ANTHRAQUINONE DYESTUFF AND GROWTH OF *GA*LIUM ODORATUM ((L.) SCOP.) RHIZOMES IN RELATION TO ECOPHYSIOLOGICAL AND ONTOGENIC CONDITIONS

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Abstract

Galium odoratum, a member of the *Rubiaceae* family is known to accumulate anthraquinone dyestuff in the rhizome. The objective of our research was to document ecophysiological variables that modulate the anthraquinone dyestuff content and the growth rate of the underground part of this species. Several variables were taken into consideration : (1) the plant population phenology and the geographic origin of the plant population, for plant material collected in 4 natural stands identified in forests of Belgium, Grand-Duchy of Luxembourg and France, (2) the plant growth conditions, i.e. natural stands *vs* controlled environment and (3) the plant propagation path, i.e. clonal *vs* seed propagation.

In natural stands, the anthraquinone dyestuff content varied with the plant phenology, being minimal at full flowering stage and the highest at leaf yellowing. It also varied depending on the geographic location of the plant population. Such a difference between plant populations was not observed when plants were grown in controlled environment. Typically, the anthraquinone dyestuff of the plant rhizome was higher in controlled environment than in natural stands, suggesting a strong impact of the growth conditions on anthraquinone biosynthesis. Additionnally, the plant propagation path does not influence the anthraquinone dyestuff content, but when newly formed rhizome was separated into fragments of increasing age –from 27 to 105 days-, a linear increase of the dyestuff with the rhizome ageing was observed.

Keywords: Galium odoratum, anthraquinones, ecophysiology, rhizome, growth condition

Introduction

Galium odoratum (L.,) Scop. Also known as sweet woodruff is a perennial herb with a creeping rhizome (Lambinon et al. 2004) growing in shady european deciduous forests, principally where beech trees are predominant. This species considered as a typical plant of ancient woodlands (Wulf 1997), has also been shown to survive and extent in recently implanted forest (Petersen and Philipp 2001). In natural conditions, the plant population extension is largely by clonal propagation and the reproduction rate measured by the seed production per individual population is low (Kolb and Lindhorst 2006). Seed recruitment from local and non-local origin can to some extent participate to the plant population growth, however the seed dispersal by seeds encroaching on the large mammal coats appears to be low (Luftensteiner 1982). Additionnally, seeds are slow to germinate, taking up to 200 days (Reppert 1985).

In natural stands, the rhizome growth over a seasonal cycle has been estimated to vary between 20-40 cm /year (Petersen and Philipp 2001; Kolb and Lindhorst 2006) but can be as much as 1m/year (Lippert-Hambacher 1992). These values, estimated by an indirect approach based on the plant population migration evaluation, must be considered as a gross estimation of the rhizome growth potential.

This plant is known for its medicinal and aromatic properties, and is marginally grown as an ornamental ground cover. It belongs to the *Rubiaceae* family, more precisely to the *Rubieae* tribe, whose several members are known to accumulate anthraquinones especially in their roots (Gilbert and Cooke 2001), and *G. odoratum* is no exception. *Rubia tinctorum* (madder) is by far the most studied member of this botanical family with regard to anthraquinone content and assortment, partly due to the long recognised use of madder root extract as a red dyeing agent for textiles, paintings (Bechtold and Mussak 2009) but also as food colorant (Derksen and Van Beek 2002). Alizarin and purpurin, respectively di- and tri-hydroxyanthraquinones, are the main dyeing stuff (Derksen and Van Beek 2002) and sweet.

Woodruff was mentioned as a substitute for madder in earlier times (Bancroft 1813).

Up to eleven anthraquinone pigments were identified in *G. odoratum* (previously *Asperula odorata)* roots, while none could be detected in the green parts (Burnett and Thomson 1968).

Besides dyeing properties, a number of biological and pharmacological activities are also associated to anthraquinone and derivatives, such as anti-oxidant, anti-microbial, anti-fungal, , anti-viral, larvicidal. Lucidin, an anthraquinone derivative is also reported to have mutagenic activity (Derksen and Van Beek 2002), while alizarin and purpurins have been shown to have a strong inhibitory effect on the genotoxicity of several carcinogens (Takahashi et al 2001).

Because no data are available on *Galium odoratum* rhizome development, the present research seeks to document ecophysiological variables that modulate the growth rate and the anthraquinone content of the rhizome of this species.

