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Metabolomics Analysis of *Galium odoratum* (L.) Scop.: Impact of the Plant Population Origin and Growth Conditions

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Abstract: *Introduction- Galium odoratum* is a plant used in traditional medicine and to prepare beverages.

Objective: To study the impact of plant origin and growth conditions on the metabolite content of the plant.

Material and methods: Aerial biomass of *Galium odoratum* was collected from five natural populations (*in situ* conditions) and from controlled environment (*ex situ* conditions). NMR-based fingerprinting method was successfully applied to the discriminating chemical profiling of the *in situ* and *ex situ* samples.

Results: Quantitative analysis of selected phytochemicals including phenylpropranoids and iridoids showed clear differences between the plants from nature and those of controlled growth conditions as well as internal variation within the group. The metabolomic approach emphasized the decrease of the secondary metabolites pool paralleled by an increase of the carbohydrates in *ex situ* conditions. The quantitative HPLC-UV points out slight variations of each of the analyzed secondary metabolites between populations in natural environment, variations maintained for three populations in the controlled conditions.

Conclusion: Metabolomics approaches using ^{1}H-NMR and HPLC are worth to consider for studying the impact of climate factors on the regulation of the phytochemical profile in relation to the origin of the plant material.

Keywords: Asperuloside, chlorogenic acid, coumarin, *Galium odoratum*, geniposidic acid, monotropein, NMR metabolomics, Rubiaceae.

INTRODUCTION

Galium odoratum (L.) Scop. also known as sweet woodruff belongs to the *Rubiaceae* family (*Rubioideae* subfamily). It is a perennial herb with a creeping rhizome [1] growing in shady European deciduous forests, principally where beech trees are predominant.

Sweet woodruff is regarded as a medicinal and aromatic plant (MAP) since the Middle Age principally in European countries [2-4]. Recorded in folk medicine in several regions of Europe it is also used for the preparation of flavored beverages, such as Maitrank or Maiwein. The plant is listed in some pharmacopoeias - as the French and Polish Pharmacopoeia - for therapeutic uses [3, 5]. *G. odoratum* is traditionally used to treat digestive and bladder disorders, hepatitis, and nervousness [6]. A phytochemical analysis of volatile compounds of *Galium odoratum* has identified over 200 compounds [7]. Several previous researches reported on the nature of different compounds produced by this plant, among which are mainly coumarin, anthraquinones, tannins, flavonoids, phenolic acids, iridoids (asperuloside, monotropein), organic acids, vitamin C, vanillin, *n*-alcanes and bitter substances [3-6, 8-13]. Coumarin (present in fresh plants at least in part as its precursor melilotoside) [13], is the most investigated pharmacologically active substance in *Galium odoratum* with concentrations ranging between 0.4 and 1.7% dry weight [9, 10]. The coumarin is also responsible of the fragrance of sweet woodruff herbs described as resembling new-mown hay or vanilla. A few studies have focused on the content of iridoids and phenolic compounds in *Galium odoratum*. Teuscher *et al*. [3]. indicate the presence of asperuloside which is largely distributed in the *Rubioideae* subfamily [14]. In addition, monotropein and geniposidic acid as iridoids, several phenylpropanoids including cinnamic, *p*-coumaric, caffeic, *p*-hydroxybenzoic and gallic acids have been reported as main phenolic compounds of the woodruff herb. Osinska and Roslon showed that content of phenolic acids ranged from 1.3 to 2%. Total content of phenolic acids varies among populations during the blooming phase and between *in situ* and *ex situ* conditions [4, 12]. The phytochemical composition of the sweet woodruff seems also to

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depend on the analyzed organ [9], stage of development [4, 8-10], environmental factors such as rainfall [5] or origin of the population [5, 15].

The species-specific secondary metabolites of *Galium odoratum* vary according to the plants adaptive strategies to their environment like any other plants [16, 17]. However, ecological controls of the phytochemical profile of *Galium odoratum* are not well understood. Despite several recent investigations on the content of secondary metabolites identified in *Galium odoratum*, little is known about the biosynthesis and the influence of the environment on this group of pharmacologically active substances. Metabolomic profiling methods have been frequently applied, these last years, to the quality assessment of medicinal plants, as by our team on *Polygonum* species [18]. The objectives of this work were to apply these methods to the evaluation of the impact of the environment on the phytochemical content of *Galium odoratum*.

