Since it is not possible to know the age of the wild plants, individuals showing a similar vegetative development were sampled.

Once cleaned, the root system of every gentian plant was divided into rhizome (RZ), principal roots (PR) and secondary roots (SR) (Figure 1) when possible, since it is a wild plant with a variable radical structure. Due to the lack of precise information on the age of the tissues and morphological descriptors, the different parts of the roots were identified by their epidermis and section physiology (colour of the epidermis and section, separation of the internodes of the roots and development grade of the endodermis).

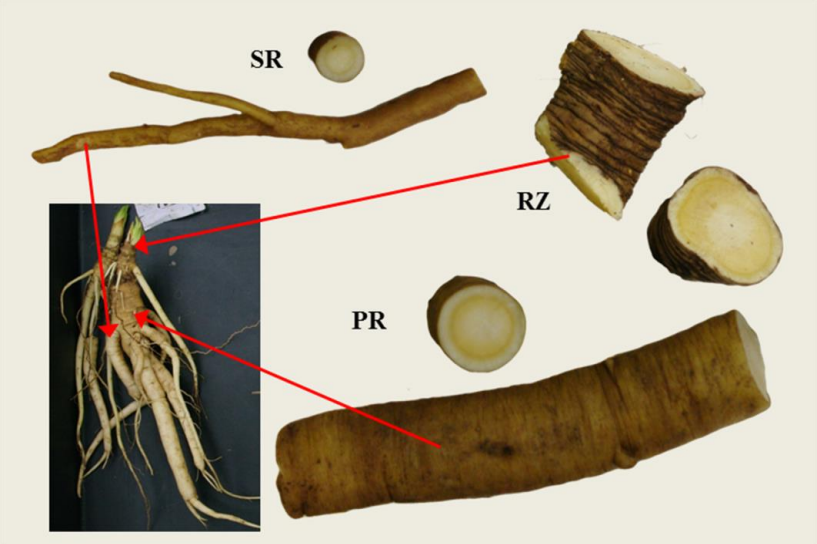

Figure 1. Division of the root system of *Gentiana lutea* subsp. *aurantiaca.* RZ: rhizome; PR: principal roots; SR: secondary roots.

Three measures were taken from representative roots for every part of the root system (RZ, PR and SR) using a digital calliper. The measurements were carried out as described in Figure 2, distinguishing between the thickness of the cortex (C) and diameter of the roots (D).

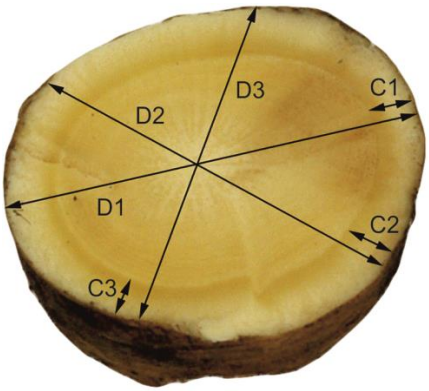

Figure 2. Measurement of gentian roots (D1, D2 and D3: total diameter, C1, C2 and C3: cortex thickness).

Rhizome, principal and secondary roots were divided carefully into cortex (including the epidermis) and vascular tissue in order to analyse both parts separately.

The roots were chopped and dried for 72 h at 38 °C in a ventilated oven (Digitronic, JP SELECTA S.A, Barcelona, Spain). The dried roots were ground in a hammer mill (Culatti AG, Steinerberg, Switzerland) using a sieve with a diameter of 1 mm.

2.1.2. Cultivated Gentian

G. lutea L. subsp. *aurantiaca* was cultivated in two different fields. One field was located in a High Mountain area (HM) in the natural habitat of the *aurantiaca* subspecies (42°52′12.37″ N 6°12′57.37″ W 1437 m), at a soil pH of 4.46. The other field, Crop Test (CT), was located in the Higher Technical School of Agrarian Engineering of the University of León, near the city of León, (5°37′24.64″ 42°34′59.64″ 819 m), at a soil pH of 7.34. Here, edaphoclimatic conditions do not allow gentian to grow wildly and irrigation is needed during summer. Although the plants survived, they showed clear symptoms of ferric chlorosis and reduced growth due to the soil properties.

The trial involved a randomised complete block (RCB) design with four replicates in every field (30 plants per replicate). The planting pattern was 0.6 × 0.3 m. The seedlings were obtained from seeds collected in the surroundings of the field HM from wild plants. Eighteen full root systems per replicate from CT and twelve from HM were randomly collected in the same moment (October) during two consecutive years (4- and 5-year-old plants).

The root system from every gentian plant was weighed, washed, chopped and ground as described before. In the case of the cultivated gentian, the full root system was used as a single, homogenised sample for analysis, since the early plant development stage does not allow for dissection into separate root parts as carried out in the case of older, wild-collected plants.

2.2. Methanolic Extracts

The protocol followed is the one described by González-López et al. [24]. Briefly, each sample (1 g of root powder) was subjected to hot methanolic extraction using a Soxhlet. The remaining methanol was evaporated at 38 °C in a vacuum concentrator. Dried extracts were re-dissolved in HPLC quality methanol and filtered using PTFE syringe filters (45 µm).