More specifically, the study aims to provide insight into the following points (1) the evolution of the anthraquinone dyestuff content with regard to both the plant phenology and the origin of the plant population, (2) the impact of the growth conditions – natural stand *(in situ)* or controlled environment *(ex situ)* - on anthraquinone dyestuff content and (3) the rhizome growth rate in relation to the plant propagation path (sexual vs clonal).

Two experimental approaches have been developed:

- anthraquinone determination of rhizomes collected from 4 natural stands identified in forested areas in Belgium, Grand-Duchy of Luxembourg and France.
- Growth rate and anthraquinone determination of newly formed rhizome of plants sexually or clonally propagated and grown in controlled environment.

Materials and methods

Study sites

Investigations were carried out on plant material collected in four different locations in Belgium (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), Differdange (Luxembourg) and Champenoux (France). The forest phytocoenoses are represented by the associations *Melico-Fagetum* for Habergy and Differdange, *Primulo-Carpinetum* for Chassepierre and *Poa chaixii-Quercetum roboris* at Champenoux according to the classification on the European database Corine Biotope (Devillers et al., 1991).

Sampling time

For the four study sites, rhizome pieces were collected randomly at the population phenological stage ‘early flowering’. Rhizome pieces were

freeze-dried and stored in the freezer at - 80°C for later phytochemical analysis.

For a single station -Habergy (Belgium) - rhizome material was collected randomly at 7 phenological stages according to the scale of Zlatnik (1978), covering the annual cycle from February to November.

Plant material propagated in controlled conditions

Clonal propagation

Rhizomes were collected randomly (cumulated total length of approximately 6 meters) at the early growth stage (March 2010) in each population and pieces of about 30 cm were set in tray (40 cm x 55 cm). The potting media is a peat based 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 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. When the plants had 6 fully expanded leaf whorl, i.e. at a phenological stage corresponding to the 'early flowering stage' of Zlatnik's scale (1978) for those shoots induced to flower (approximately 42 days of growth), newly formed rhizomes were harvested, cleaned from potting media and freeze dried.

Seedling propagation

Young seedlings were collected at the cotyledon stage - first emerging leaf whorl - on the 19th of May 2010 from the forest floor at the study site of Habergy, potted individually in tray (40 cm x 55 cm) and placed under controlled conditions as described above. It took 90 days before rhizome emission was visible (10 mm of rhizome). One week later, several rhizomes were developing below the soil surface and the three longest ones of each plant were labeled for monitoring. The growth in length was monitored with a time step of 3 to 4 weeks. After 105 days of growth, the 3 monitored rhizomes were removed, cleaned from the potting media and separated into fragments corresponding to three classes of growth age (27, 76

and 105 days of growth). Collected material was freeze-dried and stored in a freezer at -80°C.

Anthraquinone dyestuff determination

Sample extraction

Freeze-dried material was powdered by mortar and pestle, and fifty mg of powder was then soaked in 50 ml of distilled water for 16 h. This suspension was heated in a water bath at 70°C for 1 h. After the suspension was cooled, 50 ml methanol (MeOH) was added to the samples. The samples were then filtered through a 20-µm filter grade 42 (Whatman®), and the filtered volume was adjusted to 100 ml with 50% methanol.

Determination as total dye

Total dye content was estimated by reference to a standard solution (2mg/100ml) prepared as a mixture of alizarin ($C_{14}H_8O_4$, Sigma-Aldrich) and purpurin ($C_{14}H_8O_5$, Sigma-Aldrich) (1:1) dissolved in pure methanol and then adjusted to 100 ml by adding distilled water to obtain a solution of aqueous methanol 50%. The absorbance values of all methanolic extracts and standard solution were measured at 450 nm using a spectrophotometer (Beckman UV/VIS DU 800). Total dye content based on alizarin and purpurin standards was calculated according to the equation of Baghalian et al. (2010).

$$\text{Total dye content (\%)} = (100/50) \times \frac{\text{Standard concentration} \times \text{Sample absorbance value}}{\text{Standard absorbance value}}$$

Determination as alizarin and purpurin

Ten milligrams of alizarin ($C_{14}H_8O_4$, Sigma-Aldrich) and ten milligrams of purpurin ($C_{14}H_8O_5$, Sigma-Aldrich) were dissolved each in 100 ml of aqueous methanol 50%.

For each compound, 4 standard solutions (covering the range from 0.125 to 1mg/ml) were prepared and calibration curves were determined. The absorbance values of all methanolic extracts and standard solutions were measured at 429 nm for alizarin and 482 nm for purpurin (Drivas et al 2011) using a spectrophotometer (Beckman UV/VIS DU 800).