MATERIALS AND METHODS

Study Sites

The plants were collected in 2010 in five different locations in the south of Belgium (Ardennes and Belgian Lorraine), in Grand-Duchy of Luxembourg (Gutland) and in France (Lorraine). Natural populations of *Galium odoratum* were identified in forest stations next to Habergy (Belgium), Chassepierre (Belgium), Membre (Belgium), Differdange (Luxembourg) and Champenoux (France). The forest phytocoenoses are represented by the associations *Melico-Fagetum* for Habergy and Differdange, *Primulo-Carpinetum* for Chassepierre, *Tilio-Acerium* for Membre and *Poa chaixii-Quercetum roboris* at Champenoux.

Plant Material

Sampling time for plant material grown in natural conditions. For the five sites, aboveground biomass was collected randomly at the population early flowering stage (early May in our climate conditions) according to the scale of Zlatnik [19]. Biomass was freeze-dried and stored in a deep freezer at – 80 °C until they were used for phytochemical analysis.

Plant material propagation in controlled conditions. Rhizomes were collected randomly (cumulated total length of approximatetely six meters) at the early growth stage (March, 2010) in each population and pieces of about 30 cm were set in trays (40 cm x 55 cm). The potting medium was a peat-based substrate (Substrate DCM, Grobbendonk, Belgium, dry matter 30 %, organic matter 20 %, pH between 5.0 and 6.5, electric conductivity (EC) 200 µS/cm, NPK fertilizer 12-14-24 at 0.7 kg/m^3). Containers were placed in an air-conditioned room at an average temperature day/night 20 °C/15 °C. The photoperiod was 12-h long. Light was provided by fluorescent lights (Osram® Lumilux and Fluora tubes in proportion 7 to 1) with a mean photon flux density of 150 $\mu mol.m^{-2}.s^{-1}$ (PAR) (quantumètre LI-COR LI-250). The average air humidity was 70%. When young emerging shoots presented 2 to 3 leaf whorls and visible rootlets, they were separated from the main rhizome piece and put individually into plastic container (diameter 125 mm, height 100 mm). Osmocote ® type Substral fertilizer (NPK 14-13-13) was added to each pot according to the recommended dose. Experimental batches of 20 ramets were established for each population. Aboveground biomass was harvested when the plant reached the phenological stage corresponding to the 'early flowering stage' of Zlatnik's scale [19].

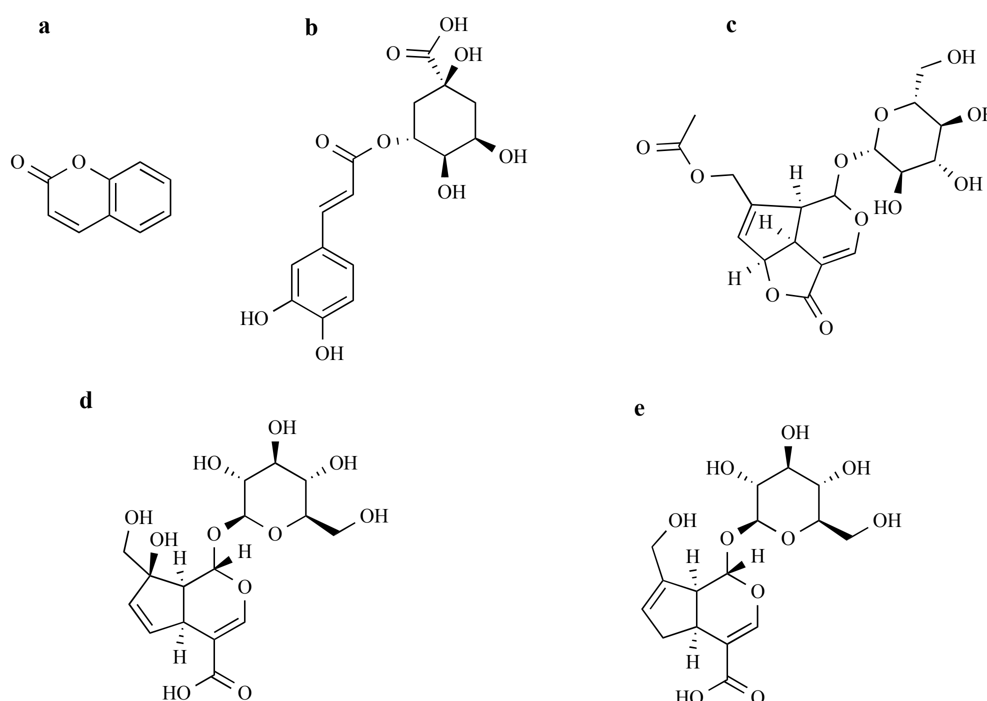

Fig. (1). Chemical structures of coumarin (**a**), chlorogenic acid (**b**), asperuloside (**c**), monotropein (**d**) and geniposidic acid (**e**) (source : www.chemicalbook.com).