2.3. HPLC Conditions

The analysis conditions have been described by González-López et al. [24]. HPLC–DAD analysis was performed on a Hewlett–Packard 1000 series HPLC instrument (Agilent Technologies, Les Ulis, France), equipped with a quaternary pump, diode array detector (DAD), and an autosampler coupled to a HP Chem Station (rev B.03.02) data-processing station. The samples (10 µL) were injected on a Waters Spherisorb® ODS2 column (250 × 4.6 mm, 5 µm particle size) at 25 °C. Double online detection was carried out in the DAD using 254.2 nm and 280 nm as preferred wavelengths. Each sample was analysed in triplicate. Two solvents were used for the separation in a gradient system. Solvent A consisted of HPLC-grade methanol while B was 5% acetic acid in water. The elution gradient applied at a flow rate of 1 mL/min was as detailed: from 0 min 10% A, 0 to 2 min 30% A, 2 to 8 min 30% A, 8 to 13 min 35% A, 13 to 20 min 50% A, 20 to 22 min 50% A, 22 to 30 min 70% A, 30 to 35 min 70%A, 35 to 45 min 85% A, 45 to 50 min 90% A, 50 to 55 min 95% A.

UV spectra and the retention times were used to identify the analysed compounds. For their quantification, calibration curves were obtained through analysing known concentrations of the analytical standards. The calibration curves were the following: amarogentin ($y = 8.9167x + 34.362$; $R^2 = 0.9999$); gentiopicroside ($y = 0.5884x + 4.0627$; $R^2 = 0.9993$); swertiamarin ($y = 8.6443x - 80.306$; $R^2 = 0.9999$); sweroside ($y = 15.902x + 158.73$; $R^2 = 0.9990$).

The overall procedure was validated by studying the accuracy, precision and reproducibility in the middle of the calibration. For the first parameter, an extraction process of a

second methanolic extract was evaluated and no signals of the analytes above the limit of detection were obtained, so the extraction could be considered quantitative. A standard mixture solution was injected six times a day (intraday precision) and once a day on five consecutive days (interday precision). Good results were obtained and the precision values ranged from 1.7 to 3.7% (RSD). Reproducibility between 0.8 and 5.09 (n = 4) was obtained for the entire procedure.

2.4. Data Analysis

Statistical significances were assessed using the Student's *t*-test or through analyses of variance using the general linear model (GLM procedure). Whenever GLM analyses showed significance, least significant differences (LSD) were computed. Differences were considered to be significant when $p \leq 0.05$. The relationship between the dry weight of the root systems and their total quantity of every bitter compound analysed was tested using regression analysis (GLM procedure). All analyses were performed using the software OriginPro 8.5 [30]. The same software was used to draw the graphs.

3. Results

3.1. Wild Gentian

3.1.1. Root Development

Significant differences were observed when analysing the dimension traits of the sections from rhizome (RZ), principal roots (PR) and secondary roots (SR) (Figure 3).

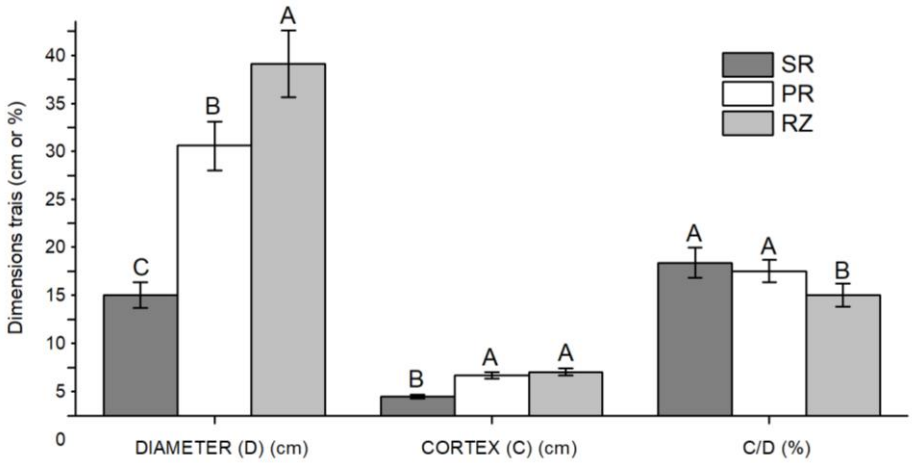

Figure 3. Diameter (D), cortex (C), and cortex/diameter % (C/D) of the root system of *Gentiana lutea* subsp. *aurantiaca* from Cantabrian Mountains. Bars with different letters for every dimension trait are significantly different ($p \leq 0.05$) according to LSD. SR: secondary roots; PR: principal roots; RZ: rhizome.

The diameter of the rhizome (36.6 mm) was significantly larger than the one of principal roots (28.1 mm), which in turn was significantly larger than the one of secondary roots (12.5 mm). Comparing the cortex thickness, the mean value of the secondary roots (1.99 mm) was significantly smaller than the principal roots and rhizome, with 4.19 and 4.55 mm, respectively (Figure 3).

On the other hand, the ratio of cortex thickness (C) to root diameter (D) (C/D (%)) showed that secondary roots and principal roots had a significantly higher percentage of cortex (15.8% and 14.9%, respectively) than the rhizome, whose mean value was only 12.4%.

3.1.2. HPLC Analyses

The protocol used for HPLC analysis allowed for a clear differentiation of the analysed compounds (Figure 4).