Statistical analysis

Data were submitted to an analysis of variance (one-way ANOVA) and mean comparison was performed using the Tukey's test at the 0.05 significance level. Data were submitted to the arcsinus transformation for the one-way ANOVA analysis of the percentage of total dyestuff. The phytochemicals analysis was conducted in eight replicates for plants propagated from seedlings and in six replicate for the other plant materials.

All analyses were performed with the software JMP® 7.0.1 (SAS Institute, Inc).

Results

Evolution of anthraquinone dyestuff with regard to the plant phenology in natural stands

Figure 1 shows the total dye, alizarin and purpurin content in rhizome of sweet woodruff over a complete growth cycle – from February to November - in natural conditions. Anthraquinone content varied over the seasonal cycle and the analysed compounds presented a similar pattern. The general phytochemical profile shows a decrease during the flowering period and a net accumulation at the leaf yellowing stage.

The total dye content ranged from 0.42% of dry weight at the full flowering stage to 1.33% at yellow leaves stage. Alizarin and purpurin contents varied respectively from 3.27 and 1.49 µg mg $^{-1}$ of dry weight at the full flowering stage to 8.22 and 5.20 µg mg $^{-1}$ at yellow leaves stage.

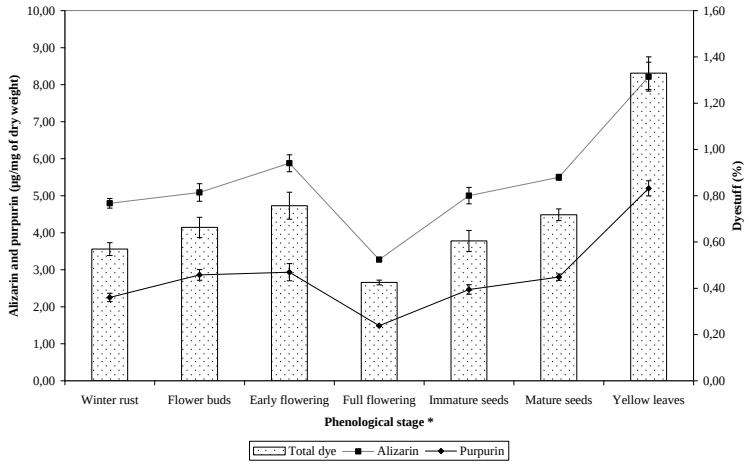

Figure 1: Evolution of the total dyestuff (% of dry weight), alizarin (µg/mg of dry weight) and purpurin (µg/mg of dry weight) content of *G. odoratum* rhizomes collected at Habergy (Belgium) during a growth season in natural conditions. Values are means and vertical bars represent the standard error. * Phenological stage according to the scale of Zlatnik (1978).

Anthraquinone dyestuff of rhizome collected in natural stands

The total dye, alizarin and purpurin content of rhizome collected in natural conditions at the early flowering stage on the 4 study sites are given in table 1. The one-way variance analysis shows a significant effect (P<0.05) of the origin of the population on anthraquinone content. The total dye content varied from 0.67% for rhizomes collected at the Differdange site to 0.85% for material harvested at the site of Chassepierre. Alizarin and purpurin contents ranged respectively from 5.31 and 2.68 µg mg $^{-1}$ of dry weight at Differdange to 6.20 and 3.30 µg mg $^{-1}$ at Chassepierre.

Table 1**:** Total dye, alizarin and purpurin content of rhizomes of G. odoratum collected at the 'early flowering' phenological stage in natural conditions with regard to the origin of the population. Values are means and standard error.

Sites	Total dye content (% of dry weight)	Alizarin (µg mg^{-1} of dry weight)	Purpurin (µg mg^{-1} of dry weight)
Chassepierre (Belgium)	**0.86 +/- 0.05 a**	**6.20 +/- 0.26 a**	**3.30 +/- 0.17 a**
Habergy (Belgium)	**0.76 +/- 0.05 ab**	**5.88 +/- 0.21 ab**	**2.93 +/- 0.21 ab**
Champenoux (France)	**0.73 +/- 0.01 ab**	**5.40 +/- 0.11 ab**	**3.08 +/- 0.04 ab**
Differdange (Luxembourg)	**0.65 +/- 0.03 b**	**5.31 +/- 0.22 b**	**2.68 +/- 0.09 b**
Overall mean	**0.75**	**5.70**	**3.00**
F value	***0.0495 ****	***0.0196 ****	***0.0485 ****

Mean values within each column followed by the same letter are not significantly different at the 0.05 probability level according to the Tukey's test. *: $P < 0.05$ significant.