Phytochemical Analysis

HPLC analysis. Extraction and sample preparation: Fifty mg of finely ground freeze-dried biomass was extracted with 5 ml of methanol during 20 min at room temperature. The extract was filtered through a GHP Acrodisc filter (0.45 µm, 13 mm, PALL corporation) and separated by HPLC-DAD technique to clearly identify asperuloside, monotropein, geniposidic acid, chlorogenic acid and coumarin. The whole analysis was then carried out in routine with an HPLC-UV to quantify the targeted secondary metabolites content. The reference molecules were from Sigma-Aldrich (Saint-Louis, MO, USA). Experimental conditions: A Waters liquid chromatograph equipped with a pump 600 (Waters, Milford, MA, USA) and a UV detector (Dual λ Absorbance Detector Waters 2487) was employed throughout this work. The analytical column was an Hypersil ODS (250 x 4.6 mm ; 5 µm, Alltech Associates Inc.) and the working temperature was 30°C. Two solvents were used: A, acetonitrile and B, trifluoroacetic acid (TFA, 0.05%). The elution profile was: 0 min A 0%, B 100%; 0-1 min A 3%, B 97% ; 1-45 min A 40% B 60%, 55-56 min A 0%, B 100% ; 56-70 min A 0%, B 100%. The UV detector was set at 254 nm for the detection of iridoids and 280 nm for the detection of coumarin and chlorogenic acid. The flow rate was 1 ml/min and the injection loop was 5 µl. All samples were analysed three times. All samples were also analysed in the same conditions, for the identity confirmation of the peaks, with an Agilent 1100 systems, equipped with a DAD detector.

Glucosidase hydrolysis of the extract. 50.0 mg of dry powdered material was extracted with 5 ml 0.1 M acetate buffer pH 4.5. After filtration through a GHP Acrodisc filter (0.45 µm,13 mm PALL corporation), the extract was separated into 2 aliquots of 2.5 ml each. One aliquot was treated with 500 µl of beta-glucosidase (β-glucosidase from almonds, lyophilized powder ≥ 2 u/mg solid, Sigma-Aldrich) in acetate buffer and incubated at 37 °C for 30 min. The reaction tube was then soaked for 3 min in boiling water. After filtration, both the treated and untreated extracts were injected with the same conditions as described above.

NMR analysis. Extraction and sample preparation: The dry powdered plant material (50.0 mg) was extracted with 1.5 ml of deuterated methanol (CD_3OD containing TMS 0.03 %) in 2 ml eppendorf tube. The extract was vortexed for 1 min at room temperature and then ultrasonicated for 10 min at room temperature. The mixture was then centrifuged at 13000 rpm for 10 min and the supernatant was transferred to 1.5 ml microtube. The mixture was centrifuged once again for 1 min at room temperature and the supernatant was then distributed into 5 mm-NMR tubes for measurements. NMR measurements: All spectra (^{1}H-NMR, COSY, HSQC and HMBC) were recorded at 300 K on a Bruker Avance 500 MHz NMR spectrometer (Bruker, Karlsruhe, Germany) operating at a proton NMR frequency of 500.13 MHz for ^{1}H, using a triple TCI ^{13}C/^{15}N 5 mm cryoprobe. Deuterated methanol was used as the internal lock. For ^{1}H-NMR, 128 scans of 32 K data points with a spectral width of 10330 Hz were recorded with the following parameters: pulse width (PW) = 30° (2.34 µs), and relaxation delay (RD) = 1.0 s. The acquisition time was 3.17 s. FIDs were Fourier transformed with LB = 0.3 Hz. The resulting spectra were manually phased and calibrated to the 0.00, using the internal standard TMS and topspin software (version 3.9, Bruker). Two replicates were measured for each plant material studied. Asperuloside and coumarin were identified in NMR spectra by using 2D NMR experiments (COSY, HSQC, HMBC).

Statistical Analysis

HPLC data were submitted to a variance analysis (one-way ANOVA) with following Tukey's test at the 0.05 significance level. A principal components analysis (PCA) was conducted to analyse the impact of the growth conditions - wild and cultivated material - among sweet woodruff populations on the basis of their phytochemicals. Analyses were performed with the software JMP® 7.0.1 (SAS Institute, Inc).

The optimised ^{1}H-NMR spectra were automatically baseline corrected and reduced to ASCII files using AMIX software (version 3.9, Bruker). Spectral intensities were normalized to total intensity and reduced to integrated regions of equal width (0.04 ppm) corresponding to the region of δ 0.40 – δ 10.00. The regions of δ 4.50 – δ 5.24 and δ 3.30 – δ 3.35 were removed prior to further analysis because of the residual signals of water and methanol, respectively. The matrix size consisted then of 220 variables. Principal component analysis (PCA) was performed with AMIX (version 3.9, Bruker); partial least square modeling-discriminant analysis (PLS-DA) and hierarchical cluster analysis (HCA) were performed with SIMCA-P software (version 11.0, Umetrics, Umeå, Sweden) with Pareto scaling of bucket variables for PCA.

