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Original paper

Dynamics of the ruscogenin biosynthesis in Ruscus aculeatus L. (Liliaceae) in vitro cultures

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Abstract

Ruscus aculeatus L. (Liliaceae) is an evergreen, slow-growing semi-shrub. The rhizomes and roots are collected for the containing steroidal saponins (ruscogenin). They are used in medicine and cosmetics for their anti-inflammatory, venotonic, and antihaemorroidal activity. Tissue culture of R. aculeatus shoot clusters was carried out through continuous passages on agar media. Production of ruscogenins (ruscogenin, neoruscogenin and two desmosides: ruscin and desglucoruscin) was quantified by HPLC in shoots and roots every three months for one year. Neoruscogenin production was reduced in the roots and increasing in the shoots. Maximum of about 1 mg/g DW was recorded on the 3rd month for the shoots and on the 12th month for the roots. In the roots, the ruscogenin content was almost constant compared to the shoots were slight elevation was observed after the sixth month. The desglucoruscin production was most variable in the roots ranging from 0.1 to 0.8 mg/g DW. The ruscin content varied insignificantly in both cultures. Obtained data imply that the development of the R. aculeatus cultures should be managed according the dynamics of ruscogenin biosynthesis as the one of the major constituents neoruscogenin showed contrasting tendencies in shoots and roots.

Keywords Steroidal saponins, HPLC analyses, ruscogenins

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Introduction

Phytosaponins is a diverse group of secondary metabolites found in a variety of plant species that are used as medicinal plants around the world (SPARG & al [1], VINCKEN & al [2]) Depending of their structure plant saponins possess number of biological and pharmacological activities like antibacterial, antifungal, anti-inflammatory, hemolytic, cytotoxic, antitumor and others (YOSHIKI & al [3], FRANCIS & al [4]). Saponin production was found to be very slow and costly in vivo especially due to the limited number of the plant families (typically monocots) that biosynthesize greater part of the popular saponin-containing pharmaceuticals nowadays. Thus, production of saponins by tissue and cell cultures is increasingly investigated as prospective way to obtain pharma- and nutraceuticals (for review see MATHUR & MATHUR [5], MURTHY & al [6]).

Ruscus aculeatus L. is naturally spread in the Mediterranean area (incl. North Africa), Central and East Europe, Caucasus and Crimea (YEO [7]). Additionally, it is found in Great Britain and United States where the northernmost populations are result from escapes from garden introductions (PRESTON, [8]). The species is used as medicinal plant since Antiquity and is gathered mainly from the wild. Ruscogenin, saponin extracted from Radix et Rhizoma Rusci, possess an anti-inflammatory, venotonic, antihaemorroidal and other properties (BLUMENTHAL & al [9]). They are used both in traditional and conventional medicine and recently in cosmetics and body shaping products (CAPRA [10]). Natural resources in Europe and Asia Minor are diminishing due to uncontrolled and unsustainable collection (LANGE & al [11], ÖZHATAY & al [12]). The species was included in Directive 92/43/EEC and the European Plant Red List. In Bulgaria, the collection of R. aculeatus from its natural accessions is regulated by Bulgarian Biodiversity Act (2007) and the Bulgarian Medicinal Plants Act (2000). In Romania, the species has a rare status in the Red list of vascular plants from Romania (OLTEAN & al [13]).

In vitro cultivation of R. aculeatus has been investigated previously covering different aspects of the propagation on media supplemented with a variety of growth regulators, biochemical and genetic characteristics and (BANCIU & al [14, 15], MOYANO & al [16],). In vitro cultivation was proposed as alternative for production of ruscogenin by callus cultures (PALAZON & al [17], VLASE & al [18]). Clone origin was pointed as important factor for the ruscogenin biosynthesis in shoot cultures (IVANOVA & al [19]). Organ differentiation and phenophase were pointed out as important factors for production of other saponins as well (MATHUR & MATHUR [5], DHARA & al [20]). Elicitation of the ruscogenin synthesis by methyl jasmonate was reported as non-effective (MANGAS & al [21]) The phenophases and seasons have shown to have influence on the ruscogenin production in R. aculeatus in vivo (NIKOLOV & GUSSEV [22]).

Current study investigates the dynamics of the ruscogenin production of in organs of in vitro-grown plants for a period of one year.

Materials and Methods

Plant material and culture conditions

In vitro cultures of R. aculeatus were obtained by cultivation of in vitro germinated seed as described previously (IVANOVA & al [23]). Rhizome explants were placed on MS (MURASHIGE & SKOOG [24]) agar media supplemented with 1 mg/l mg/L 6-Benzylaminopurine and 0.5 mg/L α-Naphthaleneacetic acid. Explants and developed plantlets were transferred on fresh media every month. Growth regulators and agar were supplied by Duchefa, Netherlands. Biomass growth was accessed every 3 months.