Rhizome anthraquinone dyestuff and growth rate of clonally propagated plant material grown in controlled environment.

The total dye, alizarin and purpurin content of rhizome collected in natural conditions at the early flowering stage on the 4 study sites are given in table 1. The one-way variance analysis shows a significant effect ($P<0.05$) of the origin of the population on anthraquinone content. The total dye content varied from 0.67% for rhizomes collected at the Differdange site to 0.85% for material harvested at the site of Chassepierre. Alizarin and purpurin contents ranged respectively from 5.31 and 2.68 µg mg^{-1} of dry weight at Differdange to 6.20 and 3.30 µg mg^{-1} at Chassepierre**.**

Table 2 **:** Total dye, alizarin and purpurin content, and dry mass of newly formed rhizomes for clonally propagated plants grown in controlled conditions. Observations realised after 42 days from potting. Values are means +/- standard error. n= 20.

Sites	Total dye content (% of dry weight)	Alizarin (µg mg^{-1} of dry weight)	Purpurin (µg mg^{-1} of dry weight)
Chassepierre (Belgium)	**0.86 +/- 0.05 a**	**6.20 +/- 0.26 a**	**3.30 +/- 0.17 a**
Habergy (Belgium)	**0.76 +/- 0.05 ab**	**5.88 +/- 0.21 ab**	**2.93 +/- 0.21 ab**
Champenoux (France)	**0.73 +/- 0.01 ab**	**5.40 +/- 0.11 ab**	**3.08 +/- 0.04 ab**
Differdange (Luxembourg)	**0.65 +/- 0.03 b**	**5.31 +/- 0.22 b**	**2.68 +/- 0.09 b**
Overall mean	**0.75**	**5.70**	**3.00**
F value	***0.0495 ****	***0.0196 ****	***0.0485 ****

Mean values within each column followed by the same letter are not significantly different at the 0.05 probability level according to the Tukey's test. NS: $P > 0.05$ not significant.

Rhizome anthraquinone dyestuff and growth rate in relation to rhizome age for plants propagated from seedlings

Growth rate

Rhizome emission was detected at the shoot base of the seedlings 90 days after planting. At the end of the experiment, a dense network of branched rhizome was formed and the rhizome mean dry weight and mean length per plant were respectively 2.75 g and 2.61m (cumulated data of the three labelled rhizome used for the monitoring). The growth monitoring presented figure 2 shows the best fitted curve characterized by the following equation: $y = 0{,}011x^2 + 5.3863x - 565.47$. The growth rate from emission to the end of the experiment was 8.2 mm/day.

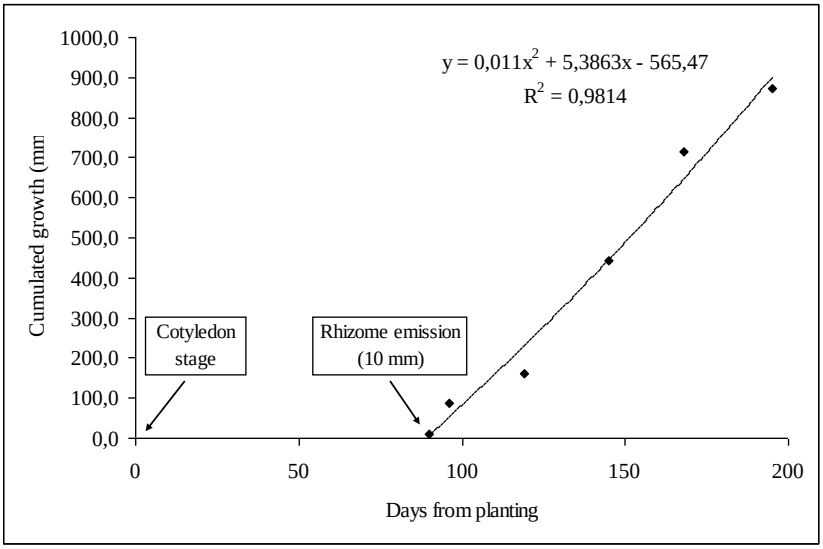

Figure 2 : Growth of the newly formed rhizome of *Galium odoratum* seedlings grown in controlled conditions. Values are means (n=8) and the line is the best fitted curve.

