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Research article

Biotechnological production of ruscogenins in plant cell and organ cultures of *Ruscus aculeatus*

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ABSTRACT

Ruscus aculeatus is a threatened medicinal plant whose main bioactive components, the ruscogenins, have long been used in the treatment of hemorrhoids and varicose veins, but recently demonstrated activity against some types of cancer. Plant cell biofactories could constitute an alternative to the whole plant as a source of ruscogenins. In this pipeline, despite the *in vitro* recalcitrance of *R. aculeatus*, after many attempts we developed friable calli and derived plant cell suspensions, and their ruscogenin production was compared with that of organized *in vitro* plantlet and root-rhizome cultures. Root-rhizomes showed a higher capacity for biomass and ruscogenin production than the cell suspensions and the yields were greatly improved by elicitation with coronatine. Although ruscogenins accumulate in plants mainly in the root-rhizome, it was demonstrated that the aerial part could play an important role in their biosynthesis, as production was higher in the whole plant than in the root-rhizome cultures.

1. Introduction

Ruscus aculeatus is a small evergreen shrub of southern and south-central Europe with thick rhizomes and erect stems bearing spiny cladodes that have taken over the function of leaves. It is pollinated by insects, and only one out of five pollinations are successful. The fruits are red berries that contain one to four seeds, usually with a very low germination capacity (less than 50%). Over-collection for medicinal steroidal saponins, together with the difficult pollinating mechanism of the flowers, low seed production and ineffective fruit/seed dispersal systems, has caused a decline in wild populations, and *R. aculeatus* is currently a threatened species, whose survival depends primarily upon vegetative reproduction from the rhizomes (Thomas and Mukassabi, 2014). Currently, *R. aculeatus* is included in the directive 92/43/EEC and the European Plant Red List (Ivanova et al., 2017). Consequently, finding new sources of steroidal saponins is a challenge for researchers in plant biotechnology.

R. aculeatus produces a range of active secondary metabolites, including triterpenes, steroids, flavonoids, coumarins, sparteine, triamine and glycolic acid. Two steroid saponins with important biological activity, ruscogenin and neoruscogenin, have been isolated from the rhizomes (de Combarieu et al., 2002), where their concentration is highest. Ruscogenin and neoruscogenin have two heterocycles with oxygen bound by a spiroacetal carbon on the D ring of the steroid core. The hydroxyl group at position 10 is glycosylated with a chain of sugars (β-D-Glc*p*-(1 → 3)-O-α-L-Rha*p*-(1 → 2)-O-α-L-Ara*p*-) to form either ruscoside or neoruscoside (Palazon et al., 2006) (Fig. 1).

Ruscus extracts are used both in traditional and conventional medicine. The therapeutic usage of ruscogenin and neoruscogenin is based on their enhancing effect on vascular permeability (Rudofsky, 1991; Bouskela et al., 1993; Svensjo et al., 1997), *in vitro* anti-elastase activity (Facino et al., 1995) and vasoconstrictor effects through different mechanisms (Bouskela et al., 1993; Bouskela and Cyrino, 1994; Bouskela et al., 1994; Svensjo et al., 1997).

R. aculeatus extracts are also used as laxatives and depuratives and for their potential cytostatic activity (Mimaki et al., 1998). In this pipeline, Ma (2014) demonstrated that the effects of ruscogenin against lung cancer were due to the suppression of the S phase of the cell cycle by the regulation of the expression of the proteins p53, p21, CDK2 and cyclin E2. More recently, Hua et al. (2018) reported ruscogenin activity

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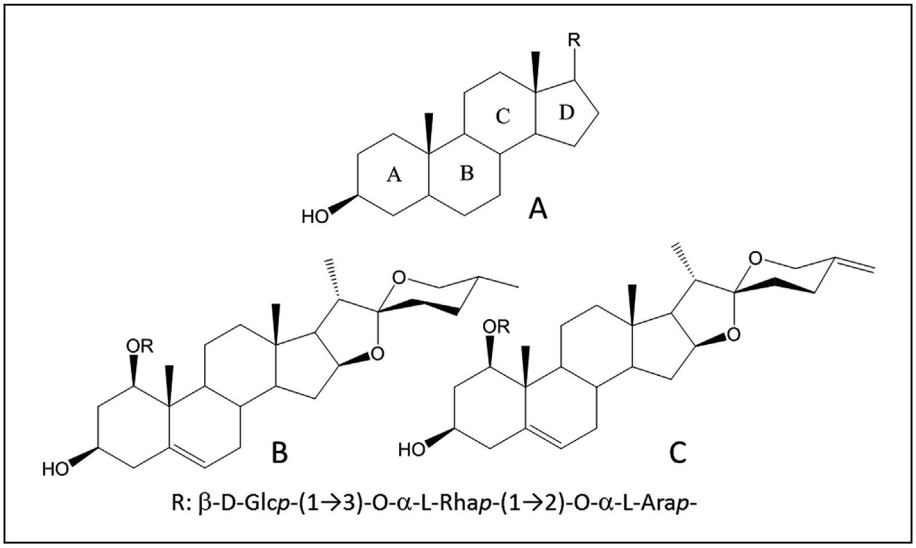

Fig. 1. Chemical structure of the steroidal backbone (A), ruscogenin (B) and neoruscogenin (C).

against hepatocellular carcinoma by the interruption of cancer metastasis through the regulation of the PI3K/Akt/mTOR signaling pathway, reducing the expression of MMP-2, MMP-9, uPA, VEGF and HIF-1a proteins. The growing interest in *R. aculeatus* saponins generated by these latest findings calls for alternative production systems such as biofactories based on plant cell and organ cultures.

There are few reports of ruscogenin production in *in vitro* cultures of *R. aculeatus*. Palazon et al. (2006) studied the changes in saponin production associated with the development of shoots or roots in *R. aculeatus* organogenic calli. The rhizome primary explants were cultured in more than 15 types of media with different hormonal treatments. In several of the media assayed, small pieces of callus appeared after 4–6 weeks of culture, but simultaneously, in the same media, a high number of rhizome explants also developed aerial shoots and/or roots. When cultured, the isolated pieces of calli also showed morphogenetic activity, developing roots and shoots. A lower saponin content was observed in calli without *versus* with organogenesis, whereas calli that developed shoots showed a higher saponin production than those developing roots. In all cases, the *in vitro* production was higher than in plants. With this background, the main objective of the current work was to develop a biotechnological system based on plant cell and root/rhizome cultures optimized for ruscogenin production and to compare the results with those achieved in plantlet *in vitro* cultures.

2. Materials and methods

2.1. Material

As starting material, we used seeds collected from *R. aculeatus* plants growing in forests of Mazandaran Province in northern Iran. The precise location was the village of Agha-Mashad, 35 km southwest of Sari, (52 56 E, 36 16 N and altitude of 620–670m). A voucher specimen has been deposited in the herbarium of Shahid Beheshti University, Tehran, Islamic Republic of Iran. The seeds were cultured in pots with a mixture of peat and perlite in the greenhouse of the Faculty of Pharmacy of the University of Barcelona. For the experiments, phylloclades, rhizomes and seeds of these plants were used.