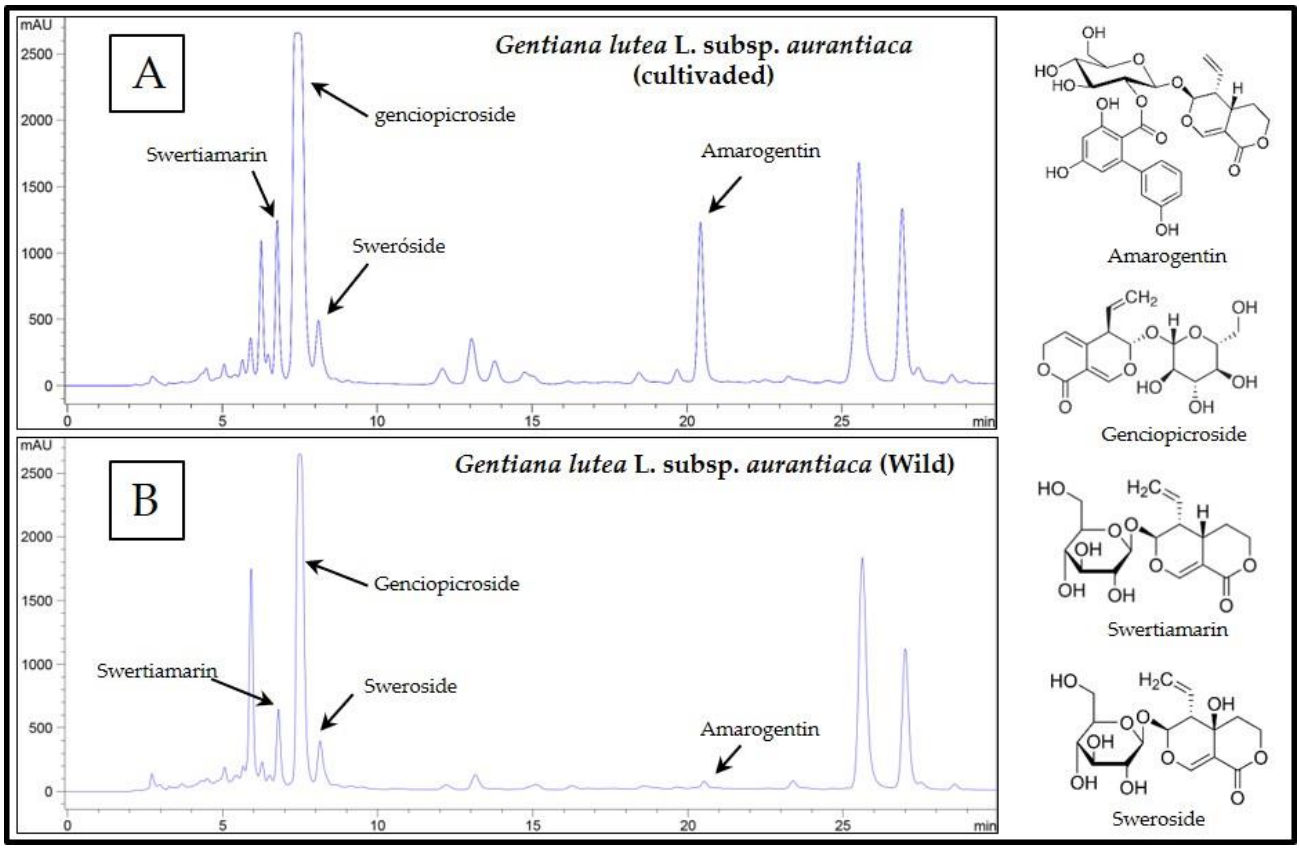

Figure 4. Representative HPLC profiles for analysed samples obtained from cultivated (**A**) and wild (**B**) *Gentiana lutea* subsp. *aurantiaca* from Cantabrian Mountains. The chemical structures of the analysed compounds are shown on the right-hand side.

In the analyses of the bitter compounds (gentiopicroside, amarogentin, sweroside and swertiamarin) of *G. lutea* L. subsp. *aurantiaca* using HPLC, significant differences in the concentration of some compounds between the three root parts were revealed (Figure 5). Concentrations are expressed in mg per gram of dry root (mg/g of d.r.).

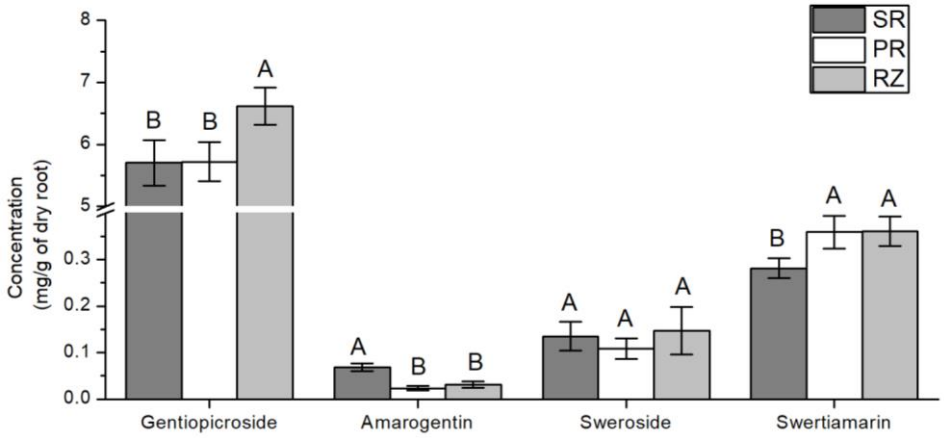

Figure 5. Concentration of bitter compounds in the different parts of the root system in *Gentiana lutea* subsp. *aurantiaca* from Cantabrian Mountains. Bars with different letters for every bitter compound are significantly different ($p \leq 0.05$) according to LSD. SR: secondary roots; PR: principal roots; RZ: rhizome. Data represent mean ± standard error.