RESULTS AND DISCUSSION

NMR Fingerprinting

The chemical profiling of *G. odoratum* was peformed by 1H NMR and the spectra showed broad range of metabolites which is useful for fingerprinting of wild type (*ex situ*) and cultivated (*in situ)* samples of the plants. The ^{1}H NMR data were subjected to PCA, PLS-DA and HCA aiming to highlight the differences among the *Galium* populations. The two wild type and cultivated populations could be easily discriminated by PCA analysis (data not shown), but their original location could not be discriminated. For more detailed discrimination PLS-DA was used. Two PLS components explains 89% of the variance with Q2 value 0.72. PLS-DA score plot shows a very clear separation of *in situ* and *ex situ* samples. By using PLS-DA component 1, all *Galium* plants were clustered in two groups (Fig. **2**). Group A, having positive component 1, was formed by cultivated (*ex situ*) *Galium* samples while group B having negative component 1, contained wild type *Galium* samples (*in situ*). The two groups were also clearly separated by a hierarchical clustering analysis (Fig. **3**). The general plants in Group A contained more carbohydrates, while the *in situ Galium* plants accumulated higher amounts of secondary metabolites, such as asperuloside and coumarin, highlighted by the statistical analysis.

HPLC Analysis

The *Galium* samples were then submitted to HPLC analysis focusing on asperuloside, coumarin and other known secondary metabolites from *Galium odoratum*

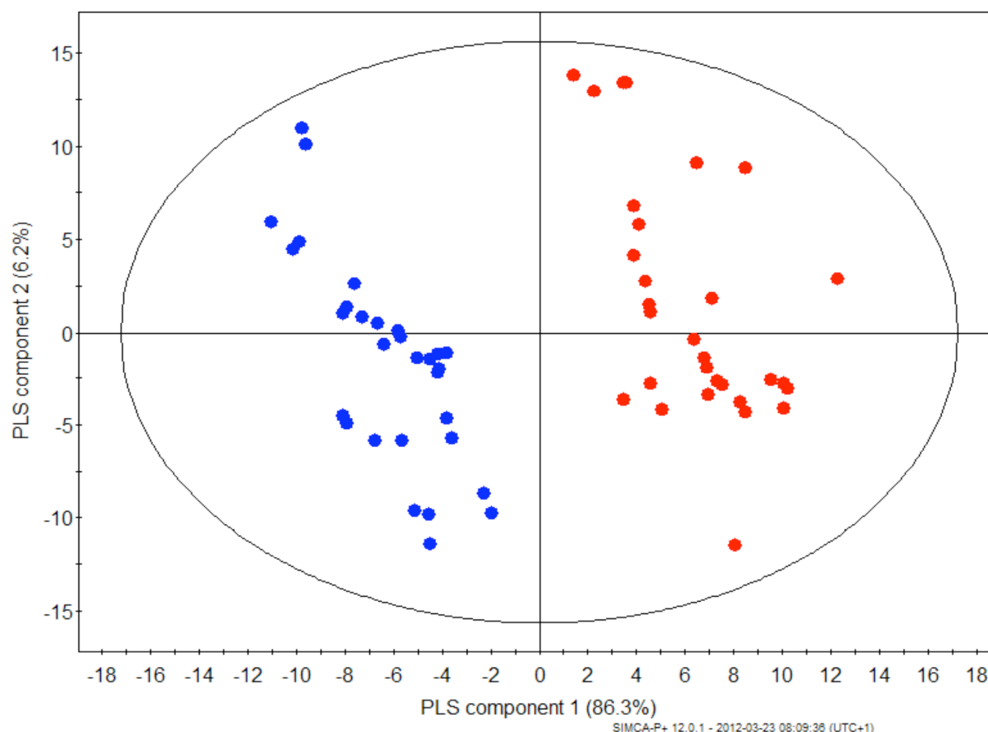

Fig. (2). Score plot of PLS-DA results obtained from all ^{1}H NMR data showing PC1 and PC2. Blue : *in situ* samples. Red : *ex situ* samples.