2.2. Optimization of a protocol for sterilizing the different plant materials

To optimize a protocol for preventing fungal and bacterial contamination without killing the plant material, the different types of explants used in this work were treated with several plant sterilizing agents. Various parameters, including the sterilizing agent, treatment duration, permeabilizing agents, and sonication treatment (with or without) were tested. The optimal treatment found for each type of explant is depicted in Supp. Fig. 1.

2.3. Plantlet in vitro cultures

Plantlet *in vitro* cultures were obtained as previously described by Mangas et al. (2006). In brief, mature embryos obtained from surface-sterilized seeds were inoculated in Petri dishes with hormone-free MS medium supplemented with 30 g L^{-1} sucrose and solidified with 0.27% (w/v) of Phytagel. Isolated embryos were cultured in a controlled climate chamber at 25 °C with a photoperiod of 16 h light/8 h dark at 28–36 μmol m^{-2} s^{-1}. 2–3 weeks after embryo germination, plantlets were transferred to Magenta (SIGMA) flasks and cultured in the same conditions. *In vitro* plants were subcultured every 6 weeks in fresh medium (Supp. Fig. 2).

2.4. Callus induction and culture

To obtain friable and non-organogenic calli, three different types of explants were used: segments of phylloclades, segments of rhizomes, and embryos. Phylloclades and rhizome segments were obtained from 4-year-old *R. aculeatus* plants growing in the greenhouse, and embryos were obtained from unripened fruits of the same plants. All the explants were sterilized as described above, and cultured in MS medium supplemented with more than 35 combinations of PGRs (see results, Tables 1 and 2). In the experiments, we tested 2,4-dichlorophenoxyacetic acid (2,4-D), indole-3-butiric acid (IBA), picloram (Pic), naphthaleneacetic acid (NAA), and indoleacetic acid (IAA) as auxins, and kinetin (Kin), (N-phenyl-N′-(2-chloro-4-pyridyl)urea (4-PU-30), meta-topolin (MT) and benzyladenine (BA) as cytokinins, as well as gibberellic acid (GA3), all of them at several concentrations. Explants were kept in a controlled climate chamber in darkness at 25 °C. After 2, 4 and 6 weeks of culture, the number of explants with calli were recorded (Supp. Fig. 3). The results were expressed as % of callus induction.

In most cases, when the induced callus was isolated and subcultured in the same medium, it showed both root and shoot organogenesis; moreover, the callus was hard and did not disintegrate when cultured in liquid medium. Therefore, to obtain friable calli without organogenesis, another set of culture media were tested, based on different combinations of PGRs, including the new cytokinin, zeatin (ZT), and vitamins and amino acids. A total of 29 new combinations were assayed (see

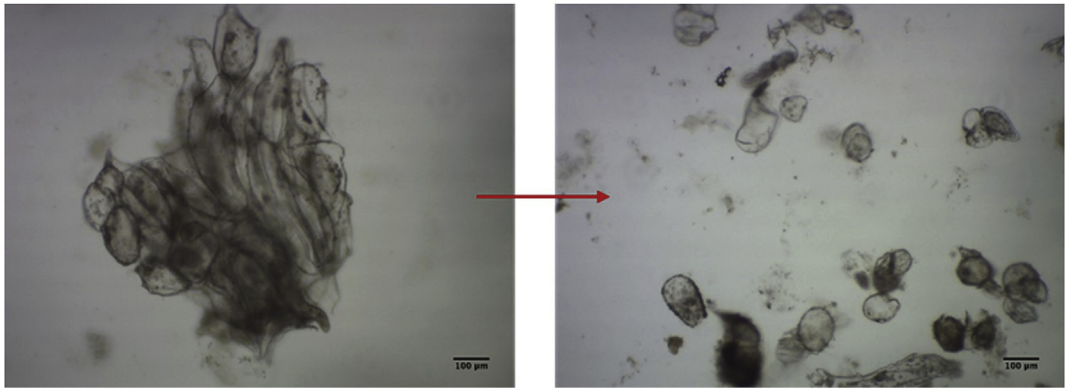

Fig. 2. Disintegration effect of the pectinase treatment in the *R. aculeatus* cell suspensions.

Table 1
Callus induction from rhizomes and phylloclades after 6–8 weeks of culture.

Explant	Mineral nutrients	Auxin	Cytokinin	% callus induction
Rhizome	*MS	2,4-D (0.5 mg L^{-1})	-	33%
	*MS	2,4-D (0.5 mg L^{-1})	Kin (0.1 mg L^{-1})	41%
	*MS	2,4-D (0.5 mg L^{-1})	Kin (1 mg L^{-1})	90%
	*MS	2,4-D (1 mg L^{-1})	-	77%
	*MS	2,4-D (1 mg L^{-1})	Kin (0.1 mg L^{-1})	67%
	*MS	2,4-D (1 mg L^{-1})	Kin (1 mg L^{-1})	82%
	*MS	2,4-D (1 mg L^{-1})	4PU-30 (1 mg L^{-1})	35%
	*MS	2,4-D (1.5 mg L-1)	-	40%
	*MS	2,4-D (1.5 mg L-1)	Kin (0.1 mg L^{-1})	37%
	*MS	2,4-D (1.5 mg L^{-1})	Kin (1 mg L-1)	50%
	MS	2,4-D (2 mg L^{-1})	4PU-30 (0.1 mg L^{-1})	24 %
	*MS	2,4-D (2 mg L^{-1})	-	30%
	*MS	2,4-D (2 mg L-1)	Kin (0.1 mg L^{-1})	40%
	*MS	2,4-D (2 mg L^{-1})	Kin (1 mg L^{-1})	71%
	MS	2,4-D (2,5 mg L^{-1})	-	0%
	MS	2,4-D (2.5 mg L^{-1})	Kin (0.1 mg L^{-1})	0%
	MS	2,4-D (2.5 mg L^{-1})	Kin (1 mg L^{-1})	30%
	*MS	2,4-D (4 mg L^{-1})	Kin (1 mg L^{-1})	19%
	MS	2,4-D (4 mg L^{-1})	Kin (1 mg L^{-1}) + GA3 (0.5 mg L^{-1})	15%
	*MS	2,4-D (4 mg L^{-1})	BA (1 mg L^{-1})	11%
	MS	2,4-D (5 mg L^{-1})	BA (0.5 mg L^{-1})	8%
	*MS	IAA (1 mg L^{-1})	Kin (0.1 mg L^{-1})	7%
	*MS	IAA (1 mg L^{-1})	Kin (0.2 mg L^{-1})	13%
	*MS	IAA (1 mg L^{-1})	Kin (1 mg L^{-1})	20%
	MS	IAA (2 mg L^{-1})	-	17%
	MS	IAA (2 mg L^{-1})	Kin (0.1 mg L^{-1})	0%
	MS	IAA (2 mg L^{-1})	Kin (1 mg L^{-1})	37%
	MS	Pic (1.2 mg L^{-1})	-	47%
	MS	Pic (2.4 mg L^{-1})	-	65%
	MS	Pic (4 mg L^{-1})	4PU-30 (3 mg L^{-1})	67%
Phylloclades	MS	2,4-D (1 mg L^{-1})	4PU-30 (1 mg L-1)	1%
	MS	2,4-D (1.5 mg L^{-1})	Kin (1 mg L^{-1})	2%
	MS	2,4-D (2 mg L-1)	-	2%
	MS	2,4-D (2 mg L^{-1})	4PU-30 (0.1 mg L^{-1})	1%
	MS	2,4-D (4 mg L^{-1})	Kin (1 mg L^{-1}) + GA3 (0.5 mg L^{-1})	10%
	MS	2,4-D (5 mg L^{-1})	BA (0.5 mg L^{-1})	2%
	MS	IAA (1 mg L^{-1})	Kin (0.2 mg L^{-1})	4%