Root System

The concentration of gentiopicroside was significantly higher in the rhizome (6.61 mg/g of d.r.) than in the principal and secondary roots (5.72 and 5.70 mg/g of d.r). In contrast, for amarogentin, the mean value of the concentration in the secondary roots (younger tissues) was significantly higher (0.068 mg/g of d.r.) than in the principal roots and rhizome (0.023 and 0.032 mg/g of d.r.). Sweroside is the only compound, whose concentration does not differ between the different plant parts.

When comparing the concentrations of bitter compounds in the cortex (including the epidermis) with the ones in vascular tissues regardless of root system division, significant differences were revealed. According to Student's *t*-test, the concentration of all analysed compounds was significantly higher in the cortex than in the vascular tissues (Figure 6).

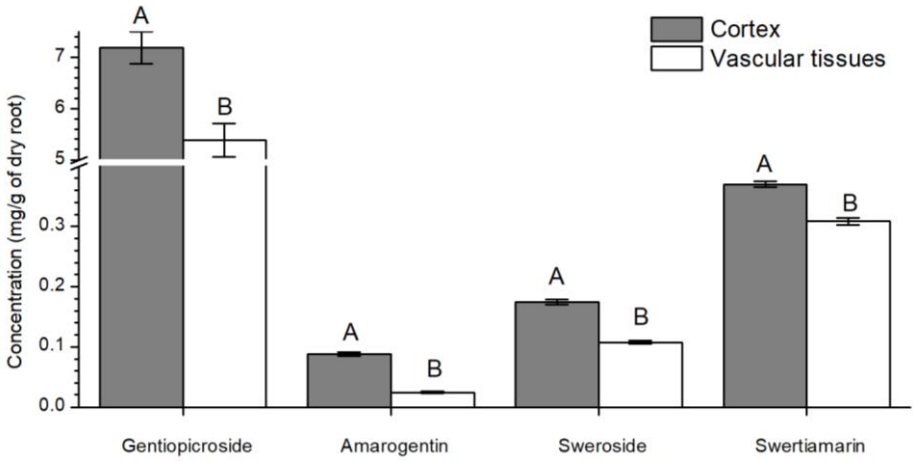

Figure 6. Mean concentration of bitter compounds in the cortex and vascular tissues of the roots of *Gentiana lutea* subsp. *aurantiaca* from Cantabrian Mountains. Bars with different letters for every bitter compound are significantly different ($p \leq 0.05$) according to Student's *t*-test. Data represent mean ± standard error.

The mean concentration of gentiopicroside in the cortex (7.19 mg/g of d.r.) was 33.6% higher than in the vascular tissues (5.38 mg/g of d.r.). For amarogentin, the concentration in the cortex (0.088 mg/g of d.r.) was nearly 400% in one of the vascular tissues (0.024 mg/g of d.r.), which constitutes the highest relative difference obtained among all the analysed compounds. For sweroside, the concentration in the cortex (0.174 mg/g of d.r.) was nearly 200% in one of the vascular tissues (0.108 mg/g of d.r.). Finally, the concentration of swertiamarin in the cortex with 0.370 mg/g of d.r. was 19.7% higher than in the vascular tissues (0.309 mg/g of d.r.).

The concentration of gentiopicroside (Figure 7A) was significantly higher in the cortex of every division of the root system (SR, PR and RZ) compared to the vascular tissues of the same division (Figure 7A, lower-case letters). The maximum difference between cortex and vascular tissues from the same division of the root system was obtained for principal roots, with concentrations of gentiopicroside being 36.12% higher in their cortex than in their vascular tissues. The highest concentration was obtained in the rhizome cortex (7.67 mg/g of d.r.), which was nearly the same as for the cortex of principal roots (7.65 mg/g of d.r.). Both are significantly higher than the concentration in the cortex of secondary roots (6.56 mg/g of d.r.) (Figure 7A, capital letters).