Ruscogenin analysis

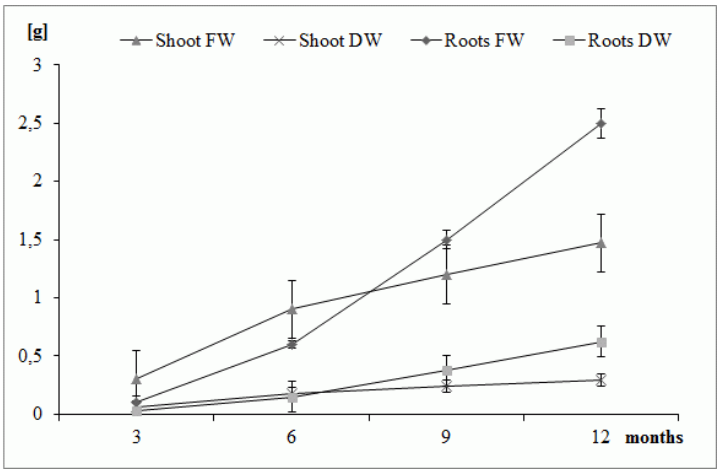
Samples for ruscogenin determination were prepared by at least 20 plants. Production of ruscogenin in shoots, rhizomes and roots of 3 to 12 months old regenerants was determined by HPLC following IVANOVA & al [19]. Samples were collected every 3 months and dried at 40°C. Samples powdered of 50 mg from respective parts were extracted with 50:50 aqueous methanol for 24 h and evaporated under vacuum at 40°C (Heidolph, Germany). Dry extracts were hydrolyzed for 4 h in butanol, deionized water and 37% HCl. Butanolic fractions were filtered and evaporated to dryness. Residues were redissolved in 200 mL methanol and filtered (0.45 mm, PTFE). Chromatographic system: column: Phenomenex Synergi MAX-RP 80A 4 µm (150x4.6 mm); detector: Waters M 996 Diode Array Detector PDA Max plot 180-800 nm. Chromatographic conditions: injection volume 10 mL; at 25 C°, Elution gradient: A (acetonitrile:water) 65:35; B (acetonitrile:water) 50:50 – 0-13 min-100% B, 13-20 min-100% B, >20- 100% B. Four standards were used

for quantitative measurement: ruscogenin, neoruscogenin, ruscin and desglucoruscin (ChromaDex, USA). Calibration curves were set between 0.2 and 3 mg/0.01 mL.

Data were processed statistically by single factor ANOVA. Person correlations were produces to access the parallels between the measured saponins.

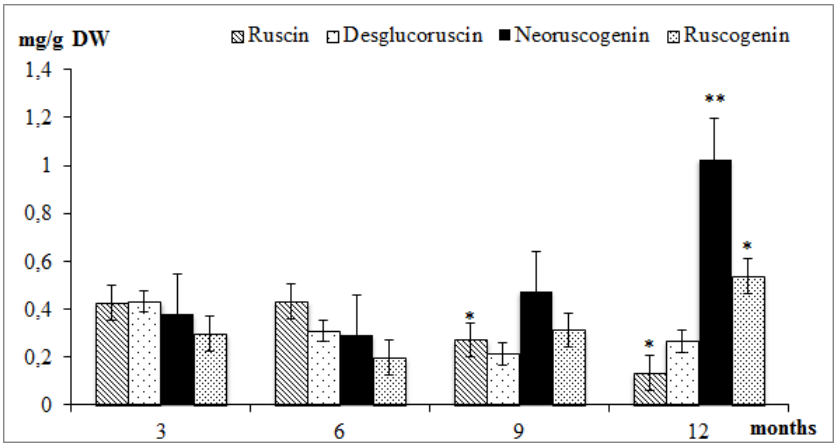
Results and discussion

Rhizome and root growth was slower in the beginning but accelerated with the advancement of the shoot development (Fig. 1). The development of *R. aculeatus* cultures was visible after the 3rd month in cultivation. The



FW- fresh weight, DW dry weight. Values are mean of five measurements, ±SE

Figure 1. Average growth of the aerial shoots and roots of *in vitro* grown *Ruscus aculeatus* for period of one year.



Sample size – minimum 20 plants. Values are mean of three measurements, ±SE
*p<0.05, ** p<0.01

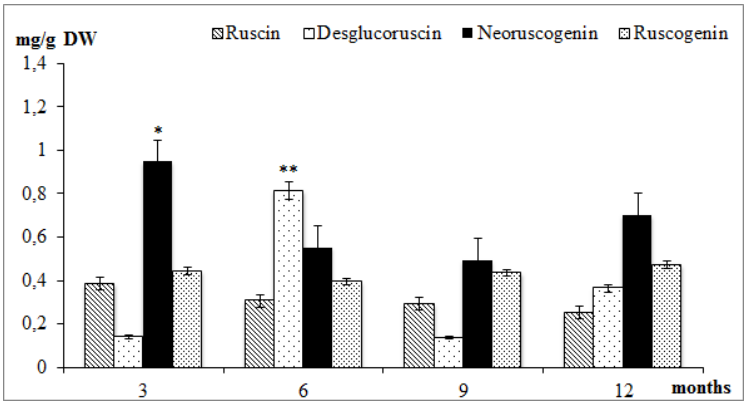
Figure 2. Dynamics of ruscugenins content in shoots of *in vitro* cultivated *Ruscus aculeatus* plants.

aerial shoots were comprised of unbranched stems with few apical cladodes. Shoots were regarded as outgrowths of meristem buds that are on the rhizome surface as it is shown in other *Ruscus* species (CURIR & al [25]).