Several growth regulator compositions were assayed using the mineral nutrition of B5 medium but are not included in the table as the results were always worse than those obtained with MS basal medium. The selected media are in grey.
* Media tested previously by Palazon et al. (2006).

Table 2
Callus induction from embryos after 8 weeks of culture.2

Mineral nutrients	Auxin	Cytokinin	% of callus induction
MS	2,4-D (0.2 mg L^{-1})	Kin (1 mg L-1)	65%
MS	2,4-D (0.5 mg L^{-1})	Kin (1 mg L^{-1})	68%
MS	2,4-D (1.5 mg L^{-1})	Kin (0.5 mg L^{-1})	70%
MS	2,4-D (1.5 mg L^{-1})	MT (0.5 mg L^{-1})	63%
MS	2,4-D (2 mg L-1)	4PU-30 (0.1 mg L^{-1})	10%
MS	2,4-D (2 mg L^{-1})	4PU-30 (3 mg L^{-1})	66%
MS	2,4-D (0.5 mg L^{-1})	BA (0.05 mg L^{-1})	89%
MS	NAA (2 mg L^{-1})	BA (2mg L^{-1})	51%
MS	NAA (5mg L^{-1})	Kin (0.5 mg L^{-1})	52%
MS	NAA (5 mg L^{-1})	Kin (5 mg L^{-1})	51%
MS	Pic (1.2 mg L^{-1})	-	82%
MS	Pic (2 mg L^{-1})	Kin (0.1 mg L^{-1}) + GA3 (0.5 mg L^{-1})	89%
MS	Pic (2.4 mg L^{-1})	-	86%
MS	Pic (4 mg L^{-1})	4PU-30 (3 mg L^{-1})	94%
MS	-	BA (0.5 mg L^{-1})	38%
The selected media are in grey.			

Fig. 3. External appearance of the different *R. aculeatus* culture systems after a 4-week growth period.

Table 3
Growth capacity, friability and organogenesis of the *R.aculeatus* calli when cultured in different media for a period of 4–6 weeks.3

Mineral nutrients	Auxin	Cytokinin	Other organic components	Growth	Organogenesis
MS	2,4D (0.2 mg L^{-1})	Kin (1 mg L^{-1})	-	++	Roots
MS	2,4D (0.2 mg L^{-1})	Kin (1 mg L^{-1})	Casein (2 mg L^{-1})	+	No
MS	2,4D (0.2 mg L^{-1})	Kin (1 mg L^{-1})	Gly (73 mg L^{-1}) + L-Gln (877 mg L^{-1}) + L-Asp. (266 mg L^{-1}) + L-Arg (228 mg L^{-1}) + Casein (2 g L^{-1}) + 0.005% pectinase	+	Roots
MS	2,4D (0.5 mg L^{-1})	Kin (1 mg L^{-1})	-	+	Roots
MS	2,4D (0.5 mg L^{-1})	Kin (1 mg L^{-1})	Casein (2 mg L^{-1})	+	No
MS	2,4D (1.5 mg L^{-1})	Kin (0,5 mg L^{-1})	-	+ (C)	Roots
MS	2,4D (1.5 mg L^{-1})	MT (0,5 mg L^{-1})	-	-	Roots
MS	2,4D (2 mg L^{-1})	BA (1 mg L^{-1})	-	-	-
MS	2,4D (2 mg L^{-1})	4PU-30(0.1 mg L^{-1})	-	+ (C)	No
MS	2,4D (2 mg L^{-1})	4PU-30 (3 mg L^{-1})	-	-	Roots
MS	2,4D (4 mg L^{-1})	Kin (1 mg L^{-1})+ GA_3 (0.5 mg L^{-1})	-	-	Roots
MS	2,4D (0.22 mg L^{-1})+ NAA (0,186 mg L^{-1})	-	Thiamine 10 mg L^{-1})	+	Roots
MS	2,4D (2 mg L^{-1}) + NAA (0,5 mg L^{-1}) + IAA (0.5 mg L^{-1})	Kin (0,2 mg L^{-1})	Nicotinic acid (1 mg L^{-1}) + Thiamine (10 mg L^{-1}) + Pyridoxine (1 mg L^{-1})	+	Roots
MS	NAA (0.3 mg L^{-1})	ZT (1 mg L^{-1})	-	+	No
MS	NAA (1 mg L^{-1})	Kin (1 mg L^{-1})	-	-	-
MS	NAA (2 mg L^{-1})	BA (2 mg L^{-1})	-	++	Roots, shoots
MS	NAA (5 mg L^{-1})	Kin (0.5 mg L^{-1})	-	+	Roots
MS	NAA (5 mg L^{-1})	Kin (5 mg L^{-1})	-	+	Roots, shoots
MS	Pic (1.2 mg L^{-1})		-	++ (C)	Clusters
MS	Pic (2.4 mg L^{-1})		-	++ (C)	Clusters
MS	Pic (2 mg L^{-1})	Kin (0.1 mg L^{-1})	-	+++ (C)	No
MS	Pic (2 mg L^{-1})	Kin (0.1 mg L^{-1}) + GA3 (0.5 mg L^{-1})	-	+++ (F)	Clusters
MS	Pic (2 mg L^{-1})	Kin (0.1 mg L^{-1}) + GA_3 (0.5 mg L^{-1})	Casein (2 mg L^{-1})	+	No
MS	Pic (2 mg L-1)	Kin (0.1 mg L^{-1}) + GA_3 (2 mg L^{-1})	-	+	No
MS	Pic (4 mg L^{-1})	4PU-30 (3 mg L^{-1})	-	+++ (C)	No
MS	IAA (0.1 mg L^{-1})	Kin (1 mg L^{-1}) + GA_3 (0.01 mg L-1)	-	+	No
MS	IAA (0.1 mg L^{-1})	Kin (1 mg L^{-1}) + GA_3 (0.1 mg L^{-1})	-	+	No
MS	-	BA (0.5 mg L^{-1})	-	+	No
B5	IBA (1 mg L^{-1})	BA (5 mg L^{-1})	-	+++ (F)	Clusters
C: Compact calli; F: Friable calli. -: no growth; +: ≈ 1 g FW; ++: ≈ 2 g FW; +++: > 2 g FW.					