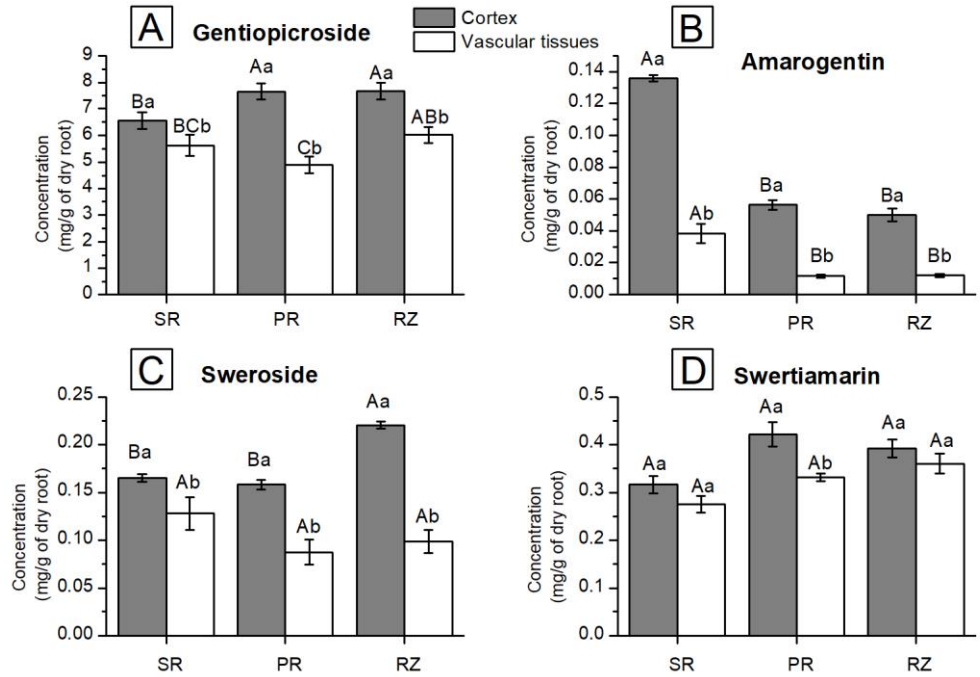

Figure 7. Concentration of bitter compounds in the cortex and vascular tissues of the different divisions of the root system in *Gentiana lutea* subsp. *aurantiaca* from Cantabrian Mountains. Bars with different capital letters for the same tissue are significantly different for each bitter compound ($p \leq 0.05$) according to LSD. Bars with different small letters for every division of the root system are significantly different ($p \leq 0.05$) according to Student's *t*-test. SR: secondary roots; PR: principal roots; RZ: rhizome.

For amarogentin (Figure 7B), the concentration in every division of the root system (SR, PR and RZ) was significantly higher in the cortex compared with the vascular tissues of the same division (Figure 7B, lower-case letters), with differences between them from 351% for the SR to 476% for the PR. When comparing the concentration of amarogentin in the different tissues derived from the different divisions of the root system (Figure 7B, capital letters), the values for SR (0.136 mg/g of d.r. for cortex and 0.039 mg/g of d.r. for vascular tissues) were significantly higher compared to the same tissues of PR and RZ. The lowest values were obtained from the vascular tissues of PR and RZ (0.012 mg/g of d.r.).

For sweroside (Figure 7C), the concentration differed significantly between the cortex of every division of the root system (SR, PR and RZ) compared to the vascular tissues of the same divisions (Figure 7C, lower-case letters). In this case, the concentration varied between the cortex and vascular tissues from 128% for the SR to 223% in the RZ. So, the concentration of sweroside in cortex and vascular tissues was characterised by an increasing variability as the tissues became older. The concentration in the cortex of the rhizome was the highest (0.220 mg/g of d.r.), significantly higher than in the cortex of the secondary and principal roots (Figure 7C, capital letters). The lowest concentration of sweroside was found in the vascular tissues of principal roots (0.087 mg/g of d.r.).

The swertiamarin concentrations were not as variable (Figure 7D). The only significant difference was shown for the principal roots (Figure 7D, lowercase letters). The maximum concentration for the cortex (0.422 mg/g of d.r.) was found in the PR and was 27% higher than in the corresponding vascular tissues.

3.2. Cultivated Gentian

Experimental Fields within (HM) and Outside (CT) the Natural Habitat of Gentian

Figure 8 shows the relation between the dry root weight and the amount of different bitter compounds for each collected root from HM and CT fields. As regards gentiopicroside and swertiamarin, the point clouds fit accurately with the regression lines for

both cultivation sites. On the other hand, when observing the scatter plot representing amarogentin and sweroside, the regression lines shift to a non-proportional and less linear behaviour (lower r-square values).

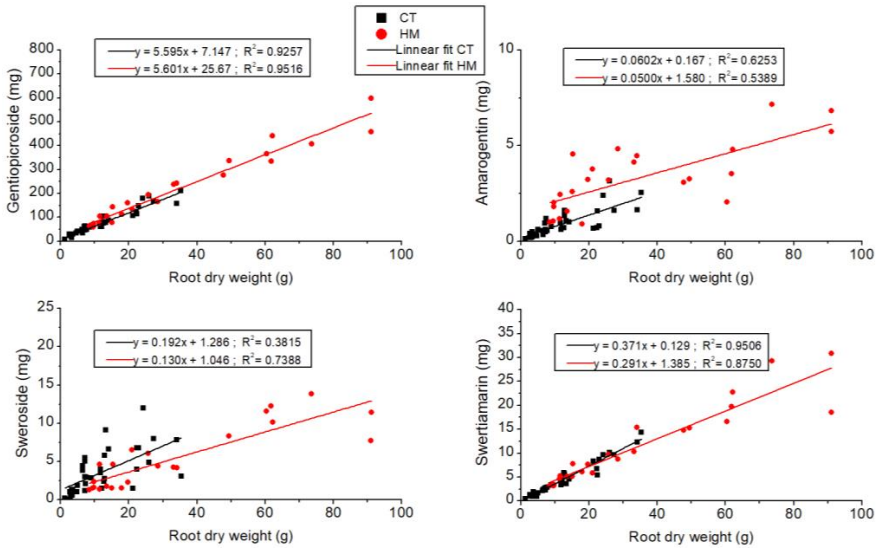

Figure 8. Relationship between dry weight of 4- and 5-year-old analysed roots systems and their total content in the bitter compounds amarogentin, gentiopicroside, sweroside and swertiamarin for cultivated *G. lutea* subsp. *aurantiaca* in the experimental fields in HM (High Mountain area; natural habitat) and CT (Crop Test; outside of natural habitat).