Rhizome anthraquinone dyestuff

Anthraquinone dyestuff varied in function of the rhizome different classes of age with content three times higher for rhizomes older than 3 months as compared to 1- month old rhizome. The results show a linear increase of the total dyestuff ($R^2 = 0.99$) with the rhizome age (Figure 3). Total dyestuff ranges from 0.97 % for newly formed rhizome (27 days) to 3.17 % for pieces of rhizome old of 105 days.

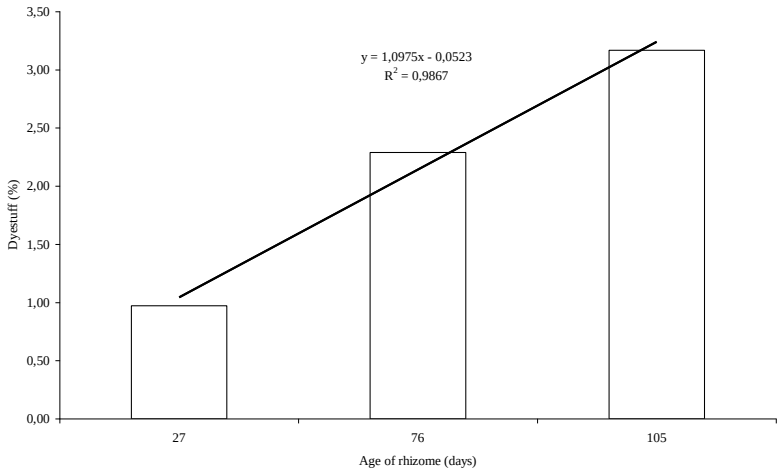

Figure 3: Anthraquinone dyestuff of newly formed rhizomes grown in controlled conditions in relation to the age of rhizomes (values are means, n=8).

The effect of the rhizome ageing on the content of anthraquinone dyestuff was very highly significant (P<0.0001). Results also showed that whatever the age of the rhizome analyzed, the content of alizarin was twice as high as the purpurin content.

Table 3 : Total dye, alizarin and purpurin content of newly formed rhizomes grown in controlled conditions in relation to the age of rhizomes. Values are means and standard error.

Age of rhizome fragment (days)	Total dye content (% of dry weight)	Alizarin (µg mg^{-1} of dry weight	Purpurin (µg mg^{-1} of dry weight
27	0.97 +/- 0.08 c	6.92 +/- 0.42 c	3.46 +/- 0.26 c
76	2.29 +/- 0.13 b	13.72 +/- 0.60 b	7.89 +/- 0.43 b
105	3.17 +/- 0.14 a	17.75 +/- 0.63 a	10.85 +/- 0.54 a
Overall mean	2.14	12.79	7.40
F value	*< 0.0001* ***	*< 0.0001* ***	*< 0.0001* ***

Mean values within each column followed by the same letter are not significantly different at the 0.05 probability level according to the Tukey's test. *** : P < 0.001 very highly significant.

Discussion

Dyestuff content

The reported results clearly show that the total dyestuff of *Galium odoratum* varies with both the growth conditions and rhizome physiology.

a) Growth conditions

The rhizome dyestuff of seedling plants grown in controlled environment is 2.14 % compared to 1.78% for clonally propagated plants grown in controlled conditions and 0.75% for rhizome pieces collected in natural stands. There are no differences of rhizome dyestuff content for

clonally propagated or seed propagated plants grown in controlled environment while in natural stands the rhizome dyestuff content is much lower.

These results suggest that the controlled environment has provided climate conditions more favorable to the anthraquinone biosynthesis. One cannot exclude that part of this difference could be related to the age of the collected rhizomes pieces in natural stands.

b) Age of the rhizome

Although it is not possible to determine the age of rhizome pieces collected in natural stands at early flowering time, -i.e. mid-May in our climate conditions- the collected samples are likely to represent pieces varying in age from 1-4 months up to one year old pieces. Indeed, in natural stands, new rhizome emission was regularly observed by the end of winter (January-February), for all study sites and rhizome older than one year, recognizable by a brownish color was avoided in the sampling.