Results, Table 3). The calli were routinely subcultured every 4 weeks.

2.5. Establishment of cell suspensions

Pieces of 2 g of friable calli were transferred to 175 mL flasks containing 20 mL of basal salt liquid media with different combinations of PGRs. The flasks were kept in darkness at 25 °C in an incubator shaker (Adolf Kuhner AG, Schweiz) at 100 rpm. The composition of the media assayed is shown in Table 3. Some callus pieces were also incubated in the same media with the addition of 0.4% pectinase for 72 h. After that, the isolated cells were recovered by 1000 g centrifugation and resuspended in the same fresh medium without pectinase. The *R. aculeatus* cell lines were maintained and periodically subcultured (every 4 weeks) according with the pouring cell culture system (Mustafa et al., 2011).

2.6. Root-rhizome cultures

Root-rhizomes from young *in vitro* plantlets were isolated and cultured in liquid MS medium with half concentration of salts supplemented with 3% (w/v) sucrose and 0.1% (w/v) myoinositol in an orbital shaker (Adolf Kuhner AG, Schweiz) at 25 °C and 115 rpm (Supp. Fig. 4). They were kept under a 16-h photoperiod and in darkness and routinely subcultured every 4 weeks.

2.7. Elicitor treatments

To compare the capacity of the different biotechnological systems (plantlets, calli, root-rhizomes and cell suspension cultures) for producing biomass and ruscogenins under elicitation, all types of cultures were treated with coronatine (COR). An aliquot of COR was added to the medium to obtain a final concentration of 1 μM at two weeks of culture, when the cultures were at the end of the exponential growth

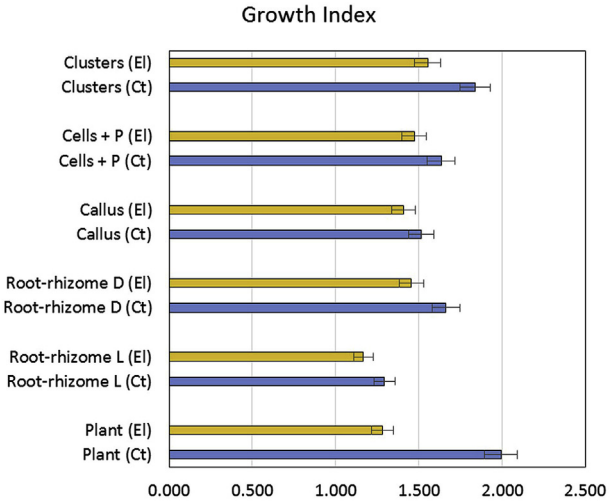

Fig. 4. Biomass production of the biotechnological systems expressed as a growth index after a 4-week growth period. El: elicitor treatment. Ct: control conditions. D: dark condition. L: Light conditions. Each value is the average of 5 biological replicates ± SE.

phase. In all the systems, samples were taken in triplicate two weeks after the elicitor treatment. Longer exposure to the elicitor could have caused the necrosis of the *R. aculeatus* cells and organs, as previously demonstrated (Mangas et al., 2006). The fresh weight (FW) of the samples was registered and after lyophilization the dry weight (DW) was also determined. Dry powder was kept for saponin extraction as described below.

2.8. Saponin extraction and quantification

The quantification of *Ruscus* saponins was based on their conversion to ruscogenin and neoruscogenin after acid hydrolysis. Saponin extraction was carried out as described previously by de Combarieu et al. (2002) with some modifications. Lyophilized powdered samples (500 mg) were extracted and sonicated for 20 min with 50% methanol (2 × 10 mL) and centrifuged at 1500 g for 10 min. After filtering through a 0.2 μm Millipore filter, the solvent was evaporated to dryness and the residue was dissolved in 4 mL isopropanol +0.5 mL H_2O + 0.55 mL HCl (37%) in a water bath under reflux for 1 h at 80 °C, in order to hydrolyze the saponins. The hydrolysate was adjusted to a pH of 9–10 with KOH (10%), and a final volume of 10 mL with methanol. The samples were filtered through a 0.2 μm Millipore filter and analyzed by HPLC. Ruscogenin and neoruscogenin quantification was performed according to Bertani and Forni (1984). 20 μL of the extract was loaded onto a Supelcosil LC8 (i.d. 4.4 × 250 mm) column connected to a Pharmacia LKB HPLC system. The solvent mixture of acetonitrile-water (6:4 v/v) was eluted at a flow rate of 1 mL min^{-1} and the saponins were detected at 200 nm. The peak areas corresponding to ruscogenin and neoruscogenin from the samples, with the same retention time as authentic saponins, were integrated by comparison with an external standard calibration curve. Ruscogenin and neoruscogenin were purchased from ChromaDex Inc.

2.9. Statistics

Statistical analysis was performed with Excel software. All data are the average of five determinations ± SD. The multifactorial ANOVA analysis followed by Tukey's multiple comparison tests was used for statistical comparisons. A P-value of < 0.05 was assumed for significant differences.

3. Results

To achieve enough friable callus biomass to establish *R. aculeatus* cell suspensions, the first step was the optimization of the culture media for callus induction. As reported by Palazon et al. (2006), callus induction from rhizomes, phylloclades and embryos of this species is challenging, and in the previously assayed media the *Ruscus* explants showed a higher capacity to regenerate organized structures such as shoots or roots than calli. Thus, to improve the callus induction from various types of primary explants, we tested more than 30 different culture media based mainly on different combinations of PGRs (Tables 1 and 2).

In a first approach, we tested the callus induction capacity of phylloclade and rhizome segments sterilized according to the protocols described in Material and Methods and supp. Fig. 1. In these conditions, only 15% of phylloclades and 25% of rhizomes showed contamination after two weeks of culture. For callus induction, segments of the sterilized plant material were used as primary explants and cultured in Petri dishes with 25 mL of MS medium supplemented with different concentrations of PGRs at 25 °C in the dark. For each treatment, more than 50 explants were inoculated in the different media tested. The results are shown in Table 1.

Of all the media tested, the best PGR combination for *R. aculeatus* callus induction was the MS medium supplemented with 2,4-D (0.5 mg L^{-1}) and Kin (1 mg L^{-1}). Using this medium, we achieved 90% callus induction from the rhizomes, although the development of roots and shoots from the explants was not completely inhibited. In contrast, the medium supplemented with Pic (4 mg L^{-1}) and 4PU-30 (3 mg L^{-1}) completely blocked organogenesis in the explants and resulted in 67% callus induction. For this reason, the latter was chosen as the most suitable culture medium for callus induction from rhizomes. In all cases, a higher rate of callus induction was obtained from rhizomes than phylloclades.