Figure 9 shows the relation between the dry weight of the total root system per plant and the concentration of the different bitter compounds depending on the cultivation site. Again, gentiopicroside and swertiamarin showed a similar behaviour, so the point clouds are rather homogeneously distributed in similar concentration values independently of the root weight. This was different to amarogentin and sweroside, where the distribution of the point clouds varied widely, indicating lower concentration values for heavier roots.

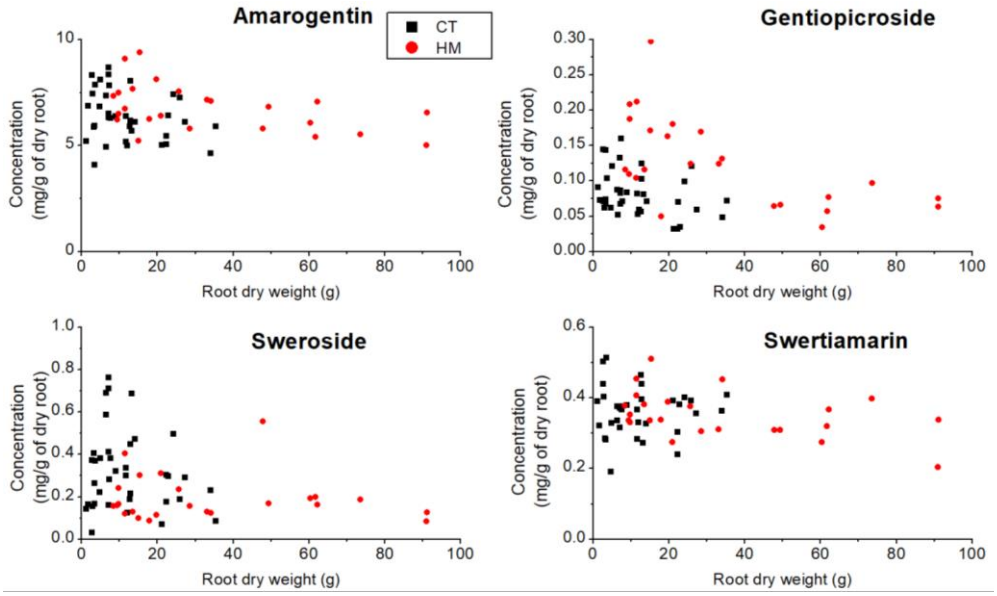

Figure 9. Relative content of bitter compounds (amarogentin, gentiopicroside, sweroside and swertiamarin) in relation to the root system total dry weight in 4- and 5-year-old cultivated *G. lutea* subsp. *aurantiaca* in the experimental fields in HM (High Mountain area; natural habitat) and CT (Crop Test; out of natural habitat).

When comparing the two cultivation sites (Figure 10), the concentration of amarogentin obtained from plants grown in the HM site were significantly higher than those from the CT site. However, the opposite was true for sweroside, which was found in lower concentrations in the roots from the HM site than in those from the CT site.

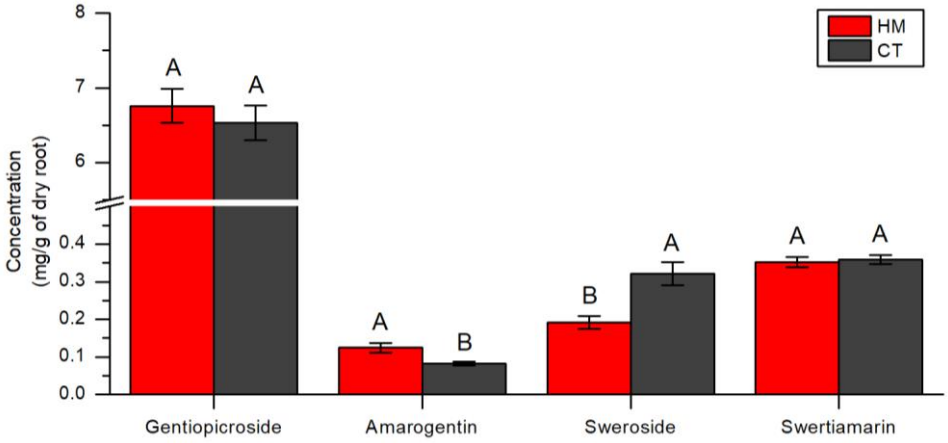

Figure 10. Mean concentration of bitter compounds in 4 and 5-year-old roots of cultivated *G. lutea* L. subsp. *aurantiaca* depending on the cultivation field (HM or CT). Bars with different letters for every bitter compound are significantly different ($p \leq 0.05$) according to Student's *t*-test. Data represent mean ± standard error.

4. Discussion

4.1. Root Development

Older root parts had a higher level of woodiness as shown in Figure 1, with a clear distinction between cortex and vascular tissues. An important observation was that the total diameter of the roots and thickness of the cortex increased with the age of the roots, but at the same time the ratio of cortex thickness to the total diameter decreased (Figure 2). Since the cortex is a hot spot especially for amarogentin and sweroside, the decreasing share of this tissue in older plants is a direct cause for the decreasing concentrations of these respective bitter compounds when analysing older and larger plant individuals. This gives way to new approaches improving the targeted cultivation of the wild species *Gentiana lutea*. Past efforts were focused on selecting individuals with a higher yield in terms of weight or the overall content of bitter compounds. However, present results suggest taking into consideration the root system structure, e.g., selecting plants with a higher number of secondary roots and thus a higher share of cortex tissue, or collecting plants in a young developmental stage, in which the cortex dominates. Focusing on a selection of plants with a significant number of secondary roots could be a resource-efficient strategy to obtain a higher yield both in terms of root weight and content of bitter compounds, especially when the focus is on amarogentin.