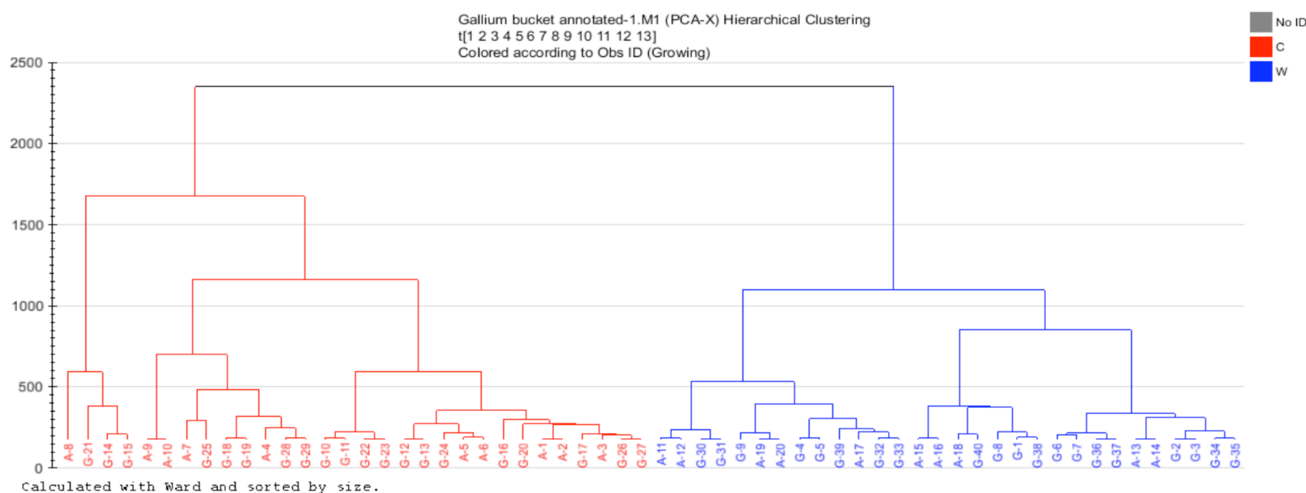

Fig. (3). Dendrogram of hierarchical cluster analysis of *Galium* samples. Red: *ex situ*. Blue: *in situ*.

(chlorogenic acid, monotropein, geniposidic acid). The aim was to target specific substances present in too low concentration to be detected by NMR.

Aerial Biomass Collected *In Situ*

The one-way ANOVA test (Table **1**) shows a very highly significant effect of the origin of the population on the content of each targeted phytochemicals. Biomass collected at Champenoux site had the highest amount for all metabolites except for geniposidic acid, more abundant in the biomass collected at Membre. Asperuloside was the most abundant secondary metabolite, regardless of the population, with values ranging from 1.87% for the biomass collected at Chassepierre to 3.85% for plant material from Champenoux. Geniposidic acid was the less abundant metabolite with values ranging from 0.03% (Chassepierre) to 0.20% (Membre).

In natural environment, coumarin and geniposidic acid were more discriminant metabolites for the investigated populations, while asperuloside showed no variation, with a clear exception for the plant material from the Champenoux site.

Aerial Biomass of Plant Material Grown in Controlled Environment (*Ex Situ*)

Results presented in Table **2** also show a very highly significant effect (P <0.0001) of the origin of the population on

Table 1. Secondary metabolites content of biomass collected *in situ* with regard to the origin of the population. Values in the table are means (n = 3). The *F* value indicates the significance of the one-way variance analysis (one-way ANOVA). Means followed by different letters within a column are significantly different (*P* = 0.05) according to Tukey test. NS = non significant. D.W. = dry weight.

Populations	Secondary metabolites (% D.W.)				
	Coumarin	Chlorogenic acid	Asperuloside	Monotropein	Geniposidic acid
Chassepierre	0.59 d	0.47 ab	1.87 b	0.27 c	0.03 c
Differdange	0.77 c	0.50 ab	1.96 b	0.35 bc	0.05 c
Membre	1.01 ab	0.44 bc	2.19 b	0.41 b	0.20 a
Habergy	0.89 bc	0.35 c	2.18 b	0.29 c	0.13 b
Champenoux	1.10 a	0.54 a	3.85 a	0.62 a	0.10 b
Mean	0.87	0.46	2.41	0.39	0.10
F	<0.0001	0.0005	<0.0001	<0.0001	<0.0001

Table 2. Secondary metabolites content of biomass grown in controlled environment (*ex situ*) with regard to the origin of the population. Values in the table are means (n = 3). The *F* value indicates the significance of the one-way variance analysis (one-way ANOVA). Means followed by different letters within a column are significantly different (*P* = 0.05) according to Tukey test. NS = non significant. D.W. = dry weight.