To obtain callus cultures from embryos, *R. aculeatus* sterilized seeds were cut in half and the embryos were isolated and inoculated in hormone-free MS medium. When the embryos started germination (7–10 days) they were cultured in different media (Table 2) to obtain *R. aculeatus* calli. In the embryos, as in the rhizome experiments, the MS medium supplemented with Pic (4 mg L^{-1}) and 4-PU-30 (3 mg L^{-1}) was optimum for callus induction, but in this case organogenesis from the calli was not completely inhibited. As the medium supplemented with 2,4-D (0.5 mg L^{-1}) and BA (0.05 mg L^{-1}) provided calli with less organogenesis, it was selected for further experiments. Embryos were a better source of explants than rhizomes, generally producing calli in less time and with less development of roots and shoots.

3.1. Optimization of the culture medium for the growth of friable calli without organization

As already mentioned, the biggest obstacle for the growth of *Ruscus* calli in the previously assayed media was a high capacity to develop shoots and roots (Palazon et al., 2006). Also, the non-organogenic calli obtained were very compact and not suitable for disintegration and establishing cell suspensions. Thus, the calli obtained from rhizomes or germinated embryos were cultured in a variety of media with the aim of completely blocking organogenesis and developing enough friable callus biomass. The assayed media and the characteristics of the established calli are shown in Table 3.

The assayed culture media resulting in better growth and friable calli were based on MS mineral nutrition with the addition of Pic (2 mg L^{-1}) and Kin (0.1 mg L^{-1}). Also, the addition of GA3 (0.5 mg L^{-1}) led to more friable calli. Consequently, the medium selected for increasing the biomass was MS mineral nutrition + Pic (2 mg L^{-1}) + Kin (0.1 mg L^{-1}) + GA3 (0.5 mg L^{-1}). However, after several subcultures in this medium, the calli began to darken and lose their growth capacity, so for further assays we used the medium B5

Table 4
Assayed media and growth capacity of the *R. aculeatus* cell suspensions.4

Mineral nutrition	Auxin	Cytokinin	Growth
MS	2,4-D (0.2 mg L^{-1})	Kin (1 mg L^{-1})	-
MS	Pic (2 mg L-1)	Kin (0.1 mg L^{-1}) + GA_3 (0.5 mg L^{-1})	**
MS	Pic (4 mg L^{-1})	4PU-30 (3 mg L^{-1})	Slow
B5	IBA (1 mg L^{-1})	BA (5 mg L^{-1})	***
AA	NAA (4 mg L^{-1})	-	Slow
, Growth Index lower than 1.5; *, Growth Index higher than 1.5.			

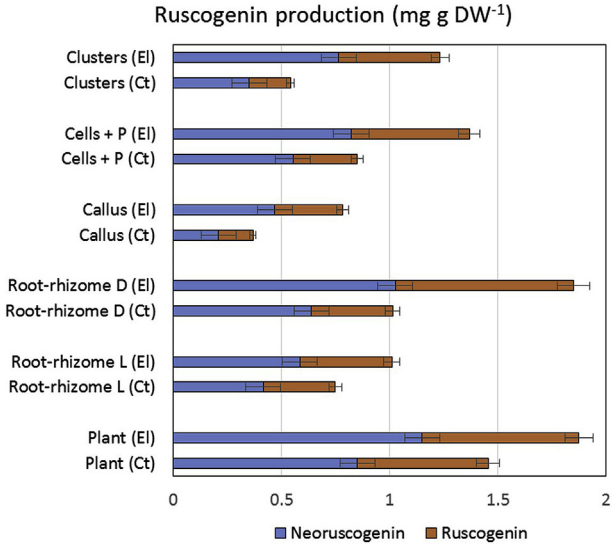

Fig. 5. Ruscogenin production expressed as mg g DW^{-1}, after 4 weeks of culture. El: elicitor treatment (COR 1 μM). Ct: control conditions (untreated cultures). D: dark condition. L: Light conditions. Each value is the average of 5 biological replicates ± SE.

supplemented with IBA (1 mg L^{-1}) and BA (5 mg L^{-1}). This resulted in virtually the same biomass production as the previous medium, but the cell growth did not decrease with the subcultures.

3.2. Callus disintegration and establishment of cell suspensions

Small pieces of callus (≈2 g FW) were transferred to 175-mL flasks (Sigma VO633) containing 20 mL of different liquid culture media. All flasks were capped with Magenta B-Caps (Sigma 38648) and incubated in the dark at 25 °C and 100 rpm in a shaker incubator. Routine maintenance of the culture was performed in the same culture conditions by transferring 10 mL of 10-day-old culture (cells plus medium) to 10 mL of fresh medium. Samples were taken periodically (every 1–2 weeks) to assess the viability and growth capacity of the cells in the different culture media assayed.

From the results shown in Table 4, we can infer that the best medium for biomass production by the cell cultures was that supplemented with IBA and BA, as in the callus cultures. Although friable calli were used as the starting material to generate the cell suspensions and a high growth capacity was achieved in this media, microscopic analysis of the cultures showed numerous clusters. We therefore decided to treat the cultures with pectinase (0.4%) to separate the plant cells and disintegrate the clusters (Fig. 2). As shown, pectinase attacked the middle lamella of the cell walls and destroyed most of the clusters, resulting in a finer cell suspension after filtration.

3.3. Elicitation treatment

To investigate the capacity of the *R. aculeatus* plant cell cultures to produce ruscogenins, a range of biotechnological platforms were established: *in vitro* plantlets (cultured in light conditions), embryo-derived calli and cell suspensions (maintained in darkness), and root-rhizomes (kept in both light and darkness conditions). All the biotechnological systems were elicited with 1 μM COR after two weeks of culture, and its effects on biomass and ruscogenin production were determined after two weeks of treatment. The obtained results were compared with those of non-elicited cultures. Among the different plant biotechnological systems (Fig. 3), a higher cell sedimentation can be seen in the cluster cultures (Fig. 3C) than in the fine cell suspensions; greening of the root-rhizomes when exposed to the light can also be observed (Fig. 3E).

Fig. 4 shows the biomass production of the different systems expressed as a growth index (GI) [(DW harvested-DW inoculum)/DW inoculum]. In control conditions (without elicitor treatment), *in vitro* plantlet cultures had the highest capacity to generate biomass, achieving a GI of 2 after 4 weeks of culture. The cluster cultures and root-rhizomes grown in the darkness also reached a GI higher than 1.5. Biomass formation decreased in root-rhizomes on transfer to light and in the cluster cultures after pectinase treatment; the derived fine cell suspensions achieved a lower GI than the cluster cultures after 4 weeks of growth (Fig. 4). In all cases, the elicitor treatment with 1 μM COR significantly ($p \leq 0.05$) reduced the growth capacity of the *in vitro* cultures, especially the plantlets, whose GI decreased by more than 35%. Taken as a whole, the results indicate that the established systems efficiently generated biomass, despite the *in vitro* recalcitrance of *R. aculeatus* and the initial difficulties in obtaining the callus and cell suspension cultures.