The levels of all tested ruscogenins were similar in start of the development of the shoots (Fig. 2)

The content of all measured saponins in 3-month old shoots varied between 0.2-0.5 mg/g DW that was about the levels previously reported for *R. aculeatus in vitro* cultures (PALAZON & al. [17], IVANOVA & al. [19]). The content of the major ruscogenins (neoruscogenin and ruscogenin) in the shoots remained almost constant until the 9th month. That could be primary related to the growth of the stems and phylloclades. Similarly, WANG &all [26] reported on low secondary metabolism in very young *Dioscorea* plants where very low expression levels of the genes concerned with biosynthesis of the steroidal sapogenins were detected. In aerial parts of *Ruscus* culture there was significant increase of neoruscogenin to average 1 mg/g DW ($p<0.01$) only in the end of the year. Ruscogenin was also increased, however not reaching half of the neoruscogenin levels. Reduction was recorded for ruscin where the content in the fully-grown plantlets was about twice as low than in the young ones ($p<0.05$).

The biosynthetic profile of the roots was significantly contrasting to those of the shoots (Fig. 3).



Sample size – minimum 20 plants. Values are mean of three measurements, ±SE
*p<0.05, ** p<0.01

Figure 3. Dynamics of ruscogenin content in roots of *in vitro* cultivated *Ruscus aculeatus* plants.

Table 1. Correlation of the saponin content in *Ruscus aculeatus in vitro* cultures

Correlations						
Plant part			Ruscin	Desglucoruscin	Neoruscogenin	Ruscogenin
Roots	Ruscin	Pearson Correlation	1	-,232	,681	-,292
		Sig. (2-tailed)		,468	,015	,357
	Desglucoruscin	Pearson Correlation	-,232	1	-,388	-,685
		Sig. (2-tailed)	,468		,212	,014
	Neoruscogenin	Pearson Correlation	,681	-,388	1	,370
		Sig. (2-tailed)	,015	,212		,237
	Ruscogenin	Pearson Correlation	-,292	-,685	,370	1
		Sig. (2-tailed)	,357	,014	,237	
Shoots	Ruscin	Pearson Correlation	1	,641	-,935	-,908
		Sig. (2-tailed)		,025	,000	,000
	Desglucoruscin	Pearson Correlation	,641	1	-,364	-,261
		Sig. (2-tailed)	,025		,245	,412
	Neoruscogenin	Pearson Correlation	-,935	-,364	1	,984
		Sig. (2-tailed)	,000	,245		,000
	Ruscogenin	Pearson Correlation	-,908	-,261	,984	1
		Sig. (2-tailed)	,000	,412	,000	

Neoruscogenin production was prevailing in the 3-month old roots (about 1 mg/g DW). Its content significantly higher than the other three ruscogenin ($p<0.05$). Similarly, NIKOLOV & GUSSEV (1997) showed that undergrown organs of the *in vivo R. aculeatus* contain higher amount of recognin during the appearance of new shoots and in flowering period. On the other hand, MANGAS & al [21] and BALICA & al [27]) stated more active ruscogenin production in the *in vitro* obtained shoots compared to the roots. This discrepancy could be attributed to the very different environmental conditions set *in vivo* and *in vitro*.

Unlike neoruscogenin, the ruscogenin content was more stable both in the shoots and roots. Elevation was observed only in the shoots after the sixth month. The desglucoruscin production was most variable in the roots ranging from 0.1 to 0.8 mg/g DW ($p<0.01$). The ruscin content varied insignificantly in roots. Cumulatively ruscogenin content in roots was higher, especially in the first half of the period. In *Ruscus* organs have limited growth potential (HIRSH [28]), thus production in a long-term culture could be intensified by stimulation of more new shoots or elicitation of the biosynthesis.

The ruscin had slight positive correlation with neoruscogenin in the roots (Table 1). In shoots ruscin showed strong negative correlation with neoruscogenin and ruscogenin. Desglucoruscin was also negatively correlated with major sapogenin but not significant. On the other hand, neoruscogenin and ruscogenin were positively correlated especially in the shoots. Both ruscin and desglucoruscin have neoruscogenin as an aglycone (DE COMBARIEU [29]). However, little is known for their biosynthesis and accumulation in different plant parts.

Conclusion

Extraction of ruscogenin form in vitro plants could be a valuable production approach for slow-growing, endangered species as R. aculeatus. The major constituents as neoruscogenin showed contrasting biosynthetic tendencies in shoots and roots during their development in vitro. Thus, the dynamics of ruscogenin biosynthesis should be regarded as important factor in production of ruscogenin by R. aculeatus in vitro cultures.

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