Populations	Secondary metabolites (% D.W.)				
	Coumarin	Chlorogenic acid	Asperuloside	Monotropein	Geniposidic acid
Chassepierre	0.48 b	0.28 c	1.28	0.04	0.10 c
Differdange	0.50 b	0.43 a	1.53	0.06	0.11 c
Membre	0.50 b	0.39 ab	1.26	0.09	0.49 a
Habergy	0.39 c	0.45 a	1.32	0.04	0.19 b
Champenoux	0.68 a	0.31 bc	1.59	0.06	0.14 bc
Mean	0.51	0.37	1.40	0.05	0.21
F	<0.0001	<0.0001	0.0469^{NS}	0.0528^{NS}	<0.0001

the content of three kinds of metabolites, coumarin and geniposidic acid being the most discriminating molecules, while asperuloside and monotropein showed no difference between plants from different sites. Asperuloside remains the most abundant compound and monotropein is, in those growth conditions, the less abundant metabolite. As noted for the *in-situ* plant material, the biomass from Membre had the highest geniposidic acid content and coumarin was more abundant in material from Champenoux.

In general, secondary metabolites content is lower for biomass grown in controlled environment (*ex situ*) compared to material collected *in situ,* except for geniposidic acid in higher amount in cultivated plants (Fig. **4**). These results show the significant influence of the plant growth conditions on the phytochemicals.

The principal component analysis (Fig. **5b**) based on HPLC results shows a strong discrimination between biomass collected *in situ* and material grown in controlled environment (*ex situ*). The principal component 2 highly correlated to the geniposidic acid content (Fig. **5a**) mostly influences the discrimination of plant material grown in controlled environment and especially plots from the site of Membre from each other. The principal component 1, highly correlated to the four other targeted phytochemicals, participates in the discrimination of plots from plant material collected in the wild and mainly plots from the site of Champenoux, as emphasized previously.

During the course of the HPLC analysis, a large unknown peak eluting shortly after the chlorogenic acid (about 3 minute later) was systematically recorded (Fig. **6a**). To tentatively identify the metabolite of this peak, an extract was submitted to a glucosidase hydrolysis. The HPLC profile of the hydrolyzed extract (Fig. **6b**) revealed the absence of the unknown compound while simultaneously, the *o*-coumaric acid peak increased, with this peak showing a similar UV spectrum to the unknown peak.

Such a profile prompted to suggest melilotoside (glucosyl-2-hydroxycinnamic acid), the glucoside of o-coumaric acid, as a component known to be present in significant amount in the fresh *Galium* extract [13].

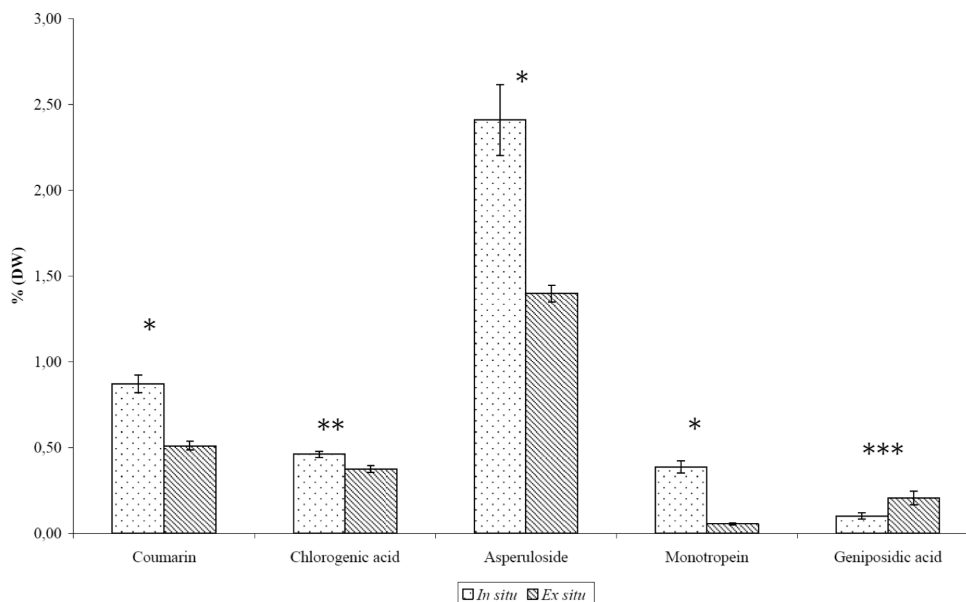

Fig. (4). Secondary metabolites content with regard to the growth conditions (*in situ* versus *ex situ*). * p <0.001, ** p < 0.005, *** p < 0.02 (t test).