4.2. HPLC Analyses

4.2.1. Root System

Although several publications describe the analysis of the concentration of bitter compounds in the roots of G. *lutea*, the approach of selecting the root samples varies. In some comparative studies, commercial samples were analysed, without specifying the part of the root system used [31–35]. Obtained results are heterogeneous due to the variable quality of these samples, depending on their origin, the subspecies, part or age of the roots. When wild collected, the part of the root system used is not described either [36,37]. In the analysis of cultivated gentian, it is possible to compare plants with the same age [38] or similar weight [39] but the root structure is typically highly variable. For comparing cultivated and wild gentian, analysing roots with similar diameters as completed by Barralis et al. [40] is one of the most suitable approaches, although the age of wild gentian remains unknown.

Until now, no studies have been carried out in *G. lutea* roots separating the different parts of the root system and therefore this has not occurred for *G. lutea* L. subsp. *aurantiaca* either, whose edaphoclimatic growing conditions are different when compared with other previously studied subspecies of *G. lutea* [24]. For example, the subsp. *aurantiaca* naturally only grows in silicic substrates with a very acidic pH. Also, the quantification of the concentration in sweroside and swertiamarin is a new objective not investigated so far.

Even though the analysis of full root systems from cultivated gentian has shown a higher concentration of amarogentin in younger plants [40], our results suggest that the age of the plant is not the only factor influencing the concentration of amarogentin. Rather, the root system development and thus the share of the different tissues is a decisive factor. Lighter root systems are composed of smaller roots and thus have a dominating share of cortex, leading to a higher concentration of amarogentin than in individuals with roots in an advanced developmental stage as indicated by higher dry weight (Figure 8).

In contrast, the concentration of gentiopicroside and swertiamarin is generally more constant regardless of the age and tissue type, as can be observed in Figure 7. In this figure, the overall content of gentiopicroside and swertiamarin in relation to the total dry weight of the analysed root system is almost perfectly adjusted to a linear performance, without further distinguishing between parts of the root system. A higher concentration of gentiopicroside in older plants was also mentioned by Franz and Fritz [41].

4.2.2. Cortex vs. Vascular Tissues (Roots)

The concentration of all of the secondary metabolites analysed is higher in the cortex than in vascular tissues. In general, amarogentin shows values opposed to the other three compounds for the various parts analysed, with a higher difference in the content between cortex and vascular tissues and a remarkable decrease in the concentration values for the older roots (PR and RZ) in both cortex and vascular tissues.

Gentiopicroside and sweroside are also more concentrated in the cortex, but in contrast to amarogentin the relative difference to the vascular tissues is lower.

Swertiamarin showed a similar concentration in all parts and tissues of the root system analysed.

Similar differences between these tissues were obtained in a study about the variability in the concentration of phenolic compounds in the outer and inner part of the stalks of *Cynara cardunculus* L. var. *altilis* carried out by Ramos et al. [42], obtaining a higher concentration in the outer part.

4.2.3. Cultivated *Gentiana* L. subsp. *aurantiaca* (HM vs. CT)

The present study is the first one to describe a comprehensive set of four relevant bitter compounds in roots of cultivated *G. lutea*, and the first one to do so for the subspecies *aurantiaca* in cultivated conditions. Therefore, the discussion will include comparative results obtained by other authors for different subspecies of *G. lutea*. The range of studies to draw comparisons to is limited, because most studies about *G. lutea* L. bitter compounds only describe gentiopicroside and amarogentin [39,43,44]. These were historically considered the most important bitter compounds—gentiopicroside due to its high concentration and amarogentin due to its bitter index [38,45]. Only the present study and Mustafa et al. [35] have investigated the concentration of amarogentin, gentiopicroside, sweroside and swertiamarin in cultivated *G. lutea*. Comparing both studies, *aurantiaca* subspecies show higher concentrations for all of the bitter compounds than roots of *G. lutea* L. (subspecies not specified) of a comparable age [35]. The mean values obtained by Mustafa et al. [35] were 0.25 mg/g of dry root of swertiamarin, 0.15 mg/g of dry root of sweroside, 0.07 mg/g of amarogentin and 3.42 mg/g of dry root of gentiopicroside.

The concentration of the characteristic bitter compounds of *G. lutea* L., analysed over the full root system of 4- and 5-year-old cultivated *G. lutea* L. subsp. *aurantiaca* within and outside of its natural environment, showed significant differences for amarogentin. This compound was more highly concentrated in gentian cultivated within its natural habitat

(HM) than outside of it (CT). For sweroside, it was the opposite, resulting in a significantly higher content when gentian was cultivated outside of its natural habitat.

Franz et al. [46] proposed that the concentration of amarogentin and gentiopicroside could depend on the variation in meteorological conditions. This could explain the different mean concentrations between root samples from the two experimental fields analysed in the present paper. On the other hand, altitude or/and edaphoclimatic characteristics also have proved to affect the concentration of bitter compounds in gentian roots. Different studies affirm that a higher altitude typically results in an increased concentration of bitter compounds [24,47,48].