All the *in vitro* cultures developed the capacity to biosynthesize ruscogenin and neoruscogenin (Fig. 5). In control conditions (without elicitation), the ruscogenin content was higher in the organogenic systems (plantlets and root-rhizomes) than in the undifferentiated cultures (callus and cluster/cell cultures). Plantlets achieved an average of 1.454 ± 0.103 mg g DW^{-1} of total saponins, 41% being ruscogenin. Production was also high in the root-rhizomes, which yielded a total saponin content of 1.014 ± 0.087 mg g DW^{-1} when cultured in darkness, whereas in light the ruscogenin production decreased significantly ($p \leq 0.05$) to 0.750 ± 0.079 mg g DW^{-1}. The ruscogenin content in the undifferentiated cultures was also significantly lower ($p \leq 0.05$), especially in the calli, where the saponin production was on average 0.369 ± 0.063 mg g DW^{-1}. The results (Fig. 5) also show that callus disintegration and establishment of the cell suspension enhanced the ruscogenin production capacity of the culture, the cell suspension achieving a yield of 0.851 ± 0.098, which was 2.3-fold higher than that of the calli.

Treatment of the different *in vitro* cultures with 1 μM COR significantly increased ($p \leq 0.05$) their capacity to produce ruscogenins, from 1.3-fold in the plantlets to 2.3-fold in the cluster cultures (Fig. 5). In general, the elicitor treatment was more effective in the undifferentiated (calli and cells) than in the well-structured cultures

(plantlets and root-rhizomes). In summary, the results demonstrate the effectiveness of COR for increasing ruscogenin production. The % of ruscogenin in the total saponin contents (rucogenin + neoruscogenin) was quite variable, ranging from 44% in the elicited root-rhizomes to 35% in the control cell and cluster cultures. In general, the proportion of ruscogenin was not significantly affected ($p \geq 0.05$) by the elicitation.

4. Discussion

As mentioned, the discovery of new biological activities of *R. aculeatus* extracts (Li et al., 2018; Hua et al., 2018), together with the threatened status of wild *R. aculeatus* populations, has stimulated interest in developing sustainable biotechnological processes for producing ruscogenins, the bioactive ingredients of the plant extract. To date, the main approach has been the development of *in vitro* micropopagation protocols (Winarto, 2017). In the only previous study on *in vitro* ruscogenin production (Palazon et al., 2006), a yield of 0.042 mg g DW^{-1} was reported in non-organogenic calli, which increased in organogenic calli, especially those that developed aerial shoots.

Approaches to developing a biotechnological platform for producing plant secondary metabolites can be empirical or rational. A rational strategy can provide new information about plant secondary metabolism, but it requires some prior knowledge of biosynthetic pathways and their regulation, which in the case of ruscogenins is very limited. In contrast, empirical approaches are based on enhancing cell growth and bioactive compound production by manipulating in-put factors, such as culture conditions and media, the use of elicitors and permeabilizing agents, and process and bioreactor design (Vidal-Limon et al., 2018). Considering the lack of knowledge about ruscogenin biosynthesis, we developed an empirical strategy based on the optimization of the culture conditions and elicitation of the production system.

Thus, in the current work, as an initial step in developing a biotechnological platform for ruscogenin production based on plant cell cultures, *R. aculeatus* calli were induced and cultured. After testing more than 70 different plant culture media, containing a variety of basal mineral nutrients and plant growth regulators at different concentrations, it was concluded that the most suitable type of explant for callus induction were the embryos. The best culture medium for this kind of explant was MS supplemented with 2,4-D (0.5 mg L^{-1}) and BA (0.05 mg L^{-1}), whereas for friable callus growth it was B5 supplemented with IBA (1 mg L^{-1}) and BA (5 mg L^{-1}). The calli were also disintegrated in different culture media, and the medium optimized for callus cultures was also found to be the best for *R. aculeatus* cell growth. Although laborious and time-consuming, this phase is essential for establishing an optimized plant secondary metabolite production system (Hidalgo et al., 2018).

Although the calli grew well when transferred to a liquid medium, resulting in a cell suspension, they generated a cluster culture formed by groups of cells (Fig. 2). The cluster cultures were therefore treated with pectinase, an enzyme that degrades the cell middle lamella, which has been previously used to obtain single cell cultures from aggregates in *Taxus* spp. and rice cell cultures with little effect on cell viability (Lee et al., 2004; Nail and Roberts, 2004). In the *R. aculeatus* cell cultures, although pectinase treatment allowed single cell cultures to be obtained from the aggregates, the growth of the system decreased (Fig. 4) because of reduced cell viability (data not shown).

Organized cultures such as roots and shoots are more genetically stable than cell suspensions and can produce the same spectrum of plant secondary products as the mother plant (Alvarez, 2014). Biorhizome cultures have been successfully utilized for the production of colchicine (Sivakumar et al., 2017). Therefore, in the second phase of this work, root-rhizomes were established and their capacity to produce biomass in hormone-free medium and ruscogenins was found to be similar to that of *in vitro* plantlets and cell and cluster cultures.

Although the cells and clusters achieved a high production of ruscogenins (0.851 ± 0.098 mg g DW^{-1} and 0.542 ± 0.033 mg g DW^{-1}, respectively), the yield of the organized cultures (root-rhizomes) was greater (1.014 ± 0.087 mg g DW^{-1}). This reflects the importance of the organization and development of specific tissues and organs for the biosynthesis of plant secondary compounds (Kumar et al., 2014), although the total ruscogenin contents of the root-rhizomes were lower than in the plants cultured in *in vitro* conditions (Fig. 5). Palazon et al. (2006) demonstrated that root development in *R. aculeatus* calli increased the ruscogenin production but to a lesser extent than shoot development. Thus, the results obtained in this work confirm those previously reported by our research group.

The commercial source of ruscogenins is the root-rhizome, the part of the plant where they mainly accumulate (Ivanova et al., 2015), but the location of ruscogenin biosynthesis is unknown. The fact that our *in vitro* plants accumulated higher ruscogenin contents than the root-rhizome cultures (Fig. 5) suggests the aerial part of the plant may play an active role in the biosynthesis of these compounds, which could subsequently accumulate in the underground organs. Mangas et al. (2006) reported a higher percentage of ruscogenin than neoruscogenin in the aerial part of *in vitro R. aculeatus* plants, as did Palazon et al. (2006). However, this was not the case in the current work, where the percentage of ruscogenin in the total ruscogenins ranged from 35% in undifferentiated systems (clusters and isolated cells) to nearly 45% in root-rhizomes, a percentage similar to that achieved in the *in vitro* plants (around 41%).

The higher ruscogenin contents of the *R. aculeatus in vitro* plants cannot be attributed to a direct effect of light, because in root-rhizomes the yield was higher in darkness than in light (Fig. 5). As mentioned, it is well accepted that the main source of isopentenyl diphosphate (IpPP) in the biosynthesis of steroidal saponins is the cytoplasmic mevalonate pathway, although there is also a contribution from the chloroplastic methylerythritol phosphate (MEP) pathway, due to crosstalk between them at the IpPP level (Upadhayay et al., 2018). This suggests that active biosynthesis of IpPP can also occur in plastids in the aerial part of the whole plant, providing an extra source of this precursor, including for steroidal saponin biosynthesis in the roots. In accordance with this hypothesis, Jang et al. (2015) demonstrated that a high photosynthetic rate due to a well-developed plastid system in leaves increases ginsenoside production in *Panax ginseng* roots.