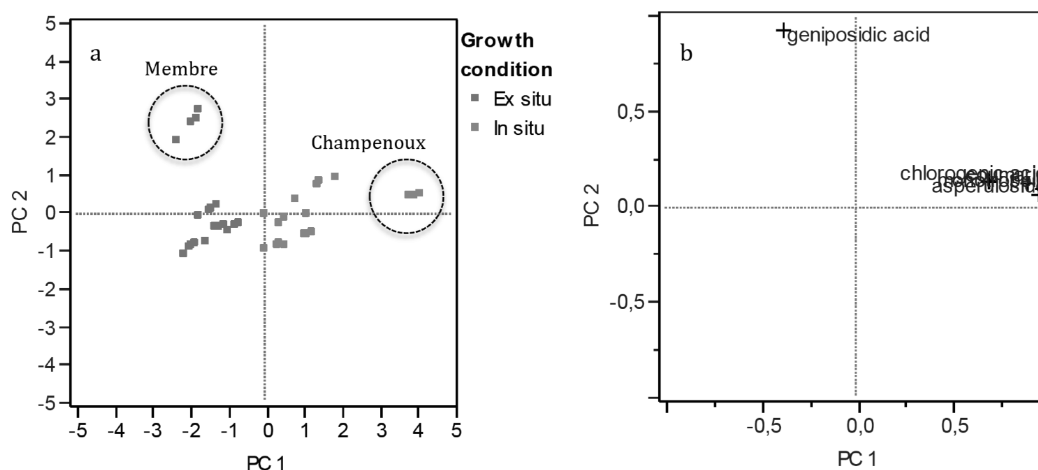

Fig. (5). Principal component analysis. **a**) score plot based on HPLC data of biomass collected in the wild (*in situ*) and material grown in controlled environment (*ex situ*). **b**) Loadings plot based on coumarin, chlorogenic acid, asperuloside, monotropein and geniposidic acid.

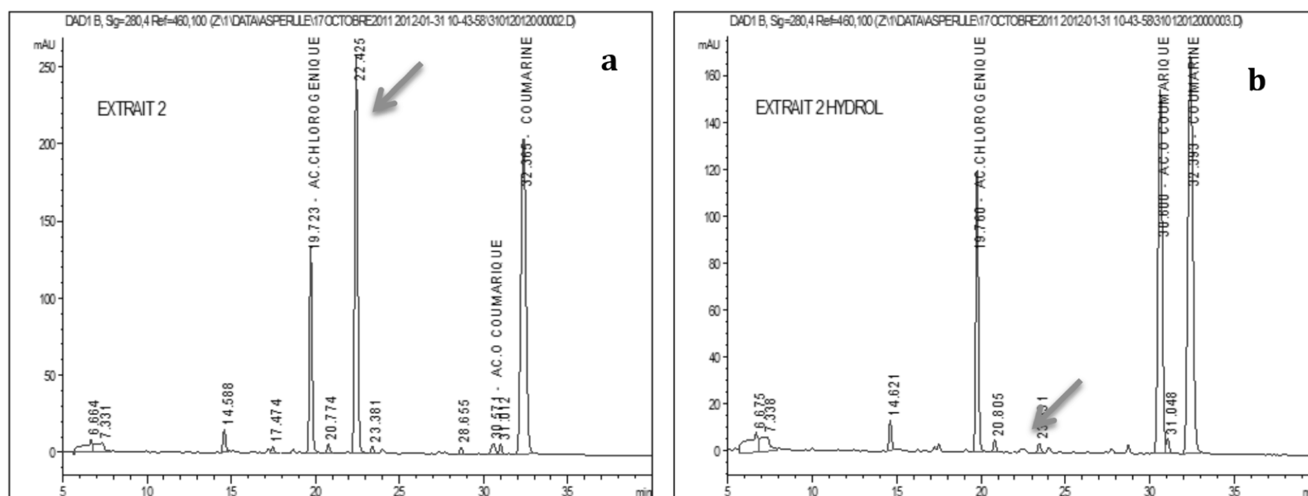

Fig. (6). HPLC chromatogram of an extract of *Galium odoratum* without hydrolysis (**a**) and after hydrolysis by β-glucosidase (**b**). The arrow indicates the melilotoside peak.

As melilotoside standard is not easily available commercially, the relative melilotoside amount was estimated from the corresponding peak surface on the HPLC profiles of the different plant materials (Table **3**). Results showed that the melilotoside content of the aerial biomass was significantly lower (P <0.0001) for plants in the wild (*in situ*) compared to plants grown in controlled conditions (*ex situ*).

It is also worth to mention that the *ex-situ* growth conditions appear to amplify the melilotoside variation between sites, the ratio between the extreme values being 1.47 and 2.73 respectively for plant material collected *in-situ* and *ex-situ*.

DISCUSSION

Quantitative analysis of the aerial biomass of natural populations of *Galium odoratum* confirmed that asperuloside and coumarin, both metabolites known as typical of *Galium* species, are the most abundant of the targeted secondary metabolites ranging respectively from 1.9 to 3.8 and 0.6 to 1.1 % depending on the ecological provenance of the collected dry biomass.