When the seedling rhizome pieces were analyzed in relation to the class of age, it clearly appears that the older pieces -105 days versus 27 days- had a dyestuff content three times as high. In fact, there was a linear relationship between the dye content and the rhizome ageing over the experiment. Whether this difference is related to an increasing capacity of the ageing underground shoot to synthesize anthraquinones is worth to consider in the future. It is interesting to mention here that in *Rubia tinctorum*, total alizarin content has been shown to be largely located into the root cortex (Angelini et al. 1997), and the increase in total dyestuff observed in *Galium* could also be related to the increase of the cortex volume relative to the stele. In that context it is interesting to mention that in *Rubia tinctorum*, based on observations made in field conditions and on a larger time scale –between 1 and 3 years-, Angelini et al. (1997) observed higher total alizarin content in the first year of growth for transplanted seedlings than later one. In an analysis of the dyestuff content with regard to the root diameter, Baydar and Karadogan (2006) observed a higher dye content in roots of smaller diameter (less than 0.5 cm) corresponding tertiary roots (3.02%) than in larger and older roots. Alizarin per unit dry weight has been shown to increase from 6.7 to 8.7 mg/g dry weight with the ageing of the root system from 2 to 3 years (Derksen 2001). It is therefore generally considered that the best yield in term of dry mass of roots, dyestuff yield is obtained for *Rubia* field harvested after 3 years.

Although those agronomic studies on *Rubia tinctorum* are indicative of physiological controls similar to those reported here for *Galium odoratum*, it is difficult due to the variations in climate, plant age and estimation methods of yield of the agronomic approaches to directly compare these two species.

c) Phenology

In natural conditions, the total dyestuff is minimal at the full flowering stage (0.42%) and increases by the end of the seasonal cycle, when the rhizome dye content is approximately twice as much as that at early season (1.33%). To our knowledge, this is the first time that a seasonal evolution of anthraquinone dyestuff is shown in *G. odoratum*. In a 3-year old field of *Rubia tinctorum*, a variation of the dye content over the annual cycle with a minimum in March (2.16%) and a maximum in August (3.25%) has be related to the local variations of the pluviometry and temperatures (Baydar and Karadogan 2006).

Anthraquinone molecules being considered as participating to the defense barrier of the plants against fungi and bacteria (Kalyoncu et al. 2006), it may be hypothesized that this variation could be an adaptative mechanism to resist to the humid conditions of the adverse season. To which extent the annual variation of anthraquinones is related to the phenological stage and/or directly under the control of the climate conditions is a question to be investigated in the future.

d) Origin of the population

In natural stands, the dye content of *Galium* rhizome pieces is rather low and varies with the plant population. Such a variation between plants materials collected from different populations was not observed when plants are grown in controlled environment, and therefore is more likely related to differences in pedo-climatic conditions between the different stands. An additional effects of the origin of the population such as shown for *Rubia tinctorum* grown in the field from different seed populations (Angelini et al. 1997; Ercan et al. 1999) cannot be excluded, and additional observations are necessary for assessing this remark.

Rhizome growth rate

Rhizome emission for plants propagated from seedlings in controlled conditions was detected after 90 days. The rhizome growth in length was slow from planting but increased rapidly after emission with a growth rate of 8.2 mm/day. Each primary rhizome develop further a branching pattern whose total length after 105 days is more than 2.5 m. Based on those values, the potential growth of the rhizome is much higher than the estimated extension of the plant population mentioned by Ziegenhagen and al. (2003) who reported a clonal propagation (i.e. rhizome growth) averaging 20 cm $year^{-1}$ and up to 40-100 cm $year^{-1}$.

The mean rhizome dry weight per plant was 2.75 g for plants propagated by seedlings. Reported values for *Rubia tinctorum* varies from 9 to 30 g/plant –depending on the origin of the seeds- in field planted with seedlings and determine over the first 5 months (Angelini et al. 1997). In

comparison, the mean dry root yield varied from 11g/plant for spring root transplant to 32 g/plant for spring seed sowing harvested after 3 years of growth in Turkey (Baydar and Karadogan 2006).

Conclusion

In our study, results show the influence the seasonal cycle as well as the origin of the population on the anthraquinone dyestuff content of rhizome collected in natural stands. The results also suggest that the controlled environment has provided climate conditions more favorable to the growth and to the anthraquinone dyestuff biosynthesis with content more important for clonally propagated plants or seed propagated plants compare to rhizome collected in natural stands.

Our research provide a better knowledge of the rhizome physiology of *Galium odoratum* in focusing on ecophysiological and ontogenic factors which could affect the growth and the anthraquinone dyestuff content of this species.

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