Plant secondary metabolite biosynthesis is part of the plant defense strategy and can be activated by elicitors (Ramirez-Estrada el al., 2016). One of the elicitors most applied to increase the production of saponins and other plant secondary metabolites in cell and organ cultures is methyl jasmonate (MeJA) (Yendo et al., 2010). However, in contrast with other triterpenes, the biosynthesis of ruscogenins is not activated by this elicitor in *R. aculeatus in vitro* plantlet cultures (Mangas et al., 2006). Thus, in this work, with the aim of improving ruscogenin production in the range of *R. aculeatus* cultures developed, we tested the new elicitor coronatine (COR). A more powerful elicitor than MeJA, COR has been used to enhance the biosynthesis of a plethora of bioactive plant secondary metabolites, such as the diterpene taxol (Sabater-Jara et al., 2014), the saponins of *Kalapanax septemlobus* (Lee et al., 2018) and phytosterols (Kim et al., 2017), among others. Several studies have reported that COR exerts its effect by activating the host's jasmonate signaling pathway. However, although the protein COI1 serves as a receptor for both COR and jasmonate (Onrubia et al., 2013), the signal transduction steps downstream of the perception machinery are different, which would explain why the elicitors act differently on plant secondary metabolism. Compared with the untreated cultures, ruscogenin and neoruscogenin production increased dramatically when COR was added to the culture medium in all the biotechnological systems established after 4 weeks of growth. The stimulatory effect of COR was most acute in the less productive cultures. After elicitation, the total ruscogenin production of the least productive system, the callus cultures, increased more than 2-fold (up to 0.784 ± 0.096 mg g DW^{-1}

compared to 0.369 ± 0.070 mg g DW^{-1} in control conditions). In contrast, in the most productive system, the plantlet *in vitro* cultures, the COR-induced increase in total ruscogenin content was only 1.3-fold (up to 1.872 mg g DW^{-1} compared to unelicited plantlets). These results demonstrate the effectiveness of the COR treatment for inducing ruscogenin production in *in vitro* plant systems, confirming those of previous studies (Onrubia et al., 2013; Ramirez-Estrada el al., 2016).

Unfortunately, the ruscogenin biosynthetic pathway is not yet fully understood and there is no evidence for any changes in the expression pattern of the genes involved or enzyme activity when *R. aculeatus* cell cultures are elicited with COR. However, there is some indication that ruscogenin formation could arise from the action of oxidases and glucosyl transferases on cholesterol and/or β-glucosidase on β-sitosterol. In this context, Kim et al. (2017) reported that β-sitosterol contents of *Lemna paucicostata* were significantly enhanced (by about 57% in relation to the unelicited plants) by the addition of COR (1 μM) to the culture medium. Thus, the clear increase of ruscogenins in COR-treated *R. aculeatus* cell cultures could be a consequence of the enhancement of the putative precursor production.

Taken as a whole, our results indicate that plant biofactories based on *R. aculeatus in vitro* cultures could provide an alternative source of ruscogenins to field-grown plants. The biotechnological system based on root/rhizome cultures grown in the dark and elicited with COR showed particular promise. In addition, although ruscogenins accumulate in the rhizome, our results suggest the aerial part of the plant could play an important role in the production of these bioactive compounds.

Contribution

Conceptualization, J. Palazon, R. Eibl and R.M. Cusidó; formal analysis, A. Khojasteh, R. Sanchez-Muñoz, E. Moyano and M. Bonfill; writing-review and editing, J. Palazon, R. Eibl, E. Moyano and R.M. Cusidó; funding acquisition, J. Palazón.

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References

Alvarez, M.A., 2014. *In vitro* plant cultures as biofactories. In: Plant Biotechnology for Health. Springer, Cham. https://doi.org/10.1007/978-3-319-05771-2_4.

Bouskela, E., Cyrino, F.Z., Marcelon, G.J., 1993. Effects of *Ruscus* extract in the internal diameter of arteriales and venules of the hamster cheek pouch microcirculation. Cardiovasc. Pharmacol. 22, 221–222. https://doi.org/10.1097/00005344-199308000-00008.

Bouskela, E., Cyrino, F.Z., Marcelon, G.J., 1994. Possible mechanism for the inhibitory effect of *Ruscus* extract on increased microvascular permeability induced by histamine in hamster cheek pouch. Cardiovasc. Pharmacol. 24, 281–285. https://doi.org/10.1097/00005344-199424020-00013.

Bouskela, E., Cyrino, F.Z., 1994. Possible mechanism for the effects of *Ruscus* extract on microvascular permeability and diameter. Clin. Hemorheol. 14, S23–S36.

de Combarieu, E., Falzoni, M., Fuzzati, N., Gattesco, F., Giori, A., Lovati, M., Pace, R., 2002. Identification of *Ruscus* steroidal saponins by HPLC-MS analysis. Fitoterapia 73, 583–596. https://doi.org/10.1016/S0367-326X(02)00220-4.

Facino, R.M., Carini, M., Stefani, R., Aldini, G., Saibene, L., 1995. Anti-elastase and anti-hyaluronidase activities of saponins and sapogenins from *Hedera helix, Aesculus hippocastanum*, and *Ruscus aculeatus*: factors contributing to their efficacy in the treatment of venous insufficiency. Arch. Pharm. (Weinheim) 328, 720–724. https://doi.org/10.1002/ardp.19953281006.

Hidalgo, D., Sanchez, R., Lalaleo, L., Bonfill, M., Corchete, P., Palazon, J., 2018. Biotechnological production of pharmaceuticals and biopharmaceuticals in plant cell and organ cultures. Curr. Med. Chem. 25, 3577–3596. https://doi.org/10.2174/0929867325666180309124317.

Hua, H., Zhu, Y., Song, Y.H., 2018. Ruscogenin suppressed the hepatocellular carcinoma metastasis via PI3K/Akt/mTOR signaling pathway. Biomed. Pharmacother. 101, 115–122.

Ivanova, T., Banciu, C., Gussev, C., Bosseva, Y., Dimitrova, D., Stoeva, T., Manole, A., 2017. Dynamics of the ruscogenin biosynthesis in *Ruscus aculeatus* L. (Liliaceae) in *in vitro* cultures. Rom. Biotechnol. Lett. 22, 2. https://doi.org/10.1016/j.biopha.2018.02.031.

Ivanova, T., Dimitrova, D., Gussev, C., Bosseva, Y., Stoeva, T., 2015. *Ex situ* conservation of *Ruscus aculeatus* L. - ruscogenin biosynthesis, genome-size stability and propagation traits of tissue-cultured clones. Biotechnol. Biotechnol. Equip. 29, 27–32. https://doi.org/10.1080/13102818.2014.984976.