This is in line with a study carried out by our group, confirming that a higher altitude leads to higher concentrations of bitter compounds—mainly gentiopicroside, swertiamarin and sweroside—in roots from several wild populations of *G. lutea* L. subsp *aurantiaca* [24]. The same study analysed soils where *aurantiaca* subspecies grow wild, showing how the content in clay, silt and sand affects the development of the roots and also the content of amarogentin.

A possible explanation for the higher content of sweroside in gentian cultivated in CT is the response of different plants to stressful conditions which trigger a higher production of secondary metabolites, as has been described for *Hypericum brasiliense* Choisy [49]. If the goal is to obtain roots with a high content in sweroside, which has high hepatoprotective and wound healing capacity [12,50,51], it would be reasonable to cultivate *G. lutea* subsp. *aurantiaca* outside of its natural habitat, even though this may result in a smaller yield in terms of root weight.

In studies about other cultivated *G. lutea* subspecies within and/or outside of their natural habitat, the concentration of amarogentin in most cases decreased when the same ecotypes of *G. lutea* L. were cultivated outside of their natural habitat [25,43,48], with an exception being the observations made by Rossetti [52]. Thus, the behaviour of the *aurantiaca* subspecies is similar to the one of other subspecies.

Bezzi et al. [48] analysed the concentration of amarogentin in roots of cultivated *G. lutea* L. (subsp. *symphyandra* and subsp. *lutea*) from several experimental fields located within and outside of its natural habitat. Amarogentin concentration was between 0.073 and 0.080 mg/g of dry root for subsp. *lutea* when cultivated within its natural habitat and 0.029 and 0.096 mg/g of dry root when cultivated out of it.

Franz et al. [43], analysed gentian roots from different ecotypes. The concentration of amarogentin in these roots varied for subsp. *lutea* ecotypes from 0.08 to 0.10 mg/g when cultivated at 1200 m and between 0.03 and 0.05 mg/g when cultivated at 450 m. In the same study, for subsp. *symphyandra*, the amarogentin concentrations were 0.51 and 0.33 mg/g, respectively.

Obtained amarogentin concentrations are higher for *G. lutea* subsp. *aurantiaca* than those obtained for other cultivated subspecies, even compared with the *symphyandra* subspecies which are considered as the subspecies richest in amarogentin (Franz et al., 1996). As regards gentiopicroside, the values are higher than the ones obtained for cultivated subspecies *symphyandra* by Kusar and Baricevic [44] (1.4–3.7 mg/g) or Mustafa et al. [35], (3.31–3.53 mg/g), but similar or lower than the ones obtained by Bezzi et al. [39] (8.35–15.12 mg/g).

The relation between the dry root weight and the total or relative content in the different bitter compounds (Figures 8 and 9) in collected gentian roots for both cultivation sites (HM and CT) indicates a very similar behaviour in both experimental fields for gentiopicroside and swertiamarin, showing a linear correlation (R^2 over 0.9) which indicates that regardless of the cultivation site, root structure and root system weight, the content of these compounds is rather stable.

Likewise, amarogentin and sweroside behaviour is similar for both fields (Figures 8 and 9). In this case, with a R^2 lower value, there is not a clear linear correlation between the dry root weight and the total or relative content in amarogentin or sweroside, which indicate that age, root structure and root system weight have an influence on the content of these compounds despite of the cultivation site.

G. lutea is a wild plant and rarely cultivated, so the individual plants are very heterogeneous, even in the same population. The growth habit of radical systems with the same weight is diverse and can range from a strong principal root to a multitude of secondary roots from a short principal root or rhizome, similarly to golden root (*Rhodiola rosea* L.). This explains why radical systems with the same weight show variable amounts of amarogentin. Radical systems composed of a multitude of secondary roots would come with a higher concentration as shown by the present results.

Obtained results prove that the distribution of the gentiopicroside, amarogentin, sweroside and swertiamarin is heterogeneous and the behaviour of amarogentin is much different compared to the other analysed compounds. This is very important if the use of the roots is focused om one single component or a mix of them. If the objective is the production of a specific bitter compound, it is possible to carry out a selection in order to cultivate plants with the optimal root structure, increasing the content of this specific compound.

5. Conclusions

The present study describes for the first time *G. lutea* subsp. *aurantiaca*, wild collected and cultivated in different environments in terms of the occurrence of the four most important bitter compounds in *G. lutea* roots. Our results show that edaphoclimatic conditions are an important factor influencing the concentration of bitter compounds in cultivated *G. lutea* subsp. *aurantiaca*, in general yielding higher concentrations and more root biomass when cultivated in its natural habitat. Moreover, we described that the concentrations are linked to the type of tissues and the root system structure, especially when analysing the content of amarogentin and sweroside, both of them being much more concentrated in young and thin roots in which the cortex dominates.

Cultivation strategies can be developed from the present results. If the focus is on amarogentin, the cultivation of gentian using a very high planting density would allow an early harvest with very high amarogentin yields. On the other hand, gentiopicroside and swertiamarin concentrations remain constant independently of the root system weight. These results reinforce the need of using selected gentian plants for cultivation in the mountainous areas rather than the collection of wild gentian, creating an exclusive economic activity and supporting the creation of a quality label as a Protected Geographical Indication (PGI). This may decrease the demand of wild growing gentian and therefore result in a higher conservation of this endangered subspecies.

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