In natural conditions, the quantitative variation of each phytochemical with the origin of the plant population suggests the participation of the selected molecules to the chemical arsenal that this species develops to adapt to different environments.

The participation of phenolic compounds to the plant adaptative mechanisms to their ecological context has been documented in several reports. In the foliage of forest trees, variations of phenolic compounds have been associated with subtle environment gradients in moisture and nutrient availability [20]. Light spectral properties and risks of photodamages were also hypothesized as causal environmental factors of phenolic compounds production in plants [21], while applied environmental stresses such as drought or cold stresses have been shown to differentially alter the production of phenolic compounds of pharmacological interest in two *Crataegus* species [22].

Our observations show that, in *Galium odoratum,* besides coumarin and chlorogenic acid, both molecules from the phenylpropanoid pathway, iridoids also participate in the adaptation of this species to its local environment. In controlled conditions, the amount of coumarin, chlorogenic acid and geniposidic acid, remains dependant on the population origin. This is, to our knowledge, the first report of intraspecific quantitative variation of phytochemicals being maintained in controlled environment, as highlighted for coumarin in the aerial biomass of plant material from the Champenoux site. These observations could be indicative of genetic mechanisms specifically regulating the metabolic networks of phytochemicals responsible for a population adaptation to its natural environment. In that respect, it is interesting to note that two sites, namely Champenoux and to a lesser extent Membre, are marginal compared to the typical habitat of *Galium odoratum* and plant material from those sites was also distinctly separated by PCA analysis from the other investigated populations.

Both groups collected from the wild and plants grown in controlled conditions are clearly discriminate based on the whole metabolome analysis as well as on the amount of the selected metabolites.

Most secondary metabolites are in higher concentration in plants collected in the wild compared to plants grown in controlled conditions, independently of the population origin, with 2 exceptions, namely geniposidic acid and melilotoside at higher concentrations in plants grown in controlled environment compared to their counterpart in natural conditions. Those results are indicative of an alteration of the biosynthetic pathway notably for coumarin as melilotisode is generally recognized as the direct precursor of coumarin [9,10]. Although the biosynthesis path of iridoïds is less documented, in their report on iridoid patterns in *Galium* species, Mitova *et al.* (2002) consider geniposidic acid as an early metabolite on the biosynthetic route to other iridoids [23].

Therefore, the reduction of coumarin and asperuloside in plants grown in controlled conditions hypothesizes for both metabolites, the impairment at some early steps along the biosynthetic paths.

This gives support to the hypothesis of specific metabolites and associated biosynthetic paths as an adaptative mechanism of a species to its local environment.

NMR-based fingerprinting strengthens the discrimination between the two plant groups at the metabolome level but does not identify the population, and multivariate analysis techniques allow a clear classification in two groups related

Table 3. Peak surface of melilotoside with regard to the origin of the plant material and growth conditions.

Populations	Peak surface of melilotoside (µV*s)	
	In situ	*Ex situ*
Chassepierre	312187	485579
Differdange	407445	975315
Membre	326596	1232180
Habergy	373901	1045300
Champenoux	460979	1328316
Mean	376222	1013338

to the growing conditions. This discrimination appears to be related to both the primary and secondary metabolites content. Most, but not all secondary metabolites are more abundant in the wild type samples while carbohydrates are more abundant in the aerial biomass of plants grown in controlled environment.

Indeed, compared to the semi-quantitative NMR technique, the quantitative analytical approach provides a more nuanced picture by pointing on some specific secondary metabolites, particularly melilotoside and geniposidic acid, as being enhanced. Such discrete modifications of specific metabolites depending on the cultural conditions may explain that, at the metabolome level, the discrimination between populations is lost. Nevertheless, the use of a more resolutive approach, such as 2D NMR techniques, and/or of statistical analysis targeted on population discrimination could allow the identification of discriminant metabolites, in relation to population.

The reported results showed the effect of geographical factor and the growth conditions on *G. odoratum* phytochemical profiles, particularly secondary metabolites.

Illustrating differences between plant populations in natural as well as in controlled conditions, the results suggest qualitative alterations of metabolic paths as well as quantitative adaptations of the secondary metabolites profile to specific environmental conditions.

They emphazise the importance of fully controlled growth conditions to test the impact of specific environmental factors on the regulation of coumarin and asperuloside, two major phytochemicals of *G.odoratum,* and to further disentangle the regulation of the biosynthetic paths of phytochemical constituents of interest. Complementarily, the growth conditions and/or the environmental factors to select in order to collect plant material enriched in specific metabolites could also be facilitated.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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