Jang, I.B., Lee, D.Y., Yu, J., Park, H.W., Mo, H.S., Park, K.C., Hyun, D.Y., Lee, E.H., Kim, K.H., Oh, C.S., 2015. Photosynthesis rates, growth, and ginsenoside contents of of 2-yr-old *Panax ginseng* grown at different light transmission rates in a greenhouse. J. Ginseng Res. 39, 345–353. https://doi.org/10.1016/j.jgr.2015.03.007.

Kim, J.Y., Kim, H.Y., Jeon, J.Y., Kim, D.M., Zhou, Y., Lee, J.S., Lee, H., Choi, H.K., 2017. Effects of coronatine elicitation on growth and metabolic profiles of *Lemna paucicostata* culture. PLoS One 12, e0187622. https://doi.org/10.1371/journal.pone.0187622.

Kumar, V., Singh, S.K., Bandopadhyay, R., Sharma, M.M., Chandra, S., 2014. *In vitro* organogenesis secondary metabolite production and heavy metal analysis in *Swertia chirayita*. Cent. Eur. J. Biol. 9, 686–698. https://doi.org/10.2478/s11535-014-0300-7.

Lee, J.K., Eom, S.H., Hyun, T.K., 2018. Enhanced biosynthesis of saponins by coronatine in cell suspension culture of *Kalopanax septemlobus*. 3 Biotech 8, 59. https://doi.org/10.1007/s13205-018-1090-9.

Lee, T.L., Shultz, R.W., Hanley-Bowdoin, L., Thompson, W.F., 2004. Establishment of rapidly proliferating rice cell suspension culture and its characterization by fluorescence-activated cell sorting analyses. Plant Mol. Biol. Rep. 22, 259. https://doi.org/10.1007/BF02773136.

Li, F., Fan, X.X., Chu, C., Zhang, Y., Kou, J.P., Yu, B.Y., 2018. A strategy for optimizing the combination of active components based on Chinese medicinal formula Sheng-Mai-San for myocardial ischemia. Cell. Physiol. Biochem. 45, 1455–1471. https://doi.org/10.1159/000487572.

Ma, A.Z., 2014. Mechanism of Ruscogenin on Suppressing Pulmonary A549 and H460 Cells. Nanjing University of Chinese Medicine.

Mangas, S., Bonfill, M., Osuna, L., Moyano, E., Tortoriello, J., Cusidó, R.M., Piñol, M.T., Palazón, J., 2006. The effect of methyl jasmonate on triterpene and sterol metabolisms of *Centella asiatica, Ruscus aculeatus* and *Galphimia glauca* cultured plants. Phytochemistry 67, 2041–2049. https://doi.org/10.1016/j.phytochem.2006.06.025.

Mimaki, Y., Kuroda, M., Kameya, A., Yokosuka, A., Sashida, Y., 1998. Steroidal saponins from the underground parts of *Ruscus aculeatus* and their cytostatic activity on HL-60 cells. Phytochemistry 48, 485–493. https://doi.org/10.1016/S0031-9422(98)00036-3.

Mustafa, N.R., de Winter, W., van Iren, F., Verpoorte, R., 2011. Initiation, growth and cryopreservation of plant cell suspension cultures. Nat. Protoc. 6, 715–742. https://doi.org/10.1038/nprot.2010.144.

Naill, M.C., Roberts, S.C., 2004. Preparation of single cells from aggregated *Taxus* suspension cultures for population analysis. Biotechnol. Bioeng. 86, 817–826. https://doi.org/10.1002/bit.20083.

Onrubia, M., Moyano, E., Bonfill, M., Cusidó, R.M., Goossens, A., Palazón, J., 2013. Coronatine, a more powerful elicitor for inducing taxane biosynthesis in *Taxus media* cell cultures than methyl jasmonate. J. Plant Physiol. 170, 211–219. https://doi.org/10.1016/j.jplph.2012.09.004.

Palazon, J., Moyano, E., Bonfill, M., Osuna, L., Cusidó, R.M., Piñol, M.T., 2006. Effect of organogenesis on steroidal saponins biosynthesis in calli cultures of *Ruscus aculeatus*. Fitoterapia 77, 216–220. https://doi.org/10.1016/j.fitote.2006.02.005.

Ramirez-Estrada, K., Vidal-Limon, H., Hidalgo, D., Moyano, E., Golenioswki, M., Cusidó, R.M., Palazon, J., 2016. Elicitation, an effective strategy for the biotechnological production of bioactive high-added value compounds in plant cell factories. Molecules 21, 182. https://doi.org/10.3390/molecules21020182.

Rudofsky, G., 1991. Effect of *Ruscus* extracts on the capillary filtration rate. In: Vanhoute, P.M. (Ed.), Retum Circulation and Norepinephine: an Update. John Libbey Eurotext, pp. 219–224.

Sabater-Jara, A.B., Onrubia, M., Moyano, E., Bonfill, Palazon, J., Pedreño, M.A., Cusido, R.M., 2014. Synergistic effect of cyclodextrins and methyl jasmonate on taxane production in *Taxus x media* cell cultures. Plant Biotechnol. J. 12, 1075–1084. https://doi.org/10.1111/pbi.12214.

Sivakumar, G., Alba, K., Phillips, G.C., 2017. Biorhizome: a biosynthetic platform for colchicine biomanufacturing. Front. Plant Sci. 8, 1137. https://doi.org/10.3389/fpls.2017.01137.

Svensjo, E., Bouskela, E., Cyrino, F.Z., Bougaret, S., 1997. Antipermeability effects of Cyclo 3 Frt in hamster with moderate diabetes. Clin. Hemorheol. Microcirc. 17, 385–388.

Thomas, P.A., Mukassabi, T.A., 2014. Biological flora of the British isles: *Ruscus aculeatus*. J. Ecol. 102, 1083–1100. https://doi.org/10.1111/1365-2745.12265.

Upadhayay, S., Jeena, G.S., Shikha, Shukla, R.K., 2018. Recent advances in steroidal saponins biosynthesis and *in vitro* production. Planta 248, 519–544. https://doi.org/10.1007/s00425-018-2911-0.

Vidal-Limon, H., Sanchez-Muñoz, R., Khojasteh, A., Moyano, E., Cusido, R.M., Palazon, J., 2018. *Taxus* cell cultures: an effective biotechnological tool to enhance and gain new biosynthetic insights into taxane production. In: Pavlov, A., Bley, T. (Eds.), Bioprocessing of Plant *in Vitro* Systems. Reference Series in Phytochemistry. Springer, Cham. https://doi.org/10.1007/978-3-319-54600-1_5.

Winarto, B., 2017. *In vitro* propagation of *Ruscus*: a review. South-west. J. Hortic. Biol. Environ. 8, 103–121.

Yendo, A.C., de Costa, F., Gosmann, G., Fett-Neto, A.G., 2010. Production of plant bioactive triterpenoid saponins: elicitation strategies and target genes to improve yields. Mol. Biotechnol. 46, 94–104. https://doi.org/10.1007/s12033-010-9257-6.